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Mitochondrial DNA Variation among Lake Trout (*Salvelinus namaycush*) Strains Stocked into Lake Ontario

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Mitochondrial DNA (mtDNA) variation was examined in 492 fish representing six lake trout (*Salvelinus namaycush*) strains used for stocking and restoring populations in Lake Ontario. mtDNA was extracted from 432 fish by a total DNA isolation protocol (CTAB). mtDNA was also extracted from 60 additional fish using the purification method of CsCl ultracentrifugation. The more rapid CTAB protocol made feasible analysis of sample sizes ($n \geq 80$ per strain) required as baseline data for future mixed-stock analysis (MSA). Restriction enzymes *AvaI*, *BamHI*, *HinfI*, and *TaqI* resolved seven mtDNA haplotypes and were used to characterize fish from each of six strains (Clearwater, Jenny, Killala, Manitou, Seneca, and Superior). Frequencies of these haplotypes were significantly different among the six strains ($p < 0.001$). Differences between haplotype frequencies of the Killala and Superior strains were striking and permit greater discrimination of these strains than allozyme data. The level of differentiation observed among strains indicates that mtDNA haplotype information will enhance the ability of MSA to determine the hatchery strains that serve as parents to lake trout fry collected from Lake Ontario.

La variation de l'ADN mitochondrial (ADNmt) a été étudiée chez 492 poissons représentant six souches de touladi (*Salvelinus namaycush*) utilisées pour l'ensemencement et le rétablissement des populations dans le lac Ontario. L'ADNmt a été extrait chez 432 poissons selon un protocole d'isolement de l'ADN total (CTAB). On a également extrait ce type d'ADNmt chez 60 autres poissons selon une méthode de purification par ultracentrifugation en CsCl. La méthode plus rapide du CTAB a permis d'analyser le nombre d'individus ($n \geq 80$ par souche) nécessaires comme données de base en vue de l'analyse future de stocks mélangés. Des enzymes de restriction *AvaI*, *BamHI*, *HinfI* et *TaqI* ont établi sept haplotypes d'ADNmt et ont été utilisées pour distinguer les caractères des poissons de chacune des six souches (Clearwater, Jenny, Killala, Manitou, Seneca et Superior). Les fréquences de ces haplotypes étaient significativement différentes dans les six souches ($p < 0,001$). Les différences entre la fréquence des haplotypes des souches Killala et Superior étaient marquées et elles ont permis de mieux distinguer ces souches que les données sur les allozymes. Le niveau de différenciation observé entre les souches montre que grâce aux données sur les haplotypes d'ADNmt, l'analyse de stocks mélangés permettra de mieux déterminer les souches provenant de piscifactoreries qui servent de parents aux alevins de touladi prélevés dans le lac Ontario.

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Lake trout (*Salvelinus namaycush*) have been stocked into Lake Ontario since 1973 as part of a program to reestablish self-perpetuating populations that disappeared from the

lake in the mid-1950's due to predation by sea lamprey (*Petromyzon marinus*), habitat degradation, and overfishing. Achievement of this species restoration goal requires agencies to reintroduce strains that will survive to maturity and successfully reproduce (Schneider et al. 1983; Eshenroder et al. 1984). Analysis of fin-clip and coded wire tag data has permitted comparison of strain performance after stocking (e.g., Elrod and

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Schneider 1986) but has not allowed assessment of reproductive success (i.e., fry production) in the wild. Comparison of reproductive success among lake trout strains in Lake Ontario has been identified by management agencies as a research priority (Schneider et al. 1983). Efforts to examine natural reproduction of lake trout in Lake Ontario have intensified since 1986 when substantial numbers of fry were collected near the eastern shore of the lake (Marsden et al. 1988).

Evaluation of reproductive success requires the use of genetic markers that can discriminate among stocked strains (e.g., allozymes or mitochondrial DNA (mtDNA); Grewe and Hebert 1988; Ihssen et al. 1988; Krueger et al. 1989). Analysis of allozyme variation among strains stocked into Lake Ontario revealed the presence of 18 polymorphic loci that could be used as informative characters to genetically differentiate strains (Krueger et al. 1989). These data were subsequently used in mixed-stock analysis (MSA) to determine the parents that contributed gametes to a sample of wild fry captured in 1986 from Stony Island reef, Lake Ontario (Marsden et al. 1989). Fry in the mixture sample could have resulted from 10 potential inter- and intrastain crosses among four strains of hatchery-origin lake trout previously stocked in Lake Ontario (Clearwater, Manitou, Seneca, and Superior strains). Since 1986, two additional strains (Jenny and Killala) have also matured. These six strains could now produce 21 potential inter- and intrastain fry types. Unfortunately, the Killala and Superior strains exhibit a low level of differentiation from each other as detected by allozyme analysis (Krueger et al. 1989). The low level of genetic differentiation in combination with an expanded number of potential fry types substantially reduces the precision of MSA estimates of parental strain contributions (Wood et al. 1987; Marsden et al. 1989). Therefore, additional genetic characters were needed to better differentiate the parental strains and improve precision of future MSA estimates.

mtDNA restriction site polymorphisms can potentially provide an additional genetic character for use in combination with allozyme markers to identify lake trout strains. The resolution capability of mtDNA studies appears to be enhanced by the rapid evolutionary rate of mtDNA, estimated for some vertebrates to be 6–10 times the rate of the nuclear genome (Brown et al. 1979). A preliminary survey of lake trout revealed 13 mtDNA haplotypes among the Clearwater, Killala, Lewis, Manitou, Seneca, and Superior hatchery strains (Grewe and Hebert 1988). However, no mtDNA studies have been conducted with large enough sample sizes ($n \geq 80$ fish per strain) to quantitatively estimate the level of differentiation among strains and provide baseline data for MSA.

The present study describes and compares mtDNA variation among six strains of lake trout stocked into Lake Ontario. The data presented herein document the mtDNA baseline data to be used in future MSA of fry mixtures collected from Lake Ontario.

Methods

Sample Size Considerations

The desired precision, and hence the sample size used, for measurement of the mtDNA haplotype variation in each lake trout strain was set to provide confidence that 95% of the time, at least one individual will be detected of every haplotype present at proportions (P) greater than or equal to 5%. In order to determine the minimum sample size (n) required to satisfy this condition, we used $(1 - P)^n$, the probability of not observing a

haplotype in a random sample of n individuals (see Schwager et al. 1990). The probability (β) of observing at least one individual with a specified haplotype in n randomly selected individuals is

$$(1) \quad \beta = 1 - (1 - P)^n.$$

Calculation of the minimum sample size required to have a certain probability or confidence (β) that at least one individual with a specified haplotype, present at $P \geq 0.05$, will be observed in a random sample of n individuals can be obtained by rearrangement of equation (1):

$$(2) \quad n = \frac{\ln(1 - \beta)}{\ln(1 - P)}.$$

Solving equation (2) by substituting the desired probability of 0.95 (95% confidence) for β and 0.05 for P gives 58.4 individuals. Thus a minimum sample size of approximately 60 individuals is required to observe with probability 0.95 at least one individual containing a haplotype that has a proportion of $P \geq 0.05$ in a population (Fig.1). In contrast, given $n = 15$, a sample size typical of many mtDNA surveys, haplotypes present at a population frequency $P < 0.181$ will be missed 5% of the time, and 50% of the time the haplotypes present at $P < 0.045$ will remain undetected. For the present study, a more conservative minimum sample size of $n = 80$ was chosen. This sample size gives 98.4% confidence that at least one individual should be observed of those haplotypes present at a true population frequency of $P \geq 0.05$.

Collections

Four hundred ninety-two fish of the Clearwater, Jenny, Killala, Manitou, Seneca, and Superior lake trout strains were analyzed. Samples of strains were obtained from the various hatcheries used to stock Lake Ontario (Table 1). These samples represented progeny from descendants of fish originally collected from various locations in the Great Lakes watershed with the exception of the Clearwater strain, which originated from Clearwater Lake in northern Manitoba, Canada (Fig.2). Most fish sampled (432 of 492 individuals) were either fingerling or yearling trout. Effort was made to randomly sample hatchery fish that were progeny of the entire broodstock (e.g., from several raceways). Fish were frozen on site in liquid nitrogen and stored at -80°C until analyzed. The 60 remaining individuals were representative of the Clearwater, Killala, and Superior lake trout strains that were previously analyzed by Grewe and Hebert (1988).

DNA Isolation and Restriction Analysis

Total DNA was isolated from frozen tissue samples (liver or white muscle) using a modified CTAB (hexadecyltrimethylammonium bromide) isolation procedure (Saghai-Marooof et al. 1984; Doyle and Doyle 1987). Frozen tissue (10–20 mg) was homogenized in 700 μL of extraction buffer (50 mM Tris (pH 8.0), 0.7 M NaCl, 10 mM EDTA, 1% CTAB, 0.1% 2-mercaptoethanol). One hundred micrograms of proteinase K was added and samples were incubated at 60°C for 30–60 min. Samples were then extracted once with chloroform – isoamyl alcohol (24:1), once with phenol – chloroform – isoamyl alcohol

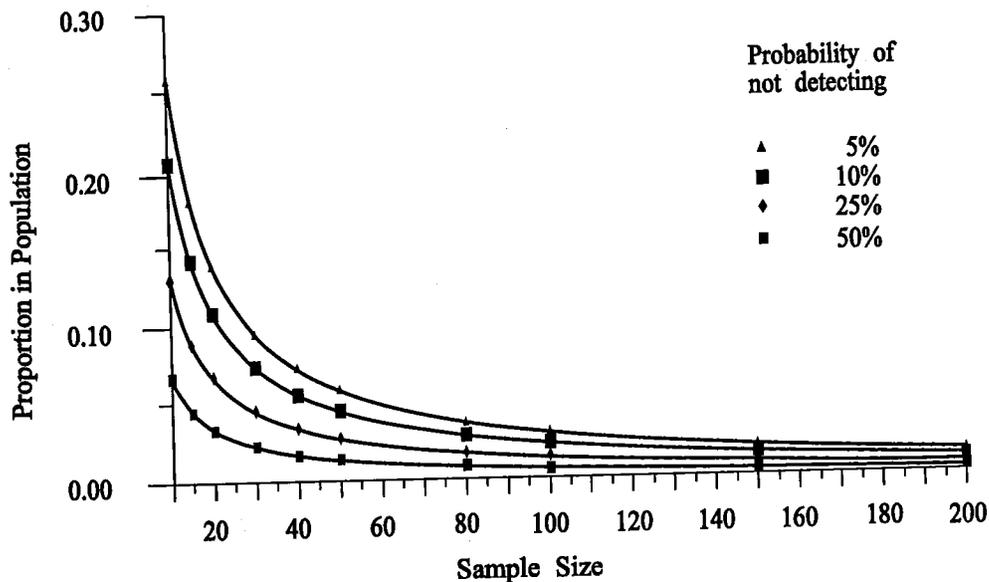


FIG. 1. Probabilities (0.05, 0.10, 0.25, and 0.50) that at least one individual of each haplotype that occurs at a proportion P in a population is observed within a sample size of n individuals.

TABLE 1. Strains, sample size, year class, and origin of lake trout samples examined.

Strain	n	Year class	Origin of fish and date obtained
Clearwater	11 ^a	1983	Clearwater Lake gamete collection, fall 1982
	71	1983	Clearwater Lake gamete collection, fall 1982
Jenny	80	1987	Allegheny National Fish Hatchery, March 1988
Killlala	11 ^a	1983	Hills Lake Provincial Fish Hatchery, August 1985
	69	1987	Harwood Provincial Fish Hatchery, March 1989
Manitou	80	1987	Harwood Provincial Fish Hatchery, March 1989
Seneca	90	1989	Bath State Fish Hatchery, Bath, N.Y., December 1989
Superior	10 ^a	1975	Jordan River National Fish Hatchery, November 1984
	8 ^a	1981	Jordan River National Fish Hatchery, November 1984
	20 ^a	1977	Jordan River National Fish Hatchery, November 1985
	42	1987	Allegheny National Fish Hatchery, March 1988

^aSamples previously analyzed by Grewe and Hebert (1988).

(25:24:1), and then a final time with chloroform – isoamyl alcohol (24:1). The aqueous phase was then collected and mixed with an equal volume of isopropanol. Samples were then placed at -20°C for a minimum of 1h after which precipitated DNA was pelleted by centrifugation in a microfuge. The DNA pellet was washed twice with 70% ethanol, dried under vacuum, and resuspended in water.

Restriction enzymes *Ava*I, *Bam*HI, *Hin*fI, and *Taq*I were chosen for this study because they had previously revealed haplotype variability among the Clearwater, Killlala, Manitou, Seneca, and Superior strains (Grewe and Hebert 1988). Restriction fragments were separated in horizontal 1.2% agarose gels submerged in a Tris–borate–EDTA (TBE) buffer system (Maniatis et al. 1982). DNA was transferred to a nylon membrane filter (Zetabind™) by southern transfer (Maniatis

et al. 1982). Nylon membrane filters were probed with lake trout mtDNA (50 ng used per eight 20×20 cm blots probed) purified by CsCl ultracentrifugation. The probe was labeled with ^{32}P using Amersham's Prime Time™ random priming kit. Filters were exposed to Kodak XAR5 X-ray film for 12–48 h. Fragment sizes of restriction profiles used to describe mtDNA haplotypes are listed in Table 2. Haplotypes were classified as described by Grewe and Hebert (1988) and Grewe (1991).

Statistical Analysis

Frequencies of mtDNA haplotypes were compared statistically among samples using contingency table analysis with the G -statistic (Sokal and Rohlf 1981). A significance level of $p \leq 0.05$ was used to reject the null hypothesis of homogeneity

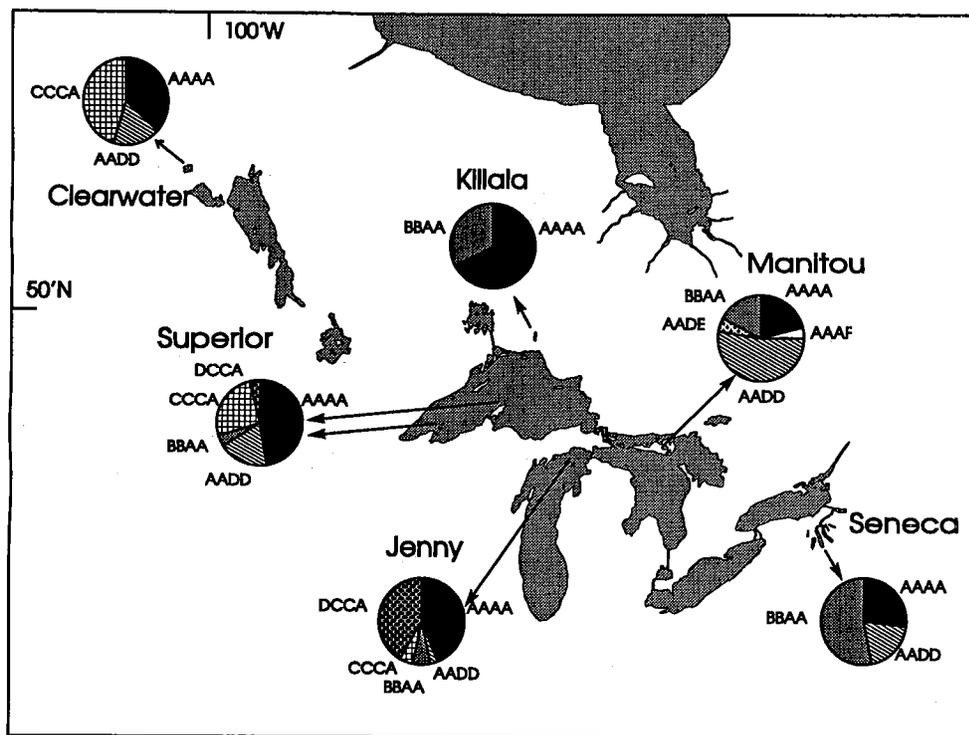


FIG. 2. Location of wild populations used to originate the Clearwater, Jenny, Killala, Manitou, Seneca, and Superior strains used for stocking into Lake Ontario. Pie diagrams display frequencies of the seven mtDNA haplotypes observed within each of the strains.

of haplotype frequencies among strains. AA AF and AA DE haplotypes, found only in the Manitou strain, were combined with the AADD haplotype category to eliminate the number of cells containing fewer than five observations in the expected contingency table.

Results

Seven different mtDNA haplotypes were resolved among the six strains using the four restriction enzymes *Ava*I, *Bam*HI, *Hin*FI, and *Taq*I (Table 3). Six of these haplotypes (designated AAAA, AADD, AADE, BBAA, CCCA, and DCCA) were previously reported by Grewe and Hebert (1988). The AADE (observed in four individuals) and newly discovered AA AF (three individuals) haplotypes were found only among individuals of the Manitou strain. The five other common haplotypes were each present in 36 or more of the 492 fish (Table 3).

The six lake trout strains exhibited significantly different frequencies of the seven mtDNA haplotypes (G -value = 477.1, $df = 25$, $p < 0.001$). All six strains possessed the AAAA haplotype at frequencies that ranged from 21 to 67%. The Clearwater, Manitou, Seneca, and Superior strains possessed the AADD haplotype at frequencies of 18, 52, 20, and 18%, respectively. The Jenny strain also contained this haplotype but at a significantly reduced frequency (3%). The Seneca and Killala strains had the highest frequencies of the BBAA haplotype (32 and 53%, respectively) as compared with the Manitou (18%), Jenny (6%), and Superior (4%) strains. The Clearwater, Jenny, and Superior strains contained the CCCA haplotype which was not observed in the Killala, Manitou, or Seneca strains. The Jenny and Superior strains were the only ones that had the DCCA haplotype (43 and 4%, respectively). Three haplotypes (AADD, CCCA,

and DCCA) found in the Superior strain at proportions of 19, 26, and 4%, respectively, were not detected in the Killala strain. Further, the BBAA haplotype occurred in the Killala strain at a frequency of 33%, but was found at a significantly lower frequency in the Superior strain (4%).

Discussion

mtDNA haplotype variation reported here among the lake trout strains helps illustrate the caution investigators should exercise when drawing conclusions from mtDNA surveys that use small sample sizes (e.g., $n < 15$; Fig. 1). Studies that examine such small sample sizes can qualitatively demonstrate that haplotype variation exists but should not be used to characterize populations and establish MSA baseline data. For example, sample sizes ($n < 15$ for eight of the nine strains) examined by the study of Grewe and Hebert (1988) were sufficient to demonstrate the presence of most of the mtDNA haplotypes observed. This previous study, however, was insufficient to estimate haplotype frequencies at a level of precision suitable for incorporation as baseline data for MSA. In the earlier survey, a sample size of 38 was used to characterize the Superior strain but was insufficient to detect BBAA individuals. This haplotype was later observed in the present survey (4%; Table 3) when the sample size was increased to 80 fish. Data from the Manitou strain represent a more drastic example where the AAAA haplotype was present at a proportion of 21% in the final sample of 80 individuals but was missed in the earlier sample of four individuals.

Time required to analyze individuals in the laboratory (both isolation of purified mtDNA and visualization of restriction fragments) has often governed the sample sizes used to characterize populations. The total DNA isolation protocol (CTAB) used for

TABLE 2. Fragment sizes (in base pairs) for each restriction profile produced by the various enzymes. Additional fragments smaller than those reported here were present in the *HinfI* and *TaqI* restriction digests. These fragments are not reported here because they did not further differentiate the restriction profiles described by this study. Dashes indicate fragments missing from a specific restriction profile.

Enzyme	Restriction profile					
	A	B	C	D	E	F
<i>AvaI</i>	5480	5480	5480	5480		
	2650	2650	2650	2650		
	2210	2210	—	—		
	2130	—	—	—		
	—	—	1840	1840		
	1680	1680	1680	1680		
	—	1520	1520	—		
	1410	1410	1410	1410		
	1100	1100	1100	1100		
	—	—	—	898		
	—	—	—	622		
	—	610	610	610		
	565	565	565	565		
	—	—	370	370		
<i>BamHI</i>	16800	—	—			
	—	9475	9475			
	—	7325	—			
	—	—	5000			
	—	—	2325			
<i>HinfI</i>	2030		2030	2030		
	1260		1260	1260		
	1090		1090	1090		
	975		975	975		
	882		882	882		
	—		827	—		
	788		788	788		
	735		—	—		
	690		690	690		
	—		—	645		
	564		564	564		
<i>TaqI</i>	3670			—	—	3670
	—			—	2250	—
	—			2170	—	—
	—			—	—	2000
				1500	1500	—
	1140			1140	1140	1140
	1050			1050	1050	1050
	990			990	990	990
	952			952	952	952
	933			933	933	933
	825			825	825	825
	670			670	670	—
	638			638	638	638

the present study permitted sufficient quantities of DNA (enough for 20 restriction digests from 10 mg of tissue) to be extracted from 80 individuals per day. Further decreases in sample processing time can be achieved through techniques that speed visualization time of restriction fragments. For example, amplification of small sections of the mitochondrial genome can be achieved through polymerase chain reaction (PCR) techniques to produce sufficient quantities of pure mtDNA. Restriction profiles of this DNA can then be visualized through ethidium bromide staining, rather than by the more time-consuming method of Southern blotting. Thus, given the speed of current techniques

now available, the time required to process individuals should not be a factor that determines the total number of individuals sampled from populations. Reduced processing time will help increase sample sizes and will permit stronger conclusions to be drawn about population structure.

mtDNA in lake trout provides another useful genetic character that improves, over use of allozyme data only, the capability to discriminate among the six hatchery strains. An example of the advantage gained with mtDNA data is illustrated by the level of differentiation between the Killala and Superior hatchery strains which originated within close proximity to each other in the Lake

TABLE 3. Numbers and frequency of mtDNA haplotypes among six lake trout strains assayed with *Ava*I, *Bam*HI, *Hinf*I, and *Taq*I. Values in parentheses are 95% confidence limits of the frequency estimates. Four-letter haplotype designations correspond to previously described fragment patterns of *Ava*I, *Bam*HI, *Hinf*I, and *Taq*I (Grewe and Hebert 1988; Grewe 1991).

Strain	Haplotype designation							Total
	AAAA	AAAF	AADD	AADE	BBA A	CCCA	DCCA	
Clearwater								
<i>n</i>	30	—	15	—	—	37	—	82
Frequency	0.366 (0.26–0.47)	—	0.183 (0.10–0.27)	—	—	0.451 (0.34–0.56)	—	
Jenny								
<i>n</i>	35	—	2	—	5	4	34	80
Frequency	0.438 (0.33–0.55)	—	0.025 (0.00–0.06)	—	0.063 (0.01–0.12)	0.050 (0.00–0.10)	0.425 (0.32–0.53)	
Killala								
<i>n</i>	54	—	—	—	26	—	—	80
Frequency	0.675 (0.57–0.78)	—	—	—	0.325 (0.22–0.43)	—	—	
Manitou								
<i>n</i>	17	3	42	4	14	—	—	80
Frequency	0.213 (0.12–0.30)	0.038 (0.00–0.08)	0.525 (0.42–0.63)	0.050 (0.00–0.10)	0.175 (0.09–0.26)	—	—	
Seneca								
<i>n</i>	24	—	18	—	48	—	—	90
Frequency	0.267 (0.18–0.36)	—	0.200 (0.12–0.28)	—	0.533 (0.43–0.64)	—	—	
Superior								
<i>n</i>	38	—	15	—	3	21	3	80
Frequency	0.473 (0.37–0.58)	—	0.188 (0.10–0.27)	—	0.038 (0.00–0.08)	0.263 (0.17–0.36)	0.038 (0.00–0.08)	

Superior basin (Fig. 2). These two strains were more similar to each other allozymically than either was to the other four strains (Krueger et al. 1989). However, differences between the mtDNA haplotype frequencies of the Killala and Superior strains were striking (Table 3). The addition of mtDNA data to the allozyme baseline data used by MSA increases the detectable genetic differences among strains and thus will improve the accuracy of MSA in determining the parental contribution to naturally produced fry in mixture samples (e.g., Pella and Milner 1987).

Construction of baseline data will be different between allozyme and mitochondrial data when MSA is to be used for second-generation analysis of fry mixtures. These differences occur because allozyme characters demonstrate typical bi-parental inheritance whereas mtDNA haplotypes are maternally inherited. For each strain, mtDNA data in the form of haplotype frequencies can be added to an allozyme baseline data set simply as an additional "locus" with haplotypes as "alleles." However, data must also be constructed to characterize potential interstrain crosses (e.g., Seneca × Superior). For allozyme data, the genotypic frequencies for interstrain crosses can be determined by using a modified Hardy-Weinberg equation as described by Marsden et al. (1989). Because of maternal inheritance, mtDNA baseline data used to characterize a potential interstrain cross instead should be the average of the haplotype frequencies of the two strains which assumes that the female to male ratio is the same for both strains.

In summary, the mtDNA haplotype variation observed among the Clearwater, Jenny, Killala, Manitou, Seneca, and Superior lake trout strains revealed a substantial level of differentiation

among strains. These data will enhance the ability of MSA to detect parental strain contributions to lake trout fry collected from Lake Ontario.

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