Mitochondrial DNA and isozyme electrophoretic analyses of the endangered Acadian whitefish, *Coregonus huntsmani* Scott, 1987[1]

L. BERNATCHEZ
Département de biologie, Université Laval, Sainte-Foy (Québec), Canada G1K 7P4
T. A. EDGE
Department of Biology, University of Ottawa, Ottawa, Ont., Canada K1N 6N5
J. J. DODSON
Département de biologie, Université Laval, Sainte-Foy (Québec), Canada G1K 7P4
AND
S. U. QADRI
Department of Biology, University of Ottawa, Ottawa, Ont., Canada K1N 6N5

Received September 7, 1989

BERNATCHEZ, L., EDGE, T. A., DODSON, J. J., and QADRI, S. U. 1991. Mitochondrial DNA and isozyme electrophoretic analyses of the endangered Acadian whitefish, *Coregonus huntsmani* Scott, 1987. Can. J. Zool. **69**: 311–316.

Electrophoretic analysis of isozymes and mitochondrial DNA (mtDNA) restriction analysis were used to study the genetic divergence between the Acadian whitefish, *Coregonus huntsmani*, and members of the subgenera *Coregonus* (lake whitefish, *C. clupeaformis*) and *Leucichthys* (Arctic cisco, *C. autumnalis*, and lake cisco, *C. artedii*). Results obtained from both studies demonstrated that the Acadian whitefish is genetically highly distinct from the other coregonines examined. mtDNA restriction analysis revealed that the Acadian whitefish possesses a unique mitochondrial genotype which is divergent from that of the two cisco species or lake whitefish. Twelve of 13 restriction enzymes used were informative in distinguishing the Acadian whitefish from the other species, and species-specific fragment patterns were observed for 10 enzymes. In isozyme analysis of five loci, the Acadian whitefish was monomorphic at two loci for alleles not found in lake whitefish. Acadian whitefish also possessed an additional isozyme at another locus that was not found in lake whitefish and Arctic cisco specimens. This isozyme is unknown from the genetic model for lake whitefish at this locus. These results provided useful genetic markers to identify the Acadian whitefish. They emphasize that the extinction of the species would represent a major loss of both genetic diversity and potential information concerning the contentious phylogeny of coregonine fishes.

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Nous avons utilisé les analyses de restriction de l'ADN mitochondrial et d'électrophorèse d'isozymes pour étudier la différenciation génétique entre le Corégone de l'Acadie et des espèces appartenant aux sous-genres *Coregonus* (Grand corégone *C. clupeaformis*) et *Leucichthys* (Cisco arctique, *C. autumnalis*, et Cisco de lac, *C. artedii*). Les résultats obtenus par les deux types d'analyse ont démontré que le Corégone de l'Acadie est génétiquement très distinct des autres corégoninés étudiés. L'analyse de restriction de l'ADN mitochondrial a révélé que le Corégone de l'Acadie possède un génotype mitochondrial unique et très divergent de ceux des ciscos et du Grand corégone. Douze des 13 enzymes de restriction utilisées ont permis de distinguer le Corégone de l'Acadie des autres espèces et des patrons de fragments spécifiques ont été obtenus pour 10 d'entre elles. Les Corégones de l'Acadie étaient tous homozygotes à deux loci pour des allèles non observés chez le Grand corégone. Ils possèdent également un isozyme additionel absent du modèle génétique du Grand corégone et non observé chez les Ciscos arctiques analysés. Les résultats présentés proposent des marqueurs génétiques utiles pour identifier le Corégone de l'Acadie. Ils stressent également le fait que l'extinction du Corégone de l'Acadie représenterait une perte importante de diversité génétique et d'information sur la phylogénie déjà assez contestée des corégoninés.

Introduction

The Acadian whitefish (we follow McAllister (1990) and Legendre (1978) in using the more geographically precise name Acadian rather than Atlantic), *Coregonus huntsmani* Scott, 1987, is a species endemic to Canada which was first reported as *Coregonus quadrilateralis* (= *Prosopium cylindraceum*) by Huntsman (1922) and subsequently as a variable form of the Sault whitefish, *Coregonus labradoricus* (= *C. clupeaformis*) by Piers (1927). It has continued to be confused with the lake whitefish, *C. clupeaformis*, despite being recognized as a new species by Leim and Scott (1966) and having nomenclatural problems resolved by Scott (1967, 1987). Its distribution is limited to the Tusket and Petite Rivière watersheds of southwestern Nova Scotia where acidification of the habitat threatens he already reduced populations with extinction (Edge 1984, 987). The Acadian whitefish was recognized as an endangered species in 1983 by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (Campbell 1987).

A taxonomic study of Acadian whitefish and lake whitefish has clearly distinguished the two species by meristic and morphometric characters (Edge 1987). However, the genetic divergence of the Acadian whitefish from other coregonines has not been investigated. In this paper we used both electrophoretic analysis of isozymes and mitochondrial DNA (mtDNA) restriction analysis to study genetic markers useful in distinguishing the Acadian whitefish from North American members of the subgenera *Coregonus* (lake whitefish, *C. clupeaformis*) and *Leucichthys* (Arctic cisco, *C. autumnalis*, and lake cisco, *C. artedii*).

The electrophoretic analysis of isozymes has proven useful in studies of genetic variation in coregonine fishes (Lindsey *et al.* 1970; Ferguson *et al.* 1978; Franzin and Clayton 1977; Ihssen *et al.* 1981; Casselman *et al.* 1981; Bodaly *et al.* 1988). Isozyme phenotypes are largely independent of environmental influences (Allendorf and Utter 1979) and hence directly reflect genetic variation. More recently, restriction analysis of mtDNA

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[1] Contribution to the program of GIROQ (Groupe Interuniversitaire de Recherches Océanographiques du Québec).

TABLE 1. Sample sizes of *C. huntsmani* from Nova Scotia used in electrophoretic analysis of five enzyme loci

	g-3-pdhA	*g-3-pdhB*	*s-mdhB*($\alpha+\beta$)	*ldhH*α	*ldhM*β
Minamkeak Lake	4	4	2	2	1
Millipsigate Lake	1	1	1	—	1
Hebb Lake	13	13	10	3	7
Annis River	2	2	1	1	—
Total	20	20	14	6	9

provided new avenues for understanding the evolutionary history of animal populations (Avise *et al.* 1987; 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, mtDNA is generally believed to provide more resolution than any other trait for the study of genetic relationships among closely related species (Avise 1986; Moritz *et al.* 1987; Thomas and Beckenbach 1989).

Material and methods

mtDNA restriction analysis

mtDNA of three Acadian whitefish from Hebb lake, N.S., was extracted from frozen livers as described in Bernatchez *et al.* (1988). mtDNA was digested with eight hexameric (*Bam*HI, *Bgl*I, *Dra*I, *Hin*dIII, *Pst*I, *Pvu*II, *Sma*I, *Xmn*I), four multihexameric (*Ava*I, *Ban*I, *Hae*II, *Hinc*II), and one multipentameric (*Ava*II) restriction enzymes. Digests were electrophoretically separated on 0.8 or 1.2% agarose gels for 16 h at 25 V. Digests of all species for a given restriction enzyme were run simultaneously on the same gel to ensure comparable mobility. DNA was then denatured, neutralized, and transferred to nitrocellulose filters by the procedure described by Maniatis *et al.* (1982). Filters were hybridized with a highly purified radiolabeled total mtDNA probe as described in Bernatchez *et al.* (1990). Filters were autoradiographed using an intensifying screen (Cronix lightning-plus) for 2–16 h.

Data analysis

Restriction fragment length polymorphisms defined in Acadian whitefish mtDNA were compared with mtDNA haplotypes observed among 141 lake cisco and 112 lake whitefish, sampled in James and Hudson bays for population genetic analyses (Bernatchez and Dodson 1990*b*; Bernatchez *et al.* 1989). In these studies, 19 and 9 different mtDNA haplotypes were observed among lake cisco and lake whitefish, respectively. An additional sample of two Arctic cisco, one captured at Arctic Red River and the other at nearby Fort McPherson, N.W.T., was included in the analysis. Nucleotide sequence divergence among all mtDNA haplotypes was estimated according to Upholt's (1977) fragment method. Sequence divergence and standard deviations were estimated independently for hexameric and multihexameric enzymes (*Ava*II was treated as multihexameric) and both estimates were then pooled following weighting for the number of base pairs sampled by each type of enzyme. The resulting distance matrix was clustered by UPGMA (Sneath and Sokal 1973) using the average linkage algorithm of the SAS statistical package.

Isozyme analysis

Electrophoretic analysis of isozymes was performed on 20 Acadian whitefish (Table 1), 20 lake whitefish from Lake George, N.S., and 35 lake whitefish from the Mira River, N.S. Whitefish were frozen (−20°C) immediately after capture. Six Arctic cisco from Cape Bathurst, N.W.T., were obtained from J. W. Clayton, Freshwater Institute, Department of Fisheries and Oceans, Winnipeg. The isozyme data for Acadian whitefish populations were pooled for each locus because of small sample sizes and the interspecific nature of the study. Three enzyme systems comprising five loci were analyzed by horizontal starch gel electrophoresis. Glycerol-3-phosphate dehydrogenase (G-3-PDH) was analyzed following Clayton *et al.* (1973), lactate dehydrogenase (LDH) following Clayton and Franzin (1970), and malate dehydrogenase (MDH) following Franzin and Clayton (1977). Phenotypes were interpreted using genetic models and enzyme nomenclature determined for lake whitefish (see Bodaly 1977; Cross and Ward 1980; Casselman *et al.* 1981; Bailey *et al.* 1976; Bodaly *et al.* 1988). The genetic basis of these loci has been shown by breeding studies (Clayton and Franzin 1970; Clayton *et al.* 1973; Imhof *et al.* 1980; Ihssen *et al.* 1981).

Results

mtDNA variation

Twelve of the restriction enzymes used were informative in distinguishing the Acadian whitefish from mtDNA lineages of the other species. The Acadian whitefish demonstrated species-specific fragment patterns for 10 enzymes. The *Bam*HI fragment pattern was shared with all lake whitefish haplotypes and the *Bgl*I fragment pattern was shared with all lake whitefish and Arctic cisco haplotypes. No pattern was shared uniquely wi the cisco species. Examples of Southern blots with fragment patterns observed are given in Fig. 1. *Pst*I gave an identical pattern for all species, which suggests that their mitochondrial genome size is the same. The length of the mtDNA molecule estimated by averaging the sums of all digestion patterns of the Acadian whitefish was 16 740 ± 560 base pairs.

The restriction enzymes used revealed an average of 75 fragments per species. This corresponds to a sampling of about 2.5% of the total mitochondrial genome. The pairwise nucleotide sequence divergence estimates calculated from the fragment presence–absence matrix are presented in Table 2. Results demonstrate that the Acadian whitefish is clearly distinct from the other three species. However, it differs more from lake cisco (5.82 ± 0.64%) and Arctic cisco (5.24 ± 0.60%) than from lake whitefish (3.77 ± 0.54%). In all cases, intraspecific variation was weak compared with interspecific levels of divergence. No variation was observed within Acadian whitefish and Arctic cisco samples. The mean intraspecific divergence was 0.52 ± 0.22% in lake cisco and 0.19 ± 0.05% in lake whitefish. In another study dealing with the intraspecific mtDNA variation in lake whitefish that included samples from the Mira River, N.S., and Grand Lake, N.B., Bernatchez and Dodson (1990*a*) observed a mean intraspecific divergence of 0.36 ± 0.16%.

The UPGMA phenogram constructed from the distance matrix indicates that the cisco species are highly genetically distinct from the phyletic line clustering lake whitefish and Acadian whitefish (Fig. 2). It also indicates that Acadian whitefish and lake whitefish are genetically very distinct. Assuming th coregonine mtDNA evolves at the rate of 2% sequence divergence per million years as estimated for mammals and birds (Brown *et al.* 1979; Shields and Wilson 1987), and

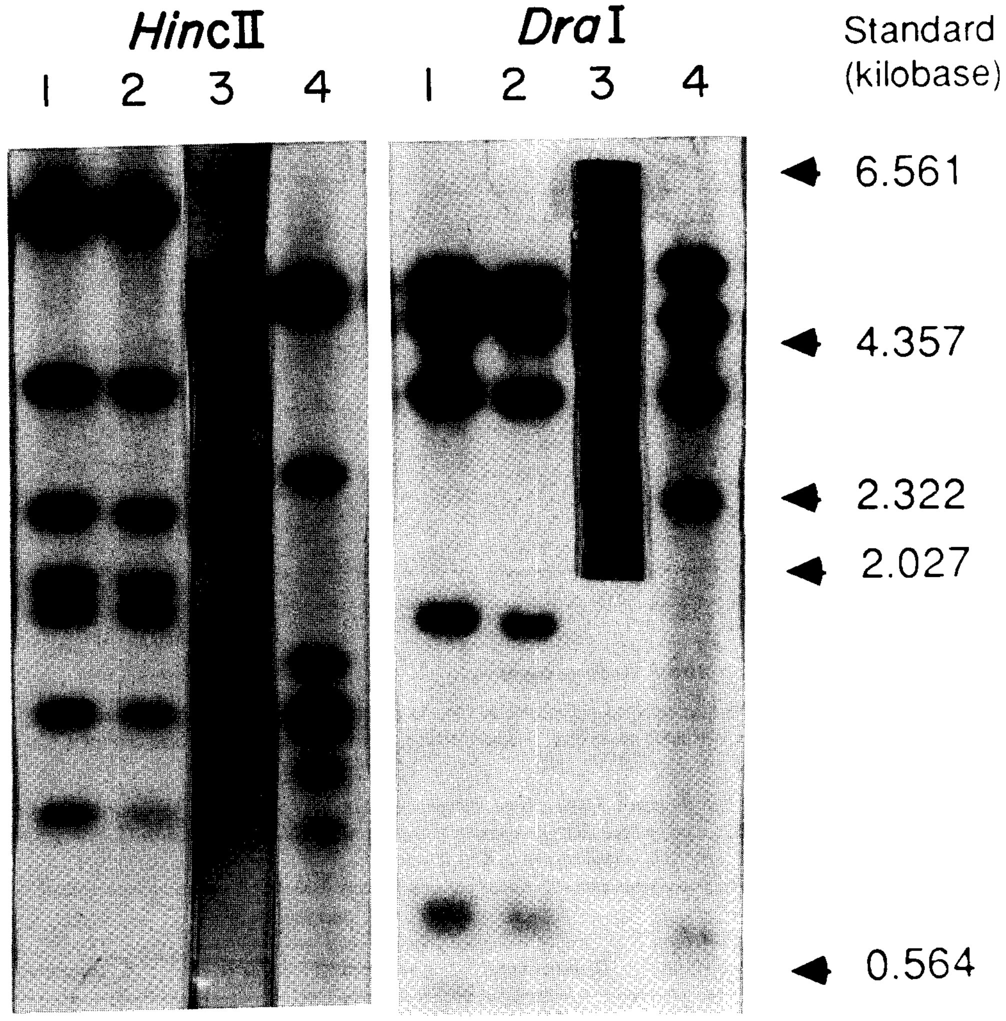

FIG. 1. Autoradiograph of 0.8% agarose gels of coregonine species mtDNA digested with restriction enzymes *Dra*I and *Hinc*II. Numbers refer to species: 1, *Coregonus autumnalis;* 2, *C. artedii*; 3, *C. huntsmani*; and 4, *C. clupeaformis*. Standard size fragments of *Hind*III-digested λ DNA are given on the right. Because of the lesser amount of mtDNA available from *C. huntsmanii*, lane 3 fragment pattern was fainter than the others. To provide comparable signal intensity for lane 3, we superimposed two autoradiographs of the same gel exposed for different periods of time, thus creating a darker background.

TABLE 2. Mean pairwise nucleotide sequence divergence estimates (below diagonal) with corresponding standard deviations (above diagonal) between *C. huntsmani* and other coregonine species

	C. huntsmani	*C. clupeaformis*	*C. autumnalis*	*C. artedii*
C. huntsmani	0	0.54	0.60	0.64
C. clupeaformis	3.77	0.19	0.57	0.65
C. autumnalis	5.24	4.67	0	0.60
C. artedii	5.82	5.94	1.44	0.52

NOTE: Values of the main diagonal are the mean sequence divergence among mtDNA lineages within each species.

currently applied to fishes (e.g., Bermingham and Avise 1986; Avise *et al.* 1987; Billington and Hebert 1988; Bentzen *et al.* 1989), the cisco species had diverged some 2.9 million years ago, whereas Acadian whitefish and lake whitefish diverged bout 1.9 million years ago.

Isozyme variation

Several isozyme markers, inferring considerable genetic differences, clearly distinguished the Acadian whitefish from the lake whitefish and the Arctic cisco. The Acadian whitefish was monomorphic at two loci (*g-3-pdhA*, *g-3-pdhB*) for alleles not found in lake whitefish examined (Table 3). These alleles were found in Arctic cisco but in much lower frequencies, especially in the case of *g-3-pdhB*. All Acadian whitefish also revealed an additional isozyme at the *s-mdhB*($\alpha+\beta$) locus that was not found in lake whitefish or Arctic cisco examined. This

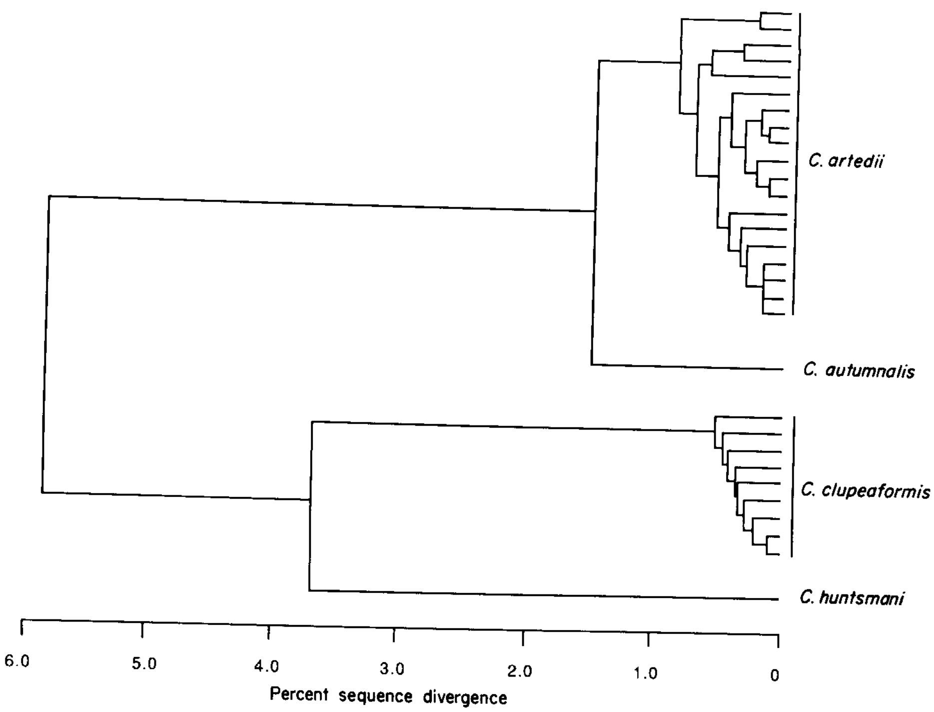

FIG. 2. Phenogram of coregonine species mtDNA genotypes generated by UPGMA cluster analysis of nucleotide sequence divergence estimates. Nineteen mtDNA haplotypes were observed in *C. artedii*, nine in *C. clupeaformis*, one in *C. huntsmani*, and one in *C. autumnalis*.

TABLE 3. Allele frequencies for glycerol-3-phosphate dehydrogenase (*g-3-pdhA* and *g-3-pdhB*), malate dehydrogenase (*s-mdhA*($\alpha+\beta$)), and lactate dehydrogenase (*ldhH*α*and ldhM*β) observed in *C. huntsmani*, *C. clupeaformis*, and *C. artedii*

	Tissue	Allele	*C. huntsmani*	*C. clupeaformis*		*C. autumnalis*
				Lake George	Mira River	
g-3-pdhA	S	1	1.0 (20)	0.0 (20)	0.0 (35)	0.67 (6)
		2	0.0	1.0	1.0	0.33
g-3-pdhB	S	1	0.0 (20)	0.90 (20)	0.44 (35)	0.33 (6)
		2	1.0	0.0	0.0	0.08
		3	0.0	0.10	0.56	0.59
s-mdhB($\alpha+\beta$)	S	1	1.0 (14)	0.67 (12)	0.31 (32)	* (5)
		2	0.0	0.33	0.69	
ldhHα	H	1	1.0 (6)	na	0.75 (6)	na
		2	0.0		0.25	
ldhMβ	S	1	0.50 (9)	0.0 (10)	0.32 (25)	0.42 (6)
		2	0.50	1.0	0.68	0.58

NOTE: For genetic models and enzyme nomenclature, see Material and methods section. The number of fish examined for each enzyme system is given in parentheses. Tissues used: S, white skeletal muscle; H, heart muscle. All specimens of Acadian whitefish had an additional isozyme at the *s-mdhB*($\alpha+\beta$) locus unknown from genetic model. *, isozyme phenotypes did not correspond to genetic model; na, data not available.

isozyme is unknown from the genetic model for this locus in lake whitefish.

Discussion

The results obtained from both mtDNA and isozyme analysis demonstrate that the Acadian whitefish is genetically very distinct from the other coregonines examined. This high level of genetic divergence of the Acadian whitefish from representatives of both *Coregonus* subgenera provides additional evidence to recognize the Acadian whitefish as a valid species.

The analysis of isozymes provided useful genetic markers to identify the Acadian whitefish. The finding at the *s-mdhB*($\alpha+\beta$) locus in all specimens of Acadian whitefish of an isozyme that was not present in the Arctic cisco examined, and is unknown from the genetic model for lake whitefish at this locus, is indicative of considerable genetic divergence from these two species. The Acadian whitefish was also monomorphic for an allele at the *g-3-pdhA* locus that is known in lake whitefish onlv at low frequencies from populations west of the Great Lake (Franzin and Clayton 1977; J. W. Clayton, unpublished data). These authors proposed that the present-day distribution of this allele in lake whitefish populations was best explained by postglacial dispersal from a Bering refugium that persisted during the Wisconsinian glaciation. In this view, the fixture of this allele in Acadian whitefish implies that the species had erged from lake whitefish well before the Wisconsinian glaciation and that the present day allele frequency differences are not the result of recent natural selection or genetic drift.

mtDNA restriction analysis also provided unambiguous genetic markers to identify the Acadian whitefish. This analysis revealed that the Acadian whitefish possesses a mitochondrial genotype which is highly divergent from that of Arctic cisco, lake cisco, and lake whitefish. The sequence divergence estimates observed between Acadian whitefish and the other coregonines (3.77–5.82%) are highly significant considering the lower sequence divergence estimates between lake trout (*Salvelinus namaycush*) and Arctic char (*S. alpinus*) (3.35%, Grewe and Hebert 1988), and between rainbow trout (*Oncorhynchus mykiss* = *Salmo gairdneri*) and chinook salmon (*O. tschawytscha*) (3.18%) or between chum (*O. keta*) and pink (*O. gorbuscha*) salmon (2.73%, Thomas *et al.* 1986). These observations strongly suggest that the Acadian whitefish does not represent a recently specialized form that evolved from either lake whitefish or ciscoes during the Wisconsinian glaciation period.

In summary, mitochondrial and nuclear genetic analyses suggest that the Acadian whitefish represents a distinct evolutionary line among coregonine fishes and that it is a key species in the understanding of the systematics of the genus *Coregonus*. The extinction of the Acadian whitefish would therefore represent a major loss of both genetic diversity and potential information concerning the contentious phylogeny of the coregonine fishes.

Acknowledgements

The authors are indebted to Dr. J. W. Clayton, D. N. Tretiak, and R. Lilly, Department of Fisheries and Oceans, Winnipeg, for assistance in performing the isozyme electrophoretic analysis and interpretation of isozyme phenotypes. We also gratefully acknowledge S. Boivin for technical assistance in mtDNA analysis and Dr. D. E. McAllister, National Museum of Natural Sciences, Ottawa, for reviewing the manuscript. This research was funded by Natural Sciences and Engineering Research Council (NSERC) grants to J.J.D. and S.U.Q. and an NSERC postgraduate scholarship to L.B.

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