A Comparative Mitogenomic Analysis of the Potential Adaptive Value of Arctic Charr mtDNA Introgression in Brook Charr Populations (*Salvelinus fontinalis* Mitchill)

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Wild brook charr populations (*Salvelinus fontinalis*) completely introgressed with the mitochondrial genome (mtDNA) of arctic charr (*Salvelinus alpinus*) are found in several lakes of northeastern Québec, Canada. Mitochondrial respiratory enzymes of these populations are thus encoded by their own nuclear DNA and by arctic charr mtDNA. In the present study we performed a comparative sequence analysis of the whole mitochondrial genome of both brook and arctic charr to identify the distribution of mutational differences across these two genomes. This analysis revealed 47 amino acid replacements, 45 of which were confined to subunits of the NADH dehydrogenase complex (Complex I), one in the *cox3* gene (Complex IV), and one in the *atp8* gene (Complex V). A cladistic approach performed with brook charr, arctic charr, and two other salmonid fishes (rainbow trout [*Oncorhynchus mykiss*] and Atlantic salmon [*Salmo salar*]) revealed that only five amino acid replacements were specific to the charr comparison and not shared with the other two salmonids. In addition, five amino acid substitutions localized in the *nad2* and *nad5* genes denoted negative scores according to the functional properties of amino acids and, therefore, could possibly have an impact on the structure and functional properties of these mitochondrial peptides. The comparison of both brook and arctic charr mtDNA with that of rainbow trout also revealed a relatively constant mutation rate for each specific gene among species, whereas the rate was quite different among genes. This pattern held for both synonymous and nonsynonymous nucleotide positions. These results, therefore, support the hypothesis of selective constraints acting on synonymous codon usage.

Introduction

Although long believed to be of limited significance (Heiser 1973), the evolutionary importance of introgressive hybridization is increasingly supported by recent molecular and ecological studies (reviewed in Arnold 1997, 215 p.). Indeed, this process represents a potent evolutionary force that may lead to the adaptive evolution of species. For instance, introgressive events, even when rare, may be more important than mutations as a source of novel genetic variability within taxa (Arnold 1992).

Evidence of interspecific mitochondrial genome introgression has been accumulating, especially in recent years. Complete mitochondrial genome (mtDNA) replacement in whole populations has been documented for various animal groups, including mammals, amphibians, insects, and fish (see Avise 2000). Most authors typically attribute the occurrence of introgression to historical demographic events, whereas relatively few consider this occurrence in light of the possible selective advantages in natural populations (Ballard and Kreitman 1994, 1995).

Patterns of nonneutral evolution in different mtDNA coding regions have been reported in humans, rats, and *Drosophila* (Nachman et al. 1996; Kennedy and Nachman 1998; Wise, Sraml, and Easteal 1998; Ballard 2000*a*, 2000*b*). Furthermore, in a recent study on mammals, Schmidt et al. (2001) observed that the mtDNA-encoded residues of cytochrome *c* oxidase that are in close physical proximity to nuclear-encoded residues evolve more rapidly than the other mitochondrial-encoded residues, which suggests positive selection. Therefore, the tacit neutral assumption of interspecific mtDNA introgression appears paradoxical when considering the evidence for nonneutrality as well as the functional importance of the 13 mtDNA-encoded genes involved in the mitochondrial respiratory chain leading to energy production. Given the high sensitivity of mitochondrial metabolism to temperature changes (Blier and Lemieux 2001) and the potential impact of single amino acid substitution on the functional properties and thermal sensitivity of enzymes (Holland, McFall-Ngai, and Somero 1997), it is conceivable that the mitochondrial genome may evolve such that animals living in different thermal regimes are able to compensate for the thermal constraints of their environment.

The recent discovery of seven allopatric populations of wild brook charr, *Salvelinus fontinalis* (Pisces, Salmonidae), in eastern Québec (Canada) that are completely introgressed with mtDNA from arctic charr, *S. alpinus*, in the apparent absence of nuclear introgression provides a unique model for investigating the possible selective importance of mtDNA introgression (Bernatchez et al. 1995; Glémet, Blier, and Bernatchez 1998). The evidence at hand suggests that the respiratory enzymes of the introgressed populations of brook charr are encoded by their own nuclear DNA and by arctic charr mtDNA (Glémet, Blier, and Bernatchez 1998). Originally, these two species evolved in distinct thermal environments: the brook charr has a more southern distribution and is typically associated with the warmer waters of lacustrine littoral zones, whereas the arctic charr is typically associated with cold water in

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arctic environments or deep lakes (or both) (Scott and Crossman 1973; Baroudy and Elliot 1994). Because introgressed populations of brook charr are found in high-altitude lakes, we could assume that the introgression of a mitochondrial haplotype that partly evolved in a cold environment could give a selective advantage to brook charr populations living at the northern limit of their distribution. The overall mtDNA nucleotide divergence between the two species has been estimated at 3% (Grewe, Billington, and Hebert 1990), a value comparable to the intraspecific level of divergence reported for most freshwater fishes with a more southern distribution (Bernatchez and Wilson 1998). Both species show highly reduced mtDNA diversity, with a single haplotype being found at a frequency of 98% (brook charr) and 95% (arctic charr) in populations surveyed in northeastern Canada, where the introgressed brook charr populations are found (Wilson et al. 1996; Danzmann et al. 1998).

In this study we perform a comparative sequence analysis of the whole mitochondrial genomes of both brook and arctic charr with the primary objective of localizing the distribution of mutational differences and determining which mtDNA-encoded peptide(s) would be likely to demonstrate specific adaptations to the thermal environment. We then performed a cladistic approach with both *S. alpinus* and *S. fontinalis* and two other salmonids, the Atlantic salmon (*Salmo salar*) and the rainbow trout (*Oncorhynchus mykiss*), to assign the derived state of the amino acid substitution observed in *Salvelinus*. We also compare the brook and arctic charr mitochondrial genomes with that of the rainbow trout to further document the relative mtDNA mutation patterns between the two *Salvelinus* species. Although the analysis of the complete mtDNA variation (mitogenomics) is becoming more common in studies of deep phylogenies (e.g., Curole and Kocher 1999), few studies, at least on animals, have focused on comparisons at lower levels of divergence, such as within-family. Thus, we quantify the extent of mutations across genes and species. This should be of particular use for future phylogenetic studies in salmonid fishes as well as for our understanding of the pattern of substitution on mtDNA evolution at relatively low levels of divergence.

Materials and Methods

DNA Source

Mitochondrial DNA was extracted from two fish using a standard phenol and chloroform extraction protocol. The brook charr possessed the same mtDNA haplotype found in 98% of all brook charr from northeastern North America, as determined by a large-scale phylogeographic survey (Danzmann et al. 1998). Arctic charr mtDNA was obtained from introgressed brook charr of Lake Alain (48°48′00″N, 69°35′30″W) and possessed the same haplotype found in native arctic charr from that area (Bernatchez et al. 1995; Wilson et al. 1996).

PCR Amplification

The entire mitochondrial genomes of brook and arctic charr were amplified using the same primers, except for the D-loop region of arctic charr, which needed different primers. PCR amplifications were performed in 50 μl total volume, with approximately 100 ng of total DNA, 0.5 μl *Taq* DNA polymerase, 15 mM $MgCl_2$, 100 mM Tris HCl buffer, 1% Triton, 500 mM KCl, 0.2 mM of each dNTP, and 0.8 μM of each primer. The thermal cycles were 3 min at 95°C followed by 32 cycles: denaturation for 1 min at 94°C, annealing for 1 min at 43–50°C (depending on the primers), and extension for 1–2.5 min (depending on fragment size) at 72°C. The last cycle was ended by a termination step of 6 min at 72°C. The first segments amplified encompassed the *cox1, cox2, nad/5-6, nad1,* and *nad2* subunits (total of 9,072 bp). We used the following primers—*cox1:* TyrCOI (5′ CTG TTT ATG GAG CTA CAA TC 3′) and SerCOI (5′ GTG GCA GAG TGG TTA TG 3′); *cox2:* AspCOII (5′ GGT CAA GGC AAA ATT GTG 3′) and LysCOII (5′ GCT TAA AAG GCT AAC GCT 3′), designed from the mtDNA sequence of rainbow trout (Zardoya, Garrido-Pertierra, and Baustita 1995); *nad5/6:* C-leu and C-glu (Park et al. 1993); *nad1:* NDI5 and NDI3, unnamed primers described in Hall and Narowrocki (1995); and *nad2:* ND2B-L (5′ AAG CTT TCG GGC CCA TAC CC 3′) and ND2E-H (5′ CCG TTT AGA GCT TTG AAG GC 3′), modified from T. Dowling (Department of Biology, Arizona State University, Tempe, Ariz.).

Direct Sequencing of PCR Products

The double-stranded PCR products were gel-purified in 1% ultra-pure low-melting agarose (GIBCO-BRL), excised from gel under UV light, and purified with a QIAquick Gel Extraction Kit (QIAGEN). The quantities of double-stranded PCR products used for the cycle sequencing reactions were determined according to the Big Dye Terminator Cycle Sequencing Ready Reaction Kit protocol (Applied Biosystems). The resulting cycle-sequencing products were purified with 3 M sodium acetate solution (pH 4.6) in 95% ethanol, followed by two consecutive 70% ethanol washings, and then run on an ABI 377 automated DNA sequencer. Sequencing of the two mtDNA molecules were completed by using the walking primer method with specific primers designed for the sequences obtained. All PCR and cycle-sequencing reactions were performed with the same primers and are available upon request.

Sequence Analysis

Sequence alignment and translation were done with Sequence Navigator (1.0.1) (Applied Biosystems). The sequence comparisons, including nucleotide differences, synonymous substitutions, and total amino acid differences, were carried out with MEGA, version 1.01 (Kumar, Tamura, and Nei 1993, 130 p.). We also quantified nonconservative amino acids substitution, i.e., the replacement of one amino acid by another belonging to a different amino acid class. These classes are based on

Table 1
Differences Between the Mitochondrial Peptide-Coding Genes of Brook and Arctic Charr

	Nucleotide Difference							Amino Acid Substitution		
						Syn.				
Gene	N_n	Total (%)	Ts	Tv	Ts/Tv	nbr	(%)	N_a	Total (%)	Non-conservative (%)
cox1	75	4.8	66	9	7.3	75	4.8	0	0	0
cox2	24	3.5	20	4	5	24	3.5	0	0	0
cox3	46	5.9	41	5	8.2	45	5.7	1	0.4	0.4
cob	58	5.1	45	13	3.5	58	5.1	0	0	0
atp8	5	3.0	4	1	4	4	2.4	1	1.8	1.8
atp6	45	6.6	43	2	21.5	45	6.6	0	0	0
nad1	51	5.2	39	12	3.3	44	4.5	7	2.2	1.2
nad2	77	7.3	60	17	3.5	66	6.3	11	3.2	2.9
nad3	21	6.0	20	1	20	19	5.4	2	1.7	1.7
nad4L	10	3.4	7	3	2.3	10	3.4	0	0	0
nad4	86	6.2	66	20	3.3	82	5.9	4	0.9	0.4
nad5	119	6.5	94	25	3.8	100	5.4	19	3.1	1.8
nad6	39	7.5	33	6	5.5	37	7.1	2	1.2	1.2
Total	656		538	118		609		47		

NOTE.—N_n, total number of nucleotide differences; N_a, total number of amino acid differences; Ts, transition; Tv, transversion; Syn., synonymous substitution.

the properties of the lateral chain of the amino acid (Lehninger, Nelson, and Cox 1993, pp. 112–116).

The nucleotide, synonymous, and nonsynonymous substitution rates were estimated for the mitochondrial protein-coding genes by comparison with the rainbow trout according to Kimura's (1980) two-parameter method (Kimura-2p) and by using the *Oncorhynchus-Salvelinus* divergence time (12%–16% sequence divergence/MYr) estimated from IgM gene introns (Andersson et al. 1995). The comparison of the mitochondrial genomes between each species, which included brook or arctic charr, rainbow trout, and Atlantic salmon, was based on the number of synonymous (K_s) and nonsynonymous (K_a) differences per site calculated with the DNAsp 3.50 program (Rozas and Rozas 2000) for protein-coding genes. The complete mtDNA sequences of brook and arctic charr have been deposited in the GenBank data library under accession numbers AF154850 and AF154851, respectively.

Results

Genome Organization, Genetic Code, and Codon Usage

The genome organization, total length, and features of all genes of the two mtDNA molecules of brook and arctic charr were identified by comparison with rainbow trout mtDNA (Zardoya, Garrido-Pertierra, and Baustita 1995). The gene arrangement is the same as in rainbow trout. The gene length is also the same as that of rainbow trout, except for *nad1* and *atp6,* which differed by one and three additional codons, respectively, in charr. In contrast to rainbow trout, the *atp6, cox3, nad3,* and *nad4* genes in both brook and arctic charr mtDNA had a complete stop codon, and only the *nad4* gene had AGA as the stop codon.

Divergence Between Brook and Arctic Charr Mitochondrial Genomes: Coding Regions

The nucleotide frequency of the light strand was equivalent between species and had the following percentages (for brook and arctic charr, respectively): A = 28.2% and 28.1%; T = 26.5% and 26.5%; C = 28.3% and 28.5%; G = 16.9% and 16.9%. The overall nucleotide divergence between the two complete mtDNA genomes was 4.6%, a value substantially higher than the 3% estimated by Grewe, Billington, and Hebert (1990) using RFLP analyses. The alignment between the two complete mtDNA sequences revealed 13 indels. One of these was found within the noncoding space between the *nad2* and *tRNA-Trp* genes, whereas others occurred within the control region. Table 1 presents the comparison of the 13 protein-coding genes between brook and arctic charr. The total nucleotide differences ranged from 3.0% in the *atp8* gene to 7.5% in the *nad6* gene, whereas the synonymous substitutions ranged from 2.4% in the *atp8* gene to 7.1% in the *nad6* gene. The transition to transversion ratio was highly variable among genes, ranging from 2.3 in the *nad4L* gene to 21.5 in the *atp6* gene.

We identified a total of 47 amino acid substitutions between brook and arctic charr. A single amino acid substitution was observed for the *cox3* gene. The total amino acid difference was higher in other genes, particularly for several genes of the NADH oxidoreductase complex, such as *nad2* (11) and *nad5* (19) (table 1). The *nad1, nad2,* and *nad5* genes also showed the largest differences in nonconservative amino acids along with *atp8.* The other genes (*cox1, cox2, atp6, nad4L,* and *cob*) revealed no difference in amino acids between the two species.

On the basis of established phylogenetic relationships within the salmonid family, with *Salvelinus* and

Table 2
Gene Position of 47 Amino Acid Substitutions Found Between Brook and Arctic Charr, and Their Comparison with Rainbow Trout and Atlantic Salmon

Gene Position	Sal	Sfo	Omy	Ssa
These are the 10 singular autapomorphies in arctic charr:				
Atp8 32	I	T	T	T
Nad1 188	T	A	A	A
Nad2 151	P	S	S	S
199	Y	S	S	S
221	A	T	T	T
313	T	A	A	A
Nad3 80	N	D	D	D
Nad4 170	D	N	N	N
Nad5 447	T	A	A	A
539	V	I	I	I
These are the 17 singular autapomorphies in brook charr:				
Cox3 44	T	A	T	T
Nad1 19	V	I	V	V
245	A	T	A	A
256	A	T	A	A
Nad2 46	Q	K	Q	Q
160	L	M	L	L
Nad4 167	L	I	L	L
Nad5 32	Q	L	Q	Q
50	L	M	L	L
77	T	M	T	T
92	I	V	I	I
165	G	A	G	G
557	S	T	S	S
563	T	A	T	T
608	L	M	L	L
Nad6 98	V	M	V	V
106	W	L	W	W
Thesse are the 10 positions with homoplasious substitutions:				
Nad1 101	I	V	V	I
246	V	I	V	I
Nad2 155	I	V	V	I
332	M	L	M	L
Nad4 121	V	I	V	I
186	L	M	L	M
Nad5 34	E	K	K	E
212	G	E	G	E
427	V	I	I	V
568	I	V	V	I
These are the five positions where both arctic and brook charr are inferred as independently autapomorphic:				
Nad2 162	I	M	T	T
285	A	M	T	T
312	M	A	T	T
Nad5 47	T	A	M	M
515	G	T	N	N
These are five positions with more than one autapomorphy otherwise:				
Nad1 263	V	M	L	V
Nad3 86	A	N	T	A
Nad5 20	T	I	I	L
28	N	S	T	N
491	S	P	H	P

Salmo genera being closer than *Oncorhynchus* (Crane, Seeb, and Seeb 1994; Lee et al. 1998; Oakley and Phillips 1999; Osinov and Lebedev 2000), we assigned the derived state of all replacement substitutions observed between brook and arctic charr with a cladistic approach using rainbow trout and Atlantic salmon as out-groups. Thus, amino acids observed in charr species were considered as derived character states if they differed from the amino acid at a given gene position that was shared by both out-groups. This exercise indicated that 27 of the 47 diverged amino acids observed between arctic and brook charr are autapomorphic characters in one of the two charr species (table 2). Seventeen of these 27 substitutions were observed only in brook charr, with the other three salmonids having the same amino acid at these positions; the other 10 substitutions are found only in arctic charr, whereas brook charr, rainbow trout, and Atlantic salmon have the same amino acid at that position. A statistical comparison of the ratios of specific substitutions for both charr species for these 27 substitutions (10/27 and 17/27, for arctic and brook charr, respectively) did not indicate any significant differences between brook charr and arctic charr (chi-square on the scaled overlap of the confidence intervals: $P = 0.257$; chi-square on frequency table: $P = 0.420$). Hence, neither brook nor arctic charr demonstrated any bias in the rate of accumulation of the amino substitutions.

In addition to the 27 replacements cited above, there are five positions inferred to have an independent, autapomorphic substitution in both charrs (each charr has a residue different from the other and from that shared by the two out-groups, table 2). These substitutions were localized in the *nad2* and *nad5* genes, which exhibited the highest amino acid replacements. In addition, if we consider the overall substitutions between the two charr species (47), seven amino acid replacements displayed a negative mutation score. A negative score denotes a nonconservative replacement for transmembrane proteins (Jones, Taylor, and Thornton 1994). Moreover, these amino acid replacements are distributed in four of the seven NADH complex genes (table 3).

Table 3
Gene Position of Amino Acid Substitutions Showing a Negative Mutation Matrix Score[a]. Substitutions are Considered from Arctic Charr to Brook Charr

Gene Position	Amino Acid Substitution	Matrix Score
nad2 151	P/S	−1
nad2 285	A/M	−1
nad2 312	M/A	−1
nad3 86	A/N	−1
nad5 32	Q/L	−2
nad5 491	S/P	−1
nad6 106	W/L	−2

[a] These scores come from the amino acid mutation matrix for transmembrane proteins (Jones, Taylor, and Thornton 1994).

Comparison with the Rainbow Trout Mitochondrial Genome

The total nucleotide divergence of the rainbow trout mtDNA genome with either brook or arctic charr was found to be very similar—10.3% and 10.1%, respectively. The relative mtDNA mutation patterns in brook and arctic charr were explored by quantifying total nucleotides, amino acids, and synonymous substitution differences for each gene and comparing the patterns of divergence with those between either charr or

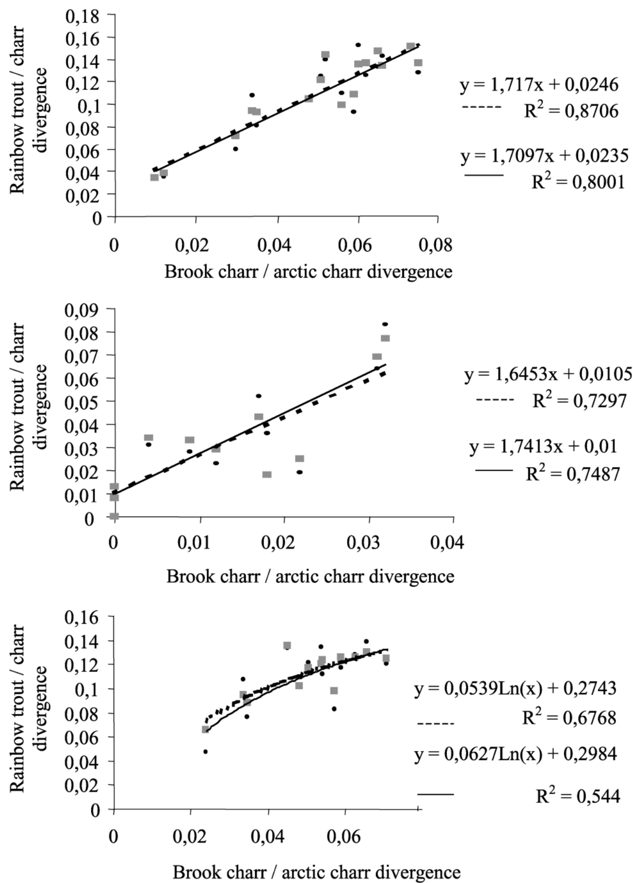

FIG. 1.—Relationships between charr divergence (in number of substitutions per site) in total nucleotide (top), amino acid (middle), and synonymous differences (bottom panel) for protein-coding genes relative to rainbow trout and charr divergences. The ordinate axis represents the divergence between rainbow trout and arctic charr (gray square and dotted line) or rainbow trout and brook charr (black dots and solid line), whereas the abscissa represents the divergence between brook and arctic charr. Each dot denotes an individual gene.

rainbow trout (fig. 1). A linear regression model best fitted the data for comparisons involving total nucleotides and amino acid changes. This model relates the divergences observed between charr species with those observed between each charr species and rainbow trout. The high correlation of divergences provides evidence of a relatively constant mutation rate for each gene (although variable among genes). In both cases the models explained a major part of the variance in the extent of genetic divergence among species for different genes when we compared either nucleotide or amino acid differences. The accumulation of silent mutations (fig. 1, bottom panel) also was highly variable among genes. But for the other two comparisons, the extent of the divergence between the charr species was highly correlated with that observed between the charr species and rainbow trout. Again, both relationships were very similar, although a negative exponential model better fit the observations, suggesting mutational saturation at silent sites with increasing divergence. Altogether, these observations suggest that whereas silent mutations accumulate at the same rate in both species for a given gene, mutational constraints vary greatly among genes.

Table 4
Estimations of Nucleotide, Synonymous, and Nonsynonymous Substitution Rates (10^{-9}/nucleotide site/year) of the Mitochondrial Protein-coding Genes for the Rainbow Trout–Arctic Charr and Rainbow Trout–Brook Charr Comparisons

		CODON POSITIONS				
Gene	Total	First	Second	Third	SYN	NSYN
Rainbow trout–arctic charr						
cox1	4.11	0.99	0	14.14	13.15	0.49
cox2	3.12	0.47	0.15	10.24	9.52	0.31
cox3	3.59	1.26	0.14	10.98	9.89	0.69
cob	4.97	1.06	0.093	17.67	15.44	0.57
atp8	2.24	1.35	0.66	5.07	4.4	1.00
atp6	5.82	1.84	0.16	21.37	19.7	0.98
nad1 . . .	5.59	1.37	0.11	20.78	18.59	0.73
nad2 . . .	6.37	3.71	0.94	17.88	15.68	2.27
nad3 . . .	6.24	4.45	3.64	16.9	15.39	2.44
nad4L . .	4.21	1.92	0	12.79	10.98	0.93
nad4 . . .	5.05	2.2	0.23	15.94	14.56	1.19
nad5 . . .	5.33	2.34	1.19	14.89	13.09	1.76
nad6 . . .	5.13	1.51	0.42	17.12	15.17	0.95
Rainbow trout–brook charr						
cox1	4.09	0.99	0	13.92	12.65	0.49
cox2	3.59	0.63	0.15	11.96	10.89	0.39
cox3	4.30	1.41	0.14	13.91	12.59	0.76
cob	4.77	0.96	0.09	16.85	14.68	0.52
atp8	2.73	1.35	0	7.9	7.57	0.33
atp6	5.41	1.67	0.16	19.34	17.86	0.9
nad1 . . .	5.81	1.84	0.11	20.6	17.98	0.96
nad2 . . .	6.16	3.68	0.84	17	14.61	2.21
nad3 . . .	5.4	3.32	0.94	14.19	12.69	2.09
nad4L . .	3.64	1.92	0	10.35	8.76	0.93
nad4 . . .	5.51	2.37	0.15	17.97	16.4	1.23
nad5 . . .	5.95	0.8	1.5	17.69	15.77	1.85
nad6 . . .	5.46	1.95	0.63	17.61	15.66	1.27

The estimations of nucleotide, synonymous, and nonsynonymous substitution rates (table 4) and the absence of a relationship between the ratios of nonsynonymous to synonymous substitution rates (K_a/K_s) among genes (fig. 2) support the existence of different mutational constraints among genes. In addition, the K_a/K_s ratios were relatively higher for the *nad2, nad3, nad5,* and *atp8* genes. Most of the species comparisons depicted a relatively similar pattern for each gene, although it was obviously divergent among genes. Interestingly, the *atp8* gene, which is among the most variable mitochondrial genes (Pesole et al. 1999), had a synonymous substitution rate 4.2 and 2.4 times lower than that of the faster *nad1* gene and a rate 3.1 and 1.8 times lower than the average rate obtained from mitochondrial genes for the rainbow trout–arctic charr and rainbow trout–brook charr comparisons, respectively. Furthermore, this gene shows a marked difference in the nonsynonymous substitution rate between the two *Oncorynchus-Salvelinus* comparisons (table 4).

Discussion

Brook and Arctic Charr Comparison

A comparison of the brook and arctic charr mitochondrial genomes indicated that most of the amino acid substitutions are localized in the NADH complex genes

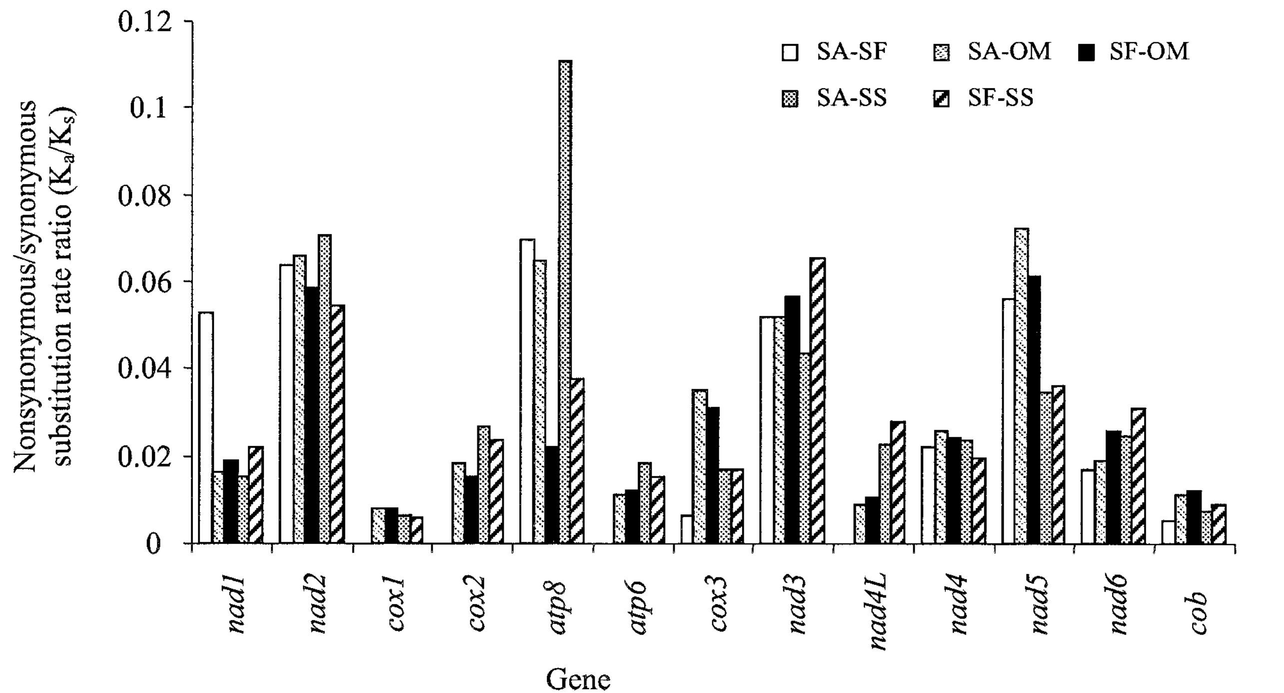

FIG. 2.—Relationship between the number of amino acid differences per site (K_a) and the number of synonymous differences per site (K_s) of the mitochondrial protein-coding genes for the brook charr–arctic charr (SA-SF), rainbow trout–brook charr (SF-OM), rainbow trout–arctic charr (SA-OM), brook charr–Atlantic salmon (SF-SS), and arctic charr–Atlantic salmon (SA-SS) comparisons. The genes appear in the order observed in the mitochondrial genome.

with others found in the *cox3* and *atp8* genes. Considering that brook and arctic charr are closely related, it was not surprising to observe an absence of amino acid substitutions in highly conserved genes (*atp6, nad4L, cob, cox1,* and *cox2*) (Meyer 1993). Considering the single amino acid substitution in *cox3,* it is unlikely that this substitution has a particular adaptive value in arctic charr relative to brook charr because this character is shared by both rainbow trout and Atlantic salmon. For instance, an amino acid that would improve the functional properties of cytochrome *c* oxidase at low temperatures in arctic charr would be of low adaptive value in a species living at higher temperatures (e.g., rainbow trout). The substitutions that showed a negative score (table 3) suggest that these amino acid substitutions may have significant consequences on the structure and functional properties of the polypeptides. These observations also suggest that complex I could represent an important target where selection could act on the charr mitochondrial genome for adaptation to specific environmental factors (for example, thermal niches). More extensive analyses are needed to clarify the potential impact associated with the substitutions observed in the mitochondrial-encoded subunits of the NADH oxidoreductase complex (tables 1–3).

The occurrence of mutations in mitochondrial-encoded peptides, which are closely associated with the nuclear-encoded genes (complexes I and IV in particular), could indicate where natural selection acts to maintain the physiological function of enzyme complexes and to favor the evolutionary coadaptation of the interaction between mitochondrial peptides (nuclear and mitochondrial encoded, see Schmidt et al. 2001). Some studies have demonstrated that amino acid substitutions may have an impact on nuclear-mitochondrial interactions and on the mechanisms regulating mitochondrial functioning (reviewed in Blier, Dufresne, and Burton 2001). Thus, future comparative analyses will be required to determine the extent of nuclear amino acid substitution divergence between the two charr to understand better the potential consequence of mitochondrial introgression between brook and arctic charr on mitochondrial functions.

Evolution of Mitochondrial Genes in Salmonids

Even when considering genes separately, our data demonstrated a highly significant level of correlation (fig. 1) and a relatively constant mutation rate (table 4) when we compared the two charr or either charr and rainbow trout (for nucleotide, amino acid, and synonymous substitution comparisons). Comparative studies of mitochondrial gene evolution have shown important differences in mutational accumulation, patterns, and rates of amino acid substitutions among mitochondrial genes and their consequences for deep phylogeny reconstruction (e.g., Mindell and Thacker 1996; Russo, Takezaki, and Nei 1996). Our results clearly show that despite the absence of recombination and the haploid nature of mitochondrial DNA, a comparison of closely related species also reveals individual patterns of evolution of the different mitochondrial genes; these patterns seem relatively independent of the phylogenetic distance of species. Therefore, the relatively constant mutational dif-

ferences observed for each gene between rainbow trout and both charr species suggest that comparable estimates of relative divergence would be obtained regardless of the selected mitochondrial gene segments used for phylogenetic reconstruction among salmonid species.

Given that synonymous substitutions appear to accumulate relatively rapidly in the *atp6, cox1,* and *cob* genes (see tables 1 and 4), it was surprising to find no amino acid substitutions in these genes between brook and arctic charr. Likewise, the highest synonymous differences were not observed in genes with higher numbers of amino acid substitutions. The patterns of divergence (nucleotide and amino acid) of each gene and the relative substitution rates between genes, established during the independent evolution of both charr species, are relatively similar to those observed during the independent evolution of the *Oncorhynchus* and *Salvelinus* genera (fig. 1). Consequently, the substitution rate does not appear to be established randomly. Furthermore, it appears that substitution patterns among different mitochondrial genes were not determined by synonymous substitution rates. Our data (fig. 2) suggest a relaxation of purifying selection or the presence of a positive selection in the *nad2, nad3,* and *nad5* genes compared with the more conservative genes such as *cox1, cox2,* and *nad4L.* In fact, several studies have reported the possible interaction of functional constraints in the evolution rate of mitochondrial genes (Ballard 2000*a,* 2000*b*). In addition, Rand and Kann (1996) mention that opposing evolutionary pressures may act on different regions of the mitochondrial genes and genomes. Accordingly, the absence of parallelism in our results between the accumulation rate of amino acids and synonymous substitutions suggests that functional constraints on the substitution rate of the different mitochondrial genes are maintained in different species. Surprisingly, 10 substitutions are inferred to be homoplasious, and eight of these 10 involve only I, V, L, and M (six of these substitutions involve V and I [table 2]). These amino acids have methylene and methyl groups, and their most important property is that their side chain is hydrophobic. This suggests important structural and functional constraints in the evolution of these sites. Because they constitute more than 20% of the amino acid substitutions observed between brook and arctic charr, these constraints could be highly significant.

There is increasing evidence that selection acts on silent sites in *Drosophila* mtDNA (Akashi and Schaeffer 1997; Moriyama and Powell 1997; Kennedy and Nachmann 1998). Although closely related species should not be affected by multiple substitutions and should provide a better representation of evolutionary events and substitution dynamics (Meyer 1993), our results suggest a mutational saturation with increasing gene divergence among closely related species (fig. 2). The data also indicate that whereas synonymous substitutions accumulate similarly in both species for a given gene, there may be a possibility of important mutational constraints at silent sites among genes, particularly in the *atp8* gene, that show a lower synonymous substitution rate than do the other genes (table 4). Akashi (1994) supports the possibility that selective constraints act on synonymous codon usage through the translation efficiency.

Conclusions

In summary, five amino acid substitutions specific to the charr comparison, localized in the *nad2* and *nad5* genes and denoting negative scores according to the functional properties of amino acids (Jones, Taylor, and Thornton 1994), could possibly have an impact on the structure and functional properties of these mitochondrial peptides. If the introgression of the mitochondrial genome of a species associated with the arctic environment (arctic charr) into a species of a more temperate environment (brook charr) can confer an adaptive advantage, future investigations should focus on the functional properties of Complex I genes in pure and hybrid populations of both species as well as in introgressed populations. Moreover, if functional property differences are detected, we will need to investigate whether they translate into modifications of the functional properties of mitochondria as well as into changes in the metabolic and physiological performance of the fish.

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