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ALLOPATRIC ORIGIN OF SYMPATRIC POPULATIONS OF LAKE WHITEFISH (*COREGONUS CLUPEAFORMIS*) AS REVEALED BY MITOCHONDRIAL-DNA RESTRICTION ANALYSIS[1]

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Abstract.—In the paper, restriction-fragment length polymorphisms in mitochondrial DNA (mtDNA) were studied to test the hypothesis that sympatric populations of lake whitefish in the Allegash basin have recently diverged through sympatric speciation. Thirteen restriction enzymes were used to analyze mtDNA of 156 specimens representing 13 populations from eastern Canada and northern Maine where normal and dwarf phenotypes of whitefish exist in sympatry and allopatry. Two monophyletic assemblages of populations that exhibit different geographic distributions were identified. One showed an eastern distribution that expands from Cape Breton to the Allegash basin and the other exhibits a more western distribution. The Allegash basin was the only area of overlap. The western assemblage exhibited the normal size phenotype in all cases, whereas the eastern assemblage exhibited the normal size phenotype in allopatric conditions and the dwarf size phenotype in sympatry. The existence of sympatric pairs in the Allegash basin result from the secondary contact of two monophyletic groups of whitefish that evolved allopatrically in separate refugia during the last glaciation events. The weak mtDNA difference of sympatric pairs suggests that speciation of lake whitefish in eastern North America was accompanied by only minor alterations of the ancestral gene pool.

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Many salmonid species are characterized by sympatric, morphologically similar populations that are ecologically specialized for distinct life histories (Behnke, 1972). In North America, sympatric populations of lake whitefish (*Coregonus clupeaformis*) are found in lakes of at least four widely distant regions from Yukon to Labrador. These sympatric pairs are primarily characterized by a bimodal distribution of gill-raker counts, a key feature used to discriminate coregonine fishes at the species and subgenus levels (Scott and Crossman, 1974). Sympatric populations usually occupy different niches, one being associated with a more pelagic (high gill-raker counts) and the other with a more benthic (low gill-raker counts) mode of life. Genetic differences and reproductive isolation have been demonstrated in some instances on the basis of allozyme frequencies (Kirkpatrick and Selander, 1979; Bodaly et al., 1988). In the context of the biological species concept, full species recognition of such populations would appear justified as they co-exist in sympatry and are reproductively isolated (Mayr, 1982). For coregonine fishes, however, species recognition of sympatric populations has been problematic for two reasons. First, the relative commonness of sympatry in coregonine fishes would lead to extensive taxonomic proliferation if specific recognition was attributed to all existing members of sympatric pairs (Behnke, 1972). On the other hand, recognizing species status for similar phenotypes (e.g., high gill-raker counts) that occupy different bodies of water may have no relation to their actual genetic affinities. Indeed, ecological and morphological similarities between coregonine populations may simply reflect phenotypic adjustments of different genotypes to similar environments (Lindsey et al., 1970). Clearly, species-level recognition of sympatric coregonine populations remains arbitrary until their segregation into monophyletic clusters is demonstrated by genetic markers (Benhke, 1972; Lindsey, 1988).

In the Allegash basin of northern Maine, at least 22 cases of sympatric pairs of lake whitefish are found (Fenderson, 1964). Two morphotypes, dwarf and normal, occur in this area. These types display significant differences in age at maturity, growth, and

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maximal age. Dwarf individuals mature by the age of 1 or 2 years and seldom live beyond their fourth year, whereas the normal form does not mature until 4 years of age and may reach an age of 12 years. Dwarf fish seldom exceed 20 cm in standard length and 100 g in weight while the normal fish commonly exceed 40 cm and 1,000 g.

Fenderson (1964) previously reported that difference in spawning time of three to four weeks provides a partial barrier to gene flow between the two forms. More recently, further evidence for distinct gene pools within one lake was found on the basis of allozyme frequencies (Kirkpatrick and Selander, 1979). The authors found significantly different electromorph frequencies at 3 of 18 loci studies between dwarf and normal fish of Second Musquacook L. All populations studied were genetically similar with a mean genetic distance (D) of 0.011 between dwarfs and normals and a mean genetic distance of 0.010 between different populations of dwarfs and between different populations of normals. Furthermore, there was no single allozyme character that distinguished all members of dwarf and normal populations. Thus, Kirkpatrick and Selander (1979) concluded that speciation of lake whitefishes in the Allegash basin has occurred with only minor alteration of the ancestral gene pool.

Because of the genetic similarity of populations sampled within and between morphotypes and lakes, allozyme analysis provided no clue to the evolutionary history of the dwarf and normal whitefishes in the Allegash basin. In particular, allozyme data were unable to confirm whether divergence of normal and dwarf whitefishes of the Allegash basin had occurred before or after the lakes or northern Maine were populated by whitefishes, approximately 12,000 years B.P. Generally speaking, this issue is intimately related to the mode of speciation that gave rise to sympatric pairs. The allopatric-speciation hypothesis suggests an origin in different glacial refugia during the Wisconsin glaciation. However, neither morphological nor genetic characteristics have provided evidence refuting the alternative sympatric-speciation hypothesis, in which ancestral populations in a series of lakes may have split into discrete, reproductively isolated sympatric pairs.

The finding of a genetic marker that could segregate the populations of a given morphotype from different lakes into monophyletic clusters could demonstrate an allopatric origin and refute the possibility of sympatric speciation within each lake. The analysis of mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) provides new avenues for understanding the genetic structure and evolutionary history of animal populations (Avise et al., 1987; Harrison, 1989; Wilson et al., 1985). Because of its faster rate of evolution, maternal mode of inheritance, and the apparent neutrality of most changes occurring in the molecule (Avise, 1986; Moritz et al., 1987; Thomas and Beckenbach, 1989), mtDNA is generally believed to provide more resolution than any other trait for the study of evolutionary history (Avise, 1987).

In this paper, mtDNA was used to test the hypothesis that the sympatric normal and dwarf morphotypes of lake whitefish in the Allegash basin have recently diverged through sympatric speciation.

Materials and Methods

Eggs of 144 female whitefish representing 13 populations from eastern Canada and northern Maine were sampled in 1988. An additional sample of 12 dwarf whitefish (6 male and 6 females), were obtained from Cliff L. in 1989 (Table 1). Fresh samples were kept on ice for up to one week and mtDNA was purified following the procedures described in Bernatchez et al. (1988). Briefly, one to five g of eggs or liver were homogenized in 15 ml TKC buffer (Tris-HCl 50 mM, KCl 1.5%, $CaCl_2$ 3 mM). The homogenate was centrifuged twice at 1,000 G for 10 min, transferred to conical tubes in which one volume of sucrose 15% was underlayered, and centrifuged for 10 min in a clinical table-top centrifuge at 2,000 rpm. The upper phase was transferred to a new tube and centrifuged at 18,000 G for 45 min. The mitochondrial pellet was resuspended in 10 ml TEK buffer (Tris-HCl 50 mM, KCl 1.5%, EDTA 10 mM) and again centrifuged at 18,000 G for 45 min. The mitochondrial pellet was resuspended in 2 ml TK buffer and 200 μl of 10% Nonidet was added. The supernatant was phenol, phenol-chloroform and chloroform extract-

TABLE 1. Locations of populations sampled and geographic distribution of whitefish clonal groups A and B. Genotypes are defined in Table 2. Letters refer to sites identified on Figure 3. * One fish from Champlain L. had a mtDNA genotype not belonging to either group A or B.

Location	Population	Morphotype	Number	mtDNA group	
				A	B
a Ontario L. (Ont.)	allopatric	normal	12	0	12
b Kipawa L. (Qué.)	allopatric	normal	12	0	12
c Manicouagan L. (Qué.)	allopatric	normal	10	0	10
*d Champlain L. (Qué.)	allopatric	normal	12	0	11
e St. Lawrence R. (Qué.)	allopatric	normal	13	0	13
f St. François L. (Qué.)	allopatric	normal	12	0	12
g Cliff L. (Maine)	sympatric	normal	12	0	12
h Musqaucook L. (Maine)	sympatric	dwarf	9	5	4
i Spider L. (Maine)	sympatric	dwarf	12	7	5
j Cliff L. (Maine)	sympatric	dwarf	16	16	0
k Témiscouata L. (Qué.)	sympatric	dwarf	12	12	0
l Grand L. (N.B.)	allopatric	normal	12	12	0
m Mira R. (N.S.)	allopatric	normal	12	12	0
Total			156	64	91

ed and mtDNA was precipitated with ethanol. MtDNA was reconstituted in 250 μl TE buffer (Tris 10 mM, EDTA 1 mM pH 8.0), and frozen at −20°C until used. MtDNA was digested separately with eight hexameric (*BamHI, BglI, DraI, HindIII, PstI, PvuII, SmaI, XmnI*), four multihexameric (*AvaI, BanI, HaeII, HincII*), and one multipentameric (*AvaII*) restriction endonucleases. Digests of mtDNA from individual fish were electrophoresed on 0.8% agarose gels at 25 V for 16 hours. Ethidium bromide was included in the gel and mtDNA bands were visualised and photographed under UV light. A *HindIII* digest and *EcoRI-HindIII* double digest of Lambda DNA were used as size standards in each gel.

All analyses were based on mtDNA fragment variation. Distinct single endonuclease patterns were identified by a specific letter in order of appearance. Considering all enzymes, each fish was assigned a composite letter code that described its observed mtDNA genotype. Sequence divergence between genotypes was estimated according to Upholt's (1977) fragment method. Sequence divergence was estimated independently for the different types of enzymes followed by pooling the estimates. The resulting distance matrix was clustered by UPGMA (Sneath and Sokal, 1973) using the average linkage algorithm of the SAS statistical package. Fragment pattern variations were sufficiently simple (involving no more than two site changes among patterns for each enzyme) to permit identification of restriction site changes among all genotypes. Therefore, the mtDNA clones were organized from a restriction site presence-absence matrix into a Wagner network by the parsimony criteria using the METRO algorithm of the Phylip package provided by Joe Felsenstein. Confidence limits on branches of the Wagner network were generated by the bootstrap method (BOOTM in Phylip package; Felsenstein, 1985). In the present case, 100 replicates of BOOTM were run.

RESULTS

The 13 restriction endonucleases used produced an average of 80 fragments per specimen, which represents a sample of 2.5% of the mtDNA genome. Nineteen mtDNA composite genotypes were resolved among 156 whitefish surveyed in 13 populations (Table 2). No length variation or heteroplasmy was detected in this study. Four of the 19 clonal lines (1, 6, 10, 11) represented 90% of all fish examined while the other 15 genotypes were observed in single individuals (two fish in the case of clonal line 13). Pairwise sequence divergence estimates among all mtDNA clones were low in all cases (mean: 0.36 ± 0.16% (SD), range: 0.07 to 0.86). Clustering of the distance matrix of sequence divergence by UPGMA revealed two major clonal groups (A, B) sep-

TABLE 2. Definition and frequency distribution of mtDNA clonal genotypes observed among whitefish samples from Northeastern America. Numbers refer to restriction enzymes: 1; *AvaI*, 2; *AvaII*, 3; *BanI*, 4; *BglI*, 5; *DraI*, 6; *HaeII*, 7; *HincII*, 8; *HindIII*, 9; *PvuII*, 10; *SmaI*, 11; *XmnI*, 12; *PstI*, 13; *BamHI*. Empty spaces in composite genotypes represent fragment patterns A.

Clonal line	Composite genotype													Number	Locale
	1	2	3	4	5	6	7	8	9	10	11	12	13		
1	A	A	A	A	A	A	A	A	A	A	A	A	A	48	a–f
2								C						1	e
3								D						1	e
4											B			1	a
5						C								1	d
6	A	A	B	A	A	A	A	A	A	A	A	A	A	61	h–m
7			B				B							1	k
8			B			E								1	k
9			B			D								1	l
10	A	B	A	A	A	A	A	A	A	A	A	A	A	22	f–i
11	A	A	A	A	A	A	B	A	A	A	A	A	A	9	b, d–f
12							B	C					A	1	e
13							B						B	2	e
14							C							1	e
15			C			B								1	d
16	B					D	D	B						1	d
17							E							1	f
18		C												1	i
19			D				B							1	d

arated by a mean sequence divergence of 0.49% (Fig. 1). These groups composed respectively 41% and 58.3% of all fish analyzed. Clonal line 16, found in a single fish from Champlain, L. represented an exception and was not clustered with either of the two clonal groups. The most parsimonious network from the Wagner analysis required a minimum of 23 site changes (Fig. 2). The analysis showed that all clones clustered in group A differed from those in group B by two *BanI* site changes. The bootstrap analysis revealed the same pattern of separation between the two groups in 89% of the bootstrapping replicates. All other enzymes were involved only in discriminating clonal lines within each group. In group A, the three rare variants (clonal lines 7, 8, 9) differed from the major clonal line 6 by a single site change. No further substructuring could be detected in clonal group B as only one site difference was involved in moving from one mtDNA genotype to the next (two site differences in the case of clonal line 15). Again, clonal line 16 was very distinct from either group A or B clonal lines.

Geographic Distribution of mtDNA Composite Genotypes

A sharp geographic pattern was observed in the distribution of clonal groups A and B (Table 1, Fig. 3). All fish sampled east of the Allegash basin (Témiscouata L., Qué.; Grand L., N.B.; and Mira R., N.S.) belonged to clonal group A, while all fish sampled west of that region belonged to clonal group B, except for one fish with the rare clonal line 16 found in Champlain L. Clonal groups A and B overlapped only in the three lakes of the Allegash basin where sympatric populations exist. In these lakes, all clonal group A fish possessed the mtDNA clonal line six and all clonal group B fish had the clonal line 10, except for one fish with the rare variant 18 derived from clonal line 10 (Fig. 2). None of the other mtDNA clonal lines were observed among the sympatric populations. The only three clonal lines of mtDNA group B that were found in more than one or two fish (1, 10, 11) were not confined to a single population but were found in many lakes west of the Allegash basin (Table 2).

MtDNA and Phenotypic Variation.

In Cliff L., a clear association was observed between mtDNA genotypes and fish phenotypes (Table 1). All 16 dwarf and all 12 normal whitefish belonged to clonal group A (clonal line 6) and clonal group B (clonal line 10) respectively. This demonstrates that the two forms are reproductively isolated in

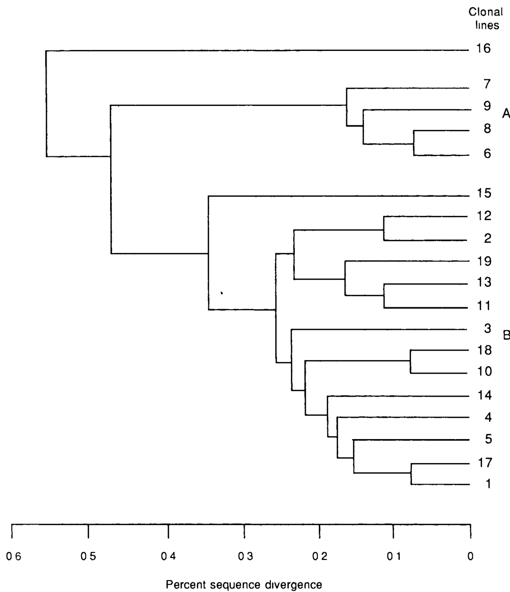

FIG. 1. UPGMA phenogram clustering the distance matrix of sequence divergence among whitefish mtDNA clonal lines observed in northeastern America. The clonal lines clustered into two major groups, A and B. Clonal line 16 was a rare variant observed in a single fish.

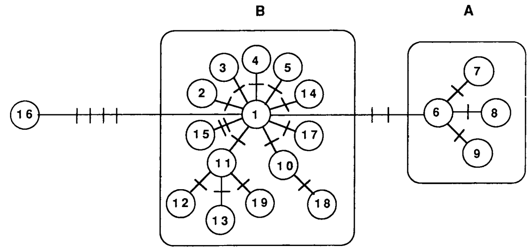

FIG. 2. Wagner parsimony network linking the 19 whitefish mtDNA clonal lines. Clonal groups A and B refer to the clusters revealed by UPGMA. Vertical bars indicate the number of site changes among clonal lines. Clonal groups A and B differ by two *BanI* site changes.

this lake (Fisher exact test, $P < 0.001$). All other populations surveyed in which only clonal group B was present (i.e., west of the Allegash basin, Fig. 3) were also of normal phenotype. In contrast, other populations with only clonal group A present (i.e., east of the Allegash basin, Fig. 3) were not fixed for a given phenotype. Hence, while all fish from Témiscouata L. were dwarf as in Cliff L., all fish from Grand L., N.B. and Mira R., N.S. were of normal phenotype. The only two populations in which both clonal groups A and B were mixed were dwarf populations of Spider L. and Second Musquacook L. in the Allegash basin.

DISCUSSION

The present study revealed the existence of two distinct mtDNA clonal groups among lake whitefish of eastern North America. The low sequence divergence estimate between the two groups (0.49%) is comparable to the level of divergence observed in two other extensive studies conducted to date on mtDNA variation among populations of northern freshwater fish species. In a survey of mtDNA variation in the walleye (*Stizostedion vitreum*), Billington and Hebert (1988) identified two mtDNA clonal groups separated by 0.5%. In another survey on lake trout (*Salvelinus namaycush*), Grewe and Hebert (1988) observed three mtDNA clonal groups separated by less than 1.0%. In both studies a clear geographic pattern in the distribution of the clonal groups was observed. These patterns were best explained by an allopatric origin of the clonal groups in separate glacial refugia known for these species.

The well-defined geographic pattern of distribution of the two mtDNA clonal groups revealed in the present study is best explained by an allopatric origin in two dif-

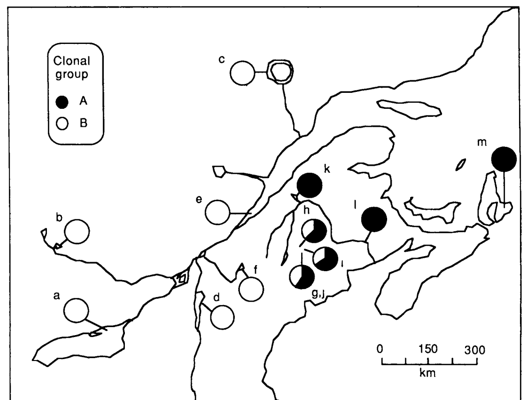

FIG. 3. Location map and geographic distribution of mtDNA clonal groups A and B identified by UPGMA and Wagner parsimony analysis. The two groups overlap in the Allegash basin only. Clonal line 16, representing a single fish from Champlain L., does not belong either to group A or B and was omitted here. Sample locations: a, Ontario L.; b, Kipawa L.; c, Manicouagan L.; d, Champlain L.; e, St. Lawrence R.; f, St. François L.; g, Cliff L. (normal); h, Musquacook L.; i, Spider L.; j, Cliff L. (dwarf); k, Témiscouata L.; 1, Grand L.; m, Mira R.

ferent glacial refugia. Lake whitefish from eastern North America are believed to have survived in two refugia; the Mississippi located west of the area studied herein and a more eastern Atlantic refugium (McAllister et al., 1986). MtDNA clonal group A was solely found in whitefish east of the Allegash basin, which suggests the origin of these populations in the Atlantic refugium. Clonal group B was observed only in western populations of the range studied, which suggests that these originated from the Mississippi refugium. The level of divergence observed between the two clonal groups is also compatible with a separation that occurred during the last glaciation events. Although there is no molecular clock calibration in fish, the use of 2% sequence divergence per million years reported for mammals and and birds (Brown et al., 1979; Shields and Wilson, 1987) is currently assumed in studies of evolutionary history of fish (Kessler and Avise, 1985; Bermingham and Avise, 1986; Gyllensten and Wilson, 1986; Avise et al., 1987; Billington and Hebert, 1988; Grewe and Hebert, 1988; Bentzen et al., 1989). If one assumes the same evolution rate for whitefish, clonal groups A and B last shared a common ancestor about 150,000 years ago which corresponds to the Illinoian glacial advance (Fulton and Andrews, 1987). This value should be considered as conservative since the rate by mtDNA evolution in cold-blooded animals appears slower than in higher vertebrates (Thomas and Beckenbach, 1989).

The Allegash basin is the only area where the two clonal groups overlapped. These results support the hypothesis that the Allegash basin is a zone of secondary contact between two groups of whitefish that evolved separately in different glacial refugia. An alternative explanation for the presence of both clonal groups A and B in this area is that these mtDNA lineages are ancestral polymorphisms and that the Allegash basin was the refugium from which both groups subsequently dispersed non-randomly. However, this hypothesis is refuted for two major reasons. The most obvious one is that the Allegash basin was glaciated during the Wisconsin glacial advance, and therefore could not act as a refugium, unless unglaciated pockets unidentified to date had persisted. Second, the two clonal lines found in the Allegash basin (clonal lines 6 and 10, Fig. 2) are separated by an intermediate lineage (clonal line 1) not found in the Allegash basin but that composed the majority of the western populations (Table 2). The distribution of this intermediate mtDNA lineage extends to Northern Hudson bay (Bernatchez et al., 1989) and west to the Mackenzie delta (Bernatchez and Dodson, unpublished data). However, neither clonal line 6 nor 10 has been observed outside the range described in the present study. These observations are not consistent with the existence of an Allegash refugium.

The Allegash basin (including Témiscouata L.) is the only area in the region studied where sympatric populations are known to exist. Furthermore, sympatric populations of Cliff L. are fixed for alternate mtDNA clonal lines, demonstrating that the two whitefish lineages can remain reproductively isolated in a sympatric situation. Therefore, we conclude that the sympatric populations in the Allegash basin and Témiscouata L. resulted from the recolonization of the area by two genetically distinct groups of whitefish that evolved allopatrically in separate refugia during the last glaciation. Thus, the present study refutes the hypothesis that sympatric populations of lake whitefish in the Allegash basin have diverged through sympatric speciation following the Wisconsin glaciation.

This study provides evidence that normal and dwarf phenotypes are not genetically fixed. Indeed, clonal group A fish may exhibit either dwarf or normal phenotypes. Interspecific competition may be the most important factor responsible for the expression of dwarfism in these fish. Among the four populations that were fixed for mtDNA clonal group A, the two found in allopatry (Grand L., N. B. and Mira R., N.S.) exhibited the normal size phenotype whereas the two existing in sympatry (Cliff L. Me. and Témiscouata L. Qué.) were dwarf. Major life history differences are associated with the dwarf phenotype relative to the normal phenotype, such as a more planktivorous food habit, a shift of several weeks in spawning time and an earlier age at maturity (Fenderson, 1964). Thus, it is possible that this niche shift from normal to dwarf phe-

notype has an important survival value for the mtDNA clonal group A whitefish group in sympatric situations. The niche shift phenomenon in the face of potential competition has been observed in many coregonine species and is partly responsible for the complexity of the systematic relationship among coregonine fish (Lindsey, 1981, 1988). Lindsey (1981) clearly demonstrated that characters that apparently render a coregonine fish population distinctive are in reality a reflection of the presence or absence of other species with which it shares its environment.

Our results also support the view of Kirkpatrick and Selander (1979) that the speciation of lake whitefish in eastern North America has occurred with only minor alteration of the ancestral gene pool. As in the case of structural nuclear gene loci, the mtDNA genotype difference in sympatric pairs is weak. Indeed, the estimate of percent sequence divergence between clonal groups A and B (0.49%) is much lower than the level of intrapopulation variation observed in several fish species. For instance, Kornfield and Bogdanowicz (1987) observed mtDNA lineages separated by over 4.0% within the same population of Atlantic herring (*Clupea harengus*). Bermingham and Avise (1986) also observed levels of divergence over 4% within populations of *Lepomis punctatus.* Avise et al. (1989) found a mean sequence divergence of 2.4% among menhaden (*Brevoortia tyrannus*) populations of the North American Atlantic coast. Therefore, the present results do not conform to the hypothesis that there is less developmental divergence between salmonids than in other fishes subject to the same temporal separation and that the developmental program of salmonids is resistant to genetic alteration due to tetraploidy (Gyllensten et al., 1985). The view that speciation can occur in the absence of significant genetic divergence is stressed by many authors (reviewed in Templeton, 1981). Rose and Doolittle (1983) suggested that a distinct class of genes may be involved in the speciation, the remainder of the genome neither contributing to the process nor being affected by it. Such a class of genes could include regulatory genes (Wilson, 1985).

According to the biological species concept, a species is defined as a reproductive community of populations, reproductively isolated from other communities of populations, that occupies a specific niche in nature (Mayr, 1982). MtDNA clonal groups A and B whitefish represent two distinct monophyletic assemblages of populations that show distinct geographic patterns of distribution. Results obtained in Cliff L. demonstrate that the two groups can remain reproductively isolated and that they occupy different niches when found in sympatry. Although these results support the view that these two groups of whitefish may represent two biological species, both clonal groups A and B were found within the dwarf whitefish populations of Spider L. and Second Musquacook L. Two alternative hypothesis can explain these observations: (1) hybridization occurs between sympatric groups A and B in these lakes; (2) both whitefish groups A and B produce dwarf forms but do not hybridize. These hypotheses are, however, not testable without the assessment of nuclear markers. Thus, a firm generalization of the biological species status of the two groups of whitefish identified in this study must await further observations on additional sympatric pairs. As conventional allozyme studies failed to reveal diagnostic nuclear markers, the test of hybridization may require finer techniques such as nuclear DNA fingerprinting.

The present study adds to the knowledge of the evolutionary history of the sympatric whitefish populations of eastern North America. First, it demonstrates for Cliff L. that dwarf and normal whitefish are reproductively isolated. Second, it confirms that the existence of sympatric pairs in the Allegash basin resulted from the recolonization of the area by two genetically and geographically distinct kinds of whitefish that evolved in allopatry. Third, it provides evidence that normal and dwarf phenotypes are not genetically fixed. Finally, this study provides another example of the difficulty of assessing the taxonomic relationships of coregonine fishes on the basis of phenotypic characteristics. The variability in phenotypic plasticity exhibited by a monophyletic assemblage of whitefish in Northeastern America shows just how well Lindsey's (1981) statement that "stocks may be cha-

meleons which shift their appearance in response to the biological colour of their surroundings" reflects the biological reality of coregonine fishes.

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