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Population structure of Atlantic salmon (*Salmo salar* L.): a range-wide perspective from microsatellite DNA variation

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Abstract

Atlantic salmon (n = 1682) from 27 anadromous river populations and two nonanadromous strains ranging from south-central Maine, USA to northern Spain were genotyped at 12 microsatellite DNA loci. This suite of moderate to highly polymorphic loci revealed 266 alleles (5–37/locus) range-wide. Statistically significant allelic and genotypic heterogeneity was observed across loci between all but one pairwise comparison. Significant isolation by distance was found within and between North American and European populations, indicating reduced gene flow at all geographical scales examined. North American Atlantic salmon populations had fewer alleles, fewer unique alleles (though at a higher frequency) and a shallower phylogenetic structure than European Atlantic salmon populations. We believe these characteristics result from the differing glacial histories of the two continents, as the North American range of Atlantic salmon was glaciated more recently and more uniformly than the European range. Genotypic assignment tests based on maximum-likelihood provided 100% correct classification to continent of origin and averaged nearly 83% correct classification to province of origin across continents. This multilocus method, which may be enhanced with additional polymorphic loci, provides fishery managers the highest degree of correct assignment to management unit of any technique currently available.

Keywords: biogeography, fisheries management, microsatellite, phylogeography, population genetics, Salmonidae

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Introduction

Analysis of neutral molecular markers has proven to be a robust method for identifying reproductive isolation among populations and for bringing an evolutionary perspective to conservation and management. A number of common circumstances can complicate identification of evolutionary patterns and processes characteristic of at-risk species. For example, independence of separate evolutionary lineages may be difficult to demonstrate if isolation has been recent. On the other hand, population fragmentation can rapidly reduce genetic variation within populations and result in significant differentiation among populations through random genetic drift. Conversely, anthropogenic disturbance and intervention can obscure historic patterns of gene flow by homogenizing populations. Furthermore, complex life histories that do not conform to common population genetic models can challenge analysis. Perhaps no fish species illustrates these conservation genetic challenges as well as the Atlantic salmon (*Salmo salar*), a species that has been eradicated from two-thirds of its habitat in the United States and is currently the focus of intensive management to avoid extinction in the United States (Mather *et al*. 1998).

Anadromous Atlantic salmon undergo extended ocean migrations but exhibit a high homing fidelity to their natal river or tributary. The substantial reproductive isolation

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between populations fostered by such precise philopatry has facilitated the evolution and persistence of the local adaptation that has been extensively documented in salmon (see Taylor 1991 for review). For instance, the number of winters spent at sea varies latitudinally in North America, with US and southern Canadian stocks comprised primarily of multisea-winter salmon while the more northerly Canadian stocks consist of one-sea-winter fish (grilse). While most North American Atlantic salmon migrate to feeding grounds in the Labrador Sea off Greenland, those spawning in rivers of the inner Bay of Fundy tend not to leave the bay, and furthermore tend to spawn as grilse rather than staying at sea for multiple winters, as do most fish from that latitude. The persistence of variation in these genetically based life history traits suggests that gene flow between stocks has been limited. However, imperfect homing to natal streams may cause neutral markers to reflect less divergence than adaptively important traits. Identifying evolutionary differences in salmon therefore may be both particularly difficult and especially important.

For more than a century, the principal management tool directed toward Atlantic salmon in Maine has been supplemental stocking with fish from larger, but often distant, populations. For example, fertilized eggs and fry from Canada's Miramichi and Saguenay Rivers which were intentionally introduced into the Penobscot River program (USFWS-NOAA 1995; Baum 1997). The extent of introgression (if any) from these fish into native Maine salmon is unknown as is the potential for the negative consequences of outbreeding depression resulting from disruption of coadapted gene complexes in locally adapted stocks (Templeton 1986).

Prompted by diminishing spawning runs and low juvenile densities, US resource managers have recently designated Atlantic salmon in seven Maine rivers as candidates for protection under the US Endangered Species Act (ESA). Moreover, federal and state (i.e. Maine) management agencies have developed an Atlantic Salmon Conservation Plan (ASCP). Knowledge of the amount of genetic diversity present and the evolutionary relationships (e.g. levels of gene exchange) among geographic populations of Atlantic salmon are essential to the most informed planning and implementation of sound management efforts (Ståhl 1987). The increased use of hatchery-reared Atlantic salmon for supplemental stocking and aquaculture in the ASCP underscores the need to characterize the genetic composition of both wild and captive populations.

Since 1992, the diminished Downeast Maine populations have been maintained as seven river-specific stocks in the hatchery. The separate river-specific stocks are intended to maintain overall genetic diversity in the face of low effective population size. Rare alleles are expected to be lost in some populations and fixed in others, resulting in overall preservation of genetic diversity. In contrast, under panmixia, alleles will be fixed or lost throughout the entire population. Because some of the river-specific strains have remained small, there has been some concern that genetic drift and demographic stochasticity may overwhelm natural selection, potentially resulting in lower genetic diversity and fitness. Documentation of the genetic relatedness among all gene pools used for various management objectives will aid in understanding the impacts such processes can have on the populations of salmon inhabiting rivers of Maine.

Previous genetic surveys of Atlantic salmon have shown little genetic divergence range-wide, due in all likelihood to the recent (8000–10 000 years BP) colonization of their present habitat (Crossman & McAllister 1986; Ståhl 1987; Davidson *et al*. 1989; Verspoor 1997). Frequency variation in transferrin proteins (Payne *et al*. 1971; Thorpe & Mitchell 1981), allozymes (Ståhl 1987; Verspoor 1988; Bourke *et al*. 1997), restriction fragment length polymorphisms of mitochondrial DNA (mtDNA) (Bermingham *et al*. 1991; King *et al*. 2000) and chromosome numbers (Hartley & Horne 1984) have suggested that some degree of genetic differentiation exists between Atlantic salmon inhabiting North America and Europe. Until recently (Fontaine *et al*. 1997; McConnell *et al*. 1997; Tessier *et al*. 1997; Beacham & Dempson 1998; King *et al*. 2000), there have been few population genetics data describing differentiation within wild Atlantic salmon from the USA or Canada, and none documenting differentiation between US and Canadian populations.

Range-wide surveys of heritable genetic information are useful for delineating evolutionarily significant lineages. We have undertaken large-scale studies of the genetic population structure of Atlantic salmon using mitochondrial (King *et al*. 2000) and microsatellite DNA variation. This line of research will ultimately help to determine the evolutionary status of Atlantic salmon in Maine relative to other portions of their range and will help verify the extent of neutral genetic variation present within the species. We report microsatellite DNA variation detected within and among Atlantic salmon populations in North America from Maine and Atlantic Canada to European populations from Iceland to Spain (Fig. 1). We summarize fragment variation at 12 microsatellite DNA loci in 1682 anadromous Atlantic salmon sampled from 27 rivers in 12 provinces or countries and two nonanadromous (landlocked) salmon strains maintained in the USA.

Methods

Tissue collection

Fin or scale tissue samples were dissected from Atlantic salmon parr, smolts, grilse and/or adults from 27 putatively wild anadromous river populations and two nonanadromous strains ranging geographically from

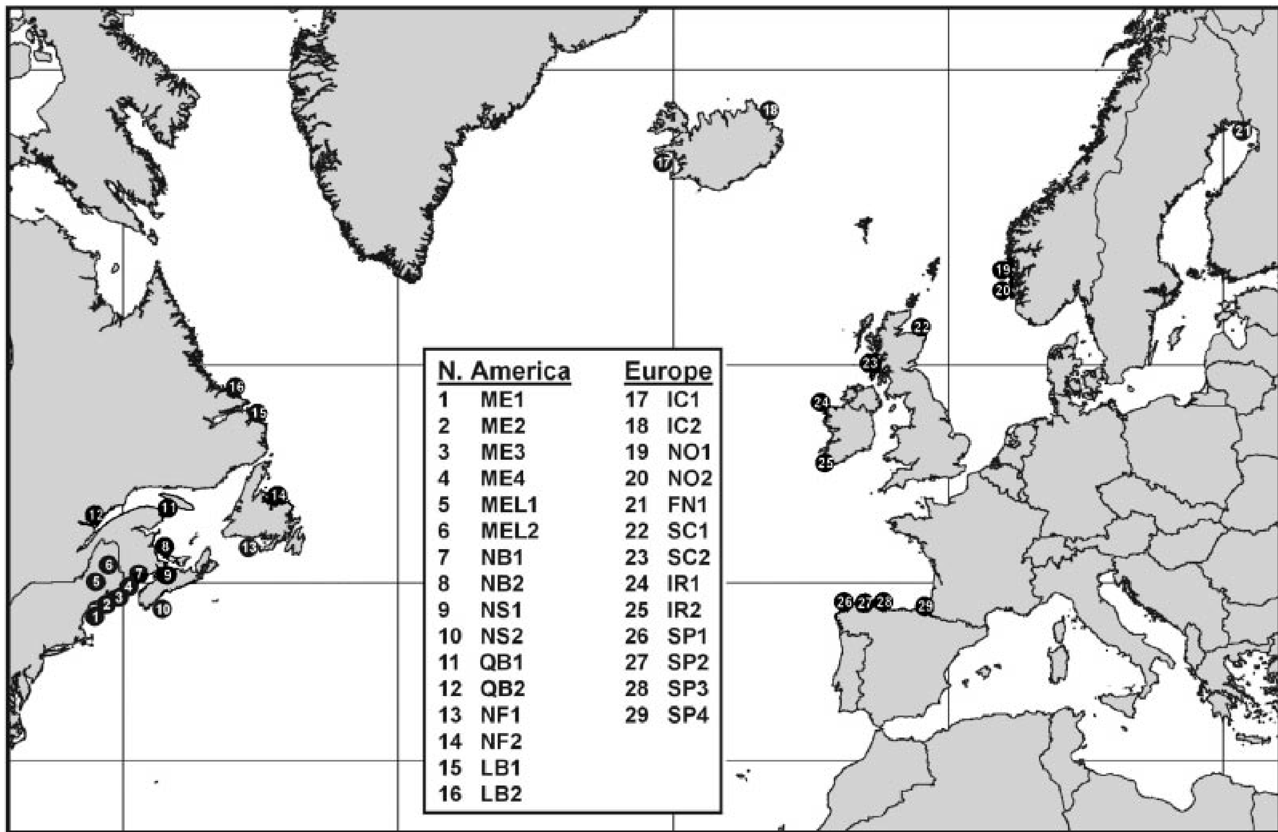

Fig. 1 Location of 29 Atlantic salmon populations sampled for variation at microsatellite loci.

south-central Maine, USA to northern Spain. Table 1 provides a general description of the river or collection location, the life stage(s) sampled, the maximum sample size surveyed at any locus and the year classes sampled. Adults from the two nonanadromous strains were sampled in hatcheries used to supplement landlocked populations in Maine. A portion of the samples from the Stewiacke and Gold Rivers of Nova Scotia, Canada and the Finland collection were taken from parr generated in a hatchery environment from wild returning adults. All other collections were from fish collected in the river and where possible they were screened to eliminate the influence of supplemental stocking or aquaculture escapees. Due to the relative scarcity of adult fish, collection efforts were directed at 0 + and 1 + parr using pulsed direct current backpack electrofishing units in Maine. To minimize the chances of collecting siblings, efforts were made to avoid collecting all fish for a given collection in a single area (or electrofishing event; Allendorf & Phelps 1981). To guarantee that selected collection sites are represented by multiple families, as many as four year-classes were sampled. Pectoral, anal, or caudal fin samples were submerged in 95% ethanol and refrigerated during fixation for at least 24 h before shipping. Scale samples were provided dried in envelopes. All samples were shipped at ambient temperature by courier to the US Geological Survey-Biological Resources Division, Leetown Science Center, Aquatic Ecology Laboratory, Kearneysville, WV.

DNA isolation

Genomic DNA was isolated from fin tissue by one of two extraction methods: the CTAB method as described by Fields *et al*. (1989) or PureGene, a commercially available DNA extraction kit (Gentra Systems). For CTAB extractions, tissue digestion and phenol–chloroform extractions were performed in a single Phase Lock Gel™I Heavy Tube (5 Prime-3 Prime, Inc.). The manufacturer's guidelines were followed for the PureGene kit. DNA quantity was determined by fluorescence assay (Labarca & Paigen 1980) and integrity of the DNA was visually determined on 1% agarose gels (Sambrook *et al*. 1989).

DNA was extracted from scales by placing one to three scales into 200 μL of InstaGene matrix (Chelex resin, Bio-Rad Laboratories), followed by vortexing and brief centrifugation. Proteinase K (10 μL) was added to the scale/chelex mixture, which was allowed to digest overnight at 65 °C. The digested solution was then boiled for 10 min and centrifuged for 3 min at 13 000 *g*. Portions (5 μL) of the resultant supernatant were used in polymerase chain reactions (PCR).

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Table 1 Collection site (with abbreviation), life stage, the maximum sample size, and year classes sampled in 1682 Atlantic salmon surveyed for variation at 12 microsatellite DNA markers

Abbr.	General collection site	Life stage*	Sample size	Year class(es) sampled
ME1	Togus Stream (Kennebec R.), Maine, USA	P	81	1993–5
ME2	Penobscot R. Maine, USA	A	195	1995–6
ME3	Narraguagus R., Maine, USA	P	230	1993–5; 1997
ME4	Dennys R., Maine, USA	P	133	1993–5; 1997
MEL1	Sebago (landlocked), Maine, USA	LA	50	1992–3; 1996
MEL2	West Grand Lake (landlocked), Maine, USA	LA	42	1992–3; 1996
NB1	Nashwaak R. (St. John R.), NB, Canada	A	66	1992–3
NB2	Miramichi R., NB, Canada	A	56	1991–2
NS1	Stewiacke R., NS, Canada	F,A	56	1984; 1993
NS2	Gold R., NS, Canada	P,F	54	1993–4
QB1	St. Jean R., Québec, Canada	A	63	1996
QB2	Saguenay R., Québec, Canada	S	59	1997
NF1	Conne R., NF, Canada	P	30	1993
NF2	Gander R., NF, Canada	A	63	1992
LB1	Sand Hill R., Labrador, NF, Canada	P	16	1995–6
LB2	Michaels R., Labrador, NF, Canada	P	29	1995–6
IC1	R. Ellidaar, Iceland	P	50	1997
IC2	R. Vesturdalsa, Iceland	P	46	1997
NO1	R. Lone, Norway	P,A	57	1994–6
NO2	R. Vosso, Norway	P,A	44	1991–5
FN1	R. Tornionjoki, Finland	F	61	1994
SC1	R. Shin, Scotland	A	24	1987–90
SC2	R. Nith, Scotland	A	29	1987–90
IR1	R. Spaddagh, Ireland	P	30	1994
IR2	R. Blackwater, Ireland	P	34	1991
SP1	R. Eo, Spain	A	26	1991–2
SP2	R. Esva, Spain	P	18	1993–4
SP3	R. Sella, Spain	A	14	1990–1
SP4	R. Bidasoa, Spain	P	26	1996
		Total	1682	

NB, New Brunswick; NS, Nova Scotia; NF, Newfoundland.
*Life stage sampled: P, wild parr; F, F_1 parr from wild adults; S, wild smolts; A, returning adult; LA, landlocked adult.

Microsatellite analysis

PCR methodology. Twelve microsatellite loci were screened in all fish (Ssa14 and Ssa289, McConnell *et al*. 1995; SSOSL25, SSOSL85, SSOSL311 and SSOSL438, Slettan *et al*. 1995, 1995; Ssa85, Ssa171, Ssa197 and Ssa202, O'Reilly *et al*. 1996; SSLEEI84 and SSLEEN82, GenBank accession nos U86703 and U86706, respectively, Schill and Walker, in preparation). Each PCR consisted of 6 ng/µL of genomic DNA, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer (forward primer labelled with TET, FAM, or HEX; Applied Biosystems), 1.0 unit of *Taq* DNA polymerase (Applied Biosystems) and deionized water to 10 µL. The amplification cycle consisted of a 2-min denaturation at 94 °C followed by 35 cycles of 94 °C denaturing for 30 s, 56 °C annealing for 30 s and 72 °C extension for 2 min. Cycling concluded with a 10-min extension at 72 °C. Thermal cycling was performed in one of three MJ DNA Engine PTC 200s (MJ Research). All thermal cyclers were configured with heated lids.

Capillary electrophoresis and scoring. For analysis, 1 µL of PCR product was diluted 3:1 with deionized water and thoroughly mixed. Then, 1 µL of this dilution was added to 12 µL of deionized formamide and 0.5 µL of the internal size standard GENESCAN-500 (Applied Biosystems). PCR products of multiple separate reactions were combined for electrophoresis into two groups of loci. Loci were identified in these combined samples by virtue of their characteristic molecular mass and attached fluorescent label. Fluorescent lablel and multiplexed electrophoresis combinations were optimized towards those presented in Table 2. The size standard contained DNA fragments labelled with the dye phosphoramidite TAMRA. This PCR product/size standard/formamide mixture was heat denatured at 95 °C for 3 min, placed immediately on ice for at least 5 min and then subjected to capillary electrophoresis on a Applied

Table 2 Summary information for 12 Atlantic salmon microsatellite loci. Recommended fluorescent dye for the forward primer and groupings (loci combined and analysed simultaneously), the number of observed alleles, the range of allele sizes, and average expected heterozygosity (H_E) overall, in North America and in Europe

			Global		North America			Europe		
Locus	Label	Group	No. of alleles	Size range of alleles	No. of alleles	Size range of alleles	H_E	No. of alleles	Size range of alleles	H_E
*Ssa*197	FAM	A	32	126–268	21	140–228	0.86	28	126–268	0.87
*Ssa*202	FAM	A	28	228–330	23	242–330	0.88	16	228–282	0.78
*Ssa*289	FAM	A	8	103–119	7	103–117	0.24	6	107–119	0.55
*Ssa*14	HEX	A	5	139–147	5	139–147	0.42	4	139–145	0.39
SSLEEI84	HEX	A	23	165–221	21	165–217	0.85	19	165–221	0.83
*Ssa*171	TET	A	37	195–285	33	207–285	0.90	24	195–267	0.80
*Ssa*85	TET	A	28	106–164	18	106–152	0.76	24	116–164	0.82
SSLEEN82	FAM	B	15	196–230	14	196–228	0.41	10	212–230	0.62
SSOSL311	FAM	B	30	108–172	18	108–166	0.14	27	118–172	0.87
SSOSL438	HEX	B	18	96–144	9	104–132	0.37	15	96–144	0.68
SSOSL25	TET	B	17	138–174	15	138–174	0.56	15	142–172	0.73
SSOSL85	TET	B	25	175–224	15	175–202	0.81	24	176–224	0.79

Biosystems PRISM 310 Genetic Analyser. Fluorescent DNA fragments were analysed, and genotype data were generated using GENESCAN software (Applied Biosystems). GENOTYPER version 2.0 (Applied Biosystems) DNA fragment analysis software was used to score, bin and output allelic (and genotypic) designations for each salmon.

Statistical analyses

Observed genotype frequencies were tested for consistency with Hardy–Weinberg and linkage equilibrium expectations using randomization tests implemented by GENEPOP 3.1 (Raymond & Rousset 1995). The Hardy–Weinberg test used the Markov chain randomization test of Guo & Thompson (1992) to estimate exact two-tailed *P*-values for each locus in each sample. Global tests combined these results over loci and sampling locations using Fisher's method (Sokal & Rohlf 1994). Bonferroni adjustments (Rice 1989) were used to determine statistical significance for these and all other tests. Linkage equilibrium tests used the randomization method of Raymond & Rousset (1995) for all pairs of loci. The amount of genetic variation in each sample was summarized by gene diversity (average expected heterozygosity) and the average frequency of unique alleles.

We examined the distribution of unique alleles at two geographical scales: regional and continental. Unfortunately, there is currently no statistical method for comparing the number of unique alleles among different samples. Two sampling issues can complicate identification of unique alleles. First, differences in sample size must be accounted for, because larger samples are expected to have a greater chance of detecting alleles. Second, the distribution of sampling locations must be considered so that alleles unique to regions but not populations are correctly identified. Both issues arise in our analysis. In order to account for differential sampling of regions, we pooled genotypes from all of the populations in the 12 regions included in the study (Table 1). In order to compensate for different sample sizes for each region, we randomly selected 40 individuals without replacement from each region and then counted the number of alleles unique to each sample. This process was repeated 1000 times and the average number of unique alleles was calculated to serve as an estimate of the number of unique alleles present within each population that this study would detect if it had a more balanced design. In order to minimize the potential effect of homoplasy, each continent was analysed independently in the regional analysis (i.e. an allele found throughout Europe but only in Labrador within North America was considered unique to Labrador). A continental analysis was performed in the same way (40 individuals randomly selected from each region) except that the number of alleles unique to each continent was recorded.

Several techniques were used to describe genetic relationships between collections. Differences between each pair of populations were summarized by pairwise genetic distance. Three methods were used to evaluate genetic distance: Nei's standard distance, Nei's D_a (Nei *et al*. 1983), and the chord distance of Cavalli-Sforza & Edwards (1967). Simulations suggested that there was less bias in the D_a estimate of genetic distance (S. Kalinowski, unpublished data) and that this distance measure estimates tree topology better than other commonly used genetic distances (Takezaki & Nei 1996), so it was used in all subsequent analyses. The statistical significance of genetic differences between each pair of samples was tested using the genetic differentiation randomization test in GENEPOP (Raymond & Rousset 1995). Results were combined over loci using

Fisher's method (Sokal & Rohlf 1994). Multidimensional scaling (MDS) with a Kruskal linear regression loss function was used to summarize relationships between sampling locations on each continent (SYSTAT 7.0). MDS was chosen to provide a perspective of the underlying structure of the genetic distance matrix without imposition of a bifurcating evolutionary history. Similarity between pairs of populations was represented by $1 - D_a$. An unrooted phylogenetic tree was fit to the data using the D_a distance matrix and neighbour-joining (NJ) algorithm. TREEVIEW (Page 1996) was used to visualize the tree. The strength of support for each node in the tree was tested by bootstrapping over loci using NJBPOP (J.-M. Cornuet, INRA, Montpellier, France). Analysis of molecular variance (AMOVA) was used to partition genetic variation in a hierarchy of groups of populations (Excoffier *et al*. 1992). ARLEQUIN 1.1 was used to quantify and test the statistical significance of differentiation between continents, among provinces and countries within each continent, and between anadromous and nonanadromous salmon in Maine.

Isolation by distance was examined by comparing D_a for each pair of samples with the geographical distance separating sampling locations. Geographic distance was estimated as the shortest ocean distance between river mouths. The distance between North American and European sampling locations was estimated via Iceland. The statistical significance of the correlation between genetic and geographical distance matrices was assessed with a Mantel randomization test performed by the MXCOMP routine in NTSYS-PC 1.8 (Rohlf 1993). The relationship between genetic distance and geographical distance was described with regression lines for each continent (SYSTAT 7.0).

Assignment tests were used to determine the likelihood of each individual's genotype being found in the collection from which it was sampled (Paetkau *et al*. 1995; Cornuet *et al*. 1999) using the programs DOH and GENECLASS available at http://gause.biology.ualberta.ca/jbrzusto/Doh.html and http://http://www.ensam.inra.fr/URLB, respectively. Values of χ^2 were generated to further test the significance of the correct assignments by comparing the observed numbers of correct classifications to the numbers of correct classifications that would be expected by chance. The expectations of numbers correctly classified by chance alone were calculated assuming equal probability of membership in any single population while the probability of membership in any grouping was assumed to be in proportion to the number of populations comprising that group. We used GENECLASS to evaluate the effects of individual loci on classification accuracy by leaving each locus out of the analysis sequentially and utilizing the remaining 11 for assignments. To investigate the relationship between the number of loci used for assignments and performance, we added loci to the analysis in a stepwise fashion. We also used GENECLASS to compare several distance measures [D_a; chord distance; Nei's standard distance; minimum distance, Nei 1987; allele-sharing, Shriver *et al*. 1997; $(\delta\mu)^2$, Goldstein *et al*. 1995] implemented in the program for their utility in making assignments.

Results

Genotypes for 12 microsatellite DNA loci were determined for 1682 juvenile and adult Atlantic salmon sampled from 29 locations covering much of the species' range and representing year classes from 1984 to 1997 (Table 1). A total of 266 alleles were observed across the 12 loci ranging from five alleles at Ssa14 to 37 at Ssa171 (Table 2). The three loci containing tetra-nucleotide repeats (Ssa197, Ssa202 and Ssa171) were hypervariable in all populations and accounted for 97 alleles. The numbers of alleles observed at a locus varied greatly between continents as indicated in the averaged expected heterozygosities (Table 3).

Randomization tests showed that most of the samples were consistent with Hardy–Weinberg expectations. When each locus from each location was analysed separately, seven (2%) were found to have *P*-values significant at the 0.05 level after correcting for the number of tests (overall $\alpha = 0.0042$): Ssa197 in ME1 (heterozygote deficiency; D) and NS1 (heterozygote excess; E); Ssa202 in NS1 (E); Ssa171 in ME1 (E); SSOSL438 in IR1 (D); and SSOSL25 in IC1 and IC2 (E). When results were combined across loci for each location, only one (3.7%) of the locations (NF1) showed significant departure from Hardy–Weinberg expectations after adjustment for multiple tests. Genotypes for each locus were consistent with expectations when combined across all locations.

Linkage disequilibrium was negligible (fewer than three pairs of loci in linkage disequilibrium, generally not the same loci involved) for most samples. Two samples, the Togus Stream tributary of the Kennebec, and the River Sella in Spain, had substantial linkage disequilibrium (19 and 11 pairs of loci in linkage disequilibrium, respectively). Both samples consisted of mixed year classes. The admixture of year classes combined with extremely small effective population sizes (and/or sampling siblings within year classes) could produce the extremes of linkage disequilibrium observed in these populations.

Estimates of genetic variation suggest that the North American collections showed consistently lower levels of gene diversity (0.60) than that observed in European populations (0.73) (Table 3). Within North America, the Maine landlocked strains (MEL1, MEL2) were found to have considerably less diversity than the anadromous salmon. Excluding these collections, only slightly lower levels of average diversity were observed between Maine (0.59) and the Canadian provinces (0.62). Within Europe, the lowest level of gene diversity was observed in the FN1 collection (0.62) while the IR2 collection showed the greatest level (0.79).

Table 3 Gene diversity (H_E) and frequency of unique alleles within each sample ($\bar{p}$) for 29 populations of Atlantic salmon

Sample	H_E	$\bar{p}$
ME1	0.59	0.0036
ME2	0.60	0.0000
ME3	0.60	0.0002
ME4	0.58	0.0006
MEL1	0.53	0.0000
MEL2	0.52	0.0000
NB1	0.62	0.0006
NB2	0.65	0.0010
NS1	0.61	0.0000
NS2	0.56	0.0008
QB1	0.67	0.0007
QB2	0.60	0.0012
NF1	0.60	0.0047
NF2	0.63	0.0012
LB1	0.61	0.0000
LB2	0.62	0.0000
IC1	0.67	0.0000
IC2	0.64	0.0009
NO1	0.75	0.0015
NO2	0.74	0.0014
FN1	0.62	0.0014
SC1	0.71	0.0035
SC2	0.78	0.0039
IR1	0.77	0.0027
IR2	0.79	0.0013
SP1	0.76	0.0063
SP2	0.71	0.0023
SP3	0.78	0.0030
SP4	0.73	0.0000
North American average	0.60	0.0009
European average	0.73	0.0022

Our unique alleles analysis of samples pooled by province or country suggests that populations within North America have fewer unique alleles than European populations. In North America, NF had the largest number of unique alleles (approximately 10) while NS had the fewest unique alleles (approximately 1). In Europe, the IR sample had the largest number of unique alleles (approximately 15) and the FN sample had the least (approximately two). For samples of 80 genes, we estimate there to be 48 alleles unique to North America and 77 alleles unique to Europe. Interestingly, the alleles unique to North America had a higher total cumulative frequency than the European alleles. In North America, unique alleles composed 21% of the simulated equal sized samples, compared to 16% in Europe. Similar trends were observed in the average frequency of unique alleles (Table 4) as European populations showed unique alleles (average frequency = 0.0022) in 11 of 13 collections and North American samples contained on average fewer unique alleles (0.0009), found in only 10 of 16 collections. Within North America, unique alleles were absent from ME2, NS1, LB1, LB2, MEL1 and MEL2. In Europe, unique alleles were absent from IC1 and SP4.

Table 4 Estimated number of unique alleles (n_a) and their total frequency (p) by country or province and continent

	n_a	p
ME	2.7	0.0040
NB	7.5	0.0096
NS	1.1	0.0013
QB	7.3	0.0129
NF	9.6	0.0172
LB	3.4	0.0044
North America	47.8	0.2136
Iceland	4.9	0.0152
Norway	5.1	0.0114
Finland	2.2	0.0046
Scotland	7.9	0.0162
Ireland	14.6	0.0264
Spain	9.3	0.0244
Europe	77.1	0.1562

Large differences in distribution of both alleles and genotypes were observed throughout the study area. Of 4872 single-locus pairwise tests of allele distributions, 3975 (81.6%) indicated departures from homogeneity ($P < 0.0001$). Similarly, 3986 (81.8%) pairwise tests of genotype distributions were observed to be heterogeneous ($P < 0.0001$). When allele and genotype heterogeneity tests were combined across all loci, highly significant heterogeneity was observed between all pairs of collections except between LB1 and LB2 ($P < 0.002$).

Pair-wise genetic distance (D_a) values were calculated between all collections to investigate evolutionary relationships in allele frequencies. The greatest genetic distances were observed between North American and European collections, ranging between 0.46 and 0.75. Among all collections, genetic distances were lowest in comparisons within countries and provinces (or state) and generally increased with geographical distance. Within continents, genetic distance between anadromous North American collections (0.02–0.19) was less than that observed among the more widely dispersed European collections (0.12–0.43). The greatest distances observed within North America were between anadromous and nonanadromous (MEL1, MEL2) collections (0.13–0.25) and the lowest were between the geographically proximate Maine collections (0.02–0.06).

A graphical representation of the genetic distance (D_a) matrix was generated using multidimensional scaling. Given the large genetic distances observed in intercontinental comparisons, North American (Fig. 2a) and European (Fig. 2b) collections are displayed using two-dimensional multidimensional scaling. A third dimension only explained 4% of the variation observed in the matrix.

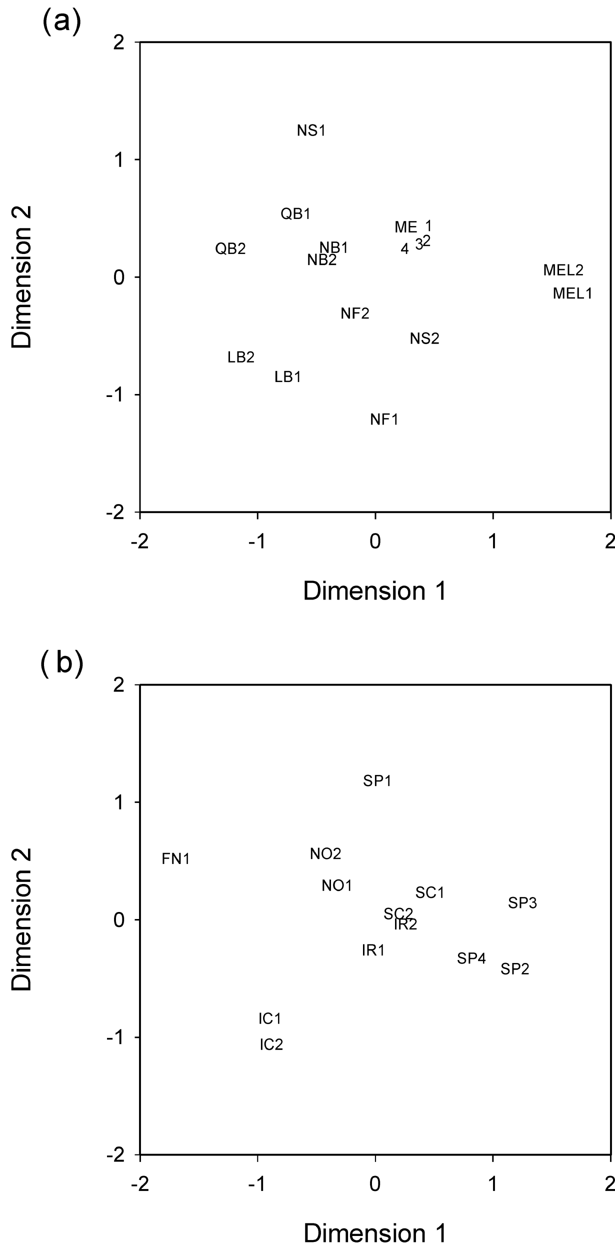

Fig. 2 Multidimensional scaling on range-wide Atlantic salmon population genetic data. (a) 1-Da similarity matrix for North American samples; (b) 1-Da similarity matrix for European samples.

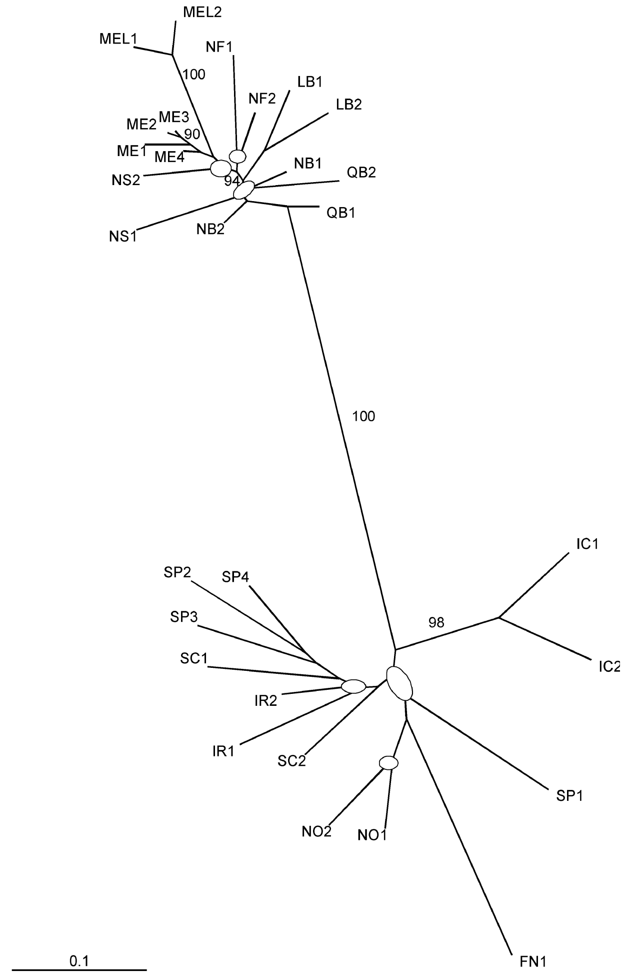

Fig. 3 Genetic distance (D_a) phenogram (neighbour-joining algorithm). White ellipses indicate nodes with bootstrap support below 50% in 5000 replications. Numbers indicate nodes with bootstrap support above 90% in 5000 replications.

In the North American comparison, collections from each province (and state) appeared to be discrete, and equally differentiated. With the exception of the clearly distinct collection from the inner Bay of Fundy (NS1), the distributions of collections appeared to mimic their geographical distribution. The nonanadromous populations were also quite distinct from the anadromous populations. The QB1 collection clustered closest to the NB collections. The rivers of the Gulf of St Lawrence (including QB1) were stocked in the early 1900s with salmon from the New Brunswick (NB) rivers sampled in this study. The anadromous collections within Maine (ME1–ME4) clustered tightly, as did the two NB collections although they came from rivers draining to separate water bodies (NB1, Bay of Fundy; NB2, Gulf of St Lawrence). These tight patterns may also reflect past stocking practices that have moved fish between rivers. Maine collections did not cluster directly with NB2 (Miramichi River) and QB2 (Saguenay River), suggesting that past transplantations of Atlantic salmon from these rivers into the Maine rivers were less successful.

The European clustering strongly corresponds to the geographical distribution of the collections. The Finland, Iceland and River Eo, Spain (SP1) collections were the most genetically distinct collections in Europe. Three clusters of genetically similar salmon were apparent: the Norway collections, the Scottish and Irish collections, and the remaining Spanish (SP2–SP4) collections.

The NJ phenogram depicting the underlying structure of the D_a distance matrix clearly illustrates the high degree of genetic differentiation between Atlantic salmon inhabiting the western and eastern regions of the North Atlantic (Fig. 3). The tree topology consists of a cluster of North American collections at one end with the European collections clustered at the other, separated by the longest branch of the tree. The branch lengths observed within the European cluster are longer than those observed within the North American cluster, suggesting distinct evolutionary histories for salmon inhabiting these two regions. The NJ phenogram depicts very similar patterns to that observed within each continent in the MDS cluster analysis. Strong bootstrap support was observed for clusters of the ME, MEL and LB collections within the North American arm of the tree, and the IC collections within the European branch.

Quantitative estimates of hierarchical gene diversity indicated significant genetic population structure at every level, with the greatest amount due to variation within collections (Table 5). Results of the range-wide analysis revealed that 21.9% of the genetic variation occurred between the continents, 5.5% was due to differentiation among collections within each continent and 72.6% was due to variation within collections. Within North America, 3.2% of the differentiation was observed among the Canadian provinces and Maine, 3.0% among collections within provinces/state and greater than 93% of the variation was observed within populations. When European countries are modelled similarly, a greater percentage of variation is attributable to differences between countries (6.1%) and among collections within countries (5.3%), leaving less of the variation originating within the collections (88.6%). In a comparison of collections representing the anadromous and nonanadromous life histories from Maine, a relatively large but not significant amount of variation (5.1%; $P > 0.06$) was observed between the groupings while a significant 1.1% variation was observed within the groupings.

A Mantel test comparing the range-wide distances identified a strong correlation ($r = 0.93$; $t = 17.3$; $P < 0.0001$) between the pairwise genetic distance (D_a) and ocean distance (km) matrices (available upon request). Figure 4 graphically depicts the raw data from this comparison showing the strong linear relationship between the distance matrices with three clusters of data points corresponding to the comparisons between continents (triangles), within Europe (open circles) and within North America (filled circles). Data from each continent depict a distinct break between collections from each continent. Among the widely distributed European collections, the relationship between genetic and geographical distance matrices represents a strong isolation-by-distance pattern ($r = 0.69$; $t = 4.0$; $P = 0.0001$). Among North American collections, the relationship was somewhat weaker but still statistically significant ($r = 0.43$; $t = 3.3$; $P = 0.0008$).

Piecewise regression lines were calculated and plotted to illustrate the pattern observed in much of the data for the within-continent comparisons for European and North American collections (Fig. 4). The fitted regression line for

Table 5 Hierarchical gene diversity analysis of Atlantic salmon among 29 collections from Maine to Spain comparing both inter and intracontinental groupings. An analysis of the variation patterns observed between the anadromous and nonanadromous (landlocked) collections in Maine is also provided

Grouping	Source of variation	Total variation	Per cent of total	F_{CT}	F_{SC}	F_{ST}
Continental	Total	5.115	100.00			
	Between continent	1.119	21.88	0.219		
	Among populations within continents	0.281	5.49		0.070	
	Within populations	3.716	72.63			0.274
North America	Total	3.788	100.00			
	Among Provinces/State	0.119	3.15	0.032		
	Among populations within Provinces	0.113	2.99		0.031	
	Within populations	3.555	93.86			0.061
Europe	Total	4.681	100.00			
	Among countries	0.287	6.13	0.061		
	Among populations within countries	0.247	5.28		0.056	
	Within populations	4.147	88.59			0.114
Maine	Total	3.735	100.00			
(anadromous vs.	Between groups	0.192	5.13	0.051^{ns}		
non-anadromous)	Among populations within groups	0.042	1.11		0.012	
	Within populations	3.502	93.76			0.063

Statistical significance of the diversity estimate was based on probabilities derived from 1023 permutations; NS denotes $P > 0.06$; all others $P < 0.0001$.

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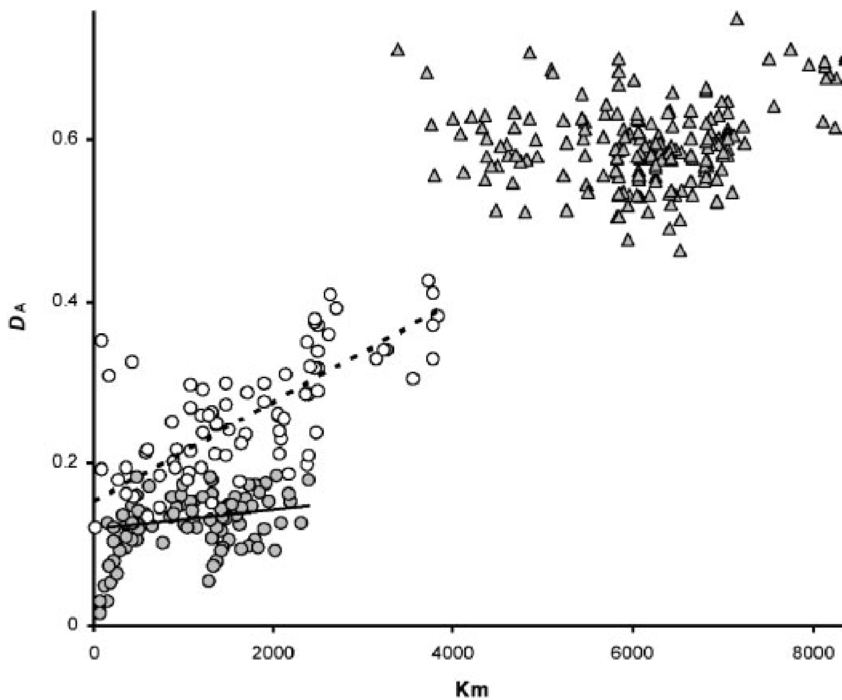

Fig. 4 Regression of geographical vs. genetic distance: triangles indicate comparisons between continents, open circles indicate comparisons within Europe, and closed circles indicate comparisons within North America.

European collections (excluding comparisons with the outlying SP1) was $D_{aE} = 6.2 \times 10^{-5}$ km + 0.15. The four Maine collections, which comprised the four smallest genetic distances in the study, exhibited a steep linear relationship that differed from the relationship observed in other North American comparisons, and were omitted from further regression analyses. The fitted regression line for North America (excluding the comparisons within Maine) was $D_{aNA} = 1.3 \times 10^{-5}$ km + 0.12. The intercepts of these within-continent comparisons are similar but the slopes appear to differ.

Assignment tests revealed that population differentiation in Atlantic salmon at microsatellite loci is sufficient to identify the origin of individual fish with a high rate of success. For this data set, the likelihood assignment methods of Paetkau *et al.* (1995) and Cornuet *et al.* (1999) performed similarly, with 1426 of 1682 (84.78%) and 1425 of 1682 (84.72%) fish correctly classified, respectively. Assignments using distance methods were less robust, but D_a gave the best results (D_a, 81.33%; chord distance, 74.55%; Nei's standard distance 73.07%; Nei's minimum distance, 73.07%; allele-sharing, 68.31%; and $(\delta\mu)^2$, 27.29%). These findings are in general agreement with those of Cornuet *et al.* (1999), with the exception that they found allele sharing and Cavalli-Sforza and Edwards chord distance to be the superior distance measures with their simulated data. We tested the effect of the number of loci on assignment accuracy by sequentially adding loci to the analysis. Assignment accuracy asymptotically approached that obtained with all 12 loci (data not shown). When eight or more loci were incorporated, classification was greater than 95% of that observed when all 12 loci were used, indicating that the number of microsatellite loci examined in the study was adequate. When single loci were individually removed from the analysis, no locus was found to contribute more than 3.02% to the classification observed with all 12 loci (Ssa85, 3.02%; SSLEEI84, 2.42%; Ssa202, 1.89%; SSOSL85, 1.83%; Ssa289, 1.83%; Ssa14, 1.33%; SSLEEN82, 1.19%; Ssa171, 1.12%; Ssa197, 1.05%; SSOSL311, 0.91%; SSOSL438, 0.57%; and SSOSL25, 0.42%). This suggests that effects detrimental to discriminatory accuracy caused by any minor perturbations in Hardy–Weinberg proportions or linkage disequilibrium at a particular locus are likely to be dampened by the information content of the other loci.

Assignments of individuals to country and/or province revealed evidence of population subdivision that is presented in Table 6. At the broadest scale, all fish were assigned to their home continent. The correct classification rates of Canadian fish to province and European fish to countries ranged from 62.3% to 80% and 71.7% to 100%, respectively. All assignments to countries and/or provinces were higher than expected by chance ($\chi^2 >= 235.3$, 2*df*). Most misclassified anadromous US fish were assigned to New Brunswick (36 of 639) and fewer were assigned to Newfoundland (12 of 639), Nova Scotia (nine of 639), Labrador (five of 639) and Quebec (one of 639) in general accord with the geographical distances involved. Landlocked salmon were correctly classified in all but two of 92 cases. We observed a relatively high rate of misclassification of New Brunswick animals to Quebec (23 of 122) as

Table 6 Results of maximum-likelihood assignment tests

Population	ME	MEL	NB	NS	QB	NF	LB	IC	NO	FN	SC	IR	SP	USA	Canada	N. America	Europe	All
ME	571	5	36	9	1	12	5	0	0	0	0	0	0	576	63	639	0	639
MEL	1	90	0	0	0	1	0	0	0	0	0	0	0	91	1	92	0	92
NB	8	0	76	7	23	5	3	0	0	0	0	0	0	8	114	122	0	122
NS	5	1	8	88	6	2	0	0	0	0	0	0	0	6	104	110	0	110
QB	0	0	25	5	89	1	2	0	0	0	0	0	0	0	122	122	0	122
NF	5	0	7	5	4	71	1	0	0	0	0	0	0	5	88	93	0	93
LB	0	2	6	1	2	2	32	0	0	0	0	0	0	2	43	45	0	45
IC	0	0	0	0	0	0	0	95	1	0	0	0	0	0	0	0	96	96
NO	0	0	0	0	0	0	0	0	99	0	2	0	0	0	0	0	101	101
FN	0	0	0	0	0	0	0	0	0	61	0	0	0	0	0	0	61	61
SC	0	0	0	0	0	0	0	0	0	0	38	8	7	0	0	0	53	53
IR	0	0	0	0	0	0	0	1	0	0	11	48	4	0	0	0	64	64
SP	0	0	0	0	0	0	0	0	7	0	5	4	68	0	0	0	84	84
Sample size	639	92	122	110	122	93	45	96	101	61	53	64	84	731	492	1223	459	1682
Observed number correctly classified	571	90	76	88	89	71	32	95	99	61	38	48	68	667	471	1223	459	1682
Per cent correctly classified	89.4	97.8	62.3	80.0	73.0	76.3	71.1	99.0	98.0	100.0	71.7	75.0	81.0	91.2	95.7	100.0	100.0	100.0
Expected number correctly classified	49.2	7.1	9.4	8.5	9.4	7.2	3.5	7.4	7.8	4.7	4.1	4.9	6.5	112.5	189.2	658.5	211.8	1682.0
χ^2	5540.2	971.6	472.9	747.7	675.4	569.8	235.3	1039.5	1071.3	675.7	282.3	376.9	586.1	2734.4	419.6	483.8	288.3	0.0

Samples from each political jurisdiction are lumped. For example, ME = ME1 + ME2 + ME3 + ME4; NB = NB1 + NB2, etc. The landlocked samples (MEL1 and MEL2) were, however, pooled into a separate category (MEL). The expectation of numbers correctly classified by chance alone were calculated assuming equal probability of membership in any single population. Probability of membership (P) in any grouping is assumed to be proportionate to the number of populations comprising that group. Specifically, for any single population $P = 1/13$; $P = 2/13$ for the United States grouping; $P = 5/13$ for the Canadian grouping; $P = 7/13$ for the North American grouping; and $P = 6/13$ for the European grouping.

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well as the reverse (25 of 122). In addition, several misclassified Spanish fish were assigned to Scotland (five of 84), Ireland (four of 84) and Norway (seven of 84). Within North America, Canadian fish were correctly assigned to Canada 95.7% (471 of 492) of the time. Correct classification of US fish to the United States was similar (667 of 731 or 91.2%).

Discussion

The microsatellite loci screened in this study appear robust for surveys of neutral genetic variation among populations of Atlantic salmon. Our survey of Atlantic salmon microsatellites has identified more genetic diversity (266 alleles at 12 loci) than other approaches applied to date. Alleles were inherited according to Mendelian expectations (unpublished data) and genotypes met Hardy–Weinberg equilibrium expectations in more than 98% of comparisons. A limited amount of linkage disequilibrium was observed throughout the study area. The disequilibrium observed in certain collections could indicate population subdivision (or mixing of distinct populations or generations) within our samples, recent recolonization by a portion of the metapopulation with a nonrandom subset of genotypes (i.e. founder effects), or habitat-specific selection acting on loci linked to the microsatellite markers used in this study, but physical linkage of loci appears unlikely.

A substantial genetic discontinuity between North American and European populations was indicated by significant genic and genotypic heterogeneity, a high degree of population subdivision measured by AMOVA, large genetic distances between populations of each continent (Fig. 3), perfect (100%) classification to continent of origin based on maximum likelihood assignment and a high number of alleles unique to each continent. Range-wide, a strongly significant relationship existed between genetic distance and geographical distance, indicating isolation by distance within continents (Fig. 4) and a greater disparity between continents, supporting the results from previous allozyme surveys (Ståhl 1987; Bourke *et al*. 1997).

Less neutral genetic differentiation was observed within North American populations than within European populations (Fig. 3), as well as fewer alleles and lower heterozygosity (Table 2). We believe this difference to have resulted from different histories of postglacial colonization of the continents rather than differing management histories, not least because six of the North American populations have no history of Atlantic salmon translocation (ME1, NB2, NF1, NF2, LB1, LB2). Evolutionary divergence has been found to be deeper for fish species from nonglaciated regions compared to more northerly taxa (Bernatchez & Wilson 1998). That our North American samples were less differentiated from each other than our European populations (Fig. 3) is not surprising in light of the more extensive history of glaciation across North America than Europe (Dawson 1992).

Before recent human disturbances, the Pleistocene glaciation probably contributed the most significant events in the evolutionary history of Atlantic salmon (Bernatchez & Wilson 1998). Atlantic salmon ascend and spawn in rivers that drain into the northern Atlantic Ocean. Their range currently includes the east coast of North America from southern Maine to Labrador, Iceland, Ireland, Great Britain and the Atlantic and Baltic coasts of Europe from northern Portugal to Russia (Scott & Crossman 1973; Hutchings & Jones 1998). All of their North American range and much of their European range was covered by ice in the most recent glaciation (Dawson 1992). The recent recolonization (8000–10 000 years BP) of most current Atlantic salmon habitat suggests that neutral genetic variation may not have reached drift–mutation–migration equilibrium.

McConnell *et al*. (1997) observed significant differentiation among multiple collections from Nova Scotia, including the Bay of Fundy, using microsatellite DNA variation. Our results suggest that the Stewiacke River sample (NS1), our lone inner Bay of Fundy collection, is clearly differentiated from all other NA collections, including that from the Gold River (NS2). The St Jean River (QB1) exhibited a lower genetic distance and a lower than average assignment rate with the New Brunswick collections (NB1, NB2). QB1 was stocked with NB1 and NB2 fish for 20 years at the turn of the century (Fontaine *et al*. 1997). While the amount of successful admixture resulting from these interdrainage translocations is unknown, our results suggest that some New Brunswick salmon may have effectively contributed to the St Jean River Atlantic salmon genome. More extensive sampling, including a temporal component, would be needed to confirm this. Interestingly, NB1, NB2, QB1 and QB2 salmon were introduced into the Penobscot River (ME2) as late as the 1960s, yet there was no significant misclassification of Maine fish to these Canadian provinces either through maximum likelihood assignment or graphical representation of genetic distance.

Our microsatellite data indicate that Maine's landlocked Atlantic salmon are differentiated from anadromous populations, raising the issue of single vs. multiple origin of North American Atlantic salmon (see Tessier and Bernatchez 2000). If nonanadromous salmon colonized North America from a temporally or spatially distinct glacial refuge than that used by ancestors of today's anadromous fish, we would expect to see alleles unique to both refugia in the modern-day descendants. The absence of unique genetic variation in the landlocked salmon from Maine observed in this study and by colleagues (Table 2; King *et al*. 2000) suggests divergence of landlocked populations after recolonization, supporting the results of Behnke (1972) and Ståhl (1987), who suggested that nonanadromous populations of Atlantic salmon were derived from anadromous stocks in postglacial times.

In European Atlantic salmon, our microsatellite data suggest three geographical groupings in the context of isolation by distance: Iceland, Finland and Atlantic Europe (western Norway, Ireland, Scotland and Spain; Fig. 2). Ståhl (1987) and Bourke *et al*. (1997) identified two major European groups, corresponding to the eastern Atlantic and the Baltic Sea, using protein polymorphism. Verspoor (1994), also using allozymes, did not identify a distinction between Baltic and eastern Atlantic populations, but did find a lone Icelandic population to be differentiated from other European populations. Bermingham *et al*. (1991) compared the distribution of mtDNA haplotype variation among 19 European hatchery groups and found distinct differences between eastern European and Baltic salmon. Haplotype differentiation was not observed between Icelandic and other eastern European salmon in that study. Taggart *et al*. (1995) described a minisatellite locus that could discriminate between European and North American populations, but not within either continent. In tying together components of variation identified by previous research, the present study illustrates the value of hypervariable microsatellite loci that have greater allelic variation than allozymes and are more representative of the genome than mtDNA or single-locus minisatellites.

In addition to the standard analyses of genetic differences described above, several analyses testing correct assignment to population were performed. With 12 microsatellite loci we were able to assign every fish surveyed to its continent of origin. Within continents, misclassifications were, as might be expected, due to geography and management history. Canadian and US fish were correctly assigned to their country of origin more than 91% of the time. Both European and North American Atlantic salmon were correctly assigned to country and/or province of origin an average of 82.7% of the time (Table 5). Simulations conducted by Cornuet *et al*. (1999) suggest that a misclassification rate of 25% with 10 microsatellite loci might reflect isolation over a period of 100–300 generations.

The relatively high rate of misclassification (32.4%) observed in New Brunswick and Quebec fish may reflect past stock transplantations (Fontaine *et al*. 1997). This contrasts with the misclassification of only 81 of 1131 (7.2%) US and Canadian anadromous fish, and suggests that stock transplants may have been more successful between the Canadian provinces than between Canada and the US. Some fish from Spain were misclassified to Ireland, Norway and Scotland, which may reflect translocation of salmon from northern Europe to Spanish rivers (de Garcia Leániz *et al*. 1989; Morán *et al*. 1994).

Management implications

Major mixed-stock Atlantic salmon fisheries exist off the western coast of Greenland, around the Faeroe Islands and in the Baltic Sea. The West Greenland fishery is composed of both North American and European origin one-sea-winter age fish. The suite of microsatellite markers reported here are believed to represent the only assay providing perfect assignment to continent of origin, and furthermore to assign individuals to province of origin with a high degree of accuracy. Additional polymorphic loci may allow identification of major river of origin with increased reliability, within the limits of translocation and stocking pursued by fishery managers.

The allelic and genotypic heterogeneity and genetic distances observed between collections on each continent suggest that each country or province should be considered an individual management unit. Recent studies have provided empirical support for a river-specific population structure (Ståhl 1987; Jordan *et al*. 1992; Morán *et al*. 1994; O'Reilly *et al*. 1996; Sanchez *et al*. 1996; Fontaine *et al*. 1997; McConnell *et al*. 1997; Tessier *et al*. 1997) and intra-river genetic structuring (Ståhl 1983; Crozier & Moffett 1989; Koljonen 1989; Verspoor & Jordan 1989; McElligott & Cross 1991; Verspoor *et al*. 1991; Hurrell & Price 1993; Galvin *et al*. 1996; Verspoor 1997; Beacham & Dempson 1998; Garant *et al*. 2000; Spidle *et al*. submitted for publication). Our data support the results of the above studies, finding significant differentiation at every level examined. Restoration efforts should take into account inter- and intra-river diversity and utilize supplementation only in a manner that does not significantly perturb the recipient population by shifting gene frequencies, influencing demographic and physiological parameters, or introducing disease.

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Tim King's research emphasis involves the application of molecular genetic markers to questions in phylogeography and conservation genetics of declining species. This contribution is yet another effort to assist natural resource managers with guidance in identifying appropriate units of conservation. Steven Kalinowski is a population geneticist whose research has applied a variety of empirical and theoretical approaches to conservation genetics. Bane Schill is a scientist at the National Fish Health Research Laboratory working primarily in molecular biology and population genetics. Adrian Spidle is working in Tim King's laboratory, with interests in the application of population genetics and evolutionary biology to fishery management. Barbara Lubinski is a biologist in King's laboratory who specializes in microsatellite DNA analysis.