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An Analysis of Diversity in Freshwater Mussels (Bivalvia: Unionidae) of the Cuyahoga and Rocky River Watersheds (Ohio, USA) Based on the 16S rRNA Gene

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ABSTRACT. *The continued loss of freshwater unionid mussel species in the Great Lakes region, and particularly from Lake Erie, raises the question of how much phylogenetic and genetic variation remain in the watershed. The introduction of molecular markers into population biology offers one reproducible technique for assessing this variation. A fragment of the 16S rRNA gene of mitochondrial DNA has previously been used to infer phylogenetic relationships in the family Unionidae. Therefore, we reanalyzed these published molecular data with the addition of 12 species from the Cuyahoga and Rocky rivers in northeast Ohio. Both rivers drain to Lake Erie. The species assessed were Pyganodon grandis, Lasmigona complanata, Lasmigona compressa, Lampsilis radiata luteola, Fusconaia flava, Potamilus alatus, Strophitus undulatus, and Toxolasma parvus, for which published data were unavailable, and northern specimens of four wide ranging species, Lasmigona costata, Leptodea fragilis, Utterbackia imbecillis, and Quadrula quadrula. The resulting phylogenetic tree remained consistent with the accepted major taxonomic divisions in the Unionidae, and it indicates that these rivers still contain a phylogenetically diverse assemblage. However, the most abundant species all are members of the Anodontinae. Intraspecific variation in these unionid species was low, even where results were compared to published sequences on individuals from distant watersheds. One exception was a unique haplotype of Q. quadrula that differed at more base sites than is generally found for many congeneric species.*

INDEX WORDS: *Conservation, evolution, genetics, population, Unionidae.*

INTRODUCTION

Freshwater mussels in Ohio (Bivalvia: Unionidae), which may have numbered as many as 80 species in the recent past, continue to decline in both abundance and geographic range (Watters 1995). The greatest loss has been the entire habitat of western Lake Erie, originally home to 34 species (La Rocque 1967), all of which were extirpated locally following invasion of the lake by the zebra mussel, *Dreissena polymorpha*. The refuge for these populations now becomes the rivers and wetlands surrounding the lake. A big problem with respect to conserving the unionid communities of these streams is that they have experienced dramatic faunal shifts since late prehistory (Tevesz *et*

al. 2002), and extant populations are largely unconnected.

Therefore, we now propose a genetic analysis of the species present within the northeastern Ohio rivers. We begin with a study of variation in the Cuyahoga and Rocky rivers, which have been heavily impacted by urbanization at their river mouths. The Cuyahoga River arises 30 km east of Cleveland, Ohio, flows southward to Akron, and then northwest to empty into Lake Erie at the harbor in downtown Cleveland. The Rocky River enters Lake Erie only about 10 km to the west of the Cuyahoga after draining much of the near western suburbs of Cleveland. The unionid fauna of the Cuyahoga watershed were recently described by Smith *et al.* (2002) and Tevesz *et al.* (2002), and like many of the Canadian rivers that empty into Lake Erie, mus-

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sel species in the Anodontinae continue to increase their representation in the Cuyahoga River fauna, probably because these species better tolerate openness following deforestation (Metcalf-Smith *et al.* 2000). No survey of the Rocky River has been published.

Molecular genetic information has proven to be valuable to clarify loss of genetic variation and to better understand unionid systematics where distantly related species often share many of the same morphological characteristics (Lydeard and Roe 1998). Furthermore, environmental variation can induce several kinds of changes in shell shape and thickness (Tevesz and Carter 1980, Mulvey *et al.* 1998) that can confound identification based on morphology. Mitochondrial DNA genes are among the best for determining relationships among closely related species. The mitochondrial DNA genome represents a complex linkage group composed of approximately 37 genes inherited normally as a single unit, without recombination (Boore 1999; but see Ladoukakis and Zouros 2001). Differences among lineages of mitochondrial ribosomal genes are restricted to independent mutational events, and the rates of mutation fixation in the these genes may be higher than those for their equivalent nuclear counterpart genes (Hillis *et al.* 1996, Skibinski *et al.* 1999). Therefore, these sequences are especially useful for determining generic and specific level relationships in molluscs (Lydeard *et al.* 1998, Stepien *et al.* 1999, Canapa *et al.* 2000). Moreover, an improved taxonomic key based on mitochondrial genes may provide a starting point to determine the taxonomic status of species with wide-spread distributions. Without clarifying phylogenetic relationships and geographic variation, unionids, which as a group are the most imperiled animals in North America (Stein and Flack 1997), cannot be evaluated accurately for protection at local levels.

In this study, we advanced a sequence database assembled by Lydeard *et al.* (1996) which provided information useful in interpreting relationships among unionids. They compared sequence data for a 5'-fragment of the mitochondrial 16S rRNA gene in 26 species from 20 genera. Their study provides valuable background for other investigations of the systematic and genetic variability among the unionids present in a given watershed. Our data extended the taxonomic use of the 16S rRNA gene for Unionids in two ways, (1) by adding several genera not previously included, and (2) by testing the effect of intraspecific variation on the resulting phy-

logeny. The latter analysis is important, even though limited by collection restrictions on these protected species, because many of these unionids are wide ranging (Mulvey *et al.* 1998).

MATERIALS AND METHODS

Sequence Procurement and Alignment

We sequenced a fragment of the 16S rRNA mtDNA gene from 41 unionids, which composed 12 different species (Genbank accession numbers AY238480- AY238491). We combined these data with 32 sequences from Lydeard *et al.* (1996), available in GenBank. Our sequences completely overlapped the published sequences. Although the region separated by our primers averaged 500 bp, only the central 400 bp could be used for a complete analysis of all species. Together these data represent 25 of 48 genera of the North American unionids, but lack specimens of only four genera, *Epioblasma*, *Obovaria*, *Quincuncina*, and *Truncilla*, that contain more than two species (Williams *et al.* 1993).

Specimen Collection

Mussels were collected from a variety of sites (Fig. 1) within the Cuyahoga River and Rocky



FIG. 1. Location of the Cuyahoga and Rocky Rivers, which flow into Lake Erie in northeast Ohio.

River watersheds (numbers indicate sample size): from Tinkers Creek, *Pyganodon grandis* (7), *Lasmigona costata* (2), *L. compressa* (2), and *Fusconaia flava* (2); from the west branch of the Cuyahoga, *Pyganodon grandis* (4), *Lampsilis radiata luteola* (2), *Lasmigona complanata* (2), *L. costata* (1), and *L. compressa* (1); and from the Cuyahoga Valley National Park, the Ohio Canal, *Pyganodon grandis* (3) and *Toxolasma parvus* (1), Kendall Lake, *Utterbackia imbecillis* (1) and Horseshoe Lake, *Pyganodon grandis* (1); from the Rocky River, *Pyganodon grandis* (2), *Lasmigona costata* (3), *L. compressa* (3), *Lampsilis radiata luteola* (2), *Leptodea fragilis* (1), *Potamilus alatus* (1), *Strophitus undulatus* (1), and *Quadrula quadrula* (1). The small populations of mussels in these rivers restrict the number available for sampling. Mussels were brought to the laboratory alive, tissue was surgically removed and placed in ice cold 70% ethanol, and it was stored at -20°C until use.

Extraction of DNA

We isolated DNA from the adductor muscle or in some specimens from gonadal tissue (females only) by a modified insect grinding protocol (Greg Spicer and Suzanne Pass, SFSU, unpublished). Unionids possess both a male-inherited form of mitochondria as well as the standard female-inherited form, as seen in other bivalves (Ladoukakis and Zouros 2001), but the male form is restricted to gonadal tissue in the Unionidae (Hoeh *et al.* 1996, Liu *et al.* 1996). Between 2–4 mg of tissue was suspended in 500 μL of grinding solution: 100 mM EDTA, 10 mM Tris pH 8.0, 1% SDS, and 0.4 mg/mL proteinase K. The tissue was subsequently homogenized with a sterile pestle and incubated at 65°C for 1 h. Following incubation, an equal volume of equilibrated phenol was added to the homogenate, the sample was vortexed 1 minute and centrifuged 5 minutes (13,000 g). The supernatant was transferred to a new tube to which we added an equal volume of chloroform. Again the sample was vortexed, centrifuged, the resultant supernatant transferred, and cleaned a second time with chloroform. After the final transfer of the supernatant, two volumes of ice-cold 100% ethanol were added to precipitate the DNA, and the sample was cooled (-20°C) overnight. The next day, the DNA was centrifuged for 5 minutes, the supernatant was discarded, the pellet dried in a vacuum for 20 minutes, and finally

re-suspended in 300 μL of 1X TE buffer, pH 7.0 (Palumbi 1997).

PCR Analysis

The universal primers 16Sar-5' (CGCCTGTT-TAACAAAAACAT) and 16Sbr-3' (CCGGTTT GAACTCAGATCATGT) that were optimized for insect use (Palumbi 1997) primed the synthesis of approximately 500 base pairs (bp) of 16S mtDNA in unionid mussels. All PCR reactions were carried out in 50 μL volumes, consisting of 5 μL buffer A (Fisher Scientific), 5 μL dNTPs (10 mM), 5 μL of each primer (2.5 mM), and generally 10 μL (0.1 M) Mg^{2+} cofactor and deionized water to a total volume of 48 μL . To this reaction mix was added 0.4 μL *Taq* polymerase before transferring to 2 μL template DNA. The reactions were amplified in a PE2400 thermal cycler set at 35 cycles: an initial denaturation phase required 5 min at 94°C , with all subsequent denaturation bouts of only 30 sec. Annealing phase also was set for 30 sec, but temperatures varied by species, from 46°C to 50°C . Extension occurred at 72°C , for 30 sec.

After amplification, primer sequences were removed by a polyethylene glycol (PEG) density centrifugation procedure. First we added 15 μL of a 20% PEG and 2.5M NaCl solution to 25 μL of PCR product. The sample was vortexed briefly, incubated at 37°C for 15 minutes, and centrifuged at 12,000 g for 25 minutes. The supernatant was discarded and the pellet (often not visible) was washed with 25 μL of fresh 80% ethanol. The sample was again centrifuged for 5 minutes, the supernatant discarded, and the pellet vacuum dried for 20 minutes. Resuspension in 18 μL of PCR water completed the procedure after which samples were delivered to Cleveland Genomics for sequencing, or for later samples, to the Cleveland State University sequencing facility. All samples were sequenced in both directions.

Sequence Analysis

All mitochondrial 16S rRNA sequences were entered into ClustalV, a package of multiple alignment programs. FASTA format was used for sequence input. Two gap penalties were offered: "fixed"—for opening up a gap; "floating"—for extending a gap. The penalty options used for both was 10 (a medium value). Higher values could reduce the sequence length, but they markedly increased tree lengths. DNA transversions were (by program de-

fault) weighed more strongly than were transitions. Alignments were compared by eye against the known structural conformation of the gene in *Mytilus* (Lydeard *et al.* 2000), which indicated that variable regions of the gene fragment predominated in loops and that the stem structures were highly conserved.

The phylogenetic relationship among sequences was inferred using the DNA parsimony algorithm in PAUP v 4.0 beta (Swofford 2002). For the specific protocol, change from an occupied site to a deletion is counted as one change; reversion from a deletion to an occupied site also counted as one change. Analyses included 51 sequences covering 36 Unionidae species and three Margeritiferidae species. Replicate sequences for each species were included only where intraspecific variation was detected.

To produce trees, three programs were used. First, a heuristic search based on parsimony produced 315 best trees. A strict consensus of these trees was produced. Second, the bootstrap protocol was run for 200 replications on the 51 sequences. If a group occurred in more than 50% of all the input trees it appeared in the consensus tree. Similar analyses used the Jackknife procedure, and the consensus tree produced was identical. The tree printed out has at each fork a number which indicates the percent of the time that the group consisted of all the species to the right of (descended from) the fork. Verification of results was obtained in a second analysis that examined the species of the Anodontinae and the Ambleminae alone because the large numbers of OTUs caused the program to produce numerous trees of equal length.

RESULTS

The total aligned data matrix (including indels, which could be either an insertion or a deletion) was 503 bp, of which 183 positions were parsimony informative. Based on these characters, a majority rule consensus parsimony tree (Fig. 2), which when run with 35 unionid species, indicated two well-supported monophyletic groups, the traditionally described subfamilies: the Anodontinae, which includes the genera (*Anodonta* + *Utterbackia* + *Pyganodon* + *Lasmigona* + *Alasmidonta* + *Anodontoides*) and the Ambleminae. The Ambleminae was divisible into two previously defined monophyletic clades: the tribe, Pleurobemini which contains further well defined groups (*Megalonaias nervosa* + *Quadrula quadrula*, + *Q. apiculata* + *Q.*

metanerva) and (*Uniomerus obesus* + *Elliptio dilatata* + *Pleurobema pyriforme* + *Elliptioideus sloatianus* + *Fusconaia flava*), each of which are strongly supported as monophyletic, and the larger tribe, Lampsilini (*Lampsilis siliquoidea* + *L. teres* + *Ptychobranthus subtentum* + *Villosa delumbis* + *Potamilus purpuratus* + *Ellipsaria lineolata* + *Lepetodea fragilis* + *Medionidus conradicus*). The position of the genera *Plectomerus*, *Obliquaria*, *Toxolasma*, and *Amblema* may be a separate monophyletic group based on a neighbor-joining analysis (data not shown), but the evidence from this one gene is weak.

A strict consensus of the 315 equally parsimonious trees yielded similar results (Fig. 3), but it highlights a few potential discrepancies between a 16S rRNA gene tree and the presently accepted phylogeny of unionid mussels. The two members of the genus *Potamilus* are no more different, and they may differ less, than do *Lampsilis siliquoidea* and *L. radiata luteola* from the congeneric species, *L. teres*. The addition of *Toxolasma parvus*, another member of the Lampsilini, to the tree proposed by Lydeard *et al.* (1996) strengthened a connection between the genera *Obliquaria* and *Plectomerus*. *Plectomerus* has traditionally been placed outside the Lampsilini based on characteristics of the marsupium, which is an outgrowth of the gills where embryos develop. Its inclusion would indicate developmental convergence of some marsupial characters (Lydeard *et al.* 1996).

Intraspecific Variation

Variation within species for the 5' fragment of the 16S rRNA gene was low. The only possible exception is for *Quadrula quadrula*. The individual from the Rocky River showed closer similarity to *Q. apiculata* (395 of 398 bp, or > 99%) than it did to the southern specimen of *Q. quadrula* (381 of 398 bp, or 96%) that was sequenced by Lydeard *et al.* (1996). Its identification was based on morphology and independently confirmed by T. Watters (Ohio Biodiversity Museum) and M. Huehner (Hiram College, OH). The 17 base differences present between the two sequences of *Q. quadrula* were significantly greater than the three variants present between individuals of *Lampsilis teres* (Table 1), which was the most differences found between any other individuals of the same unionid species (contingency test, $P < 0.01$).

Within NE Ohio, sequence diversity was very low. Only a pyrimidine transition occurred in the

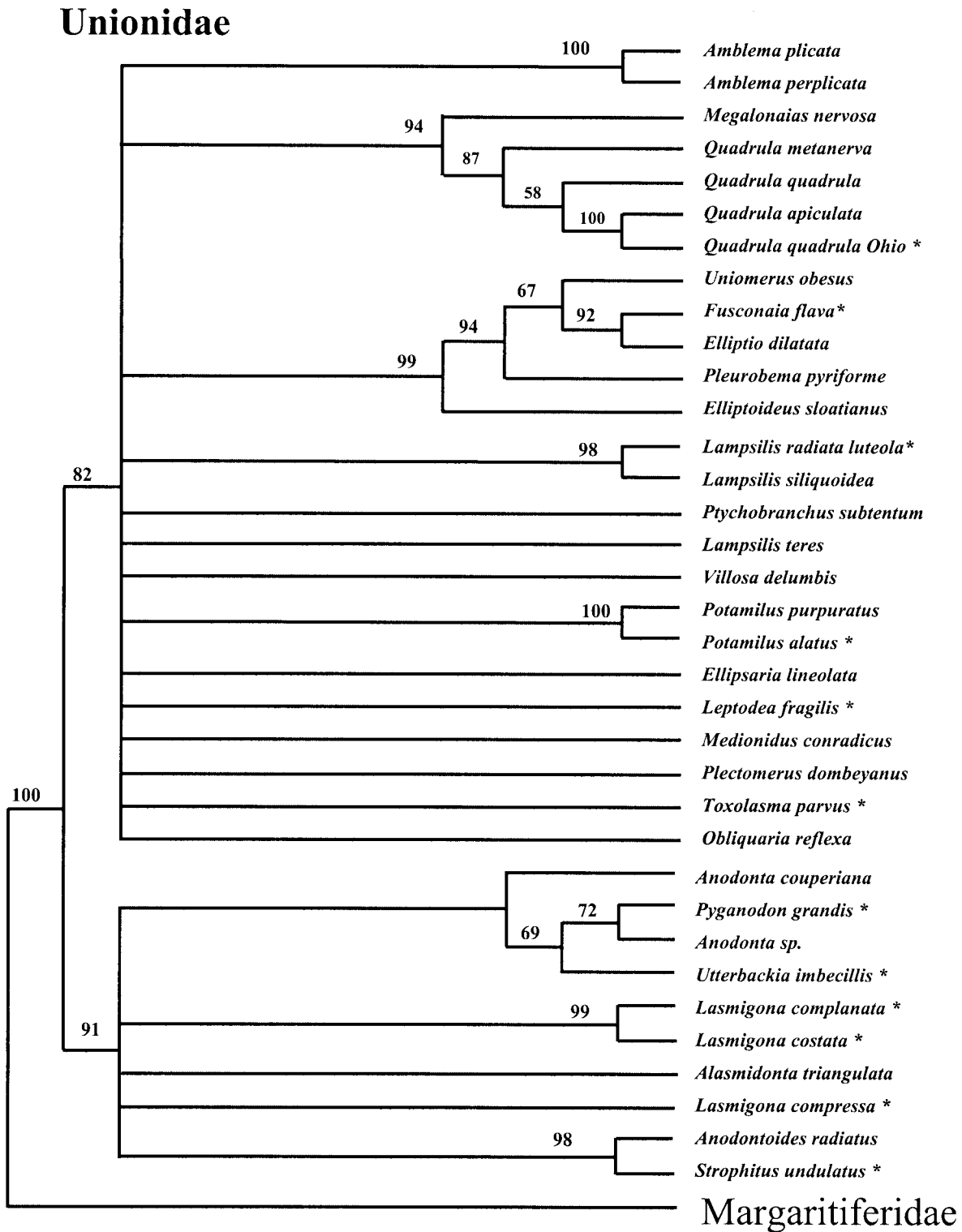


FIG. 2. A majority rule consensus tree for freshwater mussels with 200 replicate bootstrap run using a maximum parsimony analysis of the 16S rRNA sequence data (Paup beta). The number at the nodes indicate the percentage of replications that a particular clade occurred in the bootstrap analysis. Asterisks indicate new taxa added to data from Lydeard et al. (1996).

Unionidae

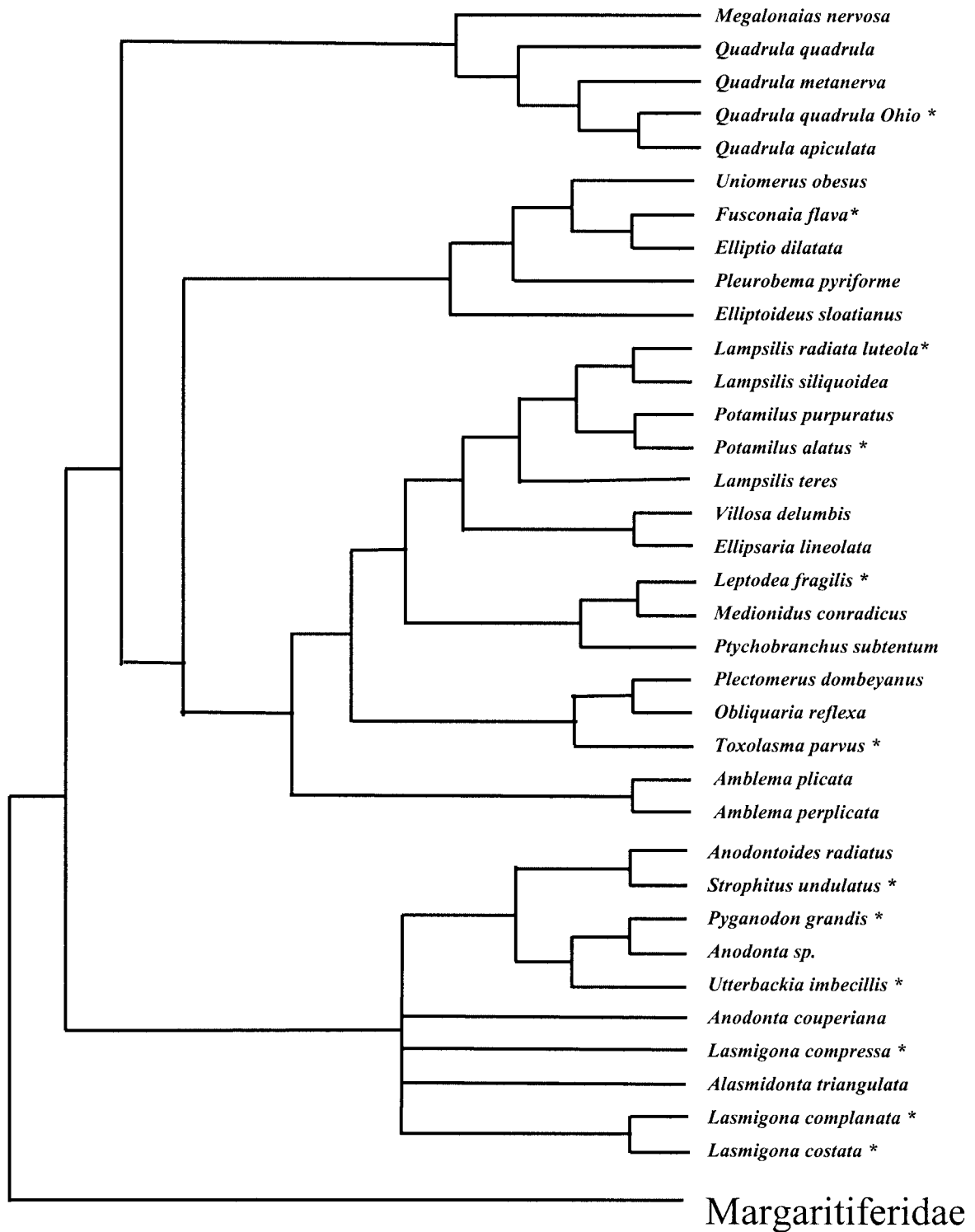


FIG. 3. A strict consensus of the 315 best trees found in a parsimony analysis of freshwater mussels using a heuristic maximum parsimony analysis of the 16S rRNA sequence data (PAUP beta). The number at the nodes indicate that all replications of the best tree included that clade. Asterisks indicate new taxa added to data from Lydeard et al. (1996).

TABLE 1. Intraspecific variation within unionid mussels based on DNA sequence of the proximal third of the 16S rRNA gene of mitochondria [Ts = transition, K = current study, L = Lydeard et al. (1996) study.

Source	Species	N	Variant type	Position	% similarity
K	<i>Pyganodon grandis</i>	17	1 Ts	152	99.8%
K	<i>Lasmigona complanata</i>	2			100%
K	<i>L. compressa</i>	5	1 Ts	144	99.8%
K&L	<i>L. costata</i>	7	1 Ts	185 ¹	99.7%
K	<i>Lampsilis radiata luteola/silivoidea</i>	5	1 indel	61 ²	99.7%
L	<i>L. teres</i>	2	3 Ts	57, 239, 303	99.2%
K&L	<i>Leptodea fragilis</i>	2	1 indel	311	99.8%
K	<i>Fusconaia flava</i>	2			100%
K&L	<i>Utterbackia imbecillis</i>	2			100%
L	<i>Quadrula apiculata</i>	2	1 Ts	195	99.7%

¹ Difference in one of the 2 sequences of Lydeard *et al.* (1996) only.

² The sequence of Lydeard *et al.* (1996) named *L. silivoidea* lacks one base present in all individuals of the present study.

486 bp gene fragment obtained for 17 *Pyganodon grandis* collected from these two watersheds. All Tinkers Creek individuals (N = 7) and the two Rocky River specimens possessed the “T” haplotype, while seven of eight from the Cuyahoga main-stream and its connecting canal or ponds carried the C haplotype. The lone T haplotype was found in one of the four headwater individuals sequenced. Similarly, one difference was found in five sequences from *Lasmigona compressa*, another transition, but this time the two specimens from the Rocky River carried the haplotype found in the upper Cuyahoga, and the two Tinkers Creek individuals differed. No variants appeared in five sequences from *Lasmigona costata*, although one variant exists in one of the two *L. costata* sequences published by Lydeard *et al.* (1996). For the three *L. radiata luteola* specimens, and the two from each of *F. flava* and *U. imbecillis*, no variants were found. The published *L. silivoidea* sequence differs from our *L. radiata luteola* sequences by a single-base indel; these species have previously been considered subspecies.

DISCUSSION

Phylogenetic Implications

Study of the phylogeny of North American unionids is ongoing because different systematic approaches and characters provide conflicting phylogenies (compare, for instance, the findings of Heard and Guckert 1971, Davis and Fuller 1981, and Lydeard *et al.* 1996). The phylogeny we present (Fig. 3) is one hypothesis, generated from a gene tree, that includes 25 different genera of the 48 gen-

era described for 281 species in the Unionidae (Williams *et al.* 1993). Fifteen of the genera not included are monotypic. The phylogeny proposed, however, represents the “true” phylogeny of unionids only if patterns of change in this one gene fragment accurately reflect evolutionary events within the family. Nevertheless, most major branch points are supported by morphological characters (Lydeard *et al.* 1996) and our reevaluation and expansion of the 16S rRNA data set enhanced the phylogeny that Lydeard *et al.* (1996) proposed. That is, despite the addition of numerous taxa, the major clades held up, and the consensus tree closely matched the strict consensus of the best trees they published previously. These results are gratifying because the addition of taxa, especially where the total number of characters is not large, can reduce support for deeper nodes in the phylogenetic tree (Huelsenbeck and Hillis 1993, Mitchell *et al.* 2000).

The 16S rRNA gene phylogeny continues to provide strong support for the subfamilies Anodontinae and Ambleminae, as well as for the tribes Pleurobimini and Lamsilini, described by Davis and Fuller (1981). One possible exception is the position of *Obliquaria*, which Lydeard *et al.* (1996) identified as a sister taxon to *Plectomerus*, and these appear to be close to *Toxolasma*. Both *Obliquaria* and *Toxolasma* are placed within the Lampsilini based on morphology (Lydeard *et al.* 1996). Therefore, our tree supports either inclusion of *Plectomerus* in the Lampsilini or the listing of *Obliquaria*, *Plectomerus*, *Toxolasma*, and *Amblema* and as a separate group either outside the Lampsilini or as a basal lineage within this tribe. As Heard and Guckert (1971) suggested, perhaps *Plectomerus* and *Am-*

blema could be grouped under the tribe Amblemini, but unquestionably, more data are required.

Conservation Implications

Although the number of individuals sequenced in all but one species were very low, these results suggest that little intraspecific variation exists in the 16S rRNA gene, even where individuals originate from different watersheds. Therefore, this fragment may be useful to identify long isolated populations or cryptic species. Two haplotypes separate *P. grandis* from Tinkers Creek and the main stem of the Cuyahoga River. None of the other species varied much, as the two *L. costata* that were collected from the Cuyahoga River system and a third from Rondout Creek in New York share one haplotype, while a fourth from the Finley River in Missouri varied in one site. Similarly, two distantly collected *U. imbecillis*, one from the Black River in South Carolina (Lydeard *et al.* 1996) and one from Kendall Lake, Ohio, are identical in sequence for the 16S rRNA. Hoeh *et al.* (1998) reported that this latter species is known for low levels of variation within a watershed, but they suggest it can be quite variable for allozymes between watersheds. The exception is for the two *Q. quadrula* individuals, which differed much more, highlighting a need for additional sampling in this group.

Little variation between sibling species mirrors results on variation among individuals from different watersheds. *Lampsilis radiata luteola* (Watters 1995), which is sometimes considered *Lampsilis siliquoidea* outside Ohio, differs from the published *L. siliquoidea* sequence by one indel early in the sequence. Likewise *Amblyma plicata* differs in a single site from *A. perplicata*, a close relative from the coast along the Gulf of Mexico, and these two taxa vary in their designation as species or subspecies (Williams *et al.* 1993, Mulvey *et al.* 1997).

Given the sound phylogeny developing above the generic level, sequence data for freshwater mussels provides a quantitative measure of biological diversity. As many as 15 species may occur in the Cuyahoga River and Rocky River watersheds, of which sequences were produced for twelve. Of the others, a number are not sufficiently common to predictably collect (i.e., *A. ferussacianus* and *A. marginata*) or the taxa receive state protection prohibiting collection (*L. nasuta*). Species belonging to all of the major unionid groups are present. *Lampsilis radiata luteola* and *Potamilus alatus* represent the Lampsilini, *Fusconaia flava* and

Quadrula quadrula represent the two putative branches of Pleurobemini, and *Toxolasma parvum* separates basally in the Ambleminae.

The relationship between biological and genetic diversity is identified by the levels of variation within the various taxa. In the Pleurobemini, which contains several basally divergent lineages, 28% of sites are polymorphic, in the Lampsilini (including *Plectomerus*), this proportion is only 10%, while in the Anodontini, 27% of sites vary. Considering data for all of the available unionid species, 45% of sites varied, and the level of variation within individuals from these two small rivers is 34% of the 505 bp of the 16S rRNA sequenced.

Of concern, however, is that the majority of the individuals found in the watershed (*Pyganodon grandis*, *Utterbackia imbecillis*, *Lasmigona costata*, and *Lasmigona compressa*) (Smith *et al.* 2002), belong to the subfamily Anodontinae, and the abundance of these taxa appears to be increasing (Tevesz *et al.* 2002). Similar trends occur in the Canadian streams on the north side of Lake Erie, which Metcalfe-Smith *et al.* (1998, 2000) address as a specific problem. They report that many of these species are more tolerant of exposed rivers, and therefore indicate change in habitat, such as a loss of Riparian zones. A specific example is the replacement of *Lampsilis siliquoidea* by *Pyganodon grandis* as the dominant species, and this change is mirrored by differences in shell versus live animal collections for *L. radiata luteola* and *P. grandis* in Tinkers Creek (Krebs *et al.* 2002), the largest tributary of the Cuyahoga. Such conditions may be a prelude to the loss of species from a watershed.

These changes therefore should raise an alarm that while the present species illustrate a phylogenetically diverse unionid fauna within the streams that flow through the urban/suburban Cleveland region, any further degradation of the habitat may result in large changes to the biota. Enough similarities are beginning to appear between research in Canada and the U.S. to guide local conservation efforts towards preserving the genetic make-up of mussel populations in and around Lake Erie. Although none of the species analyzed in this study are listed as endangered or threatened at a federal level, and only *Ligumia nasuta* is state listed, our surveys suggest several may be locally threatened. *Potamilus alatus* is known from only two specimens and a few shells in the Cuyahoga (Smith 2000, Smith *et al.* 2002), and *Quadrula* from only a single specimen. In the Rocky River (Krebs, data unpublished), *P. alatus* is present only

near the mouth of the river, and *Q. quadrula* from a single old (length = 131 mm) individual washed up on the riverbank. This detailed phylogeny indicates that only members of the Anodontinae are both abundant and dispersed throughout the watershed. Therefore, different indices of diversity may vary vastly in how this watershed is categorized, and small changes in abundance could rapidly alter any diversity index applied to the watershed based simply on species presence.

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