Biological Journal of the Linnean Society, 2003, **78**, 235–252. With 3 figures

Phylogeography and conservation genetics of endangered European Margaritiferidae (Bivalvia: Unionoidea)

ANNIE MACHORDOM*, RAFAEL ARAUJO, DIRK ERPENBECK† and MARÍA-ÁNGELES RAMOS

Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal 2, 28006 Madrid, Spain.

Received 26 April 2002; accepted for publication 29 October 2002

Margaritifera margaritifera and *M. auricularia* are among the most endangered freshwater mussels in the world, and the only species of the genus found in Europe. Our genetic study explores allozymic variability (27 loci) and differentiation at the mitochondrial sequence level (partial COI and 16S rRNA gene sequences). The Spanish *M. auricularia* population showed genetic parameters of variation that were of the same order as those of other freshwater molluscs (though at the lower end of the range), probably permitting its potential recovery. The difference between this species and *M. margaritifera* was clearly established (ten diagnostic allozymic loci, Nei = 0.462, and mean nucleotide divergence around 9.4%). The *M. margaritifera* populations analysed showed a certain degree of population genetic structure (according to allozyme data) that was not, however, related to a geographical cline. Nevertheless, two mitochondrial lineages (albeit very closely related) were identified: a northern lineage extending from Ireland to the Kola Peninsula including the western Atlantic coast, and a second cluster distributed from Ireland to the Iberian Peninsula. The phylogenetic relationships between these two species and other related taxa were established. The putative *M. m. durrovensis* could be considered an 'ecophenotype'. Palaeobiogeographical scenarios are presented and indicate unexpected 'recent' gene flow between *M. margaritifera* populations that were theoretically isolated in the early Tertiary. *Biological Journal of the Linnean Society* 2003, **78,** 235–252.

ADDITIONAL KEYWORDS: 16S rRNA – allozymes – COI – freshwater mussels – genetic variation – *Margaritifera* – mtDNA.

INTRODUCTION

The concept of 'Conservation Genetics' was developed 'to contribute to the preservation of the natural diversity of species with a view to preserve the evolutionary potential of species by exploring the relationships between today's advances in genetics and their potential contribution to the quality of wildlife (animal and plant) conservation' (Schonewald-Cox *et al*., 1983). Moritz (1994) also indicated that a prerequisite for managing biodiversity is the identification of populations with independent evolutionary histories. The different genetic or molecular tools that may be applied to endangered populations or species could provide limits for the units to manage, preserving the total diversity of the taxon. Thus, certain cryptic species or local adaptations might come to light, and the subsequent conservation programme would be designed to maintain the variability of the species, essential for their evolution and survival.

Though the last decades have seen renewed conservation interests and improved molecular tools, there is still a bias towards studies on emblematic vertebrates despite the fact that most biodiversity is provided by invertebrate species.

Freshwater mussels (Unionoidea) are among the most endangered invertebrates in the world. Their decline is the result of ever increasing human activity regarding freshwater habitats, such as the regulation and impoundment of rivers (Vaughn & Taylor, 1999). Such is the extent of their loss, that of the 344 unionoid species in the United States, 35 are extinct, 57 are

*Corresponding author. E-mail: annie@mncn.csic.es †Current address: Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, PO Box 94766, 1090 GT Amsterdam, the Netherlands.

endangered, five are threatened and 61 are candidates (Bogan, 1998). In Europe, the remarkable decline suffered by freshwater mussel populations has recently attracted the attention and concern of national and international conservation organizations (Kileen, Seddon & Holmes, 1998). However, to date, few investigations have focused on Unionoidea, and the real situation is poorly understood and especially alarming for the Margaritiferidae family.

Margaritiferidae species are presumed to be among the most primitive freshwater mussels (i.e. close to ancestors from the sea that colonized fresh waters and gave rise to all other unionaceans; Davis & Fuller, 1981; Campbell, 2000). With nearly ten species spread throughout the northern hemisphere, Margaritiferidae are among the most threatened Unionoidea and include relatively 'unknown' species such as *Margaritifera auricularia* (Spengler, 1793) in the NW Palaearctic, and *Margaritanopsis laosensis* (Lea, 1863) inhabiting only one known Asian locality. *Margaritifera auricularia* has recently been the subject of thorough investigation (Araujo & Ramos, 1998, 2000a, 2001; Araujo, Bragado & Ramos, 2000) since the 'rediscovery' of a population of some 2000 specimens in Spain and the collection of live specimens in 1991 from Moroccan rivers (Araujo & Ramos, 2000b). In Europe, populations of the widespread *Margaritifera margaritifera* (Linnaeus, 1758) were reduced by 90% over the course of the last century. This has generated much controversy with respect to the 'ecophenotype' (i.e. a morphological local adaptation) *Margaritifera margaritifera durrovensis* (Phillips, 1928) (Chesney, Oliver & Davis, 1993), an Irish hard water population inhabiting a single river, the Nore, and listed as a protected subspecies by the International Union for Conservation of Nature (IUCN).

The life history of the margaritiferids has been used as a 'book example' in biogeographical studies (e.g. the 1922 classic by Wegener *Die Entstehung der Kontinente und Ozeane*). *Margaritifera margaritifera* is considered the oldest species of the group, emerging before the Atlantic splitting of American from European populations (i.e. before the early Tertiary) (Bauer, 1997). Nevertheless, numerous conflicting hypotheses have been put forward to explain the origins of this group (Walker, 1910; Smith, 1976; Taylor, 1988). This controversy prompted our attempt to find an explanation for both the origin and the previous wide distribution of the margaritiferids through a comparison of their genetic diversity. The larvae (glochidia) of all unionoids are parasitic on the gills and/or fins of suitable fish hosts. This specificity between glochidia and fishes may provide a clue to understanding their distribution (Taylor & Uyeno, 1965; Bauer, 1997). The evolution of *M. margaritifera*, the most widespread species, *M. falcata* (Gould, 1850) and *M. laevis* (Haas, 1910) is strongly linked to salmonids (Ziuganov *et al*., 1994), while the European *M. auricularia* is thought to be associated with the acipenserids (Altaba, 1990; Araujo & Ramos, 2000b). This relationship with anadromous fishes can be interpreted as an ancestral feature for their transition from the sea to the freshwater habitat aimed at ensuring larval survival.

Moreover, the unionids in general present a morphological plasticity that sometimes impedes clear taxonomic classification. Thus, effective management could be hampered by this lack of information, which is practically non-existent at the population level (King *et al*., 1999).

Given these premises, the molecular study of available living European species of Margaritiferidae was considered to be of major interest for understanding both the evolution of this mollusc group and its conservation problems. With these objectives in mind, a combined approach (allozymic electrophoresis and mitochondrial DNA sequencing) was applied for the first time to the European species of this group.

The decline of these bivalves prevented us from dealing with large numbers of specimens from some populations, which were sometimes below the recommended figures for population structure analyses. Nevertheless, this is currently an insurmountable problem that should not dissuade us from obtaining as much data as possible on these endangered species. Thus, the main aims of this paper were: (i) to investigate the evolutionary relationships of the European *M. margaritifera* populations by testing for genetic differentiation or structure and (ii) to investigate genetic variability and phylogenetic relationships of the Spanish *M. auricularia.*

MATERIAL AND METHODS

Taxa and specimens

The samples used were portions of mussel foot. To ensure the survival of most of the organisms analysed, when possible, a small piece of foot (<1 cm^3) was dissected *in situ* and maintained in liquid nitrogen or absolute ethanol before being transported to the laboratory. The specimens were then returned alive to their original habitat (Fig. 1). Otherwise, specimens were transported to the laboratory alive.

Table 1 shows the 133 specimens included in the allozyme study [36 *M. auricularia*, 87 *M. margaritifera*, and an outgroup of ten *Potomida littoralis* (Lamarck, 1801)]. As these species are severely endangered, some populations were underrepresented. Nevertheless, the number of loci analysed (27) could, at least partly, make up for the scarce number of specimens studied (Nei, 1978).

Forty-six specimens were also used for the mitochondrial DNA sequence analysis of partial cyto-

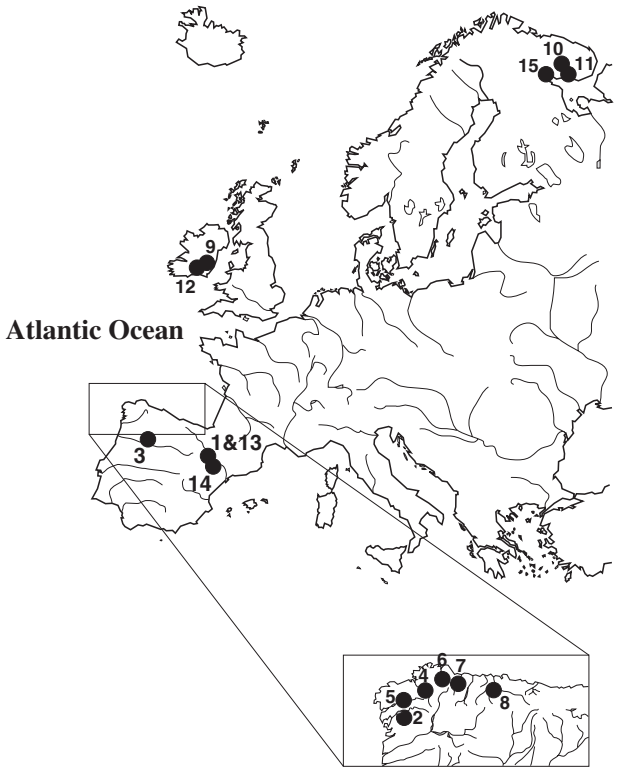

Figure 1. Sampling locations. Numbers correspond to those shown in Table 1.

chrome oxidase subunit I (COI) and 16S rRNA (16S) gene sequences (Table 1). *Potomida littoralis* and *Cumberlandia monodonta* (Say 1829) (GenBank accession numbers, A.N.: U72546 and AF156498 for the 16S and COI sequences, respectively) were used as outgroups in all the analyses. For several other analyses not shown here, the outgroup was formed by *Neotrigonia margaritacea* (Lamarck 1804) (A.N.: U56850; COI), *Amblema plicata* (Say 1817) (A.N.: U72548; 16S), *Anodonta cygnea* (Linnaeus 1758) (A.N.: U56842; COI) *and Anodonta couperiana* (Lea 1840) (A.N.: U72560; 16S). Additionally, a GenBank sequence from a North American *M. margaritifera* (Eastern Pearlshell; A.N.: U72544; 16S and U56847; COI) was included in the taxon set.

ALLOZYMES

Tissue samples were homogenized and stored at –70°C before use. Horizontal starch (11%) enzyme electrophoretic procedures were performed by combining the methods of Aebersold *et al*. (1987) and Pasteur *et al*. (1987).

Table 2 shows the 20 enzymes (27 loci) analysed and also provides details on the conditions of electrophoresis.

Allele mobility values were expressed relative to the most common allele (taken as '100'). In general, this reference value was assigned to the allele of the nominal species *M. margaritifera.* Corresponding enzyme

Table 1. Populations studied, localities, number of specimens analysed (N1 = allozymic analysis, N2 = mitochondrial sequence analysis) and GenBank accession numbers of the mitochondrial genes sequenced

					GenBank accession number	
		Locality	N1	N2	16S rRNA	COI
1	*M. auricularia*	Canal Imperial de Aragón (Zaragoza, Spain)	36	8	AF303273 to AF303279	AF303309 to AF303314
2	*M. margaritifera*	Ulla Basin (Pontevedra, Spain)	5	2	AF303281, AF303282	AF303316
3	*M. margaritifera*	Tera River (Zamora, Spain)	3	3	AF303289	AF303323 to AF303325
4	*M. margaritifera*	Mandeo River (La Coruña, Spain)	3	2	AF303285, AF303286	AF303319, AF303320
5	*M. margaritifera*	Tambre River (La Coruña, Spain)	7	2	AF303283, AF303284	AF303317, AF303318
6	*M. margaritifera*	Landro River (Lugo, Spain)	23	2	AF303287, AF303288	AF303321, AF303322
7	*M. margaritifera*	Oro River (Lugo, Spain)	4	–	–	–
8	*M. margaritifera*	Narcea River (Asturias, Spain)	10	5	AF303290 to AF303292	AF303326 to AF303330
9	*M. margaritifera*	Dereen River (Carlow, Ireland)	14	3	AF303293, AF303294	AF303331, AF303332
10	*M. margaritifera*	Pana River (Kola, Russia)	4	3	AF303297	AF303335 to AF303337
11	*M. margaritifera*	Varzuga River (Kola, Russia)	5	4	AF303298 to AF303300	AF303338 to AF303341
12	*M. m. durrovensis*	Nore River (Kilkenny, Ireland)	9	6	AF303301 to AF303306	AF303342 to AF303347
13	*Potomida littoralis*	Canal Imperial de Aragón (Zaragoza, Spain)	10	2	AF303307, AF303308	AF303348, AF303349
14	*M. auricularia*	Ebro River (Zaragoza, Spain)	–	1	AF303280	AF303315
15	*M. margaritifera*	Thurma River (Kola, Russia)	–	3	AF303295, AF303296	AF303333, AF303334

Table 2. Enzyme systems analysed by protein electrophoresis. Commission number (C.N.), name abbreviation (Abbr.), number of active loci studied and buffer used: 1 = LiOH (pH = 8.3); 2 = TBE (pH = 8.6); 3 = TME (pH = 6.9); 4 = Poulik (pH = 8.2) and 5 = TC (pH = 6.7)

Enzyme system	C. N.	Abbr.	Loci	Buffer
Aspartate aminotransferase	2.6.1.1	AAT	2	1 and 2
Catalase	1.11.1.6	CAT	1	2
Diaforase	1.6.4.3	DIA	1	1 and 2
Esterase	3.1.1.1	EST	2	1 and 3
Fumarate hydratase	4.2.1.2	FH	1	4
Glucose-6-phosphate isomerase	5.3.1.9	GPI	1	5
Glycerol-3-phosphate dehydrogenase	1.1.1.8	G3PDH	1	1 and 4
Hexokinase	2.7.1.1	HK	1	1
Isocitrate dehydrogenase	1.1.1.42	IDHP	1	1 and 3
L-lactate dehydrogenase	1.1.1.27	LDH	1	2
Leucine-alanine aminopeptidase	3.4.11	PEP-LA	3	1 and 2
Leucyl aminopeptidase	3.4.11.1	LAP	1	1 and 2
Malate dehydrogenase	1.1.1.37	MDH	2	5
Malic enzyme-NAD	1.1.1.39	ME	1	2
Mannose-6-phosphate isomerase	5.3.1.8	MPI	1	4
Phosphoglucomutase	5.4.2.2	PGM	2	5
6-phosphogluconate dehydrogenase	1.1.1.44	PGDH	1	2
Sorbitol dehydrogenase	1.1.1.14	SORD	1	1
Superoxide dismutase	1.15.1.1	SOD	1	2 and 4
Xaa-proline aminopeptidase (leucine-proline substrate)	3.4.11.9	PEP-LP	2	1 and 2

loci were similarly numbered such that higher numbers indicated a greater anode migration distance.

MITOCHONDRIAL DNA

Tissue samples, preserved in ethanol or frozen, were ground to a powder in liquid nitrogen before adding 600 μL of CTAB lysis buffer (2% CTAB, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 0.1 M TRIS [pH = 8]) and subsequently digested with proteinase K (100 μg mL^{-1}) for 2–5 h at 60°C. Total DNA was extracted according to standard phenol/chloroform procedures (Sambrook, Fritsch & Maniatis, 1989).

The COI and 16S partial sequences were amplified by polymerase chain reaction (PCR) using the following primers: LCO1490 5′-GGTCAACAAATCATAAA GATATTGG-3′ (Folmer *et al*., 1994) and COI-H 5′-TCAGGGTGACCAAAAAATCA-3′ (6 bases shorter than HCO2198 *et al.*, 1994) for COI; 16sar-L-myt 5′-CGACTGTTTAACAAAAACAT-3′ and 16sbr-H-myt 5′-CCGTTCTGAACTCAGCTCATGT-3′ (Lydeard, Mulvey & Davis, 1996) for 16S. The following cycles were conducted in the partial COI amplification: 92°C (5 min), 40 cycles of 94°C (30 s), 50°C (1 min), 72°C (1 min) and a final extension of 72°C (10 min). Amplification of the 16S gene was performed under the same conditions except at a lower annealing temperature (40°C). In a final volume of 50 μL, the PCR mix contained 1–3 μL DNA, 0.5 μM of both primers, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1 U *Tth* DNA polymerase (Biotools), the corresponding buffer and ddH_2O. The amplified fragments (around 700 bp) were purified on 'Biotools' columns prior to sequencing both strands on an ABI 377 DNA sequencer (Applied Biosystems).

The DNA sequences obtained were cleaned at the primer ends, aligned and controlled using the Sequencher program (Gene Code Corporation). The CLUSTAL W program (Thompson, Higgins & Gibson, 1994) was employed to align the 16S gene sequences. Additionally, all alignments were controlled by eye. COI translation to protein was undertaken using the package MacClade 3.05 (Maddison & Maddison, 1992). As some of the sequences obtained from GenBank were shorter than ours, it was necessary to complete the GenBank sequences by adding 'N' for each missing character.

ALLOZYME DATA ANALYSIS

Phenotype distributions of all co-dominantly expressed loci were tested for agreement with Hardy–Weinberg expectations using exact probability tests.

As two specimens of the putative *M. m. durrovensis* population 'expressed' null alleles in homozygosity, the frequency of *EST-2*100* was retrocalculated from that of the null allele. This was done by considering that the frequencies of these two alleles are in agreement with Hardy–Weinberg expectations, and taking into account the null (*EST-2*null*) homozygote frequency (p^2 = number of null homozygotes/total number of specimens analysed) to calculate the frequency of theoretically existing heterozygotes ($2pq$), and that of the *EST-2*100* homozygotes (q^2). Pairwise multilocus comparisons between samples were conducted through Nei's measure of genetic distance adapted to small sample sizes (Nei, 1978). All these computations were performed using BIOSYS-1 (Swofford & Selander, 1989), GENEPOP (Raymond & Rousset, 2000) and GENETIX (Belkhir *et al*., 2000). Genetic structure was hierarchically examined using an analysis of variance framework (Weir & Cockerham, 1984) as implemented in the ARLEQUIN package (Schneider *et al*., 1997). The significance of the isolation by distance hypothesis was statistically tested using the Mantel (1967) test. This test checks for correlation between genetic and geographical distance matrices (NTSYS program, Rohlf, 2000). In addition, maximum likelihood analyses based on allelic frequencies were performed to assess phylogenetic relationships among populations (CONTML program, PHYLIP 3.57 package, Felsenstein, 1995). We undertook ML analyses instead of recovering phenogram reconstructions from distances among populations, as the former provide more phylogenetic information.

Nucleotide data analysis

Nucleotide saturation was evaluated by plotting transition and transversion changes against uncorrected ('*p*') divergence values. Sequence analysis was based on the principles of maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML). The evolutionary model best fitting our data was selected by the Modeltest 3.06 program (Posada & Crandall, 1998). According to this, we used both GTR (General Time Reversible model, Lavane *et al*., 1984; Rodríguez *et al*., 1990) and HKY (Hasegawa, Kishino & Yano, 1985) distances. Parsimony analysis was performed by heuristic searches under TBR branch swapping and ten random replicates of taxon addition using the PAUP* 4.06 package (Swofford, 2001). Maximum likelihood analysis was performed by Quartet Puzzling (using 1000 replicates) or heuristic search. We estimated support in the phenetic and parsimony analyses by bootstrapping (1000 repetitions) (Felsenstein, 1985) and calculating decay indices (DI, Bremer, 1988, 1994; using the AutoDecay program: Eriksson, 1998). Clock-like behaviour was evaluated by calculating statistical differences between branches (PHYLTEST program, Kumar, 1996).

The analyses were performed for each separate gene. When the sequence of both genes was known, the number of nucleotides was added together. To analyse the gene pairs, congruence among tree topologies of COI and 16S rRNA genes was assessed by the partition homogeneity test in PAUP* (Mickevich & Farris, 1981; Farris *et al*., 1994).

RESULTS

Allozymes

In the initial analyses, we considered each of the 20 collection samples as a distinct population: three for *M. auricularia* and 17 for *M. margaritifera* (including the putative *M. m. durrovensis*). The three *M. auricularia* populations comprised specimens of the same population collected from different years. However, as the contingency X^2 analysis could not detect a difference in allelic frequencies, they were considered as one single population. *Margaritifera margaritifera* populations were also pooled according to river source based on the results of contingency tests, reducing the number of populations to 11 for the purpose of this analysis (Table 1).

Allelic frequencies and variability parameters of the resulting 12 populations and *Potomida littoralis* (included as an outgroup) are shown in Table 3. The low number of specimens of some populations analysed probably forced low variability indices, since polymorphism values were correlated to sample size (correlation coefficient $R = 0.65$, $P = 0.30$). In any case, P_{99} values ranged from 0 to 22.2% (maximum value shown by *M. auricularia*), while *P. littoralis*, with only ten specimens analysed, showed a value of 18.5% (higher than some of the polymorphism values recorded for *M. margaritifera* from populations with a greater number of samples analysed). There was no correlation between heterozygosity and sample size (correlation coefficient $R = -0.005$, $P < 0.05$), *Potomida* showing the highest heterozygosity value ($H_o = 0.037$).

Of the 25 tests performed (excluding the *EST-2* test for *M. m. durrovensis*) to verify the null hypothesis of Hardy–Weinberg equilibrium, significant results were recorded in three exact probability tests. Adjusting the significance levels both by standard and by sequential Bonferroni (Lessios, 1992), with an experimental error rate of 0.05, only the test involving *AAT-s* for *M. auricularia* remained significant.

Only one locus, *LDH*, proved to be monomorphic for all the species analysed. Ten diagnostic loci differentiated *M. auricularia* and *M. margaritifera*, and 23 of the 27 loci screened served to distinguish *P. littoralis* from the two *Margaritifera* species. This gave rise to high genetic distance values (Table 4) with averages of

Table 3. Allelic frequencies and parameters of variability (A = mean number of alleles per locus, P_{99} = proportion of polymorphic loci 0.99 criterion, H_o = observed heterozygosity, and H_e = expected heterozygosity) of the populations analysed by allozymic electrophoresis. Numbers in parentheses indicate the standard deviation. N = average of samples analysed for each population. Numbers assigned to populations are those indicated in Table 1

	M. auricularia					M. margaritifera							Potomida
Pop.	1	2	3	4	5	6	7	8	9	10	11	12	13
Locus allele													
AAT-m													
66	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
AAT-s													
48	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.100	0.000	0.000
100	0.944	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.964	1.000	0.900	1.000	0.050
102	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
104	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.950
CAT													
88	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
100	0.958	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
131	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
DIA													
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
114	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
EST-1													
41	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
EST-2													
84	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
87	0.986	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.529	0.000
104	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
null	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.471	0.000
FUM													
86	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000

G3PDH														
94	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
100	1.000	1.000	1.000	1.000	1.000	0.750	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
GPI														
44	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.850
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
102	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.150
107	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HK														
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
121	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
IDH														
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
145	0.986	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
155	0.014	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
192	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
LAP														
87	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.300
91	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.550
95	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.150
98	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
LDH														
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-m														
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
202	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
MDH-s														
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050
31	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.900
56	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
195	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 3. *Continued*

	M. auricularia					M. margaritifera							Potomida
Pop.	1	2	3	4	5	6	7	8	9	10	11	12	13
ME													
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
106	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
114	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MPI													
100	0.967	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
105	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
170	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
PDGH													
3	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
50	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000
95	0.972	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
100	0.000	1.000	1.000	1.000	1.000	0.977	1.000	1.000	1.000	1.000	1.000	1.000	0.000
PEP-LA-1													
97	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
PEP-LA-2													
92	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.100	0.111	0.000
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.750	0.900	0.889	0.000
PEP-LA-3													
91	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500
93	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
PEP-LP-1													
83	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
100	1.000	1.000	1.000	1.000	0.929	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

PEP-LP-2													
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
107	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
118	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
PGM-1													
87	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000
91	0.000	1.000	0.000	0.214	0.609	0.875	0.938	1.000	1.000	0.900	0.889	0.889	0.000
100	0.000	0.000	1.000	0.786	0.391	0.125	0.063	0.000	0.000	0.000	0.111	0.111	0.000
120	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
122	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGM-2													
100	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
124	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SDH													
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
144	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
SOD													
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
104	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
N	33.4	4.9	2.9	3.0	6.8	21.4	3.9	8.5	13.4	3.8	4.6	9.0	9.3
	(1.3)	(0.1)	(0.1)	(0.0)	(0.1)	(0.5)	(0.1)	(0.3)	(0.2)	(0.1)	(0.2)	(0.0)	(0.3)
A	1.3	1.0	1.0	1.0	1.1	1.1	1.1	1.0	1.0	1.0	1.1	1.1	1.3
	(0.1)	(0.0)	(0.0)	(0.0)	(0.1)	(0.1)	(0.1)	(0.0)	(0.0)	(0.0)	(0.1)	(0.1)	(0.1)
P_{99}	22.2	0.0	0.0	0.0	7.4	7.4	7.4	3.7	3.7	3.7	11.1	11.1	18.5
H_o	0.010	0.000	0.000	0.000	0.018	0.018	0.028	0.005	0.003	0.019	0.022	0.033	0.037
	(0.004)	(0.000)	(0.000)	(0.000)	(0.016)	(0.016)	(0.020)	(0.005)	(0.003)	(0.019)	(0.012)	(0.020)	(0.017)
H_e	0.013	0.000	0.000	0.000	0.020	0.020	0.025	0.005	0.003	0.016	0.022	0.037	0.063
	(0.006)	(0.000)	(0.000)	(0.000)	(0.018)	(0.018)	(0.018)	(0.005)	(0.003)	(0.016)	(0.012)	(0.017)	(0.031)

Table 4. Nei (1978) genetic distances among the populations analysed by allozyme electrophoresis (below the diagonal), and uncorrected mean nucleotide divergence (above the diagonal) for the two mitochondrial genes (16S rRNA and cytochrome oxidase subunit one) considered together. The main diagonal (in bold type) shows intrapopulation nucleotide divergence values (hyphens indicate that the calculation was not possible). Asterisks mark the absence of mitochondrial gene data for population 7. Numbers assigned to the populations correspond to those indicated in Table 1

Population	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *M. auricularia*	**0.23**	9.28	9.28	9.28	9.28	9.37	**	9.30	9.72	9.46	9.69	9.28	18.60
2 *M. margaritifera*	0.464	–	0	0	0	0.09	**	0.03	0.43	0.35	0.40	0	19.77
3 *M. margaritifera*	0.464	0.000	–	0	0	0.09	**	0.03	0.43	0.35	0.40	0	19.77
4 *M. margaritifera*	0.464	0.038	0.038	**0**	0	0.09	**	0.03	0.43	0.35	0.40	0	19.77
5 *M. margaritifera*	0.458	0.023	0.023	0.001	**0**	0.09	**	0.03	0.43	0.35	0.40	0	19.77
6 *M. margaritifera*	0.454	0.005	0.005	0.014	0.005	**0**	**	0.06	0.52	0.43	0.49	0.09	19.85
7 *M. margaritifera*	0.466	0.001	0.001	0.030	0.017	0.003	**	**	**	**	**	**	**
8 *M. margaritifera*	0.461	0.000	0.000	0.033	0.019	0.004	0.001	**0.06**	0.46	0.38	0.43	0.03	19.79
9 *M. margaritifera*	0.464	0.000	0.000	0.038	0.023	0.006	0.001	0.000	–	0.43	0.09	0.43	20.03
10 *M. margaritifera*	0.470	0.001	0.001	0.039	0.024	0.007	0.003	0.001	0.001	–	0.40	0.35	19.85
11 *M. margaritifera*	0.464	0.000	0.000	0.034	0.020	0.004	0.001	0.000	0.000	0.000	**0.17**	0.40	19.94
12 *M. m. durrovensis*	0.452	0.007	0.007	0.037	0.024	0.010	0.008	0.007	0.007	0.007	0.006	**0**	19.77
13 *Potomida littoralis*	2.540	2.148	2.148	2.148	2.162	2.138	2.135	2.146	2.147	2.140	2.138	2.130	**0**

$\overline{D}$ = 0.462 between *M. margaritifera* and *M. auricularia*, and $\overline{D}$ = 2.144 and 2.540 between these two species and *P. littoralis*, respectively. The average distance among *M. margaritifera* populations dropped to $\overline{D}$ = 0.011.

The fixation indices calculated for *M. margaritifera*, grouping the specimens by localities (populations) and the localities by 'countries' (as representing different isolated hydrographic basin systems) (F_{CT} = 0.056, F_{SC} = 0.280, F_{ST} = 0.320), revealed that the detected genetic structure is mostly (67.96%) due to percentage variation within populations, and only 5.63% due to variation among groups ('countries'). This last percentage decreased when the population of *M. margaritifera durrovensis* was considered as a different group.

The contingency test for allelic frequencies showed significant differences in four loci for *M. margaritifera*. One of these differences involved null homozygotes in *EST-2* in the Irish *M. m. durrovensis* population. However, we may have failed to detect an existing null allele in the other *M. margaritifera* populations. The three remaining differences were based on the existence of a unique allele (at a relatively high frequency) in one population or group: *G3PDH*94* in the population inhabiting the Oro River; *PEP-LA-2*96* in populations from the Russian rivers and the Irish river Nore; or to the unequal distribution of the two *PGM-1* alleles (91 and 100). The hypothesis of isolation by distance was rejected in the Mantel test correlating genetic vs. geographical distances (r = –0.11, P = 0.31). Indeed, after all the *M. margaritifera* populations were pooled and the *EST-2* locus was excluded from the Hardy–Weinberg equilibrium tests, only one case of disequilibrium (for *PGM-1,* P = 0.004) remained in this putative metapopulation.

A maximum likelihood tree based on allelic frequencies (Fig. 2) revealed great similarity among the different populations of *M. margaritifera*, including those corresponding to basins of different countries and even the putative *M. m. durrovensis*.

MITOCHONDRIAL DNA SEQUENCES

Bearing in mind that both genes could not be analysed in all the specimens, 77 sequences (41 COI and 36 16S) were obtained for the 46 specimens examined, resulting in 1163 characters (657 for COI and 506 for 16S). Only somatic tissue was sampled, since we were unable to obtain mature gonads. Nevertheless, there was no evidence of heteroplasmy or doubly uniparental inheritance (DUI, Zouros *et al*., 1994).

There were no significant differences in base composition among taxa, even though proportions of some bases were biased (Table 5). In both genes, substitutions were not randomly distributed (gamma

Table 5. Number of characters analysed, nucleotide proportions and transition/transversion (Ts/Tv) ratios in the comparison of *M. margaritifera* with *M. auricularia* and for all the taxa analysed according to COI and 16S rRNA sequences

	M. margaritifera/*M. auricularia*		Taxa analysed	
	COI	16S	COI	16S
Characters:				
Total	657	499	657	506
Constant (%)	567 (86.30)	465 (93.19)	425 (64.68)	344 (67.98)
Parsimony informative (%)	86 (13.09)	31 (6.21)	162 (24.66)	129 (25.49)
1st codon positions (%)	9 (10.46)		22 (13.58)	
2nd codon positions (%)	1 (1.16)		1 (0.62)	
3rd codon positions (%)	76 (88.37)		139 (85.80)	
A %	16.64	32.75	16.87	32.79
C %	16.50	20.98	16.40	20.93
G %	25.19	21.66	25.20	21.60
T %	41.67	24.61	41.53	24.68
Ts/Tv ratio	3.01	2.26	1.60	1.66

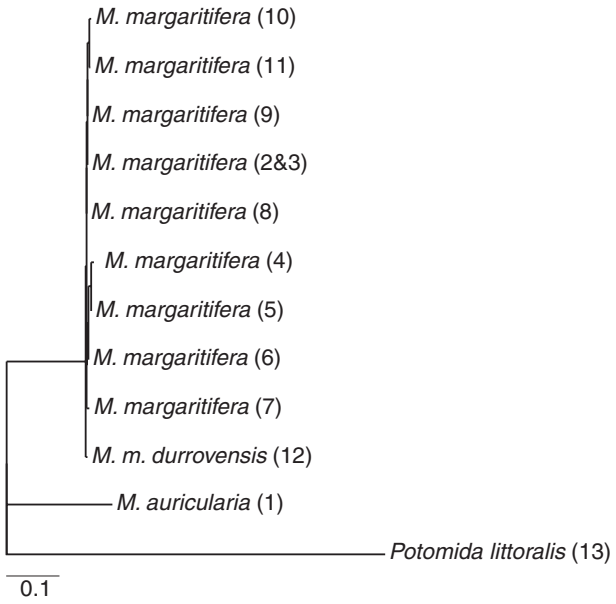

Figure 2. Maximum likelihood tree based on allozyme frequencies. The numbers assigned to the populations (in parentheses) are those indicated in Table 1

shaped parameter $\alpha = 0.2352$ for COI and $\alpha = 0.3327$ for 16S).

The 16S gene alignment required the inclusion of four gaps when the two European *Margaritifera* species were compared. Two 16S haplotypes were found for *M. auricularia* and two for the European *M. margaritifera* populations. The number of different COI haplotypes was higher: two for *M. auricularia*, and up to ten for *M. margaritifera*. A unique haplotype was found for the *Potomida* specimens in both genes. It is of note that the *M. margaritifera* specimen from the USA (GenBank) shared its COI haplotype with one Russian specimen (Varzuga River, Kola Peninsula), while the USA specimen showed three to four substitutions in the 16S sequence (more conserved), with respect to the corresponding European haplotypes. Most of the mutations in the COI gene were synonymous. Despite the mean divergence of around 12% (Table 6) between *M. auricularia* and *M. margaritifera*, there are only four amino acid substitutions (1.8%).

A plot of substitutions of each gene analysed vs. uncorrected mean ('*p*') distance showed perfect correlation (data not shown). Nevertheless, a trend towards saturation was evident for transitions in third positions of the COI gene in pairwise comparisons, with divergences greater than 15%. As divergences between outgroups and the ingroup (with the exception of *Cumberlandia*) were greater than this value (Table 6), saturation might mask the relationships between *Margaritifera* and some outgroups, since homoplastic characters could lead to the underestimation of divergence.

The partition homogeneity test (as implemented in PAUP) showed no significant differences between the phylogenies reconstructed from COI and 16S ($P = 0.63$), such that the data sets could be combined for most analyses. The phylogenetic analysis of the combined data set resulted in a gene tree with *Cumberlandia monodonta* as a well-supported sister group to monophyletic *Margaritifera* (Fig. 3, bootstrap indices 100% according to MP, NJ and ML; and DI 4). Within the genus *Margaritifera*, a supported first split separated the specimens of *M. auricularia* from those of *M. margaritifera* (bootstrap values 72% MP, 88% NJ,

Table 6. Mean nucleotide divergence values between the different taxa analysed based on 16S rRNA and COI sequences. *Mm* = *Margaritifera margaritifera*. Hyphens indicate that intrapopulation divergence could not be calculated

N	Species									
		16S								
		A								
8	**A.** *M. auricularia*	0.09	**B**							
6	**B.** *Mm durrovensis*	6.12	0	**C**						
12	**C.** *Mm* **Spain**	6.12	0	0	**D**					
2	**D.** *Mm* **Ireland**	6.12	0	0	0	**E**				
6	**E.** *Mm* **Russia**	6.15	0.03	0.03	0.03	0.07	**F**			
1	**F.** *Mm* **USA**	6.48	0.77	0.77	0.77	0.81	–	**G**		
1	**G.** *Cumberlandia*	11.74	10.63	10.63	10.63	10.67	10.01	–	**H**	
2	**H.** *Potomida*	21.99	20.73	20.75	20.75	20.79	22.11	24.53	0	**I**
1	**I.** *Anodonta couperiana*	24.29	21.41	21.41	21.41	21.45	20.73	22.03	18.34	–
1	**J.** *Amblema*	25.15	23.60	23.60	23.60	23.65	23.54	24.58	12.80	17.50
		COI								
		A								
7	**A.** *M. auricularia*	0.29	**B**							
6	**B.** *Mm durrovensis*	11.57	0	**C**						
15	**C.** *Mm* **Spain**	11.61	0.04	0.07	**D**					
2	**D.** *Mm* **Ireland**	12.33	0.76	0.80	0	**E**				
9	**E.** *Mm* **Russia**	12.05	0.61	0.65	0.39	0.38	**F**			
1	**F.** *Mm* **USA**	11.97	0.49	0.52	0.32	0.21	–	**G**		
1	**G.** *Cumberlandia*	12.93	12.75	12.79	12.90	12.65	12.84	–	**H**	
2	**H.** *Potomida*	16.14	19.03	19.07	19.48	19.16	18.98	16.59	0	**K**
1	**K.** *Anodonta cygnea*	17.22	18.23	18.26	18.55	18.20	18.27	18.40	15.73	–
1	**L.** *Neotrigonia*	23.33	23.11	23.08	23.43	23.22	23.16	21.11	23.34	23.82

98% ML; DI 4). Two groups with different support emerged from the *M. margaritifera* clade: the Spanish *M. margaritifera* and the Irish *M. m. durrovensis* were split from those from the Kola Peninsula, the North American specimen and the second Irish population.

Separate analyses of the COI and 16S fragments (figures not shown) led to similar results, although the 16S data could not resolve further intraspecific relationships of *M. margaritifera.* Internal *M. margaritifera* differentiation, as indicated by COI sequences, was mainly based on two changes: one transversion at position 244 and one transition at position 370. Nevertheless, this small difference is alternatively fixed in the 33 specimens analysed.

DISCUSSION

Genetic diversity, phylogeny and biogeography

Both nuclear and mitochondrial genetic data sets indicated similar phylogenetic relationships and population structure. The sister group relation of *M. margaritifera* and *M. auricularia* and their clear distinction were evident. While ten diagnostic nuclear loci served to differentiate these species (mean Nei genetic distance = 0.462), an average divergence of 10% was found for the mitochondrial markers. Divergence ranges found between closely related taxa frequently overlap (Mulvey *et al*., 1997; references therein). The values obtained in the present study for the two *Margaritifera* species can be considered usual (Stiven & Alderman, 1992; Roe & Lydeard, 1998) or even high (Mulvey *et al*., 1997) for Unionoidea at the intrageneric level. On the other hand, the divergence indicated by both nuclear and mitochondrial markers would be inconsistent with a DUI phenomenon as a possible explanation for the mitochondrial divergence shown by the two species. Divergences between mitochondrial male and female lineages in other bivalves are within the same range as that shown between *M. margaritifera* and *M. auricularia* haplotypes. However, in the case of these other bivalves showing DUI phenomena, no corresponding divergence has been detected at the nuclear level (Liu, Mitton & Wu, 1996).

In our mitochondrial analyses, *Cumberlandia monodonta* appeared as the sister taxon of *Margaritifera*. This has been reported by other authors (Lydeard *et al*., 1996; Graf & Foighil, 2000), although Davis & Fuller (1981) considered the two genera to be synonymous. Divergences established for *Cumberlandia*

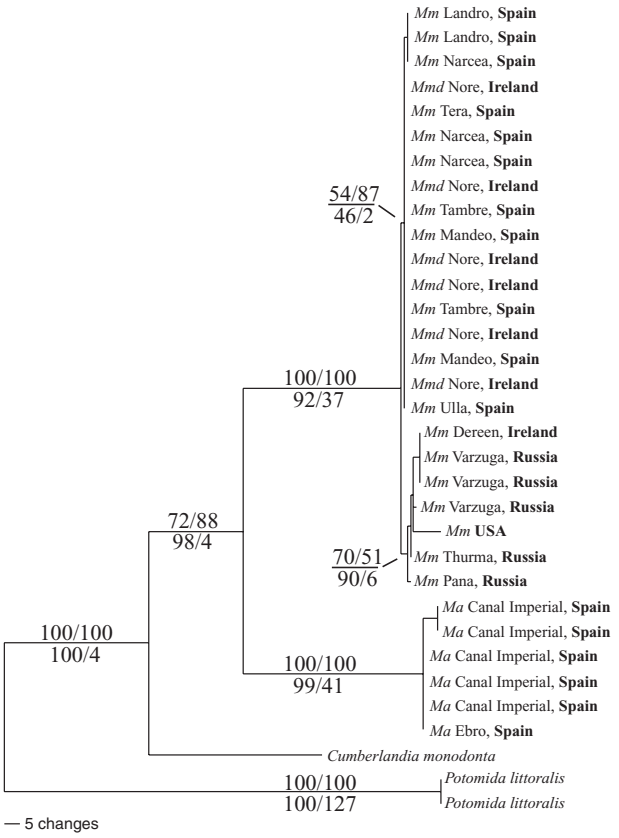

Figure 3. Maximum parsimony tree based on 16S and COI sequences. Gaps were treated as '5th' character states. Numbers above main branches represent the bootstrap values obtained for 1000 replications corresponding to MP and NJ (based on HKY85 distances); numbers below branches indicate those corresponding to ML and the decay indices. *Mm* = *Margaritifera margaritifera*; *Mmd* = *Margaritifera margaritifera durrovensis*; *Ma* = *Margaritifera auricularia*.

were almost identical with respect to *M. auricularia* ($\overline{D}$ = 12.5%) and to *M. margaritifera* ($\overline{D}$ = 12%).

At the intraspecific level, each type of marker (nuclear and mitochondrial) illustrates different features. Since nuclear protein genes are thought to evolve more slowly than mitochondrial genes, these markers may reflect different stages of the evolutionary history of the taxa analysed. No allozymic differentiation was detected among *M. auricularia* specimens from the same location obtained in different years, although the sample size was limited. Less than 0.005% mitochondrial sequence divergence was observed among these samples, or when comparing these with one specimen captured in the Ebro River. Owing to the disappearance of this species over most of its ancient distribution area (Araujo & Ramos, 2000b), there are no more data available. In any case, the population screened seems to be in equilibrium (according to tests for Hardy–Weinberg expectations) and its genetic variability parameters are within the range for freshwater bivalves (Kat, 1983a,b; van der Bank, 1995), though much lower than values estimated for the genus *Lampsilis* in North Carolina (Stiven & Alderman, 1992).

Several populations of *M. margaritifera* from distant locations were analysed. In general, great similarity was found among the populations (including the putative *M. m. durrovensis*). However, the two genetic markers yielded different results, showing some structure in one of the mitochondrial genes (COI) that was not apparent in the other (16S), probably determined by the low rates of divergence of this last gene (Lydeard *et al*., 2000). The present nuclear data indicate some interpopulation structure that could be related to the sample size of each population, although this was somewhat compensated by the considerable number of loci (27) analysed. Variability parameters such as polymorphism represent the minimum values for each population. Thus, the results indicated that some of the populations showed unique alleles (never diagnostic loci) that gave rise to differentiation indices or statistical differences that could sometimes be explained by the low number of specimens analysed (discussed in Berg & Berg, 2000). Indeed, particular cases such as the appearance of two null homozygotes in the esterases of *M. m. durrovensis* increased the differentiation index. Nevertheless, the existence of this null allele in the rest of the populations (at least in heterozygosis) cannot be precluded. Pooling the data corresponding to all *M. margaritifera* populations, the global F_{IS} for *M. margaritifera* could be indicative of a 'metapopulation' in equilibrium (Hanski & Simberloff, 1997) [with polymorphism (P_{99} = 25.9%) and heterozygosity (H_o = 0.014) slightly higher than those of *M. auricularia*]. Thus, considering the data collected as representative, differences shown by some underrepresented populations might be explained by two theories: (i) that the variation observed is a real sign of genetic structure, or (ii) that it reflects a remnant of an ancient polymorphism lost in some populations due to a decline in current populations. The lack of a geographically structured partition for this putative diversity and the known history of world decline of this species suggest the second possibility is more plausible. There is no differentiation among the populations inhabiting the Kola Peninsula, Ireland or Spain. Certain features of two of the populations from north-western Spain (mainly differences in the frequency of the *PGM-1* allele, as observed in other *M. margaritifera* populations from Ireland, Chesney *et al*., 1993) could again be interpreted as reflecting the different fixation of two alternative alleles in different populations or as a sample size error. In any

case, no differentiation was detected in these two populations at the mitochondrial level.

The mitochondrial results showed an almost total lack of differentiation of the 16S gene in all the *M. margaritifera* specimens examined (only one transition was found in one of the 26 European specimens analysed), with the exception of the North American sample (GenBank). Since the latter shared the most variable (Hoeh *et al*., 1997) COI sequence with a European specimen, we might expect no difference in the 16S sequence, though an 'artefact' may not be ruled out. The COI gene indicated the existence of two evolutionary lineages: one from Ireland (the *M. margaritifera* population) extending northwards, and the other, also from Ireland (*M. m. durrovensis*), extending to the south. In this way, the populations of '*M. m. durrovensis*' were clustered together with those from Spain (with no differentiation among specimens from different localities), and the other Irish populations were joined to those from the Kola Peninsula and USA. This cluster, based only on two substitutions but fixed in all specimens of each region, might reflect biogeographical patterns provoked by a recent phenomenon, e.g. glaciation effects. The possibility of several refugia in Europe during the last glaciation, around 18 000 years ago, has been proposed for different species (Hewitt, 1999). In the case of the brown trout *Salmo trutta*, regarded as one of the main hosts of *M. margaritifera* glochidia (Bauer, 1987), two genetic Atlantic forms have been established and at least two glacial refugia suggested: one near the coasts of Britain, and another south of this refuge (García-Marín, Utter & Pla, 1999; Machordom *et al*., 2000). At the time of refuge isolation, the two mussel haplotypes may have diverged from an ancestral one. Alternatively, if the two haplotypes coexisted at that time, subsequent brown trout recolonization may have provoked their spread to the two areas where they are currently found. Considering the possibility of coevolution or biogeographical adaptation between glochidia hosts and the different genetic lineages of *M. margaritifera* identified, the two host lineages discerned may have been responsible for separation of the two mussel groups.

The high similarity between specimens from both sides of the Atlantic Ocean implies two possible explanations. The first hypothesis considers the current distribution of *M. margaritifera* determined by its ancient existence in the Laurasia continent (Davis & Fuller, 1981; Bauer, 1997). The similarity would then suggest an almost negligible substitution rate, given the discrete differences observed between the specimen of *M. margaritifera* from the USA and those from the eastern side of the Atlantic ($\overline{D} = 0.68\%$). If we take into account the total separation of Laurasia from Gondwana approximately 50 million years ago, a substitution rate of 0.013% per million years would be deduced. The usual divergence considered for mitochondrial genes is around 1 or 2% per million years (Brown *et al*., 1982; Moritz, Dowling & Brown, 1987; Bermingham, McCafferty & Martin, 1997). Thus, according to this rationale, our deduced value is lower by 100-fold. Moreover, such a rate of change would imply that European *M. margaritifera* and *M. auricularia* diverged more than 720 million years ago (which seems highly improbable!). If, alternatively, we apply the general rate of 1–2% Myr^{-1}, the separation of these two species would have occurred 4.7–9.4 million years ago. A second hypothesis suggests much more recent gene flow between the Margaritiferids of each side of the Atlantic, and evokes glaciation periods or dispersion of glochidia by fish hosts as determining factors.

Up until the present, the general consensus has focused on this first biogeographical hypothesis. According to Walker (1910) and Smith (1976), the current occurrence of *M. margaritifera* on both sides of the Atlantic suggests that the species, as currently known, inhabited the north plate of Pangea during the Mesozoic. Irrespective of the time of colonization of each Atlantic side by *M. margaritifera*, our findings suggest recent gene flow, probably by intermediate host fish populations, impaired the differentiation of these supposed early disjunct populations. Even the restocking of North American rivers with European brown trout could account for passive migration or passage of *Margaritifera*, and explain the current resemblance of *M. margaritifera* haplotypes on each side of the Atlantic Ocean.

In order to explain this model, we need to take into account the potential dispersal of the unionids and their reproductive strategy, with a larval stage or glochidium in symbiosis with freshwater fishes. Although there are no feasible results indicating the capacity of infested fishes to enter different rivers, this possibility cannot be ruled out, at least during flood events occurring over a long period of evolutionary history. Thus, we may consider the colonization of the east side of the North American continent by juveniles of *M. margaritifera* released from infested salmonids from Europe. However, the reduced physiological efficiency of margaritiferids compared with the more recent American unionids (Davis & Fuller, 1981) and the absence of species above the North American latitude 50° would refute this theory.

To test our hypothesis under the classic palaeobiogeographical model, two important facts need to be established: first, the presence of Cretaceous fossil records of margaritiferids in Europe and North America; and, second, experimental confirmation of the ability of margaritiferids to colonize new watercourses via infested fishes under different conditions of salinity.

CONSERVATION

The genetic results presented here for the genus *Margaritifera* indicate two different situations for *M. margaritifera* and *M. auricularia*. Currently, the latter is only known to inhabit the Spanish Ebro River (about 2000 specimens) and the Moroccan Tiffrit Basin (Araujo & Ramos, 2000a,b). The size and systematic position of the extant North African population has not been fully established, but the restricted distribution area of *M. auricularia* justifies its classification as critically endangered (IUCN, 1996; Araujo & Ramos, 2000b). It should be taken into account that this diminished range is mainly (although sometimes indirectly) related to human activity, as in the case of the gradual disappearance of the suspected main host fish, *Acipenser* (Elvira, Almodóvar & Lobón-Cerviá, 1991), required for successful reproduction. Although the genetic parameters of *M. auricularia* suggest normal polymorphism and heterozygosity levels (theoretically implying good survival), its habitat and other life cycle conditions need to be urgently preserved or restored (Araujo & Ramos, 2001). As recently suggested (Araujo & Ramos, 2000b; Araujo, Bragado & Ramos, 2001), the possibility of *in vitro* reproduction might be a feasible option. Indeed, a similar genetic study of the putative Moroccan population could provide valuable information for the conservation of this endangered species.

In contrast, *M. margaritifera* populations may be considered a metapopulation, although with impoverished populations over most of its distribution area (Bauer, 1991; IUCN, 1996). The practical lack of differentiation shown here and rejection of the alternative hypothesis 'isolation by distance' could lead us to think of the different populations examined as part of a non-structured genetic entity. Considering all the markers screened here, only a slight degree of biogeographical differentiation was detected between specimens living to the north and west of Ireland, and from Ireland towards the south. Nevertheless, this differentiation was only based on two nucleotide positions in the COI gene. Management of this species may be an easier task than for *M. auricularia*. Pooling all the populations, the genetic polymorphism shown for this putative metapopulation was similar or even higher than that for *M. auricularia*. Thus, if we deal with each population as a separate unit, the main problem will stem from the low values of genetic parameters of variability (although based on a small sample size). Between these two extremes, the sequence analysis suggests the existence of two groups. This apparent dissimilarity between allozymic and mitochondrial results would be explained, as mentioned before, by the different substitution rates of the two markers employed. Moreover, we should take into account that the effective population size for mitochondrial DNA is a quarter of that corresponding to the nuclear genome. When these populations undergo sample size decay or bottlenecks, genetic drift will fix particular haplotypes, and a clearer mitochondrial structure will emerge. Thus, the present mitochondrial results could represent a preliminary commitment approach to the management of the species that avoids the risk of loss of local genetic units. Hence, two ESUs (evolutionarily significant units) could be defined based on the sequence divergence or markers found. On the other hand, our data do not support the validity of the *M. m. durrovensis* subspecies. Although it shares a mitochondrial haplotype with the Iberian populations examined, this 'hard water subspecies', might just be an 'ecophenotype' (i.e. a morphological local adaptation) as was concluded by Chesney *et al*. (1993). Whichever the case, it must be borne in mind that this lack of discrimination could be an artefact due to the small number of specimens sampled in some populations. Thus, a more conservative measure might be to continue with current protection strategies for this population until this point is clarified in future studies.

ACKNOWLEDGEMENTS

This study was supported by the MIMAM-CSIC project 'Demografía, hábitat y ciclo vital de *Margaritifera auricularia*' and the Fauna Ibérica Project (DGES PB95-0235). We thank D. Bragado and O. Soriano for their help in the field, the Department of Agriculture and the Environment of the 'Diputación General de Aragón' and the 'Consellería de Agricultura, Ganadería y Montes' of the 'Xunta de Galicia' for permission to collect the animals. We are also grateful to M. Costello, M. Speight and F. Marnell for providing the Irish specimens and the necessary permits from the Irish Natural Heritage. Russian specimens were collected by R.A. thanks to the kind invitation of V. Ziuganov to visit the Kola rivers. W. Hoeh provided the primer sequences. H. Enghoff kindly accepted to review the manuscript. We appreciate the skilful technical assistance provided by A. Montilla and L. Alcaraz. We are also indebted to A. Burton for her conscientious revision of the English. A.M. was supported by a postdoctoral grant from the 'Comunidad de Madrid'.

REFERENCES

Aebersold PB, Winans GA, Teel DJ, Milner GB, Utter FM. 1987. Manual for starch gel electrophoresis: a method for detection of genetic variation. *NOAA Technical Report, NMFS*, 1–61.

Altaba CR. 1990. The last known population of the freshwater mussel *Margaritifera auricularia* (Bivalvia, Unionidae): a conservation priority. *Biological Conservation* **52:** 271–286.

Araujo R, Bragado D, Ramos MA. 2000. Occurrence of glochidia of the endangered *Margaritifera auricularia* (Spengler, 1793) and other mussel species (Bivalvia: Unionoida) in drift and on fishes in an ancient channel of the Ebro River, Spain. *Archiv für Hydrobiologie* **148:** 147–160.

Araujo R, Bragado D, Ramos MA. 2001. Identification of the river blenny, *Salaria fluviatilis*, as a host to the glochidia of *Margaritifera auricularia*. *Journal of Molluscan Studies* **67:** 128–129.

Araujo R, Ramos MA. 1998. Description of the glochidium of *Margaritifera auricularia* (Spengler, 1793) (Bivalvia, Unionidae). *Philosophical Transactions of the Royal Society of London, B* **353:** 1553–1559.

Araujo R, Ramos MA. 2000a. Status and conservation of the relict giant European freshwater pearl mussel *Margaritifera auricularia* (Spengler, 1793). *Biological Conservation* **96:** 233–239.

Araujo R, Ramos MA. 2000b. A critic revision of the historical distribution of *Margaritifera auricularia* (Spengler, 1793) (Mollusca: Margaritiferidae) based on museum specimens. *Journal of Conchology* **37:** 49–59.

Araujo R, Ramos MA. 2001. *Action plan for Margaritifera auricularia*. Strasbourg: Nature & Environment Council of Europe Publishing.

van der Bank FH. 1995. Allozyme variation in a freshwater mussel population (*Coelatura kunensis* Mousson, 1887) from Southern Africa. *Water SA* **21:** 153–157.

Bauer G. 1987. The parasitic stage of the freshwater pearl mussel (*Margaritifera margaritifera* L.) III. Host relationships. *Archiv für Hydrobiologie* **76** (Suppl.)**:** 413–423.

Bauer G. 1991. Plasticity in life history traits of the freshwater pearl mussel- Consequences for the danger of extinction and for conservation measures. In Seitz A, Loeschcke V, eds. *Species conservation: a population-biology approach*. Basel: Birkhäuser-Verlag, 103–120.

Bauer G. 1997. Host relationships at reversed generation times: *Margaritifera* (Bivalvia) and salmonids. *Ecological Studies* **130:** 69–79.

Belkhir K *et al.* 2000. *GENETIX 4.0, Logiciel Sous Windows TM Pour la Génétique Des Populations*. Montpellier: Laboratoire Génome, Populations, Interactions, Université Montpellier II.

Berg DJ, Berg PH. 2000. Conservation genetics of freshwater mussels: comments on Mulvey *et al. Conservation Biology* **14:** 1920–1923.

Bermingham E, McCafferty SS, Martin AP. 1997. Fish biogeography and molecular clocks: Perspectives from the Panamanian Isthmus. In Kocher TD, Stepien CA, eds. *Molecular systematics of fishes.* San Diego: Academic Press, 113–126.

Bogan AE. 1998. Freshwater molluscan conservation in North America: problems and practices. *Journal of Conchology Special Publication* **2:** 223–230.

Bremer K. 1988. The limits of amino-acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42:** 795–803.

Bremer K. 1994. Branch support and tree stability. *Cladistics* **10:** 295–304.

Brown WM, Prager EM, Wang A, Wilson AC. 1982. Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *Journal of Molecular Evolution* **18:** 225–239.

Campbell DC. 2000. Molecular evidence on the evolution of the bivalvia. In Harper EM, Taylor JD, Crame JA, eds. *The evolutionary biology of the bivalvia*. London: The Geological Society, 31–46.

Chesney H, Oliver P, Davis G. 1993. *Margaritifera durrovensis* Phillips, 1928: Taxonomic status, ecology and conservation. *Journal of Conchology* **34:** 267–299.

Davis GM, Fuller SLH. 1981. Genetic relationship among recent Unionacea (Bivalvia) of North America. *Malacologia* **20:** 217–253.

Elvira B, Almodóvar A, Lobón-Cerviá J. 1991. Recorded distribution of sturgeon (*Acipenser sturio* L., 1758) on the Iberian Peninsula and actual status in Spanish waters. *Archiv für Hydrobiologie* **121:** 253–258.

Eriksson T. 1998. *Autodecay*, *Version* 4.0. Stockholm: Department of Botany, Stockholm University.

Farris JSK, Källersjö M, Kluge AG, Bult C. 1994. Testing significance of incongruence. *Cladistics* **10:** 315–319.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

Felsenstein J. 1995. *PHYLIP (Phylogeny Inference Package)*, Version 3.57c. Seattle: Department of Genetics, University of Washington,

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

García-Marín JL, Utter FM, Pla C. 1999. Postglacial colonisation of brown trout in Europe based on distribution of allozyme variants. *Heredity* **82:** 46–56.

Graf DL, Ó Foighil D. 2000. The evolution of brooding characters among the freshwater pearly mussels (Bivalvia: Unionoidea) of North America. *Journal of Molluscan Studies* **66:** 157–170.

Hanski I, Simberloff D. 1997. The metapopulation approach, its history, conceptual domain, and application to conservation. In Hanski I, Gilpin ME, eds. *Metapopulation biology. ecology, genetics, and evolution*. San Diego: Academic Press, 5–26.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **21:** 160–174.

Hewitt GM. 1999. Post-glacial re-colonisation of European biota. *Biology Journal of the Linnean Society* **68:** 87–112.

Hoeh WR, Stewart DT, Saavedra C, Sutherland BW, Zouros E. 1997. Phylogenetic evidence for role-reversals of gender-associated mitochondrial DNA in *Mytilus* (Bivalvia: Mytilidae). *Molecular Biology and Evolution* **14:** 959–967.

IUCN. 1996. *1996 IUCN red list of threatened animals.* Gland, Switzerland: IUCN.

Kat PW. 1983a. Genetic and morphological divergence among nominal species of North American *Anodonta* (Bivalvia: Unionidae). *Malacologia* **23:** 361–374.

Kat PW. 1983b. Morphology divergence, genetics, and speciation among *Lampsilis* (Bivalvia: Unionidae). *Journal of Molluscan Studies* **49:** 133–145.

Kileen IJ, Seddon MB, Holmes AM. 1998. *Molluscan conservation: a strategy for the 21st century. Journal of Conchology, special publication 2*. Dorchester, UK: Dorset Press.

King TM, Eackles MS, Gjetvaj B, Hoeh WR. 1999. Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Unionidae): conservation implications of range discontinuity. *Molecular Ecology* **8:** S65–S78.

Kumar S. 1996. *PHYLTEST: A program for testing phylogenetic hypothesis*, *Version* 2.0. Pennsylvania: Institute of Molecular Evolutionary Genetics and Department of Biology, The Pennsylvania State University.

Lavane C, Preparata G, Sacone C, Serio G. 1984. A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution* **20:** 86–93.

Lessios HA. 1992. Testing electrophoretic data for agreement with Hardy–Weinberg expectations. *Marine Biology* **112:** 517–523.

Liu H-P, Mitton JB, Wu S-K. 1996. Paternal mitochondrial DNA differentiation far exceeds maternal mitochondrial DNA and allozyme differentiation in the freshwater mussel, *Anodonta grandis grandis*. *Evolution* **50:** 952–957.

Lydeard C, Holznagel WE, Schnare MN, Gutell RR. 2000. Phylogenetic analysis of molluscan mitochondrial LSU rDNA sequences and secondary structures. *Molecular Phylogenetics and Evolution* **15:** 83–102.

Lydeard C, Mulvey M, Davis GM. 1996. Molecular systematics and evolution of reproductive traits of North American freshwater unionacean mussels (Mollusca: Bivalvia) as inferred from 16S rRNA gene sequences. *Philosophical Transactions of the Royal Society of London B* **351:** 1593–1603.

Machordom A, Suárez J, Almodóvar A, Bautista JM. 2000. Mitochondrial haplotype variation and phylogeography of Iberian brown trout populations. *Molecular Ecology* **9:** 1325–1338.

Maddison WP, Maddison DR. 1992. *Macclade: analysis of phylogeny and character evolution*, *Version* 3.05. Sunderland, MA: Sinauer.

Mantel N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* **27:** 209–220.

Mickevich MF, Farris JS. 1981. The implications of congruence in *Menidia*. *Systematic Biology* **30:** 351–370.

Moritz C. 1994. Application of mitochodrial DNA analysis in conservation: a critical review. *Molecular Ecology* **3:** 401–411.

Moritz C, Dowling TE, Brown WM. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics* **18:** 269–292.

Mulvey M, Lydeard C, Pyer DL, Hicks KM, Brim-Box J, Williams JD, Butler RS. 1997. Conservation genetics of North American freshwater mussels *Amblema* and *Megalonaias*. *Conservation Biology* **11:** 868–878.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89:** 583–590.

Pasteur N, Pasteur G, Bonhomme F, Catalan J, Britton-Davidian J. 1987. *Manuel Technique de Génétique Par Électrophorése Des Protéines*. Paris: Lavoisier.

Posada D, Crandall KA. 1998. Model test: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Raymond M, Rousset F. 2000. *GENEPOP (Version 3.2a): population genetics software for exact tests and ecumenicism*. Montpellier: Laboratoire Génétique et Environment.

Rodríguez R, Oliver JL, Marín A, Medina JR. 1990. The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology* **142:** 485–501.

Roe KJ, Lydeard C. 1998. Molecular systematics of the freshwater mussel genus *Potamilus* (Bivalvia: Unionidae). *Malacologia* **39:** 195–205.

Rohlf FJ. 2000. *NTSYS-Pc: Numerical Taxonomy and Multivariate Analysis System*, *Version* 2.1. Setauket, New York: Exeter Software.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Schneider S, Kueffer JM, Roessli D, Excoffier L. 1997. *ARLEQUIN: A software for population genetic data analysis*, *Version* 1.1. Geneva, Switzerland: Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva.

Schonewald-Cox CM, Chambers SM, Macbryde B, Thomas L. 1983. *Genetics and conservation*. Menlo Park, California: Benjamin/Cummings.

Smith DG. 1976. The distribution of the Margaritiferidae: a review and a new synthesis. *Bulletin of the American Malacological Union* **1976:** 42.

Stiven AE, Alderman J. 1992. Genetic similarities among certain freshwater mussel populations of the *Lampsilis* genus in North Carolina. *Malacologia* **34:** 355–369.

Swofford DL. 2001. *PAUP*: Phylogeny Analysis Using Parsimony (*and other methods)*, Version 4.0b6. Sunderland, MA: Sinauer Associates Inc.

Swofford DL, Selander RB. 1989. *BIOSYS. A computer program for the analysis of allelic variation in population genetics and biochemical systematics*. Illinois: Illinois Natural History Survey.

Taylor DW. 1988. Aspects of freshwater mollusc ecological biogeography. *Palaeogeographyraphy, Palaeoclimatology, Palaeoecology* **62:** 511–576.

Taylor DW, Uyeno T. 1965. Evolution of host specificity of freshwater salmonid fishes and mussels in the North Pacific region. *Venus* **24:** 199–209.

Thompson JD, Higgins DG, Gibson T. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Vaughn CC, Taylor CM. 1999. Impoundments and the decline of freshwater mussels: a case study of an extinction gradient. *Conservation Biology* **13:** 912–920.

Walker B. 1910. The distribution of *Margaritana margaritifera* (Linn) in North America. *Proceedings of the Malacological Society of London* **9:** 126–145.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38:** 1358–1370.

Ziuganov V, Zotin A, Nezlin L, Tretiakov V. 1994. *The freshwater pearl mussels and their relationships with salmonid fish*. Moscow: VNIRO Publishing House.

Zouros E, Ball AO, Saavedra C, Freeman KR. 1994. Mitochondrial DNA inheritance. *Nature* **368:** 818.