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Mitochondrial DNA sequence heterogeneity among James-Hudson Bay anadromous coregonines

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In this paper, we describe the procedures used for isolating mt DNA from frozen samples of liver and eggs, and analysing mt DNA sequence heterogeneity as a method for studying genetic divergence among coregonine fish. The estimate of mt DNA sequence divergence between three coregonine species and one salmonine representative indicates a close relationship between cisco (*Coregonus artedii*) and lake whitefish (*C. clupeaformis*) and no significant difference in the divergence of round whitefish (*Prosopium cylindraceum*) from either *Coregonus* sp. or brook charr (*Salvelinus fontinalis*). Differences in the frequency distribution of mt DNA polymorphisms were found among two populations of lake whitefish. These results suggest that mt DNA analysis is a useful tool in studying inter- and intraspecific genetic divergence of coregonine fish.

Key words: mt DNA, coregonines, genetic divergence

I. Introduction

Recent studies in James-Hudson Bay have revealed latitudinal variations in life history of sympatric populations of cisco (*Coregonus artedii*) and lake whitefish (*C. clupeaformis*) (MORIN *et al.* 1982). A main goal of our present research is to test the hypothesis that these variations are reflected in the genetic divergence of the populations and thus, population specific life history traits may represent evolutionary strategies for improving reproductive fitness. Over the past several years, the analysis of sequence heterogeneity in mitochondrial DNA (mt DNA) using restriction endonuclease digestions has been demonstrated to be a most promising technique in the analysis of relatedness between groups of organisms (AVISE *et al.* 1979a, 1979b; FERRIS *et al.* 1981; KESSLER & AVISE 1985; LANSMAN *et al.* 1983). Mt DNA is a small

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molecule (approximately 17 kilobase pairs in salmonids) and, in higher animals, lacks many of the genetic complexities that may confound attempts at reconstruction of nuclear DNA phylogenies (AVISE 1986). Mt DNA is maternally inherited and thus is not subject to segregation and recombination (de FRANCESCO *et al.* 1980). Mt DNA evolves approximately 5 to 10 times faster than nuclear DNA thus increasing the probability of detecting sequence heterogeneity within species (BROWN *et al.* 1979). Finally, mutations leading to sequence heterogeneity are of the single base substitution type rather than additions and deletions so that mt DNA accumulates mutations linearly in time allowing calibration of a molecular clock (BROWN 1983).

The most common analysis of mt DNA polymorphism involves digesting the molecule with a series of restriction endonucleases that recognize and cleave specific sequences of 4 to 6 nucleotides producing a number of fragments of different sizes which can be visualized by different methods (LANSMAN *et al.* 1981). The fragments thus obtained constitute the raw data set for further analysis.

Before undertaking an extensive survey of intra-specific geographic variability based on mt DNA polymorphisms, the method was first applied to detect sequence divergence between species. In this paper, we describe the procedures used for isolating mt DNA from frozen samples and present results of mt DNA sequence divergence between the four anadromous salmonid species that comprise the major part of the estuarine communities of eastern James-Hudson Bay: *C. artedii*, *C. clupeaformis*, *Prosopium cylindraceum* (round whitefish) and *Salvelinus fontinalis* (brook charr) that we used as a representive of the salmonine line in order to compare divergence between the two salmonid subfamilies. In addition, differences in the frequency distribution of mt DNA polymorphisms among 2 populations of *C. clupeaformis* are presented.

II. Materials and methods

1. Sampling

Populations of the major river systems located on the east coast of James-Hudson Bay (Figure 1) were sampled with gillnets from May to July 1987. Interspecific results presented here are based on fish sampled in the Povungnituk river. The intra-specific study compares adult *C. clupeaformis* from the Povungnituk and Eastmain rivers. Live fish were retained for analysis and transported on ice to shore stations. Liver and small developping gonads were removed and kept on dry-ice for 1 to 2 months before mt DNA was extracted.

2. Preparation of mitochondrial DNA

We extracted mt DNA from 8 fish per day using a modified version of the GONZALEZ—VILLASENOR *et al.* (1986) technique. All the manipulations are done at 4°C. About 3 g of frozen eggs or liver are homogenized with a teflon homogenizer in 15 ml TKC buffer, pH 7.5 (Tris-HCl 50 mM, KCl 1.5 %, $CaCl_2$ 3 mM), and EDTA is then added to a final concentration of 10 mM. The homogenate is centrifuged twice at 1000 g for 10 min in 50 ml tubes (Sorvall centrifuge, rotor SS-34) to remove most of the nuclei and cellular debris. The supernatant is poured into a 15 ml conical tube, and 10 ml of 15 % sucrose is underlayered by passing a Pasteur pipet through the homogenate. The mixture is centrifuged for 10 min in a clinical centrifuge. The upper phase is then transferred into a 50 ml tube and centrifuged for 45 min at 18,000 g. The resulting pellet is collected and resuspended in 10 ml TK (Tris-HCl 50 mM, KCl 1.5 %, EDTA 10 mM) buffer and centrifuged again at 18,000 g. The pellet is then resuspended in 2 ml TK buffer, 200 µl of 10 % Nonidet is added to lyse mitochondria, and the suspension is kept on ice for 30 min after which it is

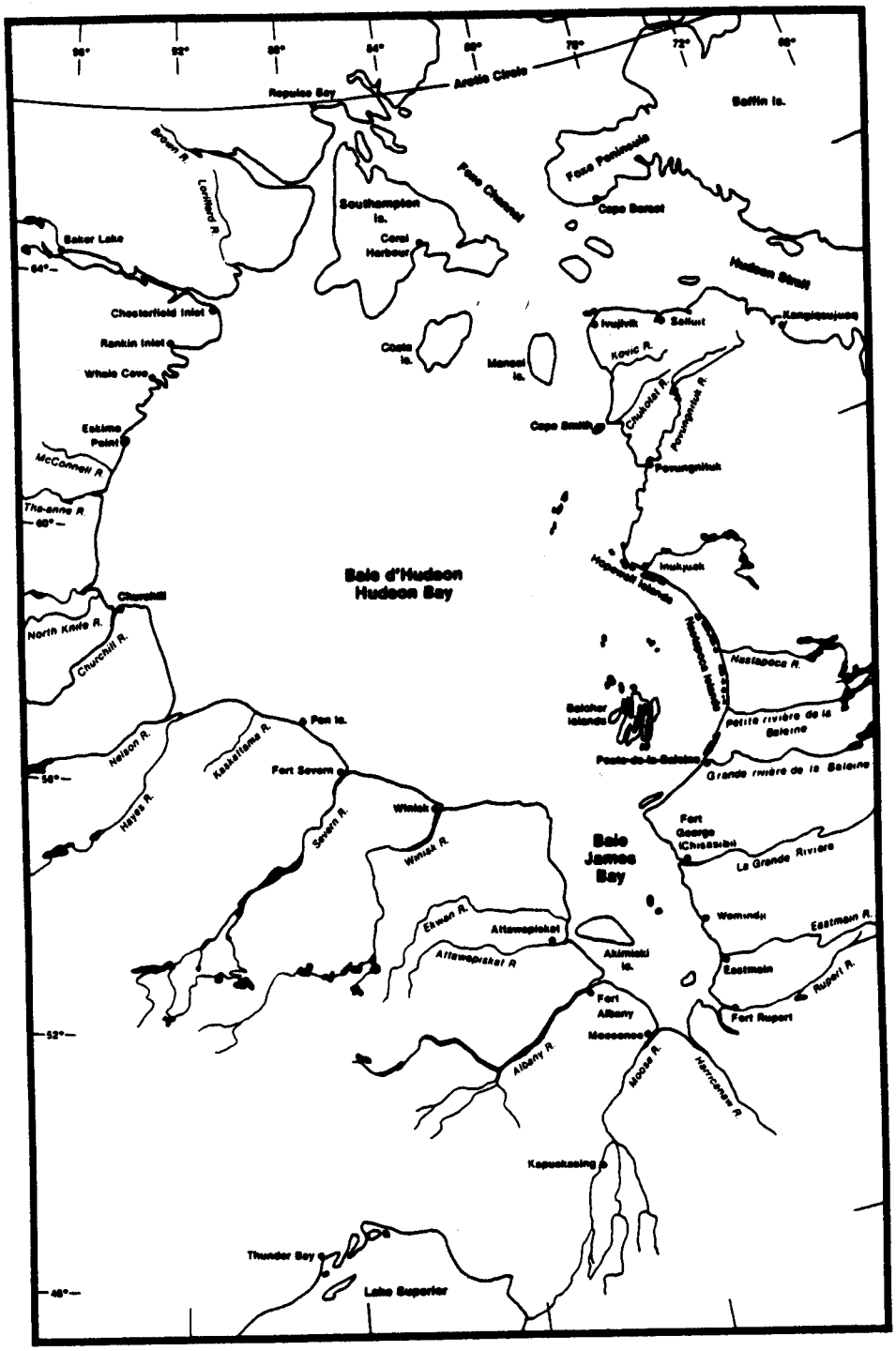

Figure 1. Location map of the stations sampled on the east coast of James and Hudson Bays from May to July 1987. Reproduced with permission from Le Naturaliste Canadien (revue d'écologie et de systématique) vol. 109(4), 1982.

Table 1. Fragment patterns of mitochondrial DNA found in the four species studied.

Enzyme	*C. clupeaformis*	*C. artedii*	*P. cylindraceum*	*S. fontinalis*
Bgl I	A	A	B	C
Xba I	A	A	B	C
Pst I	A	A	B	A
Sst II	A	A	B	A
Ava I	A	B	C	D
Dra I	A	B	C	D
Hpa I	A	B	C	D
Hinc II	A	B	C	D
Pvu II	A	B	C	D
Hind III	A	B	C	D
Acc I	A	B	C	D
Sma I	A	B	C	D
Ava II	A	B	C	D
Fnu DII	A	B	C	D

transferred to microfuge tubes and centrifuged for 5 min. The supernatant is transferred to 15 ml Pyrex tubes, and extracted successively with phenol, phenol-chloroform (1:1), and chloroform-isoamyl alcohol (24:1). Two and a half volumes of 95 % ethanol is added to the aqueous phase and mt DNA is allowed to precipitate overnight at —20°C. The sample is centrifuged at 18,000 g for 30 min. The pellet is resuspended in 225 µl TE buffer (Tris 10 mM pH 8.0, EDTA 1 mM pH 8.0) and kept at 4°C until used.

3. Restriction enzyme analysis

With each restriction enzyme used, about 100 ng (15 µl) of mt DNA was digested according to the conditions specified by the manufacturer. A total of 14 and 9 restriction endonucleases were used for the inter-and intraspecific study respectively (Tables 1,3). Many other enzymes were tried and were rejected because they produced 1 or no fragments, thus being non-informative (Aat II, Bgl II, BamH 1, Cla 1, EcoR 1,

Table 2. Estimates of mt DNA sequence divergence among the four species studied (above diagonal) with corresponding standard deviations (below diagonal).

	S. fontinalis	*P. cylindraceum*	*C. clupeaformis*	*C. artedii*
S. fontinalis	—	8.18	8.62	10.32
P. cylindraceum	1.67	—	7.84	10.77
C. clupeaformis	1.65	1.64	—	3.74
C. artedii	1.93	2.05	0.92	—

Table 3. Description of the clonal lines observed among *C. clupeaformis* sampled in the Eastmain and Povungnituk rivers in 1987. The clonal lines are labelled by numbers and refer to any combination of fragments patterns (designated by capital letters) distinguishable from all other lines.

Fish	Enzymes									
	Ava I	Dra I	Bgl I	Hpa I	Hinc II	Pvu II	Hind III	Ava II	Sma I	Clonal line
Eastmain										
1	A	A	A	A	A	A	A	A	A	1
2	A	A	A	A	A	A	A	A	A	1
3	A	A	A	A	A	A	A	A	A	1
4	A	A	A	A	A	A	A	A	A	1
5	A	A	A	A	A	A	A	A	A	1
6	A	A	A	A	A	A	A	A	A	1
7	A	A	A	A	A	A	A	A	A	1
8	A	A	A	A	A	A	A	A	A	1
9	A	A	A	A	A	A	A	A	A	1
10	A	A	A	A	A	A	A	A	A	1
11	A	—	A	A	A	A	A	B	A	5
Povungnituk										
1	A	A	A	A	A	A	A	A	A	1
2	A	A	A	A	A	A	A	A	A	1
3	A	A	A	A	A	A	A	A	A	1
4	A	A	A	A	A	A	A	A	A	1
5	A	—	A	A	A	A	A	A	A	1
6	A	A	A	A	B	A	A	A	A	2
7	A	A	A	A	B	A	A	A	A	2
8	A	A	A	A	B	A	A	A	A	2
9	A	A	A	A	C	A	A	A	A	3
10	B	B	B	A	D	B	B	C	A	4
11	A	A	A	A	C	A	A	A	A	3

Mlu 1), or often gave partial digestions (Acc 1, FnuD II) in the case of the intra-specific study.

Mitochondrial DNA digests were subjected to agarose gel electrophoresis to separate the fragments produced according to molecular weight. The electrophoresis was performed using horizontal slab gels containing 0.8 % or 1.2 % agarose, depending on the size of fragments, at 35 V overnight in a cold room (4°C). Ethidium bromide (0.1 $\mu g.ml^{-1}$) was included in the gel and mt DNA bands were visualised for photography with U.V. light.

Size estimates of fragments obtained were made by interpolation on a standard curve evaluated from fragments produced by digestion of phage DNA with Hind III and EcoR I.

4. Analysis

Two methods were used to analyse interspecific phylogenetic relationships. We first estimated the percent sequence divergence using the comparative fragment method of NEI & LI (1979). The unweighted pair group method using arithmetic average (UPGMA) was then used to cluster the species from the sequence divergence estimates (SNEATH & SOKAL 1973). In addition, we constructed a phylogenetic tree from the Wagner network parsimony technique using the presence or absence of all fragments as the character states (BERG & FERRIS 1984).

III. Results

1. Interspecific results

The 14 restriction endonucleases employed in this study produced a total of 70 to 75 fragments, depending on species, which represents a sample of about 2.5 % of the mt DNA molecule. Figure 2 gives examples of some cut-site variations observed.

The fragment pattern assignments, designated by letters for each of the 14 enzymes used, are given in Table 1. The enzymes produced a different pattern for each species. *C. clupeaformis* and *C. artedii* share identical patterns for 4 enzymes, illustrating the close affinity of the two species. No pattern is shared by *Prosopium* and *Coregonus*. Relatively close affinity of the two *Coregonus* species is also expressed by a divergence estimate of 3.74 ± 0.92 % while *P. cylindraceum* diverges from *C. artedii* and *C. clupeaformis* by 10.77 ± 2.05 % and 7.84 ± 1.64 % respectively (Table 2). Divergence estimates between *P. cylindraceum* and *S. fontinalis*, and between *P. cylindraceum* and *C. clupeaformis* do not differ significantly suggesting that the time *Prosopium* diverged from *Coregonus* is similar to its divergence from *Salvelinus fontinalis*. A UPGMA phenogram based on the sequence divergence estimates between the species is given in Figure 3. Assuming that mt DNA evolves at a rate of 2% sequence divergence/million years (BROWN 1983), *C. artedii* and *C. clupeaformis* diverged from a common ancester less than 2 million years ago during the early Pleistocene. Although *Prosopium* and *Salvelinus* were clustered together, their branching point does not differ significantly from that linking their ancestral line to the *Coregonus* line. It is suggested that separation of all three genera occurred some 4 to 5 million years ago during the late Pliocene. Results of the Wagner network represents the arrangement of the four species according to the analysis of the presence or absence of restriction fragments (Fig. 4). The

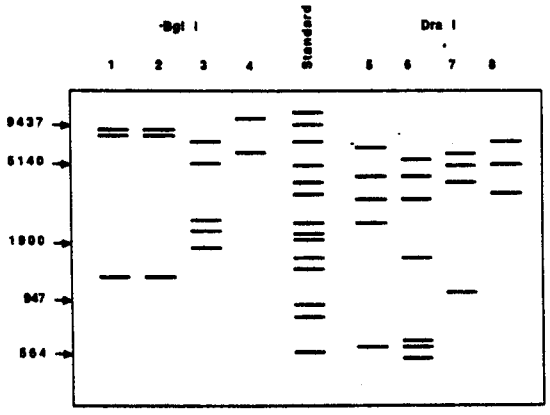

Figure 2. Mitochondrial DNA fragment patterns produced by digestion with the six base restriction endonucleases. Bgl I (columns 1—4) and Dra I (5—8). A pattern is defined as the combination of fragments produced by digestion with a particular enzyme. Pattern 1 and 5: *C. clupeaformis;* 2 and 6: *C. artedii*; 3 and 7: *P. cylindraceum;* 4 and 8: *S. fontinalis*. The standard pattern is a mixture of lambda phage digested with Hind III and double digested with Hind III and EcoR I. Fragment size is expressed as number of base pairs.

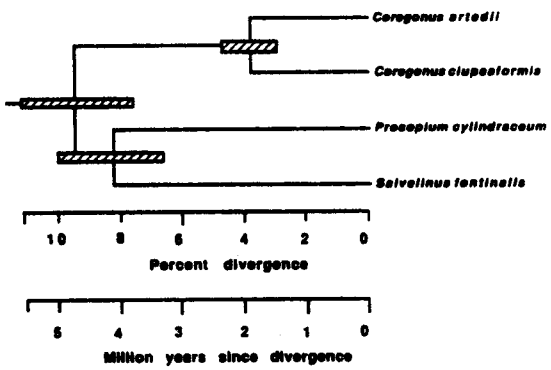

Figure 3. Phenogram showing estimated percent sequence divergence among mt DNAs of the four species studied. Estimation of time of divergence is based on Brown's calibration of mt DNA evolution determined for mammalian species (BROWN 1983). Horizontal bars represent standard deviations.

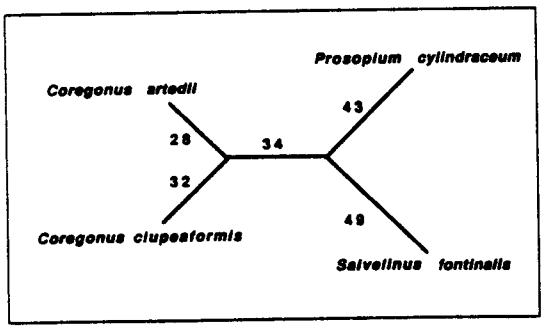

Figure 4. Arrangement of the four species studied according to the Wagner network analysis. Numbers between species refer to the fragment differences between their mt DNAs.

network illustrates the same pattern obtained from the UPGMA analysis, regrouping the 2 coregonine species close together and separating them widely from either *Prosopium* or *Salvelinus*.

2. Intraspecific divergence

Considerable Mt DNA polymorphism is observed in *C. clupeaformis* samples from the Eastmain and Povungnituk rivers (Table 3). A total of 5 clonal lines (labelled 1—5) were identified. A clonal line corresponds to a combination of fragment patterns (capital letters, Table 3) distinguishable from all other lines. The frequency distribution of the clonal lines differs between the two populations studied (Fig. 5). Fish from the Eastmain River are more homogeneous with 10 out of 11 fish belonging to the same clonal line. A maximum of 5 fish per clonal line is observed in the Povungnituk sample. Three clonal lines are exclusive to the Povungnituk River and one exclusive to the Eastmain River.

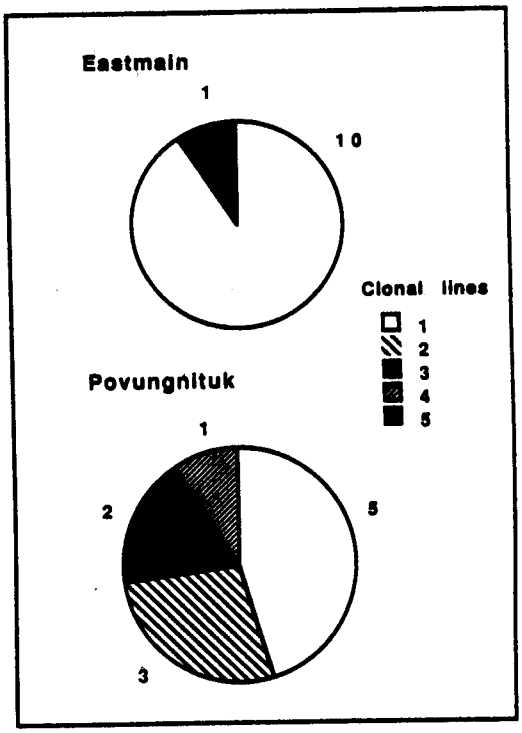

Figure 5. Distribution of the clonal lines identified among 22 *C. clupeaformis* from the Eastmain and Povungnituk rivers sampled in 1987. Description of the clonal lines are given in Table 3.

IV. Discussion

1. Interspecific results

The estimate of mt DNA sequence divergence between three coregonine species and one salmonine representative agrees with conclusions based on comparative osteology, karyology, nuclear DNA and protein analysis (NORDEN 1961, 1970; BOOKE 1968; CHELLEVOLD 1970). All tech-

niques indicate a close relationship between cisco (*C. artedii*) and whitefish (*C. clupeaformis*) and the early divergence of *Prosopium* from the main coregonine line, placing it closer to the salmonine line. The present study reveals no significant difference in the divergence of *Prosopium* from either *Coregonus* or *Salvelinus* indicating an even earlier separation of *Prosopium* than suggested to date.

Estimates of sequence divergence obtained in other studies of salmonid mt DNA add confidence to the values obtained in the present study. Our estimate of divergence between species belonging to the same genus (3.74 %) is close to that measured between *Salmo gairdneri* and *S. clarkii* (2.6 %; WILSON *et al.* 1984) and between *Salvelinus namaycush* and *S. alpinus* (3.35 %; GREWE & HEBERT 1987). Our estimates of divergence between species of different genera (from 7.84 to 10.77 %) resemble those measured between *Oncorhyncus* sp. and *Salmo trutta* and *Salvelinus fontinalis* (7.2 %, 10 %; BERG & FERRIS 1984).

2. Time of divergence

The absolute times of divergence estimated here must be considered provisional as the rate of mt DNA evolution from which they are derived was estimated from mammalian lineages (BROWN *et al.* 1979, BROWN 1983). The authors found that for mammal species separated less than 10 million years ago (based on fossil records), mt DNA sequences were calculated to accumulate differences linearly at a rate of 2 percent per million years. More recently, a similar estimate of the rate of mt DNA evolution was found in birds (SHIELDS & WILSON 1987). The ability to determine branching times in fish phylogeny is compromised by the lack of complete fossil records and insufficient knowledge concerning rates of mt DNA sequence evolution. However, from what is known about the biology of mt DNA (BROWN 1983), there appears no reason why fish mt DNA should not accumulate mutations linearly. Thus, even though the rate of fish mt DNA evolution may be different from that of mammals and birds, the estimate of time divergence provides information concerning the relative time of separation between groups of fishes.

3. Intraspecific results

Considerable polymorphism was observed in the study of *C. clupeaformis* populations from the Eastmain and Povungnituk rivers. The degree of mt DNA polymorphism in fishes documented in other studies is highly variable and does not seem to follow a general trend. For instance, GREWE and HEBERT (1987) identified only one clonal line in a sample of 15 *S. namaycush* while 5 lines were found among 17 fish from another population. THOMAS *et al.* (1986) observed a single clonal line among two separate samples of 12 *O. nerka* while WILSON *et al.* (1984) found no intrapopulation variation in 8 populations of *S. gairdneri*. Each population was distinguished by one different clonal line.

Our results suggest that the whitefish population of Povungnituk R. is more heterogenous that the Eastmain population in terms of mt DNA sequence divergence. One hypothesis that may explain differences in diversity is that the two systems were colonized by fish from different origins following deglaciation. A second hypothesis arises from a more theoretical approach. Recently, AVISE *et al.* (1984) have modeled the probability of survival of independant female lineages. They demonstrated that the number of lineages decreases in time due to random extinction associated with the vagaries of reproduction. The model predicts that within roughly 4n generations, stable-sized populations started with n females will be very likely to trace ancestries to a single female. Thus, the greater diversity of clonal lines in the Povungnituk River may be associated with a shorter time span since colonization following the northerly retreat of glaciers. However, other factors such as the number of

clonal lines initiating the population and the population size at carrying capacity may also influence clonal line diversity.

Our premilinary intraspecific studies of lake whitefish reveal a pattern of mt DNA sequence divergence between two populations of James-Hudson Bay consistent with the hypothesis of stock discreteness. However, as the two populations cannot be discriminated by the presence or absence of exclusive fragment patterns, further studies of stock discreteness in this area may have to depend on the use of frequency distributions of clonal lines among populations. In order to correctly apply this procedure we are presently investigating the number of individuals and the proportion of the mt DNA genome that must be analyzed to provide reliable estimates of intra-population mt DNA variation.

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