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MORPHOLOGICAL AND MOLECULAR DIVERSITY OF UNIONIDAE (MOLLUSCA, BIVALVIA) FROM PORTUGAL

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ABSTRACT

J. Reis, A. Machordom & R. Araujo. 2013. Morphological and molecular diversity of Unionidae (Mollusca, Bivalvia) from Portugal. *Graellsia*, 69(1): 17-36.

Freshwater mussels from the family Unionidae are known to exhibit a high level of ecological phenotypic plasticity that is reflected in their shell shape. This variation has caused uncertainty on systematics and taxonomy of the group. Several naiad populations from nine river basins from Portugal were analyzed genetically, using two mitochondrial gene fragments (16SrRNA and Cytochrome Oxidase I) and morphologically, using ANOVA analyses of shell dimensions. Molecular phylogenetic analyses were used to revise the systematics and to infer an evolutionary hypothesis for the family at the westernmost Atlantic Iberian Peninsula. Genetic and morphological data were in agreement and supported the occurrence of 5 species in the region: *Anodonta anatina*, *Anodonta cygnea*, *Potomida littoralis*, *Unio tumidiformis* and *Unio delphinus*. The differentiation of all these species, except *A. cygnea,* is thought to have taken place during the isolation of the Iberian Peninsula and formation of the current river basins in the Tertiary. The possibility of *A. cygnea* being a relatively recent introduction is discussed. Basic morphometric measures of the shell proved to be useful to separate *Unio* species, but also seem to be strongly affected by environmental conditions. The high intraspecific morphologic variation was partially related to the species' high level of phenotypic plasticity, but seems to have an important role in evolutionary processes.

Keywords: 16S; COI; Iberian Peninsula; phenotypic plasticity; mitochondrial; molecular phylogeny; morphometry; Unionidae

RESUMEN

J. Reis, A. Machordom & R. Araujo. 2013. Diversidad morfológica y molecular de los Unionidae (Mollusca, Bivalvia) de Portugal. *Graellsia*, 69(1): 17-36 (en inglés).

Las náyades de la familia Unionidae tienen gran plasticidad fenotípica, lo que se refleja en la forma de su concha. Esta variabilidad morfológica ha sido causa de gran confusión en la taxonomía y sistemática del grupo. Se han estudiado, genética y morfológicamente, numerosas poblaciones de náyades provenientes de nueve cuencas hidrográficas portuguesas. Para ello se han analizando dos fragmentos de genes mitocondriales (ARNr 16S y Citocromo Oxidasa I) así como diferentes variables morfológicas de la concha. Se han realizado además

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análisis filogenéticos para conocer la sistemática de la familia e inferir una hipótesis evolutiva de su distribución en el oeste de la península Ibérica. Los datos genéticos y morfológicos sugieren la existencia de cinco especies: *Anodonta anatina*, *Anodonta cygnea*, *Potomida littoralis*, *Unio tumidiformis* y *Unio delphinus*. La diferenciación de estas especies, con la excepción de *A. cygnea*, ha ocurrido durante el aislamiento de la Península Ibérica y posterior formación de las actuales cuencas hidrográficas en el Terciario. Se discute la posibilidad de que la presencia de *A. cygnea* se deba a una introducción reciente. Los datos morfométricos analizados pueden ser útiles para separar las especies del género *Unio*, pero son también dependientes de las condiciones ambientales. La elevada variabilidad morfológica dentro de cada especie está relacionada con su plasticidad fenotípica, pero tiene a su vez un importante papel en el proceso evolutivo.

Palabras clave: 16S; COI; península Ibérica; plasticidad fenotípica; mitocondrial; filogenia molecular; morfometría; Unionidae.

Introduction

Shells of freshwater mussels, particularly those of the family Unionidae, present an enormous morphological variability, thought to be at least partially environmentally induced (Ortman, 1920; Baker *et al.*, 2003; Zieritz & Aldridge, 2009). This variability is nowadays mostly considered a consequence of high phenotypic plasticity, which can be defined as the capability of a genotype to change its phenotype according to the prevailing environmental conditions (Bijlsma & Loeschcke, 2005). Therefore, the observed morphological diversity of freshwater mussels often does not reflect differences between evolutionary lineages with taxonomic value (Zieritz *et al.*, 2010). However, it was this variability that led the XIXth century researchers to believe in the existence of tens or hundreds of different species of unionids (Locard, 1899; Graf, 2010). Molecular markers provide important tools for determining the systematic relationships and explaining the geographic patterns of different taxa, helping to overcome the difficulties of morphological variation. Therefore they have been recently used to study several freshwater mussel groups (Lydeard *et al.*, 1996; Lydeard *et al.*, 2000; Baker *et al.*, 2003; Machordom *et al.*, 2003; Huff *et al.*, 2004; Campbell *et al.*, 2005; Källersjö *et al.*, 2005; Graf & Cummings, 2006; Araujo *et al.*, 2009a,b; Reis & Araujo, 2009; Khalloufi *et al.*, 2011).

Nevertheless, despite being amongst the most imperiled invertebrates in the world (Araujo & Ramos, 2000; Young *et al.*, 2001; Strayer *et al.*, 2004), the systematics of unionids in several European regions is still unresolved. In Portugal, the study of Unionidae started with Morelet (1845), who considered the existence of 12 species, including seven described by himself. Later authors from Bourguignat's *nouvelle école* (Castro, 1873, 1885, 1887; Locard, 1899) described a large number of new species based on morphological features of the shell. Nobre (1941) considered all the species previously identified or described for Portugal to be synonyms of one of three species: *A. cygnea*, *P. littoralis* and *U. pictorum*. Haas (1940, 1969) added to this list *U. crassus batavus* Maton & Rackett, 1807 cited by Morelet (1845). Haas (1940, 1969) also considers four species from Castro's authorship to be synonyms of *U. crassus batavus*.

Haas (1969) published a systematic revision of this family that included the Iberian Peninsula fauna. In his work, Haas (1969) considered several "races" belonging to five widespread European species to occur in the Iberian Peninsula: *Anodonta cygnea* (Linnaeus, 1758), *Potomida littoralis* (Cuvier, 1798), *Unio crassus* Retzius, 1788, *U. elongatulus* C. Pfeiffer, 1825 and *U. pictorum* (Linnaeus, 1758). The systematics of the genus *Potomida* around its Mediterranean distribution has been neglected since Haas (1969), until Araujo (2008) stated that *Potomida littoralis* is the valid name for the Iberian species. New data on freshwater mussels in Portugal were only made available by Reis (2003, 2006), Araujo *et al.* (2009c) and Reis & Araujo (2009).

In central Europe the systematics of the genus *Unio* has received some attention, with Badino *et al.* (1991), Nagel *et al.* (1998), Nagel (2000), Nagel & Badino (2001) and Källersjö *et al.* (2005) providing insights particularly about the relationships of *U. mancus* with *U. pictorum*. Based on morphological, anatomical, reproductive and genetic characters, Araujo *et al.* (2005) and Khalloufi *et al.* (2011) revealed that the two Iberian Mediterranean *Unio elongatulus* "races" considered by Haas (1969) belong to the species *U. mancus* Lamarck, 1819 and *Unio ravoisieri* Deshayes, 1847, while Reis & Araujo (2009) revealed that *Unio tumidiformis* is a

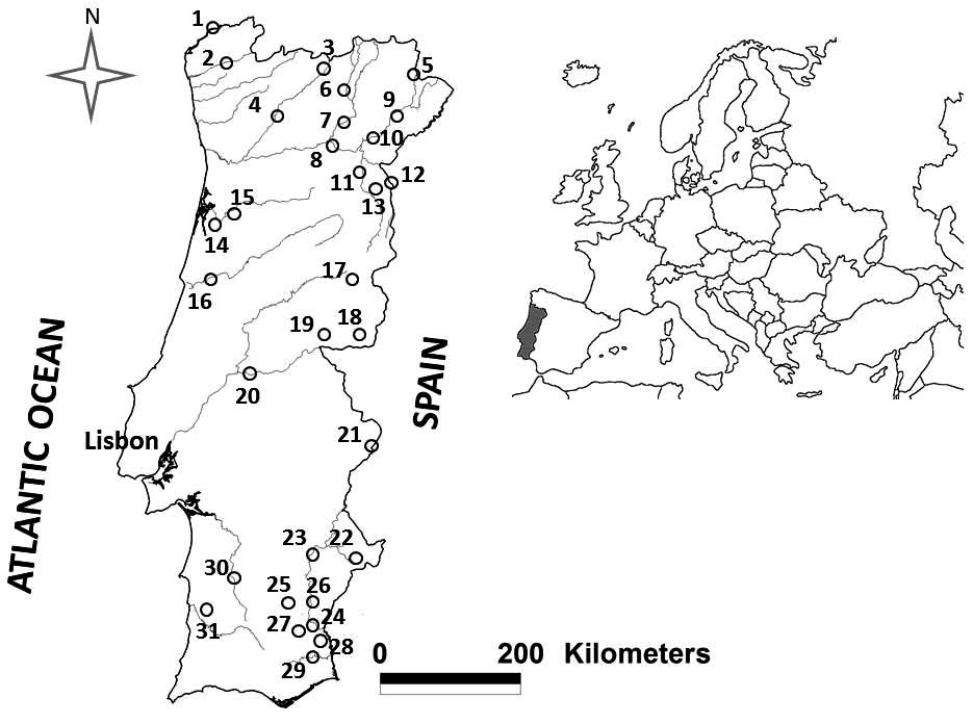

Fig. 1.– Location of sampling sites. Numbers refer to Table 1.

Fig. 1.– Localización de los puntos de muestreo. Los números son los mismos que en la Tabla 1.

valid species that corresponds to Haas' (1969) *Unio crassus batavus*. Lastly, Araujo *et al.* (2009a) consider *Unio gibbus* Spengler, 1793 a valid species from Southern Spain and Northern Africa. These studies showed that the unionid species diversity in the Iberian Peninsula (Araujo *et al.,* 2009c) is not as low as suggested by Haas (1969) nor is it as high as believed in the late XIX century. Indeed, it is not surprising that Iberian unionid endemic species do in fact exist, as freshwater fauna in the Iberian Peninsula is generally much differentiated. The Iberian freshwater fish fauna for example, to which the naiads are coupled due to their life cycle, is considered to include a significant amount of endemic species (Almaça, 1995; Elvira, 1995). This fact derives partially due to the Peninsula's historic isolation and partially for having been a refuge in Europe during glacial ages. Furthermore, some coastal historical connections between major basins and a few particularly isolated coastal systems have produced a particularly distinct freshwater fauna in Portugal. Recent studies indicate that the Atlantic Iberian *Unio* populations show some morphological differences compared to central and northern European populations (Reis, 2006; Reis & Araujo, 2009). In this paper we follow the nomenclature suggested in Araujo *et al.* (2009c) using the names *U. tumidiformis* and *U. delphinus* to refer to the Iberian Atlantic *Unio* species.

The aim of this paper is to contribute to the knowledge of the diversity of unionids from Portugal, which represents the westernmost distribution of this group in Europe, studying their genetic and morphologic variation as well as inferring their phylogenetic relationships and evolutionary history. Another objective was to evaluate if shell shape is useful for distinguishing naiad species from Portugal.

Material and methods

Taxa and Specimens

Emphasis was put on including specimens from as many different sites as possible in both the morphological and molecular analyzes. We collected

Table 1.– Portuguese populations studied, localities, number of specimens analyzed and GenBank accession numbers for each gene.

Tabla 1.– Poblaciones, localidades y número de ejemplares analizados, y código de acceso de GenBank para cada gen estudiado.

Locality	*River*	*River Basin*	*Species*	**N (Morphometry)**	**GenBank accession number (16S)**	**GenBank accession number (COI)**
1 Monção	Minho	Minho	*A. anatina*	30	-	-
			P. littoralis	13	-	-
2 Ponte-da-Barca	Lima	Lima	*U. delphinus*	20	EF571358-EF571359	EF571423-EF571424
3 Vila Verde da Raia	Tâmega	Douro	*A. anatina*	48	-	-
			U. delphinus	15	-	-
4 Mondim-de-Basto	Tâmega	Douro	*A. anatina*	11	-	-
			U. delphinus	5	EF571362-EF571363	-
5 Quintanilha	Maças	Douro	*U. delphinus*	16	EF571360-EF571361	EF571425
6 Miradeses	Rabaçal	Douro	*U. delphinus*	17	EF571373-EF571374	-
8 River mouth	Tua	Douro	*U. delphinus*	10	EF571381	EF571436-EF571437
7 Abreiro	Tua	Douro	*P. littoralis*	2	-	-
			U. delphinus	10	EF571345-EF571346	EF571413-EF571414
9 Mogadouro	Sabor	Douro	*A. anatina*	3	-	-
			P. littoralis	23	-	-
			U. delphinus	18	EF571377	-
10 Cilhade	Sabor	Douro	*U. delphinus*	2	EF571375-EF571376	EF571433
11 Castelo Melhor	Côa	Douro	*A. anatina*	26	-	-
			U. delphinus	-	EF571353	EF571418-EF571419
12 Escalhão	Águeda	Douro	*A. anatina*	32	EF571332	EF571387-EF571388
			U. delphinus	19	EF571347- EF571348	-
13 Nave Redonda	Aguiar	Douro	*A. anatina*	80	EF571335	EF571389-EF571390
14 Pateira de Fermentelos	-	Vouga	*A. cygnea*	7	-	EF571398
15 Sever do Vouga	Vouga	Vouga	*A. anatina*	4	-	-
			U. delphinus	20	EF571382-EF571383	EF571438-EF571439
16 Pereira	Mondego	Mondego	*A. anatina*	2	-	EF571391-EF571392
			P. littoralis	3	-	EF571399
			U. delphinus	31	EF571367-EF571368	EF571429
17 Capinha	Meimoa	Tagus	*U. delphinus*	-	EF571364-EF571365	EF571426-EF571427
18 Ladoeiro	Aravil	Tagus	*A. anatina*	5	EF571333-EF571334	-
			U. delphinus	25	EF571349-EF571352	EF571415-EF571417
19 Castelo Branco	Pônsul	Tagus	*U. delphinus*	36	EF571369-EF571372	EF571430-EF571432
20 Abrantes	Tagus	Tagus	*A. anatina*	29	-	EF571394-EF571395
			P. littoralis	48	EF571333	EF571401-EF571402
			U. delphinus	25	EF571380	EF571435
21 Ouguela	Xévora	Guadiana	*A. anatina*	-	EF571336	EF571396- EF571397
			P. littoralis	-	-	EF571404
			U. delphinus	20	EF571386	EF571442
22 Safara	S. Pedro	Guadiana	*A. anatina*	2	-	-
			U. tumidiformis	3	EF571341-EF571342	EF571409-EF571410
23 Moura	Guadiana	Guadiana	*U. delphinus*	28	EF571354-EF571357	EF571420-EF571422
24 Mertola	Guadiana	Guadiana	*P. littoralis*	3	-	-
			U. delphinus	30	EF571366	EF571428
25 Beja	Terges e Cobres	Guadiana	*A. anatina*	2	-	-
			U. tumidiformis	1	-	-
			U. delphinus	31	-	-
26 Pulo do Lobo	Limas	Guadiana	*A. anatina*	14	-	-
27 S. João Caldeireiros	Oeiras	Guadiana	*A. anatina*	20	-	-
			U. tumidiformis	1	-	-
			U. delphinus	24	-	-
28 Espirito Santo	Vascão	Guadiana	*A. anatina*	26	-	-
			P. littoralis	50	-	EF571403
			U. tumidiformis	15	EF571343-EF571344	EF571411- EF571412
			U. delphinus	20	EF571384-EF571385	EF571440-EF571441
29 Várzea	Odeleite	Guadiana	*U. tumidiformis*	-	EF571338	EF571405- EF571406
30 Torre Vã	Sado	Sado	*A. anatina*	29	-	EF571393
			P. littoralis	-	-	EF571400
			U. tumidiformis	5	EF571339-EF571340	EF571407- EF571408
			U. delphinus	20	EF571378-EF571379	EF571434
31 Saboia	Mira	Mira	*P. littoralis*	35	-	-

specimens belonging to the three occurring Unionidae genera in Portugal (*Anodonta, Potomida* and *Unio*) from 26 rivers or streams belonging to nine river basins, between 2001 and 2005, in the context of several projects. The location of sampling sites from where specimens were collected is shown in Fig. 1 and Table 1. We also used two specimens of *U. mancus* from the river Ebro, previously sampled for another study (Araujo *et al.*, 2005), to obtain 16S sequences for this species (GenBank accession number AN: EF536011 and EF536012). Sampling was based on searching the substrate with a glass bottom bucket and snorkelling. A number of measurements were taken from all specimens found (see below). A small tissue sample from the foot was taken from at least two specimens at each site. However, a few sites from which morphological data were available from early sampling years could not be sampled again for tissue (Table 1). We returned most specimens to the river immediately, while a few were kept in aquaria after tissue removal, to evaluate the effect of tissue removal on survival. All mussels with unusual morphological features for the considered species were kept in aquaria until molecular analyses were completed, so that the specimen was readily available if proven to be genetically distinct. Tissue samples were preserved either in pure ethanol or frozen at –80ºC. A few tissue samples were collected and sent by gracious collectors. A total of 1017 and 76 specimens where studied for morphology and genetics respectively, having been collected from 31 localities.

Morphologic data and analyses

We measured three morphometric variables as defined by Aldridge (1999): shell length (SL), shell height (SH) and shell width (SW). Measurements were made with 1/20 mm accuracy callipers. We calculated the ratios SH/SL and SW/SL (Zettler, 1997; Nagel, 1999) and performed one way ANOVAS to compare them between species, "races" and river basins (considered basic evolutionary units for freshwater organisms), which were used as grouping factors. The unequal N HSD test was used to determine the significance of differences between the ratios of specific groups. Because the ratio SW/SL is not independent of shell elongation, we performed one way ANOVAS in the same way using an independent width-ratio: SW/((SL+SH)/2). The analyzed ratios reflect certain aspects of the shell shape, such as relative height and obesity, which have been associated with environmental variables like water velocity (Eager, 1978, Aldridge, 1999; Zieritz & Aldridge, 2009). Discriminant analyses were performed using the Log-transformed table of morphometric variables for each genus and conducted on groups defined *a priori* based on the species considered by Reis (2006) and between *Unio* and *Potomida* "races" referred by Haas (1969). This was done in order to assess how effectively could the analyzed morphometric variables distinguish between species or races defined by the most recent or accepted bibliography available. For all analyses the scores from root 1 were plotted against those from root 2 with each point identified distinctly according to its taxonomic position and geographic origin. The ellipse option in STATISTICA® was used to estimate 95% confidence ellipses for the principal root scores of each group in order to visualize the overlap between the morphological characteristics of different groups. All calculations and graphics were prepared using STATISTICA® software (Statsoft, 2001).

DNA extraction and amplification

Tissue samples preserved in ethanol were ground to powder in liquid nitrogen or minced before adding 600 µl of CTAB lysis buffer (2% CTAB, 1.4 M NaCl, 0.2% ß-mercaptoethanol, 20 mM EDTA, 0.1 M TRIS [pH = 8]) and digested with proteinase K (100 µg/ml) for 1-2 days at 50 to 55 ºC. Total DNA was extracted according to standard phenol/chloroform procedures (Sambrook *et al.*, 1989).

We amplified the COI and 16S partial sequences by polymerase chain reaction (PCR) using the following primers: 16sar-L-myt and 16sbr-H-myt (Lydeard *et al*., 1996) for 16S; and LCO1490 (Folmer *et al*., 1994) and COI-H 5'-TCAGGGTGACCAAAAAATCA-3' (6 bases shorter than the HCO2198 of Folmer *et al*., 1994, as in Machordom *et al.*, 2003) for COI. The PCR conditions and the purification of the segments were similar to those described in Machordom *et al*. (2003). The amplified fragments (around 700 bp) were purified by ethanol precipitation prior to sequencing both strands using "BigDye Terminator" (Applied Biosystems, Inc., ABI) sequencing reactions. Sequence gels were run on an ABI 3730 Genetic Analyzer (Applied Biosystems).

After removing the primers regions, the forward and reverse DNA sequences obtained for each speci-

Table 2.– Taxa and respective sequences GenBank accession numbers used in phylogenetic analyses.

Table 2.– Taxa y secuencias correspondientes de GenBank utilizados en los análisis filogenéticos.

Species	Locality	COI	16S
Amblema plicata	North America	U56841	U72548
Anodonta anatina	Poland	AF462071	-
Anodonta anatina	Sweden	DQ060168	DQ060165
Anodonta cygnea	Austria	AF232824	AF232799
Anodonta cygnea	Poland	AF461419	
Anodonta cygnea	Europe	U56842	
Margaritifera margaritifera	Pontevedra, Spain	AF303316	AF303281
Neotrigonia margaritacea	Australia	U56850	DQ280034
Potomida (=Psilunio) littoralis	Canal Imperial de Aragón, Spain	AF303349	AF303308
Quadrula quadrula	North America	AF232823	AF232798
Cafferia caffra	South Africa	AF156500 and AF156501	-
Unio crassus	Poland	AF514296	-
Unio crassus	Sweden	DQ060174	DQ060162
Unio mancus	Ebro river, Spain	AY522858 and AY522857	-
Unio pictorum	Sweden	DQ060175	DQ060163
Unio pictorum	-	AF231731	-
Unio pictorum	Austria	AF156499	-
Unio tumidus	Sweden	DQ060176	DQ060161
Unio tumidus	-	AF231732	
Unio tumidus	Poland	AY074807	
Pseudanodonta complanata	Sweden	DQ060173	DQ060166

men were aligned and checked using the Sequencher program (Gene Code Corporation). CLUSTAL X (Thompson *et al*., 1994) was employed to align the 16S gene sequences, and the resulting alignments were checked by eye. Gaps were treated as missing values. COI translation to protein was also undertaken using Sequencher.

For comparison purposes we included sequences from other bivalve species or from the same species collected in other areas (Table 2). We used *Neotrigonia margaritacea* (Lamarck, 1804), *Margaritifera margaritifera* (Linnaeus, 1758), *Amblema plicata* (Say, 1817) and *Quadrula quadrula* (Rafinesque, 1820) as outgroups in all analyses.

Phylogenetic analyses

Nucleotide saturation was evaluated by plotting transition and transversion changes against uncorrected ("p") divergence values. To evaluate the phylogenetic relationships among the taxa sampled and among the populations of each taxa, the principles of parsimony (MP) and maximum likelihood (ML) were applied. The evolutionary molecular model that best fit our data was selected using MODELTEST 3.06 (Posada & Crandall, 1998) under Akaike information criterion (Akaike, 1974). According to this, we used GTR (General Time Reversible model, Lavane *et al.*, 1984; Rodríguez *et al.*, 1990) distance. Parsimony analysis was performed by heuristic searches under TBR branch swapping and random taxon addition using the PAUP* 4.0b10 package (Swofford, 2002). Maximum likelihood analyses also were run in PAUP, using the model and parameters selected by MODELTEST, through neighbor-joining or heuristic searches. We estimated support in the MP and ML analyses by bootstrapping (1000 pseudo replications) (Felsenstein, 1985).

Each gene was analyzed independently. To consider the information of both genes together, congruence among tree topologies of COI and 16S

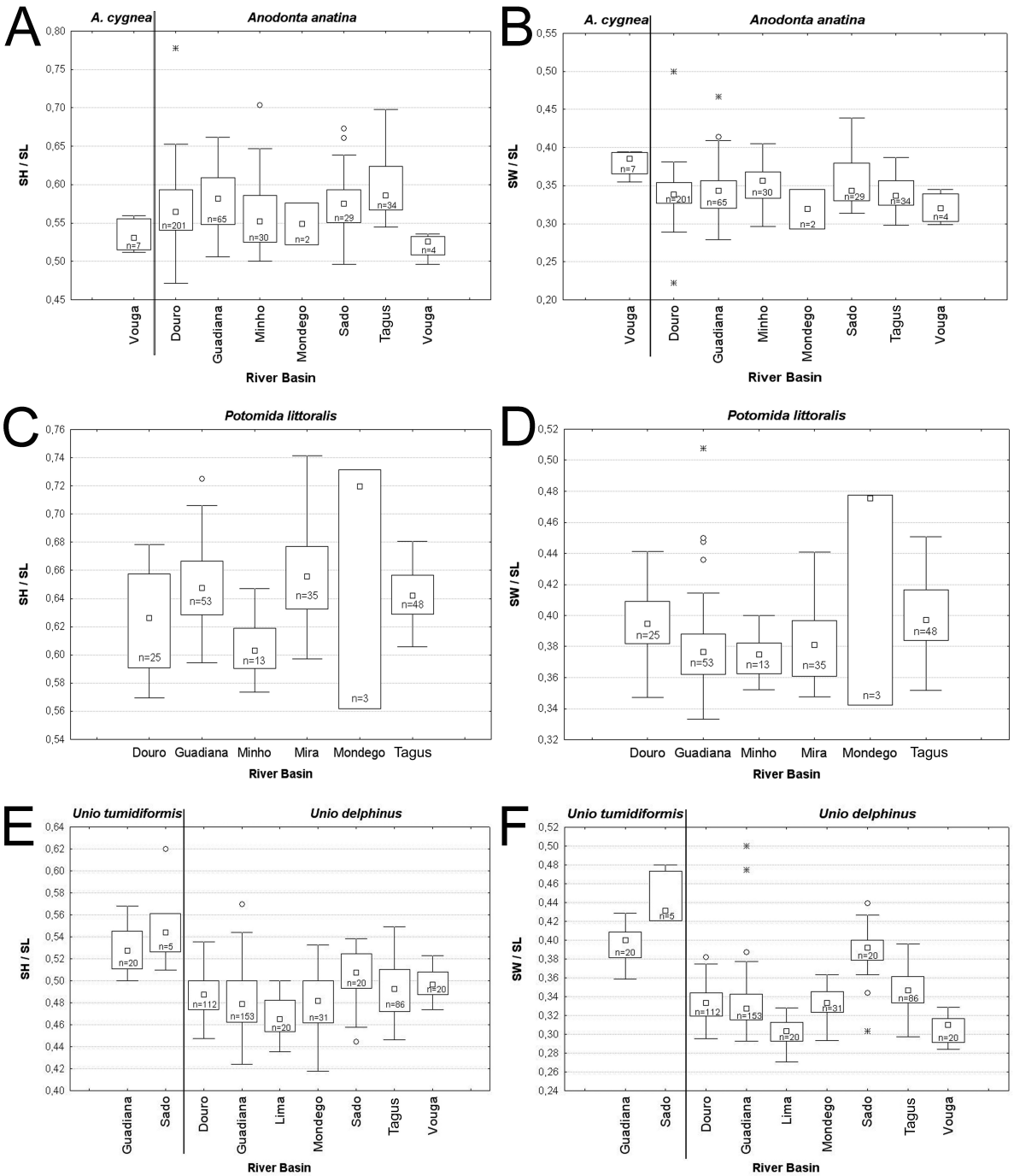

Fig. 2.– Variation of SH/SL and SW/SL ratios within river basins for each species. Boxes show variation between the 25% and 75% quartiles, inner square is the median, whiskers represent the non-outlier range, outer circles are outliers and asterisks are extremes. A-B: *Anodonta*; C-D: *Potomida*; E-F: *Unio.* SL: shell length. SH: shell height. SW: shell width.

Fig. 2.– Variación de los índices SH/SL y SW/SL de cada especie en cada cuenca. Las cajas muestran la variación entre los cuartiles 25% y 75%, la caja interior es la mediana, las barras representan el rango sin los valores atípicos, los círculos exteriores son valores atípicos y los asteriscos son valores extremos. A-B: *Anodonta*; C-D: *Potomida*; E-F: *Unio.* SL: longitud de la concha. SH: altura de la concha. SW: anchura de la concha.

rDNA genes was assessed by the partition homogeneity test in PAUP* (Mickevich & Farris, 1981; Farris *et al*., 1994).

We also performed Bayesian analyses to estimate the posterior probability of the nodes in the phylogenetic trees. MrBayes (Huelsenbeck & Ronquist, 2001) was run with 6 substitution types (nst=6), and considering gamma-distributed rate variation as well as the proportion of invariable positions for the two genes combined (but independently analyzed). For the COI gene, a partition by codon position was also taken into account. The MCMCMC (Metropolis-coupled Markov chain Monte Carlo) algorithm with four Markov chains was used for 5,000,000 generations, with a sample frequency every 100 generations, and eliminating 10% of the first trees obtained since they did not reach the stationarity of the likelihood values.

Results

Morphometry

The ratios SH/SL and SW/SL showed a high variability between populations from different river basins for all species (Fig. 2). The performed Anovas using species, "races" and river basins (per species) as grouping factors were all significant at $p<0.01$. The unequal N HSD test showed that the values for both ratios were significantly higher for *U. tumidiformis* than for *U. delphinus* ($p<0.01$; Fig. 2E,D), which can be translated in that the shell of *U. tumidiformis* is higher and wider than that of *U. delphinus*. The analyses using the ratio SW/((SL+SH)/2) fully supported these results, and the difference between *U. tumidiformis* and *U. delphinus* was significant at $p<0.01$ (means: 0.53 and 0.45 respectively). We also found that the SW/SL ratio was significantly higher in Haas' (1969) *U. p. delphinus* when compared with *U. p. mucidus* (unequal N HSD, $p<0.01$), but no significant differences were found for SH/SL. On the other hand the SH/SL ratio was significantly higher for *Potomida l. umbonatus* when compared to *P. l. littoralis* ($p<0.01$), while no significant differences for SW/SL were found between these two "races" described by Haas (1969). Despite these results, there were numerous overlapping values between the ratios of Haas' (1969) "races" of *U. pictorum* and *P. littoralis*. This was also evident when comparing river basins, which presented a highly variable pattern (Fig. 2). The trend for high inter-basin variability was maintained for *A. anatina*. Even though the shells of *A. cygnea* appeared wider than *A. anatina* (higher SW/SL ratio, Fig. 2), we found no significant differences for the two analyzed ratios using the unequal N HSD test.

All discriminant analyses gave some optimal combinations of the variables (SL, SH and SW) for $p<0.01$ (specific results not shown). However, only within the genus *Unio* did we obtain an obvious segregation of any group using 95% confidence ellipses. For the analysis of this genus, the first function (root 1) provided the best overall discrimination between groups (Wilks' lambda: 0.605; approximate $F_{(6,924)} = 44.048$; $p= 0.0$). The pooled within-group correlations of variables with the respective discriminant functions (canonical) can be considered as the factors loadings of the respective variables on the discriminant functions (Statsoft, 2001). The first canonical root (root 1) showed a moderate and unequal positive correlation with SL, SH and SW. Correlation values differed considerably between the three variables, indicating that root 1 is a measure of shape rather than overall size of the shell. The discriminant function associated to this root accounted for 90% of the total variance. The second canonical root (root 2) was strongly and nearly equally correlated to the three variables (SL, SH and SW) and thus the corresponding discriminant function is more strongly associated to the overall shell size. Therefore, shape (associated to the first discriminant function) is mainly responsible for distinguishing *U. tumidiformis* from *U. delphinus,* while the size of the shell (associated with the second discriminant function) accounts for part of the variation within each group (Fig. 3). Neither function could effectively discriminate between Haas' (1969) *U. p. mucidus* and *U. p. delphinus "*races" (Fig. 3). There was also a small overlap of the 95% confidence ellipse of *U. tumidiformis* with those of the other groups.

Sequence characteristics and variation

We obtained 113 sequences (56 COI and 57 16S) for the 76 specimens examined. A total of 1163 characters were analyzed (657 for COI and 506 for 16S). Within these sequences, 38 specimens were characterized with both genes. The 16S alignment required the inclusion of several gaps to compare all analyzed sequences. We only used

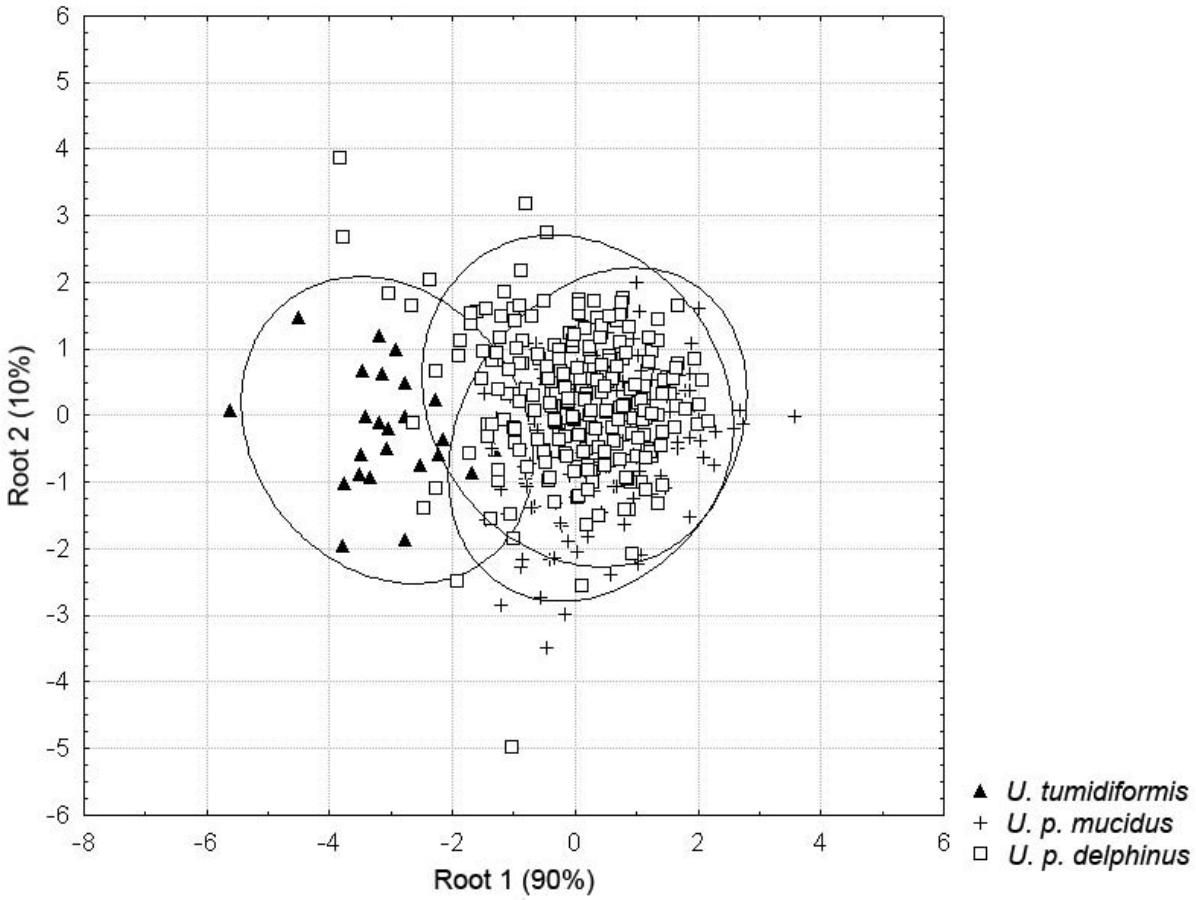

Fig. 3.– Relationship between scores on Root 1 and Root 2 for the discriminant analysis for shell measurements of Portuguese *Unio* species and "races" *sensu* Haas (1969). Ellipses encompass 95% confidence limits for each species / "race".

Fig. 3.– Relación entre los resultados de la función 1 y la función 2 para el análisis discriminante de las medidas de la concha de las especies y "razas" de *Unio* portuguesas *sensu* Haas (1969). Las elipses abarcan los intervalos de confianza de 95% para cada especie / "raza".

somatic tissue from the foot and there was no evidence of heteroplasmy or doubly uniparental inheritance (DUI, Hoeh *et al.*, 2002) among the sequences analyzed. Base composition was homogenous in all taxa analyzed, even though proportions of some bases were biased. It is worth noting the extreme conservation of the second codon position of the COI gene, with only two substitutions found. A perfect correlation was obtained plotting all substitutions against uncorrected ('p') distances for both genes (not shown). However, a trend to saturation was obtained for transitions in third position of the COI gene in pairwise comparisons, with divergences greater than 15%. As the divergences between different genera, especially between ingroups and outgroups, were sometimes above this value (Table 3), saturation might mask the relationships between them, since homoplastic characters could lead to the underestimate of divergence.

The mean sequence divergence for both genes within the same species and considered geographic unit ranged from 0% (for 16S sequences of *U. mancus*) to 0.5% (for COI sequences of *U. delphinus*), with the exception of *P. littoralis* (16.4% mean divergence between GenBank sequence AF120652

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Table 3.– Mean nucleotide divergences values between the different taxa analyzed based on 16S rRNA and COI sequences. Hyphens indicate that intrapopulation divergence could not be calculated.

Table 3.– Valores medios de divergencia de nucleótidos entre los taxa analizados basados en las secuencias de ARNr 16S y COI. Los guiones indican que no se ha calculado la divergencia intrapoblacional.

N		Species																		
			COI																	
			A																	
8	**A.**	*Anodonta anatina* (PORTUGAL-N)	0.2	**B**																
3	**B.**	*A. anatina* (PORTUGAL-S)	1.6	0.5	**C**															
1	**C.**	*A. anatina* (SWEDEN)	3.0	2.7	-	**D**														
4	**D.**	*A. cygnea*	13.5	13.7	13.8	0.1	**E**													
1	**E.**	*Pseudanodonta complanata*	11.2	11.5	11.2	11.8	-	**F**												
1	**F.**	*Amblema plicata*	16.1	16.2	16.5	14.9	15.5	-	**G**											
7	**G.**	*Potomida littoralis*	16.9	16.8	17.1	15.9	16.1	14.5	0.3	**H**										
1	**H.**	*Quadrula quadrula*	19.2	20.0	18.8	16.4	15.9	11.9	16.4	-	**I**									
1	**I.**	*Unio* sp.	15.5	15.6	14.4	16.0	14.6	15.0	16.4	16.5	-	**J**								
2	**J.**	*U. caffer*	16.1	16.1	15.8	16.0	14.0	15.0	15.7	15.7	4.9	0.2	**K**							
2	**K.**	*U. crassus*	14.8	15.3	15.6	16.2	14.5	15.3	16.0	15.6	9.8	10.3	3.2	**L**						
8	**L.**	*U. tumidiformis* (PORTUGAL)	15.5	16.3	16.0	14.5	14.4	14.8	14.8	15.9	11.3	12.4	8.7	0.6	**M**					
2	**M.**	*U. mancus*	14.4	14.8	13.7	16.1	14.3	14.7	17.1	15.9	4.8	4.8	10.9	12.0	0.2	**N**				
4	**N.**	*U. pictorum*	16.2	16.3	15.5	16.2	14.4	15.8	15.9	15.9	4.4	3.4	10.8	11.6	4.0	0.4	**O**			
30	**O.**	*U. delphinus* (PORTUGAL)	15.0	15.0	14.4	16.4	13.9	14.7	16.2	16.0	4.7	5.1	10.5	11.2	4.1	4.2	0.5	**P**		
3	**P.**	*U. tumidus*	12.5	12.8	12.4	14.5	12.8	14.3	16.0	16.1	11.6	11.2	12.2	12.3	10.8	11.3	12.3	-	**Q.**	
1	**Q.**	*Margaritifera margaritifera*	19.0	19.1	18.6	18.1	18.4	16.6	19.3	17.6	19.2	18.2	18.6	17.6	18.8	18.6	17.8	17.1	-	**R**
1	**R.**	*Neotrigonia margaritacea*	23.5	24.3	24.0	24.0	23.5	24.5	23.3	23.1	23.3	24.1	22.7	22.9	24.9	24.8	24.5	22.3	23.2	-
			16S																	
			A																	
4	**A.**	*Anodonta anatina* (PORTUGAL-N)	0.2	**B**																
1	**B.**	*A. anatina* (PORTUGAL-S)	1.5	-	**C**															
1	**C.**	*A. anatina* (SWEDEN)	1.0	1.1	-	**D**														
1	**D.**	*A. cygnea*	5.0	5.4	4.8	-	**E**													
1	**E.**	*Pseudanodonta complanata*	5.7	6.0	4.5	6.2	-	**F**												
1	**F.**	*Amblema plicata*	15.3	14.6	14.3	15.2	14.1	-	**G**											
2	**G.**	*Potomida littoralis*	15.5	15.3	13.2	16.3	14.9	13.1	0.0	**H**										
1	**H.**	*Quadrula quadrula*	14.0	14.7	14.6	14.5	13.9	15.2	17.0	-	**K**									
1	**K.**	*U. crassus*	10.6	11.6	9.6	10.7	11.4	14.2	13.4	15.3	-	**L**								
7	**L.**	*U. tumidiformis* (PORTUGAL)	10.7	11.7	9.7	11.8	11.9	15.0	12.8	15.9	4.0	0.3	**M**							
2	**M.**	*U. mancus*	8.6	9.6	7.4	9.9	9.8	12.6	12.1	14.5	3.9	5.9	0.0	**N**						
1	**N.**	*U. pictorum*	9.1	10.0	7.9	10.4	10.2	13.4	12.8	15.3	4.5	4.8	0.6	-	**O**					
42	**O.**	*U. delphinus* (PORTUGAL)	9.2	10.0	7.9	10.5	10.2	13.7	12.8	15.3	4.5	4.8	1.7	1.5	0.2	**P**				
1	**P.**	*U. tumidus*	10.7	10.7	9.0	10.0	10.0	13.2	11.9	14.6	6.8	7.2	5.2	5.0	6.2	-	**Q.**			
1	**Q.**	*Margaritifera margaritifera*	21.2	21.6	20.6	23.5	23.1	24.2	20.8	24.3	21.6	21.0	21.4	21.6	21.6	21.0	-	**R**		
1	**R.**	*Neotrigonia margaritacea*	36.1	37.7	36.1	32.3	37.0	31.5	36.6	32.9	37.3	36.5	36.9	37.1	36.5	36.4	33.2	-		

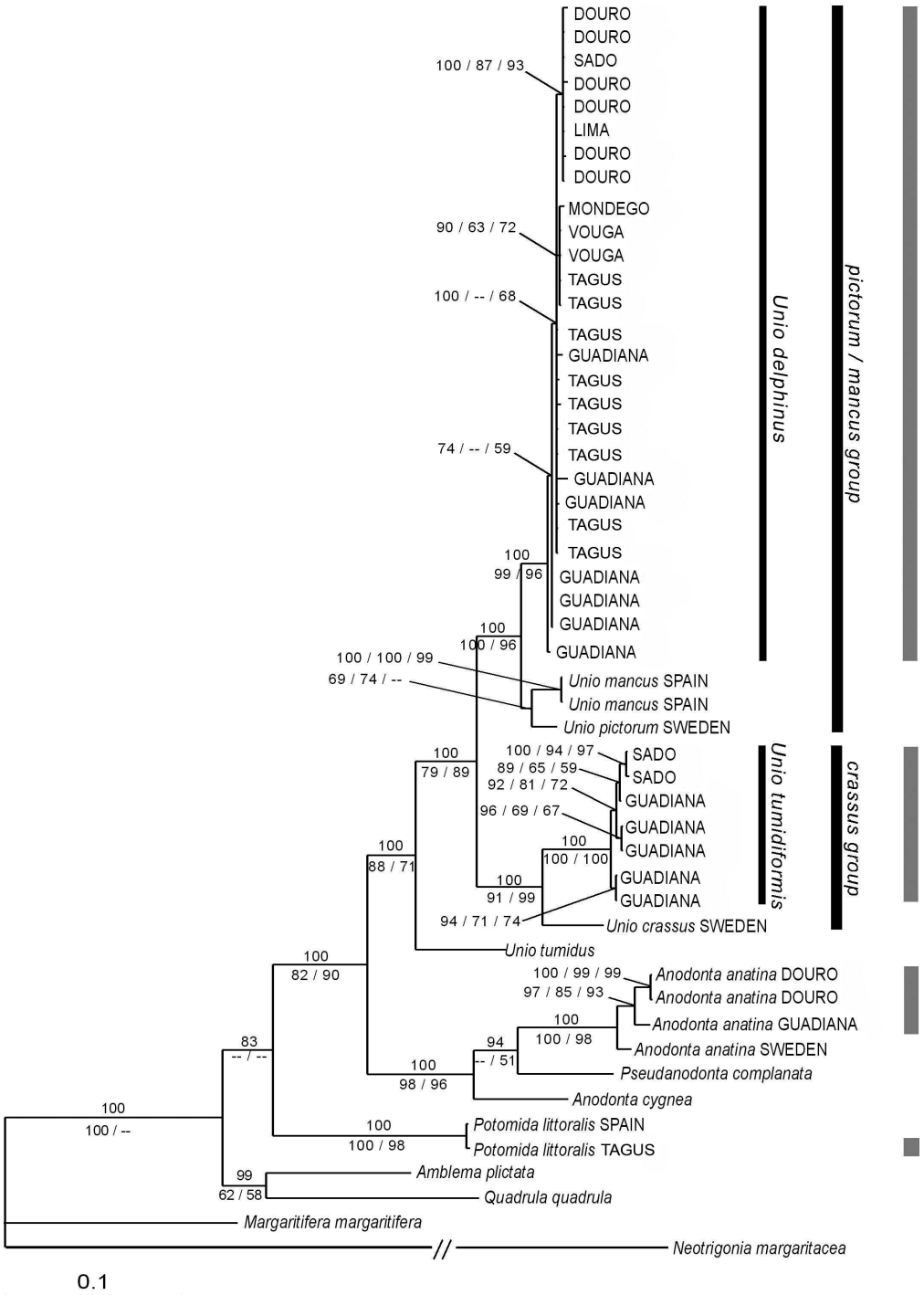

Fig. 4.– Phylogenetic relationships inferred from the combined analyses of COI and 16S. Numbers above branch or first in order represent posterior probability x 100. Numbers below branch, or respectively second and third in order, are bootstrap values for Maximum Parsimony/Maximum Likelihood. The grey bar shows the specimens sequenced for this article.

Fig. 4.– Relaciones filogenéticas según el análisis combinado de COI y 16S. Los números sobre las ramas o en primer lugar indican probabilidades posteriores x 100. Los números bajo las ramas, o en segundo y tercer lugar corresponden a valores de bootstrap de Máxima Parsimonia/Máxima Verosimilitud. La barra gris muestra los ejemplares secuenciados para este artículo.

and all other *P. littoralis* sequences) and *U. crassus* (3.2% COI sequence divergence between specimens from Poland and Sweden) (Table 3). We did not have access to the specimen corresponding to the GenBank sequence AF120652, but it clearly does not belong to the genus *Potomida* (Khalloufi *et al*., 2011). It is worth noting the high haplotype diversity found in specimens from the Southern basins (Sado and Guadiana). All analyzed specimens of *A. anatina* and *P. littoralis* from these basins showed unique haplotypes. Fifty percent of COI and 57% of 16S sequences obtained for *U. tumidiformis*, which occurs only in these basins, were unique haplotypes, contrasting with 41% of COI and 14% of 16S sequences for *U. delphinus*, a widespread species. For this last species, 41% and 50% of total COI and 16S haplotype diversity respectively were unique to the Guadiana basin.

Phylogenetic analyses

No significant differences between the topologies for COI and 16S were found using the partition homogeneity test (as implemented by PAUP) (p=0.3). We could therefore combine both data sets for most analyses. The phylogenetic analysis of the combined data set resulted in a tree where all unionids from Portugal were included in two clades: one comprising *Anodonta* and *Unio* (Bayesian posterior probability bpp=1 and bootstrap index 82% according to MP and 90% according to ML) and another well supported clade including Iberian *P. littoralis* (bpp=1, bootstrap values 100% MP and 98% ML) (Fig. 4). *Anodonta* formed with *Pseudanodonta* a well supported monophyletic clade (bpp=1, bootstrap values 98% MP and 96% ML) which included three groups, corresponding to the nominal species *A. anatina*, *A. cygnea* and *Pseudanodonta complanata*. The relationships between these three lineages were not resolved. Portuguese *A. anatina* were included in a clade (bpp=1, bootstrap values 100% MP and 98% ML) with Swedish *A. anatina* as a sister group. The genus *Unio* appeared in this study monophyletic (bpp=1, bootstrap values 88% MP and 71% ML), with *U. tumidus* as the basal branch of the clade. A second split separated the *U. crassus* group (*sensu* Haas, 1969) from the remaining species. All analyses recovered a very well supported clade that included all *U. tumidiformis* (bpp=1, bootstrap value 100% MP and ML) having *U. crassus* from Sweden as a sister group. The divergence between these two taxa was considerable (mean 8.7% COI and 4% 16S). *Unio tumidiformis* from different localities showed a tendency for grouping in separated clades. Finally, *U. mancus, U. pictorum* and *U. delphinus* formed well differentiated lineages belonging to the same clade in all analyses. The clade comprising *U. mancus* and *U. pictorum* was only supported by MP (bootstrap value 74%), and was the sister group of *U. delphinus* (bpp=1, bootstrap value 100% MP and 96% ML). Within the *U. delphinus,* specimens from basins of the same geographic area tended to cluster together, but at least a few specimens from geographically distant basins were included in those clades. Sequences from specimens corresponding to Haas' (1969) *Potomida* and *Unio* "races" did not form clades that could support the taxonomic value of those "races".

The separate analyses of the COI and 16S sequence fragments, which included a higher number of specimens, lead to similar results. The phylogenetic relationships indicated by the analyses of the 16S sequences (figure not shown) were very much like those retrieved by the combined analysis, but failed to provide support for many clades, especially within the *U. mancus* / *U. pictorum* / *U. delphinus* group. The phylogenetic tree based on the analysis of the COI sequences (Fig. 5) was similar to the one obtained by the combined analyses, but provided further information by adding one Portuguese specimen of *A. cygnea,* several sequences from *A. anatina* and *P. littoralis* from Portugal, one *U. crassus* sequence from Poland, the GenBank sequence AF120652 and an additional species, *Cafferia caffra* (Krauss, 1848). The *A. cygnea* sequence from Portugal was included in a well supported clade with *A. cygnea* sequences from other European regions (bpp=1, bootstrap values 100% MP and 98% ML) with a mean divergence of 0.1%. A split in the Portuguese *A. anatina* between southern basins (Sado and Guadiana) and central and northern basins was evident (mean divergence of 1.6%). The *U. crassus* sequence from Poland joined the one from Sweden (bpp=0.9, bootstrap values 100% MP and 92% ML) in a clade that was sister group of *U. tumidiformis*. Finally, AF120652 and *C. caffra* joined the *U. mancus* / *U. pictorum* / *U. delphinus* group, but no further insight was obtained about the relationships within this group, which presented divergences between lineages from 3.4% (*U. pictorum* vs. *C. caffra*) to 5.1% (*U. delphinus* vs. *C. caffra*).

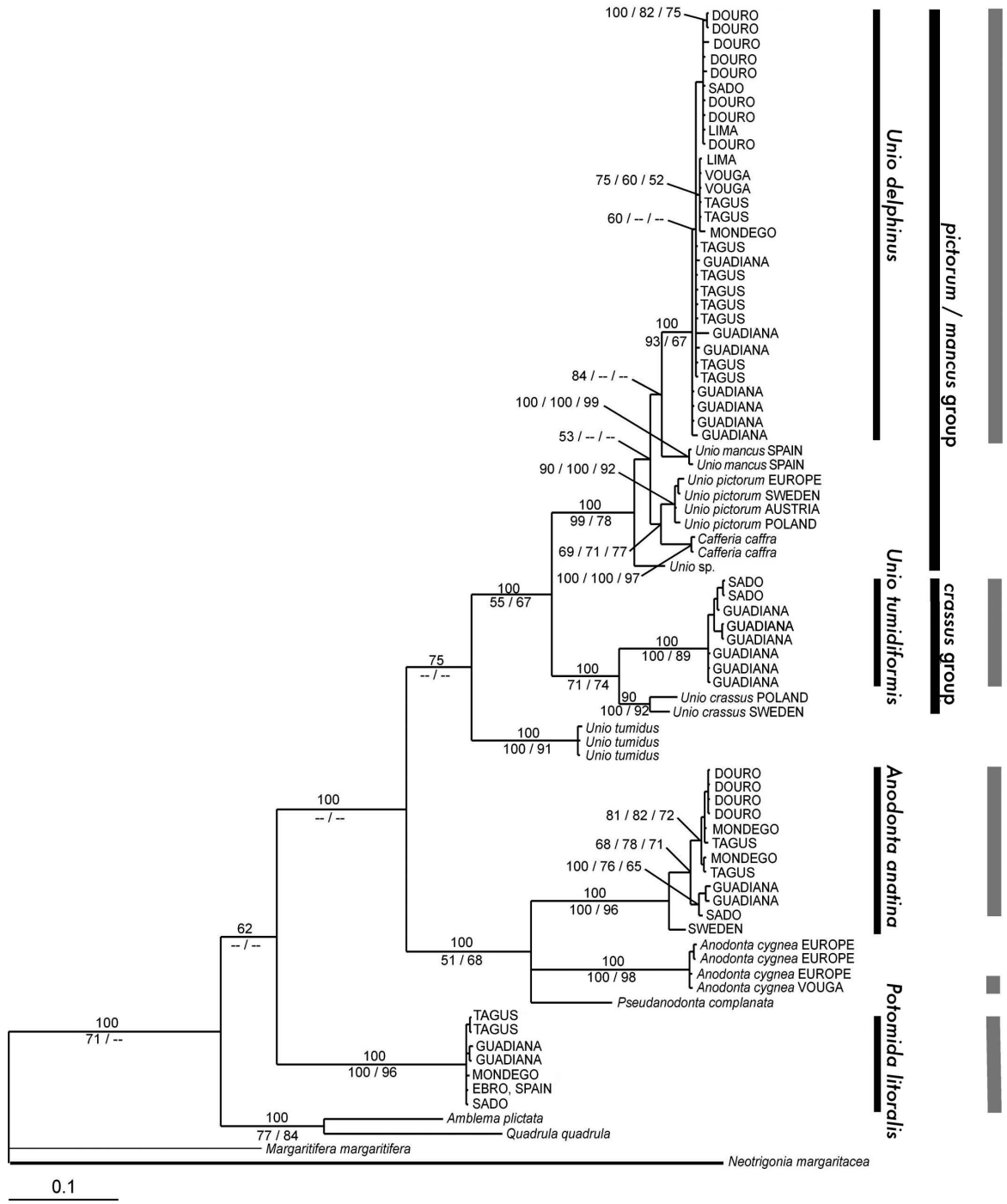

Fig. 5.– Phylogenetic relationships inferred from COI sequences. Numbers above branch or first in order represent posterior probability x 100. Numbers below branch, or respectively second and third in order, are bootstrap values for Maximum Parsimony/Maximum Likelihood. The grey bar shows the specimens sequenced for this article.

Fig. 5.– Relaciones filogenéticas basadas en las secuencias de COI. Los números sobre las ramas o en primer lugar indican probabilidades posteriores x 100. Los números bajo las ramas, o en segundo y tercer lugar corresponden a valores de bootstrap de Máxima Parsimonia/Máxima Verosimilitud. La barra gris muestra los ejemplares secuenciados para este artículo.

Discussion

Genetic diversity, phylogeny and taxonomic implications

Both genes showed a similar phylogenetic signal among the analyzed taxa, even though the 16S was considerably more conservative and showed very little intraspecific variation, a common observation within Unionoidea (Lydeard *et al.*, 2000; Machordom *et al.*, 2003; Araujo *et al.,* 2009a; Khalloufi *et al*., 2011).

Our analyses supported six well differentiated unionid lineages in Portugal, belonging to the genera *Anodonta* (3), *Potomida* (1) and *Unio* (2). Of these, only one could be confirmed beyond doubt, i.e., with negligible genetic divergence, to belong to an European widespread species: *A. cygnea*. The occurrence of *A. cygnea* in Portugal confirms the findings of Nagel *et al.* (1996), who analyzed specimens collected from the same lake system in central western Portugal, from where our specimen was collected. Although considered to be a species widespread in Portugal and the Iberian Peninsula (Azpeitia, 1933, Nobre, 1941; Haas, 1969), it was only found in one locality of our sampling scheme (Araujo *et al*., 2009c). All other *Anodonta* specimens were included in a common clade with *A. anatina* from Sweden, with divergences between Portuguese and Swedish sequences up to 3% for COI and 1% for 16S. Avise (2000) state that mitochondrial intraspecific divergences are rarely greater than 2%, so the possibility remains that these were two different species. Further studies with other molecular markers and using other characters would help clarify this issue. Portuguese *A. anatina* were found to split between two genetically distinct groups, a northern and a southern clade. This probably indicates an evolutionary trend, but the low divergences for both COI and 16S between the northern and southern clades do not allow on their own to consider that they correspond to different taxa. It is worth noting that the 16S divergence between the two Portuguese lineages (1.5%) is larger than the one between either and the *A. anatina* from Sweden (1.02 to 1.1%). Everything considered we do not have enough genetic evidence to refute the identity of these three lineages as *A. anatina*.

All analyzed *Potomida littoralis* specimens were included in a homogenous Iberian clade. Although we found a considerable haplotype diversity (six different COI haplotypes among six analyzed specimens), we found no evidence that supported the occurrence of a northern Iberian taxon (*P. littoralis littoralis*) and a southern taxon (*P. littoralis umbonatus*) as suggested by Haas (1969). Indeed, Khalloufi *et al*. (2011) have also included in this species the North African populations previously known as *P. l. fellmanni*.

The inclusion of Portuguese *Unio* in two well differentiated clades, related to some extent respectively with *U. crassus* and *U. pictorum*, confirms the classification from Haas (1969), who considered the occurrence of two taxa in Portugal, which were included in the *crassus* and *pictorum* groups. *Unio delphinus* was included in an unresolved clade containing *U. pictorum* and *U. mancus* as well. Badino *et al.* (1991), Nagel (2000) and Nagel & Badino (2001) supported the close relationship between *U. pictorum* and *U. mancus* based on protein electrophoresis analyses However, Araujo *et al.* (2005) provided solid evidence to support them as different species based on molecular studies. The similar genetic divergences between all lineages within the *delphinus / pictorum / mancus* group, associated to some known morphological differences (Haas, 1969) seem to support that the Iberian *U. delphinus* is also a distinct species, as has been already reported (Khalloufi *et al*., 2011). We were unable to determine if the unresolved phylogenetic relationships between the lineages comprised in this group corresponded to a rapid cladogenetic event or simply the lack of phylogenetic signal considering the analyzed gene fragments. If not a true polytomy, it indicates a rapid succession of independent cladogenetic events (Slowinski, 2001), that might be detected by increasing the number of gene fragments analyzed (Page & Holmes, 1998; Robalo *et al*., 2007), revealing previously hidden phylogenetic relationships. According to the analyses of COI, another two lineages were included in this problematic group: one comprising the GenBank sequence AF120652, identified as *P. littoralis* in Giribet & Wheeler (2002), but belonging to *Unio ravoisieri* (Khalloufi *et al*., 2011), and the African *Cafferia caffra*, known from the southern area of this continent. Within the *crassus* group, the phylogenetic patterns and high genetic divergences show that the Portuguese clade corresponds to a species well differentiated from its central and northern European relatives.

Origin and evolution

Biogeographical relationships between freshwater mussels are complex and integrate very distinct features over a long period of time, going back

more than 200 million years if we consider the Triassic origin of Unionoida (Haas, 1969; Graf & Cummings, 2006). Being sedentary animals, their dispersal depends largely on the hosts for their parasitic larvae. This association between freshwater mussels and their host fish certainly drives population-level processes (Graf, 1997; Vaughn & Taylor, 2000) but their phylogeny should reflect events such as the breakup of Pangaea in the Mesozoic, continental watershed evolution during the Tertiary and Pleistocene glaciations (Davis & Fuller, 1981; Graf & Cummings, 2006). The phylogenetic and biogeographic patterns observed may be often complicated by later gene flow. Nagel (2000) related the population structure of *U. pictorum* in central Europe to river connections during glacial ages and to artificial canals built in the past centuries. Machordom *et al.* (2003) suggested that there might have been recent gene flow between European and North American *M. margaritifera* populations by mean of the introduction of infected host fish. The Iberian Peninsula constituted a refuge during glaciations, and artificial connections between river systems, especially in Portugal, are not as widespread as in central Europe, so that the phylogenetic structure of Unionidae should reflect more ancient processes. Nevertheless, *A. cygnea* showed practically no divergence between Iberian and central European populations. Considering that the rate of change of COI for this species should not differ significantly from that of *A. anatina*, this would either imply a null evolution rate (not probable) or a constant and significant genetic flow between populations, which owing to its rarity in Europe (Glöer & Meier-Brook, 1998) is even less probable. As a consequence, the Portuguese populations of this species probably represent a relatively recent introduction, specially taking into account the diversity and abundance of introduced fishes in the lakes it inhabits. If this is the case, it dates back more than 160 years, as reliable accounts for the species for central Portugal exist since Morelet (1845).

The Iberian unionids were not found to be monophyletic. All taxa were either more closely related to central European species than other Iberian ones, or the relationship between them could not be resolved. This implies multiple origins for this diversity, as was suggested for other groups (Sanjur *et al.,* 2003). Many geographical sampling gaps are still needed to fill before resolving the *U. pictorum* / *U. mancus* group phylogenetic relationships, namely the Spanish Pyrenees and most of the Mediterranean European and African area. The observed polytomy between these taxa is not necessarily a "hard" one, but indicates a relatively rapid radiation (Slowinski, 2001). In fact, the similarity of genetic divergences between each taxon and the vast geographic area they occupy all together, suggest a common widespread ancestor that was isolated in several areas where it could evolve separately. This could have happened through watershed evolution: the endorrheic basins present in the Oligocene and Miocene would be the basis of the current Iberian diversity, much as they are argued to be for freshwater fish (Doadrio, 1990; Sanjur *et al*., 2003; Robalo *et al.,* 2007). Some gene flow might have occurred at different times, including the ice ages, caused by river captures. Some degree of connection with central Europe might have been maintained through the lower extremes of the Pyrenees (Vargas *et al.,* 1998). The same event can be the main factor explaining the observed diversity of *A. anatina* with the evolution of closely related Portuguese and central / northern European lineages. The southern Portuguese lineage might be related to the endorrheic basins in the isolated Betic-Riff Massif, which remained isolated until the end of the Miocene, probably with its own endemic fauna (Vargas *et al*., 1998; Machordom & Doadrio, 2001; Araujo *et al*., 2009a; Khalloufi *et al*., 2011).

Finally, the high divergence between *U. tumidiformis* and the central / northern European *U. crassus* indicate a much older origin for the first taxon, early absence of gene flow or a combination of both. We hypothesize that it can derive from an ancestor that became isolated early in the long process of watershed evolution and rise of the Pyrenees during the Tertiary, continuing its differentiation in the isolated Betic-Riff Massif (Reis & Araujo, 2009).

We can therefore identify two main speciation events under this model: the first, beginning in the Tertiary and caused by the isolation of the Iberian Peninsula and the second, later in this period, driven by the formation of the current watersheds.

Significance of morphological variability

Mollusc species are usually identified based on shell features. However, the high level of shell plasticity has led to uncertainty of the systematic

value of those characters (Aldridge, 1999; Renard *et al.,* 2000; Baker *et al.*, 2003; Zieritz & Aldridge, 2009; Zieritz *et al.*, 2010). While early freshwater bivalves' researchers such as Castro (1873, 1885, 1887) and Locard (1899) largely over-estimated species richness, there was an opposite tendency during the 1900s (for example Nobre, 1941). Haas (1969) tried to summarize all the previously described variability but avoided giving specific status to many different morphotypes. Molecular markers have proved to be a very useful tool to help resolve the systematic and taxonomic problems (Renard *et al.,* 2000; Baker *et al.*, 2003; Araujo *et al.,* 2005; Graf & Cummings, 2006; Araujo *et al.*, 2009a,b; Khalloufi *et al.*, 2011). However, this is not a fast, practical or economical way of identification and the fact that freshwater mussels are a highly endangered group means that it is not possible to sacrifice specimens to use more reliable characters for identification such as the hinge area. Therefore field identification is still largely dependent on shell shape.

In this study the morphometric variables and ratios analyzed proved to be very useful to distinguish *U. tumidiformis* from *U. delphinus*, with some overlapping values. It was less useful for differentiating the two *Anodonta* species, although *A. cygnea* seemed to be wider than *A. anatina*. Increasing the sample size for *A. cygnea* would be important to evaluate the usefulness of this character. The variation between river basins for each species showed sometimes important differences that could be associated to some of Haas' (1969) "races", although this result was not consistent for both ratios analyzed. Also, these differences, such as between *U. p. delphinus* and *U. p. mucidus*, had no correspondence in our phylogenetic results. These results support the conclusion that probably there are no cryptic species within the analyzed fauna. They may be evidence of adaptive divergence between populations that are either not isolated or are just recently so, and that given enough time may give rise to speciation processes (Lexer & Fay, 2005).

Within the genus *Unio* we found an analogous pattern of variation for both species in the Guadiana and Sado basins, with higher and wider shells in the Sado system. This suggests a clear environment influence on the shape of the shell of both species. Eager (1978) and Zieritz *et al.* (2010) suggested that shell shape develops in response to certain environmental constrains while Haas (1969) argued that variation is important due to the parasitic life stage, allowing adaptation to the unpredictable habitat where juveniles recently released from the host fish drop to. Hinch *et al.* (1989) and Watters (1994) stated that wide, globose shells are more buoyant and adapted to habitats with muddy substrate, while Hinch *et al.* (1986) related high shells to these habitats as well. Considering that the Sado river basin sites, where the mussels were collected from, are dominated by mud (J. Reis, personal observation), our results are congruent with the above mentioned statements. This variation can be more accurately related to environmental factors by studying single stream populations and microhabitat as in Zettler (1997) rather than at river basin scale. The analogous variation of morphology between basins in both *Unio* species, yet maintaining each species identity for that trait, indicates that shape is not only environmentally induced but also genetically determined, supporting the link between phenotypic plasticity and evolutionary processes suggested by Baker *et al.* (2003), Bijlsma & Loeschcke (2005), Lexer & Fay (2005) and Relyea (2005). Lexer & Fay (2005) listed evidences for this link in several organisms, ranging from plants to amphibians.

Overall our study suggests that morphological variation in unionids reflects both systematic relationships and phenotypic plasticity. Our results demonstrate how an integrated approach using morphological and molecular characters can clarify the evolutionary history of a given group. The evidence for the heritable basis of shell shape reinforces its taxonomic, phylogenetic and evolutionary value, while showing that caution should be used when attributing variation to a sole factor such as an environmental condition. Variation is often, if not always, a consequence of a complex interaction of factors that may be misleading if taken independently.

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