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

Chapter · January 2010

DOI: 10.1007/978-3-642-12425-9_19

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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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M. Glaubrecht (ed.), *Evolution in Action*,
DOI 10.1007/978-3-642-12425-9_19,

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

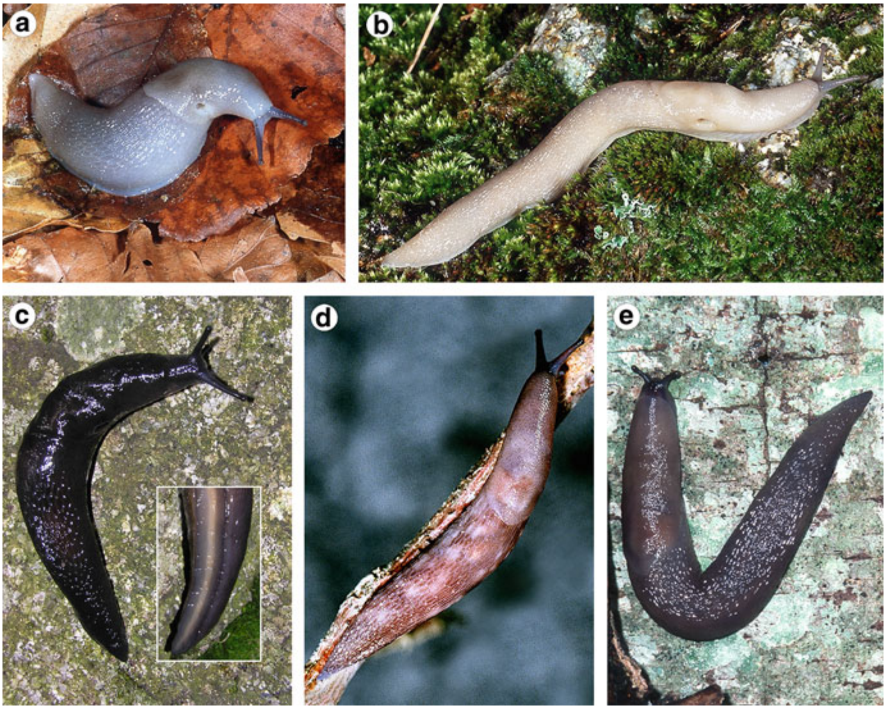

Fig. 1 Habitus photos of different colour morphs of the *Wolterstorffi*-group. All detailed pictures are approximately 2/3 natural size. (**a**, **b**) *Limax vizzavonensis* n. nom. (**a**) Specimen from Vizzavona near Cascade des Anglais (no. 12 on map, Fig. 10); (**b**) specimen from Ruine de Sorba (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 C*orsicus*-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

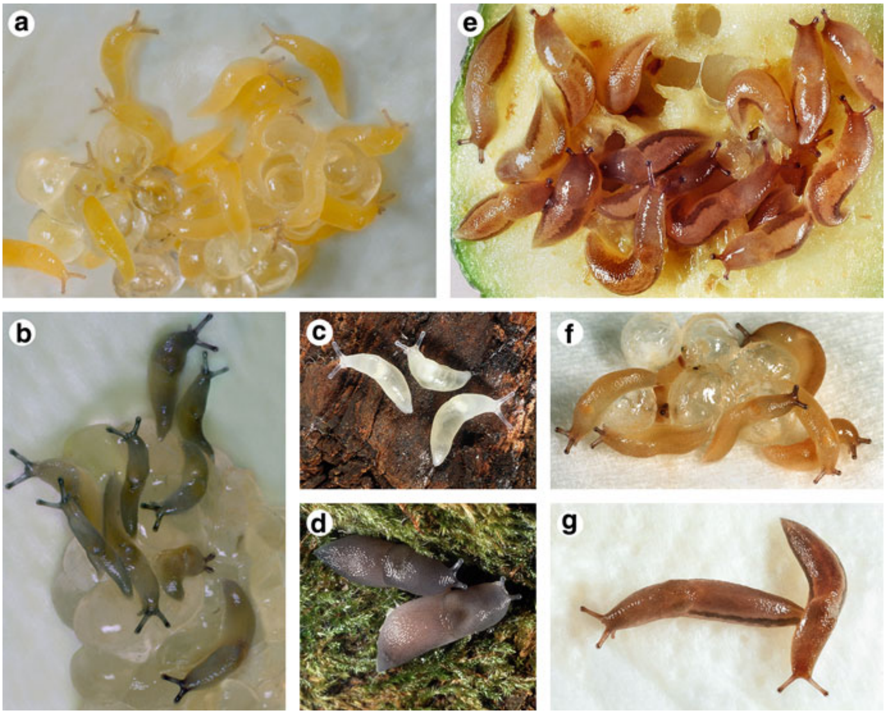

Fig. 3 Chromatic development of juveniles. All detailed pictures are approximately two times natural size. (**a**–**d**) Offspring from representatives of the Wolterstorffi-group: no traces of "Stammbinden." (**a**) F1 of a specimen from the Plateau de Coscione, photographed 1 h after the end of hatching, 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.

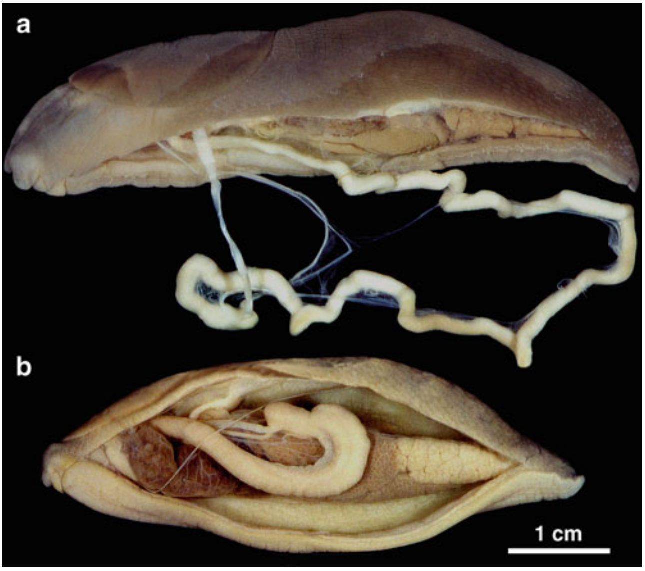

Fig. 4 Dissection photographs of the two extreme forms in the *Wolterstorffi*-group. (**a**) *Limax* sp. (Porto); the animals of this new species have an extreme long and thin penis. (**b**) *Limax wolterstorffi* (topotype): penis short and massy

(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)

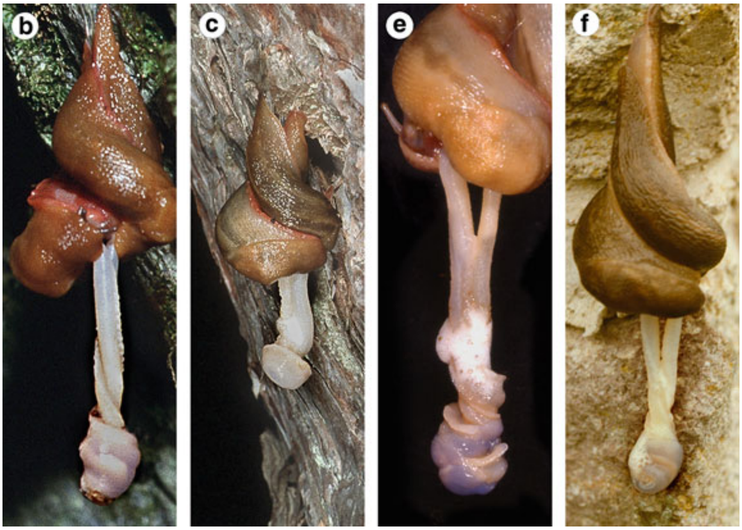

Fig. 6 Morphology of the penes in the *Corsicus*-group s. l. during or shortly after sperm exchange. *Lettering* coincides with couples in Fig. 2. All detailed pictures are approximately 2/3 natural size. Estimated penis lengths: (**b**) 5 cm; (**c**) 2 cm; (**e**) 6.5 cm; (**f**) 4 cm (**f**: photograph,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

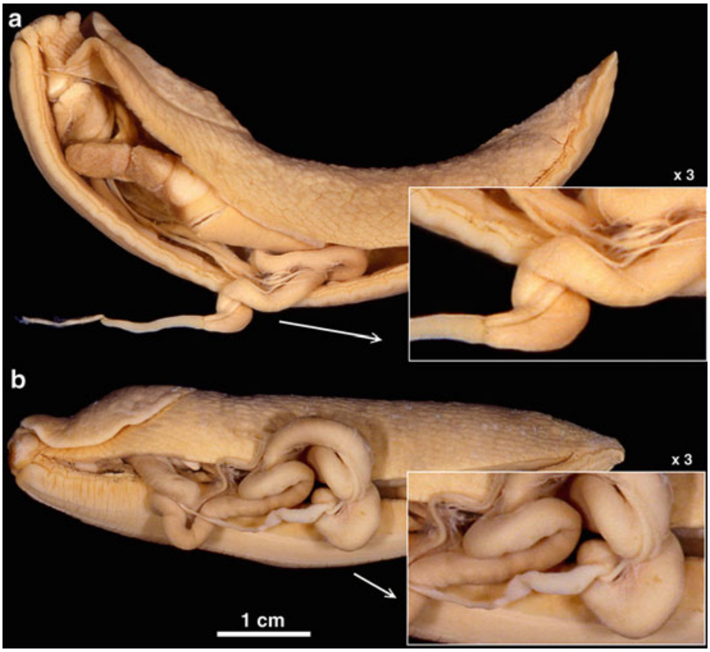

Fig. 7 Examples of anatomical specialisation in the *Corsicus*-group. (**a**) Specimen from Sperono (no. 30 on map), leg. E.Th.J. Ripken 1996; terminal insertion of retractor and vas deferens, morphology of the penis tip corresponding to *L. corsicus* s. str.; (**b**) specimen from Furiani-Marinella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

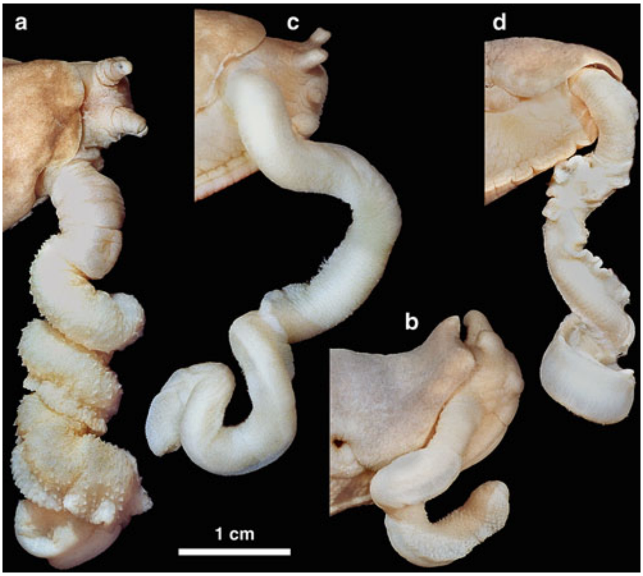

Fig. 8 Morphology of everted penes of clearly distinguishable forms of the *Corsicus*-group from different sampling places. (**a**) Truggia (no. 27 on map, Fig. 11); (**b**) Bonifatu (no. 23 on map), not fully everted; (**c,d**) Two sympatric forms from Grigione (no. 19 on map)

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%

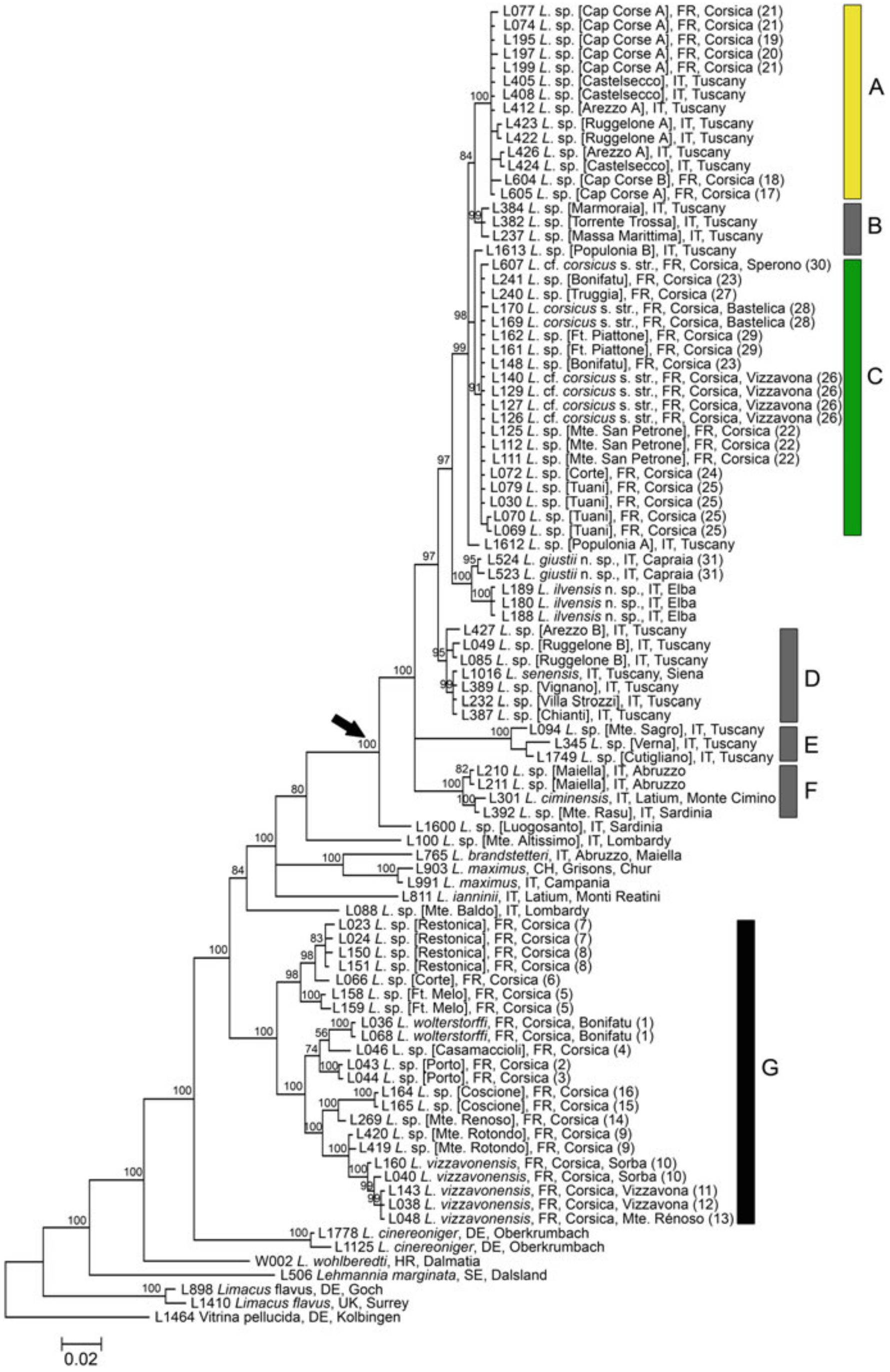

Fig. 9 Majority-rule consensus tree from the Bayesian inference analysis of the COI data. Posterior probabilities are marked above the branches. *Arrow* *Corsicus*-group sensu lato. *Bar A*

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

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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

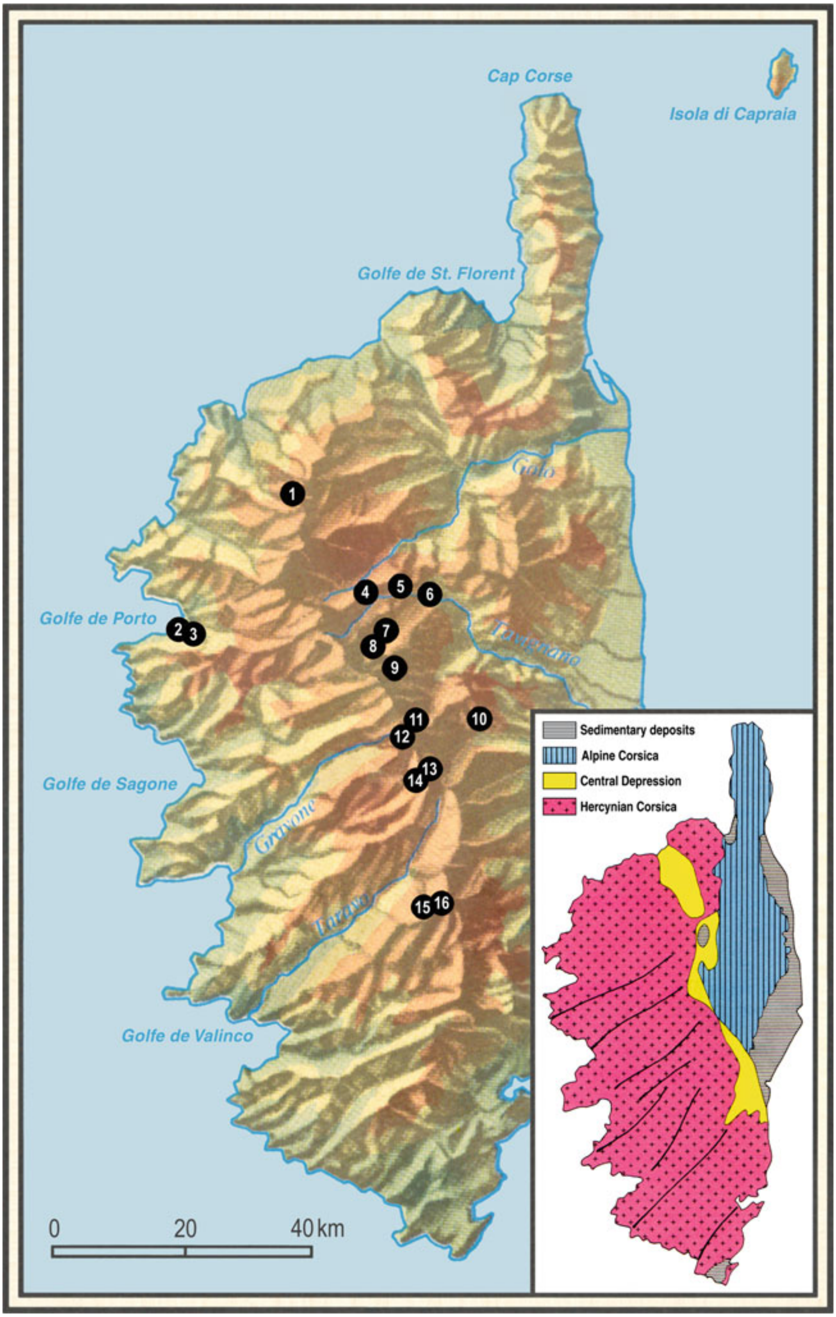

Fig. 10 Localities of the studied populations of the *Limax wolterstorffi*-group. Map base MNHN (modified). *Insert* Simplified geology after A. Gautier (1983). (*1*) Cirque de Bonifatu, 610 m (loc. typ. *wolterstorffi*); (*2*) Porto, D 81 direction Piana; (*3*) Porto, ravin du Riù; (*4*) Casamaccioli, 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

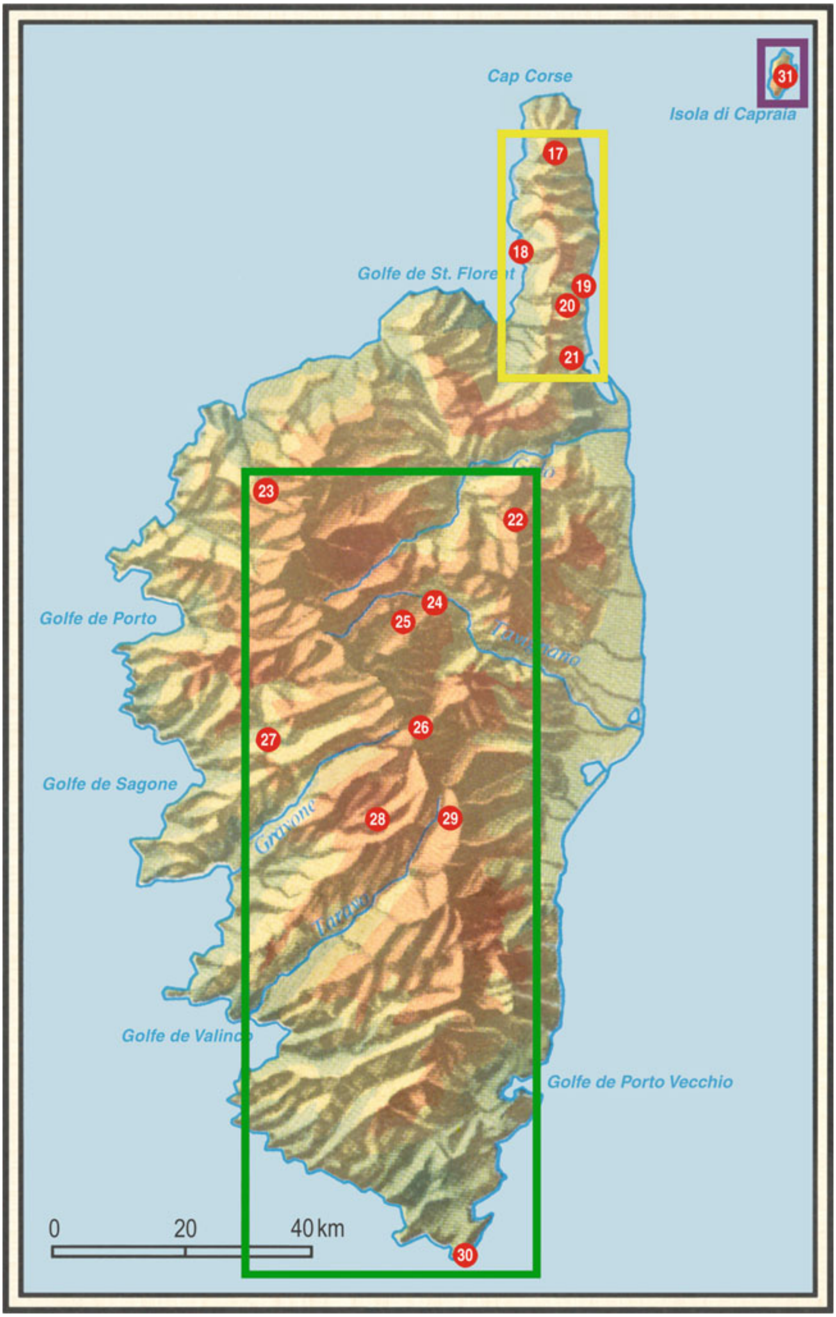

Fig. 11 Localities of the studied populations of the *Limax corsicus*- group. Map base MNHN (modified). *Green frame* Endemic *Corsicus*-group; *yellow frame* Cap Corse/Tuscany-group; *lilac frame* Capraia isolate. (*17*) Vallée de la Méria; (*18*) Nonza; (*19*) Vallée du Grigione; (*20*) Pietrabugno; (*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)

		Within species groups	Between groups		Cap Corse/ Tuscany-group
Species group	*n*		*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.

Acknowledgments This work was supported by the DFG (German Science Foundation) through the grant HA 2598/7 to G.H. within the Priority Programme “Radiations” (SPP 1127).

We have to thank a huge number of people (named in the material list) for kindly collecting or providing additional material for us. We are grateful to all collection managers and staff of various institutions for providing us with material and assistance. Dr. Isabel Hyman (Sydney, Australia) commented on the manuscript and gave us valuable input and hints for molecular work. We are extremely grateful to Dr. Markus Moser (Bayerische Staatssammlung für Paläontologie und Geologie München) for very helpful contributions on the geohistory of Corsica and the adjacent areas. Margrit Falkner gave us continuous support and a generous donation, which finally allowed the addition of crucial sequences. These three sequences have been elaborated by the commercial enterprise kmbs München.

We are very grateful to Prof. Dr. Folco Giusti (Univ. Siena) for kindly providing material and valuable comments and advices on the taxonomy of Italian *Limax*.

We acknowledge Janine Serafini (Société des Sciences historiques et naturelles de la Corse), Bernard Recorbet (DIREN Corsica), the late Bernard Roché (DIREN Corsica), Achille Pioli (Corte, ONF), Lucien Borelli (Furiani Marinella), Jean-Claude Gabrielli (ONF), and Emilio Vichera (Golfe de Sperono) for very helpful and cordial support on site.

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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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