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Molecular phylogenetic analysis of tropical freshwater mussels (Mollusca: Bivalvia: Unionoida) resolves the position of *Coelatura* and supports a monophyletic Unionidae

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ABSTRACT

In previous molecular phylogenetic analyses of the freshwater mussel family Unionidae (Bivalvia: Unionoida), the Afrotropical genus *Coelatura* had been recovered in various positions, generally indicating a paraphyletic Unionidae. However that result was typically poorly supported and in conflict with morphology-based analyses. We set out to test the phylogenetic position of *Coelatura* by sampling tropical lineages omitted from previous studies. Forty-one partial 28S nuclear rDNA and partial COI mtDNA sequences (1130 total aligned nucleotides) were analyzed separately and in combination under both maximum parsimony and likelihood, as well as Bayesian inference. There was significant phylogenetic incongruence between the character sets (partition homogeneity test, $p < 0.01$), but a novel heuristic for comparing bootstrap values among character sets analyzed separately and in combination illustrated that the observed conflict was due to homoplasy rather than separate gene histories. Phylogenetic analyses robustly supported a monophyletic Unionidae, with *Coelatura* recovered as part of a well-supported Africa–India clade (= Parreysiinae). The implications of this result are discussed in the context of Afrotropical freshwater mussel evolution and the classification of the family Unionidae.

1. Introduction

Freshwater mussels of the family Unionidae (Bivalvia: Unionoida) are well known for their complex life histories (i.e., parental care and larval parasitism) (Barnhart et al., 2008; Wächtler et al., 2001), valuable ecosystem functions (Cummings and Graf, 2009; Strayer et al., 1994; Vaughn et al., 2004), and imperiled conservation status (Lydeard et al., 2004; Strayer, 2006). The Unionidae is also among the most species-rich families of bivalves, freshwater or otherwise. The order Unionoida is composed of more than 840 species in 6 families, but the family Unionidae alone accounts for some 674 species (80%) and is broadly distributed across temperate North America and Eurasia as well as tropical Mesoamerica, Africa, and southeastern Asia (Graf and Cummings, 2007a). However, this copious diversity is less the product of a species radiation within a well defined taxon and more a consequence of our lack of success in discovering the branching pattern of the basal unionid lineages that would facilitate further subdivision of the family. Furthermore, phylogenetic analyses over the last decade have routinely recovered the Unionidae as paraphyletic (reviewed in Graf and Cummings, 2006a), but those results have been poorly supported and based upon inadequate taxon and character sampling. The objective of this paper is to test the monophyly of the Unionidae by resolving the phylogenetic position of the problematic Afrotropical unionid genus *Coelatura* Conrad, 1853.

There have been several phylogenetic studies of the Unionidae since the first 16S mtDNA phylogeny by Lydeard et al. (1996), but most analyses restricted ingroup sampling to species of the Unionidae (i.e., unionid monophyly was implicit). Only two molecular character sets have been applied to test family-group level relationships among the Unionoida: cytochrome oxidase subunit I (COI) from the mitochondrion (Bogan and Hoeh, 2000; Graf and Ó Foighil, 2000a; Hoeh et al., 2002; Hoeh et al., 2001; Roe and Hoeh, 2003; Walker et al., 2006) and the large nuclear ribosomal subunit (28S) rDNA (Graf, 2002). Phylogenetic analyses of nuclear rDNA have supported unionid monophyly, but COI analyzed independently has routinely recovered a paraphyletic Unionidae with the African *Coelatura aegyptiaca* placed as sister to a clade composed of the remainder of the sampled unionid species and the Margaritiferidae [i.e., (*Coelatura*, (Margaritiferidae, Unionidae))]. When Graf and Cummings (2006a) combined these two published molecular character sets, the problem of *Coelatura* was exacer-

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bated, and the topological instability precipitated by that species' single published COI sequence justified its removal from their analyses. The resultant ambiguity in the position of problematic taxa like *Coelatura* has contributed to disagreement over the family-group level relationships of the Unionoida, and the observation by Hoeh et al. (2009) that the phylogenetic data available to-date are insufficient to deduce any meaningful conclusions about freshwater mussel evolution. Given the importance of accurate phylogenetic reconstructions to comparative ecological research (e.g., Cadotte et al., 2010; Clarke and Warwick, 1998) and conservation biology (Cadotte and Davies, 2010; Purvis et al., 2005), such an information vacuum undermines mitigation of the global decline of freshwater mussels. Fortunately, as has been discussed elsewhere (Graf and Cummings, 2010), this particular problem is solvable.

We hypothesize that the recurrent problem of unionid paraphyly is an artifact of insufficient taxon and character sampling rather than an actual evolutionary pattern (Bergsten, 2005; Felsenstein, 2003). Among several COI-only analyses, *C. aegyptiaca* was the sole representative of the tropical Unionidae from Africa and southern Asia (a large assemblage of some 258 species, representing 40% of the family) (Graf and Cummings, 2007a). The combined 28S + COI + morphology analyses of Graf and Cummings (2006a) also suffered from a paucity of tropical taxa in addition to incomplete molecular character sampling. Whereas *Coelatura* was represented only by COI, both *Pseudodon* and *Pilsbryoconcha* from tropical Asia had only 28S (all taxa were also coded for 59 morphological characters). The lone tropical unionid with complete molecular character sampling in their analysis was *Contradens contradens*. In the current study, we set out to resolve the position of *C. aegyptiaca* relative to the other species of the Unionidae by sampling more extensively from Afrotropical and Indotropical lineages and by assembling a character set composed of both nuclear and mtDNA for all taxa. Our molecular phylogenetic analyses of the combined 28S + COI dataset finds robust support for the monophyly of the Unionidae, and the infra-familial topology has implications for the early evolution of freshwater mussels.

2. Materials and methods

2.1. Taxon sampling

Species were chosen to represent the taxa in the family-group classification of the Unionidae by Bieler et al. (2010) (Table 1). Also included were representatives from four other freshwater mussel families as well as *Neotrigonia* (the marine sister group of the Unionoida) (Giribet and Wheeler, 2002; Hoeh et al., 1998). *Mytilus edulis* (Pteriomorphia) and *Astarte castanea* (Heterodonta) comprise the outgroup. Tissue biopsies were fixed in either >95% ethanol or RNAlater (Ambion, Inc., Austin, USA; http://www.ambion.com) and stored at −20 °C until analyzed. Table 1 lists voucher specimens (shells) confirming identifications. Specimens from Burma/Myanmar were identified using Subba Rao (1989) as well as type images on the MUSSEL Project Web Site (http://www.mussel-project.net/). African taxa were identified using Mandahl-Barth (1988), and the taxonomy of Neotropical species conforms to Simone (2006).

2.2. Character sampling

Cytochrome oxidase subunit I (COI) mtDNA and large ribosomal subunit (28S) nuclear rDNA sequences were generated by standard polymerase chain reaction (PCR) and cycle sequencing methods, as described by Graf and Ó Foighil (2000a, 2000b). Partial COI sequences were amplified and sequenced using the Folmer et al. (1994) primers LCO1490 and HCO2198 or HCO700dy2, which is a modified version of the Folmer reverse primer designed specifically for freshwater mussels (Graf and Ó Foighil, 2000a; Walker et al., 2006). Domain 2 of 28S was amplified and sequenced using the D23F and D4RB primers of Park and Ó Foighil (2000). In addition, 38 sequences (19 from each locus) were obtained from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 1).

2.3. Phylogenetic analyses

Nuclear rDNA (28S) sequences were initially aligned using Clustal X (Larkin et al., 2007), with minor manual adjustments using Mesquite version 2.73 (Maddison and Maddison, 2010). In order to assess the effect of automated sequence alignment on tree topology, alternative alignments were explored using MAFFT, MUSCLE and WebPrank (as implemented through the European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/msa/). The protein-coding mtDNA (COI) was translated in Mesquite and nucleotide positions were aligned by homologous codon positions. All phylogenetic analyses were carried out upon both data partitions independently as well as in combination.

Tree searches were performed using maximum parsimony (MP) and maximum likelihood (ML). Bayesian inference (BI) was applied as an additional estimate of tree topology and branch support. MP analyses were executed in PAUP*4b10 (Swofford, 2002), with 1000 heuristic search replicates. Gaps in the alignment were treated as missing characters. Heuristic search result trees generated by random sequence addition were used as starting trees for a second heuristic search to overcome a known PAUP* bug wherein random sequence addition can lead to retention of suboptimal topologies (http://paup.csit.fsu.edu/problems.html). For COI-only and combined analyses, MP searches were performed both with and without third codon position transitions down-weighted to zero (as advocated by Hoeh et al., 2009). We refer to this weighting scheme as "3ti0." One thousand bootstrap replications (10 heuristic search replicates each) were performed in PAUP*, and MP branch support was also estimated using the Bremer–Decay Index (Bremer, 1995) facilitated by TreeRot v3 (Sorenson and Franzosa, 2007). ML searches were run with RAxML v7.0.3 (precompiled OS X executable; Stamatakis, 2006) for 1000 bootstrap replications with all partitions under separate GTR + Γ + I models, as determined using the Akaike information criterion with jModelTest (Posada, 2008). Tree searches were done both with COI as a single partition and with each codon position as its own partition (three partitions). BI analyses were performed using MrBayes 3.1.2 via the CIPRES Portal (Huelsenbeck and Ronquist, 2001; Miller et al., 2011; Ronquist and Huelsenbeck, 2003) applying the same partition options under unlinked GTR + Γ + I models (two runs, eight chains each, 24×10^6 MCMC generations, retaining every 1000th tree, and omitting the first 8000 as burn-in). Convergence of separate runs was verified using AWTY (Wilgenbusch et al., 2004) and the average standard deviation of split frequencies reported by MrBayes (<0.01).

A partition homogeneity test was performed in PAUP* (1000 replications with 100 MP heuristic search replications each) to quantify the phylogenetic incongruence between 28S and COI (Farris et al., 1995). The extent of conflict was also evaluated by plotting bootstrap values (BS) from the individual character sets versus combined BS for the same clades, allowing visualization of nodes supported by individual partitions (BS $\geqslant$ 70%) but not supported in the combined analysis (BS < 70%). The guideline of 70% for "well-supported" bootstrap clades is based upon various sources (Efron et al., 1996; Felsenstein, 2003; Hillis and Bull, 1993). Formatting and analysis of BS bipartition tables was accomplished using a custom Perl script (available from the corresponding author). Bipartition tables were generated in PAUP*, either directly from the bootstrap trees for MP or by importing the

Table 1
Taxa and sequences. Family-level classification is that of Bieler et al. (2010), and species-level nomenclature follows Graf and Cummings (2007a), with the exception of the placement of *Cafferia caffra* in the genus *Unio* (Araujo et al., 2009). Voucher specimens are deposited at the Academy of Natural Sciences of Philadelphia (ANSP) or the University of Alabama Museum of Natural History (UA).

Taxon	COI	28S	Source, including country of origin
Subclass Pteriomorphia			
Mytilus edulis (Linnaeus, 1758)	AY377727	Z29550	Okusu et al. (2003) and Littlewood (1994)
Subclass Heterodonta			
Astarte castanea (Say, 1822)	AF120662	AF131001	Giribet and Wheeler (2002) and Park and Ó Foighil (2000)
Subclass Palaeoheterodonta			
Order Trigonioida			
Neotrigonia margaritacea (Lamarck, 1804)	U56850	AF400695	Hoeh et al. (1998) and Graf (2002)
Order Unionoida			
Family IRIDINIDAE			
Mutela rostrata (Rang, 1835)	JN243862	JN243884	Egypt, ANSP 417318
Aspatharia pfeifferiana (Bernardi, 1860)	JN243863	JN243885	Zambia, ANSP A21405
Chambardia wahlbergi (Krauss, 1848)	JN243864	JN243886	Zambia, ANSP 419403
Family MYCETOPODIDAE			
Mycetopoda siliquosa (Spix & Wagner, 1827)	JN243865	JN243887	Peru, ANSP 416344
Anodontites elongata (Swainson, 1823)	JN243866	JN243888	Peru, ANSP 416347
Family HYRIIDAE			
Velesunio ambiguus (Philippi, 1847)	AF305371	AF305378	Australia, Graf and Ó Foighil (2000b)
Hyridella australis (Lamarck, 1819)	AF305367	AF305373	Australia, Graf and Ó Foighil (2000b)
Castalia ambigua Lamarck, 1819	JN243867	JN243889	Peru, ANSP 416341
Triplodon corrugatus (Lamarck, 1819)	JN243868	JN243890	Peru, ANSP 416338
Family MARGARITIFERIDAE			
Margaritifera margaritifera (Linnaeus, 1758)	JN243869	JN243891	Ireland, UA 21019
Margaritifera monodonta (Say, 1829)	AF156498	AF305382	USA, Graf and Ó Foighil (2000a, 2000b)
Family UNIONIDAE			
Subfamily COELATURINAE			
Coelatura aegyptiaca (Cailliaud, 1827) 1	JN243870	JN243892	Egypt, ANSP 416304
Coelatura aegyptiaca 2	JN243871	JN243893	Ditto
Coelatura aegyptiaca 3	JN243872	JN243894	Ditto
Coelatura gabonensis (Küster, 1862)	JN243873	JN243895	Congo, ANSP A21417
Prisodontopsis aviculaeformis Woodward, 1991	JN243874	JN243896	Zambia, ANSP 416363
Nitia teretiuscula (Philippi, 1847)	JN243875	JN243897	Egypt, ANSP 416305
Subfamily PARREYSIINAE			
Parreysia (s.s.) mandelayensis (Theobald, 1873)	JN243876	JN243900	Burma, UA 20722
Parreysia (s.s.) tavoyensis (Gould, 1843)	JN243877	JN243901	Burma, UA 20726
P. (Radiatula) bonneaudi (Eydoux, 1838)	JN243878	JN243898	Burma, UA 20714
Lamellidens generosus (Gould, 1847)	JN243880	JN243902	Burma, UA 20727
Lamellidens corrianus (Lea, 1834)	JN243881	JN243903	Burma, UA 20729
Subfamily RECTIDENTINAE			
Contradens contradens (Lea, 1838)	DQ191411	AF400692	Thailand, Graf (2002) and Graf and Cummings (2006a)
Subfamily GONIDEIANE			
Gonidea angulata (Lea, 1838)	DQ191412	AF400691	USA, Graf (2002) and Graf and Cummings (2006a)
Pseudodon vondembuschianus (Lea, 1840)	DQ206793	AF400694	Thailand, Graf (2002) and Graf and Cummings (2006a)
Pronodularia japanensis (Lea, 1859)	AB055625	AB103132	Japan, Okazaki and Ueshima (unpubl.) and Hashimoto and Matsumoto (unpubl.)
Potomida littoralis (Cuvier, 1798) 1	JN243882	JN243904	Turkey, ANSP 418428
Potomida littoralis 2	JN243883	JN243905	France, UA 21016
Subfamily AMBLEMINAE			
Tribe AMBLEMINI			
Amblema plicata (Say, 1817)	AF156512	AF305385	USA, Graf and Ó Foighil (2000a, 2000b)
Tribe QUADRULINI			
Quadrula quadrula (Rafinesque, 1820)	AF156511	DQ191417	USA, Graf and Ó Foighil (2000a), Graf and Cummings (2006a)
Tribe PLEUROBEMINI			
Pleurobema sintoxia (Rafinesque, 1820)	AF156509	DQ191418	USA, Graf and Ó Foighil (2000a) and Graf and Cummings (2006a)
Tribe LAMPSILINI			
Truncilla truncata Rafinesque, 1820	AF156513	DQ191419	USA, Graf and Ó Foighil (2000a) and Graf and Cummings (2006a)
Lampsilis cardium Rafinesque, 1820	AF156519	AF305386	USA, Graf and Ó Foighil (2000a, 2000b)
Tribe OXYNAIINI			
Oxynaia pugio (Benson, 1862)	JN243879	JN243899	Burma, UA 20739
Subfamily UNIONINAE			
Tribe UNIONINI			
Unio pictorum (Linnaeus, 1758)	AF156499	AF305383	Austria, Graf & Ó Foighil (2000a; 2000b)
Unio caffer Krauss, 1848	AF156501	AF400687	South Africa, Graf and Ó Foighil (2000a) and Graf (2002)
Tribe ANODONTINI			
Pyganodon grandis (Say, 1829)	AF156504	AF305384	USA, Graf and Ó Foighil (2000a, 2000b)
Lasmigona compressa (Lea, 1829)	AF156503	DQ191414	USA, Graf and Ó Foighil (2000a), Graf and Cummings (2006a)

1000 bootstrap trees generated by the ML analysis. PHYLIP-formatted trees from RAxML were converted to NEXUS format using Mesquite. Clades occurring in <5% of bootstrap replicates were set to 0% following PAUP* defaults. To our knowledge, this is a novel method for examining the degree of conflict/agreement among data partitions.

3. Results

Forty-one combined 28S and COI sequences representing 38 species were assembled into a matrix of 1130 characters (matrix available from the corresponding author). The Clustal alignment of the 28S partition contributed 500 aligned nucleotide positions,

although there was considerable variation in length among the taxa (399–453 nt, median = 430). Of these 500 characters, 163 (33%) contained at least one alignment gap. Gaps associated with indels occurred in 28 clusters (mean cluster length = 6.8 nt ± 6.9 SD, min = 1, max = 27), and the number of nt between gap clusters ranged from 3 to 41 (mean = 9.8 ± 8.6 SD). Among the 163 gap-sites, only 60 (37%) affected species of the Unionidae. ML trees derived from alternative MAFFT, MUSCLE and WebPrank alignments (not shown) were largely congruent with the Clustal alignment. The aligned COI partition was truncated to 630 nt. Within the ingroup, all variation in COI sequence length was the result of terminal deletions, whereas both outgroup taxa exhibited internal indels relative to *Neotrigonia* and the freshwater mussels. Taxa representing five of the six unionoid families were included, as was at least one member from each extant unionid subfamily and tribe except the Modellnaiinae (Bieler et al., 2010).

Fig. 1 depicts the optimal topologies recovered by both MP and ML analysis of the combined matrix of 28S and COI, and tree statistics for all analyses are shown in Table 2. The consensus topologies of the BI combined analyses were broadly congruent with the ML topologies. Fig. 2 depicts the support for both *a priori* clades (i.e., families and unionid subfamilies listed in Table 1) and clades determined *a posteriori* to differ in support among the separate MP, ML and BI analyses. The partition homogeneity test revealed significant phylogenetic incongruence between the two gene fragments ($p < 0.01$), and this is evident in the difference between the MP tree length of the combined analyses and the sum of individual partitions (Table 2). Nevertheless, incongruent nodes in the alternative topologies recovered by the independent data partitions were generally poorly supported. As shown in Fig. 3, for equally weighted and 3ti0 MP analyses and ML analyses with and without individual codon partitions, a total of only nine clades were supported with BS $\geqslant$ 70% by individual partitions but were not supported (BS < 70%) in the corresponding combined analysis (upper left quadrants of the graphs). These nine clades are numbered for identification and listed in Table 3 with their bootstrap values across all MP and ML analyses.

All combined topologies and attendant support values shown in Figs. 1 and 2 supported the monophyly of the Unionidae although no compelling sister-group for the family was found. *C. aegyptiaca* was recovered as part of a strictly Afrotropical clade (*Coelatura, Prisodontopsis* and *Nitia*), but neither the genus nor the species was monophyletic. The Indian–Burmese taxa (*Parreysia* (*Parreysia*), *P.* (*Radiatula*), *Oxynaia* and *Lamellidens*), representing southern Asian lineages were placed as a grade at the base of an Africa–India clade. The Africa–India clade was recovered as sister to a clade composed of southeastern Asian, Palearctic and Nearctic unionids, although the basal branching order of this "core Unionidae" clade is not well supported. In all previous molecular phylogenetic analyses, the Unionidae has been solely represented by the latter clade with the exception of *C. aegyptiaca*.

Across all analyses (Figs. 1 and 2), there is mixed support for the *a priori* family-group level relationships of freshwater mussels listed in Table 1. In the combined analyses, the monophyly of both the Palaeoheterodonta (= *Neotrigonia* + Unionoida) and the Unionoida (freshwater mussels) is well supported, although the individual partitions analyzed separately support various alternative topologies (e.g., clade #1 in Table 3). Combined analyses differ in their support for the basal lineage of the Unionoida. MP places the (Iridinidae + Mycetopodidae) clade in that position while ML supports a basal Hyriidae. The Iridinidae was not recovered as monophyletic, with *Mutela* consistently placed as sister to the Mycetopodidae (= *Mycetopoda* + *Anodontites*) rather than the (*Aspatharia* + *Chambardia*) clade. The Margaritiferidae (= *Margaritifera*), the traditional sister-group to the Unionidae, was not recovered in any of the combined analyses in that position, and there is no bootstrap support (<70%) for any alternative arrangement.

4. Discussion

The phylogenetic analyses reported herein represent the broadest sampling of the Unionidae to-date using a combination of both nuclear (28S) and mitochondrial (COI) DNA. Five of the six families of the Unionoida are represented, as are most of the infra-familial family-group taxa within the Unionidae. Although neither character set analyzed separately or in combination provides a robust estimate of inter-familial relationships, the monophyly (or not) of intra-familial lineages is well resolved (Figs. 1 and 2). Future studies applying a larger, more conserved portion of 28S and/or other nuclear loci as well as morphological characters will be useful for testing alternative topologies among the families as well as the synapomorphies that diagnose the deeper relationships among the Unionoida. The 1130 characters analyzed here are sufficient to reject the paraphyly of the Unionidae and to determine the phylogenetic position of the previously problematic Afrotropical genus *Coelatura*. The recovered topologies provide insights into the early evolution of the Unionidae, although the sister to the family remains ambiguous. The following discussion elaborates on these points to provide a context for the results of the present analyses and to emphasize areas where additional study might be fruitful.

4.1. Conflict among datasets

Graf and Cummings (2006a) assembled a combined matrix that included both 28S and COI (as well as 59 morphological characters), and they compared the relative merits of the two molecular character sets for resolving the family-level phylogeny of the Unionoida. Their matrix was also analyzed by Hoeh et al. (2009). The consensus of both of these studies was that COI is of limited utility. The amino acid sequence for the cytochrome oxidase subunit I protein is highly conserved among the Unionoida, and the combination of purifying selection and redundancy in the genetic code results in repeated synonymous substitutions (e.g., 3rd position transitions) at the same sites. This problem is well known and the solutions widely discussed (e.g., Bergsten, 2005; Swofford et al., 1996; Xia et al., 2003), including down-weighting 3rd position transitions under parsimony, applying probabilistic models that account for unobserved character transformations along relatively long branches, and breaking up long branches by including additional taxa. We have opted for all three strategies.

Our analyses of COI and 28S separately and in combination revealed significant conflict between the two character sets according to the partition homogeneity test ($p < 0.01$). The same result obtained from subsequent tests omitting individual families and unionid subfamilies, indicating that the conflict was not localized to a specific branch of the tree. However, our plots of bootstrap support for individual bipartitions under 28S and COI analyzed separately and in combination revealed that the alternative topologies of the individual character sets are, in general, poorly supported (Fig. 3). Implicit in this methodology is the assumption that mere resolution of a node on the most parsimonious or most likely topology is not sufficient to reject alternative arrangements—i.e., the result must stand up to character re-sampling as well. Only nine nodes with BS support $\geqslant$70% in either individual character set lacked support in some set of the combined MP or ML analyses (Figs. 2 and 3, Table 3). Disparity between individual and combined bipartition BS support was most evident in the equally weighted MP analysis (Fig. 3, upper left), and it improved under methods that account for rate heterogeneity among codon positions and

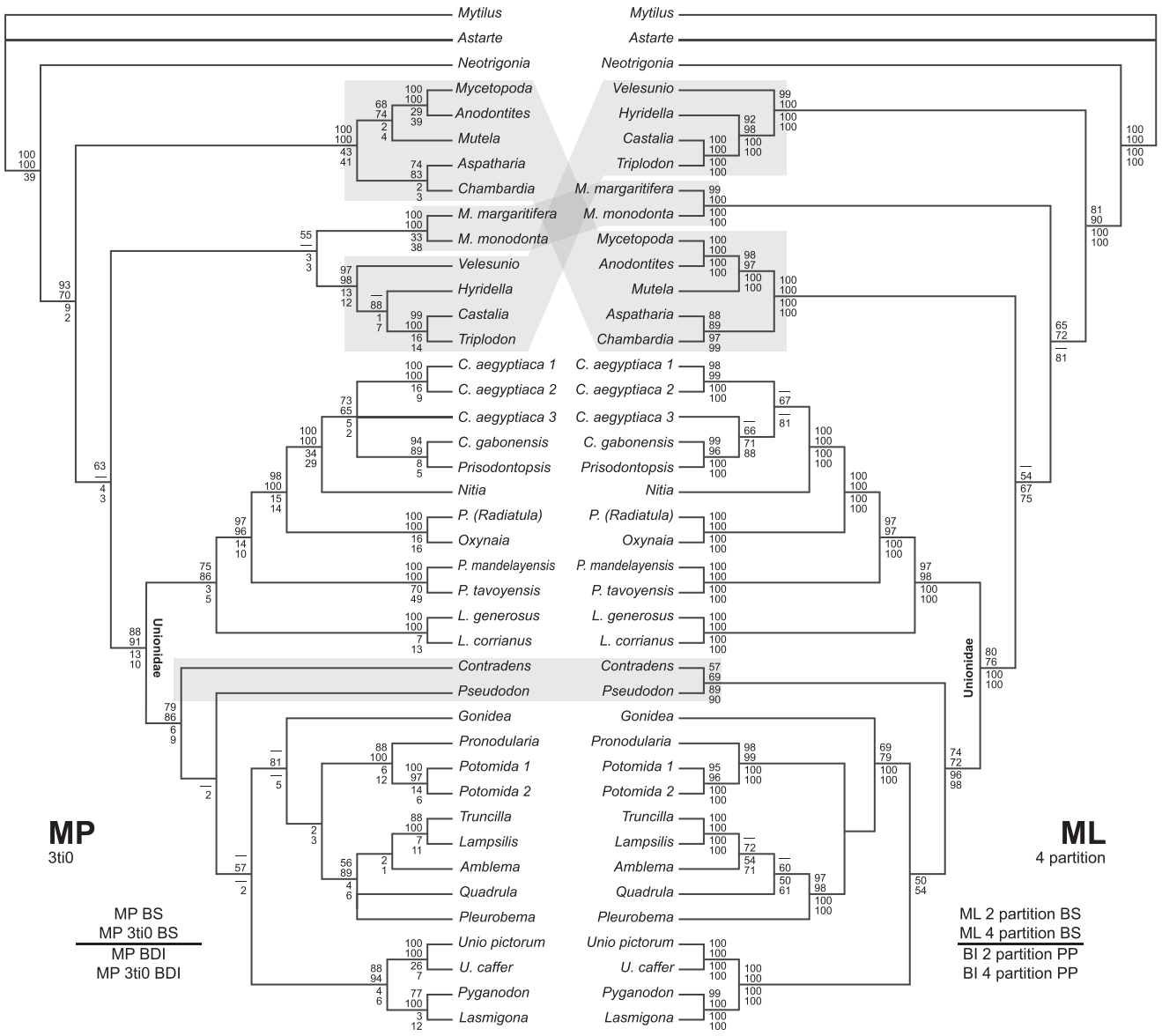

Fig. 1. Cladograms recovered from MP and ML analyses of combined 28S + COI datasets. The MP tree depicted is the strict consensus of four trees (2375 steps) recovered from the analysis of 28S and COI with 3rd codon position transitions down-weighted to 0 (3ti0). Branch support is indicated by bootstrap support above the branches and Bremer–Decay Index below. The ML tree presented was based upon 28S and COI with each codon position in its own partition (−ln 14859.423). Support values above the branches are bootstrap and below, BI posterior probabilities. Tree statistics are presented in Table 2. Gray highlighting indicates areas of topological incongruence between the two analyses. A summary of the alternative topologies recovered by different analyses is shown in Fig. 2.

multiple substitutions along long branches. ML with each COI codon position allowed to evolve under its own substitution model (Fig. 3, lower right) shows the least conflict: except for bipartitions #5 and #6 (Table 3), clades that are well supported under 28S and COI separately are also well supported in the combined analysis. These results indicate that (1) the conflict between the two data partitions is the result of copious homoplasy (i.e., noise) rather than independent histories, and (2) the signal from their shared history is manifest in the combined analyses.

4.2. Evolution of Coelatura and the African Unionidae

The previously problematic *C. aegyptiaca* in our analyses was recovered in a well-supported clade of Afrotropical unionid genera (*Coelatura*, *Prisodontopsis* and *Nitia*) (Fig. 1). Even with our limited sampling, the genus *Coelatura* was not monophyletic, nor was the species *C. aegyptiaca*. All three *C. aegyptiaca* were collected simultaneously from the Nile in Cairo, but both 28S and COI hint at the depth of the cryptic genetic diversity within this traditionally lumped species (Graf and Cummings, 2007b; Sleem and Ali, 2008). The COI sequence first analyzed by Bogan & Hoeh (2000; Genbank AF231735) is most similar to *C. aegyptiaca* 3 in our study (uncorrected $p = 0.005$). By increasing both the taxon and character sampling, we overcame the apparent homoplasy that had confounded previous analyses of this taxon (reviewed in Graf and Cummings, 2006a).

There are two distinct lineages among the African taxa included in our analyses: the *Coelatura* clade and *Unio caffer*. *U. caffer* is endemic to southern Africa (Appleton, 1979; Graf and Cummings, 2006b). Based upon morphological characteristics (Heard and Vail, 1976; Ortmann, 1918) and molecular phylogenetic analyses (Araujo et al., 2009; Graf and Cummings, 2006a, herein), *U. caffer* shares a more recent common ancestor with Palearctic species of *Unio* (Unioninae) than with the other African unionids of the *Coel-*

Table 2
Tree and character statistics for MP, ML, and BI analyses. SDSF = average standard deviation of splits frequencies, indicating the final degree of convergence among the separate BI runs.

MP	Characters			Trees	Tree length (steps)	CI	RC
	Total	3ti0	Informative				
Combo	1130	0	581	1	3539	0.3832	0.2105
Combo	1130	210	562	4	2375	0.4455	0.2878
COI	630	0	301	15	2411	0.3102	0.1279
COI	630	210	282	3	1258	0.3577	0.1882
28S	500	0	280	72	1093	0.5563	0.4224

ML	Partitions	Alpha/invariant					Tree length	−ln Likelihood
		28S	COI	COI Pos1	COI Pos2	COI Pos3		
Combo	2	1.781115	0.438261	–	–	–	8.546294	−15565.40884
		0.189198	0.207314	–	–	–		
Combo	4	1.426263	–	1.077331	46.102787	0.730166	39.034826	−14859.42261
		0.158863	–	0.274081	0.421125	0.005446		
COI	1	–	0.416266	–	–	–	102.629189	−9741.524664
		–	0.30241	–	–	–		
COI	3	–	–	1.022971	1.856751	0.7961	78.861044	−9139.134008
		–	–	0.30477	0.339361	0.007313		
28S	1	1.835327	–	–	–	–	3.469315	−5299.992698
		0.181023	–	–	–	–		

BI	Partitions	Mean alpha/invariant					Mean tree length	Mean −ln likelihood	SDSF
		28S	COI	COI Pos1	COI Pos2	COI Pos3			
Combo	2	2.092283	0.419311	–	–	–	13.474396	−15472.00	0.002
		0.185743	0.26221	–	–	–			
Combo	4	2.047001	–	0.895527	92.541262	1.176803	14.240894	−14902.80	0.002
		0.185382	–	0.204955	0.283161	0.015338			
COI	1	–	0.477832	–	–	–	15.677047	−9783.79	0.004
		–	0.289179	–	–	–			
COI	3	–	–	1.218756	94.242641	1.560725	15.539517	−9239.72	0.003
		–	–	0.248321	0.305085	0.019061			
28S	1	1.872188	–	–	–	–	4.522178	−5335.93	0.003
		0.169366	–	–	–	–			

atura clade. *U. caffer* apparently represents a separate invasion of the continent from the north, and its relationship to other African unionines (e.g., *Unio durieui* Deshayes, 1847, *U. abyssinicus* von Martens, 1866) remains to be explicitly determined (but see Khalloufi et al., 2011). The *Coelatura* clade is represented in our analyses by members of the genera *Coelatura, Prisodontopsis* and *Nitia*, and we assume that the remaining Afrotropical unionid genera (*Nyassunio, Grandidieria, Mweruella, Brazzaea* and *Pseudospatha*) will also fall into this clade based upon their morphological affinities (e.g., shell sculpture, brooding demibranchs) and geographical distributions (Graf and Cummings, 2007a; Pain and Woodward, 1968).

Two alternative hypotheses have been proposed for the history of the Unionidae in Africa: (1) the Unionidae arose on Gondwana, populating the northern continents from the south or (2) the Unionidae arose in the north and spread south into Africa with the closure of the Tethys Sea. These hypotheses have been discussed in detail by Graf and Cummings (2009) and were dubbed "Out of Africa" and "Into Africa," respectively. The results presented here contribute little to that discussion since the tree topology is consistent with either. The fact that the *Coelatura* clade is nested within a larger Africa–India clade could be the result of an early divergence associated with the breakup of Gondwana (both land masses are derived from the Mesozoic supercontinent), or it may be the result of both India and Africa being colonized by the same Asiatic lineage when those continents eventually contacted Eurasia during the Eocene (India) and Miocene (Africa) (Potter and Szatmari, 2009; Scotese et al., 1988). Additional taxon sampling of tropical Asian lineages will reveal whether the exclusive Africa–India clade is merely sampling bias or an actual historical pattern. Coupled with additional character sampling, a molecular-clock approach could be applied to estimate the divergence time of the Afrotropical *Coelatura* clade from taxa in Asia and the chronology of diversification within the *Coelatura* clade (Crisp et al., 2011). A Mesozoic date of origin would support "Out of Africa" and require a re-interpretation of the available fossil evidence (Van Damme and Pickford, 2010; Van Damme and Van Bocxlaer, 2009).

4.3. Classification of the Unionidae

The inter-familial relationships of the Unionoida are not well supported in the present analysis, and thus no new evidence can be brought to bear on the classification of the order. However, our topologies do contribute to a clearer understanding of the subfamilies and tribes of the Unionidae and a test of the classification proposed by Bieler et al. (2010) (Table 1). In their treatments of the global Unionoida, Graf and Cummings (2006a, 2007a) punted with regard to the placement of Old World "amblemines," treating those genera as *incertae sedis* at the family-group level pending further evidence. Bieler et al. (2010) were more explicit, recognizing seven subfamilies within the extant Unionidae. Only two of those families were supported as monophyletic herein (Fig. 2): Unioninae and Coelaturinae (= *Coelatura* clade). The tribes of the Ambleminae formed a clade except for the Oxynaiini, which was tentatively included by Bieler et al. (2010). The latter lineage was recovered as part of a paraphyletic Parreysiinae (India–Burma grade). No support was found for a Gonideinae clade that includes *Gonidea*,

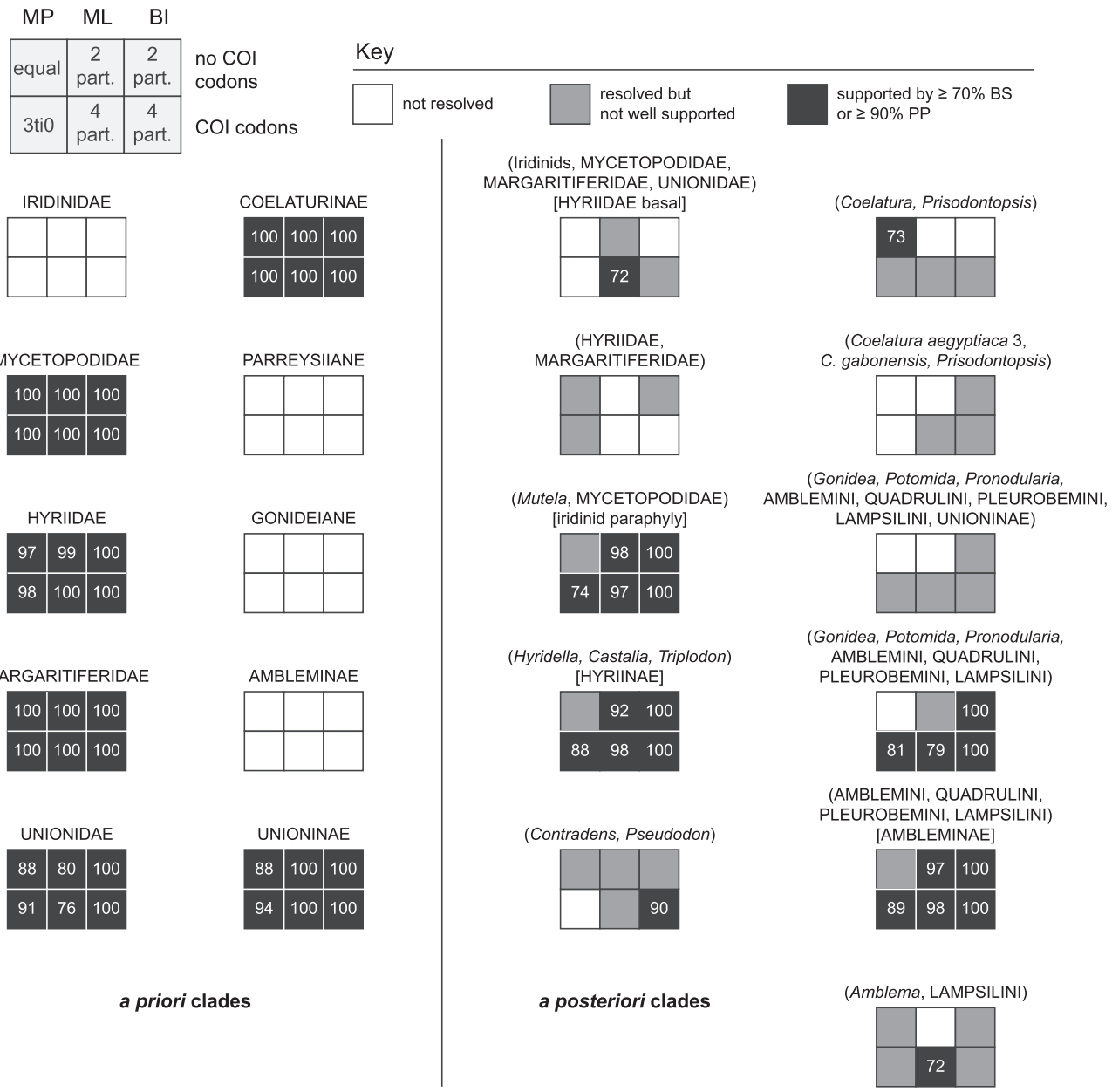

Fig. 2. Clades recovered by the combined analysis of 28S and COI under various methods and models of character evolution. *A priori* family-group clades listed in Table 1 are on the left; clades recovered *a posteriori* with differing levels of support among analyses are listed on the right. Numbers within boxes indicate bootstrap support or posterior probabilities of the clades in our analyses.

Potomida and *Pseudodon*. Only a single member of the Rectidentinae was analyzed and the Modellnaiinae was not available for study.

A revision of the family-group classification of the Unionidae is shown in Fig. 4. We recognize the Coelaturini, Oxynaiini, Parreysiini, and Lamellidentini as tribes with the Parreysiinae. All of these taxa already bear family-group level names, and thus this novel classification follows organically from recognition of a well-supported clade containing the African and Indian genera included in our analyses (Fig. 1) and the algorithm of zoological nomenclature (ICZN, 1999). This action also subdivides the unwieldy Unionidae, supporting a cladistically based subfamily (i.e., Parreysiinae) for at least 90 species in 12 genera (*Brazzaea*, *Coelatura*, *Grandidieria*, *Mweruella*, *Nitia*, *Nyassunio*, *Prisodontopsis*, *Pseudospatha*, *Lamellidens*, *Oxynaia*, *Parreysia*, *Radiatula* and *Scabies*) for which there was previously little or no phylogenetic data. This accounts for >40% of the tropical species that could not be placed by Graf and Cummings (2007a). In addition to robust molecular phylogenetic support for the monophyly of the Unionidae (Fig. 1), the species of the Parreysiinae possess a supra-anal aperture (Ortmann, 1910; Prashad, 1919a,b), a diagnostic morphological synapomorphy of the Unionidae (Graf and Cummings, 2006a: type II posterior mantle fusion, their Figs. 11 and 12). The supra-anal aperture is formed by fusion of the lateral mantle lobes dorsal to the excurrent aperture. The fusion extends for only a short distance, creating a third posterior aperture of unknown function. Morphological phylogenetic analyses are necessary to determine the synapomorphies of the infra-familial clades within the Unionidae.

4.4. Areas for future study

The strength of this study is its targeted approach. The several previous analyses (cited above) of freshwater mussel phylogeny had revealed the depth of the molecular (and morphological)

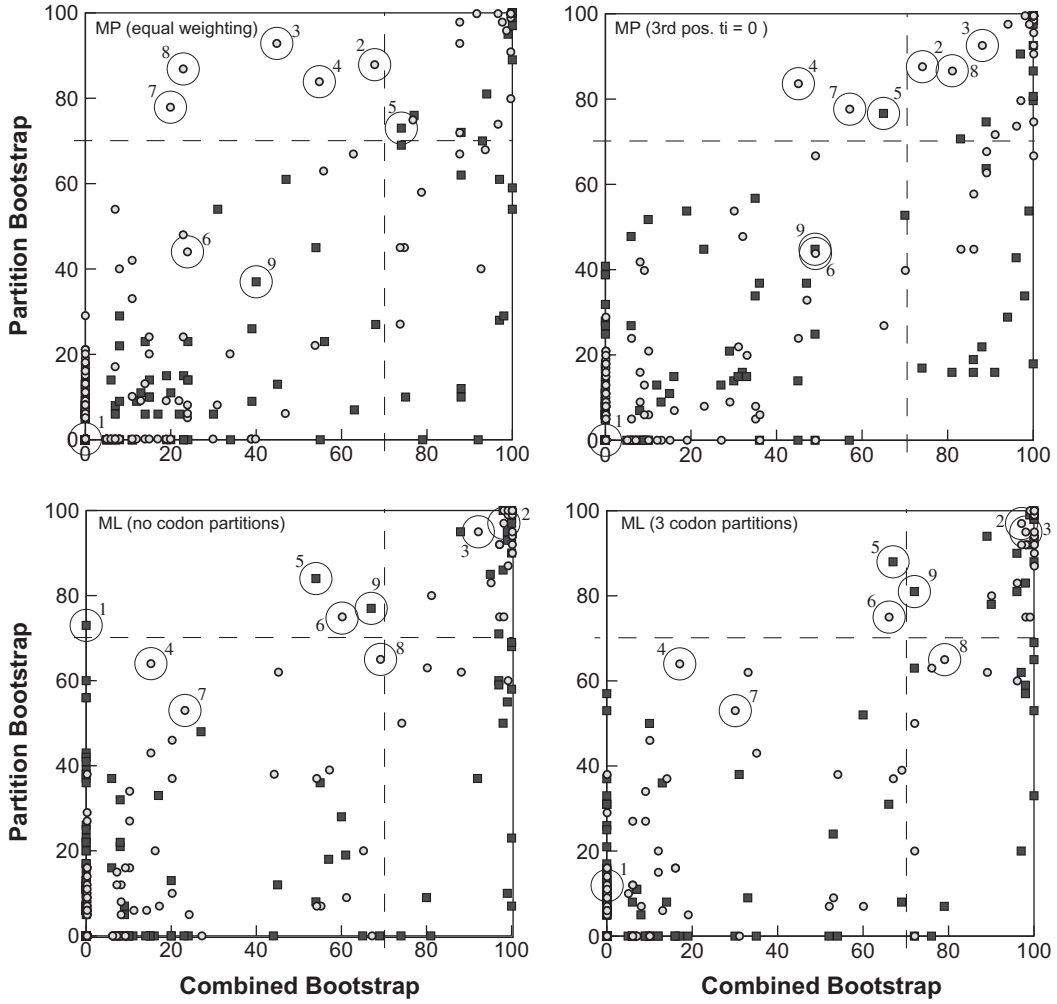

Fig. 3. Scatter plots of individual character set bootstrap values vs. combined bootstrap values under MP (equal weighting and 3ti0) and ML (COI as 1 partition, COI as 3 partitions). The upper left quadrant of each chart indicates clades with bootstrap support ($\geqslant$70%) when the partitions are analyzed separately that are not well supported in the combined analysis. Numbered clades are listed in Table 3. Circles = 28S; squares = COI.

Table 3
Clades supported by individual partitions but not in the combined analyses. Italicized values indicates the individual partition bootstrap values with support $\geqslant$70%, and the bold values indicate the combined analyses in conflict.

		MP	3ti0	3ti0			ML	Codons	Codons		
		Combo	Combo	COI	COI	28S	Combo	Combo	COI	COI	28S
1	(*Astarte*, MARGARITIFERIDAE)	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	*0.73*	0.11	0.00
2	(*Mutela*, MYCETOPODIDAE)	**0.68**	0.74	0.27	0.17	*0.88*	0.98	0.97	0.50	0.20	0.97
3	(*Hyridella*, *Castalia*, *Triplodon*)	**0.45**	0.88	0.13	0.22	*0.93*	0.92	0.98	0.37	0.57	0.95
4	(HYRIIDAE, MARGARITIFERIDAE)	**0.55**	**0.45**	0.00	0.00	*0.84*	0.15	0.17	0.00	0.00	0.64
5	(*Coelatura*, *Prisodontopsis*)	0.74	**0.65**	0.73	*0.77*	0.27	**0.54**	**0.67**	*0.84*	*0.88*	0.37
6	(*Coelatura aegyptiaca*, *C. gabonensis*, *Prisodontopsis*)	0.24	0.49	0.23	0.25	0.44	**0.60**	**0.66**	0.28	0.31	*0.75*
7	(*Gonidea*, *Potomida*, *Pronodularia*, *Amblema*, *Quadrula*, *Pleurobema*, LAMPSILINI, UNIONINAE)	**0.20**	**0.57**	0.00	0.00	*0.78*	0.23	0.30	0.00	0.00	0.53
8	(*Gonidea*, *Potomida*, *Pronodularia*, *Amblema*, *Quadrula*, *Pleurobema*, LAMPSILINI)	**0.23**	0.81	0.00	0.16	*0.87*	0.69	0.79	0.00	0.07	0.65
9	(*Amblema*, LAMPSILINI)	0.40	0.49	0.37	0.45	0.00	**0.67**	0.72	*0.77*	0.81	0.00

diversity among the species of the Unionoida and the inapplicability of the individual character sets to different levels of the tree. It is perhaps an unreasonable expectation that one data matrix should be able to provide unambiguous support for both the sequence of divergence among the families of the order and the inter-generic phylogeny of the tribes. Hypotheses of freshwater mussel relationships deduced *post-hoc* from the results of phylogenetic studies — e.g., the paraphyly of the Unionidae — should be followed up with analyses like this one with taxon and character sampling aimed at testing specific internal branches. Several

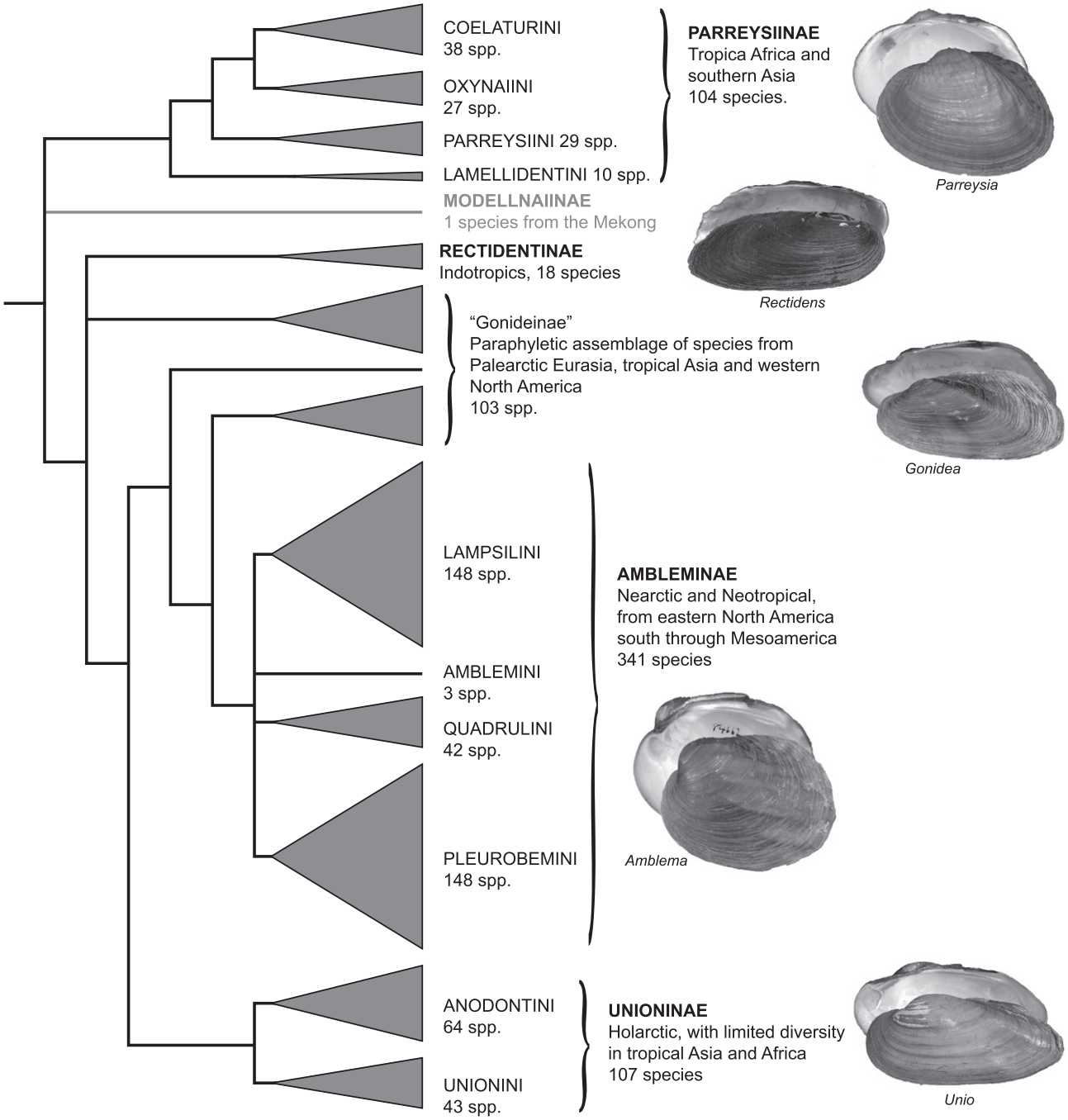

Fig. 4. Revised classification of the Unionidae. This is a cladistic representation of the classification proposed by Bieler et al. (2010) and modified to accommodate the results presented in Fig. 1. Terminal breadth is proportional to species richness (Graf and Cummings, 2007a). *Modellnaia siamensis* has never been included in a phylogenetic analysis.

contentious issues besides unionid monophyly remain to be sorted: for example, the hypothesized polyphyly of the Etheriidae (Bogan and Hoeh, 2000; Hoeh et al., 2009).

With regard to the problem of the earliest diversification of the extant Unionidae, more sampling is necessary from tropical lineages. In addition, the phylogenetic positions of *Gonidea* relative to the Ambleminae of eastern North America, Palearctic genera like *Potomida*, and *Pseudodon* in the Indotropics remain to be resolved with respect to the global Unionidae. An accurate understanding of the actual phylogenetic diversity (e.g., Cadotte et al., 2010; Purvis et al., 2005) of freshwater mussels inhabiting biodiversity hotspots like the southeastern United States (Cummings and Graf, 2009) or the Mekong and Yangtze basins (Brandt, 1974; Prozorova et al., 2005) will be valuable for informing conservation priorities for the fresh waters of those regions in general and mussels in particular. Given the variety and severity of forces impinging upon freshwater diversity worldwide (e.g., mineral and timber extraction, agriculture, infrastructure development, political strife, invasive species, climate change; Strayer and Dudgeon, 2010), we regard the phylogeny of the tropical Unionidae as among the most pressing questions in freshwater malacology. It is our hope that the results presented here will lead to further research on this important problem.

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