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INVESTIGATIONS INTO THE PHYLOGENETIC RELATIONSHIPS OF FRESHWATER PEARL MUSSELS (BIVALVIA: MARGARITIFERIDAE) BASED ON MOLECULAR DATA: IMPLICATIONS FOR THEIR TAXONOMY AND BIOGEOGRAPHY

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ABSTRACT

The phylogenetic relationships among selected members of the family Margaritiferidae are investigated using sequence data from five molecular markers. Parsimony analyses of the data support the recognition of those nominal species for which multiple samples were included in the study (*Margaritifera margaritifera, M. laevis, M. falcata* and *M. auricularia*). Although not always strongly supported, the following relationships were consistently recovered: (*Cumberlandia* + *Margaritifera auricularia*), (*M. falcata* (*M. marrianae* + *M. laevis*)) and to a lesser degree (*Dahurinaia dahurica* + *M. margaritifera*). The molecular phylogeny indicates that the taxonomy of the group is in need of revision since the genus *Margaritifera* is not monophyletic and a new taxonomy by Smith (2001) is not supported. A complicated pattern of biogeography was suggested by the three clades of Old World + New World species. It is difficult to determine whether this pattern is a reflection of extinction and contraction of an ancient, formerly widespread margaritiferid fauna, peripheral isolation of formerly widespread taxa, fish host dispersal, or even host switching.

INTRODUCTION

The family Margaritiferidae is considered to be a basal clade in the Unionoidea, a widely distributed group of freshwater pearl mussels (Haas, 1969; Smith & Wall, 1985; Smith, 2001). Margaritiferids typically grow to large sizes with some adults reaching up to 200 mm in length and have long lifespans, usually of several decades (Taylor, 1988; Ziuganov *et al.*, 1994). Many species of unionoids are endangered (Bogan, 1993); in North America alone about 70% of the 309 extant species are endangered, threatened, or candidates for extinction (Turgeon *et al.*, 1998; Roe & Hoeh, 2003). Margaritiferid species are among the most endangered unionoids (Altaba, 1990, 1997, 2000, 2003; Primack, 1998; Ziuganov *et al.*, 1994; Altaba, López & Monserrat, 2001; Nienhuis, 2003). Remaining populations are usually found aggregated in the clean pebbly or sandy bottoms of free-running rivers and streams throughout North America, Europe, Asia and North Africa (Ziuganov *et al.*, 1994; Araujo & Ramos, 2000a; Smith, 2001).

Margaritiferids rely upon specific species of fish as hosts for their parasitic glochidia larvae (Ziuganov *et al.*, 1994; Bauer, 1997; Araujo, Bragado & Ramos, 2001; Araujo, Cámara & Ramos, 2002) and several margaritiferid species form these symbiotic relationships with salmonid fish (Hendelberg, 1961; Stober, 1972; Taylor, 1988; Lucey, 1993; Buddensiek *et al.*, 1993; Watters, 1994; Ziuganov *et al.*, 1994; Bauer, 1997; Altaba *et al.*, 2001). However, *Margaritifera hembeli* uses the brown madtom (*Noturus phaeus*), a siluriform, as a host (Johnson & Brown, 1998), and *M. auricularia* is supposed to have used the sturgeon species *Acipenser sturio* (Araujo & Ramos, 2001). *Margaritifera auricularia* has also been demonstrated to be able to use the exotic Siberian sturgeon *Acipenser baeri* and the river blenny (*Salaria fluviatilis*) as the hosts for its glochidia in experimental settings (Araujo & Ramos, 2000b; Altaba *et al.*, 2001; Araujo *et al.*, 2001, 2002). These relationships appear to be crucial for the survival of the Margaritiferidae; areas in which the populations of the host fish are declining also have diminished populations of freshwater mussels (e.g. Bogan, 1993; Araujo & Ramos, 2000b).

Unionoid fossils have been found in the Triassic (Davis & Fuller, 1981; Good, 1998), and the oldest margaritiferid fossils are from the Jurassic of China (Gu, 1998). Hypotheses on the geographical distribution of the Margaritiferidae conflict; some assume that early dates of wide clade distribution suggest the break-up of the super-continent Pangea as the cause for dispersal (Smith, 1976, 2001; Davis & Fuller, 1981). Alternatively, colonization might have occurred more recently when salmonid hosts released juvenile margaritiferids onto the North American continent (Machordom *et al.*, 2003). The present distribution therefore seems to be a combination of ancient and more recent events. Indeed, post-glacial recolonization of boreal freshwaters by unionoids appears to have been greatly enhanced when anadromous fish hosts were involved (Kat & Davis, 1984). However, it is unclear whether such range extensions could have blurred ancient vicariant events.

Phylogenetic studies of unionoid mussels have been performed based on soft-tissue characters (Heard & Guckert, 1970), immunoelectrophoretic data (Davis & Fuller, 1981), overall morphology and anatomy (Graf, 2000), mitochondrial-DNA sequence data (Lydeard, Mulvey & Davis, 1996; Hoeh *et al.*, 1998–1999; Bogan & Hoeh, 2000; Graf & Ó Foighil, 2000a) and nuclear ribosomal-DNA sequences (Graf & Ó Foighil, 2000b; Graf, 2002). In most of these studies, the family Margaritiferidae is placed as the sister group to other unionoidean taxa.

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However, little work has been done to determine the relationships among the species placed within the Margaritiferidae, and only Machordom *et al.* (2003) attempted to determine the phylogenetic relationships of the European Margaritiferidae (*M. margaritifera* and *M. auricularia*) by using variation observed at 27 allozyme loci and mitochondrial sequences for cytochrome *c* oxidase subunit I (COI) and 16S rRNA of 133 specimens. Further complicating classification problems, unionoids often exhibit variable phenotypes (e.g. Lydeard, Minton & Williams, 2000), causing inaccurate designation of new species, as exemplified by the classification of the Irish hard-water ecophenotype of *Margaritifera margaritifera* as a separate species, *Margaritifera durrovensis* (Chesney, Oliver & Davis, 1993; for recent illustrations of other margaritiferid types, see Knudsen *et al.*, 2003). Also, recent molecular studies on unionids have found rampant polyphyly in the currently used genera (Lydeard *et al.*, 1996), which suggests that margaritiferid genera would benefit from further investigation.

A close phylogenetic relationship between the unionoid mussels and trigonioid bivalves has been suggested based on shell features (e.g. Newell, 1965), and has recently received support from sperm characters (Healy, 1989, 1996) and molecular data (Hoeh *et al.*, 1998; Graf & Ó Foighil, 2000a; Giribet & Wheeler, 2002; Giribet & Distel, 2003; Roe & Hoeh, 2003). However, the phylogenetic affinities of margaritiferids with other unionoids, as well as their internal affinities, are poorly understood. The phylogenetic relationships of the Margaritiferidae must be better investigated before conclusions can be made about its biogeographical history.

In this paper, sequences from the nuclear ribosomal genes 18S rRNA and 28S rRNA, the nuclear protein-coding gene histone H3, and the mitochondrial genes 16S rRNA and cytochrome *c* oxidase subunit I are used to analyse phylogenetic relationships within the family Margaritiferidae. Sequence data representing seven margaritiferid species and all currently recognized genera (*Margaritifera, Cumberlandia* and *Dahurinaia*) are included in the study. Sequences from three unionid species and two trigoniid species serve as outgroups.

MATERIAL AND METHODS

Specimens

A total of 15 specimens of margaritiferids (plus one sequence from GenBank), belonging to seven species were analysed in this study (see Figure 1 for the species distributions, Table 1 for taxonomic status, and Appendix 1 for localities and voucher numbers). These represent all three genera generally recognized as belonging to Margaritiferidae (as well as the alternative three genera recognized by Smith, 2001). Outgroups were selected among the Unionidae and the Trigoniidae, agreeing with current phylogenetic understanding of the Palaeoheterodonta (e.g. Giribet & Wheeler, 2002). All the material was collected alive and either fixed in 96% EtOH or frozen and kept at –80°C. We were not able to obtain samples from the American populations of *Margaritifera margaritifera* or from *M. hembeli.*

DNA sequence data

Genomic DNA samples were obtained from ethanol-preserved tissues using the Dneasy™ Tissue Kit from QIAGEN. The 18S rRNA gene was PCR-amplified in three overlapping fragments of size approximately of 950, 900 and 850 bp each. Primers used in amplification and sequencing (1F-4R, 3F-18Sbi and 18Sa2.0–9R) were described elsewhere (Giribet *et al.*, 1996; Whiting *et al.*, 1997). The 28S rDNA D3 fragment was amplified and sequenced using primers 28Sa and 28Sb (Whiting *et al.*, 1997). Histone H3 was amplified and sequenced using primers H3a F and H3a R (Colgan *et al.*, 1998). The mitochondrial gene fragments of cytochrome *c* oxidase subunit I (COI) and 16S rRNA were amplified using primer pairs LCO1490 and HCO2198, and 16Sar and 16Sb, respectively (Xiong & Kocher, 1991; Folmer *et al.*, 1994).

Amplifications were carried out in a 50 μl reaction volume, with 1.25 units of AmpliTaq® DNA Polymerase (Perkin Elmer), 15 mM $MgCl_2$, 200 μM of dNTPs and 1 μM of each primer. The PCR program consisted of an initial denaturing step at 94°C for

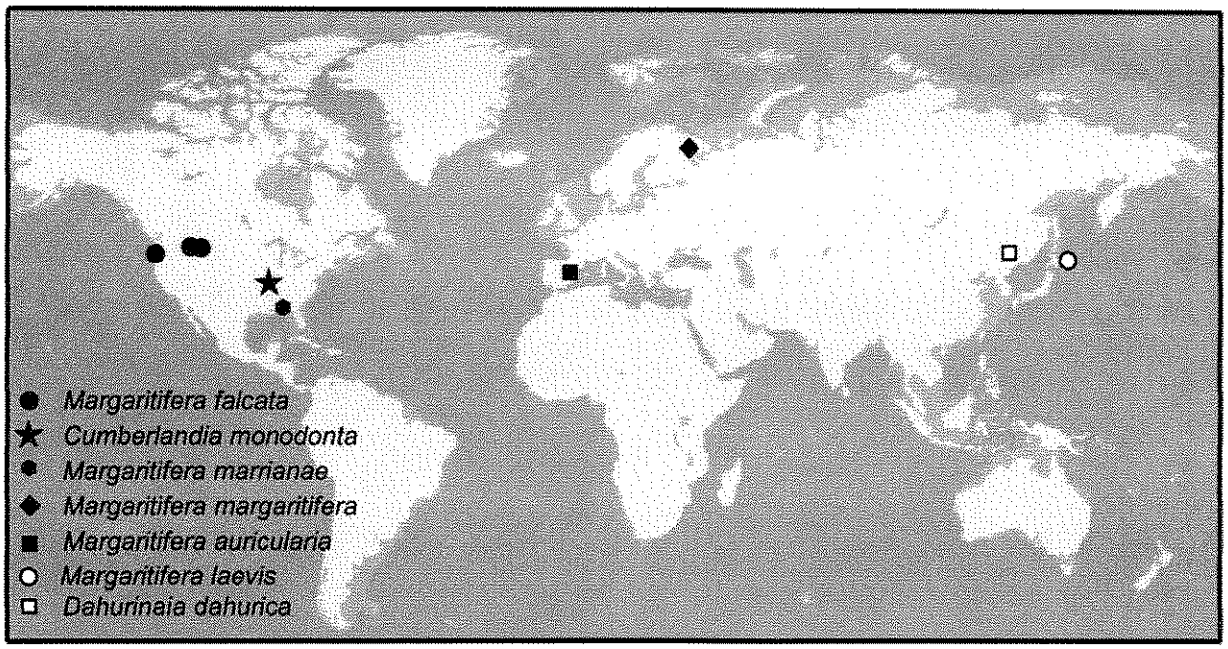

Figure 1. Map indicating the sampled localities for the margaritiferid species included in this study.

Table 1. Taxon sampling used in the analyses with MCZ DNA catalogue number and GenBank accession codes.

	Catalogue no.	18S rRNA	28S rRNA	Histone H3	COI	16S rRNA
Trigonioidea						
Trigoniidae						
Neotrigonia margaritacea	DNA1000031	AF411690*	AF411689*	AY070155*	U56950*	
Neotrigonia bednalli		AF120538*				
Unionoidea						
Unionidae						
Potomida littoralis	DNA100133	AF120652*	AF120599*		AF120536*	
Lampsilis cardium	DNA100159	AF120537*	AF120600*		AF120653*	
Anodonta sp.	DNA100644	AY579090	AY579106	AY579132	AY579122	
Margaritiferidae						
Dahurinaia dahurica	DNA100683	AY579091	AY579107	AY579133	AY579123	
Margaritifera laevis	DNA100685	AY579092	AY579108	AY579134	AY579124	AY579081
Margaritifera laevis	DNA100686	AY579093	AY579109			
Margaritifera laevis	DNA100687	AY579094	AY579110	AY579135		
Margaritifera laevis	DNA100689	AY579095	AY579111			
Margaritifera auricularia	DNA100672	AY579096	AY579112	AY579136		AY579082
Margaritifera auricularia	DNA100674	AY579097	AY579113	AY579137	AY579125	AY579083
Margaritifera falcata	DNA100670	AY579098	AY579114	AY579138	AY579126	
Margaritifera falcata	DNA100671	AY579099	AY579115	AY579139		
Margaritifera falcata	DNA100699	AY579100	AY579116	AY579140	AY579127	AY579084
Margaritifera falcata	DNA100844	AY579101	AY579117	AY579141	AY579128	AY579085
Margaritifera margaritifera	DNA100694	AY579102	AY579118	AY579142	AY579129	AY579088
Margaritifera margaritifera	DNA100697	AY579103	AY579119	AY579143	AY579130	AY579087
Margaritifera margaritifera		AF229612*				
Margaritifera marrianae	DNA100695	AY579104	AY579120			AY579086
Cumberlandia monodonta	DNA100863	AY579105	AY579121	AY579144	AY579131	AY579089

Sequences indicated with an asterisk were obtained from GenBank.

60 s, 35 amplification cycles (94°C for 15 s, 46°C for 15 s, 72°C for 15 s), and a final step at 72°C for 6 min in a GeneAmp® PCR System 9700 (Perkin Elmer). The annealing temperature to amplify the protein-encoding genes was 42–44°C.

PCR amplified samples were purified with the QIAquick® PCR Purification Kit from QIAGEN, and sequenced directly using an automated ABI Prism® 3100 Genetic Analyser. Cycle-sequencing with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer) using dye-labelled terminators (ABI PRISM™ BigDye™ v. 3 Terminator Cycle Sequencing Ready Reaction Kit) was performed in a GeneAmp® PCR System 9700 (Perkin Elmer) following the manufacturer's protocol. The BigDye-labelled PCR products were cleaned with AGTC® Gel Filtration Cartridges (Edge BioSystems).

Electropherograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher™ 4.0. Complete sequences were then edited in GDE (The Genetic Data Environment) (Smith *et al.*, 1994), where they were split according to primer delimited regions and secondary structure features (following Giribet 2001, 2002), when necessary.

The molecular loci used in this study are as follows: (1) 18S rRNA was sampled for 21 specimens, and the total length ranges between 1,765 bp in some of the outgroups and 1,776 bp in the ingroup; (2) the D3 expansion fragment of 28S rRNA for 19 specimens, of a total length ranging between 301 bp in *Lampsilis cardium* and 312 bp in *Anodonta* sp.; (3) a fragment of 327 bp of histone H3 was sequenced for 14 terminal taxa, and analysed as a pre-aligned fragment; (4) a 656 bp fragment of COI was used for 13 individuals; and (5) 16S rRNA was used for nine margaritiferid specimens, of a total length ranging between 495 and 498 bp. In total, we have generated up to *c.* 3.5 kb of unaligned sequence data per complete terminal, including multiple representatives for four of the seven sampled margaritiferid species. All the new sequences have been deposited in GenBank under accession codes AY579090 to AY579144 (see Table 1). Vouchers for all the specimens and repository institutions are listed in Appendix 1.

Analytical methods

Molecular data were analysed using the direct optimization method (Wheeler, 1996) as implemented in the computer program POY v. 3.0 (Wheeler, Gladstein & DeLaet, 2002). Eight molecular partitions were analysed independently, including each of the five loci and three combinations of loci: (1) nuclear ribosomal genes (18S rRNA + 28S rRNA); (2) mitochondrial genes (COI + 16S rRNA); and (3) all molecular data (18S rRNA + 28S rRNA + H3 + COI + 16S rRNA). A parameter space of two variables (gap/change ratio and transversion/transition ratio) was explored (Wheeler, 1995; Giribet, 2003). A total of 15 parameter sets were analysed per partition; gap/change ratio values of 1, 2 and 4 were explored ('change' refers to the highest value for a base transformation, i.e. the transversions), as well as transversion/transition ratios of 1 (equal weights), 2 (transversions receive twice as much weight as transitions), 4, 8 and infinity (transversion parsimony). Therefore, 120 independent analyses were run (8 partitions ×15 parameter sets). The tree lengths for these analyses are summarized in Table 2.

The POY analyses were run in a Beowulf cluster of 38 processors at 1–2.4 GHz at Harvard University (darwin.oeb.harvard.edu). Processes were executed in parallel using pvm and the parallel version of POY (parallel commands -parallel -dpm -jobspernode 2 in effect). The search strategy consisted of

Table 2. Tree length for the individual (18S, 18S rRNA; 28S, 28S rRNA; H3, histone H3; 16S, 16S rRNA; and COI) and combined [RIB (18S + 28S); MIT (16S + COI); MOL (18S + 28S + H3 + 16S + COI)] datasets at different parameter values, and ILDs for the combined analyses of all data, at parameter sets 110 to 481.

Parameter sets	18S	28S	RIB	H3	16S	COI	MIT	MOL	ILD
110	74	50	132	41	27	193	221	400	0.0375
111	181	78	273	101	98	551	650	1030	**0.0204**
121	255	128	406	142	124	749	873	1434	0.0251
141	401	224	664	224	178	1135	1313	2228	0.0296
181	693	416	1180	388	286	1907	2193	3816	0.0330
210	86	81	183	41	36	193	231	461	0.0521
211	195	113	330	101	107	551	658	1095	0.0256
221	280	197	515	142	144	749	895	1565	0.0339
241	450	359	878	224	218	1135	1357	2487	0.0406
281	790	683	1606	388	362	1907	2281	4332	0.0466
410	108	139	280	41	50	193	245	577	0.0797
411	219	172	432	101	121	551	672	1212	0.0396
421	328	314	719	142	172	749	922	1796	0.0507
441	546	591	1280	224	274	1135	1413	2951	0.0613
481	982	1147	2400	388	474	1907	2393	5260	0.0688

The ILD number in bold reflects the minimum incongruence among data sets.

100 random addition replicates using subtree pruning and regrafting (SPR) and tree bisection and reconnection (TBR) branch swapping. Nodal support was assessed with 1,000 jackknife replicates (Farris *et al.*, 1996), a measure comparable to bootstrap support but more conservative (for a clear comparison of both measures, see Farris, 1997).

Congruence was used as a meta-optimality criterion for choosing the combined analysis that minimized overall incongruence among partitions (Wheeler, 1995). However, a more conservative estimate of the phylogenetic hypothesis is also presented via the strict consensus of all the parameter sets. Congruence among partitions (morphological and molecular) was measured by the incongruence (ILD) metric (Mickevich & Farris, 1981; Farris *et al.*, 1995), calculated by dividing the difference between the overall tree length and the sum of its data components by the overall length: ILD = [$\text{length}_{18S+28S+H3+16S+COI}$ − (length_{18S} + length_{28S} + length_{H3} + length_{16S} + length_{COI})]/$\text{length}_{18S+28S+H3+16S+COI}$.

In order to show the amount of evolutionary change accumulated on each branch, for the combined analysis of all data under the optimal parameter set (111) we generated an implied alignment (Wheeler, 2003) and used that alignment to draw a phylogram in PAUP* (Swofford, 2002). Although several fundamental trees result in the strict consensus tree presented in Figure 5A, one of those is chosen arbitrarily to represent the branch lengths separating each species. We also calculated bootstrap support (Felsenstein, 1985) with 1,000 full heuristic replicates using this implied alignment in PAUP*, each replicate consisting of a single random addition and limiting the number of trees to 10.

RESULTS

Congruence analysis

The parameter set that minimizes incongruence between the five separate partitions is 111 (ILD = 0.0204), for which all transformations are equally weighted (Table 2). This parameter set will therefore be referred to as the 'optimal parameter set', and the trees obtained under it, the 'optimal trees'. In all cases, for all partitions analysed (the five partitions plus all the partition combinations: ribosomal, mitochondrial and molecular) and for all parameter sets, 100% of replicates yielded trees of minimal length. All the data files, output (= results) and error (= analytical parameters employed, files used, and searches) files can be downloaded from the following url: http://www.mcz.harvard.edu/Departments/InvertZoo/giribet_data.htm.

Nuclear ribosomal genes (Fig. 2)

The combined analysis of the nuclear ribosomal genes (18S rRNA and D3 region of the 28S rRNA) showed much agreement among all the parameter sets. The strict consensus of 29 shortest trees (Fig. 2B) resolved the same nodes found within the family Margaritiferidae for the optimal parameter set (111), and therefore all relationships found are parameter-independent. Analysis for parameter set 111 (Fig. 2A) yielded eight shortest trees of length 273. All these trees supported the monophyly of the Margaritiferidae (100% jackknife support) and the monophyly of all the species represented by more than one specimen with the exception of *M. margaritifera*. Jackknife values for the monophyly of each one of the species are above 90%. Relationships among species receive lower values, except for *M. marrianae* + *M. laevis*, which has a 94% jackknife value.

For the outgroup relationships, the family Unionidae (*Lampsilis* (*Potomida*, *Anodonta*)) is not resolved in the strict consensus although it is obtained under the best parameter set and receives a jackknife support value of 96% in the optimal tree. In this case, Unionidae is sister to Margaritiferidae, this relationship receiving a jackknife support of 100%.

When the two nuclear ribosomal genes are analysed independently, the 28S rRNA fragment yields no resolution while 18S rRNA gives results almost identical to those of the combined nuclear ribosomal genes.

Histone H3 (Fig. 3)

The strict consensus of the 312 trees obtained under all parameter sets (Fig. 3B) for this nuclear protein-coding gene resolved fewer nodes than the ribosomal genes; only *M. auricularia* and *M. margaritifera* were each resolved on this tree. The other two species represented by more than one individual, *M. laevis* and

M. falcata, were not resolved as monophyletic. The family Margaritiferidae is not resolved as monophyletic in the strict consensus of all parameter sets. However, the strict consensus of 32 optimal trees for parameter set 111 (101 steps; Fig. 3A) did resolve the monophyly of Margaritiferidae (jackknife value of 69%) along with the species *M. auricularia* and *M. margaritifera*. Supraspecific clades supported by the analysis under the optimal parameter set are those including (*Cumberlandia, M. falcata, M. auricularia* and *M. laevis*), and its subclade including (*M. falcata, M. auricularia* and *M. laevis*), the former receiving a jackknife value of 67%, and the latter a value below 50%.

Mitochondrial genes (Fig. 4)

The strict consensus of the 128 shortest trees obtained for the combined analyses of the mitochondrial genes 16S rRNA and COI under all parameter sets (Fig. 4B) supported the monophyly of the species *M. falcata*, and the two clades (*M. falcata, M. marrianae, M. laevis*) and (*D. dahurica, M. margaritifera*). The consensus supported neither the monophyly of Margaritiferidae nor Unionidae. For the parameter set that minimized overall incongruence (111) the strict consensus of the two optimal trees for the mitochondrial genes (650 steps; Fig. 4A) resolved monophyletic Unionidae and Margaritiferidae. The mitochondrial data supports monophyly of each of the species represented by more than one specimen with jackknife values ranging between 71 and 100%. This tree also supports a clade uniting all the margaritiferid species except *Cumberlandia* and *M. auricularia*, and another clade composed of *M. laevis* + *M. marrianae*, in addition to the clades supported in the strict consensus of all parameter sets.

The independent analyses for each of the mitochondrial genes (results not shown) support monophyly of each species

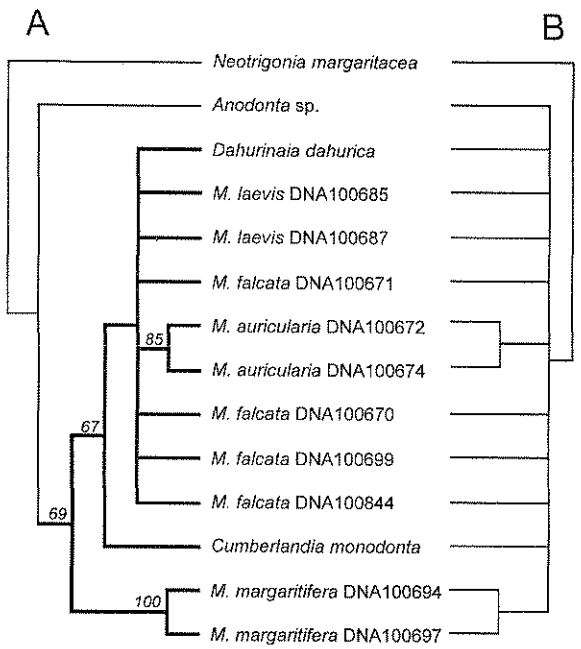

Figure 3. Cladograms based on the analyses of the histone H3 partition. **A.** Strict consensus of 32 shortest trees at 101 steps obtained for the most congruent parameter set (111). **B.** Cladogram at right is the strict consensus for all 15 parameter sets. Thicker branches in the left tree indicate margaritiferid species; numbers on branches indicate jackknife support values.

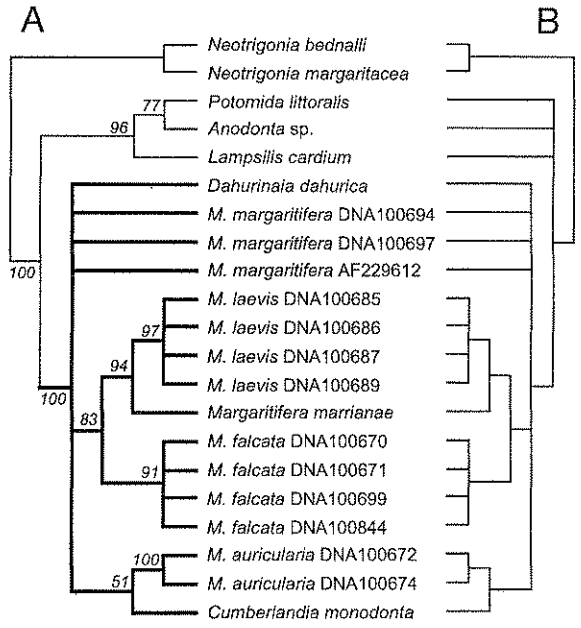

Figure 2. Cladograms based on the analyses of the nuclear ribosomal partition (18S rRNA + 28S rRNA). **A.** Strict consensus of eight shortest trees at 273 steps obtained for the most congruent parameter set (111). **B.** Cladogram at right is the strict consensus for all 15 parameter sets. Thicker branches in the left tree indicate margaritiferid species; numbers on branches indicate jackknife support values.

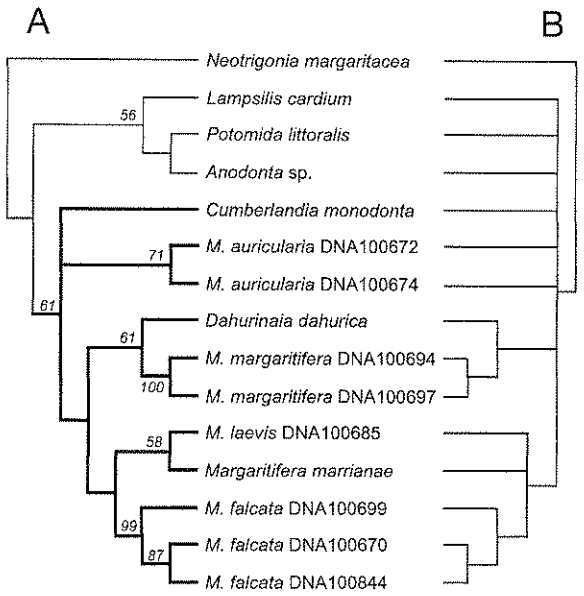

Figure 4. Cladograms based on the analyses of the mitochondrial partition (16S rRNA + COI). **A.** Strict consensus of two shortest trees at 650 steps obtained for the most congruent parameter set (111). **B.** Cladogram at right is the strict consensus for all 15 parameter sets. Thicker branches in the left tree indicate margaritiferid species; numbers on branches indicate jackknife support values.

represented by more than one specimen for all explored parameter sets. The COI data also supports monophyly of Unionoidea, Unionidae and Margaritiferidae under all conditions, and supports a stable relationship between *D. dahurica* and *M. margaritifera*, although jackknife support for this clade is low (59%). For the 16S rRNA, tree relationships are dependent on subjective rooting (we were unable to sequence 16S rRNA for outgroup taxa). The unrooted topology of the 16S rRNA data is compatible with a first split between *Cumberlandia* + *M. auricularia* and the remaining species, as suggested by other molecular partitions.

Combined molecular data (Figs 5, 6)

The combined analysis of all molecular data shows monophyly of many of the clades supported in most of the other molecular data sets. The Trigoniidae, Unionoidea, Unionidae and Margaritiferidae are each monophyletic in all analyses performed so far, and there is much agreement between the strict consensus of all parameter sets (Fig. 5B) and the strict consensus of the 12 most parsimonious trees for the best parameter set (Fig. 5A). One of these 12 trees is illustrated, with the amount of change accumulated on each branch, in Figure 6. This analysis also shows monophyly of all the species represented by multiple individuals, with the exception of the widespread species *M. margaritifera* that includes *Dahurinaia dahurica* in some of the most parsimonious trees. The clades (*M. falcata* (*M. marrianae, M. laevis*)) are found under all parameter sets (with jackknife values of 86 and 100%, respectively; bootstrap values are 100% for each). The strict consensus of all parameter sets does not resolve other clades within Margaritiferidae, but the optimal parameter set suggests a first division between *Cumberlandia* + *M. auricularia* and the remaining margaritiferid species. This analysis also supports monophyly of (*D. dahurica* + *M. margaritifera*) and a sister group relationship of this clade to the group (*M. falcata, M. marrianae, M. laevis*). The analysis under the optimal parameter set yielded 12 trees of length 1030 that were hit 100 times over 100 replicates.

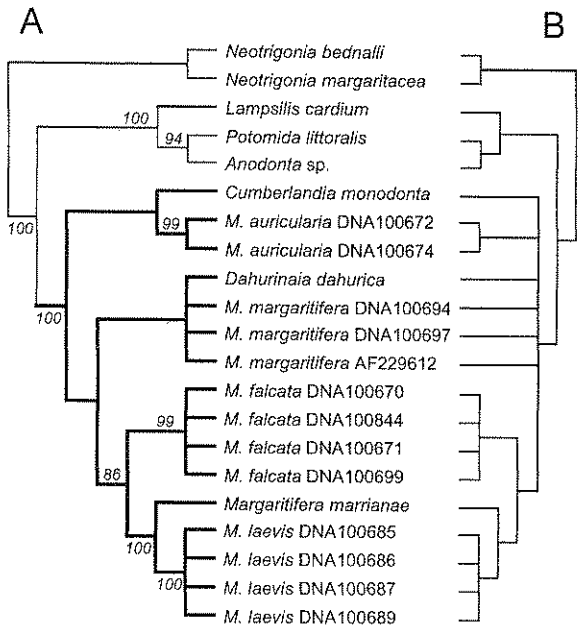

Figure 5. Cladograms based on the analyses of the combined molecular data. **A.** Strict consensus of 12 shortest trees at 1,030 steps obtained for the most congruent parameter set (111). **B.** Cladogram at right is the strict consensus for all 15 parameter sets. Thicker branches in the left tree indicate margaritiferid species; numbers on branches indicate jackknife support values.

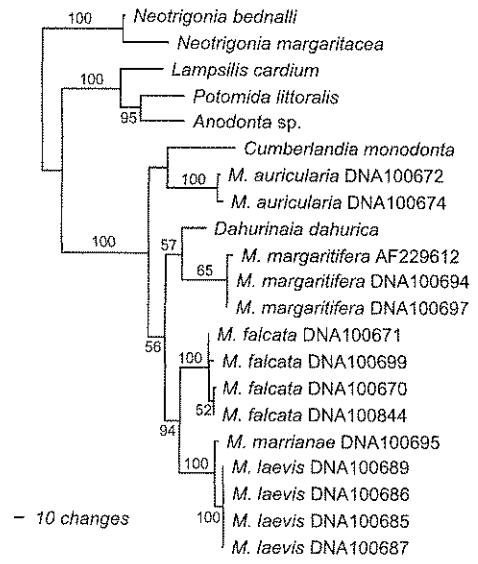

Figure 6. One of 12 shortest trees at 1,030 steps obtained after the implied alignment generated with POY is analysed in PAUP. Branch lengths are proportional to the number of evolutionary steps. Numbers on branches indicate bootstrap support values.

DISCUSSION

The data presented here show that the represented Unionoidea split into two main clades, namely the families Unionidae and Margaritiferidae. These results are consistent with previous analyses of higher relationships of the Bivalvia and Palaeoheterodonta (e.g. Hoeh *et al.*, 1998; Graf, 2000; Graf & Ó Foighil, 2000a; Giribet & Distel, 2003; Roe & Hoeh, 2003). Besides these studies that focused on higher-level relationships involving some margaritiferid species, the only one that has explored relationships within the Margaritiferidae is that of the two endangered European *Margaritifera, M. auricularia* and *M. margaritifera* (Machordom *et al.*, 2003). The most inclusive sampling of that study included representatives of the two European species plus one sequence of the North American species *Cumberlandia monodonta*, using one unionid species as outgroup. Machordom *et al.* (2003) analysed sequences from the mitochondrial genes 16S rRNA and COI, therefore their results are directly comparable with our mitochondrial partition (Fig. 4). In their tree the two species of *Margaritifera* are sister taxa to the exclusion of *Cumberlandia* (Machordom *et al.*, 2003: fig. 3). Lydeard *et al.* (1996) tested monophyly of Margaritiferidae in their 16S rRNA study, and also found *Cumberlandia* to be the sister group to *Margaritifera*, which included the species *M. margaritifera* and *M. falcata*. The results from both previous studies are consistent with our analyses based on COI data, but not with the ones based on the 16S rRNA partition. When the mitochondrial data are combined, our optimal parameter set yields two most parsimonious cladograms, one supporting a sister-group relationship of *Cumberlandia* to the remaining margaritiferids, and another supporting *Cumberlandia* as sister group to *M. auricularia*. This relationship of *C. monodonta* and *M. auricularia* has

already been suggested by Ziuganov *et al.* (1994: fig. 14). It is important to notice, however, that irrespective of the position of *Cumberlandia*, the mitochondrial data do not support monophyly of *Margaritifera*, because *Dahurinaia* nests within *Margaritifera* in all the analyses. Our data also reject monophyly of *Dahurinaia* as a subgenus as accepted by Ziuganov *et al.* (1994).

The other partitions also show that *Margaritifera* is not monophyletic. The ribosomal partition supports a clade consisting of *Cumberlandia* and *M. auricularia*, while another clade includes the North American species *M. marrianae*, *M. falcata* and the East Asian *M. laevis*. Histone H3 data support (although only for the best parameter set) a clade containing *M. falcata*, *M. auricularia*, *M. laevis* and *Dahurinaia* (*M. marrianae* was not sequenced for this locus). This result, with all the margaritiferids except *Cumberlandia* and *M. auricularia* forming a clade, is therefore obtained from the mitochondrial data, from the histone H3 partition, and from some of the shortest trees for the ribosomal partition. Not surprisingly this result is also found for the combined analysis of all data under several parameter sets (Fig. 7). Indeed, the monophyly of all margaritiferids except *Cumberlandia* and *M. auricularia* is found under seven parameter sets, and eight parameter sets support monophyly of *Dahurinaia* + *M. marrianae* + *M. falcata* + *M. margaritifera* + *M. laevis*, with the exception of the *M. margaritifera* AF229612 from GenBank (Fig. 7). This is probably so because the GenBank sequence is shorter and has several apomorphic changes. The relationship between *Cumberlandia*, *M. auricularia* and the remaining sampled margaritiferids cannot be resolved with certainty, because three topologies are obtained: ((*Cumberlandia* + *M. auricularia*) other); (*Cumberlandia* (*M. auricularia* + other)); (*M. auricularia* (*Cumberlandia* + other)). However, it seems that a clade of margaritiferids including at least the genus *Dahurinaia*, *Margaritifera margaritifera*, *M. marrianae*, *M. falcata* and *M. laevis* is found under most analytical parameters and partitions.

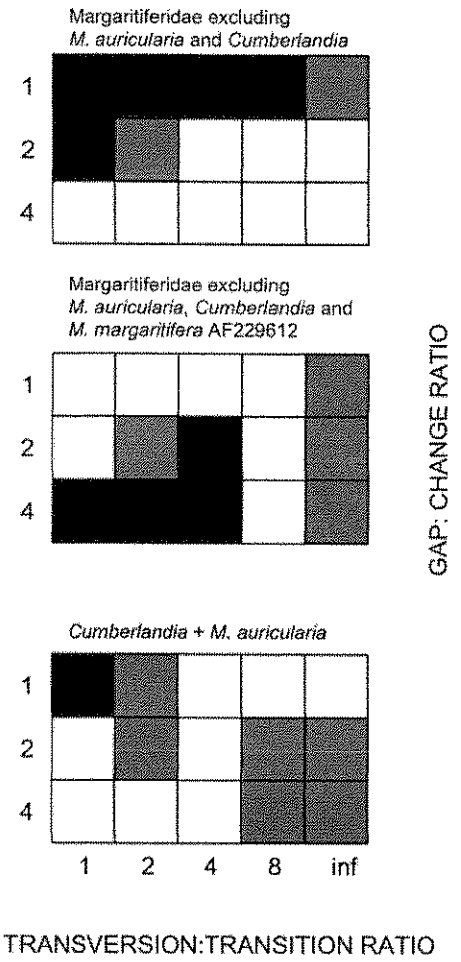

Figure 7. Graphic plots of sensitivity analyses (Navajo rugs) for combined molecular data for the clades indicated above each graph. Black squares indicate monophyly of indicated clade under gap cost and transversion: transition ratio shown along the axes; white squares indicate non-monophyly; grey squares indicate monophyly in some of a set of equally parsimonious resolutions.

Taxonomic implications

Our data suggest that the generic designations utilized in this study are not appropriateate, because the genus *Margaritifera* is not monophyletic and includes the genus *Dahurinaia*, as already proposed by Haas (1969). Furthermore, the monotypic genus *Cumberlandia* is nested within *Margaritifera* in most analyses, as suggested by Ziuganov *et al.* (1994) (see also Davis & Fuller, 1981). Alternative nomenclature, such as the one recently proposed by Smith (2001) does not match the relationships proposed here either. This may parallel the 'prodigious polyphyly' found in other unionoid genera (Lydeard *et al.*, 2000), indicating that unionoid taxonomy is in need of phylogenetic revision.

The only species that does not appear monophyletic under certain circumstances for the combined analysis of all data is *M. margaritifera*. Our study includes two samples from the Varzuga River from the Kola Peninsula (Russia) sequenced for this study and a third sample from the same locality, sequenced only for 18S rRNA from GenBank. The monophyly of *M. margaritifera*, including samples from its entire distribution range, was supported by the analyses of Machordom *et al.* (2003), and given the morphological and molecular evidence we consider the species to be monophyletic. In our case, the failure to obtain monophyly of the species is probably due to some apomorphies observed in the 18S rRNA sequence deposited in GenBank (AF229612).

An obvious alternative is that the data presented do not accurately reconstruct the phylogeny of Margaritiferidae and that some of the previous taxonomies reflect the cladistic relationships of the margaritiferid species. While this is a possibility, we would argue that the data reconstruct certain well-corroborated nodes accurately (i.e. Unionidae, Margaritiferidae, *M. auricularia*, *M. falcata* and *M. laevis* all are stable to parameter set variation and receive jackknife values between 99 and 100%). Therefore, at least the clades [*M. falcata* (*M. marrianae* + *M. laevis*)] should be considered accurate under the same criteria (nodal support and nodal stability *sensu* Giribet, 2003). Also, molecular data have been used in several studies of unionoid phylogeny and phylogeography (e.g. Lydeard *et al.*, 1996; Hoeh *et al.*, 1998; Graf & Ó Foighil, 2000a; Lydeard *et al.*, 2000; Roe, Hartfield & Lydeard, 2001; Machordom *et al.*, 2003; Serb, Buhay & Lydeard, 2003) and they have proven to be informative at many levels.

Generic designations within the Margaritiferidae have remained contentious. The genus *Dahurinaia* Starobogatov, 1970 has been dismissed by many modern workers (e.g. Smith, 1980; Ziuganov *et al.*, 1994), while the monotypic genus *Cumberlandia* has been accepted by most authors. Smith (2001: 40–43) provided the first modern attempt to revise and diagnose the margaritiferid genera, recognizing *Pseudunio* Haas, 1910, *Margaritifera* Schumacher, 1816, and *Margaritinopsis* Haas, 1912 as

Figure 8. Summary cladogram for Margaritiferidae based on the analysis for the optimal parameter set, and area cladogram of the represented specimens. The asterisk indicates that some populations of this species, not sampled in this study, occur in eastern North America (NA).

valid genera. However, the diagnoses of the genera rely strongly on conchological characters which show considerable variation among the included species. For example, *Margaritinopsis* is diagnosed by '. . . a single conchological character, reduction of the left pseudocardinal tooth, especially the anterior cusp' (Smith, 2001: 41), although Smith continues by stating that 'In a few of the included species, *M. falcata* and *M. laevis*, the reduction of the left anterior cusp is slight to moderate, whereas in other forms, e.g. *M. dahurica*, *M. monodonta*, and *M. laosensis*, the reduction is extensive and includes the complete loss of the cusp' (Smith, 2001: 43). Following that discussion differences in the adult dentition between the species of *Margaritinopsis* are also listed. None of the other characters used to designate the genera are free of homoplasy. Given the results of our analyses and the lack of clear conchological or anatomical synapomorphies supporting any current generic designations, using the generic name *Margaritifera* for all the living species of the Margaritiferidae may need to be considered.

Biogeographic implications

Besides the fact that the Margaritiferidae is a group with Laurasian distribution, little can be concluded about the biogeographic implications of the results here obtained. Figure 8 illustrates the optimal tree for the combined analysis of all data, where we have plotted the geographical area where the represented species occur. Three of the four areas represented have redundant distributions, and all five internal nodes involve geographic paralogy (*sensu* Nelson & Ladiges, 1991). No unambiguous three-area statements can be made. Even when only the nodes that are stable to parameter variation and receive high jackknife support are considered, no unambiguous biogeographic pattern emerges. A complicated pattern of biogeography was derived from the topologies obtained with three independent Old World + New World clades (and more events may be implied when the rest of the species are evaluated). The relationships between *M. laevis* and the North American species *M. marrianae* and *M. falcata* could be explained because easternmost Siberia and Alaska share faunal similarity due to Beringian land bridges. However, this only explains one possible relationship. It is difficult to determine whether this pattern is a reflection of extinction and contraction of an ancient, formerly widespread margaritiferid fauna, peripheral isolation of formerly widespread taxa, fish host dispersal or even host switching.

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APPENDIX 1

Voucher data for specimens used in molecular analyses and repository institutions for DNA samples, tissues and shells: BMNH, The Natural History Museum (London, UK); DGC, Daniel L. Gustafson Collection (Bozeman, Montana, USA); MCZ, Museum of Comparative Zoology (Cambridge, Massachusetts, USA); UAUC, University of Alabama Unionid Collection (Tuscaloosa, Alabama, USA).

Neotrigonia bednalli (Verco, 1907)
5 km NE of Rottnest Island, Western Australia; January 1996; BMNH Accession No. 2388.

Neotrigonia margaritacea (Lamarck, 1804)
D'Entrecasteau Channel, Tasmania, Australia; 19 April 2000; MCZ DNA1000031 (COI sequence data from GenBank accession code U56850).

cf. *Potomida littoralis* (Lamarck, 1801)
Llac de Banyoles, Girona, Spain; 29 April 1997; MCZ DNA100133.

Lampsilis cardium (Rafinesque, 1820)
MCZ DNA100159.

Anodonta sp.
42°04′45″N, 8°25′50″W, Rio Miño, Salvaterra de Miño, Pontevedra, Spain; 11 September 2002; MCZ DNA100644.

Cumberlandia monodonta (Say, 1829)
36°33′N, 83°03′W, Kyles Ford, River Mile 189.6, Clinch River, Hancock County, Tennessee, USA; MCZ DNA100863; UAUC 007.

Dahurinaia dahurica (Middendorff, 1850)
Komissarovka River, near Bara Bash-Levada village, Primorye Territory, Russia; 9 May 1998; MCZ DNA100683; UAUC 320.

Margaritifera auricularia (Spengler, 1793)
Ebro River, near Bítem, Tarragona, Spain; 2002; MCZ DNA100672 and MCZ DNA100674.

Margaritifera falcata (Gould, 1850)
45°17′41″N, 114°35′46″W, Middle Fork, Salmon River, Lemhi County, Idaho, USA; 1 September 2001; MCZ DNA100670; DGC W1528.
44°42′12″N, 114°02′33″W, Deer Gulch, Salmon River, Lemhi Co., Idaho, USA; 1 September 2001; MCZ DNA100671; DGC W1529.
North Umpqua River, Douglas Co., Oregan, USA; 24 June 1997; MCZ DNA100699.
44°13′12″N, 111°22′20″W, Buffalo River at US Highway 20 bridge, Fremont Co., Idaho, USA; 28 September 2002; MCZ DNA100844; DGC G1170.
Information about the collection sites can be found at http://www.esg.montana.edu/cgi-bin/aimss?g1170, 1528 and 1529.

Margaritifera laevis (Haas, 1910)
Burevestnik village, Kasatka Bay, Iturup Island, Kuril Islands, Russia; 30 July 1998 MCZ DNA100685; UAUC 324.
Ostrovnaya River, Del'fin Bay, Shikotan Island, Kuril Islands, Russia; 15 August 1998; MCZ DNA100686 (UAUC 325), DNA100687 (UAUC 326) and DNA100689 (UAUC 328).

Margaritifera margaritifera (Linnaeus, 1758)
Varzuga River, Kola Peninsula; Russia; 15 August 1997; MCZ DNA100694 (UAUC 1684) and DNA100697 (UAUC 3228) (18S rRNA sequence data from GenBank accession code AF229612, same collecting data).

Margaritifera marrianae Johnson, 1983
31°26′12″N, 87°01′12″W, Hunter Creek, Conecuh River, Conecuh Co., Alabama, USA; 22 April 1997; MCZ DNA100695; UAUC 1651.