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Molecular Approach to Identifying Three Closely Related Slug Species of the genus *Deroceras* (Gastropoda: Eupulmonata: Agriolimacidae)

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Some species of slugs belonging to the genus *Deroceras* are invasive and cause severe agricultural damage. Despite extensive knowledge about their invasiveness, data on the molecular differentiation of these morphologically similar species are lacking. Here we present a molecular approach to identifying three closely related species of the genus *Deroceras*—*D. agreste* (L., 1758), *D. reticulatum* (O. F. Müller, 1774) and *D. turcicum* (Simroth, 1894) (Gastropoda: Eupulmonata: Agriolimacidae)—based on sequences of multiple molecular markers: cytochrome *c* oxidase subunit I (*COI*), cytochrome *b* (cyt-*b*), internal transcribed spacer 2 (ITS-2) and 28S ribosomal RNA (28S rRNA). We also provide detailed photomicrographs of the penis and penial gland of the three species, as it is the latter that holds the most important phenotypic characters for distinguishing between these taxa. Since identification of the studied species based solely on morphology is considered challenging, contributing a means of molecular differentiation will aid further ecological and biodiversity surveys of these important pests.

Key words: Taxonomy, *Deroceras*, Barcoding, Gastropods, Slugs.

BACKGROUND

Species identification is central to estimating biodiversity. Traditional taxonomy is based mainly on the morphology of the investigated organism. Since the recognition of cryptic diversity (*i.e.*, complexes of morphologically similar species), which is a consequence of applying molecular techniques to the problem of species identification, morphology-based delineation became insufficient to describe biological diversity (Dayrat 2005). The modern methods for estimating species richness such as DNA-barcoding, metabarcoding and DNA-based taxonomy are now the standard and have been used in meiofauna, ants, decapod crustaceans, lizards and millipedes (Fontaneto et al. 2015; Troncoso-Palacios et al. 2018; Hosoishi and Ogata 2019; Shih et al. 2019; Nguyen et al. 2019). However, all of these methods suffer from limited correspondence between the libraries of DNA sequences and the correctly identified species.

Among terrestrial gastropods, many species of slug are especially challenging to identify, as some taxa can only be distinguished by minute morphological differences in their reproductive organs, and the anatomical sections, necessary for proper identification, are often problematic for non-specialists.

Deroceras Rafinesque, 1820 is a type genus within the family Agriolimacidae and comprises more than 120 species (Wiktor 2000). Some of the *Deroceras* species are invasive and drastically affect biodiversity and agriculture by reducing native species richness and crop infestation (Barker 2002). Among *Deroceras*

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species, *D. reticulatum* (O. F. Müller, 1774) is a major agricultural pest and is known to cause considerable damage to crops of winter rape, winter wheat and many others (Kozłowski 2010). The majority of studies are concerned with the invasiveness and significance of *D. reticulatum* in agriculture (Ferguson et al. 1988; Birkett et al. 2004; Berman et al. 2011), whereas the molecular data supporting its pattern of distribution are lacking. Additionally, sequences of two closely related and morphologically very similar species, *Deroceras turcicum* (Simroth, 1894) (*locus typicus*: Lake Ohrid in Macedonia) and *Deroceras agreste* (L., 1758) (*terra typica*: Sweden) are unknown and uncertain, respectively; what is more, these species have overlapping distribution ranges. The *COI* sequences of *D. agreste* are known from a single study about gastropods of Britain and Ireland (Rowson et al. 2014). In that study, the authors found individuals with the genital morphology characteristic of *D. reticulatum* but mtDNA sequences characteristic of *D. agreste*. These results were explained to be an example of a one-way introgression of *D. reticulatum* genes into a *D. agreste* population (Rowson et al. 2014). Notably, the population of *D. agreste* used in the study was not from the species' *terra typica*, thus the validity of the proposed conclusions remains questionable.

Deroceras reticulatum (O. F. Müller, 1774) is a widely distributed synanthropic species occurring in Europe (Wiktor 2004). The origin of this species is unknown, but it likely comes from Central Europe (Kozłowski 2010). It was described from Frideriksdal in Denmark (*locus typicus*) in 1774. Recently, it was introduced to North and South America, Tasmania, New Zealand and Central Asia (*e.g.*, Crowley and Pain 1977; Hausdorf 2002; Tulli et al. 2009; Welter-Schultes 2012). Notably, the majority of these records have not been verified genetically. For instance, Hausdorf (2002) studied terrestrial gastropods (both slugs and snails) in Colombia and determined slug specimens anatomically. Crowley and Pain (1977) published a study about molluscs of Saint Helena in the last century, identifying them based on classical taxonomic methods. Tulli et al. (2009) conducted research on predation on *D. reticulatum* by the carabid *Scarites athracinus* and collected slugs in Argentina. This study did not include any information about slug identification, thus it seems probable that species identification was based on morphological characters and/or external appearance. Finally, a book by Welther-Schultes (2012) summarizes data on the occurrence and distribution of particular species in Europe. *Deroceras reticulatum* is regarded by the DAISIE organization (Delivering Alien Invasive Species Inventories for Europe) as one of the most invasive species in Europe. It mostly occurs in cultivated areas, open habitats, meadows, gardens, and cemeteries—all in places where human activity is observed (Wiktor 2004; Welter-Schultes 2012). *Deroceras reticulatum* has a creamy or light coffee cream body pigmentation; it is sometimes blackish and spotted, in which case the dark spots behind the mantle form a reticulate pattern. However, Wiktor (1989) noted considerable variability in the distribution and pigmentation of spots and stated that the most common *D. reticulatum* individuals are spotted, even though juveniles may be uniform in color. All this makes species identification based on the external appearance extremely difficult.

The second species examined in our study, *D. agreste*, was originally described from Sweden. This species likely occurs throughout Europe, but its precise distribution is unknown (Wiktor 2000; Welter-Schultes 2012). There are currently no data relating to where *D. agreste* has been introduced and where it is indigenous. These slugs prefer open habitats, especially gardens, meadows, and less often farmland. It can be described as synanthropic and can be found together with, and in similar habitats to, *D. reticulatum* and *D. sturanyi* (Wiktor 2000). Its body is yellowish white to light greyish yellow, transparent, without spots (Welter-Schultes 2012).

Our third species, *D. turcicum*, occurs in the Balkan region (Wiktor 1989). It was originally described from Lake Ohrid in Macedonia in 1894. *Deroceras turcicum* has expanded its distribution, and is currently present in Austria, Hungary, Slovenia, Croatia, Bosnia and Herzegovina, Serbia, Montenegro and probably Albania, Greece, and Turkey (Wiktor 2000). Reise and Hutchinson (2001) reported *D. turcicum* in the Czech Republic and Slovakia, whereas Reise et al. (2005) recorded new localities of this species in Poland. *Deroceras turcicum* inhabits mostly mixed or beech woodland, parks, and gardens. Its body is dirty-cream or light cream, with dark spots covering the mantle; overall, it is extremely similar in appearance to *D. reticulatum* (Welter-Schultes 2012). Most reports about the occurrence of these slugs are based on observations and/or species identification based on traditional taxonomic methods such as anatomical sections. In the majority of reports, species identification was not supported by any molecular analysis, *e.g.,* by comparisons of *COI* sequences (Szybiak 2004; Reise et al. 2005; Sulikowska-Drozd 2007; Hutchinson and Reise 2015). Thus, cited records should be treated with caution, as the species under consideration are morphologically very similar.

In this study, we molecularly characterise and compare three closely related *Deroceras* species (*D. agreste*, *D. reticulatum*, *D. turcicum*), providing for each of them DNA sequences of four molecular markers (cytochrome *c* oxidase subunit I (*COI*), cytochrome *b* – (cyt-*b*), internal transcribed spacer 2 (ITS-2), 28S ribosomal RNA (28S rRNA)). We also provide comparative genetic analyses of *p*-distances to show differences between the studied species. Moreover, we visualise phylogenetic relationships between *Deroceras* species based on available *COI* sequences for the named species deposited in the GenBank database as well as the sequences obtained in this study. Finally, we also provide a set of photomicrographs that detail the morphology of the penis and penial gland in the studied species. Our contribution will be helpful to other researchers using modern approaches to identify the difficult to distinguish *Deroceras* species. Our findings may also have significance in biological conservation, as these slugs have been shown to have considerable economic importance in agriculture.

MATERIALS AND METHODS

Material collection and species identification

All specimens of *Deroceras agreste*, *D. turcicum* and *D. reticulatum* were obtained from a museum collection deposited in the Museum of Natural History, Wrocław University, Poland (Table 1). Gastropods were preserved in 70–75% ethyl alcohol (Wiktor and Jurkowska 2007). All specimens were identified and labelled by Professor Andrzej Wiktor (Wrocław University, Poland) and further verified by us based on anatomical characteristics under Nikon SMZ1500 stereomicroscope (Table 2). From each slug, a small piece of tissue, preserved in 99.8% ethanol, was taken for DNA extraction. The photos of penial glands were taken using a digital camera (Nikon DS-Fil-U2) associated with the stereomicroscope and later assembled into figures in Corel Photo-Paint X6, ver. 16.4.1.1281. Schematic drawings of the genitalia of each species were made using InkScape software based on Wiktor's (2000) monograph.

DNA extraction, amplification and sequencing

DNA was extracted from the collected tissue using Sherlock AX kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. This kit is intended for material with trace amounts of DNA. To obtain DNA sequences of the *COI*, ITS-2, 28S rRNA, cyt-*b* genes, PCR reactions were run using the primers listed in table 3. For *COI*, ITS-2 and 28S rRNA specific primers for *Deroceras* species were designed *de novo* based on sequences of *Deroceras* (*D. laeve*, *D. reticulatum*, *D. invadens*), *Arion* (*Arion* sp., *A. silvaticus*, *A. vulgaris*, *A. distinctus*, *A. rufus*) and *Limax* (*L. maximus*, *L. flavus*, *L. cinereoniger*) species available in GenBank (for exact accession numbers, see Table S1). A PCR cocktail and profiles/conditions for specific markers are given in the supplementary materials (Tables S2 and S3). To check the DNA quality, 3 µl sample of PCR product was run on a 1.5% agarose gel for 30 min at 100 V. PCR products were cleaned using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany). A sequencing reaction was performed using a 10-µl reaction mixture consisting of 2 µl of PCR

Table 1. Collection data on *Deroceras* specimens used in this study (N: number of sequenced individuals from each locality)

Species	N	Locality	Collector	Collection date	Fig. 1.	GenBank Accession numbers			
						COI	*cyt-b*	ITS-2	28S rRNA
D. reticulatum	3	Aragón, Pirineos Mts, Diazas near Torla (N of Huesca), Spain	A. Wiktor	07.07.1987	A	MN934414	MN931235	MT361823	MN930516
	3	Leon province, Villaturel and Villasabariego (NE of Leon), Spain	A. Wiktor	19.06.1987	B, C				
D. agreste	2	Västergötland, Sweden	H. W. Walden	30.09.1964	D	MN934413	MN931234	MT361821	MN930515
	2	Bohuslän, Sweden	H. W. Walden	10.10.1965	E				
	2	Cantabria, Spain	A. Wiktor	12.11.1988	F				
D. turcicum	2	near Maçka in Trabzon, Turkey	A. Riedel	07.12.1985	G	MN934415	MN931236	MT361822	MN930517
	2	Zafanos, Trabzon, Turkey	A. Riedel	01.12.1985	H				
	2	near Maçka in Trabzon, Turkey	A. Wiktor	29.04.2001	I				

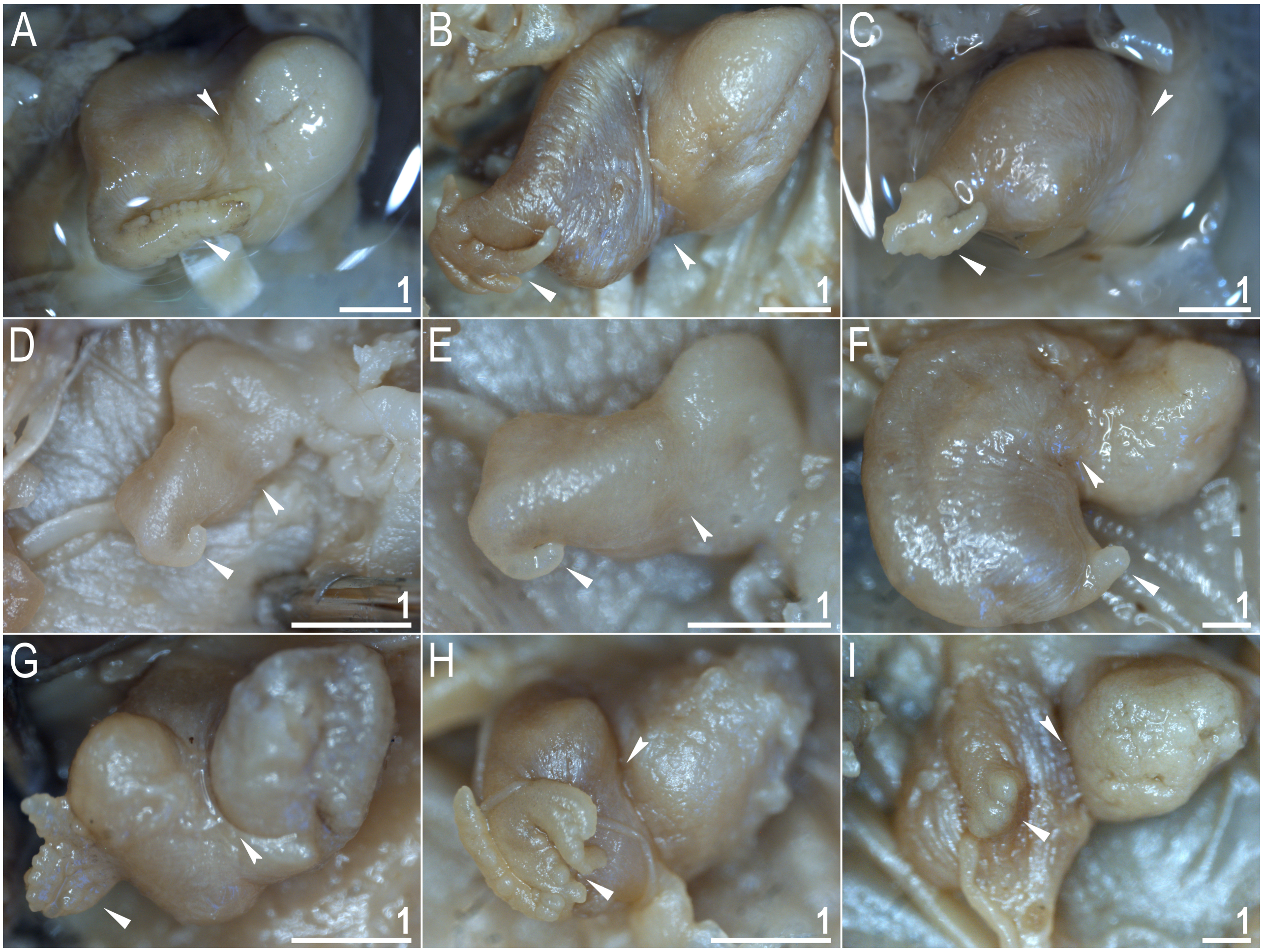

Fig. 1. Genitalia of *D. reticulatum* (A: Aragón, Pirineos Mts, Diazas near Torla (N of Huesca), Spain; B–C: Leon province, Villaturel and Villasabariego (NE of Leon), Spain), *D. agreste* (D: Västergötland, Sweden; E: Bohuslän, Sweden; F: Cantabria, Spain) and *D. turcicum* (G: near Maçka in Trabzon, Turkey; H: Zafanos, Trabzon, Turkey; I: near Maçka in Trabzon, Turkey) detailing penis and penial gland morphology. Penial gland and constriction in the penis are indicated by flat and indented arrowheads respectively. Scale bars in mm.

Table 2. Morphological differences in reproductive system between *D. reticulatum*, *D. agreste* and *D. turcicum* according to Welter-Schultes (2012)

	D. reticulatum	*D. agreste*	*D. turcicum*
penis	fleshy and with a silky sheen, in the shape of an irregular sac, divided into 2 parts by a deep lateral constriction	oval in juveniles, with lateral constriction in adults	strong swelling on the anterior part of the penis
penial gland	variable shape; usually a few branches or a single long branch	finger-shaped, never branched, smooth, without glandular papillae	variable or reduced
stimulator	large, conical and narrow	cone-shaped, with narrow base	conical
rectal caecum	large	caecum on rectum well-developed, clearly longer than wider	well-developed
vas deferens	opens into the penis wall facing the external body side	opens laterally close to penial gland	opens at the base of the penial gland

product, 0.15 µl of primer, 1 µl of sequencing buffer (BrilliantDye Terminator Sequencing Kit, Nimagen, The Netherlands), 5.85 µl of ddH_2O and 1 µl of Terminator (BrilliantDye Terminator Sequencing Kit, Nimagen, The Netherlands). The sequencing program consisted of four steps: 1 min initial denaturation at 96°C, followed by 10 s denaturation at 96°C, 5 s annealing at 55°C, 4 min elongation at 60°C for 25 cycles. Sequencing products were cleaned using ExTerminator (A&A Biotechnology, Poland) and sequenced in both directions in Genomed company (Warsaw, Poland). Obtained sequences of single haplotypes per marker and species were deposited in GenBank.

Data analysis

All obtained *COI* sequences were blasted by NCBI BLAST (Altschul et al. 1990) to verify species identification and find similar homologous sequences. In order to visualize phylogenetic relationships between the studied species, selected *COI* sequences (the longest and with published status) of six named *Deroceras* species—*D. golcheri* (Accession numbers: JN248291-293), *D. laeve* (MG421043, MG423214, KX959499-501, HM584699), *D. panormitanum* (JN248304-313), *D. reticulatum* (MG421618, MG421373, MG421157, MG421125, MG421099, MF545181, MF545161, MF545125, MF545107, FJ481179), *D. agreste* (KF894312, KF894346-247, KF894375) and *D. invadens* (JN248295-297, JN248301-303, JN248314-315, KX959490)—were downloaded from GenBank (Eskelson et al. 2011; Reise et al. 2011; Rowson et al. 2014; Araiza-Gómez et al. 2017) and aligned with sequences obtained in this study for *D. reticulatum*, *D. agreste* and *D. turcicum* in BioEdit 5.0.0 (Hall 1999) with the multiple alignment function of ClustalW (Thompson et al. 1994). Aligned sequences were trimmed and translated into protein sequences in MEGA v. 7 to check against pseudogenes. Uncorrected pairwise distances between species were calculated in MEGA7 (Kumar et al. 2016). Uncorrected genetic distances for *COI* were calculated based on all sequences obtained for *D. agreste*, *D. reticulatum* and *D. turcicum* together with those used for phylogenetic tree calculation (alignment length: 402 bp). For this molecular marker, genetic distances were calculated in two ways, first between all sequences in the dataset (see Table S4), and second between species defined as groups (Table 4). In the case of cyt-*b*, ITS-2 and 28S rRNA, only the newly obtained sequences for *D. agreste*, *D. reticulatum* and *D. turcicum* were used to calculate genetic distances (lengths of alignments: 299 bp, 280 bp and 352 bp, respectively) (Table 5). The concatenated dataset comprises 79 sequences in total (*COI* sequences of six *Deroceras* species from GenBank (42 sequences), *COI*, cyt-*b*, ITS-2 and 28S rRNA sequences for studied species obtained in this study (36 sequences) and one sequence of *Limax maximus* (Accession Number: KM612139) as the outgroup). The obtained alignment of the concatenated sequences (1619 bp) were divided into eight data blocks constituting the ITS-2, 28S rRNA as well as *COI* and cytochrome *b* that were separated into three codon positions using PartitionFinder v. 2.1.1 under the Bayesian Information Criterion (BIC) (Lanfear et al. 2016). PartitionFinder retained five out of eight predefined partitions, the best fit of which were (1) F81 for 28S rRNA, (2) TIM+G for the 2nd codon position in *COI* and 3rd codon position in cytochrome *b*, (3) TRN+G for the 3rd codon position in *COI* and 1st codon position in cytochrome *b*, (4) TRN+I for the 1st codon position in *COI* and 2nd codon position in cytochrome *b*, (5) JC for ITS-2. For the maximum-likelihood (ML) analysis in RAxML, models GTR, GTR+I, GTR+G and GTR+I+G were also tested (Stamatakis 2014). The best fit model for all partitions in this analysis was GTR+G.

ML topology was constructed using RAxML v. 8.0.19 (Stamatakis 2014). The tree branches were supported by bootstrap analysis with 1,000 replicates. Bootstrap support values ≥ 70% were regarded as significant statistical support. Bayesian inference (BI) marginal posterior probabilities were calculated in MrBayes v. 3.2 (Huelsenbeck and Ronquist 2001,

Table 3. Primers used for amplification of the four DNA fragments sequenced in the study

Fragment	Primer name	Primer sequence (5’-3’)	References
COI	COI_Der_Ff	TATATATAATTTTTGGGGTTTGATGTGG	This study
	COI_Der_Rr	CAAAAAGATGTTGATATAAAATAGG	
CytB	151-F	TGTGGRGCNACYGTWATYACTAA	Merritt et al. 1998
	270-R	AANAGGAARTAYCAYTCNGGYTG	
ITS-2	ITS2_Der_Ff	GTCGGCTAGTCWAAAGCAATCG	This study
	ITS2_Der_Rr	CCGCTTCACTCGCCGTTACT	
28S rRNA	28S_Der_Ff	GCTAAATACTTGCACGAGTCCG	This study
	28S_Der_Rr	ACGGTTGCCCAGTCTCTCC	

Huelsenbeck et al. 2001) with one cold and three heated Markov chains for 10 million generations and trees were sampled every 1000 generations. In the BI consensus tree, clades recovered with posterior probability (PP) between 0.95 and 1 were considered well supported, those with PP between 0.90 and 0.94 were considered moderately supported and those with lower PP were considered unsupported. Obtained trees were visualized in FigTree v.1.4.3, available at: http://tree.bio.ed.ac.uk/software/figtree.

RESULTS

All specimens previously identified by prof. Andrzej Wiktor were confirmed as *Deroceras reticulatum*, *D. agreste* and *D. turcicum* based on morphology of their genitalia, especially penis and penial gland appearance (Figs. 1A–I and 2A–C). *Deroceras reticulatum* has a pronounced laterally constricted penis with both parts inscribed in an oval and with a large penial gland on the posterior end of the penis (Fig. 1A–C). The penial gland can vary in shape (Fig. 1A–C). It can be represented by a single process with both lateral sides covered by easily identifiable papillae-like structures (Fig. 1A) or made up of a few branches covered by glandular papillae and merged into a common short trunk (Fig. 1B). However, these papillae-like structures or glandular papillae on the lateral sides of the penial gland can sometimes be only faintly visible (Fig. 1C). *Deroceras agreste* is characterized by sack-shaped penis, which can be laterally narrowed in older specimens. The penial gland does not show variability in its shape, it is visible as a small conical nodule or unbranched finger with a smooth surface without any papillae-like structures on the lateral sides (Fig. 1D–F). *Deroceras turcicum* has a laterally constricted penis with the anterior part heavily bloated and a narrower posterior part. The penial gland can be a complex, multifurcated structure or may be significantly reduced (Fig. 1G–I). More specifically, penial glands can consist of multiple knots, with the lateral sides covered by convexity/constrictions that can resemble papilla-like structures (but usually bigger than in *D. reticulatum*) (Fig. 1G–H), or it can be reduced and comprise only a few, usually short nodular processes set directly on the penis or on a short common stem (Fig. 1I).

Good quality sequences for all four molecular markers were obtained from all analyzed specimens. Only single haplotypes were recovered for each marker in each species. For the three analyzed species, the

Table 4. Genetic distances (%) between *Deroceras* species calculated from *COI* sequences

		1	2	3	4	5	6	7
1	*D. agreste*							
2	*D. golcheri*	17.1						
3	*D. invadens*	14.1	9.8					
4	*D. laeve*	14.4	10.3	7.8				
5	*D. panormitanum*	15.7	6.0	7.4	8.5			
6	*D. reticulatum*	5.6	15.1	14.7	13.3	13.6		
7	*D. turcicum*	9.8	16.6	14.2	14.4	15.2	8.6	

Table 5. Genetic distances (%) among three *Deroceras* species calculated from cytochrome *b*, ITS-2 and 28S rRNA sequences

			1	2	3
cytochrome *b*	1	*D. agreste*	-		
	2	*D. reticulatum*	6.8	-	
	3	*D. turcicum*	10.8	12.1	-
ITS-2	1	*D. agreste*	-		
	2	*D. reticulatum*	7.6	-	
	3	*D. turcicum*	8.0	3.5	-
28S rRNA	1	*D. agreste*	-		
	2	*D. reticulatum*	0.6	-	
	3	*D. turcicum*	1.2	1.7	-

following sequences were obtained:

- *D. agreste*: the *COI* sequence (GenBank: MN934413), 512 bp long, the cytochrome *b* sequence (GenBank: MN931234), 390 bp long, the ITS-2 sequence (GenBank: MT361821), 280 bp long, the 28S rRNA sequence (GenBank: MN930515), 400 bp long.
- *D. reticulatum*: the *COI* sequence (GenBank: MN934414), 651 bp long, the cytochrome *b* sequence (GenBank: MN931235), 299 bp long, the ITS-2 sequence (GenBank: MT361823), 352 bp long, the 28S rRNA sequence (GenBank: MN930516), 352 bp long.
- *D. turcicum*: the *COI* sequence (GenBank: MN934415), 420 bp long, the cytochrome *b* sequence (GenBank: MN931236), 299 bp long, the ITS-2 sequence (GenBank: MT361822), 350 bp long, the 28S rRNA sequence (GenBank: MN930517), 409 bp long.

Regarding the *D. reticulatum COI* sequence, the verification in BLAST resulted in fitting to *D. reticulatum* (KY765589 (Ahn et al. 2017); Query cover: 100%; E-value: 0.00; Perc. ident: 98.92%). The best hits for the *D. agreste* sequence were *D. reticulatum* and *D. agreste* (LS974196, (Kropf, unpublished) and KF894312 (Rowson et al. 2014), respectively; for both species: Query cover: 100%; E-value: 0.00; Perc. ident: 99.41%). For the *D. turcicum* sequence, the best hit was *D. reticulatum* (MG421618 (Dewaard, unpublished); Query cover: 99%; E-value: 1e-162; Perc. ident: 91.87%).

Genetic distances calculated for *COI* sequences between *Deroceras* species ranged from 5.6% (between *D. agreste* and *D. reticulatum*) to 17.1% (between *D. agreste* and *D. golcheri*) (12.01% on average) (Table 4). Regarding other molecular markers, genetic distances for cytochrome *b* range from 6.8% (between *D. agreste* and *D. reticulatum*) to 12.1% (between *D. reticulatum* and *D. turcicum*); for ITS-2, they range from 3.5% (between *D. reticulatum* and *D. turcicum*) to 8.0% (between *D. agreste* and *D. turcicum*); and for 28S rRNA, genetic distances range from 0.6% (between *D. agreste* and *D. reticulatum*) to 1.7% (between *D. reticulatum* and *D. turcicum*) (Table 5).

Maximum-Likelihood (ML) and Bayesian (BI) phylogenetic trees resulted in similar tree topologies (Fig. 3). Specimens belonging to particular species constituted seven separate clades that, in most cases, were strongly supported in BI analysis and were moderately or not supported by ML analysis (Fig. 3). The three species that we focused on in this study (*D. reticulatum*, *D. agreste* and *D. turcicum*) have been found to be distinct but closely related to one another and together form a clade that is sister to the other analyzed species (Fig. 3). However, due to polytomy, specific relationships between these three species could not be inferred. Individuals identified based on anatomical studies conducted by Wiktor and us (GenBank: MN934413, this study) and by Rowson et al. (2014) (GenBank: KF894312, KF894346-247, KF894375) as *D. agreste* clustered together, thus tentatively providing an additional and independent confirmation of species affiliations (Fig. 3). The remaining four *Deroceras* species formed another big clade, sister to (*D. reticulatum* + *D. agreste* + *D. turcicum*). Here, similarly, the specific relationships between these species could not be fully recovered due to the several weakly supported nodes that indicate polytomy (Fig. 3).

DISCUSSION

In this study, we showed that the three

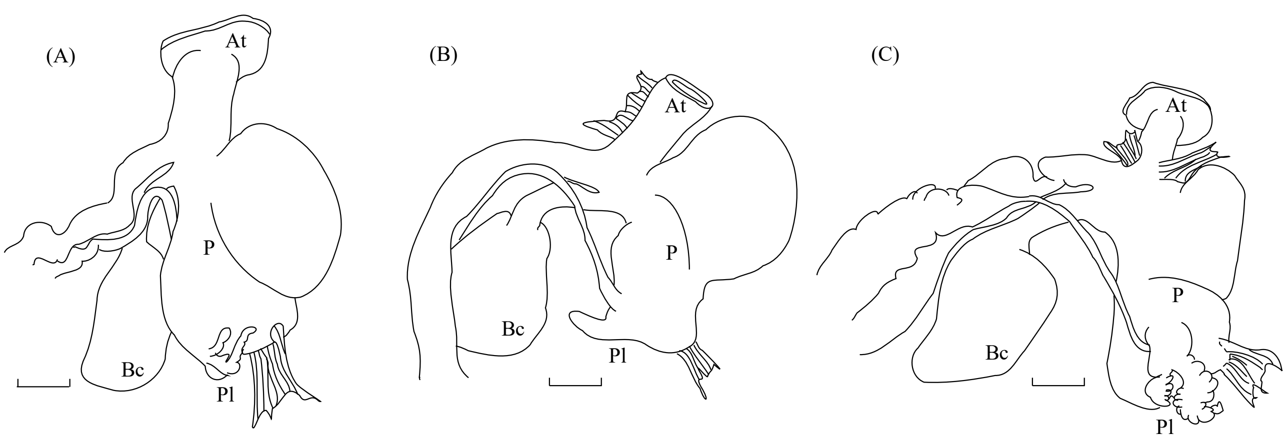

Fig. 2. Schematic drawings of genitalia of *D. reticulatum* (A), *D. agreste* (B) and *D. turcicum* (C) after Wiktor (2000). Bc, bursa copulatrix; Pl, penial gland; P, penis; At, atrium. Scale bars = 1 mm.

morphologically similar species, *Deroceras reticulatum*, *D. agreste*, *D. turcicum*, are genetically easily differentiable. The results from the BLAST search clearly underline the problems with identifying these taxa, namely that *D. agreste* and *D. turcicum* are often wrongly designated as the more common *D. reticulatum*. The DNA sequences presented in our study are valuable as they came from specimens deposited in a museum collection and were identified by Professor Andrzej Wiktor, a specialist in slug taxonomy, and verified in this study. Additionally, sequences for *D. agreste* were obtained from the specimens collected from *terra typica* in Sweden. Our phylogenetic results, presented above, further confirmed the molecular distinctives and monophyly of each studied species.

As the number of traits associated with the external appearance of slugs is usually extremely limited, the morphology of the reproductive system is considered crucial for precisely identifying many slug species (*e.g.*, Reise and Hutchinson 2001). In accordance with Wiktor (2000), we identified penis and penial gland appearance to be the most useful character for at least a provisional, and not so complicated identification (even for non-specialists), due to the ease by which these structures can be located. In concordance with previous studies, we also confirmed a considerable morphological variability in the penial glad in *D. reticulatum* (Wiktor 2000) and *D. turcicum* (Wiktor 2000; Reise and Hutchinson 2001), as well as almost no variation in *D. agreste* (Wiktor 2000). However, in dubious cases, the application of genetic data and cross-verification with the specific DNA sequences provided in our study will be of great help to other researchers. Moreover, this may have increased significance when only juveniles are present in a given sampling area, since anatomical studies of their genitalia will not be possible.

The mitochondrial *COI* and cytochrome *b* were the most variable molecular markers and thus most

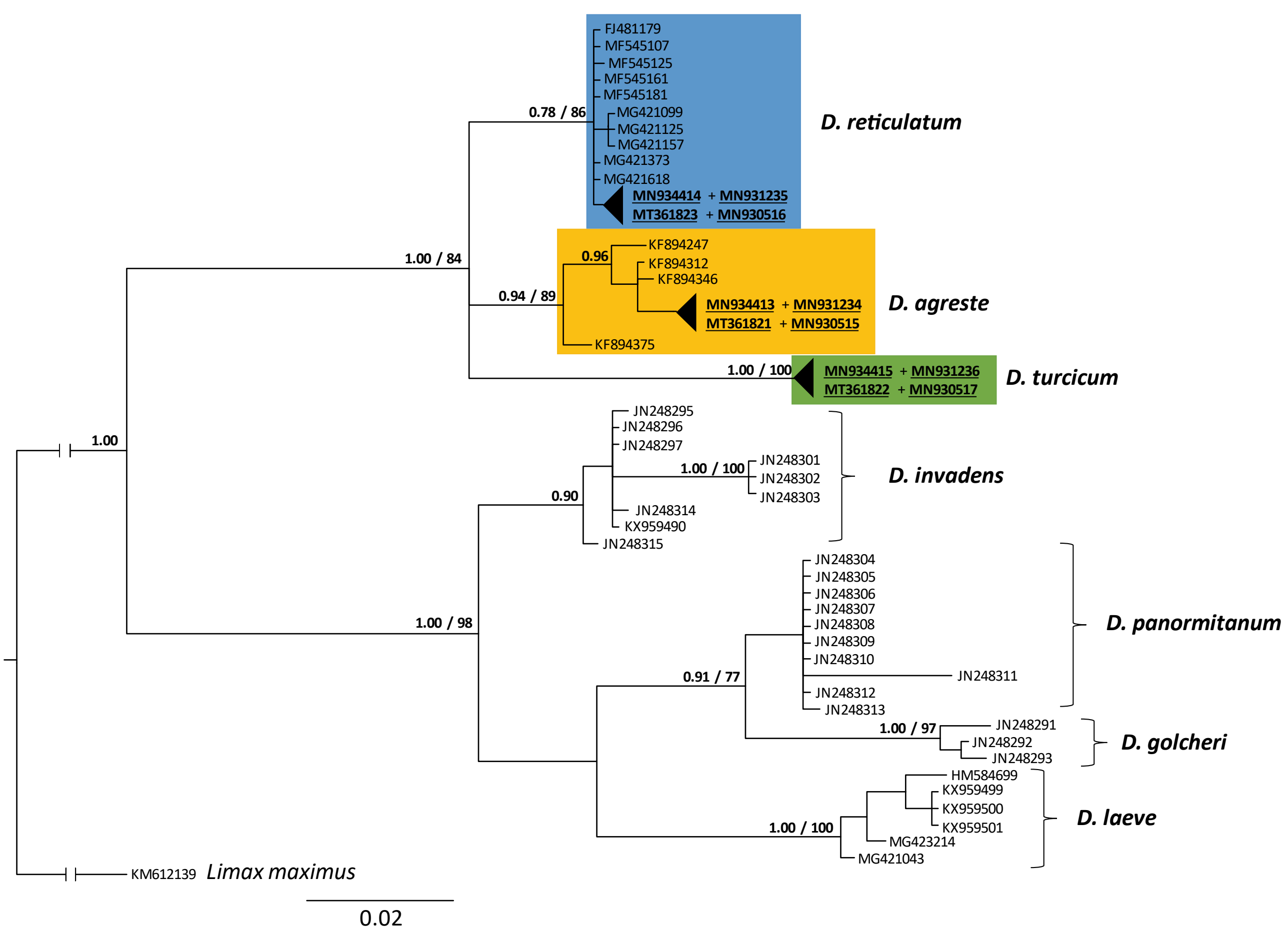

Fig. 3. A phylogenetic tree constructed from concatenated sequences of *COI*, cyt-*b*, ITS-2 and 28S rRNA of *Deroceras* species. Only topology of the Bayesian tree is shown. Bolded and underlined GenBank accession numbers indicate sequences obtained in this study. Numbers at the nodes indicate Bayesian posterior probability and bootstrap values separated by the "/" symbol. Values less than 0.90 for BI and less than 70 for ML are not shown. The scale bar represents 0.02 substitutions per nucleotide position.

informative in terms of species distinctions. The first of these is already known to be the most useful molecular marker for species identification (Fontaneto et al. 2015); however, as we show, cytochrome *b* is also variable enough to distinguish *Deroceras* species. Moreover, cytochrome *b* was successfully used for identifying species and deliminating members of other gastropod families (*e.g.*, Merritt et al. 1998; Groenenberg et al. 2016). Notably, amplification and/or sequencing of these two DNA fragments are often problematic because of their length, so obtaining good quality sequences is challenging but possible in most cases with specific primers designed for short DNA fragments. Moreover, specimens deposited in museum collections are usually old and thus their DNA is usually degraded and highly fragmented. Work with such material often requires specific kits for DNA extraction that are intended for material with trace DNA content. Therefore, by presenting reliable sequences for cyt-*b* and *COI*, we increased the chances for correct species identifications in future research. Regarding nuclear markers, ITS-2 and 28S rRNA, it has already been shown that they are more conservative than the mentioned mitochondrial cyt-*b* and *COI* (Wade et al. 2006; Zając et al. 2020). This makes them especially suitable for resolving deeper phylogenetic nodes, especially those between genera, and of higher taxonomic levels (*e.g.*, Wade et al. 2006; Neiber and Hausdorf 2015). Similar to other studies, our results show that ITS-2 is more variable than 28S rRNA. As such, ITS-2 can be used as additional support for identification by mitochondrial markers as well as in phylogeographic research (Schniebs et al. 2013; Zhou et al. 2017).

Our phylogenetic analysis revealed that the tree topology for *Deroceras* species is similar to that found in the recent study conducted by Rawson et al. (2014). The addition of new sequences of *D. turcicum* enabled us to show a close relationship with the morphologically similar *D. reticulatum* and *D. agreste*. Even though the phylogenetic position of the genus *Deroceras* is known (Bouchet and Rocroi 2005), the specific and detailed relationships between its species have only been examined superficially (*e.g.*, Koene and Schulenburg 2005). As mentioned above, molecular data regarding these slugs are extremely limited. Currently, the existing data enabled the testing of relationships among only seven named *Deroceras* taxa, from a genus comprising more than 120 species (Wiktor 2000). Importantly, many of them (including specimens of the type series) are deposited in museum collections, thereby providing an opportunity to obtain tissue for genetic studies from accurately identified specimens. This might be important, as morphological species determination is challenging and it may be difficult to obtain fresh material from the field because of the species' status (*e.g.*, endangered, endemic) or its rare occurrence. Thus, museum collections constitute a valuable source of reliable barcodes which might help enhance species identification and facilitate biodiversity estimations (Neiber and Hausdorf 2016; Neiber et al. 2017).

CONCLUSIONS

Our study presents a molecular approach to identify three closely related species of *Deroceras* slugs by providing, for each of them, definitive DNA sequences of four molecular markers. We designed *de novo* specific primers for three molecular markers (*COI*, ITS-2, 28S rRNA), which successfully amplified DNA fragments, and which can be used on the other *Deroceras* species held within museum collections. The DNA sequences of *D. turcicum* are presented in our study for the first time, providing new and compelling evidence of its close relationship with the two morphologically similar taxa (*D. reticulatum* and *D. agreste*). The sequences provided in this study are reliable barcodes which may aid in correct species identification, even by non-specialists. This study underlines the important role of natural history museums and identifies their collections as priceless resources of global biodiversity. Finally, our results have application in studies concerning the distribution, conservation biology and introgression of *Deroceras* species.

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Authors' contributions: KSZ designed the study, analyzed the data and wrote the manuscript. DS designed the primers and contributed to the data analysis and manuscript writing.

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REFERENCES

Ahn SJ, Martin R, Rao S, Choi MY. 2017. The complete mitochondrial genome of the gray garden slug *Deroceras reticulatum* (Gastropoda: Pulmonata: Stylommatophora). Mitochondrial DNA B **2:**55–256. doi:10.1080/23802359.2017.1318677.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol **215:**403–410. doi:10.1016/S0022-2836(05)80360-2.

Araiza-Gómez V, Naranjo-García E, Zúñiga G. 2017. The Exotic Slugs of the Genus *Deroceras* (Agriolimacidae) in Mexico: Morphological and Molecular Characterization, and New Data on Their Distribution. Am Malacol Bull **35:**126–133. doi:10.4003/006.035.0205.

Barker GM. 2002. Molluscs as Crop Pests. CABI Publishing, Wallingford, UK.

Berman DI, Meshcheryakova EN, Leirikh AN. 2011. Cold hardiness, adaptive strategies, and invasion of slugs of the genus *Deroceras* (Gastropoda, Pulmonata) in northeastern Asia. Biol Bull **38:**765–778. doi:10.1134/S1062359011080012.

Birkett MA, Dodds CJ, Henderson IF, Leake LD, Pickett JA, Selby MJ, Watson P. 2004. Antifeedant compounds from three species of Apiaceae active against the field slug, *Deroceras reticulatum* (Müller). J Chem Ecol **30:**563–576. doi:10.1023/B:JOEC.0000018629.58425.18.

Bouchet P, Rocroi J-P. 2005. Classification and nomenclator of gastropod families. Malacologia **47:**1–397.

Crowley TE, Pain T. 1977. Mollusca not Charopidae. *In*: Basilewsky P (ed). La faune terrestre de l'île de Sainte-Hélène. Quatrième partie. Annales du Musée Royal de l'Afrique Centrale, Zoologie **220:**1–575.

Dayrat B. 2005. Towards integrative taxonomy. Biol J Linn Soc **85:**407–415. doi:10.1111/j.1095-8312.2005.00503.x.

Eskelson MJ, Chapman EG, Archbold DD, Obrycki JJ, Harwood JD. 2011. Molecular identification of predation by carabid beetles on exotic and native slugs in a strawberry agroecosystem. Biol Control **56:**245-253. doi:10.1016/j.biocontrol.2010.11.004.

Ferguson CM, Barratt BIP, Jones PA. 1988. Control of the grey field slug (*Deroceras reticulatum* (Muller) by stock management prior to direct-drilled pasture establishment. J Agric Sci **111:**443–449. doi:10.1017/S0021859600083611.

Fontaneto D, Flot J-F, Tang CQ. 2015. Guidelines for DNA taxonomy, with a focus on the meiofauna. Mar Biodivers **45:**433–451. doi:10.1007/s12526-015-0319-7.

Groenenberg DSJ, Subai P, Gittenberger E. 2016. Systematics of Ariantinae (Gastropoda, Pulmonata, Helicidae), a new approach to an old problem. Contrib Zool **85:**37–65. doi:10.1163/18759866-08501003.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser **41:**95–98.

Hausdorf B. 2002. Introduced Land Snails and Slugs in Colombia. J Mollus Stud **68:**127–131. doi:10.1093/mollus/68.2.127.

Hosoishi S, Ogata K. 2019. Cryptic diversity of the widespread Asian ant *Crematogaster rothneyi* (Hymenoptera: Formicidae) inferred from morphological and genetic evidence. Zool Stud **58:**11. doi:10.6620/ZS.2019.58-11.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics **17:**754–755. doi:10.1093/bioinformatics/17.8.754.

Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science **294:**2310–2314. doi:10.1126/science.1065889.

Hutchinson JMC, Reise H. 2015. An invasion from Germany: *Deroceras invadens* (Pulmonata: Agriolimacidae) and other synanthropic slugs in the southwest corner of Poland. Folia Malacol **23:**301-307. doi:10.12657/folmal.023.026.

Koene JM, Schulenburg H. 2005. Shooting-darts: co-evolution and counter-adaptation in hermaphroditic snails. BMC Evol Biol **5:**25. doi:10.1186/1471-2148-5-25.

Kozłowski J. 2010. Ślimaki nagie w uprawach. Klucz do identyfikacji. Metody zwalczania. Instytut Ochrony Roślin, Państwowy Instytut Badawczy, Poland.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol **33:**1870–1874. doi:10.1093/molbev/msw054.

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B. 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Mol Biol Evol **34:**772–773. doi:10.1093/molbev/msw260.

Merritt TJS, Shi L, Chase MC, Rex MA, Etter RJ, Quattro JM. 1998. Universal cytochrome *b* primers facilitate intraspecific studies in molluscan taxa. Mol Mar Biol Biotechnol **7:**7–11.

Neiber MT, Hausdorf B. 2015. Molecular phylogeny reveals the polyphyly of the snail genus *Cepaea* (Gastropoda: Helicidae). Mol Phylogenet Evol **93:**143–149. doi:10.1016/j.ympev.2015.07.022.

Neiber MT, Hausdorf B. 2016. Molecular phylogeny and biogeography of the land genus *Monacha* (Gastropoda, Hygromiidae). Zool Scr **46:**308-321. doi:10.1111/zsc.12218.

Neiber MT, Razkin O, Hausdorf B. 2017. Molecular phylogeny and biogeography of the land snail family Hygromiidae (Gastropoda: Helicoidea). Mol Phylogenet Evol **111:**169–184. doi:10.1016/j.ympev.2017.04.002.

Nguyen AD, Nguyen MH, Nguyen TT, Phung HT. 2019. Review of dragon millipedes (Diplopoda, Polydesmida, Paradoxosomatidae) in the fauna of Vietnam, with descriptions of three new species. Zool Stud **58:**14. doi:10.6620/ZS.2019.58-14.

Reise H, Hutchinson JMC. 2001. Morphological variation in terrestrial slug *Deroceras turcicum* (Simroth, 1894) and a northern extension of its range in Central Europe. Folia Malacol **9:**63–71. doi:10.12657/folmal.009.009.

Reise H, Hutchinson JMC, Forsyth RG, Forsyth TJ. 2005. First records of the terrestrial slug *Deroceras turcicum* (Simroth, 1894) in Poland. Folia Malacol **13:**177–179.

Reise H, Hutchinson JMC, Schunack S, Schlitt B. 2011. *Deroceras panormitanum* and congeners from Malta and Sicily, with a redescription of the widespread pest slug as *Deroceras invadens* n. sp. Folia Malacol **19:**201–223. doi:10.2478/v10125-011-0028-1.

Rowson B, Anderson R, Turner JA, Symondson WOC. 2014. The Slugs of Britain and Ireland: Undetected and Undescribed Species Increase a Well-Studies, Economically Important Fauna by More Than 20%. PLoS ONE **9:**e91907. doi:10.1371/journal.pone.0091907.

Shih HT, Ng PKL, Ravichandran S, Prema M. 2019. Resurrection of

Gelasimus variegatus Heller, 1862, a fiddler crab closely related to *Austruca bengali* (Crane, 1975) and *A. triangularis* (A. Milne-Edwards, 1873) (Decapoda, Brachyura, Ocypodidae), from the Bay of Bengal, Indian Ocean. Zool Stud **58:**12. doi:10.6620/ZS.2019.58-12.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics **30:**1312–1313. doi:10.1093/bioinformatics/btu033.

Schniebs K, Gloer P, Vinarski MV, Hundsdoerfer AK. 2013. Intraspecific morphological and genetic variability in the European freshwater snail *Radix labiata* (Rossmaessler, 1835) (Gastropoda: Basommatophora: Lymnaeidae). Contrib Zool **82:**55–68. doi:10.1163/18759866-08201004.

Sulikowska-Drozd A. 2007. Malacofauna of a city park – turnover and persistence of species through 40 years. Folia Malacol **15:**75–81.

Szybiak K. 2004. Terrestrial gastropods of the park in Obrzycko. Folia Malacol **12:**73–77.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:**4673–4680.

Troncoso-Palacios J, Esquerré D, Urra FA, Díaz HA, Castro-Pastene C, Ruiz MS. 2018. The true identity of the new world iguanid lizard *Liolaemus chillanensis* Müller and Hellmich 1932 (Iguania: Liolaemidae) and description of a new species in the *Liolaemus elongatus* group. Zool Stud **57:**22. doi:10.6620/ZS.2018.57-22.

Tulli MC, Carmona DM, López AN, Manetti PL, Vincini AM, Cendoya G. 2009. Predation on the slug *Deroceras reticulatum* (Pulmonata: Stylommatophora) by *Scarites anthracinus* (Coleoptera: Carabidae). Ecología Austral **19:**55–61.

Wade CM, Mordan PB, Naggs F. 2006. Evolutionary relationships among the Pulmonate land snails and slugs (Pulmonata, Stylommatophora). Biol J Linn Soc **87:**593–610. doi:10.1111/j.1095-8312.2006.00596.x.

Welter-Schultes FW. 2012. European non-marine molluscs: a guide for species identification. Planet Poster Ed., Gottingen.

Wiktor A. 1989. Limacoidea et Zonitoidea nuda. Ślimaki pomrowiokształtne (Gastropoda: Stylommatophora). (Fauna Polska). Warszawa: Państwowe Wydawnictwo Naukowe.

Wiktor A. 2000. Ariolimacidae (Gastropoda: Pulmonata): a systematic monograph. Ann Zool **49:**347–590.

Wiktor A. 2004. Ślimaki lądowe Polski, Mantis, Olsztyn, pp. 1–303.

Wiktor A, Jurkowska J. 2007. A collection of terrestrial slugs (Gastropoda: Pulmonata) at the Museum of Natural History, Wrocław University (Poland). Folia Malacol **15:**83–93. doi:10.12657/folmal.015.010.

Zając KS, Proćków M, Zając K, Stec D, Lachowska-Cierlik D. 2020. Phylogeography and potential glacial refugia of terrestrial gastropod *Faustina faustina* (Rossmässler, 1835) (Gastropoda: Eupulmonata: Helicidae) inferred from molecular data and species distribution models. Org Divers Evol. doi:10.1007/s13127-020-00464-x.

Zhou W, Yang H, Ding H, Yang S, Lin J, Wang P. 2017. Population genetic structure of the land snail *Camaena cicatricosa* (Stylommatophora, Camaenidae) in China inferred from mitochondrial genes and ITS2 sequences. Sci Rep **7:**15590. doi:10.1038/s41598-017-15758-y.

Supplementary Materials

Table S1. GenBank accession numbers of DNA sequences used to design specific primers for three molecular markers (*COI*, ITS-2, 28S rRNA). (download)

Table S2. Concentrations of chemicals for PCR reactions. (download)

Table S3. PCR profiles. (download)

Table S4. Genetic *p*-distances between available *COI* sequences of *Deroceras* species. (download)