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Specific microsatellite loci for brook charr reveal strong population subdivision on a microgeographic scale

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We have isolated specific microsatellite loci from a partial genomic library of brook charr *Salvelinus fontinalis*. Their usefulness was investigated by measuring intra- and inter-population genetic diversity at four loci among 20 individuals from each of five lakes located 3 to 22 km apart in La Mauricie national park (Canada). These markers were moderately to highly polymorphic. A total of five, six, 16 and 18 alleles per locus were detected, and their overall expected heterozygosity was 0·53, 0·58, 0·86 and 0·87. Strong inter-population diversity was detected. Highly significant differences in allelic frequencies were found in all but two pairwise χ^2 permutation tests between populations at all loci. Numerous population unique alleles were observed in all five lakes. Consequently, a highly significant component of total genetic diversity was due to interpopulation variance, as exemplified by G_{ST} values of 0·33, 0·42, 0·47 and 0·84 for each individual locus. Altogether, the results indicated that these loci should be valuable in addressing fine scale population genetics questions in brook charr. To our knowledge, they also represent the first available microsatellites developed in the genus *Salvelinus*.

Key words: microsatellite; *Salvelinus fontinalis*; genetic variability; microgeographic differentiation.

INTRODUCTION

The brook charr *Salvelinus fontinalis* Mitchill is a native salmonid of northeastern North America. Genetic diversity of this species has previously been assessed by allozymes (Stoneking *et al.*, 1981; Ferguson *et al.*, 1991; McCracken *et al.*, 1993; Perkins *et al.*, 1993) and by mitochondrial (mt) DNA analyses (Danzmann *et al.*, 1991*a*, *b*; Ferguson *et al.*, 1991; Bernatchez & Danzmann, 1993). While these studies revealed that allozymes and mtDNA are highly polymorphic and useful markers in many situations, they may also have some limitations. For instance, low numbers of alleles per locus may hamper the ability of allozymes to reveal low levels of variability (Leberg, 1992). The invasive tissue sampling generally necessary for allozyme analysis may also render its application impossible in conservation genetics studies where death of individuals should be minimized. Some studies have also revealed that while generally polymorphic, mtDNA in brook charr was extremely low over most of the northeastern American range of distribution (Ferguson *et al.*, 1991; Danzmann *et al.*, unpublished data).

Microsatellite loci are being increasingly used to address population genetics issues (Bruford & Wayne, 1993; Taylor *et al.*, 1994; Paetkau & Strobeck, 1994; Paetkau *et al.*, 1995). Microsatellite sequences are tandemly repeated short motifs of one to five nucleotides that may display a high level of polymorphism,

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mostly due to variations in the number of basic motifs in the repeated sequence (Litt & Lutty, 1989; Tautz, 1989; Weber & May, 1989). Polymorphism can be detected by size fractioning on acrylamide gels following PCR amplification, using a pair of flanking unique primers. PCR amplification requires only a minute amount of DNA, and does not require killing the specimens. Microsatellite primers have now been developed for a variety of salmonids, including Atlantic salmon *Salmo salar* L. (Bentzen *et al.*, 1991; Slettan *et al.*, 1993), brown trout *Salmo trutta* L. (Estoup *et al.*, 1993), and rainbow trout *Oncorhynchus mykiss* (Walbaum) (Morris, 1993; Sakamoto *et al.*, 1994). However, polymorphism at a given microsatellite locus may decrease with increasing phylogenetic distance from the species for which the primers were originally designed. Thus, existing primers may amplify a corresponding locus in one species, but fail to detect polymorphism in another (Moore *et al.*, 1991). Consequently, limited success was obtained in our preliminary attempts to apply microsatellites primers developed from other salmonid genera to brook charr.

The objective of this paper was to investigate the usefulness of seven microsatellite loci isolated from a partial genomic library of brook charr. This was accomplished by documenting patterns of intra- and inter-population genetic

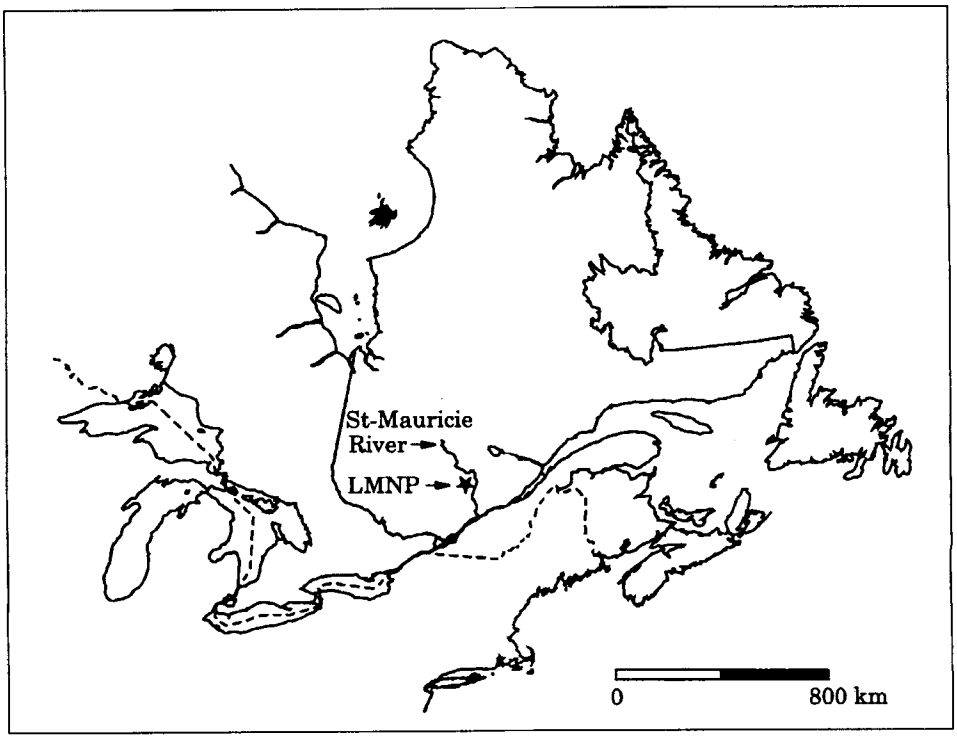

FIG. 1. Location map of the La Mauricie national park (LMNP), Canada, where brook charr were sampled.

diversity observed among five geographically proximal brook charr populations from La Mauricie national park (Canada).

MATERIALS AND METHODS

CLONING

High molecular weight brook charr DNA was digested to completion with *Taq*I. Fragments of 150 to 500 bp were inserted into the *Msp*I cohesive site of the phagemid pBluescript II SK+ (Stratagene). Recombinant plasmids were electroporated in LE392 *E. coli* using a Bio-Rad Gene Pulser apparatus and plated on LB medium for transformant selection. Transformants were screened for inserts containing repeated motifs using an equal mix of $(CT)_{10}$ and $(GT)_{10}$ oligonucleotides end-labelled with [γ^{32}P] ATP using T4 polynucleotide kinase. Plasmids from positive clones were isolated and sequenced with Sequenase 2.0 kit (USB) using T3 and T7 primers. Primers for PCR amplification

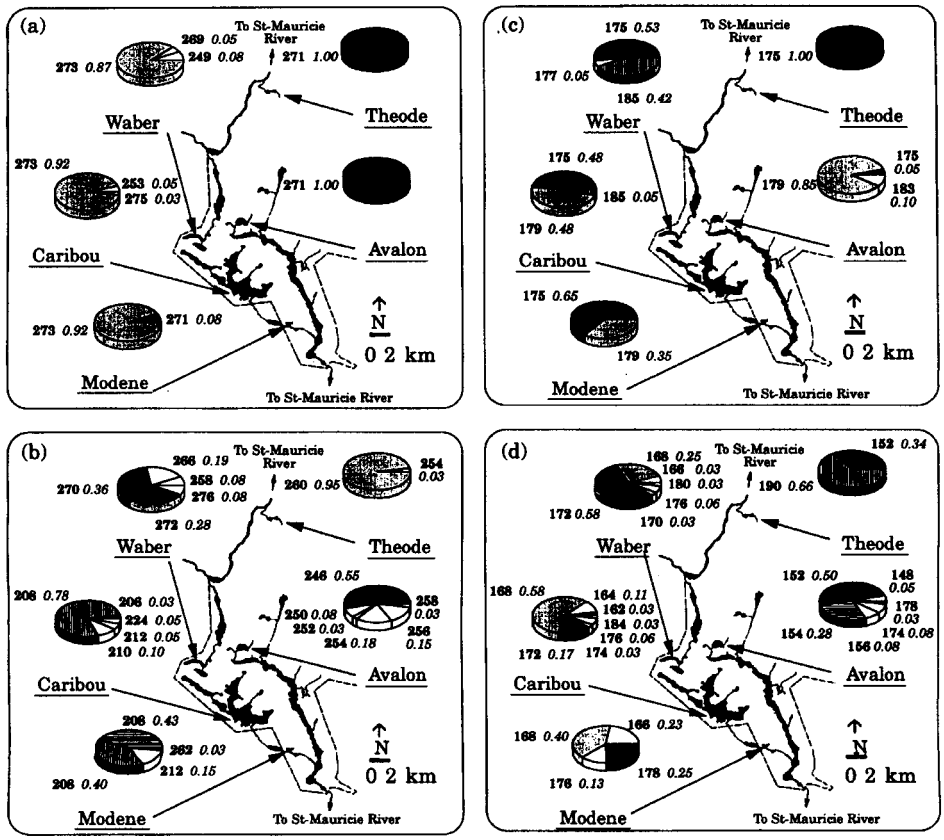

FIG. 2. Allele frequency distributions at four microsatellite loci from five wild brook charr populations from La Mauricie national park (Canada). Allele designation (bold) refers to size in base pairs of the PCR products and their relative frequency (italic) are given next to the allele designation. All alleles with relative frequency below 0·25 are illustrated in white for simplification of the figure. (a) Sfo-12; (b) Sfo-8; (c) Sfo-18; (d) Sfo-23.

TABLE I. Number of uninterrupted tandem repeats observed in the sequence of cloned alleles for brook charr microsatellite, PCR product size of these alleles, primer sequences and annealing temperature for amplification of loci by PCR

Locus	No. of repeats	Size (bp)	Primer sequence (5′–3′)	Annealing temperature (°C)
Sfo-1	52	184	ACC ATA ACC CCC CAC CAC‡ GTC CCT CCG TGG CAG ATT	65
Sfo-3	60	248	GTC ACT GGG CAG GAG TTG‡ CGA ACC TAC CAT GTC ATT	50
Sfo-8*	46	252	CAA CGA GCA CAG AAC AGG‡ CTT CCC CTG GAG AGG AAA	60
Sfo-12*	15†	271	GGT TTT GAA GAG TGA CAG CCC GTT TCA CAA TCA GAG‡	60
Sfo-18*	18	179	TGG TGT ATC CTG CTC CTG‡ TGG AAT GTG TGT CTG TTT TCT	65
Sfo-20	60	172	AAA CAC AAA GGG TTG AAC GAC AGT GAT GAG GAG ACC‡	50
Sfo-23*	42	166	GTG TTC TTT TCT CAG CCC AAT GAG CGT TAC GAG AGG‡	60

*Polymorphic loci. †The largest number of uninterrupted tandem repeats, the complete sequence of Sfo-12 is $(GT)_5$ CC $(GT)_{10}$ CC $(GT)_{15}$.
‡End-labelled primers used for PCR reactions.

were designed for the flanking regions of the repeated using the OSP program (Hillier & Green, 1991).

SAMPLING

Brook charr were collected from five lakes in La Mauricie national park, Canada (Fig. 1) during summer 1994. These populations have no recorded stocking history (Michel Plante, Canadian Park Service, La Mauricie national park, pers. comm.). Lakes were located 3–22 km apart and belonged to two drainages of the St-Maurice River watershed (Fig. 2). When possible, fish muscle tissues were obtained from anglers; in lakes where fishing was prohibited, adipose fin clips were collected non-lethally. All tissues were stored at room temperature in 95% ethanol until DNA extraction.

SCREENING AND DATA ANALYSIS

Microsatellite polymorphism was screened in polyacrylamide sequencing gels using M13-mp18 (USB) sequencing ladder as a size marker. Fifteen µl PCR reactions were performed in a thermal cycler (Perkin–Elmer), using 100 ng of DNA template, 0·2 pmol primer end-labelled with [γ^{32}P] ATP (Table I), 15 pmol of each primer, 75 mM each dNTP, 1·5 mM $MgCl_2$, 1X *Taq* buffer, and 0·25 unit of *Taq* polymerase. The following PCR profile was used: an initial denaturing step of 3 min at 95° C, 15 cycles of 60 s at 94° C, 35 s at annealing temperature (Table I) and 10 s at 72° C, and 20 other cycles of 35 s at 94° C with all other parameters unchanged.

Genetic polymorphism was measured as the number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E). Analysis of heterogeneity in allelic frequencies (Sokal & Rohlf, 1981) was assessed using the MONTE program of the REAP software package (McElroy *et al.*, 1992), which performed χ^2 randomization tests (Roff & Bentzen, 1989) for all pairwise comparison of populations at all individual loci

with 1000 randomizations. Variance in allele frequencies among samples was estimated from G_{ST} values (Nei, 1987) calculated at each individual locus. Significance of the observed G_{ST} values was tested using a random allelic permutation procedure available in the AMOVA computer package (Excoffier *et al.*, 1992).

RESULTS

BROOK CHARR MICROSATELLITE LOCI

Sequencing analysis of positive clones revealed four uninterrupted series of (GT) repeats (Sfo-3, Sfo-8, Sfo-18, Sfo-23), two perfect sequences of (CT) repeats (Sfo-1 and Sfo-20) and one imperfect structure of (GT) with intervening C nucleotides (Sfo-12). Primers for PCR amplification were designed according to the DNA sequence flanking the repeat regions (Table I).

The amplification products of the seven primer pairs were scored readily. A preliminary screening of several individuals from different populations of brook charr revealed complete monomorphism for loci Sfo-1, Sfo-3 and Sfo-20. This suggested that no mutation occurred in these loci, despite the long perfect dinucleotide repeats. Consequently, they were not considered further.

The four other microsatellites were moderately to highly polymorphic, with five alleles for Sfo-18 (overall expected heterozygosity, $H_E = 0{\cdot}58$), six alleles for Sfo-12 ($H_E = 0{\cdot}53$), 16 alleles for Sfo-23 ($H_E = 0{\cdot}86$) and 18 alleles for Sfo-8 ($H_E = 0{\cdot}87$) detected among 100 specimens (Table II). Alleles at these loci were found to vary in size by multiples of two nucleotides, suggesting that mutations occurred in the number of repeat sequences. Range of amplification products varied by five dinucleotide units for Sfo-18, 13 for Sfo-12, 21 for Sfo-23 and 36 for Sfo-8.

TABLE II. Sample size (n), number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) at four loci for five brook charr populations

Locus	Théode	Avalon	Waber	Caribou	Modène	Total
n	20	20	20	20	20	100
Sfo-8						
A	2	6	5	5	4	18
H_O	0·10	0·70	1·00	0·30	0·80	
H_E	0·10	0·64	0·74	0·38	0·62	0·87
Sfo-12						
A	1	1	3	3	2	6
H_O	0	0	0·25	0·15	0·15	
H_E	0	0	0·24	0·14	0·14	0·53
Sfo-18						
A	1	3	3	3	2	5
H_O	0	0·3	0·45	0·40	0·50	
H_E	0	0·25	0·54	0·54	0·46	0·58
Sfo-23						
A	2	6	6	7	4	16
H_O	0·35	0·75	0·55	0·77	0·70	
H_E	0·45	0·66	0·59	0·61	0·71	0·86

INTRA- AND INTER-POPULATION GENETIC DIVERSITY

We found a total of six, 12, 16, 17 and 18 alleles for Théode, Modène, Avalon, Waber and Caribou populations respectively for the four loci combined. Population allelic diversity (A) ranged between one to three alleles for each Sfo-12 and Sfo-18 loci, two to six for Sfo-8 and two to seven for Sfo-23 locus (Table II). Expected heterozygosity (H_E) ranged between 0 and 0·24 at Sfo-12, 0 and 0·54 at Sfo-18, 0·45 and 0·71 at Sfo-23 and 0·10 and 0·74 at Sfo-8. Observed heterozygosity values were comparable to expected heterozygosity in most cases (Table II).

Large differences in genetic diversity were detected among brook charr populations. Significant differences in allelic frequencies ($0{\cdot}001 < P < 0{\cdot}01$) were observed in all population χ^2 pairwise comparisons at all loci, except for comparison between Lakes Théode and Avalon, and between Lakes Caribou and Modène which did not differ at Sfo-12.

Numerous population unique alleles were observed in all five lakes (Fig. 2). For Sfo-8, allelic size distribution showed little or no overlap among lakes. Waber Lake was dominated by the largest alleles (e.g. 266, 270, 272, 274) which were found nowhere else. Théode Lake was dominated by allele 260 observed nowhere else. All alleles found in Avalon Lake were unique (e.g. 246, 250, 252, 254, 256) except alleles 254 and 258 found at low frequency in Théode and Waber Lakes respectively. Modène and Caribou populations shared alleles that were unique to both (e.g. 208, 212). For Sfo-12, Lakes Théode and Avalon were fixed for allele 271 whereas the other lakes were fixed or nearly so for allele 273. Rare alleles observed in these lakes were not shared among populations. For Sfo-18, allele 175 was dominant in all lakes, except in Avalon Lake where this allele was observed only at low frequency while allele 179 dominated. Allele 179 was also important in Caribou and Modène Lakes, but absent in Waber Lake where allele 185 was found at high frequency. For Sfo-23, Théode Lake was dominated by allele 190 observed nowhere else. Allele 152 dominated in Avalon Lake, allele 168 in Caribou and Modène Lakes and allele 172 in Waber Lake. These lakes were also characterized by numerous alleles showing low to moderate frequencies, many of which were unique to given lakes (e.g. 148, 154, 156, 162, 164, 180, 184).

The analysis of genetic variance component reflected the general pattern of high heterogeneity in allele frequency among populations surveyed. Thus, the amount of total genetic diversity explained by variance among populations

TABLE III. Interpopulation genetic variance among five brook charr populations for four microsatellite loci

Locus	d.f.	G_{st}	P
Sfo-8	4	0·474	<0·001
Sfo-12	4	0·838	<0·001
Sfo-18	4	0·423	<0·001
Sfo-23	4	0·325	<0·001

ranged from 32·52 to 83·84%, depending on the locus (Table III). The permutation procedures showed that this portion of genetic variance was highly significant ($P<0·001$) for all loci.

DISCUSSION

To our knowledge, this study provides the first report of microsatellite loci developed in the genus *Salvelinus*. Our preliminary results indicate that brook charr displays a high degree of polymorphism for these markers on a small geographic scale. They also reveal a strong pattern of population subdivision, reflected in the significantly high G_{ST} estimates. Thus, the five populations analysed differed by their patterns of intra-population diversity, and by the presence of unique alleles. We cannot ignore the possibility that such high numbers of private alleles may be due partly to the limited sample sizes and number of populations surveyed. Nevertheless, the general patterns of diversity observed illustrate the usefulness of microsatellites in monitoring population genetic diversity on a microgeographic scale.

The level of genetic variation detected in brook charr at these microsatellite loci contrast with the low mtDNA diversity generally observed in this species for the region surveyed. Preliminary analysis of mtDNA variation among brook charr populations from Québec revealed complete fixation for a single haplotype (Danzmann *et al.*, unpublished data), with the exception of one river drainage (Bernatchez *et al.*, 1995). In contrast, high mtDNA diversity has been detected in populations from other parts of their distribution range (Danzmann *et al.*, 1991*a*, *b*; Bernatchez & Danzmann, 1993). While there are no allozyme data available for the region of our study site, highly significant heterogeneity in allelic frequencies has generally been found in protein loci analyses among nearby populations in other regions (Stoneking *et al.*, 1981; Ferguson *et al.*, 1991; Perkins *et al.*, 1993). Therefore, microsatellite data reinforce the findings obtained from allozymes and mtDNA in some parts of the species range, that brook charr is among the most highly structured freshwater species (Ward *et al.*, 1994). However, because of the higher number of alleles per locus detected, and because of their population specific distributions, microsatellites may prove more valuable than allozymes in detecting demographic factors such as founder events associated with post-glacial recolonization and for estimating gene flow more accurately on a microgeographic scale. With regard to practical aspects, the use of microsatellites greatly facilitated sample acquisition in this study. Indeed, the use of invasive tissue sampling would have prevented the genetic investigation of some populations for which no killing was possible. This represents a situation routinely encountered in conservation genetics studies dealing with protected populations.

Brook charr showed levels of genetic variability comparable to other fish species screened for microsatellite variation, taking into account the microgeographic scale of this study. Estoup *et al.* (1993) detected five to six alleles per locus in four natural brown trout populations from the Atlantic and Mediterrannean basins. Screening of microsatellites for a sample population of Atlantic cod *Gadus morhua* L. revealed up to 46 alleles and heterozygosity of 92% at one

locus (Brooker *et al.*, 1994). In a study of phylogeographic variation in Californian steelhead *O. mykiss*, 20 alleles were detected at one locus (Nielsen *et al.*, 1994).

In summary, this study demonstrates the usefulness of microsatellites for assessing microgeographic differentiation among recently diverged brook charr populations. These loci exhibited allelic diversity and heterozygosities greatly in excess of those reported for protein loci or mtDNA in northeastern North America. Such diversity, coupled with the non-invasive tissue approach should make them a valuable tool in a variety of research contexts, namely conservation genetics.

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