Received: 1 August 2020 | Revised: 5 October 2020 | Accepted: 6 October 2020

DOI: 10.1111/zsc.12458

ORIGINAL ARTICLE

Zoologica Scripta | WILEY

Speciation in sympatric species of land snails from the genus *Trochulus* (Gastropoda, Hygromiidae)

Małgorzata Proćków[1] | **Elżbieta Kuźnik-Kowalska**[2] | **Joanna R. Pieńkowska**[3] | **Aleksandra Żeromska**[4] | **Paweł Mackiewicz**[4]

[1]Museum of Natural History, University of Wrocław, Wrocław, Poland

[2]Department of Invertebrate Systematics and Ecology, Wrocław University of Environmental and Life Sciences, Wrocław, Poland

[3]Department of Cell Biology, Adam Mickiewicz University, Poznań, Poland

[4]Department of Bioinformatics and Genomics, University of Wrocław, Wrocław, Poland

Correspondence
Małgorzata Proćków, Museum of Natural History, University of Wrocław, ul. Sienkiewicza 21, 50–335 Wrocław, Poland.
Paweł Mackiewicz, Department of Bioinformatics and Genomics, University of Wrocław, ul. F. Joliot-Curie 14a, 50-383 Wrocław, Poland.
Email: malgorzata.prockow@uwr.edu.pl (MP); pamac@smorfland.uni.wroc.pl (PM)

Funding information
Wrocław Centre for Networking and Supercomputing, Grant/Award Number: 307; Narodowe Centrum Nauki, Grant/Award Number: 2016/21/B/NZ8/03022

Abstract

The identification and designation of land snail species in the genus *Trochulus* on the basis of shell characteristics are problematic because of their great phenotypic plasticity. Some genetic analyses have proved inconclusive, with much variation within populations and apparent gene flow among them. We examined this issue by morphometric and molecular approaches on the morphologically similar species *T. coelomphala*, *T. hispidus* and *T. striolatus*, co-occurring in the Alpenvorland of Germany. While these species differed in shell and reproductive system morphology, there were forms that turned out intermediate in shell characters between *T. coelomphala* and *T. hispidus* but had genital morphology similar to *T. coelomphala*. Phylogenetic analysis, however, showed that these forms clustered neither with *T. coelomphala* nor *T. hispidus* but are sister to *T. striolatus* from the same region, which suggests that they evolved by way of sympatric speciation. Further, these analyses suggest that *T. coelomphala* diverged within *T. hispidus*; a crossing experiment indicated that they were interfertile. Expanding the study to include all available *Trochulus* sequences enabled us to infer evolutionary relationships between them and showed that *T. hispidus* is polyphyletic. Some *Trochulus* samples of one nominal species were grouped within others. The combination of phenotypic plasticity and possible mitochondrial DNA introgression illustrates the complex nature of evolutionary processes and the need for caution in the application of traditional taxonomic practice.

KEYWORDS

cross experiments, Europe, genital anatomy, phylogeny, shell morphology, species limits

1 | INTRODUCTION

While modern methods of molecular phylogeny have done much to increase our knowledge of the process of speciation, many confusing or unresolved issues persist. Although allopatric speciation remains the most plausible explanation in most cases and is often confirmed by the cooperation of molecular and morphological approaches, there are cases where speciation in sympatry is likely. Computer modelling revealed that sympatric speciation is an intrinsic property of the expanding populations with differentiated inbreeding, that is, higher at the edges and lower inside the occupied territory (Waga et al., 2007). Small or highly inbred populations with low crossover rate prefer a reproductive strategy complementing defective alleles by wild alleles, which could facilitate the sympatric speciation (Zawierta et al., 2008). However, uncontroversial empirical cases of this process remain scarce (e.g. Bird et al., 2012; Fruciano et al., 2016; Papadopulos et al., 2011). Recent studies have also raised questions 'how' and 'how often' speciation with gene flow (sympatric and parapatric) occurs in nature (Barluenga & Meyer, 2010), and which ecological

and genetic conditions are behind the given speciation case (Bolnick & Fitzpatrick, 2007; Kisel & Barraclough, 2010). One approach towards a better understanding of speciation is the study of incipient species and lineages that are in the process of splitting (Coyne & Orr, 2004). The roles of hybridisation and introgression are not fully understood, and any one study can provide only a single snapshot of a complex and continuously changing interaction between individuals and populations (Abbott et al., 2013). Studies on the land snail species in the genus *Trochulus* have already been used to approach these problems. Species delimitation based on shell characters has proved problematic, resulting in uncertainty about the validity of taxa described (Duda et al., 2014; Naggs, 1985; Perrin et al., 1984; Proćków, 2009; Proćków et al., 2013, 2014). This is mainly because of small interspecific differences and a high intraspecific variation reflected in a substantial diversity in shell size and shape. The morphological differences between conspecific populations can be due to environmental or adaptational modifications, which are not congruent with their phylogenetic history. In consequence, estimating species diversity of *Trochulus* over their distribution range in many cases has proven difficult (Proćków et al., 2013, 2014, 2017c). For example, the shell size of *T. striolatus* is mostly a response to prevailing local environmental and/or climate variables, and the shell features do not justify the recognition of its subspecies (Proćków et al., 2017b). Recent or ongoing gene flow between some *Trochulus* taxa may also explain an intricate evolutionary history of this genus (Proćków et al., 2017c).

Trochulus hispidus (Linnaeus, 1758) is the type and the most widespread species of its genus (Kerney et al., 1983). Its conspicuous shell variation resulted in descriptions of many species with very subtle differences; in France alone within '*Helix hispida* group', 15 species were distinguished (Locard, 1894). Although they were later synonymised to *Fruticicola hispida* (Germain, 1929), the number of species morphologically resembling *T. hispidus* is far from being agreed (Anderson, 2005; Duda et al., 2014; Welter-Schultes, 2012). Moreover, recent studies have provided an even more confusing picture, revealing that *T. sericeus/hispidus* constitutes a complex of morphologically similar but genetically divergent species in the Sarine valley in Swiss Western Prealps (Dépraz et al., 2009) and the snails morphologically resembling *T. sericeus* sometimes form genetically separate clades (Duda et al., 2014; Proćków et al., 2014, 2017c) or conversely, two morphologically different shells assigned to *T. hispidus* and *T. sericeus* do not form phylogenetically distinct clades (Duda et al., 2014; Proćków et al., 2013); hence, these snails are often described as the *T. hispidus* complex (Kruckenhauser et al., 2014). Furthermore, *T. hispidus* and *T. sericeus* appeared to be phenotypically plastic and showed no interbreeding constraints (Proćków et al., 2017a). Since their shell morphology strictly depends on microhabitat, they were regarded ecophenotypes (Proćków et al., 2018). The genus *Trochulus* appears to have a complex evolutionary history, which includes frequent interspecific gene flow and permeable species barriers (Proćków et al., 2017c). Nevertheless, the mechanisms of its speciation are still not sufficiently understood (Kruckenhauser et al., 2014).

One nominal taxon involved in the *T. hispidus* complex is *Helix coelomphala*, named by Locard (1888) to describe one of two forms of *Helix caelata* Studer, 1820, which came from many localities in Switzerland, Germany and eastern France, and most likely represented different species. This author also ascribed *Fruticicola coelata*, revised by Clessin (1874), to a newly described species. Subsequently, current *T. coelomphala* was combined with *T. caelatus* and used as a synonym of *T. hispidus* (Germain, 1929). It was also confounded with large forms of *T. hispidus* or *T. concinnus* (Ehrmann, 1933; Forcart, 1965; Geyer, 1909). Finally, unavailability of specimens of the nominal species forced Proćków (2009) to classify it as a synonym of *T. caelatus* after Germain (1929). Welter-Schultes (2012), however, tentatively classified it to *T. striolatus,* because 'Locard (1888) saw it in the vicinity of *striolata* and others'. The recent analysis of microsatellite sequences revealed a low genetic differentiation of few *T. coelomphala* populations, indicating also gene exchanges with other *Trochulus*. In addition, these populations not always are unequivocally distinguished from other *Trochulus* species in shell and genital morphometry (Proćków et al., 2017c). In his original description, Locard (1888) generally considered *T. coelomphala* shells as strongly flattened with the diameter exceeding 8 mm, and with a very wide umbilicus. While the range of this nominal species is unknown, the original description indicates that it is present in Augsburg and the Danube valley in Germany. Similar shells were also found near Dornbirn in Vorarlberg in Austria (Falkner, 1990; Forcart, 1965; Geyer, 1909). On CLECOM and Fauna Europaea checklists, *T. coelomphala* is given as occurring in Germany and Austria (Bank, 2011; Falkner et al., 2001), whereas on the Austrian Red List, this species has an annotation Data Deficient (Reischütz & Reischütz, 2007). Its occurrence in France is neither confirmed nor refuted (Welter-Schultes et al., 2011).

As a further step in elucidating the processes of speciation in *Trochulus,* we have examined the morphology and phylogeny of sympatric and syntopic populations of *T. coelomphala*, *T. hispidus* and *T. striolatus* from the Alpenvorland of Germany. We have then related these findings to the phylogeny of the genus as a whole and conducted a hybridisation experiment between the morphologically different species, *T. coelomphala* and *T. hispidus*.

2 | MATERIALS AND METHODS

2.1 | Sampling, microhabitat description, shell and genital morphometry

All apparently adult and subadult individuals of *Trochulus* were sampled along the Danube valley in Germany (Figure 1, ca. 270 km), where the potential distribution range of *T. coelomphala* was reported (Falkner, 1990). Additionally, *T. hispidus* individuals from two other sites in Germany (Bavaria) and three in Austria (Saltzkammergut, Tyrol and Vorarlberg) were analysed (Table 1, Figure 1) to compare their morphology and genetics with the Danubian snails. Altogether 20 samples were collected between June and August in 1998, 2010, 2011 and 2013 (Table 1). All the material is deposited in the Museum of Natural History in Wrocław in Poland. In order to ascertain the occurrence of *T. coelomphala* in France, the following locations originally mentioned by Locard (1888), were inspected: the vicinity of Grenoble, Sassenage and Grande-Chartreuse in Isère department; Bief-du-Fourg, Saint-Claude and Poligny in Jura department. As a result, only snails of *T. hispidus, T. phorochaetius* and *T. montanus* were found at three sites, Grande-Chartreuse, Sassenage and Poligny, and then included in previous morphological and genetic analyses (Proćków et al., 2013, 2014). We also searched Locard's (1888) locations in Germany away from the Danube valley, that is, Dillingen near Saarlouis and Dinkelsbühl, where only *T. hispidus* was found. Searches for this species in Vorarlberg in Austria were also unsuccessful. The taxonomic assignment of all individuals was done a priori, based on the morphological traits of shell and genitalia, but this information was only used a posteriori to check the consistency between the morphological and molecular approaches. The morphological identification was done based on the original description (Locard, 1888). Microhabitat conditions were assessed using the most abundant herbaceous plant species recorded at each sampling site to infer mean indicator values of light, temperature, moisture, acidity and nitrogen (Ellenberg et al., 1991).

Shells with at least five whorls were recognised as adult. From a frontal view (Figure S1a), we measured height (H), width (W), body whorl height (bwH), aperture height (h) and aperture width (w). From below (Figure S1b), we made measurements of the umbilicus major diameter (U) (i.e. the longest diameter parallel with the shell diameter, D), the umbilicus minor diameter (u) (i.e. perpendicular to the umbilicus major diameter) and the shell diameter (D) were taken. Finally, the number of whorls (whl) were counted according to Ehrmann's (1933) method. Moreover, the following coefficients of shell proportions were calculated: the height/width ratio (H/W), the relative height of body whorl = the body whorl height/shell height ratio (bwH/H), the relative umbilicus diameter = the umbilicus major diameter/shell diameter ratio (U/D) and the ratio of umbilicus minor to its major diameter (u/U).

Altogether, 510 specimens were measured in standardized views (Proćków, 2009) by the same person (M.P.), using the graduated eyepiece of a stereomicroscope with the accuracy of 0.1 mm. As the systematic measurement error with 1% error probability does not compromise results (Duda et al., 2011), the specimens were measured once, and then statistical parameters were calculated. To reduce the number of highly correlated predictors, we computed pairwise Pearson correlation coefficients involving

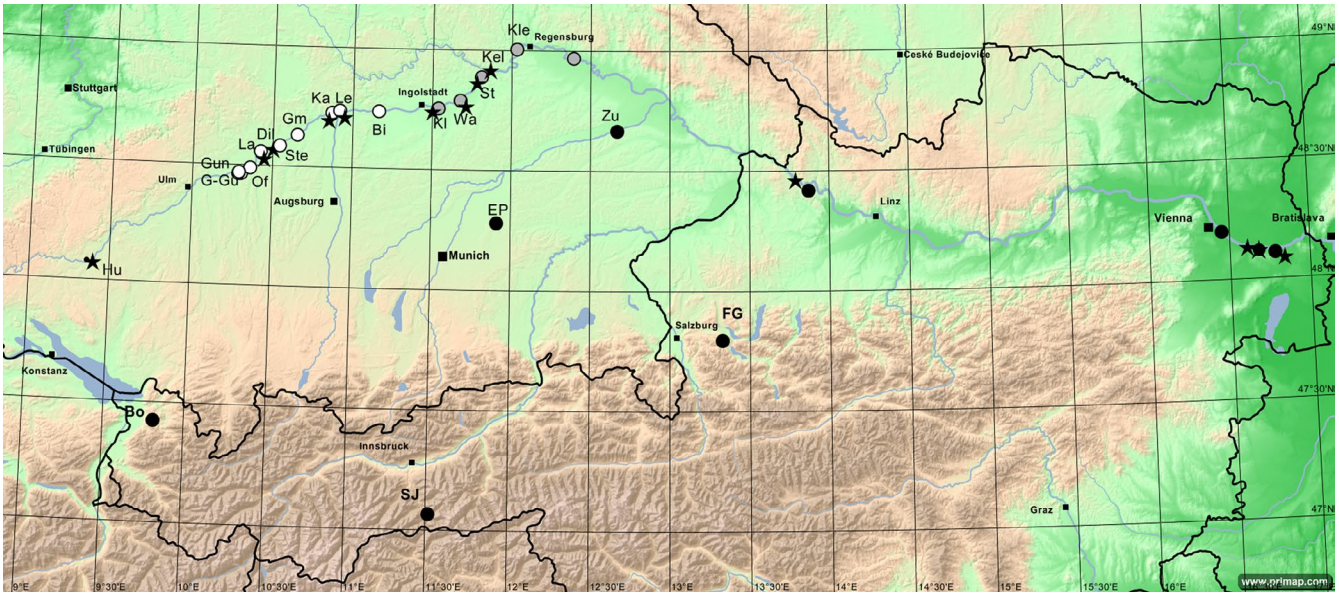


FIGURE 1 Geographical locations of new sites examined along the Danube. Acronyms are as defined in Table 1; black star – *Trochulus striolatus*, white circle – *T. coelomphala*, grey circle – *T. hispidus/coelomphala*, black circle – *T. hispidus* [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Characteristics of studied localities

Locality	Acronym	Light/moist[b]	Conchologically determined taxa	Anatomically determined taxa	Alt	Coordinates	N_g	N_m	N_a
Hundersingen	Hu	5.75/5.00	*Trochulus striolatus danubialis*	*T. striolatus*	556	48°04'14.9"N 09°23'45.9"E	5	70	16
Günzburg, Reisensburg[a]	G-Gu	4.86/6.17	*T. coelomphala*	*T. coelomphala*	436	48°27'56.8"N 10°18'05.9"E	4	8	2
Günzburg[a]	Gun	6.33/5.50	*T. coelomphala*	*T. coelomphala*	442	48°27'56.1"N 10°17'08.7"E	8	31	15
Offingen	Of	6.13/7.22	*T. coelomphala*	*T. coelomphala*	439	48°29'27.0"N 10°21'33.8"E	3	5	3
Lauingen	La	5.25/6.40 5.00/5.67	*T. coelomphala* *T. striolatus danubialis*	*T. coelomphala* *T. striolatus*	443	48°33'38.8"N 10°25'13.3"E	5 4	18 28	9 6
Dillingen a/d Donau[a]	Dil	4.00/5.67	*T. striolatus danubialis*	*T. striolatus*	392	48°34'06.4"N 10°29'59.8"E	7	35	2
Steinheim	Ste	6.75/7.17	*T. coelomphala*	*T. coelomphala*	443	48°35'04.9"N 10°32'21.7"E	4	4	1
Gremheim	Gm	5.86/6.29	*T. coelomphala*	*T. coelomphala*	445	48°37'57.5"N 10°39'03.1"E	3	7	3
Kaisheim	Ka	5.67/7.00	*T. coelomphala* *T. striolatus danubialis*	*T. coelomphala* *T. striolatus*	401	48°43'41.0"N 10°51'59.8"E	8 2	31 2	19 2
Lechsend	Le	6.56	*T. coelomphala* *T. striolatus danubialis*	*T. coelomphala* *T. striolatus*	420	48°44'18.4"N 10°54'44.8"E	3 7	13 29	2 10
Bittenbrunn	Bi	5.33/5.80	*T. coelomphala*	*T. coelomphala*	383	48°44'22.0"N 11°09'42.9"E	5	-	-
Kleinmehring	Kl	4.8/6.0	*T. hispidus/ coelomphala* *T. striolatus*	*T. coelomphala* *T. striolatus*	378	48°45'25.9"N 11°32'09.8"E	4 9	4 26	4 11
Wackerstein	Wa	6.50/7.50	*T. hispidus/ coelomphala* *T. striolatus*	*T. coelomphala* *T. striolatus*	336	48°47'19.8"N 11°40'32.4"E	7 7	33 28	9 8
Staubing	St	6.00/7.00	*T. hispidus/ coelomphala* *T. striolatus*	*T. coelomphala* *T. striolatus*	355	48°53'21.7"N 11°48'24.4"E	17 1	74 1	17 1
Kelheim	Kel	6.17/7.00	*T. striolatus*	*T. striolatus*	350	48°54'54.5"N 11°51'58.1"E	7	6	4
Zulling	Zu	-	*T. hispidus*		345	48°40'00.8"N 12°39'52.3"E	2	-	-

(Continues)

TABLE 1 (Continued)

Locality	Acronym	Light/moist[b]	Conchologically determined taxa	Anatomically determined taxa	Alt	Coordinates	N_g	N_m	N_a
Erding-Pretzen	EP	-	*T. hispidus*		464	48°16'49.6"N 11°54'27.2"E	5	-	-
Fuschl/St. Gilgen	FG	-	*T. hispidus*	*T. hispidus*	760	47°47'46.8"N 13°19'30.9"E	4	19	8
St. Jodok	SJ	-	*T. hispidus*	*T. hispidus*	1,192	47°04'00.2"N 11°30'49.0"E	3	30	7
Bödele	Bo	-	*T. hispidus*	*T. hispidus*	1,140	47°25'24.8"N 09°48'27.9"E	7	8	6

Abbreviations: N_a, the number of anatomically investigated specimens; N_g, the number of genetically investigated specimens; N_m, the number of morphologically investigated specimens.

[a]Topotypes.

[b]The mean indicator values of light and moisture (Ellenberg et al., 1991)

all variables and next reduced the number of the most correlated parameters. Based on these results, we found four shell variables (W, H, D and u) to remove because they were characterized by the largest correlation (>0.9). These procedures allowed us to reduce the number of redundant variables and leave the best predictors describing differences between the examined morphospecies. Additionally, hairs were inspected in all live-collected adults (n = 273), and their durability was recorded as: 0, no hairs; 1, present including different stages from only a few hairs to more hairs regularly covering the whole shell.

For anatomical examinations, 163 mature snails (Table 1) were dissected and theit external genital morphology was observed. Seven measurements of genitalia were taken including the length of flagellum (fl), epiphallus (ep), penis (p), bursa copulatrix (sl), bursa copulatrix duct (sd), upper vagina (=the distance between outlet of mucous glands and tips of inner dart sacs) (uv) and the width of bursa copulatrix (sw). The relative length of inner to outer dart sacs (is/os) was also recorded by measuring the distance between the tips of inner and outer dart sacs. Coefficients of the following proportions were included in the statistical analysis: flagellum/epiphallus (fl/ep), epiphallus/penis (ep/p), bursa duct length/bursa length (sd/sl) and bursa width/length (sw/sl). Additionally, cross-sections of penial papilla were examined to record the patterns of plicae. Altogether 54 snails, including 21 specimens of *T. striolatus*, 17 *T. coelomphala*, 9 *T. hispidus* and 7 *T. coelomphala/hispidus*, were analysed.

A canonical discriminant analysis (CDA) on shell and genital measurements was performed. Post hoc ANOVA analysis of group differences was performed with the Kruskal–Wallis nonparametric test. STATISTICA PL 12 (Stat Soft, Inc. 1984–2014) was used for the statistical analyses of the data.

2.2 | Cross experiments

Cross experiments were carried out between specimens belonging to 'typical' individuals of *T. hispidus* and 'typical' *T. coelomphala*, which grouped in different sub-clades in the phylogenetic trees based on mitochondrial markers. *T. hispidus* was collected from Wrocław in Poland and *T. coelomphala* from Gremheim in Germany. The snails used in the experiments were collected as juveniles (3.0–4.75 whorls) to avoid prior mating experience and thus contamination with stored sperm. They were paired and crossed in the following combinations: 30 pairs of interspecific crosses (*T. hispidus* × *T. coelomphala*) and 10 of each conspecific crosses (the control). In 13 of the 30 interspecific pairings (43%), one or both snails died before reaching sexual maturity and therefore only 17 pairs were available. To exclude a possible self-fertilization, six individuals from each cross type were raised alone.

Snails were kept in plastic containers measuring 7 × 6 × 5 cm and 12 × 7 × 5 cm, whose bottoms were covered with tissue paper and moist soil to encourage egg-laying. Additionally, litter brought from the habitat was used as substratum. Dolomite tablets were served as a supplementary source of calcium. Snails were maintained in a climate chamber on a light/dark 12/12 photoperiod at 22°C and 15°C, respectively, and 80% relative humidity. Food, for example, lettuce and carrot, was provided depending on needs.

The containers were cleaned and checked weekly for eggs. The eggs were counted and placed in separate Petri dishes lined with damp tissue paper and moist soil to avoid desiccation until they hatched, and then they were checked for hatching success. The survivorship of juveniles was assessed by counting them every four weeks. The reproductive data of all crosses were compared using Kruskal–Wallis nonparametric analysis of variance (ANOVA) with STATISTICA PL 12 (Stat Soft, Inc. 1984–2014).

2.3 | DNA extraction, PCR amplification and sequencing

The whole body of snails or foot tissue from ethanol preserved specimens were used for total DNA extraction, using Tissue Genomic DNA extraction Mini Kit (*Genoplast*) in accordance with the procedure provided by the manufacturer. In total, 141 individuals were used for this analysis, among them 21 *T. hispidus*, 43 *T. coelomphala*, 49 *T. striolatus* and 28 *T. hispidus/coelomphala* (Table 1). *COI* and *16S rDNA* sequences were obtained from 62 samples, whereas *H3*, *28S rDNA* and *ITS*2 sequences from 61 samples. Additionally, a parental pair of *T. hispidus* and *T. coelomphala* and their three offspring, bred in the laboratory, were genetically analysed. The sequences determined in this study are deposited at GenBank under the accession numbers: *COI*: MT754796-MT754857, *16S rDNA*: MT755517-MT755578, *H3*: MT758611-MT758671, *28S rDNA: MT755456-MT755516* and *ITS2: MT755395-MT55455*.

The purified total DNA was used as a template in a set of polymerase chain reactions (PCR). For subsequent phylogenetic analyses, there were amplified partial sequences of the following molecular markers: mitochondrial cytochrome *c* oxidase subunit I (*COI*) and 16S ribosomal DNA (*16S rDNA*) as well as nuclear sequences of histone 3 (*H3*), 28S ribosomal DNA (*28S rDNA*) and the whole internal transcribed spacer 2 (*ITS*2) of rDNA, flanked by 5.8S ribosomal DNA (*5.8S rDNA*) and 28S ribosomal DNA (*28S rDNA*).

The 5'-end fragment of *COI* (often called a barcode sequence) was amplified using primers and procedure described earlier (Dabert et al., 2010; Pieńkowska et al., 2018). The amplification reaction of *16S rDNA* fragment was conducted according to Manganelli et al. (2005). The DNA fragment of *H3* was amplified in accordance with Colgan et al. (1998). The fragment of *28S rDNA* was amplified with primers and the modified protocol published by Jovelin and Justine (2001). The *ITS2* sequences were amplified according to the procedure described by Almeyda-Artigas et al. (2000).

All PCR products were corroborated by 1% agarose gel electrophoresis. Prior to sequencing, samples were purified with thermosensitive Exonuclease I and FastAP Alkaline Phosphatase (Fermentas, Thermo Scientific). Finally, the amplified products were sequenced in both directions with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems) according to manufacturer's protocols.

2.4 | Phylogenetic analyses

Using BLAST searches (Camacho et al., 2009) of the GenBank database (https://www.ncbi.nlm.nih.gov/genbank), we found for the newly obtained sequences all available homologs, which were received in other studies (Caro et al., 2019; Dépraz et al., 2008; Duda et al., 2011; Kruckenhauser et al., 2014; Neiber & Hausdorf, 2015; Neiber et al., 2017; Pfenninger et al., 2005; Proćków et al., 2013, 2014; Saadi & Wade, 2019; Wade et al., 2001). We selected the sequences that were ascribed to four genera, that is, *Trochulus*, *Petasina*, *Edentiella* and *Noricella*, which are closely related and classified to Trochulini tribe (Neiber et al., 2017; Proćków et al., 2019). Short sequences were removed from the final set. The full list of the analysed sequences and their accession numbers are included in Tables S1-S4. The sequences were aligned in MAFFT (Katoh & Standley, 2013) using the slow and accurate algorithm L-INS-i with 1,000 cycles of iterative refinement and inspected in JalView (Waterhouse et al., 2009). In the case of identical sequences, we selected one representative but kept information about the annotation of all sequences, that is, taxonomic classification and locality of the found individual (Figure S2). Original taxonomic names as annotated in the database were presented in trees. We analysed in total six alignments consisting of five molecular markers, mitochondrial (*COI*, *16S rDNA*) and nuclear (*H3*, *28S rDNA*, *ITS*2), connected in various combinations: only mitochondrial, only nuclear and both types (Table 2, Tables S1-S4).

We applied three approaches in phylogenetic inferring: the maximal likelihood method in IQ-TREE (Nguyen et al., 2015), as well as two Bayesian analyses in MrBayes (Ronquist et al., 2012) and PhyloBayes (Lartillot et al., 2009). We checked the necessity of using separate nucleotide substitution models for individual partitions, that is, markers and three codon positions (Table 2). In IQ-TREE analyses, we used the substitution models proposed according to the associated ModelFinder programme (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017), whereas in MrBayes analyses,

TABLE 2 Analysed alignments and applied nucleotide substitution models in three approaches

Data set	Number of sequences	Number of sites	Nucleotide substitution models used in software		
			MrBayes	**PhyloBayes**	**IQ-TREE**
COI + *16S rDNA* (1)	314	946	mixed + I + Γ5 (1, 2 cp *COI*) mixed + Γ5 (3 cp *COI*) mixed + I+Γ5 (*16S rDNA*)	CAT-GTR + Γ5	TIM3 + F+I + Γ4 (1 cp *COI*) F81 + F+R2 (2 cp *COI*) TVM + F+Γ4 (3 cp *COI*) GTR + F+I + Γ4 (*16S rDNA*)
ITS2 + *28S rDNA* (1)	85	1,263	mixed + Γ5 (*ITS2*) mixed + I (*28S rDNA*)	CAT-GTR + Γ5	K3P + Γ4 (*ITS2*) TPM2u + F+I (*28S rDNA*)
COI + *16S rDNA* (2)	84	935	mixed + Γ5 (1 cp *COI*) mixed + I (2 cp *COI*) mixed + I+Γ5 (3 cp *COI*) mixed + I+Γ5 (*16S rDNA*)	CAT-GTR + Γ5	TN + F+Γ4 (1 cp *COI*) F81 + F+I (2 cp *COI*) GTR + F+Γ4 (3 cp *COI*) GTR + F+I + Γ4 (*16S rDNA*)
H3 + *ITS2*+*28S rDNA*	57	1529	mixed + I+Γ5 (1 cp *H3*, *28S rDNA*) mixed (2 cp *H3*) mixed + I (3 cp *H3*) mixed + I+Γ5 (*ITS2*)	CAT- Poisson + Γ5	TIM2 + F+R2 (1 cp *H3*, *28S rDNA*, *ITS2*) JC (2 cp *H3*) K3P + I (3 cp *H3*)
ITS2 + *28S rDNA* (2)	61	1,260	mixed + Γ5 (*ITS2*) mixed + I (*28S rDNA*)	CAT-GTR + Γ5	K3P + Γ4 (*ITS2*) TPM2 + F+I (*28S rDNA*)
COI + *16S rDNA* + *ITS2*+*28S rDNA*	92	2,195	mixed + Γ5 (1 cp *COI*) mixed + I (2 cp *COI*) mixed + I+Γ5 (3 cp *COI*) mixed + I+Γ5 (*16S rDNA*) mixed + I+Γ5 (*ITS2*) mixed + I+Γ5 (*28S rDNA*)	CAT-GTR + Γ5	TN + F+Γ4 (1 cp *COI*) F81 + F+I (2 cp *COI*) TPM2 + F+Γ4 (3 cp *COI*) GTR + F+I + Γ4 (*16S rDNA*) K2P + R3 (*ITS2*) TVM + F+R2 (*28S rDNA*)

Note: The alignments (1) included all available sequences, whereas the alignments (2) comprised the sequences obtained from the same individuals;

Abbreviation: cp, codon position.

based on the results of PartitionFinder (Lanfear et al., 2012). However, we applied mixed models rather than fixed ones to specify appropriate substitution models across the large parameter space (Huelsenbeck et al., 2004), but the models describing heterogeneity rate across sites (a proportion of invariant sites and the gamma-distributed rate variation) were adopted according to PartitionFinder. We considered all possible combination of partitions in PartitionFinder. In PhyloBayes, we applied the mixture model for across-site heterogeneities called CAT model (Lartillot & Philippe, 2004) for the partitioned data sets, with the number of components, weights and profiles inferred from the data.

Phylogenetic trees were calculated in IQ-TREE using a thorough and slower nearest neighbour interchange (NNI) tree search considering all possible NNIs as well as applied Shimodara-Hasegawa-like approximate likelihood ratio test (SH-aLRT) assuming 10,000 replicates and nonparametric bootstrap with 1,000 replicates. In MrBayes, we applied two independent runs starting from random trees, each using 32 and 4 (depending on the alignment set) Markov chains. The trees were sampled every 100 generations for 20,000,000 generations. In the final analysis, we selected trees from the last 5,011,000 to 13,919,000 (depending on the alignment set) generations that reached the stationary phase and convergence, that is, when the standard deviation of split frequencies stabilized and was much below the recommended threshold 0.01. In PhyloBayes, two independent Markov chains were run for 100,000 generations with one tree sampled for each generation. The last 10,000 to 95,000 trees (depending on the alignment set) from each chain were collected to compute posterior consensus trees after obtaining convergence, when the largest discrepancy observed across all bipartitions (maxdiff) was much below the proposed threshold 0.1.

Using IQ-TREE, we calculated the consensus of trees obtained in three approaches. The number of the trees supporting a given node were presented together with support values. Tests of alternative tree topologies based on the alignments of four markers were conducted in IQ-TREE assuming 1 million replicates using the RELL method. Phylogenetic trees were inspected and edited in TreeGraph (Stover & Muller, 2010).

3 | RESULTS

3.1 | Analysis of shell morphology and microhabitat conditions

Shell measurements of the examined species are shown in Table 3. The results of the canonical discriminant analysis (CDA) revealed a clear differentiation only between *T. striolatus* and 'typical' *T. coelomphala* and 'typical' *T. hispidus*, whereas intermediate forms of *T. hispidus/coelomphala* were placed roughly between these three groups. Some 'typical' *T. coelomphala* specimens also overlapped 'typical' *T. hispidus* (Figure 2). The first discriminant function captured most of the variance among the species (82.4%), which was much larger than the variance associated with the second function (15%). These two functions accounted for more than 97% of the total dispersion in ten predictor variables (Table 4). A sequential chi-square test showed that the first and the second functions significantly ($p < .001$) contributed to the population discrimination, whereas the contribution of the third function was less important but also significant ($p < .001$).

TABLE 3 Basic statistics of shell measurements (in mm) of studied *Trochulus* taxa

	T. coelomphala $n = 117$			*T. hispidus* $n = 57$			*T. hispidus/coelomphala* $n = 111$			*T. striolatus* $n = 225$		
Feature	**Range**	**Mean**	***SD***	**Range**	**Mean**	***SD***	**Range**	**Mean**	***SD***	**Range**	**Mean**	***SD***
W	7.64–12.00	9.13	0.83	6.48–8.60	7.73	0.52	7.09–11.82	9.67	0.86	9.09–14.00	11.02	0.86
H	3.82–6.18	4.69	0.47	3.70–5.38	4.62	0.47	4.0–6.73	5.31	0.51	5.64–8.18	6.74	0.54
bwH	3.27–5.64	4.12	0.37	3.19–4.51	3.85	0.36	3.63–5.45	4.54	0.38	4.73–7.09	5.58	0.43
h	2.36–4.36	3.12	0.31	2.20–3.41	2.83	0.26	2.55–4.18	3.43	0.34	3.27–5.09	4.09	0.41
w	3.09–5.45	4.19	0.38	3.19–4.51	3.81	0.30	3.64–5.82	4.67	0.46	4.36–7.09	5.56	0.48
D	7.64–11.45	8.98	0.81	6.48–8.80	7.66	0.52	7.09–11.45	9.52	0.88	9.09–14.00	10.88	0.86
U	1.64–3.09	2.23	0.35	0.99–2.00	1.43	0.24	1.27–2.91	2.03	0.35	1.09–3.45	1.72	0.38
u	1.27–2.91	1.93	0.33	0.88–1.60	1.24	0.19	1.27–2.91	1.84	0.31	0.91–2.36	1.54	0.31
whl	5.00–6.50	5.66	0.28	5.00–5.70	5.33	0.16	5.10–6.30	5.73	0.27	5.25–6.30	5.78	0.23
H/W	0.43–0.60	0.51	0.04	0.50–0.69	0.59	0.04	0.47–0.64	0.55	0.04	0.51–0.71	0.61	0.04
U/D	0.18–0.33	0.25	0.02	0.13–0.25	0.19	0.03	0.15–0.28	0.21	0.03	0.11–0.24	0.16	0.03
u/U	0.67–1.00	0.87	0.07	0.68–1.00	0.87	0.07	0.67–1.00	0.91	0.07	0.69–1.00	0.90	0.08
bwH/H	0.78–0.96	0.88	0.03	0.76–0.92	0.83	0.04	0.76–0.93	0.86	0.03	0.73–1.12	0.83	0.04

Note: For the explanation of feature abbreviations see Material and methods.

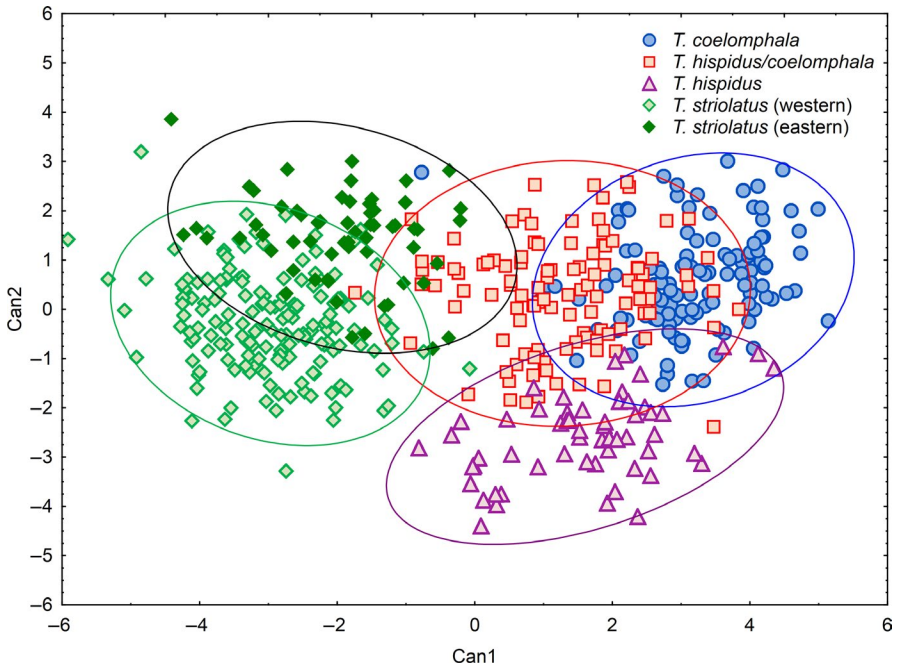


FIGURE 2 Canonical discriminant analysis (CDA) based on shell measurements of *Trochulus* taxa [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Canonical coefficients of discriminant analysis performed on shell measurements. The most contributing variables are in bold

	Standardised canonical discriminant function coefficients	
Variable	**Can 1**	**Can 2**
bwH	**−0.691**	**1.314**
U/D	**0.531**	0.283
H/W	0.099	**−1.289**
bwH/H	0.429	−0.476
whl	0.253	0.193
U	0.189	−0.006
u/U	0.147	0.199
w	0.043	−0.032
h	−0.167	0.174
W	−0.192	−0.734
Eigenvalue	6.301	1.144
Cum. Prop. (%)	82.4	97.3

Note: For the explanation of feature abbreviations see Material and methods.

The CDA plot (Figure 2) revealed conchological differences between *T. striolatus* populations collected in different regions along the Danube, that is, western group (sites Hu, La, Dil, Ka, Le) and eastern group (sites Kl, Wa, St, Kel) (Figure 1). In the plot, they created partially overlapped sets. The western group may be attributed to subspecies *T. s. danubialis* because it is characterised by a higher spire and a relatively narrower umbilicus. The group includes the type locality of this taxon, that is, Dillingen a.d. Donau (Clessin, 1874). The eastern group better corresponds to the nominal *T. striolatus,* with a larger umbilicus and more flattened spire. All these populations are located in the same climate region and inhabit very similar environments. However, considering their microhabitats, we found some differences between them. The western populations live in moister and shaded places, whereas the eastern ones are associated with drier and more illuminated microhabitats (Table 1). The other parameters, that is, temperature, acidity and nitrogen content did not differentiate these localities.

Detailed statistical analyses of *Trochulus* groups showed that the eastern group of *T. striolatus* was similar (Figure 3) to syntopic *T. hispidus/coelomphala* snails with an intermediate shell morphology. The latter differed in the largest number of shell measurements from *T. hispidus* (Table 5). *Trochulus coelomphala* and *T. striolatus* (western) were different in all but one feature. *Trochulus coelomphala* had a more flattened shell (bwH/H), larger absolute (U) and relative umbilicus diameter (U/D) than *T. hispidus/coelomphala*. The same traits also characterised *T. hispidus/coelomphala* compared to the western group of *T. striolatus*.

Considering the canonical coefficients of the first function, the highest loadings were found for the height of body whorl (bwH) and the relative umbilicus diameter (U/D) (Table 4). Using these characters, we also compared individual populations of collected samples. Based on bwH, two clear groups could be distinguished, that is, *T. striolatus* and the group consisting of other taxa (Figure 4a). The post hoc ANOVA test performed on bwH showed that only differences between *T. coelomphala* and *T. hispidus* were insignificant ($p = .195$). Regarding U/D, however, differences between all taxa were statistically significant ($p < .01$). In the box plot of the U/D ratio, four groups can be distinguished (Figure 4b). The first consists of populations

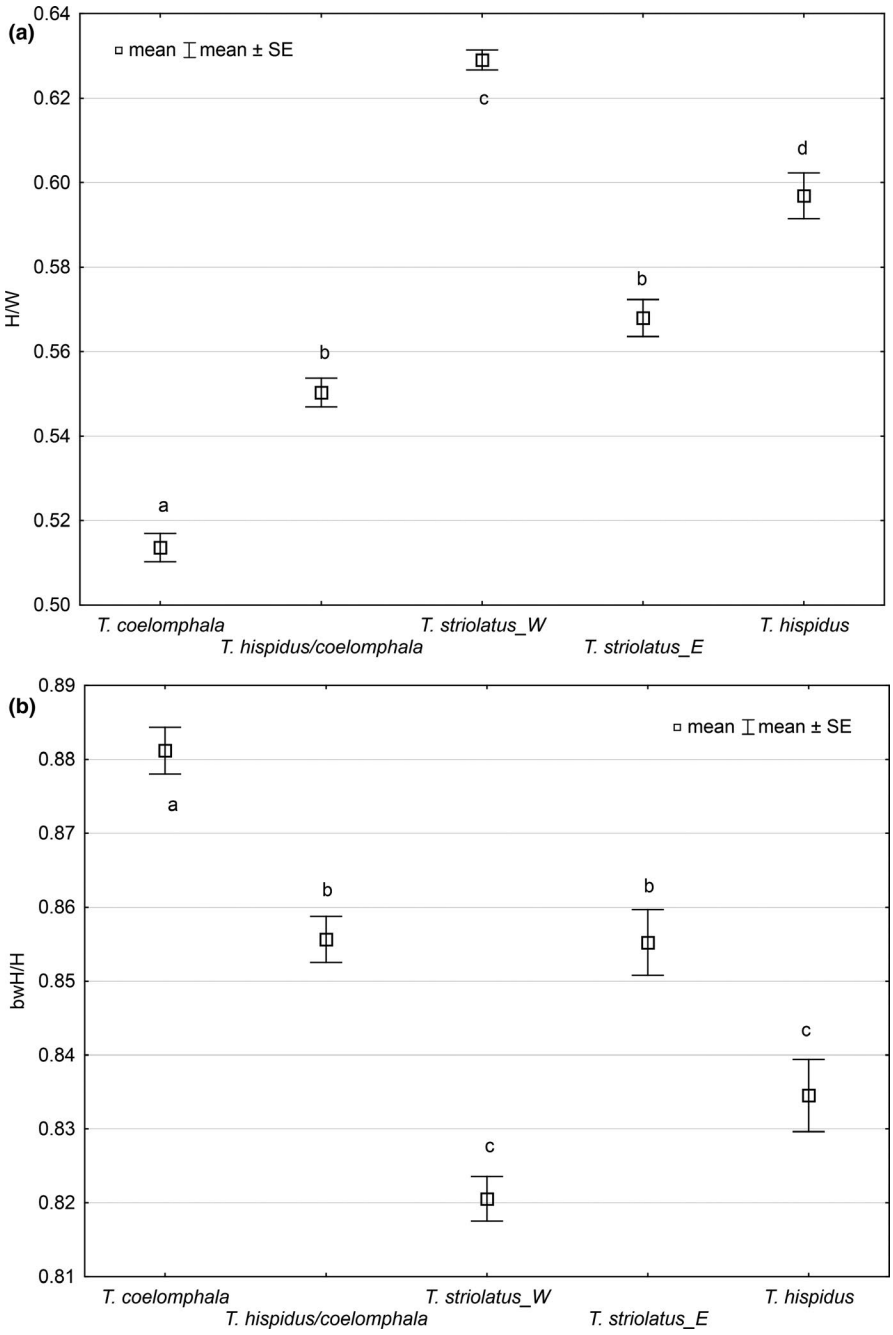


FIGURE 3 Height/width shell ratio H/W (a), and relative height of body whorl bwH/H (b) in *Trochulus*. Eastern and western groups of *T. striolatus* were considered separately. Letters a, b, c and d indicate groups that were significantly different in the Kruskal–Wallis test ($p < .05$)

belonging exclusively to 'typical' *T. coelomphala*, the second includes intermediate forms of *T. hispidus/coelomphala* and one population of 'typical' *T. hispidus*, the third groups some populations of *T. striolatus* and *T. hispidus,* and in the fourth group, there are all other populations of *T. striolatus*. The results show that 'typical' *T. coelomphala* is characterised by the largest relative umbilicus diameter and four populations of *T. striolatus* by the smallest. The intermediate position is occupied by the intermediate shell forms of *T. hispidus/coelomphala*, which originate from geographically close populations (Kl, Wa, St), from the easternmost part of the Danube river valley, in the area between Kleinmehring and Staubing (Figure 1).

The analysis of microhabitat conditions of 'typical' *T. coelomphala* and intermediate forms of *T. hispidus/coelomphala* did not reveal any differences between them considering light, temperature, moisture, acidity and nitrogen content. On the other hand, *T. striolatus* populations inhabiting more eastern and western regions at the Danube differing in local climatic conditions, were separated into two distinct groups in terms of the relative umbilicus diameter U/D (Figure 4b).

Considering hair durability, ca. 15% individuals of *T. hispidus* were completely deprived of hairs, whereas hairless specimens constituted no more than 61% and 71% of *T. coelomphala* and *T. hispidus/coelomphala* samples, respectively. No hairs were observed in 97% of live *T. striolatus*.

TABLE 5 Linear shell measurements (upper-right triangle) and coefficients (lower-left triangle) that significantly ($p < .05$) distinguish the given groups of *Trochulus*

	T. coelomphala	*T. hispidus/coelomphala*	*T. hispidus*	*T. striolatus* (western)	*T. striolatus* (eastern)
T. coelomphala		W, H, bwH, h, w, D, U, H/W, U/D, u/U, bwH/H	W, h, w, D, U, u, whl, H/W, U/D, bwH/H	W, H, bwH, h, w, D, U, u, H/W, U/D, u/U, bwH/H	W, H, bwH, h, w, D, whl, H/W, U/D, bwH/H
T. hispidus/coelomphala	0.018, 0.000, 0.000, 0.001, 0.000, 0.016, 0.017, 0.000, 0.000, 0.001, 0.000		W, H, bwH, h, w, D, U, u, whl, H/W, U/D, u/U, bwH/H	W, H, bwH, h, w, D, U, u, H/W, U/D, bwH/H	W, H, bwH, h, w, D, U/D
T. hispidus	0.000, 0.019, 0.032, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.002, 0.023, 0.007		W, H, bwH, h, w, D, u, whl, H/W, U/D	W, H, bwH, h, w, D, U, u, whl, H/W, bwH/H
T. striolatus (western)	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.007, 0.000	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.018, 0.000, 0.001, 0.000		W, D, U, u, H/W, U/D, bwH/H
T. striolatus (eastern)	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.001	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000	0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000, 0.032, 0.047	0.002, 0.003, 0.000, 0.000, 0.000, 0.000, 0.000, 0.000	

Eastern and western groups of *T. striolatus* were considered separately.

3.2 | Genital traits

The CDA of genital measurements did not reveal clearly separated groups (Figure 5). The first two canonical functions together explained 97.1% of the total variance and were statistically significant ($p < .001$), whereas the contribution of the third function was less important ($p < .05$). The length of bursa copulatrix duct (sd) appeared to be taxonomically most useful feature, which allowed to distinguish 'typical' *T. coelomphala* and *T. hispidus/coelomphala* from *T. striolatus* and 'typical' *T. hispidus* (Table 6, Figure 6a), whereas a short penis (p) discriminated 'typical' *T. hispidus* from all other taxa (Figure 6b). These results also showed that snails with intermediate shells, that is, *T. hispidus/coelomphala* correspond to 'typical' *T. coelomphala* based on both examined genital traits (Figure 5). In contrast, the length of bursa copulatrix duct (sd) in laboratory-bred hybrids (*T. hispidus* × *T. coelomphala*) is similar to *T. hispidus* (Figure 6a), whereas the length of penis (p) corresponds to *T. coelomphala* (Figure 6b).

Concerning the penial papilla structure observed in the cross-section, it was impossible to detect constant differences between all taxa examined. Its high variation in *T. striolatus* allowed to recognise three different types of fold patterns: smooth, with small protuberances and with large protuberances. Similar variation was also recorded within populations (Figure 7a-c). No differences were noticed between the western and eastern populations of *T. striolatus* differing in the relative umbilicus diameters (cf. Figure 4b). Similarly, no constant pattern could be distinguished between *T. coelomphala* and *T. hispidus*. Conversely, a high variability in both 'typical' species as well as in intermediate forms seems to be common (Figure 7d-f).

3.3 | Cross experiments

In 13 out of the 50 pairings (26%), one or both snails died before reaching sexual maturity. None of the snails kept alone reproduced. Therefore, autogamy or parthenogenesis can be excluded. Eggs were produced for all the conspecific pairs and for 8 pairs (47%) of interspecific crosses (Table 7). Life history traits were highly variable within each type of cross, but there were statistically significant differences in lifetime fecundity, number of clutches per pair and survivorship of juveniles at the 300th day (Table 7). The conspecific *T. coelomphala* pairs produced significantly more eggs and clutches compared to the control pairs of *T. hispidus* and the interspecific pairs. The intra-group variations of the eggs and clutches produced by a pair were lower for the conspecific *T. coelomphala* (ca. twofold and threefold, respectively) than for *T. hispidus* (20-fold and ninefold, respectively) and the interspecific pairs (39-fold and ninefold, respectively). The mean batch size was similar in

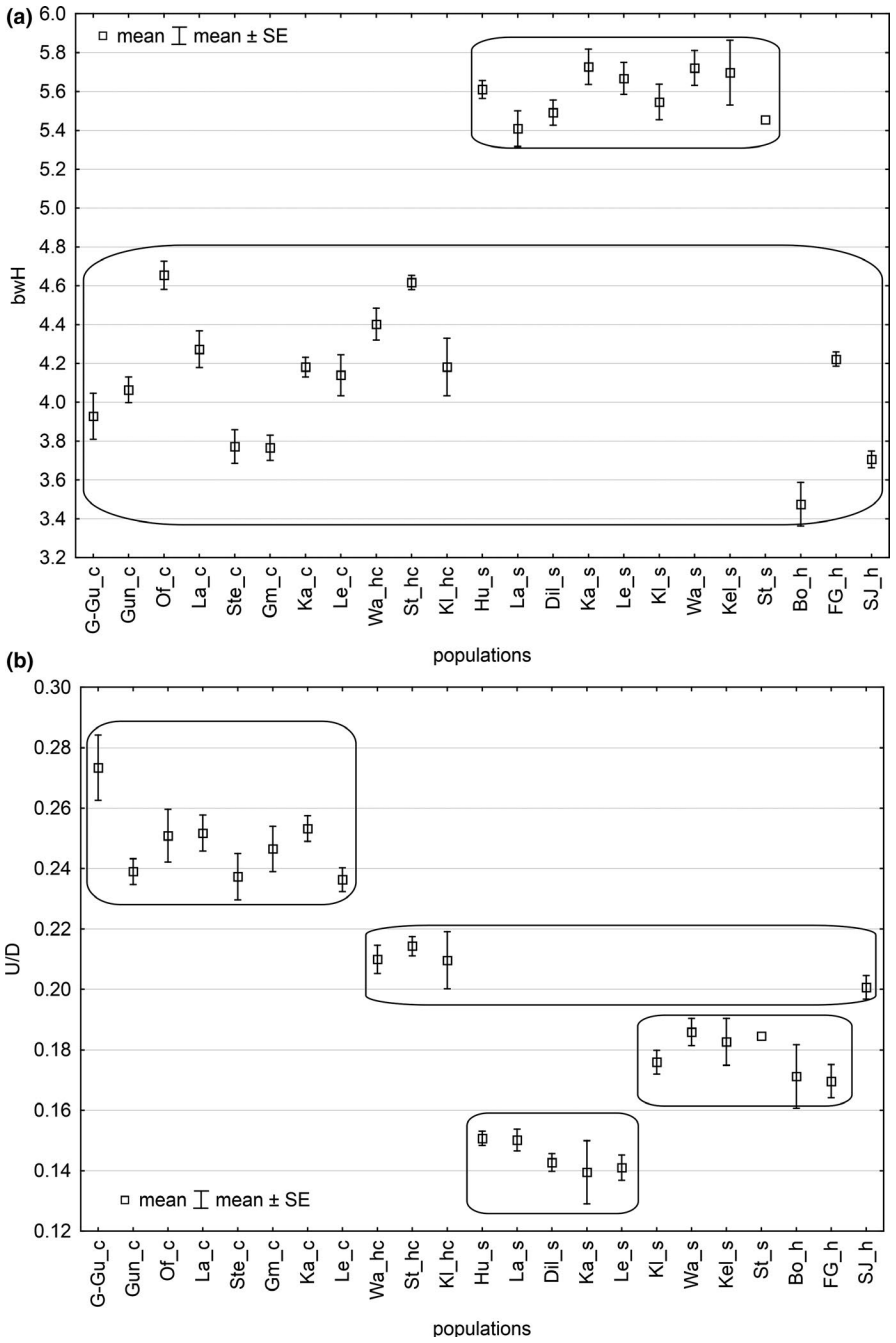


FIGURE 4 Variation of height of body whorl (a) and relative umbilicus diameter (b) in populations of *Trochulus* taxa. Abbreviations of localities are as defined in Table 1. Letters denote to: c – *Trochulus coelomphala*, h – *T. hispidus*, hc – *T. hispidus/coelomphala*, s – *T. striolatus*

all types of crosses. There were no statistically significant differences among cross types in their hatching success or survivorship until the age of the 150th day after hatching and they had similar standard variation values. It should be highlighted that the survivorship is the only life history trait that is greater in the hybrids than in both control pairs (Table 7).

3.4 | Phylogenetic analyses

In order to place the sequences obtained in a wide phylogenetic context, we assembled a comprehensive set of their homologs from Trochulini tribe. We analysed six data sets, considering mitochondrial and nuclear markers in one concatenated alignment and separately, because they could show different evolutionary histories. The alignments of *COI + 16S rDNA* and *ITS2 + 28S rDNA* were analysed in two versions assigned as (1) and (2). In the case of version (1), the sequences in each of these two alignments were obtained from the same individuals but not necessary the same individual in the both alignments. The version (2) involved individuals for which all four markers were available. Figure 8 and Figures S3–S7, show the consensus of trees obtained in three phylogenetic methods, whereas Figure S8 shows individual trees obtained in each. Figure 9

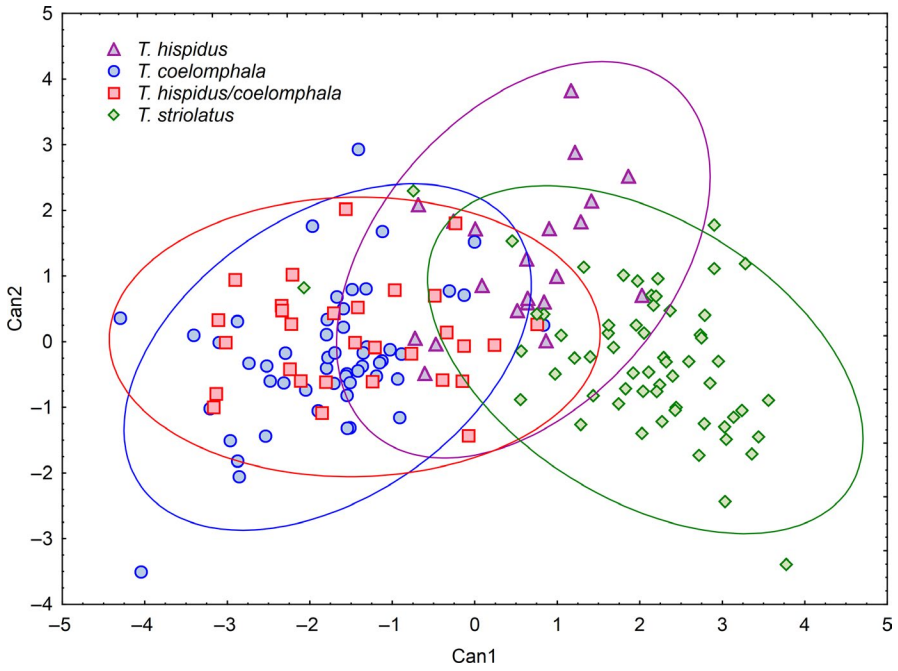


FIGURE 5 CDA based on genital measurements of *Trochulus* taxa [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 6 Canonical coefficients of discriminant analysis performed on genital measurements. The most contributing variables are in bold

	Standardised canonical discriminant function coefficients	
Variable	**Can 1**	**Can 2**
sl	−0.217	−0.278
ep/p	0.342	0.733
sd	**−0.614**	**1.306**
sd/sl	0.465	**−1.569**
ep	**−0.688**	−0.646
p	**0.754**	−0.099
uv	0.029	0.275
sw/sl	0.404	**1.064**
sw	−0.440	−0.807
Eigen value	3.098	0.253
Cum. Prop. (%)	89.7	97.1

Note: For the explanation of feature abbreviations see Material and methods.

shows the geographic distribution of all clades identified in the trees based on the newly obtained sequences as well as those studied previously (Caro et al., 2019; Dépraz et al., 2008; Duda et al., 2011; Kruckenhauser et al., 2014; Neiber & Hausdorf, 2015; Neiber et al., 2017; Pfenninger et al., 2005; Proćków et al., 2013, 2014; Saadi & Wade, 2019; Wade et al., 2001). Generally, the trees based on the mitochondrial markers were much better resolved than those inferred from nuclear ones. We obtained the best resolved phylogenies for the alignment including four markers (*COI + 16S rDNA + ITS2 + 28S rDNA*) (Figure 8) and the concatenated mitochondrial alignments (*COI + 16S rDNA*) (Figures S3 and S4). Therefore, the description below is mainly based on these data sets. We retained the original taxonomical names assigned to the sequences in the GenBank database.

Sequences assigned to *Trochulus* create a very well-supported clade separated from other members of Trochulini, that is, *Petasina*, *Noricella* and *Edentiella*, with the following support values: PP-M = 1, PP-P = .99–1, aLRT = 99, BP = 96–100, where PP-M is the posterior probability in MrBayes, PP-P is the posterior probability in PhyloBayes, aLRT is the support in Shimodara-Hasegawa-like approximate likelihood ratio test in IQ-TREE and BP is the bootstrap percentage in IQ-TREE.

Petasina is sister to *Trochulus* in all three trees, but in the tree based on the four markers, the earliest diverged lineage of *Trochulus* group includes *T. biconicus* with almost maximal support (PP-M = 1, PP-P = 1, aLRT = 99, BP = 100), whereas *Noricella* and *Edentiella* are grouped together (PP-M = 0.67, PP-P = .65, aLRT = 62, BP = 61). However, in the *COI + 16S rDNA* trees, *Noricella* is clustered with *T. biconicus* (PP-M = 0.97, PP-P = .99, aLRT = 73–88, BP = 52–81).

The next diverged clade A includes *T. clandestinus* from the Central Alps and a sequence described to *T. hispidus* from the North-western Alps in the four-markers tree (Figure 8) and *COI + 16S rDNA* (2) tree (Figure S4), and additionally, *T. clandestinus* from the North-western Alps, *T. montanus* and *T. caelatus* from the Jura Mountains and many other sequences from France, the Jura Mountains, the Rhine Valley as well as the Central and North-western Alps in the *COI + 16S rDNA* (1) tree (Figure S3). Generally, this clade has a more western distribution in comparison

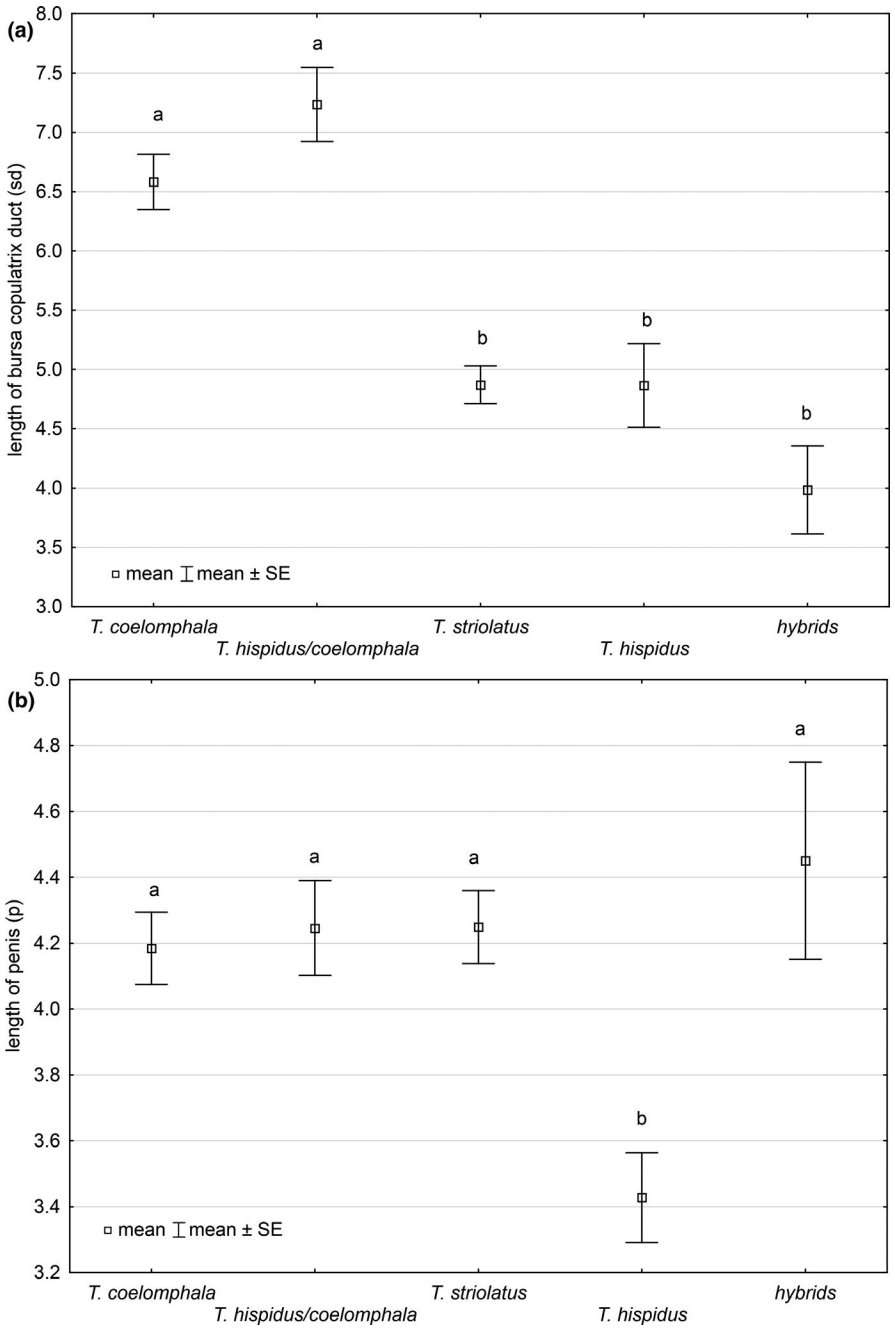


FIGURE 6 Whisker plot of the length of bursa copulatrix duct (a) and penis (b) in *Trochulus* taxa and lab hybrids *T. hispidus* × *T. coelomphala*. Letters a and b indicate groups that occurred significantly different in the Kruskal–Wallis test ($p < .05$)

to other *Trochulus* (Figure 9). Although this clade is rather moderately supported (PP-M = 0.84–0.99, PP-P = .57–0.68, aLRT = 83–88, BP = 64–76), the position of its sequences in respect to others in the tree is significant, because its sister group including the remaining *Trochulus* sequences is very well supported: PP-M = 1, PP-P = .99, aLRT = 95, BP = 87–91 in the four-markers tree (Figure 8) and *COI + 16S rDNA* (2) tree (Figure S4). The *COI + 16S rDNA* (1) tree also contains the maximally supported clade with many *T. villosus* sequences, which is sister to the highly significant group of other *Trochulus* samples (PP-M = 1, PP-P = .99, aLRT = 100, BP = 87) (Figure S3).

The branching order of several subsequently diverged clades is best resolved in the four-markers tree (Figure 8). These clades are also present and similarly supported in two-markers trees (Figures S3 and S4) but relationships between them are worse supported and the three phylogenetic methods did not always produce the same relationships between them. The clade B is maximally supported and consists of *T. hispidus* sequences from Alpenvorland and Tyrol (Figure 9). The clade C in the four-markers tree (Figure 8) includes sequences of *T. hispidus* from distant regions, that is, Spain and Sweden as well as the individuals used by us in crossbreeding experiments: *T. hispidus* (Poland) and

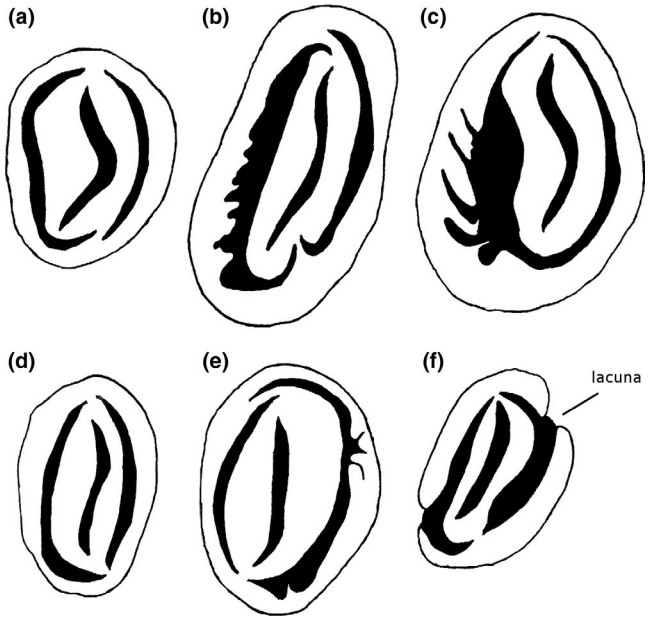


FIGURE 7 Cross-section patterns of penial papilla in *Trochulus* taxa. (a) *T. striolatus* with smooth folds (specimen Wa_36), (b) *T. striolatus* with folds containing small protuberances (specimen Wa_35), (c) *T. striolatus* with folds containing large protuberances (specimen Wa_34), (d) *T. hispidus* with smooth folds (specimen SJ_2), (e) *T. coelomphala* with folds containing small protuberances (specimen La_3), (f) *T. hispidus/coelomphala* with two lacunas on penial papilla (specimen St_4)

T. coelomphala (Alpenvorland in Germany) and their offspring (Figure 9). The position of the latter species is unexpected because it should cluster with other members of its species. This sample could be contaminated.

In the four-markers tree (Figure 8), the clade C diverged later after the clade B and is sister to other *Trochulus* sequences (PP-M = 0.90, PP-P = .91, aLRT = 96). In contrast to that, the sequences from the clades B and C are clustered together in two-markers trees (Figures S3 and S4), and the Spanish specimen is an outlier. In the tree based on *COI + 16S rDNA* (1) alignment (Figure S3), this grouping is highly supported: PP-M = 0.98, PP-P = .97, aLRT = 91, BP = 58, and also includes sequences from France and the Netherlands. Other clades of *Trochulus* are grouped together in three methods in the four-markers tree (Figure 8) but with poor support (PP-M = 0.82, PP-P = .94). The relationships between them are not well resolved and four phylogenetic methods did not always produce the same branching order. However, individual clades can be clearly recognized.

The clade D comprises *T. hispidus/sericeus* from the Vienna Basin, northern and central Germany (Figure 8 and Figure S4) as well as Hungary and Sweden in the *COI + 16S rDNA* (1) tree (Figure S3). Interestingly, there are individuals in this clade from Alpenvorland (Staubing) with an intermediate shell morphology between *T. hispidus* and *T. coelomphala*. They are significantly clustered with *T. hispidus* from the Franconian Mountains (Taubertal) with PP-M = 1, PP-P = 1, aLRT = 99–100, BP = 98–99. It suggests either an introgression of mitochondrial DNA from *T. hispidus* living in the Franconian Mountains to *T. hispidus/coelomphala* normally inhabiting the Alpenvorland region, or a similar morphological response of the same clade members to the local environment. These two localities are 190 km away. The maximally supported grouping of sequences from other remote sites, the Vienna Basin (Donauau) and Hungary (Duna-Dravak) in Figure S3 is also interesting. The distance between them is 450 km in a straight line. However, these sites are placed along the Danube valley and likely the snails were transferred with the river downstream for more than 630 km. In turn, the sample from Sweden (Västra Götaland) is clustered (PP-M = 1, PP-P = 1, aLRT = 82, BP = 77) with that from northern Germany (Lemsahl-Mellingstedt), located 540 km away. Thus, the main distribution of the clade D is likely in

TABLE 7 Results of no-choice experiments between *T. hispidus* (Th) and *T. coelomphala* (Tc)

Cross type	Th × Th	*n*	Tc × Tc	*n*	Th × Tc	*n*
Number of pairs	10		10		17	
Ovipositing pairs	10 (100%)		10 (100%)		8 ± (47.1%)	
Fecundity = eggs/pair	68.1 ± 48.8 (8–164)[a]	10	257.5 ± 55.0 (158–337)[b]	10	42.5 ± 21.9 (2–78)[a]	8
Clutches/pair	5.4 ± 3.3 (1–9)[a]	10	18 ± 5.4 (10–28)[b]	10	4.5 ± 2.4 (1–9)[a]	8
Clutch size	12.5 ± 8.5 (1–47)	55	15.3 ± 11.8 (2–53)	36	9.4 ± 6.2 (1–27)	36
Viability = hatching success	79.0% ± 28.1 (0%–100%)	55	80.2% ± 10.8 (50%–93%)	14	69.9% ± 36.2 (0%–100%)	27
Survivorship of juveniles at 150 day	43.9% ± 28.9 (0%–100%)	39	34.8% ± 24.9 (0%–100%)	14	56.2% ± 30.9 (0%–100%)	23
Survivorship of juveniles at 300 day	34.9% ± 27.4 (0%–100%)[a, b]	39	16.0% ± 9.3 (0%–31%)[b]	13	52.3% ± 31.0 (0%–100%)[a]	23

Means ± *SD* and ranges in parentheses.

Different letters (a, b) indicate groups that were statistically significantly different

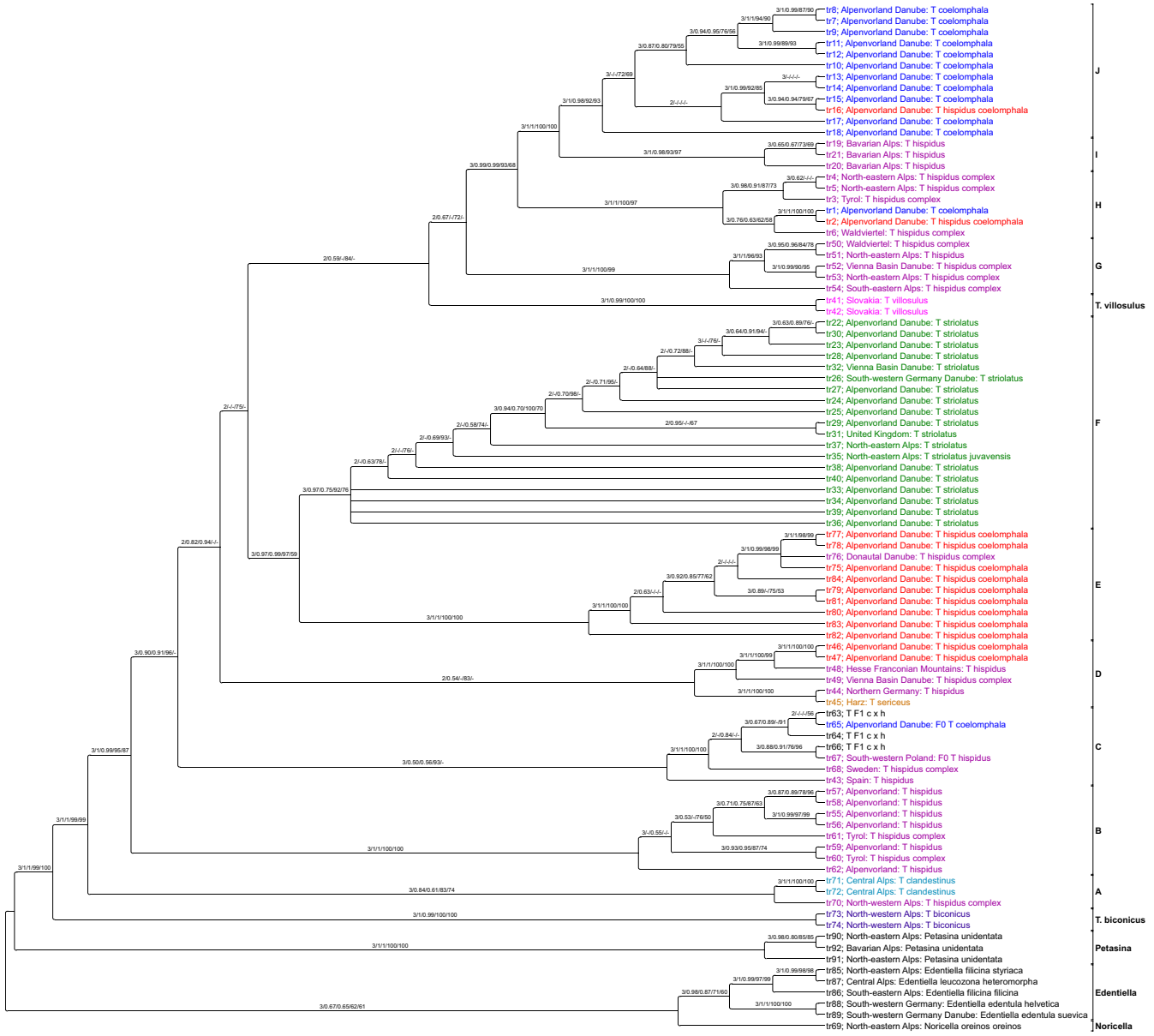


FIGURE 8 The consensus of trees obtained in three approaches for the alignment of four markers *COI + 16S rDNA + ITS2+28S rDNA*. Numbers at nodes, in the following order correspond to: the number of the trees that contained a given node, posterior probabilities estimated in MrBayes and PhyloBayes as well as support values obtained by the approximate likelihood ratio test based on a Shimodaira-Hasegawa-like procedure and bootstrap method calculated in IQ-TREE. Values of the posterior probabilities and bootstrap percentages lower than 0.50 and 50%, respectively, were indicated by a dash "-" [Colour figure can be viewed at wileyonlinelibrary.com]

central Germany, whereas the other sites were occupied secondarily (Figure 9).

Other sequences are divided into two groups clustered together in three data sets but with a very poor support (Figure 8, Figures S3 and S4). However, the first group is highly supported (PP-M = 0.97–1, PP-P = .99–1, aLRT = 97–100, BP = 59–83) and consists of the clades E and F. The clade E is maximally supported in three data sets and includes samples with a narrow geographic range along the Danube in Alpenvorland (Figure 9) and is characterised by an intermediate shell morphology between *T. hispidus* and *T. coelomphala*. Interestingly, *T. hispidus* from Donautal (Sauwald in Austria) is placed among them. In the four-markers tree (Figure 8) and *COI + 16S rDNA* (2) tree (Figure S4), the Donautal *T. hispidus* is significantly (PP-M = 1, PP-P = .99–1, aLRT = 98, BP = 96–99) clustered with *T. hispidus/coelomphala* from Staubing and Wackerstein. It could be another example of mitochondrial DNA introgression, here from *T. hispidus/coelomphala* to *T. hispidus*. Another Donautal sample of *T. hispidus* is located in clade H including samples assigned also to *T. hispidus*. However, shell morphology and genitalia of the above-mentioned specimens cannot be confirmed due to being juveniles (M. Duda, personal communication), so their taxonomic status is uncertain. We may

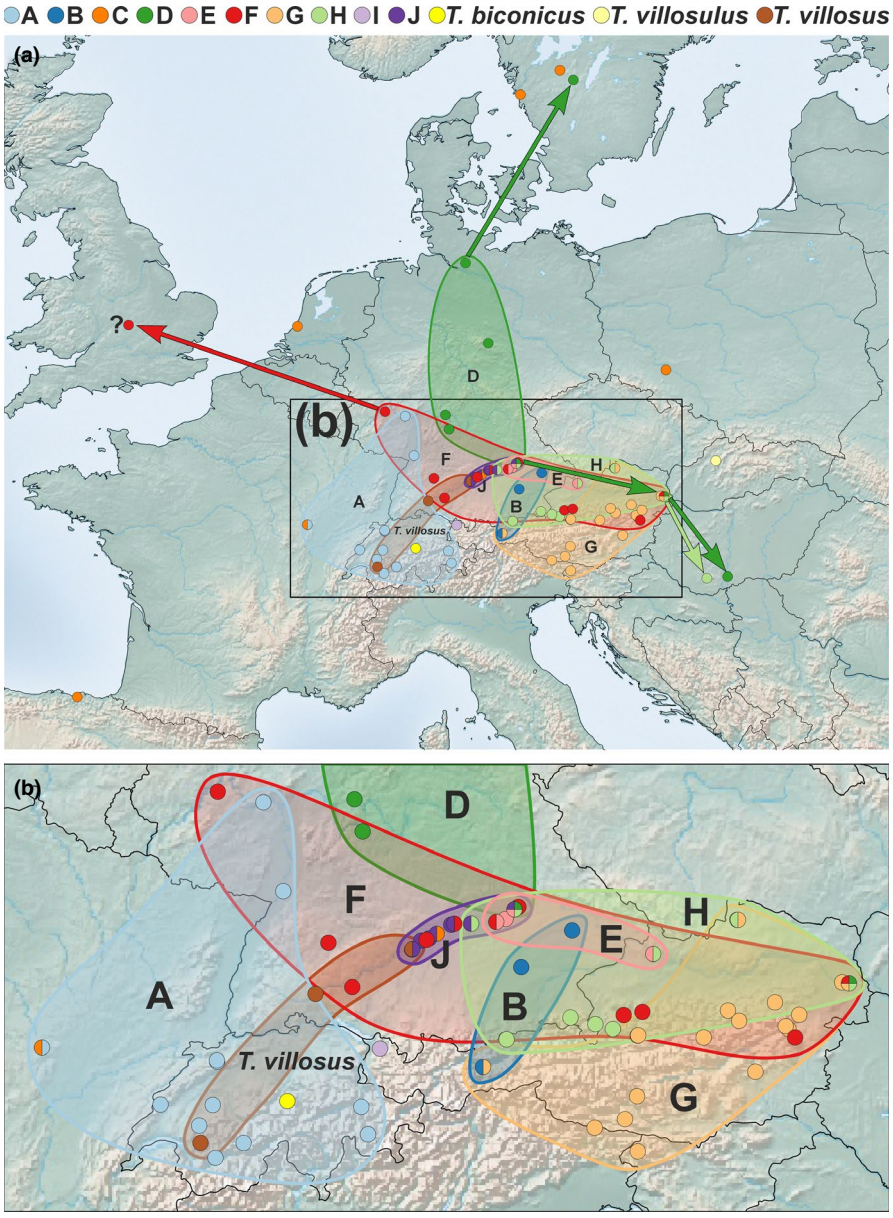
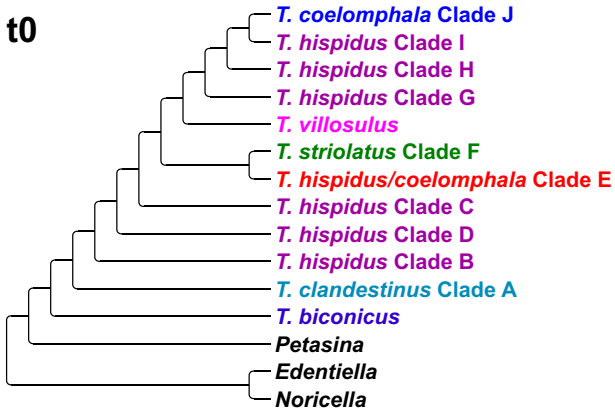


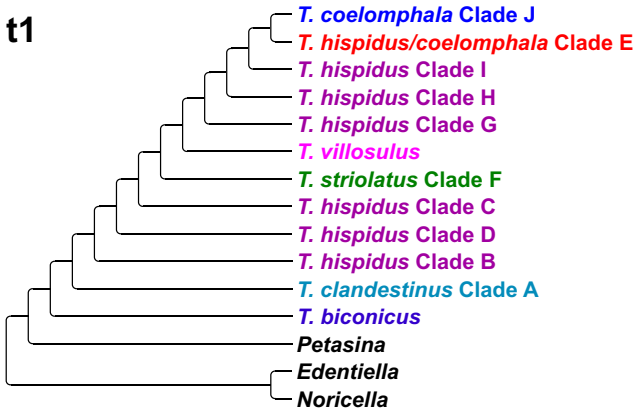
FIGURE 9 Geographic distribution of all clades identified in phylogenetic analyses. (a) General view. (b) Enlarged view on the Alps and surrounding regions. Arrows indicate potential translocations of snails from the main distribution regions of a given clade. The location of the site from the United Kingdom, in which a representative of the clade C was found, is not known precisely. The ranges of selected clades with a compact distribution or represented by a larger number of samples were presented [Colour figure can be viewed at wileyonlinelibrary.com]

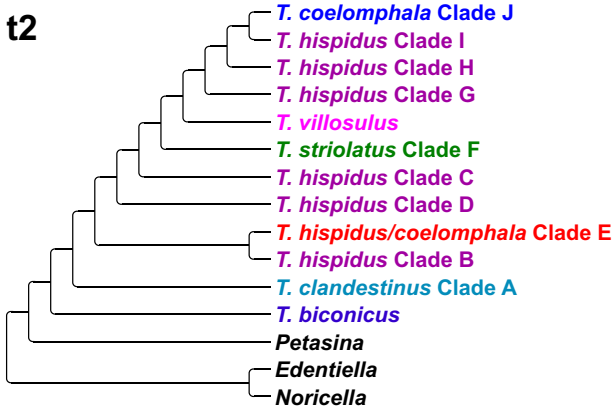
only assume that this Donautal *T. hispidus* from clade E also belongs to *T. hispidus/coelomphala* and was transported with the Danube river to Austrian Donautal about 205 km downstream.

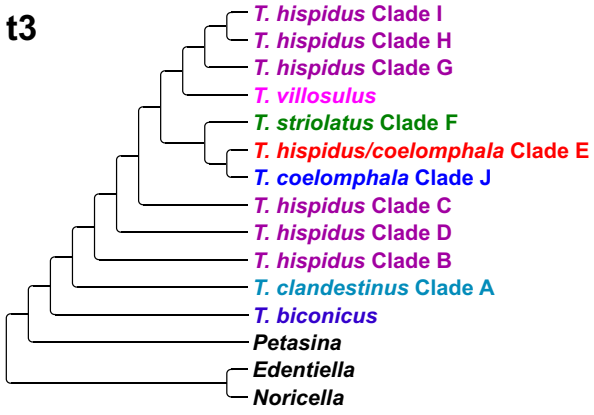
This clade E is sister to F, which contains exclusively *T. striolatus* sequences supported with PP-M = 0.93–0.97, PP-P = .63–0.75, aLRT = 92, BP = 67–76 in the four-markers tree (Figure 8) and *COI + 16S rDNA* (2) tree (Figure S4), whereas in the tree based on the *COI + 16S rDNA* (1) alignment (Figure S3), these sequences are separated into two clades and their relationship with the clade E is not resolved. The specimens in the clade F were found in the Vienna Basin, the North-eastern Alps, Alpenvorland, western Germany and one sample comes from England (Figure 9).

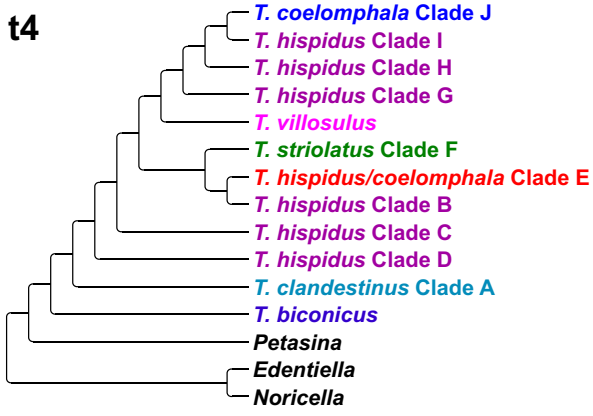
The second big group was produced consistently in the trees based on the four nuclear and mitochondrial markers (Figure 8) and two mitochondrial markers (Figures S3 and S4) with rather weak support PP-M = 0.59–0.92, PP-P = .65, aLRT = 84–91. The earliest diverged lineage includes sequences of *T. villosulus* from Slovakia. In the next almost maximally supported clade G, there are *T. hispidus* sequences from the Eastern Alps, the Vienna Basin and closely located Waldviertel in Austria (Figure 9). The clade H (supported with PP-M = 0.56–1, PP-P = .54–1, aLRT = 0.54–100, BP = 96–97) contains *T. hispidus* samples from the North-eastern Alps and sites located at the Danube, from Alpenvorland to the Vienna Basin, in the *COI + 16S rDNA* (1) tree (Figure S3). The geographic range of this clade is more northern, whereas clade G is more southern (Figure 9). Within clade H, there is also a sample from Hungary (Mecsek), which is clustered (with PP-M = 0.69, PP-P = .84, aLRT = 0.52) with that from the North-eastern Alps (Berchtesgadener Land). Such a grouping suggests a transport of snails with the Danube and its tributaries, for example, Inn, for more than 820 km.











Result of tree topology tests

Tree	logL	ΔlogL	bp-RELL	p-SH	p-WSH	c-ELW	p-AU
t0	-14661.35	0	0.976	1	0.999	0.973	0.99
t1	-14695.18	33.83	3.9E-03	0.052	0.017	4.1E-03	5.3E-03
t2	-14680.08	18.73	0.020	0.262	0.076	0.022	0.024
t3	-14813.88	152.53	0	0	0	6.8E-24	1.8E-07
t4	-14686.65	25.30	4.3E-04	0.162	0.011	6.2E-04	1.3E-03

FIGURE 10 The simplified best tree found in IQ-TREE (t0) and alternative topologies (t2-4) assuming other phylogenetic position of *T. hispidus/coelomphala* forms characterized by an intermediate shell morphology. Results of tests comparing these topologies are shown in the associated table. The table includes: log-likelihood values (logL) and their difference to the best tree (ΔlogL), bootstrap proportion using RELL method (bp-RELL) as well as p-values from Shimodaira-Hasegawa test (p-SH), weighted Shimodaira-Hasegawa test (p-WSH), Expected Likelihood Weight test (c-ELW) and an approximately unbiased test (p-AU). Values smaller than 0.05 were highlighted [Colour figure can be viewed at wileyonlinelibrary.com]

Within clade H, there are also sequences from close localities (50 km away) in Alpenvorland ascribed to *T. coelomphala* (Bittenbrunn) and *T. hispidus/coelomphala* (Staubing), which are significantly grouped together with PP-M = 1, PP-P = .99–1, aLRT = 85–100, BP = 97–100 (Figure 8 and Figure S3). They are closely related with samples from nearby regions, Waldviertel and the Vienna Basin, with PP-M = 0.74–1, PP-P = .58–1, aLRT = 62–100, BP = 58–100. The phylogeographic distribution of the sequences in this clade suggests that Alpenvorland snails originated from those from Tyrol or the North-eastern Alps, perhaps migrating via the Danube to the north-eastern part of Austria. This scenario implies that the shell shape changed from the *T. hispidus* type to that of *T. coelomphala* and *T. hispidus/coelomphala*, and next reverted to the *T. hispidus* type in Austria. This speculation may

be true if we accept that the specimens are correctly assigned to the *T. hispidus* type. Alternatively, we can presume that the mitochondrial DNA was introgressed to the Alpenvorland populations.

The clades I and J of the second group are clustered together with the maximal support. The clade J contains *T. coelomphala* (with PP-M = 1, PP-P = .98–1, aLRT = 92–100, BP = 93–100) restricted to sites at the Danube in Alpenvorland, whereas the clade I includes *T. hispidus* sequences from the Bavarian Alps (Figure 9), which are monophyletic (with PP-M = 1, PP-P = .98, aLRT = 93, BP = 97) in the four-markers tree (Figure 8) but not in two mitochondrial markers trees (Figures S3 and S4). These relationships indicate that *T. coelomphala* originated from alpine *T. hispidus* populations, which could be transferred to the Alpenvorland region with rivers flowing down from the Bavarian Alps.

One sample assigned to *T. hispidus/coelomphala* (Staubing) is directly joined with that of *T. coelomphala* from Lechsend in the four-markers tree (Figure 8) and *COI + 16S rDNA* (2) tree (Figure S4) with PP-M = 0.94, PP-P = .94–0.95, aLRT = 79–80, BP = 66–67. These localities are 70 km away. This result suggests that the intermediate shell can be developed in *T. coelomphala* or the mitochondrial DNA could be passed from *T. coelomphala* to *T. hispidus/coelomphala*. Interestingly, both taxa have the same genital morphology.

Phylogenetic trees based only on nuclear markers are much worse resolved especially at deep nodes even in the three-markers tree (Figure S5). Many *Trochulus* sequences assigned to various species (*coelomphala*, *hispidus*, *striolatus*) are mixed and usually do not form big monophyletic clades. Only in the three-markers tree (Figure S5), there is a clade including many *T. striolatus* specimens but poorly supported, and a significant clade with *T. hispidus* from the Bavarian Alps (PP-M = 1, PP-P = .95, aLRT = 96, BP = 80). The trees based on *ITS2 + 28S rDNA* alignments (Figures S6 and S7) possess maximally supported separation of *Trochulus* sequences from other members of Trochulini (*Petasina*, *Noricella* and *Edentiella*). Samples of *Edentiella* are not monophyletic and are separated into two clades.

3.5 | Testing alternative phylogenetic hypotheses

The phylogenetic position of forms with the intermediate shell morphology, assigned as *T. hispidus/coelomphala*, was unexpected because they clustered with *T. striolatus*, but not with *T. hispidus* or *T. coelomphala*. Therefore, we tested four alternative tree topologies with other relationships between these snails (Figure 10). These alternatives assumed that *T. hispidus/coelomphala* samples are related with *T. coelomphala* or *T. hispidus* found in the same geographical region, that is, Alpenvorland. In that, we considered two possibilities: (a) joining of the *T. hispidus/coelomphala* clade with that of *T. coelomphala* or *T. hispidus* (topology t1 and t2), and (b) linking of *T. coelomphala* or *T. hispidus* to *T. hispidus/coelomphala*, left in its original position as in the best tree, that is, with *T. striolatus* (topology t3 and t4). Most applied tests, including the most robust, the approximately unbiased test (AU), showed that the alternatives are significantly worse than the best found tree (Figure 10). The most conservative test Shimodara-Hasegawa (SH) did not reject three topologies but its weighted version (wSH) showed that two of them are in fact worse than the best topology. The results indicate that the closer relationship of *T. hispidus/coelomphala* with *T. striolatus* is much more probable than with *T. hispidus* or *T. coelomphala*.

4 | DISCUSSION

In the classic model of allopatric speciation (Mayr, 1963) the occurrence of hybrids was seen as arising from secondary contact between previously isolated and differentiated populations. Recent works, however, show that populations may diverge in the face of continuous gene flow or may alternate between periods of complete isolation and periods of contact and gene flow (Bennett, 1997; Bolnick & Fitzpatrick, 2007; Niemiller et al., 2008). Hence, hybrids may represent a variety of intermediate stages in speciation, possibly in sympatry or parapatry (Barton & Hewitt, 1989; Mallet, 2005). The very complex pattern of phylogeny revealed in the case of *Trochulus* presented here reflects the variety of ways in which speciation proceeds.

4.1 | Validity of *Trochulus coelomphala* as taxon and its origin

The taxonomic status of *T. coelomphala* was uncertain (Duda et al., 2014; Kruckenhauser et al., 2014; Proćków et al., 2017c). Our integrative study allowed to recognise it as a separate taxon. Previous phylogenetic analyses based on the *COI* gene showed that *T. coelomphala* is most closely related to *T. graminicola* (Falkner, 1973), an endemic species known only from its type locality in south-western Germany, about 200 km away from the *T. coelomphala* location (Proćków et al., 2017c). However, using four molecular markers, we found that *T. coelomphala* sequences are grouped within *T. hispidus* and are closely related with its samples found in the Bavarian Alps in phylogenetic trees (Figure 8, Figures S3 and S4). The close location of these regions (Figure 9 and Figure S2) indicates that *T. coelomphala* might have derived

from the snails that flowed down with rivers from the Alps to the regions with low altitude.

The samples of *T. coelomphala* from the type locality (Günzburg) and a limited geographic region in the Bavarian Danube valley extending eastward to Bittenbrunn, match the original description and distinguish it from other taxa by a flat shell with a very large relative umbilicus diameter, approximately a quarter of the total shell diameter (Figures 3 and 4b, Table 3). However, these features do not always differentiate it unambiguously when compared to *T. graminicola* and some populations of *T. hispidus* e.g. from Ruine Waldau (Proćków et al., 2017c). *Trochulus coelomphala* also differs from *T. hispidus* and *T. striolatus* in the reproductive system features by long penis and bursa copulatrix duct (Figure 6). Duda et al. (2014) also found a slender upper vagina in *T. coelomphala*. No such differences in genitalia are found between *T. coelomphala* and the morphological intermediates *T. hispidus/coelomphala* (Figure 5).

Although *T. hispidus* and *T. coelomphala* differ in both shell morphology and reproductive anatomy (Figure 3), the crossing experiment shows that they are interfertile. While long term fecundity of such hybrids remains unknown, the results indicate that *T. coelomphala* may represent an incipient species with semipermeable reproductive barrier to gene flow. Such species can differentiate despite on-going interbreeding (Hausdorf, 2011), with hybrid incompatability evolving slowly (Rieseberg et al., 2004). Permeability of species barriers may also concern other *Trochulus* species, which are most probably at different stages of evolutionary change. The processes leading to speciation do not require a period of complete allopatry (Mallet et al., 2007), and the emphasis on genetic isolation as the primary factor may be misplaced (Coyne & Orr, 2004; Drés & Mallet, 2002).

It should be also emphasised that *T. hispidus* and *T. coelomphala* may not have a chance to mate in nature because they consist of allotopic populations. Moreover, other barriers, for example, pheromones, ecological microniches and breeding habitats, may prevent mating between different species (Yanchukov et al., 2006 and references therein; König et al., 2015). This may also concern our captive breeding species, because nearly half of their interspecific pairs produced offspring. Significant differences in fecundity rate between *T. hispidus* and *T. coelomphala* (Table 7) additionally support this view.

4.2 | Origin of morphological intermediates *T. hispidus/coelomphala*

Morphometric analyses showed an overlap in the shell variation of intermediate forms of *T. hispidus/coelomphala* with all other species examined (Figure 2). These forms have mean values of height/width shell ratio (H/W), relative height of body whorl (bwH/H) and the relative umbilicus diameter (U/D) between those in *T. hispidus* and *T. coelomphala* (Table 3). It suggests that there is either gene flow between them or a little selection against the intermediate forms, limited to a narrow geographic zone. Considering other studies, where similar intermediates were observed (Duda et al., 2014; Proćków et al., 2017c), this zone can be extended to the vicinity of Regensburg, that is, between Kleinmehring and Pfatterer Au along the Danube river for ca. 100 km (Figure 1).

This morphological relationship is contradicted by molecular phylogeny. These 'intermediate' forms group with *T. striolatus*, including samples from similar localities, that is, in Alpenvorland (Figure 9). In contrast to that, sequences of *T. hispidus* and *T. coelomphala*, also found in Alpenvorland, were located in separate clades (Figure 8, Figures S3 and S4). Moreover, tree topology tests showed that the relationship of these two species with *T. hispidus/coelomphala* was significantly weaker than that of the intermediate forms with *T. striolatus* (Figure 10). Because many *T. striolatus* individuals examined here inhabit the same region as *T. hispidus/coelomphala* (Figure 9), all these results suggest that the intermediate forms originated from *T. striolatus* or its ancestor perhaps by way of the sympatric speciation in the Alpenvorland region. Fossil data indicate that these entities were present in this sympatric area in the Pleistocene. Particularly in the tufa of Regensburg, besides *T. striolatus*, three morphological variants of *T. hispidus* (noted as *H. hispida*, *H. concinna* and var. *conica*) were recorded (Taylor, 1916). *Helix concinna* Jeffreys, 1830, described as sub-depressed and wide-umbilicated, may in fact represent *T. hispidus/coelomphala*. Its restricted distribution implies evolution in situ. Interestingly, there is an apparent similarity between the intermediate forms and the eastern *T. striolatus* populations in height/width shell ratio (H/W) and relative height of body whorl (bwH/H) (Figure 3) as well as the length of penis between *T. hispidus/coelomphala* and *T. striolatus* considered as one set (Figure 6). Nevertheless, *T. hispidus/coelomphala* and *T. striolatus* are generally well conchologically differentiated in many features (Figures 2 and 4) and differ in the length of bursa copulatrix duct (Figure 6). It suggests that they should not be grouped into one species.

Coyne and Orr (2004) proposed four criteria for inferring cases of sympatric speciation: (a) the species' ranges must largely overlap; (b) speciation must be complete (i.e. two species cannot interbreed); (c) the species must be sister species (most closely related to each other) or part of a monophyletic group, which includes an ancestor and all its descendants; (d) the biogeographic and evolutionary history of the groups must make the existence of an allopatric phase very unlikely. In view of this biogeographical

concept of sympatric speciation, our case study almost meets these requirements. However, we cannot unambiguously demonstrate that the entities in question did not come into a secondary contact after allopatric distribution. This requires further studies that could profitably focus on more extended sampling of *T. striolatus* and maybe more molecular markers.

We cannot rule out either that the intermediate forms evolved from *T. hispidus* or *T. coelomphala* and their mitochondrial DNA was obtained from *T. striolatus* via introgression. Unfortunately, phylogenies based on nuclear markers are too poorly resolved (Figures S5–S7) to further verify this hypothesis, although two sequences of *T. hispidus/coelomphala* were grouped with *T. striolatus*. Because *Trochulus* snails are reciprocally mating hermaphrodites, we should expect that both the mitochondrial and nuclear genetic material would be transmitted. Other *Trochulus* lineages can hybridise to a limited extent in a small contact area (Dépraz et al., 2009).

4.3 | Taxonomic and phylogenetic position of *T. striolatus*

The distinction of *T. striolatus* from 'typical' *T. coelomphala* and 'typical' *T. hispidus* is clear based on shell features (Figures 2-4) and genetic markers. In the phylogenetic trees obtained in this study, *T. striolatus* samples create a distinct monophyletic clade, which is significantly grouped with intermediate forms of *T. hispidus/coelomphala* (Figure 8 and Figure S4). These sequences were not included in previous studies, in which *T. striolatus* was sister to *T. villosulus* in the tree based on *COI* + *16S* + *ITS1* (Pfenninger et al., 2005) and three mitochondrial genes (Kruckenhauser et al., 2014). Out of three lineages (named A, B and C) attributed to *T. striolatus/plebeius* by Pfenninger et al. (2005), only lineage C was nested within a monophyletic clade of other *T. striolatus* samples in the *COI* gene tree by Proćków et al. (2014), so apparently it represents this species. The lineages A and B were sister to C in Pfenninger et al. (2005). However, in Proćków et al. (2014), the lineage B, represented by one unique sequence from France, was grouped with *T. sericeus* from Germany. Thus, the affiliation of these lineages to *T. striolatus* remains uncertain. Although *T. striolatus* appeared to be a good species based on morphology and mitochondrial markers, microsatellite clustering suggested a gene flow with other taxa or incomplete sorting of microsatellite alleles into these lineages (Proćków et al., 2017c). An independent evolution of the same microsatellite alleles in these taxa is also a possible explanation (Proćków et al., 2017c).

A geographic pattern of genetic diversity indicates that the populations of *T. striolatus* may have been distributed over a wide range during the last glacial (Kruckenhauser et al., 2014). This is supported by ample fossil records from the Pleistocene deposits, reported from the UK, the Netherlands, Austria, Germany, Serbia, Croatia, Hungary, Slovakia (Freudentha et al., 1976; Hupuczi et al., 2010; Marković et al., 2004; Moine et al., 2005; Nenadić et al., 2010; Pazonyi et al., 2014; Schmidt et al., 1978; Taylor, 1916). However, the assignment of these fossils can be very problematic because of small conchological differences. *T. striolatus* is variable in habitat choice and morphology but quite homogeneous in mtDNA variation, which might reflect its rapid dispersal from a single refugium or only a few refugia over large parts of Europe after the last glaciation (Duda et al., 2014).

Concerning infraspecific diversity of *T. striolatus*, we found some differences in shell characters between two populations of this species (Table 5, Figures 3 and 4) but we did not observe the spatial genetic structure among them. Because these populations occupy slightly different microhabitats differing in moisture and illumination, we can assume that the differences can result from the environmental influence, which was previously reported for this species (Proćków et al., 2017b). Therefore, the recognition of *T. striolatus* subspecies is not sufficiently justified based on shell morphology (Proćków et al., 2017b) and molecular analyses (Kruckenhauser et al., 2014). In agreement with that, the separation of *T. s. danubialis* is not substantiated in our study based on constant genital anatomical characters, that is, the cross-section of penial papilla. According to Duda et al. (2014), *T. s. danubialis* cannot be also distinguished from *T. s. juvavensis* in molecular and anatomic studies. Only *T. s. striolatus* is separated from these subspecies in genetic analyses but shows only subtle anatomical difference in an additional penial plica.

4.4 | Geographic pattern and evolutionary relationships among clades of *T. hispidus* complex

Understanding the relationships among taxa in the *Trochulus* genus is a great challenge due to inconsistency between morphological and molecular data. In particular, the *T. hispidus* complex exemplifies problems in species delimitation. DNA barcoding in the absence of detailed phylogeographic relationships (Duda et al., 2014) has not been successful (Kruckenhauser et al., 2014; Pfenninger et al., 2005; Proćków et al., 2017c). Here, we included four genetic markers in an effort to obtain relationships within and among species of *Trochulus* with a greater resolution. Our results show that the samples currently assigned to *T. hispidus* are clearly polyphyletic because they are distributed into at least five main groups (clade A, B, C, D and G + H + I in Figure 8, Figures S3 and S4). Pfenninger et al. (2005) and Kruckenhauser et al. (2014) found a geographic pattern,

which suggests two old radiations of *T. hispidus* starting from unknown western and eastern regions. Our results confirm these findings, because we can also recognise two groups: the first includes clade A with *T. clandestinus*, *T. caelatus* and *T. montanus*, having the western distribution, whereas the second comprises clades B, D, G-I with *T. coelomphala*, *T. striolatus*, *T. villosulus* and *T. hispidus/coelomphala*, having a more eastern and northern distribution (Figures 8 and 9; Figures S3 and S4). Some specimens of *T. hispidus* that were grouped with unannotated specimens and *T. piccardi* (in clade A in Figure S3), may represent in fact the latter species. All these specimens are located in the western macro region: the southern Rhine Valley, the Jura Mountains, Burgundy as well as the North-western and Central Alps (Figure 9). The position of *T. piccardi* within the *T. hispidus* complex clade was interpreted as a hint of more cryptic species hidden among clades of this complex (Kruckenhauser et al., 2014).

Clade B comprises samples restricted to Alpenvorland and Tyrol (Figure 9). In the tree based on two mitochondrial markers (Figure S3), it is clustered with clade C, which includes sequences from very distant sites in Poland, Sweden, the Netherlands, France and Spain (Figures 8 and 9; Figure S3). They may have a common origin. We also found other examples of long-distance translocation of *T. hispidus*, for example, from northern Germany to Sweden (in clade D) and two independent cases from the alpine region to Hungary (in clades D and H) – Figure 9. In the latter case, snails could be transported with the Danube, which indicates the role of rivers in the distribution of these snails. Spread of land gastropods such as *Achatina fulica* or *Hygromia cinctella* along rivers has been recorded elsewhere (Beckmann & Kobialka, 2008; Defossez & Maurin, 1995; Thiengo et al., 2007; Wimmer, 2006).

Trochulus hispidus sequences, mainly from northern and central Germany as well as the Vienna Basin and Hungary, created clade D (Figure 9), which also included a *T. sericeus* sequence (Figure 8 and Figure S3). This species was recently proposed to be an ecological form of *T. hispidus* living in more humid and shaded environments (Proćków et al., 2018). The morphotype of *T. sericeus* was present in all mitochondrial *T. hispidus* clades and could not be assigned to a genetic group or any specific population (Duda et al., 2014). The vast majority of *T. hispidus* sequences were grouped in the clades G-I (Figure 8 and Figure S3). These samples are distributed in Tyrol, the Bavarian and Eastern Alps as well as in north-eastern Austria and Hungary (Figure 9). These clades differ in the geographic ranges, although they partially overlap. However, no shell and genital differences were found between them. Therefore, these samples are often described as *T. hispidus* complex (Duda et al., 2014). It cannot be excluded that at least some of them may represent cryptic species or alternatively, it is one species with the high genetic variability.

4.5 | Distinction of other *Trochulus* species

Our phylogenetic analyses have also clarified the relationships and status of other *Trochulus* species (Figure 8, Figures S3 and S4). *Trochulus biconicus*, an endemic species from the North-western Alps, may represent the earliest diverged lineage of this genus. Its distant position (Figure 8) and also close relationship to *Noricella* species (Figures S3 and S4) require further investigations. Both taxa share a similar feature, that is, pattern of plicae in the penis papilla (Duda et al., 2014; Proćków, 2009), which is different from other *Trochulus* (Proćków, 2009; Schileyko, 1978), *Petasina* and *Edentiella* species (Falkner, 1985; Schileyko, 2006).

In the presented tree (Figure S3), *T. clandestinus* from the North-western and Central Alps are clustered together in a significant monophyletic clade with *T. caelatus* and *T. montanus* from the Jura Mountains as in previous studies (Pfenninger et al., 2005; Proćków et al., 2014). However, the sequences assigned to these species are mixed in the *COI* tree including many samples (Proćków et al., 2014), so further studies should verify their annotation. *Trochulus clandestinus* requires a thorough revision. It was recently detected further east in Vorarlberg in Austria (Duda et al., 2017) and its subspecies *T. c. putonii* (Clessin, 1874), is spatially isolated in the French Vosges Mountains and the Rhine valley (Falkner et al., 2002; Falkner et al., 2011). It is known only from a laconic shell description, that is, smaller shell size and less convex last whorl (Falkner et al., 2002). *Trochulus c. putonii* shows identical reproductive system to the typical *T. clandestinus*.

Significant and separate clades were created by *T. villosulus* from Slovakia as well as *T. villosus* from the North-western Alps, Alpenvorland and South-western Germany (Figure 9). The former is sister to the main clades (G-J) of *T. hispidus* including *T. coelomphala*, whereas the latter is an early diverged lineage of *Trochulus* after clade A (Figure 8, Figures S3 and S4). Both species represent morphologically well-defined species and are monophyletic with low intraspecific distances in a pooled data set (Kruckenhauser et al., 2014; Pfenninger et al., 2005).

4.6 | Intermixed specimens

We found interesting cases in the phylogenetic trees, in which specimens of one *Trochulus* species or morphological form were located within others, for example, *T. hispidus/coelomphala* and *T. coelomphala* from Alpenvorland within *T. hispidus* from Central Germany and the Alps; *T. hispidus* from Donautal (North-eastern Austria) within *T. hispidus/coelomphala* from Alpenvorland; *T. hispidus/coelomphala* within *T. coelomphala*, both from Alpenvorland but different localities (Figure 8, Figures S3 and S4). Disregarding the incorrect assignment of the samples, these results can be interpreted

into two ways. Following our previous findings, showing the influence of local environment on shell shape in *Trochulus* taxa (Proćków et al., 2017a, 2017b, 2018), we can assume that these outstanding cases represent individuals genetically related with others in the population but having different shell morphology due to such a phenotypic plasticity and the environmental influence. It means that the same or similar shell morphology can occur independently in different genetic lineages. Accordingly, we found in this study that *T. striolatus* samples collected in the same localities as *T. hispidus/coelomphala*, that is, in more eastern sites in the Danube valley, were more similar to these intermediate forms in several shell features than to other *T. striolatus* samples inhabiting more western sites (Figure 3). Because the eastern and western sites differ in humidity and illumination, we can assume that the shell shape can be visibly modified by local climatic conditions leading to similarities between various snail species and differences between populations of the same species.

Alternatively, it cannot be excluded an occasional introgression of the mitochondrial DNA between different forms or species. The second possibility is supported by successful cross experiments between morphologically distinct taxa, presented here and elsewhere (Proćków et al., 2017a). A limited hybridisation was also found for other *Trochulus* lineages in a small contact area (Dépraz et al., 2009). However, the studied nuclear markers did not help solving this question in the case considered here because of too little variation. Nevertheless, in the case of *T. hispidus/coelomphala* within *T. coelomphala*, we can assume a gene flow due to similar genital morphology, the lack of significant differences between their microhabitats and their close geographical distribution.

5 | CONCLUSIONS

Our study demonstrates the difficulties in species identification in land gastropods from the genus *Trochulus* and the inconsistency in its species delimitation based on shell morphometrics, construction of the reproductive system and variation of genetic markers. However, the application of the interdisciplinary approach complemented with experimental hybridization enabled to solve some questions regarding this genus. The most widespread species *T. hispidus* is polyphyletic and separated in several lineages. It cannot be excluded that some of them represent cryptic species. One of species that evolved within *T. hispidus* is *T. coelomphala*, which can be quite easy recognised based on shell morphometrics and genitalia characters. However, the process of speciation is likely still ongoing because crossbreeding experiments between these species occurred successful. The most challenging findings are snails showing intermediate shell features between these two species and tentatively named *T. hispidus/coelomphala*. Unexpectedly, these forms proved to be closely related to another species, *T. striolatus*. Because the related specimens come from the same locality, Alpenvorland, we can assume that *T. hispidus/coelomphala* evolved in the way of sympatric speciation in this region. Although *T. striolatus* is generally clearly distinct from other *Trochulus* species, we found some common characters in shell and reproductive system that are shared between these intermediate forms and a subpopulation of *T. striolatus*. It can support the origin of *T. hispidus/coelomphala* from *T. striolatus*. Nevertheless, introgression of mitochondrial DNA from *T. striolatus* to the ancestor of the intermediate *Trochulus* is not inconceivable.

Speciation within *Trochulus* can be promoted by dispersion of snails with rivers translocating individuals for more than 800 km. We have also recorded several cases in which a specimen is grouped among snails with a consistently different shell size and shape in a phylogenetic tree. These results can indicate a great shell plasticity, which can be associated with an influence of environmental and climatic conditions or introgression of genetic material. In agreement with the former explanation, we found that two subpopulations of *T. striolatus*, living in microhabitats with different moisture and illumination, are distinct in some shell measures. Our findings confirm the view that processes leading to speciation are continuous and endorse Darwin's original dynamic view of speciation (Darwin, 1859). *Trochulus* species could evolve not only under allopatric speciation but also in sympatry or parapatry. The speciation process can be, however, hindered by the influence of environmental conditions on morphological features and gene flow between lineages that were not completely separated at the genetic level.

ACKNOWLEDGEMENTS

We would like to thank Paweł Jałoszyński for his valuable discussion. We are immensely grateful to two reviewers for their thoughtful remarks and linguistic corrections. This work was supported by the National Science Centre, Poland (Narodowe Centrum Nauki, Polska) under Grant number 2016/21/B/NZ8/03022. Some computations were carried out at the Wrocław Centre for Networking and Supercomputing under the grant no. 307.

ORCID

Małgorzata Proćków https://orcid.org/0000-0003-2240-7306
Elżbieta Kuźnik-Kowalska https://orcid.org/0000-0002-5509-0336
Joanna R. Pieńkowska https://orcid.org/0000-0003-0372-121X
Aleksandra Żeromska https://orcid.org/0000-0001-5243-6509
Paweł Mackiewicz https://orcid.org/0000-0003-4855-497X

REFERENCES

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C. A., Buggs, R., Butlin, R. K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S. H., Hermansen, J. S., Hewitt, G., Hudson, A. G., Jiggins, C., … Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, *26*, 229–246. https://doi.org/10.1111/j.1420-9101.2012.02599.x

Almeyda-Artigas, R. J., Bargues, M. D., & Mas-Coma, S. (2000). ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing human gnathostomiasis in the Americas. *Journal of Parasitology*, *86*, 537–544. https://doi.org/10.2307/3284869

Anderson, R. (2005). An annotated list of the non-marine Mollusca of Britain and Ireland. *Journal of Conchology*, *38*, 607–637.

Bank, R. A. (2011). *Fauna Europea: Gastropoda. Fauna Europea version 2.4*. http://www.faunaeur.org

Barluenga, M., & Meyer, A. (2010). Phylogeography, colonization and population history of the Midas cichlid species complex (*Amphilophus* spp.) in the Nicaraguan crater lakes. *BMC Evolutionary Biology*, *10*, 326. https://doi.org/10.1186/1471-2148-10-326

Barton, N. H., & Hewitt, G. M. (1989). Adaptation, speciation and hybrid zones. *Nature*, *341*, 497–503. https://doi.org/10.1038/341497a0

Beckmann, K.-H., & Kobialka, H. (2008). *Hygromia cinctella* (Draparnaud, 1801) auf dem Eroberungszug durch Deutschland (Gastropoda: Hygromiidae). *Club Conchylia Informationen*, *39*, 34–41.

Bennett, K. D. (1997). *Evolution and Ecology: The Pace of Life*. Cambridge: Cambridge University Press.

Bird, C. E., Fernandez-Silva, I., Skillings, D. J., & Toonen, R. J. (2012). Sympatric speciation in the post 'modern synthesis' era of evolutionary biology. *Evolutionary Biology*, *39*, 158–180. https://doi.org/10.1007/s11692-012-9183-6

Bolnick, D. I., & Fitzpatrick, B. M. (2007). Sympatric speciation: Models and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics*, *38*, 459–487. https://doi.org/10.1146/annurev.ecolsys.38.091206.095804

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*, 421. https://doi.org/10.1186/1471-2105-10-421

Caro, A., Neiber, M. T., Gomez-Moliner, B. J., & Madeira, M. J. (2019). Molecular phylogeny and biogeography of the land snail subfamily Leptaxinae (Gastropoda: Hygromiidae). *Molecular Phylogenetics and Evolution*, *139*, 106570. https://doi.org/10.1016/j.ympev.2019.106570

Chernomor, O., von Haeseler, A., & Minh, B. Q. (2016). Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic Biology*, *65*, 997–1008. https://doi.org/10.1093/sysbio/syw037

Clessin, S. (1874). Die Gruppe *Fruticicola* Held des Genus *Helix* L. Studien über *Helix*-Gruppe *Fruticicola* Held. *Jahrbücher Der Deutschen Malakozoologischen Gesellschaft*, *1*, 177–194, 305–336.

Colgan, D. J., McLauchlan, A., Wilson, G. D. F., Livingston, S. P., Edgecombe, G. D., Macaranas, J., Cassis, G., & Gray, M. R. (1998). Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. *Australian Journal of Zoology*, *46*, 419–437. https://doi.org/10.1071/zo98048

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sunderland, MA: Sinauer Associates.

Dabert, M., Witalinski, W., Kazimierski, A., Olszanowski, Z., & Dabert, J. (2010). Molecular phylogeny of acariform mites (Acari, Arachnida): Strong conflict between phylogenetic signal and long-branch attraction artifacts. *Molecular Phylogenetics and Evolution*, *56*, 222–241. https://doi.org/10.1016/j.ympev.2009.12.020

Darwin, C. (1859). *On the Origin of Species by Means of Natural Selection*., London: John Murray.

Defossez, J. M., & Maurin, C. (1995). A propos de la repartition de l'helicide *Hygromia cinctella* (Drap.). *Bulletin De La Societe Des Sciences Naturelles De L'ouest De La France*, *17*, 44–46.

Dépraz, A., Cordellier, M., Hausser, J., & Pfenninger, M. (2008). Postglacial recolonization at a snail's pace (*Trochulus villosus*): Confronting competing refugia hypotheses using model selection. *Molecular Ecology*, *17*, 2449–2462. https://doi.org/10.1111/j.1365-294X.2008.03760.x

Dépraz, A., Hausser, J., & Pfenninger, M. (2009). A species delimitation approach in the *Trochulus sericeus/hispidus* complex reveals two cryptic species within a sharp contact zone. *BMC Evolutionary Biology*, *9*, 171. https://doi.org/10.1186/1471-2148-9-171

Drés, M., & Mallet, J. (2002). Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *357*, 471–472. https://doi.org/10.1098/rstb.2002.1059

Duda, M., Kruckenhauser, L., Sattmann, H., Harl, J., Jaksch, K., & Haring, E. (2014). Differentiation in the *Trochulus hispidus* complex and related taxa (Pulmonata: Hygromiidae): Morphology, ecology and their relation to phylogeography. *Journal of Molluscan Studies*, *80*, 371–387. https://doi.org/10.1093/mollus/eyu023

Duda, M., Sattmann, H., Haring, E., Bartel, D., Winkler, H., Harl, J., & Kruckenhauser, L. (2011). Genetic differentiation and shell morphology of *Trochulus oreinos* (Wagner, 1915) and *T. hispidus* (Linnaeus, 1758) (Pulmonata: Hygromiidae) in the northeastern Alps. *Journal of Molluscan Studies*, *77*, 30–40. https://doi.org/10.1093/mollus/eyq037

Duda, M., Schindelar, J., Macek, O., Eschner, A., & Kruckenhauser, L. (2017). First record of *Trochulus clandestinus* (Hartmann, 1821) in Austria (Gastropoda: Eupulmonata: Hygromiidae). *Malacologica Bohemoslovaca*, *16*, 37–43.

Ehrmann, P. (1933). Mollusken (Weichtiere). In P. Brohmer, P. Ehrmann, & G. Ulmer (Eds.), *Die Tierwelt Mitteleuropas* II (1) (pp. 1–264). Leipzig: Quelle & Meyer.

Ellenberg, H., Weber, H. E., Düll, R., Wirth, V., Werner, W., & Paulissen, D. (1991). Zeigerwerte von Pflanzen in Mitteleuropa. *Scripta Geobotanica*, *18*, 1–24.

Falkner, G. (1985). *Helix liminifera* Held 1836 im Rassenkreis der *Petasina edentula* (Draparnaud) (Gastropoda: Helicidae). *Heldia*, *1*, 89–94.

Falkner, G. (1990). Binnenmollusken. In R. Fetcher, & G. Falkner (Eds.), *Weichtiere. Europäische Meeres- und Binnenmollusken, Steinbachs Naturführer* (pp. 112–280). München: Mosaik Velag.

Falkner, G., Bank, R. A., & von Proschwitz, T. (2001). CLECOM-PROJECT: Check-list of the non-marine Molluscan Species-group taxa of the States of Northern, Atlantic and Central Europe (CLECOM I). *Heldia*, *4*, 1–76.

Falkner, G., Ripken, T. H. E., & Falkner, M. (2002). Mollusques continentaux de France. Liste de référence annotée des mollusques continentaux de France. *Collection Patrimoines Naturels*, *52*, 1–350.

Falkner, G., von Proschwitz, T., & Falkner, M. (2011). *Trochulus clandestinus – The IUCN Red List of Threatened Species 2011*: e.T156515A4957060. https://doi.org/10.2305/IUCN.UK.2011-1.RLTS.T156515A4957060.en. https://www.iucnredlist.org/species/156515/4957060

Forcart, L. (1965). *New researches on Trichia hispida (LINNAEUS) and related forms*. Proceedings of the first European Malacological Congress (London 1962), 79–93.

Freudentha, M., Meijer, T., & Van der Meulen, A. J. (1976). Preliminary report on a *field* campaign in the continental Pleistocene of Tegelen (The Netherlands). *Scripta Geológica*, *34*, 1–27.

Fruciano, C., Franchini, P., Kovacova, V., Elmer, K. R., Henning, F., & Meyer, A. (2016). Genetic linkage of distinct adaptive traits in sympatrically speciating crater lake cichlid fish. *Nature Communications*, *7*, 12736. https://doi.org/10.1038/ncomms12736

Germain, L. (1929). Les Helicidae de la faune française. *Archives Du Muséum D'histoire Naturelle De Lyon*, *13*, 1–484.

Geyer, D. (1909). *Unsere Land- und Süsswasser-Mollusken. Einführung in die Molluskenfauna Deutschlands. Nebst einem Anhang über das Sammeln der Mollusken*, 2nd ed. Stuttgart: K. G. Lutz' Verlag.

Hausdorf, B. (2011). Progress toward a general species concept. *Evolution*, *65*, 923–931. https://doi.org/10.1111/j.1558-5646.2011.01231.x

Huelsenbeck, J. P., Larget, B., & Alfaro, M. E. (2004). Bayesian phylogenetic model selection using reversible jump Markov chain Monte Carlo. *Molecular Biology and Evolution*, *21*, 1123–1133. https://doi.org/10.1093/molbev/msh123

Hupuczi, J., Molnár, D., Galović, L., & Sümegi, P. (2010). Preliminary malacological investigation of the loess profile at Šarengrad, Croatia. *Central European Journal of Geosciences*, *2*, 57–63. https://doi.org/10.2478/v10085-009-0048-x

Jovelin, R., & Justine, J. (2001). Phylogenetic relationships within the polyopisthocotylean monogeneans (Platyhelminthes) inferred from partial 28S rDNA sequences. *International Journal for Parasitology*, *31*, 393–401. https://doi.org/10.1016/s0020-7519(01)00114-x

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, *14*, 587–589. https://doi.org/10.1038/nmeth.4285

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*, 772–780. https://doi.org/10.1093/molbev/mst010

Kerney, M. P., Cameron, R. A. D., & Jungbluth, J. H. (1983). *Die Landschnecken Nord- und Mitteleuropas*. Paul Parey: Hamburg und Berlin.

Kisel, Y., & Barraclough, T. G. (2010). Speciation has a spatial scale that depends on levels of gene flow. *The American Naturalist*, *175*, 316–334. https://doi.org/10.3410/f.2577956.2232054

König, K., Krimmer, E., Brose, S., Gantert, C., Buschlüter, I., König, C., Klopfstein, S., Wendt, I., Baur, H., Krogmann, L., & Steidle, J. L. M. (2015). Does early learning drive ecological divergence during speciation processes in parasitoid wasps? *Proceedings of the Royal Society B: Biological Sciences*, *282*, 20141850. https://doi.org/10.1098/rspb.2014.1850

Kruckenhauser, L., Duda, M., Bartel, D., Sattmann, H., Harl, J., Kirchner, S., & Haring, E. (2014). Paraphyly and budding speciation in the hairy snail (Pulmonata, Hygromiidae). *Zoologica Scripta*, *43*, 273–288. https://doi.org/10.1111/zsc.12046

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). Partitionfinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*, 1695–1701. https://doi.org/10.1093/molbev/mss020

Lartillot, N., Lepage, T., & Blanquart, S. (2009). PhyloBayes 3: A Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics*, *25*, 2286–2288. https://doi.org/10.1093/bioinformatics/btp368

Lartillot, N., & Philippe, H. (2004). A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Molecular Biology and Evolution*, *21*, 1095–1109. https://doi.org/10.1093/molbev/msh112

Locard, A. (1888). Contributions à la faune malacologique française. XII. Études critiques sur les *Helix* du groupe de l'*Helix rufescens* Pennant (*Helix striolata*, *H. rufescens*, *H. montana*, *H. cælata*, *H. circinata*, *H. clandestina*). *Annales De La Societe Linneenne De Lyon (Nouvelle Série)*, *34*, 309–370.

Locard, A. (1894). Conchyliologie française. Les coquilles terrestres de France. Description des familles, genres et espèces. *Avec 515 figures dessinées d'après nature et intercalées dans le texte*., Paris: J.-B. Baillière et fils.

Mallet, J. (2005). Hybridization as an invasion of the genome. *Trends in Ecology and Evolution*, *20*, 229–237. https://doi.org/10.1016/j.tree.2005.02.010

Mallet, J., Beltrán, M., Neukirchen, W., & Linares, M. (2007). Natural hybridization in heliconiine butterflies: The species boundary as a continuum. *BMC Evolutionary Biology*, *7*, 28. https://doi.org/10.1186/1471-2148-7-28

Manganelli, G., Salomone, N., & Giusti, F. (2005). A molecular approach to the phylogenetic relationships of the western palaearctic Helicoidea (Gastropoda: Stylommatophora). *Biolological Journal of the Linnean Society*, *85*, 501–512. https://doi.org/10.1111/j.1095-8312.2005.00514.x

Marković, S. B., Oches, E., Gaudenyi, T., Jovanović, M., Hambach, U., Zöller, L., & Sümegi, P. (2004). Paleoclimate record in the Late Pleistocene loess-paleosol sequence at Miseluk (Vojvodina, Serbia). *Quaternaire*, *15*, 361–368. https://doi.org/10.3406/quate.2004.1781

Mayr, E. (1963). *Animal Species and Evolution*., Cambridge, MA Belknap Press.

Moine, O., Rousseau, D.-D., & Antoine, P. (2005). Terrestrial molluscan records of Weichselian Lower to Middle Pleniglacial climatic changes from the Nussloch loess series (Rhine Valley, Germany): The impact of local factors. *Boreas*, *34*, 363–380. https://doi.org/10.1080/03009480510013060

Naggs, F. (1985). Some preliminary results of a morphometric multivariate analysis of the *Trichia* (Pulmonata: Helicidae) species groups in Britain. *Journal of Natural History*, *19*, 1217–1230.

Neiber, M. T., & Hausdorf, B. (2015). Phylogeography of the land snail genus *Circassina* (Gastropoda: Hygromiidae) implies multiple Pleistocene refugia in the western Caucasus region. *Molecular Phylogenetics and Evolution*, *93*, 129–142. https://doi.org/10.1016/j.ympev.2015.07.012

Neiber, M. T., Razkin, O., & Hausdorf, B. (2017). Molecular phylogeny and biogeography of the land snail family Hygromiidae (Gastropoda: Helicoidea). *Molecular Phylogenetics and Evolution*, *111*, 169–184. https://doi.org/10.1016/j.ympev.2017.04.002

Nenadić, D., Bogićević, K., Lazarević, Z., & Milivojević, J. (2010). Lower and Middle Pleistocene sediments of eastern Srem (Northern Serbia) - paleogeographical reconstruction. *Bulletin of the Natural History Museum*, *3*, 7–25. https://doi.org/10.5937/hnhmb1508007n

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating

maximum-likelihood phylogenies. *Molecular Biology and Evolution*, *32*, 268–274. https://doi.org/10.1093/molbev/msu300

Niemiller, M. L., Fitzpatrick, B. M., & Miller, B. T. (2008). Recent divergence-with-gene-flow in Tennessee cave salamanders (Plethodontidae: Gyrinophilus) inferred from gene genealogies. *Molecular Ecology*, *17*, 2258–2275. https://doi.org/10.1111/j.1365-294x.2008.03750.x

Papadopulos, A. S., Baker, W. J., Crayn, D., Butlin, R. K., Kynast, R. G., Hutton, I., & Savolainen, V. (2011). Speciation with gene flow on Lord Howe Island. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 13188–13193. https://doi.org/10.1073/pnas.1106085108

Pazonyi, P., Kordos, L., Magyari, E., Marinova, E., Fükőh, L., & Venczel, M. (2014). Pleistocene vertebrate faunas of the Süttő Travertine Complex (Hungary). *Quaternary International*, *319*, 50–63. https://doi.org/10.1016/j.quaint.2013.02.031

Perrin, N., Honsberger, P., & Pontet, A. (1984). Approche biochimique et morphologique des espèces du genre *Trichia* (Helicidae, Gastropoda) de Suisse occidentale. *Revue Suisse De Zoologie*, *91*, 483–495.

Pfenninger, M., Hrabáková, M., Steinke, D., & Dèpraz, A. (2005). Why do snails have hairs? A Bayesian inference of character evolution. *BMC Evolutionary Biology*, *5*, 59. https://doi.org/10.1016/j.tree.2004.07.002

Pieńkowska, J. R., Proćków, M., Górka, M., & Lesicki, A. (2018). Distribution of *Monacha claustralis* (Rossmässler, 1834) and *M. cartusiana* (O. F. Müller, 1774) (Eupulmonata: Hygromiidae) in Central European and Balkan countries: New data. *Folia Malacologica*, *26*, 103–120. https://doi.org/10.12657/folmal.026.009

Proćków, M. (2009). The genus *Trochulus* Chemnitz, 1786 (Gastropoda: Pulmonata: Hygromiidae) – a taxonomic revision. *Folia Malacologica*, *17*, 101–176. https://doi.org/10.2478/v10125-009-0013-0

Proćków, M., Duda, M., Kruckenhauser, L., Maassen, W. J. M., de Winter, A. J., & Mackiewicz, P. (2019). Redescription of the western Balkan species *Xerocampylaea waldemari* and its phylogenetic relationships to other Urticicolini (Gastropoda: Hygromiidae). *Systematics and Biodiversity*, *17*, 367–384. https://doi.org/10.1080/14772000.2019.1617365

Proćków, M., Kuźnik-Kowalska, E., & Mackiewicz, P. (2017a). Phenotypic plasticity can explain evolution of sympatric polymorphism in the hairy snail *Trochulus hispidus* (Linnaeus, 1758). *Current Zoology*, *63*, 389–402. https://doi.org/10.1093/cz/zow082

Proćków, M., Kuźnik-Kowalska, E., & Mackiewicz, P. (2017b). The influence of climate on shell variation in *Trochulus striolatus* (C. Pfeiffer, 1828) (Gastropoda: Hygromiidae) and its implications for subspecies taxonomy. *Annales Zoologici*, *67*, 199–220. https://doi.org/10.3161/00034541anz2017.67.2.002

Proćków, M., Mackiewicz, P., & Pieńkowska, J. R. (2013). Genetic and morphological studies of species status for poorly known endemic *Trochulus phorochaetius* (Bourguignat, 1864) (Gastropoda: Pulmonata: Hygromiidae) and its comparison with closely related taxa. *Zoological Journal of the Linnean Society*, *169*, 124–143. https://doi.org/10.1111/zoj.12048

Proćków, M., Proćków, J., Błażej, P., & Mackiewicz, P. (2018). The influence of habitat preferences on shell morphology in ecophenotypes of *Trochulus hispidus* complex. *Science of the Total Environment*, *630*, 1036–1043. https://doi.org/10.1016/j.scitotenv.2018.02.311

Proćków, M., Strzała, T., Kuźnik-Kowalska, E., & Mackiewicz, P. (2014). Morphological similarity and molecular divergence of *Trochulus striolatus* and *T. montanus*, and their relationship to sympatric congeners (Gastropoda: Pulmonata: Hygromiidae). *Systematics and Biodiversity*, *12*, 366–384. https://doi.org/10.1080/14772000.2014.925986

Proćków, M., Strzała, T., Kuźnik-Kowalska, E., Proćków, J., & Mackiewicz, P. (2017c). Ongoing speciation and gene flow between taxonomically challenging *Trochulus* Species complex (Gastropoda: Hygromiidae). *PLoS One*, *12*, e0170460. https://doi.org/10.1371/journal.pone.0170460

Reischütz, A., & Reischütz, P. L. (2007). Rote Liste der Weichtiere (Mollusca) Österreichs. In K. P. Zulka (Ed.), *Rote Listen gefährdeter Tiere Österreichs Teil 2: Kriechtiere, Lurche, Fische, Nachtfalter, Weichtiere* (pp. 363–433). Wien: Böhlau Verlag.

Rieseberg, L. H., Church, S. A., & Morjan, C. L. (2004). Integration of populations and differentiation of species. *New Phytologist*, *161*, 59–69. https://doi.org/10.1046/j.1469-8137.2003.00933.x

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*, 539–542. https://doi.org/10.1093/sysbio/sys029

Saadi, A. J., & Wade, C. M. (2019). Resolving the basal divisions in the Stylommatophoran land snails and slugs with special emphasis on the position of the Scolodontidae. *Molecular Phylogenetics and Evolution*, *139*, 106529. https://doi.org/10.1016/j.ympev.2019.106529

Schileyko, A. A. (1978). On the systematics of *Trichia* s. lat. *Malacologia*, *17*, 1–56.

Schileyko, A. A. (2006). Treatise on recent terrestrial pulmonate molluscs. Part 14: Helicodontidae, Ciliellidae, Hygromiidae. *Ruthenica, Supplement*, *2*, 1907–2041.

Schmidt, Z., Halouzka, R., Nosek, J., & Sixl, W. (1978). Quarternary Mollusca of the Southern Part of South Slovakian Lowland. Biologische Station Neusiedler See. *BPB –. Bericht*, *25*, 1–10.

Stover, B. C., & Muller, K. F. (2010). TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, *11*, 7. https://doi.org/10.1186/1471-2105-11-7

Taylor, J. W. (1916). *Monograph of the land and freshwater Mollusca of the British Isles, Part 22*. Leeds: Taylor Bros.

Thiengo, S. C., Faraco, F. A., Salgado, N. C., Cowie, R. H., & Fernandez, M. A. (2007). Rapid spread of an invasive snail in South America: The giant African snail, *Achatina fulica*, in Brasil. *Biological Invasions*, *9*, 693–702. https://doi.org/10.1007/s10530-006-9069-6

Wade, C. M., Mordan, P. B., & Clarke, B. (2001). A phylogeny of the land snails (Gastropoda: Pulmonata). *Proceedings of the Royal Society B: Biological Sciences*, *268*(1465), 413–422. https://doi.org/10.1098/rspb.2000.1372

Waga, W., Mackiewicz, D., Zawierta, M., & Cebrat, S. (2007). Sympatric speciation as intrinsic property of expanding populations. *Theory in Biosciences*, *126*, 53–59.

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., & Barton, G. J. (2009). Jalview Version 2–a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, *25*, 1189–1191. https://doi.org/10.1093/bioinformatics/btp033

Welter-Schultes, F. W. (2012). *European non-marine molluscs, a guide for species identification*. Göttingen: Planet Poster Editions.

Welter-Schultes, F. W., Audibert, C., & Bertrand, A. (2011). Liste des mollusques terrestres et dulcicoles de France continentale (excl. hydrobioïdes). *Folia Conchyliologica*, *12*, 4–44.

Wimmer, W. (2006). *Hygromia cinctella* (Draparnaud, 1801) auf Gut Sunder, Landkreis Celle, Niedersachsen (Gastropoda: Hygromiidae). *Braunschweiger Naturkundliche Schriften*, *7*, 671–675.

Yanchukov, A., Hofman, S., Szymura, J. M., Mezhzherin, S., Morozov-Leonov, S. Y., Barton, N. H., & Nürnberger, B. (2006). Hybridization of *Bombina bombina* and *B. variegata* (Anura, Discoglossidae) at a sharp ecotone in Western Ukraine: Comparisons across transects and over time. *Evolution*, *60*, 583–600. https://doi.org/10.1111/j.1558-5646.1991.tb04400.x

Zawierta, M., Waga, W., Mackiewicz, D., Biecek, P., & Cebrat, S. (2008). Phase transition in sexual reproduction and biological evolution. *International Journal of Modern Physics, C*, *6*(19), 917–936. https://doi.org/10.1142/S0129183108012595

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Proćków M, Kuźnik-Kowalska E, Pieńkowska JR, Żeromska A, Mackiewicz P. Speciation in sympatric species of land snails from the genus *Trochulus* (Gastropoda, Hygromiidae). *Zool Scr*. 2021;50:16–42. https://doi.org/10.1111/zsc.12458