Integrative Systematics and Biogeography of *Limax* (Gastropoda: Stylommatophora)

Dissertation

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Article II (Chapter 6)

Nitz B, Falkner G, Haszprunar G (2010) Inferring Multiple Corsican *Limax* (Pulmonata: Limacidae) Radiations: A Combined Approach Using Morphology and Molecules. Evolution in Action. Case studies in Adaptive Radiation, Speciation and the Origin of Biodiversity. Springer-Verlag, Berlin, Heidelberg: 405-435

[Including an appendix: "Two new species and one new name of peri-Tyrrhenian *Limax*" by Gerhard Falkner & Barbara Nitz]

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Declaration of contribution as co-author

Article I (Chapter 5)

B. Nitz was involved in designing the study, was responsible for lab work, sample management and molecular analyses, conducted field work and dissections and wrote the main part of the manuscript.

Article II (Chapter 6)

B. Nitz was involved in designing the study, was responsible for lab work, sample management and molecular analyses, conducted field work, contributed to dissections and wrote the main part of the manuscript.

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1 Zusammenfassung

Die Gattung *Limax* Linnaeus, 1758 (Gastropoda: Euthyneura: Stylommatophora) umfasst große (6-30 cm), terrestrische Nacktschnecken. Bisher wurden das einzigartige und sehr komplexe Paarungsverhalten und die damit in Zusammenhang stehenden morphologischen Merkmale wie Penislänge und Penisform als Basis für die Artdefinition genutzt. Allerdings ist die morphologische Artunterscheidung schwierig, weil in *Limax* eine verwirrend hohe Farbvariabilitat auftritt und nur voll geschlechtsreife Exemplare die nötigen Merkmale aufweisen.

Während der letzten Jahrzehnte wurden DNA Sequenzen zu einem gebräuchlichen Werkzeug in der Taxonomie und Phylogenie. In dieser Dissertation wurde eines der am meisten genutzen Gene, die mitochondriale Cytochrom c oxidase subunit I (COI), als zusätzliches Merkmals-Set verwendet, um *Limax* Arten zu unterscheiden und phylogenetische Analysen der Gattung *Limax* durchzuführen. Neuere Studien empfehlen den Gebrauch von DNA Sequenzen nur in Kombination mit einer soliden taxonomischen Basis und in einem integrativen taxonomischen Ansatz. Die Anwendung dieses kombinierten Forschungsansatzes mit morphologischen und molekularen Merkmalen in *Limax* ist Gegenstand dieser Arbeit. Die Möglichkeiten und Einschränkungen des integrativen Ansatzes in der Gattung *Limax* werden auf Artniveau evaluiert. Die Brauchbarkeit der kombinierten Merkmals-Sets wird im nah verwandten Korsika-*Limax*-Artsystem getestet, um die Phylogenie und die evolutionäre Geschichte dieser Radiationen aufzuklären. Weiterhin wird eine erste Interpretation der phylogenetischen Muster in der Gattung *Limax* basierend auf molekularen Daten der wichtigsten europäischen *Limax*-Linien präsentiert. Eine Diskussion über die evolutionären und biogeographischen Schlussfolgerungen dieser Ergebnisse rundet die Arbeit ab.

2 Summary

The genus *Limax* Linnaeus, 1758 (Gastropoda: Euthyneura: Stylommatophora) comprises large (6-30 cm) terrestrial slugs. The unique and complex copulation behaviour and the associated morphological characters like penis length and shape have been used up to now for species definition. However, morphological discrimination of *Limax* species is difficult due to a perplexing high colour variability and the fact that only fully mature specimens can be considered for comparisons based on genital characters. During the last few decades the use of DNA sequence variation data has become a common tool in taxonomy and phylogeny. In this study, one of the most commonly used genes, the mitochondrial cytochrome c oxidase subunit I (COI), is evaluated as a valuable character set for species identification and for subsequent phylogenetic analyses in the genus *Limax*. However, recent studies strongly suggest a use of DNA sequences only in combination with solid taxonomic foundations and in an integrative taxonomy approach. The application of this combined approach in *Limax* is emphasised and discussed in this work; an overview of the limitations and possibilities of *Limax* research based on an integrative approach of morphological and molecular characters is given. After evaluating the utility of various characters at species level, a combination of molecular techniques and morphological characters is applied to show the viability of these character sets for clearing up the phylogeny and evolutionary history of a closely related species system of Corsican *Limax* radiations. Finally, a first interpretation of the phylogenetic patterns in the genus *Limax* based on molecular data of major European *Limax* lineages is presented. Evolutionary and historical biogeographic considerations are discussed based on the results of this work.

3 Aim of the Thesis

The present Thesis should give an overview of the limitations and possibilities of *Limax* research based on an integrative approach of morphological and molecular characters. One major aim of the Thesis was to explore the usefulness of single characters for species distinction. This approach is discussed in chapter 5, using the example of a description of a new *Limax* species (*Limax sarnensis* Heim & Nitz, 2009). A second aim was to evaluate a combined approach of molecular techniques and morphological characters; here the intention is to show the viability of these character sets for clearing up the phylogeny and evolutionary history of a closely related species system (chapter 6). In chapter 7, a new species delimitation approach is tested for improvements in molecular-based species discrimination. One further aim, which is addressed in chapter 8, was to give a first interpretation of the phylogenetic patterns in the genus *Limax* based on molecular data of major European *Limax* lineages including a comparison of this molecular-based interpretation with morphological and biogeographic data. Giving initial insights into the phylogenetic relationships of European Limacidae was another intention of this chapter 8. Evolutionary and historical biogeographic considerations and the impact of an integrative approach in *Limax* research are discussed in chapter 9, the general discussion.

4 General Introduction

The genus *Limax* - background

Limax Linnaeus, 1758 (Gastropoda: Euthyneura: Stylommatophora) is a terrestrial slug genus belonging to Pulmonata, a group of air-breathing snails and slugs including mainly land and freshwater families, but also some marine families. The taxon Pulmonata, previously ranked as an order, is currently classified as an informal group according to Bouchet & Rocroi (2005). Following Holznagel *et al.* (2010), Pulmonata is still monophyletic and includes five groups; one of these groups is Eupulmonata Haszprunar & Huber, 1990. However, Jörger *et al.* (2010) redefined the clade Heterobranchia and assigned Eupulmonata as a member of the newly established taxon Panpulmonata, which itself is a member of Euthyneura. Jörger and colleagues propose based on a multi-locus molecular study that the traditional classification of Euthyeura has to be reinvestigated, since some morphological synapomorphies seem to be misinterpreted. Eupulmonata contains, among others, the taxon Stylommatophora. Although the monophyly of Stylommatophora is confirmed in the recent analyses of Holznagel *et al.* (2010), its position in Eupulmonata is still under discussion (e.g. Wade *et al.*, 2001; 2006; Dayrat *et al.*, 2011; Klussmann-Kolb *et al.*, 2008).

Stylommatophora is subdivided into two clades (Elasmognatha and Orthurethra) and the informal group Sigmurethra. Many of the slugs belonging to Sigmurethra are placed, along with semislugs and snails, in Limacoidea *sensu lato* (Hausdorf, 1998) or, as Bouchet & Rocroi, (2005) named this taxon, the “limacoid clade”. Limacoidea *sensu lato* contain several superfamilies, e.g. Helicarionidae, Gastrodontoidea, Zonitoidea, and Limacoidea (Hausdorf, 1998; Schileyko, 2003; Bouchet & Rocroi, 2005). The family Limacidae is positioned in the superfamily Limacoidea. *Limax* is the type genus of the Limacidae (common name: keelback slugs). The (phylogenetic) relationships within Limacidae have not been touched since the 1980s (Likharev & Wiktor, 1980). Schileyko 2003 provided an overview of members of the family Limacidae based on current knowledge, but without including new data. Up to now, all classifications of limacid slugs have been based on morphological characters, as follows. The members of Limacidae have a vestigial shell covered by the mantle and a tripartite sole. Body length is very variable ranging from some cm (*Malacolimax*) to more than 20 cm (own observations) in the biggest species of the genus *Limax*. Colouration is in most cases just brownish or greyish, but some genera have very colourful representatives: *Gigantomilax lederi* can have a blue pattern, and *Bielzia*

coerulans (Bielz, 1851) gleams blue, green or mauve. In the genus *Limax* a huge range of patterns like spots or stripes can be combined with various colours, including black, brown, grey, red, beige or white.

All limacids are hermaphrodites; the copulation can be quite straight forward and fast in some genera, but in others (e.g. *Limax*) it is very complicated. The prelude may take hours and *in copula* the penis reaches in some species the length of more than 90 cm (Nitz *et al.*, 2010).

The slugs of the family Limacidae as they are currently understood are mainly distributed on the Eurasian continent with emphasis on the European subcontinent and some representatives in the Caucasian mountains, Central Asia and probably in the Mediterranean areas of Northern Africa (Likharev & Wiktor, 1980). It is not clear whether records of limacid slugs in Northern Africa are species that are autochthonous species or invaders. One species belonging to Limacidae was recently described from the Himalayan Mountains (Wiktor & Bößneck, 2004). The huge geographic gaps in the known distribution patterns could be either due to unsuitable habitat types (e.g. deserts, arid mountains) or simply because parts of these areas are poorly known at all (e.g. because of political instability, lacking infrastructure). Since the different genera (see also chapter 8) are variably classified in literature and the extent of Limacidae is still to be discussed, the geographic range of distribution has to be validated.

The genus *Limax* comprises large (6-30 cm) slugs, which show exclusively nocturnal activity and feed in particular on lichens, fungi and dead plant material. The distribution range of the genus covers Europe (Falkner *et al.*, 2001; Manganelli *et al.*, 1995); the species *Limax maximus* Linnaeus, 1758 has been introduced almost worldwide. Species numbers are rather high in Southern Europe, mainly the Mediterranean region (Lessona & Pollonera, 1882; Wiktor, 2001) and in the Alpine region (e.g. Simroth, 1885, 1901, 1910; Heynemann, 1905; Hesse, 1926; Simroth & Hoffmann, 1928; Alzona, 1971; Boato *et al.*, 1989). Another hotspot of diversity seems to be the Balkan area (e.g. Rähle, 1976; Wiktor, 1983, 1996, 2001).

Knowledge of most of the *Limax* species is comparatively poor, given the fact that the animals are quite large, move slow and live in terrestrial habitats in Europe.

Morphology

Up to know species definitions in *Limax* have been based on external morphology and also on the complex genital anatomy. One major problem in slug research in general and particularly in the genus *Limax* is the apparent lack of diagnostic characters of external morphology, such as a well developed shell. Further

characters such as the copulation behaviour (e.g. Taylor, 1902-07; Peyer & Kuhn, 1928; Gerhard, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941), which is probably more informative than external characters, are poorly documented for most of the already described species. The same applies for data on the vestigial shell, jaws or the radula, which are only occasionally mentioned in the old literature. Body size, shape and colouration are all very variable and potentially misleading (Klee *et al.,* 2007). Spermatophores, used in other slugs for species discrimination (e.g. Wiktor, 1987), are indistinct in *Limax*. Morphological determinations are hampered by the high colour variability (Nitz *et al*., 2009, Heim *et al*., 2010) and by the fact that specimens have to be fully mature to be used for genital character analysis. Furthermore, the genital anatomy is influenced by nourishment, parasitism and also by changes during the developmental stages in adult slugs (male phase vs. female phase). In some studies, morphometric characters are used (e.g. Quick, 1960; Wiktor, 1983, 1996, 2001); however, due to differences in fixation and storage, they are sometimes not comparable. Another problem is the fact, that species descriptions in *Limax* are usually based on a small series of individuals, sometimes even on one specimen, therefore inter- and intraspecific variation is rarely discussed.
Thus species identifications in bioinventories and collections are often doubtful (personal observation based on museum samples) and lead in the past to problematic species lists and extensive synonymy lists, which can exhibit a high discordance (e.g. Taylor, 1902-07; Hesse, 1926; Alzona, 1971; Wiktor, 1996, 2001).
The aforementioned facts lead to a high degree of confusion in the taxonomy of the genus *Limax*. This is obvious in disagreements in estimated species numbers, ranging from about 15 species (Schileyko, 2003) up to 40 species (Wiktor, 2001). Confusion is also evident in the varying usage of terms like ‘varietates’ (Hesse, 1926) or ‘forms’. Alzona (1971) lists for instance 20 species, 72 subspecies, 10 forms and 15 synonyms for Italy. Although the nomenclatural meaning of these terms is regulated by the International Code of Zoological Nomenclature (ICZN, 1999), it is unclear which of these nominal taxa should be regarded as true species today. Wiktor states in a publication in the year 2001 that “the genus requires revision”.
For this purpose, an evaluation of the usefulness of the (morphological) characters in *Limax* is essential.

Copulation modes

The knowledge of the unique and highly complicated copulation behaviour of the genus *Limax* remained quite poor for centuries. The copulation itself was first reported and pictured quite early in the 17th century (Lister, 1678; Redi, 1684).

Further descriptions of copulations were published in the following centuries (e.g. Barbut, 1783; Adams, 1898; Dohrer, 1927). The first rather detailed studies were published by Fischer (1917) and by Peyer & Kuhn (1928) in the first half of the 20th century.

However, the most extensive studies dealing not only with the copulation of *Limax*, but with comparative sexual biology of slugs in general were carried out by Gerhardt (1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941). Gerhardt provided detailed descriptions, photographs and comparisons of the copulation of a number of *Limax* species. He defined four different copulation modes based on the copulation characters of the species *Limax maximus*, *L. cinereoniger*, *L. redii* and a new species, described by Niethammer (1936) as *L. gerhardti*. The main differences between the copulation modes are in the chronology and duration of the copulation phases, the absence or, if present, the length of the mucus thread, the length and morphology of the penis during copulation, and the position, shape and mode of transfer of the sperm mass. Gerhardt predicted in his publication of the year 1937 the existence of further copulation modes that might be defined after thorough studies of additional *Limax* species. He stated that the copulation type is the most reliable character for correct systematic assignment of species in the genus *Limax*.

In recent years, the value of copulation characters was rediscovered by René Heim (Natur-Museum Luzern - NMLU), Ulrich Schneppat (Bündner Naturmuseum Chur - BNM) and Gerhard Falkner (Staatliches Museum für Naturkunde Stuttgart - SMNS), who studied various *Limax* species on the basis of copulation observations (Nitz *et al.*, 2009, Nitz *et al.*, 2010, see chapter 5 and 6). Falkner & Niederhöfer (2008) even used a noticeable copulation mode as a reason to define a new subgenus of *Limax* in their species description of *Limax (Brachylimax) giovannellae* Falkner & Niederhöfer, 2008.

The unique and complex copulation behaviour and the associated morphological characters like penis length and shape are diagnostic criteria for each species. Observations of G. Falkner have shown, that the copulation process is highly sensitive: sometimes already a 20% difference in penis length hinders a successful copulation (G. Falkner, pers. comm.). Copulation sites are on vertical tree trunks, rocks or walls. Copulation behaviour in *Limax* involves several distinct stages (see also Hyman, 2006). In most observed *Limax* species, the copulation starts with two slugs following one another on the way to a potential copulation site (precopulation behaviour). The slugs start to form a circle with their bodies when a suitable copulation site is reached. The copulation itself starts with entwining of both slug bodies while hanging head-down and with producing a mucus thread, sail or simple

spot. In this phase, the genital pores of the partners open and the penes start to evert. The penes entwine themselves while the penes tips stay loose. Colouration of the penes is often bluish, the tips are creamy white. The length of the fully everted penes can vary between a few centimeters to nearly on meter depending on the species. Once the full extension is reached, the penes start to contract partly to form a pear-like form with the tips in contact. At the tips of the penes, the sperm mass is transfered, in most cases reciprocally. While the penes still form a mass, the partners start to separate. The penes are expanded until they loose contact and the animals start to retract them. Postcopulatory behaviour starts with cleaning each other and, in most cases, with one of the animals eating the slime thread (if present). The described phases can greatly differ between species, the same applies for the duration of the single steps and the whole copulation ranging from 15 minutes to several hours. In nearly most species, copulation takes place during the night in full darkness.

For standardising the comparison of the copulation phases in different *Limax* species, the participants (including B. Nitz) of the "First annual meeting of Task Force *Limax*" in Chur, Switzerland 2006 agreed on the following terminology of the copulation phases in German and English (Hyman, 2006):

Phases 1 - 2: Precopulation behaviour/Prelude

- Phase 1: “Hinterherkriechen/Verfolgung” - following (document timing and course)
- Phase 2: “Kreisbildung” - formation of circle (document diameter and overlap)

Phases 3 - 9: Copulation behaviour

- Phase 3: “Körperumschlingung” - body entwining (document free tails, start of mucus mass)
- Phase 4: “Abseilen” - abseiling (document length of slime threads)
- Phase 5: “Penisausstülpung” - penis eversion (document timing, length, structure,)
- Phase 6: “Penisumschlingung” - penis entwining (document type of entwining)
- Phase 7: “Birnenstadium” - pear-shape - ends with sperm mass transfer (document mode of retraction, structure of “spoon/bell”)
- Phase 8: “Penistrennung und -retraktion” - penis separation and retraction
- Phase 9: “Paarungsende” - end of copulation

Phase 10: Postcopulation behaviour

- Phase 10: “Postkopulationsverhalten” - postcopulation behaviour (document cleaning, feeding on the slime thread)

Molecular background

As outlined above, morphological discrimination of *Limax* species is a very complex task due to high variability and the fact, that only fully adult specimens are suitable for genital comparisons. Furthermore, the quick recognition of new or undetected species in the genus would be very helpful. Molecular data sets may serve as a valuable additional source of information in slug research and as a character set for subsequent identification and for phylogenetic analyses. During the last few decades the use of DNA sequence variation data has become a common tool in the reconstruction of phylogenetic relationships on various taxonomic levels. Mitochondrial genes have been shown to be a useful character set in resolving relationships among closely related species groups for a wide range of taxa (Harasewych *et al.*, 1997; see review in Avise, 1994). One of the most commonly used genes for phylogenetic tree reconstructions is the mitochondrial cytochrome c oxidase subunit I (COI).

COI is used not only for phylogenetic tree reconstruction, but also for species identification and the assignment of individuals. For most animal species, intraspecific variation of COI-sequence is far less than variation between species, making the gene a diagnostic molecular character set for systematic biology. Accordingly and as foreashadowed by Hebert *et al.* (2003a, b; Remigio & Hebert, 2003), partial COI (about 660 base pairs) has become the most established "DNA barcoding" gene and in this context is suggested for specimen (re-)identification and discovering newly encountered species. Several approaches are currently used for these purposes. Firstly, tree based methods should reveal the identity of unknown samples by their position in a previously characterised phylogeny (Hebert *et al.*, 2003a, b), assuming that the COI gene tree reflects a valid species tree. The second approach is to use a threshold value of sequence divergence to separate intraspecific from interspecific variation. This threshold value can be choosen in several ways. It can be based on a fixed threshold value, e.g. 3% sequence difference (Hebert *et al.*, 2003a, b), or, alternatively, a threshold of ten times the average of the intraspecific divergence is proposed (Hebert *et al.*, 2004). This works quite well in the majority of animal groups: more than 95% of species possess unique COI barcode sequences and species level identification is possible in most cases (Hajibabaei *et al.*, 2007; see also Waugh 2007 for a summary). Exceptions are found, for example, in Cnidaria (Hebert *et al.*, 2003b) or in insects (Whitworth *et al.*, 2007; Elias *et al.*, 2007) and in some cases in stylommatophoran land snails, where Davison *et al.* (2009) show high error rates in species identification using COI barcodes. A number of approaches have been published recently based, for

example, on character-based identification (Sarkar *et al.*, 2008) or on the barcoding gap. The barcoding gap occurs in the distribution of pairwise difference between intraspecific and interspecific divergences in a typical barcode data set (Meier *et al.*, 2008; Meyer & Paulay, 2005). One tool, which automatically searches for significant differences in the barcoding gap without an a priori species hypothesis, is ABGD (Automatic Barcoding Gap Discovery) (Puillandre *et al.*, 2011; 2012).

The question of the usefulness of barcoding in general (Taylor & Harris, 2012), the shortcomings of the current methodological approaches and the inappropriate use of barcoding, all common themes in DNA barcoding literature (Collins & Cruickshank, 2012), have recently raised a new controversy about this topic. Additionally, recent studies have shown quite high potential error rates in species identification based on DNA barcoding alone in closely related species systems (van Velzen *et al.*, 2012; Dupuis *et al.*, 2012), strongly suggesting either a multilocus approach (Dupuis *et al.*, 2012), which was not within the budget for this Thesis, or a use of DNA sequences only in combination with solid taxonomic foundations (Meyer & Paulay, 2005). One way out of this discussion is an integrative taxonomy approach (e.g. Goldstein & DeSalle, 2010; see also the review by Padial *et al.*, 2010), that takes into account not only molecular data, but also additional information like morphological or geographical data. The application of this combined approach in *Limax* is emphasised and discussed in chapters 5, 6 and 8 of the Thesis.

5 Article I: Towards a new standard in slug species descriptions: the case of *Limax sarnensis* Heim & Nitz n. sp.

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TOWARDS A NEW STANDARD IN SLUG SPECIES DESCRIPTIONS: THE CASE OF *LIMAX SARNENSIS* HEIM & NITZ N. SP. (PULMONATA: LIMACIDAE) FROM THE WESTERN CENTRAL ALPS

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ABSTRACT

The terrestrial slug *Limax sarnensis* Heim & Nitz new species is described from morphological and molecular characters, based on 298 specimens from 64 localities. Detailed descriptions of coloration, reproductive anatomy, distribution and ecology are provided. The new species differs from all other sympatric congeners by a diagnostic combination of characters: variable coloration of body with unicoloured mantle; outer fields of tripartite sole light grey to nearly black, fading from posterior to anterior and from outer edges to unpigmented middle field; penis dimension in preserved specimens about one-third to half of body length; penis interior with small transverse riblets, one longitudinal interior crest, a transverse penial crest and one longitudinal interior cord; copulates on a slime thread. It is restricted to inner alpine habitats in Switzerland and northern Italy. Phylogenetic analysis of 47 *Limax* specimens and outgroups using 1317 nucleotides of the cytochrome *c* oxidase subunit I gene supports the recognition of *L. sarnensis* as a new species. *Limax alpinus* Férussac, 1822, becomes a junior synonym of *Limax cinereoniger* Wolf, 1803, by the designation of a neotype. Genotypic and phenotypic data are concordant with copulation (behavioural observations). The combination of morphological, genetic, ecological and behavioural data should set a new standard in slug species description.

INTRODUCTION

The genus *Limax* (Stylommatophora: Limacoidea: Limacidae) consists of large, terrestrial slugs probably native to the European continent (Wiktor & Likharev, 1979; Wiktor, 1996, 2001); one species (*Limax maximus* Linnaeus, 1758) has been introduced worldwide. Two hotspots of diversity are the Mediterranean area (Lessona & Pollonera, 1882; Wiktor, 2001) and the Alps (e.g. Simroth, 1885, 1901, 1910; Heynemann, 1905; Hesse, 1926; Simroth & Hoffmann, 1928; Alzona, 1971; Boato *et al.*, 1989), but the Balkan area also contains a substantial diversity of species (e.g. Rähle, 1976; Wiktor, 1983, 1996). Nearly all species are poorly known, and many historical identifications are doubtful (personal observation based on museum samples). Accordingly, synonymy lists are extensive (e.g. Taylor, 1902–1907; Hesse, 1926; Alzona, 1971; Wiktor, 1996, 2001) and, as we will show, an undetected species new to science is present in the middle of Europe.

One of the major problems in slug research is the apparent lack of diagnostic characters of external morphology, such as a well-developed shell. The vestigial shell, body size, shape and coloration are all very variable and potentially misleading (Klee, Hyman & Haszprunar, 2007). Furthermore, spermatophores are absent, which in other slugs (Milacidae, Arionidae) can be used for species discrimination (e.g. Wiktor, 1987). Even (male) genital anatomy, hitherto regarded as diagnostic for most species, is not conclusive and is also significantly influenced by ecological factors such as nourishment or parasitism, as well as stage of development. Morphometric characters used in various studies (e.g. Quick, 1960; Wiktor, 1983, 1996, 2001) are sometimes not comparable and provide unsatisfactory results due to differences in preservation and storage techniques.

The extraordinary and complicated copulation behaviour of *Limax* species (e.g. Taylor, 1902–1907; Peyer & Kuhn, 1928; Gerhardt, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941) is certainly more informative, but data are not available for most of the described species. Additional characters such as the radula, jaws or gut anatomy are not (or only occasionally) mentioned in the old literature.

Species descriptions in the majority of slug studies are based on a small series of specimens or even on one individual. This fact hinders the estimation of the inter- and intraspecific variation present in these characters.

All these problems have caused a high degree of confusion in the taxonomy of *Limax* species, as is obvious for example in the range of estimated species numbers for this genus, ranging from *c.* 15 species (Schileyko, 2003) up to 40 species (Wiktor, 2001). Disagreements in species evaluation are also obvious in the contrasting treatment of synonyms, varieties and subspecies. For example, in Italy Alzona (1971) lists 20 species, 72 subspecies (reduced by the editor to chromatic phenotypes), 10 'forms' and 15 synonyms. Definitions of terms like 'varietates' (e.g. Hesse, 1926) are not given, and it is unclear which of these terms are considered to be equivalent to the species level

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or should be regarded as species today. Wiktor (2001) states in a recent publication that 'the genus requires revision'.

To facilitate comprehensive and comparative research on slugs, in the future descriptions should include data on biogeography, morphology, coloration and, if available, copulation behaviour. DNA sequences of the barcode gene, cytochrome *c* oxidase subunit I (COI), may serve as a valuable additional character set for subsequent identification and for phylogenetic analyses.

As part of a continuing broad study of the genus *Limax* (e.g. Hyman, 2006; Klee *et al.*, 2007), the present paper aims to describe *Limax sarnensis* new species from the Western Central Alps, including characters of morphology, copulation behaviour and mitochondrial DNA. The second, equally important aim is to set a new standard in slug species description and provide a template for future work.

MATERIAL AND METHODS

Collection and treatment of specimens

A large proportion of the specimens of the new *Limax* species were collected by the authors and by members of Task-Force-Limax (Hyman, 2006). In addition, further *Limax* species that were either similar in appearance or have overlapping distribution patterns were collected for morphological comparison and genetic differentiation: *Limax cinereoniger* Wolf, 1803, *Limax maximus* Linnaeus, 1758, *Limax* cf. n. sp. 'Blauköpfige Egelschnecke' *sensu* Turner *et al.* (1998), *Limax* cf. *engadinensis* Heynemann, 1862 and *Limax* sp. 'Southern Alps'. Also included in a phylogenetic analysis of the genus *Limax* was *Limax wohlberedti* Simroth, 1900 and outgroups were *Vitrina pellucida* (Müller, 1774) (Vitrinidae), *Lehmannia marginata* (Müller, 1774) (Limacidae) and *Limacus flavus* (Linnaeus, 1758) (Limacidae). Table 1 provides information on specimens, sampling localities, collectors and deposition of material.

Most of the mature specimens were photographed alive in dorsal, lateral and ventral views; additional photos documented the development of eggs and juveniles. Tissue samples for DNA extraction were taken from the left side of the mantle (most living specimens, some preserved specimens) or from the tip of the tail or sole (preserved material). The removal of tissue from the left side of the mantle of the living animal is only minimally invasive so that the slugs survived and sometimes even reproduced afterwards. The majority of the animals were kept alive until they were presumably adult; a smaller number were killed in earlier stages of development. The animals were relaxed and preserved using a method developed by Schneppat and Heim. This process has been developed from the traditional method of relaxing and killing the slug in water and preserving it with ethanol. For relaxation, a single slug was put into a jar slightly longer than the full length of the animal. The jar was filled with unchlorinated water and two to three drops of a solution of the synthetic tenside SUPRALAN-UF (three parts SUPRALAN-UF – a fatty alcohol polyglycol ether, supplier: Bauer Handels GmbH, Adetswil, Switzerland – to two parts water) were added and mixed by gentle shaking. After some minutes (depending on the size of the animal) the slug was narcotized, relaxed and usually stretched out with everted ommatophores. The slug was kept in the jar until dead. The amount of time this requires depended on the size of the animals as well as on the storage temperature. It was important to store the jar with the slug at or below room temperature, preferably in a refrigerator if the weather was hot, in order to prevent autolytic damage of tissue. Big animals were generally killed overnight in a refrigerator. Small- and medium-sized slugs needed 30 min to *c.* 3 h at room temperature, or overnight in a refrigerator. The advantage of this method was that the slug was anaesthetized quickly, minimizing the struggling that occurs in plain water or ethanol. This avoided common artefacts such as everted penes, contracted body and genitals, and enabled accurate comparison of slugs killed using this same technique.

The dead slug was cleaned of mucus in a sieve under cold running water, because mucus diluted the concentration of the preserving reagent and therefore could delay the preservation process.

For preservation, ethanol (96%) was gently injected with a small needle into the body cavity through the terminal tip of the sole in an acute angle between sole musculature and intestines. After injection, the specimen was put in a dish with the sole downwards and was covered with ethanol (96%) for 4–12 h depending on size. After this final step, specimens were stored in 75% ethanol. We changed the ethanol at least twice in the days following to prevent dilution of ethanol concentration.

Material was deposited in the Zoological State Collection (ZSM), Bündner Naturmuseum Chur (BNM) and Natur-Museum Luzern (NMLU) (Table 1); DNA elutions are stored in the DNA Bank of the ZSM (see www.zsm.mwn.de/dnabank/). Additional material of *Limax* species from the Alps was borrowed from the collections of BNM, Naturhistorisches Museum Bern (NMBE), Naturhistorisches Museum Basel (NMB), National Museum of Natural History (NMNH), Leiden and NMLU, and was dissected for comparison.

Eggs were preserved in unbuffered 3–4% formaldehyde solution.

Morphological studies

The total length, mantle length and width (of living and preserved animals; living animals in extended crawling position), sole length and width, and keel length (preserved animals only) of nearly 300 animals were measured using vernier callipers or a ruler. The weight of living animals was recorded.

Only animals that were either visibly mature, had copulated or had laid eggs were chosen for dissection, to ensure that characters were fully developed and comparable. Maturity was determined prior to dissection by examining the genital pore, which is easily visible and widely open in sexually mature animals, but invisible or only slightly open in juvenile or subadult animals.

The general method of dissecting follows Wiktor (2000). However, dissection of the penis is described in detail below, owing to the lack of information in the literature. In the descriptions of the genitalia the term 'distal' denotes parts closest to the genital opening.

Before starting the dissection, it was helpful carefully to widen the penis lumen by injecting ethanol (70%) at low pressure through the genital pore using a small syringe with a blunt tip. Dissection was done under a dissecting microscope. The penis wall was opened with ophthalmic scissors, usually starting from the proximal end, slightly to the right of the insertion point of the vas deferens and penis retractor muscle. This procedure was appropriate when the penis wall was thick and not transparent. If transparency of the penis wall permitted orientation and discrimination of the main internal structures (e.g. longitudinal interior penial cord and longitudinal interior penial crest), the opening cut was started at the atrium. The cut was made in a straight line towards the proximal or distal end to preserve all internal structures. It was necessary to extend the initial cut distally through the genital pore and atrium and proximally to the rounded end of the penis tip in order to free all important structures. After opening the penis, the genitalia were pinned and covered with ethanol (70%). If the animal had already copulated, the

Table 1. Locality, collector, museum registration numbers and, if sequenced, GenBank accession number of the specimens.

Species	Locality	Collector, year	Museum registration numbers	GenBank accession number	Specimens (*n*)
Limax sarnensis	Val di Campo, Ticino, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071504–20071516, 20071492, 20071528–20071532, 20071493	FJ606484 = ZSM Mol 20071492, FJ606487 = ZSM Mol 20071509	20
	Intragna, Ticino, CH	U. Schneppat & C. Howart	ZSM Mol 20071577, 20071578		2
	Lavizzarra, Ticino, CH	R. Heim, 2000	NMLU 13475, 13581, 14215–14222		10
	Airolo, Ticino, CH	L. Forcart, 1932	NMB 3159-b		1
	Airolo, Ticino, CH	R. & G. Heim, 2007	NMLU 14255, 14256		2
	Cavigliano, Ticino, CH	L. Reser, 2007	NMLU 14319		1
	Ronco Sopra Ascona, Ticino, CH	M. von Moos, 2004	NMLU 14240		1
	Prato, Ticino, CH	L. Forcart, 1932	NMB 3168-k-1, 3168-k-2		2
	Locarno, Ticino, CH	R. Heim, 2001	NMLU 14230		1
	Locarno, Ticino, CH	E. Schneppat & U. Anhorn, 2006	NMLU 14262–14269	FJ606493 = NMLU 14262	8
	Locarno, Ticino, CH	R. Cornu, 2007	BNM 54004, 54005, NMLU 14444, 14445		4
	Faido, Ticino, CH	M.-L. Kieffer, 2006	ZSM Mol 20071579–20071583		5
	Olivone, Ticino, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071503	FJ606486	1
	Astano, Ticino, CH	M. Wüthrich, 1969	NMBE 3237-1 to 3237-3		3
	Astano, Ticino, CH	J. Hassler, 2006	ZSM Mol 20071568, 20071569		2
	Astano, Ticino, CH	F. Zemp, 2007	NMLU 14318		1
	Lavertezzo, Ticino, CH	R. Heim, 2001	NMLU 14206–14214, 14223		10
	Vogorno, Ticino, CH	C. Oberer, 2001	NMLU 14225		1
	Caslano, Ticino, CH	M. Wüthrich, 1957	NMBE 1654-1, 1654-2, 1654-3		3
	Caslano, Ticino, CH	M. von Moos, 2004	NMLU 14239		1
	Morcote, Ticino, CH	M. Wüthrich, 1988	NMBE 5290		1
	Poleggio, Ticino, CH	R. Heim, 1999	NMLU 13021		1
	Lugano, Ticino, CH	M. Colling, 2006	ZSM Mol 20071549		1
	Göschenen, Uri, CH	E. Paravicini, 1944	NMB 6633d		1
	Göschenen, Uri, CH	L. Forcart, 1932	NMB 3159-t		1
	Göschenen, Uri, CH	R. & G. Heim, 2001	NMLU 14226–14229	FJ606497 = NMLU 14229	4
	Hospental, Uri, CH	J. Rüetschi, 2007	BNM 54007		1
	Hospental, Uri, CH	R. & G. Heim, 2006	NMLU 14254	FJ606499	1
	Andermatt, Uri, CH	J. Rüetschi, 2007	BNM 54026		1
	Innerkirchen, Berne, CH	D. Tschanz, 2006	NMLU 14257–14261	FJ606494 = NMLU 14257	5
	Gutlannen, Berne, CH	R. & G. Heim, 2006	NMLU 14241–14243		3
	Gadmen, Berne, CH	R. & G. Heim, 2006	NMLU 14244–14247		4
	Trient, Vallais, CH	M. Wüthrich, 1960	NMBE 5423-1 to 5423-3		3
	Salvan, Vallais, CH	M. Wüthrich, 1962	NMBE 2501-2		1
	Grächen, Vallais, CH	G. Bollinger, 1914	NMB 1060-o-1, 1060-o-2		2
	Grächen, Vallais, CH	M. Wüthrich, 1961	NMBE 2346		1
	Saas Fee, Vallais, CH	R. Walliser, 2006	ZSM Mol 20071572		1
	Saas Fee, Vallais, CH	M. Wüthrich, 1961	NMBE 2336-1 to 2336-7		7

Continued

Table 1. *Continued*

Species	Locality	Collector, year	Museum registration numbers	GenBank accession number	Specimens (*n*)
	Ried-Mörel, Vallais, CH	E. Paravicini, 1942	NMB 1060-n-1 to 1060-n-6		6
	Ried-Brig, Vallais, CH	R. Walliser, 2006	ZSM Mol 20071570, 20071571		2
	Zwischbergen, Vallais, CH	M. Wüthrich, 1980	NMBE 4300		1
	Oberwald, Vallais, CH	R. & G. Heim, 2006	NMLU 14251–14253	FJ606496 = NMLU 14251, FJ606498 = NMLU 14252	3
	Enlebuch, Lucerne, CH	R. & G. Heim, 2006	NMLU 14248–14250	FJ606495 = NMLU 14248	3
	Sarnen, Obwalden, CH	R. Heim, 1999–2007	NMLU 14200= Holotype and 43 Paratypes in NMLU, ZSM, NMBE (see description)	FJ606482 = NMLU 13438, FJ606500 = NMLU 14279, FJ606483 = NMLU 13440	44
	Tujetsch/Tavetsch, Grisons, CH	R. Levy, 2006	ZSM Mol 20071558–20071561, 20071590–20071611, 20071573, 20071574	FJ606491 = ZSM Mol 20071558	28
	Tujetsch/Tavetsch, Grisons, CH	W. Schiler, 1953	NMB 3159-n		1
	Medel, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071494–20071498		5
	Sumvitg/Somvix, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071499–20071502, 20071517–20071527, F1: ZSM Mol 20071584–20071588	FJ606485 = ZSM Mol 20071500	20
	Obersaxen, Grisons, CH	U. Schneppat & R. Cornu, 2007	BNM 54011–54015		5
	Vuorz/Waltensburg, Grisons, CH	U. Schneppat & R. Cornu, 2007	BNM 54016, 54049, 54073–54078		8
	Flond, Grisons, CH	U. Schneppat, 2007	BNM 54008–54010		3
	Mesocco, Grisons, CH	L. Forcart, 1926	NMB 3168-e		1
	Lostallo, Grisons, CH	U. Schneppat, 2006	ZSM Mol 20071575, 20071576		2
	Mesocco, Grisons, CH	U. Schneppat, 2007	ZSM Mol 20071589		1
	Bondo, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071553–20071557	FJ606490 = ZSM Mol 20071553	5
	Stampa, Grisons, CH	L. Forcart, 1928	NMB 3168-g		1
	Stampa, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071550–20071552	FJ606489 = ZSM Mol 20071552	3
	Poschiavo, Grisons, CH	L. Forcart, 1928	NMB 6633-f		1
	Poschiavo, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071533–20071542		10
	Brusio, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071543–20071548, 20071562–20071567	FJ606488 = ZSM Mol 20071543	12
	Arvier, Aoste, IT	R. & G. Heim, 2003	NMLU 14231–14238		8
	Crodo, Verbano, IT	R. & G. Heim, 2006	NMLU 14270–14274	FJ606492 = NMLU 14273	5
	Fromazza, Verbano, IT	L. Forcart, 1949	NMB 3159-k		1
	Cànero Riviera, Verbano, IT	L. Forcart, 1949	NMB 6633-g		1
Limax wohlberedti	Dalmatia, CRO	A. Wiktor, 1999	Muzeum Przyrodnicze Uniwersytetu Wroclawskiego, Coll. A. Wiktor 3004	FJ606481	
Limax cinereoniger	Sarnen, Obwalden, CH	R. Heim, 2000	NMLU 13060	FJ606459	
	Bonneville, Rhone-Alpes, F	H. & B. Nitz, 2006	ZSM Mol 20071615	FJ606464	
	Krauchthal, Berne, CH	D. Tschanz, 2006	ZSM Mol 20071631	FJ606465	
	Dresden, Saxonia, D	A. Pohl, 2006	ZSM Mol 20071616	FJ606462	
	Wicklow, Ireland, UK	R. Boyce, 2006	ZSM Mol 20071617	FJ606458	
	Limburg, NL	E. Gittenberger, 1982	RMNH 2315/EG.821009	FJ606461	

	Oberkrumbach, D	E. Klee, A. Klee & B. Nitz, 2006, 2007	ZSM Mol 20071618, 20071619		FJ606460, FJ606463
Limax sp. 'Southern Alps'	Esino, Lombardia, IT	R. Heim, 2004	NMLU 14458, 14459		FJ606473, FJ606472
	Caprino, Ticino, CH	A.J. de Winter, 1989	RMNH 106757		FJ606474
Limax cf. n. sp. 'Blauköpfige Egelschnecke'	Carona, Ticino, CH	M. Wüthrich, 1989	NMBE 5400		FJ606479
	San Salvatore, Ticino, CH	A.J. de Winter, 1989	RMNH 106761		FJ606480
Limax maximus	Sursee, Lucerne, CH	R. Heim, 2004	NMLU 13744		FJ606469
	Chur, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071620		FJ606467
	Eutin, Schleswig-Holstein, D	J. Erzold, 2006	ZSM Mol 20071621		FJ606470
	Oberlausitz, Saxonia, D	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071622		FJ606471
	Lassendorf, AU	C. Wieser, 2006	ZSM Mol 20071623		FJ606466
	Kent, UK	I. Hyman, 2006	ZSM Mol 20071624		FJ606468
Limax cf. *engadinensis*	Vinschgau, Bolzano-Bozen, IT	T. Kopf, 2006	ZSM Mol 20071625		FJ606477
	St Moritz, Grisons, CH	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071626, 20071627		FJ606475, FJ606476
	Tamins, Grisons, CH	R. Cornu & M. Kieffer, 2006	ZSM Mol 20071628		FJ606478
Vitrina pellucida	Kolbingen, D	B. Hausdorf, 2006	ZMH 51046		FJ606454
Limacus flavus	Goch, D	S. Henssen, 2006	ZSM Mol 20071629		FJ606456
Limacus flavus	Banstead, Surrey, UK	J. Hutchinson, 2007	ZSM Mol 20071630		FJ606457
Lehmannia marginata	Dalsland, Sweden	R. Heim, 2001	NMLU 14457		FJ606455

Abbreviations: AU, Austria; CH, Switzerland; CRO, Croatia; D, Germany; F, France; IT, Italy; NL, The Netherlands; UK, United Kingdom.

lumen of the penis was usually filled with a mass of mucus and sperm, often causing a swollen end. This mass usually adhered to all interior structures and had to be carefully removed to allow all the details to be seen. It was cleaned away first with fine forceps, and then with fine brushes of varying hardness.

Dissections were photographed for documentation and drawn. The radula, jaw and shell of a selection of paratypes and animals from other localities were removed and prepared for photography. Dissected radulae and jaws were sputter-coated with gold and digitally photographed using a Leo 1430VP scanning electron microscope (SEM).

The weight of eggs in a clutch was determined by calculating the mean weight of 20 normal eggs (treated in a standard way by preservation in 3–4% formaldehyde and then drained before measurement). This standardizing treatment was necessary, since fresh egg weight was affected by differing humidity levels in captivity.

For the main part, the morphological terminology used in the present study follows Wiktor (1983, 1996, 2001) and Quick (1960). However, there are two cases where we have deviated from existing terminology. First, in cases where the vas deferens and penis retractor muscle do not insert at the tip of the penis but instead insert on the side, we have named the resulting blind end of the penis the 'blind penis tip' rather than the 'blind penis appendix' or 'caecum'. This appears to be a more accurate reflection of the anatomical structures. Furthermore, it avoids confusion with the term 'caecum' or 'coecum' as commonly used in the description of the intestine of a slug. Secondly, we have adjusted the terminology for internal penial structures. Several authors (e.g. Quick, 1960; Giusti & Mazzini, 1970; Giusti, 1973; Wiktor, 1983, 1996, 2001; Falkner, 2008) have already described internal penial structures of different *Limax* species; however, a consistent terminology of these structures is lacking. Below we provide a glossary of terms describing internal penial anatomy in the genus *Limax*.

Interior penial tongue A structure situated in the proximal part of the penis. It is found enrolled or as a wrinkled mass when the penis is dissected. This tongue is able to move freely when the penis is everted. Distally it is connected to the transverse penial crest. No descriptive term or phrase has been found in the literature. Apparently in other species of the genus this structure has been considered by the authors to be a part and prolongation of the longitudinal interior penial crest.

Longitudinal interior penial cord (Quick, 1960: 'fold', 'smooth fold'; Giusti & Mazzini, 1970: 'cordone', 'cordone papillare'; Giusti, 1973: 'cordone peniale') A string-like, flattened structure beginning near the atrium and running down to near the transverse penial crest, the surface covered with numerous tiny papillae. This structure is not visible when the penis is everted.

Longitudinal interior penial crest (Falkner, 2008: 'Kamm', 'Peniskamm'; Giusti & Mazzini, 1970: 'fold', 'cresta', 'struttura laminare'; Giusti, 1973: 'cresta peniale'; Quick, 1960: 'prominent fold', 'prominent frill', 'comb'; Wiktor, 1996: 'fold', 'longitudinal fold'; Wiktor, 1983, 2001: 'wide fold'; Wiktor, 2001: 'big fold') A band-like structure beginning near the atrium and running down to the transverse penial crest where it is connected with that structure. When the penis is everted, the longitudinal penial crest is easily visible as a free-moving and erect structure.

Penis wall The muscular tube of the penis, to which all interior and exterior structures are attached. The term is given only for clear understanding and differentiation from interior structures described here.

Transverse penial crest This is the distal portion of the internal penial tongue, but is named separately because it divides the lumen of the penis into a distal and a proximal portion. No descriptive term was found in the literature.

Transverse riblets Structures built up of papillae in transverse rows, covering the interior surface of the penial wall. No clear descriptive term was found in the literature.

Transverse chamfers (Falkner, 2008: 'Riefen') Very narrow, transverse structures, covering the surface of the longitudinal interior penial crest.

DNA sequence analysis

DNA was extracted from a small piece of tissue sampled from the mantle, sole or body wall of the slugs, using a QIAGEN extraction kit (Qiagen Blood and Tissue Kit). About 1340 nucleotides of the mitochondrial COI were amplified by using PCR (Saiki *et al.*, 1985; Mullis & Faloona, 1987) for all taxa using two primer sets: mtCOI-1F-54 (5′-TTTCAACAAAYCA TAARGATATTGG-3′) and mtCOI-1R-53 (5′-AAYACCA ATAGAAATTATAGCATAAA-3′) for the first fragment and mtCOI-2F (5′-TTAGCRGGGGCAATTACTATRC-3′) and mtCOI-2R (5′-CGAAAACAGATATTAACGAACCAT-3′) for the second fragment. The primers were based on the COI universal primers (Folmer *et al.*, 1994) and the primers used by Hyman, Ho & Jermiin (2007) and were assessed using the computer program Alignment 1.2 (Engels, 1993). The PCR conditions were: 92°C for 4 min, then 40 cycles of 92°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final elongation step of 72°C for 5 min.

PCR products were purified with one of three techniques, depending on the quality and intensity of the PCR results: a Qiagen DNA purification kit, Ultra Clean Band Excision Purification kit or with ExoSapIt [PCR product was incubated at 37°C for 30 min and then at 85°C for 15 min with 5 U of exonuclease I (Amersham) and 0.5 U shrimp alkaline phosphatase (Amersham) to cleave nucleotides one at a time from the ends of excess primers and to inactivate single nucleotides (Werle *et al.*, 1994)]. The purified PCR products were amplified with the same primers as above with a BigDye v3.1 Terminator Cycle Sequencing Kit, cleaned up with SephadexG-50 Superfine columns (GE Healthcare) and sequenced using an Applied Biosystems 3730 capillary automated sequencer according to the standard protocol. Sequences were assembled and proofread using Sequencher™ (Gene Codes Corporation) and were manually aligned in the program Se-Al v. 2.0a11 (Rambaut, 1996) and deposited in GenBank (for accession numbers see Table 1). The alignment was trimmed to 1317 nucleotides, starting with position 40 of the reference taxon *Biomphalaria glabrata* (Say, 1818) (GenBank number NC 005439) and finishing at position 1356.

Prior to phylogenetic analysis, the data were partitioned into first, second and third codon sites and the compositional heterogeneity of each partition was assessed using the program Homo (L.S. Jermiin, custom software), which implements Bowker's matched-pairs test of symmetry (Ababneh *et al.*, 2006).

Model selection was made using comparisons of hierarchical Likelihood Ratio Tests and Akaike Information Criterion scores in Modeltest 3.7 (Posada & Crandall, 1998). The general time-reversible (GTR) model with eight discrete gamma (Γ) categories and a proportion of invariant (I) sites (GTR + Γ8 + I) was used. Markov Chain Monte Carlo sampling was carried out in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) for 1,000,000 generations (four simultaneous chains, sample frequency 50, burn-in 100,000 generations). The program Tracer 1.2 (Rambaut & Drummond, 2004) was used to check adequate sampling and convergence to the stationary distribution. Majority-rule consensus trees were calculated from the sampled sets of trees.

The phylogenetic trees were rooted on *V. pellucida*, because Vitrinidae appear to be the most basal family in the superfamily Limacoidea (Hausdorf, 1998).

SYSTEMATIC DESCRIPTION

Suborder Stylommatophora A. Schmidt, 1855

Superfamily Limacoidea Lamarck, 1801

Family Limacidae Lamarck, 1801

Genus *Limax* Linnaeus, 1758

Type species: *Limax maximus* Linnaeus, 1758

***Limax sarnensis* Heim & Nitz n. sp.**

(Figs 1–3)

Types: Holotype: NMLU 14200 (photograph of living animal: Fig. 1A) Rischiwald, Glaubenberg, Community Sarnen, Canton Obwalden, Switzerland (46°52′44.85″N, 08°09′27.89″E, 1080 m), leg. R. Heim 26.09.2005; dimensions (living animal): weight 22 g, total length 160 mm, sole length 155 mm, sole width 13.5 mm, mantle length 45 mm; dimensions (preserved animal): total length 140 mm; sole length 139 mm, sole width 13 mm, mantle length 42 mm, keel length 32 mm, 18 wrinkles between mid line of dorsum and pneumostome; animal adult, genital pore visible and open, not dissected. Paratypes: 43 specimens, collected at type locality (Rischiwald, Glaubenberg, Community Sarnen, Canton Obwalden, Switzerland), leg. R. Heim: NMLU 13056–13058 (1999); NMLU 13438–13441 (2000); NMLU 14189–14199 (2000–2007), NMLU 14201–14205 (2000–2006); ZSM Mol 2007 1612 (ex-NMLU 14275) (2005); Bern NMBE 26270 (ex-NMLU 14276) (2006); ZSM Mol 2007 1613, ZSM Mol 2007 1614 (ex-NMLU 14277, ex-NMLU 14278), NMLU 14279 (2006–2007); NMLU 14280–14286 (2007); NMLU 14413-14420 (2008).

Etymology: Sarnensis means from Sarnen, capital of Canton Obwalden in Switzerland. The first specimens of the new species were found in the territory of the community of Sarnen.

Material examined ($n = 298$; 64 localities): All type material (44 specimens, see above); 254 specimens from 63 localities in Switzerland and Northern Italy (for details see Table 1).

Diagnosis: A *Limax* species of variable coloration, ranging from creamy white through brownish to black, body patterning absent or with spots or stripes present, mantle coloration without any pattern; outer fields of tripartite sole monochrome light grey to nearly black, fading from posterior to anterior and from outer edges to unpigmented middle field; penis dimension in preserved specimens about one-third to half of body length; vas deferens inserted close to tip, penis retractor muscle attached to penis at same point as vas deferens; penis internally covered with weak transverse folds, one longitudinal interior penial cord, a transverse penial crest and one longitudinal interior penial crest, raised at proximal end; copulates on slime thread.

Body: Animal rather large, living animal up to 196 mm long; sole length up to 190 mm (up to 167 mm in ethanol), width up to 22 mm (up to 17 mm in ethanol), mantle length up to 58 mm (54 mm in ethanol); keel length in ethanol up to 46 mm. Weight of living animal normally *c.* 20 g, sometimes up to 50 g. One single specimen reached in captivity the length of 245 mm and a weight of 54 g. Posterior mantle edge with obtuse angled point, keel prominent. Number of wrinkles between dorsal mid-line and pneumostome: 16–24. Structure of wrinkles fine and flattened.

Coloration (Fig. 1A–E): Very variable, monochrome or patterned. Body colour uniformly black or dark through bright brown to creamy white, dorsum sometimes lighter than sides;

Figure 1. External appearance of living specimens of *Limax sarnensis* Heim & Nitz n. sp. **A.** Holotype NMLU 14200. **B.** Brightly coloured specimen, paratype NMLU 14286. **C.** Dark specimen, NMLU 14228, Göschenertal, Switzerland. **D.** Striped specimen, NMLU 14260, Innertkirchen, Nessental, Switzerland. **E.** Spotted specimen, NMLU 14257, Innertkirchen, Nessental, Switzerland. **F.** Copulation, paratypes NMLU 14419/14420. Scale bars: **A–F** = 10 mm.

contrasting pattern (if present) of distinct spots arranged in irregular or regular rows to longitudinal stripes; pattern can be dark or creamy; dark spots sometimes with a bright frame. Keel brighter than body colour, sometimes lined with rows of dark spots. Colour of the mantle similar to or darker than body, always without pattern. Sole colour (Fig. 2A, B) variable, inner field always creamy white, colour of outer fields depending on intensity of body colour, ranging from nearly creamy white in pale animals through grey to black in darker animals; pigmentation of outer fields consists of very small pigmented spots, gradually becoming less dense from outer edge of sole fields to nonpigmented middle field. Intensity of sole coloration gradually fading from posterior to anterior or sometimes of uniform intensity. Coloration of head like body or slightly lighter, darker on top than on sides, sometimes with spotted pattern on top of head, tentacles greyish to creamy. Mucus of all body parts usually colourless, in rare cases red (Oberwald, Switzerland: NMLU 14251 and 14252; Crodo, Italy: NMLU 14271, 14272 and 14274) or yellow (Aosta Valley, Italy: NMLU 14236).

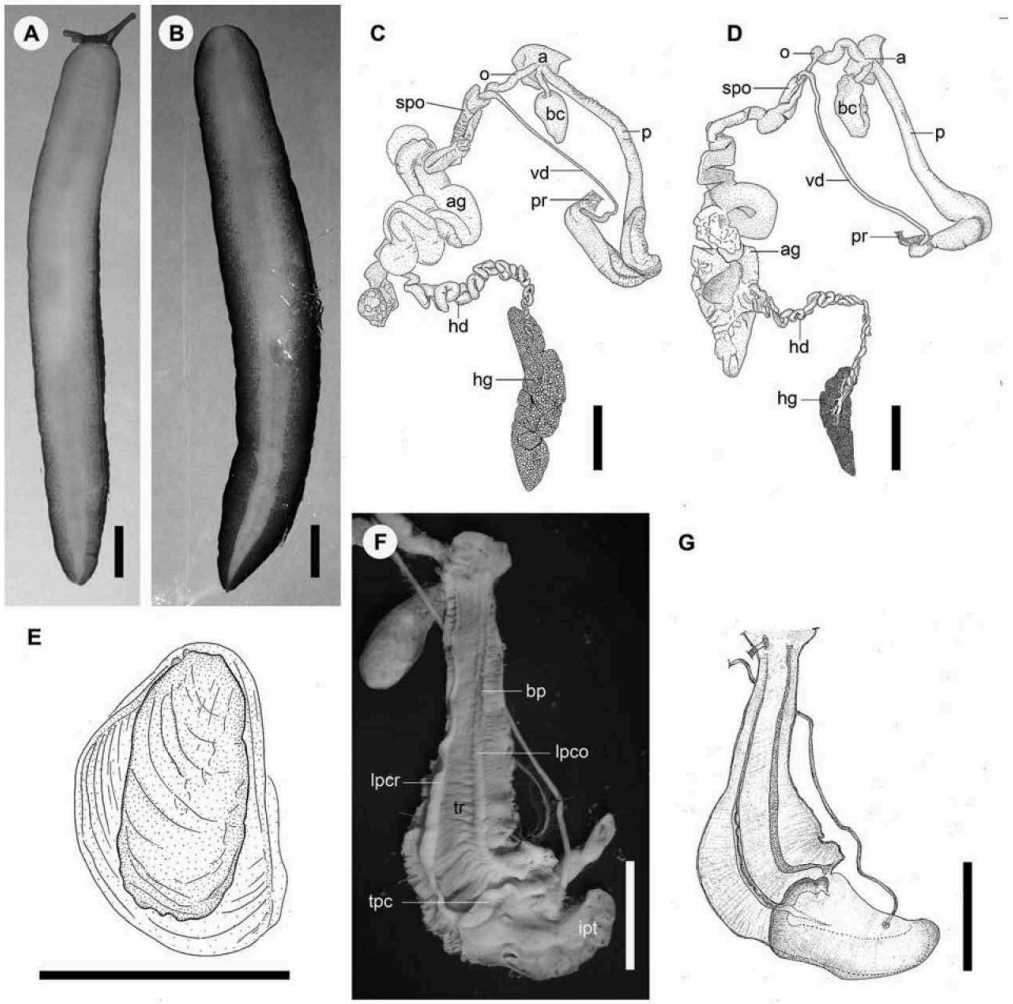

Figure 2. A, B. Sole coloration of living specimens of *Limax sarnensis* n. sp. **A.** Paratype NMLU 14419. **B.** Paratype NMLU 14415. **C, D.** Genital anatomy. **C.** Paratype NMLU 13438. **D.** Specimen ZSM Mol 20071533, Poschiavo, Switzerland. **E.** Shell, paratype NMLU 13438. **F, G.** Penial interior, paratype NMLU 14282. Scale bars: **A–G** = 10 mm. Abbreviations: a, atrium; ag, albumen gland; bc, bursa copulatrix; hd, hermaphrodite duct; hg, hermaphrodite gland; ipt, interior penial tongue; lpco, longitudinal interior penial cord; lpcr, longitudinal interior penial crest; o, oviduct; p, penis; pr, penis retractor muscle; spo, spermoviduct; tr, transverse riblets; tpc, transverse penial crest; vd, vas deferens. Drawings **C–E** by R. Kühbandner.

Genital anatomy ($n = 80$; Fig. 2C, D): Hermaphrodite gland oval or tongue-like, elongated, brown, usually fully embedded in digestive gland, sometimes positioned at end of body cavity and not fully embedded in digestive gland; hermaphrodite duct long, sometimes folded, coiled or convoluted at distal end, cream in colour; albumen gland well developed in adults in female stage, sometimes folded, yellowish, oval to triangular, size variable; spermoviduct sometimes folded; oviduct white, prostate cream; free oviduct with capsular gland well developed; vagina absent; duct of bursa copulatrix inserts into penis very near to junction of penis and free oviduct, duct and sac distinct, sac oval or pear-shaped, fixed with connecting fibres at free oviduct, atrium very short, almost invisible; penis tubular, thicker at end, 30–67 mm in adult animals, or about one-third to half length of body in preserved stage, distal part straight; proximal part nearly always bent and often hooked at end; vas deferens inserted close to penis end, leaving 1–3 mm blind round tip; penis retractor muscle attached to penis at same point as vas deferens, attached to body wall on left proximal side of pallial cavity; vas deferens enters penis with a simple pore; penis interior (Fig. 2F, G) divided into two portions by transverse penial crest towards end of penis, entry point of vas deferens contained in proximal portion; two portions connected by small openings between wrinkles of transverse penial crest; transverse penial crest may project into proximal portion and is prolonged proximally into interior penial tongue; one longitudinal interior penial crest present in distal portion of penis, beginning at opening of duct of bursa

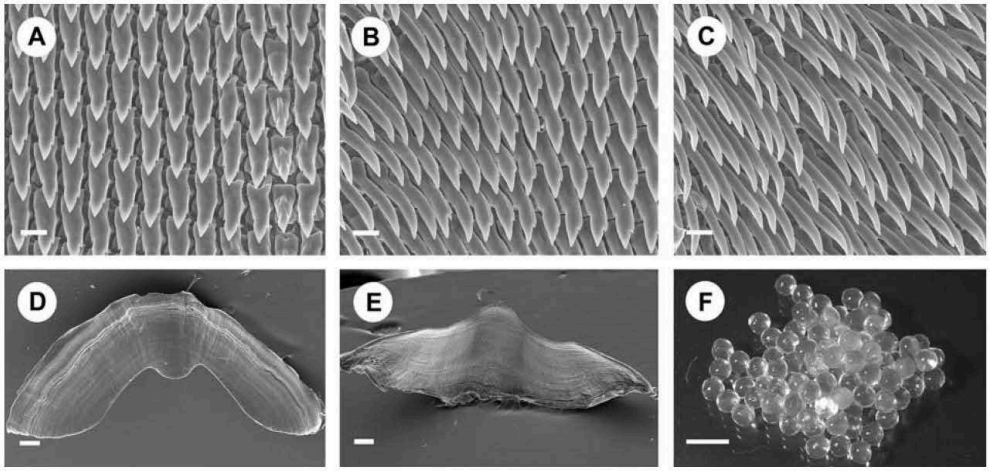

Figure 3. A–C. Radula, paratype NMLU 13438. **A.** Central and lateral teeth. **B.** Lateral and marginal teeth. **C.** Marginal teeth. **D, E.** Jaw, specimen NMLU 14240, Canton Ticino, Switzerland. **D.** Lateral view. **E.** Dorsal view. **F.** Eggs of specimen NMLU 14231, Aosta valley, Italy. Scale bars: **A–C** = 20 μm; **D, E** = 200 μm; **F** = 10 mm.

copulatrix, made up of single papillae at distal end, becoming wider and more strongly raised towards proximal end, attaching to transverse penial crest; longitudinal interior penial crest with nearly smooth surface without any visible structure of papillae but structured with numerous very fine transverse chamfers; one longitudinal interior penial cord present, running along entire length of distal portion of penis from near atrium, becoming slightly stronger towards proximal end, proximally forming a fan-like structure which does not connect to transverse penial crest, penial cord covered over entire length with numerous very small papillae, distal half with greyish or blackish pigmentation, particularly in centre; distal portion of penis wall internally covered with fine, weak transverse riblets, built up from numerous very small and short papillae; proximal portion of penis wall smooth without any visible accessory structures besides interior penial tongue, slight projection of longitudinal interior penial crest and entrance of vas deferens.

Shell (n = 33; type loc. $n = 8$, shown in brackets; Fig. 2E): Shell asymmetric, 8.2–17.2 mm (9.7–12.5 mm) long, 5.5–11.8 mm (6.5–8.8 mm) wide, thin, poorly calcified, yellowish or pale golden brown, fragile.

Radula ($n = 4$; Fig. 3A–C): Central tooth tricuspid, endocones very small, mesocone lanceolate; lateral teeth tricuspid, endocones and ectocones very small, mesocones quite short, lanceolate; marginal teeth bicuspid, endocones absent, ectocones very small, mesocones very long, narrow, dagger-like, pointed at tip.

Jaw ($n = 2$; Fig. 3D, E): Oxygnathic, with median projection.

Eggs (n of clutches = 34; Fig. 3F): Clutches consist of 30–174 eggs. Weight of single egg preserved in 3–4% formaldehyde 70–161 mg, eggs translucent, spherical (diameter: 5.1–6.3 mm) or oval (dimensions 4.8–6.3 mm × 5.3–8.5 mm), light yellowish in appearance; laid usually in one clump, sometimes in a chain.

Copulation behaviour (Fig. 1F): Copulation sites observed at the type locality are on spruce trunks (*Picea abies*). Height of copulation sites on trunks range from 80 to 180 cm ($n = 12$). The precopulation behaviour starts, as in most observed *Limax* species, with two slugs following one another on the way to a copulation site. When a suitable place is reached, both partners start to form a circle with their bodies. Copulation starts with entwining of the slug bodies and production of a mucus thread (140–700 mm, $n = 11$). Simultaneously the genital pores of both partners widen and eversion of the penes starts. While elongating, the penes themselves entwine, but the tips stay loose. The fully everted penes reach a length of 49–76% body length ($n = 5$) (*c.* 79–103 mm). Penis shape in the fully everted stage is slightly clubbed with the end of the penis thicker than the beginning. The proximal end is slightly prolate and has a faint longitudinal penis crest. Coloration of penis is bluish, with creamy white tip. After full extension the penes are contracted partially, until they form a pear-shaped mass of only 20–30 mm length and with the tips in contact. At this stage the sperm mass is probably transferred. The animals separate while the entwined penes still form a mass, so the penes are stretched before they are fully separated and retracted. The postcopulatory behaviour of the partners includes cleaning and, in most cases, one of them eats the slime thread.

Distribution (Fig. 4): The known distribution of *Limax sarnensis* is restricted to mountainous and subalpine habitats in the Swiss cantons of Lucerne, Obwalden, Ticino, Uri, Berne, Vallais, Grisons and in the northern Italian provinces of Aoste and Piemonte, covering the geographic region of central Switzerland, the upper valleys of the River Rhine, upper and lower Vallais and the upper parts of the River Ticino and its tributaries as well as Valle Poschiavo, Val Bregaglia and Valle Mesolcina. The sites ($n = 64$) cover a large altitudinal range. The lowest is at 210 m NN near Verbano, Italy, and the highest at 2200 m NN near Saas Fee, Switzerland. The majority of localities are between 1000 and 1500 m NN.

The geology of the sites varies. Soil conditions range from crystalline igneous rock to calcareous sedimentary rock with alkaline to acidic characteristics.

Population density seems to be variable and is difficult to verify, because the observed nocturnal activities of the slugs depend on various parameters such as weather, humidity, breeze, soil structure and density of vegetation. In at least some populations, surprisingly high numbers of animals were

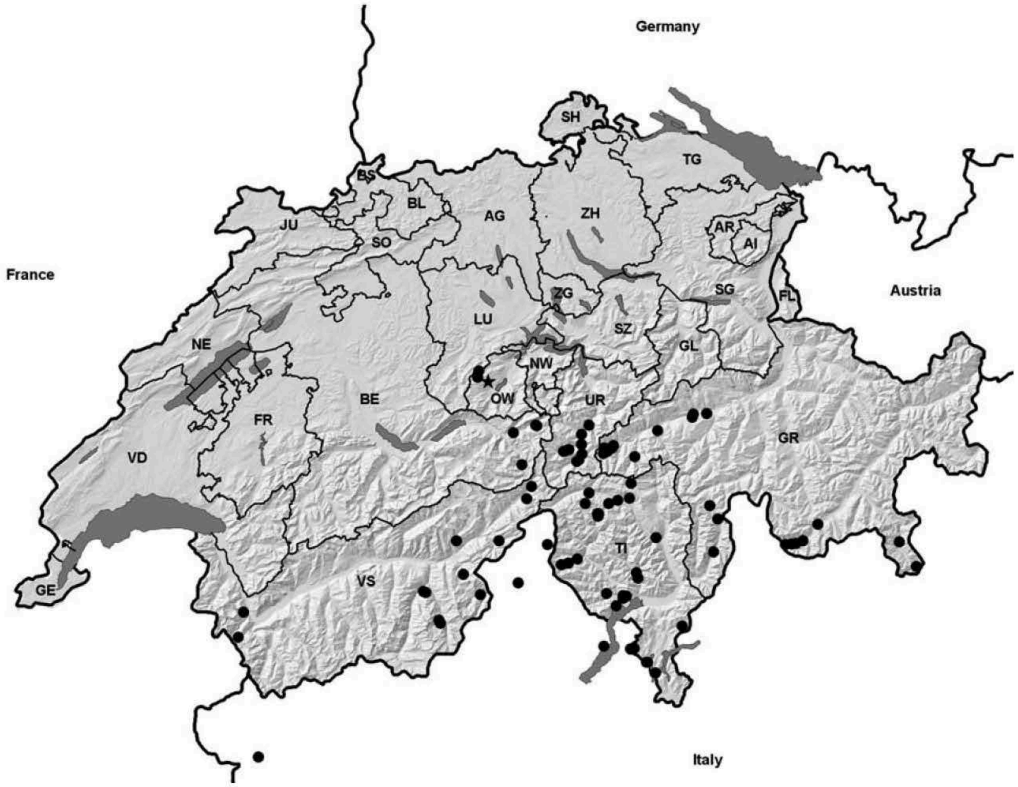

Figure 4. Map of localities. Type locality marked with a star. See Table 1 for details. Map provided by the Centre Suisse de Cartographie de la Faune (CSCF), Switzerland.

sometimes seen. In Grisons one of the authors (U.E.S.) counted 318 adult or subadult *L. sarnensis* on a 500-m transect in about an hour. At the type locality about 25 specimens can be counted in 1 h during the night in an area of *c.* 300 m^2.

Other populations of *L. sarnensis* occur in subalpine and alpine forests, dominated in the Central Alps by spruce (*P. abies*), pine (*Pinus sylvestris*), swiss stone pine (*Pinus cembra*) or mountain pine (*Pinus* mugo), and in the more southern valleys by beech (*Fagus sylvatica*), sweet chestnut (*Castanea sativa*) and sometimes by birch (*Betula pendula*).

The species was detected in 1999 near to the home of one of the authors (R.H.). The high population density in this area provided good opportunities for observations and thorough sampling. Therefore we have chosen this site as the type locality. The holotype specimen is a fully adult animal that represents the most common colour morph at the type locality. The population at the type locality comprises medium-sized *L. sarnensis* (average length of live animals 100–150 mm, maximum 180 mm), but in other populations (specimens from southern valleys, e.g. in lower parts of Canton Ticino) animals can get bigger (living animals up to 245 mm).

The southern populations from Caslano, Pollegio, Lavertezzo and Locarno in Canton Ticino differ slightly in penis size, number of wrinkles (here 17–24, at the type locality 16–20) and coloration (uniformly grey or brownish, usually without dark spots) from all other populations. The penis is in absolute terms slightly longer than in northern populations, but is shorter relative to body size; the hook at its end is weak or missing.

At the type locality of *L. sarnensis* other slug species co-occur: *Limax cinereoniger*, *Lehmannia marginata*, *Malacolimax tenellus* (O.F. Müller, 1774), *Arion* (*Mesarion*) *subfuscus* (Draparnaud, 1805), *Arion* (*Arion*) *vulgaris* Moquin-Tandon, 1855, *Arion* (*Microarion*) *intermedius* Normand, 1852, *Arion* (*Kobeltia*) *distinctus* Mabille, 1868 and *Deroceras reticulatum* (O.F. Müller, 1774). At other localities within the distribution range, additional sympatrically occurring slugs are: *Limax maximus*, *Arion* (*Carinarion*) *silvaticus* Lohmander, 1937, and *Limax* cf. *engadinensis*, *Limax* cf. n. sp. 'Blauköpfige Egelschnecke', *Tandonia rustica* (Millet, 1843) and *Limacus flavus*.

Vegetation at the type locality is subalpine forest dominated by spruce (*P. abies*), accompanied by beech (*F. sylvatica*) and fir (*Abies alba*). The understory of the habitat includes blueberry plants (*Vaccinium myrtillus*). The lichen *Pseudevernia furfuracea* occurs frequently on *P. abies* and *A. alba* as an epiphyte and is an important food source for *L. sarnensis*.

Remarks: The new species is up to now unrecognized. There are various other *Limax* species described from the geographical distribution range, but none of the available names of these can be used for the newly detected species. Some of these names are synonyms of other *Limax* species or *nomina dubia*, while others are valid species. Many of the names in use for species of the genus *Limax* require revision, so it is not possible

to compare *L. sarnensis* with all similar valid species from a sound taxonomic knowledge. Therefore we restrict comparisons to taxa that have been recorded or described from the geographic area where *L. sarnensis* occurs. Thorough revisions of *L. cinereoniger* and *L. maximus* [see also the recent nomenclatorial remarks by Von Proschwitz & Falkner (2007)] are in preparation by the authors.

The widespread species *L. cinereoniger* shows a large range of various colour morphs but, in comparison to *L. sarnensis*, it has a differing sole coloration. In adult *L. cinereoniger* the outer fields of the sole show no fading from the outer edge to the middle. The best way to distinguish this species from *L. sarnensis* is the analysis of the genitalia, especially the penis length, which is in general much longer in *L. cinereoniger* (>70% of the body length in its inverted state in preserved specimens). *Limax cinereoniger* does not copulate on a slime thread like *L. sarnensis*.

Spotted or very brightly monochrome animals of *L. sarnensis* might at first sight be confused with the type species of the genus, the common and likewise very variable (Klee *et al.*, 2007) *L. maximus*. This has happened, for example, with several samples of '*L. maximus*' at the NMBE and NMB, which have been redetermined by the authors as *L. sarnensis*. However, in contrast to *L. maximus*, spotted *L. sarnensis* have spots only on the body, not on the mantle, whereas most specimens of *L. maximus* have a spotted mantle. Even very brightly coloured *L. sarnensis* show small dark spots at the very edge of the outer sole fields; this colour pattern is not reported for *L. maximus*, in which there is no colour difference between the outer and inner fields of the sole. In addition, the blind penis tip is longer and more rounded in *L. maximus*, and the penis itself is shorter (<50% body length).

Limax engadinensis was described from St Moritz, Canton Grisons, Switzerland. Specimens of *L.* cf. *engadinensis* (validation in progress) collected by the authors at this locality resemble the original description. They are usually smaller than *L. sarnensis* and always show uniformly cream sole fields. An obvious distinguishing character is the very short penis (<25% body length) of *L.* cf. *engadinensis* compared to all other known species from this area, including *L. sarnensis* and *L. maximus*. In addition, the insertion of the vas deferens and penis retractor muscle is at the terminal end of the penis tip in *L.* cf. *engadinensis*, so they lack a blind penis tip.

Limax alpinus A. Férussac, 1821 (non *alpinus* Held, 1837) is a taxon mentioned for Switzerland (Turner *et al.*, 1998). As there might be a potential overlapping distribution range of *L. alpinus* and *L. sarnensis*, we carried out extensive investigations to clear up the taxon identity of *L. alpinus*. The name *L. alpinus* was established by Férussac (1821). He described a slug species from the Alps based on drawings sent by his colleague Studer (Férussac, 1821). It is not possible to clarify if Studer, a theologian and naturalist, collected the animals near his residence in Berne, Switzerland, or if the animals were sent to him by someone else; this is quite possible, since he was exchanging samples with other naturalists (M. Gosteli, NMBE, personal communication). Extensive search for type material in the collection of Studer (NMBE) as well as in the collections in Basel, Chur and Lucerne gave no result, therefore any type material is presumed to have been lost or destroyed, if it existed at all [neither Férussac nor Studer expressly mentioned types of *L. alpinus* (Studer, 1820; Férussac, 1821–1822)]. Personal investigations in the Alps (since 1985) have not been successful in finding any species resembling the description and colour plate of Férussac (1821) with the exception of *L. cinereoniger*, a species which shows a wide range of colour morphs including animals matching the one pictured in Férussac's description. Specimens of this special colour morph of *L. cinereoniger* have been detected at a variety of localities in the French, Swiss and Austrian Alps. This result is in agreement with Mermod (1930) and Germain (1930), who regarded *L. alpinus* as a synomym of *L. cinereoniger* or as an alpine form of this species, respectively. To prevent further confusion and to clarify the taxonomic status of *L. alpinus*, we designate a neotype for *L. alpinus* according to ICZN Art. 75. Based on the above-mentioned facts, a specimen of *L. cinereoniger* (ZSM Mol 20090150) collected in 2006 by S. Gratzer in the Alps near Ebensee, Austria, is chosen as the neotype. The specimen resembles the colour plate and the external characters mentioned in the original description by Férussac (1821). Its body is slender, the keel moderately prominent, the coloration of the dorsum yellowish-cream with some dark spots, the sides dark and the mantle brown with obtusely angled posterior mantle edge. An additional character not mentioned by Férussac, but nevertheless important for species recognition, is the coloration of the sole: the neotype has fully coloured outer sole fields and an unpigmented middle field, the characteristic sole coloration of *L. cinereoniger*. Further differentiating characters of *L. cinereoniger* are mentioned herein (see Remarks and Discussion) and in literature (e.g. Quick, 1960; Wiktor, 1996; Klee *et al.*, 2007). Accordingly, *L. alpinus* is a junior synonym of *L. cinereoniger*.

Limax albipes Dumont & Mortillet, 1853 was briefly described as a black animal with a completely white or cream sole, which contrasts with the very obvious sole coloration in dark specimens of *L. sarnensis*. However, this species has not been unequivocally recorded since its description in the year 1853. Sampling at the type locality by the authors was unsuccessful. In addition, the alpine *Limax* material of the NMBE and NMB collections was searched for matching specimens, but none resembling the description of Dumont & Mortillet were detected.

Limax subalpinus Lessona, 1880 was described as an animal with white spots on a dark mantle and should, therefore, if ever collected again, not be confused with *L. sarnensis* (which never has spots on the mantle).

Limax redii Gerhardt, 1933 and *Limax punctulatus* Sordelli, 1870 are sometimes treated as synonyms (e.g. Wiktor, 1983). According to the original descriptions, confusion with *L. sarnensis* seems quite unlikely, because the two species clearly differ in penis size from *L. sarnensis*. For *L. redii*, Gerhardt (1933) reported a penis length of at least 75 cm during copulation; *L. punctulatus* was described as a species with a penis of greater than the body length. Although these species need further research to verify their status, both have a penis size longer than that of *L. sarnensis*.

Limax dacampi Menegazzi, 1854 was described as a red-spotted slug from the southern end of Lago di Garda in Italy. The original description was poor, but the colour plate shows at least some details. *Limax dacampi* is mentioned for south Switzerland (Southern Ticino) by Turner *et al.* (1998), as well as by Hausser (2005). To date the identity of Swiss records of *L. dacampi* remains unclear and needs further research, but the red-spotted *L. dacampi sensu* Menegazzi is totally different from any colour morph of *L. sarnensis*.

Limax n. sp. 'Blauköpfige Egelschnecke' *sensu* Turner *et al.* (1998) is a taxon mentioned in the *Atlas der Mollusken der Schweiz und Liechtenstein* (Turner *et al.*, 1998) as new to science and requiring formal description. However, this has not happened to date. We found specimens that resemble the photograph given by Turner *et al.* (1998) in Canton Ticino. Specimens have a sole coloration that is quite similar to specimens of *L. sarnensis* from the same locality. However, these two species can be distinguished by internal genital morphology: the dissected specimens of *L.* cf. 'Blauköpfige Egelschnecke' lack pigmentation of the penial cord in contrast to *L. sarnensis* which shows grey or black pigmentation of the cord; the longitudinal interior penial crest is in *L.* cf. 'Blauköpfige Egelschnecke' not

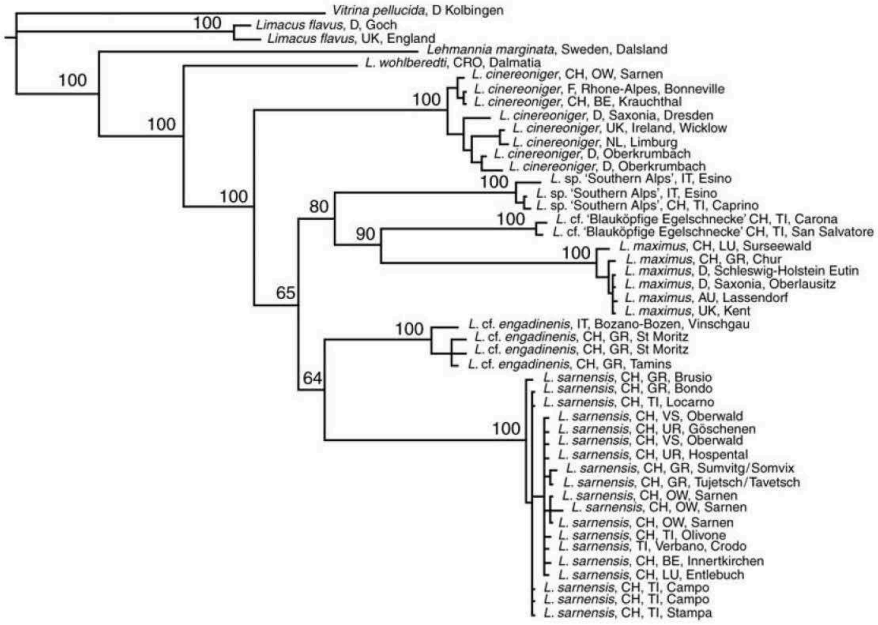

Figure 5. Majority-rule consensus tree from the Bayesian inference analysis of the COI sequence data. Posterior probabilities are marked above the branches.

connected with the transverse penial crest, whereas in *L. sarnensis* the longitudinal interior penial crest is connected with the transverse penial crest and even prolonged beyond it.

PHYLOGENETIC ANALYSIS

The matched-pairs tests of symmetry produced relatively low maximum ζ-scores of 2.062 (first codon sites), 0.893 (second codon sites) and 2.139 (third codon sites). ζ-scores of over 2.0 indicate violation of the phylogenetic assumptions of stationarity, reversibility and homogeneity. The maximum ζ-scores seen for the first and third codon sites are only slightly above 2.0, and the proportion of comparisons over this value are very small (0.49% for first codon sites, 0.08% for third codon sites), indicating that the base composition is relatively homogenous.

The results of the phylogenetic analysis (Fig. 5) strongly support the distinct status of *Limax sarnensis*. All species represented by two or more taxa in the tree (*L. sarnensis*, *Limax maximus*, *Limax cinereoniger*, *Limax* cf. *engadinensis*, *Limax* cf. n. sp. 'Blauköpfige Egelschnecke', *Limax* sp. 'Southern Alps', *Limacus flavus*) form monophyletic groups that are supported by posterior probabilities (PP) of 100. *Limax wohlberedti*, which is represented by only one specimen, is clearly distinct from its nearest neighbours. Species with strong overlap in various coloration patterns (such as *L. sarnensis*, *L. cinereoniger* and *L. maximus*) show well-supported monophyletic separation. Without exception, species occurring at least partially sympatrically with *L. sarnensis* are positioned in clearly distinct monophyletic clades. In addition to the results presented here, a maximum likelihood analysis was performed; it also showed strong support for all species groups including *L. sarnensis* (data not shown).

The phylogenetic analysis shows the genus *Limax* to be monophyletic (PP = 100). The basal part of the *Limax* clade is well resolved, with *L. wohlberedti* and *L. cinereoniger* (PP = 100) diverging at the base. However, the relationships in other parts of the tree are less well supported. The sister taxon of *L. sarnensis* is *L.* cf. *engadinensis* (PP = 64), but support for this grouping is low and in other analyses (data not shown), taxon selection affected the relationships in this part of the tree. Further sequencing of more *Limax* species and possibly additional genes will be needed to establish the relationships within *Limax* and, in particular, the sister taxon of *L. sarnensis*.

DISCUSSION

Biogeographic implications

All known habitats of *Limax sarnensis* are in areas that were covered by ice during the last glacial period. The distribution pattern shows that a lot of these sites are located near former nunataks (Imhof, 1965–1978). Nunataks are probable ice age refugial areas for a number of animals and plants (Welten & Sutter, 1982; Lepidopterologen-Arbeitsgruppe, 1997; Landolt, 2003) that show a similar distribution pattern to that of *L. sarnensis*. The distribution of *L. sarnensis* suggests that it survived the last glacial period on the ice-free edges of nunatak peaks and that it is an inner-alpine faunal element. This is also supported by the cold resistance of the species; the majority of the distribution sites are >1000 m. Personal observations by the authors reveal high activity rates of populations even at temperatures between +10 and −2°C in late autumn. The persistence of *L. sarnensis* in inner-alpine refuges over the last glacial period might also be linked with the preferred food source of the species, which is mainly lichen.

In contrast to the hypothesis of an inner-alpine survival, there is also the possibility of a refuge at the southern glacial border that enabled the survival of *L. sarnensis* during the last glacial period. In this case, *L. sarnensis* would have colonized the inner-alpine area from the south following glaciation.

However, several facts should be taken into account. (1) The borders of the distribution range are well defined by frequent collection trips of the authors (since 1985) in the Swiss Alps and in the adjacent French, German and Italian area and by comprehensive investigations of museum material from this area. Today's distribution range of *L. sarnensis* covers mainly mountainous habitats and two-thirds of the known distributions sites are situated >1000 m. The most southerly records of *L. sarnensis* are still located in an area that was covered by ice during the last glaciation period (Imhof, 1965–1978). In regions further to the south, where the former edge of the glaciers was located, there are no records for *L. sarnensis*, but only for other *Limax* species. (2) In the centre of the distribution range of *L. sarnensis* very few other species occur sympatrically; there is only overlap with other species at lower altitudes and at the edges of the distribution range.

The potential refugia of plants or animals with alpine distribution have been discussed since the early 20th century (reviewed in Brockmann-Jerosch & Brockmann-Jerosch, 1926). Recent publications have addressed this question with molecular markers and provided evidence for both hypotheses (i.e. nunatak-survival or recolonization from refugia outside the ice-shield) for various alpine plant and animal species (Schönswetter *et al.*, 2002; Stehlik *et al.*, 2002; Dépraz *et al.*, 2008). In the case of *L. sarnensis* a fine-scale sampling design with higher numbers of specimens per population and high-resolution markers such as microsatellites or AFLPs (amplified fragment length polymorphisms) would be necessary for a better understanding of the species' history.

Species identification and discrimination

Species discrimination in *Limax* cannot be based on one or two morphological character sets alone; therefore the value of various characters for identification and discrimination must be considered. The utility and limits of various characters are discussed below for the case of *L. sarnensis*.

External appearance in the genus *Limax* can be very variable; *L. sarnensis* likewise shows high variation. Therefore this species could easily be confused with other *Limax* species occurring in the same geographic area. However, the analysis above has outlined the differences between *L. sarnensis* and its sympatric congeners.

Body size. Within *Limax* this is influenced by various intrinsic or external factors including parasitism, nutrition and climatic conditions. In *L. sarnensis* we have shown a wide range of body dimensions in adults. All sympatric *Limax* species, especially the most common and widespread ones (*Limax maximus* and *Limax cinereoniger*) appear to show similar variability (Klee *et al.*, 2007), so that species identification or discrimination is not possible based on size. The only exception might be *Limax* cf. *engadinensis* which, according to our current knowledge, is in general smaller than the others.

Coloration. Variability is common in most species of the genus *Limax*, including *L. sarnensis*. However, the combination of distinct patterns or colour types allows characterization of certain species. It is not always possible to determine *Limax* species without dissection, making field identifications difficult, but at least some common and some unusual species can be discriminated. Characteristic features of *L. sarnensis* are coloration of the mantle and sole. The mantle in all specimens lacks any pattern of bright or black spots or mottling, in contrast to all known colour morphs of *L. maximus* in this area. The coloration of the outer fields of the tripartite sole is supposed to be a characteristic feature in at least some *Limax* species. The pattern seen in *L. sarnensis* (fading from the outer margins to the middle field and from posterior to anterior) is not known so far in any other alpine *Limax* species in the adult stage, except for populations of *Limax* cf. n. sp. 'Blauköpfige Egelschnecke' in Canton Ticino. Here the sole coloration can be similar to that of the sympatric *L. sarnensis*. However, these two species can be distinguished by internal genital morphology. Sympatric *L. cinereoniger*, which could be confused with *L. sarnensis* due to its similar body colour, has fully coloured outer sole fields in the adult stage and is therefore easy to distinguish. Very bright animals of *L. sarnensis* and some specimens from southern localities sometimes have a nearly monochrome sole with just a few pigment spots at the outer margin of the sole. These specimens can be distinguished from *L. maximus* (which also has a cream monochrome sole) by their lack of spots on the mantle. *Limax* cf. *engadinensis*, which occurs sympatrically at Flond (Canton Grisons), has a monochrome mantle and sole as well, but is in most cases much smaller in the adult stage and has a much shorter penis than in *L. sarnensis*. Additionally, at this locality *L. sarnensis* is represented only by the most common colour morphs with the typical sole coloration.

Genital anatomy. The size of the penis, the insertion points of the vas deferens and penis retractor muscle, and the arrangement of the bursa copulatrix are genital features that are traditionally used as the most important character complex for taxonomic discrimination. As outlined earlier, this can only be used satisfactorily if the animals are adult, in healthy condition, preserved adequately and dissected by an expert with knowledge of the variation within a species. *Limax sarnensis* has a relatively short, compact penis with a short blind tip. This allows it to be distinguished from *L. engadinensis* and *L.* cf. n. sp. 'Blauköpfige Egelschnecke' (which both have a shorter penis with no blind tip), *L. maximus* (which has a shorter penis with a longer blind tip), and *L. cinereoniger*, *Limax redii* and *Limax punctulatus* (which all have longer penes). In addition, the distinctive hook at the proximal end of the penis and the characteristic features of the penial interior are only seen in *L. sarnensis*.

Additional useful genital features include the internal structure of the penis, revealing a variety of raised structures, notably the longitudinal interior penial crest and longitudinal interior penial cord. However, most of these internal characters are poorly documented. The limited information available indicates that the longitudinal interior penial crests in *L. maximus* and *L. cinereoniger* are similar to that seen in *L. sarnensis* (Quick, 1960) (although the longitudinal interior penial crest in *L. cinereoniger* is said to be doubled at the distal end). No information is available about the presence of the longitudinal interior penial cord seen in *L. sarnensis*. However, preliminary morphological investigations of *L. maximus*, *L. cinereoniger* and *L.* cf. n. sp. 'Blauköpfige Egelschnecke' by the authors have revealed that all three species can clearly be distinguished from *L. sarnensis* and from each other based only on characters of the penial interior. This suggests that these characters may be important in species identification and discrimination, and should be examined more closely in any future investigations of the genus *Limax*.

Radula, jaw and shell. Characters relating to the hard parts, the radula, jaw and shell, are thought to have a limited taxonomic value for *Limax* and even Limacidae as a whole (e.g. Quick, 1960; Jungbluth, Likharev & Wiktor, 1981). However, there has never been a comparative study dealing with any of these characters at the species level in *Limax*. In the current study, we include SEM photographs of the radula and jaw and a drawing of the shell of *L. sarnensis* for completeness and to allow for future comparisons. Similarly, the eggs of *L. sarnensis* are described and figured herein but at present there are no data available for comparison.

Copulatory behaviour. Without doubt, copulatory behaviour is highly diagnostic for *Limax* species. However, there is little

sound documentation for comparative purposes. Most references in the literature are single observations, often from the early years of slug research, and in some cases do not even recognize that the observed phenomenon is a copulation. Many of these descriptions lack details and figures are poor or missing. Data acquisition today is still hindered by the strictly nocturnal occurrence and rarity of the event, and the sensitivity of the slugs to disturbance. *Limax sarnensis* copulates on a slime thread. Within the distribution range this behaviour is otherwise only known for *L. maximus* and *L.* cf. n. sp. 'Blauköpfige Egelschnecke' (H. Turner, personal communication, 2006). However, *L. maximus* is different in colour and has a shorter penis during copulation. Due to a lack of personal observations the differentiating copulation characters of *L.* cf. n. sp. 'Blauköpfige Egelschnecke' cannot be considered here. The other alpine species with known copulation behaviour are *L. redii* Gerhardt, 1933, *L.* cf. *engadinensis* and *L. cinereoniger*. These three species do not copulate on a slime thread.

Summary. This comparison of the most commonly used characters shows that *L. sarnensis* can easily be distinguished from all sympatric *Limax* species. The features that are characteristic for *L. sarnensis* (coloration of sole, mantle and body, penis length, position of penial retractor and vas deferens, penial interior, and copulation behaviour) have to be used in combination to give a reliable identification. Species descriptions based on single characters or only a few specimens, such as those available for *L. cinereoniger*, *Limax subalpinus* or *Limax dacampi*, might be insufficient or misleading (Wolf, 1803; Lessona, 1880; Menegazzi, 1854). The species description of the very variably coloured *L. sarnensis* shows the necessity of analysing more than a few specimens or just one or two populations. Not only coloration, but also morphological characters and ecologically influenced characters like size, require close examination to assess their variability. The range of variation within and between species can only be discovered by thorough sampling.

Molecular evidence

The molecular tree based on COI sequence data strongly supports the results based on morphology and behaviour. The species identity of *L. sarnensis* is supported by the monophyly of *L. sarnensis* and separation from all other species occurring in the same distribution range. A full study of phylogenetic relationships among *Limax* species or even of the major European lineages is beyond the scope of the present work that aims to describe *L. sarnensis*. Molecular characterization clearly adds a character set that is highly important for slug identification. Slug taxonomy, to date mainly based on very variable characters (e.g. coloration, genitalia), imprecise characters (e.g. size) or data that are difficult to collect (e.g. copulation details), badly needs the stimulus of a new, independent character set such as sequence information. It is likely that additional genes will also be needed to resolve all the phylogenetic problems in this genus, but the results presented here show that use of the COI dataset contributes to our understanding of relationships in the genus *Limax*.

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6 Article II: Corsican *Limax* radiations: Species recognition by a combined approach using morphology and molecules

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[This publication includes an appendix: "Two new species and one new name of peri-Tyrrhenian *Limax*" by Gerhard Falkner & Barbara Nitz]

Inferring Multiple Corsican *Limax* (Pulmonata: Limacidae) Radiations: A Combined Approach Using Morphology and Molecules

Barbara Nitz, Gerhard Falkner, and Gerhard Haszprunar

Abstract Slugs of the genus *Limax* (Gastropoda: Stylommatophora) show a highly complicated genital system and reproductive behaviour probably triggering radiation and speciation. Pre-studies have revealed two so far largely undescribed species groups of *Limax* in Corsica. In order to clear up the phylogeny and evolutionary history of these radiations, we used a combination of molecular techniques and morphological characters. The two independent species groups of Corsican *Limax* species are monophyletic, and consist of six to ten species each, most of them new to science. The first species group, the endemic *Wolterstorffi*-group, can be differentiated by COI-Sequences, whereas COI-sequences fail to discriminate species of the *Corsicus*-group, which also has representatives in the Apennine Peninsula. This pattern suggests a much younger radiation of the *Corsicus*-group. Two hitherto unrecognized species on the adjacent islands of Elba and Capraia are described in an appendix.

Keywords *Limax* · *Corsicus*-group · *Wolterstorffi*-group · Corsica · Elba · Capraia · Apennine Peninsula · Endemism · Radiations · COI-Sequences · Molecular systematics · DNA barcoding · New species

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1 Introduction

The genus *Limax* (Gastropoda: Pulmonata: Stylommatophora) is distributed mainly in Europe, with emphasis on southern Europe and Alpine regions (Falkner et al. 2001; Manganelli et al. 1995). These nocturnal slugs are quite large animals (6–30 cm) and feed mainly on fungi, terrestrial algae, lichens and dead plant material, but are also partly carnivorous. Up to now, species have been defined by external morphology and mainly by their complex genital anatomy.

The unique and highly complicated copulation behaviour has already been described in detail, e.g. by Gerhardt (1933, 1934, 1937). Copulation is highly sensitive: sometimes a 20% difference in penis length hinders a successful copulation (G.F., personal observation). Different species vary in the length and sculpture of their penes and also in copulation behaviour.

Estimates about species numbers vary, ranging from about 15 species (Schileyko 2003) up to 40 species (Wiktor 2001) for the whole distribution range. However, in contrast to these quite low numbers, Manganelli et al. (1995) list 18 species just for Italy. Most *Limax* species, especially the ones with a Mediterranean distribution, have small and fragmented ranges and are thus endangered by habitat destruction (burning of woods, urban development).

Current knowledge of the *Limax* fauna of Corsica is quite poor. Moquin-Tandot (1855) described *Limax corsicus* based on external characters with a type locality in Bastelica, Corsica. The name *Limax corsicus* was used by Lessona and Pollonera (1882) not only for specimens from Corsica, but they also applied the name to various *Limax* specimens from Northern Italy. However, Simroth (1910) considered *L. corsicus* to be a synonym of the common, widespread species *L. maximus* Linnaeus, 1758. Today, *L. corsicus* is regarded as a species distributed not only in Corsica but also on various Italian Islands like Sardinia and Capraia (Giusti and Mazzini 1971) and in the region of Tuscany (Giusti and Mazzini 1971). The name is generally applied for *Limax* specimens with red sole fields and brownish to creamy body colouration.

In addition to *L. corsicus*, two other *Limax* species are listed for Corsica (Holyoak 1983; Réal and Réal-Testud 1988): *L. maximus* and *L. cinereoniger* Wolf, 1803; both species have a large distribution range all over Europe, and the synanthropic *L. maximus* even occurs overseas.

Based on thorough field studies, breeding experiments, copulation observations and morphological investigations, Falkner (2001) and Falkner et al. (2002) assumed a total diversity of about nine *Limax* species probably endemic for Corsica and most of them new to science. The species form two groups, with four and five species respectively, probably representing two independent island radiations. However, morphological discrimination of *Limax* species is still difficult due to high colour variability and the fact that only fully mature specimens can be considered for genital comparisons. This leads to doubtful species identifications in collections and bio-inventories. A fast and unequivocal method of recognition of new or undetected species in the genus is required to facilitate new insights into species composition and protection of these slugs.

The standard barcode gene, cytochrome *c* oxidase subunit I (COI), is used not only for species re-identification but also proposed for the discovery of new species (Hebert et al. 2003a). This works quite well in the majority of animal groups; more than 95% of species possess unique COI barcode sequences and species level identification is possible in most cases (Hajibabaei et al. 2007; see also Waugh 2007 for a summary). Exceptions are found for example in the Cnidaria (Hebert et al. 2003b) and in insects (Whitworth et al. 2007; Elias et al. 2007).

For species discovery and (re-)identification via DNA barcoding, two general approaches are used. Firstly, tree-based methods should reveal the identity of unknown samples by their position in an established phylogeny (Hebert et al. 2003a, b). The second approach is to use a threshold value of sequence divergence to separate intraspecific from interspecific variation. This threshold value can be inferred in two ways. It may be based on a fixed threshold value, e.g. 3% sequence difference (Hebert et al. 2003 a, b), alternatively a threshold of ten times the average of intraspecific divergence has been proposed (Hebert et al. 2004). However, recent studies have shown high error rates in species delineation based on DNA barcoding alone, strongly suggesting a use of DNA sequences only in combination with solid taxonomic foundations and in an integrative taxonomy approach (Meyer and Paulay 2005; Meier et al. 2006).

In our study combining molecules and morphology, COI sequences should help to clear up the status of the Corsican *Limax* species/populations. To test the validity of new species in Corsica and to assign unidentified specimens to known species, we sequenced specimens of already described species (*L. corsicus* from its type locality on Corsica, *L. senensis* Pollonera, 1890 and *L. ciminensis* Pollonera, 1890 from their respective type localities on the Italian mainland, *L. cinereoniger* from its type locality in Germany and *L. maximus*) and specimens of the unknown and potentially new Corsican species. For comparison, we included several *Limax* species/populations from the Apennine Peninsula and from some Tyrrhenian islands, and also one of the most basal *Limax* species, *L. wohlberedti* Simroth, 1900 (B.N., personal observation).

2 Materials and Methods

2.1 Collection and Treatment of Specimens

Most Corsican *Limax* specimens were collected by the authors (G.F. and B.N.). In some cases, it was possible to document and photograph the copulation behaviour in the natural habitat and in captivity. Complementary European *Limax* specimens were collected for comparison and genetic differentiation or borrowed from other collections (see list of material in the Supplement). For the institutions from which we obtained material, the following standardised abbreviations (in brackets) are used: Istituto di Zoologia dell'Università di Siena (IZSI); Muséum National

d'Histoire Naturelle, Paris (MNHN); Museum of Natural History, Wrocław University (MNHW); Natur-Museum Luzern (NMLU); Naturhistorisches Museum Wien (NMW); Nationaal Natuurhistorisch Museum Leiden (RMNH); Staatliches Museum für Naturkunde Stuttgart (SMNS); Zoologisches Museum Hamburg (ZMH); Zoologische Staatssammlung München (ZSM).

To infer the phylogenetic position of the Corsican *Limax* species within the genus *Limax*, representatives of other limacid genera [*Lehmannia marginata* (O. F. Müller, 1774), *Limacus flavus* (Linnaeus, 1758)] and as outgroup, the vitrinid *Vitrina pellucida* (O. F. Müller 1774), were included in the genetic part of the study.

Most of the collected animals were photographed alive. Tissue samples for DNA extraction were taken alive from the left side of the mantle. This procedure is only minimally invasive so that the living slugs survived without problems. In preserved specimens, tissue was taken from either the body wall or from the left side of the mantle. For preservation, the animals were relaxed and killed in water or in a mixture of water and 2–3 drops of a solution of the synthetic tenside SUPRALAN-UF (three parts SUPRALAN-UF – a fatty alcohol polyglycol ether; Bauer Handels-GmbH, Adetswil, Switzerland – to two parts water). Preparations of slugs with everted penes were obtained with a bit of luck by drowning animals which were ready to copulate. The eversion of the penis is furthered by a quick reduction of oxygen in the drowning water obtained by drowning several animals together and slight regular movement of the jar combined with very gentle warming. The method of Colosi (1919) to use a veratric solution has not yet been tested. This method should produce slugs with everted penes and possibly also provides a procedure to study the morphology of the penial combs and the surface structures of the penes. With both methods, a complete eversion of the uttermost tip of the penis is not reached. This seems to have functional morphological reasons which also play a role in the conditioning of sperm and has to be further investigated by thin sections. All animals were fixed and preserved in ethanol. Morphological studies followed standard procedures.

Material is deposited in the ZSM, in the SMNS (Coll. Falkner) and in the MNHN. DNA elutions are stored in the DNA Bank of the ZSM (see http://www.zsm.mwn.de/dnabank/).

2.2 DNA Sequence Analysis

DNA was isolated from a small piece of tissue sampled from the mantle or body wall of the slugs using a QIAGEN extraction kit (Qiagen Blood and Tissue Kit). About 650 nucleotides of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) were amplified by polymerase chain reactions (PCR) for all taxa using the primer set: mtCOI-1F-54 (5′-TTTCAACAAAYCATAARGATATTGG-3′) and mtCOI-1R-53 (5′-AAYACCAATAGAAATTATAGCATAAA-3′). The primers were based on the COI universal primers (Folmer et al. 1994) and the primers used by

Hyman et al. (2007) and were assessed using the computer program *Alignment* 1.2 (Engels 1993). The PCR conditions were: 92°C for 4 min, then 40 cycles of 92°C for 1 min, 50°C for 1 min, 72°C for 1 min and final elongation 72°C for 5 min.

PCR products were purified with one of three techniques, depending on the quality and intensity of the PCR results: a Qiagen DNA purification kit (Ultra Clean Band Excision Purification kit) or with ExoSapIt [PCR product was incubated at 37°C for 30 min and then at 85°C for 15 min with 5 units of Exonuclease I (ExoI; Amersham) and 0.5 unit Shrimp Alkaline Phosphatase (SAP; Amersham) to cleave nucleotides one at a time from the ends of excess primers and to inactivate single nucleotides (Werle et al. 1994)]. The purified PCR products were amplified with the same primers as above with a BigDye v3.1 Terminator Cycle Sequencing Kit, cleaned up with SephadexG-50 Superfine columns (GE Healthcare) and sequenced using an Applied Biosystems 3730 capillary automated sequencer according to the standard protocol. Sequences were assembled and proofread using Sequencher™ (Gene Codes), manually aligned in the program Se-Al v. 2.0a11 (Rambaut 1996) and deposited in GenBank (for accession numbers see list of material in the Supplement). The alignment was trimmed to 615 nucleotides, starting with position 40 of the reference taxon *Biomphalaria glabrata* (Say, 1818) (GenBank number NC 005439) and finishing at position 655.

Prior to phylogenetic analysis, the data were partitioned into first, second and third codon sites. Model selection was made using comparisons of hierarchical Likelihood Ratio Tests and Akaike Information Criterion scores in *MrModeltest* 2.3 (Nylander 2004). The general time-reversible (GTR) model with eight discrete gamma (Γ) categories and a proportion of invariant (I) sites (GTR+Γ8+I) was used. Markov Chain Monte Carlo (MCMC) sampling was carried out in *MrBayes* 3.1.2 (Ronquist and Huelsenbeck 2003) for 1,000,000 generations (four simultaneous chains, sample frequency 50, burn-in 100,000 generations). Majority-rule consensus trees were calculated from the sampled sets of trees.

The phylogenetic trees were rooted on *Vitrina pellucida*, because Vitrinidae appear to be the most basal family in the superfamily Limacoidea (Hausdorf 1998).

Inter- and intra-specific genetic distances were calculated with MEGA version 4.0 (Tamura et al. 2007) using the Kimura 2-parameter model (K2P), the most effective model when distances are low (Nei and Kumar 2000).

3 Results

3.1 Morphological and Copulation Studies

Based on preliminary results, two species groups can be defined: the *Wolterstorffi*-group (Fig. 1a–e) and the *Corsicus*-group sensu lato (Fig. 2a–e).

Representatives of the *Wolterstorffi*-group (named after *L. wolterstorffi* Simroth, 1900) are generally small animals (less than 10 cm, mostly about 8 cm), mostly dark

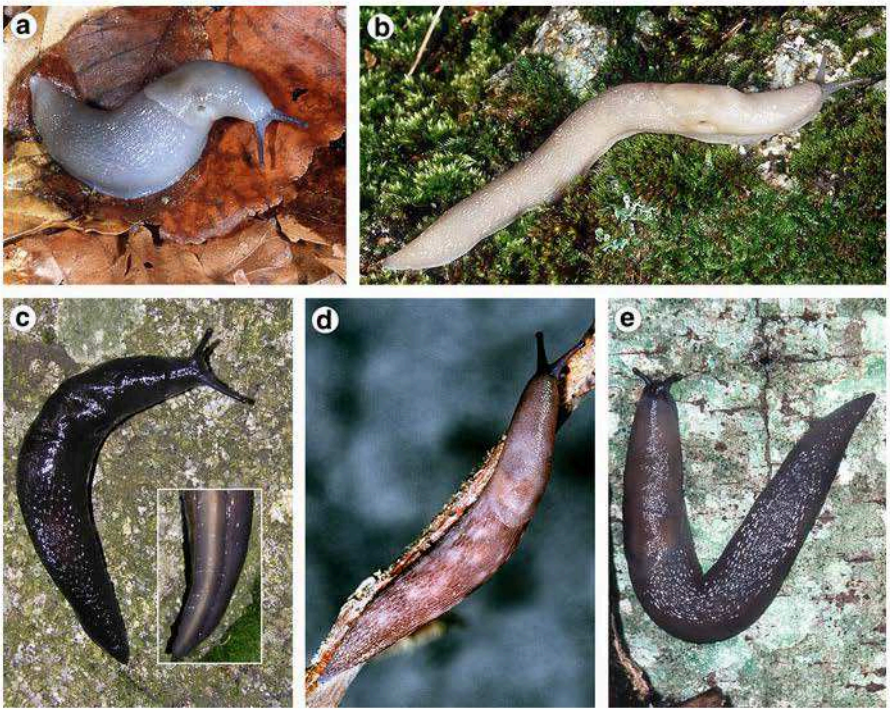

Fig. 1 Habitus photos of different colour morphs of the *Wolterstorffi*-group. All detailed pictures are approximately 2/3 natural size. (**a**, **b**) *Limax vizzavonensis* n. nom. (**a**) Specimen from Vizzavona near Cascade des Anglais (no. 12 on map, Fig. 10); (**b**) specimen from Ruine de Sorba (no. 10 on map), *creamy whitish*, but no albino; (**c**) specimen (F1) from Monte Rotondo near Petra Piana (no. 9 on map), *deep black* morph, in*sert* showing the *dark* lateral sole fields (photograph, courtesy C. M. Brandstetter); (**d**) specimen from Vallée de la Restonica at Tuani morph with irregular bright spots; (**e**) specimen from Porto, ravin du Riù (no. 3 on map), *brownish-grey* morph with metallic lustre (photograph, courtesy M. Falkner)

to uniformly black lacking distinct patterning and never show red pigmentation on body or sole. Hatchlings and early juveniles so far investigated exhibit a diffuse body colour entirely lacking lateral bandings ("Stammbinden" sensu Simroth; Fig. 3a–d). This lack has been verified by observations of eight populations (Monte Cinto, Citadelle of Corte, Porto, Vallée de la Restonica, Monte Rotondo, Bergeries de Baccialojo, Vizzavona, Plateau de Coscione), and defines for Corsica a discriminating character of the *Wolterstorffi*-group. In the whole genus *Limax*, this character has only been observed outside Corsica for *L. ianninii* Giusti, 1973, and *L. brandstetteri* Falkner, 2008, two unicoloured basal species within the *Limax maximus* group. Additionally, breeding experiments within the *Corsicus*-group with animals from Corsica (11 populations) and continental Italy (9 populations) showed the constant presence of "Stammbinden" at least in the early developmental stages (Fig. 3e–g); this character is shared with the majority of the *Limax* species. The morphological examination of the *Wolterstorffi*-group shows a huge variety in

Fig. 2 Habitus photos of different colour morphs in the *Corsicus*-group sensu lato. All detail pictures are approximately 2/3 natural size. (**a**) *Limax corsicus*, topotype from Bastelica (no. 28 on map, Fig. 11); (**b**) specimen from Vizzavona (no. 26 on map), morph with interrupted banding and diffuse bright spots on the mantle; (**c**,**d**) Vallée de la Restonica at Tuani, (no. 25 on map): (**c**) juveniles of two sympatric colour morphs which show a different phenology, although they are genetically not distinguishable: the largest specimen of the dark cohort is photographed together with the most retarded specimen of the reddish cohort; (**d**) adult specimen with *red sole*; (**e**) specimen from the Castagniccia near Croce, morph with creamy whitish sole; this rufinless morph is restricted to the central Castagnicca and is dominant at the Monte San Petrone

penis length in preserved specimens. For example, the observed penis length of L. sp. (Porto) (Fig. 4a) is approximately twice the body length. In contrast to this long and thin penis, the penis of *L. wolterstorffi* is less than the body length and very thick (Fig. 4b). Based on the findings of the morphological analyses, eight to ten species can be distinguished, although data on the reproductive behaviour are still entirely missing.

In the *Corsicus*-group s. l., specimens of Corsica and of the adjacent Apennine Peninsula are present. Although the species in this species group have specific copulation features with a huge range in penis length (Figs. 5 and 6), they share a distinct mode of sperm transfer through the extended penes, whereas in other species groups (e.g. *cinereoniger*- or *maximus*-group), the penis is everted with the sperm mass already in the tip. Up to now, morphological criteria have failed to

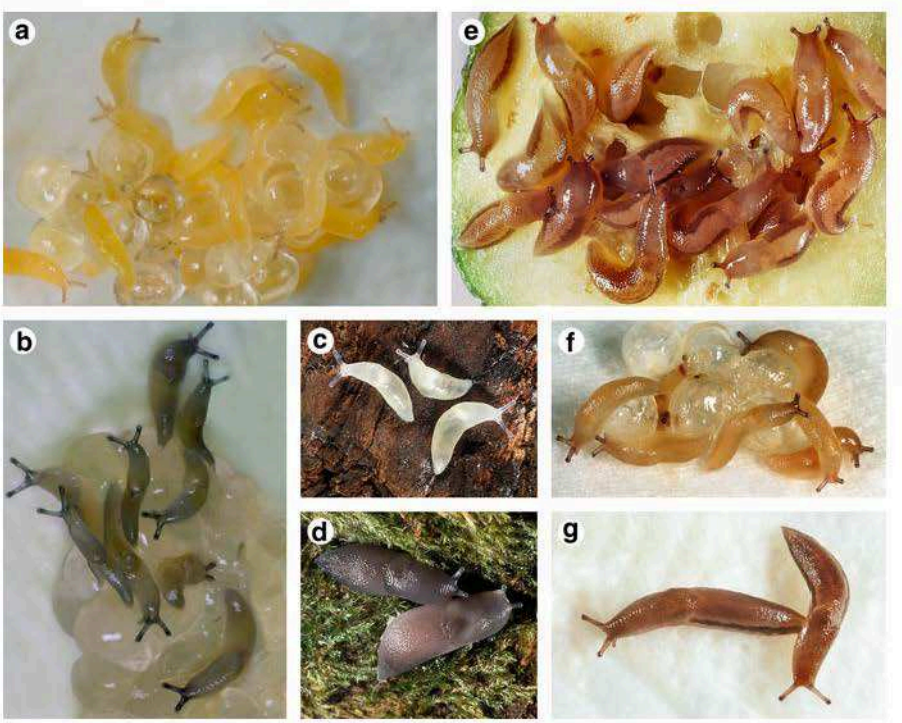

Fig. 3 Chromatic development of juveniles. All detailed pictures are approximately two times natural size. (**a**–**d**) Offspring from representatives of the Wolterstorffi-group: no traces of "Stammbinden." (**a**) F1 of a specimen from the Plateau de Coscione, photographed 1 h after the end of hatching, no pigment is developed by the embryos in the eggs; (**b**) F1 of a specimen from the Monte Rotondo, photographed shortly after hatching, the pigmentation of the body starts already in the eggs; (**c**) F1 of a specimen from the Citadelle of Corte, photographed 1 day after hatching; (**d**) the same animals as in (**c**) 4 weeks later. (**e**–**g**) Offspring from representatives of the Corsicus-group (Cap Corse/Tuscany group as an example): "Stammbinden" are always present. (**e**) F1 of a specimen from Furiani, 4 days after hatching, feeding on cucumber; (**f**) F1 of a specimen from Pietrabugno, Casevecchie, 5 days after hatching, the lateral body bands are present but very weakly developed; (**g**) F1 of a specimen from Furiani, 3 weeks after hatching

further divide this group, but molecular data (see below) distinguish an Endemic *Corsicus*-group with strictly Corsican representatives (Figs. 5b, c, d, 6b, c, and 7a) and the Cap Corse/Tuscany-group (Figs. 5e, 6e, and 7b) with representatives on Corsica and the Apennine Peninsula. For the Endemic *Corsicus*-group itself, the comparison of penis morphology and copulation modes clearly shows severe morphological differences that legitimate the assumption of at least five different species in Corsica. Species *L.* sp. (Bonifatu) and *L.* sp. (Tuani) for example, both positioned in this group, represent species with very distinct genital differences (Figs. 5b, c, 6b, c, and 8b).

Morphological characters in the Cap Corse/Tuscany-group reveal the existence of at least two species for Corsica. The specimens of the locality of Furiani *L.* sp.

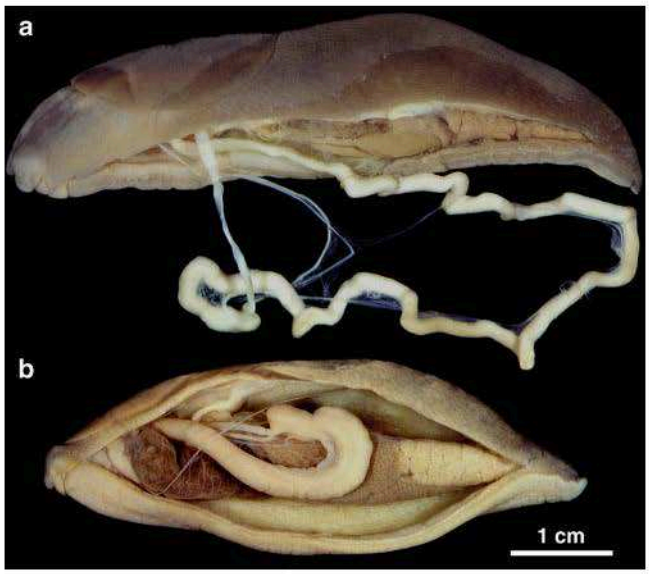

Fig. 4 Dissection photographs of the two extreme forms in the *Wolterstorffi*-group. (**a**) *Limax* sp. (Porto); the animals of this new species have an extreme long and thin penis. (**b**) *Limax wolterstorffi* (topotype): penis short and massy

(Cap Corse A), for example, show a unique copulation mode (Figs. 5e and 6e) and a very special penis morphology (Fig. 7b).

3.2 Sequence Analysis

The results of the phylogenetic analysis (Fig. 9) show monophyly for both the family Limacidae (including *Limax*, *Limacus* and *Lehmannia*) and the genus *Limax* (posterior probability, PP 100%). The basal part of the *Limax* clade is well resolved, with *L. wohlberedti* and *L. cinereoniger* diverging basally (PP 100%, 100%). All species representing European non-Corsican lineages (without the Italian relatives of the Corsicans) are clearly distinct from their nearest neighbours [*L. brandstetteri*, *L. maximus*, *L. cinereoniger*, *L. ianninii*, *L. wohlberedti*, *L.* sp. (Mte. Altissimo), *L.* sp. (Mte. Baldo), *Limacus flavus*] and form monophyletic groups that are in most cases supported by posterior probabilities of 100%. The phylogenetic reconstruction strongly supports the preliminary assumption of two independent Corsican/Tyrrhenian species groups: a mixed group from some Italian islands and the mainland and Corsica (arrow) and an endemic Corsican species group (bar G). This latter group (*Wolterstorffi*-group) is well resolved and monophyletic (PP 100%). The already described species (*L. wolterstorffi* and *L. vizzavonensis* n. nom.) and also the unnamed species [like *L.* sp. (Coscione), *L.* sp. (Porto) and *L.* sp. (Restonica)] show well -supported monophyletic separation.

Fig. 5 Maximum extension of the entwined penes during copulation in the *Corsicus*-group sensu lato. All to scale. (**a**) Montagnola Senese, Tuscany, 92.5 cm (photograph, courtesy C. M. Brandstetter); (**b**) Bonifatu (no. 23 on map, Fig. 11), 50 cm; (**c**) Tuani, Restonica Valley (no. 25 on map), 19 cm; (**d**) Marmuccio, Castagniccia, 27 cm; (**e**) Furiani-Marinella (no. 21 on map), 22 cm; (**f**) Capraia (no. 31 on map), estimated length 28.0 cm (photograph, courtesy F. Giusti)

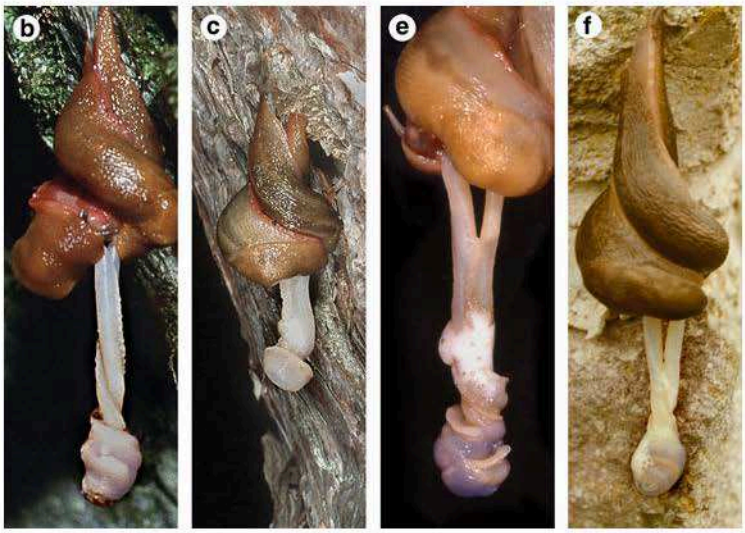

Fig. 6 Morphology of the penes in the *Corsicus*-group s. l. during or shortly after sperm exchange. *Lettering* coincides with couples in Fig. 2. All detailed pictures are approximately 2/3 natural size. Estimated penis lengths: (**b**) 5 cm; (**c**) 2 cm; (**e**) 6.5 cm; (**f**) 4 cm (**f**: photograph, courtesy F. Giusti)

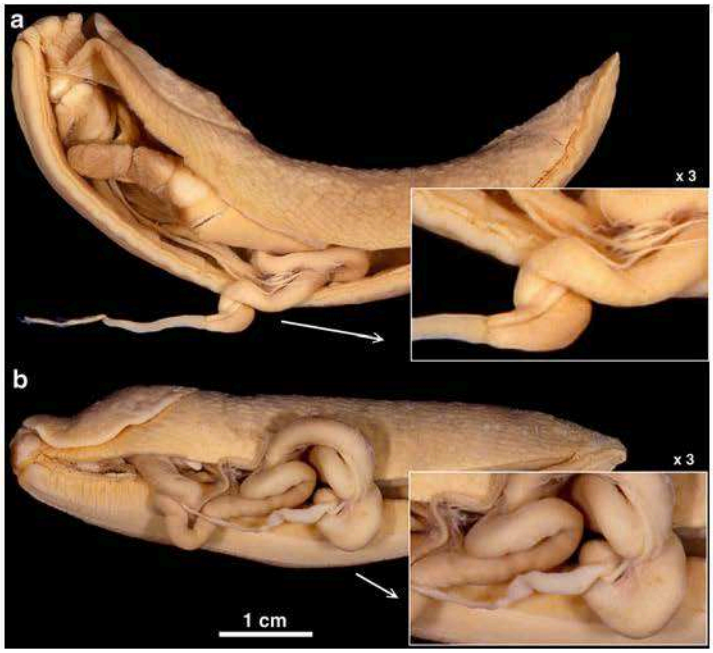

Fig. 7 Examples of anatomical specialisation in the *Corsicus*-group. (**a**) Specimen from Sperono (no. 30 on map), leg. E.Th.J. Ripken 1996; terminal insertion of retractor and vas deferens, morphology of the penis tip corresponding to *L. corsicus* s. str.; (**b**) specimen from Furiani-Marinella (no. 21 on map, Fig. 11), F1; distinct coecum and lateral insertion of retractor and vas deferens

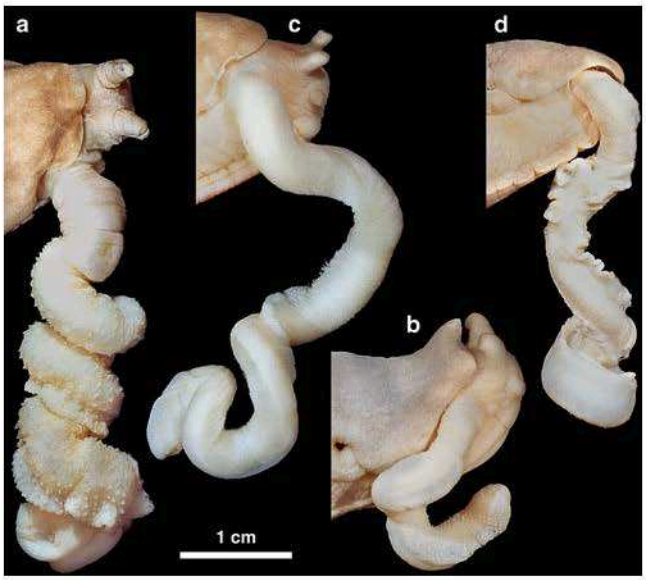

Fig. 8 Morphology of everted penes of clearly distinguishable forms of the *Corsicus*-group from different sampling places. (**a**) Truggia (no. 27 on map, Fig. 11); (**b**) Bonifatu (no. 23 on map), not fully everted; (**c,d**) Two sympatric forms from Grigione (no. 19 on map)

In contrast, species differences within the other species group (arrow) are less supported. The analysis reveals a large group of mixed species and populations from Corsica, the Apennine Peninsula, and several other Tyrrhenian islands (Sardinia, Capraia, Elba): the *Corsicus*-group sensu lato. Within this grouping, we have distinct monophyletic clades for the two species from the islands of Capraia and Elba (*L. giustii* n. sp. and *L ilvensis* n. sp., see Appendix). Further on, several non-Corsican groups are formed by specimens from the Apennine Peninsula [*ciminensis*-group: bar F, "sp. 2" of Italian Checklist (Manganelli et al. 1995): bar E, *senensis*-group: bar D, group of "Fossil Islands" ("Isole fossili" sensu Lanza 1984): bar **B**].

The Corsican specimens split into two clades: the endemic *Corsicus*-group (bar C) and the Cap Corse/Tuscany-group (bar A). This latter, monophyletic clade (PP 100%) forms an unresolved group including specimens from Tuscany (Apennine Peninsula) and specimens from Cap Corse (the most northern part of Corsica).

The Endemic *Corsicus*-group (PP 91%) comprises specimens only from Corsica, namely the whole area of Hercynian Corsica and the southern part of Alpine Corsica (see also Figs. 10 and 11).

The sequence divergence within the three species groups (*Wolterstorffi*-group, Endemic *Corsicus*-group, Cap Corse/Tuscany-group) is 3.9, 0.1 and 0.1% respectively (Table 1). The sequence divergence between the groups is 10.8% for the *Wolterstorffi*-group and the Endemic *Corsicus*-group. Between the *Wolterstorffi*-group and the Cap Corse/Tuscany-group, there is a sequence divergence of 10.8%

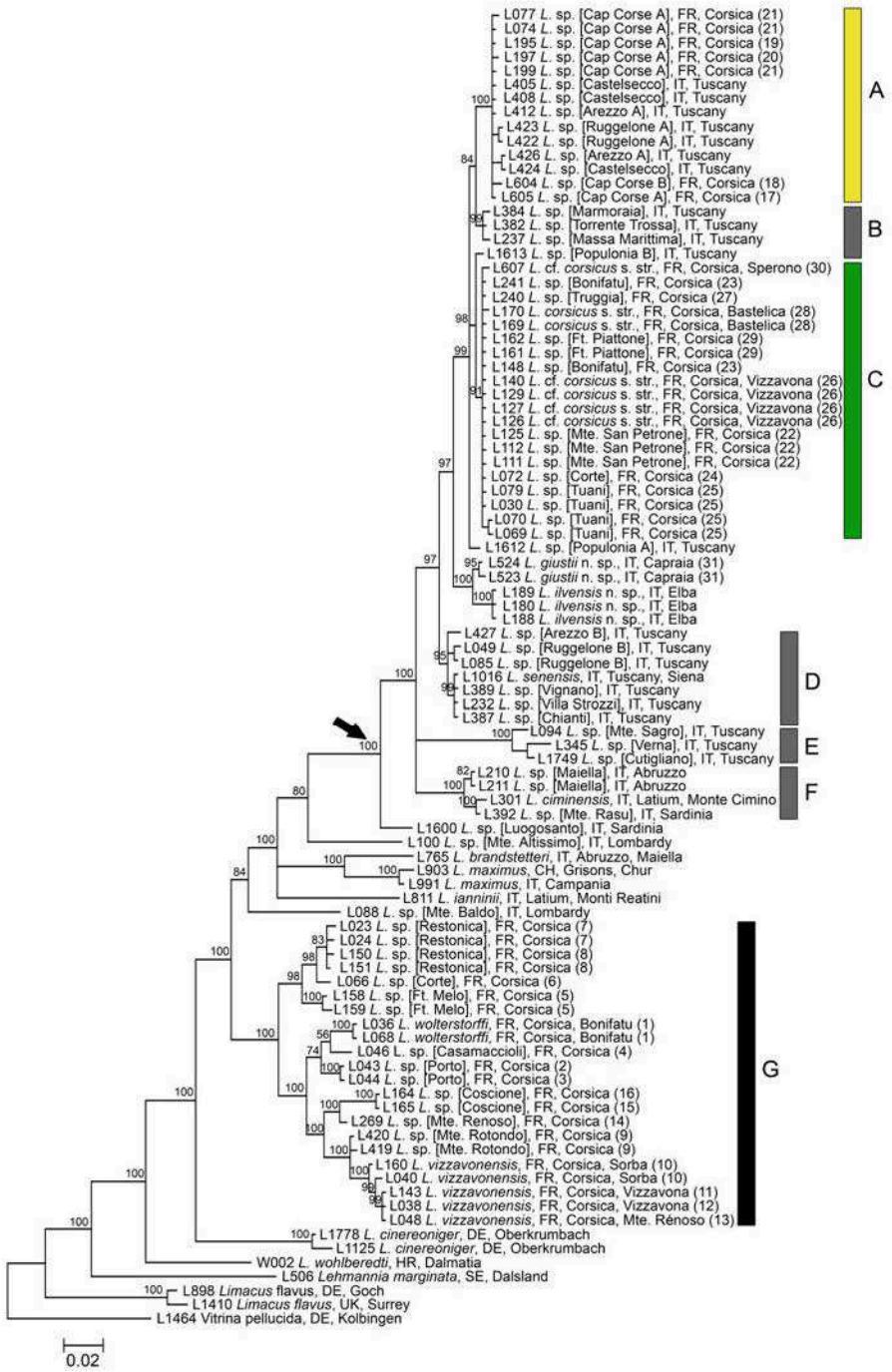

Fig. 9 Majority-rule consensus tree from the Bayesian inference analysis of the COI data. Posterior probabilities are marked above the branches. *Arrow Corsicus*-group sensu lato. *Bar A*

as well. For the Endemic *Corsicus*-group and the Corse/Tuscany-group, the sequence divergence between them is 1.4%.

Interspecific divergence within the *Wolterstorffi*-group ranges from 1.1 to 6.8% (Table 2), with an average value of 3.9%.

3.3 Distribution

All known distribution sites of the *Wolterstorffi*-group (Fig. 10) are located in mountainous habitats in the Hercynian Corsica (the geologically ancient, crystalline part of Corsica; Fig. 10 insert). Both the Endemic *Corsicus*-group and the Corsican species of the Cap Corse/Tuscany-group have their ecological preference in the montane forest zone. The Endemic *Corsicus*-group is found in the middle and southern part of Corsica, whereas the Corsican specimens of the Cap Corse/ Tuscany-group are restricted to the Cap Corse region, the most northern part of the island (Fig. 11). The distribution range of the Italian specimens of the Cap Corse/Tuscany-group also comprises habitats in Tuscany.

4 Discussion

4.1 Biogeographical Scenarios

Today's distribution pattern of the *Limax* species in the Tyrrhenian area has certainly been influenced by geological history. The polyphyly of Sardinian and Corsican groups implies that there were several independent colonisation events on the islands of Sardinia and Corsica as well as on the smaller islands closer to mainland Italy. Although a direct scaling of the splitting events in the tree is currently not possible, the geohistory of both islands suggest only a few colonisation events:

Corsica was colonised by *Limax* at least three times. The first radiation of *Limax*, the *Wolterstorffi*-group, is (according to current knowledge) endemic to the Hercynian Corsica, suggesting a very ancient colonisation from a European mainland stock. Accordingly, this group probably has its origin on the French mainland and has been split from mainland taxa by the rotation of the Corsica–Sardinia microplate (Alvarez 1972; Durand-Delga 1974). The time frame for this event is during the Eocene or Oligocene at the latest (~30–21 Mya). A test of this hypothesis would be

Fig. 9 (continued) Cap Corse/Tuscany-group. *Bar B* group of fossil islands. *Bar C* Endemic *Corsicus*-group. *Bar D senensis*-group. *Bar E* group of "sp. 2" of Italian Checklist (Manganelli et al. 1995). *Bar F ciminensis*-group. *Bar G Wolterstorffi*-group

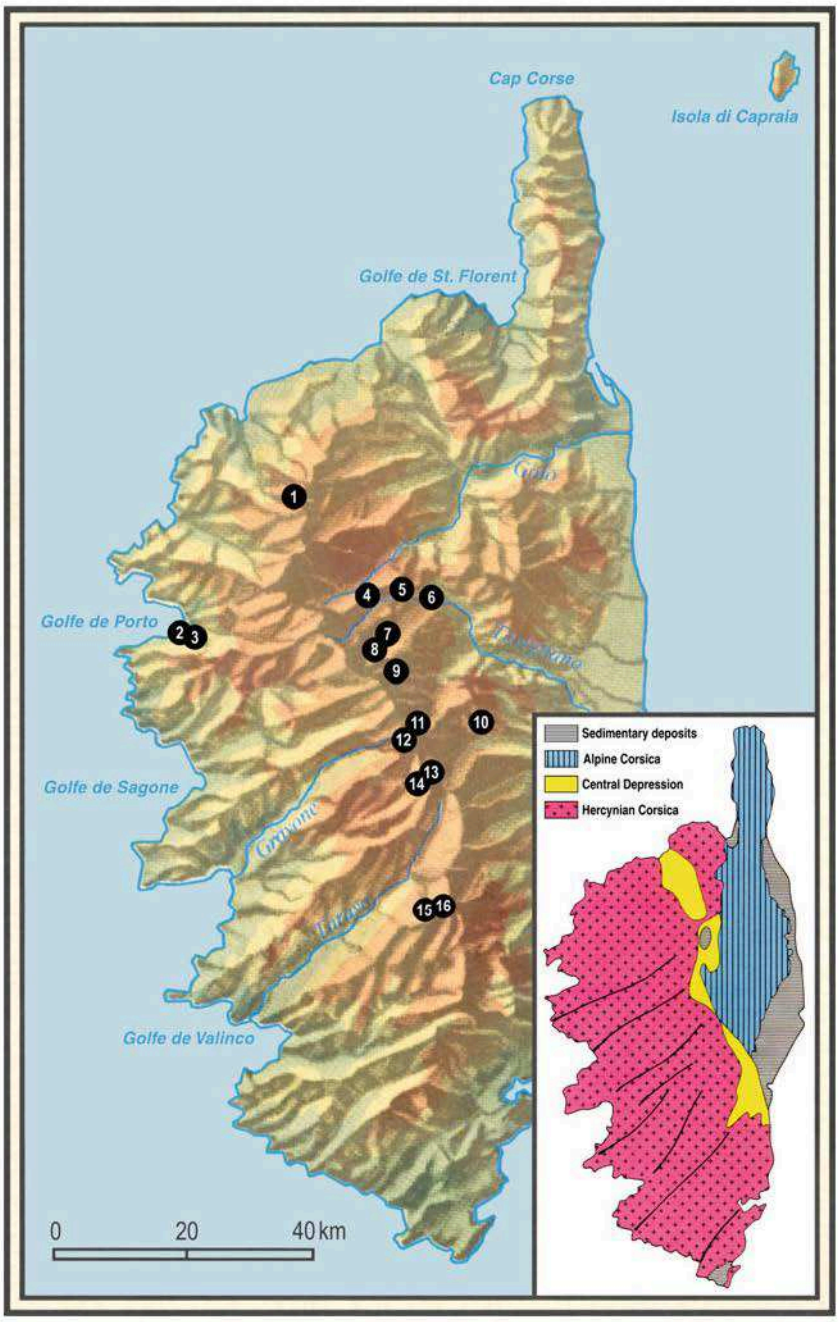

Fig. 10 Localities of the studied populations of the *Limax wolterstorffi*-group. Map base MNHN (modified). *Insert* Simplified geology after A. Gautier (1983). (*1*) Cirque de Bonifatu, 610 m (loc. typ. *wolterstorffi*); (*2*) Porto, D 81 direction Piana; (*3*) Porto, ravin du Riù; (*4*) Casamaccioli, 990 m; (*5*) Forêt de Melo, 1,300 m; (*6*) Corte, Citadelle, 450 m; (*7*) Vallée de la Restonica, 900 1,000 m;

the finding of sister taxa of the *Wolterstorffi*-group in southern France – a matter for future studies.

An additional, second lineage of *Limax* derived from the above-mentioned ancient European stock and followed the already (Miocene) formed and exposed chains of the Western and Ligurian Alps further along the Apennine chain (cf. Rook et al. 2006: Fig. 2) and radiated in middle Italy (Latium, Campania). This lineage – in our tree being represented by subsequent deviation of *Limax maximus* s. lat., *L. ianninii*, and *L.* sp. (Mte. Altissimo) (corresponding to "*Limax* sp. 3" of the Italian Checklist by Manganelli et al. 1995) – gave rise to all later colonisations of Corsica as well as the colonisation of Sardinia and the small islands close to Italy (see below).

Further land bridges enabling *Limax* to enter Corsica probably occurred only during the Pleistocene; this colonisation scenario is based on the following considerations: First, despite dense sampling, there is no Corsican taxon belonging to the above-mentioned Latium-Sardinia radiation. Accordingly, Corsica was probably not connected to either Sardinia or Latium during the late Miocene. Second, a major marine transgression during the Pliocene made any terrestrial faunistic exchange unlikely. Third, only the marine regressions following the onset of ice ages in the Pleistocene offered land connections again. Fourth, the low genetic differences of members of the Endemic *Corsicus*-group imply a recent radiation. And, fifth, the two main Corsican radiations (the *Wolterstorffi*-group and the Endemic *Corsicus*-group) are genetically clearly distinct suggesting a considerable long-term separation of these species groups.

Therefore, this second Corsican colonisation took probably place in the (Early or) Middle Pleistocene (780–130 ka). Interestingly, this now endemic group of the *Corsicus*-group seems to have initially reached only the Hercynian (i.e. older) area of Corsica, suggesting that there was still no connection to the younger Alpine Corsica (northeast Corsica). The Alpine Corsica was either separated from Hercynian Corsica and the northern Ligurian-Ocean landbridge by a small marine channel, or was still not tectonically lifted up high enough to reach the sea surface (Cavazza et al. 2001; Brunet et al. 2000; Danišík et al. 2007).

On the Italian mainland, the last remnants of this second colonisation wave are the Limaces of the "Fossil Islands" – this western Tuscan area was drowned during the Pliocene except for a number of mountain peaks above sea level (Brunet et al. 2000; Cipollari et al. 1999; Brogi 2008).

The massive regression of sea-level during the (Middle or) Late Pleistocene (probably Würm glaciation, 115,000–10,000 BP) possibly enabled the youngest, third colonisation of the Corsicus-group s. l. (the Cap Corse/Tuscany group), which

Fig. 10 (continued) (*8*) Vallée de la Restonica, 1,080 m; (*9*) Monte Rotondo, near Petra Piana, 1,850 m; (*10*) Ruine de Sorba, 1,254 m; (*11*) Vizzavona, right bank of the Vecchio, 850 m; (*12*) Vizzavona, 1,120–1,190 m (loc. typ. *minimus*); (*13*) Monte Renoso, Bastani, 2,090 m; (*14*) Monte Renoso, Vitalacia, 1,800 m; (15) Forêt de Coscione, 1,340 m; (*16*) Plateau de Coscione, 1,360 m

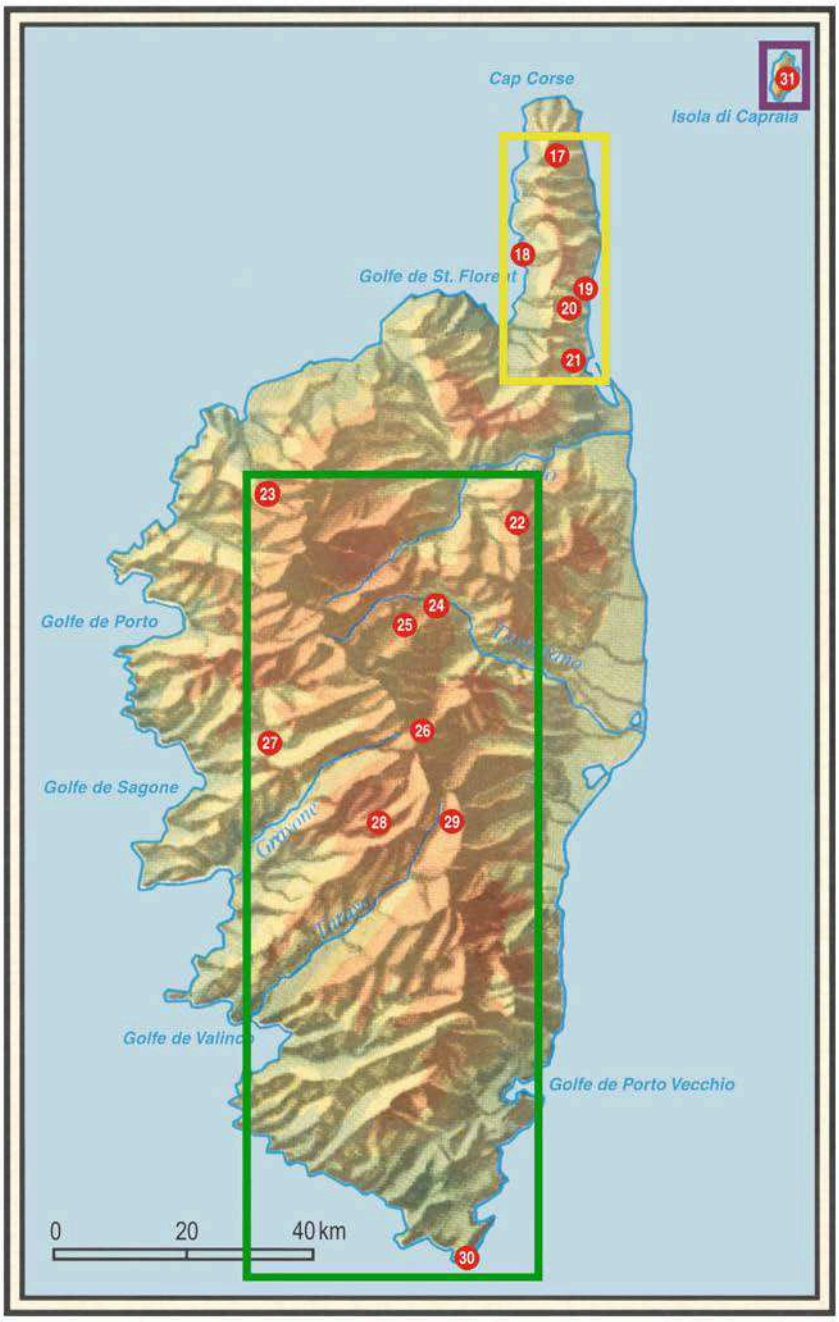

Fig. 11 Localities of the studied populations of the *Limax corsicus*- group. Map base MNHN (modified). *Green frame* Endemic *Corsicus*-group; *yellow frame* Cap Corse/Tuscany-group; *lilac frame* Capraia isolate. (*17*) Vallée de la Méria; (*18*) Nonza; (*19*) Vallée du Grigione; (*20*) Pietrabugno; (*21*) Furiani-Marinella; (*22*) Monte San Petrone, 1,060 m; (*23*) Bonifatu, 550 m;

Table 1 Percentage nucleotide sequence divergence (K2P distances) at COI within and between the Corsican/Tuscan species groups. (*n* number of specimens in each group)

Species group	*n*	Within species groups	Between groups		Cap Corse/ Tuscany-group
			Wolterstorffi-group	Endemic *Corsicus*-group	
Wolterstorffi-group	22	3.9			
Endemic *Corsicus*-group	20	0.1	10.8		
Cap Corse/ Tuscany-group	14	0.1	10.8	1.4	

presumably entered Corsica in the northeastern Alpine part of the island, the closest part of Corsica to the Italian mainland.

Because of the basal phylogenetic position of Sardinian *Limax* compared with Corsican taxa, we currently prefer the following hypothesis for the origin of the Sardinian species. Sardinia was probably colonised by two lineages of *Limax* in the late Upper Miocene (~ around 5 Mya), during the extensive period of lowest sea-level following a large-scale evaporation of the Mediterranean Sea ("Messinian salinity crisis"). Freshwater drainage systems in the shallow exposed areas and brackish conditions in deeper basins ("Lago-Mare" environment) resulted in land bridges. The origin of the colonisation of Sardinia with *Limax* was on the Italian mainland, presumably northern Latium, which was connected via the exposed northern parts of the Tyrrhenian oceanic crust (Jolivet et al. 2008; Govers et al. 2009).

The smaller islands of Capraia and Elba as well as the above-mentioned "Fossil Islands" in Tuscany remained isolated (or partly still drowned) during the Pliocene, but probably already became connected repeatedly with mainland Italy during the Early Pleistocene cooling periods (1.8–0.78 Mya) which resulted in moderate marine regressions. Both the lineage of the first *Corsicus* radiation now endemic to Hercynian Corsica and the southern part of Alpine Corsica ("endemic *Corsicus*-group") as well as the later Cap Corse/Tuscan radiation probably derived from the "Fossil Islands" area. The geographical isolation of Capraia and Elba led to the formation of two distinct sister-species: *L. giustii* n. sp. and *L. ilvensis* n. sp. (see Appendix). Another group of palaeoendemics, pulmonate snails of the genus *Tacheocampylea* L. Pfeiffer, 1877, shows a similar distribution pattern (Giusti 2007) with endemic species in Corsica, Capraia and Sardinia.

The outlined interpretation of the various colonisation events by *Limax* spp. in the west Mediterranean area are in full agreement with paleontological evidence for faunal exchange of Mammalia between paleobiogeographic provinces in Italy (Rook et al. 2006) as well as phylogenetic studies carried out with Amphibia (Zhang et al. 2008; Meijden et al. 2009; Stöck et al. 2008), and Reptilia (Mayer

Fig. 11 (continued) (*24*) Corte, west of Citadelle, 430 m; (*25*) Tuani, Vallée de la Restonica, 624 m; (*26*) Vizzavona, 860 m; (*27*) Truggia, Vallée du Liamone; (*28*) Bastelica, 770 m (loc. typ. *corsicus*); (*29*) Forêt de Piattone, 1,035 m; (*30*) Sperono, west of Bonifacio; (*31*) Capraia

Table 2 Percentage nucleotide sequence divergence (K2P distances) at COI between the species of the *Wolterstorffi*-group

Species	*L.* sp. (Restonica)	*L. wolterstorffi*	*L. vizzavonensis*	*L.* sp. (Porto)	*L.* sp. (Casamaccioli)	*L.* sp (Corte)	*L.* sp (Ft. Melo)	*L.* sp (Coscione)	*L.* sp (Mte. Renoso)	*L.* sp (Mte. Rotondo)
L. sp. (Restonica)										
L. wolterstorffi	4									
L. vizzavonensis	5.3	4.9								
L. sp. (Porto)	4.8	2.1	4.5							
L. sp. (Casamaccioli)	4.5	1.9	4.4	2.1						
L. sp (Corte)	1.2	4.1	5.5	3.7	4.3					
L. sp (Ft.Melo)	2	4.3	5.7	4.7	4.9	2.1				
L. sp (Coscione)	6.8	4.6	4.9	4.8	4.6	6.4	6.4			
L. sp (Mte. Renoso)	4.6	2.9	3.5	2.9	2.7	4.3	4.7	2.1		
L. sp (Mte. Rotondo)	4.7	3.7	1.1	3.3	3.5	4.8	5	4.2	2.3	

and Pavlicev 2007) though these studies vary in their interpretation of events and their timing.

4.2 Species Boundaries

The assumptions of species groups and species on Corsica were inferred with mutual benefit from morphology and from sequence analyses of a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I. The latter also enables tree reconstruction and provided the basis of our hypotheses of multiple colonisation of Corsica. In addition, our study provides insights into the benefits and limits of standard COI-barcoding:

In the case of the *Wolterstorffi*-group (and the majority of other *Limax* species groups; B.N., personal observation), standard species barcoding (i.e. re-identification and detection of further species by partial COI-sequencing) could be established. In all tested cases, the COI-based tree resolves the same species that were detected by morphological characters. The sequence divergences within this group are in most cases (33 of 45 pairings; cf. Table 2) higher than 3% between species, although all species can be connected by values below 3%.

However, the younger Endemic *Corsicus*-group and the Cap Corse/Tuscany-group containing specimens from Corsica and the Apennine Peninsula clearly show the limits of DNA standard barcoding concerning re-identification with COI. Despite the lack of resolution in the molecular tree, morphological and copulation characters suggest that the Endemic *Corsicus*-group comprises at least five species, including the genuine *L. corsicus* s. str. from the type locality.

Both latter mentioned *corsicus*-groups with quite recent radiation share very similar COI sequences (0.1% sequence divergence in these groups); an uncritical barcoding approach would underestimate the real number of species determined by genital anatomy and reproductive behaviour.

The current case is a significant example that, even within a single genus, species boundaries can substantially differ at the molecular level.

4.3 Evolutionary Considerations

The low genetic diversity in contrast to distinct genital anatomy and copulation features suggests an accelerated speciation rate of the two younger radiations compared to the *Wolterstorffi*-group. This acceleration might be triggered by extrinsic and intrinsic agents. First, increased rate of fragmentation of habitats of the deeper part of Corsica (contrary to the Hercynian part) by sea level changes. Alternatively, there might have been genetic exchange between populations or species in statu nascendi from the Apennine Peninsula and the island of Corsica (maybe also very recently by human influence). And second, rapid establishment of species boundaries by strong sexual selection being also reflected by an extremely

complicated copulation mode with sperm transfer through the extended penes. The unique and complex copulation behaviour and the associated morphological characters like penis length and shape are diagnostic criteria for each species. The discriminating nature of the copulatory organs is also obvious in the sympatric occurrence of different *Limax* species on Corsica.

5 Conclusions

The combined approach of morphological characters and COI-sequencing revealed multiple colonisation and three independent radiations of Corsica by *Limax*. In addition, our study provides a case showing benefits and pitfalls of COI barcoding within a single genus: except for the young radiations in Corsica and in Tuscany, standard barcoding provides sufficient resolution to identify the other *Limax* species and has lead to the molecular confirmation of two hitherto unrecognised insular endemics which are described in the Appendix. Additionally, the results establish a framework to facilitate the selection of specimens for future phylogenetic analyses with more genes. In summary, the present study shows the necessity for a combined morphological–molecular approach or an integrative taxonomy.

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Supplement: List of Material

The corresponding map points of locality in Figs. 9 and 10 are shown in parentheses.

L023, L024: *Limax* sp. (Restonica); FR, Corsica, Restonica Valley (7); leg. B. & H. Nitz, 2004; ZSM Mol 20071660, ZSM Mol 20071661, GenbankNo. GQ145497, GQ145498.

L030, L079: *Limax* sp. (Tuani); FR, Corsica, Restonica Valley (25); leg. B. & H. Nitz, 2004; ZSM Mol 20071662, ZSM Mol 20071663; GenbankNo. GQ145499, GQ145515.

L036: *Limax wolterstorffi* (Simroth, 1900); FR, Corsica, Bonifatu (1); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145500.

L038: *Limax vizzavonensis*; FR, Corsica, Vizzavona, Cascade des Anglais (12); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145501.

L040: *Limax vizzavonensis*; FR, Corsica, Sorba (10); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145502.

L043, L044: *Limax* sp. (Porto); FR, Corsica, Porto (2; 3); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145503, GQ145504.

L046: *Limax* sp. (Casamaccioli); FR, Corsica, Casamaccioli (4); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145505.

L048: *Limax vizzavonensis*; FR, Corsica, Bastani/Monte Renoso (13); leg. B. & J. Recorbet, 2003; MNHN; GenbankNo. GQ145506.

L049: *Limax* sp. (Ruggelone B); IT, Tuscany, Com. Talla, località Ruggelone; leg. W. Weidinger, 2003; SMNS ZI 0071837; GenbankNo. GQ145507.

L066: *Limax* sp. (Corte); FR, Corsica, Corte, Citadelle (6); leg. M. & G. Falkner, F1; Coll. Falkner SMNS ZI 0071838; GenbankNo. GQ145508.

L068: *Limax wolterstorffi*; FR, Corsica, Bonifatu (1); leg. M. & G. Falkner, 2002; MNHN; GenbankNo. GQ145509.

L069, L070: *Limax* sp. (Tuani); FR, Corsica, Restonica Valley (25); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145510, GQ145511.

L072: *Limax* sp. (Corte); FR, Corsica, Corte, Citadelle (24); F2; Coll Falkner; SMNS; ZI 0071839 GenbankNo. GQ145512.

L074: *Limax* sp. (Cap Corse A); FR, Corsica, Furiani (21); F1; Coll. Falkner; SMNS ZI 0071840; GenbankNo. GQ145513.

L077: *Limax* sp. (Cap Corse A); FR, Corsica, Furiani (21); F3; Coll. Falkner; SMNS ZI 0071841; GenbankNo. GQ145514.

L085: *Limax* sp. (Ruggelone B); IT, Tuscany, Com. Talla, località Ruggelone; F1; Coll. Falkner; SMNS ZI 0071842; GenbankNo. GQ145516.

L088: *Limax* sp. (Mte. Baldo); IT, Lombardy, Monte Baldo; leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071664; GenbankNo. GQ145517.

L094: *Limax* sp. (Mte. Sagro); IT, Tuscany, Alpi Apuane, Monte Sagro; leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071665; GenbankNo. GQ145518.

L100: *Limax* sp. (Mte. Altissimo); IT, Lombardy, Monte Altissimo; leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071666; GenbankNo. GQ145519.

L111, L112, L125: *Limax* sp. (Mte. San Petrone); FR, Corsica, Castagniccia, Monte San Petrone (22); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071667 - ZSM Mol 20071669; GenbankNo. GQ145520, GQ145521, GQ145522.

L126, L127, L129, L140: *Limax* cf. *corsicus* s. str.; FR, Corsica, Vizzavona (26); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071670 - ZSM Mol 20071673; GenbankNo. GQ145523, GQ145524, GQ145525, GQ145526.

L143: *Limax vizzavonensis*; FR, Corsica, Vizzavona (11); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071674; GenbankNo. GQ145527.

L148: *Limax* sp. (Bonifatu); FR, Corsica, Bonifatu (23); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071675; GenbankNo. GQ145528.

L150, L151: *Limax* sp. (Restonica); FR, Corsica, Restonica Valley (8); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071676, ZSM Mol 20071677; GenbankNo. GQ145529, GQ145530.

L158, L159: *Limax* sp. (Ft. Melo); FR, Corsica, Fôret de Melo (5); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071678, ZSM Mol 20071679; GenbankNo. GQ145531, GQ145532.

L160: *Limax vizzavonensis*; FR, Corsica, Sorba (10); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071680; GenbankNo. GQ145533.

L161, L162: *Limax* sp. (Ft. Piattone); FR, Corsica, Fôret de Piattone (29); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071681, ZSM Mol 20071682; GenbankNo. GQ145534, GQ145535.

L164: *Limax* sp. (Coscione); FR, Corsica, Plateau de Coscione (16); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071683; GenbankNo. GQ145536.

L165: *Limax* sp. (Coscione); FR, Corsica, Fôret de Coscione (15); leg. M. & G. Falkner, B. Nitz, 2004; ZSM Mol 20071684; GenbankNo. GQ145537.

L169, L170: *Limax corsicus* s. str.; FR, Corsica, Bastelica (28); leg. G. Falkner, B. Nitz & B. Recorbet, 2004; ZSM Mol 20071685, ZSM Mol 20071686; GenbankNo. GQ145538, GQ145539.

L180, L188, L189: *Limax ilvensis* n. sp.; IT, Elba; leg. E. Schwabe & J. Bohn, 2004; ZSM Mol 20071687 - ZSM Mol 20071689; GenbankNo. GQ145540, GQ145541, GQ145542.

L195: *Limax* sp. (Cap Corse A); FR, Corsica, Grigione near Bastia (19); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145543.
L197: *Limax* sp. (Cap Corse A); FR, Corsica, Pietrabugno near Bastia (20); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145544.
L199: *Limax* sp. (Cap Corse A); FR, Corsica, Furiani (21); F1; Coll. Falkner; SMNS ZI 00718; GenbankNo. GQ145545.
L210, L211: *Limax* sp. (Maiella); IT, Abruzzo, Maiella; leg. C.M. Brandstetter, 2004; SMNS ZI 0071844, ZI 0071861; GenbankNo. GQ145546, GQ145547.
L232: *Limax* sp. (Villa Strozzi); IT, Tuscany, Villa Strozzi near San Gimignano; leg. M. & G. Falkner, 1992; SMNS ZI 0071845; GenbankNo. GQ145548.
L237: *Limax* sp. (Massa Marittima); IT, Tuscany, Massa Marittima; leg. M. & G. Falkner, 1999; SMNS ZI 0071846; GenbankNo. GQ145549.
L240: *Limax* sp. (Truggia); FR, Corsica, Truggia, Liamone-Valley (27); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145550.
L241: *Limax* sp. (Bonifatu); FR, Corsica, Bonifatu (23); leg. M. & G. Falkner, 2000; MNHN; GenbankNo. GQ145551.
L269: *Limax* sp. (Mte. Renoso); FR, Corsica, Monte Renoso (14); leg. B. Recorbet, 2000; MNHN; GenbankNo. GQ145552.
L301: *Limax ciminensis*; IT, Latium, Monte Cimino; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071847; GenbankNo. GQ145553.
L345: *Limax* sp. (Verna); IT, Tuscany, Chiusi della Verna; leg. W. Weidinger, 2005; SMNS ZI 0071848; GenbankNo. GQ145554.
L382: *Limax* sp. (Torrente Trossa); IT, Tuscany, Fontebagni/Torrente Trossa; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071849; GenbankNo. GQ145555.
L384: *Limax* sp. (Marmoraia); IT, Tuscany, Montagnola Senese, Marmoraia; leg. G. Falkner & C. M. Brandstetter, 2005; SMNS ZI 0071850; GenbankNo. GQ145556.
L387: *Limax* sp. (Chianti); IT, Tuscany, Castellina in Chianti; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071851; GenbankNo. GQ145557.
L389: *Limax* sp. (Vignano); IT, Tuscany, Vignano near Siena; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071852; GenbankNo. GQ145558.
L392: *Limax* sp. (Mte. Rasu); IT, Sardinia, Monte Rasu; leg. B. & H. Nitz, 2005; ZSM Mol 20071690; GenbankNo. GQ145559.
L405, L408, L424: *Limax* sp. (Castelsecco); IT, Tuscany, Castelsecco near Arezzo; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071853, ZI 0071863, ZI 0071864; GenbankNo. GQ145560, GQ145561, GQ145567.
L412: *Limax* sp. (Arezzo A); IT, Tuscany, Arezzo, Podere Redi; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071854; GenbankNo. GQ145562.
L419, L420: *Limax* sp. (Mte. Rotondo); FR, Corsica, Monte Rotondo (9); leg. B. Recorbet, 2005; MNHN; GenbankNo. GQ145563, GQ145564.
L422, L423: *Limax* sp. (Ruggelone A); IT, Tuscany, Com. Talla, località Ruggelone; leg. W. Weidinger, 2005; SMNS ZI 0071855, ZI 0071862; GenbankNo. GQ145565, GQ145566.

L426: *Limax* sp. (Arezzo A); IT, Tuscany, Arezzo, Villa Fiorita; leg. G. Falkner & C.M. Brandstetter, 2005; ZSM Mol 20071691; GenbankNo. GQ145568.
L427: *Limax* sp. (Arezzo B); IT, Tuscany, Arezzo, Villa Fiorita; leg. G. Falkner & C.M. Brandstetter, 2005; SMNS ZI 0071856; GenbankNo. GQ145569.
L506: *Lehmannia marginata*; Sweden, Dalsland; leg. R. Heim, 2001; NMLU 14457; GenbankNo. FJ606455.
L523, L524: *Limax giustii* n. sp.; IT, Capraia (31); leg. F. Giusti, 2005; IZSI 36444/1; IZSI 36444/2; GenbankNo. GQ145582, GQ145581.
L604: *Limax* sp. (Cap Corse B); FR, Corsica, Nonza (18); leg. M. & G. Falkner, 2006; ZSM Mol 20071692; GenbankNo. GQ145570.
L605: *Limax* sp. (Cap Corse A); FR, Corsica, Vallée de la Meria (17); leg. M. & G. Falkner, 2006; ZSM Mol 20071694; GenbankNo. GQ145580.
L607: *Limax* cf. *corsicus* s. str.; FR, Corsica, Étang de Sperono, near Bonifacio, Golfcourse (30); leg. M. & G. Falkner, 2006; ZSM Mol 20071693; GenbankNo. GQ145571.
L765: *Limax brandstetteri* (Falkner, 2008); IT, Abruzzo, Maiella; leg. C.M. Brandstetter, 2005; SMNS ZI 0066222-1 ; GenbankNo. GQ145572.
L811: *Limax ianninii* (Giusti, 1973); IT, Latium, Monti Reatini, Monte Terminillo; leg. C.M. Brandstetter, 2006; SMNS 0071857-1; GenbankNo. GQ145573.
L898: *Limacus flavus*; DE, Goch; leg. S. Henssen, 2006; ZSM Mol 20071629; FJ606456.
L903: *Limax maximus*; CH, Grisons, Chur; leg. B. Nitz & U. Schneppat, 2006; ZSM Mol 20071620; GenbankNo. FJ606467.
L991: *Limax maximus*; IT, Campania, Roccamonfina; leg. C. & L. Cavegu, 2006; ZSM Mol 20071654; GenbankNo. GQ145574.
L1016: *Limax senensis* Lessona & Pollonera, 1882); IT, Tuscany, Siena; leg. M. & G. Falkner, F1; ZSM Mol 20071699; GenbankNo. GQ145575.
L1125: *Limax cinereoniger*; DE, Oberkrumbach; leg. E. Klee, A. Klee & B. Nitz, 2006; ZSM Mol 20071618; GenbankNo. FJ606460.
L1410: *Limacus flavus*; UK, Surrey, Banstead; leg. J. Hutchinson, 2007; ZSM Mol 20071630; GenbankNo. FJ606457.
L1464: *Vitrina pellucida*; DE, Kolbingen; leg. B. Hausdorf, 2006; ZMH 51046; GenbankNo. FJ606454.
L1600: *Limax* sp. (Luogosanto); IT, Sardinia, Luogosanto; leg. B. Ruthensteiner, 2007; ZSM Mol 20071695; GenbankNo. GQ145576.
L1612: *Limax* sp. (Populonia A); IT, Tuscany, Populonia; leg. J. Spelda, 2007; ZSM Mol 20071696; GenbankNo. GQ145577.
L1613: *Limax* sp. (Populonia B); IT, Tuscany, Populonia; leg. J. Spelda, 2007; ZSM Mol 20071697; GenbankNo. GQ145578.
L1749: *Limax* sp. (Cutigliano); IT, Tuscany, Cutigliano-Melo; leg. G. Bertagni, 2007; ZSM Mol 20071698; GenbankNo. GQ145579.
L1778: *Limax cinereoniger*; DE, Oberkrumbach; leg. E. Klee, A. Klee & B. Nitz, 2007; ZSM Mol 20071619; GenbankNo. FJ606463.
W002: *Limax wohlberedti*; HR, Dalmatia; leg. A. Wiktor, 1999; MNHW, Coll. A. Wiktor 3004; GenbankNo. FJ606481.

Appendix: Two New Species and One New Name of Peri-Tyrrhenian *Limax*

Gerhard Falkner and Barbara Nitz

In this appendix, we introduce names for two hitherto unrecognized species originally revealed by COI barcoding and replace a preoccupied name for a well defined species.

Limax specimens from the Tuscan islands Elba and Capraia have been described by Giusti (1969; 1976) and Giusti and Mazzini (1971) and were thought to belong to *Limax corsicus* s. str. (see also Pollonera 1905). However, our Bayesian tree reconstruction of 615 nucleotides of the cytochrome *c* oxidase subunit I gene (COI) grouped specimens of these two islands in two distinct clades with high support values (PP 100% for Elba specimens and 95% for Capraia specimens), placing *L. corsicus* from the type locality in a different group. These findings reveal the anatomical differences (especially in the internal structure of the penis) found by Giusti in a new light. In line with Code Art. 13.1.2 (ICZN 1999), we base the new names on the existing excellent descriptions. The necessary (partly unpublished) information about the type material was kindly provided by the author.

Limax giustii n. sp.

Description: Giusti 1969: Genital apparatus (Fig. 12); Giusti and Mazzini 1971: Internal structure of the penis (Fig. 13).

Derivatio nominis: Named in honor of our distinguished colleague and friend Prof. Dr. Folco Giusti di Massa, whose valuable *Limax*-studies began on his beloved island of Capraia.

Holotype: The specimen represented in Fig. 13 (Giusti and Mazzini 1971); collected in the Capraian site (very close to the village and to the locality called "La Grotta") which is called "San Leonardo"; leg. F. Giusti 14.04.1968 (1966 is a misprint – Giusti, personal communication). Body length in ethanol (after drowning) ca. 6.2 cm, width ca. 1 cm. Length of penis ca. 10.5 cm. Preparation in Giusti collection, IZSI 22001.

Paratypes: Four specimens collected in the Capraian site of "San Rocco"; leg. F. Giusti 31.10.2005. Maximum length in ethanol ca. 8 cm, width ca. 1.5 cm. The back of these specimens is predominantly uniformly dark or with a whitish band corresponding to the keel line. Preparations in Giusti collection, IZSI 36444; Tissue samples SMNS ZI 0071865 (two specimens have been sequenced: L523 and L524, DNA elutions stored in ZSM DNA-bank).

Remarks: According to Giusti's personal observations, the Capraia specimens are actually slightly smaller than other members of the so-called *L. corsicus* ("but this has not a sure relevance, due to the possibility of an insular dwarfism phenomenon"). Estimated from memory, they reach alive a length of ca. 10 cm when fully

extended. Judging from photographs, there are mainly the following colour morphs: dark brown to blackish, medium brown with contrasting yellowish-white lateral bands and keel line, and medium brown with irregular blotchy dark lateral bands which are separated from the darker back by brighter zones, sometimes the mantle shield is spotted. The reddish colouration of the sole is normally not very intense.

Several copulations were observed and photographed by Giusti in spring 1983 and 1985. The basis of comparison is not yet sufficient to draw definite taxonomical conclusions. The action follows the general scheme of the *Corsicus*-group as described for the first time by Gerhardt (1937) for Ischia. Some special features are: the penial combs are quite weakly developed, the penial bases are not in close contact [as, for example, in *L.* sp. (Bonifatu)], the bases of the bursa copulatrix are not everted [as, for example, in *L.* sp. (Tuani) and specimens from Marmuccio], in the contraction phase dense white foam is excreted (which is not the case in the Endemic *Corsicus*-group, but present in the Cape Corse/Tuscany-group), the maximum extension of the penes is between 26 and 30 cm (see Figs. 5 and 6).

The new species is endemic for the Tuscan Island of Capraia.

Limax ilvensis n. sp.

Description: Giusti 1976: Discussion of characters and internal structure of the penis (Fig. 21).

Derivatio nominis: An adjective formed from Ilva, the Roman name for Elba.

Holotype: The specimen represented in Fig. 21 (Giusti 1976); collected at the site "Portoferraio: il Forte", of the Island of Elba; leg. F. Giusti 18.02.74. Length in ethanol (after drowning) ca. 7 cm; width ca. 1.25 cm. Length of penis ca. 12 cm. Preparation in Giusti collection, IZSI 11977.

Paratypes: 1 specimen (L180), Elba, Monte Perone, ca. 600 m, biotope with chestnut and pine trees; leg. E. SCHWABE & J. BOHN 19.10.2004. ZSM Mol 20071687. – 2 specimens (L188 and L189), Elba, Capoliveri, ca. 250 m; ruderalised resting place; leg. E. SCHWABE & J. BOHN 20.10.2004. ZSM Mol 20071688 and 20071689.

Remarks: The paratypes and additional live specimens (ZSM) comprised brown and dark brown colour morphs with reddish sole, the latter characteristic for the *Corsicus*-group.

According to Giusti, the preparation of the holotype has been discoloured by ethanol, but nevertheless its colour is clearly paler than that of the Capraia specimens. The holotype shows a narrow whitish band on both sides which is bordered by interrupted blackish bands similarly narrow. The back shows a pale-brownish colour with more or less large darker lobate spots. The clypeus has a paler, almost whitish basic colour with large darker lobate spots. The lower part of the sides is similarly whitish with small, brownish lobate spots. The bleached sole is whitish throughout.

For two Elba collections with quite well preserved colours in the NMW (no. 39559, leg. Holdhaus 1904; no. 45114, leg. Paganetti 1908) the following

observations have been noted: dark to nearly black morphs are dominant, juveniles nearly black, subadults brighter with diffuse brown lateral bands and slightly darker back; in the second collection, some specimens were deep black with contrasting narrow white lateral bands. The soles were slightly reddish to yellow.

The new species is endemic for the Tuscan Island of Elba.

Limax vizzavonensis n. nom.

This new replacement name is herewith introduced for *Limax* (*Eulimax*) *cinereoniger* var. *minimus* Pollonera, 1896, which is preoccupied by *Limax minimus* Forsskål, 1775.

Nomenclatural history: Although for its time aptly described, *Limax minimus* Pollonera, 1896, was largely neglected. The name was only used by Taylor (1903) and Hesse (1926) for an infrasubspecific entity, by Caziot (1903) and Alzona (1971) at subspecific rank, and by Falkner et al. (2002) at species rank.

Following Hesse (1924), Falkner et al. overlooked the fact that the name is preoccupied by the name of a sea slug. Suppression of the older homonym *Limax minimus* Forsskål, 1775, was proposed by Lemche (1964: 37), who considered the species as unrecognisable, in order to avoid confusion. Accordingly *Limax minimus* Forsskål, 1775, was by Opinion 773 (ICZN 1966) "suppressed for the purposes of the Law of Priority but not for those of the Law of Homonymy." The consequence of the latter is that it continues to preclude the validation of its younger primary homonym which therefore must be replaced. The existing replacement name *L. obscurus* Simroth, 1900, cannot be used as it is itself preoccupied by *L. maximus* var. *obscurus* Moquin-Tandot, 1855.

The results of our foregoing morphological and genetic studies have shown that it is necessary to dispose of a valid name for this distinguishable biological entity which has already been invalidly named twice. The new name *vizzavonensis* is derived from the type locality.

Remarks: The type locality which was given by Pollonera simply as "Vizzavona" is stated more precisely by Caziot (1903), who collected the holotype, as "la Foce, près Vizzavona, à l'altitude de 1,000 m." The sequenced animal collected near Cascade des Anglais (L038) was found only about 200 m away from the type locality.

Despite the fact that the present solution of the nomenclatural problem is provided, a thorough redescription still remains a desideratum for future research.

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7 Article III: Does Automatic Barcode Gap Discovery (ABGD) improve species delimitation of a Corsican radiation of *Limax* (Gastropoda: Stylommatophora)?

This manuscript is intended to be submitted as:

Nitz B, Haszprunar G. Does Automatic Barcode Gap Discovery (ABGD) improve species delimitation of a Corsican radiation of *Limax* (Gastropoda: Stylommatophora)?

7.1 Introduction

Corsica and the Apennine Peninsula are the habitat of several mostly undescribed *Limax* species. Nitz *et al.* (2010; chapter 6) used an integrated approach combining morphological and molecular data to reveal two monophyletic species groups of six to ten species each, representing two independent radiations in the geographic region of Corsica and the adjacent mainland. One species group, the *Wolterstorffi*-group, could be differentiated by the analysis of cytochrome *c* oxidase subunit I (COI) sequences, whereas the other, the *Corsicus*-group sensu lato, was not clearly separated by molecular means. However, the molecular analyses suggested a further split of this *Corsicus*-group sensu lato into the Endemic *Corsicus*-group and the Cap Corse/Tuscany-group. To define the monophyletic groups and to further discriminate between the species within the groups, Nitz and colleagues used a tree-based approach (Bayesian inference analysis) and corroborated the findings by a comparison of sequence divergences within and between the single species groups. Shortly before and after the publication of Nitz *et al.* in the year 2010, several new approaches of species identification by utilization of (COI-) sequences were published, e.g. by Sarkar *et al.* (2008), who published a character-based identification approach, or by Puillandre *et al.* (2011; 2012), whose ABGD (Automatic Barcode Gap Discovery) method screens for barcoding gaps in a set of sequences.
In this study, the COI data set of Nitz *et al.* (2010) is used to check if the modern ABGD method improves the species discrimination in the Corsican *Limax* radiations.

7.2 Materials and Methods

7.2.1 Molecular data set

The methods and the data set used for the ABGD analysis are described in detail in Nitz *et al.* (2010). The data set comprises COI sequences (615 nucleotides) of 90 *Limax* specimens. Outgroups were removed from the initial data set.

7.2.2 ABGD analysis

For molecular-based species delineation, I calculated pairwise distances for the COI data set under the Kimura 2-parameter model in MEGA5 (Tamura *et al.*, 2011) and used the web server of ABDG (Automatic Barcode Gap Discovery, Puillandre *et al.*, 2011, http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with default options for the search for barcoding gaps.

7.3 Results

Applying ABGD with the standard options resulted in 20 groups potentially representing species at a prior maximal distance P lower than 0.002783 (named Partition I, see Table 7-1 and Figure 7-1, in which the ABGD Partitions are added to the tree in Figure 9 of Nitz *et al.*, 2010). With a prior maximal distance P at 0.001668, the software identified 41 groups (named Partition II). Partition II is in general similar to Partition I (19 groups are identical) and differs just in the splitting of one huge group (group 20 in Partition I) into 22 small groups (groups 20 II - 41 II) in Partition II.
Groups 1-19 in both partitions consist of one to five specimens each. Valid species like *Limax maximus*, *L. brandstetteri*, *L. wohlberedti*, *L. ianninii* and *L. cinereoniger* are classified correctly. Specimens of the Corsican *Wolterstorffi*-group (bar G in Fig. 7-1) are classified into nine species by ABGD. Specimens belonging to the *ciminensis*-group and "sp. 2" of Italian Checklist [Manganelli *et al.*, 1995] are divided into two groups (groups 6 and 4), which correspond bar F and bar E in Fig. 7-1.
Group 20 consists of 51 specimens from Capraia and Elba (*L. giustii* n. sp. and *L ilvensis* n. sp.), from the Appenine Peninsula (*senensis*-group, group of “Fossil Islands: bars D and B in Fig. 7-1) and from Tuscany and Corsica (Endemic *Corsicus*-group, Cap Corse/Tuscany-group: bars C and A in Fig. 7-1). This group 20 (Partition I) is split into 22 groups when applying a different prior maximal distance in ABGD (Partition II). Groups 20 II to 41 II are represented by one to 16 specimens in each group. In a lot of cases, specimens from adjacent localities are not placed into the same group, e.g. *L.* sp. [Cap Corse A] from the Cap Corse/Tuscany-group (bar A) is represented in the ABGD groups 23 II, 28 II and 24 II. Some specimens that potentially belong to one species are grouped together, e.g. all three specimens of *L. ilvensis* are arrangend into group 27 II; however, other specimens that might belong to one single species (e.g. *L. giustii*) are separated into groups with only one member (groups 40 II and 41 II). Also the two specimens of *L. corsicus* s.str. from the same sampling site are split and appear in group 20 II and 26 II.

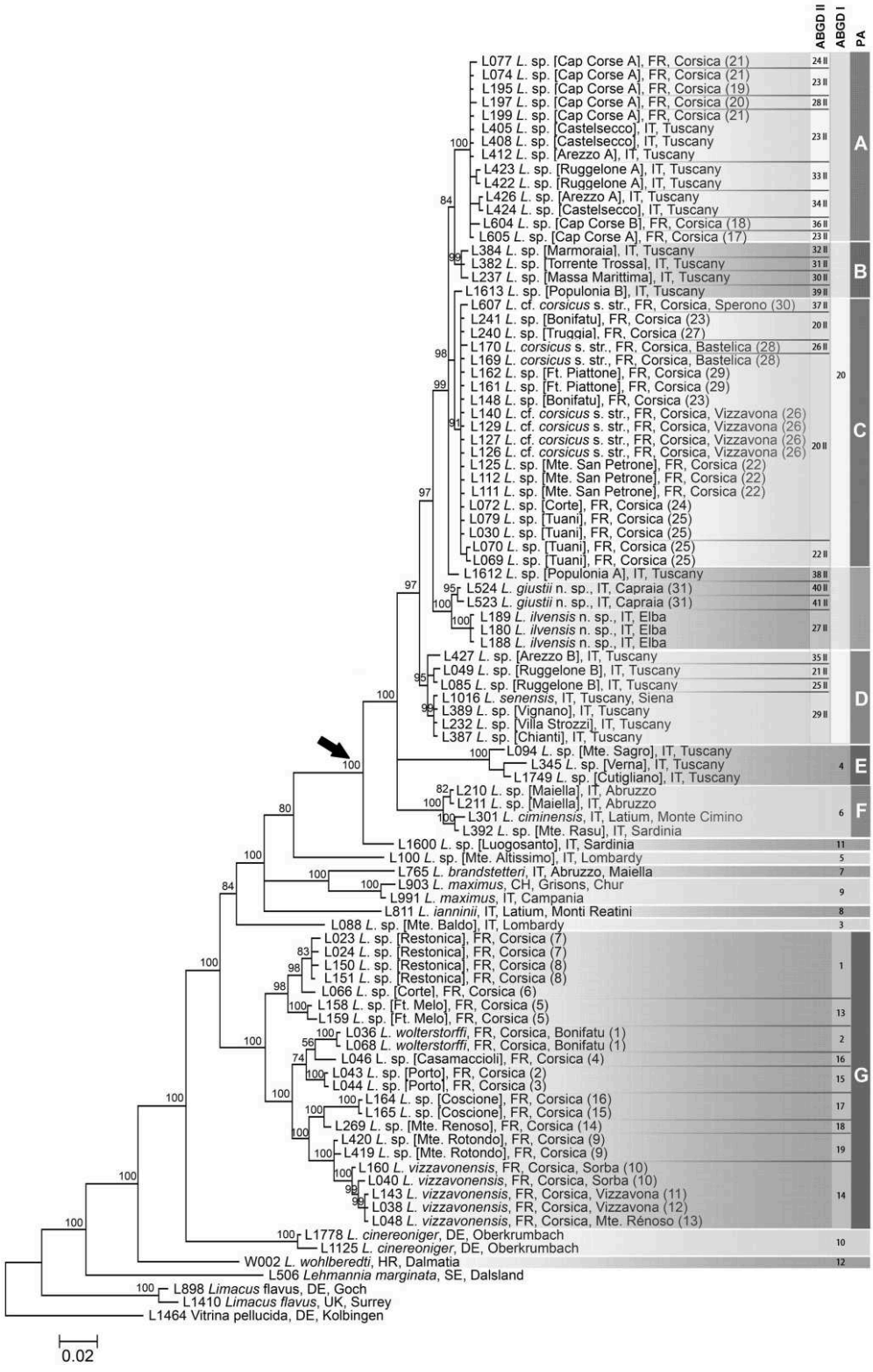

Fig. 7-1. Majority-rule consensus tree (Bayesian inference analysis) modified from Figure 9 in Nitz *et al.* (2010). Posterior probabilities are marked above the branches. Arrow: *Corsicus*-group sensu lato. Column PA (phylogenetic analysis): Bar A: Cap Corse/Tuscany-group. Bar B: group of fossil islands. Bar C: Endemic *Corsicus*-group. Bar D: *senensis*-group. Bar E: group of "sp. 2" of Italian Checklist (Manganelli *et al.* 1995). Bar F: *ciminensis*-group. Bar G: *Wolterstorffi*-group. Column ABGD I: Partition I groups resulting from the species deliniation approach with ABGD at a prior maximal distance P lower than 0.002783. Column ABGD II: Partition II groups at a prior maximal distance P at 0.001668.

Table 7-1. Molecular based species delineation with ABGD. Partition I was calculated at a prior maximal distance P lower than 0.002783. Partition II was identified at a prior maximal distance P at 0.001668.

	Partition I/II
Group 1 (n=5)	L023 *Limax* sp. [Restonica] {*Wolterstorffi*-group}, L024 *Limax* sp. [Restonica] {*Wolterstorffi*-group}, L066 *Limax* sp. Corte {*Wolterstorffi*-group}, L150 *Limax* sp. [Restonica] {*Wolterstorffi*-group}, L151 *Limax* sp. [Restonica] {*Wolterstorffi*-group}.
Group 2 (n=2)	L036 *Limax wolterstorffi* {*Wolterstorffi*-group}, L068 *Limax wolterstorffi* {*Wolterstorffi*-group}.
Group 3 (n=1)	L088 *Limax* sp. [Mte. Baldo].
Group 4 (n=3)	L094 *Limax* sp. [Mte. Sagro], L345 *Limax* sp. [Verna], L1749 *Limax* sp. [Cutigliano].
Group 5 (n=1)	L100 *Limax* sp. [Mte. Altissimo].
Group 6 (n=4)	L210 *Limax* sp. [Maiella], L211 *Limax* sp. [Maiella], L301 *Limax ciminensis*, L392 *Limax* sp. [Mte. Rasu].
Group 7 (n=1)	L765 *Limax brandstetteri*.
Group 8 (n=1)	L811 *Limax ianninii*.
Group 9 (n=2)	L903 *Limax maximus*, L991 *Limax maximus*.
Group 10 (n=2)	L1125 *Limax cinereoniger*, L1778 *Limax cinereoniger*.
Group 11 (n=1)	L1600 *Limax* sp. [Luogosanto].
Group 12 (n=1)	W002 *Limax wohlberedti*.
Group 13 (n=2)	L158 *Limax* sp. [Ft. Melo] {*Wolterstorffi*-group}, L159 *Limax* sp. [Ft. Melo] {*Wolterstorffi*-group}.
Group 14 (n=5)	L038 *Limax vizzavonensis* {*Wolterstorffi*-group}, L040 *Limax vizzavonensis* {*Wolterstorffi*-group}, L048 *Limax vizzavonensis* {*Wolterstorffi*-group}, L143 *Limax vizzavonensis* {*Wolterstorffi*-group}, L160 *Limax vizzavonensis* {*Wolterstorffi*-group}.
Group 15 (n=2)	L043 *Limax* sp. [Porto] {*Wolterstorffi*-group}, L044 *Limax* sp. [Porto] {*Wolterstorffi*-group}.
Group 16 (n=1)	L046 *Limax* sp. [Casamaccioli] {*Wolterstorffi*-group}.
Group 17 (n=2)	L164 *Limax* sp. [Coscione] {*Wolterstorffi*-group}, L165 *Limax* sp. [Coscione] {*Wolterstorffi*-group}.
Group 18 (n=1)	L269 *Limax* sp. [Mte. Renoso] {*Wolterstorffi*-group}.
Group 19 (n=2)	L419 *Limax* sp. [Mte. Rotondo] {*Wolterstorffi*-group}, L420 *Limax* sp. [Mte. Rotondo] {*Wolterstorffi*-group}.
Group 20 (n=51) (Partition I only)	L030 *Limax* sp. [Tuani] {EndemicCorsicus}, L049 *Limax* sp. [Ruggelone B], L069 *Limax* sp. [Tuani] {EndemicCorsicus}, L070 *Limax* sp. [Tuani] {EndemicCorsicus}, L072 *Limax* sp. [Corte] {EndemicCorsicus}, L074 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L077 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L079 *Limax* sp. [Tuani] {EndemicCorsicus}, L085 *Limax* sp. [Ruggelone B], L111 *Limax* sp. [Mte. San Petrone] {EndemicCorsicus}, L112 *Limax* sp. [Mte. San Petrone] {EndemicCorsicus}, L125 *Limax* sp. [Mte. San Petrone] {EndemicCorsicus}, L126 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L127 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L129 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L140 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L148 *Limax* sp. [Bonifatu] {EndemicCorsicus}, L161 *Limax* sp. [Ft. Piattone] {EndemicCorsicus}, L162 *Limax* sp. [Ft. Piattone] {EndemicCorsicus}, L169 *Limax corsicus* s. str. {EndemicCorsicus}, L170 *Limax corsicus* s. str. {EndemicCorsicus}, L180 *Limax ilvensis* n. sp., L188 *Limax ilvensis* n. sp., L189 *Limax ilvensis* n. sp., L195 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L197 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L199 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L232 *Limax* sp. [Villa Strozzi], L237 *Limax* sp. [Massa Marittima], L240 *Limax* sp. [Truggia] {EndemicCorsicus}, L241 *Limax* sp. [Bonifatu] {EndemicCorsicus}, L382 *Limax* sp. [Torrente Trossa], L384 *Limax* sp. [Marmoraia], L387 *Limax* sp. [Chianti], L389 *Limax* sp. [Vignano], L405 *Limax* sp. [Castelsecco] {Cap Corse/Tuscany-group}, L408 *Limax* sp. [Castelsecco] {Cap Corse/Tuscany-group}, L412 *Limax* sp. [Arezzo A] {Cap Corse/Tuscany-group}, L422 *Limax* sp. [Ruggelone A] {Cap Corse/Tuscany-group}, L423 *Limax* sp. [Ruggelone A] {Cap Corse/Tuscany-group}, L424 *Limax* sp. [Castelsecco] {Cap Corse/Tuscany-group}, L426 *Limax* sp. [Arezzo A] {Cap Corse/Tuscany-group}, L427 *Limax* sp. [Arezzo B], L604 *Limax* sp. [Cap Corse B] {Cap Corse/Tuscany-group}, L607 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L1016 *Limax senensis*, L1612 *Limax* sp. [Populonia A], L1613 *Limax* sp. [Populonia B], L605 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L524 *Limax giustii* n. sp., L523 *Limax giustii* n. sp.

	Partition II
Group 20 II (n=16)	L030 *Limax* sp. [Tuani] {EndemicCorsicus}, L072 *Limax* sp. [Corte] {EndemicCorsicus}, L079 *Limax* sp. [Tuani] {EndemicCorsicus}, L111 *Limax* sp. [Mte. San Petrone] {EndemicCorsicus}, L112 *Limax* sp. [Mte. San Petrone] {EndemicCorsicus}, L125 *Limax* sp. [Mte. San Petrone] {EndemicCorsicus}, L126 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L127 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L129 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L140 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}, L148 *Limax* sp. [Bonifatu] {EndemicCorsicus}, L161 *Limax* sp. [Ft. Piattone] {EndemicCorsicus}, L162 *Limax* sp. [Ft. Piattone] {EndemicCorsicus}, L169 *Limax corsicus* s. str. {EndemicCorsicus}, L240 *Limax* sp. [Truggia {EndemicCorsicus}, L241 *Limax* sp. [Bonifatu] {EndemicCorsicus}.
Group 21 II (n=1)	L049 *Limax* sp. [Ruggelone B].
Group 22 II (n=2)	L069 *Limax* sp. [Tuani] {EndemicCorsicus}, L070 *Limax* sp. [Tuani] {EndemicCorsicus}.
Group 23 II (n=7)	L074 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L195 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L199 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}, L405 *Limax* sp. [Castelsecco] {Cap Corse/Tuscany-group}, L408 *Limax* sp. [Castelsecco] {Cap Corse/Tuscany-group}, L412 *Limax* sp. [Arezzo A] {Cap Corse/Tuscany-group}, L605 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}.
Group 24 II (n=1)	L077 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}.
Group 25 II (n=1)	L085 *Limax* sp. [Ruggelone B].
Group 26 II (n=1)	L170 *Limax corsicus* s. str. {EndemicCorsicus}.
Group 27 II (n=3)	L180 *Limax ilvensis* n. sp., L188 *Limax ilvensis* n. sp., L189 *Limax ilvensis* n. sp.
Group 28 II (n=1)	L197 *Limax* sp. [Cap Corse A] {Cap Corse/Tuscany-group}.
Group 29 II (n=4)	L232 *Limax* sp. [Villa Strozzi], L387 *Limax* sp. [Chianti], L389 *Limax* sp. [Vignano], L1016 *Limax senensis*.
Group 30 II (n=1)	L237 *Limax* sp. [Massa Marittima].
Group 31 II (n=1)	L382 *Limax* sp. [Torrente Trossa].
Group 32 II (n=1)	L384 *Limax* sp. [Marmoraia].
Group 33 II (n=2)	L422 *Limax* sp. [Ruggelone A] {Cap Corse/Tuscany-group}, L423 *Limax* sp. [Ruggelone A] {Cap Corse/Tuscany-group}.
Group 34 II (n=2)	L424 *Limax* sp. [Castelsecco] {Cap Corse/Tuscany-group}, L426 *Limax* sp. [Arezzo A] {Cap Corse/Tuscany-group}.
Group 35 II (n=1)	L427 *Limax* sp. [Arezzo B].
Group 36 II (n=1)	L604 *Limax* sp. [Cap Corse B] {Cap Corse/Tuscany-group}.
Group 37 II (n=1)	L607 *Limax* cf. *corsicus* s. str. {EndemicCorsicus}.
Group 38 II (n=1)	L1612 *Limax* sp. [Populonia A].
Group 39 II (n=1)	L1613 *Limax* sp. [Populonia B].
Group 40 II (n=1)	L524 *Limax giustii* n. sp.
Group 41 II (n=1)	L523 *Limax giustii* n. sp.

7.4 Discussion

The *Wolterstorffi*-group is split into nine single ABGD-groups. For the *Wolterstorffi*-group the output of ABGD is congruent with the morphology-driven hypothesis of eight to ten species.

In the *Corsicus*-group sensu lato, morphological and copulation characters suggest a split into the Endemic *Corsicus*-group and the Cap Corse/Tuscany-group; this assumption is supported by the molecular tree with support values of 91 and 100 %

for these clades. In the ABGD analyses, the patterns in these clades are ambiguous; the *Corsicus*-group sensu lato is either lumped into one big group or split into a number of groups, which are not completely congruent with the morphological species hypotheses of about five species in the Endemic *Corsicus*-group. The morphological and molecular data presented in Nitz *et al.* (2010) also fail to clearly resolve the potential number of species in the Cap Corse/Tuscany-group containing specimens from Corsica and the Apennine Peninsula. Both latter mentioned *Corsicus*-groups with quite recent radiation share very similar COI sequences (0.1% sequence divergence in these groups) and ABGD fails to resolve the *Corsicus*-groups, either splitting them into a huge number of groups with single specimens or lumping them into one group depending on the value of the prior maximal distance. An uncritical barcoding approach without crossvalidation by additional data sets would not hit the number of morpho-species hypothesised by genital anatomy and reproductive behaviour at least in the Endemic *Corsicus*-group. The molecular results of the C*orsicus*-groups underline that recently diverged species are problematic in molecular species delineation approaches (Meyer & Paulay, 2005; Sauer & Hausdorf, 2012; van Velzen *et al.*, 2012). However, the ABGD clustering result has to be handled with care, since the method perfoms best for data sets with more than three to five sequences per species (Puillandre *et al.*, 2011), a number that was not available for most of our potential species. Our study demonstrates that a modern discrimination approach like ABGD does not further improve species discrimination in our limited data set compared to the methods used in Nitz *et al.* (2010). Thus further work including broader taxon sampling and thorough copulation observations will be needed to confirm or reject the morphology-based species hypotheses.

8 Article IV: Back to the roots of the genus *Limax*: A framework based on an integrated taxonomic approach

This manuscript is intended to be submitted as:

Nitz B, Hyman, I, Schneppat, UE, Knechtle, F, Heim, R, Haszprunar G. Back to the roots of the genus *Limax*: A framework based on an integrated taxonomic approach

8.1 Introduction

As outlined in the previous chapters of the Thesis, there is no consensus about the number of species in the genus *Limax* Linnaeus, 1758. In the course of the studies of our Task Force *Limax* team (Hyman, 2006), it became more and more apparent that species numbers in *Limax* are still severely underestimated due to a lack of research and the various management of diagnostic morphological characters. Therefore, it is difficult to get an overview of the whole genus and its possible roots and relationships. For the genus *Limax* there are no explicit phylogenetic hypotheses based on either molecular or morphological data. Up to now, all studies of limacid slugs were based on morphological characters. Hausdorf (1998) started the era of classification based on phylogenetic analysis of morphological characters in the superfamily Limacoidea in 1998. Molecular research in Stylommatophora was strongly influenced by Wade *et al.* (2001; 2006); however, no member of Limacidae was included in their rDNA study. An exhaustive molecular analysis of limacid slugs is still handicapped by the limited availability of fresh material suitable for DNA isolation for most of the species (including described species, those of unclear status and those that are cryptic and/or still undescribed). However, the activities of the Task Force *Limax* have lead to a progressive sampling of fresh *Limax* material all over Europe, enabling in the present study a first interpretation of the biogeographic patterns of this genus. Representatives of other genera of the family Limacidae Lamarck, 1801 were added to improve our understanding of the relationships in this family at the molecular level.

The previous chapters aimed to show the value of sequences as an additional character set in an intregrated taxonomy approach at the species level (Chapter 5: Nitz *et al.*, 2009) and the usefulness of a molecular character set in illuminating the relationships in a closely related species system (Chapter 6: Nitz *et al.*, 2010). In the present study I aim to (1) reconstruct phylogenetic relationships at the molecular level among a number of *Limax* species representing major European lineages and (2) to give some initial insights into the European Limacidae and their phylogenetic relationships using COI sequence variation. These results are discussed in the light of a first survey of biogeographic patterns and by appraising morphological data.

8.2 Materials and Methods

8.2.1 Collection and treatment of specimens

Most specimens were collected by BN and members of Task Force *Limax*. Complementary specimens were borrowed from other collections (see list of material, Table 8-1). For the institutions from which material was obtained, the following standardised abbreviations (in brackets) are used: Bündner Naturmuseum, Chur (BNM); Museum of Natural History, Wrocław University (MNHW); Naturhistorisches Museum Bern (NMBE); Natur-Museum Luzern (NMLU); Nationaal Natuurhistorisch Museum Leiden (RMNH); Senckenberg Museum für Naturkunde Görlitz (SMNG); Staatliches Museum für Naturkunde Stuttgart (SMNS); Zoologisches Museum Hamburg (ZMH); Zoologische Staatssammlung München (ZSM).

The selection of *Limax* specimens is intended to represent all major European lineages of the genus. Whenever possible, specimens from type localities were included in the analyses. Starting with a data set of 352 specimens (*Limax*, additional genera of Limacidae, outgroup taxa) for preliminary tree calculations (with PhyML), the initial data set was stepwise reduced to 89 *Limax* specimens so that only one to a few animals represent the major clades, reducing calculation time and producing a concise tree. The removed specimens were mainly specimens from the same locality or specimens from the same species but from different localities or different colour morphs. The *Corsicus*-group *sensu lato* and the *Wolterstorffi*-group (Nitz *et al*., 2010), which are discussed in detail in chapter 6, were restricted to two representative species each.

Seven specimens representing the European limacid genera were included in the study. All genera with Middle-European distributions belonging to Limacinae Lamarck, 1801 (*Limax* Linnaeus, 1758, *Lehmannia* Heynemann, 1863, *Malacolimax* Malm, 1868; classification after Schileyko, 2003) are covered by at least one specimen. The genus *Bielzia* Clessin, 1887, which is either grouped in the separate family Limacopsidae (Sysoev & Schileyko, 2009) or as a member of Limacidae (Limacopsinae Gerhardt, 1936; Bielziinae after Likharev & Wiktor, 1980) is included in the molecular analyses as well, represented by two specimens of *Bielzia coerulans* (Bielz, 1851).

Table 8-1. Locality, collector, museum deposition numbers and Genbank accession number of the specimens.

Species	Locality	Collector, Year	Museum Number	Genbank Number
Ambigolimax valentianus	France, Aude, Alet-les-Bains	R. Heim, 2003	NMLU 14772	JX435832
Bielzia coerulans	Romania, Fagaras Mountains	C. Komposch & K. Brandl, 2006	ZSM Mol 20071701	JX435825
Bielzia coerulans	Romania, Bucegi Mountains	C. Komposch & K. Brandl, 2006	ZSM Mol 20071705	JX435829
Boettgerilla pallens	Germany, Hörlkofen	G. Falkner, 2005	ZSM Mol 20071718	JX435886
Deroceras laeve	Switzerland, Churwalden	U. Schneppat, 2007	ZSM Mol 20071717	JX435885
Lehmannia marginata	Sweden, Dalsland	R. Heim, 2001	NMLU 14457	FJ606455
Limacus flavus	Germany, Goch	S. Henssen, 2006	ZSM Mol 20071629	FJ606456
Limacus flavus	United Kingdom, Surrey, Banstead	J. Hutchinson, 2007	ZSM Mol 20071630	FJ606457
Malacolimax tenellus	Poland, Silesian Lowlands	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071709	JX435836
Vitrina pellucida	Germany, Kolbingen	B. Hausdorf, 2006	ZMH 51046	FJ606454
Limax aff. *conemenosi*	Greece, Samos Island	E. Gittenberger, 1999	RMNH.MOL.46297	JX435837
Limax brandstetteri	Italy, Abruzzo, Maiella	C.M. Brandstetter, 2005	SMNS ZI 0066222-1	GQ145572
Limax cf. "Blauköpfige Egelschnecke"	Switzerland, Ticino, Carona	M. Wüthrich, 1989	NMBE 5400	FJ606479
Limax cf. "Blauköpfige Egelschnecke"	Switzerland, Ticino, San Salvatore	A. J. de Winter, 1989	RMNH 106761	FJ606480
Limax cf. *cephalonicus* sensu Wiktor, 2001	Greece, Ossa Mountain	D. Georgiev & I. Klimentova, 2010	BNM 060578	JX435865
Limax cinereoniger	Germany, Oberkrumbach	E. Klee, A. Klee & B. Nitz, 2006	ZSM Mol 20071618, ZSM Mol 20071619	FJ606460, FJ606463
Limax cf. *cinereoniger*	Italy, Udine	M. Giovannelli, 2006	ZSM Mol 20071703	JX435827
Limax cf. *cinereoniger*	Romania, Fagaras Mountains	C. Komposch & K. Brandl, 2006	ZSM Mol 20071704	JX435828
Limax cf. *cinereoniger*	Austria, Steiermark, Peggau	U. Schneppat, U. Stockinger & C. Komposch, 2005	BNM 063834	JX435831
Limax cf. *cinereoniger*	Romania, Bucegi Mountains	C. Komposch & K. Brandl, 2006	ZSM Mol 20071706	JX435833

Limax cf. *cinereoniger*	Romania, Fagaras Mountains	C. Komposch & K. Brandl, 2006	ZSM Mol 20071707	JX435834
Limax cf. *cinereoniger*	Italy, Udine, Lusevera	C. Dalfreddo, 2006	ZSM Mol 20071710	JX435838
Limax cf. *cinereoniger*	Italy, Südtirol, Völs	Y. Kiss & T. Kopf, 2006	ZSM Mol 20071713	JX435842
Limax cf. *cinereoniger*	Croatia, Proscansko Jezera	H. Reise, 2010	BNM 061422	JX435847
Limax cf. *cinereoniger*	Poland, Bieszady Mountains	A. Sulikowska-Drozd, 2011	BNM 062954	JX435848
Limax cf. *cinereoniger*	Croatia, Velika Paklenica	H. Reise & J. Hutchinson, 2010	SMNG p17152	JX435849
Limax cf. *cinereoniger*	Croatia, Istria	G. Wondrak, 2010	BNM 061559	JX435851
Limax cf. *cinereoniger*	Austria, Nationalpark Gesäuse	C. Komposch, 2009	BNM 058511	JX435852
Limax cf. *cinereoniger*	Hungaria, Orfü	B. Pall-Gergeli, 2010	BNM 062893	JX435853
Limax cf. *cinereoniger*	Switzerland, Wallis, Ayent Pra Combère	Y. Chittaro, 2010	BNM 062838	JX435854
Limax cf. *cinereoniger*	Spain, Aragon, Huesca	R. Heim, 2011	NMLU 14769	JX435855
Limax cf. *cinereoniger*	Slovakia, High Tatra	E. Schmid, 2011	BNM 063005	JX435856
Limax cf. *dacampi*	Croatia, Istria, Motovun	W.J.M. Maassen, 1991	Coll. W.J.M. Maassen, Leiden	JX435840
Limax cf. *dacampi*	Croatia, Istria	G. Wondrak, 2010	BNM 061546	JX435860
Limax cf. *dacampi*	Italy, Lombardy, Lago di Como, Sorico	W. Ruffieux, 2008	BNM 054151	JX435861
Limax cf. *dacampi*	Switzerland, Ticino, Coldrerio	P. Müller, 2009	BNM 062905	JX435862
Limax cf. *graecus* sensu Wiktor, 2001	Greece, Tymfi massif	F. Knechtle, 2011	BNM 062845	JX435873
Limax corsicus s. str.	France, Corsica, Bastelica	G. Falkner, B. Nitz & B. Recorbet, 2004	ZSM Mol 20071685	GQ145538
Limax engadinensis	Switzerland, Grisons, St. Moritz	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071627, ZSM Mol 20071626	FJ606476, FJ606475
Limax giovannellae	Italy, Udine	M. Giovannelli, 2006	ZSM Mol 20071702	JX435826
Limax ianninii	Italy, Latium, Monti Reatini	C.M. Brandstetter, 2006	SMNS 0071857-1	GQ145573

Limax maximus	United Kingdom, Kent	I. Hyman, 2006	ZSM Mol 20071624	J606467
Limax maximus	Switzerland, Grisons, Chur	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071620	FJ606468
Limax redii	Switzerland, Ticino, Monte San Giorgio	U. Oberli, 2009	BNM 059133	JX435877
Limax sarnensis	Switzerland, Obwalden, Sarnen	R. Heim, 1999-2007	NMLU 13438, NMLU 14279	FJ606482, FJ606500
Limax senensis	F1; Italy, Tuscany, Siena	F1; P collected by M. & G. Falkner	ZSM Mol 20071699	GQ145575
Limax sp. [Albanien]	Albania, North-Albania		ZSM Mol 20071712, ZSM Mol 20071714	JX435841, JX435844
Limax sp. [Balkan 1]	Greece, Thrakien, Toxotes	E. Burmeister, 2007	ZSM Mol 20071715, ZSM Mol 20071716	JX435845, JX435846
Limax sp. [Balkan 1]	Bulgaria, Hotnitsa	U. Schneppat, F. Knechtle & D. Georgiev, 2010	BNM 060528	JX435864
Limax sp. [Balkan 1]	Bulgaria, Plovdiv	U. Schneppat, F. Knechtle & D. Georgiev, 2010	BNM 060489, BNM 060493	JX435866, JX435870
Limax sp. [Balkan 2]	Greece, Thrakien, Keramoti	E. Burmeister, 2007	ZSM Mol 20071711	JX435839
Limax sp. [Balkan 2]	Macedonia, Dojran	W. Haberl & M. Franz	BNM 061572	JX435867
Limax sp. [Balkan 2]	Macedonia, Pestani	F. Knechtle, 2011	BNM 062849	JX435868
Limax sp. [Balkan 2]	Bulgaria, Hotnitsa	U. Schneppat, F. Knechtle & D. Georgiev, 2010	BNM 060502	JX435869
Limax sp. [Balkan 2]	F1; Bulgaria, Plovdiv	F1; P collected by D. Georgiev & S. Stoycheva, 2009	BNM 061979	JX435871
Limax sp. [Balkan 2]	Macedonia, Ohrid	F. Knechtle, 2011	BNM 062840	JX435872
Limax sp. [Cutigliano]	Italy, Tuscany, Cutigliano-Melo	G. Bertagni, 2007	ZSM Mol 20071698	GQ145579
Limax sp. [Eastern Etruskan Apennine]	F1; San Marino	F1; P collected by L. Reser-Rezbanyai & F. Schäffer, 2010	NMLU 14771	JX435882
Limax sp. [French South-Western Alps]	F1; France, Hautes-Alpes, Crots	F1; P collected by R. Heim 2010	BNM 062989	JX435881
Limax sp. [Ft. Melo]	France, Corsica, Fôret de Melo	M. & G. Falkner, B. Nitz, 2004	ZSM Mol 20071678	GQ145531
Limax sp. [Liguria]	Italy, Savona, Finale Ligure	C. Germann, 2010	NMLU 14770	JX435874
Limax sp. [Montenegro]	Montenegro, Zekova Glava	W. Paill, 2010	BNM 060818	JX435875

Limax sp. [Mte. Altissimo]	Italy, Tuscany, Alpi Apuane, Monte Altissimo	M. & G. Falkner, B. Nitz, 2004	ZSM Mol 20071666	GQ145519
Limax sp. [Piano di Chiavenna]	Italy, Sondrio, V. d. Chiavenna	B. Nitz & U. Schneppat, 2006	ZSM Mol 20071708	JX435835
Limax sp. [pseudocinereoniger]	Bulgaria, Rila Mountains	F. Knechtle, 2011	BNM 062850	JX435850
Limax sp. [pseudocinereoniger]	Bulgaria, Western Rhodope Mountains, Valley of Hremchitsa River	U. Schneppat, F. Knechtle & D. Georgiev, 2010	BNM 060529	JX435857
Limax sp. [pseudocinereoniger]	Bulgaria, Vitosha Mountains	U. Schneppat, F. Knechtle & I. Dedov, 2010	BNM 060561	JX435858
Limax sp. [pseudocinereoniger]	Montenegro, Biogradska Gora	W. Paill	BNM 060820	JX435859
Limax sp. [pseudocinereoniger]	Bulgaria, Vitosha Mountains	I. Dedov, 2010	BNM 063021, Coll. I. Dedov 7-211010-2	JX435884
Limax sp. [pseudomaximus]	Bulgaria, Sofia	U. Schneppat & F. Knechtle, 2010	BNM 060547	JX435863
Limax sp. [pseudomaximus]	Macedonia, Slatina	I. Dedov, 2010	BNM 061586	JX435878
Limax sp. [pseudomaximus]	Bulgaria, Western Rhodope Mountains, Valley of Hremchitsa River	U. Schneppat, F. Knechtle & D. Georgiev, 2010	BNM 060536	JX435879
Limax sp. [pseudomaximus]	Bulgaria, Bacho Kiro Cave	U. Schneppat, F. Knechtle & D. Georgiev, 2010	BNM 060541	JX435880
Limax sp. [pseudomaximus]	Bulgaria, Vitosha Mountains	I. Dedov, 2010	BNM 063020, Coll. I. Dedov 7-211010-1	JX435883
Limax sp. [Southern Alps]	Italy, Lombardia, Esino	R. Heim, 2004	NMLU 14459 , NMLU 14458	FJ606472, FJ606473
Limax sp. [Southern Alps]	Switzerland, Ticino, Caprino	A. J. de Winter, 1989	RMNH 106757	FJ606474
Limax sp. [Var]	France, Var, La Bastide	R. Heim, 2006	NMLU 14391, NMLU 14393	JX435830, JX435843
Limax sp. [Western Alps]	France, Isère, Chartreuse	B. & H. Nitz	ZSM Mol 20071700	JX435824
Limax vizzavonensis	France, Corsica, Vizzavona	M. & G. Falkner, B. Nitz, 2004	ZSM Mol 20071674	GQ145527
Limax wohlberedti	Croatia, Dalmatia	A. Wiktor, 1999	MNHW, Coll. A. Wiktor 3004	FJ606481
Limax wohlberedti	Montenegro, Kotor	G. Wondrak, 2009	BNM 059499	JX435876

Since preliminary tree reconstructions (by I. Hyman and me) based on combined analyses of COI and 28S have shown that *Gigantomilax* Boettger, 1883 [represented by *Gigantomilax (Vitrinoides) monticola* Boettger, 1881] and Eumilacinae Likharev & Wiktor, 1980 [represented by *Eumilax intermittens* (Boettger, 1883)] are separate from the Limacinae or even from Limacidae, no samples from these clades were considered for the present study. The same applies to the genus *Mesolimax* Pollonera 1888 [represented by *Mesolimax brauni* (Pollonera, 1888)], which was only tentatively assigned to the Limacidae by Schileyko (2003). Likharev & Wiktor (1980) placed this genus into Agriolimacidae; a position which is confirmed by our combined 28S and COI data.

Taxa with mainly Asian/eastern distribution (*Turcomilax* Simroth, 1901*; Caspilimax* Hesse, 1926; *Caucasolimax* Likharev & Wiktor, 1980 and further taxa belonging to *Gigantomilax*) could not be included due to missing samples; however, according to several authors, (Likharev & Wiktor, 1980; Schileyko, 2003; Sysoev & Schileyko, 2009) they probably belong to Limacidae.

The vitrinid *Vitrina pellucida* (O. F. Müller, 1774) was used as outgroup in the study, because Vitrinidae appears to be the most basal family in the superfamily Limacoidea (Hausdorf, 1998).

The treatment of the collected animals followed the procedures described in Nitz *et al. (*2009; 2010). All animals were killed either in water or in SUPRALAN-UF solution. They were fixed and preserved in ethanol. Morphological examinations and determinations followed the standard procedures described in the Nitz *et al.* (2009). In most cases the specimens chosen for dissection were those which were already included in the molecular part of the study, or that were from the same locality or in the same genetic clade according to the preliminary tree sets with 321 animals. In species with appropriate descriptions in the literature (e.g. *Limax brandstetteri* Falkner, 2008), morphological data was also extracted from publications.

Material was deposited in the BNM, the ZSM, the NMLU, and the SMNS (Coll. Falkner). DNA elutions are stored in the DNA Bank of the ZSM (see www.zsm.mwn.de/dnabank/).

8.2.2 DNA sequence analysis

DNA isolation, PCR (COI first and second fragment) and sequencing techniques are described in Nitz *et al. (*2009; 2010). The alignment was trimmed to 1317 nucleotides and translated into amino acids using the invertebrate mitochondrial code in MEGA5 to check manually for stop codons and shifts in reading frame.

Phylogenetic tree reconstruction based on Maximum Likelihood assumptions was calculated using *PhyML* (Guindon & Gascuel, 2003). The general time-reversible (GTR) model with eight gamma categories was applied; tree topology search was based on the SPR (subtree pruning and regrafting) algorithm. Five BioNJ trees calculated by *PhyML* were used as

starting trees. A majority-rule consensus tree was calculated based on bootstrapping (100 replications).

For Bayesian tree reconstruction, model selection was made using comparisons of hierarchical Likelihood Ratio Tests and Akaike Information Criterion scores in *MrModeltest* 2.3 (Nylander 2004). The data were partitioned into first, second and third codon sites. The general time-reversible (GTR) model with eight discrete gamma (Γ) categories and a proportion of invariant (I) sites (GTR+Γ8+I) was used.

Markov Chain Monte Carlo (MCMC) sampling was carried out in *MrBayes* 3.1.2 (Ronquist and Huelsenbeck 2003) for 4,000,000 generations (four simultaneous chains, sample frequency 100, burnin 10,000 generations). A majority-rule consensus tree was calculated from the sampled sets of trees.

The phylogenetic trees were rooted on *Vitrina pellucida*.

8.3 Results

8.3.1 Molecular results

The COI data showed no frameshift mutations or stop codons after translation of sequences using the invertebrate mitochondrial codon table. Only clades with support values higher than 70% bootstrap and 90% posterior probability (PP) are herein considered as significant. The single clades representing possibly a species or a species group (species with its next relatives) are named "lineages", since species names and species allocations have to be verified in several cases. Thirty-five lineages have been identified. Species names are only used for specimens from type localities and for specimens with clear morphological evidence.

The majority-rule consensus tree based on Bayesian inference (BI; Fig. 8-1) shows monophyly for the genus *Limax* (PP 100%). Most *Limax* species represented by more than one individual are clearly distinct from their nearest neighbours and form monophyletic groups that are in nearly all cases supported by high PP values of 100%. The basal part of the genus *Limax* is dominated by Balkan species, including *Limax wohlberedti* Simroth, 1900 (lineage 1, marked dark green in Fig. 8-1) with a specimen from the type locality, and a weakly supported clade of specimens with uncertain species assignment (lineages 2-6). This clade includes Greek, Bulgarian and Macedonian specimens.

The next clade (PP 98%) comprises the remaining included *Limax* species, which are primarily from middle and southern Europe with some additional species from the Balkans. Within this clade *Limax giovannellae* Falkner & Niederhöfer, 2008 (lineage 7), an endemic from the Julian Alps, stands as sister clade to all remaining lineages; however, the remaining taxa in this group form an unresolved polytomy consisting of seven branches. One branch (lineage 15) stands for the species *Limax ianninii* Giusti, 1973, another one (lineage 8) for

species from the Western French (Pre-)Alps, and a third one (lineage 9) for *Limax engadinensis* Heynemann, 1862, a species with central alpine distribution. The endemic Corsican *Wolterstorffi*-group (lineage 17, marked in red in Fig. 8-1; see also chapter 6), here represented by two species, and the Central Alpine *Limax sarnensis* Heim & Nitz, 2009 (lineage 10) form a clade with weak support of 86% PP. A fifth clade contains the widespread *Limax maximus* Linnaeus, 1758 (lineage 35) and the highly endemic *L. brandstetteri* (lineage 16) from the Maiella massif in the Central Appenines in Italy.

In the sixth clade, specimens from the southern edge of the Alps (lineages 11-14, marked pale blue in Fig. 8-1) and specimens from Southern Europe (lineages 18-22, marked orange in Fig. 8-1) group together with strong support (PP 99%). Within this clade, support values are high and several distinct lineages are resolved. One of these groups corresponds to the *Limax* cf. n. sp. "Blauköpfige Egelschnecke" *sensu* Turner *et al.* (1998) (lineage 11). The sister clade (PP 100%) to this species comprises three animals from quite distinct localities in the Western Alps: *L.* sp. [Liguria] from Finale Ligure, Italy (12), *L.* sp. [French South-Western Alps] from Crots, Hautes-Alpes, France (13) and *L.* sp. [Piano di Chiavenna] near Chiavenna, Italy (14). The sister group to this alpine clade consists of animals from Italy (18, 19, 21), Corsica (19), San Marino (20), the Italian-Swiss border and the Istrian peninsula (22).

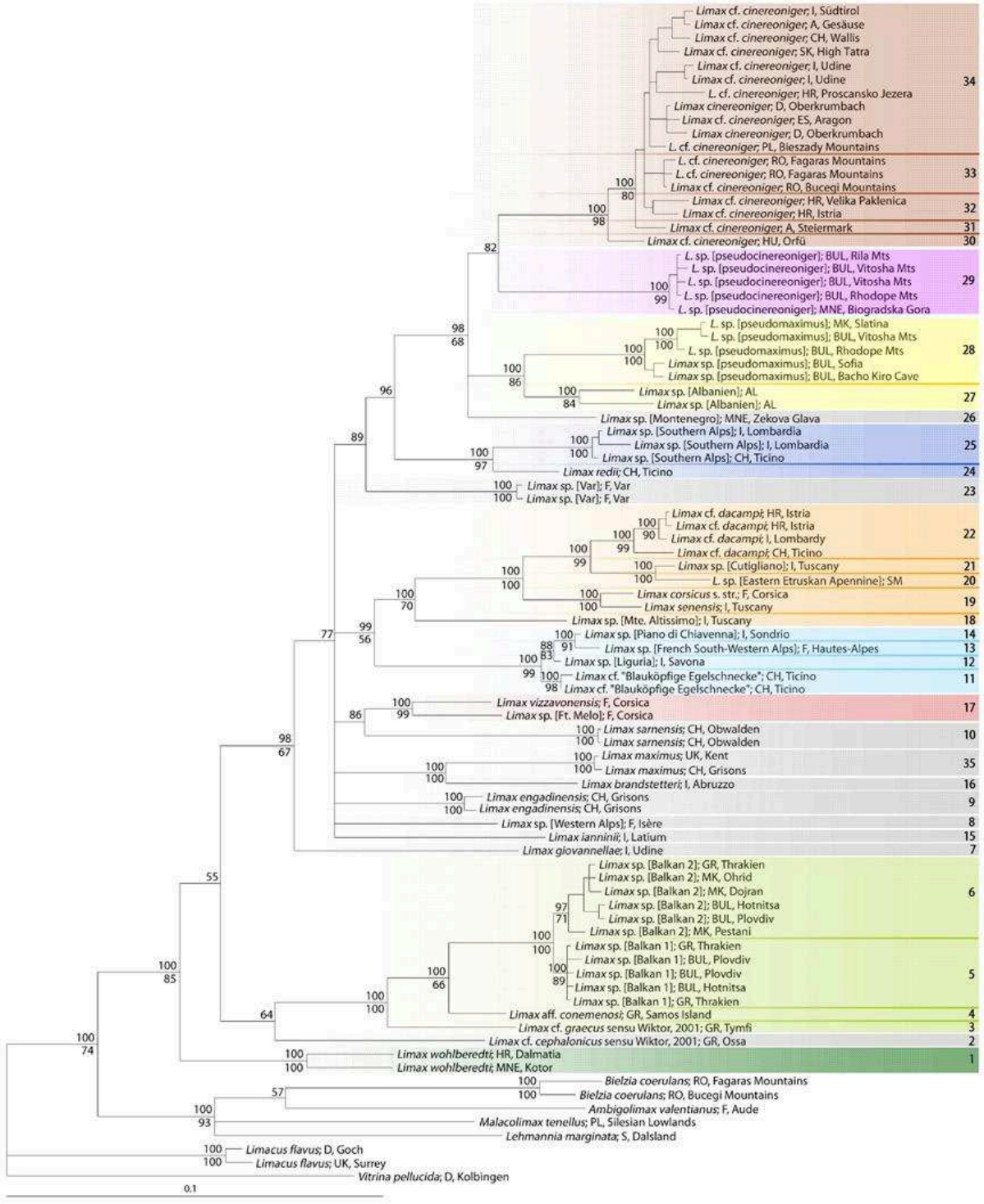

Figure 8-1. Majority-rule consensus tree from the Bayesian inference analysis of the COI sequence data. Posterior probabilities (in percent) are marked above the branches, bootstrap support values (in percent) based on Maximum Likelihood assumptions are marked below the branches

Finally, the seventh and last major clade has a borderline PP support value of 89%. Within this clade, *Limax* sp. [Var] (23) from the eastern border of France is positioned basally. The next relatives are specimens from the Italian-Swiss border (24, 25, marked dark blue in Fig. 8-1), representing probably two species according to morphological findings. The remainders of this clade are four lineages from the Balkan penisula (Macedonia, Bulgaria, Albania,

Montenegro; lineages 26-29) and the widespread *Limax cinereoniger* s.l. (lineages 30-34, marked brown in Fig. 8-1).

The bootstrap support value (in percent) based on Maximum Likelihood (ML) assumptions (in Fig. 8-1) also supports the monophyly of the genus *Limax* (85%). The Balkan taxa (lineages 1-6) are positioned basally, but the relationship between *Limax wohlberedti* (lineage 1) and lineages 2-6 (Greek, Bulgarian and Macedonian specimens) is unresolved.

The remaining specimens form a single clade containing Alpine species, species with Mediterranean distribution (Italy, Adriatic islands), Balkan specimens and the widespread species *Limax cinereoniger* s.l. and *L. maximus*. Basal nodes within this clade show limited resolution and branch support values are low. Nevertheless, several well supported species and species groups are resolved, most of them similar to the BI tree reconstruction: *Limax sarnensis* (lineage 10; 100%), *L. engadinensis* (lineage 9; 100%), *L. maximus* (lineage 35) together with *L. brandstetteri* (lineage 16) as sister group (100%), *L.* cf. "Blauköpfige Egelschnecke" (lineage 11), *L.* sp. [Liguria] from Finale Ligure (12), *L.* sp. [French South-Western Alps] from Crots (13) and *Limax* sp. [Piano di Chiavenna] from near Chiavenna, Italy (lineage 14; 100%). In addition, the specimens from Italy, the Italian-Swiss border and the Istrian peninsula are grouped together with moderate support, as they were in the BI tree (lineages 18-22; 76%). Within this group, all major lineages are supported by bootstrap values of 100%. The relationships between *L. giovannellae* (7), the endemic corsican *Wolterstorffi*-group (17), *Limax* sp. [Western Alps] (8) and *L.* sp. [Var] (23) remain unresolved. The next distinct clade consists of *L. redii* and its relative *L.* sp. [Southern Alps] (24, 25). *Limax* sp. [Montenegro] (26), *L.* sp. [Albania] (27) and two lineages of Bulgarian, Macedonian and Montenegrian specimens (28, 29) form the remaining clade together with *L. cinereoniger* s. l. (30-34) with moderate support (71%). Within this clade, the relationships between the lineages are not resolved; however, all lineages themselves are well supported (92-100%) for nearly all groups represented by more than one individual.

In both tree reconstructions (ML and BI), *Limacus flavus* (Linnaeus, 1758) is found as basal sister to all other Limacidae. Although resolution within the remaining Limacidae (without *Limax*) is not given here, preliminary data from combined COI and 28S data (not shown) strongly support the pattern of *Lehmannia* splitting into two clades. *Bielzia coerulans* is placed within Limacinae in both trees.

8.3.2 Morphological studies

Results based on dissections of members of the single lineages are presented in Table 8-2. For this study I considered data on penis length in relation to body length, the general look of the penis, the length of the blind penis tip in regard to the insertion of the vas deferens and the penis retractor muscle, the distance between the penis retractor and the vas deferens

and the overall colouration of the animals, especially the sole colouration. Information about the copulation mode was restricted to data on presence/absence of a mucus thread, spot or sail due to the absence of copulation observations in a lot of groups.

The penis length in relation to the body length was measured in preserved and dissected animals. This value ranges from about 15% of the body length to five times the body length. The shortest penis of 13 mm in the study was measured in an adult representative of *Limax engadinensis* with a total length of 61 mm (Fig.8-2A). Several other lineages show quite short and straight penes as well, e.g. the French *L.* sp. [Var] with 20% body length or *L. wohlberedti* with about 25%. Long and coiled penes are found in several lineages, including in the *Wolterstorffi*-group (twice body length, see Fig. 4 of Nitz *et al.*, 2010 in chapter 6), in the French *Limax* sp. [Western Alps] (more than twice body length), in *L.* sp. [Eastern Etruskan Apennine] with five times the body length (Fig. 8-2C) or in representatives of the *Corsicus*-group s. l., where the longest penis in copulation was documented (Fig. 5 of Nitz *et al.*, 2010 in chapter 6). Shorter penes are in most cases nearly straight, at most being folded once or twice or showing a hook at the end (Fig. 8-2D). Longer penes are in general more coiled and/or folded (Fig. 8-2B, C).

In most of the species, the penis retractor muscle and the vas deferens insert on the penis at the same point (Fig. 8-2D); sometimes there is a very short distance of 1 mm between the insertion points, but in three lineages (3, 5 and 6) the distance between the two insertion points reaches up to 8.5 mm (Fig 8-2E). The length of the blind penis tip is also variable; in most lineages the vas deferens and penis retractor muscle insert at or near the tip resulting in no blind penis tip or one that is very short (1-3 mm). The longest blind penis tip is found in lineage 3 (L. sp. [Tymfi]) with 14 mm (Fig. 8-2E).

Body length in slugs is a quite mutable character; nevertheless, there are some differences in body length in adult *Limax* slugs. Some species tend to be longer in adult stage than others, e.g. *L. wohlberedti*; in this species adult specimens can reach lengths of more than 150 mm in preserved stage. In contrast to quite large species, there are a number of species that are comparably small in adult stage: e.g. *L. engadinensis* is in most cases not longer than 70 mm in preserved stage.

Table 8-2. Morphological data of the single lineages: Penis length in approximate relation to body length, general look of the penis, length of blind penis tip in regard to the insertion of the vas deferens (VD) and the penis retractor (mrp), distance between the penis retractor and the vas deferens, mantle and overall colouration of animals, sole colouration, presence/absence of a mucus thread, spot or sail.

Lineage	Species/species group	Penis length in approx. % body	Penis look	Length of blind penis tip [mm]	MRP/VD distance [mm]	look mantle	look body	sole	keel	Copulation mode/Mucus thread
1	*L. wohlberedti*	25	nearly straight	<1	1	uni black	uni black	outer fields dark grey, inner field slightly brighter	long and pronounced, colour like body	not known
2	"Cephalonicus"	15	straight	4	one part of mrp near vd, the other strand at 7 mm distance	uni grey	uni grey	all fields pale, edges of outer fields grey	short, not pronounced, brighter than back	not known
3	"Graecus"	100	coiled	14	5	dark marbled	dark marbled	all fields pale	short, not pronounced	mucus spot
4	"Samos"	25	coiled	1-2	no distance	beige with small black spots	beige with small black spots	all fields pale	short, not pronounced	not known
5	"Balkan 1"	40-50	coiled	5-6	2;2-5	uni beige, greyish with single spots	uni beige, greyish with single spots	all fields pale	short, not pronounced	not known
6	"Balkan 2"	75-100	coiled	3,5-4,5	7-8,5	beige with small dark spots	beige with small dark spots	all fields pale	short, not pronounced	not known
7	*L. giovannellae*	30 - 100	coiled, folded	<1	no distance	uni dark grey	dark grey with single brighter wrinkles at the sides	all fields pale	pronounced at the end, brighter than back	mucus thread
8	"Western Alps"	> 200	coiled	0; mrp/VD at side of tip	no distance	uni grey/black	grey/black	outer fields dark to black, middle field pale	pronounced, white	mucus thread
9	*L. engadinensis*	20-25	(nearly) straight	2	no distance	dark brown with small brighter spots	dark brown with small brighter spots	all fields pale	pronounced at the end, little bit brighter than body	mucus spot, at least little mucus sail
10	*L. sarnensis*	30-50	nearly straight, hooked at end	1-3	no distance	depending on body colouration, but always uni	very variable, from black to creamy, with or without pattern	sarnensis type	pronounced, like body colour or brighter	mucus thread
11	"Blauköpfige Egelschnecke"	25-50	nearly straight, distal part folded	0	no distance	uni yellowish brown/creamy	uni yellowish brown/creamy	outer fields bright grey, inner field pale	pronounced at the end	mucus thread

12	"Liguria"	30	nearly straight	0; mrp/VD at side of tip	no distance	black/creamy irregular ornamentation,	pale grey with creamy wrinkles irregular and in rows along side	outer fields bright grey, fading to middle, middle field pale, whole sole with faint orange pigment dots in living specimens	pronounced, creamy wrinkles	not known
13	"French South-Western Alps"	35	nearly straight, hooked at end	<1	no distance	creamy white with black spots	creamy white with many irregular black spots and greyish pattern between wrinkles at side	outer fields bright grey, fading to middle, inner field pale	pronounced, creamy-white	mucus thread
14	Piano di Chiavenna	50	coiled	<2	no distance	uni dark cream	uni dark cream	outer fields bright grey, inner field pale	pronounced at the end	not known
15	*L. ianninii*	20	nearly straight	similar to brandstetteri?	similar to brandstetteri?	uni black	uni black	all fields pale	quite flat	not known
16	*L. brandstetteri*	30	nearly straight	1,3	no distance	uni black	uni black	all fields pale	pronounced at the end	not known
17	"Wolterstorffi"	ranging from < 100 to 200 depending on species	coiled or nearly straight	1	no distance	uni dark grey to black, like body	uni black to dark grey, sometimes brighter, rarely with irregular brighter spots ("Wolkenlimax")	outer fields darker at least in one species	short, not pronounced	not known
18	"Monte Altissimo"	80	coiled, hooked at end	1-1,5	no distance	brownish grey	brownish grey, paler at sides	all fields pale sometimes pinkish in living specimens	short, not pronounced	not known
19	*L. senensis*	100	coiled	2	no distance	redbrown with slightly brighter spotty pattern	red brown, darker stripe on side	reddish	pronounced at the end, little bit brighter than body colour	not known
19	"Corsicus"	high variability; short to more times body length depending on species	coiled	no or little blind penis tip	no distance	uni, colour like body	creamy to red, brownish with or without darker lateral bands	all fields pale to red	pronounced at the end, often little bit brighter than body colour	Sometimes mucus sail?

20	"Eastern Etruskan Apennine"	520	coiled	<1; mrp/VD at side of tip	no distance	uni dark grey	grey with black spotty row at side of keel and some creamy wrinkles at side	outer fields grey, fading to anterior end, inner field creamy	short, not pronounced creamy	mucus thread
21	"Cutigliano"	150	coiled	0	no distance	uni brown, grey, beige, black	very variable, often with spots	outer fields dark to bright grey according to body colour, middle field pale	pronounced, often brighter than body colour, sometimes with red pigmentation	not known
22	"Dacampi"	70-100	coiled	3	no distance	uni reddish to dark red	reddish to dark red, sometimes with row of black spots, sometimes wrinkles darker than body	outer fields grey, sometimes with red shimmer, sometimes not reaching end of field towards middle ; inner field pale or creamy with red shimmer	(bright) red	mucus thread
23	"Var"	20	nearly straight	0	no distance	bright brown with small black spots and dark/bright pattern	bright brown with small black spots and dark/bright pattern	all fields pale	short, not pronounced	mucus thread
24/25	*L. redii*/Southern Alps"	up to 190	coiled	no or little blind penis tip	no distance	beige to greyish or pale brown, sometimes with single dots	beige to greyish or pale brown, sometimes with dots in two rows at side	all fields pale	little brighter than back	mucus sail
26	"Montenegro"	120	coiled	<1	no distance	uni dark blackish brown	dark blackish brown, brighter between wrinkles	outer fields black, middle field pale, sometimes with pinkish hue	short, not pronounced	not known
27	"Albanien"	50	nearly straight	3	1	uni dark grey	dark grey, a little bit brighter at sides	all fields pale	short, not pronounced	not known
28	"Pseudomaximus"	90	coiled	0	no distance	dark grey with some black dots and many small white dots	dark grey with two longitudinal rows of black dots	all fields pale	short, not pronounced, bright grey	not known

29	"Pseudocinereoniger"	100	coiled, folded	0	no distance	uni dark grey	uni dark grey	outer fields dark grey to black, sometimes fading towards middle field and to anterior, middle field pale	long and pronounced, white	mucus sail
30-34	L. cinereoniger s.l.	50-80	coiled	1	no distance	in most cases uni, colour like body	creamy, brown, grey, black, sometimes red, sometimes with dark spots or spotty pattern or stripes	outer fields black to bright grey, depending on body colour, inner field pale	pronounced at the end, often brighter than body	mucus sail
35	L. maximus	<50	coiled	<2.5	no distance	in most cases similar to body colour, with a dark pattern of spots or blotches	black or dark brown to pale cream, nearly always with dark spotty pattern, sometimes with less distinct spots or longitudinal stripes on the sides	all fields pale, sometimes dark pigment dots in edges of outer fields	often pronounced at the end, sometimes weak; usually brighter than body	mucus thread

Figure 8-2. Genital anatomy. **A.** Short and straight penis*; Limax engadinensis* ZSM Mol 20071627. **B.** Coiled penis of medium size, *Limax senensis* ZSM Mol 20071699, vas deferens not dissected and still connected with penis. **C.** Very long penis; *Limax* sp. [Eastern Etruskan Apennine] NMLU 14771, vas deferens disrupted. **D.** Penis hooked at the end; *Limax* cf. *cinereoniger* L952 from Ebensee, Austria, leg. S. Gratzer. **A-D** Common insertion point of penis retractor muscle and vas deferens near tip of penis **E.** Distance between penis retractor muscle and vas deferens, long blind penis tip; *Limax* cf. *graecus* sensu Wiktor, 2001 BNM 062845. Scale bars: **A-E** = 10 mm. Abbreviations: a, atrium; ag, albumen gland; bc, bursa copulatrix; hd, hermaphrodite duct; hg, hermaphrodite gland; o, oviduct; p, penis; pr, penis retractor muscle; spo, spermoviduct; vd, vas deferens.

Colouration of *Limax* may be very variable, as mentioned earlier (e.g. Nitz *et al.,* 2009 in chapter 5). A lot of animals have a uniformly coloured body and mantle ranging from black through brown or grey to red (Fig. 8-3A-C). The body in patterned specimens (Fig. 8-3D-F, H) can be covered with sparse or very dense spots, small or big dots, often arranged in rows at the side of the body or with stripes or lateral bands. Spots can be very distinct and sharp-edged (Fig. 8-3D), but some specimens have blurry blotches, and in others a pattern of light and dark wrinkles over a contrasting background colour simply gives the impression of spots (Fig. 8-3E, F). In a lot of species, the keel is brighter than the body colour (Fig.8-3B, C, E, F,

H).The mantle of patterned specimens is often uniformly coloured (Fig. 8-3E), in most cases corresponding to the background colour of the body, but can also show a spotty pattern (Fig. 8-3D) or have brighter or marbled mantle edges (Fig. 8-3F, G, H). In some specimens the mantle can be a lot darker than the colour of the body (Fig. 8-3G). We find species that show nearly no variation between the specimens (e.g. *L. engadinensis*, where both body and mantle are brown with a paler spotty pattern, or *L.* cf. "Blauköpfige Egelschnecke" with no patterning); however in a lot of species, the variability is much higher (e.g. *L. sarnensis*, see chapter 5 or *L.* cf. *cinereoniger*, Fig. 8-3B, C, F, G, H), even between specimens from one locality.

Figure 8-3. External appearance of living specimens of *Limax.* **A.** Unicoloured dark specimen; *Limax* sp. [Western Alps] L710 from Massif Voirons, France, leg. B. & H. Nitz. **B.** Unicoloured bright specimen; *Limax* cf. *cinereoniger* ZSM Mol 20071706. **C.** Unicoloured dark red specimen; *Limax* cf. *cinereoniger* L1688 from Steiermark, Austria, leg. W. Paill. **D.** Spotted specimen; *Limax* sp. [Balkan 2] ZSM Mol 20071711. **E.** Spotted specimen with unicoloured mantle and reddish keel; *Limax* sp. [Cutigliano] L1342 from Popiglio, Italy, leg. G. Bertagni. **F.** Mantle with marbled edges, wrinkles dark and bright; *Limax* cf. *cinereoniger* ZSM Mol 20071616 from Dresden, Germany, leg. A. Pohl. **G.** Bright specimen with dark mantle; *Limax* cf. *cinereoniger* L1074 from Kärnten, Austria, leg. C. Wieser. **H.** Specimen with bands; *Limax* cf. *cinereoniger* L1719 from Genf, Switzerland, leg. J. Ruetschi. Scale bars: **A-H** = 10 mm.

The intensity of the sole colouration depends a lot on the background colour of the body; however, there are in general two types of sole colouration in *Limax*: a) the uniformly pale or creamy sole with no darker outer fields (Fig. 8-4A, B) and b) darker outer fields combined with a pale middle field (Fig. 8-4C, E). Type a) can also have pigmented spots in the edges of the sole (Fig. 8-4B; not to be confused with the outer sole fields themselves of type b). The outer fields of type b) can either be uniformly dark (Fig. 8-4C) or can fade from the outer margins to the middle field and from posterior to anterior, as in *L. sarnensis* (Fig. 8-4E). The pigmentation of the darker fields can consist of visible single pigmented spots (Fig. 8-4E) or can be monochrome (at least to the naked eye) (Fig. 8-4C). A special type of sole colouration is found in *L. wohlberedti* (midsection of the sole only a little bit brighter than the dark outer fields, Fig. 8-4F) and in the *Corsicus*-group s.l. with red pigmented sole fields (Fig. 8-4D).

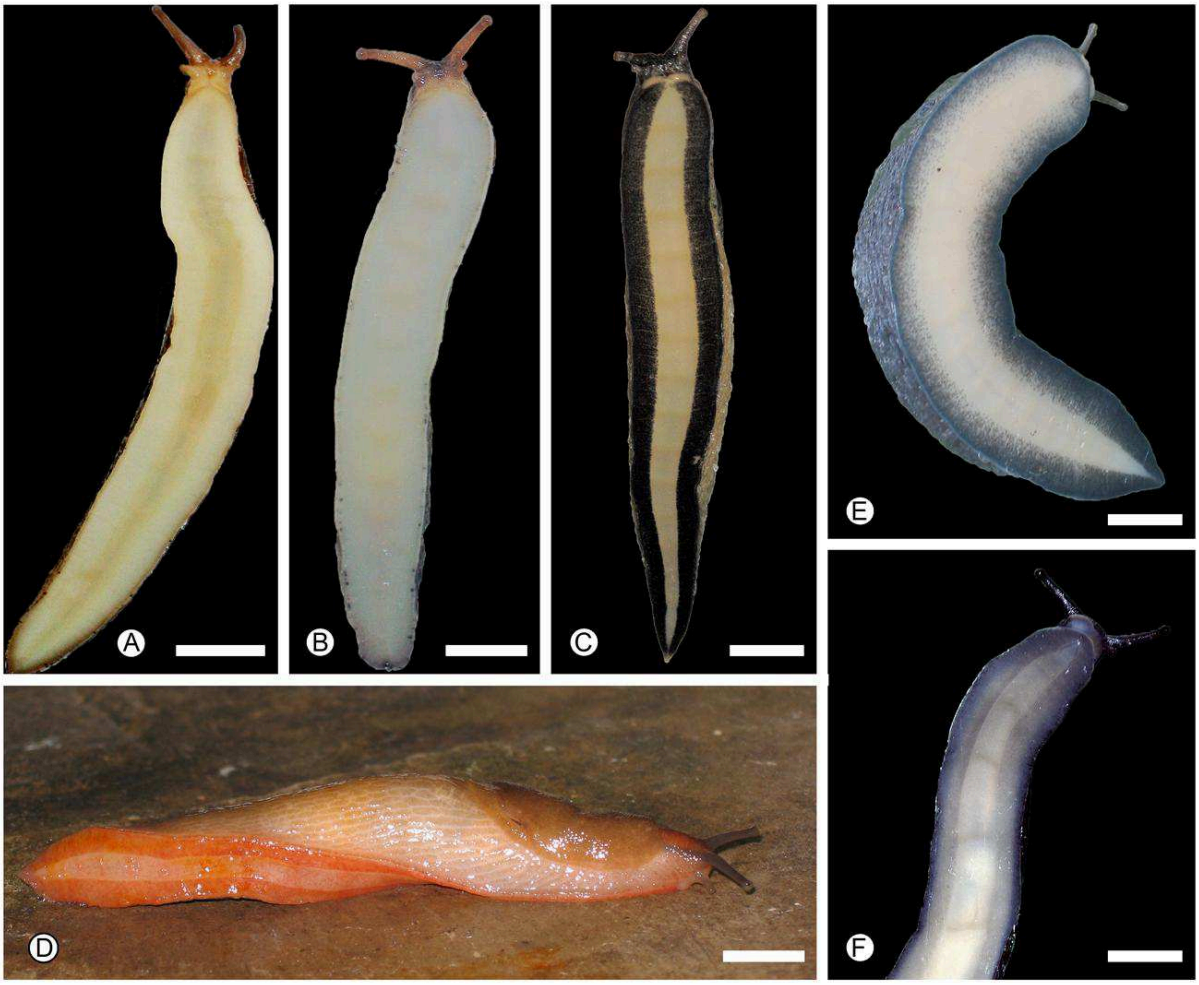

Figure 8-4. Sole colouration of living specimens of *Limax*. **A.** Uniformly pale sole; *Limax engadinensis* L1045 from Tamins, Switzerland, leg. R. Cornu & M. Kieffer. **B.** Pale sole with single spots in the sole edges; *Limax maximus* L1718 F1 from Kent, United Kingdom. **C.** Uniformly dark outer fields; *Limax cf. cinereoniger* L1034 from Kärnten, Austria, leg. C. Wieser. **D.** Red pigmented sole fields; *Limax* cf. *corsicus* L990 from Campania, Italy, leg. C.& L. Cavegu. **E.** Fading outer sole fields; *Limax sarnensis* ZSM Mol 20071503 from Olivone, Switzerland, leg. B. Nitz &

U. Schneppat. **F.** Sole type of *Limax wohlberedti* BNM 059499, photograph courtesy of U. Schneppat. Scale bars: **A-F** = 10 mm.

Due to missing or incomplete observations in a number of lineages, data on the copulation modes are still fragmented; however, for this study the presence or absence of a mucus sail or mucus thread was documented at least in some species (see also Table 8-2). Several species, for example *L. engadinensis*, produce just a small mucus spot (Fig. 8-5A, D), while others copulate hanging on a long mucus thread (e.g. *L. sarnensis*, *L. maximus*, Fig. 8-5B, E, F, H) or a mucus sail (Fig. 8-5C).

Figure 8-5. Copulation types. **A.** Copulation hanging on a mucus spot; *Limax* sp. [pseudocinereoniger], BNM 62844 + BNM 62850 Rila mountains, Bulgaria, leg. F. Knechtle, photograph courtesy of F. Knechtle. **B.** Copulation hanging on a mucus thread; *Limax maximus* Bern, photograph courtesy of M. Loosli. **C.** Copulation hanging on a mucus sail; *Limax* sp. BNM 062854 + BNM 062855 Ohrid, Macedonia, leg. F. Knechtle, photograph courtesy of F. Knechtle. **D.** Copulation with short penes; *Limax engadinensis* Tirol, Austria. **E.** Pear-like shape of penes in a copulation of *Limax maximus* Luzern, Switzerland. **F.** Medium size penes in a copulation of *Limax sarnensis* Sarnen, Switzerland. **G.** Copulation of *Limax redii* with long penes; specimens from type locality, photograph courtesy of U. Oberli. **H.** Copulation with long penes, hanging on a slime thread; *Limax* sp. [Eastern Etruskan Apennine] from San Marino, leg. R. Heim. **D-F, H:** photograph courtesy of R. Heim. Scale bars: **A-F** = 10 mm, **G, H** = 100 mm.

8.3.3 Distribution

In Figure 8-6 the locations of the specimens present in the tree are mapped (see also Table 8-1 for the single collection sites). Since in many species the distribution borders are still unknown, we cannot provide a detailed distribution map; however, we want to give a short overview of the present knowledge. The colours in the map correspond to the colours used for several clades in the tree (Fig. 8-1) and in Tables 8-1 and 8-2.

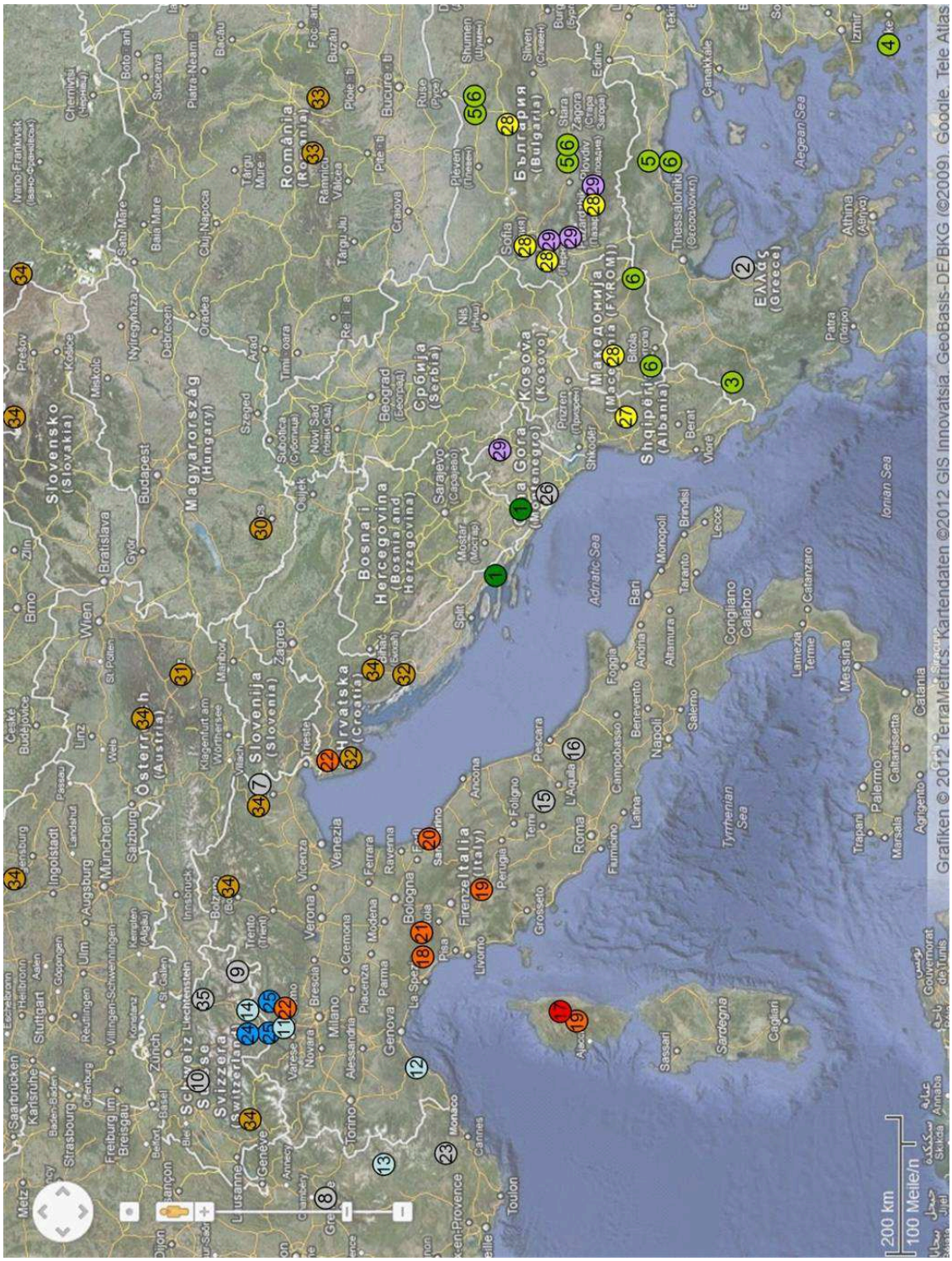

Figure 8-6. Map of localities. Collection site colouration corresponds to Fig. 8-1. See Table 8-1 for details.

Lineage 1 (*Limax wohlberedti)* is present in Montenegro and the adjacent southern border of Croatia. It seems to be restricted to mountainous habitats.

Specimens belonging to lineages 2-6 are found in Greece, Bulgaria and Macedonia. In contrast to *Limax* cf. *cephalonicus* sensu Wiktor, 2001 and *L.* cf. *graecus* sensu Wiktor, 2001 (lineage 2 and 3), which are found in woods and mountainous regions, the lineages 4-6 seem to be synanthropic at most collection sides. *Limax.* sp. [Samos] seems to be the most south eastern representative of the genus *Limax* in Europe found so far.

Limax giovannellae (lineage 7) is according to Falkner & Niederhöfer (2008) endemic for a small region in the Julian Alps and appears to be restricted to mountainous altitudes.

Limax sp. [Western Alps] (lineage 8) was found in the Chartreuse Mountains in Departement Isère in the French Prealps. However, according to the preliminary tree reconstructions and morphological results, there are probably additional species in lineage 8, which inhabit the French Prealps and Alps in the Departements Haute Savoie, Alpes-de-Haute-Provence and Alpes Maritimes.

Limax engadinensis and further specimens belonging to lineage 9 are to our current knowledge restricted to the inner alpine region in the Swiss Cantons Berne, St. Gallen and Grisons, to Vorarlberg in Austria and the Italian region Trentino-Alto Adige.

The distribution of *L. sarnensis* (lineage 10) is in the Western Central Alps; it is mainly found in subalpine and mountainous habitats (for details see Nitz *et al.*, in chapter 5).

Limax cf. "Blauköpfige Egelschnecke" (lineage 11) is currently only known from Canton Ticino, Switzerland.

The collection site of *Limax* sp. [Liguria] (lineage 12) is near Savona at the Italian Riviera.

Limax sp. [French South-Western Alps] (lineage 13) was collected in Hautes-Alpes, Valley of Durance.

Specimens belonging to lineage 14, the species *Limax* sp. [Piano di Chiavenna], are found at the Swiss-Italian border (Val Bregaglia).

Limax brandstetteri (lineage 15) and *L. ianninii* (lineage 16) are described as endemic species of the Central Appenine. They both seem to be restricted to mountainous habitats at high altitudes.

Lineage 17 stands for the *Wolterstorffi*-group (here with the representatives *Limax vizzavonensis* Falkner & Nitz, 2010 and *L.* sp. [Foret Melo]), which is endemic to mountainous habitats in Corsica (details and distribution map chapter 6).

Limax sp. [Mte. Altissimo] (lineage 18; *L.* sp. 3 according to Manganelli *et al.* 1995) was collected in Tuscany. The next relatives in the tree, *L. corsicus* Moquin-Tandon, 1855 and *L. senensis* Pollonera, 1890 (lineage 19) represent the *Corsicus*-group s.l. which is distributed on Corsica, the Appenine Peninsula and the adjacent islands Sardinia, Elba and Capraia (already discussed in detail in Nitz *et al.*, 2010, chapter 6).

Limax sp. [Eastern Etruskan Apennine] (lineage 20) is found in San Marino, located at the western side of the appenine. Specimens of *L.* sp. [Cutigliano] (lineage 21) are found in Tuscany.

Limax cf. *dacampi* (lineage 22) is found in Istria (Croatia), Sorico (Italy) and Ticino (Switzerland).

Limax sp. [Var] (lineage 23) is up to now only known from a few localities in subalpine habitats in the Departement Var, France.

Limax redii (lineage 24) and its relative, *L.* sp. [Southern Alps] (lineage 25) seem to be restricted to the area of the Swiss-Italian border (Lago di Como, Lago di Lugano).

Limax sp. [Montenegro] (lineage 26) is up to now only found in Montenegro in subalpine forests.

Limax sp. [Albania] (lineage 27) was so far collected only at one locality in Albania.

Limax sp. [pseudomaximus] and *L.* sp. [pseudocinereoniger] (lineages 28, 29) are known at present from several localities in Bulgaria, Montenegro and Macedonia. At some collection sites, both species occur sympatrically in the same habitat (mainly mountainous and subalpine forests).

Limax cf. *cinereoniger* (lineages 30-34) is a widely distributed species with records all over Central Europe to the British Isles in the west and to the Ural Mountains in the east. It is in most cases found in woodland and prefers undisturbed habitats; however, it can be present in synanthrope habitats as well.

Limax maximus (lineage 35) is another very widely distributed species, found all over Europe; it has even been introduced abroad (e.g. Australia, New Zealand, USA, South Africa). In contrast to *L. cinereoniger* s.l., it is mainly found in synanthrope habitats.

8.4 Discussion

The phylogeny of *Limax* has never been previously studied in detail, neither from a morphological nor molecular perspective. The main goal of this chapter was to provide a first step towards a phylogenetic understanding of the genus and its nearest relatives. Therefore we used a COI data set of a selection of *Limax* species (or taxonomic units) to elucidate the relationships within *Limax*.

Phylogenetic reconstruction methods

Accuracy and reliability of trees and corresponding support values are substantially improved by comparing various trees based on different approaches (Knoop & Müller, 2006). Therefore, two different methods were applied: a Maximum Likelihood and a Bayesian inference approach. The overestimation of posterior probabilities in Bayesian statistics is commonly discussed as a weakness of the method (Knoop & Müller, 2006). In contrast,

bootstrapping as used in the ML approach is regarded as an overconservative method; Maximum likelihood reconstruction in general seems to be quite robust and leads rather to an underestimation of bootstrap support values (Douady *et al.*, 2003). These observations can be confirmed within the *Limax* clade: support values are in general higher in the Bayesian reconstruction as in the ML tree. As suggested by Douady *et al.* (2003), both methods could be regarded as "potential upper and lower bound of node support". The general tree topology is quite similar in both trees, with the major groups supported by either posterior probabilities or bootstrap support values. Terminal branches are supported quite well; however, the basal nodes that should reveal the relationships between the single lineages show comparatively weak support values.

Phylogenetic and biogeographic conclusions

Limacidae Lamarck, 1801

Both trees show the genus *Limax* to be monophyletic. The relationships of other representatives of the family Limacinae to *Limax* are also congruent within the COI trees. The genus *Limacus*, often treated as subgenus of *Limax* (e.g. Wiktor, 1996; 2001; Schileyko, 2003), is clearly positioned outside of the genus *Limax*. The results therefore support the status of *Limacus* as a separate genus.

Hesse (1926) split *Lehmannia* Heynemann, 1862 into two subgenera (*Lehmannia* s.str. and *Ambigolimax*) based on anatomical differences. This split of *Lehmannia* seems to be justified also on the molecular level (see also Klee *et al.*, 2005): both trees reject monophyly of the genus *Lehmannia*, although resolution is weak. The molecular results therefore give further support of the treatment of *Lehmannia* as two distinct genera: *Lehmannia* (represented by *Lehmannia marginata* (O. F. Müller, 1774) in our analyses) and *Ambigolimax* (represented by *Ambigolimax valentianus*). The name *Ambigolimax* Pollonera, 1887 was re-used recently by Beckmann (2007) for *Ambigolimax valentianus* (Férussac, 1822).

Interestingly, *Bielzia*, which is grouped as separate family Bielziidae (Schileyko, 2003) or as subfamily Bielziinae (Likharev & Wiktor, 1980), is nested within subfamily Limacinae, close to *Lehmannia*, *Ambigolimax* and *Malacolimax*. This indicates that either Bielziidae should be synonymised with Limacidae, leaving no subfamily divisions, or that *Lehmannia*, *Ambigolimax* and *Malacolimax* belong in Bielziinae rather than Limacinae. Historical biogeographic assumptions, especially the question of the origin of the familiy Limacidae, have to be addressed in a much broader study.

These first results have to be treated as a preliminary phylogenetic interpretation and have to be verified with more data, since COI gives no sufficient resolution for deeper branchings. Aware of this, these results show the necessity of further analyses of Limacinae and Limacidae with a broader taxon sampling and extended molecular data set; however, this is

beyond the scope of the current study. Preliminary analyses (not shown) based on more genes (COI, 16S and 28S rDNA) and an enlarged taxon sampling of limacid slugs show better resolution of these deeper branchings and support the scenario shown in the COI trees with *Limacus* diverging basally and *Limax* as sister clade to all other remaining Limacinae (including *Bielzia*). However, the question for the next relatives of the family Limacidae remains open due to missing sequence data of Agriolimacidae and Boettgerillidae, which were grouped by Hausdorf (1998) as sister groups to Limacidae. Adding these taxa and potentially related groups like *Turcomilax, Caspilimax* and *Caucasolimax* might give another, more detailed picture of Limacidae in future.

Balkan lineages (lineages 1-6, 26-29)

Even though not all phylogenetic relationships among the *Limax* lineages could be unravelled in detail, our results may suggest a Balkan origin of the genus. In both tree reconstruction methods, the Balkan lineages 1-6 have a basal position. These lineages represent at least 6 species based on penis characteristics and colouration. Dissections show very short penes in *L. wohlberedti* and penes of moderate length up to body length in the other lineages. *Limax wohlberedti* from Montenegro and from the southern-most border of Croatia (Dinaric Alps) is positioned basally in the BA tree, however, in the ML reconstruction, this branch remains unresolved. Most species have a distinct blind penis tip, with the penis retractor muscle and vas deferens inserting close to the tip of the penis instead of right on the tip.

Other groups with distributions on the Balkan Peninsula include lineages 26 to 29. Some relationships among these taxa remain unresolved in either one or both of the two trees. These lineages are the nearest relatives to the widespread *L. cinereoniger* s.l.. Interestingly, the two Balkan clades (lineages 1-6 and 26-29 respectively) are only distantly related; lineages 1-6 are basal branches and 26-29 diverge (together with *L. cinereoniger* s.l.) as the most derived group compared to all other *Limax* lineages.

All these lineages found in the Balkans seem to be endemic to the Balkan Peninsula and have quite small distribution ranges; however, collecting has to be extended in this area. Considering the restricted availabilty of fresh material to date, which was collected in just six expeditions of a few days each, it is quite likely that there are additional taxa to be found. These few samples already belong to at least 10 to 15 species that have to be either matched with existing names or described as new species. Although Wiktor seems to give a quite detailed picture of *Limax* in the Balkans (Wiktor, 1983, 1996, 2001), several open questions remain. In his latest book about the slugs of Greece (2001), he states "...that the number of species can only be estimated approximately (...) and their taxonomical status needs to be established". Nevertheless, it is quite evident that the Balkan Peninsula is a very

important area for future work. The Balkan Peninsula is a hotspot region in Europe with a great richness of flora and fauna and an exceptional number of endemic and relict species (Savic, 2008). This appears to be true also for the genus *Limax*.

Alpine species (lineages 7-14, 23-25)

In addition to the Balkan Peninsula, the southern edge of the Alps seems to be another hotspot in the genus *Limax*, especially the regions at the French/Italian and the Swiss/Italian borders round the glacial lakes Como, Lugano and Maggiore and the adjacent mountains and valleys. Here we find *Limax engadinensis* (lineage 9), *L. sarnensis* (lineage 10) (both with mainly a Central Alpine distribution), *L.* cf. "Blauköpfige Egelschnecke" (lineage 11) and *L.* sp. [Piano di Chiavenna] (lineage 14). The recently described *L. giovannellae* (lineage 7) inhabits the Julian Alps and is supposed to be an endemic for this region. *Limax* sp. [Liguria] (lineage 12) occurs at the most southern foothills of the Alps in the West; *L.* sp. [French South-Western Alps] (lineage 13) inhabits the French Alpes and L. sp. [Western Alps] (lineage 8) is found at the Western edge of the Alps.

L. redii and relatives (lineage 24, 25) and *Limax* sp. [Var] (lineage 23) show a Southern Alpine distribution pattern like lineages 11, 13 or 14, however, according to the molecular results, they don't seem to be closely related to these other lineages.

Penis lengths in all these Alpine species are quite heterogenous and most of the species copulate while hanging on a mucus thread. Although the relationships among the Alpine species were not resolved in either tree, there are several distinct species or lineages that are clearly defined by molecular and/or morphological means and copulation characteristics. All these Alpine species are thought to have quite small distribution ranges.

The geological and biogeographic history of the Alps is significantly influenced by glacial periods and most of the Alps were covered with ice in the Last Glacial Maximum (LGM) 21,000 years ago (Mix *et al.*, 2001). As already mentioned in chapter 5, there are two ways for alpine fauna and flora to survive: 1) Nunatak survival or 2) recolonization from refugia outside the ice shield. For both hypotheses, there are a vast number of examples (e.g. Schönswetter *et al.*, 2002; Stehlik *et al.*, 2002; Dépraz *et al.*, 2008). For *L. sarnensis* and *L. engadinensis* it seems quite plausible that these species, which have their main distribution in the Central Alps, are "Nunatak-Survivers". For the other species in the Alpine lineages, a survival at the southern glacial border is not unlikely, since they still remain at the southern and western valleys with comparetively moderate climate and have not (re-) colonised the colder and higher parts of the Alps.

Italian/Mediterranean species (lineages 15-22)

Biogeographic conditions of the Mediterranean region of Italy and in particular of woody habitats are always severely hampered by the highly significant anthropogenic modification of this ecosystem since Etruscan times. The relationships among the Italian lineages of *Limax* were poorly resolved in our tree. Only two groups of lineages form well-supported clades. One of these is the widespread *Limax maximus* (lineage 35) and its nearest relative *L. brandstetteri* (lineage 16). The latter, highly endemic species is restricted to the Maiella massif in Italy (Falkner, 2008) and is placed by Falkner (2008) in the "L. maximus-Group", together with an additional clade ("maximus-Gruppe Maiella"). *Limax maximus* therefore probably has its roots in Italy. A thorough reappraisal of this interesting and often misinterpreted species is in preparation by Dr. Isabel Hyman *et al.* and will address these relationships in detail.

The second well-resolved clade was made up of lineages 18-22, which were grouped with the alpine lineages 11-14, The specimens in lineages 18-22 represent a reduced version of the data set from Nitz *et al.* (2010, chapter 6), and show similar relationships to the tree in this publication (Fig. 9 in chapter 6) with *Limax* sp. [Mte. Altissimo] positioned basally. One major difference is the incorporation of *L.* cf. *dacampi* and *L.* sp. [Eastern Etruskan Apennine] in the reduced data set. One characteristic feature of these lineages is a high variability in colouration and patterning, often with red pigmentation, for example in *L.* cf. *dacampi* and *L.* sp. [Cutigliano]. In the lineages 18-22 penes can be very long. In *L.* sp. [Eastern Etruskan Apennine] we measured the astonishing penis length of 52 cm in preserved stage (more than five times body length).

The *Wolterstorffi*-group, introduced in detail in chapter 6, is represented here only by two species (lineage 17). The group contains at least eight species, all of them endemic for Corsica; they are very uniformly coloured, but show a huge variety in penis length.

Widespread species (lineages 30-35)

In contrast to nearly all the above-mentioned *Limax* species, which seem to have (very) narrow distribution ranges and show a high degree of endemism, the two species *L. maximus* and *L. cinereoniger* s.l. settle nearly the whole of Europe. Interestingly, these two widely distributed species are not close relatives. In contrast to *L. maximus*, which turns out to be a kind of globetrotter with a preference for synanthropic habitats, we collected *L. cinereoniger* s. l. mainly in forests and more or less undisturbed habitats.

Synthesis

The trees enable at least a first comparison of morphological characters and molecular tree topologies. The occurence of the most basal species in the phylogeny in South Eastern

Europe indicate a Balkan origin of the whole genus. These species have short to medium penes with distinct blind penis tip. In most specimens there is an obvious distance between the insertion points of vas deferens and penis retractor muscle; this character state, which is not present in any other lineage, might be the ancestral state in *Limax*.

The sole colouration in the most basal species, *L. wohlberedti*, is quite dark in all fields with the middle field only slightly paler. The other basal Balkan species all have unicoloured creamy fields, a character which is present in several other lineages in the tree as well. The other prevalent character state with darker outer fields is present in a number of lineages as well and there is no clear evidence for assessing one of these states as ancestral or derived. Red pigmentation in the sole fields seems to be a special character of the *Corsicus*-group s.l..

The colouration of body and mantle is quite simple in basal lineages (simply black, unicolour beige or grey, sometimes with small dots on the body and mantle), however, this pattern was observed in other lineages as well. There are only a few lineages that show a high variability in body colouration within a species (*L. sarnensis*, *L. cinereoniger* s.l., *L. maximus* and the closely related lineages 20-22). The presence of red pigmentation in the body and mantle colouration is not a very common feature, but nevertheless it occurs frequently in least two distantly related groups (*L. cinereoniger* s.l., lineages 20-22 and sister clade *Corsicus*-group s.l.).

Data on copulation mode are still lacking in a number of lineages including nearly all Balkan species. The copulation, which is probably one of the most important characters in *Limax* species discrimination, is in regards of observation and documentation unfortunately also the most difficult one.

The comparison of the morphological characters in *Limax* with the characters of Limacidae in general shows a tendency of sophistication. The nearest relatives of *Limax* have short penes and no distinct sole patterning like some species in *Limax*. The body colouration is comparatively simply and lacks a pattern except in *Lehmannia*/*Ambigolimax* and in *Limacus*. Although *Malacolimax* and *Bielzia* specimens can be yellowish or blue respectively, there is not such an extraordinary variety of colours like in *Limax*. The copulation is not as complex as in *Limax*.

Conclusion

The major aim of the present study was to provide a first step towards a phylogeny of the genus *Limax*, since the relationships within the genus have not been studied in detail previously, either from a molecular or a morphological perspective. The results of this study show a high number of distinct lineages in *Limax* with an excellent concordance to the morphological results. Although these initial findings also show the limits of resolution of the

COI gene regarding relationships among a number of lineages, significant new information was generated by the input of the molecular data set, revealing the complexity of the genus and highlighting the strong need for comprehensive sampling and further studies.

9 General discussion

In this Thesis I present new insights into the genus *Limax* on the basis of novel molecular and morphological data sets. An evaluation of the utility of these character sets for species delineation, systematics, biogeography and evolutionary history of *Limax* is provided. In this last chapter, I aim to give a biogeographic synthesis and hypothetic evolutionary scenarios based on the results of this work. The impact of an integrative approach in *Limax* research is discussed.

As prerequisite for the scientific work of the Thesis, several **challenges concerning material and methods** had to be faced. One major challenge was the collection and processing of a huge number of specimens belonging to *Limax* and related groups suitable for molecular analyses. More than ten personal collection trips to Italy, France, Switzerland, Poland, Austria and in Germany, three research internships (Natural History Museum Leiden, the Netherlands; Natural History Museum London, United Kingdom; Museum of Natural History, Wrocław University, Poland) and several conference participations were undertaken. These activities resulted in nearly 2000 tissue samples, which are stored at the ZSM (see electronic supplement). Corresponding vouchers are either stored in the ZSM or belong to other museum collections.

About 600 sequences of Limacidae and Limacoidea were generated (COI, 16S, 28S). However, DNA work on the slug tissue was not straightforward and I had to newly establish some lab procedures and protocols; others had to be modified especially for my work.

Nearly 1000 specimens were raised until adult age either from the juvenile stage (when collected in the field) or from eggs (when laid in captivity). It often took nearly two years, until the animal was adult and ready to be dissected. The process of care and handling was optimized during this time as well. Fixation and dissection procedures were adapted for slugs and several hundred specimens were processed, and data on morphology were collected by me together with colleagues (R. Heim - Natur-Museum Luzern, Switzerland, I. Hyman - Australian Museum Sydney, Australia, U. Schneppat - Bündner Naturmuseum Chur, Switzerland). We took thousands of photographs to document development, morphology, the intraspecific variability and especially the copulation behaviour of limacid slugs.

A substantial collection of old literature was set up which is already scanned in part and will be electronically available for future research. Additionally, my research on *Limax* lead to the formation of the “Munich *Limax* group” headed by Prof. G. Haszprunar - ZSM and in a joint effort together with U. Schneppat (Bündner Naturmuseum Chur, Switzerland) to the “Task Force *Limax*”, an international network of up to now about 300 people interested in slug

research. In the course of these activities, additional 12,000 vouchers were collected since 2004, which are mainly stored at the BNM and NMLU.

Up to recently, all known facts about the genus *Limax* and the family **Limacidae** have been based on morphological analyses; however, the status and number of the single genera of the family are controversially discussed in literature. For example, Likharev & Wiktor (1980) listed ten genera. In contrast to that number, Wiktor (2001) distinguished twelve genera (split into the subfamilies Limacinae, Limacopsinae and Eulimacinae), but did not list all of them. Limacinae, which includes the genus *Limax*, is with seven genera the largest subfamily (Wiktor, 2001). An overview over the differing definitions of three major classifications (Hesse, 1926; Likharev & Wiktor, 1980; Schileyko, 2003) is given in Table 9-1.
A preliminary suggestion of a classification of Limacidae based on the molecular results of this Thesis and unpublished molecular analyses by I. Hyman and B. Nitz (pers. comm.) is also provided in Table 9-1. Main differences concern the position of the genus *Bielzia*, the treatment of *Lehmannia* as two different genera and the revaluation of *Limacus* as separate genus, not as subgenus of *Limax*. Interestingly, Hesse (1926) already came to quite similar results: he split *Lehmannia* into two subgenera and *Bielzia* was positioned within Limacinae. However, adding taxa like *Caspilimax*, *Turcomilax* and *Caucasolimax*, which probably also belong to Limacidae, might change this preliminary molecular-based hypothesis.

Table 9-1. Classifications of Limacidae.

Hesse 1926	**Likharev & Wiktor 1980**	**Schileyko 2003**	**Preliminary classification of this Thesis**
LIMACIDAE **L i m a c i n a e** **Gigantomilax** Gigantomilax s. s., Turcomilax **Limax** Limax s.s..: Sectio Heynemannia, Limacus; Malacolimax, Vitrinoides, Caspilimax **Lehmannia** Lehmannia s. s., Ambigolimax **Mesolimax** Mesolimax s. s., Toxolimax **Bielzia** **Monochroma** **Agriolimax** **Lytopelte** **Megalopelte** **Pseudarion** **Eumilax** Eumilax s. s., Paralimax **Metalimax** Metalimax s. s., Metalimacoides **P a r m a c e l l i n a e** **Milax** **Aspidoporus** **Boettgerilla** **Parmacella**	**LIMACIDAE** **L i m a c i n a e** **Gigantomilax** Gigantomilax s. str., Vitrinoides, Monochroma **Limax** Limax s. str., Limacus **Malacolimax** **Lehmannia** [**Mesolimax** belongs to AGRIOLIMACIDAE] **Caucasolimax** **Caspilimax** **Turcomilax** Turcomilax s. str., Michaelsia, Taulimax **E u m i l a c i n a e** **Eumilax** **Metalimax** **B i e l z i i n a e** **Bielzia**	**LIMACIDAE** **L i m a c i n a e** **Gigantomilax** Gigantomilax s. str., Vitrinoides, Monochroma **Limax** Limax s. str., Limacus **Malacolimax** **Lehmannia** **?Mesolimax** **Svanetia** =Caucasolimax according to Likharev &Wiktor, 1980 **Caspilimax** **Turcomilax** Turcomilax s. str., Kasperia=Taulimax according to Likharev &Wiktor, 1980, Michaelsia **E u m i l a c i n a e** **Eumilax** **Metalimax** **BIELZIIDAE** **Bielzia**	**LIMACIDAE** **L i m a c i n a e** **Gigantomilax** **Limax** **Limacus** **Malacolimax** **Lehmannia** **Ambigolimax** [**Mesolimax** belongs to AGRIOLIMACIDAE] **Bielzia** **E u m i l a c i n a e** **Eumilax** **?Boettgerilla** missing data for: Caspilimax Caucasolimax Turcomilax Metalimax

According to the molecular results of this Thesis, *Limax* (excluding *Limacus*) is monophyletic. Our morphology-based understanding of "what is a *Limax* species" is strongly confirmed by this result. However, there are two species that are currently allocated to *Limax*, but probably have to be removed from this genus: *Limax seticus* Wiktor & Bössneck, 2004 from the Nepalese mountains and *Limax hemmeni* Rähle, 1983 from the Greek island Samos. First molecular analyses that include these two species imply that they do not belong to *Limax*; unfortunately, the sequences were not complete enough to add them to the COI data set in chapter 8. Future analyses with a complemented data set will therefore hold some interesting surprises.

The genus *Limax* has been approached by several methodologies and various aspects have been addressed in this Thesis. **Biogeographic patterns** play a major role when trying to elucidate the evolutionary history and speciation processes in *Limax*. Today's species patterns are the result of climatic changes, extinction events, speciation and repeated vicariance and dispersal. Such influences and events have to be considered also in the evolutionary history of the genus *Limax*. The Ice Ages have been such a major influence in the flora and fauna in Europe, leading to severe changes of habitats and living conditions. The phylogeny and distribution patterns of *Limax* presented in chapter 8 seem to reflect the influence of glaciation and interglacials. A possible scenario of the evolutionary history of the genus *Limax* based on present-day species patterns in Europe and phylogenetic information is presented in Fig. 9-1. Three hotspot regions seem to be important: First, the Balkan Peninsula (marked with A in Fig. 9-1), second, the Apennine Peninsula (including adjacent islands; B) and third, the Southern Alps (C). These three regions harbour to our knowledge the highest species numbers of *Limax* in Europe and a high percentage of these species are endemics. According to the results in chapter 8, a Balkan origin is plausible. Starting in this region, *Limax* might have spread towards northwest regions (a) and then followed the Alpine Arc (b). The Apennine and the Southern Alps could have been settled by multiple events; each event was followed by speciation processes, influenced by the limited exchange between the valleys and glaciation processes (c). A number of species is restricted to southern valleys of the Alps that may have once served as glacial refuges (“Nunataks”). These species include clearly defined species, that show obvious differences in molecular or morphological data or concerning copulation characteristics, for example *L.* cf. "Blauköpfige Egelschnecke" and *L. redii*; both species seem to be restricted to single valleys in the area of the Swiss-Italian border.

Also the Balkan Peninsula has been an important refugium for a number of taxa during glacial periods (Storch, 2004; Savic, 2008). The Balkan species with a derived position in the tree in chapter 8 could therefore be the result of a secondary migration (d and e) to the

Balkan in the Last Glacial Maximum (LGM), with *Limax cinereoniger* Wolf, 1803 s. l. (re-) invading Central Europe (f) after the vanishing of the ice. The same could be true for *Limax maximus* Linnaeus, 1758, which could have survived the LGM in warmer regions of Italy and then spread out (g) once Europe became warmer again, although (additional) Nunatak survival cannot be excluded.

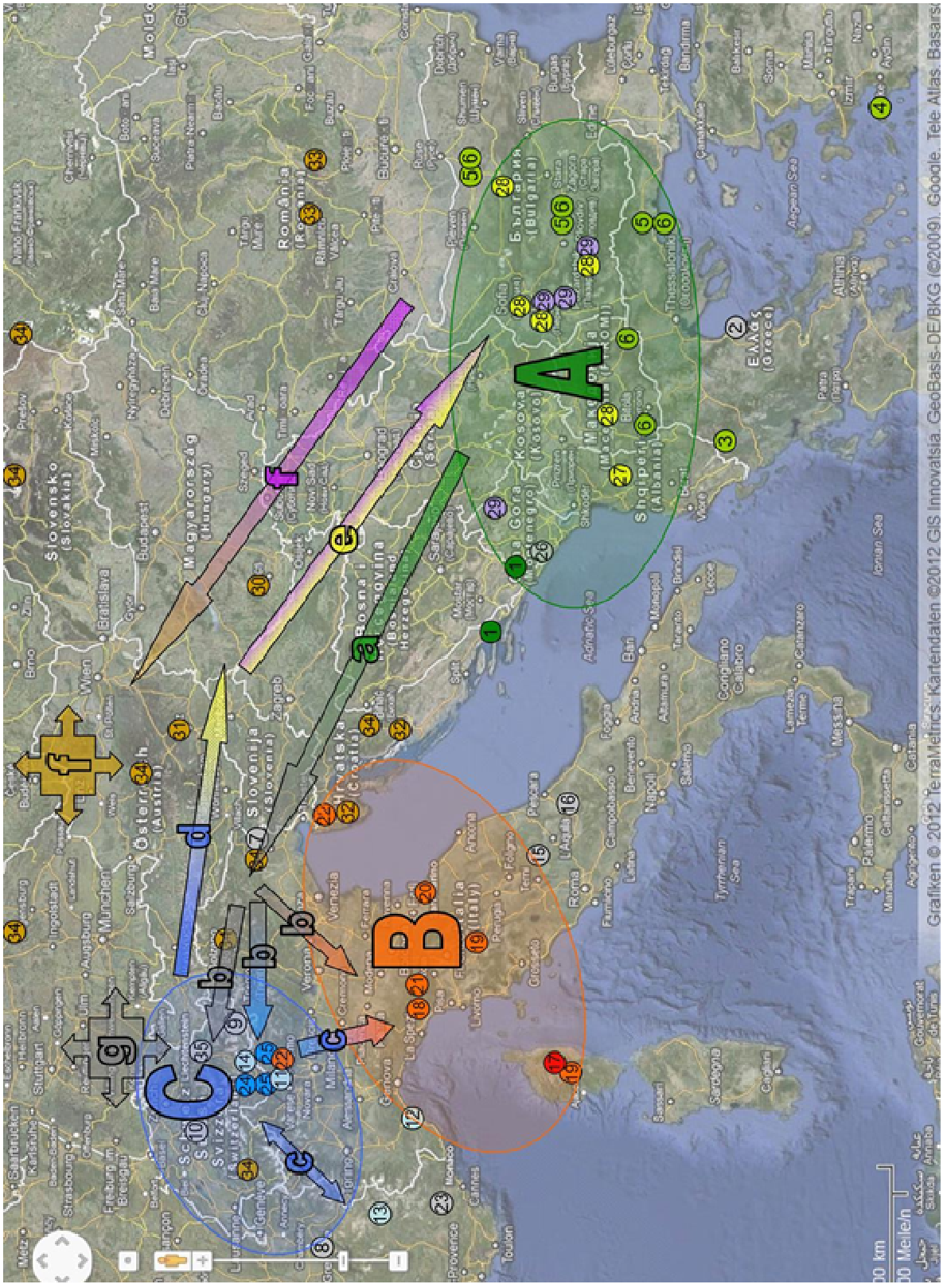

Figure 9-1. Potential scenario of the evolutionary history of the genus *Limax*. Explanations in the text. Numbers correspond to lineage numbering in chapter 8.

However, all historical biogeographic hypotheses on *Limax* are hampered by severe anthropogenic changes like deforestation, biotope destruction and forest fires leading to a substantial loss and fragmentation of *Limax* habitats. Therefore, the scattered present distribution of many *Limax* species might just be the remainder of an originally much higher diversity and a wider distribution. Especially mountainous and woody biotopes in Mediterranean regions and the Balkan area were severely diminished by human influence and became rare during the last centuries. Unfortunately, fossil records of Limacidae are missing or are of very doubtful determination, so the natural habitat and distribution of the genus *Limax* cannot be reconstructed. This leads to a further problem in slug research: the molecular clock approach with calibration of molecular phylogenies using the fossil record (e.g. Lukoschek *et al.,* 2011) is impossible. The alternative possibility, dating based on colonization after geological events such as the formation of new landbridges or new islands via volcanism or continental drift, is not applicable in the present *Limax* phylogeny either, due to the rareness of such events in Europe and to the distribution distortion after anthropogenic displacement of slugs in historical times. Therefore, the suggestions concerning the potential dating of colonization events of Corsican and Sardinian *Limax* species in chapter 6 have to be regarded for what they are: hypothetic scenarios. The interpretation of the time line of speciation and vicariance/dispersal events in *Limax* is therefore a challenge for future work.

Phylogenetic information is considered not only to reflect the colonization history, but also to help to understand the evolutionary background of organisms (e.g. Hewitt, 2000; Dayrat *et al.*, 2011; Holznagel *et al.*, 2010). However, the genetic structure of present organisms has undergone a variety of influences and events in the past, for example speciation processes, bottlenecks or expansion events. As already pointed out in the previous chapters, the analysis of the commonly used barcoding fragment COI does not automatically lead to a robust taxonomy and phylogeny. First of all, it is necessary to clarify that COI was not used in the strict sense of “barcoding” (i.e. as a tool for identification of specimens and their assignment) in this Thesis. Following Collins & Cruickshank (2012), who gave a recent definition of the terms “specimen identification” and “species delimitation”, I regard the COI sequence as one of several markers that contribute to the species delimitation process in an integrative taxonomic framework. The tree reconstructions (like in chapters 5, 6 and 8) were not utilized just for specimen identification, a field where tree-based methods perform poorly (e.g. Meier *et al.* 2006; Zhang *et al.* 2012, Goldstein & DeSalle, 2011). Instead they were used in conjunction with other data as recommended by Collins & Cruickshank (2012) and for phylogenetic purposes.

Species delimitation and phylogenetic reconstruction based on single locus DNA sequences have been shown to lead to questionable results that can substantially be improved by a

multimarker approach (e.g. Skale *et al.*, 2012, Sauer & Hausdorf, 2010, see Dupuis *et al.*, 2012 for a review). In particular, the use of mitochondrial markers like COI suffers from potential drawbacks like incomplete lineage sorting, introgression or inconsistent recombination (Funk & Omland, 2003; Rubinoff & Holland, 2005). In addition to the problems caused by single locus analyses in DNA taxonomy, rarely sampled species pose another challenge, since most methods in molecular taxonomy are designed to perform best with a higher number of sequences per clade, and tree topologies and support values are sensitive to extended taxon sampling (Puillandre *et al.*, 2011, 2012, Lim *et al.*, 2012). Even the geographical scale of sampling has been shown to critically influence the accurateness of DNA barcoding metrics like intraspecific genetic variation, interspecific genetic divergence and the proportion of monophyletic species (Bergsten *et al.*, 2012).

All these difficulties contribute to a controversial debate about the utility of DNA barcoding in taxonomy (Taylor & Harris, 2012) and should not be concealed in regard to the present Thesis; however, due to financial and logistical constraints I was not able to implement recent recommendations from literature like multimarker approaches or extended taxon sampling in the framework of my studies. In respect of the Thesis, COI sequencing outperformed a multimarker approach due to the cost effectiveness, which allowed to sequence a comparatively high number of animals and analyse not only some local populations, but to cover a geographically wide range. Thus, the efforts in this Thesis were mainly intended to (i) show the complementation of morphological and DNA sequence data, (ii) reveal a structure of clusters in the sampled European *Limax* specimens to gain first hypotheses of the biogeographic distribution, (iii) reconsider existing species delimitations in the genus and (iiii) give further hints for the direction of future investigations.
Next steps for future investigations could be, for example, the application of a multimarker approach to gain better infraspecific resolution. Another desideratum within a future multimarker approach is the incorporation of nuclear genes, which might help to infer genetic reticulation, even though hybridisation in *Limax* is not very likely, given the highly complex copulation mode. This approach in combination with new species delimitation methods especially designed for multimarker analyses (for example Bayesian Species Delineation; Yang & Rannala, 2010; Zhang *et al.*, 2011) should substantially improve the discrimination of the European *Limax* lineages. Furthermore, in some lineages, recent species radiations are to be expected and, as shown in chapter 7 for the Corsican radiation, even modern delimitation methods like ABGD fail to delineate this local radiation with the existing data set due to the limited number of sequences per species. In these cases, it would be desirable to extend not only taxon sampling and try to sequence a few more genes, but to head for next

generation sequencing technologies, which promise "the prospect of readily available, full genomic sequence data in the near future" (Taylor & Harris, 2012).

A more general problem of molecular markers is the fact that gene trees do not necessarily reflect the "true" species delimitation that is traditionally based on morphological characters (e.g. Degnan & Rosenberg, 2009; Sauer & Hausdorf, 2010); however, one can argue that the 'species' itself can be regarded as arbitrary, as the whole system of taxonomic units and therefore all species delimitations have to be considered to be hypotheses. Regarding the term 'species', there exist very contradictive points of view, which do not only comprise the scientific use of the term, but also touch philosophical questions (Hey, 2001). For example, Ereshefsky (2010) claims that the species category is not real in nature, however, he summarizes, that there are "pragmatic reasons for keeping the word 'species'". Moreover, there are a lot of different species concepts: Hey (2001) lists 24 of them. Although some species concepts like the Phylogenetic Species Concept or the Biological Species Concept are widely used, none of it fits all purposes and organisms (Hey, 2001). In my opinion, the use of the term 'species' helps to communicate scientific hypotheses. An example of the use of the term 'species' as a working hypothesis is given in chapter 6, where difficulties in the molecular and morphological delimitation of closely related entities (called species or clades) are discussed. A deeper look at these entities might lead to a new classification or a redefinition of species boundaries in these clades in future. To take into account the lack of resolution between these closely related entities, I summarized the potential species into preliminary species groups in chapter 6 and 7. Nevertheless, the usage of the term 'species' enables to communicate about hypotheses and therefore facilitates the scientific process.

However, **taxonomy and determinations** in *Limax* remain as a challenge. There are more than 250 nominal taxon names available (including a number of subspecies, varieties, etc.) that have to be validated. Descriptions often lack diagnostic characters and in most cases types are not available or in such a bad condition that important anatomical structures cannot be investigated. Collecting at the type locality could provide a first step towards the validation of a certain species; unfortunately, locality information is not specific enough in a large number of descriptions. One example is the case of *L. alpinus* Férussac, 1822 (see Nitz *et al.*, 2009, chapter 5): the only information about the type locality is "in our alps"; a term that enables different interpretations (compare Nitz *et al.*, 2009 vs. Brandstetter, 2011). Furthermore, anatomical details are not available for *L. alpinus* in the original description. It might be possible to reconstruct a type locality by searching the correspondence of the author and other old documents, but since this is still likely to lead to an ambiguous result, such effort it is appropriate in my opinion. I think that old names lacking a well-defined type locality and anatomical description should be discarded to prevent further confusion. A

similar approach is, for example, applied in a study about sea slugs of the genus *Navanax* by Ornelas-Gatdula *et al.* (2012), where the authors use the oldest name allowing a positive identification and decide against older names that are taxonomically ambiguous. Detailed redescriptions, which are needed in many cases, should only be based on topotype series (in cases with a clear type locality) or on material that corresponds unequivocally to existing names (in cases where there are type material and detailed descriptions). Due to the forementioned problems, I followed the recommendations of "open nomenclature" (Bengtson, 1988) in chapter 8 in cases where species assignment is yet not possible ("sp.") or the identification is provisional ("cf."). Nevertheless, three new species were described in the course of the Thesis: *Limax sarnensis* (chapter 5), *L. giustii* and *L. ilvensis* (chapter 6). For *L. sarnensis*, a thorough analysis of the new species is given; this high standard description is based on a combination of diagnostic characters including morphology, copulation behaviour and molecular data. Chapter 5 shows the high effort that is needed to provide a comprehensive characterisation of one species. In chapter 6 (appendix) two more species are described. For both species anatomical details were already given by Giusti (1996; 1976) and Giusti & Mazzini (1971), but they were not formally described. COI data revealed the anatomical differences in a new light and enabled the description based on the already existing morphological data. Furthermore, in the appendix of chapter 6, the name of *L. minimus* was replaced. This species, which was described by Pollonera in the year 1896, is now named *L. vizzavonensis* since the name *Limax minimus* is preoccupied. This case is a good example for a formerly well described species with details on its type locality, where it was just necessary to rename it, since all relevant data for a validation of the species were present in the original description. New morphological and molecular data were generated during the studies of the Thesis, so a thorough redescription of *L. vizzavonensis* will be possible in near future. The same applies to further species: molecular data, extensive morphological data and in a lot of cases also a detailed copulation documentation are now available and redescriptions are scheduled.

The introduction and use of subgenera in the genus *Limax* as proposed by Falkner & Niederhöfer (2008) and Falkner & Proschwitz (2009) was not applied in this Thesis. There are two reasons for that: first, characters with the potential to be used above the species level (such as differences in copulation modes, used by Falkner & Niederhöfer (2008) to justify the introduction of the new subgenus "*Brachylimax*") lack data for many species and provide only scattered information. Second, the tree topology presented in chapter 8, which with its potential clades or lineages may also serve as the basis of a genus-group system, is not stable enough yet. There are changes in tree topology due to the addition of further lineages (for example *L. cinereoniger* s. l., which is positioned quite basally in chapters 5 and 6 and is placed differently after the addition of Balkan specimens in chapter 8). Since taxon

sampling in Europe (particularly in the south and southeast), but also in the Caucasus (mainly Turkey, Georgia, Azerbaijan) still needs improvement, further changes are to be expected. However, the molecular trees will serve as a fundamental base for further molecular as well as morphological work combined in an integrative taxonomic approach that may also lead to the justification of subgenera.

Although the use of molecular data in taxonomy has increased rapidly in the last years, the value of **morphological characters** should not be underestimated. In chapter 5 all available lines of evidence are used to describe a new *Limax* species. Especially the genital system is shown to harbour a lot of taxonomic information. However, this organ is not accessible in the field and requires technical equipment and an experienced expert. For a first classification of *Limax* specimens, the combination of body and mantle and sole colouration could be used, but this preliminary identification does not replace a thorough study of internal structures. Not only the length of the penis, but also the position of the penial retractor and the vas deferens and the penial interior are important features to distinguish species in *Limax*.

The **copulation system** and the incidence of very long penes are unique for *Limax* and have not been observed in other slugs of the family Limacidae (with the exception of "*Limax*" *seticus*, which has a penis of more than body length and probably might not belong to *Limax*). The copulation mode itself certainly serves as a very valuable information source for species delimitation and evolutionary considerations, but practicality and reproducibility are limited. Gaining an appropriate number of copulation observations in full length from different populations of every species will take many years, in particular for species that do not live just around the corner in Central Europe, but on high and steep mountain ranges or in canyons of the Balkan Peninsula or the Pyrenees. The complex copulation behaviour and the high variability of penis lengths in the genus *Limax* might also have been one of the driving forces of speciation. Among the common mechanisms of selection/speciation, assortative mating and, as a result, reproductive isolation due to different penis lengths probably has been a very important one. However, the advantages of long penes and the capricious, time- and energy consuming copulation process in the light of (natural) selection and fitness still remain unclear; another matter for future research.
Once most of the species in the genus *Limax* are clearly defined, other types of analyses can be realised. Ancestral area reconstructions could help to find the possible ancestral range of the genus and might identify factors responsible for the current distribution pattern. Ancestral character reconstructions might shed light on the character evolution in *Limax*.

10 Appendix

Content Appendix

10.1 References

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