Systematics and Evolution of the Helicellinae (Gastropoda:Helicoidea) from Crete, particularly the *Xerocrassa* radiation

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Re: English Language Evaluation of the Ph.D. thesis of JAN SAUER

To whom it may concern,

As an English native speaker, I have read the thesis of Mr. Jan Sauer entitled "Systematics and Evolution of the Helicellinae (Gastropoda: Helicoidea) from Crete, particularly the *Xerocrassa* radiation". I herby confirm that the English employed in this thesis is correct and clear in both grammar and content.

Yours Sincerely,

David K A Barnes

(British Antarctic Survey, NERC, Cambridge, UK)

INHALT

SUMMARY

Radiations are systems that are especially suitable to study speciation. I investigated a radiation of xerophilous land snails on Crete to understand the underlying speciation mechanisms. Delimiting the species to be studied correctly is of outstanding importance for the analysis of speciation mechanisms. Thus, I revised the species of the subfamily Helicellinae (Gastropoda: Hygromiidae) from Crete using characters of the shell and the genitalia as basis for the evolutionary investigations in my dissertation. The revision uncovered a radiation of the genus *Xerocrassa* with ten endemic species, six of which were new to science. Additionally, the native fauna of Crete includes one more widespread *Xerocrassa* species, two *Pseudoxerophila* species and one *Xeromunda* species. Moreover, seven species of the genera *Trochoidea*, *Xerocrassa*, *Xeropicta*, *Xerotricha*, *Microxeromagna* and *Cernuella* have probably been introduced to Crete by man.

Recently, several approaches for delimiting species have been proposed, that utilize single-locus DNA sequences or multi-locus data. I compared the results of different approaches and different markers (single-locus DNA sequences, multi-locus data and morphological characters) for the delimiting species of the Cretan *Xerocrassa* radiation in the second chapter. I looked for congruence between the partitions of the examined specimens obtained by different analytical methods based on different data to infer the “true” species limits. The highest similarity between species partitions based on different datasets was found between the results of Gaussian clustering of amplified fragment length polymorphisms (AFLP) data and the morphological classification. Species delimitation based on mtDNA sequences using several different methods (e.g. fixed pairwise distance threshold or statistical parsimony anaylsis) resulted in an extensive splitting into putative species as a consequence of high substitution rates of mtDNA in helicoid land snails. However, species classifications based exclusively on single-locus data might show idiosyncrasies resulting from incomplete lineage sorting, introgression, or random phylogeographic breaks. My results demonstrate that species delimitation should be based on an analysis of several independent markers.

In the third chapter, I inferred the evolutionary history of the *Xerocrassa* radiation on Crete and the reasons for the nonmonophyly of several species in the mitochondrial gene tree. This was done by comparing this gene tree with a tree and network based on AFLP markers. In addition, an admixture analysis of these multi-locus data was performed. Whereas six of the eleven morphologically delimited *Xerocrassa* species are monophyletic in the mitochondrial gene tree, nine of these species are monophyletic in the tree based on AFLP markers. Only two morphologically delimited species could not be distinguished with the

AFLP data and might have diverged very recently or might represent extreme forms of a single species. The nonmonophyly of *X. mesostena* and *X. rhithymna* is probably the result of incomplete lineage sorting of ancestral polymorphisms, because mitochondrial haplotype groups of these species are deeply separated and there is no evidence for admixture with other species in the AFLP data. As the strongly subdivided population structure increases the effective population size, ancestral polymorphisms may persist far longer in many land snail species than in species consisting of more or less panmictic populations. The nonmonophyly of *X. franciscoi* and *X. amphiconus* might be the result of mitochondrial introgression, because the coalescences of some haplotypes of these species with some *X. mesostena* haplotypes are shallow.

In the forth chapter, I investigated the phylogeography of the endemic species *X. mesostena* from Crete using *cox1* sequences and AFLP markers. The AFLP data revealed a distinct phylogeographic subdivision of the range of *X. mesostena* that corresponds at least partly with current barriers. Within the geographical clusters, the genetic variation is structured partly by isolation by distance. The variation in the mitochondrial data is also dominated by a subdivision in geographical clusters. However, the mitochondrial haplotype groups correspond only partly with the geographical clusters delimited with the multilocus data. In some cases, phylogenetic breaks in the mitochondrial data differ only slightly from the geographic boundaries of the AFLP based clusters, whereas other phylogeographic breaks do not correspond with patterns in the multi-locus data. By excluding alternative possibilities, I corroborate the hypothesis that some of these boundaries represent random phylogenetic breaks. The comparison of the phylogeographic patterns inferred from mitochondrial *cox1* sequences and the AFLP markers in *X. mesostena* support the suggestion that phylogeographic patterns found with single locus markers - especially mitochondrial DNA - might not reflect the phylogeographic structure of a species correctly and should be supplemented by data from multiple independent loci.

In the fifth chapter, I investigated the importance of sexual selection in facilitating speciation in the *Xerocrassa* radiation on Crete. I used differences in the genitalia of the *Xerocrassa* species as potential indices of sexual selection. First, I rejected the hypothesis that differences in the genitalia can be explained by genetic drift using coalescent simulations based on the mitochondrial gene tree. Second, I showed that there is no evidence for the hypothesis that the differences in the genitalia can be explained by natural selection against hybrids under the assumption that this is more likely in geographically overlapping species pairs and clades. Thirdly, I showed that there is a positive scaling between male

spermatophore producing organs and female spermatophore receiving organs indicating sexual co-evolution. As the spermatophore enables the sperm to escape from the female gametolytic organ, the co-evolution might be a consequence of sexual conflict or cryptic female choice. Finally, I showed that the evolution of differences in the length of the flagellum that forms the tail of the spermatophore is involved in speciation.

In the sixth chapter, I investigated which additional factors might have caused the high species diversity of land snails on Crete. It has been suggested that these land snail radiations were triggered by the fragmentation of Crete in the Neogene. Contrary to the predictions of this model, the ranges of all endemic land snail species occurring on Crete are not clustered and their diversity is not higher in the areas of the Neogene paleoislands. For the genus *Xerocrassa* I showed that the geographic speciation mode was predominantly allopatric. Furthermore, I showed that the range size of sister species clades of *Xerocrassa* are asymmetric indicating that peripatric speciation was the predominant geographic speciation mode. In addition, I presented evidence that body size is involved in the competition between co-occurring species but that changes in the body size were not involved in the speciation process.

ZUSAMMENFASSUNG

Radiationen eignen sich besonders gut, um Artbildungsprozesse zu erforschen. Für meine Dissertation habe ich eine Radiation von xerophilen Landschnecken auf Kreta ausgewählt, um die Mechanismen, welche zur Artbildung führen, zu untersuchen. Für die Analyse von Artbildungsmechanismen ist die richtige Abgrenzung von Arten von größter Wichtigkeit. Deshalb habe ich die Arten der Unterfamilie Helicellinae (Gastropoda: Hygromiidae) von Kreta mit Hilfe von Gehäuse- und Genitalmerkmalen revidiert. Diese auf morphologischen Merkmalen basierte Revision bildet die Grundlage für die evolutionsbiologischen Fragestellungen. Anhand der morphologischen Revision konnte ich eine Radiation der Gattung *Xerocrassa* auf Kreta identifizieren. Diese Radiation umfasst zehn endemische *Xerocrassa* Arten, von denen sechs Arten im Rahmen meiner Dissertation neu beschrieben wurden. Des Weiteren umfasst die einheimische Landschneckenfauna Kretas eine weitere, nicht endemische *Xerocrassa* Art sowie zwei Arten der Gattung *Pseudoxerophila* und eine *Xeromunda* Art. Zusätzlich kommen weitere sieben Arten aus den Gattungen *Trochoidea*, *Xerocrassa*, *Xeropicta*, *Xerotricha*, *Microxeromagna* und *Cernuella* vor, welche durch den Menschen nach Kreta eingeführt wurden.

In den letzen Jahren wurden mehrere Methoden für die Abgrenzung von Arten vorgeschlagen, die auf Single-Locus DNA-Sequenzen bzw. Multi-Locus Daten basieren. Ich habe im zweiten Kapitel verschiedene Methoden angewendet, um die Arten der *Xerocrassa* Radiation auf Kreta abzugrenzen. Bei diesem Methodenvergleich wurden verschiedene Marker, nämlich Single-Locus DNA-Sequenzen (mitochondriale DNA), Multi-Locus Daten (AFLP) sowie morphologische Merkmale, verwendet. Übereinstimmungen der Ergebnisse, welche mit den verschiedenen Markern und Methoden erzielt wurden, deuten auf eine richtige Abgrenzung der Arten hin. Die größte Übereinstimmung ergab sich zwischen den Ergebnissen der morphologischen Klassifikation und der Gaußschen Clusterung der AFLP-Daten. Arteinteilungen basierend auf mitochondrialen DNA-Sequenzen resultierten - unabhängig von der verwendeten Methode wie z.B. „paarweise Distanzschwellenwerte“ oder „Statistische Parsimony Analyse“ - immer in einer deutlich größere Artenzahl als bei den anderen verwendeten Markern (AFLP, Morphologie). Dies kann auf die erhöhten Substitutionsraten mitochondrialer DNA in helicoiden Landschnecken zurückgeführt werden. Wenn Arteinteilung ausschließlich auf einer einzigen DNA-Sequenz beruht, können Probleme wie Introgression, Anzestrale Polymorphismen oder zufällige phylogeographische Brüche zu Fehlern in der Arteinteilung führen. Meine Ergebnisse für die Gattung *Xerocrassa*

verdeutlichen, dass es sinnvoll ist, die Einteilung von Arten durch eine Kombination mehrer unabhängiger Merkmale abzusichern.

Im dritten Kapitel wurde die Phylogenie der Gattung *Xerocrassa* auf Kreta rekonstruiert und die Gründe untersucht, die für die Para- bzw. Polyphylie mehrerer Arten in dem mitochondrialen Genbaum in Frage kommen. Dafür wurde der *cox1*-Genbaum mit einer AFLP-basierten Phylogenie und einem AFLP-basierten Netzwerk verglichen. Zusätzlich wurden „admixture" Analysen des AFLP-Datensatzes durchgeführt. Nur sechs der elf morphologisch abgegrenzten Arten sind im *cox1*-Genbaum monophyletisch, während neun Arten in der AFLP-basierten Phylogenie sowie dem AFLP-basierten Netzwerk monophyletisch sind. Nur die beiden Arten *X. amphiconus* und *X. siderensis* können anhand des AFLP Datensatzes nicht unterschieden werden. Dies deutet möglicherweise darauf hin, dass sich die beiden Arten erst kürzlich aufgespalten haben oder aber zwei extreme Formen der gleichen Art darstellen. Die Para- bzw. Polyphylie von *X. mesostena* und *X. rhithymna* im mitochondrialen Genbaum ist hingegen wahrscheinlich durch das Vorhandensein von anzestralen Polymorphismen bedingt, da die mitochondrialen „Haplotypengruppen" tief voneinander getrennt sind. Zudem deuten auch die Analysen der AFLP-Daten nicht auf Hybridisierung mit anderen Arten hin. Da stark fragmentierte Populationsstrukturen dazu führen, dass die effektive Populationsgröße zunimmt, können anzestrale Polymorphismen deutlich länger in Landschneckenpopulationen bestehen als in anderen Taxa mit besseren Ausbreitungsfähigkeiten. Die nicht vorhandene Monophylie von *X. franciscoi* und *X. amphiconus* im *cox1*-Genbaum ist wahrscheinlich durch Introgression von Mitochondrien aus *X. mesostena* zu erklären, da zwischen Haplotypen von *X. mesostena* und *X. franciscoi* bzw. *X. amphiconus* sehr junge Aufspaltungsereignisse vorliegen.

Im vierten Kapitel habe ich die phylogeographische Struktur innerhalb der endemischen Landschneckenart *X. mesostena* basierend auf *cox1* DNA-Sequenzen und AFLP-Daten untersucht. Die AFLP-Daten wiesen auf eine deutliche geographische Gliederung des Verbreitungsgebietes hin. Diese Gliederung entspricht zumindest teilweise heutigen geographischen Barrieren. Innerhalb der geographischen Cluster kann die genetische Variabilität zum Teil durch das Phänomen Isolation durch Entfernung erklärt werden. Die mitochondrialen Daten weisen ebenfalls auf eine starke geographische Gliederung hin. Jedoch stimmen die genetischen Cluster, die auf dem mitochondrialen Datensatz beruhen, nur zum Teil mit den genetischen Clustern überein, die auf den AFLP-Daten beruhen. Während manche phylogeographischen Brüche zwischen den beiden genetischen Datensätzen nur leicht von einander abweichen, weisen andere Brüche keinerlei Übereinstimmung auf. Mit meinen

Untersuchungen untermauere ich die Hypothese, dass manche dieser vorhandenen genetischen „Grenzen“ nicht auf tatsächlichen geographischen Barrieren beruhen, sondern zufällige phylogeographische Brüche sind. Durch den Vergleich zweier verschiedener molekularer Marker konnte ich zeigen, dass die phylogeographischen Verteilungsmuster von Individuen, die unter Zuhilfenahme eines Single-Locus Marker rekonstruiert wurden, nicht der tatsächlichen phylogeographischen Struktur einer Art entsprechen müssen. Dies ist insbesondere zu berücksichtigen, wenn es sich bei dem verwendeten Single-Locus Marker um mitochondriale DNA handelt. Am besten sollten phylogeographische Analysen immer auf mehreren, voneinander unabhängigen Loci beruhen.

Im fünften Kapitel habe ich untersucht, ob sexuelle Selektion eine entscheidende Rolle bei der Artbildung innerhalb der *Xerocrassa* Radiation auf Kreta gespielt hat. Dafür habe ich die Unterschiede zwischen den Genitalien verschiedener *Xerocrassa* Arten herangezogen. Mit Hilfe von verschiedenen Analysemethoden habe ich Alternativhypothesen ausgeschlossen, die ebenfalls für Unterschiede zwischen den Genitalien verantwortlich sein könnten. Durch Koaleszenz-Simulationen, die auf einer mitochondrialen Phylogenie basieren, konnte genetische Drift als Erklärung ausgeschlossen werden. Unter der Annahme, dass natürliche Selektion gegen Hybride bei sich geographisch überlappenden Arten oder Kladen wahrscheinlicher ist, konnte ich keine Hinweise dafür finden, dass die Unterschiede zwischen den Geschlechtsorganen der verschiedenen Arten durch natürliche Selektion gegen Hybride erklärt werden können. Des Weiteren war es mir möglich aufzuzeigen, dass die männlichen Spermatophoren produzierenden Organe positiv mit den weiblichen Spermatophoren aufnehmenden Organen korreliert sind. Dies deutet auf Co-Evolution hin. Da die Spermatophore den Spermien ermöglicht, aus dem gametolytischen Organ zu entkommen, deutet diese Co-Evolution darauf hin, dass es sich hierbei entweder um einen sexuellen Konflikt oder aber kryptische Weibchenwahl handelt. Die Länge des Flagellums, das den Schwanz der Spermatophore bildet, spielt für die Artbildung eine entscheidende Rolle. Das deutet darauf hin, dass vermutlich sexueller Konflikt und nicht die kryptische Weibchenwahl maßgeblich die Artbildung innerhalb der Radiation der *Xerocrassa* Arten beeinflusst hat.

Im letzten Kapitel meiner Dissertation untersuchte ich, welche Faktoren die extrem hohe Landschneckendiversität auf Kreta verursacht haben könnten. Eine Hypothese erklärt die enorme Artendiversität mit der Fragmentierung Kretas während des Neogens. Im Gegensatz zu den Annahmen dieses Modells sind die Verbreitungsgebiete der endemischen Landschneckenarten von Kreta nicht geclustert und die Anzahl der Arten in Gebieten, die im Neogen Paläoinseln entsprachen, ist ebenfalls nicht größer. Ich konnte für die Gattung

Xerocrassa auf Kreta zeigen, dass der vorherrschende geographische Artbildungsmodus allopatrisch war. Des Weiteren weist die Größe der Verbreitungsgebiete eine deutliche Asymmetrie zwischen Schwesterarten auf, was auf peripatrische Artbildung hindeutet. Außerdem konnte ich zeigen, dass Unterschiede in der Gehäusegröße wichtig für die Koexistenz von Arten sind, dieses jedoch nicht zu Artbildung geführt hat.

General Introduction

The species is the fundamental unit in biological sciences. The process by which they arise is termed speciation. When studying the systematics of a species group it is expedient to include also evolutionary aspects which are involved in the formation of species. Radiations are systems that are especially suitable to study speciation events. A radiation is the rapid diversification of a single ancestor into multiple species (Schluter 2000). Radiations can be classified as adaptive or non-adaptive, depending on whether the radiation is caused or at least accompanied by ecological specialization or not (Gittenberger 1991, 2004). Vicariance events such as rising sea level leading to the fragmentation of a larger island into smaller islets can cause allopatric speciation. The speciation processes due to the described vicariance event are not necessarily accompanied by ecological adaptation because such fragmentation will not necessarily result in different habitats on the different islands. Hence, if the habitats are very similar it is unlikely that adaptation to different ecological niches occurs. Thus, the constant maintenance of ecological niches and the occurrence of vicariance events can lead to non-adaptive radiations (Kozak *et al.* 2006). However, adaptive and non-adaptive radiations are not mutually exclusive processes. For the cichlid fishes of Lake Malawi three different stages of radiation could be shown (Kocher 2004). Within the first two phases of the radiation the cichlid fishes adapted to different habitats and to different trophic resources, whereas the third phase was associated with differentiation of male nuptial colouration, most likely caused by divergent sexual selection. This non-adaptive stage has significantly contributed to the extraordinary species richness of the group (Albertson *et al.* 1999; Danley & Kocher 2001; Kocher 2004). Although non-adaptive radiations have traditionally received less attention, evidence from recent studies suggest that divergence in reproductive phenotypes has played a critical role in several radiations (Kocher 2004; Parmakelis *et al.* 2005; Shaw 1996; Turgeon & McPeek 2002).

To study the processes driving radiations, the correct delimitation and identification of species are of outstanding importance to subsequently correctly infer the speciation processes within the radiation.

The organisms studied in this thesis are land snails from Crete (for location of the study area see Figure 1).

Fig. 1 Location of the study area.

Because of the extraordinary high species richness within land snails occurring on Crete the area and taxon are well suited to study the processes underlying radiations. Compared to other Aegean islands the species number on Crete is much higher than would have been expected for its area (Welter-Schultes & Williams 1999). One of the hypotheses which could explain this extraordinary rich land snail fauna refers to the past fragmentation of Crete into several paleoislands during the Neogene (Douris *et al.* 1998; Welter-Schultes & Williams 1999). This vicariance event may have resulted in allopatric speciation on the different paleoislands. After the reunion of the paleoislands, present day Crete inhabitants are quasi-endemics of the past paleo-islands (see Figure 2).

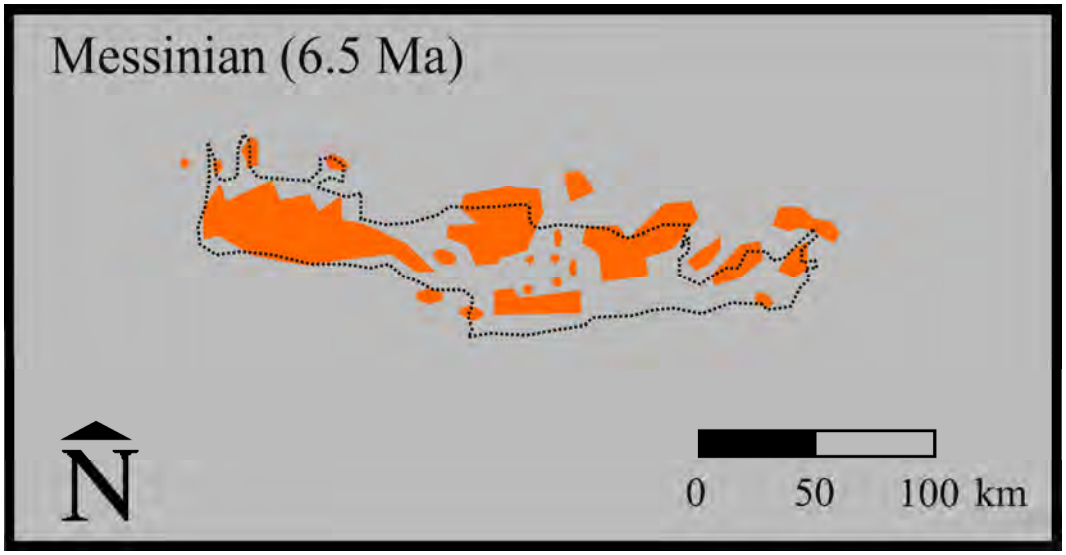

Fig. 2 Proposed land of the area of Crete during the late Miocene (modified from Welter-Schultes & Williams 1999)

The high species number of land snails on Crete is mainly due to a few radiations, e.g., of the genera *Albinaria* (Douris *et al.* 1998; Nordsieck 2004; Schilthuizen & Gittenberger 1996; Schilthuizen. *et al.* 2004; Welter-Schultes 2000a; Welter-Schultes 2000b), *Mastus* (Maassen 1995; Parmakelis *et al.* 2005) and *Orculella* (Gittenberger & Hausdorf 2004). An additional radiation occurring on Crete, the radiation of xerophilous hygromiid land snails, has been identified by Maltzan (1883, 1887). The latter, however, has been almost neglected across the literature. The aim of this thesis was to investigate the radiation of the xerophilous land snails from Crete belonging to the Helicellinae (Gastropoda: Helicoidea: Hygromiidae), with particular focus on the genus *Xerocrassa* Monterosato, 1892.

Both how many xerophilous hygromiid land snail species exist on Crete and how they can be differentiated were poorly known. Even the allocation to different genera was impossible as the anatomy has not been studied. Also little has been researched about the geographical ranges of xerophilous hygromiids species on Crete. So, in the first chapter of my thesis I focused on a morphological revision of the Helicellinae (Gastropoda: Hygromiidae) from Crete. Furthermore, a biogeographic analysis based on the distribution data of the endemic species of the land snail genera *Xerocrassa* and *Pseudoxerophila* was performed in order to test the hypothesis that the past fragmentation of Crete drove the radiation in hygromiid land snails.

The correct delimitation of species is of great importance in biology, because most empirical findings are originally referred to the specific species in which they were observed. Therefore in the second chapter I compared the performance of different methods for delimiting species using different molecular markers (mitochondrial DNA sequences and multilocus data) and morphological characters of the shell and the genitalia within the land snail genus *Xerocrassa* from Crete. Besides comparison of the different methods proposed I evaluated whether the sole use of a single mitochondrial gene fragment is suitable for species identification (DNA barcoding (Hebert *et al.* 2003)) or even as taxonomic reference system (DNA taxonomy; (Tautz *et al.* 2003) within the snail genus *Xerocrassa* from Crete.

The reconstruction of the evolutionary history of closely related species can be complicated by shared ancestral polymorphisms, introgression or inadequate taxonomy (Funk & Omland 2003). These described problems can result in discrepancies between gene trees and species classification based on other data. In the third chapter I investigated which of these problems can cause the nonmonophyly of morphologically defined species (see chapter 1) in a mitochondrial gene tree of the *Xerocrassa* species from Crete to elucidate. Therefore I compared the mitochondrial gene tree of the Cretan *Xerocrassa* radiation based on cytochrome c oxidase subunit 1 (*cox1*) sequences with the morphological species classification (chapter 1) and a tree and network based on amplified fragment length polymorphism (AFLP).

Historical geographic processes such as population division, range expansion and long distance colonization are expected to produce distinct pattern in the distribution of alleles and relationship between them (Templeton *et al.* 1995). Hence it is reasonable to infer those processes from pattern of genetic variation (Irwin 2002). In the fourth chapter I used mitochondrial DNA (*cox1*) sequences and AFLP markers to examine phylogeographic structure in the most widespread endemic *Xerocrassa* species on Crete, *Xerocrassa mesostena*. I compared the performance of the different molecular markers used to infer the phylogeographic history within *X. mesostena*. Additionally, I tested to what extent the phylogeographic patterns can be explained by recent or past geographic barriers or was shaped by other phenomena like isolation by distance or passive long-distance colonization events.

Ever since Darwin (1871), it has been suggested that sexual selection might increase the rate of reproductive divergence between populations thereby driving speciation and increasing diversity (Boul *et al.* 2007; Carson 1997; Dominey 1984; Gavrilets 2000; Gray & Cade 2000; Lande 1981; Masta & Maddison 2002; Panhuis *et al.* 2001; Price 1998; Ritchie 2007; Schluter & Price 1993; West-Eberhard 1983). In the fifth chapter I investigated whether sexual selection was associated with the radiation of *Xerocrassa* on Crete. I first tried to exclude alternative causes of changes in the genitalia, namely genetic drift and natural selection against hybrids. Afterwards I tested whether the differences in the genitalia are the result of sexual selection and whether the changes in the genitalia were associated with speciation. Additionally, I examined whether changes of the male spermatophore producing organs and the female spermatophore receiving organs are correlated indicating sexual co-evolution.

Finally, in chapter six I corroborated the results of former chapters with more detailed biogeographic analysis. I tested the predictions of different geographic speciation modes within the Cretan *Xerocrassa* radiation and discussed the results with regard to the hypothesis that the past fragmentation of Crete into several paleo-islands (Welter-Schultes & Williams 1999) caused the high species diversity of land snails there. I summarized my results about the ecology, the biogeography and the potential role of sexual selection in a previously poorly known land snail radiation on Crete.

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CHAPTER 1

REVISION OF THE HELICELLINAE OF CRETE (GASTROPODA: HYGROMIIDAE)

ABSTRACT

This chapter presents a comprehensive revision of the Helicellinae of Crete (Gastropoda: Hygromiidae) based on characters of the shell and the genitalia. The native fauna includes eleven *Xerocrassa* species, two *Pseudoxerophila* species and perhaps one *Xeromunda* species. One additional *Xerocrassa* species and six species of the genera *Trochoidea, Xeropicta, Xerotricha, Microxeromagna* and *Cernuella* were probably introduced by man to Crete. The distribution patterns of the endemic *Xerocrassa* and *Pseudoxerophila* species do not provide evidence for the hypothesis that these radiations were caused by the fragmentation of the region of present-day Crete into several palaeoislands in the late Miocene and Pliocene.

INTRODUCTION

The land snail fauna of Crete is extraordinarily rich. Compared with the land snail fauna of other Aegean islands the species number on Crete is much higher than would be expected from its area (Welter-Schultes & Williams 1999). The high species richness of land snail on Crete is mainly the result of a few radiations. The radiations of the genera *Albinaria* Vest, 1867 (Clausiliidae; Gittenberger 1991; Schilthuizen & Gittenberger 1996; Douris *et al.* 1998; Welter-Schultes 2000a; Nordsieck 2004; Schilthuizen *et al.* 2004), *Mastus* Beck, 1837 (Enidae; Maassen 1995; Welter-Schultes 2000b; Parmakelis *et al.* 2005) and *Orculella* Steenberg, 1925 (Orculidae; Gittenberger & Hausdorf 2004) on Crete have already been studied in detail. Maltzan (1883, 1887) identified at least one radiation of xerophilous hygromiids on Crete. However, after Maltzan, this radiation has been almost completely neglected. It is still unknown how many xerophilous hygromiid species live on Crete, how they can be delimited and where they are distributed. Because the anatomy of most of the described species is unknown, it is even unclear to which genera these species belong. As a first step towards a better understanding of the evolution of the Cretan xerophilous hygromiids we present a comprehensive revision of the Helicellinae *sensu stricto* of Crete.

The Helicellinae Ihering, 1909 (Gastropoda: Hygromiidae) differ from most other Hygromiidae in the course of the right ommatophoral retractor that does not cross between penis and vagina and in the whitish shell. The systematics of the Helicellinae traces back to Hesse (1926, 1934), who included three genera in the subfamily, *Helicella* Férussac, 1821, *Cochlicella* Férussac, 1821, and *Monilearia* Mousson, 1872, which is closely related to *Cochlicella*. Hesse divided *Helicella sensu lato* into several subgenera that are mainly based on differences in the genitalia. Later, *Helicella sensu lato* was further divided into several genera that were mainly based on the number of dart and accessory sacs (e.g., Zilch 1960). Some authors (e.g., Zilch 1960) included also *Monacha* Fitzinger, 1833 in the Helicellinae, because its right ommatophoral retractor usually does not cross between penis and vagina (but see Hausdorf 2000 for exceptions). Schileyko (1972; 1978; 1991) transferred *Monacha* and other genera of the Hygromiidae in which the dart apparatus is transformed to appendiculae (but in which the right ommatophoral retractor crosses between penis and vagina) into a separate subfamily of the Hygromiidae. Because of the structure of the stimulator, he excluded *Cochlicella* from the Hygromiidae and established a separate family for *Cochlicella* and *Monilearia*. Furthermore, he supposed that the course of the right ommatophoral retractor and the whitish shell are adaptations to dry habitats. He concluded that even the remaining

Helicellinae (*sensu stricto*, i.e. without *Cochlicella*, *Monilearia* and *Monacha*) are polyphyletic and that the xerophilous groups with a free right ommatophoral retractor and a whitish shell originated convergently from various more hygrophilous hygromiids which resemble the individual xerophilous groups in the structure of the dart apparatus. This view has essentially been accepted by Nordsieck (1987, 1993), who, however, included the *Cochlicella* group in the Monachainae Wenz, 1930 (1904). In contrast, Hausdorf (1988) considered the Helicellinae *sensu stricto* monophyletic, because they differ from the Hygromiinae Tryon, 1866, Monachainae and Cochlicellinae Schileyko, 1972 in the chromosome numbers (n = 26-27, rarely 24 or 25 in the Helicellinae; n = 23, rarely 21, 24 or 26 in the Hygromiinae, Monachainae and Cochlicellinae). A phylogenetic analysis of partial *cox1*, 16S rDNA, 18S rDNA and ITS-1 sequences revealed monophyly of Helicellinae inclusive of *Monacha* (Steinke *et al.* 2004). However, the bootstrap value for that clade is below 50%. Phylogenetic analyses of partial 16S rDNA sequences alone were insufficient to decide whether or not Helicellinae are monophyletic (Manganelli *et al.* 2005). However, this study suggested that *Monacha* is more closely related to Bradybaenidae than to Helicellinae. A phylogenetic analysis of partial 28S rDNA sequences (Koene & Schulenburg 2005) confirmed the monophyly of Helicellinae and showed that *Monacha* is more closely related to some Hygromiinae than to Helicellinae. Accordingly, we consider Helicellinae *sensu stricto*, i.e., without *Cochlicella* and *Monacha*, in this paper.

MATERIAL AND METHODS

The material on which this revision is based was collected on three expeditions to Crete in July/August and September/October 2004 and September/October 2005. During these expeditions land snails were sampled at about 500 localities across Crete. Among others about 1250 xerophilous hygromiid lots were collected and are kept in the Zoological Museum of the University of Hamburg (ZMH). Living specimens were killed by putting the crawling animal into boiling water. Then the soft parts were conserved in 70% ethanol.

Additional 3650 lots (mainly shells) were borrowed from the following collections: Haus der Natur, Cismar (HNC); Hungarian Natural History Museum, Budapest (HNHM; material determined as *Xerocrassa cretica*, *Xeromunda candiota* and *Cernuella virgata* has only partly been revised); collection W. J. M. Maassen, Duivendrecht (MAA); Naturhistoriska Museet, Göteborg (NMG); collection C. A. Westerlund in the NMG (NMGW); Nationaal

Natuurhistorisch Museum, Leiden, formerly Rijksmuseum van Natuurlijke Historie (RMNH); collection P. Subai, Aachen (SUB); Museum für Naturkunde, Berlin (ZMB). The material studied is listed in the supplementary Appendix S1.

The synonymy lists include only the original descriptions and quotations concerning material from Crete or relevant for the interpretation of the species.

Photographs were taken with the digital camera Leica DFC320 built on a binocular microscope (Leica MZ16) using the software IM50 version 4.0 (Leica Microsystems Imaging Solutions). Shell measurements were taken from digital photographs using the program analySIS Pro version 3.2 (Olympus Soft Imaging Solutions) or with an ocular micrometer. The counting of the shell whorls follows Kerney *et al.* (1983). Morphometric data were analysed with SPSS 15.0 (SPSS Inc.).

The terminology used for the parts of the genitalia is illustrated in Figure 1.1. The terms proximal and distal refer to the position in relation to the gonad. The measurements of the parts of the genitalia were taken with an ocular micrometer. The measurements were usually repeated once. The length of the various parts of the genitalia is correlated with the body size and, therefore, it is often not species-specific. However, the proportions of the distal parts of the genitalia proved to be important species-specific characters. In all genera except *Xerocrassa* and *Trochoidea*, the penis and the distal epiphallus up to the insertion of the penial retractor have been measured together, because the penis-epiphallus boundary cannot be recognized in these groups without an examination of the inner structure.

The genitalia were not described for each species in detail, because there are usually only morphometric differences between closely related species. However, all measurements are listed to facilitate future taxonomic work.

Most measurements of the genitalia were taken from material collected by us and fixed in the same way (see above). However, we did not note any systematic deviations of the morphometric characters that we consider species specific (especially the ratios of different parts of the genitalia) in specimens from other collections that were fixed in different ways (e.g., by drowning).

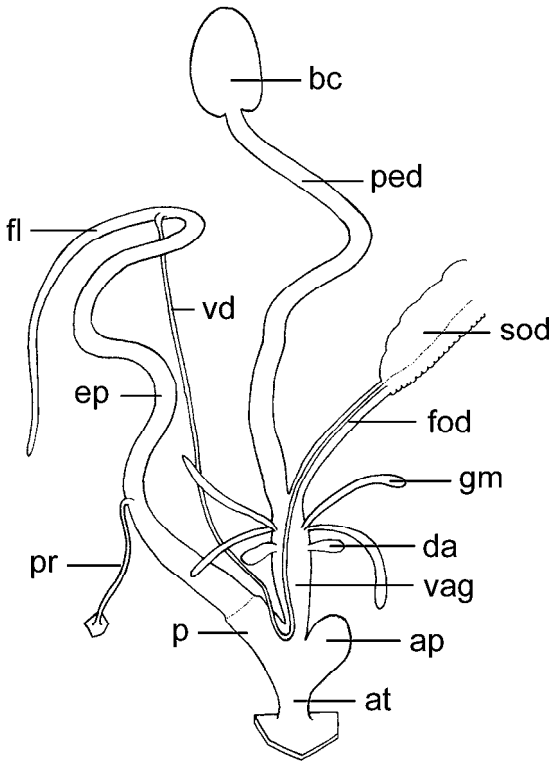

Fig. 1. Terminology of the distal parts of the genitalia: ap – appendix; at – atrium; bc – bursa of the bursa copulatrix; da – dart apparatus; ep – epiphallus; fl – flagellum; fod – free oviduct; gm – glandulae mucosae; p – penis; ped – pedunculus; pr – penial retractor; sod – spermoviduct; v – vagina; vd – vas deferens.

The spelling of the geographic names is usually taken from the "Kreta" map, 1: 140000 (Reise Know-How Verlag, Bielefeld, 2002). The localities are arranged according to Nomos (= N.; prefectures) in the locality lists. Within Nomos, localities are ordered alphanumerically according to the 10 km UTM code.

We tested the hypothesis that the distribution areas of the recent endemic species are centred on Neogene palaeoislands by means of a Monte Carlo procedure. We used the null model proposed by Hausdorf & Hennig (2003), but modified it so that the species richness distribution of the geographic cells is not considered. This null model generates range data sets such that their range size distribution and the spatial autocorrelation of the occurrences of a species approach the parameters in the real data set. We used the number of occurrences of species in 10 km UTM grids that are located on areas formerly belonging to palaeoislands as the test statistic. If the recent species originated on Neogene palaeoislands and if their ranges have not shifted, the number of occurrences of species that are located on areas formerly belonging to palaeoislands should be higher in the real data set than in the simulated data sets in which the ranges are placed independently of the position of the palaeoislands. The

position of the palaeoislands was taken from the map of Crete in the Pliocene in Fassoulas (2001: fig. 11).

SYSTEMATICS

Helicellinae Ihering, 1909

Key to the genera of the Helicellinae present on Crete

1a. With one dart apparatus 2

1b. With two symmetrical dart apparatus 4

2a. Accessory sac much smaller than dart sac, transformed into a cavity between the vagina wall, the dart sac and a tissue-layer which envelopes parts of the vagina and the dart sac . *Xeromunda*

2b. Accessory sac only slightly smaller than dart sac 3

3a. Dart apparatus with large, conical papilla inside; the penis is innervated from the right pedal ganglion; shell without hairs..... *Cernuella*

3b. Dart apparatus without large papilla inside; the penis is innervated from the right cerebral ganglion; shell with hairs ..... *Microxeromagna*

4a. Dart apparatus with dart-bearing dart sac 5

4b. Dart apparatus without dart-bearing dart sac 7

5a. Dart sac much larger than accessory sac; shell with hairs ..... *Xerotricha*

5b. Dart sac small, not much larger than accessory sac 6

6a. Accessory sacs much longer than wide, sticking out from the vagina; penis with appendix ..... *Xeropicta*

6b. Accessory sacs not much longer than wide, attached to a dilatation of the vagina; penis without appendix ..... *Pseudoxerophila*

7a. With large appendix at the atrium, two strong longitudinal folds surround each opening of the accessory sacs into the vagina at the inner side of the wall of the vagina and fuse pairwise at their distal and proximal ends ..... *Trochoidea*

7b. Without appendix or at most with a small swelling at the atrium, inner side of the wall of the vagina with irregular longitudinal folds ..... *Xerocrassa*

XEROCRASSA MONTEROSATO, 1892

Xerocrassa Monterosato, 1892: 23. Type species (by monotypy): *Helix seetzeni* L. Pfeiffer, 1847.

Diagnosis: *Xerocrassa* is characterized by a symmetrical dart apparatus consisting of two small accessory sacs and usually four branched glandulae mucosae around the vagina, irregular longitudinal folds at the inner side of the wall of the vagina and the lack of a well-developed appendix at the atrium. The penis is innervated from the right cerebral ganglion.

Remarks: The homology of the two small sacs at the vagina is doubtful. In a few *Xerocrassa sensu lato* (as used by Nordsieck, 1993) species, e.g., *X. geyeri* (Soós, 1926), there are small swellings at the base of these sacs in a position that is similar to the position of the dart sacs of *Xeropicta*. If these swellings were rudimentary dart sacs, the sacs of *Xerocrassa* and *Trochoidea* would be homologous to the accessory sacs of other Hygromiidae and not to the dart sacs. *Xerocrassa* differs from *Trochoidea* in the lack of a well-developed appendix at the atrium. However, there are structures in the atrium of several *Xerocrassa* species that are probably homologous to the inner structures of the atrial appendix of *Trochoidea* and in some *Xerocrassa* species they form even a lateral bulge at the atrium (see, e.g., Figs 1.3B, C, D, F, 1.7E). This is also the case in *X. siphnica* (Kobelt, 1883) that has therefore been erroneously included in *Trochoidea sensu stricto* by Fuchs & Käufel (1936)(see also Mylonas *et al.* 1995). *Xerocrassa* in the wide sense as used by Nordsieck (1993) is probably paraphyletic with regard to *Trochoidea* and needs a revision. Based on mitochondrial DNA sequences (Sauer & Hausdorf, unpubl. data), the *Xerocrassa* species from Crete belong to the same clade as the type species of *Xerocrassa*, *X. seetzeni* (L. Pfeiffer) from the Levant. Thus, the Cretan species will remain in *Xerocrassa* even after a revision.

Key to the *Xerocrassa* species present on Crete

1a. Proximal epiphallus: flagellum ratio ≥ 2.3 2

1b. Proximal epiphallus: flagellum ratio ≤ 2.2 6

2a. Large shell diameter ≤ 9 mm; proximal epiphallus: flagellum ratio ≥ 3.0 ..... *X. kydonia*

2b. Different 3

3a. Shell with a protruding keel 4

3b. Shell without a protruding keel 5

4a. Proximal epiphallus: flagellum ratio = 2.3-2.6; umbilicus 1.7-2.6 mm ..... *X. franciscoi*

4b. Proximal epiphallus: flagellum ratio = 3.3-4.6; umbilicus 0.2-1.0 mm .... *X. amphiconus*

5a. Penial papilla with a long, terminally open basal part and a very short conical apical part (Fig. 1.4A) ..... *X. siderensis*

5b. Penial papilla with a broader basal part and a narrower apical part with a terminal opening (Fig. 1.4E) *X. mesostena* (few specimens from the surroundings of Ano Viannos)

6a. Large shell diameter usually > 13 mm, if smaller columellar edge of aperture almost perpendicular to body whorl, umbilicus almost concentric ..... *X. cretica*

6b. Large shell diameter < 13 mm, columellar edge of aperture obliquely converging to body whorl 7

7a. Umbilicus very narrow, partly or completely obscured by the columellar edge, whorls quickly increasing, penial papilla cylindrical with a terminal opening ..... *X. meda*

7b. Umbilicus narrow or moderately wide, whorls more slowly increasing 8

8a. Umbilicus almost concentric or only slightly eccentric, penial papilla with a cylindrical basal part and a dilated apical part with a subterminal opening (Fig. 1.4C) 9

8b. Umbilicus narrow and/or eccentric, distinctly enlarged by the last whorl 10

9a. Shell striated or finely ribbed, without distinct keel; large shell diameter: umbilicus width ratio ≥ 0.15; proximal epiphallus: flagellum ratio ≤ 1.2 ..... *X. subvariegata*

9b. Shell coarsely ribbed, with a distinct keel; large shell diameter: umbilicus width ratio ≤ 0.14; proximal epiphallus: flagellum ratio ≥ 1.1 ..... *X. grabusana*

10a. Penial papilla with a long, terminally open basal part and a very short conical apical part, large shell diameter < 8.5 mm (Fig. 1.4D) 11

10b. Penial papilla with a broader basal part and a narrower apical part with a terminal opening, large shell diameter usually larger (Fig.1.4E) ..... *X. mesostena*

11a. Total vagina length: vagina up to the base of the dart apparatus ratio ≥ 1.6 ... *X. heraklea*

11b. Total vagina length: vagina up to the base of the dart apparatus ratio ≤ 1.5 12

12a. Proximal epiphallus: total vagina length ≤ 2.1 ..... *X. rhithymna*

12b. Proximal epiphallus: total vagina length ≥ 2.3 ..... *X. lasithiensis*

XEROCRASSA AMPHICONUS (MALTZAN, 1883)

(FIGS 2A, 3A, 4A, 5A; TABLES 1.1-1.3)

Helix (*Jacosta*) *amphiconus* Maltzan, 1883: 102. Locus typicus: „prope Sitia insulae Cretae“, Greece.

Helix (*Jacosta*) *amphiconus* – Kobelt, 1888: 25, pl. 98 fig. 546.

Helix (*Xerophila*) *amphiconus* – Martens, 1889: 187.

Helicella (*Trochoidea*) *syrensis amphiconus* – Fuchs & Käufel, 1936: 632.

Helix (*Jacosta*) *amphiconus* – Lindner, 1994: 78, fig. 2.

Trochoidea mesostena – Vardinoyannis, 1994: 85, 88, 130, map 45 [partim, non Westerlund, 1879].

Xerocrassa amphiconus – Hausdorf & Sauer, 2009: 376- 390, Figs 2A, 3A, 4A, 5A; Tables 1-3.

Type material: Syntypes: Greece, Kríti, N. Lasithi: Sitia - Moni Toplou, MU29 (ZMB 39695/4).

Diagnosis: *X. amphiconus* is characterized by a strongly depressed, perforate shell with a protruding keel, a relatively short flagellum (proximal epiphallus: flagellum ratio 3.3-4.6) and a penial papilla with a long, terminally open basal part and a very short conical apical part with an open channel.

Shell (Fig. 1.2A, Table 1.1): strongly depressed conical; with 4.25-4.75 whorls which are almost plain or even concave at the top side; teleoconch with regular fine ribs and irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; all whorls with a protruding keel; aperture rhombic; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus prick-like, not obscured by the columellar edge.

Genitalia (Figs 1.3A, 1.4A, Tables 1.2, 1.3): The penial papilla is divided into a long, slightly tapering, terminally open basal part and a very short conical apical part with an open channel that is adnate with the basal part at the side opposite the opening. There is a glandular belt in the penial wall near its proximal end and a longitudinal glandular field at the abvaginal inner side of the penis wall with distinct folds. There are no distinct stimulatory structures in the atrium.

Remarks: *X. amphiconus* and *X. siderensis* populate in the same region in eastern Crete, but are usually not syntopic. Whereas *X. amphiconus* is mainly distributed in the higher central region, *X. siderensis* occurs primarily in the coastal region. However, there are exceptions to this pattern, e.g. occurrences of *X. amphiconus* near Agia Fotia and Kato Zakros and records of *X. siderensis* near Ziros and Zakros. Only at Stavromenos 2.1 km towards Katsidoni a single *X. amphiconus* was found together with two *X. siderensis*. Apart from differing altitudinal preferences, we could not ascertain ecological differences between the two species. In contrast to the situation in *X. siphnica,* where keeled forms are said to aestivate only on or under stones whereas rounded forms aestivate also under shrubs (Mylonas *et al.* 1995), both *X. amphiconus* and *X. siderensis* aestivate under stones as well as in shrubs. *X. amphiconus* and *X. siderensis* are very closely related. They do not form separate clades in trees based on mitochondrial genes or AFLP markers (chapter 2, 3). This means either that they have not been separated long enough for lineage sorting to be completed or that there is still introgression. In contrast to other closely related *Xerocrassa* species from Crete, we have not found differences in the genitalia of *X. amphiconus* and *X. siderensis*. The differences in shell characteristics are no larger than differences between some extreme forms classified as *X. mesostena*. However, in some areas, e.g. north of Ziros or south of Langada, populations of both taxa occur in close vicinity without continuous morphological transitions. We have measured the large diameter of the shell, the small diameter of the shell (perpendicular to the large diameter), the diameter at three whorls, the width of the spire, the height of the shell, the height of the spire, the diameter of the aperture, the height of the aperture, the width of the umbilicus and the number of whorls of twelve specimens of three populations of *X. amphiconus* and *X. siderensis* to investigate the constancy of the conchological differences between them.

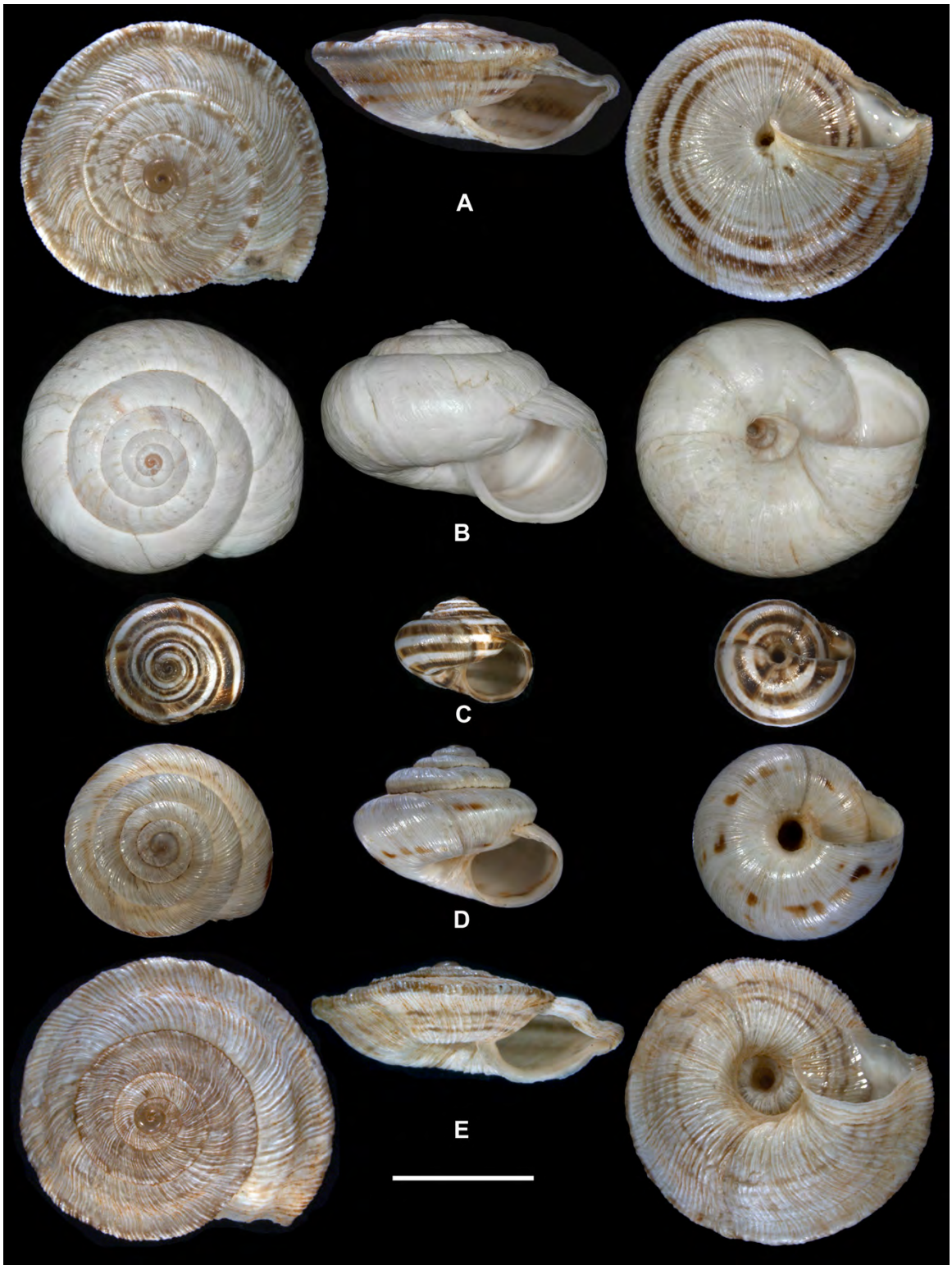

Figure 1.2. *Xerocrassa* species from Crete, shells. A, *Xerocrassa amphiconus* (Maltzan), Sitia - Moni Toplou, MU29 (syntype ZMB 39695). B, *Xerocrassa cretica* (L. Pfeiffer), Crete (syntype of *H. cretica* forma *alba* Westerlund & Blanc NMGW). C, *Xerocrassa cretica* (L. Pfeiffer), Crete (syntype of *H. curetum* Westerlund NMGW). D, *Xerocrassa cretica* (L. Pfeiffer), Elassa Island (syntype of *H. gradilis* Martens ZMB 42683). E, *Xerocrassa franciscoi* Hausdorf & Sauer, Ano Kapetaniana 7 km towards Agios Ioannis (holotype ZMH 51072). Scale bar = 5 mm for A, D, E and 10 mm for B, C.

In contrast to other closely related *Xerocrassa* species from Crete, we have not found differences in the genitalia of *X. amphiconus* and *X. siderensis*. The differences in shell characteristics are no larger than differences between some extreme forms classified as *X. mesostena*. However, in some areas, e.g. north of Ziros or south of Langada, populations of both taxa occur in close vicinity without continuous morphological transitions. We have measured the large diameter of the shell, the small diameter of the shell (perpendicular to the large diameter), the diameter at three whorls, the width of the spire, the height of the shell, the height of the spire, the diameter of the aperture, the height of the aperture, the width of the umbilicus and the number of whorls of twelve specimens of three populations of *X. amphiconus* and *X. siderensis* to investigate the constancy of the conchological differences between them. We assessed also a population of *X. siderensis* with a very angular body whorl from Agia Fotia 1.7 km towards Palekastro. Nevertheless, 100% of the specimens could be identified correctly in a discriminant analysis, even though the angularity of the body whorl, the most conspicuous difference between *X. amphiconus* and *X. siderensis*, was not considered in the measurements. The lack of morphological transitions between neighbouring populations of *X. amphiconus* and *X. siderensis* in several regions indicates that the two taxa do not or only rarely hybridise. There are also no hints that the two forms are the result of ecotypic differentiation or habitat related ecophenotypic plasticity, because there are no ecological differences between the two forms besides different altitudinal preferences and because even populations of the two taxa living in the "wrong" altitudinal zone are typical. Thus, we classify *X. amphiconus* and *X. siderensis* preliminarily as distinct species.

Distribution (Fig. 1.5A): *X. amphiconus* is restricted to the easternmost mountain range of Crete. Most records are from the central region.

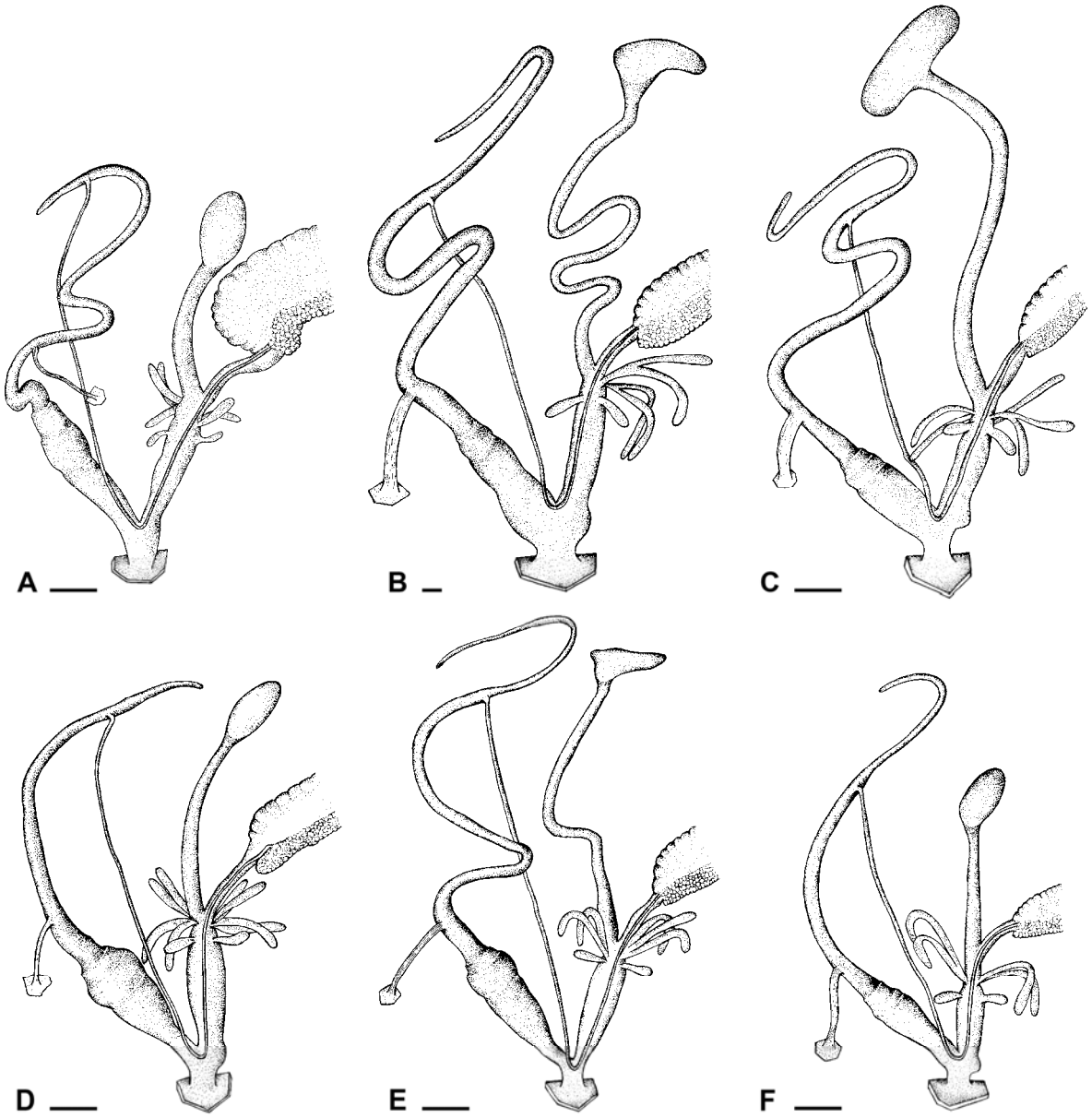

Figure 3. *Xerocrassa* species from Crete, genitalia. Scale bar = 1 mm. A, *Xerocrassa amphiconus* (Maltzan), Agia Fotia (ZMH 18645). B, *Xerocrassa cretica* (L. Pfeiffer), Palekastro 4.7 km towards Vai (ZMH 36579). C, *Xerocrassa cretica* (L. Pfeiffer) ('*gradilis*'), Palekastro 4.7 km towards Vai (ZMH 50001). D, *Xerocrassa franciscoi* Hausdorf & Sauer, Ano Kapetaniana 7 km towards Agios Ioannis (holotype ZMH 51072). E, *Xerocrassa grabusana* Hausdorf & Sauer, Kaliviani 5.5 km towards Balos (holotype ZMH 51070). F, *Xerocrassa heraklea* Hausdorf & Sauer, Stalida 3 km towards Mohos (holotype ZMH 51068).

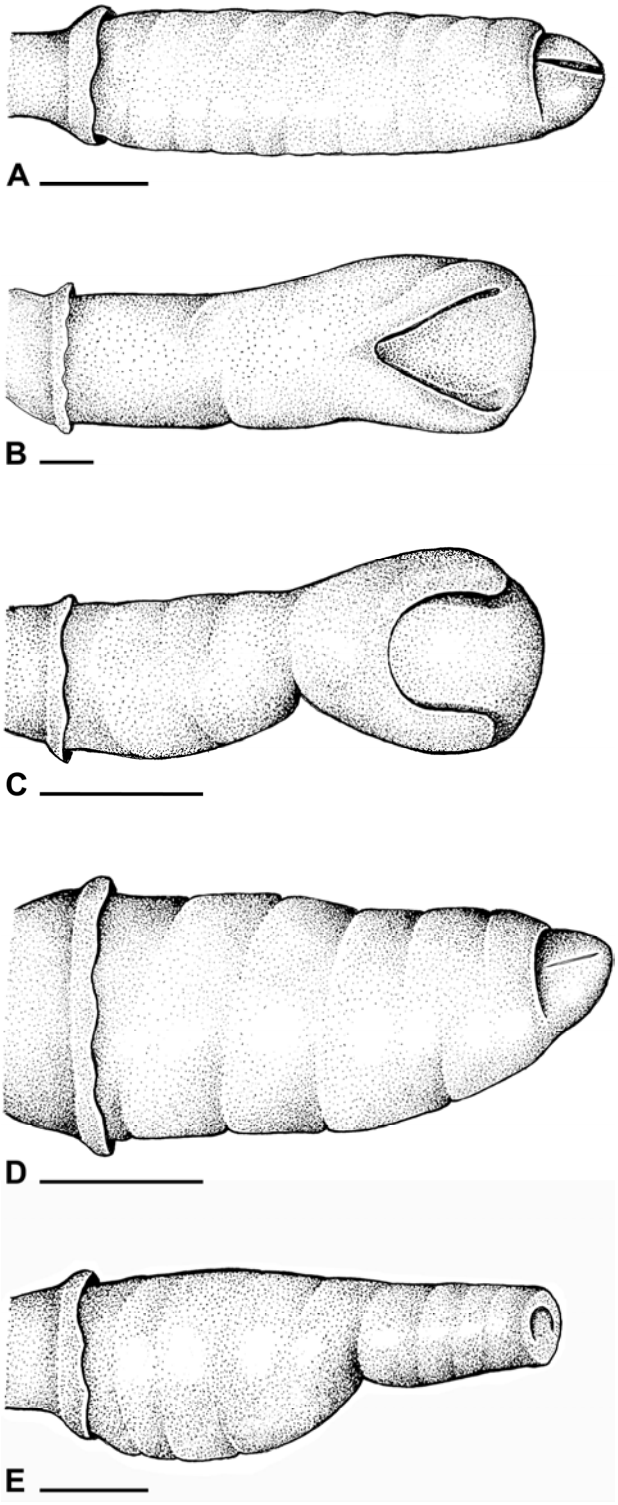

Fig. 4. *Xerocrassa* species from Crete, penial papillae. Scale bar = 0.5 mm. A, *Xerocrassa amphiconus* (Maltzan), Moni Toplou 0.7 km towards Sitia (ZMH 36606). B, *Xerocrassa cretica* (L. Pfeiffer), Zakros 3 km towards Kato Zakros (ZMH 29343). C, *Xerocrassa grabusana* Hausdorf & Sauer, 2 km N of Kaliviani (ZMH 29885). D, *Xerocrassa kydonia* Hausdorf & Sauer, Mesavlia 1.8 km towards Chania (ZMH 36893). E, *Xerocrassa mesostena* (Westerlund), Listaros 1 km towards Moni Odigitrias (ZMH 36339).

XEROCRASSA CRETICA (L. PFEIFFER, 1841)

(FIGS 1.2B-D, 1.3B-C, 1.4B, 1.5B; TABLES 1.1, 1.3, 1.4)

Helix (*Helicella*) *cretica* Férussac, 1821: 45 (Quarto Edition) [Folio Edition: 49]. Locus typicus: "L'île de Crète, près la Canée; l'île de Rhodes; Naxie, près Philoti; Standie" [nomen nudum].

Helix (*Helicella*) *cretica* L. Pfeiffer, 1841: 40. Locus typicus: "Insul. Archipelagi".

Helix cretica – L. Pfeiffer, 1848: pl. 37 figs 21-22.

Helix cretica – L. Pfeiffer, 1849: 253.

Helix cretica – Raulin, 1870: 652.

Helix (*Xerophila*) *cretica* forma *alba* Westerlund & Blanc, 1879: 63. Locus typicus: „Candie“, Greece.

Helix cretica – Kobelt, 1883: 49, pl. 18 figs 144-146.

Helix (*Xerophila*) *gradilis* Martens, 1889: 187, pl. 10, fig. 12. Locus typicus: „Insel Elasia an der Ostküste von Kreta“, Greece.

Helix (*Xerophila* [*Striatella*]) *curetum* Westerlund, 1889: 249. Locus typicus: „Arkhanes auf Creta“, Greece.

Helix (*cretica* var.) *akrotirensis* Kobelt, 1890: 87, pl. 113 figs 678-679. Locus typicus: „am Vorgebirge Akrotiri auf Creta“, Greece.

Helix (*Xerophila*) *cretica* – Martens, 1889: 187 [partim].

Helix (*Xerophila*) *mesostena* – Martens, 1889: 187 [partim, non Westerlund, 1879].

Helix cretica – Schuberth, 1892: 56, pl. 6 fig. 7.

Helix (*Xerocauta*) *cretica* – Cecconi, 1896: 219.

Helix (*Xerophila*) *cretica* – Sturany, 1904: 109.

Helicella (*Xerocrassa*) *cretica* – Haas, 1935: 111.

Helicella (*Xerocrassa*) *cretica cretica* – Fuchs & Käufel, 1936: 621, 625, 626.

Helicella (*Trochoidea*) *syrensis gradilis* – Fuchs & Käufel, 1936: 632.

Trochoidea (*Xerocrassa*) *cretica* – Frank, 1988: 87.

Trochoidea cretica – Vardinoyannis & Mylonas, 1988: 139.

Trochoidea cretica – Vardinoyannis, 1994: 85, 88, 130, map 44.

Xerocrassa cretica – Hausdorf & Sauer, 2009: 380, 383, Figs 2B-D, 3B-C, 4B, 5B; Tables 1, 3, 4.

Type material: Syntype of *H. cretica* forma *alba*: Greece, Kríti: Crete (NMGW); syntype of *H. curetum*: Greece, Kríti: N. Iraklion: Arhanes, LV30 (NMGW); syntypes of *H. gradilis*: Greece, Kríti: N. Lasithi: Elassa Island, MV30 (ZMB 42683/7).

Diagnosis: *X. cretica* is characterized by a typically large (on Crete usually 15-23 mm; at the east coast there are rare small forms > 8.3 mm), depressed conical, finely and regularly ribbed shell with a moderately wide, almost concentrical umbilicus.

Shell (Figs 1.2B-D, Table 1.1): depressed conical; with 4.25-6 convex whorls; teleoconch with regular fine ribs and irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl rounded or with an edge that becomes weaker towards the aperture; aperture almost circular; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus moderately wide, almost concentrical, not obscured by the columellar edge.

Genitalia (Figs 1.3B-C, 1.4B, Tables 1.3, 1.4): The penial papilla is longish club-shaped with a subterminal opening. A bulge extends from the subterminal opening of the penial papilla towards its apical tip. There is a glandular belt in the penial wall near its proximal end and a longitudinal glandular field at the abvaginal inner side of the penis wall with distinct folds. There are bulge-like stimulatory structures in the atrium.

Remarks: The forms of *X. cretica* living on Crete are usually large and have a rounded body whorl (Figs 1.2B-C, Table 1.1). There are also forms that remain small and often resemble juveniles in having fewer whorls and an angular body whorl (Fig. 1.2D, Table 1.1). There is continuous variation between small and large forms on some of the neighbouring islets, e.g. Koufonisi and Chrisi (Welter-Schultes 1998). Forms of intermediate size are also found on many other Aegean islands (Fuchs & Käufel 1936: fig. 52). A small form from Elassa island has been described as *H. gradilis* Martens, 1889. It also occurs in eastern Crete from Palekastro northwards to Akrotiri Sideros. It lives in close vicinity to populations of the large form of *X. cretica* without intermediate forms. It is possible that this small form originated on Elassa and has been passively dispersed from there to Crete. There is probably only limited gene flow between the adjacent populations on Crete because of the large size difference.

However, the intermediate forms on the adjacent islets demonstrate that the forms are not reproductively isolated. Actually, the forms are neither separated in a tree based on *coxI* and 16S rDNA sequences nor in a tree based on AFLP markers (chapter2, 3). *H. curetum* Westerlund, 1889 (Fig. 1.2C) is based on a juvenile of the large form of *X. cretica*.

Triantis *et al.* (2004) recorded a *Pseudoxerophila* from Paximada, Dionisades Islands north of Crete, as *Pseudoxerophila* aff. *gradilis*. However, they have not compared their material with the types of *H. gradilis* Martens from Elassa Island. There are no differences between the types of *H. gradilis* Martens from Elassa Island and the small form of *X. cretica* from the east coast of Crete vis à vis Elassa. The *Pseudoxerophila* species from the Dionisades Islands is a different species.

Distribution (Fig. 1.5B): Various forms from the Aegean Islands, eastern parts of the Greek mainland, the west coast of Turkey, Cyprus, Libya and Egypt (Brandt 1959) have been included into *X. cretica*. A detailed study is required to assess which of these forms are actually conspecific with *X. cretica* and which have to be considered separate species.

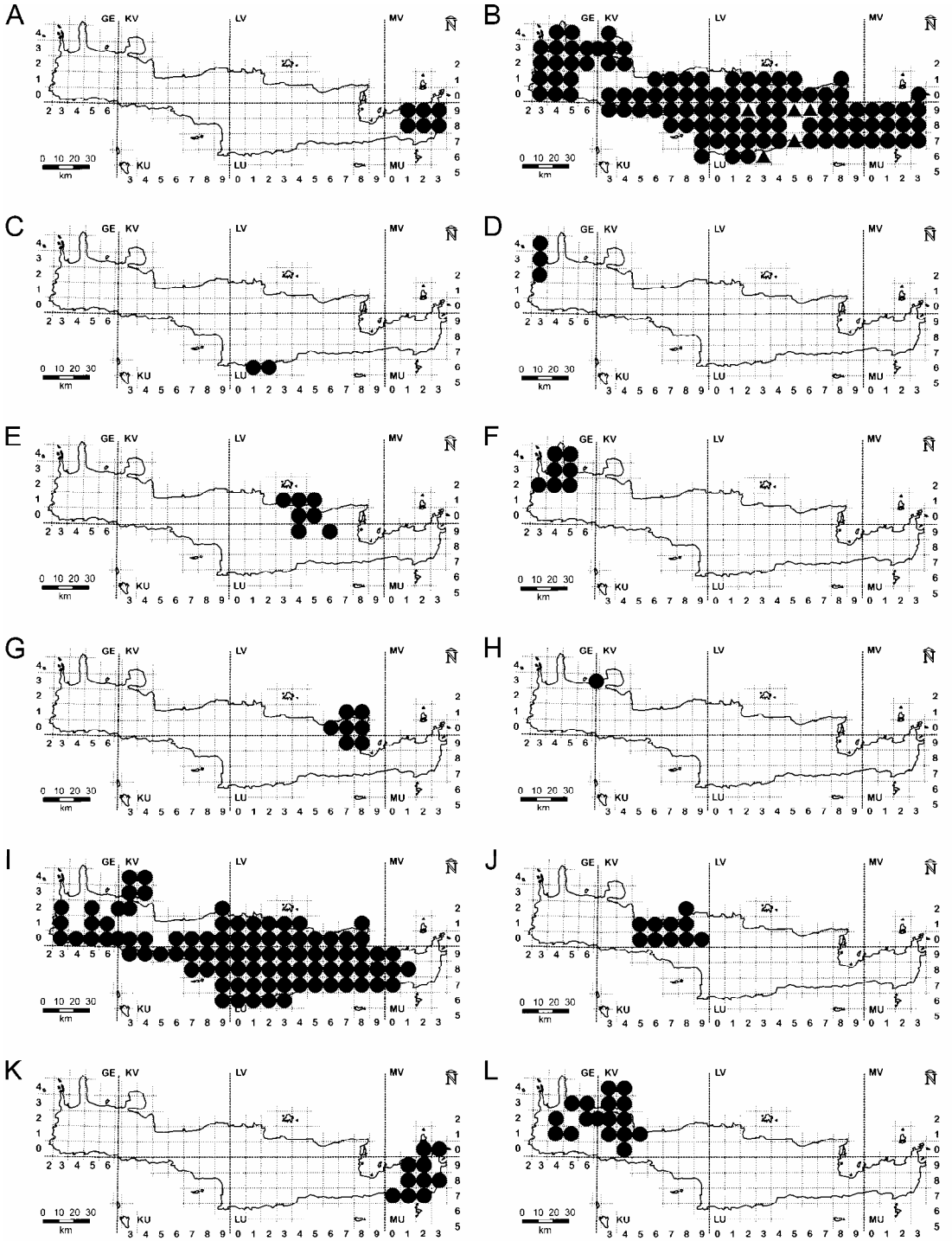

Fig. 5. Distribution of *Xerocrassa* species on Crete (UTM-grid, 10 km - squares). ● records of which vouchers have been checked, ▲ records from the literature. A, *Xerocrassa amphiconus* (Maltzan). B, *Xerocrassa cretica* (L. Pfeiffer). C, *Xerocrassa franciscoi* Hausdorf & Sauer. D, *Xerocrassa grabusana* Hausdorf & Sauer. E, *Xerocrassa heraklea* Hausdorf & Sauer. F, *Xerocrassa kydonia* Hausdorf & Sauer. G, *Xerocrassa lasithiensis* Hausdorf & Sauer. H, *Xerocrassa meda* (Porro). I, *Xerocrassa mesostena* (Westerlund). J, *Xerocrassa rhithymna* Hausdorf & Sauer. K, *Xerocrassa siderensis* (Maltzan). L, *Xerocrassa subvariegata* (Maltzan).

Table 1.1. Shell measurements (in mm) and proportions of Helicellinae from Crete (the measured *Microxeromagna lowei* are from Monemvasia in Lakonia, Greece). D – large diameter, d_U – width of umbilicus, H – height, max – maximum, min – minimum, s – width of spire, W– number of whorls.

		n	D			s			H			d_U			W			D/H			s/D			d_U/D		
			min	max	mean	min	max	Mean	Min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean
Xerocrassa amphiconus	ZMH 50609, ZMH 50597, ZMH 18645	36	9.9	12.2	11.1	5.8	7.4	6.6	4.1	5.8	5	0.2	1	0.5	4.3	5.3	4.6	1.8	2.8	2.2	0.53	0.66	0.59	0.03	0.09	0.05
Xerocrassa cretica (large form)	ZMH 50237	20	18.9	22.6	21.1	12.7	15.5	14.2	13.8	15.2	17	2	3	2.8	5.3	6.3	5.8	1.3	1.5	1.4	0.62	0.72	0.67	0.10	0.16	0.13
Xerocrassa cretica (‘*gradilis*’)	ZMH 50000	20	8.4	11.2	9.2	5.1	7.2	5.9	5.2	7.4	6.2	1.0	1.5	1.4	4.5	5.3	4.9	1.2	1.7	1.5	0.59	0.70	0.64	0.11	0.18	0.15
Xerocrassa franciscoi .	ZMH 29685	20	9.4	11.7	10.7	5.4	7.8	6.4	3.7	5.4	4.5	1.7	2.6	2.1	4.3	5.0	4.5	2.1	3.0	2.4	0.51	0.67	0.59	0.15	0.25	0.20
Xerocrassa grabusana	ZMH 36574	20	7.8	9.6	8.7	5.1	6.8	6.0	4.6	7.0	5.7	0.7	1.3	0.9	4.5	5.3	4.9	1.3	1.7	1.5	0.64	0.72	0.69	0.08	0.14	0.11
Xerocrassa heraklea	ZMH 36650	20	5.8	7.0	6.4	4.2	5.2	4.7	4.0	5.1	4.5	0.3	0.9	0.5	4.8	5.3	4.9	1.3	1.6	1.4	0.67	0.79	0.73	0.05	0.15	0.08
Xerocrassa kydonia	ZMH 36263	20	5.9	7.0	6.4	4.0	5.2	4.6	3.8	5.3	4.6	0.2	0.5	0.3	4.5	5.0	4.7	1.2	1.6	1.4	0.66	0.79	0.72	0.02	0.08	0.05
Xerocrassa lasithiensis	ZMH 29890	20	6.6	8.1	7.0	4.6	5.8	5.0	4.6	5.6	5.1	0.3	0.7	0.5	4.8	5.3	5.0	1.3	1.6	1.4	0.60	0.75	0.72	0.04	0.10	0.08
Xerocrassa meda	MAA	5	9.8	11.7	10.7	6.7	7.7	7.0	6.6	8.0	7.2	0.0	0.3	0.2	5.0	5.3	5.1	1.5	1.5	1.5	0.64	0.68	0.66	0.00	0.03	0.02
Xerocrassa mesostena	ZMH 29072	20	9.4	11.6	10.3	6.4	8.5	7.3	6.0	8.0	7.1	0.7	1.3	0.9	4.8	5.8	5.3	1.3	1.6	1.5	0.65	0.75	0.71	0.07	0.12	0.09
Xerocrassa mesostena	ZMH 29603	20	8.7	10.4	9.6	4.9	6.0	5.4	3.4	4.6	4.2	1.3	2.0	1.7	4.0	4.5	4.3	2.0	2.8	2.3	0.51	0.63	0.56	0.13	0.20	0.17
Xerocrassa rhithymna	ZMH 36273	20	6.6	8.1	7.2	4.5	5.8	5.3	4.5	5.9	5.2	0.2	0.5	0.3	4.5	5.3	5.1	1.3	1.5	1.4	0.68	0.77	0.74	0.02	0.07	0.05

Table 1.1. *Continued*

		n	D			s			H			d_U			W			D/H			s/D			d_U/D		
			min	max	mean	min	max	Mean	Min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	Mean
Xerocrassa siderensis	ZMH 36404, ZMH 29368, ZMH 18639	36	9.4	13.0	11.2	5.7	7.6	6.7	5.4	7.1	6.1	0.2	1.5	0.9	4.5	5.3	5.0	1.6	2.1	1.8	0.54	0.65	0.60	0.01	0.13	0.08
Xerocrassa subvariegata	ZMH 36019	20	7.6	9.6	8.7	5.1	6.9	6.0	5.0	6.9	5.8	1.3	2.1	1.7	4.5	5.3	4.9	1.3	1.7	1.5	0.64	0.75	0.70	0.15	0.24	0.19
Trochoidea pyramidata	ZMH 36466, MAA	20	7.2	9.9	8.3	5.3	7.5	6.1	5.9	8.2	6.9	0.2	0.7	0.5	5.0	6.3	5.5	1.1	1.3	1.2	0.69	0.77	0.74	0.02	0.08	0.06
Xeropicta krynickii	ZMH 29778	20	12.8	16.1	14.2	7.0	9.2	7.9	8.2	10.8	9.6	1.3	2.1	1.7	6.3	5.5	5.9	1.4	1.6	1.5	0.52	0.60	0.56	0.09	0.15	0.12
Pseudoxerophila bathytera	ZMH 37000	20	12.0	14.6	13.7	6.8	8.8	8.0	6.6	8.9	8.0	2.8	3.6	3.2	4.8	5.5	5.2	1.6	1.8	1.7	0.54	0.61	0.58	0.20	0.27	0.23
Pseudoxerophila oertzeni	ZMH 29719	20	7.2	8.6	7.8	4.0	5.1	4.7	3.9	4.8	4.5	1.5	2.0	1.8	4.3	4.5	4.4	1.6	1.9	1.7	0.55	0.66	0.60	0.20	0.26	0.23
Xeromunda candiota	ZMH 29990	20	9.8	11.9	10.9	6.1	7.5	7.0	8.1	10.9	9.7	0.5	0.8	0.7	4.5	5.3	5.1	1.0	1.2	1.1	0.60	0.67	0.64	0.05	0.08	0.06
Xerotricha apicina	ZMH 36954	20	5.5	6.7	6.1	3.3	4.0	3.6	3.7	4.7	4.2	0.7	1.1	0.9	4.0	4.5	4.2	1.3	1.6	1.5	0.56	0.64	0.60	0.11	0.18	0.15
Xerotricha conspurcata	ZMH 36202	14	5.4	6.3	5.8	3.2	3.9	3.6	3.5	4.2	3.9	0.7	1.0	0.8	4.3	4.8	4.4	1.4	1.6	1.5	0.58	0.64	0.61	0.11	0.16	0.13
Microxeromagna lowei	ZMH 51216	12	5.7	8.5	6.5	3.5	5.5	4.1	3.2	4.9	3.8	1.0	1.5	1.1	4.3	5.0	4.5	1.5	1.8	1.7	0.56	0.70	0.63	0.15	0.22	0.18
Cernuella (*Cernuella*) *virgata* (large form)	ZMH 36110	12	12.2	19.1	15.0	6.1	7.4	6.7	9.1	15.1	11.4	1.7	2.4	2.1	5.5	6.3	5.8	1.2	1.5	1.3	0.60	0.67	0.64	0.12	0.16	0.14
Cernuella (*Cernuella*) *virgata* (‘*cisalpina*’)	ZMH 27083	20	9.3	11.4	10.5	8.1	12.8	9.9	6.7	8.1	7.6	1.1	1.5	1.3	4.8	5.3	5.1	1.3	1.5	1.4	0.62	0.68	0.66	0.10	0.14	0.12

Table 1.2. Measurements of some parts of the genitalia of *Xerocrassa amphiconus* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Agia Fotia, MU29 (ZMH 18645)	3.3	2.4	6.6	1.4	0.6	2.1	2.5	2.9
Agia Fotia, MU29 (ZMH 18645)	3.7	2.4	6.4	1.4	0.8	1.9	2.1	2.5
Moni Toplou 0.7 km towards Sitia, MU29 (ZMH 36606)	3.5	2.5	7.6	2.0	1.0	2.4	2.8	3.2
Moni Toplou 0.7 km towards Sitia, MU29 (ZMH 36606)	3.8	2.3	7.2	1.9	0.9	2.3	2.6	2.9
Kato Zakros 0.6 km towards Zakros, MU38 (ZMH 36820)	3.2	1.6	6.3	1.9	0.8	1.4	1.6	2.0
Kato Zakros 0.6 km towards Zakros, MU38 (ZMH 36820)	2.5	1.8	6.3	1.9	0.9	2.2	2.5	2.8

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(FIGS 1.2E, 1.3D, 1.5C; TABLES 1.1, 1.3, 1.5)

Xerocrassa franciscoi Hausdorf & Sauer, 2009: 385-386, Figs 2E, 3D, 5C; Tables 1, 3, 5.

Locus typicus: Greece, Kríti, Nomos Iraklion: Ano Kapetaniana 7 km towards Agios Ioannis.

Type material: Holotype: Greece, Kríti, N. Iraklion: Ano Kapetaniana 7 km towards Agios Ioannis, 120 m alt., LU2269 (ZMH 51072, collected by B. Hausdorf & J. Sauer 19. October 2004, measurements: D = 11.6 mm, H = 4.7 mm). Paratypes: Greece, Kríti, N. Iraklion: LU1968 (HNC); LU1969 (HNC); LU2068 (HNC 29138); LU2168 (HNC 29143); LU2267 (HNC 29139); LU2268 (HNC 29162); Ano Kapetaniana 7 km towards Agios Ioannis, 120 m alt., LU2268 (ZMH 29685); LU2269 (HNC 29164); Ano Kapetaniana 4.7 km towards Agios Ioannis, 370 m alt., LU2269 (HNHM; MAA; NMG; RMNH; SUB; ZMB; ZMH 29694); LU2368 (HNC 29136); LU2369 (HNC 29142); LU2468 (HNC 29132); LU2469 (HNC 29126, 29127); LU2568 (HNC 29140).

Diagnosis: *X. franciscoi* is characterized by a discoidal shell with a broadly protruding keel and a wide umbilicus and a relatively short flagellum (proximal epiphallus: flagellum ratio 2.3-2.6).

Shell (Fig. 1.2E, Table 1.1): discoidal; with 4-4.5 whorls; teleoconch with $\pm$ regular ribs; whitish, with or without faint brown bands that might break up into spots; body whorl with a broadly protruding, well separated keel; aperture almost rhombic; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus wide, almost concentrical or slightly eccentric, not obscured by the columellar edge.

Genitalia (Fig. 1.3D, Tables 1.3, 1.5): The inner structures of the genitalia are similar to those of *X. mesostena*, but the apical part of the penial papilla is short.

Remarks: According to the AFLP data (chapter 2, 3) *X. franciscoi* is most closely related to *X. mesostena*, from which it differs in the usually higher proximal epiphallus: flagellum ratio

(Table 1.3). This ratio varies in *X. franciscoi* between 2.3 and 2.6. In the adjacent *X. mesostena* populations it is distinctly smaller. The average in *X. mesostena* is 1.3. However, there is an unusual form of *X. mesostena* on the southern slope of the Dikti Mountains with a high proximal epiphallus: flagellum ratio. Only four specimens from the surroundings of AnoVianos and Amiras of 127 measured *X. mesostena* specimens had a proximal epiphallus: flagellum ratio as high as *X. franciscoi*. The *X. mesostena* form from the southern Dikti Mountains is not related to *X. franciscoi* according to the AFLP data. A form of *X. mesostena* (Fig. 1.8C) that resembles *X. franciscoi* in the broadly protruding keel and a wide umbilicus occurs west of Paleochora, but differs from *X. franciscoi* in the longer flagellum (Tables 1.3 and 1.10).

The ranges of *X. franciscoi* and *X. mesostena* abut in LU2469 and are separated by a narrow hybrid zone (F. Welter-Schultes, pers. comm.), where six typical *X. franciscoi* have been found close to three specimens of *X. mesostena* with an angular body whorl or a not broadly protruding keel. These specimens might indicate that there is or was some hybridization between *X. franciscoi* and *X. mesostena*. No indications of hybridization were found in LU1968, where both species also occur together, or in other adjacent populations of the two taxa.

X. franciscoi is superficially similar to *X. gharlapsi* (Beckmann, 1987) from Malta. *X. franciscoi* differs from that species in the smaller shell (large diameter 9.4-11.7 mm versus 12.5-14.0 mm in *X. gharlapsi*) with a less sharp keel that is stepped from the upper side of the whorls, whereas the upper side of *X. gharlapsi* evenly passes into the very sharp keel and the in relation to the proximal epiphallus longer penis and vagina.

Distribution (Fig. 1.5C): *X. franciscoi* is restricted to the southern slope of the western Asteroussia Mountains south of Kapetaniana.

Ethymology: The species is named after Francisco W. Welter-Schultes who discovered it and recognized it as new species.

Table 1.3. Comparison of the ratios of some parts of the genitalia of *Xerocrassa* species of Crete. Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

		ep_p: p			ep_p: ep_d			ep_p: fl			ep_p: da			ep_p: v_t			v_t: v_{da}			v_t: v_{gm}		
	n	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean
Xerocrassa amphiconus	6	1.8	2.5	2.1	2.7	3.8	3.2	3.3	4.6	3.9	6.7	11.6	8.3	2.2	3.1	2.5	1.2	1.5	1.3	1.1	1.2	1.2
Xerocrassa cretica	24	1.8	3.6	2.9	4.1	8.1	5.6	0.8	1.2	1.0	6.8	15.0	11.4	2.4	5.6	3.1	1.3	1.9	1.5	1.1	1.4	1.2
Xerocrassa franciscoi	4	1.5	2.0	1.8	4.0	4.5	4.2	2.3	2.6	2.4	7.5	8.3	7.8	1.8	2.1	1.9	1.2	1.3	1.3	1.1	1.2	1.1
Xerocrassa grabusana	7	2.4	2.8	2.6	5.5	8.6	6.9	1.1	1.5	1.3	10.2	14.2	11.7	2.3	3.5	3.1	1.2	1.5	1.4	1.2	1.3	1.3
Xerocrassa heraklea	6	1.2	1.8	1.5	3.7	5.8	4.6	0.7	1.0	0.9	5.7	8.1	6.7	1.5	1.9	1.7	1.6	1.8	1.8	1.3	1.4	1.4

Table 1.3. *Continued*

		ep_p: p			ep_p: ep_d			ep_p: fl			ep_p: da			ep_p: v_t			v_t: v_{da}			v_t: v_{gm}		
	n	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean
Xerocrassa kydonia	6	2.1	2.8	2.6	3.3	5.7	4.5	3.0	4.9	3.8	5.4	9.3	7.6	1.4	2.3	1.8	1.3	1.4	1.3	1.1	1.3	1.2
Xerocrassa lasithiensis	8	1.8	3.3	2.6	2.9	4.2	3.5	0.9	1.2	1.0	8.1	14.3	10.8	2.4	3.1	2.7	1.2	1.4	1.3	1.1	1.2	1.2
Xerocrassa mesostena	127	1.0	2.7	1.7	1.8	7.1	3.3	0.9	2.8	1.3	3.1	17.5	6.9	0.9	2.8	1.8	1.1	1.4	1.2	1.0	1.2	1.1
Xerocrassa rhithymna	6	1.4	2.5	2.0	3.4	6.5	5.0	0.8	1.1	0.9	4.6	8.5	7.0	1.4	2.1	1.8	1.2	1.5	1.3	1.1	1.2	1.2
Xerocrassa siderensis	16	1.5	2.3	1.9	2.3	5.0	3.3	2.7	3.7	3.3	5.9	12.0	7.9	1.8	3.4	2.3	1.2	1.3	1.3	1.1	1.2	1.1
Xerocrassa subvariegata	20	2.2	3.8	2.9	4.6	10.0	6.7	0.9	1.2	1.1	6.4	15.0	10.1	1.8	4.2	3.1	1.2	1.5	1.3	1.1	1.4	1.2

Table 1.4. Measurements of some parts of the genitalia of *Xerocrassa cretica* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Rodovani 2 km towards Sougia, GE50 (ZMH 29588)	6.6	3.0	16.7	21.5	1.5	4.6	5.1	6.1
Kamares 2 km towards Lochria, KU99 (ZMH 37240)	6.9	2.8	17.0	16.2	1.7	3.5	4.1	5.1
Kambani 0.7 km towards Marathi, KV33 (ZMH 29486)	6.9	3.0	23.9	19.8	2.3	2.3	3.6	4.3
Agii Deka 3.4 km towards Vourvoulites, LU18 (ZMH 29900)	6.6	3.0	18.2	16.5	1.5	3.6	4.3	5.1
Moni Gorgolani near Kato Asites, LU19 (ZMH 36304)	6.1	3.1	17.8	16.5	2.6	5.0	5.8	6.6
Atsipades 1.5 km towards Loures, LU28 (ZMH 29869)	7.8	2.3	14.0	16.5	1.3	3.6	4.5	5.6
Floria 2.5 km towards Kandanos, LU41 (ZMH 37029)	7.4	3.1	19.0	23.9	1.5	4.8	5.4	6.6
Sikologos 2.0 km towards Mirtos, LU67 (ZMH 29523)	6.1	2.5	19.0	21.5	2.3	4.5	5.4	6.1
Anatoli 0.5 km towards Kalamafka, LU77 (ZMH 36267)	5.4	2.5	16.2	14.0	1.7	4.0	4.5	5.8
Anatoli 4.2 km towards Males, LU78 (ZMH 36534)	7.4	3.3	15.3	19.3	1.8	3.3	3.8	5.3
6 km E of Ierapetra, LU97 (MAA)	4.3	2.6	14.2	13.4	1.2	3.3	3.8	5.0

Table 1.4. *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Iraklion: E of the airport, LV31 (SUB)	5.8	3.3	16.5	21.5	2.1	3.6	4.3	5.1
Amnissos, LV31 (ZMH 36428)	6.6	3.3	18.2	19.0	2.0	2.8	3.5	4.6
Kokkini Chani, LV41 (ZMH 29580)	5.8	3.1	18.3	15.8	1.7	3.3	4.0	5.4
Xerokampos 3.3 km towards Zakros, MU28 (ZMH 36458)	6.6	3.5	19.8	16.5	1.5	4.1	5.3	6.4
Palekastro 4.7 km towards Vai, MU39 (ZMH 36579)	5.8	3.3	20.3	16.5	1.7	5.3	6.1	6.9
Palekastro 4.7 km towards Vai, MU39 (ZMH 50000)	1.9	1.4	5.7	5.7	0.4	1.3	1.5	1.8
Palekastro 4.7 km towards Vai, MU39 (ZMH 50000)	1.9	1.3	5.7	5.0	0.4	1.4	1.8	2.3
Palekastro 4.7 km towards Vai, MU39 (ZMH 50000)	2.2	1.1	6.0	5.8	0.4	1.3	1.6	2.0
Palekastro 4.7 km towards Vai, MU39 (ZMH 50001)	2.2	1.4	7.2	6.9	0.5	1.9	2.2	2.7
Palekastro 4.3 km towards Vai, MU39 (ZMH 50118)	2.0	1.4	6.6	5.8	0.5	1.3	1.6	2.2
Palekastro 4.3 km towards Vai, MU39 (ZMH 50118)	1.9	0.9	4.4	4.3	0.3	1.3	1.4	1.8
Zakros 3 km towards Kato Zakros, MU39 (ZMH 29343)	5.8	4.1	20.6	17.0	2.0	3.6	4.3	5.8
Palekastro 5.8 km towards Vai, MV31 (ZMH 50237)	6.9	3.5	22.3	19.0	2.0	5.3	6.1	7.1

Table 1.5. Measurements of some parts of the genitalia of *Xerocrassa franciscoi* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Ano Kapetaniana 7 km towards Agios Ioannis, LU26 (ZMH 29685)	2.8	1.4	5.7	2.2	0.8	2.2	2.5	3.0
Ano Kapetaniana 7 km towards Agios Ioannis, LU26 (ZMH 29685)	3.3	1.6	6.3	2.6	0.8	2.3	2.6	3.0
Ano Kapetaniana 7 km towards Agios Ioannis, LU26 (holotype ZMH 51072)	3.1	1.2	5.4	2.1	0.7	2.2	2.5	2.8
Ano Kapetaniana 4.7 km towards Agios Ioannis, LU26 (ZMH 29694)	3.5	1.3	5.2	2.3	0.7	2.4	2.6	2.9

XEROCRASSA GRABUSANA Hausdorf & Sauer, 2009

(FIGS 1.3E, 1.4C, 1.5D, 1.6A; TABLES 1.1, 1.3, 1.6)

Trochoidea sp. – Vardinoyannis, 1994: 86, 88, 133, map 50 [partim].

Xerocrassa grabusana Hausdorf & Sauer, 2009: 386-387, FIGS 3E, 4C, 5D, 6A; TABLES 1, 3, 6. Locus typicus: Greece, Kríti, Nomos Chania: Kaliviani 5.5 km towards Balos.

Type material: Holotype: Greece, Kríti, N. Chania: Kaliviani 5.5 km towards Balos, 130 m alt., GE3637 (ZMH 51070, collected by B. Hausdorf & J. Sauer 1. October 2004, measurements: D = 8.7 mm, H = 6.1 mm). Paratypes: Greece, Kríti, N. Chania: Limani, GE3227 (MAA); Falassarna, 5 m alt., GE3332 (HNHM; MAA; NMG; ZMH 50373); 2 km N of Falassarna, 20 m alt., GE3333 (HNHM; ZMH 29427); Platanos 3 km towards Falasarna, 65 m alt., GE3429 (ZMH 50232, 50233); E-coast of Gramvousa peninsula, car park at end of track, GE3438 (MAA); Balos Beach, GE3539 (MAA); 7 km N of Kaliviani, GE3539 (HNHM); 1 km N of Kaliviani, GE3633 (HNHM); 2 km N of Kaliviani, GE3634 (HNHM; MAA); Kaliviani 2 km towards Balos, 10 m alt., GE3635 (ZMH 29885; 36077); Kaliviani 5.5 km towards Balos, 130 m alt., GE3637 (HNC; RMNH; SUB; ZMB; ZMH 36574); 6 km N of Kalivani, GE3639 (MAA); Limin Kavounisou, 1 m alt., GE3833 (ZMH 29865, 50325); 1 km NW of Kastelli Kissamos, GE3931 (HNHM); Kastelli Kissamos 1.5 km towards Platanos, 10 m alt., GE3932 (ZMH 37023); 8 km N of Kaliviani, GE3540 (HNHM; MAA); 1 km E of Balos Beach, GE3541 (MAA).

Diagnosis: *X. grabusana* is characterized by a small, irregularly coarsely ribbed, depressed conical shell with a distinct keel that is usually separated by grooves and a moderately narrow umbilicus and a penial papilla with a cylindrical basal part and a dilated apical part and a subterminal opening.

Shell (Fig. 1.6A, Table 1.1): depressed conical; with 4.5-5 convex whorls; teleoconch with irregular ribs and irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl with a keel that is usually separated by grooves and becomes weaker towards the aperture; aperture elliptical; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded,

with a whitish internal rib; umbilicus moderately narrow, slightly eccentric, hardly obscured by the columellar edge.

Genitalia (Figs 1.3E, 1.4C, Tables 1.3, 1.6): The penial papilla is divided into a cylindrical basal part and a slightly or distinctly dilated apical part that is separated from the basal part by an incision. A bulge extends from the subterminal opening of the penial papilla towards its apical tip. There is a glandular belt in the penial wall near its proximal end and a very indistinct longitudinal glandular field at the abvaginal inner side of the penis wall. There are no distinct stimulatory structures in the atrium.

Remarks: *X. grabusana* is most similar to *X. subvariegata* from which it differs in the coarser ribs and the more distinct keel and the statistically significant (t = -4.958, $p < 0.001$ (two-sided)) higher proximal epiphallus: flagellum ratio (see Table 3). Although there is some overlap in the variation of the proximal epiphallus: flagellum ratio (as well as in the conchological variation), we consider *X. grabusana* to be specifically distinct from *X. subvariegata*, because the variation of the ratio in the *X. grabusana* population from Limin Kavounisou which is the *X. grabusana* population that is most closely situated to neighbouring *X. subvariegata* populations, lies outside of the variation in *X. subvariegata*. This indicates that *X. grabusana* and *X. subvariegata* do not or only rarely hybridize. Actually, both species are separated in a tree based on *coxI* and 16S rDNA sequences as well as in a tree based on AFLP markers (chapter 2, 3, 5).

Distribution (Fig. 1.5D): *X. grabusana* is spread in northwestern Crete from the Gramvousa peninsula to Limani in the southwest and to Kastelli Kissamos in the east.

Ethymology: The species is named after the ancient name of the Gramvousa peninsula, Grabusa (*grabusana* used as an adjective).

Fig. 1.6. *Xerocrassa* species from Crete, shells. A, *Xerocrassa grabusana* Hausdorf & Sauer, Kaliviani 5.5 km towards Balos (holotype ZMH 51070). B, *Xerocrassa heraklea* Hausdorf & Sauer, Stalida 3 km towards Mohos (holotype ZMH 51068).C, *Xerocrassa kydonia* Hausdorf & Sauer, Koutsomatados (holotype ZMH 51069). D, *Xerocrassa lasithiensis* Hausdorf & Sauer, Lenika 1 km towards Elounda (holotype ZMH 51071). E, *Xerocrassa meda* (Porro), Chania (MAA). Scale bar = 5 mm.

Table 1.6 Measurements of some parts of the genitalia of *Xerocrassa grabusana* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
2 km N of Kaliviani, GE33 (ZMH 29885)	1.7	0.8	4.7	3.8	0.4	1.5	1.6	2.1
2 km N of Kaliviani, GE33 (ZMH 29885)	1.9	0.8	5.4	3.9	0.4	1.0	1.1	1.5
Kaliviani 5.5 km towards Balos, GE33 (ZMH 36574)	2.0	0.6	5.0	4.7	0.4	1.1	1.3	1.6
Kaliviani 5.5 km towards Balos, GE33 (holotype ZMH 51070)	3.0	1.1	7.2	5.7	0.6	2.2	2.3	2.7
Limin Kavounisou, GE33 (ZMH 29865)	2.6	1.0	6.4	4.3	0.6	1.4	1.5	2.0
Limin Kavounisou, GE33 (ZMH 29865)	2.8	1.3	7.3	4.9	0.6	1.8	1.9	2.4
Limin Kavounisou, GE33 (ZMH 29865)	2.2	0.7	6.0	4.1	0.6	1.3	1.4	1.7

Table 1.7. Measurements of some parts of the genitalia of *Xerocrassa heraklea* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Vathianos Kampos 1.5 km towards Tombrouk, LV31 (ZMH 36250)	1.7	0.6	2.6	2.8	0.4	0.9	1.2	1.7
Mohos 0.7 km towards Stalida, LV 50 (ZMH 29835)	3.2	0.8	3.9	3.8	0.6	1.3	1.6	2.3
Stalida 3 km towards Mohos, LV50 (holotype ZMH 51068)	2.3	0.9	4.1	4.7	0.5	1.2	1.5	2.1
Stalida 3 km towards Mohos, LV50 (ZMH 36650)	2.6	0.9	4.0	5.4	0.7	1.5	2.0	2.5
Stalida, LV50 (ZMH 36209)	2.1	0.9	3.5	4.2	0.4	1.3	1.6	2.3
Agios Konstantinos, LU69 (ZMH 36196)	2.9	0.6	3.7	3.8	0.6	1.2	1.5	2.1

XEROCRASSA HERAKLEA Hausdorf & Sauer, 2009

(FIGS 1.3F, 1.5E, 1.6B; TABLES 1.1, 1.3, 1.7)

Xerocrassa heraklea Hausdorf & Sauer, 2009: 388-389, Figs 3F, 5E, 6B; Tables 1, 3, 7. Locus typicus: Greece, Kríti, Nomos Iraklion: Stalida 3 km towards Mohos.

Type material: Holotype: Greece, Kríti, N. Iraklion: Stalida 3 km towards Mohos, 190 m alt., LV5605 (ZMH 51068, collected by B. Hausdorf & J. Sauer 23.10.2004, measurements: D = 6.5 mm, H = 4.8 mm). Paratypes: Greece, Kríti, N. Iraklion LU4497 (HNC); LU4596 (HNC); 1 km W of Kato Karouzana LU49 (MAA); Kato Karouzana LU49 (MAA); Tombrouk 0.5 km towards Vathianos Kampos, 20 m alt., LV3711 (ZMH 29678); Vathianos Kampos 1.5 km towards Tombrouk, 20 m alt., LV3811 (ZMH 36250); Amnissos, LV31 (MAA); LV4700 (HNC); S of Kokkini Chani, LV4010 (HNC); LV4110 (HNC); Kokkini Chani, 20 m alt., LV4210 (ZMH 29586); 3 km W of Kokkini Chani, LV41 (HNHM 44203); LV5403 (HNC); LV5405 (HNC); Mohos 0.7 km towards Stalida, 410 m alt., LV5604 (ZMH 29835); Stalida 5.7 km towards Mohos, 320 m alt., LV5605 (ZMH 29302); Stalida 3 km towards Mohos, 190 m alt., LV5605 (NMG; RMNH; SUB; ZMB; ZMH 36650); Iráklion 31 km towards Agios Nikolaos, 115 m alt., LV5605 (ZMH 50470); Stalida, 10 m alt., LV5706 (ZMH 36209); 2 km SE of Potamides, LV50 (HNHM); 4 km N of Kastelli, LV50 (MAA); Limin Chersonisou, LV50 (HNHM; MAA); E of Limin Chersonisou, LV50 (HNHM); SE of Limin Chersonisou, LV50 (MAA); Limin Chersonisou, port, LV51 (MAA). – N. Lasithi: Agios Konstantinos, 810 m alt., LU6393 (ZMH 36196); SW of Agios Konstantinos, LU69 (HNHM).

Diagnosis: *X. heraklea* is characterized by a small (< 8 mm), conical, coarsely striated or ribbed shell with a blunt edge or a keel, a proximal epiphallus: dart apparatus ≤ 8.1, a proximal epiphallus: vagina length ratio ≤ 1.9, a proximal epiphallus: flagellum ratio ≤ 1.0, a total length of the vagina: vagina up to the base of the dart apparatus ratio ≥ 1.6, a total length of the vagina: vagina up to the glandulae mucosae ratio ≥ 1.3 and a penial papilla that is divided into a long, terminally open basal part and a very short conical apical part.

Shell (Fig. 1.6B, Table 1.1): conical or depressed conical; with 4.5-5.25 convex whorls; teleoconch irregular coarsely striated or ribbed, usually with irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl usually with a blunt edge or a keel that becomes weaker towards the aperture;

aperture elliptical; upper insertion of the peristome slightly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus narrow or very narrow, hardly or partly obscured by the columellar edge.

Genitalia (Fig. 1.3F): The penial papilla is divided into a long, slightly tapering or cylindrical terminally open basal part and a very short conical apical part consisting of a bulge that is adnate with the basal part at the side opposite the opening. There may be a shallow furrow in the apical part of the papilla. There is a glandular belt in the penial wall near its proximal end and a longitudinal glandular field at the abvaginal inner side of the penis wall with distinct folds. There are no distinct stimulatory structures in the atrium.

Remarks: The shells of *X. heraklea* cannot be distinguished from those of *X. lasithiensis, X. rhithymna* and *X. kydonia*. *X. heraklea* differs from *X. lasithiensis* in the smaller proximal epiphallus: dart apparatus ratio (≤ 8.1) and the smaller proximal epiphallus: vagina length ratio (≤ 1.9), from *X. rhithymna* in the lower insertion of the dart apparatus and the glandulae mucosae at the vagina length (total length of the vagina: vagina up to the base of the dart apparatus ≥ 1.6; total length of the vagina: vagina up to the glandulae mucosae ≥ 1.3) and from *X. kydonia* in the smaller proximal epiphallus: flagellum ratio (≤ 1.0; Table 1.3). *X. heraklea, X. kydonia, X. rhithymna* and *X. lasithiensis* differ from the usually larger *X. mesostena* in the penial papilla that is divided into a long, terminally open basal part and a very short conical apical part. The penial papillae of *X. heraklea, X. kydonia, X. rhithymna* and *X. lasithiensis* are similar to those of *X. amphiconus* and *X. siderensis*, but differs from the penial papillae of these species in the solid apical part without a deep channel.

Distribution (Fig. 1.5E): The range of *X. heraklea* extends in northern Crete from Tombrouk (east of Iraklion) in the west to the Lasithi plateau in the east.

Ethymology: The species is named after its distribution in the prefecture Iraklion, derived from the ancient town Heraklea (used as a noun in apposition).

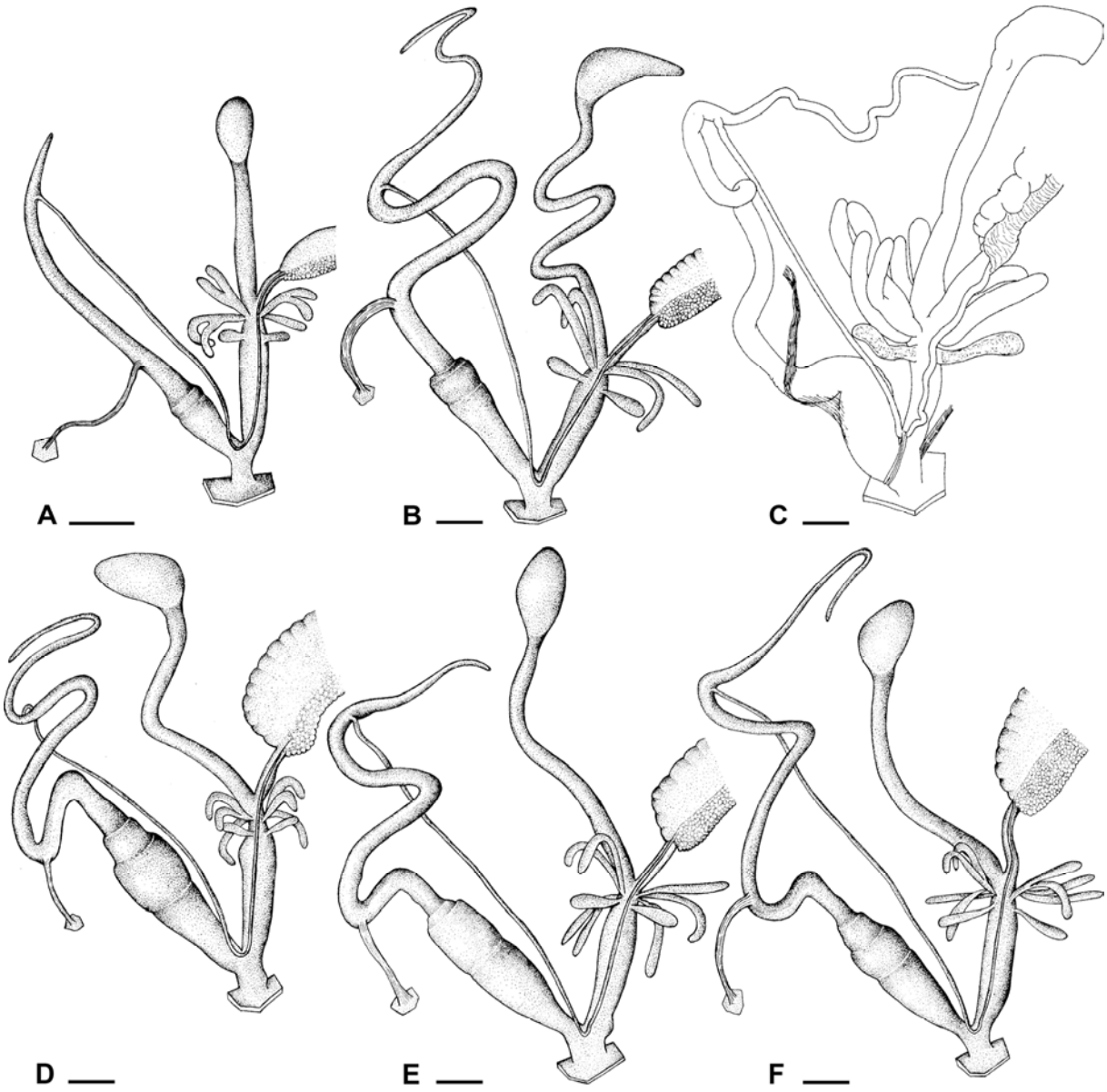

Fig. 1.7. *Xerocrassa* species from Crete, genitalia. Scale bar = 1 mm. A, *Xerocrassa kydonia* Hausdorf & Sauer, Koutsomatados (holotype ZMH 51069). B, *Xerocrassa lasithiensis* Hausdorf & Sauer, Lenika 1 km towards Elounda (holotype ZMH 51071). C, *Xerocrassa meda* (Porro), Malta, Sliema (from Giusti, Manganelli & Schembri, 1995: Fig. 542). D, *Xerocrassa mesostena* (Westerlund), Ano Arhanes: Jouchtas mountain top (ZMH 29912). E, *Xerocrassa mesostena* (Westerlund), Ano Viannos 1 km towards Mirtos (ZMH 29753). F, *Xerocrassa mesostena* (Westerlund), Moni Assomaton 0.3 km towards Vistagi (ZMH 50033).

XEROCRASSA KYDONIA Hausdorf & Sauer, 2009

(FIGS 1.4D, 1.5F, 1.6C, 1.7A; TABLES 1.1, 1.3, 1.8)

Helix (*Xerophila*) *mesostena* – Martens, 1889: 187 [partim, non Westerlund, 1879].

Trochoidea sphakiota – Vardinoyannis & Mylonas, 1988: 139 [non Maltzan, 1883].

Trochoidea mesostena – Vardinoyannis, 1994: 85, 88, 130, map 45 [partim, non Westerlund, 1879].

Xerocrassa kydonia Hausdorf & Sauer, 2009: 389-391, Figs 4D, 5F, 6C, 7A; Tables 1, 3, 8.

Locus typicus: Greece, Kríti, Nomos Chania: Koutsomatados, walking path from taverna Panorama to cave Agias Sofias, dry grassland.

Type material: Holotype: Greece, Kríti, N. Chania: Koutsomatados, walking path from taverna Panorama to cave Agias Sofias, dry grassland, 300 m alt., GE4321 (ZMH 51069, collected by B. Hausdorf & J. Sauer 03. October 2004, measurements: D = 5.8 mm, H = 4.7 mm). Paratypes: Greece, Kríti, N. Chania: Platanos 1.8 km towards Sfinari, 250 m alt., GE3526 (ZMH 29855); Platanos 2.3 km towards Sfinari, 250 m alt., GE3526 (ZMH 36484); S of Platanos, GE32 (SUB); GE4321 (HNC); Koutsomatados, near cave Agias Sofias, 300 m alt., GE4321 (MAA; ZMH 36400); Koutsomatados, walking path from taverna Panorama to cave Agias Sofias, 300 m alt., GE4321 (NMG; RMNH; ZMH 36263); Koutsomatados, GE42 (MAA); canyon S of Topolia, rocks near tunnel, GE4422 (MAA); Mesavlia 1.8 km towards Chania, 630 m alt., GE4823 (ZMH 29204, 36893, 50128); 1.3 km N of Mesavlia, GE42 (HNHM); Kakopetros 2 km towards Mesavlia, GE42 (HNHM); S of Kakopetros, near cave, GE42 (MAA); N of Kakopetros, GE42 (HNHM 44208); Kalathenes, GE42 (ZMB, specimens to Martens (1889: 187 partly)); Kaloudiana, GE43 (SUB); Rodopos 3 km towards Agios Ioannis, 420 m alt., GE4941 (ZMH 37166); Rodopos towards Agios Ioannis, GE44 (ZMH 37283); 4 km N of Rodopos, GE44 (HNHM); 7.8 km N of Rodopos, GE44 (HNHM); 2 km N of Kakopetros, GE52 (MAA); S of Afrata, towards "Evepidon Monument", GE5138 (MAA); Kolimbari 1.5 km towards Afrata, 20 m alt., GE5138 (ZMH 29628); 2 km N of Kolimbari, GE53 (HNHM); Kolimbari towards Afrata, 700 m away from the main road on a way, GE53 (SUB); Afrata 1.5 km towards Kolimbari, 60 m alt., (ZMH 50280); Afrata, 5 m alt., GE5140 (ZMH 36959, 50456); E of Afrata, GE54 (HNHM 45078); 1 km N of Afrata, GE54 (HNHM); 2 km N of Afrata, GE54 (HNHM; SUB); SE of Afrata, GE54 (HNHM); Diktynna, GE54 (MAA).

Diagnosis: *X. kydonia* is characterized by a small (< 8 mm), conical, coarsely striated or ribbed shell with a blunt edge or a keel and a narrow umbilicus, a proximal epiphallus: flagellum ratio ≥ 3.0 and a penial papilla that is divided into a long, terminally open basal part and a very short conical apical part.

Shell (Fig. 1.6C, Table 1.1): conical or depressed conical; with 3.75-5 convex whorls; teleoconch irregular coarsely striated or ribbed, sometimes with irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl usually with a blunt edge or a keel that becomes weaker towards the aperture; aperture elliptical; upper insertion of the peristome slightly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus narrow or very narrow, hardly or partly obscured by the columellar edge.

Genitalia (Figs 1.4D, 1.7A, Tables 1.3, 1.8): The inner structures of the genitalia correspond to those of *X. heraklea*.

Remarks: *X. kydonia* differs from the neighbouring populations of *X. mesostena* in the usually smaller, more conical shell with more strongly arched whorls and the shorter flagellum (Table 3). The shells of *X. kydonia* cannot be distinguished from those of *X. rhithymna*, *X. heraklea* and *X. lasithiensis*. *X. kydonia* differs from all these species in the larger proximal epiphallus: flagellum ratio (≥ 3.0; Table 1.3).

Distribution (Fig. 1.5F): *X. kydonia* is restricted to north-west Crete from Platanos in the west to Kakopetros in the east and the Rodopou peninsula in the north.

Ethymology: The species is named after its distribution in the prefecture Chania, derived from the ancient town Kydonia (used as a noun in apposition).

Table 1.8 Measurements of some parts of the genitalia of *Xerocrassa kydonia* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Platanos 1.8 km towards Sfinari, GE32 (ZMH 29855)	1.9	0.9	4.9	1.0	0.6	1.8	2.0	2.6
Platanos 2.3 km towards Sfinari, GE32 (ZMH 36484)	2.0	0.9	5.4	1.3	0.7	2.3	2.5	2.9
Koutsomatados, GE42 (holotype ZMH 51069)	1.1	0.9	3.2	0.9	0.4	1.6	2.0	2.2
Koutsomatados, GE42 (ZMH 36263)	1.5	1.1	4.1	1.3	0.4	1.4	1.5	1.8
Mesavlia 1.8 km towards Chania, GE42 (ZMH 36893)	2.3	1.0	4.7	1.1	0.9	2.2	2.5	2.8
canyon S of Topolia, GE43 (MAA)	1.5	0.9	4.0	1.3	0.6	2.0	2.3	2.7

Table 1.9 Measurements of some parts of the genitalia of *Xerocrassa lasithiensis* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Agios Konstantinos 1 km towards Agios Nikolaos, LU79 (ZMH 36919)	2.2	1.6	6.6	6.0	0.8	2.0	2.3	2.6
Sisi, LV60 (MAA)	2.0	1.6	5.4	6.0	0.6	1.4	1.6	1.8
Vrahasi 1.3 km towards Malia, LV60 (ZMH 36875)	2.8	2.2	7.8	7.2	0.7	1.9	2.2	2.5
Milatos, cave, LV70 (MAA)	2.3	1.7	5.4	5.3	0.4	1.4	1.6	2.0
Plaka 0.5 km towards Elounda, LV80 (ZMH 36126)	2.6	1.9	6.6	5.7	0.5	1.6	1.8	2.1
Lenika 1 km towards Elounda, LV80 (holotype ZMH 51071)	3.0	1.9	7.6	7.1	0.9	2.2	2.5	3.0
2 km N of Elounda, LV80 (MAA)	1.1	1.3	3.8	4.4	0.4	1.1	1.3	1.5
2 km S of Vrouhas, LV80 (MAA)	2.0	1.1	3.7	3.9	0.3	1.1	1.3	1.6

XEROCRASSA LASITHIENSIS Hausdorf & Sauer, 2009

(FIGS 1.5G, 1.6D, 1.7B; TABLES 1.1, 1.3, 1.9)

Trochoidea spec. – Schultes & Wiese, 1992: 74.

Xerocrassa lasithiensis: - Hausdorf & Sauer, 2009: 391-392 Figs 5G, 6D, 7B; Tables 1, 3, 9.

Locus typicus: Greece, Kríti, Nomos Lasithi: Lenika 1 km towards Elounda: wasteland at rocks.

Type material: Holotype: Greece, Kríti, N. Lasithi: Lenika 1 km towards Elounda: wasteland at rocks, 60 m alt., LV8400 (ZMH 51071, collected by B. Hausdorf & J. Sauer 24. October 2004, measurements: D = 7.3 mm, H = 5.7 mm). Paratypes: Greece, Kríti, N. Lasithi: Ag. Konstantinos 1 km towards Ag. Nikolaos, 310 m alt., LU7597 (ZMH 36919); Tapes 4.5 km towards Ag. Nikolaos, 280 m alt., LU7694 (ZMH 50345); Exo Lakonia 1.5 km towards Neapoli, 180 m alt., LU7797 (ZMH 50095); LU7797 (HNC); Skisma 0.6 km towards Ag. Nikolaos, LU79 (MAA); LU8094 (HNC); LU8097 (HNC); LU8099 (HNC); LU8196 (HNC); 1 km E of Lenika, 180 m alt., LU8499 (RMNH); LU8599 (HNC); 0.5 km NW of Ag. Nikolaos, LU89 (MAA); 4 km SE of Ag. Nikolaos, LU89 (MAA); Ag. Nikolaos, LU89 (MAA); LV6709 (HNC); Vrahasi 1.3 km towards Malia, 250 m alt., LV6805 (ZMH 36875); Milatos 1 km towards Epano Sisi, 80 m alt., LV6807 (MAA; NMG; ZMB; ZMH 36348); Sisi, LV60 (MAA); E of Sisi, LV60 (HNHM 44202); 0.5 km W of Sisi, LV60 (MAA); Moni Agios Georgios Selenari near Vrahasi, LV60 (MAA); 3.5 km E of Sisi, LV60 (MAA); 1 km E of Vrahasi, LV70 (MAA); Vrahasi 1.3 km towards Latsida, 340 m alt., LV7004 (ZMH 50496); Milatos 2.2 km towards Kounali, cave, 140 m alt., LV7008 (MAA; ZMH 36153); LV7304 (HNC); LV7403 (HNC); LV7404 (HNC); LV7407 (HNC); LV7501 (HNC); LV7505 (HNC); Nofalias 1.5 km towards Kourounes, 570 m alt., LV7606 (ZMH 29494); LV7705 (HNC); LV7901 (HNC); Moni Aretion, 530 m alt., LV7907 (ZMH 36186); 2 km E of Neapoli, LV70 (HNHM); 2.5 km E of Milatos, LV70 (HNHM); 6 km E of Milatos, LV70 (HNHM); Vrises, LV70 (MAA; HNHM); Kourounes 0.8 km towards Frathias, LV70 (MAA); Nofalias 2 km towards Amigdalae, LV70 (MAA); 1 km N of Nofalias, LV70 (MAA); 2 km NE of Neapoli, LV70 (MAA); 3 km S of Nofalias, LV70 (MAA); Moni Ag. Georgios Selinari, LV70 (MAA); Nofalias towards Dilakos, LV70 (ZMH 27130); LV7410 (HNC); Koudoumalo 0.3 km towards Valtos, 260 m alt., LV7810 (ZMH 36610); 2 km E of Finokalia, LV71 (MAA); LV8000 (HNC); LV8101 (HNC); LV8103 (HNC); LV8209 (HNC); LV8210

(HNC); Lenika 1 km towards Elounda, 60 m alt., LV8400 (HNHM; SUB 3882; ZMH 29890); Plaka 0.5 km towards Elounda, 0 m alt., LV8406 (ZMH 36126); LV8409 (HNC); LV8609 (HNC); Island Kolokithia, LV8703 (HNC 24928); 1.5 km W of Pines towards Neapoli, LV80 (HNHM); Spinalonga, LV80 (HNHM); Spinalonga opposite to Elounda, LV80 (HNHM); Pines, LV80 (HNHM); 1.5 km W of Pines towards Neapoli, LV80 (SUB 3881); Elounda 2.8 km towards Ag. Nikolaos, LV80 (MAA); Plaka 6 km towards Vrouhas, LV80 (MAA); 1 km E of Skinias, LV80 (MAA); 2 km N of Elounda, LV80 (MAA); 1 km S of Elounda, LV80 (MAA); 2 km W of Vrouhas, LV80 (MAA); 2 km S of Vrouhas, LV80 (MAA); Valtos, LV80 (ZMH 27072); LV8810 (HNC); LV8811 (HNC).

Diagnosis: *X. lasithiensis* is characterized by a small (< 8.2 mm), conical, coarsely striated or ribbed shell with a blunt edge or a keel and a narrow umbilicus, a proximal epiphallus: dart apparatus ratio ≥ 8.1, a proximal epiphallus: vagina length ratio ≥ 2.4, a proximal epiphallus: flagellum ratio ≤ 1.2 and a penial papilla that is usually divided into a long, terminally open basal part and a very short conical apical part.

Shell (Fig. 1.6D, Table 1.1): conical or depressed conical; with 4.25-5.25 convex whorls; teleoconch irregular coarsely striated or ribbed, usually with irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl usually with a blunt edge or a keel that becomes weaker towards the aperture; aperture elliptical; upper insertion of the peristome slightly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus narrow or very narrow, hardly or partly obscured by the columellar edge.

Genitalia (Fig. 1.7B, Tables 1.3, 1.9): The inner structures of the genitalia correspond to those of *X. heraklea*, but in one examined specimen the apical part of the penial papilla was almost absent so that the opening was almost terminal.

Remarks: The shells of *X. lasithiensis* cannot be distinguished from those of *X. rhithymna*, *X. heraklea* and *X. kydonia*. *X. lasithiensis* differs from *X. rhithymna* and *X. heraklea* in the higher proximal epiphallus: vagina length ratio (≥ 2.4) and the usually higher proximal epiphallus: dart apparatus ratio (≥ 8.1) and from *X. kydonia* in the smaller proximal epiphallus: flagellum ratio (≤ 1.2) (Table 1.3).

Distribution (Fig. 1.5G): *X. lasithiensis* is restricted to the region northeast of the Dikti mountains from Sisi to Agios Nikolaos.

Ethymology: The species is named after its distribution in the prefecture Lasithi.

XEROCRASSA MEDA (PORRO, 1840)
(FIGS 1.5H, 1.6E, 1.7C; TABLE 1.1)

Helix Meda Porro, 1840: 106. Locus typicus: „Sassari“, Sardinia, Italy.
Xerocrassa meda – Hausdorf & Sauer, 2009: 393, FIGS 5H, 6E, 7C; Table 1.

Diagnosis: *X. meda* is characterized by a depressed conical, distinctly ribbed shell with quickly increasing whorls and a very narrow, partly obscured umbilicus.

Shell (Fig. 1.6E, Table 1.1): depressed conical; with 4.75-5.25 convex whorls; teleoconch with irregular white ribs and irregular impressions (especially underneath); upper side corneous with white ribs, base almost completely whitish, with brownish bands that are more or less interrupted by the white ribs; body whorl rounded or with a blunt edge at the beginning; aperture oval; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus very narrow, partly or completely obscured by the columellar edge.

Genitalia (Fig. 1.7C): The flagellum is 0.6-0.8 times as long as the epiphallus. The penial papilla is almost cylindrical. Its opening is almost terminal. More detailed descriptions of the genitalia have been given by Hesse (1934) and Giusti *at al.* (1995).

Remarks: *X. meda* is the type species of *Xeroclausa* Monterosato, 1892. The relationships of *X. meda* to the east Mediterranean *Xerocrassa* species have to be investigated.

Distribution (Fig. 1.5H): *X. meda* is known from the Italian mainland, Sicily and Malta. Its native range is unclear, because the species is restricted to anthropogenic habitats. Originally, it was described from Sardinia, but only a single record from there is known that is possibly the result of a recent introduction (Giusti *et al.* 1995). *X. meda* has also been introduced on

Lesvos (Bank 1988; as *Cernuella* spec.) and Kos (Bank & Neuteboom, 1988; as *Trochoidea* spec.). On Crete it was found only once on the old city walls of Chania by W. J. M. Maassen in 1987. We did not find it there in 2004. Perhaps the introduced population became extinct.

XEROCRASSA MESOSTENA (WESTERLUND, 1879)

(FIGS 1.4E, 1.5I, 1.7D-F, 1.8A-D, 1.9A; TABLES 1.1, 1.3, 1.10)

Helix (*Xerophila*) *mesostena* Westerlund in Westerlund & Blanc, 1879: 71, pl. 3, fig. 23. Locus typicus: „Ile de Crète à Arkhanés“, Greece.

Helix (*Jacosta*) *Sphakiota* Maltzan, 1883: 103. Locus typicus: „in montibus „Levkaori“ dictis insulae Cretae“, Greece.

Helix (*Candidula?*) *Psiloritana* Maltzan, 1883: 105. Locus typicus: „prope Asomato montis Idae“, Greece.

Helix (*Candidula?*) *psiloritana* – Kobelt, 1888: 26, pl. 98 fig. 550.

Helix (*Xerophila*) *mesostena* – Martens, 1889: 187, pl. 10 fig. 10 [partim].

Helix (*Xerogibba*) *mesostema* [sic] – Cecconi, 1896: 219.

Helix (*Xerogibba*) *Psiloritana* – Cecconi, 1896: 220.

Helicella (*Helicopsis*?) *psiloritana* – Haas, 1935: 112.

Helicella (*Xerocrassa*) *psiloritana* – Fuchs & Käufel, 1936: 629.

“Helix” mesostena – Lindner, 1994: 74, fig. 4.

Trochoidea mesostena – Vardinoyannis, 1994: 85, 88, 130, map 45 [partim].

Trochoidea mesostena – Cameron, Mylonas & Vardinoyannis, 2000: 141.

Trochoidea mesostena – Cameron *et al.*, 2003: 95, 96.

Xerocrassa mesostena – Hausdorf & Sauer, 2009: 393, 395, 398, 399, Figs 4E, 5I, 7D-F, 8A-D, 9A; Tables 1, 3, 10.

Type material: Syntypes of *Helix mesostena*: Greece, Kríti, N. Iraklion: Arhanes, LV30 (NMGW/3); syntypes of *Helix sphakiota*: Greece, Kríti, N. Chania: Sfakia (ZMB 111956/3); syntypes of *Helix psiloritana*: Greece, Kríti, N. Rethimnon: Moni Assomaton, KV80 (ZMB 36374/16).

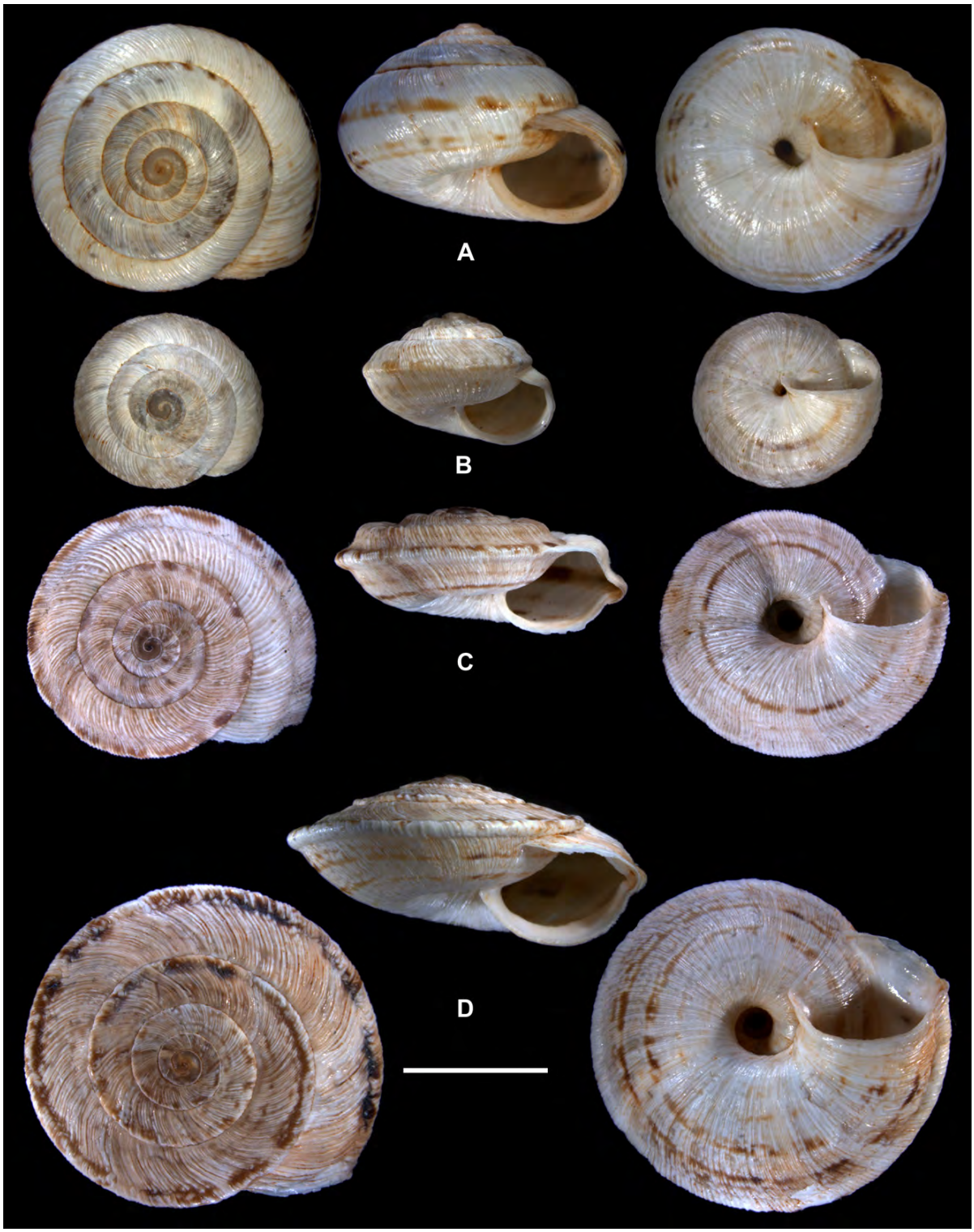

Figure 1.8 *Xerocrassa* species from Crete, shells. A, *Xerocrassa mesostena* (Westerlund), Arhanes (syntype of *H. mesostena* Westerlund NMGW). B, *Xerocrassa mesostena* (Westerlund), Sfakia (syntype of *H. sphakiota* Maltzan ZMB 111956). C, *Xerocrassa mesostena* (Westerlund), Paleochora 1.5 km towards Koundoura (ZMH 29603). D, *Xerocrassa mesostena* (Westerlund), Tapes 2.5 km towards Agios Nikolaos (ZMH 36666). Scale bar = 5 mm.

Diagnosis: *X. mesostena* is characterized by a medium-sized shell with a narrow umbilicus, a proximal epiphallus: flagellum ratio of 0.9-2.3 (rarely -2.8) and a penial papilla with a broader basal part and a narrower apical part with a terminal opening.

Shell (Figs 1.8A-D, 1.9A, Table 1.1): varying from almost discoidal to conical-globular; with 3.75-6 convex whorls; teleoconch irregularly striated or finely ribbed and with irregular impressions, especially underneath; whitish, with or without brown bands that might be fused or break up into spots; body whorl rounded or with a blunt edge or a distinct keel; aperture almost circular, elliptical or rhombic; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus varying from very narrow to wide, not or partly obscured by the columellar edge.

Genitalia (Figs 1.4E, 1.7D-F, Tables 1.3, 1.10): The penial papilla is divided into a broader basal part and a narrower apical part that is often separated from the basal part by an incision. A bulge is visible in the terminal opening of the penial papilla. There is a glandular belt in the penial wall near its proximal end and a longitudinal glandular field at the abvaginal inner side of the penis wall with more or less distinct folds. There are compact stimulatory structures in the atrium.

Remarks: *X. mesostena* is very variable. It differs from *X. heraklea, X. kydonia, X. lasithiensis* and *X. rhithymna* in the penial papilla with a broader basal part and a narrower apical part with a terminal opening and the usually larger shell with less strongly arched whorls. Where it co-occurs with *X. heraklea, X. lasithiensis* and *X. rhithymna* it can usually be distinguished from these species by shell characteristics. However, there are small forms of *X. mesostena* which can hardly be distinguished from the smaller species by shell characteristics.

Such small conical forms with a very narrow umbilicus and a blunt keel occur e.g., south of the Mesara plain and in the Sfakia (described as *Helix sphakiota*; Fig. 1.8B) and the adjacent region eastwards to Kapsodassos. Similarly keeled, but usually larger forms with a wider umbilicus occur also west of the Levka Mountains.

Depressed forms with a more or less distinct keel can be found in various regions of Crete. Usually, they have a narrow umbilicus. West of Paleochora there are populations with discoidal shells with a broadly protruding keel and a wide umbilicus (Fig. 1.8C). These populations are conchologically similar to *X. franciscoi*, but differ from that species in the

relatively longer flagellum (proximal epiphallus: flagellum ratio 0.9-1.4; see Tables 1.3 and 1.10). Another keeled, depressed form with a moderately wide umbilicus occurs at the northeastern slope of the Dikti Mountains east of Tapes (Fig. 1.8D).

On the southern slope of the Dikti Mountains between Ano Viannos and Mithi there are populations with a relatively shorter flagellum (Fig. 1.7E; proximal epiphallus: flagellum ratio 1.5-2.8) than in most other populations of *X. mesostena* (proximal epiphallus: flagellum ratio 0.8-1.6). However, specimens from the surroundings of Kroustas on the eastern slope of the Dikti Mountains have a similarly long flagellum (proximal epiphallus: flagellum ratio 1.5-2.0). These forms from the Dikti Mountains neither form a separate group in a tree based on *coxI* and 16S rDNA sequences nor in a tree based on AFLP markers (see chapter2 and 3). The AFLP tree indicates that the specimens from the surroundings of Kroustas are more closely related to other specimens from the eastern slope of the Dikti Mountains than to the specimens from the southern slope of the Dikti Mountains. Specimens from the southern slope of the Dikti Mountains preferred specimens from their own population to other *X. mesostena* specimens in courtship experiments (Sauer & Hausdorf, unpubl. data). Apparently the populations from the southern slope of the Dikti Mountains are beginning to separate from *X. mesostena*. As there is still an overlap in the morphological and genetical variability, we classify them as *X. mesostena*.

Some of the easternmost populations of *X. mesostena* resemble *X. siderensis* in possessing an initially very narrow umbilicus that is strongly enlarged only by the body whorl. Thus, *X. mesostena* cannot be distinguished from *X. siderensis* by shell characters. It differs from *X. siderensis* in the usually lower proximal epiphallus: flagellum ratio (0.9-2.3, rarely -2.8) and the penial papilla with a broader basal part and a narrower apical part with a terminal opening.

Distribution (Fig. 1.5I): *X. mesostena* is restricted to Crete. It is distributed across almost the whole island with the exception of the eastern and northwestern tip and the region between Chania and the Psiloritis mountains.

Table 1.10 Measurements of some parts of the genitalia of *Xerocrassa mesostena* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Elafonisos, GE 30 (ZMH 29329)	2.9	1.6	5.7	5.3	0.8	1.8	2.1	2.4
9 km W of Paleochora, GE 30 (ZMH 29748)	3.2	1.9	5.7	4.7	0.9	2.8	3.2	3.7
Amigdokefali 2.5 km towards Elos, GE 31 (ZMH 29332)	3.8	3.0	6.7	6.6	1.2	2.4	2.6	3.0
Paleochora 1.5 km towards Koundoura, GE40 (ZMH 50582)	2.2	1.4	5.0	4.5	0.6	1.6	1.8	2.1
Paleochora 1.5 km towards Koundoura, GE 40 (ZMH 29603)	2.5	1.6	4.8	5.4	0.7	2.6	2.8	3.1
Paleochora 1.5 km towards Koundoura, GE 40 (ZMH 29603)	2.6	1.8	5.7	5.4	0.8	2.0	2.2	2.6
Paleochora 1.5 km towards Koundoura, GE 40 (ZMH 29603)	2.8	1.4	6.0	4.7	0.6	2.3	2.5	2.8
Paleochora 0.5 km towards Chania, GE40 (ZMH 36946)	3.8	1.5	5.7	5.0	1.1	2.6	2.9	3.3
Paleochora 0.5 km towards Chania, GE40 (ZMH 36946)	2.8	0.9	5.7	5.0	0.6	2.0	2.2	2.6
1 km N of Paleochora, GE 40 (MAA)	2.1	0.9	3.2	3.5	0.8	2.8	3.0	3.2
Paleochora 2.5 km towards Chania, GE40 (ZMH 29684)	2.9	1.6	4.7	3.5	0.6	2.0	2.3	2.6

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Paleochora 2.5 km towards Chania, GE40 (ZMH 29684)	2.5	1.6	4.8	3.9	0.7	1.6	1.8	2.3
Paleochora 2.5 km towards Chania, GE40 (ZMH 29684)	2.9	1.5	5.4	5.0	0.9	1.7	2.0	2.3
Rodovani 2 km towards Sougia, GE50 (ZMH 29593)	3.5	2.5	5.4	5.7	0.8	2.3	2.5	2.9
Omalos 8 km towards Lakki, GE61 (ZMH 29814)	4.2	2.0	7.6	6.6	1.1	3.2	3.5	3.8
Theriso 2.4 km towards Drakona, GE72 (ZMH 29807)	4.3	1.9	8.1	6.0	1.0	3.7	4.0	4.3
Anopolis 2.5 km towards Chora Sfakion, KU 39 (ZMH 36673)	2.5	1.6	4.7	3.7	0.6	2.1	2.4	2.6
Chora Sfakion 2.7 km towards Askifou, KU49 (ZMH 29985)	3.2	1.6	5.2	5.0	0.6	2.0	2.3	2.5
Sellia 0.5 km towards Rodakino, KU 69 (ZMH 36913)	3.3	1.6	6.3	5.4	1.2	3.0	3.5	3.7
Plakias, KU69 (ZMH 36234)	3.7	2.1	6.6	5.4	0.9	3.1	3.3	3.7
Akoumia 2 km towards Agia Galini, 0.5 km towards "Deviation", KU89 (ZMH 29600)	3.2	2.1	6.3	5.4	1.1	2.0	2.4	2.7
Kria Vrisi 0.4 km towards Akoumia, KU89 (ZMH 29258)	3.3	2.1	6.6	5.5	0.9	2.5	2.8	3.1
Akoumia, KU89 (ZMH 29180)	3.8	1.9	6.5	4.9	0.9	2.7	3.1	3.7

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
S of Akoumia, KU89 (ZMH 27293)	3.5	2.0	5.2	4.7	0.9	2.1	2.6	2.9
SW of Akoumia, KU89 (ZMH 27291)	3.5	1.3	4.4	4.8	0.9	2.9	3.2	3.5
SE of Gerakari, KU89 (ZMH 27286)	3.8	1.3	4.7	4.1	1.0	2.2	2.6	3.0
Gerakari, KU89 (ZMH 29136)	2.9	1.4	4.4	4.4	0.9	2.0	2.3	2.8
E of Matala, KU97 (ZMH 27310)	3.3	1.9	6.0	4.5	1.2	3.4	3.6	3.8
Pitsidia, KU97 (ZMH 27306)	3.2	2.5	6.1	4.5	0.7	4.1	4.2	4.3
Pitsidia, KU97 (ZMH 27307)	3.2	2.2	4.7	3.6	0.9	2.5	2.7	2.9
Pitsidia, KU97 (ZMH 27308)	3.5	1.9	6.0	3.8	1.2	2.8	3.0	3.2
Moni Odigitrias 2 km towards Kali Limenes, KU97 (ZMH 29515)	3.1	2.0	4.7	3.8	0.7	2.1	2.5	2.8
Listaros 1 km towards Moni Odigitrias, KU97 (ZMH 36339)	4.0	1.9	6.5	4.7	1.1	3.2	3.5	3.8
Listaros 1 km towards Moni Odigitrias, KU97 (ZMH 36339)	3.9	2.1	6.6	5.0	1.3	3.8	4.1	4.5
NE of Agia Galini, KU98 (ZMH 36790)	3.3	2.2	4.7	4.9	0.9	2.5	2.8	3.2
Agia Triada, KU98 (ZMH 29614)	3.7	2.1	5.7	5.9	0.8	2.7	3.1	3.5

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Agios Ioannis, KV20 (ZMH 29743)	3.6	1.2	5.7	4.2	0.7	1.8	2.1	2.5
Anopoli, KV30 (ZMH 29787)	2.6	2.5	5.7	5.0	0.9	2.8	3.2	3.5
Mournies towards Nero Kouros rocks 0.5 km S of branch towards Malaxa, KV32 (ZMH 29705)	3.4	2.5	6.7	5.7	1.0	2.6	2.9	3.3
Moni Katholiko towards Moni Gouvernetou, KV44 (ZMH 36220)	3.7	2.8	6.9	5.4	1.2	3.5	3.9	4.2
Moni Gouvernetou, KV44 (ZMH 29013)	3.8	3.0	6.8	6.2	1.1	3.3	3.8	4.4
Imbros 4.1 km towards Asfendos, KV44 (ZMH 50222)	3.8	1.9	6.3	5.4	1.0	3.4	3.7	4.1
Spili 2.6 km towards Gerakari, KV70 (ZMH 29631)	3.4	1.8	5.9	4.5	1.0	1.9	2.3	2.6
Moni Asomaton 0.3 km towards Vistagi, KV80 (ZMH 50033)	3.5	2.8	6.2	6.3	1.3	2.5	2.8	3.3
Afrates, KV80 (ZMH 29701)	2.8	2.2	5.0	4.7	1.0	2.2	2.5	2.9
3km S of Archea Eleftherna, KV80 (ZMH 29225)	4.3	2.2	7.2	5.9	1.0	2.8	3.2	3.5
Moni Arkadiou 1 km towards Kavousi, KV81 (MAA)	2.5	0.8	5.4	3.8	0.7	3.2	3.6	4.0
Platania 0.3 km towards Vistagi, KV90 (ZMH 36928)	3.4	2.5	5.4	6.3	1.1	2.6	3.1	3.3

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Pombia 2 km towards Kali Limenes, LU07 (ZMH 36027)	3.8	2.2	6.3	4.9	1.3	3.5	3.8	4.1
Ideon Andro, LU09 (ZMH 29225)	3.0	1.8	6.9	5.3	0.9	2.7	3.0	3.6
Ideon Andro, LU09 (ZMH 29238)	2.8	2.5	6.6	4.8	0.9	3.3	3.7	3.9
Lendas, LU16 (ZMH 36062)	5.0	2.7	7.7	5.7	1.2	3.8	4.2	4.5
Ano Kapetaniana 3.5 km towards Agios Ioannis, LU26 (ZMH 29699)	3.7	1.7	5.7	5.0	0.6	2.4	2.7	3.0
Tefeli 0.5 km towards Pirgos, LU38 (ZMH 36036)	4.1	2.5	7.2	6.7	1.2	2.6	3.0	3.3
Dematia 0.5 km towards Skinias, LU47 (ZMH 29554)	4.4	2.6	7.9	7.6	1.1	4.4	4.9	5.4
Panagia 0.6 km towards Embaros, LU48 (ZMH 29926)	5.4	2.5	9.5	7.6	1.7	5.4	6.0	6.5
Chondros 2.8 km towards Arkalohori, LU57 (ZMH 50566)	4.8	2.8	9.8	5.7	1.5	4.1	4.5	5.0
Kastri 0.9 km towards Chondros, LU57 (ZMH 50055)	3.8	1.6	5.7	5.7	1.0	3.2	3.4	3.6
Kastri 3.3 km towards Chondros, LU57 (ZMH 50254)	4.1	1.9	5.7	5.5	0.7	2.5	2.8	3.0
Amiras 0.8 km towards Ano Viannos, LU57 (ZMH 29954)	3.7	1.9	5.4	3.1	0.8	2.2	2.5	2.8
Amiras 0.5 km towards Arvi, LU57 (ZMH 36948)	3.8	1.3	5.7	2.3	0.8	2.9	3.2	3.7

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Amiras 3.5 km towards Arvi, LU57 (ZMH 50400)	3.7	2.2	6.9	3.3	1.0	3.0	3.3	3.8
Ano Viannos 1 km towards Mirtos, LU57 (ZMH 29753)	4.1	2.2	6.6	3.4	1.0	2.6	3.0	3.3
Ano Viannos 1 km towards Mirtos, LU57 (ZMH 29753)	3.8	2.2	7.2	3.0	1.0	1.9	2.3	2.6
Ano Viannos 1 km towards Mirtos, LU57 (ZMH 50443)	4.4	2.5	6.3	3.1	1.1	3.2	3.5	3.8
Thomadiano 6.8 km towards Ano Viannos, LU58 (ZMH 50447)	5.7	3.7	9.5	8.2	1.4	4.1	4.5	5.0
Martha 1.5 km towards Ano Viannos, LU58 (ZMH 29849)	5.2	3.2	10.3	7.6	1.3	4.4	4.8	5.2
Xeniakos, LU58 (ZMH 29908)	5.4	3.5	8.5	7.2	1.6	3.5	3.8	4.2
1 km E of Ano Viannos, LU58 (MAA)	3.1	1.3	5.4	2.0	0.8	2.5	2.8	3.0
1 km E of Ano Viannos, LU58 (MAA)	2.3	1.4	4.7	1.6	0.6	2.5	2.8	3.1
Kastamonitsa 2.5 km towards Kastelli, LU59 (ZMH 29879)	6.0	3.5	8.2	6.9	1.2	4.6	5.0	5.6
pass Ambelos Afhin, LU59 (MAA)	2.9	1.9	7.2	5.0	0.9	3.3	3.5	3.8
1.5 km SSW of Kera, LU59 (NMG)	4.8	2.0	7.6	6.9	1.3	4.1	4.6	5.0
Rizza, bifurcation 4.4 km towards Ano Viannos, LU67 (ZMH 50054)	3.2	1.6	7.1	4.0	0.8	2.5	2.8	3.2

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Sikologos 1.5 km towards Ano Viannos, LU67 (ZMH 29540)	4.0	0.9	6.6	3.5	0.4	2.5	2.8	3.2
Sikologos 2.0 km towards Mirtos, LU67 (ZMH 29528)	2.8	1.3	5.4	3.5	0.7	2.5	2.8	3.2
Mournies, LU67 (MAA)	2.5	1.3	4.5	2.2	0.9	2.9	3.0	3.3
Mithi 4.4 km towards Ano Viannos, LU77 (ZMH 50489)	4.4	2.2	7.9	4.1	0.6	3.2	3.5	3.7
N of Mithi, LU77 (ZMH 27301)	3.7	1.6	5.0	2.8	1.3	3.8	4.0	4.1
Anatoli 0.5 km towards Kalamafka, LU77 (ZMH 36269)	3.7	1.9	6.6	5.2	0.8	2.6	3.1	3.4
Anatoli 2.2 km towards Kalogeri, LU77 (ZMH 36722)	3.9	2.2	7.2	5.4	0.9	2.6	3.0	3.3
S of Avdeliakos, LU78 (ZMH 27287)	3.2	1.8	4.4	3.2	1.2	2.3	2.7	3.1
Anatoli 4.2 km towards Males, LU78 (ZMH 36538)	4.3	1.9	6.7	4.5	1.0	2.8	3.1	3.3
NE of Males, LU78 (ZMH 27295)	3.2	2.0	4.7	3.5	0.8	2.5	2.7	3.0
Anatoli 2.9 km towards Kalamafka, LU78 (ZMH 50548)	3.6	2.2	6.0	3.9	0.8	2.5	3.0	3.4
Kalamafka 1.5 km towards Anatoli, LU78 (ZMH 36735)	4.0	2.5	6.6	5.0	1.2	3.0	3.3	3.7
0.5 km S of Kroustas, LU78 (MAA)	2.9	1.8	6.3	3.4	0.8	2.3	2.5	2.8

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
3 km S of Kroustas, LU78 (MAA)	2.7	2.8	7.3	3.7	0.8	2.2	2.5	2.6
Kroustas 1.5 km towards Prina, LU78 (ZMH 50371)	3.2	1.8	5.7	3.8	0.5	2.3	2.5	2.7
Kroustas 1.5 km towards Prina, LU78 (ZMH 50371)	4.9	1.9	8.2	4.7	1.1	3.2	3.5	4.0
Kritsa 5 km towards Avdeliakos, LU79 (ZMH 29376)	3.0	2.3	6.0	5.1	1.6	2.8	3.2	3.5
N of Tapes, LU79 (ZMH 27289)	5.4	1.6	5.3	5.2	1.7	3.5	3.8	4.2
N of Tapes, LU79 (ZMH 27289)	3.2	1.4	5.7	4.5	1.3	3.5	3.8	4.5
0.5 km N of Tapes, LU79 (ZMH 36499)	5.2	2.8	7.9	6.4	1.1	3.7	4.2	4.5
Tapes 2.5 km towards Agios Nikolaos, LU79 (ZMH 36666)	5.0	2.8	8.5	6.9	0.9	3.5	3.8	4.2
Kritsa 1 km towards Kroustas, LU79 (ZMH 50155)	4.4	1.5	6.9	3.9	1.3	3.2	3.5	3.8
Kritsa 1.5 km towards Kroustas, LU79 (ZMH 29672)	4.3	1.6	6.6	5.4	1.1	3.0	3.3	3.7
Tapes 4.5 km towards Agios Nikolaos, LU79 (ZMH 50344)	3.2	1.3	5.9	4.3	0.7	2.8	3.1	3.4
Kalo Chorio 0.6 km towards Kalamafka, LU88 (ZMH 36619)	4.0	1.8	5.7	4.4	0.8	2.1	2.5	2.8
Pachia Ammos 5 km towards Agios Nikolaos, LU88 (ZMH 36327)	4.1	1.9	7.1	4.7	0.9	3.3	3.8	4.2

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Madati 0.2 km towards Agios Nikolaos, LU89 (ZMH 36316)	4.7	1.6	7.1	4.7	1.1	3.2	3.6	3.9
Amoudara, LU89 (ZMH 29706)	3.6	1.9	5.7	3.5	0.6	2.5	2.8	3.1
Pano Chorio 0.6 km towards Thripti, LU97 (ZMH 29336)	4.7	2.1	7.6	5.4	1.0	2.8	3.3	3.7
Ierapetra 6 km towards Makrigialos, LU97 (ZMH 36467)	3.3	1.7	5.9	4.7	0.9	2.5	2.8	3.1
Koutsonari 3 km towards Agios Ioannis, LU97 (ZMH 36216)	5.0	2.2	6.9	6.3	0.9	2.6	3.2	3.7
4 km W of Pachia Ammos, LU98 (MAA)	3.0	0.9	5.7	3.5	0.6	2.5	2.5	2.8
NW of Orino, LU98 (ZMH 27300)	3.8	2.0	4.7	5.4	0.9	3.9	4.2	4.5
Anogia 6.5 km towards Ideo Andro, LV00 (ZMH 29462)	2.8	1.8	5.7	4.5	0.5	2.1	2.4	2.7
Anogia 5 km towards Ideo Andro, LV00 (MAA)	3.3	1.1	5.7	4.3	1.2	2.3	2.5	2.6
Anogia 0.5 km towards Ideo Andro, LV00 (ZMH 29076)	2.8	1.9	4.7	4.0	0.8	2.2	2.5	3.0
Sises, LV01 (MAA)	2.7	1.4	4.5	4.4	0.8	2.2	2.5	2.6
Sises, LV01 (MAA)	2.9	2.2	5.7	4.6	0.8	2.6	2.9	3.2
18 km E of Iraklion, LV11 (MAA)	3.8	2.1	5.4	4.0	1.6	3.2	3.3	3.7

Table 1.10 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Damasta 1.5 km towards Marathos, LV11 (ZMH 29149)	4.4	3.3	8.2	5.5	1.1	3.7	3.9	4.3
Paleokastro W of Iraklion, LV21 (MAA)	2.5	2.8	5.0	3.5	0.8	2.6	3.0	3.3
Prassas, LV30 (MAA)	2.6	1.6	6.6	5.0	0.7	2.3	2.6	2.8
Ano Arhanes: Jouchtas Mountain, LV30 (ZMH 29912)	3.8	3.2	6.0	5.7	0.8	2.5	2.9	3.3
Ano Arhanes: Jouchtas Mountain, LV30 (ZMH 29072)	4.0	2.8	6.2	5.7	0.9	3.1	3.3	3.5
Tombrouk 0.5 km towards Vathianos Kampos, LV21 (ZMH 29316)	4.1	2.3	7.2	6.0	1.1	2.8	3.3	3.7
Tombrouk 0.5 km towards Vathianos Kampos, LV21 (ZMH 29679)	3.2	1.3	5.2	3.7	0.4	1.9	2.1	2.5
Gonies 4.8 km towards Tzermiado, LV50 (ZMH 36492)	3.4	2.2	7.5	6.3	1.1	3.5	3.8	4.2
S of Vrahasi, LV60 (ZMH 27304)	3.3	1.3	4.7	5.0	1.2	4.4	4.8	5.3
Moni Aretiou, LV70 (ZMH 36187)	5.5	3.5	7.6	6.8	1.1	4.5	4.9	5.4
Plaka 2.5 km towards Vrouhas, LV80 (ZMH 29844)	4.5	2.8	8.2	7.6	1.0	3.8	4.2	4.6
Plaka 2.5 km towards Vrouhas, LV80 (ZMH 29101)	3.5	1.6	6.6	5.0	0.8	2.2	2.5	2.8
Vrouhas 1 km towards Plaka, LV80 (ZMH 29121)	2.9	1.5	4.5	3.8	0.6	3.0	3.3	3.7
Skordilo 2 km towards Chrissopigi, MU08 (ZMH 36120)	4.9	2.5	7.8	5.0	1.1	3.2	3.6	4.0

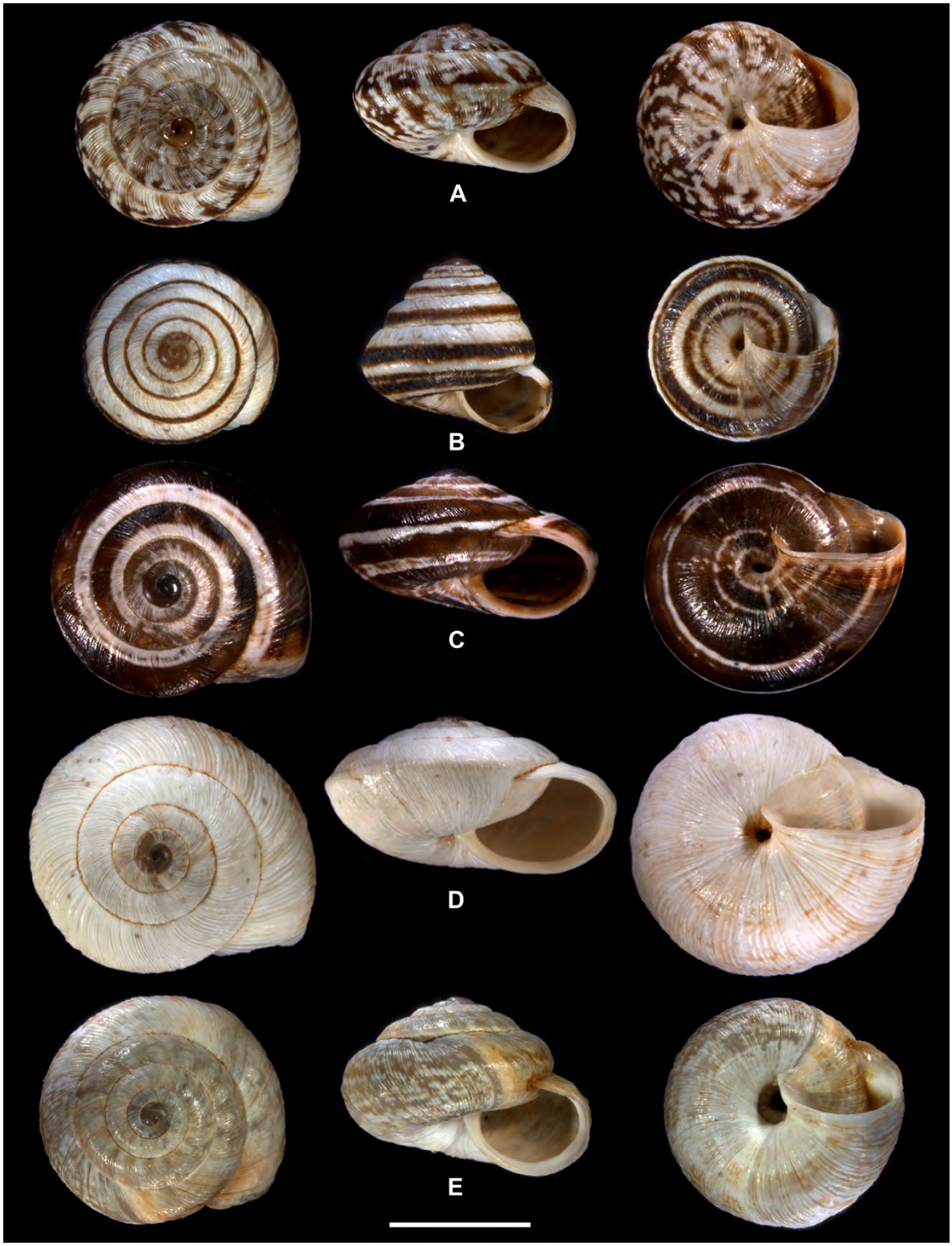

Fig. 1.9. *Xerocrassa* species from Crete, shells. A, *Xerocrassa mesostena* (Westerlund), Moni Assomaton (syntype of *H. psiloritana* Maltzan ZMB 36374). B, *Xerocrassa rhithymna* Hausdorf & Sauer, Moni Arkadiou 3 km towards Thronos (holotype ZMH 51067). C, *Xerocrassa siderensis* (Maltzan), Cape Sideros (syntype of *H. siderensis* Maltzan ZMB 111957). D, *Xerocrassa siderensis* (Maltzan), "Lassiti" (holotype of *H. euphacodes* Maltzan ZMB 101443). E, *Xerocrassa subvariegata* (Maltzan), Tuzla (syntype ZMB 36377). Scale bar = 5 mm.

XEROCRASSA RHITHYMNA Hausdorf & Sauer, 2009

(FIGS 1.5J, 1.9B, 1.10A; TABLES 1.1, 1.3, 1.11)

Xerocrassa rhithymna Hausdorf & Sauer, 2009: 399-401, Figs 5J, 9B, 10A; Tables 1, 3, 11.

Locus typicus: Greece, Kríti, Nomos Rethimnon: Moni Arkadiou 3 km towards Thronos: rocky slope N of pass.

Type material: Holotype: Greece, Kríti, N. Rethimnon: Moni Arkadiou 3 km towards Thronos: rocky slope N of pass, 600 m alt., KV8407 (ZMH 51067, collected by B. Hausdorf & J. Sauer 28. September 2004, measurements: D = 7.3 mm, H = 6.6 mm). Paratypes: Greece, Kríti, N. Chania: Kournas, NW end of lake, 20 m alt., KV5213 (ZMH 29780); Mathes 1 km towards Kournas, 40 m alt., KV5214 (ZMH 36054); Asigonia 0.8 km towards Episkopi, 300 m alt., KV5306 (ZMH 36978); Asigonia 2.5 km towards Episkopi, 210 m alt., KV5506 (ZMH 29534); Asigonia 6 km towards Kournas, 120 m alt., KV5708 (ZMH 29642); KV5708 (HNC). – N. Rethimnon: N of Episkopi, KV51 (HNHM 44209); Kali Sikea 6.7 km towards Rethimnon, 390 m alt., KV6006 (ZMH 36003); Ag. Konstantinos 0.2 km towards Episkopi, 220 m alt., KV6009 (ZMH 36227, 50150); Kali Sikea 2.7 km towards Rethimnon, 590 m alt., KV6103 (ZMH 36084); Koxare, 270 m alt., KV6900 (ZMH 29703); KV6908 (HNC); 1 km N of Koxare, KV60 (HNHM); Rethimnon 6 km towards Armeni, KV60 (HNHM); Kaloniktis 2 km towards Rethimnon, 180 m alt., KV6310 (ZMH 36294); Petres bridge 3.7 km towards Rethimnon, 5 m alt., KV6316 (ZMH 36798); KV6511 (HNC); KV6911 (HNC); Gonia, KV61 (HNHM); 3 km S of Rethimnon, KV61 (HNHM 44205); 2 km W of Ag. Andreas, KV61 (HNHM); Petres river valley, 9 km W of Rethimnon, KV61 (MAA); E of Gerani, cave, KV61 (MAA); mouth of Petres river NE of Episkopi, KV61 (SUB 10017); Armeni, KV61 (ZMH 29733); 2 km NW of Armeni, KV61 (HNHM); Rethimnon, Fortezza fortress, KV61 (HNHM 44206); KV7007 (HNC); Mixorrouma 0.5 km towards Ano Mixorrouma, 300 m alt., KV7200 (ZMH 29306); SE of Kare, KV70 (HNHM 44204); NE of Goulediana, KV70 (HNHM); 1 km NW of Kare, KV70 (HNHM); Agios Irini, 240 m alt., KV7113 (ZMH 29784); Rousopiti 1.4 km towards Mili, 250 m alt., KV7313 (ZMH 29691); E of Rethimnon, KV71 (HNHM; SUB 3879); 1 km W of Prassies, KV71 (HNHM); 5 km SE of Rethimnon, KV71 (MAA); Prassies, KV71 (ZMH 27128); KV8101 (HNC); KV8304 (HNC); KV8404 (HNC); Moni Arkadiou 5.2-6.0 km towards Mt. Dafni, 650-690 m alt., KV8407 (RMNH); KV8505 (HNC); Moni Arkadiou 2 km towards Mt. Dafni; 490-520 m alt., KV8507 (RMNH); Moni Arkadiou 3 km towards Thronos, 600 m alt., KV8407 (NMG; ZMB; ZMH 36273); Moni Assomaton school 8

km towards Psiloriti mountains, 670 m alt., KV8606 (ZMH 29161); Moni Assomaton 1.2 km towards Vistagi, 400 m alt., KV8702 (ZMH 37403, 50217); Moni Assomaton 0.3 km towards Vistagi, 365 m alt., KV8702 (ZMH 50032, 50034); KV8702 (HNC); 4.5 km NW of Apostoli, KV80 (HNHM); Amnatos 1.5 km towards Kirianna, 230 m alt., KV8212 (ZMH 29078); Kiriana 1 km towards Amnatos, 230 m alt., KV8213 (ZMH 29611); KV8318 (HNC); KV8319 (HNC); Viranepiskopi 1.8 km towards Perama, 75 m alt., KV8417 (ZMH 29655); KV8419 (HNC); Eleftherna 2.5 km towards Moni Arkadiou, 400 m alt., KV8612 (ZMH 29648); 3 km W of Eleftherna, KV81 (HNHM 44207); KV8520 (HNC); Lavris, mouth of the Geropotamos, 5 m alt., KV8621 (ZMH 29049); Moni Assomaton 12.5 km towards Psiloriti mountains, 880 m alt., KV9005 (ZMH 29130); KV9005 (HNC); KV9006 (HNC); KV9107 (HNC).

Diagnosis: *X. rhithymna* is characterized by a small (< 8.2 mm), conical, coarsely striated or ribbed shell with a blunt edge or a keel, a proximal epiphallus: dart apparatus ≤ 8.5, a proximal epiphallus: vagina length ratio ≤ 2.1, a proximal epiphallus: flagellum ratio ≤ 1.1, a total length of the vagina: vagina up to the base of the dart apparatus ratio ≤ 1.5, a total length of the vagina: vagina up to the glandulae mucosae ratio ≤ 1.2 and a penial papilla that is divided into a long, terminally open basal part and a very short conical apical part.

Shell (Fig. 1.9B, Table 1.1): conical or depressed conical; with 4.5-5.5 convex whorls; teleoconch irregular coarsely striated or ribbed, usually with irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl usually with a blunt edge or a keel that becomes weaker towards the aperture; aperture elliptical; upper insertion of the peristome slightly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus narrow or very narrow, hardly or partly obscured by the columellar edge.

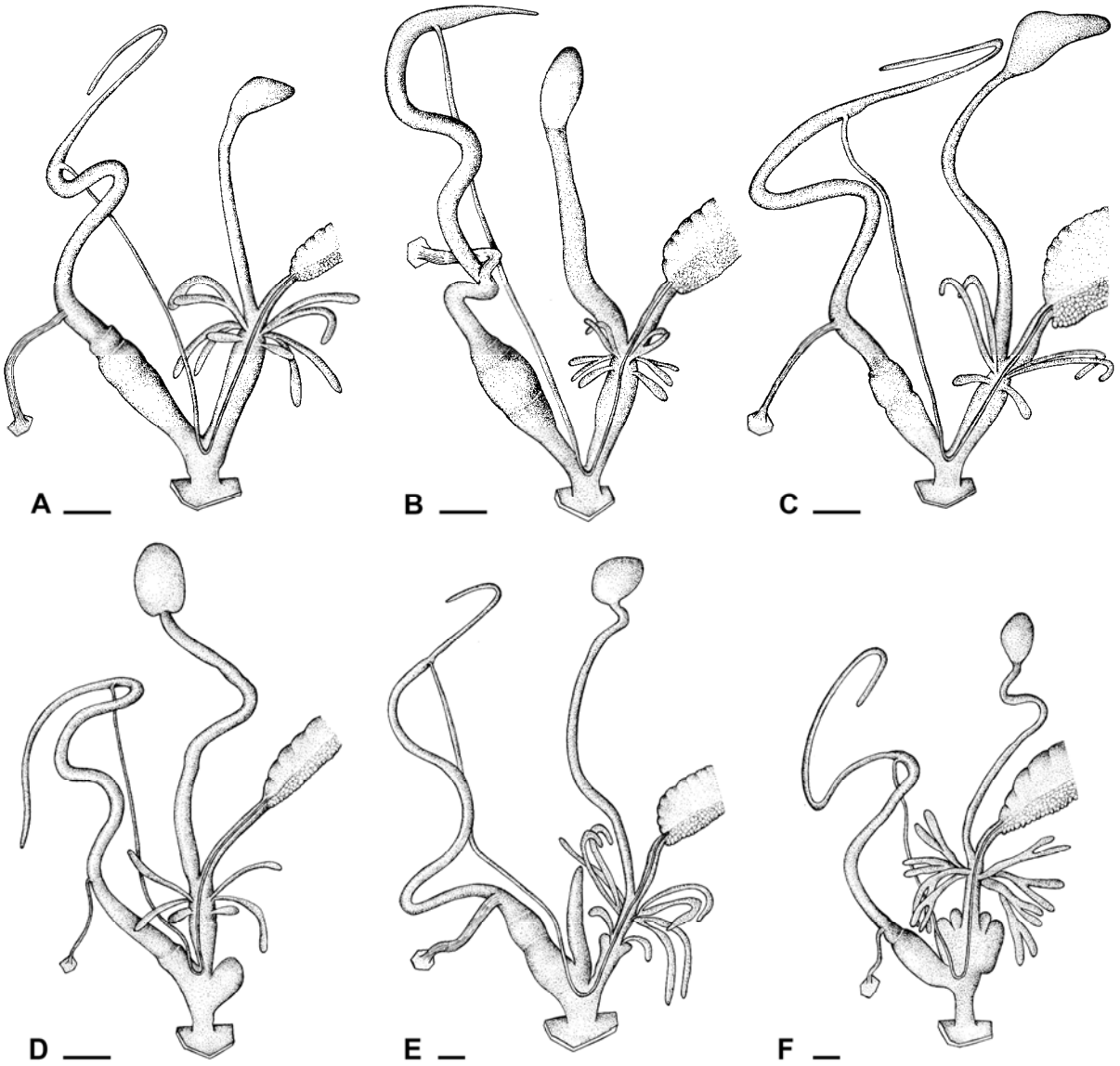

Fig. 1.10 Helicellinae from Crete, genitalia. Scale bar = 1 mm. A, *Xerocrassa rhithymna* Hausdorf & Sauer, Moni Arkadiou 3 km towards Thronos (holotype ZMH 51067). B, *Xerocrassa siderensis* (Maltzan), Vai Finikodasos (ZMH 29445). C, *Xerocrassa subvariegata* (Maltzan), Chania towards Rethimnon, at branch towards Souda (ZMH 29318). D, *Trochoidea pyramidata* (Draparnaud), Ierapetra 6 km towards Makrigialos (ZMH 36466). E, *Xeropicta krynickii* (Krynicki), Limni Kourna (ZMH 29778). F, *Pseudoxerophila bathytera* (Westerlund & Blanc), Sikologos 2.0 km towards Mirtos (ZMH 29524

Genitalia (Fig. 1.10A, Tables 1.3, 1.11): The inner structures of the genitalia correspond to those of *X. heraklea.*

Remarks: The shells of *X. rhithymna* cannot be distinguished from those of *X. lasithiensis*, *X. heraklea* and *X. kydonia*. *X. rhithymna* differs from *X. lasithiensis* in the smaller proximal epiphallus: vagina length ratio (≤ 2.1) and the usually smaller proximal epiphallus: dart apparatus ratio (≤ 8.5), from *X. heraklea* in the higher insertion of the dart apparatus and the glandulae mucosae at the vagina length (total length of the vagina: vagina up to the base of the dart apparatus ≤ 1.5; total length of the vagina: vagina up to the glandulae mucosae ≤ 1.2) and from *X. kydonia* in the smaller proximal epiphallus: flagellum ratio (≤ 1.1; Table 1.3). *X. rhithymna* differs from *X. mesostena*, with which it lives syntopically at a few sites, in the penial papilla that is divided into a long, terminally open basal part and a very short conical apical part and the usually smaller, more conical, coarsely striated or ribbed shell with a blunt edge or a keel.

Distribution (Fig. 1.5J): *X. rhithymna* is restricted to the northern part of Crete between the Lefka Ori and the Psiloriti Mountains.

Ethymology: The species is named after its distribution in the prefecture Rethimnon, derived from the ancient town Rhithymna (used as a noun in apposition).

Table 1.11 Measurements of some parts of the genitalia of *Xerocrassa rhithymna* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Kournas, KV51 (ZMH 29780)	1.5	0.9	3.8	3.5	0.5	1.3	1.4	1.8
Agios Konstantinos 0.2 km towards Episkopi, KV60 (ZMH 50150)	2.3	0.8	4.9	5.2	1.1	2.2	2.6	3.2
Moni Arkadiou 3 km towards Thronos, KV80 (holotype ZMH 51067)	3.2	0.9	5.0	6.0	0.8	2.3	2.6	3.0
Moni Arkadiou 3 km towards Thronos, KV80 (ZMH 36273)	2.8	1.1	5.7	6.6	0.9	2.0	2.3	2.6
Moni Asomaton 1.2 km towards Vistagi, KV80 (ZMH 36284)	3.7	1.6	5.4	5.0	0.6	2.0	2.2	2.6
Asomati school 12.5 km towards Psiloriti mountains, KV90 (ZMH 29130)	2.3	0.9	5.4	6.0	0.6	3.1	3.3	3.8

XEROCRASSA SIDERENSIS (MALTZAN, 1883)

(FIGS 1.5K, 1.9C-D, 1.10B; TABLES 1.1, 1.3, 1.12)

Helix (*Jacosta*) *Siderensis* Maltzan, 1883: 104. Locus typicus: „ad promontorium Sidero insulae Cretae“, Greece.

?*Helix* (*Jacosta*) *euphacodes* Maltzan, 1883: 103. Locus typicus: „in montibus „Lasethe“ (Lassiti) dictis insulae Cretae“, Greece.

Helix (*Jacosta*) *siderensis* – Kobelt, 1888: 26, pl. 98 figs 547-548.

Helix (*Jacosta*) *siderensis* – Lindner, 1994: 77, fig. 3.

Trochoidea mesostena – Vardinoyannis, 1994: 85, 88, 130, map 45 [partim, non Westerlund, 1879].

Xerocrassa siderensis – Hausdorf & Sauer, 2009: 401, 402, 403, Figs 5K, 9C-D, 10B; Tables 1, 3, 12.

Type material: Syntypes of *Helix siderensis*: Greece, Kríti, N. Lasithi: Cape Sideros, MV30 (ZMB 111957/8); holotype of *Helix euphacodes*: Greece, Kríti, N. Lasithi: "Lassiti" (ZMB 101443).

Diagnosis: *X. siderensis* is characterized by a medium-sized shell with a rounded or angular body whorl and an initially very narrow umbilicus that is often strongly enlarged by the body whorl and a relatively short flagellum (proximal epiphallus: flagellum ratio 2.7-3.7) and a penial papilla with a long, terminally open basal part and a very short conical apical part with an open channel.

Table 1.12 Measurements of some parts of the genitalia of *Xerocrassa siderensis* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Kalo Nero 0.8 km towards Makrogialos, MU17 (ZMH 36404)	4.3	2.3	7.1	2.2	1.2	2.5	2.8	3.2
Kalo Nero 0.8 km towards Makrogialos, MU17 (ZMH 36404)	4.0	2.2	7.6	2.5	1.1	2.6	3.0	3.3
Moni Kapsa, MU17 (ZMH 36740)	3.2	2.4	6.9	2.0	1.0	2.6	3.1	3.5
Moni Kapsa, MU17 (ZMH 36740)	4.3	2.0	7.2	2.6	1.2	2.9	3.2	3.7
Moni Kapsa, MU17 (ZMH 27137)	3.6	2.0	5.3	1.6	0.4	2.4	2.7	3.0
Agia Triada 5 km towards Goudouras, MU27 (ZMH 29388)	4.3	3.5	8.2	2.2	0.9	2.5	2.9	3.3
Agia Triada 5 km towards Goudouras, MU27 (ZMH 29388)	4.7	2.4	8.2	2.5	1.1	2.7	3.2	3.5
Agia Triada 1 km towards Ziros, MU27 (ZMH 29382)	3.8	2.8	8.7	2.3	1.4	2.8	3.2	3.5
Agia Triada 1 km towards Ziros, MU27 (ZMH 29382)	4.1	2.6	8.9	2.6	0.9	2.3	2.7	3.0
Xerokampos 5.3 km towards Ziros, MU27 (ZMH 36505)	4.4	1.9	7.5	2.5	1.3	3.0	3.3	3.7
Xerokampos 5.3 km towards Ziros, MU27 (ZMH 36505)	5.0	2.0	10.1	2.9	1.1	3.8	4.2	4.7

Table 1.12. *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Agia Fotia 1.7 km towards Palekastro, MU29 (ZMH 29368)	3.3	2.2	6.8	2.0	0.8	2.5	2.9	3.2
Agia Fotia 1.7 km towards Palekastro, MU29 (ZMH 29368)	3.7	2.5	8.7	2.5	1.0	2.0	2.3	2.6
Vai Finikodasos, MV30 (ZMH 29445)	3.4	2.3	6.9	2.3	0.8	2.2	2.5	2.9
Vai Finikodasos, MV30 (ZMH 29445)	3.0	1.6	5.7	1.6	0.7	1.9	2.1	2.4
Vai Finikodasos, MV30 (ZMH 50606)	3.8	2.5	7.2	2.2	1.0	2.4	2.6	3.0

Shell (Figs 1.9C-D, Table 1.1): usually depressed conical or discoidal, sometimes conical; with 4.5-5.5 convex whorls; teleoconch irregularly striated or distinctly ribbed, often with irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl usually rounded or with a blunt edge at the beginning, in some populations with a distinct edge; aperture elliptical; upper insertion of the peristome slightly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus initially very narrow, but often strongly enlarged by the body whorl, not obscured by the columellar edge.

Genitalia (Fig. 1.10B, Tables 1.3, 1.12): The inner structures of the genitalia correspond to those of *X. amphiconus*. However, in one specimen the apical part of the penial papilla was not visible (probably it was introverted into the basal part) and therefore the opening of the papilla was terminally at the basal part.

Remarks: The shells of *X. siderensis* are very variable, especially between populations. Some populations, especially east of Agia Fotia and west of Zakros are characterized by a distinct edge at the periphery of the body whorl and ribs. In the population from Agia Fotia 1.7 km towards Palekastro an extreme specimen with a distinct keel and a very narrow umbilicus was found. This specimen approaches the characters of *X. amphiconus*. However, the specimens from adjacent populations of *X. amphiconus* have a more protruding keel and are more depressed. The intermediate specimen might be evidence for an introgression between *X. siderensis* and *X. amphiconus*. Nevertheless, we classify *X. siderensis* and *X. amphiconus* as distinct species, because such specimens are very rare and even these can be referred to one of the two taxa without doubts (see remarks by *X. amphiconus*).

The westerly adjacent populations of *X. mesostena* are conchologically very similar to *X. siderensis*. Many of them also have an initially very narrow umbilicus that is strongly enlarged by the body whorl which is characteristic for typical *X. siderensis*. Thus, *X. siderensis* cannot be distinguished from *X. mesostena* by shell characters. It differs from *X. mesostena* in the higher proximal epiphallus: flagellum ratio (2.7-3.7) and the penial papilla with a long, terminally open basal part and a very short conical apical part.

X. siderensis differs conchologically from *Xeropicta krynickii* which has also a similar umbilicus in the lack of incised spiral striae.

The holotype of *Helix euphacodes* Maltzan, 1883 cannot be distinguished from forms of *X. siderensis* with a distinct edge at the periphery of the body whorl. It has been described from "in montibus „Lasethe" (Lassiti) dictis insulae Cretae". Actually, the "Lasethe" Mountains are the Dikti Mountains. However, no similar *Xerocrassa* form has been found in the Dikti Mountains. Thus, we suppose that Maltzan (1883) meant any mountains in the prefecture Lasithi (where he also collected typical *X. siderensis* and *X. amphiconus*).

Distribution (Fig. 1.5K): *X. siderensis* is restricted to the easternmost mountain range of Crete, where it occurs especially near the coast. The westernmost localities from which specimens were determined anatomically are Agia Fotia, Lithines and Kalo Nero. Near Lithines populations of *X. siderensis* on the western slopes of the eastern hills are adjacent to populations of *X. mesostena* on the eastern slopes of the foothills of the Orno Oros.

XEROCRASSA SUBVARIEGATA (MALTZAN, 1883)
(FIGS 1.5L, 1.9E, 1.10C; TABLES 1.1, 1.3, 1.13)

Helix (*Candidula*) *subvariegata* Maltzan, 1883: 105. Locus typicus: "prope Suda insulae Cretae" (more exact: "Tuzla", see Kobelt (1888)), Greece.
Helix (*Candidula*?) *subvariegata* – Kobelt, 1888: 27, pl. 98 fig. 551.
Trochoidea sp. – Vardinoyannis, 1994: 86, 88, 133, map 50 [partim].
Trochoidea sp. – Cameron *et al.*, 2000: 141.
Xerocrassa subvariegata – Hausdorf & Sauer, 2009: 403, Figs 5L, 9E, 10C; Tables 1, 3, 13.

Type material: Syntypes: Greece, Kríti, N. Chania: Tuzla near Souda (ZMB 36377/8).

Diagnosis: *X. subvariegata* is characterized by a small, irregularly striated or finely ribbed, depressed conical shell with a rounded or at most bluntly keeled body whorl and a moderately wide umbilicus and a penial papilla with a cylindrical basal part and a dilated apical part and a subterminal opening.

Shell (Fig. 1.9E, Table 1.1): depressed conical; with 4-5 convex whorls; teleoconch with irregular growth-ridges or fine ribs and irregular impressions (especially underneath); whitish, with or without brown bands that might be fused or break up into spots; body whorl usually

with an edge or a blunt keel at the beginning that becomes weaker towards the aperture; aperture elliptical; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus moderately wide, slightly eccentric, hardly obscured by the columellar edge.

Genitalia (Fig. 1.10C, Tables 1.3, 1.13): The inner structures of the genitalia correspond to those of *X. grabusana*.

Remarks: *X. subvariegata* is most similar to *X. grabusana* (see there). Some depressed forms of *X. subvariegata* can also be similar to *Pseudoxerophila oertzeni* from which it differs in the narrower umbilicus and the lack of incised spiral striae.

Distribution (Fig. 1.5L): *X. subvariegata* is spread in northwestern Crete from Tsakalaria and Kantanos in the west to Gerani in the east. West of the Lefka Ori it spreads south to the environs of Imbros.

Table 1.13 Measurements of some parts of the genitalia of *Xerocrassa subvariegata* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Floria 4 km towards Kantanos, GE41 (MAA)	2.0	0.4	4.4	4.0	0.7	1.9	2.1	2.4
Floria 4 km towards Kantanos, GE41 (MAA)	1.9	0.7	4.4	3.9	0.6	1.7	1.9	2.2
Cheretiana 0.4 km towards Pervolakia, GE42 (ZMH 29435)	1.6	0.5	5.0	5.4	0.4	0.9	1.0	1.4
Mesavlia 1.8 km towards Chania, GE42 (ZMH 36892)	2.5	0.8	5.7	4.9	0.8	1.6	1.8	2.0
Theriso 2.4 km towards Drakona, GE72 (ZMH 29806)	2.1	1.1	7.6	6.4	0.8	1.5	1.6	2.0
Theriso 2.4 km towards Drakona, GE72 (ZMH 29806)	2.1	1.1	6.9	6.0	0.8	1.6	1.7	2.0
Melidoni 1 km towards Kares, KV31 (ZMH 29456)	1.6	1.1	6.3	6.3	0.6	1.3	1.4	1.8
Pemonia 2 km towards Melidoni, KV32 (ZMH 36019)	2.1	1.0	6.2	5.4	0.4	1.6	1.8	2.0
Pemonia 2 km towards Melidoni, KV32 (ZMH 36019)	2.1	1.3	6.6	6.3	0.8	1.9	2.0	2.4
Pemonia 2 km towards Melidoni, KV32 (ZMH 36019)	2.8	1.3	8.3	7.6	0.9	2.0	2.1	2.5

Table 1.13 *Continued*

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Chania towards Rethimnon, at branch towards Souda, KV32 (ZMH 29318)	2.3	1.3	7.8	6.3	0.8	1.6	1.7	2.1
Goni, KV40 (MAA)	2.7	0.6	6.0	6.9	0.8	1.5	1.6	2.0
Imbros 0.3 km towards Chora Sfakion, KV40 (ZMH 36507)	2.1	1.1	6.9	5.9	0.6	1.4	1.6	2.0
Askifou 0.5 km towards Chora Sfakion, KV40 (ZMH 29702)	2.6	1.1	7.7	6.3	0.8	1.3	1.4	1.8
Vafes, KV41 (ZMH 36687)	2.6	1.0	7.6	7.6	0.5	1.8	2.0	2.5
Almirida 1.5 km towards Vamos, KV42 (ZMH 36865)	2.0	0.8	5.0	5.2	0.4	1.0	1.1	1.5
Almirida 1.5 km towards Vamos, KV42 (ZMH 36865)	2.5	1.1	5.4	6.0	0.6	1.9	2.0	2.3
Risoskloton, KV43 (ZMH 29468)	2.1	0.8	5.0	4.4	0.4	1.5	1.6	2.0
Kournas 1 km towards Georgioupoli, KV51 (ZMH 36051)	1.6	0.9	5.2	5.5	0.4	1.1	1.3	1.6
Kournas 1 km towards Georgioupoli, KV51 (ZMH 36051)	1.9	1.3	5.8	5.4	0.6	1.1	1.3	1.7

TROCHOIDEA BROWN, 1827

Trochoidea Brown, 1827: text for pl. 41 figs 80-81. Type species (by monotypy): *Trochus terrestris* Pennant, 1777 (= *Helix elegans* Gmelin, 1791).

Diagnosis: *Trochoidea* is characterized by a symmetrical dart apparatus consisting of two small accessory sacs and usually four branched glandulae mucosae around the vagina, two strong longitudinal folds that surround each opening of the accessory sacs into the vagina at the inner side of the wall of the vagina and fuse pairwise at their distal and proximal ends and a large rounded appendix at the atrium. Concerning the homology of the two small sacs at the vagina see *Xerocrassa*. The penis is innervated from the right cerebral ganglion.

TROCHOIDEA PYRAMIDATA (DRAPARNAUD, 1805)
(FIGS 1.10D, 1.11A, 1.12A; TABLES 1.1, 1.14, 1.15)

Helix pyramidata Draparnaud, 1805: 80, pl. 5 fig. 6. Locus typicus: "sur les plages de la Méditerranée".

Trochoidea pyramidata – Vardinoyannis, 1994: 85, 87, 129, map 43.

Trochoidea pyramidata – Hausdorf & Sauer, 2009: 404, 406, Figs 10D, 11A, 12A; Tables 1, 14, 15

Diagnosis: *Trochoidea pyramidata* is characterized by the conical shell with a more or less rounded body whorl and a narrow umbilicus.

Shell (Fig. 1.11A, Table 1.1): conical; with 4.5-7 convex whorls; teleoconch with irregular growth-ridges or fine ribs; whitish, sometimes with brown bands that might break up into spots; body whorl rounded or with a slight blunt edge at the beginning; aperture elliptical; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus narrow, not or hardly obscured by the columellar edge.

Genitalia (Fig. 1.10D, Tables 1.14, 1.15): See diagnosis of the genus. A more detailed description of the genitalia has been given by Giusti *et al.* (1995).

Distribution (Fig. 1.12A): Mediterranean region. In the eastern Mediterranean region probably introduced by man.

Fig. 1.11 Helicellinae from Crete, shells. A, *Trochoidea pyramidata* (Draparnaud), Ierapetra 6 km towards Makrigialos (ZMH 36466). B, *Xeropicta krynickii* (Krynicki), Limni Kourna (ZMH 29778). C, *Pseudoxerophila bathytera* (Westerlund & Blanc), Hagioi Pantés (syntype of *H. bathytera* NMGW). D, *Pseudoxerophila bathytera* (Westerlund & Blanc), Jouchtas mountain (syntype of *H. suspecta* NMGW). E, *Pseudoxerophila bathytera* (Westerlund & Blanc), Ierapetra (syntype of *H. hierapetrana* ZMB 39561). F, *Pseudoxerophila bathytera* (Westerlund & Blanc), Moni Toplou (syntype of *H. sitiensis* ZMB 39576). Scale bar = 5 mm for A and 10 mm for D-F.

Table 1.14 Measurements of some parts of the genitalia of *Trochoidea pyramidata* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	p	ep_d	ep_p	fl	da	v_{as}	v_{gm}	v_t
Ierapetra 6 km towards Makrigialos, LU97 (ZMH 36466)	0.6	2.1	6.0	4.7	0.5	0.6	0.9	1.2
Ierapetra 6 km towards Makrigialos, LU97 (ZMH 36466)	0.6	2.2	6.3	4.7	0.5	1.0	1.4	1.9
Ierapetra 6 km towards Makrigialos, LU97 (MAA)	0.8	2.0	6.0	4.2	0.6	0.8	1.1	1.3

Table 1.15 Comparison of the ratios of some parts of the genitalia of Helicellinae species of Crete. Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

		ep_p: ($p+ep_d$)			ep_p: fl			ep_p: da			ep_p: v_t			v_t: v_{da}			v_t: v_{gm}		
	n	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean	min	max	mean
Trochoidea pyramidata	3	2.2	2.2	2.2	1.3	1.4	1.3	9.6	12.5	11.3	3.3	5.0	4.3	1.8	1.9	1.8	1.2	1.4	1.3
Xeropicta krynickii	8	2.3	3.3	2.7	2.1	2.9	2.5	7.7	14.7	10.0	3.5	5.8	4.3	1.4	1.9	1.6	1.1	1.3	1.2
Pseudoxerophila bathytera	30	2.3	5.8	3.2	0.4	0.7	0.5	3.0	7.2	4.2	1.7	4.6	2.8	-	-	-	1.1	1.4	1.2
Pseudoxerophila oertzeni	5	2.8	6.1	3.9	0.8	1.1	0.9	4.3	5.0	4.5	2.1	3.3	2.6	-	-	-	1.1	1.3	1.2
Xeromunda candiota	11	1.6	2.8	2.1	4.3	7.5	5.3	1.5	2.3	1.9	1.5	4.1	2.4	2.8	-	-	1.1	1.3	1.2
Cernuella (*Cernuella*) *virgata*	19	2.6	5.0	3.4	2.7	4.9	3.9	2.3	3.8	3.1	5.2	8.7	6.8	-	-	-	1.2	2.0	1.6

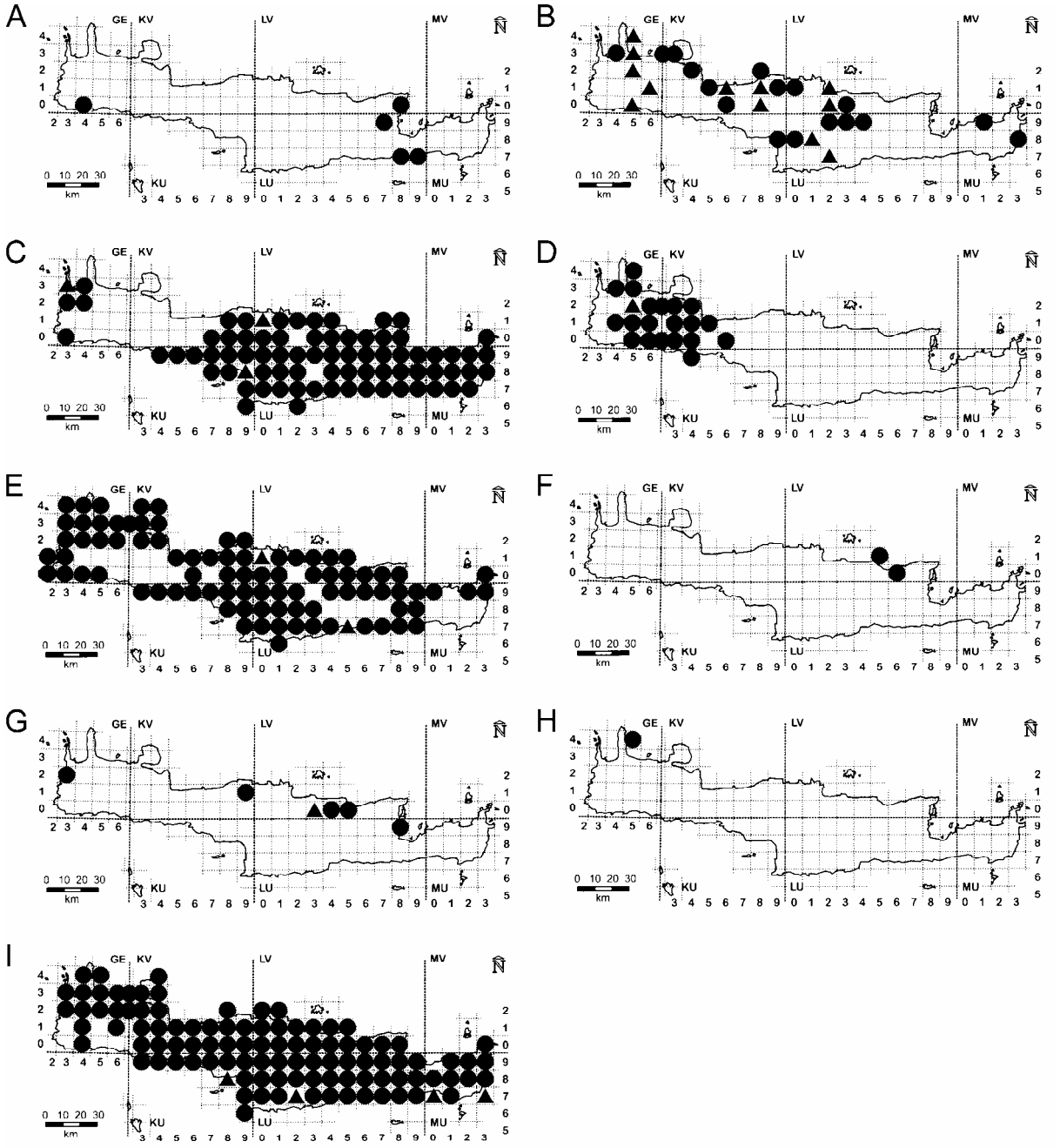

Fig. 1.12 Distribution of Helicellinae on Crete (UTM-grid, 10 km - squares). ● records of which vouchers have been checked, ▲ records from the literature. A, *Trochoidea pyramidata* (Draparnaud). B, *Xeropicta krynickii* (Krynicki). C, *Pseudoxerophila bathytera* (Westerlund & Blanc). D, *Pseudoxerophila oertzeni* (Maltzan). E, *Xeromunda candiota* (L. Pfeiffer). F, *Xerotricha apicina* (Lamarck). G, *Xerotricha conspurcata* (Draparnaud). H, *Microxeromagna lowei* (Potiez & Michaud). I, *Cernuella* (*Cernuella*) *virgata* (Da Costa).

XEROPICTA MONTEROSATO, 1892

Xeropicta Monterosato, 1892: 24. Type species (by monotypy): *Helix* (*Helicella*) *krynickii* Krynicki, 1833.

Diagnosis: *Xeropicta* is characterized by a symmetrical dart apparatus consisting of two small dart sacs, long accessory sacs which rise above the proximal end of the dart sacs and four branched glandulae mucosae around the vagina and a longish appendix at the base of the penis. The inner structure of the genitalia has been described in detail by Schileyko (1978). The penis is innervated from the right cerebral ganglion.

XEROPICTA KRYNICKII (KRYNICKI, 1833)
(FIGS 1.10E, 1.11B, 1.12B; TABLES 1.1, 1.15, 1.16)

Helix (*Helicella*) *Krynickii* Krynicki, 1833: 434. Locus typicus: "inter montes calcareos Tauriae (Sevastopoly, Inkermany. Shulya)", Ukraine.
Helix (*Pseudoxerophila*) *proteus* – Westerlund & Blanc, 1879: 59.
Helix (*Pseudoxerophila*) *krynicki* – Westerlund & Blanc, 1879: 60.
Helicopsis (*Xeropicta*) *krynickii* – Vardinoyannis, 1994: 86, 88, 131, map 46.
Xeropicta krynickii – Hausdorf & Sauer, 2009: 406: Figs 10E, 11B, 12B; Tables 1, 15, 16.

Diagnosis: *X. krynickii* is characterized by a depressed conical shell with incised spiral striae, a narrow, eccentric umbilicus and a moderately long flagellum.

Shell (Fig. 1.11B, Table 1.1): depressed conical; with 5-5.5 convex whorls; teleoconch with growth-ridges and fine incised spiral striae; upper whorls usually with fine ribs; whitish, with or without brown bands that might be fused or break up into spots; body whorl rounded or with a blunt edge at the beginning; aperture almost circular; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus narrow, strongly enlarged by the body whorl, not obscured by the columellar edge.

Genitalia (Fig. 1.10E, Tables 1.15, 1.16): See diagnosis of the genus. A more detailed description of the genitalia has been given by Schileyko (1978).

Distribution (Fig. 1.12B): *Xeropicta krynickii* is distributed from the southern Balkan Peninsula and the Aegean region around the Pontic region eastwards to Astrachan and Iran, and along the Mediterranean southwards to Egypt and Jeddah in Saudi Arabia. It was probably introduced into Crete by man and it is continuing to spread, not only on Crete, but also on mainland Greece.

Table 1.16 Measurements of some parts of the genitalia of *Xeropicta krynickii* (in mm). Abbreviations: app – appendix; da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	$p+ep_d$	ep_p	fl	app	da	v_{da}	v_{gm}	v_t
Drapanias, GE43 (MAA)	6.3	14.5	6.8	5.4	1.9	3.0	3.7	4.2
Drapanias, GE43 (MAA)	4.7	15.4	7.3	7.9	1.9	2.8	3.5	4.0
Limni Kourna, KV51 (MAA)	4.7	13.9	4.7	2.7	0.9	1.3	1.9	2.4
NW end of Limni Kourna, KV51 (ZMH 29778)	4.7	14.9	5.8	4.4	1.9	2.2	2.8	3.5
Ano Zaros, LU09 (MAA)	4.5	12.6	5.0	3.8	1.4	2.1	2.6	3.3
Ano Zaros, LU09 (MAA)	5.0	12.6	4.7	3.3	1.4	2.2	2.9	3.4
Katalagari, LU39 (MAA)	4.1	10.1	3.8	2.3	0.8	1.3	1.6	1.9
7 km W of Agios Paraskies, LU39 (MAA)	4.2	10.1	3.8	3.3	0.9	1.4	1.9	2.4

PSEUDOXEROPHILA WESTERLUND, 1879

Pseudoxerophila Westerlund in Westerlund & Blanc, 1879: 55. Type species (designated by Kobelt, 1904: 161): *Helix* (*Pseudoxerophila*) *bathytera* Westerlund & Blanc, 1879.

Diagnosis: *Pseudoxerophila* is characterized by a symmetrical dart apparatus consisting of two small dart sacs and two accessory sacs which are attached to a dilatation of the vagina and four branched glandulae mucosae around the vagina. The inner structure of the genitalia has been described in detail by Hausdorf (1988). The penis is innervated from the right cerebral ganglion.

PSEUDOXEROPHILA BATHYTERA (WESTERLUND & BLANC, 1879)
(FIGS 1.10F, 1.11C-F, 1.12C; TABLES 1.1, 1.15, 1.17)

Helix (*Pseudoxerophila*) *bathytera* Westerlund & Blanc, 1879: 55, pl. 2, fig. 16. Locus typicus: "Ile de Crète à Hagioi Pantés près de Candie", Greece.

Helix (*Pseudoxerophila*) *suspecta* Westerlund in Westerlund & Blanc, 1879: 60, pl. 2, fig. 17. Locus typicus: „Crète au Mont Iouctas“, Greece.

Helix (*Xerophila*) *Hierapetrana* Maltzan, 1887: 118. Locus typicus: „Hierapetra, Creta“, Greece.

Helix Sitiensis Maltzan, 1887: 118. Locus typicus: „To Plou, Sitia, Kreta“, Greece.

Helix (*Xerophila*) *sitiensis* – Martens, 1889: 187.

Helix (*Xerophila*) *krynickii* – Martens, 1889: 187 [partim, non Krynicki, 1833].

Helicella bathytera – Baker, 1963: 254.

Helicella itala – Curtin, 1963: 134 [non Linnaeus, 1758].

Pseudoxerophila bathytera – Hausdorf, 1988: 20, figs 14-16.

Pseudoxerophila bathytera – Lindner, 1994: 76, figs 5-6.

Helix sitiensis – Lindner, 1994: 77, fig. 1.

Helicopsis (*Helicopsis*) *bathytera* – Vardinoyannis, 1994: 86, 88, 131, map 47.

Helicopsis bathytera – Cameron *et al.*, 2000: 141.

Helicopsis bathytera – Cameron *et al.*, 2003: 95, 96.

Pseudoxerophila bathytera – Hausdorf & Sauer, 2009: 407, 408, Figs 10F, 11C-F, 12C; Tables 1, 15, 17.

Type material: Syntypes of *Helix bathytera*: Greece, Kríti, N. Iraklion: Hagioi Pantés, LV11 (NMGW/2); syntypes of *Helix suspecta*: Greece, Kríti, N. Iraklion: Jouchtas mountain, LV30 (NMGW/2); syntypes of *Helix hierapetrana*: Greece, Kríti, N. Lasithi: Ierapetra, LU87 (ZMB 39561/2); syntypes of *Helix sitiensis*: Greece, Kríti, N. Lasithi: Moni Toplou, MU29 (ZMB 39576/2).

Diagnosis: *P. bathytera* is characterized by a strongly depressed shell with fine incised spiral striae, a wide, eccentric umbilicus and a very long flagellum (proximal epiphallus: flagellum ratio 0.4-0.7). *P. bathytera* is distinctly larger than the related *P.a oertzeni*.

Shell (Figs 1.11C-F, Table 1.1): strongly depressed conical; with 4.5-5 convex whorls; teleoconch with irregular growth-ridges and fine incised spiral striae; whitish, with or without brown bands that might be fused or break up into spots; body whorl rounded or with a blunt edge at the beginning; aperture almost circular; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus wide, strongly enlarged by the body whorl, not obscured by the columellar edge.

Genitalia (Fig. 1.10F, Tables 1.15, 1.17): See diagnosis of the genus. A more detailed description of the genitalia has been given by Hausdorf (1988).

Distribution (Fig. 1.12C): *P. bathytera* is frequent in central and eastern Crete. There are also some isolated occurrences in western Crete that might be the result of anthropogenic dispersion. Vardinoyannis (1994) recorded it in western Crete only from Gramvousa. Our additional records might indicate that the species is currently expanding in western Crete. Moreover, *P. bathytera* is known from Dia (Schultes & Wiese 1990 as *Trochoidea* spec.; Vardinoyannis 1994), Chrisi (extinct?; Vardinoyannis 1994; Welter-Schultes & Wiese 1997), Mikronisi (Welter-Schultes & Wiese 1997), Paximada (Triantis *et al.* 2004) and Karpathos (Vardinoyannis 1994).

Table 1.17 Measurements of some parts of the genitalia of *Pseudoxerophila bathytera* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
Platanos 1 km towards Sfinari, GE32 (MAA)	1.5	8.8	17.6	2.2	0.0	2.0	2.3
Platanos 1 km towards Sfinari, GE32 (ZMH 4799)	1.6	5.4	13.9	1.6	0.0	1.6	2.1
Moni Arkadiou 1 km towards Kavousi, KV81 (MAA)	2.1	6.6	12.3	1.6	0.0	2.6	3.0
Kato Metohi, LU59 (MAA)	1.6	5.5	12.3	1.3	0.0	0.9	1.3
Kato Metohi, LU59 (MAA)	1.6	5.5	11.7	1.3	0.0	1.2	1.6
Sikologos 2.0 km towards Mirtos, LU67 (ZMH 29524)	3.2	7.6	13.2	1.9	0.0	3.8	4.4
Sikologos 1.5 km towards Ano Viannos, LU67 (ZMH 29538)	3.2	8.8	14.8	1.9	0.0	2.1	2.5
Kera, LU69 (MAA)	1.9	6.6	12.3	1.4	0.0	2.2	2.8
Marmaketo, LU69 (MAA)	2.1	6.3	14.8	1.6	0.0	1.9	2.5
3 km S of Kroustas, LU78 (MAA)	1.6	7.6	15.4	1.6	0.0	2.5	3.2
Vrises, LU79 (MAA)	2.3	7.0	16.8	1.7	0.0	2.0	2.4

Table 1.17 *Continued*

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
Kalamafka, LU88 (MAA)	2.1	5.9	12.0	1.8	0.0	2.2	2.6
Anogia 5 km towards Ideo Andro, LV00 (MAA)	1.8	5.4	10.5	1.8	0.0	2.2	2.6
Anogia 1.6 km towards Gonies, LV00 (MAA)	2.3	6.3	14.2	1.9	0.0	1.6	2.1
18 km W of Iraklion, LV11 (MAA)	1.3	5.7	8.4	1.3	0.0	2.2	2.5
Iraklio, E of airport, LV31 (SUB)	1.6	5.5	11.7	1.5	0.0	1.1	1.5
Vathianos Kambos, LV41 (MAA)	2.2	5.4	9.6	0.9	0.0	1.9	2.1
2 km W of Limin Chersonisou (MAA)	1.6	4.0	6.9	0.9	0.0	1.1	1.3
Milatos 2 km towards Sisi, LV60 (MAA)	1.6	4.3	7.6	1.1	0.0	1.4	1.6
1 km W of Vrahasi, LV60 (MAA)	2.0	5.2	10.4	0.9	0.0	2.2	2.5
Driros near Neapoli, LV70 (MAA)	1.6	4.4	10.1	1.1	0.0	1.3	1.5
Nofalias 2 km towards Neapoli, LV70 (MAA)	2.2	5.7	12.0	1.3	0.0	2.0	2.2
6 km SE of Milatos, LV70 (MAA)	2.1	5.7	10.7	1.3	0.0	2.5	2.8
Vrouhas, LV80 (MAA)	2.0	5.5	10.1	1.5	0.0	1.9	2.2

Table 1.17 *Continued*

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
Agios Georgios 15 km N of Agios Nikolaos, LV81 (MAA)	2.0	6.9	12.0	1.7	0.0	2.5	2.9
Agios Georgios 1 km towards beach, LV81 (ZMH 4800)	2.0	6.6	14.7	1.6	0.0	1.1	1.4
Mohlos: near bifurcation to "New Road", MU09 (MAA)	2.5	7.2	12.3	1.0	0.0	2.0	2.3
1 km N of Maronia, MU18 (MAA)	2.4	9.1	18.9	1.6	0.0	1.9	2.2
1 km E of Agia Fotia, MU29 (MAA)	2.7	6.4	11.1	2.0	0.0	2.3	2.6
0.5 km E of Paleokastro, MU39 (MAA)	3.0	8.2	12.2	2.0	0.0	2.3	2.6

PSEUDOXEROPHILA OERTZENI (MALTZAN, 1887)

(FIGS 1.12D, 1.13A-B, 1.14A; TABLES 1.1, 1.15, 1.18)

Helix (*Xerophila*) *Oertzeni* Maltzan, 1887: 117. Locus typicus: „Omalo, Kreta“, Greece.
Helix (*Xerophila*) *krynickii* – Martens, 1889: 187 [partim, non Krynicki, 1833].
Helicopsis (*Helicopsis*) sp. – Vardinoyannis, 1994: 86, 88, 132, map 48.
Helicopsis sp. – Cameron *et al.*, 2000: 141.
Pseudoxerophila oertzeni – Hausdorf & Sauer, 2009: 409, Figs 12D, 13A-B, 14A; Tables 1, 15, 18.

Type material: Syntypes: Greece, Kríti, N. Chania: Omalos, GE61 (ZMB 39570/2).

Diagnosis: *P. oertzeni* resembles *P. bathytera* from which it differs in the much smaller shell and the shorter flagellum (proximal epiphallus: flagellum ratio 0.8-1.1).

Shell (Figs 1.13A-B, Table 1.1): strongly depressed conical; with 4-4.5 convex whorls; teleoconch with irregular growth-ridges and fine incised spiral striae; whitish, with or without brown bands that might be fused or break up into spots; body whorl rounded or with an edge at the beginning that usually disappears towards the aperture; aperture almost circular; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish internal rib; umbilicus wide, strongly enlarged by the body whorl, not obscured by the columellar edge.

Genitalia (Fig. 1.14A, Tables 1.15, 1.18): The penial papilla is elongate-conical with a terminal opening. The accessory sacs are smaller than the dart sacs and more or less attached to the vagina. The openings of the two dart sacs into a dilatation of the vagina are surrounded by two strong longitudinal folds each that exrend into the proximal part of the vagina. From each dart sac another bulge extends through the inner side of dilatated part of the vagina towards the atrium. There are four branched glandulae mucosae around the vagina.

Remarks: In most populations there is only a blunt edge at the periphery of the whorls that usually disappears at the body whorl. However, in some unusually large subfossil shells from the sourroundings of Limni Kourna (Fig. 1.13B) there is a persistent keel at the body whorl. It

seems that this population is extinct. West of Kournas there are a few isolated populations in the surroundings of Agios Konstantinos and Roustika with a distinct, but not pronounced edge at the periphery of the whorls.

Distribution (Fig. 1.12D): *P. oertzeni* is restricted to western Crete. Its main centre is around the Lefka Ori. It extends eastwards to the region southwest of Episkopi.

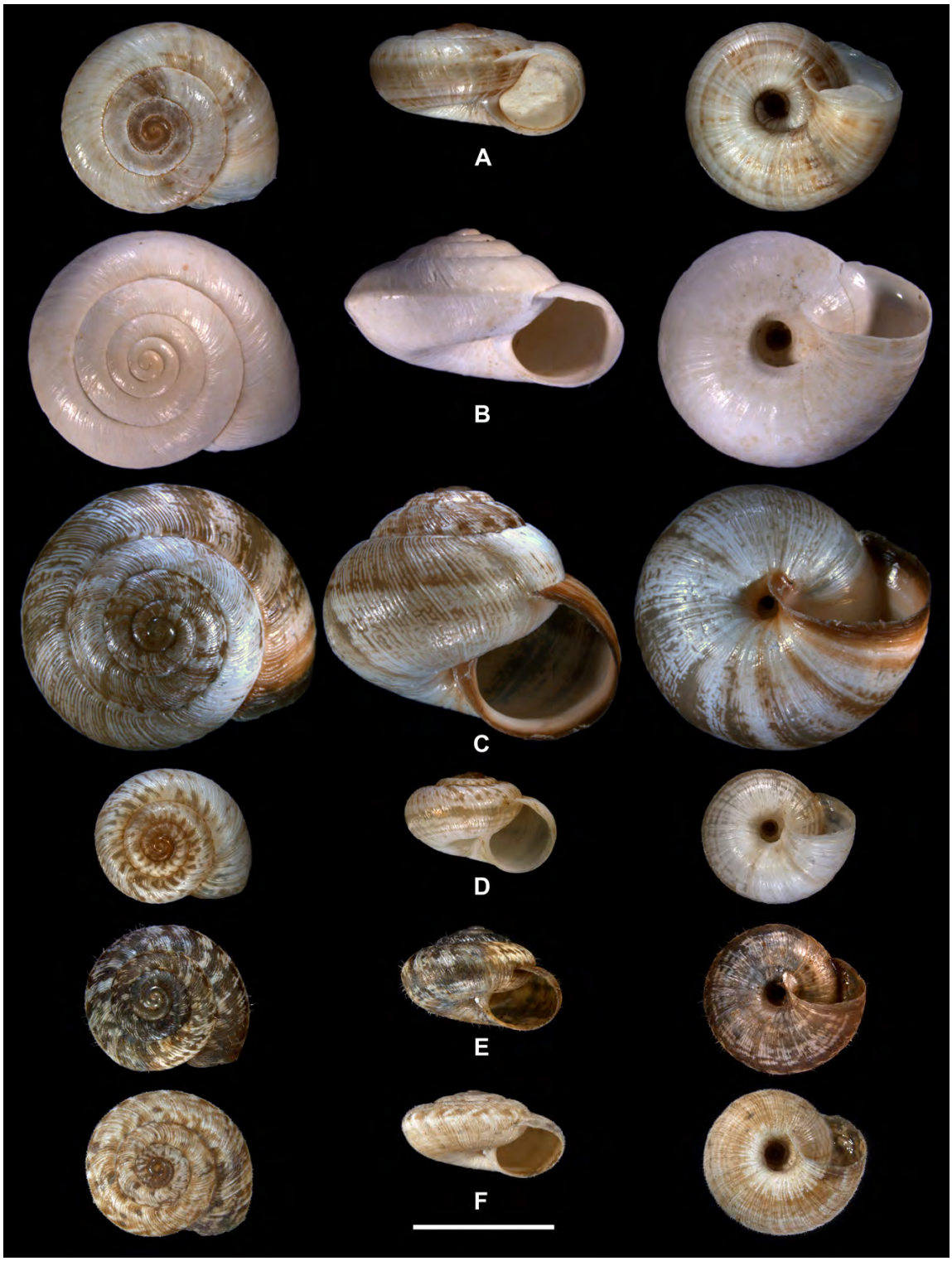

Fig. 1.13 Helicellinae from Crete, shells. A, *Pseudoxerophila oertzeni* (Maltzan), Omalos (syntype ZMB 39570). B, *Pseudoxerophila oertzeni* (Maltzan), NW side of Limni Kourna (RMNH). C, *Xeromunda candiota* (L. Pfeiffer), Mesohori (ZMH 29990). D, *Xerotricha apicina* (Lamarck), Limin Chersonisou (ZMH 36954). E, *Xerotricha conspurcata* (Draparnaud), Stalida (ZMH 36202). F, *Microxeromagna lowei* (Potiez & Michaud), Greece, Lakonia: Sgardelianika near Neapoli (ZMH 51215). Scale bar = 5 mm.

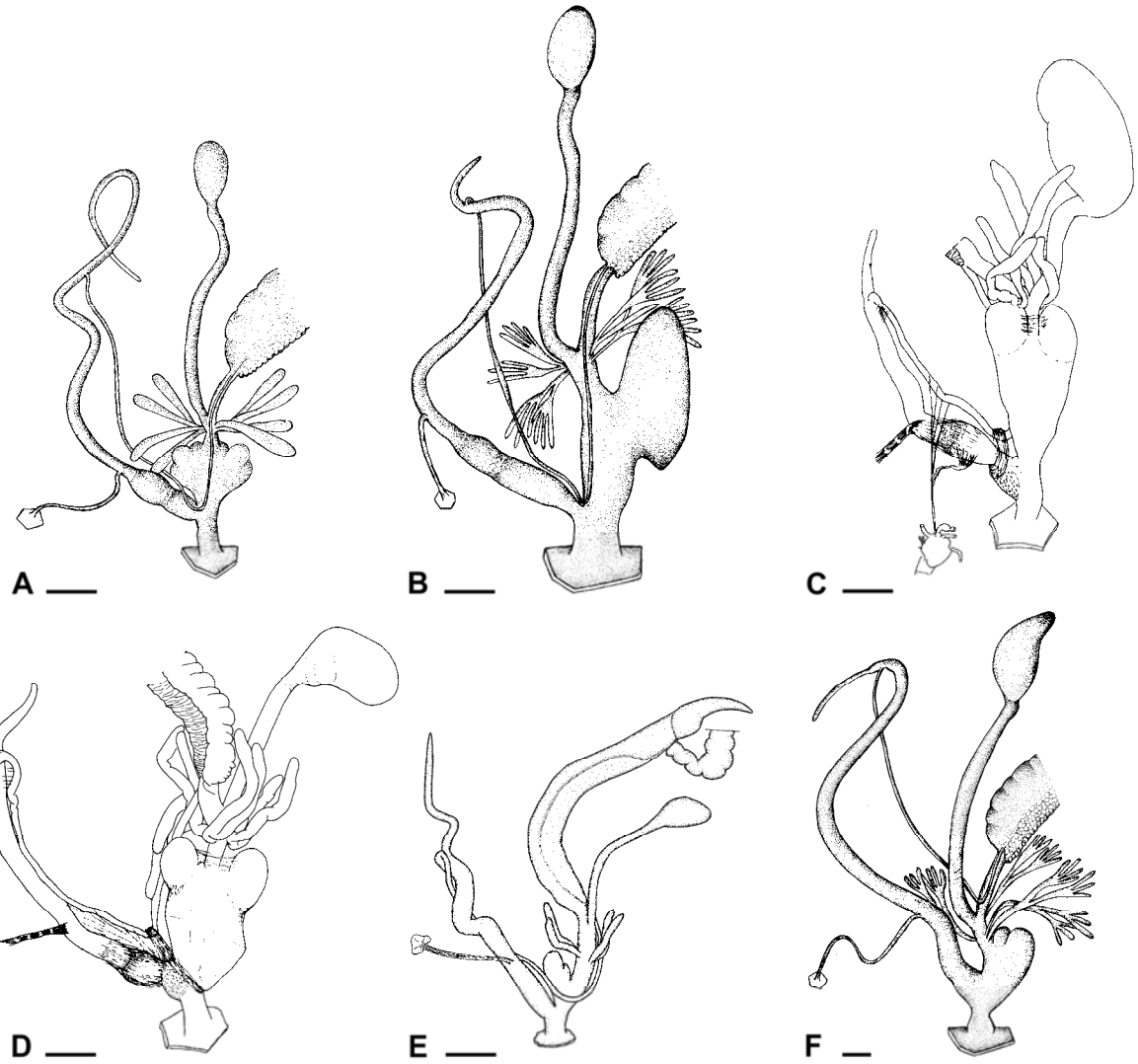

Fig. 1.14 Helicellinae from Crete, genitalia. Scale bar = 1 mm. A, *Pseudoxerophila oertzeni* (Maltzan), Omalos (ZMH 29719). B, *Xeromunda candiota* (L. Pfeiffer), Drapanos 4 km towards Kokkinio Chorio (ZMH 36255). C, *Xerotricha apicina* (Lamarck), Corsica, Cardo (from Giusti & Manganelli, 1989: Fig. 4B). D, *Xerotricha conspurcata* (Draparnaud), Sardinia, Valletta Logulentu (from Giusti & Manganelli, 1989: Fig. 2B). E, *Microxeromagna lowei* (Potiez & Michaud), Greece, Lakonia: Sgardelianika near Neapoli (from Hausdorf, 1990b: Fig. 1). F, *Cernuella* (*Cernuella*) *virgata* (Da Costa), Roustika 0.2 km towards Rethimnon (ZMH 36110).

Table 1.18 Measurements of some parts of the genitalia of *Pseudoxerophila oertzeni* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
4 km N of Omalos, GE61 (MAA)	1.6	4.4	5.6	0.9	0.0	1.5	1.9
Omalos, GE61 (ZMH 29719)	1.5	4.9	5.5	1.1	0.0	1.5	1.7
2 km S of Omalos, GE61 (MAA)	0.9	5.4	5.0	1.3	0.0	1.8	2.1
Lakki 2 km towards Omalos, GE62 (MAA)	0.9	4.1	5.0	0.9	0.0	1.1	1.3
Agios Konstantinos 0.2 km towards Episkopi, KV60 (ZMH 50148)	1.6	4.8	4.9	1.1	0.0	1.9	2.3

XEROMUNDA MONTEROSATO, 1892

Xeromunda Monterosato, 1892: 25. Type species (Opinion 1719): *Helix candiota* L. Pfeiffer, 1849.

Diagnosis: *Xeromunda* is characterized by a single large dart sac and an accessory sac which is transformed into a cavity between the vagina wall, the dart sac and a tissue-layer which envelopes parts of the vagina and the dart sac. The inner structure of the genitalia has been described in detail by Hausdorf (1988) and Manganelli & Giusti (1989). The penis is innervated from the right cerebral ganglion.

XEROMUNDA CANDIOTA (L. PFEIFFER, 1849)
(FIGS 1.12E, 1.13C, 1.14B; TABLES 1.1, 1.15, 1.19)

Helix candiota L. Pfeiffer, 1849: 255. Locus typicus (see Mousson, 1854: 10, 12): "Syra", "Canée", "Macédoine", Greece.

Helix candiota – Mousson, 1854: 10.

Helix turbinata – Raulin, 1870: 652 [non Cristofori & Jan, 1832 nec Gmelin, 1791].

Helix (*Xerophila*) *turbinata* – Westerlund & Blanc, 1879: 64 [non Cristofori & Jan, 1832 nec Gmelin, 1791].

Helix (*Xerophila*) *turbinata* var. *candiota* – Westerlund & Blanc, 1879: 64.

Helix (*Xeromunda*) *candiota* – Cecconi, 1896: 219.

Helicella (*Xeromunda*) *candiota* – Haas, 1935: 111.

Xeromunda candiota – Hausdorf, 1988: 25, figs 18-19.

Xeromunda candiota – Manganelli & Giusti, 1989: 6, figs 3-4 pl. 4 A-H.

Xeromunda candiota – Falkner, 1990: 210, fig. 211/11.

Xeromunda (*Xeromunda*) *candiota* – Hausdorf, 1990a: 109, figs 1-2, pl. 1 fig. 1.

Cernuella candiota – Vardinoyannis & Mylonas, 1988: 139.

Cernuella candiota – Vardinoyannis, 1994: 84, 87, 129, map 42.

Xeromunda candiota – Hausdorf & Sauer, 2009: 411,412, Figs 12E, 13C, 14B; Tables 1, 15, 19.

Diagnosis: *X. candiota* is characterized by a conical-globular shell with a very narrow umbilicus and a very short flagellum. It differs from the conchologically similar *Xeromunda durieui* (L. Pfeiffer, 1848) from North Africa, Puglia and Cyprus (?) in the larger dart sac, the comparatively smaller basal portion of the dart apparatus and the shorter flagellum (see Manganelli & Giusti 1989).

Shell (Fig. 1.13C, Table 1.1): conical-globular; with 4-5 convex whorls; teleoconch with irregular fine ribs, irregular impressions and partly with incised spiral striae; whitish with an irregular brownish pattern; body whorl rounded or with a blunt edge at the beginning; aperture almost circular; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, brownish on the inside, with a whitish internal rib; umbilicus very narrow, partly obscured by the columellar edge.

Genitalia (Fig. 1.14B, Tables 1.15, 1.19): See diagnosis of the genus. A more detailed description of the genitalia has been given by Hausdorf (1988) and Manganelli & Giusti (1989).

Table 1.19 Measurements of some parts of the genitalia of *Xeromunda candiota* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
Falasarna, GE33 (MAA)	1.9	4.4	1.0	2.6	0.6	2.5	3.0
Drapanos 4 km towards Kokkinio Chorio, KV42 (ZMH 36255)	3.7	6.0	1.4	3.3	0.8	2.8	3.2
Ierapetra 2.5 km towards Makrigialos, LU87 (ZMH 36717)	2.6	7.5	1.3	3.5	0.9	2.2	2.6
Ierapetra 2.5 km towards Makrigialos, LU87 (ZMH 36717)	2.9	5.4	1.1	2.6	0.6	2.0	2.3
Tholos, LU99 (MAA)	2.7	6.0	1.1	3.5	0.9	2.3	2.6
Tholos, LU99 (MAA)	2.4	5.7	1.3	3.2	0.0	1.1	1.4
18 km W of Iraklion, LV11 (MAA)	3.8	6.9	1.6	4.7	0.0	3.4	3.8
2 km W of Limin Chersonisou, LV51 (MAA)	3.0	7.1	1.3	3.5	0.8	2.6	3.1
Limin Chersonisou, LV51 (MAA)	2.8	6.1	0.8	2.6	0.3	2.0	2.5
Limin Chersonisou, LV51 (MAA)	2.3	4.4	0.7	2.8	0.2	1.7	2.1
Malia, LV60 (MAA)	3.5	7.1	1.3	3.8	0.0	1.9	2.3

Remarks: The name *Helix candiota* "Frivaldsky" was first published by Pfeiffer (1849) in the synonymy of *Helix turbinata* Cristofori & Jan, 1832. Mousson (1854) made this name available by adopting it as a valid name (Art. 11.6.1 ICZN; see also Opinion 1719). The author of this name is Pfeiffer (1849) (Art. 50.7 ICZN). According to Art. 72.4.3 ICZN, "The type series of a nominal species-group taxon of which the name was first published as a junior synonym … consists of the specimen (or specimens) cited with that name in the published synonymy, or, if none was cited there, denoted by that name when it was adopted as the name of a taxon". Pfeiffer (1849) did not cite any specimens with *H. candiota* in the synonymy. Therefore, the specimens from "Syra", "Canée" and "Macédoine" denoted by Mousson (1854) with that name are the syntypes of the taxon.

Distribution (Fig. 1.12E): Only material from the Peloponnessos and the Aegean Islands has been determined anatomically. Records from Turkey, Cyprus, Syria, the Lebanon and North Africa (see, e.g., Brandt 1959) have to be confirmed anatomically, because they might refer to *X. durieui* (L. Pfeiffer, 1848).

XEROTRICHA MONTEROSATO, 1892

Xerotricha Monterosato, 1892: 23. Type species (by monotypy): *Helix conspurcata* Draparnaud, 1801.

Diagnosis: *Xerotricha* is characterized by a symmetrical dart apparatus consisting of two large dart sacs, two small accessory sacs that are separated from the dart sacs by a laplet and four branched glandulae mucosae around the vagina. In the interior of the dart apparatus there are two tongue-like structures that are fused on one side of the vagina. Dart slightly arched, circular at its base, at the top with very narrow blades. The inner structure of the genitalia has been described in detail by Hausdorf (1988) and Giusti & Manganelli (1989). The penis is innervated from the right cerebral ganglion. The shell is small (diameter less than 10 mm) and carries hairs (or hair scars).

XEROTRICHA APICINA (LAMARCK, 1822)

(FIGS 1.12F, 1.13D, 1.14C; TABLE 1.1)

Helix apicina Lamarck, 1822: 93. Locus typicus: "environs de Brives", France.
Xerotricha apicina – Hausdorf & Sauer, 2009: 413, Figs 12F, 13D, 14C; Table 1.

Diagnosis: *X. apicina* is characterized by a small, depressed conical shell with strongly convex whorls and short hairs or hair scars and the lack of an internal rib in the aperture.

Shell (Fig. 1.13D, Table 1.1): depressed conical; with 3.75-4.25 strongly convex whorls; teleoconch with irregular growth-ridges and short hairs (or hair scars); whitish, with a spotted corneous pattern; body whorl rounded or shouldered at the beginning; aperture almost circular; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, without an internal rib; umbilicus moderately wide, slightly eccentric, not obscured by the columellar edge.

Genitalia (Fig. 1.14C): See diagnosis of the genus. A more detailed description of the genitalia has been given by Giusti & Manganelli (1989).

Distribution (Fig. 1.12F): Mediterranean region and Macaronesian Islands. The species probably originated in the western Mediterranean region and might have been distributed in the eastern Mediterranean in historical times by man.

XEROTRICHA CONSPURCATA (DRAPARNAUD, 1801)

(FIGS 1.12G, 1.13E, 1.14D; TABLE 1.1)

Helix conspurcata Draparnaud, 1801: 93. Locus typicus: "F.M. [= France méridionale] Dans les jardins, sous les haies, dans les fentes des murs", France.
Helix (*Xerophila*) *cretica* – Martens, 1889: 187 [partim, non L. Pfeiffer, 1841].
Helicella conspurcata – Vardinoyannis, 1994: 84, 88, 132, map 49.
Xerotricha conspurcata – Hausdorf & Sauer, 2009: 413, Figs 12G, 13E, 14D; Table 1.

Diagnosis: *X. conspurcata* is characterized by a small, strongly depressed conical shell with short hairs or hair scars and the lack of an internal rib in the aperture. It differs from *X. apicina* in the more depressed, usually less whitish shell with less convex whorls and a usually less eccentric, slightly narrower umbilicus and a shorter penis (shorter than the distal epiphallus in *X. conspurcata*; longer in *X. apicina*) and the dart apparatus that is less strongly tapering proximally than that of *X. apicina*.

Shell (Fig. 1.13E, Table 1.1): strongly depressed conical; with 4-4.25 convex whorls; teleoconch with irregular growth-ridges and short hairs (or distinct hair scars); whitish, with a spotted corneous pattern; body whorl rounded or shouldered at the beginning; aperture slightly elliptical; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, without an internal rib; umbilicus moderately wide, almost concentrical, not obscured by the columellar edge.

Genitalia (Fig. 1.14D): See diagnosis of the genus and the species. A more detailed description of the genitalia has been given by Hausdorf (1988) and Giusti & Manganelli (1989) .

Distribution (Fig. 1.12G): Mediterranean region and Macaronesian Islands. The species originated probably in the western Mediterranean region and might have been distributed in the eastern Mediterranean in historical times by man. The distribution in Greece and Turkey has been summarized by Hausdorf (1990b).

MICROXEROMAGNA ORTIZ DE ZÁRATE, 1950

Microxeromagna Ortiz de Zárate, 1950: 65. Type species (by monotypy): *Helix stolismena* Servain, 1880 (= *Helix lowei* Potiez & Michaud, 1835).

Diagnosis: *Microxeromagna* is characterized by a dart apparatus consisting of a dart sac without large conical papilla inside and a slightly smaller accessory sac and a small shell with hairs. The inner structure of the genitalia has been described in detail by Hausdorf (1988) and Manganelli & Giusti (1988). The penis is innervated from the right cerebral ganglion.

MICROXEROMAGNA LOWEI (POTIEZ & MICHAUD, 1835)

(FIGS 1.12H, 1.13F, 1.14E; TABLE 1.1)

Helix lowei Potiez & Michaud, 1835: 91. Locus typicus: "Madère".
Helix (*Xerophila*) *armillata* Lowe, 1852: 113. Locus typicus: "Madera".
Microxeromagna armillata – Hausdorf, 1990b: 56.
Cernuella armillata – Vardinoyannis, 1994: 84, 87, 128, map 41.
Microxeromagna lowei – Hausdorf & Sauer, 2009: 413,414, Figs 12H, 13F, 14E; Table 1.

Diagnosis: *M. lowei* is characterized by a small, strongly depressed conical shell with very short hairs or indistinct hair scars and the lack of an internal rib in the aperture. It differs from *Xerotricha conspurcata* in the shorter, more densely standing hairs or less distinct, smaller hair scars and the, on average, slightly wider umbilicus.

Shell (Fig. 1.13F, Table 1.1): strongly depressed conical; with 3.5-4.5 convex whorls; teleoconch with irregular growth-ridges and very short hairs (or indistinct hair scars), sometimes chagrinated; whitish, with a spotted corneous pattern; body whorl rounded or shouldered at the beginning; aperture slightly elliptical; upper insertion of the peristome not or slightly descending; peristome sharp, not expanded, without an internal rib; umbilicus moderately wide, slightly eccentric, not obscured by the columellar edge.

Genitalia (Fig. 1.14E): See diagnosis of the genus. A more detailed description of the genitalia has been given by Hausdorf (1988) and Manganelli & Giusti (1988).

Distribution (Fig. 1.12H): Macaronesian Islands, Iberian Peninsula, Baleares, southern France, Corsica, Italy, Greece, Turkey, Cyprus, the Lebanon, Israel. The species originated probably in the western Mediterranean region and might have been distributed in the eastern Mediterranean, in historical times by man. The distribution in Greece and Turkey has been summarized by Hausdorf (1990b). On Crete only a single specimen has been found so far.

CERNUELLA SCHLÜTER, 1838

Cernuella Schlüter, 1838: 6. Type species (designated by Gude & Woodward 1921: 182): *Helix variabilis* Draparnaud, 1801 (= *Cochlea virgata* Da Costa, 1778).

Diagnosis: *Cernuella* is characterized by a dart sac and a well developed accessory sac. On the inside the dart apparatus forms a conical structure which protrudes into the vagina and the atrium. The inner structure of the genitalia has been described in detail by Hausdorf (1988) and Manganelli & Giusti (1988). The penis is innervated from the right pedal ganglion.

CERNUELLA (*CERNUELLA*) SCHLÜTER, 1838

Diagnosis: *Cernuella sensu stricto* is characterized by three small muscles ('frenula') which connect the base of the penial papilla with the penis wall (see Manganelli & Giusti, 1988).

CERNUELLA (*CERNUELLA*) *VIRGATA* (DA COSTA, 1778)
(FIGS 1.12I, 1.14F, 1.15A-B; TABLES 1.1, 1.15, 1.20)

Cochlea Virgata Da Costa, 1778: 79, pl. 4 fig. 7. Locus typicus: "Heddington heath, in Oxfordshire"; "Hampshire"; "Cornwall"; "Newmarket heath, in Cambridgeshire", United Kingdom.

Helix (*Xerophila*) *variegata* – Westerlund & Blanc, 1879: 70.

Helix (*Xerophila*) *eugoniostoma* forma *major* Westerlund & Blanc, 1879: 73. Locus typicus: „aux environs de Candie“, Greece.

Helix (*Xerophila*) *variabilis* – Martens, 1889: 187.

Cernuella virgata – Sumner, 1983: 63.

Cernuella (*Cernuella*) *virgata* – Frank, 1988: 87.

Cernuella virgata – Vardinoyannis & Mylonas, 1988: 139.

Cernuella jonica – Vardinoyannis & Mylonas, 1988: 139.

Cernuella "virgata-ionica" – Vardinoyannis, 1994: 84, 87, 128, map 40.

Cernuella (*Cernuella*) *virgata* – Hausdorf & Sauer, 2009: 414, 415, Figs 12I, 14F, 15A-B; Tables 1, 15, 20.

Diagnosis: *C. virgata* is characterized by a depressed conical, finely and irregularly ribbed shell with an often reddish internal rib in the aperture and a moderately wide, slightly eccentric umbilicus.

Shell (Figs 1.15A-B, Table 1.1): depressed conical; with 4.5-5.5 convex whorls; teleoconch with irregular fine ribs which usually become weaker on the body whorl and irregular impressions (also on the top); whitish, with or without brown bands that might be fused or break up into spots; body whorl rounded or with a blunt edge at the beginning; aperture almost circular; upper insertion of the peristome hardly or distinctly descending; peristome sharp, not expanded, with a whitish or reddish internal rib; umbilicus narrow or moderately wide, slightly eccentric, not obscured by the columellar edge.

Genitalia (Fig. 1.14F, Tables 1.15, 1.20): See diagnosis of the genus. A more detailed description of the genitalia has been given by Hausdorf (1988) and Manganelli & Giusti (1988).

Remarks: *C. virgata* differs from *Xerocrassa cretica* in the more rapidly increasing whorls, the irregular ribbing, the more numerous irregular impressions on the top, the often reddish internal rib in the aperture and the frequently more eccentric umbilicus. Forms with a reddish internal rib can be easily identified. However, there are also forms with a whitish internal rib.

Often *Cernuella cisalpina* (Rossmässler, 1837) (= *C. jonica* (Mousson, 1854)) is distinguished from *C. virgata* as a separate species (Falkner 1990; Giusti *et al.* 1995; Kerney *et al.* 1983). Typical *C. cisalpina* differ from *C. virgata* in the smaller (see Table 1.1), more depressed and more strongly ribbed shell with a wider umbilicus. We consider *C. cisalpina* a form of the highly variable *C. virgata*, because there are no anatomical differences and various combinations of shell characters and all intermediate forms can be found. Moreover, it is worth mentioning that there are also large, smooth forms and smaller, ribbed forms in *Trochoidea pyramidata* where the forms have not been considered a distinct species.

Distribution (Fig. 1.12I): *C. virgata* is widespread in the Mediterranean region, western Europe and the Macaronesian Islands. It probably originated in the western Mediterranean region and was introduced into the eastern Mediterranean region perhaps only by man.

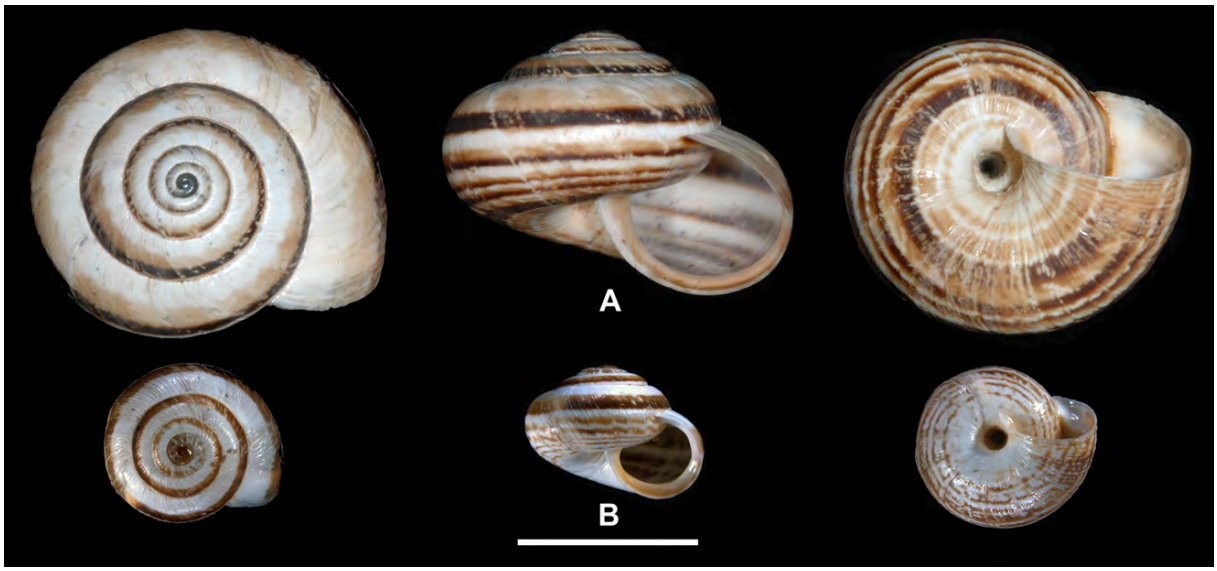

Fig. 1.15 Helicellinae from Crete, shells. A, *Cernuella* (*Cernuella*) *virgata* (Da Costa), Roustika 0.2 km towards Rethimnon (ZMH 36110). B, *Cernuella* (*Cernuella*) *virgata* (Da Costa) ('*cisalpina*'), Kato Zakros (ZMH 27083). Scale bar = 10 mm.

Table 1.20 Measurements of some parts of the genitalia of *Cernuella virgata* (in mm). Abbreviations: da – dart apparatus; ep_d – epiphallus distal of the insertion of the penial retractor; ep_p – epiphallus proximal of the insertion of the penial retractor; fl – flagellum; p – penis; v_{da} – vagina up to the base of the dart apparatus; v_{gm} – vagina up to the glandulae mucosae; v_t – total length of the vagina.

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
S of Afrata, GE53 (MAA)	2.1	7.2	2.6	2.8	0.0	0.8	1.2
Moni Agios Ioannis Theodoros near Gerakari, KU89 (MAA)	2.5	8.6	2.3	2.5	0.0	1.2	1.6
Moni Agios Ioannis Theodoros near Gerakari, KU89 (MAA)	2.1	10.4	2.8	3.2	0.0	1.2	1.6
Pitsidia, KU97 (ZMH 27312)	2.6	6.9	2.6	2.8	0.0	0.8	1.3
Roustika 0.2 km towards Rethimnon, KV60 (ZMH 36110)	3.2	11.3	3.0	3.0	0.0	1.1	2.0
4 km W of Agies Paraskies, LU39 (MAA)	2.3	7.6	1.9	2.3	0.0	0.8	1.1
Katalagari, LU39 (MAA)	2.6	10.4	2.2	2.8	0.0	0.8	1.2
1 km N of Kastelli, LU49 (MAA)	2.0	5.7	1.3	2.0	0.0	0.5	0.9
5 km E of Ferma, LU97 (MAA)	2.0	7.4	1.6	2.2	0.0	0.8	1.3
7 km W of bifurcation in direction Sisi, LV01 (MAA)	3.0	7.6	2.1	3.3	0.0	0.8	1.1
5 km W of bifurcation in direction Sisi, LV02 (MAA)	2.4	8.4	2.0	2.9	0.0	0.7	1.2

Table 20. *Continued*

	$p+ep_d$	ep_p	fl	da	v_{da}	v_{gm}	v_t
Malia, LV11 (MAA)	2.6	10.4	2.2	3.3	0.0	1.4	1.6
4 km N of Kastelli, LV50 (MAA)	2.7	10.4	3.2	3.4	0.0	0.7	1.2
Limin Chersonisou 0.5 km towards Piskopiano, LV50 (MAA)	2.8	7.4	2.0	2.0	0.0	0.8	1.2
Limin Chersonisou, LV51 (MAA)	2.1	8.2	1.9	2.8	0.0	0.6	1.1
1 km W of Vrahasi, LV60 (MAA)	2.8	10.1	3.0	2.9	0.0	0.9	1.4
Sisi, LV60 (MAA)	3.5	10.9	2.2	3.7	0.0	0.9	1.4
6 km SE of Milatos, LV70 (MAA)	2.8	10.4	2.3	3.2	0.0	0.8	1.3
Vai, MV30 (MAA)	2.1	5.9	1.6	1.8	0.0	0.6	0.9

Welter-Schultes & Williams (1999) supposed that the land snail radiations on Crete were the result of the fragmentation of the region of present-day Crete into several palaeoislands from the lower Tortonian (11 million years ago) until the late Pliocene (2-3 million years ago) (Dermitzakis 1990; Fassoulas 2001; Welter-Schultes 2000a, c; Welter-Schultes & Williams 1999). According to this vicariance model, the populations of the ancestral species of the radiations that became isolated on the palaeoislands evolved into separate species (or subspecies). Welter-Schultes & Williams (1999) thought that many recent vicariant species on Crete originated in this way on the palaeoislands.

If this hypothesis were correct, we would expect that the distribution areas of the endemic species be centred on the palaeoislands. We tested this prediction by comparing the real distribution data set of the twelve endemic *Xerocrassa* and *Pseudoxerophila* species with simulated distribution data sets generated using a null model that considers the range size distribution and the spatial autocorrelation of the occurrences of a taxon. The number of occurrences of the endemic species in 10 km UTM grids that are located in areas that were islands in the Neogene was used as test statistic. This test statistic was not significantly greater ($p = 0.612$) for the real data (194) than for 1000 Monte Carlo simulations data sets obtained by Monte Carlo simulations (mean 185.48, range 49-416). This suggests that the distribution data of the recent endemic *Xerocrassa* and *Pseudoxerophila* species on Crete do not provide evidence for the hypothesis that these radiations were caused by the fragmentation of Crete into several palaeoislands in the Neogene.

The Neogene fragmentation hypothesis has also been refuted in another case. Schilthuizen *et al.* (2004) have shown that ITS-1 sequence data are not consistent with the hypothesis that the radiation of the endemic Cretan land snail *Albinaria hippolyti* (O. Boettger) into several distinct subspecies has been caused by vicariance events in the Miocene and Pliocene as has been previously suggested (Douris *et al.* 1998; Welter-Schultes 2000b)(Douris et al., 1998; Welter-Schultes, 2000c).

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CHAPTER 2

THE PERFORMANCE OF SINGLE-LOCUS, MULTI-LOCUS AND MORPHOLOGICAL DATA AND DIFFERENT ANALYSIS METHODS IN DELIMITING SPECIES OF A CRETAN LAND SNAIL RADIATION

ABSTRACT

Because species are the fundamental units in biology, delimiting species is of outstanding importance. Recently, several approaches for delimiting species have been proposed that utilize single-locus DNA sequences or multi-locus data. However, the performance of different data and different methods for delimiting species has rarely been evaluated. In this study, we compared the results of different approaches for the delimitation of species based on single-locus DNA sequences with those of methods using dominant multi-locus data and a classification based on morphological characters. The study system was the radiation of the land snail group *Xerocrassa* on Crete. We looked for congruence between the partitions of the examined specimens obtained by different analytical methods based on different data to infer the species limits. The highest similarity between species partitions based on different datasets was found between the results of Gaussian clustering of AFLP data and the morphological classification. Species delimitation based on mtDNA sequences with fixed pairwise distance thresholds and statistical parsimony analysis resulted in an extensive splitting into putative species as a consequence of high substitution rates of mtDNA in helicoid land snails. An approach using a distance threshold based on a change in branching rates proved to be very sensitive with regard to the algorithm with which the ultrametric tree that is used for the maximum likelihood approach has been calculated and resulted in heavily under- or overestimated numbers of species. Gaussian clustering of mtDNA dataset resulted in a better agreement with the morphological classification and the Gaussian clustering of the AFLP dataset. Thus, Gaussian clustering can be recommended for species delimitation with multi-locus as well as single-locus data. However, species classifications based exclusively on single-locus data might show idiosyncrasies resulting from incomplete lineage sorting, introgression, random phylogeographic breaks or pseudogenes. Our results demonstrate that species delimitation should be based on an analysis of several independent markers. It is still reasonable to invest in morphological data, because one can look specifically for morphological characters that are directly involved in the speciation process and that may indicate the evolution of a new species before it becomes apparent in usual molecular data.

INTRODUCTION

Species are the fundamental units in biology. Most empirical findings about organisms were originally referred to certain species in which they were observed. Thus, the correct delimitation and identification of species are of outstanding importance in biology. Alpha taxonomy, the discipline dealing with the classification of organisms into species, is one of the oldest disciplines of biology. The current reference system for species of organisms has been founded by Linnaeus (1753; 1758). After 250 years approximately 1.5 million of the estimated 3 to 100 million extant species have been described (May 1999). Given the current rate of species description, it would take over 500 years to complete the catalogue (May 1999). Facing the current biodiversity crises with increasing extinction rates because of anthropogenic destruction of ecosystems and climate change, there is an urgent need to accelerate the pace of species discovery and description. Given the rapid progress in molecular techniques and the sinking costs of molecular studies, it is obvious to try to solve the taxonomic challenge by employing molecular data that have already revolutionized phylogenetic systematics. Hebert *et al.* (2003) proposed to use a DNA sequence, namely a fragment of the mitochondrial cytochrome *c* oxidase I (*cox1*) gene, as basis for a universal identification system for animals ('DNA barcoding') and Tautz *et al.* (2003) even suggested utilizing DNA sequences as such as universal taxonomic reference system ('DNA taxonomy'). However, there are strong doubts concerning the usefulness of single-locus DNA sequences for species identification and delimitation (e.g, DeSalle et al. 2005; Ferguson 2002; Lee 2004; Meier et al. 2006; Meyer & Paulay 2005; Wiemers & Fiedler 2007; Will et al. 2005; Will & Rubinoff 2004). Alternatively, multi-locus data like AFLP data (Martínez-Ortega *et al.* 2004; Parsons & Shaw 2001), multiple DNA sequences (Carstens & Knowles 2007; Dettman *et al.* 2003; Weisrock *et al.* 2006) or single nucleotide polymorphisms (Shaffer & Thomson 2007) were used to delimit species.

Beside the question whether single-locus DNA sequences are sufficient for the delimitation and identification of species, the question, which methods should be used for these purposes is also at issue. Several approaches for the delimitation of species have been developed in the last decades, especially such that utilize molecular data (Sites & Marshall 2004; Wiens 2007). However, the performance of single-locus DNA sequences versus multi-locus data and different methods for delimiting species has been compared only in a few studies (for methods using codominant multi-locus data see Marshall et al., 2006).

We investigated the performance of different methods for delimiting species based on mitochondrial sequences (*cox1*, 16S rDNA), nuclear sequences (internal transcribed spacer 2)

and multi-locus (AFLP) data as well as morphological characters of the shell and the genitalia for unravelling species limits in a radiation of the land snail genus *Xerocrassa* (Gastropoda: Helicoidea: Hygromiidae) on Crete. The *Xerocrassa* species live in open, dry habitats without obvious ecological differentiation. All species living on Crete except the more widespread *Xerocrassa cretica* are endemic to Crete and neighbouring islets. Eleven species have been formally described from Crete, but some of them proved to be synonymous, whereas some regionally restricted species were not known so far (Hausdorf & Sauer 2009).

MATERIAL AND METHODS

Sampling

Xerocrassa specimens were sampled at about 500 localities across Crete in July/August and September/October 2004 and September/October 2005. Mitochondrial *cox1* sequences and AFLP data were determined from specimens covering all morphotypes and all regions of Crete (Fig. 2.1). Of a representative subset of the specimens for which *cox1* sequences were determined, we also sequenced mitochondrial 16S rDNA to improve the resolution of the mtDNA tree. Morphological characters of the shell and the genitalia are described in detail in chapter 1.

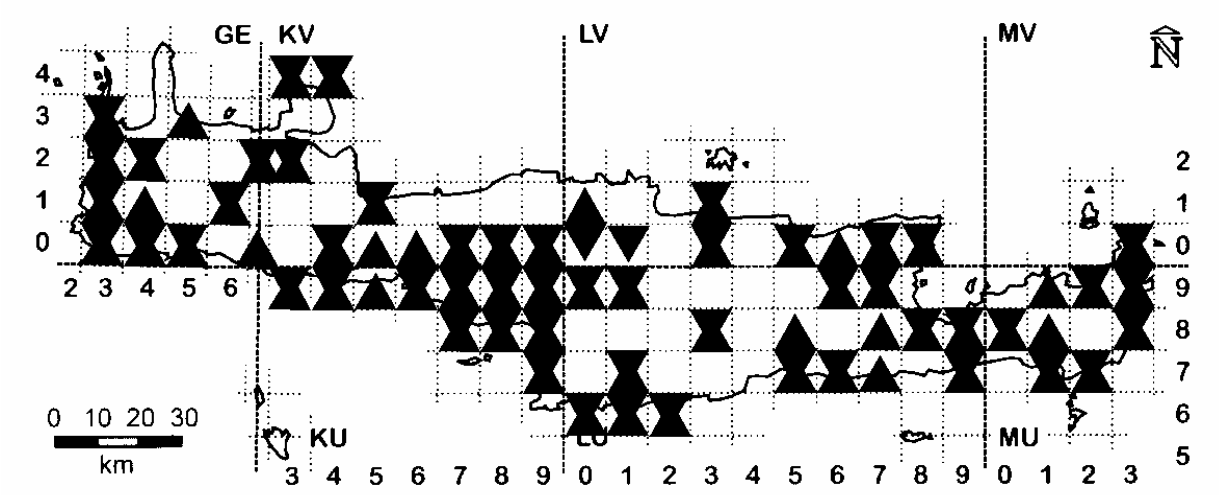

Fig. 2.1 Geographical allocation of the *Xerocrassa* specimens on Crete for which *cox1* sequences (triangles pointing downwards) or AFLP data (triangles pointing upwards) are available. The UTM 10 km grid is indicated on the map.

DNA Extraction

Usually, total genomic DNA was extracted from tissue samples of the foot preserved in 100% isopropanol following the protocol proposed by Sokolov (2000) with slight modifications. Tissue samples were minced and incubated in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulphate, 0.24 mg/ml proteinase K) at 56°C for 120 min or until complete digestion. Then, 100 µl saturated KCl was added to the lysate, gently mixed, and incubated for 5 min on ice. The samples were centrifuged at 13000 rpm for 15 min. The supernatant was brought to a clean tube and 1 ml of ice-cold ethanol (100%) and 50 µl 3M sodium acetate were added. Precipitation of DNA took place at -20 °C over night. The samples were centrifuged at 13000 rpm for 15 min and the pellet was washed in 70 % ethanol and air-dried. The pellet was resuspended in 80 µl of Tris-HCl pH 8.7. DNA content was determined with a Bio-Photometer (Eppendorf). In some cases tissue samples were minced and incubated in a Chelex extraction solution (5% Chelex 100 (Bio-Rad), 5 mM DTT and 40 µg/ml proteinase K), for 60-90 min at 56°C, centrifuged at low speed and the supernatant was directly used for PCR.

DNA Amplification and Sequencing

Fragments of two mitochondrial genes, cytochrom c oxidase subunit I (*cox1*) and 16S (large subunit) rDNA and one nuclear marker, the internal transcribed spacer 2 (ITS-2), were amplified using Polymerase Chain Reaction (PCR). The following primers were used for the amplification and sequencing of a 704 bp long fragment of *cox1*: LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and
HCO2198 5'-TACTTCAGGGTGACCAAAAAATCA-3' (modified from Folmer *et al.* 1994). Of the 16S rDNA either an approximately 470 bp long fragment was amplified with the primers 16Sar 5'-CGCCTGTTTATCAAAAACAT-3' and 16Sbr 5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi 1996) or an approximately 900 bp long fragment was amplified with the primers 16Scs1 5'-AAACATACCTTTTGCATAATGG-3' and 16Scs2 5'-AGAAACTGACCTGGCTTACG-3' (Chiba 1999). The following primers were used for the amplification and sequencing of the ITS-2 and the adjacent regions of the 5.8S and 28S rDNA: LSU1 5'-CTAGCTGCGAGAATTAATGTGA-3' and LSU3 5'-ACTTTCCCTCACGGTACTTG3' (Wade & Mordan 2000). Alternatively, the ITS-2 was amplified in a nested PCR. First, a large fragment spanning from 18S rDNA to 28S rDNA was amplified using the primers: 18S2093 5'-GGAAGTAAAAGTCGTAACAAGGT-3' and 28S200r 5'- CTGACTAATATGCTTAAATTCAG -3'. The PCR product was diluted 1:100

and again amplified with the primers 5.8S106 5'-TGAACATCGACATTTTGAACGCA-3' and 28dd. Amplifications were performed in 25 µl volumes containing 2.5 µl 10x amplification buffer (peqlab), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (peqlab), 1µl of each primer (10 pmol), 1.5 units Taq DNA polymerase (peqlab), 2.5 µl enhancer solution P (peqlab) and template DNA (usually 2.5 µl undiluted DNA extract). The *cox1* and 16S rDNA fragments were amplified under the following conditions: an initial denaturing at 94°C for 2 min, 35 cycles of PCR (94°C for 30 s, 50°C for 30 s, 72°C for 30 s), and an final extension at 72°C for 5 min. The PCRs for the long nuclear rDNA fragment were run at 94°C for 4 min, then 30 cycles (94°C for 60 s, 55°C for 60 s, 72°C for 90 s), and finally at 72°C for 7 min. The PCRs for the shorter nuclear rDNA fragment were run at 94°C for 4 min, then 30 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 60 s), and finally at 72°C for 10 min. A negative control (no template) was included in every amplification run.

The PCR products were purified using QIAquick PCR purification Kit (QIAGEN). Both strands of the amplified fragments were directly cycle-sequenced using the amplification primers and a DNA Sequencing Kit (Applied Biosystems) and electrophoresed with an automated DNA sequencer.

Sequence Analysis

Forward and reverse sequences were assembled using ChromasPro version 1.33 (Technelysium). The sequences were aligned with the CLUSTAL W algorithm (Thompson *et al.* 1994) as implemented in MEGA version 4.0 (Tamura *et al.* 2007) with the default settings and corrected by hand. Questionably aligned positions in the 16S rDNA and ITS-2 sequences were eliminated with Gblocks (Castresana 2000).

AFLP

DNA digestion – Approximately 100 ng genomic DNA were digested with 5 units EcoRI (Fermentas) at 37°C for 1 h followed by a digestion with 5 units of MseI (Fermentas) at 65°C for 1 h.

Adapter ligation – 12.5 pmol of the EcoRI-adapter, 125 pmol of the MseI-adapter and 10 units of T4 DNA ligase and its buffer (GeneCraft) were added to the digestion product and incubated at 16°C for 8 h. The ligation products were diluted 1:10 with sterile ddH_2O, and stored at -20°C.

Preselective polymerase chain reaction – Preselective PCR was carried out with one selective base on each primer (PA-MseI-C and PA-EcoRI-A, Table 2.1). 5 µl of the diluted

ligation product were added to 20 µl of the preselective PCR mastermix, consisting of 15.1 µl ddH_2O, 2.5 µl 10x PCR-buffer, 1.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTP (2 mM), 0.15 µl preselective primer mix (50 µM each), and 0.25 µl Taq-DNA polymerase (5U/µl). Preselective PCR conditions were 22 cycles of PCR (94°C for 30 s, 56°C for 30 s, 72°C for 60 s), and a final extension step at 72°C for 5 min. The quality of the preselective PCR was checked on a 1.5% agarose gel. Afterwards the products were diluted 1:20 with sterile H_2O.

Selective PCR – Five primers with two additional bases at the 3' end (Table 1) were used for selective amplifications. Six primer combinations (SMseI/SEcoRI^{DYE}) were run: AG/CA^{FAM}, AG/CC^{NED}, AG/GG^{HEX}, TG/CA^{FAM}, TG/CC^{NED} and TG/GG^{HEX}. 5 µl of the diluted preselective PCR product were added to 20 µl of the selective PCR master mix consisting of 15.05 µl ddH_2O, 2.5 µl 10x PCR-buffer, 1.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTP (2 mM), 0.2 µl dye primer mix (0.06 µM labelled selective EcoRI-Primer and 0.6 µM non-labelled selective MseI-Primer), and 0.25 µl Taq DNA polymerase (5U/µl). For the selective amplification a touch down PCR with a temperature decrease of 0.6°C of the annealing temperature each cycle was applied. The program starts with 94°C for 60 s, 65°C for 30 s and 72°C for 60 s followed by 13 cycles of 0.6°C decrease of annealing temperature and 1°C decrease of elongation temperature per cycle and 23 cycles with 94°C for 60 s, 56°C for 30 s and 72°C for 60 s.

Table 1. Primers and fluorescent dye labels used for AFLP.

Primer	Sequence	5'- labelling
PA-EcoRI-A	5'- GAC TGC GTA CCA ATT CA -3'	None
PA-MseI-C	5'- GAT GAG TCC TGA GTA AC -3'	None
SEcoRI-CA	5'- GAC TGC GTA CCA ATT CA CA -3'	FAM
SEcoRI-CC	5'- GAC TGC GTA CCA ATT CA CC -3'	NED
SEcoRI-GG	5'- GAC TGC GTA CCA ATT CA GG -3'	HEX
SMseI-AG	5'- GAT GAG TCC TGA GTA AC AG -3'	none
SMseI-TG	5'- GAT GAG TCC TGA GTA AC TG -3'	none

Electrophoresis – 1.2 µl of each of the three differently primer labelled samples were mixed with 6.2 µl Hi Dye Formamid (Applied Biosystems) and 0.2 µl GS-500 ROX size standard (Applied Biosystems). The samples were denatured at 94°C for 2 min and then cooled down on ice for 4 min. The selective PCR products were electrophoretically separated

using pop4-polymer (Applied Biosystems) on an ABI PRISM 3100 (Applied Biosystems) capillary sequencer.

AFLP data scoring – Signal detection was performed with GeneScan version 3.1 software (Applied Biosystems). Fluorescent threshold was set to 50 relative fluorescence units. The signal intensity was normalized with Genotyper version 2.5 software (Applied Biosystems). Fixed fragment categories were created. A presence/absence scoring was conducted between 70 and 322 bases with a threshold set to 50 normalized units. Category spacing was set to 1 base and the category tolerance was adjusted to +/- 0.5 bases.

Phylogenetic Analyses

Models of sequence evolution for the maximum likelihood analyses were chosen using ModelTest version 3.7 (Posada & Crandall 1998) based on the Akaike Information Criterion. Maximum likelihood analyses were conducted with Treefinder (Jobb 2007; Jobb *et al.* 2004).

Jaccard distances were calculated from the AFLP data using PhylTools version 1.32 (Buntjer 2001). These distances were used to reconstruct neighbour-joining trees with PHYLIP version 3.66 (Felsenstein 2005) and phylogenetic networks with the neighbor-net algorithm (Bryant & Moulton 2004) implemented in SplitsTree4 version 4.6 (Huson & Bryant 2006).

Confidence values for the edges of the maximum likelihood and neighbour-joining trees were computed by bootstrapping (100 replications; Felsenstein, 1985).

Species Delimitation

Fixed distance thresholds – Hebert *et al.* (2003) employed 3% distance between *cox1* sequences of lepidopterans as a threshold for species diagnosis. Sets of sequences for which each sequence in a set has at least one other sequence within a 3% threshold distance were determined using the program TaxonDNA (Meier *et al.* 2006) as approximations for species. Considering the high substitution rates of mitochondrial DNA in helicoid land snails (Chiba 1999; Hayashi & Chiba 2000; Thomaz *et al.* 1996; van Riel *et al.* 2005), we also applied a 6% sequence divergence threshold value.

Distance threshold based on change in branching rates – Pons *et al.* (2006) supposed that there is a change in branching rates at the species boundary. They proposed a maximum likelihood approach to test for the predicted change in branching rates and to optimize the distance in an ultrametric tree where this change occurred. We used a code (T. Barraclough, pers. comm.) implementing their approach in the statistical software R (R Development Core

Team, 2007) using functions from the APE library (Paradis *et al.* 2004) to test for the predicted change in branching rates and to determine the resulting putative species. Ultrametric trees were constructed using the local rate minimum deformation method implemented in Treefinder (Jobb 2007) that tries to keep the real rates as similar as possible to ideal local rates with well-defined dependencies under the assumption that rates are similar between neighboring edges and using the penalized likelihood method with the truncated Newton algorithm implemented in r8s version 1.71 (Sanderson 2002). For the penalized likelihood method a smoothing parameter of 2 for the *cox1* dataset and a smoothing parameter of 4 for the combined *cox1*/16S dataset were chosen as optimal based on the normalized chi-square-like cross-validation scores. Zero-length branches had to be collapsed prior to the estimation of the ultrametric trees with r8s. Because the R script of Barraclough needs fully resolved trees, the resulting polytomies were resolved again with zero-length branches using TreeEdit version 1.0a10 (Rambaut & Charleston 2002).

Statistical parsimony – Pons *et al.* (2006) proposed to use independent networks as identified by statistical parsimony analysis (Templeton *et al.* 1992) as initial step to delimit putative species. Statistical parsimony analysis partitions the data into independent networks of haplotypes connected by changes that are non-homoplastic with a 95% probability. We used statistical parsimony analysis as implemented in the program TCS version 1.3 (Clement *et al.* 2000) to delimit independent networks.

Gaussian clustering – Hausdorf & Hennig (in press) proposed to use Gaussian clustering for the determination of clusters of specimens as putative species. We used the implementation of Mclust (Fraley & Raftery 1998) in the program package Prabclus version 2.1-1 (Hennig & Hausdorf 2008) that is an add-on package for R (R Development Core Team, 2007). The advantage of this non-hierarchical clustering method is that it provides a decision about the number of meaningful clusters. We used the range from 0 to 100 clusters to perform the mixture estimation. Gaussian clustering operates on a dataset where the cases are defined by variables of metric scale. Therefore we performed a non-metric multidimensional scaling (Kruskal 1964) on a distance matrix. We used GTR+G+I distances calculated with PAUP* 4.0 beta 10 (Swofford 2002) for the analysis of single-locus DNA sequences and Jaccard distances for the analysis of AFLP data.

Structure – Shaffer & Thomson (2007) proposed to use Structure (Falush *et al.* 2007; Pritchard *et al.* 2000) to delimit the lower bound of potential species. We used Structure version 2.2 with the model without admixture. Following Evanno *et al.* (2005) 20 runs with 10,000 iterations after a burn-in of 10,000 iterations were carried out in order to quantify the

amount of variation of the likelihood for each *K*. According to Evanno *et al.* (2005) longer runs do not change the results significantly. We used the mean estimates of the posterior probabilities of the data for a given cluster number *K* (*L*(*K*)) and the statistic $\Delta K = m(|L(K+1)-2L(K)+L(K-1)|)/s[L(K)]$ proposed by Evanno *et al.* (2005) to estimate the number of clusters *K* for *K* between 1 and 20.

Comparison of Classifications

Because the correct species delimitation is not known a priori, we looked for congruence between the results based on different datasets and analysed with different methods to evaluate which might be reasonable approximations to the true delimiting of species. We used the corrected Rand index (Hubert & Arabie 1985) to measure the similarity of different partitions:

$$I_R = \frac{\sum_{i=1}^{k_1}\sum_{j=1}^{k_2}\binom{n_{ij}}{2} - \sum_{i=1}^{k_1}\binom{n_{i.}}{2}\sum_{j=1}^{k_2}\binom{n_{.j}}{2}\Big/\binom{n}{2}}{\left[\sum_{i=1}^{k_1}\binom{n_{i.}}{2} + \sum_{j=1}^{k_2}\binom{n_{.j}}{2}\right]/2 - \sum_{i=1}^{k_1}\binom{n_{i.}}{2}\sum_{j=1}^{k_2}\binom{n_{.j}}{2}\Big/\binom{n}{2}}$$

where k_1 is the number of clusters in the first partition, k_2 is the number of clusters in the second partition, n_{ij} is the number of specimens that are in cluster *i* in the first partition and in cluster *j* in the second partition, $n_{i.} = \sum_{j=1}^{k_2} n_{ij}$, $n_{.j} = \sum_{i=1}^{k_1} n_{ij}$. The corrected Rand index is standardized so that its expected value is 0 under random partitioning. Its maximum is 1. It becomes large if either the number of pairs of specimens is large of which both specimens are in the same cluster in both partitions, or the number of pairs is large of which both specimens are in different clusters in both partitions, which indicates similarity of the partitions.

RESULTS

We used the morphological classification as elaborated by Hausdorf & Sauer (2009) as reference. This does not mean that we consider the morphological classification to be superior a priori.

Gene Trees Based on Single-locus Sequences

The maximum likelihood tree of 124 partial *cox1* sequences (634 bps) of Cretan *Xerocrassa* species and outgroups is shown in Figure 2.2. Separate models for the three codon

positions as determined by ModelTest were used, because the resulting tree had a lower AIC value than the tree based on a uniform model for the complete dataset.

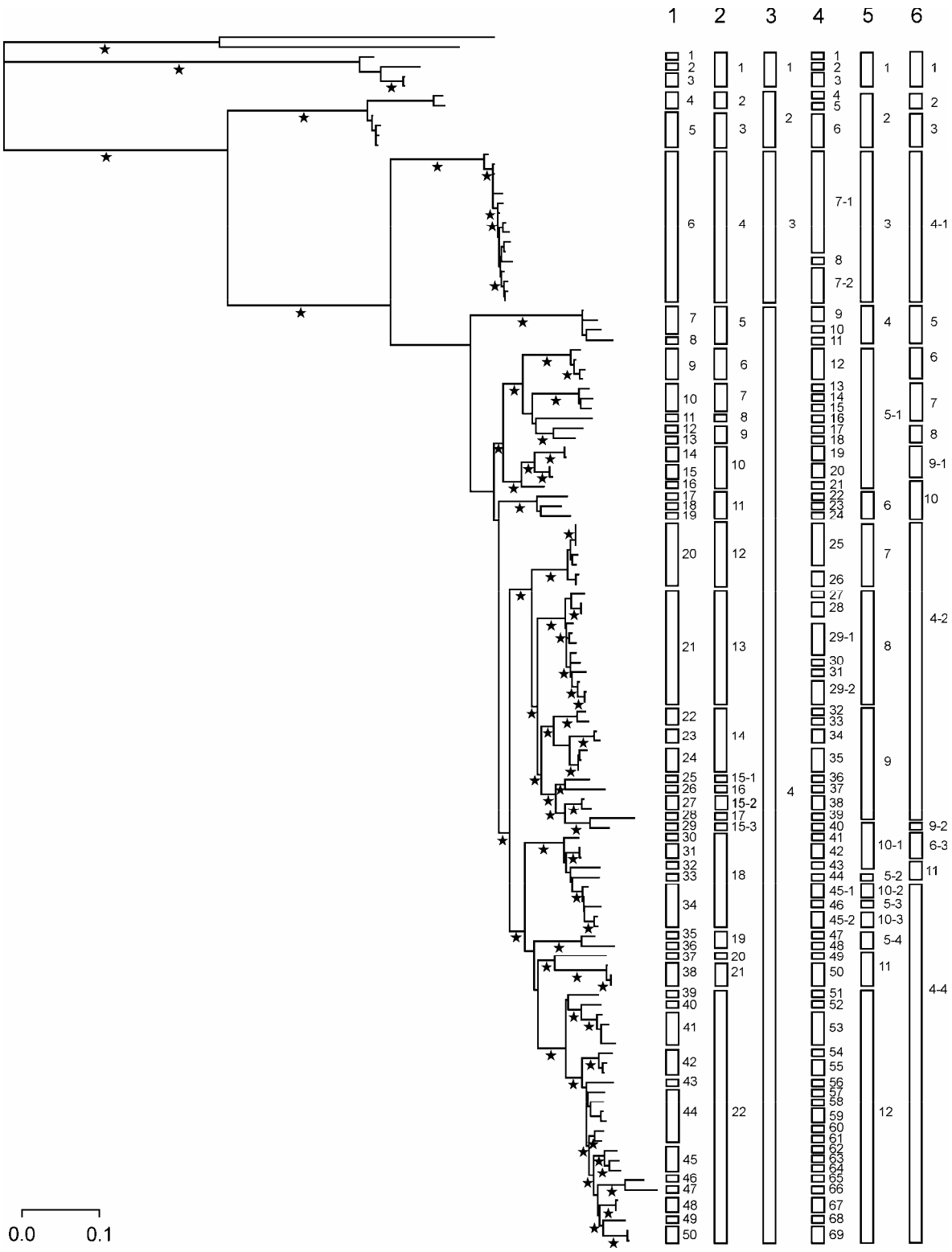

Fig. 2.2. Maximum likelihood tree of partial *cox1* sequences of Cretan *Xerocrassa*. Bootstrap support values larger than 70% are indicated by an asterisk below the branches. Uppermost branches: *Trochoidea elegans* and *Trochoidea spratti*. Partitions based on the following methods

are shown to the right of the tree: 1, 3% pairwise distance threshold; 2, 6% pairwise distance threshold; 3, distance threshold based on change in branching rates (determined using the ultrametric tree constructed with the local rate minimum deformation method); 4, parsimony networks; 5, Gaussian clustering; 6; morphological classification (partition 1, *X. cretica*;2, *X. subvariegata*; 3, *X. grabusana*; 4, *X. mesostena* (clade 4-1: specimens with '*psiloritana*' mtDNA haplotypes); 5, *X. lasithiensis*; 6, *X. heraklea*; 7, *X. rhithymna*; 8, *X. kydonia*; 9, *X. amphiconus*; 10, *X. siderensis*; 11, *X. franciscoi*). If partitions are subdivided because of the structure of the tree, a number for the subdivisions of the partitions is added to the partition number.

The maximum likelihood tree of 68 combined partial *cox1* and 16S rDNA sequences (989 bps) is shown in Figure 2.3. The combined *cox1* and 16S rDNA dataset was also partitioned into the codon positions of the *cox1* gene and the 16S rDNA fragment. Separate models for these four partitions as determined by ModelTest were used, because the resulting tree had a lower AIC value than the tree based on a uniform model for the complete dataset.

The maximum likelihood tree of 27 sequences of the nuclear internal transcribed spacer 2 (ITS-2; 392 bps) obtained with the HKY+G model as determined by ModelTest is shown in Figure 2.4. The tree is not fully resolved because of the low variability of this marker within the Cretan *Xerocrassa*. *p*-distances between the ITS-2 sequences of different Cretan *Xerocrassa* species varied from 0.0% to 5.1% (mean 2.6%). Within *Xerocrassa mesostena p*-distances varied from 0.0% to 2.0% (mean 0.6%). In comparison, *cox1 p*-distances varied from 0.3% to 21.8% (mean 13.7%) between Cretan *Xerocrassa* species and from 0.0% to 17.2% (mean 10.6%) within Cretan *Xerocrassa* species. 16S rDNA *p*-distances varied from 0.8% to 19.5% (mean 9.0%) between Cretan *Xerocrassa* species and from 0.0% to 11.8% (mean 6.1%) within Cretan *Xerocrassa* species. We have also sequenced ITS-1 of some Cretan *Xerocrassa* that show a similarly low diversity as the ITS-2 sequences (unpubl. results).

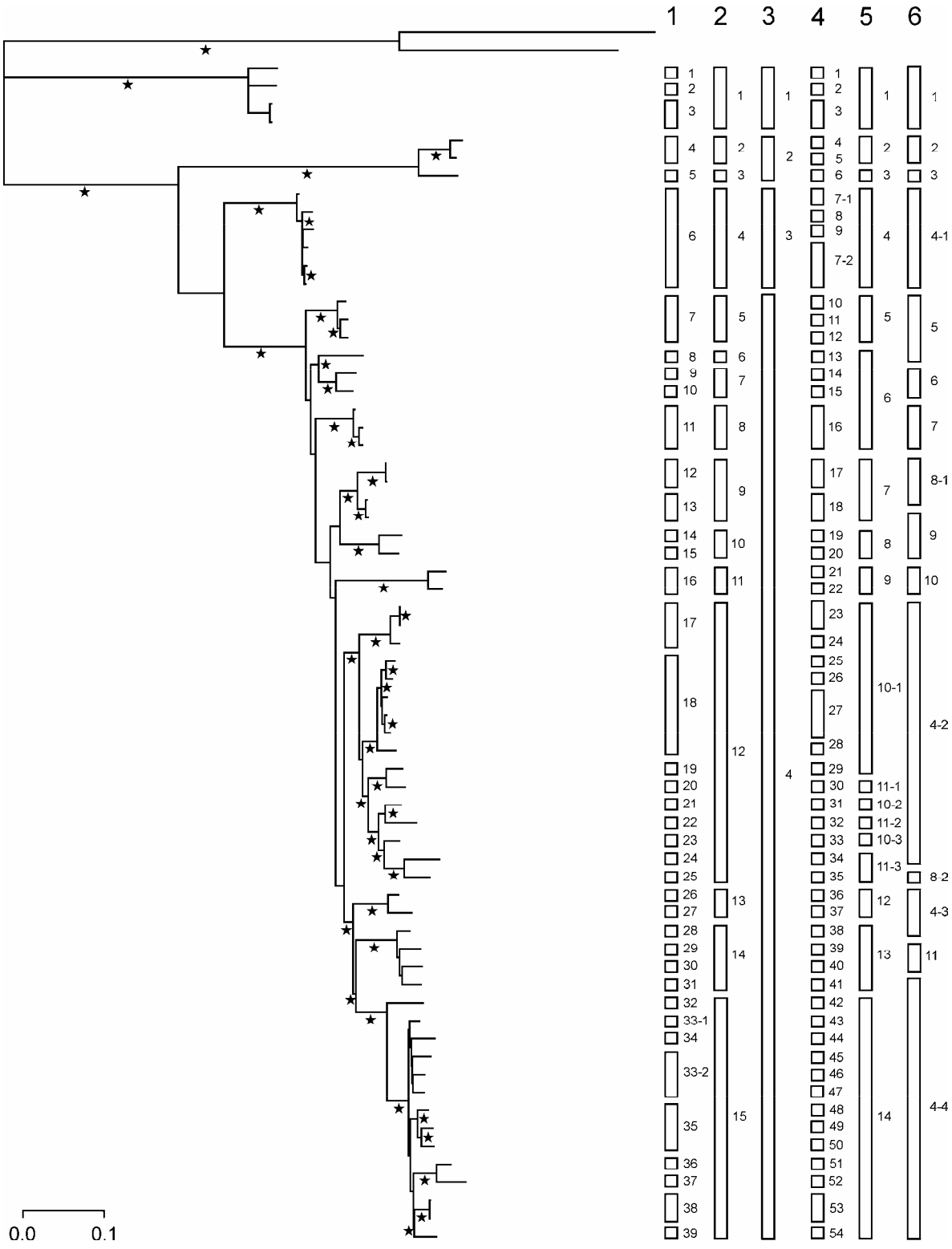

Fig. 2.3 Maximum likelihood tree of combined partial *cox1* and 16S rDNA sequences of Cretan *Xerocrassa*. Bootstrap support values larger than 70% are indicated by an asterisk below the branches. Uppermost branches: *Trochoidea elegans* and *Trochoidea spratti*. Partitions based on the following methods are shown to the right of the tree: 1, 3% pairwise distance threshold; 2, 6% pairwise distance threshold; 3, distance threshold based on change in branching rates (determined using the ultrametric

tree constructed with the local rate minimum deformation method); 4, parsimony networks; 5, Gaussian clustering; 6; morphological classification (partition 1, *X. cretica*;2, *X. subvariegata*; 3, *X. grabusana*; 4, *X. mesostena* (clade 4-1: specimens with '*psiloritana*' mtDNA haplotypes); 5, *X. lasithiensis*; 6, *X. heraklea*; 7, *X. rhithymna*; 8, *X. kydonia*; 9, *X. amphiconus*; 10, *X. siderensis*; 11, *X. franciscoi*). If partitions are subdivided because of the structure of the tree, a number for the subdivisions of the partitions is added to the partition number

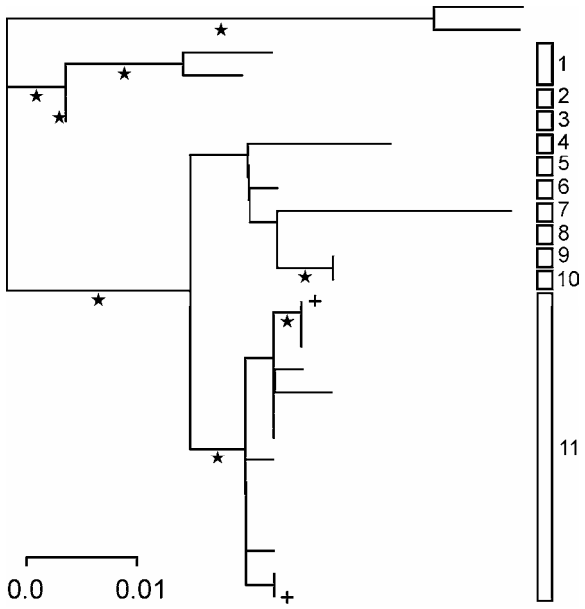

Fig. 2.4 Maximum likelihood tree of ITS-2 sequences of Cretan *Xerocrassa*. Bootstrap support values larger than 70% are indicated by an asterisk below the branches. Uppermost branches: *Trochoidea elegans* and *Trochoidea spratti*. The morphological classification is shown to the right of the tree: 1, *X. cretica*; 2, *X. subvariegata*; 3, *X. grabusana*; 4, *X. rhithymna*; 5, *X. kydonia*; 6, *X. heraklea*; 7, *X. lasithiensis*; 8, *X. franciscoi*; 9, *X. amphiconus*; 10, *X. siderensis*; 11, *X. mesostena* (specimens with '*psiloritana*' mtDNA haplotypes are marked with +).

Tree and Network Based on AFLP Data

Using six primer combinations, we scored 1476 fragments of 70-322 bases length in 151 *Xerocrassa* specimens. A maximum parsimony tree based on the AFLP data is shown in Figure 2.5 and a Neighbor-Net is shown in Figure 2.6.

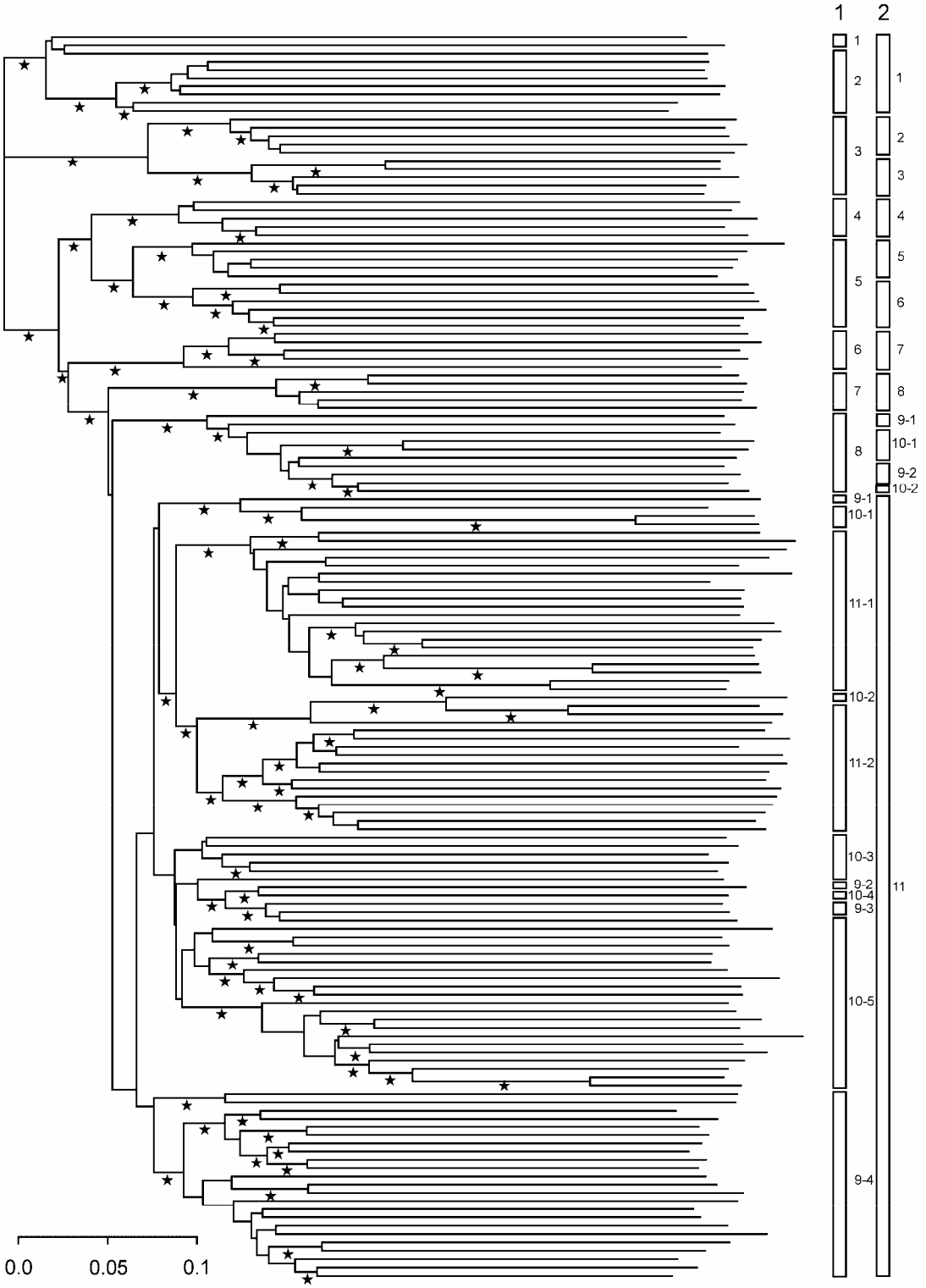

Fig. 2.5 Neighbor-joining tree based on Jaccard distances between AFLP data of Cretan *Xerocrassa*. The species classification proposed based on morphology (Hausdorf and Sauer, 2009) is shown for comparison. Bootstrap support values larger than 70% are indicated by an asterisk below the branches. Partitions based on the following methods are shown to the right of the tree: 1, Gaussian clustering; 2, morphological classification (partition 1, *X. cretica*; 2, *X. subvariegata*; 3, *X. grabusana*; 4, *X. heraklea*; 5, *X. rhithymna*; 6, *X. kydonia*; 7, *X. lasithiensis*; 8, *X. franciscoi*; 9, *X. siderensis*; 10, *X.*

X. amphiconus; 11, *X. mesostena*.. If partitions are subdivided because of the structure of the tree, a number for the subdivisions of the partitions is added to the partition number.

Species Delimitation Based on Pairwise Distance Thresholds of mtDNA Sequences

The 122 *cox1* sequences were grouped into 50 sets for which each sequence in a set has at least one other sequence within a threshold distance of 3%. 26 of these sets include only a single specimen. With a threshold value of 6%, 22 sets were found, 4 of which include only a single specimen. Because the distances between three sequences do not have to be equilateral, a fixed threshold value cannot be maintained. If the threshold distance was set to 3%, the maximum observed distance in a cluster was 4.3% and 12% of the distances within clusters were larger than 3%. If the threshold distance was set to 6%, the maximum observed distance in a cluster was 10.1% and 28% of the distances within clusters were larger than 6%.

With a 3% threshold, the 66 combined *cox1*/16S datasets were grouped into 39 sets (26 with only a single specimen), with a 6% threshold 15 sets resulted (2 with only a single specimen). If the threshold distance was set to 3%, the maximum observed distance in a cluster was 3.8% and 9% of the distances within clusters were larger than 3%. If the threshold distance was set to 6%, the maximum observed distance in a cluster was 8.6% and 39% of the distances within clusters were larger than 6%.

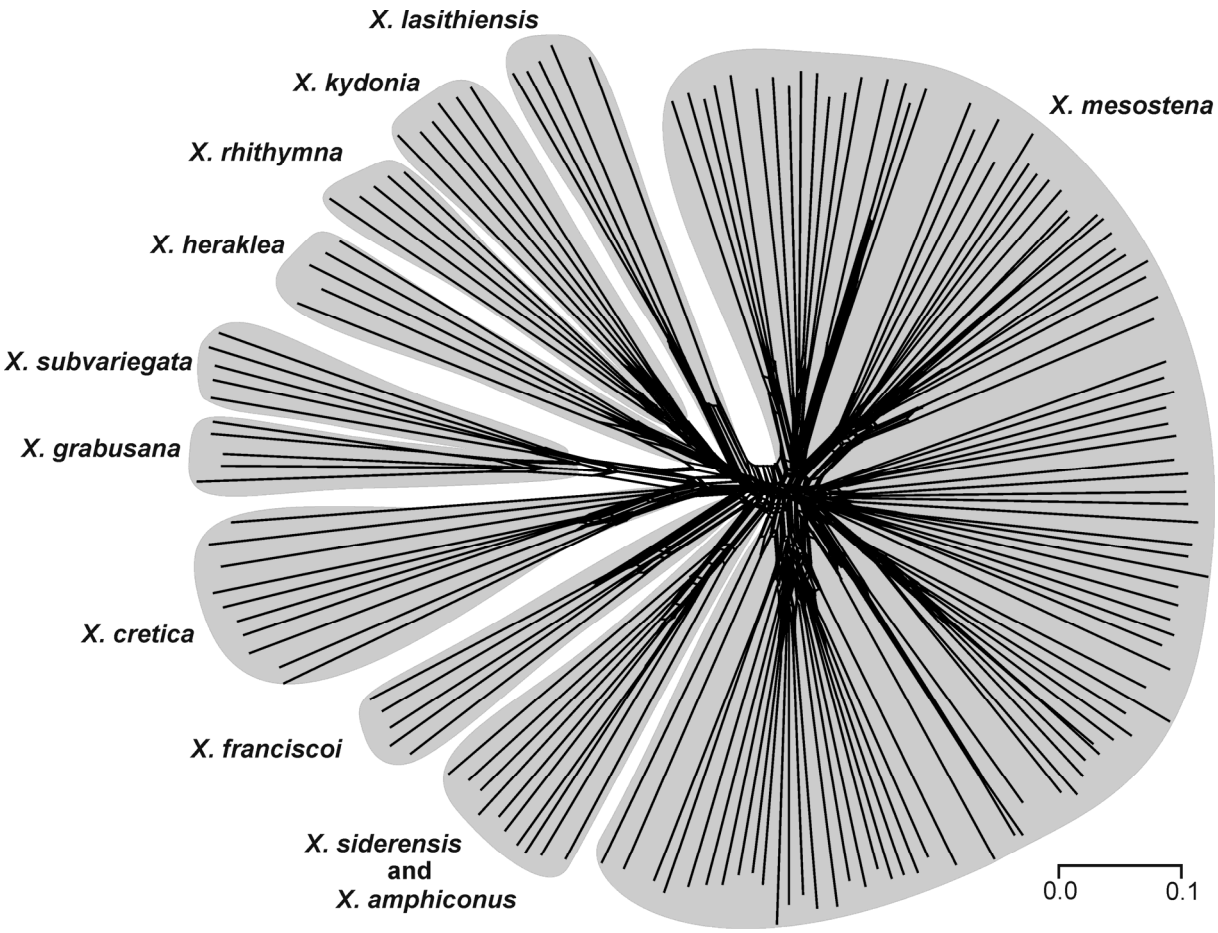

Fig. 2.6. Neighbour-net network based on Jaccard distances between AFLP data of Cretan *Xerocrassa*. The morphological classification is indicated.

Species Delimitation Based on Distance Threshold Derived from Change in Branching Rates of mtDNA Sequences

If the ultrametric *cox1* tree constructed with the penalized likelihood method was used, the general mixed Yule coalescent (GMYC) model was preferred over the null model of uniform branching rates (log$L$ = 719.0, compared to null model log$L$ = 698.1; 2ΔL = 41.8, χ^2 test, d.f. = 3, P « 0.001). Based on the shift inferred with the GMYC model assuming a single threshold the 122 sequences were grouped into 118 entities of which only 3 include more than one sequence. This was the only cluster solution within the confidence limits for the threshold. The GMYC model was also preferred over the null model of uniform branching rates, if the ultrametric *cox1* tree constructed with the local rate minimum deformation method was used (log$L$ = 179.7, compared to null model log$L$ = 165.4; 2ΔL = 28.5, χ^2 test, d.f. = 3, P « 0.001). However, the 122 sequences were grouped into only four clusters (Fig.

2.2) based on this ultrametric tree. This was the only cluster solution within the confidence limits for the threshold.

If the ultrametric *cox1*/16S tree constructed with the penalized likelihood method was used, the GMYC model was preferred over the null model of uniform branching rates (log$L$ = 310.0, compared to null model log$L$ = 305.1; 2ΔL = 9.8, χ^2 test, d.f. = 3, P = 0.020). Based on the shift inferred with the GMYC model assuming a single threshold the 66 sequences were grouped into 65 entities of which only one includes more than one sequence. There were two additional cluster solutions within the confidence limits for the threshold with 4-5 clusters of sequences. The GMYC model was not significantly better than the null model of uniform branching rates, if the ultrametric *cox1*/16S tree constructed with the local rate minimum deformation method was used (log$L$ = 13.0, compared to null model log$L$ = 9.8; 2ΔL = 6.3, χ^2 test, d.f. = 3, P = 0.097). The 66 sequences were grouped into only four clusters (Fig. 2.3) based on this ultrametric tree. There were five additional cluster solutions within the confidence limits for the threshold with 3-9 clusters of sequences.

Species Delimitation Based on Statistical Parsimony Analysis of mtDNA Sequences

Using statistical parsimony, the 122 *cox1* sequences were grouped into 76 independent networks (Fig. 2.2) based on a connection limit of 10 steps; i.e., branches of 11 steps and beyond are considered to fall outside of the 95% confidence interval for these connections to be non-homoplastic. 54 of these networks include only a single specimen. The 66 combined *cox1*/16S datasets were grouped into 54 independent networks based on a connection limit of 13 steps. 46 of these networks include only a single specimen.

Species Delimitation Based on Gaussian Clustering of mtDNA Sequences

A non-metric multidimensional scaling of GTR+G+I distances between the *cox1* sequences of 122 *Xerocrassa* specimens is shown in Figure 2.7. With Gaussian clustering twelve clusters of specimens were identified that are indicated in Figures 2.2 and 2.7. Gaussian clustering of GTR+G+I distances between the combined *cox1*/16S datasets of 66 *Xerocrassa* specimens resulted in 14 clusters (Fig. 2.3).

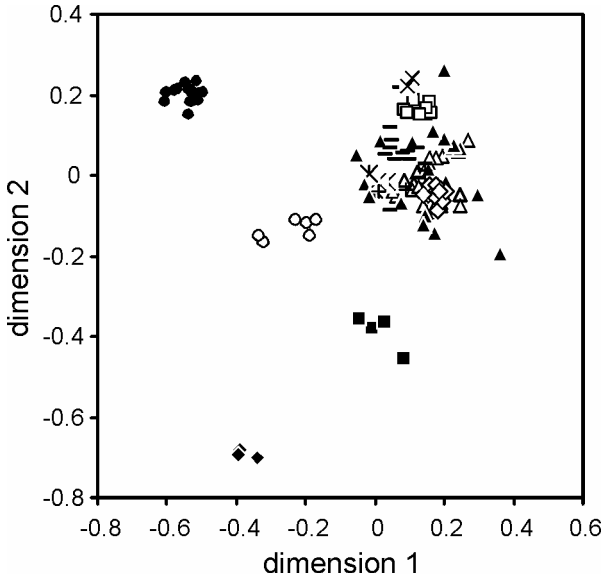

Fig. 2.7 First two dimensions of the non-metrical multidimensional scaling of GTR+G+I distances between partial *cox1* sequences of Cretan *Xerocrassa*. The clusters identified using Gaussian clustering correspond to the following morphologically delimited species: Δ, *X. mesostena*, partly; ▲, *X. mesostena*, partly, *X. amphiconus*, partly, *X. siderensis*, partly, *X. kydonia*, *X. rhithymna*, *X. heraklea* and *X. franciscoi*, partly; ●, *X. mesostena*, partly (specimens with '*psiloritana*' mtDNA haplotypes); -, *X. mesostena*, partly, and *X. amphiconus*, partly; ◇, *X. mesostena*, partly; □, *X. mesostena*, partly, and *X. franciscoi*, partly; ✳, *X. mesostena*, partly; ○, *X. subvariegata* and *X. grabusana*; ◆, *X. cretica*; ■, *X. lasithiensis*; ✕, *X. mesostena*, partly; +, *X. siderensis*, partly.

Species Delimitation Based on Gaussian Clustering of AFLP Data

A non-metric multidimensional scaling of Jaccard distances between the AFLP data of 151 *Xerocrassa* specimens is shown in Figure 2.8. With Gaussian clustering eleven clusters of specimens were identified that are indicated in Figures 2.5-2.6 and 2.8.

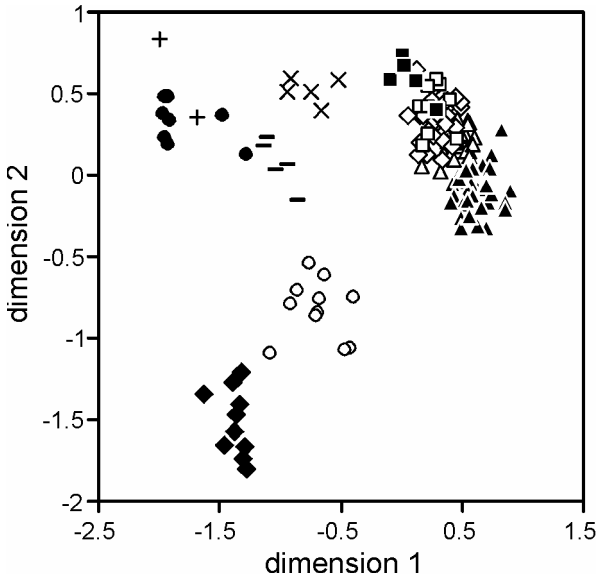

Fig. 2.8 First two dimensions of the non-metrical multidimensional scaling of Jaccard distances between AFLP data of Cretan *Xerocrassa*. The clusters identified using Gaussian clustering correspond to the following morphologically delimited species: ▲, *X. mesostena*, partly; Δ, *X. mesostena*, partly; ◇, *X. mesostena*, partly; ○, *X. kydonia* and *X. rhithymna*; ◆, *X. subvariegata* and *X. grabusana*; □, *X. amphiconus* and *X. siderensis*; ●, *X. cretica*, partly; -, *X. heraklea*; ×, *X. lasithiensis*; ■, *X. franciscoi*; +, *X. cretica*, partly.

Species Delimitation Based on Structure Analysis of AFLP Data

There is no distinct maximum of the mean estimates of the posterior probabilities of the data calculated with Structure for a given cluster number *K* for *K* between 1 and 20 (Fig. 2.9a). The statistic Δ*K* proposed by Evanno *et al.* (2005) to estimate the number of clusters *K* shows a maximum at *K*=2 (Fig. 2.9b). Note, however, that Δ*K* for *K*=1 can not be calculated. Thus, neither the mean estimates of the posterior probabilities of the data for a given cluster number *K* nor the Δ*K* values give a clear indication how many *Xerocrassa* species can be distinguished on Crete.

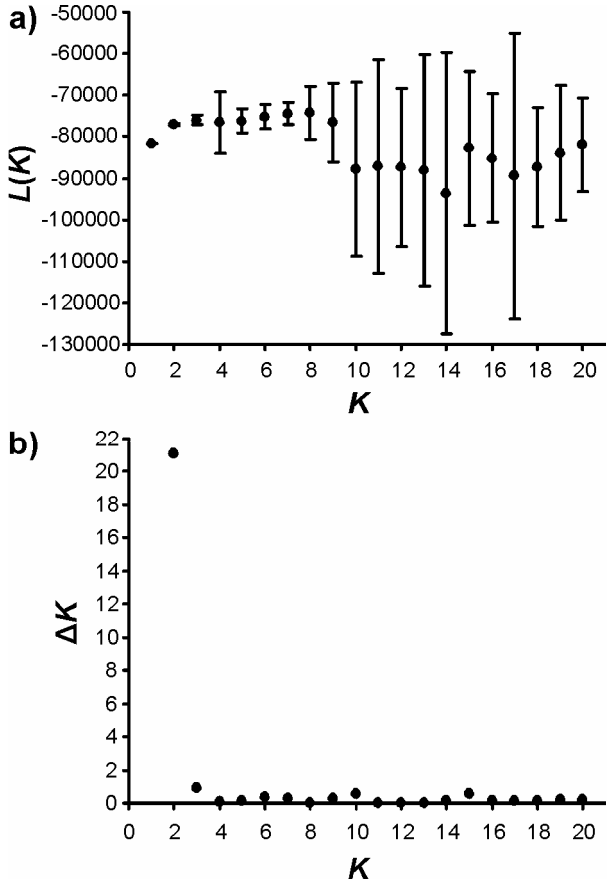

Fig. 2.9 Results of the Structure analysis of the AFLP data of Cretan *Xerocrassa* for different cluster number *K*. (a) Mean estimates of the posterior probabilities of the data for a given *K* (±SD). (b) Δ*K* (following Evanno *et al.* 2005).

Comparison of Classifications

The corrected Rand indices between partitions obtained with different methods based on different datasets are listed in Table 2.2 We did not consider the results based on the combined *cox1*/16S rDNA dataset in this table, because they resemble those based on *cox1* sequences alone, which are available from a larger set of specimens.

If only the comparisons between partitions based on different dataset are considered, the highest congruence is found between the results obtained with Gaussian clustering of AFLP data and morphology (Table 2.2). The congruence between results based on *cox1* sequences and those based on AFLP data or morphology is smaller irrespective of the method used to delimit putative species with these single-locus dataset.

Table 2.2 Corrected Rand indices between partitions obtained with different methods based on different datasets. Comparisons between results based on the different datasets are shown in bold.

	method	data	1	2	3	4	5	6	7
1	3% pairwise distance threshold	*cox1*							
2	6% pairwise distance threshold	*cox1*	0.577						
3	distance threshold based on change in branching rates[a]	*cox1*	0.034	0.014					
4	distance threshold based on change in branching rates[b]	*cox1*	0.044	0.104	0.001				
5	statistical parsimony analysis	*cox1*	0.777	0.409	0.053	0.028			
6	Gaussian clustering	*cox1*	0.457	0.831	0.010	0.137	0.322		
7	Gaussian clustering	AFLP	**0.207**	**0.304**	**0.003**	**0.042**	**0.175**	**0.254**	
8	morphology		**0.071**	**0.154**	**0.001**	**0.131**	**0.045**	**0.124**	**0.373**

[a]determined based on the ultrametric tree constructed with the penalized likelihood method

[b]determined based on the ultrametric tree constructed with the local rate minimum deformation method

The results of Gaussian clustering of AFLP data (Fig. 2.8) differ from the morphological classification in combining the three morphologically differentiated species pairs *X. subvariegata* and *X. grabusana*, *X. kydonia* and *X. rhithymna*, and *X. amphiconus* and *X. siderensis* each and in splitting the *X. mesostena* complex into three groups and *X. cretica* into two clusters. However, *X. subvariegata*, *X. grabusana*, *X. kydonia*, and *X. rhithymna* as defined morphologically are monophyletic in the tree based on the AFLP data (Fig. 2.5).

Although five of the morphologically delimited species (*X. subvariegata*, *X. grabusana*, *X. kydonia*, *X. heraklea*, and *X. lasithiensis*) are monophyletic in the mitochondrial gene trees (Figs. 2.2-2.3), the partitions obtained with the methods for the analysis of single-locus sequences do not correspond well with the morphological classification. The low corrected Rand indices (Table 2.2) between the partitions obtained with methods using arbitrarily fixed distance thresholds or independent networks determined by statistical parsimony based on *cox1* sequences and the morphological classification are the result of the extensive splitting of

the mitochondrial dataset into putative species by these methods because of the high sequence divergences. The method using a distance threshold based on a change in branching rates (Pons *et al.* 2006) yielded very different results depending on the algorithm with which the ultrametric tree that is used for the maximum likelihood approach has been calculated. Using the ultrametric tree calculated with the local rate minimum deformation method it underestimated the number of species in comparison with the morphological classification and the Gaussian clustering of the AFLP data, whereas it heavily overestimated the species diversity on the basis of the ultrametric tree calculated with the penalized likelihood method. The number of species estimated by Gaussian clustering of the mitochondrial dataset is in a better agreement with the morphological classification.

DISCUSSION

Comparison of Trees Based on Single-locus and Multi-locus Data

A comparison of the trees of the *Xerocrassa* land snail species from Crete based on the mitochondrial sequences (Figs. 2.2-2.3) with the tree based on the AFLP markers (Fig. 2.5) shows some striking differences. We use the morphological classification as reference system for this comparison.

One difference concerns a well-supported clade of specimens from an area southwest of the Psiloritis Mountains in the mitochondrial trees (‘*psiloritana*’, see Figs. 2.2-2.3). Morphologically these specimens cannot be distinguished from *X. mesostena*. However, in the mitochondrial trees they are separated from the main clade of *X. mesostena* haplotypes by several other species. On the other hand, they belong to three clades within the *X. mesostena* complex in the AFLP tree (Fig. 2.5), in which they intermingle with specimens with ‘normal’ *X. mesostena* haplotypes from the same region. ‘*psiloritana*’ and ‘normal’ *X. mesostena* haplotypes have even been found together in a population near Agia Galini. This presence of topologically widely separated haplotypes within one species might be an extreme case of incomplete lineage sorting. The subdivision of the mitochondrial haplotypes of *X. mesostena* into two widely separated clades resembles the subdivision of the mitochondrial haplotypes of the salamander *Ambystoma ordinarium* (Weisrock *et al.* 2006). One possible explanation for this deep subdivision is that the *X. mesostena* populations in the area southwest of the Psiloritis Mountains were isolated for a considerable time and that lineage sorting in that area resulted in the fixation of a rather old haplotype lineage. Since these populations met other *X. mesostena* populations again, there is gene flow, indicating that these groups are not

reproductively isolated. Thus, the '*psiloritana*' haplotype group might be a paradigm for one of the causes of high mitochondrial sequence diversity within land snail species discussed by Thomaz *et al.* (1996), namely ancient isolation of populations that consequently diverged, though this process is certainly not the only cause for the unusually high intraspecific mitochondrial sequence diversity. If only the mitochondrial tree would be known and sampling would not be dense enough to show that there are a few 'normal' *X. mesostena* haplotypes scattered in the area mainly occupied by the '*psiloritana*' haplotypes, the populations with the '*psiloritana*' haplotypes would certainly be classified as a cryptic species.

Another confusing case concerns *X. amphiconus* from eastern Crete. Most specimens of this species have haplotypes that group with *X. siderensis*, a closely related species from the same region in eastern Crete. However, one *X. amphiconus* specimen falls within the main *X. mesostena* haplotype grouping in the *cox1* tree as well as in a separate analysis of the 16S rDNA sequences. In the AFLP tree, this specimen groups with other *X. amphiconus* as expected based on morphology. The *X. mesostena* specimen with which this specimen forms a clade in the mitochondrial DNA trees is from central Crete. Mitochondrial DNA can be easily transferred across species boundaries by introgression (Chan & Levin 2005; Funk & Omland 2003; Takahata & Slatkin 1984). However, to explain the occurrence of an apparent central Cretan *X. mesostena* haplotype in *X. amphiconus* would require additionally a long distance dispersal event. Incomplete lineage sorting does not appear to be more plausible in this case, although it cannot be excluded. Sequence ambiguities, frameshift mutations, or stop codons that might indicate that the *X. amphiconus cox1* and 16S rDNA sequences are nuclear mitochondrial pseudogenes (Bensasson *et al.* 2001) have not been found in these sequences.

The tree based on the ITS-2 (Fig. 2.4) is not fully resolved because of the low variability of this marker within the Cretan *Xerocrassa*. Moreover, direct sequencing resulted in superimposed sequences in several cases. This indicates that there is often intra-individual variation in the *Xerocrassa* ITS as has been described from other land snails (Armbruster & Korte 2006) and other organisms (Harris & Crandall 2000; Odorico & Miller 1997; Tang *et al.* 1996; Vogler & DeSalle 1994; Wesson *et al.* 1992) because of incomplete homogenisation of the copies of the rDNA cluster. Thus, the intra-individual variation in the ITS should be examined thoroughly before conclusions about the delimitation of closely related species or their relations are based on this marker. One remarkable result of the phylogenetic analysis of the ITS-2 sequences is the monophyly of all specimens morphologically classified as *X. mesostena* inclusive two specimens that have the very distinct '*psiloritana*' mitochondrial

haplotype, but do not form a clade in the ITS-2 tree (Fig. 2.4). This corroborates that the specimens with the '*psiloritana*' haplotype actually belong to *X. mesostena*.

These results confirm other studies (e.g., Ballard *et al.* 2002; Machado & Hey 2003; Patton & Smith 1994; Shaw 2002; Sota & Vogler 2001; Weisroch *et al.* 2006) that have reported large distinctions between mitochondrial gene trees and trees based on nuclear DNA sequences and/or species classifications based on morphology. The idiosyncratic results based on single-locus data, especially mitochondrial DNA sequences, may have several reasons. Theory predicts that the haplotypes or alleles of recently separated species will often not form monophyletic groups because of incomplete lineage sorting of ancestral polymorphisms (Avise & Wollenberg 1997; Hudson & Coyne 2002; Neigel & Avise 1986; Rosenberg 2003). Actually, it has been shown that the individuals of about a quarter of the examined species do not form exclusive groups in gene trees (Funk & Omland 2003). In our study, incomplete lineage sorting may also be the major cause of the non-monophyly of species in the tress based on mitochondrial sequences (Figs. 2.2-2.3). It is the most likely reason for the reciprocal non-monophyly of *X. amphiconus* and *X. siderensis*, as well as *X. mesostena* and *X. franciscoi*, and the paraphyly of *X. rhithymna* with respect to *X. kydonia*. Incomplete lineage sorting may result in an overestimation of the number of species if basal clades of a paraphyletic species are separated as distinct species or in an underestimation if species whose haplotypes or alleles are not exclusive due to incomplete lineage sorting are lumped together. Moreover, it is has been shown that phylogeographic breaks in the distribution of haplotypes or alleles that may imply species boundaries can form within a continuously distributed species even when there are no barriers to gene flow (Irwin 2002; Kuo & Avise 2005; Neigel & Avise 1993; Rauch & Bar-Yam 2004) and that introgression of single markers, especially mitochondrial DNA, into related species can easily occur (Chan & Levin 2005; Funk & Omland 2003; Takahata & Slatkin 1984).

Species Delimitation with Methods Using Dominant Multi-locus Data

We compared the results of different approaches for the delimiting species based on single-locus DNA sequences with those of methods using dominant multi-locus data and a classification based on morphological characters of land snails from Crete belonging to *Xerocrassa*. The problem of an evaluation of the performance of different methods and different data for delimiting species with empirical data is that the correct species delimitation is not known a priori. Therefore, we looked for congruence between the partitions of the

examined specimens obtained by different analytical methods based on different data to infer the true species limits.

The highest similarity between species partitions based on the different datasets (as quantified by the corrected Rand index; see Table 2.2) was found between the results of Gaussian clustering of the AFLP data and the morphological classification (Hausdorf & Sauer 2009). The results of Gaussian clustering of the AFLP data differ from the morphological classification in combining four morphologically differentiated species pairs and in splitting the two most widespread species into two, respectively three groups. However, four of the combined morphologically defined species are monophyletic in the tree based on the AFLP data (Fig. 2.5) indicating that the separation of these species is not a fault of the morphological study, but has not been realized in the analysis of the AFLP data because of a lack of resolution of the Gaussian clustering. This lack of resolution of the Gaussian clustering may be the result of the low number of sampled specimens of the rarer species compared with the intensively sampled widespread *X. mesostena* complex.

The remaining two morphologically defined species pairs might represent comparatively recently diverged units in which lineage sorting of the nuclear AFLP markers as well as the mitochondrial lineages is not yet completed and/or between which there might be introgression. However, further investigations are necessary to corroborate the species status of these units, especially of the sympatrically distributed *X. amphiconus* and *X. siderensis* that differ only in shell characters. Nevertheless, we consider them preliminarily as distinct species, because among hundreds of examined shells there was only one specimen that was clearly intermediary and that might indicate limited hybridisation between these taxa.

The attempt to determine the number of Cretan *Xerocrassa* species based on the AFLP data with Structure (Falush *et al.* 2007; Pritchard *et al.* 2000) as proposed by Shaffer & Thomson (2007) failed. Structure is a model-based clustering method using multi-locus genotype data to infer population structure and assign individuals to populations under the assumption of Hardy-Weinberg equilibrium within populations. With the Cretan *Xerocrassa* AFLP data neither the mean estimates of the posterior probabilities of the data for a given cluster number K as calculated by Structure nor the statistic ΔK proposed by Evanno *et al.* (2005) provided a clear indication how many species may be involved. Inference with Structure may depend heavily on the modelling assumption, i.e. multi-locus Hardy-Weinberg equilibrium within populations (Pritchard *et al.* 2000). No distinct ‘populations’ with approximate Hardy-Weinberg equilibrium can be found in our AFLP dataset. This may be due to two main reasons, unfounded modelling assumption and inadequate sampling. The

general question is to what extent species, especially such with a distinctly subdivided population structure as expected in poor dispersers like land snails, will be overall in Hardy-Weinberg equilibrium. Even if they were approximately in Hardy-Weinberg equilibrium, an extensive sampling within populations across the whole range of a species might be necessary to recognize a group of populations as a coherent species different from other population groups. Such a sampling is hardly feasible in most groups. These problems have not been considered by Shaffer & Thomson (2007) when they stated that units that are not identified by Structure lack the evidential support to be even candidates for species recognition. We doubt that the use of population genetic approaches based on the assumption of Hardy-Weinberg equilibrium is appropriate for delimiting species under usual circumstances, i.e., for species that do not approximately represent a single (meta-)population and that are not extensively sampled throughout their range.

The success of morphological investigations in recognizing species that are difficult to delimit with molecular data is partly due to the fact we can look specifically for morphological (or behavioural) characters that are directly involved in the speciation process (e.g., characteristics of the copulatory organs or courtship behaviour) (Lee 2004) and weight such characters more heavily than other characters. Actually, this is what classical taxonomists often do: features of the copulatory organs are the most important characters for species delimitation and identification in many taxa. On the other hand, the genes that are directly involved in the speciation process are known only in a few cases (Wu & Ting 2004). Note, moreover, that most complex morphological characters are influenced by several loci (Will *et al.* 2005), and thus can be understood as a sort of coding of multi-locus data. Another advantage of morphological characters is that many of them can still be screened more rapidly than molecular data can be produced. This is of great importance because the often very rare unknown species have to be sorted out of the often very abundant known species before they can be further investigated.

Species Delimitation with Methods Using Single-Locus Data

We applied different approaches for species delimitation using the mitochondrial DNA sequence data of the Cretan *Xerocrassa*. The resulting partitions of all methods based on single-locus data are less similar to those of the Gaussian clustering of the AFLP data or to the morphological classification than the partition obtained by Gaussian clustering of the AFLP data is to the morphological classification (Table 2.2). Given the differences between gene trees based on different markers, it is not surprising that there are large disagreements

between molecular operational taxonomic units (MOTU) based on different markers (Blaxter *et al.* 2005). Because of the idiosyncrasies of any single genetic marker, taxonomy should not rely exclusively on single-locus markers. Previous studies (Meier *et al.* 2006; Meyer & Paulay 2005; Wiemers & Fiedler 2007) and our results show that the assertion of Hebert *et al.* (2003) that a 'COI-based identification system will undoubtedly provide taxonomic resolution that exceeds that which can be achieved through morphological studies' is wrong.

In some 'DNA barcoding' studies (e.g., Blaxter *et al.* 2004; Blaxter *et al.* 2005; Floyed *et al.* 2002; Hebert *et al.* 2003) arbitrarily fixed distance thresholds were used for the delimitation of putative species or MOTU. However, this approach is internally inconsistent, because three sequences can have two pairwise distances conforming to and one exceeding a given threshold (Meier *et al.* 2006). Moreover, the choice of threshold value for distinguishing intra- from interspecific distances is largely arbitrary because of the large overlap of intra- and interspecific variability (DeSalle *et al.* 2005; Ferguson 2002; Lee 2004; Meyer & Paulay 2005; Wiemers & Fiedler 2007; Will & Rubinoff 2004). Our analyses confirm, not surprisingly, that the choice of the threshold has a strong influence on the number of recognized putative species. Different authors of molecular taxonomic surveys have used different threshold for the same gene. Hebert *et al.* (2003) employed 3% distance between *cox1* sequences of lepidopterans as a threshold for species diagnosis, whereas Blaxter *et al.* (2005) used a threshold of 0.4% distance between *cox1* sequences of tardigrades to diagnose MOTU without justification of the choice of the threshold. The consequences of the choice of the threshold on the diversity estimate have also not been discussed. Blaxter *et al.* (2004) reported an 'unexpected diversity' of tardigrades at three sites within Scotland. They defined 32 MOTU based on 0.4% difference between partial 18S rDNA sequences and claimed that this is a 'surprising abundance' given that the documented British fauna is 68 species. Actually, without any knowledge of the variation of 18S rDNA sequences within tardigrade species, their figure is meaningless. If we use a threshold of 0.4% distance between the *cox1* sequences of the Cretan *Xerocrassa* species, we find 106 MOTU. This would roughly double the complete land snail fauna of Crete.

One reason for the high numbers of putative species found based on arbitrarily fixed distance thresholds and statistical parsimony analyses of *Xerocrassa cox1* sequences is the high substitution rate of mitochondrial DNA in helicoid land snails (Chiba 1999; Hayashi & Chiba 2000; Thomaz *et al.* 1996; van Riel *et al.* 2005). The contrast between the high distances between the mtDNA haplotypes (Figs. 2.2-2.3) and the almost invariable sequences of the nuclear ITS-2 (Fig. 2.4), which is approximately as variable as mtDNA sequences in

other taxa (e.g., in *Drosophila*: 1.2% per million years average substitution rate of ITS ((Schlötterer *et al.* 1994) and 1% per million years average substitution rate of mtDNA (DeSalle *et al.* 1987)), corroborates that the substitution rate in the helicoid land snail mtDNA is extraordinary high. Even if a high fixed distance threshold (6%) is used, species delimitation based on *cox1* sequences results in highly split partitions that differ strongly from those based on AFLP and morphological data (Table 2.2). This highlights the strong influence of differences in substitutions rates between taxa on species delimitation based on fixed distance threshold and statistical parsimony analyses. Examples for the influence of varying substitutions rates on the observed 'diversity' can also be found in 'DNA barcoding' studies. For example, Floyd *et al.* (2002) noted that taxa classified as different genera within cephalobid nematodes have similar or identical 18S rDNA sequences, whereas species within one genus in the Rhabditidae have distinguishable sequences. Their phylogramss (Floyd *et al.* 2002: Fig. 2.4) shows that branch length are much longer in the Rhabditidae than in the Cephalobidae indicating that the difference in taxonomic splitting might not be a fault in traditional taxonomy, but an effect of different substitutions rates between the two families. Variations in substitutions rates decouple the 'diversity' estimated with distance threshold and statistical parsimony analyses from species diversity. If variations in substitution rates are not accounted for, single-locus surveys across larger taxonomic groups will not give any comparable diversity estimates beyond sequence variation itself. Hebert *et al.* (2004) proposed to use 10x the mean intraspecific variation in the group under study as threshold for delimiting species. This proposal accounts for a combination of variations in substitution rates and variations in speciation rates. However, simulations based on neutral coalescent theory and the Bateson-Dobzhansky-Muller model of speciation have shown that this threshold can delimit species with error rates of less than 10% only when they have been isolated for more than 4 million generations (Hickerson *et al.* 2006), and thus fails to recognize many reproductively isolated species. Our study confirms this conclusion. The mean *p*-distance of *cox1* sequences within Cretan *Xerocrassa* species is 10.6%. Obviously, the 10x rule cannot yield reasonable results in such a case.

The approach to optimize a distance threshold based on a change in branching rates proposed by Pons *et al.* (2006) proved to be very sensitive with regard to the algorithm with which the ultrametric tree that is used for the maximum likelihood approach has been calculated and resulted in heavily under- or overestimated numbers of species.

The number of species estimated by Gaussian clustering of the mitochondrial sequences is in better agreement with the morphological classification and the Gaussian clustering of the

AFLP dataset than that of any of the other methods using single-locus data. The partition of the individuals to species is slightly less similar to the morphological classification and the result of Gaussian clustering of the AFLP dataset than the partition obtained with a 6% distance threshold. However, the 6% threshold is arbitrary and might miss many species in datasets with sequences with lower substitution rates, whereas the Gaussian clustering will recognize separate clusters independent of the absolute distances between them. Thus, Gaussian clustering can be recommended for species delimitation with multi-locus data as well as single-locus data. However, it must be considered that analyses of single-locus data generally do not allow a reliable classification of closely related species.

Pons *et al.* (2006) proposed to avoid the problems caused by the bad correspondence of species limits defined by the sequence variation of a single genetic marker and the limits defined by other methods by using a taxonomic system based on the sequence information itself as the primary information source for establishing group membership and defining species boundaries. The implementation of this proposal would make an inappropriate method a governing principle and would result in a taxonomic disaster.

Nevertheless, we admit that single-locus markers like mitochondrial DNA may be helpful to obtain a first idea about the taxonomy of cryptic groups like meiofaunal taxa for which currently no other framework is available (Blaxter *et al.* 2005). Single-locus markers may also be used for identifications, especially in cases in which other identification methods fail (e.g. identification of larval stages or food components), if they have been shown to agree with species delimitations based on other evidence.

We have not applied here some methods for species delimitation that are based on character or haplotype profiles obtained from local 'populations', i.e., population aggregation analysis (Davis & Nixon 1992) and cladistic haplotype aggregation (Brower 1999). These methods have two major drawbacks. The most serious problem is that they offer no possibility to distinguish co-occurring species. If one cannot distinguish co-occurring species a priori, they will be merged in a 'population' profile that shows such a large variability, respectively dispersion across a haplotype tree that many other species might be lumped in the aggregate. For example, *X. cretica* co-occurs syntopically with almost all other Cretan *Xerocrassa* species. If such syntopic occurrences of these congeneric species are not recognized, this will result in the lumping of all Cretan *Xerocrassa* species. On the other hand, if one is able to distinguish potentially co-occurring species, 'population' profile based methods can at best be used to obtain a hint how to classify strictly allopatric populations, if an intensive sampling of such populations is available. The necessity for an intensive sampling of local populations to

ascertain whether certain characters or lineages actually co-occur in a local population is the second major problem of these approaches. One can either concentrate in intensively sampling some populations or in sampling more equally across larger regions, because effort is limited in every study. Concentrating sampling effort to a few populations can result in missing some regionally restricted species at all. Moreover, the lack of common haplotypes in some widely dispersed populations might not be very informative, because these populations might be connected by interjacent, unsampled populations in which the haplotypes co-occur. Therefore, we recommend taking samples from a larger number of populations across the whole study region (Fig. 2.1) to assure not to miss regionally restricted species and intermediate populations. Where unusual markers occur sampling can be intensified to elucidate the significance of the variation.

Wiens & Penkrot (2002) proposed a protocol for tree-based species delimitation using DNA data that requires that focal species be defined *a priori*. It investigates whether a focal species corresponds to a single species or to multiple species or is conspecific with another species. The rules designed by Wiens & Penkrot (2002) may be helpful to obtain a first idea about the status of a focal species. However, the approach is limited by the idiosyncrasies of single markers discussed above. Incomplete lineage sorting and introgression may result is the incorrect synonymization of species and phylogeographic breaks in the distribution of haplotypes may result in the erroneous recognition of populations as species even when there are no barriers to gene flow.

Pons *et al.* (2006) tried to solve the first problem of the 'population' profile based methods, the inability to recognize co-occurring species, and the necessity of the definition of focal species in the Wiens & Penkrot (2002) approach by restricting the use of these approaches to subdivide independent networks as determined by statistical parsimony further. They did not question the status of independent networks as putative species. However, our results indicate that independent networks are not necessarily a meaningful approximation of species, at least not in taxa with high substitution rates. In a study of ambystomatid salamanders (Weisrock *et al.* 2006), statistical parsimony also subdivided mitochondrial DNA sequences of a species, the monophyly of which has been substantiated by multiple nuclear makers, into several independent networks. Thus, we advise against using statistical parsimony for delimiting species.

CONCLUSIONS

This study has shown that Gaussian clustering can be recommended for species delimitation with single-locus sequence data as well as dominant multi-locus data. However, species delimitation based on single-locus sequences can differ strongly from that based on multi-locus or morphological data that are in better agreement with each other and that can be considered a reasonable approximation to the true species limits. Thus, any serious attempt to establish the taxonomy of a group should be based on an analysis of several independent markers. Unfortunately, routinely sequencing of several independent markers is technically or at least financially not yet possible for large numbers of specimens from taxonomically diverse groups. Thus, other techniques for screening multiple independent markers like AFLP are currently the best choice for gathering molecular genetic data sufficient for taxonomic analyses of closely related species.

Despite the increasing availability of molecular data, it is reasonable to invest in morphological data, because one can look specifically for morphological characters that are directly involved in the speciation process and that may indicate the evolution of a new species before it becomes apparent in usual molecular data. Thus, we plead for an integrative taxonomy (Will *et al.* 2005) combining morphological and molecular approaches to unravel biodiversity.

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CHAPTER 3

RECONSTRUCTING THE EVOLUTIONARY HISTORY OF THE RADIATION OF THE LAND SNAIL GENUS *XEROCRASSA* ON CRETE BASED ON MITOCHONDRIAL SEQUENCES AND AFLP MARKERS

ABSTRACT

The reconstruction of the evolutionary history of closely related species can be complicated by shared ancestral polymorphisms, introgression or inadequate taxonomy and is especially challenging in rapid radiations. We inferred the evolutionary history of the radiation of the land snail genus *Xerocrassa* on Crete and the reasons for the nonmonophyly of several species in the mitochondrial gene tree. This was done by comparing this gene tree with a tree and a network based on AFLP markers as well as an admixture analysis of these multilocus data. Whereas six of the eleven morphologically delimited *Xerocrassa* species from Crete are monophyletic in the mitochondrial gene tree, nine of these species are monophyletic in the tree based on AFLP markers. Only two morphologically delimited species could not be distinguished with the AFLP data and might have diverged very recently or might represent extreme forms of a single species. The nonmonophyly of *X. mesostena* and *X. rhithymna* is probably the result of incomplete lineage sorting of ancestral polymorphisms, because the mitochondrial haplotype groups of these species are deeply separated and there is no evidence for admixture with other species in the AFLP data. Because the strongly subdivided population structure increases the effective population size, ancestral polymorphisms may persist far longer in many land snail species than in species representing more or less panmictic populations. The nonmonophyly of *X. franciscoi* and *X. amphiconus* might be the result of mitochondrial introgression, because the coalescences of some haplotypes of these species with some *X. mesostena* haplotypes are shallow.

INTRODUCTION

The reconstruction of the evolutionary history of closely related species can be complicated by shared ancestral polymorphisms, introgression or inadequate taxonomy (Funk & Omland 2003). These problems may result in discrepancies between gene trees and a species classification based on other data, which may became apparent if several individuals of each species are sequenced. The probability that ancestral polymorphisms are shared between species increases with decreased time between speciation events (Avise & Wollenberg 1997; Hudson & Coyne 2002; Maddison 1997; Neigel & Avise 1986; Rosenberg 2003). The likelihood that isolation mechanisms are incomplete and introgression (Chan & Levin 2005; Funk & Omland 2003; Takahata & Slatkin 1984) is possible is also higher if species originated in a short time span and, hence, were at least initially genetically similar. Inadequate taxonomy, the third cause for discrepancies between gene trees and a species classification, is more likely, if several species originated in a short period of time and are morphologically not strongly differentiated. Thus, the delimitation of species and the reconstruction of their relationships are especially challenging in species radiations (Erwin 1992; Gittenberger 1991; Schluter 2000). Species in diverse groups that are nonmonophyletic in gene trees have been found, e.g., in radiations of East African cichlids (Albertson *et al.* 1999; Egger *et al.* 2007; Moran & Kornfield 1993; Nagl *et al.* 1998), Darwin's finches (Petren *et al.* 2005; Sato *et al.* 1999), Japanese carabid beetles (Sota & Vogler 2001), Hawaiian swordtail crickets (Shaw 2002), North American lycaenid butterflies (Gompert *et al.* 2008), ambystomatid salamanders (Weisrock *et al.* 2006), Argentinean liolaemid lizards (Morando *et al.* 2004), North American crotaphytid lizards (McGuire *et al.* 2007), and barley (Jakob & Blattner 2006).

We investigated the radiation of the land snail genus *Xerocrassa* (Gastropoda: Helicoidea: Hygromiidae) on Crete. Eleven native *Xerocrassa* species can be distinguished on Crete based on morphological characters of the shell and the genitalia (Hausdorf & Sauer 2009). All native species living on Crete except the more widespread *Xerocrassa cretica* are endemic to Crete. Only six of the morphologically defined species are monophyletic in a mitochondrial gene tree (Sauer & Hausdorf 2009). We checked the morphological species delimitation using a tree and a network based on AFLP markers and used an integrative approach combining several criteria to discriminate between incomplete lineage sorting and

introgression as causes for the nonmonophyly of species in the mitochondrial gene tree of the *Xerocrassa* radiation on Crete.

MATERIAL AND METHODS

Sampling

Snails were sampled at about 500 localities across Crete in July/August and September/October 2004 and September/October 2005. AFLP data were determined from 151 *Xerocrassa* specimens covering all morphotypes and all regions of Crete (Fig. 3.1).

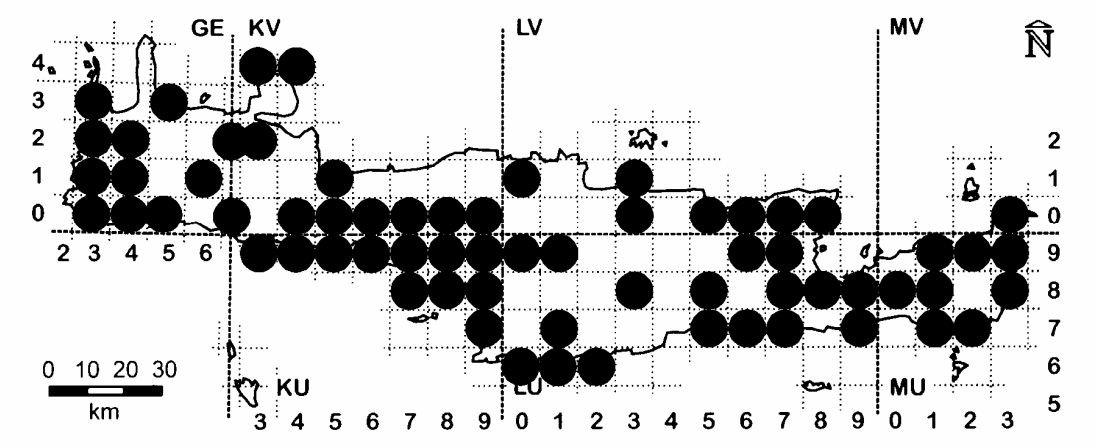

Fig. 3.1 Geographical allocation of the *Xerocrassa* specimens on Crete for which AFLP data were determined. The UTM 10 km grid is indicated on the map.

DNA extraction

Total genomic DNA was extracted from tissue samples of the foot preserved in 100% isopropanol following the protocol proposed by Sokolov (2000) with slight modifications as detailed in chapter 2.

Mitochondrial sequences

Fragments of the cytochrome oxidase subunit 1 (*cox1*) gene were amplified and aligned as described in chapter 2.

AFLP

Approximately 100 ng genomic DNA were digested with 5 units EcoRI (Fermentas) at 37°C for 1 h followed by a digestion with 5 units of MseI (Fermentas) at 65°C for 1 h. 12.5 pmol of

the EcoRI-adapter, 125 pmol of the MseI-adapter and 10 units of T4 DNA ligase and its buffer (GeneCraft) were added to the digestion product and incubated at 16°C for 8 h. The ligation products were diluted 1:10 with sterile ddH_2O, and stored at -20°C.

Preselective PCR was carried out with one selective base on each primer (PA-MseI-C and PA-EcoRI-A, Table 3.1). 5 µl of the diluted ligation product were added to 20 µl of the preselective PCR mastermix, consisting of 15.1 µl ddH_2O, 2.5 µl 10x PCR-buffer, 1.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTP (2 mM), 0.15 µl preselective primer mix (50 µM each), and 0.25 µl Taq-DNA polymerase (5U/µl). Preselective PCR conditions were 22 cycles of PCR (94°C for 30 s, 56°C for 30 s, 72°C for 60 s), and a final extension step at 72°C for 5 min. The quality of the preselective PCR was checked on a 1.5% agarose gel. Afterwards the products were diluted 1:20 with sterile H_2O.

Five primers with two additional bases at the 3' end (Table 3.1) were used for selective amplifications. Six primer combinations (SMseI/SEcoRI^{DYE}) were run: AG/CA^{FAM}, AG/CC^{NED}, AG/GG^{HEX}, TG/CA^{FAM}, TG/CC^{NED} and TG/GG^{HEX}. 5 µl of the diluted preselective PCR product were added to 20 µl of the selective PCR master mix consisting of 15.05 µl ddH_2O, 2.5 µl 10x PCR-buffer, 1.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTP (2 mM), 0.2 µl dye primer mix (0.06 µM labelled selective EcoRI-Primer and 0.6 µM non-labelled selective MseI-Primer), and 0.25 µl Taq DNA polymerase (5U/µl). For the selective amplification a touch down PCR with a temperature decrease of 0.6°C of the annealing temperature each cycle was applied. The program starts with 94°C for 60 s, 65°C for 30 s and 72°C for 60 s followed by 13 cycles of 0.6°C decrease of annealing temperature and 1°C decrease of elongation temperature per cycle and 23 cycles with 94°C for 60 s, 56°C for 30 s and 72°C for 60 s.

Table 3.1 Primers and fluorescent dye labels used for AFLP.

Primer	Sequence	5'- labelling
PA-EcoRI-A	5'- GAC TGC GTA CCA ATT CA -3'	None
PA-MseI-C	5'- GAT GAG TCC TGA GTA AC -3'	None
SEcoRI-CA	5'- GAC TGC GTA CCA ATT CA CA -3'	FAM
SEcoRI-CC	5'- GAC TGC GTA CCA ATT CA CC -3'	NED
SEcoRI-GG	5'- GAC TGC GTA CCA ATT CA GG -3'	HEX
SMseI-AG	5'- GAT GAG TCC TGA GTA AC AG -3'	none
SMseI-TG	5'- GAT GAG TCC TGA GTA AC TG -3'	none

1.2 µl of each of the three differently labelled primer samples were mixed with 6.2 µl Hi Dye Formamid (Applied Biosystems) and 0.2 µl GS-500 ROX size standard (Applied Biosystems). The samples were denatured at 94°C for 2 min and then cooled down on ice for 4 min. The selective PCR products were electrophoretically separated using pop4-polymer (Applied Biosystems) on an ABI PRISM 3100 (Applied Biosystems) capillary sequencer.

Signal detection was performed with GeneScan version 3.1 (Applied Biosystems). Fluorescent threshold was set to 50 relative fluorescence units. The signal intensity was normalized with Genotyper version 2.5 (Applied Biosystems). Fixed fragment categories were created. A presence/absence scoring was conducted between 70 and 322 bases with a threshold set to 50 normalized units. Category spacing was set to 1 base and the category tolerance was adjusted to +/- 0.5 bases.

Phylogenetic analyses

Models of sequence evolution for the maximum likelihood analyses were chosen using ModelTest version 3.7 (Posada & Crandall 1998) based on the Akaike Information Criterion. Maximum likelihood analyses were conducted with Treefinder (Jobb 2007; Jobb *et al.* 2004). Confidence values were computed by bootstrapping (100 replications; Felsenstein 1985).

We checked the homogeneity of base frequencies across taxa using the chi-square test implemented in PAUP* 4.0 beta 10 (Swofford 2002). However, this test ignores correlation due to phylogenetic structure. Therefore, we also measured the probability that the base composition of two sequences is homogeneous for each pair of sequences using the matched-pairs test of symmetry as implemented in SeqVis version 1.4 (Ho *et al.* 2006).

To reduce compositional heterogeneity at the third codon positions we recoded these positions into 2-state categories by pooling purines (adenine and guanine: R) and pyrimidines (cytosine and thymine: Y) (RY-coding, see Phillips and Penny 2003) using the GTR2 model in Treefinder. In addition we analyzed the data set using the nonstationary model of evolution of Galtier & Gouy (1998) as implemented in nhPhyML-Discrete (Boussau & Gouy 2006), limited to 5 base content frequency categories and with 6 categories for a discrete gamma model of among-site rate variation. Trees obtained by nhPhyML-Discrete were then compared using the approximately unbiased test (Shimodaira 2002) implemented in CONSEL (Shimodaira & Hasegawa 2001).

Jaccard distances were calculated from the AFLP data using PhylTools version 1.32 (Buntjer 2001). These distances were used to reconstruct neighbor-joining trees with PHYLIP version 3.66 (Felsenstein 2005) and phylogenetic networks with the neighbor-net algorithm (Bryant & Moulton 2004)implemented in SplitsTree4 version 4.6 (Huson & Bryant 2006). Confidence values for the edges of the neighbor-joining tree were computed by bootstrapping (1000 replications).

Bayesian analysis of genetic structure and admixture based on multilocus markers

We used the Bayesian program Structure (Falush *et al.* 2007; Pritchard *et al.* 2000) to investigate the genetic structure of the *Xerocrassa* radiation on Crete based on the AFLP data without a priori grouping of individuals into species. We explored the pattern of admixture by varying the number of groups *K* in the dataset and assigning proportions of each individual to these groupings using Structure version 2.3.1 with the model with admixture. We carried out 5 separate runs with 100,000 iterations after a burn-in of 10,000 iterations for each cluster number *K* from 2 to 20. Distruct version 1.1 (Rosenberg 2004) was used to create a figure of the Structure output of the run with the highest likelihood.

RESULTS

Mitochondrial gene tree

Separate models for the three codon positions as determined by ModelTest based on the AIC were used for the maximum likelihood analysis (1. codon positions: TrN+I+G, 2. codon positions TrN+I, 3. codon positions TVM+G), because the resulting tree had a lower AIC value than the tree based on a uniform model for the complete dataset. The maximum likelihood tree of 122 partial *cox1* sequences (634 bps) of Cretan *Xerocrassa* species and two *Trochoidea* species as outgroups is shown in Figure 3.2a (see also chapter 5).

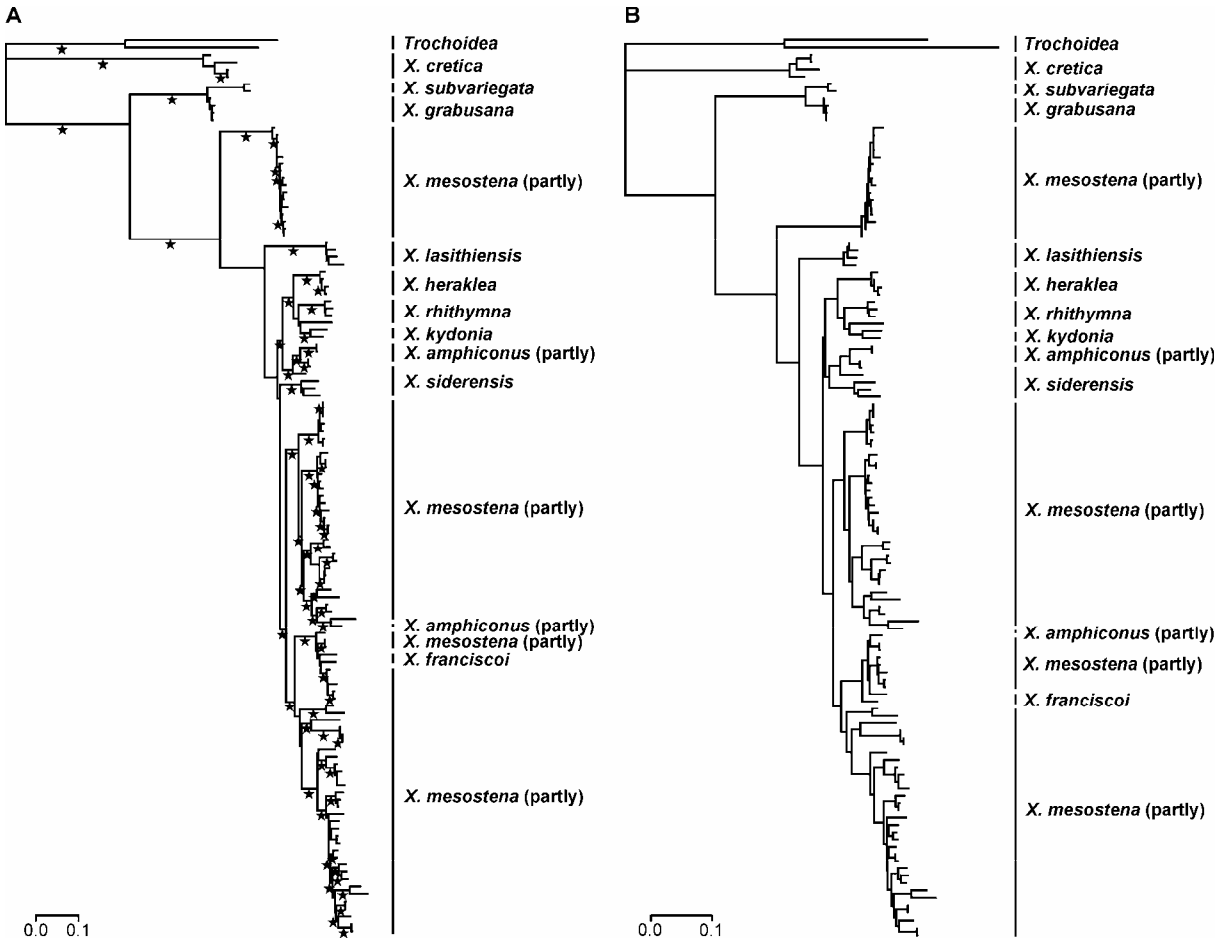

Fig. 3.2 Maximum likelihood trees of partial *cox1* sequences of 122 Cretan *Xerocrassa* species and two *Trochoidea* species. (A) Maximum likelihood tree calculated with a stationary model. Bootstrap support values larger than 70% are indicated by asterisks below the branches. (B) Maximum likelihood tree calculated with a nonstationary model.

According to chi-square tests the base composition at the first and second codon positions of the used *cox1* sequences are not heterogeneous (p=1.000), but there is significant heterogeneity at the third codon positions (p=0.016). The results of the matched-pairs tests of symmetry are compatible with these results. According to the matched-pairs tests of symmetry 37.5% of the pairwise comparisons of the nucleotides at the third codon positions indicate significant (p<0.050) heterogeneity, whereas only 4.0% of the pairwise comparisons of the nucleotides at the first codon positions and 0.0% of the pairwise comparisons of the nucleotides at the second codon positions indicate significant heterogeneity.

To reduce the compositional heterogeneity at the third codon positions we recoded the nucleotides at the third codon positions as purines and pyrimidines. This RY-recoding

resulted in a loss of information so that the resulting tree was badly supported. The early branching Psiloritis haplotype group (see below) of *X. mesostena* became nested in *X. siderensis* haplotypes from eastern Crete.

The analysis with the nonstationary model implemented in nhPhyML-Discrete requires a starting tree. We used the maximum likelihood tree obtained with the unmodified dataset as well as the maximum likelihood tree obtained with the RY-recoding of the third codon positions as starting trees. The Psiloritis haplotype group of *X. mesostena* has the same basal position as in the maximum likelihood tree obtained with the unmodified dataset and the stationary model (Fig 3.2a) in both resulting trees. According to the approximately unbiased test, the tree obtained using the maximum likelihood tree calculated based on the unmodified dataset as starting tree (Fig.3.2b) was significantly (p<0.0001) better than the tree obtained using the other starting tree.

Both reconstructions of the mitochondrial gene tree (Fig. 3.2) show that the haplotypes of *X. cretica, X. subvariegata, X. grabusana, X. lasithiensis, X. heraklea* and *X. kydonia* form monophyletic groups, but the haplotypes of the other five species do not. The haplotypes of the widespread *X. mesostena* are paraphyletic with respect to all other endemic *Xerocrassa* species with the exception of *X. subvariegata* and *X. grabusana* and form two deeply separated main groups. Whereas the majority of the *X. mesostena* individuals have haplotypes that form a terminal bush-like group, most individuals living in a region southwest of the Psiloritis Mountains have mitochondrial haplotypes that form a strongly supported early branch in the mitochondrial gene tree, which we call the 'Psiloritis group'. The haplotypes of *X. rhithymna* are paraphyletic with respect to *X. kydonia*. The haplotypes of *X. franciscoi* are nested in the major *X. mesostena* group. In the tree calculated with the stationary model (Fig. 3.2a) most haplotypes of *X. amphiconus* form a clade that is nested in some haplotypes of *X. siderensis*, which together form the sister group of the *X. heraklea*/*rhithymna*/*kydonia* clade. The second group of *X. siderensis* haplotypes forms the sister clade of the major *X. mesostena* group. In contrast, the two haplotypes clades of *X. amphiconus* and *X. siderensis* form a single clade, which is the sister group of the *X. heraklea*/*rhithymna*/*kydonia* clade, in the tree calculated with the nonstationary model (Fig. 3.2b). In both reconstructions, one haplotype of *X. amphiconus* is nested in the major *X. mesostena* group.

Tree and network based on AFLP data

Using six primer combinations, we scored 1476 fragments of 70-322 bases length in 151 *Xerocrassa* specimens. A neighbor-joining tree based on Jaccard distances between AFLP data of Cretan *Xerocrassa* is shown in Figure 3.3 and a neighbor-net is shown in Figure 3.4. Nine of the eleven morphologically defined species are monophyletic in the AFLP tree. This is also true for *X. rhithymna*, *X. franciscoi* and *X. mesostena*, which are nonmonophyletic in the mitochondrial gene tree. Of the morphologically defined species, only *X. amphiconus* and *X. siderensis* are polyphyletic in the mitochondrial gene tree as well as in the neighbor-joining tree and the neighbor-net based on the AFLP data. However, *X. amphiconus* and *X. siderensis* together form a separate group in the tree and the neighbor-net based on the AFLP data, whereas the haplotypes of the *X. amphiconus*/*siderensis* group are polyphyletic in the mitochondrial gene tree (Fig. 3.2). The *X. mesostena* individuals that are characterized by Psiloritis haplotypes are intermingled between individuals with other haplotypes in the tree (Fig. 3.3) and the neighbor-net (Fig. 3.4) based on AFLP markers.

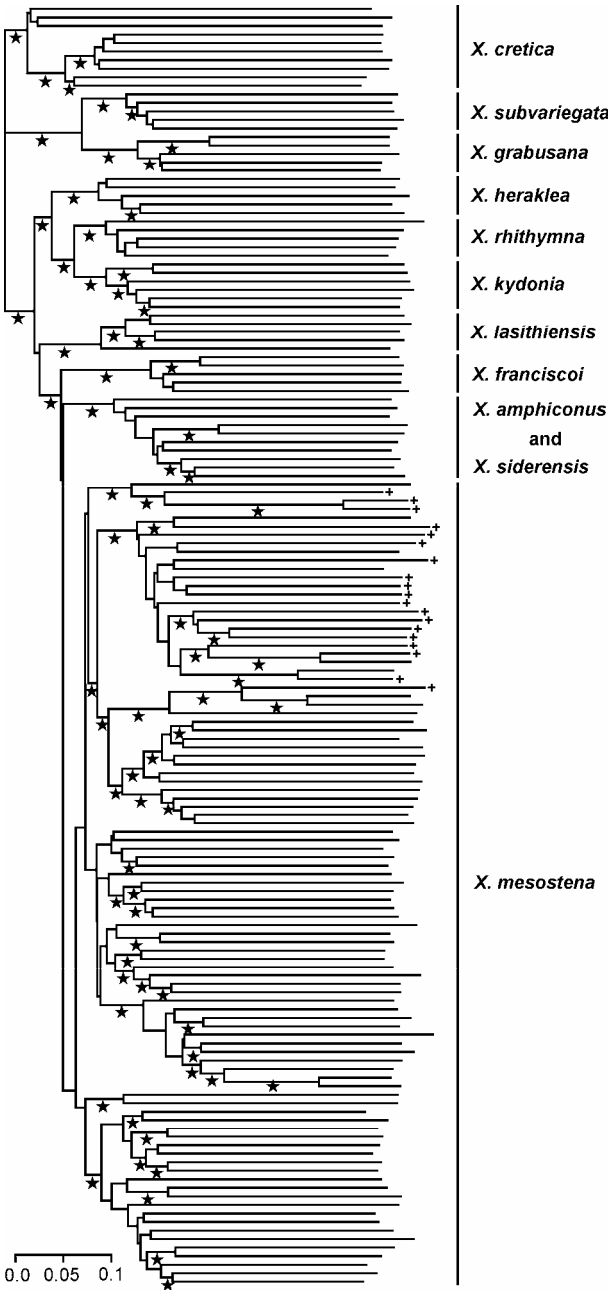

Fig. 3.3 Neighbor-joining tree based on Jaccard distances between AFLP data of Cretan *Xerocrassa*. Bootstrap support values larger than 70% are indicated by an asterisk below the branches. The *X. mesostena* individuals that are characterized by a mitochondrial haplotype of the Psiloritis group are indicated by +.

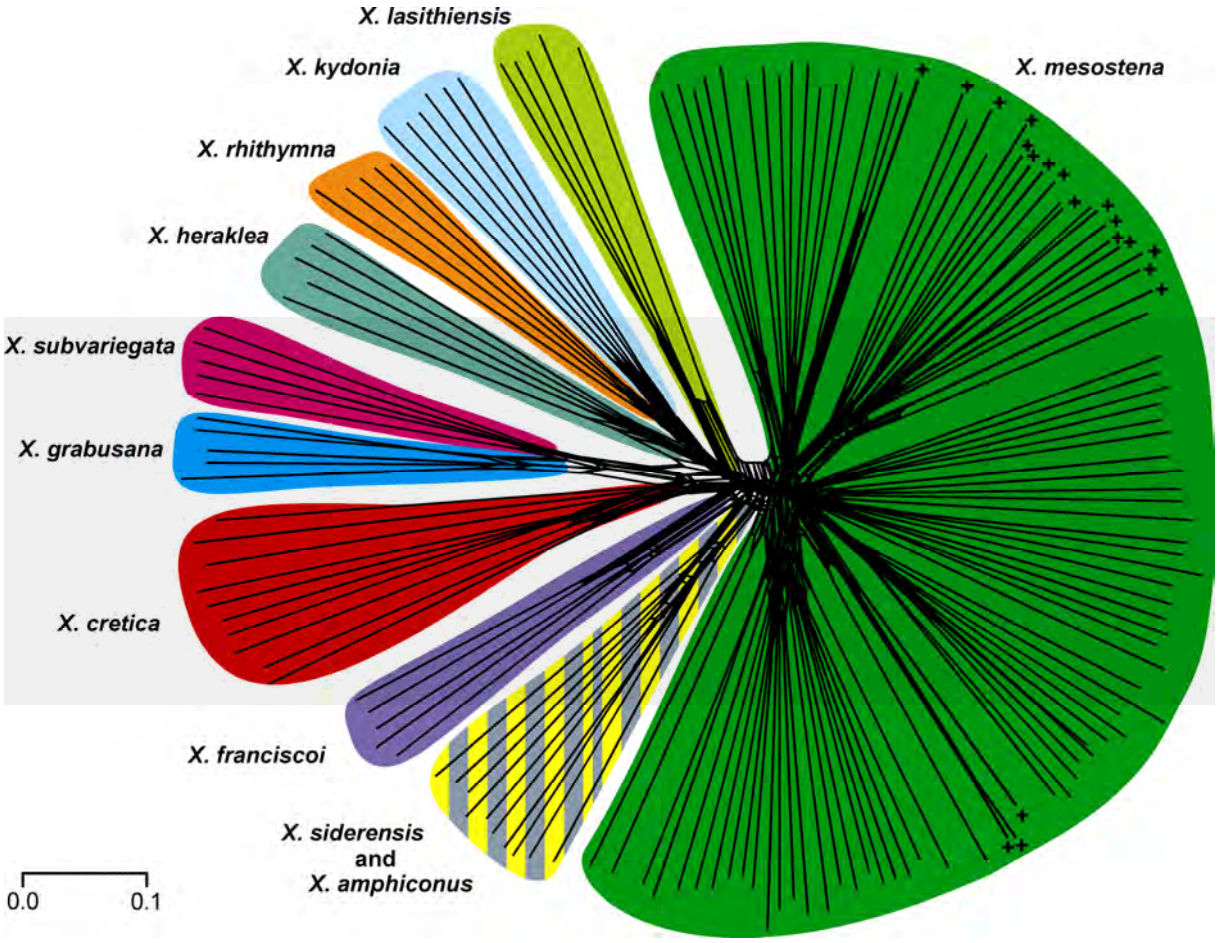

Fig. 3.4 Neighbor-net network based on Jaccard distances between AFLP data of Cretan *Xerocrassa*. The morphological classification is indicated. The *X. mesostena* individuals that are characterized by a mitochondrial haplotype of the Psiloritis group are indicated by +.

Bayesian analysis of genetic structure and admixture based on multilocus markers

The likelihoods of the runs of the admixture analyses with Structure for K from 2 to 20 are shown in Figure 3.5. For K from 2 to 13 $L(K)$ increased. For $K \geq 10$ the variance of $L(K)$ of the different runs increased strongly. This indicates that it is difficult to determine the most likely grouping of individuals at high K values and that it is important to perform several runs for each K. The highest likelihood has been obtained in a run with K=13. The result of this run is shown in Figure 3.6. The four runs with next highest likelihoods were found with K=11.

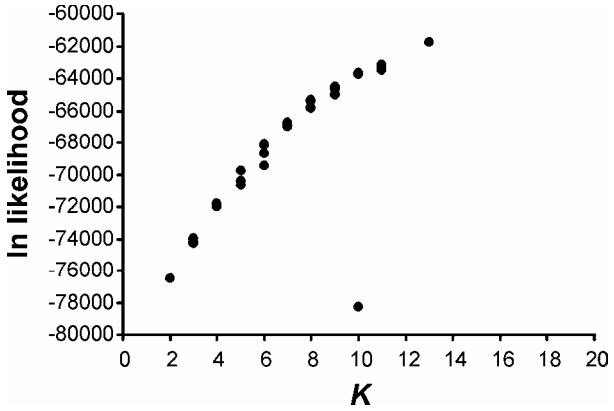

Fig. 3.5 Ln likelihoods of each five runs of admixture analyses with the AFLP data of Cretan *Xerocrassa* with Structure for cluster number *K* from 2 to 20. Only values in the surrounding of the maximum are displayed.

In the run with the highest likelihood *X. cretica*, *X. subvariegata*+*X. grabusana*, *X. heraklea*+*X. rhithymna*+*X. kydonia*, *X. lasithiensis*, *X. franciscoi* and *X. amphiconus*+*X. siderensis* were recognized as separate 'populations'. *X. mesostena*, which has been most extensively sampled, because it is the most widespread and most variable species, has been subdivided into several 'populations'. However, the admixture analyses indicated that these 'populations' intergrade into each other (Fig. 3.6). The *X. mesostena* individuals with the Psiloritis haplotypes did not form a separate cluster, but belonged to different 'populations' that are admixed with each other and with other 'populations' classified as *X. mesostena* (Fig. 3.6). The results obtained in the runs with *K*=11 differ only in recognizing *X. kydonia* as a separate 'population' and in subdividing *X. mesostena* into fewer 'populations'.

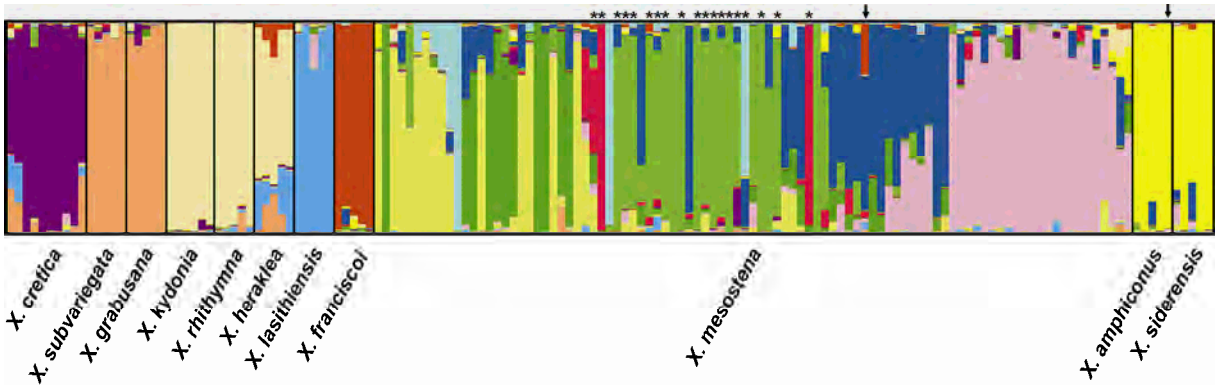

Fig. 3.6 Results of the admixture analysis with the AFLP data of Cretan *Xerocrassa* with Structure for *K*=13. Individuals are grouped into morphologically defined species and are ordered from west to east within species. The *X. mesostena* individuals that are characterized by a mitochondrial haplotype of the Psiloritis group are indicated by *. The *X. mesostena* individual from the population adjacent to the range of *X. franciscoi* and the *X. amphiconus* individual with a haplotype nested in the main *X. mesostena* haplotype group are indicated by arrows.

The morphological species classification is more compatible with the tree (Fig. 3.3) and network (Fig. 3.4) based on the AFLP data than with the 'population' classification obtained with Structure (Fig. 3.6). However, Structure provides additional information on the admixture of the examined individuals. The Structure analysis showed increasing admixture of the easternmost representatives of *X. mesostena* with the neighbouring *X. amphiconus*/*siderensis* group towards the boundary between the ranges of these groups (Fig. 3.6). Although at least a part of the mitochondrial haplotypes of the *X. amphiconus*/*siderensis* group is sister to the *X. heraklea*/*rhithymna*/*kydonia* clade, *X. amphiconus* and *X. siderensis* did not show admixture with *X. heraklea*, *X. rhithymna* or *X. kydonia*. *X. kydonia* showed admixture with the geographically adjacent *X. lasithiensis*, although they are not sister species. The Structure analyses also indicated some admixture between *X. franciscoi* and neighboring individuals of *X. mesostena*.

Because *X. rhithymna* and *X. kydonia* were not recognized as separate ‘populations’ in the admixture analysis with the complete data set (Fig. 3.6), we run admixture analyses, in which only these two species were considered, with *K*=2 to infer the reasons for the paraphyly of the mitochondrial haplotypes of *X. rhithymna* with respect to those of *X. kydonia*. In the run with the highest likelihood, all *X. rhithymna* individuals including the specimen with the haplotype that is sister to the *X. kydonia* haplotypes had an inferred ancestry of 99.5-99.8% in their own cluster. That means that there is no evidence for introgression of *X. kydonia* alleles

into *X. rhithymna* individuals. The inferred ancestry of five *X. kydonia* individuals was also between 99.3-99.7% in their own cluster, but one individual had an inferred ancestry of only 86.5% in its own cluster and 13.5% in the *X. rhithymna* cluster.

DISCUSSION

Comparison of the mitochondrial gene tree and the tree and the network based on AFLP data

The reconstruction of the evolutionary history of radiations is challenging, because incomplete lineage sorting of ancestral polymorphisms (Avise & Wollenberg 1997; Hudson & Coyne 2002; Maddison 1997; Neigel & Avise 1986; Rosenberg 2003), introgression resulting from interspecific gene flow in the early stages of speciation (Chan & Levin 2005; Funk & Omland 2003; Takahata & Slatkin 1984) or inadequate taxonomy may result in discrepancies between species classification and gene trees as well as between different gene trees.

There were strong discrepancies between a species classification based on morphological characters of the shell and the genitalia (Hausdorf & Sauer 2009) and a mitochondrial gene tree in a study of the radiation of the land snail genus *Xerocrassa* on Crete (Sauer & Hausdorf 2009). In the mitochondrial gene tree based on *cox1* sequences (Fig. 3.2) only six of the eleven morphologically defined *Xerocrassa* species living on Crete are monophyletic. A topology test showed that the lack of monophyly of the morphologically delimited species is not the result of a poor resolution of the mitochondrial gene tree (Sauer & Hausdorf 2009). We could also exclude the possibility that the lack of monophyly of the morphologically delimited species in the mitochondrial gene tree is an artefact resulting from mistakes in tree reconstruction due to compositional bias by using a nonstationary model for the maximum likelihood analyses (Fig. 3.2b).

We generated a multilocus dataset using AFLP markers (Vos *et al.* 1995) to investigate whether the discrepancies between the morphologically based species classification and the mitochondrial gene tree are artefacts resulting from inadequate taxonomy or whether they can be explained by evolutionary processes like incomplete lineage sorting or introgression. Nine of the eleven morphologically delimited Cretan *Xerocrassa* species form separate groups in the tree (Fig. 3.3) and the neighbor-net (Fig. 3.4) based on 1476 AFLP markers. Thus, the AFLP data corroborate the morphological species classification with the exception of the separation of *X. amphiconus* and *X. siderensis*, which together form a separate clade, but are intermingled within this clade. This species pair is also exceptional with regard to

morphology and distribution. Whereas most other *Xerocrassa* species can be distinguished by characters of the genitalia, the genitalia of *X. amphiconus* and *X. siderensis* show no differences. These two species differ only in shell characters. Whereas the other endemic species have largely allopatric distribution areas, the ranges of *X. amphiconus* and *X. siderensis* broadly overlap. Nevertheless, they usually do not occur together. They tend to prefer different altitudinal zones (see chapter 1), though there are several populations of each species occurring in the zone preferred by the other species. Only few individuals show intermediate shell characters indicating possible hybridization. The lack of clear genetic differentiation of the two species might indicate that the species diverged only very recently. Given the broad overlap of their current distribution areas, this would indicate sympatric speciation. However, it is unclear whether the diffuse ecological differentiation of the two taxa might be the basis for strong disruptive selection resulting in sympatric speciation (Gavrilets 2003; Rundle & Nosil 2005; Schluter 2000). At present, we cannot rule out the possibility that the two forms actually represent only extreme morphs of a single species.

In other respects the results of the phylogenetic analyses based on *cox1* sequences (Fig. 3.2) and those based on the AFLP data (Fig. 3.3) are similar. Minor discrepancies concern the position of *X. lasithiensis* and *X. franciscoi*. *X. lasithiensis* is sister to all endemic species except *X. grabusana* and *X. subvariegata* in the mitochondrial gene tree, but branches off only after the split of the *X. heraklea*/*rhithymna*/*kydonia* clade in the AFLP tree. The Structure analysis of the AFLP data indicated that there is admixture between *X. lasithiensis* and *X. heraklea* (Fig. 3.3), but not between *X. lasithiensis* and *X. rhithymna* or *X. kydonia*. Since *X. heraklea* is the sister species of *X. rhithymna* and *X. kydonia* in the AFLP tree as well as in the mitochondrial gene tree, this admixture cannot easily be explained by shared ancestral polymorphisms, but is probably the result of hybridization between these geographically neighbouring species. The position of *X. lasithiensis* in the AFLP tree might have been influenced by the introgression of markers as a result of hybridization. Thus, the position of *X. lasithiensis* in the mitochondrial gene tree might reflect the historical branching sequence accurately.

In the AFLP tree *X. franciscoi* is sister to *X. mesostena* and the *X. amphiconus*/*siderensis* group. However, *X. franciscoi* and *X. mesostena* + the *X. amphiconus*/*siderensis* group are separated only by a very short branch, which is not supported by the bootstrap analysis. In the mitochondrial gene tree the haplotypes of *X. franciscoi* are nested in the major haplotype

group of *X. mesostena* and are separated from the most closely related haplotypes of *X. mesostena* only by shallow distances. In the maximum likelihood tree of the *cox1* sequences calculated with the stationary model (Fig. 3.2a) most haplotypes of the *X. amphiconus*/*siderensis* group (except one that is nested within the major group of *X. mesostena*) form two distinct clades and are separated from those of *X. mesostena* by larger distances. In the maximum likelihood tree calculated with the nonstationary model (Fig. 3.2b) these two haplotype clades of the *X. amphiconus*/*siderensis* group form one clade that is sister to the *X. heraklea*/*rhithymna*/*kydonia* clade. These patterns are compatible with the hypothesis that *X. franciscoi* originated rather recently from a southern isolate of the *X. mesostena* complex, whereas the *X. amphiconus*/*siderensis* group represents eastern populations that begun to separate from the *X. mesostena* complex (perhaps together with the *X. heraklea*/*rhithymna*/*kydonia* clade) earlier. It is possible that the two speciation processes overlapped temporarily. Such an independent origin of species from the same stem species cannot easily be depicted in a species tree. The mitochondrial gene tree hints to the phylogenetic origin of *X. franciscoi* from neighbouring populations of the *X. mesostena* complex, whereas the AFLP tree reflects the genetic cohesion of the individuals of each of the species caused by intraspecific gene flow and recombination that, on the other hand, obscured the details of the relations between the species. Thus, both marker types supply complementary information with regard to the phylogenetic history of the *Xerocrassa* radiation on Crete, similar to the situation concerning the radiation of the cichlid genus *Tropheus* in Lake Tanganyika (Egger *et al.* 2007).

Incomplete lineage sorting versus introgression as cause of the discrepancies between the mitochondrial gene tree and the species classification

We analyzed the potential mechanisms resulting in the nonmonophyly of species in the mitochondrial gene tree by applying several criteria that have been proposed to discriminate between incomplete lineage sorting and introgression. These criteria are the depth of the coalescences in the mitochondrial tree (Funk & Omland 2003; Holder *et al.* 2001; Jakob & Blattner 2006; Joly *et al.* 2009; McGuire *et al.* 2007; Morando *et al.* 2004), the geographical occurrence of the individuals of species that are nonmonophyletic in the gene tree (Donnelly *et al.* 2004; Funk & Omland 2003; Gompert *et al.* 2008; Jakob & Blattner 2006; McGuire *et*

al. 2007; Morando *et al.* 2004) and concordance with results of admixture analyses of nuclear multilocus markers (Berthier *et al.* 2006; Gompert *et al.* 2008).

The nonmonophyly of *X. mesostena, X. franciscoi* and *X. rhithymna* in the mitochondrial gene tree (Fig. 3.2) cannot be explained by inadequate taxonomy, because they form separate groups in the tree (Fig. 3.3) and the neighbor-net (Fig. 3.4) based on AFLP markers. Thus, these cases of nonmonophyly originated by evolutionary processes. The most remarkable pattern is seen in *X. mesostena,* the most widespread of the endemic species. Whereas individuals classified morphologically as *X. mesostena* living in a region southwest of the Psiloritis Mountains have mitochondrial haplotypes that form a strongly supported early branch in the *cox1* gene tree (Fig. 3.2), the *X. mesostena* individuals from other regions of the island have haplotypes that form a terminal bush-like group. In the tree (Fig. 3.3) and the neighbor-net (Fig.3.4) based on AFLP markers individuals with Psiloritis haplotypes are intermingled with individuals with other haplotypes. The admixture analysis indicated that the individuals with mitochondrial haplotypes belonging to the early branch do not form a separate cluster, but belong to different 'populations' that are admixed with each other and with other 'populations' classified as *X. mesostena* (Fig. 3.6) indicating that there is gene flow between the groups characterized by different mitochondrial haplotype groups. There is no indication that one of the two haplotype groups found in *X. mesostena* was introduced into that species by introgression, because these haplotype groups were not found in other species, with the exception of *X. franciscoi* and one *X. amphiconus* specimen that have haplotypes that are nested in the major *X. mesostena* haplotype group. The divergences within the Psiloritis haplotype group of *X. mesostena* are shallow. This indicates that this group might have been derived form an isolated population and might have spread more widely only recently. The population structure of land snail species that often consist of many more or less isolated populations (Boato 1988; Goodacre 2002; Schilthuizen & Lombaerts 1994; Ursenbacher *et al.* in press) that sometimes can reach high densities in favourable patches of habitat in conducive to the persistence of such ancestral polymorphisms, because such a population structure increases the effective population size (Slatkin 1991; Wakeley 2000). The Psiloritis haplotype group of *X. mesostena* can be considered a paradigm for one of the causes of the high mitochondrial sequence diversity within land snail species discussed by Thomaz *et al.* (1996), namely long-term persistence of ancient haplotypes resulting from the strongly subdivided

population structure, though this process is certainly not the only cause for the unusually high intraspecific mitochondrial sequence diversity in land snails.

The second case of nonmonophyly of a morphologically delimited species in the mitochondrial gene tree (Fig. 3.2) concerns *X. rhithymna*. In the *cox1* gene tree *X. rhithymna* is paraphyletic with respect to *X. kydonia*. The two species are sister species according to the AFLP tree. The ranges of the two species are separated by more than 40 km (see chapter 1). Thus, dispersal of *X. rhithymna* individuals to the range of *X. kydonia* and hybridization between the two species are rare events at most. This is also confirmed by the Structure analysis of the AFLP data that did not provide any evidence for admixture, neither for the *X. rhithymna* individual with the haplotype that is sister to the *X. kydonia* haplotypes nor for any other *X. rhithymna* individual. A small amount of admixture in one *X. kydonia* individual is not necessarily the result of introgression, but might be due to shared ancestral polymorphisms. Thus, is it more likely that the nonmonophyly of *X. rhithymna* in the mitochondrial gene tree is the result of incomplete lineage sorting rather than of an introgression of a *X. kydonia* haplotype into *X. rhithymna*. This is further supported by the position of the *X. rhithymna* haplotype that is more closely related to the *X. kydonia* haplotypes than to the other *X. rhithymna* haplotypes. A haplotype originating from a *X. kydonia* lineage by introgression might be found in any position relative to the *X. kydonia* haplotypes. The more recent the introgression was, the shallower is the expected coalescence with *X. kydonia* haplotypes. On the other hand, an ancestral polymorphism can be expected with a higher likelihood in a basal position. Thus, a phylogenetically basal position like that of the *X. rhithymna* haplotype in relation to the *X. kydonia* haplotypes indicates incomplete lineage sorting as cause of this case of nonmonophyly in the mitochondrial gene tree.

The last species that is monophyletic in the AFLP tree (Fig. 3.3), but not in the mitochondrial gene tree (Fig. 3.2), is *X. franciscoi*. The small range of this endemic species borders directly on the range of *X. mesostena*. Its *cox1* haplotypes are nested within the main *cox1* haplotype group of *X. mesostena* and are separated from *X. mesostena* haplotypes only by shallow distances. This might indicate introgression. Actually, the Structure analysis of the AFLP data also shows admixture between *X. franciscoi* and a representative of the neighbouring *X. mesostena* population (Fig. 3.6). However, the nonmonophyly of the mitochondrial haplotypes of *X. franciscoi*, the shallow distances between them and *X. mesostena* haplotypes and the admixture of *X. franciscoi* and neighbouring *X. mesostena* can

also be explained by a recent origin of *X. franciscoi* from *X. mesostena* by peripatric speciation. It is difficult to determine the relative roles of incomplete lineage sorting and hybridization in generating nonmonophyly of relatively recently separated species in gene trees.

The shallow divergence of the single haplotype of *X. amphiconus* that is nested in the main clade of *X. mesostena* (Fig. 3.2) might indicate that there is some introgression between *X. mesostena* and the *X. amphiconus*/*siderensis* group. However, according to the Structure analysis of the AFLP data, this *X. amphiconus* individual shows no admixture with *X. mesostena* (Fig. 3.6). Nevertheless, we cannot exclude that there was introgression of mitochondrial DNA from *X. mesostena* into *X. amphiconus*, because maternally inherited DNA like mitochondrial DNA may introgress much more rapidly through prezygotic barriers than biparentally inherited DNA (Chan & Levin 2005; Takahata & Slatkin 1984).

Actually, the Structure analysis of the AFLP data shows that there is admixture between the *X. amphiconus*/*siderensis* group and geographically adjacent easternmost *X. mesostena* individuals (Fig. 3.6). The pattern of increasing admixture with decreasing distance from the distribution area of the *X. amphiconus*/*siderensis* group observed in the easternmost *X. mesostena* would be compatible with the hypothesis that there is introgression of genes from the *X. amphiconus*/*siderensis* group into *X. mesostena*. However, the admixture between the *X. amphiconus*/*siderensis* group and the adjacent *X. mesostena* individuals might have been resulted also from an east-west cline of markers in the eastern populations of the stem species of *X. mesostena* and the *X. amphiconus*/*siderensis* group.

The discrimination of incomplete lineage sorting of ancestral polymorphisms and introgression as causes of nonmonophyly of species in gene trees is difficult (Berthier *et al.* 2006; Donnelly *et al.* 2004; Funk & Omland 2003; Gompert *et al.* 2008; Holder *et al.* 2001; Jakob & Blattner 2006; Joly *et al.* 2009; McGuire *et al.* 2007; Morando *et al.* 2004). Our study showed that the most likely cause of nonmonophyly can be inferred at least in some cases by a combination of several criteria, namely the depth of the coalescences in the gene tree, the geographical distribution of shared genetic markers, and concordance with results of admixture analyses of nuclear multilocus markers. However, all these criteria have limitations. Randomly distributed genetic markers shared with allopatric species with limited dispersal abilities might indicate incomplete lineage sorting. However, the expectation that genetic markers that are concentrated geographically near species boundaries indicate

introgression (e.g., Funk & Omland 2003; McGuire et al. 2007) is not necessarily true, because such a pattern might also be derived from a pre-existing cline in the stem species. Likewise, introgression of mitochondrial DNA cannot be completely excluded, even if there is no evidence for admixture of multilocus markers, because maternally inherited DNA like mitochondrial DNA may introgress much more rapidly through prezygotic barriers than biparentally inherited DNA (Chan & Levin 2005; Takahata & Slatkin 1984). Such shortcomings of individual criteria are ameliorated by using several criteria in an integrative approach.

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CHAPTER 4

DISCORDANT AND CONCORDANT PHYLOGEOGRAPHIC PATTERNS OF MITOCHONDRIAL AND AFLP MARKERS ACROSS THE RANGE OF AN ENDEMIC LAND SNAIL SPECIES ON CRETE

ABSTRACT

We investigated phylogeographic patterns inferred from mitochondrial *cox1* sequences and AFLP markers in the endemic helicoid land snail species *Xerocrassa mesostena* from Crete. The AFLP data revealed a distinct phylogeographic subdivision of the range of *Xerocrassa mesostena* that corresponds at least partly with current barriers. Within the geographical clusters the genetic variation is structured partly by isolation by distance. The variation in the mitochondrial data is also dominated by a subdivision in geographical clusters. However, the mitochondrial haplotype groups correspond only partly with the geographical clusters delimited with the multilocus data. In some cases phylogenetic breaks in the mitochondrial data differ only slightly from the geographic boundaries of the AFLP based clusters. This pattern can be explained by limited dispersal across barriers. However, there are also phylogeographic breaks in the mitochondrial data that do not correspond with patterns in the multilocus data. We corroborate the hypothesis that some of these boundaries represent random phylogenetic breaks by excluding alternative possibilities. Mitochondrial DNA can provide a first insight in the phylogeographic structure of a species. However, the comparison of the phylogeographic patterns inferred from mitochondrial *cox1* sequences and the AFLP markers in *Xerocrassa mesostena* support suggestions that phylogeographic patterns found with single locus markers, especially mitochondrial DNA, might not reflect the phylogeographic structure of a species correctly and should be supplemented by data from multiple independent loci.

INTRODUCTION

The aim of phylogeography (Avise 2000; Avise *et al.* 1987) is the reconstruction of the historical relationships between genealogical units within a species or within a group of closely related species and their distribution areas and the inference of the causes of these relationships. Most phylogeographic studies used mitochondrial DNA sequences as genetic markers (Avise 2000; Avise *et al.* 1987; Zink & Barrowclough 2008). There are several theoretical arguments for using mitochondrial sequences for phylogeographic studies. First, mitochondrial sequences are on average more variable than nuclear coding sequences. Thus, more informative sites for shallow phylogenies can be gathered by sequencing mitochondrial genes than by sequencing nuclear genes using the same amount of effort (Hare 2001). Second, the effective population size of mitochondrial DNA is usually smaller than that of nuclear genes, because it is haploid and maternally inherited in most organisms. Thus, mitochondrial DNA is sorted more rapidly than nuclear loci after a subdivision of a population so that the resulting populations (or species) become reciprocally monophyletic more quickly. Third, there is no recombination in mitochondrial DNA so that hierarchical matrilineal genealogical patterns can be reconstructed.

However, several studies have cautioned against the use of a single genetic marker for reconstructing the phylogeographic history of species because of processes that may cause discordances between the pattern shown by a single locus marker and the phylogeographic history of species (Bensch *et al.* 2006). Single markers may not reflect the phylogeographic history of a species correctly because of natural selection (Boissinot & Boursot 1997; Mishmar *et al.* 2003), hybridization (Barton 2001; Gompert *et al.* 2008; Good *et al.* 2008; Irwin *et al.* 2009; Pidancier *et al.* 2006; Smith & Green 2004) or random phylogeographic breaks (Hoelzer 2001; Irwin 2002; Kuo & Avise 2005).

We investigated whether such processes have affected the phylogeographic pattern inferred from mitochondrial *cox1* sequences in the endemic helicoid land snail species *Xerocrassa mesostena* from Crete by comparing this pattern with that found using multilocus markers. We used amplified fragment length polymorphism (AFLP) as multilocus markers (Vos *et al.* 1995). These have been shown to be powerful tools for investigating the phylogeographic structure of species (Bensch & Åkesson 2005; Despres *et al.* 2002; Milá *et al.* 2010). Land snails are particularly suitable for phylogeographic studies, because species with small dispersal distances are more likely than those with large dispersal distances to show phylogeographic breaks caused by geographic barriers (Avise 2000; Bond *et al.* 2001). However species with small dispersal distances are also more likely to show genealogical

breaks that are not the result of geographic barriers (Hoelzer 2001; Irwin 2002; Kuo & Avise 2005). The radiation of *Xerocrassa* on Crete is thought to have been triggered by sexual selection(Sauer & Hausdorf 2009). This radiation resulted in ten endemic species of which *X. mesostena* is the most widespread one that is missing only in the easternmost part of Crete (Hausdorf & Sauer 2009). Just as the other *Xerocrassa* species, *X. mesostena* is xerophilic and lives in open, dry habitats that are currently available almost throughout Crete.

MATERIAL AND METHODS

Sampling

Xerocrassa mesostena specimens were sampled in July/August and September/October 2004 and September/October 2005. Mitochondrial *cox1* sequences and AFLP data were determined from 95 specimens from 84 localities across Crete covering all known morphotypes.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from tissue samples of the foot preserved in 100% isopropanol following the protocol proposed by Sokolov (2000) as modified by Sauer & Hausdorf (2009). Fragments of the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene were amplified using Polymerase Chain Reaction (PCR) and sequenced as described by in chapter 2.

Sequence Analysis

Forward and reverse sequences were assembled using ChromasPro version 1.33 (Technelysium). The sequences were aligned with the CLUSTAL W algorithm (Thompson *et al.* 1994) as implemented in MEGA version 4.0 (Tamura *et al.* 2007) with the default settings.

AFLP

DNA digestion – Approximately 100 ng genomic DNA were digested with 5 units EcoRI (Fermentas) at 37°C for 1 h followed by a digestion with 5 units of MseI (Fermentas) at 65°C for 1 h.

Adapter ligation – 12.5 pmol of the EcoRI-adapter, 125 pmol of the MseI-adapter and 10 units of T4 DNA ligase and its buffer (GeneCraft) were added to the digestion product and

incubated at 16°C for 8 h. The ligation products were diluted 1:10 with sterile ddH_2O, and stored at -20°C.

Preselective polymerase chain reaction – Preselective PCR was carried out with one selective base on each primer (PA-MseI-C and PA-EcoRI-A, Table 4.1). Five µl of the diluted ligation product were added to 20 µl of the preselective PCR mastermix, consisting of 15.1 µl ddH_2O, 2.5 µl 10x PCR-buffer, 1.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTP (2 mM), 0.15 µl preselective primer mix (50 µM each), and 0.25 µl Taq-DNA polymerase (5U/µl). Preselective PCR conditions were 22 cycles of PCR (94°C for 30 s, 56°C for 30 s, 72°C for 60 s), and a final extension step at 72°C for 5 min. The quality of the preselective PCR was checked on a 1.5% agarose gel. Afterwards the products were diluted 1:20 with sterile H_2O.

Selective PCR – Five primers with two additional bases at the 3' end (Table 4.1) were used for selective amplifications. Six primer combinations (SMseI/SEcoRI^{DYE}) were run: AG/CA^{FAM}, AG/CC^{NED}, AG/GG^{HEX}, TG/CA^{FAM}, TG/CC^{NED} and TG/GG^{HEX}. 5 µl of the diluted preselective PCR product were added to 20 µl of the selective PCR master mix consisting of 15.05 µl ddH_2O, 2.5 µl 10x PCR-buffer, 1.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTP (2 mM), 0.2 µl dye primer mix (0.06 µM labelled selective EcoRI-Primer and 0.6 µM non-labelled selective MseI-Primer), and 0.25 µl Taq DNA polymerase (5U/µl). For the selective amplification a touch down PCR with a temperature decrease of 0.6°C of the annealing temperature each cycle was applied. The program starts with 94°C for 60 s, 65°C for 30 s and 72°C for 60 s followed by 13 cycles of 0.6°C decrease of annealing temperature and 1°C decrease of elongation temperature per cycle and 23 cycles with 94°C for 60 s, 56°C for 30 s and 72°C for 60 s.

Electrophoresis – 1.2 µl of each of the three different primer labelled samples were mixed with 6.2 µl Hi Dye Formamid (Applied Biosystems) and 0.2 µl GS-500 ROX size standard (Applied Biosystems). The samples were denatured at 94°C for 2 min and then cooled down on ice for 4 min. The selective PCR products were electrophoretically separated using pop4-polymer (Applied Biosystems) on an ABI PRISM 3100 (Applied Biosystems) capillary sequencer.

AFLP data scoring – Signal detection was performed with GeneScan version 3.1 software (Applied Biosystems). Fluorescent threshold was set to 50 relative fluorescence units. The signal intensity was normalized with Genotyper version 2.5 software (Applied Biosystems). Fixed fragment categories were created. A presence/absence scoring was conducted between 70 and 322 bases with a threshold set to 50 normalized units. Category spacing was set to 1 base and the category tolerance was adjusted to +/- 0.5 bases.

Table 4.1 Primers and fluorescent dye labels used for AFLP.

Primer	Sequence	5'- labelling
PA-EcoRI-A	5'- GAC TGC GTA CCA ATT CA -3'	None
PA-MseI-C	5'- GAT GAG TCC TGA GTA AC -3'	None
SEcoRI-CA	5'- GAC TGC GTA CCA ATT CA CA -3'	FAM
SEcoRI-CC	5'- GAC TGC GTA CCA ATT CA CC -3'	NED
SEcoRI-GG	5'- GAC TGC GTA CCA ATT CA GG -3'	HEX
SMseI-AG	5'- GAT GAG TCC TGA GTA AC AG -3'	none
SMseI-TG	5'- GAT GAG TCC TGA GTA AC TG -3'	none

Phylogenetic Analyses

Models of sequence evolution for the distance calculations and the maximum-likelihood analysis based on the *cox1* sequences were chosen using ModelTest version 3.7 (Posada & Crandall 1998) based on the Akaike Information Criterion (AIC). The maximum-likelihood analysis was conducted with Treefinder (Jobb 2007; Jobb *et al.* 2004). Individuals of *Xerocrassa subvariegata* and *X. grabusana* were used as an outgroup for the phylogenetic reconstruction. Confidence values for the edges of the maximum-likelihood tree were computed by bootstrapping (100 replications; Felsenstein 1985)

Based on the AFLP data Jaccard distances were calculated with PhyloTools version 1.32 (Buntjer 2001). These distances were used to reconstruct a neighbour-net with SplitsTree4 version 10 (Huson & Bryant 2006).

Test for neutral evolution of the mtDNA

We tested whether the *cox1* sequences are evolving neutrally with the McDonald & Kreitman (1991) test as implemented in DnaSP version 4.10.9 (Rozas *et al.* 2003).

Inferring Phylogeographic Structure based on AFLP data

We used the Bayesian program Structure (Falush *et al.* 2007; Pritchard *et al.* 2000) to investigate the phylogeographic structure of *Xerocrassa mesostena* based on the AFLP data without a priori grouping of individuals into populations. We explored the pattern of admixture by varying the number of groups *K* in the dataset and assigning proportions of each individual to these groupings using Structure version 2.3.1 with the model with admixture.

Following Evanno *et al.* (2005) 20 runs with 10,000 iterations after a burn-in of 10,000 iterations were carried out for each cluster number *K* from 2 to 15. We used the mean estimates of the posterior probabilities of the data for a given cluster number *K* (*L*(*K*)) and the statistic $\Delta K = m(|L(K+1)-2L(K)+L(K-1)|)/s[L(K)]$ proposed by Evanno *et al.* (2005) to estimate the number of clusters *K*. For the *K* determined in that way, a run with a burn-in of 20,000 iterations followed by 100,000 iterations was performed to infer the admixture.

As alternative to Structure, we investigated the phylogeographic structure based on the AFLP data using Gaussian clustering (Fraley & Raftery 1999) as implemented in the program package PRABCLUS version 2.1-1 (Hennig & Hausdorf 2008); this is an add-on package for R (R Development Core Team, 2009) This non-hierarchical clustering method does not depend on the assumption of Hardy-Weinberg equilibrium within populations and provides a decision about the number of meaningful clusters. Gaussian clustering operates on a dataset where the cases are defined by variables of metric scale. Therefore we performed a non-metric multidimensional scaling (Kruskal 1964) based on Jaccard distances.

Identifying Barriers to Gene Flow and Long Distance Dispersal Events

Barriers to gene flow are indicated by high genetic distances across short geographical distances. We calculated geographic distances with ArcGis v.9.3 (ESRI 2008) and used Jaccard distances for the AFLP data and GTR+G distances calculated with PAUP*4.0 beta 10 (Swofford 2002) for the *cox1* sequences. We determined the pairs of individuals that belong to both, the quartile of the highest genetic distances and the quartile of the lowest geographic distances. Barriers to gene flow were visualized by plotting lines between these specimen pairs on a map.

Long distance dispersal events will result in low genetic distances across large geographical distances. Thus, we determined the pairs of individuals that belong to both, the quartile of the lowest genetic distances and the quartile of the highest geographic distances and visualized potential long distance dispersal events by plotting lines between these specimen pairs on a map.

Isolation by Distance and Subdivision into Clusters

We used distance-based redundancy analysis (dbRDA, Legendre & Anderson 1999, McArdle & Anderson 2001) as implemented in DISTML (Anderson 2004) to investigate whether the phylogeographic structure of *X. mesostena* can be better described as isolation by distance (Wright 1943) or as subdivision into clusters. We performed a multivariate multiple

regression analysis of the genetic distance matrices (Jaccard distances based on AFLP data respectively GTR+G distances based on *cox1* sequences) and latitude and longitude as spatial variables to infer how much of the genetic variability can be explained by isolation by distance. *P* values estimated from 9999 permutations of the spatial variables. Then a set of *n*-1 dummy variables (*n* = number of AFLP based clusters or *cox1* haplotype clusters) indicating the genetic cluster affiliation of each individual (a 0/1 table) was constructed. The cluster affiliation of the individuals belonging to the last (*n*th) cluster would be redundant information. The genetic distance matrices were analysed using dbRDA with the set of variables indicating the genetic cluster affiliation of each individual as predictor variable set and with the spatial variables set as covariates to determine the importance of the subdivision into clusters. We used 9999 permutations of the cluster affiliations to estimate *P* values. Additionally, the within cluster variation was analysed separately for the clusters either based on the AFLP data or the *cox1* sequences.

Concordance of genetic clusters with geography

The geographical separation of the AFLP based clusters and *cox1* haplotype groups was assessed using one-way analysis of similarities (ANOSIM) as implemented in PAST v.1.95 (Hammer *et al.* 2001). ANOSIM is a non-parametric test of differences between two or more groups that is based on comparing distances between groups with distances within groups (Clarke 1993). Any distance measure may be used. To quantify the geographical separation of the genetic clusters, Euclidean distances were calculated based on the UTM coordinates of the localities of the examined specimens. Large positive values of the test statistic *R* (up to 1) signify dissimilarity between groups. The significance was computed by 10,000 permutations of group membership. The *P* values were Bonferroni corrected for multiple comparisons.

RESULTS

Mitochondrial Gene Tree and Phylogeographic Structure Based on cox1 Sequences

Separate models for the three codon positions as determined by ModelTest were used for the maximum-likelihood analysis (1. codon positions: TN+G, 2. codon positions HKY+I, 3. codon positions GTR+I+G), because the resulting tree had a lower AIC value than the tree based on a uniform model for the complete dataset. The maximum-likelihood tree of partial *cox1* sequences (634 bp) of 95 *Xerocrassa mesostena* individuals and two outgroup individuals (*Xerocrassa subvariegata and Xerocrassa grabusana*) can be divided into eight major haplotype groups (Fig. 4.1). The deepest split separates haplotypes from individuals

inhabiting a region southwest of the Psiloritis Mountains (clade 4) from all other haplotypes of *X. mesostena*. The haplotype groups are restricted to subregions of Crete, but several of them have overlapping ranges (Fig. 4.2).

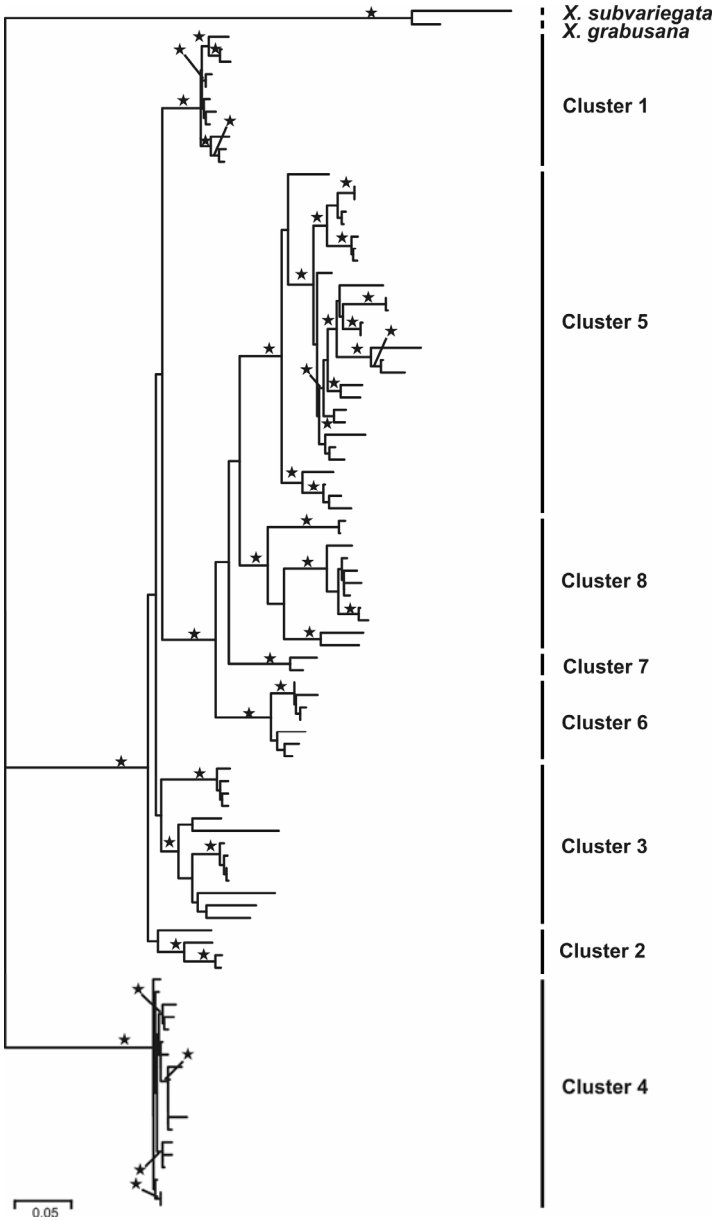

Fig. 4.1 Maximum-likelihood tree based on partial *cox1* sequences of 95 *Xerocrassa mesostena* individuals and *X. subvariegata* and *X. grabusana* as outgroups. Haplotype clades are labelled to the right of the tree. Bootstrap support values larger than 75% are indicated by asterisks above the branches.

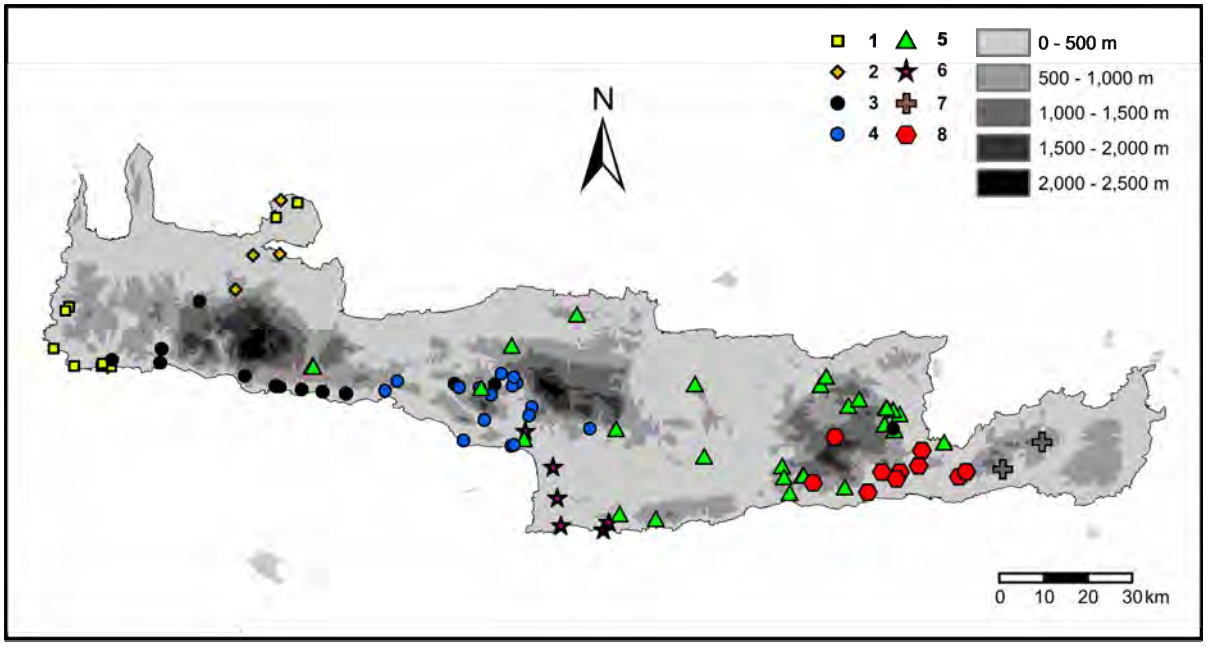

Fig. 4.2 Geographic distribution of the mitochondrial haplotype groups.

The ratio of nonsynonymous to synonymous polymorphisms within *X. mesostena* and within the outgroups was not significantly different from the ratio of nonsynonymous to synonymous polymorphisms fixed between these groups (Fisher's exact test $P = 1.000$). Therefore, the McDonald-Kreitman test result was consistent with neutral evolution of the *cox1* gene.

Network and Phylogeographic Structure based on AFLP data

1328 AFLP fragments of 70-322 bases length resulting from six primer combinations were scored in 95 individuals of *X. mesostena* and one individual of *X. subvariegata* and of *X. grabusana* as outgroups. The main groupings in the neighbor-net (Fig. 4.3) based on these data are in better concordance with geography than the clades in the mitochondrial gene tree (Fig. 4.1). The *X. mesostena* individuals possessing 'Psiloritis group' *cox1* haplotypes are intermingled between individuals with other haplotypes.

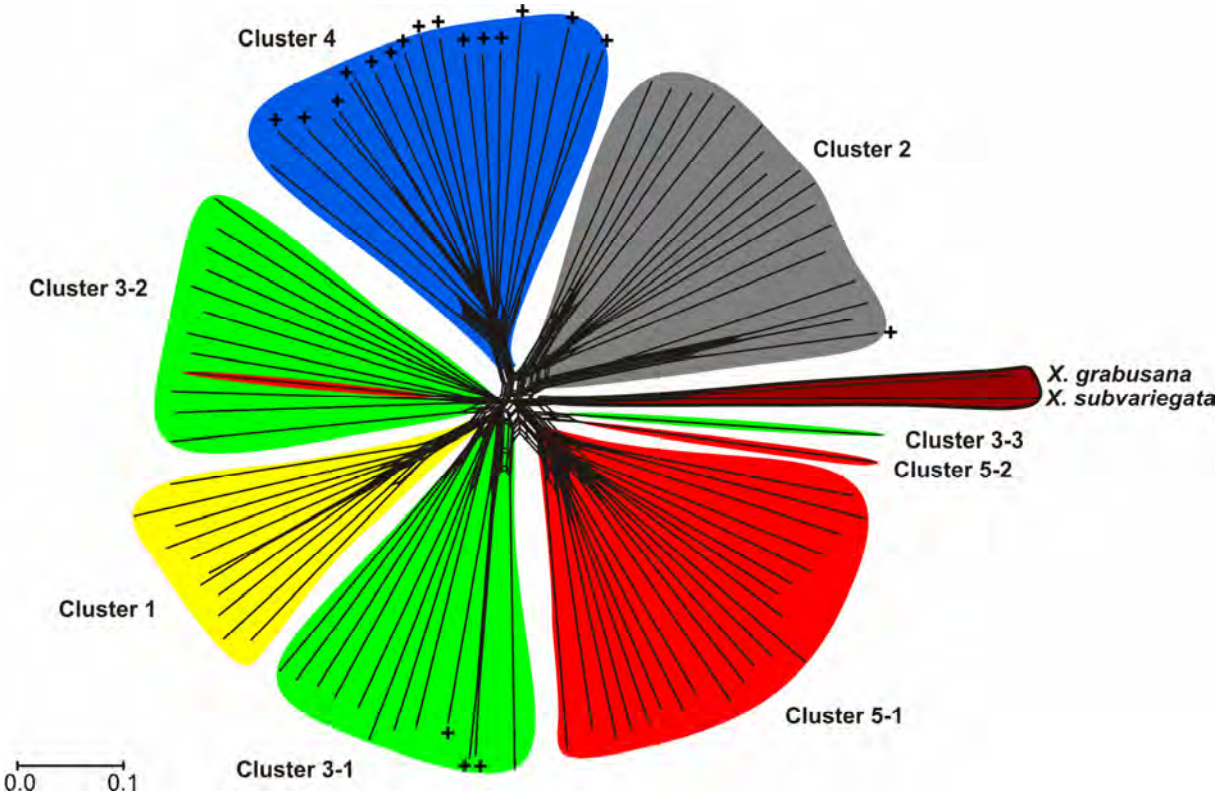

Fig. 4.3 Neighbor-net network based on Jaccard distances between AFLP data of 95 *Xerocrassa mesostena* individuals and *X. subvariegata* and *X. grabusana* as outgroups. The clusters delimited using Structure are shown for comparison. The *X. mesostena* individuals that are characterized by mitochondrial haplotypes of the 'Psiloritis' group are indicated by +.

Both the mean estimates of the posterior probabilities of the data calculated in the admixture analysis with STRUCTURE (Fig. 4.4A) and the statistic ΔK proposed by Evanno *et al.* (2005) to estimate the number of clusters K (Fig. 4.4B) showed a distinct maximum for $K = 5$. The admixture of the individuals of *X. mesostena* found in an extended run with $K = 5$ is displayed in Figure 4.5. The distribution of the five clusters is shown in Figure 4.6. The five clusters are in good accordance with the main groupings in the neighbor-net (Fig.4.3).

The first of the five clusters is distributed from the Akrotiri Peninsula to the north and to the east of the Lefka Mountains. The second cluster is found along the south coast of western Crete and extends into the region southwest of the Psiloritis Mountains. To the east a widespread cluster is spread from southeast of the Lefka Mountains towards an area east of the Dikti Mountains. This cluster is divided in the neighbour-net (Fig. 4.3) into two groups. The one group (cluster 3-1) includes the specimens from southern-central Crete eastwards to the south-western slope of the Dikti Mountains as well as one individual from the region north of the Psiloritis Mountains and one individual from the region east of the Dikti Mountains. The other group (cluster 3-2) includes the specimens from the region between the Lefka and

the Psiloritis Mountains eastwards to the north-western slope of the Dikti Mountains. Finally, there are two more restricted clusters, one in the region southwest of the Psiloritis Mountains and one in the region from the Dikti Mountains eastwards.

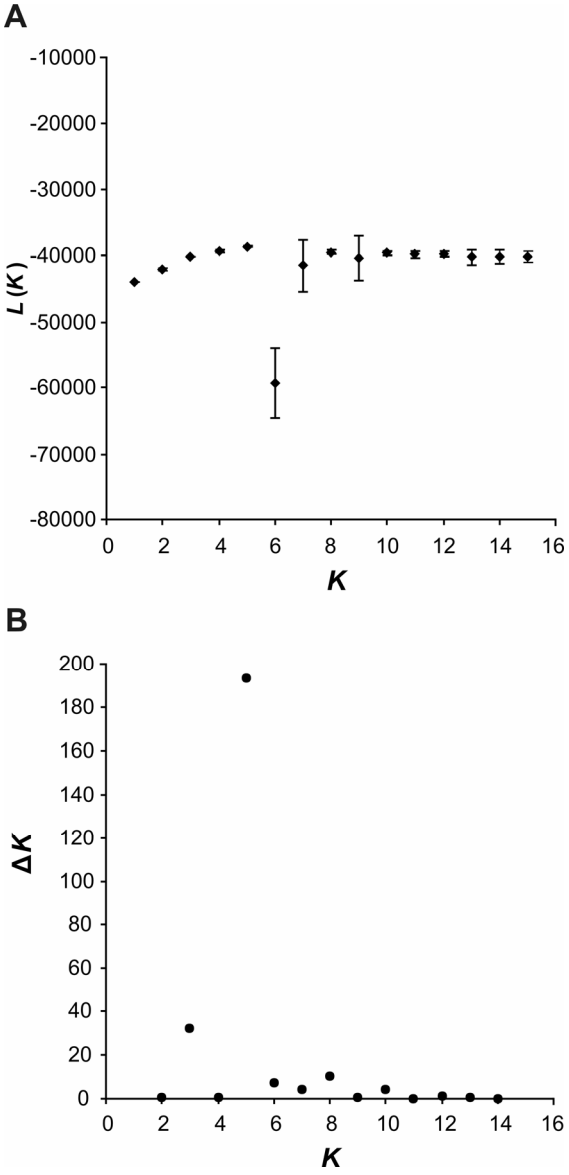

Fig. 4.4 Results of the Structure analysis of the AFLP data of *Xerocrassa mesostena* for different cluster numbers *K* from 2 to 15. A. Mean estimates of the posterior probabilities of the data for a given *K* (±SD). B. Δ*K* (following Evanno *et al*. 2005).

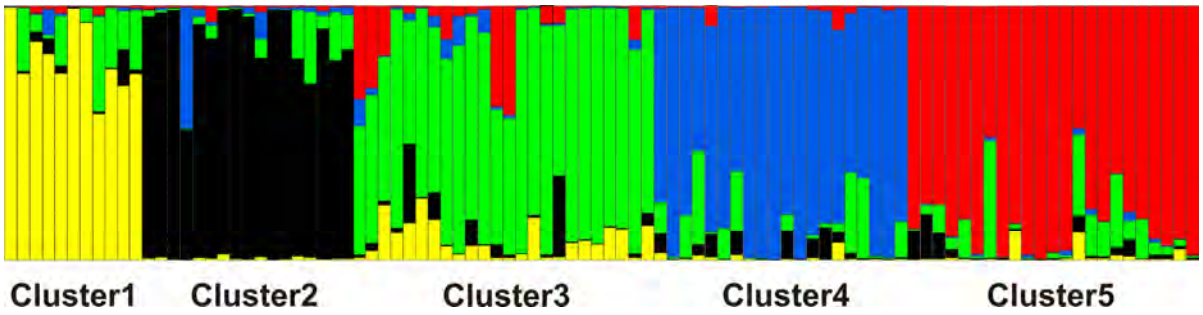

Fig. 4.5 Results of the admixture analysis with the AFLP data of *Xerocrassa mesostena* with Structure for *K*=5. Individuals are sorted into clusters and the clusters are ordered from west to east.

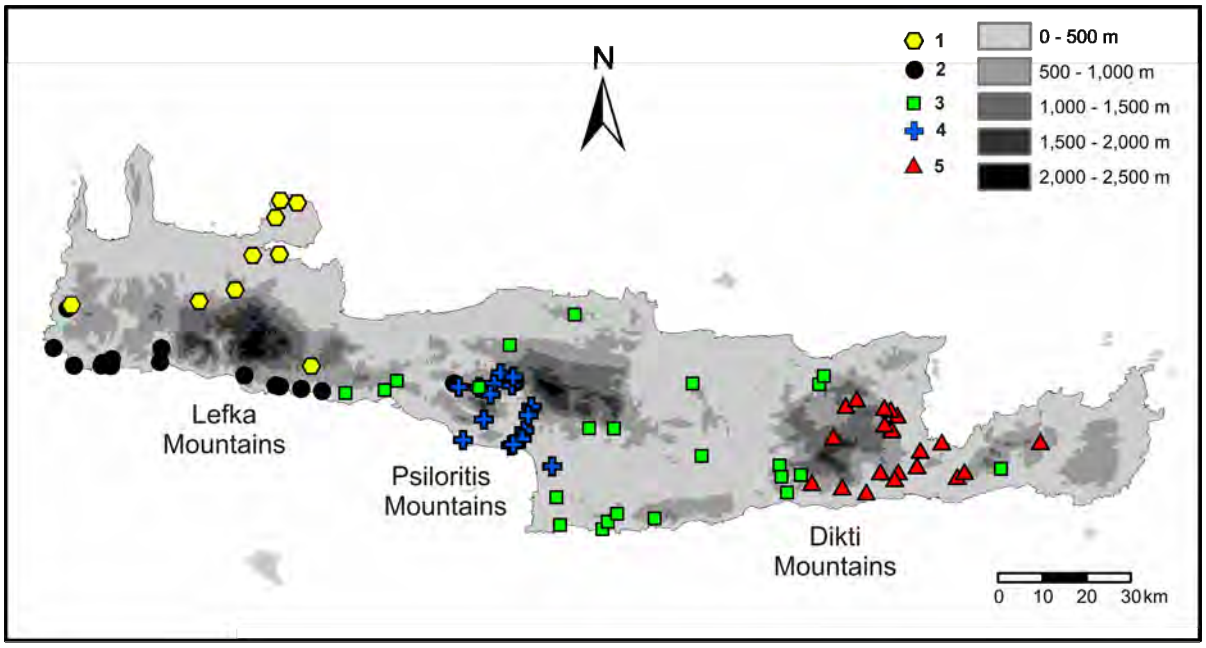

Fig. 4.6 Geographic distribution of the AFLP based clusters delimited using Structure.

The Gaussian clustering based on the AFLP data of the 95 *X. mesostena* specimens resulted also in five clusters (Fig.4.8). The clustering is almost identical with that found in the Structure analysis. Only three individuals from areas where clusters abut were assigned into different clusters. These three individuals have high degrees of admixture according to the Structure analysis. The second largest admixture component is always from the cluster occurring in the adjacent region to which Gaussian clustering assigned the particular individual.

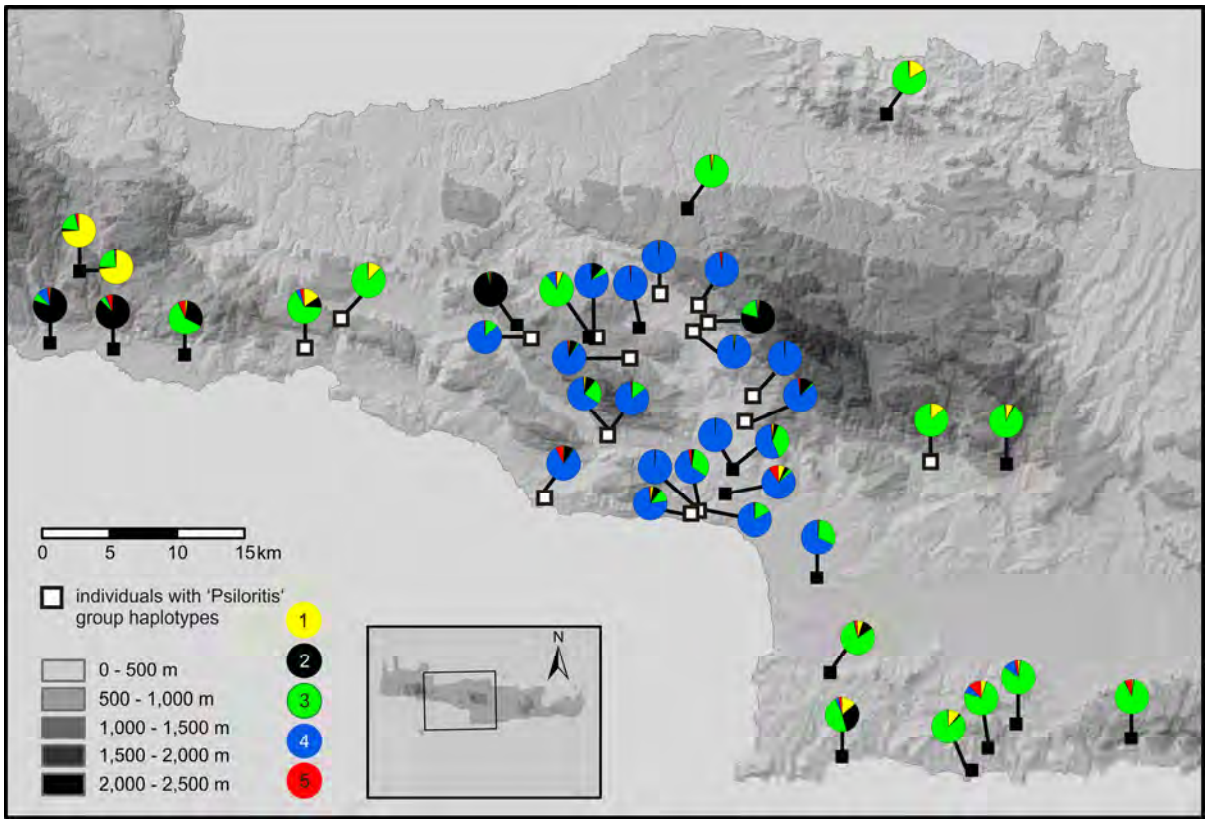

Fig. 4.7 Admixture of the *Xerocrassa mesostena* individuals in the region around the Psiloritis Mountains calculated based on the AFLP data using Structure. Colours refer to the AFLP based clusters shown in Figures 4.2 and 4.6. Individuals possessing 'Psiloritis group' *cox1* haplotypes are indicated by white squares, individuals with other haplotypes by black squares.

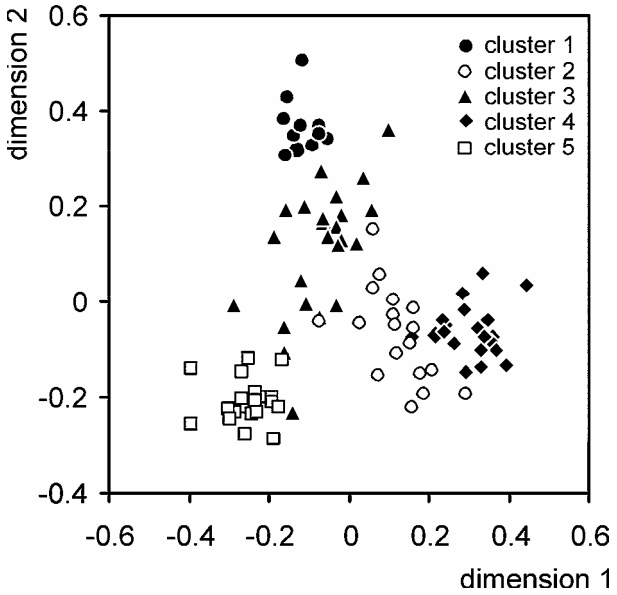

Fig. 4.8 First two dimension of a non-metric multidimensional scaling based on Jaccard distances between the AFLP data of the *Xerocrassa mesostena* individuals. Clusters delimited with Gaussian clustering are shown.

Identifying Barriers to Gene Flow and Long Distance Dispersal Events

High genetic distances based on AFLP data across short geographical distances that indicate barriers to gene flow are concentrated in three regions of Crete: in the Lefka Mountains, southwest of the Psiloritis Mountains and in the Dikti Mountains (Fig. 4.9A). With regard to *cox1* sequences, the only region where high genetic distances are concentrated across short geographical distances is the region southwest of the Psiloritis Mountains (Fig. 4.9B). However, in this region more pairs of individuals show high genetic distances across short geographical distances with regard to the *cox1* sequences than with regard to the AFLP data.

Low genetic distances based on AFLP data across large geographical distances that indicate potential long distance dispersal events have been found between 63 pairs of individuals. Most of these pairs consist of specimens from western Crete and specimens from the region between the Psiloritis and the Dikti Mountains (Fig. 4.10A). Many more pairs of individuals (134) show low genetic distances across large geographical distances with regard to *cox1* sequences (Fig. 4.10B). Most of these pairs of individuals include also one specimen from western Crete. However, the counterparts are not only found in the region between the Psiloritis and the Dikti Mountains, but frequently also in southern central Crete or the region east of the Dikti Mountains.

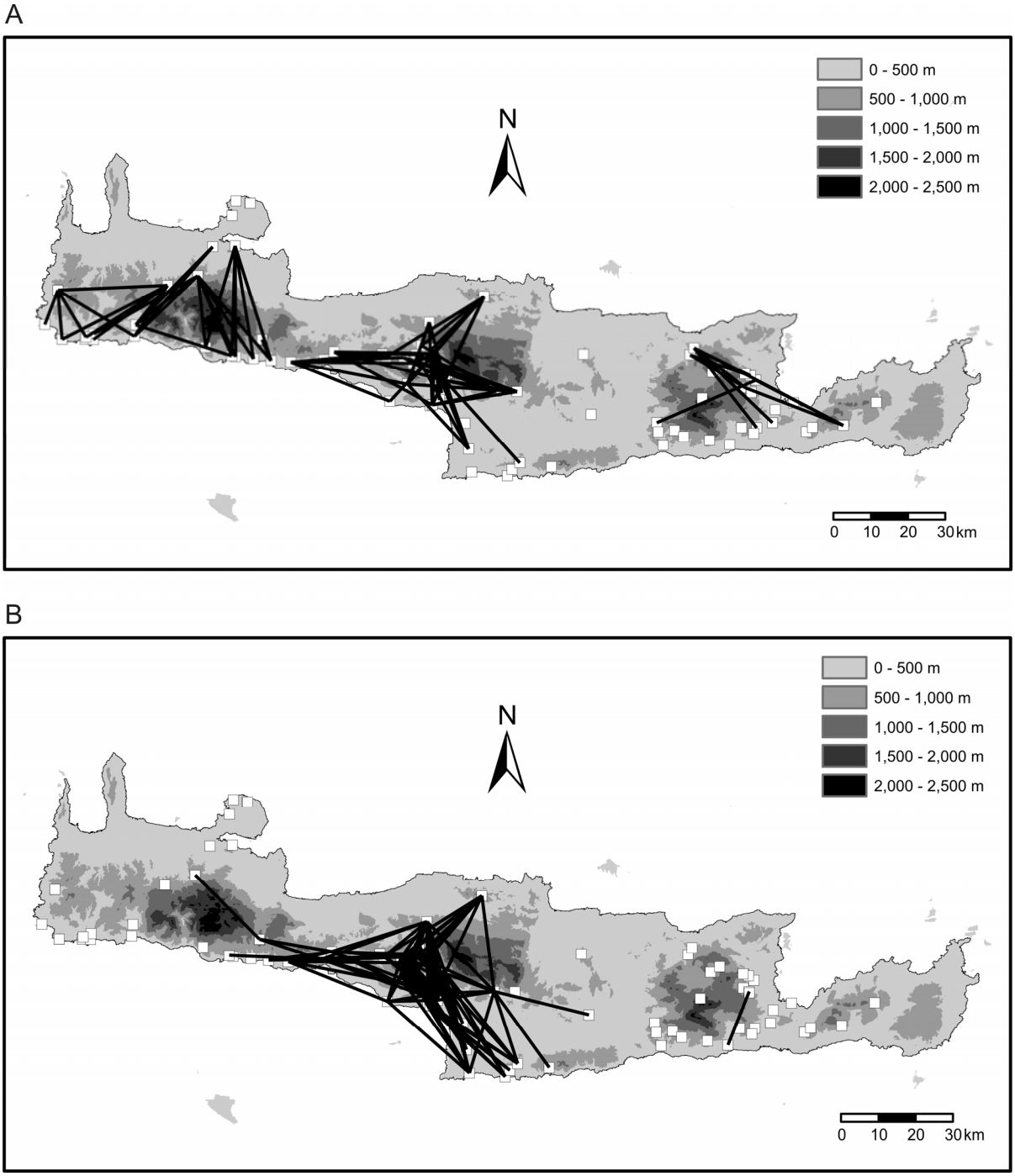

Fig. 4.9 Visualization of geographic barriers by plotting lines between the pairs of individuals that belong to both, the quartile with the highest genetic distances and the quartile with the lowest geographic distances. A. Using Jaccard distances based on AFLP data. B. Using GTR+G distances between *cox1* sequences.

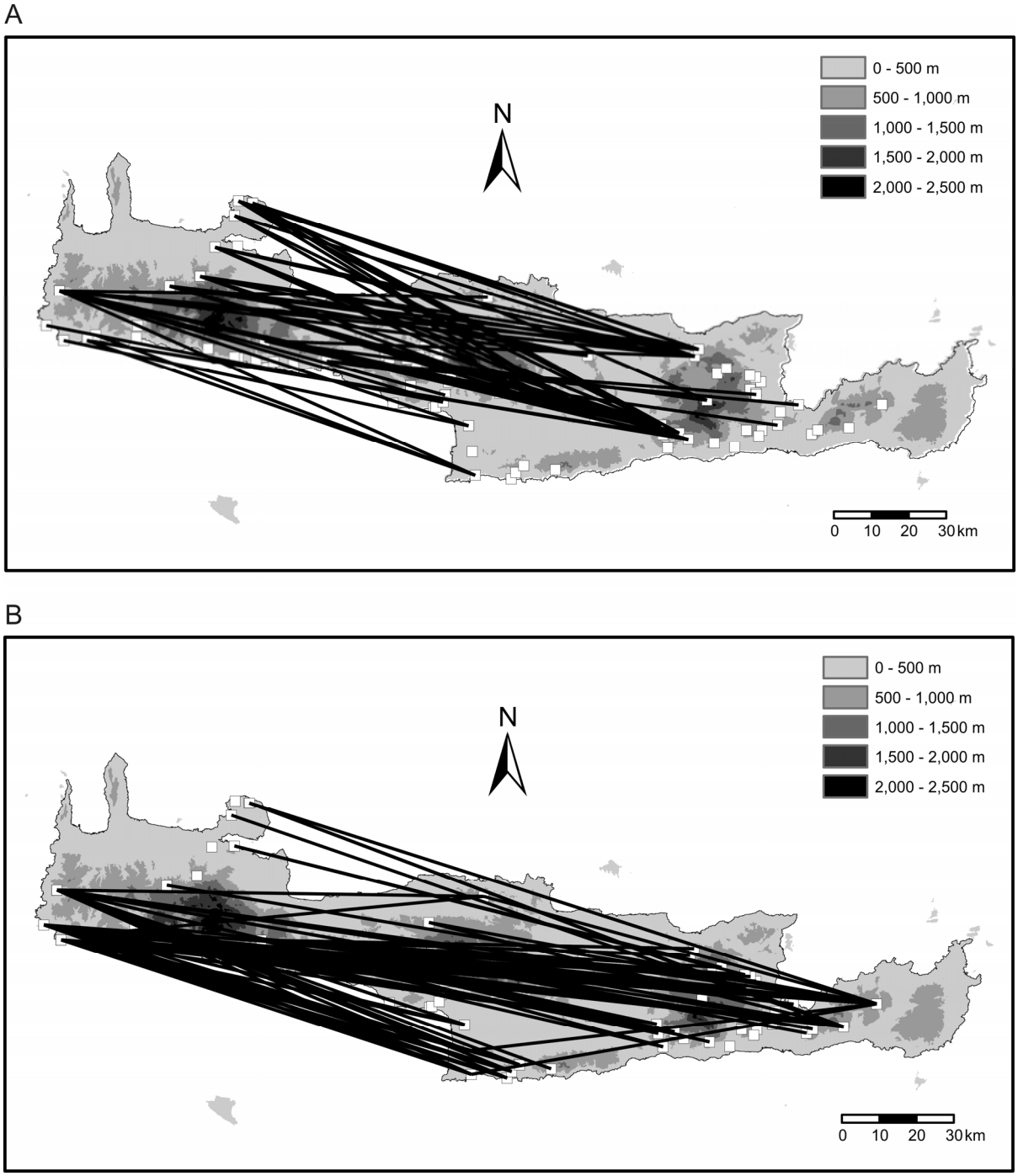

Fig. 4.10 Visualization of potential long distance dispersal events by plotting lines between the pairs of individuals that belong to both, the quartile with the lowest genetic distances and the quartile with the highest geographic distances. A. Using Jaccard distances based on AFLP data. B. Using GTR+G distances between *cox1* sequences.

Isolation by Distance and Subdivision into Clusters

The distance based redundancy analysis with the genetic distance matrices either based on the AFLP data or the *cox1* sequences as response variables and the geographic coordinates of the sample sites as predictor variables indicated that the proportion of variances of the genetic distances explained by isolation by distance was significant, but low (AFLP: proportion of variation explained was 0.105, pseudo-F = 5.371, $p < 0.0001$; *cox1*: proportion of variation explained was 0.130, pseudo-F = 6.85, $p < 0.0001$). A dbRDA with the cluster affiliation of the individuals as predictor variable set and the spatial variables set as covariates showed that the subdivision into clusters explains a higher proportion of genetic variation (AFLP: proportion of variation explained was 0.190, pseudo-F = 5.909, $p < 0.0001$; *cox1*: proportion of variation explained was 0.560, pseudo-F = 18.96, $p < 0.0001$). A dbRDA with the genetic distance matrices as response variables and the geographic coordinates of the sample sites as predictor variables restricted to the individual AFLP based clusters or *cox1* haplotype groups showed that isolation by distance explains an additional proportion of the genetic variation within most of the clusters (Tables 4.2 and 4.3), This proportion is much higher than the proportion of the genetic variation explained by isolation by distance in the complete dataset.

Table 4.2 Results of the dbRDA within the clusters delimited using Structure based on the AFLP data. Significances were obtained by 9,999 permutations.

	n	Pseudo-F	*P*	Proportion of variation explained
Cluster 1	11	0.881	0.723	0.181
Cluster 2	17	1.797	0.002	0.204
Cluster 3	24	1.938	0.000	0.156
Cluster 4	20	1.310	0.022	0.134
Cluster 5	23	2.060	0.000	0.171

Table 4.3 Results of the dbRDA within the *cox1* haplotype groups. Significances were obtained by 9,999 permutations.

	n	Pseudo-F	*P*	Proportion of variation explained
Cluster 1	11	4.181	0.001	0.511
Cluster 2	4	1.165	0.539	0.700
Cluster 3	13	3.305	0.001	0.398
Cluster 4	19	2.351	0.023	0.227
Cluster 5	28	1.934	0.036	0.134
Cluster 6	7	4.075	0.011	0.671
Cluster 8	11	2.373	0.064	0.372

Concordance of genetic clusters with geography

The ANOSIM showed that the clusters determined with Structure based on the AFLP data are geographically well separated (global $R = 0.7568$, $p < 0.0001$). The pairwise comparisons confirmed that all five clusters are significantly separated (Table 4.4). The *cox1* clades are also geographically separated (global $R = 0.5928$, $p < 0.0001$), although the in comparison with the ANOSIM results for the AFLP clusters lower global *R* value indicates that the *cox1* clusters overlapped more geographically. This was also confirmed by the relatively fewer significant pairwise comparisons (Table 4.5).

Table 4.4 Pairwise *R* values calculated using an ANOSIM of the AFLP based clusters delimited using Structure. Significances were obtained by 10,000 permutations and are indicated by asterisks (** < 0.01 and *** < 0.001). The *P* values were Bonferroni corrected for multiple comparisons.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Cluster 1		0.311**	0.760***	0.988***	1.000***
Cluster 2	0.311**		0.729***	0.781***	0.996***
Cluster 3	0.760***	0.729***		0.335***	0.532***
Cluster 4	0.988***	0.781***	0.335***		1.000***
Cluster 5	1.000***	0.996***	0.532***	1.000***	

Table 5 Pairwise *R* values calculated using an ANOSIM of the *cox1* haplotype groups. Significances were obtained by 10,000 permutations and are indicated by asterisks (* < 0.05, ** < 0.01 and *** < 0.001). The *P* values were Bonferroni corrected for multiple comparisons.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Cluster 8
Cluster 1		0.160	0.268	0.989***	0.885***	1.000***	1.000	1.000***
Cluster 2	0.160		0.151	0.998	0.789***	1.000	1.000	1.000*
Cluster 3	0.268	0.151		0.633***	0.598***	0.590**	0.849	0.816***
Cluster 4	0.989***	0.998**	0.633***		0.478***	0.467*	1.000	1.000***
Cluster 5	0.885***	0.789***	0.598***	0.478***		0.242	0.323	0.004
Cluster 6	1.000***	1.000	0.590**	0.467*	0.242		1.000	1.000***
Cluster 7	1.000	1.000	0.849	1.000	0.323	1.000		0.714
Cluster 8	1.000***	1.000**	0.816***	1.000***	0.004	1.000***	0.714	

DISCUSSION

Comparison of phylogeographic patterns found based on AFLP markers and on cox1 sequences

In the current study the 95 examined individuals of the Cretan endemic land snail species *Xerocrassa mesostena* were classified into five clusters in an admixture analysis with Structure based on the AFLP dataset (Fig. 4.5). These clusters form geographically coherent units that are well separated from each other (Fig. 4.6, Table 4.4). The geographical boundaries of the clusters correspond at least partly with current barriers, namely the Lefka, Psiloritis and Dikti Mountains as well as the Messara Plain. The importance of the mountain ranges as geographic barriers is also visualized by plotting pairs of individuals that are separated by large AFLP based genetic distances, but small geographical distances on a map (Fig. 4.9A).

The *cox1* haplotypes of the same 95 *X. mesostena* individuals can be classified in eight clades based on a maximum-likelihood tree (Fig. 4.1). The most basal group, the 'Psiloritis' group, is so deeply separated from the rest of the *X. mesostena* haplotypes (average GTR+G distance to individuals of other clusters was 18.8%) that it might be taken for a cryptic species, if only mitochondrial sequences were available. Most of the *cox1* clades form geographically coherent units (Fig. 4.2), but they are less well separated from each other than the AFLP based clusters (Table 4.5). The distribution of some clades extends across the mountain ranges, especially in western Crete. Accordingly, fewer pairs of individuals that are separated by large *cox1* based genetic distances, but small geographical distances indicate geographic barriers (Fig. 4.9B). Such pairs are mainly found in the region southwest of the Psiloritis Mountains, where the 'Psiloritis' haplotype group encounters other *cox1* haplotype groups.

A comparison of the distribution of the AFLP based clusters and the *cox1* clusters shows that the distribution of the clusters southwest of the Psiloritis Mountains, the clusters east of the Dikti Mountains and the more widespread central clusters is somewhat similar. However, a more detailed comparison shows that the boundaries between the clusters are usually not congruent. This means that a haplotype group is not only found in individuals belonging to the AFLP cluster that corresponds geographically to the haplotype clade, but haplotypes of one group can also be found in individuals belonging to adjacent AFLP clusters. For example, the most divergent 'Psiloritis' haplotype group occurs not only in individuals belonging to the geographically corresponding AFLP cluster, but also in individuals belonging to two additional AFLP clusters (Figs. 4.3, 4.6, 4.7). On the other hand, two other haplotype groups

can also be found in the AFLP cluster that is geographically corresponding to the 'Psiloritis' haplotype group. This is what might be expected from adjacent conspecific metapopulations between which there is some gene flow and this also demonstrates that these haplotype groups do not represent distinct cryptic species despite the large genetic distances separating them.

There are some clearly separated haplotype clades that do not correspond with AFLP clusters. For example, the haplotype clade distributed in southern central Crete (Fig. 4.2, cluster 6) can be found in individuals belonging to two adjacent AFLP clusters. A lack of congruence between the distributions of AFLP clusters and haplotype clades is also obvious in western Crete. There are two AFLP clusters that are separated by the Lefka Mountains and its foothills and that meet at the west coast of Crete (Fig. 4.6, clusters 1 and 2). In contrast, there are three mitochondrial haplotype clades of which two occur both north and south of the Lefka Mountains and its foothills (Fig. 4.2, clusters 1-3). The individuals belonging to the southern AFLP cluster are split into a western and an eastern group based on their *cox1* haplotypes. The individuals belonging to the northern AFLP cluster carry even three different *cox1* haplotype clades two of which occur on the Akrotiri Peninsula. There are no obvious geographic barriers between the individuals carrying the different haplotype clades.

Processes resulting in phylogeographic structure in the multilocus data

The distance based redundancy analyses of the AFLP data as well as the *cox1* sequences showed that the subdivision into clusters explains more of the variance in the genetic data of *X. mesostena* than isolation by distance. Within the clusters the genetic variability is structured by isolation by distance in addition (Tables 4.2 and 4.3). The distinct geographical structuring of the variation in the AFLP multilocus markers (Fig. 4.6) is most likely explained by barriers to gene flow. Actually, several geographical boundaries of the AFLP clusters are coincident with the most prominent current barriers on Crete, the Lefka, Psiloritis and Dikti Mountains and the Messara Plain. However, not all boundaries of the AFLP clusters can be explained by the current barriers. Probably historical barriers contributed to the origin of these clusters.

It is important to consider the time frame for the evolution of the phylogeographic pattern of *X. mesostena* to constrain the nature of these barriers. Unfortunately, there are no estimates for substitution rates for *Xerocrassa*. The only estimate of mitochondrial substitution rates available for Helicellinae is a substitution rate of 0.056 changes per site per million years that has been estimated for 16S rDNA of *Candidula* (Pfenninger *et al.* 2003). For other Hygromiidae (*Leptaxis*) the substitution rate of *cox1* has been estimated as 0.028-0.130

changes per site per million years (van Riel *et al.* 2005). Similar high substitution rates, ca. 0.05 changes per site per million years, were reported for 16S rDNA of other Helicoidea (Chiba 1999; Hayashi & Chiba 2000). The highest pairwise GTR+G distance between *cox1* sequences of individuals of *X. mesostena* in the current study was 23.85 %. Given a substitution rate of 0.05 changes per site per million years, this would indicate an early Pleistocene origin of the deepest splits within *X. mesostena*. Thus, the fragmentation of Crete into several paleoislands from the lower Tortonian (11 Mya) until the late Pliocene (2-3 Mya) (Dermitzakis 1990; Fassoulas 2001; Welter-Schultes 2000a; Welter-Schultes 2000b; Welter-Schultes & Williams 1999) cannot explain the origin of the phylogeographic pattern in *X. mesostena*. Actually, neither the distribution of the AFLP based clusters nor the distribution of the *cox1* haplotype clades are in good concordance with the supposed positions of the paleoislands. For example, the area where the deepest branching *cox1* haplotype clade, the 'Psiloritis' clade, and the corresponding AFLP cluster are located was part on a larger paleoisland in the Neogene. If this phylogeographic group were already present in the Pliocene, it is questionable, why it did not disperse across the paleoisland (e.g., along the coasts). It has also been claimed that the phylogeographic structure of other endemic land snail species, e.g. *Albinaria hippolyti*, has been caused by the fragmentation of Crete into several paleoislands in the Neogene (Douris *et al.* 1998). However, Schilthuizen *et al.* (2004) have shown that *Albinaria hippolyti* probably originated only after the fusion of the paleoislands so that its phylogeographic structure can not be explained by the Neogene fragmentation of Crete. Using Monte Carlo simulations, Hausdorf & Sauer (2009) have shown that the distribution areas of the endemic species of the *Xerocrassa* radiation on Crete do not correspond with the Pliocene paleoislands any better than should be expected by chance. Thus, it is unlikely that this radiation was triggered by the past fragmentation of Crete into several paleoislands. Monte Carlo simulations have also shown that the known distribution areas of all 74 endemic land snail species on Crete are not significantly clustered (Sauer & Hausdorf in press). Thus, the biogeographic data provide no evidence that phylogeographic units or even the complete recent land snail fauna of Crete was shaped by a common sequence of vicariance events like the fragmentation into several paleoislands in the Neogene.

Large parts of Crete were repeatedly forested during more humid periods over the last million years (Bottema 1980; Frenzel *et al.* 1992; Quade *et al.* 1994). Thus, the xerophilous *Xerocrassa* species that occur only in open environments were at least temporarily restricted to woodless patches during these periods. Forest areas may have formed barriers to gene flow

that contributed to the formation of the AFLP clusters and the corresponding *cox1* haplotype clades.

Processes resulting in incongruence between the distribution of mitochondrial haplotype groups and AFLP based clusters

Distinct phylogeographic breaks in a multilocus dataset usually reflect present or past barriers to gene flow. It is unlikely that selection is acting on so many of a number of randomly chosen loci that it determines a distinct phylogeographic pattern shown by multiple markers. It is even more unlikely that many markers show a congruent phylogeographic break by chance. The exact geographic position of a phylogeographic break in a single locus marker caused by a barrier to gene flow may slightly differ from the position of the corresponding break in other markers or in multilocus data because of limited dispersal across the barrier or dispersal after the erosion of the barrier.

Such limited dispersal may explain the slight differences between the mitochondrial haplotype groups of *Xerocrassa mesostena* occurring in the region southwest of the Psiloritis Mountains and east of the Dikti Mountains (Fig. 4.2, clusters 4 and 8) and the corresponding clusters delimited with the multilocus data (Fig. 4.6, clusters 4 and 5). The corresponding clusters were probably caused by the same barriers. The specimens that were not classified in corresponding clusters using the two different datasets (Fig. 4.7) may have been affected by gene flow across these barriers. Dispersal across barriers or the erosion of barriers can result in the transfer and the establishment of mitochondrial haplotypes in populations characterized by a different nuclear background. Each migrating individual can contribute to mitochondrial gene flow, because the land snails examined were hermaphrodites. In contrast, migrating individuals are not likely to drive a noticeable lasting effect on the multilocus pattern characterizing the population where an immigrant arrives, even if this immigrant will propagate successfully, because its multilocus genotype will be disintegrated by recombination. The much higher number of potential long distance dispersal events recognized with the mitochondrial data (Fig. 4.10B) than with the multilocus data (Fig. 4.10A) of the same individuals aids visualization of the different effects of migration on single gene patterns and multilocus patterns. Within clusters, where dispersal is more frequent, gene flow will result in isolation by distance as we have found with both the AFLP data and the mitochondrial sequences (Tables 4.2 and 4.3).

There may also be phylogeographic breaks in a single locus marker that do not correspond with breaks in the multilocus dataset. Such phylogeographic breaks may have

several reasons: (1) they may be due to selection on the locus (Boissinot & Boursot 1997; Mishmar *et al.* 2003); (2) they may result from introgression (Barton 2001; Gompert *et al.* 2008; Good *et al.* 2008; Irwin *et al.* 2009; Pidancier *et al.* 2006; Smith & Green 2004); (3) they may be the result of barriers to gene flow that are so young that most other markers have not yet been sorted (which may become more apparent when a marker with a lower effective population size, like mitochondrial DNA, is studied); (4) in the case of mitochondrial DNA they may be the result of barriers to gene flow that are no longer visible in multilocus data, because strong male-driven gene flow has obscured the phylogeographic patterns in the nuclear genome, but not in the mitochondrial DNA (Yang & Kenagy 2009); (5) they may be a random phylogeographic breaks (Hoelzer 2001; Irwin 2002; Kuo & Avise 2005).

We found such breaks in the distribution of the mitochondrial haplotype groups of *X. mesostena* that do not correspond with the pattern shown by the multilocus data. For example, the distribution of the haplotype group of *X. mesostena* in southern central Crete (Fig. 4.2, cluster 6) does not have a multilocus equivalent, but runs across the boundaries of the AFLP delimited clusters. There is no evidence that the distribution of this haplotype group is the result of selection, because the result of a McDonald-Kreitman test is consistent with the neutral evolution of the investigated *cox1* sequences. It cannot be ascribed to introgression, because this haplotype group is not shared with other *Xerocrassa* species (Sauer & Hausdorf 2009). The distribution of the haplotype group of *X. mesostena* in southern central Crete can also not be ascribed to a very recent barrier to gene flow. Rather the distribution of this haplotype group extends across a current barrier, the Messara Plain, that is the southern border for 'Psiloritis' haplotype group (Fig. 4.2, cluster 4) and the corresponding AFLP based cluster (Fig. 4.6, cluster 4). The lack of a multilocus based cluster corresponding to the *cox1* haplotype group cannot be explained by a homogenization of the nuclear markers as a result of male dispersal, because *X. mesostena* is a hermaphrodite in which each individual contributes to the transmission of mitochondria. Thus, it seems that the haplotype group of *X. mesostena* in southern central Crete resulted from a random phylogeographic break. Computer simulations have shown that such random phylogenetic breaks in the distribution of single locus markers have to be expected and that they originate especially easily in species with low dispersal abilities like land snails (Arter 1990; Schilthuizen & Lombaerts 1994) even when they are continuously distributed and even when there are no barriers to gene flow (Hoelzer 2001; Irwin 2002; Kuo & Avise 2005). Nevertheless, phylogenetic breaks in the distribution of single locus markers that do not correspond with current barriers to gene flow have been ascribed with few exceptions (e.g., Irwin *et al.* 2005) to past barriers to gene flow.

If there were past barriers to gene flow, other markers should also show a phylogeographic break in that region. This is not the case for the haplotype group of *X. mesostena* in southern central Crete as we could show with the help of the AFLP data.

Conclusions

An analysis of AFLP data revealed a distinct phylogeographic subdivision of the range of the endemic land snail *Xerocrassa mesostena* from Crete that corresponds at least partly with current barriers. The distribution of mitochondrial haplotype groups agrees only to a small extent with the phylogeographic pattern of the multilocus markers. The conclusion of the current study is that this was the result of dispersal across barriers and of random phylogeographic breaks. The frequent cases in which mitochondrial haplotype groups were found in different nuclear backgrounds in *X. mesostena* support the caution by several authors against using single genetic marker for reconstructing the phylogeographic history of species (Bensch *et al.* 2006). Conclusions about the phylogeographic structure should not be based on single genetic markers alone. This is especially important if decisions, e.g. concerning conservation issues like breeding programs or re-introductions, will be based on the phylogeographic structure. Obviously, the strongest possible conclusion, namely that two groups characterized by different haplotype clades represent separate species should also be based on multilocus data. Molecular taxonomy (Hebert *et al.* 2003; Tautz *et al.* 2003) based on mitochondrial DNA would have resulted in a splitting of *Xerocrassa mesostena* into several 'species' that are neither isolated from each other nor reflect the phylogeographic structure revealed by the multilocus data.

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CHAPTER 5

SEXUAL SELECTION IS INVOLVED IN SPECIATION IN A LAND SNAIL RADIATION ON CRETE

ABSTRACT

We investigated the importance of sexual selection in facilitating speciation in a land snail radiation on Crete. We used differences in the genitalia of the Cretan *Xerocrassa* species as potential indices of sexual selection. First, we rejected the hypothesis that differences in the genitalia of the *Xerocrassa* species can be explained by genetic drift using coalescent simulations based on a mitochondrial gene tree. Second, we showed that there is no evidence for the hypothesis that the differences in the genitalia can be explained by natural selection against hybrids under the assumption that this is more likely in geographically overlapping species pairs and clades. Thirdly, we showed that there is a positive scaling between male spermatophore producing organs and female spermatophore receiving organs indicating sexual co-evolution. The spermatophore enables the sperm to escape from the female gametolytic organ. Thus, the co-evolution might be a consequence of sexual conflict or cryptic female choice. Finally, we showed that the evolution of differences in the length of the flagellum that forms the tail of the spermatophore is concentrated towards the tips of the tree indicating that it is involved in speciation. If speciation is facilitated by sexual selection, niches may remain conserved and non-adaptive radiation may result.

INTRODUCTION

Ever since Darwin (1871), it has been suggested that sexual selection might increase the rate of reproductive divergence between populations thereby driving speciation and increasing diversity (Boul *et al.* 2007; Carson 1997; Dominey 1984; Gavrilets 2000; Gray & Cade 2000; Lande 1981; Masta & Maddison 2002; Panhuis *et al.* 2001; Price 1998; Ritchie 2007; Schluter & Price 1993; West-Eberhard 1983). There are few experimental studies that support this hypothesis (Martin & Hosken 2003; Rice & Hostert 1993; Uy & Borgia 2000). The hypothesis received more support from comparative studies that found correlations between species diversity and indices of sexual selection (Arnqvist *et al.* 2000; Barraclough *et al.* 1995; Katzourakis *et al.* 2001; Møller & Cuervo 1998; Owens *et al.* 1999; Seddon *et al.* 2008; Stuart-Fox & Owens 2003). However, the selection of the taxa in these studies is strongly biased towards birds and insects and similar comparative studies of mammals, butterflies, and spiders (Gage *et al.* 2002; Isaac *et al.* 2005) and even birds (Morrow *et al.* 2003) did not find significant correlations between species diversity and indices of sexual selection. Thus, the generality of the importance of sexual selection in speciation and radiation is still an open question.

We investigated whether sexual selection was associated with the radiation of the hermaphroditic land snail genus *Xerocrassa* on Crete (Hausdorf & Sauer 2009). There are eleven *Xerocrassa* species on Crete, ten of which are endemic. The *Xerocrassa* species are xerophilic and live in open, dry habitats. During the summer they aestivate under stones and bushes or, more rarely, attached to the vegetation. At the beginning of the rainy season at the end of September or the beginning of October they became active and mate. At the end of the winter the adults usually die. The *Xerocrassa* species feed on decaying plants. Differential adaptation of the Cretan species to different habitats could not be ascertained. Thus, the *Xerocrassa* radiation might be a non-adaptive radiation as has also been suggested for other land snail radiations on Crete (Gittenberger 1991; Parmakelis *et al.* 2005). Most Cretan *Xerocrassa* species can be distinguished by characters of the genitalia. Differences in the genitalia are often a product of sexual selection (Arnqvist 1998; Eberhard 1985; 2001; Hosken & Stockley 2004). Some recent studies indicate that the evolution of the genitalia is also strongly influenced by sexual selection and male-female counter-adaptation in hermaphroditic gastropods (Anthes *et al.* 2008; Beese *et al.* 2009; Beese *et al.* 2006; Koene & Schulenburg 2005). The lack of adaptation to different niches and the fact that several of the

Cretan *Xerocrassa* species can be distinguished only by characters of the genitalia implies that sexual selection might have driven the *Xerocrassa* radiation on Crete.

The genitalia of *Xerocrassa* are shown in Figure 5.1. Mating lasts 1–2 hours. During mating the sperm are transported from the vesicula seminalis in the hermaphroditic duct, where it is stored, through the spermoviduct and the vas deferens into the epiphallus.

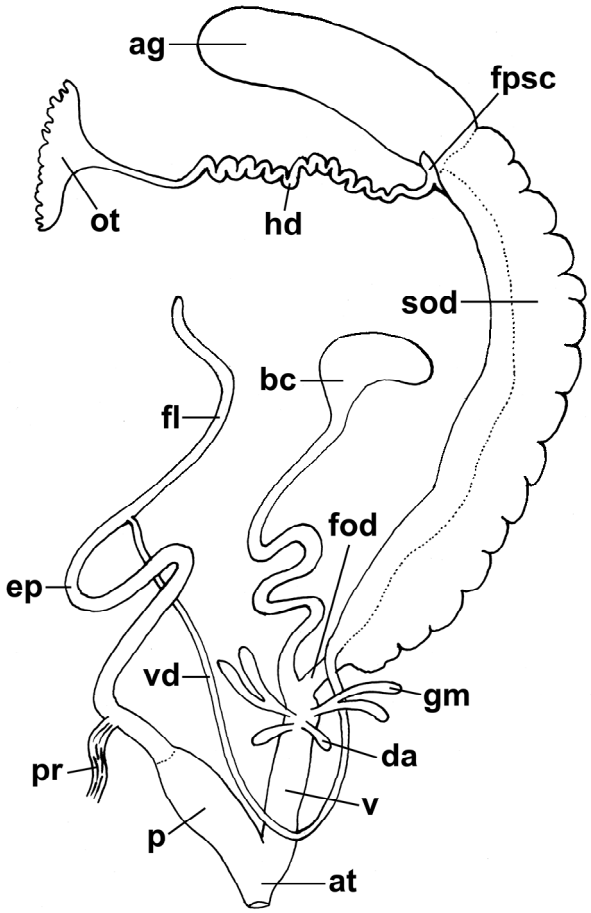

Fig. 5.1 Schematic representation of the genitalia of *Xerocrassa*; ag: albumen gland, at: genital atrium, bc: bursa copulatrix, da: vestigial sacs of the dart apparatus, ep: epiphallus, fl: flagellum, fod: free oviduct, fpsc: fertilization pouch-spermatheca complex, gm: mucus glands of the dart apparatus, hd: hermaphroditic duct, ot: ovotestis, p: penis, pr: penis retractor, sod: spermoviduct, v: vagina, vd: vas deferens.

The epiphallus forms the broader anterior part of the spermatophore (Fig. 5.2) containing most of the sperm. The narrow tail of the spermatophore is formed by the blind ending flagellum. Both, sperm container and tail are furnished with hook-like structures that point towards the anterior end so that they impede and delay the transfer of the spermatophore. Copulation is reciprocal and after simultaneous intromission of the penis into the vagina of the mating partner each snail transfers the spermatophore into the partner's bursa copulatrix,

the female gametolytic organ. In the bursa copulatrix the spermatophore of the mating partner with the vast majority of sperm (99.98% in *Cornu aspersum*; Rogers and Chase 2001) is digested. Sperm have to actively swim out via the tail of the spermatophore to avoid digestion (Lind 1973).

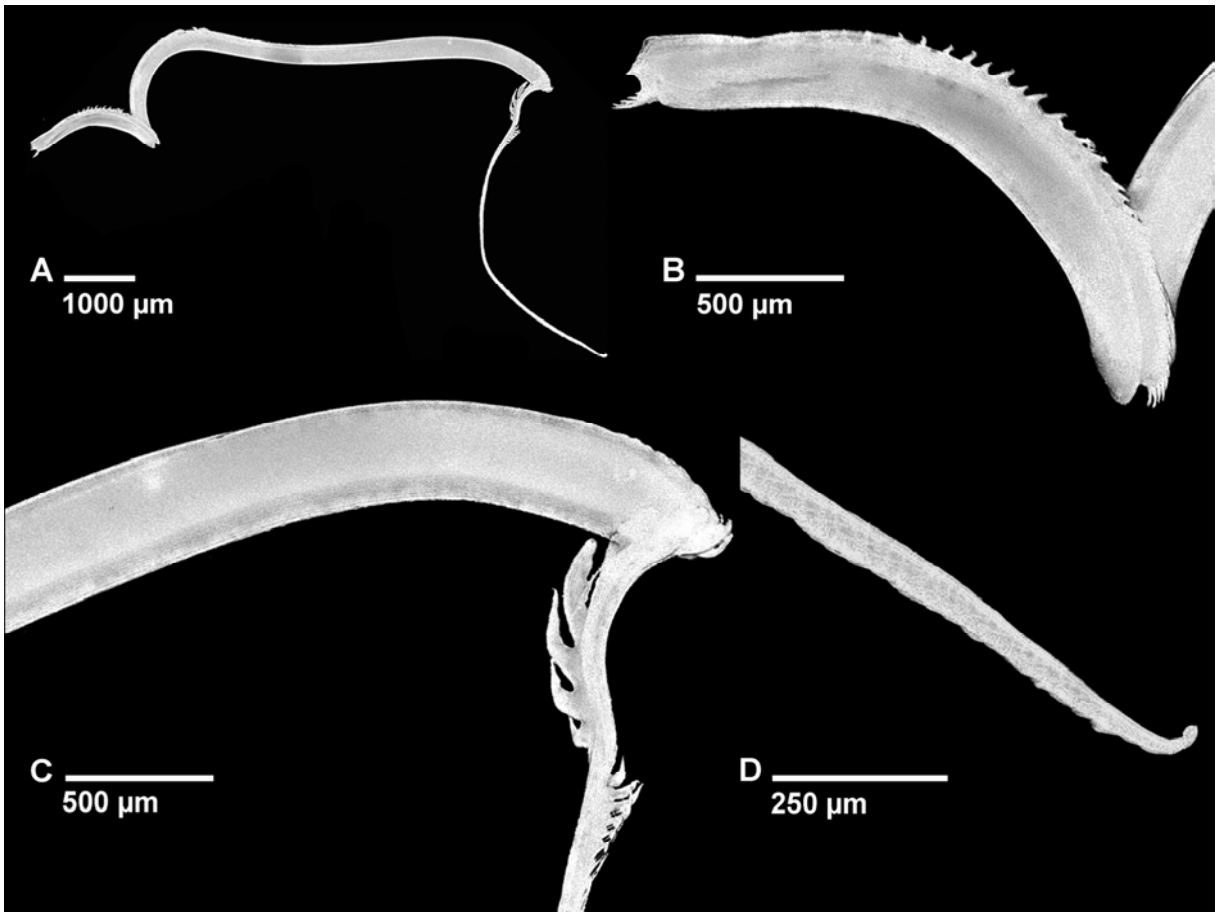

Fig. 5.2 Spermatophore of *Xerocrassa mesostena* from Xeniakos (Crete, Nomos Iraklion). (A) Complete spermatophore. (B) Anterior end. (C) Transition from anterior part to tail. (D) Posterior end of the tail.

Sperm are most successful at reaching the spermathecae when the tail of the spermatophore is protruding into the vagina. Thus, a lengthening of the flagellum might increase paternity success. Koene & Schulenburg (2005) found correlations between the length of the flagellum and the spermatophore receiving organ in helicoid land snails indicating co-evolution probably as a result of counter-adaptation between male and female reproductive organs that should secure control over fertilization. Moreover, most helicoid land snails possess a dart apparatus. In *Cornu aspersum* it has been shown that the donor of the spermatophore can influence the partner's bursa copulatrix with an allohormone from the mucus glands of the dart apparatus to enhance paternity (Chase & Blanchard 2006; Koene &

Chase 1998). The allohormone reconfigures the spermatophore receiving organ of the mating partner in such a manner as to allow more of the donated sperm to escape digestion, precede to the spermathecal storage sacs and fertilize eggs. Most helicoid land snails can transfer the mucus produced by the glands of the dart apparatus with a calcareous dart into the hemolymph of the mating partner. The *Xerocrassa* species possess a vestigial dart apparatus with glands, but without darts. It is not clear whether the snails apply the substance produced by the mucus glands onto the body surface or directly onto the inner surface of the copulatory organs of the partner during mating.

We investigated whether the differences in the genitalia of the Cretan *Xerocrassa* species are the result of sexual selection and whether the changes in the genitalia were associated with speciation. First, we tried to exclude two alternative causes of changes in the genitalia, namely genetic drift and natural selection against hybrids. We tested whether differences in the genitalia of the *Xerocrassa* species can be explained by genetic drift using coalescent simulations based on a mitochondrial gene tree. We tested whether the differences in the genitalia can be explained by natural selection against hybrids under the assumption that this is more likely in geographically overlapping species pairs and clades. Then we investigated whether changes in male spermatophore producing organs and female spermatophore receiving organs are correlated indicating sexual co-evolution. Finally, we investigated whether the changes in the genitalia facilitated speciation using randomisations of phylogenetic independent contrasts across the species tree.

MATERIALS AND METHODS

Sampling

Xerocrassa specimens were sampled at about 500 localities across Crete in July/August and September/October 2004 and September/October 2005. Mitochondrial cytochrom oxidase subunit 1 (*cox1*/COI) sequences were determined of 122 *Xerocrassa* specimens covering all eleven species distinguished by Hausdorf & Sauer (2009) and all regions of Crete and two *Trochoidea* species used as outgroups.

Morphological data

Measurements of the shell and the parts of the genitalia of the Cretan *Xerocrassa* species were taken from Hausdorf and Sauer (in press). In addition, the bursa copulatrix has been measured in a subsample (n=63). The small '*gradilis*' form of *Xerocrassa cretica* has not been

considered in the calculations, because its distribution on Crete is restricted to a few square kilometres whereas the typical large form is distributed across the whole island.

Shell measurements were taken from digital photographs using the program analySIS Pro v3.2 (Olympus Soft Imaging Solutions) or with an ocular micrometer. The measurements of parts of the genitalia were taken with an ocular micrometer and were usually repeated once. Penis, epiphallus, flagellum, vagina and bursa copulatrix (Fig. 5.1) were used for the analyses, because they are copulatory organs or organs that are involved in spermatophore production or spermatophore uptake and might be affected by sexual selection and because species specific differences were observed in these parts (Hausdorf & Sauer 2009).

Body size has been measured as shell volume calculated as the mean of the volume of a cone and of a cylinder based on the small diameter of the shell and shell height (1/2 x (1/4 π x small diameter^2 x height + 1/12 π x small diameter^2 x height)), the best approximation for shell volume according to J. Heller (pers. comm.).

DNA extraction

Usually, total genomic DNA was extracted from tissue samples of the foot preserved in 100% isopropanol following the protocol proposed by Sokolov (2000) with slight modifications as described in chapter 2.

DNA amplification and sequencing

Fragments of the cytochrom oxidase subunit 1 (*cox1*) gene were amplified using Polymerase Chain Reaction (PCR) with the following primers LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TACTTCAGGGTGACCAAAAAATCA-3' (modified from Folmer *et al.* 1994) . Amplifications were performed in 25 µl volumes containing 2.5 µl 10x amplification buffer (peqlab), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (peqlab), 1µl of each primer (10 pmol), 1.5 units Taq DNA polymerase (peqlab), 2.5 µl enhancer solution P (peqlab) and template DNA (usually 2.5 µl undiluted DNA extract). The *cox1* fragments were amplified under the following conditions: an initial denaturing at 94°C for 2 min, 35 cycles of PCR (94°C for 30 s, 50°C for 30 s, 72°C for 30 s), and an final extension at 72°C for 5 min. A negative control (no template) was included in each amplification run. The PCR products were purified using QIAquick PCR purification Kit (QIAGEN). Both strands of the amplified fragments were

directly cycle-sequenced using the amplification primers and a DNA Sequencing Kit (Applied Biosystems) and electrophoresed with an automated DNA sequencer.

Sequence analyses

Forward and reverse sequences were assembled using ChromasPro version 1.33 (Technelysium). The sequences were aligned with the CLUSTAL W algorithm (Thompson *et al.* 1994) as implemented in MEGA version 4.0 (Tamura *et al.* 2007) with the default settings. The sequences analyzed in this chapter have been deposited in GenBank under the accession numbers FJ627054-FJ627177.

Phylogenetic analyses

Models of sequence evolution for the maximum likelihood analyses were chosen using ModelTest version 3.7 (Posada & Crandall 1998) based on the Akaike Information Criterion. Maximum likelihood analyses were conducted with Treefinder (Jobb 2007; Jobb *et al.* 2004). Confidence values were computed by bootstrapping (100 replications; Felsenstein 1985).

An ultrametric species tree is necessary for some of the following analyses for which relative datings of the speciation events are required. The species tree has been constructed from the *cox1* gene tree using a modification of the shallowest divergence clustering method (Maddison & Knowles 2006) that is based on the observation that the order of interspecific coalescences provides a high probability of consistency with the actual species history (Takahata 1989). We determined the shallowest interspecific divergences in an ultrametric gene tree to estimate the order of interspecific coalescences and to get relative datings of these events. The ultrametric tree has been constructed using the penalized likelihood method with the truncated Newton algorithm implemented in r8s version 1.71 (Sanderson 2002). Zero-length branches had to be collapsed prior to the estimation of the ultrametric tree with r8s. The deepest node has been arbitrarily calibrated with 1.

Test for differentiation by genetic drift

We used the approach proposed by Masta & Maddison (2002) to test whether the divergence of traits can be explained by genetic drift or whether it must have been accelerated by selection. The probability of fixation of phenotypic traits under genetic drift is estimated using coalescent based simulations of neutrally evolving mitochondrial haplotypes. For this test we did not consider *Xerocrassa cretica* and the species pair *Xerocrassa subvariegata* –

Xerocrassa species 1. These taxa diverged much earlier than the other species (Fig. 5.3, 5.4) so that they do not provide power in distinguishing between drift and selectively driven divergence, because species that diverged more than $4N$ generations (where N is the population size of each sister taxon) ago are expected to be monophyletic with high probability (Neigel & Avise 1986) irrespective of whether selection was involved. All other Cretan *Xerocrassa* diverged in a comparatively short period of time. All Cretan *Xerocrassa* species except the species pair *Xerocrassa amphiconus – Xerocrassa siderensis* that cannot be distinguished by genital characters show fixed differences in genital characters (Hausdorf & Sauer 2009). Thus, we considered the species pair *Xerocrassa amphiconus – Xerocrassa siderensis* to be one unit for this test.

The method assumes that nuclear genes code for the phenotypic characters of interest and tests whether the fixation of phenotypes is likely to occur under the assumption of genetic drift. The approach consists of five steps: (i) it is shown that the lack of monophyly of the morphologically defined species units in the mitochondrial gene tree is consistent with a pattern of incomplete lineage sorting and not the result of a poor resolution of the mitochondrial gene tree, (ii) it is shown that the used *cox1* haplotypes evolve neutrally, (iii) a measure of incomplete lineage sorting among species, namely s of Slatkin & Maddison (1989), is calculated for the reconstructed mitochondrial gene tree, (iv) this s is compared against s values from simulated gene trees assuming a star phylogeny to estimate time since divergence, and (v) nuclear gene trees in populations with the estimated divergence times are simulated to determine the probability of fixation of differences in nuclear-encoded phenotypic characters under the assumption of neutrality.

To show that the lack of monophyly of the morphologically defined species in the mitochondrial gene tree is not the result of a poor resolution of the mitochondrial gene tree, we calculated the maximum-likelihood tree under the constraint that the sequences of each recognized species form a clade using the ‘resolve multifurcations’ option of Treefinder. Then we investigated whether this tree can be rejected in comparison with the unconstrained maximum-likelihood tree by applying the approximately unbiased test (Shimodaira 2002) implemented in Treefinder.

Second, we used the McDonald & Kreitman (1991) test implemented in DnaSP version 4.10.9 (Rozas *et al.* 2003) to determine whether the *cox1* haplotypes evolve neutrally. We compared the ratio of nonsynonymous to synonymous polymorphisms in *cox1* within the seven recently diverged Cretan *Xerocrassa* species used for the genetic drift analysis and

within the three most basal *Xerocrassa* species (*Xerocrassa cretica*, *Xerocrassa subvariegata*, *Xerocrassa* species 1) with the ratio of nonsynonymous to synonymous polymorphisms fixed between these groups. These two ratios should be the same if the gene is evolving in accordance with the neutral theory of Kimura (1983).

Thirdly, an imaginary character state was assigned to each mitochondrial haplotype representing the species unit to which it belongs. The number of parsimony steps *s* in this imaginary character in the mitochondrial gene tree was calculated to assess incompleteness of lineage sorting among the seven species units that show fixed differences in genital characters. Larger *s* values indicate greater levels of incomplete lineage sorting and suggest a more recent divergence of species.

Fourthly, gene trees were simulated to estimate the upper 95% confidence limit for the number of generations since population divergence that would be expected to give the observed *s* value for the mitochondrial gene tree. 10,000 gene trees were generated with the program MESQUITE (Maddison & Maddison 2006) with an effective population size N_e of each species = 10,000 (probably lower than would be realistic for the widespread species, but all calculations are scaled by N_e). Generations since isolation (branch length of the population tree) were estimated, conservatively, as the greatest time that would yield at least a 5% probability of producing a gene tree with an *s* as high as or higher than that observed (the longer the time, the lower the expected *s*).

Finally, the estimated lengths of branches in the species tree were divided by two (equivalent to multiplying the population size by 2) to consider nuclear genes with two times the population size of mitochondrial genes (because the examined snails are hermaphrodites and, thus, all individuals of a population can pass mitochondrial DNA on to the next generation). Coalescent simulations with these branch lengths were run to estimate the probability of complete fixation of genital differences (s = 6 for seven species units) by genetic drift.

Test of effects of natural selection against hybrids

There are allopatric species that are widely separated, but nevertheless differ distinctly in their genitalia. Thus, differences in the genitalia cannot be exclusively the result of natural selection against hybrids. Nevertheless, natural selection against hybrids, i.e. reinforcement, might contribute to the observed differences in the genitalia. We would expect effects of natural selection against hybrids especially in areas where the ranges of two species overlap

so that there is an increased chance of hybridization. We investigated the importance of natural selection against hybrids in creating differences in the genitalia by testing whether the differences are larger between species with geographically overlapping ranges than between species that are not in contact or have only slightly overlapping ranges. Following Barraclough *et al.* (1999), we test the prediction that the differences are larger between co-occurring species by randomising phylogenetic independent contrasts among nodes, holding the degree of geographical overlap fixed for every node. The association between morphological changes and the degree of overlap is expressed as the sum across all nodes of the change in character X_i multiplied by the degree of geographical overlap S_i at each node, $\sum_{i=1}^{i=m} X_i S_i$. If larger morphological changes occur between geographically overlapping sister clades, the observed association between character changes and degree of overlap is expected to be larger than that under the null model of no association between morphological change and geographical overlap. Standardized phylogenetic independent contrasts between morphological characters were calculated with CAIC (Purvis & Rambaut 1995). Logarithms of the length of the penis, the epiphallus, the flagellum, the vagina and the bursa copulatrix were standardized by the logarithm of the body size before the contrasts were calculated. The degree of geographical overlap between two species was calculated as the ratio of the area of overlap to the area of the smaller of the two ranges. Following Fitzpatrick & Turelli (2006), we calculated the nested average degree of overlap at node *i* that separates clades C1 and C2 as

$$\bar{o}_i = \sum_{j \in C_1} \sum_{k \in C2} \left(\frac{1}{2}\right)^{n_{jk}-1} o_{jk}$$

where the double sum is over all species in the two clades, o_{jk} denotes the degree of geographical overlap between species *j* and *k*, and n_{jk} is the number of nodes separating the two species on the tree. Geographical overlap between ranges was calculated from distribution maps based on the 10 km x 10 km UTM grid (Hausdorf & Sauer 2009).

Correlated evolution of spermatophore producing and spermatophore receiving organs

For the analysis of correlated evolution of male and female reproductive organs we used the general least squares approach (Pagel 1997, 1999) implemented in the BayesContinuous module of BayesTraits (Pagel & Meade 2007). We tested the influence of phylogeny on the

correlation between the log-transformed length of the spermatophore producing organs, the sum of the length of epiphallus and flagellum, and the log-transformed length of the bursa copulatrix (including pedunculus) by comparing a model in which the parameter lambda that estimates the extent to which character similarities match the degree of shared ancestry between species is set to 0.0 with one in which lambda is allowed to take its maximum likelihood value. If a likelihood ratio test comparing the log-likelihoods of the two models is significant some sort of phylogenetic correction is required. We included log-transformed body size (as measured by the shell volume) as a covariate in the correlation analyses, because the size of the reproductive organs scales with body size.

Influence of evolution of genitalia and body size on speciation

Following Barraclough *et al.* (1999), we used randomisations to compare the observed pattern of character changes across the tree to that expected under a null model of no association with cladogenesis. If morphological change is associated with speciation, recently split species should display greater divergence than expected under the null model. In contrast, if morphological differences promote persistence and/or subsequent radiation, divergence should be greater between more distantly related lineages. Hence, we test for a concentration of changes towards either the tips or the root of the tree. The null model is implemented by randomly shuffling phylogenetic independent contrasts among branches of the tree and recording where changes occur on the tree in each trial. The pattern of variability in each character with respect to relative node age is expressed as the sum over all nodes of the amount of change occurring across each node X_i multiplied by the relative age of that node A_i $\sum_{i=1}^{i=m} X_i A_i$. For each test, the two-tailed probability of the observed value was calculated from the null distributions obtained by 1000 randomisations.

RESULTS

Phylogenetic analyses

The maximum likelihood tree of 122 partial *cox1* sequences (634 bps) of Cretan *Xerocrassa* species and two *Trochoidea* species as outgroups is shown in Figure 5.3. Separate models for the three codon positions as determined by ModelTest were used, because the resulting tree had a lower AIC value than the tree based on a uniform model for the complete dataset.

The mitochondrial haplotypes of six of the eleven species recognized by Hausdorf & Sauer (2009) form distinct clades in the *cox1* gene tree, but the sequences of the other five

species do not form monophyletic groups. In particular, sequences of the widespread *Xerocrassa mesostena* are paraphyletic with respect to most of the other endemic *Xerocrassa* species.

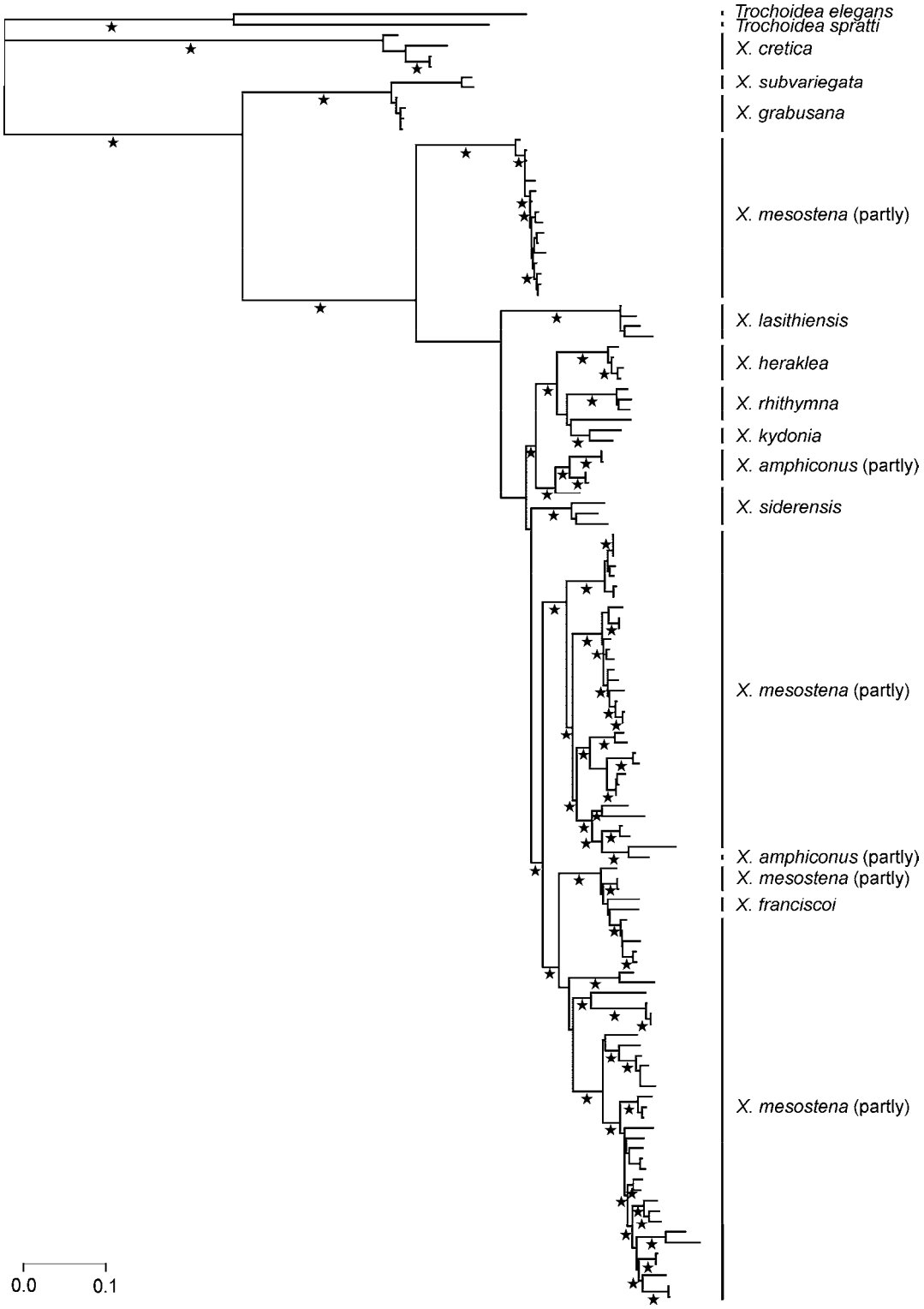

Fig. 5.3 Maximum likelihood tree of 124 partial *cox1* sequences of Cretan *Xerocrassa* species. Bootstrap support values larger than 70% are indicated by asterisks below the branches.

The mitochondrial gene tree was made ultrametric using the penalized likelihood method with a smoothing parameter of 2 chosen as optimal based on the normalized chi-square-like cross-validation scores. The species tree shown in Figure 5.4 has been derived from the ultrametric mitochondrial gene tree using a modification of the shallowest divergence clustering method as described in methods.

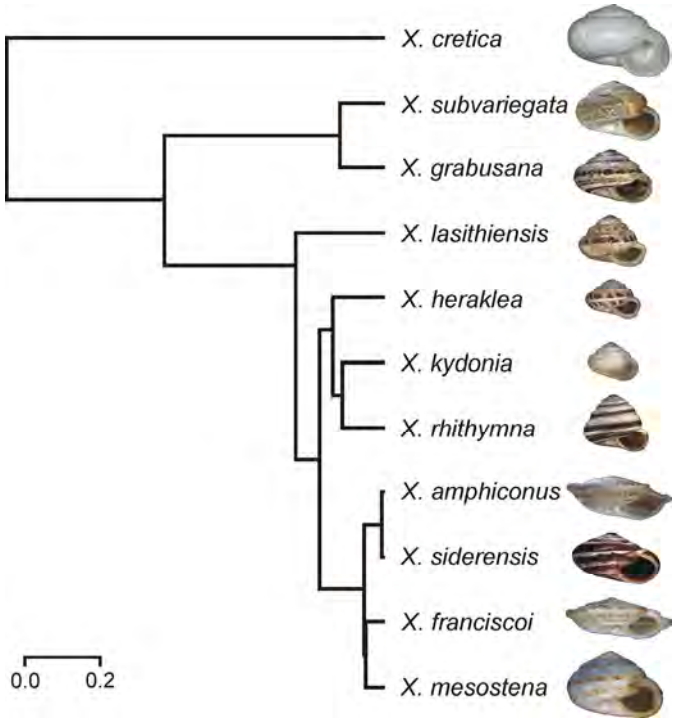

Fig. 5.4 Ultrametric species trees of Cretan *Xerocrassa* species based on *cox1* sequences.

Can differences in the genitalia be explained by genetic drift?

We used coalescent simulations based on the *cox1* sequence data of the six most recently diverged endemic *Xerocrassa* species and the species pair *Xerocrassa amphiconus* – *Xerocrassa siderensis* (that show no fixed differences in genital characters) to test whether the fixation of differences in the genitalia between these species can be explained by genetic drift. The mitochondrial haplotypes were not completely sorted by species, suggesting relatively recent divergence of species (Fig. 5.3). The lack of monophyly of five of the morphologically defined species in the mitochondrial gene tree is not the result of a poor resolution of the mitochondrial gene tree, because the hypothesis that the mitochondrial haplotypes of each species form separate clades could be rejected with the approximately unbiased test (P<0.001). Thus, the lack of monophyly of the morphologically defined species in the mitochondrial gene tree is consistent with a pattern of incomplete lineage sorting.

The *cox1* sequences contained 3 synonymous to 0 nonsynonymous fixed differences between the seven recently diverged Cretan *Xerocrassa* species and the three most basal *Xerocrassa* species (*Xerocrassa cretica*, *Xerocrassa subvariegata*, *Xerocrassa* species 1) versus 304 synonymous to 9 nonsynonymous polymorphisms within these two groups. The ratio of nonsynonymous to synonymous polymorphisms within the groups is not significantly different from the ratio of nonsynonymous to synonymous polymorphisms fixed between the groups (Fisher's exact test $P = 1.000$). Therefore, the McDonald and Kreitman test results are consistent with neutral evolution of the *cox1* gene.

There are fixed differences in genital characters between the seven investigated species units implying rapid fixation of genital differences, assumed to be encoded by nuclear genes. The minimum conceivable *s* value for seven species units is 6, which is equivalent to every species being fixed for a different phenotype. The observed *s* contrasting the seven species units in the *cox1* tree was 9. The coalescent simulations indicate that $s = 9$ corresponds with an upper 95% confidence limit on branch lengths of $1.75N$ generations. An *s* as low as 6 occurred rarely (in 162 of 10,000 coalescent simulations) in coalescent simulations with branch lengths reduced to $0.875N$ generations to mimic nuclear genes. Thus, the observed fixation of differences in the genitalia among species units is highly unlikely to have arisen under neutrality, assuming the fixed differences reflect fixed differences in underlying nuclear genes. Consequently, the evolution of the genitalia is better explained by divergent selection.

Can differences in the genitalia be ascribed to natural selection against hybrids?

The randomisation tests with respect to degree of geographical overlap show that standardized phylogenetic independent contrasts in body size are significantly larger between geographically overlapping clades in the Cretan *Xerocrassa* radiation than expected under the null model of no association between morphological change and geographical overlap (Table 5.1). This indicates that competition between co-occurring species resulted in ecological character displacement with regard to body size. On the contrary, contrasts in the length of penis, epiphallus, flagellum, vagina and bursa copulatrix standardized by body size are not larger between geographically overlapping clades than expected under the null model (Table 5.1). Thus, there is no evidence for selection against hybrids that resulted in larger contrasts between geographically overlapping species.

Table 5.1. Positive associations between standardized contrasts in characters and the degree of geographical overlap and associations between standardized contrasts in characters and relative node age in the Cretan *Xerocrassa* radiation according to randomisation tests. Positive signs of association of contrasts in characters with relative node age suggest that changes are concentrated towards the root and negative signs suggest that changes occur near the tips.

	geographical overlap	node age	
	p	sign	*p*
log body size	0.021	+	0.372
log penis: log body size	0.134	-	0.270
log epiphallus: log body size	0.775	-	0.092
log flagellum: log body size	0.458	-	<0.002
log vagina: log body size	0.598	-	0.102
log bursa copulatrix: log body size	0.192	-	0.122

Correlated evolution of spermatophore producing and spermatophore receiving organs

For the correlation between the log-transformed length of the male spermatophore producing organs and the log-transformed length of the female bursa copulatrix the maximum likelihood value of lambda calculated with BayesTraits (Pagel & Meade 2007) is 0.0. This indicates that the correlation evolves among species as if they were independent (Pagel 2000). Thus, phylogenetic correction can be dispensed with. The partial correlation between the log-transformed length of the spermatophore producing organs and the log-transformed length of the bursa copulatrix with log-transformed body size as a covariate that corrects for the scaling with body size is highly significant (Pearson coefficient R=0.933, two-sided p<0.001) indicating co-evolution between male spermatophore producing organs and female spermatophore receiving organs.

Influence of evolution of genitalia and body size on speciation

The randomisation tests with respect to node age show that the distributions of the contrasts in the length of penis, epiphallus, vagina and bursa copulatrix standardized by body size as well as in body size across the species tree do not differ from random distributions (Table 5.1). On the contrary, changes in the length of the flagellum standardized by body size are

significantly concentrated towards the tips of the tree indicating that the evolution of differences in flagellum length is involved in speciation in the Cretan *Xerocrassa* radiation.

DISCUSSION

The results of our tests indicate that sexual selection affecting flagellum length is involved in speciation in the land snail genus *Xerocrassa* on Crete. First, we could exclude two alternative causes of the changes in the genitalia, namely genetic drift and natural selection against hybrids. Coalescent simulations based on the degree of incomplete lineage sorting in a mitochondrial gene tree and the assumption that differences in the genitalia of the *Xerocrassa* species as potential indices of sexual selection are nuclear-encoded showed that the fixation of these differences between species cannot be ascribed to genetic drift. Whereas ten of the eleven *Xerocrassa* species on Crete can be distinguished by diagnostic differences in the genitalia, only five of the species can be distinguished by shell characters that might reflect alternative niches or predation pressures. The degree of fixation in the shell characters is similar to that in the mitochondrial DNA haplotypes. Nevertheless, the significantly larger contrasts in body size between geographically overlapping clades indicate that selection is involved in the evolution of shell size. It is possible that some other shell characters evolve mainly by genetic drift.

Randomisation tests demonstrated that the differences in the genitalia are not larger between co-occurring groups than between geographically separated taxa. Thus, there is no evidence that the differences in the genitalia are the result of natural selection against hybrids that might originate especially in areas where groups co-occur. Rather, it is likely that the differences in the genitalia are a product of sexual selection as has been shown in other groups (Arnqvist 1998; Eberhard 1985; Eberhard 2001; Hosken & Stockley 2004). An important role of sexual selection is also indicated by mating experiments with Cretan *Xerocrassa* species that demonstrated assortative mating even between populations belonging to the same species (Sauer and Hausdorf, unpubl. data) as has also been found by Baur & Baur (1992) in *Arianta arbustorum*, an other helicoid land snail.

The significant positive scaling of male spermatophore producing organs and female spermatophore receiving organs in the Cretan *Xerocrassa* species indicates sexual co-evolution. Koene & Schulenburg (2005) have already noted a correlation between the length of the flagellum that forms the tail of the spermatophore and the spermatophore receiving organ in helicoids land snails at a coarser taxonomic scale. The tail of the spermatophore

enables sperm to leave the spermatophore after it has been deposited in the partner's bursa copulatrix, the female gametolytic organ, during copulation (Lind 1973). Sperm are most successful at leaving the spermatophore and reaching the spermathecae when the tail of the spermatophore is protruding into the vagina. Thus, a lengthening of the spermatophore producing organs might increase paternity success. The co-evolution of male spermatophore producing organs and female spermatophore receiving organs might be the result of an evolutionary arms race over the control of fertilization, i.e. of sexual conflict (Arnqvist & Rowe 2005; Bergsten & Miller 2007; Chapman *et al.* 2003). Alternatively, changes in the length of the spermatophore producing organs might be the result of cryptic female choice (Eberhard 1985; Eberhard 2001) for sperm that are better in escaping sperm digestion or for larger spermatophores as nutritional nuptial gifts (Gwynne 1984; Vahed 1998) or as signals of donor quality or condition (Anthes *et al.* 2008) and the co-evolution might be the result of the necessity to process larger spermatophores (Anthes *et al.* 2008). Furthermore, natural selection might counter increasing spermatophore length because of increasing predation or desiccation risk resulting from long copulation times required for the transfer of long spermatophores.

Finally, randomisations of the contrasts in genital characters across nodes in the species tree (Table 5.1) showed that changes in the length of the flagellum standardized by body size are significantly concentrated towards the tips of the tree. If a lock-and-key mechanism (Shapiro & Porter 1989) would have triggered the radiation, we would expect that changes in those parts of the genitalia that directly interact during copulation, namely penis and vagina, are concentrated towards the tips of the tree. This is not the case. Rather, the evolution of differences in flagellum length that are probably the result of sexual selection is involved in speciation in the Cretan *Xerocrassa* radiation. The length of the spermatophore is determined by the length of the flagellum and the length of the epiphallus. The epiphallus produces the broad part of the spermatophore with the sperm container, whereas the narrow tail of the spermatophore is produced by the flagellum. That only changes in the flagellum are concentrated towards the tips of the tree and not changes in the epiphallus indicates that the meaning of these changes is not to transfer a larger amount of sperm or to provide more nutritious substance, but to optimize the length of the spermatophore with a modicum of extra cost. This is better compatible with the hypothesis that the driving force of the changes is rather sexual conflict than cryptic female choice. The hook-like structures at the spermatophore (Fig. 5.2) that point towards the anterior end and impede and delay the transfer

of the spermatophore and so enable more sperm to leave the spermatophore before digestion also indicate that sexual conflict is involved. Divergence in shape and size aspects of spermatophore morphology in allopatry might also have triggered the radiation in the land snail genus *Mastus* (Parmakelis *et al.* 2005).

Our analyses show that speciation in *Xerocrassa* on Crete was associated with and perhaps driven by sexual selection. This result is in accordance with other studies that have shown that sexual selection can promote speciation (Boul *et al.* 2007; Carson 1997; Darwin 1871; Dominey 1984; Gavrilets 2000; Gray & Cade 2000; Lande 1981; Masta & Maddison 2002; Panhuis *et al.* 2001; Price 1998; Ritchie 2007; Schluter & Price 1993; West-Eberhard 1983). This hypothesis received previously support from comparative studies that found correlations between species diversity and indices of sexual selection in birds (Barraclough *et al.* 1995; Møller & Cuervo 1998; Owens *et al.* 1999; Seddon *et al.* 2008), lizards (Stuart-Fox & Owens 2003) and insects (Arnqvist *et al.* 2000; Katzourakis *et al.* 2001). However, similar comparative studies of mammals, butterflies, and spiders (Gage *et al.* 2002; Isaac *et al.* 2005) and birds (Morrow *et al.* 2003) did not find significant correlations between species diversity and indices of sexual selection and challenge the generality of the importance of sexual selection in speciation and diversification. One reason for the heterogeneous results may be that the coarse taxonomic scale at which most studies were performed may result in comparisons of taxa with very different ecology and biogeographic history so that the effect of sexual selection on speciation may be obscured by such factors (Seddon *et al.* 2008). Thus, analyses of groups of closely related species considering their phylogenetic relationships as done in our study might be a more powerful approach to investigate the generality and importance of sexual selection in speciation.

In the Cretan *Xerocrassa* radiation, sexual selection seems to be the initial mechanism resulting in speciation. Ecological differentiation of the lineages as indicated by different body sizes is generally not associated with recent speciation (lineage splitting close to the tips of the tree), but has been achieved when clades came into contact. This is consistent with the pattern found in some Nicaraguan crater lake cichlids in which sexual selection contributes more strongly or earlier during speciation than ecological separation (Wilson *et al.* 2000) and with the results of the comparative analysis of Barraclough *et al.* (1999) who also did not find evidence for an association of speciation with ecological disparity in tiger beetles. However, separation of lineages as a result of sexual selection does not always precede ecological differentiation in radiations. Based on the distribution of ecological and morphological

characteristics across the phylogeny of the cichlid fishes of Lake Malawi, Danley & Kocher (2001) suggested that this radiation has proceeded in three major bursts of cladogenesis of which the first two episodes resulted in adaptation to different niches, whereas the third episode was associated with differentiation of male nuptial colouration, most likely in response to divergent sexual selection. Also studies of other taxa suggest that ecological divergence is common in the early stages of a radiation (Losos *et al.* 1998; Schliewen *et al.* 1994; Schluter 1998, 2000a, b, 2001; Schluter & McPhail 1993; Sturmbauer 1998). Although it is plausible, that niche space might be subdivided early in the history of a radiation, it is unclear why the importance of sexual selection should vary in the history of a radiation. An alternative explanation of the observed patterns might be that an appreciable fraction of the speciation events is always the result of sexual selection, but that lineages that became adapted to different niches during or after speciation have a higher chance of persistence. As the geographical pattern of body size differences in the *Xerocrassa* radiation indicates, differentiation in ecologically important properties is associated with sympatry. Lineages that do not differ in adaptive characteristics may become more easily extinct if they become sympatric. The differential extinction of lineages that differ only in non-adaptive characteristics will result in an apparently almost exclusive adaptive phase in the early history of a radiation and more frequent cases of speciation as a result of sexual selection towards the present.

Barraclough *et al.* (1999) could not establish a role of body size in interactions between North American tiger beetle species of the subgenus *Cicindella* (*Ellipsoptera*). They suggested that one reason for the lack of evidence for the importance of body size in interspecific interactions in *Ellipsoptera* might be that the strength and direction of species interactions may have been highly variable over time, because communities represent transient groups of species. This may explain the difference between the significant association of contrasts in body size and sympatry in the Cretan *Xerocrassa* radiation and the lack of evidence in the North American tiger beetles. The ranges of the Cretan land snails were probably only slightly affected by the Pleistocene glacials and Cretan land snail communities were therefore more stable than North American insect communities. Thus, the strength and direction of interactions between land snail species on Crete did not vary as much as in North American insect communities.

Without evidence that the observed phenotypic differences reflects adaptation to different niches in *Xerocrassa*, as with other land snail radiations on Crete (Gittenberger 1991;

Parmakelis *et al.* 2005), our results suggest that the *Xerocrassa* radiation was facilitated by sexual selection rather than adaptation. If speciation is facilitated by sexual selection, niches may remain conserved (Peterson *et al.* 1999; Wiens 2004; Wiens & Graham 2005) and non-adaptive radiation (Cameron *et al.* 1996; Dominey 1984; Gittenberger 1991; Kozak *et al.* 2006; Kozak & Wiens 2006; Parmakelis *et al.* 2005; Turgeon & McPeek 2002) may result.

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CHAPTER 6

PALAEOGEOGRAPHY OR SEXUAL SELECTION – WHICH FACTORS PROMOTED CRETAN LAND SNAIL RADIATIONS?

ABSTRACT

The high land snail diversity of Crete is the result of a few radiations. It has been suggested that these radiations were triggered by the fragmentation of Crete in the Neogene. Contrary to the predictions of this model, the ranges of the endemic species are not clustered and their diversity is not higher in the areas of the Neogene palaeoislands. We investigated the radiation of the helicoid genus *Xerocrassa* in detail. The asymmetry between the range sizes of sister species and clades of *Xerocrassa* indicates that peripatric speciation was the predominant speciation mode. Coalescent simulations show that the differences in the genitalia of the *Xerocrassa* species cannot be explained by genetic drift. They can also not be explained by natural selection against hybrids, because they are not larger between geographically overlapping groups than between allopatric groups. The evolution of differences in flagellum length is concentrated towards the tips of the tree indicating that sexual selection might have facilitated the radiation. If speciation is driven by sexual selection, niches may remain conserved and non-adaptive radiation may result.

INTRODUCTION

The land snail fauna of Crete is extraordinarily rich. Approximately 140 land snail species are known from Crete. This number is much higher than would be expected considering the area of Crete, if it were compared with other Aegean islands (Welter-Schultes & Williams 1999). The high land snail diversity on Crete is mainly the result of a few radiations, namely of *Mastus* (Maassen 1995; Parmakelis *et al.* 2005), *Orculella* (Gittenberger & Hausdorf 2004), *Albinaria* (Douris *et al.* 1998; Nordsieck 2004; Schilthuizen & Gittenberger 1996; Schilthuizen. *et al.* 2004; Welter-Schultes 2000a; Welter-Schultes 2000b) and *Xerocrassa* (Hausdorf & Sauer 2009).

Gittenberger (1991) noted that the *Albinaria* species occupy more or less the same or only a narrow range of habitats. Thus, he suggested that the *Albinaria* radiation on Crete is a non-adaptive radiation. Parmakelis *et al.* (2005) could not find evidence for differential adaptation in the Aegean *Mastus* species and classified the *Mastus* radiation also as non-adaptive. Which processes resulted in the land snail radiations on Crete, if they were not caused by divergent natural selection and differential adaptation? Welter-Schultes & Williams (1999) suggested that the land snail radiations on Crete were the result of the fragmentation of the region of present-day Crete into several palaeoislands from the lower Tortonian (11 million years ago) until the late Pliocene (2-3 million years ago) (Dermitzakis 1990; Fassoulas 2001; Welter-Schultes 2000a; Welter-Schultes 2000b; Welter-Schultes & Williams 1999). Parmakelis *et al.* (2005) supposed that the radiation of *Mastus* was triggered by a diversification of the spermatophore morphology in allopatry, perhaps by sexual selection.

Considering these hypotheses, we investigated the systematics, the ecology, the biogeography and the potential role of sexual selection in a so far only insufficiently known radiation on Crete, that of the helicoid genus *Xerocrassa*.

Systematics of the Xerocrassa radiation on Crete

In the nineteenth century ten nominal species belonging to *Xerocrassa* have been described from Crete. Afterwards this group has not been revised. Because tests of evolutionary hypotheses require robust taxonomic and phylogenetic hypotheses, we revised the Cretan *Xerocrassa* species based on morphological characters, AFLP markers and mitochondrial *cox1* sequences.

Eleven native Cretan *Xerocrassa* species can be delimited based on morphological characters of the shell and the genitalia (Hausdorf & Sauer 2009). With the exception of *X. cretica*, which is widespread in the Eastern Mediterranean, all species are endemic to Crete.

All species except the species pair *X. siderensis* and *X. amphiconus* differ in characters of the genitalia.

The morphological classification is largely supported by multi-locus AFLP data. Nine of the eleven morphologically defined species are monophyletic in the neighbor-joining tree based on Jaccard distances between AFLP data of 151 *Xerocrassa* specimens (Fig. 6.1). Only *X. amphiconus* and *X. siderensis* that are sympatric, but rarely syntopic cannot be distinguished based on the AFLP data.

On the contrary, the mitochondrial haplotypes of only six of the eleven morphologically defined species form monophyletic groups in a maximum likelihood tree of 122 partial *cox1* sequences (634 bps) of Cretan *Xerocrassa* species (Fig. 6.2), whereas the sequences of the other five species do not form distinct clades. In particular, sequences of the widespread *X. mesostena* are paraphyletic with respect to most of the other endemic *Xerocrassa* species. These results confirm other studies (e.g., Sota & Vogler 2001; Shaw 2002; Machado & Hey 2003; Weisrock *et al.* 2006) that have reported large distinctions between mitochondrial gene trees and trees based on nuclear markers and/or species classifications based on morphology. If sets of sequences for which each sequence in a set has at least one other sequence within a 3% threshold distance would be considered as approximations for species following Hebert *et al.* (2003), the 122 *cox1* sequences would represent 50 species. This excessive splitting is at least partly the result of high substitution rates of mitochondrial DNA in helicoid land snails (Chiba 1999; Hayashi & Chiba 2000; Thomaz *et al.* 1996; van Riel *et al.* 2005). This result challenges the generality and applicability of approaches to identify and delimit putative species on single signature sequences (e.g., Floyd *et al.* 2002; Blaxter *et al.* 2003; Blaxter *et al.* 2005; Hebert *et al.* 2003).

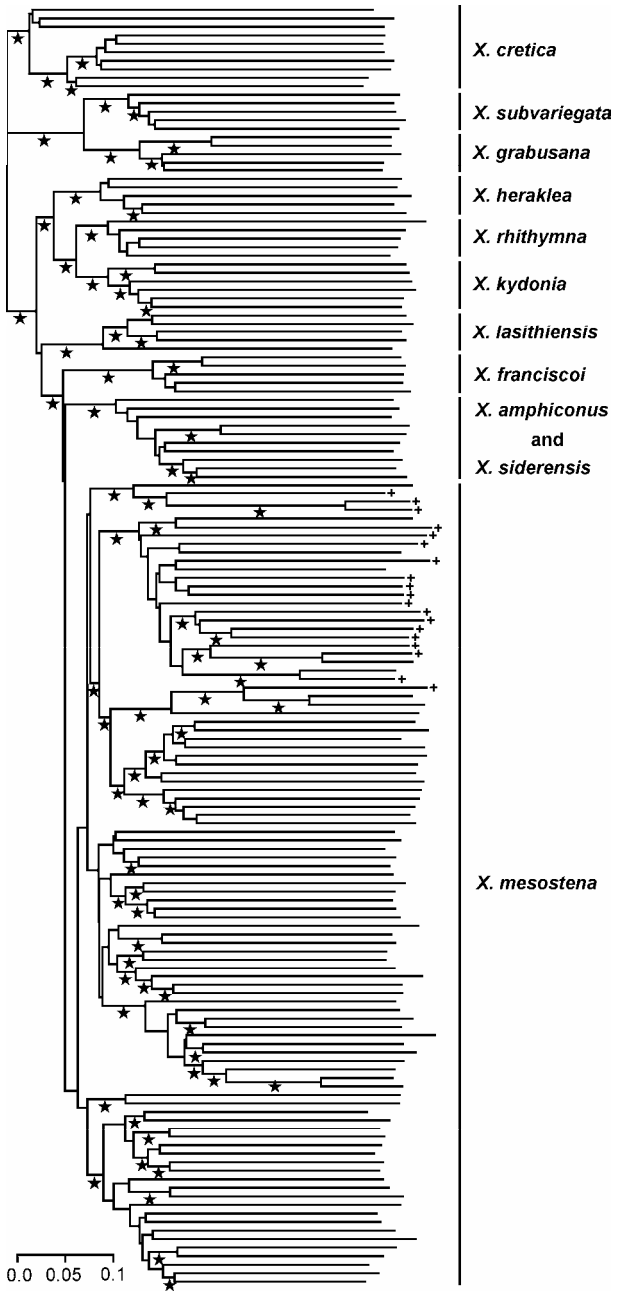

Fig. 6.1 Neighbor-joining tree based on Jaccard distances between AFLP data of Cretan Xerocrassa. Bootstrap support values larger than 70% are indicated by an asterisk below the branches. The *X. mesostena* individuals that are characterized by a mitochondrial haplotype of the Psiloritis group are indicated by +.

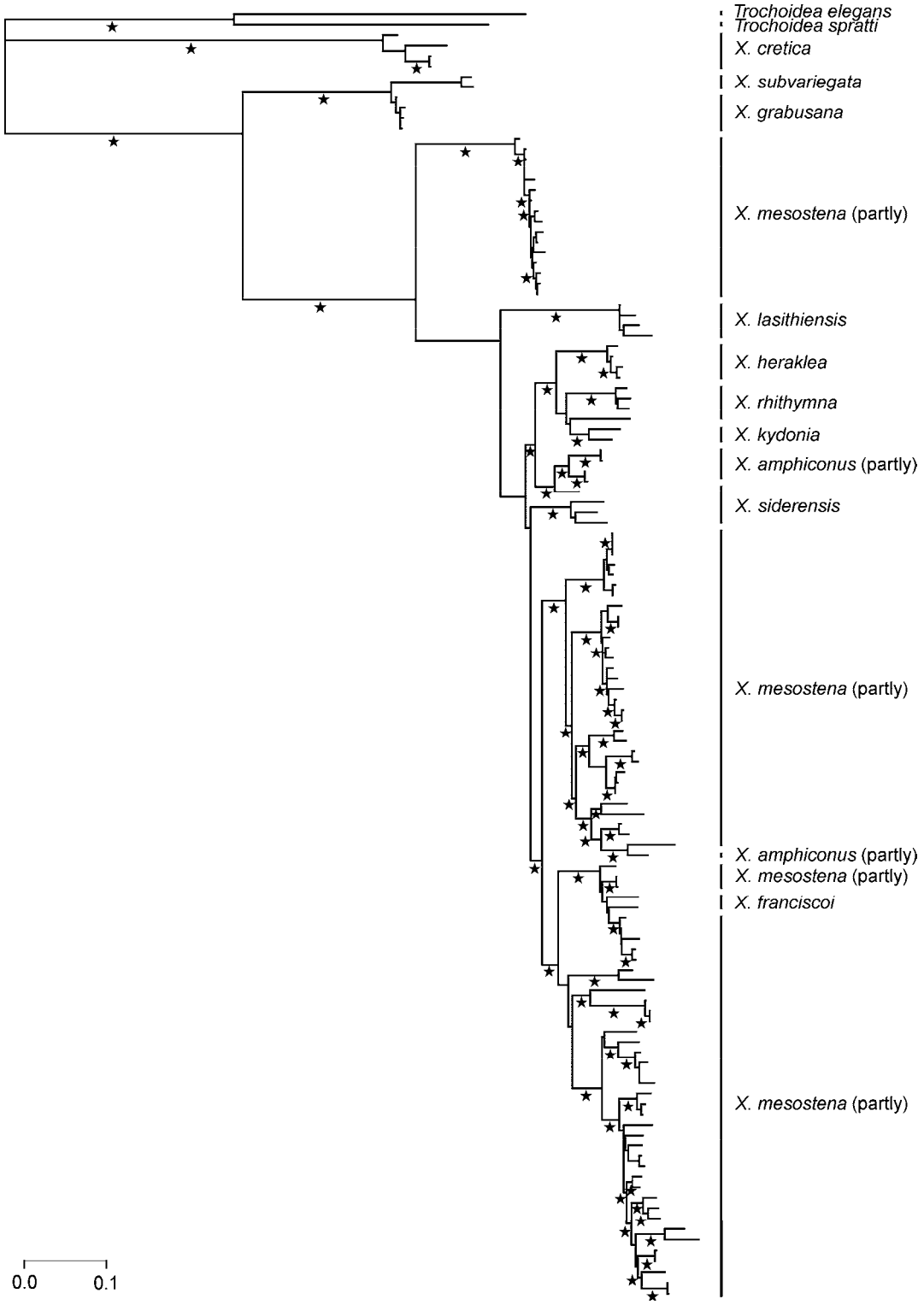

Fig. 6.2 Maximum likelihood tree of 122 partial *cox1* sequences of Cretan *Xerocrassa* species and two *Trochoidea* species. Bootstrap support values larger than 70% are indicated by asterisks below the branches.

Ecological differentiation of the Cretan Xerocrassa species

All *Xerocrassa* species are xerophilic and live in open, dry habitats. During the summer they aestivate under stones and bushes or, more rarely, attached to the vegetation. They are generalists feeding on decaying plants. There are no obvious adaptations to different habitats or lifestyles. Usually only one or two *Xerocrassa* species live in the same place, just as in *Albinaria* (Gittenberger 1991). The only species that regularly co-occur with other species is the widespread *X. cretica* that is much larger than all other Cretan *Xerocrassa* species. In the few cases in which two of the other species co-occur, there are also conspicuous differences in body size. Thus, we have investigated, if a differentiation in body size might have triggered the radiation. First we tested whether the differentiation in body size might be the result of competition between the species. If this were the case, we would expect that the differences are larger between species with geographically overlapping ranges than between species that are not in contact or have only slightly overlapping ranges. Following Barraclough *et al.* (1999), we tested this prediction by randomising phylogenetic independent contrasts in body size (measured as shell volume) among nodes, holding the degree of overlap fixed for every node. Standardized phylogenetic independent contrasts were calculated based on a species tree derived from the *cox1* gene tree. The AFLP tree could not be used, because it did not provide meaningful branch length. First we made the *cox1* gene tree ultrametric with the penalized likelihood method (Sanderson 2002) and then we constructed the species tree using a modification of the shallowest divergence clustering method (Maddison & Knowles 2006). Randomisation tests based on the resulting tree (see Fig. 6.3) showed that contrasts in body size are significantly larger between geographically overlapping clades in the Cretan *Xerocrassa* radiation than expected under the null model of no association between morphological change and geographical overlap (Table 6.1). This indicates that competition between co-occurring species resulted in ecological character displacement with regard to body size.

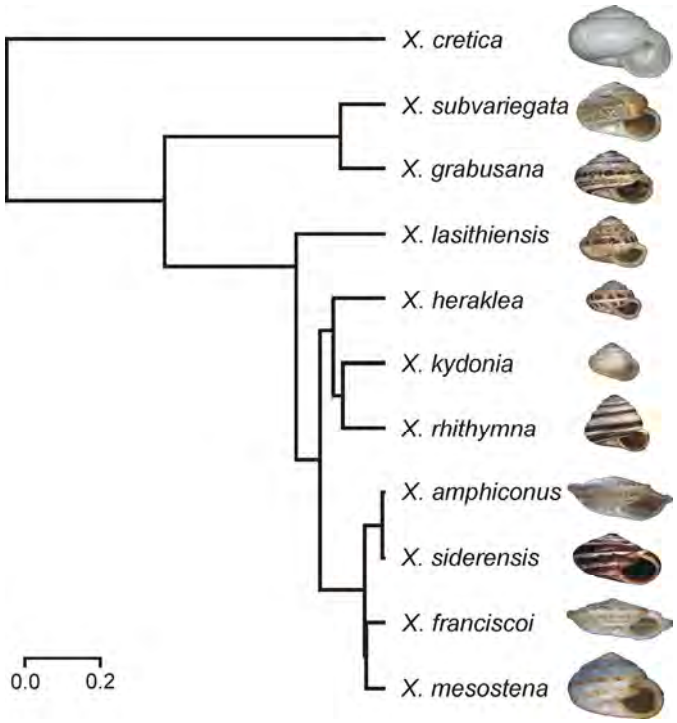

Fig. 6.3 Ultrametric species trees of Cretan *Xerocrassa* species based on *cox1* sequences.

In the next step we tested whether the changes in body size were associated with speciation. If this were the case, recently split species should display greater divergence in body size than expected under a null model of no association of body size changes with cladogenesis. In contrast, if differences in body size promote persistence and/or subsequent radiation, divergence should be greater between more distantly related lineages. Hence, we test for a concentration of changes towards either the tips or the root of the species tree by randomly shuffling phylogenetic independent contrasts among branches of the tree and recording where changes occur on the tree in each trial as proposed by Barraclough *et al.* (1999). This test showed that the distribution of the contrasts in body size across the species tree does not differ from random distributions (Table 6.1) indicating that body sizes changes have not triggered speciation.

Table 6.1. Positive associations between standardized contrasts in characters and the degree of geographical overlap and associations between standardized contrasts in characters and relative node age in the Cretan *Xerocrassa* radiation according to randomisation tests. Positive signs of association of contrasts in characters with relative node age suggest that changes are concentrated towards the root and negative signs suggest that changes occur near the tips.

	geographical overlap	node age	
	p	sign	*p*
log body size	0.021	+	0.372
log penis: log body size	0.134	-	0.270
log epiphallus: log body size	0.775	-	0.092
log flagellum: log body size	0.458	-	<0.002
log vagina: log body size	0.598	-	0.102
log bursa copulatrix: log body size	0.192	-	0.122

Geographic mode of speciation

Organisms with low vagility like land snails are more promising as model groups in studies investigating the geographical modes of speciation than more mobile groups, because frequent range shifts will obscure the geographical pattern of speciation (Barraclough & Vogler 2000).

To test whether the fragmentation of the region of present-day Crete into several palaeoislands in the late Miocene and Pliocene might have triggered the land snail radiations on Crete as suggested by Welter-Schultes & Williams (1999), we first investigated whether speciation was predominantly allopatric. If this were the case, recently diverged sister species are expected to display little or no overlap in geographic ranges (Barraclough & Vogler 2000). Alternatively, if speciation is predominantly sympatric, one range of recently diverged sister species is expected to enclose the other entirely. The intercept of a regression of the degree of sympatry between sister clades, the ratio of the area of overlap and the range size of the clade with the smaller range, against node age as approximated by the branch lengths in the ultrametric species tree indicates the predominant geographic mode of speciation. It is expected to be close to 0, if speciation is predominantly allopatric, and close to 1, if speciation is predominantly sympatric. The intercept of the linear regression of the arcsine transformed degree of sympatry against branch length in species tree as approximation for node age is 0.202 ($\pm$0.176) (Fig. 6.4A) indicating that allopatric speciation was at least predominant.

If the land snail radiations on Crete were the result of the fragmentation of Crete into several palaeoislands from the late Miocene until the Pliocene as hypothesized by Welter-Schultes & Williams (1999), we would expect that the ranges of the species are clustered and that the ranges are centred on the positions of the Neogene palaeoislands. However, the distribution areas of the 74 endemic land snail species belonging to genera with at least two endemic species are not significantly clustered (p=0.311) according to the test for clustering proposed by Hausdorf & Hennig (2003). The number of occurrences of the endemic land snail species in 10 km UTM grids that are located in areas formerly belonging to palaeoislands, was not significantly greater (p=0.595) for the real data (742) than for 1000 data sets obtained by Monte Carlo simulations (mean 723.21, range 431-1156) as described by Hausdorf & Sauer (2009). These results indicate that there is no evidence for the hypothesis that the land snail radiations on Crete are a result of the fragmentation of Crete in the late Miocene and Pliocene.

Peripatric speciation in small isolated populations at the periphery of a range is an alternative to vicariance. Following Barraclough & Vogler (2000) we used range symmetry as indicator for peripatric speciation. It can be predicted that the geographic ranges of recently split sister species tend to display asymmetry of range size, if peripatric speciation is the predominant diversification mode. The two null models proposed by Barraclough & Vogler (2000) were used to produce random range fragments. According to the phylogenetic broken stick model, the range of an ancestral species was split successively into two randomly sized fragments according to the phylogeny for each group. This model produces an even distribution of range size symmetry immediately after speciation that ranges from 0.0 to 0.5 with a mean value of 0.25. Lower values would suggest a tendency towards range size asymmetry indicating the possible importance of peripatric speciation. Alternatively, we also used the simultaneous broken stick model according to which a stick of given length is broken at n-1 uniform random points to produce n randomly sized pieces.

The intercept of the linear regression of the doubled and arcsine transformed degree of symmetry of the Cretan *Xerocrassa* species against relative node age (Fig. 6.4B) is significantly smaller than expected under the phylogenetic broken stick model (p = 0.043) as well as under the simultaneous broken stick model (p = 0.004). This result indicates that peripatric speciation was the predominant geographic speciation mode in the *Xerocrassa* radiation on Crete.

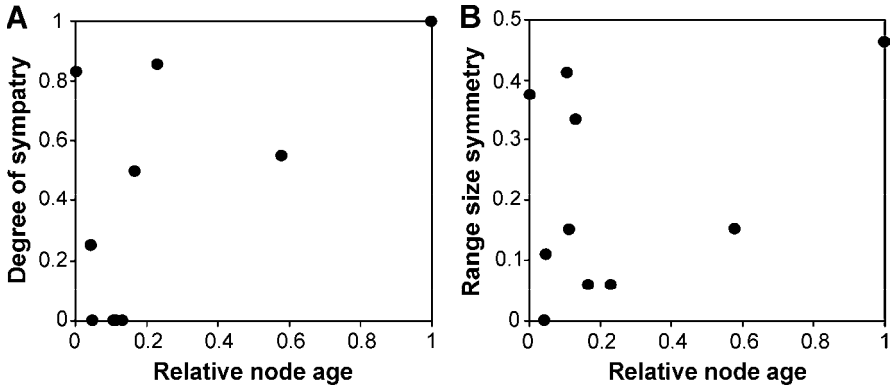

Fig. 6.4 A. Plot of the arcsine transformed degree of sympatry of *Xerocrassa* clades against relative node age based on the ultrametric species tree constructed from the *cox1* gene tree. B. Plot of range size symmetry of *Xerocrassa* clades against relative node age.

Evolution of genitalia by genetic drift versus selection

As already noted, the Cretan *Xerocrassa* species do not differ by adaptation to different niches. Which factors might have triggered the radiation, if not differential adaptation by natural selection? Several theories assume that random genetic drift plays an important role for speciation in peripheral isolates (Carson & Templeton 1984; Mayr 1963, 1982; Slatkin 1996; Templeton 1996). Most of the Cretan *Xerocrassa* species differ in the proportions of different parts of the genitalia. We tested whether the phenotypic differences between the more recently diverged species can be explained by genetic drift using the coalescent simulation approach proposed by Masta & Maddison (2002) based on the *cox1* tree (Fig. 6.2). According to this approach, the observed phenotypic differentiation is unlikely (p=0.016) to have arisen under neutrality, assuming the fixed phenotypic differences among species reflect fixed differences in underlying nuclear genes (chapter 5). Thus, the evolution of the genitalia is better explained by divergent selection.

Lock-and-key hypothesis versus sexual selection

Differences in genitalia may result from natural selection against hybrids as supposed by the lock-and-key hypothesis (Shapiro & Porter 1989) or from sexual (Arnqvist 1998; Eberhard 1985; Eberhard 2001; Hosken & Stockley 2004).

If differences in the genitalia are the result of natural selection against hybrids, we would expect that the differences are larger between species with geographically overlapping ranges than between species that are not in contact or have only slightly overlapping ranges, because selection against hybrids can happen only where two species co-occur. We test this prediction by randomising phylogenetic independent contrasts among nodes, holding the degree of

overlap fixed for every node as described above for body size. This test showed that independent contrasts in the length of penis, epiphallus, flagellum, vagina and bursa copulatrix standardized by body size are not larger between geographically overlapping clades in the Cretan *Xerocrassa* radiation than expected under the null model of no association between differences in the genitalia and geographical overlap (Table 6.1; Sauer & Hausdorf 2009). Thus, there is no evidence for selection against hybrids that resulted in larger contrasts between geographically overlapping species.

Influence of evolution of genitalia on speciation in Xerocrassa

If changes in the genitalia were associated with speciation, recently split species should display greater divergence than expected under a null model of no association of changes in the genitalia with cladogenesis. We tested this prediction using the approach proposed by Barraclough *et al.* (1999). The tests showed that the distributions of the contrasts in the length of penis, epiphallus, vagina and bursa copulatrix standardized by body size across the species tree do not differ from random distributions (Table 6.1; Sauer and Hausdorf 2009). On the contrary, changes in the length of the flagellum standardized by body size are significantly concentrated towards the tips of the tree indicating that the evolution of differences in flagellum length facilitated speciation in the Cretan *Xerocrassa* radiation (Sauer & Hausdorf 2009).

If a lock-and-key mechanism (Shapiro & Porter 1989) would have triggered the radiation, we would expect that changes in those parts of the genitalia that directly interact during copulation, namely penis and vagina, are concentrated towards the tips of the tree. This is not the case. Rather, speciation in the Cretan *Xerocrassa* radiation has been facilitated by the evolution of differences in flagellum length. The flagellum forms the tail of the spermatophore. During copulation spermatophores are exchanged and transferred into the partner's bursa copulatrix, the female gametolytic organ. In the bursa copulatrix the spermatophore of the mating partner is digested. Sperm have to actively swim out via the tail of the spermatophore formed by the flagellum to avoid digestion (Lind 1973). Sperm are most successful at reaching the spermathecae when the tail of the spermatophore is protruding into the vagina. Thus, a lengthening of the flagellum might increase paternity success. Koene & Schulenburg (2005) found correlations between the length of the flagellum and the spermatophore receiving organ in helicoids land snails indicating counter-adaptation. We also found a positive scaling of male spermatophore producing organs and female spermatophore receiving organs in the Cretan *Xerocrassa* species indicates sexual co-evolution. The co-

evolution of male spermatophore producing organs and female spermatophore receiving organs might be the result of an evolutionary arms race over the control of fertilization, i.e. of sexual conflict (Arnqvist & Rowe 2005; Chapman *et al.* 2003). Alternatively, changes in the length of the spermatophore producing organs might be the result of cryptic female choice (Eberhard 1985; Eberhard 2001) for sperm that are better in escaping sperm digestion or for larger spermatophores as nutritional nuptial gifts (Gwynne 1984; Vahed 1998) or as signals of donor quality or condition (Anthes *et al.* 2008) and the co-evolution might be the result of the necessity to process larger spermatophores (Anthes *et al.* 2008). Natural selection might counter increasing spermatophore length because of increasing predation or desiccation risk resulting from long copulation times required for the transfer of long spermatophores. Divergence in size and shape aspects of spermatophore morphology in allopatry might also have triggered the radiation in the land snail genus *Mastus* (Parmakelis *et al.* 2005).

Sexual selection and non-adaptive radiation

Our analyses show that speciation in *Xerocrassa* on Crete was associated with and perhaps driven by sexual selection. This result is in accordance with other studies that have shown that sexual selection can promote speciation (Darwin 1871; Dominey 1984; Gavrilets 2000; Gray & Cade 2000; Masta & Maddison 2002; Panhuis *et al.* 2001; Ritchie 2007; West-Eberhard 1983). This hypothesis received previously support from comparative studies that found correlations between species diversity and indices of sexual selection in birds (Barraclough *et al.* 1995; Møller & Cuervo 1998; Owens *et al.* 1999; Seddon *et al.* 2008), lizards (Stuart-Fox & Owens 2003) and insects (Arnqvist *et al.* 2000; Katzourakis *et al.* 2001). However, similar comparative studies of mammals, butterflies, and spiders (Gage *et al.* 2002; Isaac *et al.* 2005) and birds (Morrow *et al.* 2003) did not find significant correlations between species diversity and indices of sexual selection and challenge the generality of the importance of sexual selection in speciation and diversification. One reason for the heterogeneous results may be that the coarse taxonomic scale at which most studies were performed may result in comparisons of taxa with very different ecology and biogeographic history so that the effect of sexual selection on speciation may be obscured by such factors (Seddon *et al.* 2008). Thus, analyses of groups of closely related species considering their phylogenetic relationships as done in our study might be a more powerful approach to investigate the generality and importance of sexual selection in speciation.

In the Cretan *Xerocrassa* radiation, sexual selection seems to be the initial mechanism resulting in speciation. On the contrary, ecological differentiation of the lineages as indicated

by different body sizes is generally not associated with recent speciation (lineage splitting close to the tips of the tree), but has been achieved when clades came into contact. This is consistent with the pattern found in some Nicaraguan crater lake cichlids in which sexual selection contributes more strongly or earlier during speciation than ecological separation (Wilson *et al.* 2000) and with the results of the comparative analysis of Barraclough *et al.* (1999) who also did not find evidence for an association of speciation with ecological disparity in tiger beetles. However, separation of lineages as a result of sexual selection does not always precede ecological differentiation in radiations. Based on the distribution of ecological and morphological characteristics across the phylogeny of the cichlid fishes of Lake Malawi, Danley & Kocher (2001) suggested that this radiation has proceeded in three major bursts of cladogenesis of which the first two episodes resulted in adaptation to different niches, whereas the third episode was associated with differentiation of male nuptial colouration, most likely in response to divergent sexual selection. Also studies of other taxa suggest that ecological divergence is common in the early stages of a radiation (Losos *et al.* 1998; Schluter 2000a, b, 2001; Schluter & McPhail 1993; Sturmbauer 1998). Although it is plausible, that niche space might be subdivided early in the history of a radiation, it is unclear why the importance of sexual selection should vary in the history of a radiation. An alternative explanation of the observed patterns might be that an appreciable fraction of the speciation events is always the result of sexual selection, but that lineages that became adapted to different niches during or after speciation have a higher chance of persistence. As the geographical pattern of body size differences in the *Xerocrassa* radiation indicates, differentiation in ecologically important properties is associated with sympatry. Lineages that do not differ in adaptive characteristics may become more easily extinct if they become sympatric. The differential extinction of lineages that differ only in non-adaptive characteristics will result in an apparently almost exclusive adaptive phase in the early history of a radiation and more frequent cases of speciation as a result of sexual selection towards the present.

Without evidence that the observed phenotypic differences reflects adaptation to different niches in *Xerocrassa*, as with other land snail radiations on Crete (Gittenberger 1991; Parmakelis *et al.* 2005), our results suggest that the *Xerocrassa* radiation was facilitated by sexual selection rather than adaptation. If speciation is facilitated by sexual selection, niches may remain conserved (Peterson *et al.* 1999; Wiens 2004; Wiens & Graham 2005) and non-adaptive radiation (Cameron *et al.* 1996; Dominey 1984; Gittenberger 1991; Kozak *et al.* 2006; Kozak & Wiens 2006; Parmakelis *et al.* 2005; Turgeon & McPeek 2002) may result.

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General Discussion

The Cretan endemic land snail species with particular focus on the Xerocrassa radiation from Crete

Radiations, whether they are adaptive ore non-adaptive, provide a good model system to study speciation events. As a first step for the better understanding of the evolutionary processes within the Helicellinae occurring on Crete, I presented a morphological revision based on characters of the shell and the genitalia (chapter 1). The results of this morphological revision show that the native fauna on Crete includes eleven *Xerocrassa* species, two *Pseudoxerophila* species and one *Xeromunda* species. Additionally there are seven species of the genera, *Trochoidea*, *Xerocrassa*, *Xeropicta*, *Xerotricha*, *Microxeromagna* and *Cernuella* that were anthropogenically introduced to Crete.

Further investigations and conclusions focus on the members of the genus *Xerocrassa*. The ranges of the eleven endemic species of the *Xerocrassa* radiation are mainly allopatric. Besides one sister species pair, they can all be distinguished by differences in the male and female parts of the genitalia. *X. amphiconus* and *X. siderensis* is the only species pair that differs only in shell characters, but have not been distinguished using characters of the genitalia or molecular data. The ranges of these species are broadly overlapping, but they rarely occur synoptically. They tend to occur at prefer different altitudinal zones (chapter 1), though there are several populations of each species occurring in the zone preferred by the other species. A few individuals show intermediate shell characters indicating possible hybridization. A possible explanation for the lack of clear genetic differentiation of these two species might be a very recent species divergence. But the results of my thesis could also be argued to indicate that the two forms only represent two extreme morphs of a single species.

Besides the former mentioned exception, the morphological classification of the *Xerocrassa* species described in chapter 1 is largely supported by multi-locus amplified fragment length polymorphism (AFLP) data (chapter 2 and 3). Nine of the eleven morphologically defined species are monophyletic in the neighbor-joining tree based on Jaccard distances between AFLP data of 151 *Xerocrassa* specimens (chapter 2 Fig. 2.1). In contrast, the mitochondrial haplotypes of only six of the eleven morphologically defined species form monophyletic groups in a maximum likelihood tree of 122 partial cytochrome c oxidase subunit 1 (*cox1*) sequences (634 bps) of Cretan *Xerocrassa* species (chapter 2, Fig. 2.2), whereas the sequences of the other five species do not form distinct clades. In particular,

sequences of the widespread *X. mesostena* are paraphyletic with respect to most of the other endemic *Xerocrassa* species.

The non-monophyly of species in a gene tree can be ascribed to different reasons namely incomplete lineage sorting of ancestral polymorphisms (Avise & Wollenberg 1997; Hudson & Coyne 2002; Maddison 1997; Neigel & Avise 1986; Rosenberg 2003), introgression resulting from interspecific gene flow in the early stages of speciation (Chan & Simon 2005; Funk & Omland 2003; Takahata & Slatkin 1984) or inadequate taxonomy. Furthermore, problems like the lack of resolution in the molecular marker used or artefacts resulting from mistakes in tree reconstruction can lead to the apparent non-monophyly of morphologically delimited species. By using either a topology test (see chapter 5) or a non-stationary model for the maximum likelihood analysis (see chapter 3), I could show that the observed non-monophyly of species in the *cox1* based gene tree was not caused by the described problems.

When identifying the cause of non-monophyly of species in a gene tree, it is difficult to discriminate between incomplete lineage sorting of ancestral polymorphisms and introgression (Berthier *et al.* 2006; Donnelly *et al.* 2003; Funk & Omland 2003; Gompert *et al.* 2008; Holder *et al.* 2001; Jakob & Blattner 2006; Joly *et al.* 2009; McGuire *et al.* 2007; Morando *et al.* 2004). In some cases the combination of several criteria, namely the depth of the coalescences in the gene tree, the geographical distribution of shared genetic markers, and concordance with results of admixture analyses of nuclear multilocus markers, allow the identification of the most likely causes of non-monophyly (see chapter 3). For example, the non-monophyly of *X. mesostena* in the *cox1* based gene tree is most probably the result of incomplete lineage sorting of ancestral polymorphisms. In contrast, none of the results presented in chapter 1 to 4 supported the hypotheses that an introgressed haplotype is the cause of the observed non-monophyly of this species.

To further elucidate the question of an inadequate taxonomy I compared the results of different approaches for delimiting species based on single-locus DNA sequences with those of methods using dominant multi-locus data and the classification based on morphological characters of the *Xerocrassa* radiation derived in chapter 1. The problem of an evaluation of the performance of different methods and different data for delimiting species with empirical data is that the correct species delimitation is not known a priori. Therefore, I looked for congruence between the partitions of the examined specimens obtained by different analytical methods based on different data to infer the true species limits. For estimating the congruence between the resulting different data partitions, I used the corrected rand index (see chapter 2; Table 2.2). When comparing the species partitions based on the morphology to the

classification based on different molecular markers and methods the highest similarity between data partitions based on different methods and markers is found between the morphological partition and the multi locus data partition (AFLP) derived from Gaussian Clustering. Contrary to the good fit of the amplified fragment length polymorphism (AFLP) data with the morphological partition the different methods based on mitochondrial DNA sequences results either in much higher or much lower putative species numbers (see chapter 2; Fig. 2.2), depending on the method used to infer putative species. For example Hebert *et al.* (2003) proposed a 3 % fixed distance threshold for species diagnosis in lepidopterans based on *cox1* sequences, when I used this threshold to infer putative species of the *Xerocrassa* radiation it resulted in a total of 50 candidate species (see chapter 2; Fig. 2.2). In contrast the analysis proposed by Pons *et al.* (2006) which is based on changes in branching rates in an ultrametric tree resulted in only 4 putative species based on the same *cox1* sequences. The latter method was in addition sensitive to the algorithm used to calculate the ultrametric tree. Hence, the single locus sequence data was neither consistent when comparing the different methods based on the same marker nor congruent to the AFLP data (see chapter 2; Table 2.2).

From the congruence between the data partitions derived from Gaussian clustering based on the multilocus data (AFLP) and the morphological partition, and that nine out of 11 *Xerocrassa* species are monophyletic in the AFLP based neighbor-joining tree, I conclude that inadequate taxonomy is not responsible for the non-monophyly of *Xerocrassa* species in the gene tree.

Despite the increasing availability of molecular data, it is reasonable to invest in morphological data, because one can look specifically for morphological characters that are directly involved in the speciation process and that may indicate the evolution of a new species before it becomes apparent in usual molecular data.

Biogeography of Cretan endemic land snail species and causes of the Cretan Xerocrassa radiation

Because of the extraordinary high species richness of land snails occurring on Crete, the area and taxon are well suited to study the processes underlying radiations. Organisms with low dispersal capabilities like land snails are more suited as model groups in studies investigating the geographical modes of speciation than more mobile groups, because frequent range shifts will obscure the geographical pattern of speciation (Barraclough & Vogler 2000). Compared to other Aegean islands the species number on Crete is much higher

than would have been expected for its area (Welter-Schultes & Williams 1999). One of the hypotheses which could explain this extraordinary rich land snail fauna refers to the past fragmentation of Crete into several paleoislands during the Neogene (Douris *et al.* 1998; Welter-Schultes & Williams 1999). This vicariance event may have resulted in allopatric speciation on the different paleoislands. After the reunion of the paleoislands, present day Crete inhabitants are quasi-endemics of the past paleoislands.

As a first step to investigate the biogeography and the geographic modes of speciation I tested if the speciation of the endemic *Xerocrassa* radiation was predominantly allopatric following an approach of Barraclough & Vogler (2000; see chapter 6 for detailed methods). If this were the case one would expect that recently diverged sister species show little or no overlap in geographic ranges. Alternatively, if speciation is predominantly sympatric, one range of the recently diverged sister species is expected to enclose the other entirely. The results indicate that the geographic mode of speciation was at least predominantly allopatric.

Vicariance events like the fragmentation of Crete into smaller islands could be expected to have affected different groups of organisms in a similar way. Thus a reasonable hypothesis is that the ranges of different endemic species occurring on Crete are clustered and that the ranges are centred on the positions of the Neogene palaeoislands. However analysis using Hausdorf & Hennig (2003)'s test the distribution ranges of 74 endemic land snails are not significantly clustered according (see chapter 6). A further analysis using Monte Carlo simulations shows that the ranges of the endemic species belonging to the genera *Xerocrassa* and *Pseudoxerophila* are not significantly concentrated towards areas which were formerly paleoislands (see chapter 1). An alternative to vicariance as a geographic mode of speciation would be peripatric speciation in small isolated population at the periphery of a range. Using a randomization approach proposed by Barraclough & Vogler (2000) I could show that peripatric speciation was the predominant geographic speciation mode in the *Xerocrassa* radiation (see chapter 6 for details). This means that distribution ranges of sister species are asymmetric significantly more often than expected by chance.

For a better understanding of the processes which influence the fragmentation of land snails on Crete I evaluated the phylogeographic structure of the widespread endemic *Xerocrassa mesostena*. To infer the phylogeographic structure I used different molecular markers, namely multilocus dominant markers (AFLP) and single locus mtDNA sequences (*cox1*). The AFLP data revealed a distinct phylogeographic subdivision of the range of *Xerocrassa mesostena* that corresponds partly with current geographic barriers like mountain ranges and the Messara Plain (see chapter 4). Whereas the distribution of mitochondrial

haplotype groups agrees only to a small extent with the phylogeographic pattern of the multilocus markers and thus only to a small extent with geographic barriers (see chapter 4; Fig.4.2 and 4.6). A potential different explanation for the observed mitochondrial haplogroups could be the earlier mentioned paleoislands. Given a substitution rate of 0.05 changes per site per million years, derived from closely related hygromiid taxa, this would indicate an early Pleistocene origin of the deepest splits within *X. mesostena*. Hence, the fragmentation of Crete into several paleoislands from the lower Tortonian (11 Mya) until the late Pliocene (2-3 Mya) (Dermitzakis 1990; Fassoulas 2001; Welter-Schultes 2000a; Welter-Schultes 2000b; Welter-Schultes & Williams 1999) cannot explain the *cox1* based phylogeographic pattern of *X. mesostena*. Actually, neither the distribution of the AFLP based clusters nor the distribution of the *cox1* haplotype clades are in good concordance with the supposed positions of the paleoislands.

What else has influenced the speciation processes of the Cretan Xerocrassa radiation

To further investigate the Cretan endemic *Xerocrassa* radiation I tested for additional mechanisms that might be responsible for the diversification of the *Xerocrassa* species. Was the radiation mainly driven by adaptive mechanisms or by non adaptive mechanisms as already proposed for the *Albinaria* radiation (Gittenberger 1991) and the *Mastus* radiation (Parmakelis *et al.* 2005) occurring on Crete? The *Xerocrassa* species show no obvious adaptations to different habitats or lifestyles (see chapter 2, 5 and 6). Seldom more than two *Xerocrassa* species are known to co-occur. If two species co-occur they differ remarkably in shell size. Following an approach by Barraclough *et al.* (1999) I could show that differences in shell size are important for lineage persistence of co-occurring species (ecological character displacement with regard to body size) but that changes in body size did not facilitate speciation processes in the Cretan *Xerocrassa* radiation (see chapter 5 and 6). Most of the endemic *Xerocrassa* species can only be distinguished by characters of their genitalia (see chapter 1). Using a test of correlated evolution I could show that the length of the male spermatophore producing organs and the length of the female spermatophore receiving organs in the Cretan *Xerocrassa* species are significantly positive correlated which indicates sexual co-evolution. Koene & Schulenburg (2005) noted a correlation between the length of the flagellum that forms the tail of the spermatophore and the spermatophore receiving organ in helicoids land snails at a coarser taxonomic scale. Thus, sexual selection is probably involved in the speciation process of the *Xerocrassa* species. Additional processes which can result in differences in the genitalia are natural selection against hybrids (Shapiro & Porter 1989) and

genetic drift (Carson & Templeton 1984; Mayr 1963, 1982; Slatkin 1996; Templeton 1996). I could exclude natural selection against hybrids using a test proposed by Barraclough *et al.* (1999), and genetic drift using a test proposed by Masta & Maddison (2002) for being responsible for the differences in genitalia (detailed description see chapter 5). In contrast the results indicate that the changes in the flagellum length of the male genitalia are important for speciation processes in the *Xerocrassa* radiation. The flagellum is part of the male genitalia system and forms the tail of the spermatophore but is not directly involved in the copulatory processes. An elongation of the flagellum facilitates the sperm escaping from digestion out of the gametolytic organ. Thus sexual conflict rather than cryptic female choice is the driving force of the changes in flagellum length.

The evidence presented suggests that the Cretan *Xerocrassa* radiation was facilitated by sexual selection in allopatry and not by adaptation to different ecological niches as already proposed for the *Albinaria* and *Mastus* radiations on Crete (Gittenberger 1991; Parmakelis *et al.* 2005).

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