Conservation Genetics of North American Freshwater Mussels *Amblema* and *Megalonaias*

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Abstract: *Freshwater bivalves are among the most endangered groups of organisms in North America. Efforts to protect the declining mussel fauna are confounded by ambiguities associated with recognition of distinct evolutionary entities or species. This, in part, is due to the paucity of reliable morphological characters for differentiating taxa. We have employed allozymes and DNA sequence data to search for diagnosably distinct evolutionary entities within two problematic genera of unionid mussels,* Amblema *and* Megalonaias. *Within the genus* Amblema *three species are recognized based on our DNA sequence data for the mitochondrial 16S rRNA and allozyme data (*Amblema neislerii, A. plicata, *and* A. elliotti*). Only one taxonomically distinct entity is recognized within the genus* Megalonaias—M. nervosa. Megalonaias boykiniana *of the Apalachicolan Region is not diagnosable and does not warrant specific taxonomic status. Interestingly,* Megalonaias *from west of the Mississippi River, including the Mississippi, exhibited an allozyme and mtDNA haplotype frequency shift suggestive of an east-west dichotomy. The results of this study eliminate one subspecies of* Amblema *and increase the range of* A. plicata. *This should not affect the conservation status of "currently stable" assigned to* A. plicata *by Williams et al. (1993). The conservation status of* A. elliotti *needs to be reexamined because its distribution appears to be limited to the Coosa River System in Alabama and Georgia.*

Conservación Genética de las Ostras Americanas de Agua Dulce *Amblema* y *Megalonaias*

Resumen: *Los bivalbos de agua dulce se encuentran entre los grupos de organismos mas amenazados de Norte América. Los esfuerzos para proteger la disminución de poblaciones de mejillones son confusos debido a ambiguedades associadas con el reconocimineto de entidades evolutivas o especies distintivas. Esto se debe en parte a la escasés de caracteres morfológicos confiables para la diferenciación de taxas. Nosotros hemos empleado datos de alozimas y secuencias de ADN para buscar entidades evolutivas distintivas diagnosticables dentro de dos generos de mejillones uniónidos,* Amblema *y* Megalonaias. *Dentro del género* Amblema, *tres especies son reconocidas en base a datos de secuencias de ADN del ARNr mitocondrial 16S y datos de alozimas (*Amblema neislerii, A. plicata *y* A. ellioti*). Unicamente una entidad taxonómica distintiva es reconocida dentro del género* Megalonaias - M. nervosa. Megalonaias boykiniana *de la región de los Apalaches no es diagnosticable y no garantiza un estatus taxonómico específico. Sin embargo,* Megalonaias *del oeste del río Mississippi, incluyendo el mismo Mississippi, exhiben una alozima y una desviación en la frecuencia de haplotipo del DNAmt sugiriendo una dicotomía este-oeste. Los resultados de este estudio eliminan una subespecie de* Amblema *e incrementan el rango de* A. plicata. *Esto no debería afectar el estatus de conservación de "actualmente estable" asignado a* A. plicata *por Williams, et al. (1993). El estatus de conservación para* A. ellioti *necesita ser reexaminado puesto que su distribución parece estar limitada a la Coosa Río System de Alabama y Georgia.*

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Introduction

Bivalve mollusks represent an important element in the faunal diversity of freshwater habitats and may exceed by an order of magnitude the biomass of all other benthic organisms in a stream (Negus 1966). The greatest freshwater mussel diversity in the world is found in North America and consists of nearly 300 species and subspecies in two families (Unionidae and Margaritiferidae) (Turgeon et al. 1988). Much of this fauna is concentrated in the southeastern United States (Lydeard & Mayden 1995; Williams & Neves 1995). Currently, 21 taxa (7% of the fauna) are presumed extinct and an additional 120 taxa (40% of the fauna) are considered threatened (Williams & Neves 1995). Habitat alterations including impoundments, channelization, sedimentation, pollution, and the introduction of nonindigenous bivalves (Asian clam [*Corbicula fluminea* Müller 1774], and zebra mussel [*Dreissena polymorpha* Pallas 1771]) contribute to the decline of this fauna (Williams et al. 1993).

Freshwater mollusks are among the most endangered groups of animals in North America (Master 1990), and efforts for their conservation depend upon the recognition of evolutionarily significant units. Nevertheless, recognition of species among freshwater bivalves has been a contentious area because traditional classifications are conchologically based. Shell characteristics, such as shape, dimensions, sculpture, and degree of inflation, are highly variable and known to vary geographically and in response to environmental conditions (Johnson 1970). Nineteenth century investigators such as Isaac Lea, who described over 800 nominal species of *Unionacea* and *Mutelacea*, applied names to each shell phenotype (Johnson 1970). Other researchers explained shell variation as phenotypic plasticity and synonymized many phenotypes (Simpson 1914; Johnson 1970). Delineation of freshwater mussel taxa based on shell morphology can present some perplexing taxonomic problems and complicate conservation efforts (Williams & Mulvey 1994). As discussed by Davis (1994), molluscan taxonomic issues might be better resolved if molecular techniques were used in conjunction with ecological, anatomical, and distributional data, an approach rarely applied to unionid mussels.

We examined two widespread genera from the southeastern United States: *Amblema* and *Megalonaias*. These genera, both members of the subfamily Ambleminae (*sensu* Lydeard et al., 1996), were chosen because they are widely distributed and thought to be comprised of two or more evolutionarily distinct entities that have been a source of taxonomic confusion and because recognition of these species may have an impact on their conservation or listing status. Additionally, both are commercially harvested for use as seeds for the Indo-Pacific pearl trade, a three billion dollar a year industry.

Currently two species are recognized in the genus *Amblema*. The most widespread species, *A. plicata*, is generally recognized as having two subspecies (Turgeon et al. 1988). The northern subspecies, the threeridge (*A. p. plicata* Say 1817), occurs from the St. Lawrence River drainage throughout the upper Mississippi, Ohio, Cumberland, and Tennessee rivers. A southern subspecies, the roundlake (*A. p. perplicata* Conrad 1841), is described from the lower Mississippi River drainage and Gulf Coast rivers from west Florida (Choctawhatchee River) to central Texas. The second species, the fat threeridge (*A. neislerii* Lea 1858), is endemic to the Apalachicola River drainage in Florida and Georgia (Clench & Turner 1956; Williams & Butler 1995).

Two species are assigned to the genus *Megalonaias* (Turgeon et al. 1988). The washboard (*Megalonaias nervosa* Rafinesque 1820) occurs throughout the western Gulf of Mexico drainages from the Alabama River drainage in Alabama and Georgia west to Nuevo Leon, Mexico, and historically was found throughout the entire Mississippi River system. The round washboard (*M. boykiniana* Lea 1840) ranges from the Escambia River drainage east to the Ochlockonee River drainage, including the Apalachicola, Chatahoochee, and Flint drainages.

Based on shared morphological and conchological features, Hurd (1974) did not recognize the subspecies *A. plicata plicata* and *A. p. perplicata*. Additionally, the highly plastic shell form of *Amblema* has made it difficult to determine whether *A. neislerii* is conspecific (an ecophenotype) with *A. plicata* (Frierson 1927). Similarly, *M. boykiniana* has not been uniformly recognized as a taxon distinct from *M. nervosa* (Burch 1975). Confounding the issue further, immunological evidence reported by Davis and Fuller (1981) suggested that *Amblema* and *Megalonaias* were congeneric.

The fat threeridge and the round washboard are two of approximately 30 mussel taxa endemic to the Apalachicolan Region (Butler 1989), which consists of river systems flowing into the Gulf of Mexico from the Escambia to the Suwannee. Recent surveys suggested that both species were in decline, with restricted distributions and generally small population sizes (Butler 1993). In 1989 and 1991 the U.S. Fish and Wildlife Service (USFWS) published two Animal Notices of Review (USFWS 1989, 1991) including *Amblema neislerii* as a category 2 candidate. Although *Megalonaias boykiniana* was not included in the 1989 or 1991 Notices, a conservation status survey of mussels in the Apalachicola and Ochlockonee rivers revealed that *M. boykiniana* had disappeared from much of its range (Butler 1993). In August 1994 the USFWS proposed *A. neislerii* as endangered under the Endangered Species Act (USFWS 1994).

We have undertaken an analysis of genetic variation using protein electrophoresis and DNA sequencing of *A.*

neislerii and *M. boykiniana* to clarify their status and relationships to their respective congeners. Specifically, we describe genetic variation among populations within currently recognized species and then describe genetic differentiation between sister taxa. Recognition of genetically and evolutionarily distinct entities is an essential aspect of conservation efforts for these freshwater mussels.

Methods

Taxa Examined and Specimens Collected

Amblema neislerii is conchologically similar yet distinct from its currently recognized congeners, *A. p. plicata* and *A. p. perplicata*, both of which are widespread and abundant. *Amblema neislerii* reaches a length of about 100 mm (Williams & Butler 1995). The seven to nine horizontal parallel ridges and the highly inflated appearance of larger specimens make *A. neislerii* one of the most distinctive unionids in the Apalachicolan Region and easily distinguishable from *A. plicata sensu latu*. The soft anatomy of this species has not been described, although Heard and Guckert (1971) noted that members of the *Ambleminae* had septa and water-tubes that were well-developed and continuous but not perforated, and they considered this subfamily to be tachytictic (short-term breeders).

Megalonaias boykiniana is the largest freshwater mussel found in the Apalachicolan Region and reaches lengths of over 200 mm (Williams & Butler 1995). In the course of examining museum material to determine historic distributions, it became evident that conchological characters alone did not unambiguously distinguish *M. boykiniana* from the widely distributed *M. nervosa* (J. D. Williams and J. Brim-Box, unpublished data). The anatomy of this species has not been well described, but Heard and Guckert (1971) noted that the septa and water-tubes of members of the genus *Megalonaias* are well developed and continuous as in other amblemines. Utterback (1915), Coker et al. (1921), and Heard and Guckert (1971) suggested that members of this genus were bradytictic (long-term breeders), whereas Howard (1914) and Lefevre and Curtis (1910) have suggested a tachytictic breeding period. In addition, Woody and Holland-Bartels (1993) considered *M. nervosa* to be a late, short-term breeder in Wisconsin, with fertilization and glochidia formation occurring between July and August and glochidia release occurring between September and November.

Specimens representing the currently recognized species and subspecies were obtained from sources shown in Table 1. The anodontine (*Utterbackia imbecillis* Say

Table 1. Localities for *Amblema* and *Megalonaias* specimens and sample labels for the allozyme phenogram.

Species[a]	*Drainage*	*Location*[b]	*Label*[b]
Amblema neislerii	Apalachicola River	Gadsden Co., FL	FL
Amblema plicata	Pearl River	Hinds Co., MS	MS
	Amite River	Baton Rouge Parish, LA	LA
	Neches River	Jasper Co., TX	TX1
	Guadaloupe River	Gonzoles Co., TX	TX3
	Mississippi River	Vernon Co., WI	WI
	Muskingum River	Washington Co., OH	OH
	Big Darby Creek	Pickaway Co., OH	OH1
	Duck River	Marshall Co., TN	TN2
	Conech River	Escambia Co., AL	AL1
	Pascagoula River	Jackson Co., MS	MS1
	Bogue Chitto	Dallas Co., AL	AL2
	Big Swamp Creek	Lowndes Co., AL	AL3
Amblema elliotti	Conasauga River	Murray/Whitfield Co., GA	GA
(formerly *A. p. perplicata*)	Coosa River	Shelby Co., AL	AL
Megalonaias boykiniana	Apalachicola River	Gadsden Co., FL	FL
(= *nervosa*)	Ochlockonee River	Gadsden/Leon Co., FL	FL1
Megalonaias nervosa	Coosa River	Shelby Co., AL	AL
	Alabama River	Monroe Co., AL	AL1
	Alabama River	Baldwin/Monroe Co., AL	AL2
	Kentucky Lake	TN	TN1
	Little Brazos River	Robertson Co., TX	TX2
	Guadaloupe River	Gonzoles Co., TX	TX3
	Neches River	Jasper Co., TX	TX1
Utterbackia imbecillis	Black River	Williamsburg Co., SC	SC

[a] *Species names are based on analyses presented in the text.* Utterbackia imbecilis *served as the outgroup.*
[b] *FL, Florida; MS, Mississippi; LA, Louisiana; TX, Texas, WI; Wisconsin; OH, Ohio; TN, Tennessee; GA, Georgia; SC, South Carolina.*

Table 2. Allozyme frequencies for *Amblema* and *Megalonaias* samples.[a]

	Amblema[b]									Megalonaias[b]									Utterbackia imbecillis[b]
	neislerii	elliottii		plicata						boykiniana		nervosa							
	FL	*AL*	*GA*	*MS*	*TX1*	*TX3*	*LA*	*WI*	*OH*	*FL*	*FL1*	*AL*	*AL1*	*AL2*	*TN1*	*TX1*	*TX3*	*TX2*	*SC*
Acon																			
1	0.07																		
2	0.93	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
3																			1.00
6pgd																			
1	0.06																		
2	0.75	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.90	1.00	1.00	1.00	1.00	0.90	1.00	1.00	0.50	1.00
3	0.19									0.10								0.50	
4															0.10				1.00
ppPep																			
1	0.19	1.00	1.00	1.00	1.00	0.90	1.00	1.00											
2	0.81					0.10			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3																			
lcd																			
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.12									
2																			
3										0.88	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
4																			0.75
5																			0.25
f-Est																			
1	0.12																		
2	0.88	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00										
3										1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
4																			1.00
Mdh-1																			
1	1.00				0.10					1.00	1.00	0.75	0.75	1.00	1.00	1.00	1.00	1.00	
2		0.50		1.00	0.90	1.00	0.57		1.00				0.25						
3		0.50	1.00																1.00
4												0.25							
5							0.43	1.00											
Mdh-2																			
1																			1.00
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
p-Est																			
1																			
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
3																			1.00
Me																			
1	1.00	0.25								1.00	1.00	0.75	1.00	0.50					
2		0.75	1.00	1.00	0.10									0.50	1.00	1.00	1.00	1.00	1.00
3					0.90	1.00	1.00	1.00	1.00										
4												0.25							
Gpd																			
2	0.25	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.80	1.00	0.75	0.83	1.00	1.00	1.00	1.00	1.00	1.00
3	0.75									0.20		0.25	0.17						
Mpi																			
1	0.13		0.33																
2	0.87	1.00	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.80	0.50	0.50	1.00	1.00
3															0.20				
Pgm-1																			
1	0.06			0.50															1.00
2	0.94	0.50	1.00	0.50	1.00	1.00	0.86												
3		0.50					0.14	1.00	1.00	0.87	1.00	1.00	1.00	1.00	0.90	1.00	1.00	1.00	
4															0.10				
5										0.13									

Table 2. Continued.

	Amblema[b]									Megalonaias[b]									Utterbackia imbecilis[b]
	neislerii	elliottii		plicata						boykiniana		nervosa							
	FL	*AL*	*GA*	*MS*	*TX1*	*TX3*	*LA*	*WI*	*OH*	*FL*	*FL1*	*AL*	*AL1*	*AL2*	*TN1*	*TX1*	*TX3*	*TX2*	*SC*
Pgm-2																			
1	0.87	1.00	1.00																1.00
2	0.13												0.17						
3				0.92	1.00	1.00	0.79		1.00						0.10				
4				0.08			0.21	1.00		1.00	1.00	0.50	0.50		0.90	1.00	1.00	1.00	
5												0.50	0.33	1.00					
Gpi																			
1			1.00				0.07												
2	1.00	1.00		1.00	1.00	1.00	0.93	1.00	1.00										
3										1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
4																			1.00
N	7.80	2.00	2.80	11.50	4.90	4.90	6.90	1.00	1.00	4.20	3.90	2.00	2.80	1.90	4.90	1.90	1.90	2.00	1.90
	(0.2)	(0)	(0)	(0.3)	(0.1)	(0.1)	(0.1)	(0)	(0)	(0.1)	(0)	(0)	(0.2)	(0)	(0)	(0.1)	(0.1)	(0)	(0.1)

[a] *N = mean sample size per locus.*
[b] *FL, Florida; MS, Mississippi; LA, Louisiana; TX, Texas; WI, Wisconsin; OH, Ohio; TN, Tennessee; GA, Georgia; SC, South Carolina.*

1829) was used as an outgroup. Systematic relationships of *Utterbackia*, *Amblema*, and *Megalonaias* have been discussed in Davis and Fuller (1981) and are used in Table 1; we do not, however, synonymize *Amblema* and *Megalonaias*. Sample sizes for some populations were small due to the difficulty in obtaining material; therefore, not every population is represented in both the allozyme and DNA analyses. Foot tissue samples were taken from all specimens and stored at −70°C. Voucher specimens have been deposited at The Academy of Natural Sciences, Philadelphia, Pennsylvania.

Protein Electrophoresis

Starch gel electrophoresis was conducted to determine allozyme genotype for 14 enzyme-determining loci. The following combinations of buffers and stains were used: amine-citrate, pH 6.0 (Clayton & Tretiak 1972) for isocitrate dehydrogenase (Icd) and NAD-malate dehydrogenase (Mdh); tris-citrate-EDTA, pH 7.1 (Ayala et al. 1972) for aspartate aminotransferase (Aat), β-naphthyl-propionate esterase (p-Est), and aconitate hydratase (Ah); tris-borate-EDTA, pH 8.0 (Selander et al. 1971) for α-glycerophosphate dehydrogenase (Gpd), NADP-malate dehydrogenase (Me), fluorescent esterase (f-Est), and glucosephosphate isomerase (Gpi); tris-citrate, pH 8.0 (Selander et al. 1971) for phosphoglucomutase (Pgm), mannose phosphate isomerase (Mpi), and 6-phosphogluconate dehydrogenase (Pgd); and lithium hydroxide, pH 8.2 (Selander et al. 1971) for peptidases with glycylleucine (gl-Pep) and phenylalanylproline (pp-Pep) substrates. For multilocus systems loci are numbered in order of decreasing anodal mobility. Similarly, allozymes of polymorphic loci are designated in decreasing anodal mobility (e.g., allozyme 1 faster than allozyme 2). Samples of *A. neislerii* and *M. boykiniana* were run on all gels to serve as reference material for allozyme mobilities. Additionally, samples from several populations and taxa were run on each gel to facilitate comparison of allozyme mobilities. Allozyme data were analyzed using BIOSYS-1 (Swofford & Selander 1981). Genetic distance values among populations and taxa were generated using the method of Nei (1978) and a phenogram made using UPGMA.

Sequence Procurement, Alignment, and Analysis

Genomic DNA was isolated from foot tissue by standard phenol extraction (Sambrook et al. 1989). Mitochondrial DNA 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). Approximately 100 ng of genomic DNA provided template for double-stranded reactions via the polymerase chain reaction in a 25-μL reaction solution containing each dNTP at 0.1 mM, each primer at 1.0 μM, 40 mM $MgCl_2$, 2.5 μL 10x Taq buffer, and 0.6 units of AmpliTaq polymerase. Reactions were amplified for 30 cycles at 92°C for 40 seconds, 50°C for 1 minute, and 72°C for 1.5 minutes.

Single-stranded DNA was obtained by asymmetric amplification, purified and concentrated with Millipore Ultrafree-MC filters, and sequenced using the Sequenase version 2 kit (U. S. Biochemical) and ^{35}S-labeled dATP. The two sequencing primers used were the 16sbr-H-myt5′-GCGGAACTTTACCTTTTC-3′ and 16Sint2-H-5′-RGRTTGCCCCAATCHHHC.

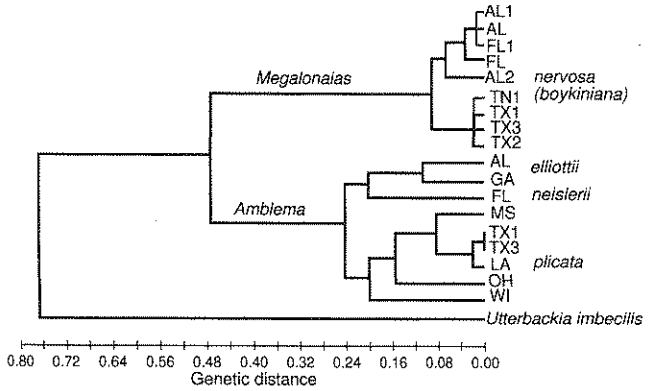

Figure 1. UPGMA phenogram based on Nei (1978) genetic distance values. Abbreviations are given in Table 1. Utterbackia imbecillis *was used as an outgroup.*

Sequences were entered into a database using ESEE (Cabot & Beckenbach 1989) and then were aligned by eye and with CLUSTAL (Higgins & Sharp 1989). Phylogenetic analyses were conducted using maximum parsimony of the orthologous sequences using the branch-and-bound procedure of PAUP (version 3.1; Swofford 1993), with all substitutions given equal weight. A bootstrap analysis with 500 iterations was conducted to estimate the internal stability of the data matrix (Felsenstein 1985). To examine data structure a skewness test statistic (g1) based on the distribution of tree lengths of a random sample of 100,000 topologies was calculated.

Results

Allozyme and Sequence Variation

Significant genetic variation was found within and among nominal taxa (Table 2). Genetic distance values (Nei 1978) for all pairs of populations were used to generate the phenogram (Fig. 1). The cophenetic correlation for the UPGMA phenogram was 0.97, indicating a good fit of the phenogram to the original data matrix. As summarized in the phenogram, *Amblema* and *Megalonaias* species form distinct clusters and both are distinct from *Utterbackia*. Genetic distance between clusters representing *Amblema* and *Megalonaias* was 0.516. Between these two genera and the outgroup, *Utterbackia*, the genetic distance value was 0.801. Between species of *Amblema* and *Megalonaias*, genetic distances ranged from 0.273 to 0.317. Distances between species within a genus ranged from 0.012 to 0.276 and 0.0 to 0.122, for *Amblema* and *Megalonaias*, respectively. Additionally, *A. neislerii* was distinct from *A. plicata*, and both were distinct from *A. elliottii*. Unique alleles were observed for *A. atrocostata* (Mdh-1) and *A. neislerii* (Acon, 6-Pgd, Mpi, Pgm-2) which distinguish them from *A. plicata* and from each other. In contrast, *M. boykiniana* did not occupy a distinct branch from *M. nervosa* populations; *M. boykiniana* was genetically similar to populations of *M. nervosa* taken from nearby locations.

Nucleotide sequences of 405 to 407 bases in length were obtained for a portion of the 16S rRNA gene from 42 specimens representing *Megalonaias* (n = 18), *Amblema* (n = 22), and the outgroup, *Utterbackia imbecillis* (n = 2). The total aligned data matrix, including all insertions and deletions (indels), was 413 base pairs (bp). Ninety of the 413 bp were variable and involved base substitutions, although some minor indel variation was observed (sequences are available from the authors). Sixty-one of the 90 variable positions were phylogenetically informative. Of the remaining 29 variable sites, 26 were unique to the outgroup *Utterbackia*, 2 were unique to 2 different specimens within *Amblema*, and 1 was unique to a single specimen within *Megalonaias*.

Within the genus *Megalonaias,* four variable sites were observed, which defined five unique intra-generic mitochondrial haplotypes (Fig. 2a). The M1 haplotype was widely distributed and was found in all of the eastern locales. In addition, M1 was found in specimens of both *M. nervosa* and *M. boykiniana*. Indeed, M1 was the only haplotype found for *M. boykiniana*. Two other haplotypes were found within these easternmost locales, one in the Alabama River (M2), and one in Kentucky Lake, Tennessee (M3). Each of these haplotypes differed from M1 by one base pair substitution. The three locales from Texas all possessed the M5 haplotype, and one Neches River specimen had an additional, unique haplotype, M4. Both M4 and M5 differ from M1 by one base pair substitution.

The genus *Amblema* possessed 12 variable sites, which defined seven unique intra-generic haplotypes (Fig. 2b). The A1 haplotype was found for all specimens of *A. neislerii*. A second haplotype (A2) differed from *A. neislerii* by a single transversion and was found in all specimens from both locales of *A. elliottii*. The remaining four haplotypes were restricted to *A. plicata* and differed by at least eight base pair substitutions from those found in *A. elliottii* and *A. neislerii*. The most common haplotype (A3) was found in all six locales throughout the range of *A. plicata*. Three of the four localities, which had sequence data from more than one individual, possessed an additional haplotype that differed by one mutation from the common haplotype.

Percent sequence differences (uncorrected for multiple hits) within *Megalonaias* ranged from 0.2% to 0.5% including differences within *M. nervosa* and between *M. boykiniana* and *M. nervosa*. Within *Amblema* sequence differences ranged from no variation within *A. neislerii* and *A. elliottii* to a maximum of 0.5% within *A. plicata*. Percent sequence difference was 0.2% to 0.4% between *A. elliottii* and *A. neislerii* and increased to 2.2% to 2.5% between *A. plicata* and either *A. elliottii* or *A. neislerii*.

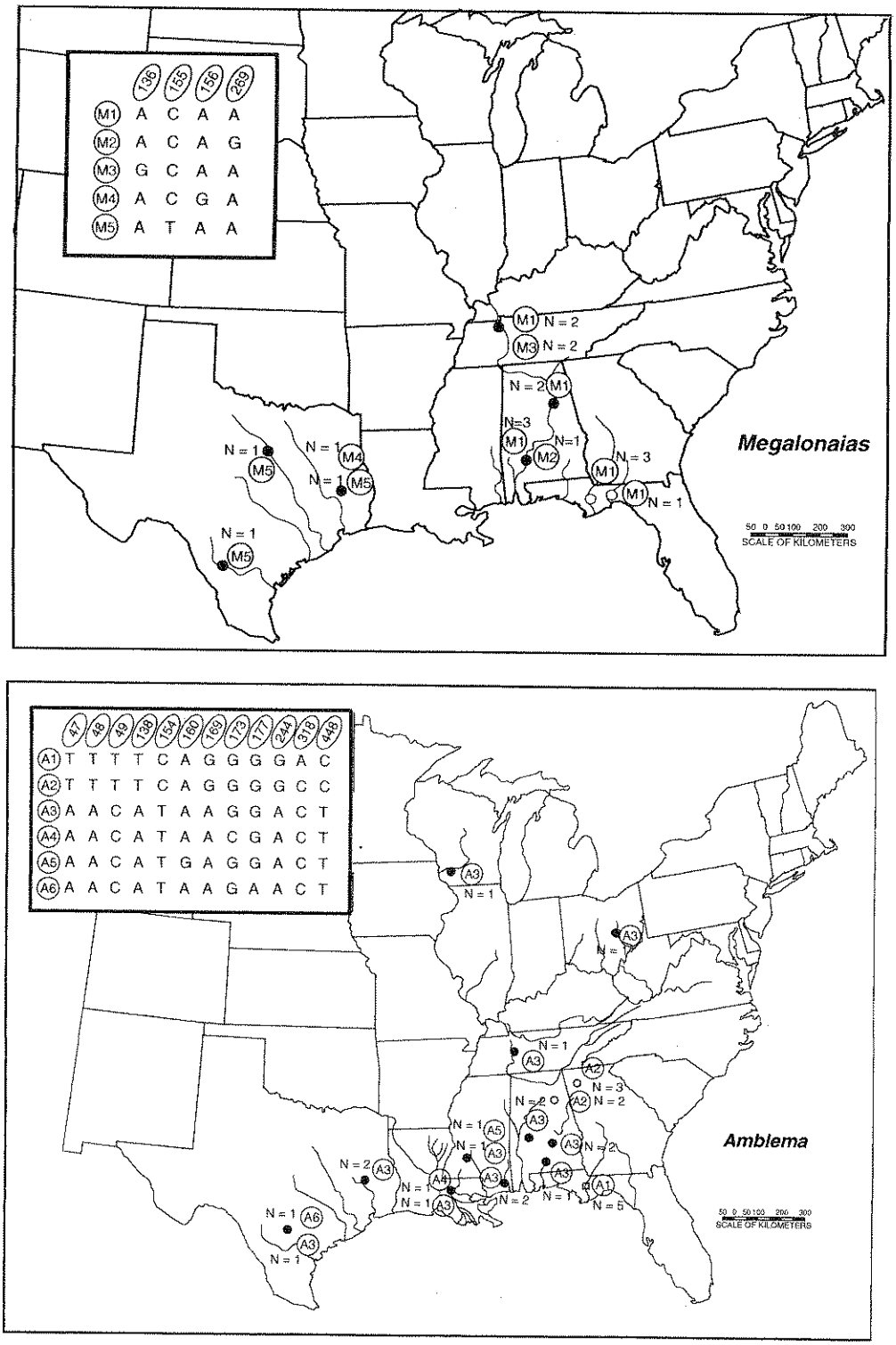

Figure 2. Sample locations and 16s rRNA sequence haplotype distributions for Megalonaias boykiniana *and* M. nervosa *(top) and* Amblema neislerii, A. plicata, *and* A. elliottii *(bottom).*

Between the two genera the percent sequence difference was 11.7% to 13.7%, which was lower than the percent sequence difference found between the outgroup and the ingroup taxa (14.5% to 15.2%).

Phylogenetic Analysis of mtDNA Haplotypes

The random tree length distributions of 100,000 randomly generated trees were significantly skewed ($g1 = -0.47$), suggesting the 16S rRNA gene data possessed a

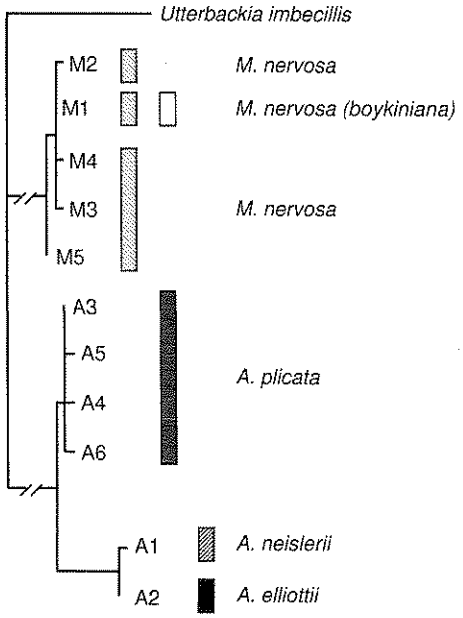

Figure 3. One of three equally parsimonious trees for Megalonaias *and* Amblema *based on sequence analysis of a 423 bp segment of the 16s rRNA gene.*

strong phylogenetic signal (Hillis & Huelsenbeck 1992). A maximum parsimony analysis of the 11 haplotypes analyzed with the branch-and-bound option in PAUP yielded two equally parsimonious trees of 104 steps with a consistency index (CI) of 0.981 (CI = 0.97, excluding uninformative characters). In one of the two equally parsimonious trees (Fig. 3), two main branches were recognized in the cladogram comprising the *Megalonaias* haplotypes M1 through M5 and the *Amblema* haplotypes A1 through A6, respectively. Within *Amblema* haplotypes A1, representing *A. neislerii*, and A2, representing *A. elliottii*, were sister haplotypes and were grouped together by six unique and one homoplastic synapomorphy. Haplotypes A3 through A6, representing *A. plicata*, were diagnosed by one synapomorphy; however, the second equally parsimonious tree depicts *A. plicata* as paraphyletic. The haplotypes of *Megalonaias* are grouped together by 21 unique synapomorphies. Within *Megalonaias* haplotypes M1 through M4 were sister to M5. Haplotypes M1 through M4 were grouped together by one homoplastic synapomorphy. Haplotype M1 was found in all *M. boykiniana* specimens and all eastern locales of *M. nervosa*.

Discussion

Genetic data presented for species of freshwater mussels in two genera, *Amblema* and *Megalonaias*, have clarified the evolutionary entities to which conservation efforts should be directed. Recognition of evolutionarily significant units is critical for species conservation. This recognition has been particularly troublesome in the mollusks, where estimates of the total number of species have varied widely, ranging from 40,000 to over 150,000 species (Boss 1971). In freshwater mussels Boss (1971) pointed out that in the most extreme example, over several hundred names were suggested for the freshwater mussels of France, a faunal group where only seven species are recognized today.

Table 3. Genetic distance values reported for unionids based on allozyme data and compared with values obtained in the present study.

Unionid	*No. taxa*	*Genetic distance*	*study*
Among species			
Elliptio	7 species	0.210 + 0.017	Davis et al. 1981
Uniomerus	3 species	0.308 + 0.165	Davis 1983
Utterbackia	3 species	0.457 + 0.073	Kat 1983*a*
Lampsilis	6 species	0.609 + 0.478	Kat 1983*b*
Amblema	3 species	0.237, 0.201	this study
Megalonaias	2 species	all <0.100	this study
Among genera			
Amblemini	4 genera	0.651 + 0.275	Davis et al. 1981
Pleuroblemini	3 genera	0.243 + 0.086	Davis et al. 1981
Amblema vs *Megalonaias*		0.469	this study

Confusion in unionid systematics is a result of conchologically-based species descriptions. The two genera examined are typical of the ambiguity in freshwater mussel taxonomy. Prior to Utterback (1916), all of the species examined here were placed in the genus *Amblema*. Utterback noted that the genus *Amblema* needed a thorough revision because of the plasticity of the shell characters and proposed the creation of a new genus *Megalonaias* to account for differences noted in shell and reproductive characters of *M. heros* Say 1929 (=*nervosa*). He cited unique anatomical features and differences in the breeding season as the special characters of the genus that separated it from *Amblema*. Utterback (1916) was also the first to suggest that *Unio boykiniana* Lea 1840, based on its shell characters, probably belonged to the genus *Megalonaias*. Nevertheless, Davis and Fuller (1981) suggested that the genera *Amblema* and *Megalonaias* were congeneric, based on immunological data and eight shell and anatomical characters.

Genetic distances are phenetic criteria, and values reported at various taxonomic ranks differ widely among taxa. For comparison, values for Nei's genetic distance reported for species and genera of unionid bivalve mollusks are summarized in Table 3. Observed genetic distances between *Amblema* and *Megalonaias* were within the range reported for between-genera distances. These genera are clearly distinct and should not be synonymized as suggested by Davis and Fuller (1981). Indeed, molecular data suggest that they are not even sister taxa (Lydeard et

al., 1996). In addition, Davis and Fuller (1981) provide morphological characters (i.e., beak sculpture and perforate gillsepta) that support the separation of these taxa.

Amblema

At the species level the taxonomic history of *Amblema* is complicated by the description of numerous nominal species and "varieties" of *Amblema plicata* from the upper Mississippi and Ohio rivers. Conrad (1841) described an additional species, *A. perplicata*, from streams near Jackson, Louisiana (Mississippi River drainage). Simpson (1914) delineated the distribution of *A. plicata* as south-central Canada, Lake Erie (type locality), upper Mississippi River south to the Tennessee and Arkansas rivers, and *A. perplicata* as occurring in streams draining into the Gulf of Mexico from the Alabama River west to central Texas and north to southern Kansas. Subsequent to Simpson (1914) *A. plicata* and *perplicata* have been variously treated as species, subspecies, or one wide-ranging species—*plicata*. The name *perplicata*, as a species or subspecies, has generally been applied to those populations inhabiting Gulf Coast drainages from Florida west to Texas, although some treatments (e.g., Hurd 1974) have considered *A. plicata* and *A. perplicata* conspecific. Since its original description, *Amblema neislerii* has been recognized as a distinct species endemic to the Apalachicola River drainage in Florida and Georgia.

Genetic data reported here support *A. neislerii* as a distinct taxon. Populations of *Amblema* inhabiting the coastal plain portion of Gulf drainages from the Escambia River in Florida west to Texas and northward in the Mississippi River drainage represent a single species, *A. plicata*. Because the name *perplicata* is based on a population from the lower Mississippi River drainage, near Jackson, West Filiciana Parish, Louisiana, this name becomes a synonym of *plicata*. The genetically distinct populations of *Amblema* from the Coosa and Conasauga rivers (Mobile Basin) in Alabama and Georgia, previously referred to as *perplicata*, require a new name. The oldest available name within the range of this genetically distinct entity is *Unio elliottii* Lea 1856. The description of *Unio elliottii* was based on material from Othcalooga Creek, Gordon County, Georgia [Coosa River Drainage]. Johnson (1974) designated the figured specimen in the Smithsonian Institution, United States National Museum (USNM) 84019, as the type. We reexamined the specimen, the lectotype USNM 84019, and determined that it is the figured specimen, and it is from the Othcalooga Creek, Gordon County, Georgia (Fig. 4). Two other species orginally described by Isaac Lea, *Unio latecostatus* Lea 1845 from the Black Warrior River and *Unio atrocostata* Lea 1845 from the Alabama River, Alabama, become synonyms of *A. plicata*. The vernacular name Coosa fiveridge is suggested for *A. elliottii* because its distribution is confined to the Cossa River drainage of Alabama and Georgia.

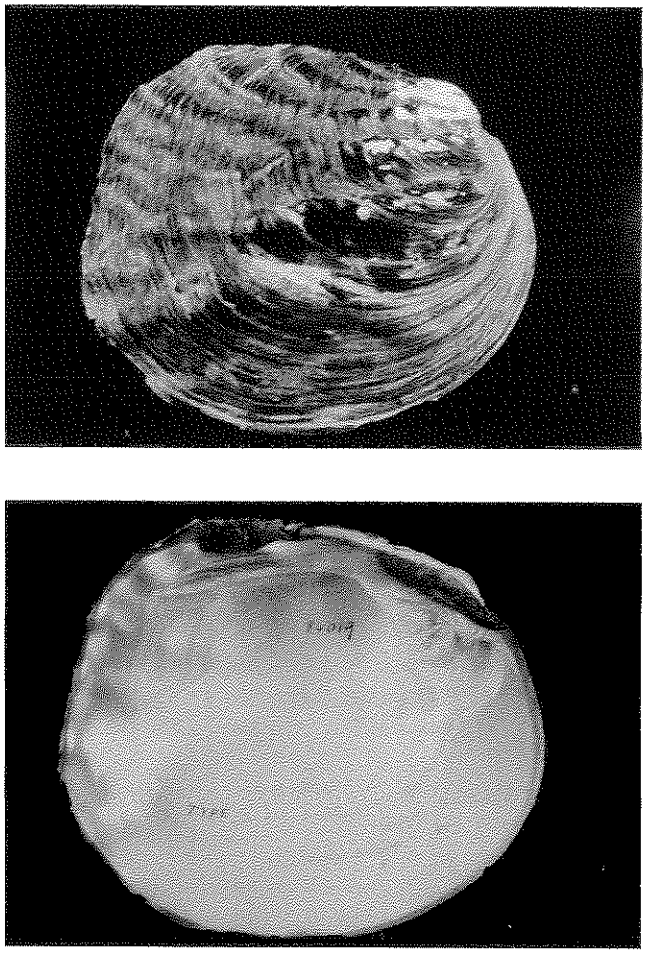

Figure 4. Amblema elliottii *figured holotype USNM 84019.*

Amblema neislerii is a genetically identifiable, unique evolutionary entity. Given the limited genetic difference (sequence data), it appears to have only recently diverged from *A. elliottii*. Although the extent of sequence variation falls within that observed for intraspecific variation of *A. plicata*, this variation is unique to all individuals recognized as *A. neislerii*. Low genetic distance would be expected between recently diverged taxa. Lydeard et al. (1996) report comparable divergences between other closely related, congeneric species of unionids.

Genetic distance based on allozymes between *A. plicata* and *A. elliottii* is 0.237. This is within the range of typical values reported for species level differentiation for bivalves and is greater than the distance observed for *A. plicata* and *A. neislerii*.

Megalonaias

Megalonaias boykiniana was not genetically diagnosable from *M. nervosa* populations because no sequence

differences consistent with a separation of these two taxa were observed. The DNA sequence data and allozyme data are consistent. This is not surprising because *M. boykiniana* has not been uniformly recognized as a taxon distinct from the widespread and abundant *M. nervosa*. Western populations of *M. nervosa* were distinct, however, from eastern populations, and these genetic differences should be considered in future management decisions. Additional study is needed to delineate the distributions of the western and eastern haplotypes.

Conservation efforts directed toward these mussels are directly affected by the data obtained in this study. *Megalonaias boykiniana* was considered for federal protection by the USFWS (Butler 1993), but was subsequently dropped from a proposed rule seeking protection for seven Apalachicolan Region species (USFWS 1994) because of the uncertainty of its taxonomic status. *Megalonaias nervosa* is one of the most widely harvested mussels for the Indo-Pacific pearl culture industry and is the preferred commercial species in the upper Mississippi River (Fritz 1986), comprising over 60% of the commercial harvest in Illinois and Wisconsin. Synonymizing *M. boykiniana* under *M. nervosa* increases the range of the latter species and should not affect its conservation status of currently stable, as assigned by Williams et al. (1993) in a review of the conservation status of freshwater mussels in the United States and Canada.

Amblema neislerii is clearly a distinct taxon and is in need of efforts to protect it and its limited habitat. Recent surveys indicate this species is found only in the Apalachicola and Chipola rivers in Florida and is extirpated from the Flint River in Georgia. An Apalachicola River system endemic, it is found only in the main channel (and not tributaries) of these rivers and, unlike *A. plicata*, does not tolerate impounded conditions. This species has been recognized as rare and endangered throughout its range for over two decades (Athearn 1970; Stansbery 1971), but was only formally proposed for federal protection (as endangered) in 1994.

The results of our study eliminate one subspecies of *Amblema* and increase the range of *A. plicata*. The range increase for this species should not affect its conservation status of currently stable. The conservation status of *A. elliottii* needs to be reexamined because its distribution appears to be limited to the Mobile Basin and Escambia and Choctawhatchee rivers.

Genetic data provided a useful means by which unique evolutionary entities or phylogenetic unionid species could be diagnosed. The delineation of mussel taxa should include shell morphology, soft tissue anatomy, ecology, and genetics because phenotypic variation reflects the interaction of environmental and genetic factors and is the basis for evolution and adaptation (Williams & Mulvey 1994). Molecular data, in conjunction with morphological and life history data, should increase our ability to resolve many unionid taxonomic enigmas and allow for more informed decisions on the conservation status of mussel species.

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