Aquatic Invaders

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DISTRIBUTION & DISPERSAL

Current status and potential establishment range for veined rapa whelks *Rapana venosa* on the U.S. East coast

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Introduction

What is a rapa whelk? A rapa whelk or *Rapana venosa* is a large predatory marine snail (Figure 1). These gastropods may reach sizes in excess of 165 mm shell length (SL) and may live for more than ten years. Rapa whelk shells are easily distinguished from other large gastropods on the basis of morphological features including 1) a thick shell with similar length and width dimensions, 2) a broad, flat columella, 3) horizontal black veins visible on the exterior of the brown or tan shell in specimens of all ages and on the interior of the opercular opening in young animals, 4) a 'D' shaped opercular opening that is bright red or orange in older specimens (Figure 1)

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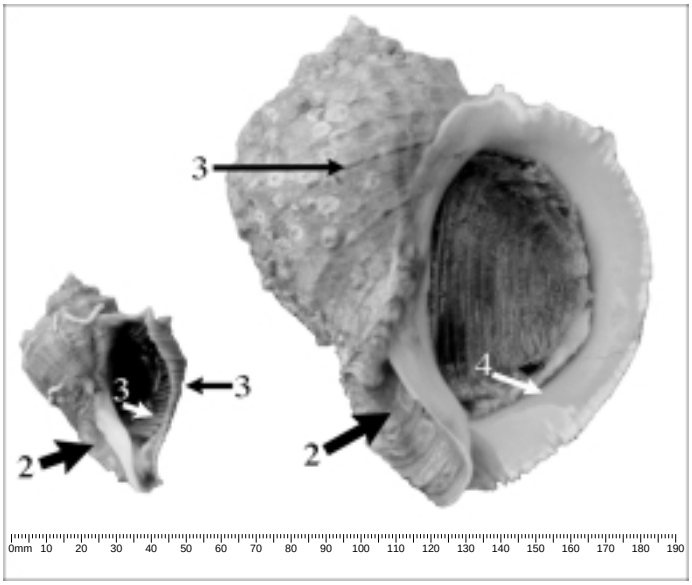

Figure 1. Rapa whelks collected from Chesapeake Bay, U.S.A. Specimens are identified following the morphological characteristics described in text including a distinctive columella (2), horizontal black veins visible in the shell (3) and the opercular opening of younger individuals (3), and bright red or orange colored opercular opening in older individuals (4).

TAXONOMY

DNA and Systematic Analysis of Invasive and Native Dreissenid Mussels: Is *Dreissena bugensis* really *D. rostriformis*?

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Key Words: DNA, cytochrome b gene, *Dreissena, Dreissena bugensis, Dreissena rostriformis,* quagga mussel, 16S RNA, systematics, zebra mussel

Abstract

Prior studies by our Great Lakes Environmental Genetics Laboratory have shown the utility of DNA characters in resolving the systematic relationships and identifications of the bivalve family Dreissenidae. The present study analyzes the phylogenetic relationships among four extant species of *Dreissena* — including the zebra mussel *D. polymorpha*, the quagga mussel *D. bugensis*, the Lake Okhrid native *D. stankovici*, and the Caspian Sea native *D. rostriformis*. We compare results from

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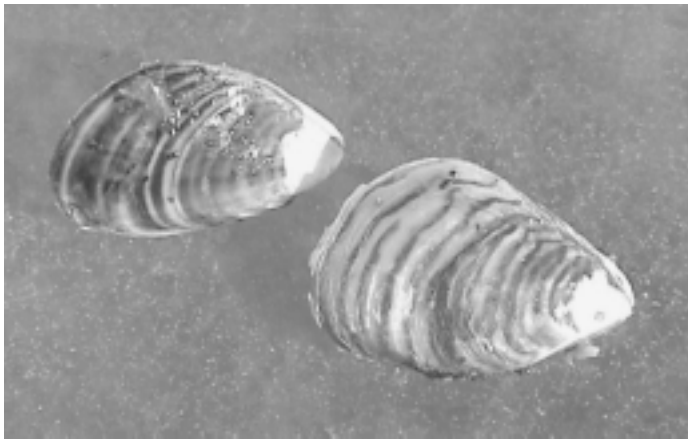


Quagga mussel, *Dreissena bugensis*. Photo: J. Ellen Marsden

DNA and Systematic Analysis of Invasive and Native Dreissenid Mussels... *continued from p. 1*

sequences of the mitochondrial 16S ribosomal DNA region and the cytochrome b gene. This is the first published DNA sequence analysis of the species *D. stankovici* and *D. rostriformis.* We also examine intraspecific divergence levels among invasive and native ranges, and discuss the phylogeographic history of the genus and component taxa. Results show that the genus diverged into two clades in the region of the Paratethys Sea — the ancestral *D. polymorpha/stankovici* and the *D. rostriformis/bugensis* lineages about 9 to 11 million years ago. The former clade has been restricted to freshwater habitats, while the latter inhabited fresh, brackish, and saltwater areas. *Dreissena stankovici* diverged from *D. polymorpha* about 3 to 4 million years ago, and *D. bugensis* (restricted to the Dnieper-Bug estuarine region before 1940) and *D. rostriformis* (endemic to mesohaline waters of the Caspian Sea area, 10-12.7 ppt) differentiated about 300,000 years ago during the mid-Pleistocene Epoch. We also recognize a divergent form of *D. polymorpha* in the Volga River delta dating to about 430,000 years ago, and dispute taxonomic validity of the putative subspecies of *D. rostriformis* in the Caspian Sea. Genetic evidence shows that the invasions of *D. polymorpha* and *D. bugensis* in the Great Lakes each were founded by large numbers of genotypes from multiple sources.

Introduction

The systematic relationships and identification of the species comprising the dreissenid mussel genus *Dreissena* are characterized by a history of confusion and controversy (reviewed by Rosenberg and Ludyanskiy 1994). This has led to considerable difficulty in correctly identifying species that have been "on the move" in recent decades, invading new habitats in both their native Eurasia and their nonindigenous continent of North America (see Figure 1). As some of the species and putative "subspecies" intergrade and overlap in morphological characters, and traditional characters preclude their correct identification at early life history stages, DNA sequencing offers resolution for these systematic problems (see Stepien et al. 1999, 2001, 2002). For example, our prior work developed rapid diagnostic DNA tools for distinguishing the zebra mussel *D. polymorpha* and the quagga mussel *D. bugensis*, and determined that the two species diverged about 9 to 11 million years ago (Stepien et al. 1999). In the present study, we analyze DNA sequence data from two mitochondrial DNA gene regions (16S ribosomal DNA and cytochrome b) for four extant species of *Dreissena* from their native and invasive ranges — *D. bugensis*, *D. polymorpha*, *D. rostriformis*, and *D. stankovici*.

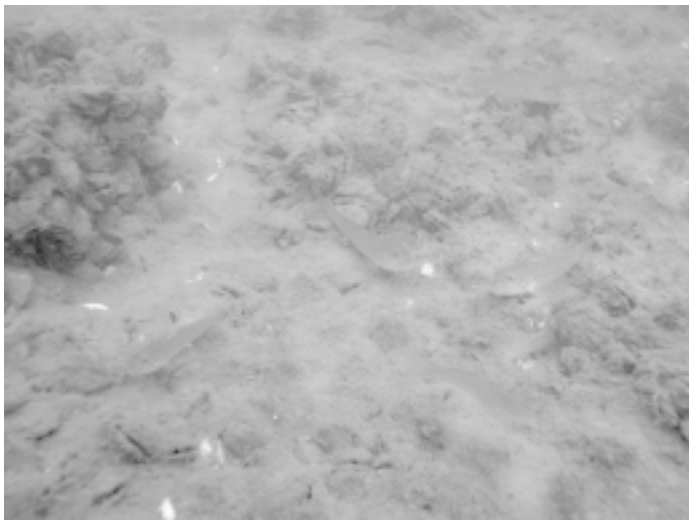


Figure 1. Underwater photograph of the invasive dreissenid-goby community taken off Ohio State University's Stone Laboratory, Gibraltar Island, Lake Erie at 6 m on August 1, 2002 by Carol Stepien. Samples of dreissenids from this area comprised about 90% quagga mussels *Dreissena bugensis* and 10% zebra mussels *D. polymorpha*. Several round gobies *Neogobius melanostomus*, which also are invaders from the Ponto-Caspian region and prey on dreissenids, are shown.

The quagga mussel *D. bugensis*, endemic to the estuarine region of the Dnieper and Southern Bug Rivers, presently is regarded as a separate species from *D. rostriformis* — but often has been classified as the subspecies *D. rostriformis bugensis* (Rosenberg and Ludyanskiy 1994). The extant range of the species *D. rostriformis* is believed to be restricted to the Caspian Sea region (Figure 2). Rosenberg and Ludyanskiy (1994) were in doubt as to the taxonomic separation of *D. rostriformis* and *D. bugensis*, describing considerable morphological overlap but noted that the two taxa characteristically inhabit different salinity regimes, reflecting different tolerance ranges — with *D. bugensis* in fresh and oligohaline waters (0.1-4.0 ppt) and *D. rostriformis* in mesohaline waters (10-12.7 ppt). The fossil record indicates that the Early Pliocene marked the earliest appearance of ancestral *D. rostriformis/bugensis* Caspian lineages in the Black Sea and Caspian depressions. The freshwater *D. bugensis* subsequently diverged in the northwestern Black Sea depression (Figure 2), while the *D. rostriformis* lineage disappeared from the Black Sea region.

Since 1940 *Dreissena bugensis* has been extending its range in the Black Sea river drainages through shipping and construction of canals (Zhuravel 1967) and recently appeared in Volga River reservoirs, the Volga delta, and low-saline waters in the northern Caspian Sea (Antonov 1996). The first recorded specimens of *D. bugensis* in the North American Great Lakes date to Port Colborne in Lake Erie in 1989 (Mills et al. 1993), a year following the discovery of *D. polymorpha* in 1988 (May and Marsden 1992). *Dreissena bugensis* originally became established in the Erie Canal, Lake Erie, and Lake Ontario, where it remained relatively rare except in deeper waters until the last few years. *Dreissena polymorpha* originally greatly outnumbered *D. bugensis*, and the former quickly spread throughout waterways in eastern North America (Figure 3). Recent field studies in the eastern, central, and western basins of Lake Erie indicate that *D. bugensis* has greatly increased its abundance in what was formerly zebra mussel *D.*

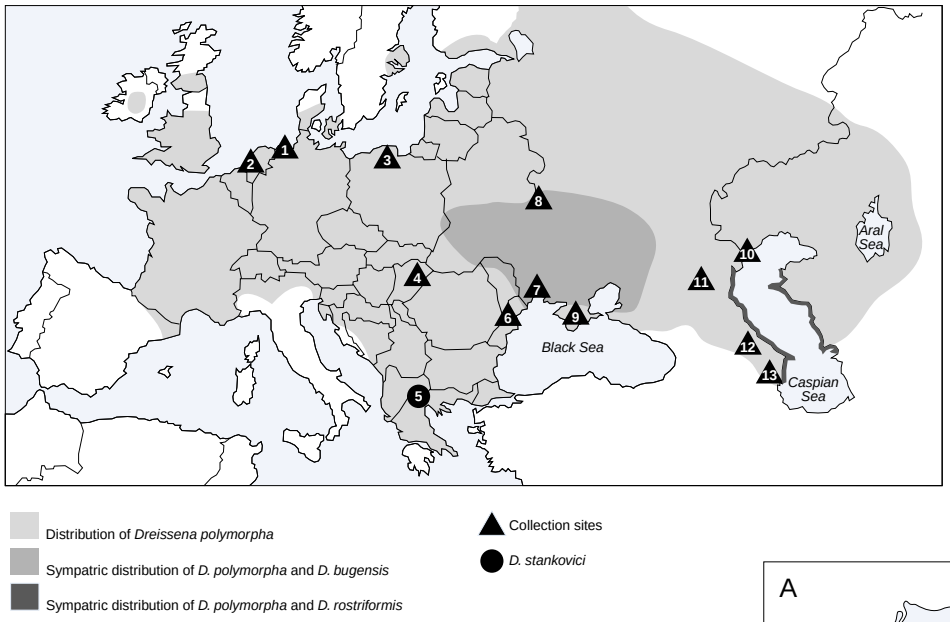
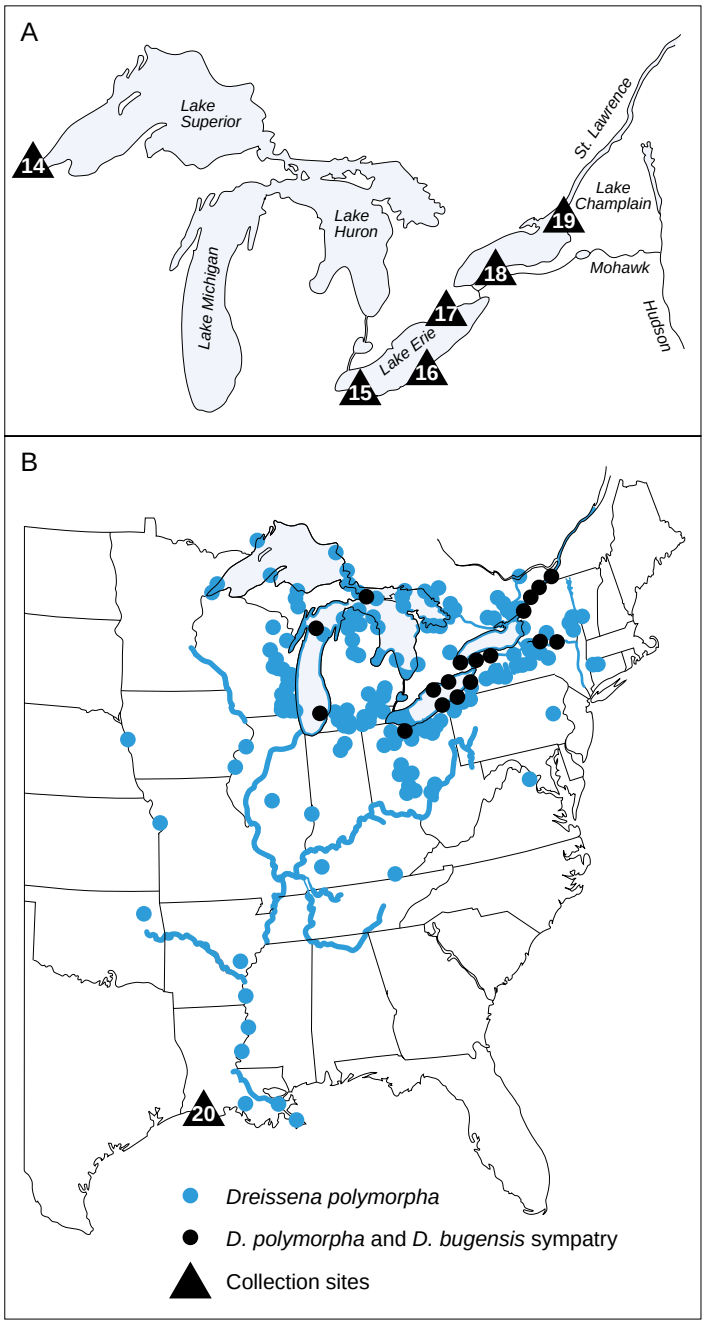


Figure 2 (left). Species distributions and collection sites in Eurasia.

Figure 3 (below). Species distributions and collection sites in North America. (Range data from the National Zebra Mussel and Aquatic Nuisance Species Clearinghouse, 2003).



polymorpha habitat, resulting in the progressive displacement of the latter (Berkman et al. 2000; Jarvis et al. 2000). In 2002 one of us (CAS) found that *D. bugensis* comprised about 90% of the dreissenid mussels collected off Stone Laboratory, Gibraltar Island (near Put-In-Bay, South Bass Island), with *D. polymorpha* only numbering about 10% (Figure 1). In 2001, the proportion of *D. bugensis* and *D. polymorpha* in the same region was about 50:50. In 1997 *D. bugensis* was found in northern Lake Huron and appeared in Lake Michigan in 2001, where it is still rare (Nalepa et al. 2001 and Figure 3).

Dermott and Munawar (1993) described a morphologically distinguishable "profundal" (deepwater) ecotype of *D. bugensis* inhabiting waters 10 to 30 m deep in eastern Lake Erie. This ecotype is characterized by its white color, elliptical shell with a distinctive basal knob and a dorsal swelling, straight ventral shell margin, and more anterior location of the foot and byssal threads. The question of whether the profundal mussel is genetically distinguishable from the quagga mussel has been controversial (Baldwin et al. 1996; Claxton et al. 1997). Prior studies by our laboratory indicated that the profundal ecotype is not genetically separable from *D. bugensis* using 16S rDNA sequences (Stepien et al. 1999) and variation at 52 nuclear RAPDs loci (Stepien et al. 2002). Here, we further test samples of the profundal variant identified by Ronald Dermott using cytochrome b sequences (which evolve more rapidly than does 16S rDNA). We also compare the relationship of the profundal variant to *D. rostriformis,* which it resembles in color, using both 16S rDNA and cytochrome b sequences.

Four subspecies of the species *D. rostriformis* have been described as endemic to the Caspian Sea (Starobogatov 1994; Rosenberg and Ludyanskiy 1994), including: *D. r. grimmi, D. r. distincta, D. r. pontocaspica*, and *D. r. compressa*. These putative taxa occupy slightly different depths and ranges, and display slight variations

in shell morphology — but overlap in all (Logvinenko and Starobogatov 1968; Starobogatov 1994). Three of these putative subspecies (*D. r. grimmi, D. r. distincta*, and *D. r. compressa*) are tested in this investigation.

Dreissena stankovici has the smallest known range among dreissenids, encompassing Lake Okhrid and possibly other lakes in the Vardar and Vistritsa River drainages on the border of Albania and Macedonia (L'vova and Starobogatov 1982; Figure 2). This taxon has been variously classified as either belonging to *D. polymorpha* or as a separate species (*D. stankovici*) due to divergence in shell morphology (L'vova and Starobogatov 1982). Its differentiation from *D. polymorpha* and the other taxa thus is examined here.

Materials and Methods

Samples and sampling sites are indicated in Table 1, with approximate locations shown on the maps in Figures 2 and 3. The present study focuses on relationships among the species of *Dreissena*, and is part of a larger ongoing analysis of the extent of variation within each species.

Mussels were either frozen live in liquid nitrogen or on dry ice and stored at -80° C, or were placed directly in 95% ethanol while alive and then stored at room temperature. The shells were archived. Guts were removed and genomic DNA was extracted from mantle tissues and purified using the DNeasy QIAquick tissue kit and protocol (Qiagen, Inc., Valencia, CA). We amplified the mitochondrial DNA 16S ribosomal and cytochrome b regions using PCR (the polymerase chain reaction) as described by Stepien et al. 1999 and 2001, and then purified the products with a PCR purification Quiaquik kit (Qiagen, Inc., Valencia, CA). DNA sequencing reactions were performed separately in both directions (for independent verification) using the PCR primers and cycle sequencing (CEQ DTCS kit, Beckman-Coulter, Inc., Fullerton, CA). Sequences were determined using a Beckman-Coulter CEQ 8000 capillary autosequencer at the Cleveland State University DNA Analysis Facility. Results for the 16S rDNA sequences are compared to our prior analyses (Stepien et al. 1999, 2001).

We augmented our previous data sets for the mitochondrial 16S ribosomal DNA region (Stepien et al. 1999, 2001) — which included 20 representative *D. polymorpha*, 20 representative *D. bugensis,* 9 *Congeria kusceri,* 10 *Mytilopsis leucophaeta*, and 10 *Corbicula fluminea* — with 7 *D. rostriformis* (including the subspecies *D. r. distincta, D. r. grimmi,* and *D. r. compressa*) and 2 *D. stankovici* for the present study. We then sequenced a 429 bp portion of the more rapidly evolving mitochondrial cytochrome b gene for 22 representative *D. polymorpha* (from North America and Eurasia), 19 representative *D. bugensis* (from North America and Eurasia), 7 *D. rostriformis* (including the subspecies *D. r. distincta, D. r. grimmi,* and *D. r. compressa*), and 2 specimens of *D. stankovici*. A list of specimens and locations is found in Table 1 and collection sites are mapped on Figures 2 and 3. Newly determined sequences for the related living fossil *Congeria* (see Stepien et al. 2001) were used for comparison as an outgroup for the cytochrome b sequences. Cytochrome b gene sequences have not been previously reported for dreissenids.

We analyzed the data using the neighbor joining algorithm with Kimura (1980) 2-parameter genetic distances in MEGA 2.1 (Kumar et al. 2001) and 1000 bootstrap replications. Maximum parsimony analyses and trees were also constructed in MEGA, with 1000 bootstrap replications and consensus analysis of the most-parsimonious trees.

Results

Our sequence results for the 16S rDNA gene are deposited in GenBank for *Dreissena stankovici* as AY302247, for *D. rostriformis* as AY302248, and those obtained previously by our laboratory are AF038996 for *D. bugensis*, AF038997 for *D. polymorpha*, AF038998 for *Mytilopsis leucophaeta*, AF038999 for *Corbicula fluminea*, and AF320601 for *Congeria kusceri* (Stepien et al. 1999 and 2001). Our sequences for the cytochrome b gene will be deposited in GenBank pending publication of our next paper in this series. Kimura (1980) 2-parameter genetic distances among taxa are reported in Table 2. Figures 4 and 5 shows the phylogenetic relationships among the taxa for neighbor-joining and maximum parsimony analyses for each gene region, with bootstrap support from 1000 replications.

16S rDNA

A single nucleotide substitution separated all *D. rostriformis* from all *D. bugensis* sequences in the 16S rDNA region. Similarly, a single nucleotide separation occurred between the two haplotypes found for the living fossil species *Congeria kusceri* from a single cavern system (see Figure 2 and Stepien et al. 2001). All other taxa were represented by single haplotypes. Calibration of the 16S rDNA genetic distances using a divergence of 0.0074 per my (see Stepien et al. 1999 for calibration methodology using the fossil record) reveals an estimated 270,000 years (+/- 135,000 years) of separation between haplotypes of *D. bugensis* and *D. rostriformis,* as well as between the two haplotypes of *Congeria kusceri* (Stepien et al. 2001). In contrast, *D. polymorpha* and *D. stankovici* diverged an estimated 3.38 +/- 1.89 million years ago (d= 0.025 +/- 0.014). Thus, the 16S rDNA data indicate that *D. bugensis* and *D. rostriformis* may either be subspecies or recently diverged species.

The phylogeny based on the 16S rDNA sequences shows that *D. stankovici* is the sister species to *D. polymorpha* and this clade then comprises the sister group to the *D. rostriformis/bugensis* clade (Figure 4). The species *D. stankovici* and *D. polymorpha* share 4 substitution synapomorphies (2 transitions and 2 transversions). In addition, *D. stankovici* is distinguished from the other taxa by a unique DNA substitution. In turn, *D. polymorpha* diverges from all other *Dreissena* species at 4 nucleotide positions, including 2 transitional substitu-

Continued on p. 14

Table 1. Collection sites (refer to Figure 2 and Figure 3)

Species	Id#	Body of Water	Location	Latitude	Longitude	Map site	Haplotype
D. polymorpha	TB6	Lake Ijsselmeer	Amsterdam, Netherlands	52.46° N	5.14° E	1	p-2
D. polymorpha	UG9	Rhine River	Vuren, Netherlands	51.82° N	5.05 ° E	2	p-2
D. polymorpha	ZS11	(Small pond)	Naar Rupel, Belgium	51.00° N	4.00 ° E	2	p-2
D. polymorpha	TQ8	Lake Dybrzk	Poland	52.46°N	19.00° E	3	p-4
D. polymorpha	UK9	Danube River	Budapest, Hungary	47.30° N	19.08° E	4	p-2
D. polymorpha	TJ2	Danube River	Budapest, Hungary	47.30° N	19.08° E	4	p-1
D. stankovici	ABW3	Lake Okhrid	Pestani, Macedonia	41.02° N	20.80° E	5	s-1
D. stankovici	ADH7	Lake Okhrid	Pestani, Macedonia	41.02° N	20.80° E	5	s-1
D. polymorpha	ADH3	Dniester Liman	Belgorod-Dniestrovskii, Ukraine	46.20° N	30.36° E	6	p-2
D. polymorpha	ADH5	Dniester Liman	Belgorod-Dniestrovskii, Ukraine	46.20° N	30.36° E	6	p-2
D. polymorpha	ADH6	Dniester Liman	Belgorod-Dniestrovskii, Ukraine	46.20° N	30.36° E	6	p-4
D. bugensis	ADH8	Dniester Liman	Belgorod-Dniestrovskii, Ukraine	46.20° N	30.36° E	6	b-1
D. polymorpha	ACY1	s. Dnieper River	Kherson, Ukraine	46.41° N	32.60° E	7	p-2
D. bugensis	AAW1	s. Dnieper River	Kherson, Ukraine	46.41° N	32.60° E	7	b-6
D. bugensis	ACY10	s. Dnieper River	Kherson, Ukraine	46.41° N	32.60° E	7	b-1
D. polymorpha	ACY3	Southern Bug R.	Nikolaev, Ukraine	46.88° N	31.98° E	7	p-6
D. polymorpha	ADH9	Southern Bug R.	Nikolaev, Ukraine	46.88° N	31.98° E	7	p-2
D. polymorpha	ACY11	Southern Bug R.	Nikolaev, Ukraine	46.88° N	31.98° E	7	p-2
D. bugensis	ACY6	Dnieper River	Kiev, Ukraine	46.30° N	34.00° E	8	b-7
D. bugensis	ACY7	Dnieper River	Kiev, Ukraine	46.30° N	34.00° E	8	b-1
D. polymorpha	ADH4	Kakhovskii Canal	Crimea, Ukraine	45.46° N	34.77° E	9	p-9
D. polymorpha	ACY5	Kakhovskii Canal	Crimea, Ukraine	45.46° N	34.77° E	9	p-7
D. bugensis	ADH2	Kakhovskii Canal	Crimea, Ukraine	45.46° N	34.77° E	9	b-1
D. polymorpha	ABW5	s. Volga River	Obukhovskaya Protoka, Russia	45.73° N	48.07° E	10	p-8
D. bugensis	AAW3	s. Volga River	Obukhovskaya Protoka, Russia	45.73° N	48.07° E	10	b-1
D. bugensis	ADF1	n. Caspian Sea	Lagan, Russia	45.35° N	47.45° E	11	b-8
D. bugensis	ABW4	n. Caspian Sea	Lagan, Russia	45.35° N	47.45° E	11	b-9
D. rostriformis grimmi	AAJ3	c. Caspian Sea	Central Basin	40.82° N	49.82° E	12	r-4
D. rostriformis distincta	AAJ1	s. Caspian Sea	Southern Basin	39.63° N	49.77° E	13	r-1
D. rostriformis distincta	AAJ2	s. Caspian Sea	Southern Basin	39.63° N	49.77° E	13	r-2
D. rostriformis distincta	ABW6	s. Caspian Sea	Southern Basin	39.63° N	49.77° E	13	r-3
D. rostriformis compressa	AAJ4	s. Caspian Sea	Southern Basin	39.63° N	49.77° E	13	r-5
D. polymorpha	VP5	w. Lake Superior	Duluth-Superior Harbor	46.50° N	92.07° W	14	p-5
D. polymorpha	WK4	w. Lake Superior	Duluth-Superior Harbor	46.50 °N	92.07° W	14	p-2
D. polymorpha	US6	w. Lake Erie	Put-in-Bay, OH	41.50° N	83.00° W	15	p-2
D. bugensis	TS33	w. Lake Erie	Put-in-Bay, OH	41.50° N	83.00° W	15	b-1
D. bugensis	US12	w. Lake Erie	Put-in-Bay, OH	41.50° N	83.00° W	15	b-2
D. polymorpha	QI1	c. Lake Erie	Cleveland, OH	41.30° N	81.42° W	16	p-3
D. bugensis	TD15	c. Lake Erie	Cleveland, OH	41.30° N	81.42° W	16	b-1
D. bugensis	TJ12	e. Lake Erie	Mid e. Basin, Profundal	42.26° N	79.50° W	17	b-4
D. bugensis	TJ15	e. Lake Erie	Mid e. Basin, Profundal	42.26° N	79.50° W	17	b-4
D. bugensis	VR14	e. Lake Erie	Mid e. Basin, Profundal	42.26° N	79.50° W	17	b-5
D. bugensis	WQ3	e. Lake Erie	Mid e. Basin, Profundal	42.26 °N	79.50° W	17	b-3
D. bugensis	YI8	w. Lake Ontario	Niagara Bar, NY	43.19° N	78.52° W	18	b-1
D. polymorpha	YK3	e. Lake Ontario	Cape Vincent, NY	44.20° N	76.10° W	19	p-2
D. bugensis	TB11	e. Lake Ontario	Cape Vincent, NY	44.20° N	76.10° W	19	b-4
D. bugensis	YL15	e. Lake Ontario	Cape Vincent, NY	44.20° N	76.10° W	19	b-10
D. polymorpha	TG9	s. Mississippi R.	Baton Rouge, LA.	30.24° N	91.11° W	20	p-2

Table 2. Kimura (1980) 2-Parameter Pairwise Genetic Distances among *Dreissena* Taxa and Outgroups (+/- standard error) calculated using MEGA 2.1 (Kumar et al. 2001). Above diagonal: Cytochrome b gene. Below diagonal: 16S ribosomal DNA. Note: Distance between *D. bugensis* and *D. rostriformis* is the same as that between the two haplotypes of *Congeria kusceri* for 16S rDNA. X=Data not presently available for other outgroups.

	Dreissena bugensis	*D. polymorpha*	*D. rostriformis*	*D. stankovici*	*Mytilopsis leucophaeta*	*Congeria kusceri* a	*C. kusceri* b	*Corbicula fluminea*	*Mytilus edulis*
Dreissena bugensis	———	0.276 +/- 0.033	0.010 +/- 0.006	0.276 +/- 0.021	X	0.435 +/- 0.045	X	X	X
D. polymorpha	0.079 +/- 0.014	———	0.276 +/- 0.033	0.130 +/- 0.033	X	0.429 +/- 0.042	X	X	X
D. rostriformis	0.002 +/- 0.001	0.082 +/- 0.014	———	0.284 +/- 0.034	X	0.429 +/- 0.045	X	X	X
D. stankovici	0.074 +/- 0.013	0.025 +/- 0.014	0.076 +/- 0.013	———	X	0.450 +/- 0.047	X	X	X
Mytilopsis leucophaeta	0.130 +/- 0.018	0.125 +/- 0.018	0.128 +/- 0.018	0.112 +/- 0.016	———	X	X	X	X
Congeria kusceri a	0.112 +/- 0.016	0.109 +/- 0.016	0.109 +/- 0.016	0.099 +/- 0.015	0.058 +/- 0.011	———	X	X	X
C. kusceri b	0.114+/- 0.016	0.112 +/- 0.016	0.112 +/- 0.015	0.101 +/- 0.015	0.060 +/- 0.011	0.002 +/- 0.001	———	X	X
Corbicula fluminea	0.402 +/- 0.036	0.423 +/- 0.038	0.406 +/- 0.037	0.417 +/- 0.038	0.399 +/- 0.035	0.405 +/- 0.035	0.405 +/- 0.035	———	X
Mytilus edulis	0.639 +/- 0.054	0.638 +/- 0.054	0.634 +/- 0.052	0.618 +/- 0.052	0.638 +/- 0.054	0.617 +/- 0.051	0.617 +/- 0.051	0.613 +/- 0.052	———

tions and 2 indels (with *D. polymorpha* having 2 separate deletions that are pleisiomorphies shared with ancestral taxa). The *D. rostriformis/bugensis* clade is distinguished from the *D. polymorpha/stankovici* lineage by 7 synapomorphies characterizing the former, including 3 transversions, 3 transitions, and an insertion.

Cytochrome b

The cytochrome b gene in dreissenids correspondingly appears to evolve about 3.8 times the rate of 16S rDNA region, at an estimated 0.028 per million years. We found 9 haplotypes in 22 samples of *D. polymorpha*, with 3 haplotypes occurring in North America (of 6 individuals examined from 5 sites to date) and 6 in Eurasia (of 16 individuals examined from 9 sites to date). These diverged intraspecifically by genetic distances of 0.003 to 0.017 (the latter for the southern Volga River versus the other sites; see Figure 5), or an estimated range of 107,000 to 670,000 years ago.

There were 2 unique haplotypes of *D. polymorpha* found in North America (of the 6 individuals analyzed to date); one from Lake Superior, and the other in central Lake Erie (Cleveland, Ohio). The other 4 individuals analyzed from North America (from western Lake Superior, western Lake Erie, eastern Lake Ontario, and the southern Mississippi River) had a common haplotype that was shared with 9 Eurasian individuals (from the Danube River at Budapest, Hungary; Lake IJesselmer, the Netherlands; Naar Rupel, Belgium; the Rhine River at Vuren, the Netherlands; the Dnieper River at Kherson, Ukraine; the Dniester River at Belgorod-Dniestrovskii, Ukraine (2 individuals); and the Southern Bug River at Nikolaev, Ukraine (2 samples). In addition, there were 6 other haplotypes that have only been identified from locations in Eurasia to date (in the Danube River at Budapest, Hungary; in both Lake Dybrzk, Poland and the Dniester River at Belgorod-Dniestrovskii, Ukraine; in the Southern Bug River at Nikolaev, Ukraine; 2 separate types from the Kakhovskii Canal in the Crimea, Ukraine; and a single type in the Obukhovskaya Protoka Channel of the Volga River delta, Russia). The Volga River haplotype differs by 4 nucleotide substitutions from all other haplotypes of *D. polymorpha.* It diverges from the other haplotypes by a genetic distance of .012, equivalent to about 429,000 years. Its divergence is shown by its longer branch on the tree (p-8 on Figure 5).

A total of 43 nucleotide sites in the cytochrome b gene region sequences separate *D. stankovici* from *D. polymorpha,* of which 25 are apomorphies uniquely distinguishing *D. stankovici.* The *D. rostriformis/bugensis* lineage shares 18 characters with *D. stankovici*, diverging from *D. polymorpha*. The species *D. polymorpha* is characterized by 16 apomorphies. A genetic distance of 0.130 +/- 0.033 separates *D. stankovici* and *D. polymorpha* (Table 2), which is equivalent to about 4.64 +/- 1.18 million years. The species *D. stankovici* and *D. polymorpha* share 57 sequence characters, and their clade diverges from the *D. rostriformis/bugensis* lineage by an estimated 9.86 +/- 1.18 million years.

There were six haplotypes of *D. bugensis* among 10 individuals from 5 locations in North America, including a widespread type found in western Lake Erie (2 individuals), central Lake Erie, and western Lake Ontario (that was also widespread in Eurasia); another widely distributed type in the eastern basin of Lake Erie (2 individuals of the profundal variant), and a single individual from eastern Lake Ontario; and 3 unique (singleton) haplotypes respectively found in western Lake Erie, eastern Lake Erie (profundal variant), and eastern Lake Ontario. The profundal variants did not cluster together and were not distinguished by one or more common synapomorphies, indicating that they do not comprise a divergent lineage or a valid taxonomic group.

There were 5 haplotypes in Eurasia among 9 individuals sampled from 6 locations, revealing similar levels of genetic variation in North America and across Eurasia to date. The widespread haplotype found in North America also occurred in the upper (Kiev) and lower reaches of the Dniester River; in the Kakhovskii Canal in the Crimea, Ukraine; and in the Volga River delta, Russia. Four singleton haplotypes unique to specific Eurasian locations were found in the southern Dnieper River estuary, the Dnieper River at Kiev, and in the northern Caspian Sea (2 divergent types), respectively.

Intraspecific divergences among haplotypes of *D. bugensis* ranged from 0.006 to 0.014 (the upper number was for the northern Caspian Sea samples), corresponding to 214,000 to 500,000 years. The divergence of *D. rostriformis* from *D. bugensis* was estimated to be 0.010 +/- 0.006, within this range, or about 357,000 years. All *D. rostriformis* sequenced differed from all *D. bugensis* at a single nucleotide site position, resulting in monophyletic lineages in the most-parsimonious tree from a maximum parsimony analysis (Figure 5). In addition, the 4 haplotypes of *D. rostriformis* varied from each other at 2 other nucleotide positions (reflecting variation within this taxon). The 3 subspecies of *D. rostriformis* analyzed did not group according to their subspecies definitions. For example, the 3 haplotypes of *D. rostriformis distincta* were separated in 2 groups on the tree (Figure 5), indicating that it is not a valid taxon. The present results also confirm the presence of *D. bugensis* in the Volga River delta and the northern Caspian Sea today (Antonov 1996; see Table 1 and Figures 3 and 5).

Both the neighbor-joining and most-parsimonious maximum parsimony trees yielded the same phylogenetic relationships among species as found for 16S rDNA (Figures 4 and 5). The cytochrome b and 16S rDNA sequence data congruently showed that *D. stankovici* and *D. polymorpha* are sister species and their clade is sister to the *D. rostriformis/bugensis* lineage. Parsimony analyses supported the monophyly of *D. rostriformis*, indicating that it has been separated from the *D. bugensis* lineage and there is no apparent ongoing gene flow between them. The tree did not support recognition of the subspecies *D. r. grimmi*, *D. r. compressa*, or *D. r. distincta* (Figure 5).

Discussion

The present study produced a fully resolved and well-supported phylogeny of the evolutionary relationships among *Dreissena* species (Figures 4 and 5), revealing that there are two primary clades – *D. polymorpha/stankovici* and *D. rostriformis/bugensis*. The clades

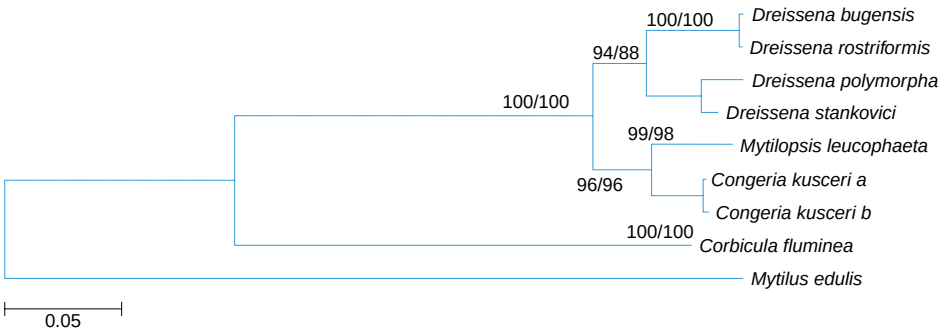


Figure 4. Neighbor-joining tree of dreissenid relationships from mitochondrial 16S ribosomal DNA sequence data. Tree was constructed with Kimura (1980) 2-parameter genetic distances in MEGA 2.1 (Kumar et al. 2001), with support from 1000 bootstrap replications indicated (above diagonal). This tree was congruent with the most parsimonious tree from a maximum parsimony analysis, and results from 1000 bootstrap replications (below diagonal) were similar. C.I. = 0.90, C.I. for parsimony informative sites = 0.80.

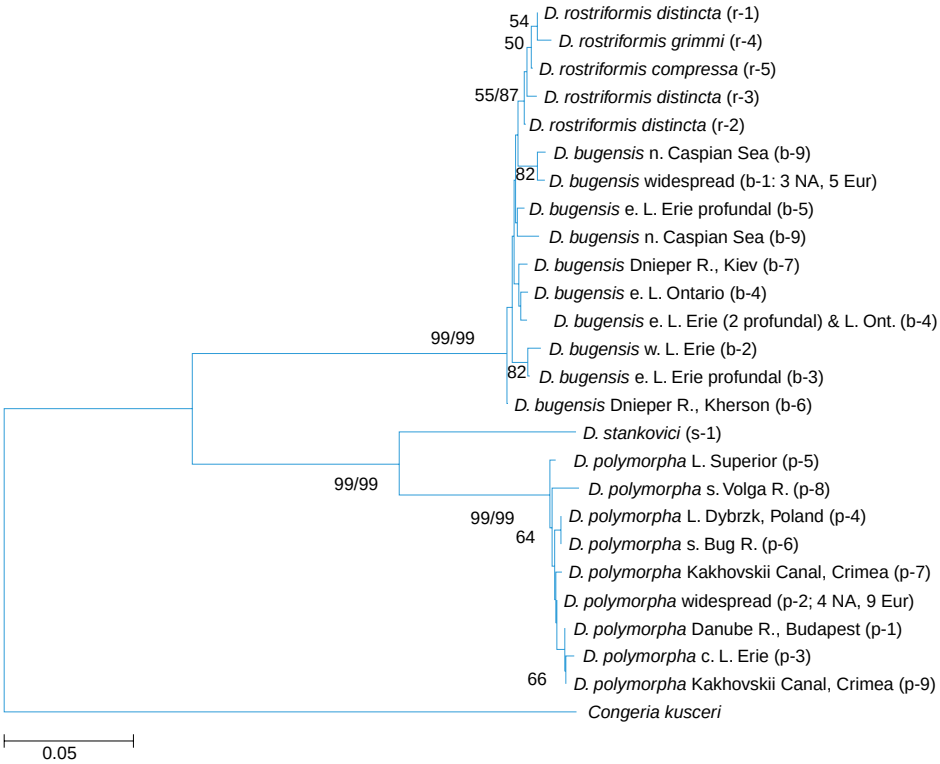


Figure 5. Neighbor-joining tree of *Dreissena* relationships from mitochondrial cytochrome b DNA sequence data. Tree was constructed with Kimura (1980) 2-parameter genetic distances in MEGA 2.1 (Kumar et al. 2001). Numbers indicated support for relationships from 1000 bootstrap replications for those greater or equal to 50% (above diagonal or single values). Haplotypes are designated in parentheses on the tree, according to Table 1. This tree was congruent with the most parsimonious trees from a maximum parsimony analysis, and support values for those relationships from 1000 bootstrap replications (below diagonal) were similar. C.I. = 0.93, C.I. for parsimony informative sites = 0.88. Relationships among all species, including *D. polymorpha*, *D. bugensis*, *D. stankovici*, and *D. rostriformis* were supported by 100% consensus in maximum parsimony.

differentiated about 9.86 +/- 1.18 million years ago according to the cytochrome b sequence calibration, which is similar to the estimated separation of 10.69 +/- 1.89 million years from the 16S rDNA data. The species *D. stankovici* diverged from the lineage shared with *D. polymorpha* about 4.64 +/- 1.50 million years ago according to the cytochrome b data, which is within the range of 3.38 +/- 1.89 million years obtained from the 16S rDNA sequences (see Stepien et al. 1999). In contrast, *D. bugensis* and *D. rostriformis* diverged only about 347,000 +/- 117,000 years ago according to the cytochrome b gene, which is close to the 270,000 +/- 140,000 years estimated from 16S rDNA.

Diversification of the lineage *Dreissena* was shaped by geological and climatic changes and corresponding salinity fluctuations in the Paratethys Region (Briggs 1974; Zaitsev and Mamaev 1997), which includes the modern Black, Azov, Caspian and Aral Seas (see Figure 2). Hutchinson (1957) commented that few species are able to transition the formidable barrier between saline and freshwater habitats. Salinity tolerance differences characterize *Dreissena* taxa (Nevesskaya 1965; Rosenberg and Ludyanskiy 1994), and salinity changes presumably resulted in vicariant barriers to *Dreissena* taxa, leading to their extirpation or genetic divergence and eventual speciation.

By the late Miocene Epoch, from 5 to 7 million years ago, crust movements led to the formation of the Eurasian mountain ranges and the shrinking of the Paratethys Seas into a number of brackish basins (Zaitsev and Mamaev 1997). By this time, our divergence calibrations indicate that the two major lineages of *Dreissena* had diverged — with the *D. polymorpha/stankovici* ancestral clade restricted to freshwater and the ancestral *D. rostriformis/bugensis* lineage inhabiting fresh, brackish, and saline waters. The distributions of the dreissenid lineages changed over the course of the late Miocene to early Pliocene as a result of decreasing salinity from river inflow, followed by increase due to reestablishment of an oceanic connection (3 to 5 million years ago; Zaitsev and Mamaev 1997). During this time, *D. stankovici* diverged in the lakes of Albania/Macedonia (see Figure 2). During the Pliocene (1.5 to 3 million years ago), the Paratethys Seas again became increasingly freshwater (Banarescu 1992; Zaitsev and Mamaev 1997), leading to the expansion and contraction of ranges for *D. polymorpha* and the ancestral *D. rostriformis/bugensis* lineage.

During the Pliocene Epoch, the future Black and Caspian Seas were connected through the present-day northern Caucasus region, which then rose to form mountains, gradually isolating the Caspian Sea (Banarescu 1992). During this time, the lineages of *D. bugensis* and *D. rostriformis* diverged – gradually becoming separate species, with *D. bugensis* restricted to fresh waters to the west in the Black Sea and *D. rostriformis* in saline waters to the east in the Caspian Sea region. During the Pleistocene interglacials, the waters of the Aral and Caspian Seas were higher than today and they again were connected to the Black Sea (Zaitsev and Mamaev 1997), producing fluctuations in the distributions of the two species. Most extant haplotypes of *Dreissena* species diversified during the mid-Pleistocene, presumably due to vicariant changes in salinity and range contractions and expansions during the glacial and interglacial periods (see Figure 5).

During the late Pleistocene, the Black Sea again became connected to the Mediterranean Sea and the world ocean through the Sea of Marmara (Banarescu 1992). Marine flora and fauna recolonized the future Black Sea and the brackish and freshwater species were forced into bays, limans, and river estuaries having reduced salinities. This rise in salinity likely isolated *D. bugensis* in the upper rivers of the middle Black Sea region and led to the disappearance of any remnants of the *D. rostriformis* lineage from this area. Throughout the Pleistocene, the salinity of the Black Sea fluctuated — with freshening during the glacial advances and incursions of salt water during the interglacial periods — and two connections and severances from the Mediterranean Sea (Briggs 1974; Zaitsev and Mamaev 1997). Following the last glaciation (18,000-20,000 ya); the oceanic connection was lost, salinity was lowered by the melting waters, many marine species became extinct, and brackish and freshwater species reinvaded the Black Sea region (Banarescu 1992; Zaitsev and Mamaev 1997). The geological and climatic history of the Paratethys region thus produced the phylogenetic patterns of *Dreissena* diversification seen today.

Our data sets show that the level of genetic divergence between the *D. bugensis* and *D. rostriformis* clades is similar to that distinguishing haplotypes within other dreissenid species, indicating their recent separation during the mid Pleistocene Epoch. However, all D. *rostriformis* and *D. bugensis* are clearly separable by a synapomorphy in the 16S rDNA sequence data and by another in the cytochrome b data. Although 5 different haplotypes of *D. rostriformis* occur in our cytochrome data set, all are united by this synapomorphy and form a monophyletic clade, which is the sister group to the *D. bugensis* haplotypes. There also is apparently relatively long-term geographic separation of the two taxa in the Black versus the Caspian Sea regions, with recent expansion of *D. bugensis* into the northern Caspian Sea region and Volga River delta (Antonov 1996) — as confirmed by the present study. There is no evidence that *D. bugensis* and *D. rostriformis* interbreed today.

According to Rosenberg and Ludyanskiy (1994), *D. bugensis* reaches a larger size, has a more pronounced byssal groove, usually has a broader, more inflated shell, and characteristically is darker in color than *D. rostriformis*. *Dreissena bugensis* lives in salinities up to 1 ppt, and *D. rostriformis* lives in salinities 10 to 12.7 ppt (Logvinenko and Starobogatov 1968). Babak (1983) described *D. bugensis* from Quaternary deposits of the Black Sea, indicating that this taxon diverged during the period estimated by our study. Since *D. bugensis* is

currently recognized by most investigators as a separate species from *D. rostriformis*, we recommend that they continue to be recognized as separate species despite their smaller level of genetic divergence. The level of divergence suggests their relatively recent separation, which also is indicated by the fossil record and by the geological history of the Black and Caspian Sea basins. We thus agree with Rosenberg and Ludyanskiy (1994) that although the two species are closely related, it is appropriate to retain them as separate species based on the evidence currently available. However, our analysis of sequence diversity at cytochrome b and 16S rDNA for the three "subspecies" of *D. rostriformis* did not validate their recognition described by Logvinenko and Starobogatov (1968). Since these putative taxa overlap in morphological characters, ranges, and depths (Logvinenko and Starobogatov 1968; Rosenberg and Ludyanskiy 1994) — and do not show patterns of genetic divergence — they need to be synonymized.

Our cytochrome b sequences show that levels of genetic diversity in North American populations of *D. bugensis* and *D. polymorpha* are remarkably high, with numbers of haplotypes per samples similar to those found across their respective Eurasian distributions. These results are congruent to findings by Stepien et al. (2002) for levels of genetic variation at 63 nuclear RAPDs loci in *D. polymorpha* and 52 in *D. bugensis*. Results from both studies indicate that the invasions of both species each involved a large number of individuals from multiple founding sources. Both the cytochrome b sequences (Figure 5) as well as the nuclear DNA data (Stepien et al. 2002) reveal that the *D. polymorpha* population in Lake Superior significantly differs from samples in the lower Great Lakes, suggesting separate founding sources and events.

Findings from our cytochrome b sequences as well as nuclear RAPD divergences (Stepien et al. 2002) reveal that *D. polymorpha* from the Volga River are quite different from other sites (note the long length of the branch for haplotype p-8 in the tree shown in Figure 5), suggesting long historic separation. In fact, this taxon has been named as the subspecies *D. polymorpha andrusovi*, which has higher salinity tolerance than does *D. polymorpha* (Logvinenko and Starobogatov 1968; Rosenberg and Ludyanskiy 1994). We will further test this genetic divergence with additional samples, in order to determine its phylogenetic status. Provisionally, it may be a valid taxon, diverged by about 429,000 +/- 214,000 years.

Individuals representing the profundal ecotype of *D. bugensis* (Dermott and Munawar 1993) do not form a clade separable from the shallow water ecotype in Lake Erie (Figure 5). Results of the present study agree with our previous work based on nuclear DNA RAPDs markers (Stepien et al. 2002) and 16S rDNA sequences (Stepien et al. 1999), indicating that samples of the profundal variant are not genetically distinguishable from *D. bugensis* and should not be taxonomically recognized.

We presently are working to increase the number of samples and population sites in our survey of variation in the cytochrome b gene, in order to resolve additional population relationships and elucidate the founding source populations for the invasions. Results of the present investigation indicate that the mitochondrial cytochrome b gene holds considerable promise for resolving population genetic and systematic relationships in dreissenid mussels.

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References

Andrusov N. 1897. Fossile und lebende Dreissensidae Eurasiens. Trav. Soc. Nat. St.-Pétersbourg. Sect. Géol. Minérol. Vol. 25. Merkushevs, St.-Pétersbourg, Russia (In Russian)

Antonov PI. 1996. The bivalve species *Dreissena bugensis* (Ant.) as new to the Volga fauna. pp 9-10. In: Kutikova LA (ed) Problemy gidrobiologii kontinental'nikh vod i ikh malakofauna (International conference. Problems of aquatic ecology in inland waters and their malakofauna). Book of Abstracts. (in Russian)

Babak EV. 1983. The Pliocene and Quaternary dreissenids (Dreissenidae, Bivalvia) of the Evksinsk Basin. *Akad. Nauk SSSR Tr. Paleontol. Inst*. Vol. 204. Nauka, Moscow, Russia (In Russian)

Baldwin BS, Black M, Sanjur O, Gustafsom R, Lutz RA, Vrijenhoek RC. 1996. A diagnostic molecular marker for zebra mussels (*Dreissena polymorpha*) and potentially co-occurring bivalves: mitochondrial COI. *Mol. Mar. Biol. Biotech.* 5:9-14.

Banarescu P. 1992. Zoogeography of Fresh Waters. Vol. 2. Distribution and Dispersal of Freshwater Animals in North America and Eurasia. AULA-Verlag, Germany.

Berkman PA, Garton DW, Haltuch MA, Kennedy GW, Febo LR. 2000. Habitat shift in invading species: Zebra and quagga mussel population characteristics on shallow soft substrates. *Biol. Invasions,* 2:1-6.

Briggs JC. 1974. Marine Zoogeography. McGraw-Hill, Inc., New York.

Claxton WT, Martel A, Dermott RM, Boulding EG. 1997. Discrimination of field-collected juveniles of two introduced dreissenids (*Dreissena polymorpha* and *Dreissena bugensis*) using mitochondrial DNA and shell morphology. *Can. J. Fish. Aquat. Sci.* 54:1280-1288.

Dermott R, Munawar M. 1993. Invasion of Lake Erie offshore

sediments by *Dreissena*, and its ecological implications. *Can. J. Fish. Aquat. Sci.* 50:2298-2304.

Grigorovich IA, MacIsaac HJ, Shadrin NV, Mills EL. 2002. Patterns and mechanisms of aquatic invertebrate introductions in the Ponto-Caspian region. *Can. J. Fish. Aquat. Sci.* 59:1189-1208.

Jarvis P, Dow J, Dermott R, Bonnell R. 2000. Zebra (*Dreissena polymorpha*) and quagga mussel (*Dreissena bugensis*) distribution and density in Lake Erie, 1992-1998. *Can. Tech. Rept. Fish. Aquat. Sci.* 2304:1-46.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-120.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. *MEGA2.1*: *Molecular Evolutionary Molecular Evolutionary Genetics Analysis software*, Arizona State University, Tempe, Arizona.

Logvinenko BM, Starobogatov YI. 1968. Phillum Mollusca. In *Atlas of invertebrates of the Caspian Sea*. (eds. YA Birshtein, LG Vinogradov, NN Kondakov, MS Astakhova, NN Romanova), pp 308-385. Pishchevaya Promyshlennost', Moscow, Russia. (In Russian)

L'vova AA, Starobogatov YaI. 1982. A new species of the genus *Dreissena* (Bivalvia, Dreissenidae) from Lake Ohrid. *Zool. Zhurn.* 61:1749-1752.

May B, Marsden JE. 1992. Genetic identification and implications of another invasive species of dreissenid mussel in the Great Lakes. *Can. J. Fish. Aquat. Sci*. 49:1501-1506.

Mills EL, Leach JH, Carlton JT, Secor CL. 1993. Exotic species in the Great Lakes: A history of biotic crises and anthropogenic introductions. *Journal of Great Lakes Research*, 19:1-54.

Mills EL, Chrisman JR, Baldwin B, Owens RW, O'Gorman R, Howell T, Roseman EF, Raths MK. 1999. Changes in the dreissenid community in the lower Great Lakes with emphasis on southern Lake Ontario. *J. Great Lakes Res*. 25:187-197.

Nalepa TF, Schloesser DW, Pothover S, Hondorp D, Fanslow DL, Tuchman M, Fleischer G. 2001. First finding of the amphipod *Echinogammarus ischnus* and the mussel *Dreissena bugensis* in Lake Michigan. *J. Great Lakes Res*. 27:384-391.

National Zebra Mussel and Aquatic Nuisance Species Clearinghouse. 2003. New York Sea Grant Extension Program. [WWW document]. URL http://cce.cornell.edu/seagrant/nansc/zmaps.html

Nevesskaya LA. 1965. (Late Quaternary bivalve mollusks of the Black Sea, their systematics and ecology). *Tr. Paleontol. Inst.* 105:1-391. (In Russian)

Rosenberg G, Ludyanskiy ML. 1994. A nomenclatural review of *Dreissena* (Bivalvia: Dreissenidae), with identification of the quagga mussel as *Dreissena bugensis. Can. J.Fish. Aquat. Sci.* 51:1474-1484.

Starobogatov Ya I (ed.) 1994. Freshwater zebra mussel, *Dreissena polymorpha* (Pall.) (Bivalvia, Dreissenidae): Systematics, ecology, and practical meaning. Nauka, Moscow, Russia (In Russian).

Stepien CA, Hubers AN, Skidmore J. 1999. Diagnostic genetic markers and evolutionary relationships among invasive dreissenoid and corbiculoid bivalves: Phylogenetic signal from mitochondrial 16S rDNA. *Molecular Phylogenetics and Evolution* 13(1):31-49.

Stepien CA, Morton B, Dabrowska KA, Guarnera R, Radja T, Radja B. 2001. Genetic diversity and evolutionary relationships of the troglodytic "living fossil" *Congeria kusceri* (Bivalvia: Dreissenidae). *Molecular Ecology,* 10:1873-1879.

Stepien CA, Taylor CD, Dabrowska KA. 2002. Genetic variability and phylogeographic patterns of a nonindigenous species invasion: A comparison of exotic versus native zebra and quagga mussel populations. *Journal of Evolutionary Biology,* 15(2):314-328.

Swofford DL. 2001. *PAUP (Phylogenetic Analysis Using Parsimony)* vers. 4.0b5. Sinauer Assoc. Sunderland, Massachusetts

Vanderploeg HA, Nalepa TF, Jude DJ, Mills EL, Holeck K, Liebig JR, Grigorovich IA, Ojaveer H. 2002. Dispersal and emerging ecological impacts of Ponto-Caspian species in the Laurentian Great Lakes. *Can. J. Fish. Aquat. Sci.* 59:1209-1228.

Williamson M. 1996. *Biological Invasions*. Chapman and Hall, New York, New York.

Zaitsev Y, Mamaev V. 1997. Marine Biological Diversity in the Black Sea: A study of Change and Decline. United Nations Publications, New York, NY.

Zhuravel PA. 1967. On the colonization of artificial waterbodies by *Dreissena bugensis*. *Gidrobiol. Zhurn.* 3(2):87-90. (In Russian).