


Lifting the curtain on the freshwater mussel diversity of the Italian Peninsula and Croatian Adriatic coast

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Abstract Freshwater mussels of the order Unionida have been dramatically declining globally. Despite their ecological importance, conservation of these animals has been hindered by unresolved taxonomy and a lack of data on the distribution and status of populations, especially in southern Europe. Although the Italian Peninsula has been noted as a centre of endemism and one of the major refugia of the glacial ages for several taxa, few studies have been performed on the genetic diversity of Unionida. Most importantly, the taxonomic status of several freshwater mussel populations of the Italian Peninsula is still unresolved. Here we present the first comprehensive dataset for the Unionida of the region spanning Italy and the coastal Croatian region (west of the Dinaric Alps). In total,

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191 specimens were collected (85 *Anodonta*, 64 *Unio*, 17 *Microcondylaea bonellii* and 25 *Sinanodonta woodiana*) from 34 sites across the Italian Peninsula and coastal Croatian river basins for molecular identification (COI, 16S and 28S). Genetic analyses were performed to understand major phylogenetic and phylogeographic patterns. Seven species were detected: three *Anodonta* species (*A. anatina*, *A. cygnea* and *A. exulcerata*), two *Unio* species (*U. mancus* and *U. elongatulus*), *Microcondylaea bonellii*, and the invasive *Sinanodonta woodiana*. The presence of three endemic species (*A. exulcerata*, *U. elongatulus* and *M. bonellii*) confirms the importance of the region as a centre of endemism for freshwater mussels. The Apennine Mountains act as an important biogeographic barrier.

Keywords Unionida · Italy · Croatia · Biogeography · Genetic diversity · Conservation

Introduction

Any conservation management plan requires at the very least the following information: (1) clearly defined target species, and data on the species' (2) distribution and (3) population size. However, the taxonomic status of several European freshwater mussel taxa of the order Unionida is still unresolved, despite these animals' importance in providing a range of ecosystem functions and services (nutrient cycling, water purification, bioturbation of the sediments, substrate for other species, among others; Lopes-Lima et al. 2014). The available information on freshwater mussels in Europe is therefore unevenly distributed, with considerable differences in data quality and quantity among countries (Lopes-Lima et al. 2017b). For example, the Mediterranean basins of southern Europe, with the exception of those of Iberia, are still understudied. This is particularly unfortunate, as these basins are of high conservation priority because of their high levels of endemism and the growing demand for water (for agricultural, industrial and domestic activities), which has already caused a decline and loss of endemic biodiversity in many freshwater ecosystems (Albrecht et al. 2006; Benejam et al. 2010).

A particularly high level of freshwater endemism has been described for the Italian Peninsula (for a review see Fochetti 2012). Unfortunately, knowledge of this region's biodiversity, including that of freshwater mussels, is largely incomplete (Lopes-Lima et al. 2017b). Current data indicate that four unionid genera occur in Italy and the Northern Adriatic coast, three of which are native (*Anodonta*, *Unio*, *Microcondylaea*) and one non-

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native (*Sinanodonta*) (Haas 1969; Manganelli et al. 1998; Nagel and Badino 2001; Lopes-Lima et al. 2017b). However, because of the lack of data on the diversity and phylogeographic patterns of freshwater mussels in this region, the real number of freshwater mussel species remains unclear, rendering evaluation of the conservation status of this faunal group a particularly challenging task.

One example of the urgent need for more research in this important geographic area is provided by the genus *Anodonta*. In western and central Europe, two species are currently recognized, i.e., *A. anatina* (Linnaeus 1758) and *A. cygnea* (Linnaeus 1758) (Froufe et al. 2014), but Nagel et al. (1996) suggested the presence of two additional *Anodonta* taxa of uncertain rank in Italy based on allozyme data. Although *A. cygnea* and *A. anatina* are currently globally listed by the IUCN as Least Concern (Lopes-Lima 2014a, b), *A. cygnea* has suffered a dramatic decline and it was assessed as Near Threatened in Europe (Killeen and Aldridge 2011). While morphological distinction between the two recognized *Anodonta* species is extremely difficult because of the high morphological plasticity within each of them (Zieritz and Aldridge 2011) and morphological convergence between them, molecular identifications have been shown to produce more reliable results (e.g. Froufe et al. 2014). A comprehensive molecular study comprising all European *Anodonta* spp., including the Italian populations, is, however, still lacking. Recently, an extensive analysis of the genetic variability of *A. anatina* across Europe identified three major mtDNA clades (Froufe et al. 2014): one formed by all the individuals from Iberia, except those from the Ebro basin; another including all the European non-Iberian and non-Italian samples; and a third grouping the Italian individuals with those from the Ebro basin. This latter clade was suggested as corresponding to one of the previously identified Italian taxa of uncertain rank (i.e., *Anodonta* I; Nagel et al. 1996). However, as Froufe et al. (2014) only included two Italian populations, they refrained from drawing any taxonomic conclusions, as further sampling was still needed.

The genus *Unio* also exhibits extreme intraspecific phenotypic plasticity and regional variation in shell shape. Recent molecular works have updated the phylogeny of this genus, which currently encompasses four main lineages: *pictorum*, *crassus*, *tumidus* and *gibbus* (Froufe et al. 2016a; Lopes-Lima et al. 2017b). Many doubts still exist about which *Unio* species inhabit the Italian Peninsula. Although Lopes-Lima et al. (2017b) suggested that *U. mancus* and *U. cf. elongatulus* may co-occur in Italy and Croatia, no study has yet clarified this issue. Additionally, the question of the presence of *U. mancus* in south-eastern Europe has still not been answered. Despite all these uncertainties, *U. mancus* was listed as Near Threatened in 2014 by the IUCN (Lopes-Lima and Seddon 2014) and is protected in Spain under the name *U. elongatulus*. On the other hand, no IUCN conservation assessment of *U. elongatulus* exists to date, since this name has only recently been revived for the *Unio* population in Lake Maggiore (Prié and Puillandre 2014). There are only a few molecular studies that have included Italian *Unio* populations. Earlier studies based on allozymes, performed on several *Unio* populations across Europe, including Italy, were inconclusive as they revealed a mixed cluster containing several *Unio* species (Badino 1982; Nagel and Badino 2001). A recent study, based on COI sequences, analysed 25 specimens from 13 Italian sites, resulting in only eight distinct haplotypes that clustered in a single haplotype network, thus leading the authors to conclude that they were all *U. elongatulus* (Riccardi et al. 2016).

One of the most understudied European unionid species is *Microcondylaea bonellii* (Férussac 1827). This species is endemic to the Italian Peninsula and the Adriatic drainages of the Balkans (Lopes-Lima et al. 2017b). It is listed in Annex V of the European Community Habitats and Species Directive as a protected species and has been assessed as Vulnerable in Europe by the IUCN (Albrecht et al. 2011). However, information on *M. bonellii* is limited. The only molecular study available so far, detected no genetic variability

in one north-eastern Italian population, based on data from seven allozyme loci from 12 specimens (Nagel and Badino 2001). Reproduction, growth and habitat preferences have also been studied in this population (Nagel et al. 2007). Altogether, it is currently assumed that the few extant Italian populations are likely to be isolated (Nagel and Badino 2001).

Finally, the Chinese pond mussel *Sinanodonta woodiana* (Lea 1834) is a freshwater mussel originating from Asia. It was first introduced to Europe via Romania in 1979 (Sárkány-Kiss 1986) from where it quickly spread to several other European countries (Lajtner and Crnčan 2011). It was first reported in Italy in 1996 (Manganelli et al. 1998) and since has rapidly colonized several Italian regions, including the south (Cianfanelli et al. 2007; De Vico et al. 2007). In Croatia, this species was recorded in 2001 in the river Danube, which forms the border between Croatia and Serbia (Paunović et al. 2006). In the meantime, *S. woodiana* has become widespread in the eastern, central and north-western parts of Croatia (Lajtner and Crnčan 2011, 2014) and it is expected to continue spreading further into the south.

European phylogeographical studies have improved our knowledge of refugial areas and the patterns of recolonization routes. Although the majority of published studies on freshwater mussels have evaluated genetic diversity based mainly on partial distributions (e.g. Nagel et al. 1996; Machordom et al. 2003; Araujo et al. 2005, 2009; Geist et al. 2010; Prié and Puillandre 2014), recent ones have included detailed phylogeographic data covering entire species ranges (Froufe et al. 2014, 2016a, b). These studies highlighted Iberia, North Africa and the Balkans as primary refugia, in accordance with what has been described for the majority of other taxa (e.g. Schmitt 2007). Therefore, the deficit of Italian studies is a significant shortfall, given the role of this region in the context of Europe's Pleistocene phylogeography. In most cases, this region is characterized by simpler phylogeographic patterns compared to other known European refugia: several species exhibit a genetic subdivision into northern and southern lineages within the continuous Italian range (e.g. Schmitt 2007). Moreover, while the southern part of the Italian Peninsula has been identified as a major hotspot of genetic diversity, as a glacial/interglacial refugium and as a source for later (re)colonizations of northern areas (e.g. Magri 2008; Canestrelli et al. 2010), its northern part has long been acknowledged as an area of postglacial (re)colonization (e.g. Salvi et al. 2013; Vilaça et al. 2014). Although the majority of the studies that support the refugia-within-refugia scenario (Gómez and Lunt 2007) have been undertaken in the Iberian Peninsula, including those on freshwater mussels (e.g. Araujo et al. 2005, 2009; Froufe et al. 2014, 2016a), recent studies have evidenced patterns of population genetic differentiation that could also be explained by the occurrence of this scenario in Italy (e.g.; Giovannotti et al. 2010; Bogdanowicz et al. 2015; Mezzasalma et al. 2015). In this context, we aim to provide information for the conservation of freshwater mussels in the Italian Peninsula and Croatian Adriatic coast. In order to do that we intend to: (i) clarify the systematics of the extant freshwater mussel species, (ii) detect the presence of potentially endemic lineages, and (iii) identify potential biogeographic areas within the region.

Materials and methods

Sample collection

In total, 191 unionid (i.e., 85 *Anodonta*, 64 *Unio*, 17 *Microcondylaea bonellii* and 25 *Sinanodonta woodiana*) specimens were collected from 34 sites across the Italian Peninsula and coastal Croatian river basins during 2014–2015 (Fig. 1).



Fig. 1 Map of the 34 sites at which *Unionida* were sampled across the Italian Peninsula and coastal Croatian river basins

A small tissue sample from the foot was collected in the field (following Naimo et al. 1998) and placed directly in 99% ethanol for subsequent molecular analysis. Each individual was identified morphologically to the genus level and photographed, and immediately returned to the substrate. In addition, we preserved one to two voucher specimens (i.e., whole animal) per genus per population in 95% ethanol to be deposited in selected natural history museums, i.e., the Museo de La Specola-Florence, the Naturhistorisches Museum der Bürgergemeinde Bern, and at the North Carolina Museum of Natural Sciences. Genomic DNA was extracted from tissue samples using a standard high-salt protocol (Sambrook and Fritsch 1989).

Sequencing, alignment and phylogenetic analyses

The F-type COI gene was amplified for all specimens using LCO_22me and HCO_700dy primers (Walker et al. 2006), applying annealing temperatures ranging from 50 to 55 °C and PCR conditions as described by Froufe et al. (2014). Also, 28S rDNA and 16S rDNA were amplified from two individuals of each of the main COI mtDNA clades recovered (see results), using 28S-RD1.3f and 28S-rD4b (Whiting 2002), and 16SL and 16SH primer pairs (Palumbi et al. 1991), and applying annealing temperatures ranging from 45 to 48 °C (28S) and 45 °C (16S), and the same PCR conditions as for COI. Sequences were obtained

using the BigDye sequencing protocol (Applied Biosystems 3730xl) by Macrogen Inc., Korea. Forward and reverse sequences were edited and assembled using ChromasPro 1.7.4 (Technelysium, Tewantin, Australia). As the divergence between female and male mitochondrial genomes of the same species in the family Unionidae is no less than 30% (e.g. Froufe et al. 2016c), this threshold was used as a reference to assure that all obtained COI sequences were F-type.

Considering common morphological misidentifications between *A. anatina* and *A. cygnea*, a phylogenetic analysis was carried out including (1) all *Anodonta* COI sequences obtained in the present study, (2) all available COI sequences of *A. cygnea* and (3) previously reported European and Ebro+Italian *A. anatina* clades (Froufe et al. 2014) (Online Resource 1), using *Cristaria plicata* (EU698940) as outgroup. The dataset was aligned with ClustalW, in Bioedit 7.2.5 (Hall 1999) and the best-fit models of nucleotide substitution were selected under the corrected Akaike Information Criterion using JModelTest 2.1.8 (Darriba et al. 2012) for each codon position. Model GTR+I was optimal for the first COI codon position, model F81 was optimal for the second, and model GTR was optimal for the third. Bayesian Inference (BI) was performed in MrBayes v3.2.5 (Ronquist et al. 2012) using the previously selected models. Analyses were initiated with program-generated trees and four heated Markov chains with default incremental heating. Two independent runs of 24×10^6 generations were sampled at intervals of 1000 generations producing a total of 24,000 trees. Burnin was determined upon convergence of log likelihood and parameter values using Tracer 1.6 (Rambaut et al. 2014).

For the *Unio* dataset, all available sequences from *U. mancus*, *U. pictorum* and *U. elongatulus* were included (Online Resource 2) and aligned as described above. The same procedure was applied for the *M. bonellii* alignment dataset (Online Resource 3). Concerning the *Sinanodonta woodiana* data set, all newly sequenced individuals were aligned with all available *S. woodiana* sequences and other *Cristariini* species in order to account for existing taxonomic uncertainties, using appropriate outgroups (Online Resource 4). The best-fit models of each COI codon (1: GTR+G; 2: F81; 3: HKY+G) and posterior BI analysis were performed as described above.

Four additional phylogenetic analyses were performed on the COI, 16S, 28S and the concatenated alignment datasets, respectively, obtained from two individuals of each of the main COI mtDNA clades recovered (see results). The best-fit models were selected as described above for COI (GTR+I), 16S (GTR+G) and 28S (GTR+G) gene fragments. BI was performed as described above. Detailed information, including the individuals and outgroups analysed are given in Online Resource 5.

All sequences were deposited in GenBank (Online Resources 1–4) and haplotype networks were calculated using TCS 1.21 (Clement et al. 2000). Sequence divergences (uncorrected *p*-distance) were assessed using MEGA 6.0 (Tamura et al. 2013) and sequence and nucleotide diversity measures were calculated using DnaSP v5.1.0.1. To test for molecular signatures of demographic expansion and hypothesizing post-Pleistocene range expansions, Fu's *F*_s (1997) and Tajima's *D* (1989) statistics were calculated.

Distribution maps

Geographic distribution maps were constructed using genetic data (coloured circles in maps) and the published dataset (Lopes-Lima et al. 2017b) for the native unionid species (shaded light colours in maps). For *S. woodiana*, distribution data were compiled from the literature (Yurishinets and Kornishin 2001; Paunović et al. 2006; von Proschwitz 2008;

Pou-Rovira et al. 2009; Lajtner and Crnčan 2011; Klenovšek et al. 2012; Mienis 2012; Colomba et al. 2013; Tomović et al. 2013; Reischütz et al. 2014; Renda and Niero 2014).

Results

All mtDNA COI sequences obtained were considered F-type, as no sequences similar to the M-type were detected.

Anodonta spp.

The final *Anodonta* spp. COI alignment included 160 sequences (Online Resource 1) and was 567 bp long. No insertions, deletions or stop codons were observed after translating all sequences to amino acids. The BI tree showed two clearly defined and well supported clusters (Fig. 2). One corresponded to *A. anatina* and was split into two groups matching the previously reported European and Ebro+Italian clades (Froufe et al. 2014), with a mean distance of 2.3% (uncorrected *p*-distance) and with the newly collected Italian *A. anatina* individuals falling in either of these two groups (Online Resource 1). The other main cluster retrieved in the phylogeny comprised two species with a divergence of 8.3% (uncorrected *p*-distance), i.e., *A. cygnea* and *A. exulcerata* (Fig. 2).

Anodonta anatina was present at only four sites (Online Resource 1; Fig. 3a, left). At two of these sites, only individuals belonging to the Ebro+Italian clade were found, whereas at the other two sites, individuals from both the European and the Ebro+Italian clades were present in sympatry (Fig. 3a, right). The European clade, represented by 42 analysed sequences and 10 unique haplotypes, and the Ebro+Italian clade, represented by 41 analysed sequences and 8 haplotypes, were separated by a minimum of 14 mutations (Fig. 3a, centre). Neither of the haplotype networks revealed any geographic structure of genetic variability.

Anodonta cygnea was present at only three sites (Online Resource 1; Fig. 3b, left), whereas *A. exulcerata* was found at 15 sites, including two coastal Croatian sites (Online Resource 1; Fig. 3c, left), and thus was the most widespread *Anodonta* species and endemic to the region (from Italian Peninsula to Croatia west of the Dinaric Alps). Only in Lake Maggiore were the two species found in sympatry. Both species networks revealed star-shaped topologies but once again without geographic structure of genetic variability (Fig. 3b, c, right). However, with 12 haplotypes in a total of 35 individuals sequenced, haplotypic diversity of *A. exulcerata* was higher ($Hd = 0.697$) than that of *A. cygnea* ($Hd = 0.274$) across its entire distribution. Moreover, most of the newly sequenced Italian *A. cygnea* individuals correspond to the most common haplotype found throughout Europe. Tests of demographic history for *A. cygnea* yielded significantly negative results for *D* (Tajima's) and *F_s* (Fu's): $D = -2.10158$ ($p < 0.05$) and $F_s = -3.37300$ ($p < 0.02$), respectively.

Unio spp.

The final *Unio* spp. COI alignment included 250 sequences (Online Resource 2), was 600 bp long, and no insertions, deletions or stop codons were observed after translating all sequences to amino acids. Two *Unio* spp. were found in Italy and coastal Croatian sites, i.e., *U. mancus* and *U. elongatulus*, while the *U. pictorum* sequences analysed ($n = 54$)

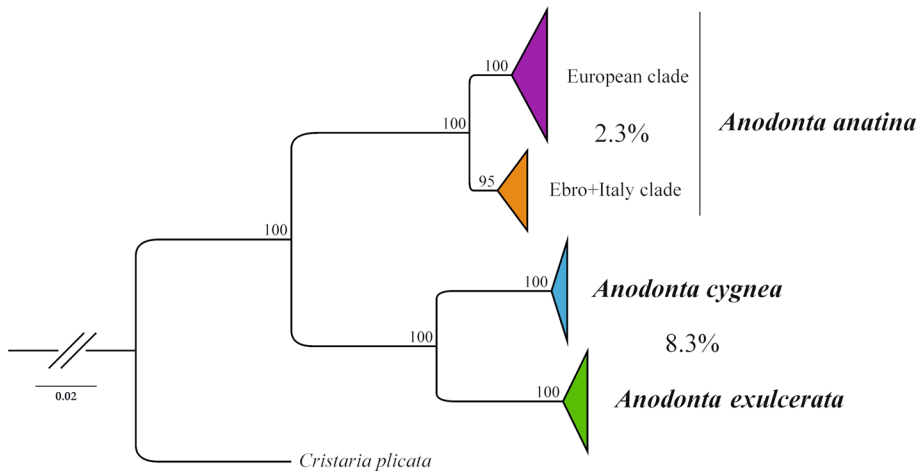


Fig. 2 *Anodonta* phylogenetic tree obtained by Bayesian Inference analysis of the COI fragment (details in the main text and Online Resource 1). Support values are given as Bayesian posterior probability above nodes. The tree was rooted with *Cristaria plicata*. Percentage values refer to uncorrected *p*-distance between the adjacent clades. *Anodonta anatina* sampling sites and distribution map of the two previously described *A. anatina* mtDNA clades

were restricted to populations outside this geographical area (Online Resource 2; Fig. 4a, left). *Unio elongatulus* was present at 18 sites in Italy and one in Croatia, *U. mancus* was found only at three south-west Italian sites, and both species never occurred in sympatry (Online Resource 2; Fig. 4b, left). *Unio mancus* was represented by 143 analysed sequences that were collapsed into 15 unique haplotypes, being 3.8% divergent (uncorrected *p*-distance) from *U. elongatulus* (Fig. 4a, right). *Unio elongatulus* was represented by 53 analysed sequences that were collapsed into 11 unique haplotypes (Fig. 4a, right). Once again, no geographic structure of genetic variability was depicted in either of the species' haplotype networks. In more detail, the *U. mancus* network revealed that only the subspecies *U. m. requienii* and *U. m. turtonii*, as described by Prié and Puillandre (2014), are present in Italy (Fig. 4b, right).

Microcondylaea bonellii

The *M. bonellii* COI alignment comprised 26 sequences (Online Resource 3) and was 600 bp long, and no insertions, deletions or stop codons were observed after translating all sequences to amino acids. *Microcondylaea bonellii* was only present at four sites (Online Resource 3; Fig. 5 left), and only four haplotypes were retrieved (Fig. 5 right).

Sinanodonta woodiana

The final *Sinanodonta* spp. COI alignment comprised 65 sequences (Online Resource 4) and was 626 bp long, and no insertions, deletions or stop codons were observed after translating all sequences to amino acids.

The non-native *S. woodiana* was widespread across the Italian Peninsula and present at nine sites (Online Resource 4; Fig. 6 left). The BI tree revealed that all the 28 Italian individuals analysed in the present study shared the same haplotype, which is also shared

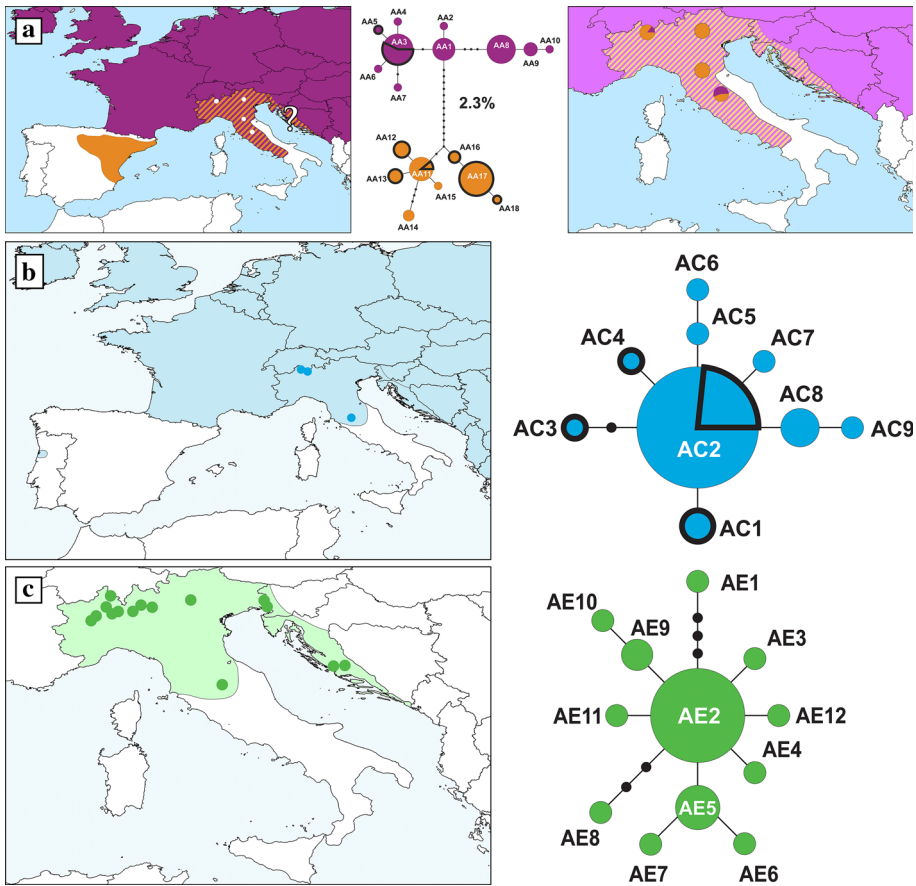


Fig. 3 **a** Left *Anodonta anatina* sampling sites (this study) and distribution map of the two previously described *A. anatina* mtDNA clades (based on Froufe et al. 2014; Lopes-Lima et al. 2017b; and this study). Centre COI haplotype (TCS) network showing the relationships of the two *A. anatina* clades. Circle size is proportional to the observed haplotype frequencies, *thick outlines* represent individuals sampled in Italy and *black points* represent unobserved haplotypes and potential intermediates. Uncorrected *p*-distance between the two major haplogroups is also shown. Right Geographic distribution of *A. anatina* sampling sites with pie charts depicting the ratio of individuals from both clades in each site. Colour codes: *purple* European clade; *orange* Italy+Ebro clade. *Question mark* indicates where no genetic information is available. **b** Left *Anodonta cygnea* sampling sites (this study) and distribution (Lopes-Lima et al. 2017b and this study). Right COI haplotype (TCS) network showing the relationships of the *A. cygnea* haplotypes. Circle size is proportional to the observed haplotype frequencies, *thick outlines* represent individuals sampled in Italy+Croatia, and *black points* represent unobserved haplotypes and potential intermediates. **c** Left: Geographic distribution of *A. exulcerata* sampling sites (this study) and distribution (Lopes-Lima et al. 2017b and this study). Right COI haplotype (TCS) network showing the relationships of the *A. exulcerata* haplotypes. Circle size is proportional to the observed haplotype frequencies and *black points* represent unobserved haplotypes and potential intermediates. (Color figure online)

by all available sequences from Europe and four individuals from the Yangtze basin, China (Online Resource 4; Fig. 6 right). A very well supported sister clade, here referred to as *Sinanodonta* sp. 1, was recovered, containing four Chinese and two Japanese specimens (Online Resource 4; Fig. 6 right). Two individuals from South Korea clustered in a separate group, here referred to as *Sinanodonta* sp. 2, and which was sister to *S. woodiana* and

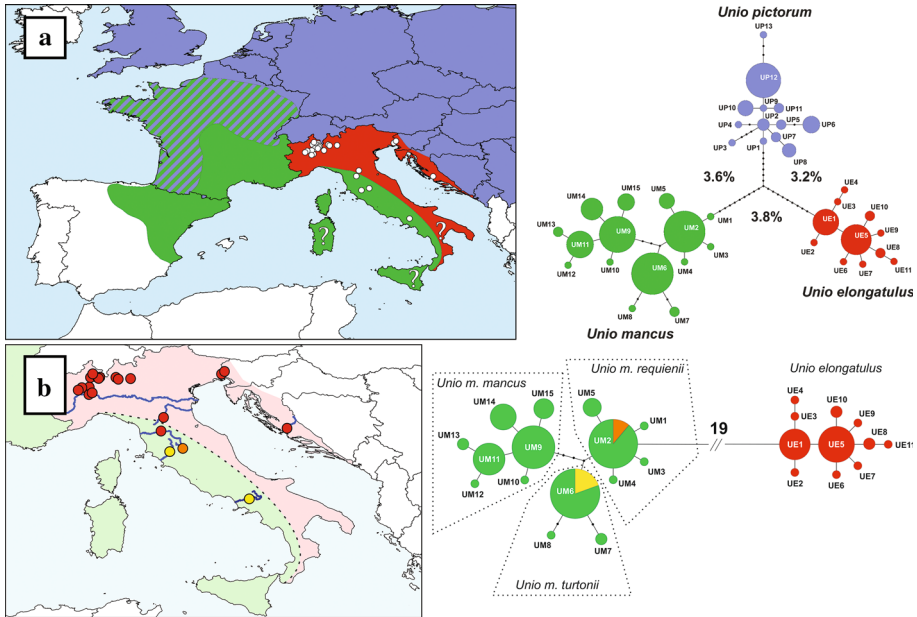


Fig. 4 **a** Left *Unio mancus*, *Unio elongatulus*, and *Unio pictorum* sampling sites and distributions. Right COI haplotype (TCS) network showing the relationships of the three *Unio* species. Circle size is proportional to the observed haplotype frequencies, and black points represent unobserved haplotypes and potential intermediates. Uncorrected *p*-distance between the three species is also shown. Colour codes; green *U. mancus*; red *U. elongatulus*; blue *U. pictorum*. Question mark indicates where no genetic information is available. **b** Left *U. mancus* (green) and *U. elongatulus* (red) distribution maps in the study area. Circles represent sampling sites: orange *U. mancus* Arno population, yellow *U. mancus* Volturno and Ombrone populations; red *U. elongatulus* populations. Right COI haplotype (TCS) network showing the relationships of the three *U. mancus* subspecies (delimited by dashed lines) previously described by Prié et al. (2012). In both haplotype networks, circle size is proportional to the observed haplotype frequencies, and black points represent unobserved haplotypes and potential intermediates. (Color figure online)

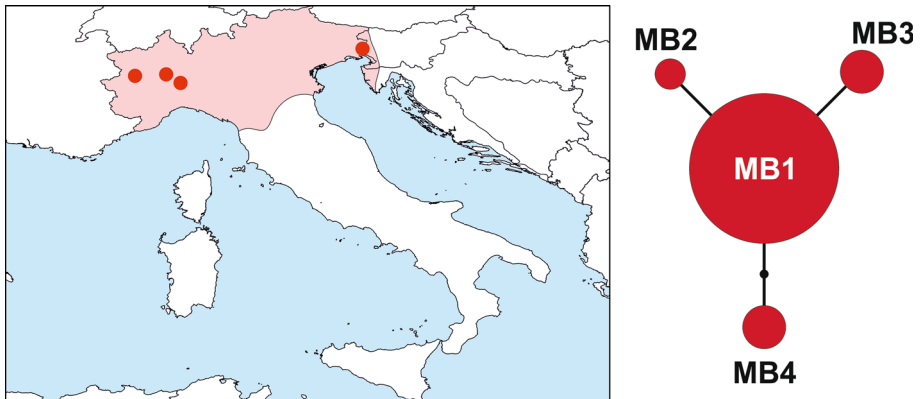


Fig. 5 *Microcondylaea bonellii* sampling sites, distribution map and COI haplotype (TCS) network showing the relationships of the four haplotypes retrieved

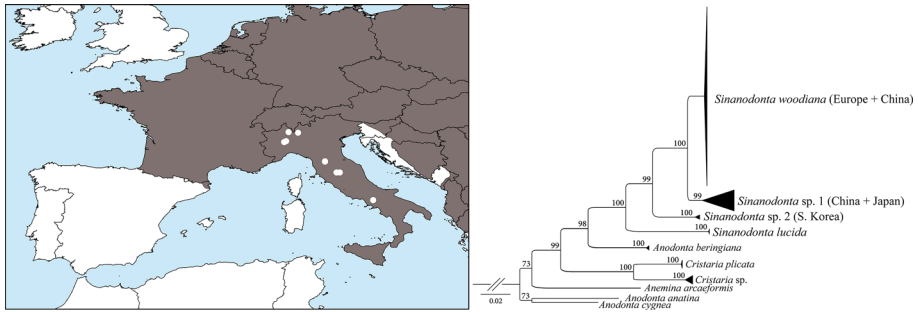


Fig. 6 *Left* *Sinanodonta woodiana* sampling sites and invaded European distribution. *Right* *Sinanodonta* phylogenetic tree obtained by Bayesian Inference analysis (see details in the text and Online Resource 4). Support values are given as Bayesian posterior probabilities above nodes

Sinanodonta sp. 1 (Online Resource 4; Fig. 6 right). The other *Sinanodonta* species analysed, i.e., *S. lucida*, forms a distinct individual clade (Online Resource 4; Fig. 6 right).

Combined phylogeny

The final COI alignment, performed on two individuals of each of the main COI mtDNA clades recovered, comprised 18 sequences (Online Resource 5) and was 593 bp long, and no insertions, deletions or stop codons were observed after translating all sequences to amino acids. The corresponding 16S and 28S alignments were 491 and 702 bp long, respectively. All individual phylogenetic BI trees were congruent (data not shown), with the concatenated BI tree (alignment 1786 bp long) presented in Fig. 7. The COI+16S+28S phylogeny recovered revealed close affinity of *A. exulcerata* with *A. cygnea*. Moreover, *A. anatina* clustered with these two species and *S. woodiana* is the sister species of the *Anodonta* clade (Fig. 7). As expected, all the *Unio* species clustered together, whilst *M. bonellii* is closer to *Potomida littoralis* (Cuvier, 1798).

Discussion

The present study represents the first comprehensive investigation of the Unionida of the Italian Peninsula and in a coastal Croatian river basin. Mitochondrial and nuclear sequence data collected in representative surveys across the study region revealed the presence of six native and one non-native species: three *Anodonta* species (*A. anatina*, *A. cygnea*, *A. exulcerata*), two *Unio* species (*U. mancus*, *U. elongatulus*), *Microcondylaea bonellii* and the non-native *Sinanodonta woodiana*. Our data resolve previous taxonomic uncertainties. This, combined with the detailed distribution data presented here, is essential for conservation management plans as well as future ecological research. Previous relevant scientific literature from this region was unfortunately based on incorrect taxonomy; for these studies, covering a range of scientific fields, including ecology (e.g. Ravera and Sprocati 1997; Ravera et al. 2007), physiology (Campanella et al. 2005; Carella et al. 2016), toxicology (Guidi et al. 2010; Poletini et al. 2015; Sabbioni et al. 2015), and aquaculture (Panini et al. 2008; Sicuro et al. 2010, 2015), reanalysis using the taxonomy and distribution revealed in the present study might be necessary. For example, the study species of Ravera and Sprocati (1997), Ravera et al. (2007), and Sicuro et al. (2010) is *U. elongatulus*

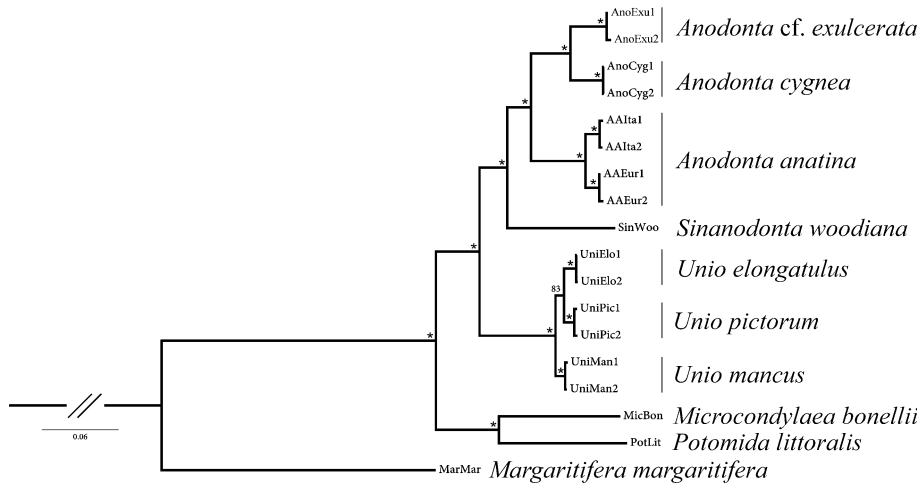


Fig. 7 Bayesian Inference phylogenetic (COI+16S+28S) tree of the Unionida from the Italian Peninsula and a coastal Croatian river basin here investigated: six native and one invasive species. *Unio pictorum* and *Potomida littoralis* specimens were added for phylogenetic comparison and *Margaritifera margaritifera* was included as the outgroup (see details in the text and Online Resource 1). Support values are given as Bayesian posterior probability above nodes

rather than *U. mancus*, which is only present in southern Italy, and when describing the host fishes of *Anodonta cygnea*, Giusti et al. (1975) were not aware that three *Anodonta* species occurred in Trasimeno Lake instead of one.

Anodonta

Phylogenetic analyses of *Anodonta* populations sampled in the present study recovered unexpected results. First, the Italian Peninsula hosts an unexpectedly high species diversity for this genus, with three species that exhibited COI genetic divergence values of 14.5% (uncorrected *p*-distance) between *A. anatina* and *A. exulcerata*, and 8.3% between *A. cygnea* and *A. exulcerata*. Moreover, *A. exulcerata* seems to be the most common and widespread Anodontinae in Italy (found in 15 sites), also occurring in a coastal Croatian river basin (Fig. 3c, left). 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 Bürgergemeinde Bern (NMBE 549733 and NMBE 549734), and two at the North Carolina Museum of Natural Sciences (NCSM 102851 and NCSM 102852). Since *Anodonta exulcerata* (Porro, 1838) is the oldest available name for the *Anodonta* taxa in the studied region (Haas 1969; Graf and Cummings 2017), *A. exulcerata* is used herein for this newly detected *Anodonta* species. The shell morphology of the *A. exulcerata* sampled in this study is consistent with the holotype of *A. exulcerata* (Natural History Museum, London: Holotype NMNHUK 41-5-6-127). Furthermore, in its type locality (Lake Annone) it was the only *Anodonta* species found in the present study. Nevertheless, the taxonomy of *A. exulcerata* should be further investigated.

In a previous study (Froufe et al. 2014), all Italian *A. anatina* individuals analysed clustered within an exclusive Ebro+Italian mtDNA clade, with no Italian individuals falling in the other two main mtDNA clades, i.e., Iberia and Europe. However, the

inclusion of additional *A. anatina* populations and individuals in the present study resulted in some Italian individuals clustering in the European clade (Figs. 2, 3a). Moreover, individuals from two distinct *A. anatina* clades were for the first time found in sympatry, i.e., individuals from both European and Ebro+Italian clades were collected in both Trasimeno (56% European) and Maggiore (15% European) lakes. Only two haplotypes were retrieved from the newly collected Italian individuals that clustered in the European clade, while six haplotypes were retrieved from the Ebro+Italian clade (in a total of eight described) (Fig. 3a, centre). This rather striking difference in haplotype diversity between sympatric *A. anatina* clades could indicate that the two European *A. anatina* haplotypes actually represent introductions into these Italian lakes, possibly in the larval stage through fish host stocking. This hypothesis is supported by the fact that Lake Trasimeno and Lake Maggiore are rich in non-native fish species (Gherardi et al. 2008; Volta and Jepsen 2008; Boggero et al. 2016), originating mainly from Central Europe. Our observation that the Ebro+Italy mtDNA clade contains higher genetic diversity than the European clade, despite occupying a more restricted geographical area, supports the idea that Italy served as an important refugium during the Pleistocene glaciations, as has been documented for other taxa (e.g. Taberlet et al. 1998; Hewitt 1999).

The available literature suggests the presence of *A. anatina* in the River Volturno in the south of Italy and coastal Croatia (Lopes-Lima et al. 2017b). However, despite intensive surveying efforts, we were unable to find this species as far south as Volturno in Italy (the southernmost locality was Lake Trasimeno) nor in the same coastal Croatia river basin where *A. exulcerata* was found, although more extensive sampling in this country is needed to confirm our observations. Contrary to global assessments of *A. anatina*, which is considered a widespread and common species that can attain high densities (currently listed by the IUCN as Least Concern; Lopes-Lima 2014a, b), the species is not very common in Italy. Moreover, the recent decline in Italy (Cappelletti et al. 2009) might be accelerated by the spread of *S. woodiana*, which negatively affects *A. anatina* populations in other regions (Donrovich et al. 2017). Therefore, a systematic and comprehensive future survey is needed to properly assess the species' threatened status in the region.

The haplotype network for *A. cygnea*, which occurs all over Europe, revealed a typical star-shape, suggesting that this species has undergone a recent bottleneck followed by rapid expansion. This scenario is further supported by the tests of demographic history. Also, most of the newly sequenced Italian individuals possess the most common haplotype, found throughout Europe (Fig. 3b, right). The question of the theoretical center of diversity of *A. cygnea* thus remains unanswered. Although *A. cygnea* was the rarest *Anodonta* species in the present study, genetic diversity of the specimens analysed was considerable. Of the nine known *A. cygnea* haplotypes, we retrieved four haplotypes (three private) in Italy alone. The probability that this species was recently introduced in the region is consequently low. The very low number of *A. cygnea* populations found in the Italian Peninsula (i.e., only three sites) is more likely a result of the declining global distribution of this species, which was assessed as Near Threatened within its European range in 2011 (Killeen and Aldridge 2011).

Anodonta exulcerata appears to be endemic to the region ranging from the Italian Peninsula to Croatia west of the Dinaric Alps. The discovery of a third *Anodonta* species in the region, *A. exulcerata*, in fact the most common *Anodonta* species in Italy, was unexpected. The reason why this species has not been discovered before is possibly related to its conchological similarity to the other two co-existent congeneric species, *A. anatina* and *A. cygnea*. Morphological identification is notoriously difficult in almost all Unionida because of the high morphological variability within species and morphological convergences

between species, but the problem is particularly common in Anodontinae, which lack diagnostic teeth (e.g. Zieritz et al. 2010; Lopes-Lima et al. 2017a). As a result, Haas (1969), for example, incorrectly lumped all the European *Anodonta* species—including all Italian and Dalmatian types—under a single species, *A. cygnea*. However, based on enzyme electrophoresis data, Nagel et al. (1996) identified two Italian *Anodonta* taxa of uncertain rank: *Anodonta* I, which corresponds to the Ebro+Italian mtDNA clade of *A. anatina* (Froufe et al. 2014), and *Anodonta* II, probably corresponding to *A. exulcerata* of the present study. Despite a lack of comprehensive surveys or long-term monitoring studies on *Anodonta* species, our data indicate that *A. exulcerata* is widely distributed in the region and locally abundant, which might suggest that its conservation status is not worrisome. However, as for other European Unionida, the lack of basic ecological baseline data (e.g. distribution, density and population structure) makes quantification of population change challenging. This can give the impression of population stability, and thus more research is needed for a precise assessment of the conservation status of this species.

Unio

Only two *Unio* species were found in Italy and Croatia, *U. mancus* and *U. elongatulus*. The two species have never been found in sympatry and lack geographic structure of genetic variability.

Unio elongatulus was only recently discovered by Prié and Puillandre (2014) in Lake Maggiore in Italy. In a recent review of the freshwater mussels of Europe (Lopes-Lima et al. 2017b), this distinct *Unio* species was listed as *Unio* cf. *elongatulus* because the type locality of the species is the North Adriatic rivers (Haas 1969), although only specimens from Lake Maggiore were known at the time. In the present study, we confirm the presence of this species in Croatia as well as in Italy, thereby confirming the validity of the name *U. elongatulus* for this taxon. *Unio elongatulus* is distributed from north of the Apennines in Italy to coastal Croatia in the east, although one population of *U. elongatulus* was found in Florence (Bisenzio; Online Resource 2) south of the Apennines (Fig. 4). However, presence of the invasive *S. woodiana* in the same locality might suggest that both species were introduced to this highly disturbed and urbanised site. As previously described for the pre-Alpine Lakes in Italy (Riccardi et al. 2016), the present haplotype network does not reveal any geographic structure and seems to indicate that *U. elongatulus* has stable populations in the region.

The other *Unio* species found in Italy, *U. mancus*, seems to be restricted to the river basins south of the Apennines. *Unio mancus* has previously been recorded from eastern Iberia, France and Corsica, as well as from Sardinia and Sicily, though the latter records were published in the absence of molecular data (Lopes-Lima et al. 2017b). Prié et al. (2012) divided *U. mancus* populations of Iberia and France into three subspecies. However, this division was based on very low COI divergence and allopatric distribution, and did not include the Italian populations. In the present study, we confirm the presence of *U. mancus* in Italy and reveal that Italian populations fall within two of the subspecies of Prié et al. (2012): the Arno population corresponds to *Unio m. requienii*, while the Ombrone and Volturno populations correspond to *Unio m. turtonii* (Fig. 4). The lack of geographic structure between the newly analysed individuals and the remaining populations (i.e., France and Spain) gives further support to the integration of these two subspecies into one (i.e., *Unio m. turtonii*) present in eastern France, Corsica and Italy, leaving *Unio m. mancus* restricted to its western distribution. Further studies increasing the sampling coverage in south-eastern France and eastern Italy and using additional markers (e.g. nuclear and male

mtDNA) could shed some light on this taxonomic issue. Finally, the Sardinian and Sicilian *U. mancus* populations need further research, including molecular analyses to resolve the genetic diversity patterns across the species' entire range.

Due to the until recently unresolved taxonomic status of *U. elongatulus* and *U. mancus*, the two species have been regularly misidentified or synonymised. This has resulted in a lack of clarity concerning their conservation status and legal protection. *Unio elongatulus* is listed as a protected species in the Bern Convention and as a species of community interest in the Habitats Directive, and therefore legally protected in EU countries. *Unio mancus*, on the other hand, is protected as a synonym of *U. elongatulus* in Spain. Due to these taxonomic complications, there still needs to be an IUCN assessment for *U. elongatulus*, and the assessment for *U. mancus* should be revised to include all the Italian *Unio* populations. The present study highlights the urgent need for new assessments for both species.

Microcondylaea bonellii

In a recent phylogeny *M. bonellii* clustered with the Middle Eastern *Leguminaia wheatleyi* and the western North American *Gonidea angulata* within the tribe Gonideini (Lopes-Lima et al. 2017a). *Microcondylaea bonellii* populations showed a disjunctive and fragmented distribution in that analysis, suggesting that the remaining populations are relicts of a much wider distribution in the northern hemisphere. At present, *M. bonellii* is considered endemic to the Italian Peninsula and the Adriatic drainages of the Balkans, and currently known only from three isolated populations in the Po basin (west) and in one population in the east, in the Isonzo river (Online Resource 3, Fig. 5 left). The 17 individuals analysed from the west (Po basin) possessed only three haplotypes (COI; only one for both 16S and 28S), while the Isonzo River Basin in the east exhibited one additional haplotype (Online Resource 3, Fig. 5 right). Thus, the overall genetic diversity was very low, confirming the findings of a previous molecular study using allozymes (Nagel and Badino 2001), in which a population from the Isonzo River showed no genetic diversity. *Microcondylaea bonellii* is one of the most understudied European unionid species and is known only from a small number of isolated sites, with one of the lowest genetic diversities recorded in a European freshwater mussel species (Froufe et al. 2014, 2016a, b; Lopes-Lima et al. 2017b). Therefore, *M. bonellii* is likely to be even more threatened than presently considered (i.e., Vulnerable; Albrecht et al. 2011) and its conservation status should be urgently reassessed.

Sinanodonta woodiana

The non-native *S. woodiana* is now established in most of the Italian Peninsula and is present in Croatia (Lajtner and Crnčan 2011). As expected for a recent introduction, no genetic diversity was detected (COI), not only in Italy, but in its entire invaded European range. The *S. woodiana* sequences available on GenBank seem to correspond to three distinct species (COI uncorrected *p*-distance >5%) (Fig. 6 right). All Italian individuals sequenced in the present study share the same haplotype with all other available *S. woodiana* sequences from Europe and four individuals from the Yangtze basin (China), here referred to as *S. woodiana*. Since this species has a negative impact on several native unionids in Europe, especially on *A. anatina* (Sousa et al. 2014), the present study highlights the need for a thorough generic taxonomic revision that should include all of the genetic diversity now revealed.

Conclusions and future directions

The present study highlights Italy and coastal Croatia as a biogeographic region of particular importance for freshwater mussel diversity. The six unionid species present in this region display two main phylogeographic patterns: (i) no geographic genetic structure, detected in the three *Anodonta* species; and (ii) the Apennines as a major barrier, separating *Unio elongatulus* and *Microcondylaea bonellii* in the north from *Unio mancus* exclusively in the south. Of the three endemic species present in the area, i.e., *A. exulcerata*, *U. elongatulus* and *M. bonellii*, the latter two are restricted to the northern region between the Alps and the Apennines. This situation strongly suggests that this area acted as a glacial refugium for *U. elongatulus* and *M. bonellii*. Remarkably, neither of these two species was successful in colonising river basins outside their glacial refugium. On the other hand, *A. cygnea*, *A. anatina* and *U. mancus* have a wider European distribution, but without a comprehensive sampling scheme across their entire range, no detailed phylogeographic patterns can be discussed.

It is well established that at the European scale, Unionida have suffered dramatic declines (Lopes-Lima et al. 2017b). The southern Mediterranean areas are of special concern because of their intrinsic geo-climatic characteristics, increased human development, biological invasions, land-use, and particularly high susceptibility to climate change (Fenoglio et al. 2010). Against this background, the low number of studies focussing on freshwater mussels of the Italian Peninsula is a significant shortfall. In fact, the real number of species present in the region was not known until the present study and many important ecological characteristics of these animals are poorly understood, e.g. density, growth, demographics, population structure, reproduction, primary fish hosts, habitat preferences and local threats. Future studies focusing on this important group should include multi-disciplinary approaches (ecology, molecular biology, physiology) that would provide information that is crucial for more effective conservation. The results presented here represent a first step towards this end by resolving taxonomic problems and assessing the broad-scale distribution and genetic diversity of the species in the region.

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