MARGARITIFERA MAROCANA (PALLARY, 1918): A VALID SPECIES INHABITING MOROCCAN RIVERS

R. ARAUJO1, C. TOLEDO1, D. VAN DAMME2, M. GHAMIZI3 AND A. MACHORDOM1

1Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain; 2Unit of Research on Paleontology, Gent University, Krijgslaan 281, B-9000 Gent, Belgium; and 3Muséum d’Histoire Naturelle de Marrakech, Université Cadi Ayyad, Faculté des Sciences, Sémlika, B.P. 2290 Marrakech, Morocco

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ABSTRACT

Within the Unionoida the family Margaritiferidae is a small but widely distributed group, whose number of genera and species is under discussion. Using molecular and morphological characters, the present paper redescribes several Moroccan specimens of Margaritifera, previously classified as M. auricularia marocana. The shell and anatomical features of the taxon are similar to those observed in European specimens of M. auricularia auricularia. Notwithstanding, the two subspecies can be separated by certain hinge characters. Partial sequences of the mitochondrial genes COI and 16S rRNA support recognition of the Moroccan populations as a different species: Margaritifera marocana. We examined the phylogenetic relationships of the Margaritiferidae. The COI data indicated two monophyletic clades: one including M. margaritifera, M. dahurica, M. falcata and M. laevis, and the second comprising M. auricularia from the Iberian Peninsula and the Moroccan specimens of M. marocana as an independent lineage. Cumberlandia monodonta was recovered as the basal margaritiferid, sister to Margaritifera.

INTRODUCTION

Within the Unionoida, or freshwater mussels, the family Margaritiferidae is a small but widely distributed group whose systematics and taxonomy remain chaotic (Haas, 1969; Smith, 2001; Huff et al., 2004). The currently valid species and subspecies are: in North America, Cumberlandia monodonta (Say, 1829), Margaritifera falcata (Gould, 1850), M. hembeli (Conrad, 1838), M. mariana Johnson, 1983 and M. margaritifera (Linnæus, 1758); in Europe, M. auricularia auricularia (Spengler, 1793) and M. margaritifera; in Africa, M. auricularia marocana (Pallary, 1918); and in Asia, Margaritifera laevis (Haas, 1910), Margaritifera dahurica (Middendorff, 1850) and Margaritarianopsis laoensis (Lea, 1863).

There are no clear shell characters exclusive to margaritiferids, although according to Smith (2001) the presence of an elongated and compressed shell, arcuate in older individuals, hinge teeth always present in juvenile stages and small mantle attachment scars on the inner shell surface are, among others, diagnostic characters of the family. Among their anatomical characters, interlamellar septa reduced to scattered connections in the gills, the incomplete diaphragm between the infrabranchial and suprabranchial chambers, and the absence of a supra-anal opening separate from the exhalant opening, are valid synapomorphies of the group (Smith, 2001; Graf & Cummings, 2006). According to these features, the Margaritiferidae have been considered a basal clade in the Unionoidea (Smith, 2001; Huff et al., 2004), but their supposedly primitive characters have been discussed by Graf (2002) and Graf & Cummings (2006). Phylogenetic studies to date have recovered the Margaritiferidae as the sister group to the Unionidae (Graf & Cummings, 2006).

Huff et al. (2004) examined phylogenetic relationships within the group, working with seven species and using sequence data for five molecular markers. Although not all these markers recovered robust phylogenies, their results indicated that the taxonomy of the group was in need of revision, and these authors rejected the previous taxonomy of the group established by Smith (2001). With regard to biogeographic implications, relations among New and Old World species inferred by Huff et al. (2004) have suggested a complex pattern with four possible explanations: extinction and contraction of an ancient, formerly widespread margaritiferid fauna, peripheral isolation of formerly widespread taxa, host fish dispersal or host switching.

Of the nine previously mentioned margaritiferid species, only two inhabit the Western Palaearctic region: Margaritifera margaritifera, originally ranging from Scandinavia to central Spain, and M. auricularia, originally ranging from Central Europe to Morocco. Both have been described as endangered across their respective distribution areas (Araujo & Ramos, 2001). Already extirpated from the main river systems of Western Europe, M. auricularia auricularia survives only in the Ebro basin in Spain and in the Loire and Garonne basins in France (Araujo & Ramos, 2001; Nienhuis, 2003). The Moroccan Margaritifera populations originally described by Pallary (1918, 1923, 1928) as three endemic species (Fig. 1) were formerly found in the Fez River (M. marocana Pallary, 1918), Redom River (M. redomica Pallary, 1923), Sebou basin (both), and the Derna River (M. darnaica Pallary, 1928) (Oum er Rhia basin). These three taxa were subsequently synonymized by Haas (1969) as a single subspecies, M. auricularia marocana, on the basis of conchological characters. Other known localities for M. a. marocana are the Beth and Tiflet Rivers (Araujo & Ramos, 2000), both tributaries of the Sebou.

As part of a large, ongoing study of the phylogeny of the Palaearctic unionoid genera based on molecular and morphological characters, the present paper describes several specimens of Moroccan Margaritifera and provides information on the taxonomy and phylogeny of the margaritiferids.
MATERIAL AND METHODS

To identify the historical distribution of the North African populations of *Margaritifera*, we reviewed the following literature: Bourguignat (1864), Pallary (1918, 1920, 1923, 1928), Haas (1969), Van Damme (1984), Daget (1998) and Araujo & Ramos (2000). Live specimens were obtained by field sampling of several Moroccan rivers in June 2007. Specimens were collected by wading, sometimes using a water-scoop, or by snorkelling. The few specimens that were not returned to their habitat were transported alive, chilled and without water, to the laboratory where they were dissected and photographed. We took length measurements of 40 specimens, and described their shell shape, colour, outline and hinge characteristics. All relevant features were photographed. Anatomical studies were conducted on both living and preserved specimens, paying close attention to characters that are taxonomically relevant in unionoids (Ortmann, 1911; Haas, 1924; Nagel, 1999; Graf & Cummings, 2006).

Foot tissue samples were obtained from 20 specimens and preserved in absolute ethanol for molecular analysis. This procedure does not harm the mussels. We extracted genomic DNA from 12 individuals (one from the Derna River, three from the Oum er Rhia and eight from the Abid). Total DNA was extracted from absolute ethanol preserved tissue using the ChargeSwitch gDNA Micro Tissue (Invitrogen) extraction kit. We selected the two mitochondrial genes used previously by Huff *et al.* (2004); these showed the greatest phylogenetic resolution power for relationships among margaritiferids. Partial sequences of cytochrome oxidase subunit I (COI) and 16S rRNA (16S) were amplified by polymerase chain reaction (PCR) using the same primers as described in Machordom *et al.* (2003). The PCR mix contained 3 μl DNA, 5 μl of the corresponding buffer (with 10 × 2 mM MgCl2), 1 μl of dNTPs mix (10 mM), 0.8 μl of both primers (10 μM), 0.3 μl *Taq* DNA polymerase (5 U/μl) (Biotools) and ddH2O for a total volume of 50 μl. For both genes, the following PCR conditions were used in the amplifications: 94°C (4 min), 40 cycles of 94°C (45 s), 45°C (1 min), 72°C (1 min) and a final extension at 72°C (10 min).

The products were visualized with blue light on 0.8% agarose gels stained with SYBR Safe (Invitrogen), with co-migrating 100 bp or 1 kb ladder molecular weight markers to confirm the correct amplification. The amplified fragments (around 700 bp) were purified by ethanol precipitation prior to sequencing both strands using BigDye Terminator kits (Applied Biosystems, ABI). Products were electrophoresed on an ABI 3730 genetic analyser (Applied Biosystems). The forward and reverse DNA sequences obtained for each specimen were aligned and checked using the Sequencher program (Gene Code Corporation) after removing primer regions. When necessary, Clustal X (Thompson *et al.*, 1997) was used to align the obtained sequences, but the final alignment was adjusted by eye.

To examine phylogenetic relationships within the *Margaritiferidae*, we completed our data set (Table 1) with sequences for *Margaritifera auricularia* and *M. margaritifera* previously established by Machordom *et al.* (2003) and with the downloaded sequences for the North American *M. margaritifera*.
We collected several specimens of Moroccan *Margaritifera* from one tributary (Abid River) of the Oum er Rbia and from the main river itself. Although we obtained other naïved species from rivers of the Sebou basin, we were unable to find specimens of *Margaritifera*. In the Abid River, a large population of hundreds of specimens buried in the gravel and mud was found (Fig. 2). Only three live specimens were found in the Oum er Rbia River, buried in the gravel of a side channel, where the empty shells of seven specimens were also found. Most live specimens were returned to their biotope. A few were preserved and deposited in the collections of the Museo Nacional de Ciencias Naturales (Madrid, Spain) and the Muséum d’Histoire Naturelle de Marrakech (Morocco) with the permission of the Université Cadi Ayyad (Faculté des Sciences, Semlalia, Marrakech). We found no specimens in the Derna River, but had access to preserved mantle and foot samples from two individuals collected in this river in November 2006.

The size range of living specimens was 7–15 cm. No gravid specimens were found at the two sites sampled. Shell and anatomical features were similar to those observed in European specimens of *M. a. auricularia*. Notwithstanding, we noted some hinge characters that served to separate the two subspecies. In the left valve of *M. a. marocana*, the two cardinal teeth are much weaker and less protruding than in *M. a. auricularia* and are sometimes flattened or even absent in small specimens. Also, in *M. a. marocana* a lamellar extension bridges the gap between the posterior cardinal and the lateral tooth, whereas in *M. a. auricularia* the large, conical, posterior cardinal is clearly separate from the lateral. Similar differences can be observed between the dentition of the right valves, the anterior cardinal tooth being more robust and conical in *M. a. auricularia*. Only in one of the specimens from the Oum er Rbia River was the nacre purple (Fig. 2), a character absent in the Abid population. Although not clearly visible in all specimens, small mantle muscle scars appeared on the central inner side of the valves.

The analyses of the partial sequences of the mitochondrial genes, cytochrome oxidase subunit I (COI, 657 bp) and 16S rRNA (16S, 508 bp), show the Moroccan populations as a different lineage (GenBank accession numbers: EU429676 to EU429697) (Fig. 3). Although not revealing any geographic structure, the Moroccan specimens showed two haplotypes for the 16S gene (divergence 0.20%) and four for COI (divergence between 0.15 and 0.56%). Nevertheless, divergences among the Moroccan samples from the Derna, Abid and Oum Er Rbia rivers were very low, probably because they belong to the same river basin.

Genetic divergences found between the Moroccan and Spanish populations were 9.22% for COI and 7.05% for
16S, comparable to the distance between *M. falcata* and *M. laevis* (7.9% for COI and 4.1% for 16S) or between *M. margaritifera* and *M. laevis* (9.9% for COI and 4.7% for 16S). Moreover, the phylogenetic relationships inferred here point to the independence of the Moroccan lineage as an evolutionary unit.

**Figure 2.** *Margaritifera marocana*. A. Collection site in Abid River. B. Papillae of the inhalant siphon (Oum er Rbia River). C. A sample of the population from the Abid River. D. Specimen from the Abid River. E. Specimen from the Oum er Rbia River.
Although phylogenetic reconstruction using 16S sequences was less resolved (tree not shown), the partition homogeneity test indicated no significant conflicts among trees obtained using data from the two genes once non-shared species had been eliminated ($P = 0.94$). The topologies found were stable for COI, the different treatments always yielding the same topology and a single tree in the case of the parsimony analyses (tree length = 369, consistency index CI = 0.6938, homoplasy index HI = 0.3062). In these trees the Moroccan specimens appeared as a sister clade of the Spanish $M. a. auricularia$ (Fig. 3). However, the 16S data provided two contrasting topologies according to the different analyses, differing only in the position of $M. a. marocana$, which appeared either as sister group of $M. a. auricularia$ as before or in a basal position within the genus. The most resolved relationships were given by the COI data, indicating two clades (Fig. 3): one including $Margaritifera$ margaritifera, $M. dahurica$, $M. falcata$ and $M. laevis$, and a second comprising $M. auricularia$ from the Iberian Peninsula and the Moroccan specimens, $Cumberlandia$ monodonta occupied a basal position except in some of the 16S analyses in which the position of this species was unresolved. Two of the methods (parsimony and neighbour-joining) applied to the 16S data supported a sister relationship between $M. laevis$ and $M. marrianae$, both sister to $M. falcata$. The lack of COI data for $M. marrianae$ determined that $M. laevis$ and $M. falcata$ were recovered as sister species in analyses based on the COI matrix (Fig. 3).

**DISCUSSION**

As mentioned above, there are no clear shell or anatomical features that can be used to discriminate between specimens of $M. a. marocana$ and $M. a. auricularia$. Although the African specimens might seem smaller, only 13 of 175 specimens of $M. a. auricularia$ collected across its entire distribution area were larger than 15 cm (Araujo & Ramos, 2000), which is the size of the largest specimens of $M. a. marocana$. Given the wide variability in shell shape of the two subspecies, as is common among freshwater mussels, only a few hinge characters seem to be diagnostic. It should be noted that these differences between the hinges of the two subspecies and the presence of mantle-attachment scars in $M. a. marocana$ were sufficient for Smith (2001) to separate the two taxa.

Nacre colour is of less taxonomical value. Notwithstanding, Pallary (1920) cited a pink-violet nacre as a common character among hundreds of specimens from the Fez River, and we noted purple nacre in a specimen in the Muséum National d’Histoire Naturelle, Paris, from the Sebou River. However, we observed this character only in one specimen from the Oum Er Rbia River; the specimens of the Abid population lacked this feature. It is clear that nacre colour is highly variable and convergent in naiads and is not useful for taxonomic purposes.

The presence of young specimens of $M. a. marocana$, at least in the Abid River, indicates recent recruitment in this population. Representatives of the genus Sturio (sturgeons) and Salaria (bennies) have been reported as the hosts for $M. a. auricularia$ (Araujo & Ramos, 2001). Given the absence of these fish established in recent surveys (Azeroual, 2003) in the rivers in which we found $M. a. marocana$, this naiad must depend on other host fish. The only benthic fish recently cited for the Moroccan rivers examined here are the common eel (genus Anguilla) and several barbel species (genera Barbus and Varicorhinus) (Azeroual, 2003). Though Anguilla and Barbus have been shown to be unsuitable hosts for $M. auricularia$ (Araujo et al., 2001), there is always the possibility that they could nevertheless be appropriate hosts for the endemic Moroccan margaritifid. We could also guess that if the host fish were a salmonid, as in the case of $M. margaritifera$, it would most likely be Salmoides macrostigma, since it is a very abundant trout in the Abid River (Ghamizi, personal observation). There is a need for further research on the host fish of $M. a. marocana$.

The present data on phylogenetic position as well as genetic divergence support the recognition of the specimens from Morocco as a distinct species. According to the taxonomic recommendations of Haas (1969) and Smith (2001), in which the former author synonymized the three Moroccan species described by Pallary, until specimens from the type locality of $M. marocana$ (Pallary, 1918) become available for molecular analysis, we ascribe the Moroccan populations of $Margaritifera$ to this species.

We have found two large clades within the Margaritiferidae: one including $Margaritifera$ margaritifera, $M. dahurica$ (as was hypothesized by Huff et al., 2004, when they suggested that this species should be included in $Margaritifera$), $M. falcata$, $M. laevis$, and $M. marrianae$, and the other comprising $M. auricularia$ and $M. marocana$. The former clade is currently distributed across Laurasia, the latter has a more restricted distribution in the southwestern Palaearctic.

The current distribution of $M. auricularia$ is very restricted with respect to its natural or historical one; up until 100 years ago this species was found along all the European Atlantic coasts, as well as in several peri-Mediterranean localities (Araujo & Ramos, 2000). The Pyrenees represent a barrier for a great number of species, and some vicariant sister species exist on both sides of these mountains. This could be the case here, if the Spanish and French populations of $M. auricularia$ (the only two countries where the species survives) were reproductively isolated in geological time, as occurred between the Spanish and Moroccan ones. A strong relationship between the Iberian and North African floras and faunas has been demonstrated in a series of papers (e.g. Perdices, Machordom & Doadrio, 1995; Buckley et al., 1996; García-Paris, Alcobendas & Alberch, 1998; Álvarez et al., 2000; Pardo, Cubas & Tahiri, 2008). Such relationships have been attributed to the existence of the Betic-Rifian Massif during most of the Palaeogene (Steininger & Rögl, 1984). On the other side of the world, the close relationship between margaritiferid species on both sides of the Pacific has been explained by Huff et al. (2004) as the outcome of the possible connection between these two areas.
via Beringian land bridges. A possible relation between North America and Europe was also suggested by the analysis of Huff et al. (2004), with Cunemondia placed as sister to M. auricularia in their ‘summary cladogram’. Given the very ancient origin of this family (Davis & Fuller, 1981; Delvene & Araujo, in press), the apparent low mutation rate and the relation with anadromous host fish that were potentially useful for their dispersion, it is possible that evidence of its ancient history has been masked by episodes of dispersion, expansion, range restrictions and extinction, hindering the unravelling of the biogeographic history of the family.

To substantially improve our knowledge of relict populations of this imperilled group, a similar comparative study should address the relationship between the two surviving populations of M. auricularia in Spain and France. Similarly, more information is needed on M. hemelii and especially on the Asian species M. laevis, before we can elucidate the biogeographical history of the Laurasian family Margaritiferidae.

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