

**Genetic Differentiation
of
Rainbow Trout (*Oncorhynchus mykiss*)
in the
Togiak National Wildlife Refuge, Alaska**

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Abstract: The purpose of this study was to genetically compare rainbow trout (*Oncorhynchus mykiss*) within and among drainages in the Togiak National Wildlife Refuge (NWR). Mitochondrial DNA variation was analyzed in rainbow trout collected from four refuge streams and compared to two collections made from the Kisaralik River (Yukon Delta NWR). Large differences were detected among the six collections based on an overall *G* test ($P < 0.001$). All Togiak NWR collections were highly different from the Yukon Delta NWR collections in pair-wise comparisons ($P < 0.001$). Genetic differences also existed among the four Togiak NWR collections ($P < 0.001$). Genotypic frequencies in collections were different between drainages (e.g., Arolik versus Gechiak rivers) as well as within a drainage (Togiak River). Multiple populations of genetically distinct rainbow trout exist in the Togiak NWR based on this analysis. This study provided preliminary evidence of a complex population structure for rainbow trout organized among and within major river drainages. To conserve the natural diversity of rainbow trout, populations should be the units of focus for refuge fish management. Future studies should further characterize the population structure within and among each major river system in the refuge.

Introduction

The Togiak National Wildlife Refuge (NWR) encompasses a vast area with several major drainages that have intact native biodiversity and retain original ecosystem function. The Togiak NWR is located about 640 km southwest of Anchorage, Alaska, and comprises 1.7 million ha, equivalent in size to the states of Connecticut and Rhode Island (U.S. Fish and Wildlife Service 1986). Several major drainages flow into Kuskokwim Bay and northwestern Bristol Bay on the coastal perimeter of the refuge and provide more than 20 million ha of pristine freshwater habitat. The annual cycle of transport via Pacific salmon (*Oncorhynchus* sp.) of marine-origin nutrients and energy is a dominant characteristic of these systems (e.g., Levy 1997). A total of 33 species of fish occur in Togiak NWR streams, lakes, and coastal ponds and sloughs (U.S. Fish and Wildlife Service 1990). For example, self-sustaining populations of five species of Pacific salmon, rainbow trout (*Oncorhynchus mykiss*), three species of char (*Salvelinus* sp.), Arctic grayling

(*Thymallus arcticus*), northern pike (*Esox lucius*), and burbot (*Lota lota*) occur in the refuge. Rainbow trout were chosen as an indicator species for the resident freshwater fish community within the refuge (U.S. Fish and Wildlife Service 1990). An indicator species is used as a logistically practical means to monitor the status of the fish community as a whole. Changes in population dynamics of indicator species would denote that important changes in the fish community were occurring.

Rainbow trout are present throughout the refuge and contribute to important sport and subsistence fisheries. The species is present in the Kuskokwim Bay drainages such as the Kanektok, Arolik, and Goodnews rivers and in Bristol Bay drainages such as the Togiak, Ungalikthluk-Negukthlik, Osviak, and Igushik rivers (U.S. Fish and Wildlife Service 1990). Rainbow trout in western Alaska have a freshwater resident life cycle, typically mature at age 5-7 at 400-500 mm forklength (FL), and spawn from late April through June. Adult fish migrate to spawning areas in

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the spring and then move to food sources (e.g., eggs from spawning salmon) during the summer. Maximum age for these fish exceeds 11 years with a maximum length of about 720 mm (Irving and Faustini 1994; Lisac and MacDonald 1995; Lisac 1996; MacDonald 1997; MacDonald and Lisac 1998).

Rainbow trout, in combination with other salmonid species, support important sport fisheries in the refuge. The State of Alaska establishes harvest regulations for refuge waters. Commercial operators within the refuge are limited by a competitive permit process and provide fixed-camp fishing, guided and non-guided float-trip fishing, and daily fly-in fishing (U.S. Fish and Wildlife Service 1991). Subsistence fishing is conducted by local villages inhabited primarily by Yup'ik Eskimos. In the villages of Togiak and Manokotak, many households (48-60%) in 1994-1995 used as a food source rainbow trout; however, this species comprised less than 5% by weight of the total freshwater fish consumed annually (Bristol Bay Native Association and Alaska Department of Fish and Game 1996). Dolly Varden char (*S. malma*), smelt (*Osmerus* sp.), and northern pike were the three most important subsistence fishes in these two villages, having comprised 73 and 91% of total weights consumed.

Conservation of the natural diversity of fish and wildlife is a primary purpose of the Togiak NWR. The first purpose listed in the legislation that established the refuge (Alaska National Interest Lands Conservation Act 1980) was:

... to conserve fish and wildlife populations and habitats in their natural diversity including, but not limited to, salmonoids, marine birds and mammals, migratory birds and large mammals. (Section 303 (6) (B) (I))

Supporting this purpose, the goal of public use management at the Togiak NWR is "...to provide high quality fish and wildlife oriented recreation, subsistence, interpretive and educational opportunities consistent with the refuge's resource oriented purposes." (U.S. Fish and Wildlife Service 1991).

Populations are an important organizational unit of biodiversity because they can accumulate and maintain genetically based adaptations that enhance survival in their particular environments due to their low or negligible reproduction with other populations.

For example, steelhead (anadromous rainbow trout) populations possess traits for ocean survival whereas some inland rainbow trout populations are well-known to be adapted to the harsh, arid climates of the Great Basin region of Nevada, California, Idaho, and Oregon (Behnke 1992). The term population here means a local group of fish of the same species that breed among themselves. The sub-division of rainbow trout into populations results from reproductive-isolating mechanisms that prevent interbreeding, such as homing to natal spawning areas and waterfalls that prevent upstream movement. Homing capabilities in rainbow trout have been well documented (e.g., Lindsey et al. 1959) and may include the use of olfactory cues (e.g., Cooper and Scholz 1976; Scholz et al. 1978). Disruption of natural population structure can lead to lost genetic diversity and lower production of fish, affecting ecosystem function and human use. Potential threats to the natural diversity of rainbow trout can include overharvest (sport, commercial, subsistence), habitat degradation, and interbreeding with hatchery fish. Refuge managers need to be able to identify populations if they are to maintain biodiversity and ensure sustainable production.

Rainbow trout over its native range is organized into semi-discrete populations that exhibit anadromous or freshwater resident life history forms. For example, populations of steelhead trout (anadromous) were genetically identifiable among streams along the coast of British Columbia based on allelic frequencies for protein-coding loci (Parkinson 1984). Similarly, rainbow trout from inland areas of resident or anadromous life histories have been reported to be geographically organized among streams into genetically identifiable populations (e.g., Allendorf 1975; Wishard et al. 1984; Taylor 1995; Bagley and Gall 1998). Large differences among regions in allozyme, minisatellite, and microsatellite allele frequencies, and mitochondrial DNA (mtDNA) genotype frequencies have also been noted in studies that examined rainbow trout populations over broad geographic areas (e.g., Parkinson 1984; Nielsen et al. 1994; Taylor 1995).

Developing and maintaining populations seems to be a strong characteristic within rainbow trout. Rainbow trout were introduced into the Great Lakes

Table 1. Locations of rivers, dates, lengths (mm), ages, and sample sizes (N) of rainbow trout analyzed from the Togiak and Yukon Delta National Wildlife Refuges (NWR), Alaska. Mean ages were only for subsets of the collections because an age could not be assigned to every fish due to regenerated scales. NA indicates that data were not available.

Collection	Approximate Location (°N, °W)	Date	Mean Length (range, mm)	Mean Age (range, years)	N
Togiak NWR					
Arolik River	59° 35'N, 161° 35'W	June 8-11	476 (315-570)	5.8 (4-8)	52
Gechiak Creek	59° 18'N, 160° 17'W	May 30–June 1	454 (395-640)	5.4 (4-8)	52
Pungokepuk Creek	59° 17'N, 160° 02'W	May 20–22	503 (393-663)	5.5 (4-7)	25
Negukthlik River	59° 04'N, 160° 08'W	May 26–June 26	465 (414-545)	6.0 (6)	10
Yukon Delta NWR					
Kisaralik-Upper	60° 30'N, 160° 15'W	August 5–7	NA	NA	44
Kisaralik-Lower	60° 45'N, 160° 30'W	August 14–15	NA	NA	48

approximately 100 years ago and already show detectable levels of population differentiation based on both nuclear and mitochondrial genetic data (Krueger and May 1987; Danzmann et al. 1993; Krueger et al. 1994; Dueck and Danzmann 1996; O'Connell et al. 1997). Although this species shows a high level of genetic differentiation in areas less affected by human activities, hatchery stocking appears to have caused the loss of genetic diversity in U.S. coastal areas (Reisenbichler and Phelps 1989; Reisenbichler et al. 1992) and in some inland resident populations (Allendorf et al. 1980; Williams et al. 1996; Nielsen et al. 1997; Williams et al. 1997). Detecting losses of biodiversity has often been hindered by the lack of genetic data prior to the adverse human activities (stocking) that affected the populations.

The purpose of this study was to genetically compare rainbow trout within and among streams in the Togiak NWR. The primary question to be answered was, "Do rainbow trout randomly spawn with each other in refuge waters or are they subdivided into separate spawning populations?" Mitochondrial DNA variation was analyzed in rainbow trout collected from four refuge streams. The fish

used in this study came from refuge waters that are viewed as being relatively pristine with few serious adverse human activities affecting populations. The relative level of genetic differentiation observed among the four streams was then compared to two out-group collections from the Kisaralik River (Yukon Delta NWR), located approximately 200 km from the nearest Togiak NWR collection site. This study was viewed as a preliminary step to determine whether a more detailed investigation of rainbow trout population structure would be warranted.

Methods

Collections

Scales and portions of fins (~1 cm²) were collected from 10 to 52 adult rainbow trout caught from each of six sites by angling (Table 1; Figure 1). Fins were stored in 70–100% ethanol until analyzed. Scales were stored dry in envelopes. Adult fish (assumed to be mature based on length and age) were sampled in the spring as close to the spawning period as possible to increase the probability that a collection represented a single population rather than a mixture of populations. Fish ages ranged from 4 to 8 years old (based on scales). The two out-group collections were

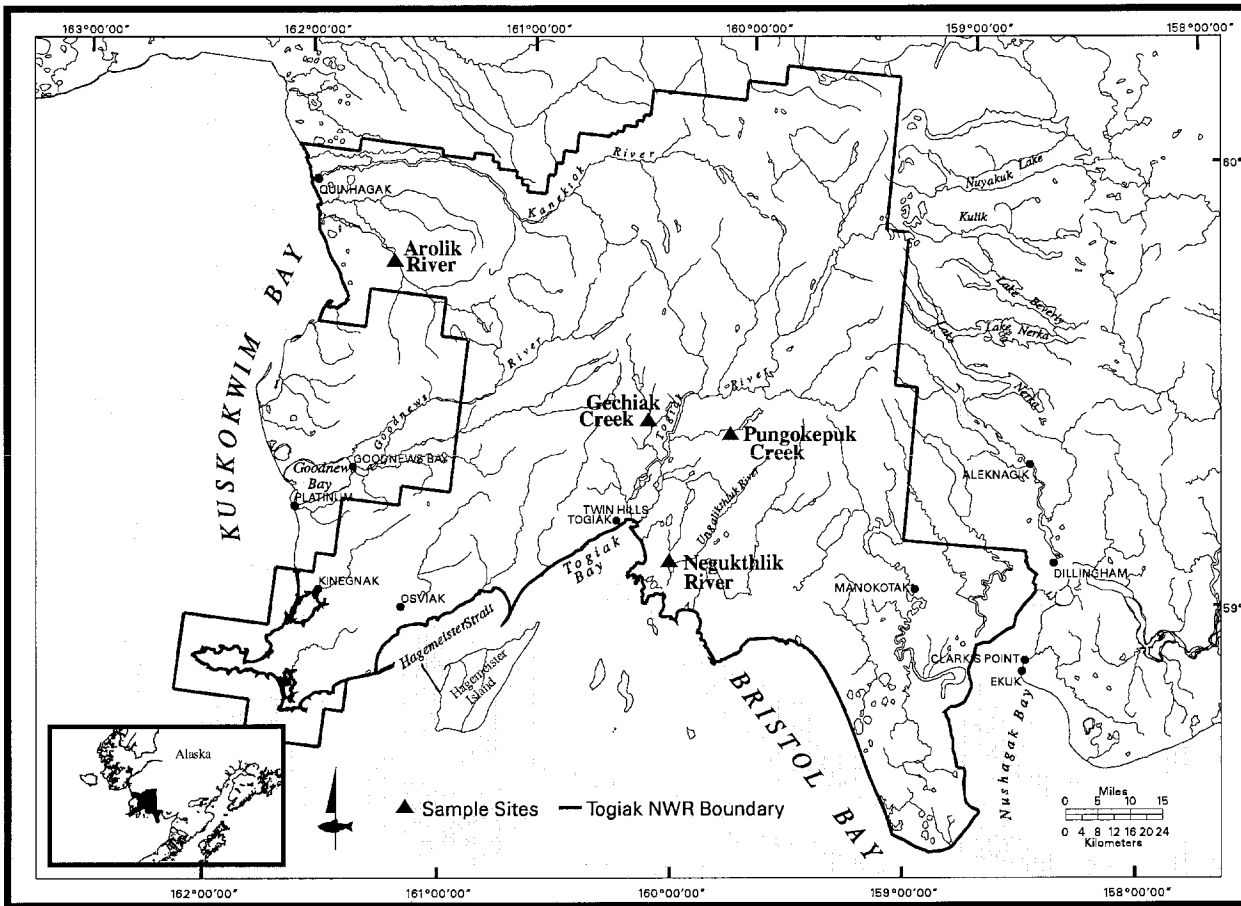


Figure 1. Locations of collections of rainbow trout made in the Togiak National Wildlife Refuge and analyzed for mitochondrial DNA variation.

not sampled during the spawning season. These collections were made in August from the Kisaralik River in the Yukon Delta NWR.

Collections were taken to examine genetic variation within and among river systems. Fish from Gechiak and Pungokepuk creeks represented rainbow trout from two locations within the same drainage, the Togiak River (Figure 1). The other two Togiak NWR collections were not hydrogeographically related by freshwater to one another nor to the Togiak River. One of these collections came from the Negukthlik River that connects with the Unglikthluk River, approximately 3 km upstream from Togiak Bay. The mouth of this system is approximately 20 km from the mouth of the Togiak River in Togiak Bay. The other collection came from the Arolik River located near the northwestern boundary of the Togiak NWR. This river flows into Kuskokwim Bay 7 km south of the Kanektok River and approximately 270 km by ocean

from the mouth of the Togiak River.

The two outgroup collections were made from the Kisaralik River (Yukon Delta NWR) at sites approximately 35 km apart. One collection was designated as “upper” (from Golden Gate Falls to Quartz Creek) and the other as “lower” (from a 9 km stretch beginning 35 km downstream of Quartz Creek). The Kisaralik River flows into the Kuskokwim River, approximately 200 km by river and ocean north of the mouth of the Arolik River.

mtDNA analysis

Nucleic acids were extracted from about 25 mg of scale or fin tissue. Scales were incubated at 65°C overnight in 500 µL of STE buffer (0.1 M NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 8.0), 50 µL 20% SDS and 30 µL proteinase K (10 mg/mL). Five hundred µL of buffered phenol: chloroform: isoamyl (25:24:1) was then added to the sample, vortexed, and

centrifuged for 15 min at 13,800 RCF (relative centrifugal force). Five hundred μL of supernatant were transferred to 1.5 mL tubes and the phenol process and centrifugation repeated. Five hundred μL of supernatant were then transferred to 1.5 mL tubes and 500 μL of chloroform:isoamyl (24:1) added. Tubes were then vortexed, and spun at 13,800 RCF for 15 min. Five hundred μL of supernatant were then transferred to 1.5 mL tubes and 15 μL 5 M NaCl and 1 mL 100% ethanol were added, mixed by inverting the tubes several times, and then centrifuged at 8,000 RCF for 5 min. The supernatant was then discarded. The DNA pellet was washed with 70% ethanol and air dried for > 60 min. The DNA pellet was then diluted in 100 μL of TE buffer, pH 7.4 (10 mM Tris, pH 7.4; 1 mM EDTA, pH 8.0) and heated to 65°C for 60 min, and stored at -20°C.

DNA from fin tissue was isolated by using Puregene™². Tissues were placed in 500 μL of cell lysis buffer and 30 μL proteinase K (10 mg/mL), then incubated overnight at 65°C. Three μL of RNAase A solution (4 mg/mL) were added to cell lysate and incubated for an additional 30 min at 37°C, cooled to room temperature, and 200 μL protein precipitation buffer added and vortexed vigorously. The solution was then placed on ice for 60 min and then centrifuged at 13,800 RCF for 3 min. Supernatant was poured into 1.5 mL tubes and 500 μL of isopropanol (2-propanol) added. Tubes were inverted several times to mix the alcohol and the supernatant, then were centrifuged at 13,800 RCF for 1 min. Supernatant was poured off and the pellet washed with 600 μL 70% ethanol, and then centrifuged for 1 min at 13,800 RCF. The supernatant was decanted and the DNA pellet was air dried at ambient temperature for 60 min. The DNA pellet was then hydrated in 100 μL TE buffer as described above.

DNA samples (5 μL) were electrophoresed to assess the DNA quantity and quality in 0.8% agarose gels cast in TBE buffer (Sambrook et al. 1989), stained with ethidium bromide, and photographed with Polaroid™ 667² film on an ultraviolet (UV) light table.

One mtDNA segment, cytochrome-B (*cytB*; Bickham et al. 1995), was amplified using the polymerase chain reaction (PCR) with the following

²Mention of a commercial product does not represent an endorsement.

primers:

LGL765 5' -GAAAAACCAAYCGTTGTWATTCAACT-3'
LGL766 5' -GTTTAATTAGAATYTYAGCTTTGGG-3'

Each PCR reaction comprised 3 μL (~150 ng) total genomic DNA, 2.5 μL of 10X buffer (Sigma or Perkin Elmer; 500 mM KCl, 100 mM Tris, pH 9.5), 1.5 μL MgCl₂ (25 mM), 2.5 μL of dNTP mix (2 mM each of dATP, dTTP, dCTP, dGTP in 10 mM Tris-HCL, pH 8.0), 0.5 μL of a 10 mM solution of each of two primers, 1.5 units of *Taq* polymerase, and deionized H₂O added for a final volume of 25 μL . The amplification cycle consisted of 95°C for 3 min for 1 cycle; 95°C for 45 sec, 50°C for 50 sec, 70°C for 2 min 30 sec for 32 cycles; 70°C for 5 min for 1 cycle.

Three restriction enzymes (*DdeI*, *DpnII*, and *MspI*) were used to identify different mtDNA genotypes (haplotypes). Each restriction enzyme recognizes a unique sequence of four or five bases and cuts the DNA at that site. Restriction digests consisted of 5 units of a restriction enzyme (*DdeI* 5 base; *DpnII* 4 base; or *MspI* 4 base), 5 μL of amplified PCR product, 1.5 μL of each enzyme's 10X buffer, and deionized H₂O added to a final volume of 15 μL . Digests were electrophoresed in 2.5% agarose gels, stained with ethidium bromide, and photographed under UV light. Sizes of restriction fragments and uncut *cytB* were estimated by comparison to a 100 basepair (bp) ladder. Restriction fragment patterns were visually identified from gels and photographs. Each fish was assigned a composite genotype based on the individual genotypes observed from each of the three restriction enzymes.

Data analysis

Counts of composite genotypes were used to genetically characterize each collection and to make comparisons among collections. Differences among collections were determined by two approaches. First, the simplest approach compared the presence and absence of composite genotypes among collections, and compared among collections which genotype was most common and most rare. Second, frequencies of mtDNA composite genotypes were used to perform statistical tests to compare collections, to calculate measures of genotypic variation (*h* and *F_{ST}*), and to calculate genetic distance between collections.

Statistical comparisons of genotypic data used the

Table 2. Mitochondrial DNA genotype frequencies, nucleon diversity (h), and sample sizes (N) of collections of rainbow trout from rivers in the Togiak and Yukon Delta National Wildlife Refuges (NWR), Alaska. Gechiak and Pungokepuk creeks are tributaries to the Togiak River. Upper (-U) and lower (-L) reaches of the Kisaralik River were sampled.

Collection	mtDNA Composite Genotype			h	N
	AAA	ABB	ABA		
Togiak NWR					
Arolik	0.385	—	0.615	0.473	52
Gechiak	0.096	0.077	0.827	0.301	52
Pungokepuk	—	0.538	0.462	0.497	26
Negukthlik	—	—	1.000	0.000	10
Yukon Delta NWR					
Kisaralik-U	1.000	—	—	—	44
Kisaralik-L	0.958	—	0.042	0.080	48

log-likelihood ratio G -test (Sokal and Rohlf 1981) compared to a χ^2 distribution to provide hierarchical tests of heterogeneity among collections. The null hypothesis of no evidence of heterogeneity among genotypic frequencies was rejected at $P < 0.05$. The critical values used in the pair-wise G -test comparisons were modified to account for the increase in Type-I errors when multiple tests of the same hypothesis were made (Cooper 1968).

Measurement of mtDNA variability within collections used nucleon diversity h (Nei and Tajima 1981). This measure is mathematically equivalent to expected heterozygosity at nuclear loci, and uses the frequency of composite mtDNA genotypes in its calculation. Nucleon diversity varies between zero (low) and one (high), and provides a relative measure of the amount of mtDNA variation observed within each collection.

Genotypic variability among collections was partitioned with F_{ST} (Wright 1965; Nei 1977). F_{ST} measures the amount of total variation observed that is attributable to differences among collections. F_{ST} varies between zero (no variation among collections) to one (all variation exists among collections; all fish in each collection have the same unique genotype, a genotype not observed in any other collection).

Genetic distances were calculated between each

pair of collections (chord distance; Cavalli-Sforza and Edwards 1967) and were then subjected to unweighted pair-group-method cluster analysis based on arithmetic averages (UPGMA; Sneath and Sokal 1973) to generate a dendrogram to assess differences among populations. Distance measures of zero indicate no differences and larger distances indicate larger genetic differences. Thus, the greater the length of a horizontal line between two collections in a dendrogram, the greater the genetic distance.

Analyses of the data (data input modified because of their haploid character) were performed with "Genes in Populations" version 2.1 designed by B. May and C. Krueger and written in the programming language C by W. Eng and E. Paul. Calculation of genetic distances and UPGMA cluster analysis were performed using PHYLIP 3.57c (Felsenstein 1995).

Results

Fragment pattern variation

Genetic variation among individuals was revealed by the fragment patterns of *cytB* for *DdeI* (one genotype A), *DpnII* (two genotypes A and B), and *MspI* (two genotypes A and B; see Appendix 1). These genotypes occurred in three combinations (AAA, ABB, and ABA) to provide the composite genotypes used in subsequent analyses (Table 2). The

Table 3. Mitochondrial DNA genotype differentiation (F_{st}) and heterogeneity tests (G ; Sokal and Rohlf 1981) among collections of rainbow trout from the Togiak and Yukon Delta National Wildlife Refuges, Alaska. The total G test for all collections is subdivided into G values contributed by genotype variation within and among refuges. The G test for the Togiak collections are further subdivided between variation within the Togiak River (Gechiak vs. Pungokepuk) and the other two collections. Probability values are given for the H_0 that genotype frequencies were not different among collections.

Comparison	F_{st}	G	df	P
Within the Togiak NWR				
Gechiak vs. Pungokepuk	0.18	22.5	2	< 0.001
Arolik vs. Negukthlik vs. Pooled Gechiak and Pungokepuk	0.19	43.2	4	< 0.001
Total Togiak (4 collections)	0.27	65.7	6	< 0.001
Within the Yukon Delta NWR				
Kisaralik River Upper vs. Lower	0.021	2.64	2	> 0.3
Between Refuges				
Togiak NWR pooled vs. Yukon Delta NWR pooled	0.52	172	2	< 0.001
Among all collections	0.61	240	10	< 0.001

uncut *cytB* segment was approximately 1300 bp long. The sums of the fragments from *DpnII* and *MspI* were comparable to the size of the uncut segment (Appendix 1). The sum of the fragments for *DdeI* approximated 1012 bp. This value was less than the uncut size (1300 bp) and was possibly due to small fragments (< 50 bp) that were not observed.

Genotypic frequencies

Out of three mtDNA genotypes observed, only ABA was present in each of the collections from the Togiak NWR (Table 2). The other two genotypes were not observed in two of four collections. Only rainbow trout from the Arolik and Gechiak had detectable frequencies of the AAA genotype (0.385 and 0.096, respectively). The ABB genotype occurred only in fish from the Gechiak (0.077) and Pungokepuk creeks (0.54), tributaries to the Togiak River. Fish from the Negukthlik River showed no variation and were all the common ABA genotype. However, this collection contained only ten fish and the power to detect rare genotypes was low. Nucleon diversity (h) was highest in the Pungokepuk River (0.497) and lowest in Negukthlik River (0.000). The two Yukon Delta collections had high frequencies of the AAA

genotype (1.00 and 0.96), no ABB genotypes, and low frequencies of the ABA genotype (0.00 and 0.04) which was the common genotype in Togiak NWR fish.

Differences among collections

Large genetic differences were detected among the six collections from Togiak and Yukon Delta NWRs based on the total G test ($P < 0.001$; Table 3). Genetic differences were also detected among the four collections from the Togiak NWR ($P < 0.001$) and contributed approximately 27% to the total G value. Genotypic frequencies among drainages (Arolik versus Negukthlik versus Togiak-pooled Gechiak and Pungokepuk) were different ($P < 0.001$) and contributed 66% to the Togiak total G value of 65.7 (Table 3). Differences in mtDNA genotypic frequencies occurred between all paired comparisons of the Arolik, Gechiak, and Pungokepuk collections, and between the Negukthlik and Pungokepuk fish (Table 4). Most (72%) of the balance of the total G value (172 of 240) was due to the difference between the Togiak and Yukon Delta NWR fish (Table 3). All Togiak NWR collections were highly different from the Yukon Delta NWR collections in pair-wise

Table 4. Pair-wise G tests and probability values (P) for heterogeneity of mitochondrial DNA genotype frequencies between rainbow trout collections from the Togiak (four sites) and Yukon Delta (two sites) National Wildlife Refuges, Alaska. Upper (-U) and lower (-L) reaches of the Kisaralik River were sampled. ** indicates $P < 0.01$ and *** $P < 0.001$ for the H_0 that the two collections were not different from each other. Probabilities were adjusted to accommodate multiple tests of the same H_0 . G could not be calculated (undefined) for the Kisaralik-U vs. Negukthlik comparison because the collections were fixed for different haplotypes and the G formula requires values greater than zero in each cell of a R x C table.

Collection	Togiak NWR			Yukon Delta NWR	
	Gechiak	Pungokepuk	Negukthlik	Kisaralik-U	Kisaralik-L
Togiak NWR					
Arolik	16.8**	47.7***	8.68	52.9***	42.3***
Gechiak	—	22.5***	3.49	100***	89.4***
Pungokepuk		—	12.20*	92.4***	84.5***
Negukthlik			—	Undefined	42.5***
Yukon Delta NWR					
Kisaralik-U				—	2.64

comparisons (Table 4; $P < 0.001$).

Diversity analysis of Togiak NWR fish (F_{ST} ; used to partition the total variation) indicated that 27% of the variation observed was due to differences among collections (Table 3). The balance (73%) was due to differences among individuals within collections. A high level of differentiation within a single drainage, the Togiak River, was revealed by the difference in mtDNA genotypic variation between the Gechiak and Pungokepuk fish (Table 3; $P < 0.001$; $F_{ST} = 0.18$). A within-drainage difference was not observed between

the upper and lower Kisaralik River collections from the Yukon Delta NWR ($P > 0.3$).

Cluster analysis of genetic distances organized the collections into two distinct refuge groups (Table 5; Figure 2). The first group contained the four rainbow trout collections from the Togiak NWR. Within this group, Pungokepuk Creek was the most divergent of the four collections and illustrated a high level of genetic diversity within the Togiak River drainage. The second group included the upper and lower Kisaralik River samples from the Yukon Delta NWR.

Table 5. Genetic distances (chord distance; Cavalli-Sforza and Edwards 1967) between rainbow trout collections from the Togiak and Yukon Delta National Wildlife Refuges, Alaska, based on mtDNA genotype frequencies. Upper (U) and lower (L) reaches of the Kisaralik River were sampled.

Collection	Togiak NWR			Yukon Delta NWR	
	Gechiak	Pungokepuk	Negukthlik	Kisaralik-U	Kisaralik-L
Togiak NWR					
Arolik	0.189	0.934	0.432	0.759	0.464
Gechiak	—	0.357	0.181	1.38	1.02
Pungokepuk		—	0.641	2.00	1.72
Negukthlik			—	2.00	1.59
Yukon Delta NWR					
Kisaralik-U				—	0.042

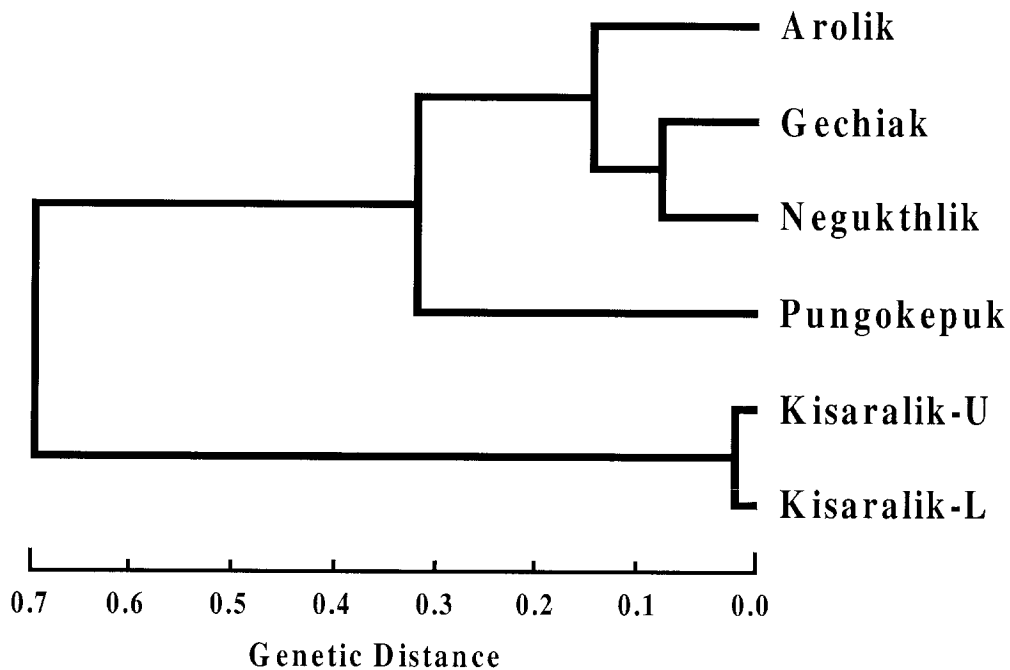


Figure 2. Dendrogram generated by cluster analysis of genetic distance (chord distance; Cavalli-Sforza and Edwards 1967) between rainbow trout collections from Togiak National Wildlife Refuge.

These two refuge groups were joined by a large genetic distance (relative to the distances within the two groups; Figure 2).

Discussion

Population structure

Rainbow trout collections from the Togiak NWR were genetically different from one another, indicating the presence of multiple populations in the refuge. Collections between drainages (e.g., Arolik versus Gechiak) as well as within a drainage (Togiak River) were different (Table 4). This study provided evidence (based on four collections) of a complex population structure for rainbow trout within the Togiak NWR, organized not only among major rivers but also within a drainage.

Population diversity within a drainage was demonstrated by genetic differences between the Gechiak and Pungokepuk collections (Tables 3 and 4). These tributaries empty into the Togiak River approximately 10 km from each other. The Togiak River is hydrogeographically complex, being more than 90 km long with five major tributaries (two were sampled) and nine major lakes (U.S. Fish and Wildlife Service 1990). The extensive habitat available to rainbow trout in this drainage makes it likely that

more than just two populations (Gechiak and Pungokepuk creeks) exist within the drainage. Similar hydrogeographical complexity within other drainages in the refuge, including Kanektok and Goodnews drainages suggests the possibility of multiple populations within each major refuge drainage. Multiple populations within a drainage have been reported for rainbow trout based on ecological or genetic data for resident fish in lakes (Lindsey et al. 1959), steelhead from coastal areas of British Columbia (Parkinson 1984), and for trout above and below waterfalls (Northcote et al. 1970; Currens et al. 1990).

Maintenance of the genetic differences between rainbow trout in Gechiak and Pungokepuk creeks requires that the fish have some mechanism that isolates them during the spawning season and prevents interbreeding of the populations. Because of the lack of physical barriers to movement such as waterfalls in this drainage, natal homing is the most likely mechanism to cause separate populations. Though natal homing in rainbow trout has not been investigated in the Togiak NWR, movement patterns coinciding with the spawning season have been documented. Past radio-tracking studies of rainbow trout have indicated seasonal movements between

spring spawning and summer feeding associated with spawning Pacific salmon (feeding on eggs and decaying flesh; Lisac and MacDonald 1995; Adams 1996; Faustini 1996; Lisac 1996). Similar movement patterns were observed for rainbow trout from Pungokebuk and Gechiak creeks that were anchored during the spring and later recaptured in the main stem and tributaries of the Togiak River during the summer and winter (Mark Lisac, Togiak National Wildlife Refuge, personal communication).

More than one population could occupy the Kisaralik River system (Yukon Delta NWR), even though no evidence was provided by the mtDNA genotypic frequencies (Tables 3 and 4). Genotypic diversity of mtDNA based on the restriction enzymes we used was low in these collections; thus, the ability to detect differences between the two sites was low. In addition, the timing and main-stem locations of these collections reduced the power to detect the presence of multiple populations in this river system. The two Kisaralik collections were made in August after the spawning season when seasonal movements associated with feeding could have resulted in a mixture of several populations within each collection. The locations of the Kisaralik collections were confined to the lower two-thirds of the river separated by only 35 km. Fish from the lower river may have originated from a combination of main stem, tributary, and upstream spawning sites.

Description of the population structure within the Kisaralik system will require the use of additional genetic markers to compare adult collections made during the spring spawning season from upstream areas, tributaries, and the previously sampled downstream areas. Future collections should be from areas that may geographically define populations. For example, waterfalls or rapids that restrict fish movement or tributaries with geographically isolated spawning areas can define population boundaries and cause more than one population to occur in a watershed (e.g., Currens et al. 1990). These features can help identify strategic locations from which to make collections. Both collections from the Kisaralik River were made downstream of three stretches of falls and rapids that could restrict fish movement and that may define a boundary to an upstream population. In addition, a number of tributaries exist within this

system that could serve as spawning areas for separate populations. Fish from the upstream location and from tributaries should be analyzed and compared to help describe the population structure for this river.

Management implications

The genetic data from this project indicated that rainbow trout in the Togiak NWR are not one large random-mating population but occur in multiple populations, semi-isolated from each other. Such differences in mtDNA genotypic frequencies cannot exist if the rainbow trout from different locations freely interbreed with one another each spawning season. The populations in the Togiak NWR appear geographically organized by major watershed and likely also by tributary within a watershed. Thus, the Togiak NWR probably has several populations of rainbow trout within each major drainage.

To conserve the natural diversity of rainbow trout, populations should be the units of focus for management actions. Two fundamental reasons support this management approach. First, populations often contain genetically encoded adaptations that are required for survival and reproduction, and this has been shown to be true for rainbow trout (e.g., Kelso and Northcote 1981). These adaptations, as products of natural selection, accumulate and are maintained within populations over time through semi-reproductive isolation from other populations. New generations or year classes of rainbow trout are produced through successful spawning, incubation, and survival in part due to these adaptations. Often these adaptations (e.g., spawning site selection) help to temporally synchronize movement, feeding, and spawning behaviors of a population to take advantage of local environmental cycles. Thus, the extinction of a population represents the loss of adaptations essential for population survival that cannot be replaced except over long periods of time. Restoration of the population would be difficult when specialized genetic adaptations are required for survival and reproduction in a unique habitat.

The second major reason for using the population as the unit of focus for management is that each population may have different population dynamics (e.g., mortality, natality, and growth rates). Populations organized within watersheds can function

as separate ecological units. The degree of their ecological independence depends on the amount of population mixing that may occur during non-reproductive parts of their life cycle such as on feeding grounds during the summer or in overwintering areas. Geographical separation during critical life history stages means that geographically localized mortality or habitat loss could severely affect one population but not others. For example, geographically localized fishing mortality could severely depress the abundance of one population (even to extinction) and not affect all other populations in the refuge or even within a drainage. Thus, detection of a population in trouble requires that assessment studies must analyze and interpret data for each population and not on data from mixtures of populations. Similarly, management actions in response to a population problem must be able to be implemented at a fine-enough geographic and temporal scale to address population-specific issues.

Information about individual populations is required if managers are to be effective in conserving the diversity of rainbow trout populations within the Togiak NWR as prescribed by ANILCA. Sex-specific age, size, growth, and mortality data should be collected from populations, as this will provide key information about interaction of the population with its environment. Mortality that exceeds year class recruitment, the breakdown of population isolating mechanisms, and habitat loss are three processes that commonly threaten populations and should be monitored. Thus, knowledge of the population structure of rainbow trout within each major watershed is essential for the design of future population studies. This population-specific information would form the basis for the development of management plans, the regulation of harvests, and the protection of habitat.

Genetic description of the population structure of rainbow trout in the Togiak NWR should be the focus of the next investigation. New collections should be analyzed for mtDNA variation and for variation at nuclear loci (e.g., allozymes or microsatellite, Wenberg et al. 1996). Collections should also be repeated at the sites used in the present study to determine the temporal stability of the genetic markers. Foundational to the success of such studies

is the quality of the collections of fish to be compared. Key characteristics of the collections are as follows:

- collections should be made of adults on spawning grounds to avoid mixtures of populations (Allendorf and Phelps 1981);
- each collection should comprise a minimum of 60 individuals if possible (Grewe et al. 1993);
- locations of collections should be based on hydrogeographical features that may define population boundaries (e.g., upstream and downstream of waterfalls, spawning tributaries); and
- each major drainage (e.g., Kanektok, Goodnews) should be represented by two or more collections to facilitate hierarchical analysis of genetic data within and among drainages.

The genetic description of population structure will also provide baseline data for the refuge that can be used to monitor changes in genetic diversity over time and be used for studies that use mixed-stock analysis techniques to estimate the population origins of collections that represent mixtures of stocks (e.g., collections from overwintering areas).

The best approach to describe population structure would be to integrate a study that describes patterns of genetic variation among populations as described above with a study of adult spawning movements. These two data types relate directly to the patterns of gene flow that define population structure and thus will yield a more accurate description of population structure than if only one data type was used. This approach would also help identify the locations of critical habitats (e.g., spawning, overwintering) that should be monitored and protected. After the population structure has been described, other studies should be conducted to geographically assess the distribution and amount of fishing mortality in relation to population structure, to identify critical habitats, and to describe the role of population-isolating mechanisms in maintaining the population structure (e.g., natal homing, water temperature, physical barriers to fish movement). Population-based studies will provide the information required for effective decision making to conserve the population diversity of rainbow trout in the Togiak NWR.

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References

- Adams, F. J. 1996. Status of the rainbow trout in the Kanektok River, Togiak National Wildlife Refuge, Alaska, 1993-94. U.S. Fish and Wildlife Service, Alaska Fisheries Technical Report Number 39, Region 7, Anchorage.
- Allendorf, F. W. 1975. Genetic variability in a species possessing extensive gene duplication: genetic interpretation of duplicate loci and examination of genetic variation in populations of rainbow trout. Ph.D. thesis. University of Washington, Seattle.
- Allendorf, F. W., D. M. Espeland, D. T. Scow, and S. Phelps. 1980. Coexistence of native and introduced rainbow trout in the Kootenai River drainage. Proceedings of the Montana Academy of Science 39:28-36.
- Allendorf, F. W., and S. R. Phelps. 1981. Use of allelic frequencies to describe population structure. Canadian Journal of Fisheries and Aquatic Sciences 38:1507-1514.
- Bagley, M. J., and G. A. E. Gall. 1998. Mitochondrial and nuclear DNA sequence variability among populations of rainbow trout (*Oncorhynchus mykiss*). Molecular Ecology 7:945-961.
- Behnke, R. J. 1992. Native trout of western North America. American Fisheries Monograph 6, Bethesda, Maryland.
- Bickham, J.W., C.C. Wood, and J.C. Patton. 1995. Biogeographic implications of cytochrome b sequences and allozymes in sockeye salmon (*Oncorhynchus nerka*). Journal of Heredity 86: 140-144.
- Bristol Bay Native Association and Alaska Department of Fish and Game. 1996. The harvest and use of freshwater fish in Togiak and Manokotak, 1994-95. Alaska Fish and Game, Division of Subsistence, Anchorage.
- Cavalli-Sforza, L. L., and A. W. F. Edwards. 1967. Phylogenetic analysis: models and estimation procedures. Evolution 21:550-570.
- Cooper, D. W. 1968. The significance level in multiple tests made simultaneously. Heredity 23:614-617.
- Cooper, J. C., and A. T. Scholz. 1976. Homing of artificially imprinted steelhead (rainbow trout), *Salmo gairdneri*. Journal of the Fisheries Board of Canada 33:826-829.
- Currens, K. P., C. B. Schreck, and H. W. Li. 1990. Allozyme and morphological divergence of rainbow trout (*Oncorhynchus mykiss*) above and below waterfalls in the Deschutes River, Oregon. Copeia 1990(3):730-746.
- Danzmann, R. G., M. M. Ferguson, and S. K. A. Arndt. 1993. Mitochondrial DNA variability in Ontario and New York rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Zoology 71:1923-1933.
- Dueck, L. A., and R. G. Danzmann. 1996. Matriarchal population structure of introduced

- rainbow trout (*Oncorhynchus mykiss*) in the Lake Ontario watershed. *Canadian Journal of Zoology* 53:2100-2114.
- Faustini, M. A. 1996. Status of rainbow trout in the Goodnews River, Togiak National Wildlife Refuge, Alaska, 1993-1994. U.S. Fish and Wildlife Service, Alaska Fisheries Technical Report Number 36, Region 7, Anchorage.
- Felsenstein, J. 1995. PHYLIP (Phylogeny Inference Package) Version 3.57c. University of Washington, Seattle.
- Grewe, P. M., C. C. Krueger, C. F. Quadro, E. Bermingham, H. L. Kincaid, and B. May. 1993. Mitochondrial DNA variation among lake trout (*Salvelinus namaycush*) strains stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Sciences* 50:2397-2403.
- Irving, D. B., and M. A. Faustini. 1994. Status of rainbow trout in the Goodnews River, Togiak National Wildlife Refuge, Alaska, 1988-1989. U.S. Fish and Wildlife Service, Alaska Fisheries Technical Report Number 24, Region 7, Anchorage.
- Kelso, B.W., and T.G. Northcote. 1981. Current response of young rainbow trout from inlet and outlet spawning stocks of a British Columbia lake. *International Association of Theoretical and Applied Limnology* 21:1214-1221.
- Krueger, C. C., D. L. Perkins, R. J. Everett, D. R. Schreiner, and B. May. 1994. Genetic variation in naturalized rainbow trout (*Oncorhynchus mykiss*) from Minnesota tributaries to Lake Superior. *Journal of Great Lakes Research* 20:299-316.
- Krueger, C. C., and B. May. 1987. Genetic comparison of naturalized rainbow trout populations among Lake Superior tributaries: differentiation based on allozyme data. *Transactions of the American Fisheries Society* 116:795-806.
- Levy, S. 1997. Pacific salmon bring it all home. *Bio Science* 47:657-660.
- Lindsey, C. C., T. G. Northcote, and G. F. Hartman. 1959. Homing of rainbow trout to inlet and outlet spawning streams at Loon Lake, British Columbia. *Journal of the Fisheries Research Board of Canada* 16:695-719.
- Lisac, M. J. 1996. Length frequency, age distribution and movements of rainbow trout in the Negukthlik and Ungalikthluk rivers, Togiak National Wildlife Refuge, Alaska, 1989-90. U.S. Fish and Wildlife Service, Alaska Fisheries Technical Report Number 35, Region 7, Anchorage.
- Lisac, M. J., and R. MacDonald. 1995. Length frequency, age distribution and movements of rainbow trout in the Arolik River, Togiak National Wildlife Refuge, Alaska, 1991-1994. U.S. Fish and Wildlife Service, Alaska Fisheries Technical Report Number 34, Region 7, Anchorage.
- MacDonald, R. 1997. Length frequency and age distribution of resident fish collected from rivers within Togiak National Wildlife Refuge, Alaska, 1996. U.S. Fish and Wildlife Service, Fisheries Data Series Number 97-4, Region 7, Anchorage.
- MacDonald, R., and M. J. Lisac. 1998. Species occurrence, length frequency and age distribution of resident fish collected from rivers within Togiak National Wildlife Refuge, Alaska, 1997. U.S. Fish and Wildlife Service, Fisheries Data Series Number 98-5, Region 7, Anchorage.
- Nei, M. 1977. F-statistics and analysis of gene diversity in subdivided populations. *Annals of Human Genetics* 41:225-233.
- Nei, M., and F. Tajima. 1981. DNA polymorphism detectable by restriction endonucleases. *Genetics* 97:145-163.
- Nielsen, J. L., C. Carpanzano, M. C. Fountain, and C. A. Gan. 1997. Mitochondrial DNA and nuclear microsatellite diversity in hatchery and wild *Oncorhynchus mykiss* from freshwater habitats in southern California. *Transactions of the American Fisheries Society* 126:397-417.
- Nielsen, J. L., C. A. Gan, J. M. Wright, D. B. Morris, and W. K. Thomas. 1994. Biogeographic distributions of mitochondrial and nuclear markers for southern steelhead. *Molecular Marine Biology and Biotechnology* 3:281-293.
- Northcote, T. G., S. N. Williscroft, and H. Tsuyuki. 1970. Meristic and lactate dehydrogenase genotype differences in stream populations of rainbow trout below and above a waterfall. *Journal of the Fisheries Research Board of*

- Canada 27:1987-1995.
- O'Connell, M., R. G. Danzmann, J.-M. Cornuet, J. M. Wright, and M. M. Ferguson. 1997. Differentiation of rainbow trout (*Oncorhynchus mykiss*) populations in Lake Ontario and the evaluation of the stepwise mutation and infinite allele mutation models using microsatellite variability. *Canadian Journal of Fisheries and Aquatic Sciences* 54:1391-1399.
- Parkinson, E. A. 1984. Genetic variation in populations of steelhead trout (*Salmo gairdneri*) in British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences* 41:1412-1420.
- Reisenbichler, R. R., J. D. McIntyre, M. F. Solazzi, and S. W. Landino. 1992. Genetic variation in steelhead of Oregon and northern California. *Transactions of the American Fisheries Society* 121:158-169.
- Reisenbichler, R. R., and S. R. Phelps. 1989. Genetic variation in steelhead (*Salmo gairdneri*) from the north coast of Washington. *Canadian Journal of Fisheries and Aquatic Sciences* 46:66-73.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning, a laboratory manual*. 2nd edition, Cold Spring Harbor, NY.
- Scholz, A. T., C. K. Gosse, J. C. Cooper, R. M. Horrall, A. D. Hasler, R. I. Daly, and R. J. Poff. 1978. Homing of rainbow trout transplanted in Lake Michigan: a comparison of three procedures used for imprinting and stocking. *Transactions of the American Fisheries Society* 107:439-443.
- Sokal, R. R., and F. J. Rohlf. 1981. *Biometry*, 2nd edition. San Francisco, Freeman.
- Sneath, P. H. A., and R. R. Sokal. 1973. *Numerical taxonomy*. San Francisco, Freeman.
- Taylor, E. B. 1995. Genetic variation at minisatellite DNA loci among north Pacific populations of steelhead and rainbow trout (*Oncorhynchus mykiss*). *Journal of Heredity* 86:354-363.
- U.S. Fish and Wildlife Service. 1986. Togiak National Wildlife Refuge Final Comprehensive Conservation Plan. Region 7, Anchorage, Alaska.
- U.S. Fish and Wildlife Service. 1990. Fishery management plan Togiak National Wildlife Refuge fiscal year 1990-1994. King Salmon Fishery Assistance Office, King Salmon, Alaska and Togiak National Wildlife Refuge, Dillingham, Alaska.
- U.S. Fish and Wildlife Service. 1991. Togiak National Wildlife Refuge final public use management plan and environmental assessment. Dillingham, Alaska.
- Wenburg, J. K., J. B. Olsen, and P. Bentzen. 1996. Multiplexed systems of microsatellites for genetic analysis in coastal cutthroat trout (*Oncorhynchus clarki clarki*) and steelhead (*Oncorhynchus mykiss*). *Molecular Marine Biology and Biotechnology* 5:273-283.
- Williams, R. N., R. F. Leary, and K. P. Currens. 1997. Localized genetic effects of a long-term hatchery stocking program on resident rainbow trout in the Metolius River, Oregon. *North American Journal of Fisheries Management* 17:1079-1093.
- Williams, R. N., D. K. Shiozawa, J. E. Carter, and R. F. Leary. 1996. Genetic detection of putative hybridization between native and introduced rainbow trout populations of the upper Snake River. *Transactions of the American Fisheries Society* 125:387-401.
- Wishard, L. N., J. E. Seeb, F. M. Utter, and D. Stefan. 1984. A genetic investigation of suspected redband trout populations. *Copeia* 1984:120-132.
- Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution* 19:395-420.

Appendix 1. Restriction fragment patterns for the mitochondrial DNA cytochrome-B segment in rainbow trout from Togiak NWR. The numbers in the table are the fragment sizes in base pairs (bp) for each enzyme. Each + indicates the presence of a fragment.

Fragment Size	<i>cytB</i>					
	Uncut	<i>DdeI</i>	<i>DpnII</i>		<i>MspI</i>	
		A	A	B	A	B
1300	+	—	—	—	—	—
1048	—	—	—	—	+	+
667	—	—	+	+	—	—
387	—	—	+	+	—	—
346	—	+	—	—	—	—
282	—	+	—	—	—	—
262	—	+	—	—	—	—
215	—	—	+	—	—	—
208	—	—	—	—	—	—
182	—	—	—	—	+	—
167	—	—	—	+	—	—
122	—	+	—	—	—	—
108	—	—	—	—	—	+
96	—	—	—	—	+	+
82	—	—	—	—	—	+
80	—	—	+	+	—	—
62	—	—	—	+	—	—
50	—	—	—	—	—	—
Total	1300	1012	1349	1363	1326	1334