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Unravelling morphological overlap of the rock-dwelling snails *Pyramidula saxatilis* (Hartmann, 1842) and *P. pusilla* (Vallot, 1801)

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ABSTRACT

Pyramidula saxatilis and *P. pusilla* are two Central European rock-dwelling snail species that frequently co-occur and show substantial overlap in overall shell morphology. The two species can be separated from each other by differences in mitochondrial and nuclear DNA (mtDNA and nDNA) sequences. Recent studies have not shown consistent differences between these species in shell shape but have suggested possible species-specific features in shell microstructure. We investigated this issue by studying variation in the microsculpture of the upper shell surface (based on five shells per population from a total of nine populations for each species) and by analysing the geometric morphometrics of shell shape (based on 51 individuals and 12 populations of *P. saxatilis* and 54 individuals and 14 populations of *P. pusilla*), with species identifications based on mtDNA and nDNA markers. While geometric morphometrics and canonical variance analysis did reveal some statistical differences in overall shell shape, these differences were too subtle to be consistently detected by the eye. However, the density and development of growth ridges on the upper shell surface of the two species were found to be statistically different, with *P. saxatilis* having denser and very regular ridges. As a final verification of the utility of shell miscrosculpture, we were able to separate these two species even in two mixed populations with highly overlapping shell phenotypes, the shell-based identifications being confirmed by cytochrome *c* oxidase subunit I sequence data for all of the collected individuals. We therefore recommend using shell microsculpture to distinguish these two species.

INTRODUCTION

The minute land snails, *Pyramidula saxatilis* (Hartmann, 1842) and *P. pusilla* (Vallot, 1801), are calciphilous species that, like all other members of the genus *Pyramidula*, are exclusively restricted to surfaces of calcium-rich rocks, mostly limestones (Gittenberger & Bank, 1996). Their shells do not exceed 3 mm in both shell height and diameter. The shells are typically dome-shaped with a broad open umbilicus and simple aperture, and are yellowish reddish to dark brown in colour (Kerney & Cameron, 1979; Gittenberger & Bank, 1996). The reproductive anatomy is simple (Martínez-Ortí, Gómez-Moliner & Prieto, 2007), which is why prior to the DNA analysis, species identification relied on readily observable conchological characters (Gittenberger & Bank, 1996).

During the last three decades, there have been substantial advances in the taxonomic knowledge of the European representatives of *Pyramidula*. After more than 100 years of all the species-level names being synonymised with *P. rupestris* (Draparnaud, 1801), Gittenberger & Bank (1996) concluded that there are at least six conchologically defined *Pyramidula* species in Europe. Based on a molecular phylogenetic study of the Western Palearctic *Pyramidula*, Razkin *et al.* (2016, 2017) delimited nine well-supported species clades. However, shell height and width for several of them largely overlapped, and no unambiguous morphological characters for the reliable separation of these species were identified (Kirchner *et al.*, 2016; Razkin *et al.*, 2017). Our previous study (Horsáková *et al.*, 2022) of four central and southwest European species, representing two pairs of highly convergent shell forms, provided, for the first time, reliable diagnostic characters based on shell morphology. Within these two species pairs, shell microsculpture appeared to be the main distinguishing character. *Pyramidula saxatilis* and *P. pusilla* were one of those two pairs, with the distribution of the former being entirely nested in the much wider range of the latter. While these species highly overlapped in shell morphospace (i.e. this is why *P. saxatilis* has not been recognized a separate species prior to molecular analysis), less variation was found in shell microsculpture within each species. When characteristically developed, reliable identification seems to be possible, although this has not been rigorously examined (Horsáková *et al.*, 2022). However, due to the frequent co-occurrence of the two species, discriminating between the two in some mixed populations can be challenging without DNA-based confirmation. It is not clear how the characters in shell microstructure will perform in these problematic cases.

Here, we re-examine in detail variations in shell shape and microsculpure in these two syntopic species based on specimens whose

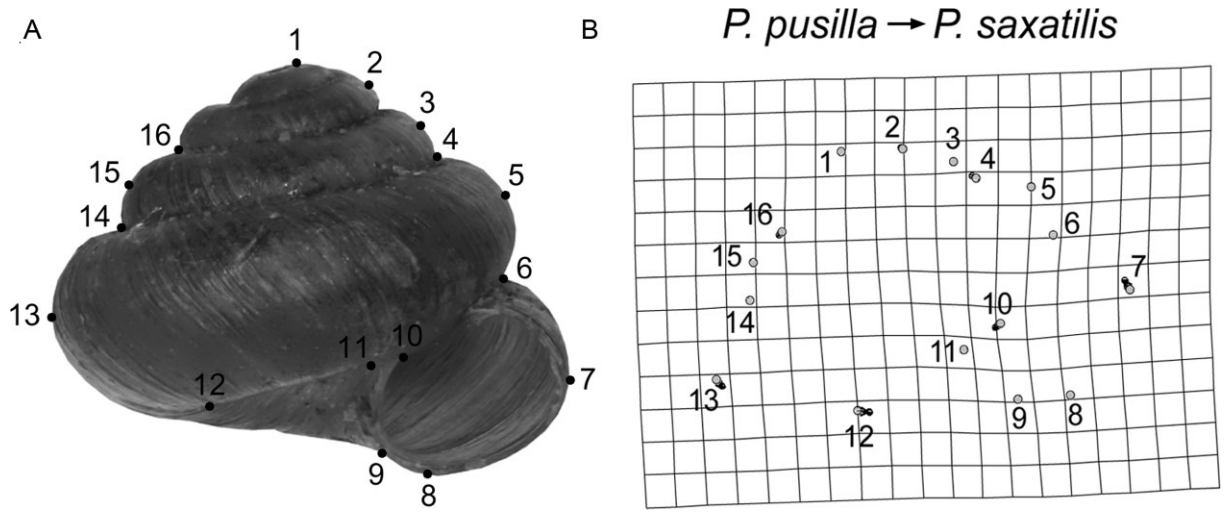

Figure 1. Position of 16 landmarks used in geometric morphometrics (**A**) and a thin-plate spline, illustrating the transitions in shape (i.e. landmark vector shifts) between *Pyramidula pusilla* and *P. saxatilis* (**B**).

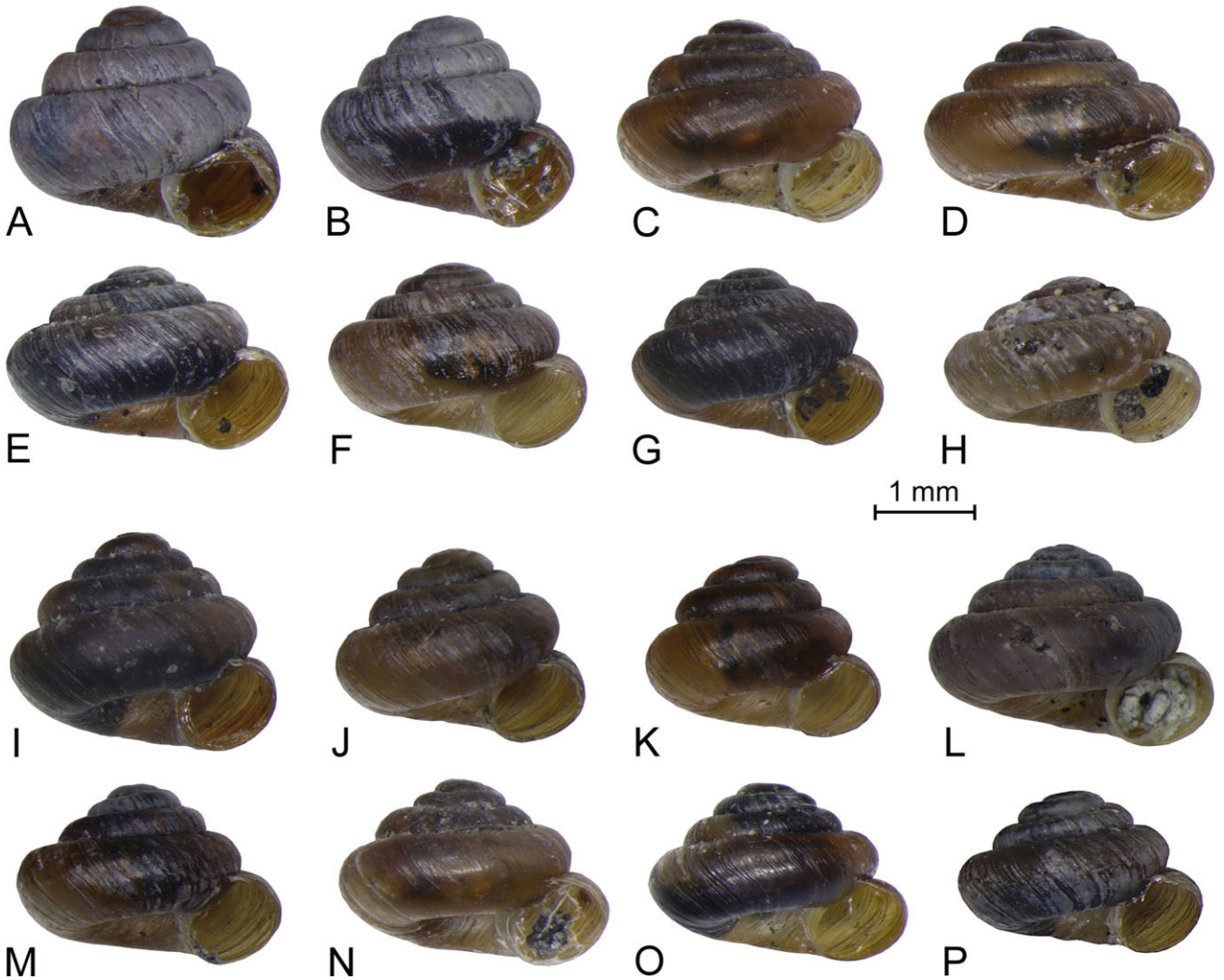

Figure 2. Variation in shell shape among populations of *Pyramidula saxatilis* and *P. pusilla* as identified by both mtDNA and nDNA markers. **A–H.** *Pyramidula saxatilis*. **A,B.** Hochschwab, Austria (P189). **C.** Lunz am See, Austria (P231). **D.** Nods, Switzerland (P229). **E.** Villeret, Switzerland (P208). **F.** Selva di Val Gardena, Italy (P249). **G.** Schladming, Austria (P206). **H.** San Marino, RSM (P209). **I–P.** *Pyramidula pusilla*. **I.** Spital am Pyhrn, Austria (P193). **J–K.** Tatranská Kotlina, Slovakia (P213). **L.** Valentová, Slovakia (P205). **M.** Johnsbach, Austria (P196). **N.** Štramberk, Czech Republic (P214). **O.** Motyčky, Slovakia (P369). **P.** Horný Jelenec, Slovakia (P203).

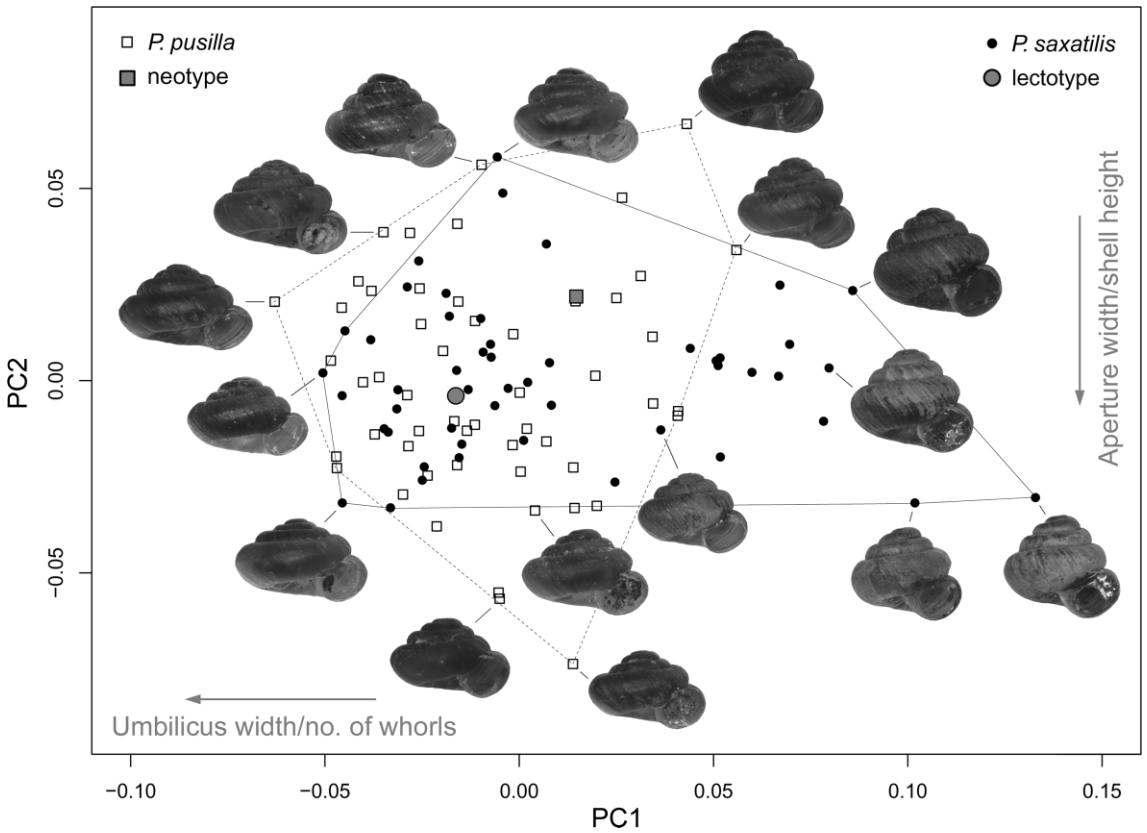

Figure 3. Position of 54 shells of *Pyramidula pusilla* and 51 shells of *P. saxatilis* along the first two PC axes based on Procrustes' shape coordinates of the 16 landmarks on the shell apertural view. Convex polygons were added to the diagram to highlight the overlap between the species. Neotype and lectotype specimens of the respective species were also used in the analysis and are shown by the grey symbols. Shell scores on the axes were associated with shell measurements (Table 2), with those measurements most closely associated with each axis shown in grey colour along each axis.

identification was validated by DNA sequence data. We aim to explore the applicability of using the microsculptural differences, first recognized by Horsáková *et al.* (2022), based on a representative dataset allowing for statistical testing. In addition, we test the validity of shell microsculpture for reliable identification using two mixed populations of highly overlapping shell phenotypes. First, we sorted all individuals into the species and then verified the identification by DNA barcoding of each individual. We believe that detailed *post hoc* statistical verifications of newly discovered morphological traits are crucial to evaluate their consistency and utility for species-level identifications, especially in otherwise 'cryptic' species for which no other or only a few diagnostic characters are available.

MATERIAL AND METHODS

Shell examination and morphometry

We used 51 individuals (from 12 populations) of *Pyramidula saxatilis* and 54 individuals (14 populations) of *P. pusilla*. The two species were identified using mitochondrial and nuclear DNA (mtDNA and nDNA) markers (Supplementary Material Appendix S1); the neotype of *P. pusilla* was the only exception, as it could not be DNA barcoded. The selection was restricted to shells of >4 whorls, which returns an average of four shells per population (Supplementary Material Appendix S1). Along with these, we analysed the lectotype of *P. saxatilis* (voucher no. M562, museum loan, Natural History Museum St. Gallen, Switzerland; see Horsáková *et al.*, 2022) and the neotype of *P. pusilla* based on photographs (voucher no. 210.996.MO.1, Natural History Museum, Dijon, France; photographs provided by E. Fara). We measured shell width and height, aperture width and height, body whorl height and umbilicus width, using microscope images of the dorsal, ventral and apertural views of each shell, as generated by an Olympus SZX7 microscope with Olympus C-7070 Wide Zoom camera and QuickPHOTO MICRO software v. 3.1 (PROMICRA, s.r.o.). The photographs were generated via the Deep Focus function by stacking four to nine sequential in-focus images, taken at regular intervals along the shell axis. The number of whorls was counted according to Cameron (2003). Geometric morphometrics (GM) analysis using 16 landmarks on the apertural view was conducted (Fig. 1A), as this is a comprehensive analytical approach that evaluates morphometric variation independently of the effect of size, position and rotation of the shell (Rohlf & Marcus, 1993; Schilthuizen & Haase, 2010). DNA-based identification and morphometrics of these specimens followed Horsáková *et al.* (2022).

Principal Component Analysis (PCA) based on Procrustes shape coordinates of the landmark data was applied to visualize overall variation in shell shape. To associate the first three PCA axes with measured shell characteristics, standardized shell measurements were linearly fitted into the ordination space using the envfit function in the R package vegan v. 2.5–6 (Oksanen *et al.*, 2019). These shell measurements included the following ratios: shell width/number of whorls, shell height/number of whorls, shell height/shell width, body whorl height/shell height, aperture width/shell width, aperture width/shell height, aperture height/body whorl height and umbilicus width/number of whorls. Ratios, rather than single shell measurements, were used to account for potentially different growth stages of individuals (Horsáková *et al.*, 2022).

Table 1. Descriptive statistics for shell measurements of the studied material, including type specimens, of *Pyramidula pusilla* (54 inds from 12 populations in Austria, the Czech Republic, Italy and Slovakia) and *P. saxatilis* (51 inds from 11 populations in Austria, Italy, San Marino and Switzerland).

Shell measurements	Minimum	1st quartile	Median	Mean	3rd quartile	Maximum
Pyramidula saxatilis						
Shell height/no. of whorls	0.38	0.43	0.45	0.46	0.48	0.54
Shell width/no. of whorls	0.53	0.60	0.62	0.62	0.64	0.70
Shell height/shell width	0.63	0.68	0.73	0.74	0.78	0.89
Body whorl height/shell height	0.70	0.75	0.77	0.77	0.78	0.83
Aperture height/body whorl height	0.60	0.62	0.64	0.65	0.66	0.73
Aperture width/shell height	0.43	0.50	0.52	0.51	0.54	0.59
Aperture width/shell width	0.32	0.36	0.37	0.38	0.40	0.45
Umbilicus width/no. of whorls	0.12	0.16	0.18	0.17	0.19	0.22
No. of whorls	3.8	4.1	4.1	4.1	4.2	4.9
Pyramidula pusilla						
Shell height/no. of whorls	0.37	0.42	0.43	0.43	0.45	0.51
Shell width/no. of whorls	0.49	0.59	0.62	0.61	0.64	0.70
Shell height/shell width	0.64	0.67	0.70	0.71	0.73	0.81
Body whorl height/shell height	0.72	0.74	0.76	0.76	0.78	0.82
Aperture height/body whorl height	0.58	0.64	0.66	0.66	0.67	0.77
Aperture width/shell height	0.41	0.49	0.52	0.52	0.55	0.61
Aperture width/shell width	0.33	0.35	0.37	0.37	0.39	0.42
Umbilicus width/no. of whorls	0.13	0.17	0.19	0.19	0.20	0.24
No. of whorls	3.9	4.0	4.2	4.1	4.2	4.6

Table 2. Multiple regressions of shell characteristics and specimen scores on the first two PC axes.

	PC 1	PC 2	Adj. R^2 (%)	*P*
Shell height/no. of whorls	**0.838**	0.546	38.14	<0.001
Shell width/no. of whorls	**−0.886**	−0.463	19.14	<0.001
Shell height/shell width	**0.860**	0.510	86.90	<0.001
Body whorl height/shell height	−0.313	**−0.950**	26.26	<0.001
Aperture height/body whorl height	−0.405	**−0.914**	39.53	<0.001
Aperture width/shell height	−0.258	**−0.966**	63.22	<0.001
Aperture width/shell width	**0.716**	−0.698	54.55	<0.001
Umbilicus width/no. of whorls	**−0.979**	0.204	56.28	<0.001

The table shows the regression coefficients, the fit of each shell characteristic into the ordination space (i.e. the variation in the character explained by specimen scores on the first two PC axes in a multiple linear regression; Adj. R^2) and the significance (*P*) of the result based on 4,999 permutations. The highest regression coefficients are in bold.

To test the difference between shape variations of the two *a priori* predefined species-level groups (identified by mtDNA and nDNA markers), canonical variance analysis (CVA) of the landmark data was performed with a permutation test of the Mahalanobis distances (from the pooled within-group covariance matrix) and Euclidean distances (between group means) using 10,000 runs (CVA function in the R package Morpho v. 2.5; Schlager, 2017). All R-based analyses were done using R v. 3.5.3 (R Core Team, 2019).

Microsculpture variation

We examined shell microsculpture of nine populations per species identified by mtDNA and nDNA markers, covering the species' distribution and shell variation (Supplementary material Appendix S1). Five individuals with undamaged shell surfaces and having at least 3.5 whorls were selected from each population. We counted the number of growth ridges on the upper shell surface for the 0.5-mm wide section, starting at the point where the shell has three whorls. A ridge was counted if it ran continuously from the suture to the shell perimeter. Differences in ridge density variation between the two studied species were tested using generalized equation estimates with Gaussian error structure (GEE-g). Because the five individuals from each site are actually pseudoreplicates, the model with an autocorrelation structure was applied. The site was used as a random factor and species as an explanatory variable. For the ridge density counts, we used the microscope imaging approach detailed earlier; for high resolution imaging of the microsculpture, we used the Keyence VHX-5000 digital microscope with ZS-20 and ZS-200 objective lenses.

DNA barcode and identification error

We studied two mixed Slovakian populations exhibiting very similar shell phenotypes for both species. For each population, we first identified all individuals based on shell microsculpture and then determined the identification error using analyses of COI sequences. In the first population (Motyčky I), all 39 collected specimens were morphologically assigned to three groups: (1) unambiguous *P. saxatilis* (15 inds), (2) unambiguous *P. pusilla* (15 inds) and (3) individuals that were hard to assign unambiguously to either species (9 inds). In the second population (Motyčky II), all 27 individuals were morphologically identified as either *P. saxatilis* (3 inds) or *P. pusilla* (24 inds). After morphology-based identification, all individuals were DNA barcoded.

DNA analysis of mixed populations

DNA was extracted from all specimens from the Motyčky I and II populations (see above) using the E.Z.N.A. Mollusc DNA Kit (Omega BioTek), following manufacturer's instructions, and extracts were stored at −20 °C. PCR amplification of mitochondrial cytochrome *c* oxidase subunit I (COI) was performed using the universal primers LCO1490 (5′–AAATAATGCTATTTCATGAYCAYGC–3′) and HCO2198

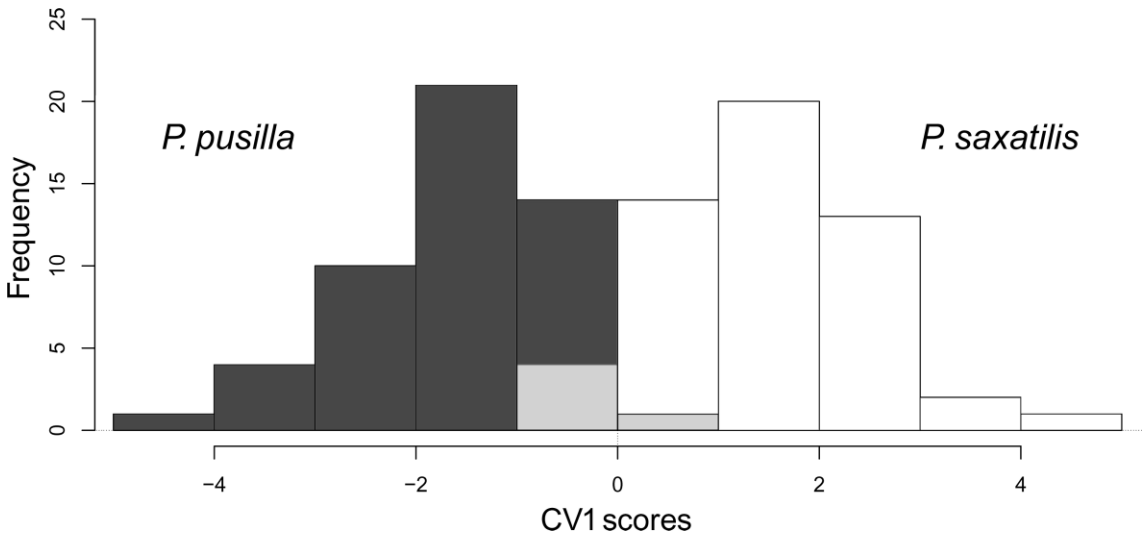

Figure 4. Position of 54 shells of *Pyramidula pusilla* and 51 shells of *P. saxatilis* on the first CV axis based on the Procrustes shape coordinates of the 16 landmarks on the shell apertural view. Pale grey areas show the overlap between species. The overall classification accuracy was 95.2%.

(5′–GCTCCGCAAATCTCTGARCAYTG–3′) (Folmer *et al.*, 1994). PCR products were cleaned using ExoSAP (Affymetrix) and cycle sequenced at SEQme s.r.o., Dobříš, Czech Republic. Sequences were assembled in Geneious v. 8.0.2 (Biomatters Ltd.) and visually checked for potential misreads. The sequences were translated into amino acids to check for erroneous stop codons and aligned using the Mafft v.7 online server (Kuraku *et al.*, 2013; Katoh, Rozewicki & Yamada, 2019). We applied four different phylogeny reconstruction methods (neighbour-joining, maximum likelihood, maximum parsimony and Bayesian inference) to examine which clades are robustly supported (see Horsáková, Nekola & Horsák, 2019 for details). All analyses unambiguously sorted the specimens into two well-supported clades (strong support was indicated by bootstrap values > 75% and Bayesian posterior probabilities > 95%), corresponding to *Pyramidula pusilla* and *P. saxatilis* (data not shown). COI validated identifications were used to evaluate whether microsculpture could be used to discriminate between the two species.

RESULTS

Both species have highly variable shell shapes (Fig. 2), with most of the variation overlapping between the species (Fig. 3, Table 1). The variation was related mainly to the variation in umbilicus width and the aperture width/shell height ratio (Table 2). A high-spired form of *Pyramidula saxatilis* with a narrow umbilicus was the only exception that likely does not have a parallel in *P. pusilla*. The existence of some consistent differences in shell shape was, however, suggested by the CVA (Fig. 4). In the CVA run on 16 landmarks, only 3 of 54 shells of *P. pusilla* were erroneously classified as *P. saxatilis* and only 2 of 51 shells of *P. saxatilis* were erroneously classified as *P. pusilla*. Although significant ($P = 0.048$), these minute differences were not visible to the naked eye, and this is also suggested by the negligible shifts in landmark vectors (Fig. 1B).

In contrast to shell shape, shell microsculpture was found to be a reliable character for identifying the two species by eye (Fig. 5). While *P. saxatilis* seems to be characterized by fine and very regular growth ridges, *P. pusilla* shows mostly coarse and clearly irregular ridges. The two species differ significantly in ridge density, with almost no overlap in the variation of this character (Fig. 6), although counts are arbitrary in some cases due to the unclear development of the ridges. *Pyramidula pusilla* also typically possesses at least some anastomosing ridges, while this feature is virtually absent in *P. saxatilis* (Fig. 7). However, even microsculpture shows some variability, with the existence of intermediate individuals in some mixed populations, usually those from the Western Carpathian Mountains. Unfortunately, ridges can gradually vanish in full-grown individuals. When the utility of ridge development and density was tested for populations with some individuals that were difficult to assign morphologically to either species, it appeared that *P. pusilla* was more often misidentified than *P. saxatilis*. In the Motyčky I population, two individuals of *P. pusilla* were erroneously identified as *P. saxatilis* (Supplementary Material Appendix S2), and eight of the nine ambiguous individuals also belonged to *P. pusilla*. In the Motyčky II population, one shell was morphologically identified as *P. pusilla* (Supplementary Material Appendix S3) but, based on COI data, clearly clustered with *P. saxatilis*. Using nDNA, we excluded the possibility of mitochondrial introgression (data not shown).

DISCUSSION

Although our analysis confirmed that *Pyramidula pusilla* and *P. saxatilis* have strongly overlapping shell shapes (Horsáková *et al.*, 2022), consistent differences were revealed by CVA. As this analysis incorporates data on the assignment of each specimen to the species, it allows a highly accurate classification. However, if the entire variation is analysed (i.e. with the PCA, not incorporating information about species identity), the level of overlap is very high (Fig. 3). This can be recognized as the variation observable to the human eye. The minute differences in shell shape are associated with slightly wider mouth, higher spire, narrower umbilicus and also visually higher body whorl in *P. saxatilis* as compared with *P. pusilla*. Although these shell characters can aid with identification, unfortunately, they are simply not readily detectable by the eye, as is confirmed by the negligible shifts in landmark positions (Fig. 1). Luckily, there are diagnostic morphological differences in ridge development and density that can be seen by the eye and that allow unambiguous identification of the two species in most cases. While some miscrosculpture variation was noted (chiefly in *P. pusilla*), this character appears to be especially useful at the population level when a series of individuals are being identified. The utility of such microsculptural traits in distinguishing species is to be expected given that they are likely more selectively neutral than shell shape, which has been repeatedly shown to be under a strong selective pressure (e.g. Goodfriend, 1986; Haase, Esch & Misof, 2013; Dowle *et al.*, 2015). As a result,

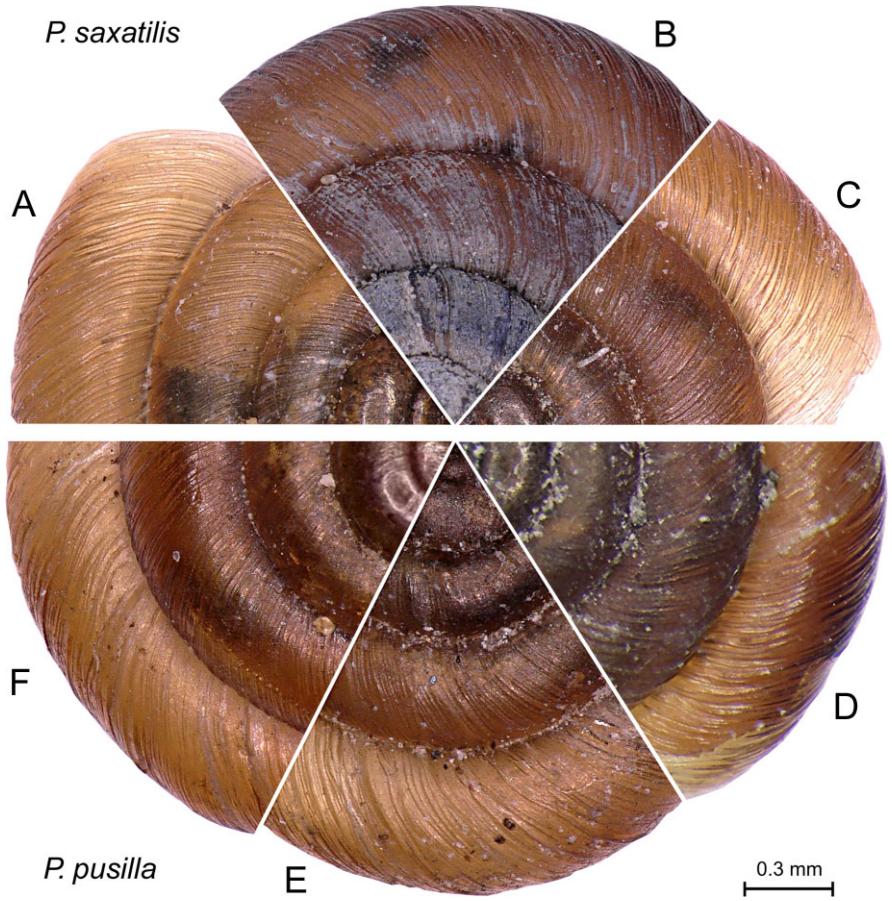

Figure 5. Variation in shell microsculpture among populations of *Pyramidula saxatilis* and *P. pusilla* identified by COI sequences. **A–C.** *Pyramidula saxatilis*. **A.** Nods, Switzerland (P229). **B.** Hochschwab, Austria (P189). **C.** Villeret, Switzerland (P208). **D–F.** *Pyramidula pusilla*. **D.** Motyčky, Slovakia (P361). **E.** Pavlov, Czech Republic (P211). **F.** Tatranská Kotlina, Slovakia (P213).

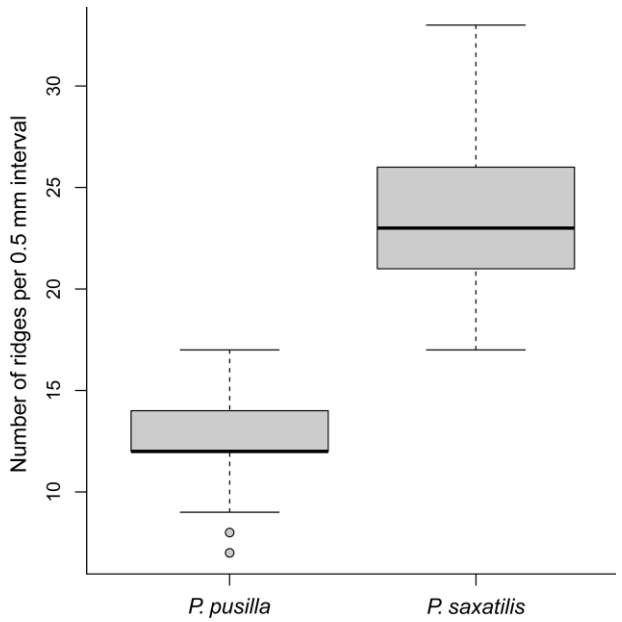

Figure 6. Variation in the number of growth ridges per 0.5 mm section on the upper shell surface (measured from the end of the third whorl). All measured populations were identified by mtDNA and nDNA markers. The difference between species is significant at $P << 0.001$ (GEE-g).

shell microsculpture has been found to provide useful diagnostic features in other land snails (Horsák & Pokryszko, 2010; Nekola, Coles & Horsak, 2015; Horsáková *et al.*, 2019). When microsculpture and shell shape are combined, the probability of correct identification is greatly increased. If there is a possibility of misidentification, it is most likely to occur in *P. pusilla* populations with a fine and more regular growth ridge pattern (Fig. 7AA). Such populations seem not to be very common. It is thus always necessary to inspect several individuals and consider the development of ridges across the entire upper shell surface. In *P. pusilla*, there are parts of the shell surface showing some segments of irregular and anastomosing ridges, while *P. saxatilis* has a very constant ridge density (Fig. 5).

We purposefully focused on two mixed populations with individuals representing variation that includes the extremes as well as the continuous morphological overlap between the two species. Therefore, the boundary between the two species was not clear-cut as it was for most of the other examined populations. In general, the problem was that individuals of *P. pusilla* with close to regularly developed ridges were easily misidentified as *P. saxatilis*. A useful approach for tackling this problem is to evaluate the degree of coarseness and regularity of the ridges. In *P. saxatilis*, the ridges are finer and very regular, while in *P. pusilla* they always show at least some irregularities (Fig. 5). While there is some variation in *P. saxatilis*, in general, the microsculpture is rather stable; in contrast, in *P. pusilla*, the microsculpture varies from coarse and very irregular to fine and more regular. Old and corroded shells in which the microsculpture is difficult to evaluate or which are falsely assumed to be *P. pusilla*

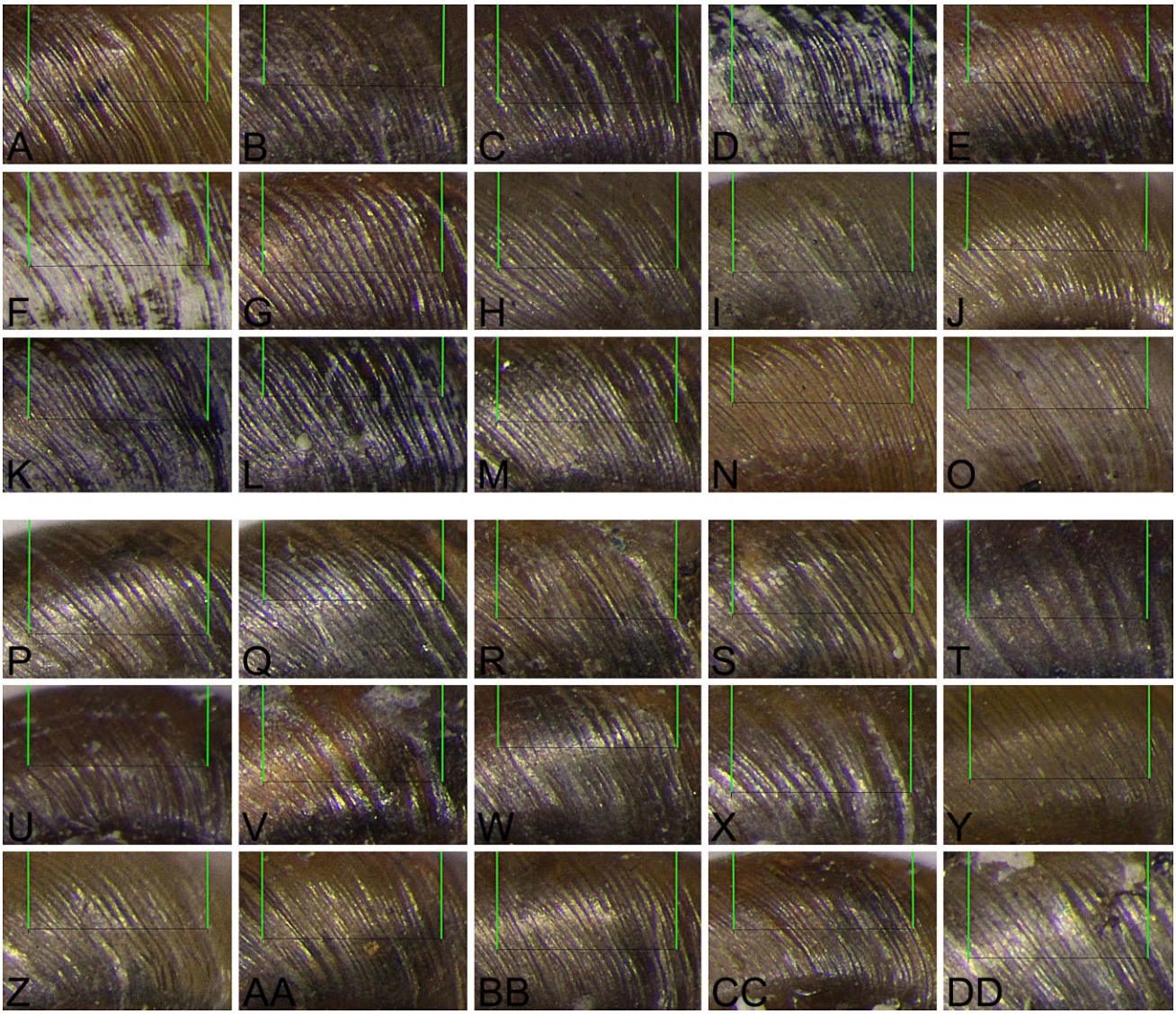

Figure 7. Variation in the development of growth ridges per 0.5 mm section on the upper shell surface (defined from the end of the third whorl) of *Pyramidula saxatilis* (**A–O**) and *P. pusilla* (**P–DD**). All images are from populations identified using mtDNA and nDNA markers. **A.** Hochschwab, Austria (P189). **B, C.** Spital am Pyhrn, Austria (P195). **D, E.** Schladming, Austria (P206). **F, G.** Villeret, Switzerland (P208). **H, I.** San Marino, RSM (P209). **J, K.** Bytča, Slovakia (P222). **L, M.** Selva di Val Gardena, Italy (P224). **N, O.** Type locality, St. Gallen, Switzerland (P378). **P, Q.** Cogne, Italy (P190). **R, S.** Zádiel, Slovakia (P191). **T, U.** Spital am Pyhrn, Austria (P193). **V, W.** Johnsbach, Austria (P196). **X, Y.** Pavlov, Czech Republic (P211). **Z.** Štramberk, Czech Republic (P212). **AA, BB.** Tatranská Kotlina, Slovakia (P213). **CC.** Horný Jelenec, Slovakia (P218). **DD.** Liptovské Revúce, Slovakia (P369).

because of the absence of fine ridges pose another problem. In such cases, careful study of the microsculpture on the penultimate whorl is important. In general, the microsculpture is more distinctive and easier to observe on juveniles and pre-adults. However, if only juveniles are studied, it may lead to the impression that *P. pusilla* consistently shows very regular ridges. In addition, in fresh shells, the surface is dull in *P. saxatilis* and often shiny in *P. pusilla* (Horsáková *et al.*, 2022). Overall, we urge caution and recommend that doubtful morphology-based identifications be verified by DNA barcoding. This is especially important when these species are reported from a country or region for the first time.

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SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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