1 Phylogeny of European Anodontini (Bivalvia: Unionidae) with re-description of *Anodonta exulcerata*
2 Porro 1838
3
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7 Running title: Phylogeny of European Anodontini
8 Word count for main text (excluding refs, tables and figures) Total: 6799

ABSTRACT

Freshwater bivalves are highly threatened and globally declining due to multiple anthropogenic impacts, making them important conservation targets. Because conservation policies and actions generally occur at the species level, accurate species identification and delimitation is critical. A recent phylogenetic study of Italian mussel populations revalidated one *Anodonta* species bringing the number of known European Anodontini species from 3 to 4. The current study contributes to the clarification of the taxonomy and systematics of European Anodontini, using a combination of molecular, morphological and anatomical data, and constructs phylogenies based on complete mitogenomes. A re-description of *A. exulcerata* Porro, 1838 and a comparative analysis of the morphological and anatomical characters with respect to the other two species of *Anodonta* present in the area are provided. No reliable diagnostic character have emerged from the comparative analysis of the morphometric characters of 109 specimens from 16 sites across the Italian peninsula. In fact, the discriminant analysis resulted in a greater probability of correct assignment to the site of origin than to the species. This confirms the difficulties of an uncritical application of visual characters for the delimitation of species, especially for the Anodontinae.

Keywords: conservation - freshwater mussels – mitogenome - morphological plasticity - revalidated species.

35 INTRODUCTION

36 Conservation of freshwater mussels (Bivalvia: Unionida) is essential to maintain the important
37 ecosystem functions and services they provide (Bogan 1993; Lopes Lima et al., 2017a; Vaughn,
38 2018). Similar to other freshwater taxonomic groups, these bivalves are highly threatened and
39 globally declining due to multiple anthropogenic impacts (e.g. Lopes-Lima et al., 2018, Rodriguez et
40 al., 2019), making them important for conservation. However, for many freshwater mussel species,
41 effective conservation measures are hindered by our incomplete understanding of biological species
42 delineations and/or current inability to identify them correctly by morphology (Prié et al., 2012). This
43 is due to the exceptionally high phenotypic plasticity within freshwater mussel species and
44 morphological convergences between species, reflecting an adaptive phenotypic response to habitat
45 factors (Zieritz & Aldridge, 2009; Zieritz et al, 2010; Hornbach et al., 2010; Reis et al., 2013; Guarneri
46 et al., 2014).

47 Taxonomic misidentifications are particularly common for species within the tribe Anodontini, which
48 generally lack diagnostic hinge teeth (Lopes-Lima et al, 2017a). As a result, the Anodontini include
49 some of the most over-described species on the planet (e.g. at least 549 synonyms are available for
50 *Anodonta cygnea,* Linnaeus, 1758; Graf & Cummings, 2019), whilst morphologically cryptic species
51 have recently been revealed through molecular data in other genera of this tribe (Smith et al., 2018).
52 The Anodontini *sensu* Froufe et al. (2019) have a Holarctic distribution from western North America
53 to Europe, parts of northern Africa and the Middle East until Transbaikalia (note that Pfeiffer et al
54 (2019) also include the Cristariini *sensu* Froufe et al. (2019) into the Anodontini, with an East
55 Asian/western North American distribution, but since this clade is consistently separated, here we
56 adopt Lopes-Lima et al.'s (2017b) narrower definition).

57 With increasing molecular sequence data and taxon sampling, the phylogeny and taxonomy of the
58 Anodontini has been considerably revised over the past few years but is still unresolved (Lopes-Lima
59 et al, 2017b; Williams et al., 2017; Smith et al., 2018; Pfeiffer et al., 2019). Current molecular
60 evidence places at least 12 genera in this tribe, and an additional two genera (*Pegias* Simpson, 1900

from North America and *Simpsonella* Cockerell, 1903 from the Philippines) are usually regarded as Anodontini despite the lack of molecular evidence (Lopes-Lima et al., 2017b). Ten of these genera (*Alasmidonta* Say, 1818, *Anodontoides* Simpson in F.C. Baker, 1898, *Arcidens* Simpson, 1900, *Lasmigona* Rafinesque, 1831, *Pseudodontoideus* Frierson, 1927, *Pyganodon* Crosse & Fischer, 1894, *Simpsonaias* Say, 1825, *Strophitus* Rafinesque, 1820, *Utterbackia* F.C. Baker, 1927, and *Utterbackiana* Frierson, 1927) are confined to the east coast basins of North America, one (*Pseudanodonta* Bourguignat, 1877) is confined to the Palearctic, and one (*Anodonta* Lamarck, 1799) is present in the west coast basins of North America, Palearctic, northern Africa and the Middle East. This disjunct distribution of the *Anodonta*-clade is difficult to explain from a biogeographical perspective and may indicate insufficient character sampling of phylogenies to date, which adopted a two-marker approach (Lopes-Lima et al., 2017b). Next-generation sequencing technology has enabled fast and cost-effective generation of multilocus (phylogenomic) sequence data (McCormack, 2013), but whilst phylogenomics have successfully resolved deep-nodes of freshwater mussel phylogenies (Froufe et al. 2019; Lopes-Lima et al. 2017b; Pfeiffer et al. 2019), this tool has yet to be applied for resolving relationships at the tribe level.

In Europe, the total number of Anodontini species is still unknown and therefore their phylogenetic relationship uncertain (Lopes-Lima et al., 2017a). Until recently, three Anodontini species were recognised from Europe, i.e, *Anodonta anatina* (Linnaeus, 1758), *Anodonta cygnea* (Linnaeus, 1758) and *Pseudanodonta complanata* (Rossmässler, 1835), all with a widespread distribution across the continent which, in the case of *A. anatina*, extends to Transbaikalia (Zieritz et al., 2018). Building on preliminary work by Nagel et al. (1996) and Froufe et al. (2014), a fourth species, *Anodonta exulcerata* Porro, 1838 was recently resurrected by Froufe et al. (2017) based on high genetic distance (>8% in COI sequence) from its sister species *A. cygnea*. *A. exulcerata* is restricted to the Adriatic river basins and delimited by the Italian Alps in the north, Apennine Mountains in the west and Dinaric Alps in the east (Froufe et al., 2017). In addition, the authors confirmed the presence of two

genetically distinct *A. anatina* clades, one restricted to the Ebro and Adriatic basins, and one distributed across Europe and parts of Asia except the Iberian Peninsula.

Froufe et al.'s (2017) molecular re-assessment finally resolved uncertainties regarding the identity and number of *Anodonta* species present in Italy (i.e. *A. anatina*, *A. cygnea* and *A. exulcerata*), which have resulted in several incongruences in the scientific literature as well as between national and regional species inventories (Bon & Mezzavilla, 2000; Bodon et al., 2005; Cosolo, 2008; Autorità di Bacino dei fiumi dell'Alto Adriatico, 2010; Boggero et al., 2016). However, conservation work on the ground, including field surveys, requires the ability to identify species unequivocally through distinguishing morphological (ideally conchological) characters that can be quickly assessed in the field. Unfortunately, no such distinguishing characters are currently known for *A. exulcerata*, which exhibits strong conchological similarity to both *A. anatina* and *A. cygnea*.

The phylogenies in Froufe et al. (2017) did not include any member of the *Anodonta* genus from western North America nor the remaining recognized European Anodontini (*P. complanata*) and was therefore limited to reveal the phylogenetic relationships of the European Anodontini. In this context, the aims of this study are to a) re-assess the species diversity, phylogenetic relationships and systematics of European Anodontini using molecular data; (b) unravel the global Anodontini phylogeny using phylogenomics; and (c) identify morphometric, morphological and/or anatomical characters for distinguishing Italian *Anodonta* spp. in the field.

MATERIAL AND METHODS

SAMPLE COLLECTION

Anodonta specimens (N= 109) were collected from 16 sites across the Italian Peninsula river basins during 2014-2016 (Table 1). A small biopsy from the foot was collected in the field (following Naimo et al., 1998) and placed directly into 99% ethanol for subsequent molecular analysis. Whole specimens were also collected and transported alive to the laboratory for anatomical observations.

111 All individuals have been barcoded previously for molecular species identification (using COI)
112 published in Froufe et al. (2017).
113
114 DNA EXTRACTIONS AND SEQUENCING
115 Genomic DNA was extracted from the tissue samples, using a standard high-salt protocol (Sambrook
116 et al., 1989). F-type mitogenome sequencing and assemblage followed Gan et al. (2014), whilst
117 annotations were performed following Fonseca et al. (2016). All mitogenomes were deposited in the
118 GenBank database under the accession numbers (submitted; Supplementary Table S1).
119 Two datasets were constructed: one for COI and another for the mitogenomes. The COI dataset
120 included all European Anodontini sequences available in GenBank, with *Sinanodonta woodiana*
121 (Lea, 1834) and *Anemina arcaeformis* (Heude, 1877) as outgroups (Supplementary Table S2). The
122 mitogenome dataset included all the Anodontini specimens with sequences available on GenBank,
123 with *Pseudunio marocanus* (Pallary, 1918) as outgroup, plus the seven newly sequenced species:
124 *Unio elongatulus* (Pfeiffer, 1825), *Unio mancus* (Lamarck, 1819), Northwest Iberian Lineage
125 *Anodonta anatina*; *Anodonta cygnea*; *Anodonta exulcerata*; *Anodonta nuttalliana* (Lea, 1838);
126 *Pseudanodonta complanata* and *Pseudunio auricularius* (Spengler, 1793) (Supplementary Table S1).
127 For each dataset, sequences of additional specimens were downloaded from GenBank (details in
128 Supplementary Table S1).
129 The COI data set was aligned with the MAFFT multiple sequence alignment algorithm (Katoh &
130 Standley, 2013,) and the final alignment was then restricted to its unique haplotypes, using DnaSP
131 v5.1.0.1 (Librado & Rozas, 2009).
132 Mitogenomes were visualized using GenomeVx (Conant & Wolf, 2008). Sequences of all mtDNA
133 protein-coding genes (PCG), except ATP8 and the gender-specific open reading frames (H-ORF and
134 F-ORF; Breton et al., 2011), were used in the phylogenetic analyses. The sequences of each gene
135 were aligned using MAFFT software (version 7.304, Katoh & Standley, 2013) and trimmed with
136 GUIDANCE2 (Sela et al., 2015) following Froufe et al. (2016c). The gene alignments were then

concatenated with 12,959 nucleotides (nt). PartitionFinder v. 2.1.1 software (Lanfear et al., 2016) was used to retrieve the optimal partitioning scheme under the greedy algorithm with proportional branch lengths across partitions. Finally, the best substitution models of DNA evolution for each partition were selected under BIC ranking method (Schwarz, 1978) with both the codon positions of the protein-coding genes and each rRNA being defined as the initial data blocks for the partitioning schemes search. MEGA v7 (Kumar et al., 2016) was used to estimate the whole mitogenome divergence.

PHYLOGENETIC ANALYSES

Maximum Likelihood (ML) and Bayesian Inference (BI) methods were used for all the phylogenetic analyses. ML analyses were performed using RAxML (v. 8.2.10, Stamatakis, 2014) with 100 rapid bootstrap replicates and 20 ML searches. The BI was applied using MrBayes v. 3.2.6 (Ronquist et al., 2012) with two independent runs (10^7 generations with a sampling frequency of 1 tree for every 100 generations), each with four chains (3 hot and 1 cold). All runs reached convergence (average standard deviation of split frequencies below 0.01). The posterior distribution of trees was summarized in a 50% majority rule consensus tree (burn-in of 25%).

For the COI dataset, the models used for BI were: cod 1: K80+I; cod 2: F81; cod 3: HKY+G; while the GTR+G was employed for the ML analyses. As for the Mitogenome data set, models used included GTR+I+G, HKY+G, SYM+I+G, and GTR+G, for the ML analyses.

MOLECULAR BASED SPECIES DELINEATION METHODS

Three distinct molecular methods were applied to determine the number of Molecular Operational Taxonomic Units (MOTUs). For the first, i.e. the BIN system implemented in BOLD (Ratnasingham & Hebert, 2013), the COI dataset was analyzed with the Cluster Sequences tool implemented in BOLD 4 (http://v4.boldsystems.org) (Ratnasingham & Hebert, 2013). The second species delineation method used the 95% statistical parsimony connection limit in TCS 1.21 (Clement et al., 2000). For

the third, i.e. bPTP (Zhang et al., 2013), the BI phylogenies obtained before were used for the input tree. Species delimitation analysis was performed using the python code (available at: www.exelixis-lab.org/software.htm, Zhang et al., 2013) with 1 x 10^6 iterations of MCMC and 25% burn-in.

COMPARATIVE ANATOMY AND CONCHOLOGY

Morphological analyses of the specimens collected during this study were carried out on shells and living animals. Living specimens were kept in aquaria to observe the external morphology of incurrent, excurrent [anal] and supra-anal apertures. The live specimens were then sacrificed for more comprehensive anatomical and morphological analyses. These included a visual examination of each specimen, noting the shell shape, umbo sculpture, and the soft body anatomy (only whole specimens). Digital callipers were used to measure shell dimensions to the nearest 0.1 mm. Shell length was measured as the maximum anterior-posterior dimension of the shell parallel to the hinge ligament. Shell height was the maximum dorso-ventral dimension taken perpendicular to the length. Shell width was the maximum lateral dimension, again taken perpendicular to the length. To standardize the variables for size, we calculated the height/length (H/L), width/length (W/L), and width/height (W/H) ratios for all specimens. Since the index of convexity (W/H), which is often used to discriminate between anodontine species, is not independent of shell elongation, it was standardized over length to obtain an independent width-ratio [(W/H)/L]. The angle between dorsal margin and posterior margin was measured to the nearest five degrees with a goniometer. The normal distribution was verified for each parameter using Shapiro-Wilk test, optimized for small sample sizes ($N<50$). Analysis of Variance (ANOVA) with a Tukey's test post-hoc comparison on the angle and the H/L, W/L, W/H and (W/H)/L ratios were performed using StatPlus Pro (6.1.7.5). Discriminant Analysis (DA) was then employed to assess how accurately individual shells had been assigned to the genetically identified species.

SHELL MORPHOMETRY

For a geometric-morphometric analysis of inter- and intraspecific variation in shell morphology of the *Anodonta* species native to Italy, we used Fourier shape analysis, as developed and explained by Crampton & Haines (1996). This method decomposes xy-coordinates of a shell outline into a number of harmonics, each of which is in turn explained by two Fourier coefficients. We analysed 109 specimens collected by the authors (Table 1) and 29 specimens collected by Nagel et al. (1996). The xy-coordinates of the sagittal shell outline of each specimen were obtained from digital photographs using the program IMAGEJ (Rasband, 2008) and subjected to fast Fourier transformation using the program HANGLE, applying a minimum smoothing normalization of 2 to eliminate high-frequency pixel noise. Preliminary analysis indicated that the first 10 harmonics described the outlines with sufficiently high precision. Discarding of the first harmonic, which does not contain any shape information, resulted in a set of 18 Fourier coefficients per individual. After rotating outlines to maximum overlap by program HTREE, principal component analysis (PCA) was performed on the 18 Fourier coefficients using program PAST (Hammer & Harper, 2006). The number of principal components to be retained was determined using the broken stick model of the scree plot. Synthetic outlines of extreme and average shell shapes were drawn using program HCURVE as explained in Crampton & Haines (1996).

To test for statistically significant differences in sagittal shell shape between species, separate ANOVA were run on each of the significant principal components, fitting species as a factor with three levels. Tukey's posthoc test was performed to identify significant differences between each population pair. Finally, we assessed the rate of accurate species identification based on Fourier Shape Analysis using DA on the set of 18 Fourier coefficients. Statistical analyses were performed in R.3.1.1.

215 RESULTS

216 MOLECULAR PHYLOGENY AND SPECIES DELINEATION

217 The final haplotype COI alignment was 567 nucleotides long and included 143 haplotypes (including
218 two as outgroup). The best ML and BI trees retrieved had similar topologies, thus only the BI is
219 shown in Figure 1. As previously reported (Froufe et al., 2017), *A. exulcerata* clusters with *A. cygnea*
220 in a well-supported clade. All the *A. anatina* COI clades are grouped in another well-supported clade,
221 while the phylogenies failed to cluster *P. complanata* with support (Figure 1**).** All the three species
222 delineation methods applied retrieved the same results, i.e., identifying the following MOTUs: *A.*
223 *cygnea, A, exulcerata*, *P. complanata*, and four within *A. anatina* (Fig. 1).

224 The length of the newly sequenced mitogenomes is within the expected F-type range of the freshwater
225 mussels and all present the same previously described gene order, UF1 (Lopes-Lima et al., 2017c).
226 Their main characteristics, *i.e.* size, gene composition and order, morphological features of the
227 lectotype and paratype (Figs 1S and 2S), of representative specimens (Fig. 3S), and of the specimens
228 examined for this study are shown in Supplementary Figures 4S-9S and in Table 3S. The best ML
229 and BI trees retrieved had similar topologies, with the exception of the phylogenetic relationship of
230 the *Lanceolaria* sp. clade. The phylogenomic tree shows the monophyly of Anodontini and its sister
231 status to the Cristariini clade (Fig. 2). The genus *Anodonta* is not monophyletic due to the paraphyletic
232 positions of *A. anatina*, *P. complanata*, *A. nuttalliana* and *A. cygnea*+*A. exulcerata* clades (Fig. 2).
233 As expected, the phylogenomics also joins *A. cygnea* with *A. exulcerata* with high support. *P*-distance
234 between these two species was 10% for the whole mitogenome (Supplementary Table 4S).

235

236 COMPARATIVE ANATOMY AND CONCHOLOGY

237 SOFT TISSUES MORPHOLOGY

238 The inner and outer gills have the same form and size across the three taxa (i.e., *A. cygnea*, *A.*
239 *exulcerata*, and *A. anatina*). The form and size of labial palps are similar in the three species. Main
240 interspecific differences are only found in the papillae morphology of the incurrent aperture and in

241 the pigmentation of the mantle surface in the excurrent aperture (Fig. 4S), characters that were
242 proposed for reliably separating other mussel species (Glöer & Meier-Brook, 1994; Sayenko, 2007;
243 Sayenko et al., 2009). In the present study, *A. anatina* can be reliably discriminated from other
244 *Anodonta* species by internal morphology only in living specimens through its apertural anatomy.
245 Compared to other *Anodonta* species, *A. anatina* exhibits a longer excurrent aperture, a greater
246 protrusion of papillae from the edge of the shell and a brownish colour of mantle edge and papillae
247 (Fig. 4S). In contrast, the apertural anatomy of *A. exulcerata* and *A. cygnea* is similar and
248 characterised by a short excurrent aperture without marginal and papillae coloration. Living or freshly
249 dissected *A. exulcerata* and *A. cygnea* specimens present a clear irregular tan band at the insertion of
250 papillae (Fig. 4S and 5S). The papillae showed a distinct pattern being arranged in 4-5 series in *A.*
251 *anatina* (4 series in 81 and 5 in 19% of the specimens), only 2-3 series in *A. cygnea* (2 series in 27%
252 and 3 in 73 % of the specimens) and in *A. exulcerata* (2 series in 42 % and 3 in 58% of the specimens).
253 Another discriminant character is foot and mantle colour, which has been shown to be useful to
254 differentiate *A. cygnea* from *A. anatina* (Mordan & Woodward, 1990; Mezhzherin et al., 2014).
255 Indeed, *A. anatina* and *A. exulcerata* present a light brown/creamy-white colour, whereas *A. cygnea*
256 is generally bright-orange coloured (Fig. 6S).
257
258 UMBONAL SCULPTURE
259 *Anodonta cygnea* umbo sculpture consists of thin concentric lines while *A. anatina* presents wavy
260 rugae (Fig. 6S). *Anodonta exulcerata* is more similar to *A. anatina* than to *A. cygnea* (Fig. 6S),
261 generally presenting wavy rugae. Rugae in *A. exulcerata* and especially in *A. anatina* are thicker and
262 more widely spaced when compared to *A. cygnea*.
263
264 SHELL MORPHOMETRY
265 LINEAR MORPHOMETRIC ANALYSIS

Analyses of morphometric shell indexes H/L, W/L, W/H showed substantial intraspecific variability, with a wide overlap between the three species. The only two indexes with discriminating value are the angle between dorsal and posterior margin, and the convexity index standardized by length. Both the angle (ANOVA: $F=10.9122$, $df=2$, $p<0.001$) and the standardized convexity index (ANOVA: $F=30.382$, $df=2$, $p<0.001$) were significantly different among species. While the standardized convexity index was significantly different among the three species (Tukey's pairwise comparisons significant at <0.05 level), differences in the angle were only significant between *A. cygnea* and each of the other two species, but not significant between *A. anatina* and *A. exulcerata*. However, the wide intraspecific variability of biometric parameters (Table 3) does not allow a reliable discrimination of these species, displaying largely overlapping characters. The PCA eigenvalues described >99% of the total variability between species. The PC1 axis described 97.3 % and the PC2 axis described 2.97 % of the total variation (Fig. 3A, B). The first component is mainly weighted by lateral inflation and width of the angle between dorsal and posterior margin. The PCA, with group assigned by species, showed a wide morphological range for all species (Fig. 3A) with a large overlap of the three species clusters, including 82% of the total individuals. The limited usefulness of the biometric characters is confirmed by the Discriminant Analysis (Table 4) with only 67% of the specimens being correctly assigned to each species. The major contributors to the principal discriminant factor were the angle between the dorsal and posterior margins and the index of convexity standardized by length (Fig. 3B). The more obtuse angle and the lower lateral inflation of *A. cygnea* (Table 3) allowed a 90% correct assignment, with the remaining 10% of the specimens being misidentified with *A. exulcerata*. Conversely, *A. anatina* was the most misidentified with 28% and 18% erroneous assignment to *A. exulcerata* and *A. cygnea*, respectively.

GEOMETRIC MORPHOMETRIC ANALYSIS

The first two principal components obtained by the PCA on the 18 Fourier coefficients were retained by the broken stick model and together explained 38% of the total variance in sagittal shell shape

(Fig. 4). The three *Anodonta* species overlapped considerably in their sagittal shell shape, so that PC1 values were not significantly different between any of the three species pairs (ANOVA: F=2.665, df=2, P=0.0733). However, PC2 values were significantly different among species (ANOVA: F=41.86, df=2, P<0.0001), with significant differences between all three pairs of species (Tukey's pairwise comparisons significant at <0.05 level). As illustrated by synthetic outlines of extreme shell forms in the PCA plot, *A. anatina* shells tend to have a more triangular outline with a more developed wing and straighter ventral margin than *A. exulcerata* and *A. cygnea* (Fig. 4). A large proportion of the *A. cygnea* specimens included in our dataset displayed a particularly convex dorsal margin and pointed posterior margin.

Despite the statistically significant differences in PC2 scores between all three Italian *Anodonta* species, the power of discriminating *A. exulcerata* from the other two *Anodonta* species based on shell shape was relatively poor. Thus, only 71% of specimens were assigned to the correct species based on DA of the morphometric dataset (Table 5A). While the discrimination between *A. anatina* and *A. cygnea* in this respect was very reliable, both of these species were often misidentified as *A. exulcerata* and vice versa. As a result, the proportion of correctly identified specimens was particularly low for *A. cygnea* (59%), but also far from satisfactory for *A. exulcerata* (67%) and *A. anatina* (80%). On the other hand, the morphometric dataset was relatively powerful in correctly assigning specimens to sites of collection, as 81% of specimens were correctly assigned to their site non-regarding the species (Table 5B).

A complete re-description of the species is presented in the systematics section.

DISCUSSION

The Anodontini has consistently been retrieved as monophyletic, encompassing several North American genera along with *Anodonta* and *Pseudanodonta* species (Lopes-Lima et al. 2017b; Williams et al., 2017). In the most recent classification systems for Europe and North America, the *Anodonta* genus included two to four species (*Anodonta californiensis* Lea, 1852, *A. kennerlyi* Lea,

318 1860, *A. nuttalliana* Lea, 1838 *A. oregonensis* Lea, 1838) restricted to western North America
319 (Williams et al., 2017) and three species (*Anodonta anatina*, *A. cygnea*, *A. exulcerata*) present in
320 Europe (Lopes-Lima et al., 2017b; Froufe et al., 2017). However, their phylogenetic relationships are
321 still unresolved. The first comprehensive synthesis of the world unionoid fauna placed many North
322 American species in the genus *Anodonta* (Simpson 1900, 1914). Since then, all eastern North
323 American *Anodonta* species have been reassigned to other genera (e.g. *Utterbackia*, *Utterbackiana*,
324 *Pyganodon*) (Williams et al., 2017). On the other hand, the western North American species are still
325 considered *Anodonta* but their phylogenetic relationship with their European congeneric species is
326 still contentious (Chong et al., 2008; Blevins et al., 2017; Lopes Lima et al., 2017b). Until now, the
327 phylogenetic position of *A. nuttalliana*, based on two marker approach, was clustering inside the two
328 European *Anodonta* species, i.e. *A. anatina* and *A. cygnea* (Lopes-Lima et al.,2017b). However, in
329 the present study this species clusters for the first time with all Eastern North American Anodontini
330 species, suggesting its separation from *Anodonta* and the need for future multi-marker molecular
331 studies including the other western North American Anodontini. As for European species, the results
332 of the first mitogenome analysis confirm the close relationship between *A. cygnea* and *A. exulcerata*
333 but suggest that *A. anatina* is not congeneric. Furthermore, the status of *Pseudanodonta* is not
334 conclusive (Fig. 2). Again, the inclusion of more taxa and/or nuclear molecular markers is needed to
335 solve this issue.
336 The three species delineation methods applied here suggest the division of *A. anatina* into four
337 separate species. However, due to the low divergence levels seen in the COI uncorrected *p*-distance
338 among these clades (between 1.7% and 3.7%) and lack of sampling in some regions (e.g., Southeast
339 Europe and Tunisia) we refrained from drawing taxonomic conclusions. These should be addressed
340 in the future using a holistic approach, i.e. combining multi marker molecular analyses with
341 morphological, ecological and biogeographical parameters. The present study confirmed the species
342 status of *A. exulcerata* based on the high genetic unc-*p* divergence (8.5% for COI and 10% for the
343 whole mitogenome) between *A. exulcerata* and its sister species *A. cygnea*.

344 The high genetic divergence between these species was not reflected by any major morphological
345 and/or morphometric differences in the analysed characters. This is probably the reason why *A.*
346 *exulcerata* has been unrecognized until now, being erroneously assigned either to its sister species,
347 *A. cygnea*, or to *A. anatina*. Indeed, PCA and DA analyses revealed a wide morphological overlap
348 among the *Anodonta* species, leading to 29% of specimens being incorrectly assigned in the field.
349 From the results of geometric morphometric analysis, *A. cygnea* is more easily misidentified with *A.*
350 *exulcerata,* due to its closer morphological similarity. In fact, *A. anatina* showed the highest
351 percentage of correct assignments by geometric morphometric comparison (80%), while *A.*
352 *exulcerata* and *A. cygnea* were confused with each other in more than 28% of the cases. On the
353 contrary, *A. cygnea* is correctly identified in 80% of cases when linear biometric characters are used,
354 while *A. anatina* is more frequently misidentified with *A. exulcerata*. Indeed, *A. cygnea* tends to be
355 more laterally compressed and posteriorly pointed, with a more obtuse angle between dorsal and
356 posterior margin compared to the other two species. Although erosion smoothed the umbonal
357 ornamentation in 64% of the specimens examined, when visible, this feature can help discriminating
358 between *A. cygnea* and *A. exulcerata*. However, umbo sculpture is useless for discriminating *A.*
359 *exulcerata* and *A. anatina*, which present very similar double-looped lines.
360 No clear discriminating character can be identified in the wide and largely overlapping variability of
361 shell shapes of *A. exulcerata*, *A. cygnea* and *A. anatina*, demonstrating once more that shell plasticity
362 evolved as an adaptation to local conditions (e.g. Walker et al., 2001; Hornback et al., 2010; Zieritz
363 et al., 2010; Inoue et al., 2013) hindering the conchological identification of species. This is especially
364 evident in the anodontine mussels (Reis et al., 2013; Mezhzherin et al., 2014; Klishko et al., 2018)
365 which display considerable intraspecific shell shape variation caused by shifts of metabolism at sexual
366 maturity, changes in allometric growth and other physiological characteristics (Zieritz & Aldridge,
367 2011; Klishko et al., 2016). Moreover, the morphometric analyses were more powerful in
368 discriminating between sites of collection of the specimens than between species. This result confirms
369 that shell shape is more environmentally than genetically controlled, which is congruent with the

370 hypothesis that phenotypic plasticity allowing survival in a wide range of environments could be
371 under positive selection in many freshwater mussel species (Baker et al., 2003; Reis et al., 2013).
372 Equally, only minimal differences were present in anatomical characters between *A. exulcerata* and
373 the other two species. The easiest-to-use quantitative character is the number of papillae series, which
374 is similar in *A. exulcerata* and the closely related *A. cygnea*, but useful to distinguish both species
375 from *A. anatina*. All the other morphological differences revealed in the present study are purely
376 qualitative and concern mainly the pigmentation of the tissues. Pigmentation was creamy-yellowish
377 in 59% of *A anatina* and 79% of *A. exulcerata* specimens, while it tended to brownish in the remaining
378 41% and 21% respectively. The papillae have similar coloration in *A. cygnea* and *A. exulcerata,* while
379 those in *A. anatina* are darker*,* but the most conspicuous difference is the bright orange pigmentation
380 of the tissues in *A. cygnea* (100% of specimens examined). One might argue that the colouring might
381 be excessively tied to external factors to use it as a taxonomic discriminant. However, this
382 distinguishing character was reported for many *A. cygnea* and *A. anatina* populations from other
383 environments and has therefore been previously proposed as a character suitable to separate both
384 species (Mordan & Woodward, 1990; Mezhzherin et al., 2014). Differences in pigmentation seem to
385 be associated with the amount and distribution of orange-yellow extracellular calcified granules in
386 interstitial tissues (Coville & Lim, 2003). Being determined by anatomical and physiological features,
387 it has been suggested that the distribution of granules may be a useful character for phylogenetic
388 analyses (Byrne, 2000). Furthermore, shell and mantle-edge pigmentation seems to be mainly under
389 genetic control (Brake et al., 2004; Wen et al., 2013) although susceptible to dietary induced
390 modifications (Liu et al., 2009). However, unlike the traditionally used conchological characteristics,
391 the plasticity of this feature is poorly documented (e.g. Colville & Lim, 2003; Prié, 2017) and we
392 failed to find any study specifically addressing the variability of soft tissue pigmentation in relation
393 to environmental conditions. While the reliability of such qualitative characters remains to be verified,
394 our study provides further evidence that ecophenotypic plasticity hinders shell morphology-based
395 identification. However, despite the variability and overlap of morphometric characters, they support

much more the separation of *A. cygnea* from *A. anatina*, than that of *A. exulcerata* from any of the other two species. The overlap in morphologies and lack of reliable distinctive characters between *A. exulcerata* and *A. cygnea* could be partially explained by the presence of hybrids. Hybridization has been documented in populations of co-occurring congeneric *Pyganodon* species in eastern North America that have similar levels of differentiation at COI (9-11%; Cyr et al. 2007; Doucet-Beaupré et al. 2012) to the difference reported between *A. exulcerata* and *A. cygnea*. Since hybrids are infrequently detected when we sequence m-lineage COI (Cyr et al. 2007; Zanatta, personal communication), we cannot rule out potential hybridization of intermediate forms of *A. exulcerata* and *A. cygnea.* Additionally, it has been shown that *A. cygnea* is typically hermaphroditic, lacking the DUI typical dioecious forms of the F- and M-ORFs within their mitogenomes, but instead possessing an H-ORF exclusive of hermaphrodite species (Chase et al. 2018). Since *A. exulcerata* also presents an H-ORF, this strongly suggests that the species is also a true hermaphrodite. If intermediate forms between *A. cygnea* and *A. exulcerata* are the result of hybridization then it would be between two hermaphroditic species, a topic that has never been addressed and would be interesting to investigate further.

SYSTEMATICS SECTION

Class: Bivalvia Linnaeus, 1758

Order: Unionida Gray, 1854

Family: Unionidae Rafinesque 1820

Subfamily: Unioninae Rafinesque 1820

Tribe: Anodontini Rafinesque 1820

Genus: *Anodonta* Lamarck 1799

Species: *Anodonta exulcerata*, 'Villa' Porro, 1838: 111, pl. 2, fig. 12

Common Name: Fretted Anodonta (Sowerby, 1870)

Type locality: "Nei piccoli laghi di Oggiono, Alserio, e più ancora di Pusiano in Brianza" (In the small lakes of Oggiono (= Lake Annone), Alserio, and even more in Pusiano, Brianza, Italy)

Type: NHMUK1841.5.6.127; Lectotype

Chresonymy:

Anodonta exulcerata "Villa" Porro 1838

Anodonta piscinalis exulcerata — Drouët, 1883

Anodonta exulcerata — C.B. Adams, 1847

Margaron (*Anodonta*) *cygnea* (Drap.) [in part] — Lea, 1852

Anodon exulceratus — Sowerby, 1870

Margaron (*Anodona*) *cygnea* (Linn).[in part] — Lea, 1870

Anodonta (*Acalliana*) *exulcerata* — Bourguignat, 1881

Anodonta (*Acalliana*) *exulcerata* — Bourguignat, 1882

Anodonta exulcerata — Bourguignat, 1883

Anodonta exulcerata — Catlow & Reeve, 1845; Clessin, 1874

Anodonta (Groupe de l'*A. acallia*) *exulcerata* — Locard, 1890

Anodonta (*Euanodonta*) *exulcerata* — Westerlund, 1890

Anodonta (Groupe de l'*A. acallia*) *exulcerata* — Locard, 1893

Anodonta cygnea (Linnaeus, 1758) [in part] — Simpson, 1900; Simpson, 1914

Anodonta anatina (Linnaeus, 1758) [in part] — Germain, 1931

Anodonta palustris exulcerata — Modell, 1945

Anodonta (*Anodonta*) *cygnea* (Linnaeus, 1758) [in part] — Haas, 1969

Anodonta exulcerata — Froufe et al., 2017

Comments:

We present only a chresonymy for *A. exulcerata* and determined the earliest described *Anodonta* from Northern Italy. We have included *Anodonta idrina* Spinelli, 1851 as the next available taxon for this species. However, due to the confusion of shell forms of *A. anatina, A. cygnea* and *A. exulcerata,* we have not attempted a complete review of all of the *Anodonta* taxa described from Italy in the later

448 part of the 19th and early 20th century. This list of taxa includes at least 56 taxa described from Italy
449 (e.g. Alzona, 1971).
450 Based on the similarity of the shell and on the coincidence of the sampling spots (including one of
451 the type localities, i.e. Lake Oggiono) the re-discovered species was recognized as *Anodonta*
452 *exulcerata*, ('Villa') Porro, 1838, using the oldest available name for the *Anodonta* taxa in the studied
453 region (Haas 1969; Graf & Cummings, 2019). The shells of the lectotype specimen of *A. exulcerata*
454 deposited in British Natural History Museum (NHMUK1841.5.6.127; Fig. 1S) and of the paratype
455 specimens from the "original series" (Zilch 1967: 111; Senckenberg Museum, N°5166) were analysed
456 in detail before attributing this name to the species erroneously synonymized. Johnson (1971) in
457 reviewing the unionid types in NHMUK, found a specimen labeled *Anodon exulceratus* and listed it
458 as the specimen from Ziegler figured in Sowerby (1870). Ziegler is listed in the Malacology ledger
459 as the donor of *Anodon exulceratus* (Dr. T. White pers. comm. 2/4/2019). Sowerby credited the name
460 to a "Villa" manuscript in the British Museum, indicating that it was Sowerby's figured type. Johnson
461 credited the species description to Sowerby (1870). Sowerby (1870: species 131 page [48], Plate 33
462 sp. 131) listed "Villa. MS in Mus. Brit." Johnson (1971) cited *Anodon exulceratus* "Porro" Sowerby,
463 1870. Thus, Johnson was aware of the citation of Villa manuscript by Sowerby but chose to ignore it
464 and claim it was a Porro manuscript name, ignoring Porro's (1838) description of *Anodonta*
465 *exulcerata*. Listing of that specimen figured by Sowerby as the figured holotype represents an
466 inadvertent lectotype fixation under Art. 74.6 of the Code (ICZN 1999). However, Porro (1838)
467 mentioned in his description "the plurality of individuals" observed. He also listed three lakes in his
468 distribution. This documents that the description of *A. exulcerata* by Porro was based on multiple
469 individuals. Thus, the inadvertent lectotype designation by Johnson, (1971) for *A. exulcerata* 'Porro'
470 Sowerby, 1870 may be valid, but the application of the lectotype to *A. exulcerata* Porro, 1838 by
471 assumption of holotype is invalid as Porro mentions multiple specimens in his description. This
472 NHMUK specimen, NHMUK 1841.5.6.127 is here designated as the lectotype for *Anodonta*
473 *exulcerata* "Villa" Porro, 1838.

474

475 *Shell Description:*

476 Shell generally thin, equivalve and inequilateral, large (max length 103 mm, N = 109) elliptical to
477 sub-oval, moderately inflated. Angle between dorsal margin and posterior margin 124° to 147° (mean
478 = 135°). Anterior margin broadly rounded, posterior margin narrowly rounded to bluntly pointed;
479 ventral margin convex, occasionally flat straight in the middle nearer to the posterior edge; dorsal
480 margin straight to slightly convex in passing from the posterior margin, occasionally extending into
481 a low dorsal wing; posterior ridge rounded, occasionally weakly bi-angulated distally; posterior slope
482 moderately steep, flat to slightly convex; umbo broad, moderately inflated, elevated slightly above
483 hinge line; umbo sculpture with thin wavy rugae; umbo cavity wide, shallow. Pseudocardinal and
484 lateral teeth absent. Adductor muscles scars rather light shallow (not deep). Nacre is white to bluish
485 white, usually iridescent. Periostracum tawny to olive or brown; small individuals yellowish brown
486 to dark olive, large individuals brownish black with dark green rays of varying width and intensity.
487 Morphological shell features correspond quite well to the first description of the species (Porro, 1838)
488 and to the lectotype made available from the Natural History Museum of London (Fig. 1S). One
489 discrepancy lies in the fact that, contrary to what is indicated by Porro, we cannot argue that "in the
490 majority of individuals the upper and lower margins are parallel, and only in a few individuals are
491 distant posteriorly". On the contrary, the shape of the shell is so variable that it appears haphazard to
492 draw any generalization (Fig 4, 5, and 8S).
493 Umbo sculpture also appears to be highly variable ranging from a clearly double-looped to a finely
494 concentric lines arrangement (Fig. 7S and 9S).

495

496 *Soft Anatomy Description:*

497 In life the mantle is creamy-white to yellowish or light brownish (respectively 79 and 21% of
498 individuals examined), brownish or tan at the openings of the apertures, mantle outside of apertures

transparent white to grey; visceral mass creamy-white to pink powder, may be pale-orange adjacent to foot; foot pale orange to creamy-white.

Gills creamy to gold; dorsal margin sinuous to concave, ventral margin convex; anterior margin of inner gills slightly longer and wider than outer gills. Outer gills marsupial; glochidia held across gill length; well-padded when gravid; light brownish to brownish orange.

Labial palps creamy white; straight to concave dorsally, convex ventrally, pointed distally; with a smooth external surface and a finely canaliculated internal surface.

Incurrent aperture longer than excurrent and supra-anal apertures; supra-anal and incurrent apertures occasionally of similar length. Incurrent aperture creamy white to grey within; greyish or brownish basal to papillae; papillae in 2 to 3 rows (respectively 47 and 63% of individuals examined), inner row usually larger, longer, thick; papillae white-creamy to light tan; whitish in living animals. Excurrent aperture smooth, whitish at the external margin, with darkly coloured irregular band at the base. Supra-anal aperture smooth, creamy white within, without marginal coloration.

Voucher Specimens:

Six voucher specimens of this species were deposited: two at the Museo de La Specola-Florence (catalogue numbers: MZUF BC/51405 and MZUF BC/51406), two at the Naturhistorisches Museum der Burgergemeinde Bern (NMBE 549733 and NMBE 549734), and two at the North Carolina Museum of Natural Sciences (NCSM 102851 and NCSM 102852) (Table 2; Froufe et al., 2017). Since *Anodonta exulcerata* Porro, 1838 is the oldest available name for the *Anodonta* taxa in the studied region (Haas, 1969; Graf & Cummings 2019), *A. exulcerata* is used herein for this newly detected *Anodonta* species. The shell morphology of the *A. exulcerata* sampled in this study (Fig. 3S) is consistent with the lectotype of *A. exulcerata* (Natural History Museum, UK: Lectotype NMNHUK 1841.5.6.127) and with the paratype specimens of the Senckenberg Museum, Frankfurt/Main (Zilch, 1967). Furthermore, in one of its type localities (Lake Annone) it was the only *Anodonta* species found (Froufe et al., 2017).

525 *Distribution: Anodonta exulcerata* is endemic from the Italian Peninsula to Croatia west of the
526 Dinaric Alps (Froufe et al., 2017), which confirms the distribution reported by Clessin (1876). In
527 Northern Italy it appears to be the most common *Anodonta* species.
528 *Habitat and biology: Anodonta exulcerata* occur in waters with little or no current and substrates
529 typically composed of mud or muddy sand, often with detritus. Due to misidentification with the
530 other *Anodonta* species, information on biology is scarce. Gravid individuals brooding glochidia at
531 different stages of development have been observed from early September to late December in Lake
532 Maggiore and Lake Varese (Riccardi N. pers. obs.). Glochidial host fish species are unknown.
533 *Conservation status:* The fact that *A. exulcerata* has not been previously recognized has precluded
534 any assessment of its conservation status. However, it is widely distributed in the region and locally
535 abundant, which might suggest that currently the species is not at risk.
536
537 *Comparison with similar species:*
538 Close conchological similarity and wide shell plasticity make the use of shell shape for the
539 discrimination of *A. exulcerata* from coexisting congeneric species (i.e. *A. anatina* and *A. cygnea*)
540 unreliable. Like *A. anatina*, *A. exulcerata* tends to be more swollen than *A. cygnea* slightly posterior
541 to the umbo. However, the difference, whenever it exists, may be masked by the wide shell plasticity.
542 Indeed, except for the index of convexity standardized over length, the mean values of the shell
543 measurement ratios were not significantly different (Table 3). To the extent that reliable external
544 features could be identified to distinguish the two Central, Northern and Eastern European *Anodonta*
545 species (Gallenstein 1895, Möller 1933, Bloomer 1937, Franz 1939), it also became apparent that the
546 Italian forms could not be clearly identified (Gallenstein 1894, Falkner 1994). Rather, a mixture of
547 the otherwise species-specific characteristics was often found. Only through the analysis of further
548 characters (allozymes, DNA) a new view on this problem became possible and older assumptions
549 about the peculiarities of the Italian unionid fauna receive an objective basis.

Clessin (1874) already stressed the close similarity of *A. exulcerata* and *A. anatina* ["belongs to the Formenkreis of *Anodonta anatina* Rossm. "] and attributed *A. exulcerata*, as well as the closely similar *A. idrina* (Spinelli, 1851), to the *A. anatina* "group". Kobelt (1876) reiterates that *A. idrina*, *A. exulcerata* and *A. gibba* (a *nomen nudum*, however) should not be separated, and emphasizes the enormous difficulties and uncertainties in separating the species of *Anodonta*. This is the only final message to be drawn after getting lost in the enormous variety of conflicting opinions among the malacologists of the time.

For the determination of live animals or shells in the field, however, a diagnosis based on external characters is highly desirable. For this purpose, a larger number of molecularly determined forms must be examined anatomically and conchologically. This step is reserved for later investigation.

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841 FIGURE CAPTIONS

842 Fig. 1 Anodontini phylogenetic trees obtained by Bayesian Inference (BI) and Maximum Likelihood
843 (ML) analyses of the cytochrome c oxidase I (COI) gene fragment. The values nodes indicate
844 Bayesian posterior probability percentage / Maximum Likelihood bootstrap values, respectively.
845 Values over 95% are represented by an asterisk. Vertical bars correspond to molecular operational
846 taxonomic units by various species delimitation methods: red - BINS of BOLD; green - TCS (95%);
847 blue - bPTP; black - consensus.

848

849 Fig. 2 Unionida phylogenetic tree obtained by Bayesian Inference (BI) and Maximum Likelihood
850 (ML) analyses estimated from 14 concatenated individual mtDNA gene sequences (12 protein-coding
851 and 2 rRNA genes). The values nodes indicate Bayesian posterior probability percentage / Maximum
852 Likelihood bootstrap values, respectively. Values over 95% are represented by an asterisk.

853

854 Fig. 3 Scatterplot and 95% confidence ellipses of 108 specimens comprising three *Anodonta* species
855 collected from sites in Italy displaying the first two Principal Component scores obtained by
856 Discriminant Analysis based on linear biometric values.
857 Aa = *A. anatina*; Ac = *A. cygnea*; Ae = *A. exulcerata*. W/H = width/height; H/L = height/length; W/L
858 = width/length; W/H = width/height ratios. [(W/H)/L] = index of convexity standardized over length;
859 angle = measure of the angle formed by lines tangent to the posterior and dorsal margins.

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861

862 Fig. 4 Scatterplot and 95% confidence ellipses of 138 specimens comprising three *Anodonta* species
863 collected from sites in Italy displaying the first two Principal Component scores obtained by Principal
864 Component Analysis of 18 Fourier coefficients. Synthetic shell outlines of “extreme” morphotypes
865 are displayed with the anterior margin facing to the left and the dorsal margin to the top of the page.
866 Aa = *A. anatina*; Ac = *A. cygnea*; Ae = *A. exulcerata*.

Fig. 1S Lectotype of *Anodonta exulcerata* Porro, 1838 – N° 1841.5.6.127, Natural History Museum, London.

Fig. 2S Paratypes of *Anodonta exulcerata* Porro, 1838 – N° 5166, Senckenberg Museum of Natural History, Frankfurt am Main.

Fig. 3S –Representative specimens of *Anodonta exulcerata* collected in Lake Maggiore, at location Monvalle, Gureé beach close to the reeds belt (left and center), and at location Magadino, inside the Porto Patriziale (right).

Fig. 4S - Aspect of excurrent aperture and papillae in living (left) and freshly dissected (right) *A. anatina* (top), *A. exulcerata* (intermediate) and *A. cygnea* (bottom).

Fig. 5S – Arrangement of papillae in *A. anatina* (left), *A. exulcerata* (center) and *A. cygnea* (right).

Fig. 6S - Coloration of soft tissues in freshly dissected *A. anatina* (left), *A. exulcerata* (center) and *A. cygnea* (right).

Fig. 7S Umbonal sculpture of *A. anatina* (*Aa*), *A. exulcerata* (*Ae*), and *A. cygnea* (*Ac*). LT = Lake Trasimeno; LCA = Lake Castel dell'Alpi; LC = Lake Caldonazzo; LL = Lake Levico; LMA = Lake Maggiore; LMO = Lake Montepulciano; LLU = Lake Lugano.

Fig. 8S Variability of shell shape of *A. exulcerata* specimens.

Fig. 9S Variability of umbo sculpture in *A. anatina* (Aa), *A. exulcerata* (Ae) and *A. cygnea* (Ac) specimens. LT = Lake Trasimeno; LCA = Lake Castel dell'Alpi; LC = Lake Caldonazzo; LL = Lake Levico; LMA = Lake Maggiore; LMO = Lake Montepulciano; LLU = Lake Lugano.

TABLES

Table 1 Geographic locations of sampled sites, numbers of individuals used in morphometric and molecular analyses, species identified (*Aa* = *A. anatina*; *Ac* = *A. cygnea*; *Ae* = *A. exulcerata*)

Table 2 - Voucher specimens of *A. exulcerata*; MZUF = Museo de La Specola-Florence, NMBE = Naturhistorisches Museum der Bürgergemeinde-Bern, NCSM = North Carolina Museum of Natural Sciences.

Table 3 Biometric measurements (mean ± SD, range in brackets) of *A. exulcerata*, *A. cygnea* and *A. anatina* shells.

Table 4 Confusion matrix of Disciminant Analysis of biometric variables (angle; H/L; W/L; W/H) of Italian *Anodonta* specimens, showing the proportion of specimens correctly/incorrectly assigned to each species (based on 87 specimens; specimens with broken shells were omitted).

Table 5 Confusion matrix of Disciminant Analysis of 18 Fourier coefficients obtained by Fourier Shape Analysis of Italian *Anodonta* specimens, showing the proportion of specimens correctly/incorrectly assigned to (A) species (based on 137 specimens and including specimens collected by Nagel (1996) and (B) site of collection (based on 97 specimens collected by the authors and excluding sites from which fewer than five specimens were available for analysis).

Abbreviations: LC, Lake Castel Dell'Alpi; LE, Lake Endine; LL, Lake Levico; LMa, Lake Maggiore; LMo, Lake Montepulciano; LT, Lake Trasimeno.

Table S1 List of specimens analysed for the mitogenomes, GenBank references, and country. *original identification.

Table S2 List of all individual haplotypes, species and GenBank accession codes.

Table S3 Main structural features of mitochondrial genomes from newly sequenced specimens.

FIGURES

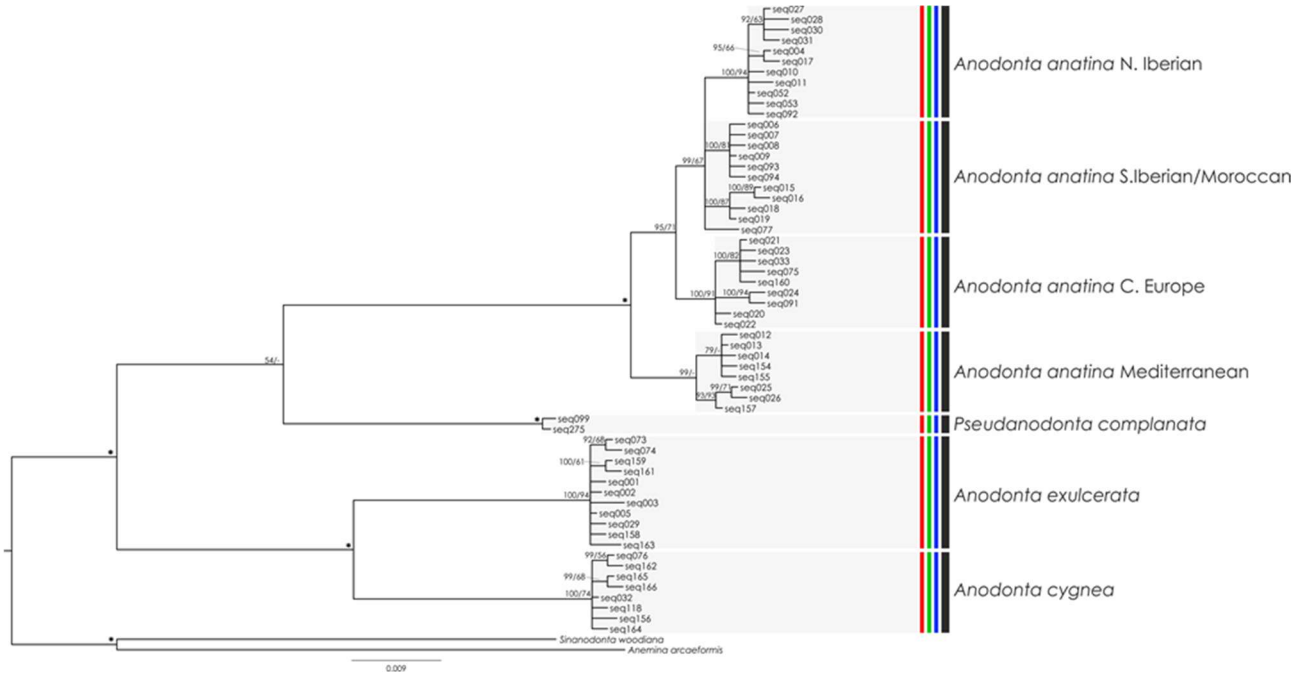

Fig. 1 Anodontini phylogenetic tree obtained by Bayesian Inference (BI) and Maximum Likelihood (ML) analyses of the cytochrome c oxidase I (COI) gene fragment. The values nodes indicate Bayesian posterior probability percentage / Maximum Likelihood bootstrap values, respectively. Values over 95% are represented by an asterisk. Vertical bars correspond to molecular operational taxonomic units by various species delimitation methods: red - BINS of BOLD; green - TCS (95%); blue - bPTP; black - consensus.

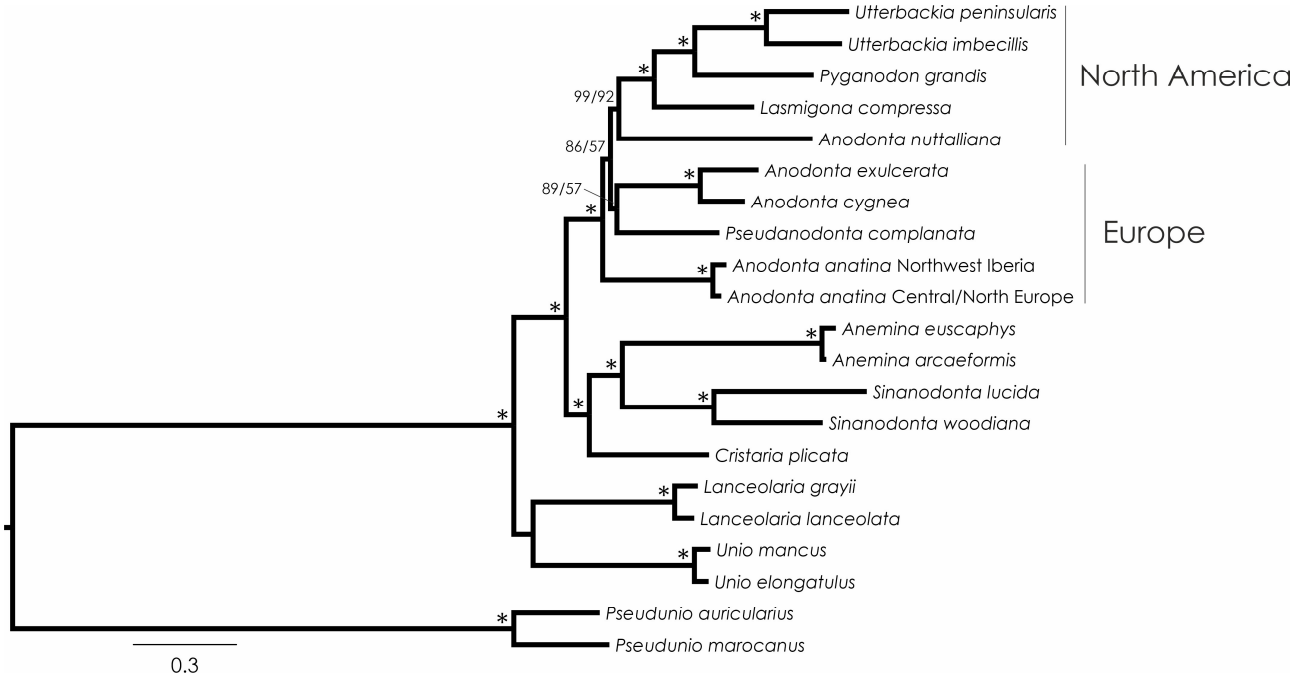

Fig. 2 Unionida phylogenetic tree obtained by Bayesian Inference (BI) and Maximum Likelihood (ML) analyses estimated from 14 concatenated individual mtDNA gene sequences (12 protein-coding and 2 rRNA genes). The values nodes indicate Bayesian posterior probability percentage / Maximum Likelihood bootstrap values, respectively. Values over 95% are represented by an asterisk.

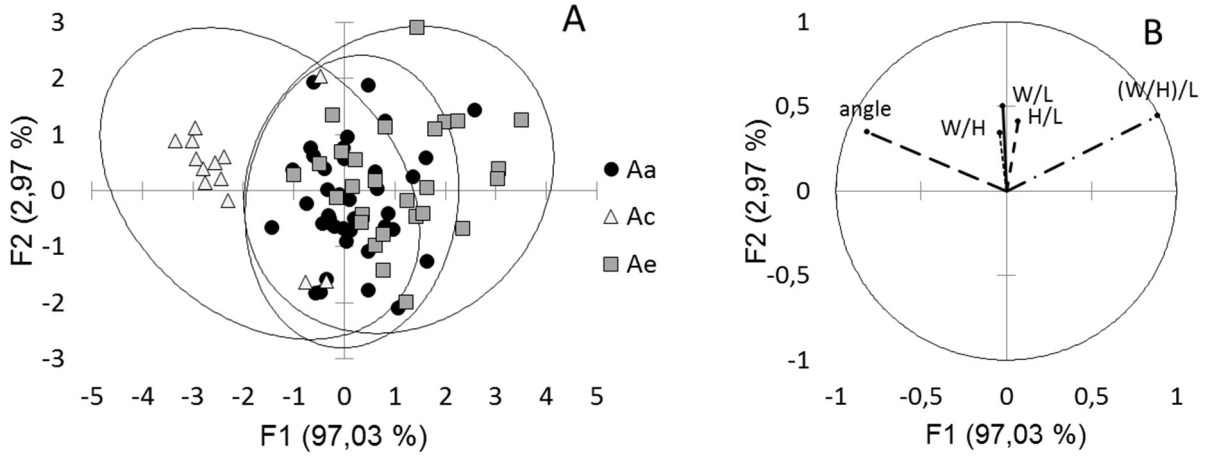

Fig.3 Scatterplot and 95% confidence ellipses of 108 specimens comprising three *Anodonta* species collected from sites in Italy displaying the first two Principal Component scores obtained by Discriminant Analysis based on linear biometric values.

Aa = *A. anatina*; Ac = *A. cygnea*; Ae = *A. exulcerata*. W/H = width/height; (H/L) = height/length; (W/L) = width/length; W/H = width/height ratios. [(W/H)/L] = index of convexity standardized over length; angle = measure of the angle formed by lines tangent to the posterior and dorsal margins.

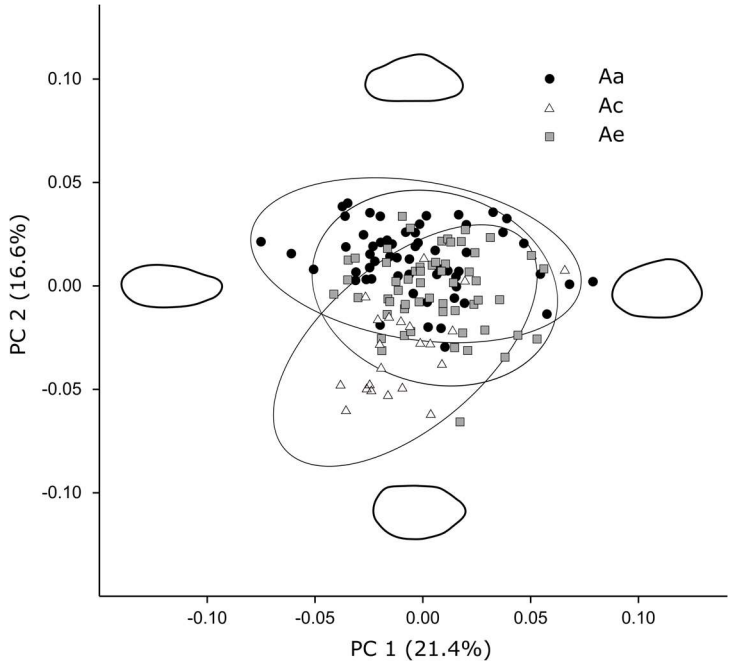

Fig. 4 Scatterplot and 95% confidence ellipses of 138 specimens comprising three *Anodonta* species collected from sites in Italy displaying the first two Principal Component scores obtained by Principal Component Analysis of 18 Fourier coefficients. Synthetic shell outlines of "extreme" morphotypes are displayed with the anterior margin facing to the left and the dorsal margin to the top of the page.

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995 TABLES

996 Table 1 Geographic locations of sampled sites, numbers of individuals used in morphometric and
997 molecular analyses, species identified (*Aa = A. anatina*; *Ac = A. cygnea*; *Ae = A. exulcerata*). In the
998 morphometric analysis 29 additional specimens collected by Nagel et al. (1996) were included.

Catchment	Site	Latitude	Longitude	Morphometrics	mtDNA	Species
Po River	Lake Lugano	45.956475	8.965843	4	4	*Ac*
Po River	Lake Maggiore	45.980342	8.644341	51	51	*Ac, Aa, Ae*
Po River	Lake Varese	45.801208	8.736260	1	1	*Ae*
Po River	Lake Monate	45.796366	8.669498	-	2	*Ae*
Po River	Lake Comabbio	45.767263	8.700858	-	4	
Po River	Lake Viverone	45.412818	8.048182	1	1	*Ae*
Po River	Lake Candia	45.321452	7.914937	-	1	*Ae*
Po River	Lake Annone	45.814254	9.359094	1	1	*Ae*
Po River	Lake Pusiano	45.796396	9.279407	-	1	*Ae*
Po River	Lake Endine	45.760005	9.920562	6	6	*Ae*
Brenta River	Lake Caldonazzo	46.005170	11.258318	3	3	*Ae*
Brenta River	Lake Levico	46.014029	11.286210	5	5	*Aa, Ae*
Isonzo River	Isola Morosini (unnamed channel)	45.763785	13.436075	2	2	*Ae*
Reno River	Lake Castel dell'Alpi	44.184531	11.275864	16	16	*Aa*
Arno River	Lake Montepulciano	43.087531	11.928983	10	10	*Ac*
Tiber River	LakeTrasimeno	43.089545	12.153232	9	9	*Aa, Ae*

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1000 Table 2 Voucher specimens of *A. exulcerata*; MZUF = Museo de La Specola-Florence, NMBE =
1001 Naturhistorisches Museum der Bürgergemeinde-Bern, NCSM = North Carolina Museum of Natural
1002 Sciences.

Catalog Number	locality	Latitude	Longitude	river basin	shell length (mm)
MZUF GC/51405	Lake Maggiore	45°50'59.9"N	8°37'06.9"E	Po	97,25
MZUF GC/51406	Lake Levico	46°00'31.7"N	11°17'06.5"E	Brenta	72,61
NMBE 549733	Lake Maggiore	46°08'55.9"N	8°51'32.2"E	Po	89,80
NMBE 549734	Lake Caldonazzo	46°00'25.5"N	11°15'53.1"E	Brenta	82,96
NCSM 102851	Lake Maggiore	45°50'59.9"N	8°37'06.9"E	Po	86,22

NCSM 102852	Lake Caldonazzo	46°00'25.5"N	11°15'53.1"E	Brenta	80,49

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Table 3 Biometric measurements (mean ± SD, range in brackets) of *A. exulcerata*, *A. cygnea* and *A. anatina* shells.

	A. exulcerata	*A. cygnea*	*A. anatina*
Length (mm) of shell	82.82 ± 10.78 (65.41–103.77)	126.39 ± 31.52 (82.05–168.41)	95.66 ± 17.69 (65.93–152.92)
Height (mm) of shell	48.11 ± 6.10 (38.48–58.56)	72.71 ± 18.40 (46.66–98.11)	54.45 ± 8.20 (41.55–79.81)
Width (mm) of shell	29.08 ± 5.87 (19.52–41.13)	45.31 ± 16.43 (23.29–68.16)	32.82 ± 8.87 (21.30–65.26)
H/L	0.58 ± 0.02 (0.53–0.63)	0.57 ± 0.02 (0.54–0.61)	0.57 ± 0.03 (0.51–0.63)
W/L	0.35 ± 0.05 (0.26–0.45)	0.35 ± 0.05 (0.26–0.41)	0.34 ± 0.05 (0.27–0.43)
W/H	0.60 ± 0.08 (0.47–0.76)	0.61 ± 0.08 (0.44–0.72)	0.58 ± 0.12 (0.45–0.82)
(W/H)/L	0.007±0.001** (0.006–0.010)	0.005±0.0009** (0.004–0.007)	0.006±0.001** (0.005–0.010)
Angle (°) between dorsal and posterior margin	135 ± 6 (124–147)	144± 9 ** (116–153)	136 ± 7 (115–148)

** $p<0.0001$

Table 4 Confusion matrix of Disciminant Analysis of biometric variables (angle; H/L; W/L; Wmax/Hmax; Wmax/Hmax/L) of Italian *Anodonta* specimens, showing the proportion of specimens correctly/incorrectly assigned to each species (based on 87 specimens; specimens with broken shells were omitted).

Species					
Given group		Predicted group			
	A. anatina	*A. cygnea*	*A. exulcerata*	Total	% correct
A. anatina	21	7	11	39	54
A. cygnea	0	19	2	21	90
A. exulcerata	5	4	18	27	67
Total	17	33	37	87	67

Table 5 - Confusion matrix of Discriminant Analysis of 18 Fourier coefficients obtained by Fourier Shape Analysis of Italian *Anodonta* specimens, showing the proportion of specimens correctly/incorrectly assigned to (A) species (based on 138 specimens and including specimens collected by Nagel (1996) and (B) site of collection (based on 97 specimens collected by the authors and excluding sites from which fewer than five specimens were available for analysis). Abbreviations: LC, Lake Castel Dell'Alpi; LE, Lake Endine; LL, Lake Levico; LMa, Lake Maggiore; LMo, Lake Montepulciano; LT, Lake Trasimeno.

(A) Species

Given group	Predicted group				
	A. anatina	*A. cygnea*	*A. exulcerata*	Total	% correct
A. anatina	47	0	12	59	80
A. cygnea	1	13	8	22	59
A. exulcerata	10	9	38	57	67
Total	58	22	58	138	71

(B) Sites

Given group	Predicted group							
	LC	LE	LL	LMa	LMo	LT	Total	% correct
LC	14	1	0	1	0	0	16	88
LE	0	5	0	1	0	0	6	83
LL	0	0	4	0	0	1	5	80
LMa	3	6	1	39	0	2	51	76
LMo	0	0	0	0	10	0	10	100
LT	0	0	1	1	0	7	9	78
Total	17	12	6	42	10	10	97	81

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