

## AQUATIC CONSERVATION: MARINE AND FRESHWATER ECOSYSTEMS

*Aquatic Conserv: Mar. Freshw. Ecosyst.* **24**: 561–574 (2014)Published online 23 April 2014 in Wiley Online Library  
([wileyonlinelibrary.com](http://wileyonlinelibrary.com)). DOI: 10.1002/aqc.2456

# ***Genetic diversity of the pan-European freshwater mussel *Anodonta anatina* (Bivalvia: Unionoidea) based on CO1: new phylogenetic insights and implications for conservation***

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## ABSTRACT

1. The duck mussel, *Anodonta anatina*, has been described as abundant and widespread in Europe. However, it is listed as near threatened or threatened in several countries owing to severe declines in abundance and/or spatial distribution.

2. Despite its potential ecological importance and conservation status almost nothing is known regarding its genetic diversity.

3. As a preliminary analysis, variation within the cytochrome oxidase 1 mitochondrial gene was determined from European specimens from Portugal in the south west, to Ukraine in the east, and Sweden in the north.

4. Three major mtDNA clades were retrieved: clade 1 includes all the individuals from Iberia, except those from the Ebro basin; clade 2 includes all the European non-Iberian and non-Italian samples; and clade 3 includes all the individuals from Italy and from the Ebro basin. AMOVA analysis revealed significant genetic differences among the three clades. Within each of the major clades, several geographically related haplogroups were also retrieved, especially in Iberia, where four genetically distinct groups (North-West, South-Central, South-West and Ebro) were revealed.

5. Given the evidence of regional declines of *A. anatina* in Europe, the confirmation of geographically distinct genotypes indicates a need for the development of management strategies directed towards the conservation of localized populations.

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Received 18 May 2013; Revised 29 January 2014; Accepted 23 February 2014

KEY WORDS: *Anodonta anatina*; river; genetics; invertebrates; molluscs

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## INTRODUCTION

Unionoid mussels are among the most critically endangered fauna and are globally in decline (Strayer, 2008). Conserving unionoid populations has wider importance because these animals are responsible for important ecosystem functions and services (e.g. food resource to higher trophic levels, clearing the water, control of the amount and composition of suspended particles, nutrient cycling and provision of habitat for other organisms; Aldridge *et al.*, 2007; Vaughn, 2010; Sousa *et al.*, 2011, 2012). In addition, their unusual life cycle, that requires a host fish species for larval (glochidia) development and dispersal (Barnhart *et al.*, 2008), has the potential to drive genetic isolation between populations (Douda *et al.*, 2013), which may lead to phylogeographic patterns of conservation importance.

While North America is known as a global centre of endemism for freshwater mussels, with over 300 species and subspecies described (Williams *et al.*, 1993; Graf and Cummings, 2007), in Europe the real number of species is still unknown but is considered to be relatively low (about 16). It is interesting to note that the number of phylogeographic and/or genetic diversity studies on European freshwater mussels is surprisingly low (Nagel *et al.*, 1996; Machordom *et al.*, 2003; Araujo *et al.*, 2005, 2009a, b; Geist *et al.*, 2010; Skidmore *et al.*, 2010), especially when compared with those from North America (King *et al.*, 1999; Serb *et al.*, 2003; Chong *et al.*, 2008; Elderkin *et al.*, 2008; Zanatta and Murphy, 2008; Mock *et al.*, 2010, 2013; Zanatta and Harris, 2013; Inoue *et al.*, 2014). Understanding the spatial patterns of unionoid mussel lineages may be especially interesting in southern European peninsulas because these have been shown to have served as refugia during cold periods in the Pleistocene, acting both as centres of origin of endemisms and as sources from which formerly glaciated areas were recolonized during interglacial periods (see Weiss and Ferrand, 2007, for a review). In southern Europe, the main Pleistocene glacial refugia include the Balkans, the Carpathian Mountains, the Italian Peninsula and Iberia (Hewitt, 2000). It is generally accepted that many

temperate European species were forced to shift their distributions into the three Mediterranean refugia because of repeated glaciations and climatic fluctuations (Hewitt, 1999). For example, in the Iberian Peninsula, many vertebrate, invertebrate and plant species show strong genetic subdivisions, reflecting at least two refuges for each species that indicate not only the occurrence of different glacial refuges in the Iberian Peninsula, but also different glacial refuges for the same species (see Gómez and Lunt, 2007, for a review). However, several other studies have also revealed northern refugia (i.e. non-Mediterranean) in a number of vertebrate and invertebrate European species (Bilton *et al.*, 1998; Bunje, 2005; Pauls *et al.*, 2006; Benke *et al.*, 2009).

The duck mussel, *Anodonta anatina* (Linnaeus, 1758), is a convenient model organism for testing biogeographical hypotheses concerning European freshwater ecosystems, especially owing to its occurrence throughout most of the continent. This freshwater mussel occurs in Europe and Asia below 65°N latitude down to Portugal, Sicily and Turkey and extending as far east as Siberia (Lake Baikal) (Graf, 2007). *Anodonta anatina* is a habitat generalist, inhabiting both lentic and lotic waters, ranging from small streams to large rivers, lakes and reservoirs (Hinzmann *et al.*, 2013). While little is known on the global conservation status of *A. anatina*, some regional studies indicate population declines around Europe, resulting in the species being protected in some European countries (e.g. Germany and Luxembourg) and listed as near threatened or threatened in Austria, Germany, Ireland and Romania (Sárkány-Kiss, 2003; Reischütz and Reischütz, 2007; Byrne *et al.*, 2009; Binot-Hafke *et al.*, 2011). Moreover, as with most unionoids, *A. anatina* presents great phenotypic plasticity having had more than 400 synonyms assigned (Nagel *et al.*, 1996; Graf and Cummings - <http://www.mussel-project.net>; March 2013). For example, the major morphological differences observed among *A. anatina* populations from distinct rivers or basins has resulted in more than 20 synonyms being described for Iberia alone (Araujo *et al.*, 2009b). Further complicating this picture, synonyms of this species are generally mixed with

those of *Anodonta cygnea* (Linnaeus, 1758) with which it has frequently been misidentified. In fact, the inappropriate use of shell characters such as contour line, thickness, or colouring has been a persistent and contentious problem that allowed taxonomists to exaggerate the extant number of bivalve species. Reliable conchological characters traditionally used to identify taxonomic units are very limited because of the high morphological plasticity of shells, which often reflect different ecophenotypes (Sousa *et al.*, 2007; Zieritz and Aldridge, 2009; Zieritz *et al.*, 2010).

Thus, despite being the most widespread European unionid species, there are few data available on the autecology of *A. anatina*, and no molecular studies have encompassed the species' supposed entire range.

In the present study, the Iberian Peninsula was chosen for several reasons as a detailed study area to investigate the genetic variation of *A. anatina*. First, it has a low number of artificial connections between river systems compared with central Europe. Second, despite the fact that *A. anatina* has been generally considered to have a wide host fish spectrum (Bauer, 2001), recent studies conducted in Iberia and central Europe suggest a narrow host range that is restricted to native fishes, mainly cyprinids (Douda *et al.*, 2013). Third, fish faunas in Iberia are quite distinct from those in Central Europe (73% of species are endemic to Iberia) and even inside the Iberian Peninsula major river basins have different fish assemblages (Maceda-Veiga, 2013). Moreover, phylogeographical studies in Iberian freshwater fishes, including cyprinids, have demonstrated the existence of evolutionarily distinct groups within each species, which suggests multiple refugia during glacial periods (Callejas and Ochando, 2000; Carmona *et al.*, 2000; Coelho *et al.*, 2005; Doadrio *et al.*, 2013). Given the potential for the biogeography of host fishes to affect the genetic structure of unionoid populations, *A. anatina* in Iberia would be predicted to display phylogeographical patterns reflective of host fish isolation. Thus, the genetic diversity of this species was investigated using novel and already available cytochrome c oxidase subunit I (CO1) sequence data. In addition, by comparing the Iberian data

with other European populations across the species range, the study aimed to investigate how *A. anatina* populations are genetically structured across its range and how this might facilitate future conservation planning.

## METHODS

### Specimens examined

All *A. anatina* CO1 sequences available on GenBank were downloaded, providing 23 sequences from Portugal, Spain, Sweden, Poland and Ukraine (Table 1). In addition, *A. anatina* individuals (total 61) were sampled from the following 10 major Iberian Peninsula river basins: Minho, Douro, Vouga, Mondego, Tejo, Guadiana, Sado, Guadalquivir, Barbate and Ebro (Table 1). Additional material was provided by collaborators, across the species range, in 29 samples from the following areas: Danube Basin and Lake Balaton (Hungary); Elbe Basin (Czech Republic); Lake Maggiore (Italy); Thames and Bure Basins (UK) and Dniester Basin (Ukraine) (Table 1).

The distribution of *A. anatina* samples previously available on GenBank and those newly sequenced is summarized in Figure 1.

### DNA extraction, amplification and sequencing

Whole genomic DNA was extracted from small tissue pieces preserved in 96% ethanol, using a standard high-salt protocol (Sambrook *et al.*, 1989). A fragment of ~700 bp of CO1 gene was amplified by PCR using the universal primer HCO2198 (Folmer *et al.*, 1994) paired with UNIOCOII.2 (Walker *et al.*, 2006) and the modified versions, i.e., LCO22me2 and HCO700dy2 (Walker *et al.*, 2006, 2007). The PCR conditions (25  $\mu$ L reactions) were as follows and identical for both primer pairs: each reaction contained 2.5  $\mu$ L 10 $\times$  Invitrogen PCR Buffer, 0.5  $\mu$ L 10 mmol L<sup>-1</sup> of each primer, 1.5  $\mu$ L 50 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 0.5  $\mu$ L 10 mmol L<sup>-1</sup> dNTPs, 0.1  $\mu$ L Invitrogen Taq DNA Polymerase and approximately 2  $\mu$ L DNA template. The cycle parameters were: initial denaturation at 94°C for 3 min, denaturation at 94°C (30 s), annealing at 45°C (HCO2198 and UNIOCOII.2) or 48°C

Table 1. List of samples examined with respective GenBank accession codes and information about population, river basin, country, haplotype and clades. All samples are from *Anodonta anatina* except the ones used for outgroups, i.e. *Pseudanodonta complanata* (JQ253891, JQ253892, DQ060172, DQ060173) and *Anodonta cygnea* (GU230749, AA388)

Code	Population	River Basin	Country	Haplotype	Clade
AA42 <sup>c</sup>	P1	Minho	Portugal	H4	1
AA51 <sup>c</sup>	P1	Minho	Portugal	H3	1
AA61 <sup>c</sup> , AY579122 <sup>a</sup>	P1	Minho	Portugal	H1	1
AA1 <sup>c</sup> , AA3 <sup>c</sup> , AA31 <sup>c</sup> , AA33 <sup>c</sup>	P2	Douro	Portugal	H1	1
AA11 <sup>c</sup> , AA12 <sup>c</sup> , AA13 <sup>c</sup> , EF571387 <sup>c</sup> , EF571388 <sup>c</sup> , EF571389 <sup>c</sup> , EF571390 <sup>c</sup>	P2	Douro	Portugal	H11	1
AA32 <sup>c</sup>	P2	Douro	Portugal	H12	1
AC1 <sup>c</sup> , AC9 <sup>f</sup> , V1 <sup>f</sup> , AA87 <sup>f</sup>	P3	Vouga	Portugal	H1	1
AC3 <sup>c</sup>	P3	Vouga	Portugal	H5	1
AA83 <sup>f</sup>	P3	Vouga	Portugal	H2	1
Ai1 <sup>f</sup> , Ai12 <sup>f</sup> , Ai13 <sup>f</sup> , Ai15 <sup>f</sup> , Ai21 <sup>f</sup> , EF571391 <sup>c</sup> , EF571392 <sup>c</sup>	P4	Mondego	Portugal	H6	1
Ai20 <sup>f</sup>	P4	Mondego	Portugal	H7	1
AA229 <sup>f</sup> , AA233 <sup>f</sup> , AA237 <sup>f</sup> , EF571395 <sup>c</sup>	P5	Tejo	Portugal	H6	1
AA234 <sup>f</sup>	P5	Tejo	Portugal	H10	1
AA242 <sup>f</sup>	P5	Tejo	Portugal	H9	1
EF571394 <sup>c</sup>	P5	Tejo	Portugal	H8	1
AA14 <sup>c</sup>	P6	Guadiana	Portugal	H18	1
AA15 <sup>f</sup> , AA17 <sup>c</sup>	P6	Guadiana	Portugal	H17	1
AA16 <sup>c</sup>	P6	Guadiana	Portugal	H16	1
AA19 <sup>f</sup>	P6	Guadiana	Portugal	H13	1
EF571396 <sup>c</sup>	P6	Guadiana	Portugal	H14	1
EF571397 <sup>c</sup>	P6	Guadiana	Portugal	H15	1
AA173 <sup>f</sup> , AA176 <sup>f</sup> , AA179 <sup>f</sup>	P7	Sado	Portugal	H19	1
EF571393 <sup>c</sup> , AA180 <sup>f</sup> , AA181 <sup>f</sup> , AA182 <sup>f</sup> , AA183 <sup>f</sup> , AA287 <sup>f</sup> , AA288 <sup>f</sup> , AA291 <sup>f</sup> , AA292 <sup>f</sup> , AA293 <sup>f</sup>	P7	Sado	Portugal	H13	1
AA289 <sup>f</sup>	P8	Guadalquivir	Spain	H21	1
AA294 <sup>f</sup>	P8	Guadalquivir	Spain	H13	1
AA339 <sup>f</sup> , AA341 <sup>f</sup> , AA343 <sup>f</sup> , AA344 <sup>f</sup> , AA342 <sup>f</sup> , AA345 <sup>f</sup> , AA346 <sup>f</sup> , AA257 <sup>f</sup> , AA258 <sup>f</sup>	P9	Barbate	Spain	H22	1
AA259 <sup>f</sup> , AA260 <sup>f</sup> , AA261 <sup>f</sup> , AA264 <sup>f</sup>	P9	Barbate	Spain	H20	1
AA263 <sup>f</sup>	P10	Ebro	Spain	H36	3
AA70 <sup>f</sup> , AA72 <sup>f</sup> , ACGOM3 <sup>f</sup>	P10	Ebro	Spain	H37	3
AA71 <sup>f</sup> , ACGOM1 <sup>f</sup>	P10	Ebro	Spain	H40	3
ACGOM2 <sup>f</sup>	P11	Danube	Hungary	H24	2
AA369 <sup>f</sup>	P11	Danube	Hungary	H26	2
AA370 <sup>f</sup> , AA375 <sup>f</sup>	P11	Danube	Hungary	H28	2
AA371 <sup>f</sup>	P12	Lake Balaton	Hungary	H25	2
AA372 <sup>f</sup>	P12	Lake Balaton	Hungary	H26	2
AA73 <sup>f</sup>	P12	Lake Balaton	Hungary	H24	2
AA74 <sup>f</sup> , AA75 <sup>f</sup> , AC79 <sup>f</sup>	P12	Lake Balaton	Hungary	H27	2
AA76 <sup>f</sup> , AC77 <sup>f</sup>	P13	Elbe	Czech Republic	H24	2
AA387 <sup>f</sup> , AA389 <sup>f</sup> , AA390 <sup>f</sup> , AA391 <sup>f</sup> , AA392 <sup>f</sup> , AA394 <sup>f</sup>	P13	Elbe	Czech Republic	H30	2
AA393 <sup>f</sup>	P13	Elbe	Czech Republic	H26	2
JF496764 <sup>b</sup>	P14	Lake Maggiore	Italy	H38	3
AA379 <sup>f</sup> , AA380 <sup>f</sup>	P14	Lake Maggiore	Italy	H39	3
AA381 <sup>f</sup>	P14	Lake Maggiore	Italy	H35	3
DQ060168 <sup>h</sup>	P15	Lake Castel dell'Alpi	Italy	H30	2
AF494102 <sup>i</sup> , GU230745 <sup>j</sup>	P16	Bure	UK	H30	2
EF440346 <sup>i</sup>	P17	Thames	UK	H30	2
GU230744 <sup>j</sup>	P18	Lake Stavsjon	Sweden	H31	2
EF440347 <sup>i</sup>	P19	Vistula	Poland	H31	2
GU230742 <sup>j</sup>	P20	Odra	Poland	H29	2
DG3 <sup>f</sup> , DG4 <sup>f</sup>	P20	Odra	Poland	H34	2
JQ253884 <sup>k</sup>	P21	Lake Wdzydze	Poland	H34	2
JQ253885 <sup>k</sup>	P22	Lake Hamrzysko	Poland	H29	2
JQ253883 <sup>k</sup>	P23	Dniester	Ukraine	H29	2
JQ253891 <sup>k</sup>	P24	Dniester	Ukraine	H32	2
JQ253892 <sup>k</sup>	P24	Dniester	Ukraine	H41	2
DQ060172 <sup>h</sup> , DQ060173 <sup>h</sup>	P25	Salgir	Ukraine	H33	2
GU230749 <sup>j</sup>	--	Severskiy Donec	Ukraine	--	--
AA388 <sup>f</sup>	--	Danube	Ukraine	--	--
	--	Silveran	Sweden	--	--
	--	Lake Hamrzysko	Poland	--	--
	--	Lake Maggiore	Italy	--	--

<sup>a</sup>listed on GenBank as *Anodonta* sp.; <sup>b</sup>listed on GenBank as *A. cygnea*; <sup>c</sup>Hinzmann *et al.*, 2013 (GenBank n<sup>o</sup>: KC583446–KC583463); <sup>d</sup>Huff *et al.*, 2004; <sup>e</sup>Reis *et al.* 2013, (GenBank n<sup>o</sup>: EF571397.1–EF571387.1); <sup>f</sup>This study (GenBank n<sup>o</sup>: KC583464–KC583519); <sup>g</sup>Plazzi *et al.*, 2011; <sup>h</sup>Källersjö *et al.*, 2005; <sup>i</sup>Soroka, 2008; <sup>j</sup>Soroka, 2010; <sup>k</sup>Yanovich (unpublished).

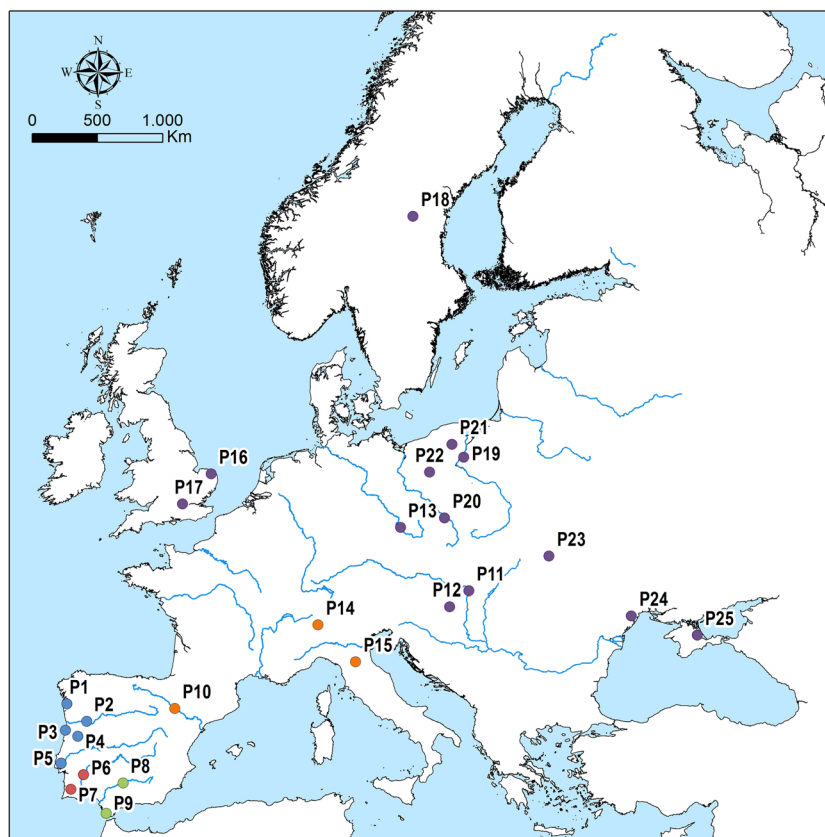


Figure 1. Map showing the location of *Anodonta anatina* samples from Europe. Population codes (P) follow Table 1. Colours represent the geographic distribution of the major groups as in the phylogeny (Figure 2).

(LCO22me2 and HCO700dy2) (40 s) and extension at 72°C (60 s) repeated for 40 cycles and a final extension at 72°C for 10 min. Amplified DNA templates were purified and sequenced by a commercial company, Macrogen, using the same primers.

### Phylogenetic analyses and genetic diversity

Owing to the common misidentification between *A. cygnea* and *A. anatina*, all the *A. cygnea* CO1 sequences available were downloaded. A preliminary analysis on these sequences showed conclusively that all but one were *A. cygnea* (see Results). Selected outgroups included one *A. cygnea* individual previously published, one sequenced for this study, and four individuals of *Pseudanodonta complanata* (Rossmässler, 1835) (Table 1).

Chromatograms were checked by eye using ChromasPro 1.41 (technelysium.com.au) and the alignment was performed using Bioedit v.5.0.9.

(Hall, 1999). This data set was then analysed using maximum likelihood (ML) and Bayesian inference (BI) methods. The best-fit model of nucleotide substitution evolution under the corrected Akaike Information Criterion was estimated using JModelTest 0.1.1 (Posada, 2008). The Tamura and Nei (1993) model of sequence evolution with the proportion of invariable sites estimated from the data (TrN + G; G = 0.3380) was chosen. ML trees were built in PhyML (Guindon and Gascuel, 2003) with 1,000 bootstrap replicates and searching for the best-scoring ML tree. Phylogenetic BI was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Two independent runs  $10^7$  generations long were sampled at intervals of 100 generations producing 100 000 trees. Burn-in was determined upon convergence of log likelihood and parameter estimation values using Tracer 1.4 (Rambaut and Drummond, 2007). For each of the major clades

obtained in the phylogeny, sequence divergence was assessed using MEGA5 software (Tamura *et al.*, 2011).

### Population geographic structure

To evaluate relationships among closely related haplotypes, sequences were joined in a statistical parsimony network constructed under a 95% criterion using TCS 2.1 (Clement *et al.*, 2000). This method is intended to detect potential spatial patterns according to the distribution of haplotypes. Moreover, this technique allows a more detailed display of population information than strictly bifurcating trees, being particularly suited to the analysis of single-species gene genealogies (Posada and Crandall, 2001).

ARLEQUIN 3.5 software (Excoffier and Lischer, 2010) was used to run the analysis of molecular variance (AMOVA) to estimate the hierarchical distribution of mtDNA genetic differentiation within and among populations, using  $\Phi_{ST}$  (from the absolute number of nucleotide differences) and with 10 000 permutations. AMOVA was used to assess possible differences among populations of *A. anatina* for (i) Iberia alone; and (ii) its entire range. To assess the levels of genetic differentiation among Iberian *A. anatina* populations, pairwise population differentiation values ( $\Phi_{ST}$ ) were calculated using the same software and associated probability values were quantified using uncorrected p-distance and 10 000 permutations.

## RESULTS

### Phylogenetic analyses and genetic diversity

The CO1 alignment consisted of 113 DNA sequences of *A. anatina* specimens, four of *P. complanata* and two of *A. cygnea* (Table 1). Aligned sequences had a total length of 590 bp, with 61 polymorphic and 53 parsimony informative sites. No indels and no unexpected stop codons were observed after translating all sequences to amino acid.

The tree topologies resulting from the single tree recovered from ML and BI approaches were congruent, with a sole exception between the two phylogenies (see below). The results of both

analyses are shown in Figure 2 and it seems that the phylogeny of the *Anodonta* genus needs further evaluation, because *A. cygnea* and *P. complanata* form a sister clade to *A. anatina*. Three major mtDNA clades were retrieved with high support: the first includes all the individuals from Iberia, except those from the Ebro basin (clade 1); the second, all the European non-Iberian and non-Italian samples (clade 2); and the third, basally related to all the other *A. anatina* samples, includes all the individuals from Italy and from the Ebro Basin (clade 3). The divergence between them (uncorrected distances) varied from 1.9% between clade 1 and clade 2 to 3.1% between clade 1 and clade 3 (Table 2).

A further analysis of each clade showed additional geographic patterns. Clade 1 is divided into three major well-supported groups: one corresponded to all north-west Iberian populations (1–5, Figure 1 and Table 1); another included south-west populations (6 and 7, Figure 1 and Table 1); and the third encompassed all the south-central Iberian populations (8 and 9, Figure 1 and Table 1). As explained above, the Ebro population does not cluster inside clade 1, but inside clade 3. Thus, within the Iberian Peninsula, these results demonstrate the existence of four geographically related groups, with an average genetic distance (uncorrected distances) between them, ranging from 1.0% to 3.5% (Table 2).

Non-Iberian populations all cluster inside the geographically diverse clade 2, with the exception of the Italian samples. This clade includes 32 individuals collected in central and northern Europe (Table 1), without clear geographically related groups (Figure 2).

In clade 3, all the Italian samples from Lake Maggiore (population 14, Figure 1 and Table 1) grouped together inside a well-supported group. It is interesting that its sister taxa were revealed to be the Iberian Ebro Basin samples (population 10, Figure 1 and Table 1). A previously published sequence from Lake Castel dell'Alpi, Italy (population 15, Figure 1 and Table 1) and listed as *A. cygnea* on GenBank (JF496764) was grouped with the Ebro Basin, Iberia, inside clade 3 in the BI tree, but not in the ML tree (the sole exception between the two phylogenies), where it was placed

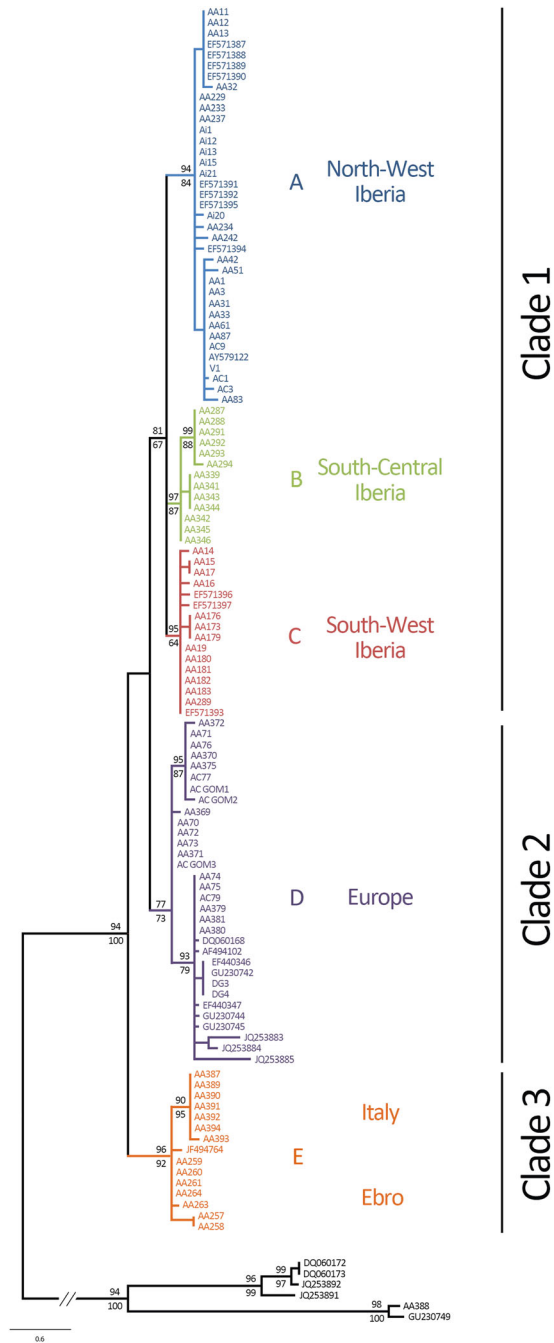


Figure 2. Phylogenetic tree obtained by BI analysis of 113 COI sequences (590 bp) of *Anodonta anatina* specimens with *Pseudanodonta complanata* and *Anodonta cygnea* individuals as outgroups. Support values (%) are given as Bayesian posterior probability (BI) above nodes and bootstrap support (ML) below nodes. Some values were omitted for clarity at short nodes within clades. The tree topologies resulting from ML and BI approaches were congruent, with one sole exception found between BI and ML phylogenies (JF496764), which was the grouping with Ebro Basin (Iberia) inside clade 3 in the BI tree, but not in the ML tree, where it was placed as sister to all the other samples in clade 3. Colours highlight the major mtDNA groups found that correspond to the geographic distribution as seen in Figure 1; and letters A–E relate to the major haplotypes groups as in Figure 3.

as sister to all the other samples in clade 3. From all the sequences available on GenBank under study here, this was the only mislabelled specimen detected.

**Population geographic structure**

At the 95% confidence limit, TCS produced a single network shown in Figure 3. From all the *A. anatina* sequences analysed, 41 distinct haplotypes were resolved, 23 of which occurred only once with the most frequent haplotype (H6) occurring in 11 individuals across the species range (Table 1). The distribution of Iberian haplotypes in the network reveals further interesting spatial patterns: the north-western Iberian populations are at one of the tips of the network, and comprise the two most frequent haplotypes (H1 and H6) across it. The individuals from south-central Iberia appear between the north-western (seven mutations from it) and the south-western (four mutations from it) populations. This latter group includes one haplotype (H13) that is shared among individuals from three distinct populations (6, 7, and 8; Table 1 and Figure 1) including the Guadalquivir basin (south-central Iberia). As seen in the phylogeny, the fourth Iberian group (Ebro) is closer to those from Italy (H35, H38 and H39; Figure 3), with its haplotypes (H36-H37-H40) being 27 mutations from the closest Iberian populations (south-west group).

The vast majority of the European non-Iberian and non-Italian haplotypes from the central and north European populations cluster close together in an internal position in the species network. Interestingly, the three supported groups depicted in the phylogeny were not represented in the same way in the network; although all the haplotypes from Hungary (including those from Lake Balaton) and three from the River Elbe in the Czech Republic (H24-28 - Figure 3) were distinct from the individuals from the UK, Sweden, Czech Republic, Poland and Ukraine (H29-H33; Figure 3). In between these European and Italian +Ebro haplotypes (at exactly seven mutations each way) appears H34, a haplotype present in only three individuals from Poland. Another interesting result was the position in the network of one

Table 2. Left: Mean genetic divergence between the main *Anodonta anatina* mitochondrial clades (Figure 2). Right: Mean genetic divergence between the Iberian *A. anatina* mitochondrial groups (Figure 2). The number of base differences per site from averaging over all sequence pairs, individuals (n) and haplotypes (k) are shown; standard error estimate(s) are shown above the diagonal, in bold. The analysis involved 113 COI mtDNA sequences

	Clade 1	Clade 2	Clade 3	n	K		North-West	South-Central	South-West	Ebro	n	k
Clade 1		<b>0.006</b>	<b>0.009</b>	66	23	North-West				0.007	37	12
Clade 2	0.019		<b>0.008</b>	32	12	South-Central	0.016		0.003	0.006	13	5
Clade 3	0.031	0.024		15	6	South-West	0.014	0.010		0.006	16	7

unique haplotype from Ukraine (H41), which appears at the other tip of the network and eight mutations away from the Iberian (Ebro Basin) haplotype H35. Moreover, the other five individuals from Ukraine have four distinct haplotypes (Table 1), with H33 (Figure 3) being also several mutations from the closest haplotype in the network.

Although some care must be taken owing to the small sample size, the results from AMOVA showed that 54.25% of the overall variation in *A. anatina* across its range (major groups corresponding to the three major clades obtained in the phylogeny) was found among regions. In contrast, only 9.93% of total mitochondrial haplotype variation occurred within populations, with all structured levels presenting highly significant genetic differences (Table 3). When testing for structure inside Iberia alone, 71.92% of overall variation of the species was found among the four regions tested but 21.37% occurred within populations. Again, all structured levels presented highly significant genetic differences (Table 3).

All pairwise  $\Phi_{ST}$  values showed significant genetic differences among the Iberian groups visualized in the network of haplotypes and phylogenetic tree (Table 4): the smallest pairwise  $\Phi_{ST}$  was found between south central and south west ( $\Phi_{ST}$  0.70297,  $P < 0.01$ ; Table 4) and the highest  $\Phi_{ST}$  was found between the north west and the Ebro ( $\Phi_{ST}$  0.93775,  $P < 0.01$ ; Table 4). Moreover, the pairwise  $\Phi_{ST}$  values assessed for individual Iberian populations showed significant genetic differences among them, with only three pairs showing no significant genetic differences (Table 5).

## DISCUSSION

This study represents the first attempt to uncover the genetic diversity of *A. anatina* across its

European distribution range, based on COI sequences. The detailed analysis of the mtDNA genetic variation clearly revealed three major mtDNA clades, and within each one, several geographically related haplogroups with generally non-overlapping geographic distributions. These results were further supported by the hierarchical analyses of molecular variance that showed significant genetic differences among the three mtDNA clades. However, it would be desirable for future studies to supplement the number of individuals and populations used in this present one, in order to retrieve a stronger statistical hierarchical analysis of genetic variation.

The results obtained in this study strongly indicate that *A. anatina* has a complex phylogeographic history with high genetic diversity found within its range. Several mtDNA allopatric lineages were revealed, which can be explained by the occurrence of multiple refugia for this species during the last glacial periods, so that considerable genetic diversity has been conserved. In addition, the results seem to support the idea of multiple refugia within the Iberian Peninsula, with the existence of at least three (north west, south and Ebro) during Pleistocene glaciations.

Following a similar pattern to Iberia, other taxa have shown a high level of endemism in Italy, especially in the Calabria region (southern Italy) (e.g. red squirrel: Grill *et al.*, 2009; pygmy shrews: Vega *et al.*, 2010). Nagel *et al.* (1996) using allozymes, described the presence of two Italian *Anodonta* groups (groups I and II) which exist in sympatry with little or no gene flow. These *Anodonta* groups might correspond to the findings of the present study (for Lake Maggiore and Lake Castel dell'Alpi; Table 1 and Figure 1) but further sampling is needed. This will be important not only to clarify the number of possible genetically distinct groups that may exist in Italy but will also



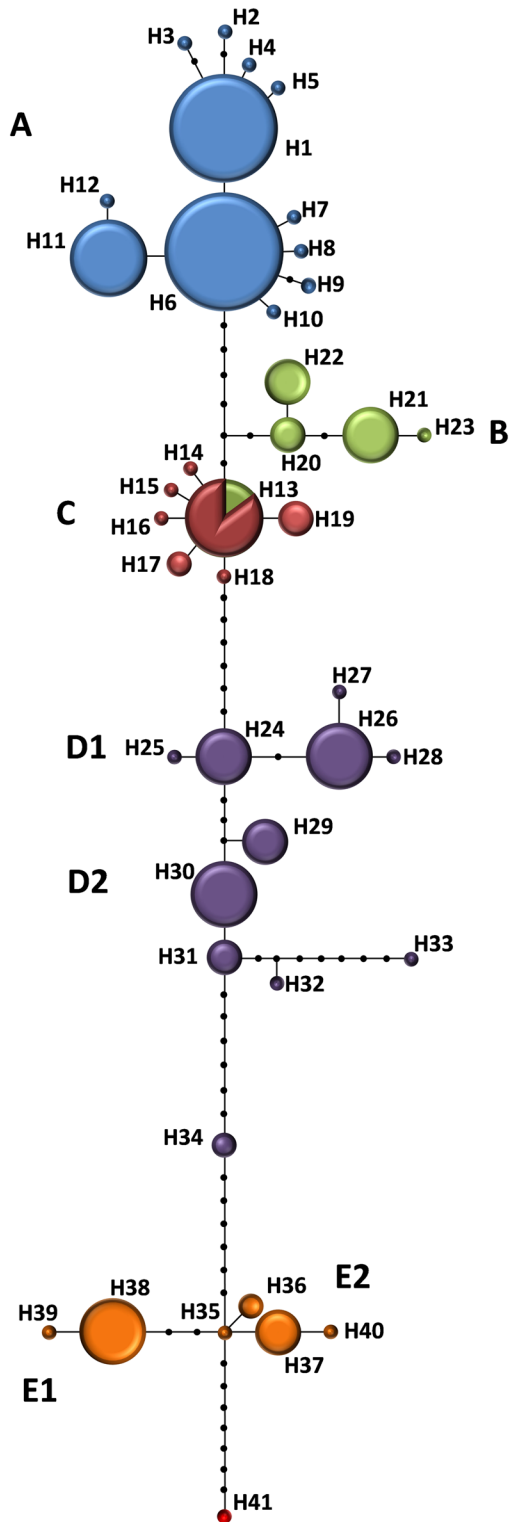


Figure 3. Haplotype (TCS) network showing the relationships of *Anodonta anatina* specimens, inferred from the same individuals sequenced. Circle size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates. Colours correspond to the major mtDNA groups found in the phylogeny (Figure 2).

Table 3. Analysis of molecular variance (AMOVA) measured among populations of *Anodonta anatina* for its entire range and for Iberia (see text for details)

Structure tested	Source of variation	% Variance	Fixation indices	P
Entire range	Among regions	54.25	0.54248	<0.01
	Among populations / within regions	35.82	0.78300	<0.01
	Within populations	9.93	0.90072	<0.01
Iberia	Among regions	71.92	0.71918	<0.01
	Among populations / within regions	6.71	0.23897	<0.01
	Within populations	21.37	0.78628	<0.01

Table 4. Genetic differentiation of *Anodonta anatina* ( $\Phi_{ST}$ ) among the Iberian population groups. Associated p-values were all highly significant ( $P < 0.01$ ; probability that a random value obtained from 10 000 permutations is greater than the observed value)

	North-West	South-Central	South-West
North-West			
South-Central	0.81575		
South-West	0.84095	0.70297	
Ebro	0.93775	0.88215	0.91813

be fundamental in understanding the genetic closeness of the Italian and Ebro (Iberia) populations. Although never observed in native European freshwater mussels, the close affinity between Italian and Ebro haplotypes (0.6% uncorrected distances for this study) has been described for other freshwater taxa (e.g. freshwater fish and amphibians: Vargas *et al.*, 1998; and aquatic snails: Bunje, 2005), where it is postulated to have been maintained through the lower extremes of the Pyrenees (Vargas *et al.*, 1998). Only by thorough analyses of the French Mediterranean populations (not included in this study) can this be clarified.

Regarding the rest of the species distribution, Nagel *et al.* (1996) using allozymes described two closely related groups within *A. anatina* from the central and western European populations. One included all the populations from the drainage basins of the rivers Seine, Rhine, and from rivers that drain to the North Sea, and this was closely related to a second group that encompassed the populations of the rivers Loire, Rhône, Danube and their respective tributaries. In addition to this finding, *Anodonta* group I (from Italy; see above)

Table 5. Genetic differentiation of *Anodonta anatina* ( $\Phi_{ST}$ ) among the Iberian populations. Associated *P*-values were all highly significant ( $P < 0.01$ ; probability that a random value obtained from 10 000 permutations is greater than observed value) except for bold values with \*

	Minho	Douro	Vouga	Mondego	Tejo	Guadiana	Sado	Guadalquivir	Barbate
Minho									
Douro	0.42129								
Vouga	<b>0.01967*</b>	0.42758							
Mondego	0.63492	0.38434	0.64405						
Tejo	0.44797	0.31148	0.48135	<b>0.01260*</b>					
Guadiana	0.83794	0.85389	0.85771	0.89014	0.83719				
Sado	0.90950	0.89514	0.91863	0.94819	0.89822	<b>0.13742*</b>			
Guadalquivir	0.82964	0.85499	0.85265	0.88230	0.83472	0.70696	0.78515		
Barbate	0.90563	0.89369	0.91643	0.94871	0.89582	0.79964	0.88918	0.57054	
Ebro	0.54280	0.67524	0.60090	0.62978	0.58689	0.45740	0.51711	<b>0.25032*</b>	0.37189

was more related to the central and western European groups than to *Anodonta* group II (also from Italy). The new mtDNA results presented here are somewhat concordant with those obtained by Nagel *et al.* (1996) and provide additional details on the structure of the species: not only do the southern European peninsulas, Iberia and Italy, seem to have acted as important glacial refugia for *A. anatina* (isolation within refugia during Pleistocene glaciations), but at least another glacial refugium, somewhere in central-east Europe, seems to have also existed. The haplotype distinctiveness and position in the network, especially of both the Polish and Ukrainian samples, point out the necessity for further sampling in these regions. As an example, in the aquatic snail *Theodoxus fluviatilis* (Linnaeus, 1789) several independent mtDNA lineages were found in different drainages of the Black Sea, Ukraine (Bunje, 2005), which indicates that further sampling for *A. anatina* may also provide evidence for refugia within the refugium in this region. A number of missing haplotypes were detected for the European group (clade 2). This situation may result from incomplete geographical sampling or alternatively by missing haplotypes that may now be extinct. Either way, the mtDNA genetic variation reveals a complex phylogeographic history of *A. anatina* in Europe, especially in central regions.

In North America, the Pleistocene glaciations also had major impacts on the genetic structure of many species (Hewitt, 1996). Several studies on freshwater mussels describe populations in unglaciated regions that show greater genetic diversity than more northerly regions (King *et al.*, 1999; Serb *et al.*, 2003; Zanatta and Murphy,

2008; Zanatta and Harris, 2013; Inoue *et al.*, 2014). Moreover, studies using co-occurring mussel species indicate that they may have a very different genetic structure in the same habitats (Elderkin *et al.*, 2008; Zanatta and Harris, 2013). The genus *Anodonta* also occurs in western North America where it contains three highly divergent lineages (Chong *et al.*, 2008). In recent studies, Mock *et al.* (2010, 2013) described the phylogeographical subdivision in the most widespread and diverse of these lineages and concluded that the observed genetic structure corresponded to the major hydrologic basins. Unfortunately, studies on the genetic diversity of freshwater mussels in Europe are still very scarce. An exception are the studies conducted in Iberia on the genus *Unio*, that although reporting much smaller total sample sizes, point to the existence of endemisms (Araujo *et al.*, 2009a, b; Reis and Araujo, 2009; Reis *et al.*, 2013). Conversely, in populations of *Margaritifera margaritifera* (Linnaeus, 1758) from several drainages in Spain (Iberia) only two variable sites were found in a 657 bp segment of the COI gene (Machordom *et al.*, 2003). In addition, the pattern obtained in the present study for *A. anatina* is very different from the one seen for *P. complanata* within the UK, where levels of genetic differentiation are modest with surprisingly good gene flow between widely dispersed populations (Skidmore *et al.*, 2010). Those patterns were explained by human activities such as fish stocking and the interconnectivity of the different drainages.

The phylogeography of Iberian freshwater fish species strongly reflects the geomorphological history of Iberian water drainages and formation

of mountain ranges, with several areas being identified as harbouring distinct native fish lineages (Antunes *et al.*, 2006; Filipe *et al.*, 2009; Doadrio *et al.*, 2013). Unfortunately, 52% of Iberian fish species are now catalogued as threatened (according to IUCN criteria; Maceda-Veiga, 2013). Mussel species such as *A. anatina*, considered as host generalists, are often supposed to be safe from host limitation despite their observed population declines. However, Douda *et al.* (2013) found that the host fish range in *A. anatina* is more restrictive than first assumed. As conservation efforts must consider both the mussels and their native hosts, the confirmation of geographically distinct genotypes in *A. anatina* indicates a need for the development of management strategies directed towards the conservation of localized populations as well as their hosts. Furthermore, future host studies should include each of the major geographically distinct genotypes of *A. anatina* depicted in the present study.

Additional research efforts are needed and they must include more markers and individuals from the whole range. Even so, three major groups can be defined based on the present results, with each deserving individual attention from a conservation perspective. Although *A. anatina* is still the most common European unionoid and the one with the widest distribution range, the geographical coverage and conservation status of the three major groups depicted in this study is very different. In Iberia (clade 1) the species has decreased dramatically over the last two decades, mainly in Spain where it is becoming rare and is already protected in some regions (CMADS, 2007; Araujo *et al.*, 2009b). The majority of the European populations fall into clade 2, which seems to be generally stable although the species is already threatened and protected in some countries within this clade (e.g. Austria, Germany, Ireland and Romania) (Sárkány-Kiss, 2003; Reischütz and Reischütz, 2007; Byrne *et al.*, 2009; Binot-Hafke *et al.*, 2011). Clade 3 includes only the Ebro and the Italian populations, thus covering a small distributional range in which *A. anatina* is decreasing dramatically and facing multiple threats (e.g. eutrophication, water abstraction and the introduction of invasive

species; Cianfanelli *et al.*, 2007; Cappelletti *et al.*, 2009; Halcon, 2011).

In summary, the results of this study have several direct consequences for the conservation of *A. anatina* because several mtDNA lineages were found that represent geographically distinct genotypes. Although this preliminary study was based solely on CO1, the results are of great interest in conservation planning because the data suggest that *A. anatina* has at least three management units. The analysis of mitochondrial DNA was the primary tool used in this phylogeographic study, owing to the fast mutation rate of mitochondrial genes and because maternal inheritance makes it possible to determine where a species was able to establish populations (Avice, 2000). However, the lower mutation rate and slower fixation rate of nuclear genes, when compared with the mitochondrial genome, means that nuclear genealogies may be more indicative of older demographic events (Avice, 2004). Therefore, in future analyses, the distribution of ancestral nuclear alleles could be useful in identifying refugial areas for this species. In addition, using microsatellites to estimate effective population sizes and to reflect current gene flow will also be of great utility. Finally, and as noted previously (Chong *et al.*, 2008), it seems that the phylogeny of the *Anodonta* genus needs further evaluation as it appears not to be monophyletic, with *A. cygnea* and *P. complanata* forming a sister clade to *A. anatina*.

#### ACKNOWLEDGEMENTS

This work was supported financially by the Portuguese Foundation for Science and Technology (FCT) project PTDC/AAC-AMB/117688/2010, and partly by the European Regional Development Fund through COMPETE, under the project PEst-C/MAR/LA0015/2011. We would like to thank our collaborators who kindly provided us with tissue samples: Erika Bódis, Karel Douda, Oksana Stolyar, Arpad Benko-Kiss, Nicoletta Riccardi. Marianna Soroka and Larissa Yanovich gave us the exact locations of their previously sequenced animals. We thank

José María Irurita, José Miguel Barea Azcón, Keiko Nakamura, Pablo Redin and Esther Penin for all their help during the Spanish field campaigns. Official capture and sampling licences were issued by Junta de Castilla y León (Consejería de Fomento y Medio Ambiente EP/CYL/331/2012, de 17 de Abril de 2012); Junta de Andalucía (Consejería de Medio Ambiente- SGYB/FOA/AFR/SFS, de 17 de Julio de 2012); Xunta de Galicia (Consellería de Medio Ambiente- 539/2012 de 15 de Setembro de 2012); Gobierno de Aragón (Dirección General de Conservación del Medio Natural- 136.071/2012 de 12 de Julio de 2012).

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