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Diagnostic Genetic Markers and Evolutionary Relationships among Invasive Dreissenoid and Corbiculoid Bivalves in North America: Phylogenetic Signal from Mitochondrial 16S rDNA

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Diagnostic genetic markers from 486 aligned nucleotide sequences of mitochondrial 16S ribosomal DNA were developed for the four closely related species of dreissenoid and corbiculoid bivalves that have invaded North America; the zebra mussel *Dreissena polymorpha,* the quagga mussel *D. bugensis,* and the dark false mussel *Mytilopsis leucophaeata* of the superfamily Dreissenoidea, and the Asian clam *Corbicula fluminea* of the sister superfamily Corbiculoidea. Evolutionary relationships were examined among the four genera and comparisons were made with native Eurasian populations of *D. polymorpha* and *D. bugensis.* Tests were conducted for gender-specific mitochondrial lineages, which occur in some other bivalves. Genetic variability and divergence rates were tested between stem (paired) and loop (unpaired) regions of secondary structure. There were 251 variable nucleotide sites, of which 99 were phylogenetically informative. Overall transition to transversion ratio was 0.76: 1.00 and both accumulated linearly in stem and loop regions, suggesting appropriate phylogenetic signal. Genetic distance calibration with the fossil record estimated the pairwise sequence divergence as 0.0057 ± 0.0004 per million years. *Mytilopsis* and *Dreissena* appear to have diverged about 20.7 ± 2.7 million years ago. *D. bugensis* and *D. polymorpha* appear separated by about 13.2 ± 2.2 million years. No intraspecific variation was found, including between Eurasian and North American populations, among shallow and deep morphotypes of *D. bugensis* and between the sexes. Restriction endonuclease markers were developed to distinguish among the species at all life history stages, allowing rapid identification in areas of sympatric distribution. © 1999 Academic Press

Key Words: **16S rDNA; bivalves; *Corbicula;* Corbiculoidea; *Dreissena;* Dreissenoidea; MtDNA; *Mytilopsis.***

INTRODUCTION

The development of diagnostic genetic markers for making rapid discriminations among species provides an important tool in conservation biology, which is just now beginning to be realized (DeSalle and Birstein, 1996; Amato *et al.,* 1998; Palumbi and Cipriano, 1998). Molecular genetic data also offer new suites of characters to augment and test traditional morphological classifications and to delineate phylogenetic species (Davis and Nixon, 1992; Stepien and Kocher, 1997). In this paper, we explore use of mtDNA sequence markers from the mitochondrial 16S ribosomal DNA region for rapidly distinguishing among related invasive freshwater bivalves, which constitute a growing problem in North American freshwater ecosystems. We also investigate their systematic relationships, possible evolutionary divergence times, and the influence of secondary structural constraints on the rate of evolution of mt 16S RNA.

The invasion and spread of exotic bivalves belonging to the sister superfamilies Dreissenoidea (including the zebra mussel *Dreissena polymorpha,* the quagga mussel *Dreissena bugensis,* and the dark false mussel *Mytilopsis leucophaeata*) and Corbiculoidea (the Asian clam *Corbicula fluminea*) constitute a growing problem in North American freshwater systems (Morton, 1997). These exotic bivalves have altered food web pathways, converted soft benthos into hard substrate, bioamplified contaminants, resulted in the decline of native bivalves, and produced serious fouling problems (Mills *et al.,* 1996; Morton, 1997). The most pronounced effects occurred during the last decade from the Eurasian zebra mussel, which became established in the Great Lakes from a ballast water introduction (Herbert *et al.,* 1989). From an initial population in Lake St. Clair (founded about 1986; Hebert *et al.,* 1989; Morton, 1997), *D. polymorpha* has undergone a broad and rapid range expansion throughout the Great Lakes region and beyond (Fig. 1; National Zebra Mussel and Aquatic Nuisance Species Clearinghouse, 1999). Its present North American range extends east to the Hudson

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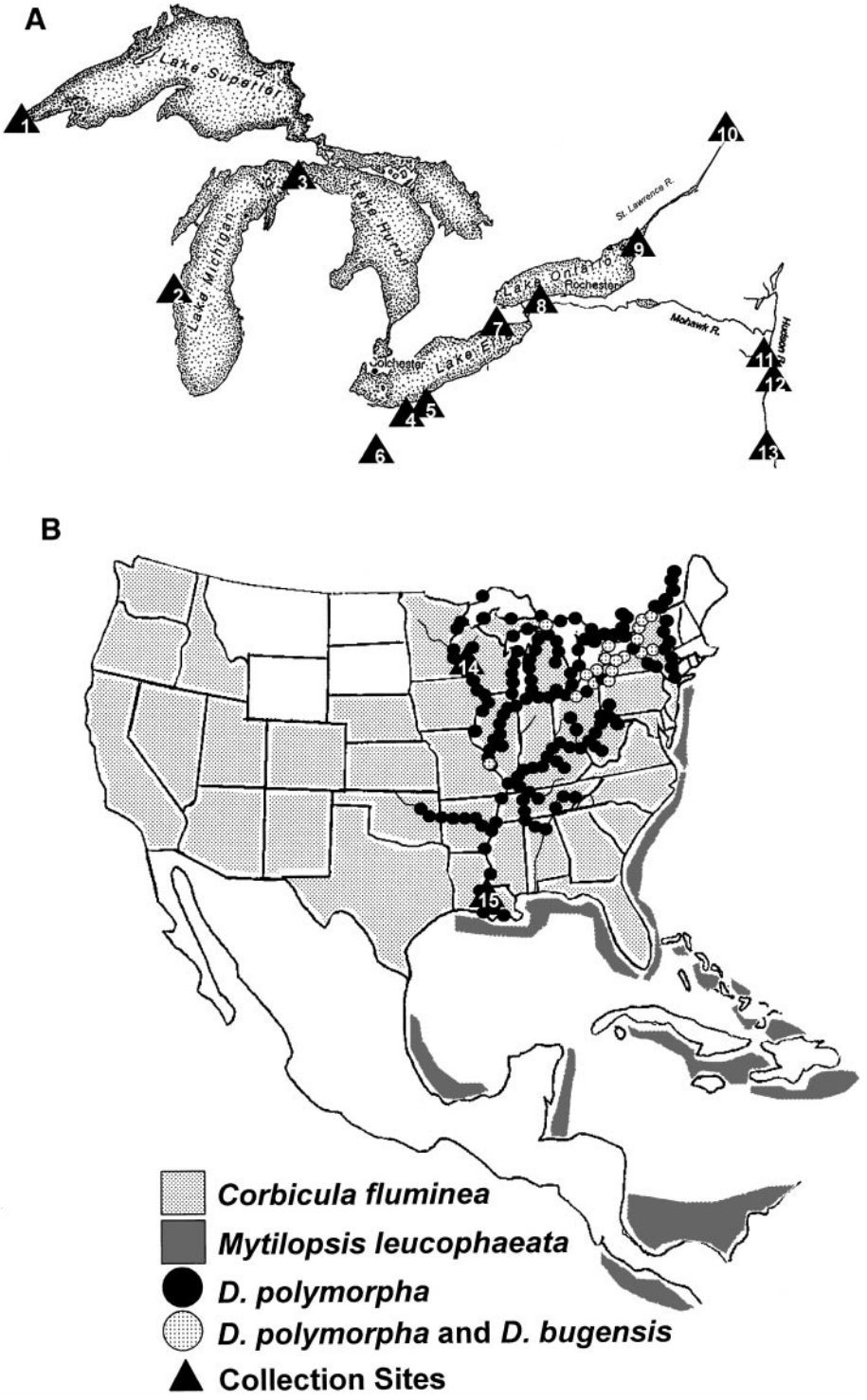

FIG. 1. (A) Sampling sites for mussels in the North American Great Lakes region. Z, zebra mussel, *Dreissena polymorpha;* Q, quagga mussel, *D. bugensis;* P, profunda variant of *D. bugensis;* M, *Mytilopsis leucophaeata;* C, *Corbicula fluminea.* Site 1 (Z), Duluth, MN; 2 (Z), Sheboygan, WI; 3 (Z), Mackinaw Straits, MI; 4 (Z), Lorain, OH; 5 (Z and Q), Eastlake, OH; 6 (C), Mohican River, OH; 7 (P), Mid-Eastern basin, Lake Erie; 8 (Z and Q), Olcott, NY; 9 (Z and Q), Cape Vincent, NY; 10 (Z), Gentilly, Quebec; 11 (Z), Stuyvesant, NY; 12 (Z), Catskill, NY; 13 (M), Newburgh, NY. (B) Distribution of *Dreissena, Mytilopsis,* and *Corbicula* in the New World (National Zebra Mussel and Aquatic Nuisance Species Clearinghouse, 1999). Sample site 14 (Z), Lake Pepin, MN; 15 (Z and M), Baton Rouge, LA.

River estuary, west to the Arkansas River, and throughout the Mississippi River drainage basin (Fig. 1; National Zebra Mussel and Aquatic Nuisance Species Clearinghouse, 1999).

A second dreissenid species, the quagga mussel *D. bugensis* was discovered in the Great Lakes in 1992 (May and Marsden, 1992). The quagga mussel now is common in Lakes Erie and Ontario and the St. Lawrence River and is expanding westward (Fig. 1; National Zebra Mussel and Aquatic Nuisance Species Clearinghouse, 1999). In the past few years, it has become as numerous as the zebra mussel in some regions of the lower Great Lakes (Claxton *et al.,* 1997). The quagga mussel has also been reported in the Mississippi River at St. Louis, Missouri (National Zebra Mussel and Aquatic Nuisance Species Clearinghouse, 1999).

Dermott and Munawar (1993) described a deep water morphotype of *D. bugensis,* termed the "profunda," from >40 m in the eastern basin of Lake Erie. The profunda type shares a rounded ventral shell margin with *D. bugensis* (which is pointed in *D. polymorpha*). The ventral margin of the profunda type is less convex and the shell is less pigmented than in *D. bugensis* from shallower waters (Dermott and Munawar, 1993). Mackie and Schloesser (1996) suggested that apparent differences in morphology between deep and shallow water forms of *D. bugensis* may be the result of ecophenotypic variation.

Assessing the early life history stages of freshwater mussel populations in North America is difficult since *Dreissena* spp. occur sympatrically with *M. leucophaeata* and *C. fluminea* in some areas (Fig. 1). Like *Dreissena, M. leucophaeata* and *C. fluminea* disperse via free-swimming veliger larvae in the water column and are aquatic nuisance species (Nichols and Black, 1994; Morton, 1997). *Corbicula, Mytilopsis,* and *Dreissena* are difficult to distinguish at early life history stages prior to shell formation (Nichols and Black, 1994; Claxton *et al.,* 1997). Claxton *et al.* (1997) determined that the amount of shell overlap, position of the dorsal point of curvature, angle of the shell at the hinge, and degree of flatness of the ventral region can be used to microscopically discriminate between *D. polymorpha* and *D. bugensis* after shell formation (>300 μm). Denson *et al.* (1998) described differences between *Mytilopsis* and *Dreissena* in spermatozoan external morphology.

Some bivalves have separate male and female mtDNA lineages associated with a "doubly uniparental" (DUI) mode of inheritance, which results in a female mitotype in the female offspring and both female and male mitotypes in the male offspring (Zouros *et al.,* 1994a,b; Skibinski *et al.,* 1994; Quesada *et al.,* 1998). The heteroplasmic male condition may confound the use of mtDNA polymorphisms to discern among species (Heath *et al.,* 1995; Quesada *et al.,* 1995), and its presence/absence thus was tested in the present study. Claxton *et al.* (1997) did not find DUI in dreissenids in a study of restriction sites in the mtDNA COI gene.

Evolutionary Relationships, Morphology, and Historic Distributions of the Exotic Bivalves

The bivalve superfamily Dreissenoidea is believed to have diverged during the late Mesozoic Era from an ancestor shared with its sister superfamily Corbiculoidea (Morton, 1993), based on shared shell microstructure characters (Taylor *et al.,* 1973). The Dreissenoidea contains a single family, the Dreissenidae, with two subfamilies; the extinct Dreissenomyinae and the partially extant Dreisseninae (Rosenberg and Ludyanskiy, 1994). The subfamily Dreisseninae has four genera that are believed to have diverged during the late Miocene Epoch and one is extinct (Nuttall, 1990). The relationships of the two widespread extant genera are examined in this study, including the freshwater *Dreissena* and the estuarine and marine *Mytilopsis* (Rosenberg and Ludyanskiy, 1994). The genus *Congeria* was believed to be extinct since the Miocene (Nuttall, 1990) but *C. kusceri* recently was described living in underground caves in the former Yugoslavia (Morton *et al.,* 1998). Stepien and Skidmore have obtained specimens from Morton and are sequencing them to compare with the results of the present study.

The genus *Dreissena* is hypothesized to have evolved in the Paratethys Sea during the late Miocene Epoch, based on the fossil record (Iljina *et al.,* 1976 in Nuttall, 1990). *Dreissena* is characterized by a rounded dorsal shell margin, pointed umbo, broad myophore plate, rounded pallial line, and the absences of an apophysis and a pallial sinus (Pathy and Mackie, 1992). During the Quaternary Ice Ages, *D. polymorpha* was restricted to the northwestern basin of the Dnieper River basin (Ukraine; Fig. 2), which was drained by the Paleodanube River (Kinzelbach, 1992). Construction of canals during the Industrial Revolution led to the anthropogenic recolonization of Europe by *D. polymorpha* (Morton, 1993).

Dreissena bugensis has been described from Quaternary deposits near the Black Sea (Babak, 1983 in Rosenberg and Ludyanskiy, 1994). Its present-day Eurasian distribution in the Ukraine is given in Fig. 2 (Mills *et al.,* 1996). The primary characteristic that distinguishes *D. bugensis* from *D. polymorpha* is a less pronounced angle between the side of the valve and the flat ventral surface of the shell (Pathy and Mackie, 1992).

The genus *Mytilopsis* is hypothesized to be ancestral to *Dreissena* and to have evolved in Europe during the Eocene Epoch (Nuttall, 1990). Both *Dreissena* and *Mytilopsis* have byssal threads that are used to attach to hard substrates (Morton, 1997). Overlap in shell coloration and external morphology between *Dreissena* spp. and *M. leucophaeata* may lead to misidentifications (Pathy and Mackie, 1992). A flattened dorsal

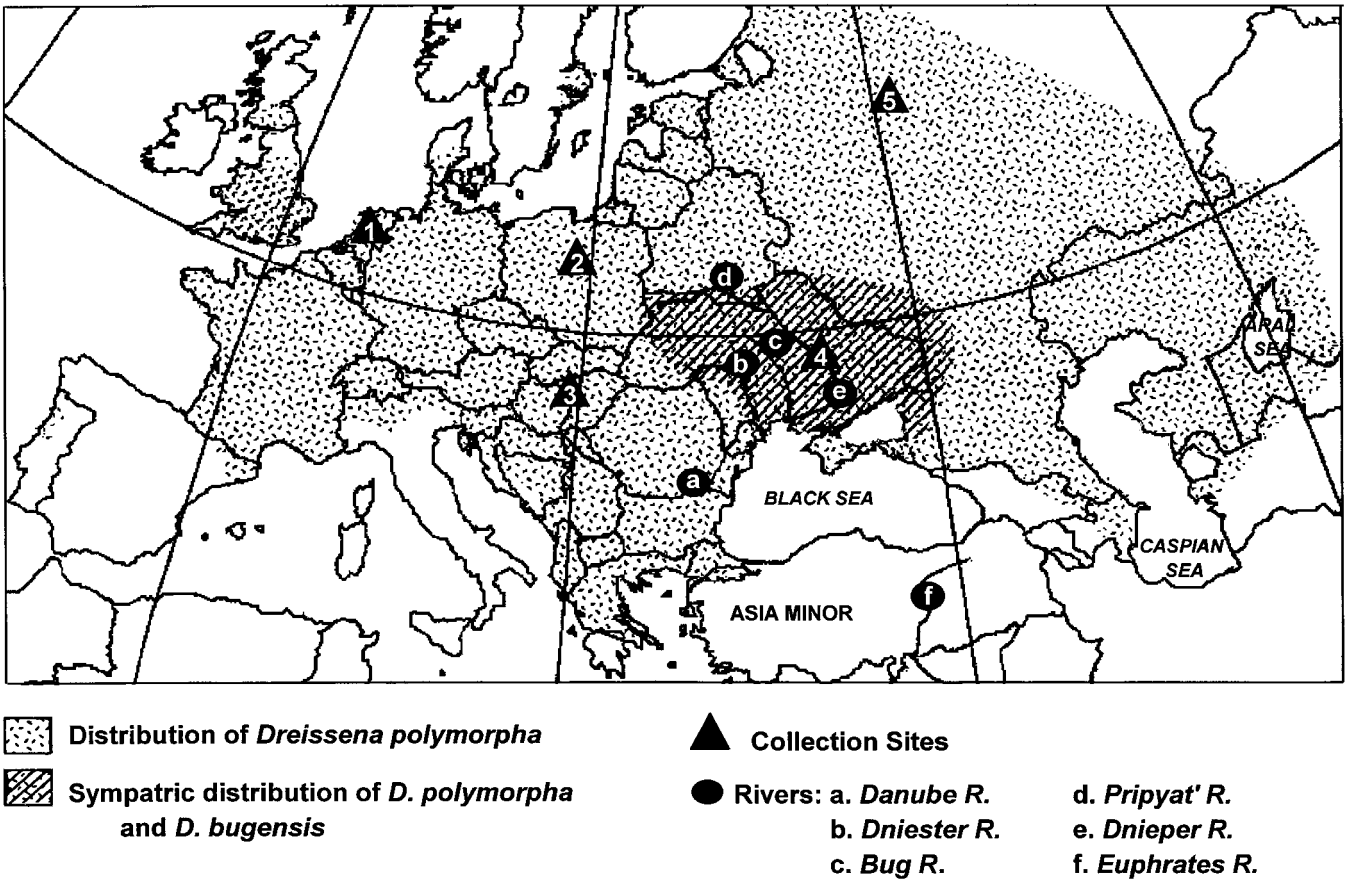

FIG. 2. Distribution and collection sites in Eurasia for the zebra mussel (Z) *Dreissena polymorpha* and the quagga mussel (Q) *D. bugensis.* Site 1 (Z), Lake IJsselmeer, Netherlands; 2 (Z), Wtoctawek Reservoir, Poland; 3 (Z), Danube River, Hungary; 4 (Z and Q), Dnieper River, Ukraine; 5 (Z), Volga River, Russia. Distribution was obtained from Kinzelbach (1992), Ludyanskiy (1993), Mills *et al.* (1996), and Marsden *et al.* (1996).

margin, a rounded umbo, a narrow myophore plate, an apophysis, and a pallial sinus characterize *Mytilopsis.* The number of species in the genus *Mytilopsis* in North America is controversial due to their variable shell morphology and coloration patterns (Morton, 1993, 1997).

Fossil evidence from coastal Peru (of *M. trigalensis;* Olsson, 1931) and western Panama (of *M. dalli;* Joukowsky, 1906) indicates that *Mytilopsis* colonized Central America and tropical South America during the late Oligocene Epoch (Nuttall, 1990). During the Neogene Epoch, *Mytilopsis* spread northward to the Gulf of Mexico and Atlantic seaboard regions of the United States. *M. leucophaeata* colonized the Hudson River during the 1930s (Jacobson, 1953) and has been reported from the upper Mississippi River (Koch, 1989). In both areas, it now is sympatric with introduced populations of *D. polymorpha* (Pathy and Mackie, 1992; Strayer *et al.,* 1996; Fig. 1). *M. leucophaeata* poses an economic and ecological threat similar to, but probably not as severe as, that of *Dreissena* spp. (Pathy and Mackie, 1992; Morton, 1997).

A member of the related freshwater superfamily Corbiculoidea, *C. fluminea* is an exotic bivalve pest that was introduced to North America in the Columbia River, Washington State in approximately 1938 (Morton, 1997). It has spread throughout much of the United States (Fig. 1; Hornbach, 1992; Morton, 1997) and occurs sympatrically with dreissenids in the Great Lakes region and the southern United States (Fig. 1; Janech and Hunter, 1995; Morton, 1997). *C. fluminea* has a small byssus only as a juvenile and burrows as an adult. Like dreissenids and *Mytilopsis,* it is highly exploitative, opportunistic, constitutes a biofouling problem, and negatively affects native bivalves (Morton, 1997).

Approach and Objectives of the Present Study

Mitochondrial DNA markers have been shown to be useful for analyzing relationships among bivalve species (Foighil *et al.,* 1995; Lydeard *et al.,* 1996) and populations (Boulding *et al.,* 1993; Hare and Avise, 1996; Rawson and Hilbish, 1998). The invertebrate mtDNA 16S rRNA gene has been relatively well stud-

ied due to availability of universal primers (Kocher *et al.,* 1989; Palumbi, 1996) and has been useful for resolving species through family level relationships among bivalves (Canapa *et al.,* 1996, Foighil *et al.,* 1995; Lydeard *et al.,* 1996).

The objectives of the present investigation were: (1) to develop molecular markers to allow rapid discrimination among taxa at all life history stages, (2) to analyze the systematic relationships among dreissenoid and corbiculoid bivalves from mtDNA 16S rDNA sequences, and (3) to compare the possible constraints imposed by secondary structure (stem and loop regions) on the mutation rate of this gene. Taxa included *D. polymorpha* and *D. bugensis* from North America and Europe, the profunda and shallower water variants of *D. bugensis, M. leucophaeata,* and *C. fluminea.* Genetic distances were calibrated with the fossil record and comparisons were made with other taxa and with results from the COI gene (Baldwin *et al.,* 1996), in order to interpret the relative rate of divergence of mt 16S rDNA and possible substitution constraints. Sequences from male and female mussels were compared to test for the presence of gender-specific mtDNA lineages.

MATERIALS AND METHODS

Sampling

Samples of *Dreissena polymorpha* ($N = 24$) and *D. bugensis* ($N = 20$) represented the extents of their North American (Fig. 1) and European (Fig. 2) distributions. Sample sites of *D. polymorpha* from North America (Fig. 1) included Lake Superior at Duluth, Minnesota (site 1, 46.5°N, 92.07°W, $N = 1$); Lake Michigan at Sheboygan, Wisconsin (site 2, 43.45°N, 87.44°W, $N = 1$); the Mackinaw Straits between Lakes Michigan and Huron (site 3, 44.9°N, 85°W, $N = 1$); Lake Erie at Eastlake, Ohio (site 5, 41.5° N, 81.5°W, $N = 3$); Lake Ontario at Olcott, New York (site 8, 43°N, 79°W, $N = 1$) and Cape Vincent, New York (site 9, 44.2°N, 76.1°W, $N = 1$); the St. Lawrence River at Gentilly, Ontario (site 10, 43°N, 78.5°W, $N = 1$); the Hudson River at Stuyvesant, New York (site 11, 42.2°N, 73.5°W, $N = 2$) and Catskill, New York (site 12, 42.1°N, 73.5°W, $N = 1$); Lake Pepin, Minnesota (site 14, 43°N, 93°W, $N = 1$); and the Mississippi River at Baton Rouge, Louisiana (site 15, 30°N, 90°W, $N = 1$). Samples of *D. polymorpha* from Eurasia (Fig. 2) included Lake Ijsselemeer, the Netherlands (site 1, 52.46°N, 5.14°E, $N = 2$); Wtoctawek Reservoir, Poland (site 2, 52.5°N, 19.0°E, $N = 2$); the Danube River, Budapest, Hungary (site 3, 47.3°N, 19.0°E, $N = 2$); the Dnieper River, Ukraine (site 4, 48.23°N, 34.0°E, $N = 2$); and the Volga River, Russia (site 5, 58.0°N, 42.0°E, $N = 2$). Samples of *D. bugensis* from North America (Fig. 1) included Lake Erie at Lorain, Ohio (site 4, $N = 1$) and Eastlake, Ohio (site 5, $N = 3$); and Lake Ontario at Olcott, New York (site 8, $N = 4$) and Cape Vincent, New York (site 9, $N = 2$). Samples of *D. bugensis* from Eurasia were tested from the Dnieper River, Ukraine (site 4, $N = 10$; Fig. 2). The profunda variant of *D. bugensis* was sampled from the eastern basin of Lake Erie (site 7, 42.26°N, 79.5°W, $N = 10$; Fig. 1).

Mytilopsis leucophaeata was examined from the Mississippi River at Baton Rouge, Louisiana (site 15, $N = 3$) and the Hudson River at Newburgh, New York (site 13, 42.2°N, 73.5°W, $N = 5$), representing the extremes of its North American range (Fig. 1). *Corbicula fluminea* (the common white form; see Hillis and Patton, 1982) was sampled from the Mohican River, Ohio (site 6, 41.0°N, 82.5°W, $N = 7$).

Specimens were either preserved frozen at −80°C or placed directly in 95% ethanol at room temperature. Shells were dried and stored as voucher specimens. Sex was determined by examining the gonads at 100× magnification using an Olympus Model BHS microscope and comparing them to photographs of dreissenid sperm and eggs (Nichols, 1993; Walker *et al.,* 1996).

DNA Extraction, Amplification, and Sequencing

Genomic DNA was isolated and purified from the adductor and byssal retractor muscles and/or mantle tissue, following methods described in Stepien (1995). The universal primers 16Sar-L (5′CGCCTGTTTAACAAAAACAT3′) and 16Sbr-H (5′CCGGTCTGAACTCAATCACG3′; Palumbi, 1996) were used to amplify a portion (about 470 bp) of the mt 16S rDNA using the polymerase chain reaction (PCR; Mullis *et al.,* 1986). 16Sbr-H was end-labeled with biotin at the 5′ end (Hultman *et al.,* 1989) for later separation of the strands. The amplification program was 39 cycles at 94°C for 40 s, 52°C for 30 s, and 72°C for 1 min. A final cycle was run at 72°C for 5 min to insure that strands were fully polymerized. Quality, quantity, and size of the PCR products were assessed on agarose minigels (see Fig. 6 for photograph of amplified products).

The PCR products were separated into single strands (Hultman *et al.,* 1989; Uhlen, 1989) using Dynabeads M-280 streptavidin (product 112.05; Dynal Corp., Oslo, Norway). Sanger dideoxy sequencing (Sanger *et al.,* 1977) incorporating [^{35}S]dATP radioactive labeling (product NEG-734H, Dupont, New England Nuclear, Boston, MA) was performed separately on each strand using Sequenase Version 2.0 sequencing kits (product 70770, Amersham/U.S. Biochemical Corp., Cleveland, OH) and 7 µL of the 0.2 µM complementary PCR primer. Two 6% polyacrylamide wedge spacer sequencing gels (product S2S, Owl Scientific Inc., Woburn, MA) were run for each reaction for 2.5 and 7 h at 75 W and 55°C. Bands were visualized with autoradiography (film product 011014, Fuji Inc., Tokyo), after 48 to 72 h of exposure.

Restriction Digests and Species-Discriminating Markers

Sequences for mt 16S rDNA were entered into the DNASTAR program (DNASTAR, Inc., Madison, WI) to determine the placement of species-specific restriction sites, allowing rapid discrimination. DNA samples from five individuals each of *D. polymorpha, D. bugensis, M. leucophaeata,* and *C. fluminea* were tested with the diagnostic restriction enzymes. The diagnostic endonucleases were Dde I (from *Desulfovibrio desulfuricans,* strain Norway, product 113-250; Boehringer Mannheim, Indianapolis, IN), which recognizes C*TNAG, and Acs I (from *Arthrobacter citreus* 310, product 113-259, Boehringer Mannheim), which recognizes A/G*AATTT/C. Restriction digests included 5 µL of the amplified PCR product, 6.5 µL of ddH_20, 1.5 µL of buffer H (from the Boehringer Mannheim kits; 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol), and 1 µL of the restriction enzyme. The solution was incubated at 37°C for 4 h. Samples were heated to 65°C for 5 min and run on a 2% TBE agarose minigel at 65V for 1 to 2 h. The fragments were compared to the migration of DNA molecular weight marker VI (product 062-590, Boehringer Mannheim).

Data Analysis

Mitochondrial 16S rDNA sequences from this study and for *Mytilus edulis* (Hoffman *et al.,* 1992) were aligned with KODAK/IBI AssemblyLIGN software (IBI, 1992). Sequences were compared to 16S rDNA secondary structure models for *Drosophila* (Guttel and Fox, 1988; Guttel *et al.,* 1992) and *M. edulis* and *Pecten maximus* (Lydeard *et al.,* 1996) to identify stem (paired) and loop (unpaired) regions. A secondary structure model of mt 16S rRNA was constructed for *D. bugensis.* Designation of the stem and loop regions was based on homology to a model for *M. edulis* by Lydeard *et al.* (1996).

Nucleotide composition for each species was computed from the entire data set and separately for stems and loops. χ^2 tests (Sokal and Rohlf, 1981; EXCEL 97, Microsoft, 1997) were used to examine nucleotide biases within species for the entire data set and separately for stem and loop categories. Contingency table tests (Sokal and Rohlf, 1981) were used to determine whether nucleotide distributions differed among the taxa.

Mutational saturation in the mt 16S rRNA genes was tested by conducting χ^2 and contingency tests (Sokal and Rohlf, 1981) within and between the stem and loop structural categories for the numbers of (1) variable versus invariable sites, (2) phylogenetically informative versus uninformative nucleotide substitutions determined in PAUP* 4.0 (d64, Swofford, 1998), and (3) transitional versus transversional substitutions. χ^2 tests (Sokal and Rohlf, 1981) were used to determine whether transitions (ts) and transversions (tv) occurred at similar frequencies within species. A contingency table test (Sokal and Rohlf, 1981) examined whether ts and tv occurred at similar proportions among taxonomic levels. For this analysis, the numbers of transitional and transversional events were determined, following parsimony methodology, by minimizing the number of substitutions that occurred at each nucleotide position. For each variable position, bases were marked on the phylogenetic tree and the phylogenetic level at which each substitution occurred was recorded. In a separate analysis, the numbers of ts and tv occurring among each pairwise combination of taxa, for the entire data set and separately for stem and loop regions, were plotted against pairwise (p) genetic distances (in EXCEL 97, Microsoft, 1997) to evaluate possible mutational saturation (following Lydeard *et al.,* 1996). The correlation coefficient "r" (Sokal and Rolhf, 1981) was used to compare the pairwise numbers of ts and tv versus pairwise genetic distances for stems, loops, and the entire data set.

Kimura (1980) two-parameter genetic distances, which correct for multiple substitutions per site, and their standard errors were estimated using MEGA (Kumar *et al.,* 1993). Separate runs (1) coded insertions/deletion events (indels) as characters, (2) examined tv and indels only (due to possible saturation of ts; see Kocher and Carleton, 1997), and (3) omitted sites that had indels. Both pairwise (p) and Kimura (1980) two-parameter distances (d) were used to compare our genetic distances with those from other studies of bivalves (e.g., Baldwin *et al.,* 1996; Canapa *et al.,* 1996; Lydeard *et al.,* 1996). Divergence times were calculated by calibrating the genetic distances to the fossil record estimate of the separation between the superfamilies Dreissenoidea and Corbiculoidea as 54 mya (million years ago), at the beginning of the Eocene Epoch (Morton, 1993). This method of calibration also was used to compare our results with those for the COI gene by Baldwin *et al.* (1996). In all calibrations and comparisons, the distance between *C. fluminea* and *M. leucophaeata* was set at 54 my.

Mytilus edulis was used as an outgroup for comparing genetic divergences, rooting the trees, and polarizing characters because it was the bivalve taxon nearest to the dreissenoid/corbiculoid lineage for which mt 16S rDNA sequences had been published (Hoffman *et al.,* 1992; Table 1). Maximum parsimony trees (in PAUP*; Swofford, 1998) and neighbor joining (NJ; Saitou and Nei, 1987) genetic distance trees were used to test relationships among the dreissenoid and corbiculoid taxa. NJ trees (Saitou and Nei, 1987) were constructed from the Kimura (1980) two-parameter and p-distances, using MEGA (Kumar *et al.,* 1993). Separate runs were made including all types of substitutions (with indels coded as an additional character state), with tv and indels only (excluding ts), and excluding indels. Maximum parsimony analyses were conducted using the exhaustive search algorithm in PAUP* 4.0) (d64, Swofford, 1998) from the entire data set and

exclusion of phylogenetically uninformative characters. Bootstrap analyses (Felsenstein, 1985, Swofford *et al.,* 1996) with 1000 replications tested the support of the data set for the nodes of the maximum parsimony (using the branch-and-bound algorithm; Swofford, 1998) and genetic distance (neighbor joining in MEGA; Kumar *et al.,* 1993) trees.

RESULTS

Sequence Variation and Secondary Structure of mt 16S rRNA

The mt 16S rDNA data set (Table 1) consisted of 486 aligned nucleotides (reported in GenBank as AF038996 for *Dreissena bugensis,* AFO389997 for *D. polymorpha,* AFO38998 for *M. leucophaeata,* and AFO38999 for *C. fluminea*). No intraspecific polymorphisms were found. Morphotypes of *D. bugensis* from deep (the profunda variant) and shallower waters had identical sequences. Figure 3 shows the secondary structure of mt 16S rDNA for *D. bugensis,* based on a model for *M. edulis* by Lydeard *et al.* (1996).

Base composition showed significant bias in the dreissenoids and corbiculoids, which was statistically similar in stem and loop regions and among taxa (Table 2). *Mytilus edulis* had fewer adenine nucleotides (29%) than did the other taxa, and the proportions of nucleotides in the stem regions were more evenly distributed.

A total of 251 (52%) nucleotide sites were variable (Table 3A) and 99 (20%) of these were phylogenetically informative (Table 3B). The relative proportions of variable versus invariable sites were statistically similar in stems and loops (Table 3A). The distribution of phylogenetically informative and uninformative sites differed significantly between stem and loop regions, with stems having a greater proportion of informative sites (Table 3B).

There were 145 ts (94 in stems and 51 in loops) and 191 tv (112 in stems and 79 in loops), with each substitution event counted only once in the data set and interpreted according to the phylogenetic tree shown in Fig. 5 (a parsimonious approach; Table 3C). The absolute proportional numbers of ts and tv did not differ statistically between stems and loops, but tv were more prevalent in loops (Table 3C). At the different levels of taxonomic comparisons (with each substitution event counted only once, at the level it occurred on the phylogenetic tree), tv significantly outnumbered ts only at the level of order (Table 4).

Regression analysis of the pairwise numbers of ts and tv with pairwise genetic distances showed a linear relationship in both stem and loop regions (Fig. 4). Significantly more tv occurred in the higher level taxonomic comparisons (Table 4). The ts:tv ratio was greater at the species level (1.4:1), equal at the generic (1:1) level, and lower at the superfamily (0.7:1) and order (0.7:1) levels. Overall, the relative proportions of ts and tv did not differ significantly among the various taxonomic levels (Table 4).

Genetic Distance and Parsimony Analyses

Kimura (1980) two-parameter and p-distances, calculated from the entire data set and for tv and indels alone, are given in Table 5. Kimura (1980) two-parameter distances among the taxa ranged from $d = 0.079 \pm 0.014$ to 0.602 ± 0.053, based on all types of substitutions, and from $d = 0.034 \pm 0.009$ to 0.348 ± 0.041, based on tv and indels (and excluding ts; Table 5). Pairwise distances ranged from $p = 0.075 \pm 0.012$ to 0.413 ± 0.023, based on all types of substitutions, and from 0.033 ± 0.008 to 0.251 ± 0.021, based on tv and indels only (Table 5). Pairwise and Kimura (1980) two-parameter distances were almost identical (within 0.00 to 0.02 for each pairwise comparison) with deletion of all sites with indels and thus are not given.

Genetic distance NJ trees based on all substitutions (Fig. 5A), with tv and indels only (Fig. 5B) and excluding indels (not shown), were identical in topology and similar in proportional distances. An exhaustive maximum parsimony search in PAUP* (Swofford, 1998) produced a single most parsimonious tree (Fig. 5C) that was identical to the NJ trees in topology, having 383 steps, a consistency index (C. I.) excluding uninformative characters of 0.81, and a g1 skewness of -1.30. When sites with indels were excluded, an identical most parsimonious tree was obtained, having 318 steps and a C.I. of 0.81. The next most parsimonious tree had 388 steps based on all characters and 323 steps with indels excluded.

D. polymorpha and *D. bugensis* clustered together in the genetic distance and maximum parsimony trees, with 97 and 88%, respective, bootstrap support (Figs. 5A and 5C). The *Dreissena* taxa examined were linked by 17 nucleotide synapomorphies (Table 1). A total of 8 autopomorphies and two insertions defined *D. bugensis. D. polymorpha* was characterized by 11 nucleotide autopomorphies.

Mytilopsis leucophaeata was the sister group to the dreissenids tested, supported by 100% of the bootstrap replications in the genetic distance and parsimony trees (Figs. 5A and 5C). Fifty-nine nucleotide synapomorphies and three indels linked *Mytilopsis* and *Dreissena* (Table 1). Twenty autapomorphies defined *M. leucophaeata* (Table 1).

Rates of Evolutionary Divergences

The rate of the mt 16S rDNA molecular clock was calibrated to the divergence of *M. leucophaeata* and *C. fluminea* from the fossil record as 54 mya (Morton, 1970; Table 6). The divergence rate for p-distances was calculated as $p = 0.0057$ per my for the entire data set and $p = 0.0033$ for tv and indels only. Divergence rates from Kimura (1980) two-parameter distances were d =

TABLE 1

Aligned 16S rDNA Sequences

Taxon	Base position
	0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	A A A G A A T A A G G A T C G C G C C T G C C C G G T G A - - - - - - - - T G A G A G - - - - - - T T
D. polymorpha	A A A G A A T A A G G A T A G C G C C T G C C C G G T G A - - - - - - - - C G A G A G - - - - - - T T
M. leucophaeata	A A A T A A T A G A G G T C T G G C C T G C C C A G T G A - - - - - - - - T G A G A A - - - - - - T T
C. fluminea	A A T T A A T A A A G G T T T G G C C T G C C C G G T G A - - - - - T C T G A A A T G G - - - - T T
M. edulis	G T A A G T A A A A G G T A G T C C C T G C C C A G T G C A A C T A G A G T A A T G T C T G T T G T
Structural category	L L L L L S S S S S S S S L L S S S S L S S S L L S S S S S S S S S S S L L L L L L S S S S S S S S
Informative	U U U I U U U U U I U I U I I I U U U U U U U U I U U U U U U U U U U U I I U U I U U I I U U U U U U
	1
	5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 0
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	A A A C G G C C G C A G T T A A - - T A G C T G T G C T A A G G T A G C G C A A T C A A T T G T C C
D. polymorpha	A A A C G G C C G C A G T T A A - - T A G C T G T G C T A A G G T A G C G C A A T C A A T T G T C C
M. leucophaeata	A A A C G G C C G C A G T T A A - - T A G C T G T G C T A A G G T A G C G C A A T C A A T T G T C C
C. fluminea	T A A C G G C T G C - G A T T G - - A A A C C G T A C T A A G G T A G C A T A A T A A T T T G C C C
M. edulis	A A A C G G C G G C - G T T A A C G T C A A C G T C C T A A C G C G C C A T A A T T T G C T T
Structural category	L L S S S S S S S S S S S S L L L L L S S S S S S S S L L S S S S L L L L L L L L L S S S S S S S S
Informative	U U U U U U U I U U I U U U U U U U U U I U I U U I U U U U U U U U U U U U U I U I U U U I U U
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
	0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	T T T A A T A A G G G A A T G G T A T G A A T G G C T G T A C G C G A G A T A A G C T G T C T C T A
D. polymorpha	C T T A A T T G A G G A A T G G T A T G A A T G G C T G T A C G T G A G A T A A G C T G T C T C T A
M. leucophaeata	C T T A A T A G A G G A A T G G T A T G A A T G G C T G G A C G T G A G A T A A G C T G T C T C T A
C. fluminea	T T T A A T T G G G G G G A G A G A A T G A A T G G T T T G A C G G T A A A A A A G C T G T - T T T A
M. edulis	T T C A A T T G A A G G A T G G T A T G A A A G G G T T A A C G A A G A A G G T G C T G T G T C T A
Structural category	S S S L L L S S S S S S S S L L L L L L L L L L S S S S L L L S S S S S S S S S S S L L L L S S S S S S
Informative	I U U U U U I U I U U I U U U U U U U U U U U U I U I I U U U I I U I U I U U U U U U U I U U U U
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2
	5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 0
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	T T A T A T T A G C A A G A A A T T T C C T T T A A G T G A A A A G A C T T A G A T T T T T T T A
D. polymorpha	T T A T A A T A A C A T G A A A T T T C C T T T G A G T G A A A A G A C T T A G A T T T T T T T A
M. leucophaeata	T T A C A A C A G C A T G A A A T T T C C T T T A A G T G A A A A G A C T T A A A T T T T T A T A
C. fluminea	A A A A T A A A A A A A A G A A A G T T T A A C T T T T A A G T G A A A A G G C T T A A G T T T T T A T A
M. edulis	A A A A T T C A A T T T A A A - C T A A C T T T A A G G T G A A G A G G C C T T T A T G T A A A A G
Structural category	S S S S S S S S S S L L L L L L L L L L L S S S S S L L L L L L S S S S S L L L L L L S S S S S
Informative	I I U I I I I U I I U I U U U U U U I I U I U U U U U U U U U U U U U I U I U U I U U U U U U I U U
	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
	0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	A A A G A C G A G A A G A C C C T A T C G A A C T T A A T C A T T T T T A A A G A A C T C A - - T
D. polymorpha	A A A G A C G A G A A G A C C C T A T C G A A C T T A A T A G T T T T T A A G A G A A A T A G - - C
M. leucophaeata	A A A G A C G A G A A G A C C C T A T C G A A C T T A A T A A T C A A T T A A A A C A A A A A - - T
C. fluminea	A A A G A C G A G A A G A C C C C G T C G A G C T T A A T T A G A A T A A C T T A A A T G T A - - G
M. edulis	A A G G A C G A C A A G A C C C T A T C A A T C T T T A T C T T A A T T G G A G C T - C T C A G G C
Structural category	S L S S S L S S L L L L L S S S S S L S S S S S S S S S S S S S S S S S S S S S S S L L L L L L L L L S
Informative	U U U U U U U U U U U U U U U U U U U U U U U U I U U U U U U I I U I I U U U I U U I U U I U U U U I
	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3
	5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 0
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	T T A A A A A G A A T T T T T A C T G G G G C A G T A - - A G A A G A A A A A A A T A A C T C T T C
D. polymorpha	T T A G A A A G A A G T T T T A C T G G G G C A G T A - - A G A A G A A A A A A A T A A T T C T T C
M. leucophaeata	T A A T T G A G A A A T T T T A C T G G G G C A G T A - - A G A A G A A A A A A A T A A T T C T T C
C. fluminea	G T T A T T T A A A A T T T T T A T T G G G G C A A T A - - G A A A A T G A A A T G A A T C A T T T T
M. edulis	T C T A T A C G A T - - T T T G A T G G G A G A T C A G T A G A A - - - - - - - - A T A A G T C T T C
Structural category	S S S S S S S S S S S S S S S S S S S L L L S S S S S S S S S S S S S S S L L L L L L L L L S S S S S
Informative	U I I U I U I U U U U U U U U U I U U U U U U U I U U U U U U U I I U U U U I U U U U I U U U U U

TABLE 1—*Continued*

Aligned 16S rDNA Sequences

Taxon	Base position
	3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
	0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	C – – T T A T – – – – A A A A A A – – – – A A G A T C C C T C A A T G A G – G A G A A A A A G A A A
D. polymorpha	C – – T T G A – – – – A A A A A A – – – – – – G A T C C C T T A T T A A G – G A C A A A A – G A A A
M. leucophaeata	C – – A A A A – – – – T C C A A A – – – – – – G A T C C C T T A A T A A G – G A A A A A A – G A A A
C. fluminea	T – – T T T A – – – – T T A T A A – – – – – G G A T C C A G T T T T G A C T G A A A A A A – G C A A
M. edulis	T A C T A T C A T A T T A A T C T T A C T A G T A T T T C C T A A C T T T A T A T G T G T G G C – –
Structural category	S S S S S L L L L L L L S S L L L L L L L L L L L L L L S S S L L L L L L L S S S L L L L L L S S S
Informative	I U U U U U I U U U U I U U I U U U U U U U U U U U U U U I U U U U U U U I U U U U U U U U U I U U
	3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 4
	5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 9 0
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	A A G T T A C C G T A G G G A T A A C A G C G T T A T C G T T T T T A A G A G A T C T A A T C G A A
D. polymorpha	A A G T T A C C G T A G G G A T A A C A G C G T T A T C G T T T T T A A G A G A A C T A A T C G A A
M. leucophaeata	A A G T T A C C G T A G G G A T A A C A G C G T T A T C A T T T T T A A G A G A A C T A A T C G A A
C. fluminea	A A G C T A C C G C G G G G A T A A C A G G G T A A T T T T T T C T G A G A G T T C A T A T T T A A
M. edulis	T A G C T A C T C T A G G G A T A A C A G C G C A A T T T C T C C C G A A A G A T G G T A T T G G A
Structural category	S S S S S L L S S S S S S S L L L L L L L S S S L S S S S S S S S S L L S S L L L L L L L S S L L S
Informative	U U U I U U U U U U U U U U U U U U U U U U U I U U I I U U U I U I U U U U U I U I I U U I U U U U
	4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4
	0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
D. bugensis	G A A A C G G T T T G C G A C C T C G A T G T T G G A T T T A A A T T T C T T C C T G A G C G C A G
D. polymorpha	G A A A C G G T T T G C G A C C T C G A T G T T G G A T T T A A A T T T C T T C C T T G G C G C A G
M. leucophaeata	A A A A T G G T T T G C G A C C T C G A T G T T G G A T T C A A G T T T C T T C C T T G G C G C A G
C. fluminea	G A A G A A G T T T G C T A C C T C G A T G T T G G A T T A G A A T T T C T T T A T G C T T G C A G
M. edulis	G G G G A A G A T T G C G A C C T C G A T G T T G G C T T T A G A T A T C C T A G A G G C G G – A G
Structural category	S S S S S S S S L L S S S L L L L L L L L L L S S S S S S S S S S S S S S S S S S S S L L L L S S S
Informative	U U U I I I U U U U U U U U U U U U U U U U U U U U U U U U U U U U U U U U U I I U I U I I U U U U
	4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4
	5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 8 8 8 8 8 8 8
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6
D. bugensis	G A G C T A G G A A A A G T A G G T C T G T C C G C C C T T G A A T T G
D. polymorpha	T A G T T T G G A A A A G T A G G T C T G T T C G C C C T T G A A T T G
M. leucophaeata	G A G C T A G G A A A A G T A G G T C T G T T C G C C C T T T A A C T G
C. fluminea	C A G G T A T A A A T A G T A G G A C T G T T C G T C C T T T A A T T T
M. edulis	A A G C T T C T G A T G G T G G G T C T G T T C G C C C T T T A A A A T
Structural category	S S L L L S S L S S S L L L S S S S S L L L L L S S S S S L L L L L S S
Informative	U U U U U I I I U U I U U U U U U U U U U U U U U U U U U U I U U U I I

Note. Deletions are indicated with a dash. Nucleotide sites are designated as either stems (S) or loops (L) from the secondary structure model and as either phylogenetically informative (I) or uninformative (U) from the PAUP analysis. Restriction sites used in Fig. 6 are underlined.

0.0074 per my for the entire data set and d = 0.0041 for tv and indels only. The values from the two types of distances overlapped in standard errors in all cases for comparisons of the entire data set (Table 6A), those based on tv and indels (Table 6B), or those omitting indels (not shown).

The p-distance divergence between *D. bugensis* and *D. polymorpha* was estimated as 13.2 ± 2.2 mya from the entire data set and 10.06 ± 2.6 mya using tv and indels only, occurring during the middle to late Miocene Epoch. Using Kimura (1980) two-parameter distances, this estimate was 10.8 ± 1.9 my from the entire data set and 8.5 ± 2.2 from tv and indels only. The average divergence of *Dreissena* and *Mytilopsis* occurred earlier in the Miocene Epoch, at 20.7 ± 2.7 mya calculated from the entire data set using p-distances. Their divergence was 17.8 to ± 3.3 mya, based on tv and indels. Using Kimura (1980) two-parameter distances, these estimates were 17.8 ± 2.5 my from the entire data set and 15.3 ± 3.1 from tv and indels.

Restriction Digest Analysis and Species Discrimination

Digestions of PCR-amplified mt 16S rDNA with the endonucleases Dde I and Acs I yielded species-specific patterns for *D. bugensis, D. polymorpha, M. leucophaeata,* and *C. fluminea,* as shown in Fig. 6. The Dde I digest (Fig. 6A) cleaved the amplified mt 16S rDNA

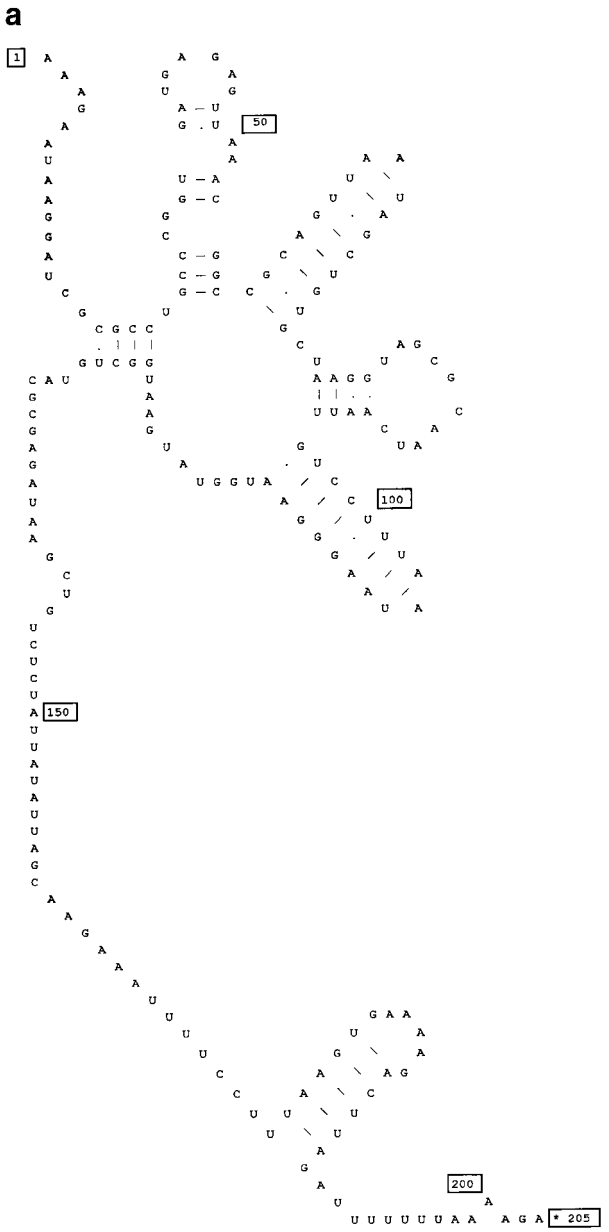

FIG. 3. Model of mtDNA 16S rDNA secondary structure for *Dreissena bugensis.* Numbers correspond to nucleotide sites in Table 1. Stems are paired nucleotides; loops are unpaired. (A) 5′ end that joins the 3′ side (B) at the asterisk. (B) 3′ end that joins the 5′ side (A) at the asterisk.

piece from *C. fluminea* twice, producing fragments that were 289, 103, and 65 bp in length. *M. leucophaeata* contained one Dde restriction site, yielding fragments of 391 and 62 bp. *D. bugensis* was defined by three Dde I restriction sites and four fragments of 239, 110, 62, and 45 bp (the latter is not visible on our gel). *D. polymorpha* was cleaved twice, producing fragments that were 281, 110, and 62 bp.

In the Acs I digest (Fig. 6B), the amplified mt 16S rDNA piece from *C. fluminea* was cleaved twice, producing fragments that were 244, 158, and 55 bp in length. *M. leucophaeata* was cleaved into four pieces of 211, 149, 67, and 26 bp (the latter is not visible on our gel). *D. bugensis* was cleaved three times, producing fragments of 160, 149, 92, and 55 bp. *D. polymorpha* was cut twice, yielding fragments that were 249, 149, and 55 bp.

DISCUSSION

Patterns of Molecular Evolution of mt 16S rDNA

Lack of intraspecific variation in the present study and others (summarized by Simon *et al.,* 1994; Palumbi, 1996) indicates that the mt 16S rDNA gene is highly conserved in many taxa, apparently due to its functional role in protein assembly (De Rijk *et al.,* 1995). Unlike results for *Mytilus edulis* from the same region of mt 16S rDNA, which has male and female mitotypes (Rawson and Hilbish, 1998), no intraspecific variability

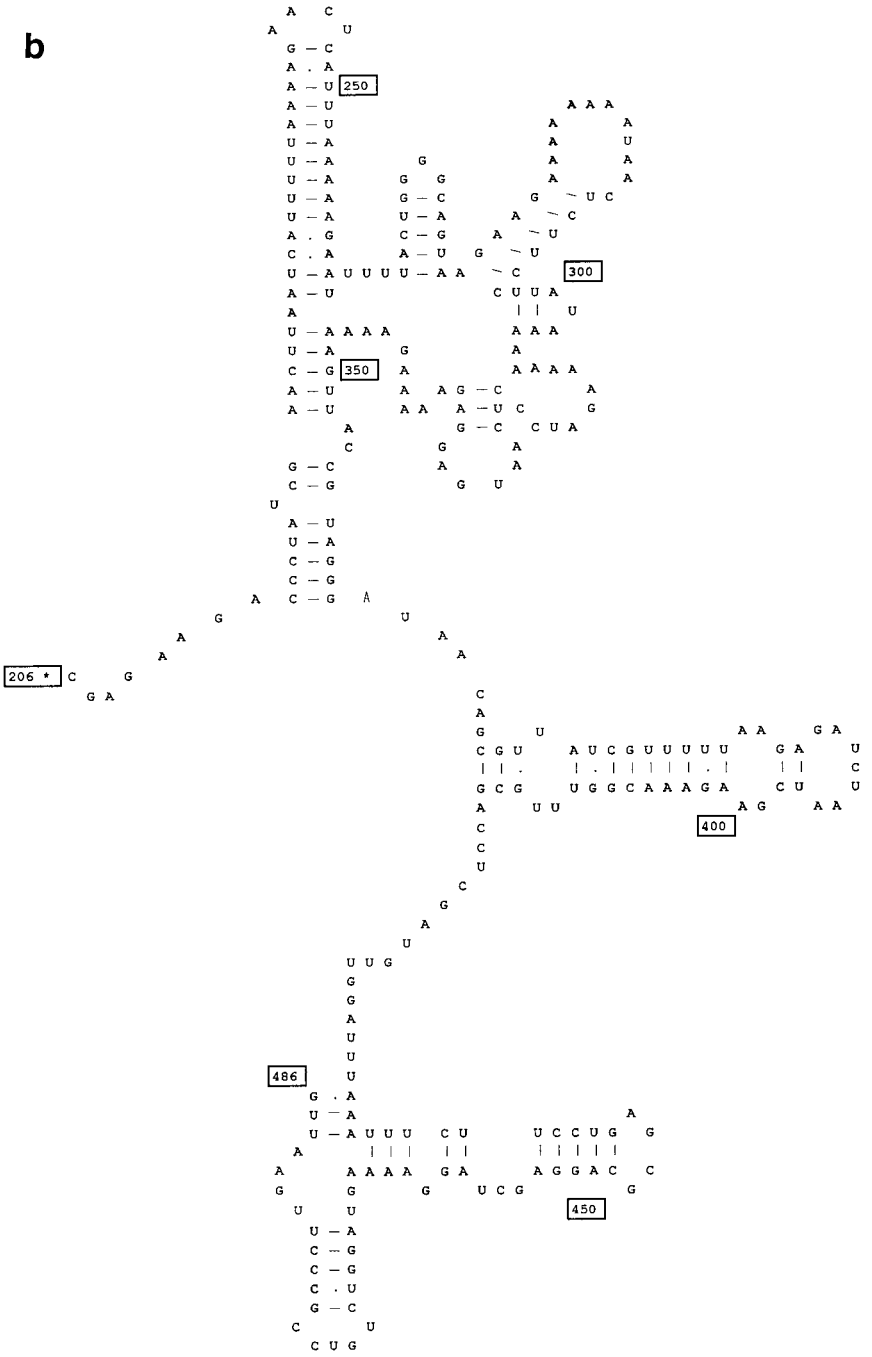

FIG. 3—*Continued*

was found in our study. This may be due to differential rates of evolution in mytiloids versus the dreissenoid/corbiculoid lineage, resulting from different historical effective population sizes (summarized by Avise, 1994), and/or a result of the doubly uniparental inheritance in mytiloids (Rawson and Hilbish, 1998; Quesada *et al.,* 1998). A nuclear RAPDs study in progress by Skidmore and Stepien for these taxa (including the same individuals and much larger sample sizes) indicates high levels of genetic variability and population genetic divergence and structure from European and North American populations.

Our study found significant adenine and thymine biases in both stem and loop regions for all species examined, except for stems in *M. edulis* (Table 2). Analysis of mt 16S rDNA for unionids showed significant adenine, but not thymine, nucleotide bias (Lydeard *et al.,* 1996). Similar to our results, Baldwin *et al.* (1996) found significant adenine and thymine biases in the mtDNA COI gene of *D. bugensis, D. polymorpha,*

TABLE 2

Nucleotide Composition of the mt 16S rDNA Gene among (A) Stem Regions, (B) Loop Regions, and (C) the Entire Data Set (Stems and Loops)

A. Stem regions

Taxa	Nucleotides G	A	T	C	X^2 *P* value
D. bugensis	59	82	82	46	$X^2 = 14.2$
	22%	31%	30%	17%	$P = 0.003$*
D. polymorpha	60	79	84	46	$X^2 = 13.7$
	22%	30%	31%	17%	$P = 0.003$*
M. leucophaeata	59	86	76	48	$X^2 = 12.9$
	22%	32%	28%	18%	$P = 0.005$*
C. fluminea	61	88	87	35	$X^2 = 28.0$
	22%	33%	32%	13%	$P < 0.001$*
M. edulis	64	76	82	56	$X^2 = 5.9$
	24%	27%	29%	20%	$P = 0.116$
Mean	60.6	82.2	82.2	46.2	$X^2 = 13.8$
(+/−) s.e.	0.9	2.2	1.8	3.4	$P = 0.003$*
	23%	30%	30%	17%	
X^2 for differences in distribution among taxa					$X^2 = 6.84$
					$P = 0.868$

B. Loop regions

Taxa	Nucleotides G	A	T	C	X^2 *P* value
D. bugensis	40	78	44	25	$X^2 = 31.1$
	21%	42%	24%	13%	$P < 0.001$*
D. polymorpha	42	76	46	20	$X^2 = 34.6$
	22%	41%	26%	11%	$P < 0.001$*
M. leucophaeata	34	81	46	23	$X^2 = 41.2$
	18%	44%	25%	13%	$P < 0.001$*
C. fluminea	37	70	61	18	$X^2 = 35.8$
	20%	37%	33%	10%	$P < 0.001$*
M. edulis	45	62	60	26	$X^2 = 17.3$
	23%	32%	31%	14%	$P < 0.001$*
Mean	39.6	73.4	51.4	22.4	$X^2 = 29.5$
(+/−) s.e.	1.9	3.4	3.7	1.5	$P < 0.001$*
	21%	39%	28%	12%	
X^2 for differences in distribution among taxa					$X^2 = 12.0$
					$P = 0.444$

C. Entire region

Taxa	Nucleotides G	A	T	C	X^2 *P* value
D. bugensis	99	160	126	71	$X^2 = 38.0$
	22%	35%	27%	16%	$P < 0.001$*
D. polymorpha	102	155	130	66	$X^2 = 38.7$
	22%	34%	29%	15%	$P < 0.001$*
M. leucophaeata	93	167	122	71	$X^2 = 45.6$
	20%	37%	27%	16%	$P < 0.001$*
C. fluminea	98	158	148	53	$X^2 = 61.9$
	21%	35%	32%	12%	$P < 0.001$*
M. edulis	109	138	142	82	$X^2 = 20.0$
	23%	29%	31%	17%	$P < 0.001$*
Mean	100.2	155.6	133.6	68.6	$X^2 = 38.1$
(+/−) s.e.	2.6	4.8	4.9	4.7	$P < 0.001$*
	22%	34%	29%	15%	
X^2 for differences in distribution among taxa					$X^2 = 13.8$
					$P = 0.312$

Note. *Significant difference at $P < 0.05$.

TABLE 3

Distributions of Nucleotide Sites in Stem and Loop Regions of mt 16S rDNA that are (A) Variable versus Invariable, (B) Phylogenetically Informative versus Uninformative, and have (C) Transitional versus Transversional Substitutions

A. Distribution of variable and invariable sites

	Variable sites	Invariable sites	χ^2 Tests
Stems	156 (55%)	130 (45%)	$\chi^2 = 2.36$
	60%	57%	$P = 0.124$
Loops	95 (47%)	105 (53%)	$\chi^2 = 0.50$
	40%	43%	$P = 0.480$
Overall	251 (52%)	235 (48%)	
Test for difference in distribution of sites between stems and loops			$\chi^2 = 2.34$ $P = 0.126$

B. Distribution of informative and uninformative sites

	Informative sites	Uninformative sites	χ^2 Tests
Stems	67 (23%)	219 (77%)	$\chi^2 = 80.8$
	68%	56%	$P < 0.001$*
Loops	32 (16%)	168 (84%)	$\chi^2 = 92.5$
	32%	44%	$P < 0.001$*
Overall	99 (20%)	387 (80%)	
Test for differences in the distribution of sites between stems and loops			$\chi^2 = 4.00$ $P = 0.045$*

C. Distribution of transitional and transversional substitutions

	N Transitions	N Transversions	Ratio	χ^2 Tests
Stems	94 (46%)	112 (54%)	0.84:1.0	$\chi^2 = 1.57$
	65%	59%		$P = 0.210$
Loops	51 (39%)	79 (61%)	0.65:1.0	$\chi^2 = 6.03$
	35%	41%		$P = 0.014$*
Overall	145 (43%)	191 (57%)	0.76:1.0	
Test for difference in distribution of transitions and transversions between stems and loops				$\chi^2 = 1.33$ $P = 0.249$

Note. *Significant difference at $P < 0.05$.

M. leucophaeata, and *C. fluminea.* Adenine and thymine biases thus appear to characterize the mtDNA of dreissenoid and corbiculoid bivalves. Nucleotide bias in mtDNA appears to vary among different bivalve orders.

Secondary structure of the mt 16S rDNA gene influences the distribution and utility of nucleotide variability, with stem regions having lower mutation rates than loop regions (Simon *et al.,* 1994; Fig. 3). At higher level taxonomic comparisons, proportionally more variable characters are often found in stem regions when loop regions became saturated (Guttel *et al.,* 1994). Our finding of a significantly greater number of variable nucleotides and a greater proportion of ts in stem regions (Table 3A) suggests some saturation, although

the proportion of ts:tv did not differ significantly between stems and loops (Table 3C). The numbers of phylogenetically informative versus uninformative sites also were significantly greater in stems than in loops, indicating saturation (Table 3B). Informative characters comprised 20% of the overall data set (and 39% of the polymorphic characters; Table 3A) in our study, slightly less than the proportion found in unionid mussels (29%; Lydeard *et al.,* 1996) for mt 16S rDNA and greater than that found in the marine clam genus *Mercenaria* (5%; Foighil *et al.,* 1996).

Transitional bias has been found in most mtDNA studies (Brown *et al.,* 1982) and proportions of the ts:tv may be used to interpret the relative degree of mutational saturation (Hillis *et al.,* 1996). Multiple substitutions accumulate progressively at given nucleotide sites with evolutionary time, resulting in progressively lower ts:tv ratios (Kocher and Carleton, 1997). In our data set, ts:tv ratios decreased with increasing level of taxonomic comparison, suggesting increased saturation and phylogenetic noise. The ts:tv ratio was significantly lower for the deepest evolutionary comparison (between orders), indicating some saturation (Table 4). Lydeard *et al.* (1996) found a much higher transitional bias for unionids, with ts:tv ratios close to 22:1 for closely related species and close to 1:1 for distantly related species. Ts and tv continued to accumulate linearly with genetic distance in our data set (Fig. 4), suggesting that saturation did not obscure the phylogenetic signal (Lydeard *et al.,* 1996; Hillis *et al.,* 1996). Similarly, Lydeard *et al.* (1996) found that the number of ts continued to increase with genetic distance in unionid mussels. In our study, numbers of both ts and tv were significantly correlated with genetic distance at all taxonomic levels in both stem and loop regions (Fig. 4). The relatively low levels of saturation and divergence detected in the present study are unlikely to appreciably affect the phylogenetic utility of 16S rDNA for discerning these relationships (Yang, 1998).

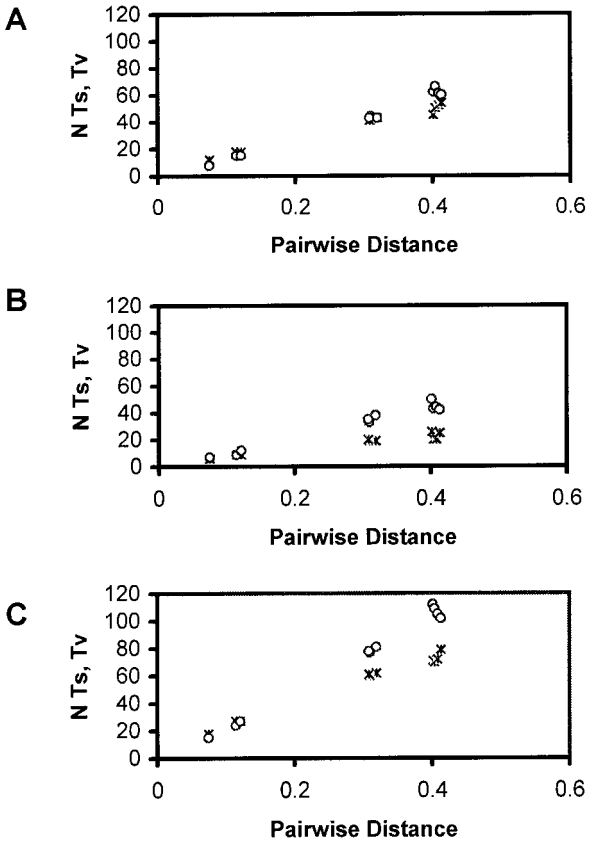

FIG. 4. Regression analysis of the number of transitions (Ts, crosses) and transversions (Tv, circles) versus p-distances among each pair of taxa. (A) Stem regions (Ts: $14.5\times + 4.5$, $r^2 = 0.98$; Tv: $161.7\times - 4.8$, $r^2 = 0.99$). (B) Loop regions (Ts: $49.3\times + 3.1$, $r^2 = 0.92$; Tv: $117.2\times - 2.4$, $r^2 = 0.97$). (C) Entire data set (Ts: $163.8\times + 7.6$, $r^2 = 0.99$; Tv: $278.9\times - 7.2$, $r^2 = 0.99$).

TABLE 4

Numbers of Transitions and Transversions within and among Taxonomic Levels

Taxonomic comparison	Transitions	Transversions	Ratio	χ^2 Test
Species *D. bugensis* vs *D. polymorpha*	21 (58%)	15 (42%)	1.4:1.0	$\chi^2 = 1.00$ $P = 0.317$
Genus *Dreissena* vs *Mytilopsis*	14 (50%)	14 (50%)	1.0:1.0	$\chi^2 = 0.00$ $P = 1.000$
Superfamily Dreissenoidea vs Corbiculoidea	39 (41%)	56 (59%)	0.7:1.0	$\chi^2 = 3.04$ $P = 0.081$
Order Veneroida vs Mytiloida	70 (39%)	106 (61%)	0.7:1.0	$\chi^2 = 7.36$ $P = 0.007$*
Overall	144 (43%)	191 (57%)	0.8:1.0	$\chi^2 = 6.59$ $P = 0.010$*
χ^2 contingency test among taxonomic levels				$\chi^2 = 4.91$ $P = 0.179$

Note. *Significant difference at $P < 0.05$.

Studies of mussels (Lydeard *et al.,* 1996), insects (DeSalle *et al.,* 1987), and vertebrates (Mindell and Honeycutt, 1990) found that mt 16S rDNA sequences accumulated substitutions linearly up to 200 to 300 my. The sequence divergences in our study corresponded to a maximum of 86 my of total evolutionary divergence time (Table 6), indicating appropriate variability to address these systematic relationships. The high g1 skewness from maximum parsimony analyses of our data also indicated appreciable phylogenetic signal (Hillis and Huelsenbeck, 1992; Swofford *et al.,* 1996).

Phylogenetic Relationships and Divergences

Prior to molecular studies, systematic relationships among bivalues were based predominantly on shell characters, which often displayed convergence (Morton, 1996). Molecular genetic data, such as the mt 16S rDNA gene in this study, offer new suites of characters

TABLE 5

Genetic Distances among Taxa

A. Based on all substitution types

Taxon	*D. bugensis*	*D. polymorpha*	*M. leucophaeata*	*C. fluminea*	*M. edulis*
D. bugensis	—	0.079 +/− 0.014	0.133 +/− 0.018	0.401 +/− 0.038	0.580 +/− 0.052
D. polymorpha	0.075 +/− 0.012	—	0.125 +/− 0.018	0.418 +/− 0.039	0.584 +/− 0.052
M. leucophaeata	0.121 +/− 0.015	0.115 +/− 0.015	—	0.399 +/− 0.037	0.593 +/− 0.052
C. fluminea	0.310 +/− 0.022	0.319 +/− 0.022	0.308 +/− 0.022	—	0.602 +/− 0.053
M. edulis	0.401 +/− 0.023	0.404 +/− 0.023	0.409 +/− 0.024	0.413 +/− 0.023	—

B. Based on transversional substitutions and indels (excluding transitions)

Taxon	*D. bugensis*	*D. polymorpha*	*M. leucophaeata*	*C. fluminea*	*M. edulis*
D. bugensis	—	0.034 +/− 0.009	0.066 +/− 0.013	0.217 +/− 0.028	0.302 +/− 0.037
D. polymorpha	0.033 +/− 0.008	—	0.059 +/− 0.012	0.230 +/− 0.029	0.318 +/− 0.038
M. leucophaeata	0.062 +/− 0.013	0.055 +/− 0.011	—	0.219 +/− 0.028	0.340 +/− 0.041
C. fluminea	0.177 +/− 0.018	0.184 +/− 0.018	0.178 +/− 0.018	—	0.348 +/− 0.041
M. edulis	0.227 +/− 0.020	0.235 +/− 0.020	0.247 +/− 0.021	0.251 +/− 0.021	—

Note. Kimura (1980) two-parameter genetic distances +/− s.e. (above the diagonal) and pairwise distances +/s.e. (below the diagonal).

to augment and test traditional morphological classifications. In another example, relationships through the familial level were discerned by Lydeard *et al.* (1996) among unionid mussels from mt 16S rDNA sequences. Evolutionary relationships among higher bivalve taxa have been resolved from nuclear 18S rDNA sequences, which evolve more slowly than does mt 16S rDNA (Adamkewicz *et al.,* 1997).

Phylogenetic relationships from mt 16S rDNA sequences in our study (Fig. 5) were congruent with those from the mtDNA COI gene (Baldwin *et al.,* 1996) for these dreissenoid and corbiculoid taxa. In both studies, *M. leucophaeata* was separated from *Dreissena* by about twice the genetic distance as that between *D. polymorpha* and *D. bugensis* (Table 5; Baldwin *et al.,* 1996). The mt 16S rDNA sequence for the profunda variant was identical to that of *D. bugensis* from shallower waters, supporting the conclusion from allozyme and COI data that they are the same species (Spidle *et al.,* 1994a; Claxton *et al.,* 1997).

Heteroplasmy and Hybridization Questions

Our data from the mt 16S rDNA gene in the Dreissenoidea do not indicate doubly uniparental inheritance (DUI), since all samples (males and females) yielded a single sequence for each taxa. DUI may occur and not be visible in our study due to the relatively slow rate of mt 16S rDNA evolution. However, Geller and Powers (1994) and Rawson and Hilbish (1998) sequenced the same region of the mt 16S rDNA gene and found DUI in *Mytilus spp.,* with female haplotypes of different species being more closely related to each other than to male conspecifics. DUI was not found in the study of the mtCO1 gene of dreissenids (Claxton *et al.,* 1997; Claxton and Boulding, 1998). These results suggest that DUI does not occur in dreissenoids or corbiculoids.

Hybrids of *D. polymorpha* and *D. bugensis* have occurred in laboratory crosses but did not survive to the settling stage (Nichols and Black, 1994). Evidence of species-specific sperm attractants (Miller *et al.,* 1994), the large Nei's (1972) genetic distance between *D. polymorpha* and *D. bugensis* from allozymes ($D = 1.2$ to 1.7; May and Marsden, 1992; Spidle *et al.,* 1994a), and the genetic distance in our mt 16S rDNA sequences (0.075 ± 0.012; Table 5B) suggest considerable barriers to hybridization. Spidle *et al.* (1994b) using diagnostic allozyme loci tested for potential hybrids between *D. bugensis* and *D. polymorpha* in North America. No evidence for hybridization was found by Spidle *et al.* (1994b) or in our results.

Relationships between Genetic Distance and Evolutionary Time

The calibrated rate for mtDNA COI sequence divergence was estimated as $p = 0.006$ per my, based on all substitutions (Baldwin *et al.,* 1996), which was very close to the rate for the mt 16S rDNA divergence calculated in our study based on all substitutions (0.0057 per my; Table 6). These rates of evolution are slower than the overall rates of $p = 0.01$ to 0.02 per my that have been cited for mtDNA as a whole (Brown, 1982; Avise, 1994). Recent studies have shown that different regions of animal mtDNA have markedly different rates of sequence evolution (Rand, 1994). The mt 16S rDNA and COI genes evolve more slowly than does the mt genome as a whole (Cann *et al.,* 1984; Palumbi, 1996). DeSalle *et al.* (1987) suggested that the high adenine and thymine content in insect mt 16S rDNA sequences, as was found in the present study,

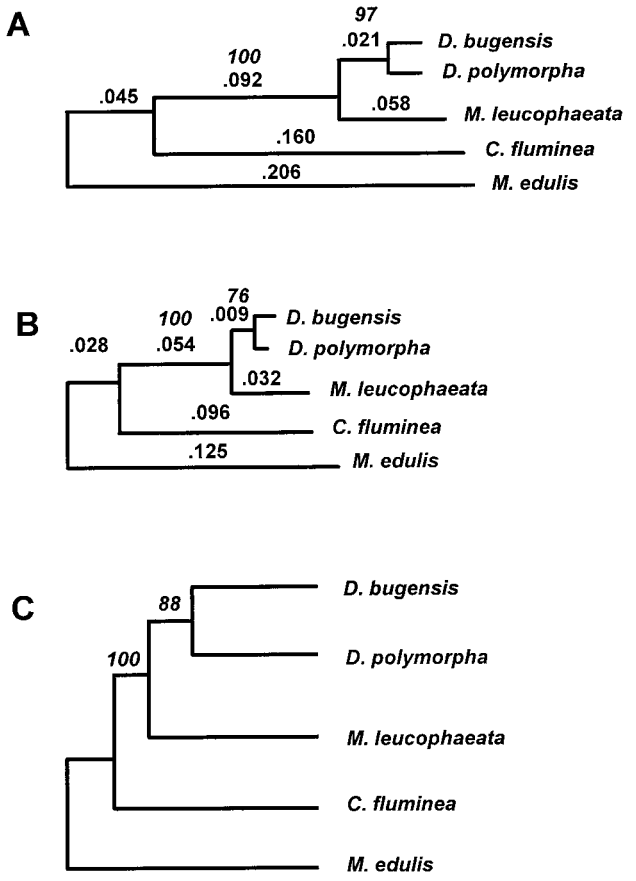

FIG. 5. Phylogenetic trees of relationships among the dreissenoid and the corbiculoid taxa from the mt 16S rDNA sequences. Bootstrap values from 1000 replications are italicized. Branch lengths (decimals) for the distance trees are not italicized. (A) Neighbor joining (NJ) distance tree from MEGA (Kumar *et al.,* 1993), based on Kimura (1980) two-parameter distances and all substitution types. (B) NJ tree based on transversions and indels only. (C) Most parsimonious tree from maximum parsimony analysis and an exhaustive search using all characters with PAUP*4.0, vers. d64 (Swofford, 1998). Length = 383 steps, Consistency index (C. I.) excluding uninformative characters = 0.81, Homoplasy index (H. I.) = 0.19, g1 skewness = −1.30. With exclusion of all nucleotide sites with indels, this tree was also the most parsimonious, with 318 steps and a C.I. = 0.81.

constrains the rate of sequence evolution. Venerid clams, which are in the same order as Dreissenoidea and Corbiculoidea, had similar rates of mt 16S rDNA sequence divergence to that in our study. Estimates for venerids ranged from p = 0.0014 to 0.0036 per my, based on transversional p-distances (Canapa *et al.,* 1996), overlapping the rate calculated in our study from tv and indels of p = 0.0033 per my (Table 6). Three independent fossil calibrations found rates of sequence divergence that ranged from p = 0.0008 to 0.0020 per my for unionid mussels for all substitutions (Lydeard *et al.,* 1996), suggesting a slower rate than ours. Variations in rates of sequence divergence in the mt 16S rDNA gene suggest that different lineages of bivalves evolve at different rates. Alternatively, these discrepancies may be the result of calibration and distance errors (Avise, 1994).

D. bugensis fossils are known only from Quaternary deposits (Babak, 1983, in Rosenberg and Ludyanskiy, 1994), supporting their relatively recent origin, as found in our study (Table 6; calculated as 13.2 ± 2.2 mya from all substitutions). Estimated divergences between *D. polymorpha* and *D. bugensis* from allozyme data (May and Marsden, 1992; Spidle *et al.,* 1994a) may be as high as 22 to 32 my using the calibration rate of Carlson *et al.* (1978) and Grant (1987) for Nei's (1972) $D = 1.0$ as equal to 19 my of divergence. However, when Nei's (1972) D is larger than 1.0, the variance is also large and time estimates are highly inaccurate (Nei, 1987). These divergences calculated from allozymes were beyond the level of signal saturation (Avise, 1994). The allozyme dates were roughly congruent with the divergence estimate of 27 mya from the COI data (Baldwin *et al.,* 1996), which may be a result of high variation in third codon positions.

Based on our mt 16S rDNA data, *Dreissena* and *Mytilopsis* diverged about 20.7 ± 2.7 mya, which was roughly congruent with fossil evidence that places the origin of the genus *Dreissena* during the Miocene Epoch (Iljana *et al.,* 1976, in Nuttall, 1990). Uncorrected COI sequence data from Baldwin *et al.* (1996) and our method of calibration (see Materials and Methods) estimated the divergence of *Dreissena* and *Mytilopsis* at about 32 mya (Baldwin *et al.,* 1996), predating the fossil estimates (Nuttall, 1990) and our estimate. Although the fossil record is advocated for calibrating a molecular clock in a lineage (Avise, 1994), the actual divergence may predate its first fossil appearance, underestimating the age of the splitting event (Hillis *et al.,* 1996). Although the relative divergences of taxa can be compared, time estimates such as these based on a molecular clock are highly speculative due to problems with calibration and obtaining reliable confidence intervals (see Hillis *et al.,* 1996).

Diagnostic Molecular Markers

PCR amplification and restriction enzyme digests are well suited for identifying species at early life history stages, since only a small amount of tissue is required and alcohol preservation can be used (Palumbi, 1996; Stepien and Kocher, 1997). PCR amplification and restriction enzyme digests have been used to discern among larval species of sea cucumbers (Olsson *et al.,* 1991) and spiny lobsters (Silberman and Walsh, 1992). Kenchington *et al.* (1993) described nuclear 18S rDNA RFLP markers that delineated between adductor muscles of the sea scallop (*Placopecten magellanicus*) and the Iceland scallop (*Chlamys islandica*). Foighil *et al.* (1995) successfully utilized mt 16S rDNA RFLPs to discriminate among the American (*Crassostrea virginica*) and Asian oysters (*C. gigas* and *C. ariakensis*). In addition, mtDNA PCR/RFLP markers for the COI

TABLE 6

Estimated Times for Genetic Divergences (my) from Kimura (1980) Two-Parameter Genetic Distances (above the Diagonal) and from Pairwise Distances (below the Diagonal)

A. Based on all substitution types

Taxon	*D. bugensis*	*D. polymorpha*	*M. leucophaeata*	*C. fluminea*	*M. edulis*
D. bugensis	—	10.8 +/− 1.9	18.7 +/− 2.5	54.4 +/− 5.1	78.6 +/− 7.0
D. polymorpha	13.2 +/− 2.2	—	17.0 +/− 2.4	56.6 +/− 5.3	79.2 +/− 7.0
M. leucophaeata	21.3 +/− 2.7	20.1 +/− 2.6	—	54.0 +/− 5.1	80.3 +/− 7.1
C. fluminea	54.2 +/− 3.8	55.9 +/− 3.9	54.0 +/− 3.8	—	81.6 +/− 7.1
M. edulis	70.3 +/− 4.1	70.1 +/− 4.1	71.6 +/− 4.1	72.4 +/− 4.1	—

B. Based on transversional substitutions and indels

Taxon	*D. bugensis*	*D. polymorpha*	*M. leucophaeata*	*C. fluminea*	*M. edulis*
D. bugensis	—	8.5 +/− 2.2	16.3 +/− 3.2	53.8 +/− 6.9	74.4 +/− 9.0
D. polymorpha	10.1 +/− 2.6	—	14.4 +/− 3.0	56.5 +/− 7.1	78.3 +/− 9.4
M. leucophaeata	18.8 +/− 3.4	16.8 +/− 3.3	—	54.0 +/− 6.9	83.7 +/− 9.9
C. fluminea	53.8 +/− 5.5	55.9 +/− 5.5	53.9 +/− 5.5	—	85.6 +/− 9.9
M. edulis	68.9 +/− 6.1	71.5 +/− 6.2	75.0 +/− 6.3	76.2 +/− 6.3	—

gene were used for distinguishing between *D. bugensis* and *D. polymorpha* (Baldwin *et al.,* 1996, Claxton *et al.,* 1997). A species-specific PCR primer screening technique to identify larval *D. polymorpha* from *D. bugensis* for the mtDNA COI gene was developed by Claxton and Boulding (1998). In this study, we developed markers that can be used alone or in conjunction with those of Baldwin *et al.* (1996), Claxton *et al.* (1997), and Claxton and Boulding (1998) in order to rapidly discern between *D. bugensis* and *D. polymorpha* at all life history stages. Our diagnostic molecular markers also may be used (Fig. 6) to discriminate among *D. bugensis, D. polymorpha, M. leucophaeata,* and *C. fluminea* in areas of sympatry (Fig. 1B).

Future Directions

The systematic relationships among additional taxa, including other species of *Mytilopsis, Corbicula, Dreissena,* and their relatives may be elucidated further with mt 16S rDNA sequences. For example, *Corbicula* has been hypothesized to comprise either one (Britton and Morton, 1979; based on allozymes) or two species in North America (Hillis and Patton, 1982; based on allozymes and morphology). In this study, the common white form *C. fluminea* was tested. The origin of *Corbicula* in Asia has not been determinable from allozyme (Smith *et al.,* 1979; McLeod and Sailstad, 1980; Hillis and Patton, 1982) or morphological (Morton, 1996) data. Sequence data hold promise for elucidating the relationships of the heteromyarian dreissenoid genus *Congeria,* which was believed extinct since the Miocene Epoch (Nuttall, 1990), with *C. kuscheri* recently described as living in underground caves in Slovakia (Morton *et al.,* 1998). *Congeria* and *Mytilopsis* are hypothesized to be sister taxa (Nuttall, 1990; Morton *et al.,* 1998), which can be tested with DNA sequence data. Mitochondrial 16S rDNA also may be useful for clarifying the number of species and evolutionary relationships in the genus *Mytilopsis,* which have been enigmatic (Morton, 1993, 1997).

Mitochondrial 16S rDNA sequences may be useful for elucidating the genetic relationships among dreissenids (Kinzelbach, 1992; Rosenberg and Ludyianskiy, 1994). As more dreissenid specimens become available, a taxonomic key based on mt 16S rDNA molecular characters could be established, as has been done for unionid mussels based on nuclear ITS-1 regions (White *et al.,* 1996). Distinguishing among populations of *D. polymorpha* and *D. bugensis* will require a molecular marker that is evolving at a faster rate than mt 16S rDNA. Research by Skidmore and Stepien (in progress) using nuclear DNA RAPD analysis has generated data that distinguish among populations of *D. polymorpha* and *D. bugensis* within and between regions of North America and Eurasia.

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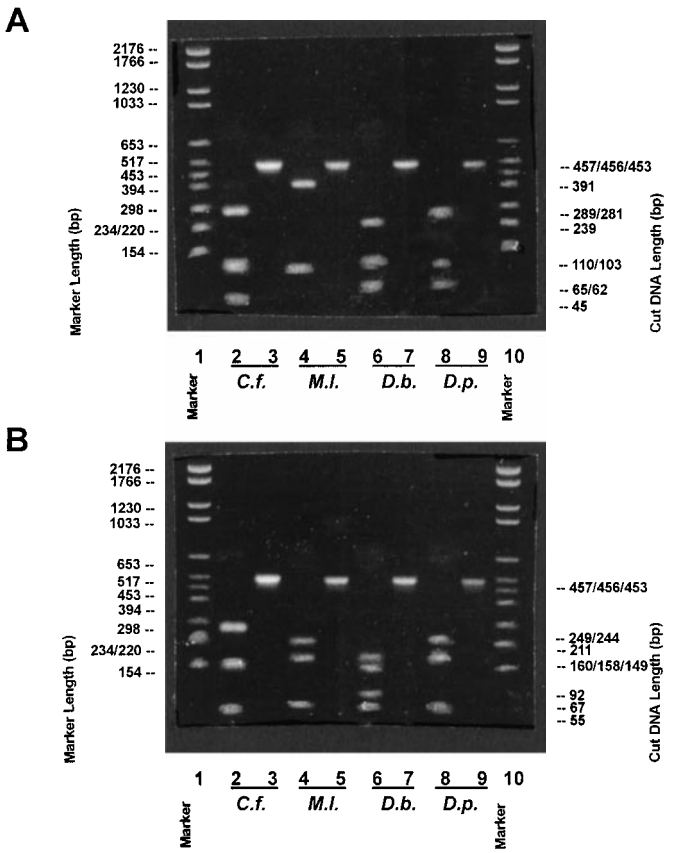

FIG. 6. Agarose gel electrophoresis of the mt 16S rDNA fragments amplified by PCR and digested with the restriction endonucleases Dde I (A) and Acs I (B). Sizes of the digested fragments are given on the right axis. For each taxon, amplified fragments that were not digested follow each digest. The molecular marker is DNA MWM VI, located at the far lanes (1 and 10). Lengths of the molecular marker fragments are given on the left axis. Taxa are abbreviated: *C. f.* = *Corbicula fluminea* (lanes 2-cut and 3-uncut), *M. l.* = *Mytilopsis leucophaeata* (lanes 4-cut and 5-uncut), *D. b.* = *Dreissena bugensis* (lanes 6-cut and 7-uncut), and *D. p.* = *D. polymorpha* (lanes 8-cut and 9-uncut).

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