

Two Genetically Distinct Forms of Rougheye Rockfish Are Different Species

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Abstract.—The variation in mitochondrial DNA (mtDNA) and at eight microsatellite loci was analyzed in 700 rougheye rockfish *Sebastes aleutianus* sampled along the Pacific Rim from the Oregon coast to the western Aleutian Islands and Bering Sea. The program STRUCTURE was used to analyze the microsatellite genotypes and determine that the samples probably came from two genetically distinct sources (type I and type II) by minimizing the strong Hardy–Weinberg and gametic disequilibria observed in the total sample. The two types had nearly fixed differences at one microsatellite locus ($\mu Sma 6$), which corresponded to divergent mtDNA haplotype clusters. We conclude that these two types are distinct species. The ranges of the two types overlapped but were not coincident; in some areas, one or the other predominated. For example, most of the Aleutian Island samples were type I fish. Although both types were caught in the same hauls in some regions, often one or the other species was predominant in a haul. The differences in distributions suggest a historic vicariant or ecological basis for their divergence. No phenotypic characteristics have been confirmed that can be used to distinguish the two species visually. Rougheye rockfish have high commercial value and their incidental catch has the potential to greatly affect larger fisheries. The discovery of cryptic speciation in a commercially important species underscores our need to learn more about the distribution and biology of these and other groundfish species.

The rougheye rockfish *Sebastes aleutianus* is a commercially valuable species caught in Alaskan longline and trawl fisheries. Consequently, most of our sparse knowledge of these and most other Alaskan marine species comes from information acquired in their management and conservation. Rougheye rockfish are targeted by fishermen when possible and are often harvested to the maximum level allowed by regulations. In Alaskan waters, they have been managed as part of a species assemblage with shorttraker rockfish *S. borealis*.

Originally described by Pallas in 1811 as *Perca variabilis*, the rougheye rockfish was described and named *Sebastes aleutianus* by Jordan and Evermann (pages 1795–1796 in Jordan and Ever-

mann 1898 and pages 445–448 in Jordan 1899) from four specimens caught in Shelikof Strait near Kodiak, Alaska, in 1896 during a survey of the northern fur seal *Callorhinus ursinus* in western Alaskan waters. Their descriptions suggest that Jordan had previously (e.g., Jordan 1885) confused rougheye rockfish with the Asian species, akoudai *S. matsubarae*. The description (Jordan and Evermann 1898) also included a footnote expressing concern about confusing *S. aleutianus* with a species previously described from Sitka, Alaska (Richardson 1844). Subsequent samples from Sitka confirmed that Richardson's fish was the copper rockfish *S. caurinus*. Prior to the 1970s, Russian biologists included what are now identified as rougheye, shorttraker, and blackgill rockfish *S. melanostomus* as a single species, *S. introniger* (Love et al. 2002). During the 1896 survey, numerous specimens of *S. introniger* were collected, which may have included additional rougheye rockfish. Other historic synonyms of *S. aleutianus* include *S. kawardai* (Matsubara 1934), *S. melanostictus* (Matsubara 1934), and *Sebastes swifti* (Evermann and Goldsborough 1907). The systematics

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of rockfish has been revised several times in the last century, usually by elevating and restoring subgeneric names. At different times, rougheye rockfish have been assigned to the genera *Sebastes*, *Sebastes*, and *Eosebastes* (Kendall 2000; Love et al. 2002).

The confusion encountered in describing rougheye rockfish is in part a consequence of the large number of rockfish species in the northern Pacific Ocean and their close similarities. A present-day indication of the problem is that the intrageneric phylogeny of *Sebastes* has not yet been resolved (Kendall 2000). Situations that result in descriptions of multiple synonymous species may also hide cryptic species, morphologically similar species that cannot be readily delineated from their appearances and that are often distinguished by genetic differences (e.g., Gómez et al. 2002; McGovern and Hellberg 2003).

In the last four decades, during which genetic methods have been applied to studies of fish, large genetic differences within rougheye rockfish that may be consistent with the existence of distinct species have been noted in three separate instances. Electrophoretic studies of hemoglobin in *Sebastes* resolved four banding patterns, three of which (labeled A, B, and C) were distinct, whereas the fourth (labeled D) appeared to be from a hybrid between types A and B (Tsuyuki et al. 1968). After additional genetic and morphological studies, Tsuyuki and Westrheim (1970) described type C as *S. caenaemeticus*, which they later synonymized with *S. borealis* (Westrheim and Tsuyuki 1972); *S. borealis* had been described earlier in 1970 (Barsukov 1970). The other three hemoglobin patterns received little subsequent attention, although in three separate collections (Tsuyuki and Westrheim 1970), types A and B were predominant and the presumed hybrid was relatively rare, far from Hardy–Weinberg expectations.

In an allozyme-based phylogenetic survey of the genus *Sebastes*, Seeb (1986) also observed two genetically distinct rougheye rockfish types in a sample of 47 fish. Several loci, namely, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH**), β -*N*-acetylgalactosaminidase (*bGALA**), and acid phosphatase (*ACP**) (following the enzyme nomenclature of Shaklee et al. 1990), had fixed differences (23 of one and 24 of the other), and other loci displayed parallel frequency differences. Seeb noted that the two groups of specimens she examined differed in the extent of pigmentation and suborbital spination. With a single exception, the loci of each type met Hardy–Weinberg expecta-

tions for genotypic frequencies, whereas the sample as a whole did not. Seeb suggested that the two allozyme forms might warrant consideration as separate species.

A third allozyme study undertaken to analyze the population-genetic structure of rougheye rockfish also observed two strongly differentiated types of rockfish (Hawkins et al. 1997). In this study's much larger sample size (about 700), there were no fixed differences but there was substantial departure from Hardy–Weinberg expectations, with a surplus of homozygotes and two types that could be separated by principal components analysis. The genetically different types had distinct but overlapping distributions, which led the authors to speculate that oceanographic currents isolate two large Gulf of Alaska populations.

In a preliminary study of population-genetic structure in Gulf of Alaska and Bering Sea rougheye rockfish, we also observed two divergent genetic forms of rougheye rockfish, which we describe here. We observed strong, but concordant, genetic differences for both mitochondrial DNA (mtDNA) and at microsatellite locus markers. Microsatellite alleles and mtDNA haplotypes are transmitted independently by two different modes. Microsatellites are Mendelian, diploid traits whereas the mitochondrial genome is inherited clonally along matrilineal lines (Hutchinson et al. 1974) and does not undergo recombination (Gyllenstein et al. 1985, 1991). Consequently, each mtDNA haplotype carries its mutational history and the number of mutational differences between two haplotypes reflects the elapsed time since they diverged.

Gametic disequilibrium at diploid loci and correlation of mtDNA haplotypes and nuclear allele frequencies can provide evidence of reproductive isolation. Fixed differences for both marker types would reflect an advanced stage of lineage sorting and argue that such genetic divergence is a consequence of speciation, especially in species that have overlapping spatial distributions. Here we evaluate the genetic compositions of rougheye rockfish sampled from much of their Alaskan and northeastern Pacific ranges to determine whether there is evidence that fish currently identified and managed as *S. aleutianus* include two distinct species. We also contrasted the genetic compositions of the two *S. aleutianus* species with that of the shorttraker rockfish, its presumed closest relative (Kendall 2000).

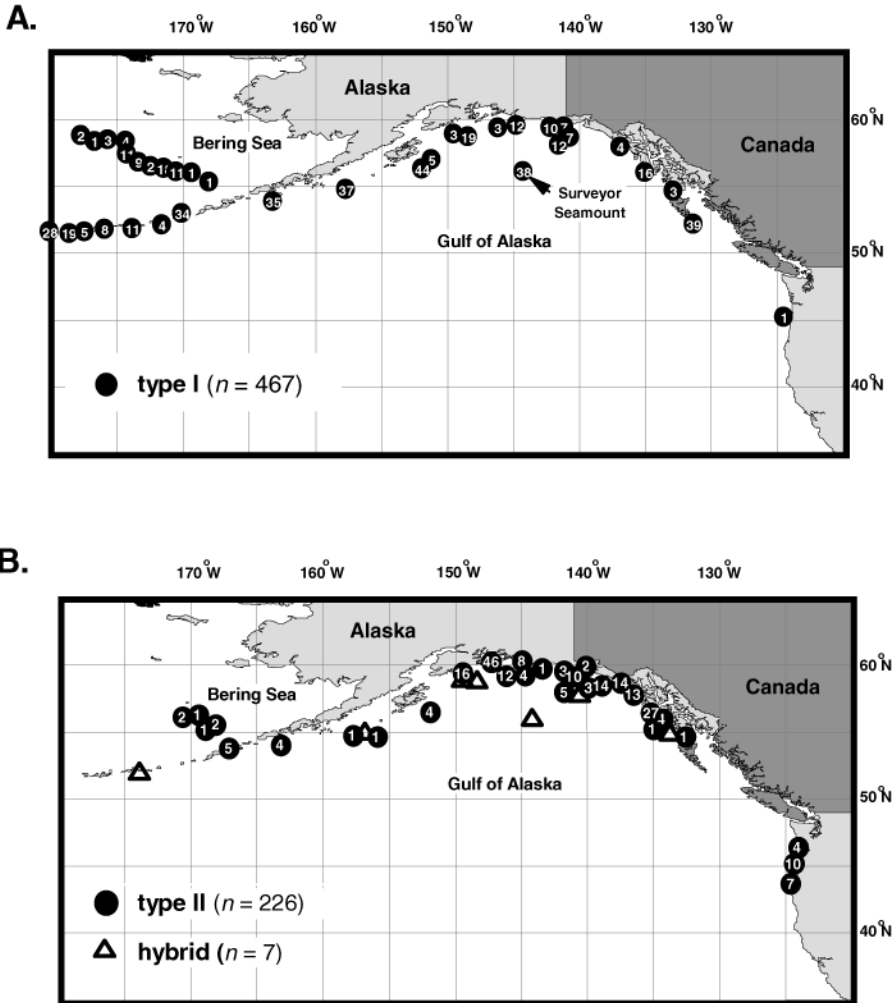


FIGURE 1.—Locations of rougheye rockfish surveyed for mtDNA and microsatellite variation. Panel (A) shows the locations of type I fish and panel (B) the locations of type II fish (circles) and presumed hybrids (triangles). The numbers within the circles are the sample sizes from each site; each triangle represents a single fish. Sites include all collections taken within 50 km.

Methods

Samples.—Rougheye rockfish were collected for genetic analysis by trawl and longline during stock assessment surveys conducted between 1993 and 2000 by the U.S. National Marine Fisheries Service, the Canadian Department of Fisheries and Oceans, and the Alaska Department of Fish and Game. The 84 sites at which they were collected extended along the Pacific Rim from the Oregon coast (46°16'55"N, 124°19'05"W) to the western edge of the Aleutian Islands (52°29'20" N, 179°58'48"E) as well as along the continental slope in the Bering Sea and from the Surveyor Seamount (Figure 1A). A sample of heart or muscle tissue

from each individual was preserved in a solution of 20% dimethyl sulfoxide and 80% 0.25-M ethylenediaminetetraacetic acid (EDTA) at pH 8, saturated with NaCl (Seutin et al. 1991). Total cellular DNA was isolated using Puregene DNA isolation kits.

Mitochondrial DNA analysis.—In a preliminary survey, we screened a subsample of collections that were geographically far apart to obtain data for constructing an mtDNA haplotype tree. That initial screening surveyed restriction site variation in three mtDNA regions: (1) the *ND5/ND6* region, which begins in the leucyl (CUN) tRNA and extends through the NADH-dehydrogenase subunit-

5 and NADH-dehydrogenase subunit-6 genes into the glutamyl tRNA gene; (2) the *ND3/ND4* region, which begins in the glycyl tRNA gene and spans the NADH-dehydrogenase subunit-3, arginyl tRNA, NADH-dehydrogenase subunit-4 L and NADH dehydrogenase subunit-4 genes, extending into the histidyl tRNA gene; and (3) the *12S/16S* region, which extends from near the phenylalanyl tRNA end of the *12S* rRNA gene through the valyl tRNA gene to near the leucyl tRNA end of the *16S* rRNA gene. The three mtDNA regions were amplified by polymerase chain reaction (PCR) from total genomic DNA using primers and methods described in Gharrett et al. (2001a, 2001b). Sub-samples of the amplified DNA were digested with the following restriction endonucleases: *Ase* I, *Ban* II, *Bst*N I, *Bst*U I, *Cfo* I, *Dde* I, *Hind* II, *Hinf* I, *Mbo* I, *Msp* I, *Nla* IV, *Rsa* I, *Sau*96 I, *Sty* I, and *Taq* I.

Based on the results of the preliminary survey, we conducted a second survey that used fewer restriction enzymes but a larger number of fish. The enzymes used for the second survey were *Bst*N I, *Bst*U I, *Cfo* I, *Dde* I, *Hind* II, *Hinf* I, *Mbo* I, *Msp* I, *Rsa* I, and *Sty* I. The first two surveys allowed us to identify the mtDNA regions and restriction sites that were the primary determinants of the haplotype genealogy, and we resolved most of the haplotypes observed in the first two surveys for application in a third survey that had larger numbers of collections and sample sizes. The discriminating mtDNA region and site combinations used for the third, more extensive screening were *Hinf* I in the *12S/16S* region; *Bst*N I, *Dde* I, and *Hinf* I in the *ND3/ND4* region; and *Bst*N I, *Mbo* I, and *Rsa* I in the *ND5/ND6* region.

Restriction fragments produced by restriction endonuclease digestion were separated by electrophoresis through 1.5% agarose (a mixture composed of one part Ultra Pure agarose and two parts Synergel) in 0.5× TBE buffer (TBE is 90 mM tris-boric acid and 2 mM EDTA, pH 8.3), and the fragments in the gels were stained with ethidium bromide and digitally photographed on an ultraviolet light transilluminator. One-kilobase and 100-base-pair (bp) ladders served as references to estimate restriction fragment sizes. In the preliminary survey, fragments of digested samples that could not be adequately resolved using agarose gels were separated in a 7% polyacrylamide gel (29:1 acrylamide: bisacrylamide) in 1× TBE using a mixture of 25-bp and 100-bp ladders (BRL Gibco, Grand Island, New York) as molecular weight markers to estimate restriction fragment sizes.

Those gels were stained with SYBR Green 1 Nucleic Acid Gel Stain (Molecular Probes, Eugene Oregon).

Microsatellite analyses.—We analyzed eight microsatellite loci (μ *Sma* 3, μ *Sma* 5, μ *Sma* 6, μ *Sma* 7, μ *Sma* 11 [Wimberger et al. 1999], μ *SR7-2*, μ *SR7-7*, and μ *SR7-25* [M. Westerman, National Marine Fisheries Service, Southwest Fisheries Science Center, La Jolla, California, unpublished data; GenBank accession numbers AF269054, AF269055, and AF269056]) to quantify rougheye rockfish variation at nuclear loci.

The microsatellite sequences were PCR-amplified in 10- μ L reactions containing 1 μ L of 10× reaction buffer (Promega, Madison, Wisconsin), 1.25 mM of each deoxynucleotide triphosphate, 1.875 mM MgCl₂, 0.35 μ M forward primer, 0.4 μ M reverse primer, 0.04 M forward labeled primer, 0.05 μ L (about 2 units) of *Taq* polymerase, and about 1 μ L template DNA. The amplification regime was 95°C for 3.75 min, followed by 1 cycle of 94°C for 1.5 min, 58°C for 0.5 min, and 72°C for 0.75 min; 30 cycles of 94°C for 0.5 min, 58°C for 0.5 min, and 72°C for 0.75 min; an elongation cycle at 68°C for 1 min; and cooling to 4°C. Amplified, labeled fragments were analyzed with a LICOR sequencer using allele ladders prepared for the species and loci or a 50–350-bp LI-COR standard. Alleles were scored using LI-COR software.

Data analysis.—A minimum-spanning tree for mtDNA haplotypes was constructed from the haplotypes observed in the preliminary survey of mtDNA variation with version 4.001 of the program Network (Bandelt et al. 1995; available at www.fluxus-engineering.com). Because the preliminary survey examined the largest number of sites, it was used for estimates of divergence and as the foundation for the subsequent surveys, which used fewer restriction endonucleases. New haplotypes observed in the later surveys were positioned on the original tree. In some cases, additional enzymes were used to eliminate ambiguities. Only eight (singleton) haplotypes observed in the two initial surveys were undetectable with the reduced suite of restriction enzymes and mtDNA regions.

At diploid (nuclear) loci, mixtures of genetically divergent populations can result in apparent departures from the genotypic frequencies predicted for randomly mating organisms (Hardy–Weinberg equilibrium) and gametic disequilibrium among loci. We tested microsatellite genotypic distributions for conformance to Hardy–Weinberg expectations using GENEPOP 3.3 (Raymond and Rous-

set 1995); locus pairs were tested for gametic equilibrium with Arlequin 2.000 (Schneider et al. 2000). An excess of homozygotes will yield a significantly positive inbreeding coefficient (F_{is} ; Weir and Cockerham 1984), which was estimated with GENEPOP 3.3. Genetic divergence between alleles or genotypes was also tested using GENEPOP 3.3.

The program Structure (version 2; Pritchard et al. 2000) sorts individuals in a mixture into a specified number of contributing populations (k) by minimizing the Hardy–Weinberg and gametic disequilibrium. Analyses can be compared using likelihood values estimated for each input k . Starting with $k = 1$, the likelihood increases precipitously when the most likely number of contributors is reached and plateaus at higher values of k . We used STRUCTURE to analyze our microsatellite data to provide information on the number of contributing sources, to assign individuals to the most likely source among contributing populations, and to identify possible hybrids. The parameters used to run the program were 100,000 dememorization steps and 500,000 iterations of the program for each value of k tested (1–4). The program was run assuming the existence of an admixture, and fitting the model took into account three possible generations of hybridization. STRUCTURE also estimates the probability of hybrid ancestry. We compared fish assigned to groups by STRUCTURE with mtDNA haplotype groups to identify potential hybrids.

Nucleotide divergences (\hat{d} ; Nei and Tajima 1983; Nei 1987; Nei and Miller 1990) and the average number of sites and nucleotides surveyed were estimated with REAP (McElroy et al. 1990). Pairwise fixation coefficients (F_{ST} s) were estimated for the two rougheye rockfish species and shortraker rockfish from the mtDNA variation (ϕ -statistics; Excoffier et al. 1992) and the microsatellite variation (θ -statistics; Weir and Cockerham 1984) using Arlequin 2.000 (Schneider et al. 2000), which conducted 20,000 permutations to estimate significance. Microsatellite data for shortraker rockfish were taken from a previous study (Matala et al. 2004); our shortraker rockfish mtDNA data are unpublished.

Results

Mitochondrial DNA Analysis

Restriction site analysis was conducted on 39 rougheye rockfish to obtain detailed information on the general structure of a haplotype tree. The

fish analyzed included 20 individuals from Southeast Alaska and 19 from the western half of the Aleutian chain in order to sample the geographic range broadly. Digests of 15 restriction endonucleases were used to obtain restriction site information from each of the *ND3/ND4*, *ND5/ND6*, and *12S/16S* mtDNA regions. The survey detected 197 different restriction sites, which included 813.33 nucleotides. A haplotype tree constructed from the restriction site data had 15 composite haplotypes resulting from 21 variable restriction sites. The haplotypes clustered into two groups, one around haplotype A and the other around haplotype B (Figure 2A). Haplotypes A and B were separated by six restriction site differences. Only cluster A fish were observed in the western samples, but fish from both clusters were observed in the eastern samples. We noted that microsatellite variation at the μ *Sma* 6 locus was limited to the *183/183 and *177/177 homozygotes. Only the *183/183 genotype was observed in fish of mitochondrial cluster B and only *177/177 genotypes were observed in cluster A.

A second survey of restriction site variation in the *ND3/ND4*, *ND5/ND6*, and *12S/16S* regions was conducted using 10 restriction endonucleases on DNA amplified from an additional 81 fish. The additional fish increased total sample sizes from the western Aleutian Islands ($n = 61$) and the eastern Gulf of Alaska, including Kodiak Island ($n = 59$). The restriction sites cut by the 10 restriction endonucleases included four of the six sites separating haplotypes A and B (Figure 2A). Haplotypes C, E, O, and G, which were observed in the preliminary survey, could not be resolved using these restriction endonucleases. The second survey confirmed the distinctness of the cluster A and B haplotypes and resolved an intermediate group of haplotypes centered on haplotype b (Figure 2B). The mitochondrial haplotypes remained strongly correlated with μ *Sma* 6 genotypes: the cluster B mtDNA haplotypes were homozygous for the *183 allele except for a single *185/181 heterozygote, and cluster A haplotypes were homozygous for the μ *Sma* 6*177 allele except for a single *183/183 homozygote. The four newly observed haplotype b individuals were homozygous for the μ *Sma* 6*177 allele.

The haplotype tree obtained in the second analysis was used to choose seven enzyme-by-mtDNA region combinations that revealed the restriction sites that defined the general topology and resolved most of the haplotypes. In that analysis, 49 composite haplotypes were observed from 48 variable

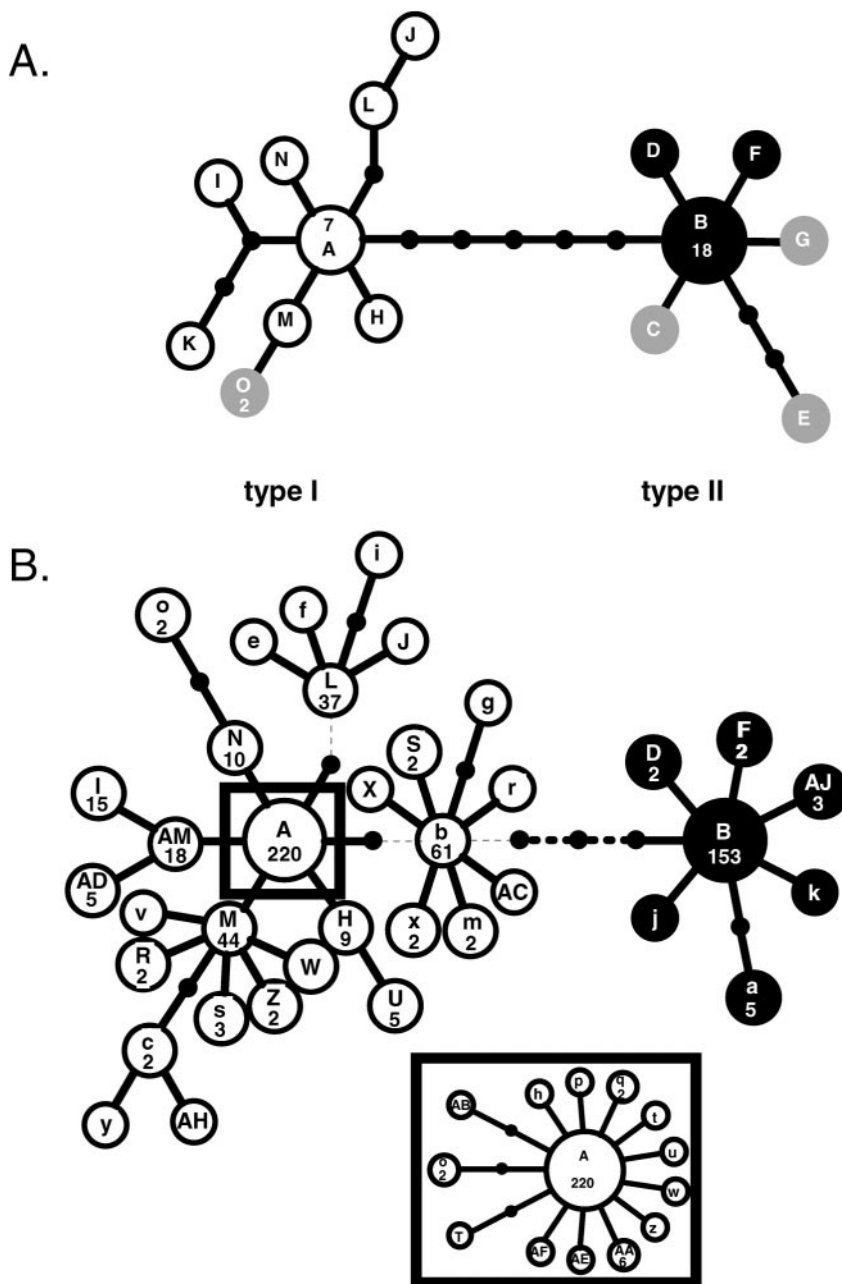


FIGURE 2.—Panel (A) presents a minimum-spanning haplotype tree (NETW4001; Bandelt et al. 1995) based on restriction site differences in the *ND3/ND4*, *ND5/ND6*, and *12S/16S* mtDNA regions for 15 restriction endonucleases. The numbers in circles representing haplotypes are the numbers of those haplotypes observed in this preliminary survey. Type I haplotypes corresponded to μSma 6*177/177 and type II haplotypes to μSma 6*183/183 haplotypes. Panel (B) presents a minimum-spanning haplotype tree based on restriction site variation using seven enzyme–mtDNA region combinations, which recognize sites that represent the major divergences in the topology and most of the haplotypes observed in two preliminary surveys. The fine-dotted lines indicate site differences that were observed in the first preliminary survey (panel A) but that were possible to detect in this survey. Heavily dotted lines represent sites screened in the second survey (not shown), but not in this survey. Haplotypes represented by circles filled with gray in panel A could not be detected in this survey. The inset is an expansion that includes additional haplotypes connected simply to haplotype A.

restriction sites (corresponding to 206.2 nucleotides) among the 637 fish analyzed for mtDNA variation (Tables A.1, A.2). In the haplotype tree resulting from this analysis, separation of cluster A haplotypes from the newly observed haplotype b group was reduced to a single restriction site and cluster B haplotypes differed by a single restriction site on the opposite side of the haplotype b group (Figure 2B) because the other defining sites were not analyzed.

Microsatellite Analysis

Eight microsatellite loci were surveyed for variability in 698 rougheye rockfish (Table A.3). The number of alleles observed at each locus ranged from 4 to 39 and averaged 21. All loci except μSma 5 had genotypic frequencies that departed substantially from Hardy–Weinberg expectations (Table 1); all departures were the result of too many homozygotes. In addition, except for four locus pairs, severe gametic disequilibrium was observed for all pairs of loci (Table 1), even after correction for multiple testing.

The survey of rougheye rockfish microsatellite variation revealed that most fish were homozygous at μSma 6 for either the *177 or *183 allele. The exceptions were two heterozygotes, *177/183, as well as two *181/183, one *181/185, and three *185/183 heterozygotes in a total of 698 fish. Both homozygous types were captured in some of the hauls from the northeastern Gulf of Alaska. If these samples of rougheye rockfish were from a single panmictic population, one would expect to see many more heterozygotes (more than 300 if the entire sample were from a panmictic population) between the *177 allele and the *183 (or *181 and *185) alleles.

We obtained both mtDNA and microsatellite data for 633 fish. All of the 165 haplotype cluster B fish carried only the *183, *181, and *185 alleles, except for a single *183/177 heterozygote. Four *183/183 homozygotes and one *183/177 heterozygote were observed in the sample of 468 haplotype cluster A and cluster b fish, which were otherwise fixed for the *177 allele.

Resolution of Genetically Distinct Sources of Rougheye Rockfish

The combination of strong departure from Hardy–Weinberg and gametic equilibria and the nearly perfect correlation between μSma 6 genotypes and mtDNA haplotypes suggested a mixture of two (or more) genetically distinct types. Consequently, we used the program STRUCTURE (Pritchard et

al. 2000) both to estimate the number of genetically distinct rougheye rockfish sources that had contributed to our sample and to assign individuals to the sources. The mtDNA haplotype data were reduced to three pools: the haplotype A cluster, the haplotype B cluster, and the haplotype b cluster (Figure 2B). We made a priori assignments of the source of each individual based on its mtDNA type. The program STRUCTURE evaluates such assignments in terms of the probability that each fish came from its assigned group or had ancestors from the other group. In the simulations, the natural logarithm of the probability of the data given k strongly increased from $k = 1$ [$\log_e(P) = -19,499.8$] to $k = 2$ [$\log_e(P) = -16,859.8$]. There was relatively little increase for $k = 3$ [$\log_e(P) = -16,875.9$] and a small decrease for $k = 4$ [$\log_e(P) = -16,861.8$], which were within the suggested guidelines (Pritchard et al. 2000). The two groups generated by STRUCTURE were separated nearly perfectly by Sma 6*177/177 homozygotes (which we will call type I) and μSma 6*183/183 homozygotes (plus *181 and *185 alleles, which we will call type II). These differences followed closely the mtDNA haplotype differences; haplotype clusters A and b corresponded to type I and haplotype cluster B corresponded to type II. Fitting the model to three or four sources resulted in the assignment of very few fish to the third and fourth sources.

Seven fish had nonconcordant mtDNA haplotype and STRUCTURE assignments. A single haplotype A– μSma 6*177/177 individual was assigned to type II based on genotypes at other microsatellite loci. In addition, STRUCTURE assigned three μSma 6*183/183 fish that had haplotype A to type II and suggested that a fourth haplotype A– μSma 6*183/183 fish may have had type I ancestry ($P = 0.758$). The program STRUCTURE indicated that the heterozygous (*183/177) fish that possessed haplotype B had type I ancestry based on its microsatellite genotypes and that the haplotype A–*183/177 fish may have had type II ancestry ($P = 0.477$).

The seven possibly hybrid fish were removed and the type I and type II data were analyzed separately using STRUCTURE. Each analysis identified a single source population, which supports the conclusion that k is probably two for the rougheye rockfish data set and that haplotype A and b fish are not differentiated with respect to microsatellite genotypes. In addition, there was no simple and obvious population structure within the two types.

Because the assignments STRUCTURE makes

TABLE 1.—Probability of Hardy–Weinberg equilibrium (on the diagonal) and gametic equilibrium for pairs of loci (below the diagonal) for all rougheye rockfish and type I and II (see text) fish. Although no correction was made for multiple testing in the table values, the adjusted α (for an overall α of 0.05) for the tests of Hardy–Weinberg and gametic equilibrium are 0.00639 and 0.00244, respectively; significant entries are in bold italics.

Micro-satellite	$\mu Sma\ 3$	$\mu Sma\ 5$	$\mu Sma\ 6$	$\mu Sma\ 7$	$\mu Sma\ 11$	$\mu Sr7-2$	$\mu Sr7-7$	$\mu Sr7-25$
Total								
$\mu Sma\ 3$	<i><10⁻⁴</i>							
$\mu Sma\ 5$	0.137	0.636						
$\mu Sma\ 6$	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>					
$\mu Sma\ 7$	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>				
$\mu Sma\ 11$	<i><10⁻⁴</i>	0.001	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>			
$\mu Sr7-2$	<i><10⁻⁴</i>	0.335	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	0.002		
$\mu Sr7-7$	0.001	0.001	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	
$\mu Sr7-25$	0.006	<i><10⁻³</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>	<i><10⁻⁴</i>
Type I								
$\mu Sma\ 3$	<i><10⁻⁴</i>							
$\mu Sma\ 5$	0.502	0.929						
$\mu Sma\ 6$	1	1	1					
$\mu Sma\ 7$	0.147	0.116	1	0.006				
$\mu Sma\ 11$	0.253	0.497	1	0.015	0.001			
$\mu Sr7-2$	0.473	0.922	1	0.631	0.004	0.317		
$\mu Sr7-7$	0.498	0.040	1	0.002	0.030	0.966	0.158	
$\mu Sr7-25$	0.880	0.013	1	0.025	0.108	0.335	0.083	0.072
Type II								
$\mu Sma\ 3$	<i><10⁻⁴</i>							
$\mu Sma\ 5$	0.945	0.495						
$\mu Sma\ 6$	0.868	0.252	0.047					
$\mu Sma\ 7$	0.822	0.515	1	0.098				
$\mu Sma\ 11$	0.493	0.773	0.079	0.376	<i><10⁻⁴</i>			
$\mu Sr7-2$	0.092	0.444	0.868	0.471	0.894	0.166		
$\mu Sr7-7$	0.624	0.785	0.551	0.420	0.153	0.077	0.018	
$\mu Sr7-25$	0.482	0.084	0.009	0.597	0.159	0.460	0.370	0.824

are based on reductions of Hardy–Weinberg and gametic disequilibria, the resulting type I and II data sets should exhibit reduced disequilibria relative to that observed in the combined data set (Table 1). Disequilibria were, in fact, much lower within the type I and type II data sets (Table 1). After adjustment was made for multiple testing, gametic disequilibrium between loci was eliminated except between the locus pair $\mu Sma\ 7$ and $\mu Sr7-7$ in type I data (Table 1) and departure from Hardy–Weinberg expectations was reduced in both type I and II data sets at all loci that were significant in the test of the total data. Hardy–Weinberg departures were eliminated at many of the loci. The extent of departure from Hardy–Weinberg equilibrium at $\mu Sma\ 3$ and $\mu Sma\ 11$ was much greater than we have seen resulting from population mixtures and probably indicates the existence of nonpriming (null) alleles at those loci.

Examination of the type I and II data sets shows that the mtDNA haplotype and $\mu Sma\ 6$ differences are not the only indication of genetic divergence between the two types of rougheye rockfish. The distributions of allele frequencies also differ

strongly at all eight loci ($P < 10^{-5}$), and the differences at $\mu Sma\ 7$, $Sma\ 11$, $\mu Sr7-2$, $\mu Sr7-7$, and $\mu Sr7-25$ are nearly diagnostic (Figure 3). Divergences in genotypic frequencies at $\mu Sma\ 3$, $\mu Sma\ 7$, and $\mu Sma\ 11$ were also significant ($P < 10^{-5}$).

Type I rougheye rockfish were predominant in western waters but extended throughout the range sampled (Figure 1). In contrast, there were no type II fish in the western Aleutian Island and western Bering Sea collections and only scattered observations in the eastern Bering Sea and Aleutian Islands. Type II fish were most prevalent in the central and eastern Gulf of Alaska (Figure 1).

Pairwise Comparisons

The mtDNA nucleotide divergence (\hat{d}) estimated between type I and type II fish from the preliminary survey, which included the largest sample of restriction sites, averaged 0.0063 substitutions per nucleotide, about three times the estimates of average within-cluster divergences ($\hat{d} = 0.0023$, 0.0018, and 0.0024 for type I and type II rougheye rockfish and shorttraker rockfish, respectively). In contrast, the average divergence between type I

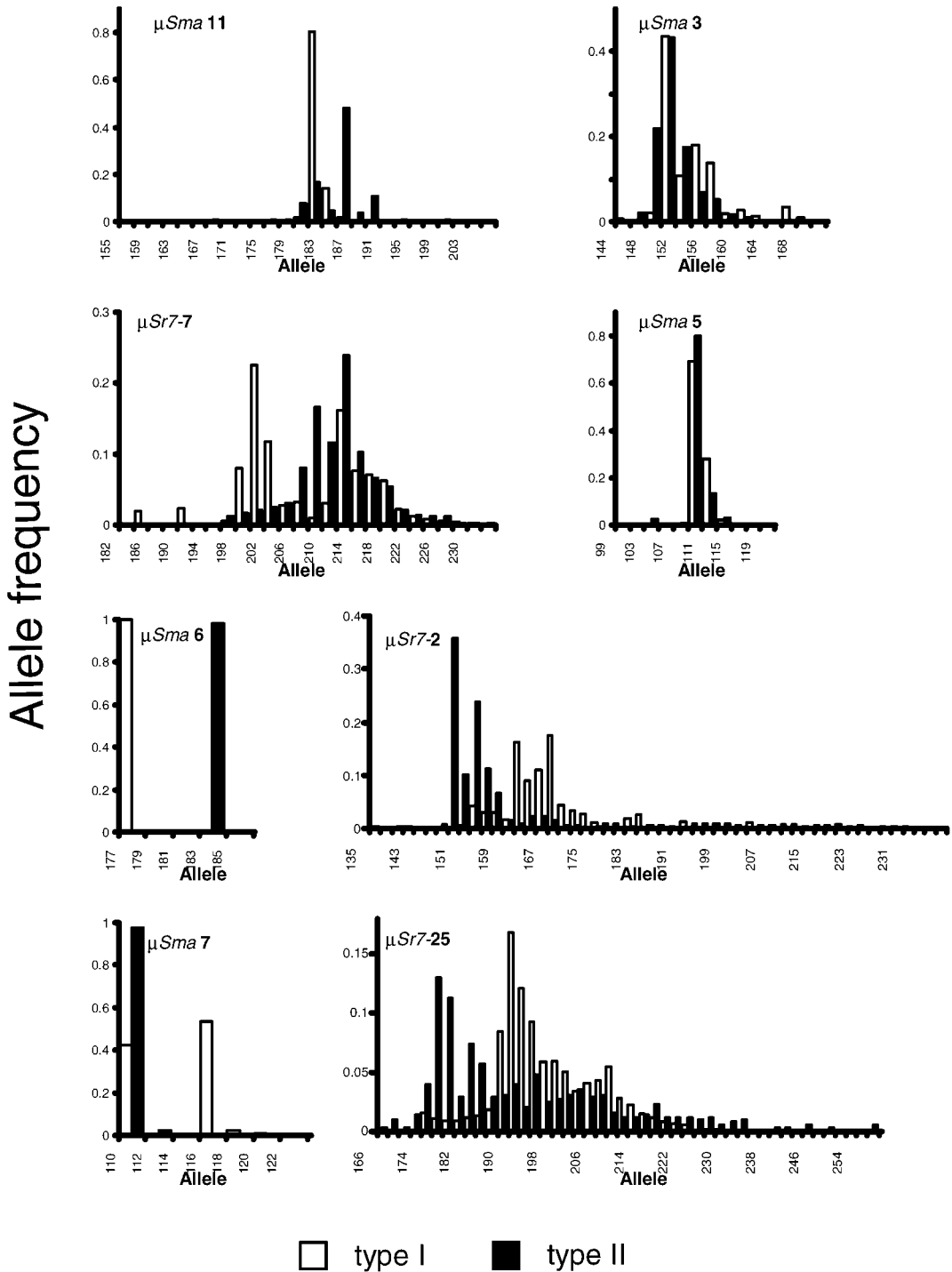


FIGURE 3.—Distributions of allele frequencies for rougheye rockfish types I and II at eight microsatellite loci. The frequencies reflect the total observed for 225 type II (filled bars) and 467 type I (unfilled bars) fish analyzed from collections ranging from Oregon to the Aleutian Islands.

TABLE 2.—Pairwise ϕ -statistics (Excoffier et al. 1992; below the diagonal) and θ -statistics (Weir and Cockerham 1984; above the diagonal) for type I and type II rougheye rockfish (see text) and shortraker rockfish. All significance levels (based on 20,000 permutations in Arlequin 2.000 [Schneider et al. 2000]) are less than 0.0001.

Fish	Type I	Type II	Shortraker rockfish
Type I		0.301	0.310
Type II	0.839		0.422
Shortraker rockfish	0.958	0.970	

rougheye rockfish and shortraker rockfish (surveyed with the same enzymes in the same mtDNA regions) was 0.0233 and the average divergence between type II rougheye rockfish and shortraker rockfish was 0.0228, nearly three times higher than the divergence between types I and II.

Pairwise fixation coefficients for the mtDNA paralleled the results of nucleotide divergence (Table 2). The high ϕ -statistics for mtDNA reflect stronger divergence between shortraker rockfish and the two rougheye rockfish species. In contrast, the θ -statistics for the microsatellite data suggest that type I and type II rougheye rockfish are more divergent than type II rougheye rockfish and shortraker rockfish.

Discussion

Genetic Divergence of Type I and II Rougheye Rockfish

The strong genetic divergence of both mitochondrial and nuclear genes and the observation of very few obvious heterozygotes, coupled with the broad but incomplete overlap in geographic distributions, strongly indicate the existence of two rougheye rockfish species. The differences at four restriction sites observed between the distinct mtDNA haplotype lineages of the two types of rougheye rockfish exceeds the divergence we observed between several pairs of closely related rockfish species (Li et al., unpublished).

For example, some haplotypes of species within each of the three following groups cannot be distinguished by restriction site differences (for the same enzymes and mtDNA regions we used for rougheye rockfish): gopher rockfish *S. carnatus* and black-and-yellow rockfish *S. chrysomelas*, the trio of dusky rockfish *S. ciliatus*, northern rockfish *S. polyspinis*, and darkblotched rockfish *S. crameri*, and the trio of harlequin rockfish *S. variegatus*, pygmy rockfish *S. wilsoni*, and Puget Sound rockfish *S. emphaeus*. This suggests that type I and type

II rougheye rockfish diverged earlier than these other groups of similar rockfish species.

The coincidence of mtDNA haplotype and μSma 6 genotypes indicates that lineage sorting is complete or nearly complete for both. The other microsatellite loci have not completed lineage sorting. Although the allele frequencies differ significantly at μSma 3 and μSma 5, they are similar, whereas allele frequency profiles differ substantially at μSma 7, μSma 11, $\mu Sr7$ -2, $\mu Sr7$ -7, and $\mu Sr7$ -25 (Figure 3).

An estimate of the time since divergence of the two rougheye rockfish types might provide clues about the causes of divergence. Although no generally accepted mitochondrial (molecular) clock is available for rockfish and our data include only a portion of the mitochondrial genome, we can make a crude estimate by applying a rate of 1% nucleotide divergence per million years. Smith (1992) suggested that rate as an upper limit for salmonids. Using that value, the divergence between types I and II rougheye rockfish ($d = 0.0063$) probably occurred more than 630,000 years ago. In contrast, the divergence of rougheye rockfish from shortraker rockfish probably occurred more than 2.3 million years ago. Using this clock, the average intraspecific divergence is about 200,000 years or more, that is, about two glacial cycles (Porter 1989; Petit et al. 1999).

Another approach to estimating divergence time is based on the times required for lineage sorting. From that approach, we can also make some deductions about their recent (in geological time) demographic history. Mitochondrial haplotype lineage sorting should be substantially complete after $4N_{ef}$ generations, where N_{ef} is the effective number of females (Avice et al. 1984; Neigel and Avice 1986). Rougheye rockfish can live and reproduce for more than 100 years; they mature at about 20 years (Love et al. 2002), and the average generation time may exceed 30 years. A rough estimate of the current number of rougheye rockfish in the Gulf of Alaska and Bering Sea is between 4 and 10×10^7 , that is, $2-5 \times 10^7$ females. Unless the effective number of rougheye rockfish females dropped to about 1,000 during the late Pleistocene, it is likely that the divergence of types I and II occurred well before the Wisconsin Epoch because $4 \times 1,000 (N_{ef}) \times 30$ (years per generation) equals 120,000 years. Longer generation spans or a larger N_{ef} would increase the divergence time. In addition, both lineages would have had to experience severe population declines or one lineage would still carry substantial numbers of haplotypes ob-

served in the other lineage. The star patterns in the haplotype tree are consistent with recent population expansion, but there is more structure, particularly in the haplotype A cluster, than would be expected from a recent expansion from such a small number of predecessors. Divergence of diploid loci would require a longer time, and the microsatellite loci show varying degrees of divergence. The results of the two approaches to assigning a time frame to the divergence of types I and II roughey rockfish are consistent and indicate that the divergence probably took place between several hundred thousand and 1 million years ago. It is unlikely that the divergence resulted from one of the last two glaciations.

Use of shortraker rockfish as an outgroup exposes two problems in using outgroups that are too divergent from the taxa of interest. The extent of divergence between the two roughey rockfish types and shortraker rockfish was similar based on estimates of nucleotide divergence and paralleled those observed for the ϕ -statistics (indicating a homogeneous clock; Table 2). However, it was not possible to root unequivocally the roughey rockfish haplotype to the shortraker rockfish haplotypes because of critical homoplastic sites. The second problem arose in the estimates of θ -statistics from the microsatellite data. In that analysis, the type I roughey rockfish were about equally divergent from the type II fish and shortraker rockfish, whereas the type II fish were more divergent from the shortraker rockfish (Table 2). These results probably occurred because the shortraker and roughey rockfish are too divergent for microsatellite data to be useful in comparisons. A combination of a high mutation rate, numerous alleles, and the influences of random drift and the strong likelihood that common allele sizes arose independently appear to make microsatellites a poor source of data for phylogenetic comparisons of divergent taxa. Although quillback rockfish *S. maliger* and brown rockfish *S. auriculatus* appear to be appropriate outgroups for copper rockfish, those three species are much more closely related than roughey and shortraker rockfish (e.g., Rocha-Olivares et al. 1999a; Gharrett et al. 2001b) and appear to hybridize on occasion (Seeb 1998).

Although hybridization between closely related *Sebastes* species may not be unusual (Seeb 1998), there appeared to be relatively little hybridization between types I and II roughey rockfish. The single potential hybrid identified in collections from the far western Aleutians carried a cluster A mtDNA haplotype but had one or two microsat-

ellite alleles that were rare in type I fish and abundant in type II fish. Given that all the other fish in the western Aleutian Islands were type I fish and it was collected a substantial distance from type II fish, we think that individual is a type I fish. All other possible hybrids were collected in the eastern Gulf of Alaska. Four fish had cluster A haplotypes but type II allelic composition (including a μ *Sma* 6*183/183 genotype), and STRUCTURE assigned them to type II. If the contribution of the mtDNA haplotype is considered, it is more likely that these fish have a type I female ancestor or that lineage sorting is not quite complete for the mtDNA haplotype in type II fish. One of the two fish heterozygous at μ *Sma* 6 (*183/177) has a cluster A haplotype and the other had a cluster B haplotype. They are probably hybrids between types I and II roughey rockfish.

Conservation and Management Implications

Both types I and II roughey rockfish species appear to be sympatric in some areas of the Gulf of Alaska and Bering Sea but do not have coincident distributions. In particular, the observations that most of the samples from the western Aleutian Islands and Bering Sea are type I and many hauls throughout the range were entirely or predominantly one type or the other suggest either a historic vicariant or ecological basis for their divergence.

Roughey rockfish have been combined with shortraker rockfish for fisheries management and together they are caught incidentally in fisheries for other species, particularly Pacific ocean perch *S. alutus*, Atka mackerel *Pleurogrammus monopterygius*, Greenland halibut (market name, Greenland turbot) *Reinhardtius hippoglossoides*, Pacific cod *Gadus macrocephalus*, sablefish *Anoplopoma fimbria*, and Pacific halibut *Hippoglossus stenolepis*. Roughey rockfish are rarely able to survive the barotrauma that they experience in being brought to the surface from the depths they inhabit, so tagging studies cannot be used to study their population structure.

Populations of roughey rockfish are quite sensitive to overharvest and are slow to rebuild, as are populations of most long-lived species. Consequently, groundfish management pays careful attention to the abundance assessments, harvest levels, and in-season harvest trends of these desirable species. However, little is known about the life histories of most Alaskan rockfish species. In particular, the numerous species and morphological changes that occur during their early life histories

make them difficult to identify and study. The absence of knowledge about their biology and life histories introduces considerable uncertainty into conservation and management. If the two rough-eye rockfish species differ in life history traits, habitat preferences, or abundance, overharvest of one type is a risk, particularly where they overlap. Our inability to visually identify larval and juvenile rockfish impedes efforts to learn more about them.

It would be advantageous to identify phenotypic characters by which the fisheries community can distinguish between the two species of rough-eye rockfish. Two color phases were observed in the northeastern Gulf of Alaska (Seeb 1986) that correlated with distinct allozyme genotypes. In the fish that we collected and for which we noted coloration, all of the fish in a sample collected from the western Gulf of Alaska had dark coloration. A sample of fish collected near Yakutat included both dark and light phenotypes, although the color phases were not discrete and there were some intermediate fish. The correlation between coloration and mtDNA- μ Sma 6 genetic composition was imperfect, although type II fish usually had the light color phase. Larger sample sizes will be needed to address the possibility of coloration differences. Whether or not phenotypic or morphometric characters exist by which the species can be visually delineated, conservation and management actions require more knowledge about their biology and life histories.

Two crucial questions relating to the conservation and management of the rough-eye rockfish species are (1) what are the distributions of the two species and what factors determine those distributions? and (2) what is the geographic scale of their population structure? These questions precede questions about population dynamics characteristics, such as natural mortality, age at maturity, and growth. Ecological factors constitute one set of forces that may cause genetic divergence between the species. These factors may be related to choice of habitat and influences on spawning location and time as well as on migration and dispersal patterns. In looking at the distributions of the two rough-eye rockfish species, we note that samples collected within or near the more protected, possibly shallower inside waters (Prince William Sound and southeast Alaska) included more of type II fish. Also, five of the six Aleutian Island type II fish were collected from an embayment. One hypothesis that merits testing is that at some period of their life history the two species

are separated by either water masses or depth. Therefore, the species distribution and genetic variation of rough-eye rockfish collected within Prince William Sound, the inside waters of southeast Alaska and Cook Inlet, and the inside waters of British Columbia may provide some insight into the species' distributions.

The term "cryptic species" often evokes an image of a small or rare fish that has escaped notice by being reclusive. In the past, genetic differences have been used to detect cryptic species of fish. For example, the all-female atherinid, Texas silverside *Menidia clarkhubbsi*, studied by Echelle et al. (1983) is a cryptic species that is found in brackish waters with the inland silverside *M. beryllina* and the tidewater silverside *M. peninsulae*. The species was recognized and subsequently described because its allozyme profile differs from that of both of the other species with which it was observed. In addition, the observation of fixed differences at allozyme loci in Hawaiian bonefishes *Albula* spp. led to a thorough morphological examination in which two species possessing different morphotypes clearly corresponding to the two different genetic profiles were identified (Shaklee and Tamaru 1981). Rough-eye rockfish are abundant, have been known for more than a century, and have been harvested during much of that time. Little, however, is known about their early life history and the extent of their larval and adult dispersal. Recently two cryptic *Sebastes* species have been described. Two forms of dusky rockfish have been resolved from shape and coloration differences (*S. ciliatus* and *S. variabilis*; Orr and Blackburn 2004), and mtDNA analysis has detected cryptic rockfish species in the southern hemisphere (Rocha-Olivares et al. 1999b). Although we are surprised by the discovery of a second species "hiding" within a well-known taxon, perhaps we should not be given the limited extent of our knowledge about the biology of rough-eye rockfish. The addition of a new cryptic species to a management complex underscores the need to learn more about the distribution and biology of these and other groundfish species.

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References

- Avise, J. C., J. E. Neigel, and J. Arnold. 1984. Demographic influences on mitochondrial DNA lineage survivorship in animal populations. *Journal of Molecular Evolution* 20:99–105.
- Bandelt, H.-J., P. Forster, B. C. Sykes, and M. B. Richards. 1995. Mitochondrial portraits of human populations. *Genetics* 141:743–753.
- Barsukov, V. V. 1970. [Species composition of the genus *Sebastes* in the North Pacific and description of a new species.] *Doklady Akademii Nauk SSSR* 195: 994–997. (In Russian; English translation in *Proceedings of the Academy of Sciences USSR [Biology]* 195:760–763).
- Echelle, A. A., A. F. Echelle, and C. D. Crozier. 1983. Evolution of an all-female fish, *Menidia clarkhubbsi* (Atherinidae). *Evolution* 37:772–784.
- Evermann, B. W., and E. L. Goldsborough. 1907. The fishes of Alaska. U.S. Bureau of Fisheries. 26(1906):219–360.
- Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular inference from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479–491.
- Gharrett, A. J., A. K. Gray, and V. Brykov. 2001a. Phylogeographic analysis of mitochondrial DNA variation in Alaskan coho salmon, *Oncorhynchus kisutch*. *Fishery Bulletin* 99:528–544.
- Gharrett, A. J., A. K. Gray, and J. Heifetz. 2001b. Identification of rockfish (*Sebastes* spp.) by restriction site analysis of the mitochondrial ND-3/ND-4 and 12S/16S gene regions. *Fishery Bulletin* 99:49–62.
- Gómez, A., M. Serra, G. R. Carvalho, and D. H. Hunt. 2002. Speciation in ancient cryptic species complexes: evidence from molecular phylogeny of *Branchionus plicatilis* (Rotifera). *Evolution* 56: 1431–1444.
- Gyllensten, U., D. Wharton, A. Josefsson, and A. C. Wilson. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature (London)* 352:255–257.
- Gyllensten, U., D. Wharton, and A. C. Wilson. 1985. Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *Journal of Heredity* 76:321–324.
- Hawkins, S., J. Heifetz, J. Pohl, and R. Wilmot. 1997. Genetic population structure of rougheye rockfish (*Sebastes aleutianus*) inferred from allozyme variation. Pages 1–10 in *National Marine Fisheries Service, Alaska Fishery Science Quarterly Report* July–August–September 1997, Seattle, Washington.
- Hutchinson, C. A., III, J. C. Newbold, S. S. Potter, and M. H. Edgell. 1974. Maternal inheritance of mammalian mitochondrial DNA. *Nature (London)* 251: 536–538.
- Jordan, D. S. 1885. A catalogue of the fishes known to inhabit the waters of North America, north of the Tropic of Cancer, with notes on species discovered in 1883 and 1884. Report to the U.S. Fish Commission 13:789–973. [Report published in 1887; page 108 in separate publication in 1885.]
- Jordan, D. S. 1899. The fur seals and fur-seal islands of the North Pacific Ocean, part III. Special papers relating to the fur seal and to the natural history of the Pribilof Islands. U.S. Government Printing Office, Washington, D.C.
- Jordan, D. S., and B. W. Evermann. 1898. The fishes of north and middle America: a descriptive catalog of the species of fishlike vertebrates found in the waters of North America, north of the Isthmus of Panama, part II. U.S. National Museum Bulletin 47: 1861–1958.
- Kendall, A. W. 2000. An historical review of *Sebastes* taxonomy and systematics. *Marine Fisheries Review* 62:1–23.
- Love, M. S., M. Yoklavich, and L. Thorsteinson. 2002. The rockfishes of the northeast Pacific. University of California Press, Berkeley.
- Matsubara, K. 1934. [Studies on the scorpaenoid fishes of Japan, I. Descriptions of one new genus and five new species.] *Journal of the Imperial Fisheries Institute of Tokyo* 30:199–210. (In Japanese.)
- Matala, A. P., A. K. Gray, J. Heifetz, and A. J. Gharrett. 2004. Population structure of Alaskan shortraker rockfish, *Sebastes borealis*, inferred from microsatellite variation. *Environmental Biology of Fishes* 69:201–210.
- McElroy, D. M., P. Moran, E. Bermingham, and I. Kornfield. 1990. REAP: an integrated environment for the manipulation and phylogenetic analysis of restriction data. *Journal of Heredity* 83:157–158.
- McGovern, T. M., and M. E. Hellberg. 2003. Cryptic species, cryptic endosymbionts, and geographical variation in chemical defences in the bryozoan *Bugula neritina*. *Molecular Ecology* 12:1207–1215.
- Nei, M. 1987. *Molecular evolutionary genetics*. Columbia University Press, New York.
- Nei, M., and J. C. Miller. 1990. A simple method for estimating average number of nucleotide substitutions within and between populations from restriction data. *Genetics* 125:873–879.
- Nei, M., and F. Tajima. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. *Genetics* 105:207–217.
- Neigel, J., and J. C. Avise. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. Pages 513–534 in E. Nevo and S. Karlin, editors. *Evolutionary process and theory*. Academic Press, New York.
- Orr, J. W., and J. E. Blackburn. 2004. The dusky rockfishes (Teleostei: Scorpaeniformes) of the North Pa-

- cific Ocean: resurrection of *Sebastes variabilis* (Pallas, 1814) and a redescription of *Sebastes ciliatus* (Tilesius, 1813). Fishery Bulletin 102:328–348.
- Petit, J. R., and 18 coauthors. 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature (London) 399:420–436.
- Porter, S. C. 1989. Some geological implications of average quaternary glacial conditions. Quaternary Research 32:245–261.
- Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.
- Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–249.
- Richardson, J. 1844. Ichthyology, part 1. Pages 51–70 in R. B. Hinds, editor. The zoology of the voyage of H. M. S. *Sulphur*, under the command of Captain Sir Edward Belcher, R.N., C.B., F.R.G.S., etc., during the years 1836–42, No. 5. Smith, Elder, and Company, London.
- Rocha-Olivares, A., R. H. Rosenblatt, and R. D. Vetter. 1999a. Evolution of a mitochondrial cytochrome *b* gene sequence in the species-rich genus *Sebastes* (Teleostei, Scorpaenidae) and its utility in testing the monophyly of the subgenus *Sebastomus*. Molecular Phylogenetics and Evolution 11:426–440.
- Rocha-Olivares, A., R. H. Rosenblatt, and R. D. Vetter. 1999b. Cryptic species of rockfishes (*Sebastes*: Scorpaenidae) in the southern hemisphere inferred from mitochondrial lineages. Journal of Heredity 90:404–411.
- Schneider, S., J.-M. Kueffer, D. Roessleri, and L. Excoffier. 2000. Arlequin version 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- Seeb, L. W. 1986. Biochemical systematics and evolution of the scorpaenid genus *Sebastes*. Doctoral dissertation. University of Washington, Seattle.
- Seeb, L. W. 1998. Gene flow and introgression within and among three species of rockfishes, *Sebastes auriculatus*, *S. caurinus*, and *S. maliger*. Journal of Heredity 89:393–403.
- Seutin, G., N. N. White, and P. T. Boag. 1991. Preservation of avian blood and tissue samples for DNA analysis. Canadian Journal of Zoology 69:82–90.
- Shaklee, J. B., F. W. Allendorf, D. C. Morizot, and G. S. Whitt. 1990. Gene nomenclature for protein-coding loci in fish. Transactions of the American Fisheries Society 119:2–15.
- Shaklee, J. B., and C. S. Tamaru. 1981. Biochemical and morphological evolution of Hawaiian bone fishes (*Albula*). Systematic Zoology 30:125–146.
- Smith, G. R. 1992. Introgression in fishes: significance for paleontology, cladistics, and evolutionary rates. Systematic Biology 41:41–57.
- Tsuyuki, H., E. Roberts, R. H. Lowes, W. Hadaway, and S. J. Westrheim. 1968. Contribution of protein electrophoresis to rockfish (Scorpaenidae) systematics. Journal of the Fisheries Research Board of Canada 25:2477–2501.
- Tsuyuki, H., and S. J. Westrheim. 1970. Analyses of the *Sebastes aleutianus*–*S. melanostomus* complex, and description of a new scorpaenid species, *Sebastes caenaematicus*, in the Northwest Pacific Ocean. Journal of the Fisheries Research Board of Canada 27:2233–2254.
- Weir, B. S., and C. C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.
- Westrheim, S. J., and H. Tsuyuki. 1972. Synonymy of *Sebastes caenaematicus* with *Sebastes borealis*, and range extension record. Journal of the Fisheries Research Board of Canada 29:606–607.
- Wimberger, P., J. Burr, A. Gray, A. Lopez, and P. Bentzen. 1999. Isolation and characterization of twelve microsatellite loci for rockfish (*Sebastes*). Marine Biotechnology 1:311–315.

Appendix: Detailed Genetic Results for Roughey Rockfish

TABLE A.1.—Composite mtDNA haplotypes of roughey rockfish. Fragment patterns are given in Table A.2; a minimum-spanning tree is shown in Figure 2B.

Haplotype	12S/16S			ND3/ND4		ND5/ND6		
	Hinf I	BstNI	Dde I	Hinf I	BstNI	Mbo I	Rsa I	
A	b	b	b	a	a	a	a	
B (C,E,G)	b	b	b	a	a	b	b	
D	b	b	b	d	a	b	b	
F	b	b	c	a	a	b	b	
H	a	b	b	a	a	a	a	
I	b	b	e	a	a	a	a	
J	b	b	a	a	a	d	a	
K	b	b	d	a	a	a	a	
L	b	b	b	a	a	d	a	
M (O)	b	b	b	a	b	a	a	
N	b	a	b	a	a	a	a	
R	b	b	b	a	b	a	c	
S	b	b	b	a	d	a	b	
T	b	b	b	a	f	a	a	

TABLE A.1.—Continued.

Haplotype	<i>12S/16S</i>		<i>ND3/ND4</i>			<i>ND5/ND6</i>		
	<i>Hinf</i> I	<i>Bst</i> N I	<i>Dde</i> I	<i>Hinf</i> I	<i>Bst</i> N I	<i>Mbo</i> I	<i>Rsa</i> I	
U	a	b	b	a	a	a	d	
W	b	b	a	a	b	a	a	
X	b	b	d	a	a	a	b	
Z	b	c	b	a	b	a	a	
a	b	b	b	b	a	b	b	
b	b	b	b	a	a	a	b	
c	b	b	b	a	e	a	a	
e	b	b	b	b	a	d	a	
f	b	b	b	c	a	d	a	
g	b	b	b	a	a	d	g	
h	b	b	b	a	a	e	a	
i	b	b	g	a	a	g	a	
j	b	b	b	a	a	b	f	
k	b	b	b	a	a	b	g	
m	a	b	b	a	a	a	b	
o	b	a	b	b	a	a	a	
p	b	b	b	a	a	a	c	
q	b	b	b	a	a	a	e	
r	b	b	b	a	a	a	i	
s	b	b	b	a	c	a	a	
t	b	b	b	a	g	a	a	
u	b	b	b	a	h	a	a	
v	b	b	b	a	j	a	a	
w	b	b	b	a	k	a	a	
x	b	b	b	b	a	a	b	
y	b	b	b	b	e	a	a	
z	b	b	b	c	a	a	a	
AA	b	b	c	a	a	a	a	
AB	b	e	a	a	a	a	a	
AC	b	f	b	a	a	a	b	
AD	b	b	d	a	a	c	a	
AE	b	b	b	a	a	c	a	
AF	b	b	f	a	a	a	a	
AJ	b	b	b	a	f	b	b	

TABLE A.2.—Restriction fragments (base pairs) for enzyme-by-region combinations used to construct the haplotype tree in Figure 2A. The binary code is the presence/absence of inferred restriction sites; invariant sites are sites observed in all individuals.

mtDNA region enzyme haplotype	<i>ND3/ND4</i>								
	<i>Bst</i> NI					<i>Hinf</i> I			
	a	b	c	e	f	a	b	c	d
Fragment sizes	717	717	717	717	1,102	1,261	1,261	1,738	1,041
	865	385	385	385					220
		480	433	480	480	477	477		477
			47			494	364	494	494
	631	631	631	611	631		130		
				20		153	153	153	153
	112	112	112	112	112				
	60	60	60	60	60				
Sum	2,385	2,385	2,385	2,385	2,385	2,385	2,385	2,385	2,385
Binary Code	1000	1100	1110	1101	0100	010	011	000	110
Invariant sites	3	3	3	3	3	2	2	2	2

TABLE A.2.—Extended.

mtDNA region enzyme haplotype	<i>ND5/ND6</i>						
	<i>Mbo</i> I						<i>Rsa</i> I
	a	b	c	d	e	g	a
Fragment sizes	1,575	845	1,575	800	1,575	800	860
		730		775		775	
	330	330	330	330	720	720	
	390	390	680	390			253
	290	290		290	290	290	204
							26
							245
							150
							750
Sum	2,585	2,585	2,585	2,585	2,585	2,585	2,488
Binary code	0011	0111	0010	1011	0001	1001	000111
Invariant sites	1	1	1	1	1	1	3

TABLE A.2.—Extended.

mtDNA region enzyme haplotype	<i>ND5/ND6</i>					
	<i>Bst</i> NI					
	a	b	c	d	e	f
Fragment sizes	1,250	670	670	1,250	670	1,250
		580	385		580	
		195				
	140	140	140	200	140	140
	60	60	60		60	60
	690	690	690	690	470	690
					220	
	360	360	360	360	360	320
						40
Sum	2,500	2,500	2,500	2,500	2,500	2,500
Binary code	000110010	010110010	011110010	000010010	010110110	000110011
Invariant sites	1	1	1	1	1	1

TABLE A.2.—Extended.

MtDNA region enzyme haplotype	<i>ND3/ND4</i>						
	<i>Dde</i> I						
	a	b	c	d	e	f	g
Fragment sizes	1,011	1,011	1,011	1,011	1,011	831	1,011
						180	
	462	462	462	462	462	462	432
							30
	311	190	190	190	190	190	190
		121	121	121	121	121	121
	254	254	216	254	216	254	254
			38		38		
	195	195	195	195	195	195	195
	103	103	103	71	71	103	103
				32	32		
	38	38	38	38	38	38	38
	11	11	11	11	11	11	11
Sum	2,385	2,385	2,385	2,385	2,385	2,385	2,385
Binary code	00000	00100	00110	00101	00111	10100	01100
Invariant sites	6	6	6	6	6	6	6



TABLE A.2.—Extended.

mtDNA region enzyme haplotype	<i>ND5/ND6</i>						
	<i>Rsa</i> I						
	b	c	d	e	f	g	i
Fragment sizes	350	860	565	860	350	350	350
	510				510	510	260
			295				250
	253	457	253	253	457	253	253
	204		204	204		230	204
	26	26	26	26	26		26
	245	245	245	395	245	245	245
	150	150	150		150	150	150
	750	750	750	750	750	750	750
Sum	2,488	2,488	2,488	2,488	2,488	2,488	2,488
Binary code	100111	000011	010111	000110	100011	100101	101111
Invariant sites	3	3	3	3	3	3	3

TABLE A.2.—Extended.

mtDNA region enzyme haplotype	<i>ND5/ND6</i>				<i>12S/16S</i>	
	<i>Bst</i> N I				<i>Hinf</i> I	
	g	h	j	k	a	b
Fragment sizes	1,250	1,250	505	1,250	1,291	1,291
			165		1,139	803
			580			336
	140	140	140	140		
	750	60	60	60		
		1,050	690	395		
				295		
	360		360	360		
Sum	2,500	2,500	2,500	2,500	2,430	2,430
Binary code	000100010	000110000	110110010	000111010	0	1
Invariant sites	1	1	1	1	1	1

TABLE A.3.—Frequencies of microsatellite alleles for 692 type I and type II roughey rockfish. The two types were determined using the program STRUCTURE (Pritchard et al. 2000) and do not include eight fish that STRUCTURE identified as possible hybrids. Alleles are designated by estimates of their size (base pairs). The sample size is n , H_{Obs} is the observed heterozygosity, H_{Exp} is the expected heterozygosity, and F_{is} is an estimate of the inbreeding coefficient (Weir and Cockerham 1984).

Microsatellite and statistic	Allele	Type I	Type II	
$\mu Sma\ 3$	144	0.008	0	
	146	0.003	0.021	
	148	0.020	0.219	
	150	0.435	0.437	
	152	0.109	0.173	
	154	0.180	0.069	
	156	0.138	0.054	
	158	0.018	0.017	
	160	0.028	0.010	
	162	0.013	0	
	164	0.003	0	
	166	0.035	0	
	168	0.009	0	
	170	0.003	0	
	n		467	225
	H_{Obs}		0.620	0.518
	H_{Exp}		0.743	0.723
F_{is}		0.165	0.284	
$\mu Sma\ 5$	99	0	0.004	
	103	0	0.026	
	107	0	0.008	
	109	0.693	0.802	
	111	0.279	0.134	
	113	0.021	0.028	
	115	0.005	0	
	121	0.002	0	
	n		467	225
	H_{Obs}		0.442	0.335
	H_{Exp}		0.440	0.332
F_{is}		-0.0074	-0.008	
$\mu Sma\ 6$	177	1.000	0	
	181	0	0.008	
	183	0	0.982	
	185	0	0.010	
	n		466	224
H_{Obs}		1.000	0.027	
H_{Exp}		1.000	0.036	
F_{is}		0	0.135	
$\mu Sma\ 7$	110	0.425	0.977	
	112	0.006	0.023	
	116	0.535	0	
	118	0.025	0	
	120	0.007	0	
	122	0.002	0	
	n		466	225
H_{Obs}		0.488	0.036	
H_{Exp}		0.531	0.048	
F_{is}		0.081	0.183	
$\mu Sma\ 11$	155	0.002	0	
	157	0	0.006	
	161	0	0.006	
	163	0.002	0	
	165	0.002	0.003	

TABLE A.3.—Continued.

Microsatellite and statistic	Allele	Type I	Type II
	167	0.002	0.010
	171	0	0.003
	173	0.003	0.003
	175	0.006	0.008
	177	0	0.010
	179	0.018	0.077
	181	0.807	0.166
	183	0.140	0.045
	185	0.015	0.484
	187	0.004	0.038
	189	0	0.106
	191	0	0.003
	193	0	0.008
	195	0.002	0.006
	199	0	0.008
	201	0	0.003
	203	0	0.003
	205	0	0.003
n		467	224
H_{Obs}		0.330	0.552
H_{Exp}		0.324	0.708
F_{is}		-0.026	0.221
$\mu Sr7-2$	135	0	0.003
	137	0.002	0
	139	0.002	0.003
	141	0.003	0.003
	143	0.002	0
	147	0.002	0.008
	149	0	0.359
	151	0.006	0.102
	153	0.042	0.237
	155	0.029	0.113
	157	0.030	0.067
	159	0.017	0.014
	161	0.164	0.010
	163	0.090	0.023
	165	0.110	0.023
	167	0.174	0.014
	169	0.043	0.006
	171	0.034	0.006
	173	0.028	0
175	0.011	0.003	
177	0.010	0	
179	0.009	0	
181	0.018	0.003	
183	0.025	0	
185	0.006	0	
187	0.006	0	
189	0.004	0	
191	0.013	0	
193	0.009	0	
195	0.010	0	
197	0.010	0	
199	0.009	0	
201	0.006	0	
203	0.011	0	
205	0.005	0	
207	0.006	0	
209	0.007	0	
211	0.007	0	
213	0.003	0	
215	0.006	0	
217	0.005	0	

TABLE A.3.—Continued.

Microsatellite and statistic	Allele	Type I	Type II
	219	0.008	0
	221	0.003	0
	223	0.005	0
	225	0.002	0
	227	0.004	0
	229	0.003	0
	231	0.003	0
	233	0.002	0
	237	0.002	0
<i>n</i>		465	224
<i>H</i> _{Obs}		0.905	0.771
<i>H</i> _{Exp}		0.910	0.780
<i>F</i> _{is}		0.005	0.011
μ Sr7-7	182	0.002	0
	184	0.019	0
	188	0.002	0
	190	0.024	0
	194	0.002	0
	196	0.006	0.012
	198	0.080	0.017
	200	0.227	0.021
	202	0.118	0.025
	204	0.027	0.032
	206	0.032	0.080
	208	0.010	0.168
	210	0.031	0.115
	212	0.161	0.238
	214	0.076	0.102
	216	0.070	0.067
	218	0.062	0.054
	220	0.022	0.021
	222	0.012	0.014
	224	0.008	0.012
	226	0.005	0.012
	228	0.004	0.003
	230	0.003	0.003
	232	0	0.003
<i>n</i>		467	223
<i>H</i> _{Obs}		0.891	0.647
<i>H</i> _{Exp}		0.883	0.874
<i>F</i> _{is}		-0.009	0.030
μ Sr7-25	166	0	0.003
	168	0	0.010
	170	0	0.003
	172	0	0.014
	174	0.016	0.040
	176	0.011	0.129
	178	0.009	0.112
	180	0.009	0.029
	182	0.012	0.074
	184	0.013	0.057
	186	0.017	0.029
	188	0.085	0.031
	190	0.168	0.040
	192	0.121	0.020
	194	0.092	0.048
	196	0.059	0.025
	198	0.061	0.027
	200	0.051	0.033
	202	0.034	0.035
	204	0.041	0.029
	206	0.043	0.031
	208	0.054	0.016

TABLE A.3.—Continued.

Microsatellite and statistic	Allele	Type I	Type II
	210	0.028	0.012
	212	0.022	0.012
	214	0.015	0.014
	216	0.012	0.023
	218	0.008	0.012
	220	0.008	0.012
	222	0.006	0.014
	224	0	0.010
	226	0.002	0.012
	228	0.002	0.006
	230	0	0.008
	232	0	0.010
	236	0	0.003
	240	0	0.003
	244	0	0.006
	248	0	0.003
	256	0	0.006
<i>n</i>		466	223
<i>H</i> _{Obs}		0.877	0.932
<i>H</i> _{Exp}		0.921	0.944
<i>F</i> _{is}		0.047	0.012