

Electrophoretic Identification of Juvenile Rockfish (Genus *Sebastes*) Recruiting to Drifting Algae and Seagrass Habitats Off the Washington Coast

Abstract

Drifting algae and seagrasses floating on the surface of Washington's marine waters form a unique and ecologically important habitat for the early life history stages of some species of Pacific rockfishes (genus *Sebastes*). Investigations aimed at understanding the significance of this habitat as an intermediary step toward the benthic and midwater environments favored by adult rockfish have been plagued by difficulties in accurately identifying the species of juvenile rockfish encountered during habitat sampling. The difficulty stems from the many sympatric species that share similar morphological features and overlapping meristics as larvae and juveniles. In this study, we successfully used allozyme data obtained from 37 presumptive gene loci in 11 reference species to confirm the identities of 149 juvenile splitnose rockfish (*S. diploproa*) and six black rockfish (*S. melanops*). Genotypes from 29 juveniles did not match those from any of the reference species and could not be positively identified. Future efforts employing molecular techniques for the identification of rockfish species during early life history phases would benefit from the application of recent advances in DNA methodologies such as the analysis of mtDNA or microsatellites.

Introduction

The study presented here is part of an ongoing ecological investigation of juvenile fish recruitment to drifting habitat off the coast of Washington, and in the Strait of Juan de Fuca and the San Juan Archipelago. The habitat often harbors juvenile fishes from a wide variety of taxa, with juvenile rockfishes (Scorpaenidae; *Sebastes* spp.) being the most abundant in species and numbers, and an important group of fishes both ecologically and in fishery harvests. Pelagic juveniles of some rockfish species recruit to drifting habitat where they feed and grow; however, the extent to which this habitat provides a recruitment pathway to benthic and midwater environments favored by the adults of most species is incompletely understood. Studies conducted by Boehlert (1977), Buckley et al. (1995), and Kokita and Omori (1998), suggest that for some rockfishes, drifting habitat is at least important, and may be an obligate step in the recruitment pathway.

In order to understand the importance of drifting habitats to the early life history stages of rockfishes, it is necessary to identify to species each of the fish collected during habitat sampling. Identification methods for juvenile *Sebastes* rely heavily on differences in pigmentation and meristics (Moser et al. 1985, Matarese et al. 1989, Kendall 1991, Moreno 1993, Sakuma and Laidig 1995);

however, these methods are often unreliable due to similarities in gross morphology and overlapping meristics among the many species known to occur sympatrically in the northeast Pacific. Identification is further hampered by a lack of complete descriptive early life history series for some species. More novel approaches using bony structures such as otoliths (Laidig and Ralston 1995), cleithra (Silberberg 1991), and the bones of the caudal complex (Sanchez and Acha 1988, Laidig et al. 1991) have proven useful for distinguishing some species, but rarely lead to unequivocal identification, especially in the absence of other identifying features.

Electrophoretic analysis of protein allozymes can provide unambiguous identification when fixed allelic differences occur among species at one or more loci, or, nearly certain identification when the frequency of shared alleles at one or more loci differ substantially among species. Further, while morphologic and meristic features may change throughout the life history of an animal, numerous studies conducted over a wide range of fish taxa substantiate the tendency for allozyme electromorphs to be ontologically stable (Morgan 1975, Smith and Crossland 1977, Seeb and Kendall 1991). Thirty-two loci examined by Seeb and Kendall (1991) in 10 species of *Sebastes* exhibited similar levels of activity and resolution between adults and juveniles.

Allozymes have been described from the adults of several *Sebastes* species by Johnson et al. (1970a, 1970b, 1971, 1972, 1973), Wishard et al. (1980), Jungmann (1983), and Seeb (1986). Seeb and Kendall (1991) and Seeb (1993) discussed the relative merits of classical taxonomic and modern molecular approaches to rockfish species identification and used allozymes and mtDNA haplotypes to identify larval and juvenile *Sebastes*. More recently, Seeb (1998) used allozymes and mtDNA to investigate genetic differentiation and introgression within and among three species of *Sebastes* from the Pacific Northwest and Sévigny et al. (2000) used a single allozyme locus to distinguish between, and describe the distribution of, two species of north Atlantic *Sebastes* from the Gulf of St. Lawrence. In this study, we used allozyme data obtained from 37 presumptive gene loci in 11 reference species to identify 155 juvenile rockfish of unknown or uncertain identity.

Methods

Field Sampling

Juvenile *Sebastes* spp. were collected by dip net from drifting mats of vegetation off the coasts of Washington and southern British Columbia. For details on sampling methods and species composition see Buckley et al. in preparation. Forty-one juveniles ranging in size from 20 - 66 mm (TL) were captured over a 7-day period beginning on July 10, 1997; and 149 juveniles ranging in size from 16 - 67mm (TL) were captured over an 8-day period beginning on June 27, 1998. Each juvenile fish was placed in an air-tight plastic bag and kept frozen in the field with dry ice for up to 10 days before being transferred to a -70°C freezer for storage. Juveniles were tentatively identified to species based on gross morphology (Matarese et al. 1989), known geographic distribution (Hart 1980) and previously reported associations with drift habitat (Hitz 1961, Mitchell and Hunter 1970, Boehlert 1977, Brodeur and Pearcy 1986, Moser 1991, Buckley et al. 1995). Six individuals were later positively identified as *S. nigrocinctus* based on distinguishing morphologic characteristics observed in previous aquarium grow-out studies conducted by the second author and were not electrophoresed.

Reference species were selected based on the tentative identification of each juvenile and on the relative abundance of species landed in com-

mercial and sport fisheries in the study area. Adults of the following species were sampled: *S. alutus*, *S. entomelas*, *S. melanops*, *S. flavidus*, *S. pinniger*, *S. helvomaculatus*, *S. babcocki*, *S. paucispinis*, *S. aleutianus*, *S. diploproa*, and *S. caurinus*. *Sebastes caurinus* were spearfished by SCUBA divers on an artificial reef in southern Hood Canal, Washington. All others were obtained from commercial landings of trawl and longline vessels at Westport, Washington. Approximately 1cc of muscle, heart, liver, and retinal tissue were dissected from each reference species, placed into test tubes, and kept frozen with dry ice for up to 8 hours before being transferred to a -70°C freezer. In addition, pectoral fin tissue from each fish was archived in ethanol for possible future DNA studies. Spearfished samples were dissected immediately after the fish were killed. Whole fish from commercial harvests were stored at sea on wet ice for up to 5 days prior to dissection.

Laboratory Analysis

We used horizontal starch-gel electrophoresis following the general methods of Acbersold et al. (1987). Muscle, liver, and retinal tissue from a single representative of each reference species was assayed on multiple buffers in order to optimize screening protocols and to identify potentially fixed allelic differences. Thirty-seven loci were adequately resolved and subsequently screened in up to twenty adults from each species to determine if observed among-species differences were maintained in larger samples. The loci, along with the buffers on which they were screened, are listed in Table 1.

A locus was judged polymorphic when the frequency of the most common allele within any of the species screened was ≤ 0.95 . Alleles that occurred at frequencies greater than 0.95 in only one of the reference species were considered diagnostic. Alleles were defined in terms of electrophoretic mobility relative to the mobility of the most common allele at each locus in a reference sample of *S. alutus*.

Skeletal muscle was dissected from each juvenile and electrophoresed alongside muscle from selected adult reference samples with known genotypes. In addition, liver tissue was dissected from six juveniles whose identity, based on gross morphology and meristics, was believed to be either *S. melanops* or *S. flavidus* (see results for explanation).

TABLE 1. Enzymes, loci, and buffers used in the electrophoretic analysis of 11 species of Pacific rockfish (genus *Sebastes*). Except where noted, all loci were resolved from skeletal muscle.

Enzyme (abbreviation)	EC number (IUBMBNC 1992)	Locus	Buffer ^a
Aspartate aminotransferase (AAT)	2.6.1.1	<i>mAAT-1*</i> <i>sAAT-1*</i> ^{b,c}	V II
Acid phosphatase (ACP)	3.1.3.2	<i>ACP*</i> ^c	V
Alcohol dehydrogenase (ADH)	1.1.1.1	<i>ADH*</i> ^c	V
Aconitate hydratase (AH)	4.2.1.3	<i>AH-1*</i> ^c <i>AH-2*</i> <i>AH-3*</i>	V V V
Alanine aminotransferase (ALAT)	2.6.1.2	<i>ALAT*</i>	I
beta-N-Acetylgalactosaminidase (bGALA)	3.2.1.53	<i>bGALA-2*</i>	IV
Creatine kinase (CK)	2.7.3.2	<i>CK-A*</i>	I
Esterase-D (ESTD)	3.1.1.56	<i>ESTD*</i>	I
Fructose-bisphosphate (FBP)	3.1.3.11	<i>FBP-1*</i> ^c <i>FBP-2*</i>	III III
Formaldehyde dehydrogenase (FDHG)	1.2.1.1	<i>FDHG*</i>	I
Fumarate hydratase (FH)	4.2.1.2	<i>FH*</i>	V
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	1.2.1.12	<i>GAPDH*</i>	V
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	<i>G3PDH*</i>	III
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	<i>GPI-A*</i> <i>GPI-B*</i>	I I
L-Iditol dehydrogenase (IDDH)	1.1.1.14	<i>IDDH*</i> ^c	II
Isocitrate dehydrogenase (IDHP)	1.1.1.42	<i>IDHP-1*</i> ^{b,c} <i>IDHP-2*</i>	V V
L-Lactate dehydrogenase (LDH)	1.1.1.27	<i>LDH-A*</i> <i>LDH-C*</i> ^b	V V
Malate dehydrogenase (MDH)	1.1.1.37	<i>mMDH*</i> <i>sMDH-A*</i> <i>sMDH-B*</i>	V V V
Mannose-6-phosphate dehydrogenase (MPI)	5.3.1.8	<i>MPI*</i>	I
Cytosol nonspecific dipeptidase (PEPA)	3.4.13.18	<i>PEPA*</i>	I
Tripeptide aminopeptidase (PEPB)	3.4.11.4	<i>PEPB*</i>	V
Phosphogluconate dehydrogenase (PGDH)	1.1.1.44	<i>PGDH*</i>	V
Phosphoglycerate kinase (PGK)	2.7.2.3	<i>PGK*</i>	V
Phosphoglucomutase (PGM)	5.4.2.2	<i>PGM-1*</i>	I
Pyruvate kinase (PK)	2.7.1.40	<i>PK-2*</i> ^c	IV
Superoxide dismutase (SOD)	1.15.1.1	<i>SOD*</i>	III
Triose-phosphate isomerase (TPI)	5.3.1.1	<i>TPI-1*</i> <i>TPI-2*</i>	I I

^aI = TRIS-GLY (tris-glycine) pH 8.5 (Holmes and Masters 1970); II = LiOH-RW (lithium hydroxide, modified by Ridgway) electrode pH 8.0, gel pH 8.2 (Ridgway et al. 1970); III, IV, V = CAME (citrate-amine) pH 5.9, 6.5, and 6.8 respectively (Clayton and Tretiak 1972) modified with 1 mM EDTA.

^bResolved from retinal tissue.

^cResolved from liver tissue.

Results

The most common allele at eight nonpolymorphic loci (*mAAT**, *FBP-2**, *FDHG**, *IDHP-2**, *LDH-C**, *mMDH**, *sMDH-A**, and *TPI-1**) exhibited the same electrophoretic mobility in each of the 11 reference species. Significant ($P = 0.05$) allele frequency differences were detected among species at seven nonpolymorphic loci (*CK-A**, *GAPDH**, *LDH-A**, *sMDH-B**, *PGK**, *PK-2**,

and *SOD**) and 22 polymorphic loci (*sAAT**, *ACP**, *ADH**, *AH-1**, *AH-2**, *AH-3**, *ALAT**, *bGALA-2**, *ESTD**, *FBP-1**, *FH**, *G3PDH**, *GPI-A**, *GPI-B**, *IDDH**, *IDHP-1**, *MPI**, *PEPA**, *PEPB**, *PGDH**, *PGM-1**, *TPI-2**). Of these, 10 exhibited alleles that were diagnostic for seven species (three for *S. paucispinus*, two each for *S. alutus*, *S. entomelas*, and *S. helvomaculatus*, and one each for *S. aleutianus*, *S. caurinus*, and *S. diploproa*) (Table 2).

TABLE 2. Allele frequencies from 10 diagnostic loci (frequency of allele >0.95 in only one species) observed in an electrophoretic analysis of 11 species of Pacific rockfish (genus *Sebastes*). Alleles that were diagnostic are listed in bold. Alleles are designated by their mobility relative to the most common allele (*100) in *Sebastes alutus*. A minus sign preceding an allele indicates cathodal migration.

Locus, number of fish successfully scored (N), and allele	Species										
	<i>S. alutus</i>	<i>S. entomelas</i>	<i>S. flaviventris</i>	<i>S. caurinus</i>	<i>S. melanops</i>	<i>S. paucispinis</i>	<i>S. habrococki</i>	<i>S. pinniger</i>	<i>S. aleutianus</i>	<i>S. diploproa</i>	<i>S. helvo-maculatus</i>
AH-2*											
N	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*53	0.000	0.000	1.000	0.000	1.000	0.000	1.000	0.000	0.000	0.929	0.000
*75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000
*81	0.000	1.000	0.000	1.000	0.000	1.000	0.000	0.975	0.000	0.000	1.000
*98	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000
*102	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000
AH-3*											
N	19	20	20	18	20	15	20	20	12	12	20
*100	0.974	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*109	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*111	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.950	0.000	0.000	0.000
*119	0.000	1.000	1.000	1.000	0.975	1.000	1.000	0.050	1.000	1.000	1.000
CK-A*											
N	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
*78	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
FH*											
N	19	20	20	18	20	15	20	20	19	13	20
*100	1.000	0.000	1.000	1.000	1.000	0.933	1.000	1.000	1.000	1.000	1.000
*71	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
*88	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000
GAPDH*											
N	19	20	20	18	20	10	12	20	7	13	20
*-100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
*-1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
IDHP-1**^a											
N	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	1.000	0.925	0.028	0.175	1.000	0.000	1.000	1.000	0.857	0.025
*60	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025
*69	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000
*72	0.000	0.000	0.075	0.000	0.825	0.000	1.000	0.000	0.000	0.071	0.000
*93	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.950
*121	0.000	0.000	0.000	0.972	0.000	0.000	0.000	0.000	0.000	0.000	0.000
LDH-A*											
N	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
*300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000

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TABLE 2. Continued

Locus, number of fish successfully scored (<i>N</i>), and allele	Species										
	<i>S. atunus</i>	<i>S. entomelas</i>	<i>S. flavidus</i>	<i>S. caurinus</i>	<i>S. melanops</i>	<i>S. paucispinis</i>	<i>S. habrebecki</i>	<i>S. pinniger</i>	<i>S. aleutianus</i>	<i>S. diploproa</i>	<i>S. helveticus</i>
<i>sMDH-B*</i>											
<i>N</i>	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	0.000	1.000	0.000
*56	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
*76	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	1.000
<i>PEPB*</i>											
<i>N</i>	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	0.000	1.000	0.000
*56	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
*76	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	1.000
*55	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.026	0.000	0.025
*63	0.000	0.000	0.000	0.000	0.000	0.967	0.000	0.000	0.000	0.000	0.000
*87	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000
*108	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000
*111	0.000	1.000	0.000	0.667	0.150	0.000	0.000	0.000	0.000	0.000	0.000
<i>SOD*</i>											
<i>N</i>	19	20	20	18	20	15	20	20	19	14	20
*100	1.000	1.000	1.000	0.000	1.000	0.000	1.000	1.000	0.000	0.000	0.000
*40	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
*152	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	1.000	1.000

* IDH-2 in Seeb (1985).

As previously noted, six juveniles were tentatively identified as either *S. flavidus* or *S. melanops*. Diagnostic alleles were not observed among the reference samples from these two species; however, the magnitude of the difference in allele frequencies at one locus, *IDHP-1**, was highly significant ($P < 10^{-5}$). Although *IDHP-1** is electrophoretically detectable in both eye and liver, the presence of an additional IDHP locus (*IDHP-2**) that exhibits nearly the same electrophoretic mobility as *IDHP-1** makes scoring either locus in eye difficult. Electrophoretic banding patterns obtained from the livers of all six juveniles were consistent with those observed most frequently in adult *S. melanops*. The identities of 149 juveniles that were field-identified as *S. diploproa* were confirmed through subsequent electrophoretic analysis alongside adult reference samples from that species. One allele (*108**) was diagnostic at the *PEPB** locus for *S. diploproa*, though its electrophoretic mobility was very near that of another

allele (*111**) at the same locus that was observed in three other species. On some occasions, the two alleles were difficult to distinguish; however, the other three species could be excluded based on genotypes at other loci. Multilocus genotypes from the remaining 29 juveniles, all of which exhibited similar morphologic features, did not match those of any of the reference species.

Discussion

An ideal molecular marker for species identification of early life history stages would exhibit the following attributes: 1) high interspecific variability; 2) low intraspecific average heterozygosity; 3) consistent expression throughout all life history stages; and 4) availability from a wide variety of tissue sources and quantities. With respect to these attributes, none of the three most widely used molecular approaches—mtDNA, microsatellite DNA, or allozymes—are ideal for *Sebastes*.

Amplification of microsatellite DNA loci using the polymerase chain reaction and flanking regions (primers) that are conserved among congeners may offer some advantages over the other two techniques. For instance, many allozymes are known to be expressed differentially among different organs. Dissecting organs from larvae or small juveniles can be problematic and the amount of tissue available may not yield electrophoretically detectable amounts of enzyme. Microsatellites, on the other hand, can be amplified from very small quantities of tissue from a wide variety of tissue sources, and, different tissues have the same DNA. Further, while dozens of published histochemical staining recipes exist for use in allozyme surveys, the number of loci resolved by them in any single species is typically on the order of 10-20. Microsatellites are believed to occur along the entire length of the chromosome. Thus, the number of loci is potentially very high, though their detection is limited by the availability of suitable primer sequences. Allozyme electromorphs are normally read by eye and proper genotyping of individuals relies heavily on the interpretive skills of the scorer and on maintaining an archive of high quality electromorph standards. Analyses of microsatellite loci generally involves the use of automated sequencing and imaging devices that can reduce the incidence of scoring error and the need for archived voucher specimens.

Digestion profiles obtained from mtDNA offer several of the same advantages as microsatellites and form the basis for much of the recently published literature on *Sebastes* systematics and population structure (Seeb 1998, Rocha-Olivares 1998, Rocha-Olivares et al. 1999a, 1999b, 1999c, 2000). Higher substitution rates in mtDNA, relative to nuclear DNA, may help distinguish among the most closely related *Sebastes* species. Disadvantages of mtDNA include fewer potential loci and longer fragment lengths that are more susceptible to degradation. A potential disadvantage of microsatellites lies in their tendency toward hypervariability. Greater within-species variability may render identifications less certain by increasing

the likelihood of not encountering low frequency alleles that are shared among species. This concern is especially pronounced in studies of *Sebastes* due to the large number of sympatrics. Assaying several loci would decrease the risk of mis-identification.

Although we've succeeded in employing allozymes to positively identify two species of *Sebastes*, our failure to identify all individuals underscores the importance of acquiring genotypic reference data from as many species as possible. Our assembly of reference data was based largely on the assumption that those species that occurred in greatest abundance as adults were the species most likely to recruit to drifting habitat as juveniles. Future studies would benefit from the inclusion of a larger suite of reference species. Further, future larval and juvenile *Sebastes* identification efforts would benefit from the application of DNA methodologies, especially in those instances where discernible differences among morphologically similar species can be resolved with allozymes only through the analysis of internal organs and/or where fixed allelic differences are not detected. Regardless of the molecular technique used, a thorough morphological assessment should be conducted prior to molecular analysis in order to narrow the field of possible species to a manageable few.

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