

Original Article

A never-ending story of Western Palaearctic *Pyramidula* taxonomy and phylogeny

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ABSTRACT

The genus *Pyramidula* (Gastropoda: Pupilloidea) has represented one of the most taxonomically puzzling groups of Western Palaearctic micro land snails owing to a high level of morphological conservatism and shell convergence. We reconstructed *Pyramidula* phylogeny across the entire Western Palaearctic, using both mitochondrial (COI) and nuclear (ITS1 + ITS2) markers, based on 123 individuals from 89 localities. Unlike earlier efforts, we treated each distinct ITS indel area as a separate binary presence/absence variable that could be used as a genetic character state. Quantitative and qualitative shell features were also coded from 355 individuals sourced from genetically verified populations. Bayesian analysis and maximum likelihood both generated trees with nine well-supported species-level clades, with ITS1 + ITS2 tree resolution being notably improved at shallow phylogenetic levels through formal inclusion of indel information. Each of these clades was also found to possess distinct shell morphology and biogeography. Two are described as new species: *Pyramidula koshka* Horsák & Leonov sp. nov. and *Pyramidula pygmaea* Horsák & Kafimola sp. nov. Our results establish the first comprehensive phylogenetic framework for the genus across the Western Palaearctic. We document how indels can affect phylogenetic inference at multiple evolutionary scales and provide practical guidelines for incorporating them into Bayesian inference and maximum likelihood analyses.

Keywords: molecular phylogeny; Palaearctic; Gastropoda; indel inclusion; taxonomic revision; species description

INTRODUCTION

Pyramidula Fitzinger, 1833 consists of 18 extant species (MolluscaBase 2025) of small (shell <3 mm) land snails strictly associated with calcareous rock faces (Welter-Schultes 2012). Although principally distributed from Europe to the Himalayas, they also extend into northern Africa, Southeast Asia archipelagos, and Japan. Traditionally, a single species, *Pyramidula rupestris* (Draparnaud, 1801), was recognized in Europe (Kerney *et al.* 1983). However, Gittenberger and Bank (1996) split it into six putative species based on conchological differences in observed museum lots. Molecular analyses demonstrated that only five of these were supported, with a sixth apparently cryptic species, *Pyramidula saxatilis* (Hartmann, 1842), being hidden within *Pyramidula pusilla* (Vallot, 1801), a common and widespread species (Razkin *et al.* 2016, 2017). Although Horsáková *et al.* (2022) documented the conchological traits distinguishing the four

western and central European species, showing that none was truly cryptic, all other taxa remained unresolved.

Here, we continue our integrative revision of *Pyramidula*, aiming to resolve taxonomy and phylogeny of species that extend east from the Adriatic to the Hyrcanian ecoregion in northern Iran. For each putative taxon, we separately consider both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) markers, in addition to conchometrics, ecology, and biogeography. We also formally code indel (insertion/deletion) information, i.e. the presence or absence of alignment gaps, in a way that allows them to inform phylogenetic reconstructions, a procedure rarely used in land snail phylogenetics (but see Goulding *et al.* 2023). Through this process, we aim to resolve species-scale taxonomy within the *Pyramidula jaenensis* (Clessin, 1882) complex and to validate hypotheses on the existence of several undescribed species in the Hyrcanian ecoregion. Based on these new results, we have

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constructed an identification key to all species-level entities currently recognized from the Western Palearctic.

MATERIALS AND METHODS

Specimen set

Razkin *et al.* (2017) provided sequences for the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA genes, and for the nuclear 5.8S ribosomal RNA (rRNA), internal transcribed spacer 2 (ITS2), and 28S rRNA genes from specimens collected at 183 sites. They also provided shell width, shell height, and umbilicus width measurements from most of these samples. Earlier, Razkin *et al.* (2016) had also conducted RADSeq (Restriction site–Associated DNA sequencing) analyses on a subset of this material by selecting a single individual per presumed species-level clade. Horsáková *et al.* (2022) and Horsák *et al.* (2022) expanded on these data by considering additional 62 populations centred on western and central Europe, using COI, internal transcribed spacer 1 (ITS1) and ITS2 (Horsáková *et al.* 2022) or only COI (Horsák *et al.* 2022), also collecting shell measurements and qualitative shell traits.

Although Razkin *et al.* (2017) did not recover ITS1 sequence information from their samples, these data are crucial to generating monophyletic species-level clades and robust species delimitation in this group. Therefore, our principal set of 49 genetically analysed specimens (Fig. 1; Supporting Information, Table S1) is limited only to those where COI, ITS1, and ITS2 sequences existed, or where it was possible to generate these sequences owing to the physical existence of samples in our collection. To this, we have added 16 additional populations ranging from the Balkans and Aegean east into northern Iran (Supporting Information, Table S1), representing not only *Pyramidula* ‘*jaenensis*’, but also *Pyramidula cephalonica* (Westerlund, 1898), *Pyramidula chorismenostoma* (Westerlund & Blanc, 1879) and the poorly understood *Pyramidula hierosolymitana* (Bourguignat, 1852). Additionally, *Plagyrona placida* (Shuttleworth, 1852) from the Canary Islands was used to root trees. Given that its ITS sequence is inter-alignable with *Pyramidula* and that *Plagyrona* also resembles *Pyramidula* in shell features, it is likely to represent phylogenetically the closest clade.

DNA amplification and sequencing

DNA extraction and PCR amplification of COI, ITS1, and ITS2 for all newly analysed individuals follows methods detailed by Horsáková *et al.* (2022). GenBank accession numbers for all new sequences are provided in the Supporting Information (Table S1). ITS1 and ITS2 were concatenated and aligned with MAFFT v.7 (Kuraku *et al.* 2013, Katoh *et al.* 2019) using the Q-ins-i algorithm, which considers secondary rRNA structure (Katoh and Toh 2008). All alignments were inspected visually for potential errors and are available upon request.

Phylogenetic reconstruction

We have observed in other groups that indels reflect species-level clades (e.g. Nekola *et al.* 2015, Horsáková *et al.* 2019). Given that this also appeared true in *Pyramidula* based on a preliminary visual inspection of aligned sequences, we desired a formal way to use it to inform trees; an approach rarely taken in land snail phylogenetics. Unfortunately, in their standard configurations both Bayesian inference (BI) and maximum likelihood (ML; via RAxML) treat indels as missing data (Ronquist *et al.* 2012, Stamatakis 2014). Although ML in MEGA (Kumar *et al.* 2024) can be forced to consider indels (through use of the ‘use all sites’ option within ‘data subset to use’), the resultant trees suggest that this harvests only a limited amount of indel information. To consider indels formally within the BI and ML framework, we therefore followed an established indel-coding strategy (Redelings *et al.* 2024), in which indels within multiple sequence alignments are coded using a custom R script based on the *seqinr* package (Charif and Lobry 2007). This method follows the approach of Simmons and Ochoterena (2000) by encoding each insertion–deletion event with different start and/or end positions as binary presence/absence data. The resultant matrix is exported in NEXUS and PHYLIP formats, allowing it to inform BI/ML reconstructions. BI and ML procedures followed Horsáková *et al.* (2022). These were applied independently to both the COI and ITS1 + ITS2 alignments, both for sequence only and for sequence + indel coded data. Genetically validated species were considered present when they possessed highly supported, reciprocally monophyletic clades in both the nDNA and mtDNA across both reconstruction

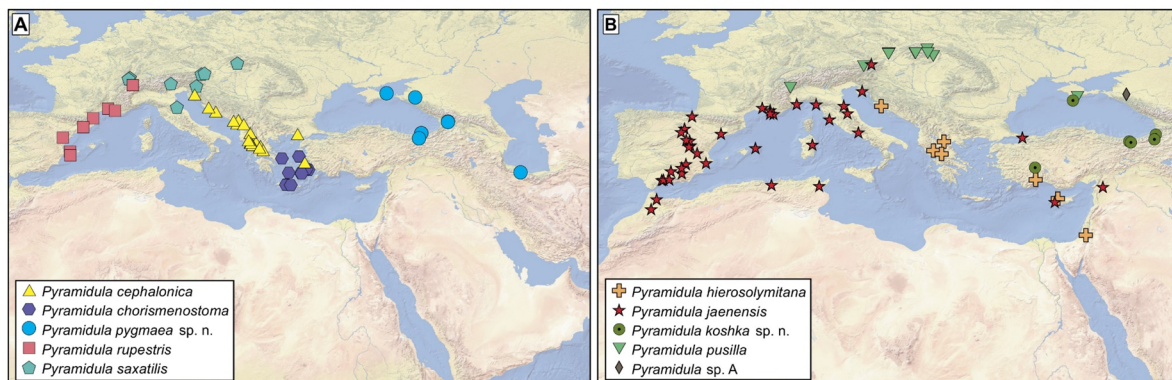


Figure 1. Position of samples used in phylogenetic reconstruction based on the full dataset; a few samples with only conchological signal, as for type material, are also plotted (see Supporting Information, Table S2). Note that samples of *Pyramidula pusilla* cover only a part of its distribution (see Horsáková *et al.* 2022 for mapping the full range). The map was created in ArcGIS 10.8 (Esri, Redlands, CA, USA) using the Natural Earth GeoTIFF raster dataset.

methods, while also possessing distinct morphological and biogeographical traits.

Because Razkin *et al.* (2017) provided additional ITS2 sequences, along with conchological data, these could be used to improve species delineation and maximize biogeographical coverage for a previously unresolved group of taxa closely related to *Pyramidula jaenensis*. We therefore constructed a separate ITS2 alignment extended by 69 non-identical sequences of these taxa (Supporting Information, Table S1). Note that for the species requiring the most taxonomic rigour, i.e. *Pyramidula hierosolymitana*, *Pyramidula koshska*, *Pyramidula pygmaea*, and *Pyramidula* sp. A, we excluded identical sequences only if they came from the same population. Sequences from the same set of 69 additional individuals were also used to extend COI alignment (Supporting Information, Table S1), to document congruence between nDNA and mtDNA signals. BI and ML analyses were run separately on the extended COI and ITS2 alignments using the same methods as described above. Analyses of ITS2 were again run both with and without indel coding. Specimen placement within the resultant trees, in addition to their shell dimensions, were used to identify the species and provide a more complete documentation of its shell variation and ranges.

Conchological morphometrics

Shell width and height, and umbilicus width were gathered from the papers by Razkin *et al.* (2017) and Horsáková *et al.* (2022) and newly measured from 353 genetically confirmed individuals across all taxa (Supporting Information, Table S2). For populations present in our collection (Supporting Information, Table S1: ITS1 exists), top, bottom, and front images of each were created using an Olympus SZX 10 microscope with Olympus C-7070 Wide Zoom camera and QuickPHOTO MICRO software to provide deep focus. These were used to record the number of coils, coil expansion rate, and suture depth. In addition, surface features were imaged using a Keyence VHX-5000 digital microscope with ZS-20 and ZS-200 objective lenses. These were used to record apex shape, lustre and colour, protoconch and teleoconch microsculpture, presence and arrangement of ribs/striae/growth ridges, ridge angle, and ridge density per 0.5 mm of teleoconch surface. Subsequent analyses were conducted in R v.3.5.2 using the 'ade4' (Dray and Dufour 2007) and 'ggplot2' (Wickham 2016) packages.

Only mature or nearly mature (≥ 3.5 whorls) shells were considered for the above-mentioned exploration. Because whorl information was not provided by Razkin *et al.* (2017), an attempt was made to remove immature shells from their data by eliminating shells from the lower quartile of the height/width measurements. We recognize that juveniles from larger individuals might be retained using this approach and also that small adults could be eliminated. For a comparison free of differences related to the absolute size of species, we also consider shell width/height and umbilicus width/shell width ratios.

We also included images of types for the above listed analysis. They include: *Pyramidula hierosolymitana* (lectotype, Museum d'Histoire Naturelle, Geneva, Switzerland, MHNG-MOLL-116903, imaged and provided by Emmanuel Tardy, © MHN); *Pyramidula jaenensis* (type series, S. Clessin collection, State Museum of Natural History Stuttgart, Germany, SMNS-ZI0142153, provided by D. Wanke and M. Pallmann, ©

SMNS); *Pyramidula pusilla* (neotype, Natural History Museum, Dijon, France, No. 210.996.mo.1, provided by E. Fara); *Pyramidula rupestris* (lectotype, Natural History Museum, Vienna, Austria, Draparnaud collection xxvi 97a, provided by I. Gallmetzer, © NHMW), and *Pyramidula saxatilis* (type series, J.D.W. Hartmann collection, Natural History Museum St. Gallen, Switzerland, MS62, imaged by the authors).

RESULTS

The new ITS1 + ITS2 phylogenetic reconstructions, based on alignments either excluding (Fig. 2) or including (Fig. 3A) indel information, differed greatly in their treatment of the crown *Pyramidula jaenensis* complex. Although the tree reconstruction without indel coding (Fig. 2) remains poorly resolved, with *Pyramidula jaenensis* appearing to be polyphyletic, the tree informed by indel coding (Fig. 3A) shows *Pyramidula jaenensis* to be monophyletic with very high support. These results are replicated within the mtDNA data (Fig. 3B), albeit with some examples of introgression/incomplete sorting being evident. Although all five deeper nodes were supported in the nDNA tree without indel coding (Fig. 2), two of them were not when indel data were used (Fig. 3A). Only a single node, the sister position of *Pyramidula rupestris* and *Pyramidula saxatilis*, was also supported in the mtDNA tree (Fig. 3B).

Combining results of the two nDNA trees (Figs 2, 3A), nine species-level clades are highly supported, including six previously well-defined species: *Pyramidula cephalonica*, *Pyramidula chorismenostoma*, *Pyramidula jaenensis*, *Pyramidula pusilla*, *Pyramidula rupestris*, and *Pyramidula saxatilis*. Three other lone branches of species-scale length are also recognized: *Pyramidula koshska* and *Pyramidula hierosolymitana* are positioned outside the *Pyramidula jaenensis* complex, whereas *Pyramidula pygmaea* is positioned within it. Across all these analyses, the crown-group consisting of *Pyramidula jaenensis*, *Pyramidula chorismenostoma*, *Pyramidula cephalonica*, and *Pyramidula pygmaea* represents a highly supported clade. The extended ITS2 and COI trees (Fig. 4; Supporting Information, Figs S1, S2) also suggested the presence of a 10th species, representing an individual from the Krasnodar region along the northeastern Black Sea coast, which is rooted between *Pyramidula hierosolymitana* and *Pyramidula koshska*. Information used from these three extended trees also allowed assignment of all analysed individuals into the 10 above-mentioned species-level clades, enabling exploration of their conchometry and distribution.

Comparison of qualitative (Figs 5, 6) and quantitative (Fig. 7) shell traits among the nine genetically recognized and well-supported clades document that each exhibits a unique suite of features, with shell height/width ratios, umbilicus width, and teleoconch microsculpture being among the most informative (Figs 5, 7). Of interest are the multiple instances of species that are separated by high vs. low spire shell, by wide vs. narrow umbilicus, or by fine/regular vs. coarse/irregular growth ridges.

SPECIES DESCRIPTIONS

Because Horsáková *et al.* (2022) have already provided detailed information for *Pyramidula jaenensis* s.s., *Pyramidula pusilla*, *Pyramidula rupestris*, and *Pyramidula saxatilis*, we provide updated versions in Supporting Information (Appendix S1) for interested

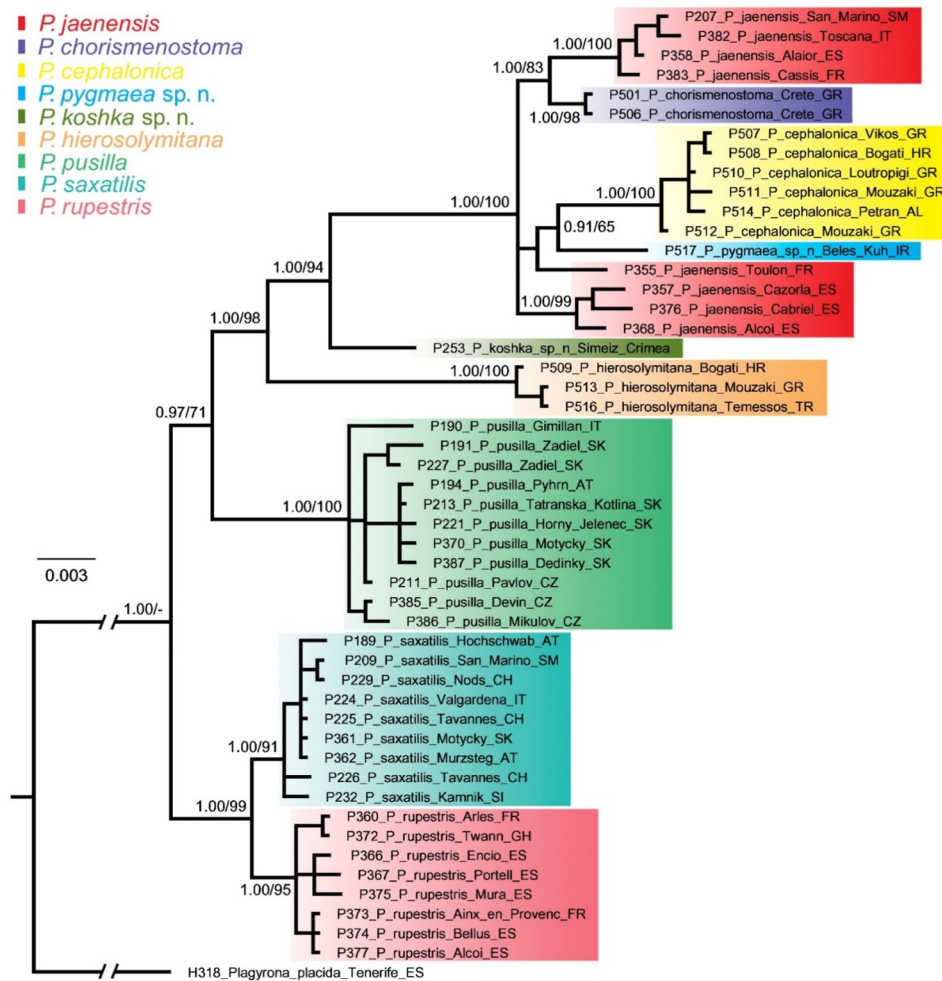


Figure 2. Bayesian inference (BI) tree for *Pyramidula* species based on nuclear DNA (ITS1 + ITS2). Posterior probability values from BI are shown by the respective nodes, followed by maximum likelihood (ML) rapid bootstrap values. The support values are displayed only for species-level and higher clades, and only if clades are considered supported, i.e. BI ≥ 0.90 or ML $\geq 70\%$.

readers. The updates relate mainly to shell variation and notes on identification and distribution. Newly considered taxa are detailed below in the same fashion.

Pyramidula cephalonica (Westerlund, 1898)

Patula (*Pyramidula*) *rupestris* var. *cephalonica* Westerlund, 1898: 156.

Type locality: Greece, Kefallinia [=Cephalonia island]. Lectotype designated by Gittenberger and Bank (1996: 72).

Material analysed: Conchology and genetics: 24 individuals from 20 populations covering Croatia, Montenegro, Albania, and Greece.

Diagnosis: Medium- to high-spire conical shell, with whorls regularly increasing in width, broader than high. Aperture relatively small and umbilicus wide open, mostly just below one-third of the total shell width to broader (Figs 5, 7). Teleoconch with (very) prominent growth lines; in well-preserved specimens, these may have the character of riblets. Shell colour brown to yellowish brown (in non-corroded individuals), with dull appearance.

Genetics: Well-defined and highly distinct monophyletic clade in both mtDNA and nDNA, closely related to *Pyramidula jaenensis*.

Variation and identification notes: Although mostly stable, some populations are less conical. It is similar to some *Pyramidula jaenensis* morphotypes but differs in possessing coarse growth lines, and their ranges do not overlap. Although it is sympatric or even syntopic with *Pyramidula hierosolymitana*, it is easy to differentiate because the latter has a very narrow umbilicus, high spire, and more slowly expanding whorls (Figs 5, 7).

Distribution: Confirmed populations limited from northern Dalmatia southwards into Greece.

Other notes: Although Gittenberger and Bank (1996) and Bank et al. (2025) also report this species from Turkey, it has not been confirmed there genetically by neither Razkin et al. (2016) nor our work. Although Razkin et al. (2017) did display one *Pyramidula cephalonica* individual from western Turkey, in their sample list (Data S2 in Razkin et al. 2017), nothing is reported within the *Pyramidula cephalonica* clade (G6-B) from Turkey. Images of Turkish *Pyramidula* ‘*cephalonica*’ in the study by Bank

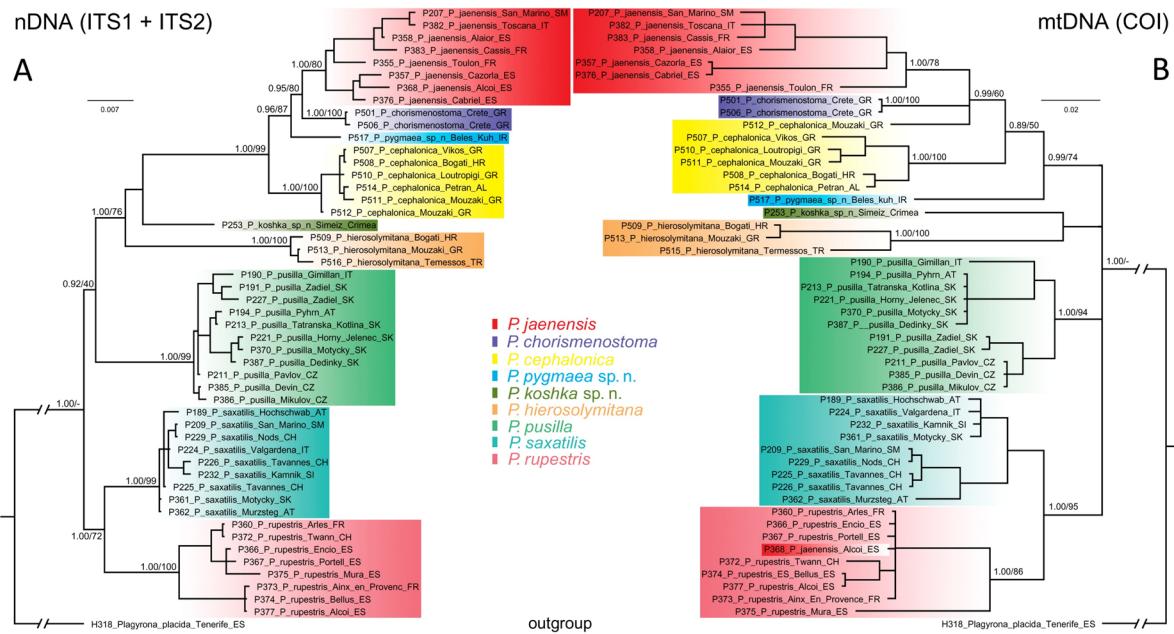


Figure 3. Bayesian inference (BI) tree for *Pyramidula* species based on nuclear DNA (ITS1 + ITS2) with indel partition included (A) and mitochondrial DNA (COI) (B). Posterior probability values from BI are shown by the respective nodes, followed by maximum likelihood (ML) rapid bootstrap values. The support values are displayed only for species-level and higher clades, and only if clades are considered supported, i.e. BI ≥ 0.90 or ML $\geq 70\%$.

et al. (2025; plate 40, figs 6, 7), are clearly a different species, given their very wide umbilicus (>0.4 of shell diameter, as opposed to <0.3 for genetically verified *cephalonica* populations within the known range). These *Pyramidula* ‘*cephalonica*’ are also notably flatter, with a smaller aperture and more tightly coiled whorls. These shell features, combined with fine, regular, and sharp growth ridges, correspond to the description of *Pyramidula humilis* (W. H. Benson, 1838) from the Western Himalayas (cf. lectotype UMZC I.102010.A; Preece *et al.* 2022). Genetic data will thus be necessary to determine whether the Turkish *Pyramidula* ‘*cephalonica*’ represent disjunct *Pyramidula humilis* populations or a new species with similar conchology.

Pyramidula chorismenostoma (Westerlund & Blanc, 1879)

Helix (*Patula*) *rupestris* *chorismenostoma* Westerlund & Blanc, 1879: 32.

Type localities: Greece: (i) Kikladhes, island Siros, ‘St. Georges’ [= Ag. Georgios, = Kinion]; and (ii) Voiotia, ‘Mont Macolessos’ (near Ritsona) in the study by Gittenberger and Bank (1996: 72).

Material analysed: Conchology and genetics: nine individuals from eight populations covering six Aegean islands and Crete.

Diagnosis: Adult shell scalariform. In most individuals, whorls start detachment after the second revolution, but in some rare individuals they remain attached up to the third. Teleoconch has prominent growth lines, similar to those for *Pyramidula cephalonica* (Fig. 5); protoconch with rugged microstructure. Shell colour light to dark brown, always of dull appearance; older shells corrode towards brownish-blue colour.

Genetics: Well-defined and highly distinct monophyletic clade in both mtDNA and nDNA, closely related to *Pyramidula jaenensis*.

Distribution: Southern two-thirds of Greece through the Aegean islands to Crete and eastern coast of Turkey (Bank *et al.* 2025).

Pyramidula hierosolymitana (Bourguignat, 1852)

Helix hierosolymitana Bourguignat, 1852: 13–14.

Synonym: *Patula rupestris* var. *dalmatina* Clessin, 1887, type locality: Omiš (Almissa), Croatia.

Type locality: Valley of Jehoshaphat near Jerusalem, Israel. Lectotype: MHNG-MOLL-116903, Museum d’Histoire Naturelle, Geneve, Switzerland. Lectotype designated by Gittenberger and Bank (1996: 76).

Material analysed: Conchology: 15 individuals from three populations covering Croatia, Turkey, and Israel (only the lectotype); genetics: Greece and Cyprus.

Diagnosis: High-spined conical shell, with rapidly expanding whorls. Shell almost as tall as wide, umbilicus very narrow, mostly just below one-fifth of the total shell width. Teleoconch with prominent irregular growth ridges (Figs 5, 6). Shell colour dark brown to yellowish brown (in non-corroded individuals), with a rather dull appearance.

Genetics: Well-defined and highly distinct monophyletic clade in both mtDNA and nDNA. Given that the nearest genetically analysed population originates from Cyprus, sequences from (or in proximity) to the type locality are needed to confirm epithet assignment. However, several unique shell features held in common between the lectotype and genetically analysed *Pyramidula hierosolymitana* provide high confidence for our use of this nomen.

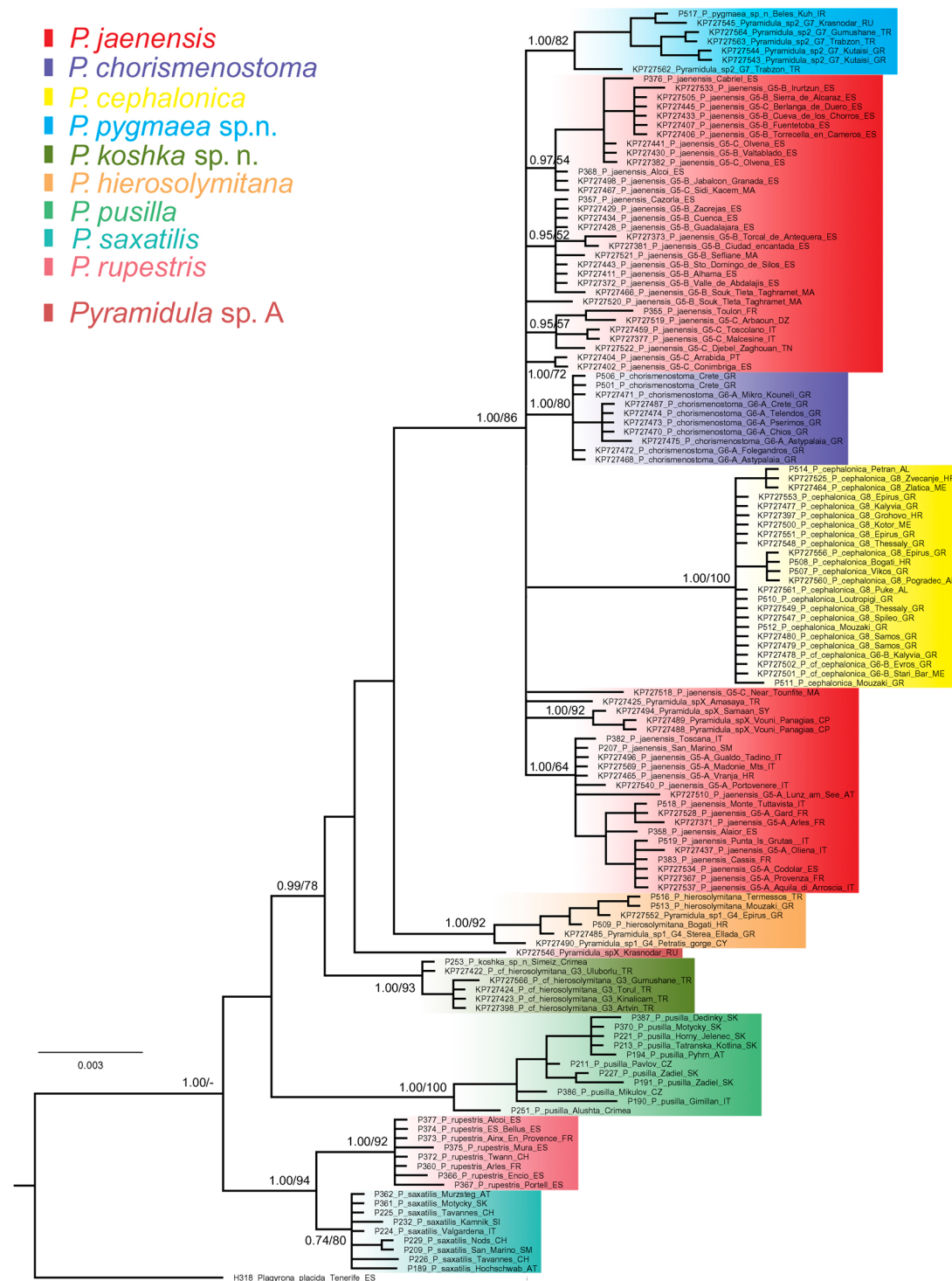


Figure 4. Bayesian inference (BI) tree for all *Pyramidula* sequences based on nuclear DNA ITS2 marker (standard settings without indel partition included). Posterior probability values from BI are shown by the respective nodes, followed by maximum likelihood (ML) rapid bootstrap values. The support values are displayed only if clades are considered supported, i.e. BI ≥ 0.90 or ML $\geq 70\%$.

Variation and identification notes: Although its variability remains poorly known, it appears that shell height and umbilicus width can range from tall/very narrow to flatter/more open. This species is easy to distinguish from all other sympatric *Pyramidula* species by its very narrow umbilicus and coarse irregular growth ridges (Figs 5–7). Although also similar to *Pyramidula rupestris*, it has a

less open aperture and more tightly coiled whorls. Moreover, their ranges are distinctly parapatric (see below).

Distribution: Ranges from Central Croatia (Krka NP) across Greece, Cyprus, and Turkey to Georgia. This species is separated from the similar-shelled *Pyramidula rupestris* by a 750 km gap

Identification key for Western Palaearctic *Pyramidula*

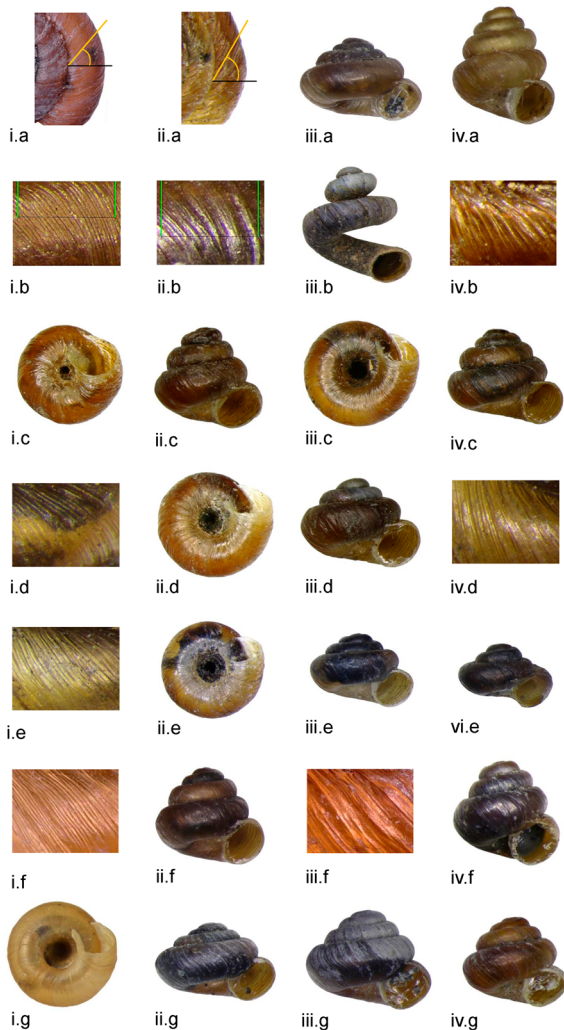
- 1(2) Angle of growth ridges $<45^\circ$ (i.a), shell flat (iii.a, ii.g; but iii.g), umbilicus wide (i.g), suture shallow.....go to 3
- 2(1) Angle of growth ridges $>45^\circ$ (ii.a), shell mostly conical (iv.a), umbilicus narrow (i.c) to wide (iii.c), suture deep.....go to 5
- 3(4) Upper shell microsculpture fine and regular without anastomose growth ridges (i.b, ii.g, iii.g); mainly the Alps.....*P. saxatilis*
- 4(3) Upper shell microsculpture irregular, with coarser and anastomose growth ridges (ii.b, i.a); widespread.....*P. pusilla*
- 5(6) Shell notably scalariform (iii.b); S Greece, Crete and W Turkey.....*P. chorismenostoma*
- 6(5) Shell normal, not scalariform.....go to 7
- 7(8) Umbilicus $<1/5$ of shell width (i.c), high spire (ii.c), coarse growth ridges (iv.b); Croatia to Israel.....*P. hierosolymitana*
- 8(7) Umbilicus $>1/5$ of shell width.....go to 9
- 9(10) Umbilicus $>1/4$ of shell width.....go to 11
- 10(9) Umbilicus $<1/4$ of shell width.....go to 15
- 11(12) Umbilicus $>1/3$ of shell width (iii.c), growth ridges sharp (iv.c, i.d); Croatia to Greece.....*P. cephalonica*
- 12(11) Umbilicus $1/3$ to $1/4$ of shell width (ii.d., ii.e), growth ridges flat and finer (iv.d, i.e).....go to 13
- 13(14) Height/width >0.8 (iii.d), around Black Sea.....*P. koshka* sp. n.
- 14(13) Height/width <0.7 (iii.e), nipple-like apex in subadults (iv.e), eastern Black Sea coast to N Iran.....*P. pygmaea* sp. n.
- 15(16) Upper shell growth ridges fine and regular (i.f), slow expansion rate (ii.a), aperture less open; shell variable in height and umbilicus width (ii.f); widely distributed with several distinct genetic and geographic races.....*P. jaenensis*
- 16(15) Upper shell growth ridges coarse and irregular (iii.f), faster expansion rate (iv.f), aperture widely open; shell rarely also flat with narrow aperture (iv.g); W Spain to W Alps.....*P. rupestris*
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Figure 5. Identification key to Western Palaearctic *Pyramidula* species. Notes on variation in critical characters: for *Pyramidula saxatilis*, some populations of higher spire and narrower umbilicus resemble *Pyramidula rupestris*, but differences in microsculpture are stable; for *Pyramidula pusilla*, in some populations with finer and more regular microsculpture, differentiation from *Pyramidula saxatilis* can be very hard, and DNA barcoding is sometimes necessary; for *Pyramidula rupestris*, some populations are close to *Pyramidula jaenensis* by flatter shell, lower expansion rate, and narrower aperture, also microsculpture can be fine, and in those cases differentiation can be difficult and DNA barcoding is recommended. (ii.g, iii.g *Pyramidula saxatilis*, two forms).

covering the Alps and Apennine peninsula. Shell features of *Patula rupestris* var. *dalmatina* Clessin, 1887 topotypes (RMNH. MOL.245337) suggest that this nomen might be a junior synonym of *Pyramidula hierosolymitana*.

Other notes: Razkin *et al.* (2017) associated the name *Pyramidula hierosolymitana* with their clade G3, claiming that only G3 possesses conical shells with a narrow umbilicus. However, their shell images of the clade G3 (Razkin *et al.* 2017: fig. 2A) show a flat shell with obviously open umbilicus. It seems that the clade designation was misassigned (also by Razkin *et al.* 2016, named as '*Pyramidula* sp.1'), because the data from the clade G4 (by Razkin *et al.* 2016, named as '*Pyramidula* sp.2') cluster with our samples from Croatia, Greece, and Turkey (Fig. 4; Supporting Information, Fig. S1), expressing shells characteristic of *Pyramidula hierosolymitana* (Fig. 6).

Pyramidula koshka Horsák & Leonov sp. nov

Type locality: Crimea: 44.4031N, 33.9925E; Koshka Mt. near Simeiz, limestone rocks; 170 m a.s.l.; 11 July 1992 and 23 August 1997; V. N. Popov lgt.

Material analysed: Conchology: 18 adults and 36 subadult or juvenile individuals from the type locality; shell measurements and genetic sequences for five individuals were adopted from Razkin *et al.* (2017).

Holotype: Shell height (SH) 1.98 mm, shell width (SW) 2.65 mm, umbilicus width (UW) 0.81 mm, 3.8 whorls (Fig. 6) [NMPC (Prague): P6M 044124]; eight paratypes from type locality (Supporting Information, Fig. S3) [four ex. NMPC (Prague): P6M 044125; four ex. SMF (Frankfurt am Main): SMF 383162].

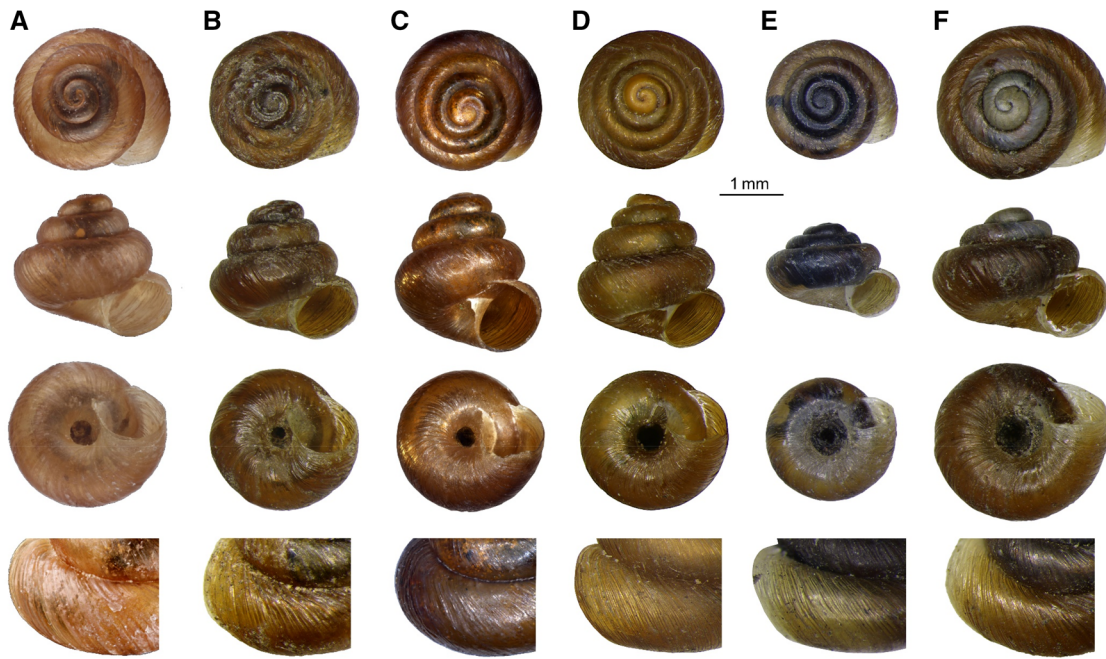


Figure 6. *Pyramidula* types and genetically verified specimens. A, *Pyramidula hierosolymitana*, lectotype, MHNG-MOLL-116903. B, *Pyramidula hierosolymitana*, P509, Croatia. C, *Pyramidula rupestris*, lectotype, MO14800. D, *Pyramidula jaenensis*, topotype, P357, Spain. E, *Pyramidula pygmaea*, holotype, P517, Iran. F, *Pyramidula koshka*, holotype, P253, Crimea.

Shell description: Shell colour reddish to dark brown, always of dull appearance even in non-corroded individuals. Low-spired shell, broader than wide, with rather wide umbilicus (typically 0.25–0.30 of shell width), tumid whorls, and deep suture, with rather fast coil expansion rate. Growth ridge inclination angle $>45^\circ$ (see Figs 5, 6), with the whorls almost flattened on top along the suture. One of the largest taxa within the genus: SH 2.0–2.4 mm and SW 2.5–2.7 mm; ≤ 4 whorls.

Differential diagnosis: This species is characterized by its larger, flat, rapidly expanding shells, with a dominate body whorl (Fig. 6; Supporting Information, Fig. S3). Its range is sympatric with *Pyramidula pusilla*, *Pyramidula hierosolymitana*, *Pyramidula pygmaea*, and possibly *Pyramidula cephalopnica* (but see note by this species in its distribution). Species with the closest-appearing shells are reported from the western Himalayas (*Pyramidula humilis* and *Pyramidula salimalli*). Both differ by possessing a much wider umbilicus (more than one-third of the shell width); *Pyramidula przewalskii* from western China has, in contrast, a very narrow umbilicus (approximately one-fifth of the shell width).

Genetics: Well-defined and highly distinct monophyletic clade in both mtDNA and nDNA. More data are needed to consider genetic variation fully, because three samples from Turkey are rather homogeneous and differ from the Crimean sample. In the study by Razkin et al. (2016), samples from this clade are called ‘*Pyramidula* cf. *hierosolymitana*’ but in the paper by Razkin et al. (2017) group ‘G3’.

Variation and identification notes: The combination of growth ridge inclination angle $>45^\circ$ with low-spired shell and its large size is unique.

Etymology: The specific epithet comes from Mt. Koshka near Simeiz (Crimea), which represents the type locality.

Distribution: Insufficiently known, currently confirmed only from the southernmost tip of Crimea, several sites along the SE coast of the Black Sea in Turkey, and near Uluborlu in SW Turkey. Based on images in the paper by Bank et al. (2025: figs 1, 3; plate 40), more localities might exist elsewhere in Turkey.

Pyramidula pygmaea Horsák & Kafimola sp. nov

Type locality: Iran: 36.6536N, 50.7912E; Beles Kuh NR, Tonekabon city in Mazandaran province, limestone rocks; 570 m a.s.l.; 31 July 2024 and 10 August 2025; S. Kafimola and F. Nejat lgt.

Material analysed: Nine adult and 35 juvenile individuals from the type locality; shell measurements and genetic sequences for five other individuals, each originating from a distinct population, were adopted from Razkin et al. (2016): Turkey, Trabzon (two individuals) and Gümüşhane (one), Georgia, Imereti (one), and Krasnodar, Russia (one). One sample from Crimea (two dead, adult shells among five *Pyramidula pusilla*) could not be verified through DNA, and it is assigned here based solely on conchology (Supporting Information, Fig. S3B–D).

Holotype: SH 1.98 mm, SW 2.65 mm, UW 0.81 mm, 3.8 whorls (Fig. 6) [NMPC (Prague): P6M 044126]; four paratypes from type locality (Supporting Information, Fig. S3) [two ex. NMPC (Prague): P6M 044127; two ex. SMF (Frankfurt am Main): SMF 383163].

Shell description: Shell colour yellowish grey to dark brown, always of dull appearance even in non-corroded individuals. Low-spired

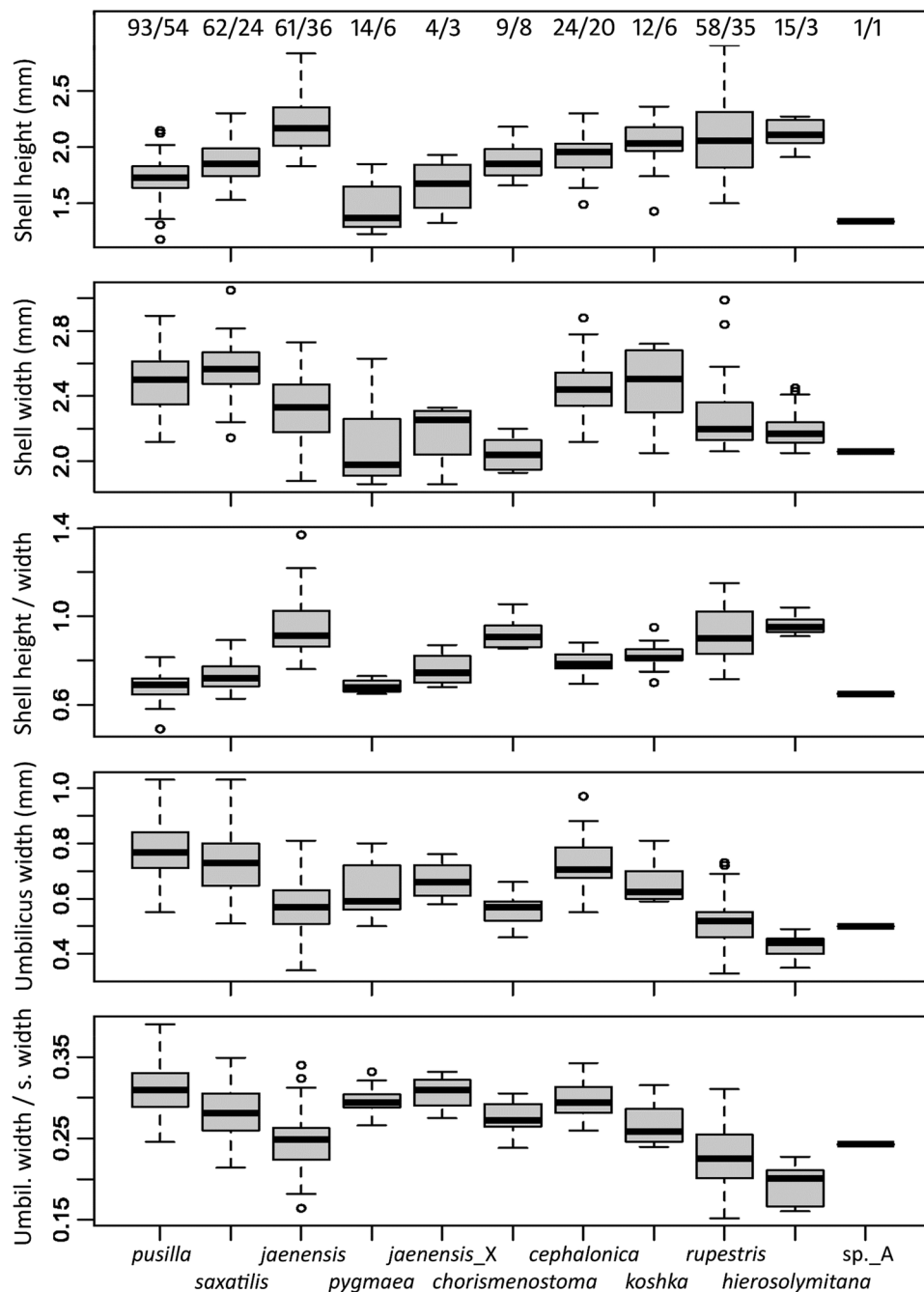


Figure 7. Variation in shell measurements and their differences between species. Numbers of measured shells and studied populations (separated by the slash) are shown above each boxplot. Note that the eastern Mediterranean *Pyramidula jaenensis* populations are labelled ‘*jaenensis_X*’.

shell, broader than wide, with wide umbilicus (typically 0.3 of the shell width), tumid whorls and deep suture, medium coil expansion rate. Growth ridge inclination angle $>45^\circ$ (see Figs 5, 6), with the whorls almost flattened on top along the suture. Juvenile/subadult shells (~ 3.25 whorls) with a notably pointed apex (Fig. 5; Supporting Information, Fig. S3). One of the smallest taxa within the genus: SH 1.4–1.8 mm and SW 1.9–2.6 mm (Fig. 6); ≤ 4.3 whorls (Supporting Information, Fig. S3).

Differential diagnosis: Smaller than *Pyramidula jaenensis*, with a flatter shell and wider umbilicus (Figs 6, 7). Juvenile/subadult shells possess a notably pointed apex. The conchologically nearest

species appear to be from the western Himalayas (*Pyramidula humilis* and *Pyramidula salimalli*), both of which differ by possessing a wider umbilicus (more than one-third of the shell width) and larger shell diameter (>2.0 – 2.5 mm); *Pyramidula przewalskii* from western China is also larger (>2.5 mm) and has a very narrow umbilicus (approximately one-fifth of the shell width).

Genetics: Distinct lineage in mtDNA and nDNA, closely related to *Pyramidula jaenensis*. All markers (COI, ITS1 + ITS2) are available for only one specimen, but monophyly is confirmed by multiple sequences included in ITS2 phylogeny reconstruction (Fig. 4; Supporting Information, Fig. S1). However, the species is

polyphyletic in mtDNA, represented by at least three distinct mitochondrial lineages (Supporting Information, Fig. S2).

Variation and identification notes: A combination of growth ridge inclination angle $>45^\circ$, small size, and low-spired shell is unique.

Etymology: The specific epithet *pygmaea* is derived from the Latin adjective *pygmaeus* (= dwarf, very small) in feminine form, referring to the fact that this new species is the smallest known member of the genus in the Western Palearctic.

Distribution: Insufficiently known, currently ranging from north-eastern coast of Black Sea (Crimea, Russia, Georgia, and Turkey) to southern coast of the Caspian Sea (Iran). In the study by Razkin *et al.* (2016) it is referred to as '*Pyramidula* sp.2' and by Razkin *et al.* (2017) as group 'G7'.

DISCUSSION

Pyramidula taxonomy has been challenging for decades (Gittenberger and Bank 1996, Welter-Schultes 2012). With this study, we expand the area where its phylogenetics, biodiversity, and biogeography are reasonably resolved eastwards to northern Iran. In comparison to the phylogeny of Razkin *et al.* (2017), our work resolves several previously ambiguous nodes, primarily by separate reconstruction of mitochondrial and nuclear phylogenies and by permitting indel information to affect ITS1 + ITS2 trees. At least nine species can be validated empirically from the Atlantic to the Caspian based on DNA sequence, conchometrics, and biogeography. These include *Pyramidula jaenensis*, *Pyramidula pusilla*, *Pyramidula rupestris*, and *Pyramidula saxatilis*, which are largely restricted to central and western Europe, and *Pyramidula cephalonica*, *Pyramidula chorismenostoma*, *Pyramidula hierosolymitana*, *Pyramidula koska*, and *Pyramidula pygmaea*, which range from the Balkans east to the Caspian Sea. *Pyramidula cephalonica*, *Pyramidula chorismenostoma*, *Pyramidula jaenensis*, and *Pyramidula pygmaea* represent a genetically distinct group, whose constituent species possess largely non-overlapping ranges. Additional diversity might well exist, in particular among the eastern Mediterranean *Pyramidula* 'jaenensis' populations, given their location far from the main continuous distribution in the western Mediterranean. The very limited data from these that we have at our disposal might suggest existence of conchometric differences, but genetic data are incongruent and do not support them as monophyletic species-level clades. Also, based on a single long-branch DNA sequence and shell images illustrating odd traits, it seems likely that more taxa exist east of the Aegean (e.g. *Pyramidula* sp. A in Fig. 4; Supporting Information, Figs S1, S2). Given the frequency of Ponto-Caspian endemism in other groups (Copilaş-Ciocianu *et al.* 2022), such a result would not be surprising.

It is interesting that some shell features have seemingly diverged multiple times within the western Palearctic *Pyramidula* fauna, making any attempts to draw evolutionary relationships among species based on shell architecture erroneous. For instance, *Pyramidula rupestris* and *Pyramidula saxatilis* differed greatly in all recorded shell features, while representing closely related sister species. In contrast, the pairs *Pyramidula rupestris* vs. *Pyramidula jaenensis* and *Pyramidula saxatilis* vs. *Pyramidula pusilla* are

conchologically so similar that in some cases using a DNA barcode is required for their reliable identification. The same would probably be true for *Pyramidula rupestris* and *Pyramidula hierosolymitana* if they had a sympatric distribution. Nonetheless, none of these pairs shares the nearest common ancestor. Horsáková *et al.* (2022) suggested that the western European evolution of these traits might be a response to heat conservation. This might also be the case for the eastern species. For instance, the generation of a scalariform shell in *Pyramidula chorismenostoma* could be a physiological adaptation to increase heat dissipation within its very hot summer climate.

Correct interpretation of mitochondrial and nuclear signals Razkin *et al.* (2017) generated trees based on concatenated mtDNA and nDNA data (COI + 5.8S/ITS2/28S). Although done to generate a single 'robust' phylogeny (with the added benefit of increased support values simply owing to larger sample size), this action violates many of the assumptions underlying tree recovery algorithms. Not the least is the requirement that each data stream reflects the same basic topology. This should not be expected, given that mitochondrial and nuclear loci evolve under different constraints and can possess different histories. As a result, they often generate conflicting signals (Ballard and Whitlock 2004, Toews and Brelsford 2012). Because of the matrilineal-only nature of mtDNA heredity, its signal tends to be biased towards bottleneck history. This becomes especially important over the full-glacial to interglacial, when multiple separate refugia might exist (Taberlet *et al.* 1998, Haase *et al.* 2013, Korábek *et al.* 2018, 2023). In concatenated data, such lineages might swamp nDNA divergence, confusing the monophyletic nature of a species. Additionally, high land snail hybridization rates (Gould and Woodruff 1986) have frequently led to mitochondrial introgression (e.g. Nekola *et al.* 2015, 2018, 2025, Horsáková *et al.* 2019). Lastly, typically used mitochondrial markers (e.g. COI, CytB) evolve at a substantially faster rate than even the most active regions (ITS1 and ITS2) within the very highly conserved rRNA gene complex. As a result, mtDNA will easily make up $\geq 90\%$ of the variability in a concatenated mtDNA + ITS dataset. This overrides or obscures the phylogenetic signal provided by the more slowly evolving nuclear loci, regardless of whether these signals are congruent (e.g. Ballard and Whitlock 2004).

As a result, even when their signals appear congruent, separate presentation of mtDNA and nDNA trees offers transparency in terms of branch support, genetic distances, evolutionary history, and their potential discordances (e.g. Horsáková *et al.* 2019, Haase *et al.* 2021, Nekola *et al.* 2025, Kafimola *et al.* 2026). Given that the traits coding for observed differences between species are almost completely carried in the nuclear genome, and with both parents passing these along to their offspring, nuclear-derived topologies, if well resolved, will almost always provide a clearer picture of phylogeny than mtDNA or concatenated mtDNA + nDNA signal (e.g. Lorencová *et al.* 2021, Nekola *et al.* 2015, 2025, 2026).

Although explicit instances of mitochondrial introgression (e.g. the introgressed individual has mtDNA essentially identical to the current sequence found in the mother's species) have proved to be very rare in *Pyramidula* (a single case between *Pyramidula rupestris* and *Pyramidula jaenensis* out of all >300 individuals analysed), we

note that mtDNA and nDNA topological incongruence related to past introgression events is rather frequent. For example, although monophyletic in nDNA, *Pyramidula cephalonica* possesses two mitochondrial clades, which led to confusion in the concatenated trees of Razkin *et al.* (2017). One of these (their 'G6-B clade', labelled as '*P. cf. cephalonica*') represents individuals with mtDNA originating from *Pyramidula chorismenostoma*. The founder of this clade was almost certainly an offspring from a *Pyramidula chorismenostoma* mother and a *Pyramidula cephalonica* father. However, enough time has elapsed since this mating that the mtDNA sequence of its descendants has diverged from the current *Pyramidula chorismenostoma* mtDNA gene pool. *Pyramidula pygmaea* represents another example of mtDNA–nDNA discordance, possessing a single, highly supported nDNA clade but being distributed across three mtDNA clades. Such relicts of past introgression are not limited to *Pyramidula*, having also been seen in *Paralaoma* (Nekola *et al.* 2025), *Pupilla* (Nekola *et al.* 2015), and *Vertigo* (Nekola *et al.* 2018); in all these cases, mtDNA and nDNA concatenation would generate the false appearance of these as representing unique species-scale entities, rooted outside of the diagnostic clade of that species.

Mind the gap! Creating accurate phylogenies in *Pyramidula*

Especially within the *Pyramidula jaenensis* group, it is essential for indel information not to be discarded (as done by default in most phylogenetic analyses), but to be used to inform tree topology. Without ITS1 + ITS2 indel information (Razkin *et al.* 2016, 2017) not only did *Pyramidula jaenensis* represent a polytomy of three highly supported separate clades, but also the other species related to it (*Pyramidula cephalonica*, *Pyramidula chorismenostoma*, and *Pyramidula pygmaea*) were grouped together or nested within these clades, without support for them representing distinct species. This result aligns neither with conchology nor with biogeography and creates a conundrum as to whether they all represent separate species-level units or rather a single highly diverse entity. Use of indel information, however, resolves this situation and clearly shows that *Pyramidula jaenensis* s.s. is monophyletic, with it also being member of a highly supported clade consisting of three other distinct, monophyletic taxa. Each possesses unique shell features and biogeographical ranges. This result highlights that indels in the ITS1 + ITS2 region provide a strong and reliable phylogenetic signal rather than analytical noise. It became obvious when compared directly with a reconstruction based on the same alignment, but without indel information. Interestingly, the ITS1 + ITS2 tree architecture is largely concordant with the RADseq data (Razkin *et al.* 2016), with *Pyramidula pusilla* being the only exception. Although *Pyramidula pusilla* appeared as sister to the *Pyramidula rupestris* + *Pyramidula saxatilis* clade in the RADseq tree, our ITS1 + ITS2 phylogeny placed it as sister to the clade comprising all other analysed taxa, excluding *Pyramidula rupestris* + *Pyramidula saxatilis*, which formed a separate basal lineage. This topological difference might reflect the use of an explicit outgroup in our analysis, as opposed to the unrooted RADseq tree of Razkin *et al.* (2016).

Many studies have shown that indels represent a strong phylogenetic signal, often saturating more slowly than nucleotide substitutions and thus helping to resolve both deep and shallow evolutionary relationships (Simmons and Ochoterena 2000,

Redelings *et al.* 2024). Several approaches have been proposed for incorporating indels into phylogenetic inference, including indel coding schemes and probabilistic models of indel dynamics, and multiple studies have demonstrated that phylogenies are less resolved if indels are not included (Simmons *et al.* 2007, Ashkenazy *et al.* 2014, Redelings *et al.* 2024). Other studies also showed that their utility depends strongly on the evolutionary scale under consideration (Simmons and Ochoterena 2000, Dessimoz and Gil 2010), being informative particularly at shallower scales among closely related species (Ogden and Rosenberg 2007, Löytynoja and Goldman 2008). At this level, indels typically represent recent evolutionary changes that have not been obscured by multiple substitutions, and their homology can often be established confidently owing to the reliability of sequence alignments. Even a single indel may serve as a clear synapomorphy, supporting the monophyly of a given clade (e.g. Nekola *et al.* 2015). In contrast, at deeper phylogenetic levels the reliability of indels as phylogenetic markers declines (Löytynoja and Goldman 2008, Dessimoz and Gil 2010, Redelings *et al.* 2024). In these cases, the homology of indels becomes increasingly uncertain owing to high sequence divergence, and multiple independent indels (homoplasy) may occur at the same sites or are overwritten by subsequent evolutionary events. As a result, indels at deep evolutionary scales frequently carry more noise than signal. These conclusions are fully congruent with our results.

We therefore strongly recommend the use of indel information to inform phylogenetic reconstructions, especially in non-coding regions and for phylogenies across shallow evolutionary scales. Shared indels may carry stronger evidence for common ancestry than shared substitutions owing to their lower probability of homoplasy (Redelings *et al.* 2024). As is seen here and in other land snail genera (e.g. *Cochlicopa*, Armbruster and Bernhard 2000; *Euconulus*, Horsáková *et al.* 2019; *Pupilla*, Nekola *et al.* 2015; *Vallonia*, Korte and Armbruster 2003), this information represents an important source of phylogenetic information. What makes the approach by Simmons and Ochoterena (2000) so elegant is that it allows this information to be assessed quantitatively within the same analytical framework as base-pair substitutions, thereby allowing indel and base-pair information to be incorporated into a single tree rather than in multiple outputs (e.g. trees and haplotype tables). Although several land snail phylogenetic studies have treated gaps as an additional (fifth) character state (e.g. Schilthuizen *et al.* 2004, Mejía and Zuniga 2007), only Goulding *et al.* (2023) used formal simple indel coding implemented in FASTGAP v.1.2 (Borchsenius 2009). Because FASTGAP occasionally failed to generate valid binary matrices for our datasets, we developed an R-based implementation of the method of Simmons and Ochoterena (2000) to ensure full compatibility with BI and RAXML workflows (Supporting Information, Appendix S2).

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Natuurhistorisch Museum, Leiden) of *Patula rupestris* var. *dalmatina* Clessin, 1887 topotype (RMNH.MOL.245337). We are thankful also to E. Neubert, B. van der Bijl, E. Fara, O. Razkin, K. Urfer, S. Puissant, I. Gallmetzer, D. Wanke, M. Pallmann, I. Richtling, S. Hof, E. Gittenberger, and E. Schwabe for consultations, museum loans and/or help with imaging and measurements of type material originally used in our previous studies and repeatedly used also in the present study. Comments from two anonymous reviewers helped to improve the quality of this work.

AUTHOR CONTRIBUTIONS

All authors collected the samples. M.H. and S.K. conceived the idea. M.H. led data curation. S.K. ran all phylogenetic analyses and created R script for indel coding. M.H. and S.K. led the writing, with the support of J.C.N. and V.H. All authors reviewed and approved the final version of the manuscript.

SUPPLEMENTARY DATA

Supplementary data is available at *Zoological Journal of the Linnean Society* online.

CONFLICT OF INTEREST

None declared.

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DATA AVAILABILITY

All data generated during this study are included in this published article, its Supporting Information files and deposited in GenBank (PX924888–PX924903, PX921658–PX921674, and PX921676–PX921691).

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