AQUATIC CONSERVATION: MARINE AND FRESHWATER ECOSYSTEMS
Aquatic Conserv: Mar. Freshw. Ecosyst. **26**: 307–318 (2016)
Published online 19 June 2015 in Wiley Online Library
(wileyonlinelibrary.com). DOI: 10.1002/aqc.2575

Newly developed microsatellite markers for the pan-European duck mussel, Anodonta anatina: *revisiting the main mitochondrial lineages*

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ABSTRACT

1. Freshwater mussels of the family Unionidae are one of the most threatened groups worldwide and have suffered severe decline over recent decades. Although the freshwater duck mussel, *Anodonta anatina* (Linnaeus, 1758), is still widespread, this species has shown evidence of recent declines and is already protected in some European countries.

2. Informed conservation efforts must take into account patterns in genetic diversity and phylogeography. In the present study, 20 newly developed polymorphic loci were described and tested in seven populations of *A. anatina*, belonging to three previously detected divergent mtDNA lineages. The genetic diversity patterns, within and among *A. anatina* populations, were evaluated to test their congruence with those lineages.

3. A high genetic differentiation (F_{ST}) was found among all populations, with the exception of those in Central Europe (Germany) and the UK, which were not strongly structured.

4. The present study confirms the division of the species into three evolutionarily significant units corresponding to the three previously detected mtDNA lineages, which should be managed independently. Furthermore, owing to the high differentiation among southern European populations, the establishment of distinct management units for the Guadalquivir, Guadiana and Douro populations in the Iberian Peninsula is also proposed.

Received 18 October 2014; Revised 17 March 2015; Accepted 27 April 2015

KEY WORDS: *Anodonta anatina*; anodontini; genetic diversity; microsatellites; Unionidae; conservation genetics

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INTRODUCTION

Freshwater bivalves of the order Unionoida, also known as freshwater mussels, are among the most threatened faunal groups worldwide (Strayer *et al.*, 2004; Lopes-Lima *et al.*, 2014). Unionoid mussels are widespread, colonizing freshwater ecosystems of all continents except Antarctica. They can attain very high densities in some habitats (Bogan, 2008) which, together with their filter-feeding behaviour, make them particularly important in the transfer of matter and energy from the water column to the benthos (Strayer *et al.*, 1999). In addition, these bivalves are important ecosystem engineers (Gutiérrez *et al.*, 2003), not only through the indirect effects of filter feeding (e.g. increasing water clarity), but also by bioturbation and consequent changes in the sediments (e.g. oxygen, organic matter, redox potential) and the production of shells, which function as a substrate for other species (Vaughn and Hakenkamp, 2001; Aldridge *et al.*, 2007; Spooner *et al.*, 2013). Unionoids also provide important services to humans, such as purification of water, as prey for several commercial fishes and as a source of food and other valuable materials (shells and pearls) (Haag, 2012).

Anodonta anatina (Linnaeus, 1758) is the most widespread freshwater mussel species in the Palaearctic, occurring from Portugal and the British Isles in the west to the Transbaikal region in the east of Russia (Douda *et al.*, 2013; Hinzmann *et al.*, 2013). Although this species is considered common and widespread in most countries, severe declines have been reported in some regions and consequentially this species is 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). However, the taxonomy of this species is still contentious, which may impair the application of possible conservation measures. Indeed, until the middle of the 20th century, many malacologists had split *A. anatina* into hundreds of regional species, mainly based on its highly variable shell characters (Graf, 2010). Nevertheless, by the end of the 20th century most of these synonymies had been resolved, merging all these putative species into *A. anatina* (Araujo, 2013).

Only recently has the genetic diversity of *A. anatina* been described across a large part of its entire range. Froufe *et al.* (2014) described the existence of at least three major mtDNA lineages in most of its European range, with non-overlapping geographic distributions and with significant genetic differences among them. One lineage includes all Atlantic Iberian populations, the second includes individuals from the Mediterranean Iberian Peninsula (Ebro) and Italy and the third includes the remaining European populations, from the British Isles in the west to Russia in the east. Furthermore, the results indicated that *A. anatina* has a pronounced genetic substructure within the Atlantic Iberian lineage, consistent with the existence of multiple Pleistocene refugia within this region (for a review see Gómez and Lunt, 2007). Since the mitochondrial DNA represents only the maternal lineage and its exclusive use may introduce errors in phylogenetic studies owing to phenomena such as hybridization and introgression (Alves *et al.*, 2006), data from nuclear markers should be used to complement these results.

Microsatellite markers are ideal for inferring population structure and dynamics and have been used frequently in conservation genetics studies of freshwater mussels with many applications: for example, to understand the spatial patterns of genetic diversity, including isolation, gene flow and migration (Geist *et al.*, 2010a; Mock *et al.*, 2013); to establish phylogeographical patterns and the establishment of evolutionary and management units (Zanatta and Murphy, 2008); to establish co-evolution patterns between a freshwater mussel species and its host fish (Zanatta and Wilson, 2011); to investigate the occurrence and frequency of multiple paternity (Bai *et al.*, 2012); and to investigate maximum fertilization distance among female and male freshwater mussels (Ferguson *et al.*, 2013).

To date, microsatellite markers have been developed for few European freshwater mussel species, i.e. *Margaritifera margaritifera* (Linnaeus, 1758) (Geist *et al.*, 2003), *Unio crassus* Philipsson, 1788 (Sell *et al.*, 2013), *Potomida littoralis* (Cuvier, 1798) (Froufe *et al.*, 2013) and *Anodonta cygnea*

(Linnaeus, 1758) (Geist *et al*., 2010b). Moreover, with the exception of *M*. *margaritifera* (Geist and Kuehn, 2005; Bouza *et al*., 2007; Geist *et al*., 2010a), no conservation or phylogeographic studies using these markers have been published so far. Therefore, the present study aimed: (i) to develop effective and polymorphic microsatellite markers for *A*. *anatina*; (ii) to compare their usefulness in identifying discrete evolutionary and management units with lineages previously described by Froufe *et al*. (2014) using mtDNA; and (iii) to characterize the genetic diversity patterns of the species.

METHODS

Specimens examined and DNA extraction

Small samples from the foot were non-destructively excised (following Naimo *et al*., 1998) and placed directly into 99% ethanol, relocating the mussel back to its previous position. In total, 140 *A*. *anatina* specimens were collected from seven different ecosystems (Figure 1, Table 1). Total genomic DNA was extracted from all individuals using a standard high-salt protocol following Sambrook *et al*. (1989).

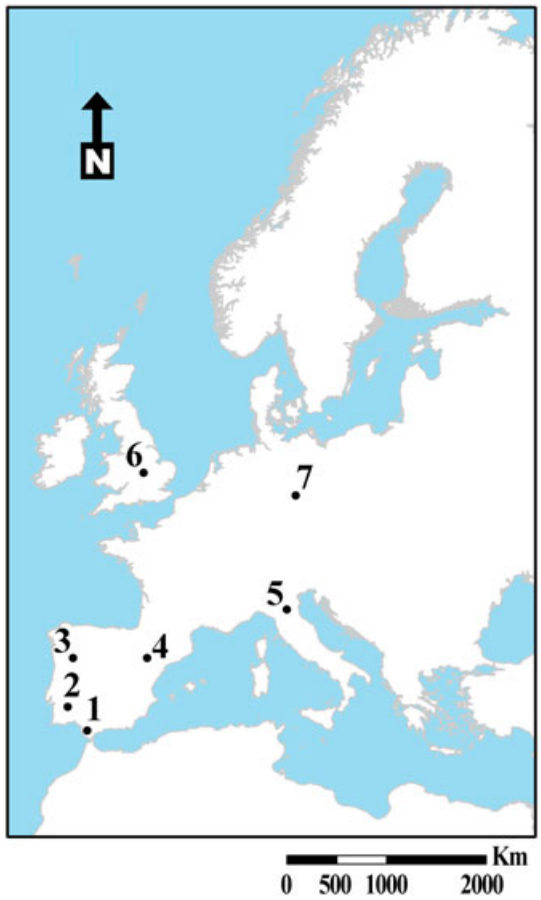

Figure 1. Map showing the locations of *Anodonta anatina* populations analysed with the microsatellite markers: 1 – Guadalquivir, 2 – Guadiana, 3 – Douro, 4 – Ebro, 5 – Reno, 6 – Medway, 7 – Rhine.

Microsatellite development

Total genomic DNA from one *A*. *anatina* individual was sent to Genoscreen (Lille, France) for microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science) using the method described by Malausa *et al*. (2011). Multiple sequences were received in FASTA format with the respective quality files. PCR reactions were then designed to amplify the new loci to improve genotyping throughput as well as cost-effectiveness.

Four sets of nine primer pairs each were chosen and initially tested, following Froufe *et al*. (2013), on 21 individuals (three per population) with PCR reactions in simplex to validate selected loci and ascertain optimal annealing temperatures. After these trials, 20 loci were selected, combined in

Table 1. Location of the analysed *Anodonta anatina* populations and corresponding microsatellite statistics; N = number of samples; N_A = number of observed alleles per population; N_{PA} = number of private alleles per population; H_E = mean expected heterozygosity; H_O = mean observed heterozygosity; F_{IS} = mean inbreeding coefficient

Population (basin)	Site	Latitude	Longitude	Country	N	N_A	N_{PA}	H_E	H_O	F_{IS}
Guadalquivir	River Corbones	37°35'14.51"N	5°38'56.96"W	Spain	20	88	23	0.40	0.38	0.07
Guadiana	River Oeiras	37°36'43.83"N	7°49'53.76"W	Portugal	20	79	10	0.35	0.32	0.12
Douro	River Sabor	41°14'22.65"N	6°58'04.59"W	Portugal	20	31	1	0.07	0.05	0.38
Ebro	River Ebro	41°50'11.79"N	1°14'02.06"W	Spain	20	62	10	0.26	0.29	0.04
Reno	Lake Castel dell'Alpi	44°11'06.98"N	11°16'34.19"E	Italy	20	114	34	0.52	0.43	0.19
Medway	River Medway	51°24'48.43"N	0°42'20.16"E	United Kingdom	20	114	16	0.52	0.55	−0.03
Rhine	Reinhardswinden pond	49°58'31.49"N	10°27'10.54"E	Germany	20	123	17	0.58	0.58	0.03

three multiplex-PCR reactions (eight in MixA, seven in MixB, and five in MixC; Table 2) and tested for polymorphism in all the 140 *A. anatina* individuals. PCR reactions were performed on a DNA Engine DyadW Peltier Thermal Cycler (Bio-Rad Laboratories), consisting of a denaturing step at 95 °C for 15 min followed by 11 cycles of denaturation at 95 °C for 30 s, 90 s annealing at 58 °C where the annealing temperature was lowered by 0.5 °C with each consecutive cycle, and 30 s elongation at 72 °C; 29 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 60 s, and extension at 72 °C for 30 s; and a final extension at 60 °C for 30 min. Labelled PCR amplicons were resuspended in 10 mL Hi-DiTM Formamide and their sizes determined in an Applied Biosystems 3100 DNA analyser, with LIZ 500 size standard as an internal size standard.

Microsatellite analyses

In total, 140 individuals were genotyped for all 20 loci. Allele frequencies, observed (H_O) and expected heterozygosity (H_E), were estimated in Genetix v.4.0.5.2 (Belkhir *et al.*, 2004). Linkage disequilibrium (LD), inbreeding coefficients (F_{IS}) and deviations from the Hardy-Weinberg equilibrium (HWE) were tested in Genepop-on-the-Web (http://genepop.curtin.edu.au/index.html; Raymond and Rousset, 1995) using exact tests with significance estimated by a Markov chain method after 10 000 randomizations. Sequential Bonferroni correction was employed to account for multiple testing (Holm, 1979). The presence and frequency of null alleles were tested in each locus using MICROCHECKER (Van Oosterhout *et al.*, 2004) and FREENA (Chapuis and Estoup, 2007), respectively. Alleles were considered as private alleles if they exhibited a frequency of more than 5% in one population and did not occur in any other population.

Global, single-locus and pairwise genetic differentiation among samples and their putative genetic structuring were assessed with the F_{ST} fixation index (Weir and Cockerham, 1984) in FSTAT 2.9.3.2 (Goudet, 1995), with significance being assessed with 1000 permutations. In addition, Jost's actual measure of differentiation D_{est} (Jost, 2008) was estimated using SMOGD v.1.2.5 (Crawford, 2010). The impact of null alleles was assessed by comparing F_{ST} estimates before and after correction for null alleles using the ENA (Excluding Null Alleles) method implemented in FREENA. As the null alleles had minimal or no impact on F_{ST} estimates (data not shown), all subsequent analyses were conducted on data uncorrected for null alleles.

A neighbour-joining dendrogram (NJ) based on pairwise Nei's (D_A) genetic distances (Nei *et al.*, 1983) was constructed with POPULATIONS v.1.2.32 software (Langella, 1999). Confidence estimates of tree topology were calculated by 1000 bootstrap replicates of loci. A NJ tree was drawn and visualized using Seaview v.4.4.3 (Gouy *et al.*, 2010). Population structure was analysed using the Bayesian model-based clustering approach implemented in STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000). Fifteen independent runs were made for K = 1–8, with each run consisting of a burn-in of 10^5 Markov-chain Monte Carlo steps followed by 5×10^5 steps. Selection of the most likely number of genetic clusters (K) was based on the second-order rate of change in probability between successive K values as described in Evanno *et al.* (2005) and implemented in STRUCTURE HARVESTER (Earl and von Holdt, 2012). Values from different run replicates under K = 3 and K = 6 were combined with CLUMPP v.1.1.2 (Jakobsson and Rosenberg, 2007) followed by DISTRUCT v.1.1 (Rosenberg, 2004) to graphically display each individual's membership coefficients for each cluster. Tests for genetic variation within groups (F_{ST}), among populations within groups (F_{SC}) and among populations (F_{CT}) were conducted with Analysis of Molecular Variation (AMOVA) with ARLEQUIN v.3.5.1.3 (Excoffier and Lischer, 2010), where the groups corresponded to the most likely number of genetic clusters (K = 3 and K = 6) identified in STRUCTURE analyses. The significance of F-statistics of the AMOVA was tested using 10 000 permutations.

RESULTS

A summary of the microsatellite results is provided in Table 2. In total, 243 alleles were observed

Table 2. Characteristics of 20 polymorphic microsatellite loci in *Anodonta anatina*; Primer sequences (*F* = forward primer sequence; *R* = reverse primer sequence); fluorescent dyes; repeat motif; *Na* = number of observed alleles per locus; sizes of amplified fragments; H_E = mean expected heterozygosity; H_O = mean observed heterozygosity; F_{IS} = mean inbreeding coefficient; HWE = Hardy–Weinberg deviation (*P*); and GenBank accession references

Locus	Primer sequences (5′-3′)	Fluorescent dye	Repeat	*Na*	Allele size range (bp)	H_E	H_O	F_{IS}	HWE	GenBank Accession
MixA										
AA2	F: TCAGCAAAGCTAATTAGATATCACAC R: TGACACCATCCCTACGTGAC	VIC	$(tg)_{14}$	23	124-184	0.62	0.47	0.24	<0.01	KP052829
AA3	F: GTGAGTGCATTGATTGTTTCG R: CTCGGAGAGGTGTAATTGCTG	VIC	$(ttgtg)_{12}$	13	210-295	0.51	0.36	0.30	<0.01	KP052830
AA6	F: GCATGATACCGAGTGTTCGAT R: CTGCGAAGTCGGGAAGTAGT	PET	$(tacac)_{12}$	16	93-183	0.64	0.68	−0.03	0.62	KP052831
AA7	F: GCCTGTGCAGATAGTGGTGA R. GAACCATGAACTTTGGTTTGTAA	FAM	$(ac)_{13}$	27	218-288	0.71	0.71	0.01	<0.01	KP052832
AA21	F: CATTTTGCTGTTGGACTTGTCT R: TGCTGAAAATGTTCCACTCG	VIC	$(tg)_9$	5	183-193	0.11	0.07	0.34	-	KP052833
AA26	F: CATGTGATGTACATTCCACCAGT R: TCCTGAGATCCATGTGTTGTG	NED	$(gt)_{11}$	18	140-188	0.35	0.34	0.05	0.86	KP052834
AA35	F: AGCTCCAGCCAGAATTCCTA R: CCCAAGACTCCACTACTCTTGC	PET	$(ct)_8$	36	193-275	0.91	0.91	0.00	0.12	KP052835
AA37	F: TGTTTGTACAGAGGGTGCCA R: GTAAGTTGAGTATGGGTGACATGC	FAM	$(tg)_7$	6	104-116	0.23	0.26	−0.10	0.53	KP052836
MixB										
AA13	F: TGAATAGTTCAAAACGCACGC R: TGTCCCTTCAACGGTCTTACA	PET	$(ca)_{13}$	17	99-131	0.52	0.51	0.01	0.31	KP052837
AA16	F: GATAACGTTCTTAATGTCTGACAAGG R: TTGCAATTGTTTCCTTTCTTTC	FAM	$(gt)_{16}$	18	201-259	0.48	0.32	0.30	<0.01	KP052838
AA17	F: TGATCCCATTTGTCAGACCC R: TGCTAGGAGATAAGCAGGTTCC	FAM	$(ga)_{11}$	15	98-156	0.40	0.36	0.10	<0.01	KP052839
AA20	F: CCTATCTTAATTGTATGAGCAATCACA R: CAAGTTACACAATGTGGCAGC	VIC	$(ca)_{12}$	42	192-298	0.65	0.61	0.06	0.01	KP052840
AA34	F: CATGCTAGCAGAGAAGATCCG R: GGAGTGTATATGTATGTGTCTGCG	PET	$(ac)_{11}$	2	251-253	0.06	0.07	−0.12	-	KP052841
AA36	F: CTCATCAGGACAAGATTGTATGC R: ACAGCGCTTTAGACCACTCG	VIC	$(ac)_8$	3	168-172	0.15	0.10	0.34	0.02	KP052842
AA38	F: TCAGTTGTGTTGTTAGTGTCCTTG R: CCTCTTTGCTCACGTCCTGT	FAM	$(ac)_7$	2	162-164	0.04	0.04	−0.10	-	KP052843
MixC										
AA41	F: CATATCATGCCCTTGAGCTGT R: CAAGTGTTGGCTTTTGTCCA	NED	$(ag)_{10}$	10	208-226	0.42	0.40	0.05	0.25	KP052845
AA42	F: TTGGAACCAGGAGAAAGCAC R: TTGTTTGGCATGTATTCCCTC	NED	$(ac)_9$	5	111-121	0.37	0.47	−0.27	<0.01	KP052848
AA44	F: TTTTATTTTAGATGCGGAGTCCA R: TGCCAATTACTTGAACCTTCC	FAM	$(tg)_8$	4	208-214	0.26	0.28	−0.09	0.16	KP052844
AA45	F: AGGGGTGTGGGATAGGTTTG R: CAACACCAGTAACCAACACAAG	VIC	$(gt)_7$	6	116-130	0.28	0.26	0.06	0.04	KP052846
AA46	F: AACAGTAGATTGTTGTTCTTCATGC R: CGTAGCTAGATTTGGCACAGG	VIC	$(ct)_7$	5	175-183	0.26	0.20	0.23	0.01	KP052847

across the 20 loci, ranging from two (AA34, AA38) to 42 (AA20) alleles per locus. Six loci deviated from Hardy–Weinberg equilibrium, and significant inbreeding coefficients (F_{IS}) ranged from 0.01 to 0.34. Null alleles were detected in very few loci in very few populations (Table 3). However, the Reno population was an exception presenting null alleles in seven alleles. Curiously, one locus (AA6) failed to amplify on almost all individuals from the Ebro and Reno populations, which clustered together in the NJ tree (Figure 2). Nevertheless, this locus was not excluded from further analyses, as its removal affected neither the F_{ST} values nor the final population structure results (data not shown). Finally, linkage equilibrium was rejected for only one comparison (Medway; AA7/AA37) after Bonferroni correction (Holm, 1979), indicating that all loci could be considered as independent markers. Population microsatellite statistics are shown in Table 1; observed heterozygosity frequencies ranged from 0.05 (Douro) to 0.58 (Rhine), while expected heterozygosity frequencies ranged from 0.07 (Douro) to 0.58 (Rhine). The significant F_{IS} inbreeding coefficients varied from −0.03 (Medway) to 0.38 (Douro). All pairwise D_{est}-values (Table 4), and F_{ST} -values were significant when corrected for multiple tests, these values indicating pronounced differentiation among all populations with the exception of Medway and Rhine. Four well

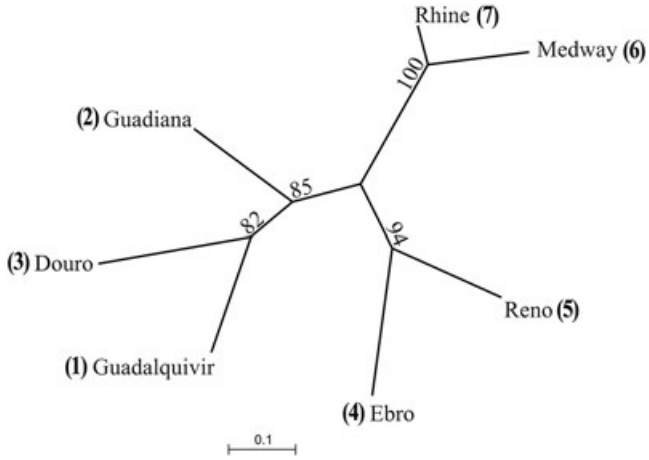

Figure 2. Neighbour-joining tree representing the *Anodonta anatina* populations analysed (bootstrap values above the branches).

supported groups are shown in the NJ tree (Figure 2); one with the Guadiana Basin, the second with the remaining Atlantic Iberian Basins (Douro and Guadalquivir), the third with the Ebro and Reno Basins and the fourth with Medway and Rhine Basins (Figure 2). The plots of ΔK (Evanno *et al.*, 2005) based on the STRUCTURE analysis indicated that three (K = 3) is the most likely number of clusters present in the full dataset. Cluster one included the populations from Guadalquivir, Guadiana, and Douro, cluster two included the populations from Ebro and Reno and cluster three integrated the populations from

Table 3. Null allele frequencies for 20 microsatellite loci across all populations of *Anodonta anatina*. Null allele presences determined with MICROCHECKER (Van Oosterhout *et al.*, 2004) are indicated in bold

Locus	Guadalquivir	Guadiana	Douro	Ebro	Reno	Medway	Rhine
AA2	-	-	-	-	**0.11**	-	**0.27**
AA3	-	0.36	-	-	**0.15**	-	0.12
AA6	-	-	-	-	-	-	-
AA7	-	-	-	-	-	-	-
AA13	-	-	-	-	-	-	-
AA16	-	-	0.20	-	**0.39**	**0.13**	-
AA17	-	-	-	-	**0.26**	-	-
AA20	-	-	-	-	**0.11**	-	-
AA21	-	-	-	-	**0.14**	-	-
AA26	-	-	-	-	-	-	-
AA34	-	-	-	-	-	-	-
AA35	-	-	0.15	-	-	-	-
AA36	-	-	-	-	**0.19**	-	-
AA37	-	-	-	-	-	-	-
AA38	-	-	-	-	-	-	-
AA41	-	0.42	-	-	-	-	-
AA42	-	-	-	-	-	-	-
AA44	-	-	-	-	-	-	-
AA45	-	-	-	-	-	-	-
AA46	0.17	-	-	-	-	-	-

Table 4. Pairwise estimates of F_{ST} (above diagonal) and Jost's D_{EST} (below diagonal) between all populations sampled for *Anodonta anatina*. All values are significant after sequential Bonferroni correction ($P < 0.003$)

	Guadalquivir	Guadiana	Douro	Ebro	Reno	Medway	Rhine
Guadalquivir		0.408	0.550	0.551	0.411	0.464	0.399
Guadiana	0.293		0.613	0.611	0.394	0.444	0.357
Douro	0.219	0.264		0.808	0.637	0.661	0.607
Ebro	0.418	0.434	0.597		0.352	0.492	0.448
Reno	0.545	0.480	0.581	0.293		0.350	0.299
Medway	0.714	0.539	0.763	0.551	0.645		0.081
Rhine	0.605	0.410	0.686	0.548	0.597	0.079	

Medway and Rhine (Figure 3(A)). With the second most likely number of clusters (K = 6), each of the clusters, one to five, included only one population, while cluster six included the Medway and Rhine populations (Figure 3(B)). The results from AMOVA for K = 3 showed that 25% of the genetic variation was explained by differences among groups with high and significant F_{ST}-values. Furthermore, the AMOVA results for K = 6 still explain 41% of the variation among groups, also with high and significant F_{ST}-values.

DISCUSSION

The 20 microsatellite markers developed here resulted in successful amplifications of divergent populations of *A. anatina* across a wide geographical range.

A high level of population structure was found among all populations, but with considerable similarity between the populations in Central Europe (Rhine, Germany) and the British Isles (Medway, United Kingdom). The levels of differentiation among populations found in the present study are also congruent with the previously published mtDNA lineages (Froufe *et al.*, 2014). Three distinct ESUs are proposed in the present study together with three MUs that should be managed independently.

Population genetics

The significant deviations from HWE detected in some loci have been commonly reported for Unionoid mussels (Eackles and King, 2002; Jones *et al.*, 2006; Zanatta and Murphy, 2006). Possible explanations include the effect of null alleles, recent population bottlenecks or significant levels of close inbreeding, which may arise because of hermaphroditic reproduction known to occur in some populations of *A. anatina* (Hinzmann *et al.*,

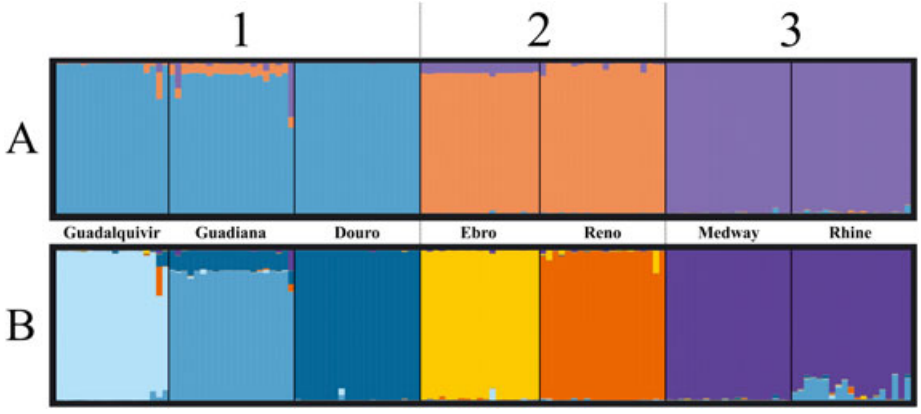

Figure 3. Results of the STRUCTURE Bayesian clustering analysis on *Anodonta anatina* populations: (A) with the most likely number of clusters (K = 3); (B) with the second most likely number of clusters (K = 6). Each individual is represented by a vertical bar in K coloured segments with the length of each bar being proportional to the estimated membership coefficient. Black lines separate individuals from different geographic regions. Numbers over the graphics correspond to the main mtDNA clades proposed by Froufe *et al.* (2014): 1 – Atlantic Iberian clade, 2 – Ebro + Italian clade; and 3 - Central European clade.

2013). Since the primers were developed using DNA from animals from the Iberian clade, the number of null alleles in the Italian lineage (Ebro + Reno) might be related to the high genetic divergence of the Italian from the Iberian lineage with probable changes in the flanking region. The Douro and Reno populations had high F_{IS} values. In the case of the Reno population, this can be attributed to founder effects since it is a very recent (1951) small lake created by a natural landslide. As for the Douro population, the high F_{IS} values could be caused by the declining population resulting from recent bottlenecks due to human activities such as dam construction (Sousa *et al*., 2012).

Relatedness of populations

The most likely number of clusters from the Bayesian assignment and AMOVA analysis indicates a strong genetic structure among the studied *A. anatina* populations corresponding to the pattern previously found with mtDNA markers. One group includes all Atlantic Iberian basins (Gudalquivir, Guadiana and Douro), a second includes the Mediterranean Iberian (Ebro) and Italian (Reno) populations, and a third integrates the British (Medway) and German (Rhine) populations. Substructure of the Iberian populations is also confirmed with the Bayesian, AMOVA and NJ analysis. The pairwise F_{ST}/D_{EST} comparison generated some of the highest values of genetic differentiation found so far for freshwater mussels (Zanatta and Murphy, 2007; Zanatta and Wilson, 2011). Although populations of *M. margaritifera* (family Margaritiferidae) in Central Europe have comparably high levels of differentiation, in the family Unionidae this highly structured pattern is only equivalent to those of the congeneric species *A. californiensis/nuttalliana* in the western US ($D_{EST} = 0.610$ among all populations; Mock *et al*., 2010).

Correspondence with mtDNA analysis

In the present study, the high values of genetic differentiation among the populations belonging to the previously detected mtDNA lineages (Froufe *et al*., 2014) are congruent with an ancient divergence event. In addition, the results from the NJ tree, with four supported clades, confirm the existence of genetic substructure in the Iberian populations. On the other hand, the mtDNA analysis has found no evident genetic structure in northern, central and eastern Europe from the British Isles through Germany and Poland all the way to Ukraine, including the Scandinavian countries. Similarly, a lower genetic differentiation between Medway (UK) and Rhine (Germany) populations was detected in this study using the new microsatellite markers. By contrast, in Germany a distinct pattern was found for the freshwater mussel *M. margaritifera* with highly differentiated populations among central European drainages (Geist *et al*., 2010a). Although the present study only analysed two *A. anatina* populations belonging to the northern European mtDNA clade, a probable explanation for the divergent pattern between the two species might be related to the fact that while *Margaritifera* spp. have a very narrow range of habitat preference and host fish for dispersal, *Anodonta* spp. may use a wide range of hosts and are able to occupy habitats subjected to different abiotic conditions. These features could significantly increase the dispersal ability and colonization success of *A. anatina* (Douda *et al*., 2013; Mock *et al*., 2013).

Conservation implications

Based on the present nuclear data, the previously identified mtDNA lineages of *A. anatina* (Froufe *et al*., 2014) are proposed here as three distinct evolutionarily significant units (ESUs) since they are geographically and historically separated, being reciprocally monophyletic for mtDNA haplotypes and showing, at the same time, significant divergence of allele frequencies at the nuclear loci (Moritz, 1994; Palsbøll *et al*., 2007; Frankham, 2010). Furthermore, all the southern European populations analysed can be defined as distinct management units since they present a high among-population differentiation, mainly at nuclear loci (Hedrick *et al*., 2001; Caballero *et al*., 2012).

Although more populations are needed to gain a fully comprehensive picture across the whole range of *A. anatina*, the populations from the British

Isles, and central and northern Europe can be viewed as belonging to the same ESU. This ESU has a wide range and is therefore considered to have a more favourable conservation status than those from Southern Europe, which are more limited in distribution. Even so, considerable declines in spatial distribution and density of *A. anatina* have been recorded in some central European countries owing to pollution and habitat modification (Lopes-Lima, 2014), which means that this ESU will require monitoring. The ESU that includes the Italian and Ebro populations is mainly threatened by habitat degradation, water abstraction and invasive species and is declining rapidly (Cianfanelli *et al*., 2007; Halcon, 2011). As for the Atlantic Iberian ESUs, the Guadiana and Guadalquivir MUs on the south of the Peninsula are affected by historic mining pollution and more recent human impacts such as dam construction, urban development and agriculture (Company *et al*., 2008). Water shortage in some of the temporary streams of this ESU is possibly the most acute threat to this and other aquatic species. In the Douro MU the *A. anatina* populations are affected mainly by dam construction and pollution (Sousa *et al*., 2012).

All of the ESUs and MUs identified in this study deserve independent management attention. The genetic differentiation and divergence between ESUs should be considered in any study involving the physiology and toxicology of these animals. Distinct physiological traits such as host fish requirements, temperature resistance and other local adaptation characteristics should be assessed individually in each ESU. Furthermore, captive breeding programmes that aim to release or reintroduce *A. anatina* into natural habitats, should take account of specific genetic stocks with respect to the original genetic differentiation among the MUs detected in this study.

Unionoid populations are decreasing at an alarming pace, having been severely affected by many human activities that have resulted in habitat loss and fragmentation, pollution, overexploitation of resources, climate change and introduction of invasive species (Strayer *et al*., 2004; Lopes-Lima *et al*., 2014). Informed conservation strategies require high-resolution genetic analyses to identify populations with unique, divergent or low diversity (Geist *et al*., 2003). The microsatellites described here are valuable in fine-scale geographic studies and also provide a broadly useful set of tools for ecological and population genetic studies. For example, these markers may be applied to studies of dispersal rates (Berg *et al*., 2007), reproductive patterns such as identifying gamete dispersal and distance of reproduction (Ferguson *et al*., 2013), and the assessment of multipaternity, hermaphroditism and self-fertilization ratios (Bai *et al*., 2012). They also allow identification of co-evolutionary patterns, since these mussels have a parasitic larval phase in which they need a host fish for metamorphosis and dispersal (Geist and Kuehn, 2008; Zanatta and Wilson, 2011).

Throughout its range, *A. anatina* is exposed to increasing human pressure including increasing demands for water. This is especially pronounced in the Mediterranean biodiversity hotspot region (e.g. Portugal, Spain and Greece), which has severely impaired freshwater ecosystems, with a widespread decline and loss of endemic freshwater species (Albrecht *et al*., 2006; Benejam *et al*., 2010). Therefore, it is important to study patterns of genetic diversity of *A. anatina* in other biogeographical areas of southern Europe, such as the Balkans, Turkey or the Ponto-Caspian region, to assess whether they support further distinct and vulnerable ESUs. The microsatellite loci described in this study offer considerable utility in this respect.

ACKNOWLEDGEMENTS

We would like to thank our collaborators who have kindly provided us with tissue samples: Juergen Geist, Katharina Stoeckl, and Irene Guarneri. Financial support was provided by the Portuguese Foundation for Science and Technology (FCT) project PTDC/AAC-AMB/117688/2010. We would like to thank Susana Lopes for help with fragment analysis and acknowledge the assistance of staff at Genoscreen for generation of 454 sequence reads and DNA sequencing. 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 Andalucia (Consejería de Medio Ambiente- SGYB/FOA/AFR/SFS, de 17 de Julio de 2012); and Gobierno de Aragón (Dirección General de Conservación del Medio Natural- 136.071/2012 de 12 de Julio de 2012).

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