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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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 Dreissenoidae (including the zebra mussel *Dreissena polymorpha*, the quagga mussel *D. bugensis*, and the dark false mussel *Mytilopsis leucophaeata*) and Corbiculoidae (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; Amato et al., 1998; Palumbi and Cipriano, 1998). 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
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 spermatozoon 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 Dreissenioidea is believed to have diverged during the late Mesozoic Era from an ancestor shared with its sister superfamily Corbiculoidae (Morton, 1993), based on shared shell microstructure characters (Taylor et al., 1973). The Dreissenioidea contains a single family, the Dreissenidae, with two subfamilies; the extinct Dreissenomyinae and the partially extant Dreisseniinae (Rosenberg and Ludynskiy, 1994). The subfamily Dreisseniinae 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 Ludynskiy, 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 (Ilyina 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 absence 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 Ludynskiy, 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...
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 dreissenid 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 calculated 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, 85.5°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* 6, 43.5°N, 79.5°W, *N* = 1) and Cape Vincent, New York (*site* 7, 44.2°N, 76.1°W, *N* = 1); the St. Lawrence River at Gentilly, Ontario (*site* 10, 43.5°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, 43.5°N, 93°W, *N* = 1); and the Mississippi River at Baton Rouge, Louisiana (*site* 15, 30.5°N, 90°W, *N* = 1). Samples of *D. polymorpha* from Eurasia (Fig. 2) included Lake Jissemeer, the Netherlands (*site* 1, 52.46°N, 5.14°E, *N* = 2); Wtocałek 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).

*Mylitopsis 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 Stepieen (1995). The universal primers 16Sar-L (5′ CGCCTGTITAAACAAAACAT3′) and 16Sbr-H (5′ CCGGTCTGACTCAATCAGGC3′; 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 110.07; Dynal Corp., Oslo, Norway). Sanger dideoxy sequencing (Sanger et al., 1977) incorporating [35S]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 S2S, Owl Scientific Inc., Woburn, MA) was performed separately on each strand using Sequenase Version 2.0 sequencing kits (product 550770, Amersham/U.S. Biochemical Corp., Cleveland, OH) and 7 µL of the 0.2 µM complementary PCR primer. Two 6% polyacrylamide wedge 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*TAILG, and Acs I (from *Arthrobacter citreus* 310, product 113-259, Boehringer Mannheim), which recognizes A/G*KATT/C. Restriction digests included 5 µL of the amplified PCR product, 6.5 µL of ddH2O, 1.5 µL of buffer H (from the Boehringer Mannheim kits; 50 mM Tris–HCl, 10 mM MgCl2, 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 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. χ2 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 χ2 and contingency tests (Sokal and Rohlf, 1981) within and between the stem and loop structural categories for the numbers of (1) variable versus invariant sites, (2) phylogenetically informative versus uninformative nucleotide substitutions determined in PAUP* 4.0 (d64, Swofford, 1998), and (3) transitional versus transversional substitutions. χ2 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 Dreissenoida and Corbiculoida 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 mya.

*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. edulis, 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 invariant 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 superfamly (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 ± 0.014 to 0.602 ± 0.053, based on all types of substitutions, and from d = 0.034 ± 0.009 to 0.348 ± 0.041, based on tv and indels (and excluding ts; Table 5). Pairwise distances ranged from p = 0.075 ± 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) 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 autopomorphies 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 = 0.023, based on all types of substitutions, and from d = 0.012 to 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.

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### TABLE 1

#### Aligned 16S rDNA Sequences

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Base position</th>
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</thead>
<tbody>
<tr>
<td>D. bugensis</td>
<td>AAAGAATAAGGATCGCGCCTGCCCGGTGA----TTGAGAGTTTT</td>
</tr>
<tr>
<td>D. polymorpha</td>
<td>AAAGAATAAGGATCGCGCCTGCCCGGTGA----TTGAGAGTTTT</td>
</tr>
<tr>
<td>M. leucophaeata</td>
<td>AATAATAGAGGTCTGGCCTGCCCAGTGCACTAGAGTAATGTCTGTTGT</td>
</tr>
<tr>
<td>C. fluminea</td>
<td>AAATAATAGAGGTCTGGCCTGCCCAGTGCACTAGAGTAATGTCTGTTGT</td>
</tr>
<tr>
<td>M. edulis</td>
<td>GTAAGTAAAAGGTAGTCCCTGCCCAGTGCACTAGAGTAATGTCTGTTGT</td>
</tr>
</tbody>
</table>

**Structural category**

| L L L L S S S S S S S L L L L L L L L 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 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 |

---

| D. bugensis         | AAACGGCCGCAGTTAA----TAGCTGTGCTAAGGTAGCGCAATCAATTGTCC |
| D. polymorpha       | AAACGGCCGCAGTTAA----TAGCTGTGCTAAGGTAGCGCAATCAATTGTCC |
| M. leucophaeata     | AAACGGCCGCAGTTAA----TAGCTGTGCTAAGGTAGCGCAATCAATTGTCC |
| C. fluminea         | TAACGGCTGC--GATTG----AAACCGTACTAAGGTAGCATAATAATTTGCCC |
| M. edulis           | AAACGGCGGC--GTTAACGTCAACGTCCTAACCTAGCGCCATAATTTGCTT |

**Structural category**

| L L L L S S S S S S S L L L L L L L L 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 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 |

---

| D. bugensis         | TTTAATAAGGGAATGGTATGAATGGCTGTACGCGAGATAAGCTGTCTCTA |
| D. polymorpha       | CTTAATTGAGGAATGGTATGAATGGCTGTACGTGAGATAAGCTGTCTCTA |
| M. leucophaeata     | CTTAATAGAGGAATGGTATGAATGGCTGGACGTGAGATAAGCTGTCTCTA |
| C. fluminea         | TTTAATTGGGGGAGAGAATGAATGGTTTGACGGTAAAAAAGCTGT--TTTA |
| M. edulis           | TTTCAATTGAAGGATGGTATGAAAGGGTTAACGAAGAAGGTGCTGTGTCTA |

**Structural category**

The p-distance divergence between *D. bugensis* and *D. polymorpha* was estimated as 13.2 
\( \pm 6.2 \) mya from the entire data set and 10.0 
\( \pm 6.6 \) mya using tv and indels only, occurring during the middle to late Miocene Epoch. Using Kimura (1980) two-parameter distances, these estimates were 17.8 
\( \pm 2.5 \) mya from the entire data set and 15.3 
\( \pm 3.1 \) 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. leuco- phaeata*, and *C. fluminea*, as shown in Fig. 6. The Dde I digest (Fig. 6A) cleaved the amplified mt 16S rDNA...
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 cut 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. 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.** 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.
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 Stepień 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 unionoids 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)

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. Stem regions</strong></td>
<td></td>
</tr>
<tr>
<td>D. bugensis</td>
<td>59</td>
</tr>
<tr>
<td>D. polymorpha</td>
<td>60</td>
</tr>
<tr>
<td>M. leucophaeata</td>
<td>59</td>
</tr>
<tr>
<td>C. fluminea</td>
<td>61</td>
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<tr>
<td>M. edulis</td>
<td>64</td>
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</table>

**B. Loop regions**

<table>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>D. bugensis</td>
<td>40</td>
</tr>
<tr>
<td>D. polymorpha</td>
<td>42</td>
</tr>
<tr>
<td>M. leucophaeata</td>
<td>34</td>
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<tr>
<td>C. fluminea</td>
<td>37</td>
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<tr>
<td>M. edulis</td>
<td>45</td>
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</table>

**C. Entire region**

<table>
<thead>
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<th>Taxa</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>D. bugensis</td>
<td>99</td>
</tr>
<tr>
<td>D. polymorpha</td>
<td>102</td>
</tr>
<tr>
<td>M. leucophaeata</td>
<td>93</td>
</tr>
<tr>
<td>C. fluminea</td>
<td>98</td>
</tr>
<tr>
<td>M. edulis</td>
<td>109</td>
</tr>
</tbody>
</table>

**P**-value

- **D. bugensis**
  - X^2: 14.2
  - P-value: 0.000
- **D. polymorpha**
  - X^2: 13.7
  - P-value: 0.000
- **M. leucophaeata**
  - X^2: 12.9
  - P-value: 0.000
- **C. fluminea**
  - X^2: 20.0
  - P-value: 0.000
- **M. edulis**
  - X^2: 38.0
  - P-value: 0.000

**Transitions and Transversions**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>D. bugensis</td>
<td>100</td>
</tr>
<tr>
<td>D. polymorpha</td>
<td>104</td>
</tr>
<tr>
<td>M. leucophaeata</td>
<td>99</td>
</tr>
<tr>
<td>C. fluminea</td>
<td>101</td>
</tr>
<tr>
<td>M. edulis</td>
<td>109</td>
</tr>
</tbody>
</table>

**P**-value

- **D. bugensis**
  - X^2: 38.1
  - P-value: 0.000
- **D. polymorpha**
  - X^2: 20.0
  - P-value: 0.000
- **M. leucophaeata**
  - X^2: 12.9
  - P-value: 0.000
- **C. fluminea**
  - X^2: 20.0
  - P-value: 0.000
- **M. edulis**
  - X^2: 38.1
  - P-value: 0.000

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 corbiculid 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).

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).

### Table 4

<table>
<thead>
<tr>
<th>Taxonomic comparison</th>
<th>Transitions</th>
<th>Transversions</th>
<th>Ratio</th>
<th>χ² Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. bugensis vs D. polymorpha</td>
<td>21 (58%)</td>
<td>15 (42%)</td>
<td>1.4:1.0</td>
<td>χ² = 1.00, P = 0.317</td>
</tr>
<tr>
<td>Genus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dreissenida vs Mytilopsis</td>
<td>14 (50%)</td>
<td>14 (50%)</td>
<td>1.0:1.0</td>
<td>χ² = 0.00, P = 1.000</td>
</tr>
<tr>
<td>Superfamily</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dreissenoida vs Corbiculoida</td>
<td>39 (41%)</td>
<td>56 (59%)</td>
<td>0.7:1.0</td>
<td>χ² = 3.04, P = 0.081</td>
</tr>
<tr>
<td>Order</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veneroida vs Mytiloida</td>
<td>70 (39%)</td>
<td>106 (61%)</td>
<td>0.7:1.0</td>
<td>χ² = 7.36, P = 0.007</td>
</tr>
<tr>
<td>Overall</td>
<td>144 (43%)</td>
<td>191 (57%)</td>
<td>0.8:1.0</td>
<td>χ² = 6.59, P = 0.010</td>
</tr>
</tbody>
</table>

χ² contingency test among taxonomic levels: χ² = 4.91, P = 0.179

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

**Heteroplasy and Hybridization Questions**

Our data from the mt 16S rDNA gene in the Dreissenoida 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 dreissenoids (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,

---

**Table 5**

<table>
<thead>
<tr>
<th>Taxon</th>
<th><em>D. bugensis</em></th>
<th><em>D. polymorpha</em></th>
<th><em>M. leucophaeata</em></th>
<th><em>C. fluminea</em></th>
<th><em>M. edulis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. bugensis</em></td>
<td>—</td>
<td>0.079 +/- 0.014</td>
<td>0.133 +/- 0.018</td>
<td>0.401 +/- 0.038</td>
<td>0.580 +/- 0.052</td>
</tr>
<tr>
<td><em>D. polymorpha</em></td>
<td>0.075 +/- 0.012</td>
<td>—</td>
<td>0.125 +/- 0.018</td>
<td>0.418 +/- 0.039</td>
<td>0.584 +/- 0.052</td>
</tr>
<tr>
<td><em>M. leucophaeata</em></td>
<td>0.121 +/- 0.015</td>
<td>0.115 +/- 0.015</td>
<td>—</td>
<td>0.399 +/- 0.037</td>
<td>0.593 +/- 0.052</td>
</tr>
<tr>
<td><em>C. fluminea</em></td>
<td>0.310 +/- 0.022</td>
<td>0.319 +/- 0.022</td>
<td>0.308 +/- 0.022</td>
<td>—</td>
<td>0.602 +/- 0.053</td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>0.401 +/- 0.023</td>
<td>0.404 +/- 0.023</td>
<td>0.409 +/- 0.024</td>
<td>0.413 +/- 0.023</td>
<td>—</td>
</tr>
</tbody>
</table>

**Note.** Kimura (1980) two-parameter genetic distances +/- s.e. (above the diagonal) and pairwise distances +/− s.e. (below the diagonal).
Venerids ranged from p sequence divergence to that in our study. Estimates for and Corbiculiformes, had similar rates of mt 16S rDNA clams, which are in the same order as Dreissenidae. Constraints the rate of sequence evolution. Venerid (NJ) distance tree from MEGA (Kumar et al., 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 Dreissenidae and Corbiculiformes, 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 Ludzanski, 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 allopzyme 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 allopzyme were beyond the level of signal saturation (Avise, 1994). The allopzyme 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 (Iijima 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; Stepken and Kocher, 1997). PCR amplification and restriction enzyme digests have been used to discern among larval species of sea cucumbers (Olsson et al., 1993), 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
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 sympathy (Fig. 1B).

**Future Directions**

The systematic relationships among additional taxa, including other species of *Mytilopsis*, *Corbicula*, *Dreissenia*, 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 dreissenid genus *Congeria*, which was believed extinct since the Miocene Epoch (Nuttall, 1990), with *C. kusceri* 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 Ludynianski, 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.

**ACKNOWLEDGMENTS**

This research was supported by the Ohio Sea Grant College Program, Project No. RZM-9, under Grant NA36RG0473 from the National Sea Grant College Program, National Oceanic and Atmospheric Administration to C. A. Stepien. J. L. Skidmore was supported by a graduate student fellowship in systematics of nonindigenous species from the National Fish and Wildlife Foundation, A. N. Hubers, and J. L. Skidmore were partially supported by the Department of Biology, CWRU. We thank the following individuals for providing specimens; Renata Claudi, Ronald Dermott, David W. Garton, John Hageman, Henk A. Jenner, Douglas Jensen, Joseph F. Koone, J. Ellen Marsden, Bernie May, Gary Rosenberg, Charles E. Rozeek, Adrian P. Spidle, III, David Stein, David L. Strayer, Bruce A. Thompson, and Timothy Zak. David W. Garton, Leroy Hushak, Amy Benson, Yves DeLafontaine, Ed Mills, and Charles O'Neill gave

### 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)**

<table>
<thead>
<tr>
<th>Taxon</th>
<th><em>D. bugensis</em></th>
<th><em>D. polymorpha</em></th>
<th><em>M. leucophaeata</em></th>
<th><em>C. fluminea</em></th>
<th><em>M. edulis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. bugensis</em></td>
<td>—</td>
<td>10.8 +/- 1.9</td>
<td>18.7 +/- 2.5</td>
<td>54.4 +/- 5.1</td>
<td>78.6 +/- 7.0</td>
</tr>
<tr>
<td><em>D. polymorpha</em></td>
<td>13.2 +/- 2.2</td>
<td>—</td>
<td>17.0 +/- 2.4</td>
<td>56.6 +/- 5.3</td>
<td>79.2 +/- 7.0</td>
</tr>
<tr>
<td><em>M. leucophaeata</em></td>
<td>21.3 +/- 2.7</td>
<td>20.1 +/- 2.6</td>
<td>—</td>
<td>54.0 +/- 5.1</td>
<td>80.3 +/- 7.1</td>
</tr>
<tr>
<td><em>C. fluminea</em></td>
<td>54.2 +/- 3.8</td>
<td>55.9 +/- 3.9</td>
<td>54.0 +/- 3.8</td>
<td>—</td>
<td>81.6 +/- 7.1</td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>70.3 +/- 4.1</td>
<td>70.1 +/- 4.1</td>
<td>71.6 +/- 4.1</td>
<td>72.4 +/- 4.1</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Taxon</th>
<th><em>D. bugensis</em></th>
<th><em>D. polymorpha</em></th>
<th><em>M. leucophaeata</em></th>
<th><em>C. fluminea</em></th>
<th><em>M. edulis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. bugensis</em></td>
<td>—</td>
<td>8.5 +/- 2.2</td>
<td>16.3 +/- 3.2</td>
<td>53.8 +/- 6.9</td>
<td>74.4 +/- 9.0</td>
</tr>
<tr>
<td><em>D. polymorpha</em></td>
<td>10.1 +/- 2.6</td>
<td>—</td>
<td>14.4 +/- 3.0</td>
<td>56.5 +/- 7.1</td>
<td>78.3 +/- 9.4</td>
</tr>
<tr>
<td><em>M. leucophaeata</em></td>
<td>18.8 +/- 3.4</td>
<td>16.8 +/- 3.3</td>
<td>—</td>
<td>54.0 +/- 6.9</td>
<td>83.7 +/- 9.9</td>
</tr>
<tr>
<td><em>C. fluminea</em></td>
<td>53.8 +/- 5.5</td>
<td>55.9 +/- 5.5</td>
<td>53.9 +/- 5.5</td>
<td>—</td>
<td>85.6 +/- 9.9</td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>68.9 +/- 6.1</td>
<td>71.5 +/- 6.2</td>
<td>75.0 +/- 6.3</td>
<td>76.2 +/- 6.3</td>
<td>—</td>
</tr>
</tbody>
</table>
information on species distributions. Brian Bargmeyer helped to extract some of the DNA. Charles Lydeard generously provided bivalve 16S rDNA models for comparison. We also thank Rob DeSalle, Brian Morton, Joseph Koonce, Charles Rozek, Christopher Cullis, and an anonymous reviewer for valuable suggestions that improved the manuscript.

REFERENCES


DIAGNOSTIC GENETIC MARKERS AMONG INVASIVE BIVALVES


