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
	Lavizarra, 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
	Innertkirchen, Berne, CH	D. Tschanz, 2006	NMLU 14257–14261	FJ606494 = NMLU 14257	5
	Guttannen, 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, 1980	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
	Entlebuch, 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. Schlier, 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 Wrocławskiego, 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. Etzold, 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 ($GTR + \Gamma 8 + I$) 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).

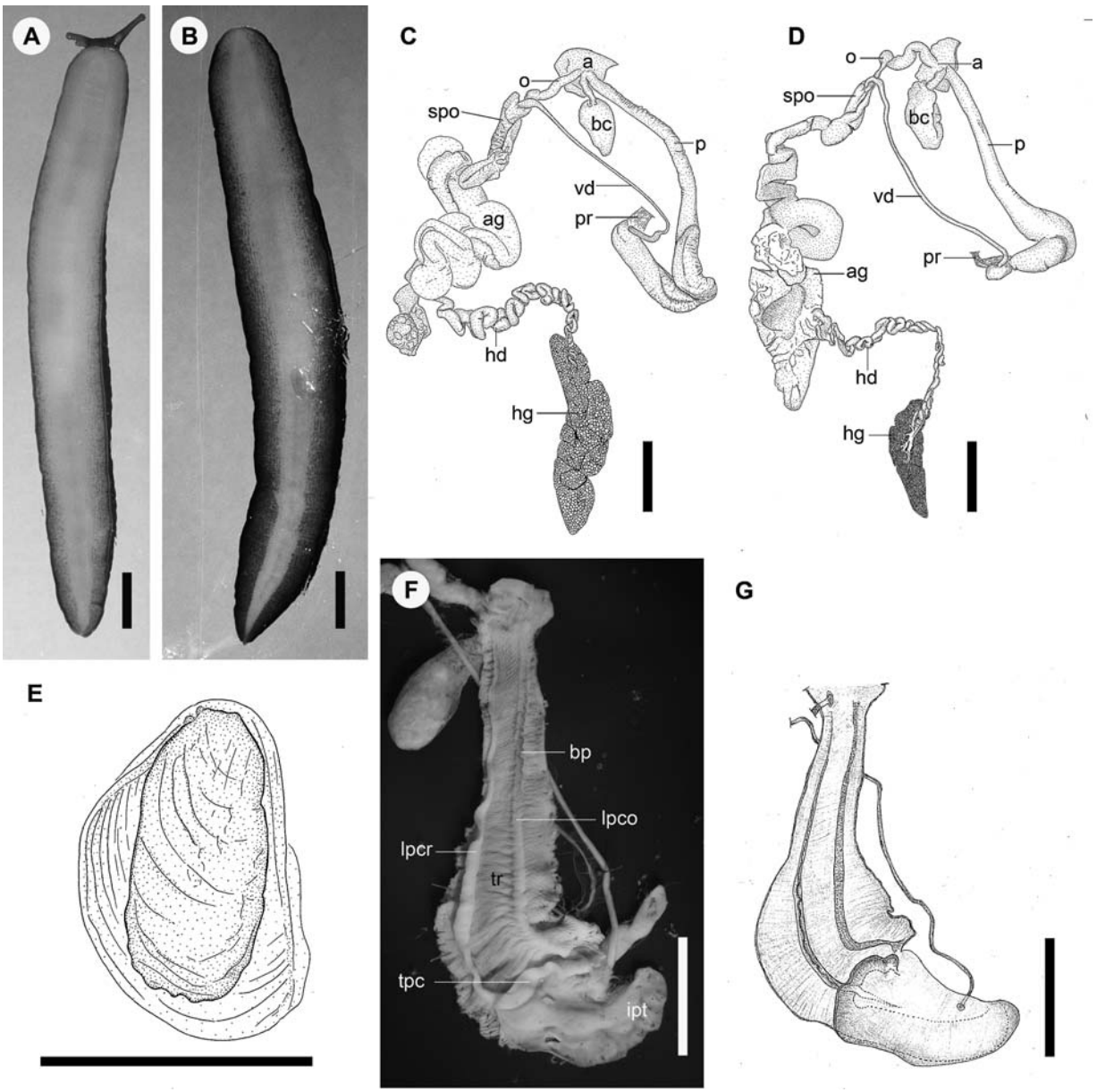

Figure 2. A, B. Sole coloration of living specimens of *Limax sarnensis* n. sp. **A.** Paratype NMLU 14419. **B.** Paratype NMLU 14415. **C, D.** Genital anatomy. **C.** Paratype NMLU 13438. **D.** Specimen ZSM Mol 20071533, Poschiavo, Switzerland. **E.** Shell, paratype NMLU 13438. **F, G.** Penial 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

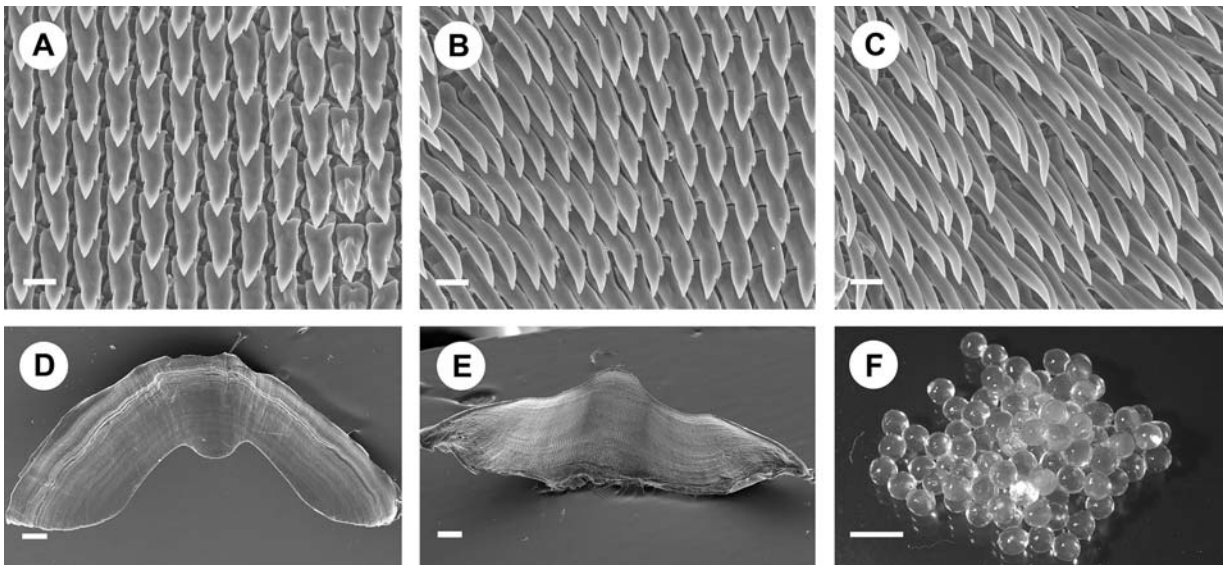

Figure 3. A–C. Radula, paratype NMLU 13438. **A.** Central and lateral teeth. **B.** Lateral and marginal teeth. **C.** Marginal teeth. **D, E.** Jaw, specimen NMLU 14240, Canton Ticino, Switzerland. **D.** Lateral view. **E.** Dorsal view. **F.** Eggs of specimen NMLU 14231, Aosta valley, Italy. Scale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

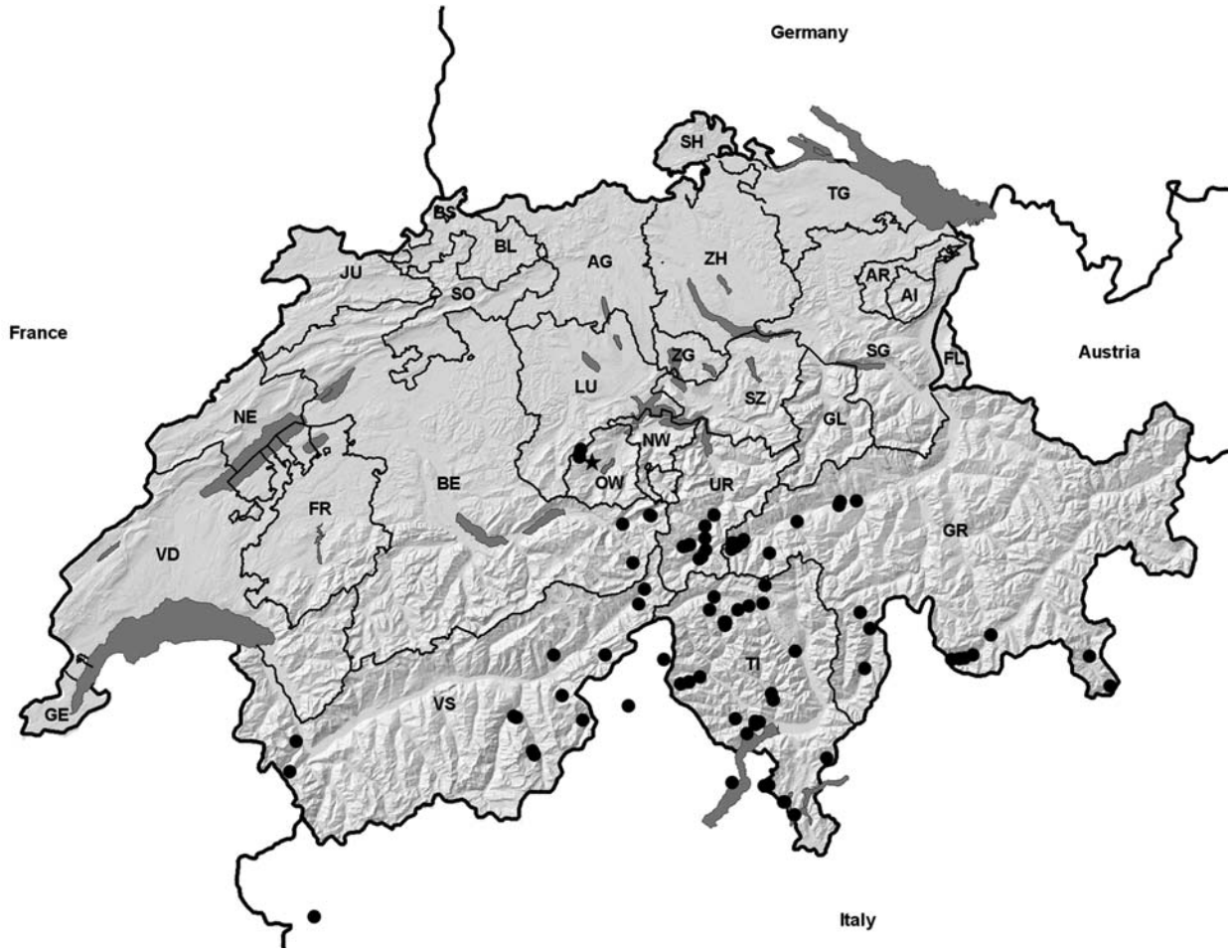

Figure 4. Map of localities. Type locality marked with a star. See Table 1 for details. Map provided by the Centre Suisse de Cartographie de la Faune (CSCF), Switzerland.

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

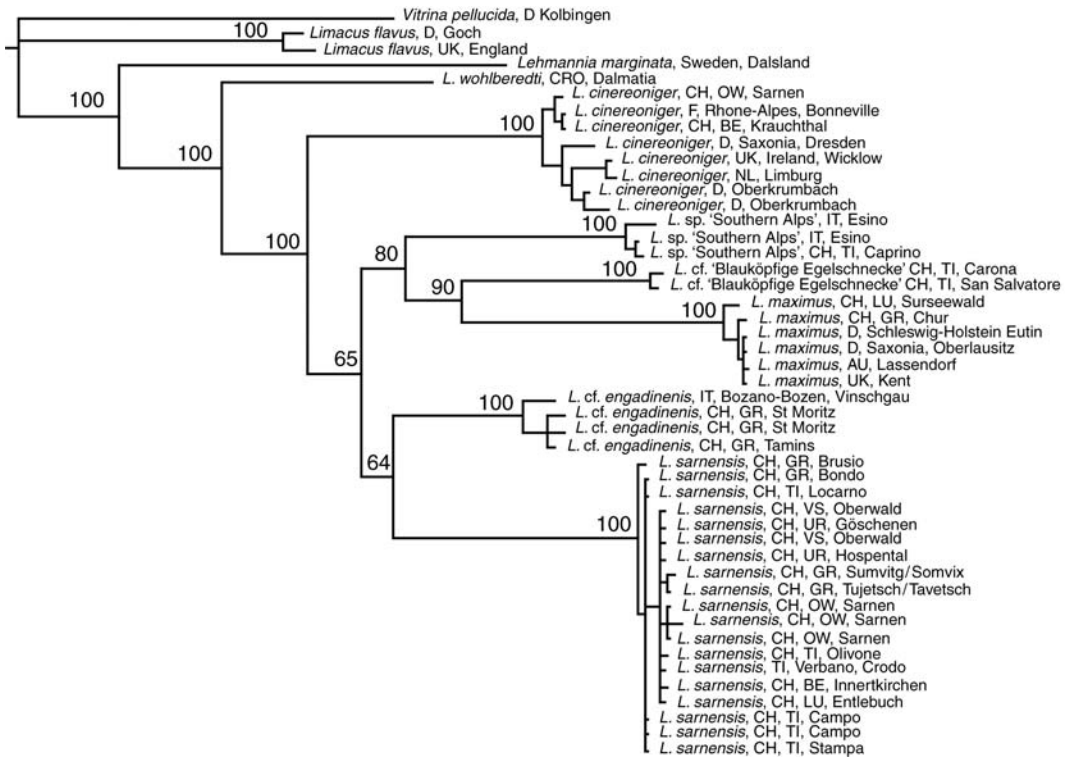

Figure 5. Majority-rule consensus tree from the Bayesian inference analysis of the COI sequence data. Posterior probabilities are marked above the branches.

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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