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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

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The taxonomic status of *Trochulus phorochaetius* and its phylogenetic relationships to *Trochulus plebeius* and *Trochulus hispidus* were evaluated based on molecular, morphological, and genital anatomy data. The canonical discriminant analysis (CDA) of shell morphology allowed the clear differentiation between these three nominal species, whereas the genitalia revealed their high similarity. Analyses of cytochrome *c* oxidase subunit I (*COI*) sequences were not always congruent with the differentiation between these three species by shell characters. None of them formed a monophyletic group covering all its sequences. Instead, many sequences obtained from individuals classified to the same morphospecies, and/or usually collected from the same region or country, created highly supported separate clades and delimited clusters. Three distinct clades containing sequences of two morphospecies originating from the same country were identified in molecular phylogenetic and species delimitation studies: (1) *T. plebeius* + *T. hispidus* from Great Britain; (2) *T. plebeius* + *T. hispidus* from Poland; and (3) *T. phorochaetius* + *T. hispidus* from France. In the latter case some of the sequences were even identical. Their genetic similarity could indicate the ability to hybridize, which may be evidenced by the lack of major differences in their reproductive system. The assignment of distinctive morphospecies, and thus existing taxonomic names, to genetically defined evolutionary lineages is premature and arbitrary to some extent at this stage.

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ADDITIONAL KEYWORDS: ABGD – canonical discriminant analysis – correspondence analysis – cytochrome *c* oxidase subunit I – France – GMYC – morphospecies – phylogenetic analyses – species delimitation – *Trochulus hispidus* – *Trochulus plebeius*.

INTRODUCTION

The delimitation of species and reconstruction of their phylogenetic relationships are two major aims of systematics (Mayr & Ashlock, 1991; Coyne & Orr, 2004). Species are routinely used as fundamental units in biogeography, ecology, conservation, and evolutionary biology (Avise, 2000; Goldstein *et al*., 2000; Hey *et al*., 2003; Weiss & Ferrand, 2007). Delimiting species is important in the context of understanding speciation processes. Therefore, resolving species boundaries is necessary to study evolution (Sites & Marshall, 2003).

Taxonomic conclusions in molluscs are often hindered by the lack of morphological and anatomical diversity between different lineages, especially in cryptic species or species with overlapping variability

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(Liu, Hershler & Clift, 2003; Pinceel *et al*., 2004; Pfenninger, Cordellier & Streit, 2006; Pfenninger & Schwenk, 2007; Dépraz, Hausser & Pfenninger, 2009). In other cases, mollusc taxonomy is also ambiguous because of the high phenotypic plasticity of shell morphology or other traditional taxonomic characters (Giusti & Manganelli, 1992; Goodacre, 2001; Uit de Weerd, Piel & Gittenberger, 2004). In such cases, an integrative approach, using molecular and morphological data, can be useful to distinguish between species-level taxa, or to reveal whether differences in morphology correspond with different structured populations of a polymorphic taxon (Jordaens, Van Riel & Backeljau, 2003; Korte & Armbruster, 2003; Parmakelis *et al*., 2003; Elejalde *et al*., 2008).

This integrative approach seems to be the most appropriate for some species of the genus *Trochulus*, as they are very similar to each other and an individual conchological variation does not allow for species to be determined unambiguously (Proćków, 2009). Even genital anatomical differences are not diagnostic enough to allow for unequivocal identification (Hesse, 1931): several authors have reported inconsistent details in the reproductive organs, which have often relied only on subtle differences in size ratios (Wagner, 1915; Forcart, 1965; Schileyko, 1978; Proćków, 2009). Even molecular methods have not solved taxonomic problems for some cases: for example, they have indicated several distinct mitochondrial lineages in *Trochulus hispidus* and the probable occurrence of cryptic species (Pfenninger *et al*., 2005; Dépraz *et al*., 2009; Kruckenhauser *et al*., 2011).

This study is focused on the poorly known endemic species of *Trochulus phorochaetius* (Bourguignat, 1864: 52), described from the Chartreuse Mountains, which is the southernmost range in French Jura. As originally stated (Bourguignat, 1864), this species resembles a small-sized *Trochulus villosus* (Studer, 1820) in the conspicuous long hairs, and probably because of this trait, *T. phorochaetius* was used as the synonym of *T. villosus* by Hesse (1921) and Germain (1929, 1930). Subsequently, the unavailability of specimens of the nominal species forced Proćków (2009) to classify it as a synonym of *T. villosus* after Germain (1929). However, Winter's (1990) studies, based on conchological and genital characters, revealed the shell similarity of *T. phorochaetius* to either *Trochulus sericeus* (Draparnaud, 1801) or *Trochulus plebeius* (Draparnaud, 1805), whereas its genitalia resembled the illustration and measurements of *Trichia 'sericea'* from Zürichberg (Switzerland) included in Klöti-Hauser (1920). It was also reported that *T. phorochaetius* differs from East German and Czechoslovakian specimens of *T. 'plebeia'*, illustrated by Schileyko (1978), in the much shorter bursal duct and the lower portion of the vagina, which is longer and well differentiated from the dart-sac complex (Winter, 1990).

The status of both *T. sericeus* and *T. plebeius* also remains uncertain (Schileyko, 1978; Wiktor, 2004; Anderson, 2005; Pfenninger *et al*., 2005). According to Falkner (1982, 1990), the name *T. plebeius* refers to a quite different species from the Swiss and French Jura. The snails are larger than *T. sericeus*, with more prominent growth lines, less and shorter hairs, last whorl weakly keeled, and aperture with a strong lip inside. They live in dry and warm forests and shrubs. In contrast, *T. sericeus* is distinguished by a globose shell, with long curved hairs, a weak lip, and a narrow umbilicus, as well as by more convex whorls, with coarser growth lines than in another similar *Trochulus* species, *T. hispidus*. *Trochulus sericeus* inhabits the herb layer in damp forests and shrubs. On the other hand, significant shell variation within and between populations of *T. hispidus* has been reported, and the synonymy of *T. hispidus* and *T. plebeius* has been proposed (Proćków, 1997, 2009).

Because the traditional classification based on conchological and genital characters is ambiguous and insufficient, it should be verified by molecular methods based on DNA analyses. Molecular studies of *Trochulus* taxa have been recently used to test species-level taxonomies based on morphological characters (Duda *et al*., 2011). Molecular methods have enabled the objective and rigorous genetic analysis of differences in populations and between higher-level taxa, as well as providing essential data to address many issues of speciation (Coyne & Orr, 2004).

Here, we have compared the endemic *T. phorochaetius* with other congeners, i.e. *T. plebeius* and *T. hispidus*, using the combination of molecular and morphological data. Because *T. plebeius* and *T. hispidus* are both controversial, and the latter is clearly a complex of cryptic taxa (Dépraz *et al*., 2009), we provisionally adopted the classification of these taxa provided by Kerney, Cameron & Jungbluth (1983).

MATERIAL AND METHODS

Sampling, shell, and genital morphology

Samples of the species were collected from 16 different localities in France, Germany, England, and Poland (Fig. 1; Table 1). *Trochulus phorochaetius* (Fig. 2A) was collected from the type locality in the Chartreuse Mountains and the adjacent area. Because of the lack of unequivocal distinction between *T. plebeius* and *T. sericeus*, both English and

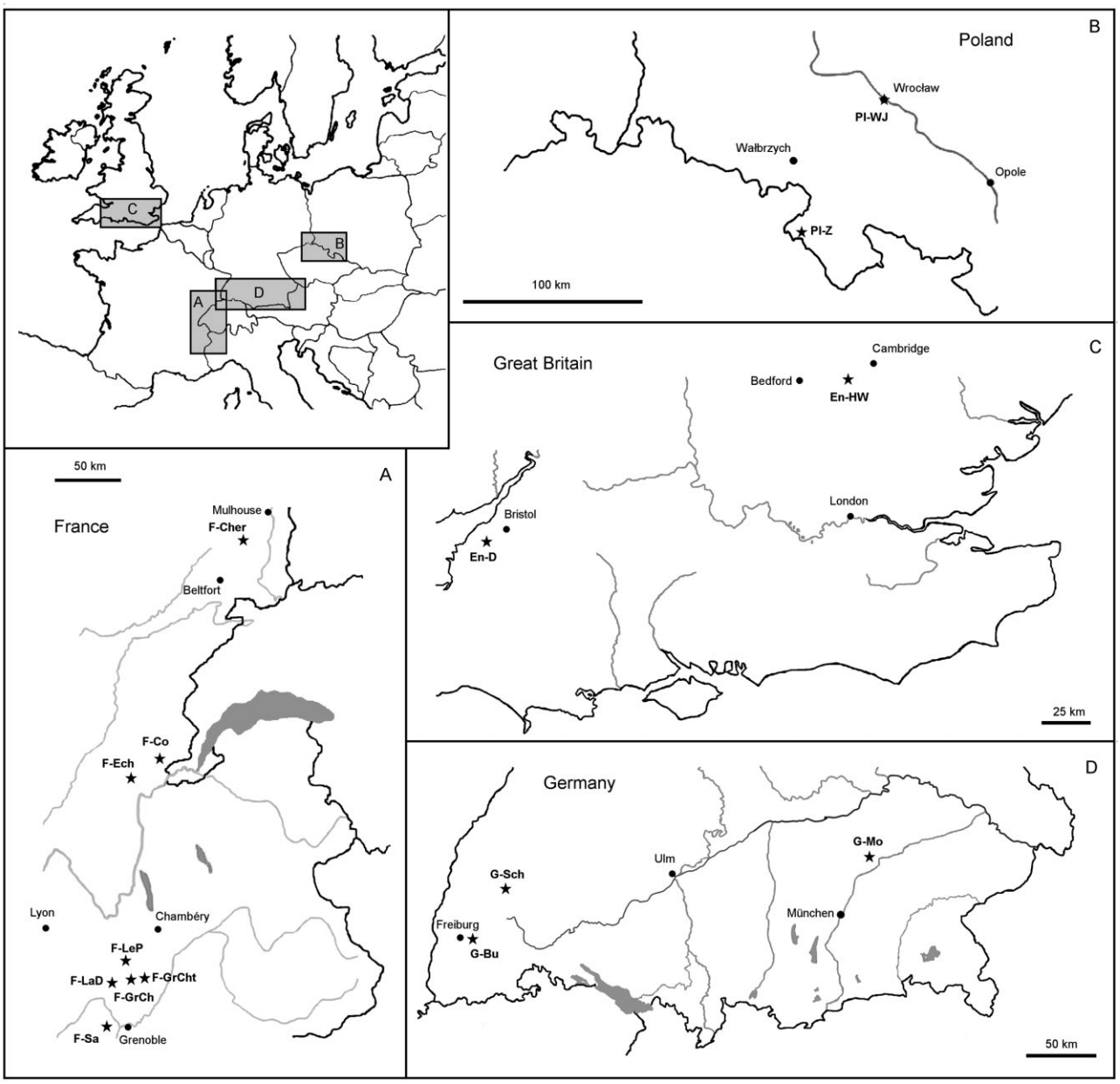

Figure 1. Geographical locations of the populations sampled (★) in France (A), Poland (B), Great Britain (C), and Germany (D); •, main cities. See Table 1 for abbreviations.

Polish samples taken from the locations given by Wiktor (1964) and Paul (1967) were determined as *T. plebeius* (this name is used for snails morphologically similar to both *T. plebeius* and *T. sericeus* throughout our paper). Because the locations of the first descriptions for these two nominal species were imprecise, the French populations were considered as topotypical material (Fig. 2B–C). *Trochulus hispidus* (Fig. 2D) was identified based on the morphological traits commonly used in malacological studies (Kerney *et al*., 1983; Wiktor, 2004). Only French populations of *T. hispidus* co-occurred with *T. plebeius* or *T. phorochaetius*. In the genetic analyses we also included three specimens of *Trochulus coelomphala* (Locard, 1888). All snails were collected in two seasons between April and August in 2009 and 2010. Initially, all the specimens were determined as three presumed species based on conchological traits, and then morphometrically and genetically examined.

Shells with at least five whorls were recognized as adult, and from the side perspective we measured

Table 1. Localities of samples used in the study

Locality	Acronym	Alt	Coordinates	N_g	N_m	N_a
Trochulus hispidus						
Wrocław-Jarnołtów, Lower Silesia, Poland	Pl-WJ	130	51°07′16.9″N 16°50′38.7″E	3	30	5
Downside, North Somerset, England	En-D	160	51°23′28.4″N 02°43′06.9″W	4	9	3
Moosburg a/d Isar, Bavaria, Germany	G-Mo	376	11°55′45.5″N 48°12′26.5″E	3	4	3
Schramberg, Schwarzwald, Germany	G-Sch	508	08°22′46.0″N 47°58′14.8″E	1	7	3
Buchenbach, Schwarzwald, Germany	G-Bu	518	47°58′14.8″N 08°03′34.8″E	–	10	6
Échallon, Ain, France	F-Ech1	518	46°11′59.2″N 05°44′44.6″E	3	3	3
Grande Chartreuse monastery, Isère, France	F-GrCht	851	45°21′56.1″N 05°47′32.9″E	4	42	5
near Grande Chartreuse monastery, Isère, France	F-GrCh	786	45°21′32.0″N 05°45′15.0″E	2	1	–
Trochulus plebeius						
Hayley Wood, Cambridgeshire, England	En-HW	84	52°09′31.0″N 0°06′54.9″W	4	31	5
Zieleniec, Sudetes, Poland	Pl-Z	686	50°20′07.7″N 16°24′34.6″E	2	32	5
Échallon, Ain, France	F-Ech2	518	46°11′59.2″N 05°44′44.6″E	2	7	3
Coiserette, Jura, France	F-Co	611	46°20′21.0″N 05°49′56.3″E	2	13	2
Chèrmenille, Vosges, France	F-Cher	581	47°59′16.1″N 06°50′13.4″E	2	–	–
Trochulus phorochaetius						
Sassenage, Isère, France	F-Sa	252	45°12′31.9″N 05°39′11.3″E	5	28	5
Le Pont du Lac, Savoie, France	F-LeP	750	45°26.020′N 05°52.513′E	4	13	5
near Grande Chartreuse monastery, Isère, France	F-GrCh	786	45°21′32.0″N 05°45′15.0″E	3	1	–
Là Diat, Isère, France	F-LaD	722	45°20′35.6″N 05°48′02.9″E	4	5	2
Trochulus coelomphala						
Günzburg-Reisensburg, Bavaria, Germany	G-Gu	436	48°27′56.8″N 10°18′05.9″E	3	–	–

Abbreviations: Alt, altitude (m a.s.l.); N_a, total number of anatomically investigated specimens; N_g, total number of genetically investigated specimens; N_m, total number of morphologically investigated specimens.

shell height (H), shell width (W), body whorl height (bwH), aperture height (h), and aperture width (w). From below, measurements of the umbilicus major diameter (U) (i.e. the longest diameter parallel with the shell diameter, D), 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: height/width ratio (H/W), relative height of body whorl = body whorl height/shell height ratio (bwH/H), umbilicus relative diameter = umbilicus major diameter/shell diameter ratio (U/D), and ratio of umbilicus minor to its major diameter (u/U). Altogether, 236 specimens from 15 sample sites were measured in standardized views (Proćków, 2009) by the same person (M.P.), using the graduated eyepiece of a stereomicroscope with an 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.

Three additional shell traits were recorded as binary (present/absent) characters: lip, internal rib, and light spiral band running along the body whorl at approximately half of its height (Fig. 3A, B). Hairs were inspected in all live-collected adults and juveniles ($N = 276$), 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) (Fig. 3C). In order to quantify the hair length, digital microscopic images were taken from seven hairs of 29 specimens (eight from each species analysed and five from Coiserette individuals). Hair lengths were measured by using TPSdig 2.16 (Rohlf, 2010) and then assigned to two categories: long ($\geq$ 0.41 mm) or short hairs ($\leq$0.4 mm) (Fig. 3D–G).

The definition of adulthood from shell apertural traits in *T. hispidus* is problematic, because adult specimens often lack a lip (Frömming, 1954; Proćków, pers. observ.). Therefore, all snails with at least five whorls were regarded as adults because it was the smallest number of whorls seen in an individual with a fully developed lip.

For anatomical examinations, between two and six adult snails from 13 populations (Table 1) were dissected, and external genital morphology was observed. Seven measurements of genitalia were taken, including the lengths of the flagellum (fl), epiphallus (ep), penis (p), spermatheca (= bursa copulatrix) (sl), spermathecal duct (sd), upper vagina (i.e. distance between outlet of mucous glands and tips of inner dart sacs) (uv), and the width of spermatheca (sw). Additionally,

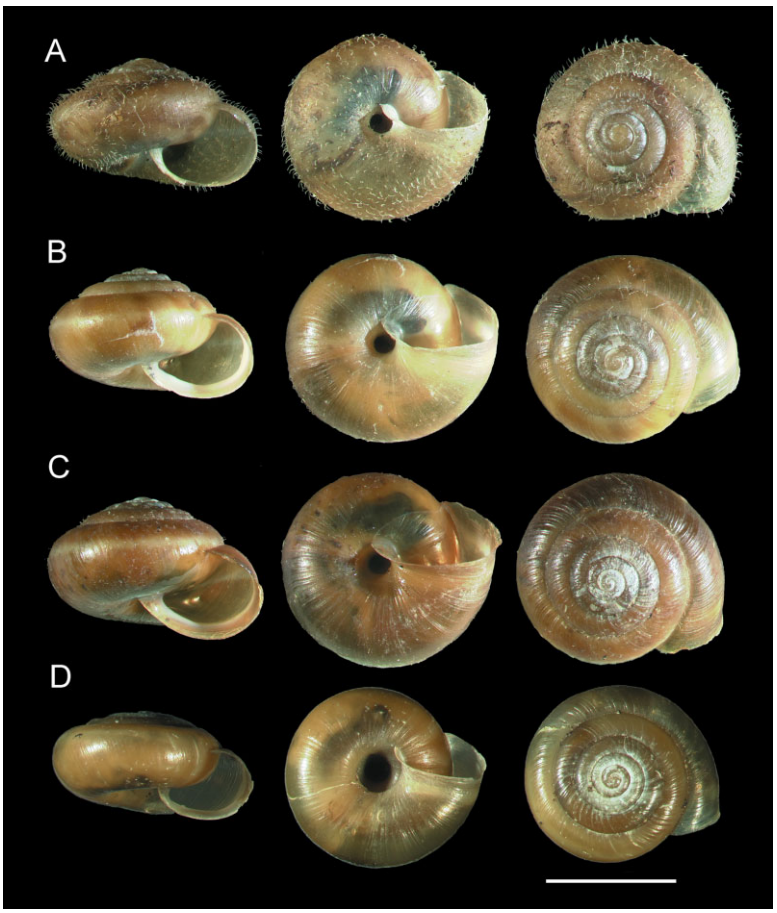

Figure 2. A, *Trochulus phorochaetius* specimen from Sassenage; B, *Trochulus plebeius* specimen from Échallon; C, *T. plebeius* specimen from Coiserette; D, *Trochulus hispidus* specimen from Schramberg. Scale bar: 5 mm.

coefficients of the following proportions were included in statistical analysis: flagellum/epiphallus (fl/ep), epiphallus/penis (ep/p), spermatheca length/spermathecal duct length (sl/sd), and spermatheca width/spermatheca length (sw/sl).

Measurements were log-transformed to obtain the normal distribution before being used in canonical discriminant analysis (CDA). Qualitative data were subjected to correspondence analysis (CA). Variables contributing most to CDA, i.e. the most promising in population distinction, were further used in Kruskal–Wallis non-parametric analysis of variance (ANOVA). Statistical analyses were performed with STATISTICA 8 (Stat Soft, Inc. 1984–2007).

Genetic analysis

DNA extraction, PCR amplification, and sequencing

Snail feet or, in the case of small specimens, the entire animals preserved in ethanol were used for DNA extraction. The extraction method, with little modification, is based on Sokolov's (2000) method, elaborated for mucopolysaccharide-rich molluscan tissues. The genomic DNA solution obtained was diluted to 100 ng μL^{-1} and used in the polymerase chain reaction (PCR).

The degenerate primers designed by J.P. (unpubl. data) to amplify the 5′ end of the cytochrome *c* oxidase subunit I (*COI*) gene (often used as a barcode sequence) were: bcsmF1, 5′-AAYCATAAAGAYATTGG DACWTTDTA-3′, and bcsmR1, 5′-TAWACYTCWGGR TGACCAAAAAAYCA-3′ [the nucleotides and ambiguity codes were determined according to the International Union of Pure and Applied Chemistry (IUPAC)]. The region located between the primers was 650 base pair (bp) in length. All PCR reactions were run under the following thermal cycle programme: 1 min at 94 °C, followed by 42 cycles of 40 s at 94 °C, 40 s at 53 °C, and 1 min at 72 °C, and finally 5 min at 72 °C. The PCR was carried out in a 25-μL volume following a modified protocol prepared by the Biodiversity Institute of Ontario for Consortium for the Barcode of Life (http://barcoding.si.edu/PDF/Protocols_for_High_Volume_DNA_Barcode_Analysis.pdf). The PCR

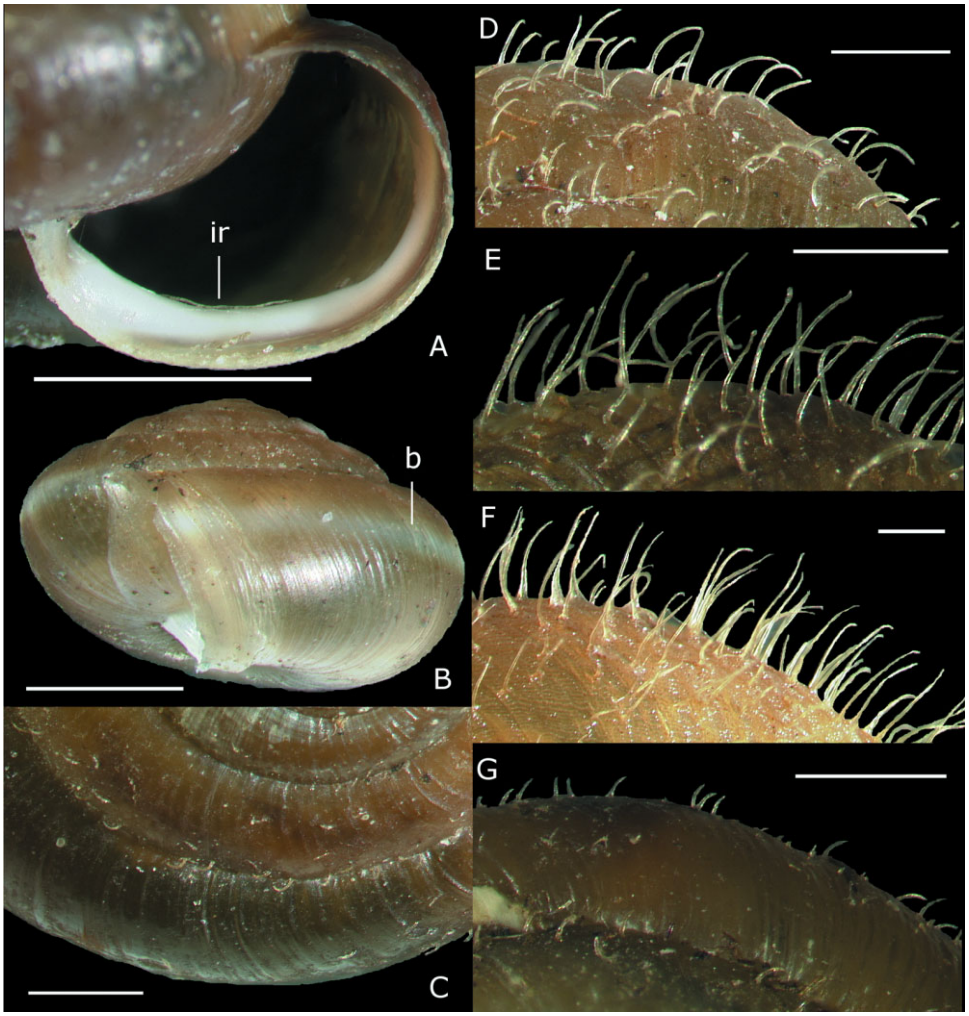

Figure 3. Shell traits: A, internal rib (ir) in *Trochulus plebeius* specimen from Zieleniec; B, band (b) in *Trochulus hispidus* specimen from Échallon; C, short hairs in *T. plebeius* specimen from Coiserette; D, long hairs in *Trochulus phorochaetius* specimen from Le Pont du Lac; E, very long hairs in *Trochulus villosulus* specimen from Wojtkowa, south-east Poland; F, very long hairs in *Trochulus villosus* specimen from Fützen, south Germany; G, short hairs in *T. hispidus* specimen from Buchenbach. Scale bars: A, B, 2.5 mm; C–G, 1 mm.

products were visualized on 1% agarose gels and sequenced in both directions on an Applied Biosystems Hitachi 3130xl Genetic Analyser automated sequencer. Full-length sequences were assembled and edited by eye using BioEdit 7.0.5. (Hall, 1999). All new 51 sequences have been deposited in GenBank under accession numbers JX475050–JX475100.

Molecular phylogenetic analyses

To study the phylogenetic relationships of the DNA sequences obtained, they were aligned together with other *COI* gene sequences assigned to *Trochulus* collected from BLAST searches of the GenBank database, including other *Trochulus* species, such as *Trochulus oreinos oreinos*, *Trochulus oreinos scheerpeltzi*, and *Trochulus villosus*. Two sequences of *Trochulus lubomirskii* (= *Trichia lubomirskii* = *Plicuteria lubomirskii*) and one from *Petasina bielzi* (Hygromiidae), were also included as out-groups. The alignment was obtained in MAFFT 6.857 using the slow and accurate algorithm L-INS-i with 1000 cycles of iterative refinement (Katoh & Toh, 2008). After the exclusion of incomplete and redundant sequences, the final alignment with the length of 567 nucleotides was created by 56 sequences (including 19 sequences derived from GenBank and 37 non-identical sequences obtained from the 51 new sequences).

Phylogenetic trees were inferred by six methods using four programs: Bayesian inference (BI) with MrBayes 3.2.1 (Ronquist *et al*., 2012); maximum likelihood (ML) with TreeFinder (Jobb, von Haeseler & Strimmer, 2004); and morePhyML (Guindon &

Gascuel, 2003; Criscuolo, 2011), maximum parsimony (MP), neighbour joining (NJ), minimum evolution (ME), and weighted least squares (WLS) with PAUP* 4.0b (Swofford, 1998).

In MrBayes analyses, we assumed three separate mixed + Γ + I models for three codon positions to sample appropriate models across the substitution model space in the Bayesian Markov chain Monte Carlo (MCMC) analysis itself (Huelsenbeck, Larget & Alfaro, 2004), escaping the need for a priori model testing. In the Bayesian analysis, we applied two independent runs starting from random trees using four Markov chains each. Trees were sampled every 100 generations for 10 000 000 generations. In the final analysis we selected trees from the last 3 145 000 generations that reached the stationary phase and convergence (i.e. the standard deviation of split frequencies stabilized, and was lower than 0.004, much below the proposed threshold of 0.01).

In TreeFinder, we also applied separate substitution models for three codon positions: TN + Γ (for the first codon position), HKY + Γ (for the second codon position), and J2 + Γ (for the third codon position), as suggested by the Propose Model module in accordence with corrected Akaike Information Criterion (AICc). The ML tree constructed with morePhyML, and the trees based on three distance methods (NJ, ME, and WLS) in PAUP, were calculated using the best-fit substitution model TPM2uf + Γ + I, as proposed in jModeltest 0.1.1 (Posada, 2008), according to all three criteria: AIC, AICc and Bayesian Information Criterion (BIC).

We assumed a search depth of 2 in TreeFinder and used the best heuristic search algorithms, i.e. NNI and SPR in morePhyML. In the case of ME, WLS, and MP methods, the final trees were searched from ten starting trees obtained by stepwise addition with a random-addition sequence followed by the tree bisection and reconnection (TBR) branch-swapping algorithm.

The non-parametric bootstrap analyses were performed on 1000 replicates for TreeFinder, PhyML, and each of the PAUP methods. Additionally, we applied the Local Rearrangements–Expected Likelihood Weights (LR-EWL) method in TreeFinder. In all analyses among-site rate variation was modelled on a gamma distribution with five category rates.

Species delimitation procedures

To delimit species based on *COI* sequences we applied two approaches: the Automatic Barcoding Gap Detection (ABGD) method (Puillandre *et al*., 2011) and the General Mixed Yule-Coalescent model (GMYC) (Pons *et al*., 2006; Monaghan *et al*., 2009). ABGD was carried out via a web interface (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html) using a distance matrix obtained from the phylogenetic tree that was inferred by MrBayes (see the Molecular phylogenetic analyses section). GMYC analyses were performed in the R environment (R Core Team, R: A Language and Environment for Statistical Computing, http://www.R-project.org) using the Splits package (Ezard, Fujisawa & Barraclough, 2009). We applied both single (Pons *et al*., 2006) and multiple threshold models (Monaghan *et al*., 2009). The input tree was obtained by the conversion of the MrBayes tree to the ultrametric one using the chronopl command from the Analyses of Phylogenetics and Evolution (Ape) package in R (Paradis, Claude & Strimmer, 2004), which implements the penalized likelihood method (Sanderson, 2002).

RESULTS

Shell and genital morphology

Shell measurements in *T. phorochaetius*, *T. plebeius*, and *T. hispidus* are shown in Table 2. The results of the canonical discriminant analysis (CDA) (Fig. 4) indicated that the first discriminant function captured most of the variance among species (77.3%), which was much larger than the variance associated with the second function (22.2%). These two functions accounted for more than 99% of the total dispersion in ten predictor variables. Considering the canonical coefficients making up the first function, the highest loading was found for umbilicus relative diameter (U/D) (Table 3). A sequential chi-square test showed that the first ($\chi^2 = 872$, d.f. = 30, $P < 0.001$) and the second ($\chi^2 = 324$, d.f. = 18, $P < 0.001$) functions contributed to population discrimination to a very large extent, whereas the contribution of the third function was not significant.

A clear differentiation between *T. hispidus* and the remaining two *Trochulus* species could be noticed (Fig. 4). *Trochulus plebeius* and *T. phorochaetius* also seem to be separate sets, but the differences between them are much smaller. When the specimens from Coiserette were included in the analysis, several of them were placed in such a position that *T. plebeius* and *T. phorochaetius* appeared to overlap (Fig. 4). Even if the Coiserette specimens were not considered, the gap between these species was still very small, and may reflect a sampling issue. The second function of CDA was generally determined by umbilicus major and minor diameter (positive U and negative u) and their ratio (positive u/U) (Table 3). However, none of these characters was specific enough to distinguish *T. phorochaetius* and *T. plebeius* unambiguously, because their ranges overlapped among populations. Nevertheless, it was possible to recognize two distinct groups (Fig. 5). The first consisted of

Table 2. Shell measurements (in mm) of *Trochulus phorochaetius*, *Trochulus plebeius*, and *Trochulus hispidus*

	T. phorochaetius, $N = 47$			*T. plebeius*, $N = 83$			*T. hispidus*, $N = 106$		
Feature	Range	Mean	*SD*	Range	Mean	*SD*	Range	Mean	*SD*
W	6.6–8.7	7.82	0.49	7.1–8.8	7.91	0.49	5.7–9.8	7.88	0.66
H	4.3–5.6	4.86	0.32	4.3–6.3	5.19	0.43	3.1–5.5	4.48	0.48
bwH	3.5–4.6	4.10	0.25	3.7–4.9	4.28	0.28	2.7–4.4	3.75	0.32
h	2.5–3.6	3.02	0.21	2.6–3.6	3.15	0.23	2.2–3.5	2.80	0.30
w	3.3–4.7	4.13	0.32	3.5–4.8	4.19	0.30	2.6–4.4	3.68	0.31
D	6.6–8.6	7.69	0.47	5.3–8.9	7.74	0.68	5.7–9.7	7.79	0.65
U	0.6–1.1	0.79	0.13	0.5–1.4	0.83	0.22	1.1–2.4	1.67	0.27
u	0.6–1.0	0.73	0.11	0.5–1.4	0.75	0.20	1.0–2.1	1.52	0.25
whl	5.0–5.5	5.19	0.15	5.0–5.9	5.36	0.20	5.0–6.2	5.50	0.27
H W^{-1}	0.54–0.72	0.62	0.04	0.57–0.76	0.66	0.04	0.46–0.71	0.57	0.04
U/D	0.07–0.14	0.10	0.02	0.07–0.18	0.11	0.03	0.16–0.27	0.21	0.02
u/U	0.71–1.00	0.94	0.08	0.64–0.81	0.76	0.08	0.77–1.00	0.91	0.06
bwH/H	0.78–0.95	0.84	0.03	0.75–0.93	0.83	0.03	0.76–0.97	0.84	0.04
hair length	0.23–0.67	0.46	0.10	0.11–0.61	0.26	0.10	0.15–0.36	0.24	0.06

For abbreviations, see Material and methods.

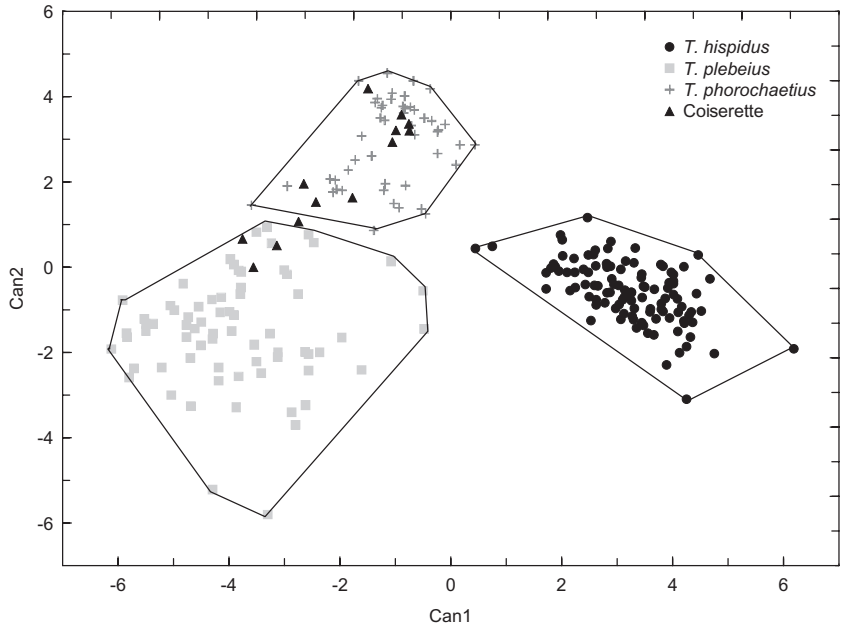

Figure 4. Canonical discriminant analysis based on shell measurements of *Trochulus hispidus*, *Trochulus plebeius*, *Trochulus phorochaetius*, and the Coiserette specimens. Wilks' lambda = 0.0229, $F_{30,664} = 58.009$, $P < 0.00001$.

populations belonging to *T. plebeius* and the second included *T. phorochaetius* samples. The differences between them turned out to be statistically significant (Kruskal–Wallis test, $P < 0.001$). Specimens from the Coiserette site appeared to be very similar to *T. phorochaetius* ($P = 0.32$).

Given the CDA results, an ANOVA was further performed on the umbilicus relative diameter (U/D) for all species (Fig. 6). *Trochulus hispidus* populations showed significant differences from *T. plebeius* and *T. phorochaetius* (Kruskal–Wallis test, $P < 0.001$), whereas *T. plebeius* and *T. phorochaetius* were not statistically different ($P = 1.00$). Although the umbilicus relative diameter represented a strong diagnostic character for *T. hispidus*, it could not be used alone as an unequivocal discriminator because its ranges overlapped with *T. plebeius* (Fig. 6). On the other hand, this trait clearly separated *T. hispidus* and

Table 3. Canonical coefficients of discriminant analysis performed on shell measurements

	Standardized canonical discriminant function coefficients	
Variable	Can 1	Can 2
U/D	1.963	–1.035
u/U	1.043	1.356
u	–1.381	–2.844
U	0.047	3.583
h	0.008	–2.374
bwH	–0.177	0.603
whl	0.114	–0.471
D	1.312	–0.496
W	–0.692	0.332
w	–0.036	0.097
Eigenvalue	9.766	2.811
Cum. Prop. (%)	77.25	99.49

For abbreviations, see Material and methods.

T. phorochaetius because of the non-overlapping ranges of the U/D coefficient, i.e. 0.16–0.27 (mean 0.21) and 0.07–0.14 (mean 0.1), respectively (Fig. 6). Note that the problematic Coiserette specimens did not differ from those representing *T. plebeius* or *T. phorochaetius*.

The canonical discriminant analysis correctly classified 100% of *T. hispidus*, 92.9% of *T. plebeius*, 93.8% of *T. phorochaetius*, and 76.9% of Coiserette individuals. The overall classification accuracy was 92.5%.

The correspondence analysis (CA) of the qualitative data (Fig. 7) revealed that the first dimension explained 89.42% of the total inertia. The positive side of the first axis handled such characters as combinations of lip, internal rib, and band, whereas on the negative side there were traits describing hairs (i.e. their length and durability). These two groups of characters represented two distinct poles, between which the three species examined and Coiserette specimens were placed. *Trochulus plebeius* and *T. hispidus* showed quite strong development of the apertural traits (i.e. internal rib and lip/internal rib combination). In nearly 70% of individuals of both species these characters were present. Hairs and especially their length might be a helpful character to distinguish *T. phorochaetius*: a little more than 64% of its specimens had long hairs ($\geq$ 0.41 mm), whereas only 12% of *T. plebeius* specimens (collected in Zieleniec) and none of the *T. hispidus* or Coiserette individuals shared this character. Considering hair durability, more than 53% individuals of *T. hispidus* were deprived of hairs at all, whereas such specimens constituted no more than 21 and 10% in *T. plebeius* and *T. phorochaetius*, respectively. Grande Chartreuse monastery (F-GrCht) was the only population in which no hairs were observed in any live adult snails, whereas 58.3% of the 36 juveniles inspected lacked hairs, and the rest appeared to have single hairs that were hardly visible, even under a stereomicroscope. On the other hand, hairs were present in all live specimens from the Coiserette site ($N = 6$). The band, and traits in combination with the band, strongly dominated in all adult Coiserette individuals studied (96%); however, these characters could not be considered as constant because of the low sample size ($N = 13$). The band was also observed in *T. hispidus*, *T. plebeius*, and *T. phorochaetius*, but in lower frequency (37, 21, and 8% of their individuals, respectively).

An additional correspondence analysis excluding the Coiserette specimens was performed (data not shown). It revealed that long hairs constituted an even more essential discriminant trait for *T. phorochaetius*, whereas hair durability was characteristic of ~80 and 90% live individuals assigned to *T. plebeius* and *T. phorochaetius*, respectively.

The CDA of genital measurements (Fig. 8) did not show any clear tendency to group the individuals according to their species assignment, in contrast with the CDA based on shell measurements (Fig. 4). However, the first two canonical functions together explained 90.4% of the total variance. The only two Coiserette specimens included in the analysis, clearly separated from the rest, and many individuals of *T. hispidus* did not mix with *T. phorochaetius* and *T. plebeius* specimens. Genital measurements and significant differences between all taxa studied are shown in Table 4. The most distinctive genital feature appeared to be the length of the spermathecal duct. This trait reached more than 7 mm in the two specimens dissected from Coiserette, whereas in *T. hispidus*, which could also be more or less distinguished from the other species, it ranged between 1.3 and 5.1 mm. In *T. phorochaetius* and *T. plebeius* there was much overlap and the spermathecal duct reached 2.5–6.6 mm and 2.4–5.7 mm, respectively (Table 4).

Molecular phylogenetic analysis

The Bayesian phylogenetic tree based on *COI* gene sequences is presented in Figure 9. A similar topology was also obtained with maximum-parsimony and maximum-likelihood methods (both in Treefinder and PhyML), whereas the distance trees were slightly different. Generally, deep relationships were poorly resolved, but a lot of terminal branches were very well supported by several methods (see Figure S1 for the cladogram with the support values obtained in all approaches).

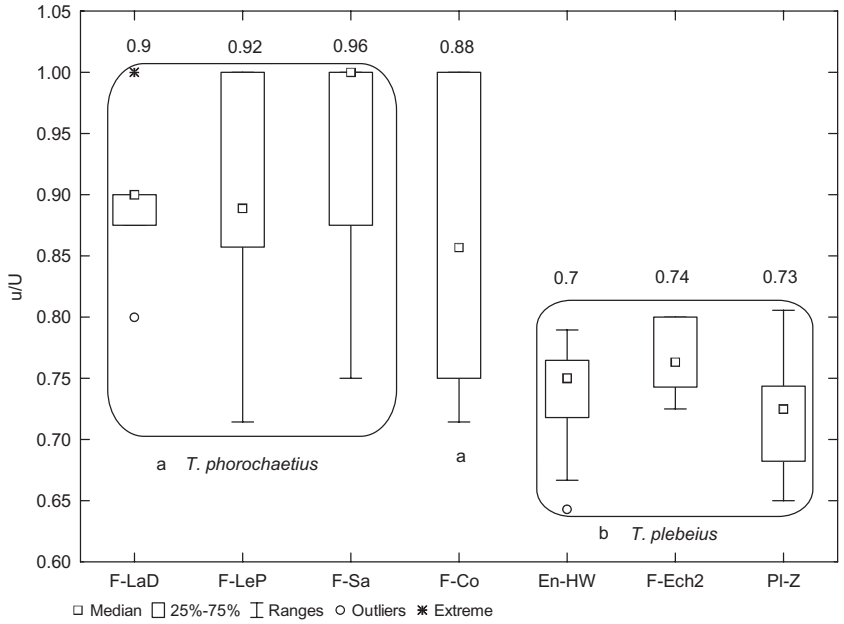

Figure 5. Variation of the ratio of the umbilicus minor to its major diameter (u/U) in *Trochulus phorochaetius* and *Trochulus plebeius*. Numbers over the box plots indicate mean values. Letters 'a' and 'b' indicate significant differences determined by the Kruskal–Wallis test ($P < 0.001$). Abbreviations are as defined in Table 1.

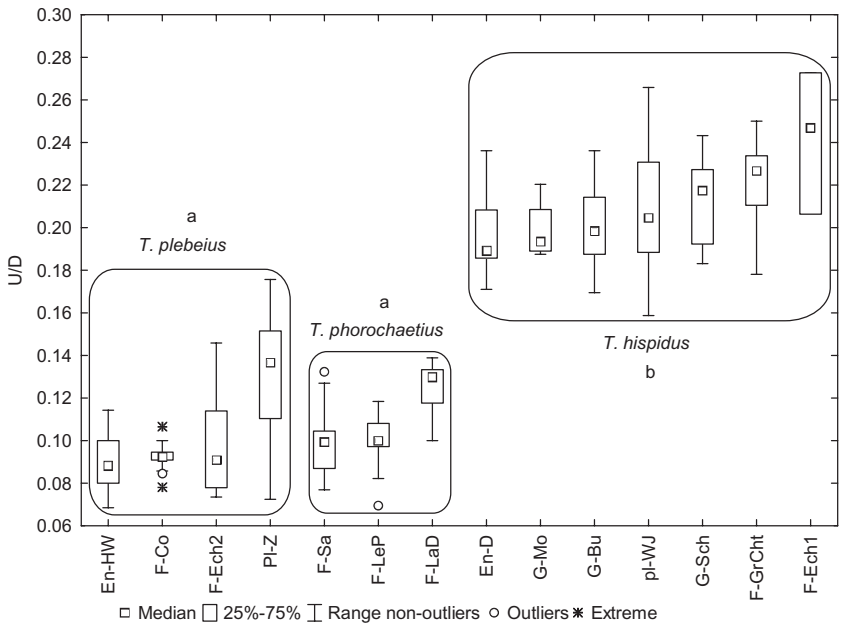

Figure 6. Variation of populations of *Trochulus* taxa in umbilicus relative diameter (U/D). Letters 'a' and 'b' indicate significant differences determined by the Kruskal–Wallis test ($P < 0.001$). Abbreviations are as defined in Table 1.

None of the morphologically recognized *Trochulus* species was grouped in a single clade that would contain all sequences assigned to the given species collected from different localities or countries (Fig. 9). Only, *T. hispidus* found in Germany (Moosburg, G-Mo) and England (Downside, En-D) created one group significantly supported by bootstrap and posterior probability values. Instead, there were many highly supported separate clades that consisted of very similar (Tables S1, S2) sequences isolated from individuals classified to the same species usually collected from the same locality. In most cases,

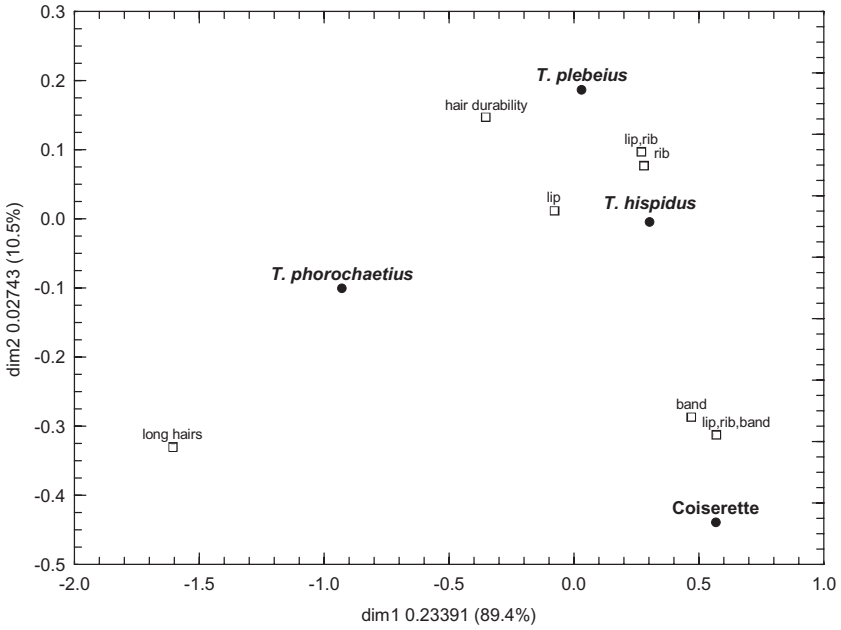

Figure 7. Correspondence analysis of qualitative data in *Tochulus* taxa and the Coiserette specimens.

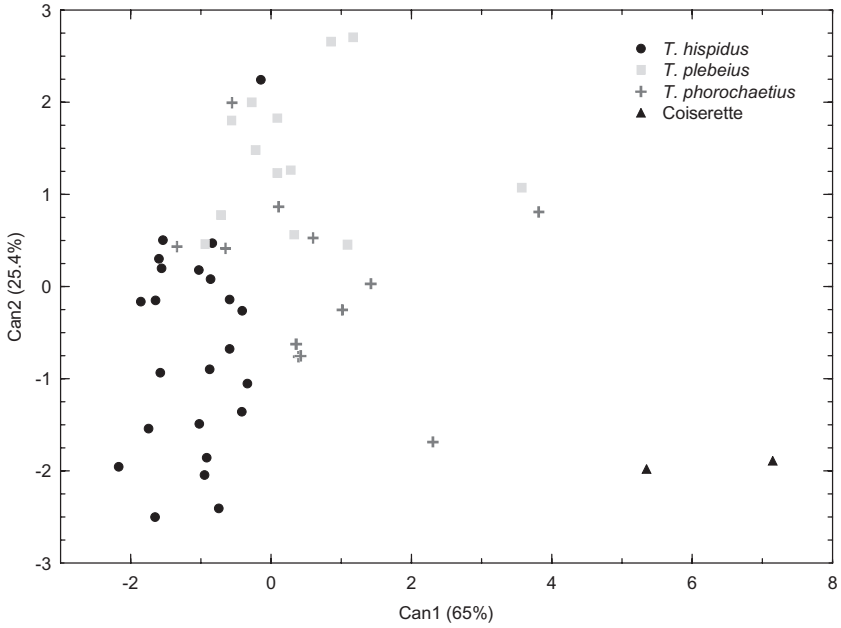

Figure 8. Canonical discriminant analysis based on genital measurements of *Trochulus hispidus*, *Trochulus plebeius*, *Trochulus phorochaetius*, and the Coiserette specimens. Wilks' lambda = 0.10933, $F_{21,115} = 6.3833$, $P < 0.00001$.

sequences of one species even coming from the same country did not form one monophyletic group. Many of the *T. phorochaetius* samples analysed that were found in different places in France (F-LaD, F-Sa, and F-LeP) were clustered in one well-supported clade, but excluded other French sequences assigned to this species (F-GrCh and F-LaD) that cluster with *T. hispidus*. One common group was also formed by French *T. plebeius* sequences from Coiserette (F-Co) and Échallon (F-Ech), but sequences from Chèrmenille samples (F-Cher) were separated from them. Similarly, in the case of English (En-D), German (G-Mo and G-Sch), and Polish (Pl-WJ) *T. hispidus*, at least one sequence did not cluster with the other sequences from the same country. Two of the Polish *T. plebeius* sequences analysed (Pl-Z) were separated

Table 4. Genital measurements (in mm) of *Trochulus phorochaetius*, *Trochulus plebeius*, and *Trochulus hispidus*

	T. phorochaetius, $N = 12$			*T. plebeius*, $N = 13$*			*T. hispidus*, $N = 28$		
Feature	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
fl	2.42–5.40	4.21	0.99	2.53–5.05	3.71	0.93	2.31–5.05	3.78	0.73
ep	2.42–4.40	3.00	0.61	2.42–4.61	3.19	0.64	1.76–4.61	2.68	0.59
p	1.98–3.85	2.73	0.61	1.87–4.29	3.01	0.79	1.65–4.18	2.40	0.66
sd	2.53–6.59	4.36	1.19	2.42–5.71	3.57	1.13	1.32–5.05	3.18	1.03
sl	1.32–2.42	1.81	0.26	1.32–3.30	2.20	0.54	1.32–3.52	2.19	0.64
sw	0.77–1.65	1.25	0.25	1.32–4.27	2.34	0.77	0.66–2.09	1.06	0.40
uv	0.00–1.11	0.18	0.37	0.00–1.32	0.49	0.49	0.00–1.32	0.03	0.36
fl/ep	1.00–2.00	1.42	0.31	0.96–1.43	1.17	0.14	0.86–1.90	1.43	0.22
ep/p	0.89–1.39	1.12	0.17	0.85–1.32	1.08	0.16	0.77–1.61	1.15	0.22
sl/sd	0.28–0.67	0.44	0.14	0.32–1.20	0.62	0.23	0.36–1.42	0.73	0.23
sw/sl	0.50–0.93	0.70	0.14	0.38–1.00	0.65	0.16	0.30–0.72	0.49	0.12

Statistically significant differences were found between *T. hispidus* and *T. plebeius* for p, sw, uv, fl/ep, sw/sl; between *T. hispidus* and *T. phorochaetius* for sd, sl/sd, sw/sl; between *T. plebeius* and *T. phorochaetius* for fl/ep; and between the Coiserette specimens and all three taxa for sd. Abbreviations: SD, standard deviation; *data excluding Coiserette specimens; for other abbreviations, see Material and methods.

from each other too. The separated sequences of one species from the same country showed relatively high genetic distances between each other (Tables S1, S2). Only in the case of Austrian *T. hispidus* (HQ2044**) and English *T. plebeius* (En-HW) were all analysed sequences of the given species collected in one country grouped together.

Interestingly, four clades comprised sequences from two different species found in the territory of one country. All analysed sequences of *T. plebeius* (En-HW) and two of *T. hispidus* (En-D) from England were significantly grouped together. One well-supported clade was formed by Polish *T. plebeius* (Pl-Z3) and two *T. hispidus* sequences (Pl-WJ). In addition, sequences of *T. hispidus* (F-GrCht, F-GrCh, and F-Ech) and *T. phorochaetius* (F-GrCh and F-LaD) from different places in France not only created one branch with very high support values but also some were identical (Tables S1, S2). It should be noted that a *T. hispidus* sequence (G-Sch4) also grouped significantly with a *T. coelomphala* sequence (G-Gu8), both of which were isolated from German snails.

Molecular delimitation of species boundaries

Species boundaries were established using two molecular delimitation methods: ABGD and GMYC (Fig. 10). Both the single- and multiple-threshold GMYC models fitted the data significantly better (LR test, $P < 10^{-7}$) than the null models, assuming that the entire sample derives from a single species with uniform branching, in contrast to the alternative supposition of several independently evolving species populations. However, the comparison of the single and multiple threshold models showed that the latter model was not significantly better than the former (χ^2 test, $P = 0.95$).

Generally, the methods applied gave a very similar picture of species delimitation (Fig. 10), and were congruent with the statistical significance of the corresponding clades defined in the phylogenetic analyses (Figs 9, S1). The results obtained with the ABGD method only slightly differed in the dependence of a priori threshold values. For a prior maximal distance from 0.001 to 0.022, this approach (called A1) revealed 13 species clusters and nine singletons, whereas for the distance from 0.036 to 0.100 it recognized 12 clusters and seven singletons (A2 approach). Similar delimitations were obtained in GMYC models. The single-threshold model (G1) indicated 12 clusters and ten singletons, whereas the multiple-threshold model (G2) indicated 13 clusters and 12 singletons.

The singletons recognized by all methods were: *T. coelomphala* (G-Gu10), *T. plebeius* (Pl-Z5, F-Cher3/Cher4), *T. hispidus* (Pl-WJ1), and taxa included in the out-group. Their sequences did not group significantly with any other samples, and were represented by early diverging and relatively long branches in the phylogenetic tree (Figs 9, 10).

At least three approaches delimited five clusters, each containing taxa that were described under the same species name: *T. hispidus* (HQ2044**), *T. villosus* (EU025***), French *T. plebeius* (F-Co and F-Ech), *T. oreinos oreinos* (HQ2044**), and *T. oreinos scheerpeltzi* (HQ2043**). The clade of *T. hispidus* from

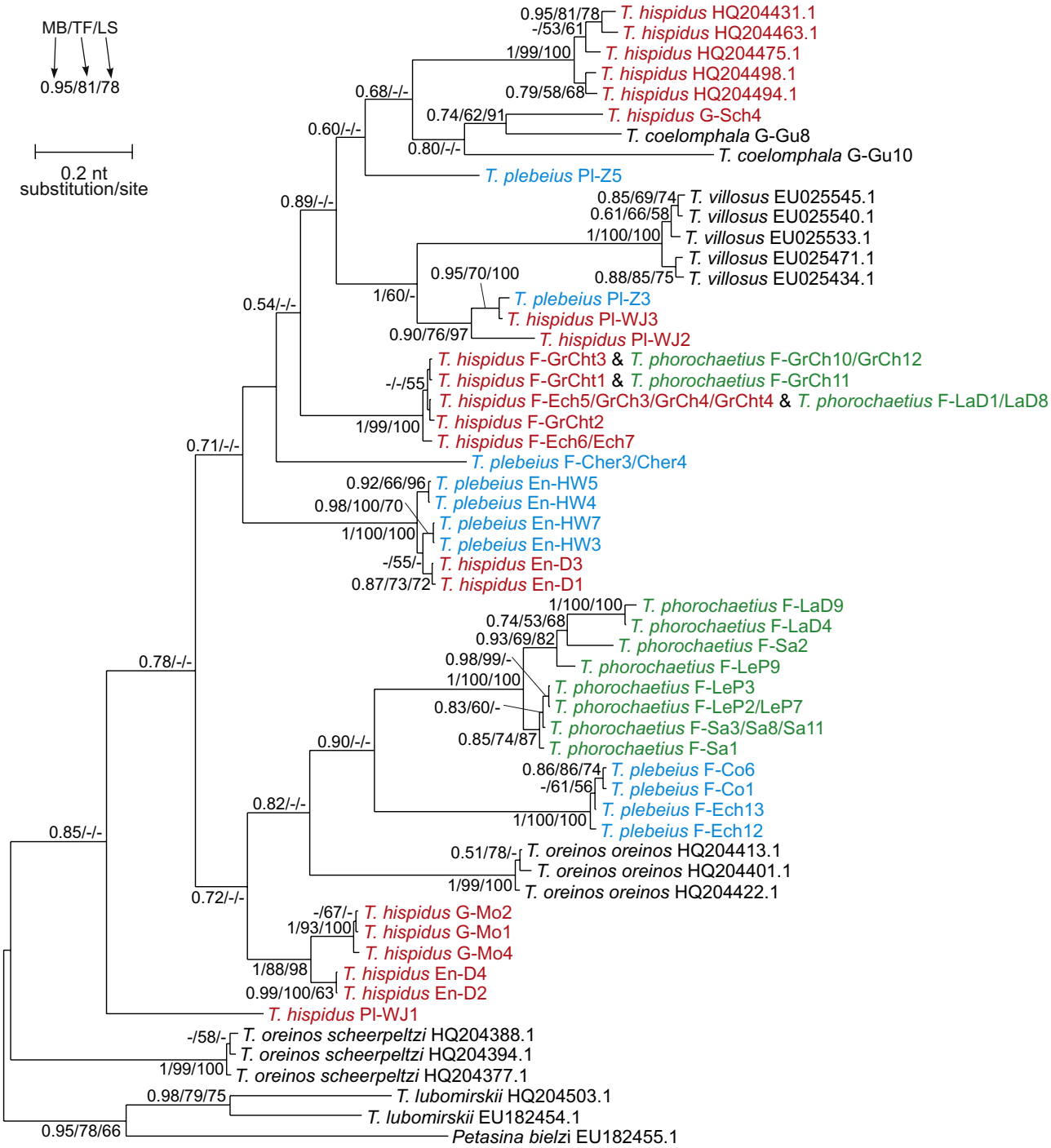

Figure 9. The Bayesian tree for *COI* gene sequences assigned to *Trochulus*. Two sequences of *Trochulus lubomirskii* (= *Trichia lubomirskii* = *Plicuteria lubomirskii*) and one *Petasina bielzi* were also included as the out-group. Labels for *Trochulus hispidus*, *Trochulus plebeius*, and *Trochulus phorochaetius* were coloured in red, blue, and green, respectively. Additional acronyms beside the species name or an ampersand symbol '&' indicate 100% sequence identity for the joined names. Numbers at nodes, in the order shown, correspond to posterior probabilities estimated in MrBayes (MB), as well as bootstrap support values calculated in TreeFinder (TF), and PAUP using the weighted least squares (LS) method. Values of the posterior probabilities and bootstrap percentages lower than 0.50 and 50%, respectively, were omitted or indicated by a dash '-'.

Germany (G-Mo) and England (En-D) that was strongly supported in the phylogenetic analyses was split into two clusters according to their geographic locality, respectively, by four delimitation approaches. Samples of *T. phorochaetius* collected from various localities in France (F-LaD, F-Sa, and F-LeP), and grouped in one clade of the phylogenetic tree, were also divided into two groups by three delimitation methods; however, this split correlated with phylogenetic relationships rather than with geographic distribution. Only the A2 approach recognized this *T. phorochaetius* clade as one delimited cluster.

In agreement with the phylogenetic studies, four delimiting methods consistently identified English samples assigned to the different morphospecies *T. plebeius* (En-HW) and *T. hispidus* (En-D) as one species. Similarly, French sequences of *T. hispidus* (F-GrCht, F-GrCh, and F-Ech) and *T. phorochaetius* (F-GrCh and F-LaD) were treated as one cluster by four methods. *Trochulus plebeius* (Pl-Z3) and two *T. hispidus* (Pl-WJ) found in Poland constituted one species according to the ABGD delimitation, but GMYC methods clustered *T. plebeius* (Pl-Z3) with only one *T. hispidus* (Pl-WJ3). Two ABGD approaches also joined the German samples of *T. hispidus* (G-Sch4) and *T. coelomphala* (G-Gu) in one group.

DISCUSSION

Morphological comparison of *T. phorochaetius* with *T. hispidus* and *T. plebeius*

The shell size of *T. phorochaetius* examined in this study stayed in accordance with those given by Winter (1990), although measurements alone were not sufficient for reliable differentiation (Table 2). Our results showed that *T. phorochaetius* was very similar to *T. plebeius*, whereas both differed from *T. hispidus*. Umbilicus relative diameter (U/D) discriminated *T. hispidus* from the other two taxa, although the differentiation from *T. plebeius* was less distinct than from *T. phorochaetius*. This character was less variable in *T. phorochaetius* than in *T. hispidus*: 0.07–0.14 and 0.16–0.27, respectively (Table 2). The conchological similarity of *T. phorochaetius*, *T. plebeius* and *T. sericeus* has already been mentioned by Winter (1990), who identified many characters possibly specific for *T. plebeius* from Belgium, Luxemburg, and southern Germany, i.e. smaller shells with about the same number of whorls, a higher spire, and often more narrow umbilicus. These shells also possessed a dense cover of curved hairs, which were clearly shorter and finer than in *T. phorochaetius*. In contrast to this study, we have found no such differences, except for hair morphology. Hair length could be used to distinguish *T. phorochaetius*, but with caution; admittedly, hairs were not as long as in *T. villosus* or even *T. villosulus* (Fig. 3E–F), but were still conspicuous compared with *T. plebeius* or *T. hispidus* (Fig. 3C, G). Furthermore, one of the characters quoted in the diagnosis of *T. phorochaetius* was short, whitish, and weakly lost hairs ('petits poils blancs, courts, recourbés et peu caducs'; Bourguignat, 1864). Thus, in this study we confirm that hair durability is distinctive for *T. phorochaetius*, similar to *T. villosus* and *T. villosulus* (Proćków, 2009). However, when only weathered shells with no hairs are available, recognizing these species is difficult.

The ratio of umbilicus minor to its major diameter (u/U) appeared to be another significant character, which may help in distinguishing *T. phorochaetius* from *T. plebeius*. Indeed, in the respective populations, the mean values differed among both species, but their ranges still overlapped (Fig. 5), reaching 0.71–1.0 in *T. phorochaetius* and 0.64–0.81 in *T. plebeius*. However, some morphological tendencies could be noticed, i.e. the umbilicus in *T. phorochaetius* seemed to be round, whereas in *T. plebeius* the umbilicus was more oval. The most distinctive features distinguishing three *Trochulus* morphospecies are presented in Table 5. Differentiation of *T. phorochaetius* from both *T. plebeius* and *T. hispidus* was impossible on the basis of the anatomical characters examined. No constant trait that would be useful for the reliable identification of the species has been found. Winter (1990), describing the genital system of *T. phorochaetius*, did not give the measurements but only referred to the figures of *T. 'plebeia'* (Schileyko, 1978) and measurements of *T. 'sericea'* (Klöti-Hauser, 1920). He particularly pointed out the distinction of *T. phorochaetius* from the East German and Czechoslovakian specimens of *T. 'plebeia'* examined by Schileyko (1978), and simultaneously showing its resemblance to the Swiss *T. 'sericea'* investigated by Klöti-Hauser (1920). Likewise, our studies did not reveal anatomical differences between *T. plebeius* and *T. hispidus*, possibly suggesting that they are not different species. As genital morphology is strongly associated with mating success in land snails (Gómez, 2001), our results may provide additional evidence for a lack of reproductive isolation and thus the ability to crossbreed (see later in the Discussion). In agreement with this, there were two significant clades containing *COI* sequences of both *T. plebeius* and *T. hispidus* from the same countries: Great Britain and Poland, respectively (Fig. 9). Similarly, laboratory studies of the Sicilian helicid *Marmorana* showed that snails with different shell shapes (i.e. flat keeled versus globular) mated and produced viable offspring (Rensch, 1937), and that the different populations had similar genitalia (Fiorentino *et al.*, 2008). Furthermore, neither substantial sexual isolation barriers nor genital differentiation were found between

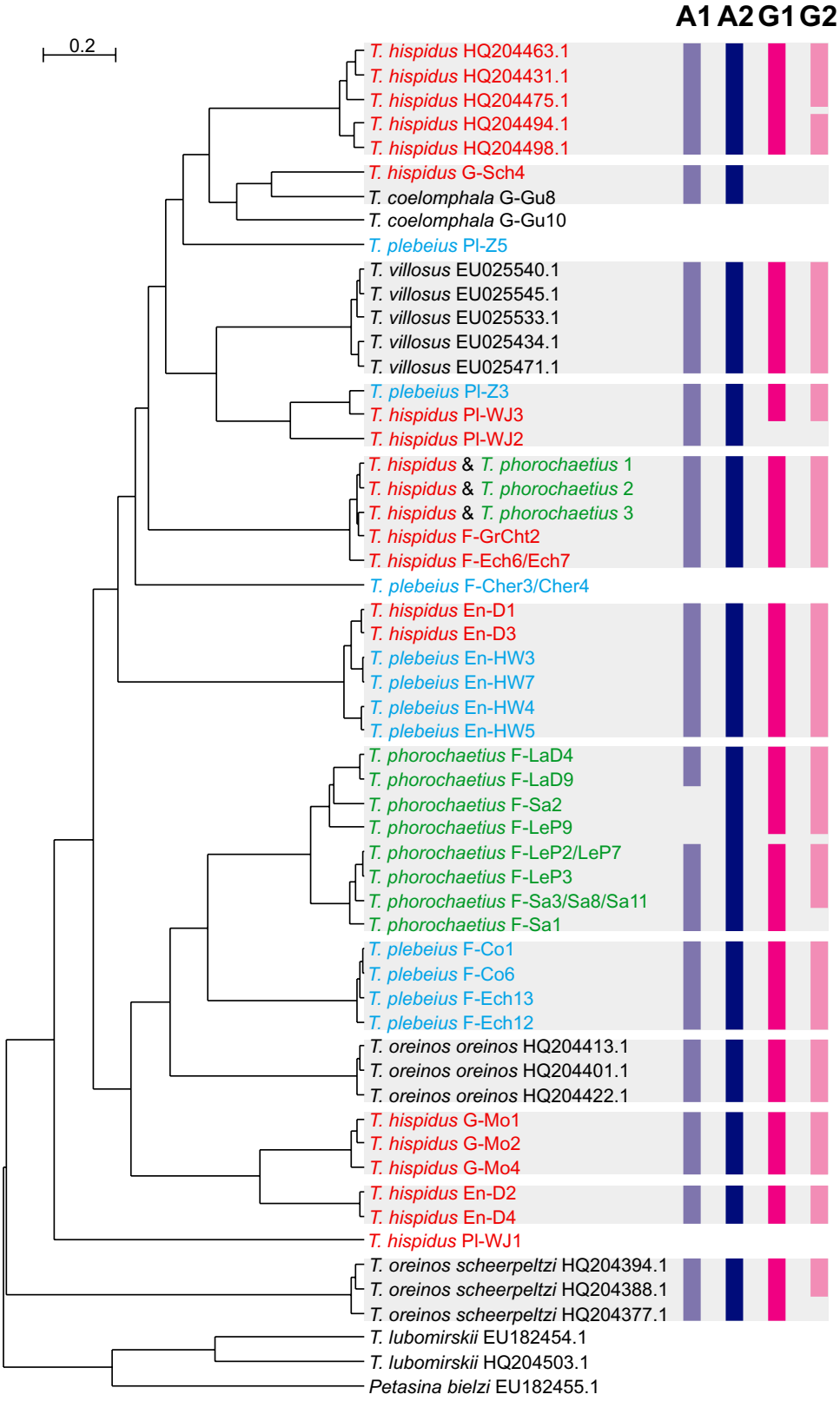
A1 A2 G1 G2
0.2
T. hispidus HQ204463.1
T. hispidus HQ204431.1
T. hispidus HQ204475.1
T. hispidus HQ204494.1
T. hispidus HQ204498.1
T. hispidus G-Sch4
T. coelomphala G-Gu8
T. coelomphala G-Gu10
T. plebeius Pl-Z5
T. villosus EU025540.1
T. villosus EU025545.1
T. villosus EU025533.1
T. villosus EU025434.1
T. villosus EU025471.1
T. plebeius Pl-Z3
T. hispidus Pl-WJ3
T. hispidus Pl-WJ2
T. hispidus & T. phorochaetius 1
T. hispidus & T. phorochaetius 2
T. hispidus & T. phorochaetius 3
T. hispidus F-GrCht2
T. hispidus F-Ech6/Ech7
T. plebeius F-Cher3/Cher4
T. hispidus En-D1
T. hispidus En-D3
T. plebeius En-HW3
T. plebeius En-HW7
T. plebeius En-HW4
T. plebeius En-HW5
T. phorochaetius F-LaD4
T. phorochaetius F-LaD9
T. phorochaetius F-Sa2
T. phorochaetius F-LeP9
T. phorochaetius F-LeP2/LeP7
T. phorochaetius F-LeP3
T. phorochaetius F-Sa3/Sa8/Sa11
T. phorochaetius F-Sa1
T. plebeius F-Co1
T. plebeius F-Co6
T. plebeius F-Ech13
T. plebeius F-Ech12
T. oreinos oreinos HQ204413.1
T. oreinos oreinos HQ204401.1
T. oreinos oreinos HQ204422.1
T. hispidus G-Mo1
T. hispidus G-Mo2
T. hispidus G-Mo4
T. hispidus En-D2
T. hispidus En-D4
T. hispidus Pl-WJ1
T. oreinos scheerpeltzi HQ204394.1
T. oreinos scheerpeltzi HQ204388.1
T. oreinos scheerpeltzi HQ204377.1
T. lubomirskii EU182454.1
T. lubomirskii HQ204503.1
Petasina bielzi EU182455.1

Figure 10. Clusters of delimited species obtained with the ABGD and GMYC methods, based on the ultrametric MrBayes tree. The clusters were marked by bars differently coloured for four approaches: ABGD methods assuming a prior maximal distance from 0.001 to 0.022 (A1), and from 0.036 to 0.100 (A2), as well as GMYC single- (G1) and multiple-threshold (G2) models. Labels for *Trochulus hispidus*, *Trochulus plebeius*, and *Trochulus phorochaetius* were coloured in red, blue, and green, respectively. Additional acronyms at the species name or an ampersand symbol '&' indicate 100% sequence identity for the joined names; *T. hispidus* & *T. phorochaetius* 1 denotes *T. hispidus* F-GrCht1 & *T. phorochaetius* F-GrCh11; *T. hispidus* & *T. phorochaetius* 2 denotes *T. hispidus* F-GrCht3 & *T. phorochaetius* F-GrCh10/GrCh12; *T. hispidus* & *T. phorochaetius* 3 denotes *T. hispidus* F-Ech5/GrCh3/GrCh4/GrCht4 & *T. phorochaetius* F-LaD1/LaD8.

Table 5. The most diagnostic morphometric features differentiating the three *Trochulus* morphospecies

Feature	*T. phorochaetius*	*T. plebeius*	*T. hispidus*
Umbilicus relative diameter (U/D)	Smaller **0.10** (0.07–0.14)	Smaller **0.11** (0.07–0.18)	Bigger **0.21** (0.16–0.27)
Ratio of umbilicus minor to its major diameter (u/U)	Bigger **0.94** (0.71–1.00)	Smaller **0.76** (0.64–0.81)	Bigger **0.91** (0.77–1.00)
Umbilicus morphology	Round	Oval	Round
Hair length (in mm)	Longer **0.46** (0.23–0.67)	Shorter **0.26** (0.11–0.61)	Shorter **0.24** (0.15–0.36)
Hair durability	Bigger	Bigger	Smaller
Development of the apertural traits	Weaker	Stronger	Stronger
Length of the spermathecal duct (in mm)	Longer **4.36** (2.53–6.59)	Shorter **3.57** (2.42–5.71)	Shorter **3.18** (1.32–5.05)

Values given in bold represent means, whereas values in parentheses denote ranges.

Albinaria species (Schilthuizen & Lombaerts, 1995; Giokas, Mylonas & Rolán-Alvarez, 2006).

Molecular phylogenetic and species delimitation studies

The phylogenetic and species delimitation analyses performed were not entirely congruent with the morphological differentiation in three species defined by shell characters. The delimitation methods applied suggest that there are many more species than are currently recognized from morphological studies. At least eight delimited clusters included very similar sequences that were assigned to the same morphospecies and collected from the same locality or country (e.g. *T. hispidus* HQ2044**; *T. plebeius* F-Co and F-Ech; *T. hispidus* G-Mo; *T. hispidus* En-D). Even samples of *T. phorochaetius* from France were divided into two clusters by most methods. Several early diverging taxa represented by one sequence were also considered as separate species by all methods. Generally, *COI* sequences did not divide into three separate clades corresponding to the particular morphological species (Figs 9, 10). In contrast, we found two separate significant clades for English or Polish specimens, respectively, that grouped sequences from *T. hispidus* and *T. plebeius*, i.e. species occupying distinct morphospaces in the CDA. In agreement with phylogenetic studies, all ABGD and GMYC approaches recognized English *T. hispidus* and *T. plebeius* as one species. Polish *T. hispidus* and *T. plebeius* were also one ABGD cluster and GMYC methods grouped Polish *T. plebeius* with one Polish *T. hispidus*. What is more, sequences of French *T. hispidus* and *T. phorochaetius* not only significantly clustered together but some of them were even identical. All these sequences were consistently identified as one species by all four delimitation approaches. German *T. hispidus* and *T. coelomphala* sequences were also clearly grouped and considered as one species by the ABGD method. Similar results were obtained by Kruckenhauser *et al*. (2011), who found no genetic differentiation between individuals resembling the *T. sericeus* or the *T. hispidus* phenotypes morphologically, and who also reported an intermixing of *T. hispidus* with other related taxa (e.g. *T. coelomphala*). Thus, the mitochondrial data do not support morphologically determined species of the taxa analysed. Alternatively, the very close phylogenetic relationships of *Trochulus* specimens from the

same country assigned to different species indicate hybridization and a flow of genetic material between them. Interestingly, such a view is consistent with the lack of significant differences in their genital morphology.

On the other hand, both shell morphology and mtDNA results allowed the clear differentiation between *T. hispidus* and *T. oreinos* from the north-eastern Austrian Alps (Duda *et al*., 2011). These two species and their subspecies included in our analyses were also located in significantly distinct clades of the phylogram (Fig. 9), and were identified as separate species according to ABGD and GMYC methods (Fig. 10). Similarly, *T. villosus* sequences taken from GenBank constituted a separate well-defined phylogenetic clade, as well as an ABGD and GMYC cluster, supporting its distinctness from *T. phorochaetius*.

Populations from East France and their taxonomic considerations

Having the established the presence of genetically and morphologically distinct evolutionary lineages, we wanted to know if there are arguments that allow assigning existing taxonomic names to them. The CDA carried out on the morphometric data predicted that the three well-recognized morphotypes corresponded to three names/morphospecies, i.e. *T. phorochaetius*, *T. plebeius*/*sericeus*, and *T. hispidus* (Fig. 4). However, the correspondence of these morphotypes with the mitochondrial lineages was not clear when all of the material examined was considered (Figs 9, 10). Although all French *T. plebeius* sequences significantly grouped together and were delimited as one cluster, the second clade of French snails assigned to *T. phorochaetius* did not include all sequences of this species. In fact, three of the *T. phorochaetius* sequences were identical to some of those of *T. hispidus*, and with them formed the third significant phylogenetic clade and delimited cluster of French snails. It must be emphasized that this case refers to individuals from syntopic populations. The genetic similarity between them may strongly suggest the ability for hybridization, which has been recorded in land snails. Recent studies of the *Trochulus sericeus*/*hispidus* complex recognized genetically divergent but morphologically cryptic lineages. In the small contact area, however, the lineages hybridized to a limited extent (Dépraz *et al*., 2009). Additional evidence, like crossbreeding experiments, could confirm the status of the inferred morphospecies; however, this did not appear to be an easy task. The attempts to crossbreed snails from Lubawka (*T. hispidus* morphotype) and Hayley Wood (*T. plebeius* morphotype) with snails from Wrocław (*T. hispidus* morphotype) and Zieleniec (*T. plebeius* morphotype) failed because all individuals died before reaching maturity (Proćków, unpubl. data).

With respect to shell morphology, specimens from Coiserette (Fig. 2C) appeared to be the most problematic. Three of them corresponded to *T. plebeius*, whereas the others corresponded to *T. phorochaetius* (Fig. 4). In considering hair length, which mostly differentiates the two species, the Coiserette specimens may presumably belong to *T. plebeius*. None of these specimens had hairs longer than 0.34 mm, with a mean of 0.19 mm (Fig. 3C), and in the initial investigation, delimiting snails by eye, we identified them as *T. plebeius*. Genital morphological investigations of Coiserette specimens revealed their distinctness from other *T. plebeius* populations with respect to the length of the spermathecal duct, which reached more than 7 mm. In the remaining specimens, morphologically determined as *T. plebeius*, it was up to 5.7 mm. As only two adults from Coiserette were available for dissection, confirmation of their anatomical separation using more extensive material is required. Genetically, Coiserette specimens were closely related to the Échallon population assigned to *T. plebeius*, and formed a common significant mitochondrial lineage and species cluster (Figs 9, 10). The two sites are situated only ca. 23 km apart (Fig. 1). Moreover, Échallon is the only locality with a syntopic population of morphologically delimited specimens of either *T. plebeius* or *T. hispidus*, which appeared to belong to the distinct mitochondrial lineages of East France (Fig. 9).

Concluding remarks

The assignment of distinctive morphospecies, and thus existing taxonomic names, to genetically identified evolutionary lineages is difficult and arbitrary to a certain degree. The recognition of some clades as separate species in the light of the data presented depends largely on the applied species concept (de Queiroz, 2007). The results obtained indicate that hybridization is possible between morphologically different taxa (e.g. *T. phorochaetius* and *T. hispidus*), which is contrary to the biological species concept (BSC). The unified species concept (USC), however, is based on the idea that spatially separated metapopulation lineages that differentiate in various evolutionary directions can be considered species, whereas secondary species criteria (e.g. morphological, genetic, or behavioural) are treated as different evidences relevant to assessing lineage separation (de Queiroz, 2007). Three mtDNA phylogroups, each containing two morphospecies living in the same geographical region, could represent three such metapopulations: (1) *T. plebeius* + *T. hispidus* from Great Britain; (2) *T. plebeius* + *T. hispidus* from Poland; and (3)

T. phorochaetius + *T. hispidus* from France. An integrative approach is a prerequisite for the future taxonomy (Dayrat, 2005). Studies concerning nuclear DNA could provide additional details about the hybridization events in the wild and should be compared with the mtDNA data. However, nuclear sequences did not differentiate between any of the mitochondrial clades, except *T. oreinos*, and provided no argument for species status of any of the *T. hispidus* lineages from the Eastern Alps and surrounding areas (Kruckenhauser *et al*., 2011). For crucial conclusions on species boundaries, it is essential to investigate potential gene flow between syntopic populations.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. The Bayesian cladogram for *COI* gene sequences assigned to *Trochulus*.
Table S1. Percentage identity between the *COI* gene sequences used in the study.
Table S2. Mean number of substitutions per site between the *COI* gene sequences used in the study.