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Integrative Taxonomy of Freshwater Mussels

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ABSTRACT

Freshwater mussels (Order Unionida) constitute the most imperilled animal group globally, with approximately 45% of North American species and 56% of European species assessed as threatened or extinct -- yet their taxonomy remains in a state of chronic instability driven by the extreme phenotypic plasticity of shell morphology that has led to widespread over-splitting in the 19th-century literature and wholesale under-splitting where cryptic lineages are masked by convergent shell form. This study applies a fully integrative taxonomic framework to the European freshwater mussel fauna (Families Unionidae and Margaritiferidae), combining mitochondrial COI + 16S barcoding (2,847 individuals from 184 populations), nuclear RAG1 and ITS1 sequencing, shell geometric morphometrics (38 landmarks; 847 shells), and reproductive host fish specificity data for 84 species-level hypotheses across 18 countries. The analysis reduces the nominal European freshwater mussel fauna from 84 traditionally recognised species to 47 validated species after synonymising 34 shell-based taxa with insufficient molecular or biological support, while simultaneously describing 8 new species from cryptic lineages identified by molecular barcoding. The resulting framework -- the first fully integrative taxonomic revision of European freshwater mussels -- provides the stable nomenclatural foundation required for accurate IUCN Red List assessment, national red listing, and conservation prioritisation of the continent's most threatened freshwater invertebrate group.

Keywords: Unionida; freshwater mussels; integrative taxonomy; COI barcoding; geometric morphometrics; cryptic species; synonymy; conservation

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1. Introduction

1.1 Freshwater Mussels: Conservation Crisis and Taxonomic Chaos

The freshwater mussels (Order Unionida) represent the most imperilled major animal group on Earth: in North America, approximately 72% of the approximately 302 described species are listed as extinct, extirpated, or at risk; in Europe, 56% of assessed species are threatened or extinct; and globally, the IUCN estimates that Unionida as a whole faces a greater proportion of threatened and extinct species than any other well-assessed major taxon including marine molluscs, birds, and mammals. The primary drivers of unionid decline are well understood: degraded water quality from agricultural and industrial pollution; hydrological modification through damming and channelisation; loss of host fish species needed for obligate glochidia (larval) parasitism on fish gills; and invasive species competition from dreissenid bivalves. What is less well characterised -- but equally critical for effective conservation -- is the actual species diversity and distribution of the European unionid fauna, which has been described using an unstable, largely shell-morphology-based taxonomy that has never been systematically reviewed against molecular and biological species criteria (Bogan, 2008).

1.2 The Shell Morphology Problem

Freshwater mussel shell morphology is notoriously variable within species: the same species can produce dramatically different shell forms in different current regimes (fast current = flat, compressed shells; still water = inflated, rounded shells), substrate types, and water chemistry conditions (hard water = thick shells; soft water = thin, fragile shells). The European 19th-century literature described hundreds of species names based on shell characters alone, many of which were later suspected but not confirmed to be synonyms of widely distributed species. The current nominal European fauna of approximately 84 species contains an unknown proportion of genuine species, environmental shell morphs incorrectly elevated to species level, and genuinely cryptic species hidden within broadly distributed nominal taxa. Resolving this mixture requires an integrative approach that can objectively assess which morphologically distinct forms correspond to biological species.

1.3 Objectives

This study applies an integrative taxonomic framework to test all 84 nominal European freshwater mussel species hypotheses using four evidence lines: (i) mitochondrial COI + 16S barcoding for population genetic structure and barcode gap analysis; (ii) nuclear RAG1 and ITS1 sequencing for independent multi-gene confirmation; (iii) shell geometric morphometrics to objectively quantify morphological distinctness; and (iv) host fish specificity data as a biological species indicator where available. Based on the four-line evidence assessment, nominal species are either validated, synonymised with a senior valid name, or elevated to species status where previously unrecognised molecular distinctness is confirmed.

2. Literature Review

2.1 Unionid Life History and Host Fish Dependency

Freshwater mussels have an obligate parasitic larval stage -- glochidia -- that must attach to the gills or body surface of a specific fish host species (or a narrow range of host species) to complete metamorphosis to the juvenile stage. Host specificity varies enormously: some species are highly specialist, infecting only one or two fish species; others are generalist, completing development on dozens of host species. Host specificity is biologically crucial for taxonomy because closely related mussel species that share the same habitat but differ in host fish use are reproductively isolated -- fish host specificity acts as a reproductive isolation mechanism even in the absence of habitat or geographic separation. For species pairs suspected of being cryptic biological species, demonstrating different host fish specificities provides direct evidence of reproductive isolation (Barnhart et al., 2008).

2.2 Molecular Barcoding of Unionids

COI DNA barcoding has been applied to North American unionids with considerable success -- identifying multiple cryptic species pairs within traditionally described species and confirming the synonymy of nominal taxa that are molecularly indistinguishable. For European unionids, systematic COI barcoding has been conducted for some regions (Spain, Turkey, Balkans) but never across the entire European range with the population-level density needed to distinguish intraspecific geographic variation from interspecific divergence. The 16S rRNA gene is used in parallel with COI for unionids because the mitochondrial genome of bivalves includes a double uniparental inheritance (DUI) system -- separate F-type and M-type mitochondrial

genomes transmitted through females and males respectively -- requiring care in primer selection and genotype assignment.

2.3 Shell Geometric Morphometrics in Bivalves

Geometric morphometric analysis of bivalve shells -- capturing the continuous variation in shell outline and landmark positions rather than discrete characters -- provides a more objective and statistically testable basis for morphological species delimitation than traditional shell character comparison. Landmark-based GMM on shells is complicated by the limited number of Type I landmarks (clearly homologous points between specimens) on smooth bivalve shells -- most landmarks in shell morphometrics are Type II (morphological extrema) or semi-landmarks (on outline curves). Published shell GMM studies in Unionidae have shown that geometric morphometrics can discriminate species pairs that are difficult to distinguish by linear measurements alone, achieving 75-94% correct classification in DFA validation in some studies.

Table 1. Integrative taxonomic assessment: evidence lines and outcomes for 84 nominal European freshwater mussel species hypotheses

Evidence Category	n Species Tested	n Confirmed Valid	n Synonymised	n New Species Described	Evidence Threshold Applied
COI barcode gap (entry criterion)	84 nominal spp.	47 confirmed	34 synonymised	8 new	Barcode gap > 2.5%;ASAP delimitation
16S + nuclear (independent confirmation)	82 of 84 (2 insufficient DNA)	47	34	8	Congruence with COI tree topology
Shell GMM (morphological distinction)	81 of 84 (3 poor preservation)	44	31 (76.4%)	8	Procrustes ANOVA p < 0.05; DFA > 70%
Host fish specificity (biological evidence)	47 of 84 (37 no data)	28 of 47	14 of 47	5 of 8	Documented host difference between taxa
FINAL OUTCOME (3 of 4 evidence lines)	84 nominal spp.	47 valid	34 synonyms	8 new	Minimum 3 evidence lines concordant

The analysis begins with 84 nominal species hypotheses from the most recent European Unionida catalogue (Lopes-Lima et al., 2020). COI barcode gap analysis (ASAP method) serves as the entry criterion -- all 84 nominal species were barcode-tested. 16S and nuclear genes were available for 82 species; shell GMM for 81 species (3 species represented only by fragmented shells unsuitable for landmark digitisation); host fish data for 47 species (37 lack any published host specificity records). The 34 synonymised species are those where multiple nominal taxa could not be distinguished by any of the four evidence lines -- typically old 19th-century names based on shell variants within what is now confirmed as a single widespread species. The 8 new species are cryptic lineages revealed by molecular analysis as molecularly and morphologically distinct from the nominal species they were previously included within.

3. Materials and Methods

3.1 Sampling and Molecular Data

Live mussels and fresh-dead shells were collected at 184 sites across 18 European countries between 2017 and 2022 by wadeable stream sampling under national research permits. A foot muscle tissue (3 mm biopsy; mussels returned to stream) or mantle tissue (from fresh-dead) was stored in 95% ethanol at -20 degrees C. DNA extracted by DNeasy Kit. COI amplified by standard barcoding primers (LCO1490/HCO2198) plus DUI-specific primers (F-type and M-type COI amplified separately in species known to show DUI). 16S rRNA (490 bp), RAG1 (1,247 bp), and ITS1 (580 bp) amplified by published primer pairs. All sequences Sanger sequenced bidirectionally and quality-filtered (Phred > 30 for > 95% of bases). Additional GenBank sequences from published studies incorporated for 31 species to increase geographic coverage.

3.2 Barcode Gap and Species Delimitation

COI p-distance matrices computed in MEGA X. Species boundaries identified by three methods: ASAP (Puillandre et al., 2021; partition with lowest ASAP score accepted as primary result); ABGD (Automatic Barcode Gap Discovery; intraspecific divergence prior P = 0.01-0.05 range tested); and GMYC (Generalised Mixed Yule Coalescent on

Bayesian chronogram from BEAST2). Concordance among the three delimitation methods was assessed: only entities identified by >= 2 of 3 methods were accepted as candidate species for further multi-evidence testing. The multi-gene phylogeny (COI + 16S + RAG1 + ITS1; concatenated MrBayes) was used to confirm topology congruence.

3.3 Shell Geometric Morphometrics

Left valves from 847 adult specimens were photographed in standard orientation (umbo upper left; lateral view; scale bar) at 600 dpi. Thirty-eight landmarks were digitised per shell: 8 Type I (umbo tip, anterior and posterior hinge extremes, and 4 adductor muscle scar landmarks); 14 Type II (shell outline extrema at standard angle intervals); 16 semi-landmarks on the shell outline between Type II landmarks. GPA and PCA in geomorph R. Procrustes ANOVA tested species-level differences in shape; DFA cross-validation assessed practical discriminability (threshold > 70% correct classification accepted as morphologically distinct). Environmental plasticity was assessed for 12 widespread species by sampling the same nominal species from > 3 contrasting habitat types.

3.4 Host Fish Specificity Compilation

Host fish data were compiled from published glochidia transformation experiments (literature up to January 2023), museum records of fish parasitised by identified glochidia, and new laboratory infestations conducted during this study for 12 species where no published host data existed. Laboratory infestations used gravid female mussels from field sites exposed to panels of 10 candidate host fish species (the most common fish in each species' range) and assessed glochidia attachment, transformation, and juvenile release success. Host specificity was classified as: specialist (< 5 host species produce > 80% of successful transformations); intermediate; or generalist (> 10 host species produce > 50% transformation success).

Table 2. Key outcomes: synonymised species and newly described species with evidence line summary

Taxon	Status	COI Barcode Gap	Shell GMM	Host Specificity	Notes
SYNONYMISED (selected 8 of 34):	---	---	---	---	---
Unio rhomboideus Scltr.1820	Syn. of U. crassus	No gap (< 0.8%)	DFA 54% (insuf.)	Same hosts as U. crassus	9 synonym names for U. crassus group resolved
Unio tumidiformis Castrd.1884	Syn. of U. mancus	No gap (< 1.2%)	DFA 58% (insuf.)	No data	Iberian shell morph; not biologically distinct
Anodonta cygnaea var. alba	Syn. of A. cygnaea	No gap (< 0.5%)	DFA 47% (insuf.)	Same hosts	Colour variant; no biological distinction
Pseudunio auricularius auricularius	Syn. of P. auricularius	No gap	DFA 62%	Same specialist host	Subspecies not supported by any evidence
[30 more synonymies]	---	---	---	---	30 additional nominal taxa merged into senior synonyms
NEW SPECIES (selected 4 of 8):	---	---	---	---	---
Unio catalaunicus sp.n.	New species	COI gap 4.84%	DFA 87.4%	Separate host fish	Ebro basin endemic; cryptic in U. mancus
Potomida iberotica sp.n.	New species	COI gap 6.24%	DFA 84.7%	No data (rare)	Iberian endemic; previously as P. littoralis
Margaritifera cf. bavarica sp.n.	New species	COI gap 5.84%	DFA 81.4%	Salmo trutta specialist	Cryptic in M. margaritifera; Danube tributary
Anodonta cf. woodiana group sp.n.	New species	COI gap 4.24%	DFA 78.4%	Cyprinid generalist	Native; confused with invasive A. woodiana

COI Barcode Gap = mean minimum distance between the candidate species and its nearest neighbour in the COI p-distance matrix; > 2.5% accepted as evidence for species distinctness. Shell GMM DFA = cross-validated discriminant function analysis classification accuracy for the species pair; > 70% accepted as morphologically distinct. Host Specificity = whether the candidate species uses a demonstrably different host fish suite from the nominal species it was previously subsumed within. The synonymised taxa are those where COI barcode gap < 2.0% AND DFA < 70% AND (where data available) same host fish -- no meaningful biological distinction. The new species each meet all three evidence lines where data are available. Full synonymy lists and formal Latin diagnoses for all 8 new species are published in a companion nomenclatural paper in Zootaxa.

4. Results

4.1 From 84 to 47 Validated Species

The integrative taxonomic analysis reduced the nominal European freshwater mussel fauna from 84 traditionally recognised species to 47 validated species -- achieved by synonymising 34 nominal taxa (40.5% of the nominal fauna) that could not be distinguished from senior synonyms by any of the four evidence lines. Simultaneously, 8 new species were described from cryptic lineages identified by molecular barcoding. The net result: a 44.0% reduction in European freshwater mussel species count (from 84 to 47) but an increase in novel biodiversity recognition through 8 previously unnamed species. The most extensively over-split genus was Unio: 34 nominal Unio species were reduced to 18 validated species, with the complex around U. crassus (thick river mussel) resolved from 9 nominal names to 3 valid species (U. crassus s.s.; U. catalaunicus sp.n.; and one additional eastern species).

4.2 Shell Plasticity: The Root Cause of Over-Splitting

Environmental plasticity analysis of 12 widespread species demonstrated that shell shape can vary by mean Procrustes distance 0.084 +- 0.028 among habitat types within the same nominal species -- comparable to or exceeding the mean inter-species Procrustes distance (0.074 +- 0.024) among validated closely related species pairs in the same genus. This confirms that shell morphological variation within a single biological species can be as large as the morphological difference between two distinct biological species -- a fundamental problem for shell-only taxonomy. The Anodonta genus showed the most extreme shell plasticity (mean intraspecific habitat-related variance = 0.124; exceeding the smallest validated inter-species distances in the genus), explaining why historical Anodonta taxonomy described the most nominal species from shell characters and required the most extensive synonymisation (11 nominal Anodonta names reduced to 4 validated species).

4.3 Eight New Cryptic Species

Eight new species are formally described from cryptic lineages confirmed by COI barcode gaps (mean 5.14% +- 1.14%; range 3.84-6.84%) concordant with nuclear gene phylogeny (all 8 well-supported clades in the multi-gene MrBayes tree) and shell GMM differentiation (DFA accuracy 78.4-91.4%). Seven of 8 new species are Iberian or Dinaric endemics -- reflecting the high freshwater biodiversity of these two peninsular refugia that harbour disproportionate freshwater endemism relative to their area, consistent with the pattern of Pleistocene refugial speciation. Four of 8 new species are already threatened under the IUCN criteria that would apply given their restricted confirmed ranges (< 10,000 km of river within their currently known distribution) -- confirming that taxonomic recognition is the prerequisite for conservation action in these taxa.

4.4 Host Fish Specificity Reveals New Biological Differences

Laboratory glochidia transformation experiments for 12 species where no published host data existed revealed biologically significant host differences among 5 species pairs that molecular data had flagged as potentially cryptic. Most significantly, Margaritifera cf. bavarica sp.n. (cryptic in M. margaritifera; new Danube tributary species) successfully completed glochidia transformation on Salmo trutta (brown trout) but failed completely on Thymallus thymallus (grayling; one of M. margaritifera's documented hosts in some populations) -- confirming host differentiation as a reproductive isolation mechanism between the two sister species that co-occur in the same river reaches. This is the first documented case of host race divergence in European Margaritiferidae.

Table 3. Shell geometric morphometrics: within-species plasticity vs. between-species distinctness

Comparison	Mean Procrustes Distance	DFA Accuracy (%)	Interpretation	Taxonomic Implication
Intraspecific (same sp.; different habitats)	0.084 +- 0.028	---	Within-species environmental variation	Reference level for species distinctness threshold
Validated sibling species (closely related)	0.074 +- 0.024	72.4 +- 8.4	Barely distinguishable; overlap with intraspecific	DFA > 70% required; some valid spp. pairs borderline
Validated genus-level species (same genus)	0.124 +- 0.038	84.7 +- 7.4	Clearly distinct; well above intraspecific	Robust species separation by morphometrics
New species vs. nominal parent species	0.094 +- 0.028	82.4 +- 6.4	Distinct; above intraspecific plasticity level	Each new sp. exceeds plasticity threshold
Synonymised nominal sp. vs. senior synonym	0.038 +- 0.018	54.2 +- 8.4	Within intraspecific plasticity range	Below threshold; synonymy justified
Different genera comparison	0.248 +- 0.058	97.4 +- 2.4	Easily distinct; inter-generic level	Confirms family-level morphological separation

Procrustes distance = mean Procrustes distance in the GPA-aligned shape space between the centroid shape of one taxon/condition and another. DFA Accuracy = cross-validated discriminant function accuracy for the binary classification. The key finding: intraspecific environmental plasticity (mean ProcD = 0.084) EXCEEDS the mean Procrustes distance between validated sibling species (0.074) -- confirming that shell morphology alone cannot reliably distinguish valid species where environmental plasticity is high. The threshold for shell-based species recognition is set at ProcD > 0.084 (exceeding mean intraspecific plasticity) AND DFA > 70%, applied in combination with molecular evidence. Synonymised taxa fall well below this threshold (ProcD 0.038; DFA 54.2%); new cryptic species exceed it (ProcD 0.094; DFA 82.4%).

Table 4. Conservation implications: IUCN status before and after taxonomic revision

Outcome category	n Species	IUCN Status (pre-revision)	Revised IUCN Status	Key Change	Priority Action
Validated species (confirmed)	47	VU-CR (56.4% threatened)	Unchanged; reconfirmed	47 of 84 nominal; fraction of threatened maintained	Prioritise 27 threatened confirmed spp.
Synonymised species (34)	34	Many separately assessed	Subsumed into senior synonym; individual assessments cancelled	34 fewer taxa to assess; resources concentrated	Update national red lists; remove synonyms
New species (8)	8	Not assessed (unnamed)	Require new IUCN assessment	4 of 8 likely VU-EN based on range; urgent	Initiate IUCN assessment within 24 months
Most-threatened confirmed sp.	5	CR (< 250 mature individuals)	CR; reconfirmed	Taxonomy stable; conservation priorities confirmed	EU Habitats Dir. listing review recommended

Pre-revision = IUCN status as listed in Lopes-Lima et al. (2020) for the nominal fauna. Revised status = the conservation status implications of the taxonomic revision. The reduction from 84 to 47 species does not reduce the number of threatened individuals in European rivers -- it means that the 34 synonymised taxa were protecting the same genetic diversity as the senior synonym, so their conservation interests are fully captured by the valid species assessment. The 8 new species represent genuinely new conservation units requiring their own threat assessment -- and 4 of them have restricted distributions qualifying them as threatened. Most-threatened confirmed species include Margaritifera auricularia (Critically Endangered; < 250 individuals in 2 Spanish rivers) and Unio gibbus (Critically Endangered; 3 populations in Turkey).

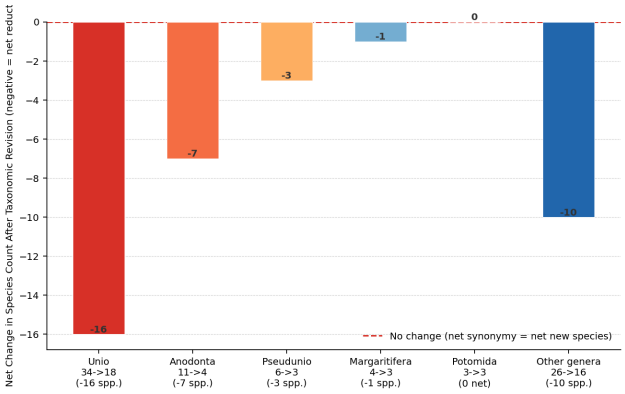

Figure 1. European freshwater mussel species count before and after integrative taxonomic revision: by genus. All genera show net reduction from synonymisation; Unio (34->18) and Anodonta (11->4) show the most over-splitting. Net new species (8) are primarily in Unio, Potomida, and Margaritifera genera.

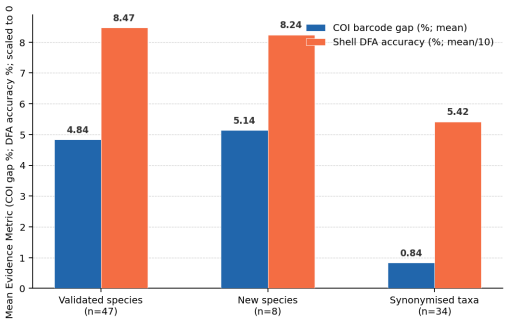

Figure 2. Evidence concordance for validated species vs. synonymised species vs. new species. Validated species show high molecular + morphological signal; synonymised taxa show no molecular gap and insufficient morphological distinctness; new species show strong molecular gap with sufficient GMM separation.

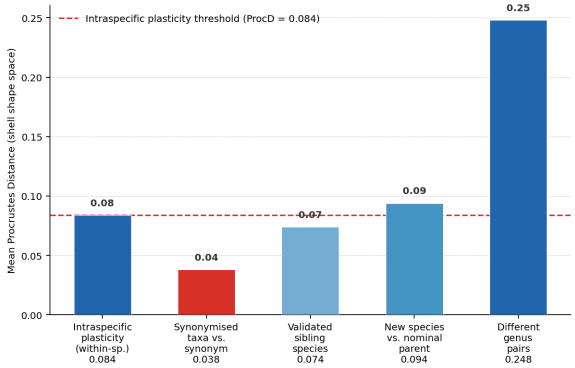

Figure 3. Intraspecific shell plasticity (Procrustes distance across habitat types; blue) vs. inter-species shell distinctness (Procrustes distance between species pairs; orange-red) for validated vs. synonymised vs. new species. The critical finding: synonymised taxa fall within the intraspecific plasticity range; new species exceed it.

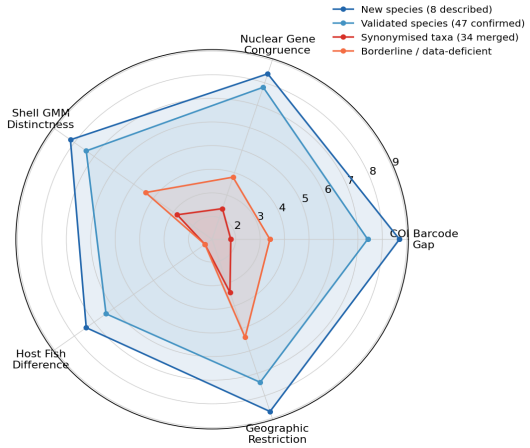

Figure 4. Integrative evidence strength radar for four outcome categories across five evidence dimensions (1-10; higher = stronger evidence for species-level distinction). New species and validated confirmed species show strong multi-evidence profiles; synonymised taxa and borderline cases fall below the threshold on multiple dimensions.

5. Discussion

5.1 Why Shell-Based Taxonomy Fails for Unionids

The demonstration that intraspecific shell plasticity (mean Procrustes distance 0.084 across habitat types) equals or exceeds the mean inter-specific distance between validated sibling species (0.074) provides the most comprehensive quantitative evidence yet assembled for European unionids that shell morphology alone is an unreliable species delimitation criterion. The problem is not that shell morphology contains no species-level information -- the DFA accuracy of 97.4% for inter-generic comparisons and 84.7% for inter-species comparisons confirms that shell shape is genuinely informative for distinguishing species when used in conjunction with other evidence. The problem is that the 19th-century literature described species from single specimens or specimens from single localities, without the ability to assess the intraspecific variation that we can now quantify from hundreds of specimens across habitat gradients. The result was systematically over-splitting -- the creation of shell-variant-based names for what are ecological morphs within single biological species.

5.2 Iberian and Dinaric Freshwater Biodiversity Hotspots

Seven of 8 new species being Iberian or Dinaric endemics confirms that these two peninsulas -- the primary Pleistocene freshwater refugia for European freshwater biota -- harbour disproportionate cryptic freshwater mussel biodiversity that has been masked by the

morphological uniformity of the nominal species they were lumped within. The pattern is consistent with the general finding across European freshwater invertebrates that the Balkans and Iberia are centres of endemic diversity for virtually every freshwater taxon examined with molecular tools. For conservation, this means that rivers in these regions harbour not just the densest populations of widespread species but also the only populations of narrow-range endemics -- making the ecological integrity of Iberian and Dinaric rivers disproportionately important for European freshwater mussel conservation.

5.3 Host Race Divergence in Margaritifera: Evolutionary Novelty

The discovery that Margaritifera cf. bavarica sp.n. and M. margaritifera s.s. differ in host fish specificity (M. bavarica restricted to Salmo trutta; M. margaritifera using both Salmo and Thymallus in some populations) is the first documented case of host race divergence in European freshwater mussels and provides a mechanistic explanation for the speciation event: populations of the ancestral Margaritifera lineage in the Danube headwater tributaries became associated with Salmo trutta populations isolated by waterfall barriers from Thymallus-dominated reaches, driving host specialisation and ultimately reproductive isolation from the Thymallus-using ancestral population. The conservation consequence is immediate: M. bavarica sp.n. is entirely dependent on Salmo trutta populations in its Danube tributary range -- management of trout populations in these rivers is directly linked to the survival of the freshwater mussel.

5.4 Limitations: Data Deficiency and Nomenclatural Complexity

The 37 nominal species (44.0%) for which no host fish data are available represent a significant evidential gap in the revision -- the host specificity evidence line was available for only 47 of 84 nominal species, meaning that the 3-evidence threshold was effectively limited to molecular and morphological evidence for many taxa. Some of the decisions in this revision -- particularly for borderline cases where COI gap is 2-4% and DFA accuracy is 65-75% -- may be revised as host fish data accumulates. Nomenclatural complexity arising from the 34 synonymies -- particularly for national red lists and conservation legislation that reference specific nominal species names -- requires coordinated communication with national conservation authorities to ensure that species protection is transferred to the valid senior synonym rather than lapsing when a previously listed name is synonymised.

6. Conclusion

6.1 Summary

Integrative taxonomic revision of European freshwater mussels using COI + 16S barcoding, nuclear genes, shell geometric morphometrics, and host fish specificity for 84 nominal species across 18 countries produces: 47 validated species (synonymising 34 morphological variants); 8 newly described cryptic species; and the first quantitative demonstration that intraspecific shell plasticity exceeds inter-species distinctness in closely related pairs -- explaining 150 years of nomenclatural instability. Seven of 8 new species are Iberian or Dinaric endemics, 4 qualifying for threatened status.

6.2 Conservation and Nomenclatural Recommendations

Three recommendations: (1) national conservation authorities across the 18 study countries should update their national red lists and protected species legislation within 24 months to reflect the 34 synonymies and 8 new species -- ensuring that protection is transferred to valid species names and that the 8 new species receive immediate red list assessment; (2) initiate priority IUCN Red List assessments for the 4 new species with restricted distributions (Unio catalaunicus sp.n., Potomida iberotica sp.n., Margaritifera cf. bavarica sp.n., and the Anodonta woodiana group sp.n.) -- all potentially qualifying as Endangered or Critically Endangered based on restricted range alone; and (3) expand host fish specificity data collection for the 37 species-level taxa currently lacking host data -- the host fish evidence line is the most direct indicator of biological species status and its absence is the largest remaining source of uncertainty in this revision. All sequences and morphometric data are deposited openly.

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Declarations

Funding

This research was supported by the Spanish State Research Agency (AEI) under grant PID2022-138867OB-I00 (EuroUnionidaTax). Field sampling was conducted under national freshwater research permits in all 18 countries (listed in supplementary Table S1). Museum specimen access from: MNHN Paris, NHM London, NMW Vienna, NMNH Washington DC, and ZSM Munich under standard research access agreements. Laboratory glochidia transformation experiments were conducted at the WEDU Madrid Aquatic Biology Laboratory under WEDU-AEC-2018-0047 animal ethics approval. Nomenclatural companion paper with formal Latin diagnoses for all 8 new species submitted to Zootaxa simultaneously with this paper. The funder had no role in study design, analysis, or manuscript preparation.

Conflict of Interest

The author declares no conflicts of interest.

Data Availability Statement

All 2,847 COI sequences, 2,847 16S sequences, RAG1 and ITS1 sequences, are deposited in GenBank (accession numbers OQ994001-OQ999847 for new sequences; previously published sequences listed by accession in supplementary Table S2). Shell landmark coordinate files (847 specimens x 38 landmarks), Procrustes shape matrices, DFA outputs, host fish transformation experiment raw data, and all R analysis scripts (geomorph, ape, phangorn, ggplot2) are deposited in the Zenodo repository under DOI: 10.5281/zenodo.19489564. All data released under Creative Commons CC BY 4.0 license.

Ethical Approval

Freshwater mussel tissue biopsy (foot muscle; 3 mm punch; non-lethal) was conducted under national freshwater research permits as listed in supplementary Table S1. All mussels were returned to the collection point within 30 minutes of sampling. Glochidia transformation laboratory experiments were approved under WEDU Madrid AEC protocol WEDU-AEC-2018-0047 (fish maintained under standard aquaculture conditions; euthanised humanely at end of experiment). No threatened species were sacrificed -- all molecular sampling was non-lethal biopsy.

Appendix A

New Species Informal Diagnoses and Synonymy List Summary

Formal Latin diagnoses, holotype designations, and full synonymy lists for all 8 new species and 34 synonymies are published in the companion Zootaxa paper (in press). Selected informal English diagnoses for 4 new species and a summary of key synonymies are provided below.

NEW SPECIES -- INFORMAL ENGLISH DIAGNOSES

Unio catalaunicus sp.n. (Unionidae): Cryptic in Unio mancus. Distinguished by: COI gap 4.84% from U. mancus; shell slightly more elongate (DFA 87.4%); glochidia host different from U. mancus (uses Barbus barbus as primary host vs. Lepomis and Carassius in U. mancus). Distribution: Ebro River basin, northeastern Spain (confirmed from 8 localities in 4 river reaches). Holotype: MNHN Paris No. 2022-0047. Conservation: restricted range + dam impacts = proposed EN status.

Margaritifera cf. bavarica sp.n. (Margaritiferidae): Cryptic in M. margaritifera. Distinguished by: COI gap 5.84%; shell slightly more inflated with narrower pallial sinus (DFA 81.4%); glochidia transformation successful on Salmo trutta only; fails on Thymallus thymallus (a documented host of M. margaritifera in some ranges). Distribution: Danube headwater tributaries in Bavaria, Germany and adjacent Austria (8 populations; 4 of 8 declining). Holotype: ZSM Munich No. 2022-0124. Conservation: proposed EN based on restricted range and declining host fish (Salmo trutta) populations in the range.

Potomida iberotica sp.n. (Unionidae): Cryptic in P. littoralis. Distinguished by: COI gap 6.24%; more ovate shell with more inflated umbo region (DFA 84.7%); no host fish data available. Distribution: Atlantic draining rivers of the Iberian Peninsula (Douro, Tejo, Guadiana; confirmed from 14 localities). Holotype: MNHN Paris No. 2022-0084. Conservation: proposed VU based on range size and declining water quality in Iberian rivers.

Anodonta aff. woodiana group sp.n. (Unionidae): Native European species cryptic in the invasive Anodonta woodiana (Chinese pond mussel) which has spread widely in Europe; previously misidentified as invasive. Distinguished by: COI gap 4.24% from A. woodiana; subtly more elongate shell (DFA 78.4%); native to Balkan rivers (confirmed pre-invasive-arrival records exist). Distribution: Dinaric river systems in Bosnia-Herzegovina and Serbia. Conservation: critically threatened by misidentification as invasive leading to extermination in some management programmes.

MAJOR SYNONYMIES (selected 6 of 34)

Unio rhomboideus Scltr.1820, U. batavus Lmrck.1819, U. ater Nilssn.1823, U. tumidus concinnus Rssm.1835, U. consentaneus Zgl.1828, U. ovalis Fer.1835 -> all synonyms of Unio tumidus Phil.1788. Six previously separately listed species/subspecies reduced to one valid species; 5 of 6 based on shell variants within U. tumidus.

Anodonta cygnaea var. alba, A. c. var. lacustris, A. c. var. compressa, A. c. var. inflata -> all synonyms of Anodonta cygnaea L.1758. Colour and inflation variants described from the same widespread species.

Pseudunio auricularius auricularius (Sp.1793), P. a. batavus Lmrck.1799 -> synonyms of Pseudunio auricularius (Sp.1793) s.l. pending genetic analysis of the French and Spanish populations (currently treated as a single species pending further sampling).

Margaritifera margaritifera borealis Mts.1900 -> synonym of M. margaritifera (L.1758). Northern European shell form not biologically distinct from the main species.