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Molecular systematics and evolution of reproductive traits of North American freshwater unionacean mussels (Mollusca: Bivalvia) as inferred from 16S rRNA gene sequences

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SUMMARY

North American freshwater unionacean bivalves are a diverse group of nearly 300 species. Unionaceans exhibit an array of conchological, anatomical, life history, and reproductive characteristics that have figured prominently in proposed classification schemes. Recently, two very different classifications of North American unionaceans have been proposed. Depending on the classification system utilized, a very different evolutionary trajectory of anatomical and reproductive features is obtained. The lack of a robust, well corroborated phylogeny of North American unionacean bivalves hinders the progress of evolutionary and ecological studies involving these species. Here we present a mitochondrial DNA (mtDNA) based phylogeny for North American unionacean mussels and compare it to previously proposed classifications. In addition, we present a 'total evidence' phylogeny which incorporates both the mtDNA sequence data and available morphological data. The molecular and total evidence phylogenies agree largely with the conclusions of a previous study based largely on immunoelectrophoretic data. North American unionaceans can be divided into two families: the Unionidae, which is comprised of most of the species and the Margaritiferidae. Within the Unionidae are two subfamilies, the Anodontinae and Ambleminae. The resultant phylogeny was used to examine the evolution of several key anatomical features including the number of gills (demibranchs) used by females to brood developing embryos, incubation length (bradytictic vs tachytictic), larval (glochidial) tooth structures, and shell texture. Both molecular and total evidence phylogenies indicate several of the aforementioned characters evolved independently or were subsequently lost or gained in several lineages.

1. INTRODUCTION

The greatest diversity of the near-cosmopolitan freshwater unionacean mussels, naiades, or clams (Mollusca: Bivalvia: Unionacea) occurs in North America. Of the roughly 297 recognized North American taxa, most are found in the southeastern United States (Burch 1973, 1975; Williams *et al.* 1993; Lydeard & Mayden 1995). Unionaceans exhibit an array of conchological, anatomical, life history, and reproductive characteristics, many of which are associated with their fascinating mode of reproduction. North American unionaceans are ovoviviparous and embryo development occurs in the interlamellar spaces of the gills (demibranchs), which are modified to form so-called marsupia (McMahon 1991). Bivalved larvae (glochidia) are released and typically ecto-parasitize a fish host before metamorphosing into juveniles. Many unionid species have evolved a number of adaptations that increase the likelihood that glochidia will come into contact with fish hosts. For example, females, particularly in the genera *Lampsilis* and *Villosa*, have developed highly modified mantle flaps that resemble small, actively swimming prey fish (Kraemer 1970; Haag *et al.* 1995). Some mussels are short-term breeders (i.e. tachytictic), whose glochidial development and release take place between April and August, while other species are long-term breeders (i.e. bradytictic), whose glochidia are retained throughout the year and released during the summer (Kat 1984).

Despite a century of systematic studies of North American Unionacea, little consensus has been reached regarding their evolutionary relationships. Classification schemes proposed near the beginning of the 20th century were based on the examination of all morphological features including shell characteristics and soft-part anatomy (Simpson 1896, 1900, 1914; Sterki 1898, 1903; Ortmann 1910, 1912). Many later investigators, however, tended to advocate the use of either conchological characters (e.g. Frierson 1927; Modell 1942, 1949, 1964; Haas 1969*a*, *b*) or soft-part anatomy, particularly that associated with reproduction (Heard and Guckert 1971).

Heard & Guckert (1971) considered that similar shell shape and type among naiades was probably due to convergent or parallel evolution rather than shared

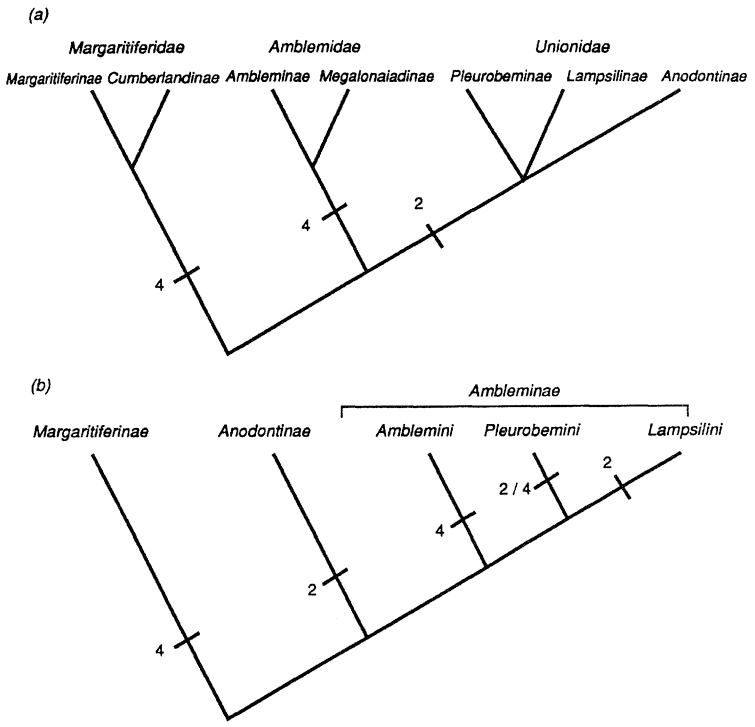

Figure 1. (*a*) Phylogenetic hypothesis of North American freshwater mussels based on Heard & Guckert's (1971) classification scheme. (*b*) Phylogenetic hypothesis based on Davis & Fuller's (1981) classification scheme. Numbers at nodes refer to the number of marsupial demibranchs (4 – tetragenous, 2 – ectobranchous).

common ancestry. They argued that reproductively related characteristics would accurately reflect natural, evolutionary affinities of North American naiades. Heard & Guckert (1971) primarily used two reproductive characters in their classification (figure 1*a* and table 1): the number of marsupial demibranchs (4, both outer and inner demibranchs (tetragenous); versus 2, only outer demibranchs (ectobranchous)), and the length of the breeding season used as a marsupium (tachytictic vs bradytictic). With some changes in taxonomic ranks, Heard & Guckert's (1971) classification of unionacean clams has been followed in important publications (e.g. Burch 1973, 1975; Thorp & Covich 1991).

Davis & Fuller (1981) proposed a novel classification of North American unionaceans based largely on a phenetic analysis of immunoelectrophoretic data of 52 species (figure 1*b* and table 1). They recognized three phenetically distinct subfamilies within the Unionidae: the Magaritiferinae, the Anodontinae, and the Ambleminae. Davis & Fuller (1981; figure 1*b* and table 1) indicated that the number of marsupial demibranchs and length of breeding season do not define higher taxa as hypothesized by Heard & Guckert (1971). Their classification suggested that the tetragenous and ectobranchous condition evolved independently several times (figure 1*b*). Later allozyme based studies (Davis *et al.* 1981; Davis 1984) supported the recognition of three phenetically distinct taxonomic groups (i.e. Margaritiferinae, Anodontinae, and Ambleminae) of Davis & Fuller (1981). However, relationships within and among the three groups remained uncertain. For instance, Davis (1984) suggested Anodontinae might be the most basal subfamily instead of Margaritiferinae, based on a phenetic analysis of allozyme genetic distances.

Recently, Rosenberg *et al.* (1994) presented a preliminary molecular phylogeny of molluscs including Unionoidean bivalves based on sequences of about 150 nucleotides in the D6 region of the large (28S) nuclear ribosomal (rRNA) gene. Their phylogeny provided evidence for the distinction of margaritiferid species from other unionaceans, but only one phylogenetically informative site was found among the remaining 17 unionid species examined, indicating the limited utility of the D6 region of the 28S rRNA gene at this hierarchical level. Despite the efforts of many investigators, no robust, cladistically based phylogenetic hypothesis of North American unionacean mussels is available.

The pattern of macroevolutionary diversification within the North American Unionacea involved a singular history, but historic to current schemes present widely disparate views. The lack of a phylogeny and its historical content hinders progress of evolutionary and ecological studies of these species. Our central purpose was to estimate a phylogeny of North American unionacean mussels derived from DNA sequences of a portion of the mitochondrial 16S rRNA gene and compare it to classifications proposed by Heard & Guckert (1971) and Davis & Fuller (1981). We also used a 'total evidence' (Miyamoto 1985; Kluge 1989) approach wherein the molecular data are analysed in conjunction with summarized morphological data (Davis & Fuller 1981). As a result, we provide the first cladistically based phylogenetic hypothesis of North American unionaceans that incorporates both molecular and morphological data.

Table 1. *Systematic position of genera used in this study following the classification of North American unionacean mussels of* (*a*) *Heard & Guckert* (*1971*), *and* (*b*) *Davis & Fuller* (*1981*). *Actual species and sample size* (*if greater than one*) *are shown in b.*

(a) Unionacea	(b) Unionidae
Margaritiferidae	Margaritiferinae
Margaritiferinae	*Margaritifera* (*falcata*, *margaritifera*)
Margaritifera	*Cumberlandia*[1] (*monodonta*)
Cumberlandinae	Anodontinae
Cumberlandia	*Alasmidonta* (*triangulata*)
Amblemidae	*Anodonta* (*couperiana*, sp.)
Ambleminae	*Anodontoides* (*radiatus* n = 2)
Amblema	*Lasmigona* (*costata* n = 2)
Elliptoideus	*Utterbackia* (*imbecilis* n = 2)
Plectomerus	Ambleminae
Quadrula	Pleurobemini[2]
Megalonaiadinae	*Elliptio* (*dilatata*)
Megalonaias	*Pleurobema* (*pyriforme* n = 6)
Unionidae	*Uniomerus* (*obesus*)
Pleurobeminae	Lampsilini
Elliptio	(heterogenae)
Pleurobema	*Ellipsaria* (*lineolata*)
Uniomerus	*Lampsilis* (*teres* n = 2, *siliquoidea*)
Anodontinae	*Leptodea* (*fragilis* n = 2)
Alasmidonta	*Medionidus* (*conradicus*)
Anodonta	*Potamilus* (*purpuratus* n = 2)
Anodontoides	*Villosa* (*delumbis*)
Lasmigona	(mesogenae)
Utterbackia	*Obliquaria* (*reflexa* n = 2)
Lampsilinae	(ptychogenae)
(heterogenae)	*Ptychobranchus* (*subtentum*)
Ellipsaria	Amblemini[3]
Lampsilis	*Amblema* (*plicata*, *perplicata*)
Leptodea	*Elliptoideus* (*sloatianus*)
Medionidus	*Megalonaias* (*nervosa* n = 2)
Potamilus	*Plectomerus* (*dombeyanus* n = 3)
Villosa	*Quadrula* (*metanerva*, *quadrula*,
(mesogenae)	*apiculata* n = 2)
Obliquaria	
(ptychogenae)	
Ptychobranchus	

[1] Davis & Fuller (1981) recommended synonymizing *Cumberlandia* with *Margaritifera*.
[2] Although the Pleurobeminae and Pleurobemini of Heard & Guckert (1971) and Davis & Fuller (1981) appear identical based on the taxa examined, Davis & Fuller (1981) include *Fusconaia* in Pleurobemini; Heard & Guckert (1971) place *Fusconaia* in Ambleminae. In addition, Heard & Guckert (1971) include *Cyclonaias* in Pleurobeminae; Davis & Fuller (1981) place it in Amblemini.
[3] Davis and Fuller (1981) place *Cyclonaias* in their Amblemini, but leave *Fusconaia* in Pleurobemini. Heard & Guckert (1971) place *Cyclonaias* in Pleurobeminae, but leave *Fusconaia* in Ambleminae. Davis & Fuller (1981) recommend synonymizing *Megalonaias* and *Plectomerus* with *Amblema*.

2. MATERIALS AND METHODS

(*a*) *Specimen collection and taxa examined*

Twenty-three genera, representing 29 species, as currently classified (Heard & Guckert 1971; Davis & Fuller 1981) were examined (table 1). Forty-five specimens were sequenced. Localities, sources, and voucher material are available for all specimens from the authors.

(*b*) *Sequence procurement, alignment, and analysis*

Genomic DNA was isolated from frozen or 70–95 % ethanol preserved specimens by standard phenol extraction. Mitochondrial DNA(MtDNA) sequences were obtained for an amplified segment of the 16S rRNA gene using primers 16sar-L-myt (5′-CGACTGTTTAACAAAAACAT-3′) and 16sbr-H-myt (5′-CCGTTCTGAACTCAGCTCATGT-3′). These primers were designed by substituting nucleotides of 16sar-L and 16sbr-H of Palumbi *et al.* (1991) with those from homologous nucleotides determined from *Mytilus edulis* (Hoffmann *et al.* 1992). This region corresponds to positions 12887 to 13398 of the complete mitochondrial DNA sequence of *Drosophila yakuba* (Clary & Wolstenholme 1985). Approximately 100 ng of genomic DNA provided template for double stranded reactions via the polymerase chain reaction (PCR). PCR reactions were done in a 25 µl solution containing each dNTP at 0.1 mM, each primer at 1.0 µM, 40 mM $MgCl_2$, 2.5 µl 10 × Taq buffer, and 0.6 units of AmpliTaq polmerase. Reactions were amplified for 30 cycles at 92 °C for 40 sec, 50 °C for 1 min, and 72 °C for 1.5 min.

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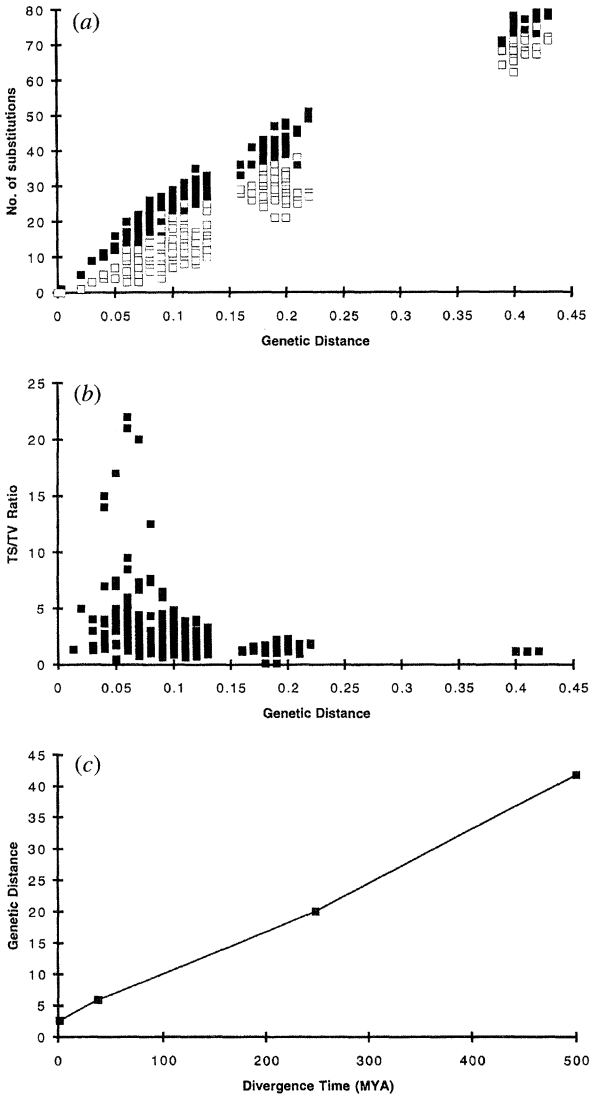

Figure 2. (*a*) Absolute number of transitions (closed boxes) and transversions (open boxes) for all pairwise comparisons plotted against percent sequence difference (genetic distance). (*b*) Ratio of transitions/transversions (TS/TV ratio) plotted against percent sequence difference. (*c*) Per cent sequence difference plotted against estimated time of divergence based on the oldest known fossils for each group.

Single-stranded DNA was obtained by asymmetric amplification, concentrated on Millipore Ultrafree-MC filters, and sequenced using the Sequenase version 2.0 kit (U.S. Biochemical) and ^{35}S-labeled dATP. Two sequencing primers were employed; the 16sbr-H-myt primer and an internal primer designed from actual sequences of unionid mussels (16Sint1-H: 5′-GAAAA-RGTAAAGYTCCGC-3′ or 16Sint2-H 5′-RGRTTG-CCCCAATCHHHC).

Sequences were entered in the software program ESEE (Cabot & Beckenbach 1989) and subsequently aligned using CLUSTAL (Higgins & Sharp 1989). A consensus sequence of the ingroup taxa was also superimposed over the hypothesized secondary structure (Gutell & Fox 1988; Gutell *et al.* 1992) of the fruitflies *Drosophila yukuba* and *Drosophila melanogaster* to further refine the alignment and identify regions corresponding to hypothesized loops and stems.

Bivariate plots of the number of transitions (TS) and number of transversions (TV) versus genetic distance (uncorrected for multiple hits) for all pairwise comparisons (figure 2) were made to examine nucleotide substitution patterns exhibited among the taxa. Based on the observed pattern of substitution, decisions were made to determine appropriate step matrices to be employed in the phylogenetic analysis.

Phylogenetic analyses were conducted using maximum parsimony (MP) of the orthologous sequences and the heuristic search option with 10 replications of random stepwise additions of PAUP (version 3.1; Swofford 1993). We employed the following options in PAUP: uninformative characters were ignored, only minimal trees were kept, and zero length branches were collapsed. A bootstrap analysis (Felsenstein 1985) with 200 iterations was conducted to estimate the internal stability of the data matrix. The marine bivalve, *Mytilus edulis*, was used as an outgroup (Hoffmann *et al.* 1992). Higher level relationships among bivalves are uncertain and we recognize the need for determining the sister group to North American unionacean mussels. A skewness test statistic (g_1) was also calculated based on the distribution of tree lengths of a random sample of 10000 topologies. Data matrices with a strong phylogenetic signal are significantly more structured than random data (Hillis & Huelsenbeck 1992).

Neighbour-joining analysis (NJ) (Saitou & Nei 1987) was done with the software program MEGA (Kumar *et al.* 1993). Distances used were absolute genetic distance values (p) and genetic distance values estimated by Kimura's (1980) two-parameter method, which corrects observed dissimilarity for multiple substitutions in sequences evolving with a transition bias. Bootstrapping (200 replicates) was done with MEGA to estimate the internal stability of the nodes of tree nodes.

3. RESULTS

(*a*) *DNA sequence variation*

The total aligned data matrix including insertions and deletions (indels) was 414 bp. Two regions (32 bp and 19 bp in length, including indels) could not be aligned unambiguously and were excluded from subsequent analyses. In all, 197 (47%) positions varied among all taxa; 65 of the 197, were restricted to differences between ingroup and outgroup taxa. A total of 120 sites (29%) were phylogenetically informative. Some variation due to indels was also detected. Of the 21 indels found among all taxa, 9 were restricted to differences between ingroup and outgroup taxa. Five indels were phylogenetically informative. Complete sequences are available from GenBank under accession numbers (U-72544 to U-72576). An aligned data matrix can be obtained from C.L.

Comparison of the mussel DNA sequence data with the proposed secondary structure for *Drosophila* (Gutell & Fox 1988; Gutell *et al.* 1992) resulted in identification of 159 stem sites, 210 loop sites, and 45 uncertain sites (additional sequence data is needed to ascertain whether uncertain sites represent loops or stems).

Approximately 43% and 39% of the variable sites were found in stems and loops, respectively. The two ambiguous areas of alignment for mussel DNA sequence data corresponded to the two larger hairpin loops of regions 928–958 and 1013–1030 in *D. yakuba* (as numbered in Guttell & Fox 1988).

Percentage sequence differences (uncorrected for multiple hits) ranged from 0% (*Plectomerus dombeyanus* $n = 3$; *Pleurobema pyriforme* $n = 6$; *Anodontoides radiata* $n = 2$; *Utterbackia imbecilis* $n = 2$; *Leptodea fragilis* $n = 2$; *Potamilus purpuratus* $n = 2$; *Obliquaria reflexa* $n = 2$) to 0.8% (*Lampsilis teres* $n = 2$) for intraspecific comparisons; 0.31% (*Amblema plicata* vs *Amblema perplicata*) to 5% (*Quadrula apiculata* vs *Quadrula metanerva*) for intrageneric comparisons; and 2.4% (within Pleurobemini and Lampsilini *sensu* Davis & Fuller 1981) to 21.6% (margaritiferid species vs other ingroup taxa) for all other interspecific comparisons among the ingroup taxa. Genetic distance values between the ingroup and outgroup taxa ranged from 39 to 43%. For phylogenetic analyses, only one sequence was included for species exhibiting no intraspecific variation.

The observed substitution patterns conformed to prior evolutionary findings for the mitochondrial 16S rRNA gene (Brown *et al.* 1979; Mindell & Honeycutt 1990). Nucleotide substitutions were the most frequently observed mutations; indels were less frequently observed. The absolute number of both TS and TV increased linearly as genetic distance increased; TS tended to outnumber TV (figure 2*a*). Between closely related species (up to roughly 10% sequence difference), TS/TV ratios indicated greater variation with some ratios approaching 22; however, among more distantly related taxa the TS/TV ratios were closer to one (figure 2*b*). As TS/TV ratios approach one, saturation due to multiple substitutions at a site may be a problem. However, TS showed no sign of levelling off, suggesting saturation may not be substantial. Typically, TS is expected to level off while TV increases linearly as genetic distances increase (Mindell & Honeycutt 1990).

Mindell & Honeycutt (1990) showed in a comparison of 16S rRNA sequence data among vertebrates that transitions did not exhibit saturation up to an estimated time of divergence of up to 300 million years. Estimates of divergence for mussels examined here are not precisely known and are based on the oldest known fossils for each group (Haas 1969*b*). A bivariate plot of genetic distance versus time of divergence reveals a linear relationship (figure 2*c*). Desalle *et al.* (1987) and Desalle (1992) found similar results. They reported the 16S rRNA gene showed little if any saturation effect with rates staying linear for approximately 200 million years as judged by comparison of sequences from *Drosophila* species and the mosquito, *Aedes albopictus*.

(*b*) *Molecular phylogenetic analysis*

Based on our analysis of nucleotide substitution patterns and the recommendation of Dixon & Hillis (1993) to downweight stem characters relative to loops due to the effects of compensatory mutations, the data were treated as follows: (1) all substitutions received equal weighting; (2) TV were weighted twice TS and (3) stems were downweighted 20% of loops. In addition, indels were treated as unique characters and assigned a zero or one for an insertion or deletion, respectively.

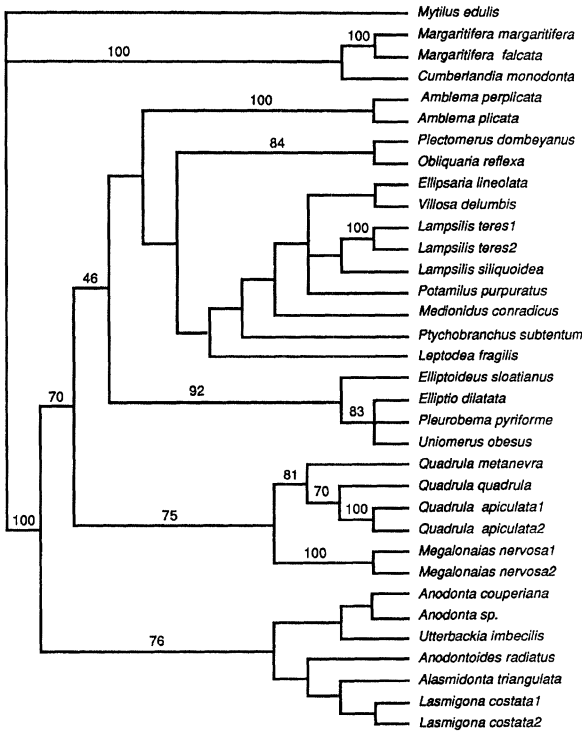

Figure 3. A strict consensus tree of four equally parsimonious trees obtained using maximum parsimony analysis of the 16S rRNA sequence data when substitutions received equal weight. The numbers at the nodes indicate the percent of replications (200 replicates) that a particular clade occurred in the bootstrap analysis.

Using maximum parsimony, four equally parsimonious trees (consistency index (I_{CON}) = 0.572 and 0.472 including and excluding uninformative characters, respectively; total length (L_{TOT}) = 538; $g_1 = -0.912$, $p < 0.01$) were obtained when all substitutions received equal weight. Figure 3 shows the strict consensus tree of the four equally parsimonious trees obtained when substitutions received equal weight. A strict consensus tree of the four most parsimonious trees (figure 3) clearly identifies two strongly supported clades: *Margaritifera* + *Cumberlandia* (Margaritiferidae), and all other unionaceans (Unionidae). Although malacologists disagree about the taxonomic ranking of these two groups, our results corroborate views recognizing their taxonomic distinction (e.g. Ortmann 1910, 1912; Heard & Guckert 1971; Davis & Fuller 1981). The family Unionidae is comprised of two well-supported monophyletic groups – the Anodontinae and Ambleminae (figure 3). The phylogenetic hypothesis depicted in figure 3 corroborates Davis & Fuller's (1981) proposed phylogeny (figure 1*b*); however, we elevated Margaritiferinae and Anodontinae + Ambleminae to family level (Margaritiferidae and Unionidae, respectively) to more accurately reflect phylogeny (Wiley 1981).

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Table 2. *Comparison of bootstrap values for the following clades: Margaritiferidae, Unionidae, Anondontinae, Ambleminae, Lampsilini, and Pleurobemini*[1] *using different analyses.*

(EW = equal weighting, TV2 × TS = transitions weighted twice transversions, ST0.8/LPS1.0 = stems weighted 20% less than loops; NJ = neighbour joining (p) absolute genetic distance, (K) Kimura's (1980) two-parameter method; TE = 'Total evidence' (16S rRNA + anatomical data set).)

	EW	TV2 × TS	ST0.8/LPS1.0	NJ (*p*)	NJ(K)	TE
Margaritiferidae	100	99	99	100	98	100
Unionidae	100	98	99	100	96	100
Anodontinae	76	84	73	99	84	98
Ambleminae	70	69	72	83	79	87
Lampsilini	< 50	< 50	< 50	< 50	< 50	75
Pleurobemini	83	82	88	92	91	96
Megalonaias + *Quadrula*	75	81	79	83	86	91

[1] Includes *Elliptio* + *Uniomerus* + *Pleurobema*.

Table 3. *Anatomical and reproductive characters from Davis and Fuller (1981) used for the total evidence analysis.*

(Characters and character states are as follows: 1 = number of marsupial demibranchs (0 = 4, 1 = 2); 2 = length of incubation (0 = bradytictic, 1 = tachytictic); 3 = septa (0 = no true septa, 1 = true septa present); 4 = water tubes (0 = no water tubes, 1 = water tubes not tripartite, 2 = tubes tripartite); 5 = supra-anal opening (0 = excurrent aperature entire, 1 = with supra-anal opening); 6 = diaphragm (0 = grossly incomplete, 1 = slightly incomplete); 7 = connective tissue at distal margin (0 = no connective tissue, 1 = additional connective tissue); 8 = glochidial teeth (0 = irregular teeth, 1 = without hooks or teeth, 2 = hooks); 9 = glochidial spines (0 = without numerous spines, 1 = with numerous spines); 10 = glochidial shape (0 = subspherical, 1 = variable, 2 = subtriangular); 11 = glochidial size (0 = small, 1 = medium, 2 = large); 12 = marsupia confined to region (0 = not confined to region, 1 = confined to region); 13 = mantle structures (0 = no specialized structures, 1 = specialized structures); 14 = shell texture (0 = smooth, 1 = heavily sculptured).)

	Anatomical Characters													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mytilus edulis	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Margaritifera margaritifera	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Margaritifera falcata	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cumberlandia monodonta	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amblema perplicata	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Amblema plicata	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Elliptoideus sloatianus	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Plectomerus dombeyanus	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Quadrula quadrula	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Quadrula metanevra	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Quadrula apiculata	0	1	1	1	1	1	0	1	0	1	1	0	0	1
Megalonaias nervosa	0	0	1	1	1	1	0	1	0	1	1	0	0	1
Elliptio dilatata	1	1	1	1	1	1	0	1	0	1	1	0	0	0
Pleurobema pyriforme	1	1	1	1	1	1	0	1	0	1	1	0	0	0
Uniomerus obesus	1	1	1	1	1	1	0	1	0	1	1	0	0	0
Anodonta couperiana	1	0	1	2	1	1	1	2	1	2	2	0	0	0
Utterbackia imbecilis	1	0	1	2	1	1	1	2	1	2	2	0	0	0
Anodontoides radiatus	1	0	1	2	1	1	1	2	1	2	2	0	0	0
Alasmidonta triangulata	1	0	1	2	1	1	1	2	1	2	2	0	0	0
Lasmigona costata	1	0	1	2	1	1	1	2	1	2	2	0	0	0
Anodonta sp.	1	0	1	2	1	1	1	2	1	2	2	0	0	0
Ellipsaria lineolata	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Lampsilis teres	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Leptodea fragilis	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Lampsilis siliquoidea	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Medionidus conradicus	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Potamilus purpuratus	1	0	1	1	1	1	0	2	0	1	1	1	1	0
Villosa delumbis	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Obliquaria reflexa	1	0	1	1	1	1	0	1	0	1	1	1	1	0
Ptychobranchus subtentum	1	0	1	1	1	1	0	1	0	1	1	1	1	0

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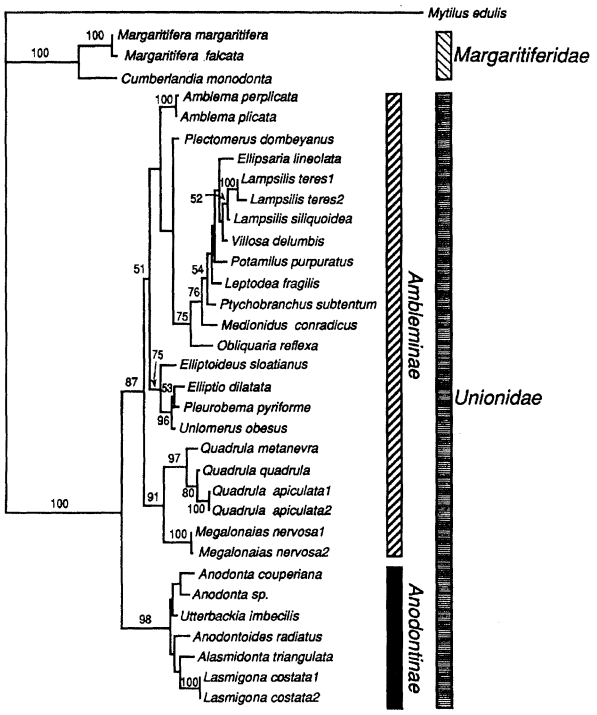

Figure 4. The single most parsimonious tree obtained using maximum parsimony analysis of the combined 16S rRNA sequence data and morphological data after one round of successive weighting. The numbers at the nodes indicate the percent of replications (200 replicates) that a particular clade occurred in the bootstrap analysis.

Within the Ambleminae (sensu Davis & Fuller 1981), three distinct clades are apparent: a basal *Megalonaias* + *Quadrula* clade, an *Elliptoideus* + *Elliptio* + *Pleurobema* + *Uniomerus* clade, and an *Amblema* + *Plectomerus* + *Obliquaria* + *Leptodea* + *Ptychobranchus* + *Medionidus* + *Potamilus* + *Ellipsaria* + *Villosa* + *Lampsilis* clade. The *Megalonaias* + *Quadrula* clade is strongly supported (boostrap frequency of 75%); however, neither Davis & Fuller (1981) nor Heard & Guckert (1971) recognized such a group exclusive of *Amblema*. With the exception of *Elliptoideus*, the *Elliptio* + *Pleurobema* + *Uniomerus* clade supports the monophyly of the Pleurobemini. Additional taxa (e.g. *Fusconaia* and *Cyclonaias*) need to be sequenced to determine their relationships to the Pleurobemini. The monophyly of the Lampsilini was supported including *Plectomerus* as sister to *Obliquaria*. The former had been placed in in Amblemini (Davis & Fuller 1981) or Ambleminae (Heard & Guckert 1971). Together *Plectomerus* and *Obliquaria* are sister to the remaining Lampsilini genera including *Leptodea* + *Ptychobranchus* + *Medionidus* + *Potamilus* + *Ellipsaria* + *Villosa* + *Lampsilis*.

Eight trees of equal parsimony ($L_{TOT} = 685$; $g_1 = -0.901$, $p < 0.01$) were found when TV was weighted twice TS. A strict consensus tree of these eight trees is similar to the tree shown in figure 3, however, the clade comprised of *Megalonaias* + *Quadrula* is depicted as sister to *Elliptoidius* + *Elliptio* + *Pleurobema* + *Uniomerus*. This alternative placement is not surprising given the generally weak support, based on low bootstrap values, for higher relationships within the Ambleminae. In addition, the TV weighted twice TS strict consensus tree is less resolved, with *Megalonaias* + *Quadrula* + *Elliptoidius* + *Elliptio* + *Pleurobema* + *Uniomerus*, *Amblema plicata* + *Amblema perplicata*, and *Leptodea* + *Ptychobranchus* + *Medionidus* + *Potamilus* + *Ellipsaria* + *Villosa* + *Lampsilis* forming an unresolved trichotomy. The lack of resolution obtained when weighting TV may indicate the loss of valuable phylogenetic information provided by TS and supports our view that saturation is not a substantial problem.

Two equally parsimonious trees ($L_{TOT} = 4578$, $I_{CON} = 0.559$, 0.468 including and excluding uninformative characters, respectively; $g_1 = -0.8789$, $p < 0.01$) were obtained when stems were downweighted relative to loops. The strict consensus tree of the two equally parsimonious trees based on downweighting stems is identical to the tree shown in figure 3 except it resolves the unresolved trichotomy of *Elliptio*, *Pleurobema*, and *Uniomerus* with *Elliptio* and *Uniomerus* being sister taxa.

A neighbour-joining analysis (Saitou & Nei 1987) using genetic distances *p* and Kimura's two-parameter method resulted in trees similar to the tree shown in figure 3. Differences include switching in the placement of Lampsilini members (*Medionidus*, *Ptychobranchus*, *Leptodea*), and *Amblema* being sister to the *Elliptoideus* + Pleurobemini members. High bootstrap values were obtained for several clades, particularly the clade including all Ambleminae members exclusive of *Megalonaias* + *Quadrula*. Table 2 summarizes bootstrap values obtained for the various analyses employed. Although bootstrap values are not actual confidence limits, they indicate the internal stability of the data set. Hillis & Bull (1993) showed under certain conditions that bootstrap values of 70% offer substantial support for a given clade.

The molecular phylogeny of North American unionaceans is incongruent with the classification of Heard & Guckert (1971) (figure 1*a*), but supports that of Davis & Fuller (1981; figure 1*b*). Davis & Fuller (1981) hypothesized that Ambleminae and Anodontinae were sister groups, which in turn are sister to the Margaritiferinae. Although we changed the taxonomic ranks of the taxa to accurately reflect their phylogenetic relationships, our molecular phylogeny supports their hypothesized phylogeny.

(*c*) *Total evidence*

A data matrix comprised of 14 anatomical or reproductive characters (table 3) was constructed from data in Davis & Fuller (1981; tables 10 and 11). Maximum parsimony analysis of the 14 morphological characters combined with the 16S rRNA DNA sequence data (equally weighted for all substitutions) yielded 138 equally parsimonious trees ($L_{TOT} = 568$; $g_1 = -0.888$, $p < 0.01$) with little overall resolution. For instance, Margaritiferidae, Anodontinae, and Ambleminae are depicted as monophyletic, but within the Ambleminae only three monophyletic groups were identified by all trees: *Amblema perplicata* + *Amblema plicata*, *Pleurobema* + *Elliptoidius*, and *Quadrula* + *Megalonaias*. Breakdown in resolution relative to the molecular phylogeny (figure 3) is likely due to homoplasy in several anatomical characters. We chose to apply

'successive weighting' (Farris 1969; Carpenter 1988; Williams & Fitch 1989) to the complete data set in which the more consistent characters are weighted more heavily *a posteriori*. Characters were re-weighted on the basis of rescaled consistency index (Farris 1989) using a base weight of 1000. After one round of successive weighting one tree was obtained that is equivalent to one of the 138 most parsimonious trees. The single most parsimonious tree is similar to the molecular phylogeny (figure 3) with the exception of minor differences within the Lampsilinae taxa. Including the anatomical data breaks the sister relationship of *Obiquaria reflexa* + *Plectomerus dombeyanus* and places *P. dombeyanus* as sister to the Lampsilini taxa with *O. reflexa* as the basal-most member.

4. DISCUSSION

(*a*) *Evolution of select anatomical features in North American unionaceans*

The phylogeny based on the combined molecular and anatomical data (figure 5), depicts the evolution of a number of anatomical characters that have figured prominently in the systematics of North American freshwater unionaceans. The family Unionidae (figure 5) is diagnosed by three unique, unreversed synapomorphies – presence of true septa, presence of a supra-anal opening, and the presence of a slightly incomplete diaphragm. This agrees with the classification schemes of Ortmann (1910), Heard & Guckert (1971), and Davis & Fuller (1981). Traditionally, the Margaritiferidae were diagnosed largely on the absence of derived characters found in ostensibly advanced unionaceans. This tendency is revealed by the absence of anatomical synapomorphies uniting the family, but the monophyly of Margaritiferidae is is strongly supported by the 16S rRNA gene.

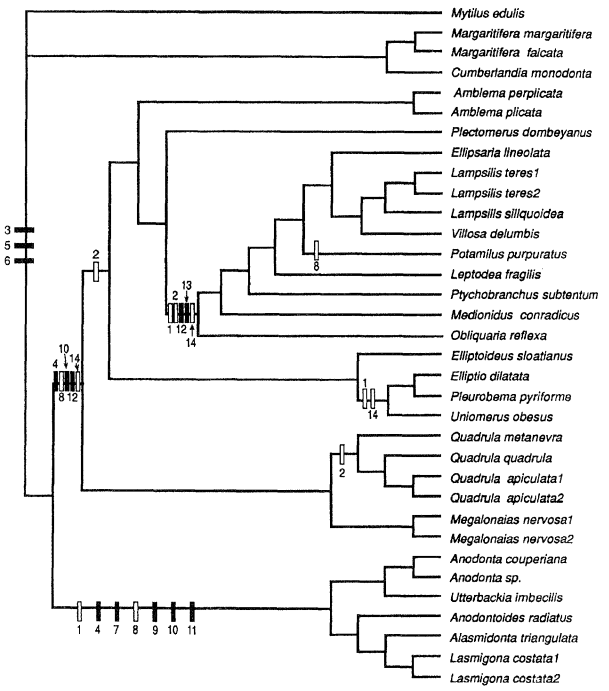

Figure 5. The single most parsimonious tree obtained using maximum parsimony analysis of the combined 16S rRNA sequence data and morphological data after one round of successive weighting. The numbers at the nodes correspond to unique anatomical synapomorphies (dark bars) or homoplasious synapomorphies (open bars) as indicated in table 3.

Both subfamilies within the Unionidae are diagnosed by the presence of several anatomical synapomorphies. The subfamily Anodontinae is recognized by five unique, unreversed synapomorphies: tripartite water tubes, additional connective tissue at the distal margin of the marsupial demibranch, presence of numerous spines on the glochidium, a subtriangular-shaped glochidium, and a large glochidium. In addition, two homoplasious synapomorphies (ectobranchous and glochidium with hooks) unite the subfamily as well. The subfamily Ambleminae is diagnosed by three unique, unreversed synapomorphies – non-tripartite water tubes, variable shaped glochidium, and a medium-sized glochidium, and two homoplasious synapomorphies – glochidia without hooks or teeth and shell texture heavily sculptured. The molecular phylogeny provides independent support for the recognition of these clades.

Two tribes within the Ambleminae can be distinguished based on anatomical characters, the Lampsilini and the Pleurobemini. The Pleurobemini is diagnosable by the ectobranchous condition and possessing smooth shells. Lampsilini is characterized by two unique, unreversed synapomorphies (marsupia confined to a restricted region and presence of specialized mantle structures), and three homoplasious synapomorphies (ectobranchous, bradytictic, smooth shell structure). The 16S rRNA gene also supports the monophyly of these two tribes, but *Obliquaria* is sister to *Plectomerus*. Together these two taxa are sister to the remaining Lampsilini members (figure 3). *Plectomerus* is not a *Lampsilini* member and its sister-species status with *Obliquaria* would require an additional evolutionary step for five anatomical features. *Obliquaria* is placed in an informal, unusual subtribal taxon called Mesogenae, meaning its marsupial demibranch is located in the middle section of the demibranch. Most Lampsilini members are Heterogenae, where the posterior section of the demibranch is marsupial. The strong molecular support for the *Obliquaria* + *Plectomerus* sister status suggests, the homology of marsupial characters needs to be re-addressed. On the other hand, the mtDNA gene tree may not fully reflect the gene tree (Avise 1994). Until further data are examined, we tentatively recognize the monophyly of the Lampsilini and the phylogeny depicted by the total evidence approach (figure 4, 5).

Based on the total evidence tree (figure 4, 5), four anatomical characters exhibited homoplasy among North American unionaceans: number of marsupial demibranchs, length of the incubation period, hooked or hookless glochidia, and shell texture. Two characters were pivotal for classification decisions of Heard & Guckert (1971): ectobranchous condition and length of the incubation period. Heard & Guckert (1971) hypothesized that the ectobranchous condition evolved once in a lineage they classified as Unionidae (figure 1*a*; table 1*a*). The total evidence phylogeny indicates

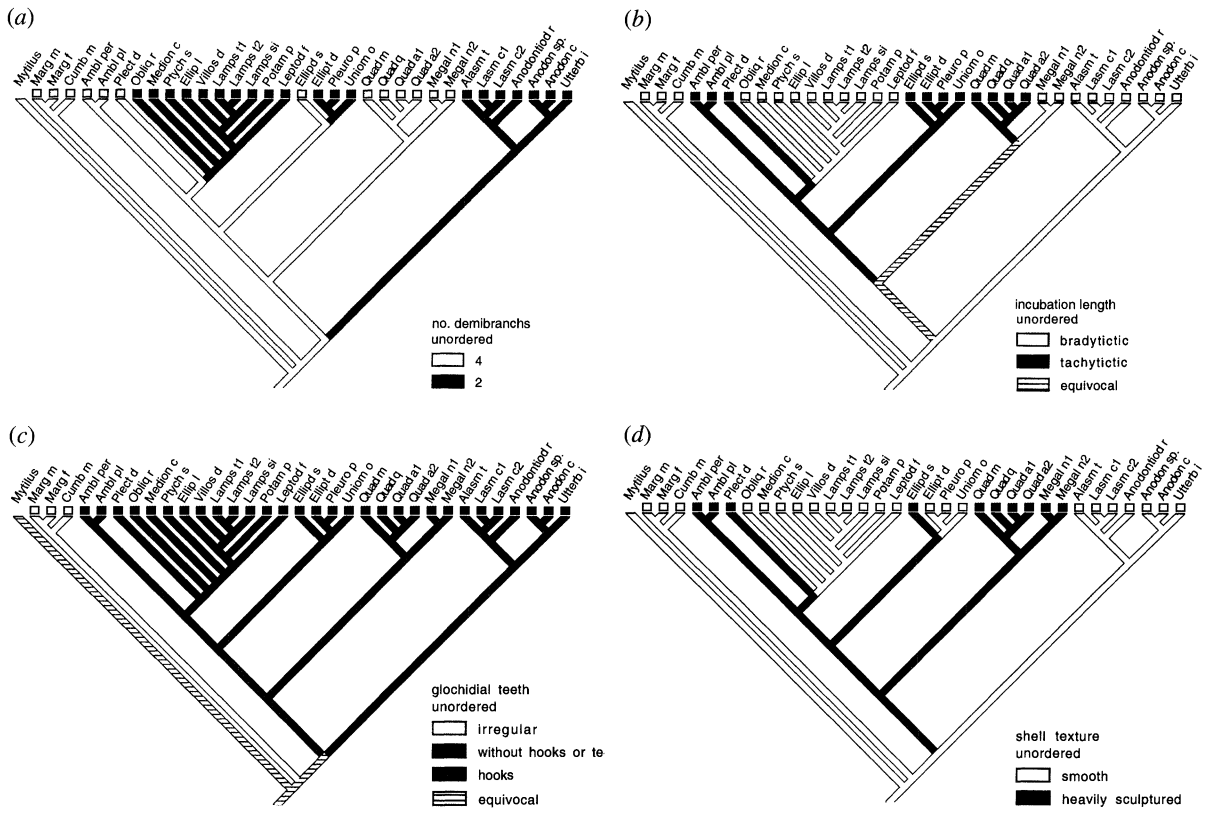

Figure 6. Anatomical character evolution based on the total evidence phylogeny. (*a*) number of demibranchs (*b*) length of incubation (*c*) glochidial teeth (*d*) shell texture. See text for discussion.

the ectobranchous condition evolved independently three times from the tetragenous condition (figure 6*a*): once in the Lampsilini, the Pleurobemini, and the Anodontinae. Heard & Guckert (1971) recognized the bradytictic and tachytictic condition evolved independently in different lineages but still relied on the character to construct their classification. For example, their family Amblemidae is principally tachytictic, except for their subfamily Megalonaiadinae, which was diagnosed by the bradytictic condition. As indicated by the total evidence phylogeny (figure 6*b*), the bradytictic and tachytictic conditions have evolved several times, supporting Davis & Fuller's (1981) argument of the unreliability of the character for estimating unionacean relationships. The remaining homoplasious characters are hooked or hookless glochidia and shell texture. Hookless glochidia evolved from glochidia with irregular teeth in the subfamily Ambleminae, and hooked glochidia is a synapomorphy of the subfamily Anodontinae (figure 6*c*). The Lampsiline genus *Potamilus* (Hoggarth 1988) has evolved hooks independently. However, closer examination of the placement of the hooks and hook morphology indicates the types of hooks found in *Potamilus* and the Anodontinae are not homologous (Davis & Fuller 1981; Hoggarth 1988) and should be coded accordingly. Davis & Fuller (1981) proposed that heavily sculptured shells were a synapomorphy uniting their tribe Amblemini (table 1*b*, figure 1*b*). However, the total evidence phylogeny (figure 6*d*) indicates that heavily sculptured shells evolved once in the ancestor of the Ambleminae, and subsequently, was lost twice, once in the Pleurobemini and once in the Lampsilini. Davis & Fuller's (1981) proposed synapomorphy of Amblemini is a symplesiomorphy and does not indicate shared common ancestry.

(*b*) *Phylogeny of North American unionacean mussels*

The phylogenetic hypothesis of North American unionaceans based on the 16S rRNA gene data and combined anatomical data and mtDNA sequence data concurs with the classification scheme of Davis & Fuller (1981). Strong support was found for the monophyly of the families Margaritiferidae and Unionidae, the subfamilies Anodontinae and Ambleminae, and the tribe Pleurobemini. Weaker support was found for the monophyly of Lampsilini.

Equally noteworthy are differences associated with taxa placed in Davis & Fuller's Amblemini (table 1*a*) and Heard & Guckert's (1971) Amblemidae (table 1*b*). Heard & Guckert (1971) divided Amblemidae into three subfamilies – Gonideinae, Ambleminae, and Megalonaiadinae. Gonideinae was recognized as distinct by the presence of perforated septa and included only *Gonidea angulata*. Ambleminae was diagnosed by non-perforated septa, and tachytictic reproduction. The genus *Megalonaias* was placed in the subfamily Megalonaiadinae because it was bradytictic. Davis & Fuller (1981) suggested Heard & Guckert's Amblemidae was artificial due to placing all tetragenous species, exclusive of Margaritiferidae, into one family, and for diagnosing subfamilies based on length of breeding season. In addition, they removed *Fusconaia* and placed it into Pleurobemini. The remaining

taxa *Amblema* + *Megalonaias* + *Plectomerus* + *Cyclonaias* + *Quadrula* + *Quincuncina* + *Tritogonia* constituted their tribe Amblemini, which was largely diagnosed by the presence of heavily sculptured shells and being mostly tetragenous. Our molecular and total evidence phylogenies do not support either of these classifications, although position of *Gonidea* and *Fusconaia* cannot be assessed at this time. It is evident that both Heard & Guckert's (1971) Amblemidae and Davis & Fuller's (1981) Amblemini were diagnosed by plesiomorphic (e.g. tetragenous or heavily sculptured shells) characters.

Based on a shared anatomical character (i.e. simple incurrent papillae) and relatively low immunological distances, Davis & Fuller (1981) recommended synonimizing *Megalonaias* and *Plectomerus* with *Amblema*. No support was found for the monophyly nor the recommendation of synonymizing the genera. Simple incurrent papillae also are found in other genera, suggesting this character does not provide evidence of monophyly. Our findings also are supported by a recent examination of intra-generic DNA and allozyme variation throughout the range of two of the three genera, *Megalonaias* and *Amblema* (Mulvey *et al.* 1996).

(*c*) *Utility of the 16S rRNA gene in assessing relationships among unionaceans*

The 16S rRNA gene is useful for assessing higher relationships among North American unionaceans. The 16S rRNA gene provided strong evidence for the recognition and monophyly of the unionacean sister families Margaritiferidae and Unionidae. The DNA sequence data also supported the monophyly of the subfamilies Anodontinae and Ambleminae within the Unionidae. No substantial degree of saturation was indicated by an analysis of nucleotide substitution patterns, suggesting the gene should be of use systematic studies within the Unionacea. However, it is likely the gene evolves too quickly to assess relationships among all bivalves. For these phylogenetic questions, the more conservative nuclear ribosomal genes (Davis 1994; Rosenberg *et al.* 1994) is recommended.

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