Reader:

Please note the following erratum for this article.

Page 56, line 5

Read "*S. melanops*" instead of "*S. maliger*" so that the sentence begins

"Similarly, the haplotypes of *S. melanops* and *S. flavidus* (subgenus *Sebastosomus*) were tightly clustered…".

Identification of rockfish (*Sebastes* spp.) by restriction site analysis of the mitochondrial ND-3/ND-4 and 12S/16S rRNA gene regions

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Abstract—Species-specific restriction site variation in the 12S/16S rRNA and ND-3/ND-4 mtDNA regions was used to distinguish among 15 rockfish species of the genus *Sebastes* common to the waters of Alaska. Intraspecific variation exhibited by eight of the species (based on five individuals of each species) did not obscure the interspecific variation, except possibly between *S. zacentrus* and *S. variegatus*. Intraspecific nucleotide diversity averaged 0.0024 substitutions per nucleotide, whereas interspecific nucleotide divergence averaged 0.0249. In contrast, the average nucleotide divergences between *Sebastes* and two other scorpaenid species, *Helicolenus hilgendorfi* and *Sebastolobus alascanus*, were 0.0805 and 0.1073, respectively. Cladistic and phenetic analyses supported some, but not all, of the subgenera assignments of *Sebastes*. A scheme for distinguishing among the species studied was presented. Restriction sites of 10 restriction endonucleases were mapped in the two PCR-amplified mtDNA regions by using double digests. In all, we detected 153 sites corresponding to 640 (13.5%) of the 4815 nucleotides in the two regions combined. The ND-3/ND-4 region exhibited substantially more intraspecific, interspecific, and intergeneric variation than the 12S/16S rRNA region.

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The species-rich genus of *Sebastes* rockfish has challenged both fisheries scientists and ichthyologists since they were first described from Alaskan waters by Tilesius (*S. ciliatus*; 1813, cited in Eschmeyer, 1998) and Richardson (*S. caurinus*; 1845). Both the large number of species, about 100 worldwide (Ishida, 1984; Kendall, 1991), and the metamorphoses that occur in larval and juvenile fish produce a confusing number of forms. The diversity of species and forms combine to limit our knowledge of the biology, including life histories, of rockfishes. To date, identification to species is not possible for many larvae and juveniles (e.g. Kendall, 1991; Moser, 1996), and distinguishing between some adult species may be difficult. For example, adult *S. variegatus* is similar to *S. zacentrus* and adult *S. mystinus*, *S. melanops*, and *S. ciliatus* are often misidentified (Love[1]). The inability to identify species constrains surveys of larval abundance and, consequently, ecological studies that are important for conservation and management of rockfish and other species. In addition, the questions facing biologists and fishery managers require tools that can resolve intraspecific population (stock) structure, as well as methods for identifying species.

The size of the genus and the paucity of information about some of the species have also contributed to a chaotic history of their systematics and many aspects of the phylogeny have not been resolved (Kendall[2] and reviewed in Cramer [1895] and Phillips [1957]. Cuvier (1829, cited in Eschmeyer,1998) first described the genus *Sebastes* for northern Atlantic specimens. The number of genera recognized for the species presently placed in *Sebastes* has expanded and contracted repeatedly, reaching a maximum of 15 (Jordan et al., 1930) and now these genera are generally considered subgenera. When combined with five northwestern Pacific Ocean (Matsubara, 1943) and one northern Atlantic Ocean subgenus, *Sebastes* comprises about 22 subgenera (Kendall, 1991).

Identification and systematics of fish depend largely on morphological characters; morphology alone, however, does not always provide sufficient criteria, especially for identification of larval and juvenile forms. Genetic information, obtained by using biochemical or molecular methods, has been used to address systematic problems. In some

[1] Love, M. 2000. Personal commun. Marine Sciences Institute, University of California, Santa Barbara, CA. 93106.

[2] Kendall, A.W. 2000. Personal commun. NMFS Alaska Fisheries Center, 7600 Sandpoint Way NE, Seattle, WA 98115.

instances, genetic differences can be used to differentiate between species that have overlapping morphologies. For example, cryptic species of southern Atlantic Ocean *Sebastes* species were recognized from mtDNA analysis (Rocha-Olivares et al., 1999a). Genera and many species of rockfish can be distinguished from protein electrophoresis differences (e.g. Tsuyuki et al., 1968; Johnson et al., 1972). More recently, allozyme data (Seeb, 1986) and mtDNA variation (Johns and Avise, 1998; Rocha-Olivares, 1998a; Seeb, 1998; Rocha-Olivares et al. 1999b, 1999c) have been used to address questions about the evolution and systematics of *Sebastes*. Genetic differences may provide the means for identifying rockfish larvae and juveniles that cannot be identified from their morphology (Seeb and Kendall, 1991). Recently, Rocha-Olivares (1998b) devised a PCR-based approach for identification of *Sebastes* species. The advantage of his approach is that it is fast. The disadvantage is that failed PCR reactions are part of the identification scheme. However, failed reactions can also result from poor quality DNA or intraspecific variation and lead to misidentification of the specimens. Intraspecific genetic variation can also provide information about population structure (e.g. Wishard et al., 1980; Seeb et al., 1988; Rocha-Olivares and Vetter, 1999).

Vertebrate mitochondrial DNA (mtDNA) is compact (about 16,500 base pairs) and has been completely sequenced in a variety of organisms including carp (*Cyprinus carpio*; Chang et al., 1994) and rainbow trout (*Oncorhynchus mykiss*; Zardoya et al., 1995). Because mitochondria are transmitted primarily through maternal genes (Gyllensten et al., 1991), mtDNA is haploid and clonally inherited (Meyer, 1993). Restriction fragment analyses of PCR-amplified regions of mtDNA provide a rapid and practical method for detecting nucleotide sequence variation in mtDNA between individuals or species. Sequence variation detected by restriction enzymes produces binary character-state data that can be used in phylogenetic analyses (e.g. Dowling et al., 1992). An advantage of restriction site surveys over sequencing is that they are practical for detecting variation in large sequence spans. The number of nucleotides screened in restriction site surveys depends on the number of restriction enzymes used and their match with the DNA sequence.

We have developed primers that can be used to PCR amplify regions of *Sebastes* mtDNA. The amplified regions provide material for addressing species identification and stock identification questions about rockfish. In addition, the haplotypes observed provide information for addressing systematic relationships among *Sebastes*.

Our objective in this study was to examine the potential that restriction fragment analyses of PCR-amplified mtDNA regions have for the study of rockfish biology. We asked the following specific questions: 1) Is there interspecific haplotype variation? 2) Is there intraspecific haplotype variation? 3) Does intraspecific variability compromise the use of mtDNA restriction fragments in species identification? 4) Can a simple strategy for identifying species be devised? 5) If there is interspecies variation, how do similarities between species correlate with (presumed) systematic relationships? To answer these questions, we conducted restriction site analyses on five individuals from each of 15 different *Sebastes* species common in Alaskan waters and mapped the sites using double digests to determine individual-based haplotypes. From these data, we examined intra- and inter-specific divergences and used both phenetic and cladistic procedures to examine relationships among the haplotypes. We also mapped the sites for shortspine thornyhead (*Sebastolobus alascanus*) and *Helicolenus hilfendorfi* to facilitate analysis. Finally, we developed a mtDNA restriction fragment-based strategy for identifying *Sebastes* species.

Materials and methods

Adult specimens of 15 different species of *Sebastes* rockfish and *Sebastolobus alascanus* were collected from the eastern Gulf of Alaska (Table 1). These species are the most abundant of the approximately 25 species reported in the region. In the field, species identification was confirmed by using the pictoral guide of Kramer and O'Connell (1988) and the key and descriptions in Hart (1973). H. Ida (Kitasato University, Sanriku, Japan) provided samples of *Helicolenus hilgendorfi* from Japanese coastal waters. Samples of heart tissue from each specimen were preserved in 95% ethanol or a solution of 20% dimethyl sulfoxide (DMSO), 0.25M ethylenediaminetetraacetic acid (EDTA) at pH 8 and saturated with NaCl (Seutin et al., 1991).

Total cellular DNA was isolated by phenol-chloroform extraction (Wallace, 1987) or with Puregene DNA^{TM} isolation kits (Gentra Systems, Inc., Minneapolis, MN). Two target regions were PCR-amplified from total cellular DNA with primers that we developed for coho salmon (*Oncorhynchus kisutch*) mtDNA studies. The ND3/ND4 region begins in the glycyl tRNA gene and spans the NADH-dehydrogenase subunit-3, arginyl tRNA, NADH-dehydrogenase subunit-4L, and NADH-dehydrogenase subunit-4 genes, ending in the histidyl tRNA gene. The 12S/16S region 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 (Table 2). From restriction digests, we estimated that the ND3/ND4 and 12S/16S regions comprised 2385 and 2430 base pairs (bp), respectively, as compared with 2331 and 2402, respectively, for *O. mykiss*. Target sequences were amplified by heating to 94°C for 5 min, followed by 30 cycles for 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C using Taq polymerase from Perkin Elmer (Norwalk, CT) according to manufacturer's directions. ND3/ND4 amplification required 3mM $MgCl_2$, whereas amplification of 12S/16S required 2mM $MgCl_2$.

Single digests of subsamples of the PCR-amplified mtDNA regions were made by using 10 restriction endonucleases. *Bst*U I, *Cfo* I, *Dde* I, *Hin*f I, *Mbo* I, *Msp* I, and *Rsa* I have 4-nucleotide recognition sites; *Bst*N I recognizes an ambiguous 5-nucleotide site; and *Hin*d II and *Sty* I recognize ambiguous 6-nucleotide sites. Digestions were carried out under conditions recommended by the manufacturers. Fragments were separated by electrophoresis through 1.5% agarose (a mixture composed of one part

Table 1

Rockfish and related species and subgenera of *Sebastes* spp. used in mitochondrial DNA haplotype comparisons. The number designates the species and the letter indicates the particular composite haplotype observed.

Designation	Common name	Species	Subgenus
1, a and b	Pacific ocean perch	*Sebastes alutus*	*Acutomentum*
2, a and b	rosethorn rockfish	*Sebastes helvomaculatus*	*Sebastomus*
3	quillback rockfish	*Sebastes maliger*	*Pteropodus*
4, a and b	redbanded rockfish	*Sebastes babcocki*	*Rosicola*
5, a and b	black rockfish	*Sebastes melanops*	*Sebastosomus*
6	yellowtail rockfish	*Sebastes flavidus*	*Sebastosomus*
7, a–d	sharpchin rockfish	*Sebastes zacentrus*	*Allosebastes*
8	harlequin rockfish	*Sebastes variegatus*	*Allosebastes*
9	redstripe rockfish	*Sebastes proriger*	*Allosebastes*
10, a and b	rougheye rockfish	*Sebastes aleutianus*	*Zalopyr*
11, a and b	yelloweye rockfish	*Sebastes ruberrimus*	*Sebastopyr*
12	shortraker rockfish	*Sebastes borealis*	*Zalopyr*
13	light dusky rockfish	*Sebastes ciliatus*	*Sebastosomus*
14	silvergray rockfish	*Sebastes brevispinis*	*Acutomentum*
15, a and b	copper rockfish	*Sebastes caurinus*	*Pteropodus*
16	helicolenus	*Helicolenus hilgendorfi*	
17, a–d	shortspine thornyhead	*Sebastolobus alascanus*	

Table 2

Primers used for polymerase chain reaction amplification of rockfish (*Sebastes*, *Helicolenus,* and *Sebastolobus* spp.) mtDNA regions. a = Thomas and Beckenbach (1989); b = Cronin et al. (1993); c = Gharrett[1]; d = Anderson et al. (1981); e = Anderson et al. (1982); f = Roe et al. (1985); g = Chang et al., 1994; h = Zardoya et al. (1995).

Region amplified	Sequence	Location in *O. mykiss*[h]	Source
ND3/ND4	5′ TAACGCGTATAAGTGACTTCCAA 3′	bp 10574–10596	from a (similar to b)
	5′ TTTTGGTTCCTAAGACCAATGGAT 3′	bp 12881–12904	from a and c (similar to b)
12S/16S	5′ AATTCAGCAGTGATAAACATT 3′	bp 1234–1254	consensus: d, e, f, g
	5′ AGATAGAAACTGACCTGGATT 3′	bp 3615–3635	consensus: d, e, f, g

[1] Gharrett, A. J. 2000. Unpubl. *Oncorhynchus kisutch* sequences. Fisheries Division, Univ. Alaska, Fairbanks, 11120 Glacier Hwy., Juneau, AK 99801.

Ultra Pure™ agarose [BRL Gibco, Grand Island, NY] and two parts Synergel™ [Diversified Biotech Inc., Boston, MA]) in 0.5×TBE buffer (TBE is 90 mM tris-boric acid, and 2 mM EDTA, pH 7.5). DNA in the gel was stained with ethidium bromide and photographed on an ultraviolet light transilluminator. Digests that produced small unresolvable fragments on agarose gels were subjected to electrophoresis on 8% polyacrylamide gels (29:1 acrylamide:bisacrylamide) in 2×TAE (TAE is 40 mM tris-acetic acid and 1 mM EDTA, pH 8.0). DNA in polyacrylamide was stained with SYBR Green 1 Nucleic Acid Stain™ (Molecular Probes, Eugene, OR). Molecular weight markers used to estimate restriction fragment sizes were 100 base pair (bp) or 25-bp ladders (BRL Gibco, Grand Island, NY). Restriction sites were mapped by using double digests. Double digests were examined both in agarose and polyacrylamide by using 100- and 25-bp ladders. Composite haplotypes for all 10 restriction enzymes and both mtDNA regions were determined for each individual.

Generalized (relaxed Dollo) parsimony trees (Swofford et al., 1996) were computed from shared restriction sites by a heuristic search with PAUP* 4.0 (Swofford, 1998), which assumed unordered states. Because the likelihood of the loss of a site is higher than the restoration of a lost site, we conducted analyses that assumed 1) no added cost, 2) twice the cost, and 3) four-times the cost for restoring a site. Multiple maximum parsimony trees from each analysis were combined to produce a majority consensus tree using PAUP* 4.0 (Swofford, 1998). A maximum-likelihood tree was estimated with the program RESTML in

PHYLIP 3.57c (Felsenstein[3]), assuming that all restriction sites were 4 bp long (PHYLIP, Felsenstein[3]). Nucleotide divergences (proportion of nucleotide substitutions) and their standard errors were estimated according to Nei and Tajima (1983), Nei (1987), and Nei and Miller (1990) by using REAP (McElroy et al., 1990).

Results

Restriction fragment patterns from double digests were used to construct restriction site maps for comparisons of species and detection of intraspecies variation (Appendix 1). The map includes 153 restriction sites, 36 of which were common in all haplotypes and 28 of which were cladistically uninformative because the presence or absence occurs only in a single haplotype. Many of the cladistically uninformative sites, however, were useful in species delineation. These data represent 153 restriction sites (79.3 on average) corresponding to 640 nucleotides (332.05 on average) per haplotype.

Among the 85 fish examined were 30 different composite haplotypes (Table 3); each species had haplotypes that were distinct from those of other species, although *S. variegatus* composite haplotype 8 differed at a single site from *S. zacentrus* composite haplotype 7c (Table 4). All other pairs of species differed by 5 or more sites. Intraspecific variation was observed in nine of the seventeen species even when only five specimens of each species were analyzed. The most variable species were *S. zacentrus* and *Sebastolobus alascanus,* each of which had four haplotypes. In the study, differences between haplotypes ranged from a single site difference or 0.0014 nucleotide substitutions per site to 65 restriction site differences and 0.120 nucleotide substitutions per site (Table 4). Nucleotide divergence within variable species averaged 0.0024 subsitutions (1.56 site changes), whereas divergences between *Sebastes* species averaged ten-fold higher, 0.0249 (15.4 site changes), ranging from 0.0015 (1 site change) to 0.0384 (25 site changes). Nucleotide divergences between *Sebastes* species and *Sebastolobus alascanus* averaged 0.1073 (59.2 site changes) and divergences between *Sebastes* species and *H. hilgendorfi* averaged 0.0805 (43.5 site changes).

Distribution of the variation between the two different mtDNA regions (ND3/ND4 and 12S/16S) reflects their rates of evolution. In the 12S/16S region, which is more conservative, 27 of 58 restriction sites were shared by all haplotypes. Nucleotide diversities between *Sebastes* species averaged 0.0094 nucleotide changes per nucleotide (a total of 3.29 sites differences in the region), divergences between *Sebastes* and *H. hilgendorfi* averaged 0.0641 (12.67 site differences), and divergences between *Sebastes* and *Sebastolobus alascanus* averaged 0.0561 (18.03 site differences). In contrast, in the ND3/ND4 region only 9 of 95 sites were common to all haplotypes; and nucleotide divergences between *Sebastes* species averaged 0.0471 (12.11 site differences) and divergences between *Sebastes* and *H. hilgendorfi* and between *Sebastes* and *Sebastolobus alascanus* averaged 0.1373 (31.75 site differences) and 0.1929 (40.93 site differences), respectively. The maximum likelihood and majority consensus tree for the 60 maximum parsimony trees that imposed a cost of two for regained restriction sites had identical topologies (Fig. 1). The topologies of parsimony trees, which had either no additional cost or a cost of four, were somewhat different. Several groups of species were present in all three parsimony topologies. The *S. zacentrus*-*S. variegatus* pair, mentioned above, and each of four species pairs—*S. melanops*-*S. flavidus*, *S. babcocki*-*S. helvomaculatus*, *S. proriger*-*S. brevispinis,* and *S. maliger*-*S. caurinus*—clustered tightly at subterminal nodes. A more interior cluster of species included *S. melanops*, *S. flavidus*, *S. babcocki*, and *S. helvomaculatus*. In addition, *S. maliger* and *S. caurinus* clustered separately from all other *Sebastes* species and the *Sebastes* species were distinct from *H. hilgendorfi* and *Sebastolobus alascanus*.

The mtDNA variation we observed among *Sebastes* species provides a tool for identifying species. From our data, numerous schemes could be devised that distinguish among the *Sebastes* species examined. We propose a simple scheme that minimizes the number of digests required and involves separation of restriction fragments from the ND3/ND4 PCR product on an agarose-Synergel™ gel using only four restriction enzymes. *Mbo* I digests produce 11 different haplotypes (haplotypes A–K; Figure 2A; Table 3); *S. alutus* (B), *S. melanops* (E), *S. babcocki* (G and H), *S. ruberrimus* (I), and *S. caurinus* (J) are species specific. If *Mbo* I haplotypes A (*S. helvomaculatus* or *S. flavidus*) or C (*S. maliger* or *S. caurinus*) are observed, digest the ND3/ND4 PCR product with *Hin*d II; *Hin*d II haplotype B is specific for *S. helvomaculatus* and *Hin*d II haplotype C is specific for *S. maliger* (Fig. 2B; Table 3). If *Mbo* I haplotypes F (*S. ciliatus* or *S. borealis*) or K (*S. aleutianus*, *S. proriger*, or *S. brevispinis*) are observed, digest the ND3/ND4 PCR product with *Bst*N I; *Bst*N I haplotype A is specific for *S. ciliatus* and *Bst*N I haplotype G is specific for *S. brevispinis* (Fig. 2C; Table 3). *Mbo* I and *Bst*N I haplotypes do not distinguish between *S. aleutianus* and *S. proriger*, but *Cfo* I haplotype B is specific for *S. aleutianus* (Fig. 2D, Table 3). The combined haplotype of *Mbo* I, *Hin*d II, *Bst*N I, and *Cfo* I can be used to identify *S. borealis* (KAFD) and *S. proriger* (FAFD) (Fig. 2). The single difference between *S. zacentrus* and *S. variegatus* is the presence of a 123-bp fragment in *Rsa* I digests of *S. zacentrus* (Table 2; Appendix 1).

This simple scheme takes advantage of unique single-site differences for several of the species. Although a neighbor-joining tree (Saitoh and Nei, 1987) appeared stable to intraspecific variation for increased sample sizes of three species (data not shown), a single site change that produces apparent convergence between taxa in our scheme is conceivable. Increased certainty can be achieved by conducting digests with all four enzymes. With this strategy there will be at least two site differences between every pair of species, except *S. proriger* and *S. brevispinis*, which

[3] Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.57c. Distributed by the author. Department of Genetics, Box 357360, Univ. Washington, Seattle, WA 98195-7360.

Table 3

Composite haplotypes for *Sebastes* spp., *Helicolenus hilgendorfi*, and *Sebastolobus alascanus* in the 12S/16S and ND3/ND4 mtDNA regions. The species codes are listed in Table 2. The haplotype codes refer to haplotypes in Table 4. Five individuals were analyzed for each species. Where intraspecific variation was observed, alternative haplotypes are presented.

	12S/16S haplotypes									
Species	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
1a	A	A	A	D	A	B	C	B	A	A
1b	A	A	A	D	A	A	C	B	A	A
2a	A	A	A	C	A	B	B	A	D	A
2b	A	A	A	C	A	B	C	A	D	A
3	A	A	A	D	A	B	A	A	B	A
4a	A	A	A	C	A	B	C	B	B	A
4b	A	A	A	C	A	B	C	B	B	A
5a	A	A	A	D	A	B	C	B	D	A
5B	A	A	A	D	A	B	C	B	D	A
6	A	A	A	A	A	B	C	B	D	A
7a	A	A	A	B	A	B	C	A	C	A
7b	A	A	A	B	A	B	C	A	C	A
7c	A	A	A	B	A	B	C	A	C	A
7d	A	A	A	B	A	B	C	A	C	A
8	A	A	A	B	A	B	C	A	C	A
9	A	A	A	D	A	B	C	A	C	A
10a	A	A	A	D	A	B	C	B	A	A
10b	A	A	A	D	A	B	C	B	A	A
11a	A	A	A	D	A	C	C	A	A	A
11b	A	A	A	D	A	C	C	A	A	A
12	B	A	A	A	A	B	C	A	C	A
13	A	A	A	D	A	B	C	A	A	A
14	A	A	A	D	A	A	C	A	C	A
15a	A	A	A	D	A	B	A	A	A	A
15b	A	A	A	D	A	B	A	A	A	A
16	D	B	B	F	B	B	F	B	A	A
17a	C	B	B	E	A	D	E	B	E	B
17b	C	B	B	E	A	D	E	B	E	B
17c	C	B	B	E	A	D	E	B	E	B
17d	C	B	B	E	A	D	E	B	E	B

	ND3/ND4 haplotypes									
Species	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
1a	B	C	A	J	A	A	B	B	C	C
1b	B	C	A	J	A	A	B	B	C	C
2a	F	C	D	K	B	A	A	B	C	C
2b	F	C	D	K	B	A	A	B	E	C
3	F	B	D	L	C	D	C	C	B	A
4a	F	C	D	G	A	A	G	B	B	C
4b	F	C	D	F	A	A	H	B	B	C
5a	D	C	D	F	A	B	E	A	A	C
5B	D	C	D	F	A	B	E	B	A	C
6	E	C	D	H	A	C	A	B	A	C
7a	F	A	D	E	A	A	D	B	C	C
7b	F	A	D	A	A	A	D	B	C	C
7c	F	C	D	E	A	A	D	B	C	C

continued

Table 3 (continued)

Species	ND3/ND4 haplotypes									
	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hin*f I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
7d	F	A	D	E	A	A	D	B	C	B
8	F	C	D	E	A	A	D	B	B	C
9	F	C	D	E	A	A	K	E	B	C
10a	F	C	B	N	A	E	K	D	D	C
10b	F	C	B	N	A	F	K	D	D	C
11a	C	C	A	B	A	D	I	B	D	C
11b	C	C	D	B	A	D	I	B	D	C
12	F	C	D	D	A	A	F	B	B	C
13	A	D	A	M	A	A	F	B	F	C
14	G	C	D	C	A	A	K	D	B	D
15a	F	B	C	L	A	G	C	C	B	A
15b	F	B	C	L	A	G	J	C	B	A
16	J	G	F	Q	A	A	N	G	H	A
17a	H	E	E	O	A	H	M	F	G	F
17b	I	E	E	O	A	H	M	F	G	F
17c	H	F	E	O	A	H	M	F	G	E
17d	H	E	E	P	A	H	M	F	G	F

can be resolved by using *Msp* I, and *S. zacentrus* and *S. variegatus* (see above). We do not recommend using *Dde* I because it has many sites, often produces small fragments requiring both agarose and polyacrylamide gels for resolution, and is, therefore, time consuming to analyze. However, the restriction patterns of *Dde* I are nearly species specific.

Discussion

Sufficient interspecies restriction site variation occurred in the ND3/ND4 and 12S/16S mtDNA regions in *Sebastolobus alascanus*, *Helicolenus hilgendorfi*, and 15 *Sebastes* species to distinguish among them. Intraspecific variation was observed in nine of the seventeen species, but it did not interfere with our ability to distinguish between species. We used the interspecific variation to devise a strategy to identify the species we studied. Intraspecific variability can serve as a basis for stock identification.

A broader survey, particularly for *S. zacentrus* and *S. variegatus*, might reveal overlaps in haplotype compositions that compromise the ability to distinguish between some species pairs. This would be most likely if there were gene flow between the species or if the species had recently diverged. Otherwise, extending the analysis to other mtDNA regions and additional restriction endonucleases should increase resolution. Of course, additional intraspecific variation has the potential to obscure the topology of trees. To test this possibility, we examined trees that included the additional haplotypes observed in samples of 40 to 126 individuals each from *S. caurinus* (n=79), *S. aleutianus* (n=126), and *S. borealis* (n=40) (data not shown). The additional haplotypes (5, 13, and 5, respectively) increased the number of branches at the tip of the species limbs but did not influence or obscure relationships with other species. We are currently investigating the population structure of *S. aleutianus*, *S. borealis*, S. *alutus*, *S. caurinus*, and *Sebastolobus alascanus* by using mtDNA restriction site variation.

Because of the similarity of many *Sebastes* species, there is a chance that very similar species can be misidentified. In fact, a young dusky rockfish (*S. ciliatus*) and a young yellowtail rockfish (*S. flavidus*) were misidentified in the field as black rockfish (*S. melanops*) prior to our mtDNA analysis. Also, it is possible that closely related species may hybridize (e.g. Seeb, 1998). Because hybrids carry only the maternal lineage and because only the maternal contributor can be identified, mtDNA analysis is a poor tool for identifying hybrids.

In addition to providing a tool that can distinguish among a variety of rockfish species, the data appear to provide criteria that may prove useful in unraveling some questions about rockfish systematics. Both outgroups are distinct from *Sebastes*; *H. hilgendorfi* is more closely related than *Sebastolobus alascanus*. The 15 *Sebastes* species studied include eight subgenera, five of which were represented by two or more species. Despite the uncertainty in some of the subgenus assignments,[2] our analyses of mtDNA restriction sites show some concordance with subgeneric assignments. Unfortunately, the only recently reviewed subgenus is *Sebastomus* (Chen, 1971), for which we have only a single representative (*S. helvomaculatus*). A phylogeny of subgenera is unavailable.

Several species pairs were persistent in the analyses. Within *Sebastes, S. maliger* and *S. caurinus* (subgenus *Pteropodus*) were distinct from the other *Sebastes* species.

Table 4

Differences between haplotypes (see Table 3 and Appendix 1) of rockfish (*Sebastes, Helicolenus,* and *Sebastolobus* spp.). Above the diagonal are the number of restriction site differences. Below the diagonal are estimates of evolutionary differences (nucleotide substitutions per site; Nei and Tajima 1981; Nei and Miller 1990). An average of 79.3 sites and 332.05 bases were examined for each haplotype (McElroy et al.1990).

Species	1a	1b	2a	2b	3	4a	4b	5a	5b	6
1a		1	14	14	21	13	13	16	15	16
1b	0.0015		15	15	22	14	14	17	16	17
2a	0.0224	0.0243		2	19	9	9	14	13	14
2b	0.0222	0.0240	0.0031		19	9	9	14	13	14
3	0.0335	0.0354	0.0306	0.0302		16	18	21	20	21
4a	0.0208	0.0226	0.0143	0.0141	0.0253		2	11	10	13
4b	0.0210	0.0229	0.0145	0.0143	0.0290	0.0031		11	10	13
5a	0.0260	0.0279	0.0225	0.0222	0.0334	0.0175	0.0177		1	6
5b	0.0242	0.0260	0.0207	0.0204	0.0315	0.0158	0.0160	0.0015		5
6	0.0267	0.0286	0.0230	0.0227	0.0340	0.0213	0.0216	0.0094	0.0078	
7a	0.0192	0.0210	0.0194	0.0191	0.0306	0.0246	0.0249	0.0260	0.0242	0.0265
7b	0.0210	0.0229	0.0212	0.0210	0.0325	0.0265	0.0269	0.0280	0.0260	0.0285
7c	0.0174	0.0192	0.0176	0.0174	0.0287	0.0227	0.0230	0.0242	0.0223	0.0246
7d	0.0210	0.0228	0.0213	0.0210	0.0327	0.0264	0.0268	0.0279	0.0260	0.0284
8	0.0192	0.0210	0.0194	0.0191	0.0272	0.0211	0.0214	0.0226	0.0208	0.0231
9	0.0205	0.0223	0.0207	0.0204	0.0283	0.0190	0.0193	0.0206	0.0188	0.0243
10a	0.0223	0.0242	0.0295	0.0256	0.0336	0.0208	0.0211	0.0223	0.0239	0.0296
10b	0.0238	0.0257	0.0310	0.0272	0.0351	0.0224	0.0227	0.0206	0.0221	0.0312
11a	0.0261	0.0281	0.0333	0.0293	0.0339	0.0280	0.0283	0.0333	0.0313	0.0376
11b	0.0277	0.0296	0.0313	0.0274	0.0320	0.0260	0.0264	0.0313	0.0294	0.0356
12	0.0208	0.0226	0.0209	0.0206	0.0285	0.0192	0.0194	0.0210	0.0192	0.0216
13	0.0190	0.0208	0.0329	0.0325	0.0302	0.0277	0.0281	0.0336	0.0317	0.0381
14	0.0339	0.0324	0.0342	0.0337	0.0383	0.0324	0.0328	0.0270	0.0285	0.0308
15a	0.0321	0.0340	0.0325	0.0321	0.0075	0.0272	0.0311	0.0355	0.0336	0.0361
15b	0.0351	0.0371	0.0356	0.0351	0.0104	0.0303	0.0342	0.0385	0.0366	0.0391
16	0.0740	0.0767	0.0835	0.0779	0.0745	0.0724	0.0735	0.0827	0.0843	0.0885
17a	0.1043	0.1028	0.1148	0.1084	0.1043	0.0981	0.1041	0.0996	0.1011	0.1055
17b	0.1055	0.1041	0.1161	0.1097	0.1057	0.0995	0.1055	0.1010	0.1025	0.1070
17c	0.1031	0.1016	0.1136	0.1071	0.1029	0.0968	0.1029	0.0983	0.0998	0.1043
17d	0.1028	0.1013	0.1133	0.1069	0.1029	0.0966	0.1026	0.0981	0.0996	0.1040

Species	7a	7b	7c	7d	8	9	10a	10b	11a	11b
1a	12	13	11	13	12	13	14	15	16	17
1b	13	14	12	14	13	14	15	16	17	18
2a	12	13	11	13	12	13	18	19	20	19
2b	12	13	11	13	12	13	16	17	18	17
3	19	20	18	20	17	18	21	22	21	20
4a	15	16	14	16	13	12	13	14	17	16
4b	15	16	14	16	13	12	13	14	17	16
5a	16	17	15	17	14	13	14	13	20	19
5b	15	16	14	16	13	12	15	14	19	18
6	16	17	15	17	14	15	18	19	22	21
7a		1	1	1	2	7	14	15	14	13
7b	0.0016		2	2	3	8	15	16	15	14
7c	0.0015	0.0031		2	1	6	13	14	13	12
7d	0.0016	0.0032	0.0032		3	8	15	16	15	14
8	0.0031	0.0047	0.0015	0.0048		5	12	13	12	11
9	0.0109	0.0126	0.0093	0.0126	0.0078		9	10	11	10

continued

None of the other subgenera were as coherent. The haplotypes of *S. zacentrus* and *S. variegatus* (subgenus *Allosebastes*) were very similar and the haplotype of a third member, *S. proriger*, generally clustered nearby. Similarly, the haplotypes of *S. maliger* and *S. flavidus* (subgenus *Sebastosomus)* were tightly clustered, but the branch for the haplotype of the third member, *S. ciliatus*, was distal; and different tree construction methods inconsistently placed

Table 4 (continued)

Species	7a	7b	7c	7d	8	9	10a	10b	11a	11b
10a	0.0227	0.0246	0.0208	0.0245	0.0193	0.0140		1	14	13
10b	0.0243	0.0262	0.0224	0.0260	0.0208	0.0155	0.0015		15	14
11a	0.0229	0.0248	0.0210	0.0247	0.0195	0.0174	0.0226	0.0242		1
11b	0.0210	0.0229	0.0192	0.0228	0.0177	0.0157	0.0208	0.0223	0.0015	
12	0.0143	0.0161	0.0126	0.0160	0.0111	0.0093	0.0174	0.0189	0.0211	0.0193
13	0.0227	0.0246	0.0243	0.0245	0.0227	0.0207	0.0292	0.0308	0.0266	0.0282
14	0.0239	0.0258	0.0221	0.0258	0.0206	0.0122	0.0236	0.0252	0.0274	0.0255
15a	0.0292	0.0311	0.0272	0.0311	0.0257	0.0269	0.0322	0.0338	0.0361	0.0340
15b	0.0322	0.0342	0.0303	0.0342	0.0288	0.0299	0.0353	0.0368	0.0391	0.0371
16	0.0795	0.0823	0.0811	0.0821	0.0839	0.0843	0.0740	0.0756	0.0801	0.0817
17a	0.1151	0.1136	0.1166	0.1186	0.1151	0.1101	0.0996	0.1011	0.1014	0.1029
17b	0.1164	0.1149	0.1179	0.1199	0.1164	0.1115	0.1010	0.1025	0.1031	0.1046
17c	0.1140	0.1124	0.1155	0.1173	0.1140	0.1090	0.0983	0.0998	0.1002	0.1017
17d	0.1136	0.1120	0.1151	0.1170	0.1136	0.1086	0.0981	0.0996	0.0999	0.1014

Species	12	13	14	15a	15b	16	17a	17b	17c	17d
1a	13	12	21	20	22	41	58	59	57	57
1b	14	13	20	21	23	42	57	58	56	56
2a	13	20	21	20	22	45	62	63	61	61
2b	13	20	21	20	22	43	60	61	59	59
3	18	19	24	5	7	42	59	60	58	58
4a	12	17	20	17	19	40	55	56	54	54
4b	12	17	20	19	21	40	57	58	56	56
5a	13	20	17	22	24	45	56	57	55	55
5b	12	19	18	21	23	46	57	58	56	56
6	13	22	19	22	24	47	58	59	57	57
7a	9	14	15	18	20	43	62	63	61	61
7b	10	15	16	19	21	44	61	62	60	60
7c	8	15	14	17	19	44	63	64	62	62
7d	10	15	16	19	21	44	63	64	62	62
8	7	14	13	16	18	45	62	63	61	61
9	6	13	8	17	19	46	61	62	60	60
10a	11	18	15	20	22	41	56	57	55	55
10b	12	19	16	21	23	42	57	58	56	56
11a	13	16	17	22	24	43	56	57	55	55
11b	12	17	16	21	23	44	57	58	56	56
12		13	14	17	19	44	61	62	60	60
13	0.0212		19	18	20	43	62	63	61	61
14	0.0221	0.0307		23	25	50	59	60	58	58
15a	0.0271	0.0288	0.0369		2	41	62	63	61	61
15b	0.0301	0.0317	0.0399	0.0030		43	64	65	63	63
16	0.0811	0.0786	0.0928	0.0734	0.0765		61	62	60	60
17a	0.1117	0.1135	0.1055	0.1118	0.1147	0.1145		1	3	1
17b	0.1131	0.1150	0.1070	0.1131	0.1160	0.1158	0.0014		4	2
17c	0.1105	0.1124	0.1045	0.1105	0.1135	0.1129	0.0043	0.0057		4
17d	0.1102	0.1120	0.1040	0.1104	0.1133	0.1130	0.0014	0.0029	0.0058	

S. ciliatus on the tree (not shown). Haplotypes of *S. aleutianus* and *S. borealis* (subgenus *Zalopyr*) were found in the same general region of the tree, but are not sister taxa. Likewise, the two representatives of *Acutomentum*, *S. alutus* and *S. brevispinis,* were not monophyletic sister taxa. Disparities, such as we observed between relationships of haplotype and assignments of subgenera, have also been reported for allozyme comparisons (Seeb, 1986) and mtDNA cytochrome *b* sequences (Johns and Avise, 1998; Rocha-Olivares, 1998a; Rocha-Olivares et al., 1999a, 1999b). The members of subgenera *Acutomentum* and *Allosebastes*, in particular, seem discordant with trees. It is important to recall that the systematics is not unequivocal and controversies date back more than a century (e.g. Cramer, 1895). Therefore, discrepancies between the molecular-based comparisons and current systematic place-

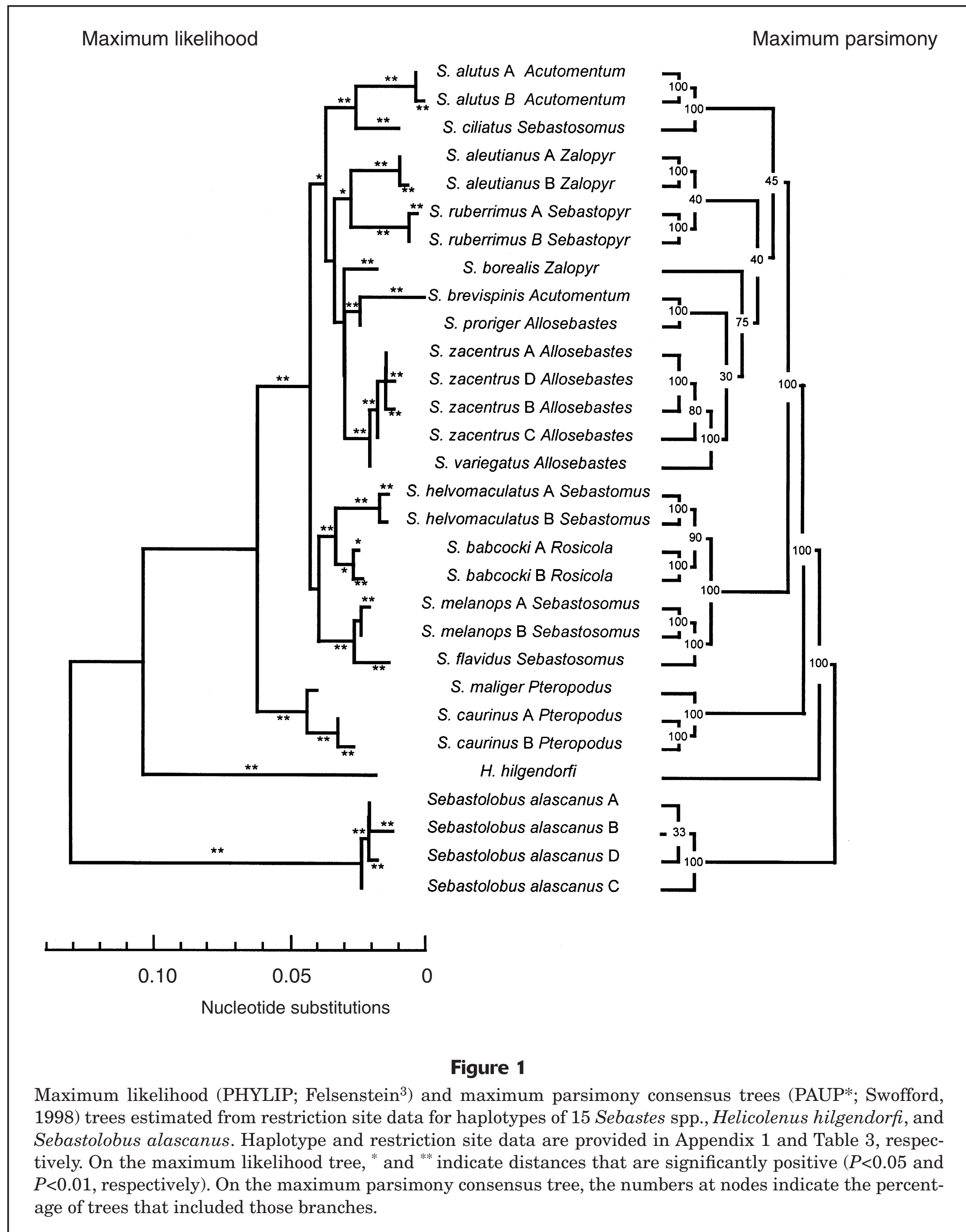

Figure 1

Maximum likelihood (PHYLIP; Felsenstein[3]) and maximum parsimony consensus trees (PAUP*; Swofford, 1998) trees estimated from restriction site data for haplotypes of 15 *Sebastes* spp., *Helicolenus hilgendorfi*, and *Sebastolobus alascanus*. Haplotype and restriction site data are provided in Appendix 1 and Table 3, respectively. On the maximum likelihood tree, * and ** indicate distances that are significantly positive (P<0.05 and P<0.01, respectively). On the maximum parsimony consensus tree, the numbers at nodes indicate the percentage of trees that included those branches.

ments do not necessarily discredit the validity of the molecular comparisons.

Use of restriction site data in mtDNA holds promise for the identification and systematics of *Sebastes* and suggests the possibility of applications for stock identification. Larval and juvenile rockfish carry mtDNA that is adequate for PCR amplification (e.g. see Seeb and Kendall, 1991; Rocha-Olivares 1998b). Combining molecular identification with morphometry may solve many of the problems of identification that accompany rockfish studies. The apparent coherence of closely related rockfish species that we observed in both cladistic and phenetic analyses suggests that we should focus our applications on groups of species that are presumed to be close relatives. The consensus tree depicting relationships among interior clades within the *Sebastes* parsimony tree did not unequivocally position those clades either in this study or analyses of the cytochrome *b* region (Johns and Avise, 1998; Rocha-Olivares, 2000). Consequently, determination of higher level relationships among *Sebastes* requires analysis of additional mtDNA regions. Moreover, because the divergence of mtDNA sequences provides only one perspective of the evolution of *Sebastes* divergence, the relationships inferred by mtDNA analyses must be corroborated by analysis of the interspecific divergence of nuclear genes.

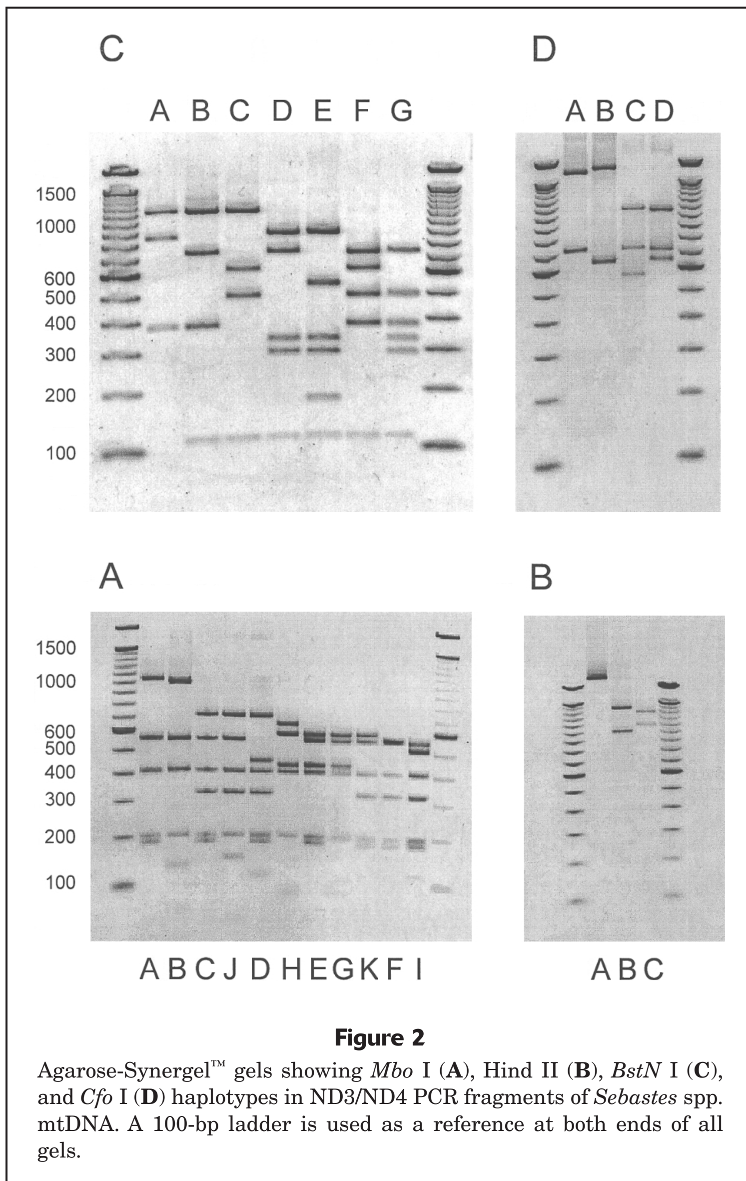

Figure 2

Agarose-Synergel™ gels showing *Mbo* I (**A**), Hind II (**B**), *BstN* I (**C**), and *Cfo* I (**D**) haplotypes in ND3/ND4 PCR fragments of *Sebastes* spp. mtDNA. A 100-bp ladder is used as a reference at both ends of all gels.

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Appendix 1

Restriction site locations for *Sebastes* spp., *Helicolenus hilgendorfi*, and *Sebastolobus alascanus* in the 12S/16S and ND3/ND4 mtDNA regions. The *Sebastes* species are listed in Table 2; five individuals of each species are represented. Sites were mapped by double digests. Haplotypes for each restriction endonuclease are presented for each mtDNA region. X's denote presence and O's denote absence of a site. (X)'s are sites that occur in the primers and were present in all PCR products. They were not used in the analysis.

12S/16S

Rsa I hapotypes

sites	A	B	C	D	E
293	O	O	O	O	X
507	O	O	X	X	O
588	O	O	O	O	X
761	X	X	X	X	X
950	O	O	O	O	X
1000	X	X	X	X	O
1071	X	X	X	X	O
1263	O	O	O	O	X
1308	O	X	O	X	X
1358	X	X	X	X	X
2164	O	O	O	O	X

Mbo I haplotypes

sites	A	B	C	D	E	F
201	O	X	X	O	O	O
849	X	X	X	X	O	X
1015	X	X	X	X	X	O
1403	X	X	X	X	X	X
1507	X	O	X	X	X	X
1746	O	O	O	X	O	O
1984	X	X	X	X	X	X
2059	X	X	X	X	X	X
2228	X	X	X	X	X	X
2318	X	X	X	X	X	X
2388	X	X	X	X	X	X

Msp I haplotypes

sites	A	B
65	X	X
766	X	X
1259	O	X
1390	X	X
1535	X	X
2226	X	X
2403	X	X

Dde I haplotypes

sites	A	B	C	D	E	F
44	X	X	X	X	X	X
55	X	X	X	X	X	X
976	X	X	X	X	X	O
1043	X	X	O	X	X	O
1056	X	X	O	X	O	O
1304	X	X	X	X	X	O
1735	O	X	X	X	X	O
2181	X	O	X	X	X	O
2393	X	X	X	X	X	X

Cfo I haplotypes

sites	A	B
537	X	X
602[1]	X	O
665[1]	X	X
1898	X	X
2268	X	X

Hinf I haplotypes

sites	A	B	C	D
982	O	O	X	O
1291	X	X	X	O
2094	O	X	X	O

Hind II haplotypes

sites	A	B
1717	X	O

Sty I haplotypes

sites	A	B
295	X	O
2053	O	X
2254	X	X

*Bst*N I haplotypes

sites	A	B	C	D
326	X	X	X	X
988	O	O	O	X
1687	O	O	X	O
1741	O	X	O	O
2416	(X)	(X)	(X)	(X)

*Bst*U I haplotypes

sites	A	B
87	X	X
600[1]	X	O
664[1]	X	X
729	X	X
1872	X	X
2128	X	X

ND3/ND4

Cfo I haplotypes

sites	A	B	C	D	E	F
709	X	O	X	X	X	X
1221	O	O	O	O	X	X
1436	O	O	O	O	X	O
1741	O	X	X	X	O	O
1813	O	O	X	O	O	O

*Bst*U I haplotypes

sites	A	B	C	D	E	F	G
4	(X)	(X)	(X)	(X)	(X)	(X)	(X)
344	O	O	O	O	O	X	O
1499	O	O	O	O	X	O	O
1854	O	O	O	X	O	O	O
2025	O	X	X	O	O	O	O
2306	O	X	O	O	X	X	X

Hinf I haplotypes

sites	A	B	C	D	E	F	G	H
130	O	X	O	O	O	X	O	O
389	O	X	X	O	O	O	O	X
494	X	X	X	X	X	X	X	X
853	O	O	O	O	O	O	O	X
1448	O	O	O	O	O	O	O	X
1537	O	O	O	O	O	O	X	O
1755	O	O	O	O	X	X	O	O
1888	O	O	O	X	O	O	O	X
2232	X	X	X	X	X	X	X	X

Sty I haplotypes

sites	A	B	C	D	E	F
49	X	X	X	X	X	X
194	O	O	O	X	X	X
365	X	X	X	X	O	O
391	O	O	O	O	X	X
534	X	O	X	X	X	X
1258	O	O	O	O	O	X
1570	O	X	X	X	X	X
2311	X	X	X	X	X	X

Msp I haplotypes

sites	A	B	C	D	E	F	G
30	X	X	X	X	X	X	X
933	O	O	O	O	O	X	O
1199	O	O	O	O	O	X	O
1261	O	O	O	O	O	O	X
1738	X	X	X	X	X	X	X
1826	X	X	O	X	X	O	O
1844	O	O	O	X	X	O	O
2073	O	O	O	O	O	X	O
2110	O	X	X	O	X	O	O

Rsa I haplotypes

sites	A	B	C	D	E	F	G	H
346	O	O	O	X	X	O	X	X
742	O	O	O	O	O	O	O	X
1077	O	O	O	O	O	X	O	O
1231	O	O	O	O	O	O	X	O
1492	O	O	O	O	O	O	O	X
1863	O	O	X	O	X	O	O	X
1985	O	X	X	X	X	X	X	X

continued

Appendix 1 (continued)

	Dde I haplotypes																
sites	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
65	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X
195	X	O	O	X	X	X	X	X	X	X	X	X	X	X	O	O	O
266	O	O	O	O	O	O	X	O	X	O	O	X	O	O	X	X	X
298	X	X	O	X	X	X	X	O	X	X	X	X	X	X	O	O	X
336	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
374	X	X	X	O	X	O	O	O	O	X	O	O	X	O	O	O	O
590	O	X	X	X	X	X	X	X	X	X	X	X	X	X	O	O	X
624	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O
663	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X
695	O	O	O	O	O	O	O	O	X	O	O	X	O	O	O	O	O
754	O	O	O	O	O	O	O	O	X	O	O	X	X	O	O	O	X
1257	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X
1409	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O
1560	O	O	O	O	O	O	O	O	X	O	O	X	O	O	O	O	O
1577	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	X	X
1601	X	O	X	X	X	X	X	X	X	X	X	X	X	X	O	O	O
1696	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X
1722	O	O	O	O	O	O	O	O	O	X	O	O	O	X	X	X	X
1779	O	O	X	O	O	O	O	O	X	O	O	X	X	O	O	O	O
1795	O	O	O	O	O	X	X	X	X	X	X	X	X	O	O	O	X
1912	X	X	X	X	X	X	X	X	O	X	X	X	X	X	O	O	X
2089	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O
2188	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	X	O
2374	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)

	Mbo I haplotypes													
sites	A	B	C	D	E	F	G	H	I	J	K	L	M	N
198	X	X	X	X	X	X	X	X	X	X	X	X	O	O
272	O	O	O	O	O	O	O	O	O	O	O	O	X	O
302	O	O	O	O	O	O	O	O	O	O	O	O	O	X
648	O	O	O	X	O	O	O	O	O	O	O	O	O	O
746/ 210	O	O	O	O	O	O	O	O	O	O	O	X	O	O
758	X	X	X	X	X	X	X	O	X	X	X	X	X	O
797	O	O	O	O	O	O	O	O	O	O	O	O	X	O
861	O	O	O	O	O	O	X	X	O	O	O	O	O	O
886	O	X	O	O	O	O	O	O	O	O	O	O	O	O
900	O	O	O	O	O	O	O	O	O	X	O	O	O	O
940	X	X	X	X	X	X	X	X	X	X	X	X	X	X
971	O	X	O	O	O	X	O	O	O	O	O	O	O	O
1029	O	O	O	O	O	O	O	O	X	O	O	O	O	O
1534	O	O	O	O	X	X	X	X	X	O	X	X	X	X
1647	O	O	X	X	O	X	O	O	X	X	X	X	O	X
1979	X	X	X	X	X	X	X	X	X	X	X	X	O	X
2016	O	O	O	O	O	O	O	O	O	O	O	O	O	X

	*Bst*N I haplotypes									
sites	A	B	C	D	E	F	G	H	I	J
112	O	X	X	X	X	X	X	X	X	X
291	O	O	O	O	X	O	O	O	O	O
551	O	O	O	O	O	O	O	X	X	O
829	X	X	O	X	X	X	X	X	X	X
1035	O	O	O	O	O	O	O	O	O	X
1214	X	X	X	O	O	X	X	O	O	O
1261	O	O	O	O	O	O	O	O	X	O
1694	O	O	X	X	X	X	X	O	O	O
1982	O	O	O	X	X	O	X	O	O	O
2325	X	X	X	X	X	X	X	X	X	X

	*Hin*d II haplotypes	
sites	A	B
1007	O	X
1103	O	O

[1] Two *Cfo* I and *Bst*U I sites are the same.