J. Great Lakes Res. 27(3):267–280
Internat. Assoc. Great Lakes Res., 2001

Genetic and Biogeographic Relationships of the Invasive Round (*Neogobius melanostomus*) and Tubenose (*Proterorhinus marmoratus*) Gobies in the Great Lakes Versus Eurasian Populations

Alison K. Dillon[1] and Carol A. Stepien*

Great Lakes Environmental Genetics Laboratory
Center for Environmental Science, Technology and Policy
Cleveland State University
1899 East 22nd Street, MC 219
Cleveland, Ohio 44114-4435

ABSTRACT. *Population genetic structure and systematic relationships were investigated for two exotic fishes introduced to the Great Lakes in 1990, the round goby* Neogobius melanostomus *and the tubenose goby* Proterorhinus marmoratus*, using DNA sequences from the left domain of the mitochondrial DNA control region. Samples of round gobies were compared from different sites in the Great Lakes, an introduced population from the Gulf of Gdansk in Poland, and a native population from the northern Black Sea. The round goby was characterized by relatively high genetic variability, and 17 haplotypes were identified from 64 individuals. Levels of genetic variation for the round goby were similar in the invasive and native sampling sites, suggesting relatively large founding populations and lack of bottlenecks. The northern Black Sea was eliminated as a probable founding source for both the Great Lakes and the exotic population in Poland. Substitutions in the left domain of the control region revealed significant differences among samples from the Great Lakes and Eurasia, and between Lakes Erie and St. Clair, suggesting non-random mating. No variation was detected in the tubenose goby population in the Great Lakes, which has been less successful in terms of spread and population growth. A molecular clock calibration suggested that the genera* Neogobius *and* Proterorhinus *diverged about 5.2 ± 1.0 million years ago, apparently separating from a common ancestor shared with* Gobius *during the isolation of the Paratethys basin from the Mediterranean Tethys Sea.*

INDEX WORDS: *DNA, exotic species, founder effect, genetics, Gobiidae, Great Lakes,* Neogobius melanostomus, Proterorhinus marmoratus.

INTRODUCTION

The repeated successes of nonindigenous species in the Great Lakes have had serious ecological impacts, resulting in many permanent changes to the ecosystem (Mills *et al.* 1993, Ricciardi and MacIsaac 2000). Levels of genetic variability in populations of exotic species may be positively correlated with invasive success (Williamson 1996), which was examined in the present study and through other investigations by our laboratory (Stepien *et al.* 1998, Stepien and Dillon in press). Comparisons of levels of genetic variability, spread patterns, and founding sources for exotic species may help in understanding the common factors regulating their relative successes.

In 1990, the Eurasian round *Neogobius melanostomus* and tubenose *Proterorhinus marmoratus* gobies (Teleostei: Gobiidae) were discovered in the St. Clair River (Fig. 1) of the Laurentian Great Lakes (Crossman *et al.* 1992, Jude *et al.* 1992), where they presumably had been introduced from foreign ballast water discharge (Mills *et al.* 1993). The round goby has spread to all five Great Lakes—faster than any previously introduced fish and its population sizes are large in the lower Great Lakes (Fig. 1, Jude *et al.* 1992, Marsden *et al.* 1997). The tubenose goby has not spread to other locations as rapidly, and presently reaches northwestern Lake Erie (Fig. 1, U.S. Geological Survey 2001). This

*Corresponding author and all reprint requests: E-mail: c.stepien@csuohio.edu
[1]Present Address: Athersys, Inc., 3201 Carnegie Ave., Cleveland, Ohio 44115

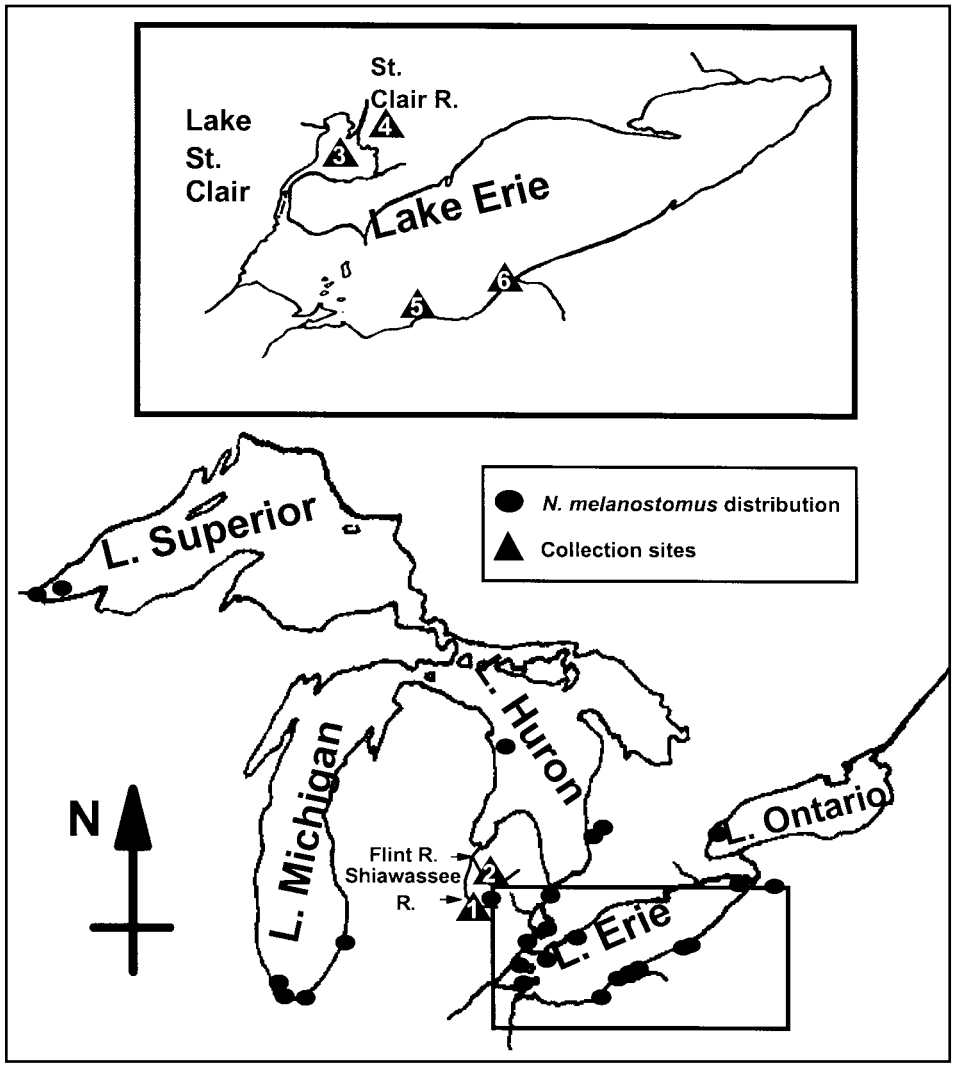

FIG. 1. North American distribution and collection sites for* Neogobius melanostomus *and* Proterorhinus marmoratus*. Sites for* N. melanostomus *are 1 = Shiawassee River, near Linden, Michigan, 2 = Flint River., near Russelville, Michigan, 3 = Lake St. Clair, 5 = Lake Erie, off Avon Point, Ohio, and 6 = Lake Erie, off the Chagrin River, Ohio.* Proterorhinus marmoratus *was collected at sites 3 = Lake St. Clair and 4 = St. Clair River. (Distribution information is from Biological Resources Division, USGS 2001).

study primarily concentrated on the round goby, since it now presents a greater ecological problem.

Systematic and Biogeographic Relationships

The genera *Neogobius* and *Proterorhinus* comprise the subfamily Neogobiinae, which is endemic to the Ponto-Caspian region (including the Marmara, Black, Azov, Caspian, and Aral seas, Fig. 2) (Simonovic *et al.* 1996). The neogobiins have fused pelvic fins and elongated dorsal and anal fins (Miller 1986) and are distinguished from the Atlantic and Mediterranean basin *Gobius* spp. by the absence of a swimbladder and location of the uppermost rays of the pectoral fins within the fin membrane (Pinchuk 1991). The Neogobiinae is hypothesized to have descended from an ancestor

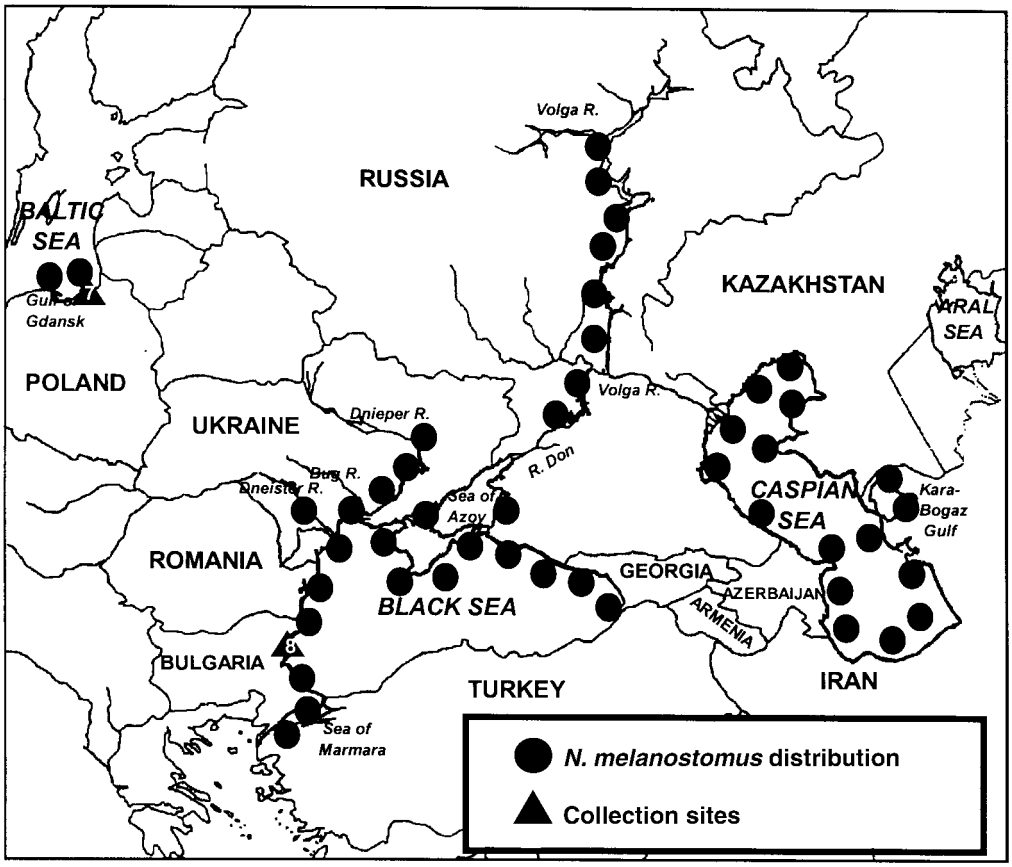

FIG. 2. European collection sites for* Neogobius melanostomus *are numbered 7 and 8. 7 = Gulf of Gdansk, Poland, and 8 = Black Sea, near Varna, Bulgaria. The Ponto-Caspian region includes the Marmara, Black, Azov, Caspian, and Aral seas.

shared with *Gobius*, which either entered the Black Sea basin during Quaternary interglacial connections with the Mediterranean Sea (Stevanovic 1982) or earlier during the mid-Miocene Epoch (McKay and Miller 1991). The monotypic *Proterorhinus* is distinguished from *Neogobius* by its unique long anterior nostrils (Miller 1986). The native distribution of *P. marmoratus* is limited to the Black and Caspian seas, where it is threatened by anthropogenic habitat disturbance (Lelek 1989).

A previous DNA study by Dougherty *et al.* (1996) sequenced 365 base pairs (bp) of the mitochondrial (mt) DNA cytochrome *b* gene from 44 round gobies and two tubenose gobies finding 63 nucleotide differences between the species (p_n = 0.173) and four intraspecific polymorphisms in the former (p_n = 0.011). Cytochrome *b* evolves relatively slowly in fishes (Kocher *et al.* 1989, Stepien and Kocher 1997) and the Dougherty *et al.* (1996) study did not discern enough variation to evaluate differences among sample sites. Dougherty *et al.* (1996) found five haplotypes (one of which was shared among sites), each differing by a single nucleotide, for round gobies in the Black Sea (n = 15, n = 3 haplotypes) and the St. Clair and Detroit rivers (n = 29, n = 3 haplotypes, one shared with the Black Sea). They concluded that more than one female round goby type was introduced to the Great Lakes basin and excluded the northern Black Sea as the founding source (Dougherty *et al.* 1996). The Dougherty *et al.* (1996) samples were made available in this study, allowing a direct comparison of the levels of resolution obtained from the mtDNA control region and cytochrome *b* sequences.

Objectives

The purpose of this investigation was to genetically characterize the exotic round and tubenose goby populations in the Great Lakes using sequences from the left domain of the mtDNA control region. Specific objectives were to: (1) evaluate the possible origin(s) of the founding population(s), (2) compare levels of genetic variation in round gobies from native versus introduced areas of their range, (3) contrast the relative levels and distribution of intra- versus interspecific genetic variation, and (4) provide a data base for evaluating whether future colonization sites are due to spread from nearby locations or new introductions from Eurasia.

Hypotheses for the above objectives (1 to 4) were: (1, A) the exotic round goby population in the North American Great Lakes did/did not originate from the northern Black Sea, (1, B) the exotic population in the Gulf of Gdansk did/did not originate from the northern Black Sea, (1, C) the two introduced populations did/did not share a common origin, (2, A) levels of genetic variability were similar/different in the exotic populations versus the native population sample, (2, B) levels of genetic variability were similar/different between the two introduced populations, (3, A) levels of genetic variability were similar/different between the round and tubenose gobies, (3, B) significant differences occurred/ did not occur among sampling locations of the round goby in North America. Results of this investigation will aid understanding of the relative genetic variability and patterns of dispersal of the gobies, in comparison with other exotic species.

MATERIALS AND METHODS

Samples Collected

The left domain (~750 bp) of the mtDNA control region was sequenced for *N. melanostomus* from five sites in the Great Lakes (Fig. 1), including: two tributaries of Saginaw Bay, Lake Huron; the Shiawassee River (site 1, 45° 15′ N, 84° 05′ W, n = 5) and the Flint River (site 2, 43° 00′ N, 83° 45′ W, n = 5); Lake St. Clair (site 3, 42° 22′ N, 82° 39′ W, n = 10); and two sites in Lake Erie, Avon Point (site 5, 41° 50′ N, 82° 02′ W, n = 10) and the mouth of the Chagrin River (site 6, 41° 44′ N, 81° 25′ W, n = 10). Eurasian sampling locations (Fig. 2) included the Gulf of Gdansk, Poland (site 7, 54° 20′ N, 18° 40′ E, n = 20) and the Black Sea, Bulgaria (site 8, 43° 14′ N, 27° 58′ E, n = 15). Twelve *P. marmoratus* were analyzed from the St. Clair River (site 4, 42° 45′ N, 82° 25′ W, n = 11) and Lake St. Clair (site 3, n = 1) in the Great Lakes (Fig. 1). Samples were stored frozen at -80°C or in 95% ethanol at room temperature.

DNA Extraction, Amplification, and Sequencing

Methodology for extracting and purifying DNA from the samples followed Stepien (1995). The left domain of the control region, extending from the tRNA-proline gene to the central conserved section (Fig. 3), was amplified using the polymerase chain reaction (PCR) with the oligonucleotide primers L16007 (5′-CCC-AAA-GCT-AAA-ATT-CTA-A-3′, Kocher *et al.* 1989) and H16498 (5′-CCT-GAA-GTA-GGA-ACC-AGA-TG-3′, Meyer *et al.* 1990) and then sequenced according to Stepien (1995).

Data Analysis

Proportions of polymorphic nucleotides (p_n) were calculated according to Nei (1987). Population genetic analyses were conducted in two ways: (1) by analyzing evolutionary relationships among haplotype sequences using genetic distance and cladistic phylogenetic trees, and (2) by comparing levels of genetic variability within and among sampling sites, and testing for geographic heterogeneity (Ferris and Berg 1987, Weir 1996).

The first approach included maximum parsimony in PAUP* 4.0 (Phylogenetic Analysis Using Parsimony, Swofford 2001) and neighbor-joining (NJ) genetic distance analyses (Saitou and Nei 1987) for 651 bp (bases 90 to 740). Parsimony analyses employed the branch-and-bound algorithm (Hendy and Penny 1982) and 50% majority rule consensus of the most parsimonious trees (Margush and McMorris 1981). Kimura's (1980) two-parameter and pairwise (p-) genetic distances (Nei 1987), their standard errors, and neighbor-joining (NJ) trees were calculated with MEGA (Molecular Evolutionary Genetics Analysis, vers. 1.01, Kumar *et al.* 1993). Kimura's (1980) two-parameter distances were used to correct for the unequal rate of transitional versus transversional substitutions that occur in animal mtDNA sequences (Kocher and Carlton 1997). Pairwise distances were used to estimate possible divergence times, using a hypothesized rate of sequence divergence of 2% per million years (reviewed by Avise 2000). This rate is slower than that of mammals (Avise 2000) but average for fishes, whose mtDNA evolves more slowly appar-

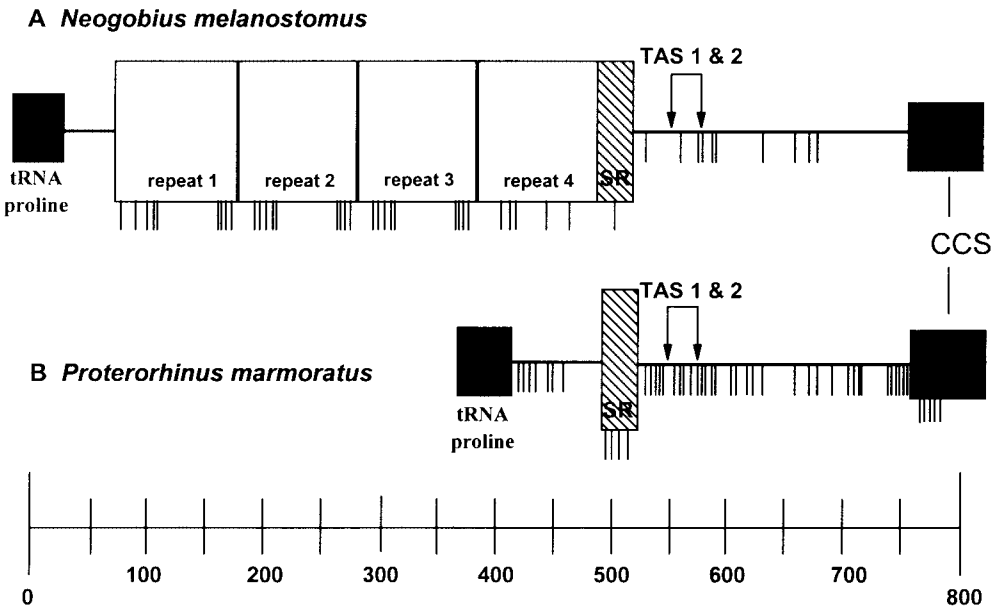

FIG. 3. A schematic diagram of the left domain of the control region for (A)* Neogobius melanostomus *and (B)* Proterorhinus marmoratus. *Locations of the repeated sequences, short repeat (SR), termination associated sequences (TAS), and the central conserved section (CCS) are shown. Vertical lines: A = intraspecific substitutions, B = interspecific differences.

ently due to poikilothermy (Bernatchez *et al.* 1992, Bernatchez and Danzmann 1993, Stepien and Kocher 1997). Bootstrap support values were determined from 1,000 permutations for the nodes of the distance and parsimony trees.

The second approach compared the relative frequencies of the haplotypes and their geographic divergences, using haplotype diversity (*h*, which is equivalent to heterozygosity, Nei and Tajima 1983) and maximum-likelihood analysis within (nucleotide diversity—Π, Nei 1987) and among sampling locations (nucleotide divergence—*d*xy, Nei and Tajima 1983) from the DA2 program in REAP (the Restriction Enzyme Analysis Package, vers. 5.0, McElroy *et al.* 1992).

Phylogenetic utility of the repeated sequence arrays versus the unique sequences (see Faber and Stepien 1998) were compared through separate analyses of: (1) the entire data set (17 haplotypes), (2) the data subset of the repeated arrays (11 haplotypes for nucleotide positions 090 to 595), and (3) the data subset comprising the unique sequences (11 haplotypes for nucleotide positions 000 to 089 and 596 to 733). The purpose was to test whether the repeated and the unique sequence regions revealed similar results and to compare their relative levels of phylogenetic resolution.

Frequency differences in the geographic distributions of the haplotypes also were analyzed with modified chi-square tests, with a Monte Carlo simulation approach with 10,000 randomizations (Roff and Bentzen 1989) and the MONTE program in REAP (McElroy *et al.* 1992). A Bonferroni correction that divided the *P*-value by the number of pairwise comparisons was used for the multiple post-hoc tests (Fry *et al.* 1993).

Hierarchical AMOVA (Analysis of MOlecular VAriance, vers. 1.53, Excoffier *et al.* 1992, Excoffier 1995) analyses, based on Euclidean distances among pairs of haplotypes and their relative frequencies, tested the partitioning of variation between regions (North America and Europe), and among sampling sites. Significance of the φ_{ST} (F_{ST} analog) values was determined with 1,000 random permutations (Excoffier *et al.* 1992).

Tajima's (1989) test of selective neutrality based on the infinite-site model was performed using the Arlequin software package (vers. 1.1, Schneider *et al.* 1997). This test evaluated whether the observed number of polymorphic sites and the average num-

ber of pairwise nucleotide differences deviated significantly from theoretically predicted values (Tajima 1989).

RESULTS

Structure and Substitutions in the Left Domain of the mtDNA Control Region

A single haplotype discerned for *P. marmoratus* and the five most common *N. melanostomus* haplotypes (aa, cc, dd, ee, and gg) were reported in GenBank (accession numbers AF082969 to AF082974). No intraspecific variation was found in *P. marmoratus* (n = 12).

Locations of the inter- and intraspecific substitutions were mapped on a structural diagram of the left domain for the mtDNA control region (Fig. 3).

Two putative Termination Associated Sequences (TAS, Doda *et al.* 1981, Foran *et al.* 1988) were identified in each species. The region immediately flanking the 5′ tRNA-proline end (bases 1 to 89) differed in sequence and length between the species, and was omitted in all analyses due to alignment uncertainty. The left domain of *N. melanostomus* was approximately 400 bp longer than that of *P. marmoratus*, resulting from four nonidentical tandemly repeated sequences (Table 1, Fig. 3). Faber and Stepien 1998 discuss tandem repeats in the mtDNA control region of fishes. A fifth shortened version (26 bp) of the repeated sequence immediately followed, and also occurred in *P. marmoratus* (Fig. 3).

Interspecific Differences

The species *N. melanostomus* and *P. marmoratus* differed by 26 transitional (S) and 23 transversional (V) substitutions within 306 bp of aligned sequence data (excluding the four repeats, S:V ratio = 1.13). They also diverged by nine insertion/deletions encompassing a total of 50 nucleotide positions. Mean genetic distances between the two species were Kimura (1980) two parameter d = 0.112 ± 0.024 and pairwise p = 0.104 ± 0.020.

Intraspecific Variation in the Round Goby *N. melanostomus*

Round goby haplotypes varied in both the repeated array (Table 1) and the unique sequences of the mtDNA control region (Table 2). Sequence similarity among the repeated variants (lettered A through K) alone ranged from 87% to 99%. Thirteen transitions (S), four transversions (V), and a two-base insertion occurred within the four tandem repeats (S:V ratio = 3.25, Table 1).

The unique sequences differed by eight transitions (S), two transversions (V), and a single insertion (S:V ratio = 4.0). Most of this variation (10 of 11 polymorphic sites) occurred in the Eurasian samples (Table 2). Two of the transitions (bases 575 and 588) occurred in single individuals sampled from the Black Sea (Table 2). Nucleotide position 671 was fixed in both of the Eurasian populations (G), and a transversion (53% T - 47% G) distinguished some North American samples.

The complete data set, including repeated and non-repeated information, revealed 17 round goby haplotypes and 43 polymorphic nucleotide sites (p_n = 0.07, S:V ratio = 13.0; Table 2, Fig. 3). Nucleotide polymorphism within the four repeats (p_n = 0.08) was higher than in the non-repetitive regions (p_n = 0.05). Intrasite polymorphisms were detected in five of the seven sampling locations (the Shiawassee and Flint rivers of Lake Huron were monomorphic). Nucleotide polymorphism (p_n), haplotype diversity (*h*), and nucleotide diversity (Π) values (Table 3) were largest in the native population from the Black Sea (p_n = 0.05, *h* = 0.96 ± 0.04) and the Lake St. Clair sampling site (Π = 0.010 ± 0.007). Haplotype diversity (*h*) averaged 0.46 ± 0.02 overall. Nucleotide divergences (*d*xy) between pairs of sampling sites ranged from 0.0002 (between Poland and the Black Sea) to 0.0185 (between Poland and Lake Huron). Within the three Great Lakes, *d*xy was smallest between Lakes Huron and St. Clair (0.0009), intermediate between Lakes Erie and St. Clair (0.0015), and larger between Lakes Erie and Huron (0.0060).

Variance components and associated φ statistics showed significant hierarchial geographic partitioning of genetic variation (Table 4, Excoffier *et al.* 1995). Variation between the North American and Eurasian regions was similar to that found among sampling sites within regions. Modified chi-square tests (Roff and Bentzen 1989, McElroy *et al.* 1992) also revealed significant differences in the distributions of the haplotypes between North America and Eurasia and among the five sampling sites overall (Table 5). Pairwise comparisons showed a significant difference between samples from Lakes Erie and St. Clair.

Tajima's (1989) test supported selective neutrality (*D* = –0.23, *P* = 0.43) when all samples of round gobies were grouped as a single population. When the sample sites were analyzed separately, selective

TABLE 1. ***Alignment of repeated sequences (coded A through K) identified in the left domain of*** **Neogobius melanostomus** ***mtDNA control region sequences. Dots denote conserved nucleotides that are identical to those shown in haplotype aa. - = nucleotide deletion. Variable-base positions among types of repeats are lettered. Substitutions are labeled as S = transition, V = transversion, and I = insertion/deletion.***

```
                          *                                *                                *
A ATATATGTATTATCACCATTAATAGACTTTAACCATTCAAGAGTACATAT--ATATATGTTTTATCAACATATATATATATTAACCATATATAATAATGTTCTAGGAC
B .....................C.............G.G.....--.....................................................
C ...............................T.............--.....................................................
D .........C.....................................--.....................................................
E ...............................G.............--.....................................................
F ...............................G.............--.....................................C.T..A..T
G .....................................................--.....................................C.T..A..T
H .....................................................AT..GA....AC.................A.............C.T..A..T
I .....................................................AT..GA....ACC................A.............C.T..A..T
J .....................................................AT..GA....AC.................GA............C.T..A..T
K .....................C.............G.G.....AT..GA....AC.................A.............C.T..A..T
          S           S     S S     S S       I   SV   VSS                VV              S S  S  S
```

TABLE 2. Haplotype data for intraspecific variation in the mtDNA control region of* Neogobius melanostomus. *Dots denote conserved nucleotides that are identical to those in haplotype aa. Variable nucleotide positions are lettered. n = the frequency of each haplotype.

		Nucleotide position											Repeats				Distribution frequency						
Haplotype	n	528	539	575	588	589	595	599	639	662	672	679	1	2	3	4	Point	Avon River	Chagrin River	Shiawassee River	Flint St. Clair	Lake Poland	Black Sea
aa	10	C	-	T	T	A	T	C	A	G	T	C	A	A	F	I	1	8	0	0	1	0	0
bb	3	.	-	.	.	.	.	.	.	.	.	.	A	E	F	I	1	2	0	0	0	0	0
cc	8	.	-	.	.	.	.	.	.	.	.	.	A	F	F	I	0	0	0	5	3	0	0
dd	10	.	-	.	.	.	.	.	.	.	G	.	G	G	G	I	2	0	5	0	3	0	0
ee	7	.	-	.	.	.	.	.	.	.	G	.	A	A	G	H	6	0	0	0	1	0	0
ff	4	.	-	.	.	.	.	.	.	.	G	.	A	A	A	H	0	0	0	0	2	0	2
gg	8	.	-	.	.	.	.	.	.	.	G	T	A	A	A	H	0	0	0	0	0	7	1
hh	3	T	T	.	.	.	.	.	T	A	G	T	D	A	A	H	0	0	0	0	0	3	0
ii	2	.	-	.	.	G	.	.	T	.	G	T	A	A	A	H	0	0	0	0	0	0	2
jj	2	.	-	.	.	.	.	T	.	.	G	T	A	A	A	H	0	0	0	0	0	0	2
kk	1	.	-	.	.	.	C	.	.	.	G	T	C	C	C	H	0	0	0	0	0	1	0
ll	1	.	-	.	C	.	.	.	.	.	G	.	B	B	B	K	0	0	0	0	0	0	1
mm	1	T	-	C	.	.	C	.	T	A	G	T	A	A	A	H	0	0	0	0	0	0	1
nn	1	.	-	.	.	.	.	.	T	.	G	T	A	A	A	J	0	0	0	0	0	0	1
oo	1	.	-	.	.	.	.	.	T	.	G	T	A	A	A	H	0	0	0	0	0	0	1
pp	1	.	-	.	.	.	.	.	T	A	G	T	A	A	A	H	0	0	0	0	0	0	1
qq	1	.	-	.	.	.	.	.	.	.	G	.	A	G	G	H	0	0	0	0	0	0	1
Total	64																10	10	5	5	10	11	13

TABLE 3. Nucleotide polymorphism (p_n), haplotype diversity ($h \pm SE$), and nucleotide diversity ($\Pi \pm SE$) of* Neogobius melanostomus *within sampling sites (REAP vers. 2.0, McElroy* et al. *1992)

Sampling location	p_n	$h \pm$ SE	$\Pi \pm$ SE
Avon Point	0.018	0.644 ± 0.152	0.007 ± 0.005
Chagrin River	0.002	0.356 ± 0.159	0.001 ± 0.001
Shiawassee River	0.000	0.000 ± 0.000	0.000 ± 0.000
Flint River	0.000	0.000 ± 0.000	0.000 ± 0.000
Lake St. Clair	0.025	0.844 ± 0.080	0.012 ± 0.007
Poland	0.014	0.564 ± 0.134	0.004 ± 0.003
Black Sea	0.046	0.962 ± 0.041	0.009 ± 0.004

TABLE 4. Hierarchical analyses of molecular variance among haplotypes of* N. melanostomus *(AMOVA, Excoffier* et al. *1992). * = significant. (A) Values based on the entire sequence data, (B) Values using haplotypes from the repeated sequence region, (C) Values using haplotypes from the nonrepeated sequences.

Variance component		Variance	% Total variance	Φ statistic	P
		(A) Entire Data Set			
Between regions	Φ_a^2	1.04	40.7%	$\Phi_{ct} = 0.41$	< 0.001*
Among sampling sites	Φ_b^2	0.55	21.7%	$\Phi_{sc} = 0.37$	< 0.001*
Within sampling sites	Φ_c^2	0.96	37.6%	$\Phi_{st} = 0.62$	< 0.001*
		(B) Haplotypes from the tandem repeat region			
Between regions	Φ_a^2	0.47	33.8%	$\Phi_{ct} = 0.34$	< 0.001*
Among sampling sites	Φ_b^2	0.38	27.2%	$\Phi_{sc} = 0.41$	< 0.001*
Within sampling sites	Φ_c^2	0.55	39.0%	$\Phi_{st} = 0.61$	< 0.001*
		(C) Haplotypes from the unique sequences			
Between regions	Φ_a^2	0.56	48.0%	$\Phi_{ct} = 0.48$	< 0.001*
Among sampling sites	Φ_b^2	0.10	08.8%	$\Phi_{sc} = 0.17$	< 0.001*
Within sampling sites	Φ_c^2	0.51	43.2%	$\Phi_{st} = 0.57$	< 0.001*

TABLE 5. Modified chi-square values from Monte Carlo tests (Roff and Bentzen 1989) for the distribution of mtDNA control region sequence haplotypes of* N. melanostomus *(REAP vers. 2.0, McElroy* et al. *1992). * = significant, using a Bonferroni correction for multiple post-hoc tests (Fry* et al. *1993).

Locations Compared	χ^2	*P*
Between regions (North America and Eurasia)	59.7	< 0.001*
Among five sampling sites (two Eurasian populations and Lakes Erie, Huron, and St. Clair)	159.9	< 0.001*
Among Lakes Erie, Huron, and St. Clair	32.9	< 0.001*
Between Lakes Erie and St. Clair	23.6	< 0.001*
Between Lakes Erie and Huron	16.7	< 0.001*
Between Lakes St. Clair and Huron	5.0	0.198

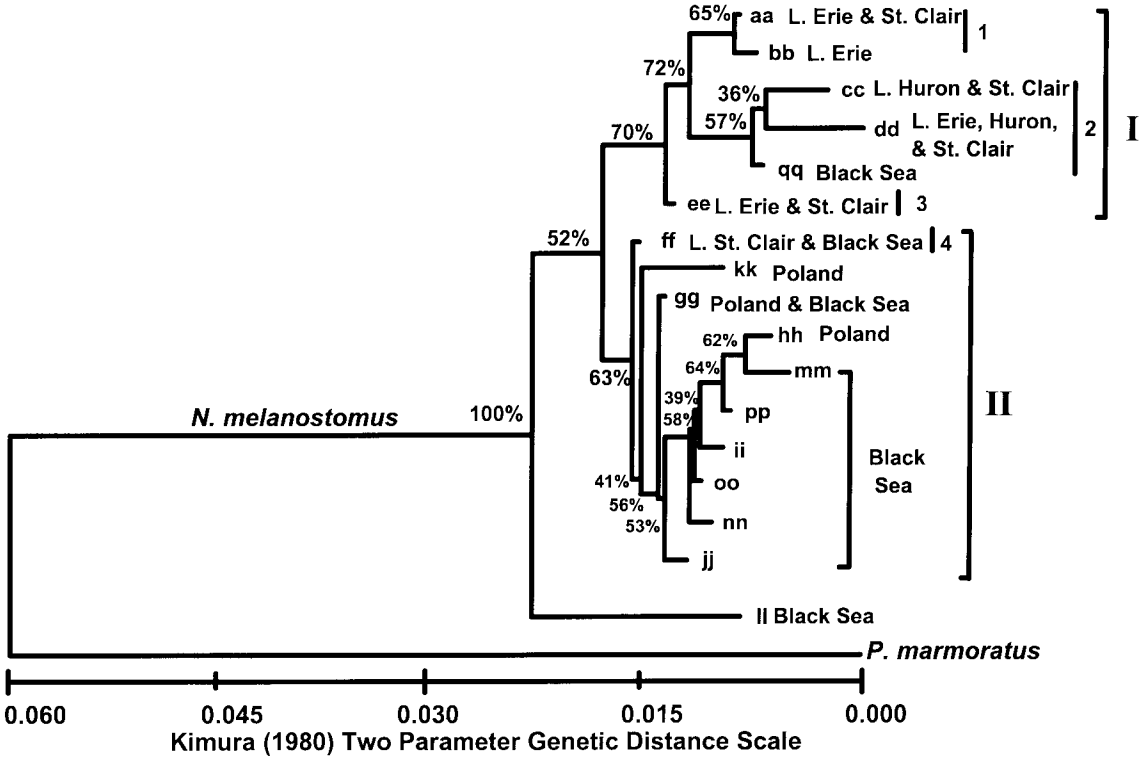

FIG. 4. Neighbor-joining tree for haplotypes of* Neogobius melanostomus *using Kimura (1980) two-parameter distances from the computer program MEGA 1.01 (Kumar* et al. *1993). Genetic distances may be calculated by adding horizontal branch lengths between pairs. Percentages indicate bootstrap support for nodes computed from 1,000 replicates. The major types present in the Great Lakes are labeled 1–4, and the primary groups are denoted as I and II (see Results). This tree was congruent with results from maximum parsimony analysis using PAUP* 4.0 (Swofford 2001).

neutrality also was supported in four of the five locations (Lake Erie, $D = -0.16$, $P = 0.46$; Lake Huron, $D = 2.42$, $P = 0.99$; Lake St. Clair, $D = 1.45$, $P = 0.91$; and Poland, $D = 0.27$, $P = 0.61$). There was a deviation in the sample from the Black Sea ($D = -1.80$, $P = 0.02$).

Genetic Divergences

Kimura's (1980) two-parameter and p-distances among round goby haplotypes were similar, with overlapping standard errors. The NJ tree (Fig. 4) showed that haplotype ll from the Black Sea was basal to all other round gobies, due to its unique repeat sequences (Tables 1 and 2). The remaining haplotypes formed two groups, one containing the majority of the North American haplotypes (83% of the haplotypes and 97% of the individuals, designated as I), and the other containing mainly Eurasian types (90% exclusively Eurasian haplotypes and 92% of the Eurasian individuals, designated as II; Fig. 4). Group I contained three major types, including two from Lakes Erie and St. Clair (type 1, haplotypes aa and bb; type 3, haplotype ee) and another found in Lakes Erie, Huron, and St. Clair (type 2, haplotypes cc and dd). One Black Sea haplotype (qq) clustered with the North American type 3 haplotypes. Type 4 in Lake St. Clair also was identified in the Black Sea, and was the sister taxon to a cluster of exclusively Eurasian haplotypes.

Genetic distances among the haplotypes overall ranged from d = 0.002 to 0.042, averaging 0.014 ± 0.005. Haplotypes in group I (Fig. 4) were separated by an average genetic d = 0.009 ± 0.004, and group II by d = 0.006 ± 0.003. The four types identified in the North American population (Fig. 4) diverged by a mean d = 0.011 ± 0.004. Maximum parsimony analysis with PAUP* 4.0 (Swofford

2000) found 12 most parsimonious trees, having 497 steps and consistency indices (excluding uninformative characters) of 0.86. The 50% majority rule consensus tree was congruent with the NJ tree (Fig. 4).

DISCUSSION

Interspecific Divergences and Biogeography

It has been hypothesized that the *Gobius* (represented by the outgroup in this study) and the neogobiin (*Proterorhinus* and *Neogobius*) lineages diverged due to the isolation of the Paratethys from the Mediterranean Tethys Sea basins, about 10 to 12 million years ago (Steininger and Rogl 1984). Congruent with this hypothesis, genetic divergences in this study suggest that the neogobiins diverged from the *Gobius* lineage during the mid-Miocene, and then speciated into *Neogobius* and *Proterorhinus* during the late Miocene/early Pliocene Epochs (an average sequence p-distance of 0.104 ± 0.020, corresponding to an estimated 5.2 ± 1.0 million years). These results refute the hypothesis that the neogobiins diverged from *Gobius* later during Quaternary interglacial connections between the basins of the Black and Mediterranean seas (Stevanovic 1982).

Population Divergences and Invasion Biogeography

Polymorphisms in the mtDNA control region of the round goby fit a selective neutrality hypothesis. A single deviation found in the Black Sea population may be due to sampling error and small sample size, lack of random mating, or selection (Tajima 1989). Levels of haplotypic and nucleotide diversity appeared similar overall in samples from Lake St. Clair (the North American founding population) and a native population from the Black Sea, indicating that the round goby introduction may have been relatively large and did not undergo a genetic bottleneck (a marked reduction in population size). Dougherty *et al.* (1996) also found that samples from the Great Lakes and the Black Sea housed similar levels of genetic diversity in the mtDNA cytochrome *b* gene—although that gene was less variable overall than the control region. In this study, the Lake St. Clair sample was less variable in the unique sequences and more diverse in the repeated array, compared with the Black Sea population. In contrast, the exotic population found in the Gulf of Gdansk of the Baltic Sea, Poland was less genetically variable, suggesting a possible founder effect or a less diverse source population. The Gulf of Gdansk population was identified in 1990 (the same year as the appearance in North America) and presumably was introduced through ballast water discharge from ships originating in the Black or Caspian Seas (Skora and Stolarski 1993).

Only one mtDNA haplotype was shared between the samples from Lake St. Clair and the Black Sea, indicating that the North American population did not originate from this region of the northern Black Sea. This corroborates the results of Dougherty *et al.* (1996), who likewise found little similarity between samples from the Great Lakes and the northern Black Sea. In this study, only a single haplotype (not found in the Great Lakes) was shared between the Black Sea and the Gulf of Gdansk population, indicating that the Black Sea also was not its founding source. The exotic populations in the Gulf of Gdansk and the Great Lakes apparently were founded by independent sources, since they shared no haplotypes.

The presence of four divergent groupings of haplotypes in the North American Great Lakes suggests that the introduction was founded by a large number of individuals from one or more unknown source populations. Estimates suggest the haplotype groups differentiated during the mid-Pleistocene, presumably due to geographic isolation in Eurasian glacial refugia (Hewitt 1996, 2000; Stepien *et al.* 1998). Other freshwater fishes in Europe exhibit similar levels and patterns of genetic divergences related to glacial refugia (Bernatchez and Osinov 1995, Haenfling and Brandl 1998, Stepien *et al.* 1998). Differences among sites in the Great Lakes are consistent with the possibilities of multiple founding sources, low gene flow among sites, and differential lineage extinction.

All mtDNA haplotypes from Lakes Erie and Huron also were present in the Lake St. Clair site, except for one (haplotype bb, n = 3, Lake Erie). The presence of a unique haplotype in Lake Erie may have been the result of an independent introduction or to sampling error. Five of the six Great Lakes haplotypes were found in Lake St. Clair fish, suggesting that populations in the other sites were the result of intra- and inter-lake spread. Sampling locations in Lakes Erie and Huron (presumably areas of spread) had fewer haplotypes and lower haplotypic diversity, possibly due to founder effects. The two tributaries of Saginaw Bay, Lake Huron each contained a single haplotype, suggesting that they were founded by different genetic types or experienced differential lineage extinction. It has been hy-

pothesized that round gobies from Lake Huron tributaries (Flint and Shiawasse rivers) may have originated from transport of bait fish from Lake St. Clair (Jude 2000). The results of this study were consistent with the hypothesis of separate bait-fish introductions (Jude 2000), as both genetic types were found in Lake St. Clair.

In contrast to the cytochrome *b* study by Dougherty *et al.* (1996), mtDNA control region sequences revealed greater variability and significant differences among sampling locations of the round goby. Both the repeated and nonrepeated mtDNA control region sequences revealed phylogeographic utility and yielded congruent relationships. This study found significant differences among samples from Lakes Erie and St. Clair, which may suggest barriers to gene flow or non-random mating due to localized spawning sites. Lack of a pelagic larval stage (Marsden *et al.* 1997) may limit gene flow among sites by limiting dispersal opportunities. Male round gobies are aggressive, actively recruit mates, and defend the nest (Jude 1997). Reports indicate that males reproduce only once at a large size (about age 3), do not feed during nest-guarding, and then may die (Marsden *et al.* 1997). These life history features indicate highly selective mate choice and substantial fitness investment per reproduction. These characteristics also would tend to preserve local population genetic differences and limit gene flow over geographic distances.

All tubenose gobies surveyed from the St. Clair region were a single haplotype, suggesting that the source population had low genetic variability or the founding population was small. Investigations of native Eurasian tubenose goby populations are necessary to address this question.

The round goby has undergone a more rapid expansion in the Great Lakes and has a higher level of genetic variability than the tubenose goby. Differences in relative success between the two species also may be related to the larger size and more aggressive nature of the round goby (Jude *et al.* 1995, Marsden *et al.* 1997). Expansion of the round goby population may be augmented by the abundance of its zebra mussel prey, for which there are few competitors (Jude 1997). The smaller tubenose goby primarily consumes smaller prey, including aquatic insects and benthic zooplankton, for which there are more native competitors (Jude *et al.* 1995). This study provides a data base that will allow populations of the round and tubenose gobies to be genetically monitored, in order to elucidate whether additional introductions occur and evaluate their future patterns of spread.

ACKNOWLEDGMENTS

This study was supported by the Ohio Sea Grant Program development fund #RF-729753 (1997–98), and the Lake Erie Protection Fund #SG 50-97 (1997–98) to C. Stepien. This project was part of the Environmental Risk Analysis Program at Cleveland State University's Center for Environmental Science, Technology and Policy. Specimens were provided by the Michigan Department of Natural Resources (B. Haas and M. Thomas), the Ohio Division of Wildlife (C. Knight and K. Kayle), D. Jude, J. Ram, S. Rudnicka, P. Simonovich, and M. Sapoto.

REFERENCES

Avise, J.C. 2000. *Phylogeography: The history and formation of species.* Cambridge: Harvard Univ. Press.

Bernatchez, L., and Danzmann, R.G. 1993. Intraspecific DNA sequence variation of the mitochondrial DNA of brook char (*Salvelinus fontinalis* Mitchill). *Mol. Biol. Evol.* 10:1002–1014.

———, and Osinov, A. 1995. Genetic diversity of trout (genus *Salmo*) from its most eastern native range based on mitochondrial DNA and nuclear gene variation. *Mol. Ecol.* 4:285–297.

———, Guyomard, R., and Bonhomme, F. 1992. DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Mol. Ecol.* 1:161–173.

Crossman, E.J., Holm, E., Cholmondeley, R., and Tuininga, K. 1992. First record for Canada of the rudd, *Scardinius erythrophthalmus*, and notes on the introduced round goby, *Neogobius melanostomus*. *The Canadian Field-Naturalist* 106:206–209.

Doda, J.N., Wright, C.T., and Clayton, D.A. 1981. Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. *Proc. Nat. Acad. Sci. USA* 78:6116–6120.

Dougherty, J.D., Moore, W.S., and Ram, J.L. 1996. Mitochondrial DNA analysis of round goby (*Neogobius melanostomus*) and tubenose goby (*Proterorhinus marmoratus*) in the Great Lakes basin. *Can. J. Fish. Aquat. Sci.* 53:474–480.

Excoffier, L. 1995. *AMOVA, Analysis of molecular variance*, vers. 1.55. Geneva, Switzerland: Univ. of Geneva.

———, Smouse, P.E., and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances

among DNA haplotypes: application to human mitochondrial DNA data. *Genetics* 131:479–491.

Faber, J.E., and Stepien, C.A. 1998. Tandemly repeated sequences in the mitochondrial DNA control region and phylogeography of the pike-perches *Stizostedion*. *Mol. Phyl. Evol.* 10:310–322.

Ferris, S.D., and Berg, W.J. 1987. The utility of mitochondrial DNA in fish genetics and fishery management. In *Population genetics and fishery management*, eds. N. Ryman and F. Utter, pp. 277–299. Seattle, WA: Univ. of Washington Press.

Foran, D.R., Hixson, J.E., and Brown, W.M. 1988. Comparison of ape and human sequences that regulate mitochondrial DNA transcription and D-loop DNA synthesis. *Nucleic Acids Res.* 16:5841–5861.

Fry, J.C., Iles, T.C., Randerson, P.F., Bridge, P.D., Dunstan, F.D.J., Bowker, D.W., and Weingert, R.G. 1993. *Biological data analysis: a practical approach*. New York, NY: Oxford Univ. Press.

Haenfling, B., and Brandl, R. 1998. Genetic differentiation of the bullhead *Cottus gobio* L. across watersheds in Central Europe: evidence for two taxa. *Heredity* 80:110–117.

Hendy, M.D., and Penny, D. 1982. Branch and bound algorithms to determine minimal evolutionary trees. *Math. Biosci.* 59:277–290.

Hewitt, G.M. 1996. Some genetic consequences of Ice Ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* 58:247–27.

_____. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405:907–915.

Jude, D.J. 1997. Round gobies: Cyberfish of the third millennium. *Great Lakes Res. Rev.* 3:27–34.

_____. 2000. Round and tubenose gobies: 10 years with the latest Great Lakes phantom menace. *Dreissena! The Digest of the National Aquatic Nuisance Clearinghouse* 11(4): 1–13.

_____, Reider, R.H., and Smith, R. 1992. Establishment of Gobiidae in the Great Lakes basin. *Can. J. Fish. Aquat. Sci.* 49:416–421.

_____, Janssen, J., and Crawford, G. 1995. Ecology, distribution, and impact of the newly introduced round and tubenose gobies in the biota of the St. Clair and Detroit Rivers. In *The Lake Huron Ecosystem: Ecology, Fisheries and Management*, eds. M. Munawar, T. Edsall, and J. Leach, pp. 447–460. Amsterdam: SPB Academic Publishing.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111–120.

Kocher, T. D., and Carlton, K. L. 1997. Base substitution in fish mitochondrial DNA: patterns and rates. In *Molecular Systematics of Fishes*, eds. T. D. Kocher and C. A. Stepien, pp. 13–24. San Diego, CA: Academic Press.

_____, Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., and Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Nat. Acad. Sci. USA* 86:6196–6200.

Kumar, S., Tamura, K., and Nei, M. 1993. *MEGA: Molecular Evolutionary Genetics Analysis*, version 1.02. University Park, PA: Institute of Molecular Evolutionary Genetics, Pennsylvania State University.

Lelek, A. 1989. Threatened fishes of Europe, volume 9. In *The Freshwater Fishes of Europe*, eds. Council of Europe: European Committee for the conservation of nature and natural resources. Wiesbaden: AULA Verlag.

Margush, T., and McMorris, F.R. 1981. Consensus n-trees. *Bull. Math. Biol.* 42:239–244.

Marsden, J.E., Charlebois, P., Wolfe, K., Jude, D., and Rudnicka, S. 1997. The round goby (*Neogobius melanostomus*): a review of European and North American literature with notes from the Round Goby Conference, Chicago. *Illinois Natural History Survey. Special Pub.* No. 20, Champaign, IL.

McElroy, D.P., Moran, P., Bermingham, P.E., and Kornfield, I. 1992. REAP: An integrated environment for the manipulation and phylogenetic analysis of restriction data. *J. Hered.* 83:157–158.

McKay, S.I., and Miller, P.J. 1991. Isozyme criteria in the testing of phyletic relationships between species of *Gobius* and related eastern Atlantic-Mediterranean genera (Teleostei: Gobiidae). *J. Fish Biol*. 39 (Suppl. A):291–299.

Meyer, A., Kocher, T.D., Basatbwaki, P., and Wilson, A.C. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature* 347:550–553.

Miller, P.J. 1986. Gobiidae, p. 1019–1095. In *Fishes of the Northeast Atlantic and Mediterranean*, eds. P.J. P. Whitehead, M.L. Bauchot, J.C. Hureau, J. Nielsen, and E. Tortonese. Paris: UNESCO.

Mills, E.L., Leach, J.H., Carlton, J.T., and Secor, C.L. 1993. Exotic species in the Great Lakes: a history of biotic crises and anthropogenic introductions. *J. Great Lakes Res.* 19:1–54.

Nei, M. 1987. *Molecular Evolutionary Genetics*. New York, N.Y.: Columbia Univ. Press.

_____, and Tajima, F. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction-site data. *Genetics* 105:207–217.

Pinchuk, V.I. 1991. Kvoprosy grupirovkakh vidov v predelakh roda *Neogobius* (Perciformes) (About speciation within the genus *Neogobius* (Perciformes)). *Voprosy Ikhtiologii* 31:380–393.

Ricciardi, A., and H.J. MacIsaac. 2000. Recent mass invasion of the North American Great Lakes by Ponto-Caspian species. TREE 15:62–65.

Roff, D.A., and Bentzen, P. 1989. The statistical analysis of mitochondrial DNA polymorphisms: X^2 and the problem of small samples. *Mol. Biol. Evol.* 6:539–545.

Saitou, N., and Nei, M. 1987. The neighbor joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.

Schneider, S., Kueffer, J., Roessli, D., and Excoffier, L. 1997. *Arlequin vers. 1.1: a software for population genetic data analysis.* Univ. of Geneva, Switzerland: Genetics and Biometry Laboratory

Simonovic, P.D., Nikolic, V.P., and Skora, K.E. 1996. Vertebral number in Ponto-Caspian gobies: phylogenetic relevance. *J. Fish Biol.* 49:1027–1029.

Skora, K.E., and Stolarski, J. 1993. New fish species in the Gulf of Gdansk, *Neogobius* spp. [cf. *Neogobius melanostomus* (Pallas 1811)]. *Bull. Sea Fish. Inst.* 1:83.

Steininger, F.F., and Rogl, R. 1984. Paleogeography and palinspastic reconstruction of the Neogene of the Mediterranean and Paratethys, In *The Geological Evolution of the eastern Mediterranean,* eds. J.E. Dixon and A.H.F. Robertson, pp. 659–686. Oxford: Blackwell Sci. Pub., Inc.

Stepien, C.A. 1995. Population genetic divergence and geographic patterns from DNA sequences: examples from marine and freshwater fishes, In *Evolution and the Aquatic Ecosystem: Defining Unique Units in Population Conservation,* ed., J.L. Nielsen, pp. 263–287. Bethesda, MD: American Fisheries Society, Symposium 17.

———, and Dillon, A.K. 2001. Phylogeography of the invasive ruffe *Gymnocephalus cernuus* (Percidae): glacial refugia, the leading edge hypothesis, and exotic introductions. *Evolution* in press.

———, and Kocher, T.D. 1997. Molecules and morphology in studies of fish evolution,. In *Molecular Systematics of Fishes,* eds. T.D. Kocher and C.A. Stepien, pp. 1–11. San Diego, CA: Academic Press.

———, Dillon, A.K., and Chandler, M.D. 1998. Genetic identity, phylogeography, and systematics of ruffe *Gymnocephalus* in the North American Great Lakes. *J. Great Lakes Res.* 24:361–378.

Stevanovic, P.M. 1982. *Istorijska Geologija-Kenozoik (Geological History-Cenozoic).* Belgrade: Univ. of Belgrade.

Swofford, D.L. 2001. *Phylogenetic analysis using parsimony (PAUP)* 4.0*. vers. b5. Sunderland, Massachusetts: Sinauer Assoc., Inc.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:858–595.

U. S. Geological Survey (Biological Resources Division). 2001. Online (range maps). http://nas.er.usgs.gov/fishes/images/goby_map.gif (round goby) http://nas.er.usgs.gov/fishes/images/pr_marmo.gif (tubenose goby)

Weir, B.S. 1996. Genetic analysis II: methods for discrete population genetic data. In *Molecular Systematics, 2nd ed.,* eds. D.M. Hillis, C. Moritz, and B.K. Mable, pp. 385–405. Sunderland, MA: Sinauer Assoc., Inc.

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

Submitted: 16 January 2000
Accepted: 14 December 2000
Editorial handling: David J. Jude