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VERSITA

Radix spp.: Identification of trematode intermediate hosts in the Czech Republic

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Abstract

Lymnaeid snails of the genus *Radix* serve as intermediate hosts of some schistosomes and fasciolids. In Europe, delineation of species within the genus *Radix* is unresolved and, therefore, spectrum of snail hosts susceptible to trematode infections is under discussion. We used and compared three criteria for species delineation using snails collected at 43 localities. (a) Sequence analysis of ITS-2 rDNA disclosed that the collected snails belong to four species – *R. auricularia* (Linnaeus, 1758), *R. peregra* (Müller, 1774), *R. lagotis* (Schrank, 1803) and *R. labiata* (Rossmaessler, 1835) (criteria and names are based on the work of Bargues *et al*. 2001). Occurrence of *R. peregra* in the Czech Republic was confirmed by molecular data for the first time. (b) Characterization of reproductive system disclosed differences in location, size and shape of bursa copulatrix and its ductus. Unfortunately, some *R. labiata* specimens shared morphological features of reproductive organs with *R. lagotis*. (c) Statistical analysis of shell morphology proved that significant differences exist among particular species. One prediction model showed that correct classification of species may be achieved in 82–84% of cases. However, identification of individual snails in the field (without knowledge of respective snail population and use of statistical tools) still remains a complicated issue due to overlaps of shell characteristics. Concerning the role in trematode transmission, *R. lagotis*, *R. labiata* and *R. peregra* are susceptible to *Trichobilharzia regenti*. Also, successful experimental infections of *R. lagotis* and *R. labiata* by *Fascioloides magna* were accomplished.

Keywords

Lymnaeidae, *Radix*, *Trichobilharzia*, *Fascioloides*, trematodes, species delineation

Introduction

Freshwater snails of the genus *Radix* Montfort, 1810 (Lymnaeidae) are distributed worldwide. In the past, species determination was based on shell morphology (e.g., Jackiewicz 2000a), anatomy of reproductive system (Hubendick 1951; Jackiewicz 1998, 2000b; Glöer 2002; Vinarski and Glöer 2007; Vinarski 2009) and color of the mantle (Jackiewicz 1993), and these criteria are continuously used for description of new species (e.g. Kruglov and Staroboratov 1993, Glöer 2002, Glöer and Pešic 2008). Due to different emphasis on particular morphological characters several names for the same *Radix* species were introduced. Recently, molecular biology brought a new insight into the taxonomy of *Radix* snails (Bargues *et al*. 2001, Remigio 2002, Pfenninger *et al*. 2006, Correa *et al*. 2010).

Conchological characters for species delineation in *Radix* include shape of the shell, shape of the shell mouth, ratio of the shell and mouth heights, form of whorls, etc. (Uličný 1892, Hubendick 1951, Ložek 1956, Glöer and Meier-Brook 1998, Glöer 2002). According to some authors, however, these parameters are influenced by environmental conditions. For example, the shape of the shells changed in offspring generations of snails collected in the field and bred under laboratory conditions (Wullschleger and Jokela 2002, Pfenninger *et al*. 2006), and is also subject to change during snail development (Ložek 1956). In central Europe, *R. auricularia* (Linnaeus, 1758), *R. peregra* (Müller, 1774), *R. ovata* (Draparnaud, 1805), *R. lagotis* (Schrank, 1803) and *R. ampla* (Hartmann, 1821) have been recognized according to shell morphology (Uličný 1892, Ložek 1956, Glöer and Meier-Brook 1998). Some authors (Bargues *et al*. 2001) state that *R. peregra sensu*

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Table I. Sampled localities and summary of morphological and molecular examinations

Snail species (based on ITS-2 sequences)	GenBank accessions numbers	Locality	Number of collected/ dissected/ sequenced snails	Dissection of gonads (type)	Coordinates of locality
R. auricularia	GU574297, GU574301, GU574302	Červený pond 1	40/5/3	A	49042´19.26″N, 1406´25.27″E
R. auricularia	GU574317, GU574320, GU574321	Červený pond 2	51/5/3	A	49042´20.73″ N,1406´29.99″E
R. auricularia	GU574285	Brdy, pond3	23/5/1	A	*
R. auricularia	GU574306, GU574310, GU574311, GU574312	Prager creek	42/5/4	A	5000´38.69″N, 14030´35.17″E
R. auricularia	GU574316	Labutí pond	2/1/1	A	49052´27.96″N, 12043´7.48″E
R. auricularia	GU574323, GU574318, GU574319	Bonětický pond	54/5/3	A	49040´3.49″N, 12048´30″E
R. auricularia	GU574298, GU574299, GU574300	Lhotka pond	6/3/3	A	48051´26.38″N, 14041´31.81″E
R. auricularia	GU574303, GU574304, GU574305	Velký Klínský pond	28/5/3	A	*
R. auricularia	GU574294, GU574295, GU574296	Linda pond	105/5/3	A	48053´36.72″N, 14040´15.45″E
R. auricularia	GU574313, GU574314, GU574315	Kunžak pond	13/4/3	A	4907´1.66″N, 15011´54.89″E
R. auricularia	GU574286, GU574287, GU574288	Pětidomý pond	237/5/3	A	49050´19.13″N, 13046´50.44″E
R. auricularia	GU574277, GU574289, GU574290	Cekovský pond	234/5/3	A	49049´9.21″N, 13045´27.86″E
R. auricularia	GU574307, GU574308, GU574309	Budkovan pond	15/5/3	A	49019´50.79″N, 16046´33.99″E
R. auricularia	GU574322	Vondra pond	3/1/1	A	4905´9.94″N, 15025´18.15″E
R. auricularia	GU574291, GU574292, GU574293	Protivský pond	32/5/3	A	*
R. labiata	GU574234, GU574251, GU574255	Poněšice-Bedrná 81 (pond)	20/3/3	B	*
R. labiata	GU574262, GU574269, GU574270	Poněšice-Bedrná 84 (pond)	15/3/3	B	*
R. labiata	GU574244, GU574245	Poněšice-Bedrná (pond)	18/2/2	B	*
R. labiata	GU574246, GU574247, GU574248, U574249	Poněšice,spring of the Černý creek	38/5/4	B	*
R. labiata	GU574250, GU574252, GU574253	Poněšice, Novoborský seník pond	36/5/3	B	*
R. labiata	GU574241, GU574243	Laboratory strain, Faculty of Science, České Budějovice	20/2/2	B	48058´41.58″N, 14026´47.26″E
R. labiata	GU574239, GU574240	Boletice 6 (pond)	12/2/2	B	*
R. labiata	GU574258, GU574259, GU574260, GU574261	Sedliště S1(creek)	26/5/4	B	*
R. labiata	GU574263, GU574264, GU574265	Sedliště S3 (creek)	23/5/3	B	*
R. labiata	GU574279	Poněšice – Libochovka (pond)	2/1/1	B	4904´46.18″N, 14029´9.01″E
R. labiata	GU574236, GU574237, GU574238	Poněšice (pond)	23/4/3	B	*
R. labiata	GU574266, GU574267, GU574268	Military area Brdy, Octárna creek	65/5/3	C	5004´20.08″N, 14025´27.03″E
R. labiata	GU574271, GU574276	Military area Brdy, Octárna pool	5/2/2	C	5004´20.08″N, 14025´27.03″E
R. labiata	GU574235, GU574242, GU574280	Poněšice - Bedrná 83 (pond)	31/5/3	C	49024´32.65″N, 13047´37.32″E
R. labiata	GU574254, GU574256, GU574257	Boletice 12 (pond)	24/5/3	C	49024´43.11″N, 13049´12.06″E
R. labiata	GU574272, GU574273, GU574274, U574275	Suchá Rudná creek	20/5/4	C	49042´56.37″N, 13055´32.63″E
R. labiata	GU574281	Pasečná pond	29/4/1	C	49043´0.59″, 13055´35.12″E

R. labiata	GU574278, GU574282, GU574283, GU574284	Krásná Lípa, reek	42/5/41	C	*
R. lagotis	GU574227, GU574228, GU574229	Laboratory strain (2), Faculty of Science, Prague	15/4/3	B	*
R. lagotis	GU574230, GU574231, GU574232	Laboratory strain (1), Faculty of Science, Prague	15/4/3	B	5003´47.73´N, 17021´43.47´E
R. lagotis	GU574233	Podkadovský pond	5/1/1	B	48036´35.66´N, 1406´15.08´E
R. lagotis	GU574224, GU574225, GU574226	Žoldánka pond	86/5/3	B	50054´30.14´N, 14030´5.55´E
R. peregra	GU574218, GU574219, GU574220, GU574221, GU574222, GU574223	Úpa (Mladé Buky), river	12/6/6	D	50036´7.62´N, 15050´25.93´E
R. peregra	GU574212	Opnur - Iceland	6/2/2	x	**
R. peregra	GU574213, GU574214	Osland - Iceland	5/2/2	x	**
R. peregra	GU574215, GU574216	Kriutjörn - Iceland	Kriutjörn - Iceland	x	**
R. peregra	GU574217	Faunty Porde - Iceland	5/2/2	x	**

Columns contain results of sequencing, GenBank accessions numbers, locality names, numbers of collected/dissected/sequenced snails, results of dissections, locality coordinates. Abbreviations: A, B, C, D – morphological types of gonads; x –data not available; *military area, coordinates were not available; **coordinates were not recorded.

Müller (1774) is synonymous with *R. balthica* (Linnaeus, 1758) and *R. ovata*.

Snails of the genus *Radix* are hermaphrodites. For species determination within the genus *Radix*, the shape of bursa copulatrix and the length of its ductus are important (Hubendick 1951, Jackiewicz 2000b, Glöer 2002), e.g., *R. peregra* has a pyriform bursa copulatrix and short ductus (Jackiewicz 2000b).

In molecular phylogenetic analyses of lymnaeid snails the following markers are used: within the rDNA operon, the 18S gene, ITS-1 and ITS-2, and within the mtDNA genome, the 16S and *cox*1 (e.g., Mas-Coma *et al*. 2009, Correa *et al*. 2010, Pfenninger *et al*. 2011); also microsatellites have been analyzed (Salinger and Pfenninger 2009). Due to its variability, the ITS-2 region was recommended as one of the most relevant markers for establishment of valid species within the family Lymnaeidae (Mas-Coma *et al*. 2009). Unfortunately, morphological and molecular analyses are not always in accord and, therefore, further comparative studies focused on morphological and molecular variability of particular species are desirable.

Besides being a zoological problem, species delineation of *Radix* snails has a practical impact on parasitology. Particular species of the genus *Radix* may differ in their susceptibility to medically and/or veterinary important trematodes belonging to bird schistosomes (family Schistosomatidae, especially the genus *Trichobilharzia* Skrjabin et Zakharow, 1920) (Horák *et al*. 1998, 2002; Ferte *et al*., Horák and Kolářová 2011) and fasciolids (family Fasciolidae, especially the genus *Fascioloides* Ward, 1917) (Dreyfus *et al*. 1997, Faltýnková *et al*. 2006, Caron *et al*. 2007, Lotfy *et al*. 2008, Mas-Coma *et al*. 2009). Only susceptible snails play a role in parasite transmission and have impact on epidemiological/epizootiological situation.

Worldwide distributed bird schistosomes of the genus *Trichobilharzia* cause cercarial dermatitis (swimmer´s itch) in humans, an allergic reaction against larvae penetrating the human skin (Kolářová *et al*. 1997, Horák *et al*. 2002). Cercarial dermatitis is considered to be a re-emerging disease, causing severe problems in recreational areas in all around the world (Horák and Kolářová 2011). *Fascioloides magna* (Bassi, 1875) was originally distributed in North America, but recently has spread in Europe and its veterinary importance is growing also in the Czech Republic (Novobilský *et al*. 2007).

There were two main aims of our contribution: (a) apply different tools (molecular and morphological methods) for identification of specimens of the genus *Radix* collected in the Czech Republic; (b) perform infection experiments and assess susceptibility of selected *Radix* isolates to sympatric schistosomes and fasciolids.

Materials and methods

Collection and processing of snails

During the years 2005–2008, snails representing the genus *Radix* were collected at 39 localities in the Czech Republic; in

addition DNA of four isolates of *R. peregra* from Icelandic localities was analyzed for comparative purposes. All specimens of *R. peregra* analyzed in this study were *R. peregra sensu* Müller (1774). The number of collected, dissected and sequenced snails is presented in Table I. Snails having shell height less than 10 mm were not sampled, because their shells and gonads were not fully developed. Snails were fixed in 70% ethanol. Each snail was taken from ethanol and placed on a separate Petri dish. The soft body was removed from the shell and, subsequently, the reproductive system was analyzed (see below). The empty shell was marked by a number and stored for later determination based on shell characteristics. A small piece of the tissue (posterior part of foot and anterior part of mantle) was cut for later DNA analyses; these samples were post-fixed in 96% ethanol.

ITS-2 sequencing

DNA extraction was performed using Qiamp DNA MiniKit (Qiagen) according to manufacturer instructions. Concentration and purity of DNA were evaluated by using ND-1000 V3.7 (NanoDrop) spectrophotometer. Extracted DNA was stored at –20°C. Polymerase chain reaction (PCR) was performed in 25 µl volumes. The PCR solution was composed of 2.5 µl 10× NH_4 Reaction Buffer (Bioline), 1 µl 50 mM $MgCl_2$ (Bioline), 2 µl 2.5 mM dNTP (Bioline), 1µl Taq polymerase (BioTaq Red DNA Polymerase, concentration 1U/µl, Bioline), 0.5 µl 10 µM primer NEWS (5´-TGTGTCGATGAAGAACGCAG-3´; Almeyda-Artigas *et al*. 2000), 0.5 µl 10 µM primer RIXO (5´-TTCTATGCTTAAATTCAGGGG-3´; Almeyda-Artigas *et al*. 2000), 50 ng DNA and deionized water. Amplification was performed according to Bargues *et al*. (2001) and PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen). Concentration and purity of the samples were evaluated using ND-1000 V3.7 spectrophotometer. Purified PCR products were sequenced using primers NEWS and RIXO, and 3130 Genetic Analyzer (Applied Biosystems).

Alignments containing ITS-2 sequences and sequences downloaded from GenBank were obtained using the ClustalW algorithm implemented in BioEdit (Hall 1999) and manually refined. Phylogenetic trees were constructed in PAUP 4.0 (Swofford 1998) by neighbor-joining method (bootstrap with 1000 repetitions). ITS-2 sequences were deposited in GenBank under accession numbers GU574212-GU574323.

Dissection of snail reproductive system

Only adult snails were used. Observation was focused only on anatomy of the female part of the reproductive system, as there are the most important determination characteristics. The following two criteria were selected: (1) shape and location of bursa copulatrix with respect to corpus pyriforme, (2) length of ductus of bursa copulatrix. Examining the soft body of snails, the anterior part of the mantle was removed under the stereo microscope and female reproductive organs were uncovered. From corpus pyriforme, a thin coat of lacunary system covering the surface of bursa copulatrix and its ductus was removed. Bursa copulatrix and its ductus were described and photographed.

Morphology of shells

The following parameters were measured by caliper: height (HS) and width (WS) of the shell, height (HA) and width (WA) of the aperture. From these absolute values three ratios (HS/HA, WS/WA, HS/WS) and their reciprocal values describing proportions of the shell were calculated. Importance of the above parameters for species delineation was assessed by statistical methods (Kruskall-Wallis test) using SPSS program, version 16. For statistical evaluation, in some cases, also auxiliary ratios (e.g. HA/WA) were calculated. In addition, prediction systems using (a) absolute values, (b) their ratios and (c) logarithmic values were proposed; these prediction systems were based on data obtained by canonical discriminant analysis of the samples.

Infection experiments

Three species of the genus *Radix*, namely *R. lagotis*, *R. peregra* and *R. labiata* (Rossmaessler, 1835), were tested as possible intermediate hosts of *T. regenti* Horák, Kolářová et Dvořák, 1998 and *F. magna*. *Radix labiata* and *R. peregra* were collected in Czech water bodies, kept under laboratory conditions, their infection-free F1 progeny molecularly characterized/identified (as described above) and used for infection experiments. *Radix lagotis* was maintained under laboratory conditions for several years. For all three species, individual snails of 3–5 mm in length were exposed to 3–20 miracidia for 4–5 hours (Table V), kept for several weeks in aquaria, fed *ad libitum*, and subsequently examined for infection (larvae developing in hepatopancreas).

Miracidia of *T. regenti* were obtained from ducks infected by cercariae of the laboratory maintained strain of *T. regenti* (Kolářová *et al*. 2010). Eggs of *F. magna* were isolated from liver of wild game (*Cervus elaphus* Linnaeus, 1758); they were incubated at 30°C for 14 days when first miracidia started to hatch. Viability of miracidia and ability to infect snails were tested with the already proven intermediate hosts of *T. regenti* and *F. magna*, *R. lagotis* and *Pseudosuccinea columella* (Say, 1817), respectively.

Results

Species delineation based on ITS-2 sequences

ITS-2 sequences (n = 111) were obtained from 107 specimens collected at 42 localities (including those in Iceland). Sequences clustered into four different species: *R. auricularia*,

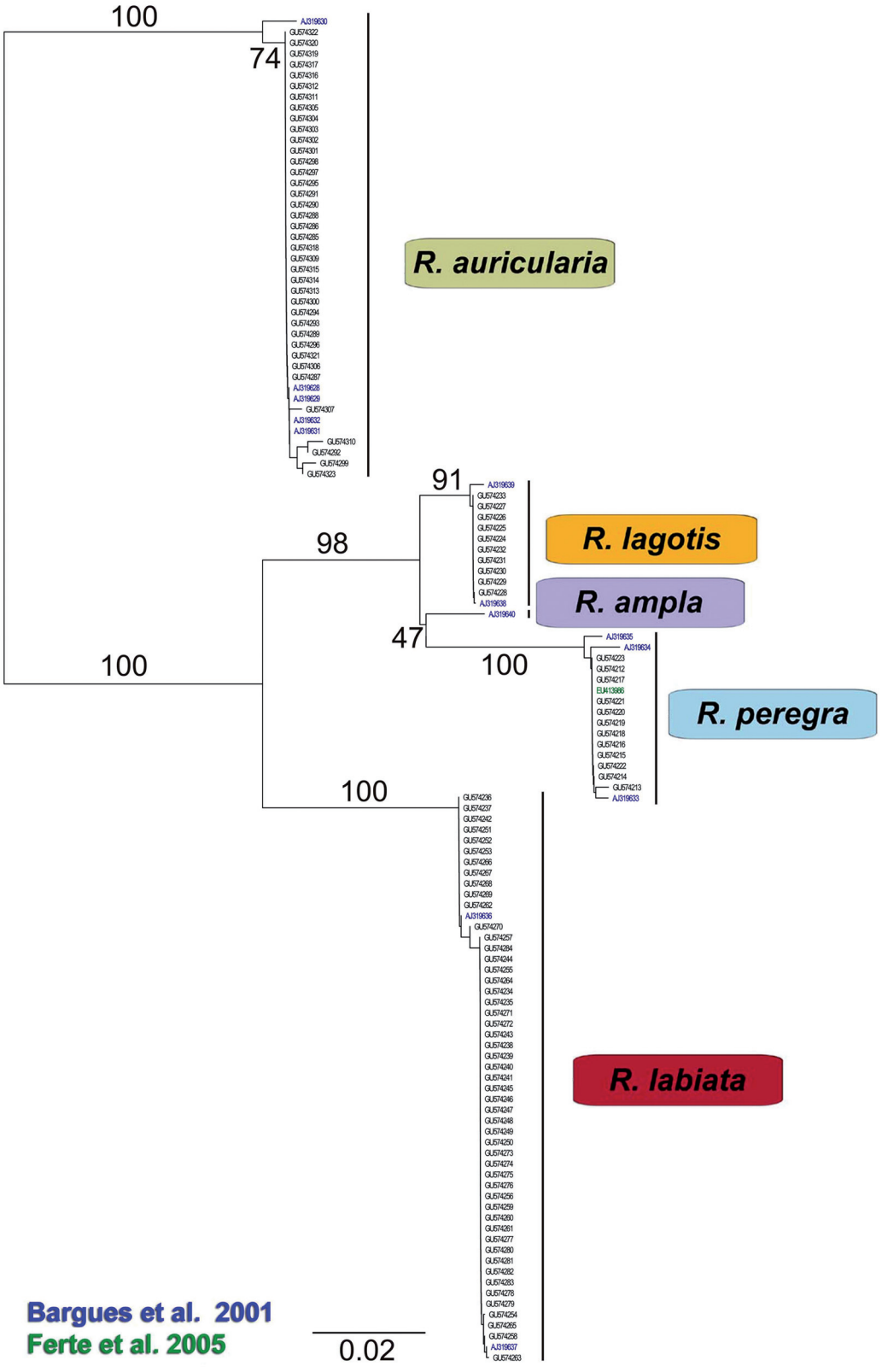

Fig. 1. Phylogenetic tree of the analyzed *Radix* spp. isolates; based on ITS-2 sequences. A close relation of *R. lagotis* and *R. peregra* (*sensu* Müller, 1774) is shown; *R. auricularia* seems to be the most separated species. GenBank accession numbers of sequences already published by other authors are in color corresponding with the color of citation. The branch with *R. ampla* is not supported by a bootstrap value, because it is based on only one sequence obtained from GenBank. The tree was constructed using Neighbor-joining method with LogDet distances; bootstrap with 1000 repetitions

R. lagotis, *R. peregra* and *R. labiata* (Fig. 1; names used according to Bargues *et al*. 2001). Particular sequences of *R. auricularia*, *R. lagotis*, *R. peregra* and *R. labiata* differed as shown in Table II. The highest level of sequence similarity was found between *R. lagotis* and *R. peregra* (and *R. ampla* not sampled in this study). On the other hand, *R. auricularia* seems

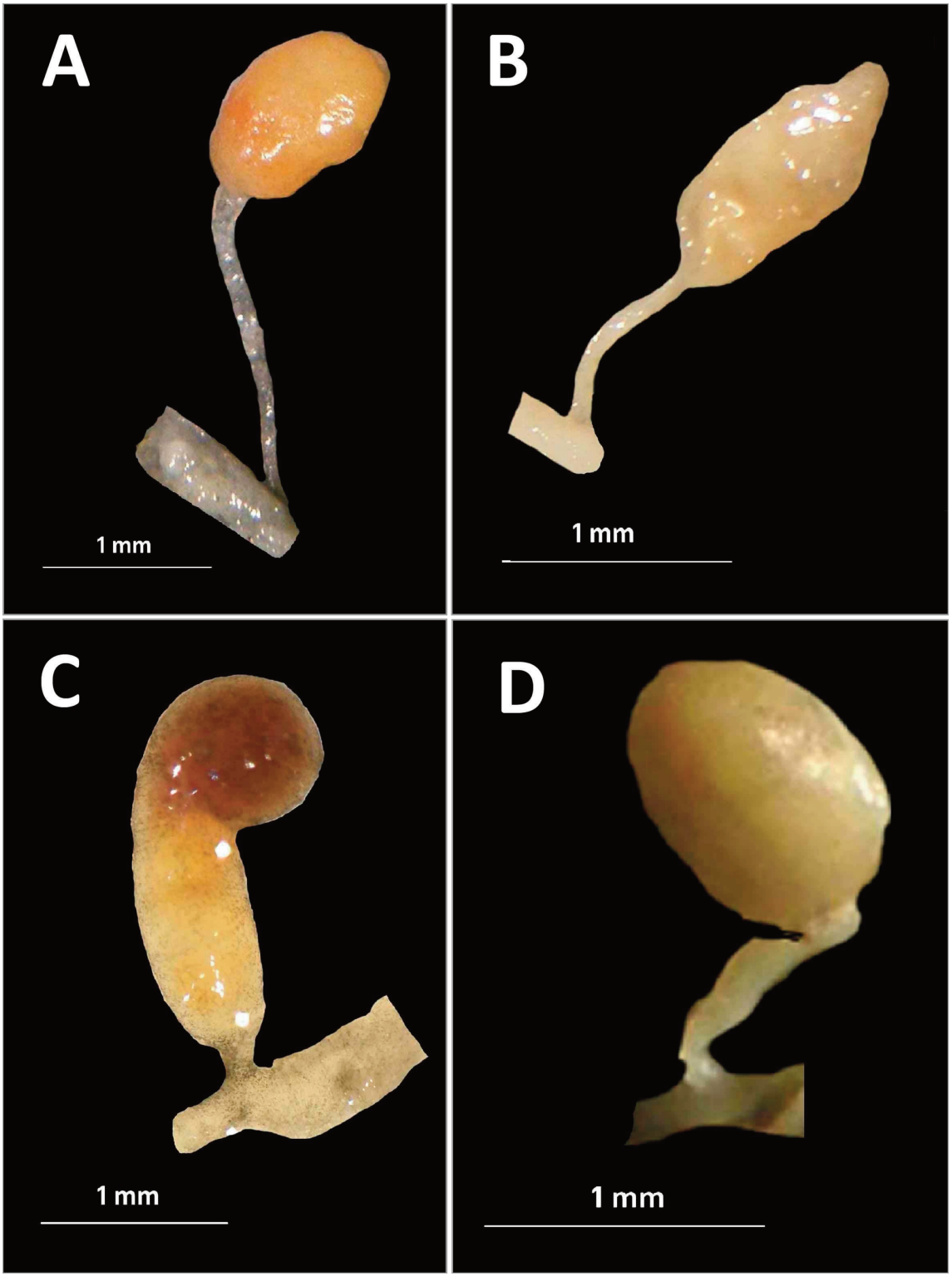

Fig. 2. Comparison of selected parts of the snail reproductive system. Shape and size of bursa copulatrix and ductus of bursa copulatrix is shown. **A** – *R. auricularia*, **B** – *R. lagotis*, **C** – *R. labiata* (type C), **D** – *R. peregra* (*sensu* Müller, 1774)

Table II. Evaluation of sequence variations between *Radix* species

	R. peregra	***R. lagotis***	***R. labiata***	***R. auricularia***
R. peregra	X	X	X	X
R. lagotis	0.034	X	X	X
R. labiata	0.087	0.070	X	X
R. auricularia	0.132	0.115	0.117	X

Differences are expressed as "p" distances, i.e. proportion of different nucleotides, between pairs of species. Every species was represented by its most common sequence variant – GU574322, GU574274, GU574233 and GU574220. Program PAUP was used for the calculation.

to be the most distinct species. *Radix peregra* from the Czech Republic and that from Iceland are practically identical; they differ in substitution of one base only.

Species delineation based on dissection of reproductive system

Altogether, 158 snails from 43 localities (including those in Iceland) were dissected; four morphological types (A-D) were recognized:

Type A – *R. auricularia* (Fig. 2A): Globular bursa copulatrix is variably colored (white, light pink, yellow) and situated aside the top of corpus pyriforme. Ductus is thin and very long, encircling corpus pyriforme. Mantle is black and yellow; in the posterior part, big light yellow spots occur on black background, while in the anterior part the yellow color dominates over small black spots.

Type B – *R. lagotis* and *R. labiata* (Fig. 2B): Pyriform bursa copulatrix is white or yellow, situated on the surface of corpus pyriforme, between vagina and prostate gland. Length of its ductus is between half and full length of bursa. Mantle is black with small or big yellowish spots.

Type C – *R. labiata* (Fig. 2C): Elongated bursa copulatrix is brown or yellow, situated on the surface of corpus pyriforme, ductus is shorter than half-length of bursa copulatrix. Mantle is black with small yellowish spots.

Type D – *R. peregra* (Fig. 2D): Globular bursa copulatrix is yellow, situated on the surface of corpus pyriforme under vagina. Its ductus is of the same length as bursa copulatrix. Mantle is black with large yellowish spots in the posterior part; the anterior part is rather yellow with small black spots.

Comparison of snails with regard to the morphology of reproductive system and ITS-2 sequences revealed some over-

Table III. Evaluation of conchological parameters of *Radix* spp.

Ratio	Species	Mean	Standard deviation	N
HS/HA	*R. lagotis*	1.363	0.053	10
	R. auricularia	1.265	0.115	67
	R. labiata	1.524	0.084	63
	R. peregra	1.183	0.025	5
	Total	1.381	0.161	145
HS/WS	*R. lagotis*	1.736	0.077	10
	R. auricularia	1.550	0.143	67
	R. labiata	1.736	0.093	63
	R. peregra	1.517	0.130	5
	Total	1.643	0.151	145
WS/WA	*R. lagotis*	5.569	1.226	10
	R. auricularia	7.590	2.396	67
	R. labiata	3.636	0.480	63
	R. peregra	7.121	0.468	5
	Total	5.717	2.535	145

Mean values and standard deviations of ratios between selected shell values (measurements) demonstrate partial overlaps of conchological parameters of *Radix* spp. Descriptive statistics for ratios of shell values (measurements) was performed using SPSS program, version 16. Abbreviations: N – total number of analyzed specimens, HS/HA – ratio height of the shell/height of the aperture, HS/WS – ratio height of the shell/width of the shell, WS/WA – ratio width of the shell/width of the aperture.

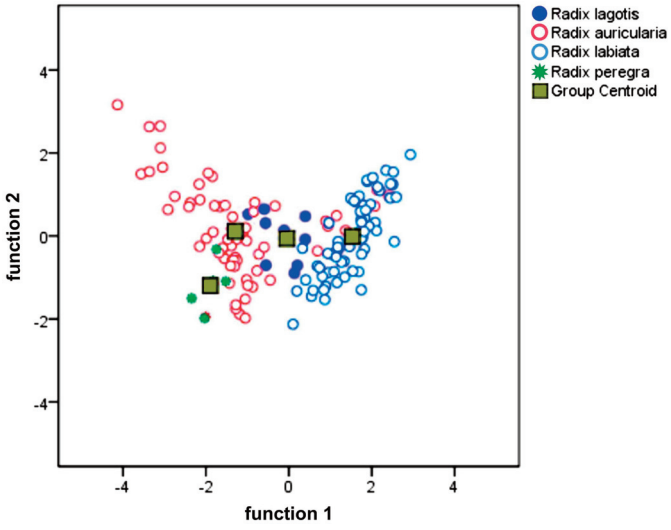

Fig. 3. Canonical discriminant analysis of differences in shell morphology within the genus *Radix*. Canonical discriminant analysis showed that the ratios HS/HA, WS/WA, HS/WS can be used as markers of species delineation within the genus *Radix*; clearly separated group centroids can be demonstrated. However, the values for some specimens overlapped, e.g., the values of *R. auricularia* and *R. peregra* (*sensu* Müller, 1774). Tests were performed using SPSS program, version 16. Abbreviations: HS – height of the shell, HA – height of the aperture, WS – width of the shell, WA – width of the aperture

laps: Snails belonging to *R. lagotis* had always one morphological type of reproductive system (type B, see above), while *R. labiata* had either type B or type C. The other species, *R. auricularia* and *R. peregra*, always corresponded to types A and D, respectively.

Species delineation based on shell morphology

A set of 145 shells from 39 Czech localities was used for species delineation based on shell morphology. Table III contains mean values of ratios between selected shell values (measures) for each species. Statistical evaluation (Kruskall-Wallis test) showed that particular species differed significantly in absolute values of four parameters (HS, HA, WS, WA; $p<0.001$), as well as in ratios of these parameters ($p = 0.029$ for HA/WA, $p<0.001$ for the remaining ratios). Canonical discriminant analysis showed that the ratios HS/HA, WS/WA, HS/WS can be used as markers of species delineation within the genus *Radix* (Fig. 3 shows clearly separated group centroids); nevertheless, the values for some specimens overlapped, e.g., the values of *R. auricularia* and *R. peregra* (Fig. 3).

Quality and practical use of these significant differences were further tested in prediction systems. The highest prediction success rate, direct as well as cross-validated (leave-one-out classification), was reached for the system using ratio values – 84.1% and 82.1% of correct classifications in direct and cross-

Table IV. Prediction system (direct and cross-validated) based on ratios of absolute shell values (basic ratios HS/HA, WS/WA, HS/WS, and auxiliary ratios HS/WA, HA/WA, HA/WS

			Classification of predicted group membership				
		Species	***R. lagotis***	***R. auricularia***	***R. labiata***	***R. peregra***	**Total**
Original	Count	*R. lagotis*	1	8	1	0	10
		R. auricularia	1	59	7	0	67
		R. labiata	1	0	62	0	63
		R. peregra	0	5	0	0	5
	%	*R. lagotis*	10	80	10	0	100
		R. auricularia	1.5	88.1	10.4	0	100
		R. labiata	1.6	0	98.4	0	100
		R. peregra	0	100	0	0	100
Cross-validated	Count	*R. lagotis*	1	8	1	0	10
		R. auricularia	0	58	9	0	67
		R. labiata	2	1	60	0	63
		R. peregra	0	5	0	0	5
	%	*R. lagotis*	10	80	10	0	100
		R. auricularia	0	86.6	13.4	0	100
		R. labiata	3.2	1.6	95.2	0	100
		R. peregra	0	100	0	0	100

Prediction system was based on data obtained by canonical discriminant analysis of the samples. Prediction success rates reached 84.1% and 82.1% of correct classifications in direct and cross-validated systems. *Radix auricularia* and *R. labiata* could in most cases be distinguished one from another. Tests were performed using SPSS program, version 16). Abbreviations: HS – height of the shell, HA – height of the aperture, WS – width of the shell, WA – width of the aperture

Table V. Infection experiments showing susceptibility/suitability of *Radix* spp. to *Fascioloides magna* and *Trichobilharzia regenti*

Parasite/snail species*	Number of exposed snails	Exposure to parasites (No. of miracidia/snail)	Examination (days post exposure)	Number and percentage of infected snails
Fascioloides magna				
R. lagotis	42	3–5	44	2 (4.8%)
R. lagotis	30	8–10	57	0
R. peregra	11	5–6	40	0
R. labiata	20	20	35	1 (5%)
Trichobilharzia regenti				
R. peregra	47	3–5	32	30 (64%)
R. labiata	81	3–4	35	72 (89%)
R. labiata	80	3–4	34	71 (89%)
*R. lagotis***	97	3–4	38	88 (91%)
*R. lagotis***	81	3–4	37	73 (90%)

*Snail identification was based on ITS-2 sequences (names according to Bargues *et al*. 2001). **For many years, *R. lagotis* served as a susceptible snail species for routine laboratory maintenance of *T. regenti*; however, the level of susceptibility (in %) was not identified before.

validated systems, respectively (Table IV). As shown, *R. auricularia* and *R. labiata* could in most cases be distinguished one from another. On the other hand, the same model produced poor results for less common snail species (e.g., *R. peregra*).

Infection experiments

Exposure of snails to miracidia of *T. regenti* and *F. magna* (Table V) showed that *R. peregra*, *R. lagotis* and *R. labiata* are susceptible to *T. regenti* and can serve as vectors (intermediate hosts) of this parasite under conditions of the Czech Republic. Infections of *R. lagotis* and *R. labiata* by *F. magna* proved that this liver fluke is able to infect the above two snail species and develop to the stage of rediae. Although the percentage of infected snails of both species was about 5%, the parasite was more developed in *R. labiata* where young cercariae inside rediae were observed.

Discussion

Spectrum of intermediate hosts (freshwater snails) needs to be identified in order to understand the modes of transmission of medically and veterinary important flukes. Therefore, we tried to determine species of the genus *Radix* occurring in the Czech Republic and serving as presumptive intermediate hosts of European schistosomes and fasciolids. The study incorporated comparison/determination of snails by three approaches – DNA sequence variation in ITS-2, morphology of reproductive system and shell morphometry.

As the ITS-2 region was used as a recommended marker for monitoring species spectrum within the family Lymnaeidae (Ferte *et al*. 2005, Mas-Coma *et al*. 2009), we also used it for delineation of snail species in this study. In the past, Bargues *et al*. (2001) analyzed lymnaeids from a large part of Europe (including 9 isolates from the Czech Republic); *R. labiata* was separated from the group of snails described generally as *R. peregra* in different countries, and populations referred to as *R. ovata*/*R. balthica* were synonymized with *R. peregra sensu* Müller (1774); in total, six valid European species were listed – *R. auricularia*, *R. ampla*, *R. peregra*, *R. labiata*, *R. lagotis* and *Radix* sp.

Radix peregra (Müller, 1774) and *R. ovata* (Draparnaud, 1805) are recently treated as junior synonyms of *R. balthica* (Linnaeus, 1758). However, the use of the latter name varies, and *R. peregra* is mentioned simultaneously (Lewin 2006, Muñoz-Antoli *et al*. 2007). Moreover, there is data that *R. ovata* and *R. peregra* are separate species, because juvenile growth rate and reproductive schedule under the same conditions differ (contrary to the convergence of shell shape) (Wullschleger and Jokela 2002), and snails from the sympatric location avoided maiting with the opposite species (Wullschleger *et al*. 2002). In this study we preferred the name of *R. peregra* (Müller, 1774) also for practical reasons, because GenBank records related to the crucial publication of Bargues *et al*. (2001) still refer to *R. peregra*.

In the present study, samples from 39 localities in the Czech Republic and 4 localities from Iceland (as comparative samples) were evaluated. Four species *sensu* Bargues *et al*. (2001) were recognized for the Czech Republic (*R. auricularia*, *R. lagotis*, *R. peregra* and *R. labiata*); the most common species among our samples were *R. auricularia* and *R. labiata*.

The finding of *R. peregra* at the locality Mladé Buky in eastern part of the Czech Republic (in Krkonoše mountains) is of particular interest. Sequences obtained from these specimens were the same as those from the Icelandic snails referred to as *R. peregra* by Bargues *et al*. (2001). These authors declared that *R. peregra* is distributed only in western and north-

ern parts of Europe and in Iceland, but not in central Europe. Now, we have shown that *R. peregra* is likely present in central Europe, but it is relatively rare if compared to other species. The snails which have been presented in the past as the commonly occurring *R. peregra* from central Europe, including Germany (e.g., Ložek 1956, Glöer and Meier-Brook 1998, Beran 2002), most likely belong to *R. labiata* according to Bargues *et al*. (2001) and Glöer (2002).

Radix ampla was not found in the Czech Republic, although its occurrence has been listed by various authors (e.g., Ložek 1956, Beran 2002). Based on molecular analyses, Bargues *et al*. (2001) supported validity of this species by finding a unique ITS-2 sequence which differed from that of *R. auricularia*; the same has been confirmed by the study of *cox*1 (Albrecht *et al*. 2008).

Comparative morphology of reproductive systems represents a less commonly used approach for *Radix* species delineation. In our study, four different morphological types (A-D) of reproductive systems were found. Jackiewicz (2000b) and Glöer (2002) mentioned type A for *R. auricularia*. Jackiewicz (2000b) assigned type C to *R. peregra* which in fact was rather *R. labiata* according to the more recent nomenclature (Glöer 2002). Group B was identical with *R. lagotis* (according to Dr. K. Schniebs, personal communication). Description of a reproductive system similar to our group D was not found in literature; however, ITS-2-based molecular identification showed that it belongs to *R. peregra*. The occurrence of two morphological types (B and C) of reproductive system in particular isolates of *R. labiata* was unusual, because there is no reference describing this phenomenon. Changes in size/shape of reproductive organs might be influenced by actual snail physiology/reproduction activity.

In at least three accounts, mantle coloration has been used for species delineation in *Radix* (Uličný 1892, Hubendick 1951, Jackiewicz 1993). In our samples, mantle coloration was similar in the pairs *R. labiata*-*R. lagotis* and *R. auricularia*-*R. peregra*. However, characterizations were made using fixed specimens; the fixative could change mantle coloration as observed in long-term-stored samples.

Comparison of shell morphology is the most often and generally applied method of species delineation in *Radix*. If statistical analyses are performed it seems that conchological parameters used in this study may be useful in most cases for species delineation. However, one should be aware due to the fact that shell morphology of particular specimens (individual snails) does not always reflect results produced by molecular methods, and shell plasticity seems to be the reason of this incongruence. This is in accord with some other papers (e.g., Pfenninger *et al*. 2006). Molecular analysis (preferred here as the main criterion) divided our samples into four clades, but the groups formed by individuals sharing the same shell morphotype were not identical with these clades; overlaps of parameters can be observed in some cases. Search for some other morphological criteria being entirely in accordance with molecular data is therefore advisable and vice versa (i.e. also other molecular markers need to be tested for their appropriateness to delineate species within the polymorphic group of *Radix*).

Concerning infection experiments, it has been shown that the neurotropic fluke of *T. regenti* develops in *R. peregra*, *R. labiata* and *R. lagotis*. *Radix peregra* has already been mentioned in the original description of *T. regenti* (Horák *et al*. 1998), but at that time no identification of snails by molecular tools was applied. Later on, *R. lagotis* and *R. labiata* were identified as the intermediate hosts in the Czech Republic (*R. lagotis* served for routine laboratory maintenance of the parasite for many years, but without determination of the level (in %) of its susceptibility); the role of *R. peregra* in *T. regenti* transmission was weakened. Now it is re-confirmed that *R. peregra*, besides *R. lagotis* and *R. labiata*, can play a role as a vector of *T. regenti*; this is in agreement with the results from France (Jouet *et al*. 2008). Interestingly, in Iceland, *R. peregra* represents the dominant snail species and hosts several schistosomes (Skírnisson *et al*. 2009), showing that both the schistosomes and the snails can adapt to each other under specific local conditions.

Fascioloides magna as a recently introduced parasite relies on *Galba truncatula* as the intermediate host. Nevertheless, there are reports on its development in *Radix* sp. (Faltýnková *et al*. 2006). In our work, for the first time, we exposed molecularly identified *Radix* snails to *F. magna* and showed that the parasite is able to undergo larval development in these snails (namely in two species – *R. lagotis* and *R. labiata*). Probably due to the date of examination (only 35–57 days post exposure) the parasite was found in the stage of rediae and young cercariae. On the other hand, *R. peregra* might be resistant, as no experimental infection of these snails was recorded.

In conclusion, the taxonomy of *Radix* species is not stable; rather, it is under reconstruction. The four species characterized in this contribution (incl. *R. peregra*) likely occur in a large part of Europe. Although statistical analysis of selected conchological parameters showed to be applicable to species delineation if groups of snails are compared, it does not guarantee an error-free identification of individual snails in the field. Therefore, further search for reliable identification markers is advisable in the future. Among them, molecular markers (ITS-2 in our study) seem to represent a powerful tool. However, molecular analyses of *Radix* spp. need to be based on representative sampling of specimens and genes to come to relevant biogeographic conclusions (as noted above for the geographic distribution of *R. peregra*). In terms of transmission of economically important trematodes via freshwater snails, the infection experiments and collections of larval stages of flukes in the field should be supported by a relevant identification of host snails; whenever possible, their characterization should comprise morphological and molecular data. That remains to have a clear view on host-parasite associations and transmission modes of *Trichobilharzia* and *Fascioloides* in the Czech Republic.

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