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Molecular resolution of the family Dreissenidae (Mollusca: Bivalvia) with emphasis on Ponto-Caspian species, including first report of *Mytilopsis leucophaeata* in the Black Sea basin

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Abstract

Considerable uncertainty exists in determination of the phylogeny among extant members of the Dreissenidae, especially those inhabiting the Ponto-Caspian basin, as multiple systematic revisions based on morphological characteristics have failed to resolve relationships within this group of bivalves. In this study we use DNA sequence analyses of two mitochondrial gene fragments, 16S rRNA and cytochrome *c* oxidase subunit I (COI), to determine phylogenetic relationships among *Dreissena rostriformis*, *D. bugensis*, *D. polymorpha*, *D. stankovici*, *Congeria kusceri*, and *Mytilopsis leucophaeata*. *Dreissena stankovici* was determined to represent a sister taxa to *D. polymorpha* and both are more closely related to other extant *Dreissena* species than *Congeria* or *Mytilopsis*. Sequence divergence between *D. rostriformis* and *D. bugensis* was relatively low (0.3–0.4%), suggesting that these two taxa constitute a single species. However, environmental differences suggest two races of *D. rostriformis*, a brackish water race (*rostriformis*) and a freshwater race (*bugensis*). Spread of *bugensis*-type individuals into habitats in the Caspian Sea that are occupied by *rostriformis*-type individuals may create novel hybridization opportunities. Species-specific molecular markers also were developed in this study since significant intraspecific variation in morphological features complicates dreissenid identification. Using two gene fragments (nuclear 28S and 16S), we identified restriction fragment length polymorphisms (RFLPs) that distinguish among *D. rostriformis*/*bugensis*, *D. polymorpha*, and *D. stankovici* and revealed the presence of a cryptic invader to the Black Sea basin, *Mytilopsis leucophaeata*. This is the first report of this North American native in southern Europe.

Keywords: 16S; COI; *Dreissena*; *Mytilopsis*; *Congeria*; Mitochondrial DNA; RFLP; Phylogenetics; Nonindigenous species; Invasion; Hybridization

1. Introduction

1.1. Traditional taxonomy and phylogenetics

Dreissenid molluscs are an important group of biofouling bivalves that are rapidly invading habitats around the world (e.g., Hebert et al., 1989; May and Marsden, 1992; Nuttall, 1990). Dreissenids have been reclassified many times, at many levels (i.e., genus, subgenus, species, subspecies, and variety). These analyses have resulted in confusion regarding phylogenetic relationships within the family. For example, Russian systematists have been unable to develop a uniform taxonomic history for the genus *Dreissena*. Andrusov (1897) outlined the first major taxonomic classification scheme for dreissenids and included *Dreissena polymorpha* (Pallas), *D. rostriformis* (Deshayes), and *D. bugensis* (Andrusov), amongst others, as legitimate species. Zhadin (1952) did not recognize *D. rostriformis* as a species but subsequent reclassifications by Logvinenko (1965), Logvinenko and Starobogatov (1968), and Starobogatov (1994) did. Zhadin (1952) recognized *D. bugensis* as a species, while Mordukhai-Boltovskoi

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(1960) classified *D. bugensis* as a subspecies of *D. rostriformis*. Starobogatov (1994) re-elevated *D. bugensis* to species level within the subgenus *Pontodreissena* and maintained species level classification for both *D. polymorpha* (subgenus *Dreissena*) and *D. rostriformis* (subgenus *Pontodreissena*) and included the newly identified *D. stankovici* (subgenus *Carinodreissena*). Rosenberg and Ludyanskiy's (1994) comparative review of dreissenid taxonomy included additional species, subspecies, and varieties based primarily on Russian accounts (e.g., Andrusov, 1897; Babak, 1983; Logvinenko and Starobogatov, 1968; Nevesskaya, 1963; Starobogatov, 1970; Starobogatov, 1994; Taktakishvili, 1973).

Some workers have suggested that *Dreissena* and *Mytilopsis* evolved from extinct branches of the genus *Congeria* (Andrusov, 1897; Babak, 1983; Mackie et al., 1989; Starobogatov, 1994), while others maintain that *Dreissena* and *Congeria* arose from *Mytilopsis* (Marelli, 1994). *Mytilopsis* was considered a subgenus of *Congeria* by Russian taxonomists (Andrusov, 1897; Babak, 1983; Starobogatov, 1970), but was elevated to genus level by Nuttall (1990), a classification scheme later supported by Rosenberg and Ludyanskiy (1994). Marelli and Gray (1985) suggested that there exist only five extant species of *Mytilopsis*, including *M. leucophaeata*. Unfortunately, as with *Dreissena*, traditional taxonomic classification of the genus *Mytilopsis* is complex, discordant and variable through time. We argue that the use of molecular techniques can help clarify the phylogenetics of this group.

1.2. Distribution of dreissenids

Two of four *Dreissena* species used herein, *D. rostriformis* and *D. stankovici*, have never been reported outside their historical ranges (Table 1). *Dreissena rostriformis* occurs in the Middle and South Caspian Sea at salinities between 12 and 13.5‰, while the closely related *D. bugensis* is typical of freshwater or oligohaline habitats both within its historical range in the Black Sea basin, and in its introduced range in the Volga River. *D. polymorpha* occurs in similar habitats as those reported for *D. bugensis*, but is capable of inhabiting mesohaline waters typical of the northern Caspian Sea (Table 1).

1.3. Invasion history

Human activities are rapidly changing aquatic ecosystems. Most notable are activities related to transoceanic shipping and canal creation, both of which link water bodies and allow transfer of nonindigenous species between previously isolated aquatic ecosystems. The Dreissenidae have undergone considerable global redistribution as a result of shipping activities (Nuttall, 1990). Typically considered Ponto-Caspian "endemics" (Geary et al., 2000), two dreissenids have recently invaded the Laurentian Great Lakes. *Dreissena polymorpha*, the zebra mussel, was first discovered in Lake St. Clair in 1988 (Hebert et al., 1989), while *D. bugensis*, the quagga mussel, was first reported from Lake Ontario in 1991 (May and Marsden, 1992). A "profundal" variety was reported from deep-water habitats in Lake Erie in 1992 (Dermott and Munawar, 1993) and later identified as *D. bugensis* using allozymes (Marsden et al., 1996; Spidle et al., 1994). Another Dreissenidae, the dark false mussel *Mytilopsis leucophaeata*, is native to the Gulf of Mexico, but invaded the Hudson River, New York in the 1930s. The species also has recently been identified in the Upper Mississippi River (Koch, 1989) and at several locations in southern New England (Smith and Boss, 1996). *Mytilopsis leucophaeata* also has been reported from European waters as early as 1835 (Wolff, 1999) and is found along North Sea coasts from Germany to France (Marelli and Gray, 1983; Oliver et al., 1998) and

Table 1
Historical ranges of extant dreissenids including distribution patterns related to salinity and depth

Species	Historical range	Salinity[a]		Depth		Source
		Native	Introduced	Native	Introduced	
D. polymorpha	Estuaries and lower reaches of Ponto-Caspian rivers and Northern Caspian Sea, coastal shallows of Middle and South Caspian Sea[b]	Oligohaline Freshwater Mesohaline	Oligohaline Freshwater Mesohaline	Shallow water	>60 m	Starobogatov and Andreeva (1994), Karataev et al. (1998), Karpevich (1955)
D. bugensis	Dnieper–Bug Liman and lower reaches of Inguletz River (Black Sea basin)	Oligohaline (<3‰) Freshwater	Oligohaline (2‰) Freshwater	0–28 m	>130 m	Starobogatov and Andreeva (1994), Markovskii (1954), Orlova et al. (1998)
D. rostriformis	Middle and South Caspian Sea	Mesohaline (12–13‰) Freshwater	NA	20–80 m	NA	Starobogatov and Andreeva (1994)
D. stankovici	Lake Ohrid		NA		NA	Starobogatov and Andreeva (1994)

[a] Salinity in freshwater zone is up to 1‰, oligohaline zone from 1 to 5‰, and mesohaline zone from 5 to 18‰.
[b] Prior to establishment of *Mytilaster lineatus* (Starobogatov and Andreeva, 1994).

the River Thames estuary, England (Bamber and Taylor, 2002). European populations occupy both freshwater and brackish estuary habitats (Reise et al., 1999).

Dreissena polymorpha has an extensive distribution in both European and North American freshwaters (Nalepa and Schloesser, 1993). In contrast, *D. bugensis* has a more restricted distribution in European and North American freshwaters, but is currently undergoing range expansion in the Volga River, Russia, and is replacing *D. polymorpha* in the lower Great Lakes (Berkman et al., 2000; Mills et al., 1999). Dreissenids are nuisance species in many invaded habitats owing to biofouling (Kharchenko, 1995; Marelli and Gray, 1983), but are considered beneficial in some habitats where they improve water quality (Reeders et al., 1993). Owing largely to human-induced range expansion, co-occurrence of dreissenid species is increasing globally and the ability to discern morphologically similar species has become increasingly important. *Dreissena polymorpha*, *D. bugensis*, *D. rostriformis*, *D. stankovici*, and *M. leucophaeata* share many life-history characteristics (e.g., use of byssal threads for attachment, and possession of a free-swimming veliger larva) and exhibit strong morphological and shell colour similarities (Biochino, 1994; Lukashev, 2000; May and Marsden, 1992; O'Neill, 1990; Pathy and Mackie, 1993; Protasov and Gorpinchuk, 2000). Moreover, each species may exhibit pronounced intraspecific variability. Genetic markers may prove particularly useful for discrimination of species, such as dreissenids, with high intraspecific variability or small larval or juvenile size (Claxton et al., 1997, 1998; Skurikhina et al., 2001). Identification of a few individuals early on in an incipient invasion may allow for implementation of rapid control measures. For example, shortly after *M. sallei* arrived in Darwin, Australia, a comprehensive eradication campaign was undertaken before the species could become established (Pyne, 1999).

In this study, we use mitochondrial gene sequencing to assess the phylogenetic relationships among members of the family Dreissenidae identified from Ponto-Caspian and Mediterranean regions including the genera *Dreissena*, *Mytilopsis*, and *Congeria*. This is the first study to use molecular techniques to resolve the placement of *D. stankovici* and *D. rostriformis* within the family Dreissenidae. In addition, we attempt to resolve the relationship between *D. rostriformis* and *D. bugensis* using sequence data. The relationship between these taxa has been highly discordant over time based on traditional taxonomical accounts as some authors consider each a species while others consider *D. bugensis* a subspecies of *D. rostriformis* (e.g., Andrusov, 1897; Mordukhai-Boltovskoi, 1960; Starobogatov, 1994; Zhadin, 1952). Furthermore, we use nuclear and mitochondrial DNA restriction digests to identify species that are difficult to distinguish based on morphological characteristics alone.

2. Materials and methods

2.1. DNA isolation and PCR amplification

Specimens used in sequencing were collected from their current European ranges (Table 2). Two cryptic dreissenid specimens were collected from the Dniester Liman, Black Sea, and included in our analyses. External shell morphology of these individuals was similar but not identical to those of *D. bugensis* and *D. polymorpha*. Total DNA was extracted from mantle muscle tissues of specimens preserved in 95% ethanol or frozen using either a standard phenol–chloroform method or a DNA purification kit (Wizard, Promega). Extracted genomic DNA was used as a template for DNA amplification using polymerase chain reaction (PCR). We amplified the nuclear ribosomal RNA gene 28S using the primer pair 5′-TCC GAT AGC GCA CAA GTA CC-3′ and 5′-TTG CAC GTC AGA ATC GCT AC-3′. The 28S primers were specifically designed for dreissenid molluscs based on published sequence data (Park and Ó'Foighil, 2000). Also, we amplified two mitochondrial genes: cytochrome *c* oxidase subunit I (COI) using the primer pair 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ (Folmer et al., 1994); and the mitochondrial gene fragment 16S rRNA using the primer pair 5′-CGC CTG TTT ATC AAA AAC AT-3′ and 5′-CCG GTC TGA ACT CAG ATC ACG T-3′ (Schubart et al., 2001). The balance of the PCRs was double-distilled water, 10× manufacture-supplied PCR buffer, 25 mM $MgCl_2$, 0.2 mM each of four dNTPs, and 0.5 U *Taq* DNA polymerase (Gibco-BRL or Promega). Reactions were run on a PTC-225 Programmable Thermal Controller (MJ Research, Inc.) using an initial denaturation cycle at 94 °C (120 s) followed by 40 cycles consisting of a denaturation cycle at 94 °C (60 s), an annealing cycle that depended on the primer pair (46.5 °C for 28S, 48.5 °C for 16S, and 56.5 °C for COI) (60 s), an extension cycle at 72 °C (90 s). A 5-min extension was added after cycling. PCR products were run on 1.8–2% agarose gels using electrophoresis in standard TBE buffer for 2–4 h at 80–100 V and visualized using UV-transillumination of ethidium bromide-stained gels. Photographs were stored as digitized images for subsequent analyses.

2.2. DNA sequencing

Phylogenies were constructed using the mitochondrial 16S gene fragment and COI; both genes were sequenced using the DTCS Quick Start cycle sequencing kit (Beckman Coulter) and CEQ2000XL automated sequencer. Sequences were aligned by eye, and comparisons were made on 456–458 bp of 16S

Table 2
Dreissenid species collection locations and GenBank Accession numbers for species used in this study

Species	Sample location	GenBank Accession Number	
		16S	COI
D. rostriformis	Caspian Sea, Russia	AF 507048	AF 510505 (type 1) (A) AF 510506 (type 2) (B) AF 510507 (type 3) (C)
D. bugensis	Dniester Liman, Ukraine	AF 507047	AF 510504 (D)
	Kuybyshev Reservoir, Russia	AF 507047	AF 510504 (D)
	Bug Liman, Ukraine		AF 510504 (D)
	Dnieper Liman, Ukraine		AF 510504 (D)
	Volga Delta, Russia		AF 510504 (D)
	Gorky Reservoir, Russia		AF 510504 (D)
	Kakhovka Reservoir, Russia		AF 510504 (D)
D. polymorpha	Gorky Reservoir, Russia	AF 507049	AF 510509 (type 2) (E)
	Ingul River, Ukraine	AF 507049	AF 510509 (type 2) (E)
	Dnieper Liman, Ukraine		AF 510509 (type 2) (E)
	Dniester Liman, Ukraine		AF 510509 (type 2) (E)
	Bug Liman, Ukraine		AF 510508 (type 1) (F)
	Volga Delta, Russia		AF 510510 (type 3) (G)
	Obukhorskoga Channel, Russia		AF 510510 (type 3) (G)
D. stankovici	Lake Ohrid, Macedonia	AF 507050	
	Lake Prespa, Macedonia	AF 507050	
M. leucophaeata	Dniester Liman, Ukraine	AF 507051 (type 1)	
	Dniester Liman, Ukraine	AF 507052 (type 2)	

Identical Accession numbers indicate identical sequences, while letters in brackets correspond to different haplotypes in Fig. 2.

sequence and 555 bp of the COI gene. Where comparisons included *M. leucophaeata*, *Congeria kusceri*, and *Corbicula fluminea* (from Stepien et al., 1999, 2001), sequences were limited 450 bp for 16S and 537 bp for COI. Using the Molecular Evolutionary Genetics Analysis (MEGA) program (version 1.01) developed by Kumar et al. (1993), genetic distances were estimated using Kimura's two-parameter distance model and phylogenetic relationships inferred using the neighbor-joining (NJ) algorithm. Support for each branch point was tested using 1000 bootstrap replications. All sequences obtained in this study have been submitted to GenBank (Accession numbers in Table 2). A maximum parsimony model also was evaluated using a close-neighbor-interchange (CNI) algorithm and 1000 bootstrap replications for both 16S and COI but since topology was identical, only NJ trees are shown.

2.3. Restriction digests

The PCR products of the 28S gene were screened with a variety of restriction enzymes until readily identifiable restriction fragment length polymorphisms (RFLPs) were detected (Table 3). Based on 16S sequence differences between *D. bugensis* and *D. rostriformis* (see below) *Msp*I and *Hpa*II were used to cut 16S. Digests were carried out according to manufacturer's instructions and fragments were visualized on 1.8–2% agarose gels.

Table 3
Dreissenid restriction digest band patterns for both nuclear and mitochondrial genes using multiple restriction enzymes

Gene and restriction enzyme	Species				
	D. rostriformis	*D. bugensis*	*D. stankovici*	*D. polymorpha*	*M. leucophaeata*
Nuclear DNA					
28S and *Hin*fI	225, 240	225, 240	140, 160, 225	140, 160	225, 290
28S and *Hae*III	160, 165, 185, 190	160, 165, 185, 190	90, 170	90, 155, 160, 190	160, 185, 190
28S and *Msp*I	130, 150, 210	130, 150, 210	130, 160, 185	130, 150, 210	130, 150, 210
28S and *Rsa*I	225, 325	225, 325	125, 320	125, 225, 325	125, 325
Mitochondrial DNA					
16S and *Msp*I	525	50, 475			
16S and *Hpa*II	525	50, 475			

Approximate size (bp) of fragments is given.

3. Results

3.1. Phylogenetic analysis

The NJ tree based on 16S showed that *D. rostriformis* and *D. bugensis* differed by a single nucleotide (Fig. 1). Bootstrap support for distinct nodes in this part of the tree was weak, providing the first molecular evidence that these individuals might represent a common species. However, both NJ and maximum parsimony analyses maintained each taxon as monophyletic. Considering *D. rostriformis* and *D. bugensis* as a single species with two possible races (see below), intraspecific differences ranged from 0.00 to 0.23%. Intraspecific differences in each of *D. polymorpha* and *D. stankovici* were 0.00%, while variation within *M. leucophaeata*, including the two cryptic individuals, was 0.7 and 0.9%. Interspecific differences were much greater as differences between *D. rostriformis* and *D. polymorpha* and *D. stankovici* were 7.6 and 6.5%, respectively. *D. polymorpha* and *D. stankovici* were maintained as separate species based on 16S analyses (Fig. 1), distinct from the *D. rostriformis–D. bugensis* branch. *C. kusceri* was more similar to *M. leucophaeata* than the *Dreissena* species that are monophyletic (Fig. 1). Differences between *Mytilopsis* and *Dreissena* ranged from 9.7 to 11.5% while differences between *Mytilopsis* and *Congeria* were 6.0%. Sequence data suggest the two cryptic individuals were *M. leucophaeata*, confirming the first record for this species in Southern Europe.

The NJ tree based on COI sequences of *D. rostriformis*, *D. bugensis*, and *D. polymorpha* was developed to provide higher resolution on the *D. rostriformis–D. bugensis* relationship (Fig. 2) since COI appears to evolve at a faster rate than 16S. *C. kusceri*, *M. leucophaeata*, and *C. fluminea* (outgroup) were included to maintain tree topology. *D. rostriformis* maintained a distinct cluster within this branch, but bootstrap estimates of differentiation were weak (Fig. 2). Intraspecific differences were 0–0.36% for *D. rostriformis*, 0–0.54% for *D. bugensis* and 0–1.1% for *D. polymorpha*; intraspecific differences within the *D. rostriformis–D. bugensis* group ranged from 0 to 1.1%, the same range as that of *D. polymorpha*.

3.2. Restriction digests

Restriction digests of both nuclear and mitochondrial genes were consistent with the phylogenetic analyses. Restriction digests of both nuclear and mitochondrial genes using multiple restriction enzymes produced consistent patterns. Each nuclear restriction digest produced characteristic banding patterns for *D. polymorpha*, *D. rostriformis* (including *D. bugensis*), *D. stankovici*, and *M. leucophaeata* (initially a cryptic species), patterns considered diagnostic for these four species (Table 3).

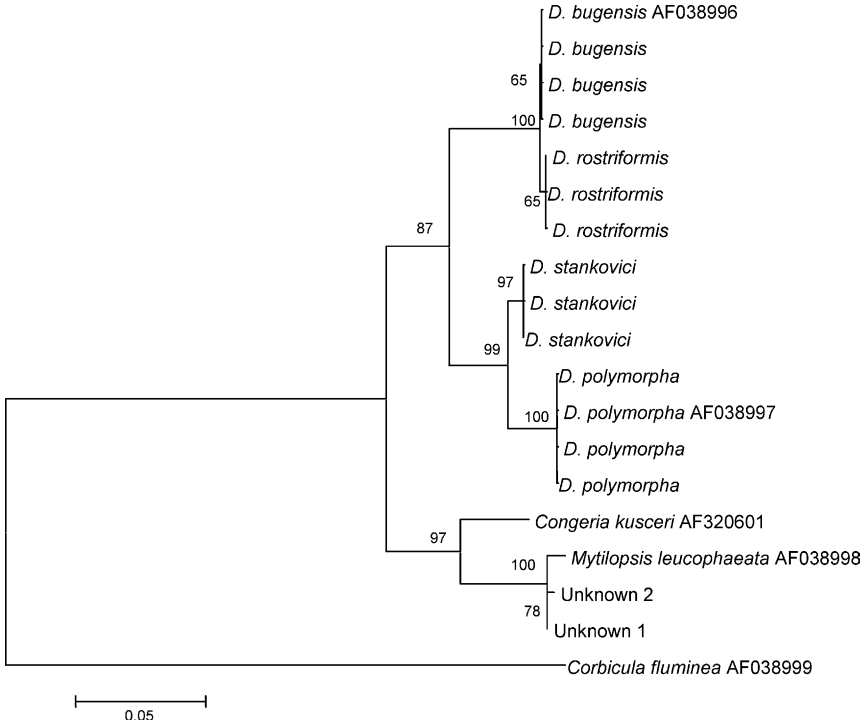

Fig. 1. Neighbor-joining (NJ) tree comparing dreissenid species using 450 bp of 16S sequence; *Corbicula fluminea*, the Asian clam, is the outgroup. Numbers at nodes indicate bootstrap confidence levels (1000 bootstrap replicates). Sequences retrieved from GenBank are indicated by their Accession numbers. *Corbicula fluminea* sequence published and aligned by Stepien et al. (1999).

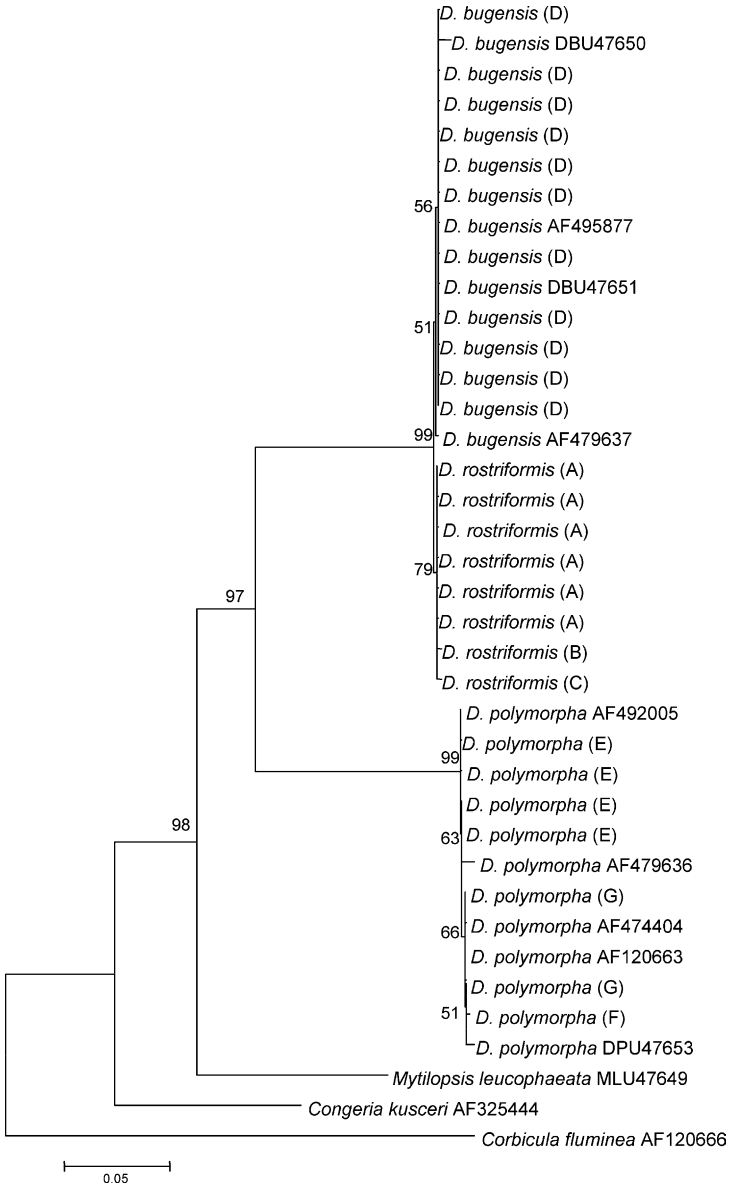

Fig. 2. Neighbor-joining (NJ) tree comparing dreissenid species using 537 bp of COI sequence. Numbers at nodes indicate bootstrap confidence levels (1000 bootstrap replicates). Sequences retrieved from GenBank are indicated by their Accession numbers, while Accession numbers for our individuals are found in Table 2 based on observed haplotypes in brackets.

Furthermore, restriction digests of the 16S mitochondrial gene using *Msp*I or *Hpa*II could be used to discriminate between *D. rostriformis* races (Table 3).

4. Discussion

4.1. Dreissenid phylogenetics

In contrast to traditional dreissenid taxonomy based principally on morphological attributes, our molecular analyses, in combination with environmental tolerances, suggest that *D. bugensis* and *D. rostriformis* may represent a single species with two distinct races. This view is supported by only a single base pair difference (0.23%) between *D. bugensis* and *D. rostriformis* in the 16S gene, and by 2–3 bp differences (0.36–0.54%) in the COI gene. Consequently, we suggest the ancestral name of *D. rostriformis* Deshayes, 1838 be used in taxonomic descriptions, in compliance with established nomenclature rules. Furthermore, identification of the Great Lakes quagga mussel as *D. bugensis* (Spidle et al., 1994) should be reconsidered in light of our results. We propose that the quagga mussel be renamed *D. rostriformis*. If we consider the existence of two races (*bugensis* and *rostriformis*), the taxonomic classification scheme proposed by Mordukhai-Boltovskoi (1960) is supported by our genetic analyses.

There are two alternate hypotheses that could explain the observed sequence divergence between *D. rostriformis* and *D. bugensis*. The first is possible asymmetric introgression of the mitochondrial genome in these dreissenids. Asymmetric introgression arises due to an ancient divergence event coupled with mitochondrial DNA "leakage." This phenomenon has been reported for *Mytilus* spp. (e.g., Hilbish et al., 1994; Rawson and Hilbish, 1998) but not for other bivalves, due to the high degree of hybridization observed within the *Mytilus* species complex. However, this hypothesis is not supported by our RFLP data that show *D. rostriformis* and *D. bugensis* are not anciently divergent in their nuclear genome, as opposed to their mitochondrial genome, as no differences were detected in restriction digest patterns (Table 3). The second alternate hypothesis is that *D. rostriformis* and *D. bugensis* represent two recently formed species. Starobogatov (1994) argued *D. bugensis* evolved from an extinct, endemic Black Sea subspecies of *D. rostriformis* that inhabited the basin between the early Pleistocene and early Holocene. However, Babak (1983) argued all endemic Pontodreissena within the ancient Black Sea basin became extinct during the late Pleistocene due to intrusion of saline water. Furthermore, Babak (1983) argued that *D. bugensis* in the modern Black Sea basin arose due to an invasion by *D. rostriformis* during the Novoeuxinian Epoch. This view would support our genetic findings that *D. rostriformis* and *D. bugensis* are potentially conspecific, supporting two distinct races.

Our finding that *D. bugensis* clustered separately within the *D. rostriformis–D. bugensis* branch supports Mordukhai-Boltovskoi's (1960) classification, which recognized *D. bugensis* as a subspecies of *D. rostriformis*. Also, our results are consistent with Baldwin et al. (1996), who reported 0.65% sequence divergence in COI between populations of *D. bugensis*. Observed intraspecific sequence divergence in *D. rostriformis* (including *D. bugensis*) based on COI sequences (up to 1.1%), is identical to that observed for *D. polymorpha*. Our results are in accordance with other mollusc studies. For example, Arnaud et al. (2000) likewise found 0.12–1.3% sequence divergence between COI haplotypes of pearl oyster *Pinctada mazatlanica*. Intraspecific variability based on COI sequences in *Calyptogena magnifica*, *C. elongata*, *C. phaseoliformis*, *Calyptogena* spp., and *Ectenagena extenta* ranged from 0.59 to 1.38% (Peek et al., 1997). Similarly, intraspecific variability in four species of *Lampsilis* clams ranged between 0 and 2.8% (Roe et al., 2001). Furthermore, other studies have reported intraspecific variability in COI sequences greater than or equal to those reported here for the *D. rostriformis* group. For example, Meyran et al. (1997) observed up to 8.2% intraspecific COI variability in the amphipod *Gammarus pulex*. Similarly, Baldwin et al. (1998) and Cristescu et al. (2001) observed up to 3 and 1.62% intraspecific variability in COI sequences of marine shrimp (genus *Penaeus*) and *Cercopagis pengoi* waterfleas, respectively. COI sequence data have been used to resolve other taxonomic debates. For example, Claxton et al. (1998) suggested the COI gene was suitable to resolve species identity within dreissenids, and Therriault et al. (2002) used it to resolve the number of species belonging to the genus *Bythotrephes*.

Phylogenetic studies on bivalve molluscs using the16S gene also have found greater intraspecific variability than that observed here between *D. bugensis* and *D. rostriformis* (see Lydeard et al., 1996; Rawson and Hilbish, 1995, 1998; Roe et al., 2001). For example, 16S mitochondrial gene sequences for *Lampsilis altilis* exhibited no intraspecific variation within drainages, but small intraspecific variation between drainages (Roe et al., 2001). Thus, the variability observed between the *rostriformis* race and the *bugensis* race is comparable to the geographic variability observed for *Lampsilis*. Intraspecific variability in *Mytilus* mussels also is considerably greater than in the *D. rostriformis* group surveyed here (e.g., Hilbish et al., 1994; Hoeh et al., 1997; Rawson and Hilbish, 1998).

Dreissena polymorpha, *D. rostriformis* (especially the *bugensis* race), and *M. leucophaeata* each have been reported from salinity concentrations ranging from freshwater to oligohaline (e.g., MacNeill, 1991; Strayer and Smith, 1993; Walton, 1996; Table 1). Salinity has fluctuated in the Ponto-Caspian basin over time, possibly affecting tolerances in extant taxa (see Dumont, 1998). The haplotypic heterogeneity in 16S observed for the *D. rostriformis* group may result from, or be related to, differences in salinity in the Black and Caspian Seas. Koehn et al. (1980) and Gardner and Palmer (1998) demonstrated that genotypic heterogeneity in *Mytilus galloprovincialis* corresponded to salinity fluctuations. Similarly, Sarver and Foltz (1993) demonstrated that salinity affected the macrogeographic distribution of *M. trossulus* and *M. galloprovincialis*, while genetic variability in *Littorina* gastropods varied across salinity regimes (Yaroslavtseva and Sergeeva, 2001).

Dreissena rostriformis and *D. bugensis* are extremely similar based on mitochondrial gene sequences (Figs. 1 and 2), raising the possibility of potential hybridization. Spidle et al. (1995) were unable to detect natural hybridization between *D. polymorpha* and *D. bugensis* in the Great Lakes. However, genetic divergence between these species is much more extensive than within the *D. rostriformis* group (Figs. 1 and 2). *D. rostriformis* and *D. bugensis* had very restricted distributions during the last century. However, the *bugensis* race is rapidly colonizing habitats throughout the Volga River system (M.I. Orlova, unpublished data). Because the Volga River drains into the Caspian Sea, there exists a strong possibility for future range overlap and interbreeding between native *D. rostriformis* and introduced *bugensis*-type individuals.

Hilbish et al. (2002) demonstrated that water circulation patterns (or lack thereof) might prevent larval dispersal for *Mytilus*. Such a dispersal barrier would be unlikely in the Volga River since veligers could disperse downstream into the Caspian Sea.

According to the phylogenetic species concept (PSC), any monophyletic group can technically be considered a "species." Thus, by definition, *D. bugensis* and *D. rostriformis* could be considered "species" based on the 16S and COI sequence data (Figs. 1 and 2). However, the level of differentiation observed in the mitochondrial genome does not support two distinct "species." Intraspecific versus inter-specific differences noted for *D. bugensis* and *D. rostriformis* are consistent with conspecific "species," at least at the level typically employed for bivalve molluscs. Since most bivalve species diverged a long time ago, deep phylogenetic divergences are often considered a prerequisite for species-level classification. This deep phylogenetic divergence was not identified in this study between *D. rostriformis* and *D. bugensis* but was identified for *D. polymorpha*, *C. kusceri*, and *M. leucophaeata* (Fig. 2). Admittedly, *D. rostriformis* and *D. bugensis* occupy different ecological niches so it would be prudent to consider them as distinct races. Thus, until further genetic data are available, we argue that *D. bugensis* should be considered as a freshwater race of *D. rostriformis*.

4.2. Implications for invasion biology

In this study, we identify *M. leucophaeata* from the Dniester Liman, Black Sea basin for the first time. This species spread from its native Gulf of Mexico to the Hudson River in New York via transfer of ship ballast water (Jacobson, 1953). Ballast water transfer also appears to have been responsible for the transfer of this species from North America to Europe (Bamber and Taylor, 2002; Oliver et al., 1998), and possibly to the Black Sea region. Canal development has opened invasion corridors between the previously disjunct regions of the Black–Azov and Caspian Seas, creating pathways that were previously unavailable (Ricciardi and MacIsaac, 2000). With its broad ecological tolerances, *M. leucophaeata* may be poised to spread throughout the Ponto-Caspian region, as has the ctenophore *Mnemiopsis leidyi* (see Reise et al., 1999). It took about 30 million years for *Mytilopsis* to expand its range from Europe to North America during the Oligocene, but considerably less time to re-invade Europe during recent times (Nuttall, 1990). This trend is likely to continue as global shipping activities increase (Ruiz et al., 2000). *Mytilopsis sallei*, the only other extant *Mytilopsis* species, also has undergone considerable range expansion due to shipping (Chu et al., 1997). For example, *Mytilopsis* has successfully invaded brackish water ports in Fiji, India, Hong Kong, Japan, West Africa, the Rhine-Scheldt delta (Nuttall, 1990) and, more recently, England (Bamber and Taylor, 2002). The ability to adapt to a wide range of salinity concentrations appears to be partially responsible for these invasions.

Globally, mussel populations are declining in many freshwater ecosystems owing to exploitation, cultural eutrophication and introduction of nonindigenous species (Ricciardi and Rasmussen, 1999; Strayer, 1999). The Black Sea is similarly experiencing declining mussel populations (Shurova, 2001). Introduced species are one mechanism contributing to the decline of native fauna in general and native mussels in particular (Martel et al., 2001; Ricciardi et al., 1996). The arrival of *M. leucophaeata* to the Black Sea may herald further loss of native molluscs in the region, the effects of which may not be apparent for many years.

In summary, our study clearly shows that *D. polymorpha*, *D. stankovici*, and *D. rostriformis* are distinct species, but that *D. bugensis* appears to be only a race of the latter species. Based on 16S and COI sequence data, species-level status does not appear justified. It appears that salinity differences, and possibly isolation, have resulted in different races of *D. rostriformis* in different basins.

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