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Gene diversity analysis of mitochondrial DNA, microsatellites and allozymes in landlocked Atlantic salmon

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This study investigates the patterns of genetic diversity detected in allozymes, mtDNA, and microsatellites, in order to assess their relative efficacy to differentiate sympatric landlocked salmon populations and to estimate changes in genetic diversity between wild and first-generation hatchery fish. Overall, the three genetic markers indicated a genetic differentiation between two sympatric populations of Lake Saint-Jean, Québec. MtDNA and microsatellites also showed significant differences between wild and first-generation hatchery fish originating from the same river. Allozyme analysis was the most limited approach due to the low genetic diversity detected and the necessity to kill specimens. Although low polymorphism was found in mtDNA, it was the most discriminant marker between wild populations. Microsatellite analysis appears to be a promising approach due to its high sensitivity in differentiating wild populations, in detecting changes in allele composition between wild and first-generation hatchery fish and its potential for increased resolution by augmenting the number of polymorphic loci. Given the benefits and disadvantages of the three methods, the combination of mtDNA and microsatellite analyses will best address our research objectives.

Key words: conservation genetics; Atlantic salmon; stock identification; fishery management.

INTRODUCTION

Stocking is being used as a major management tool to enhance exploited fish populations facing declines in abundance. It should be designed to maintain both inter- and intra-population components of a species' genetic diversity. A better understanding of stock structure, as well as estimates of differentiation and the degree of natural gene exchange among populations, can provide guidelines to optimize population enhancement practices to conserve genetic resources (Ryman, 1991).

Many genetic approaches can provide information upon which to base management decisions. Allozyme analysis has been a method of choice for many management purposes (Ferguson, 1994). To a lesser extent, the analysis of mitochondrial DNA variation has also been used successfully (Billington & Hebert, 1991; Bernatchez, 1994). More recently, the application of variable number of tandem repeats (VNTR) analysis has been introduced as a potentially powerful method for fine-scale studies of genetic diversity in fishes (Taggart & Ferguson, 1990; Wright & Bentzen, 1994). It is obvious from the literature that the relative usefulness of different methods depends largely upon the species and geographic scale to which they are applied, as well as the questions asked. Therefore, it is valuable to compare the information generated by different

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molecular approaches. Yet, this is rarely achieved (Carvalho & Hauser, 1994).

Lake Saint-Jean is a moderate-sized lake (1000 km^2) located in central Québec (approximately 200 km north of Québec city, Canada) which harbours landlocked Atlantic salmon *Salmo salar* L. Since the mid-1980s, several spawning runs have been declining to less than 30 spawning adults being counted in some. Consequently, a supportive-breeding programme has been undertaken in the early 1990s for the four major tributaries of the lake used for spawning. Recently we initiated a genetic study with the main objective of providing guidelines for the conservation of salmon genetic diversity in this lake. This preliminary study compares genetic diversity among the same samples using allozymes, mtDNA, and microsatellites, in order to assess their relative usefulness.

MATERIALS AND METHODS

Thirty-six wild spawning (not issued from stocking programmes) fish were collected in the Métabetchouane and Mistassini River, draining 35 km apart into Lake Saint-Jean. Adipose fins were clipped and preserved in 95% ethanol. Thirty-six first-generation hatchery parr whose parents originated from both rivers were also obtained and preserved at −80° C.

Allozyme analysis involved only comparisons between hatchery samples as killing of wild fish was not possible. Analysis of muscle and liver was carried out on cellulose acetate (Hebert & Beaton, 1989) for seven structural (*MDH-1**, *MDH-2**, *MDH-3**, *MDH-4**, *AAT-3**, *IDDH-1**, *IDDH-2**) and one regulatory (*PGM-1r**) loci, known to be the most polymorphic for salmon in North America (Pollard, 1992; Verspoor, 1994).

MtDNA RFLP analysis was performed on three polymerase chain reaction (PCR) amplified segments using primers developed by Cronin *et al.* (1993) and Bernatchez & Danzmann (1993). These encompassed the ND-5/6 (2·4 kb), the ND-1 (2·0 kb), and the cytochrome *b* gene/D-loop (2·1 kb) regions. PCR conditions were as in Bernatchez *et al.* (1995) except that the annealing temperature was 45° C. Aliquots of the PCR products were digested with 19 restriction enzymes (*Alu*I, *Ava*II, *Ban*I, *Ban*II, *Bsp1286*I, *Cfo*I, *Dde*I, *Hae*II, *Hae*III, *Hin*cII, *Hin*fI, *Hpa*II, *Mbo*I, *Mbo*II, *Msp*I, *Nci*I, *Rsa*I, *Sau3a*I, *Taq*I). Electrophoresis and detection procedures were as described previously (Bernatchez *et al.*, 1995). Genotypes were defined by composite patterns of restriction site variation across all enzymes and segments.

Microsatellite polymorphism was analysed by specific PCRs at four loci using primers developed for *Salmo trutta* L. (μ15: Estoup *et al.*, 1993; μ3, μ85, μ79·1: Presa, unpublished data), and one locus (*SFO-8*) using primers developed for *Salvelinus fontinalis* Mitchill (Angers *et al.*, 1995). PCR reactions with *S. trutta* primers were performed in 12 μl reaction volume containing 1 unit of *Taq* polymerase, 1·25 μl reaction buffer, 15 pmol each primers, 500 μM of dNTP, 0·15 μl of α^{35}S-dATP and 1 μl (50–100 ng) of total DNA. We used the following profile in PCR: one denaturation at 95° C for 2 min; 35 cycles of 35 s at 94° C, 35 s at annealing temperature (μ3 = 57° C, μ15 = 58° C, μ79·1 = 59° C, μ85 = 55° C), and 10 s at 72° C. Conditions for the SFO-8 locus followed Angers *et al.* (1995). Migration, fixation, drying, and autoradiography followed standard procedures. Alleles were sized by comparison with the standard M13 sequence.

Genetic polymorphism for each sample was measured by Hardy–Weinberg expected heterozygosity (H_E) at nuclear loci and by nucleon diversity index (h) for mtDNA (Nei, 1987). The significance of differences in allele frequencies among samples was estimated by χ^2 test of homogeneity. Variance in allele frequencies among samples was also estimated from G_{st} calculations (Nei, 1987). The significance of G_{st} values was tested using a random allelic permutation procedure (Excoffier *et al.*, 1992).

TABLE I. Sample size, allele frequencies, expected heterozygosity (H_E), nucleon diversity (h) in Métabetchouane (MET) and Mistassini (MIST) rivers for wild (W) and hatchery (H) stocks

River	Allozyme								mtDNA					
	*MDH-3**				*PGM-1r**									
	N	100	120	H_E	*N*	a	b	H_E	*N*	AAA	ABB	BBB	BAB	*h*
MET (W)	N.A.				N.A.				27	0·92	0·04	0·04	0·00	0·14
MET (H)	32	0·30	0·70	0·42	31	0·57	0·43	0·49	32	0·81	0·19	0·00	0·00	0·32
MIST (W)	N.A.				N.A.				36	0·17	0·08	0·72	0·03	0·46
MIST (H)	30	0·10	0·90	0·18	29	0·49	0·51	0·50	30	0·00	0·00	1·00	0·00	0·00

For *PGM-1r**, allelic frequency were calculated assuming the genotypes to be in Hardy–Weinberg proportions. Restriction enzymes for mtDNA haplotypes are in order: *Alu*I, *Cfo*I, *Hae*II.

N.A., Not available.

RESULTS

Low variation was detected in allozymes between hatchery samples originating from the Mistassini and Métabetchouane Rivers. Only two loci were polymorphic ($P_{0.99}$) with two alleles detected in each case (Table I). Significant allele frequency difference was observed between tributaries only at *MDH-3** ($\chi^2 = 7{\cdot}46$; $P = 0{\cdot}007$) which also translated into a significant G_{st} estimate of 0·099 (Table III).

Low polymorphism was also observed in mtDNA. Only three of the 19 restriction enzymes used were variable. The ND-1 segment was polymorphic for *Cfo*I and *Hae*II as was the cytochrome *b*/D-loop segment for *Alu*I. No variation was detected in the ND-5/ND-6 segment. Only four mtDNA composite genotypes were resolved such that nucleon diversity was low in all samples. However, genotypes segregated strongly between rivers ($\chi^2 = 36{\cdot}25$; $P < 0{\cdot}001$). Salmon from the Métabetchouane River were highly dominated by genotype AAA whereas those from the Mistassini River were dominated by genotype BBB (Table I). Consequently, a strong component (61·4%) of the total mtDNA gene diversity was due to population subdivision. A significant difference in the frequency of mtDNA genotypes was observed also between wild and hatchery

TABLE II. Allele frequencies and expected heterozygosity (H_E) for μ85, μ15, SFO-8, μ79·1 and μ3 microsatellite loci in Métabetchouane (MET) and Mistassini (MIST) rivers for wild (W) and hatchery (H) stocks

Locus and population	*N*	Alleles (named by size in base pairs) and their proportions								
μ85		162	170	H_E						
MET (W)	30	0·00	1·00	0·00						
MET (H)	32	0·00	1·00	0·00						
MIST (W)	36	0·18	0·82	0·30						
MIST (H)	32	0·03	0·97	0·06						
μ15		218	220	H_E						
MET (W)	30	0·15	0·85	0·26						
MET (H)	33	0·35	0·65	0·46						
MIST (W)	36	0·19	0·81	0·31						
MIST (H)	30	0·17	0·83	0·28						
SFO-8		200	206	210	H_E					
MET (W)	19	0·05	0·00	0·95	0·10					
MET (H)	36	0·01	0·00	0·99	0·02					
MIST (W)	31	0·00	0·03	0·97	0·06					
MIST (H)	31	0·06	0·02	0·92	0·15					
μ79·1		149	151	153	155	H_E				
MET (W)	28	0·57	0·39	0·00	0·04	0·53				
MET (H)	32	0·75	0·25	0·00	0·00	0·38				
MIST (W)	35	0·20	0·03	0·56	0·21	0·61				
MIST (H)	22	0·43	0·05	0·39	0·14	0·66				
μ3		202	204	206	208	210	212	214	216	H_E
MET (W)	19	0·02	0·19	0·03	0·29	0·31	0·00	0·03	0·12	0·78
MET (H)	36	0·30	0·12	0·03	0·12	0·17	0·10	0·17	0·00	0·83
MIST (W)	31	0·00	0·00	0·00	0·07	0·61	0·25	0·03	0·04	0·57
MIST (H)	31	0·00	0·00	0·02	0·28	0·48	0·21	0·00	0·02	0·66

TABLE III. Pairwise comparison of interpopulation diversity (G_{st}) estimated individually for each allozyme, mtDNA and microsatellite loci in Métabetchouane (MET) and Mistassini (MIST) rivers for wild (W) and hatchery (H) stocks

	MET *v.* MIST	MET (W) *v.* MET (H)	MIST (W) *v.* MIST (H)
Allozyme			
*MDH-3**	0·099**	N.A.	N.A.
*PGM-1r**	0·000	N.A.	N.A.
mtDNA	0·614**	0·042	0·164**
Microsatellite			
μ85	0·155**	0·000	0·094*
μ15	0·000	0·084*	0·000
SFO-8	0·006	0·008	0·015
μ79·1	0·341**	0·041*	0·050**
μ3	0·146**	0·086**	0·035*

MET *v.* MIST only included comparison between first-generation hatchery parr for allozyme.
Significance levels: *$0{\cdot}001 < P < 0{\cdot}05$; **$P < 0{\cdot}001$.
N.A., Not available.

samples from the Mistassini River ($\chi^2 = 9{\cdot}82$; $P = 0{\cdot}006$), translating into a significant G_{st} value (Table III).

Microsatellites were polymorphic to various extents depending on locus (Table II). The number of alleles per locus varied between 2 and 8 while sample heterozygosity varied between 0 and 83%. Highly significant differences ($P < 0{\cdot}001$) in allele frequencies were observed between rivers at three loci (μ3: $\chi^2 = 50{\cdot}12$; μ79·1: $\chi^2 = 71{\cdot}98$; μ85: $\chi^2 = 12{\cdot}02$). Consequently, a significant component (14·6 to 34·1%) of the total genetic diversity was due to population subdivision (Table III). River-specific alleles were also detected at moderate frequencies for the three loci. For μ3, alleles 202 and 204 were observed only in Métabetchouane River, as were allele 162 at μ85 and allele 153 at μ79·1 for the Mistassini River. Significant changes in allele frequencies between wild and first-generation hatchery salmon were observed also. Thus, allelic frequencies differed at loci μ3 ($\chi^2 = 40{\cdot}86$; $P < 0{\cdot}001$), μ15 ($\chi^2 = 6{\cdot}53$; $P = 0{\cdot}007$) and μ79·1 ($\chi^2 = 5{\cdot}64$; $P = 0{\cdot}035$) between samples originating from the Métabetchouane River, whereas Mistassini samples differed at loci μ3 ($\chi^2 = 13{\cdot}16$; $P = 0{\cdot}013$) and μ85 ($\chi^2 = 7{\cdot}70$; $P = 0{\cdot}004$). Significant G_{st} estimates were obtained also in all cases although the values were always smaller than inter-river comparisons (Table III).

No general trend in patterns of intra-sample genetic diversity was observed between wild and first-generation hatchery fish. For the Mistassini River, loss or reduction of less frequent alleles and lower diversity was detected in hatchery fish for mtDNA and microsatellite μ85, whereas no obvious changes in number of alleles and similar or higher diversity was observed at other loci and for the Métabetchouane fish at all loci (Tables I, II). However, important shifts in allele frequencies were observed in both rivers, for mtDNA and microsatellite μ15 and μ3.

DISCUSSION

Altogether, the three genetic markers used indicated an important genetic differentiation between two landlocked salmon populations of Lake Saint-Jean.

Thus, unique or dominant alleles, highly significant differences in allele frequencies and high G_{st} values were observed. This supported the hypothesis that the two rivers, perhaps through homing behaviour, harbour distinctive genetic populations that fulfil the main prerequisite for local adaptation (Hindar *et al.*, 1991; Carvalho, 1993). Highly significant shifts in allele frequency between wild and hatchery stocks were detected also. Other studies indicated that breeding in the hatchery was responsible for such changes (Verspoor, 1988). Such conclusions cannot be made in the present case as allelic fluctuation through genetic drift is expected in small populations. Therefore, a firmer assessment of the breeding effect on genetic diversity of landlocked salmon from Lake Saint-Jean must await further comparisons among generations of wild populations.

Allozyme analysis showed very low polymorphism. Only two loci were variable, allowing the determination of weak differences in allele frequencies and a relatively low G_{st} value. We cannot refute the possibility that we observed low variation because comparisons were made on hatchery fish only. Nevertheless, these results fit the general pattern of low variation at protein loci in the species (Davidson *et al.*, 1989; Verspoor, 1994). However, the main constraint with the use of allozymes in our study was that the method necessitates killing which was not compatible with the goal of preserving already threatened populations. This may represent a general drawback of the technique for any study dealing with endangered populations (Park & Moran, 1994).

Very little variation in the number of haplotypes was observed in mtDNA, as usually reported for Atlantic salmon (e.g. Bermingham, 1990; King *et al.*, 1993). It may be argued that the low variability observed in mtDNA was due to the use of RFLP analysis as it is increasingly reported that direct sequencing provides greater resolution (Carr & Marshall, 1991). However, this may not be exclusively so in salmonids. For instance, sequencing of the D-loop, generally considered the most variable region in the mitochondrial genome, was monomorphic among *S. trutta* populations of the European North Atlantic basin, yet RFLP analysis revealed many haplotypes among the same populations (Hall, 1992; Hall & Nawrocki, 1995). In a study of brook charr no difference was observed in the number of mtDNA genotypes detected by RFLP or sequence analysis of the D-loop region (Bernatchez & Danzmann, 1993). Therefore it appears unlikely that sequencing would have generated more variation than RFLP, unless intense analytical effort was applied. Despite its low diversity, mtDNA was the most discriminating marker between wild populations, as exemplified by the high G_{st} value observed. This can be explained partly by the fact that maternally inherited mtDNA is more sensitive to genetic drift than nuclear DNA due to its smaller effective population size (Takahata & Slatkin, 1984).

Microsatellite analysis proved to be useful, both for detecting variation between populations and changes in allelic composition between wild and hatchery stocks. In some loci, population unique alleles were found at moderate frequencies which enhanced their discrimination. The polymophic loci were useful in detecting changes in the number of alleles and were more sensitive to find variation between wild and hatchery stocks. Although high genetic diversity was detected with the loci used, microsatellite analysis could be improved by including new loci and by using specific microsatellite primers for Atlantic salmon (Wright & Bentzen, 1994).

In summary, allozyme analysis was the most limited approach to reach our specific objectives as both sufficient resolution and the sample acquisition were limited. Although low variation was detected in mtDNA, it was the most discriminatory locus between wild populations. Microsatellites appear to be promising due to their high sensitivity in differentiating wild populations, in detecting changes in allele composition, and potential for increased resolution. Microsatellite and mtDNA methods also had the advantage of requiring only minute and non-destructive amounts of tissue. Given the benefits and disadvantages of the three methods, it appears that a combination of mtDNA and microsatellite analyses would address best the objectives of our ongoing research on the conservation genetics of *S. salar*.

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