Abstract—Larval and juvenile rockfishes (*Sebastes* spp.) are difficult to identify using morphological characters. We developed a key based on sizes of restriction endonuclease fragments of the NADH dehydrogenase-3 and -4 (ND3/ND4) and 12S and 16S ribosomal RNA (12S/16S) mitochondrial regions. The key makes use of variation in the ND3/ND4 region. Restriction endonuclease *Dde* I variation can corroborate identifications, as can 12S/16S variation. The key, based on 71 species, includes most North American taxa, several Asian species, and *Sebastolobus alascanus* and *Helicolenus hilgendorfi* that are closely related to rockfishes. Fifty-eight of 71 rockfish species in our database can be distinguished unequivocally, using one to five restriction enzymes; identities of the remaining species are narrowed to small groups: 1) *S. polyspinis*, *S. crameri*, and *S. ciliatus or variabilis* (the two species could not be distinguished and were considered as a single species) ; 2) *S. chlorostictus*, *S. eos*, and *S. rosenblatti*; 3) *S. entomelas* and *S. mystinus*; 4) *S. emphaeus*, *S. variegatus*, and *S. wilsoni*; and 5) *S. carnatus* and *S. chrysomelas*.

A key to selected rockfishes (*Sebastes* spp.) based on mitochondrial DNA restriction fragment analysis

Zhuozhuo Li[1]
Andrew K. Gray[1]
Milton S. Love[2]
Akira Goto[3]
Takashi Asahida[4]
Anthony J. Gharrett[1]

[1] Fisheries Division, School of Fisheries and Ocean Sciences
University of Alaska Fairbanks
11120 Glacier Highway
Juneau, Alaska 99801
Email address (for A. J. Gharrett, contact author): ffajg@uaf.edu

[2] Marine Science Institute
University of California
Santa Barbara, California 93106-6150

[3] Graduate School of Fisheries Sciences
Hokkaido University
3-1-1 Minato-cho, Hakodate
Hokkaido 041-8611, Japan

[4] School of Fisheries Sciences
Kitasato University
Sanriku, Ofunato
Iwate 022-0101, Japan

Manuscript submitted 11 October 2004 to the Scientific Editor's Office.

Manuscript accepted for publication 1 August 2005 by the Scientific Editor.

Fish. Bull. 104:182–196 (2006).

More than 100 species of rockfishes (genus *Sebastes*) are found worldwide (Kendall, 2000), and the majority are distributed along the Pacific coast of North America and in the northwest Pacific from the western Bering Sea south to Japan and Korea (Love et al., 2002). Sixty-five rockfish species are found along the California coast (Moser, 1996). Within the genus, there is a high degree of similarity of the morphological characters among many species. These similarities are in part due to recent divergence but also may have resulted from convergence of congeners occupying similar habitats.

Historically, species identification of *Sebastes* has been based on morphology; however, this approach is often insufficient, especially for identifying sympatric species, where there is considerable similarity in size and physical features and in pigmentation, as well as overlap in meristic characters. The difficulty in identifying *Sebastes* to species level exists for all developmental stages, but larvae are especially difficult to identify because they lack diagnostic characters (Kendall, 1991). Currently, only 15 species at the larval stage can be separated by physical characters such as body shape, pigmentation patterns, and head spine development (Love et al., 2002).

Rockfishes are important ecologically, and most species are economically valuable. *Sebastes* larvae form a large portion of ichthyoplankton collections and rank third or fourth in abundance among all fish larvae taken during California Cooperative Fisheries Investigations (CalCOFI) surveys that have covered the entire length of the California and Baja California coast and now cover waters off southern California. The ability to identify *Sebastes* accurately and efficiently at all developmental stages will greatly improve both our ability to learn about their life histories and our management and conservation ef-

forts. An increased understanding of life history variation can improve the systematic descriptions of *Sebastes* species, which has mostly been based on morphology.

Several methods have been developed to obtain species-specific information that supplements the use of morphological and meristic characters for species identification. Otolith microstructure and other hard structures have been used to distinguish the late-stage larvae and young-of-the-year pelagic juveniles of some *Sebastes* species (Laidig and Adams[1]; Laidig and Ralston, 1994; Laidig et al., 1996). Biochemical genetic methods have been used to identify adults (Barrett et al., 1966; Seeb, 1986), and may also be used to identify juveniles of some species (Seeb and Kendall, 1991). LeClair and Buckley (2001) used allozyme variation at 37 loci to positively identify 155 individuals of juvenile *Sebastes diploproa* and *Sebastes melanops*; but they were unable to identify another 29 individuals, partly because of a limited database. Despite some success with their use, allozymes often have low resolution, and there are complexes of species that cannot be distinguished by using allozymes alone (Seeb and Kendall, 1991).

DNA sequence-based methods have some advantages over the use of allozymes for the identification of juvenile fish species. They often have greater resolution, due in part to the fact that expression of some metabolic enzymes changes during development, whereas DNA sequences generally do not. DNA is less susceptible to degradation than the enzymes used in allozyme studies. Also, DNA sequence-based methods require a small amount of tissue sample, which is especially suited for work with samples from early life stages (e.g., Gray et al., 2006). Because it has a relatively high rate of substitution (Moritz et al., 1987), mitochondrial DNA (mtDNA) can be useful for distinguishing closely related species. Mitochondrial DNA is usually inherited maternally in vertebrates and does not recombine with paternal mtDNA if it is leaked into the zygote (Gyllensten et al., 1991; but see Rokas et al., 2003). Thus, the mtDNA sequence represents a matrilineal phylogeny and is often useful in delineating phylogenetic relationships of closely related species. Sequences of the mitochondrial cytochrome *b* gene were used to identify the pelagic young of *Sebastes constellatus* and *Sebastes ensifer* (Rocha-Olivares et al., 2000). Multiplex PCR of haplotype-specific regions of mtDNA has also been used to identify early stages of *Sebastes* species (Rocha-Olivares, 1998).

The objective of this study was to devise a key for species identification using mtDNA restriction fragment data from both the ND3/ND4 and 12S/16S regions. In a previous study, data for species-specific mtDNA restriction site variation in the ND3/ND4 region were presented for 15 *Sebastes* species (Gharrett et al., 2000). We have included data for 56 additional species using the ND3/ND4 and 12S/16S regions of the mtDNA as the target region of PCR-amplification. We have included specimens of *Helicolenus hilgendorfi* and *Sebastolobus alascanus* for contrast.

Materials and methods

Adult specimens of 71 species of rockfishes were collected from the Gulf of Alaska, the coastal waters of California and Baja California, and from the coast of Japan. Samples of *H. hilgendorfi* and *Sebastolobus alascanus*, species from two sister genera, were collected from Japanese coastal waters and the Gulf of Alaska, respectively, to provide outgroup comparisons. A sample of heart tissue from each specimen was preserved in either 95% ethanol or a solution that was 80% 0.25M ethylenediaminetetraacetic (EDTA) acid at pH 8 and saturated with NaCl (Seutin et al., 1991) and 20% dimethyl sulfoxide (DMSO). At least five individuals of each species were analyzed, except for a few species for which less than five specimens were available. We did not distinguish between *S. ciliatus* and *S. variabilis*, which had not yet been described (Orr and Blackburn, 2004), when we collected samples.

Total genomic DNA was isolated by using a Purgene DNA™ isolation kit (Gentra Systems, Inc., Minneapolis, MN). Two target regions of mtDNA were amplified by using the polymerase chain reaction. 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 starts near the phenylalanyl tRNA end of the 12S rRNA gene, and runs through the valyl tRNA gene to near the leucyl tRNA end of the 16S rRNA gene. Primers for target regions have been used to amplify these regions in northern Pacific rockfish (Gharrett et al., 2001). The lengths for the amplified ND3/ND4 and 12S/16S regions are about 2385 and 2430 base pairs, respectively, based on the aggregate restriction maps.

Subsamples of the PCR products of each individual were digested with one or more of the restriction endonucleases *Bst*N I, *Bst*U I, *Cf*o I, *Dd*e I, *Hin*d II, *Hin*f I, *Mbo* I, *Msp* I, *Rsa* I, and *Sty* I. Fragments were separated electrophoretically in 1.5% agarose gels (one part agarose [Sigma-Aldrich, St. Louis, MO] and two parts Synergel™ [Diversified Biotech Inc., Boston MA]) in 0.5×TBE buffer (TBE is 90mM Tris-boric acid, and 2 mm EDTA, pH 7.5). A 100 base-pair ladder provided molecular weight markers to estimate restriction fragment sizes. Gels were stained with ethidium bromide and photographed on an ultraviolet light transilluminator. Restriction fragments that could not be accurately measured from agarose gels were separated on 8% polyacrylamide gels and stained with SYBR Green I Nucleic Acid Stain™ (Molecular Probes, Eugene, OR) using a 25-bp ladder for a molecular weight standard.

[1] Laidig, T. E., and P. B. Adams (eds.). 1991. Methods used to identify pelagic juvenile rockfish (genus *Sebastes*) occurring along the coast of central California. NOAA-TM-NMFS-SWFSC-166, 180 p. NMFS Southwest Fisheries Science Center, 110 Shaffer Rd., Santa Cruz, CA 95060.

A restriction site map was created for each endonuclease by using all observed restriction fragment patterns and necessary double digests.

Results

Baseline data

Interspecific variation was observed for all enzymes in both mitochondrial regions, except for *Hin*d II in the 12S/16S region. Intraspecific variation was observed for several enzymes. A total of 215 restriction sites were detected in the ND3/ND4 and 12S/16S regions (Appendix 1). Of the 215 sites, 97 were unique to *Sebastes* species, 21 were unique to *Sebastolobus alascanus*, seven were unique to *H. hilgendorfi*, and one was shared by *Sebastolobus alascanus* and *H. hilgendorfi*. The ND3/ND4 region had 141 sites, and the 12S/16S region had 74 sites. In the ND3/ND4 region, 36 sites were common in all haplotypes, whereas 46 occurred in only a single haplotype.

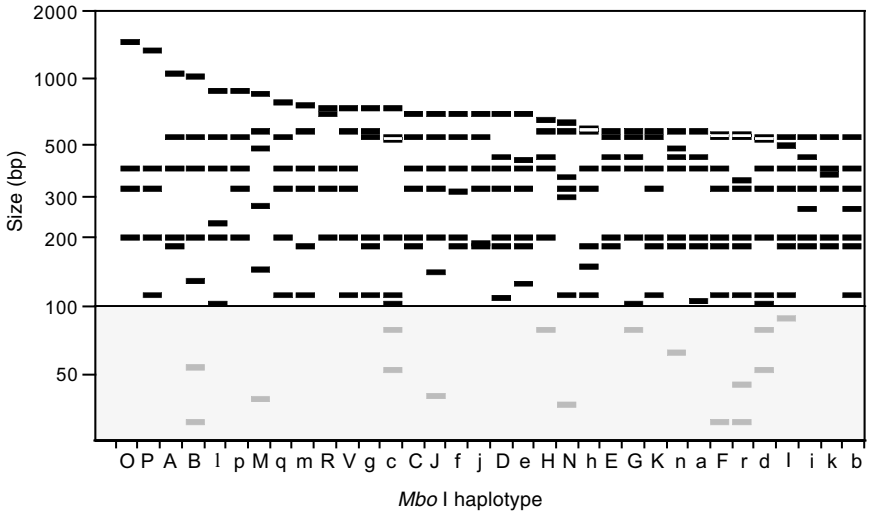

Figure 1

A mock gel showing expected fragment patterns for rockfish (*Sebastes* spp.) haplotypes as a result of digestion of the mitochondrial ND3/ND4 PCR product by restriction endonuclease *Mbo* I. The mobilities are the logarithm of the fragment sizes and separation was assumed to have taken place in 1.5% agarose gels (one part agarose and two parts Synergel^{Tm}). The haplotypes correspond to the fragment sizes in Appendix 1 and to the haplotypes in Table 1 and the identification key. A haplotype may occur in more than one species. Most of the fragments that occurred in the shaded region would probably not be well resolved in an agarose gel.

A total of 132 composite haplotypes resulted from site differences in the two mtDNA regions (Table 1). The individuals of *Sebastes* species had 127 haplotypes; *Sebastolobus alascanus* had four haplotypes; and *H. hilgendorfi* had a single haplotype. Thirty-four of the 71 species displayed intraspecific variation and were represented by more than one composite haplotype; the remaining 37 species had a single composite haplotype.

Two pairs and three triplets of *Sebastes* species had identical composite haplotypes and could not be separated at the species level with this restriction site information (see identification key). These groups were 1) *S. carnatus* and *S. chrysomelas*; 2) *S. chlorostictus*, *S. eos*, and *S. rosenblatti*; 3) *S. ciliatus* or *variabilis*, *S. crameri*, and *S. polyspinis*; 4) *S. emphaeus*, *S. variegatus*, and *S. wilsoni*; and 5) *S. entomelas* and *S. mystinus*. Several pairs of haplotypes between variable *Sebastes* species were separated by a single restriction site. These included 1) *S. hopkinsi* and *S. ovalis—Dde* I; 2) *S. zacentrus* and the *S. emphaeus-S. variegatus-S. wilsoni* complex—*Rsa* I; and 3) *S. reedi* and the *S. ciliatus or variabilis-S. crameri-S. polyspinis* complex—*Mbo* I. The most variable species was *S. mystinus*, with five haplotypes. Five *Sebastes* species, *S. dalli*, *S. hubbsi*, *S. polyspinis*, *S. trivittatus*, and *S. zacentrus*, as well as *Sebastolobus alascanus*, had four haplotypes each. The remaining 58 *Sebastes* species have species-specific markers that allow unambiguous identification.

Use of the key

The key we developed for *Sebastes* species was based exclusively on variation in the ND3/ND4 region because it required only a single PCR amplification and variation in the 12S/16S region contributed no additional resolution between species to that provided by the ND3/ND4 region. The key is not a dichotomous key, but it is applied in the same way that taxonomic keys are applied. The first step is to digest PCR-amplified DNA from the ND3/ND4 region with restriction endonuclease *Mbo* I and to estimate the sizes of the fragments produced by separating the fragments on an agarose-Synergel^{TM} gel. The best results will be achieved by using molecular weight markers, digitally photographing the gels, and estimating the fragment sizes with appropriate software. Alternatively, visual recognition of the fragment pattern can be accomplished by constructing a graphic key (e.g., Fig. 1 for *Mbo* I fragments) from fragment sizes predicted by the restriction site maps in Appendix 1. Note that in the figure, the logarithm of the size of the fragment (in base pairs) is used to estimate the mobility of a fragment (Sambrook et al., 1989). When the *Mbo* I haplotype has been identified, proceed to the next step. For example if the *Mbo* I digest results in haplotypes D or e, proceed to step g. in the key, which specifies digestion of another subsample of the ND3/ND4 PCR

Table 1

Composite haplotypes for *Sebastes* spp., *Helicolenus hilgendorfi*, and *Sebastolobus alascanus* in the ND3/ND4 and 12S/16S mtDNA regions. The haplotype codes refer to haplotypes in Appendix 1.

	ND3/ND4										12S/16S									
Species	*BstN* I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hinf* I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I	*BstN* I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hinf* I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
aleutianus A	F	C	B	N	A	E	K	D	D	C	A	A	A	D	A	B	C	B	A	A
aleutianus B	F	C	B	N	A	F	K	D	D	C	A	A	A	D	A	B	C	B	A	A
alutus A	B	C	A	J	A	A	B	B	C	C	A	A	A	D	A	B	C	B	A	A
alutus B	B	C	A	J	A	A	B	B	C	C	A	A	A	D	A	A	C	B	A	A
atrovirens	O	B	D	b	C	G	j	C	B	I	A	A	A	D	A	B	A	A	A	A
auriculatus	F	B	D	v	A	G	k	C	B	A	A	A	A	D	A	B	A	A	A	A
aurora	B	C	D	m	A	A	b	L	A	e	A	A	A	D	A	A	C	B	A	A
babcocki A	F	C	D	G	A	A	G	B	B	C	A	A	A	C	A	B	C	B	B	A
babcocki B	F	C	D	F	A	A	H	B	B	C	A	A	A	C	A	B	C	B	B	A
borealis	F	C	D	D	A	A	F	B	B	C	B	A	A	A	A	B	C	A	C	A
brevispinis	G	C	D	C	A	A	K	D	B	D	A	A	A	D	A	A	C	A	C	A
capensis A	F	C	D	G	B	A	E	B	D	O	A	A	A	C	A	A	C	A	D	A
capensis B	F	C	D	F	B	A	E	B	D	O	A	A	A	C	A	A	A	A	D	A
capensis C	F	C	D	G	B	A	E	B	D	O	A	A	A	D	A	A	C	A	D	A
carnatus A	F	a	A	I	C	G	C	a	B	A	A	A	A	D	A	B	A	A	A	A
carnatus B	F	a	A	I	C	A	C	a	B	A	A	A	A	D	A	B	A	A	A	A
carnatus C	F	a	a	I	C	G	C	a	B	A	A	A	A	D	A	B	A	A	A	A
caurinus A	F	B	C	L	A	G	C	C	B	A	A	A	A	D	A	B	A	A	A	A
caurinus B	F	B	C	L	A	G	J	C	B	A	A	A	A	D	A	B	A	A	A	A
chlorosticus	F	B	D	G	B	A	E	B	C	C	A	A	A	C	A	B	C	A	D	A
chrysomelas A	F	a	A	I	C	G	C	a	B	A	A	A	A	D	A	B	A	A	A	A
chrysomelas B	F	a	A	I	C	G	C	a	B	A	A	A	A	D	A	B	A	A	C	A
ciliatus variabilis	A	D	A	M	A	A	F	B	F	C	A	A	A	D	A	B	C	A	A	A
constellatus A	F	C	D	G	F	A	E	E	D	M	G	A	A	C	A	A	C	E	D	A
constellatus B	F	C	D	G	F	A	E	E	D	M	A	A	A	C	A	A	C	A	D	A
crameri	A	D	A	M	A	A	F	B	F	C	A	A	A	D	A	B	C	A	A	A
dalli A	F	C	D	bb	b	G	p	C	B	g	A	A	A	D	A	H	A	A	A	A
dalli B	F	C	D	bb	b	G	l	C	B	g	A	A	A	D	A	H	A	A	A	A
dalli C	F	C	D	bb	b	G	p	C	a	b	A	A	A	D	A	H	A	A	A	A
dalli D	F	C	D	bb	b	G	p	C	B	g	A	A	A	D	A	B	A	A	A	A
diploproa	g	B	C	aa	A	A	C	M	C	C	A	A	A	C	A	B	G	F	A	A
elongatus A	W	C	I	h	A	A	C	M	P	C	A	A	A	D	A	B	I	A	A	A
elongatus B	W	C	I	h	A	A	A	M	P	C	A	A	A	D	A	B	I	A	A	A
emphaeus A	F	C	D	E	A	A	D	B	D	C	A	A	A	B	A	B	C	A	C	A
emphaeus B	F	C	D	E	A	A	D	B	B	C	A	A	A	B	A	B	C	A	C	A
ensifer A	F	C	D	F	B	Q	E	B	C	C	A	A	A	C	A	B	A	A	C	A
ensifer B	F	C	D	F	B	A	E	B	C	C	A	A	A	C	A	B	C	A	C	A
entomelas	F	C	J	l	A	A	E	a	B	C	A	A	A	D	A	A	A	F	B	A
eos	F	B	D	G	B	A	E	B	C	C	A	A	A	C	A	B	C	A	D	A
exsul	F	C	D	D	B	A	i	B	D	M	A	A	A	C	A	B	C	A	B	A
flavidus	E	C	D	H	A	C	A	B	A	C	A	A	A	A	A	B	C	B	D	A
gilli A	b	C	D	x	A	P	m	A	R	N	A	E	D	D	A	B	C	B	A	A
gilli B	b	C	D	x	A	P	K	A	R	N	A	E	D	D	A	B	C	B	A	A
goodei	Y	C	D	p	A	O	a	B	D	D	A	A	A	D	A	I	C	A	A	A
helvomaculatus A	F	C	D	K	B	A	A	B	C	C	A	A	A	C	A	B	B	A	D	A

continued

Table 1 (continued)

	ND3/ND4										12S/16S									
Species	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hinf* I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hinf* I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
helvomaculatus B	F	C	D	K	B	A	A	B	E	C	A	A	A	C	A	B	C	A	D	A
hopkinsi A	Z	C	A	n	A	g	K	B	C	e	A	A	A	C	A	B	C	A	B	A
hopkinsi B	Z	C	A	n	A	g	K	A	C	e	A	A	A	C	A	B	C	A	B	A
hopkinsi C	Z	C	A	u	A	g	g	B	C	e	A	A	A	C	A	A	C	A	B	A
hubbsi A	L	I	D	R	A	I	O	A	C	G	A	C	A	D	A	A	C	A	A	A
hubbsi B	M	I	D	S	A	I	O	A	C	G	A	C	A	D	A	A	C	A	A	A
hubbsi C	M	I	D	T	A	I	P	A	C	G	A	C	A	D	A	B	C	A	A	A
hubbsi D	M	I	D	S	A	F	O	A	C	G	A	C	A	D	A	A	C	A	A	A
inermis A	F	I	D	X	A	A	D	e	J	I	A	A	A	A	A	B	C	A	C	A
inermis B	F	H	D	X	A	A	D	e	J	I	A	A	A	A	A	B	C	A	C	A
inermis C	F	I	D	cc	A	I	D	e	J	I	A	A	A	A	A	B	C	A	C	A
jordani A	a	C	D	c	E	A	c	A	P	C	F	A	A	H	A	B	C	A	D	A
jordani B	a	C	D	c	E	A	d	A	P	C	F	A	A	H	A	B	C	A	D	A
jordani C	a	C	D	q	E	A	c	A	P	C	F	A	A	H	A	B	C	A	D	A
joyneri A	O	H	D	a	A	A	D	e	L	A	A	A	A	A	A	B	C	A	C	A
joyneri B	O	H	D	a	A	I	D	e	L	A	A	A	A	A	A	B	C	A	C	A
lentignosus	F	C	D	F	B	A	n	B	D	M	A	A	A	C	A	B	C	A	B	A
levis	B	A	g	d	A	A	q	d	B	C	A	A	A	D	A	B	C	A	D	A
macdonaldi	P	b	A	e	A	b	C	g	D	C	B	A	A	G	A	G	C	A	A	D
maliger	F	B	D	L	C	D	C	C	B	A	A	A	A	D	A	B	A	A	B	A
melanops A	D	C	D	F	A	B	E	A	A	C	A	A	A	D	A	B	C	B	D	A
melanops B	D	C	D	F	A	B	E	B	A	C	A	A	A	D	A	B	C	B	D	A
melanostomous A	O	C	D	g	A	A	K	A	O	C	A	A	A	D	A	B	C	B	C	A
melanostomous B	O	C	D	g	A	A	K	A	B	C	A	A	A	D	A	B	C	B	C	A
miniatus A	F	C	K	r	C	A	e	B	A	C	A	A	A	C	A	A	C	A	A	A
miniatus B	F	C	K	r	C	N	e	B	A	C	A	A	A	C	A	A	C	A	A	A
mystinus A	F	C	J	l	A	A	E	a	B	C	A	A	A	D	A	A	A	B	B	A
mystinus B	B	C	J	l	A	C	E	D	B	C	A	A	A	D	A	A	A	A	B	A
mystinus C	F	C	J	l	A	A	E	a	B	e	A	A	A	D	A	A	A	B	B	A
mystinus D	B	C	J	l	A	A	E	D	B	C	A	A	A	D	A	A	A	B	B	A
mystinus E	B	C	J	l	A	C	E	D	B	C	A	A	A	D	A	A	A	B	B	A
nebulosus A	F	G	A	Z	A	G	C	C	B	A	A	A	A	D	A	B	A	A	A	A
nebulosus B	F	G	A	Z	B	G	C	C	B	A	A	A	A	D	A	B	A	A	A	A
nigrocinctus	F	C	D	c	A	A	G	B	B	C	A	A	A	C	A	B	C	A	B	A
nivosus	N	A	D	U	A	I	C	B	I	A	A	A	A	G	A	A	C	A	A	C
ovalis	Z	C	A	o	A	g	K	B	C	e	A	A	A	C	A	B	C	A	B	A
paucispinis A	F	A	b	c	A	A	V	A	b	d	A	A	A	D	A	B	C	A	B	A
paucispinis B	F	A	b	c	g	A	V	A	b	d	A	A	A	D	A	B	C	A	B	A
phillipsi	B	C	D	m	A	A	b	B	B	e	A	A	A	D	A	B	C	B	A	A
pinniger	F	C	D	s	A	A	C	B	B	A	A	A	A	D	A	A	C	A	A	A
polyspinis A	A	D	A	M	A	A	F	B	F	C	A	A	A	D	A	B	C	A	A	A
polyspinis B	A	J	A	f	A	A	F	A	F	C	A	A	A	D	A	B	C	A	A	A
polyspinis C	A	D	A	M	A	A	F	B	F	C	A	A	A	I	A	B	C	A	A	A
polyspinis D	V	D	A	M	A	A	F	B	N	C	A	A	A	D	A	B	C	A	A	A
proriger	F	C	D	E	A	A	K	E	B	C	A	A	A	D	A	B	C	A	C	A
rastrelliger	c	C	C	v	A	a	A	C	B	A	A	A	A	D	A	B	A	A	A	A
reedi	A	D	A	M	A	A	r	B	F	C	A	A	A	D	A	B	C	A	A	A

continued

Table 1 (continued)

	ND3/ND4										12S/16S									
Species	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hinf* I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I	*Bst*N I	*Bst*U I	*Cfo* I	*Dde* I	*Hin*d II	*Hinf* I	*Mbo* I	*Msp* I	*Rsa* I	*Sty* I
rosaceus	F	C	D	k	B	A	E	B	B	C	A	A	A	C	A	B	C	A	B	A
rosenblatti	F	B	D	G	B	A	E	B	C	C	A	A	A	C	A	B	C	A	D	A
ruberrimus A	C	C	A	B	A	D	I	B	D	C	A	A	A	D	A	C	C	A	A	A
ruberrimus B	C	C	D	B	A	D	I	B	D	C	A	A	A	D	A	C	C	A	A	A
rubrivinctus	g	C	D	D	A	A	E	b	B	C	A	A	A	C	A	B	C	B	B	A
rufus A	F	C	D	aa	A	A	F	B	B	e	E	A	A	C	A	B	C	D	D	A
rufus B	F	C	D	aa	A	A	F	B	B	e	E	A	A	C	A	B	C	D	B	A
rufus C	F	C	D	aa	A	A	C	B	B	e	E	A	A	C	A	B	C	D	B	A
saxicola	O	C	D	t	A	G	f	B	C	C	G	F	C	D	A	B	C	A	A	A
semicinctus	X	C	D	j	A	C	p	A	Q	L	A	D	A	A	A	A	H	A	A	A
serrenoides A	D	C	D	D	B	C	f	B	P	C	A	A	A	D	A	B	C	B	D	A
serrenoides B	D	C	C	F	B	C	f	B	P	C	A	A	A	D	A	B	C	B	D	A
serriceps A	F	C	D	y	A	g	E	B	B	C	A	A	A	C	A	B	C	A	B	A
serriceps B	F	C	D	z	A	g	E	B	B	C	A	A	A	C	A	B	C	A	B	A
simulator	F	K	D	F	A	A	E	B	C	C	A	A	A	C	A	B	C	A	D	A
spinorbis	O	C	D	D	B	A	E	B	D	M	A	A	A	C	A	B	C	A	B	A
taczanowski A	P	A	D	W	A	I	R	H	C	I	A	A	A	A	A	D	C	B	A	C
taczanowski B	P	A	D	W	C	I	R	H	C	I	A	A	A	A	A	D	C	B	A	C
taczanowski C	P	A	D	W	C	I	R	H	C	I	A	A	A	A	A	D	C	A	A	C
thompsoni	O	H	D	Y	A	A	D	e	L	I	A	A	A	A	A	B	C	A	C	A
trivittatus A	F	A	D	V	A	F	K	E	C	H	A	A	A	A	A	E	B	B	A	C
trivittatus B	F	A	D	V	A	D	K	E	C	H	E	A	A	A	A	E	B	C	A	C
trivittatus C	F	A	D	V	B	D	K	E	B	H	E	A	A	A	A	E	B	C	A	C
trivittatus D	F	A	D	V	B	D	K	E	C	H	E	A	A	A	A	E	B	C	A	C
umbrosus	F	C	D	w	B	A	n	B	D	M	A	A	A	C	A	B	A	A	B	A
variegatus	F	C	D	E	A	A	D	B	B	C	A	A	A	B	A	B	C	A	C	A
vulpes A	B	D	D	V	A	A	h	B	C	G	A	C	A	A	A	E	B	B	A	B
vulpes B	B	D	D	V	A	A	h	B	C	H	A	C	A	A	A	E	B	B	A	B
vulpes C	B	D	D	i	A	A	h	B	C	H	A	C	A	A	A	E	B	B	A	B
wilsoni A	F	C	D	E	A	A	D	B	B	C	A	A	A	B	A	B	C	A	C	A
wilsoni B	F	C	A	i	A	A	D	B	B	C	A	A	A	B	A	B	C	A	C	A
zacentrus A	F	A	D	E	A	A	D	B	C	C	A	A	A	B	A	B	C	A	C	A
zacentrus B	F	A	D	A	A	A	D	B	C	C	A	A	A	B	A	B	C	A	C	A
zacentrus C	F	C	D	E	A	A	D	B	C	C	A	A	A	B	A	B	C	A	C	A
zacentrus D	F	A	D	E	A	A	D	B	C	B	A	A	A	B	A	B	C	A	C	A
Helicolenus hilgendorfi	J	G	F	Q	A	A	N	G	H	A	D	B	B	F	B	B	F	B	A	A
Sebastolobus alascanus A	H	E	E	O	A	H	M	F	G	F	C	B	B	E	A	D	E	B	E	B
Sebastolobus alascanus B	I	E	E	O	A	H	M	F	G	F	C	B	B	E	A	D	E	B	E	B
Sebastolobus alascanus C	H	F	E	O	A	H	M	F	G	E	C	B	B	E	A	D	E	B	E	B
Sebastolobus alascanus D	H	E	E	P	A	H	M	F	G	F	C	B	B	E	A	D	E	B	E	B

Identification key

Key for distinguishing *Sebastes* spp. using ND3/ND4 restriction site information. This is one of many possible schemes to identify unknown specimens to species level or to small groups of species. Because new intraspecific variation may be encountered, it is wise to confirm the identification by cutting the DNA using an additional one or two enzymes.

1. Digest mtDNA with *Mbo* I.
2. Place resulting restriction fragment patterns (haplotypes) in one of the following categories (see Appendices 1 and 2 for details).
   - a. O—*S. hubbsi* A, B, D.
   - b. P—*S. hubbsi* C.
   - c. A or B.
     Digest mtDNA with *Bst*N I.
     - i. B—*S. alutus*
     - ii. W—*S. elongatus* A
     - iii. E—*S. flavidus*
     - iv. F—*S. helvomaculatus*
     - v. C—*S. rastrelliger*
   - d. p or l.
     Digest mtDNA with *Bst*N I.
     - i. X—*S. semicinctus*
     - ii. F—*S. dalli*
   - e. R—*S. taczanowski*
   - f. q, V, C, J, f, j.
     Digest with *Bst*N I.
     - i. B—*S. levis*
     - ii. N—*S. nivosus*
     - iii. O.
       Digest mtDNA with *Bst*U I.
       1. B—*S. atrovirens*
       2. C—*S. saxicola*
     - iv. W—*S. elongatus*
     - v. g—*S. diploproa*
     - vi. F.
       Digest mtDNA with *Bst*U I.
       1. A—*S. paucispinis*
       2. C.
          Digest mtDNA with *Dde* I.
          - *a.* s—*S. pinniger*
          - *b.* aa—*S. rufus* C
       3. a—*S. carnatus*, *S. chrysomelas*
       4. B.
          Digest mtDNA with *Cfo* I.
          - *a.* D—*S. maliger*
          - *b.* C—*S. caurinus*
       5. G—*S. nebulosus*
     - vii. P—*S. macdonaldi*
   - g. D or e.
     Digest mtDNA with *Bst*N I.
     - i. F.
       Digest mtDNA with *Dde* I.
       1. A—*S. zacentrus* B
       2. E.
          Digest mtDNA with *Rsa* I.
          - *a.* C—*S. zacentrus* A,C,D
          - *b.* B—*S. emphaeus* B, *S. variegatus*, *S. wilsoni* A (identical)
          - *c.* D—*S. emphaeus* A
       3. cc—*S. inermis* C
       4. X or r.
          Digest mtDNA with *Bst*U I.
          - *a.* C—*S. miniatus*
          - *b.* H—*S. inermis* A
          - *c.* I—*S. inermis* B
       5. i—*S. wilsoni* B
     - ii. O.
       Digest mtDNA with *Dde* I.
       1. Y—*S. thompsoni*
       2. a—*S. joyneri*
   - h. c—*S. jordani* A
   - i. H—*S. babcocki* B
   - j. g—*S. hopkinsi* A
   - k. h—*S. vulpes*
   - l. E.
     Digest mtDNA with *Bst*N I.
     - i. B—*S. mystinus* A
     - ii. O—*S. spinorbis*
     - iii. D—*S. melanops*
     - iv. F or g.
       Digest mtDNA with *Dde* I.
       1. l .
          Digest mtDNA with *Bst*N I.
          - *a.* F—*S. entomelas*/ *S. mystinus* A, C
          - *b.* B—*S. mystinus* B, D
       2. D—*S. rubrivinctus*
       3. G.
          Digest mtDNA with *Bst*U I.
          - *a.* C—*S. constellatus*
          - *b.* B—*S. chlorostictus*, *S. eos*, *S. rosenblatti* (identical)
       4. z—*S. serriceps* A
       5. k or y.
          Digest mtDNA with *Hin*d II.
          - *a.* A—*S. serriceps* B
          - *b.* B—*S. rosaceus*
   - m. G—*S. babcocki* A
   - n. K.
     Digest mtDNA with *Bst*N I.
     - i. Z—*S. hopkinsi* B
     - ii. O—*S. melanostomus*
     - iii. F.
       Digest mtDNA with *Hin*f I.
       1. A—*S. proriger*
       2. D—*S. trivittatus*
       3. E—*S. aleutianus* A
       4. B or F.
          Digest mtDNA with *Bst*U I.
          - *a.* A—*S. trivittatus*
          - *b.* C—*S. aleutianus* B
     - iv. G—*S. brevispinis*
   - o. a—*S. goodei*
   - p. F.
     Digest mtDNA with *Bst*N I.
     - i. A—*S. crameri*, *S. ciliatus*, *S. polyspinis* A, B (first 2 spp. and *S. polyspinis* A identical)
       Digest mtDNA with *Bst*U I.
       1. D—*S. crameri*, *S. ciliatus*, *S. polyspinis* A
       2. J—*S. polyspinis* B
     - ii. F.
       Digest mtDNA with *Dde* I.
       1. D—*S. borealis*
       2. aa—*S. rufus* A, B
     - iii. V—*S. polyspinis* C
   - q. d—*S. jordani* B
   - r. I—*S. ruberrimus*
   - s. i—*S. exsul*
   - t. b—*S. aurora*
   - u. k—*S. auriculatus*
   - v. r—*S. reedi*

product with endonuclease *Bst*N I. Continue digesting with the specified restriction endonucleases, identifying the resultant haplotype, and continue to the next step until the species has been identified. Between one and five restriction enzymes will be needed to achieve the separation. Examining the fragment patterns of additional restriction enzymes can increase confidence in the identifications (see key).

Although variation observed in the 12S/16S region was not used in the key, it does provide alternatives to species identification that may be useful for resolving the identification of some species or for corroborating identifications, especially when there is intraspecific variation that has not been previously observed for the enzymes applied in resolving species. In particular, *Rsa* I, *Dde* I, and *Mbo* I exhibited substantial interspecific variation in the 12S/16S region. In addition, if one has reduced the possible species by other means, a single 12S/16S digest can be used in some instances. For example, *S. aleutianus* and *S. borealis* differ in fragments produced by *Bst*N I and by *Rsa* I, and *S. caurinus* and *S. maliger* also differ in fragments produced by *Rsa* I.

Discussion

Restriction site analysis of mtDNA is a simple and effective tool for identifying juveniles of *Sebastes* species. Because inexpensive equipment is used to obtain data from restriction fragment patterns, the analyses can be conducted in most laboratories, including many high school laboratories.

Although the restriction fragment key identified most (58 or 81.7%) of the 71 different rockfish species we evaluated, it failed to identify 13 species. The identities of those 13, however, were narrowed to five small groups of species. One group, *S. carnatus* and *S. chrysomelas*, are very closely related; they differ obviously only in body coloration as adults: *S. carnatus* has flesh-colored blotches on an olive-brown background, and *S. chrysomelas* has yellow blotches on a black background (Love et al., 2002). They are included in the subgenus *Pteropodus* (Kendall, 2000). Another group includes *S. chlorostictus*, *S. eos*, and *S. rosenblatti*, which are also morphologically similar, occur sympatrically, and are closely related (Chen, 1971; Love, 1996; Rocha-Olivares et al., 1999; Love et al., 2002); all are members of the subgenus *Sebastomus*. Estimates of divergence times of the subgenus *Sebastomus* suggest that the three species are the result of the most recent speciation events within the subgenus, which may have begun less than 140,000 years ago (Rocha-Olivares et al., 1999). Members of a third group, *S. emphaeus-S. variegatus-S. wilsoni*, are assigned to the subgenus *Allosebastes* (Kendall, 2000). The relationships between species in the other two unresolved groups are not as clear because the subgenera of the members differ. In one of those groups, *S. entomelas* is in subgenus *Acutomentum* and *S. mystinus* is in subgenus *Sebastosomus*. In the other subgroup, *S. polyspinis* remains unassigned to a subgenus, *S. crameri* is in *Eosebastes*, and *S. ciliatus* (subgenus *Sebastosomus*) has only recently been separated from *S. variabilis* (Orr and Blackburn, 2004). The similarities observed may actually reflect genetic relationships because the subgenera assignments are probably inaccurate reflections of phylogeny (Kendall, 2000). Our inability to resolve within those five groups of rockfish to species indicates the need for additional markers. Approaches for obtaining such markers include screening additional regions of the mtDNA and application of additional restriction enzymes. If additional mtDNA regions and restriction enzymes do not provide species-specific information, other molecular techniques such as microsatellites should be considered.

We applied the baseline data from which our key was developed to identification of recently extruded rockfish larvae in Southeast Alaskan waters (Gray et al., 2006). That application, which was made while the key presented in this article was being developed, evolved over subsequent years of application. The key was also used to delineate juvenile rockfishes from the southern California Bight (Li et al., in press). We were able to identify all the specimens to species or to narrow identification to one of the five small groups of genetically similar species. The identifications of juvenile rockfish were concordant with genetic and morphological criteria, except that the genetic key did resolve species in the closely related subgenus *Sebastomus*, which could not be resolved with morphological criteria. The larval specimens, in contrast, could not be identified to species with morphological criteria. We detected previously unobserved intraspecific variation in both studies as well. Neither the variation observed in the larval and juvenile studies, the intraspecific variation observed in developing the key, nor intraspecific variation observed among large numbers of individuals of several species examined as part of a population genetic analysis (Li, 2004) obscured species detection.

Molecular genetic keys can remove much of the guesswork for species determination in ichthyoplankton and juvenile surveys. Because DNA-based characters remain constant throughout the life of an individual, genetic divergence can provide unequivocal markers for species delineation. For specific questions involving the identification of small numbers of species, such as distinguishing between the two sibling species included in *S. aleutianus* (Gharrett et al., 2005), it is possible to develop assay methods (e.g., single nucleotide polymorphism—referred to as SNP (Collins et al., 1996), that can rapidly identify large numbers of specimens. Finally, such a key can be used to identify larval and juvenile species of *Sebastes* so that the variation in the morphological characters can be determined.

Acknowledgments

This work represents, in part, the master's thesis work of Z. Li at the University of Alaska Fairbanks. The project was supported by funding from the U.S. Geological

Survey (Biological Resources Division), Western Regional Office in Seattle, WA (R.W.O. 32) to AJG. AJG was at Hokkaido University and Kitasato University and was supported by the Japan Society for Promotion of Science when he contributed some of the effort to this study.

Literature cited

Barrett, I., J. Joseph, and H. G. Moser.
1966. Electrophoretic analysis of hemoglobins of California rockfish (genus *Sebastodes*). Copeia 1966:489–494.

Chen, L.-C.
1971. Systematics, variation, distribution, and biology of rockfishes of the subgenus *Sebastomus* (Picses, Scorpaenidae, *Sebastes*). Bull. Scripps. Inst. Oceaogr. 18:1–115.

Collins, F. S., M. S. Guyer, and A. Chakravarti.
1996. Variations on a theme: cataloging human DNA sequence variation. Science 278:1580–1581.

Gharrett, A. J., A. K. Gray, and J. Heifetz.
2001. Identification of rockfish (*Sebastes* spp.) from restriction site analysis of the mitochondrial ND-3/ND-4 and 12S/16S rRNA gene regions. Fish. Bull. 99:49–62.

Gharrett, A. J., A. P. Matala, E. L. Peterson, A. K. Gray, Z. Li, and J. Heifetz.
2005. Two genetically distinct forms of rougheye rockfish are different species. Trans. Am. Fish. Soc. 134:242–260.

Gray, A. K., A. W. Kendall Jr., B. L. Wing, M. G. Carls, J. Heifetz, Z. Li, and A. J. Gharrett.
In press. Identification of larval rockfishes (*Sebastes* spp.) in Southeast Alaskan waters was possible using mitochondrial markers but not pigmentation patterns. Trans. Am. Fish. Soc. 134.

Gyllensten, U., D. Wharton, A. Josefsson, and A. C. Wilson.
1991. Paternal inheritance of mitochondrial DNA in mice. Nature 352:255–257.

Kendall, A. W., Jr.
1991. Systematics and identification of larvae and juveniles of the genus *Sebastes*. Environ. Biol. Fish. 30:173–190.
2000. An historic review of *Sebastes* taxonomy and systematics. Mar. Fish. Rev. 62:1–23.

Laidig, T. E., and S. Ralston.
1994. The potential use of otolith characters in identifying larval rockfish (*Sebastes* spp.). Fish. Bull. 93:166–171.

Laidig, T. E., K. M. Sakuma, and M. M. Nishimoto.
1996. Description of pelagic larval and juvenile *Sebastes saxicola* (Family Scorpaenidae), with an examination of larval growth. Fish. Bull. 94:289–299.

LeClair, L. L., and R. M. Buckley.
2001. Electrophoretic identification of juvenile rockfish (Genus *Sebastes*) recruiting to drifting algae and seagrass habitats off the Washington coast. Northwest Sci. 75:53–60.

Li, Z.
2004. Phylogenetic relationship and identification of juveniles of the genus *Sebastes*. M.S. thesis, 146 p. Univ. Alaska Fairbanks, Fairbanks AK.

Li, Z., M. M. Nishimoto, M. S. Love, and A. J. Gharrett.
In press. Comparing the identification of southern California juvenile rockfishes (genus *Sebastes* spp.) by restriction site analysis of the mitochondrial ND3/ND4 region and by morphological characteristics. Fish. Bull. 104(3).

Love, M. S.
1996. Probably more than you want to know about the fishes of the Pacific coast, 386 p. Really Big Press. Santa Barbara, CA.

Love, M. S., M. Yoklavich, and L. Thorsteinson (eds.).
2002. The rockfishes of the Northeast Pacific, 405 p. Univ. California Press, Berkeley, CA.

Moritz, C., T. E. Dowling, and W. M. Brown.
1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18:269–292.

Moser, H. G.
1996. Scorpaenidae: scorpionfishes and rockfishes. *In* The early stages of fishes in the California Current region (H. G. Moser, ed.), p. 793–795. CalCOFI Atlas 33.

Orr, J. W., and J. E. Blackburn.
2004. The dusky rockfishes (Teleostei: Scorpaeniformes) of the North Pacific Ocean: resurrection of *Sebastes variabilis* (Pallas, 1814) and a redescription of *Sebastes ciliatus* (Tilesius, 1813). Fish. Bull. 102:328–348.

Rocha-Olivares, A.
1998. Multiplexing haplotype-specific PCR: a new approach for species identification of the early life stages of rockfishes of the species-rich genus *Sebastes* Cuvier. J. Exp. Mar. Biol. Ecol. 231:279–290.

Rocha-Olivares, A., H. G. Moser, and J. Stannard.
2000. Molecular identification and description of pelagic young of the rockfishes *Sebastes constellatus* and *Sebastes ensifer*. Fish. Bull. 98:353–363.

Rocha-Olivares, A., R. H. Rosenblatt, and R. D. Vetter.
1999. Molecular evolution, systematics, and zoogeography of the rockfish subgenus *Sebastomus* (*Sebastes*, Scorpaenidae) based on mitochondrial cytochrome *b* and control region sequences. Mol. Phy. Evol. 11:441–458.

Rokas, A., E. Ladoukakis, and E. Zouros.
2003. Animal mitochondrial DNA recombination revisited. Trends Ecol. Evol. 18:411–417.

Sambrook, J., E. F. Fritsch, and T. Maniatis.
1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seeb, L. W.
1986. Biochemical systematics and evolution of the Scorpaenidae genus *Sebastes*. Ph.D. diss., 176 p. Univ. Washington, Seattle, WA.

Seeb, L. W., and A. W. Kendall Jr.
1991. Allozyme polymorphisms permit the identification of larval and juvenile rockfishes of the genus *Sebastes*. Environ. Biol. Fish. 30:191–201.

Seutin, G., B. N. White, and P. T. Boag.
1991. Preservation of avian blood and tissue samples for DNA analysis. Can. J. Zool. 69:82–90.

Appendix 1

Restriction site locations for *Sebastes* spp., *Sebastolobus alascanus,* and *Helicolenus hilgendorfi* in the ND3/ND4 and 12S/16S mtDNA regions. Letters denote haplotypes (restriction digest patterns). "X" denotes presence of site. "O" denotes absence of site.

ND3/ND4 *Bst*N I sites	haplotypes																							
	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	V	W	X	Y	Z	a	b	c	g
112	O	X	X	X	X	X	X	X	X	X	O	X	X	X	X	O	O	X	X	O	X	X	X	X
291	O	O	O	O	X	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	X	X	O
551	O	O	O	O	O	O	O	X	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
829	X	X	O	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O	O	O	O	X	X	X
1035	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1214	X	X	X	O	O	X	X	O	O	O	O	O	X	O	X	O	X	O	X	O	X	X	X	X
1261	O	O	O	O	O	O	O	O	X	O	X	X	X	O	O	O	O	O	O	O	O	O	O	O
1495	O	O	O	O	O	O	O	O	O	O	X	X	O	O	O	O	O	O	X	O	O	O	O	O
1694	O	O	X	X	X	X	X	O	O	O	O	O	O	X	X	O	X	X	O	X	O	O	X	X
1982	O	O	O	X	X	O	X	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O
2010	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
2121	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	X	O	O
2255	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
2325	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	O	X	O

ND3/ND4 *Bst*U I sites	haplotypes												
	A	B	C	D	E	F	G	H	I	J	K	a	b
4[1]	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)
344	O	O	O	O	O	X	O	O	O	O	O	O	O
668	O	O	O	O	O	O	O	X	O	O	O	O	O
710	O	O	O	O	O	O	O	O	X	O	O	O	X
1499	O	O	O	O	X	O	O	O	O	O	O	O	O
1854	O	O	O	X	O	O	O	O	O	X	X	O	O
2025	O	X	X	O	O	O	O	X	O	O	X	X	X
2109	O	O	O	O	O	O	O	O	X	X	O	X	O
2306	O	X	O	O	X	X	X	O	O	O	O	X	O

[1] The site at 4 is in the primer region.

Cfo I sites	haplotypes											
	A	B	C	D	E	F	I	J	K	a	b	g
709	X	O	X	X	X	X	X	X	X	X	X	X
1221	O	O	O	O	X	X	X	O	O	O	O	O
1436	O	O	O	O	X	O	O	O	O	O	O	O
1464	O	O	O	O	O	O	O	X	O	X	O	O
1472	O	O	O	O	O	O	O	O	O	O	X	X
1741	O	X	X	X	O	O	X	X	X	O	X	X
1762	O	O	O	O	O	O	O	O	X	O	O	O
1813	O	O	X	O	O	O	O	O	O	O	X	O

ND3/ND4 *Dde* I sites	haplotypes																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	
65	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	
195	X	O	O	X	X	X	X	X	X	X	X	X	X	X	O	O	O	O	O	O	X	X	O	
266	O	O	O	O	O	O	X	O	X	O	O	X	O	O	X	X	X	O	O	O	O	O	O	
298	X	X	O	X	X	X	X	O	X	X	X	X	X	X	O	O	X	O	O	O	O	X	X	
336	X	X	X	X	X	X	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	X	X	X	O	X	O	

continued

Appendix 1 (continued)

ND3/ND4 (continued) *Dde* I sites	haplotypes																						
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
590	O	X	X	X	X	X	X	X	X	X	X	X	X	X	O	O	X	X	X	X	X	X	X
624	O	O	X	O	O	O	O	O	O	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	O	O	O	O	O	O
695	O	O	O	O	O	O	O	O	X	O	O	X	O	O	O	O	O	O	O	O	O	O	O
754	O	O	O	O	O	O	O	O	X	O	O	X	X	O	O	O	X	O	O	O	X	X	X
1144	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1190	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	X	X	O	O	O
1257	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O
1409	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O
1560	O	O	O	O	O	O	O	O	X	O	O	X	O	O	O	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	O	O	O	O	O	O
1587	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1601	X	O	X	X	X	X	X	X	X	X	X	X	X	X	O	O	O	X	X	X	X	X	X
1696	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O
1722	O	O	O	O	O	O	O	O	O	X	O	O	O	X	X	X	X	O	O	O	O	O	O
1779	O	O	X	O	O	O	O	O	X	O	O	X	X	O	O	O	O	X	X	X	O	O	X
1795	O	O	O	O	O	X	X	X	X	X	X	X	X	O	O	O	X	O	O	O	X	X	X
1912	X	X	X	X	X	X	X	X	O	X	X	X	X	X	O	O	X	X	X	O	X	X	X
2091	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	X	O	O
2122	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
2188	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	X	O	O	O	O	O	O	O
2374[2]	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)

ND3/ND4 *Dde* I sites	haplotypes (continued)																						
	X	Y	Z	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t
65	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
195	O	O	X	X	O	X	X	O	X	X	X	X	X	X	X	X	X	X	O	X	X	X	X
266	O	O	X	O	X	O	O	O	O	O	O	O	X	O	O	O	O	O	X	O	O	O	X
298	X	O	X	O	X	X	X	X	X	X	O	X	X	X	O	X	X	X	X	X	O	X	O
336	X	O	X	O	X	X	X	X	X	X	X	X	X	X	O	X	O	O	X	X	X	X	X
374	X	X	O	X	O	O	O	X	X	X	O	X	O	O	O	O	O	O	O	O	X	X	O
590	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
624	O	O	O	O	O	O	O	O	O	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	O	O	O	O	O	O	O
695	O	O	X	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
754	X	O	X	O	O	X	O	O	X	O	O	X	O	X	O	X	X	X	O	X	O	O	O
1144	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O
1190	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1257	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1409	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1560	X	X	X	X	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1577	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1587	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O
1601	X	X	X	X	X	O	X	O	X	X	X	X	O	X	X	X	X	X	O	O	X	X	X
1696	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1722	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1779	X	X	X	X	X	O	O	O	X	O	X	O	X	O	O	O	O	O	O	O	O	O	X
1795	X	X	X	X	X	X	X	X	X	X	X	O	X	O	X	X	O	X	X	X	X	X	X
1912	X	X	X	X	O	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2091	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
2122	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
2188	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
2374[2]	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)

continued

Appendix 1 (continued)

ND3/ND4 *Dde* I sites	haplotypes (continued)									Hind II sites	haplotypes						
	u	v	w	x	y	z	aa	bb	cc		A	B	C	E	F	b	g
65	O	O	O	O	O	O	O	O	O	308	O	O	O	O	O	O	X
195	X	X	X	O	X	X	X	X	X	320	O	O	O	O	X	O	O
266	O	X	O	O	O	O	O	X	O	324	O	O	O	O	O	X	O
298	X	X	X	O	X	X	X	X	O	978	O	O	O	O	O	X	O
336	O	X	X	X	X	X	X	X	O	1007	O	X	O	O	X	O	O
374	O	O	O	O	O	O	O	O	X	1071	O	O	O	X	O	O	O
590	O	X	X	X	X	X	X	X	X	1091	O	O	X	O	O	O	O
624	O	O	O	X	O	O	O	O	O								
663	O	O	O	O	O	O	O	O	O								
695	O	X	O	O	O	O	O	X	O								
754	X	O	O	O	O	O	X	O	O								
1144	O	O	O	O	O	O	O	O	O								
1190	O	O	O	O	O	O	O	O	O								
1257	O	O	O	O	O	O	O	O	O								
1409	O	O	O	O	X	X	O	O	O								
1560	O	X	O	O	O	O	O	O	O								
1577	O	O	O	O	O	O	O	O	O								
1587	O	O	O	O	O	O	O	O	O								
1601	X	X	O	X	X	O	X	X	X								
1696	O	O	O	O	O	O	O	O	O								
1722	O	O	O	O	O	O	O	O	O								
1779	O	X	O	O	O	O	X	X	X								
1795	X	X	X	X	O	O	X	X	X								
1912	X	X	X	X	X	X	X	X	X								
2091	O	O	O	O	O	O	O	O	O								
2122	O	O	O	O	O	O	O	O	O								
2188	O	O	O	O	O	O	O	O	O								
2374[2]	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)	(X)								

[2] The site at 2374 is in the primer region.

ND3/ND4 *Hinf* I sites	haplotypes															
	A	B	C	D	E	F	G	H	I	N	O	P	Q	a	b	g
43	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O
130	O	X	O	O	O	X	O	O	X	O	X	X	O	O	O	O
183	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O
389	O	X	X	O	O	O	O	X	O	O	O	O	O	O	O	O
472	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X
494	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
621	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O
853	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O
999	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O
1017	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O
1342	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O
1448	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O
1537	O	O	O	O	O	O	X	O	O	O	X	O	O	X	O	O
1755	O	O	O	O	X	X	O	O	O	O	O	O	O	O	O	O
1888	O	O	O	X	O	O	O	X	O	O	O	O	O	O	O	O
2011	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O
2232	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

continued

Appendix 1 (continued)

ND3/ND4 *Mbo* I sites	haplotypes																					
	A	B	C	D	E	F	G	H	I	J	K	M	N	O	P	R	V	a	b	c	d	e
150	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
190	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
198	X	X	X	X	X	X	X	X	X	X	X	O	O	X	X	X	X	X	X	X	X	X
261	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
272	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O
302	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	X	O	O	O	O
632	O	O	O	O	O	O	O	O	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	O	O	O	O	O	O	O	O
758	X	X	X	X	X	X	X	O	X	X	X	X	O	O	O	O	O	X	X	X	X	X
797	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O
861	O	O	O	O	O	O	X	X	O	O	O	O	O	O	O	O	O	O	O	X	X	O
886	O	X	O	O	O	O	O	O	O	O	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	O	O	O	O	O	O	O	O
940	X	X	X	X	X	X	X	X	X	X	X	X	X	O	O	X	X	X	X	X	X	X
971	O	X	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1029	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O	O	O
1270	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O
1481	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	X	O
1534	O	O	O	O	X	X	X	X	X	O	X	X	X	O	X	O	X	X	X	X	X	O
1647	O	O	X	X	O	X	O	O	X	X	X	O	X	X	X	X	X	O	X	X	X	X
1658	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1748	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
1979	X	X	X	X	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X	O	X	X
2016	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O
2339	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

ND3/ND4 *Mbo* I sites	haplotypes (continued)											
	f	g	h	i	j	k	l	m	n	p	q	r
150	O	O	X	O	O	O	O	O	O	O	O	O
190	O	O	O	O	X	O	O	O	O	O	O	O
198	X	X	O	X	X	X	X	O	X	X	X	X
261	O	O	O	O	O	O	O	O	X	O	O	O
272	O	O	O	O	O	O	O	O	O	O	O	O
302	O	O	O	O	O	O	O	O	O	O	O	O
632	O	O	O	O	O	O	O	O	O	O	O	O
648	O	O	O	O	O	O	O	O	O	O	O	O
758	X	X	X	X	X	X	X	X	X	X	X	X
797	O	O	O	O	O	O	O	O	O	O	O	O
861	O	O	O	O	O	O	O	O	O	O	O	O
886	O	O	O	O	O	O	O	O	O	O	O	O
900	O	O	O	O	O	O	O	O	O	O	O	O
940	X	X	X	X	X	X	O	X	X	O	O	X
971	O	O	O	O	O	O	O	O	O	O	O	X
1029	O	O	O	O	O	O	O	O	O	O	O	O
1270	O	O	O	X	O	X	O	O	O	O	O	O
1481	O	O	O	O	O	O	O	O	O	O	O	O
1534	O	X	X	X	O	O	O	X	X	O	X	X
1647	X	X	X	O	X	X	X	X	O	X	X	X
1658	X	O	O	O	O	O	O	O	O	O	O	O
1748	O	O	O	O	O	O	X	O	O	O	O	O
1979	X	O	X	X	X	X	X	X	X	X	X	X
2016	O	O	O	O	O	O	O	O	O	O	O	O
2339	O	O	O	O	O	O	O	O	O	O	O	X

continued

Appendix 1 (continued)

ND3/ND4 *Msp* I	haplotypes														
sites	A	B	C	D	E	F	G	H	L	M	a	b	d	e	g
30	X	X	X	X	X	X	X	X	X	O	X	X	X	X	X
933	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O
1199	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O
1230	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O
1261	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O
1541	O	O	O	O	O	O	O	X	O	O	O	X	O	O	X
1624	O	O	O	O	O	O	O	O	X	O	O	O	O	X	O
1738	X	X	X	X	X	X	X	O	X	X	X	X	X	X	X
1826	X	X	O	X	X	O	O	X	X	X	O	X	X	X	X
1844	O	O	O	X	X	O	O	O	O	O	O	O	X	O	O
2073	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O
2110	O	X	X	O	X	O	O	O	O	O	O	O	X	X	X

Rsa I	haplotypes																
sites	A	B	C	D	E	F	G	H	I	J	L	N	O	P	Q	R	a	b
346	O	O	O	X	X	O	X	X	O	O	X	X	O	X	O	O	O	O
561	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O
742	O	O	O	O	O	O	O	X	X	O	O	O	O	O	O	O	O	O
1077	O	O	O	O	O	X	O	O	O	O	X	X	O	O	O	O	O	O
1231	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O
1321	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O
1339	O	O	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O
1357	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O
1433	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O
1492	O	O	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O
1863	O	O	X	O	X	O	O	X	X	X	X	O	O	O	O	O	O	X
1985	O	X	X	X	X	X	X	X	X	X	X	X	X	O	X	X	X	O
2063	O	O	O	O	O	O	O	O	O	O	O	O	O	O	X	O	O	O

ND3/ND4 *Sty* I	haplotypes																
sites	A	B	C	D	E	F	G	H	I	L	M	N	O	b	d	e	g
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	O	O	O	O	O	O	O	O	O	O	X	O	O	X	O	O	X
194	O	O	O	X	X	X	O	O	X	O	O	X	O	X	O	O	O
365	X	X	X	X	O	O	X	X	X	X	X	X	O	X	O	X	X
391	O	O	O	O	X	X	O	O	O	X	O	O	O	O	O	O	O
534	X	O	X	X	X	X	O	O	X	X	X	X	X	X	O	X	X
1258	O	O	O	O	O	X	O	O	O	O	O	O	O	O	O	O	O
1570	O	X	X	X	X	X	O	O	O	X	X	O	X	O	X	X	O
1725	O	O	O	O	O	O	X	O	O	O	O	X	O	O	O	X	O
2311	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

12S/16S *Bst*N I	haplotypes						
sites	A	B	C	D	E	F	G
326	X	X	X	X	O	X	X
988	O	O	O	X	O	X	O
1105	O	O	O	O	O	X	O
1530	O	O	O	O	O	O	X
1687	O	O	X	O	O	O	O
1741	O	X	O	O	O	O	O
2416	X	X	X	X	X	X	X

Appendix 1 (continued)

12S/16S *Bst*U I sites	haplotypes A	B	C	D	E	F
87	X	X	X	X	X	X
600	X	O	X	X	O	X
664	X	X	X	X	X	X
729	X	X	O	O	X	X
1633	O	O	O	O	X	O
1872	X	X	X	X	X	X
2128	X	X	X	X	X	X
2191	O	O	O	X	O	X

Cfo I sites	haplotypes A	B	C	D
537	X	X	O	X
602	X	O	X	X
665	X	X	X	X
1630	O	O	O	X
1898	X	X	X	X
2268	X	X	X	X

Dde I sites	haplotypes A	B	C	D	E	F	G	H	I
44	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X
976	X	X	X	X	X	O	O	O	X
1043	X	X	O	X	X	O	O	O	X
1056	X	X	O	X	O	O	X	O	X
1304	X	X	X	X	X	O	X	X	X
1339	O	O	O	O	O	O	O	O	X
1735	O	X	X	X	X	O	X	X	X
2181	X	O	X	X	X	O	O	X	X
2393	X	X	X	X	X	X	X	X	X

Hinf I sites	haplotypes A	B	C	D	E	G	H	I
982	O	O	X	O	O	O	O	X
1181	O	O	O	O	O	X	O	O
1291	X	X	X	O	O	X	X	X
1700	O	O	O	O	O	O	X	X
2094	O	X	X	O	X	O	X	X

Hind II sites	haplotypes A	B
1717	X	O

12S/16S *Mbo* I sites	haplotypes A	B	C	E	F	G	H	I
201	O	X	X	O	O	X	X	X
849	X	X	X	O	X	X	X	X
1015	X	X	X	X	O	O	X	X
1210	O	O	O	O	O	O	O	X
1403	X	X	X	X	X	X	X	X
1507	X	O	X	X	X	X	X	X
1637	O	O	O	O	O	O	X	O
1984	X	X	X	X	X	X	X	X
2059	X	X	X	X	X	X	X	X
2228	X	X	X	X	X	X	X	X
2318	X	X	X	X	X	X	X	X
2388	X	X	X	X	X	X	X	X

Msp I sites	haplotypes A	B	C	D	E	F
65	X	X	X	X	X	X
241	O	O	O	O	O	X
330	O	O	X	X	O	O
766	X	X	X	X	X	X
1259	O	X	X	O	O	X
1390	X	X	X	X	X	X
1535	X	X	X	X	O	X
2226	X	X	X	X	X	X
2403	X	X	X	X	X	X

Rsa I sites	haplotypes 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

Sty I sites	haplotypes A	B	C	D
295	X	O	X	X
982	O	O	O	X
1748	O	O	X	O
2053	O	X	O	O
2254	X	X	X	X