ON THE IDENTITY OF *RADIX PEREGRA* (O. F. MÜLLER) (GASTROPODA: BASOMMATOPHORA: LYMNAEIDAE) IN THE AZORES

Katrin Schniebs[1], Ulrich Bössneck†[2] & Anna K. Hundsdoerfer[1]

[1]Senckenberg Natural History Collections Dresden, Königsbrücker Landstraße 159, 01109 Dresden, Germany
[2]formerly Stendaler Straße 2, 99092 Erfurt, Germany

Abstract *Specimens of the freshwater gastropod genus* Radix *Montfort, 1810 were collected in March 2015 in two São Miguel Island creeks, the largest island in the Portuguese archipelago of the Azores. Based on the shape of their shells they could have been assigned to two different* Radix *species. Because it is now known that morpho-anatomical studies of the shells and genitalia do not allow for a reliable determination of most* Radix *species, DNA markers were used as the best tools for identification. Four specimens, two from each location, were subject to molecular identification. Their cyt–b and ITS-2 sequences formed one cluster with the sequences of the* Radix balthica *specimens used in the analysis. This is the first identification of this species, a potential intermediate host of the* Fasciola hepatica *Linnaeus, 1758, based on molecular genetics for the Azores.*

Key words Radix balthica, *Lymnaeidae, Azores, molecular genetics, morphology*

Introduction

In 1975 Backhuys mentioned the first record for *Lymnaea* (*Radix*) *peregra* (O. F. Müller, 1774) on the Azores from São Miguel Island. Records of this species from the Azores in later publications (Raposeiro *et al*. 2007, 2011; Cunha *et al*. 2010) only cite Backhuys (1975) without adding further information. Teixeira *et al*. (2012) refer to this species as a potential intermediate host of the liver fluke *Fasciola hepatica* Linnaeus, 1758 on this archipelago and therefore these snails are not only of interest to specialists of freshwater molluscs, but also of veterinary and human medical importance.

In earlier literature, the name *R. peregra* was commonly used as the synonym of *R. labiata* (Rossmaessler, 1835) (Schniebs *et al*. 2013) but was also used for *R. balthica* (Linnaeus, 1758) (e. g. Glöer 2002; Lawton *et al*. 2015) as well as for *R. lagotis* (Schrank, 1803) (Schniebs *et al*. 2015). Although *Seddon et al*. (2014) indicate in the IUCN Red List an occurrence of *R. balthica* in the Azores, the authors of the present work do not know on which basis the assignment of *R. peregra* to this species took place. In our opinion a thorough clarification is necessary to which *Radix* species the snails occurring on São Miguel Island belong according to valid taxonomy. However, the molecular identification is the only reliable method to identify *Radix* species, as shell and other anatomical features are morphologically plastic and most *Radix* species share morphological characters as a result of convergent adaptations to local environments (Lawton *et al*. 2015).

In March 2015 one of the authors, the late U. Bössneck, was able to collect some *Radix* specimens from two creeks on São Miguel Island: from the creek Ribeira de Praia (mentioned in Backhuys 1975 for *L. (R.) peregra*) as well as from a creek to the northwestern bank of the Lagoa das Furnas. Four of these (two each from one creek) were donated to the Senckenberg Natural History Collections Dresden, Museum of Zoology (SNSD) for molecular genetic analyses. The aim of this study was to identify these individuals by the analysis of the nuclear marker ITS-2 as well as the mitochondrial marker cyt–b, molecular phylogeny and comparative morphology.

Material and methods

All specimens used for molecular genetic studies are listed in Table 1. Specimens are stored either in the mollusc collection of the Senckenberg Natural History Collections, Dresden if used for morphological analyses as well or in the tissue collection of the SNSD (if tissue samples only).

The snails were fixed in 70–80% ethanol or isopropyl alcohol. Shell morphology, mantle pigmentation and anatomy were documented from

Contact author : katrin.schniebs@senckenberg.de
†Deceased.

Table 1 Specimens used for molecular genetic studies

Code	Collection No. SNSD	Locality	ENA/GenBank No. cyt-b	ENA/GenBank No. ITS-2	References
Lymnaea stagnalis (LINNAEUS 1758)					
Lymnaea stagnalis 1	Moll 49239	Germany, Saxony, Dresden-Zschieren, old branch of river Elbe, N 50°59'50'' E 13°52'28''	HE573102	HE573064	Schniebs *et al*. 2011
Lymnaea stagnalis 3	Moll 53108	Germany, Baden-Württemberg, Konstanz- Egg, ditch Hockgraben, N 47°40'57.3'' E 9°11'34.2''	FR797894	FR797834	Vinarski *et al*. 2011
Radix auricularia (LINNAEUS 1758)					
Radix auricularia 1	Moll 51980	Bulgaria: river Kamchia 2,5km north of Staro Oryahovo, N 43,0195° E 27,8245°	LS974261	LS974218	Schniebs *et al*. 2019
Radix auricularia 2	Moll 52857	Russia, Novosibirsk Region, Novosibirsk, Reservoir near Kirza River, N 54°14.224' E 81°39.63114'	HE557667	HE557647	Schniebs *et al*. 2011
Radix auricularia 3	Moll 53070	Germany, Bavaria, Weichering, pond in riverside forest, N 48°43'34.1'' E 11°19'23.6''	FR797902	FR797842	Vinarski *et al*. 2011
Radix auricularia 4	Moll 53086	Switzerland, Lake Constance near Güttingen	LT623597	LT623582	Schniebs *et al*. 2018
Radix auricularia 5	Moll S6815	Russia, Republic of Buryatia, Lake Baikal, Kotovo Bay near Monakhovo, N 53° 39.011' E 108° 58.587'	LS974264	LS974219	Schniebs *et al*. 2019
Radix auricularia 6	Moll S7384	Denmark: Soro, lake Degnemosen, N 55.699417 E 12.504774	LS974263	LS974221	Schniebs *et al*. 2019
Radix labiata (ROSSMÄSSLER 1835)					
Radix labiata 1	Moll 51863	Montenegro, Žabljak, Black Lake, sand pools, 43°08'50''N 19°05'42''E	HE798507	HE798455	Schniebs *et al*. 2013
Radix labiata 2	Moll 52415	Austria, Carinthia, Hermagor, N 46°37' E 13°22'	HE798484	HE798457	Schniebs *et al*. 2013
Radix labiata 3	Tissue 3956	Spain: Santa Marina del Valdeon, Picos de Europa, Vega de Liordes, marshy headwater region, 1940m, 43°09'11''N 4°50'09''W	HE798491	HE798464	Schniebs *et al*. 2013
Radix labiata 4	Moll S172	France, Bourgogne, Département Cote-d'Or	HE798492	HE798465	Schniebs *et al*. 2013
Radix labiata 5	Moll S284	Switzerland, Basel City, Riehen, well Nollenbrunnen, 47°34'41''N 7°40'17''E	HE798493	HE798466	Schniebs *et al*. 2013
Radix labiata 6	Moll S2904	Germany, Saxony, small brook north of Tharandt, N 51°00'08'' E 13°34'19''	HE798496	HE798469	Schniebs *et al*. 2013
Radix labiata 7	Tissue 9527	Portugal: Vila Real District, Frades (8km west of Montalegre), Rio Cavado River	LR732142	LR732158	This paper
Radix dolgini (GUNDRIZER AND STAROBOGATOV 1979)					
Radix dolgini 1	Moll 52861	Russia: Tomsk Region, District Teguldet, lake near Novoshumilovo village, N 57°25'30''E 88°31'13''	LS974268		Schniebs *et al*. 2019
				KT030064	Vinarski *et al*. 2016

Table 1 (*Continued*)

Code	Collection No. SNSD	Locality	ENA/GenBank No. cyt-b	ITS-2	References
Radix dolgini 2	Moll 52862	Russia: Tomsk Region, District Teguldet, lake near Novoshumilovo village, N 57°25'30'' E 88°31'13''	LS974269		Schniebs *et al*. 2019
				KT030065	Vinarski *et al*. 2016
Radix dolgini 3	Moll S5217	Russia: Altay Territory, a swamp in the floodplain of Kulunda River, N 50°59'50'' E 80°00'07''	LS974271		Schniebs *et al*. 2019
				KT030061	Vinarski *et al*. 2016
Radix dolgini 4	Moll S5218	Russia: Altay Territory, a swamp in the floodplain of Kulunda River, N 50°59'50'' E 80°00'07''	LS974272		Schniebs *et al*. 2019
				KT030062	Vinarski *et al*. 2016
Radix lagotis (SCHRANK 1803)					
Radix lagotis 1	Moll 49868	Germany, Saxony, pond Vierteich near Freiteilsdorf, 13°41'57'' E 51°15'43''	HE573114	HE573076	Schniebs *et al*. 2011
Radix lagotis 2	Moll 51858	Germany, Brandenburg, Strodehne, N 51.74555° E 12.22396°	LN874262	LN874255	Schniebs *et al*. 2015
Radix lagotis 3	Moll S1777	Ukraine: Donetsk Region, Staromarjevka, Kalmius River	LS974274	LS974226	Schniebs *et al*. 2019
Radix lagotis 4	Moll S3765	Bulgaria. Dragoman marshland 40km west of Sofia, N 42°56'11.7'' E 22°57'9.3''	LN874264	LN874257	Schniebs *et al*. 2015
Radix ampla (HARTMANN 1821)					
Radix ampla 1	Moll 51112	Russia, Chelyabinsk Region, river Miass near Dynamo village, N 45°57' E 60°02'	HE798470	HE798448	Schniebs *et al*. 2013
Radix ampla 2	Moll 51113	Russia, Chelyabinsk Region, river Miass near Dynamo village, N 45°57' E 60°02'	HE798471	HE798449	Schniebs *et al*. 2013
Radix ampla 3	Moll 53082	Germany, Baden-Württemberg: Lake Constance, subbasin Überlinger See	LS974278	LS974229	Schniebs *et al*. 2019
Radix ampla 4	Moll 53285	Germany, Mecklenburg-Western Pomerania, lake Tollensesee near Klein Nemerow, N 53.4909° E 13.2146°	LS974280	LS974231	Schniebs *et al*. 2019
Radix ampla 5	Moll S2924	Switzerland: Lake Biel, near bridge to St. Peter's Island	LS974281	LS974232	Schniebs *et al*. 2019
Radix ampla 6	Moll S5186	Czech Republic, Františkov nad Ploučnicí, Ploučnice River near the bridge, N 50°43'26'' E 14°19'27.5''	LS974283	LS974234	Schniebs *et al*. 2019
Radix ampla 7	Moll S6511	Croatia, Zrmanja River near Bilišane, N 44°11'38'' E 15°47'35''	LS974291	LS974242	Schniebs *et al*. 2019
Radix ampla 8	Moll S8731	Russia, Moscow region, river Oka near Turovo, 54°50.836'N, 37°52.799'E	LS974307	LS974258	Schniebs *et al*. 2019

Table 1 (*Continued*)

Code	Collection No. SNSD	Locality	ENA/GenBank No.		References
			cyt-b	ITS-2	
Radix balthica (LINNAEUS 1758)					
Radix balthica 1	Moll 51139	Russia, Tomsk Region, Lake Motshishtshe, N 56°13'10.2" E 84°54'48.0"	LR732143		This paper
				HG931938	Vinarski *et al*. 2014 (unpublished)
Radix balthica 2	Moll 51292	Switzerland, canton Basel City, Riehen, Wiesengriener, 47°35'21'' N 07°38'32''E	HE573134	HE573083	Schniebs *et al*. 2011
Radix balthica 3	Moll 51860	Sweden, Øland, east shore near Lille Seby, 56.345°N 16.565°E	HE573141	HE573090	Schniebs *et al*. 2011
Radix balthica 4	Moll 52412	Croatia, Lake Milanovac near Plitvica, 44°53'45''N 15°36'34''E	HE573139	HE573089	Schniebs *et al*. 2011
Radix balthica 5	Moll 52663	Germany, Baden-Württemberg, river Danube near Sigmaringendorf, 48°03'45.54'' N 09°15'49.36''E	HE573120	HE573080	Schniebs *et al*. 2011
Radix balthica 6	Moll 52907	Germany, Saxony: pond Rummelteich southwest of Trebsen, R 4552057 H 5683105	LS974275	LS974227	Schniebs *et al*. 2019
Radix balthica 7	Moll S135	France, Region Centre, Thenay, small creek N 47°23'22'' E 01°17'31''	HE573130		Schniebs *et al*. 2011
				LR732159	This paper
Radix balthica 8	Moll S147	France, Merignac near Bordeaux, N 45°47'20.96'' E 01°10'04.79''	HE573168		Schniebs *et al*. 2011
				LR732166	This paper
Radix balthica 9	Moll S1743	Spain, Mallorca, Serra de Tramuntana, La Granja de Esporles, sintered spring streams	LR732144	LR732160	This paper
Radix balthica 10	Moll S2151	Germany, Mecklenburg-Western Pomerania, lake Torgelower See, N 53°34.252' E 12°46.622'	HE573159	HE573096	Schniebs *et al*. 2011
Radix balthica 11	Moll S2174	Germany, Mecklenburg-Western Pomerania, lake Tiefwarensee, N 53°32.332' E 12°41.258'	HE573160	HE573097	Schniebs *et al*. 2011
Radix balthica 12	Moll S2825	Sweden, Fårö, shore of the Baltic Sea	LR732145	LR732165	This paper
Radix balthica 13	Moll S2827	Sweden, Gotland, shore of the Baltic Sea near Lickershamn	LR732146	LR732164	This paper
Radix balthica 14	Moll S4443	Italy, Sardinia, Province Nuoro, Gola su Gorrupu, Rio Flumineddo, N 40°11'07.14 E 9°30'08.57''	LR732147	LR732161	This paper
Radix balthica 15	Moll S5621	Germany, Bavaria, bank of the Lake Chiemsee in Chieming, N 47°53'46'' E 12°31'38''	LR732148	LR732162	This paper
Radix balthica 16	Moll S5795	Switzerland, Feldmeilen, bank oft he Lake Zurich, N 47°16'18.16'' E 08°37'40.46''	LR732149	LR732163	This paper
Radix balthica 17	Tissue coll. 9526	Portugal, Frades (8km west of Montalegre), Rio Cavado	LR732150	–	This paper
Radix balthica 18	Tissue coll. 9528	Portugal, Peso, (4km west of Melgaco), Rio Minho	LR732151	–	This paper

Table 1 (*Continued*)

Code	Collection No. SNSD	Locality	ENA/GenBank No. cyt-b	ITS-2	References
Radix balthica 19	Tissue coll. 9529	Portugal: Castrelos between Vinhais and Braganca	LR732152	–	This paper
Radix balthica 20	Tissue coll. 9530	Portugal: Aveleda near Braganca, Rio Calabor	LR732153	–	This paper
Radix Azores 1	Moll S7967	Portugal, Azores, São Miguel, Agua de Alto, Ribeira de Praia (creek), N 37°43.938' W 25°28.193'	LR732154	LR732167	This paper
Radix Azores 2	Moll S7968	Portugal, Azores, São Miguel, Agua de Alto, Ribeira de Praia (creek), N 37°43.938' W 25°28.193'	LR732155	LR732168	This paper
Radix Azores 3	Moll S7969	Portugal, Azores, São Miguel, Furnas, creek to the northwestern bank of the Lagoa das Furnas, N 37°45.990' W 25°19.996'	LR732156	LR732169	This paper
Radix Azores 4	Moll S7970	Portugal, Azores, São Miguel, Furnas, creek to the northwestern bank of the Lagoa das Furnas, N 37°45.990' W 25°19.996'	LR732157	LR732170	This paper

the specimens studied (Fig. 1). Genital organs were dissected and measured using stereo microscope (Nikon SMZ18). Photographs were taken with a digital camera system (Nikon DS-Fi2). Samples of tissue taken from the foot were fixed in absolute ethanol for analysis. They were registered in the tissue collection of the SNSD by assigning a tissue voucher number and a corresponding collection number in the mollusc collection of SNSD, and are stored at –80°C.

Characters examined from the four *Radix* specimens collected on São Miguel Island are: shell morphology, mantle pigmentation, shape and position of the bursa copulatrix, length and position of the bursa duct, and length ratio of praeputium to penial sheath.

Sequence data of the nuclear ITS-2 spacer and the mitochondrial cyt–b gene (329bp fragment) were obtained to exclude inconsistent results concerning the identification of the *Radix* specimens from São Miguel Island.

For the taxonomy of the freshwater molluscs used in the molecular genetic analyses, we followed the current European checklists (Falkner *et al*., 2001; Bank, 2011).

Molecular techniques and sequence analyses

For the molecular analyses, we obtained sequence data of the nuclear ITS-2 spacer (partial, with 28S ribosomal RNA gene, partial sequence), which is 349 bp long in *Radix labiata* and up to 489 bp in *Lymnaea stagnalis* (the length of the ITS-2 spacer varies within genera and families) and a 329 bp fragment of the mitochondrial cyt–b gene.

For primers and protocols of DNA extraction, PCR sequencing, see Schniebs *et al*. (2011). All DNA-sequences have been placed in the European Nucleotide Archive (ENA, see http://www.ebi.ac.uk/ena/), available also from GenBank (Table 1).

We analysed the new sequences together with published sequences from our earlier publications (Schniebs *et al*. 2011, 2013, 2018, 2019; Vinarski *et al*. 2011, 2016). For outgroup comparison, we used the Palaearctic species *Lymnaea stagnalis* (Linnaeus, 1758) belonging to the same freshwater gastropod family Lymnaeidae Rafinesque, 1815. We included sequences of *Radix auricularia* (Linnaeus, 1758), *R. dolgini* (Gundrizer & Starobogatov, 1979), *R. labiata, R. lagotis, R. balthica,* and *R. ampla* (Hartmann, 1821) in the ingroup for inter- and intraspecific comparisons.

Alignments were performed using the sequence alignment editor BioEdit (Hall, 1999). The ITS-2 alignment was obtained using the Clustal algorithm of MEGA4 (Tamura *et al*., 2007)

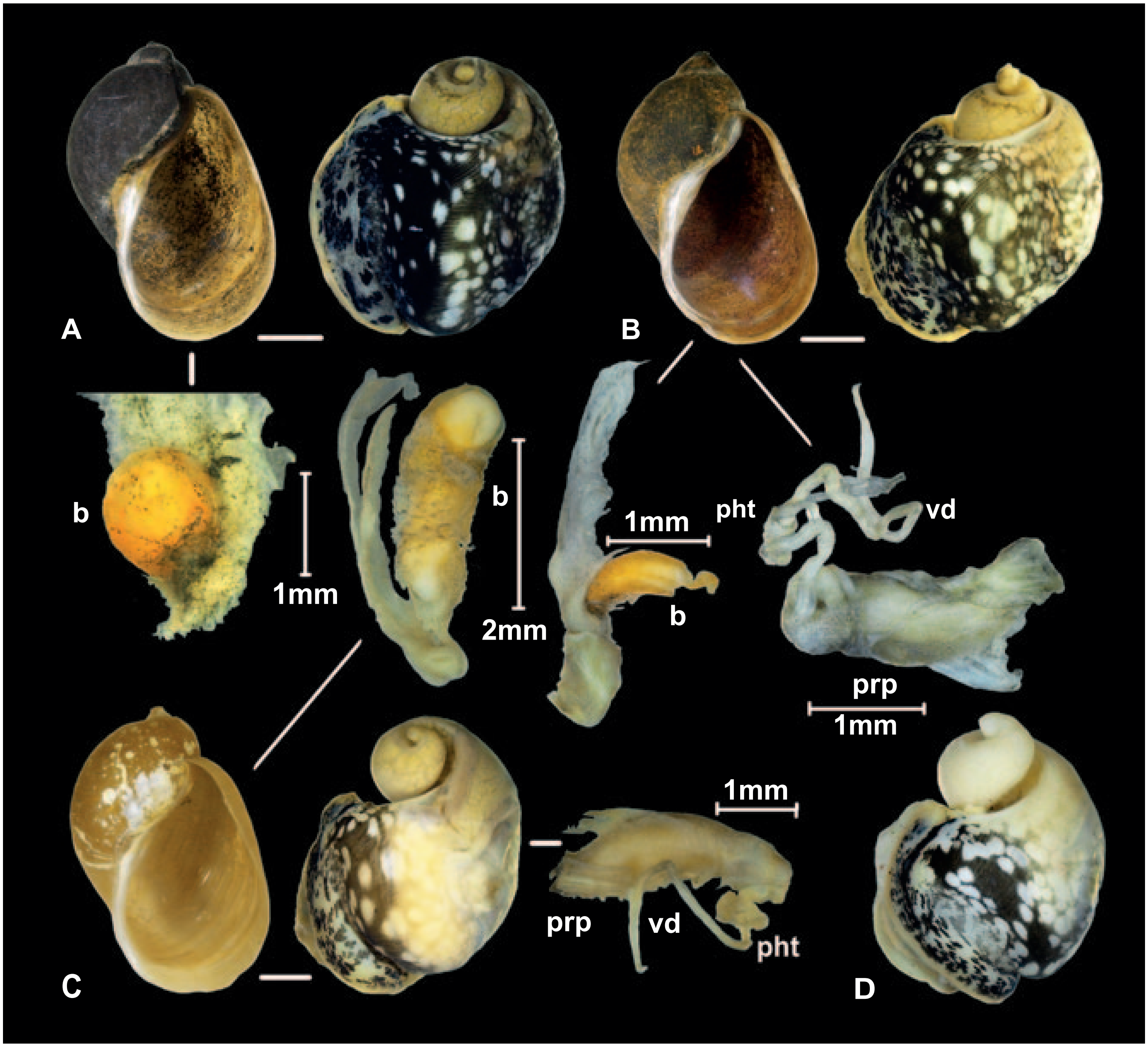

Figure 1 Shells, mantle pigmentation and anatomy of the *Radix balthica* specimens from São Miguel Island (Azores). **A, B**: Specimens from the creek Ribeira de Praia, SNSD Moll S7967 (*Radix* Azores 1) and SNSD S7968 (*Radix* Azores 2); **C, D**: specimens from a creek to the northwestern bank of the Lagoa das Furnas, SNSD Moll S7969 (*Radix* Azores 3) and SNSD S7970 (*Radix* Azores 4). Abbreviations: b – bursa copulatrix, pht – phallotheca, prp – praeputium, vd – vas deferens.

and improved by eye. For post-alignment editing, see Schniebs *et al*. (2017). MEGA 4 was also used to check the mitochondrial sequences for stop codons. We chose an analytical approach under the maximum parsimony (MP) criterion to be able to include the gap code information. Losing this information by analysing under distance or maximum likelihood criteria would mean losing the greatest part of the phylogenetic signal. The phylogenetic analyses for the ITS-2 spacer and cyt–b fragment were carried out using PAUP (version 4.0b10; Swofford, 2002; settings: gapmode=NewState, addseq=closest, maxtree=10000; number of bootstrap replicates=10000). For presentation of the MP results for ITS-2 and cyt–b, one of the 10000 and 2613 best trees respectively was chosen to be able to illustrate branch lengths (a tree showing the same overall topology as the majority rule consensus tree was chosen).

RESULTS

Morphology

Shell The two specimens collected in the creek Ribeira de Praia have complete, thin-walled conical ovoid shells of black to brownish-black colour with three whorls (Fig. 1A, B). The height of the shell ranges from 10.3 to 10.8mm and the height of the aperture from 8.2 to 9.3mm.

The shells of the specimens from the creek at the northwestern bank of the Lagoa das Furnas are both very fragile, light brown conical egg-shaped and the first two whorls were eroded (Fig. 1C). The height of the two shells could therefore not be measured. The height of the aperture ranges from 5.3 to 7.8mm. The shell of the smaller specimen with the collection number SNSD Moll S7970 broke during extraction of the soft body.

In the shells from both localities the aperture slopes steeply downwards from the upper approach (Fig. 1A, B, C).

Mantle pigmentation The mantle pigmentation of the four specimens sequenced shows a broad polymorphism (Fig. 1). Even the individuals collected in the same creek show a different pigmentation. The two specimens from the creek Ribeira de Praia (Fig. 1A, B) have a deep bluish black or yellowish black mantle with numerous distinct bluish white or yellowish white spots of various sizes. The number of irregular bluish black or black patches on the mantle collar varies from a few on a bluish grey background (Fig. 1A) to many on a greyish yellow green background (Fig. 1B). The mantle edge is yellowish grey in both specimens.

One specimen from the creek to the northwestern bank of the Lagoa das Furnas (Fig. 1C) has a relatively light mantle pigmentation from yellowish grey and yellow with a small number of yellowish blotches. The light yellowish grey mantle collar shows numerous irregular grey or greyish black patches. The mantle edge is yellowish grey. The mantle pigmentation of the other specimen (Fig. 1D) shows more and smaller white spots on greyish black. The bluish grey mantle collar also has numerous irregular patches, but in black. The mantle edge is greyish yellow green.

Male genitalia Measurements of praeputium and phallotheca could only be taken in two specimens (Fig. 1 B,C). The ratio was 0.96 and 1,2. The male genitalia are pigmented bluish grey green (Fig. 1B) or yellowish grey (Fig. 1C).

Bursa copulatrix The bursa copulatrix could be examined in three specimens only. The shape of the bursa was very variable, from nearly spherical (Fig. 1A) to elongate (Fig. 1C). The bursa duct was not visible (Fig. 1B) or very short (Fig. 1A, C). In all three specimens examined, the bursa duct entered on the ventral side into the provagina above the female vent.

Molecular phylogeny

The maximum-parsimony (MP) tree of the mitochondrial cyt–b marker (tree length=319, CI=0.6207, RI=0.8905) is illustrated as one of the best 2613 trees in Fig. 2. These phylogenetic relationships of the four *Radix* specimens from two creeks on São Miguel Island hypothesize them in one cluster of 98% bootstrap support together with 20 *R. balthica* specimens from different locations in the Palaearctic. Although basal branches have less than 59% bootstrap support, the clades of the species themselves have full or nearly full support (99%) in most cases except for *R. labiata* (86%) and *R. ampla* (65%).

We found no matches of the two haplotypes obtained from the four *R. balthica* specimens collected on São Miguel Island with that of four *R. balthica* specimens from different sampling sites in Portugal (*Radix balthica* 17–20). One haplotype (*Radix* Azores 4) even matches that of a specimen (*Radix balthica* 11) from Germany (Mecklenburg-Western Pomerania).

The hypothesis of phylogenetic relationship of the four *Radix* specimens from São Miguel Island based on the nuclear marker ITS-2 (tree length=789, CI=0.8580, RI=0.9575) is illustrated as one of the 10000 best maximum parsimony (MP) trees in Fig. 3. As in the trees of the mitochondrial marker they group together with the 20 *R. balthica* specimens included in this analysis. Most of the basal branches show very low support. This very low support effectively leads to a polytomy of *R. lagotis*, *R. ampla*, and *R. balthica*. The clades of the other species show 82% bootstrap support for *R. auricularia*, 100% for *R. dolgini*, and 87% for *R. labiata*.

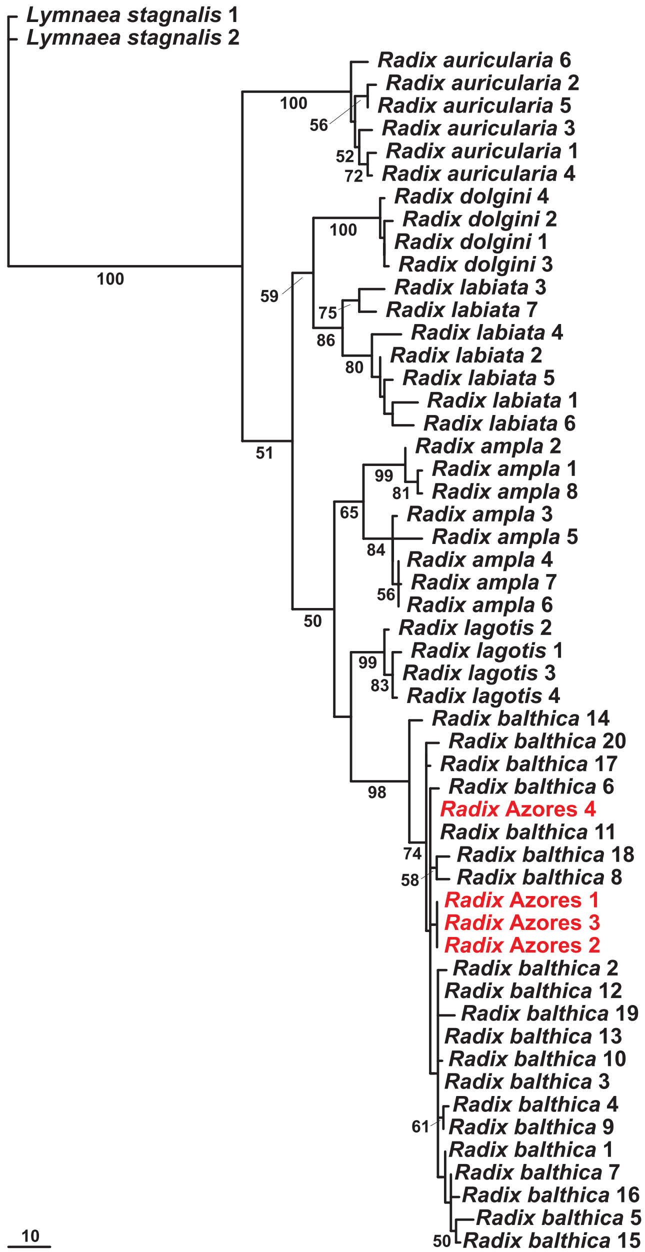

Figure 2 Hypothesis of species affiliation of the four *Radix* specimens from São Miguel Island (Azores) based on one of the 2613 best maximum-parsimony trees of the mitochondrial marker cyt–b (fragment of 329 bp) (tree length=319, CI=0.6207, RI=0.8905). The overall topology corresponds to that of the strict consensus tree. Branch lengths are proportional to the number of substitutions. Bootstrap support values above 50% are reported below nodes. The *Radix* specimens from São Miguel Island are marked red.

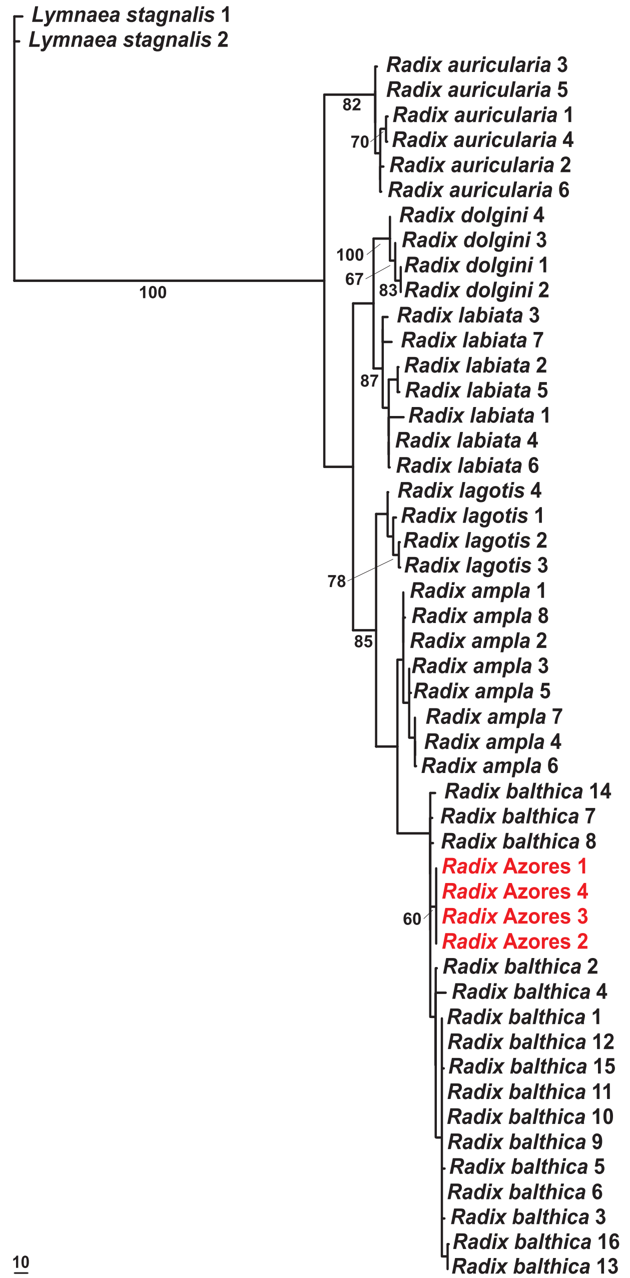

Figure 3 Hypothesis of species affiliation of the four *Radix* specimens from São Miguel Island (Azores) based on one of the 10000 best maximum-parsimony trees of the nuclear marker ITS-2 (tree length=789, CI=0.8580, RI=0.9575). The overall topology corresponds to that of the strict consensus tree. Branch lengths are proportional to the number of substitutions. Bootstrap support values above 50% are reported below nodes. The *Radix* specimens from São Miguel Island are marked red.

Discussion

Morphology

Shell The shells of the four analysed specimens do not reach the maximum height (13.2mm) and maximum width (9.6mm) reported by Backhuys (1975).

The shapes of the shells of the two specimens analysed from the creek Ribeira de Praia (Fig. 1A, B) as well as from the two specimens from the creek on the northwestern bank of the Lagoa das Furnas (Fig. 1C) are similar to the description and the figure in Backhuys 1975 (p. 63 and Fig. 40) but the aperture slopes more steeply downwards from the upper approach (Fig. 1A, B, C) than that in the shell in Backhuys' Fig. 40. Shells with such an aperture have been reported from specimens of *R. balthica* analysed by molecular genetics from Switzerland (Schniebs *et al*. 2011, their Fig. 4, shells 7 and 9).

Unfortunately, Backhuys (1975) did not provide a description of the mantle pigmentation and the genitalia of the specimens he collected.

Mantle pigmentation The mantle pigmentation is different in each of the four specimens studied (Fig. 1). It even differs in the specimens collected in the same creek (Fig. 1 A, B and C, D). Similar patterns of mantle pigmentations were found for *R. balthica* from Gemany (Schniebs *et al*. 2011, their Fig. 5) as well as for *R. ampla* (Schniebs *et al*. 2019, their Fig. 2).

Male genitalia With 0.96 and 1,2 the ratio of praeputium and phallotheca of the two specimens analysed is within the variability found in *R. balthica* by Schniebs *et al*. (2011), but also within the variability found in *R. labiata* (Schniebs *et al*. 2013) and *R. ampla* (Schniebs *et al*. 2019).

Bursa copulatrix A similar shape of the bursa as well as similar lengths of the bursa duct could be found in *R. balthica* (Schniebs *et al*. 2011, their Fig. 7, specimens 2 and 7) as well as in *R. ampla* (Schniebs *et al*. 2019, their Fig. 5D).

Each morphological character (shell, mantle pigmentation, ratio of praeputium and phallotheca, shape of the bursa and length of the bursa duct) taken alone does not allow any reliable conclusions about the species affiliation. But the combination of the individual features suggests a designation to *R. balthica*. Only a molecular genetic analysis can, however, yield certainty.

Molecular phylogeny
The molecular genetic analyses of the mitochondrial marker cyt–b (Fig. 2) and the nuclear ITS-2 spacer (Fig. 3) show that the four Radix specimens from two creeks on São Miguel Island group together with the R. balthica specimens included. We thus conclude that the four specimens belong to R. balthica. The two specimens from the creek Ribeira de Praia (Radix Azores1 and Radix Azores 2) have the same cyt–b haplotype as the specimen Radix Azores 3 from the creek to the northwestern bank of the Lagoa das Furnas (Fig. 2).

Biogeography
The Azores are purely oceanic island of volcanic origin that at some period in the Miocene arose above the level of the sea (Backhuys 1975). That is why these islands have received their fauna and flora by accidental transport (birds, driftwood etc.) and by human activity in the last few centuries (Backhuys 1975). The malacofauna of the Azores contains Holarctic, Nearctic, Palaearctic as well as Macaronesian elements (Backhuys 1975). In the opinion of Waldén (1984) the depauperate malacofauna of the Azores is probably a result of Pleistocene cooling. Although Backhuys (1975) realized that "nothing can be said with certainly about the exact history of the colonization by land and freshwater molluscs" (page 288) he considered *R. balthica* as introduced by man as well as *Galba truncatula* (O. F. Müller, 1774), another representative of the family Lymnaeidae. Raposeiro *et al*. (2012) described the introduction of *G. truncatula* as accidental by sheep from mainland Portugal. This also appears to be the most likely way of introduction of *R. balthica*, but introduction by Palaearctic migratory water birds occurring on the Azores in autumn and spring or during the winter time (Barcelos *et al*. 2015) cannot be excluded.

The facts that there are no matches of the two haplotypes obtained from the four *R. balthica* specimens collected on São Miguel Island with that of four *R. balthica* specimens from different sampling sites in Portugal (*Radix balthica* 17–20) as well as that one haplotype (*Radix* Azores 4) even matches that of a specimen (*Radix balthica* 11) from Germany (Mecklenburg-Western Pomerania) could be estimated as confirmation of our previous results: The analysis of haplotype network relationship of mitochondrial cyt–b haplotypes showed no distinct correlation of genetic variability to the geographic distribution pattern in Palaearctic *R. balthica* (Schniebs *et al.* 2011).

CONCLUSIONS

This is the first species identification of *R. balthica* based on molecular genetics for the Azores.

Since some *Radix* species (*R. balthica*, *R. labiata*) were reported as a potential intermediate host of *Fasciola hepatica* (e.g. Bargues *et al*. 2003; Caron *et al*. 2007, 2014) in Europe, it is of veterinary and human medical importance to know exactly which species of *Radix* occurs on São Miguel Island.

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