Arthropod-Borne Viruses in Danube Delta: Surveillance, Phylogeography and Vector Feeding Patterns

Dissertation with the aim of achieving a doctoral degree at the

Faculty of Mathematics, Informatics and Natural Sciences

Department of Biology

of Hamburg University

Submitted by

Alexandru Tomazatos

Hamburg, 2020

Arthropod-Borne Viruses in Danube Delta: Surveillance, Phylogeography and Vector Feeding Patterns

Dissertation with the aim of achieving a doctoral degree at the

Faculty of Mathematics, Informatics and Natural Sciences

Department of Biology

of Hamburg University

Submitted by

Alexandru Tomazatos

Hamburg, 2020

Thesis Assessors:
Professor Dr. Tim-Wolf Gilberger, co-supervisor
Professor Dr. Iris Bruchhaus

Eidesstattliche Versicherung
Declaration on oath

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hamburg, den 22. Mai 2020

Abstract

One of the visible effects of today's environmental changes and globalization is the increasing impact of zoonotic pathogens. Of particular concern to public health are the arthropod-borne viruses (arboviruses). This category comprises almost exclusively RNA viruses, known for their complex life cycles shaped by fast evolution and arthropod vectors with high ecological plasticity. Because arbovirus maintenance is linked inextricably to vector and host ecology, some geographic regions have a higher risk of arbovirus introduction, persistence or emergence.

The Danube Delta Biosphere Reserve is a well-preserved and biodiverse complex of ecosystems, with high densities of blood-sucking arthropods and vertebrate hosts. This dissertation resulted from a surveillance program implemented in the eastern parts of DDBR (Romania) and is focused on three aspects of the arboviral life cycle: (i) communities of mosquitoes (Diptera: Culicidae) and *Culicoides* biting midge vectors (Diptera: Ceratopogonidae), briefly touching upon tick vectors (Acari: Ixodidae); (ii) detection and characterization of arboviruses in mosquitoes, ticks, reptiles and the reconstruction of spatiotemporal migration patterns of West Nile virus (WNV) in Romania by phylogeographic analysis; (iii) blood feeding patterns of mosquitoes and biting midges as interactions with host communities and their implications on virus ecology. In this last part, blood feeding was used to test the possibility of West Nile virus xenosurveillance by detection of host antibodies in mosquito blood meals.

Vector surveillance in the DDBR during 2014-2016 revealed a rich and abundant mosquito fauna composed of 16 mosquito taxa, including two new records in Romania. Competent vectors of WNV dominated the total collection, both in terms of species composition and abundance. Eight taxa of *Culicoides* biting midge were recorded in 2017. As in the case of mosquitoes, some species were new records for the Romanian entomofauna. Morphological identification was combined with molecular barcoding of mitochondrial DNA. This strategy improved overall taxonomic

assignment and gave some interesting insights into the intra- and inter-specific relationships of mosquitoes and *Culicoides* from Danube Delta.

In the second part, dedicated to virus detection and characterization, the screening of a large number of mosquitoes found a very small infection rate of WNV. The phylogeographic reconstruction conducted with sequence data of European and African strains showed that WNV was independently introduced at least two times in Romania. The intercontinental virus migration patterns overlapped the flyway network used by birds between Africa and Eurasia, highlighting DDBR's suitability and role for the regional dissemination of this virus.

A metagenomic survey of sera collected from reptiles discovered Letea virus, a new orbivirus infecting grass snakes (*Natrix natrix*). The majority of the nine assembled Letea virus genomes had reassorted cognate genes and also recombined gene fragments. Although phylogenetic analysis placed Letea virus in the *Culicoides*/sand fly-borne orbivirus clade, the screening of mosquitoes, biting midges and ticks did not confirm the arthropod vector of this virus.

A second unknown virus, tentatively named Sulina virus, was detected in *Ixodes ricinus* ticks from two rural sites, near the shore of the Black Sea. Sulina virus belongs to the *Orthonairovirus* genus (*Nairoviridae*) and did not replicate in mammal cells lines or in adult mice.

Extensive trapping of mosquitoes and biting midges resulted in large sets of bloodfed individuals. The analysis of their blood meals revealed wide host ranges for both vector groups. Mammals dominated the feeding spectra, with cattle, horses, humans and wild boars as most utilized hosts. Despite being fed upon less frequently, birds were the most diverse host group and accounted for >70% of *Culicoides* host species and half of detected host species for mosquitoes. Overall, host selection in these vector communities appeared to be driven by extrinsic factors like host availability and abundance.

Flavivirus-specific antibodies were detected in blood meals taken by several abundant mosquito vectors on horses and dogs. This xenosurveillance approach

complemented molecular screening and phylogeographic reconstruction, enabling a comprehensive analysis of WNV ecology in the region. At the same time, xenosurveillance of antibodies in blood meals confirmed the potential of domestic dogs as sentinel animals for WNV surveillance.

These results illustrate a dynamic circulation of arboviruses sustained by rich vector and host assemblages in Danube Delta's diverse ecosystems. This dissertation summarizes the first molecular studies of vector blood feeding, detection of an orthonairvirus and phylogeographic analysis of WNV in the country, as well as the first report of orbivirus infection in reptiles. Many gaps remain in the cycles of these newly characterized viruses; more genetic and ecological data of WNV strains from Romania are needed for a better phylogeographic resolution. However, these results may provide useful baseline data for the study of vector populations and, perhaps, a model of arbovirus surveillance to be further developed in this underresearched area with high risk and burden of vector-borne diseases.

Zusammenfassung

Einer der offensichtlichen Effekte der sich wandelnden Umweltbedingungen und Globalisierung unserer Zeit ist der zunehmende Einfluss von zoonotischen Pathogenen auf die so genannte «public health». Von besonderer Bedeutung sind hierbei die von Arthropoden übertragenden Viren (englisch: arthopod-borne viruses, kurz Arboviren). Diese Kategorie wird beinahe ausschließlich von Viren mit einem RNA Genom gebildet, deren komplexe Lebenszyklen durch rasche Evolution und den Interaktionen mit ihren Vektororganismen, mit der ihrerseits hohen ökologischen Plastizität, gekennzeichnet sind. Die Aufrechterhaltung dieses Lebenszyklus ist untrennbar mit der Ökologie dieser Vektoren sowie der Wirtsorganismen verbunden. Daher sind bestimmte geographische Regionen für die Einführung, Aufrechterhaltung oder dem Entstehen von Arboviren von besonderer Bedeutung.

Das Biosphärenreservat Donaudelta (eng.: Danube Delta Biosphere Reserve, kurz DDBR) ist ein gut erhaltenes und biodiverses Ökosystem mit einer hohen Dichte an blutsaugenden Arthropoden sowie deren vertebratischen Wirten. Die vorliegende Dissertation ist das Ergebnis eines Beobachtungsprogrammes des östlichen Teiles des DDBR (Rumänien) und fokussiert sich auf drei wesentlichen Aspekte des Lebenszyklus der Arboviren: (i) den Gemeinschaften der Stechmücken (Diptera: Culicidae) und *Culicoides* Gnitzen (Diptera: Ceratopogonidae) sowie exkursiv der Zecken (Acari: Ixodidae); (ii) der Detektion und Charakterisierung von Arboviren der Stechmücken, Zecken und Reptilien sowie der Rekonstruktion der raum-zeitlichen Muster der Migration des West Nil Virus (WNV) in Rumänien durch eine phylo-geographische Analyse; (iii) der Analyse der Blutmahlzeiten von Stechmücken und Gnitzen als Surrogaten der Interaktionen mit Wirtsgemeinschaften und der daraus folgenden Implikationen für die Virusökologie. In diesem letzten Abschnitt wird der Versuch unternommen zu beschreiben, in wie fern die Analyse von wirtsspezifische Antikörper in den Blutmahlzeiten der Stechmücken als Mittel zur sog. «Xenoüberwachung» des WNV genutzt werden könnte.

Die Beprobung des DDBR in den Jahren 2014 – 2016 offenbarte eine üppige Stechmückenfauna, gebildet durch 16 verschiedene Taxa, mit zwei Erstbeschreibungen für die Region Rumänien. Für WNV kompetente Vektoren bildeten, mit Hinsicht auf Komposition sowie Häufigkeit, die Mehrheit der gesammelten Arten. Zudem konnten im Jahr 2017 acht Taxa der Gnitzen beschrieben werden. Wie schon bei den Stechmücken, zeigten sich auch hier weitere Erstbeschreibungen für die rumänische Entomofauna. Zur taxonomischen Bestimmung wurden Methoden der klassischen Morphologie sowie des molekularen «Barcodings» der mitochondrialen DNA herangezogen und miteinander kombiniert. So konnte nicht nur die Bestimmungen im Allgemeinen verbessert werden, sondern es offenbarten sich auch einige interessante Zusammenhänge der intra- bzw. interspezifischen Beziehungen von Stechmücken und Gnitzen des DDBR.

Der zweite Teil der Dissertation behandelt die Detektion und Charakterisierung von Viren in einer Großzahl von Stechmücken. Dabei zeigte sich das nur ein geringer Teil mit dem WNV infiziert war. Eine phylo-geographische Rekonstruktion mit den Daten der europäischen und afrikanischen Linien des WNV zeigt, dass dieses wenigstens zweimal unabhängig in Rumänien eingeführt wurden sein muss. Die interkontinentalen Migrationsbewegungen des Virus überlappen mit den Zugwegen der Vögel zwischen Afrika und Eurasien. Dies hebt die Bedeutung des DDBR in seiner Rolle für die regionale Verteilung des Virus hervor.

Eine metagnomische Analyse von Serumproben aus Reptilien der Region um Letea zeigte das Vorhandensein eines unbekannten Orbivirus der Ringelnatter (*Natrix natrix*). Zwar ordneten phylogenetische Analysen den Letea Virus der Klade der *Culicoides*/Sandmücken-übertragenden Orbiviren zu, es konnte jedoch nicht in direkt in Stechmücken, Gnitzen oder Zecken nachgewiesen werden.

Ein weiteres unbekanntes Virus, vorläufig Sulina Virus genannt, wurde in *Ixodes ricinus* Zecken aus zwei ländlichen Gegenden nahe der Schwarzmeerküste entdeckt. Das Sulina Virus gehört zu den *Orthonairoviren* (*Nairoviridae*) und repliziert nicht in Säugetierzellen oder adulten Mäusen.

Das ausgiebige Fangen von Stechmücken und Gnitzen resultierte in einer großen Sammlung von Individuen mit aufgenommenen Blutmahlzeiten. Deren Analyse zeigte ein weites Wirtsspektrum beider Vektorgruppen an. Säugetiere wie Rinder, Pferde, Menschen und Wildschweine waren die am häufigsten identifizierten Wirte. Zwar gab es absolut weniger Blutmahlzeiten die Vögeln zugeordnet werden konnten, diese zeigten dann jedoch eine hohe Diversität und bildeten mehr als 70% der aus Gnitzen bzw. die Hälfte der aus Stechmücken identifizierten Wirte. Zusammengenommen impliziert die Wirtsselektion dieser Vektoren eine extrinsische Steuerung durch Faktoren wie der Verfügbarkeit und Anzahl von Wirten.

Spezifische Antikörper gegen Falviviren (*Flaviviridae*) konnten in Blutmahlzeiten, stammend von Pferden und Hunden, nachgewiesen werden. Dieser Ansatz der «Xenoüberwachung» ergänzt die anderen molekularen und phylo-geopraphischen Ansätze zum Verständnis der Ökologie des WNV in der Region. Des Weiteren bestätigte es das Potential von Hunden als sog. «Sentinals» zur Überwachung der WNV Zirkulation.

Die Ergebnisse dieser Arbeit illustrieren die dynamische Zirkulation von Arboviren durch die große Menge von Vektoren und Wirten in den verschiedenen Ökosystemen des DDBR. Weiterhin werden in dieser Dissertation die Ergebnisse der ersten Studien zur molekularen Analysen von Vektorblutmahlzeiten, zur Detektion eines Ortonairovirus und phylo-geopgrahischen Analyse des WNV in Rumänien sowie des ersten Berichtes eines Orbiviruses in einem Reptil zusammengefasst. Jedoch verbleiben einige Lücken im Verständnis der Lebenszyklen dieser neu charakterisierten Viren. Weiterhin sind weitere genetische und ökologische Daten der rumänischen WNV Linien für eine bessere Auflösung der phylo-geopraphischen Rekonstruktion von Nöten. Nichtsdestotrotz stellten die Ergebnisse dieser Arbeit den Ausgangspunkt für weitere Studien zu Vektorpopulationen und für zu entwickelnde Unternehmungen zur Überwachung von Arboviren in dieser wenig untersuchten Region, mit ihrer besonderen Anfälligkeit und Vorbelastungen an Vektor-übertragenden Krankheiten, da.

Acknowledgements

Many people contributed in many ways to this project and to the longer journey. None of this would be possible without the support and patience of my family. I am very grateful for their constant support and patience, especially in the early years of dealing with wildlife around our home.

I wish to thank to Professor Dr. Jonas Schmidt-Chanasit for his trust, generosity and humor. Without the initiative of Dr. Daniel Cadar there would be no project in Danube Delta and paths would not have crossed. I am indebted to him for teaching me, for his friendship, support and rough rides through swamps and storms, failed experiments and long nights in doubt.

Because self-doubt and excitement are inherent in this work, I kept my sanity with the help of Alexandra Bialonski, Heike Baum, Jonny Schulze, Mathis Petersen, Leonie Meya, Jessica Börstler, Corinna Thomé, Alexander Schlaphof, Branka and Bernhard Zibrat, Anucha Ponyian and Claudia Poggensse. I am grateful to them for teaching, listening and offering me their advice.

A significant part of my work was supervised by Dr. Renke Lühken. I wish to thank him for his patience during my first manuscripts, for guidance and company in the tropics and during long stopovers. In this regard, I am thankful to Dr. Stephanie Jansen and Dr. Hanna Jöst for their help with insect work, to Professor Dr. Egbert Tannich for supporting the study of mosquitoes in Danube Delta and to Dr. Andreas Krüger for fruitful discussion on entomological problems.

Family played an important part in this project and the memorable time spent on the field would not have been so fruitful without the help of my cousin, Iulia Maranda. Her composure and diligence set the bar from the first trip. We discovered and learned together. Also, this project would not have developed the way it has without the help of Liviu Pârâu, Vasile Suhov and Patricia Iftene. Thank you!

Table of Contents

List of Abbreviations

Abbreviation		Full description
Ae. aegypti	-	*Aedes aegypti*
Ae. albopictus	-	*Aedes albopictus*
Ae. atropalpus	-	*Aedes atropalpus*
Ae. caspius	-	*Aedes caspius*
Ae. detritus	-	*Aedes detritus*
Ae. hungaricus	-	*Aedes hungaricus*
Ae. japonicus	-	*Aedes japonicus*
Ae. koreikus	-	*Aedes koreikus*
Ae. vexans	-	*Aedes vexans*
AHSV	-	African horse sickness virus
AHV	-	Abu Hammad virus
AIC	-	Akaike Information Criterion
aLRT	-	approximate Likelihood Ratio Test
AMV	-	Abu Mina virus
An. algeriensis	-	*Anopheles algeriensis*
An. hyrcanus	-	*Anopheles hyrcanus*
An. maculipennis s.l.	-	*Anopheles maculipennis sensu lato*
ARTSV	-	Artashat virus
ASFV	-	African swine fever virus
BDAV	-	Bandia virus
BF	-	Bayes factor
BG-GAT	-	Biogents Gravid Aedes trap
BOLD	-	Barcode of Life database
BTV	-	Bluetongue virus
BUKV	-	Bukakata virus
C. griseidorsum	-	*Culicoides griseidorsum*
C. kibunensis	-	*Culicoides kibunensis*
C. pallidicornis	-	*Culicoides pallidicornis*
C. punctatus	-	*Culicoides punctatus*
C. puncticollis	-	*Culicoides puncticollis*
C. riethi	-	*Culicoides riethi*
C. subfasciipennis	-	*Culicoides subfasciipennis*
C/SBOV	-	*Culicoides*/sand fly-borne orbivirus
CCHFV	-	Crimean-Congo hemorrhagic fever virus
cDNA	-	complementary deoxyribonucleic acis
CGLV	-	Changuinola virus

CGV	-	Chobar Gorge virus
CHIMV	-	Chim virus
CHUV	-	Chuzan virus
CNUV	-	Chenuda virus
CO_2	-	carbon dioxide
COI	-	cytochrome c oxidase subunit I
Coq. richiardii	-	*Coquillettidia richiardii*
CORV	-	Corriparta virus
Cx. modestus	-	*Culex modestus*
Cx. pipiens s.l./torrentium	-	*Culex pipiens* sensu lato/*torrentium*
DDBR	-	Danube Delta Biosphere Reserve
DENV	-	Dengue virus
DGKV	-	Dera Ghazi Khan virus
DNA	-	deoxyribonucleic acid
(ds)RNA	-	double-stranded ribonucleic acid
DUGV	-	Dugbe virus
ECSA	-	East/Central/South African
EEC1	-	East European Clade 1
EEV	-	Equine encephalosis virus
EHDV	-	Epizootic hemorrhagic disease virus
ELISA	-	Enzyme-linked Immunosorbent Assay
ERV	-	Estero Real virus
ERVEV	-	Erve virus
EU	-	European Union
EUBV	-	Eubenangee virus
EVS	-	Encephalitis Vector Surveillance
FOMV	-	Fomede virus
GC	-	guanine-cytosine
GERV	-	Geran virus
GIV	-	Great Island virus
GPC	-	glycoprotein precursor
HAZV	-	Hazara virus
HUGV	-	Hughes virus
IBOV	-	insect-borne orbivirus
IFEV	-	Ife virus
IgG	-	Immunoglobulin G
ISKV	-	Issyk-Kul virus
ISV	-	insect-specific virus
JAPV	-	Japanaut virus

KASOV	-	Kasokero virus
KEMV	-	Kemerovo virus
KTRV	-	Keterah virus
KUPEV	-	Kupe virus
L	-	large genomic segment
LEAV	-	Letea virus
LEBV	-	Lebombo virus
LIPV	-	Lipovnik virus
LPHV	-	Leopard Hill virus
M	-	medium genomic segment
MBOV	-	mosquito-borne orbivirus
MCC	-	maximum clade credibility
MCMC	-	Markov Chain Monte Carlo
mDNA	-	mitochondrial deoxyribonucleic acid
ML	-	maximum likelihood
MLD	-	mucin-like domain
MOBV	-	Mobuck virus
N	-	nucleocapsid
NaCl	-	sodium chloride
NGS	-	next-generation sequencing
NJ	-	neighbor-joining
NSDV	-	Nairobi sheep disease virus
ORF	-	open reading frame
ORUV	-	Orungo virus
OTU	-	ovarian tumor domain
PALV	-	Palyam virus
PATAV	-	Pata virus
PCR	-	polymerase chain reaction
PCTN	-	Pacific coast tick nairovirus
PHSV	-	Peruvian horse sickness virus
PLV	-	Parry's Lagoon virus
PMSF	-	phenylmethanesulfonyl fluoride
QYBV	-	Qalyub virus
RdRp	-	RNA-dependent RNA polymerase
RNA	-	ribonucleic acid
RT-PCR	-	reverse transcription-polymerase chain reaction
S	-	small genomic segment
s.l.	-	sensu lato
SAKV	-	Sakhalin virus

SAPV	-	Saphire ll virus
SBV	-	Schmallenberg virus
SCRV	-	St. Croix River virus
SKI-1/S1P	-	subtilisin/kexin-isozyme-1/site-1 protease
SOLV	-	Soldado virus
(ss)RNA	-	single-stranded ribonucleic acid
SULV	-	Sulina virus
SVIV	-	Sathuvachari virus
SySV-1	-	Shāyáng spider virus 1
TAGV	-	Taggert virus
TAMV	-	Tamdy virus
TBEV	-	Tick-borne encephalitic virus
TBOV	-	tick-borne orbivirus
TcTV-1	-	Tǎchéng tick virus 1
TFAV	-	Thiafora orthonairovirus
TFLV	-	Tofla virus
TIBOV	-	Tibet orbivirus
TILLV	-	Tillamook virus
TOV	-	tick orbivirus
TRBV	-	Tribeč virus
UMAV	-	Umatilla virus
Ur. unguiculata	-	*Uranotaenia unguiculata*
UTR	-	untranslated region
USUV	-	Usutu virus
UZAV	-	Uzun-Agach virus
WALV	-	Wallal virus
WARV	-	Warrego virus
WEC1	-	West European clade 1
WMV	-	Wad Medani virus
WNV	-	West Nile virus
WzTV	-	Wēnzhōu tick virus
YFV	-	Yellow fever virus
YOUV	-	Yunnan virus
ZIKV	-	Zika virus

List of Figures

List of Tables

1 Introduction

1.1 Background

Almost two decades ago it was estimated that zoonoses account for over 60% of human infectious diseases and zoonotic pathogens have a double potential for emergence compared to the non-zoonotic ones [2]. More recent estimates discuss about approximately two-thirds of human infections as being shared with other animals [3]. The intimate connection between human and animal health was observed for a long time. Towards the end of the 20th century the concept of "health" progressed from a focus on human health to inclusion of the domesticated species' health, to wildlife and flora, and eventually to ecological systems and the environment [4]. In the wake of outbreaks of West Nile virus (WNV), Severe Acute Respiratory syndrome (SARS), Ebola virus disease (EVD), monkey pox and avian influenza, the biomedical community called for unity within an inter- and transdisciplinary framework which today hopes to include other issues, such as antimicrobial resistance [5]. The name of this holistic approach combining conservation with human and veterinary medicine eventually became the "One Health" concept.

Over a long history of interaction with humans, arthropod-borne viruses (arboviruses) have been causing a great deal of morbidity and mortality. Chinese historical records from 3rd and 10th century CE contain clinical descriptions resembling a dengue-like illness [6]. More recently, reports of similar clinical pictures, describing acute illness with prolonged convalescence, appeared in the French West Indies and Panama in the 1600s [6]. By the 1700s, Dengue virus (DENV) had spread globally, from Asia and Africa to North America, probably helped by sailing ships and the development of global trade [7]. Similarly to DENV, Yellow fever virus (YFV) and its mosquito vector, *Aedes aegypti*, are very likely to have been introduced from Africa to South America in the bilges of the sailing vessels during the slave trade (around 300-400 years ago) [8,9]

The last two centuries since the First Industrial Revolution painted a very complex picture of today's world; over a background of industrialization and globalisation, human population increased and the biosphere became dominated by human activity. The conversion of natural areas and human migration related to conflict and climate change are rapidly altering the profile of infectious diseases [10,11]. The connection between a rapidly changing world and the increasing impact of infectious diseases seems part of a logical progression and is evident perhaps for many. However, it is as evident that fast changes in ecosystems and population dynamics will entail complex epidemiological scenarios, as both hosts and pathogens seek to adapt to a changing environment. Eco-climatic changes, marked by urbanization, intensification of agriculture and expanded networks for movement of people and goods, favor the movement of infected hosts and invasive species acting as disease vectors. Habitat fragmentation by deforestation, mining, expansive agriculture or dam building is causing alterations in the vector-host-parasite relationship [12]. In many cases, epidemics and epizootics are caused by human encroachment on ecosystems where arboviral transmission cycles occur naturally (i.e. sylvatic cycles) [13]. The change of land use initiated by deforestation can help mosquito vectors to invade and thrive in new habitats [14]. For example, the colonization of the Amazon region, with the development of road networks and increase in human population, has led to the emergence and spread of numerous new viruses. Human biting rates of the primary vector of malaria in the Amazon, *Anopheles darlingi*, were more than 270 times higher in deforested areas associated with road development than in areas with intact forest coverage [15]. Contacts between humans and DENV have increased during and after the Second World War, when large-scale ecologic and demographic disruptions were followed by rapid urbanization with inadequate housing and sewage. This facilitated dispersal and high densities of *Ae. aegypti*, leading to an increase in magnitude and frequency of dengue epidemics in the following decades. Today, over half of the world's human population is living in areas of dengue risk [6,7,16]. Other arboviruses, such as WNV, Zika virus (ZIKV) and Chikungunya virus (CHIKV),

achieved comparable spread by emergence and reemergence from previously restricted foci [17], even despite efficient human vaccines for some viruses (e.g., YFV) [18].

Diversity of arboviruses

Arboviruses are not a proper taxonomical group, but rather a category defined around biological and ecological characteristics. The natural arboviral cycle is a complex interplay between the virus, a primary hematophagous arthropod vector (acting also as reservoir) and a primary vertebrate amplification host (further referred to as host). After being ingested with a blood meal taken from an infected vertebrate host, the virus escapes anatomical and physiological barriers inside the vector's body and disseminates systemically during an interval known as the extrinsic incubation period. The viral load becomes high in some tissues, particularly in the salivary glands from where the virus is ready to be transmitted to another vertebrate during a next feeding bout [19]. The main arthropod vectors are mosquitoes (Diptera: Culicidae) [20] sand flies (Diptera: Psychodidae) [21], biting midges (Diptera: Ceratopogonidae) [22] and ticks (Ixodida: Ixodidae and Argasidae) [23]. Other arthropods may be involved in arbovirus transmission to a lesser extent: louse flies (Diptera: Hippoboscidae) [24,25], lice (Phthiraptera: Echinophthiriidae) [26], mites (Acari: Mesostigmata) [27], bed bugs (Hemiptera: Cimicidae) [28] and fleas (Phthiraptera) [29]. For the natural cycle (biological transmission) to be completed, the virus must replicate in both arthropod and vertebrate. The vertebrates capable to develop viraemia and sustain transmission belong to all classes of terrestrial vertebrates. Most commonly these are birds, bats, rodents and primates [30]. Arbovirus cycles are remarkably complex, as highlighted by their evolutionary strategies for spread and persistence into new areas with new host populations. Changes in vector and host ecology (e.g., caused by encroachment on natural habitats) may allow the virus to escape the primary cycle and establish a secondary cycle, involving other vector and host species (**Figure 1**). In many cases, secondary hosts (e.g., humans, domestic animals) become incidental hosts with limited or no contribution to arbovirus maintenance ("dead end" hosts) [31]. The distinction

between "reservoir" and "amplifier" has become blurred and the two terms referring to a vertebrate host are often used interchangeably [32,33]. Based on data about the habitat and type of arboviral transmission, vertebrate hosts classified into reservoirs are those almost exclusively involved in enzootic transmission in sylvatic environments, while amplifiers are those acting in epidemic/epizootic transmission in urban or anthropic environments [33,34]. However, the arthropod vectors are often considered reservoirs and/or amplifiers of the virus, while the vertebrates retain only the status of amplifier. These intricate aspects of transmission roles add to the difficulty of disentangling the transmission networks of arboviruses.

The capacity to perpetuate in multiple ecological niches suggests an enormous number of arboviruses, of which only little over 500 are known [31]. Almost 150 species are known as causal agents of human disease [19]. Known pathogenic arboviruses for humans and other animals belong mainly to nine families: *Asfarviridae* (genus *Asfarvirus* with a single species, *African swine fever virus*), *Nairoviridae* (genus *Orthonairovirus*), *Peribunyaviridae* (genus *Orthobunyavirus*), *Phenuiviridae* (genus *Phlebovirus*), *Flaviviridae* (genus *Flavivirus*), *Reoviridae* (genus *Orbivirus*) *Rhabdoviridae* (genera *Ephemerovirus* and *Vesiculovirus*), *Orthomyxoviridae* (genus *Thogotovirus*) and *Togaviridae* (genus *Alphavirus*). Because arboviruses are defined on the basis of their biological properties and complex natural cycles, classification and species definition was subject of many changes during the last several decades. Viral taxonomy was based primarily on morphology and antigenic properties. Members of a virus species were defined based on a consensus around genome

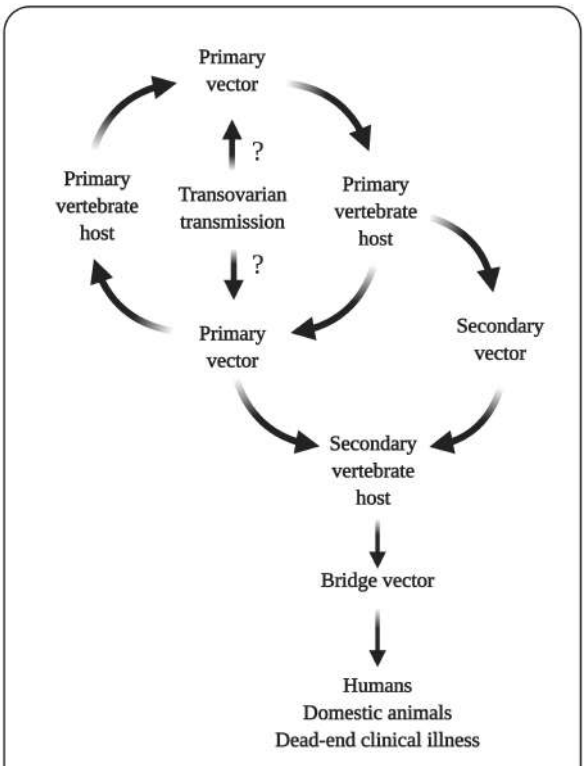


Figure 1. Hypothetical arbovirus life cycle (source: Gubler & Vasilakis [31])

sequence relatedness, natural host range, cell and tissue tropism, pathogenicity, mode of transmission and antigenic properties ("polythetic" species definition) [35]. The definition of a virus species has changed in the last decade, from the concept of a polythetic class to "a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria" [36], emphasizing phylogenetic relatedness as the defining property. With no other property to simultaneously differentiate between members of a virus species, the delineation of species often becomes an arbitrary task along the lines of genetic similarity [37], with a continual need for modification as new viruses are discovered.

Relatively recent advances in molecular biology, in particular the development of high-throughput sequencing and metagenomics, had a profound effect on virus taxonomy. Molecular and bioinformatic tools have improved our ability to detect and characterize viral communities from a large variety of biological samples [38–42]. This was showcased also by the discovery of "insect-specific viruses" (ISV) that infect insects and insect cells, but do not replicate in vertebrate or vertebrate cells [43]. Many of discovered ISVs belong to viral families of known arboviruses [44–48]. A close association of ISVs with their insect hosts is assumed to be an ancient characteristic, because many of them are vertically transmitted [49] and some have become integrated within the genomes of their arthropod hosts [50]. Mechanisms of ISV transmission and maintenance in nature are so far unclear. The most prevalent assumption supported by experimental [49,51] and field studies [52] is that these viruses are maintained by vertical transmission. Discovery of ISVs and their interactions with other components of a vector's microbiome is relevant from the perspective of vaccine development [53] or their potential as biological control agents influencing vector competence (i.e., decreasing the vector's ability to transmit pathogenic arboviruses) [54–57].

Unique aspects of arbovirus evolution

With the exception of ASFV, arboviruses have RNA genomes with a high mutation rates as a result of replication via the low fidelity RNA polymerase. The

outcome is a wide range of genetic variation subjected to natural selection and a much faster evolution in comparison to DNA organisms [58]. RNA viruses are also characterized by small genomes [59]**.** The mutation rates of RNA viruses are orders of magnitude higher in comparison to those of most DNA viruses (single-stranded DNA viruses provide some exceptions). Both RNA and DNA viruses using a low fidelity polymerase have mutation rates estimated in the range of 10^{-3}–10^{-5} changes introduced per nucleotide copied, i.e. approximately one substitution per genome per replication cycle for those viruses using RNA-dependent RNA polymerase for replication [59,60].

Molecular evolution of viral genomes depends also on factors other than the inherent differences between RNA and DNA replication machineries, with some DNA viruses shown to evolve at rates close to those of RNA viruses [61]. The fast evolution of RNA viruses (including arboviruses), with rapid growth kinetics and selection acting at the level of populations tending to near-infinite sizes is a challenge to the traditional paradigm of population genetics. As a result, the use of the species concept in virus taxonomy presents some difficulties [58]. In this regard, the adoption of the quasispecies proved useful. The quasispecies theory is a mathematical formulation conceived for describing the fast-evolving and self-organizing RNA-like molecules [62–64]. The current meaning of a viral quasispecies is a population of closely related, continuously mutating, thus non-identical virus genomes (a cloud or swarm of mutants), in competition and under selection for the fittest group of variants in a given landscape [65–67]. Although a high mutation rate results in many nonviable progenies, such rates create a "cloud" or "swarm" of potentially favorable mutations at population level, increasing the chances for adaptation in new hosts [63]. Single genomes influence the replication of others in a negative or positive manner, making selection operational at the population (cloud) level, akin to group selection [65]. Thus, the strains that are part of the viral quasispecies determine collectively the phenotype of the viral population (e.g., virus fitness, host response/disease) through their interaction [68–71].

The low fidelity of the RNA polymerase creates a background of random point mutation (insertion, deletion, substitution), over which arboviral genomes use additional mechanisms for genetic diversification. Whereas genetic drift operates on the viral genome incrementally, generating diversity and adaptation in a step-wise fashion, viruses can evolve also by recombination and reassortment. Genetic recombination occurs between closely related viral strains when they co-infect the same host cell. The resulting hybrid RNA molecule can be produced by homologous or non-homologous recombination; the latter means that genetic material is exchanged between different genomic regions [72]. Because this exchange requires the parental strains to be in close proximity within the same host cell during replication, the probability of recombination increases with multiplicity of infection. Given the very wide range of hosts and tissues that arboviruses infect, the frequency of recombination in nature is difficult to quantify [73]. Reassortment (genetic shift) is a mechanism used frequently by arboviruses with segmented genomes. It consists of swapping cognate genes among relatively close strains during co-infection, thus generating progenies with new genetic combinations [74]. Perhaps the most common example of a reassortant is influenza A virus (*Orthomyxoviridae*), a non-arbovirus which caused three major pandemics in the last half of century [75,76]**.** In arboviruses, gene reassortment is commonly detected among orbiviruses like Bluetongue virus (BTV) [77–79] and Epizootic hemorrhagic fever virus (EHDV) [80,81] and is pervasive in bunyaviruses [74].

The arboviral cycle requires at least two distinct hosts (the arthropod and the vertebrate), resulting in a dual host tropism of the virus. In many competent hosts and vectors, viral amplification can be very efficient and lead to high viral loads, sometimes of over a billion new genomes. In such cases, a vast sequence space can be explored during a single infection [82]. Adaptive evolution of arboviruses that facilitates emergence and spread is well exemplified by some mosquito-borne viruses (moboviruses). For example, in the New World, WNV displaced previously circulating strains by attaining a shorter extrinsic incubation period in *Culex* mosquitoes [83,84].

Also, the differential virulence of WNV upon its arrival in the USA (being particularly devastating to crows) was found to rest on a single amino acid substitution of the NS3 helicase; the resulting motif enhanced viral replication, increased viral load and the efficiency of transmission [85]. In the case of CHIKV, adaptive evolution enabled the infection of a local, secondary vector (*Ae. albopictus*), propelling CHIKV from Africa to the Indian Ocean and further to global spread [86,87].

1.2 Arbovirus surveillance in Europe

The amplification levels of arboviruses are highly variable in space and time. The natural (enzootic) transmission cycle can be completed at a very low level, oftentimes undetected [88,89]. When competent vectors feed on susceptible hosts within a permissive environment, amplification can quickly reach epidemic levels. Like in the other regions of the world, global connectedness and changing eco-climatic patterns have facilitated the spread and endemisation of some arboviruses and their vectors in Europe. This led to an increase of outbreak frequency and intensity in humans and other animals [90]. The presence of WNV is firmly established throughout most of southern and southeastern Europe, being detected in the last years in Austria, Bulgaria, Croatia, Cyprus, Czech Republic, France, Germany, Greece, Hungary, Italy, Portugal, Romania, Serbia, Slovenia, Spain, Turkey [91]. Vector surveillance can reveal the presence of pathogens prior to emergence and offer crucial information about pathogen circulation, so that control measures (e.g., vaccination, livestock biosecurity) can be timely implemented. Because the eco-epidemiological situation is very dynamic, knowledge gained from surveillance programs should be complemented by studies addressing key issues regarding local vectors, such as population genetics and vector competence. Arboviruses infecting vertebrates throughout Europe have increased both in diversity and in geographical range: Batai virus (BATV) in mosquitoes [92] and antibodies in bovines [93], Sindbis virus (SINV) in mosquitoes [94,95] and birds [96], Usutu virus (USUV) in mosquitoes [97], birds [98] and bats [99]. Not much time elapsed from detection of antibodies of USUV [100], until viral infections were confirmed in blood donors [101,102]. Furthermore, the hot

summers of 2018 and 2019 boosted the intensity of WNV outbreaks and its expansion northwards. The virus arrived in Germany and was first detected in birds, then in equids and humans [103]. Because its epidemiology is similar, if not identical to that of WNV, human cases of USUV infection saw a spectacular increase during these hot summers [104–106].

Efficient vector surveillance is difficult to implement for many reasons, especially limited financial support, but also for training much-needed specialist entomologists [107]. In the case of moboviruses, one of the main concerns for European public and veterinary health, only a few countries are using an integrated vector-human-animal WNV surveillance approach (e.g., Austria, France, Greece, Italy, United Kingdom and Germany) [108–111]. Some other countries where outbreaks have become severe are developing such programs (e.g., Hungary and Serbia) [112–114].

A complicating factor for disease control is the spread of invasive vector species. Five invasive species of container-breeding *Aedes* mosquitoes are well established in Europe: *Ae. albopictus*, *Ae. aegypti*, *Ae. japonicus, Ae. atropalpus* and *Ae. koreikus* [115–122]. *Aedes triseriatus* was reported only once in France in 2004 [123]. Since it was first detected in Albania in 1970s, *Ae. albopictus* has spread to at least 20 European countries, including the United Kingdom [124,125] and Germany [126]. Although *Ae. aegypti* was driven out of continental Europe since the 1970s, in 2012 this species triggered a dengue outbreak in Madeira [127] and is back on the eastern shores of the Black Sea [128].

It is not only invasive mosquito vectors that ride the commercial networks of the world. *Culicoides* biting midges are vectors of bluetongue, a disease with a well-documented history of intercontinental dispersal and devastating effects on ruminants [129]. Nationwide surveillance of this vector group is performed by fewer countries, mostly in the Mediterranean, where BTV is endemic [130]. Consequently, we have much less data on the bionomics of BTV vectors. In 2006, the spread (and subsequent overwintering) of sub-Saharan BTV8 serotype to northern Europe, over 900 km further

north than any known European limit, caused great loses to the European agriculture [131]. A few years later, the emergence of a new orthobunyavirus designated Schmallenberg virus (SBV) affecting cattle in western Europe, emphasised the necessity of *Culicoides* surveillance [132]. These outbreaks motivated research on vector ecology [133–136], vector competence [137–140] and taxonomy [141–143]; the last topic is notoriously difficult, as entomological training still benefits from little support [144].

The spread of non-flying parasites such as ticks makes no exception in the context of changes to the biosphere impacting vectors and hosts. Among many important bacterial and parasitic pathogens, ticks harbor highly pathogenic arboviruses [145–147] and a very diverse microbiome from which virus emergence can be initiated [148–151]. Ticks can reach new habitats by attaching to migratory birds, livestock or exotic pets [152,153]. Infested birds can translocate parasites between distant habitats along intercontinental flyways between Africa and Europe [154–159]. Studies from migration hotspots or at stopover sites found birds carrying ticks positive for CCHFV [160], TBEV [161] or WNV [162]. The parasites can detach and establish new natural foci of vector-borne diseases if local conditions permit their survival [155]. This hypothesis received support in the recent years from studies documenting the arrival of *Hyalomma* spp. in western and northern parts of Europe [163–166].

1.2.1 Arbovirus surveillance in Romania

Several arboviruses were reported in Romania since the 1950s (e.g., Tick-borne encephalitis virus, West Nile virus, Kemerovo virus, Tahyna virus) [167–180]. In 1996, Romania experienced one of the first and largest outbreaks of West Nile disease in Europe [181,182], followed by a second major outbreak in 2010 [183]. Since 1996, surveillance is mostly passive, with veterinary and occasional vector surveys at county level, usually following clinical outbreaks [182,184–186]. WNV is the main targeted arbovirus, predominantly in the south and east of the country [185–192]. As a result, data on arbovirus diversity, seroprevalence, as well as information about vector diversity and ecology is outdated or available for restricted areas or groups of species

where foci of disease were recorded [175,179,180,182,186,193]. Sporadic cases of ZIKV or DENV are occasionally imported via travellers [194–197].

Populations of *Ae. albopictus* were found in the last decade (2012-2014) in the capital city, located in the south of the country [198]. A few years later (2017-2018), this species was found across a wider area in the south (Bucharest, Giurgiu, Ilfov, Prahova), but also in the east (Constanța), southwest (Mehedinți) and northwest (Bihor) [199].

Information about tick vectors is more up-to-date than for insect vectors and relies on more comprehensive sampling [172,200–204]. A recent and wider serological survey of TBEV in Transylvania confirmed that this virus is endemic to the region [205]. Some studies conducted in eastern Dobrogea found migratory birds infested with ticks that tested positive for various tick-borne microbial pathogens [158,206,207] and WNV [162].

The first outbreak of bluetongue on the territory of Romania occurred in 2014 [208]. The entomological surveillance for BTV vectors was instituted in addition to serological testing of livestock. However, it was performed only in several sites around the country. With the exception of known vectors, such as *C. imicola* or *C. nubeculosus* [209,210], other potential vectors are recorded as ”species complex” (e.g., *Culicoides obsoletus* complex) or as “other *Culicoides*” [211,212]. The most comprehensive work about the Romanian *Culicoides* fauna was published in the 1970s [213] and updated two decades ago [214]. To this day, comprehensive studies of *Culicoides* diversity and a quantification of the risk they pose to the animal health do not exist in the country. Therefore, knowledge about *Culicoides* species composition and ecology is missing for most parts of the country.

1.3 Arboviruses relevant to public health in Europe

1.3.1 Mosquito-borne viruses

***West Nile virus* (WNV, *Flaviviridae*: *Flavivirus*)**

Found in temperate and tropical latitudes on every continent except Antarctica, WNV is the most widespread encephalitic arbovirus and second only to DENV in its global distribution [215]. The virus was first isolated from a case of human febrile illness in Uganda in 1937 and is taxonomically positioned within the JEV serocomplex [216]. Of the nine possible WNV lineages documented, the most important for public health are lineages 1 and 2 [217]. WNV is one of the most important viral pathogens of Europe and in the European Union it is a notifiable disease in humans and equids.

In nature, the virus circulates in an enzootic cycle between ornithophilic *Culex* mosquitoes and birds, especially passerines (order *Passeriformes*) [218,219]. Bird migration can play an important role in spreading WNV between distant geographic locations, either as biological or mechanical carriers [162,220,221]**.** The virus was also detected in *Aedes*, *Anopheles*, *Coquillettidia* and *Uranotaenia* mosquitoes [186,222,223].

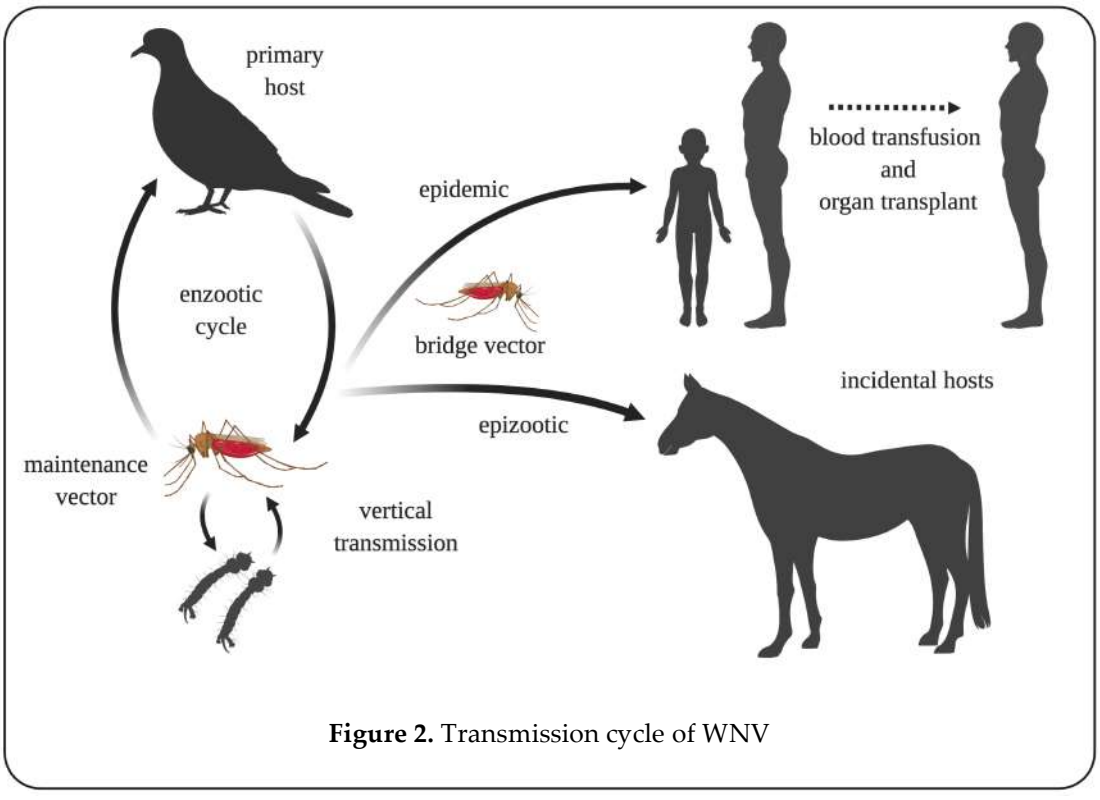


Figure 2. Transmission cycle of WNV

Aside from mosquito vectorship, certain *Hyalomma*, *Argas* and *Ornithodoros* ticks are suspected to play a role in transmission [27,217]. Vertical transmission and overwintering in vectors or hosts can lead to persistence and endemisation of WNV without the need of yearly reintroduction into temperate areas [224–226]. Spillover from its enzootic cycle results in infection of mammals [227,228], reptiles [229] and amphibians [230], hosts that develop viraemia below the threshold for further maintenance (dead-end hosts).

Usutu virus (USUV, *Flaviviridae: Flavivirus*)

Isolated from *Culex neavei* in South Africa near Usutu river, USUV is another member of the JEV antigenic group [231]. Studies on USUV evolutionary history and European circulation indicate an African origin of the virus [232,233]. Ornithophilic mosquitoes transmit the virus to birds and *Culex pipiens* s.l. is considered the most competent vector [234].

On European territory, the virus was first detected in the capital city of Austria in 2001, but retrospective screening of tissue from Italian birds found that USUV had entered Europe years prior to initial detection [235]. USUV has received a great deal of attention since its emergence in Europe, characterized by episodes of

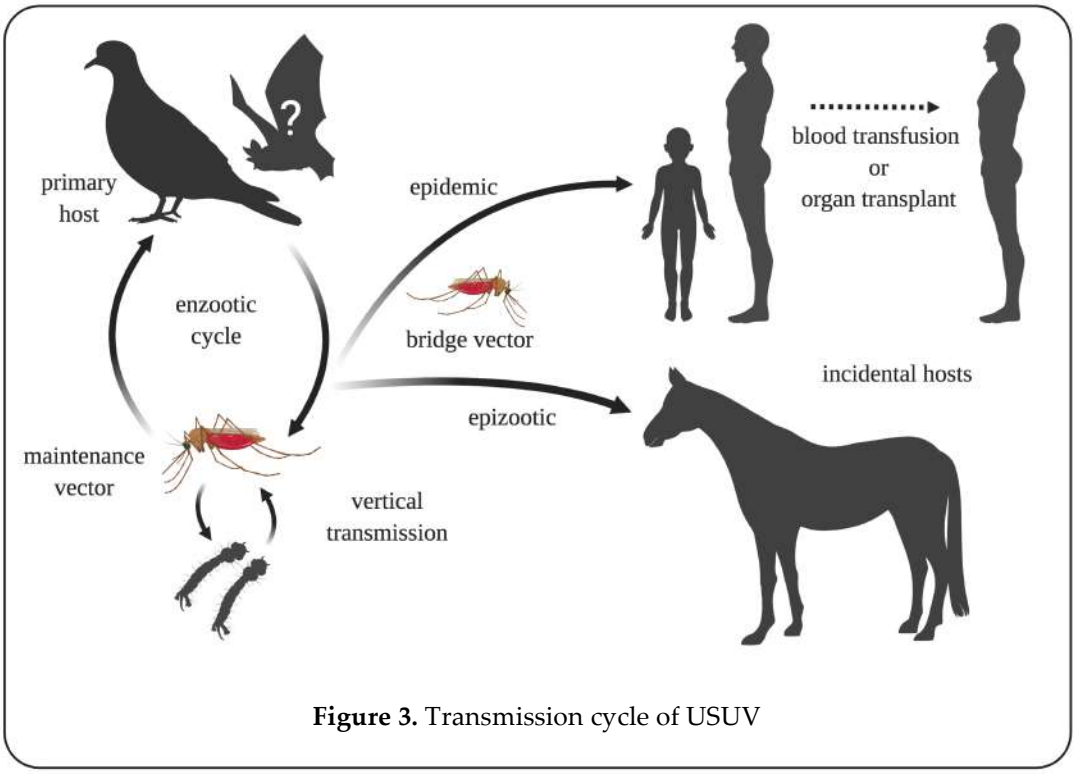


Figure 3. Transmission cycle of USUV

spectacular mortality in birds from Austria [236], Germany [237], France [238] and the Netherlands [239]. The virus was also found in the organs of *Pipistrellus* bats from Germany [99] and rodents from Senegal [240]. Some other mammals (dogs, horses, swine) tested positive for USUV-specific antibodies in the Mediterranean and Balkans [241]. After the recurrent epizootics from Europe since the middle of 1990s, USUV was detected in blood donors [101,102,104] and was recently found to be a cause of neuroinvasive disease in humans [106,242]. Moreover, its prevalence in humans could be underestimated, owing to the absence of symptoms and cross-reactivity in routine serological screening.

***Dengue virus* (DENV, *Flaviviridae*: *Flavivirus*)**

DENV comprises four serotypes (DENV1-4) and is a flavivirus closely related to Japanese encephalitis serocomplex. Unlike the epornitic flaviviruses (e.g., USUV and WNV), DENV is using humans as amplification hosts and is the most important arboviral pathogen [243]. It is also the most widespread arbovirus in the world, being endemic to most tropical and subtropical regions [244]. The spread of DENV serotypes relies on its primary and secondary vectors, *Ae. aegypti*, respectively *Ae. albopictus*, which are very successful at using global networks of transport and adapting to urban/periurban environments. After a major dengue epidemic that affected Greece in 1928 (almost one million cases and 1000 deaths) [245], local transmission in Europe

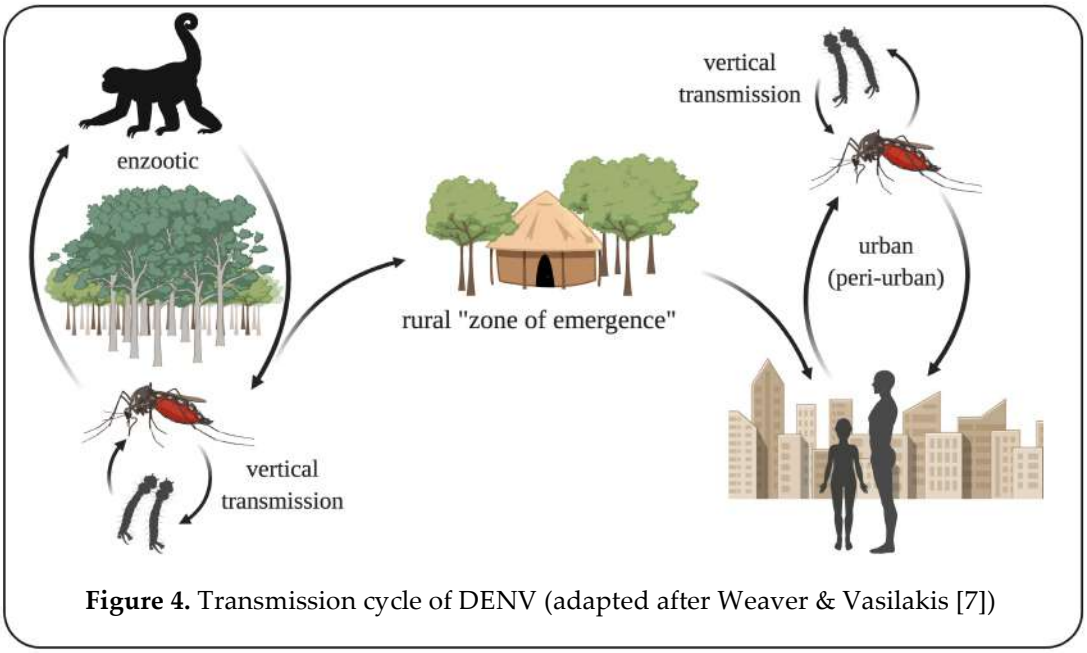


Figure 4. Transmission cycle of DENV (adapted after Weaver & Vasilakis [7])

ceased for more than 70 years. Autochthonous transmission resumed a decade ago (2010) when France, Croatia and Madeira archipelago reported autochthonous cases of dengue [127,246]; additional cases, comprising the majority of recorded DENV infections in Europe, are imported via returning travellers [247].

***Chikungunya virus* (CHIKV, *Togaviridae*: *Alphavirus*)**

Chikungunya virus has most likely an African origin, due to its close relationship with the African sister species O'nyong'nyong virus. Three lineages of CHIKV have been documented: West African, East/Central/South African (ECSA) and Asian. The African lineages are enzootic in the sub-Saharan jungles, vectored by arboreal *Aedes* mosquitoes among non-human primate hosts [87,248]. The virus received much attention when a new lineage originating from the ECSA phylogroup emerged on islands of the Indian Ocean. A single amino acid mutation in the E1 glycoprotein was favorable for the transmission of CHIKV by *Aedes albopictus*, a secondary vector that was locally abundant [249]. The Indian Ocean lineage reached Asia, from where it was soon introduced in Europe, presumably by infected travellers returning to Italy. There, local *Ae. albopictus* initiated an outbreak with more than 200 cases [250]. Autochthonous outbreaks followed in southern France in 2010, 2014 and 2017 [251,252]. Temperate climate is a key factor in CHIKV cycle interruption and there is no indication so far of virus overwintering [250,253]. However, probable

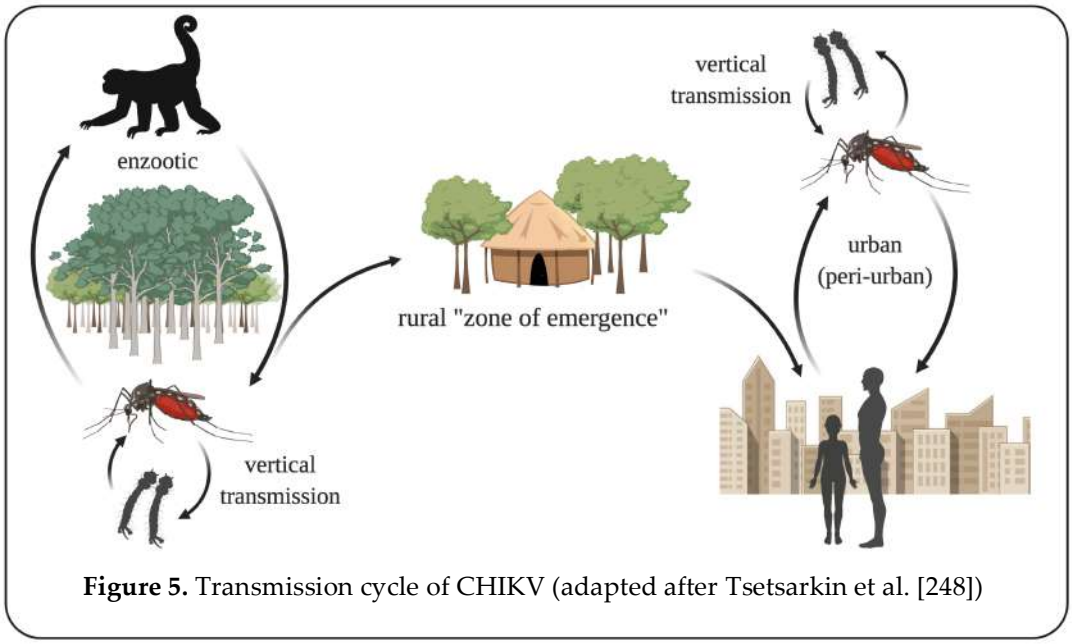


Figure 5. Transmission cycle of CHIKV (adapted after Tsetsarkin et al. [248])

vertical transmission by transovarial dissemination was reported in Indian wild-caught *Ae. albopictus* and the primary vector *Ae. aegypti* [254,255].

1.3.2 Tick-borne viruses

***Tick-borne encephalitis virus* (TBEV, *Flaviviridae: Flavivirus*)**

Three classical subtypes of TBEV have been described: (i) European subtype (TBEV-Eu, also named Western/Central European or "ricinus" subtype) circulating in mainland Europe, (ii) the Siberian subtype (TBEV-Sib or "persulcatus" subtype) found in the rest of Russia, reaching eastern Europe and (iii) the Far East subtype (TBEV-FE) from Asia, mainly northern China to eastern Russia [256]. Two other Asian subtypes, the Baikalian subtype and the Himalayan subtype, have been proposed recently [257,258]. Main vectors are *Ixodes ricinus* (TBEV-Eu) and *Ixodes persulcatus* (TBEV-Sib and TBEV-FE) [259], but so far at least 22 species of hard ticks have been found to carry the virus [260]. Amplifying hosts of TBEV are small-medium mammals, like

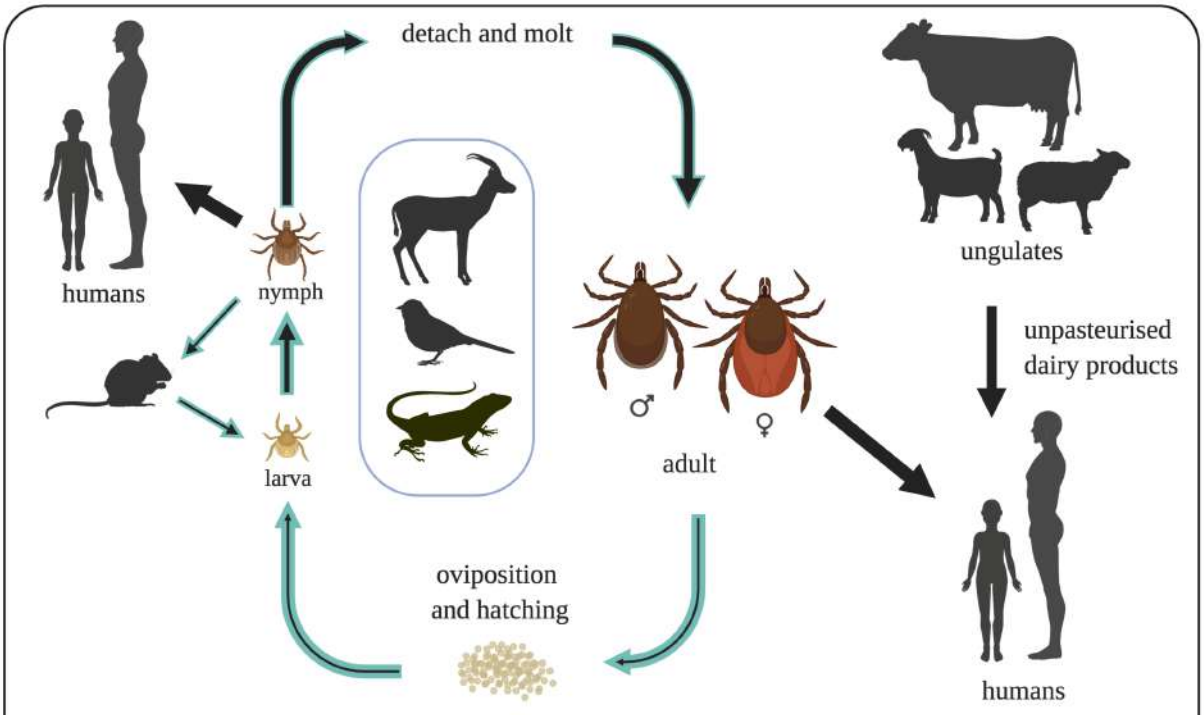


Figure 6. Transmission cycle of TBEV. Some rodents may be involved in non-viraemic transmission of virus to uninfected immatures. Large hosts are necessary for completion of the tick life cycle, without reported involvement in the virus cycle. Green arrows indicate tick life cycle. Black arrows indicate possible transmission of TBEV between ticks and mammals or directly between ticks (source: Estrada-Peña & de la Fuente [261]).

woodland rodents (*Apodemus* spp., *Myodes* spp.) and terrestrial insectivores (*Talpa europaea, Sorex araneus* and *Erinaceus concolor*). The virus is also maintained in vector populations through transovarial and transstadial transmission [217]. The interactions between the virus, vector and vertebrate hosts underscore the enzootic TBEV transmission as one of the most complex arboviral cycles [261]. TBEV can alter the tick's questing behavior, making the vector more tolerant to repellents [262]. The virus can also cross the placenta of some rodents and, possibly, infect their offspring through maternal milk [263]. Vertical transmission of TBEV was also reported in passerine birds, although the role of birds in TBEV transmission remains unclear [260]. Uninfected ticks can acquire virions while co-feeding with TBEV-infected ticks without systemic viraemia in the vertebrate host (non-viraemic transmission). This transmission mechanism makes ticks both vectors and reservoirs, while the mammal host becomes a transient host [264,265]. Human TBE cases are widespread in central and northern Europe [259]. Most of these infections are acquired through tick bites, though outbreaks caused by consumption of raw milk and dairy products from infected livestock are not rare [266,267]. Large animals (e.g., ungulates) can influence spatial and temporal distribution of TBE foci indirectly by sustaining tick populations [268].

Crimean-Congo hemorrhagic fever virus
(CCHFV, *Nairoviridae*: *Orthonairovirus*)

Crimean-Congo hemorrhagic fever (CCHF) is probably the most important tick-borne viral disease of humans. The virus was first discovered at the end of World War II in the Crimean region of the former Soviet Union [269]. Despite its name, the virus has a very wide distribution, reaching more than 30 countries across southern Asia, the Middle East, southeastern Europe and Africa [270,271]. Infected humans show a wide spectrum of symptoms, from mild fever to multi-organ failure and hemorrhage, generally leading to fatality in ~30% of cases [269].

Typical for tick-borne viruses, the complex ecology of tick vectors is reflected in the maintenance cycle of CCHFV. Ticks are both vectors and reservoirs of CCHFV,

being able to maintain the virus by transovarial, transstadial and rarely, by venereal transmission [272]. A transient viraemia in vertebrate hosts makes CCHFV available to uninfected ticks. Furthermore, due to the aggregated patterns of tick infestation, uninfected ticks may acquire the virus by co-feeding with CCHFV-infected ticks, while the host remains non-viraemic [273]. Noteworthy is the potential of CCHFV for nosocomial outbreaks among medical staff caring for CCHF patients [270].

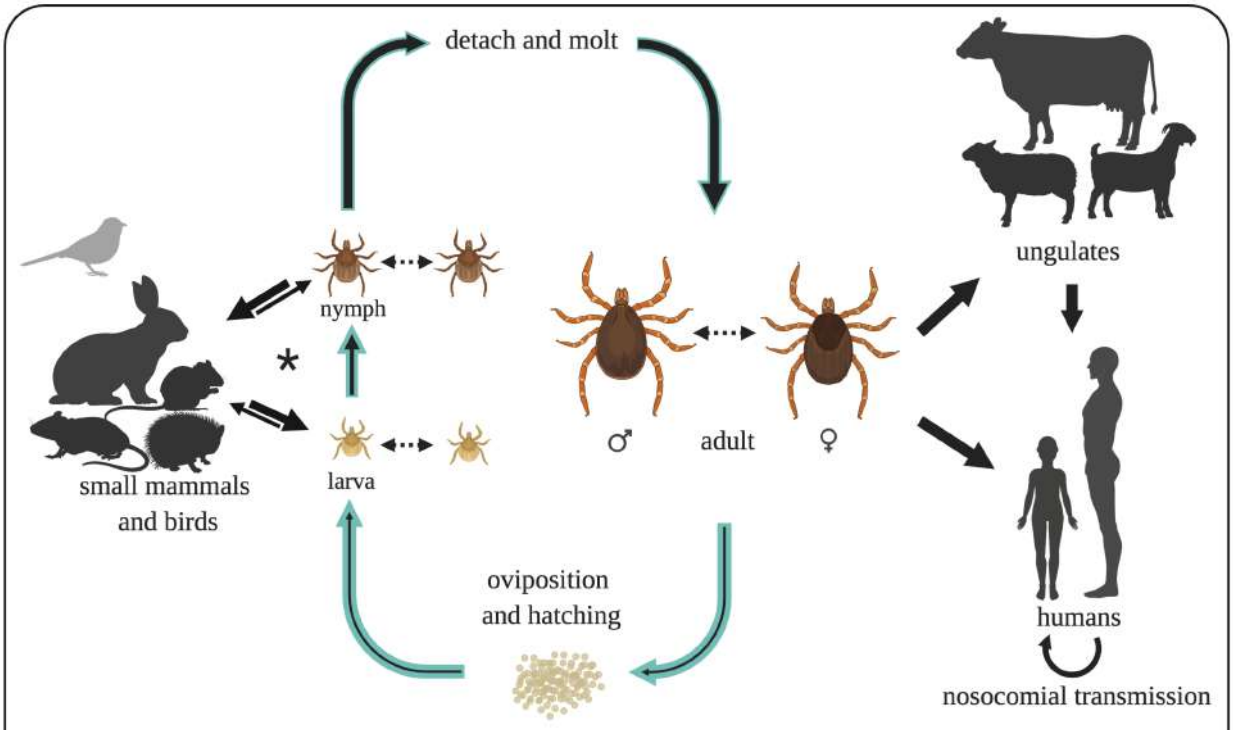


Figure 7. Transmission of CCHFV by *Hyalomma* ticks. Green arrows indicate tick life cycle. Larvae remain attached on small host and molt (two-host tick) or detach and molt (three-host ticks) (transition marked by asterisk). Nymphs follow a similar pattern. Adults feed on large hosts. Black arrow indicates the efficiency of virus transmission between ticks and mammals (solid) or between co-feeding ticks (dashed). Humans are infected by tick bite or exposure to bodily fluids of viraemic vertebrates. (source: Bente et al. [269])

The main vectors of CCHFV are *Hyalomma* ticks [269], but transmission was reported also in other tick genera (e.g., *Rhipicephalus* or *Dermacentor*) [274]. Research into the diversity of vertebrate hosts has been more strenuous, mostly because many vertebrates develop a transient viraemia without visible illness. Thus, the identification of potential CCHFV hosts relies largely on specific antibodies collected from livestock and wildlife [271]. A distinct host preference was observed by Spengler and Estrada-Peña [275] between adult and immature *Hyalomma* spp. Immatures feed

largely on rodents, lagomorphs and birds, while the adults are associated with large ungulates and, to a lesser extent, with lagomorphs and swine. The hosts tightly associated with *Hyalomma* immatures (i.e. small mammals, but not birds) are known to develop variable/transient viraemia and drive CCHFV transmission [276]. The main vector, *Hyalomma marginatum*, is known for its ecological plasticity and preference for birds may enable the expansion of its geographic range. There are several possible scenarios regarding the migration of expansion of ticks via migratory hosts. Infected ticks can be imported into new areas and infect local hosts. Uninfected ticks may also establish new populations and sustain local transmission upon subsequent introduction of the virus or if the area is already endemic [277]. Indeed, sampling of ticks along migration routes has revealed the presence of the *Hyalomma* ticks on migratory birds [156,165] and on some occasions, the vectors where positive for CCHFV or other pathogens [160,162,278].

1.3.3 *Culicoides*-borne viruses

***Bluetongue virus* (BTV, *Reoviridae: Orbivirus*)**

BTV is the type species of the *Orbivirus* genus, having so far 28 documented serotypes (BTV1-28) of varying virulence [279,280]. The segmented genome is prone to genetic reassortment and recombination [79,281] and serotypes circulating in livestock can interact with live vaccines leading to new genetic combinations [282]. The last serotype described to date (BTV28) was detected after vaccine contamination [280].

The bluetongue disease was first described in the early 1900s in South Africa after European sheep were introduced in the late eighteenth century [217]. BTV is transmitted between ruminants by competent *Culicoides* midges [283], but seminal shedding in viraemic rams and bulls, transplacental and contact transmission were demonstrated for some serotypes [217,279,280]. Sheep are known to be the most affected hosts of BTV; other domestic or wild ruminants are often asymptomatic potential reservoir hosts [284,285]. Some carnivores may become infected with BTV after eating infected meat [286], something that was also reported for African horse

sickness virus (AHSV), another *Culicoides*-bornes orbivirus causing disease in equids [287,288].

The most important vector of BTV in Africa, southern Europe and the Middle East is *Culicoides imicola*. In northern Europe, potential vectors of BTV are *Culicoides chiopterus*, *Culicoides obsoletus*, *Culicoides scoticus*, *Culicoides dewulfi* (Obsoletus complex) or *Culicoides pulicaris* and *Culicoides punctatus* (Pulicaris complex) [129]. The spread of bluetongue disease is also influenced by wind, as passive dispersal of competent vectors [289,290]. Wind-enabled dispersal can also be the key to colonisation of new areas. One such example is the spread of the main BTV vector, *Culicoides imicola*, in the Mediterranean region [291,292].

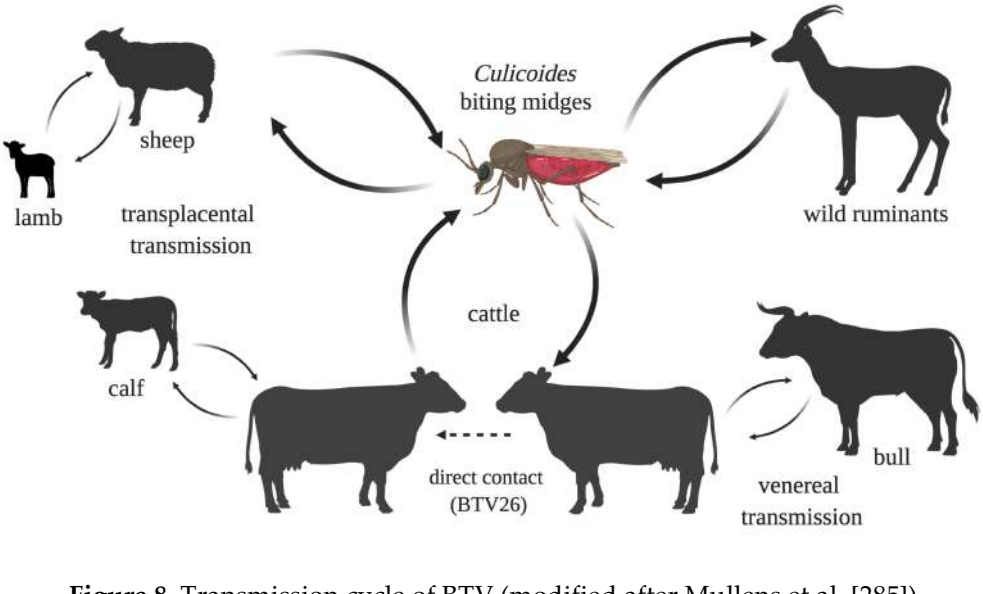


Figure 8. Transmission cycle of BTV (modified after Mullens et al. [285])

***Schmallenberg virus* (SBV, *Perybuniaviridae*: *Orthobunyavirus*)**

SBV is a member of the Simbu serogroup, a diverse orthobunyavirus group with a worldwide distribution and of major veterinary importance [293]. It is the first member of this serogroup to emerge in Europe and it was discovered during the summer of 2011 in dairy cattle near the German-Dutch border [132]. Infection in adult ruminants results mainly in mild clinical signs (e.g., fever, temporary decrease in milk production). When dams are infected during gestation, SBV infection often leads to abortion, stillbirth or severe congenital musculo-skeletal and neurological malformations [294]. Soon after the first outbreak, SBV range expanded rapidly across

southern, western and central Europe [295,296]. In temperate regions, the virus emerges periodically and several *Culicoides* species were found SBV-positive. In France, the virus was detected in nine *Culicoides* species, most of which are also BTV vectors [297]. Similar results were reported in Italy [298] and Poland [299], where SBV was found also in nulliparous midges, suggesting vertical transmission [300]. The rapid spread of SBV into new areas inhabited by different *Culicoides* spp. is very similar to the northward spread of BTV, suggesting rapid adaptation of the virus to new populations of vectors [301].

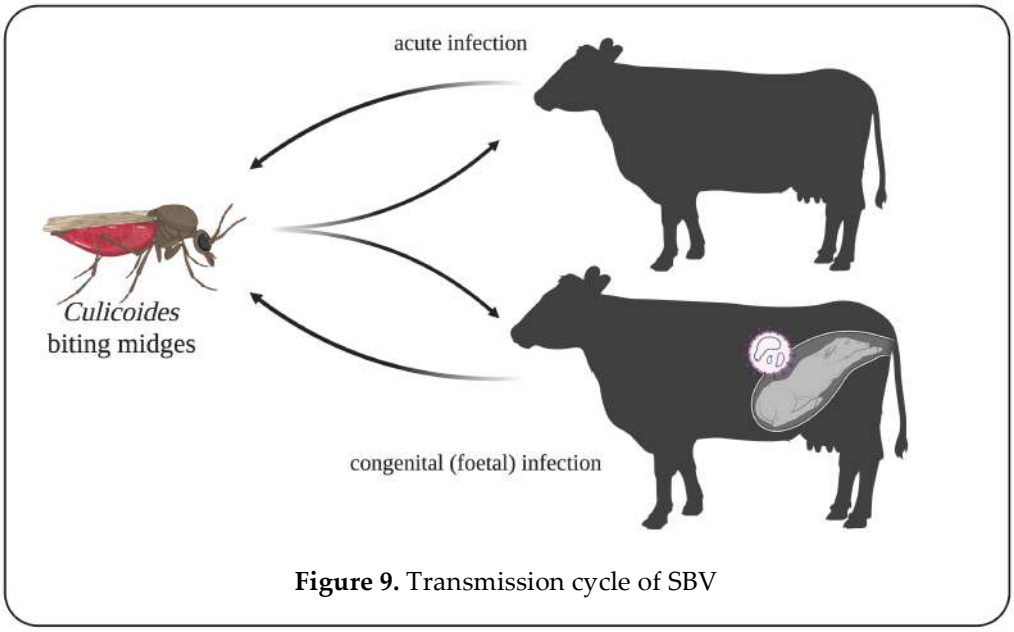


Figure 9. Transmission cycle of SBV

1.4 The importance of vector feeding patterns

In the natural transmission cycle, the arthropod vector becomes the link between host and virus during blood feeding. The vector's necessity for blood meals is satisfied on a multitude of vertebrate species, wild and domestic, some of which can be unknown arbovirus amplifiers [302]. The contact frequency between vectors and hosts has a direct impact on the pathogen's ecology, amplifying or diluting transmission in relation to vector host-preference, host susceptibility to infection and relative abundance [219,303,304]. For these reasons, blood-feeding studies of disease vectors are needed in order to estimate the efficiency and probability of pathogen transmission. To this end, research has focused on biting midges that transmit BTV, SBV and avian malaria [305,306], ticks that transmit *Borrelia* spp. [307,308], triatomine

bugs that transmit Chagas disease [309], horse flies that transmit *Besnoitia* spp. [310], tsetse flies that are vectors of African trypanosomes [311], black flies that transmit *Leucocytozoon* spp. [312,313] or sand flies that transmit *Leishmania* spp. and phleboviruses [314,315]. However, the majority of such studies addressed the blood feeding behavior of mosquitoes, owing to their huge diversity, invasive potential and medical importance: *Culex* mosquitoes that transmit WNV [219,316], *Aedes* mosquitoes that transmit DENV and CHIKV [317,318] or *Anopheles* mosquitoes that transmit human malaria [319]. Feeding behavior is very plastic and the preference for certain hosts is generally not a clearly defined trait. Some factors influencing feeding behavior are intrinsic (genetic), like the anthropophily of malaria vectors *Anopheles gambiae* sensu lato (s.l.) [320], or extrinsic (e.g., host infections, availability and reproductive phenology) [321–323]. However, it is more likely that a combination of both intrinsic and extrinsic factors will determine which hosts and how frequent will they be fed upon. In the case of mosquitoes, some species tend to feed predominantly on a certain host group (the primary hosts involved in the enzootic cycle), such as *Culex* mosquitoes feeding on birds [324] or *Aedes* mosquitoes biting mammals [325]. Even strong preferences are overruled when environmental or physiological factors compel the vector to feed on the hosts readily available (e.g., potential secondary hosts). Feeding patterns that shift from a primary host to a secondary host can increase the probability of a pathogen escaping its primary enzootic cycle into a secondary cycle (**Figure 1**); in this scenario, an enzootic vector becomes a bridge-vector and the spillover of pathogens in the community of secondary hosts can initiate an epidemic or epizootic [326,327]. These shifts in host selection can be driven by natural factors, like migration of primary hosts (e.g., bird hosts of *Culex* spp.) [304], though often they are initiated also by human modifications of the landscape [14,15].

Host-feeding studies in Europe have focused mostly on mosquitoes [108,316,328–335]. Similar analyses of biting midges were motivated by outbreaks of BTV and SBV in the last 10-15 years [137,336–343]. Comparatively, fewer such studies exist for sand flies [344–349], ticks [307,308,350,351] or black flies [312,313].

Furthermore, the majority of these studies were conducted in the western half of Europe, for example in the north where black flies are abundant or in the Mediterranean basin where sand fly-borne *Leishmania* spp. and phleboviruses are endemic. In the central and eastern parts of the continent, blood feeding studies are still scarce and they were conducted on populations of biting midges from Bulgaria [352,353] and Serbia [354], mosquitoes from Serbia and Hungary [355,356] or various vectors from Czech Republic [343,357]. There are still regions or countries where data on this aspect of vector ecology is not available or is outdated. This is also the situation for Romania, where host-feeding data is largely missing. The only blood meal analysis available was conducted by Fălcuță et al. [358], who used serological tools to detect the mammal hosts of *An. maculipennis* s.l. in the south of the country. Given the geographic expansion of arboviruses and other vector-borne pathogens, there is an increasing need for eastern and southeastern European countries to generate knowledge about this crucial aspect of vector ecology and integrate it in their surveillance programs.

1.4.1 Xenosurveillance: disease identification using blood meals

When an arthropod feeds on a host, it takes a blood meal that contains the host's infection status and immunological record. Surveillance of pathogens in a population by exploiting the arthropods' hematophagous behavior is a technique named "xenosurveillance". Although the term was recently coined by Grubaugh et al. [359], the concept is not new. Several variations have been used for disease identification. Xenomonitoring consists in surveying pathogens in vectors rather than hosts [314]. When a host is suspected of an infection that is under the sensitivity threshold of available assays, researchers can use xenodiagnosis. This can be performed by feeding a non-infected, competent vector (reared in a sterile conditions) on the host, allowing the pathogen to incubate and reach detectable levels [360,361]. The blood feeding behavior of vectors can also be used to obtain serial blood samples from small vertebrates that are difficult to sample by venipuncture or when

measurements of blood hormones would be artificially influenced by the stress caused during classical procedures [362–364].

Advances in sequencing capabilities are making xenosurveillance increasingly popular for studying the ecology of vectors and associated pathogens. Bitome-Essono et al. [365] found malaria parasites in engorged biting flies (tsetse flies, stable flies and horse flies), while other studies of mosquito blood meals found myxoma virus [330], herpesviruses and papillomaviruses [366], *Plasmodium falciparum*, parasitic worms and Hepatitis B virus [367], H5N1 avian influenza virus [368], *Anaplasma* sp. and Lobuck virus [369]. The blood meal analysis can also trace the recent history of the host's immune responses. Thus, the technique can also be employed using serological tools for detection of specific antibodies [370].

1.5 Study rationale and aims

Romania lies at the geographical center of Europe, being one of the most biogeographically diverse country of the continent [371]. In the easternmost part of the country, the Danube river discharges into the Black Sea creating Danube Delta, Europe's second largest and best preserved wetland. Taken together with the Razim-Sinoe lagoon complex situated the southwestern side, this complex of ecosystems was designated the Danube Delta Biosphere Reserve (DDBR) and listed as a World Heritage Site in 1998 [372]. Located at the 45° parallel north, DDBR is a biodiversity hotspot and an important hub for migratory birds in the Southeastern European flyway of the greater Palearctic - Afrotropical migration system [373,374]. Birds, especially migratory species, are an important group from the perspective of public health. Many species can play role in the transport of pathogens as biological carriers (natural reservoir/amplifying hosts), either as mechanical carriers or as hosts and vehicles of infected ectoparasites [375]. Danube Delta has diverse and abundant vector communities that parasitize indigenous and migrant host species [158,162,206,207,376–378]. Given that arbovirus circulation is mostly focal and a permissive environment conditions their dispersal, the study area is propitious for arbovirus import and emergence.

By analyzing a diverse collection of samples from the eastern part of DDBR between 2014 and 2017, the present work attempted to shed light on the diversity and ecology of some arboviruses and their vectors in several ecosystems of this wetland. To this end, the specific aims of this dissertation were:

1) To study the composition, diversity and phenology of resident mosquito fauna and to obtain information about biting midge species composition in the study area.

2) To detect and characterise arboviruses infecting vectors and some vertebrate hosts. In the case of WNV, an additional aim was to analyze its spatiotemporal dispersal patterns using a phylogeographic approach.

3) To obtain information about feeding patterns of mosquitoes and biting midges by molecular analysis of their blood meals; in the case of mosquitoes, xenosurveillance was used to detect flavivirus-specific host antibodies and complement molecular and computational methods to better understanding WNV ecology in the DDBR.

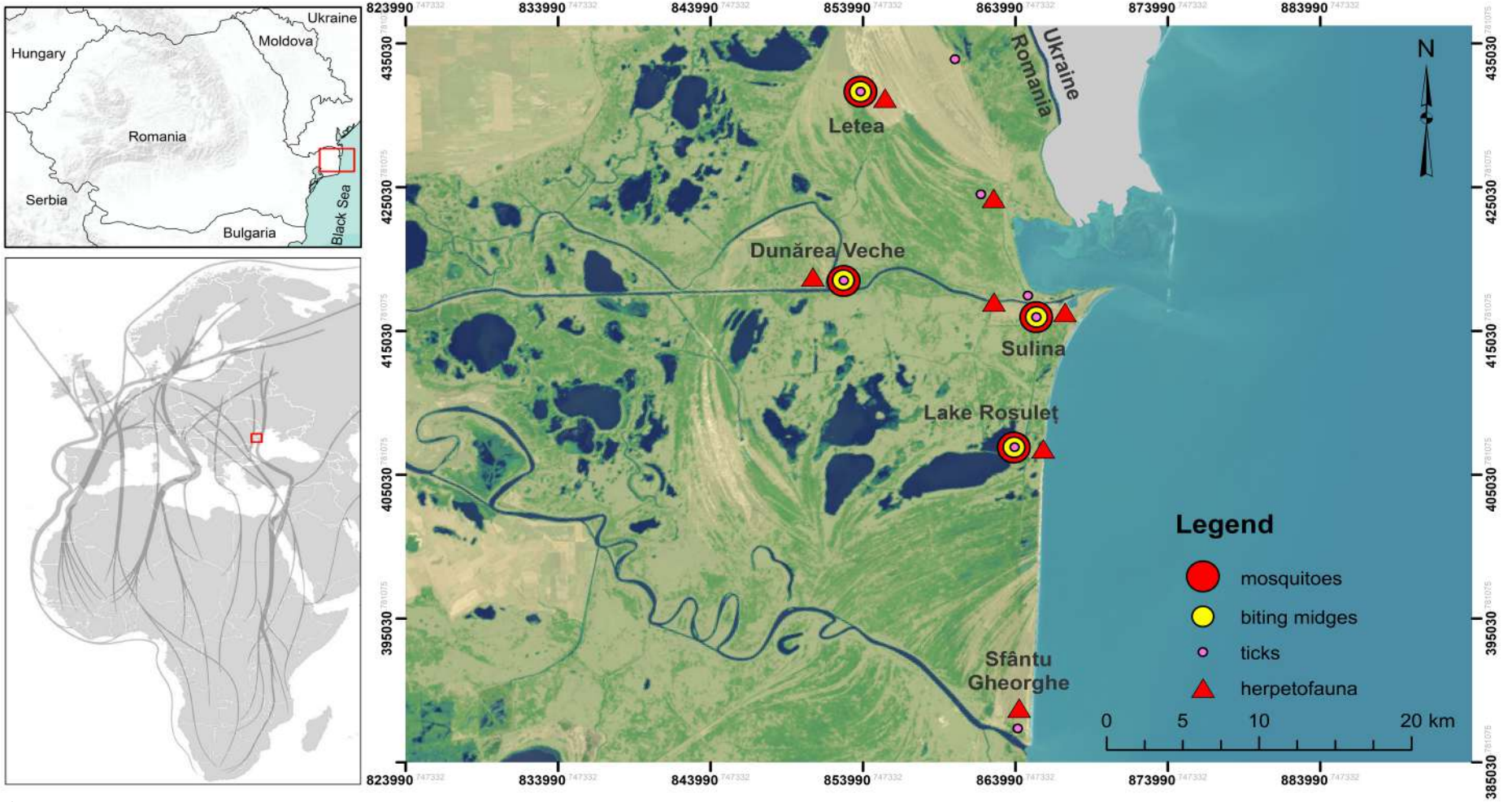


Figure 10. Study area in the Danube Delta Biosphere Reserve (DDBR) with sampling sites used between 2014 and 2017. Vector trapping targeted mosquitoes (2014-2016), biting midges (2017) and ticks (2014-2017). Potential arbovirus hosts (herpetofauna) investigated were *Pelophylax* frogs, European pond turtles (*Emys orbicularis*) (2014-2016) and snakes (*Natrix natrix* and *Natrix tessellata*) (2014-2017). Lower left grey map depicts the African-Eurasian bird migration flyways as described by Bairlein et al. [1]. Red rectangles localize the study area at national (higher left) and continental scales (lower left).

1.6 List of publications

General Discussion is based on the following published articles **(**personal contribution is stated under each research article):

a) Török, E., Tomazatos, A., Cadar, D., Horváth, C., Keresztes, L., Jansen, S., Becker, N., Kaiser, A., Popescu, O., Schmidt-Chanasit, J., Jöst, H., Lühken, R (2016). **Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of *Anopheles algeriensis* Theobald, 1903 and *Aedes hungaricus* Mihályi, 1955 for Romania**. *Parasites & Vectors, 9:196.*

- I participated in study design, performed fieldwork, participated in sample processing, collected statistical data and wrote parts of the Methods section.

b) Tomazatos, A., Jansen, S., Pfister, S., Török, E., Maranda, I., Horváth, C., Keresztes, L., Spînu, M., Tannich, E., Jöst, H., Schmidt-Chanasit, J., Cadar, D., Lühken, R. (2019) **Ecology of West Nile Virus in the Danube Delta, Romania: Phylogeography, Xenosurveillance and Mosquito Host-Feeding Patterns**. *Viruses, 11:1159.*

- I participated in study design, performed fieldwork, host identification by PCR and antibody screening by immunofluorescence (xenosurveillance); I participated in sample processing, virus screening by PCR, data analysis and manuscript drafting.

c) Tomazatos, A., Jöst, H., Schulze, J., Spînu, M., Schmidt-Chanasit, J., Cadar, D., Lühken, R. (2020) **Blood-meal analysis of *Culicoides* (Diptera: Ceratopogonidae) reveals a broad host range and new species records for Romania**. *Parasites & Vectors, 13:79.*

- I designed the study, performed fieldwork, morphological identification of insects and sample processing; I participated in PCR barcoding, I analyzed the data and drafted the manuscript.

d) Tomazatos, A., Marschang, R.E., Maranda, I., Baum, H., Bialonski, A., Spinu, M, Lühken, R., Schmidt-Chanasit, J., Cadar, D. (2020) **Letea Virus: Comparative Genomics and Phylogenetic Analysis of a Novel Reassortant Orbivirus Discovered in Grass Snakes (*Natrix natrix*)**. *Viruses, 12:243*

- I participated in study design, performed fieldwork and identified the biting midges and ticks. I participated in tick processing and the initial cell culture experiments. I performed the screening of biting midges by PCR, analyzed the data and drafted most of the manuscript (without the Sequencing subsection of Methods)

The Additional Chapter 2 is based on unpublished results (supplementary material for the original manuscript is available in **Appendix**).

e) Tomazatos, A., von Possel, R., Pekarek, N., Holm, T., Rieger, T., Baum, H., Bialonski, A., Maranda, I., Erdelyi-Molnár, I., Spînu, M., Lühken, R., Jansen, S., Emmerich, P., Schmidt-Chanasit, J., Cadar, D. **Discovery and Genetic Characterization of a Novel Orthonairovirus Infecting *Ixodes ricinus* Ticks from Danube Delta.**

(submitted manuscript)

- I participated in the study design, performed fieldwork and identified most of the ticks. I participated in sample processing and virus screening by PCR; I analyzed the data and drafted most of the manuscript (without the Sequencing and Serology subsections of Methods).

Alexandru Tomazatos

Prof. Dr. med. Dr. med. habil. Jonas Schmidt-Chanasit, M.D.

2 Additional Chapter: Discovery and Genetic Characterization of Sulina Virus, a Novel Orthonairovirus Infecting *Ixodes ricinus* Ticks from Danube Delta

(submitted manuscript)

2.1 Background

The unique biology of ticks (Ixodida) enables the transmission of a large variety of pathogens, among which a consistent part is represented by arthropod-borne viruses (arboviruses). Of particular interest is the genus *Orthonairovirus* (family *Nairoviridae*, order *Bunyavirales*), a group almost exclusively vectored by ticks and which comprises some of the most important tick-borne pathogens [379]. To date, 43 orthonairoviruses are officially classified into 15 viral species [380], with additional taxa described by recent work [381,382].

The genome of orthonairoviruses is a tri-partite, negative-sense single-stranded RNA (-ssRNA) genome, consisting of a small segment (S) encoding the nucleocapsid (N), a medium segment (M) encoding a glycoprotein precursor (GPC) and the large segment (L), longest of all bunyaviruses, encoding an RNA-dependent RNA polymerase (RdRp) [383].

Most of orthonairoviruses are maintained in arthropods or transmitted by ticks to rodents, bats, eulipotyphla and birds [146]. Crimean-Congo hemorrhagic fever virus (CCHFV) is the most prominent orthonairovirus and causes probably the most important tick-borne viral disease of humans. It occurs in about 30 countries from Africa and Eurasia and can cause a high mortality rate (usually up to 40%) [270]. The most important orthonairovirus of veterinary interest is Nairobi sheep disease virus (NSDV, also known as Ganjam virus (GANV) in India), which causes highly lethal hemorrhagic gastroenteritis in small ruminants of Africa and India [384]. Despite the medical relevance of orthonairoviruses, their diversity and potential of emergence, only recently has sequence data become available [146,385,386] for a better understanding of the genus' evolution and ecology (e.g. relation within the large

Bunyavirales order and associations with hosts and vectors). Most of our understanding on orthonairovirus structure and function comes from studies of pathogens like CCHFV [387–390]. Fewer studies addressed these matters in other orthonairoviruses (e.g. Hazara virus, HAZV) [391–393] or used a comparative approach [394–396].

Very little is known about tick-borne viruses in Romania. Most of the available data was produced by serosurveys from the 1970s until early 1990s [168,169,179,397–399]. During this period, several tick-borne viruses were isolated (e.g. Tick-borne encephalitis virus, TBEV; Kemerovo group orbiviruses; Bhanja virus) [169,172,398,399]. The presently available data indicate that orthonairoviruses have not been detected or isolated in Romania. Ceianu et al., [400] reported the only data pertaining to orthonairovirus circulation, observing a high seroprevalence (>27%) of anti-CCHFV antibodies in sheep from northern Dobrogrea and bordering areas of the DDBR. The biogeographical conditions and habitat suitability are known to be conducive to the establishment of tick-borne virus foci [401], especially in the southern areas of Romania, where vectors reach high abundance and diversity [203]. Furthermore, most neighboring countries and some in regional proximity (e.g. Serbia, Bulgaria, Ukraine, Kosovo, Greece, Turkey) are known for a wider circulation of tick-borne viruses and endemicity of CCHFV [153,402–406].

As part of a longer program of arbovirus discovery and surveillance program, we subjected to transcriptome sequencing ticks collected from their hosts in rural and natural sites of DDBR. A previously unknown orthonairovirus, provisionally designated Sulina virus (SULV), was detected in *Ixodes ricinus* ticks from two rural sites. Here we report the predictive genetic and protein analysis, inferred phylogenetic relationship with the *Orthonairovirus* genus and an evaluation of its infection potential in vertebrates.

2.2 Materials and methods

2.2.1 Tick sampling and processing

A batch of 385 ixodid ticks collected directly from their hosts in several sites of DDBR in 2016 (**Figure 10**) was subjected to bulk RNA-sequencing. An additional cohort comprising 930 bloodfed ticks collected in the same manner in 2014, 2015 and 2017 was included in the study for serological analysis (**Table S1**). Most of the parasites were collected from domestic hosts. A small set of ticks (2016) was obtained from wild birds during a migration study conducted in Sfântu Gheorghe [373]. Ticks of each host were collected in separate vials, frozen in the field and transported on dry ice. In the laboratory, samples were stored at -80°C and identified on chill tables using morphological keys [407,408].

For homogenization, single ticks were first placed into a sterile 2 mL Eppendorf tube with two 5 mm steel beads and kept in liquid nitrogen for 1 min. The samples were loaded into a TissueLyser (Qiagen, Hilden, Germany) and the frozen ticks were pulverized at 50 Hz for 4 min. To each sample we added 0.6 mL of high-glucose (4.5 g/L) Dulbeco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich, St. Louis, USA) with L-glutamine, 12.5% head-inactivated fetal bovine serum, 100 μg/mL streptomycin, 100 μg/mL penicillin and 100 μg/mL amphotericin B. The final mix was homogenized using the TissueLyser at 50 Hz for 2 min and clarified by centrifugation (10,000 rpm/2 min) at 8 ◦C.

2.2.2 Sulina virus discovery and genome sequencing

The tick collection of 2016 was subjected and processed for next-generation sequencing, as described elsewhere [409]. Briefly, the tick homogenates were filtered through 0.45 μm pore-sized columns to reduce the volume of bacteria and other contaminant and were treated with a mixture of nucleases (Turbo DNase, Ambion, Carlsbad, CA, USA; Baseline-ZERO, Epicenter, Madison, WI, USA; Benzonase, Novagen, San Diego, CA, USA; RNAse One, Promega, Fitchburg, WI, USA) to digest unprotected nucleic acids including host DNA/RNA. Viral RNA/DNA was extracted

with the MagMAX Viral RNA Isolation Kit (Thermo Fisher) according to the manufacturer's instructions. After random RT-PCR amplification, the extracted viral nucleic acids were subjected to library preparation using a QIAseq FX DNA Library Kit (Qiagen, Hilden, Germany) and sequenced using (2 × 150 bp paired-end) MiSeq Reagent Kits v3 (Illumina, San Diego, CA, USA) on a MiSeq platform. Raw reads were first trimmed and filtered to remove polyclonal and low-quality reads, de novo assembled and compared with a non-redundant and viral proteome database using BLASTx with a cut-off E-value of 0.001. The virus-like contigs and singlets were further compared to all protein sequences in non-redundant protein databases with a default E-value cutoff of 0.001.

2.2.3 Prevalence of Sulina virus in ticks

For the prevalence of the Sulina virus, we screened aliquots of the individual homogenates using an RT-PCR assay with the specific primers DD352F: ACCTCCGTCATTGCCTGTGT and DD352R: TGAGGTTCCCGACACACCAC, using a Superscript III one-step RT-PCR kit (Invitrogen, Carlsbad CA, USA). Amplification reactions were performed in a volume of 10.8 μL containing 3 μL sample, 4 μL reaction mix, 0.5 μL Mg_2SO_4 (0.25 μmol), 1 μL ddH_2O, 1 μL of each primer (10 pmol) and 0.3 μL Enzyme Mix. Reverse transcription at 60 ◦C for 1 min, 50 ◦C for 45 min and 94 ◦C for 2 min was followed by 45 cycles of amplification at 94 ◦C for 15 sec, 55 ◦C for 30 sec and 68 ◦C for 30 sec. Final extension was performed at 68 ◦C for 7 min.

2.2.4 Virus isolation

30 μL of the supernatant from the positive pools were inoculated into 24 wells cell culture plate with monolayer cultures of C6/36 and Vero cells. The cultures were maintained at 28 °C and 37 °C respectively, for 6–7 days and examined every 2 days for evidence of viral cytopathic effect (CPE). This procedure was repeated until passage 4. Supernatant was subjected to Sulina virus-specific RT-PCR assay and selected positive homogenates were used for intraperitoneal inoculation of adult mice, which were checked daily for clinical signs. Following observation, the mice were

euthanized and organs along with sera were harvested aseptically and screened as described in the previous section.

2.2.5 Serological screening of engorged ticks and host sera

2.2.5.1 Cloning, expression and purification of recombinant antigen

A cDNA of the Sulina virus nucleoprotein was amplified by PCR and cloned into the procaryotic expression vector pOPIN-F (N-terminal His tag with 3C protease cleavage site) using the In-Fusion HD EcoDry cloning kit (Clontech). The nucleoprotein of Sulina virus was expressed with N-terminal His-Tag and 3C protease cleavage site in E. coli strain BL21 (DE3) in TB medium with 100 μM carbenicillin at 17 °C overnight after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. After pelleting the cells were re-suspended in lysis buffer containing 80 mM sodium phosphate, pH 7.4, 7 M urea, 150 mM NaCl, 5% glycerol, 10 mM imidazole and 0.1 mM PMSF. Cells were disrupted by sonication and the denaturated proteins were purified by nickel affinity chromatography from the soluble fraction after centrifugation. The protein was eluted in buffer containing 80 mM sodium phosphate, pH 7.4, 4 M urea, 150 mM NaCl, 5% glycerol and 250 mM imidazole. The imidazole was removed by dialysis over night at 4 °C. The purified protein was concentrated using centrifugal filter devices, flash frozen in liquid nitrogen and stored at -80 °C.

2.2.5.2 Enzyme-linked immunosorbent assay (ELISA) for detection of SULV-specific antibodies

For serological testing we included the entire tick collection (2014-2017; n=1305) and blood samples from 39 dogs (2-15 dogs per site) (**Table S1**) obtained in a different, parallel study [410]. The recombinant Sulina virus nucleoprotein was prepared and subjected to ELISA using a protocol published elsewhere [411].

2.2.6 Sequence data analysis

Genome assembly, sequence analysis, genomic organization and multiple alignments were performed using Geneious v9.1.8. Open reading frames (ORFs) were predicted with Geneious v9.1.7 (Biomatters, Auckland, New Zealand) and ORFfinder

[412]. Putative functions of Sulina virus proteins were assigned by BLASTx searches in GenBank. Protein structure and targeting predictions were generated with NetOGlyc 4.0, NetNGlyc1.0, SignalP 4.1, ProP 1.0 and TMHMM 2.0 of the Center for Biological Sequence Analysis portal [413], PredictProtein [414], InterProScan database [415] accessed through Geneious and the HMMER web server [416].

Protein sequences of representatives from recognized orthonairovirus species sensu International Committee on Taxonomy of Viruses (ICTV) [380] were downloaded from GenBank (**Table S2**) and aligned with Sulina virus homologs using the MAFFT algorithm implemented in Geneious. Alignments of individual genes and concatenated genomes of Sulina virus were scanned with Simplot v3.5.1 [417] and RDP package v4.95 [418] for detection of potential recombination and reassortment events. Recombination search was performed with default settings, i.e. 200bp window size and a Bonferroni correction of the *p*-value of 0.01. Results were further considered if significant signal of recombination was obtained with at least three different methods in RDP.

Evolutionary relationships of Sulina virus within the *Orthonairovirus* genus were analyzed by constructing phylogenetic trees for each of the three proteins (N, GPC and RdRp). Using the Akaike information criterion (AIC) computed by Prottest v3.4.2 [419] and MegaX [420], we found that the best-fit amino acid substitution models are LG+Γ+I for nucleocapsid (N) and glycoprotein precursor (GPC), respectively LG+Γ+I+F for the RNA-dependent RNA polymerase (RdRp). We built phylogenetic trees by maximum likelihood in PhyML 3.0 [421], with Subtree Pruning and Regrafting (SPR) branch-swapping and approximate likelihood ratio test (aLRT) for assessment of specific node support. The maximum likelihood trees were compared to a second set of phylogenies inferred by Bayesian Monte Carlo Markov Chain (MCMC) sampling method implemented in BEAST v1.10.3 [422]. The models were run for 10^7 generations, with sampling every 10^4 steps and 10% burn-in. The resulting trees were summarized with TreeAnnotator v1.10.1 [423] and the phylogenies were displayed with FigTree v1.4.3 [424]. Pairwise amino acid sequence

identity was plotted as color-coded matrix with Sequence Demarcation Tool (SDT) v1.2 [425].

2.3 Results

2.3.1 Tick identification, molecular and serological screening of Sulina virus

We identified six taxa in the samples collected in 2016 from Sulina (29.9%), Letea (27%), Dunărea Veche (26%), Sfântu Gheorghe (8.1%), Lake Roșuleț (3.4%), Sfiștofca (4.2%) and Sulina Levee (1.6%) (**Figure 10**). A seventh taxon, *Dermacentor marginatus* (n=2) was found in 2017 on dogs and was included in the serological screening only (**Table S1).** The most abundant group screened was *Rhipicephalus* spp. (n=298, 77.4%), followed by *Ixodes ricinus* (n=57, 14.8%), *Hyalomma marginatum* (n=21, 5.5%) and *Dermacentor reticulatus* (n=9, 2.3%) (**Table 1**).

Table 1. Ticks collected in DDBR (2016) and screened by SULV-specific RT-PCR, with information on host species and number of SULV-positive *Ixodes ricinus*.

Host/Tick sp.	*Dermacentor reticulatus*	*Hyalomma marginatum*	*Ixodes ricinus*	*Rhipicephalus annulatus*	*Rhipicephalus rossicus*	*Rhipicephalus sanguineus* s.l.
Bos taurus			1	63		3
Canis l. familiaris	7		48 (19)		95	132
Equus caballus				3		
Homo sapiens	1		1			1
No host (unattached)	1	4	5 (1)			
Acrocephalus palustris		2				
Ficedula albicolis		1				
Iduna pallida		1				
Ixobricus minutus		2				
Lanius collurio		2				
Phoenicurus phoenicurus		1				
Saxicola rubetra		1				
Sturnus vulgaris			1			
Sylvia atricapilla		1				1
Sylvia communis		4	1			
Sylvia curuca		1				
Turdus merula		1				
Total = 385 (20)	**9 (0)**	**21 (0)**	**57 (20)**	**66 (0)**	**95 (0)**	**137 (0)**

Upon detection of SULV by NGS, the individual tick homogenates were screened by RT-PCR revealing that 20 of 57 *I. ricinus* were positive for Sulina virus RNA (35%, 5.2% of the 2016 collection). Of these positive ticks, 19 individuals were collected from domestic dogs in Sulina and one specimen was found without a host in Sfântu Gheorghe.

Attempted isolation failed on Vero E6, C6/36 cells, as well as in mice. The ELISA test of the larger sample collection (2014-2017) did not find specific antibodies in blood meals of ticks or blood of domestic dogs.

2.3.2 Sulina virus genome

We obtained the complete genome sequences of three Sulina virus strains, which exhibited a typical bunyaviral, negative-sense (ss)RNA genome with three single-ORF genes of different lengths. The S gene is 1,512 bp long, the M gene is 4,092 bp long and the L gene has 11,823 bp. As in other orthonairoviruses, the S segment is coding for a nucleocapsid (N), the M segment encodes a glycoprotein precursor (GPC) and the RNA-dependent RNA polymerase (RdRp) is encoded by the L segment. BLASTx searches of SULV ORFs found the highest similarity with Kasokero virus (KASOV) for the nucleocapsid (~34.9%) and with Tamdy virus for glycoprotein precursor (~34.4%) and RdRp (~45.4%). Complete coding sequences for SULV generated in the present study were deposited in GenBank with the accession numbers MT263282-263290.

2.3.2.1 Nucleocapsid protein (N)

The putative N protein of SULV is 503 aa long, having a size similar with the other homologs included in our analysis (479-516 aa). It differs substantially from Erve virus (ERVEV) and Thiafora virus (TFAV), which have long C-terminal extensions (630 aa and 673 aa, respectively). The molecular weight (57.6 kDa) is also close to the average known for orthonairoviruses (53kDa). In –ssRNA viruses the nucleocapsid is responsible for RNA binding and oligomerisation, forming ribonucleoprotein complexes that are associated with the RdRp for viral RNA synthesis and encapsidation [388]. The N protein of CCHFV is also known to interact with host

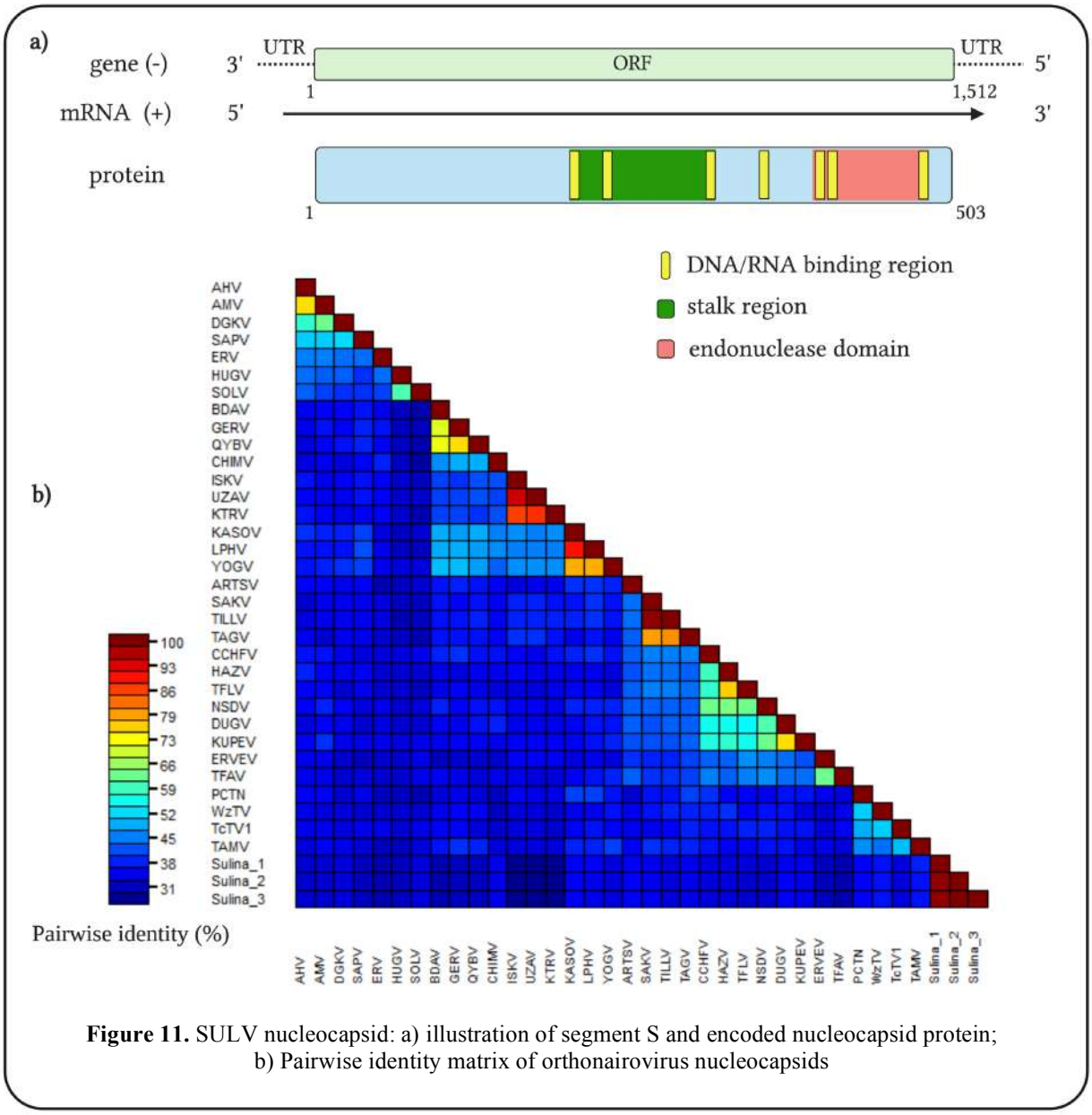


Figure 11. SULV nucleocapsid: a) illustration of segment S and encoded nucleocapsid protein; b) Pairwise identity matrix of orthonairovirus nucleocapsids

antiviral defense factors [426]. Although CCHFV provides much of the structural knowledge of the nairoviral nucleocapsid protein [388,390], the structures of ERVEV, HAZV and KUPEV have also been published [395]. These studies found that the N protein has two major domains: a globular head and an extended stalk. As in the case of CCHFV nucleocapsid [390], SULV was predicted to have two regions active in RNA binding **(Figure 11a**, **Figure S1**, **S2**). First region is located at mid-point, corresponding to stalk region of CCHFV and contains residues of positive charge responsible for RNA binding. Sequence alignment revealed fully conserved (K229, Q314) or conservatively changed (H202N) amino acids. The second region is delimited by sites involved in endonuclease activities [395], having most of the binding residues fully

conserved among orthonairoviruses (H390, R398, H467, H470) or conservatively changed (R425K). Also, at the C-terminus of the stalk region is located a proteolitic cleavage site for the apoptosis mediator caspase-3, present in the CCHFV nucleocapsid as the ^{266}DEVD^{269} motif [427]. This specific sequence is present with some changes in other orthonairoviruses species, such as HAZV (^{269}DQ(L/V)D^{272}), or TFAV (^{261}D(I/V)LD^{264}), but is not present in SULV nucleocapsid, neither in most of the analyzed orthonairoviruses. The experiments of Carter et al. [388] identified three CCHFV N residues essential in replicon activity (K132, Q300, K411) and another two residues (K90 and H456) whose mutation significantly reduce N functionality. Although the alignment of SULV N protein showed identical residues corresponding to CCHFV residues K90 and K135, only residues Q300 and H456 were fully conserved among nairoviral nucleocapsids.

Genetic distance matrices computed using a MAFFT algorithm showed identity values of ~25% (ERVEV) to 33% (TAMV) between SULV nucleocapsid and orthonairovirus homologs (**Figure 11b**).

2.3.2.2 Glycoprotein precursor (GPC)

The M segment of orthonairoviruses contains a single ORF encoding a polyprotein that is co- and post-translationally processed into mature glycoproteins of different sizes [383]. The two glycoproteins, Gn and Gc, are responsible for host receptor recognition, hemagglutination process and induction of immune response by the vertebrate host [428]. Consequently, the GPC is the most variable product of the orthonairovirus genome. The GPC of Sulina virus is 1363 aa long (151.6 kDa), similar to GPCs of *Tamdy orthonairovirus* and significantly shorter than that of CCHFV (1700 aa, 188 kDa) (**Figure 12a**). SULV GPC was predicted to have an N-terminal signal peptide between positions 18 and 19 (IWA-SD), followed by a mucin-like domain (MLD). This domain is characterized by high level of *O*-glycosylation (16 sites), similar to that of DGKV (18 sites). Predicted *N*-linked glycosylation were present in relatively large number (15 sites) when compared to other GPCs, fewer only than in SAKV and KTRV (17, respectively 20 *N*-linked glycans). The MLD length is smaller than in other

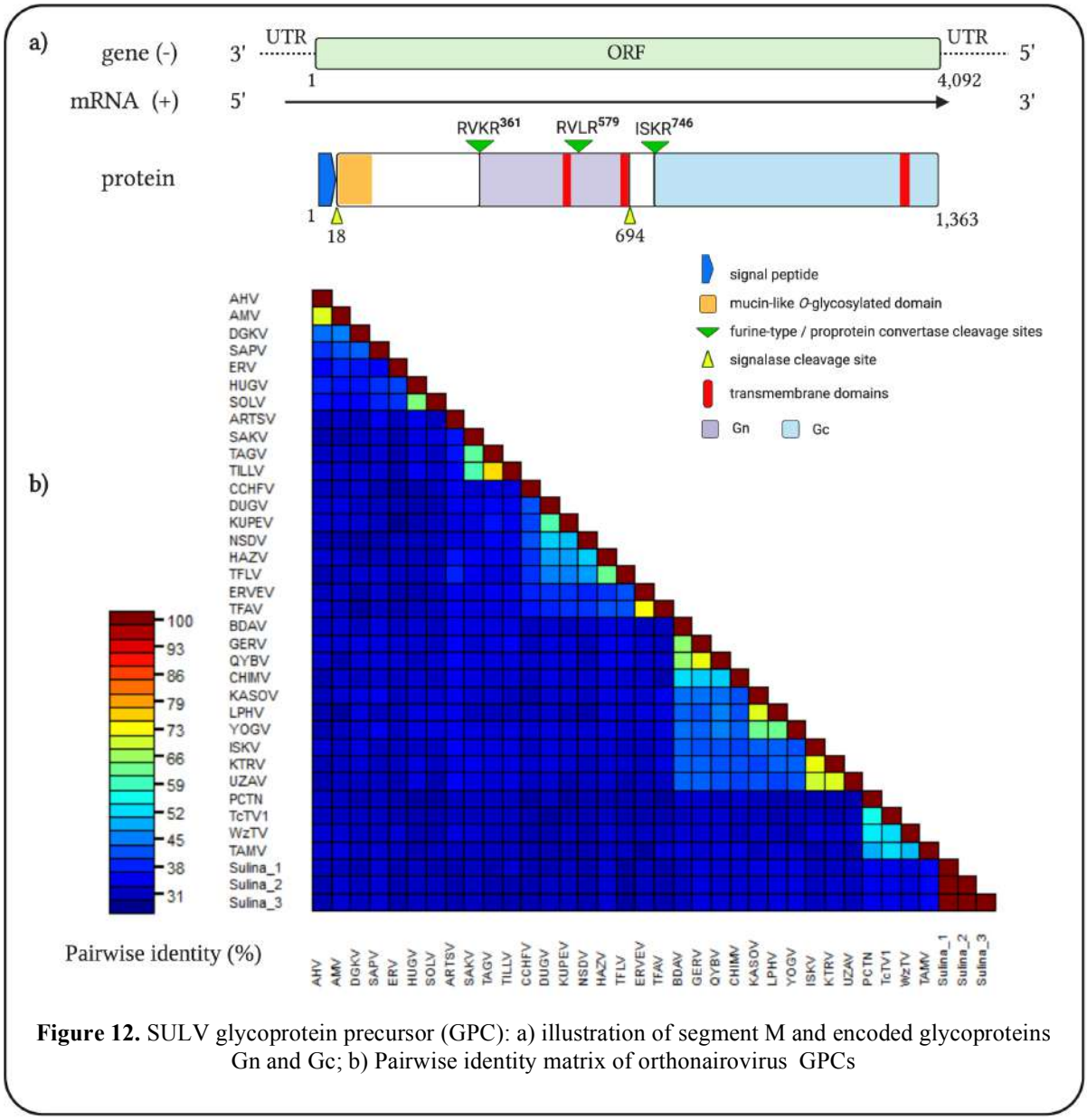


Figure 12. SULV glycoprotein precursor (GPC): a) illustration of segment M and encoded glycoproteins Gn and Gc; b) Pairwise identity matrix of orthonairovirus GPCs

orthonairoviruses (53 aa compared to 56 aa in DGKV or 243 aa in CCHFV). As expected, the MLD showed very low amino acid sequence identity, being located in the most variable region of the polyprotein [383].

GPC is proteolytically processed by a series of signalases, proteases, furin, subtilisin/kexin-isozyme-1/site-1 protease (SKI-1/S1P) and convertases [383]. Predicted proteolytic sites in the GPC of Sulina virus are shown in **Figure 12a**. The number of transmembrane domains in the GPC of orthonairoviruses varies between one (QYBV) and five (CCHFV, HAZV, DUGV). Sulina virus was predicted to have a total of three transmembrane helices: two around the polyprotein's mid-point (C-terminal of Gn), and a third domain at the C-terminus of Gc.

Two zinc finger domains formed by conserved cysteine residues can be observed in orthonairoviruses with similar number and localization of transmembrane domain (**Figure S3**). These structures were shown to be involved in protein-protein interaction, while the double zinc finger located at the C-terminus of Gn (between the transmembrane domains) could interact with ribonucleoproteins for virus assembly and budding [429].

Pairwise identity of SULV GPC is between ~20% (CCHFV) and 30% (Tamdy virus) (**Figure 12b**), lower than what we observed in the other orthonairoviral homologs.

2.3.2.3 RNA-dependent RNA polymerase (RdRp)

All bunyaviruses encode their RdRp on the large (L) segment, the longest of which is found in orthonairoviruses [383]. The L segment of SULV encodes a 3940 aa long polymerase sequence (**Figure 13a**).

Four conserved regions (I-IV) have been identified in the bunyaviral L protein [430]. One particular region (III) is known as the functional core of the polymerase, containing six conserved motifs (premotif A and motifs A-E) [430,431], and was predicted to have catalytic roles in template positioning and priming [430] (**Figure S4**). The order and spacing of the motifs is conserved in all bunyaviral polymerases [431], having the sixth specific motif named premotif A (or motif F) and located upstream from motif A [432]. Region I contain an endonuclease domain, presumably involved in cap-snatching [433], while the role of region II is yet to be described. Two transmembrane domains are located at the C-terminus of this region (**Figure 13a**).

Region IV may have a role in cleaving capped primers and RNA binding [434]. The alignment of SULV L protein with orthonairovirus homologs showed these regions to be highly conserved. The lowest similarity was found in the region I of orthonairovirus RdRp, while the highest similarity was exhibited by region III with its six motifs.

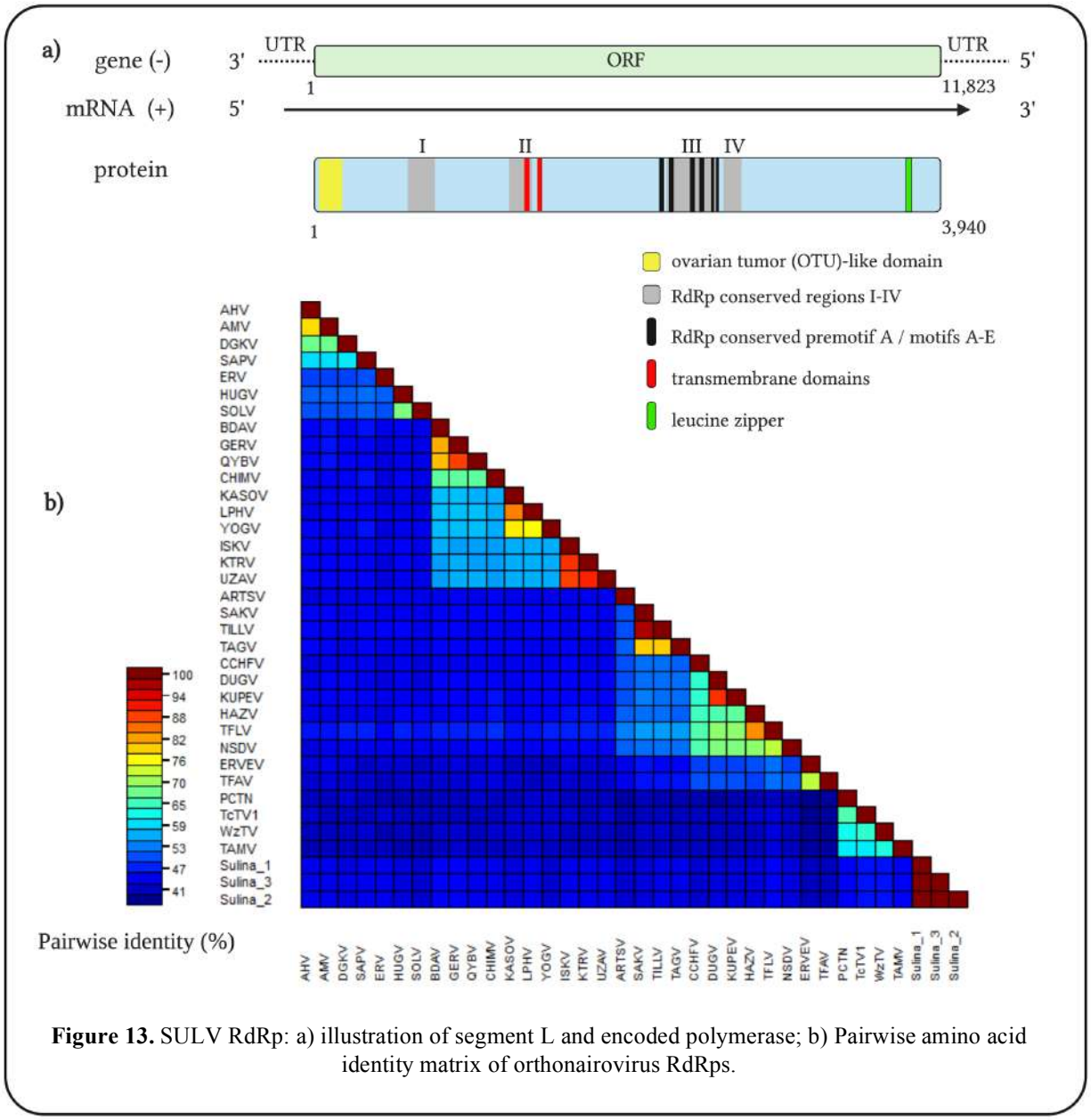


Figure 13. SULV RdRp: a) illustration of segment L and encoded polymerase; b) Pairwise amino acid identity matrix of orthonairovirus RdRps.

Another particularity of the orthonairovirus polymerase that we observed in the L protein of SULV is an N-terminal ovarian tumor (OTU)-like protease domain (**Figure S5**). This structure is found in eukaryotes, pathogenic bacteria and several groups of viruses [435]. Unlike the eukaryotic version of this domain, the OTU-like protease domain of the CCHFV was implicated as a potential virulence factor due to its ability to interfere with host immune regulation and antiviral signaling pathways [394,436]. Its role as a virulence determinant was also inferred from observing the functional differences between CCHFV and the less virulent DUGV [437]. The amino acid sequence alignment showed that most key residues of OTU's catalytic site are shared by all orthonairoviruses [394]: D41, G42, C44, Y91, W101, W121 and H152,

while P39 is present in most species (**Figure S5**). The L proteins of orthonairoviruses are also known to contain leucine zippers, structural motifs of transcription factors [438]. The position of leucine zipper varies in orthonairovirus RdRps. For example, the RdRp of CCHFV contains this structure upstream of region III. In SULV a leucize zipper was predicted at the C-terminal region of the L protein (**Figure 13a**).

The amino acid sequence alignment showed that the highest pairwise amino acid identity between SULV and representative orthonairoviruses ranged from 37% (ERVEV) to ~44% (TAMV) (**Figure 13b**).

2.3.3 Phylogenetic analysis

Both maximum likelihood (**Figures 14-16**) and Bayesian MCMC (**Figure S6a-c**) methods produced phylogenies with very similar topologies and overall strong node support. For each protein, Sulina virus displayed a similar pattern of clustering and formed a well-supported monophyletic clade with *Tamdy orthonairovirus*. The trees were congruent with previous, more extensive phylogenetic analyses of the genus [146,386], as well as with a more recent reorganization of the genus [380].

Although frequent reassortment is known to occur in orthonairoviruses [439,440], we found no evidence for segment reassortment or signals of recombination.

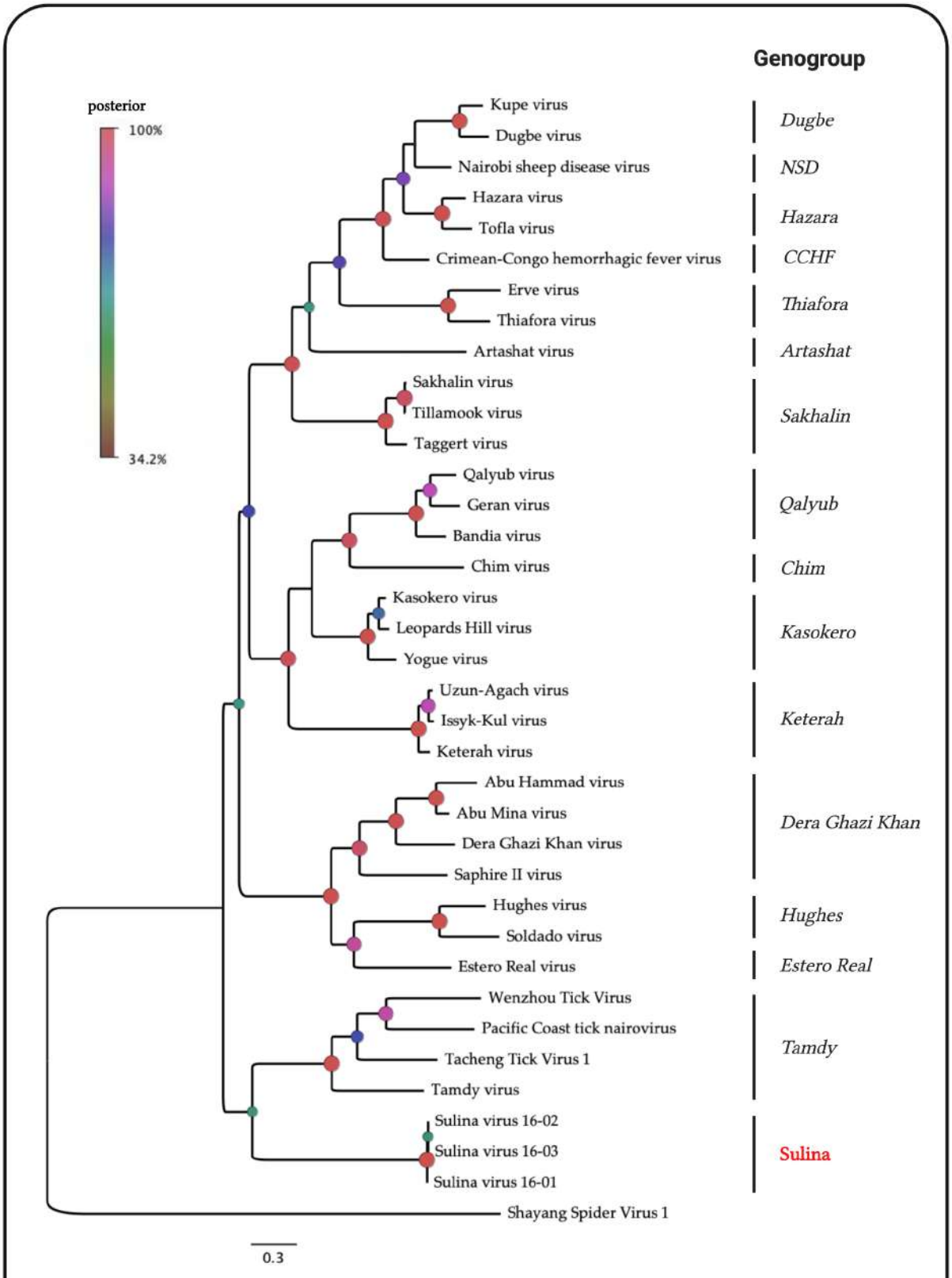


Figure 14. Maximum likelihood phylogenetic tree based on the S protein (nucleocapsid) of representative orthonairoviruses and Sulina virus (in red). The color and size of node circles indicate statistical support by approximate likelihood ratio test (aLRT) in PhyML 3.0. Shayang virus was used as outgroup and the scale bar indicates substitutions per site.

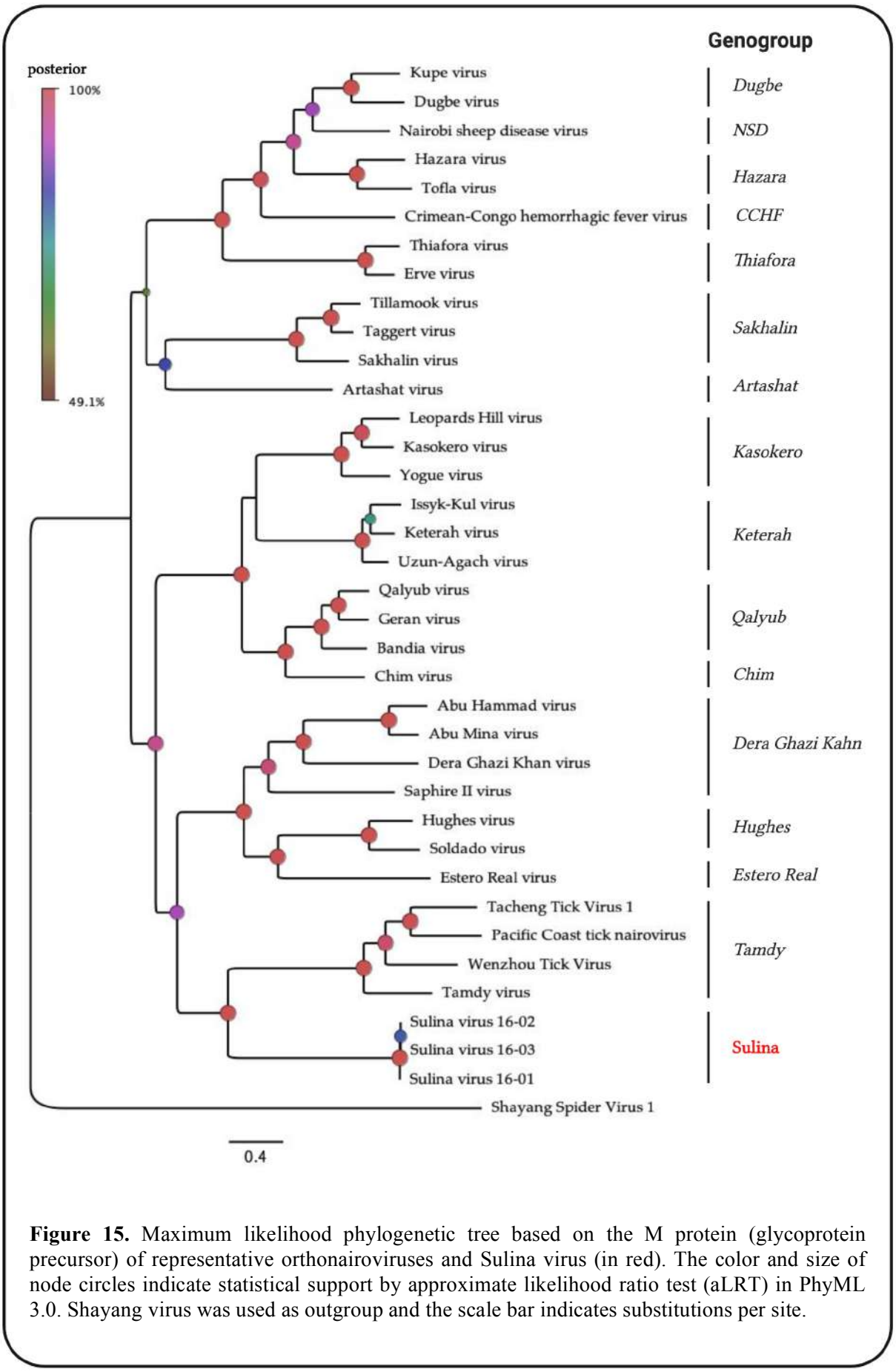


Figure 15. Maximum likelihood phylogenetic tree based on the M protein (glycoprotein precursor) of representative orthonairoviruses and Sulina virus (in red). The color and size of node circles indicate statistical support by approximate likelihood ratio test (aLRT) in PhyML 3.0. Shayang virus was used as outgroup and the scale bar indicates substitutions per site.

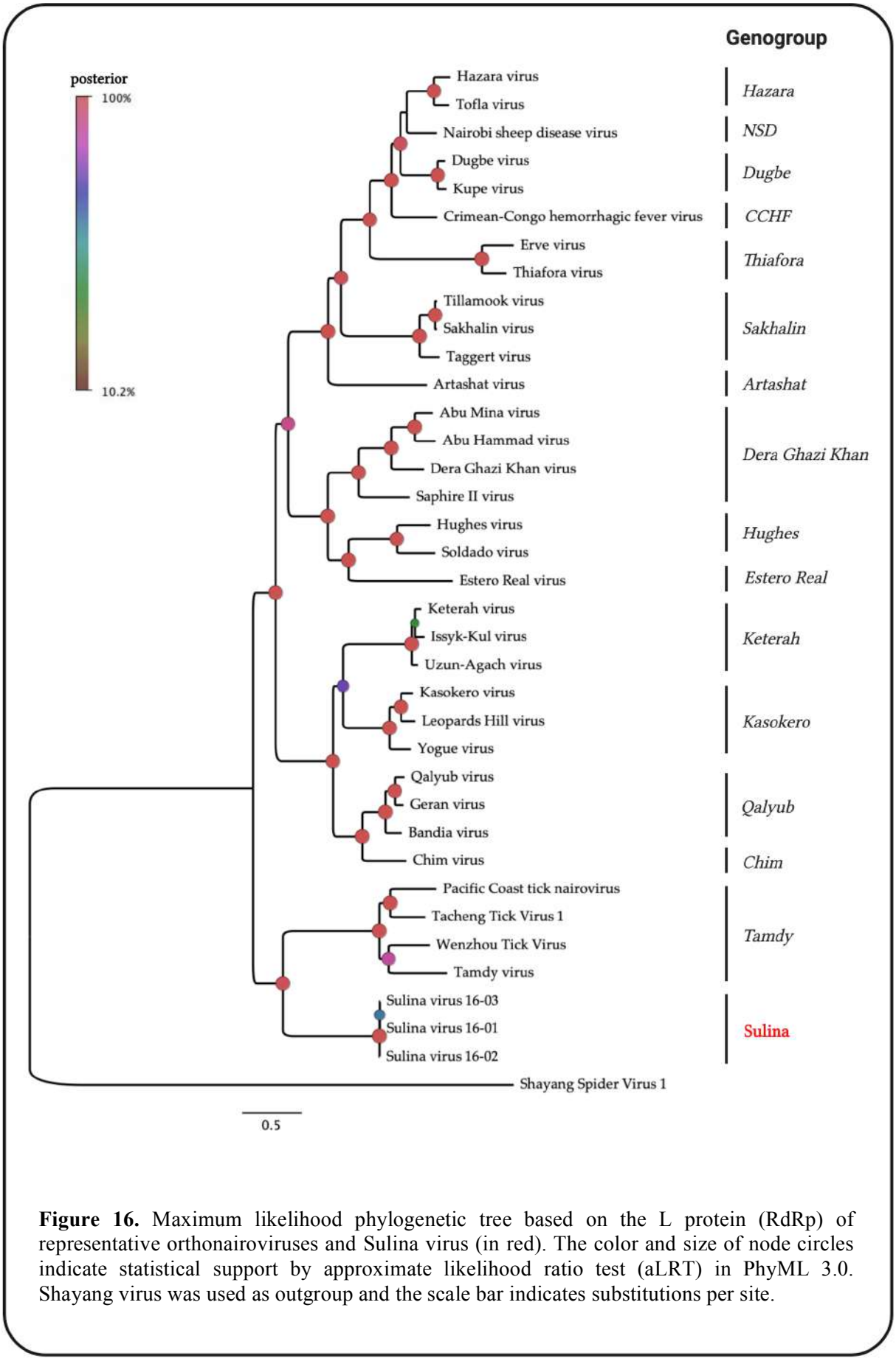


Figure 16. Maximum likelihood phylogenetic tree based on the L protein (RdRp) of representative orthonairoviruses and Sulina virus (in red). The color and size of node circles indicate statistical support by approximate likelihood ratio test (aLRT) in PhyML 3.0. Shayang virus was used as outgroup and the scale bar indicates substitutions per site.

2.4 Discussion

Although ticks are disease vectors of the highest importance, in Romania they are mostly responsible for transmitting pathogenic bacteria (e.g. *Borrelia burgdorferi* s.l.) [441] or protozoa (e.g. apicomplexans) [442,443], while tick-borne viruses do not generally contribute much to the burden of communicable diseases. A noteworthy exception is Tick-borne encephalitis virus (TBEV), a flavivirus that is endemic in some areas of Transylvania [205]. Our study found seven of the 29 species of ixodid ticks known in Romania [203,407] and subjected ticks of six taxa to deep transcriptome sequencing (the seventh taxa was included in serological screening only, see **Table S1**). Surprisingly, SULV RNA was present only in *I. ricinus*, with 35% of the analyzed ticks infected. The prevalence of SULV seems striking when compared to that of pathogenic orthonairoviruses. For example, CCHFV was detected in ticks from Turkey, the Balkans or Iberian Peninsula in 1.4%-8.8% of the analyzed cohorts [402,406,444]. Similar or substantially higher detection rates were reported for the less pathogenic TcTV-1 (4.8%-14.3%) [445] or SOLV (~20%) [446]. Much higher levels were detected for PCTN (of the *Tamdy orthonairovirus*), an orthonairovirus of unknown pathogenicity that was found in 57.6% of screened *Dermacentor occidentalis* [382].

Arboviruses vectored by ticks have complex life cycles owing to the complex ecology of their vectors. In the absence of sufficient data, the reasons behind the high prevalence in *I. ricinus* will probably remain unknown. However, several factors may be responsible for this result. When co-feeding on some nonviremic hosts, infected ixodid ticks can transmit the virus to noninfected ticks [447]. Tick saliva contributes to the enabling of nonviremic transmission [448], a mode that plays an important role in the amplification of CCHFV [269] and of *I. ricinus*-borne flaviviruses [449]. However, even in the case of an immune host, this possibility seems unlikely since we did not detect specific antibodies in the dogs infested with Sulina virus-positive ticks. Another possibility may be that SULV can be transmitted transstadially during metamorphosis or transovarially to tick progenies. In such cases, ticks could be both vectors and long-term reservoirs of the virus [450]. Furthermore, we could not

isolate SULV in Vero or C6/36 cells and adult mice. Thus, based on the available data we must consider the possibility of Sulina virus being a possible tick-specific virus.

Because of the developments in metagenomics and their current relative accessibility, numerous viruses have been and are going to be discovered from diverse groups of vectors and hosts [38,40]. Unlike the already huge variety of insect-specific viruses documented mainly in mosquitoes [451,452], a similar approach for tick viruses is only now becoming popular. An almost identical picture of tick virus discovery is offered by the *Phlebovirus* genus (family *Phenuiviridae*), conordinal viruses with similar genome organization, vectored also by hematophagous dipterans (e.g. the pathogenic tick-borne Heartland virus and Severe fever with trombocytopaenia syndrome virus). In the recent years, surveys of tick populations in both the Old World and the New World revealed a greater diversity of phleboviruses comparatively to that known for orthonairoviruses. As in the case of latter, some phleboviruses detected in Europe could not replicate on various cell lines [453,454] and lacked infectivity of vertebrates [455]. Some of these studies also reported wide intervals of detection rate (2.1%-88.9%) [404,405,454,456]. As expected, a number of studies showed that some tick phleboviruses do grow in cell cultures and thus, were successfully isolated [150,457,458], hinting at the immense viral diversity underlying their phenotypical variation.

The three SULV strains analyzed have >97% nucleotide sequence similarity (~99% for amino acid sequence) across their entire genomes. Such homogeneity is another striking characteristic when comparing SULV to orthonairoviruses like CCHFV (20%-31%) [440], SAKV (up to 27%-33%) [459] or NSDV (11%-56%) [384]. The low sequence diversity may relate to the small number of sequences analyzed and may imply also geographic and temporal sampling bias. The role of the M segment in cell attachment and the positive selection exerted by host immune response is usually reflected in greater sequence variation, as opposed to the necessity of conservation for the replication machinery (i.e. the polymerase). Also, the wider variation seen in glycoproteins may be linked to their complex structure and expression strategy. Still,

such high sequence homogeneity (91%-99%) is exhibited by orthonairovirus M proteins in PCTN [382], TAMV [460] or KUPEV [461].

Interestingly, the *Tamdy orthonairovirus* genogroup comprises viruses associated with the tick's microbiome (PCTN, [382]), but also with vertebrate infection or human illness. TAMV is pathogenic for newborn mice and is known to cause disease in humans [462,463]. TcTV-1 was associated with febrile illness in humans and infection of livestock [445]. These associations illustrate the potential of orthonairoviruses to emerge as pathogens. Genetic variation is molded by numerous ecological and physiological factors, like vector feeding preferences, host availability and immunity, and can accumulate at different structural levels of the genome. This is especially the case for orthonairoviruses in particular [439,440,459], and bunyaviruses in general [74], due to the segmented nature of their genomes. Indeed, much of the ecological and clinical data available for orthonairoviruses is patchy and focused on the few taxa known as agents of severe disease (e.g. CCHFV, NSDV). Sequence data needed for basic comparative analysis has only recently been made available [146,386].

Both of our phylogenetic inference methods yielded similar results (**Figures 14-16**, see **Figure S6a-c** in Appendix) and are in agreement with those of more comprehensive analyses of the genus [146,386]. The status of Sulina virus as a new species or genogroup within the *Orthonairvirus* genus is also supported by the pairwise identity of orthonairovirus proteins. The pairwise identity of Sulina virus nucleocapsid (segment S) is lower than the threshold of 52% indicated by Walker et al., [386], indicating that the virus characterized herein is as a separate species (genogroup) (**Figure 11b**). Membership of Sulina virus for the *Orthonairovirus* genus is indicated also by the 37%-44% protein identity of the RdRp (segment L) (**Figure 13b**), slightly higher than the limits based on the analysis of Kuhn et al. [146]. The same authors proposed a limit of ~26% identity for the orthonairoviral glycoproteins (segment M) that is above the minimum observed between Sulina virus and CCHFV (~20%), but higher when comparing it to TAMV (~30%) **(Figure 12b)**. It is very likely that in the future, both inter-specific and inter-generic thresholds will have to be

revised as we increase the known sequence space for bunyaviruses and reach to deeper nodes in their evolutionary tree.

Romania is one of the most biogeographically diverse countries within the European space [371], with very suitable conditions for the establishment of tick-borne virus foci, especially in the southern half of the country [401]. The regions encompassed therein also correspond to the northern distribution limit of *Hyalomma marginatum*, the main vector of CCHFV. However, *H. marginatum* is known for its ecological plasticity and in recent years was found further north, across the Carpathian range [203,464]. Countrywide surveys found *Ixodes ricinus* to be the most common and widely distributed tick in Romania [202], having a high diversity of hosts on account of habitat variety [204]. Although the expanse and biodiversity of Danube Delta are unique for the region, eastern European countries have many ecosystems (e.g. wetlands) located along migratory pathways and used by millions of migratory birds. The potential of avifauna to carry pathogens or translocate infected parasites is by now well known, both in the DDBR [158,162] and in other European hotspots of migration [160,278]. Our study included parasites of migratory birds netted in Sfântu Gheorghe. Despite testing negative for viral RNA, it is noteworthy that most of the ticks collected from birds were *Hyalomma marginatum* (17/21, 81%).

Unlike our knowledge of tick-borne viruses, vector diversity and ecology has been considerably advanced in Romania during the last decade [200–203]. Considering the biogeographical and eco-epidemiological similarities between Romania and neighboring countries with known orthonairovirus endemicity (e.g. Turkey, Bulgaria, Kosovo, Ukraine, Greece) [402–404,406], further efforts of virus discovery and characterization of vectors' microbiome will help to understand dynamics and risks of tick-borne pathogens, as well as inform necessary public health measures.

In conclusion, the present study described Sulina virus (SULV), a new tick orthonairovirus, circulating in feeding and questing *Ixodes ricinus* ticks from Danube Delta. The three SULV strains belong to a single species and we found no evidence of host infection or replication in vertebrates. The pathogenesis of SULV in wildlife and

livestock or its infectivity for humans or other vertebrates need to be further investigated, justifying continued surveillance for a better understanding of SULV natural cycle. The discovery and characterization of Sulina virus will be added to the growing body of work necessary for thorough analysis of orthonairovirus structure, ecology and evolution.

3 General Discussion

3.1 Vector surveillance in Danube Delta Biosphere Reserve

3.1.1 Longitudinal mosquito surveillance

(based on Török et al., 2016; Tomazatos et al., 2019)

Extensive mosquito collection between April and September of 2014-2016 identified 16 mosquito taxa, representing approximately 29% of the 55 taxa known for the Romanian fauna [465–469]. The first detailed analysis of DDBR's mosquito fauna was based on the 2014 collection. The results confirmed 12 known taxa and revealed two new species for Romania's entomofauna: *Aedes hungaricus* and *Anopheles algeriensis*. In the following seasons (2015 and 2016), four additional known species were added to the taxa list (**Table 2**). Despite the small number of individuals per new respective taxa, the extrapolated taxa richness indices showed a fairly good coverage obtained with Encephalitis Vector Surveillance (EVS) traps in the study area.

Barcoding of mitochondrial DNA (mDNA) markers in addition to morphological identification proved very useful for species identification. The maximum likelihood (ML) phylogeny of partial cytochrome c oxidase subunit I (COI) sequences was congruent with the results of morphological taxa discrimination. The well-supported phylogenetic clustering (≥95%) offered some information about the evolutionary relationships of DDBR's mosquitoes with other European populations (**Figure 17**). Trapped at all four sites and in larger numbers among the rarer taxa (n = 2280, 0.37%), *An. algeriensis* is known as a common occurrence in central Europe, parts of the Mediterranean basin as far north as Germany and the United Kingdom [470]**.** Its breeding habitat is characteristic of marshlands, with slow-flowing waters and dense vegetation. It is know as a mammophilic species and a potential malaria vector [470]. Considering its low frequency in the study area, it is quite probable that *An. algeriensis* has only a minor role in pathogen transmission. The phylogenetic clustering of *An. algeriensis* COI sequence contained three distinct branches. Accordingly, the genetic distances calculated for this species reached a divergence level of 6%. This is higher

than the 2-3% inter-species cut-off considered by some authors for mosquitoes [474] or the 4% threshold for biting midges [475]. The unbalanced number of conspecific sequences compared, small sampling area and the use of a single marker preclude firm conclusions regarding the existence of new species. However, the phylogeny shows that a very heterogeneous population of *An. algeriensis* (possibly indicative of a speciation event) can be found in the study area (~160 km^2). Such degree of divergence is important if we consider that different populations can have different vector competence [476]. Population subdivision can be observed also in the case of *Ae. detritus*, although to a smaller extent (3% intra-specific divergence). In contrast with the two above-mentioned species, the phylogenetic clustering of *Aedes hungaricus* tends to indicate high homogeneity between DDBR (Romanian) and Hungarian populations, although the small number of available sequences is again problematic. In the case of mosquitoes belonging to "species complexes", for example *Culex pipiens* s.l./*torrentium* or *Anopheles maculipennis* s.l., we managed to identify a small proportion of specimens from both taxa. However, the barcoding may not be a good strategy for species/biotype identification. Rather, mass screening by specific PCR would be a better strategy to discriminate these taxa [477]**.**

The collection was dominated clearly by *Coquillettidia richiardii* and *Anopheles hyrcanus* with 55.88%, respectively 24.81% of all identified mosquitoes. Danube Delta is an important region for their distribution in Romania [469], providing excellent breeding conditions for these multivoltine species. Due to their dominance, representing over three quarters of the total collection, the general phenology and temporal pattern of mosquito functional groups follow the patterns of the two dominant species (**Figure 18, Figure 19**). Seven mosquito taxa were dominant during the sampling period, each represented by >15,000 individuals: *Coq. richiardii* (57.88%), *An. hyrcanus* (24.81%), *An. maculipennis* s.l. (4.32%), *Ae. caspius* (3.92%), *Cx. pipiens* s.l./*torrentium* (3.52%), *Ae. vexans* (2.54%) and *Cx. modestus* (2.30%). A clear separation can be observed between these taxa and the less frequent mosquitoes, which make up for 0.66% of the collected specimens (**Table 2**).

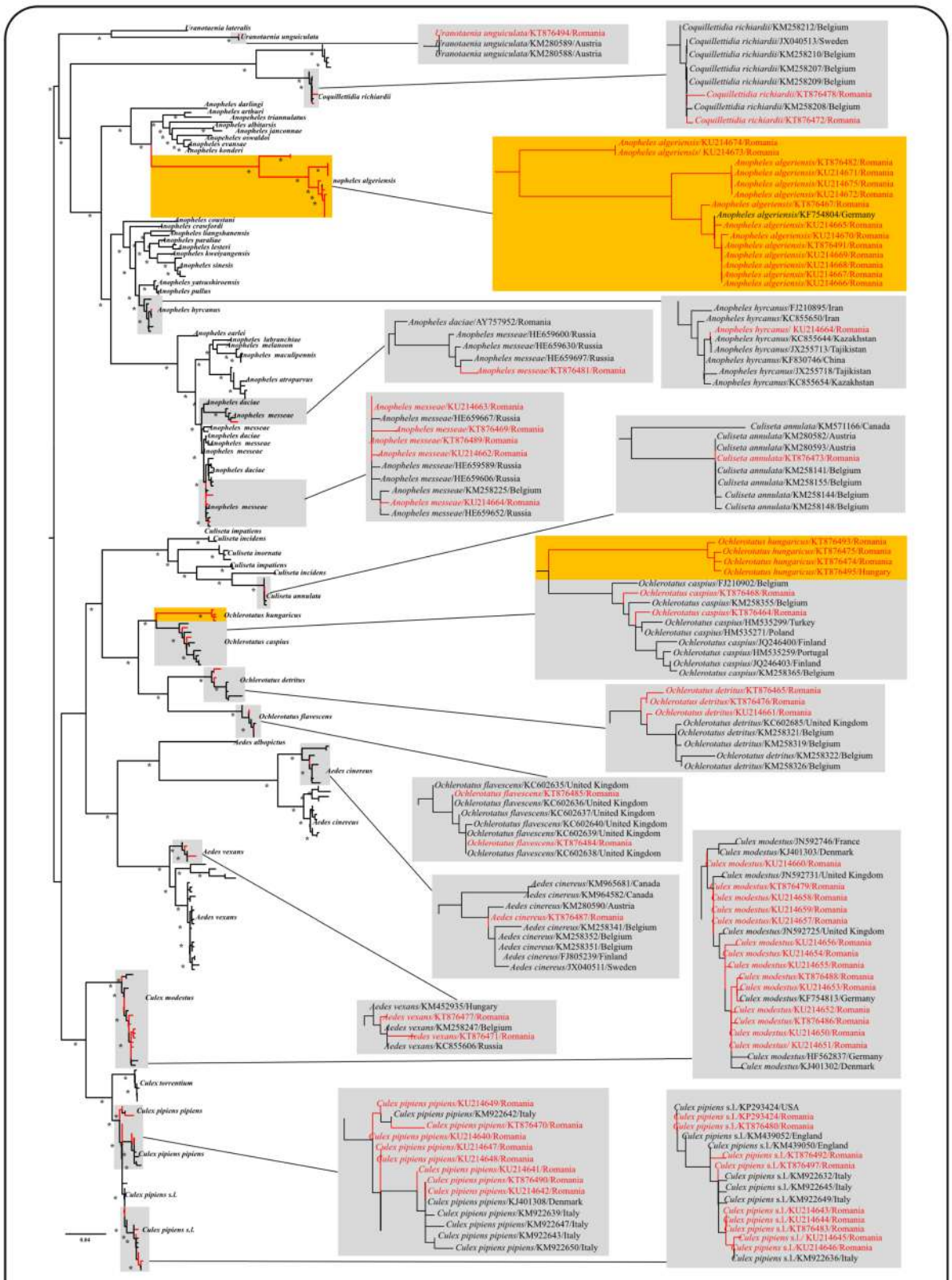


Figure 17. Maximum likelihood (ML) phylogeny of the COI fragments from 14 mosquito species collected in DDBR (2014, red font). Red portions of the tree and magnified areas in grey/orange indicate the location of the mosquito species detected in this study. *Ae. hungaricus* and *An. algeriensis* are highlighted in orange. The ML and parallel NJ bootstrap values above 70% (1000 replicates) are marked by asterisk. The scale-bar indicates the genetic distance as the mean number of nucleotide substitutions per site.

The results of this entomological survey are most likely biased because they were obtained with a single type of trap designed for adult mosquitoes and operated in a small number of sites. DDBR is a very heterogeneous complex of ecosystems, having around 30 ecosystem types [478]. One solution for avoiding biased trapping could be the design of surveys with more sampling sites and more trap types. That is also because trap performance varies relative to the mosquito species targeted [479], mainly due to different host preferences among mosquito species. Such bias in mosquito collection in Danube Delta is well illustrated by the live-bait trapping employed by previous studies. Sampling by bird-baited traps resulted in more *Cx. pipiens* s.l., whereas *Coq. richiardii* was present in greater numbers when using the human landing catch [186,480]. During the 2016 sampling season, BG-GATs (Biogents Gravid *Aedes* Traps) were used with the intention of collecting egg-laying mosquitoes. The traps were set at the main and secondary sites along the itinerary. The results were extremely poor as regards the number of collected mosquitoes. The most plausible explanation one can think of is that BG-GATs cannot compete with the ubiquitous natural oviposition sites in the study area.

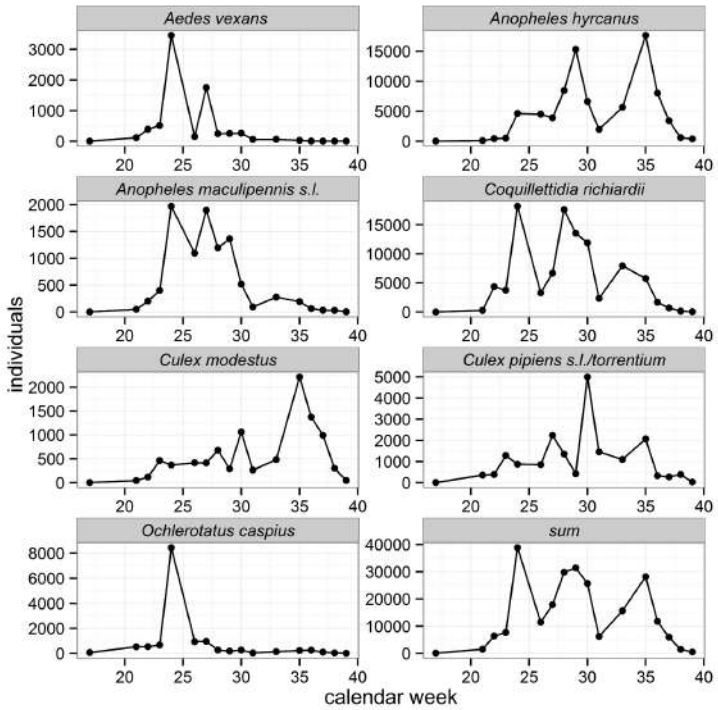


Figure 18. Number of specimens of the seven most common mosquito taxa (>2000 specimens) detected per calendar week in the DDBR (2014).

All seven dominant taxa have been previously implicated in the transmission of WNV and six of these taxa were found positive for the virus in Romania or DDBR [182,186,188,481]. The principal WNV vectors in Europe are *Cx. pipiens* s.l./*torrentium* and *Cx. modestus* [182,329]**.** These taxa can be found in the study area in various proportions, considering trapping methods. Overall, the study found that most of the collected mosquitoes are potential WNV vectors (**Table 2**).

Conducting entomological surveillance in a very heterogeneous landscape, such as a wetland, will always pose logistical challenges when attempting to sample vector populations in longitudinal manner. In DDBR, sampling sites can be reached only by boat. Therefore, operating even one type of mosquito traps in parallel with targeting other vector groups or vertebrate hosts requires more human resources and time coordination. Although a more detailed picture of the vector populations and associated risks of pathogen transmission can be obtained by a more diverse trapping strategy (e.g., including collection of immature stages), the data generated by this study offers a first detailed overview of the mosquito communities in the eastern parts of DDBR.

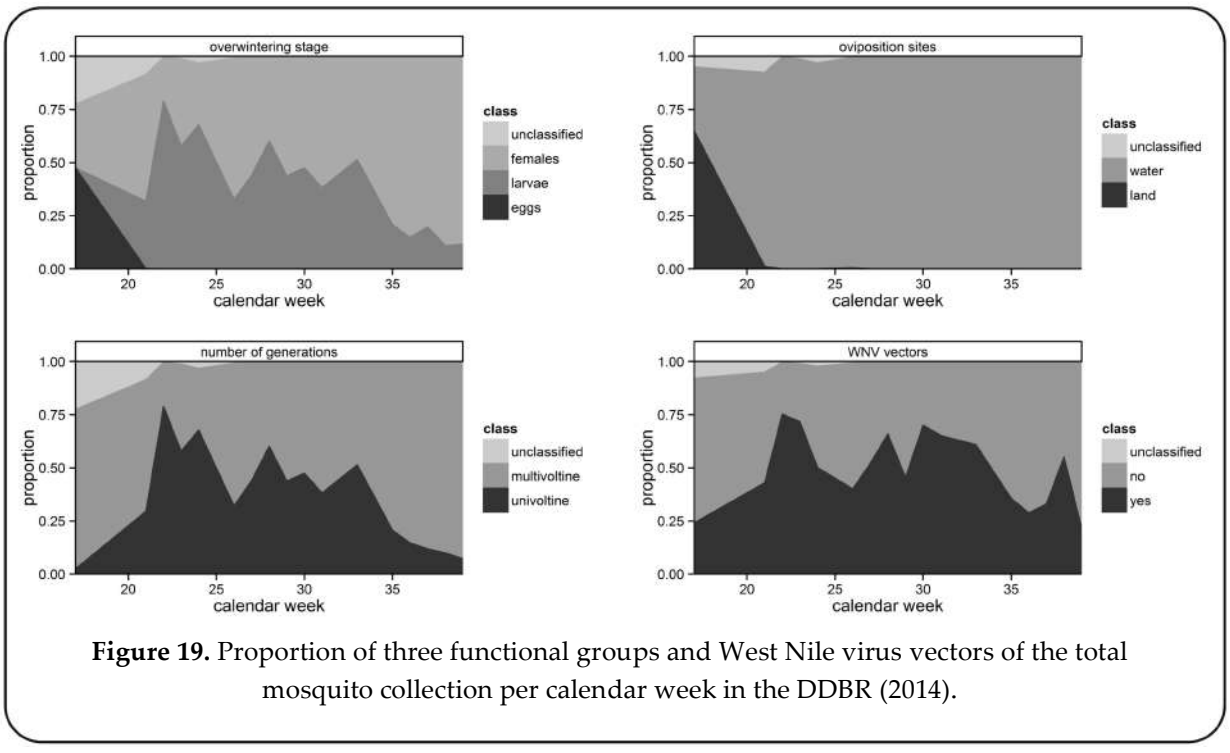


Figure 19. Proportion of three functional groups and West Nile virus vectors of the total mosquito collection per calendar week in the DDBR (2014).

Table 2. Mosquito taxa recorded in the study area of DDBR between 2014 and 2016, with the number of specimens collected, overall proportion and information on their involvement in WNV transmission, in Romania or elsewhere.

Mosquito taxa	Sum	%	WNV-Positive in Romania/DDBR	Involved in WNV transmission elsewhere
Coquillettidia richiardii Ficalbi, 1889	379,513	57.88	yes[1]	yes[3]
Anopheles hyrcanus Pallas, 1771	162,725	24.81	yes[1]	yes[3]
Anopheles maculipennis (s.l.) Meigen, 1818[a]	28,363	4.32	yes[1]	yes[3]
Aedes caspius Pallas, 1771	25,767	3.92	yes[1]	yes[3]
Culex pipiens Linnaeus, 1758 s.l. /*torrentium* Martini, 1925[b]	23,134	3.52	yes[1,2]	yes[3]
Aedes vexans Meigen, 1830	16,673	2.54	no	yes[3]
Culex modestus Ficalbi, 1890	15,135	2.30	yes[1]	yes[3]
Anopheles algeriensis Theobald, 1903	2,280	0.34	no	no
Aedes flavescens Müller, 1764	529	0.088	no	no
Aedes detritus Haliday, 1833	246	0.037	no	no
Aedes cinereus Meigen, 1818	51	0.0077	no	yes[3]
Culiseta annulata Schrank, 1776	12	0.0018	no	yes[4]
Aedes hungaricus Mihályi, 1955	4	0.0006	no	no
Uranotaenia unguiculata Edwards, 1913	3	0.00046	yes[1]	yes[5]

Table 2. (continued)

Mosquito taxa	Sum	%	WNV-Positive in Romania/DDBR	Involved in WNV transmission elsewhere
Aedes geniculatus Olivier 1791	1	0.00015	no	yes[4]
Aedes sticticus Meigen 1838	1	0.00015	no	yes[4]
Unidentified	1,041	0.17	-	-
Aedes sp.	169	0.026	-	-
Culex sp.	17	0.0025	-	-
Anopheles. sp.	3	0.00046	-	-
Total	**655,667**	**100**		

[a] Selected specimens from 2014 were identified as *Anopheles messeae* Falleroni, 1926 by DNA barcoding
[b] *Culex pipiens* (s.l.) and *Culex torrentium* were not differentiated for most of the collected specimens. Selected specimens were identified as *Culex pipiens* Linnaeus, 1758 (s.l.) and *Culex pipiens pipiens* Linnaeus, 1758 by DNA barcoding (see **Figure 17**)
[1] [186]
[2] [182]
[3] [471]
[4] [472] (laboratory experiment)
[5] [473]

3.1.2 Species composition of *Culicoides* biting midges

(based on Tomazatos et al., 2020**c**)

The small flies of *Ceratopogonidae* family (Diptera) are a very diverse group, of which the *Culicoides* genus comprises the main arboviral vectors [482]. There is very little information about *Culicoides* in Romania (as in most of eastern Europe). The sampling of biting midges during the 2017 season identified eight taxa at two rural sites (Sulina and Letea) and two natural sites with reduced human presence (Dunărea Veche and Lake Roșuleț) **(Table 3)**. As in the case of mosquitoes, DNA barcoding of mDNA proved very helpful for detection of known and unknown *Culicoides* taxa. The barcoding had a relatively high rate of success (91.7%), confirming the usefulness of a DNA barcoding strategy in biodiversity studies [483–486]. Unlike the barcoding of mosquitoes where mDNA barcoding was used to confirm the morphological identification of a much larger collection, the mDNA barcoding was the primary strategy for identification of *Culicoides* spp. Molecular assays were combined with wing morphology from a smaller subset of insects (n=37). The main reason for this approach was the physical state of the insects. Important characters (e.g., wings, legs, antennae) were often missing after shipping on dry ice and storage at -80 °C, necessary for keeping viruses viable for replication. It is important to note however, that greater reliance on molecular libraries to the detriment of traditional, morphology-based taxonomy could be problematic, due to some misidentified sequences used as references [487]. The popularity of genetic markers in biodiversity studies has shown some of their pitfalls, as well [488,489]. This holds true especially in the case of insects [488,490,491]. Ideally, both molecular and morphological methods should be used in complementarily, in what is currently known as integrative taxonomy [492].

We found eight *Culicoides* taxa, of which seven were identified at species level. Three species, *C. griseidorsum*, *C. puncticollis* and *C. submaritimus*, were recorded for the first time in Romania, increasing the number of known *Culicoides* spp. in this country to 49 species [214]. *Culicoides submaritimus* has been considered a synonym of *C. maritimus* by some authors [493,494], while recent studies mentioned *C.*

submaritimus as a distinct species [142,495]. The present study identified *C. submaritimus* by comparison with COI sequences from Turkey, the only sequences available in molecular databases. Both *C. submaritimus* and *C. maritimus* are absent from the inventory of biting midges of Romania by Damian-Georgescu [214], although some recent studies include Romania in the distribution maps of *C. maritimus* [142,496]. *Culicoides griseidorsum, C. kibunensis*, *C. punctatus* and *C. riethi* were confirmed by wing morphology, as well. *Culicoides puncticollis* was identified first by wing morphology, being present only in the small subset used for morphological analysis; subsequently, this species was confirmed by mDNA barcoding. Wing morphology was also examined for the "unknown *Culicoides"*, a species that was confirmed by intra- and inter-specific genetic distances. For this species, no identification could be made with the interactive key of Mathieu et al. [142] or by searches in molecular databases (GenBank and BOLD, respectively).

The purpose of the ML analysis (**Figure 20**) was to verify the identity of taxa against conspecifics from the Palearctic realm. All specimens clustered in well-defined terminal clades with high bootstrap support, with the exception of *C. subfasciipennis*/*C. pallidicornis* clade. These two species are being morphologically discriminated by the presence of a variable light spot on the annal cell of *Culicoides subfasciipennis* [142]. The inability of COI sequences to distinguish between these two species was observed also by Sarvašová and her colleagues [497] in central European populations. This pattern of phylogenetic clustering and the unreliable variability of wing morphology led some taxonomists to doubt the status of *C. subfasciipennis* and *C. pallidicornis* as two separate species [497,498]. Another notable clustering pattern was that of *C. punctatus.* Although forming a well-supported clade, almost half (45.5%) of the analyzed *C. punctatus* diverged from the main group as a distinct haplotype (designated *C. punctatus* P). The genetic distance between the two groups of *C. punctatus* is approximately 4%, which some authors consider to be within intra-specific boundaries when using COI markers [475]. This outcome further confirms the utility of DNA barcoding in detection of sibling (or cryptic) species, undistinguishable by

morphology alone [475,499,500]. Similarly to mosquitoes, differentiating between isomorphic sibling species is important for finding potential variations in vector capacity [129]. With the exception of *C. punctatus* P haplotype (~97% identity score in GenBank searches), all *Culicoides* taxa identified in this study showed >98% to 100% identity with GenBank references. The unknown *Culicoides* sequence diverged by 15.6-16.3% from the closest described species, forming a monophyletic clade with *C. kibunensis*. Such genetic distances are similar to those found between other *Culicoides* spp. [487,501] or between different mosquito species [474]. Therefore, the unknown *Culicoides* could be considered a distinct, new species or even a species without any reference in GenBank or Barcode of Life database (BOLD).

The study of *Culicoides* spp. discussed herein offers an interesting, albeit incomplete picture of the biting midge communities from DDBR. Despite the fact that sample analysis was biased in favor of natural sites with a higher diversity of wild hosts (Dunărea Veche and Lake Roșuleț), the results suggest that *C. punctatus* is one of the dominant taxa. Probably the most compelling case for further exploration of *Culicoides* diversity is made by the unknown *Culicoides* and the newly recorded taxa. Furthermore, *C. punctatus* was found positive for BTV and SBV in southern and central Europe [300,502]. Given the potential of *C. punctatus* as a vector and its feeding behavior (see section **3.2.2**), a more detailed, longitudinal study could be useful in characterization of biting midge diversity of DDBR and their potential as bridge vectors between wild and domestic hosts.

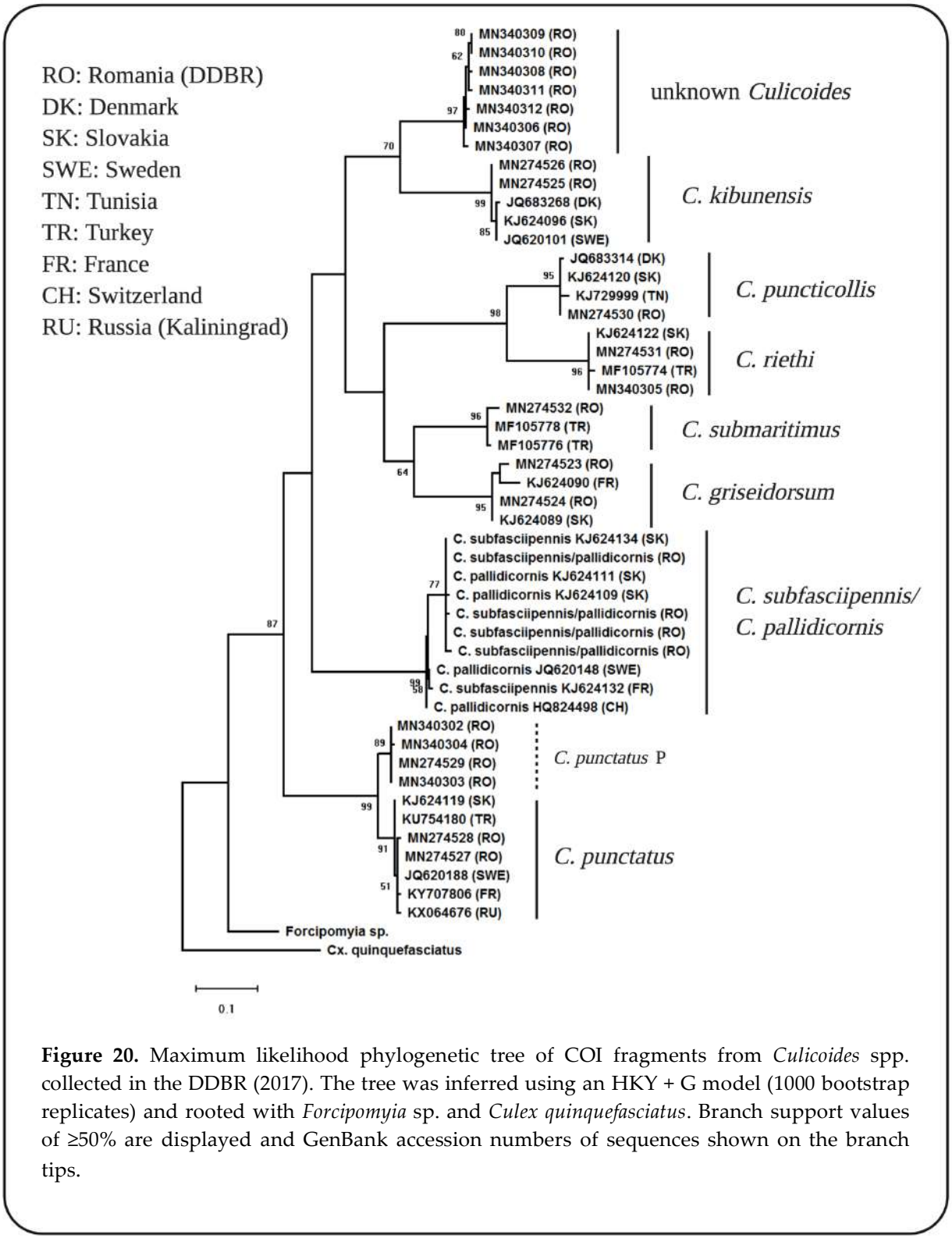


Figure 20. Maximum likelihood phylogenetic tree of COI fragments from *Culicoides* spp. collected in the DDBR (2017). The tree was inferred using an HKY + G model (1000 bootstrap replicates) and rooted with *Forcipomyia* sp. and *Culex quinquefasciatus*. Branch support values of ≥50% are displayed and GenBank accession numbers of sequences shown on the branch tips.

Table 3. Biting midge taxa identified in DDBR in 2017

Midge taxa	**Sulina**	**Letea**	**Dunărea Veche**	**Lake Roșuleț**	**Sum**	**%**	**Identified by COI barcoding**	**Identified by wing morphology**
Culicoides griseidorsum Kieffer, 1918	21	212	41	2	**276**	24.3	yes	yes
Culicoides kibunensis Tokunaga, 1937	1	10	87	4	**102**	9	yes	yes
Culicoides punctatus Kieffer 1914	26	342	74	13	**455**	40	yes	yes
Culicoides puncticollis Becker 1903[a]	0	0	1	0	**1**	>0.1	yes	yes
Culicoides riethi Kieffer 1914	1	11	0	0	**12**	1.1	yes	yes
Culicoides subfasciipennis Kieffer, 1919/ *Culicoides pallidicornis* Tokunaga & Murachi, 1959	102	87	36	17	**242**	21.3	yes	no
C. submaritimus Tokunaga & Murachi, 1959	0	0	2	6	**8**	0.7	yes	no
unknown *Culicoides*[b]	12	1	17	9	**39**	3.4	yes	yes
Total	**163**	**663**	**257**	**51**	**1,135**	**100**		

[a]Identified only in the subset of morphological vouchers.

[b]Morphological identification was inconclusive; taxon authentication was based on intra-and inter-specific genetic comparisons among specimens from the morphological voucher subset and the larger collection

3.2 Detection and characterization of arboviruses

3.2.1 West Nile virus: phylogeography of an endemic pathogen

(based on Tomazatos et al., 2019)

The screening of over 655,000 mosquitoes from 16 taxa revealed the presence of WNV RNA in only two pools of *Cx. pipiens* s.l./*torrentium* (n = 99) collected at Lake Roșuleț and Sulina in June 2014. The WNV-positive mosquito pools contained two similar WNV strains with 99.5% pairwise identity of their genomes and 99.8% identity between their polyprotein sequences.

For inferring the possible origin and pattern of spatial-temporal spread of WNV between DDBR and other locations we used two datasets: one composed of full WNV genomes and another one of partial NS5 sequences. The phylogeographic analysis of the partial NS5 dataset identified at least two distinct introductions of WNV lineage 2 in Romania. These WNV variants compose two subclades that overlap in southeastern Romania: East European clade 1 (EEC1, with variants from DDBR and Bucharest) and West European clade 1 (WEC1, with variants from Bucharest). The analysis of both datasets suggests that the EEC1 has African progenitors, either from South Africa or Senegal. The analysis of the NS5 dataset indicated that the WEC1 strains originate probably in Greece, entering Romania over the Balkan Peninsula (**Figure 21, Figure 23b**).

Although the close relationship of the Russian strain Volgograd/2007 with the EEC1 was expected, it is interesting that an Italian strain discovered also in 2014 in northeastern Italy clustered within the EEC1 [503] (**Figure 22b**). This pattern is suggestive of an independent introduction of the EEC1 in south/central part of Europe. Like eastern Europe, the Italian Peninsula is crossed by major Palearctic - Afrotropical flyways [374]. Also, the region where this Italian EEC1 strain was found has similar eco-geographical characteristics, with masses of birds visiting three main wetlands in the vicinity of the Adriatic coast. In the context of intercontinental dispersal of WNV, bird migration is considered the most plausible explanation, with short-distance

migration as a potential dispersal mechanism for spread within Europe [220,221,504,505].

Several mutations observed in proteins C, prM, E, NS2A, NS3 and NS5 are specific to EEC1. Of these particular mutations, S1839T (NS3) and G2932R (NS5) are also shared with two South African strains. More extensive similarity supporting the African origin of WNV strains from DDBR is showcased by African-specific residue changes R835K (NS1), I1462M (NS2B), R1516K (NS3), T2296A (NS4), N2305S (NS4) and R2719K (NS5). Although the impact of mutations on the nonstructural genes is unclear (likely some occurred after the introduction of WNV in the country), similar changes modulated the host antiviral response by inhibiting interferon signaling [506].

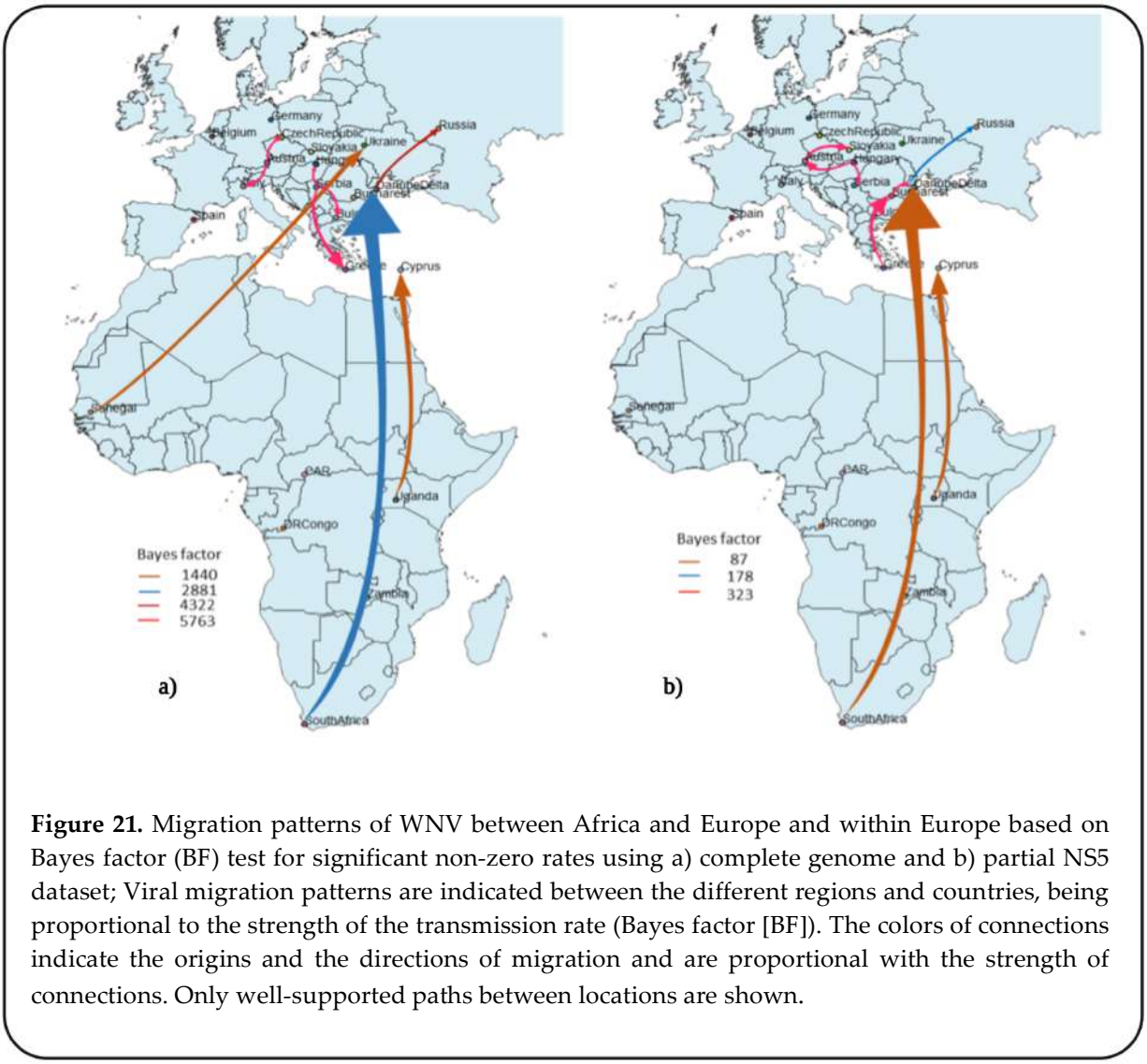


Figure 21. Migration patterns of WNV between Africa and Europe and within Europe based on Bayes factor (BF) test for significant non-zero rates using a) complete genome and b) partial NS5 dataset; Viral migration patterns are indicated between the different regions and countries, being proportional to the strength of the transmission rate (Bayes factor [BF]). The colors of connections indicate the origins and the directions of migration and are proportional with the strength of connections. Only well-supported paths between locations are shown.

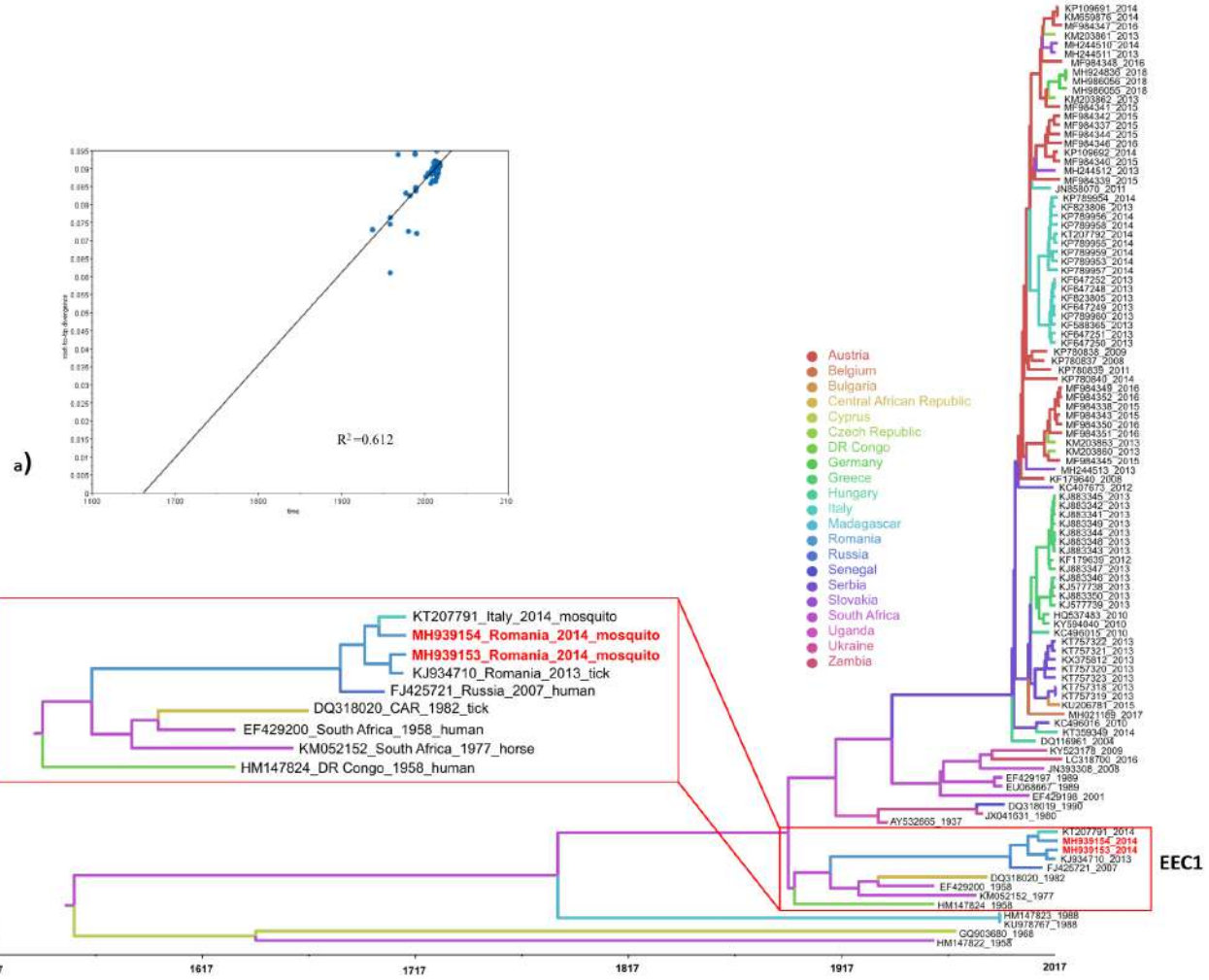


Figure 22. a) Root-to-tip regression analysis of the West Nile virus (WNV) complete genome-based maximum likelihood tree. Plots of the root-to-tip genetic distance against sampling time are shown; b) Bayesian maximum clade credibility (MCC) tree representing the timescale phylogeny of WNV lineage 2, based on complete genome sequences, including the EEC1 clade. The colored branches of the MCC tree represent the most probable geographic location of their descendant nodes (see color codes). Time is reported in the axis below the tree and represents the year before the last sampling time (2018).

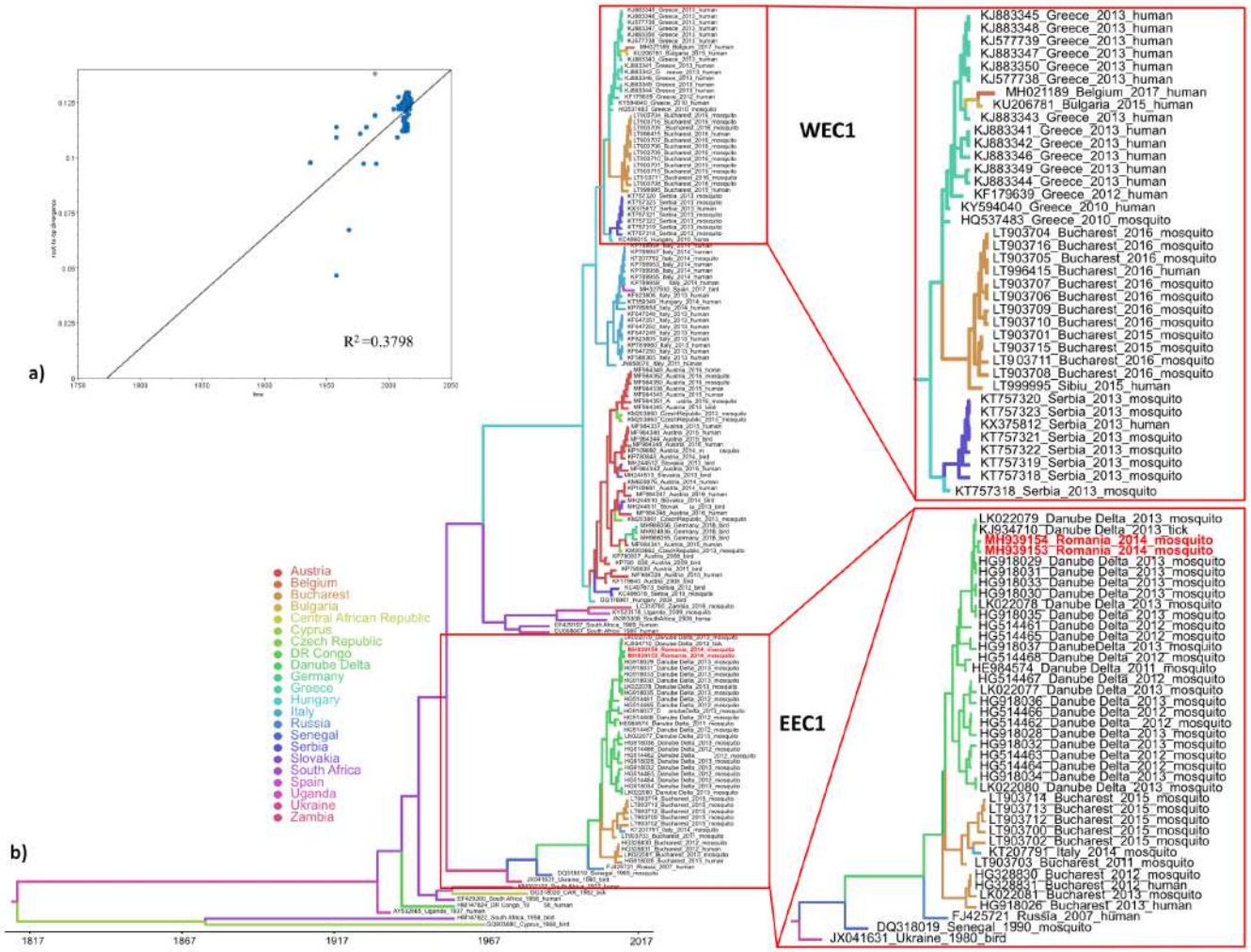


Figure 23. a) Root-to-tip regression analysis of the WNV partial NS5-based maximum likelihood tree. Plots of the root-to-tip genetic distance against sampling time are shown; b) Bayesian maximum clade credibility (MCC) tree representing the timescale phylogeny of WNV lineage 2, based on NS5 gene sequences, including the EEC1 and WEC1 clades. The colored branches of the MCC tree represent the most probable geographic location of their descendant nodes (see color codes). Time is reported in the axis below the tree and represents the year before the last sampling time (2018).

During and after the 2010 WNV outbreak, variants of EEC1 were detected in the east and southeast of Romania (DDBR, Constanța, Bucharest, Ilfov), but also across the Carpathian range (Cluj, Alba, Sibiu) [183,186] (data from published reports, without suitable sequences available for analysis). Foci and periodic outbreaks of WNV are known in the southeast since the first major outbreak of 1996 [182,185,186]. The presence of a second geographically distinct clade (the WEC1) is likely to have resulted from a more recent introduction from south of the Danube, followed by adaptation to the local ecological conditions and rapid spread. The 2010 outbreak of WNV revealed the extent of EEC1 circulation within the country, therefore it is no surprise that EEC1 reached Bucharest/Ilfov area from DDBR at least twice (**Figure 23b)**, co-circulating with WEC1 in the Danube Floodplain. In August 2015, Cotar and her collaborators [481] detected the co-circulation of the two clades not only around Bucharest, but also in the south of DDBR, near Razim-Sinoe lagoon. In the respective report, WNV strains of both clades were detected in mosquitoes caught in 2015 at two sampling sites (Bucharest and south of DDBR). However, having found only WEC1, but no EEC1 at both sites next year (2016), the authors concluded that the EEC1 was "completely replaced […] in mosquito populations". Despite the fact that the infection rate was considerably higher than what we have found during 2014-2016, and that the circulation of WNV is known to be very dynamic, the conclusion was based on <20,000 mosquitoes trapped in only two sites. Overwintering can support viral persistence and endemisation [218], yet this mechanism apparently was not considered. Detection of RNA in males of *Culex pipiens* s.l./*torrentium* [186] indicated vertical transmission of the virus, which may be crucial for virus persistence in the absence of new introductions. Overwintering of WNV in Romania is strongly suggested, but so far we do not have sufficient data to confirm it. It is very likely that periodical reintroduction together with local overwintering contribute to the maintenance of this virus in Romania. Furthermore, a dynamic circulation of WNV does not imply only rapidly growing outbreaks (e.g., seasons of 2018 and 2019), but also a silent, "under-the-radar" transmission, traceable by serosurveys conducted years prior to abundant case reports

[507]. Other phylodynamic studies from countries where WNV is repeatedly introduced, also pointed to a period of enzootic cycling before the spillover to dead-end hosts reveals a potential outbreak [508,509]. One major limitation of phylodynamic reconstructions in Romania is the scarcity of suitable WNV sequences. Intense WNV transmission is described in neighboring states (e.g. Serbia and Hungary), an unsurprising fact considering the trends of arbovirus activity at regional and continental level [112–114]. WNV could be penetrating Romanian borders also from west of the Carpathian range, possibly from Hungary, where recently it has shown an outstanding increase in human infections [105]. Thus, the dispersal patterns and eco-epidemiology of WNV in Romania are more complex than what we can infer with the currently available data.

The results of mosquito sampling and WNV detection between 2014 and 2016 were determined by a combination of environmental and sampling factors. The number of WNV-positive mosquitoes found by this surveillance study may seem very low if we consider the number of trap nights, of collected mosquitoes and access to the core areas of DDBR. Precipitation is a key element for the development of mosquito populations; during the seasons of mosquito sampling, precipitation was reduced in comparison with preceding and following years. During summer, water level was low on the Danube, main canals and lakes. Consequently, adjacent marshes and plains were flooded to a lesser extent and for shorter time, while some even remained completely dry. Another issue could be the use of a single trap type. The low proportion of *Cx. pipiens* s.l. (3,53% of total collection) was likely the result of using a single trapping method [479,510]. Other studies in the DDBR and elsewhere obtained greater numbers of the main WNV vector by using different techniques (e.g., live bird baits) [188,511]. During the first and second sampling seasons (2014 and 2015), the number of human WN disease cases reported in Romania was low and the case number increased 3-fold in 2016 (>90 reported cases) [91]. After mosquito collection in the field was concluded, the number of infections in the country decreased again (2017), but increased by 4-fold during the hot summer of 2018 (18% of all locally

acquired infections reported in the EU) [91]. Besides the two virus isolates obtained from *Cx. pipiens* s.l., we found flavivirus-specific IgG in blood meals of taxa known as secondary vectors of WNV (see section **3.3.1.1**). Overall, these results support a silent circulation of WNV in natural and rural sites from DDBR.

3.2.2 Letea virus: a reassortant orbivirus discovered in grass snakes (*Natrix natrix*)

(based on Tomazatos et al., 2020**d**)

The rationale for the inclusion of reptiles in this project was provided by the growing, yet still limited and fragmentary evidence for their role in circulation of arboviruses [229,230,512-516]. Apart from sera of grass snakes (*Natrix natrix*) and dice snakes (*Natrix tessellata*), the project initially included sera collection from other herpetofauna commonly found in the study area: the European pond turtle (*Emys orbicularis*) and water frogs of the genus *Pelophylax*. The absence of arbovirus sequences in the sera of pond turtles and amphibians shifted the focus in 2017 entirely on the snake hosts. Next-generation sequencing and reverse transcription-PCR (RT-PCR) revealed the presence of an unknown segmented virus showing significant identity scores with viruses of the genus *Orbivirus.* The new virus was named Letea virus (LEAV), after the eponymous village from DDBR, and represents the first documentation of an orbivirus infecting reptiles.

Inclusion and demarcation of species within the *Orbivirus* genus consider several criteria, such as sequence identity of segments encoding the polymerase (VP1) and major subcore shell protein (T2), gene reassortment between close strains, high levels of serological cross-reaction against conserved antigens like the T13 protein, conservation of UTR terminal nucleotides, range of hosts and vectors or the clinical signs produced by orbivirus infection [517].

The genome of LEAV has a structure typical for orbiviruses, with 10 double-stranded (ds)RNA segments delimited by untranslated regions (UTR) with conserved terminal sequences similar to those of other orbiviruses. Unlike the hexanucleotides of BTV and AHSV, the UTRs of LEAV contain heptanucleotides with minor variations

between genomic segments. In each UTR, distal dinucleotides at both ends are inverse complements, as found in other *Orbivirus* species [37,518–522].

The amino acid identity observed in polymerase sequences is above the 30% threshold defined by Attoui et al. [523] for inclusion in the *Orbivirus* genus. The comparative analysis of the T2 protein sequence showed identity levels much lower (22%-53%) than the 91% cut-off proposed [37], confirming that LEAV is a distinct orbivirus species. Moreover, the nine different LEAV strains belong to the same species, as their T2 amino acid sequences are >98% identical. The separation of LEAV as a separate serogroup (species) within the genus is confirmed also by the comparison of segments determining orbivirus antigenic properties: VP2 and VP7 [524]. The orbiviral VP7 protein (also known as T13 or the core surface protein) forms the outer layer of the viral core, defining the virus serogroup (species) [286]. This structural role makes the sequence of T13 to be relatively conserved among orbiviruses [525]. LEAV T13 sequence exhibited low identity levels when compared with orbivirus homologs, confirming LEAV as a new, separate serogroup. Like in other *Culicoides*-/sand fly-borne orbiviruses (C/SBOV), the VP2 of LEAV is encoded by segment-2 and is homologous to the VP3 of mosquito-borne orbiviruses (MBOV) and the VP4 of tick-borne/tick orbiviruses (TBOV/TOV) [520]. In contrast with T13, the VP2 (outer capsid protein 1) is one of the most variable proteins and significant identity values (10%-15%) were observed only between LEAV and insect-borne viruses (IBOV, i.e. C/SBOV and MBOV). Due to its neutralizing epitopes and role in cell attachment, the VP2 protein is subjected to intense selective pressures by the host's immune response. Thus, it is one the most variable orbiviral proteins [286]. The high amino acid identity (>98%) found when comparing the nine LEAV strains indicated that all the analyzed LEAV genomes belong to a single LEAV serotype.

The NS4 is a nonstructural protein found in some orbiviruses and the last one described to date [526,527]. In LEAV, the NS4 protein is of similar size and position as in other C/SBOV [526,528,529], having also the lowest sequence identity among all compared orbiviral proteins.

The evolutionary relationships between LEAV and other orbiviruses were analyzed using the amino acid and nucleotide sequences of the polymerase (VP1), T2 (VP2/VP3) and T13 (VP7) (**Figure25a-c**). The three proteins have been used extensively as markers for taxonomic and phylogenetic studies due to their high degree of conservation [519,520,522,525,530]. Phylogenetic analyses of VP1 and T13 (amino acid and nucleotide) agree with previous studies, insofar as the virus clustering defines three vector-specific clades (C/SBOV, MBOV and TBOV) [520,528,529,531–534]. However, these phylograms did not show the IBOVs as a monophyletic group descending from TBOV as previously shown, although a common descent from the distant tick-associated St. Croix River virus (SCRV) was well supported. The phylogenetic analysis of T2 proteins revealed two major clades corresponding to the genomic segments encoding this protein (segment-3 of C/SBOV and segment-2 of MBOV/TBOV) (**Figure 25c**). This is in agreement with results of Fagre et al. [533], but the distinct topology suggests that IBOV are not a monophyletic group as proposed in the past [525,529,535]. The pairwise comparison of amino acid sequence identity indicated also that individual conserved proteins are related within the vector-defined clusters, as shown by the phylogenetic trees. Therefore, the sequences and their coding assignments may be useful for associating potential vectors to orbiviruses.

Apart from the high mutation rate owing to a polymerase without proofreading activity, reassortment of cognate genomic segments is another important driver of genetic diversity in viruses with segmented RNA genomes. This process can generate novel phenotypes with fundamental implications for immune escape, host or vector range, virulence and pathogenicity [536–539]. The ability to reassort genomic segments is also a primary criterion for inclusion in the *Reoviridae* family and it may have contributed to the great evolutionary success of these viruses. Most natural cases of orbivirus reassortment have been described in BTV, probably due to its antigenic diversity, wide geographic range and economic importance [77–79]. Additionally, reassortment has been described in Epizootic hemorrhagic disease virus (EHDV) [80,81], Corriparta virus (CORV) [540], Changuinola virus (CGLV) [534] or Banna

orbivirus (BAOV) [541]. We found that reassortment between LEAV strains involved segment-4 (VP4), segment-7 (T13) and segment-9 (VP6) in eight of the nine genomes analyzed (**Figure 24**). An additional reassortment event may have occurred in the NN28SUL16 strain, as a receiver of its the segment-3 (T2) from an unidentified LEAV donor strain. This is suggested by the striking sequence divergence of NN28SUL16 segment-3 within a highly similar (>98%) VP3 dataset. When translated to protein sequence, the identity of this segment with the rest of the LEAV homologs was ≥98.9%, confirming the necessity of its structural conservation.

Intragenic recombination between segments of LEAV was detected in the majority of LEAV strains (**Figure 24**). As in the case of sand fly-borne CGLV serogroup, the strain biodiversity could be an important factor for the exchange of RNA segment/fragment in LEAV [534]. This is indicated by detections of reassortment and intragenic recombination in eight, respectively seven, of nine LEAV strains. This is all the more clear for orbiviruses with great antigenic diversity like BTV [79,281,542], AHSV [543], but also for orthoreoviruses [544,545] and rotaviruses [546].

Although the phylogenetic analyses suggest that LEAV is possibly a midge-borne orbivirus, we found no viral RNA in *Culicoides* midges, mosquitoes and ticks from the study area. Additionally, no ectothermic hosts were detected in bloodfed *Culicoides* midges and mosquitoes (with the exception of an amphibian in the case of *Ur. unguiculata*).

Reoviruses that are known to infect reptiles belong to the turreted group of the family (subfamily *Spinareovirinae*, e.g. *Reptilian orthoreovirus*) and they cause severe illness of digestive and respiratory systems [547–549]. The prevalence of LEAV in grass snakes was 7.89% and the animals displayed no sign of disease. Curiously, the virus was absent from the sister species, *Natrix tessellata.* All attempts of growing and isolating LEAV on insect or vertebrate cell lines failed. The cells showed no cytopathic effect or silent replication of the virus. Many known reoviruses (and most orbiviruses) grow easily on vertebrate cells *in vitro*, while a few are restricted to insect cells [44,524,550]. Although LEAV isolation could be further attempted on additional cell

lines, it is possible that a shift in cell tropism underlies the inability of LEAV to infect certain cell types. A similar case is Parry's Lagoon virus (PLV), a serotype of CORV. In contrast to the wide range of vertebrate hosts of CORV [551], the antigenically-related PLV showed a distinct cell tropism and replicated only on insect cells [552]. With the available data one may speculate that such shifts in cell tropism could be the result of successive changes through recombination, genetic drift and shift. *Reoviridae* is a very successful family of segmented (ds)RNA viruses using a wide range of hosts, across various econiches. The family's repertoire of evolutionary strategies includes also deletion [532,553], gene duplication and concatemerisation [529,554]. These strategies were also observed previously in aquareoviruses [555], rotaviruses [556], phytoreoviruses [557] and even in a cross-family heterologous recombinant bat coronavirus containing reovirus genomic components [558]. These aspects seem to confirm the potential of reoviruses for "species jumps" and adaptation to new vectors and hosts [532,555].

Previous orbivirus research found that the overall GC content and the UTR proportion relative to the genome's length reflect three groups similar to those illustrated by phylogenetic clustering. The GC content was reported to be highest in TBOV with 52%-57.3% GC, followed by the C/SBOV with 39.9%-45.9% GC and the MBOV with 36.7%-41.6% GC [521,525,528,529]. The GC content of LEAV is below these intervals, having 34.6%-34.9% GC. In the case of UTRs, those of C/SBOV span over 3.5%-4.1% of their total genome length; in TBOV the UTRs are between ~4.5%-5% and in MBOV at ~5%-5.7% of the genome length [525,559,560]. Again, LEAV remains outside these limits, having the proportion of UTRs at 6.64% of the genome's length, which is higher than those reported in other orbiviruses.

The conserved Arg-Gly-Asp (^{167}RGD^{171}) motif on the T13 protein of BTV was associated with cell attachment in *Culicoides* vectors [561,562]. This conserved motif was also found by later studies in some species closely related to BTV [528,560,563]. Sequence analysis did not identify this motif in LEAV. As with other orbivirus species, this may reflect the higher divergence relative to BTV. Although the phylogenetic

analyses indicate LEAV as a potentially *Culicoides*-borne orbivirus, it is interesting to note some general inconsistencies in vector associations of IBOV (C/SBOV and MBOV). For example, phylogenetic analyses place Orungo virus (ORUV), Lembombo virus (LEBV), Pata virus (PATAV) and Japanaut virus (JAPV) in the C/SBOV clade, even though they were discovered in mosquitoes [517,564–567]. For ORUV, follow-up studies on mosquito transmission were inconclusive [566]. Interestingly, Tracambé virus (TRV), a serotype of the sand fly-borne CGLV serogroup was isolated from anopheline mosquitoes [568]. Some serotypes of C/SBOV have been isolated from both mosquitoes and biting midges: Eubenangee virus (EUBV) [517,567,569], Palyam virus (PALV) [570], Warrego virus WARV [517], Wongorr virus (WGRV) [571] and Tibet orbivirus (TIBOV) [522,560,572]. The associations between some viruses and more than one vector family could be the results of "species jumps" permitted by the fast evolution characteristic of RNA segmented viruses. Such occurrences in orbiviruses would not necessarily run counter to the "co-evolution" hypothesis, but rather complement it [528,529,535,555].

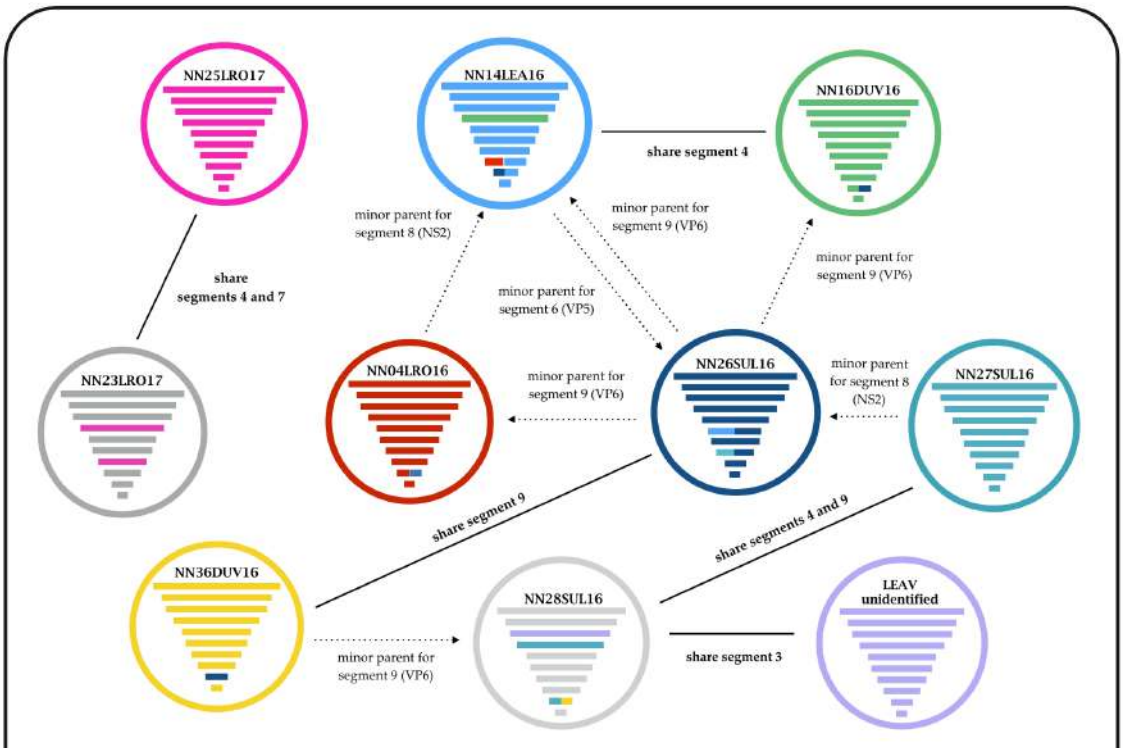


Figure 24. Graphical representations of gene reassortment and intragenic recombination between LEAV strains. Each colored circle represents a different LEAV strain. The 10 horizontal lines inside the circles represent the 10 genomic segments. Solid lines indicate reassortments of genes between different LEAV variants. Dashed arrows show the origins of gene fragments that have potentially been derived through recombination.

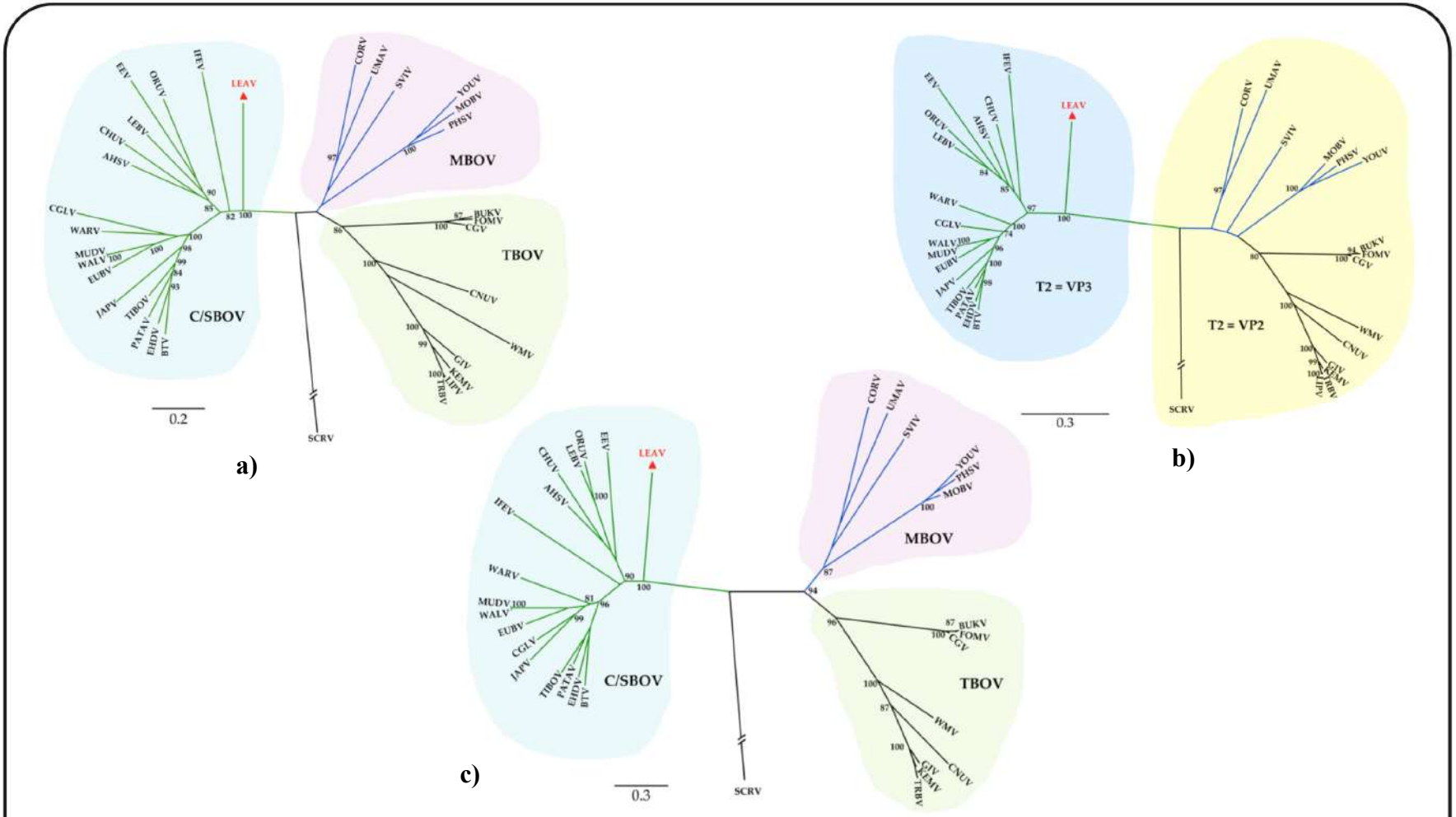


Figure 25. Maximum likelihood phylogenetic trees of the VP1 a), T13 (VP7) b) and T2 c) orbivirus proteins constructed using maximum likelihood inference and 100 bootstrap replicates. Bootstrap support of ≥80% is displayed at the nodes. Letea virus (LEAV) is indicated by the red triangle. C/SBOV stands for *Culicoides*-/sand fly-borne orbiviruses, MBOV stands for mosquito-borne orbiviruses and TBOV stands for tick-borne orbiviruses.

3.3 Vectors feeding patterns

3.3.1 Host-feeding pattern of mosquitoes

(based on Tomazatos et al., 2019)

Of the total mosquito collection, 3842 (0.59% of 655,677 individuals) were engorged specimens from 12 of the 16 identified taxa. The six dominant mosquito taxa that were found engorged had a relatively similar representation in the total collection. The successful analysis of 2331 individuals (60.7% of all engorged individuals) revealed a wide range of hosts fed upon by mosquitoes in the eastern parts of the DDBR (**Table 4, Table 5**). Large mammals (cattle, horses and wild boars) dominated the host spectrum. *Sus scrofa*, a frequently detected host, is considered to be wild boar, rather than domestic pig. The reason is that in the last years, at least in the study area, the pig became a rarity in local homesteads and the wild boar remains one of the most abundant wild mammals in the DDBR. Five of the six dominant mosquito taxa, *Coquillettidia richiardii, Anopheles hyrcanus, Anopheles maculipennis* s.l., *Aedes vexans* and *Aedes caspius,* were generalists with similar patterns of feeding. Their mammophilic feeding character was followed to smaller extents by anthropophily and ornithophily. These observations confirm what other studies have reported in North America [573,574] and Europe [108,329,334,355]. *Culex pipiens* s.l./*torrentium* (identified as *Cx. pipiens pipiens* f. *pipiens* in 51 of 88 females) fed on all three main host groups, overall taking more blood meals from non-human mammals than from birds and humans altogether. However, the feeding pattern of *Cx. pipiens* s.l. differs significantly from that of the other dominant taxa. Bird hosts were detected for this taxon in more than a third of the sequenced blood meals (**Figure 26a**). This taxon is considered the main vector of WNV in Romania and Europe [182,217] and was described by numerous studies as primarily ornithophilic [219,304,316,334,575,576]. Even so, the proportions of host groups detected for *Culex pipiens* s.l./*torrentium* are in agreement with other studies from Africa [302] and North America [573].

The host selection depends on numerous factors, both intrinsic (e.g., genetics, larval ecology) and extrinsic (e.g., available hosts). Even if genetic factors have

considerable weight in host selection, the choice might ultimately be determined by the physiological state or host abundance and availability [577]. Adult mosquitoes may also lack host specialization, feeding opportunistically on different host groups [578]. This would mean that the availability of vertebrate weighs more for host choice than intrinsic factors, and ultimately would be more important for pathogen transmission than species-specific host preferences [579]. Interestingly, when comparing the four sites, more blood meals originated from humans at Lake Roșuleț and Dunărea Veche, than in rural sites (Sulina and Letea) (**Figure 26b**). The sampling in natural sites was conducted at an old, isolated fishing cabin at Lake Roșuleț and at the edge a crop field on the banks of Danube (Dunărea Veche). The several workers who are present days on a row keep dogs and cats as permanent residents of these sites. Although wildlife hosts from the natural sites were detected most frequently, the proportion of detected human, cat and dog blood was larger compared to the inhabited (rural) sites (Letea and Sulina). This indicates that vertebrate availability plays an important role in mosquito host selection in DDBR. As regards dominance of abundant vertebrates among the overall detected hosts, it is noteworthy for this study that in the DDBR live several thousand feral horses and free-ranging cattle. To date, there is no official census, though an estimated four thousand horses and a few thousand free or owned cattle roam and reproduce freely in the DDBR. The authorities estimated the number of feral horses at 5000 individuals within the DDBR, of which 1000-2000 live around Letea [580]. However, these estimates have not been updated in the recent years. The feral animals have their origins in pre-1990 state-owned collective farms, but also private ones, from where they were released in recent decades. Cattle and horses were the most common species, accounting for more than 50% of the detected hosts. The high abundance in combination with large body size and defensive behavior might explain why both mammal species were facilitated so often [581].

The frequent feeding contacts with humans and other mammals can facilitate the transmission of other parasites, like filarial nematodes, especially in the case of

dominant taxa like *Coq. richiardii*, *An. hyrcanus*, *Cx. pipiens* f. *pipiens*, *Ae. vexans* and *Ae. caspius*. These mosquitoes were found positive for *Dirofilaria* spp. and *Setaria* spp. in DDBR [410,582] and in other European regions [356].

The success rate of host DNA amplification is known to vary widely, usually depending on the digestion status of the blood or storage method [583,584]. Amplification and sequencing of host DNA can also be problematic when a blood meal was acquired from different host species (mixed blood meals). Direct sequencing usually results in high background noise and ambiguous electropherograms, often with low sequence quality. Cloning is unfeasible for large sets of blood meals, being both labor-intensive and expensive. Although there are PCR strategies for overcoming limitations associated with mixed blood meals [328], the high-throughput sequencing could emerge as a methodological trend for the characterization of blood meals in unprecedented detail [585].

Danube Delta is a biodiverse and heterogeneous ecosystem complex that is known for its dynamic and rich bird and mosquito fauna [376,478]. For such reasons, it is surprising that birds were the least utilized group of hosts (n=85, 3.6% of all detected hosts). However, this study collected few specimens of the bird-biting *Cx. pipiens* s.l./*torrentium*. In previous studies conducted in the DDBR, >95% of mosquitoes captured with bird-baited traps belonged to this taxon, while the most abundant species in our study (*Coq. richiardii*) was absent or in very small numbers [188,191,480]. Thus, it is likely that the general sampling bias previously discussed for mosquito surveillance is also reflected in the collection of bloodfed mosquitoes.

Table 4. Frequency and percentage (in brackets) of detected hosts for the six most abundant mosquitoes and information on the overall proportion of each host detected in DDBR between 2014 and 2016

Host/mosquito spp.	***Coquillettidia richiardii***	***Anopheles hyrcanus***	***Anopheles maculipennis* s.l.**	***Aedes vexans***	***Aedes caspius***	***Culex pipiens* s.l./ *torrentium***	**Sum**
Anas platyrhynchos		3 (0.4)	1 (0.4)		1 (0.9)		5 (0.2)
Anatidae	1 (0.1)	12 (1.5)	4 (1.4)		1 (0.9)		18 (0.8)
Ardea purpurea	10 (1.2)		1 (0.4)				11 (0.5)
Circus aeroginosus			1 (0.4)				1 (0)
Corvus corone	1 (0.1)						1 (0)
Corvus fragilegus				1 (0.4)			1 (0)
Cyanistes caeruleus	5 (0.6)					3 (5.4)	8 (0.3)
Cygnus olor	1 (0.1)						1 (0)
Dendrocopos syriacus	1 (0.1)						1 (0)
Egretta garzetta	1 (0.1)						1 (0)
Falco tinnunculus						1 (1.8)	1 (0)
Gallus gallus	1 (0.1)			1 (0.4)			2 (0.1)
Hirundo rustica	2 (0.2)						2 (0.1)
Ixobrychus minutus						3 (5.4)	3 (0.1)
Locustella luscinoides						3 (5.4)	3 (0.1)
Motacilla alba	1 (0.1)						1 (0)
Netta rufina	1 (0.1)						1 (0)
Nycticorax nycticorax	4 (0.5)	2 (0.3)	2 (0.7)			7 (12.5)	15 (0.6)
Parus major						2 (3.6)	2 (0.1)
Pelecanus onocrotalus	1 (0.1)		1 (0.4)				2 (0.1)
Phalacrocorax carbo	1 (0.1)						1 (0)
Streptopelia orientalis			1 (0.4)				1 (0)
Strix aluco						2 (3.6)	2 (0.1)
Upupa epops						1 (1.8)	1 (0)
Homo sapiens	55 (6.7)	132 (16.7)	40 (14.3)	8 (3.5)	14 (13.2)	7 (12.5)	271 (11.5)

Table 4. (continued)

Host/mosquito spp.	*Coquillettidia richiardii*	*Anopheles hyrcanus*	*Anopheles maculipennis* s.l.	*Aedes vexans*	*Aedes caspius*	*Culex pipiens* s.l./ *torrentium*	Sum
Bovidae	2 (0.2)						2 (0.1)
Canis aureus	1 (0.1)	1 (0.1)			1 (0.9)		3 (0.1)
Canis lupus familiaris	62 (7.5)	10 (1.3)	19 (6.8)	7 (3)	4 (3.8)	8 (14.3)	111 (4.7)
Capra hircus	1 (0.1)	1 (0.1)					2 (0.1)
Capreolus capreolus	1 (0.1)		1 (0.4)				2 (0.1)
Chiroptera	2 (0.2)	1 (0.1)	1 (0.4)				4 (0.2)
Equus caballus	140 (16.9)	78 (9.9)	21 (7.5)	120 (52.2)	25 (23.6)	2 (3.6)	391 (16.7)
Erinaceus europaeus	1 (0.1)						1 (0)
Felis catus	34 (4.1)	3 (0.4)	7 (2.5)			1 (1.8)	47 (2)
Lepus europaeus	3 (0.4)			1 (0.4)	1 (0.9)	1 (1.8)	6 (0.3)
Lutra lutra	2 (0.2)	1 (0.1)					3 (0.1)
Microtus levis						1 (1.8)	1 (0)
Mustela lutreola	1 (0.1)						1 (0)
Mustela nivalis			1 (0.4)				1 (0)
Nyctereutes procyonoides	1 (0.1)						1 (0)
Ovis aries	8 (1)	4 (0.5)		2 (0.9)	2 (1.9)		16 (0.7)
Pipistrellus kuhlii	1 (0.1)						1 (0)
Rattus norvegicus	4 (0.5)					1 (1.8)	7 (0.3)
Rhinolophus hipposideros		1 (0.1)					1 (0)
Sus scrofa	299 (36.2)	28 (3.5)	25 (8.9)	12 (5.2)	11 (10.4)	2 (3.6)	382 (16.3)
Bloodfed specimens	**1,054**	**1,454**	**568**	**343**	**234**	**88**	**3,741**
Succesfully analyzed specimens[1]	**827**	**791**	**280**	**230**	**106**	**56**	**22,90**
Identified hosts per mosquito species[1]	**834**	**792**	**283**	**230**	**106**	**62**	**2,307**
Identified host taxa	**30**	**13**	**15**	**9**	**9**	**17**	

[1] the difference between the number of successfully analyzed specimens and identified hosts resulted from 17 mixed blood meals

Table 5. Frequency and percentage (in brackets) of detected hosts for the six least abundant mosquitoes and information on the overall proportion of each host detected in DDBR between 2014 and 2016.

Host/mosquito spp.	***Anopheles algeriensis***	***Culex modestus***	***Aedes flavescens***	***Aedes detritus***	***Aedes cinereus***	***Uranotaenia unguiculata***
Homo sapiens	9 (50)	3 (37.5)	2 (33.3)			1 (50)
Bos taurus	5 (27.8)	1 (12.5)		4 (80)	1 (33.3)	
Canis lupus		1 (12.5)				
Equus caballus	2 (11.1)			1 (20)	2 (66.7)	
Felis catus	2 (11.1)					
Rattus norvegicus		2 (25)				
Sus scrofa		1 (12.5)	4 (66.7)			
Pelophylax ridibundus						1 (50)
Bloodfed specimens	**29 (0.8)**	**36 (0.9)**	**7 (0.2)**	**6 (0.2)**	**3 (0.1)**	**2 (<0.1)**
Succesfully analyzed specimens	**18**	**8**	**6**	**5**	**3**	**2**
Identified hosts per mosquito species	**18**	**8**	**6**	**5**	**3**	**2**
Identified host taxa	**4**	**5**	**2**	**2**	**2**	**2**

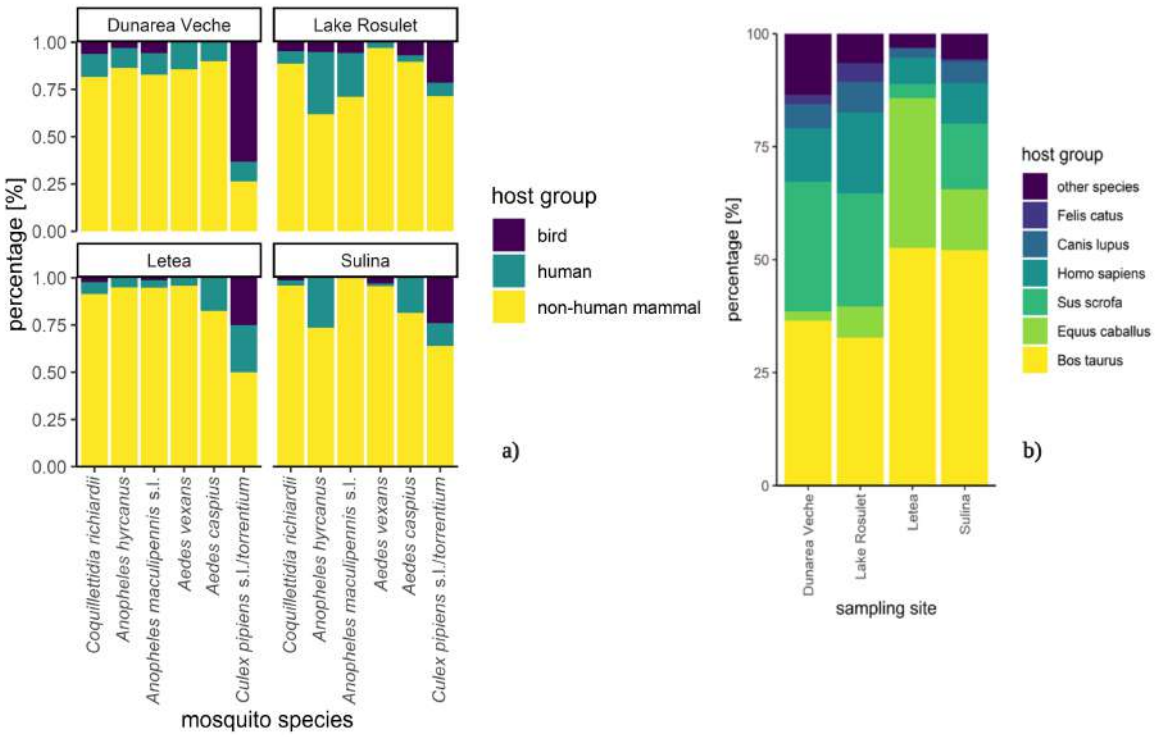


Figure 26. a) Percentage of host-feeding groups (birds, human, non-human mammals) of the six most abundant bloodfed mosquito taxa and b) percentage of main host species detected in two rural sites (Letea, Sulina) and the two natural sites (Dunărea Veche and Lake Roșuleț) in DDBR between 2014 and 2016.

3.3.1.1 Xenosurveillance of WNV using mosquito blood meals

(based on Tomazatos et al., 2019)

The use of haematophagous insects as "flying syringes" enables blood sampling in diverse vertebrate communities and the tracing of vector-host contacts in a natural context. By extending the concept from detection of the pathogen's nucleic acid to specific antibodies in hosts, the analysis of mosquito blood meals was able to inform on the circulation of WNV in the study area. Flavivirus-specific immunoglobulin G (IgG) was detected in all four sampling sites and for every year of mosquito trapping (2014-2016). Mosquito blood meals originating from horses and dogs were flavivirus-seropositive, but no antibodies were detected in blood meals taken from humans or birds. Seroprevalence in blood meals taken from dogs (6.3%) and horses (4.34%) was similar to direct observations made in dogs and horses from southern Europe [586–588]. Comparing our results with direct sampling conducted in

Romania, we found that the seroprevalence in horses was lower than what Popescu et al. [589] reported in horses (15.1%) or what Crivei et al. [189] found in dogs from eastern counties (42.1%).

Bloodfed mosquitoes containing flavivirus-specific antibodies belonged to taxa that dominated the entomological collection (**Table 6**). These mosquitoes were found positive for WNV RNA in previous work from DDBR, with the exception of *Ae. vexans*, which was found WNV-positive elsewhere and probably is a minor vector of this virus [112,186,481,576]. Considering the WNV-positive *Cx. pipiens* s.l./*torrentium* detected in 2014, active circulation of WNV in DDBR is also supported by flavivirus-specific antibodies detected in blood meals of these known WNV secondary vectors. Furthermore, the study confirmed the reliability of dogs as alternative sentinel species for WNV surveillance, as described by previous research [189,590].

Table 6. Bloodfed mosquitoes positive for flavivirus (WNV) IgG antibodies with information on the host species, sampling year and collection sites in the DDBR between 2014 and 2016.

Host	Mosquito	Year	Dunărea Veche	Lake Roșuleț	Letea	Sulina	Sum
horse	*Aedes vexans*	2016		1			1
	Anopheles hyrcanus	2016		3			3
	Anopheles maculipennis s.l.	2015			1	1	2
	Coquillettidia richiardii	2015			3	1	4
		2016	1	1			2
dog	*Aedes caspius*	2016	1				1
	Anopheles hyrcanus	2014			1		1
		2016		1		1[1]	2
	Anopheles maculipennis s.l.	2014		1			1
	Coquillettidia richiardii	2014				1	1
		2015		1			1
	Sum		**2**	**8**	**5**	**4**	**19**

[1]also positive for USUV-specific IgG

Xenosurveillance has obvious advantages, offering solutions to some logistical or ethical questions that may arise when attempting to sample diverse host communities and elusive or protected vertebrates. Using the mosquito blood meals collected between 2014 and 2016, we found some mammals known to be rare or which

have a cryptic behavior: raccoon dog (*Nyctereutes procyonoides*), European mink (*Mustela lutreola*), Eurasian otter (*Lutra lutra*) and Golden jackal (*Canis aureus*). One interesting bird host was the oriental turtle dove (*Streptopelia orientalis*). This species is considered a very rare occurrence in Europe [591]. Although its sequence identity showed 99% identity to references from Genbank, this specimen could be a hybrid with between *Streptopelia orientalis* and *Streptopelia turtur*. Such records provide a compelling argument for the use of a non-invasive sampling method like the blood meal analysis for identification and monitoring of species (and their infections), similar to that described by Martínez-de la Puente et al. [592] for the rare and elusive Iberian lynx (*Lynx pardinus*).

3.3.2 Blood meal analysis of *Culicoides* biting midges

(based on Tomazatos et al., 2020**c**)

Most of the analyzed *Culicoides* taxa had a broad host-feeding range, similar to that of mosquitoes. Mammal hosts were detected only for *Culicoides rieth*i, but the small sample size (n=12) does not allow an accurate conclusion on the species' feeding preferences. Both mammal and avian hosts were detected to various extents for all the other biting midges. The broad host choice matches previous studies, which reported similar results for different *Culicoides* spp. [593,594]. Humans and carrion crows were the only hosts of *C. submaritimus* (n=8) and cattle, wild boar or goat dominated the hosts spectra of the three most frequent *Culicoides* taxa: *C. punctatus*, *C. subfasciipennis*/*C. pallidicornis* and *C. griseidorsum*. The high frequency of cattle detection relates probably to the large number of free-roaming animals available in the DDBR and their large body mass [323]. However, despite this distinct dominance of mammal hosts, different avian hosts were detected for these three *Culicoides* taxa, similarly to previous reports [338,353,501,595].

Culicoides kibunensis is considered predominantly ornithophilic [305,357,596,597]. With 24 species of birds and nine species of mammals, this vector of avian malaria [305,597] showed the highest host diversity. The diversity of bird hosts is not surprising, considering the abundance and diversity of this group in the DDBR.

Nevertheless, the observed generalist host-feeding pattern that included humans was also observed by other studies in Europe [305,339,597]. Interestingly, the unknown *Culicoides* species showed a similar host range as *C. kibunensis* and both formed a monophyletic clade in the phylogenetic tree of taxa identification (**Figure 20**). These observations could indicate a positive correlation between phylogenetic relatedness and feeding behavior, as proposed by some authors [341,598]. In contrast, other studies speculated that such similarities in host-feeding patterns are not necessarily driven by phylogeny, but might be the result of other factors (e.g., body size-driven host choice due to larger emissions of CO_2 or volatile compounds) [593]. Although the monophily shown in **Figure 20** and similarities in host feeding are suggestive of such correlations, the analysis of a larger amount of samples could clarify these aspects.

The results indicate that host availability is a factor that shapes the observed host-*Culicoides* associations. Although no host census was conducted in the sampling sites, the number of humans and their pets in natural habitats (Lake Roșuleț and Dunărea Veche) is known and was constant during the sampling seasons. Humans, dogs and cats had low abundance at these sites compared to birds or free-ranging cattle and feral horses. Nonetheless, these hosts were detected for all analyzed *Culicoides* species, meaning that distribution of vectors and their potential hosts should be considered when interpreting host-feeding patterns of vectors. For example, a high proportion of *C. griseidorsum* were found to have fed on goats, but this host was widely available at Letea, where most of these biting midges were collected.

A small number of insects from Dunărea Veche were engorged with blood from buffalo and goat (**Table 8**). These hosts were only available in the nearest village, perhaps more than 4 km from the trapping site. Winds over the delta's flat landscape could help passive dispersal, as distances of more than 3 km covered during one night have been reported [289,290,599,600]. In this way, wind dispersal can enable the long-distance dispersal of *Culicoides* vectors and their associated pathogens [601].

The broad host range indicates that most of the analyzed *Culicoides* spp. are potential bridge vectors, despite their actual vector competence being largely

unknown. Similar to the study of mosquitoes, regular trapping of *Culicoides* spp. in core areas of DDBR revealed taxa new for the country and probably for the *Culicoides* genus (i.e. the unknown *Culicoides*). Cows and goats are known as BTV reservoirs, oftentimes asymptomatically infected [284,306]. Although other ruminants (goat, buffalo, roe deer) were far less bitten, the abundance and open range of cows could supplant the role of wild ruminants as BTV reservoirs in the area. *Culicoides punctatus* is targeted by surveillance as a potential vector of BTV in Italy [502] and SBV in Poland [300]**.** Considering the growing documentation of cryptic and new species [475,499,500,602] and the differences in BTV susceptibility between different *C. punctatus* populations [129], the risk of transmission of *Culicoides*-borne pathogens affecting both livestock and wildlife should not be ruled out in Danube Delta.

Table 7. Frequency and percentage (in brackets) of identified *Culicoides* biting midges and their vertebrate hosts in DDBR in 2017

Host	*C. griseidorsum*	*C. kibunensis*	*C. punctatus*	*C. riethi*	*C. subfasciipennis/ C. pallidicornis*	*C. submaritimus*	Unknown *Culicoides*	Host without *Culicoides* identity	Total hosts
Bos taurus	170 (63.9)	4 (5.2)	370 (81.3)	8 (80)	188 (83.2)	1 (11.1)	2 (11.1)	74 (68.5)	817 (70.7)
Bubalus bubalis			1 (0.2)		1 (0.4)				2 (0.2)
Canis lupus familiaris		4 (5.2)			3 (1.3)				7 (0.6)
Capra hircus	46 (17.3)	1 (1.3)	4 (0.9)	1 (10)	1 (0.4)				53 (4.6)
Capreolus capreolus		1 (1.3)							1 (0.1)
Equus caballus	15 (5.6)		12 (2.6)	1 (10)	4 (1.8)		1 (5.6)	5 (4.6)	38 (3.3)
Felis catus		1 (1.3)			1 (0.4)				2 (0.2)
Homo sapiens	3 (1.1)	10 (13)	9 (2)		4 (1.8)	5 (55.6)	2 (11.1)	10 (9.3)	43 (3.7)
Sus scrofa	28 (10.5)	3 (3.9)	45 (9.9)		22 (9.7)			3 (2.8)	101 (8.7)
Acrocephalus arundinaceus		1 (1.3)			1 (0.4)				2 (0.2)
Acrocephalus scirpaceus		13 (16.9)							13 (1.1)
Ardea cinerea		1 (1.3)							1 (0.1)
Ardea purpurea		6 (7.8)						1 (0.9)	7 (0.6)
Columba palumbus		1 (1.3)					1 (5.6)		2 (0.2)
Coracias garrulus		2 (2.6)					3 (16.7)		5 (0.4)
Corvus corone	1 (0.4)	6 (7.8)				3 (33.3)		6 (5.6)	16 (1.4)
Cyanistes caeruleus		4 (5.2)	1 (0.2)				1 (5.6)		6 (0.5)
Emberiza schoeniclus								1 (0.9)	1 (0.1)
Falco tinnunculus							1 (5.6)	1 (0.9)	2 (0.2)
Gallinula chloropus		3 (3.9)							3 (0.3)
Gallus gallus	2 (0.75)	2 (2.6)					2 (11.1)		6 (0.5)
Hirundo rustica							2 (11.1)		2 (0.2)
Meleagris gallopovo								1 (0.9)	1 (0.1)
Motacilla alba		1 (1.3)							1 (0.1)
Nycticorax nycticorax		1 (1.3)			1 (0.4)		1 (5.6)		3 (0.3)

Table 7. (continued)

Host	*C. griseidorsum*	*C. kibunensis*	*C. punctatus*	*C. riethi*	*C. subfasciipennis/ C. pallidicornis*	*C. submaritimus*	Unknown *Culicoides*	Host without *Culicoides* identity	Total hosts
Parus major		1 (1.3)						3 (2.8)	4 (0.4)
Passer montanus		1 (1.3)							1 (0.1)
Phalacrocorax carbo								2 (1.9)	2 (0.2)
Streptopelia decaocto							2 (11.1)		2 (0.2)
Strix aluco		1 (1.3)							1 (0.1)
Sylvia borin	1 (0.4)	4 (5.2)							5 (0.4)
Tito alba		1 (1.3)							1 (0.1)
Asio otus		2 (2.6)							2 (0.2)
Tito alba/Asio otus		2 (2.6)						1 (0.9)	3 (0.3)
Failed host detection	**11 (4)**	**26 (25.5)**	**13 (2.8)**	**2 (16.7)**	**19 (7.9)**	**1 (12.5)**	**21 (53.8)**		
Total biting midges	**276[a] (21.8)**	**102[b] (8.1)**	**455 (36)**	**12 (0.9)**	**242[c] (19.1)**	**8[d] (0.6%)**	**39[e] (3.1)**		

[a]contained one mixed blood meal: *B. taurus* + *G. gallus*
[b]contained one mixed blood meal: *S. scrofa* + *H. sapiens*
[c]contained three mixed blood meals: *B. taurus* + *C. l. familiaris; S. scrofa* + *A. arundinaceus; B. taurus* + *N. nycticorax*
[d]contained two mixed blood meals: *C. corone* + *H. sapiens*
[e]contained one mixed blood meal: *E. caballus* + *H. rustica*

4 Conclusions and outlook

The diversity, abundance and new taxonomic records of *Culicoides* and mosquitoes reflect the extent of research conducted so far in this delta, thereby highlighting both the gap and the need for further exploration. The new taxonomic records should not be surprising, even though the number of mosquito taxa recorded in this work is half of that known for DDBR [376]. This is probably related to landscape heterogeneity and the different ecological requirements of mosquitoes inhabiting the region. Previous entomological work has been conducted mainly in the western (Tulcea), southern (Razim-Sinoe lagoon) and central (Maliuc) parts of DDBR. Importantly, the logistics of surveillance studies are usually complex and expensive. This is especially the case for Danube Delta, where everything depends on a long and slow chain of supply, and travel or sampling is possible only by water.

Although this work provides a detailed overview of mosquito communities, the data is likely to be biased in favor of some of the less specialized taxa. The main reason is the use of one trap type in several ecosystems. A diversification of attractants and sampling sites may reduce this bias. This issue is not so evident in the case of *Culicoides* spp., mainly because the species composition was assessed using a limited number of bloodfed individuals plus a very small set of morphological vouchers. Considering the new records, the bird-biting unknown *Culicoides* and the presence of *C. puncticollis* only among the 37 (unfed) morphological vouchers, one may safely assume that the number of biting midge taxa in Danube Delta is substantially larger. As for their importance in pathogen transmission, the gap remains to be filled by future work.

The large mosaic of ecosystems, the number, type and location of traps can underestimate arbovirus presence, considering their focal character in non-epidemic years. As suggested also by epidemiological reports [91], the low detection rate WNV in our study resulted from sampling during the drier seasons of 2014-2016, when virus circulation was reduced in comparison to previous and latter transmission seasons.

The detection in only two sites during the second half of June 2014 was possible due to systematic trapping of a very large number of mosquitoes. Romania has an operational surveillance system for WNV, however full genome sequence data that would allow a higher phylogeographic resolution is missing. The implementation of an efficient integrated animal-human-vector surveillance program may be a challenging undertaking, requiring inter-institutional coordination, capacity building and long-term funding, but it is very much needed in the current epidemiological context. As seen throughout Europe (e.g., Italy, United Kingdom, Germany, Serbia, Hungary), such programs need to be tailored on the eco-epidemiological situation of the country. The activity must be scaled accordingly and surveillance conducted in a targeted manner, in order to save resources. DDBR remains a key area for the surveillance of arboviruses and other vector-borne pathogens due to its eco-geographical attributes, including its importance for long-distance migratory birds. Although this dissertation dealt with migratory birds only opportunistically, the collection of ticks during bird ringing found passerines to be frequently infested with *Hyalomma marginatum*. This CCHFV vector is known to travel with birds over long distances. In DDBR, an illustrative example is a WNV-positive immature attached to a song thrush (*Turdus philomelos*), that was mist netted at Enisala [162]. Such findings justify a special attention for birds and their parasitic associations, especially in the context of migration and environmental changes.

Virus discovery could strengthen surveillance work in biodiverse and little researched regions. The technological advancements of sequencing that allow unbiased deep sequencing, high-throughput sample screening and/or species barcoding have an unprecedented power for finding new zoonotic pathogens and disentangling transmission networks. In this dissertation, the discovery and characterization of an orbivirus in reptiles (LEAV) and an orthonairovirus in ticks (SULV) are two examples that offer a small, but interesting preview of the viral diversity harbored by the biotopes of Danube Delta. Unbiased characterization of viral diversity was outside the scope of this dissertation, but the results herein discussed

could offer some starting points for probing into the viromes of vectors and hosts. For example, blood-feeding patterns indicate the most frequent host-vector contacts and offer a blood sample of sufficient volume and quality (in most cases). This way, xenosurvaillance can be performed by specifically targeting associations that are frequent or known to sustain transmission. Data on the abundance, distribution, mobility or exposure of preferred hosts (dogs, cattle, feral and domestic horses, birds, humans and wild boars) could and should be used for prediction of outbreaks and guidance in virus discovery at rural-natural interface, where exposure and risk of zoonotic/enzootic transfer is higher.

5 References

1. Bairlein, F.; Dierschke, J.; Dierschke, V.; Salewski, V.; Geiter, O.; Hüppop, K.; Köppen, U.; Fiedler, W. *Atlas des Vogelzugs: Ringfunde Deutscher Brut- und Gastvögel*. **2014,** Aula-Verlag: Wiebelsheim, Germany.
2. Taylor, L.H.; Latham, S.M.; Woolhouse, M.E.J. Risk factors for human disease emergence. *Philos. Trans. R. Soc. B Biol. Sci.* **2001**, *356*, 983–989.
3. Karesh, W.B.; Dobson, A.; Lloyd-Smith, J.O.; Lubroth, J.; Dixon, M.A.; Bennett, M.; Aldrich, S.; Harrington, T.; Formenty, P.; Loh, E.H.; et al. Ecology of zoonoses: Natural and unnatural histories. *Lancet* **2012**, *380*, 1936–1945.
4. Bresalier M.; Cassidy A.; Woods A.; One Health in History. In *One Health: The Theory and Practice of Integrated Health Approaches*; Zinsstag, J.; Schelling, E.; Waltner-Toews, D.; Whittaker, M.; Tanner, M., Eds. CABI: Croydon, United Kingdom, **2015**; pp 1-15.
5. Destoumieux-Garzón, D.; Mavingui, P.; Boetsch, G.; Boissier, J.; Darriet, F.; Duboz, P.; Fritsch, C.; Giraudoux, P.; Roux, F. Le; Morand, S.; et al. The one health concept: 10 years old and a long road ahead. *Front. Vet. Sci.* **2018**, *5*:14.
6. Gubler, D.J. Dengue and dengue hemorrhagic fever. *Clin. Microbiol. Rev.* **1998**, *11*, 89–99.
7. Weaver, S.C.; Vasilakis, N. Molecular Evolution of Dengue Virus. *Infect. Genet. Evol.* **2009**, 523–540.
8. Bryant, J.E.; Holmes, E.C.; Barrett, A.D.T. Out of Africa: A Molecular Perspective on the Introduction of Yellow Fever Virus into the Americas. *PLoS Pathog.* **2007**, *3(5): e75.*
9. Chippaux, J.P.; Chippaux, A. Yellow fever in Africa and the Americas: A historical and epidemiological perspective. *J. Venom. Anim. Toxins Incl. Trop. Dis.* **2018**, *24:20.*
10. Castelli, F.; Sulis, G. Migration and infectious diseases. *Clin. Microbiol. Infect.* **2017**, *23*, 283–289.
11. McMichael, C. Climate change-related migration and infectious disease. *Virulence* **2015**, *6*, 548–553.
12. Vasconcelos, P.F.; Travassos da Rosa, A.P.; Rodrigues, S.G.; Travassos da Rosa, E.S.; Dégallier, N.; Travassos da Rosa, J.F. Inadequate management of natural ecosystem in the Brazilian Amazon region results in the emergence and reemergence of arboviruses. *Cad. saúde pública / Ministério da Saúde, Fundação Oswaldo Cruz, Esc. Nac. Saúde Pública* **2001**, *17 Suppl*, 155–164.
13. Patz, J.A.; Graczyk, T.K.; Geller, N.; Vittor, A.Y. Environmental Changes & Parasitic Diseases. *Int. J. Parasitol.* **2000**, *30*, 1395–1405.
14. Burkett-Cadena, N.D.; Vittor, A.Y. Deforestation and vector-borne disease: Forest conversion favors important mosquito vectors of human pathogens. *Basic and Applied Ecology* **2018**, *26*, 101-110.
15. Vittor, A.Y.; Gilman, R.H.; Tielsch, J.; Glass, G.; Shields, T.; Lozano, W.S.; Pinedo-Cancino, V.; Patz, J.A. The effect of deforestation on the human-biting rate of *Anopheles darlingi*, the primary vector of *Falciparum* malaria in the Peruvian Amazon. *Am. J. Trop. Med. Hyg.* **2006**, *74*, 3–11.
16. Gubler, D.J. Dengue, urbanization and globalization: The unholy trinity of the 21st century. *Trop. Med. Health* **2011**, *39*, 3–11.
17. Chancey, C.; Grinev, A.; Volkova, E.; Rios, M. The global ecology and epidemiology of West Vile virus. *Biomed Res. Int.* **2015**, 376230.
18. Klitting, R.; Fischer, C.; Drexler, J.F.; Gould, E.A.; Roiz, D.; Paupy, C.; de Lamballerie, X. What does the future hold for Yellow fever virus? (II). *Genes (Basel).* **2018**, *9*, 12–16.
19. Young, P.R. Arboviruses: A Family on the Move. *Adv. Exp. Med. Biol.* **2018**, *1062*, 165–173.
20. Eldridge, B.F. Mosquitoes, the *Culicidae*. In *The Biology of Disease Vectors*, 2nd ed.; Marquardt, W.C., Ed.; Elsevier Academic Press: Burlington, MA; **2005**, pp. 95-111.
21. Munstermann, L.E. Phlebotomine Sand Flies, the *Psychodidae*. In *The Biology of Disease Vectors*, 2nd ed.; Marquardt, W.C., Ed.; Elsevier Academic Press: Burlington, MA; **2005**; pp. 141-151.
22. Borkent, A. The biting midges, the *Ceratopogonidae* (Diptera). In *The Biology of Disease Vectors*, 2nd ed.; Marquardt, W.C., Ed.; Elsevier Academic Press: Burlington, MA; **2005**; pp. 113-126.

23. Klompen, H. Ticks, the *Ixodida*. In *The Biology of Disease Vectors*, 2nd ed.; Marquardt, W.C., Ed.; Elsevier Academic Press: Burlington, MA; **2005**; pp. 45-55.
24. Farajollahi, A.; Crans, W.J.; Nickerson, D.; Bryant, P.; Wolf, B.; Glaser, A.; Andreadis, T.G. Detection of West Nile Virus RNA From the Louse Fly *Icosta Americana* (Diptera: Hippoboscidae). *J. Am. Mosq. Control Assoc.* **2005**, *21*, 474–476.
25. Gancz, A.Y.; Barker, I.K.; Lindsay, R.; Dibernardo, A.; McKeever, K.; Hunter, B. West Nile virus outbreak in North American owls, Ontario, 2002. *Emerg. Infect. Dis.* **2004**, *10*, 2135–2142.
26. La Linn, M.; Gardner, J.; Warrilow, D.; Darnell, G.A.; McMahon, C.R.; Field, I.; Hyatt, A.D.; Slade, R.W.; Suhrbier, A. Arbovirus of Marine Mammals: a New Alphavirus Isolated from the Elephant Seal Louse, *Lepidophthirus macrorhini*. *J. Virol.* **2001**, *75*, 4103–4109.
27. Mumcuoglu, K.Y.; Banet-Noach, C.; Malkinson, M.; Shalom, U.R.I.; Galun, R. Argasid Ticks as Possible Vectors of West Nile Virus in Israel. *Vector-Borne Zoonotic Dis.* **2005**, *5*, 65–71.
28. Adelman, Z.N.; Miller, D.M.; Myles, K.M. Bed Bugs and Infectious Disease: A Case for the Arboviruses. *PLoS Pathog.* **2013**, *9(8): e1003462.*
29. Gage, K.L. Fleas, the *Siphonaptera*. In *The Biology of Disease Vectors*, 2nd ed.; Marquardt, W.C., Ed.; Elsevier Academic Press: Burlington, MA; **2005**; pp. 77-92.
30. Hall, R.A.; Hall-Medelin, S.; Hobson-Peters, J.; Prow, N.A; Mackenzie, J.S. Ecological and Epidemiological Factors Influencing Arbovirus Diversity, Evolution and Spread. In *Arboviruses: Molecular Biology, Evolution and Control*; Vasilakis, N., Gubler, D.J, Eds. Poole, UK: Cairster Academic Press, **2016**; pp. 135-166.
31. Gubler, D.J; Vasilakis, N. The Arboviruses: Quo Vadis? In *Arboviruses: Molecular Biology, Evolution and Control*; Vasilakis, N., Gubler, D.J, Eds. Poole, UK: Cairster Academic Press, **2016**; pp. 1-6.
32. Carver, S.; Bestall, A.; Jardine, A.; Ostfeld, R.S. Influence of hosts on the ecology of arboviral transmission: Potential mechanisms influencing dengue, Murray Valley encephalitis, and Ross River virus in Australia. *Vector-Borne Zoonotic Dis.* **2009**, *9*, 51–64.
33. Kuno, G.; Mackenzie, J.S.; Junglen, S.; Hubálek, Z.; Plyusnin, A.; Gubler, D.J. Vertebrate reservoirs of arboviruses: Myth, synonym of amplifier, or reality? *Viruses* **2017**, *9, 185*.
34. Go, Y.Y.; Balasuriya, U.B.R.; Lee, C. Zoonotic encephalitides caused by arboviruses: transmission and epidemiology of alphaviruses and flaviviruses. *Clin. Exp. Vaccine Res.* **2014**, *3*, 58–77.
35. Arrigo, N.C.; Weaver, S.C.; Calisher, C. The Taxonomy of Arboviruses. In *Arboviruses: Molecular Biology, Evolution and Control*; Vasilakis, N., Gubler, D.J, Eds. Cairster Academic Press: Poole, UK; **2016**; pp. 9-29.
36. King, A.M.Q.; Adams, M.J.; Carstens, E.B.; Lefkowitz, E.J. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*; Elsevier Academic Press: San Diego, CA, **2012**; ISBN 9780123846846.
37. Attoui, H.; Stirling, J.M.; Munderloh, U.G.; Billoir, F.; Brookes, S.M.; Burroughs, J.N.; de Micco, P.; Mertens, P.P.C.; de Lamballerie, X. Complete sequence characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of *Ixodes scapularis*. *J. Gen. Virol.* **2001**, *82*, 795–804.
38. Li, C.X.; Shi, M.; Tian, J.H.; Lin, X.D.; Kang, Y.J.; Chen, L.J.; Qin, X.C.; Xu, J.; Holmes, E.C.; Zhang, Y.Z. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. *Elife* **2015**, *4:e05378*.
39. Porter, A.F.; Shi, M.; Eden, J.; Zhang, Y.; Holmes, E.C. Diversity and Evolution of Novel Invertebrate DNA Viruses Revealed by Meta-Transcriptomics. *Viruses* **2019**, *11*, 1092.
40. Shi, M.; Lin, X.D.; Tian, J.H.; Chen, L.J.; Chen, X.; Li, C.X.; Qin, X.C.; Li, J.; Cao, J.P.; Eden, J.S.; et al. Redefining the invertebrate RNA virosphere. *Nature* **2016**, *540*, 539–543.
41. Shi, M.; Zhang, Y.Z.; Holmes, E.C. Meta-transcriptomics and the evolutionary biology of RNA viruses. *Virus Res.* **2018**, *243*, 83–90.
42. Zhang, Y.Z.; Shi, M.; Holmes, E.C. Using Metagenomics to Characterize an Expanding Virosphere. *Cell* **2018**, *172*, 1168–1172.
43. Blitvich, B.J.; Firth, A.E. Insect-specific flaviviruses: A systematic review of their discovery, host

range, mode of transmission, superinfection exclusion potential and genomic organization; *Viruses* **2015**, *7*, 1927-1959

44. Auguste, A.J.; Kaelber, J.T.; Fokam, E.B.; Guzman, H.; Carrington, C.V.F.; Erasmus, J.H.; Kamgang, B.; Popov, V.L.; Jakana, J.; Liu, X.; et al. A Newly Isolated Reovirus Has the Simplest Genomic and Structural Organization of Any Reovirus. *J. Virol.* **2015**, *89*, 676–687.
45. Calzolari, M.; Zé-Zé, L.; Růžek, D.; Vázquez, A.; Jeffries, C.; Defilippo, F.; Osório, H.C.; Kilian, P.; Ruíz, S.; Fooks, A.R.; et al. Detection of mosquito-only flaviviruses in Europe. *J. Gen. Virol.* **2012**, *93*, 1215–1225.
46. Junglen, S.; Korries, M.; Grasse, W.; Wieseler, J.; Kopp, A.; Hermanns, K.; León-Juárez, M.; Drosten, C.; Kümmerer, B.M. Host Range Restriction of Insect-Specific Flaviviruses Occurs at Several Levels of the Viral Life Cycle. *mSphere* **2017**, *2*, e00375-16.
47. Marklewitz, M.; Zirkel, F.; Rwego, I.B.; Heidemann, H.; Trippner, P.; Kurth, A.; Kallies, R.; Briese, T.; Lipkin, W.I.; Drosten, C.; et al. Discovery of a Unique Novel Clade of Mosquito-Associated Bunyaviruses. *J. Virol.* **2013**, *87*, 12850–12865.
48. Nasar, F.; Palacios, G.; Gorchakov, R. V.; Guzman, H.; Travassos Da Rosa, A.P.; Savji, N.; Popov, V.L.; Sherman, M.B.; Lipkin, W.I.; Tesh, R.B.; et al. Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 14622–14627.
49. Bolling, B.G.; Olea-Popelka, F J.; Eisen, L.; Moore, G.M.; Blair, C. Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. *Virology* **2012**, *427*, 90–97.
50. Fort, P.; Albertini, A.; Van-Hua, A.; Berthomieu, A.; Roche, S.; Delsuc, F.; Pasteur, N.; Capy, P.; Gaudin, Y.; Weill, M. Fossil rhabdoviral sequences integrated into arthropod genomes: Ontogeny, evolution, and potential functionality. *Mol. Biol. Evol.* **2012**, *29*, 381–390.
51. Saiyasombat, R.; Bolling, B.G.; Brault, A.C.; Bartholomay, L.C.; Blitvich, B.J. Evidence of Efficient Transovarial Transmission of *Culex* Flavivirus by *Culex pipiens* (Diptera: Culicidae) . *J. Med. Entomol.* **2011**, *48*, 1031–1038.
52. Bolling, B.G.; Eisen, L.; Moore, C.G.; Blair, C.D. Insect-specific flaviviruses from *Culex* mosquitoes in Colorado, with evidence of vertical transmission. *Am. J. Trop. Med. Hyg.* **2011**, *85*, 169–177.
53. Nasar, F.; Gorchakov, R. V.; Tesh, R.B.; Weaver, S.C. Eilat Virus Host Range Restriction Is Present at Multiple Levels of the Virus Life Cycle. *J. Virol.* **2015**, *89*.
54. Hall-Mendelin, S.; McLean, B.J.; Bielefeldt-Ohmann, H.; Hobson-Peters, J.; Hall, R.A.; Van Den Hurk, A.F. The insect-specific Palm Creek virus modulates West Nile virus infection in and transmission by Australian mosquitoes. *Parasites and Vectors* **2016**, *9*, 1–10.
55. Kenney, J.L.; Solberg, O.D.; Langevin, S.A.; Brault, A.C. Characterization of a novel insect-specific flavivirus from Brazil: Potential for inhibition of infection of arthropod cells with medically important flaviviruses. *J. Gen. Virol.* **2014**, *95*, 2796–2808.
56. Öhlund, P.; Lundén, H.; Blomström, A.L. Insect-specific virus evolution and potential effects on vector competence. *Virus Genes* **2019**, *55*, 127–137.
57. Vasilakis, N.; Tesh, R.B. Insect-specific viruses and their potential impact on arbovirus transmission. *Curr. Opin. Virol.* **2015**, *15*, 69–74.
58. Lauring, A.S.; Andino, R. Quasispecies theory and the behavior of RNA viruses. *PLoS Pathog.* **2010**, *6(7): e1001005*.
59. Holmes, E.C. *The Evolution and Emergence of RNA Viruses*. Oxford University Press: New York, USA, **2009**, pp 37-52.
60. Gregori, J.; Perales, C.; Rodriguez-Frias, F.; Esteban, J.I.; Quer, J.; Domingo, E. Viral quasispecies complexity measures. *Virology* **2016**, *493*, 227–237.
61. Sanjuán, R.; Domingo-Calap, P. Mechanisms of viral mutation. *Cell. Mol. Life Sci.* **2016**, *73*, 4433–4448.
62. Eigen, M. Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften* **1971**, *58*, 465–523.

63. Eigen, M. Viral quasispecies. *Sci. Am.* July **1993**.
64. Eigen, M. *The Hypercycle: A Principle of Natural Self-Organization*; Springer-Verlag: Heidelberg-Berlin, Germany, **1979**; pp. 15-25.
65. Andino, R.; Domingo, E. Viral quasispecies. *Virology* **2015**, *479–480*, 46–51.
66. Biebricher, C.K.; Eigen, M. What is a quasispecies? *Curr. Top. Microbiol. Immunol.* **2006**, *299*, 1–31.
67. Eigen, M. On the nature of virus quasispecies. *Trends Microbiol.* **1996**, *4*, 216–218.
68. Grubaugh, N.D.; Weger-Lucarelli, J.; Murrieta, R.A.; Fauver, J.R.; Garcia-Luna, S.M.; Prasad, A.N.; Black, W.C.; Ebel, G.D. Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching. *Cell Host Microbe* **2016**, *19*, 481–492.
69. Grubaugh, N.D.; Fauver, J.R.; Rückert, C.; Weger-Lucarelli, J.; Garcia-Luna, S.; Murrieta, R.A.; Gendernalik, A.; Smith, D.R.; Brackney, D.E.; Ebel, G.D. Mosquitoes Transmit Unique West Nile Virus Populations during Each Feeding Episode. *Cell Rep.* **2017**, *19*, 709–718.
70. Jerzak, G.V.S.; Bernard, K.; Kramer, L.D.; Shi, P.Y.; Ebel, D. The West Nile virus mutant spectrum is host-dependant and a Determinant of Mortality in Mice. **2007**, *360*, 469–476.
71. Vignuzzi, M.; Stone, J.K.; Arnold, J.J.; Cameron, C.E.; Andino, R. Quasispecies diversity determines pathogenesis through Cooperative Interactions Within a Viral Population. *Nature* **2006**, *439*, 344–348.
72. Worobey, M.; Holmes, E.C. Evolutionary aspects of recombination in RNA viruses. *J. Gen. Virol.* **1999**, *80*, 2535–2543.
73. Stapleford K.A.; Moratorio, G.; Vignuzzi, M. Genetic Diversity of Arboviruses. In *Arboviruses: Molecular Biology, Evolution and Control*; Vasilakis, N., Gubler, D.J, Eds. Cairster Academic Press: Poole, UK; **2016**; pp. 121-134.
74. Briese, T.; Calisher, C.H.; Higgs, S. Viruses of the family *Bunyaviridae*: Are all available isolates reassortants? *Virology* **2013**, *446*, 207–216.
75. McDonald, S.M.; Nelson, M.I.; Turner, P.E.; Patton, J.T. Reassortment in segmented RNA viruses: Mechanisms and outcomes. *Nat. Rev. Microbiol.* **2016**, *14*, 448–460.
76. Varsani, A.; Lefeuvre, P.; Roumagnac, P.; Martin, D. Notes on recombination and reassortment in multipartite/segmented viruses. *Curr. Opin. Virol.* **2018**, *33*, 156–166.
77. Hornyák, Á.; Malik, P.; Marton, S.; Dóró, R.; Cadar, D.; Bányai, K. Emergence of multireassortant bluetongue virus serotype 4 in Hungary. *Infect. Genet. Evol.* **2015**, *33*, 6–10.
78. Nomikou, K.; Hughes, J.; Wash, R.; Kellam, P.; Breard, E.; Zientara, S.; Palmarini, M.; Biek, R.; Mertens, P. Widespread Reassortment Shapes the Evolution and Epidemiology of Bluetongue Virus following European Invasion. *PLoS Pathog.* **2015**, *11(8): e1005056.*
79. Shaw, A.E.; Ratinier, M.; Nunes, S.F.; Nomikou, K.; Caporale, M.; Golder, M.; Allan, K.; Hamers, C.; Hudelet, P.; Zientara, S.; et al. Reassortment between Two Serologically Unrelated Bluetongue Virus Strains Is Flexible and Can Involve any Genome Segment. *J. Virol.* **2013**, *87*, 543–557.
80. Allison, A.B.; Goekjian, V.H.; Potgieter, A.C.; Wilson, W.C.; Johnson, D.J.; Mertens, P.P.C.; Stallknecht, D.E. Detection of a novel reassortant Epizootic hemorrhagic disease virus (EHDV) in the USA containing RNA segments derived from both exotic (EHDV-6) and endemic (EHDV-2) serotypes. *J. Gen. Virol.* **2010**, *91*, 430–439.
81. Allison, A.B.; Holmes, E.C.; Potgieter, A.C.; Wright, I.M.; Sailleau, C.; Breard, E.; Ruder, M.G.; Stallknecht, D.E. Segmental configuration and putative origin of the reassortant orbivirus, Epizootic hemorrhagic disease virus serotype 6, strain Indiana. *Virology* **2012**, *424*, 67–75.
82. Ciota, A.T., Ebel, G.D. Role of Inter- and Intra-host Genetics in Arbovirus Evolution. In *Arboviruses: Molecular Biology, Evolution and Control*; Vasilakis, N., Gubler, D.J, Eds. Cairster Academic Press: Poole, UK; **2016**; pp. 167-174.
83. Goldberg, T.L.; Anderson, T.K.; Hamer, G.L. West Nile virus may have hitched a ride across the Western United States on *Culex tarsalis* mosquitoes. *Mol. Ecol.* **2010**, *19*, 1518–1519.
84. Moudy, R.M.; Meola, M.A.; Morin, L.L.L.; Ebel, G.D.; Kramer, L.D. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes. *Am. J. Trop.*

Med. Hyg. **2007**, *77*, 365–370.
85. Brault, Aaron C, Claire Y-H Huang, Stanley A Langevin, Richard M Kinney, Richard A Bowen, Wanichaya N Ramey, Nicholas A Panella, Edward C Holmes, Ann M Powers, B.R.M. A single positively selected West Nile viral mutation confers increased virogenesis in American crows. *Nat. Genet*. **2007**; 39(9): 1162–1166.
86. De Lamballerie, X.; Leroy, E.; Charrel, R.N.; Ttsetsarkin, K.; Higgs, S.; Gould, E.A. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: A sign of things to come? *Virol. J.* **2008**, *5:33*.
87. Weaver, S.C.; Chen, R.; Diallo, M. Chikungunya Virus: Role of Vectors in Emergence from Enzootic Cycles. *Annu. Rev. Entomol.* **2019**, *65: 15.1–15.20*.
88. Boyles, S.M.; Mavian, C.N.; Finol, E.; Ukhanova, M.; Stephenson, C.; Hamerlinck, G.; et al. Under-the-radar dengue virus infections in natural populations of *Aedes aegypti* mosquitoes. *mSphere* **2020**, 5(2): e00316-20
89. Bustamante, D.M.; Lord, C.C. Sources of error in the estimation of mosquito infection rates used to assess risk of arbovirus transmission. *Am. J. Trop. Med. Hyg.* **2010**, *82*, 1172–1184.
90. Barzon, L. Ongoing and emerging arbovirus threats in Europe. *J. Clin. Virol.* **2018**, *107*, 38–47.
91. European Centre for Disease Prevention and Control. West Nile virus infection. In: ECDC. Annual Epidemiological Report for 2018. Stockholm: ECDC, **2019**.
92. Jöst, H.; Bialonski, A.; Schmetz, C.; Günther, S.; Becker, N.; Schmidt-Chanasit, J. Short report: Isolation and phylogenetic analysis of Batai virus, Germany. *Am. J. Trop. Med. Hyg.* **2011**, *84*, 241–243.
93. Hofmann, M.; Wiethölter, A.; Blaha, I.; Jöst, H.; Heinemann, P.; Lehmann, M.; Miller, T.; Cadar, D.; Yanase, T.; Kley, N.; et al. Surveillance of Batai virus in bovines from Germany. *Clin. Vaccine Immunol.* **2015**, *22*, 672–673.
94. Hesson, J.C.; Verner-Carlsson, J.; Larsson, A.; Ahmed, R.; Lundkvist, Å.; Lundström, J.O. Culex torrentium mosquito role as major enzootic vector defined by rate of Sindbis virus infection, Sweden, 2009. *Emerg. Infect. Dis.* **2015**, *21*, 875–878.
95. Jöst, H.; Bialonski, A.; Storch, V.; Günther, S.; Becker, N.; Schmidt-Chanasit, J. Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. *J. Clin. Microbiol.* **2010**, *48*, 1900–1903.
96. Ziegler, U.; Fischer, D.; Eiden, M.; Reuschel, M.; Rinder, M.; Kerstin, M.; Schwehn, R.; Schmidt, V.; Groschup, M.H.; Keller, M. Sindbis virus – a wild bird associated zoonotic arbovirus circulates in Germany. *Vet. Microbiol.* **2019**, S0378-1135(19)30670-4
97. Jöst, H.; Bialonski, A.; Maus, D.; Sambri, V.; Eiden, M.; Groschup, M.H.; Günther, S.; Becker, N.; Schmidt-Chanasit, J. Isolation of Usutu virus in Germany. *Am. J. Trop. Med. Hyg.* **2011**, *85*, 551–553.
98. Roesch, F.; Fajardo, A.; Moratorio, G.; Vignuzzi, M. Usutu Virus: An Arbovirus on the Rise. *Viruses* **2019**, *11, 640*.
99. Cadar, D.; Becker, N.; de Mendonca Campos, R.; Börstler, J.; Jöst, H.; Schmidt-Chanasit, J. Usutu virus in bats, Germany, 2013. *Emerg. Infect. Dis.* **2014**, *20*, 1771–1773.
100. Allering, L.; Jöst, H.; Emmerich, P.; Günther, S.; Lattwein, E.; Schmidt, M.; Seifried, E.; Sambri, V.; Hourfar, K.; Medizinische, E.; et al. Detection of Usutu virus infection in a healthy blood donor from south-west Germany , 2012. *Euro Surveill.* **2012**, *17(50):pii=20341.*
101. Bakonyi, T.; Jungbauer, C.; Aberle, S.W.; Kolodziejek, J.; Dimmel, K.; Stiasny, K.; Allerberger, F.; Nowotny, N. Usutu virus infections among blood donors, Austria, July and August 2017 – Raising awareness for diagnostic challenges. *Euro Surveill.* **2017**, *22(41):pii=17-00644.*
102. Zaaijer, H.L.; Slot, E.; Molier, M.; Reusken, C.B.E.M.; Koppelman, M.H.G.M. Usutu virus infection in Dutch blood donors. *Transfusion* **2019**, 9999;1–7.
103. Ziegler, U.; Santos, P.D.; Groschup, M.H.; Hattendorf, C.; Eiden, M.; Höper, D.; Eisermann, P.; Keller, M.; Michel, F.; Klopfleisch, R.; et al. West Nile Virus Epidemic in Germany Triggered by Epizootic Emergence , 2019. *Viruses* **2020**, *12, 448*.
104. Aberle, S.W.; Kolodziejek, J.; Jungbauer, C.; Stiasny, K.; Aberle, J.H.; Zoufaly, A.; Hourfar, M.K.;

Weidner, L.; Nowotny, N. Increase in human West nile and Usutu virus infections, Austria, 2018. *Euro Surveill.* **2018**, *23(43):pii=1800545*.

105. Nagy, A.; Mezei, E.; Nagy, O.; Bakonyi, T.; Csonka, N.; Kaposi, M.; Koroknai, A. Extraordinary increase in West Nile virus cases and first confirmed human Usutu virus infection in Hungary, 2018. *Euro Surveill.* **2019**, *24(28):pii=1900038*.
106. Pacenti, M.; Sinigaglia, A.; Martello, T.; Rui, M.E. De; Franchin, E.; Pagni, S.; Riccetti, S.; Milani, A.; Montarsi, F.; Capelli, G.; et al. Clinical and virological findings in patients with Usutu virus infection, northern Italy , 2018. *Euro Surveill.* **2019**, *24(47):pii=1900180*.
107. Engler, O.; Savini, G.; Papa, A.; Figuerola, J.; Groschup, M.H.; Kampen, H.; Medlock, J.; Vaux, A.; Wilson, A.J.; Werner, D.; et al. European surveillance for West Nile virus in mosquito populations. *Int. J. Environ. Res. Public Health* **2013**, *10*, 4869–4895.
108. Börstler, J.; Jöst, H.; Garms, R.; Krüger, A.; Tannich, E.; Becker, N.; Schmidt-Chanasit, J.; Lühken, R. Host-feeding patterns of mosquito species in Germany. *Parasites and Vectors* **2016**, *9*:318.
109. Gossner, C.M.; Marrama, L.; Carson, M.; Allerberger, F.; Calistri, P.; Dilaveris, D.; Lecollinet, S.; Morgan, D.; Nowotny, N.; Paty, M.; et al. West Nile virus surveillance in Europe: Moving towards an integrated animal-human-vector approach. *Euro Surveill.* **2017**, *22(18):pii=30526*.
110. Michel, F.; Fischer, D.; Eiden, M.; Fast, C.; Reuschel, M.; Müller, K.; Rinder, M.; Urbaniak, S.; Brandes, F.; Schwehn, R.; et al. West Nile virus and Usutu virus monitoring of wild birds in Germany. *Int. J. Environ. Res. Public Health* **2018**, *15, 171*.
111. Cristina Radaelli, M.; Verna, F.; Pautasso, A.; Bellavia, V.; Ballardini, M.; Mignone, W.; Masoero, L.; Dondo, A.; Picco, L.; Moschi, R.; et al. Mosquito-Borne Diseases and 'One Health': The Northwestern Italian Experience. *Curr. Top. Trop. Emerg. Dis. Travel Med.* **2018**, *52-80.*
112. Petrić, D.; Petrović, T.; Hrnjaković Cvjetković, I.; Zgomba, M.; Milošević, V.; Lazić, G.; Ignjatović Ćupina, A.; Lupulović, D.; Lazić, S.; Dondur, D.; et al. West Nile virus 'circulation' in Vojvodina, Serbia: Mosquito, bird, horse and human surveillance. *Mol. Cell. Probes* **2017**, *31*, 28–36.
113. Petrović, T.; Šekler, M.; Petrić, D.; Lazić, S.; Debeljak, Z.; Vidanović, D.; Ćupina, A.I.; Lazić, G.; Lupulović, D.; Kolarević, M.; et al. Methodology and results of integrated WNV surveillance programmes in Serbia. *PLoS One* **2018**, *13(4): e0195439*.
114. Zana, B. Multi-Approach Investigation Regarding the West Nile Virus Situation in Hungary, 2018. *Viruses* **2020**, *12, 123.*
115. Ballardini, M.; Ferretti, S.; Chiaranz, G.; Pautasso, A.; Riina, M.V.; Triglia, G.; Verna, F.; Bellavia, V.; Radaelli, M.C.; Berio, E.; et al. First report of the invasive mosquito *Aedes koreicus* (Diptera: Culicidae) and of its establishment in Liguria, northwest Italy. *Parasites and Vectors* **2019**, *12*:*334*.
116. Capelli, G.; Drago, A.; Martini, S.; Montarsi, F.; Soppelsa, M.; Delai, N.; Ravagnan, S.; Mazzon, L.; Schaffner, F.; Mathis, A.; et al. First report in Italy of the exotic mosquito species *Aedes* (*Finlaya*) *koreicus*, a potential vector of arboviruses and filariae. *Parasites and Vectors* **2011**, *4*:*188*.
117. Cunze, S.; Kochmann, J.; Klimpel, S. Global occurrence data improve potential distribution models for *Aedes japonicus japonicus* in non-native regions. *Pest Manag. Sci.* **2020**.
118. Osório, H.C.; Zé-Zé, L.; Neto, M.; Silva, S.; Marques, F.; Silva, A.S.; Alves, M.J. Detection of the invasive mosquito species *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in Portugal. *Int. J. Environ. Res. Public Health* **2018**, *15*, 1–9.
119. Pichler, V.; Kotsakiozi, P.; Caputo, B.; Serini, P.; Caccone, A.; Torre, A. Della Complex interplay of evolutionary forces shaping population genomic structure of invasive *Aedes albopictus* in southern Europe. *PLoS Negl. Trop. Dis.* **2019**, *13(8): e0007554*.
120. Schaffner, F.; Medlock, J.M.; Van Bortel, W. Public health significance of invasive mosquitoes in Europe. *Clin. Microbiol. Infect.* **2013**, *19*, 685–692.
121. Suter, T.; Flacio, E.; Fariña, B.F.; Engeler, L.; Tonolla, M.; Müller, P. First report of the invasive mosquito species *Aedes koreicus* in the Swiss-Italian border region. *Parasites and Vectors* **2015**, *8:402.*
122. Zielke, D.E.; Ibáñez-Justicia, A.; Kalan, K.; Merdić, E.; Kampen, H.; Werner, D. Recently discovered *Aedes japonicus japonicus* (Diptera: Culicidae) populations in the Netherlands and northern Germany resulted from a new introduction event and from a split from an existing

population. *Parasites and Vectors* **2015**, *8:40*
123. Medlock, J.M.; Hansford, K.M.; Schaffner, F.; Versteirt, V.; Hendrickx, G.; Zeller, H.; Bortel, W. Van A review of the invasive mosquitoes in Europe: Ecology, public health risks, and control options. *Vector-Borne Zoonotic Dis.* **2012**, *12*, 435–447.
124. Dallimore, T.; Hunter, T.; Medlock, J.M.; Vaux, A.G.C.; Harbach, R.E.; Strode, C. Discovery of a single male *Aedes aegypti* (L.) in Merseyside, England. *Parasites and Vectors* **2017**, *10:309.*
125. Medlock, J.M.; Hansford, K.M.; Versteirt, V.; Cull, B.; Kampen, H.; Fontenille, D.; Hendrickx, G.; Zeller, H.; Van Bortel, W.; Schaffner, F. An entomological review of invasive mosquitoes in Europe. *Bull. Entomol. Res.* **2015**, *105*, 637–663.
126. Lühken, R.; Heitmann, A.; Jansen, S.; Schmidt-Chanasit, J.; Börstler, J.; Werner, D.; et al. Microsatellite typing of *Aedes albopictus* (Diptera: Culicidae) populations from Germany suggests regular introductions. *Infect. Genet. Evol.* **2020**, 104237.
127. Wilder-Smith, A.; Quam, M.; Sessions, O.; Rocklov, J.; Liu-Helmersson, J.; Franco, L.; Khan, K. The 2012 dengue outbreak in Madeira: Exploring the origins. *Euro Surveill.* **2014**, *19(8):pii=20718*.
128. European Centre for Disease Prevention and Control. *Aedes aegypti* - current known distribution: July 2019. Stockholm: ECDC, **2019**.
129. Carpenter, S.; Wilson, A.; Mellor, P.S. *Culicoides* and the emergence of Bluetongue virus in northern Europe. *Trends Microbiol.* **2009**, *17*, 172–178.
130. Ribeiro, R.; Wilson, A.J.; Nunes, T.; Ramilo, D.W.; Amador, R.; Madeira, S.; Baptista, F.M.; Harrup, L.E.; Lucientes, J.; Boinas, F. Spatial and temporal distribution of *Culicoides* species in mainland Portugal (2005-2010). Results of the Portuguese entomological surveillance programme. *PLoS One* **2015**, *10(4): e0124019*.
131. Maan, S.; Maan, N.S.; Ross-smith, N.; Batten, C.A.; Shaw, A.E.; Anthony, S.J.; Samuel, A.R.; Darpel, K.E.; Veronesi, E.; Oura, C.A.L.; et al. Sequence analysis of Bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. *Virology* **2008**, *377*, 308–318.
132. Hoffmann, B.; Scheuch, M.; Höper, D.; Jungblut, R.; Holsteg, M.; Schirrmeier, H.; Eschbaumer, M.; Goller, K. V; Wernike, K.; Fischer, M.; et al. Novel Orthobunyavirus in Cattle, Europe, 2011. *Emerg. Infect. Dis.* **2012**, *18*, 469–472.
133. Cuéllar, A.C.; Kjær, L.J.; Kirkeby, C.; Skovgard, H.; Nielsen, S.A.; Stockmarr, A.; Andersson, G.; Lindstrom, A.; Chirico, J.; Lühken, R.; et al. Spatial and temporal variation in the abundance of *Culicoides* biting midges (Diptera: Ceratopogonidae) in nine European countries. *Parasites and Vectors* **2018**, *11:112.*
134. Garros, C.; Viennet, E.; Rossi, S.; Balenghien, T. Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae : Diptera): applications on Palaearctic biting midge species , vectors of Orbiviruses. *Infect. Genet. Evol.* **2011**, *11*, 1103–1110.
135. Lühken, R.; Kiel, E.; Steinke, S.; Fladung, R. Topsoil conditions correlate with the emergence rates of *Culicoides chiopterus* and *Culicoides dewulfi* (Diptera: Ceratopogonidae) from cowpats. *Parasitol. Res.* **2015**, *114*, 1113–1117.
136. Rigot, T. The space-time distribution of Palearctic *Culicoides* spp., vectors of Bluetongue disease in Europe. *Univ. Libr. Bruxelles* **2011**, *Doctoral t*.
137. Barber, J.; Harrup, L.E.; Silk, R.; Veronesi, E.; Gubbins, S.; Bachanek-Bankowska, K.; Carpenter, S. Blood-feeding, susceptibility to infection with Schmallenberg virus and phylogenetics of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. *Parasites and Vectors* **2018**, *11:116.*
138. Carpenter, S.; Veronesi, E.; Mullens, B.; Venter, G. Vector competence of *Culicoides* for arboviruses: three major periods of research, their influence on current studies and future directions. *Rev. Sci. Tech. l'OIE* **2015**, *34*, 97–112.
139. Veronesi, E.; Antony, F.; Gubbins, S.; Golding, N.; Blackwell, A.; Mertens, P.P.C.; Brownlie, J.; Darpel, K.E.; Mellor, P.S.; Carpenter, S. Measurement of the Infection and Dissemination of Bluetongue Virus in *Culicoides* Biting Midges Using a Semi-Quantitative RT-PCR Assay and

Isolation of Infectious Virus. *PLoS One* **2013**, *8(8): e70800.*
140. Pagès, N.; Talavera, S.; Verdún, M.; Pujol, N.; Valle, M.; Bensaid, A.; Pujols, J. Schmallenberg virus detection in *Culicoides* biting midges in Spain: First laboratory evidence for highly efficient infection of *Culicoides* of the Obsoletus complex and *Culicoides imicola*. *Transbound. Emerg. Dis.* **2017**, *65*, e1–e6.
141. Augot, D.; Sauvage, F.; Jouet, D.; Simphal, E.; Veuille, M.; Couloux, A.; Kaltenbach, M.L.; Depaquit, J. Discrimination of *Culicoides obsoletus* and *Culicoides scoticus*, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis. *Infect. Genet. Evol.* **2010**, *10*, 629–637.
142. Mathieu, B.; Cêtre-Sossah, C.; Garros, C.; Chavernac, D.; Balenghien, T.; Carpenter, S.; Setier-Rio, M.L.; Vignes-Lebbe, R.; Ung, V.; Candolfi, E.; et al. Development and validation of IIKC: An interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic region. *Parasites and Vectors* **2012**, *5*:*137*.
143. Nolan, D. V.; Carpenter, S.; Barber, J.; Mellor, P.S.; Dallas, J.F.; Mordue (Luntz), A.J.; Piertney, S.B. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Vet. Microbiol.* **2007**, *124*, 82–94.
144. Harrup, L.E.; Bellis, G.A.; Balenghien, T.; Garros, C. *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: Current challenges and future directions. *Infect. Genet. Evol.* **2015**, *30*, 249–266.
145. Ahmeti, S.; Berisha, L.; Halili, B.; Ahmeti, F.; von Possel, R.; Thomé-Bolduan, C.; Michel, A.; Priesnitz, S.; Reisinger, E.C.; Günther, S.; et al. Crimean-congo hemorrhagic fever, Kosovo, 2013–2016. *Emerg. Infect. Dis.* **2019**, *25*, 321–324.
146. Kuhn, J.H.; Wiley, M.R.; Rodriguez, S.E.; Bào, Y.; Prieto, K.; Travassos da Rosa, A.P.A.; Guzman, H.; Savji, N.; Ladner, J.T.; Tesh, R.B.; et al. Genomic characterization of the genus Nairovirus (Family Bunyaviridae). *Viruses* **2016**, *8, 164.*
147. Kuivanen, S.; Levanov, L.; Kareinen, L.; Sironen, T.; Jääskeläinen, A.J.; Plyusnin, I.; Zakham, F.; Emmerich, P.; Schmidt-Chanasit, J.; Hepojoki, J.; et al. Detection of novel tick-borne pathogen, Alongshan virus, in *Ixodes ricinus* ticks, south-eastern Finland, 2019. *Euro Surveill* **2019**, *24, 24(27):pii=1900394*.
148. Gondard, M.; Temmam, S.; Devillers, E.; Pinarello, V.; Bigot, T.; Chrétien, D.; Aprelon, R.; Vayssier-taussat, M. RNA Viruses of *Amblyomma variegatum* and *Rhipicephalus microplus* and Cattle Susceptibility in the French Antilles. *Viruses* **2020**, *12, 144*.
149. Temmam, S.; Chrétien, D.; Bigot, T.; Dufour, E.; Petres, S.; Desquesnes, M.; Devillers, E.; Dumarest, M.; Yousfi, L.; Jittapalapong, S.; et al. Monitoring silent spillovers before emergence: a pilot study at the tick/human interface in thailand. *Front. Microbiol.* **2019**, *10:2315*.
150. Torii, S.; Matsuno, K.; Qiu, Y.; Mori-Kajihara, A.; Kajihara, M.; Nakao, R.; Nao, N.; Okazaki, K.; Sashika, M.; Hiono, T.; et al. Infection of newly identified phleboviruses in ticks and wild animals in Hokkaido, Japan indicating tick-borne life cycles. *Ticks Tick. Borne. Dis.* **2019**, *10*, 328–335.
151. Wille, M.; Harvey, E.; Shi, M.; Edward, C.; Hurt, A.C. Sustained virome diversity in Antarctic penguins and their ticks: geographical connectedness and no evidence for low pathogen pressure. *ISME J.* **2019**.
152. Mihalca, A.D. Ticks imported to Europe with exotic reptiles. *Vet. Parasitol.* **2015**, *213*, 67–71.
153. European Centre for Disease Prevention and Control Crimean-Congo haemorrhagic fever Annual Epidemiological Report for 2018. Stockholm: ECDC, **2019**
154. De Liberato, C.; Frontoso, R.; Magliano, A.; Montemaggiori, A.; Autorino, G.L.; Sala, M.; Bosworth, A.; Scicluna, M.T. Monitoring for the possible introduction of Crimean-Congo haemorrhagic fever virus in Italy based on tick sampling on migratory birds and serological survey of sheep flocks. *Prev. Vet. Med.* **2018**, *149*, 47–52.
155. Estrada-Peña, A.; Jameson, L.; Medlock, J.; Vatansever, Z.; Tishkova, F. Unraveling the ecological complexities of tick-associated Crimean-congo hemorrhagic fever virus transmission: A gap

analysis for the western palearctic. *Vector-Borne Zoonotic Dis.* **2012**, *12*, 743–752.
156. Jameson, L.J.; Morgan, P.J.; Medlock, J.M.; Watola, G.; Vaux, A.G.C. Importation of *Hyalomma marginatum*, vector of Crimean-Congo haemorrhagic fever virus, into the United Kingdom by migratory birds. *Ticks Tick. Borne. Dis.* **2012**, *3*, 95–99.
157. Kaiser, M.N.; Hoogstraal, H.; Watson, G.E. Ticks (Ixodoidea) on migrating birds in Cyprus, fall 1967 and spring 1968, and epidemiological considerations. In *Proceedings of the 3rd International Congress of Acarology*, Prague, Czech Republic, 1971; Daniel, M. et al., Eds; Springer Science+Business Media: Dordrecht, Germany, **1973**, pp. 473-478.
158. Mărcuțan, I.-D.; Kalmár, Z.; Ionică, A.M.; D'Amico, G.; Mihalca, A.D.; Vasile, C.; Sándor, A.D. Spotted fever group rickettsiae in ticks of migratory birds in Romania. *Parasit. Vectors* **2016**, *9*:*294*.
159. Pascucci, I.; Di Domenico, M.; Capobianco Dondona, G.; Di Gennaro, A.; Polci, A.; Capobianco Dondona, A.; Mancuso, E.; Cammà, C.; Savini, G.; Cecere, J.G.; et al. Assessing the role of migratory birds in the introduction of ticks and tick-borne pathogens from African countries: An Italian experience. *Ticks Tick. Borne. Dis.* **2019**, *10*:6.
160. Palomar, A.M.; Portillo, A.; Santibáñez, P.; Mazuelas, D.; Arizaga, J.; Crespo, A.; Gutiérrez, Ó.; Cuadrado, J.F.; Oteo, J.A. Crimean-congo hemorrhagic fever virus in ticks from migratory birds, Morocco. *Emerg. Infect. Dis.* **2013**, *19*, 260–263.
161. Waldenström, J.; Lundkvist, Å.; Falk, K.I.; Garpmo, U.; Bergström, S.; Lindegren, G.; Sjöstedt, A.; Mejlon, H.; Fransson, T.; Haemig, P.D.; et al. Migrating Birds and Tick-borne Encephalitis Virus. *Emerg. Infect. Dis.* **2007**, *13*, 1215–1218.
162. Kolodziejek, J.; Marinov, M.; Kiss, B.J.; Alexe, V.; Nowotny, N. The complete sequence of a West Nile virus lineage 2 strain detected in a *Hyalomma marginatum marginatum* tick collected from a song thrush (*Turdus philomelos*) in Eastern Romania in 2013 revealed closest genetic relationship to strain Volgograd 2007. *PLoS One* **2014**, *9(10): e109905*.
163. Chitimia-Dobler, L.; Nava, S.; Bestehorn, M.; Dobler, G.; Wölfel, S. First detection of *Hyalomma rufipes* in Germany. *Ticks Tick. Borne. Dis.* **2016**, *7*, 1135–1138.
164. Chitimia-Dobler, L.; Schaper, S.; Rieß, R.; Bitterwolf, K.; Frangoulidis, D.; Bestehorn, M.; Springer, A.; Oehme, R.; Drehmann, M.; Lindau, A.; et al. Imported *Hyalomma* ticks in Germany in 2018. *Parasites and Vectors* **2019**, *12:134*.
165. England, M.E.; Phipps, P.; Medlock, J.M.; Atkinson, P.M.; Atkinson, B.; Hewson, R.; Gale, P. *Hyalomma* ticks on northward migrating birds in southern Spain: Implications for the risk of entry of Crimean-Congo haemorrhagic fever virus to Great Britain. *J. Vector Ecol.* **2016**, *41*, 128–134.
166. Grandi, G.; Chitimia-Dobler, L.; Choklikitumnuey, P.; Strube, C.; Springer, A.; Albihn, A.; Jaenson, T.G.T.; Omazic, A. First records of adult *Hyalomma marginatum* and *H. rufipes* ticks (Acari: Ixodidae) in Sweden. *Ticks Tick. Borne. Dis.* **2020**, 101403.
167. Drăgănescu, S. The problem of panencephalitis in the People's Republic of Romania. *Minerva Med.* **1957**, *48*, 1313–1316.
168. Drăgănescu, N. Study of arboviruses in Romania (1958--1978). Results obtained in the 'Stefan S. Nicolau' Institute of Virology. *Virologie* **1979**, *30*, 283–293.
169. Duca, M.; Duca, E.; Buiuc, D.; Luca, V. A seroepidemiologic and virological study of the presence of arboviruses in Moldavia in 1961-1982. *Rev. Med. Chir. Soc. Med. Nat. Iasi* **1989**, *93*, 719–733.
170. Drăgănescu, N.; Girjabu, E.; Iftimovici, R.; Klebleev, E. Serological studies for the presence of arboviruses in the forest-steppe region of Tulcea. *Rev. Roum. Virol.* **1991**, *42*, 47–51.
171. Petrescu, A.; Copelovici, Y.; Drăgănescu, N.; Birnaure, F.; Girjabu, E.; Mihail, A.; Zaharia, C.N.; Topârceanu, F.; Teleguță, M.; Marin, C. The evidence of circulation of some viruses in a great town of Romania during the first half of 1992. *Rev. Roum. Virol.* **1993**, *44*, 243–251.
172. Topciu, V.; Rosiu, N.; Arcan, P.; Csaky, N. Isolation of a new arbovirus strain (Kemerovo group) from *Ixodes ricinus* ticks in the South-western region of Romania. *Acta Virol.* **1970**, *14*, 412.
173. Drăgănescu, N.; Popescu, G. Clinico-epidemiologic and serologic studies in certain loci of tick-borne encephalitis in Socialist Republic of Rumania. *Neuropatol. Pol.* **1966**, *4*, 333–344.

174. Drăgănescu, N.; Popescu, G.; Dilgeanu, I. Serological investigation of the infection caused by tick virus in workers in the field of zootechnics in Dobruja. *Stud. Cercet. Inframicrobiol.* **1966**, *17*, 7–11.
175. Drăgănescu, N.; Gheorghiu, V.; Birt, E.; Birzu, I.; Pop, C.; Toldan, I. On infections produced by some arboviruses of the B group in men and some domestic animals in Rumania. *Stud. Cercet. Inframicrobiol.* **1969**, *20*, 375–378.
176. Drăgănescu, N.; Girjabu, E.; Iacobescu, V. Serologic investigations on the presence of B group arbovirus infections in apparently healthy subjects of several districts in Romania. *Rev. Roum. Virol.* **1974**, *11*, 141–146.
177. Drăgănescu, N.; Iftimovici, R.; Girjabu, E.; Iacobescu, V.; Busila, A.; Cvasniuc, D.; Tudor, G.; Manastireanu, M.; Lăpușneanu Investigations on the presence of antibodies to several alphaviruses in humans and domestic animals of a region with elevated epidemiological potential. *Virologie* **1975**, *26*, 99–102.
178. Drăgănescu, N.; Duca, M.; Girjabu, E.; Popescu-Pretor, I.; Răducanu, S.; Deleanu, L.; Totescu, E. Epidemic outbreak caused by West Nile virus in the crew of a Romanian cargo ship passing the Suez Canal and the Red Sea on route to Yokohama. *Virologie* **1977**, *28*, 259–262.
179. Drăgănescu, N.; Girjabu, E.; Iftimovici, R.; Totescu, E.; Iacobescu, V.; Tudor, G.; Manastireanu, M. Investigations on the presence of antibodies to alphaviruses, flaviviruses, Bunyavirus and Kemerovo virus in humans and some domestic animals. *Virologie* **1978**, *29*, 107–111.
180. Drăgănescu, N.; Iftimovici, R.; Mutiu, A.; Girjabu, E.; Iacobescu, V.; Lăpușneanu, C.; Ignatescu, B.; Manolescu, G. Detection of antibodies to alphaviruses and flaviviruses in some migratory birds of the Danube Delta. *Virologie* **1978**, *29*, 113–116.
181. Han, L.L.; Popovici, F.; Alexander Jr, J.P.; Laurentia, V.; Tengelsen, L. a; Cernescu, C.; Gary Jr, H.E.; Ion-Nedelcu, N.; Campbell, G.L.; Tsai, T.F.; et al. Risk factors for West Nile virus infection and meningoencephalitis, Romania, 1996. *J Infect Dis* **1999**, *179*, 230–233.
182. Savage, H.M.; Ceianu, C.; Nicolescu, G.; Karabatsos, N.; Lanciotti, R.; Vladimirescu, A.; Laiv, L.; Ungureanu, A.; Romanca, C.; Tsai, T.F. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. *Am. J. Trop. Med. Hyg.* **1999**, *61*, 600–611.
183. Sîrbu, A.; Ceianu, C.S.; Pănculescu-Gâtej, R.I.; Vazquez, A.; Tenorio, A.; Rebreanu, R.; Niedrig, M.; Nicolescu, G.; Pistol, A. Outbreak of West Nile virus infection in humans, Romania, July to October 2010. *Euro Surveill.* **2011**, *16(2):pii=19762*.
184. Camp, J. V.; Haider, R.; Porea, D.; Oșlobanu, L.E.; Forgách, P.; Nowotny, N. Serological surveillance for Tahyna virus (California encephalitis orthobunyavirus, Peribunyaviridae) neutralizing antibodies in wild ungulates in Austria , Hungary and Romania. *Zoonoses Public Health* **2018**, *65*, 459–463.
185. Ceianu, C.S.; Ungureanu, A.; Nicolescu, G.; Cernescu, C.; Nitescu, L.; Tardei, G.; Petrescu, A.; Piţigoi, D.; Martin, D.; Ciulacu-Purcărea, V.; et al. West Nile virus surveillance in Romania: 1997-2000. *Viral Immunol.* **2001**, *14*, 251–262.
186. Dinu, S.; Cotar, A.I.; Pănculescu-Gâtej, I.R.; Fălcuţă, E.; Prioteasa, F.L.; Sîrbu, A.; Oprişan, G.; Bădescu, D.; Reiter, P.; Ceianu, C.S. West Nile virus circulation in south-eastern Romania, 2011 to 2013. *Euro Surveill.* **2015**, *20(20):pii=21130*.
187. Cernescu, C.; Nedelcu, N.; Ruta, S.; Tsai, T.F. Continued Transmission of West Nile Virus to Humans in Southeastern Romania , 1997 – 1998. *J. Infect. Dis.* **2000**, *181*, 710–712.
188. Cotar, A.I.; Fălcuță, E.; Prioteasa, L.F.; Dinu, S.; Ceianu, C.S.; Paz, S. Transmission Dynamics of the West Nile Virus in Mosquito Vector Populations under the Influence of Weather Factors in the Danube Delta, Romania. *Ecohealth* **2016**, *13*, 796–807.
189. Crivei, L.A.; Rățoi, I.; Răileanu, C.; Porea, D.; Anița, D.; Savuța, G.; Oșlobanu, L. First Record of West Nile Virus Specific Seroconversion in Dogs From Eastern Romania. *Bull. UASVM Vet. Med.* **2018**, *75(2)*.
190. Marinov, E.M.; Kiss, J.B.; Răileanu, Ș.; Condac, M.; Doroftei, M.; Prioteasa, L.; Alexe, V. Preliminary results of the research on the West Nile Virus from the Danube Delta Biosphere Reserve. *Sc. Annals of Danube Delta Institute* **2009**, *13*:59-66.

191. Prioteasa, F.L. Evaluarea potențialului vectorial al speciilor de culicidae (Diptera-Insecta) din Delta Dunării, pentru virusul West-Nile (Flaviviridae). Doctoral thesis, University of Bucharest, Bucharest, **2011**.
192. Purcărea-Ciulacu, V.; Alexse, A.; Prioteasa, L.; Fălcuță, E.; Vladimirescu, A.; Coipan, E.C.; Popescu, D.; Dumitrescu, G.; Nicolescu, G. The Transmission Cycles of West Nile Virus in Romania and the Detection of Virus Presence. *Int J Infect Dis.* **2008**, 12, 1:49.019 E331–E332.
193. Campbell, G.L.; Ceianu, C.S.; Savage, H.M. Epidemic West Nile encephalitis in Romania: waiting for history to repeat itself. *Ann. N. Y. Acad. Sci.* **2001**, *951*, 94–101.
194. Eckerle, I.; Briciu, V.T.; Ergonül, Ö.; Lupșe, M.; Papa, A.; Rădulescu, A.; Tsiodras, S.; Tsitou, C.; Drosten, C.; Nussenblatt, V.R.; et al. Emerging souvenirs of clinical presentation of the returning traveller with imported arbovirus infections in Europe. *Clin. Microbiol. Infect.* **2018**, *24*, 240–245.
195. Dinu, S.; Pănculescu-Gâtej, I.R.; Florescu, S.A.; Popescu, C.P.; Sârbu, A.; Oprișan, G.; et al. Molecular epidemiology of dengue fever cases imported into Romania between 2008 and 2013. *Travel Med. Infect. Dis.* **2014**, .
196. Lustig, Y.; Cotar, A.I.; Ceianu, C.S.; Castilletti, C.; Zelena, H.; Burdino, E.; Tienen, C. Van; Avsic, T.; Aarons, E.; Reusken, C. Lack of Zika virus antibody response in confirmed patients in non-endemic countries. *J. Clin. Virol.* **2017**, *3919*.
197. Florescu, S. A.; Cotar, A.I.; Popescu, P.C.; Ceianu, C.S.; Zaharia, M.; Vancea, G.; Codreanu, D.; et al. First Two Imported Cases of Zika Virus. *Vector-Borne Zoonotic Dis.* **2017**, *20(20)*, 4–7.
198. Prioteasa, L.F.; Dinu, S.; Fălcuță, E.; Ceianu, C.S. Established population of the invasive mosquito species *Aedes albopictus* in Romania, 2012-14. *J. Am. Mosq. Control Assoc.* **2015**, *31*, 177–181.
199. Fălcuţă, E.; Prioteasa, L.F.; Horváth, C.; Mihalca, A.D. The invasive Asian tiger mosquito *Aedes albopictus* in Romania: towards a country-wide colonization? *Parasitol. Res.* **2020**.
200. Briciu, V.T.; Titilincu, A.; Cârstina, D.; Lefkaditis, M.; Mihalca, A.D. First survey on hard ticks (Ixodidae) collected from humans in Romania : possible risks for tick-borne. *Exp. Appl. Acarol.* **2011**, 199–204.
201. Dumitrache, M.O.; Gherman, C.M.; Cozma, V. Hard ticks (Ixodidae) in Romania: surveillance, host associations, and possible risks for tick-borne diseases. *Parasitol. Res.* **2012**, *110*, 2067–2070.
202. Mihalca, A.D.; Gherman, C.M.; Magdas, C.; Dumitrache, M.O.; Doms, C.; Sa, A.D.; Gyo, A.; Oltean, M.; Mircean, V.; Cozma, V.; et al. *Ixodes ricinus* is the dominant questing tick in forest habitats in Romania : the results from a countrywide dragging campaign. *Exp. Appl. Acarol.* **2012**, 175–182.
203. Mihalca, A.D.; Dumitrache, M.O.; Magda, C.; Dom, C.; Mircean, V.; Pocora, I.V.G. V; Barabási, S.S.; Sándor, V.C.A.D. Synopsis of the hard ticks (Acari: Ixodidae) of Romania with update on host associations and geographical distribution. *Exp. Appl. Acarol.* **2012**, 183–206.
204. Kalmár, Z.; Mihalca, A.D.; Dumitrache, M.O.; Gherman, M.; Mircean, V.; Oltean, M.; Doms, C.; Matei, I.A.; Daniel, I.M.; Sándor, A.D.; et al. Geographical distribution and prevalence of *Borrelia burgdorferi* genospecies in questing *Ixodes ricinus* from Romania : A countrywide study. *Ticks Tick. Borne. Dis.* **2013**, *4*, 403–408.
205. Salat, J.; Mihalca, A.D.; Mihaiu, M.; Modrý, D.; Ruzek, D. Tick-Borne Encephalitis in Sheep, Romania. *Emerg. Infect. Dis.* **2017**, *23*, 2065–2067.
206. Sándor, A.D.; Mărcuţan, D.I.; D'Amico, G.; Gherman, C.M.; Dumitrache, M.O.; Mihalca, A.D. Do the ticks of birds at an important migratory hotspot reflect the seasonal dynamics of *Ixodes ricinus* at the migration initiation site? A case study in the Danube Delta. *PLoS One* **2014**, *9(2): e89378*.
207. Mărcuțan, I.D.; Sándor, A.D.; Mihalca, A.D.; Gherman, C.M.; Kalmár, Z.; Amico, G.D.; Dumitrache, M.O.; Cozma, V. Prevalence of *Anaplasma phagocytophilum* in ticks collected from migratory birds in Danube Delta , Romania. *Parasit. Vectors* **2014**, *7(Suppl 1):P16*.
208. Tilibașa, E.M.; Popescu, D.; Badea, C.; Hora, F. Ștefan; Dărăbuș, G. A report regarding first occurence of bluetongue in Romania, 2014. *Sci. Work. Ser. C. Vet. Med.* **2015**, *LXI(2)*, 277–280.
209. Tilibaşa, E.M.; Dărăbuş, G. Preliminary studies on dynamics of *Culicoides* spp. In western Romania in conjunction with some environmental factors. *Parasit. Vectors* **2014**, *7(Suppl 1):O7*.

210. Dărăbuş, G.; Tilibaşa, E.M.; Oprescu, I.; Morariu, S.; Mederle, N.; Ilie, M.; Sujic, T. The abundance of *Culicoides* (Diptera: Ceratopogonidae) in Timiș county. *Lucr. Ştiinţifice Med. Vet.* **2017**, *2*, 1689–1699.
211. Ioniță, M.; Mitrea, I.L.; Buzatu, M.C.; Dascălu, L.; Seasonal dynamics of haematophagous arthropod populations (ticks and *Culicoides* spp.) —vectors of pathogens in animals and humans, in different areas of Romania. Scient Works Med Vet. **2009**, *52:629–36*.
212. Ilie, A.; Șerban, C.; Imre, M.; Sorescu, D.; Ilie, M.; Imre, K.; et al. A survey (or presences, dynamics, prevalences) of *Culicoides* (Diptera: Ceratopogonidae) in Gorj county, Romania, preliminary results of entomological surveillance for bluetongue. *Lucr. Stiint. Stiint. Agric. Med. Vet.* **2013**, *46*, 5–9.
213. Damian-Georgescu, A. New species of *Ceratopogonidae* (Diptera) for the Romanian fauna. Studii si Cercetări de Biologie, Seria Zoologie **1972**; 24:423–32
214. Damian-Georgescu, A. Familia Ceratopogonidae, Genul *Culicoides*. Fauna României, Insecta; Editura Academiei Române: Bucharest, Romania **2000**, Fasc. 14 Diptera, vol. 11
215. Gould, E.; Pettersson, J.; Higgs, S.; Charrel, R.; de Lamballerie, X. Emerging arboviruses: Why today? *One Heal.* **2017**, *4:1–13*.
216. Smithburn, K.C.; Hughes, T.P.; Burke, A.W.; Paul, J.H. A Neurotropic Virus Isolated from the Blood of a Native of Uganda. *Am. J. Trop. Med.* **1940**, *20*, 471–472.
217. Hubálek, Z.; Rudolf, I.; Nowotny, N. Arboviruses pathogenic for domestic and wild animals. In *Advances in Virus Research*; Maramorosch, K., Murphy, F.A., Eds.; Elsevier Inc., **2014**; Vol. 89; pp. 201-275.
218. Reisen, W.K. Ecology of West Nile virus in North America. *Viruses* **2013**, *5*, 2079–2105.
219. Marm Kilpatrick, A.; Daszak, P.; Jones, M.J.; Marra, P.P.; Kramer, L.D. Host heterogeneity dominates West Nile virus transmission. *Proc. R. Soc. B Biol. Sci.* **2006**, *273*, 2327–2333.
220. Malkinson, M.; Banet, C.; Weisman, Y.; Pokamunski, S.; King, R.; Drouet, M.T.; Deubel, V. Introduction of West Nile virus in the Middle East by migrating white storks. *Emerg. Infect. Dis.* **2002**, *8*, 392–397.
221. Jourdain, E.; Toussaint, Y.; Leblond, A.; Bicout, D.J.; Sabatier, P.; Gauthier-Clerc, M. Bird Species Potentially Involved in Introduction, Amplification, And Spread of West Nile Virus in A Mediterranean Wetland, The Camargue (Southern France). *Vector-Borne Zoonotic Dis.* **2007**, *7*, 15–33.
222. Hubálek, Z. Mosquito-borne viruses in Europe. *Parasitol. Res.* **2008**, *103:S29–S43*.
223. Murgue, B.; Zeller, H.; Deubel, V. The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. *Curr. Top. Microbiol. Immunol.* **2002**, *267*, 195–221.
224. Rudolf, I.; Betášová, L.; Blažejová, H.; Venclíková, K.; Straková, P.; Šebesta, O.; Mendel, J.; Bakonyi, T.; Schaffner, F.; Nowotny, N.; et al. West Nile virus in overwintering mosquitoes, central Europe. *Parasites and Vectors* **2017**, *10:452*.
225. Montecino-Latorre, D.; Barker, C.M. Overwintering of West Nile virus in a bird community with a communal crow roost. *Sci. Rep.* **2018**, *8:6088*.
226. Calistri, P.; Giovannini, A.; Hubalek, Z.; Ionescu, A.; Monaco, F.; Savini, G.; Lelli, R. Epidemiology of west nile in europe and in the mediterranean basin. *Open Virol. J.* **2010**, *4*, 29–37.
227. Farfán-Ale, J.A.; Blitvich, B.J.; Marlenee, N.L.; Loroño-Pino, M.A.; Puerto-Manzano, F.; García-Rejón, J.E.; Rosado-Paredes, E.P.; Flores-Flores, L.F.; Ortega-Salazar, A.; Chávez-Medina, J.; et al. Antibodies to West Nile virus in asymptomatic mammals, birds, and reptiles in the Yucatan Peninsula of Mexico. *Am. J. Trop. Med. Hyg.* **2006**, *74*, 908–914.
228. Jeffrey Root, J. West Nile virus associations in wild mammals: A synthesis. *Arch. Virol.* **2013**, *158*, 735–752.
229. Dahlin, C.R.; Hughes, D.F.; Meshaka, W.E.; Coleman, C.; Henning, J.D. Wild snakes harbor West Nile virus. *One Heal.* **2016**, *2*, 136–138.
230. Kostiukov, M.A.; Gordeeva, Z.E.; Bulychev, V.P.; Nemova, N. V; Daniiarov, O.A. The lake frog (*Rana ridibunda*)-one of the food hosts of blood-sucking mosquitoes in Tadzhikistan-a reservoir of the West Nile fever virus. *Med. Parazitol. (Mosk).* **1985**, 49–50.

231. Williams, M.C.; Simpson, D.I.H.; Haddow, A.J.; Knight, E.M. The Isolation of West Nile Virus from Man and of Usutu Virus from the Bird-Biting Mosquito *Mansonia aurites* (Theobald) in the Entebbe Area of Uganda. *Ann. Trop. Med. Parasitol.* **1964**, *58*, 367–374.
232. Cadar, D.; Bosch, S.; Jöst, H.; Börstler, J.; Garigliany, M.M.; Becker, N.; Schmidt-Chanasit, J. Putative lineage of novel African Usutu virus, central Europe. *Emerg. Infect. Dis.* **2015**, *21*, 1647–1650.
233. Engel, D.; Jöst, H.; Wink, M.; Börstler, J.; Bosch, S.; Garigliany, M.M.; Jöst, A.; Czajka, C.; Lühken, R.; Ziegler, U.; et al. Reconstruction of the evolutionary history and dispersal of Usutu virus, a neglected emerging arbovirus in Europe and Africa. *MBio* **2016**, *7(1):e01938-15*.
234. Fros, J.J.; Miesen, P.; Vogels, C.B.; Gaibani, P.; Sambri, V.; Martina, B.E.; Koenraadt, C.J.; van Rij, R.P.; Vlak, J.M.; Takken, W.; et al. Comparative Usutu and West Nile virus transmission potential by local *Culex pipiens* mosquitoes in north-western Europe. *One Heal.* **2015**, *1*, 31–36.
235. Weissenböck, H.; Bakonyi, T.; Rossi, G.; Mani, P.; Nowotny, N. Usutu Virus, Italy, 1996. *Emerg. Infect. Dis.* **2013**, *19*, 1996–1999.
236. Chvala, S.; Kolodziejek, J.; Nowotny, N.; Weissenböck, H. Pathology and viral distribution in fatal Usutu virus infections of birds from the 2001 and 2002 outbreaks in Austria. *J. Comp. Pathol.* **2004**, *131*, 176–185.
237. Becker, N.; Jöst, H.; Ziegler, U.; Eiden, M.; Höper, D.; Emmerich, P.; Fichet-Calvet, E.; Ehichioya, D.U.; Czajka, C.; Gabriel, M.; et al. Epizootic emergence of Usutu virus in wild and captive birds in Germany. *PLoS One* **2012**, *7(2): e32604*.
238. Lecollinet, S.; Blanchard, Y.; Manson, C.; Lowenski, S.; Laloy, E.; Quenault, H.; Touzain, F.; Lucas, P.; Eraud, C.; Bahuon, C.; et al. Dual emergence of Usutu virus in common blackbirds, Eastern France, 2015. *Emerg. Infect. Dis.* **2016**, *22*, 2225–2227.
239. Rijks, J.M.; Kik, M.; Slaterus, R.; Foppen, R.; Stroo, A.; Ijzer, J.; Stahl, J.; Gröne, A.; Koopmans, M.; van der Jeugd, H.; et al. Widespread Usutu virus outbreak in birds in The Netherlands, 2016. *Euro Surveill* **2016**, *21(45):pii=30391*.
240. Diagne, M.; Ndione, M.; Di Paola, N.; Fall, G.; Bedekelabou, A.; Sembène, P.; Faye, O.; Zanotto, P.; Sall, A. Usutu Virus Isolated from Rodents in Senegal. *Viruses* **2019**, *11, 181*.
241. Clé, M.; Beck, C.; Salinas, S.; Lecollinet, S.; Gutierrez, S.; Van de Perre, P.; Baldet, T.; Foulongne, V.; Simonin, Y. Usutu virus: A new threat? *Epidemiol. Infect.* **2019**, *147, e232, 1–11*.
242. Gaibani, P.; Cavrini, F.; Gould, E.A.; Rossini, G.; Pierro, A.; Landini, P.; Sambri, V. Comparative Genomic and Phylogenetic Analysis of the First Usutu Virus Isolate from a Human Patient Presenting with Neurological Symptoms. *PLoS One* **2013**, *8(5): e64761*.
243. Weaver, S.C.; Reisen, W.K. Present and future arboviral threats. *Antiviral Res.* **2010**, *85*, 328–345.
244. Bhatt, S.; Gething, P.W.; Brady, O.J.; Messina, J.P.; Farlow, A.W.; Moyes, C.L.; Drake, J.M.; Brownstein, J.S.; Hoen, A.G.; Sankoh, O.; et al. The global distribution and burden of dengue. *Nature* **2013**, *496*, 504–507.
245. Rosen, L. Dengue in Greece in 1927 and 1928 and the pathogenesis of dengue hemorrhagic fever: new data and a different conclusion. *Am. J. Trop. Med. Hyg.* **1986**, *35*, 642–653.
246. Tomasello, D.; Schlagenhauf, P. Chikungunya and dengue autochthonous cases in Europe, 2007-2012. *Travel Med. Infect. Dis.* **2013**, *11*, 274–284.
247. Kuna, A.; Gajewski, M.; Biernat, B. Selected arboviral diseases imported to Poland – current state of knowledge and perspectives for research. *Ann. Agric. Environ. Med.* **2019**, *26*, 385–391.
248. Tsetsarkin, K.A.; Chen, R.; Weaver, S.C.; Diseases, I. Interspecies transmission and chikungunya virus emergence. *Curr Opin Virol.* **2016**, *16*, 143–150.
249. Schuffenecker, I.; Iteman, I.; Michault, A.; Murri, S.; Frangeul, L.; Vaney, M.C.; Lavenir, R.; Pardigon, N.; Reynes, J.M.; Pettinelli, F.; et al. Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med.* **2006**, *3*, 1058–1070.
250. Rezza, G. Infection with Chikungunya virus in Italy : an outbreak in a temperate region. *Lancet* **2007**, *370*, 1840–46.
251. Amraoui, F.; Failloux, A.B. Chikungunya: an unexpected emergence in Europe. *Curr. Opin. Virol.* **2016**, *21*, 146–150.

252. Calba, C.; Guerbois-galla, M.; Franke, F.; Jeannin, C.; Auzet-caillaud, M.; Grard, G.; Pigaglio, L.; Decoppet, A.; Weicherding, J.; Munoz-Riviero, M.; et al. Preliminary report of an autochthonous chikungunya outbreak in France , July to September 2017. *Euro Surveill.* **2017**, *22(39):pii=17-00647*.
253. Vairo, F.; Di Pietrantonj, C.; Pasqualini, C.; Mammone, A.; Lanini, S.; Nicastri, E.; Castilletti, C.; Ferraro, F.; Di Bari, V.; Puro, V.; et al. The surveillance of Chikungunya virus in a temperate climate: Challenges and possible solutions from the experience of Lazio Region, Italy. *Viruses* **2018**, *10, 501*.
254. Jain, J.; Kushwah, R.B.S.; Singh, S.S.; Sharma, A.; Adak, T.; Singh, O.P.; Bhatnagar, R.K.; Subbarao, S.K.; Sunil, S. Evidence for Natural Vertical Transmission of Chikungunya Viruses in Field Populations of *Aedes aegypti* in Delhi and Haryana States in India − A Preliminary Report. *Acta Trop.* **2016**.
255. Niyas, K.P.; Abraham, R.; Unnikrishnan, R.N.; Mathew, T.; Nair, S. Molecular characterization of Chikungunya virus isolates from clinical samples and adult *Aedes albopictus* mosquitoes emerged from larvae from Kerala , South India. *Virol. J.* **2010**, *7:189*.
256. Ecker, M.; Allison, S.L.; Meixner, T.; Heinz, F.X. Sequence analysis and genetic classification of Tick-borne encephalitis viruses from Europe and Asia. *J. Gen Virol.* **1999**, *80*, 179–185.
257. Dai, X.; Shang, G.; Lu, S.; Yang, J.; Xu, J.; Dai, X.; Shang, G.; Lu, S.; Yang, J.; Xu, J. A new subtype of eastern Tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau , China. *Emerg. Microbes Infect.* **2018**, *7:1, 1-9*.
258. Kovalev, S.Y.; Mukhacheva, T.A. Reconsidering the classification of Tick-borne encephalitis virus within the Siberian subtype gives new insights into its evolutionary history. *Infect. Genet. Evol.* **2017**, 10.1016/j.meegid.2017.09.014 .
259. Lindquist, L.; Vapalahti, O. Tick-borne encephalitis. *Lancet* **2008**, *371*, 1861–1871.
260. Michelitsch, A.; Wernike, K.; Klaus, C.; Dobler, G.; Beer, M. Exploring the Reservoir Hosts of Tick-Borne Encephalitis Virus. *Viruses* **2019**, *11*, 669.
261. Estrada-Peña, A.; de la Fuente, J. The ecology of ticks and epidemiology of tick-borne viral diseases. *Antiviral Res.* **2014**, *108*, 104–128.
262. Belova, O.A.; Burenkova, L.A.; Karganova, G.G. Ticks and tick-borne diseases different Tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged ixodid ticks – Evidence of virus replication and changes in tick behavior. *Ticks Tick. Borne. Dis.* **2012**, *3*, 240–246.
263. Bakhvalova, V.N.; Potapova, O.F.; Panov, V. V; Morozova, O. V Vertical transmission of Tick-borne encephalitis virus between generations of adapted reservoir small rodents. *Virus Genes* **2009**, *140*, 172–178.
264. Labuda, M.; Kozuch, O.; Zuffova, E.; Eleckova, E.; Hails, R.S.; Nuttall, P.A. Tick-Borne Encephalitis Virus Transmission between Ticks Cofeeding on Specific Immune Natural Rodent Hosts. *Virology* **1997**, *235, 138–143*.
265. Randolph, S.E. Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. *Ticks Tick. Borne. Dis.* **2011**, *2*, 179–182.
266. Król, M.E.; Borawski, B.; Nowicka-Ciełuszecka, A.; Tarasiuk, J.; Zajkowska, J. Outbreak of alimentary Tick-borne encephalitis in Podlaskie voivodship, Poland. *Przegl Epidemiol* **2019**, *73(2)*, 239–248.
267. Makenov, M.; Karan, L.; Shashina, N.; Akhmetshina, M.; Zhurenkova, O.; Kholodilov, I.; Karganova, G.; Smirnova, N.; Grigoreva, Y.; Yankovskaya, Y.; et al. First detection of Tick-borne encephalitis virus in *Ixodes ricinus* ticks and their rodent hosts in Moscow, Russia. *Ticks Tick. Borne. Dis.* **2019**, *10*, 101265.
268. Jaenson, T.G.T.; Hjertqvist, M.; Bergström, T.; Lundkvist, Å. Why is Tick-borne encephalitis increasing? A review of the key factors causing the increasing incidence of human TBE in Sweden. *Parasites and Vectors* **2012**, *5:184*.
269. Bente, D.A.; Forrester, N.L.; Watts, D.M.; McAuley, A.J.; Whitehouse, C.A.; Bray, M. Crimean-Congo hemorrhagic fever: History, epidemiology, pathogenesis, clinical syndrome and genetic diversity. *Antiviral Res.* **2013**, *100*, 159–189.
270. Ergönül, Ö. Crimean-Congo Hemorrhagic Fever. *Lancet Infect Dis* **2006**; *6: 203–14*.

271. Nasirian, H. Crimean-Congo hemorrhagic fever (CCHF) seroprevalence: A systematic review and meta-analysis. *Acta Trop.* **2019**, *196*, 102–120.
272. Gonzalez, J.P.; Camicas, J.L.; Cornet, J.P.; Faye, O.; Wilson, M.L. Sexual and transovarian transmission of Crimean-Congo haemorrhagic fever virus in *Hyalomma truncatum* ticks. *Res. Virol.* **1992**, *143*, 23–28.
273. Estrada-Peña, A.; Ruiz-Fons, F.; Acevedo, P.; Gortazar, C.; De la Fuente, J. Factors driving the circulation and possible expansion of Crimean-Congo haemorrhagic fever virus in the western Palearctic. *J. Appl. Microbiol.* **2013**, *114*, 278–286.
274. Gargili, A.; Estrada-Peña, A.; Spengler, J.R.; Lukashev, A.; Nuttall, P.A.; Bente, D.A. The role of ticks in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus: A review of published field and laboratory studies. *Antiviral Res.* **2017**, *144*, 93–119.
275. Spengler, J.R.; Estrada-Peña, A. Host preferences support the prominent role of *Hyalomma* ticks in the ecology of Crimean-Congo hemorrhagic fever. *PLoS Negl. Trop. Dis.* **2018**, *12(2): e0006248*.
276. Hoogstraal, H. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. *J. Med. Entomol.* **1979**, *15*, 307–417.
277. Spengler, J.R.; Bergeron, E.; Spiropoulou, C.F. Crimean-Congo hemorrhagic fever and expansion from endemic regions. *Curr Opin Virol.* **2019**, *34*, 70–78.
278. Mancuso, E.; Toma, L.; Polci, A.; D'Alessio, S.G.; Luca, M. Di; Orsini, M.; Domenico; Marcacci, M.; Mancini, G.; Spina, F.; et al. Crimean-congo hemorrhagic fever virus genome in tick from migratory birds, Italy. *Emerg. Infect. Dis.* **2019**, *25*, 1418–1420.
279. Belbis, G.; Zientara, S.; Bréard, E.; Sailleau, C.; Caignard, G.; Vitour, D.; Attoui, H. Bluetongue Virus: From BTV-1 to BTV-27. *Adv. Virus Res.* **2017**, *99*, 161–197.
280. Bumbarov, V.; Golender, N.; Jenckel, M.; Wernike, K.; Beer, M.; Khinich, E.; Zalesky, O.; Erster, O. Characterization of Bluetongue virus serotype 28. *Transbound. Emerg. Dis.* **2019**, 1–12.
281. He, C.-Q.; Ding, N.-Z.; He, M.; Li, S.-N.; Wang, X.-M.; He, H.-B.; Liu, X.-F.; Guo, H.-S. Intragenic Recombination as a Mechanism of Genetic Diversity in Bluetongue Virus. *J. Virol.* **2010**, *84*, 11487–11495.
282. Jacquot, M.; Rao, P.P.; Yadav, S.; Nomikou, K.; Maan, S.; Jyothi, Y.K.; Reddy, N.; Putty, K.; Hemadri, D.; Singh, K.P.; et al. Contrasting selective patterns across the segmented genome of Bluetongue virus in a global reassortment hotspot. *Virus Evol.* **2019**, *5(2): vez027*.
283. Foxi, C.; Delrio, G.; Falchi, G.; Marche, M.G.; Satta, G.; Ruiu, L. Role of different Culicoides vectors (Diptera: Ceratopogonidae) in Bluetongue virus transmission and overwintering in Sardinia (Italy). *Parasites and Vectors* **2016**, *9:440*.
284. Jiménez-Clavero, M.A. Animal viral diseases and global change: Bluetongue and West Nile fever as paradigms. *Front. Genet.* **2012**, *3*, 105.
285. Mullens, B.; Loomis, E.; Anderson, J. Clues to control of Bluetongue virus. *Calif. Agric.* **1986**, 23.
286. Mertens, P.; Mohd Jaafar, F.; Attoui, H. Orbiviruses and Bluetongue Virus. *eLS*. John Wiley & Sons, Ltd: Chichester **2015**.
287. Alexander, K.A.; Kat, P.W.; House, J.; House, C.; O'Brien, S.J.; Laurenson, M.K.; McNutt, J.W.; Osburn, B.I. African horse sickness and African carnivores. *Vet. Microbiol.* **1995**, *47*, 133–140.
288. O'Dell, N.; Arnot, L.; Janisch, C.E.; Steyl, J.C.A. Clinical presentation and pathology of suspected vector transmitted African horse sickness in South African domestic dogs from 2006 to 2017. *Vet. Rec.* **2018**, *182*, 175.
289. Johansen, C.A.; Farrow, R.A.; Morrisen, A.; Foley, P.; Bellis, G.; Van Den Hurk, A.F.; Montgomery, B.; Mackenzie, J.S.; Ritchie, S.A. Collection of wind-borne haematophagous insects in the Torres Strait, Australia. *Med. Vet. Entomol.* **2003**, *17*, 102–109.
290. Kluiters, G.; Swales, H.; Baylis, M. Local dispersal of palaearctic *Culicoides* biting midges estimated by mark-release-recapture. *Parasites and Vectors* **2015**, *8:86*.
291. Jacquet, S.; Garros, C.; Lombaert, E.; Walton, C.; Restrepo, J.; Allene, X.; Baldet, T.; Cetre-Sossah, C.; Chaskopoulou, A.; Delecolle, J.C.; et al. Colonization of the Mediterranean basin by the vector biting midge species *Culicoides imicola*: An old story. *Mol. Ecol.* **2015**, *24*, 5707–5725.
292. Jacquet, S.; Huber, K.; Pagès, N.; Talavera, S.; Burgin, L.E.; Carpenter, S.; Sanders, C.; Dicko,

A.H.; Djerbal, M.; Goffredo, M.; et al. Range expansion of the Bluetongue vector, *Culicoides imicola*, in continental France likely due to rare wind-transport events. *Sci. Rep.* **2016**, *6:27247*.
293. Sick, F.; Beer, M.; Kampen, H.; Wernike, K. Culicoides Biting Midges—Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance. *Viruses* **2019**, *11*, 376.
294. Doceul, V.; Gouzil, J.; Vitour, D.; Zientara, S. Schmallenberg Virus. *eLS* John Wiley & Sons, Ltd: Chichester **2015**.
295. Collins, Á.B.; Doherty, M.L.; Barrett, D.J.; Mee, J.F. Schmallenberg virus: a systematic international literature review (2011-2019) from an Irish perspective. *Ir. Vet. J.* **2019**, *72*, 1–22.
296. Koenraadt, C.J.M.; Balenghien, T.; Carpenter, S.; Ducheyne, E.; Elbers, A.R.W.; Fife, M.; Garros, C.; Ibáñez-Justicia, A.; Kampen, H.; Kormelink, R.J.M.; et al. Bluetongue, Schmallenberg - what is next? *Culicoides*-borne viral diseases in the 21st century. *BMC Vet. Res.* **2014**, *10:77*.
297. Ségard, A.; Gardès, L.; Jacquier, E.; Grillet, C.; Mathieu, B.; Rakotoarivony, I.; Setier-Rio, M.L.; Chavernac, D.; Cêtre-Sossah, C.; Balenghien, T.; et al. Schmallenberg virus in *Culicoides* Latreille (Diptera: Ceratopogonidae) populations in France during 2011-2012 outbreak. *Transbound. Emerg. Dis.* **2018**, *65*, e94–e103.
298. Goffredo, M.; Capelli, G.; Quaglia, M.; Federici, V.; Catalani, M. Schmallenberg virus in Italy : a retrospective survey in *Culicoides* stored during the bluetongue Italian surveillance program. *Prev. Vet. Med.* **2013**, *111*, 230–236.
299. Kęsik-Maliszewska, J.; Larska, M.; Collins, Á.B.; Rola, J. Post-Epidemic Distribution of Schmallenberg Virus in *Culicoides* Arbovirus Vectors in Poland. *Viruses* **2019**, *11*, 447.
300. Larska, M.; Lechowski, L.; Grochowska, M.; Zmudziński, J.F. Detection of the Schmallenberg virus in nulliparous *Culicoides obsoletus/scoticus* complex and *C. punctatus* - The possibility of transovarial virus transmission in the midge population and of a new vector. *Vet. Microbiol.* **2013**, *166*, 467–473.
301. Balenghien, T.; Pagès, N.; Goffredo, M.; Carpenter, S.; Augot, D.; Jacquier, E.; Talavera, S.; Monaco, F.; Depaquit, J.; Grillet, C.; et al. The emergence of Schmallenberg virus across *Culicoides* communities and ecosystems in Europe. *Prev. Vet. Med.* **2014**, *116*, 360–369.
302. Omondi, D.; Masiga, D.K.; Ajamma, Y.U.; Fielding, B.C.; Njoroge, L.; Villinger, J. Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. *PLoS One* **2015**, *10(7): e0134375*.
303. Keesing F.; Holt, R.D.; Ostfeld, R.S. Effects of species diversity on disease risk. *Ecol. Lett.* **2006**, *9*, 485–498.
304. Kilpatrick, A.M.; Kramer, L.D.; Jones, M.J.; Marra, P.P.; Daszak, P. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biol.* **2006**, *4(4): e82*.
305. Santiago-Alarcon, D.; Havelka, P.; Schaefer, H.M.; Segelbacher, G. Bloodmeal Analysis Reveals Avian *Plasmodium* Infections and Broad Host Preferences of *Culicoides* (Diptera: Ceratopogonidae) Vectors. *PLoS One* **2012**, *7(2): e31098*.
306. Talavera, S.; Muñoz-Muñoz, F.; Verdun, M.; Pujol, N.; Pagès, N. Revealing potential bridge vectors for BTV and SBV: a study on *Culicoides* blood feeding preferences in natural ecosystems in Spain. *Med. Vet. Entomol.* **2017**, *32*, 35–40.
307. Pichon, B.; Rogers, M.; Egan, D.; Gray, J. Blood-Meal Analysis for the Identification of Reservoir Hosts of Tick-Borne Pathogens in Ireland. *Vector-Borne Zoonotic Dis.* **2005**, *5*, 172–180.
308. Wodecka, B.; Skotarczak, B. Identification of host blood-meal sources and *Borrelia* in field-collected *Ixodes ricinus* ticks in north-western Poland. *Ann. Agric. Environ. Med.* **2016**, *23*, 59–63.
309. Vinauger, C.; Pereira, M.H.; Lazzari, C.R. Learned host preference in a Chagas disease vector, *Rhodnius prolixus*. *Acta Trop.* **2012**, *122*, 24–28.
310. Baldacchino, F.; Gardès, L.; Stordeur, E. De; Jay-robert, P.; Garros, C. Blood-feeding patterns of horse flies in the French Pyrenees. *Vet. Parasitol.* **2014**, *199*, 283–288.
311. Simo, G.; Njiokou, F.; Mbida, J.A.M.; Njitchouang, G.R. Tsetse fly host preference from sleeping sickness foci in Cameroon: Epidemiological implications. *Infect. Genet. Evol.* **2008**, *8*, 34–39.
312. Hellgren, O.; Bensch, S.; Malmqvist, B. Bird hosts, blood parasites and their vectors - Associations uncovered by molecular analyses of blackfly blood meals. *Mol. Ecol.* **2008**, *17*, 1605–

1613.
313. Malmqvist, B.; Strasevicius, D.; Hellgren, O.; Adler, P.H.; Bensch, S. Vertebrate host specificity of wild-caught blackflies revealed by mitochondrial DNA in blood. *Proc. R. Soc. B Biol. Sci.* **2004**, *271*, 152–155.
314. Muñoz, C.; Martínez-de la Puente, J.; Figuerola, J.; Pérez-Cutillas, P.; Navarro, R.; Ortuño, M.; Bernal, L.J.; Ortiz, J.; Soriguer, R.; Berriatua, E. Molecular xenomonitoring and host identification of Leishmania sand fly vectors in a Mediterranean periurban wildlife park. *Transbound. Emerg. Dis.* **2019**; doi: 10.1111/tbed.13319.
315. Yared, S.; Gebresilassie, A.; Abbasi, I.; Aklilu, E.; Kirstein, O.D.; Balkew, M.; Brown, A.S.; Clouse, R.M.; Warburg, A.; Hailu, A.; et al. A molecular analysis of sand fly blood meals in a visceral leishmaniasis endemic region of northwestern Ethiopia reveals a complex host-vector system. *Heliyon* **2019**, *5*, e02132.
316. Rizzoli, A.; Bolzoni, L.; Chadwick, E.A.; Capelli, G.; Montarsi, F.; Grisenti, M.; De La Puente, J.M.; Muñoz, J.; Figuerola, J.; Soriguer, R.; et al. Understanding West Nile virus ecology in Europe: *Culex pipiens* host feeding preference in a hotspot of virus emergence. *Parasites and Vectors* **2015**, *8:213*.
317. Faraji, A.; Egizi, A.; Fonseca, D.M.; Unlu, I.; Crepeau, T.; Healy, S.P.; Gaugler, R. Comparative Host Feeding Patterns of the Asian Tiger Mosquito, *Aedes albopictus*, in Urban and Suburban Northeastern USA and Implications for Disease Transmission. *PLoS Negl. Trop. Dis.* **2014**, *8(8): e3037.*
318. Richards, S.L.; Ponnusamy, L.; Unnasch, T.R.; Hassan, H.K.; Apperson, C.S. Host-Feeding Patterns of *Aedes albopictus* (Diptera: Culicidae) in Relation to Availability of Human and Domestic Animals in Suburban Landscapes of Central North Carolina. *J Med Entomol* **2006**, *43*, 543–551.
319. Russell, T.L.; Beebe, N.W.; Bugoro, H.; Apairamo, A.; Cooper, R.D.; Collins, F.H.; Lobo, N.F.; Burkot, T.R. Determinants of host feeding success by *Anopheles farauti*. *Malar. J.* **2016**, *15*.
320. Dekker, T.; Takken, W.; Braks, M.A. Innate preference for host-odor blends modulates degree of anthropophagy of *Anopheles gambiae* sensu lato (Diptera: Culicidae). *J. Med. Entomol.* **2001**, *38*, 868–871.
321. Burkett-Cadena, N.D.; McClure, C.J.W.; Ligon, R.A.; Graham, S.P.; Guyer, C.; Hill, G.E.; Ditchkoff, S.S.; Eubanks, M.D.; Hassan, H.K.; Unnasch, T.R. Host reproductive phenology drives seasonal patterns of host use in mosquitoes. *PLoS One* **2011**, *6(3): e17681*.
322. Shoemaker, L.G.; Hayhurst, E.; Weiss-Lehman, C.; Strauss, A.T.; Krause-Porath, A.; Borer, E.T.; Seabloom, E.W.; Shaw, A.K. Pathogens manipulate the preference of vectors, slowing disease spread in a multi-host system. *Ecol. Lett.* **2019**, *22*, 1115–1125.
323. Viennet, E.; Garros, C.; Gardès, L.; Rakotoarivony, I.; Allène, X.; Lancelot, R.; Crochet, D.; Moulia, C.; Baldet, T.; Balenghien, T. Host preferences of palaearctic *Culicoides* biting midges: Implications for transmission of orbiviruses. *Med. Vet. Entomol.* **2013**, *27*, 255–266.
324. Molaei, G.; Andreadis, T.G.; Armstrong, P.M.; Anderson, J.F.; Vossbrinck, C.R. Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission, northeastern United States. *Emerg. Infect. Dis.* **2006**, *12*, 468–474.
325. Egizi, A.; Healy, S.P.; Fonseca, D.M. Rapid blood meal scoring in anthropophilic *Aedes albopictus* and application of PCR blocking to avoid pseudogenes. *Infect. Genet. Evol.* **2013**, *16*, 122–128.
326. Hamer, G.L.; Kitron, U.D.; Brawn, J.D.; Loss, S.R.; Ruiz, M.O.; Goldberg, T.L.; Walker, E.D. *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. *J Med Entomol* **2008**, *45*, 125–128.
327. Janssen, N.; Fernandez-Salas, I.; Díaz González, E.E.; Gaytan-Burns, A.; Medina-de la Garza, C.E.; Sanchez-Casas, R.M.; Börstler, J.; Cadar, D.; Schmidt-Chanasit, J.; Jöst, H. Mammalophilic feeding behaviour of *Culex quinquefasciatus* mosquitoes collected in the cities of Chetumal and Cancun, Yucatán Peninsula, Mexico. *Trop. Med. Int. Heal.* **2015**, *20*, 1488–1491.
328. Alcaide, M.; Rico, C.; Ruiz, S.; Soriguer, R.; Muñoz, J.; Figuerola, J. Disentangling vector-borne transmission networks: A universal DNA barcoding method to identify vertebrate hosts from

arthropod bloodmeals. *PLoS One* **2009**, *4(9): e7092*.
329. Balenghien, T.; Fouque, F.; Sabatier, P.; Bicout, D.J. Horse-, Bird-, and Human-Seeking Behavior and Seasonal Abundance of Mosquitoes in a West Nile Virus Focus of Southern France. *J. Med. Entomol.* **2006**, *43*, 936–946.
330. Brugman, V.A.; Hernández-triana, L.M.; Prosser, S.W.J.; Weland, C.; Westcott, D.G.; Fooks, A.R.; Johnson, N. Molecular species identification, host preference and detection of myxoma virus in the *Anopheles maculipennis* complex (Diptera: Culicidae) in southern England, UK. *Parasit. Vectors* **2015**, 8:421.
331. Brugman, V.A.; England, M.E.; Stoner, J.; Tugwell, L.; Harrup, L.E.; Wilson, A.J.; Medlock, J.M.; Logan, J.G.; Fooks, A.R.; Mertens, P.P.C.; et al. How often do mosquitoes bite humans in southern England? A standardised summer trial at four sites reveals spatial, temporal and site-related variation in biting rates. *Parasites and Vectors* **2017**, *10:420.*
332. Camp, J. V.; Bakonyi, T.; Soltész, Z.; Zechmeister, T.; Nowotny, N. *Uranotaenia unguiculata* Edwards, 1913 are attracted to sound, feed on amphibians, and are infected with multiple viruses. *Parasites and Vectors* **2018**, *11:456*.
333. Díez-Fernández, A.; Martínez-de la Puente, J.; Gangoso, L.; Ferraguti, M.; Soriguer, R.; Figuerola, J. House sparrow uropygial gland secretions do not attract ornithophilic, nor mammophilic mosquitoes. *Med. Vet. Entomol.* **2019**, doi: 10.1111/mve.12401.
334. Muñoz, J.; Ruiz, S.; Soriguer, R.; Alcaide, M.; Viana, D.S.; Roiz, D.; Vázquez, A.; Figuerola, J. Feeding patterns of potential West Nile virus vectors in South-West Spain. *PLoS One* **2012**, *7(6): e39549*.
335. Vogels, C.B.F.; Fros, J.J.; Pijlman, G.P.; van Loon, J.J.A.; Gort, G.; Koenraadt, C.J.M. Virus interferes with host-seeking behaviour of mosquito. *J. Exp. Biol.* **2017**, *220*, 3598–3603.
336. Bartsch, S.; Bauer, B.; Wiemann, A.; Clausen, P.H.; Steuber, S. Feeding patterns of biting midges of the *Culicoides obsoletus* and *Culicoides pulicaris* groups on selected farms in Brandenburg, Germany. *Parasitol. Res.* **2009**, *105*, 373–380.
337. Elbers, A.R.W.; Meiswinkel, R. *Culicoides* (Diptera: Ceratopogonidae) host preferences and biting rates in the Netherlands: Comparing cattle, sheep and the black-light suction trap. *Vet. Parasitol.* **2014**, *205*, 330–337.
338. Lassen, S.B.; Nielsen, S.A.; Skovgård, H.; Kristensen, M. Molecular identification of bloodmeals from biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) in Denmark. *Parasitol. Res.* **2011**, *108*, 823–829.
339. Lassen, S.B.; Nielsen, S.A.; Kristensen, M. Identity and diversity of blood meal hosts of biting midges in Denmark. *Parasites and Vectors* **2012**, *5:143*.
340. Martínez-De La Puente, J.; Martínez, J.; Ferraguti, M.; Morales-De La Nuez, A.; Castro, N.; Figuerola, J. Genetic characterization and molecular identification of the bloodmeal sources of the potential bluetongue vector *Culicoides obsoletus* in the Canary Islands, Spain. *Parasites and Vectors* **2012**, *5:147*.
341. Martínez-de la Puente, J.; Figuerola, J.; Soriguer, R. Fur or feather? Feeding preferences of species of *Culicoides* biting midges in Europe. *Trends Parasitol.* **2015**, *31*, 16–22.
342. MartÍnez-de la Puente, J.; Navarro, J.; Ferraguti, M.; Soriguer, R.; Figuerola, J. First molecular identification of the vertebrate hosts of *Culicoides imicola* in Europe and a review of its blood-feeding patterns worldwide: implications for the transmission of bluetongue disease and African horse sickness. *Med. Vet. Entomol.* **2017**, *31*, 333–339.
343. Votýpka, J.; Synek, P.; SvobodovÁ, M. Endophagy of biting midges attacking cavity-nesting birds. *Med. Vet. Entomol.* **2009**, *23*, 277–280.
344. Bongiorno, G.; Di Muccio, T.; Bianchi, R.; Gramiccia, M.; Gradoni, L. Laboratory transmission of an Asian strain of *Leishmania tropica* by the bite of the southern European sand fly *Phlebotomus perniciosus*. *Int. J. Parasitol.* **2019**, *49*, 417–421.
345. Chaskopoulou, A.; Giantsis, I.A.; Demir, S.; Bon, M.C. Species composition, activity patterns and blood meal analysis of sand fly populations (Diptera: Psychodidae) in the metropolitan region of Thessaloniki, an endemic focus of canine leishmaniasis. *Acta Trop.* **2016**, *158*, 170–176.

346. Cotteaux-Lautard, C.; Leparc-goffart, I.; Berenger, J.M.; Plumet, S.; Pages, F. Phenology and host preferences *Phlebotomus perniciosus* (Diptera: Phlebotominae) in a focus of Toscana virus (TOSV) in South of France. *Acta Trop.* **2016**, *153*, 64–69.
347. Maia, C.; Dionísio, L.; Afonso, M.O.; Neto, L.; Cristóvão, J.M.; Campino, L. *Leishmania* infection and host-blood feeding preferences of phlebotomine sandflies and canine leishmaniasis in an endemic European area, the Algarve Region in Portugal. *Mem. Inst. Oswaldo Cruz* **2013**, *108*, 481–487.
348. Pérez-Cutillas, P.; Muñoz, C.; Martínez-De La Puente, J.; Figuerola, J.; Navarro, R.; Ortuño, M.; Bernal, L.J.; Ortiz, J.; Soriguer, R.C.; Berriatua, E. A spatial ecology study in a high-diversity host community to understand blood-feeding behaviour in *Phlebotomus* sandfly vectors of *Leishmania*. *Med. Vet. Entomol.* **2020**.
349. Rossi, E.; Bongiorno, G.; Ciolli, E.; Di Muccio, T.; Scalone, A.; Gramiccia, M.; Gradoni, L.; Maroli, M. Seasonal phenology, host-blood feeding preferences and natural *Leishmania* infection of *Phlebotomus perniciosus* (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. *Acta Trop.* **2007**, *105*, 158–165.
350. Cadenas, F.M.; Rais, O.; Humair, P.; Douet, V.; Moret, J.; Gern, L.; Cadenas, N.; Rais, O. Identification of Host Bloodmeal Source and *Borrelia burgdorferi* Sensu Lato in Field-Collected *Ixodes ricinus* Ticks in Chaumont (Switzerland). *J. Med. Entomol.* **2007**, *44*, 1109–1117.
351. Humair, P.-F.; Douet, V.; Cadenas, F.M.; Schouls, L.M.; Van De Pol, I.; Gern, L. Molecular Identification of Bloodmeal Source in *Ixodes ricinus* Ticks Using 12S rDNA As a Genetic Marker. *J. Med. Entomol.* **2007**, *44*, 869–880.
352. Bobeva, A.; Ilieva, M.; Dimitrov, D.; Zehtindjiev, P. Degree of associations among vectors of the genus *Culicoides* (Diptera: Ceratopogonidae) and host bird species with respect to haemosporidian parasites in NE Bulgaria. *Parasitol. Res.* **2014**, *113*, 4505–4511.
353. Bobeva, A.; Zehtindjiev, P.; Ilieva, M.; Dimitrov, D.; Mathis, A.; Bensch, S. Host preferences of ornithophilic biting midges of the genus *Culicoides* in the Eastern Balkans. *Med. Vet. Entomol.* **2015**, *29*, 290–296.
354. Vasić, A.; Zdravkovi, N.; Bojkovski, J.; Marinov, M.; Mathis, A.; Niculaua, M.; Luanda, E.; Pavlovi, I.; Pflüger, V.; Pudar, D.; et al. Species diversity, host preference and arbovirus detection of *Culicoides* (Diptera: Ceratopogonidae) in south-eastern Serbia. *Parasit. Vectors* **2019**, *12:61*.
355. Kurucz, K.; Kepner, A.; Krtinic, B.; Hederics, D.; Földes, F.; Zana, B.; Jakab, F.; Kemenesi, G. Blood-meal analysis and avian malaria screening of mosquitoes collected from human-inhabited areas in Hungary and Serbia. *J. Eur. Mosq. Control Assoc.* **2018**, *36*, 3–13.
356. Zittra, C.; Kocziha, Z.; Pinnyei, S.; Harl, J.; Kieser, K.; Laciny, A.; Eigner, B.; Silbermayr, K.; Duscher, G.G.; Fok, É.; et al. Screening blood-fed mosquitoes for the diagnosis of filarioid helminths and avian malaria. *Parasites and Vectors* **2015**, *8:16*.
357. Černý, O.; Votýpka, J.; Svobodová, M. Spatial feeding preferences of ornithophilic mosquitoes, blackflies and biting midges. *Med. Vet. Entomol.* **2011**, *25*, 104–108.
358. Fălcuță, E.; Toty, C.; Prioteasa, F.; Nicolescu, G.; Teodorescu, I.; Purcărea-Ciulacu, V.; Fontenille, D. Blood-meal preferences for *Anopheles maculipennis* (Diptera: Culicidae) complex species in Comana, Giurgiu county (Romania). *Rom. J. Biol.* **2010**, *55*, 49–55.
359. Grubaugh, N.D.; Sharma, S.; Krajacich, B.J.; Fakoli, L.S.; Bolay, F.K.; Diclaro, J.W.; Johnson, W.E.; Ebel, G.D.; Foy, B.D.; Brackney, D.E. Xenosurveillance: A Novel Mosquito-Based Approach for Examining the Human-Pathogen Landscape. *PLoS Negl. Trop. Dis.* **2015**, *9(3): e0003628*.
360. Marsden, P.D.; Barreto, A.C.; Cuba, C.C.; Gama, M.B.; Ackers, J. Improvements in routine xenodiagnosis with first instar *Dipetalogaster maximus* (Uhler 1894) (Triatominae). *Am. J. Trop. Med. Hyg.* **1979**, *28*, 649–652.
361. Molina, R. Indirect xenodiagnosis of visceral leishmaniasis in 10 HIV-infected patients using colonized *Phlebotomus perniciosus* 1993, 277–278.
362. Arnold, J.M.; Oswald, S.A.; Voigt, C.C.; Palme, R.; Braasch, A.; Bauch, C.; Becker, P.H. Taking the stress out of blood collection: comparison of field blood-sampling techniques for analysis of baseline corticosterone. *J. Avian Biol.* **2008**, *39*, 588–592.

363. Kading, R.C.; Biggerstaff, B.J.; Young, G.; Komar, N. Mosquitoes used to draw blood for arbovirus viremia determinations in small vertebrates. *PLoS One* **2014**, *9(6): e99342*.
364. Voigt, C.C.; Peschel, U.; Wibbelt, G.; Fro, K. An Alternative , Less Invasive Blood Sample Collection Technique for Serologic Studies Utilizing Triatomine Bugs (Heteroptera; Insecta). *J. Wildl. Dis.* **2006**, *42*, 466–469.
365. Bitome-Essono, P.Y.; Ollomo, B.; Arnathau, C.; Durand, P.; Mokoudoum, N.D.; Yacka-Mouele, L.; Okouga, A.P.; Boundenga, L.; Mve-Ondo, B.; Obame-Nkoghe, J.; et al. Tracking zoonotic pathogens using blood-sucking flies as "flying syringes". *Elife* **2017**, *6:e22069*.
366. Ng, T.F.F.; Willner, D.L.; Lim, Y.W.; Schmieder, R.; Chau, B.; Nilsson, C.; Anthony, S.; Ruan, Y.; Rohwer, F.; Breitbart, M. Broad surveys of DNA viral diversity obtained through viral metagenomics of mosquitoes. *PLoS One* **2011**, *6(6): e20579*.
367. Fauver, J.R.; Weger-Lucarelli, J.; Fakoli, L.S.; Bolay, K.; Bolay, F.K.; Diclaro, J.W.; Brackney, D.E.; Foy, B.D.; Stenglein, M.D.; Ebel, G.D. Xenosurveillance reflects traditional sampling techniques for the identification of human pathogens: A comparative study in West Africa. *PLoS Negl. Trop. Dis.* **2018**, *12(3): e0006348*.
368. Barbazan, P.; Thitithanyanont, A.; Missé, D.; Dubot, A.; Bosc, P.; Luangsri, N.; Gonzalez, J.P.; Kittayapong, P. Detection of H5N1 avian influenza virus from mosquitoes collected in an infected poultry farm in Thailand. *Vector-Borne Zoonotic Dis.* **2008**, *8*, 105–109.
369. Batson, J.; Dudas, G.; Haas-stapleton, E.; Kistler, A.L.; Lucy, M.; Logan, P.; Ratnasiri, K.; Retallack, H.; Biohub, Z.; St, I.; et al. Single mosquito metatranscriptomics recovers mosquito species , blood meal sources , and microbial cargo , including viral dark matter. **2020**. *bioRxiv*, doi.org/10.1101/2020.02.10.942854
370. Barbazan, P.; Palabodeewat, S.; Nitatpattana, N.; Gonzalez, J.-P. Detection of Host Virus-Reactive Antibodies in Blood Meals of Naturally Engorged Mosquitoes. *Vector-Borne Zoonotic Dis.* **2009**, *9*, 103–108.
371. Convention on Biological Diversity. Available online: https://www.cbd.int/countries/profile/?country=ro (accessed on (20.05.2020).
372. World Heritage Convention. Available online: https://whc.unesco.org/en/list/588/ (accessed on 28.05.2010).
373. Droz, B.; Joye, S.; Titta, A. de; Schneider, F.; Antoniazza, S. Phenology of Passerine bird migration in the Danube Delta, Romania. *Ring* **2017**, *38*, 25–42.
374. Newton, I. *The Migration Ecology of Birds*, 1st ed. Elsevier Academic Press: London, UK, **2008**; pp. 699-729.
375. Hubálek, Z. An annotated checklist of pathogenic microorganisms associated with migratory birds. *J. Wildl. Dis.* **2004**, *40*, 639–659.
376. Prioteasa, F.-L.; Fălcuță, E. An annotated checklist of the mosquitoes (Diptera: Culicidae) of the Danube Delta Biosphere Reserve. *Eur. Mosq. Bull. 28* **2010**, *28*, 240–245.
377. Sándor, A.D.; Dumitrache, M.O.; D'Amico, G.; Kiss, B.J.; Mihalca, A.D. *Rhipicephalus rossicus* and not *R. sanguineus* is the dominant tick species of dogs in the wetlands of the Danube Delta, Romania. *Vet. Parasitol.* **2014**, *204*, 430–432.
378. Dumitrache, M.O.; Kiss, B.; Dantas-Torres, F.; Latrofa, M.S.; D'Amico, G.; Sándor, A.D.; Mihalca, A.D. Seasonal dynamics of *Rhipicephalus rossicus* attacking domestic dogs from the steppic region of southeastern Romania. *Parasites and Vectors* **2014**, *7:97*.
379. Labuda, M.; Nuttall, P.A. Tick-borne viruses. *Parasitology* **2004**, *129*, S221–S245. DOI: 10.1017/S0031182004005220.
380. Abudurexiti, A.; Adkins, S.; Alioto, D.; Alkhovsky, S. V.; Avšič-Županc, T.; Ballinger, M.J.; Bente, D.A.; Beer, M.; Bergeron, É.; Blair, C.D.; et al. Taxonomy of the order Bunyavirales: update 2019. *Arch. Virol.* **2019**, *164*, 1949–1965.
381. Gauci, P.J.; McAllister, J.; Mitchell, I.R.; Cybinski, D.; St George, T.; Gubala, A.J. Genomic characterisation of Vinegar hill virus, an Australian nairovirus isolated in 1983 from *Argas robertsi* ticks collected from cattle egrets. *Viruses* **2017**, *9*, 373.
382. Bouquet, J.; Melgar, M.; Swei, A.; Delwart, E.; Lane, R.S.; Chiu, C.Y. Metagenomic-based

Surveillance of Pacific Coast tick *Dermacentor occidentalis* Identifies Two Novel Bunyaviruses and an Emerging Human Ricksettsial Pathogen. *Sci. Rep.* **2017**, *7: 12234*.

383. Lasecka, L.; Baron, M.D. The molecular biology of nairoviruses, an emerging group of tick-borne arboviruses. *Arch. Virol.* **2014**, *159*, 1249–1265.
384. Yadav, P.D.; Vincent, M.J.; Khristova, M.; Kale, C.; Nichol, S.T.; Mishra, A.C.; Mourya, D.T. Genomic analysis reveals Nairobi sheep disease virus to be highly diverse and present in both Africa, and in India in the form of the Ganjam virus variant. *Infect. Genet. Evol.* **2011**, *11*, 1111–1120.
385. Walker, P.J.; Widen, S.G.; Firth, C.; Blasdell, K.R.; Wood, T.G.; Travassos Da Rosa, A.P.A.; Guzman, H.; Tesh, R.B.; Vasilakis, N. Genomic characterization of Yogue, Kasokero, Issyk-kul, Keterah, Gossas, and Thiafora viruses: Nairoviruses naturally infecting bats, shrews, and ticks. *Am. J. Trop. Med. Hyg.* **2015**, *93*, 1041–1051.
386. Walker, P.J.; Widen, S.G.; Wood, T.G.; Guzman, H.; Tesh, R.B.; Vasilakis, N. A global genomic characterization of nairoviruses identifies nine discrete genogroups with distinctive structural characteristics and host-vector associations. *Am. J. Trop. Med. Hyg.* **2016**, *94*, 1107–1122.
387. Barnwal, B.; Karlberg, H.; Mirazimi, A.; Tan, Y.J. The non-structural protein of Crimean-Congo hemorrhagic fever virus disrupts the mitochondrial membrane potential and induces apoptosis. *J. Biol. Chem.* **2016**, *291*, 582–592.
388. Carter, S.D.; Surtees, R.; Walter, C.T.; Ariza, A.; Bergeron, E.; Nichol, S.T.; Hiscox, J.A.; Edwards, T.A.; Barr, J.N. Structure, Function, and Evolution of the Crimean-Congo Hemorrhagic Fever Virus Nucleocapsid Protein. *J. Virol.* **2012**, *86*, 10914–10923.
389. Wang, Y.; Dutta, S.; Karlberg, H.; Devignot, S.; Weber, F.; Hao, Q.; Tan, Y.J.; Mirazimi, A.; Kotaka, M. Structure of Crimean-Congo Hemorrhagic Fever Virus Nucleoprotein: Superhelical Homo-Oligomers and the Role of Caspase-3 Cleavage. *J. Virol.* **2012**, *86*, 12294–12303.
390. Guo, Y.; Wang, W.; Ji, W.; Deng, M.; Sun, Y.; Zhou, H.; Yang, C.; Deng, F.; Wang, H.; Hu, Z.; et al. Crimean-Congo hemorrhagic fever virus nucleoprotein reveals endonuclease activity in bunyaviruses. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 5046–5051.
391. Fuller, J.; Surtees, R.A.; Slack, G.S.; Mankouri, J.; Hewson, R.; Barr, J.N. Rescue of Infectious Recombinant Hazara Nairovirus from cDNA Reveals the Nucleocapsid Protein DQVD Caspase Cleavage Motif Performs an Essential Role other than Cleavage. *J. Virol.* **2019**, *93*: e00616-19.
392. Matsumoto, Y. A Minigenome Study of Hazara Nairovirus Genomic Promoters Yusuke. *J. Virol.* **2019**, 93:e02118-18.
393. Surtees, R.; Ariza, A.; Punch, E.K.; Trinh, C.H.; Dowall, S.D.; Hewson, R.; Hiscox, J.A.; Barr, J.N.; Edwards, T.A. The crystal structure of the Hazara virus nucleocapsid protein. *BMC Struct. Biol.* **2015**, *15*:24.
394. Capodagli, G.C.; Deaton, M.K.; Baker, E.A.; Lumpkin, R.J.; Pegan, S.D. Diversity of Ubiquitin and ISG15 Specificity among Nairoviruses' Viral Ovarian Tumor Domain Proteases. *J. Virol.* **2013**, *87*, 3815–3827.
395. Wang, W.; Liu, X.; Wang, X.; Dong, H.; Ma, C.; Wang, J.; Liu, B.; Mao, Y.; Wang, Y.; Li, T.; et al. Structural and Functional Diversity of Nairovirus-Encoded Nucleoproteins. *J. Virol.* **2015**, *89*, 11740–11749.
396. Dzimianski, J. V.; Beldon, B.S.; Daczkowski, C.M.; Goodwin, O.Y.; Scholte, F.E.M.; Bergeron, É.; Pegan, S.D. Probing the impact of nairovirus genomic diversity on viral ovarian tumor domain protease (vOTU) structure and deubiquitinase activity. PLoS Pathog. **2019**; *15(1): e1007515*.
397. Drăgănescu, N.; Girjabu, E.; Iftimovici, R. Serological studies in man and different species of domestic animals from the forest-steppe region of Romania for the detection of Togaviridae and Bunyaviridae. *Rev. Roum. Virol.* **1993**, *44*, 207–210.
398. Drăgănescu, N. Research on arbovirus infections. *Virologie* **1989**, *40*, 257–269.
399. Ungureanu, A.; Popovici, V.; Catanas, F.; Ioniță, I.; Tutoveanu, A.; Safta, M. Isolation of Bhanja virus in Romania. *Arch. Roum. Pathol. Exp. Microbiol.* **1990**, *49*, 139–145.
400. Ceianu, C.S.; Pănculescu-Gâtej, R.I. First Serologic Evidence for the Circulation of Crimean-Congo Hemorrhagic Fever Virus in Romania. *Vector-Borne Zoonotic Dis.* **2012**, *12*, 718–721.

401. Hubálek, Z. Biogeography of Tick-Borne Bhanja Virus (Bunyaviridae) in Europe. *Interdiscip. Perspect. Infect. Dis.* **2009**, *2009*, 372691; doi:10.1155/2009/372691.
402. Tekin, S.; Bursali, A.; Mutluay, N.; Keskin, A.; Dundar, E. Crimean-Congo hemorrhagic fever virus in various ixodid tick species from a highly endemic area. *Vet. Parasitol.* **2012**, *186*, 546–552.
403. Papa, A.; Christova, I.; Papadimitriou, E.; Antoniadis, A. Crimean-Congo hemorrhagic fever in Bulgaria. *Emerg. Infect. Dis.* **2004**, *10*, 1465–1467.
404. Papa, A.; Kontana, A.; Tsioka, K.; Chaligiannis, I.; Sotiraki, S. Novel phleboviruses detected in ticks, Greece. *Ticks Tick. Borne. Dis.* **2016**, *7*, 690–693.
405. Papa, A.; Kontana, A.; Tsioka, K.; Saratsis, A.; Sotiraki, S. Novel phlebovirus detected in *Haemaphysalis parva* ticks in a Greek island. *Ticks Tick. Borne. Dis.* **2017**, *8*, 157–160.
406. Sherifi, K.; Cadar, D.; Muji, S.; Robaj, A.; Ahmeti, S.; Jakupi, X.; Emmerich, P.; Krüger, A. Crimean-Congo Hemorrhagic Fever Virus Clades V and VI (Europe 1 and 2) in Ticks in Kosovo, 2012. *PLoS Negl. Trop. Dis.* **2014**, *8:e3168*.
407. Estrada-Peña, A.; Mihalca, A.D.; Petney, T., Eds. *Ticks of Europe and North Africa. A Guide to Species Identification*; 1st ed.; Springer International Publishing: Cham, Switzerland, **2017**.
408. Jane B. Walker, J.E.K. and I.G.H. *The Genus Rhipicephalus (Acari, Ixodidae): A Guide to the Brown Ticks of the World*; 2nd ed.; Cambridge University Press: Cambridge, UK, **2005**.
409. Fernández-Correa, I.; Truchado, D.A.; Gomez-Lucia, E.; Doménech, A.; Pérez-Tris, J.; Schmidt-Chanasit, J.; Cadar, D.; Benítez, L. A novel group of avian astroviruses from Neotropical passerine birds broaden the diversity and host range of *Astroviridae*. *Sci. Rep.* **2019**, *9*, 9513.
410. Tomazatos, A.; Cadar, D.; Török, E.; Maranda, I.; Horváth, C.; Keresztes, L.; Spinu, M.; Jansen, S.; Jöst, H.; Schmidt-chanasit, J.; et al. Circulation of *Dirofilaria immitis* and *Dirofilaria repens* in the Danube Delta Biosphere Reserve , Romania. *Parasit. Vectors* **2018**, *11:392.*
411. Schmitz, H.; Gabriel, M.; Emmerich, P. Specific detection of antibodies to diferent flaviviruses using a new immune complex ELISA. *Med. Microbiol. Immunol.* **2011**, *200*, 233–239.
412. ORFfinder; Available online: https://www.ncbi.nlm.nih.gov/orffinder/
413. CBS Prediction Server; Available online: http://www.cbs.dtu.dk/services/
414. PredictProtein Server; Available online: https://predictprotein.org/
415. Jones, P.; Binns, D.; Chang, H.Y.; Fraser, M.; Li, W.; McAnulla, C.; McWilliam, H.; Maslen, J.; Mitchell, A.; Nuka, G.; et al. InterProScan 5: Genome-scale protein function classification. *Bioinformatics* **2014**, *30*, 1236–1240.
416. Potter, S.C.; Luciani, A.; Eddy, S.R.; Park, Y.; Lopez, R.; Finn, R.D. HMMER web server: 2018 update. *Nucleic Acids Res.* **2018**, *46*, W200–W204.
417. Lole, K.S.; Bollinger, R.C.; Paranjape, R.S.; Gadkari, D.; Kulkarni, S.S.; Novak, N.G.; Ingersoll, R.; Sheppard, H.W.; Ray, S.C. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* **1999**, *73*, 152–160.
418. Martin, D.P.; Murrell, B.; Golden, M.; Khoosal, A.; Muhire, B. RDP4: Detection and analysis of recombination patterns in virus genomes. *Virus Evol.* **2015**, *1(1): vev003*.
419. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. ProtTest 3 : fast selection of best-fit models of protein evolution. *Bioinformatics* **2017**, *27*, 1164–1165.
420. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549.
421. Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst. Biol.* **2010**, *59*, 307–321.
422. Suchard, M.A.; Lemey, P.; Baele, G.; Ayres, D.L.; Drummond, A.J.; Rambaut, A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol.* **2018**, *4(1): vey016*.
423. Drummond, A.J.; Rambaut, A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* **2007**, *7:214*.
424. Figtree sofware; Available online: http://tree.bio.ed.ac.uk/software/figtree/

425. Muhire, B.M.; Varsani, A.; Martin, D.P. SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS One* **2014**, *9(9): e108277*.
426. Andersson, I.; Bladh, L.; Mousavi-jazi, M.; Magnusson, K.; Haller, O.; Mirazimi, A.; Preparedness, M.; Control, D.; Solna, S.; Irol, J. V Human MxA Protein Inhibits the Replication of Crimean-Congo Hemorrhagic Fever Virus. *J. Virol.* **2004**, *78*, 4323–4329.
427. Karlberg, H.; Tan, Y.J.; Mirazimi, A. Induction of caspase activation and cleavage of the viral nucleocapsid protein in different cell types during Crimean-Congo hemorrhagic fever virus infection. *J. Biol. Chem.* **2011**, *286*, 3227–3234.
428. Charrel, R.N.; Attoui, H.; Butenko, A.M.; Clegg, J.C.; Deubel, V.; Frolova, T. V.; Gould, E.A.; Gritsun, T.S.; Heinz, F.X.; Labuda, M.; et al. Tick-borne virus diseases of human interest in Europe. *Clin. Microbiol. Infect.* **2004**, *10*, 1040–1055.
429. Gamsjaeger, R.; Liew, C.K.; Loughlin, F.E.; Crossley, M.; Mackay, J.P. Sticky fingers: zinc-fingers as protein-recognition motifs. *Trends Biochem. Sci.* **2007**, *32*, 63–70.
430. Muller, R.; Poch, O.; Delarue, M.; Bishop, D.H.L.; Bouloy, M. Rift valley fever virus L segment: Correction of the sequence and possible functional role of newly identified regions conserved in RNA-dependent polymerases. *J. Gen. Virol.* 1994, *75*, 1345–1352.
431. Poch, O.; Sauvaget, I.; Delarue, M.; Tordo, N. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* **1989**, *8*, 3867–3874.
432. Gerlach, P.; Malet, H.; Cusack, S.; Reguera, J. Structural Insights into Bunyavirus Replication and Its Regulation by the vRNA Promoter. *Cell* **2015**, *161*, 1267–1279.
433. Reguera, J.; Gerlach, P.; Rosenthal, M.; Gaudon, S.; Coscia, F.; Günther, S.; Cusack, S. Comparative Structural and Functional Analysis of Bunyavirus and Arenavirus Cap-Snatching Endonucleases. *PLoS Pathog.* **2016**, *12*, e1005636.
434. Aquino, V.H.; Moreli, M.L.; Moraes Figueiredo, L.T. Analysis of Oropouche virus L protein amino acid sequence showed the presence of an additional conserved region that could harbour an important role for the polymerase activity. *Arch. Virol.* **2003**, *148*, 19–28.
435. Amroun, A.; Priet, S.; de Lamballerie, X.; Quérat, G. Bunyaviridae RdRps: structure, motifs, and RNA synthesis machinery. *Crit. Rev. Microbiol.* **2017**, *43*, 753–778.
436. James, T.W.; Frias-Staheli, N.; Bacik, J.-P.; Levingston Macleod, J.M.; Khajehpour, M.; García-Sastre, A.; Mark, B.L. Structural basis for the removal of ubiquitin and interferon-stimulated gene 15 by a viral ovarian tumor domain-containing protease. *Proc. Natl. Acad. Sci.* **2011**, *108*, 2222 LP – 2227.
437. Bakshi, S.; Holzer, B.; Bridgen, A.; McMullan, G.; Quinn, D.G.; Baron, M.D. Dugbe virus ovarian tumour domain interferes with ubiquitin/ISG15-regulated innate immune cell signalling. *J. Gen. Virol.* **2013**, *94*, 298–307.
438. Alber, T. Structure of the leucine zipper. *Curr. Opin. Genet. Dev.* **1992**, *2*, 205–210.
439. Lukashev, A.N. Evidence for recombination in Crimean-Congo hemorrhagic fever virus. *J. Gen. Virol.* **2005**, *86*, 2333–2338.
440. Deyde, V.M.; Khristova, M.L.; Rollin, P.E.; Ksiazek, T.G.; Nichol, S.T. Crimean-Congo Hemorrhagic Fever Virus Genomics and Global Diversity. *J. Virol.* **2006**, *80*, 8834–8842.
441. Răileanu, C.; Moutailler, S.; Pavel, I.; Porea, D.; Mihalca, A.D.; Savuța, G.; Vayssier-Taussat, M. *Borrelia* diversity and co-infection with other tick borne pathogens in ticks. *Front. Cell. Infect. Microbiol.* **2017**, *7:36*.
442. Andersson, M.O.; Tolf, C.; Tamba, P.; Stefanache, M.; Radbea, G.; Rubel, F.; Waldenström, J.; Dobler, G.; Chițimia-Dobler, L. *Babesia*, *Theileria*, and *Hepatozoon* species in ticks infesting animal hosts in Romania. *Parasitol. Res.* **2017**, *116*, 2291–2297.
443. Leica, L.; Mitrea, I.L.; Ionita, M. Clinical Occurrence of Canine Babesiosis in the Coastal Area of the Black Sea (Dobrogea) in Southeastern Romania and Associated Epidemiological Implications. *J. Parasitol.* **2019**, *105*, 491–496.
444. Negredo, A.; Habela, M.Á.; de Arellano, E.R.; Diez, F.; Lasala, F.; López, P.; Sarriá, A.; Labiod, N.; Calero-Bernal, R.; Arenas, M.; et al. Survey of Crimean-congo hemorrhagic fever enzootic focus, Spain, 2011-2015. *Emerg. Infect. Dis.* **2019**, *25*, 1177–1184.

445. Liu, X.; Zhang, X.; Wang, Z.; Dong, Z.; Xie, S.; Jiang, M.; Song, R.; Ma, J.; Chen, S.; Chen, K.; et al. A Tentative Tamdy Orthonairovirus Related to Febrile Illness in Northwestern China. *Clin. Infect. Dis.* **2019**, *70*, 2155–2160.
446. Johnson, B.K.; Chanas, A.C.; Shockley, P.; Squires, E.J.; Varma, M.G.; Leake, C.J.; Simpson, D.I. Investigation of the ecology of Soldado virus on Puffin Island, North Wales. *Acta Virol.* **1979**, *23*, 428–432.
447. Jones, L.D.; Davies, C.R.; Steele, G.M.; Nuttall, P.A. A novel mode of arbovirus transmission involving a nonviremic host. *Science* **1987**, *237*, 775–777.
448. Jones, L.D.; Hodgson, E.; Nuttall, P.A. Enhancement of virus transmission by tick salivary glands. *J. Gen. Virol.* **1989**, *70*, 1895–1898.
449. Labuda, M.; Nuttall, P.A.; Kozuch, O.; Elecková, E.; Williams, T.; Zuffová, E.; Sabó, A. Non-viraemic transmission of Tick-borne encephalitis virus: a mechanism for arbovirus survival in nature. *Experientia* **1993**, *49*, 802–805.
450. Hubálek, Z.; Rudolf, I. Tick-borne viruses in Europe. *Parasitol. Res.* **2012**, *111*, 9–36.
451. Bolling, B.G.; Weaver, S.C.; Tesh, R.B.; Vasilakis, N. Insect-specific virus discovery: Significance for the arbovirus community. *Viruses* **2015**, *7*, 4911–4928.
452. Roundy, C.M.; Azar, S.R.; Rossi, S.L.; Weaver, S.C.; Vasilakis, N. Insect-Specific Viruses: A Historical Overview and Recent Developments. In *Advances in Virus Research*; Kielian, M., Mettenleiter, T.C.; Roossinck, M., Eds.; Elsevier Inc., **2017**; Vol. 98; pp. 119-146.
453. Pereira, A.; Figueira, L.; Nunes, M.; Esteves, A.; Cotão, A.J.; Vieira, M.L.; Maia, C.; Campino, L.; Parreira, R. Multiple *Phlebovirus* (Bunyaviridae) genetic groups detected in *Rhipicephalus*, *Hyalomma* and *Dermacentor* ticks from southern Portugal. *Ticks Tick. Borne. Dis.* **2017**, *8*, 45–52.
454. Ohlendorf, V.; Marklewitz, M.; Kopp, A.; Yordanov, S.; Drosten, C.; Junglen, S. Huge diversity of phleboviruses in ticks from Strandja Nature Park, Bulgaria. *Ticks Tick. Borne. Dis.* **2019**, *10*, 697–703.
455. Emanet, N. Novel Tick Phlebovirus Genotypes Lacking Evidence for Vertebrate Infections in Anatolia and Thrace, Turkey. *Viruses* **2019**, *11, 703.*
456. Pimentel, V.; Afonso, R.; Nunes, M.; Vieira, M.L.; Bravo-Barriga, D.; Frontera, E.; Martinez, M.; Pereira, A.; Maia, C.; Paiva-Cardoso, M. das N.; et al. Geographic dispersal and genetic diversity of tick-borne phleboviruses (*Phenuiviridae, Phlebovirus*) as revealed by the analysis of L segment sequences. *Ticks Tick. Borne. Dis.* **2019**, *10*, 942–948.
457. Matsuno, K.; Kajihara, M.; Nakao, R.; Nao, N.; Mori-Kajihara, A.; Muramatsu, M.; Qiu, Y.; Torii, S.; Igarashi, M.; Kasajima, N.; et al. The Unique Phylogenetic Position of a Novel Tick-Borne Phlebovirus Ensures an Ixodid Origin of the Genus *Phlebovirus*. *mSphere* **2018**, *3*: e00239-18.
458. Ejiri, H.; Lim, C.K.; Isawa, H.; Yamaguchi, Y.; Fujita, R.; Takayama-Ito, M.; Kuwata, R.; Kobayashi, D.; Horiya, M.; Posadas-Herrera, G.; et al. Isolation and characterization of Kabuto Mountain virus, a new tick-borne phlebovirus from *Haemaphysalis flava* ticks in Japan. *Virus Res.* **2018**, *244*, 252–261.
459. Safonova, M. V.; Shchelkanov, M.Y.; Khafizov, K.; Matsvay, A.D.; Ayginin, A.A.; Dolgova, A.S.; Shchelkanov, E.M.; Pimkina, E. V.; Speranskaya, A.S.; Galkina, I. V.; et al. Sequencing and genetic characterization of two strains Paramushir virus obtained from the Tyuleniy Island in the Okhotsk Sea (2015). *Ticks Tick. Borne. Dis.* **2019**, *10*, 269–279.
460. Zhou, H.; Ma, Z.; Hu, T.; Bi, Y.; Mamuti, A.; Yu, R.; Carr, M.J.; Shi, M.; Li, J.; Sharshov, K.; et al. Tamdy virus in ixodid ticks infesting bactrian camels, Xinjiang, China, 2018. *Emerg. Infect. Dis.* **2019**, *25*, 2136–2138.
461. Crabtree, M.B.; Sang, R.; Miller, B.R. Kupe virus, a new virus in the family Bunyaviridae, genus Nairovirus, Kenya. *Emerg. Infect. Dis.* **2009**, *15*, 147–154.
462. Lvov, D.K.; Sidorova, G.A.; Gromashevskiĭ, V.L.; Skvortsova, T.M.; Aristova, V.A. Isolation of Tamdy virus (Bunyaviridae) pathogenic for man from natural sources in Central Asia, Kazakhstan and Transcaucasia. *Vopr. Virusol.* **1984**, *29*, 487–490.
463. Lvov, D.K.; Sidorova, G.A.; Gromashevsky, V.L.; Kurbanov, M.; Skvoztsova, L.M.; Gofman, Y.P.; Berezina, L.K.; Klimenko, S.M.; Zakharyan, V.A.; Aristova, V.A.; et al. Virus 'Tamdy'-a new

arbovirus, isolated in the Uzbee S.S.R. and Turkmen S.S.R. from ticks *Hyalomma asiaticum asiaticum* Schulee et Schlottke, 1929, and *Hyalomma plumbeum plumbeum* Panzer, 1796. *Arch. Virol.* **1976**, *51*, 15–21.

464. Santos-Silva, M.M.; Vatansever, Z. *Hyalomma marginatum* Koch, 1844. In *Ticks of Europe and North Africa. A Guide to Species Identification*; 1st ed; Estrada-Peña, A., Mihalca, A.D., Petney, T., Eds.; Springer International Publishing: Cham, Switzerland, **2017**. pp. 343-354.
465. Nicolescu, G.; Vladimirescu, A.; Ciolpan, O. The distribution of mosquitoes in Romania (Diptera: Culicidae). Part III: Detailed maps for *Anopheles, Aedes* and *Culex*. *Eur Mosq Bull.* **2003**, *14*, 25–31.
466. Nicolescu, G.; Vladimirescu, A.; Ciolpan, O. The distribution of mosquitoes in Romania (Dipera: Culicidae). Part IV: Detailed maps for *Coquillettidia, Culiseta, Ochlerotatus, Orthopodomyia* and *Uranotaenia*. *Eur Mosq Bull.* **2003**, *15*, 16–27.
467. Nicolescu, G. The mosquitoes (Diptera:Culicidae) from Romania: an annotated checklist and bibliography. *Roum. Arch. Microbiol. Immunol.* 1994, *54*, 75–109.
468. Nicolescu G, Vladimirescu A, C.O.T. The distribution of mosquitoes in Romania (Diptera: Culicidae). Part I: *Anopheles, Aedes* and *Culex*. *Eur Mosq Bull.* **2002**, *13*, 17–26.
469. Nicolescu G, Vladimirescu A, C.O.T. The distribution of mosquitoes in Romania (Diptera: Culicidae). Part II: *Culiseta, Coquillettidia, Ochlerotatus, Orthopodomyia* and *Uranotaenia*. *Eur Mosq Bull.* **2003**, *14*, 1–15.
470. Becker, N.; Petric D,; Zgomba, M.; Boase, C.; Madon, M.; Dahl, C.; Kaiser, A. *Mosquitoes and Their Control*, 2nd ed.; Springer: Heidelberg, Germany, **2010**.
471. Medlock, J.M.; Snow, K.R.; Leach, S. Potential transmission of West Nile virus in the British Isles: An ecological review of candidate mosquito bridge vectors. *Med. Vet. Entomol.* **2005**, *19*, 2–21.
472. Martinet, J.P.; Ferté, H.; Failloux, A.B.; Schaffner, F.; Depaquit, J. Mosquitoes of north-western Europe as potential vectors of arboviruses: A review. *Viruses* **2019**, *11, 1059*.
473. Pachler, K.; Lebl, K.; Berer, D.; Rudolf, I.; Hubalek, Z.; Nowotny, N. Nile Virus Lineage in *Uranotaenia unguiculata* mosquitoes, Austria, 2013. *Emerg. Infect. Dis.* **2014**; 20:2119–22.
474. Chan, A.; Chiang, L.P.; Hapuarachchi, H.C.; Tan, C.H.; Pang, S.C.; Lee, R.; Lee, K.S.; Ng, L.C.; Lam-Phua, S.G. DNA barcoding: Complementing morphological identification of mosquito species in Singapore. *Parasites and Vectors* **2014**, *7:569*
475. Nielsen, S.A.; Kristensen, M. Delineation of *Culicoides* species by morphology and barcode exemplified by three new species of the subgenus *Culicoides* (Diptera: Ceratopogonidae) from Scandinavia. *Parasites and Vectors* **2015**, *8:151*.
476. Hardy, J.L.; Houk, E.J.; Kramer, L.D.; Reeves, W.C. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annu. Rev. Entomol. Vol. 28* **1983**, 229–262.
477. Rudolf, M.; Czajka, C.; Börstler, J.; Melaun, C.; Jöst, H.; von Thien, H.; Badusche, M.; Becker, N.; Schmidt-Chanasit, J.; Krüger, A.; et al. First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype *pipiens/molestus* hybrids in Germany. *PLoS One* **2013**, *8(9): e71832*.
478. Hanganu, J.; Dubyna, D.; Zhmud, E.; Grigoraș, I.; Menke, U.; Drost, H.; Ştefan, N.; Sărbu, I. Vegetation of the Biosphere Reserve "Danube Delta" - with Transboundary Vegetation Map on a 1:150,000 scale; Danube Delta National Institute, Romania, Kholodny MG - and Institute of Botany, Danube Delta Biosphere Reserve, Ukraine and RIZA, Eds. RIZA rapport 2002.049: Lelystad, The Netherlands, **2002**.
479. Lühken, R.; Pfitzner, W.P.; Börstler, J.; Garms, R.; Huber, K.; Schork, N.; Steinke, S.; Kiel, E.; Becker, N.; Tannich, E.; et al. Field evaluation of four widely used mosquito traps in Central Europe. *Parasites and Vectors* **2014**, *7:268*.
480. Reiter, P. West Nile virus in Europe: undertsanding the present to gauge the future. *Euro Surveill.* **2010**, *15(10):pii=19508*.
481. Cotar, A.I.; Fălcuţă, E.; Dinu, S.; Necula, A.; Bîrluţiu, V.; Ceianu, C.S.; Prioteasa, F.L. West Nile virus lineage 2 in Romania, 2015-2016: Co-circulation and strain replacement. *Parasites and Vectors* **2018**, *11:562*.
482. Carpenter, S.; Groschup, M.H.; Garros, C.; Felippe-Bauer, M.L.; Purse, B. V. *Culicoides* biting

midges, arboviruses and public health in Europe. *Antiviral Res.* **2013**, *100*, 102–113.
483. Hernández-Triana, L.M.; Brugman, V.A.; Nikolova, N.I.; Ruiz-Arrondo, I.; Barrero, E.; Thorne, L.; de Marco, M.F.; Krüger, A.; Lumley, S.; Johnson, N.; et al. DNA barcoding of British mosquitoes (Diptera, Culicidae) to support species identification, discovery of cryptic genetic diversity and monitoring invasive species. *Zookeys* **2019**, *832*, 57–76.
484. Pires, A.C.; Marinoni, L. DNA barcoding and traditional taxonomy unified through integrative Taxonomy: A view that challenges the debate questioning both methodologies. *Biota Neotrop.* **2010**, *10*, 339–346.
485. Hebert, P.D.N.; Cywinska, A.; Ball, S.L.; DeWaard, J.R. Biological identifications through DNA barcodes. *Proc. R. Soc. B Biol. Sci.* **2003**, *270*, 313–321.
486. Hajibabaei, M.; Singer, G.A.C.; Hebert, P.D.N.; Hickey, D.A. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends Genet.* **2007**, *23*, 167–172.
487. Ander, M.; Troell, K.; Chirico, J. Barcoding of biting midges in the genus *Culicoides*: A tool for species determination. *Med. Vet. Entomol.* **2013**, *27*, 323–331.
488. Beebe, N.W. DNA barcoding mosquitoes: Advice for potential prospectors. *Parasitology* **2018**, *145*, 622–633.
489. Whitworth, T.L.; Dawson, R.D.; Magalon, H.; Baudry, E. DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proc. R. Soc. B Biol. Sci.* **2007**, *274*, 1731–1739.
490. Borkent, A. The Subgeneric Classification of Species of *Culicoides* - thoughts and a warning; Last revised: May 16, 2016. *R. Br. Columbia Museum, Am. Museum Nat. Hist. Inst. Nac. Biodiversidad, Salmon Arm, Br. Columbia, Canada* **2016**.
491. Garros, C.; Mathieu, B.; Balenghien, T.; Cêtre-Sossah, C.; Delécolle, J.C. Suggesting synonymies? Comments on Kiehl et al. (2009) 'the European vectors of Bluetongue virus: Are there species complexes, single species or races in Culicoides obsoletus and C. pulicaris detectable by sequencing ITS-1, ITS-2 and 18S-rDNA?' *Parasitol. Res.* **2010**, *107*, 731–734.
492. Schlick-Steiner, B.C.; Steiner, F.M.; Seifert, B.; Stauffer, C.; Christian, E.; Crozier, R.H. Integrative Taxonomy: A Multisource Approach to Exploring Biodiversity. *Annu. Rev. Entomol.* **2010**, *55*, 421–438.
493. Dik, B.; Yağci, Ş.; Linton, Y.M. A review of species diversity and distribution of *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) in Turkey. *J. Nat. Hist.* **2006**, *40*, 1947–1967.
494. Rawlings, P. A key based on wing patterns of biting midges (genus *Culicoides* Latreille - Diptera: Ceratopogonidae) in the Iberian Peninsula, for use in epidemiological studies. *Graellsia* **1996**, *52*, 57–71.
495. Talavera, S.; Muñoz-Muñoz, F.; Pagès, N. New insights on diversity, morphology and distribution of *Culicoides* Latreille 1809 (Diptera: Ceratopogonidae) from northeast Spain. *Ann. la Soc. Entomol. Fr.* **2011**, *47*, 214–231.
496. Szadziewski, R.; Borkent, A.; Dominiak, P.; Fauna Europaea: Ceratopogonidae. In *Fauna Europaea: Diptera, Nematocera. Fauna Europaea version 2.6; 2013*. Beuk P., Pape T., Eds. Available online: https ://fauna -eu.org/cdm_datap ortal /taxon /71618 29a-94f3-4c36-943d-0d15f 8fa1f 77 (accessed on 15 Aug 2019).
497. Sarvašová, A.; Delecolle, J.C.; Halan, M.; Mathieu, B.; Kočišová, A. Morphological and molecular analysis of the genus *Culicoides* (Diptera: Ceratopogonidae) in Slovakia with five new records. *Zootaxa* **2014**, *3872*, *541*.
498. Filatov, S.; Szadziewski, R. Annotated checklist and distribution of *Culicoides* biting midges of Ukraine (Diptera: Ceratopogonidae). *J. Nat. Hist.* **2017**, *51*, 487–511.
499. Pagès, N.; Muñoz-Muñoz, F.; Talavera, S.; Sarto, V.; Lorca, C.; Núñez, J.I. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. *Vet. Parasitol.* **2009**, *165*, 298–310.
500. Sarvašová, A.; Kočišová, A.; Candolfi, E.; Mathieu, B. Description of *Culicoides* (*Culicoides*) *bysta* n. sp., a new member of the Pulicaris group (Diptera: Ceratopogonidae) from Slovakia. *Parasites*

and Vectors **2017**, *10:279*.
501. Pettersson, E.; Bensch, S.; Ander, M.; Chirico, J.; Sigvald, R.; Ignell, R. Molecular identification of bloodmeals and species composition in *Culicoides* biting midges. *Med. Vet. Entomol.* **2013**, *27*, 104–112.
502. Goffredo, M.; Catalani, M.; Federici, V.; Portanti, O.; Marini, V.; Mancini, G.; Quaglia, M.; Santilli, A.; Teodori, L.; Savini, G. Specie di *Culicoides* coinvolte nell'epidemia di Bluetongue 2012-2014 in Italia. *Vet. Ital.* **2015**, *51*, 131–138.
503. Ravagnan, S.; Montarsi, F.; Cazzin, S.; Porcellato, E.; Russo, F.; Palei, M.; Monne, I.; Savini, G.; Marangon, S.; Barzon, L.; et al. First report outside Eastern Europe of West Nile virus lineage 2 related to the Volgograd 2007 strain, northeastern Italy, 2014. *Parasites and Vectors* **2015**, *8:418*.
504. Ciccozzi, M.; Peletto, S.; Cella, E.; Giovanetti, M.; Lai, A.; Gabanelli, E.; Acutis, P.L.; Modesto, P.; Rezza, G.; Platonov, A.E.; et al. Epidemiological history and phylogeography of West Nile virus lineage 2. *Infect. Genet. Evol.* **2013**, *17*, 46–50.
505. Rappole, J.H.; Derrickson, S.R.; Hubálek, Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerg. Infect. Dis.* **2000**, *6*, 319–328.
506. Liu, W.J.; Chen, H.B.; Wang, X.J.; Huang, H.; Khromykh, A.A. Analysis of Adaptive Mutations in Kunjin Virus Replicon RNA Reveals a Novel Role for the Flavivirus Nonstructural Protein NS2A in Inhibition of Beta Interferon Promoter-Driven Transcription. *J. Virol.* **2004**, *78*, 12225–12235.
507. Papa, A. West Nile Virus – Neutralizing Antibodies in Humans in Greece. *Vector-Borne Zoonotic Dis.* **2010**, *10*, 10–13.
508. Veo, C.; Della Ventura, C.; Moreno, A.; Rovida, F.; Percivalle, E.; Canziani, S.; Torri, D.; Calzolari, M.; Baldanti, F.; Galli, M.; et al. Evolutionary Dynamics of the Lineage 2 West Nile Virus That Caused the Largest European Epidemic: Italy 2011-2018. *Viruses* **2019**, *11, 814*.
509. Zehender, G.; Veo, C.; Ebranati, E.; Carta, V.; Rovida, F.; Percivalle, E.; Moreno, A.; Lelli, D.; Calzolari, M.; Lavazza, A.; et al. Reconstructing the recent West Nile virus lineage 2 epidemic in Europe and Italy using discrete and continuous phylogeography. *PLoS One* **2017**, *12(7): e0179679*.
510. L'Ambert, G.; Ferré, J.; Schaffner, F.; Fontenille, D. Comparison of Different Trapping Methods for Surveillance of Mosquito Vectors of West Nile Virus in Rhône Delta, France Comparison of different trapping methods for surveillance of mosquito vectors of West Nile virus in Rhône Delta , France. *J. Vect. Ecol.* **2012** *37*, 269–275.
511. Victoriano Lopis, I.; Tomassone, L.; Grego, E.; Serrano, E.; Mosca, A.; Vaschetti, G.; Andrade, D.; Rossi, L. Evaluating the feeding preferences of West Nile virus mosquito vectors using bird-baited traps. *Parasites and Vectors* **2015**, *9:479*.
512. Bingham, A.M.; Graham, S.P.; Burkett-Cadena, N.D.; White, G.S.; Hassan, H.K.; Unnasch, T.R. Detection of Eastern Equine Encephalomyelitis Virus RNA in North American Snakes. *Am. J. Trop. Med. Hyg.* **2012**, *87*, 1140–1144.
513. Graham, S.P.; Hassan, H.K.; Chapman, T.; White, G.; Guyer, C.; Unnasch, T.R. Serosurveillance of Eastern equine encephalitis virus in amphibians and reptiles from Alabama, USA. *Am. J. Trop. Med. Hyg.* **2012**, *86*, 540–544.
514. Steinman, A.; Banet-noach, C.; Simanov, L.; Grinfeld, N.; Aizenberg, Z.; Levi, O.; Lahav, D.A.N.; Malkinson, M.; Perk, S.; Shpigel, N.Y. Experimental Infection of Common Garter Snakes (*Thamnophis sirtalis*) with West Nile Virus. *Vector-Borne Zoonotic Dis.* **2006**, *6(4)*: 361-368
515. Whitney, E.; Jamnback, H.; Means, R.G.; Watthews, T.H. Arthropod-borne-virus survey in St. Lawrence County, New York. Arbovirus reactivity in serum from amphibians, reptiles, birds, and mammals. *Am. J. Trop. Med. Hyg.* **1968**, *17*, 645–650.
516. Marschang, R.E. Viruses infecting reptiles. *Viruses* **2011**, *3*, 2087–2126.
517. Attoui, H. ; Mertens, P.; Becnel, J.; Belaganahalli, S.; Bergoin, M.; Brussaard, C.; Chappell, J.; Ciarlet, M.; del Vas, M.; Dermody, T. Orbiviruses, Reoviridae. In *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*; King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., Eds. Elsevier Academic Press: San Diego, CA, USA, **2012**.
518. Attoui, H.; Jaafar, F.M.; Belhouchet, M.; Aldrovandi, N.; Tao, S.; Chen, B.; Liang, G.; Tesh, R.B.; de Micco, P.; de Lamballerie, X. Yunnan orbivirus, a new orbivirus species isolated from *Culex*

tritaeniorhynchus mosquitoes in China. *J. Gen. Virol.* **2005**, *86*, 3409–3417.
519. Attoui, H.; Mendez-lopez, M.R.; Rao, S.; Hurtado-Alendes, A.; Lizaraso-Caparo, F.; Jaafar, F.M.; Samuel, A.R.; Belhouchet, M.; Pritchard, L.I.; Melville, L.; et al. Peruvian horse sickness virus and Yunnan orbivirus, isolated from vertebrates and mosquitoes in Peru and Australia. *Virology* **2009**, *394*, 298–310.
520. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Brownlie, J.; Tesh, R.; Attoui, H.; Mertens, P.P.C. Genetic characterization of the tick-borne orbiviruses. *Viruses* **2015**, *7*, 2185–2209.
521. Cooper, E.; Anbalagan, S.; Klumper, P.; Scherba, G.; Simonson, R.R.; Hause, B.M. Mobuck virus genome sequence and phylogenetic analysis: Identification of a novel Orbivirus isolated from a white-tailed deer in Missouri, USA. *J. Gen. Virol.* **2014**, *95*, 110–116.
522. Li, M.; Zheng, Y.; Zhao, G.; Fu, S.; Wang, D.; Wang, Z.; Liang, G. Tibet Orbivirus, a novel Orbivirus species isolated from *Anopheles maculatus* mosquitoes in Tibet, China. *PLoS One* **2014**, *9(2): e88738*.
523. Attoui, H.; Fang, Q.; de Lamballerie, X.; Cantaloube, J.-F.; Jaafar, F.M. Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus *Aquareovirus*, family *Reoviridae*). *J. Gen. Virol.* **2002**, *83*, 1941–1951.
524. Attoui, H.; Mohd Jaafar, F.; Belhouchet, M.; Biagini, P.; Cantaloube, J.F.; De Micco, P.; De Lamballerie, X. Expansion of family *Reoviridae* to include nine-segmented dsRNA viruses: Isolation and characterization of a new virus designated Aedes pseudoscutellaris reovirus assigned to a proposed genus (*Dinovernavirus*). *Virology* **2005**, *343*, 212–223.
525. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Tesh, R.; Attoui, H.; Mertens, P.P.C. Umatilla virus genome sequencing and phylogenetic analysis: Identification of Stretch Lagoon orbivirus as a new member of the *Umatilla virus* species. *PLoS One* **2011**, *6(8): e23605*.
526. Belhouchet, M.; Jaafar, F.; Firth, A.E.; Grimes, J.M.; Mertens, P.P.C.; Attoui, H. Detection of a fourth orbivirus non-structural protein. *PLoS One* **2011**, *6(10): e25697*.
527. Ratinier, M.; Caporale, M.; Golder, M.; Franzoni, G.; Allan, K.; Nunes, S.F.; Armezzani, A.; Bayoumy, A.; Rixon, F.; Shaw, A.; et al. Identification and characterization of a novel non-structural protein of Bluetongue virus. *PLoS Pathog.* **2011**, *7(12): e1002477*.
528. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Nomikou, K.; Pritchard, I.; Lunt, R.; Kirkland, P.D.; Attoui, H.; Brownlie, J.; Mertens, P.P.C. Full genome sequencing and genetic characterization of Eubenangee viruses identify Pata virus as a distinct species within the genus *Orbivirus*. *PLoS One* **2012**, *7(3): e31911*.
529. Mohd Jaafar, F.; Belhouchet, M.; Belaganahalli, M.; Tesh, R.B.; Mertens, P.P.C.; Attoui, H. Full-genome characterisation of Orungo, Lebombo and Changuinola viruses provides evidence for co-evolution of orbiviruses with their arthropod vectors. *PLoS One* **2014**, *9(1): e86392*.
530. Dilcher, M.; Weidmann, M. Confusions in orbivirus protein classification. *Virol. J.* **2012**, *9:166*.
531. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Pritchard, I.; Kirkland, P.D.; Brownlie, J.; Attoui, H.; Mertens, P.P.C. Full genome characterization of the *Culicoides*-borne marsupial orbiviruses: Wallal virus, Mudjinbarry virus and Warrego viruses. *PLoS One* **2014**, *9(10): e108379*.
532. Dilcher, M.; Hasib, L.; Lechner, M.; Wieseke, N.; Middendorf, M.; Marz, M.; Koch, A.; Spiegel, M.; Dobler, G.; Hufert, F.T.; et al. Genetic characterization of Tribeč virus and Kemerovo virus, two tick-transmitted human-pathogenic Orbiviruses. *Virology* **2012**, *423*, 68–76.
533. Fagre, A.C.; Lee, J.S.; Kityo, R.M.; Bergren, N.A.; Mossel, E.C.; Nakayiki, T.; Nalikka, B.; Nyakarahuka, L.; Gilbert, A.T.; Peterhans, J.K.; et al. Discovery and Characterization of Bukakata orbivirus (Reoviridae: Orbivirus), a Novel Virus from a Ugandan Bat. *Viruses* **2019**, *11, 209*.
534. Silva, S.P.; Dilcher, M.; Weber, F.; Hufert, F.T.; Weidmann, M.; Cardoso, J.F.; Carvalho, V.L.; Chiang, J.O.; Martins, L.C.; Lima, C.P.S.; et al. Genetic and biological characterization of selected Changuinola viruses (*Reoviridae, Orbivirus*) from Brazil. *J. Gen. Virol.* **2014**, *95*, 2251–2259.
535. Belhouchet, M.; Jaafar, F.M.; Tesh, R.; Grimes, J.; Maan, S.; Mertens, P.P.C.; Attoui, H. Complete sequence of Great Island virus and comparison with the T2 and outer-capsid proteins of Kemerovo, Lipovnik and Tribec viruses (genus *Orbivirus*, family *Reoviridae*). *J. Gen. Virol.* **2010**,

91, 2985–2993.
536. McDonald, S.M.; Patton, J.T. Assortment and packaging of the segmented rotavirus genome. *Trends Microbiol.* **2011**, *19*, 136–144.
537. Mukherjee, A.; Dutta, D.; Ghosh, S.; Bagchi, P.; Chattopadhyay, S.; Nagashima, S.; Kobayashi, N.; Dutta, P.; Krishnan, T.; Naik, T.N.; et al. Full genomic analysis of a human group A rotavirus G9P[6] strain from Eastern India provides evidence for porcine-to-human interspecies transmission. *Arch. Virol.* **2009**, *154*, 733–746.
538. Horimoto, T.; Kawaoka, Y. Influenza: Lessons from past pandemics, warnings from current incidents. *Nat. Rev. Microbiol.* **2005**, *3*, 591–600.
539. Taubenberger, J.K.; Kash, J.C. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* **2010**, *7*, 440–451.
540. Gonzales, H.A.; Knudson, D.L. Orbivirus Species and Speciation: Genetic Reassortment between Corriparta Serogroup Viruses. *Intervirology* **1987**, *28*, 126–133.
541. Xing, S.; Guo, X.; Zhang, X.; Zhao, Q.; Li, L.; Zuo, S.; An, X.; Pei, G.; Sun, Q.; Cheng, S.; et al. A novel mosquito-borne reassortant orbivirus isolated from Xishuangbanna, China. Supplement. *Virol. Sin.* **2017**, *32*, 159–162.
542. Coetzee, P.; Van Vuuren, M.; Stokstad, M.; Myrmel, M.; Venter, E.H. Bluetongue virus genetic and phenotypic diversity: Towards identifying the molecular determinants that influence virulence and transmission potential. *Vet. Microbiol.* **2012**, *161*, 1–12.
543. Ngoveni, H.G.; van Schalkwyk, A.; Koekemoer, J.J.O. Evidence of Intragenic Recombination in African Horse Sickness Virus. *Viruses* **2019**, *11*, *654*.
544. Farkas, S.L.; Marton, S.; Dandár, E.; Kugler, R.; Gál, B.; Jakab, F.; Bálint, Á.; Kecskeméti, S.; Bányai, K. Lineage diversification, homo- and heterologous reassortment and recombination shape the evolution of chicken orthoreoviruses. *Sci. Rep.* **2016**, *6:36960*.
545. Naglič, T.; Rihtarič, D.; Hostnik, P.; Toplak, N.; Koren, S.; Kuhar, U.; Jamnikar-Ciglenečki, U.; Kutnjak, D.; Steyer, A. Identification of novel reassortant mammalian orthoreoviruses from bats in Slovenia. *BMC Vet. Res.* **2018**, *14:264*.
546. Marthaler, D.; Suzuki, T.; Rossow, K.; Culhane, M.; Collins, J.; Goyal, S.; Tsunemitsu, H.; Ciarlet, M.; Matthijnssens, J. VP6 genetic diversity, reassortment, intragenic recombination and classification of rotavirus B in American and Japanese pigs. *Vet. Microbiol.* **2014**, *172*, 359–366.
547. Day, J.M. The diversity of the orthoreoviruses: Molecular taxonomy and phylogentic divides. *Infect. Genet. Evol.* **2009**, *9*, 390–400.
548. Kugler, R.; Marschang, R.E.; Ihász, K.; Lengyel, G.; Jakab, F.; Bányai, K.; Farkas, S.L. Whole genome characterization of a chelonian orthoreovirus strain identifies significant genetic diversity and may classify reptile orthoreoviruses into distinct species. *Virus Res.* **2016**, *215*, 94–98.
549. Bányai, K.; Borzák, R.; Ihász, K.; Fehér, E.; Dán, Á.; Jakab, F.; Papp, T.; Hetzel, U.; Marschang, R.E.; Farkas, S.L. Whole-genome sequencing of a green bush viper reovirus reveals a shared evolutionary history between reptilian and unusual mammalian orthoreoviruses. *Arch. Virol.* **2013**, *159*, 153–158.
550. Hermanns, K.; Zirkel, F.; Kurth, A.; Drosten, C.; Junglen, S. Cimodo virus belongs to a novel lineage of reoviruses isolated from African mosquitoes. *J. Gen. Virol.* **2014**, *95*, 905–909.
551. Doherty, R.L.; Whitehead, R.H.; Wetters, E.J.; Gorman, B.M.; Carley, J.G. A survey of antibody to 10 arboviruses (Koongol group, Mapputta group and ungrouped) isolated in Queensland. *Trans. R. Soc. Trop. Med. Hyg.* **1970**, *64*, 748–753.
552. Harrison, J.J.; Warrilow, D.; McLean, B.J.; Watterson, D.; O'Brien, C.A.; Colmant, A.M.G.; Johansen, C.A.; Barnard, R.T.; Hall-Mendelin, S.; Davis, S.S.; et al. A new orbivirus isolated from mosquitoes in North-Western Australia shows antigenic and genetic similarity to Corriparta virus but does not replicate in vertebrate cells. *Viruses* **2016**, *8, 141*.
553. Van Gennip, R.G.P.; Drolet, B.S.; Rozo Lopez, P.; Roost, A.J.C.; Boonstra, J.; Van Rijn, P.A. Vector competence is strongly affected by a small deletion or point mutations in Bluetongue virus. *Parasites and Vectors* **2019**, *12:470*.

554. Anthony, S.J.; Darpel, K.E.; Belaganahalli, M.N.; Maan, N.; Nomikou, K.; Sutton, G.; Attoui, H.; Maan, S.; Mertens, P.P.C. RNA segment 9 exists as a duplex concatemer in an Australian strain of Epizootic haemorrhagic disease virus (EHDV): Genetic analysis and evidence for the presence of concatemers as a normal feature of orbivirus replication. *Virology* **2011**, *420*, 164–171.
555. Mohd Jaafar, F.; Goodwin, A.E.; Belhouchet, M.; Merry, G.; Fang, Q.; Cantaloube, J.F.; Biagini, P.; de Micco, P.; Mertens, P.P.C.; Attoui, H. Complete characterisation of the American grass carp reovirus genome (genus *Aquareovirus*: family *Reoviridae*) reveals an evolutionary link between aquareoviruses and coltiviruses. *Virology* **2008**, *373*, 310–321.
556. Tian, Y.; Tarlow, O.; Ballard, A.; Desselberger, U.; McCrae, M.A. Genomic concatemerization/deletion in rotaviruses: a new mechanism for generating rapid genetic change of potential epidemiological importance. *J. Virol.* **1993**, *67*, 6625–32.
557. Murao, K.; Uyeda, I.; Ando, Y.; Kimura, I.; Cabauatan, P.Q.; Koganezawa, H. Genomic rearrangement in genome segment 12 of rice dwarf phytoreovirus. *Virology* **1996**, *216*, 238–240.
558. Huang, C.; Liu, W.J.; Xu, W.; Jin, T.; Zhao, Y.; Song, J.; Shi, Y.; Ji, W.; Jia, H.; Zhou, Y.; et al. A Bat-Derived Putative Cross-Family Recombinant Coronavirus with a Reovirus Gene. *PLoS Pathog.* **2016**, *12(9): e1005883*.
559. Kapoor, A.; Tesh, R.B.; Duraisamy, R.; Popov, V.L.; da Rosa, A.P.A.T.; Lipkin, W.I. A novel mosquito-borne Orbivirus species found in South-east Asia. *J. Gen. Virol.* **2013**, *94*, 1051–1057.
560. Wang, J.; Li, H.; He, Y.; Zhou, Y.; Xin, A.; Liao, D.; Meng, J. Isolation of Tibet orbivirus from *Culicoides* and associated infections in livestock in Yunnan, China. *Virol. J.* **2017**, *14:105*.
561. Basak, A.K.; Gouet, P.; Grimes, J.; Roy, P.; Stuart, D. Crystal structure of the top domain of African horse sickness virus VP7: comparisons with bluetongue virus VP7. *J. Virol.* **1996**, *70*, 3797–806.
562. Tan, B.H.; Nason, E.; Staeuber, N.; Jiang, W.R.; Monastryrskaya, K.; Roy, P. RGD tripeptide of Bluetongue virus VP7 protein is responsible for core attachment to *Culicoides* cells. *J. Virol.* **2001**, *75*, 3937–3947.
563. Anthony, S.J.; Maan, N.; Maan, S.; Sutton, G.; Attoui, H.; Mertens, P.P.C. Genetic and phylogenetic analysis of the core proteins VP1, VP3, VP4, VP6 and VP7 of Epizootic haemorrhagic disease virus (EHDV). *Virus Res.* **2009**, *145*, 187–199.
564. Brown, S.E.; Morrison, H.G.; Karabatsos, N.; Knudson, D.L. Genetic relatedness of two new Orbivirus serogroups: Orungo and Lebombo. *J. Gen. Virol.* **1991**, *72*, 1065–1072.
565. Tomori, O.; Fabiyi, A.; Murphy, F. Characterization of Orungo virus, an orbivirus from Uganda and Nigeria. *Arch. Virol.* **1976**, *51*, 285–298.
566. Tomori, O.; Aitken, T.H.G. Orungo Virus: Transmission Studies with *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **1978**, *14*, 523–526.
567. Center for Disease Control and Prevention. Available online: https://wwwn.cdc.gov/Arbocat/VirusDetails.aspx?ID=144&SID=2 (accessed on 8.04.2020).
568. ReoID. Available online: https://www.reoviridae.org/dsRNA_virus_proteins/ReoID/orbiviruses.htm (accessed on 8.04.2020).
569. Mellor, P.S.; Jennings, M. Replication of Eubenangee virus in *Culicoides nubeculosus* (Mg.) and *Culicoides variipennis* (Coq.). *Arch. Virol.* **1980**, *63*, 203–208.
570. Ebersohn, K.; Coetzee, P.; Snyman, L.P.; Swanepoel, R.; Venter, E.H. Phylogenetic characterization of the Palyam serogroup orbiviruses. *Viruses* **2019**, *11, 446*.
571. Parkes, H.; Gould, A.R. Characterisation of Wongorr virus, an Australian orbivirus. *Virus Res.* **1996**, *44*, 111–122.
572. Lei, W.; Guo, X.; Fu, S.; Feng, Y.; Nie, K.; Song, J.; Li, Y.; Ma, X.; Liang, G.; Zhou, H. Isolation of Tibet orbivirus, TIBOV, from *Culicoides* collected in Yunnan, China. *PLoS One* **2015**, *10(8): e0136257*.
573. Apperson, C.S.; Hassan, H.K.; Harrison, B.A.; Harry, M.; Aspen, S.E.; Farajollahi, A.R.Y.; Crans, W.; Thomas, J.; Falco, R.C.; Benedict, M.; et al. Host Feeding Patterns of Established and Potential Mosquito Vectors of West Nile Virus in the Eastern United States. *Vector Borne Zoonotic Dis.* ***2004***;

4(1): 71–82.

574. Greenberg, J.A.; Lujan, D. A.; DiMenna, M. A; Wearing, H.J.; Hofkin, B. V. Identification of blood meal sources in *Aedes vexans* and *Culex quinquefasciatus* in Bernalillo County, New Mexico. *J. Insect Sci.* **2013**, *13*:75.
575. Gomes, B.; Sousa, C.A.; Vicente, J.L.; Pinho, L.; Calderón, I.; Arez, E.; Almeida, A.P.; Donnelly, M.J.; Pinto, J. Feeding patterns of *molestus* and *pipiens* forms of *Culex pipiens* (Diptera: Culicidae) in a region of high hybridization. *Parasites and Vectors* **2013**, *6:93*.
576. Hubálek, Z.; Halouzka, J. West Nile fever - a reemerging mosquito-borne viral disease in Europe. *Emerg. Infect. Dis.* **1999**, *5*, 643–650.
577. Takken, W.; Verhulst, N.O. Host Preferences of Blood-Feeding Mosquitoes. *Annu. Rev. Entomol.* **2013**, *58*, 433–453.
578. Chaves, L.F.; Harrington, L.C.; Keogh, C.L.; Nguyen, A.M.; Kitron, U.D. Blood feeding in patterns of mosquoitoes: random or structured? *Front. Zool.* **2010**, *7*:3
579. Burkett-Cadena, N.D.; McClure, C.J.W.; Estep, L.K.; Eubanks, M.D. What drives the spatial distribution of mosquitoes? *Ecosphere* **2013**, *4*(2):30.
580. Danube Delta Biosphere Reserve Administration. Press release: The situation of abandoned horses around Letea Forest in Danube Delta Biosphere Reserve (in Romanian). Available online: http://www.ddbra.ro/informatii-publice/media/comunicate-de-presa/situatia-cailor-abandonati-in-zona-padurii-letea-din-rezervatia-biosferei-delta-dunarii-a811 (accessed on 8.04.2020).
581. Port, G.R.; Boreham, P.F.L.; Bryan, J.H. The relationship of host size to feeding by mosquitoes of the *Anopheles gambiae* Giles complex (Diptera: Culicidae). *Bull. Entomol. Res.* **1980**, *70*, 133–144.
582. Ionică, A.M.; Zittra, C.; Wimmer, V.; Leitner, N.; Votýpka, J.; Modrý, D.; Mihalca, A.D.; Fuehrer, H.P. Mosquitoes in the Danube Delta: Searching for vectors of filarioid helminths and avian malaria. *Parasites and Vectors* **2017**, *10:324*.
583. Martínez-De La Puente, J.; Ruiz, S.; Soriguer, R.; Figuerola, J. Effect of blood meal digestion and DNA extraction protocol on the success of blood meal source determination in the malaria vector *Anopheles atroparvus*. *Malar. J.* **2013**, *12:109*.
584. Reeves, L.E.; Holderman, C.J.; Gillett-Kaufman, J.L.; Kawahara, A.Y.; Kaufman, P.E. Maintenance of host DNA integrity in field-preserved mosquito (Diptera: Culicidae) blood meals for identification by DNA barcoding. *Parasites and Vectors* **2016**, *9:503*.
585. Logue, K.; Keven, J.B.; Cannon, M. V.; Reimer, L.; Siba, P.; Walker, E.D.; Zimmerman, P.A.; Serre, D. Unbiased Characterization of *Anopheles* Mosquito Blood Meals by Targeted High-Throughput Sequencing. *PLoS Negl. Trop. Dis.* **2016**, *10(3): e0004512*.
586. García-Bocanegra, I.; Busquets, N.; Napp, S.; Alba, A.; Zorrilla, I.; Villalba, R.; Arenas, A. Serosurvey of West Nile Virus and Other Flaviviruses of the Japanese Encephalitis Antigenic Complex in Birds from Andalusia, Southern Spain. *Vector-Borne Zoonotic Dis.* **2011**, *11*, 1107–1113.
587. García-Bocanegra, I.; Jurado-Tarifa, E.; Cano-Terriza, D.; Martínez, R.; Pérez-Marín, J.E.; Lecollinet, S. Exposure to West Nile virus and Tick-borne encephalitis virus in dogs in Spain. *Transbound. Emerg. Dis.* **2018**, 00:1–8.
588. Maquart, M.; Dahmani, M.; Marié, J.-L.; Gravier, P.; Leparc-Goffart, I.; Davoust, B. First Serological Evidence of West Nile Virus in Horses and Dogs from Corsica Island, France. *Vector-Borne Zoonotic Dis.* **2017**, *17*, 275–277.
589. Popescu, D.; Necșulescu, M.; Alexse, A.; Purcărea-Ciulacu, V. West Nile virus infection of horses in anthropic ecosystems in Romania. *Rev. Rom. Med. Vet.* **2008**, *18*, 39–46.
590. Durand, B.; Haskouri, H.; Lowenski, S.; Vachiery, N.; Beck, C.; Lecollinet, S. Seroprevalence of West Nile and Usutu viruses in military working horses and dogs, Morocco, 2012: Dog as an alternative WNV sentinel species? *Epidemiol. Infect.* **2016**, *144*, 1857–1864.
591. BirdLife International. 2016. *Streptopelia orientalis*. *The IUCN Red List of Threatened Species* 2016: e.T22690439A93273750.https://dx.doi.org/10.2305/IUCN.UK.20163.RLTS.T22690439A93273750.en (accessed on 20.05.2020)
592. Martínez-De la Puente, J.; Méndez, M.; Ruiz, S.; Godoy, J.A.; Soriguer, R.C.; Figuerola, J. Individual identification of endangered species using mosquito blood meals: a proof-of-concept

study in Iberian lynx. *Parasitol. Res.* **2015**, *114*, 1607–1610.
593. Hopken, M.W.; Ryan, B.M.; Huyvaert, K.P.; Piaggio, A.J. Picky eaters are rare: DNA-based blood meal analysis of *Culicoides* (Diptera: Ceratopogonidae) species from the United States. *Parasites and Vectors* **2017**, *10:169*.
594. Tempelis, C.H.; Nelson, R.L. Blood-feeding patterns of midges of the *Culicoides variipennis* complex in Kern County, California. *J. Med. Entomol.* **1971**, *8*, 532–534.
595. Calvo, J.H.; Berzal, B.; Calvete, C.; Miranda, M.A.; Estrada, R.; Lucientes, J. Host feeding patterns of *Culicoides* species (Diptera: Ceratopogonidae) within the Picos de Europa National Park in northern Spain. *Bull. Entomol. Res.* **2012**, *102*, 692–697.
596. Martinez-De La Puente, J.; Merino, S.; Toms, G.; Moreno, J.; Morales, J.; Lobato, E.; Talavera, S.; Sarto I Monteys, V. Factors affecting *Culicoides* species composition and abundance in avian nests. *Parasitology* **2009**, *136*, 1033–1041.
597. Santiago-Alarcon, D.; Havelka, P.; Pineda, E.; Segelbacher, G.; Schaefer, H.M. Urban forests as hubs for novel zoonosis: blood meal analysis, seasonal variation in *Culicoides* (Diptera: Ceratopogonidae) vectors and avian haemosporidians. *Parasitology* **2013**, 140, 1799–1810.
598. Augot, D.; Hadj-Henni, L.; Strutz, S.E.; Slama, D.; Millot, C.; Depaquit, J.; Millot, J.-M. Association between host species choice and morphological characters of main sensory structures of *Culicoides* in the Palaeartic region. *PeerJ* **2017**, *5:e3478*.
599. Jacob A. Greenberg, Mark A. DiMenna, Ben Hanelt, and Hofkin, B.V. Analysis of post-blood meal flight distances in mosquitoes utilizing zoo animal blood meals. *J Vector Ecol.* **2012**, *37*, 83–89.
600. Sanders, C. J.; Selby, R.; Carpenter, S.; Reynolds, D.R. High-altitude flight of *Culicoides* biting midges. *Vet. Rec.* **2011**, *169(8):208*.
601. Sedda, L.; Rogers, D.J. The influence of the wind in the Schmallenberg virus outbreak in Europe. *Sci. Rep.* **2013**, *3:3361*.
602. Talavera, S.; Muñoz-Muñoz, F.; Verdún, M.; Pagès, N. Morphology and DNA barcoding reveal three species in one: description of *Culicoides cryptipulicaris* sp. nov. and *Culicoides quasipulicaris* sp. nov. in the subgenus *Culicoides*. *Med. Vet. Entomol.* **2017**, *doi: 10.1111/mve.12228*.

Appendix

Török et al. Parasites & Vectors (2016) 9:196
DOI 10.1186/s13071-016-1484-7

Parasites & Vectors

RESEARCH Open Access

Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of *Anopheles algeriensis* Theobald, 1903 and *Aedes hungaricus* Mihályi, 1955 for Romania

Edina Török[1,2,3†], Alexandru Tomazatos[4†], Daniel Cadar[4], Cintia Horváth[1], Lujza Keresztes[1], Stephanie Jansen[4], Norbert Becker[5,6], Achim Kaiser[5], Octavian Popescu[2,3], Jonas Schmidt-Chanasit[4,7], Hanna Jöst[4,7] and Renke Lühken[4*]

Abstract

Background: Mosquito-borne viruses (moboviruses) are of growing importance in many countries of Europe. In Romania and especially in the Danube Delta Biosphere Reserve (DDBR), mosquito and mobovirus surveillance are not performed on a regular basis. However, this type of study is crucially needed to evaluate the risk of pathogen transmission, to understand the ecology of emerging moboviruses, or to plan vector control programmes.

Methods: We initiated a longitudinal mosquito surveillance study with carbon dioxide-baited Heavy Duty Encephalitis Vector Survey traps at four sampling sites to analyse the spatio-temporal pattern of the (i) mosquito species composition and diversity, (ii) functional groups of mosquitoes (oviposition sites, overwintering stage, and number of generations), and (iii) the occurrence of potential West Nile virus (WNV) vectors.

Results: During 2014, a total of 240,546 female mosquitoes were collected. All species were identified using morphological characteristics and further confirmed by mitochondrial cytochrome *c* oxidase subunit I (COI) gene analysis of selected specimens. The two most common taxa were *Coquilettidia richiardii* (40.9 %) and *Anopheles hyrcanus* (34.1 %), followed by *Culex pipiens* (*sensu lato*) (*s.l.*)/*Cx. torrentium* (7.7 %), *Aedes caspius* (5.7 %), *Cx. modestus* (4.0 %), *An. maculipennis* (*s.l.*) (3.9 %), and *Ae. vexans* (3.0 %). A further seven species were less common in the area studied, including two new records for Romania: *An. algeriensis* and *Ae. hungaricus*. Phylogenetic analysis of COI gene demonstrated the evolutionary relatedness of most species with specimens of the same species collected in other European regions, except *Ae. detritus* and *An. algeriensis*, which exhibited high genetic diversity. Due to the dominance of *Cq. richiardii* and *An. hyrcanus* (75 % of all collected specimens), the overall phenology and temporal pattern of functional groups basically followed the phenology of both species. A huge proportion of the mosquito population in the course of the entire sampling period can be classified as potential WNV vectors. With 40 % of all collected specimens, the most frequent species *Cq. richiardii* is probably the most important vector of WNV in the DDBR.

(Continued on next page)

* Correspondence: luehken@bnitm.de
†Equal contributors
[4]Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research National Reference Centre for Tropical Infectious Diseases, Hamburg, Germany
Full list of author information is available at the end of the article

(Continued from previous page)

Conclusion: This is the first DNA-barcoding supported analysis of the mosquito fauna in the DDBR. The detection of two new species highlights the lack of knowledge about the mosquito fauna in Romania and in the DDBR in particular. The results provide detailed insights into the spatial-temporal mosquito species composition, which might lead to a better understanding of mobovirus activity in Romania and thus, can be used for the development of vector control programs.

Keywords: Romania, Danube Delta Biosphere Reserve, Mosquito surveillance, Mitochondrial cytochrome *c* oxidase subunit I, *Aedes hungaricus*, *Anopheles algeriensis*

Background

In Europe, at least ten different mosquito-borne viruses (moboviruses) are circulating [1] and especially members of the family Flaviviridae, i.e. dengue virus, West Nile virus (WNV), and Usutu virus (USUV), are of growing public health and veterinary importance [2]. Although mosquito and pathogen surveillance in Romania is not performed on a regular basis, the presence of several moboviruses is well known (e.g. WNV, Sindbis virus, Tahyna virus, Lednice virus) [1]. Since the first large WNV outbreak in 1996, with several hundred human cases in Southern Romania [3], WNV has a high relevance for the country. In 2010, another WNV epidemic with more than 50 human cases demonstrated that the virus is widely distributed and established in the country [4].

The Danube Delta is situated in eastern Romania and was formed by Europe's second largest river discharging into the Black Sea [5]. Under protection since 1991, the Danube Delta Biosphere Reserve (DDBR) covers 580,000 hectares in Romania and 4600 hectares in the Ukraine. The biological diversity in the DDBR is huge, comprising over 1800 species of flora and 3500 species of fauna [6]. Located halfway between the Equator and North Pole, the DDBR is an important hub for migratory birds from Africa and Asia. These circumstances strongly suggest a high risk of introduction of bird associated zoonotic pathogens such as WNV or USUV.

Pathogens imported by migratory birds find a diverse mosquito fauna, which have excellent breeding habitats in this ecologically heterogeneous wetland. Covering more than 30 different ecosystems [5], the DDBR is characterized by vast natural marshes and fresh water bodies, mainly lakes and channels, providing excellent conditions for a diverse and very abundant mosquito fauna [7]. The checklist of the mosquitoes in the DDBR consist of 31 species [7], compromising 56.4 % of the 55 species known for Romania [8–12].

However, regular mosquito monitoring programmes are missing in Romania. As already highlighted by Prioteasa & Falcuta [7], in the DDBR, these types of studies are predominantly hampered by transportation problems, as many areas can only be reached by boat. However, a detailed knowledge on the species composition and phenology are crucially needed to evaluate the risk of pathogen transmission, plan vector control programmes, and to understand the ecology of circulating moboviruses. Therefore, this longitudinal mosquito surveillance study in the DDBR was conducted in order to evaluate the spatio-temporal pattern of the (i) mosquito species composition and diversity, (ii) functional groups of mosquitoes (oviposition sites, overwintering stage, and number of generations), and (iii) the occurrence of potential WNV vectors.

Methods

Study area and mosquito sampling

Four mosquito trapping sites were selected in the DDBR within an area of about 160 km^2 and a minimal linear distance of ten kilometres between the sites (Fig. 1). Research permits and approval (9/25.04.2014; 10692/ARBDD/25.04.2014) were issued by the Danube Delta Biosphere Reserve Authority. Between April and September 2014, four carbon dioxide-baited Heavy Duty Encephalitis Vector Survey (EVS) traps (Bioquip Products Inc., California, USA) were operated at each site for one night every tenth day on average. The annual mean temperature of the area is 11 °C (-1 °C in January and 22 °C in July), with a mean precipitation about 350 mm per year (see Fig. 2 for weather conditions during the sampling year 2014).

Trapping site Letea is situated between a channel and a swamp. The biotope is characterized by a few black locusts (*Robinia pseudoacacia*) and mulberry trees (*Morus nigra*) between a small field covered with grasses and a swampy area with reed (*Phragmites australis*) and bulrush (*Typha angustifolia*). Trapping site Dunărea Veche lies on an old, natural branch of the Danube. The high spring water levels flood the area until mid-June connecting the channel with swamps around it in many places. The channel flows very slowly and the soil is permanently moist. Vegetation is dominated by *T. angustifolia*, *P. australis*, *Urtica dioica* and *Fraxinus pallisae*. The trapping site Sulina lies in a black locust tree grove (*R. pseudoacacia*) between a private garden and a stagnant waterbody. The surrounding flora also consists of vines (*Vitis vinifera*) and various species of ruderal herbaceous species. The trapping site Lacul Roșuleț is a platform surrounded by stagnant or very slow flowing water. *T. angustifolia*, *P. australis* and *Salix alba* dominate

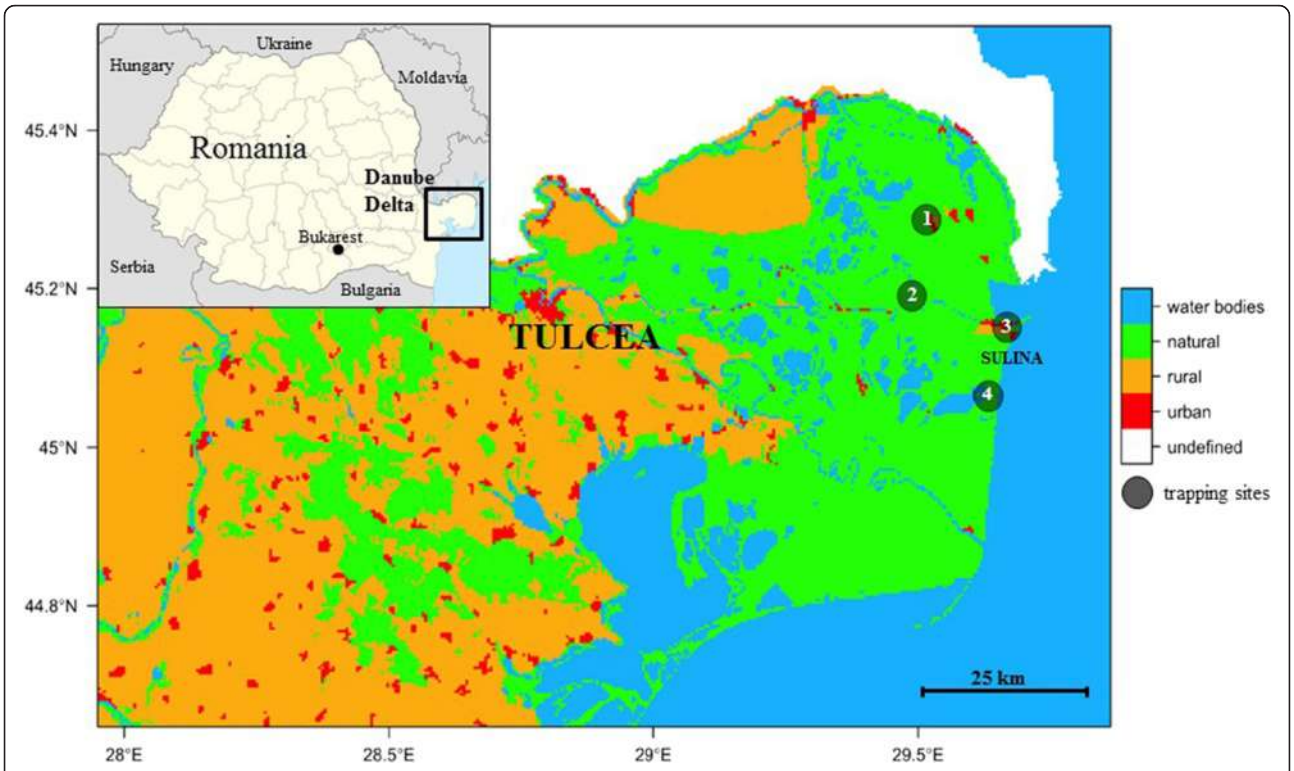


Fig. 1 Sampling sites (1: Letea, 2: Dunărea Veche, 3: Sulina, 4: Lake Roşuleţ), of mosquitoes in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014. Landcover variables are aggregated land cover data [Corine Land Cover (CLC) 2006 raster data, http://www.eea.europa.eu]. CLC-codes: water bodies = 511-523, natural = 311-423, rural = 211-244, urban = 111-142

the surrounding area, which is bordered by big trees which stand up in the flat landscape of vast marshes and lakes.

Morphological and molecular identification of mosquitoes

Collected mosquitoes were stored, transported to the laboratory on dry ice and morphologically identified on chill tables [13, 14]. Due to transportation or storage, some specimens were damaged and missed relevant characters for the species identification. These were only identified to the genus level or classified as "unidentified". Selected specimens of all collected species were double-checked by another person without knowing previous identification results. The morphological identification of these specimens was confirmed by the analysis of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene [15]. Mosquitoes were placed in sterile 2 ml reaction tubes and 1.5 ml of cell culture medium (high-glucose Dulbecco's modified Eagle's medium [Sigma-Aldrich, St. Louis, MO] with 10 % heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B) and 0.75 µl Zirconia beads (Biospec; 2.0 mm beads) were added for homogenization in a TissueLyser (Qiagen, Hilden, Germany) for 2 min at 50 oscillation/s. The suspensions were clarified by centrifugation (5000 g for 1 min), and the supernatant was used for DNA extraction with the RTP Pathogen Kit (Stratec Biomedical AG, Birkenfeld, Germany) according to the manufacturer's instructions. The extracted DNA of each sample was used as a template for the amplification of ~ 560 bp fragment of the COI gene using the C1-N-2191:5'-GGTAAAATTAAAATATAAACTTC-3'/C1-J-1632:5'-TGATCAAATTTATAAT-3' primers [15]. Each PCR reaction was performed with the HotStartTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) according the manufacturer's protocol. PCR products were sequenced at least twice in each direction by conventional Sanger technology (LGC, Berlin, Germany).

Genetic diversity and phylogenetic analysis

Sequence assembly, analysis, and multiple alignments were performed using Geneious v7.1.8 (Biomatters, Auckland, New Zealand). The species-level identification based on COI was conducted with BOLD (http://www.boldsystems.org) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In order to investigate the evolutionary relationship of the mosquito species collected during this study with those previously reported worldwide and available in

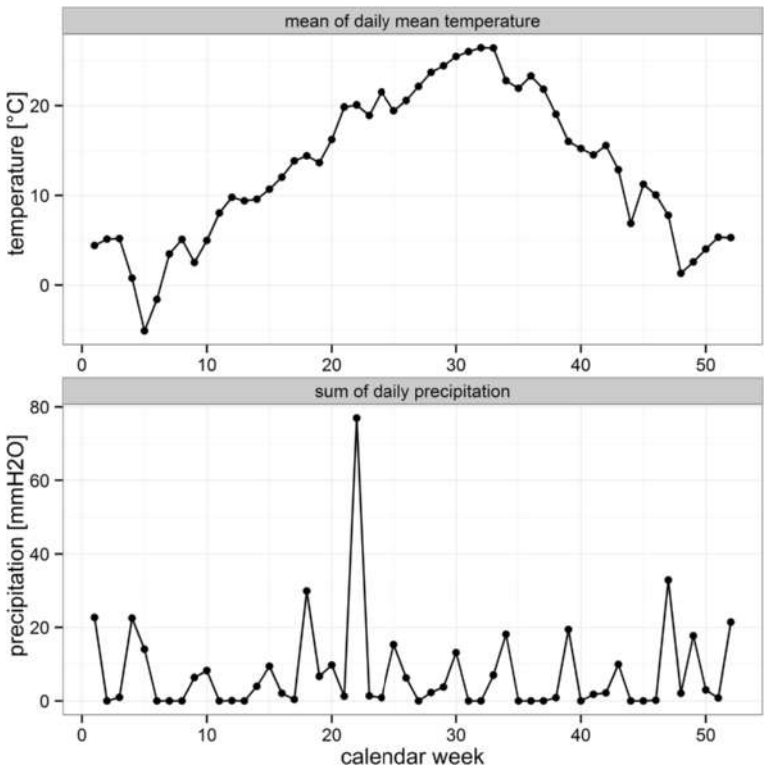


Fig. 2 Climate data (mean of the daily mean temperature [°C] and sum of the daily precipitation [mm] per calendar week) for the Danube Delta Biosphere Reserve (Romania) for 2014 downloaded from http://www.ecad.eu/ [41]

GenBank, a maximum likelihood (ML) analysis was performed using PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/versions.php) with 1000 pseudoreplicates. To assess the robustness of ML phylogenetic groupings, a bootstrap resampling analysis was conducted using 1000 replicate neighbor-joining (NJ) tree and Kimura-2 distance model in MEGA6 [16]. The Akaike information criterion was chosen as the model selection criterion and the general time-reversible model of sequence evolution with gamma distributed rate variation among sites and a proportion of invariable sites (GTR + I + Γ) as the best model. Sequences were deposited in the GenBank database with the accession numbers KU214640–KU214675 and KT876464–KT876495.

Data analysis

All other data analysis was conducted with R [17]. The packages plyr [18] and lubridate [19] were used for data manipulation and the packages ggplot2 [20] and gridExtra [21] for data visualization. Due to small variations of the sampling intervals per trapping site, the data were summarized per calendar week. Taxa information on functional characteristics (overwintering stage, oviposition sites, number of generations) and the classification as potential WNV vectors based on the feeding preference were extracted from the literature (Tables 1 and 2). Abundance-based Coverage Estimator (ACE) and Chao1 were used to determine sampling efficiency of mosquito taxa [22–24]. This procedure was performed with the function "EstimateR" from the R package vegan [25].

Results

Mosquito species composition

A total of 240,546 female mosquito specimens belonging to 8 genera and 14 taxa were successfully identified by morphological characteristics (Tables 1 and 2). The seven dominant taxa, with more than 2000 individuals each, were *Coquilettidia richiardii* (40.9 %), *Anopheles hyrcanus* (34.1 %), *Culex pipiens* (*sensu lato*) (*s.l.*)/*Cx. torrentium* (7.7 %), *Aedes caspius* (5.7 %), *Cx. modestus* (4.0 %), *An. maculipennis* (*s.l.*) (3.9 %), and *Ae. vexans* (3.0 %). Among the rare species, representing 0.7 % of all collected individuals, were *Ae. detritus, Ae. flavescens*, *Ae. cinereus*, *Culiseta annulata*, and *Uranotaenia unguiculata*. In addition, we detected two new species for Romania: *An. algeriensis* and *Ae. hungaricus*, which both have been morphologically and genetically confirmed.

In Letea, three females of *Ae. hungaricus* were trapped between 29th June and 9th July 2014. These were

Table 1 Mosquito taxa recorded in the study area of the Danube Delta Biosphere Reserve (DDBR) in Romania during the sampling period in 2014 with the number of specimens collected, their respective overall proportion, information if the species was previously known from Romania and the DDBR, and three functional characteristics for each taxon

Taxa	Specimens (percentage)	Previously known for Romania and the DDBR	Oviposition sites	Overwintering stage	No. of generations	Source for functional classification
Coquilettidia richiardii (Ficalbi, 1889)	98276 (40.8552 %)	yes	water	larvae	univoltine	[42]
Anopheles hyrcanus (Pallas, 1771)	82073 (34.1193 %)	yes	water	females	multivoltine	[43]
Culex pipiens Linnaeus, 1758 (*s.l.*)/Cx. *torrentium* (Martini, 1925)[a]	18416 (7.6559 %)	yes	water	females	multivoltine	[42]
Aedes caspius (Pallas, 1771)	13709 (5.6991 %)	yes	land	eggs	multivoltine	[42]
Culex modestus Ficalbi, 1890	9534 (3.9635 %)	yes	water	females	multivoltine	[43]
Anopheles maculipennis Meigen, 1818 (*s.l.*)[b]	9380 (3.8994 %)	yes	water	females	multivoltine	[42]
Aedes vexans (Meigen, 1830)	7295 (3.0327 %)	yes	land	eggs	multivoltine	[42]
Unidentified	1041 (0.4328 %)	-	-	-	-	-
Anopheles algeriensis Theobald, 1903	697 (0.2898 %)	no	water	larvae	multivoltine	[43]
Aedes sp.	71 (0.0295 %)	-	-	-	-	-
Aedes detritus (Haliday, 1833)	31 (0.0129 %)	yes	land	eggs	multivoltine	[42]
Culex sp.	10 (0.0042 %)	-	-	-	-	-
Aedes flavescens (Müller, 1764)	5 (0.0021 %)	yes	land	eggs	univoltine	[42]
Aedes hungaricus Mihályi, 1955	3 (0.0012 %)	no	land	-	-	[14]
Aedes cinereus Meigen, 1818	2 (0.0008 %)	yes	land	eggs	multivoltine	[42]
Culiseta annulata (Schrank, 1776)	1 (0.0004 %)	yes	water	females	multivoltine	[42]
Uranotaenia unguiculata Edwards, 1913	1 (0.0004 %)	yes	water	females	multivoltine	[43]

[a]Selected specimens were identified as *Culex pipiens* Linnaeus, 1758 (*s.l.*) and *Culex pipiens pipiens* Linnaeus, 1758 by DNA-barcoding (Fig. 5), [b]selected specimens were identified as *Anopheles messeae* Falleroni, 1926 by DNA-barcoding (Fig. 5).

identified according to the following morphological characteristics [14, 26]: small species, with blackish brown scaled proboscis and palps, occiput with narrow whitish scales dorsally, broad whitish scales and scattered dark scales laterally, scutum covered with greyish white scales and a median stripe of dark brown scales, scutellum with pale narrow scales, hypostigmal scale patch absent, upper mesepisternal scale patch reaches the anterior angle of the mesepisternum, mesepimeral scale patch does not reach the lower margin of the mesepimeron, femora of the fore legs predominately pale scaled in the basal half, tibiae of the hind legs with dark scales on the anterior surface, tarsomeres dark scaled without pale basal rings, wing veins covered with dark scales, abdominal terga with blackish brown scales and pale basal bands, which are slightly narrower in the middle and connected with pale lateral triangular patches (Fig. 3). Not all characteristics could clearly be seen on each specimen, because of damage due to transportation and storage. Therefore, a reference adult female collected as larva in 1998 on the Tisa river close to Mártély in Hungary was taken for morphological comparison. The specimen from Hungary was independently identified as *Ae. hungaricus* from three entomologists and the overall appearance was in agreement with the three specimens from Romania.

A second new species, *An. algeriensis*, was found with 697 females (0.3 % of all collected mosquito specimens) at all four sampling sites between April and September 2014. Typical morphological characteristics have been observed [14]: head antennal ornamentation rare and poorly developed whorls without a tuft or long white scales on interocular apse, maxillary palpus is entirely dark, no white rings, thorax covering of scutum with setae only, hind leg colour of tarsomeres entirely dark and mostly with a small apical ring, wings ornamentation entirely dark and without spot on the costal margin (Fig. 4).

Table 2 Mosquito taxa recorded in the study area of the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014 and the host preference determining the possibility to be a potential bridge vector of West Nile virus

Taxa	Involved in West Nile virus transmission elsewhere	Ornithophilic (bird-biting)	Anthropophilic (human-biting)	Potential bridge vector (readily bites both birds and humans)	Source for classification
Coquilettidia richiardii (Ficalbi, 1889)	yes	yes	yes	yes	[44]
Anopheles hyrcanus (Pallas, 1771)	yes	no	yes	no	[14]
Culex pipiens Linnaeus, 1758 (*s.l.*) / Cx. *torrentium* (Martini, 1925)[a]	(yes)[c]	(yes)[c]	(yes)[c]	yes	[44]
Aedes caspius (Pallas, 1771)	yes	no	yes	no	[44]
Culex modestus Ficalbi, 1890	yes	yes	yes	yes	[44]
Anopheles maculipennis Meigen, 1818 (*s.l.*)[b]	yes	no	yes	no	[44]
Aedes vexans (Meigen, 1830)	yes	no	yes	no	[44]
Unidentified	-	-	-	unclassified	-
Anopheles algeriensis Theobald, 1903	no	no	yes	no	[44]
Aedes sp.	-	-	-	unclassified	-
Aedes detritus (Haliday, 1833)	no	yes	yes	yes	[44]
Culex sp.	-	-	-	unclassified	-
Aedes flavescens (Müller, 1764)	no	no	yes	no	[44]
Aedes hungaricus Mihályi, 1955	no	no	yes	no	[14]
Aedes cinereus Meigen, 1818	yes	yes	yes	yes	[44]
Culiseta annulata (Schrank, 1776)	no	yes	yes	yes	[44]
Uranotaenia unguiculata Edwards, 1913	yes	no	no	no	[14, 45]

[a]Selected specimens were identified as *Culex pipiens* Linnaeus, 1758 (*s.l.*) and *Culex pipiens pipiens Linnaeus*, 1758 by DNA-barcoding (Fig. 5), [b]selected specimens were identified as *Anopheles messeae* Falleroni, 1926 by DNA-barcoding (Fig. 5), [c]*Culex pipiens* (*s.l.*) and Cx. *torrentium* were not differentiated for most of the collected specimens

All mosquito specimens were homogenized for further pathogen screening. Extracted DNA is stored in the Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research National Reference Centre for Tropical Infectious Diseases, Hamburg, Germany.

DNA barcoding and phylogeny of mosquito species

COI sequences of ~ 550 bp were successfully amplified from 66 mosquito specimens from the DDBR and compared with those currently available in databases. Four sequences of *Ae. hungaricus* are submitted as the first records for public databases. The alignment was unambiguous without gaps and stop codons in amino acid translation. Comparisons of the COI sequence alignment indicated point mutations for all detected mosquito species with the highest number observed in *An. algeriensis* (number of point mutations [npms] = 40), followed by *Cx. pipiens* (*s.l.*) (npms = 15) and *Ae. detritus* (npms = 13). No deletion or insertion among the sequenced samples have been observed. Gene sequences of *Ae. cinereus* ($n = 1$), *Ae. vexans* ($n = 2$), *An. hyrcanus* ($n = 1$), *An. messeae* (morphologically identified as *An. maculipennis* (*s.l.*) ($n = 5$), *Cs. annulata* ($n = 1$), *Cq. richiardii* ($n = 2$), *Cx. pipiens pipiens* (n = 8), *Cx. pipiens* (*s.l.*) (morphologically identified as *Culex pipiens* (*s.l.*)/*Cx. torrentium*) ($n = 9$), *Cx. modestus* ($n = 14$), *Ae. flavescens* ($n = 2$), *Ae. caspius* ($n = 2$), and *Ur. unguiculata* ($n = 1$) from the DDBR were very similar to sequences obtained from mosquitoes collected in other European regions, except for *An. algeriensis* ($n = 14$) and *Ae. detritus* ($n = 3$), which exhibited relatively high intraspecific divergence (6 and 3 %, respectively). These results are supported by the phylogenetic analysis, which demonstrated the close evolutionary relatedness and a similar clustering of the above mentioned species with specimens of the same taxon from other regions (Fig. 5). Due to missing COI or other gene sequences of *Ae. hungaricus* in the databases, the phylogenetic clustering of this particular species should be interpreted with caution. However, the analysed specimens of this species formed a distinct and highly supported monophyletic clade, which is clustered with *Ae. caspius* in a distinct group within the *Aedes* phylogeny (Fig. 5). It is important to note that the sequences of the *Ae. hungaricus* specimens

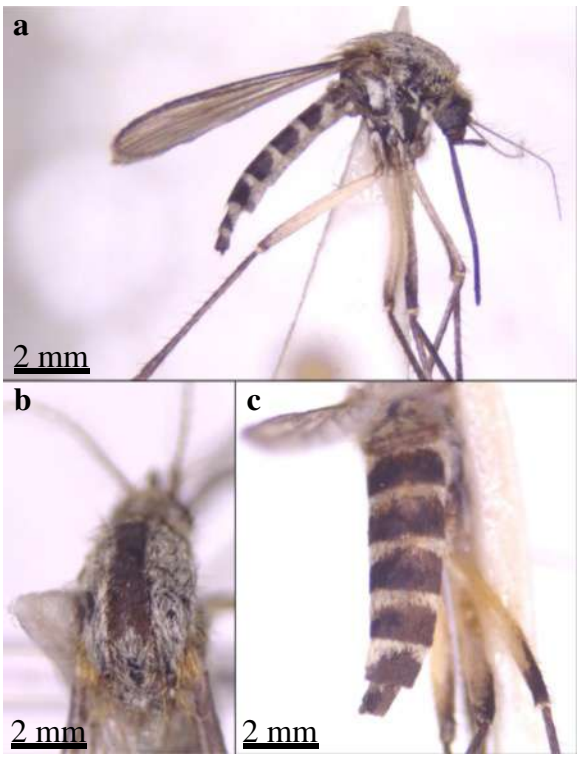


Fig. 3 Specimen of *Aedes hungaricus* detected in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014. **a** Lateral view; **b** Scutum; **c** Abdomen

from DDBR and the reference specimen from Hungary were almost identical. *Anopheles algeriensis* forms a highly divergent paraphyletic group with several lineages (likely new subspecies) within the genus *Anopheles*. The overall clustering pattern of the phylogenetic tree was similar to that of NJ tree (data not shown), and all species branched with their respective subfamilies.

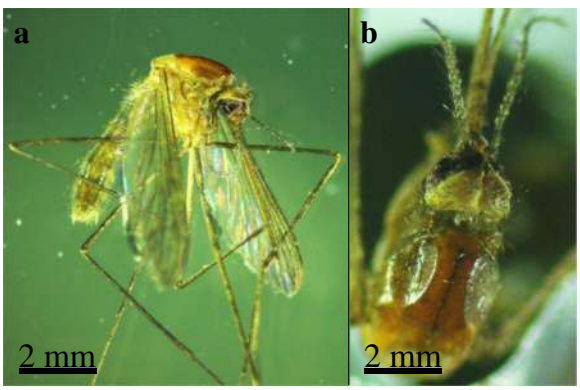


Fig. 4 Specimen of *Anopheles algeriensis* detected in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014. **a** Lateral view; **b** Scutum and head

Data analysis

Between eight and twelve taxa were recorded at the four sampling sites (Table 3). Except for the sampling site Sulina, with 12 observed and 15 estimated taxa (20 % difference), the ACE and Chao1 indices estimated the same number of taxa as observed, suggesting a good coverage of the taxa present in the study area.

The number of detected taxa per sampling site and calendar week varied from three to ten with the lowest taxa richness for the first sampling in April and highest number of detected taxa in June (Fig. 6). The highest numbers of mosquito specimens per calendar week were collected at the beginning of June, followed by two peaks at the end of June and August. The detected taxa showed different phenological patterns (Fig. 7). For example, the highest number of specimens for *Ae. vexans* and *Ae. caspius* were trapped early in the year, whereas most *Cx. modestus* were sampled in the late summer. Another example is the number of observed generations, e.g. *Ae. caspius* showed a single population peak, while *Cq. richiardii* and *An. hyrcanus* had three and two population peaks, respectively (Fig. 7).

The mosquito population over the sampling period was dominated by taxa, which lay their eggs on the water surface, whereas taxa laying their eggs on the soil were only present at the beginning of the sampling period (Fig. 8). The overwintering stages of the taxa followed a series with the highest proportion of species overwintering in the egg stage at the beginning of the year, followed by taxa overwintering in the larval stage, and were finally dominated by taxa, which overwinter as females. Univoltine taxa had their highest proportion during the summer months, whereas multivoltine taxa were present during the entire sampling period. Potential WNV vectors were also present during the entire sampling period, accounting for more than 50 % of the total number of collected specimens and exceeding 50 % of all collected specimens for most calendar weeks in the summer.

Discussion

This study confirmed 12 previously recognized mosquito species for Romania by combining morphological identification and sequencing of the COI gene, representing one fifth (21.8 %) of the known 55 species of the country [8–12]. Both indices for extrapolated taxa richness, bias-corrected Chao and ACE, indicated a relatively good coverage of the mosquito taxa collected with EVS traps for the studied area. However, at the same time, the first reports of two mosquito species for Romania, *An. algeriensis* and *Ae. hungaricus*, highlight the lack of knowledge about the mosquito fauna of the country and the DDBR in particular. These new records were demonstrated, because a huge number of specimens of nearly

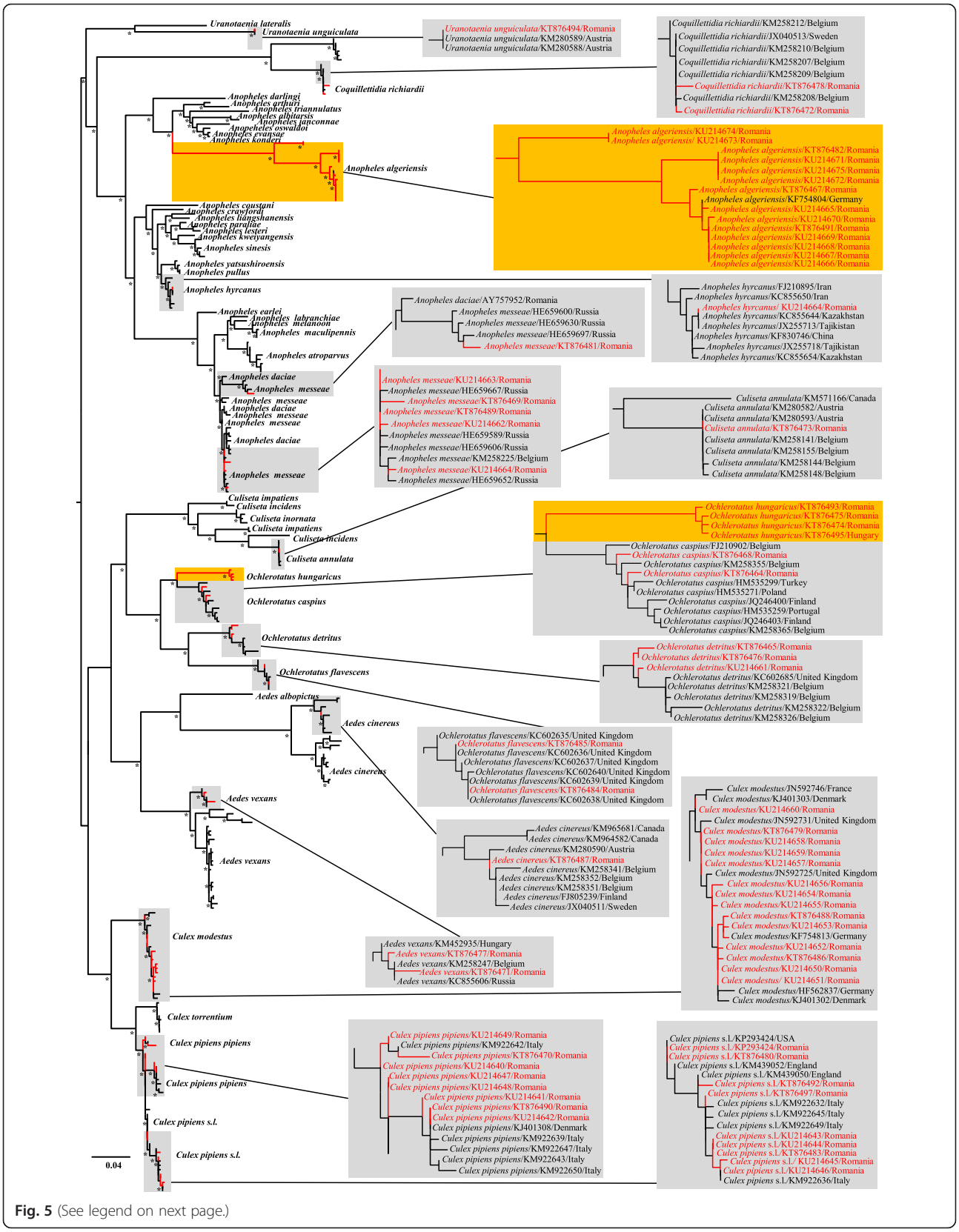


Fig. 5 (See legend on next page.)

(See figure on previous page.)
Fig. 5 Maximum-likelihood phylogeny of the COI gene sequences for selected specimens of the 14 collected mosquito species detected in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014 (red font) and additional sequences retrieved from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov). Red marked sections of the tree and the magnified areas in grey/orange indicate the location of the mosquito species detected in this study. The clades including *Aedes hungaricus* and *Anopheles algeriensis* (first reports for Romania) are highlighted in orange. The maximum likelihood bootstrap replicates (≥70 %) and parallel NJ bootstrap values above 70 (1000 replicates) are indicated with an asterisk at the nodes. The scale-bar indicates the genetic distance scale expressed as mean number of nucleotide substitutions per site

one quarter of a million mosquitoes was collected over the entire vegetation period. Furthermore, the sampling sites included remote areas of the DDBR only accessible by boat. Only three specimens of *Ae. hungaricus* were found. Due to its general rarity in Europe, the ecology of this species is largely unknown [14]. It was only described that the larvae develop in floodwater pools in river valleys and probably have several generations per year [27]. With 697 specimens, *An. algeriensis* was trapped more frequently, but representing only 0.3 % of all collected mosquito specimens. The species is widespread in Europe with a distribution centre in the Mediterranean region, but was also found in central Europe as far to the north in England or Germany [14, 28–30]. Larval breeding sites are generally located in marshes and slow running brooks covered with dense vegetation [14], which are also present in the study area. Females of the species bite mammals outside, near their breeding sites and are susceptible to *Plasmodium* spp. [14]. However, due to their scarcity, both new species probably do not play an important role as vectors of pathogens in Romania [30].

In order to avoid incorrect mosquito species identification, selected specimens of each morphological identified mosquito species were used for a DNA-barcoding approach. The analysis of the intraspecific sequence variation (6 %) of the *An. algeriensis* COI gene revealed the existence of at least three new relatives. This result is supported by the phylogenetic analysis suggesting the occurrence of a heterogeneous *An. algeriensis* population within a relatively small region. Such differentiation might be especially important if the different subpopulations have a different vector competence [31]. The congruence between morphology-based identification and DNA-barcode grouping based on phylogenetic clustering with high bootstrap support (≥95 %) was found for all morphologically identified taxa. Therefore, morphology-based identification is appropriate to identify the mosquito species in the study area. However, especially the detection of cryptic species (e.g. *Culex pipiens* (*s.l.*)/*Cx. torrentium* or the members of the *Anopheles maculipennis* complex) probably require a mass screening *via* specific PCRs [32], [33] rather than a DNA-barcoding approach.

Table 3 Estimated taxa richness according the abundance-based coverage estimator (ACE) and Chao1 for the four study sites in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014

	Dunărea Veche	Lacul Roşule	Letea	Sulina
Observed number of taxa	8.000	9.000	12.000	12.000
Chao1	8.000	9.000	12.000	15.000
Chao1 standard error	0.000	0.000	0.000	4.517
ACE	8.000	NaN[a]	13.380	NaN[a]
ACE standard error	0.935	NaN[a]	1.708	NaN[a]

[a]Calculation of the ACE not possible, because all rare species (<10 specimens) contained only a single specimen

The main difficulty in the phylogenetic tree reconstruction was the unbalanced amount of available nucleotide sequences from other countries. However, the mitochondrial gene (COI) based phylogeny clearly related the DDBR mosquito species to those collected in other European countries and provided evidence for population subdivision in *An. algeriensis* and *Ae. detritus*. Such differences suggest allopatric speciation evolvement or mixing of different mosquito populations, which developed in distinct geographic regions. Another interesting point worth mentioning here, is the phylogenetic clustering of *Ae. hungaricus*. Although the latter seems to be a homogenous species, almost identical with the reference specimen from Hungary, further studies on genetic diversity of this rare species from other countries are necessary for a final assessment.

The mosquito fauna of the trapping sites was clearly dominated by two species: *Cq. richiardii* and *An. hyrcanus*. For Romania, both species were previously reported to have their main distribution in the DDBR and surrounding floodplains [10]. *Coquillettidia richiardii* has a specialized life-cycle with larvae and pupae living permanently submerged and obtaining oxygen from the aerenchyma of various aquatic plants in permanent water bodies, finding perfect conditions in the DDBR. Similar breeding site preferences for stagnant water bodies with rich aquatic vegetation were described for *An. hyrcanus*. Both species are multivoltine [14, 34] and had two (*An. hyrcanus*) and three populations peaks (*Cq. richiardii*) during the study year. Due to their dominance, representing over three quarters of all collected specimens, the overall phenology and temporal pattern of functional groups basically followed the pattern

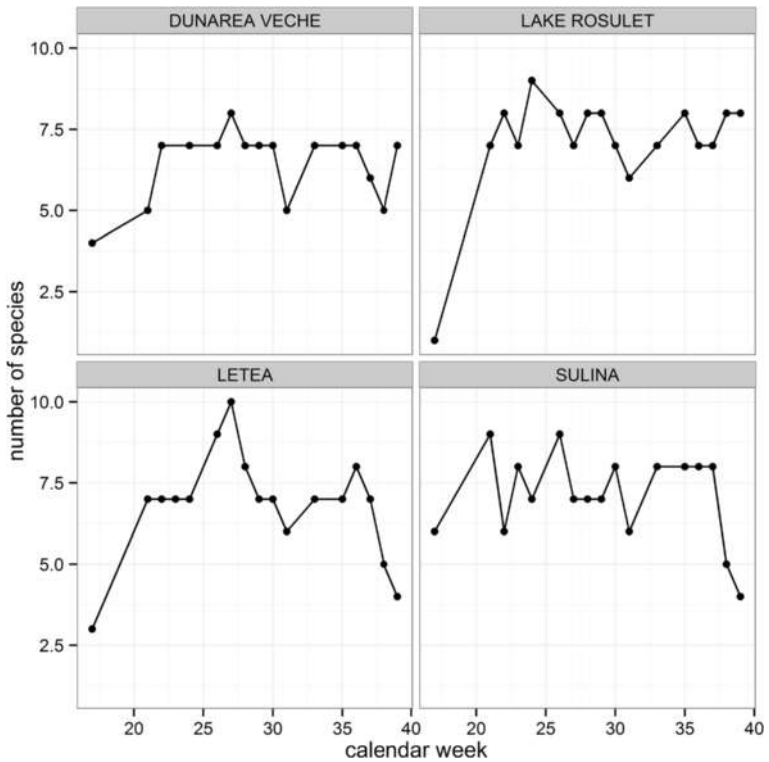


Fig. 6 Number of detected mosquito taxa per calendar week for the four sampling sites in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014

of both species with three distinct population peaks, domination of the oviposition site "water" (both species) and domination of the overwintering stage "larvae" (*Cq. richiardii*) and "female" (*An. hyrcanus*).

Between 2011 and 2013, different mosquito species in Romania have been tested WNV-positive [35]. *Culex pipiens* (*s.l.*) is considered to be the most important WNV vector in the country [35, 36], and together with *Cx. modestus* considered to be the main vector species of WNV in Europe [37, 38]. However, in Romania, WNV was also detected in mosquito pools of the species *Cq. richiardii*, *An. hyrcanus*, *Ur. unguiculata*, *Ae. caspius*, and *An. maculipennis* (*s.l.*). Nicolescu [36] highlighted that these species might play an important role in the transmission cycle of WNV, if the principal vector species are missing or present only with low densities. During the entire sampling period, a huge proportion of the mosquito population can be classified as potential WNV vectors. With 40 % of all collected specimens, the most frequent species *Cq. richiardii* is probably the most important vector of WNV in the DDBR, followed by *Cx. pipiens* (*s.l*)/*Cx. torrentium*, *Ae. caspius* and *Cx. modestus*, which were all found WNV-positive in Romania [35]. *Anopheles hyrcanus* was the second most frequent species and also detected WNV-positive in the country [35, 39]. However, due to the generally assumed host preference for mammals, the species probably does not play an important role as bridge vector.

Conclusion

The data generated during this study is likely biased, because it only included four sampling sites and one type of adult trap (e.g. different types of adult traps are known to have a different trapping performance) [40]. Therefore, an increase of sampling sites and the use of diverse trapping methods (e.g. different types of adult traps or gravid traps) including the collection of immature stages might allow the detection of more mosquito species. Nevertheless, these data from one vegetation period provide a first, but detailed overview of the mosquito communities in the DDBR. Thereby, the detection of two new mosquito species highlights the lack of knowledge about the composition and genetic diversity of the mosquito fauna in Romania and in the DDBR in particular. The greatest proportion of collected specimens could be classified as potential WNV vectors, which can account for up to 70 % of all sampled mosquitoes per calendar week. The extension of the entomological surveillance programme will provide baseline data, which are necessary to better understand mobovirus

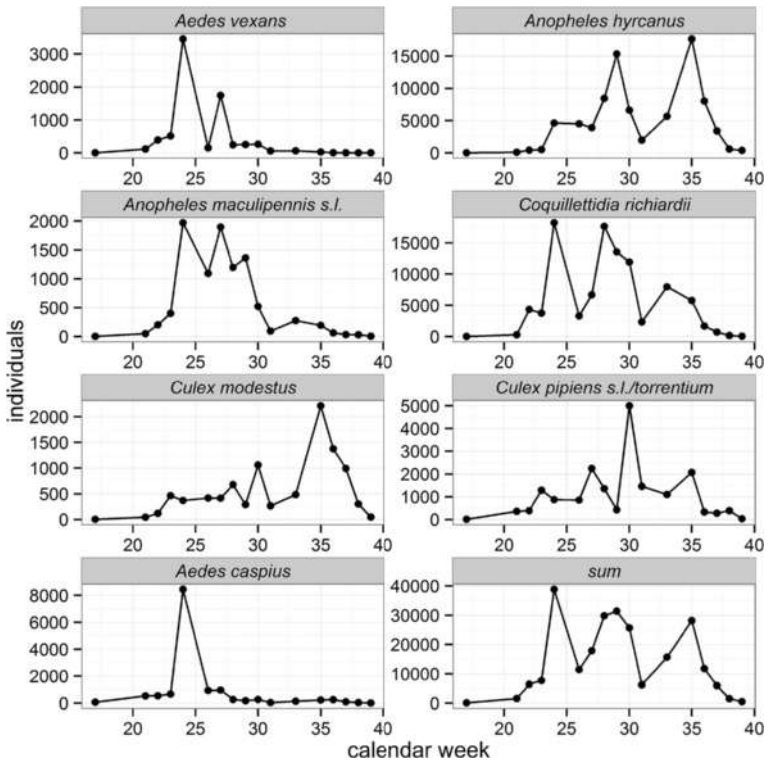


Fig. 7 Number of detected specimens per calendar week of the seven most common mosquito taxa (>2000 specimens) detected in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014

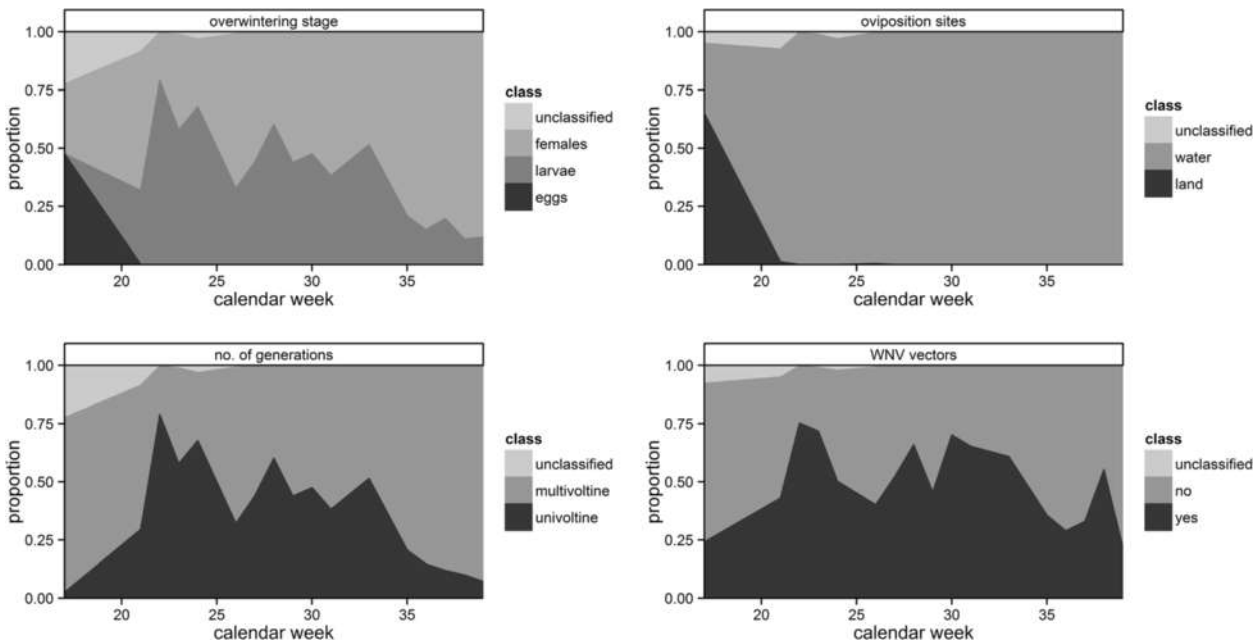


Fig. 8 Proportion of three functional groups and West Nile virus vectors of the total catch of mosquitoes per calendar week in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2014

activity and the phylogeography of a medically important mosquito vector species. Finally, this information can also help to implement vector control programmes, e.g. to adjust the timing of interventions.

Abbreviations

ACE: abundance-based coverage estimator; Chao1: estimator of species richness named after the inventor of the index (Chao [27, 28]); COI: cytochrome oxidase I; DDBR: Danube Delta Biosphere Reserve; DNA: deoxyribonucleic acid; EVS: Heavy Duty Encephalitis Vector Survey; mobovirus: mosquito-borne virus; PCR: polymerase chain reaction; RNA: ribonucleic acid; USUV: Usutu virus; WNV: West Nile virus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the study: DC, SJ, HJ, LK, RL, OP, JSC. Collected the data: HJ, CH, SJ, AT, ET. Analysed the data: DC, HJ, RL. Provided reference specimens of *Aedes hungaricus*: NB, AK. Drafted the manuscript: DC, HJ, RL. Critically revised the manuscript: SJ, AK, JSC, ET. All authors approved the final version of the manuscript.

Acknowledgements

This research was supported by a grant of the Ministry of National Education, CNCS-UEFISCDI, PN-II-ID-2012-4-0595 and the Sectoral Operational Programme Human Resources Development, financed from the European Social Fund and by the Romanian Government under the POSDRU/187/1.5/S/156069/. We acknowledge the E-OBS dataset from the EU-FP6 project ENSEMBLES (http://ensembles-eu.metoffice.com) and the data providers in the ECA&D project (http://www.ecad.eu).

Author details

[1]Department of Biology and Ecology, Babeş-Bolyai University, Cluj-Napoca, Romania. [2]Romanian Academy Institute of Biology, Bucharest, Romania. [3]Molecular Biology Center, Institute for Interdisciplinary Research in Bio-Nano-Sciences, Babes-Bolyai University, Cluj-Napoca, Romania. [4]Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research National Reference Centre for Tropical Infectious Diseases, Hamburg, Germany. [5]German Mosquito Control Association (KABS), Institute for Dipterology, Speyer, Germany. [6]University of Heidelberg, Heidelberg, Germany. [7]Centre for Infection Research (DZIF), Partner site Hamburg-Luebeck-Borstel, Hamburg, Germany.

Received: 15 January 2016 Accepted: 31 March 2016
Published online: 11 April 2016

References

1. Hubálek Z. Mosquito-borne viruses in Europe. Parasitol Res. 2008;103:29–43.
2. Rizzoli A, Jiménez-Clavero M, Barzon L, Cordioli P, Figuerola J, Koraka P, Martina B, Moreno A, Nowotny N, Pardigon N, Sanders N, Ulbert S, Tenorlo A. The challenge of West Nile virus in Europe: knowledge gaps and research priorities. Euro Surveill. 2015;20:21135.
3. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu I. West Nile encephalitis epidemic in southeastern Romania. Lancet. 1998;352:767–71.
4. Sirbu A, Ceianu CS, Panculescu-Gatej RI, Vázquez A, Tenorio A, Rebreanu R, Niedrig N, Nicolescu G, Pistol A. Outbreak of West Nile virus infection in humans, Romania, July to October 2010. Euro Surveill. 2011;16.
5. Hanganu J, Dubyna D, Zhmud E, Grigorş I, Menke U, Drost H, Ştefan N, Sârbu I. Vegetation of the Biosphere Reserve "Danube Delta" - with Transboundary Vegetation Map on a 1:150,000 Scale. Danube Delta National Institute, Romania, Kholodny MG - and Institute of Botany, Danube Delta Biosphere Reserve, Ukraine and RIZA, editors. The Netherlands, Lelystad: RIZA rapport 2002.049; 2002.
6. Administraţia Rezervaţiei Biosferei Delta Dunării. 2016. http://www.ddbra.ro/en/danube-delta-biosphere-reserve/danube-delta/biodiversity. Accessed 1 Mar 2016.
7. Prioteasa F-L, Falcuta E. An annotated checklist of the mosquitoes (Diptera: Culicidae) of the Danube Delta Biosphere Reserve. Eur Mosq Bull. 2010;28:240–45.
8. Nicolescu G. The mosquitoes (Diptera: Culicidae) from Romania an annotated checklist and bibliography. Roum Arch Microbiol Immunol. 1994;54:75–109.
9. Nicolescu G, Vladimirescu A, Ciolpan O. The distribution of mosquitoes in Romania (Diptera: Culicidae). Part I: *Anopheles*, *Aedes* and *Culex*. Eur Mosq Bull. 2002;13:17–26.
10. Nicolescu G, Vladimirescu A, Ciolpan O. The distribution of mosquitoes in Romania (Diptera: Culicidae). Part II: *Culiseta*, *Coquillettidia*, *Ochlerotatus*, *Orthopodomyia* and *Uranotaenia*. Eur Mosq Bull. 2003;14:1–15.
11. Nicolescu G, Vladimirescu A, Ciolpan O. The distribution of mosquitoes in Romania (Diptera: Culicidae). Part III: Detailed Maps for *Anopheles*, *Aedes* and *Culex*. Eur Mosq Bull. 2003;14:25–31.
12. Nicolescu G, Vladimirescu A, Ciolpan O. The distribution of Mosquitoes in Romania (Dipera: Culicidae). Part IV: Detailed maps for *Coquillettidia*, *Culiseta*, *Ochlerotatus*, *Orthopodomyia* and *Uranotaenia*. Eur Mosq Bull. 2003;15:16–27.
13. Schaffner F, Geoffroy B, Hervy JP, Rhaiem A, Brunhes J. The Mosquitoes of Europe/Les Moustiques d'Europe. An identification and training Programme/Logiciel D'identification et D'enseignement. Montpellier: IRD Editions & EID Méditerranée; 2001.
14. Becker N, Petric D, Zgomba M, Boase C, Madon M, Dahl C, Kaiser A. Mosquitoes and Their Control. 2nd ed. Heidelberg: Springer; 2010.
15. Kambhampati S, Smith PT. PCR Primers for the amplification of four insect mitochondrial gene fragments. Insect Mol Biol. 1995;4:233–36.
16. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–29.
17. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2015. http://www.R-project.org. Accessed 1 Dec 2015.
18. Wickham H. The split-apply-combine strategy for data analysis. J Stat Softw. 2011;40:1–29.
19. Grolemund G, Wickham H. Dates and times made easy with lubridate. J Stat Softw. 2011;40:1–25.
20. Wickham H. ggplot2: elegant graphics for data analysis. New York: Springer; 2009.
21. Auguie B. gridExtra: miscellaneous functions for "grid" graphics. R package version 2.0.0. 2015. http://CRAN.R-project.org/package=gridExtra. Accessed 1 Dec 2015.
22. Chao A. Non-parametric estimation of the number of classes in a population. Scand J Stat. 1984;11:265–70.
23. Chao A. Estimating the population size for capture-recapture data with unequal catchability. Biometrics. 1987;43:783–91.
24. Colwell RK, Coddington JA. Estimating terrestrial biodiversity through extrapolation. Phil Trans Roy Soc London B. 1994;345:101–18.
25. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner, H. vegan: Community Ecology Package. R package version 2.3-2. 2015. http://CRAN.R-project.org/package=vegan. Accessed 1 Dec 2015.
26. Mihalyi F. *Aëdes hungaricus* n. sp. (Culicidae, Diptera). Ann Hist Nat Mus Nat Hun. 1955;6:343–5.
27. Mihályi F. Description of the larva of *Aëdes* (*Ochlerotatus*) *hungaricus* Mihályi (Diptera: Culicidae). Acta Zool Acad Sci Hung. 1961;7:231–33.
28. Scholte E-J, den Hartog W, Reusken C. A report of *Anopheles algeriensis* (Diptera: Culicidae) from The Netherlands. Entomolog Ber. 2011;71:39–42.
29. Tóth S, Kenyeres Z. Revised checklist and distribution maps of mosquitoes (Diptera, Culicidae) of Hungary. Eur Mosq Bull. 2012;30:30–65.
30. Krüger A, Tannich E. Rediscovery of *Anopheles algeriensis* Theob. (Diptera: Culicidae) in Germany after half a century. J Eur Mosq Control Assoc. 2013;31:14–6.
31. Hardy JL, Houk EJ, Kramer LD, Reeves WC. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Ann Rev Entomol. 1983;28:229–62.
32. Rudolf M, Czajka C, Börstler J, Melaun C, Jöst H, von Thien H, et al. First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype *pipiens/molestus* hybrids in Germany. PLoS One. 2013;8:e71832.
33. Lühken R, Czajka C, Steinke S, Jöst H, Schmidt-Chanasit J, Pfitzner W, et al. Distribution of individual members of the mosquito *Anopheles maculipennis* complex in Germany identified by newly developed real-time PCR assays. Med Vet Entomol. 2016; doi: 10.1111/mve.1216.
34. Gutsevich AV, Monchadskii AS, Shtakelberg AA. Mosquitoes. Family Culicidae. In: Fauna of the USSR. Jerusalem: Keter Publishing House; 1974.
35. Dinu S, Cotar A, Păunculescu-Gătej I, Fălcuţă E, Prioteasa F, Sîrbu A, Oprişan G, Bădescu D, Reiter P, Ceianu C. West Nile virus circulation in south-eastern Romania, 2011 to 2013. Euro Surveill. 2015;20:21130.

36. Nicolescu G. A General characterisation of the mosquito fauna (Diptera: Culicidae) in the epidemic area for West Nile virus in the South of Romania. Eur Mosq Bull. 1998;2:13–8.
37. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, Laiv L, Ungureanu A, Romanca C, Tsai TF. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg. 1999;61:600–11.
38. Balenghien T, Vazeille M, Grandadam M, Schaffner F, Zeller H, Reiter P, Sabatier P, Fouque F, Bicout DJ. Vector competence of some French *Culex* and *Aedes* mosquitoes for West Nile virus. Vector Borne Zoonotic Dis. 2008; 8:589–96.
39. Lvov DK, Butenko AM, Gromashevsky VL, Kovtunov AI, Prilipov AG, Kinney R, Aristova VA, Dzharkenov AF, Samokhvalov EI, Savage HM, Shchelkanov MY, Galkina IV, Deryabin PG, Gubler DJ, Kulikova LN, Alkhovsky SK, Moskvina TM, Zlobina LV, Sadykova GK, Shatalov AG, Lvov DN, Usachev VE, Voronina AG. West Nile virus and other zoonotic viruses in Russia: examples of emerging-reemerging situations. In: Calisher CH, Griffin DE, editors. Emergence and control of zoonotic viral encephalitides. Vienna: Springer; 2004. p. 85–96.
40. Lühken R, Pfitzner W, Börstler J, Garms R, Huber K, Schork N, et al. Field evaluation of four widely used mosquito traps in Central Europe. Parasit Vectors. 2014;7:268.
41. Haylock MR, Hofstra N, Klein Tank AMG, Klok EJ, Jones PD, New M. A European daily high-resolution gridded dataset of surface temperature and precipitation. J Geophys Res (Atmos). 2008;113:D20119.
42. Schäfer ML, Lundstrom JO, Pfeffer M, Lundkvist E, Landin J. Biological diversity versus risk for mosquito nuisance and disease transmission in constructed wetlands in southern Sweden. Met Vet Entomol. 2004;18:256–67.
43. Bueno-Marí R, Jiménez-Peydró R. Classification of Spanish mosquitoes in functional groups. J Am Mosq Control Assoc. 2011;27:1–7.
44. Medlock JM, Snow KR, Leach S. Potential transmission of West Nile virus in the British Isles: an ecological review of candidate mosquito bridge vectors. Med Vet Entomol. 2005;19:2–21.
45. Pachler K, Lebl K, Berer D, Rudolf I, Hubalek Z, Nowotny N. Putative new West Nile virus lineage in *Uranotaenia unguiculata* mosquitoes, Austria, 2013. Emerg Infect Dis. 2014;20:2119–22.

viruses

MDPI

Article

Ecology of West Nile Virus in the Danube Delta, Romania: Phylogeography, Xenosurveillance and Mosquito Host-Feeding Patterns

Alexandru Tomazatos [1,†], Stephanie Jansen [1], Stefan Pfister [2], Edina Török [3], Iulia Maranda [1], Cintia Horváth [4], Lujza Keresztes [5], Marina Spînu [4], Egbert Tannich [1,6], Hanna Jöst [1], Jonas Schmidt-Chanasit [1,7], Daniel Cadar [1,†] and Renke Lühken [1,7,*,†]

1 Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, 20359 Hamburg, Germany; alex_tomazatos@yahoo.com (A.T.); jansen@bnitm.de (S.J.); maranda.iulia@gmail.com (I.M.); tannich@bnitm.de (E.T.); joest@bnitm.de (H.J.); jonassi@gmx.de (J.S.-C.); danielcadar@gmail.com (D.C.)

2 Humboldt-Universität zu Berlin, 10117 Berlin, Germany; stefan.pfister@hu-berlin.de

3 "Lendület" Landscape and Conservation Ecology, Institute of Ecology and Botany, MTA Centre for Ecological Research, 2163 Vácrátót, Hungary; torok.edina@okologia.mta.hu

4 Department of Clinical Sciences-Infectious Diseases, University of Agricultural Sciences and Veterinary Medicine, 400372 Cluj-Napoca, Romania; cintia.horvath@usamvcluj.ro (C.H.); marina.spinu@gmail.com (M.S.)

5 Center of Systems Biology, Biodiversity and Bioresources, Faculty of Biology and Geology, Babeș-Bolyai University, 400372 Cluj Napoca, Romania; keresztes2012@gmail.com

6 German Centre for Infection Research (DZIF), partner site Hamburg-Luebeck-Borstel-Riems, 20359 Hamburg, Germany

7 Faculty of Mathematics, Informatics and Natural Sciences, Universität Hamburg, 20148 Hamburg, Germany

* Correspondence: renkeluhken@gmail.com

† These authors contributed equally to this work.

Received: 5 November 2019; Accepted: 11 December 2019; Published: 14 December 2019

Abstract: The ecology of West Nile virus (WNV) in the Danube Delta Biosphere Reserve (Romania) was investigated by combining studies on the virus genetics, phylogeography, xenosurveillance and host-feeding patterns of mosquitoes. Between 2014 and 2016, 655,667 unfed and 3842 engorged mosquito females were collected from four sampling sites. Blood-fed mosquitoes were negative for WNV-RNA, but two pools of unfed *Culex pipiens* s.l./*torrentium* collected in 2014 were tested positive. Our results suggest that Romania experienced at least two separate WNV lineage 2 introductions: from Africa into Danube Delta and from Greece into south-eastern Romania in the 1990s and early 2000s, respectively. The genetic diversity of WNV in Romania is primarily shaped by in situ evolution. WNV-specific antibodies were detected for 19 blood-meals from dogs and horses, but not from birds or humans. The hosts of mosquitoes were dominated by non-human mammals (19 species), followed by human and birds (23 species). Thereby, the catholic host-feeding pattern of *Culex pipiens* s.l./*torrentium* with a relatively high proportion of birds indicates the species' importance as a potential bridge vector. The low virus prevalence in combination with WNV-specific antibodies indicate continuous, but low activity of WNV in the Danube Delta during the study period.

Keywords: West Nile virus; virus genetics; phylogeography; xenosurveillance; blood meal

1. Introduction

Emerging or re-emerging mosquito-borne viruses (moboviruses) are of growing concern in Europe [1]. Several moboviruses circulate on the European continent [2]. Thereby, West Nile virus

(WNV, genus *Flavivirus*, family *Flaviviridae*) is of particular importance. This zoonotic virus belongs to the Japanese encephalitis serocomplex and is one the most widespread moboviruses in the world [3–5]. Enzootic transmission takes place between birds as amplifying hosts and mosquitoes as vectors. WNV can cause high mortalities in birds, but spillover events also have significant public health consequences, e.g., headache, rash and even neurological complication [6,7]. Various outbreaks of WNV infections have been reported in southern and southeastern Europe, resulting in several thousand human cases with dozens of fatal outcomes [8,9]. Thereby, Romania is a hotspot for WNV circulation [6,8,10–14]. Over the last two decades, the country has experienced at least three large outbreaks of WNV (1996, 2010, 2018) with a mortality rate of up to 20%. Genetic and phylogenetic analyses grouped the WNV strains into eight distinct evolutionary lineages, from which the most spread worldwide and associated with disease and outbreaks belong to lineages 1 and 2 [15,16]. The virus is transmitted and maintained in the natural cycle by mosquitoes (mostly of the *Culex* genus) as vectors with birds as the main amplifying hosts, while humans and horses are considered incidental or dead-end hosts. Nowadays, West Nile virus exhibits a worldwide distribution throughout Africa, the Middle East, Europe, western Russia, southwestern Asia, and Australia [15]. Starting early 1990s, the frequency, severity and geographic range of human WNV outbreaks increased with the appearance of new viral strains in Romania, Russia, Israel, and Greece [17,18]. In the western hemisphere, West Nile virus spread from its 1999 appearance in New York City throughout the Pacific Coast and Argentina in 2005 [19–21]. Nowadays, the severity, magnitude and geographic location of the WNV outbreaks differs greatly, being instrumented by the local ecological conditions and increased anomalies of seasonal temperature. The Danube Delta Biosphere Reserve (DDBR) is the second largest wetland in Europe. This complex of ecosystems is predominantly located in Romania, with small parts also located in the Ukraine. The heterogeneous area of the DDBR has a high biodiversity with an important function as a major hub for bird migration [22,23]. Thus, the area has a high risk for the introduction of zoonotic pathogens. Introduced moboviruses find an abundant and diverse mosquito fauna [9,23]. Most of the DDBR is only accessible by boat. This makes comprehensive studies on mosquito fauna and associated viruses a difficult task, e.g., showcased by a recent pilot study, detecting two new mosquito species for Romania (*Aedes hungaricus* and *Anopheles algeriensis*) [9].

The circulation of WNV in the DDBR was reported before 2014 [12,24]. WNV dynamics in the Danube Delta are positively correlated with temperature and negatively correlated with rainfall. However, there is still a lack of knowledge driving the risk of WNV transmission under near-natural conditions as present in the DDBR. Therefore, in this study, classical virus screening of mosquitoes was combined with a xenosurveillance approach. Testing for WNV-specific antibodies in sentinel horses or chicken is a common monitoring tool in Europe [25]. However, such a surveillance system is difficult to implement under the remote conditions of a wetland system and the results might not reflect the natural transmission cycle. In addition, comprehensive sampling from wild animals needs a lot of effort. In this study, mosquitoes were used as "biological syringes", i.e., blood-fed specimens were screened for WNV-specific antibodies and viral RNA. Experimental studies by Leigthon et al. [26] demonstrated the potential of mosquitoes for sero-epidemiological studies. This was further supported by a field-study in Thailand detecting antibodies against dengue virus and Japanese encephalitis virus in blood-fed mosquitoes in two different mosquito species [27]. However, a broad application of this method for different wild mosquito species, which feed on different vertebrate hosts, was missing.

Host-feeding patterns of blood-sucking arthropods shape the transmission cycle of vector-borne pathogens, offering direct insights into the interaction between vectors and hosts. However, there is still a lack of knowledge about the host spectrum of mosquitoes in Europe [28]. Previous studies predominantly investigated certain combinations of vector species and pathogens, e.g., *Culex* spp./WNV [29–32], *Culex* spp./avian malaria [33], *Anopheles* spp./malaria [33,34] and *Aedes albopictus* as an invasive vector species for a variety of pathogens [35]. Only few European studies analyzed the host-feeding patterns of a wide range of species [36–38], which is required to better understand pathogen circulation.

Thus, in order to get comprehensive insight into the ecology of WNV in the DDBR, the mosquito fauna was studied in a longitudinal surveillance program over three years. Molecular assays were applied to (i) screen for WNV infections in mosquitoes, analyze the evolutionary mechanism of the virus and its dispersal patterns in Europe, in particular in Romania and the DDBR, (ii) detect WNV-specific antibodies in the blood meals from horses, dogs, humans and birds and (iii) identify potential vector species by analyzing the host-feeding patterns of the blood-fed mosquitoes.

2. Materials and Methods

Mosquitoes were collected within a longitudinal arbovirus surveillance program between 2014 and 2016 at two sampling sites in a rural/urban environment (Letea, Sulina) and two near-natural sampling sites (Dunărea Veche and Lake Roșuleț) in the DDBR. Each year, on average, every tenth day between April and September, three to four (2014, 2015) or one (2016) carbon dioxide-baited Heavy Duty Encephalitis Vector Survey trap(s) (Bioquip Products Inc., CA, USA) were installed at each site. A detailed description of the collection sites can be found in Török et al. [9]. The DDBR Authority issued research permits (9/25.04.2014, 10692/ARBDD/25.04.2014; 7717/ARBDD/28.04.2016, 11/28.04.2016). The collected specimens were transported on dry ice, stored in the freezer and identified by morphology on chill tables using a stereomicroscope (Olympus SZX12, Tokyo, Japan) [39]. Blood-fed mosquitoes were separated from unfed specimens. Furthermore, morphologically identified *Culex pipiens* specimens were typed to species level (*Cx. pipiens pipiens* f. *pipiens*, *Cx. pipiens pipiens* f. *molestus* or *Cx. torrentium*) using a molecular assay [40].

For the WNV screening, mosquito pools between 1 and 250 specimens were pooled per sampling site and sampling date. Mosquitoes were put in 2 mL safe-lock tubes (Eppendorf, Hamburg, Germany) or 50 mL centrifuge tubes (Sarstedt, Nümbrecht, Germany) with zirconia beads (2 mm, BioSpec Products, Bartlesville, OK, USA) and 0.5 or 3 mL chilled high-glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA). Mosquitoes were homogenized in a TissueLyser or TissueLyser II (Qiagen, Hilden, Germany) for 2 min at 30–50 Hz. The suspension was clarified by centrifugation for 1 min at 8000 rpm and 4 °C. RNA was extracted with a KingFisher Flex 96 Deep-Well Magnetic Particle Processor using the MagMAX CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, Waltham, MA, USA). Samples were tested with pan-flavivirus RT-PCR modified from Chao et al. [41] as described in detail by Becker et al. [42]. WNV-positive mosquito pools were subjected to Sanger sequencing (LGC Genomics, Berlin, Germany) for complete genome sequencing [43].

The blood-fed specimens were individually placed into 2 mL safe-lock tubes. Homogenization and extraction were conducted using the same protocol as described above. Thereby, 30 µL supernatant from each of ten specimens was pooled for WNV screening. Detection of WNV-RNA was conducted with the RealStar WNV RT-PCR Kit 1.0 (altona Diagnostics, Hamburg, Germany).

For the host identification, the supernatant of individual blood-fed specimens was heat-inactivated at 99 °C for 1 min in a Peqlab thermocycler (VWR International GmbH, Darmstadt, Germany) for the reduction of possible inhibitors. The PCR assay used the Phusion Blood Direct Master Mix (Thermo Fisher Scientific, MA, USA), 5 µL of the homogenate was used in a total of 30 µL reaction volume for PCR amplification of the cytochrome b gene [44,45]. Amplification was conducted by incubation for 5 min at 98 °C, followed by 40 cycles of 1 s at 98 °C, 5 s at 57 °C and 30 s at 72 °C, ending with incubation for 1 min at 72 °C. If the reaction with the first primer set yielded no result, the PCR reaction was repeated using another pair of vertebrate-specific primers targeting the 16S rRNA gene fragment [46]. The same applied to potential mixed blood meals, as indicated by double peaks in the sequence electropherograms at different positions, resulting in unreadable chromatograms. For this PCR, amplification was conducted by incubation for 5 min at 98 °C, followed by 40 cycles of 1 s at 98 °C, 5 s at 50 °C and 30 s at 72 °C, concluded by incubation for 1 min at 72 °C. The amplicons were sequenced (LGC Genomics, Berlin, Germany) and analyzed with Geneious v9.1.7 (Biomatters, Auckland, New Zealand). Sequences were compared to available sequences from GenBank database

(https://blast.ncbi.nlm.nih.gov/). Host species were identified if the percentage identity was 95% or higher. The statistical computer program R [47] was used for all data analyses. Data manipulation and visualization was conducted with functions from the packages plyr [48], dplyr [49], magrrittr [50] and ggplot2 [51]. Spearman's rank correlation was used to analyze the statistical relationship between the number of analyzed specimens per mosquito species and the number of detected host species. For each mosquito species, higher order taxa (e.g., Anatidae, Bovidae, Chiroptera) were only considered for the calculations of host species, if no corresponding taxa of lower ranks were detected. The frequencies of detected birds, non-human mammals or humans between the six most abundant mosquito species and between the four sampling sites were compared with Chi-square tests with Bonferroni corrected p-values for multiple pairwise comparisons.

Horse-, human-, dog- and bird-derived blood meals were tested for WNV-specific IgG/IgY, using an indirect immunofluorescence (IIF) assay as described previously [52]. Host species were selected, which are important amplifying hosts (bird), known to become critically ill from WNV infections (human, horse) or were previously identified to be suitable sentinel species for WNV (dog, horse) [6,7,53,54]. In brief, Vero cells infected with WNV NY99 were seeded on microscope slides with 12 reaction wells (Marienfeld, Lauda-Königshofen, Germany). Slides were treated with Acetone (99%), 15 µL of each sample (single mosquito homogenized in 500 µL) was transferred into one reaction well. Cells were washed with PBS and stained with Alexa Fluor® 488-conjugated Alpaca Secondary Antibodies (Jackson ImmunoResearch, West Grove PA, USA 1:200 in 1% Evans blue solution), namely goat anti-human IgG, goat anti-horse IgG, rabbit anti-chicken IgY and rabbit anti-dog IgG antibodies, depending on the identified blood-meal source. In order to test for cross-reactivity with heterologous flaviviruses potentially circulating in the sampling area, the WNV IgG positive samples were also tested for Usutu virus- (USUV) and tick-borne encephalitis virus- (TBEV) specific IgG using the same assay with the respective virus.

Genomes obtained for WNV strains from Danube Delta were compared with all complete and partial publicly available NS5 gene sequences from Europe and Africa. Phylogenetic trees were inferred using the Bayesian Markov chain Monte Carlo (MCMC) approach available in BEAST v1.10 [55]. Analyses were performed under the best fit nucleotide substitution model identified as the GTR +Γ for complete genome and TN93+Γ for partial NS5 datasets using jModelTest 2 [56] and a prior MCMC was chosen by testing all models and determining Bayes factors ($\log_{10}$ BF). We employed TempEst for an interactive regression approach to explore the association between genetic divergence through time and sampling dates [57]. In order to assess the spatial temporal dynamics of WNV, the time to most recent common ancestor (tMRCA), and the effective population dynamics of WNV, we employed a relaxed uncorrelated log normal (UCLN) molecular clock, a flexible demographic model (coalescent Gaussian Markov Random field Bayesian Skyride model, GMRF) as the best demographic scenario detected. In all cases, each of the MCMC chain lengths was run for 10^8 generations (with 10% burn-in) and subsampled every 10^4 iterations to achieve convergence. The Bayesian maximum clade credibility (MCC) trees were visualized using FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/). To test the hypothesis that WNV is periodically imported from Africa into Europe, a phylogeographic analysis was conducted using a discrete model attributing state characters represented by the detection locality of each strain and the Bayesian stochastic search variable (BSSV) algorithm implemented in BEAST v1.10 [55].

3. Results

3.1. Mosquitoes and WNV in the Danube Delta

In total, 655,667 mosquitoes representing 14 species and four unspecified taxa (unidentified, *Aedes* spp., *Culex* spp. and *Anopheles* spp.) were collected (Table S1). The mosquitoes were dominated by six species: *Coquilettidia richiardii* (57.9%), *Anopheles hyrcanus* (24.8%), *Anopheles maculipennis* s.l. (4.3%), *Aedes caspius* (3.9%), *Culex pipiens* s.l./*torrentium* (3.5%) and *Aedes vexans* (2.5%). Other mosquito species

were represented by 0.0002% to 2.3% individuals per taxa. WNV-RNA was detected in two pools of unfed *Cx. pipiens* s.l./*torrentium* specimens, while all blood-fed mosquito specimens were tested negative. Both WNV-positive pools were collected in the second half of June 2014 at Lake Roșuleț (4 specimens), a near-natural site, and Sulina (95 specimens), the only town in the DDBR.

3.2. Genome Characterization of WNV in the DDBR

Both WNV positive mosquito pools have been subjected to Sanger sequencing for complete genome sequencing as described elsewhere [43] and deposited in GenBank under the accession numbers MH939153 and MH939154. Sequence comparison between the two sequenced genomes revealed 51 nucleotide (identity rate 99.5%) and 8 amino acid (identity rate 99.8%) differences almost all of them distributed along the polyprotein (Figure S1). Several structural and nonstructural genes of the WNV from Danube Delta exhibited unique or similar amino acid changes exclusively with African WNV strains (Figure S2).

3.3. Phylogeography and Spatio-Temporal Dispersal Pattern of WNV

The Bayesian phylogenetic tree of the complete coding sequence of WNV showed that the strains from Danube Delta clustered in the Eastern European clade 1 together with the strains Hyalomma/Romania/2013, Volgograd/2007 and Italy 792/14 (Figure 1). Given that the majority of available sequences from Romania are partial NS5 gene fragments, we have inferred Bayesian MCC phylogenies with similar topologies as for the complete genome-based tree. In addition, the phylogenies revealed that the Romanian WNV strains fell into two distinct monophyletic clades within WNV phylogeny, suggesting two distinct introductions into Romania (Figure 2b). One clade designated as Eastern European clade 1 (EEC1) included all WNV strains from Danube Delta and some from south-east Romania (Bucharest), while the second clade designated as Western European clade 1 (WEC1), which forms also a distinct monophyletic clade with WNV strains from south-east Romania, but not from DDBR (Figure 1b, Figure 2b). To assess the viral migration and origin of the WNV in Romania, a discrete-trait phylogeography analysis [58] using the complete genome and NS5 datasets was used to reconstruct the WNV movements between continents/countries. Both datasets exhibited a strong temporal signal and the coefficient of rate variation supported the use of a relaxed clock model (Figure 1a, Figure 2a). The phylogenetic analysis revealed that the long-distance movement pattern of WNV between Africa and Europe occurred. We estimated at least 6 intercontinental and 10 continental (Europe) viral migration events (Figure 3). For Romania, we observed at least 2 distinct introduction events (Figure 1b, Figure 2b). The limited number of available sequences from Romania and the lack of WNV data from several European and African countries make it difficult to infer with confidence the spatiotemporal pattern of WNV EEC1. The time to the most recent common ancestor (tMRCA) of the Romanian WNV strains from WEC1 and EEC1 clades indicates a very recent emergence. These strains were most likely introduced into Romania during the 1990s and 2000s as two distinct introduction events (Figures 1–3). The EEC1 clade seems to be a descendant of an ancestor that probably emerged in South Africa around 1910 (95% HPD for 1901–1920; posterior probability 0.96) (Figure 1b), while the WEC1 clade shares a common ancestor that probably emerged in Greece around 1999 (95% HPD for 1994–2003; posterior probability 0.99) (Figure 2b). The spatial origin and diffusion patterns of the WNV were reconstructed using a BSSV analysis. The earliest introduction and migration event of WNV lineage 2 in Romania (Danube Delta) was detected from South Africa (analysis based on complete genome) or Senegal (based on NS5) between 1992 and 2001, after which the virus dispersed to Russia, Italy and southeast Romania (Bucharest) (Figures 1–3). The second origin and introduction of WNV in Romania was detected to be from Greece (based on NS5) between 2001 and 2002 (Figures 1–3). Furthermore, the phylogeographic analysis also revealed the co-circulation of both EEC1 and WEC1 in south-east Romania, but not in Danube Delta (Figures 1–3).

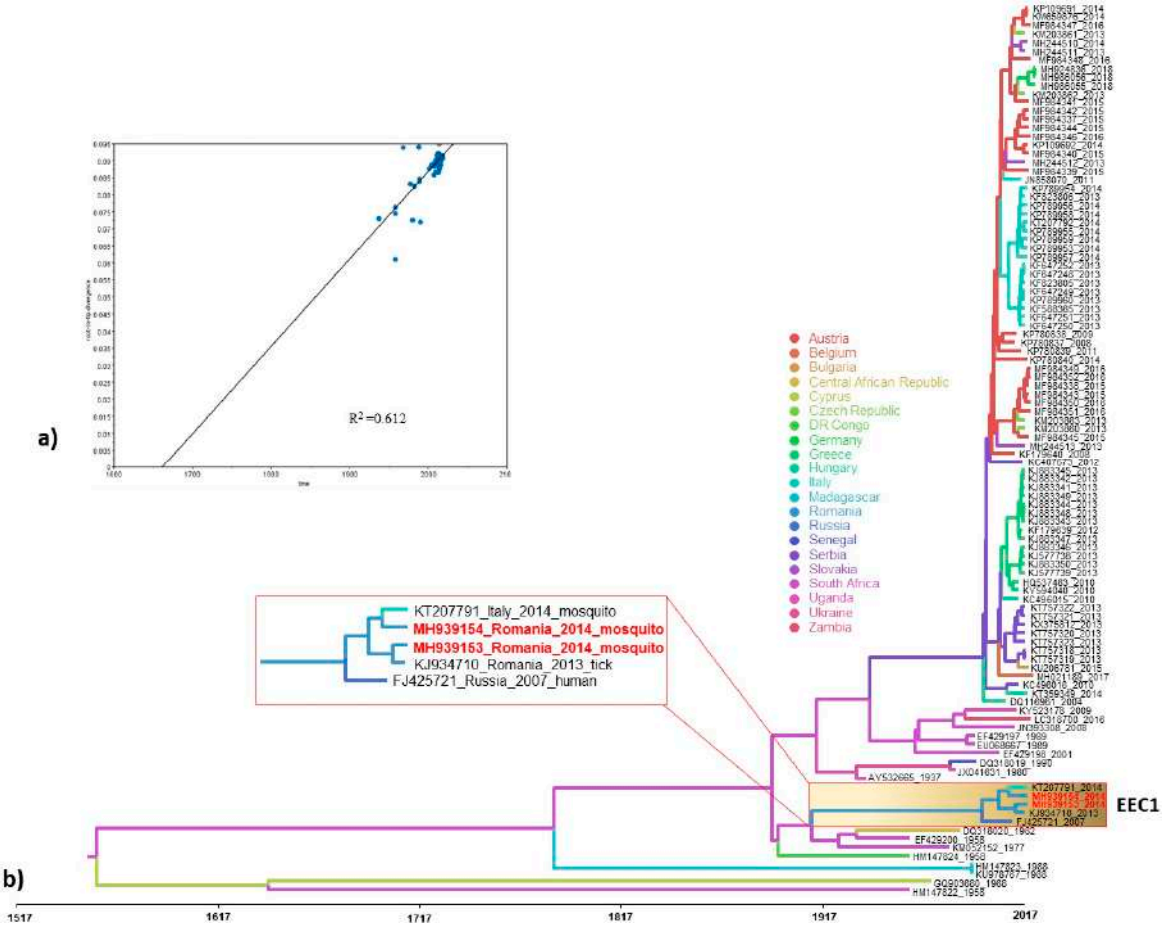


Figure 1. (**a**) Root-to-tip regression analysis of the West Nile virus (WNV) complete genome based maximum likelihood tree. Plots of the root-to-tip genetic distance against sampling time are shown; (**b**) Bayesian maximum clade credibility (MCC) tree representing the time scale phylogeny of WNV lineage 2 based on complete genome sequences, including the EEC1 clade. The colored branches of the MCC tree represent the most probable geographic location of their descendant nodes (see color codes). Time is reported in the axis below the tree and represents the year before the last sampling time (2018).

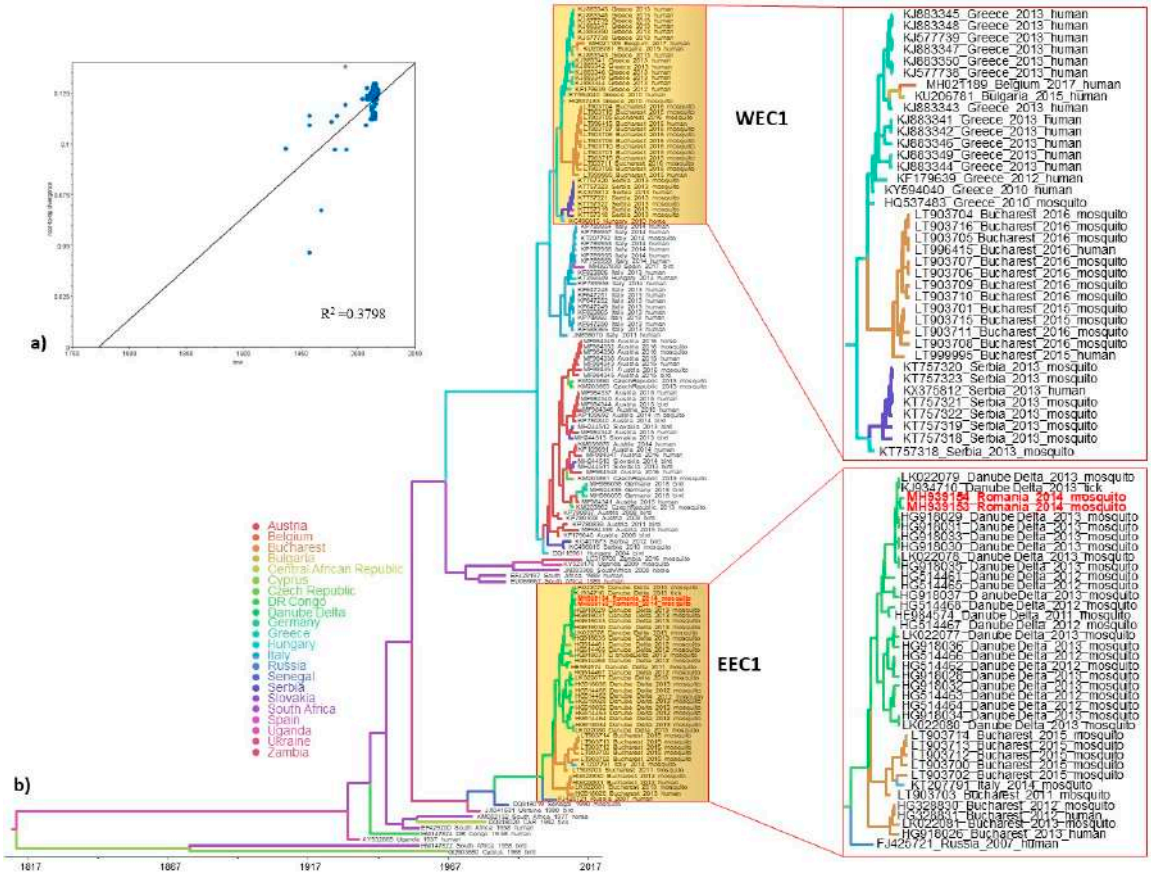


Figure 2. (**a**) Root-to-tip regression analysis of the WNV partial NS5 based maximum likelihood tree. Plots of the root-to-tip genetic distance against sampling time are shown; (**b**) Bayesian maximum clade credibility (MCC) tree representing the time scale phylogeny of WNV lineage 2 based on NS5 gene sequences, including the EEC1 and WEC1 clades. The colored branches of the MCC tree represent the most probable geographic location of their descendant nodes (see color codes). Time is reported in the axis below the tree and represents the year before the last sampling time (2018).

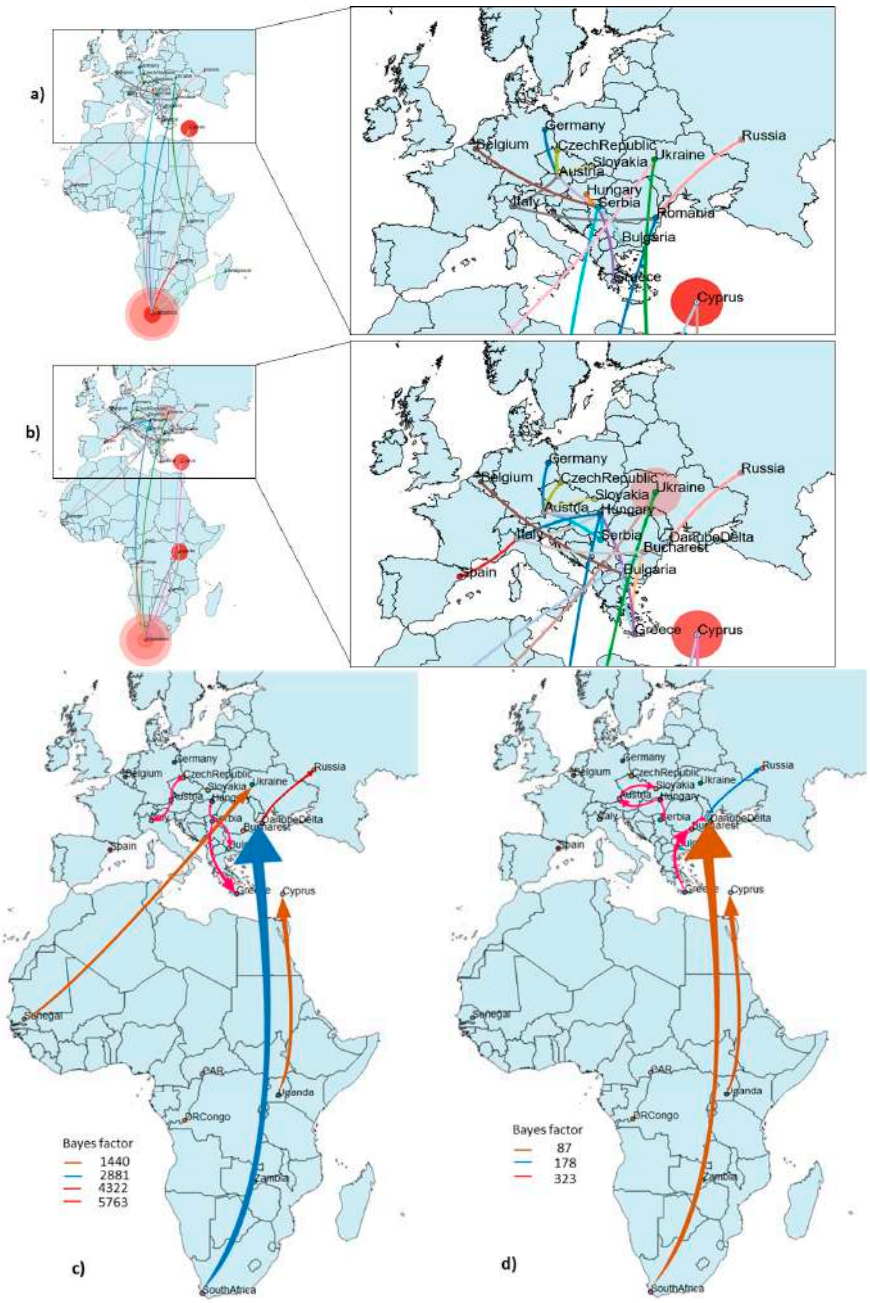


Figure 3. Spatial dynamics of the WNV lineage 2 reconstructed from the (**a**) complete genome and (**b**) partial NS5 based on MCC tree, a flexible demographic prior (coalescent Gaussian Markov Random field Bayesian Skyride model, GMRF) with location states and a Bayesian Stochastic Search Variable Selection (BSSVS) with location states. The directed lines between locations connect the sources and target countries (color coded) of viral strains and represent branches in the MCC tree along which the relevant location transition occurs. Location circle diameters are proportional to the square root of the number of MCC branches maintaining a particular location state at each time-point. Migration pattern of WNV between Africa and Europe and within Europe based on Bayes factor (BF) test for significant non-zero rates using complete genome (**c**) and partial NS5 dataset (**d**). Viral migration patterns are indicated between the different regions and countries and are proportional to the strength of the transmission rate (Bayes factor [BF]). The color of the connections indicates the origin and the direction of migration and are proportional with the strength of connections. Only well supported paths between locations are shown.

The analysis of the complete genome sequences revealed nonsynonymous geographic and clade-specific mutations in all members of EEC1 including some African ancestral specific amino acid residues which further strengthen the African origin of the WNV circulating in Danube Delta (Figure S2).

3.4. Screening for WNV-Specific IgG Antibodies

Nineteen blood meals (2.2%, n = 858 analyzed mosquito specimens) contained WNV-specific antibodies (Table 1, Table S2). Seven of these samples originated from dogs (6.3%, n = 111) and 12 from horses (3.1%, n = 391). All blood meals from birds (n = 85) and humans (n = 271) were WNV IgY/IgG negative. Positive samples were detected for all four sampling sites. WNV IgG positive samples were also tested for USUV- and TBEV-specific IgG. Only one WNV IgG positive blood meal from a dog was also tested positive for USUV-specific IgG.

Table 1. Samples of blood-fed mosquito species positive for West Nile virus-specific IgG and IgY antibodies with information on the host species, mosquito species, sampling site with the respective number of tested mosquito specimens (in brackets).

Host-Species	Mosquito Species	Dunărea Veche	Lake Roșuleț	Letea	Sulina	Sum
dog	*Aedes caspius*	1 (1)	0 (0)	0 (2)	0 (1)	1 (4)
	Aedes vexans	0 (1)	0 (3)	0 (1)	0 (2)	0 (7)
	Anopheles hyrcanus	0 (2)	1 (2)	1 (5)	1 (1) [1]	3 (10)
	Anopheles maculipennis s.l.	0 (3)	1 (13)	0 (2)	0 (1)	1 (19)
	Coquillettidia richiardii	0 (5)	1 (40)	0 (7)	1 (10)	2 (62)
	Culex modestus	0 (0)	0 (1)	0 (0)	0 (0)	0 (1)
	Culex pipiens s.l./*torrentium*	0 (1)	0 (5)	0 (0)	0 (2)	0 (8)
horse	*Aedes caspius*	0 (2)	0 (6)	0 (10)	0 (7)	0 (25)
	Aedes cinereus	0 (0)	0 (0)	0 (2)	0 (0)	0 (2)
	Aedes detritus	0 (0)	0 (0)	0 (0)	0 (1)	0 (1)
	Aedes vexans	0 (0)	1 (20)	0 (92)	0 (8)	1 (120)
	Anopheles algeriensis	0 (0)	0 (0)	0 (1)	0 (1)	0 (2)
	Anopheles hyrcanus	0 (1)	3 (6)	0 (65)	0 (6)	3 (78)
	Anopheles maculipennis s.l.	0 (0)	0 (1)	1 (12)	1 (8)	2 (21)
	Coquillettidia richiardii	1 (2)	1 (32)	3 (90)	1 (16)	6 (140)
	Culex pipiens s.l./*torrentium*	0 (0)	0 (0)	0 (1)	0 (1)	0 (2)
human	*Aedes caspius*	0 (1)	0 (1)	0 (7)	0 (5)	0 (14)
	Aedes flavescens	0 (1)	0 (1)	0 (0)	0 (0)	0 (2)
	Aedes vexans	0 (1)	0 (1)	0 (5)	0 (1)	0 (8)
	Anopheles algeriensis	0 (0)	0 (1)	0 (0)	0 (8)	0 (9)
	Anopheles hyrcanus	0 (7)	0 (96)	0 (20)	0 (9)	0 (132)
	Anopheles maculipennis s.l.	0 (4)	0 (33)	0 (3)	0 (0)	0 (40)
	Coquillettidia richiardii	0 (12)	0 (28)	0 (11)	0 (4)	0 (55)
	Culex modestus	0 (0)	0 (1)	0 (0)	0 (2)	0 (3)
	Culex pipiens s.l./*torrentium*	0 (2)	0 (1)	0 (1)	0 (3)	0 (7)
	Uranotaenia unguiculata	0 (1)	0 (0)	0 (0)	0 (0)	0 (1)
bird	*Aedes caspius*	0 (0)	0 (2)	0 (0)	0 (0)	0 (2)
	Aedes vexans	0 (0)	0 (0)	0 (0)	0 (2)	0 (2)
	Anopheles hyrcanus	0 (2)	0 (15)	0 (0)	0 (0)	0 (17)
	Anopheles maculipennis s.l.	0 (2)	0 (8)	0 (1)	0 (0)	0 (11)
	Coquillettidia richiardii	0 (6)	0 (19)	0 (4)	0 (2)	0 (31)
	Culex pipiens s.l./*torrentium*	0 (12)	0 (3)	0 (1)	0 (6)	0 (22)
	Sum	2 (69)	8 (339)	5 (343)	4 (107)	19 (858)

[1] also positive for USUV-specific IgG.

3.5. Host-Feeding Patterns

From the total mosquito collection, 3842 mosquitoes (0.6%) were blood-fed, belonging to 13 mosquito species (Table 2, Table S3). The blood-fed mosquito species were dominated by six species: *An. hyrcanus* (37.8%), *Cq. richiardii* (27.4%), *An. maculipennis* s.l. (14.8%), *Ae. vexans* (8.9%), *Ae. caspius* (6.1%) and *Cx. pipiens* s.l./*torrentium* (2.3%). Other mosquito species were represented by 1 (0.03%) to

36 (0.94%) specimens per taxon (Table S3). Fifty-one of the collected 88 blood-fed specimens of *Cx. pipiens* s.l./*torrentium* were identified as *Cx. pipiens pipiens* f. *pipiens*.

Table 2. Frequency and percentage (in brackets) of detected host taxa for the six most abundant species and information on the overall proportion of each host.

	Coquillettidia richiardii	*Anopheles hyrcanus*	*Anopheles maculipennis* s.l.	*Aedes vexans*	*Aedes caspius*	*Culex pipiens* s.l./*torrentium*	Sum
Anas platyrhynchos		3 (0.4)	1 (0.4)		1 (0.9)		5 (0.2)
Anatidae	1 (0.1)	12 (1.5)	4 (1.4)		1 (0.9)		18 (0.8)
Ardea purpurea	10 (1.2)		1 (0.4)				11 (0.5)
Circus aeroginosus			1 (0.4)				1 (0)
Corvus corone	1 (0.1)						1 (0)
Corvus fragilegus				1 (0.4)			1 (0)
Cyanistes caeruleus	5 (0.6)					3 (5.4)	8 (0.3)
Cygnus olor	1 (0.1)						1 (0)
Dendrocopos syriacus	1 (0.1)						1 (0)
Egretta garzetta	1 (0.1)						1 (0)
Falco tinnunculus						1 (1.8)	1 (0)
Gallus gallus	1 (0.1)			1 (0.4)			2 (0.1)
Hirundo rustica	2 (0.2)						2 (0.1)
Ixobrychus minutus						3 (5.4)	3 (0.1)
Locustella luscinoides						3 (5.4)	3 (0.1)
Motacilla alba	1 (0.1)						1 (0)
Netta rufina	1 (0.1)						1 (0)
Nycticorax nycticorax	4 (0.5)	2 (0.3)	2 (0.7)			7 (12.5)	15 (0.6)
Parus major						2 (3.6)	2 (0.1)
Pelecanus onocrotalus	1 (0.1)		1 (0.4)				2 (0.1)
Phalacrocorax carbo	1 (0.1)						1 (0)
Streptopelia orientalis			1 (0.4)				1 (0)
Strix aluco						2 (3.6)	2 (0.1)
Upupa epops						1 (1.8)	1 (0)
Homo sapiens	55 (6.7)	132 (16.7)	40 (14.3)	8 (3.5)	14 (13.2)	7 (12.5)	271 (11.5)
Bos taurus	185 (22.4)	515 (65.1)	157 (56.1)	78 (33.9)	46 (43.4)	17 (30.4)	1009 (43)
Bovidae	2 (0.2)						2 (0.1)
Canis aureus	1 (0.1)	1 (0.1)			1 (0.9)		3 (0.1)
Canis lupus	62 (7.5)	10 (1.3)	19 (6.8)	7 (3)	4 (3.8)	8 (14.3)	111 (4.7)
Capra hircus	1 (0.1)	1 (0.1)					2 (0.1)
Capreolus capreolus	1 (0.1)		1 (0.4)				2 (0.1)
Chiroptera	2 (0.2)	1 (0.1)	1 (0.4)				4 (0.2)
Equus caballus	140 (16.9)	78 (9.9)	21 (7.5)	120 (52.2)	25 (23.6)	2 (3.6)	391 (16.7)
Erinaceus europaeus	1 (0.1)						1 (0)
Felis catus	34 (4.1)	3 (0.4)	7 (2.5)			1 (1.8)	47 (2)
Lepus europaeus	3 (0.4)			1 (0.4)	1 (0.9)	1 (1.8)	6 (0.3)
Lutra lutra	2 (0.2)	1 (0.1)					3 (0.1)
Microtus levis						1 (1.8)	1 (0)
Mustela lutreola	1 (0.1)						1 (0)
Mustela nivalis			1 (0.4)				1 (0)
Nyctereutes procyonoides	1 (0.1)						1 (0)
Ovis aries	8 (1)	4 (0.5)		2 (0.9)	2 (1.9)		16 (0.7)
Pipistrellus kuhlii	1 (0.1)						1 (0)
Rattus norvegicus	4 (0.5)					1 (1.8)	7 (0.3)
Rhinolophus hipposideros		1 (0.1)					1 (0)
Sus scrofa	299 (36.2)	28 (3.5)	25 (8.9)	12 (5.2)	11 (10.4)	2 (3.6)	382 (16.3)
blood-fed specimens	1054	1454	568	343	234	88	3741
succesful analyzed specimens [1]	827	791	280	230	106	56	2290
identified hosts per mosquito species [1]	834	792	283	230	106	62	2307
identified host taxa	30	13	15	9	9	17	

[1] differences between the number of successful analysed mosquito specimens and identified hosts results from a total of 17 mixed blood-meals.

The success rate for the identification of the blood sources was 60.7% (2331 specimens), amounting to 2348 identified hosts from 43 species and three unspecified taxa (Anatidae, Bovidae, Chiroptera). The difference of 17 specimens between the number of detected hosts and analyzed mosquito specimens results from mixed blood-meals (Table 2). Hosts were detected for 12 (92.3%) out of the 13 analyzed blood-fed mosquito species, with no successful PCR amplification for a single specimen of *Culex martinii*. The largest number of host taxa was detected for *Cq. richiardii* (30 species, 827 successfully analysed specimens), followed by *Cx. pipiens* s.l./*torrentium* (17 species, 56 specimens), *An. maculipennis* s.l. (15 species, 280 specimens) and *An. hyrcanus* (13 species, 791 specimens). Both, *Ae. vexans* and *Ae. caspius* fed on a moderate number of host species (nine host species each, 230 and 106 specimens)

(Table S3). The other mosquito species with two to 18 specimens fed on two to five host species. Not surprisingly, the number of collected specimens and detected host species were statistically significantly correlated (Spearman rho = 0.90, $p < 0.001$).

Cattle (*Bos taurus*) were the most common host (n = 1009, 43.0% of the detected hosts), followed by horse (*Equus caballus*, n = 391, 16.7%), wild boar (*Sus scrofa*, n = 382, 16.3%), humans (n = 271, 11.5%) and dog (n = 111, 4.7%) (Table 2). The non-human mammalian host group (19 host species and two taxa of higher order) was the most numerous group (n = 1992, 85.0% of the detected hosts), followed by humans (n = 271, 11.5% of all collected mosquitoes). Birds represented the smallest (n = 85, 3.6% of all mosquito specimens) but most diverse host group (23 species and one unspecified taxa).

These three most common host groups were determined for all six most abundant mosquito species (Figure 4). The ratios of the three host groups were statistically significantly ($\chi 2 = 252.72$, df = 10, $p < 0.001$) different between the six most abundant mosquito species. All five taxa except *Cx. pipiens* s.l./*torrentium* showed similar host-feeding patterns with clear preference for non-human mammals (81.2–95.7% of detected blood meal sources), followed by the host groups human and bird with 3.5–16.7% and 0.9–3.9%, respectively. However, *Ae. vexans* had statistically significantly different host group proportions with a higher proportion of non-human mammals compared to *An. hyrcanus*, *An. maculipennis* s.l. and *Ae. caspius* (adjusted p values < 0.05, Table S4). Similarly, we observed a higher proportion of non-human mammals for *An. maculipennis* s.l. and *An. hyrcanus* compared to *Cq. richiardii* (adjusted p values < 0.01, Table S4). Furthermore, the host-feeding pattern of *Cx. pipiens* s.l./*torrentium* was found to be statistically different (adjusted p values < 0.001, Supplementary Table S4) compared to all other five abundant species, with 53.2% non-human mammals, followed by 35.5% birds and 11.3% human.

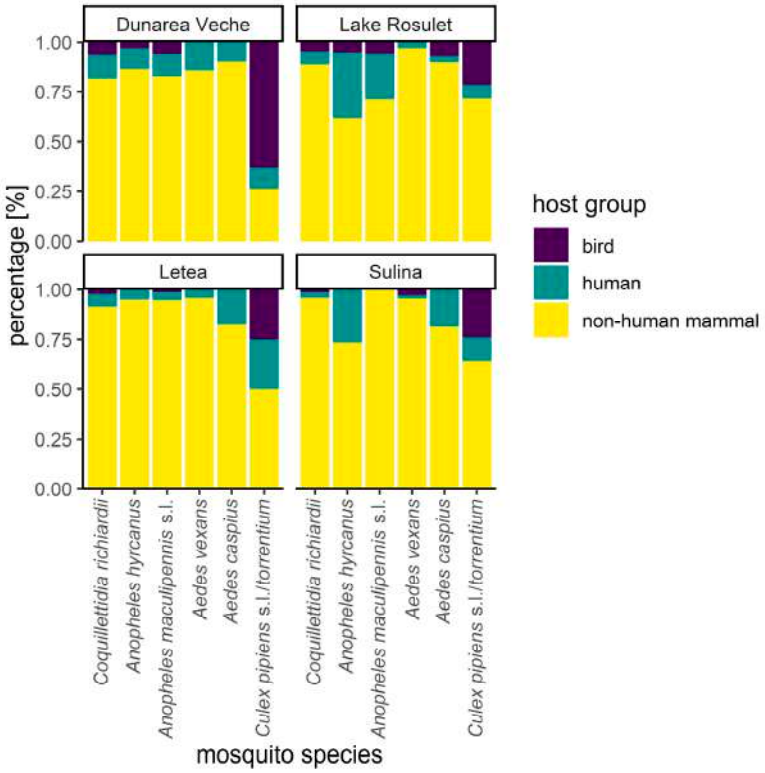


Figure 4. Percentage of host-feeding groups (birds, human, non-human mammals) of the six most abundant blood-fed mosquito species for two sampling sites in a rural/urban environment (Letea, Sulina) and two near-natural sampling sites (Dunărea Veche and Lake Roșuleț) in the DDBR, Romania (2014–2016).

The sampling sites had statistically significant different compositions of detected host groups (Figure 4, $\chi 2 = 114.41$, df = 6, $p < 0.001$). There were no statistical differences between the two near-natural sites Dunărea Veche and Lake Roșuleț (adjusted p values > 0.05, Table S5), but all other combinations were statically significantly different (adjusted p values < 0.05, Table S5, Figure S3). We observed higher percentages of non-human mammals and lower percentages of humans for the two more anthropogenic influenced sites, Letea and Sulina. Furthermore, we detected higher relative proportions of birds and humans for Sulina compared to Letea.

4. Discussion

In this study, we elucidated the possible origin, pattern of spatial-temporal dynamics, and eco-epidemiological factors of WNV in the ecosystem of DDBR. Our phylogeographic analysis identified at least two distinct introduction events of WNV lineage 2 to Romania. It circulates under a number of different virus variants (EEC1 and WEC1) with South Africa/Senegal and Greece as a possible hub for the progenitor of WNV strains involved in the outbreaks in Romania. The presence of a geographically distinct WNV clade (WEC1) is likely due to very recent introduction, adaptation to the local ecological conditions and some geographic barriers such as climate, vegetation, and vector species. Furthermore, the long-term circulation (EEC1) and adaptation of the virus to the host populations and its enzootic maintenance lead to spread into new geographic regions and local virus variants (in situ evolution).

Although the overlap between the phylogenetic and geographical clustering of the Romanian and Russian members of the Eastern European clade of WNV lineage 2 was expected, it is interesting to note that the clade also contains an Italian strain. This suggests a new, independent introduction of the EEC1 in the south-central part of the continent. Similarly to Eastern Europe, the Italian Peninsula is crossed by major Afro-European bird migration routes. To date, the dispersion pattern of WNV into temperate Eurasia can be best explained by bird migration [59–62], with short-distance migratory species as potential mode of WNV spread within Europe [62]. Interestingly, we found evidence of adaptive evolution in the WNV from Danube Delta also in non-structural genes, which likely indicates that the host immune selection pressure does not cause increases in viral fitness [63]. Mutations observed at amino acid positions T108I (C), S196P and R361K (E), I1192V (NS2A) and G2932R (NS5) have been found to be involved in the formation of EEC1. Although the impact of these mutations mostly from the nonstructural genes is unclear (likely occurred due to introduction of WNV in this country), similar changes modulated the host antiviral response by inhibition of interferon signaling [64]. The residue alternations R851K (NS1), I1462M (NS2B), R1516K (NS3), T2296A (NS4), N2305S (NS4) and R2719K (NS5) are specific for African variants. Similar patterns of convergent evolution have been described for WNV and suggest that a limited number of residue changes are permitted due to functional constraints [65].

This study successfully used a xenosurveillance approach to monitor the presence of WNV-specific antibodies in different host species. As demonstrated previously [66], mosquito-based surveillance allows non-invasive blood-sampling from free-roaming vertebrate hosts (e.g., feral horse) and from species which are rare or have a cryptic behavior (e.g., raccoon dog (*Nyctereutes procyonoides*), European mink (*Mustela lutreola*), Eurasian otter (*Lutra lutra*) or Golden jackal (*Canis aureus*)). This study supports previous studies, which identified horses and dogs as suitable sentinel species for WNV [53,54]. WNV seroprevalence in dogs (6.3%) and horses (3.1%) was similar to conventional sampling of the species in different areas with WNV activity [67–69]. Nevertheless, the seroprevalence in horses was markedly lower than in southeastern Romania (15.1%) [70]. WNV-specific antibodies were found in blood meals from horses and dogs in all four sampling sites, but not in mosquito blood-meals from human or bird. Thus, this indicate widespread, continuous WNV circulation, but probably only on a low level. In addition, due to potential cross-reactivity of the applied serological assay, we cannot exclude the possibility that one of the samples was also positive for USUV, a virus with a similar transmission cycle to WNV.

Feral horses and free-ranging cattle were the two most commonly detected host species, accounting for more than 50% of the detected hosts. These animals have their origins in pre-1990 state-owned collective farms and private homesteads from where they were released in more recent years. There is no official census published, but it is estimated that 4000 horses and a few other thousand cattle roam and reproduce freely in the DDBR. The high abundance in combination with the relatively huge body size [71] might explain why both host species are facilitated so often. However, there were differences between the sampling sites. Non-human mammals dominated the detected hosts for the two sampling sites located in the interface between anthropic and natural landscapes (Sulina and Letea), i.e., homesteads in direct proximity of livestock. In contrast, the other two near-natural sampling sites (Lake Roșuleț and Dunărea Veche) were both located deep inside the Danube Delta and only insignificantly anthropogenically influenced. However, humans are commonly present in a fishing cabin and an agricultural holding. In the absence of high abundances of cattle and horse, mosquitoes might rather select other available hosts, e.g., birds and humans. Thus, host availability probably is a decisive factor for the host-selection of mosquitoes, influencing the risk of local pathogen transmission.

At the same time, this study highlights the importance of *Cx. pipiens* s.l./*torrentium* as a WNV vector in Europe [72]. The only two WNV positive pools belonged to this taxon. Previous studies described the species complex as predominantly ornithophilic [30–32,60,73]. However, this study again demonstrates its catholic host-feeding pattern. As the other five most abundance taxa, *Cx. pipiens* s.l./*torrentium* predominantly fed on non-human mammals and humans but had the highest proportion of birds, i.e., a more than nine times higher proportion, making the species a potential bridge vector. This is in line with studies from Africa [74], Middle East [75], Europe [37], and North America [76]. Although several other collected mosquito species (e.g., *An. hyrcanus*) have been found positive for WNV-RNA in Romania [11,13,24], members of the *Culex pipiens* complex are considered the main vectors for WNV in both urban and rural/natural transmission cycles [13].

Active WNV circulation in the DDBR is strongly implicated by WNV infections of unfed mosquito specimens and serological evidence of WNV-specific antibodies in the hosts. However, this study collected only few specimens of the most competent vector *Cx. pipiens* s.l./*torrentium*. In previous studies in the DDBR [24,77], >95% of mosquitoes captured with bird-baited traps belonged to this species complex, while the most abundant species in our study (*Cq. richiardii*) was absent. The usage of a single type of trap in only a few sampling sites across the delta's heterogenous landscape is likely to have contributed to a biased sampling outcome [78,79]. In addition, mobovirus transmission is generally restricted to small foci in non-epidemic years [80]. Therefore, the presence of WNV can be underestimated depending on the number, type and location of traps. Further studies are needed to identify and further understand the driving factors of landscape and time.

5. Conclusions

The detection of WNV-RNA and WNV-specific antibodies confirms the circulation of this important mobovirus in the DDBR. Serological evidence for WNV circulation confirms the applicability of mosquito-based surveillance in sero-epidemiological studies. Host identification for blood-fed mosquitoes allows the usage of host-specific conjugates. In addition, host-feeding patterns of *Cx. pipiens* s.l./*torrentium* underly the relevance of the taxon as an enzootic and bridge vector for WNV in Europe, which was further confirmed by the detection of WNV lineage 2 RNA in two pools of unfed specimens from the same taxon. Local overwintering or reintroduction of the virus could be considered decisive factors for the evolution, dispersal and endemisation of WNV in temperate Europe. Thus, to better understand the impact of ecological/immunological factors on WNV evolution, studies based on more comprehensive genetic data, including those from previously unsampled geographic regions, are required.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/12/1159/s1, Figure S1: Schematic representation of the nucleotide (a) and amino acid (b) differences between WNV strains from Danube Delta generated during this study, Figure S2: Schematic representation of the specific amino acid replacements in the Danube Delta WNV genomes, Figure S3: Percentage of host-feeding groups (birds, human, non-human mammals) for the four sampling sites in the DDBR, Romania (2014–2016), Table S1: Frequency and percentage of mosquitoes collected at four sampling sites in the DDBR, Romania (2014–2016), Table S2: Samples of blood-fed mosquito species positive for West Nile virus-specific IgG antibodies with information on the host species, mosquito species, sampling year collected at four sampling sites in the DDBR, Romania (2014–2016), Table S3: Number and percentage (in brackets) of detected host taxa for the six less abundant species, Table S4: Results of Chi-square tests for the comparison of host-feeding groups between the six most abundant mosquito species with adjusted p-values collected at four sampling sites in the DDBR, Romania (2014–2016), Table S5: Results of Chi-square tests for the comparison of host-feeding groups of mosquitoes between the four sampling sites in the DDBR, Romania (2014–2016) with adjusted p-values.

Author Contributions: Conceptualization, S.J., L.K., E.T. (Egbert Tannich), M.S., H.J., J.S.-C., D.C., R.L.; formal analysis, A.T., D.C., R.L.; investigation, A.T., S.P., E.T. (Edina Török), I.M., C.H., H.J., D.C., R.L.; writing—original draft preparation, A.T., D.C., R.L.; writing—review and editing, S.J., J.S.-C.; visualization, A.T., D.C., R.L.

Funding: This research received no external funding.

Acknowledgments: We would like to thank Heike Baum, Alexandra Bialonski, Mathis Petersen, Michelle Helms and Claudia Poggensee for excellent technical assistance on sample processing and virus screening, Corinna Thomé and Alexander Schlaphof for their advice and help regarding serological assays, and to the people of Danube Delta who facilitated fieldwork in the wild or on their properties.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zeller, H.; Marrama, L.; Sudre, B.; Bortel, W.V.; Warns-Petit, E. Mosquito-borne disease surveillance by the European Centre for Disease Prevention and Control. *Clin. Microbiol. Infect.* **2013**, *19*, 693–698. [CrossRef] [PubMed]
2. Hubálek, Z. Mosquito-borne viruses in Europe. *Parasitol. Res.* **2008**, *103*, 29–43. [CrossRef] [PubMed]
3. Chancey, C.; Grinev, A.; Volkova, E.; Rios, M. The global ecology and epidemiology of West Nile virus. *BioMed Res. Int.* **2015**, *2015*, 1–20. [CrossRef] [PubMed]
4. Gould, E.; Pettersson, J.; Higgs, S.; Charrel, R.; de Lamballerie, X. Emerging arboviruses: Why today? *One Health* **2017**, *4*, 1–13. [CrossRef] [PubMed]
5. Weaver, S.C.; Reisen, W.K. Present and future arboviral threats. *Antivir. Res.* **2010**, *85*, 328–345. [CrossRef] [PubMed]
6. Tsai, T.; Popovici, F.; Cernescu, C.; Campbell, G.; Nedelcu, N. West Nile encephalitis epidemic in southeastern Romania. *Lancet* **1998**, *352*, 767–771. [CrossRef]
7. Ziegler, U.; Lühken, R.; Keller, M.; Cadar, D.; van der Grinten, E.; Michel, F.; Albrecht, K.; Eiden, M.; Rinder, M.; Lachmann, L.; et al. West Nile virus epizootic in Germany, 2018. *Antivir. Res.* **2019**, *162*, 39–43. [CrossRef]
8. European Centre for Disease Prevention and Control. Historical Data by Year—West Nile Fever Seasonal Surveillance. 2019. Available online: https://www.ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical (accessed on 5 November 2019).
9. Török, E.; Tomazatos, A.; Cadar, D.; Horváth, C.; Keresztes, L.; Jansen, S.; Becker, N.; Kaiser, A.; Popescu, O.; Schmidt-Chanasit, J.; et al. Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of *Anopheles algeriensis* Theobald, 1903 and *Aedes hungaricus* Mihályi, 1955 for Romania. *Parasit. Vectors* **2016**, *9*, 196. [CrossRef]
10. Han, L.L.; Popovici, F.; Alexander, J.P., Jr.; Laurentia, V.; Tengelsen, L.A.; Cernescu, C.; Gary, H.E., Jr.; Ion-Nedelcu, N.; Campbell, G.L.; Tsai, T.F. Risk factors for West Nile virus infection and meningoencephalitis, Romania, 1996. *J. Infect. Dis.* **1999**, *179*, 230–233. [CrossRef]
11. Savage, H.M.; Ceianu, C.; Nicolescu, G.; Karabatsos, N.; Lanciotti, R.; Vladimirescu, A.; Laiv, L.; Ungureanu, A.; Romanca, C.; Tsai, T.F. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. *Am. J. Trop. Med. Hyg.* **1999**, *61*, 600–611. [CrossRef]

12. Sirbu, A.; Ceianu, C.S.; Panculescu-Gatej, R.; Vasquez, A.; Tenorio, A.; Rebreanu, R.; Niedrig, M.; Nicolescu, G.; Pistol, A. Outbreak of West Nile virus infection in humans, Romania, July to October 2010. *Euro Surveill.* **2010**, *16*, 19762.
13. Dinu, S.; Cotar, A.; Pănculescu-Gătej, I.; Fălcuţă, E.; Prioteasa, F.; Sîrbu, A.; Oprişan, G.; Bădescu, D.; Reiter, P.; Ceianu, C. West Nile virus circulation in south-eastern Romania, 2011 to 2013. *Euro Surveill.* **2015**, *20*, 21130. [CrossRef] [PubMed]
14. Popescu, C.P.; Florescu, S.A.; Cotar, A.I.; Badescu, D.; Ceianu, C.S.; Zaharia, M.; Tardei, G.; Codreanu, D.; Ceausu, E.; Ruta, S.M. Re-emergence of severe West Nile virus neuroinvasive disease in humans in Romania, 2012 to 2017–implications for travel medicine. *Travel Med. Infect. Dis.* **2018**, *22*, 30–35. [CrossRef] [PubMed]
15. Petersen, L.R.; Brault, A.C.; Nasci, R.S. West Nile virus: Review of the literature. *JAMA* **2013**, *310*, 308–315. [CrossRef]
16. Fall, G.; Di Paola, N.; Faye, M.; Dia, M.; de Melo Freire, C.C.; Loucoubar, C.; de Andrade Zanotto, P.M.; Faye, O. Biological and phylogenetic characteristics of West African lineages of West Nile virus. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0006078. [CrossRef]
17. May, F.J.; Davis, C.T.; Tesh, R.B.; Barrett, A.D. Phylogeography of West Nile virus. *J. Virol.* **2011**, *85*, 2964–2974. [CrossRef]
18. Sambri, V.; Capobianchi, M.; Charrel, R.; Fyodorova, M.; Gaibani, P.; Gould, E.; Niedrig, M.; Papa, A.; Pierro, A.; Rossini, G.; et al. West Nile virus in Europe: Emergence, epidemiology, diagnosis, treatment, and prevention. *Clin. Microbiol. Infect.* **2013**, *19*, 699–704. [CrossRef]
19. Nash, D.; Mostashari, F.; Fine, A.; Miller, J.; O'Leary, D.; Murray, K.; Huang, A.; Rosenberg, A.; Greenberg, A.; Sherman, M.; et al. The outbreak of West Nile virus infection in the New York City area in 1999. *N. Engl. J. Med.* **2001**, *344*, 1807–1814. [CrossRef]
20. Lindsey, N.P.; Staples, J.E.; Lehman, J.A.; Fischer, M. Surveillance for human West Nile virus disease. *MMWR Surveill. Summ.* **2010**, *59*, 1–17.
21. Petersen, L.R.; Hayes, E.B. West Nile virus in the Americas. *Med. Clin. North Am.* **2008**, *92*, 1307–1322. [CrossRef]
22. Hanganu, J.; Dubyna, D.; Zhmud, E.; Grigoraş, I.; Menke, U.; Drost, H.; Ştefan, N.; Sârbu, I. *Vegetation of the Biosphere Reserve "Danube Delta"—With Transboundary Vegetation Map on a 1:150,000 Scale*; Danube Delta National Institute, Romania, M.G. Kholodny Institute of Botany & Danube Delta Biosphere Reserve, Ukraine and RIZA, Eds.; RIZA Rapport: Lelystad, The Netherlands, 2002.
23. Prioteasa, F.L.; Falcuta, E. An annotated checklist of the mosquitoes (Diptera: Culicidae) of the Danube Delta Biosphere Reserve. *Eur. Mosq. Bull.* **2010**, *28*, 240–245.
24. Cotar, A.I.; Falcuta, E.; Prioteasa, L.F.; Dinu, S.; Ceianu, C.S.; Paz, S. Transmission dynamics of the West Nile virus in mosquito vector populations under the influence of weather factors in the Danube Delta, Romania. *EcoHealth* **2016**, *13*, 796–807. [CrossRef]
25. Gossner, C.M.; Marrama, L.; Carson, M.; Allerberger, F.; Calistri, P.; Dilaveris, D.; Lecollinet, S.; Morgan, D.; Nowotny, N.; Paty, M.C.; et al. West Nile virus surveillance in Europe: Moving towards an integrated animal-human-vector approach. *Euro Surveill.* **2017**, *22*, 30526. [CrossRef]
26. Leighton, B.J.; Roitberg, B.D.; Belton, P.; Lowenberger, C.A. Host antibodies in mosquito bloodmeals: A potential tool to detect and monitor infectious diseases in wildlife. *J. Med. Entomol.* **2008**, *45*, 470–475. [CrossRef]
27. Barbazan, P.; Palabodeewat, S.; Nitatpattana, N.; Gonzalez, J.P. Detection of host virus-reactive antibodies in blood meals of naturally engorged mosquitoes. *Vector Borne Zoonotic Dis.* **2009**, *9*, 103–108. [CrossRef]
28. Kurucz, K.; Kepner, A.; Krtinic, B.; Hederics, D.; Foldes, F.; Brigetta, Z.; Jakab, F.; Kemenesi, G. Blood-meal analysis and avian malaria screening of mosquitoes collected from human-inhabited areas in Hungary and Serbia. *J. Eur. Mosq. Control Assoc.* **2018**, *36*, 3–13.
29. Balenghien, T.; Fouque, F.; Sabatier, P.; Bicout, D.J. Horse-, Bird-, and Human-Seeking Behavior and Seasonal Abundance of Mosquitoes in a West Nile Virus Focus of Southern France. *J. Med. Entomol.* **2006**, *43*, 936–946. [CrossRef]
30. Gomes, B.; Sousa, C.A.; Vicente, J.L.; Pinho, L.; Calderón, I.; Arez, E.; Almeida, A.P.; Donnelly, M.J.; Pinto, J. Feeding patterns of *molestus* and *pipiens* forms of *Culex pipiens* (Diptera: Culicidae) in a region of high hybridization. *Parasit. Vectors* **2013**, *6*, 93. [CrossRef]

31. Muñoz, J.; Ruiz, S.; Soriguer, R.; Alcaide, M.; Viana, D.S.; Roiz, D.; Vázquez, A.; Figuerola, J. Feeding patterns of potential West Nile virus vectors in south-west Spain. *PLoS ONE* **2012**, *7*, e39549. [CrossRef]
32. Rizzoli, A.; Bolzoni, L.; Chadwick, E.A.; Capelli, G.; Montarsi, F.; Grisenti, M.; de la Puente, J.M.; Muñoz, J.; Figuerola, J.; Soriguer, R.; et al. Understanding West Nile virus ecology in Europe: *Culex pipiens* host feeding preference in a hotspot of virus emergence. *Parasit. Vectors* **2015**, *8*, 213. [CrossRef]
33. Martínez-de la Puente, J.; Ferraguti, M.; Ruiz, S.; Roiz, D.; Soriguer, R.C.; Figuerola, J. *Culex pipiens* forms and urbanization: Effects on blood feeding sources and transmission of avian *Plasmodium*. *Malar. J.* **2016**, *15*, 589. [CrossRef] [PubMed]
34. Fălcuță, E.; Toty, C.; Prioteasa, F.L.; Nicolescu, G.; Teodorescu, I.; Purcarea-Ciulacu, V.; Fontenille, D. Blood-meal preferences for Anopheles maculipennis (Diptera: Cuicidae) complex species in Comana, Giurgiu county (Romania). *Rom. J. Biol. Zool.* **2010**, *55*, 1–110.
35. Martínez-de la Puente, J.; Muñoz, J.; Capelli, G.; Montarsi, F.; Soriguer, R.; Arnoldi, D.; Rizzoli, A.; Figuerola, J. Avian malaria parasites in the last supper: Identifying encounters between parasites and the invasive Asian mosquito tiger and native mosquito species in Italy. *Malar. J.* **2015**, *14*, 32. [CrossRef] [PubMed]
36. Hernández-Triana, L.M.; Brugman, V.A.; Prosser, S.W.J.; Weland, C.; Nikolova, N.; Thorne, L.; Marco, M.F.D.; Fooks, A.R.; Johnson, N. Molecular approaches for blood meal analysis and species identification of mosquitoes (Insecta: Diptera: Culicidae) in rural locations in southern England, United Kingdom. *Zootaxa* **2017**, *4250*, 67. [CrossRef] [PubMed]
37. Börstler, J.; Jöst, H.; Garms, R.; Krüger, A.; Tannich, E.; Becker, N.; Schmidt-Chanasit, J.; Lühken, R. Host-feeding patterns of mosquito species in Germany. *Parasit. Vectors* **2016**, *9*, 318. [CrossRef] [PubMed]
38. Schönenberger, A.C.; Wagner, S.; Tuten, H.C.; Schaffner, F.; Torgerson, P.; Furrer, S.; Mathis, A.; Silaghi, C. Host preferences in host-seeking and blood-fed mosquitoes in Switzerland: Host preferences in mosquitoes. *Med. Vet. Entomol.* **2015**, *30*, 39–52. [CrossRef]
39. Becker, N.; Petric, D.; Zgomba, M.; Boase, C.; Madon, M.; Dahl, C.; Kaiser, A. *Mosquitoes and Their Control*, 2nd ed.; Springer: Berlin/Heidelberg, Germany, 2010; ISBN 978-3-540-92873-7.
40. Rudolf, M.; Czajka, C.; Börstler, J.; Melaun, C.; Jöst, H.; von Thien, H.; Badusche, M.; Becker, N.; Schmidt-Chanasit, J.; Krüger, A.; et al. First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype *pipiens/molestus* hybrids in Germany. *PLoS ONE* **2013**, *8*, e71832. [CrossRef]
41. Chao, D.Y.; Davis, B.S.; Chang, G.J.J. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. *J. Clin. Microbiol.* **2007**, *45*, 584–589. [CrossRef]
42. Becker, N.; Jöst, H.; Ziegler, U.; Eiden, M.; Höper, D.; Emmerich, P.; Fichet-Calvet, E.; Ehichioya, D.U.; Czajka, C.; Gabriel, M.; et al. Epizootic emergence of Usutu virus in wild and captive birds in Germany. *PLoS ONE* **2012**, *7*, e32604. [CrossRef]
43. Jungbauer, C.; Hourfar, M.K.; Stiasny, K.; Aberle, S.W.; Cadar, D.; Schmidt-Chanasit, J.; Mayr, W.R. West Nile virus lineage 2 infection in a blood donor from Vienna, Austria, August 2014. *J. Clin. Virol.* **2015**, *64*, 16–19. [CrossRef]
44. Burkett-Cadena, N.D.; Graham, S.P.; Hassan, H.K.; Guyer, C.; Eubanks, M.D.; Katholi, C.R.; Unnasch, T.R. Blood feeding patterns of potential arbovirus vectors of the genus culex targeting ectothermic hosts. *Am. J. Trop. Med. Hyg.* **2008**, *79*, 809–815. [CrossRef] [PubMed]
45. Kitano, T.; Umetsu, K.; Tian, W.; Osawa, M. Two universal primer sets for species identification among vertebrates. *Int. J. Leg. Med.* **2007**, *121*, 423–427. [CrossRef] [PubMed]
46. Kocher, T.D.; Thomas, W.K.; Meyer, A.; Edwards, S.V.; Paabo, S.; Villablanca, F.X.; Wilson, A.C. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6196–6200. [CrossRef] [PubMed]
47. *R Core Team R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2013.
48. Wickham, H. The split-apply-combine strategy for data analysis. *J. Stat. Softw.* **2011**, *40*, 1–29. [CrossRef]
49. Wickham, H.; François, R.; Henry, L.; Müller, K. Dplyr: A Grammar of Data Manipulation. 2019. Available online: https://rdrr.io/cran/dplyr/ (accessed on 5 November 2019).
50. Bache, S.M.; Wickham, H. Magrittr: A Forward-Pipe Operator for R. 2014.

51. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2009; ISBN 978-0-387-98140-6.
52. Tappe, D.; Schmidt-Chanasit, J.; Ries, A.; Ziegler, U.; Müller, A.; Stich, A. Ross River virus infection in a traveller returning from northern Australia. *Med. Microbiol. Immunol. (Berl.)* **2009**, *198*, 271–273. [CrossRef]
53. Crivei, A.L.; Răți (Anton), I.; Răileanu, C.; Porea, D.; Aniţa, D.; Savuța, G.; Oșlobanu, L. First record of West Nile virus specific seroconversion in dogs from Eastern Romania. *Bull. Univ. Agric. Sci. Vet. Med. Cluj Napoca Vet. Med.* **2018**, *75*, 163. [CrossRef]
54. Durand, B.; Haskouri, H.; Lowenski, S.; Vachiery, N.; Beck, C.; Lecollinet, S. Seroprevalence of West Nile and Usutu viruses in military working horses and dogs, Morocco, 2012: Dog as an alternative WNV sentinel species? *Epidemiol. Infect.* **2016**, *144*, 1857–1864. [CrossRef]
55. Suchard, M.A.; Lemey, P.; Baele, G.; Ayres, D.L.; Drummond, A.J.; Rambaut, A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol.* **2018**, *4*, vey016. [CrossRef]
56. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
57. Rambaut, A.; Lam, T.T.; Max Carvalho, L.; Pybus, O.G. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol.* **2016**, *2*, vew007. [CrossRef]
58. Lemey, P.; Rambaut, A.; Drummond, A.J.; Suchard, M.A. Bayesian phylogeography finds its roots. *PLoS Comput. Biol.* **2009**, *5*, e1000520. [CrossRef] [PubMed]
59. Jourdain, E.; Toussaint, Y.; Leblond, A.; Bicout, D.J.; Sabatier, P.; Gauthier-Clerc, M. Bird species potentially involved in introduction, amplification, and spread of West Nile virus in a mediterranean wetland, the Camargue (Southern France). *Vector Borne Zoonotic Dis.* **2007**, *7*, 15–33. [CrossRef] [PubMed]
60. Hubálek, Z.; Halouzka, J. a West Nile fever - a reemerging mosquito-borne viral disease in Europe. *Emerg. Infect. Dis.* **1999**, *5*, 643–650. [CrossRef] [PubMed]
61. Malkinson, M. Introduction of West Nile virus in the Middle East by Migrating White Storks. *Emerg. Infect. Dis.* **2002**, *8*, 392–397. [CrossRef]
62. Ciccozzi, M.; Peletto, S.; Cella, E.; Giovanetti, M.; Lai, A.; Gabanelli, E.; Acutis, P.L.; Modesto, P.; Rezza, G.; Platonov, A.E.; et al. Epidemiological history and phylogeography of West Nile virus lineage 2. *Infect. Genet. Evol.* **2013**, *17*, 46–50. [CrossRef]
63. Di Giallonardo, F.; Geoghegan, J.L.; Docherty, D.E.; McLean, R.G.; Zody, M.C.; Qu, J.; Yang, X.; Birren, B.W.; Malboeuf, C.M.; Newman, R.M.; et al. Fluid spatial dynamics of West Nile virus in the United States: Rapid spread in a permissive host environment. *J. Virol.* **2016**, *90*, 862–872. [CrossRef]
64. Liu, W.J.; Chen, H.B.; Wang, X.J.; Huang, H.; Khromykh, A.A. Analysis of adaptive mutations in Kunjin virus replicon RNA reveals a novel role for the flavivirus nonstructural protein NS2A in inhibition of beta interferon promoter-driven transcription. *J. Virol.* **2004**, *78*, 12225–12235. [CrossRef]
65. Armstrong, P.M.; Vossbrinck, C.R.; Andreadis, T.G.; Anderson, J.F.; Pesko, K.N.; Newman, R.M.; Lennon, N.J.; Birren, B.W.; Ebel, G.D.; Henn, M.R. Molecular evolution of West Nile virus in a northern temperate region: Connecticut, USA 1999–2008. *Virology* **2011**, *417*, 203–210. [CrossRef]
66. Martínez-de la Puente, J.; Méndez, M.; Ruiz, S.; Godoy, J.A.; Soriguer, R.C.; Figuerola, J. Individual identification of endangered species using mosquito blood meals: A proof-of-concept study in Iberian lynx. *Parasitol. Res.* **2015**, *114*, 1607–1610. [CrossRef]
67. García-Bocanegra, I.; Jurado-Tarifa, E.; Cano-Terriza, D.; Martínez, R.; Pérez-Marín, J.E.; Lecollinet, S. Exposure to West Nile virus and tick-borne encephalitis virus in dogs in Spain. *Transbound. Emerg. Dis.* **2018**, *65*, 765–772. [CrossRef]
68. García-Bocanegra, I.; Arenas-Montes, A.; Napp, S.; Jaén-Téllez, J.A.; Fernández-Morente, M.; Fernández-Molera, V.; Arenas, A. Seroprevalence and risk factors associated to West Nile virus in horses from Andalusia, Southern Spain. *Vet. Microbiol.* **2012**, *160*, 341–346. [CrossRef] [PubMed]
69. Maquart, M.; Dahmani, M.; Marié, J.L.; Gravier, P.; Leparc-Goffart, I.; Davoust, B. First serological evidence of West Nile virus in horses and dogs from Corsica Island, France. *Vector Borne Zoonotic Dis.* **2017**, *17*, 275–277. [CrossRef] [PubMed]
70. Popescu, D.; Necșulescu, M.; Alexse, A.; Purcărea-Ciulacu, V. West Nile virus infection of horses in anthropic ecosystems in Romania. *Rev. Rom. Med. Vet.* **2008**, *18*, 39–46.
71. Port, G.R.; Boreham, P.F.L.; Bryan, J.H. The relationship of host size to feeding by mosquitoes of the *Anopheles gambiae* Giles complex (Diptera: Culicidae). *Bull. Entomol. Res.* **1980**, *70*, 133–144. [CrossRef]

72. Hubalek, Z.; Rudolf, I.; Nowotny, N. Arboviruses pathogenic for domestic and wild animals. *Adv. Virus Res.* **2014**, *89*, 201–275. [PubMed]
73. Kilpatrick, A.M.; Kramer, L.D.; Jones, M.J.; Marra, P.P.; Daszak, P. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. *PLoS Biol.* **2006**, *4*, e82. [CrossRef]
74. Omondi, D.; Masiga, D.K.; Ajamma, Y.U.; Fielding, B.C.; Njoroge, L.; Villinger, J. Unraveling host-vector-arbovirus interactions by two-gene high resolution melting mosquito bloodmeal analysis in a Kenyan wildlife-livestock interface. *PLoS ONE* **2015**, *10*, e0134375. [CrossRef]
75. Shahhosseini, N.; Friedrich, J.; Moosa-Kazemi, S.H.; Sedaghat, M.M.; Kayedi, M.H.; Tannich, E.; Schmidt-Chanasit, J.; Lühken, R. Host-feeding patterns of *Culex* mosquitoes in Iran. *Parasit. Vectors* **2018**, *11*, 669. [CrossRef]
76. Apperson, C.S.; Hassan, H.K.; Harrison, B.A.; Savage, H.M.; Aspen, S.E.; Farajollahi, A.; Crans, W.; Daniels, T.J.; Falco, R.C.; Benedict, M.; et al. Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. *Vector Borne Zoonotic Dis.* **2004**, *4*, 71–82. [CrossRef]
77. Prioteasa, F.L. Evaluating the Vectorial Potential of Danube Delta Culicids (Diptera: Insecta) for West Nile Virus (Flaviviridae). Ph.D. Thesis, Faculty of Biology, University of Bucharest, Bucharest, Wallachia, 2011.
78. L'Ambert, G.; Ferré, J.B.; Schaffner, F.; Fontenille, D. Comparison of different trapping methods for surveillance of mosquito vectors of West Nile virus in Rhône Delta, France. *J. Vector Ecol.* **2012**, *37*, 269–275. [CrossRef]
79. Lühken, R.; Pfitzner, W.; Börstler, J.; Garms, R.; Huber, K.; Schork, N.; Steinke, S.; Kiel, E.; Becker, N.; Tannich, E.; et al. Field evaluation of four widely used mosquito traps in Central Europe. *Parasit. Vectors* **2014**, *7*, 268. [CrossRef] [PubMed]
80. Ceianu, C.S.; Ungureanu, A.; Nicolescu, G.; Cernescu, C.; Nitescu, L.; Tardei, G.; Petrescu, A.; Pitigoi, D.; Martin, D.; Ciulacu-Purcarea, V.; et al. West Nile virus surveillance in Romania: 1997–2000. *Viral Immunol.* **2001**, *14*, 251–262. [CrossRef] [PubMed]

Parasites & Vectors

RESEARCH Open Access

Blood-meal analysis of *Culicoides* (Diptera: Ceratopogonidae) reveals a broad host range and new species records for Romania

Alexandru Tomazatos[1], Hanna Jöst[1], Jonny Schulze[1], Marina Spînu[2], Jonas Schmidt-Chanasit[1,3], Daniel Cadar[1] and Renke Lühken[1,3*]

Abstract

Background: *Culicoides* biting midges are potential vectors of different pathogens. However, especially for eastern Europe, there is a lack of knowledge on the host-feeding patterns of this vector group. Therefore, this study aimed to identify *Culicoides* spp. and their vertebrate hosts collected in a wetland ecosystem.

Methods: *Culicoides* spp. were collected weekly from May to August 2017, using Biogents traps with UV light at four sites in the Danube Delta Biosphere Reserve, Romania. Vectors and hosts were identified with a DNA barcoding approach. The mitochondrial cytochrome *c* oxidase subunit 1 was used to identify *Culicoides* spp., while vertebrate hosts were determined targeting cytochrome *b* or *16S* rRNA gene fragments. A maximum likelihood phylogenetic tree was constructed to verify the biting midge identity against other conspecific Palaearctic *Culicoides* species. A set of unfed midges was used for morphological confirmation of species identification using slide-mounted wings.

Results: Barcoding allowed the species identification and detection of corresponding hosts for 1040 (82.3%) of the 1264 analysed specimens. Eight *Culicoides* spp. were identified with *Culicoides griseidorsum*, *Culicoides puncticollis* and *Culicoides submaritimus* as new species records for Romania. For 39 specimens no similar sequences were found in GenBank. This group of unknown *Culicoides* showed a divergence of 15.6–16.3% from the closest identified species and clustered in a monophyletic clade, i.e. a novel species or a species without reference sequences in molecular libraries. For all *Culicoides* spp., nine mammalian and 24 avian species were detected as hosts. With the exception of *C. riethi* ($n = 12$), at least one avian host was detected for all *Culicoides* spp., but this host group only dominated for *Culicoides kibunensis* and the unknown *Culicoides* sp.. The most common host group were mammals ($n = 993$, 87.6% of all identified blood sources) dominated by cattle ($n = 817$, 70.6%).

Conclusions: Most *Culicoides* spp. showed a broad host-feeding pattern making them potential bridge vectors. At the same time, new records of biting midge species for Romania, as well as a potentially unknown *Culicoides* species, highlight the lack of knowledge regarding the biting midge species and their genetic diversity in eastern Europe.

Keywords: *Culicoides*, Barcoding, Host-feeding patterns, Danube delta, Romania

Background

Biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) are vectors of a variety of pathogens. These include protozoans [1–3], filarial worms [4] and numerous viruses [5]. Their relevance as vectors is primarily related to veterinary health, though

*Correspondence: renkeluhken@gmail.com
[1] WHO Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany
Full list of author information is available at the end of the article

outbreaks of the *Culicoides*-borne Oropouche virus in humans regularly occur in the Neotropics [6]. In Europe, several biting midge species are able to transmit bluetongue virus (BTV), African horse sickness virus and Schmallenberg virus (SBV) [7]. These viruses are responsible for outbreaks of non-contagious diseases in ruminants, causing huge economic losses, e.g. due to restrictions on animal trade [8].

The expansion of BTV from the Mediterranean basin to central Europe up to Scandinavia [9–11] prompted studies on *Culicoides* taxonomy [12–14], ecology [15–17] and vector competence [18–20]. In contrast, only few studies focused on the *Culicoides* fauna in southeastern Europe. Severe BTV outbreaks were observed between 2014 and 2015 in the Balkan Peninsula [21, 22]. In Romania, BTV was confirmed for the first time in 2014 [23]. The most comprehensive studies on the *Culicoides* fauna conducted in Romania date back to the end of the 20th century [24, 25]. More recent studies of the *Culicoides* fauna in Romania only focused on the known vectors of BTV. Thus, with the exception of *C. imicola* Kieffer, 1913 or *C. nubeculosus* (Meigen 1830) [26, 27], biting midges were recorded as species groups considered the most important vectors of BTV/SBV, i.e. *C. obsoletus* group and *C. pulicaris* group, or as "other *Culicoides*" [28, 29]. Currently, species-specific information on the distribution of other *Culicoides* taxa in Romania is missing.

The identification of blood sources from engorged vectors is a useful method to understand vector-host interactions and the ecology of associated pathogens [30, 31]. The host-feeding patterns of *Culicoides* have received much less attention compared to other vector groups (e.g. mosquitoes and ticks) [32, 33]. In Europe, most of the vertebrate hosts identified from engorged biting midges are ruminants [34–36]. However, other mammalian species such as humans and pigs can also be frequent [37–39]. In comparison, avian hosts are generally a more diverse, but less frequent group compared to mammals [34, 37, 38, 40]. Information about hosts of *Culicoides* species from eastern Europe was obtained by recent efforts undertaken in natural areas of Bulgaria [41] and Serbia [42]. In Serbia, blood-meal analysis predominantly detected ruminant hosts, whereas in Bulgaria, a large diversity of avian hosts was recorded for ornithophilic biting midges. To the best of our knowledge, such studies do not exist for Romania. Therefore, the aim of this study was to investigate the host-feeding patterns of *Culicoides* species collected from four sampling sites in the Danube Delta Biosphere Reserve (DDBR).

Methods

Trapping methods and study sites

Biting midges were collected at four sites in the DDBR as part of a pilot longitudinal arbovirus surveillance programme [43] (Fig. 1, Additional file 1: Text S1). The trapping site Letea is characterized by a semi-open enclosure for cattle and goats built of wood, reeds and rushes, located a short distance from a small canal and almost 1 km from a deciduous forest. In Sulina, the sampling site was a covered cow stable with two or three animals kept at night with a stagnant water body (canal) and a large dung heap in close proximity. The local host communities of both anthropogenic sites (Letea and Sulina) are predominantly characterized by cattle, horse, cat, poultry and humans accompanied by dogs. In contrast, the site at Dunărea Veche lays at the confluence of two branches of the Danube and adjacent small canals; a large crop field is bordered by these waters. The site Lake Roșuleț is an old fishery surrounded by a shallow, stagnant canal and rows of trees isolating the area from the surrounding marshland. Only few humans (farmers and fishermen) with dogs and cats are present in Dunărea Veche and Lake Roșuleț. The host community of both sites is predominantly characterized by a high diversity of wild mammals and birds.

Between May and August 2017, one Biogents Sentinel trap (BG trap; Biogents, Regensburg, Germany (http://www.biogents.com/)) equipped with an ultraviolet lamp was operated at each site for one night per week resulting in a total of 60 trap nights. The climate of the study area is continental with an annual mean temperature of 11 ℃ (− 1 ℃ in January and 22 ℃ in July) and around 350 mm of mean precipitation per year. Sampling in the present study was conducted during a hot and dry summer. A mean temperature of 21 ℃ and mean precipitation under 30 mm was recorded in the Danube Delta between May and August 2017 (http://www.meteoromania.ro/clima/monitorizare-climatica/).

Sample processing

Insects were frozen, shipped on dry ice and stored at − 80 ℃ in the laboratory. Due to the large amount of non-engorged and engorged *Culicoides*, only a random subsample of 1264 engorged specimens from all four sampling sites and every month of collection were selected. During the progress of sequencing, a dominance of cattle was observed for the sites Sulina and Letea. Therefore, we focused specifically on the engorged *Culicoides* from the sites Dunărea Veche and Lake Roșuleț, where a wider range of wildlife host blood meals were likely to be detected. Dry, frozen storage was preferred over ethanol storage to allow virus isolation and characterization at a later time. Biting midges were separated

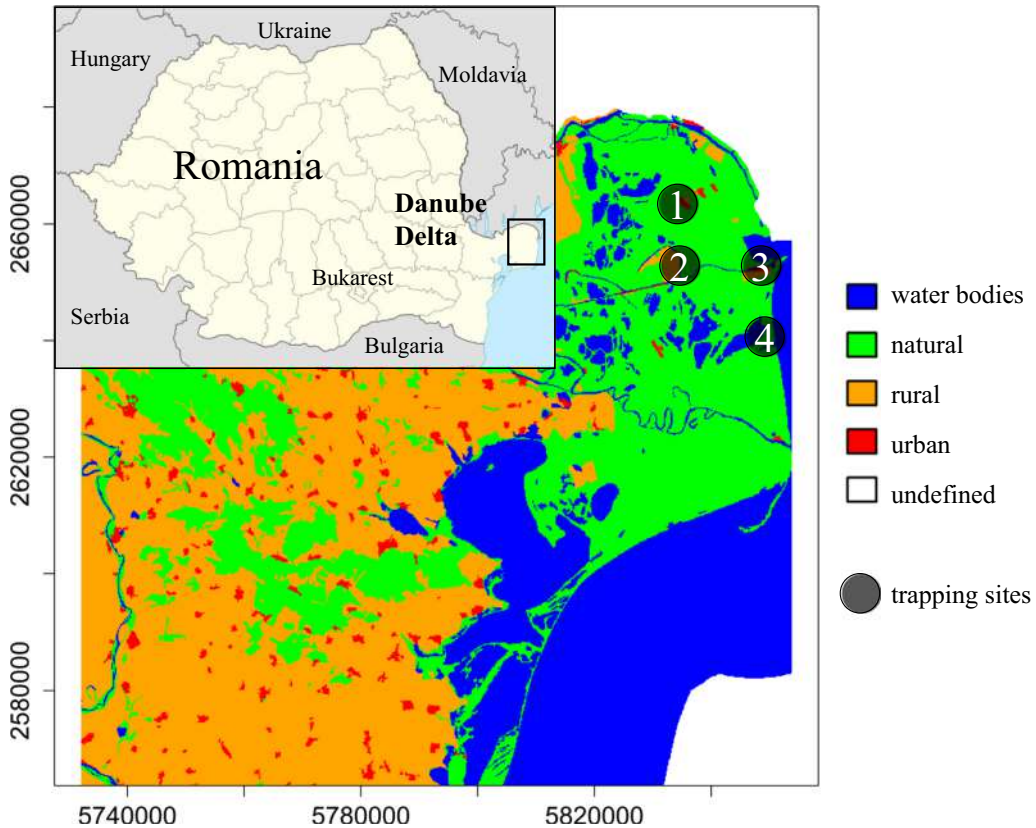


Fig. 1 Sampling sites (1: Letea; 2: Dunărea Veche; 3: Sulina; 4: Lake Roşuleţ) of *Culicoides* in the Danube Delta Biosphere Reserve (Romania) during the sampling period in 2017. Landcover variables are aggregated land cover data (Corine Land Cover (CLC) 2018 raster data, https://land.copernicus.eu/). CLC codes: water bodies, 511–523; natural, 311–423; rural, 211–244; urban, 111–142

by engorged status and wing patterns under a stereomicroscope (Olympus ZSX12, Tokyo, Japan). In addition, a small set of unfed specimens ($n=37$) from each sampling site (Sulina, $n=10$; Letea, $n=9$; Dunărea Veche, $n=10$; Lake Roşuleţ, $n=8$) were used for morphological identification, which were selected as morphologically representative for the different *Culicoides* species in the samples. Wings were mounted on slides in Euparal (Carl Roth, Karlsruhe, Germany) and species identified by morphology using the key of Mathieu et al. [14].

For DNA extraction, each specimen was placed into an individual sterile 2 ml tube (Eppendorf, Hamburg, Germany) with 5–9 zirconium beads (1 mm, Carl Roth) and 200 µl of Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) with 100 µg/ml streptomycin (PAN-Biotech, Aidenbach, Germany) and 2.5 µg/ml amphotericin B (PAN-Biotech). The samples were homogenised with a TissueLyser II (Qiagen, Hilden, Germany) twice for 3 min at 30 Hertz. The suspension was clarified by centrifugation at 8000× *rpm* for 2 min at 4 ℃. Total nucleic acid was extracted from 100 µl of supernatant, using the MagMAX™RNA/DNA Pathogen Kit with a KingFisher™ Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular identification of biting midges

A 658-bp fragment of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) was amplified PCR, using the primers HCO2198 and LCO1490 [44]. One microliter template was added to a 10 µl reaction mix, containing 6.6 µl of Hotstar Taq Master Mix (Qiagen), 2.2 µl of molecular grade water (included in the Master Mix kit) and 0.6 µl of each 10 µM primer. The following cycling program was used: initial denaturation at 95 ℃ for 15 min, followed by 40 cycles of 30 s denaturation at 94 ℃, 45 s annealing at 40 ℃ and 1 min extension at 72 ℃, and final extension step for 10 min at 72 ℃. Each PCR run included DNA of *Culex quinquefasciatus* Say, 1823 (positive control) and ultrapure water (negative control). All amplicons were visualised on 2% agarose gels and PCR products sequenced with LGC Genomics (Berlin, Germany).

Molecular identification of *Culicoides* hosts

Hosts were identified using two PCR protocols targeting the cytochrome *b* (*cytb*) and *16S* rRNA gene fragment [45–47]. Both protocols were described in detail in a previous study by Börstler et al. [32]. If the amplification with the first pair of primers failed [45, 46], another PCR was applied using the second pair of primers [47]. The same applied to potential mixed blood meals as indicated by double peaks at different positions in the sequence electropherograms. These samples were also analysed with both PCRs. As observed in our previous studies [32, 33], the PCR targeting the *cytb* gene fragment generally has a higher amplification rate for mammals, and the PCR targeting the *16S* rRNA gene fragment a higher amplification rate for birds. We used the DNA of a mammal (African green monkey, *Chlorocebus sabaeus* (L.)) and a bird (European blackbird, *Turdus merula* L.) as positive controls. The negative control was ultrapure water, which was included in each PCR run. These amplicons were also visualised and sequenced as described above.

Data analysis

Sequences were visualised and edited with Geneious version 9.1.7 (Biomatters, Auckland, New Zealand). The resulting sequences were submitted for species identification using the basic alignment search tool (BLAST) in the GenBank DNA sequence database (https://blast.ncbi.nlm.nih.gov/) and the Barcode of Life Database [48]. In order to rule out potential contamination, samples indicating human host DNA were repeated separately in an individual PCR reaction. Identity values for the *Culicoides* and host species generally ranged between 98 and 100%. Sequences with lower identity values were repeated. One exception was the newly described haplotype of *C. punctatus* (Meigen, 1804), which showed identity values between 96 and 97%. In addition, information on the fauna of the DDBR were used to interpret the sequences. For example, domestic pig has become quite a rarity in the study area (Additional file 1: Text S1). Therefore, these sequences were classified as wild boar, which is a common wild mammal in the DDBR.

To assess the phylogenetic relationship of *Culicoides* identified in the DDBR with other previously reported species in the Palaearctic, a maximum likelihood tree was constructed with MEGAX [49] with additional conspecific and outgroup sequences (*Forcipomyia* spp. and *Cx. quinquefasciatus*) from GenBank (Additional file 2: Table 1). The HKY + G model was identified as the best-fit model of nucleotide substitution by Jmodeltest 2.1.10 [50] based on calculations of Bayesian and Akaike's information criteria. Robustness of nodes was assessed by 1000 bootstrap replicates. The *Culicoides* spp. sequences generated in this study were deposited in the GenBank database under the accession numbers MN274523-274532 and MN340302-340312.

Results

Molecular identification of biting midges

Sequencing a fragment of the *cox*1 gene allowed the molecular identification of 1134 (89.7%) of the analysed 1264 engorged *Culicoides* (Table 1). Five species were identified for engorged biting midges: *C. griseidorsum* Kieffer, 1918; *C. kibunensis* Tokunaga, 1937; *C. punctatus*; *C. riethi* Kieffer 1914; and *C. submaritimus* Tokunaga & Murachi, 1959. *Culicoides subfasciipennis* Kieffer, 1919/*C. pallidicornis* Tokunaga & Murachi, 1959 were not differentiated to the species level. Furthermore, 39 sequences (3.1% of the analysed specimens) could not be identified to species level by comparison with other *Culicoides* sequences available on GenBank. The sequences of these specimens had a high similarity indicative of belonging to the same species and represent the seventh taxon hereafter referred to as "unknown *Culicoides*". The eighth taxon detected was *C. puncticollis* (Becker, 1903), only present in the non-engorged fed biting midges selected for morphological identification. Four of the seven detected engorged species were confirmed by morphology: *C. griseidorsum*; *C. kibunensis*; *C. riethi*; and *C. punctatus*. In contrast, engorged *C. submaritimus* and *C. subfasciipennis*/*C. pallidicornis* were identified solely by barcoding and were not found in the small set of unfed specimens. *Culicoides puncticollis* was identified by morphology and *cox*1 barcoding, but only from the same subset of 37 unfed specimens (Additional file 3: Figure S1). As the *cox*1 sequences are not suitable to differentiate between *C. subfasciipennis* and *C. pallidicornis* [51, 52], these specimens were classified as *C. subfasciipennis*/*C. pallidicornis*. The unknown *Culicoides* species had similar wing patterns to *C. kibunensis* (Fig. 2).

In order to perform a identity verification of the generated *Culicoides cox*1 sequences, we constructed a maximum likelihood phylogenetic tree including conspecific *Culicoides* and outgroup sequences (Fig. 3). A distinct haplotype of *C. punctatus* (designated as *C. punctatus* P) was identified in almost half ($n = 207$, 45.5%) of the 454 *C. punctatus* specimens analysed. These clustered within a separate monophyletic clade showing a genetic distance of approximately 4% to *C. punctatus* (Fig. 3). For the unknown *Culicoides* we could not find any similar sequences in the databases. This group of specimens showed a divergence of 15.6–16.3% from the closest identified *Culicoides* species (data not shown). The sequences of these specimens had a high similarity with each other and clustered with *C. kibunensis* in a monophyletic clade (Fig. 3).

Table 1 Frequency of detected hosts per *Culicoides* spp. with corresponding percentage collected in the Danube Delta Biosphere Reserve (Romania) during 2017

Host	*C. griseidorsum* *n* (%)	*C. kibunensis* *n* (%)	*C. punctatus* *n* (%)	*C. punctatus* P *n* (%)	*C. riethi* *n* (%)	*C. subfasciipennis/C. pallidicornis* *n* (%)	*C. submaritimus* *n* (%)	Unknown *Culicoides* sp. *n* (%)	Host information without *Culicoides* identification *n* (%)	Total *n* (%)
Mammals (*n* = 1064, 92%)										
Bos taurus L.	170 (63.9)	4 (5.2)	207 (85.9)	163 (81.1)	8 (80.0)	188 (83.2)	1 (11.1)	2 (11.1)	74 (68.5)	817 (70.7)
Bubalus bubalis (Kerr)			1 (0.4)			1 (0.4)				2 (0.2)
Canis lupus familiaris (L.)		4 (5.2)				3 (1.3)				7 (0.6)
Capra hircus L.	46 (17.3)	1 (1.3)	3 (1.2)	1 (0.5)	1 (10.0)	1 (0.4)				53 (4.6)
Capreolus capreolus (L.)		1 (1.3)								1 (0.1)
Equus caballus L.	15 (5.6)		9 (3.7)	3 (1.5)	1 (10.0)	4 (1.8)		1 (5.6)	5 (4.6)	38 (3.3)
Felis catus L.		1 (1.3)				1 (0.4)				2 (0.2)
Homo sapiens L.	3 (1.1)	10 (13.0)	5 (2.1)	4 (2.0)		4 (1.8)	5 (55.6)	2 (11.1)	10 (9.3)	43 (3.7)
Sus scrofa L.	28 (10.5)	3 (3.9)	16 (6.6)	29 (14.4)		22 (9.7)			3 (2.8)	101 (8.7)
Birds (*n* = 92, 8%)										
Acrocephalus arundinaceus (L.)		1 (1.3)				1 (0.4)				2 (0.2)
Acrocephalus scirpaceus (Hermann)		13 (16.9)								13 (1.1)
Ardea cinerea L.		1 (1.3)								1 (0.1)
Ardea purpurea L.		6 (7.8)							1 (0.9)	7 (0.6)
Columba palumbus L.		1 (1.3)						1 (5.6)		2 (0.2)
Coracias garrulus L.		2 (2.6)						3 (16.7)		5 (0.4)
Corvus corone L.	1 (0.4)	6 (7.8)					3 (33.3)		6 (5.6)	16 (1.4)
Cyanistes caeruleus (L.)		4 (5.2)		1 (0.5)				1 (5.6)		6 (0.5)
Emberiza schoeniclus (L.)									1 (0.9)	1 (0.1)
Falco tinnunculus L.								1 (5.6)	1 (0.9)	2 (0.2)
Gallinula chloropus (L.)		3 (3.9)								3 (0.3)
Gallus gallus (Gmelin)	2 (0.8)	2 (2.6)						2 (11.1)		6 (0.5)
Hirundo rustica L.								2 (11.1)		2 (0.2)

Table 1 (continued)

Host	*C. griseidorsum* *n* (%)	*C. kibunensis* *n* (%)	*C. punctatus* *n* (%)	*C. punctatus* P *n* (%)	*C. riethi* *n* (%)	*C. subfasciipennis/C. pallidicornis* *n* (%)	*C. submaritimus* *n* (%)	Unknown *Culicoides* sp. *n* (%)	Host information without *Culicoides* identification *n* (%)	Total *n* (%)
Meleagris gallopovo L.									1 (0.9)	1 (0.1)
Motacilla alba L.		1 (1.3)								1 (0.1)
Nycticorax nycticorax (L.)		1 (1.3)				1 (0.4)		1 (5.6)		3 (0.3)
Parus major L.		1 (1.3)							3 (2.8)	4 (0.3)
Passer montanus (L.)		1 (1.3)								1 (0.1)
Phalacrocorax carbo (L.)									2 (1.9)	2 (0.2)
Streptopelia decaocto (Frivaldszky)								2 (11.1)		2 (0.2)
Strix aluco L.		1 (1.3)								1 (0.1)
Sylvia borin (Boddaert)	1 (0.4)	4 (5.2)								5 (0.4)
Tito alba (Scopoli)		1 (1.3)								1 (0.1)
Asio otus (L.)		2 (2.6)								2 (0.2)
Tito alba/Asio otus		2 (2.6)							1 (0.9)	3 (0.3)
Culicoides specimens without host identification	11	26	7	6	2	19	1	21		
Total biting midge specimens	276[a]	102[b]	248	207	12	242[c]	8[d]	39[e]		

[a] Including one mixed blood meal: *Bos taurus* + *Gallus gallus*

[b] Including one mixed blood meal: *Sus scrofa* + *Homo sapiens*

[c] Including three mixed blood meals: *Bos taurus* + *Canis lupus familiaris*; *Sus scrofa* + *Acrocephalus arundinaceus*; *Bos taurus* + *Nycticorax nycticorax*

[d] Including two mixed blood meals: *Corvus corone* + *Homo sapiens*

[e] Including one mixed blood meal: *Equus caballus* + *Hirundo rustica*

Fig. 2 Two wing pictures for the unknown *Culicoides* species collected in the Danube Delta Biosphere Reserve (Romania) during 2017

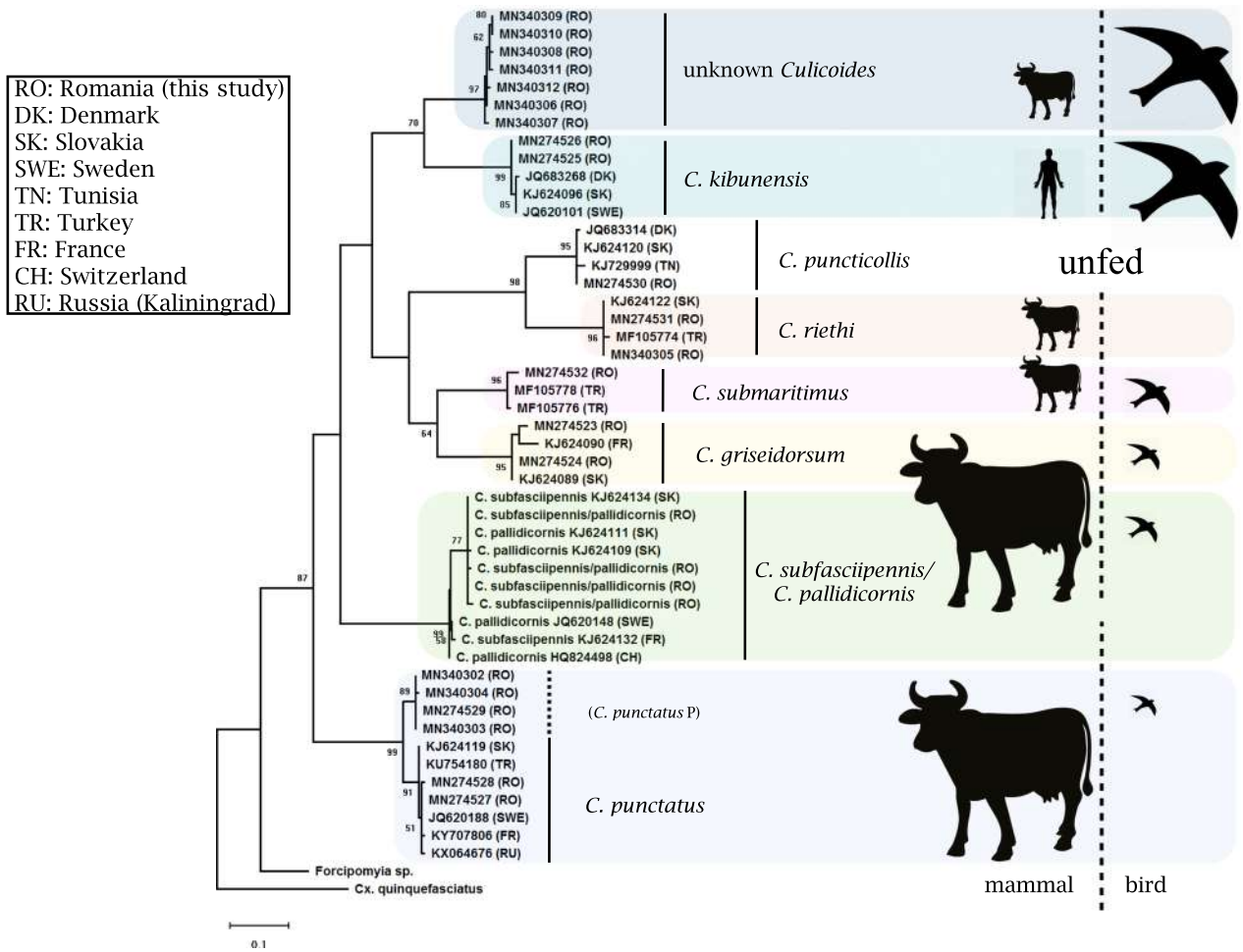


Fig. 3 Maximum likelihood phylogenetic tree of *cox*1 sequences for *Culicoides* species collected in the Danube Delta Biosphere Reserve (Romania) during 2017. Silhouettes indicate observed host-feeding patterns regarding the relative frequencies of mammalian and avian hosts. The tree was inferred using an HKY + G model (1000 bootstrap replicates) and rooted with *Forcipomyia* sp. and *Culex quinquefasciatus*. Branch support values of ≥ 50% are displayed and GenBank accession numbers of sequences shown on the branch tips

Culicoides punctatus ($n=455$, 36.0% of all analysed specimens), *C. griseidorsum* ($n=276$, 21.8%), *C. subfasciipennis*/*C. pallidicornis* ($n=242$, 19.1%) and *C. kibunensis* ($n=102$, 8.1%) were the most frequent taxa identified (Table 1, Additional file 4: Table S2). *Culicoides riethi* ($n=12$, 0.95%) was found in the traps set near livestock in the sites Sulina and Letea, while *Culicoides submaritimus* ($n=8$, 0.63%) was only found for Dunărea Veche and Lake Roșuleț, respectively.

Molecular identification of *Culicoides* hosts

Overlapping the two sets of sequences obtained for *Culicoides* identification and their hosts, information was available for 1040 (91.7%) of the 1134 molecular identified biting midges (Table 1). Blood-meal identification was not possible for 93 specimens due to failed PCR amplification. In addition, eight mixed blood meals were detected. With the exception of *C. punctatus* ($n=455$) and *C. riethi* ($n=12$), mixed blood meals where found for engorged specimens of all five *Culicoides* spp. Two *Culicoides* specimens contained blood from two mammalian hosts, while the other six specimens had mixed blood meals from a bird and a mammal.

A total of 33 vertebrate species were identified including nine species of mammals (27.3%) and 24 species of birds (72.7%) (Table 1). Mammals dominated the host spectrum ($n=1064$, 92.0% of all 1156 identified blood sources). Cattle (*Bos taurus*) was the most abundant species ($n=817$, 70.7%), followed by wild boar ($n=101$, 8.7%). Other mammalian hosts were each found at a rate below 5%. Birds amounted to 8% of all the identified hosts with the Eurasian reed warbler (*Acrocephalus scirpaceus*; $n=13$, 1.12%) and the carrion crow (*Corvus corone*; $n=16$, 1.38%) as most frequent.

With the exception of *C. riethi* ($n=12$), at least one avian host was detected for all *Culicoides* spp. Birds dominated the blood-meal sources of *C. kibunensis* and the unknown *Culicoides* sp. (68.8% and 72.2% of the detected hosts, respectively) (Table 1). *Culicoides kibunensis* had the highest diversity of hosts, with seven (77.8%) of the nine mammalian hosts and 18 (75%) of 24 species of avian hosts. Furthermore, humans were the most frequent mammalian host for this species ($n=10$, 13.0% of all identified hosts). In contrast, the three most frequent *Culicoides* spp. (*C. griseidorsum*, *C. punctatus* and *C. subfasciipennis*/*C. pallidicornis*) showed high proportions of cattle (between 63.9 and 85.9% of all identified blood sources per taxon). The second most frequent hosts were goat (*Capra hircus*) for *C. griseidorsum* (17.3%) and wild boar for *C. punctatus* (6.6%), *C. punctatus* P (14.4%) and *C. subfasciipennis*/*C. pallidicornis* (9.7%) (Table 1). No differences were observed between *C. punctatus* and its distinct haplotype *C. punctatus* P. Furthermore, for *C. submaritimus* ($n=8$) only blood meals from humans ($n=5$), carrion crows ($n=3$) and cattle ($n=1$) were detected.

Discussion

The relevance of *Culicoides* spp. as important vectors of pathogens is well known. Thus, information about their diversity and host-feeding patterns is crucial to understand parasite-host interactions and the ecology of associated pathogens [30]. DNA barcoding is an important tool in biodiversity studies [53–57]. Thereby, barcoding also helped to identify cryptic and new *Culicoides* species [58–60]. In this study, successful sequencing of 1040 engorged insects demonstrated that barcoding is a useful tool for both, *Culicoides* and host identification. However, it must be considered that the different genetic markers can have pitfalls and do not necessarily reflect morphological differences [56, 61], i.e. using a single marker might be insufficient for an accurate identification of species.

A total of seven *Culicoides* species-level taxa were detected for the four sites in the DDBR. In the phylogenetic tree, specimens of the same taxon clustered in well-supported terminal clades. The only exception was *C. subfasciipennis*/*C. pallidicornis*. The separation between these two species is based on a variable light spot on the wing's anal cell of *C. subfasciipennis* [14]. However, the analysis indicated no sequence differences of the *cox*1 gene. The discriminatory characters on the wing might be unreliable and further studies are required to clarify the status of both species [51, 52].

Culicoides griseidorsum, *C. puncticollis* and *C. submaritimus* were recorded for the first time in Romania, increasing the number of known *Culicoides* species for the country to 49 species [25]. *Culicoides submaritimus* has been considered a synonym of *C. maritimus* Tokunaga, 1940 by some authors [62, 63], while recent studies treated *C. submaritimus* as a distinct species [14, 64]. In the present study, *C. submaritimus* was identified by its similarity with *cox*1 sequences from Turkey, which are the only sequences available on GenBank for this species, while no *cox*1 sequences were available for *C. maritimus*. Neither *C. submaritimus*, nor *C. maritimus* are included in the inventory of *Culicoides* biting midges of Romania [25], although more recent studies include the country in the distribution of *C. maritimus* [14, 65].

The observed genetic variation for the analysed *C. punctatus* in two distinct clades is within intraspecific boundaries [59]. Such sibling species may vary in their vectorial capacity [66], e.g. vector competence

or host-feeding patterns of members in the *Anopheles gambiae* complex. However, we did not detect differences in the host-feeding patterns between either taxa. Furthermore, the specimens clustering within the clade designated as "unknown *Culicoides*" showed genetic distances of 15.6–16.3% from the closest described species. These distances are similar to those observed between the other *Culicoides* species in our study. Comparable distances were found in other *Culicoides* spp. [67, 68] or mosquitoes [69], indicating that these specimens belong to a separate new species or a species without reference sequences in molecular libraries.

The overall host spectrum covered species expected for the DDBR, including livestock species like buffalo (*Bubalis bubalis*). Therefore, most of the analysed *Culicoides* spp. had a broad host-feeding range. Only mammalian hosts were detected for *C. riethi*, but the small sample size of only 12 engorged specimens does not allow an accurate conclusion on the species' host-feeding pattern. Both, mammalian and avian hosts were detected for all other biting midge taxa to various extents. The broad host choice matches previous studies, which find similar results for different *Culicoides* spp. [70, 71]. Humans and carrion crow were the only hosts of *C. submaritimus* ($n = 8$). Cattle, wild boar or goat dominated the hosts of the three most frequent *Culicoides* taxa (*C. punctatus*, *C. subfasciipennis*/*C. pallidicornis* and *C. griseidorsum*). The high frequency of cattle probably relates to the large number of free-roaming cattle available in the DDBR and their large body mass [72]. However, as observed before [41, 67, 73, 74], despite this distinct dominance of mammalian hosts, different avian hosts were detected for the three *Culicoides* taxa.

Culicoides kibunensis is considered predominantly ornithophilic [37, 38, 75, 76]. With 18 species of birds and seven species of mammals, this vector of avian malaria [37, 38] showed the highest overall host diversity. The wide range of bird species is not surprising, considering the diversity of this vertebrate group in the DDBR. Nevertheless, the observed generalist host-feeding pattern including humans match previous studies [34, 37, 38]. Interestingly, the unknown *Culicoides* species showed a similar host-feeding pattern as *C. kibunensis*, with which it formed a monophyletic clade in the phylogenetic tree. These observations support the hypothesis of a positive correlation between biting midge phylogenetic relatedness and their feeding behaviour [40, 77]. In contrast, other studies speculated that such similarities in host-feeding patterns are not necessarily driven by phylogenetic relatedness, but might be the result of other factors (e.g. body size-driven host choice due to larger emissions of CO_2 or volatile compounds) [71].

Host availability probably has a significant impact on the observed host-feeding patterns of *Culicoides* spp. Although no quantitative information on the host community is available, the prevalence of humans and domestic animals at Dunărea Veche and Lake Roșuleț is known. Humans, dogs and cats had relative low abundance at both sites compared to birds or free-ranging cattle and horses. Nevertheless, humans, dogs or cats were detected as hosts for all analysed *Culicoides* species. Thus, caution regarding the distribution of biting midges and the potential host has to be considered when interpreting host-feeding patterns of *Culicoides*. For example, a high proportion of *C. griseidorsum* were found to have fed on goats, but this host was widely available at Letea, where most of this species were collected (Additional file 4: Table S2, Additional file 5: Table S3).

Information on the host-feeding patterns can be also used to estimate dispersal distances of *Culicoides* spp. [77]. Biting midges from the sampling site Dunărea Veche were engorged with blood from buffalo and goat. These hosts are only available in the nearest village more than 4 km from the trapping site, which is in the range of a previous study on *Culicoides* [78]. Maximum dispersal distances of more than 3 km over one night were recorded regularly. Winds over the delta's flat landscape might favour passive dispersal [79–82]. Thereby, besides active midge movement, wind dispersal is considered an important mode of long-distance dispersal for *Culicoides*-borne pathogens [83–85].

Conclusions

The broad host range of different mammalian and avian species indicates that most of the analysed *Culicoides* species in the DDBR are potential bridge vectors. However, the actual vector competence of these species is largely unknown. Of the dominant *Culicoides* species analysed, *C. punctatus* was previously indicated as a potential vector of BTV and SBV [86, 87]. Free roaming cattle, the most abundant and most frequently detected hosts in the region, could have an important role in amplification and spread of pathogens between wild ruminants and livestock [88]. At the same time, the new records of biting midge taxa for the country presented here and the detection of a potentially unknown *Culicoides* taxon highlight the lack of knowledge regarding the biting midge species and their genetic diversity in Europe.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-3938-1.

Additional file 1: Text S1. Description of the sampling sites with information on vegetation, surrounding environment and available hosts.

Additional file 2: Table S1. Accession numbers of *Culicoides* spp., *Forcipomyia* spp. and *Culex quinquefasciatus* used for phylogenetic analysis.

Additional file 3: Figure S1. Wing patterns for *C. punctatus*, *C. punctatus* P, *C. kibunensis*, *C. puncticollis*, *C. riethi* and *C. griseidorsum* collected in this study.

Additional file 4: Table S2. Overview of the *Culicoides* species per sampling site.

Additional file 5: Table S3. Overview of the frequency of each molecularly identified *Culicoides* host species per sampling site.

Abbreviations

DDBR: Danube delta biosphere reserve; DNA: Deoxyribonucleic acid; BTV: Bluetongue virus; SBV: Schmallenberg virus; BG trap: Biogents sentinel trap; PCR: Polymerase chain reaction.

Acknowledgements

We would like to thank to Vasile Suhov and the residents of Sulina and Letea, who granted access to their properties. In addition, we are very grateful to Dr Andreas Krüger for his support and fruitful discussion. In addition, we thank Patricia Iftene for help during the field work, Daniel Truchado for his comments on the manuscript and to Daniel Ciobanu for help with literature research.

Authors' contributions

AT, HJ, MS, JSC, DC and RL conceived and designed the study. AT, HJ and JS collected the data. AT, HJ, JSC, DC and RL analyzed the data. AT, DC and RL drafted the manuscript. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The data supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate

The DDBR administration approved all research activities for trapping at specific study sites (9/19.04.2017, 5627/ARBDD/13.04.2017).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] WHO Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. [2] University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania. [3] Faculty of Mathematics, Informatics and Natural Sciences, Universität Hamburg, Hamburg, Germany.

Received: 24 September 2019 Accepted: 3 February 2020
Published online: 17 February 2020

References

1. Linley JR. Biting midges as vectors of nonviral animal pathogens. J Med Entomol. 1985;22:589–99.
2. Chagas CRF, Bukauskaite D, Ilgunas M, Iezhova T, Valkiunas G. A new blood parasite of leaf warblers: molecular characterization, phylogenetic relationships, description and identification of vectors. Parasit Vectors. 2018;11:538.
3. Svobodová M, Dolnik OV, Ivan Č, Rádrová J. Biting midges (Ceratopogonidae) as vectors of avian trypanosomes. Parasit Vectors. 2017;10:224.
4. Debrah LB, Nausch N, Opoku VS, Owusu W, Mubarik Y, Berko DA, et al. Epidemiology of *Mansonella perstans* in the middle belt of Ghana. Parasit Vectors. 2017;10:15.
5. Borkent A. The biting midges, the *Ceratopogonidae* (Diptera). In: Marquardt WC, editor. Biology of disease vectors. 2nd ed. Burlington MA: Elsevier Academic Press; 2005. p. 113–26.
6. da Rosa JF, de Souza WM, de Paula Pinheiro F, Figueiredo ML, Cardoso JF, Acrani GO, et al. Oropouche virus: clinical, epidemiological, and molecular aspects of a neglected orthobunyavirus. Am J Trop Med Hyg. 2017;96:1019–30.
7. Sick F, Beer M, Kampen H, Wernike K. *Culicoides* biting midges—underestimated vectors for arboviruses of public health and veterinary importance. Viruses. 2019;11:376.
8. Pinior B, Firth CL, Loitsch A, Stockreiter S, Hutter S, Richter V, et al. Cost distribution of bluetongue surveillance and vaccination programmes in Austria and Switzerland (2007–2016). Vet Rec. 2018;182:257.
9. Maan S, Maan NS, Ross-smith N, Batten CA, Shaw AE, Anthony SJ, et al. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology. 2008;377:308–18.
10. Foxi C, Delrio G, Falchi G, Marche MG, Satta G, Ruiu L. Role of different *Culicoides* vectors (Diptera: Ceratopogonidae) in bluetongue virus transmission and overwintering in Sardinia (Italy). Parasit Vectors. 2016;9:440.
11. Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PPC, Baylis M. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol. 2005;3:171–81.
12. Nolan DV, Carpenter S, Barber J, Mellor PS, Dallas JF, Mordue AJ, et al. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. Vet Microbiol. 2007;124:82–94.
13. Augot D, Sauvage F, Jouet D, Simphal E, Veuille M, Couloux A, et al. Discrimination of *Culicoides obsoletus* and *Culicoides scoticus*, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis. Infect Genet Evol. 2010;10:629–37.
14. Mathieu B, Cêtre-Sossah C, Garros C, Chavernac D, Balenghien T, Carpenter S, et al. Development and validation of IIKC: an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic region. Parasit Vectors. 2012;5:137.
15. Cuéllar AC, Kjær LJ, Kirkeby C, Skovgard H, Nielsen SA, Stockmarr A, et al. Spatial and temporal variation in the abundance of *Culicoides* biting midges (Diptera: Ceratopogonidae) in nine European countries. Parasit Vectors. 2018;11:112.
16. Lühken R, Kiel E, Steinke S, Fladung R. Topsoil conditions correlate with the emergence rates of *Culicoides chiopterus* and *Culicoides dewulfi* (Diptera: Ceratopogonidae) from cowpats. Parasitol Res. 2015;114:1113–7.
17. Garros C, Viennet E, Rossi S, Balenghien T. Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of orbiviruses. Infect Genet Evol. 2011;11:1103–10.
18. Carpenter S, Veronesi E, Mullens B, Venter G. Vector competence of *Culicoides* for arboviruses: three major periods of research, their influence on current studies and future directions. Rev Sci Tech. 2015;34:97–112.
19. Barber J, Harrup LE, Silk R, Veronesi E, Gubbins S, Bachanek-Bankowska K, et al. Blood-feeding, susceptibility to infection with Schmallenberg virus and phylogenetics of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. Parasit Vectors. 2018;11:116.
20. Pagès N, Talavera S, Verdún M, Pujol N, Valle M, Bensaid A, et al. Schmallenberg virus detection in *Culicoides* biting midges in Spain: first laboratory evidence for highly efficient infection of *Culicoides* of the obsoletus complex and *Culicoides imicola*. Transbound Emerg Dis. 2018;65:e1–6.
21. Kyriakis CS, Billinis C, Papadopoulos E, Vasileiou NGC, Athanasiou LV, Fthenakis GC. Bluetongue in small ruminants: an opinionated review, with a brief appraisal of the 2014 outbreak of the disease in Greece and the south-east Europe. Vet Microbiol. 2015;181:66–74.

22. Stojmanovski Z, Tabakovski B. Spatio-temporal characteristics of the bluetongue epizooty in the Balkan Peninsula from 2014 to february 2015. Maced Vet Rev. 2018;41:65–72.
23. Tilibașa EM, Popescu D, Badea C, Hora FȘ, Dărăbuș G. A report regarding the first occurrence of bluetongue in Romania, 2014. Scientific Works. Series C. Vet Med. 2015;61:277–80.
24. Damian-Georgescu A. New species of Ceratopogonidae (Diptera) for the Romanian fauna. Studii si Cercetari de Biologie, Seria Zoologie. 1972;24:423–32.
25. Damian-Georgescu A. Familia Ceratopogonidae, Genul *Culicoides*. Fauna României, insecta, Fasc 14 diptera, vol. 11. Bukuresti: Editura Academiei Romane; 2000.
26. Tilibașa EM, Dărăbuș G. Preliminary studies on dynamics of *Culicoides* spp. in western Romania in conjunction with some environmental factors. Parasit Vectors. 2014;7(Suppl. 1):O7.
27. Dărăbuș G, Tilibașa E, Oprescu I, Morariu S, Mederle N, Ilie M, Sujic T, Imre M. The abundance of *Culicoides* (Diptera: Ceratopogonidae) in Timiș county. Scient Works Med Vet. 2017;50:1689–99.
28. Ioniță M, Mitrea IL, Buzatu MC, Dascălu L, Ionescu A. Seasonal dynamics of haematophagous arthropod populations (ticks and *Culicoides* spp.)—vectors of pathogens in animals and humans, in different areas of Romania. Scient Works Med Vet. 2009;52:629–36.
29. Ilie A, Șerban C, Imre M, Sorescu D, Ilie M, Imre K, et al. A survey (or presences, dynamics, prevalences) of *Culicoides* (Diptera: Ceratopogonidae) in Gorj county, Romania preliminary results of entomological surveillance for bluetongue. Scient Works Med Vet. 2013;46:5–9.
30. Kent RJ. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. Mol Ecol Resour. 2009;9:4–18.
31. Bitome-Essono PY, Ollomo B, Arnathau C, Durand P, Mokoudoum ND, Yacka-Mouele L, et al. Tracking zoonotic pathogens using blood-sucking flies as 'flying syringes'. Elife. 2017;6:e22069.
32. Börstler J, Jöst H, Garms R, Krüger A, Tannich E, Becker N, et al. Host-feeding patterns of mosquito species in Germany. Parasit Vectors. 2016;9:318.
33. Shahhosseini N, Friedrich J, Moosa-Kazemi SH, Sedaghat MM, Kayedi MH, Tannich E, et al. Host-feeding patterns of *Culex* mosquitoes in Iran. Parasit Vectors. 2018;11:669.
34. Lassen SB, Nielsen SA, Kristensen M. Identity and diversity of blood meal hosts of biting midges in Denmark. Parasit Vectors. 2012;5:143.
35. Hadj-Henni L, De Meulemeester T, Depaquit J, Noël P, Germain A, Helder R, et al. Comparison of vertebrate cytochrome b and prepronociceptin for blood meal analyses in *Culicoides*. Front Vet Sci. 2015;2:15.
36. Talavera S, Muñoz-Muñoz F, Verdun M, Pujol N, Pagès N. Revealing potential bridge vectors for BTV and SBV: a study on *Culicoides* blood feeding preferences in natural ecosystems in Spain. Med Vet Entomol. 2017;32:35–40.
37. Santiago-Alarcon D, Havelka P, Schaefer HM, Segelbacher G. Bloodmeal analysis reveals avian *Plasmodium* infections and broad host preferences of *Culicoides* (Diptera: Ceratopogonidae) Vectors. PLoS ONE. 2012. https://doi.org/10.1371/journal.pone.0031098.
38. Santiago-Alarcon D, Havelka P, Pineda E, Segelbacher G, Schaefer HM. Urban forests as hubs for novel zoonosis: blood meal analysis, seasonal variation in *Culicoides* (Diptera: Ceratopogonidae) vectors, and avian haemosporidians. Parasitology. 2013;140:1799–810.
39. Ninio C, Augot D, Delecolle JC, Dufour B, Depaquit J. Contribution to the knowledge of *Culicoides* (Diptera: Ceratopogonidae) host preferences in France. Parasitol Res. 2011;108:657–63.
40. Martínez-de la Puente J, Figuerola J, Soriguer R. Fur or feather? Feeding preferences of species of *Culicoides* biting midges in Europe. Trends Parasitol. 2015;31:16–22.
41. Bobeva A, Zehtindjiev P, Ilieva M, Dimitrov D, Mathis A, Bensch S. Host preferences of ornithophilic biting midges of the genus *Culicoides* in the eastern Balkans. Med Vet Entomol. 2015;29:290–6.
42. Vasic A, Zdravkovi N, Bojkovski J, Marinov M, Mathis A, Niculaua M, et al. Species diversity, host preference and arbovirus detection of *Culicoides* (Diptera: Ceratopogonidae) in south-eastern Serbia. Parasit Vectors. 2019;12:61.
43. Török E, Tomazatos A, Cadar D, Horváth C, Keresztes L, Jansen S, et al. Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of *Anopheles algeriensis* theobald, 1903 and *Aedes hungaricus* Mihályi, 1955 for Romania. Parasit Vectors. 2016;9:196.
44. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.
45. Burkett-Cadena ND, Graham SP, Hassan HK, Guyer C, Eubanks MD, Katholi CR, et al. Blood feeding patterns of potential arbovirus vectors of the genus *Culex* targeting ectothermic hosts. Am J Trop Med Hyg. 2008;79:809–15.
46. Kitano T, Umetsu K, Tian W, Osawa M. Two universal primer sets for species identification among vertebrates. Int J Legal Med. 2007;121:423–7.
47. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci. 1989;86:6196–200.
48. Ratnasingham S, Hebert PDN. The barcode of life data system (www.barcodinglife.org). Mol Ecol Notes. 2007; 7: 355–64.
49. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9.
50. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772.
51. Sarvašová A, Delecolle JC, Halan M, Mathieu B, Kočišová A. Morphological and molecular analysis of the genus *Culicoides* (Diptera: Ceratopogonidae) in Slovakia with five new records. Zootaxa. 2014;3872:541–60.
52. Filatov S, Szadziewski R. Annotated checklist and distribution of *Culicoides* biting midges of Ukraine (Diptera: Ceratopogonidae). J Nat Hist. 2017;51:487–511.
53. Pires AC, Marinoni L. DNA barcoding and traditional taxonomy unified through integrative taxonomy: a view that challenges the debate questioning both methodologies. Biota Neotrop. 2010;10:339–46.
54. Hebert PDN, Cywinska A, Ball SL, DeWaard JR. Biological identifications through DNA barcodes. Proc R Soc B Biol Sci. 2003;270:313–21.
55. Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 2007;23:167–72.
56. Hernández-Triana LM, Brugman VA, Nikolova NI, Ruiz-Arrondo I, Barrero E, Thorne L, et al. DNA barcoding of British mosquitoes (Diptera, Culicidae) to support species identification, discovery of cryptic genetic diversity and monitoring invasive species. ZooKeys. 2019;832:57–76.
57. Whitworth TL, Dawson RD, Magalon H, Baudry E. DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). Proc R Soc B Biol Sci. 2007;274:1731–9.
58. Pagès N, Muñoz-Muñoz F, Talavera S, Sarto V, Lorca C, Núñez JI. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. Vet Parasitol. 2009;165:298–310.
59. Nielsen SA, Kristensen M. Delineation of *Culicoides* species by morphology and barcode exemplified by three new species of the subgenus *Culicoides* (Diptera: Ceratopogonidae) from Scandinavia. Parasit Vectors. 2015;8:151.
60. Sarvašová A, Kočišová A, Candolfi E, Mathieu B. Description of *Culicoides* (*Culicoides*) *bysta* n. sp., a new member of the Pulicaris group (Diptera: Ceratopogonidae) from Slovakia. Parasit Vectors. 2017;10:279.
61. Beebe NW. DNA barcoding mosquitoes: advice for potential prospectors. Parasitology. 2018;145:622–33.
62. Dik B, Yağci Ş, Linton YM. A review of species diversity and distribution of *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) in Turkey. J Nat Hist. 2006;40:1947–67.
63. Rawlings P. A key based on wing patterns of biting midges (genus *Culicoides* Latreille—Diptera: Ceratopogonidae) in the Iberian Peninsula, for use in epidemiological studies. Graellsia. 1996;52:57–71.
64. Talavera S, Muñoz-Muñoz F, Pagès N. New insights on diversity, morphology and distribution of *Culicoides* Latreille 1809 (Diptera: Ceratopogonidae) from northeast Spain. Ann Soc Entomol Fr. 2011;47:214–31.
65. Szadziewski R, Borkent A, Dominiak P. Fauna Europaea: Ceratopogonidae. In: Beuk P, Pape T, ed. Fauna Europaea: Diptera, Nematocera. Fauna Europaea version 2.6; 2013. https://fauna-eu.org/cdm_dataportal/taxon/7161829a-94f3-4c36-943d-0d15f8fa1f77. Accessed 15 Aug 2019.
66. White BJ, Collins FH, Besansky NJ. Evolution of *Anopheles gambiae* in relation to humans and malaria. Annu Rev Ecol Evol S. 2011;42:111–32.

67. Pettersson E, Bensch S, Ander M, Chirico J, Sigvald R, Ignell R. Molecular identification of bloodmeals and species composition in *Culicoides* biting midges. Med Vet Entomol. 2013;27:104–12.
68. Ander M, Troell K, Chirico J. Barcoding of biting midges in the genus *Culicoides*: a tool for species determination. Med Vet Entomol. 2013;27:323–31.
69. Chan A, Chiang LP, Hapuarachchi HC, Tan CH, Pang SC, Lee R, et al. DNA barcoding: complementing morphological identification of mosquito species in Singapore. Parasit Vectors. 2014;7:569.
70. Tempelis CH, Nelson RL. Blood-feeding patterns of midges of the *Culicoides variipennis* complex in Kern county, California. J Med Entomol. 1971;8:532–4.
71. Hopken MW, Ryan BM, Huyvaert KP, Piaggio AJ. Picky eaters are rare: DNA-based blood meal analysis of *Culicoides* (Diptera: Ceratopogonidae) species from the United States. Parasit Vectors. 2017;10:169.
72. Viennet E, Garros C, Gardès L, Rakotoarivony I, Allène X, Lancelot R, et al. Host preferences of palaearctic *Culicoides* biting midges: implications for transmission of orbiviruses. Med Vet Entomol. 2013;27:255–66.
73. Lassen SB, Nielsen SA, Skovgård H, Kristensen M. Molecular identification of bloodmeals from biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) in Denmark. Parasitol Res. 2011;108:823–9.
74. Calvo JH, Berzal B, Calvete C, Miranda MA, Estrada R, Lucientes J. Host feeding patterns of *Culicoides* species (Diptera: Ceratopogonidae) within the Picos de Europa National Park in northern Spain. Bull Entomol Res. 2012;102:692–7.
75. Černý O, Votýpka J, Svobodová M. Spatial feeding preferences of ornithophilic mosquitoes, blackflies and biting midges. Med Vet Entomol. 2011;25:104–8.
76. Martinez-de La Puente J, Merino S, Toms G, Moreno J, Morales J, Lobato E, et al. Factors affecting *Culicoides* species composition and abundance in avian nests. Parasitology. 2009;136:1033–41.
77. Augot D, Hadj-Henni L, Strutz SE, Slama D, Millot C, Depaquit J, et al. Association between host species choice and morphological characters of main sensory structures of *Culicoides* in the Palaeartic region. PeerJ. 2017;5:e3478.
78. Sanders CJ, Harrup LE, Tugwell LA, Brugman VA, England M, Carpenter S. Quantification of within- and between-farm dispersal of *Culicoides* biting midges using an immunomarking technique. J Appl Ecol. 2017;54:1429–39.
79. Greenberg JA, DiMenna MA, Hanelt B, Hofkin BV. Analysis of post-blood meal flight distances in mosquitoes utilizing zoo animal blood meals. J Vector Ecol. 2012;37:83–9.
80. Sanders CJ, Selby R, Carpenter S, Reynolds DR. High-altitude flight of *Culicoides* biting midges. Vet Rec. 2011;169:208.
81. Johansen CA, Farrow RA, Morrisen A, Foley P, Bellis G, Van Den Hurk AF, et al. Collection of wind-borne haematophagous insects in the Torres Strait, Australia. Med Vet Entomol. 2003;17:102–9.
82. Kluiters G, Swales H, Baylis M. Local dispersal of palaearctic *Culicoides* biting midges estimated by mark-release-recapture. Parasit Vectors. 2015;8:86.
83. Ducheyne E, De Deken R, Bécu S, Codina B, Nomikou K, Mangana-Vougiaki O, et al. Quantifying the wind dispersal of *Culicoides* species in Greece and Bulgaria. Geospat Health. 2007;1:177.
84. Sedda L, Brown HE, Purse BV, Burgin L, Gloster J, Rogers DJ. A new algorithm quantifies the roles of wind and midge flight activity in the bluetongue epizootic in northwest Europe. Proc R Soc B. 2012;279:2354–62.
85. Burgin LE, Gloster J, Sanders C, Mellor PS, Gubbins S, Carpenter S. Investigating incursions of bluetongue virus using a model of long-distance *Culicoides* biting midge dispersal: investigating bluetongue virus incursions. Transbound Emerg Dis. 2013;60:263–72.
86. Goffredo M, Catalani M, Federici V, Portanti O, Marini V, Mancini G, et al. Specie di *Culicoides* coinvolte nell'epidemia di Bluetongue 2012–2014 in Italia. Vet Ital. 2015;51:131–8.
87. Larska M, Lechowski L, Grochowska M, Zmudziński JF. Detection of the Schmallenberg virus in nulliparous *Culicoides obsoletus/scoticus* complex and *C punctatus*-the possibility of transovarial virus transmission in the midge population and of a new vector. Vet Microbiol. 2013;166:467–73.
88. Conraths FJ, Gethmann JM, Staubach C, Mettenleiter TC, Beer M, Hoffmann B. Epidemiology of bluetongue virus serotype 8, Germany. Emerg Infect Dis. 2009;15:433–5.

Publisher's Note

viruses

MDPI

Article

Letea Virus: Comparative Genomics and Phylogenetic Analysis of a Novel Reassortant Orbivirus Discovered in Grass Snakes (*Natrix natrix*)

Alexandru Tomazatos [1], Rachel E. Marschang [2], Iulia Maranda [1], Heike Baum [1], Alexandra Bialonski [1], Marina Spînu [3], Renke Lühken [1,4], Jonas Schmidt-Chanasit [1,4] and Daniel Cadar [1,*]

1 Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, 20359 Hamburg, Germany; alex_tomazatos@yahoo.com (A.T.); maranda.iulia@gmail.com (I.M.); baum@bnitm.de (H.B.); bialonski@bnitm.de (A.B.); renkeluhken@gmail.com (R.L.); jonassi@gmx.de (J.S.-C.)

2 Cell Culture Lab, Microbiology Department, Laboklin GmbH & Co. KG, 97688 Bad Kissingen, Germany; rachel.marschang@googlemail.com

3 Department of Clinical Sciences-Infectious Diseases, University of Agricultural Sciences and Veterinary Medicine, 400372 Cluj-Napoca, Romania; marina.spinu@gmail.com

4 Faculty of Mathematics, Informatics and Natural Sciences, Universität Hamburg, 20148 Hamburg, Germany

* Correspondence: danielcadar@gmail.com

Received: 17 January 2020; Accepted: 20 February 2020; Published: 21 February 2020

Abstract: The discovery and characterization of novel arthropod-borne viruses provide valuable information on their genetic diversity, ecology, evolution and potential to threaten animal or public health. Arbovirus surveillance is not conducted regularly in Romania, being particularly very scarce in the remote and diverse areas like the Danube Delta. Here we describe the detection and genetic characterization of a novel orbivirus (*Reoviridae*: *Orbivirus*) designated as Letea virus, which was found in grass snakes (*Natrix natrix*) during a metagenomic and metatranscriptomic survey conducted between 2014 and 2017. This virus is the first orbivirus discovered in reptiles. Phylogenetic analyses placed Letea virus as a highly divergent species in the *Culicoides*-/sand fly-borne orbivirus clade. Gene reassortment and intragenic recombination were detected in the majority of the nine Letea virus strains obtained, implying that these mechanisms play important roles in the evolution and diversification of the virus. However, the screening of arthropods, including *Culicoides* biting midges collected within the same surveillance program, tested negative for Letea virus infection and could not confirm the arthropod vector of the virus. The study provided complete genome sequences for nine Letea virus strains and new information about orbivirus diversity, host range, ecology and evolution. The phylogenetic associations warrant further screening of arthropods, as well as sustained surveillance efforts for elucidation of Letea virus natural cycle and possible implications for animal and human health.

Keywords: arbovirus; reptile; orbivirus; Danube Delta; metatranscriptomics; surveillance

1. Introduction

The *Reoviridae* family is a large and diverse group of nonenveloped, icosahedral viruses with genomes composed of 9–12 linear molecules of double-stranded RNA (dsRNA). Reoviruses are divided between the *Spinareovirinae* subfamily (species with turrets on the core particle) and *Sedoreovirinae* subfamily (species with smooth, nonturreted core particles). They infect numerous host species, from plants to crustaceans, insects, aquatic and terrestrial vertebrates [1]. Among the 16 *Reoviridae*

genera, the *Orbivirus* genus (subfamily: *Sedoreovirinae*) is the largest, having 22 species recognized by the International Committee on Taxonomy of Viruses (ICTV) and a significant number of species proposals [2]. Orbiviruses are vector-borne pathogens, primarily transmitted by ticks and other hematophagous insects (mosquitoes, *Culicoides* biting midges and sand flies). Their wide host range includes wild and domestic ruminants, camelids, equids, humans, marsupials, bats, sloths and birds [1]. The most studied orbiviruses are the *Culicoides*-borne *Bluetongue virus* (BTV, type species), *African horse sickness virus* (AHSV) and *Epizootic hemorrhagic disease virus* (EHDV), all known as important pathogens of livestock and wildlife [3]. Some orbiviruses such as Tribeč virus, Kemerovo, Lebombo and Orungo viruses have been detected in human infections and are considered human pathogens [4].

Orbiviral genomes consist of 10 linear segments of dsRNA designated by their decreasing molecular weight. They encode seven structural proteins (VP1–VP7) and three to four nonstructural proteins (NS1, NS2, NS3/NS3a and NS4) [1]. The high conservation degree of certain structural core proteins (e.g., polymerase, major core and subcore proteins) recommends them for comparative and phylogenetic analyses of different *Orbivirus* species [5,6]. In contrast, the proteins of the outer capsid are highly variable and their specificity to the host's neutralizing antibody response can be used to distinguish between different serotypes of the same orbivirus species [7,8]. The phylogenetic clustering of *Orbivirus* members results in clades indicating their putative or potential arthropod vectors: *Culicoides*- or sand fly-borne (C/SBOV), mosquito-borne (MBOV) and tick-borne orbiviruses (TBOV) [9]. One exception to this classification is *St. Croix River virus* (SCRV), a distant member of the genus considered to be a "tick orbivirus" (TOV), having no known vector [5].

As one of Europe's largest wetlands, the Danube Delta Biosphere Reserve (DDBR) located in the southeast of Romania, is a very biodiverse and heterogeneous complex of ecosystems [10]. The region is a major hub for bird migration along main African–Eurasian fly corridors, with ecoclimatic conditions suitable for abundant and diverse populations of arthropod vectors [11–14], which may allow pathogen import and maintenance [15–18].

During an arbovirus survey in DDBR, we identified a novel orbivirus in grass snakes (*Natrix natrix* Linnaeus 1758), tentatively named *Letea virus* (LEAV) after the eponymous village from the study area. The aims of this study were to characterize the genome of LEAV and its evolutionary relationship with other members of the *Orbivirus* genus. This is the first report of reptiles as orbivirus hosts. The present study expands our knowledge of orbivirus host range, ecology and the complete genomic data may help understand the evolutionary relationship among species of the *Orbivirus* genus.

2. Materials and Methods

2.1. Snake Capture and Sampling

Clinical apparently healthy grass snakes (n = 190) and dice snakes (*Natrix tessellata* Laurenti 1768, n = 63) were captured by hand along transects in several areas of DDBR from 2014 to 2017, as part of an arbovirus survey (Figure 1, Table S1**)**. A blood sample of ~1 mL was collected in a 2 mL sterile Eppendorf tube from the caudal vein of adults and subadults from both species (total n = 253). After clot formation and centrifugation for 2 min at 1000 rpm, the serum was carefully transferred to cryogenic vials using a 100 µL pipette with sterile filter tips. Samples were frozen at −28 °C in the field, shipped to the laboratory on dry ice and stored at −80 °C without interruption of the cold chain. All snakes were released immediately after blood collection back into their habitats. The DDBR Administration Authority issued research permits for all research activities (9/25.04.2014, 10692/ARBDD/25.04.2014; 7717/ARBDD/28.04.2016, 11/28.04.2016; 9/19.04.2017, 5627/ARBDD/13.04.2017).

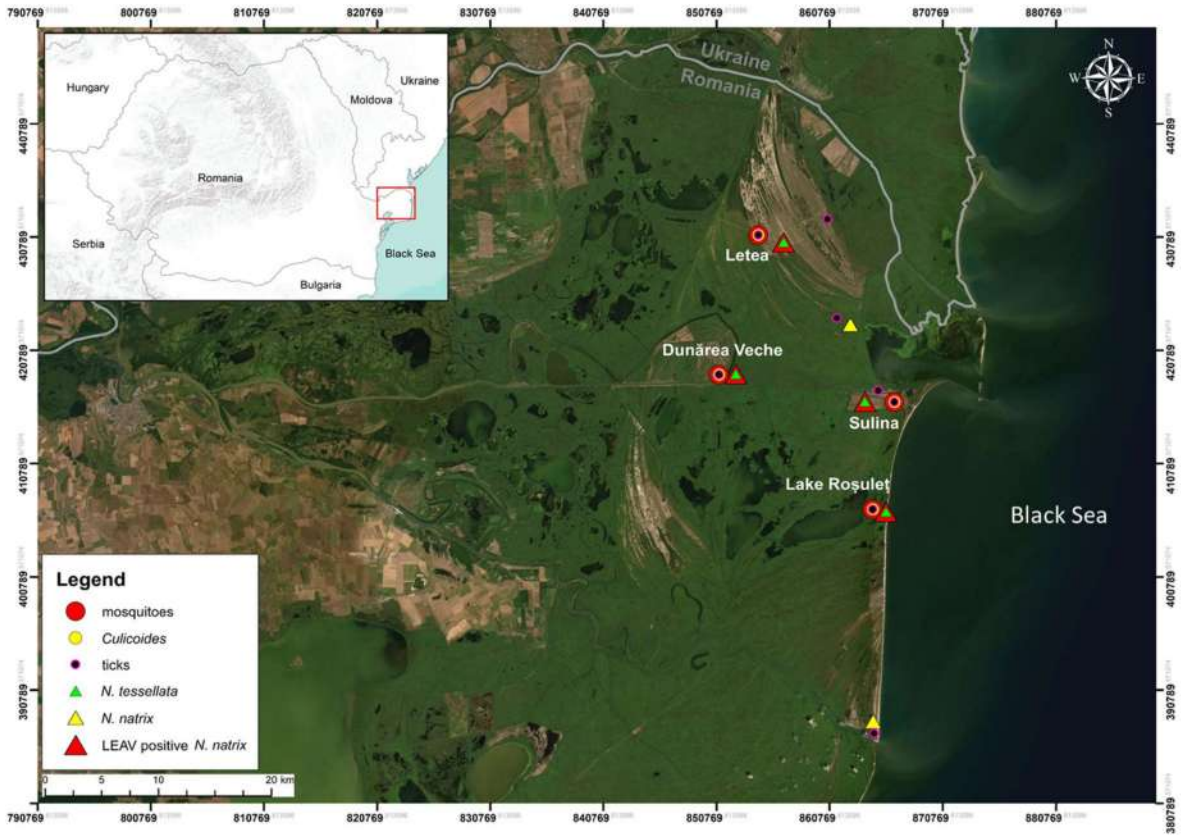


Figure 1. Satellite image of the study area (source: Esri) with sampling sites of grass and dice snakes and arthropods in Danube Delta Biosphere Reserve (Romania) during the study period 2014–2017. The various arthropod vectors were collected during 2014–2016 (mosquitoes), 2014–2017 (ticks) and 2017 (*Culicoides* midges).

2.2. Letea Virus Discovery and Genome Sequencing

The protocol used to perform metagenomic and metatranscriptomic on snake sera for virus discovery has been previously described [19]. Briefly, 100 µL sera serum samples used for deep-sequencing were filtered through a 0.45 µm filter (Millipore, Darmstadt, Germany) in order to remove larger debris and some bacteria. The filtrates were treated with a mixture of nucleases (Turbo DNase, Ambion, Carlsbad, CA, USA; Baseline-ZERO, Epicenter, Madison, WI, USA; Benzonase, Novagen, San Diego, CA, USA; RNAse One, Promega, Fitchburg, WI, USA) to digest unprotected nucleic acids including host DNA/RNA. Enriched viral particles were then subjected to RNA/DNA extraction by using MagMAX™ Viral RNA Isolation Kit (Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. After random RT-PCR amplification, the extracted viral RNA and DNA were subjected for library preparation by using a QIAseq FX DNA Library Kit (Qiagen, Hilden, Germany). Normalized samples were pooled and sequenced on a MiSeq or NextSeq550 platform. The generated raw reads were first qualitatively checked with Phred quality score <20 trimmed and filtered to remove polyclonal and low-quality reads (<55 bases long) using CLC workbench (Qiagen, Hilden, Germany). The remaining filtered raw reads were de novo assembled separately using Trinity v2.6.64239 and CLC workbench and compared with a nonredundant and viral proteome database (NCBI) using BLASTx with an E-value cutoff of 0.001. The virus-like contigs and singlets were further compared to all protein sequences in nonredundant protein databases with a default E-value cutoff of 0.001. The viral metagenomics and metatranscriptomics output have been visualized and analyzed in MEGAN [20].

2.3. Genetic Characterization and Phylogenetic Analysis

Genome finishing, sequence assembly, and analysis were performed using Geneious v9.1.7. (Biomatters, Auckland, New Zealand) (Table S2). Open reading frames (ORF) of the LEAV genome were detected with Geneious v9.1.7 and ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). Putative functions of LEAV proteins were assigned by comparison to sequences in Genbank, using BLASTx. Pairwise distances for nucleotide and amino acid sequences were calculated in Geneious v9.1.7 using MAFFT.

Evolutionary relationship of LEAV with representative members of the *Orbivirus* genus were analyzed by inferring phylogenetic trees with amino acid and nucleotide ORF sequences of conserved genes encoding the polymerase (VP1), the subcore shell protein T2 (VP2/VP3) and the major core surface protein T13 (VP7) [5,6]. Nucleotide and amino acid sequences were aligned with MAFFT in Geneious v9.1.5. Phylogenetic analyses were performed with the best-fit nucleotide and amino acid substitution models selected by their lowest AIC (Akaike information criterion) or BIC (Bayesian information criterion) scores using jModelTest v2.1.10 [21,22] and ProtTest v3.4.2 [22,23], respectively. Amino acid phylogenetic trees were constructed using the maximum likelihood (ML) method in SeaView v4 [24] with LG+I+G+F for T2 and T13 (VP7) and for VP1 with the LG+I+G substitution models. The robustness of tree nodes was assessed by 500 bootstrap replicates. Nucleotide phylogenies were constructed by ML (500 bootstrap replicates) and by Bayesian Inference using the Markov Chain Monte Carlo (MCMC) method implemented in Beast v1.10.3 [25]. The output consisted of two combined MCMC chains, each of 10^7 generations with sampling every 1000 steps and 20% burn-in. Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used for visualization of tree output files.

Nucleotide sequences of LEAV genes obtained in the present study were submitted to GenBank and were assigned accession numbers MN873603–MN873692. The accession numbers of the other orbivirus sequences used for phylogenetic analysis are listed in Table S3.

2.4. Detection of Gene Reassortment and Intragenic Recombination

In order to screen LEAV for potential gene reassortment, we assembled complete genomes by segment concatenation and aligned them with MAFFT. Simplot v3.5.1 [26] was used to screen for potential reassortment between LEAV genomes (n = 9) using a 90% cutoff value for tree permutation across a given genomic segment.

For detection of intragenic recombination, we inspected individual gene alignments in the Recombination Detection Program (RPD) package v4.95 and the tests therein (Bootscan, MaxChi, Chimaera, SiScan, PhylPro, 3seq and GENECONV) [27]. These tests were performed with default settings: a 200 bp window size and a Bonferroni correction of the p-value of 0.01. Recombination events were further considered upon detection of significant signals from at least three methods (Table S4).

2.5. Screening of Potential LEAV Vectors

We retrospectively and concurrently analyzed arthropods collected within the same arbovirus surveillance program at the respective sites in DDBR (Figure 1), with the scope to identify a potential LEAV vector. In total, 18,093 *Culicoides* (16,829 unfed/gravid and 1264 engorged), 3973 engorged mosquitoes and 469 ticks were screened for detection of LEAV RNA (Tables S5 and S6). The unfed/gravid *Culicoides* (n = 16,829) and a part of the tick samples (n = 385) were screened using an RT-PCR assay. The engorged dipterans (1264 *Culicoides* and 3973 mosquitoes) and the rest of the ticks (n = 84) were subjected to metagenomic and metatranscriptomic analyses.

The collection, processing and nucleic acid extraction from engorged mosquitoes and biting midges has been described in previous studies [14,28]. In the case of unfed/gravid *Culicoides* midges, insects were pooled as 1–118 specimens with the rest of the process being the same as in the above-referenced work. Ticks were collected from their hosts using fine tweezers and identified using morphological keys [29,30]. For homogenization, ticks were placed into a sterile 2 mL Eppendorf tube individually

or as pooled nymphs, according to host, site and date of collection (6–9 specimens). Two 5 mm steel beads were added inside and the tube was then kept in liquid nitrogen for 1 min. The samples were loaded into a Tissue Lyser (Qiagen, Hilden, Germany) and the frozen ticks were pulverized at 50 Hz for 4 min. To each sample we added 0.6 mL of high-glucose (4.5 g/L) Dulbeco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich, St. Louis, USA) with L-glutamine, 12.5% head-inactivated fetal bovine serum, 100 µg/mL streptomycin, 100 µg/mL penicillin and 100 µg/mL amphotericin B. The final mix was homogenized using the TissueLyser at 50 Hz for 2 min and clarified by centrifugation (10,000 rpm) for 2 min at 8 °C. Total RNA was extracted using the MagMax RNA/DNA Pathogen kit on a KingFisher Flex Magnetic Particle Processor (ThermoFisher Scientific, USA), according to the manufacturer's instructions.

In order to detect the presence of LEAV in the reptile and arthropod samples, we designed a specific primer pair, 234F: AGGCAAAACAGTAGGATCAG and 234R: GGGCTAAGTGGATCTGAAAC, which amplifies a fragment of the outer capsid 2 protein (VP5). All PCR amplifications were performed in 10.8 µL consisting of 3 µL RNA, 4 µL reaction mix, 0.5 µL Mg_2SO_4 (0.25 µmol), 1 µL ddH_2O, 1 µL of each primer (10 pmol) and 0.3 µL EnzymMix. The reactions comprised a first reverse transcription at 60 °C for 1 min, 50 °C for 45 min, 94 °C for 2 min, followed by 45 cycles of amplification at 94 °C for 15 sec, 55 °C for 30 sec and 68 °C for 30 sec. Final extension was at 68 °C for 7 min. RT-PCR was carried out using a Superscript III one-step RT-PCR kit (Invitrogen, Carlsbad CA, USA).

2.6. Isolation of LEAV

Sera samples from the LEAV RT-PCR positive snakes were used for attempted virus isolation on C6/36 (*Aedes albopictus*), Vero E6 (African green monkey kidney), BHK-21 (baby hamster kidney) and several reptile cell lines, including iguana heart cells (IgH-2, ATCC: CCL-108), Russell's viper heart cells (VH-2, ATCC: CCL-140), *Terrapene* heart cells (TH-1, ATCC: CCL-50) and checked for viral cytopathic effect (CPE). Briefly, sera samples, undiluted and 1:10 diluted were inoculated onto the above-mentioned cell lines and were observed daily for cytopathic effects (CPE). All cultures were harvested 10 to 14 days later and subjected to the LEAV specific PCR test. This procedure was repeated until passage 5.

3. Results

3.1. Detection and Genomic Analysis of LEAV

Of the 190 *N. natrix* sera, 15 specimens of *N. natrix* (7.89%) were found positive for LEAV RNA. All samples collected from *N. tessellata* (n = 63) tested negative (Table S1), as did all arthropod samples (ticks, mosquitoes and *Culicoides*) analyzed for the presence of LEAV RNA. Attempts to isolate the LEAV strains in several cell line cultures of different vertebrate and insect origins were not successful.

We obtained all 10 genomic segments of LEAV and assembled a total of nine complete genomes. BLASTx searches showed that the proteins encoded by LEAV genome match orbivirus homologs. Each segment is monocistronic with the encoded protein spanning most of the positive strand. One exception is segment-9, which additionally to VP6, contains a fourth nonstructural protein (NS4) on a smaller (+2) ORF. All nine LEAV strains have a genome length of 19,983 nucleotides and a GC content of 34.6–34.9%. Gene sizes range from 4010 bp (VP1) to 751 bp (NS3) and their coding asignments are homologous to BTV [1,31]. Descriptions of each LEAV gene with the closest relatives as retrieved by the BLASTx are found in Table 1.

Table 1. Characteristics of the dsRNA genome segments of LEAV (NN04LRO16).

Segment	Protein Encoded	Segment Length (bp)	Segment ORF (with Stop Codon)	Predicted Protein (aa)	Predicted Protein Mass (kDa)	5′ UTR (bp)	5′ Conserved Terminus	3′ UTR (bp)	3′ Conserved Terminus	%GC	Top Blastp Results (ORF)% Pairwise Identity, Accession no.
1	RNA-dependent RNA polymerase (VP1)	**4010**	3855	**1284**	**147.84**	44	**5′-GUGAAAG**	111	**CAUUUAC-3′**	32.4%	VP1 AHSV-5 55.4%, AKP19848
2	similar to outer shell VP2 of BTV, neutralization epitope (OC1)	**3060**	2979	**992**	**115.13**	32	**5′-GUAAUUA**	49	**UUGUUAC-3′**	31.8%	VP2 BTV-5 25.6%, CAE51147
3	major subcore protein (T2/VP3)	**2851**	2727	**908**	**104.30**	68	**5′-GUAAAUG**	56	**GACUUAC-3′**	34.9%	VP3 LEBV 54.2%, YP_009507713
4	minor core and capping enzyme (CaP/VP4)	**2058**	1941	**646**	**75.50**	51	**5′-GUAAAAC**	66	**AAAGUAC-3′**	36.2%	VP4 PALV 50.5%, QCU80098
5	tubules (TuP/NS1)	**1960**	1851	**616**	**71.06**	29	**5′-GUAGAAG**	80	**GAUUUAC-3′**	37.0%	NS1 AHSV-8 32.1%, AKP19783
6	outer capsid protein (OC2/VP5)	**1684**	1605	**534**	**60.03**	41	**5′-GUAAAAA**	38	**GAAUUAC-3′**	36.0%	VP5 CGLV 48%, AGZ91957
7	major core surface protein (T13/VP7)	**1164**	1053	**350**	**39.30**	46	**5′-GUAUAAC**	65	**CACUUAC-3′**	37.7%	VP7 WALV 46.2%, AIT55708
8	nonstructural protein, viral inclusion bodies (ViP/NS2)	**1281**	1107	**368**	**41.04**	85	**5′-GUAAAUA**	89	**GACUUAC-3′**	36.8%	NS2 CGLV 33.5%, ACZ91977
9	minor core protein, helicase (Hel/VP6)	**1164**	936	**311**	**34.71**	61	**5′-GUAAUGA**	167	**AGCGUAC-3′**	33.6%	VP6 CGLV 32.1%, AGZ91984
	nonstructural protein (NS4)		246	**81**	**10.03**	-	**-**	-	**-**	40.2%	no results
10	nonstructural, virus release (NS3)	**751**	603	**200**	**21.62**	111	**5′-GUAAAAG**	37	**UCAUUAC-3′**	36.8%	NS3 IFEV 35.8%, QBL15286
Total genome length		**19,983**									

Sequencing of LEAV untranslated regions (UTRs) revealed that the segments share seven conserved nucleotides at both 3′ and 5′ termini. The first and last two nucleotides of all LEAV segments are inverted complements (Table 1) and identical to those found in most orbiviruses [1].

Comparison of main LEAV protein sequences to homologs of representative orbiviruses (Table 2) revealed identity values of 10–54% with *Culicoides*-/sand fly-borne orbiviruses (C/SBOV), 12–47% with tick and tick-borne orbiviruses (TBOV), and 11–46% with mosquito-borne orbiviruses (MBOV). The sequence identity between the polymerase (VP1) of LEAV and that of other orbiviruses was 33% (SCRV) to 54% (C/SBOV), above the 30% threshold proposed by [5] for viruses within a single genus of the *Reoviridae* family. Analysis of the VP3 protein indicated that it is the T2 protein forming the subcore shell, homologous to the VP3 of BTV and of other C/SBOV [31]. Similarly to VP1, the T2 is highly conserved and the level of identity relative to other orbiviruses ranged from 22% (SCRV) to 53% (C/SBOV).

Table 2. Nucleotide (nt) and amino acid (aa) identities between LEAV and some representative orbiviruses: *Culicoides*-borne (AHSV, African Horse Sickness virus; WALV, Wallal virus), sandfly-borne (CGLV), mosquito-borne (PHSV, Peruvian horse sickness virus), tick-borne (CGV, Changuinola virus) and tick orbivirus (SCRV, St. Croix River virus).

Segment	Protein	AHSV		CGLV		WALV		PHSV		CGV		SCRV	
		nt	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**
1	VP1 (Pol)	58	**54**	58	**53**	60	**54**	56	**46**	50	**47**	42	**33**
2	VP2 (OC1)	30	**15**	31	**10**	33	**11**	31	**11**	19	**NSI**	21	**NSI**
3	VP3 (T2)	57	**52**	57	**53**	58	**53**	50	**36**	46	**37**	37	**22**
4	VP4 (CaP)	54	**50**	53	**49**	53	**48**	52	**44**	44	**40**	44	**35**
5	NS1 (TuP)	42	**26**	39	**24**	40	**22**	42	**21**	28	**16**	28	**15**
6	VP5 (OC2)	51	**41**	54	**46**	54	**45**	47	**34**	42	**31**	38	**27**
7	VP7 (T13)	52	**41**	52	**42**	53	**45**	41	**22**	37	**25**	32	**18**
8	NS2 (Vip)	42	**27**	45	**30**	45	**25**	34	**17**	34	**21**	25	**12**
9	VP6 (Hel)	36	**22**	38	**26**	37	**24**	43	**24**	33	**20**	24	**14**
	NS4	32	**NSI**	30	**11**	40	**NSI**	27	**NSI**	21	**NSI**	N/A	
10	NS3	35	**23**	37	**21**	36	**21**	32	**15**	36	**21**	29	**20**

NSI: no significant identity, N/A: not applicable.

Segments 2 and 6 encode the outer capsid proteins VP2 and VP5 in LEAV (Table 1). VP2 is the most variable protein of C/SBOV, located in the first line of contact with host cells and a major determinant of virus serotype [31]. Significant levels of identity of the hypervariable VP2 were observed only between LEAV and insect-borne orbiviruses (IBOV, 10–15%), while within the same group VP5 comparison revealed values similar to T13 (Table 2).

3.2. Phylogenetic Analysis

The orbivirus VP1, T2 and T13 proteins are used in phylogenetic studies and for classification of *Reoviridae* members at both species and genus level [5,6]. LEAV was placed in the C/SBOV clade by all phylogenetic analyses, consistent with the levels of sequence identity revealed by comparisons with the other orbivirus proteins (Figure 2, Figure 3 and Figure S1).

The phylogenetic trees based on VP1 and T13 amino acid (Figure 2a,b) and nucleotide sequences (Figure S1) displayed a clustering typical for the *Orbivirus* genus, with main clades indicative of their arthropod vectors [9]. The three main branches are rooted by SCRV, an orbivirus isolated from tick cells which is considered a tick-associated orbivirus (TOV) [5].

The subcore shell protein T2 is encoded by segment 3, coresponding to the VP3 protein of the C/SBOV clade (Figure 2c). As in the VP1 and T13 phylogenies, LEAV is basal within this clade. The main difference is that the T2 tree splits between two clades instead of three, having a clear separation between the orbiviruses encoding T2 on segment-2 (MBOV, TBOV and SCRV (TOV)) and those encoding T2 on segment-3 (C/SBOV).

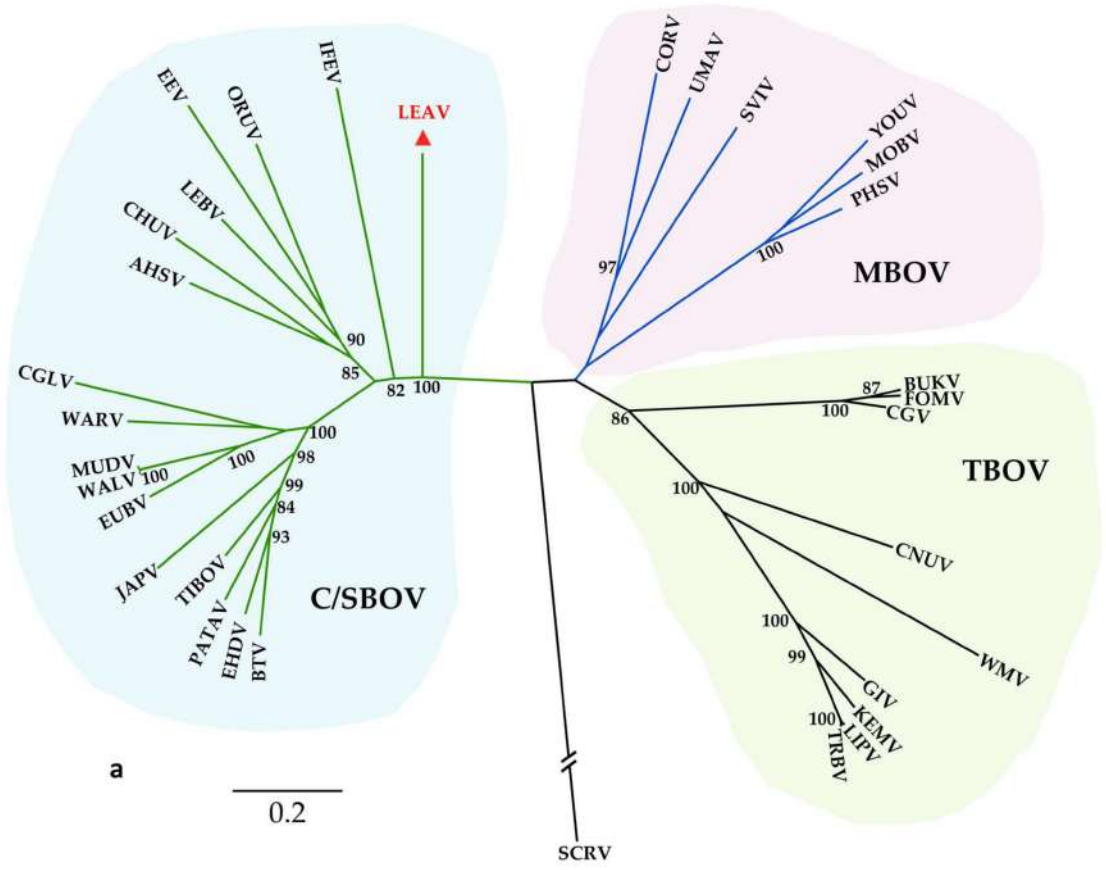


Figure 2. *Cont.*

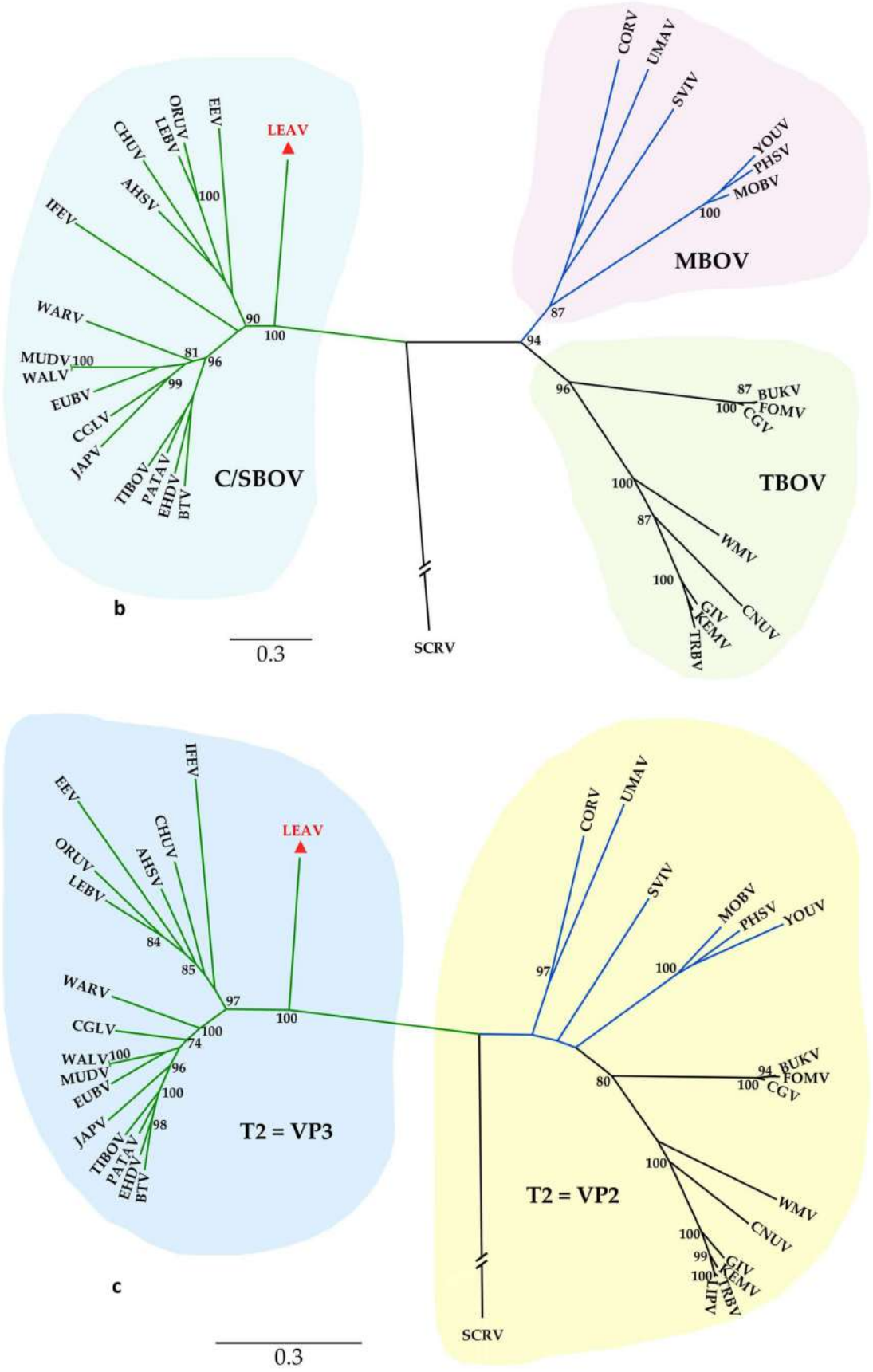


Figure 2. Maximum likelihood phylogeny of the VP1 (**a**), T13 (VP7) (**b**) and T2 (**c**) orbivirus proteins constructed using maximum likelihood inference and 100 bootstrap replicates.

Bootstrap supports ≥80% are displayed at the nodes. Letea virus (LEAV) is indicated by the red triangle. C/SBOV stands for *Culicoides*-/sand fly-borne orbiviruses: African Horse Sickness virus (AHSV), Bluetongue virus (BTV), Changuinola virus (CGLV), Chuzan virus (CHUV), Epizootic hemorrhagic disease virus (EHDV), Equine encephalosis virus (EEV), Eubenangee virus (EUBV), Ife virus (IFEV), Japanaut virus (JAPV), Lebombo virus (LEBV), Mudjinabarry virus (MUDV), Orungo virus (ORUV), Pata virus (PATAV), Tibet orbivirus (TIBOV), Wallal virus (WALV), Warrego virus (WARV); MBOV stands for mosquito-borne orbiviruses: Corriparta virus (CORV), Mobuck virus (MOBV), Peruvian horse sickness virus (PHSV), Sathuvachari virus (SVIV), Umatilla virus (UMAV), Yunnan orbivirus (YOUV); TBOV stands for tick-borne orbiviruses: Bukakata virus (BUKV), Chenuda virus (CNUV), Chobar Gorge virus (CGV), Fomede virus (FOMV), Great Island virus (GIV), Kemerovo virus (KEMV), Lipovnik virus (LIPV), Tribeč virus (TRBV), Wad Medani virus (WMV); St. Croix River virus (SCRV).

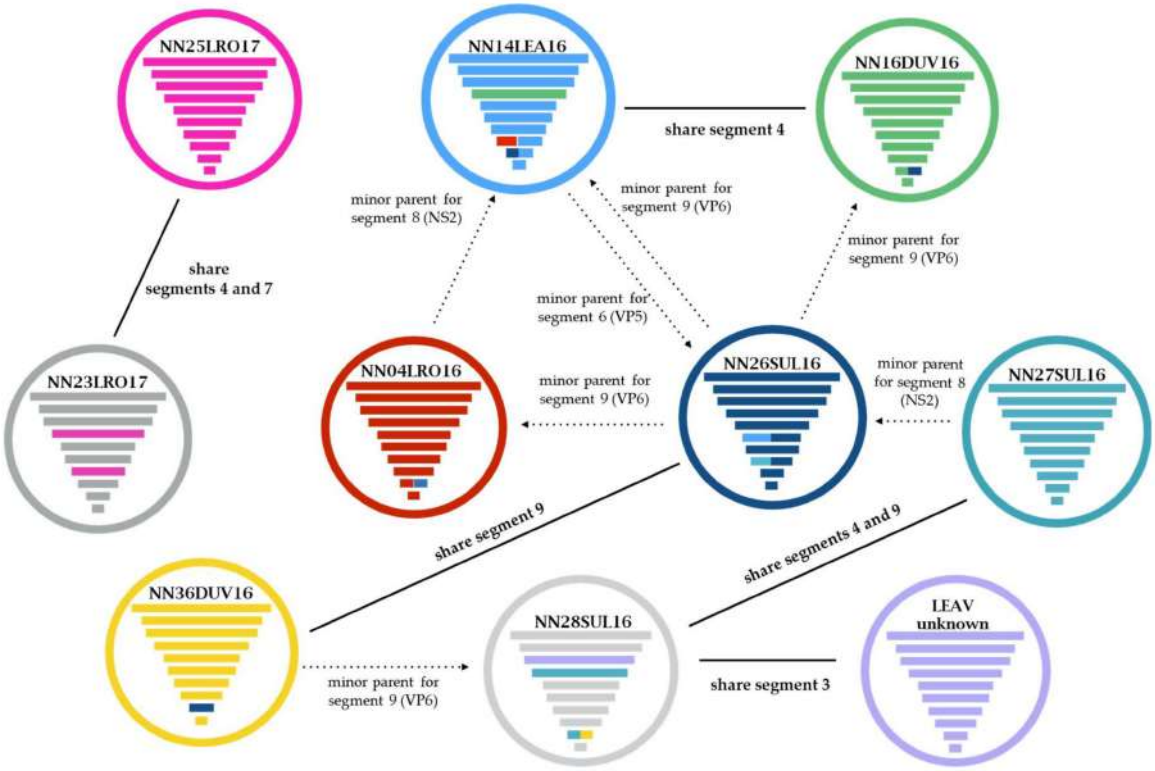


Figure 3. Graphical representation of gene reassortment and intragenic recombination between LEAV strains. Each colored circle represents a different LEAV strain. The 10 horizontal lines inside the circles represent the 10 genomic segments. Solid lines indicate reassortments of genes between different LEAV variants. Dashed arrows show the origins of gene fragments that have potentially been derived through recombination.

3.3. Detection of Gene Reassortment and Intragenic Recombination

Putative reassortment events involving LEAV segments were detected by Simplot (Bootscan) in eight of the nine LEAV genomes (Figure 3). Segment-7 (T13) was exchanged between NN23LRO17 and NN25LRO17, while the other instances indicated exchanges of segment-4 (VP4) and -9 (VP6) (Figure 3 and Figure S2). Significant signals of recombination among LEAV genes (≥3 methods) were found by RDP for segment-6 (VP5), segment-8 (NS2) and segment-9 (VP6) (Figure 3, Table S4).

4. Discussion

Reptiles are known as hosts of numerous viruses. However, limited or fragmentary evidence is available regarding their role in arboviral cycles [32–37]. The present study investigated the possibility

that natricine snakes harbor arboviruses by screening sera collected from sympatric populations of grass snakes (*N. natrix*) and dice snakes (*N. tessellata*) from the Danube Delta Biosphere Reserve in Romania. Thus, we described for the first time the discovery and genetic characterization of a novel orbivirus species (Letea virus, LEAV) infecting reptiles.

Inclusion and demarcation of species within the *Orbivirus* genus considers several criteria, such as sequence identity of segments encoding the polymerase (VP1) and major subcore shell protein T2, gene reassortment between close strains, high levels of serological cross-reactivity against conserved antigens like the T13 protein, conservation of UTR terminal nucleotides, range of hosts and vectors or the clinical signs associated with orbivirus infection [1]. We propose that LEAV should be included in the *Orbivirus* genus as a separate species, based on the comparative and phylogenetic analyses reported herein. In addition to a typical orbivirus genomic architecture of 10 linear segments of dsRNA, the UTRs of LEAV include conserved terminal sequences similar to other orbiviruses. The LEAV terminal nucleotides are not conserved hexanucleotides as in the case of BTV or AHSV, but heptanucleotides showing little variation among the 10 segments. Distal dinucleotides at both of the UTR ends are inverted complements (Table 1), as shown in other orbiviruses [1,3,6,9,38–40].

The NS4 is a nonstructural protein found in some orbiviruses and the last one described to date [41,42]. In LEAV, we found NS4 to be of similar size and position as in other C/SBOV [9,41,43], showing also the lowest sequence identity among all compared orbiviral proteins (Table 2).

The amino acid identity observed in the polymerase is above the 30% threshold defined by [5,44] for inclusion in the *Orbivirus* genus (Table 2). The protein sequence of LEAV T2 (VP3) showed identity levels significantly lower than the 91% cutoff indicated for this protein [5], confirming that LEAV is a distinct orbivirus species. Furthermore, the nine different LEAV strains belong to the same species, as their T2 amino acid sequences are >98% identical. Additional taxonomical markers of orbiviruses are the VP2 (outer capsid 1 in C/SBOV) and VP7 (T13) proteins, determining the serotype and serogroup, respectively [45]. The core surface protein T13 forms the outer layer of the viral core and is the primary antigenic constituent of virus serogroup (species) [31]. The low amino acid identity of LEAV T13 (Table 2) to other T13 proteins confirms that this virus belongs to a distinct serogroup. VP2 is encoded on segment-2 in LEAV and functionally equivalent to VP3 of MBOV and VP4 of TBOV/TOV [46]. Due to its neutralizing epitopes and role in cell attachment, the VP2 (OC1) protein is subjected to intense selective pressures by the host's immune responses. Thus, it is one of the most variable orbiviral proteins [31]. Unsurprisingly, we found significant levels of identity for LEAV VP2 (10–15%) only in comparison with IBOV proteins. Moreover, the high amino acid identity (>98%) found between VP2 of the nine LEAV strains showed that all sequences belong to the same LEAV serotype.

Previous studies noted that the overall GC content and the UTR proportion relative to the genome's length reflect three groups similar to those illustrated by phylogenetic analyses. First, the GC content is highest in TBOV with 52–57.3% GC, followed by the C/SBOV with 39.9–45.9% GC and the MBOV with 36.7–41.6% GC [6,9,39,43]. The GC content of LEAV is 34.6–34.9%, therefore below these intervals. Second, the UTRs of C/SBOV span 3.5–4.1% of their total genome length, in TBOV the UTRs are between ~4.5–5% and in MBOV ~5–5.7% of the virus genome [6,47,48]. Again, LEAV falls outside these limits, having the proportion of UTRs at 6.64% of the genome's length, which is higher than in other orbiviruses.

Apart from the high mutation rate owing to a polymerase lacking proofreading activity, reassortment of cognate genomic segments is an important driver of genetic diversity in viruses with segmented RNA genomes [49]. This process can generate novel phenotypes with fundamental implications for immune escape, host or vector range, virulence and pathogenicity [50–53]. The ability to reassort genomic segments is a primary criterion for inclusion in the *Reoviridae* family [31] and it may have contributed to the great evolutionary success of this family. Most natural cases of orbivirus reassortment have been described in BTV studies, mostly due to its antigenic diversity, wide geographic range, but also economic importance [54–58]. Additionally, reassortment has been described in EHDV [59–62], CORV [63], CGLV [64] and Banna orbivirus (BAOV) [65]. We found that

reassortment between LEAV strains involved segment-4 (VP4), segment-7 (T13) and segment-9 (VP6) in eight of nine genomes analyzed. We speculate on another segment exchange where LEAV strain NN28SUL16 received its segment-3 (T2) from an unidentified LEAV parental strain, due to the striking sequence divergence of NN28SUL16 segment-3 within a highly similar (>98%) VP3 dataset (Figure 3 and Figure S3). When translated to protein sequence, the identity of this segment to the rest of the LEAV homologs was ≥98.9%, confirming the necessity of its structural conservation.

Intragenic recombination between segments of LEAV was detected in the majority of LEAV strains (Figure 3, Table S4). The effective contribution of each mechanism generating diversity in segmented viruses is far from being understood. Against a backdrop of rapid mutation, the formation of mosaic genes along with reassortment can have compounding effects on viral fitness. As in the case of sand fly-borne Changuinola (CGLV) serogroup [63], the strain biodiversity could be an important factor for the occurrence of RNA segment/fragment exchange in LEAV. This is indicated by detections of reassortment and intragenic recombination in eight, respectively seven, of nine LEAV strains. This is all the more clear for orbiviruses with great antigenic diversity like BTV [57,66,67] and AHSV [68], but also for orthoreoviruses [69–71] and rotaviruses [72].

Despite field efforts parallel to sera collection and the significant LEAV prevalence in grass snakes (7.89%), none of the screened arthropod pools or individuals tested positive for LEAV RNA. Also, recent analyses of mosquito and *Culicoides* host-feeding patterns in DDBR did not indicate ectothermic species as hosts of these insects (with very few exceptions provided by frogs fed upon by mosquitoes) [18]. Reoviruses known to infect reptiles belong to the turreted group of the family (subfamily *Spinareovirinae*, e.g., *Reptilian orthoreovirus*) and cause severe illness of digestive and respiratory organs [73–76]. The grass snake and dice snake occur sympatrically across the study area. In Dunărea Veche and Sulina we could observe some ecological features (bank structure, water body type, prey, microhabitat usage) very similar to those described for other European populations, indicating ecological partitioning in syntopic populations [77]. Although we encountered more *N. tessellata* in the aforementioned sites, LEAV was not detected in the sera of this species. Infected grass snakes showed no sign of disease. To our knowledge, this is the first report of orbivirus detection in reptiles.

All attempts of growing and isolating LEAV on insect or vertebrate cell lines showed no cytopathic effect or silent replication of the virus. Most known orbiviruses grow easily in vertebrate cells in vitro, while a few are known to be restricted to insect cells [78,79]. Although LEAV isolation could be further attempted on additional cell lines, we may speculate a shift in cell tropism underlying the inability of LEAV to infect certain cell types. A similar case is Parry's Lagoon virus (PLV), a serotype of CORV. In contrast to the wide vertebrate host range of CORV [80], the antigenically related PLV showed a distinct cell tropism and replicated only in insect cells [81]. With the available data we may speculate that such shifts in cell tropism could be the result of successive changes through recombination, genetic drift and shift. *Reoviridae* is a very successful family of segmented dsRNA viruses, having wide host ranges across various econiches. The family's repertoire of evolutionary strategies also includes deletion [82,83], gene duplication and concatemerisation [9,84,85]. These strategies were also observed previously in aquareoviruses [86], rotaviruses [87], phytoreoviruses [88] and even in a cross-family heterologous recombinant virus containing reovirus genomic components [89]. This suggests an increased potential for "species jumps" and adaptation to new vectors and/or hosts [81,86].

Earlier studies associated a conserved Arg-Gly-Asp ($_{167}$RGD$_{171}$) motif on the T13 protein of BTV with the attachment to *Culicoides* cells [90,91]. This conserved motif was also found by later studies in some species closely related to BTV [43,48,70], but we did not observed it in LEAV. As with other orbivirus species, this may reflect the higher divergence relative to BTV. Although the phylogenetic analyses indicate LEAV as a potentially *Culicoides*-borne orbivirus, it is interesting to note some general inconsistencies in vector associations of IBOV (C/SBOV and MBOV). For example, phylogenetic analyses place Orungo virus (ORUV), Lembombo virus (LEBV), Pata virus (PATAV) and Japanaut virus (JAPV) in the C/SBOV clade, even though they were discovered in mosquitoes [1,92,93]. For ORUV,

follow-up studies on mosquito transmission were inconclusive [93]. Interestingly, Tracambé virus (TRV), a serotype of the sand fly-borne CGLV serogroup, was isolated from mosquitoes of the genus *Anopheles* (Reoviridae.org). Some serotypes of C/SBOV have been isolated from both mosquitoes and biting midges: Eubenangee virus (EUBV) [1,94], Palyam virus (PALV) [1,95], Warrego virus WARV [1], Wongorr virus (WGRV) [1], and Tibet orbivirus (TIBOV) [48]. The associations between some viruses and more than one vector family could be the result of "species jumps" permitted by fast evolution characteristic of RNA segmented viruses. Such occurrences in members of the *Orbivirus* genus would not necessarily run counter to the "coevolution" hypothesis [11,14,43,86], but possibly complement it.

In conclusion, a novel orbivirus (LEAV) was identified and characterized, expanding the known host range of orbiviruses and revealing its genetic relationship to the *Orbivirus* genus. Phylogenetic analysis indicates LEAV as a potentially *Culicoides*-borne orbivirus, although this was not confirmed by the screening of *Culicoides* midges and other arthropods from the DDBR. LEAV failed to replicate in vitro in several types of cells, which may warrant further attempts using additional cell types. The discovery and characterization of LEAV offers valuable information, expanding our knowledge about the evolution and host range of orbiviruses. The phylogenetic associations can justify further screening of arthropods and continued surveillance in order to describe the natural cycle of LEAV and its possible impact on vertebrate hosts.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/2/243/s1, Figure S1: Maximum likelihood phylogenies of main conserved orbivirus genes, Figure S2: Analysis of genetic reassortment (BootScan) performed with Simplot 3.5.1 using the concatenated genomic segments of Letea virus, Figure S3: Multiple alignments of Letea virus (LEAV) genes highlighting disagreements of their nucleotide sequences (LEAV is indicated by the red triangle), Table S1: Snake sampling in DDBR 2014–2017, Table S2: Summary of next-generation sequencing data for the LEAV genomes, Table S3: Genbank accession numbers for Orbivirus nucleotide sequences, Table S4: Results of RDP scanning for detection of recombination in LEAV, Table S5: List of ticks screened for the presence of LEAV RNA, Table S6: List of biting midges screened for the presence of LEAV RNA.

Author Contributions: Conceptualization, A.T., M.S., R.L., J.S.-C. and D.C.; Methodology, A.T., R.E.M., I.M., H.B., A.B. and D.C.; Formal Analysis, A.T., R.E.M., R.L., J.S.-C. and D.C.; Investigation, A.T., R.E.M. and D.C.; Writing—Original Draft Preparation, A.T. and D.C.; Writing—Review and Editing, A.T., R.E.M., R.L., J.S.-C. and D.C.; Visualization, A.T., I.M. and D.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors wish to thank Andrei Tomazatos and Patricia Iftene for their help during fieldwork and to Anucha Ponyiam for excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Attoui, H.; Mertens, P.; Becnel, J.; Belaganahalli, S.; Bergoin, M.; Brussaard, C.; Chappell, J.; Ciarlet, M.; del Vas, M.; Dermody, T. Orbiviruses, Reoviridae. In *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*; Elsevier Academic Press: San Diego, CA, USA, 2012; ISBN 9780123846846.
2. Attoui, H.; Mohd Jaafar, F. Zoonotic and emerging orbivirus infections. *OIE Rev. Sci. Tech.* **2015**, *34*, 353–361. [CrossRef] [PubMed]
3. Attoui, H.; Mohd Jaafar, F.; Belhouchet, M.; Aldrovandi, N.; Tao, S.; Chen, B.; Liang, G.; Tesh, R.B.; de Micco, P.; de Lamballerie, X. Yunnan orbivirus, a new orbivirus species isolated from Culex tritaeniorhynchus mosquitoes in China. *J. Gen. Virol.* **2005**, *86*, 3409–3417. [CrossRef] [PubMed]
4. Drolet, B.S.; Van Rijn, P.; Howerth, E.W.; Beer, M.; Mertens, P.P. A Review of Knowledge Gaps and Tools for Orbivirus Research. *Vector Borne Zoonotic Dis.* **2015**, *15*, 339–347. [CrossRef] [PubMed]
5. Attoui, H.; Stirling, J.M.; Munderloh, U.G.; Billoir, F.; Brookes, S.M.; Burroughs, J.N.; de Micco, P.; Mertens, P.P.C.; de Lamballerie, X. Complete sequence characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of Ixodes scapularis. *J. Gen. Virol.* **2001**, *82*, 795–804. [CrossRef] [PubMed]

6. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Tesh, R.; Attoui, H.; Mertens, P.P.C. Umatilla virus genome sequencing and phylogenetic analysis: Identification of Stretch Lagoon orbivirus as a new member of the Umatilla virus species. *PLoS ONE* **2011**, *6*, e23605. [CrossRef]
7. Huismans, H.; Erasmus, B.J. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J. Vet. Res.* **1981**, *48*, 51–58.
8. Mertens, P.P.C.; Pedley, S.; Cowley, J.; Burroughs, J.N.; Corteyn, A.H.; Jeggo, M.H.; Jennings, D.M.; Gorman, B.M. Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virology* **1989**, *170*, 561–565. [CrossRef]
9. Mohd Jaafar, F.; Belhouchet, M.; Belaganahalli, M.; Tesh, R.B.; Mertens, P.P.C.; Attoui, H. Full-genome characterisation of Orungo, Lebombo and Changuinola viruses provides evidence for co-evolution of orbiviruses with their arthropod vectors. *PLoS ONE* **2014**, *9*, e86392. [CrossRef]
10. Hanganu, J.; Dubyna, D.; Zhmud, E.; Grigoras, I.; Menke, U.; Drost, H.; Ştefan, N.; Sărbu, I. *Vegetation of the Biosphere Reserve "Danube Delta"—With Transboundary Vegetation Map*; Danube Delta National Institute: Tulcea, Romania, 2002; ISBN 9036954797.
11. Cotar, A.I.; Fălcuță, E.; Prioteasa, L.F.; Dinu, S.; Ceianu, C.S.; Paz, S. Transmission Dynamics of the West Nile Virus in Mosquito Vector Populations under the Influence of Weather Factors in the Danube Delta, Romania. *Ecohealth* **2016**, *13*, 796–807. [CrossRef]
12. Prioteasa, F.-L.; Fălcuță, E. An annotated checklist of the mosquitoes (Diptera: Culicidae) of the Danube Delta Biosphere Reserve. *Eur. Mosquito Bull.* **2010**, *28*, 240–245.
13. Sándor, A.D.; Dumitrache, M.O.; D'Amico, G.; Kiss, B.J.; Mihalca, A.D. Rhipicephalus rossicus and not R. sanguineus is the dominant tick species of dogs in the wetlands of the Danube Delta, Romania. *Vet. Parasitol.* **2014**, *204*, 430–432. [CrossRef]
14. Török, E.; Tomazatos, A.; Cadar, D.; Horváth, C.; Keresztes, L.; Jansen, S.; Becker, N.; Kaiser, A.; Popescu, O.; Schmidt-Chanasit, J.; et al. Pilot longitudinal mosquito surveillance study in the Danube Delta Biosphere Reserve and the first reports of Anopheles algeriensis Theobald, 1903 and Aedes hungaricus Mihályi, 1955 for Romania. *Parasit. Vectors* **2016**, *9*, 196. [CrossRef]
15. Kolodziejek, J.; Marinov, M.; Kiss, B.J.; Alexe, V.; Nowotny, N. The complete sequence of a West Nile virus lineage 2 strain detected in a Hyalomma marginatum marginatum tick collected from a song thrush (Turdus philomelos) in Eastern Romania in 2013 revealed closest genetic relationship to strain Volgograd 2007. *PLoS ONE* **2014**, *9*, e109905. [CrossRef] [PubMed]
16. Mărcuţan, I.-D.; Kalmár, Z.; Ionică, A.M.; D'Amico, G.; Mihalca, A.D.; Vasile, C.; Sándor, A.D. Spotted fever group rickettsiae in ticks of migratory birds in Romania. *Parasit. Vectors* **2016**, *9*, 294. [CrossRef] [PubMed]
17. Sándor, A.D.; Mărcuţan, D.I.; D'Amico, G.; Gherman, C.M.; Dumitrache, M.O.; Mihalca, A.D. Do the ticks of birds at an important migratory hotspot reflect the seasonal dynamics of Ixodes ricinus at the migration initiation site? A case study in the danube delta. *PLoS ONE* **2014**, *9*, e89378. [CrossRef]
18. Tomazatos, A.; Jansen, S.; Pfister, S.; Török, E.; Maranda, I.; Horv, C.; Keresztes, L.; Spînu, M.; Tannich, E.; Jöst, H.; et al. Ecology of West Nile Virus in the Danube Delta, Romania: Phylogeography, Xenosurveillance and Mosquito Host-Feeding Patterns. *Viruses* **2019**, *11*, 1159. [CrossRef] [PubMed]
19. Truchado, D.A.; Diaz-Piqueras, J.M.; Gomez-Lucia, E.; Doménech, A.; Milá, B.; Pérez-Tris, J.; Schmidt-Chanasit, J.; Cadar, D.; Benítez, L. A Novel and Divergent Gyrovirus with Unusual Genomic Features Detected in Wild Passerine Birds from a Remote Rainforest in French Guiana. *Viruses* **2019**, *11*, 1148. [CrossRef] [PubMed]
20. Huson, D.H.; Beier, S.; Flade, I.; Górska, A.; El-Hadidi, M.; Mitra, S.; Ruscheweyh, H.J.; Tappu, R. MEGAN Community Edition—Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLoS Comput. Biol.* **2016**, *12*, e1004957. [CrossRef] [PubMed]
21. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and high-performance computing Europe PMC Funders Group. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
22. Guindon, S.; Gascuel, O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol.* **2003**, *52*, 696–704. [CrossRef]
23. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. ProtTest 3: Fast selection of best-fit models of protein evolution. *Bioinformatics* **2017**, *27*, 1164–1165. [CrossRef] [PubMed]
24. Gouy, M.; Guindon, S.; Gascuel, O. SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. *Mol. Biol. Evol.* **2010**, *27*, 221–224. [CrossRef] [PubMed]

25. Suchard, M.A.; Lemey, P.; Baele, G.; Ayres, D.L.; Drummond, A.J.; Rambaut, A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol.* **2018**, *4*, vey016. [CrossRef] [PubMed]
26. Lole, K.S.; Bollinger, R.C.; Paranjape, R.S.; Gadkari, D.; Kulkarni, S.S.; Novak, N.G.; Ingersoll, R.; Sheppard, H.W.; Ray, S.C. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* **1999**, *73*, 152–160. [CrossRef]
27. Martin, D.P.; Murrell, B.; Golden, M.; Khoosal, A.; Muhire, B. RDP4: Detection and analysis of recombination patterns in virus genomes. *Virus Evol.* **2015**, *1*, vev003. [CrossRef]
28. Tomazatos, A.; Jöst, H.; Schulze, J.; Spînu, M.; Schmidt-Chanasit, J.; Cadar, D.; Lühken, R. Blood-meal analysis of Culicoides (Diptera: Ceratopogonidae) reveals a broad host range and new species records for Romania. *Parasit. Vectors* **2020**, *13*, 79. [CrossRef]
29. Estrada-Peña, A.; Mihalca, A.D.; Petney, T. (Eds.) *Ticks of Europe and North Africa: A Guide to Species Identification*, 1st ed.; Springer International Publishing: Cham, Switzerland, 2017.
30. Walker, J.B.; Keirans, J.E.; Horak, I. *The Genus Rhipicephalus (Acari, Ixodidae): A Guide to the Brown Ticks of the World*, 2nd ed.; Cambridge University Press: Cambridge, UK, 2005.
31. Mertens, P.; Mohd Jaafar, F.; Attoui, H. *Orbiviruses and Bluetongue Virus*; John Wiley & Sons, Ltd.: Chichester, UK, 2015.
32. Bingham, A.M.; Graham, S.P.; Burkett-Cadena, N.D.; White, G.S.; Hassan, H.K.; Unnasch, T.R. Detection of Eastern Equine Encephalomyelitis Virus RNA in North American Snakes. *Am. J. Trop. Med. Hyg.* **2012**, *87*, 1140–1144. [CrossRef]
33. Farfán-Ale, J.A.; Blitvich, B.J.; Marlenee, N.L.; Loroño-Pino, M.A.; Puerto-Manzano, F.; García-Rejón, J.E.; Rosado-Paredes, E.P.; Flores-Flores, L.F.; Ortega-Salazar, A.; Chávez-Medina, J.; et al. Antibodies to West Nile virus in asymptomatic mammals, birds, and reptiles in the Yucatan Peninsula of Mexico. *Am. J. Trop. Med. Hyg.* **2006**, *74*, 908–914. [CrossRef]
34. Graham, S.P.; Hassan, H.K.; Chapman, T.; White, G.; Guyer, C.; Unnasch, T.R. Serosurveillance of Eastern equine encephalitis virus in amphibians and reptiles from Alabama, USA. *Am. J. Trop. Med. Hyg.* **2012**, *86*, 540–544. [CrossRef]
35. Steinman, A.; Banet-noach, C.; Simanov, L.; Grinfeld, N.; Aizenberg, Z.; Levi, O.; Lahav, D.A.N.; Malkinson, M.; Perk, S.; Shpigel, N.Y. Experimental Infection of Common Garter Snakes (Thamnophis sirtalis) with West Nile Virus. *Vect Born Zoo Dis.* **2006**, *6*, 361–368. [CrossRef]
36. Whitney, E.; Jamnback, H.; Means, R.G.; Watthews, T.H. Arthropod-borne-virus survey in St. Lawrence County, New York. Arbovirus reactivity in serum from amphibians, reptiles, birds, and mammals. *Am. J. Trop. Med. Hyg.* **1968**, *17*, 645–650. [CrossRef] [PubMed]
37. Marschang, R.E. Viruses infecting reptiles. *Viruses* **2011**, *3*, 2087. [CrossRef]
38. Attoui, H.; Mendez-lopez, M.R.; Rao, S.; Hurtado-Alendes, A.; Lizaraso-Caparo, F.; Mohd Jaafar, F.; Samuel, A.R.; Belhouchet, M.; Pritchard, L.I.; Melville, L.; et al. Peruvian horse sickness virus and Yunnan orbivirus, isolated from vertebrates and mosquitoes in Peru and Australia. *Virology* **2009**, *394*, 298–310. [CrossRef] [PubMed]
39. Cooper, E.; Anbalagan, S.; Klumper, P.; Scherba, G.; Simonson, R.R.; Hause, B.M. Mobuck virus genome sequence and phylogenetic analysis: Identification of a novel Orbivirus isolated from a white-tailed deer in Missouri, USA. *J. Gen. Virol.* **2014**, *95*, 110–116. [CrossRef] [PubMed]
40. Li, M.; Zheng, Y.; Zhao, G.; Fu, S.; Wang, D.; Wang, Z.; Liang, G. Tibet Orbivirus, a novel Orbivirus species isolated from *Anopheles maculatus* mosquitoes in Tibet, China. *PLoS ONE* **2014**, *9*, e88738. [CrossRef] [PubMed]
41. Belhouchet, M.; Jaafar, F.; Firth, A.E.; Grimes, J.M.; Mertens, P.P.C.; Attoui, H. Detection of a fourth orbivirus non-structural protein. *PLoS ONE* **2011**, *6*, e25697. [CrossRef] [PubMed]
42. Ratinier, M.; Caporale, M.; Golder, M.; Franzoni, G.; Allan, K.; Nunes, S.F.; Armezzani, A.; Bayoumy, A.; Rixon, F.; Shaw, A.; et al. Identification and characterization of a novel non-structural protein of bluetongue virus. *PLoS Pathog.* **2011**, *7*, e1002477. [CrossRef]
43. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Nomikou, K.; Pritchard, I.; Lunt, R.; Kirkland, P.D.; Attoui, H.; Brownlie, J.; Mertens, P.P.C. Full genome sequencing and genetic characterization of Eubenangee viruses identify Pata virus as a distinct species within the genus Orbivirus. *PLoS ONE* **2012**, *7*, e31911. [CrossRef]

44. Attoui, H.; Fang, Q.; de Lamballerie, X.; Cantaloube, J.-F.; Mohd Jaafar, F. Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus Aquareovirus, family Reoviridae). *J. Gen. Virol.* **2002**, *83*, 1941–1951. [CrossRef]
45. Attoui, H.; Mohd Jaafar, F.; Belhouchet, M.; Biagini, P.; Cantaloube, J.F.; De Micco, P.; De Lamballerie, X. Expansion of family Reoviridae to include nine-segmented dsRNA viruses: Isolation and characterization of a new virus designated aedes pseudoscutellaris reovirus assigned to a proposed genus (Dinovernavirus). *Virology* **2005**, *343*, 212–223. [CrossRef]
46. Belaganahalli, M.N.; Maan, S.; Maan, N.S.; Brownlie, J.; Tesh, R.; Attoui, H.; Mertens, P.P.C. Genetic characterization of the tick-borne orbiviruses. *Viruses* **2015**, *7*, 2185–2209. [CrossRef] [PubMed]
47. Kapoor, A.; Tesh, R.B.; Duraisamy, R.; Popov, V.L.; da Rosa, A.P.A.T.; Lipkin, W.I. A novel mosquito-borne Orbivirus species found in South-east Asia. *J. Gen. Virol.* **2013**, *94*, 1051–1057. [CrossRef] [PubMed]
48. Wang, J.; Li, H.; He, Y.; Zhou, Y.; Xin, A.; Liao, D.; Meng, J. Isolation of Tibet orbivirus from Culicoides and associated infections in livestock in Yunnan, China. *Virol. J.* **2017**, *14*, 1–9. [CrossRef] [PubMed]
49. McDonald, S.M.; Nelson, M.I.; Turner, P.E.; Patton, J.T. Reassortment in segmented RNA viruses: Mechanisms and outcomes. *Nat. Rev. Microbiol.* **2016**, *14*, 448–460. [CrossRef]
50. McDonald, S.M.; Patton, J.T. Assortment and packaging of the segmented rotavirus genome. *Trends Microbiol.* **2011**, *19*, 136–144. [CrossRef]
51. Mukherjee, A.; Dutta, D.; Ghosh, S.; Bagchi, P.; Chattopadhyay, S.; Nagashima, S.; Kobayashi, N.; Dutta, P.; Krishnan, T.; Naik, T.N.; et al. Full genomic analysis of a human group A rotavirus G9P[6] strain from Eastern India provides evidence for porcine-to-human interspecies transmission. *Arch. Virol.* **2009**, *154*, 733–746. [CrossRef]
52. Horimoto, T.; Kawaoka, Y. Influenza: Lessons from past pandemics, warnings from current incidents. *Nat. Rev. Microbiol.* **2005**, *3*, 591–600. [CrossRef]
53. Taubenberger, J.K.; Kash, J.C. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* **2010**, *7*, 440–451. [CrossRef]
54. Hornyák, Á.; Malik, P.; Marton, S.; Dóró, R.; Cadar, D.; Bányai, K. Emergence of multireassortant bluetongue virus serotype 4 in Hungary. *Infect. Genet. Evol.* **2015**, *33*, 6–10. [CrossRef]
55. Maan, S.; Maan, N.S.; van Rijn, P.A.; van Gennip, R.G.P.; Sanders, O.; Wright, I.M.; Batten, C.; Hoffmann, B.; Eschbaumer, M.; Oura, C.A.L.; et al. Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. *PLoS ONE* **2010**, *5*, e10323. [CrossRef]
56. Nomikou, K.; Hughes, J.; Wash, R.; Kellam, P.; Breard, E.; Zientara, S.; Palmarini, M.; Biek, R.; Mertens, P. Widespread Reassortment Shapes the Evolution and Epidemiology of Bluetongue Virus following European Invasion. *PLoS Pathog.* **2015**, *11*, e1005056. [CrossRef] [PubMed]
57. Shaw, A.E.; Ratinier, M.; Nunes, S.F.; Nomikou, K.; Caporale, M.; Golder, M.; Allan, K.; Hamers, C.; Hudelet, P.; Zientara, S.; et al. Reassortment between Two Serologically Unrelated Bluetongue Virus Strains Is Flexible and Can Involve any Genome Segment. *J. Virol.* **2013**, *87*, 543–557. [CrossRef]
58. Qin, S.; Yang, H.; Zhang, Y.; Li, Z.; Lin, J.; Gao, L.; Liao, D.; Cao, Y.; Ren, P.; Li, H.; et al. Full genome sequence of the first bluetongue virus serotype 21 (BTV-21) isolated from China: Evidence for genetic reassortment between BTV-21 and bluetongue virus serotype 16 (BTV-16). *Arch. Virol.* **2018**, *21*, 1–4. [CrossRef] [PubMed]
59. Allison, A.B.; Goekjian, V.H.; Potgieter, A.C.; Wilson, W.C.; Johnson, D.J.; Mertens, P.P.C.; Stallknecht, D.E. Detection of a novel reassortant epizootic hemorrhagic disease virus (EHDV) in the USA containing RNA segments derived from both exotic (EHDV-6) and endemic (EHDV-2) serotypes. *J. Gen. Virol.* **2010**, *91*, 430–439. [CrossRef] [PubMed]
60. Allison, A.B.; Holmes, E.C.; Potgieter, A.C.; Wright, I.M.; Sailleau, C.; Breard, E.; Ruder, M.G.; Stallknecht, D.E. Segmental configuration and putative origin of the reassortant orbivirus, epizootic hemorrhagic disease virus serotype 6, strain Indiana. *Virology* **2012**, *424*, 67–75. [CrossRef]
61. Anbalagan, S.; Cooper, E.; Klumper, P.; Simonson, R.R.; Hause, B.M. Whole genome analysis of epizootic hemorrhagic disease virus identified limited genome constellations and preferential reassortment. *J. Gen. Virol.* **2014**, *95*, 434–441. [CrossRef]
62. Anthony, S.J.; Maan, N.; Maan, S.; Sutton, G.; Attoui, H.; Mertens, P.P.C. Genetic and phylogenetic analysis of the non-structural proteins NS1, NS2 and NS3 of epizootic haemorrhagic disease virus (EHDV). *Virus Res.* **2009**, *145*, 211–219. [CrossRef]

63. Gonzales, H.A.; Knudson, D.L. Orbivirus Species and Speciation: Genetic Reassortment between Corriparta Serogroup Viruses. *Intervirology* **1987**, *28*, 126–133. [CrossRef]
64. Silva, S.P.; Dilcher, M.; Weber, F.; Hufert, F.T.; Weidmann, M.; Cardoso, J.F.; Carvalho, V.L.; Chiang, J.O.; Martins, L.C.; Lima, C.P.S.; et al. Genetic and biological characterization of selected Changuinola viruses (Reoviridae, Orbivirus) from Brazil. *J. Gen. Virol.* **2014**, *95*, 2251–2259. [CrossRef]
65. Xing, S.; Guo, X.; Zhang, X.; Zhao, Q.; Li, L.; Zuo, S.; An, X.; Pei, G.; Sun, Q.; Cheng, S.; et al. A novel mosquito-borne reassortant orbivirus isolated from Xishuangbanna, China. *Virol. Sin.* **2017**, *32*, 159–162. [CrossRef]
66. Coetzee, P.; Van Vuuren, M.; Stokstad, M.; Myrmel, M.; Venter, E.H. Bluetongue virus genetic and phenotypic diversity: Towards identifying the molecular determinants that influence virulence and transmission potential. *Vet. Microbiol.* **2012**, *161*, 1–12. [CrossRef] [PubMed]
67. He, C.-Q.; Ding, N.-Z.; He, M.; Li, S.-N.; Wang, X.-M.; He, H.-B.; Liu, X.-F.; Guo, H.-S. Intragenic Recombination as a Mechanism of Genetic Diversity in Bluetongue Virus. *J. Virol.* **2010**, *84*, 11487–11495. [CrossRef] [PubMed]
68. Ngoveni, H.G.; van Schalkwyk, A.; Koekemoer, J.J.O. Evidence of Intragenic Recombination in African Horse Sickness Virus. *Viruses* **2019**, *11*, 654. [CrossRef] [PubMed]
69. Farkas, S.L.; Marton, S.; Dandár, E.; Kugler, R.; Gál, B.; Jakab, F.; Bálint, Á.; Kecskeméti, S.; Bányai, K. Lineage diversification, homo- and heterologous reassortment and recombination shape the evolution of chicken orthoreoviruses. *Sci. Rep.* **2016**, *6*, 36960. [CrossRef] [PubMed]
70. Naglič, T.; Rihtarič, D.; Hostnik, P.; Toplak, N.; Koren, S.; Kuhar, U.; Jamnikar-Ciglenečki, U.; Kutnjak, D.; Steyer, A. Identification of novel reassortant mammalian orthoreoviruses from bats in Slovenia. *BMC Vet. Res.* **2018**, *14*, 1–10. [CrossRef] [PubMed]
71. Zhang, X.L.; Shao, J.W.; Li, X.W.; Mei, M.M.; Guo, J.Y.; Li, W.F.; Huang, W.J.; Chi, S.H.; Yuan, S.; Li, Z.L.; et al. Molecular characterization of two novel reoviruses isolated from Muscovy ducklings in Guangdong, China. *BMC Vet. Res.* **2019**, *15*, 1–9. [CrossRef]
72. Marthaler, D.; Suzuki, T.; Rossow, K.; Culhane, M.; Collins, J.; Goyal, S.; Tsunemitsu, H.; Ciarlet, M.; Matthijnssens, J. VP6 genetic diversity, reassortment, intragenic recombination and classification of rotavirus B in American and Japanese pigs. *Vet. Microbiol.* **2014**, *172*, 359–366. [CrossRef]
73. Bányai, K.; Borzák, R.; Ihász, K.; Fehér, E.; Dán, Á.; Jakab, F.; Papp, T.; Hetzel, U.; Marschang, R.E.; Farkas, S.L. Whole-genome sequencing of a green bush viper reovirus reveals a shared evolutionary history between reptilian and unusual mammalian orthoreoviruses. *Arch. Virol.* **2013**, *159*, 153–158. [CrossRef]
74. Day, J.M. The diversity of the orthoreoviruses: Molecular taxonomy and phylogentic divides. *Infect. Genet. Evol.* **2009**, *9*, 390–400. [CrossRef]
75. Duncan, R.; Corcoran, J.; Shou, J.; Stoltz, D. Reptilian reovirus: A new fusogenic orthoreovirus species. *Virology* **2003**, *319*, 131–140. [CrossRef]
76. Kugler, R.; Marschang, R.E.; Ihász, K.; Lengyel, G.; Jakab, F.; Bányai, K.; Farkas, S.L. Whole genome characterization of a chelonian orthoreovirus strain identifies significant genetic diversity and may classify reptile orthoreoviruses into distinct species. *Virus Res.* **2016**, *215*, 94–98. [CrossRef]
77. Janev Hutinec, B.; Mebert, K. Ecological Partitioning between Dice Snakes (Natrix tessellata) and Grass Snakes (Natrix natrix) in Southern Croatia. *Mertensiella* **2011**, *18*, 225–233.
78. Hermanns, K.; Zirkel, F.; Kurth, A.; Drosten, C.; Junglen, S. Cimodo virus belongs to a novel lineage of reoviruses isolated from African mosquitoes. *J. Gen. Virol.* **2014**, *95*, 905–909. [CrossRef] [PubMed]
79. Auguste, A.J.; Kaelber, J.T.; Fokam, E.B.; Guzman, H.; Carrington, C.V.F.; Erasmus, J.H.; Kamgang, B.; Popov, V.L.; Jakana, J.; Liu, X.; et al. A Newly Isolated Reovirus Has the Simplest Genomic and Structural Organization of Any Reovirus. *J. Virol.* **2015**, *89*, 676–687. [CrossRef] [PubMed]
80. Doherty, R.L.; Whitehead, R.H.; Wetters, E.J.; Gorman, B.M.; Carley, J.G. A survey of antibody to 10 arboviruses (Koongol group, Mapputta group and ungrouped) isolated in Queensland. *Trans. R. Soc. Trop. Med. Hyg.* **1970**, *64*, 748–753. [CrossRef]
81. Harrison, J.J.; Warrilow, D.; McLean, B.J.; Watterson, D.; O'Brien, C.A.; Colmant, A.M.G.; Johansen, C.A.; Barnard, R.T.; Hall-Mendelin, S.; Davis, S.S.; et al. A new orbivirus isolated from mosquitoes in North-Western Australia shows antigenic and genetic similarity to corriparta virus but does not replicate in vertebrate cells. *Viruses* **2016**, *8*, 141. [CrossRef] [PubMed]

82. Dilcher, M.; Hasib, L.; Lechner, M.; Wieseke, N.; Middendorf, M.; Marz, M.; Koch, A.; Spiegel, M.; Dobler, G.; Hufert, F.T.; et al. Genetic characterization of Tribeč virus and Kemerovo virus, two tick-transmitted human-pathogenic Orbiviruses. *Virology* **2012**, *423*, 68–76. [CrossRef]
83. Van Gennip, R.G.P.; Drolet, B.S.; Rozo Lopez, P.; Roost, A.J.C.; Boonstra, J.; Van Rijn, P.A. Vector competence is strongly affected by a small deletion or point mutations in bluetongue virus. *Parasit. Vectors* **2019**, *12*, 470. [CrossRef]
84. Anthony, S.J.; Maan, N.; Maan, S.; Sutton, G.; Attoui, H.; Mertens, P.P.C. Genetic and phylogenetic analysis of the core proteins VP1, VP3, VP4, VP6 and VP7 of epizootic haemorrhagic disease virus (EHDV). *Virus Res.* **2009**, *145*, 187–199. [CrossRef]
85. Anthony, S.J.; Darpel, K.E.; Belaganahalli, M.N.; Maan, N.; Nomikou, K.; Sutton, G.; Attoui, H.; Maan, S.; Mertens, P.P.C. RNA segment 9 exists as a duplex concatemer in an Australian strain of epizootic haemorrhagic disease virus (EHDV): Genetic analysis and evidence for the presence of concatemers as a normal feature of orbivirus replication. *Virology* **2011**, *420*, 164–171. [CrossRef]
86. Mohd Jaafar, F.; Goodwin, A.E.; Belhouchet, M.; Merry, G.; Fang, Q.; Cantaloube, J.F.; Biagini, P.; de Micco, P.; Mertens, P.P.C.; Attoui, H. Complete characterisation of the American grass carp reovirus genome (genus Aquareovirus: Family Reoviridae) reveals an evolutionary link between aquareoviruses and coltiviruses. *Virology* **2008**, *373*, 310–321. [CrossRef] [PubMed]
87. Tian, Y.; Tarlow, O.; Ballard, A.; Desselberger, U.; McCrae, M.A. Genomic concatemerization/deletion in rotaviruses: A new mechanism for generating rapid genetic change of potential epidemiological importance. *J. Virol.* **1993**, *67*, 6625–6632. [CrossRef] [PubMed]
88. Murao, K.; Uyeda, I.; Ando, Y.; Kimura, I.; Cabauatan, P.Q.; Koganezawa, H. Genomic rearrangement in genome segment 12 of rice dwarf phytoreovirus. *Virology* **1996**, *216*, 238–240. [CrossRef] [PubMed]
89. Huang, C.; Liu, W.J.; Xu, W.; Jin, T.; Zhao, Y.; Song, J.; Shi, Y.; Ji, W.; Jia, H.; Zhou, Y.; et al. A Bat-Derived Putative Cross-Family Recombinant Coronavirus with a Reovirus Gene. *PLoS Pathog.* **2016**, *12*, e1005883. [CrossRef] [PubMed]
90. Basak, A.K.; Gouet, P.; Grimes, J.; Roy, P.; Stuart, D. Crystal structure of the top domain of African horse sickness virus VP7: Comparisons with bluetongue virus VP7. *J. Virol.* **1996**, *70*, 3797–3806. [CrossRef]
91. Tan, B.H.; Nason, E.; Staeuber, N.; Jiang, W.R.; Monastryrskaya, K.; Roy, P. RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to *Culicoides* cells. *J. Virol.* **2001**, *75*, 3937–3947. [CrossRef]
92. Tomori, O.; Fabiyi, A.; Murphy, F. Characterization of Orungo virus, an orbivirus from Uganda and Nigeria. *Arch. Virol.* **1976**, *51*, 285–298. [CrossRef]
93. Tomori, O.; Aitken, T.H.G. Orungo Virus: Transmission Studies with *Aedes Albopictus* and *Aedes Aegypti* (Diptera: Culicidae)1. *J. Med. Entomol.* **1978**, *14*, 523–526.
94. Mellor, P.S.; Jennings, M. Replication of Eubenangee virus in *Culicoides nubeculosus* (Mg.) and *Culicoides variipennis* (Coq.). *Arch. Virol.* **1980**, *63*, 203–208. [CrossRef]
95. Ebersohn, K.; Coetzee, P.; Snyman, L.P.; Swanepoel, R.; Venter, E.H. Phylogenetic characterization of the Palyam serogroup orbiviruses. *Viruses* **2019**, *11*, 446. [CrossRef]

Figure S1: Protein sequence alignment of orthonairovirus nucleocapsid. Green arrows represent the predicted stalk region. Nucleic acid binding sites are annotated with yellow boxes

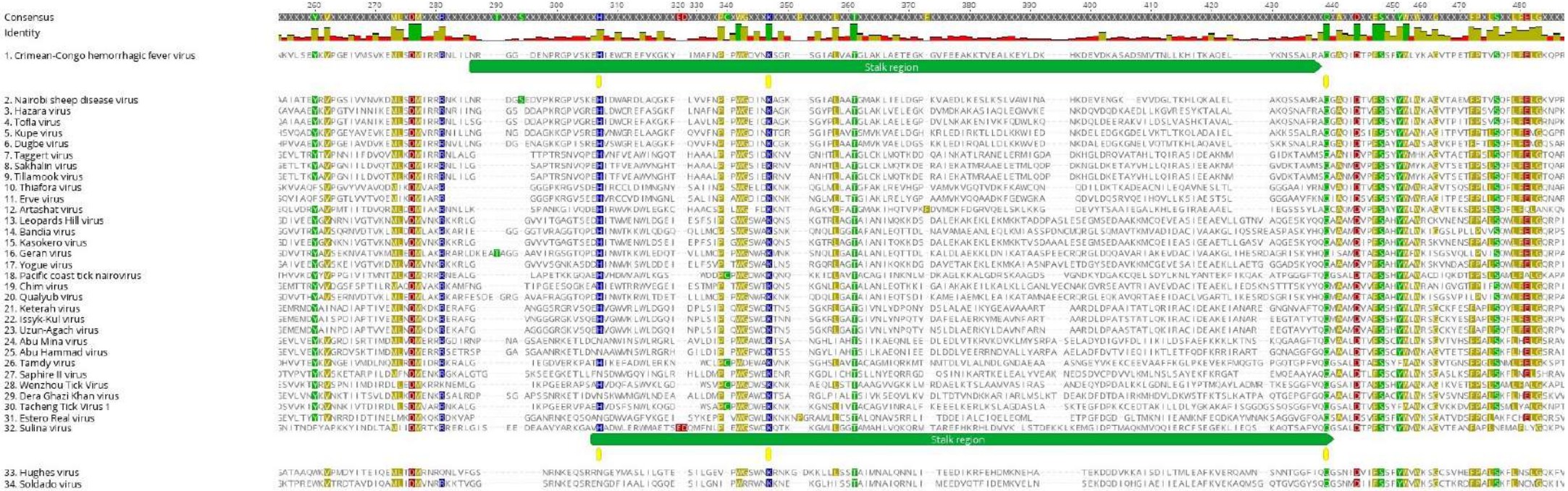


Figure S2: Protein sequence alignment of orthonairovirus nucleocapsid's domain with predicted endonuclease activity. Nucleic acid binding sites are annotated with yellow boxes

Figure S3: Protein sequence alignment of orthonairovirus Gn. Turquoise arrows represent predicted zinc fingers.

Figure S4-1: Protein alignment of orthonairovirus RdRp: conserved region III (N-terminal).

Figure S4-2: Protein alignment of orthonairovirus RdRp: conserved region III (middle) - premotif A and motif A

Figure S4-3: Protein alignment of orthonairovirus RdRp: conserved region III (C-terminal) - motifs B-E

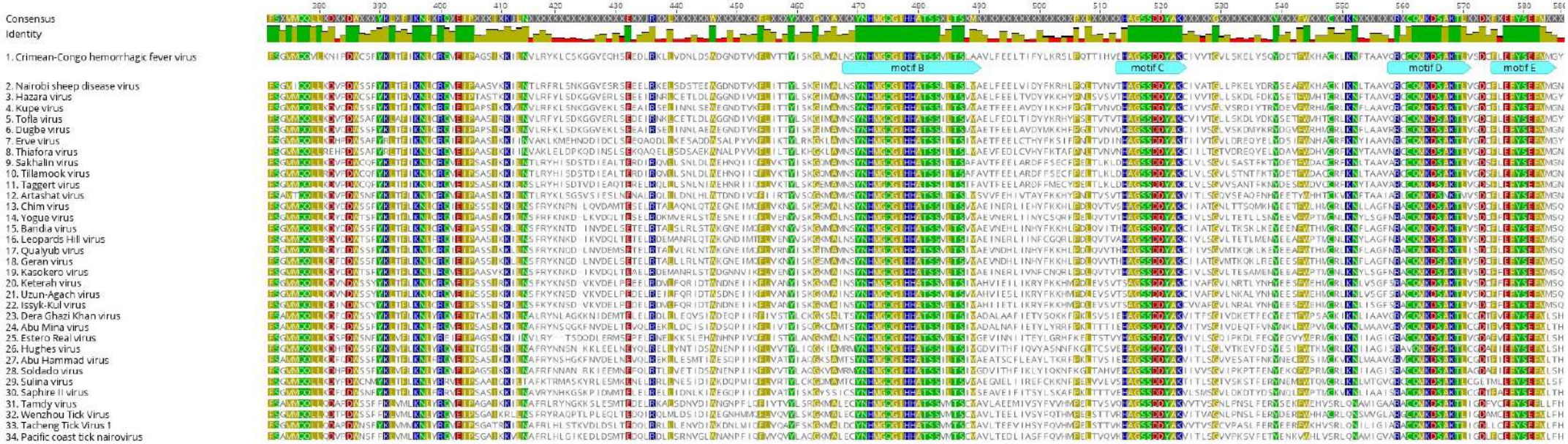
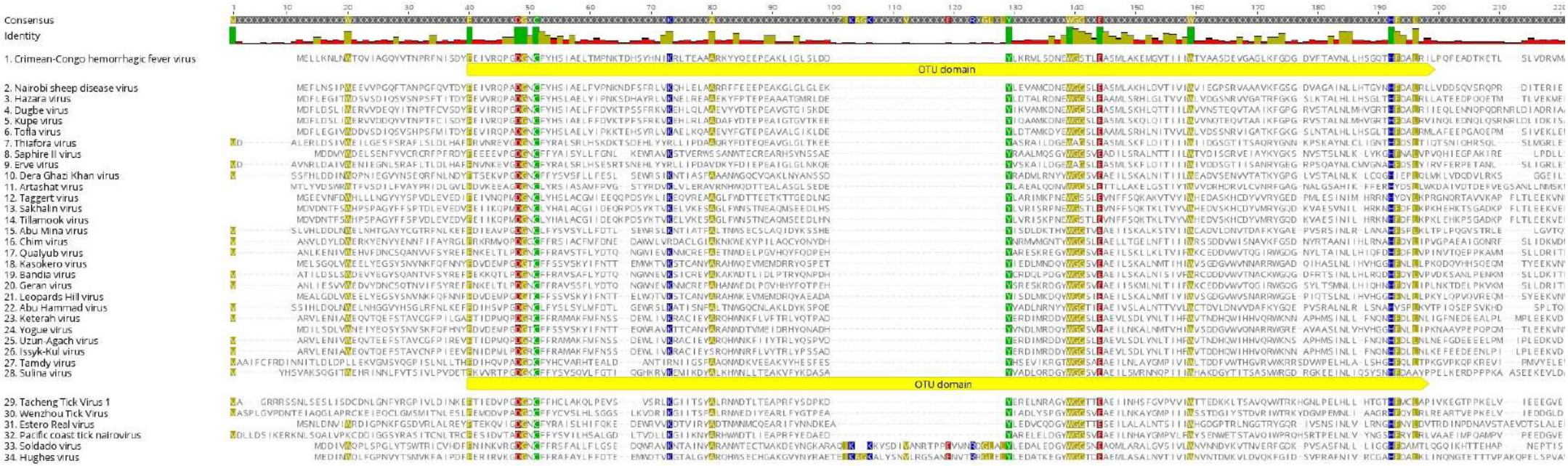


Figure S5: Protein alignment of orthonairovirus RdRp: ovarian tumor (OTU)-like protease domain



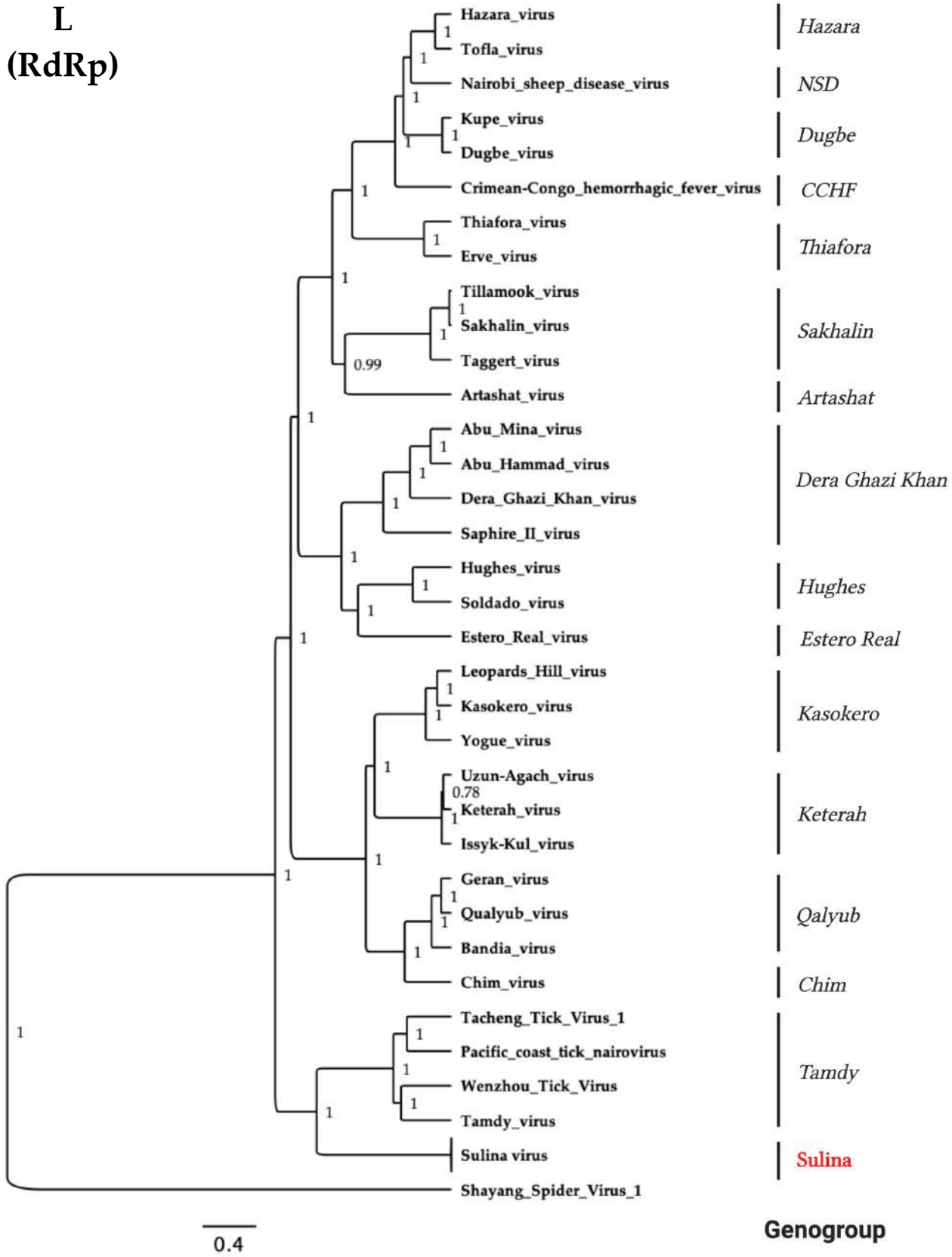


Figure S6a: Bayesian phylogenetic tree based on L protein sequences of orthonairovirus representatives and Sulina virus (in red). The tree was inferred using the LG+I+G+F substitution model and scale bar indicates substitutions per site.

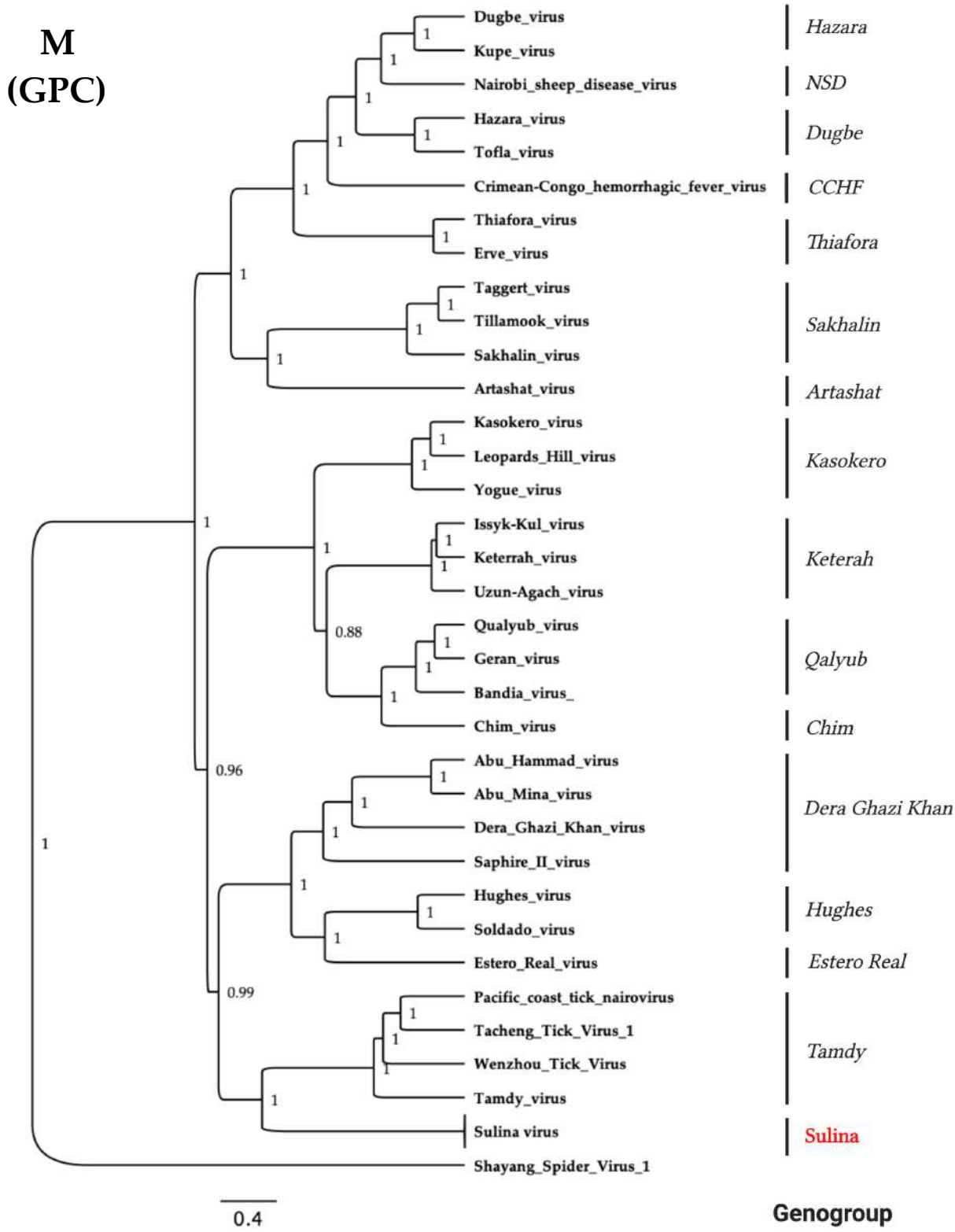


Figure S6b: Bayesian phylogenetic tree based on M protein sequences of orthonairovirus representatives and Sulina virus (in red). The tree was inferred using the LG+I+G substitution model and scale bar indicates substitutions per site.

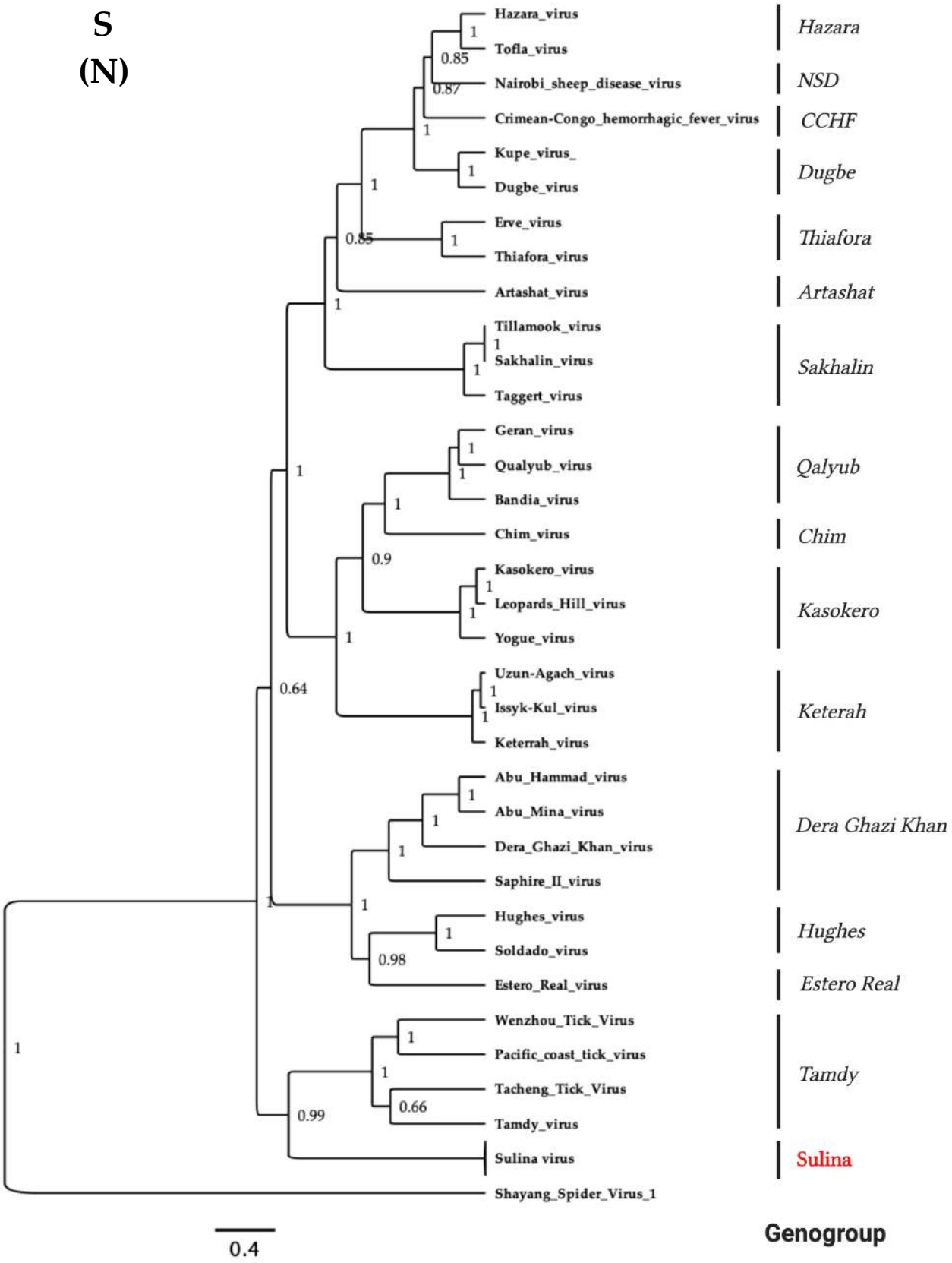


Figure S6c: Bayesian phylogenetic tree based on S protein sequences of orthonairovirus representatives and Sulina virus (in red). The tree was inferred using the LG+I+G substitution model and scale bar indicates substitutions per site.

Table S1. Overview of ticks (2014-2017) and dog blood samples (2016) analysed by ELISA for detection of Sulina virus antibodies

species / n		*Canis lupus familiaris*	*Felis catus*	*Erinaceus europaeus*	*Lacerta agilis*
Rhipicephalus sanguineus s.l.	115	105	6	4	-
Rhipicephalus rossicus	85	82	-	3	-
Rhipicephalus annulatus	0	0	-	-	-
Ixodes ricinus	19	0	-	-	19
Hyalomma marginatum	10	10	-	-	-
Dermacentor reticulatus	1	1	-	-	-
unidentified nymphae	19	19	-	-	-
total ticks, 2014	249	217	6	7	19

species / n		*Canis lupus familiaris*	*Felis catus*	*Equus caballus*
Rhipicephalus sanguineus s.l.	341	339	1	1
Rhipicephalus rossicus	157	157	-	-
Dermacentor reticulatus	4	4	-	-
total ticks, 2015	502	500	1	1

species / n		*Canis lupus familiaris*	*Equus caballus*	*Erinaceus europaeus*	*Bos taurus*	*Homo sapiens*	*Acrocephalus palustris*	*Ficedula albicolis*	*Iduna pallida*	*Ixobricus minutus*	*Lanius collurio*	*Phoenicurus phoenicurus*	*Saxicola rubetra*	*Sturnus vulgaris*	*Sylvia atricapilla*	*Sylvia communis*	*Sylvia curuca*	*Turdus merula*
Rhipicephalus sanguineus s.l.	136	132	-	-	3	1	-	-	-	-	-	-	-	-	-	-	-	-
Rhipicephalus rossicus	95	95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhipicephalus sp.	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Rhipicephalus annulatus	66	-	3	-	63	-	-	-	-	-	-	-	-	-	-	-	-	-
Ixodes ricinus	52	48	-	-	1	1	-	-	-	-	-	-	-	1	-	1	-	-
Hyalomma marginatum	17	-	-	-	-	-	2	1	1	2	2	1	1	-	1	4	1	1
Dermacentor reticulatus	8	7	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
total ticks, 2016	**375**	**282**	**3**	**0**	**67**	**3**	**2**	**1**	**1**	**2**	**2**	**1**	**1**	**1**	**2**	**5**	**1**	**1**

species / n		*Canis lupus familiaris*	*Bos taurus*	*Lacerta agilis*
Rhipicephalus sanguineus s.l.	67	56	11	-
Rhipicephalus rossicus	83	83	-	-
Rhipicephalus annulatus	3	-	3	-
Ixodes ricinus	21	-	5	16
Hyalomma marginatum	2	-	2	-
Dermacentor reticulatus	1	1	-	-
Dermacentor marginatus	2	2	-	-
total ticks, 2017	**179**	**142**	**21**	**16**

Blood samples collected from domestic dogs (2016)

Collection site	n	%
Sulina	15	*38.5*
Letea	13	*33.5*
Dunărea Veche	7	*17.9*
Lake Roșuleleț	2	*5.1*
Sfiștofca	2	*5.1*
total blood samples	**39**	***100***

Table S2: GenBank accession numbers of orthonairovirus sequences used for phylogenetic analyses

Species	Virus	S segment	M segment	L segment
Artashat orthonairovirus	**Artashat virus (ARTSV)**	AKC89354	YP_009666121	AKC89352
Chim orthonairovirus	**Chim virus (CHIMV)**	YP_009666114	YP_009666115	YP_009666113
CCHF orthonairovirus	**CCHFV**	ABB30040	ABB30026	ABB30024
Dera Ghazi Khan orthonairovirus	**Abu Hammad virus (AHV)**	AMT75373	AMT75372	AMT75371
	Abu Mina virus (AMV)	AMT75376	AMT75375	AMT75374
	Dera Ghazi Khan virus (DGKV)	AMT75391	AMT75390	AMT75389
	Saphire II virus (SAPV)	AMT75424	AMT75423	AMT75422
Dugbe orthonairovirus	**Dugbe virus (DUGV)**	AAL73397	AMT75393	NP_690576
	Kupe virus (KUPEV)	ABY82500	ABY82501	ABY82502
Estero Real orthonairovirus	**Estero Real virus (ERV)**	AXP33557	AXP33569	AXP33563
Hazara orthonairovirus	**Hazara virus (HAZV)**	YP_009507852	YP_009507851	YP_009507850
	Tofla virus (TFLV)	YP_009227120	YP_009227121	YP_009227122
Hughes orthonairovirus	**Hughes virus (HUGV)**	AMT75409	AMT75408	AMT75407
	Soldado virus (SOLV)	AMT75427	AMT75426	AMT75425
Kasokero orthonairovirus	**Kasokero virus (KASV = KASOV)**	YP_009246487	YP_009246488	YP_009449567
	Leopards Hill virus (LPHV)	YP_009111286	BAP90969	BAP90968
	Yogue virus (YOGV)	YP_009246491	YP_009246490	YP_009246486
Keterah orthonairovirus	**Issyk-kul virus (ISKV)**	ALD84348	ALD84347	ALD84346
	Keterah virus (KTRV)	YP_009361834	YP_009361833	YP_009361838
	Uzun-Agach virus (UZAV)	AKC89315	AKC89314	AKC89313
Nairobi sheep disease orthonairovirus	**Nairobi sheep disease virus (NSDV)**	AED88237	YP_009361837	YP_009361832
Qalyub orthonairovirus	**Bandia virus (BDAV)**	AMT75385	AMT75384	AMT75383
	Geran virus (GERV)	AKC89342	AKC89341	AKC89340
	Qalyub virus (QYBV)	AMT75415	AMT75414	AMT75413
Sakhalin orthonairovirus	**Sakhalin virus (SAKV)**	AMT75421	AKC89335	AMT75419
	Taggert virus (TAGV)	AMR73397	AMT75429	AMR73395
	Tillamook virus (TILLV)	AMT75433	AMT75432	AMT75431
Tamdy orthonairovirus	**Tǎchéng tick virus 1 (TcTV-1)**	QEI22773	QEI22769	QEI22765
	Tamdy virus (TAMV)	QFU19354	QFU19353	QFU19352
	Wēnzhōu tick virus (WzTV)	YP_009304991	YP_009304990	YP_009304993
Thiafora orthonairovirus	**Erve virus (ERVEV)**	AFH89034	AFH89033	AFH89032
	Thiafora orthonairovirus (TFAV)	ALD84357	ALD84356	YP_009513191
	Pacific coast tick nairovirus (PCTN)	ARF07705	ARF07703	ARF07704
Spider shaspivirus	**Shāyáng spider virus 1 (SySV-1)**	YP_009300679	AJG39282	YP_009300680