North American Journal of Aquaculture 62:285–289, 2000

COMMUNICATIONS

The Use of Microsatellite DNA Loci for Genetic Monitoring of Atlantic Salmon Populations

JOSE L. MARTINEZ

Departamento de Biologia Funcional, Universidad de Oviedo, C/Julian Claveria, s/n. 33006-Oviedo, Spain

STEVE GEPHARD

Connecticut Department of Environmental Protection, Fisheries Division, Post Office Box 719, Old Lyme, Connecticut 06371, USA

FRANCIS JUANES

Department of Forestry and Wildlife Management, University of Massachusetts, Holdsworth Hall, Amherst, Massachusetts 01003-4210, USA

EVA GARCIA-VAZQUEZ*

Departamento de Biologia Funcional, Universidad de Oviedo, C/Julian Claveria, s/n. 33006-Oviedo, Spain

Abstract.—This work addresses the application of a new group of molecular markers, microsatellite loci, to monitor genetic diversity in stocks used in fish enhancement programs. To illustrate the potential use of these markers, we examined the genetic variation associated with the Connecticut River and Penobscot River populations of Atlantic salmon *Salmo salar* in the United States. Both rivers are stocked annually with fry from captive or cultured stocks that are one generation removed from sea-run adults. We analyzed microsatellite loci variation among sea-run adults and the derived cultured stock for the Connecticut River in a given year, along with two consecutive cohorts of cultured age-0 juveniles for the Penobscot River. Our results reveal subtle differences in allele frequencies between the two samples for each stock and some potential increases of homozygotes. These results demonstrate the potential value of microsatellite loci for routine genetic monitoring of cultured Atlantic salmon populations associated with restoration programs in the eastern United States.

Introduction

The Connecticut and the Penobscot rivers are being systematically stocked with fry of Atlantic salmon *Salmo salar* obtained from a cultured stock. Artificial stocking is necessary to maintain desired population sizes (Beland 1984; Rideout and Stolte 1989). The wild population of the Penobscot River was seriously depleted during the 1950s and has been the subject of a restoration program since 1965 (Beland 1984). The Connecticut River population was extirpated 200 years ago and has been under restoration during the last 30 years since initial release of fry from other North American rivers (Moffitt et al. 1982; Rideout and Stolte 1989). Sea-run adults returning to each river are trapped and artificially spawned. The breeding strategy for sea-run adults usually consists of fertilizing eggs from one female with sperm from one male. Crosses are not systematically planned. For the Penobscot River population, males and females ripening at the same time are mated at random within size classes (i.e., large salmon with large salmon, and recently small grilse with grilse). Systematic surveys of genetic variability for the two river populations have not been conducted. It has been assumed that the genetic variability in the sea-run stocks will be maintained in the cultured stocks through random selection and mating. Although most of the fertilized eggs are used to produce young fish for restocking, some are retained in hatcheries and raised to maturity for additional fry production. Those fry are then used to restock the river in larger numbers than would be possible with only sea-run adults.

Routine surveys of genetic variability in other cultured stocks have usually been carried out by electrophoretic analysis at protein loci (Verspoor 1988; Davidson et al. 1989). Nevertheless, other types of loci are potentially more powerful tools to assess changes in genetic variability. For ex-

* Corresponding author: egv@sauron.quimica.uniovi.es

Received June 24, 1999; accepted March 20, 2000

ample, variable number of tandem repeat loci (minisatellites and microsatellites) are highly polymorphic (Bentzen et al. 1991; McConnell et al. 1995) and are assumed to be neutral with respect to natural selection. They are widely applied for studies of population dynamics (Perez et al. 1997) and structure (Fontaine et al. 1997; McConnell et al. 1997), genetic stock identification for the assessment of impacts of supportive breeding (Galvin et al. 1995; Tessier et al. 1997), and for historical studies of genetic changes to Atlantic salmon populations over many decades (Nielsen et al. 1997).

The aim of the work described here is two-fold. First, we describe the application of a new group of molecular markers, microsatellite loci, to monitor genetic diversity of two stocks of Atlantic salmon used in fish enhancement programs: the Connecticut River and Penobscot River stocks. Second, by comparing the genetic pattern at microsatellite loci of sea-run adults and the cultured stocks we evaluate the effect of the present management strategy (crosses of returning sea-run adults to yield cultured stocks for repopulating tributaries) on genetic variability. We discuss the preservation of genetic variability (e.g., similarity of the Connecticut River domestic stock with the sea-run adults from which it derives) and temporal stability of the genetic composition of domestic stocks (similarity of juveniles released in Penobscot River in two consecutive years).

Methods

Tissue samples were obtained in 1997 from randomly sampled sea-run adults (age 3, $N = 41$) at the Whittemore Salmon Station (WSS) located on the Connecticut River in Barkhamsted, Connecticut. Tissue samples of the cultured Connecticut River stock from the Kensington State Salmon Hatchery (KSSH, Berlin, Connecticut) were taken in 1997 from randomly selected adults of the same age and cohort (age 3, $N = 40$) as the sea-run adults at WSS. For the Penobscot River cultured stock, reared at the Green Lake National Fish Hatchery (Ellsworth, Maine), we analyzed tissue samples of age-0 juveniles planned for release in the Penobscot River over two consecutive years, 1997 ($N = 41$) and 1998 ($N = 58$).

Immediately after sampling, tissues were frozen at −20°C until laboratory analyses were performed. We studied samples variation at three microsatellite loci: *SSOSL311*, *SSOSL417*, and *SSOSL436* (Slettan et al. 1995a, 1995b). Chelex-based DNA extraction was performed according to Estoup et al. (1996); polymerase chain reaction (PCR) amplifications were carried out on reaction mixtures containing approximately 50 ng of extracted Atlantic salmon DNA template, 10 mM tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 20 pmol of each primer, 1 unit Dynazyme II DNA polymerase (Finnzymes Oy), and 250 μM of each dNTP in a final volume of 20 μL. Amplifications were performed with an initial denaturing step (5 min at 95°C) followed by 35 cycles of denaturation at 95°C (20 s), annealing at 52°C for *SSOSL417*, 55°C for *SSOSL311*, and 54°C for *SSOSL416* (20 s), and extension at 72°C (20 s), followed by a final extension at 72°C (7 min). The PCR products were separated electrophoretically on denaturing polyacrylamide sequencing gels, and allele sizes were established by comparison with a pUC sequence reaction (Martinez et al. 1999) after DNA silver staining (Promega).

Statistical analyses were performed with the Genepop 3.1b program, an updated version of Raymond and Rousset (1995); the analyses include exact *P*-values for testing conformation of genotypes to Hardy–Weinberg proportions, exact *P*-values for testing heterozygote deficits, and exact *P*-values for allelic frequency homogeneity between stocks. Global tests across populations were constructed by using Fisher's method, provided the microsatellite loci were independent (Slettan et al. 1997). Exact *P*-values were estimated by the Markov chain method (1,000 dememorization steps, 50 batches, 1,000 iterations/batch). Significance probabilities were adjusted for the number of tests performed simultaneously (i.e., number of loci) by the Bonferroni method (Sokal and Rohlf 1995).

Results

The three loci revealed different amounts of genetic variation (Table 1). All four samples revealed low variation at the *SSOSL311* locus (three or four alleles). In contrast, all samples were much more variable at the *SSOSL417* and *SSOSL436* loci, where 6–13 and 15–18 alleles, respectively, were observed. For the Connecticut River samples, differences in allelic frequencies between WSS and KSSH samples were statistically significant only at the *SSOSL436* locus ($P < 0.001$) but not when all loci are considered together after a Bonferroni correction. For the Penobscot River, allelic frequencies differed between the juveniles sampled in 1997 and 1998 at *SSOSL417* ($P < 0.001$) and *SSOSL436* ($P < 0.001$) loci. These differences were still significant after a Bonferroni correction ($P < 0.001$).

Table 2 presents a summary of the genetic var-

TABLE 1.—Genetic variation for three loci in samples from the Connecticut and Penobscot rivers. Results are presented as allele frequencies. Abbreviations are as follows: Ho = observed heterozygosity, He = expected heterozygosity; H–W = conformity with Hardy–Weinberg genotypic proportions; WSS = Whittemore; KSSH = Kensington; GL = Green Lake. Number of individuals are in parentheses.

Locus/ allele	Connecticut River		Penobscot River	
	WSS (41)	KSSH (40)	GL 97 (41)	GL 98 (58)
SSOSL311				
122	0.94	0.89	0.96	0.89
124	0.05	0.06	0.01	0.04
150				0.02
156	0.01	0.05	0.03	0.05
Ho–He	0.10–0.12	0.18–0.20	0.07–0.07	0.10–0.10
H–W	Yes	Yes	Yes	Yes
SSOSL417				
159	0.17	0.11	0.18	0.15
163				0.01
167	0.06	0.03		0.03
171	0.15	0.20	0.14	0.04
173	0.07	0.07	0.24	0.08
175	0.30	0.43	0.32	0.41
179				0.02
183	0.07			
185				0.01
191	0.02			
193	0.03	0.04		0.10
195				0.01
197			0.05	0.03
203		0.03		
205	0.07	0.03	0.07	0.08
207	0.04	0.06		0.04
211	0.02			
Ho–He	0.92–0.84	0.71–0.76	0.62–0.79	0.66–0.79
H–W	Yes	Yes	Yes	No ($P < 0.001$)
SSOSL436				
152		0.01		
154	0.03	0.06	0.04	
156		0.01	0.03	0.07
158		0.07	0.07	
160				0.13
164	0.03		0.04	
166	0.09	0.20	0.12	
168	0.04	0.04	0.04	0.09
170				0.10
172	0.10	0.01	0.03	
174	0.04			
176	0.01	0.07	0.05	0.02
178	0.08	0.17	0.18	
180	0.04	0.03		0.10
182			0.01	
184	0.03		0.03	0.04
186	0.03		0.07	0.03
188				0.1
190	0.04	0.03	0.04	
192	0.14	0.07	0.07	0.11
194				0.08
196	0.07	0.17	0.05	0.02
198				0.01
200	0.07	0.03		
202	0.08	0.03		
204	0.03		0.04	0.07
206			0.01	0.02
210	0.05			
212			0.08	0.01
Ho–He	0.85–0.93	0.61–0.89	0.84–0.93	0.60–0.92
H–W	Yes	No ($P < 0.001$)	Yes	No ($P < 0.001$)

TABLE 2.—Genetic variability of populational parameters of the analysed samples. Abbreviations are as follows: H = mean heterozygosity; NA = mean number of alleles per locus; H–W = conformity with Hardy–Weinberg; chi = chi-square goodness-of-fit to H–W genotypic proportions; *P* = exact *P*–value (Markov chain).

	Connecticut River		Penobscot River	
	Whittemore	Kensington	Green Lake (1997)	Green Lake (1998)
H	0.636	0.486	0.496	0.439
NA	10.7	9.3	9	11
H–W	Yes	No	Yes	No
Chi	10.4	Infinity	5.5	Infinity
P	0.11	<0.001	0.487	<0.001

iability found for the four samples at the three analyzed loci. Although the Connecticut River samples were not statistically different with respect to allelic frequencies, differences between WSS and KSSH with respect to genetic variability were evident. Genotypes for the WSS sample did not significantly depart from Hardy–Weinberg proportions when all three analyzed loci are combined (chi-square 10.4, $P = 0.110$), whereas genotypes for the KSSH sample deviated significantly ($P < 0.001$), presenting many more homozygotes than expected at many alleles for all three loci. In addition, both mean number of alleles (9.3) and mean heterozygosity (0.49) were lower for the KSSH sample than for the WSS sample (10.7 and 0.64, respectively).

Mean heterozygosities of the two samples of the Penobscot River cultured stock (0.50 in 1997 and 0.44 in 1998) were in the same range as the cultured Connecticut River stock (0.49). Both Penobscot samples exhibited significantly higher number of homozygotes than expected ($P < 0.001$, for heterozygote deficit in both cases). When combining all the loci to test conformity with Hardy–Weinberg, the 1997 sample was at Hardy–Weinberg equilibrium (chi-square = 5.5, $P = 0.49$), whereas the 1998 sample clearly was not ($P < 0.001$). Both domestic stocks (KSSH and Green Lake) deviated significantly from Hardy–Weinberg equilibrium due to increased proportion of homozygotes, in spite of their very different genetic compositions.

Discussion

Use of hypervariable loci such as microsatellites is encouraged for managers in routine surveys because it has been shown here to be a very useful tool for monitoring genetic variability of populations or stocks. We found two important results that can be directly related to management. One was the loss of heterozygosity observed in the domestic stock of the Connecticut River compared with sea-run adults. This observed trend for the Connecticut River stock is similar to the findings of Verspoor (1988) for Canadian hatchery stocks. However, the cultured fish (KSSH) had a higher heterozygosity (0.18) at *SSOSL311* than did the sea-run fish (WSS; 0.10). This suggests the observed differences reflect random variation rather than nonrandom mating or reduced effective population size.

The second important result was the discovery of significant differences in allele frequencies between year-classes in the Penobscot River stock. As previously indicated, the Green Lake domestic stock is annually created from sea-run crosses within size-classes (probably age-classes); by mating individuals from the same cohorts, overlap among generations is precluded. Because the Penobscot River population restoration was based on introductions from different Canadian egg sources in different years (Beland 1984), it is possible that a complete mixture of the different founder stocks into a single common gene pool has still not been achieved and that the Penobscot River population consists of several sub-populations not fully overlapping in time. Alternatively, another explanation could lie in the exaggerated influence of a small group of individuals or even a single individual on the composition of the 1998 Green Lake stock, a likely occurrence in cultured stocks. This seems unlikely, however, because of the relatively large number of parents used for the Green Lake stock: 199 males and 199 females in 1997 and 639 males and 639 females in 1998. In any case, interbreeding different age-classes could help to homogenize the gene pool among years and would decrease substantially the likelihood of mating closely related individuals (e.g., full siblings). This interbreeding could be accomplished by mating individuals from different cohorts and using mature parr and reconditioning kelts.

Utter (1991) emphasized the relevance of continued genetic studies on Atlantic salmon for stock identification and enhancement programs. Our results of two such enhancement programs suggest that genetic monitoring is still necessary. Careful attention should be given to mating strategies, genetic typing of returning adults, and to maximizing genetic variability in the F_1 cultured stock to avoid drastic changes in the genetic pattern of populations undergoing restoration. Genetic monitoring of cultured stocks is especially important in res-

toration programs when these stocks are being employed to enhance wild populations and will constitute a large part of the future wild sea runs.

Acknowledgments

We are grateful to Paul Gaston, manager of the Green Lake National Fish Hatchery (U. S. Fish and Wildlife Service), for his very helpful comments and information. We are indebted to the Asturian Regional Government (Consejeria de Agricultura), the U. S. Forest Service, the University of Massachusetts, and a Hatch Grant for financial support. We thank the Green Lake National Fish Hatchery and Joseph Ravita (Whittemore Salmon Station) and Albin Sorek Sonski (Kensington State Salmon Hatchery), both of the Connecticut Department of Environmental Protection, Fisheries Division, for providing fish and samples. Juliana Perez kindly collaborated in all the laboratory work. Eva Garcia-Vazquez was recipient of a Spanish FPU DGICYT grant.

References

Beland, K. 1984. Strategic plan for management of Atlantic salmon in the State of Maine. Atlantic Sea-Run Salmon Commission, Bangor, Maine.

Bentzen, P., A. S. Harris, and J. M. Wright. 1991. Cloning of hypervariable minisatellite and simple sequence microsatellite repeats for DNA fingerprinting of important aquacultural species of salmonids and *Tilapia.* Pages 243–262 *in* T. Burke, G. Dolf, A. J. Jeffreys, and R. Wolff, editors. DNA fingerprinting approaches and applications. Birkhäuser Verlag, Basel, Switzerland.

Davidson, W. S., T. P. Birt, and J. M. Green. 1989. A review of genetic variation in Atlantic salmon, *Salmo salar* L., and its importance for stock identification, enhancement programmes and aquaculture. Journal of Fish Biology 34:547–560.

Estoup, E., C. R. Lagiader, E. Perrot, and D. Chourrout. 1996. Rapid one-tube extraction for reliable PCR detection of fish polymorphic markers and transgenes. Molecular Marine Biology and Biotechnology 5:295–298.

Fontaine, P-M., J. J. Dodson, L. Bernatchez, and A. Slettan. 1997. A genetic test of metapopulation structure in Atlantic salmon (*Salmo salar*) using microsatellites. Canadian Journal of Fisheries and Aquatic Sciences 54:2434–2442.

Galvin, F., S. McKinnell, J. B. Taggart, A. Ferguson, M. O'Farrell, and T. F. Cross. 1995. Genetic stock identification of Atlantic salmon using single locus minisatellite profiles. Journal of Fish Biology 47(Supplement A):186–199.

Martinez, J. L., P. Moran, A. M. Pendas, J. B. Taggart, and E. Garcia-Vazquez. 1999. Genetic characterization and chromosomal location of a single locus GT microsatellite from Atlantic salmon. Animal Genetics 30:399–400.

McConnell, S., L. Hamilton, D. Morris, D. Cook, D. Paquet, P. Bentzen, and J. M. Wright. 1995. Isolation of salmonid microsatellite loci and their application to the population genetics of Canadian east coast stocks of Atlantic salmon. Aquaculture 137: 19–30.

McConnell, S. J. K., D. E. Ruzzante, P. T. O'Reilly, L. Hamilton, and J. M. Wright. 1997. Microsatellite loci reveal highly significant genetic differentiation among Atlantic salmon (*Salmo salar* L.) stocks from the east coast of Canada. Molecular Ecology 6: 1075–1089.

Moffitt, C. M., B. Kynard, and S. Rideout. 1982. Fish passage facilities and anadromous fish restoration in the Connecticut River basin. Fisheries 7(6):2–11.

Nielsen, E. E., M. M. Hansen, and V. Loeschcke. 1997. Analysis of microsatellite DNA from old scale samples of Atlantic salmon *Salmo salar*: a comparison of genetic composition over 60 years. Molecular Ecology 6:487–492.

Perez, J., P. Moran, A. M. Pendas, and E. Garcia-Vazquez. 1997. Applications of single-locus DNA minisatellite probes to the study of Atlantic salmon (*Salmo salar* L.) population genetics. Journal of Heredity 88:79–82.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–249.

Rideout, S. G., and L. W. Stolte. 1989. Restoration of Atlantic salmon to the Connecticut and Merrimack rivers. Pages 67–81 *in* R. H. Stroud, editor. Present and future Atlantic salmon management. Atlantic Salmon Federation and National Coalition for Marine Conservation, Marine Recreational Fisheries 12, Savannah, Georgia.

Slettan, A., I. Olsaker, and Ø. Lie. 1995a. Atlantic salmon, *Salmo salar*, microsatellites at the SSOSL25, SSOSL85, SSOSL311, SSOSL417 loci. Animal Genetics 26:281–282.

Slettan, A., I. Olsaker, and Ø. Lie. 1995b. A polymorphic dinucleotide repeat microsatellite in Atlantic salmon, *Salmo salar* (SSOSL436). Animal Genetics 26:368.

Slettan, A., I. Olsaker, and Ø. Lie. 1997. Segregation studies and linkage analysis of Atlantic salmon microsatellites using haploid genetics. Heredity 78: 620–627.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry, 2nd edition. Freeman, New York.

Tessier, N., L. Bernatchez, and J. M. Wright. 1997. Population structure and impact of supportive breeding inferred from mitochondrial and microsatellite DNA analyses in land-locked Atlantic salmon, *Salmo salar* L. Molecular Ecology 6:735–750.

Utter, F. M. 1991. Biochemical genetics and fishery management: a historical perspective. Journal of Fish Biology 39:1–21.

Verspoor, E. 1988. Reduced genetic variability in first-generation hatchery populations of Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences 45:1686–1690.