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Phylogeny of European Anodontini (Bivalvia: Unionidae) with a redescription of *Anodonta exulcerata*

NICOLETTA RICCARDI[1*], ELSA FROUFE[2], ARTHUR E. BOGAN[3], ALEXANDRA ZIERITZ[4], AMÍLCAR TEIXEIRA[5], ISABELLA VANETTI[6], SIMONE VARANDAS[7], SERENA ZACCARA[6], KARL-OTTO NAGEL[8] and MANUEL LOPES-LIMA[2,9,10]

[1]*CNR – Institute of Water Research, Verbania Pallanza (VB), Italy*
[2]*CIIMAR/CIMAR—Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N 4450-208 Matosinhos, Portugal*
[3]*North Carolina Museum of Natural Sciences, 11 West Jones St., Raleigh, NC 27601, USA*
[4]*School of Geography, University of Nottingham, University Park, NG7 2RD, Nottingham, UK*
[5]*CIMO-ESA-IPB - Mountain Research Centre, School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301–854 Bragança, Portugal*
[6]*DISTA – Department of Theoretical and Applied Sciences, University of Insubria, Varese, Italy*
[7]*Forestry Department, CITAB-UTAD – Centre for Research and Technology of Agro-Environment and Biological Sciences, University of Trás-os-Montes and Alto Douro, Apartado 1013, 5001–811 Vila Real, Portugal*
[8]*Senckenberg Forschungsinstitut Frankfurt, Malacology, Senckenberganlage 25, 60325 Frankfurt am Main, Germany*
[9]*CIBIO/InBIO – Research Center in Biodiversity and Genetic Resources, Universidade do Porto, Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485–661 Vairão, Portugal*
[10]*IUCN SSC Mollusc Specialist Group, c/o IUCN, David Attenborough Building, Pembroke St, Cambridge, UK*

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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 an *Anodonta* species bringing the number of known European Anodontini from three to four species. 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 redescription of *A. exulcerata* and a comparative analysis of morphological and anatomical characters with respect to the other two species of *Anodonta* present in the area are provided. No reliable diagnostic character has emerged from 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 Anodontinae.

KEYWORDS: conservation – freshwater mussels – mitogenome – morphological plasticity – revalidated species.

INTRODUCTION

Conservation of freshwater mussels (Bivalvia: Unionida) is essential to maintain important ecosystem functions and services that they provide (Bogan 1993; Lopes-Lima

*Corresponding author. E-mail: nicoletta.riccardi@irsa.cnr.it

et al., 2017a; Vaughn, 2018). Similar to other freshwater taxonomic groups, these bivalves are highly threatened and globally declining due to multiple anthropogenic impacts (e.g. Lopes-Lima *et al.*, 2018, Ferreira-Rodriguez *et al.*, 2019), raising their conservation importance. However, for many freshwater mussel species, effective conservation measures are hindered by our incomplete understanding of biological species delineations and/or current inability to identify them correctly based on morphology (Prié *et al.*, 2012). This is due to the exceptionally high phenotypic plasticity within freshwater mussel species and morphological convergences between species, reflecting an adaptive phenotypic response to habitat factors (Zieritz & Aldridge, 2009; Hornback *et al.*, 2010; Zieritz *et al*, 2010; Reis *et al.*, 2013; Guarneri *et al.*, 2014).

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

With increasing molecular sequence data and taxon sampling, the phylogeny and taxonomy of Anodontini have been considerably revised over the past few years, but are still unresolved (Lopes-Lima *et al*, 2017b; Williams *et al.*, 2017; Smith *et al.*, 2018; Pfeiffer *et al.*, 2019). Current molecular 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 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 Palaearctic and one (*Anodonta* Lamarck, 1799) is present in the west coast basins of North America, across the Palaearctic, 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 (Lopes-Lima *et al.*, 2017b; Froufe *et al.* 2019; Pfeiffer *et al.* 2019), this tool has yet to be applied to resolve relationships at the tribe level.

In Europe, the total number of Anodontini species is still unknown and, therefore, their phylogenetic relationship remains uncertain (Lopes-Lima *et al.*, 2017a). Until recently, three Anodontini species were recognized in 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*. *Anodonta exulcerata* is restricted to 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 reassessment 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, and 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 (1) reassess the species diversity, phylogenetic relationships and systematics of European Anodontini using molecular data, (2) unravel the global Anodontini phylogeny using phylogenomics and (3) identify morphometric, morphological and/or anatomical characters to distinguish Italian *Anodonta* species in the field.

MATERIAL AND METHODS

Sample collection

Anodonta specimens (N = 109) were collected from 16 sites across the Italian Peninsula river basins during 2014–16 (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.

All individuals had been barcoded previously for molecular species identification (using *COI*) published in Froufe *et al.* (2017).

DNA extractions and sequencing

Genomic DNA was extracted from the tissue samples, using a standard high-salt protocol (Sambrook *et al.*, 1989). F-type mitogenome sequencing and assemblage followed Gan *et al.* (2014), whilst annotations were performed following Fonseca *et al.* (2016). All mitogenomes were deposited in the GenBank database under the accession numbers (submitted; Supporting Information, Table S1).

Two datasets were constructed: one for *COI* and another for the mitogenomes. The *COI* dataset included all European Anodontini sequences available in GenBank, with *Sinanodonta woodiana* (Lea, 1834) and *Anemina arcaeformis* (Heude, 1877) as outgroups (Supporting Information, Table S2). The mitogenome dataset included all the Anodontini specimens with sequences available from GenBank, with *Pseudunio marocanus* (Pallary, 1918) as outgroup, plus the eight newly sequenced species: *Unio elongatulus* (Pfeiffer, 1825), *U. mancus* (Lamarck, 1819), north-west Iberian lineage *Anodonta anatina*; *A. cygnea*; *A. exulcerata*; *A. nuttalliana* (Lea, 1838); *Pseudanodonta complanata* and *Sinanodonta woodiana* (Supporting Information, Table S1). For each dataset, sequences of additional specimens were downloaded from GenBank (details in Supporting Information, Table S1).

The *COI* dataset was aligned with the MAFFT multiple sequence alignment algorithm (Katoh & Standley, 2013) and then the final alignment was restricted to its unique haplotypes, using DnaSP v.5.1.0.1 (Librado & Rozas, 2009).

Mitogenomes were visualized using GenomeVx (Conant & Wolfe, 2008). Sequences of all mtDNA protein-coding genes (PCG), except ATP8 and the gender-specific open reading frames (H-ORF

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*). In the 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	*Ae*
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*

and F-ORF; Breton *et al.*, 2011), were used in the phylogenetic analyses. The sequences of each gene were aligned using MAFFT software (v.7.304; Katoh & Standley, 2013) and trimmed with 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 v.7 (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 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 one tree for every 100 generations), each with four chains (three hot and one 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 GTR+G was employed for the ML analyses. As for the mitogenome dataset, 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 analysed 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 1×10^6 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 dorsoventral 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 the 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 the 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 ten 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 the 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 post hoc test was performed to identify significant differences between each population pair. Finally, we assessed the rate of accurate species identification based on the Fourier Shape Analysis using DA on the set of 18 Fourier coefficients. Statistical analyses were performed in R.3.1.1.

RESULTS

Molecular phylogeny and species delineation

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

The length of the newly sequenced mitogenomes is within the expected F-type range of the freshwater mussels, and all present the same previously described gene order, UF1 (Lopes-Lima *et al.*, 2017c). Their main characteristics, i.e. size, gene composition and order, morphological features of the lectotype and paratype (Supporting Information, Figs S1, S2) of representative specimens (Supporting Information, Fig. S3) and of the specimens examined for this study are shown in the Supporting Information, Figures S4–S9 and in Table S3. The best ML and BI trees retrieved have similar

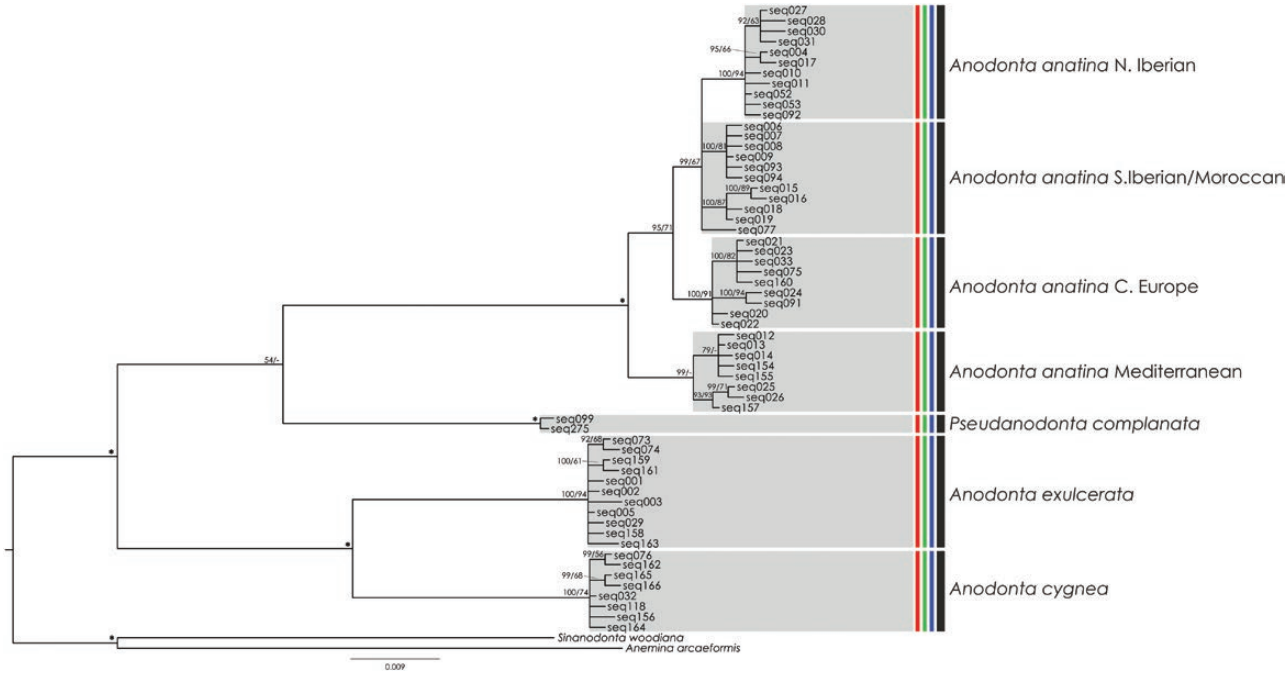

Figure 1. Anodontini phylogenetic trees obtained by Bayesian inference (BI) and maximum likelihood (ML) analyses of the cytochrome *c* oxidase I (*COI*) gene fragment. The values nodes indicate Bayesian posterior probability percentage / maximum likelihood bootstrap values, respectively. Values over 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.

topologies, with the exception of the phylogenetic relationship of the *Lanceolaria* sp. clade. The phylogenomic tree shows the monophyly of Anodontini and its sister-status to the Cristariini clade (Fig. 2). The genus *Anodonta* is not monophyletic due to the paraphyletic positions of *A. anatina*, *P. complanata*, *A. nuttalliana* and *A. cygnea*+*A. exulcerata* clades (Fig. 2). As expected, the phylogenomics also joins *A. cygnea* with *A. exulcerata* with high support. *P*-distance between these two species was 10% for the whole mitogenome (Supporting Information, Table S4).

Comparative anatomy and conchology

Soft tissues morphology

The inner and outer gills have the same form and size across the three taxa (i.e. *A. anatina*, *A. cygnea* and *A. exulcerata*). The form and size of labial palps are similar in the three species. Main interspecific differences are only found in the papillae morphology of the incurrent aperture and in the pigmentation of the mantle surface in the excurrent aperture (Supporting Information, Fig. S4), characters that were proposed for reliably separating other mussel species (Glöer & Meier-Brook, 2003; Sayenko, 2007; Sayenko *et al.*, 2009). In the present study, *A. anatina* can be reliably discriminated from other *Anodonta* species by internal morphology only in living specimens through its apertural anatomy. Compared to other *Anodonta* species, *A. anatina* exhibits a longer excurrent aperture, a greater protrusion of papillae from the edge of the shell and a brownish colour of mantle edge and papillae (Supporting Information, Fig. S4). In contrast, the apertural anatomy of *A. exulcerata* and *A. cygnea* is similar and characterized by a short excurrent aperture without marginal and papillae coloration. Living or freshly dissected *A. exulcerata* and *A. cygnea* specimens present a clearly irregular tan band at the insertion of papillae (Supporting Information, Figs S4, S5). The papillae show a distinct pattern, being arranged in four or five series in *A. anatina* (four series in 81 and five in 19% of the specimens), only two or three series in *A. cygnea* (two series in 27% and three in 73% of the specimens) and in *A. exulcerata* (two series in 42% and three in 58% of the specimens).

Another discriminant character is foot and mantle colour, which has been shown to be useful to differentiate *A. cygnea* from *A. anatina* (Mordan & Woodward, 1990; Mezhzherin *et al.*, 2014). Indeed, *A. anatina* and *A. exulcerata* present a light-brown/creamy-white colour, whereas *A. cygnea* is generally bright-orange coloured (Supporting Information, Fig. S6).

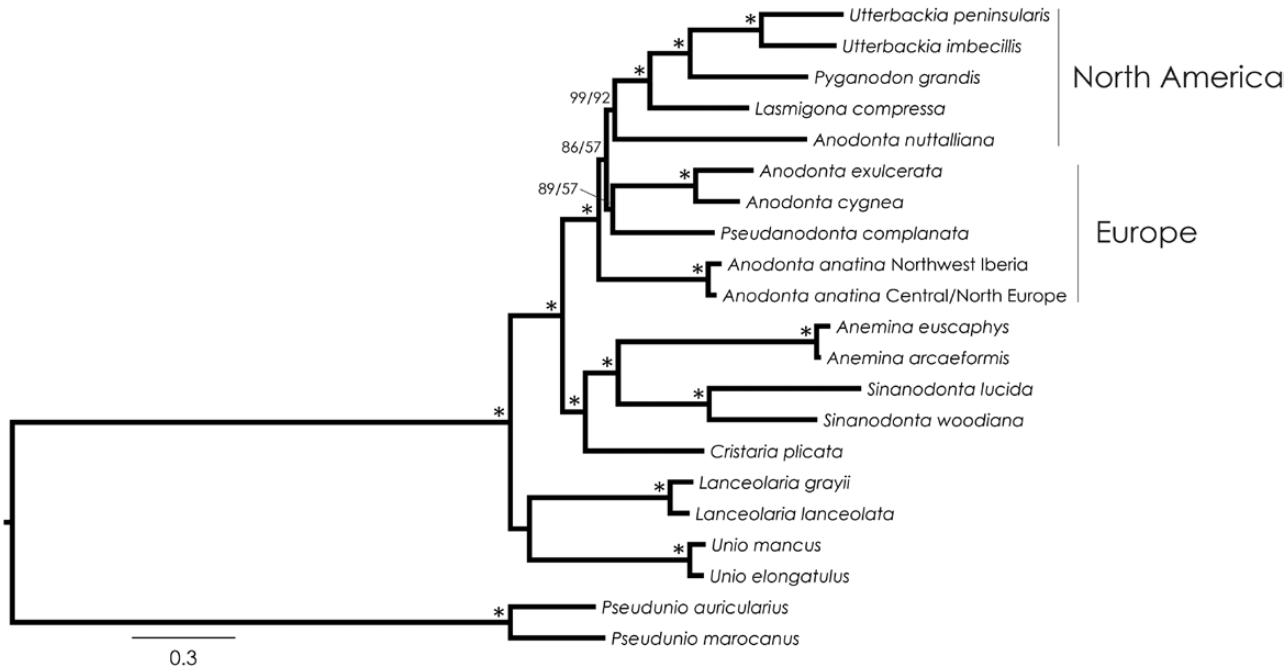

Figure 2. Unionida phylogenetic tree obtained by Bayesian inference (BI) and maximum likelihood (ML) analyses estimated from 14 concatenated individual mtDNA gene sequences (12 protein-coding and two rRNA genes). The values nodes indicate Bayesian posterior probability percentage / maximum likelihood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

Table 2. 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)

** $P < 0.0001$.

Umbonal sculpture

Anodonta cygnea umbo sculpture consists of thin concentric lines, while *A. anatina* presents wavy rugae (Supporting Information, Fig. S6). *Anodonta exulcerata* is more similar to *A. anatina* than to *A. cygnea* (Supporting Information, Fig. S6), generally presenting wavy rugae. Rugae in *A. exulcerata*, and especially in *A. anatina*, are thicker and more widely spaced when compared to *A. cygnea*.

Shell morphometry

Linear morphometric analysis

Analyses of morphometric shell indexes H/L, W/L, W/H shows 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, $P < 0.001$) are significantly different among species. While the standardized convexity index is significantly different among the three species (Tukey's pairwise comparisons significant at <0.05 level), differences in the angle are 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 2) does not allow a reliable discrimination of these species, displaying largely overlapping characters. The PCA eigen-values describe >99% of the total variability between species. The PC1 axis describes 97.3% and the PC2 axis describes 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, shows 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 3) with only 67% of the specimens being correctly assigned to each species. The major contributors to the principal discriminant factor are 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 2) allow a 90% correct assignment, with the remaining 10% of specimens being misidentified as *A. exulcerata*. Conversely, *A. anatina* is the most misidentified with 28% and 18% erroneous assignments to *A. exulcerata* and *A. cygnea*, respectively.

Geometric morphometric analysis

The first two principal components obtained by the PCA on the 18 Fourier coefficients are retained by the broken stick model, and together explain 38% of the total variance in sagittal shell shape (Fig. 4). The three *Anodonta* species overlap considerably in their sagittal shell shape, so that PC1 values are not significantly different between any of the three species pairs (ANOVA: F = 2.665, df = 2, $P = 0.0733$). However, PC2 values are 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 display 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 is relatively poor. Thus, only 71% of specimens are assigned to

Table 3. 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

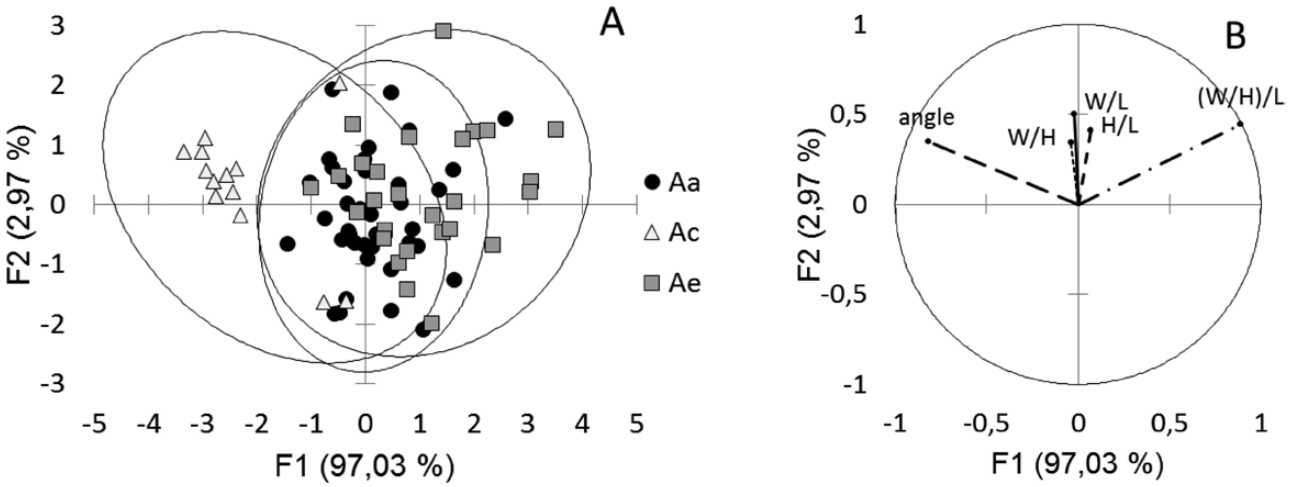

Figure 3. Scatterplot and 95% confidence ellipses of 108 specimens comprising three *Anodonta* species collected from sites in Italy displaying the first two principal component scores obtained by discriminant analysis based on linear biometric values. Aa = *A. anatina*; Ac = *A. cygnea*; Ae = *A. exulcerata*. 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.

the correct species based on DA of the morphometric dataset (Table 4A). While the discrimination between *A. anatina* and *A. cygnea* in this respect was reliable, both of these species were often misidentified as *A. exulcerata* and vice versa. As a result, the proportion of correctly identified specimens is 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 is relatively powerful in correctly assigning specimens to sites of collection, as 81% of specimens are correctly assigned to their site non-regarding the species (Table 4B).

A complete redescription of the species is presented in the systematics section below.

DISCUSSION

Anodontini is consistently 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, 1860, *A. nuttalliana* Lea, 1838 and *A. oregonensis* Lea, 1838) restricted to western North America (Williams *et al.*, 2017) and three species (*Anodonta anatina*, *A. cygnea* and *A. exulcerata*) present in Europe (Froufe *et al.*, 2017; Lopes-Lima *et al.*, 2017b). However, their phylogenetic relationships are still unresolved. The first comprehensive synthesis of the global unionoid fauna placed many North American species in the genus *Anodonta* (Simpson 1900, 1914). Since then, all eastern North American *Anodonta* species have been reassigned to other genera (e.g. *Pyganodon*, *Utterbackia*, *Utterbackiana*; Williams *et al.*, 2017). However, western North American species are still considered to belong to *Anodonta*, but their phylogenetic relationship with European

Table 4. Voucher specimens of *A. exulcerata*; MZUF = Museo de La Specola-Florence, NMBE = Naturhistorisches Museum der Bürgergemeinde-Bern, NCSM = North Carolina Museum of Natural 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

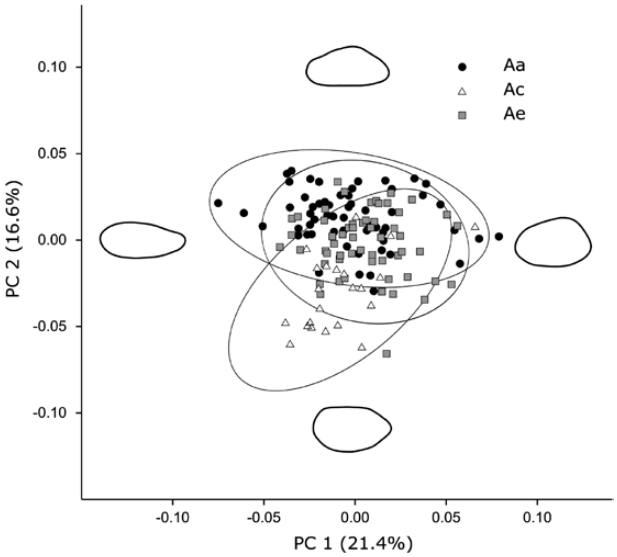

Figure 4. Scatterplot and 95% confidence ellipses of 138 specimens comprising three *Anodonta* species collected from sites in Italy displaying the first two principal component scores obtained by principal component analysis of 18 Fourier coefficients. Synthetic shell outlines of 'extreme' morphotypes are displayed with the anterior margin facing to the left and the dorsal margin to the top of the page. Aa = *A. anatina*; Ac = *A. cygnea*; Ae = *A. exulcerata*.

congeneric species remains contentious (Chong *et al.*, 2008; Blevins *et al.*, 2017; Lopes-Lima *et al.*, 2017b). Until now, the phylogenetic position of *A. nuttalliana*, based on a two-marker approach, clustered with the two European *Anodonta* species, i.e. *A. anatina* and *A. cygnea* (Lopes-Lima *et al.*,2017b). However, in the present study, this species clusters for the first time with all eastern North American Anodontini species, suggesting a separation from *Anodonta* and the need for future multimarker molecular studies including the other western North American Anodontini. As for the European species, the results of the first mitogenome analysis confirms the close relationship between *A. cygnea* and *A. exulcerata* but suggest that *A. anatina* is not congeneric. Furthermore, the status of *Pseudanodonta* is not conclusive (Fig. 2). Again, the inclusion of more taxa and/or nuclear molecular markers is needed to solve this issue.

The three species delineation methods applied here suggest the division of *A. anatina* into four separate species. However, due to the low divergence levels seen in the *COI* uncorrected *p*-distance among these clades (between 1.7% and 3.7%) and lack of sampling in some regions (e.g. south-eastern Europe and Tunisia), we refrained from drawing taxonomic conclusions. These should be addressed in the future using a holistic approach, i.e. combining multimarker molecular analyses with morphological, ecological and biogeographical parameters. The present study confirms the species status of *A. exulcerata* based on the high genetic unc-*p* divergence (8.5% for *COI* and 10% for the whole mitogenome) between *A. exulcerata* and its sister-species *A. cygnea*.

The high genetic divergence between these species was not reflected by any major morphological and/or morphometric differences in the analysed characters. This is probably the reason why *A. exulcerata* has not been accepted until now, being erroneously assigned either to *A. cygnea* or *A. anatina*. Indeed, PCA and DA analyses reveal a broad morphological overlap among *Anodonta* species, leading to 29% of specimens being incorrectly assigned in the field. From the results of geometric morphometric analysis, *A. cygnea* is more easily misidentified with *A. exulcerata*, due to its closer morphological similarity. *Anodonta anatina* shows the highest percentage of correct assignments by geometric morphometric comparison (80%), while *A. exulcerata* and *A. cygnea* are confused with each other in more than 28% of cases. On the contrary, *A. cygnea* is correctly identified in 80% of cases when linear biometric characters are used, while *A. anatina* is more frequently misidentified with *A. exulcerata*. *Anodonta cygnea* tends to be more laterally compressed and posteriorly pointed, with a more obtuse angle between the dorsal and posterior margin compared to the other two species. Although erosion smoothed the umbonal ornamentation in 64% of the specimens examined, when visible, this feature can help

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	Predicted group						
Given 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

discriminating between *A. cygnea* and *A. exulcerata*. However, umbo sculpture is useless for discriminating *A. exulcerata* and *A. anatina*, which present similar double-looped lines.

No clear discriminating character can be identified in the wide and largely overlapping variability of shell shapes of *A. anatina*, *A. cygnea* and *A. exulcerata*, demonstrating once more that shell plasticity evolved as an adaptation to local conditions (e.g. Walker *et al.*, 2001; Hornback *et al.*, 2010; Zieritz *et al.*, 2010; Inoue *et al.*, 2013) hindering the conchological identification of species. This is especially evident in anodontine mussels (Reis *et al.*, 2013; Mezhzherin *et al.*, 2014; Klishko *et al.*, 2018), which display considerable intraspecific shell-shape variation caused by shifts of metabolism at sexual maturity, changes in allometric growth and other physiological characteristics (Zieritz & Aldridge, 2011; Klishko *et al.*, 2016). Moreover, the morphometric analyses were more powerful in discriminating between sites of collection of the specimens than between species. This result confirms that shell shape is more environmentally than genetically controlled, which is congruent with the hypothesis that phenotypic plasticity, allowing survival in a wide range of environments, could be under positive selection in many freshwater mussel species (Baker *et al.*, 2003; Reis *et al.*, 2013).

Equally, only minimal differences were present in anatomical characters between *A. exulcerata* and the other two species. The easiest-to-use quantitative character is the number of papillae series, which is similar in *A. exulcerata* and the closely related *A. cygnea*, but useful to distinguish both species from *A. anatina*. All the other morphological differences shown here are purely qualitative and concern mainly the pigmentation of tissues. Pigmentation is creamy-yellowish in 59% of *A. anatina* and 79% of *A. exulcerata* specimens, while it tends to be brownish in the remaining 41% and 21%, respectively. The papillae have similar coloration in *A. cygnea* and *A. exulcerata*, while those in *A. anatina* are darker, but the most conspicuous difference is the bright orange pigmentation of the tissues in *A. cygnea* (100% of specimens examined). One could argue that the colouring might be excessively tied to external factors to use it as a taxonomic discriminant. However, this distinguishing character was reported for many *A. cygnea* and *A. anatina* populations from other environments and has, therefore, been previously proposed as a character suitable to separate both species (Mordan & Woodward, 1990; Mezhzherin *et al.*, 2014). Differences in pigmentation seem to be associated with the amount and distribution of orange-yellow extracellular calcified granules in interstitial tissues (Colville & Lim, 2003). Being determined by anatomical and physiological features, it has been suggested that the distribution of granules may be a useful character for phylogenetic analyses (Byrne, 2000). Furthermore, shell and mantle-edge pigmentation seems to be mainly under genetic control (Brake *et al.*, 2004; Wen *et al.*, 2013), although susceptible to dietary-induced modifications (Liu *et al.*, 2009). However, unlike the traditionally used conchological characteristics, the plasticity of soft tissue pigmentation is poorly

documented (e.g. Colville & Lim,2003; Prié, 2017) and we fail to find any study specifically addressing the variability of this feature in relation to environmental conditions. While the reliability of such qualitative characters remains to be verified, our study provides further evidence that ecophenotypic plasticity hinders shell morphology-based identification. However, despite the variability and overlap of morphometric characters, they better support the separation of *A. cygnea* from *A. anatina*, than that of *A. exulcerata* from either of the two species. The overlap in morphology 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 have 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 further investigate.

SYSTEMATICS

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, here designated.

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 determine 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 *Anodonta* taxa described from Italy in the later part of the 19th and early 20th centuries. This list of taxa includes at least 56 taxa described from Italy (e.g. Alzona, 1971).

Based on the similarity of the shell and on the coincidence of the sampling spots (including one of the type localities, i.e. Lake Oggiono), the rediscovered species was recognized as *Anodonta exulcerata*, ('Villa') Porro, 1838, using the oldest available name for the *Anodonta* taxa in the studied region (Haas 1969; Graf & Cummings, 2019). The shells of the lectotype specimen of *A. exulcerata* deposited in the Natural History Museum, London (NHMUK1841.5.6.127;

Supporting Information, Fig. S1) and of the paratype specimens from the 'original series' (Zilch, 1967: 111; Senckenberg Museum, N°5166) were analysed in detail before attributing this name to the erroneously synonymized species. Johnson (1971), in reviewing the unionid types in NHMUK, found a specimen labelled *Anodon exulceratus* and listed it as the specimen from Ziegler figured in Sowerby (1870). Ziegler is listed in the Malacology ledger as the donor of *Anodon exulceratus* (Dr T. White, pers. comm. 2/4/2019). Sowerby credited the name to a 'Villa' manuscript in the British Museum, indicating that it was Sowerby's figured type. Johnson credited the species description to Sowerby (1870). Sowerby (1870: species 131 page [48], pl. 33 species 131, page 48, plate 33) listed 'Villa. MS in Mus. Brit'. Johnson (1971) cited *Anodon exulceratus* 'Porro' Sowerby, 1870. Thus, Johnson was aware of the citation of the Villa manuscript by Sowerby, but chose to ignore it and claim it was a Porro manuscript name, ignoring Porro's (1838) description of *Anodonta exulcerata*. Listing of that specimen figured by Sowerby as the figured holotype represents an inadvertent lectotype fixation under Art. 74.6 of the Code (ICZN, 1999). However, Porro (1838) mentioned in his description 'the plurality of individuals' observed. He also listed three lakes in his distribution. This documents that the description of *A. exulcerata* by Porro was based on multiple individuals. Thus, the inadvertent lectotype designation by Johnson, (1971) for *A. exulcerata* 'Porro' Sowerby, 1870 may be valid, but the application of the lectotype to *A. exulcerata* Porro, 1838 by assumption of holotype is invalid as Porro mentions multiple specimens in his description. This NHMUK specimen, NHMUK 1841.5.6.127 is here designated as the lectotype for *Anodonta exulcerata* 'Villa' Porro, 1838.

Shell description: Shell generally thin, equivalve and inequilateral, large (max. length 103 mm, N = 109) elliptical to suboval, moderately inflated. Angle between dorsal margin and posterior margin 124° to 147° (mean = 135°). Anterior margin broadly rounded, posterior margin narrowly rounded to bluntly pointed; ventral margin convex, occasionally flat straight in the middle nearer to the posterior edge; dorsal margin straight to slightly convex in passing from the posterior margin, occasionally extending into a low dorsal wing; posterior ridge rounded, occasionally weakly biangulated distally; posterior slope moderately steep, flat to slightly convex; umbo broad, moderately inflated, elevated slightly above hinge line; umbo sculpture with thin wavy rugae; umbo cavity wide, shallow. Pseudocardinal and lateral teeth absent. Adductor muscle scars rather light shallow (not deep). Nacre is white to bluish white, usually iridescent. Periostracum tawny to olive or brown; small individuals yellowish brown to dark olive, large individuals brownish black with dark green rays of varying width and intensity. Morphological shell features correspond well to the first description of the species (Porro, 1838) and to the lectotype made available from the Natural History Museum, London (Supporting Information, Fig. S1). One discrepancy lies in the fact that, contrary to what is indicated by Porro, we cannot argue that 'in the majority of individuals the upper and lower margins are parallel, and only in a few individuals are distant posteriorly'. On the contrary, the shape of the shell is so variable that it appears haphazard to draw any generalization (Fig 4; Supporting Information, Fig. S8).

Umbo sculpture also appears to be highly variable, ranging from a clearly double-looped to a finely concentric lines arrangement (Supporting Information, Figs S7S, S9).

Soft anatomy description: In life the mantle is creamy white to yellowish or light-brownish (respectively, 79 and 21% of 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 two to three rows, 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 5; 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 *A. exulcerata* specimens sampled in this study (Supporting Information, Fig. S3) 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 am Main (Zilch, 1967). Furthermore, in one of its type localities (Lake Annone), it was the only *Anodonta* species found (Froufe *et al.*, 2017).

Distribution: Anodonta exulcerata is found from the Italian Peninsula to Croatia west of the Dinaric Alps (Froufe *et al.*, 2017), which confirms the distribution reported by Clessin (1876). In northern Italy it appears to be the most common *Anodonta* species.

Habitat and biology: Anodonta exulcerata occur in waters with little or no current and substrates typically composed of mud or muddy sand, often with detritus. Due to misidentification with other *Anodonta* species, information on biology is scarce. Gravid individuals brooding glochidia at different stages of development have been observed from early September to late December in Lake Maggiore and Lake Varese (N. Riccardi, pers. obs.). Glochidial host fish species are unknown.

Conservation status: The fact that *A. exulcerata* has not been previously recognized has precluded any assessment of its conservation status. However, it is widely distributed in the region and locally abundant, which might suggest that currently the species is not at risk.

Comparison with similar species: Close conchological similarity and wide shell plasticity make the use of shell shape for the discrimination of *A. exulcerata* from coexisting congeneric species (i.e. *A. anatina* and *A. cygnea*) unreliable. Like *A. anatina*, *A. exulcerata* tends to be more swollen than *A. cygnea* slightly posterior to the umbo. However, the difference, whenever it exists, may be masked by the broad shell plasticity. Indeed, except for the index of convexity standardized over length, the mean values of the shell measurement ratios were not significantly different (Table 2). To the extent that reliable external features could be identified to distinguish the two central, northern and eastern European *Anodonta* species (Gallenstein 1895, Möller 1933, Bloomer 1937, Franz 1939), it also became apparent that the Italian forms could not be clearly identified (Gallenstein 1894, Falkner 1994). Rather, a mixture of the otherwise species-specific characteristics was often found. Only the analysis of further characters (allozymes, DNA) contributed a new view on this problem providing an objective basis to older assumptions about the peculiarities of the Italian unionid fauna.

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 similar *A. idrina* (Spinelli, 1851), to the *A. anatina* 'group'. Kobelt (1876) reiterated that *A. idrina*, *A. exulcerata* and *A. gibba* (a *nomen nudum*) should not be separated, and emphasizes the enormous difficulties and uncertainties in distinguishing the species of *Anodonta*. This is the only final message to be drawn after getting lost in the enormous variety of conflicting opinions among malacologists of the time.

For the determination of live animals or shells in the field, 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 future investigation.

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SUPPORTING INFORMATION

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

Figure S1. Lectotype of *Anodonta exulcerata* – N° 1841.5.6.127, Natural History Museum, London.
Figure S2. Paratypes of *Anodonta exulcerata* – N° 5166, Senckenberg Museum of Natural History, Frankfurt am Main.
Figure S3. 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).
Figure S4. Aspect of excurrent aperture and papillae in living (left) and freshly dissected (right) *A. anatina* (top), *A. exulcerata* (intermediate) and *A. cygnea* (bottom).
Figure S5. Arrangement of papillae in *A. anatina* (left), *A. exulcerata* (centre) and *A. cygnea* (right).
Figure S6. Coloration of soft tissues in freshly dissected *A. anatina* (left), *A. exulcerata* (center) and *A. cygnea* (right).
Figure S7. 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.
Figure S8. Variability of shell shape of *A. exulcerata* specimens.
Figure S9. 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.
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.