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Biochemical Genetics Of Southern California Basses Of The Genus Paralabrax - Specific Identification Of Fresh And Ethanol-Preserved Individual Eggs And Early Larvae

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Abstract. – Three species of basses of the genus *Paralabrax* are found in southern California coastal waters. Adults can be identified on the basis of morphological characters, but the eggs and early larval stages of the three species are extremely similar in appearance. This paper reports an investigation of biochemical genetic characters for specific identification. An electrophoretic analysis of 43 presumptive gene loci demonstrated several genetic differences between any two of the three species, but no single locus was able to unambiguously distinguish the three species. In contrast, an analysis of *Paralabrax* mitochondrial DNA (mtDNA) demonstrated that 8 of 13 informative restriction endonuleases produced species-specific fragment patterns. A technique is described for the relatively rapid enrichment and analysis of mtDNA from both fresh and ethanol-preserved individual eggs and early larvae which allows specific identification on a cost-effective basis.

Biochemical Genetics of Southern California Basses of the Genus *Paralabrax*: Specific Identification of Fresh and Ethanol-preserved Individual Eggs and Early Larvae

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The serranid genus *Paralabrax* is endemic to the eastern Pacific Ocean and comprises seven species. Three species, the kelp bass *P. clathratus*, barred sand bass *P. nebulifer*, and spotted sand bass *P. maculatofasciatus*, are common in southern California coastal waters and all are important components of the California sport fishery (Oliphant 1979). Adults of these three species can be separated on the basis of morphological differences (Miller and Lea 1972); however, it is not possible at this time to determine the specific identity of field-caught *Parlabrax* eggs, larvae, or early juveniles using morphological characters (Butler et al. 1982; R. Lavenberg, Los Ang. Cty. Mus. Nat. Hist., Los Angeles, CA 90007, pers. commun., June 1988). Ecological studies of the early life history of the three species, as well as stock assessment studies based on the early-life-history stages, have been hampered by the inability to specifically identify *Paralabrax* eggs and larvae.

A variety of biochemical characters have been used to identify the eggs and larval forms of closely related fishes in the absence of discriminating morphological characters. Electrophoresis of water soluble proteins (allozyme analysis) has been used to differentiate species of marine fishes which have morphologically similar eggs, larvae and early juvenile stages (Morgan 1975, Smith and Crossland 1977, Sidell et al. 1978, Smith et al. 1980, Mork et al. 1983, Graves et al. 1989). However, due to the small sizes of eggs and larvae, and the possibility of low enzyme activity during early-life-history stages, some studies have reported difficulty in resolving the electrophoretic bands of eggs and very small larvae (Smith and Crossland 1977, Sidell et al. 1978, Smith et al. 1980, Graves et al. 1989).

Restriction endonuclease analysis of mitochondrial DNA (mtDNA) has

been used to demonstrate intra- and interspecific genetic relationships among and within various marine and freshwater fishes (Ferris and Berg 1987). This technique provides an additional tool for the investigation of egg and larval identities. Although original protocols were costly, time-consuming, and generally not applicable to the small amount of tissue available with early-life-history forms, a modification of existing methodologies has provided a relatively simple method for distinguishing the eggs and larvae of closely related (congeneric) species. In this paper, we report the results of an investigation of water-soluble protein and mtDNA restriction fragment differentiation among the three southern California species of *Paralabrax* to find a reliable genetic marker to discriminate the early-life-history stages of the three species. A cost-effective means of analyzing mtDNA restriction fragments of both fresh and ethanol-preserved individual eggs is presented.

Materials and methods

Adult specimens of the three *Paralabrax* species were collected by hook and line, pole spear, and beach seine off San Diego and La Jolla, California. After collection, fish were transported to the laboratory alive or on ice. Samples of liver, eye, and muscle tissue were removed and frozen at −25°C until electrophoretic analysis was undertaken. Gonads and livers were removed and used fresh or stored at −70°C for mtDNA analysis.

Eggs and larvae of the three species were obtained from a captive brood stock maintained at the Southern California Edison research facility in Redondo Beach, California. Eggs were collected within 12 hours of spawning and either transported to the laboratory in San Diego in seawater or preserved in 95% ethanol.

Protein electrophoresis

Four specimens each of *Paralabrax maculatofasciatus* and *P. nebulifer* were collected for the electrophoretic investigation. The sample of *P. clathratus* (45) was larger because individuals were collected as part of a more extensive investigation of gene flow within southern California coastal fishes (Waples and Rosenblatt 1987). Samples of muscle, liver, and eye tissue were individually macerated in an approximately equal volume of 0.1 M potassium phosphate buffer, pH 7.0, before centrifugation for 10 minutes at 16,000 g, 5°C. Supernatants were loaded on horizontal starch gels using procedures similar to those described by Selander et al. (1971). Staining recipes for the 24 enzyme and protein systems studied (Table 1) were modified from Shaw and Prasad (1970) and Harris and Hopkinson (1976). A more detailed description of the electrophoretic procedures, including all staining recipes, is presented in Waples (1986).

Proteins encoded by multiple genes (isozymes) were numbered according to decreasing anodal mobility. For each gene locus the mobility of the most common allele in *Paralabrax clathratus* was arbitrarily designated 100, and alternate alleles were numbered in accordance with their mobility relative to this standard. Genetic similarity and genetic distance were computed from the allele frequency data using Nei's (1978) method, which corrects for small sample size.

Mitochondrial DNA analysis

Mitochondrial DNA for restriction analysis was purified from fresh or frozen gonad or liver samples from six individuals of each species following the protocols for equilibrium density gradient ultracentrifugation of Lansman et al. (1981), with minor modifications. Typical yields were 1 microgram of mtDNA per gram of fresh tissue.

A mini-prep procedure was developed for isolating mtDNA from small tissue samples based on the techniques described by Chapman and Powers (1984). Tissue samples as small as an individual egg (0.6 mg) were homogenized in 0.15 mL 10 mmol/L TRIS, 10 mmol/L EDTA, pH 7.4 (TE buffer) in a 0.2-mL ground glass homogenizer. The homogenate was transfered to a 1.5 mL microfuge tube and an additional 0.5 ml of TE buffer added. The tube was spun at 800 g, 4°C, for 3 minutes to remove nuclei and cellular debris. The supernatant was transferred to a second microfuge tube and centrifuged at 12,000 g, 4°C, for 20 minutes to pellet mitochondria. The mitochondrial pellet was resuspended in 0.4 mL TE buffer and lysed with 0.04 mL 10% SDS. The mitochondrial lysate was extracted with an equal volume of a 25:24:1 phenol/chloroform/isoamyl alcohol solution and then an equal volume of 24:1 chloroform/isoamyl alcohol. The DNA from the aqueous layer, after the addition of ammonium acetate, was precipitated in two volumes of 95% ethanol at −70°C for at least 2 hours.

The following restriction endonucleases employed in this study were purchased from Bethesda Research Laboratories (BRL) and used according to the supplier's instructions: *Ava*I, *Ava*II, *Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Eco*RI, *Hinc*II, *Hind*III, *Hin*fI, *Hpa*II, *Kpn*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Sst*I, *Xba*I, and *Xho*I. Endlabelling was performed according to the protocols of Brown (1980), with the exception that an ethanol precipitation was not necessary for digestions with restriction endonucleases that recognized six base pairs. Electrophoresis of restriction fragments was performed on both large and mini-submarine horizontal

Table 1

Enzymes and proteins surveyed and tissues and buffers used in the electrophoretic analysis of three species of *Paralabrax*.

Protein (EC number)	Locus	Buffer*	Tissue**
Aconitate hydratase (4.2.1.3)	Acon	1	l
Adenosine deaminase (3.5.4.4)	Ada	1	m
Adenylate kinase (2.7.4.3)	Ak	1	l
Alcohol dehydrogenase (1.1.1.1)	Adh-1	2	l
	Adh-2	2	l
Aspartate aminotransferase (2.6.1.1)	Aat-1,2	1,2	l
Creatine kinase (2.7.3.2)	Ck-A	2	m
	Ck-B	2	e
Esterase (3.1.1.-); α-naphthyl acetate	Est-1,2,3	3	m
Fumarte hydratase (4.2.1.2)	Fum	1	l
Glucose-6 phosphate dehydrogenase (1.1.1.49)	G6pdh	1	l
Glucosephosphate isomerase (5.3.1.9)	Gpi-A	2,3	m,l
	Gpi-B	2,3	m
Glutamate dehydrogenase (1.4.1.2)	Gdh	1	l
Glyceraldehyde-phosphate dehydrogenase (1.2.1.12)	Gapdh-1	1	l
	Gapdh-2	1	m
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	G3pdh-A	1	m
	G3pdh-B	1	l
Iditol dehydrogenase (1.1.1.14)	Iddh	1	l
Isocitrate dehydrogenase (NADP) (1.1.1.42)	Icdh-s	1	m
	Icdh-m	1	l
Lactate dehydrogenase (1.1.1.27)	Ldh-A	1	m,e
	Ldh-B,C	1	e
Malate dehydrogenase (1.1.1.37)	Mdh-A,B,m	1	m
Mannosephosphate isomerase (5.3.1.8)	Mpi	1	l
Phosphoglucomutase (5.4.2.2)	Pgm	2	m
Phosphogluconate dehydrogenase (1.1.1.44)	Pgdh	1	m
Peptidase (3.4.11.-); leucyl-tyrosine	Pep-1	3	m
Peptidase (3.4.11.-); leucylglycyl-glycine	Pep-2	3	m
Peptidase (3.4.11.-); non-specific	Pep-3	3	m
Superoxide dismutase (1.15.1.1)	Sod	2	l
Xanthine dehydrogenase (1.1.1.204)	Xdh	1	l
General proteins	Pro-1,2,3,4,5	3	m

**Buffers: 1 = Tris-citric acid, pH 6.9 (Whitt 1970); 2 = Tris-citric acid, pH 8.0 (Selander et al. 1971); 3 = Lithium hydroxide (Selander et al. 1971).

**m = muscle, l = liver, e = eye.

agarose gels (0.8–1.2%) and vertical polyacrylamide gels (3.5–5.0%) at 3 volts/cm.

The Southern transfer and hybridization protocols of Maniatis et al. (1982) were followed. Hybridization probe mtDNA (purified *Paralabrax* mtDNA) was nick translated with biotinylated dATP using the procedures of the BRL Nick Translation System. Visualization of the fragments followed the procedures included with the BRL BluGene Gene Detection Kit.

The mean mtDNA nucleotide sequence divergence between the three *Paralabrax* species was calculated from the number of shared fragments using the algorithm of Nei and Tajima (1983).

Results

In the electrophoretic analysis, 43 presumptive gene loci could be resolved in all three species (Table 1). The genetic interpretation of observed banding patterns was guided by expectations based on known patterns of tissue specificity of expression and subunit composition of enzymes in fishes and other vertebrates. Discussion of banding patterns for each enzyme system can be found in Waples (1986). Twenty-two loci were monomorphic (fixed for the same allele) in all individuals

Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 2
Allele frequencies at variable loci in three *Paralabrax* species.

Locus/Allele		*P. clathratus*	*P. nebulifer*	*P. maculatofasciatus*
Aat-1	130	—	—	0.167
	100	1.0	1.0	0.833
	2N	90	6	6
Ada	100	0.878	—	—
	90	0.111	—	—
	82	0.011	0.167	1.0
	60	—	0.833	—
	2N	90	6	8
Adh-2	-100	1.0	—	0.833
	-300	—	1.0	—
	-500	—	—	0.167
	2N	70	4	6
Ck-2	120	—	1.0	1.0
(Ck-A)	100	1.0	—	—
	2N	62	8	2
Est-3	108	—	—	1.0
	100	0.988	1.0	—
	94	0.012	—	—
	2N	82	6	6
Gapdh-2	100	0.989	—	1.0
	-1200	0.011	1.0	—
	2N	90	8	6
G3pdh-2	100	1.0	0.125	1.0
(G3pdh-A)	60	—	0.875	—
	2N	90	8	8
Gpi-2	150	—	0.25	—
(Gpi-B)	100	1.0	0.75	1.0
	2N	90	8	8
G6pdh	100	1.0	—	1.0
	90	—	1.0	—
	2N	50	4	4
Icdh-1	100	1.0	—	—
(Icdh-s)	95	—	1.0	1.0
	2N	10	6	6
Icdh-2	100	1.0	0.875	0.875
(Icdh-m)	95	—	0.125	0.125
	2N	40	8	8
Iddh	100	1.0	1.0	0.75
	-500	—	—	0.25
	2N	10	4	4
Mpi	100	0.986	—	—
	95	0.014	1.0	1.0
	2N	70	4	4
Pgdh	124	—	1.0	1.0
	100	1.0	—	—
	2N	40	6	4
Pgm	100	0.989	0.25	—
	73	0.011	0.75	1.0
	2N	90	8	8
Pep-1	108	0.012	—	—
	100	0.988	1.0	1.0
	2N	82	8	8
Pep-2	110	—	0.125	—
	100	1.0	0.875	1.0
	2N	42	8	8
Pep-3	110	0.011	—	—
	100	0.989	1.0	1.0
	2N	90	8	8
Pro-2	110	—	—	1.0
	100	1.0	1.0	—
	2N	90	8	8
Pro-4	100	1.0	—	1.0
	80	—	1.0	—
	2N	78	6	6
Sod	105	—	1.0	1.0
	100	1.0	—	—
	2N	80	8	8

sampled: Aat-2, Ak, Acon, Adh-1, Ck-B, Est-1, Est-2, Fum, Gpi-A, Gdh, Gapdh-1, G3pdh-B, Ldh-A, Ldh-B, Ldh-C, Mdh-A, Mdh-B, Mdh-m, Pro-1, Pro-3, Pro-5, and Xdh. The remaining 21 loci were polymorphic either within or between species (Table 2).

Genetic similarity values for the three pairwise comparisons (Table 3) were between 0.70 and 0.85, which is near the high end of the range of genetic similarity values found between congeneric fish species (Shaklee et al. 1982, Thorpe 1983). Nevertheless, each species pair can be distinguished on the basis of apparent fixed differences at multiple gene loci. The samples of *Paralabrax clathratus* and *P. nebulifer* shared no alleles at seven presumptive gene loci (Adh-2, Ck-A, G6pdh, Icdh-S, Pgdh, Pro-4, Sod); *P. clathratus* and *P. maculatofasciatus* were distinct at six loci (Ck-A,

Table 3
Values of genetic distance (above diagonal) and mtDNA nucleotide sequence differentiation (below diagonal) between three *Paralabrax* species. Standard errors of the estimates are in parentheses.

	P.c.	*P.n.*	*P.m.*
Paralabrax clathratus	—	0.304 (0.090)	0.265 (0.085)
Paralabrax nebulifer	0.145 (0.026)	—	0.165 (0.063)
Paralabrax maculatofasciatus	0.142 (0.027)	0.069 (0.019)	—

Est-3, Icdh-S, Pgdh, Pro-2, Sod); and *P. nebulifer* and *P. maculatofasciatus* were distinguished at six loci (Adh-2, Est-3, Gapdh-2, G6pdh, Pro-2, Pro-4). A number of loci were also identified that are capable of separating one species from each of the other two. For example, *P. clathratus* is fixed for alleles not found in *P. nebulifer* or *P. maculatofasciatus* at four gene loci (Ck-A, Icdh-S, Pgdh, Sod). Thus all three species can be distinguished using only two loci: one of the four for which *P. clathratus* has unique alleles, and one of the six that separates *P. nebulifer* and *P. maculatofasciatus*. A similar procedure can be used with the two loci (Adh-2, G6pdh) that distinguish *P. nebulifer* from the other two species or the two loci (Est-3, Pro-2) for which *P. maculatofasciatus* has unique alleles. No single locus was identified that by itself completely distinguishes all three *Paralabrax* species. The closest to a completely diagnostic locus is Ada; at this locus, a different allele predominates in each of the species at a frequency of 0.80 or more.

Considerable mtDNA sequence differentiation was demonstrated between the three *Paralabrax* species (Table 4). Six of the restriction endonucleases (*Bgl*II, *Cla*I, *Kpn*I, *Sal*I, *Sma*I, and *Xba*I) failed to cleave the circular mtDNA molecule more than once in each of three species and were therefore uninformative. Of the 13 restriction endonucleases which produced two or more fragments in the *Paralabrax* species, eight (*Ava*I, *Ava*II, *Bst*EII, *Hinc*II, *Hind*III, *Hinf*I, *Hpa*II, and *Xho*I) were able to distinguish all three species, while three enzymes (*Bam*HI, *Eco*RI, and *Sst*I) were able to distinguish one of the species from the other two, and two enzymes (*Pst*I and *Pvu*II) produced similar mtDNA fragments in all three species. Thus 8 of the 13 informative restriction endonucleases were diagnostic for all three species.

The mean nucleotide sequence divergences between the three *Paralabrax* species, in pairwise comparisons, are presented in Table 3. Because digestions with *Hinf*I, *Hpa*II, and *Ava*II (restriction endonucleases which recognize four or five nucleotide base pairs) produced so many fragments, it was not possible to assure homology of similarly migrating fragments, and the results from these enzymes were not used in the calculation of interspecific divergences.

Little intraspecific mtDNA nucleotide sequence variation was detected in this study. Of the 18 fish analyzed with the 13 informative restriction endonucleases, only two variants were encountered, each in a single individual (Table 4).

The species-specific fragment patterns produced from the digestion of *Paralabrax* mtDNA with the restriction endonuclease *Hind*III (Fig. 1) were considered the most practical to use for specific identification because there were diagnostic differences in the larger (most easily visualized) bands. Fresh or frozen tissue samples as small as 0.01 g could be identified from a *Hind*III digestion of the mtDNA enriched in a miniprep procedure and run on a 1.0% agarose minigel stained with ethidium bromide.

It was not possible to identify tissue samples smaller than 0.01 g on ethidium bromide-stained minigels

Table 4

Restriction fragment sizes (in kilobase pairs) in three species of *Paralabrax*. Not all fragments were resolved in digestions with *Ava*II.

Restriction endonuclease	*Paralabrax* species	Fragment sizes
*Ava*I	*clathratus*	7.4, 4.1, 2.1, 1.9
	maculatofasciatus	11.9, 2.6, 2.3
	nebulifer	9.2, 3.6, 2.7, 1.3
*Ava*II	*clathratus*	4.9, 2.7, 1.8, 1.2, 1.1, 0.7, 0.7, 0.5
	maculatofasciatus	2.6, 2.2, 1.7, 1.5, 1.5, 1.5, 1.3, 1.1, 1.0
	nebulifer	5.7, 1.9, 1.8, 1.5, 1.5, 1.3, 1.1, 1.0
*Bam*HI	*clathratus*	16.8
	maculatofasciatus	10.0, 6.8
	nebulifer	10.0, 6.8
*Bst*EII	*clathratus*	6.5, 5.3, 5.0
	maculatofasciatus	9.4, 7.4
	nebulifer	14.3, 2.5 (5) or 10.1, 4.2, 2.5 (1)
*Eco*RI	*clathratus*	8.8, 7.9
	maculatofasciatus	8.8, 7.9
	nebulifer	7.9, 4.6, 2.4, 1.9
*Hinc*II	*clathratus*	4.2, 2.8, 2.7, 2.4, 1.3, 1.2, 1.0, 0.7, 0.3
	maculatofasciatus	4.6, 3.6, 3.0, 2.7, 1.5, 0.7, 0.6
	nebulifer	4.0, 3.5, 3.0, 2.4, 1.6, 1.5, 0.7
*Hind*III	*clathratus*	6.2, 4.0, 2.2, 1.8, 0.7, 0.6
	maculatofasciatus	6.2, 4.7, 2.2, 2.0, 1.8
	nebulifer	6.2, 6.2, 2.2, 1.8, 0.4
*Pst*I	*clathratus*	12.6, 4.2
	maculatofasciatus	12.6, 4.2 (5) or 9.4, 4.2, 3.0 (1)
	nebulifer	12.6, 4.2
*Pvu*II	*clathratus*	11.6, 5.2
	maculatofasciatus	11.6, 5.2
	nebulifer	11.6, 5.2
*Sst*I	*clathratus*	14.0, 2.8
	maculatofasciatus	16.8
	nebulifer	16.8
*Xho*I	*clathratus*	12.1, 2.9, 1.8
	maculatofasciatus	16.8
	nebulifer	9.8, 4.8, 2.2

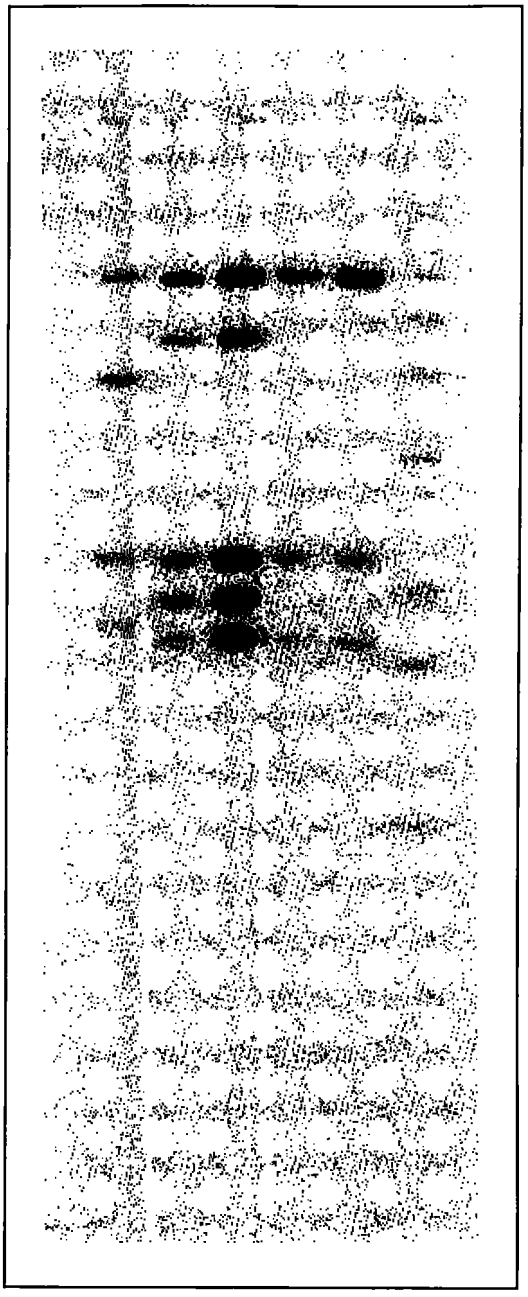

Figure 1
Species-specific fragment patterns of mtDNA isolated from three species of *Paralabrax* and digested with the restriction endonuclease *Hind*III. Note that the upper (larger) bands distinguish the three species. From left to right: *P. clathratus* (1), *P. maculatofasciatus* (2), *P. nebulifer* (2), and the size standard (BRL 1 Kb ladder). The mtDNA restriction fragments were endlabeled with radioactive nucleotide triphosphates, separated on a 1.0% agarose gel and visualized by autoardiography.

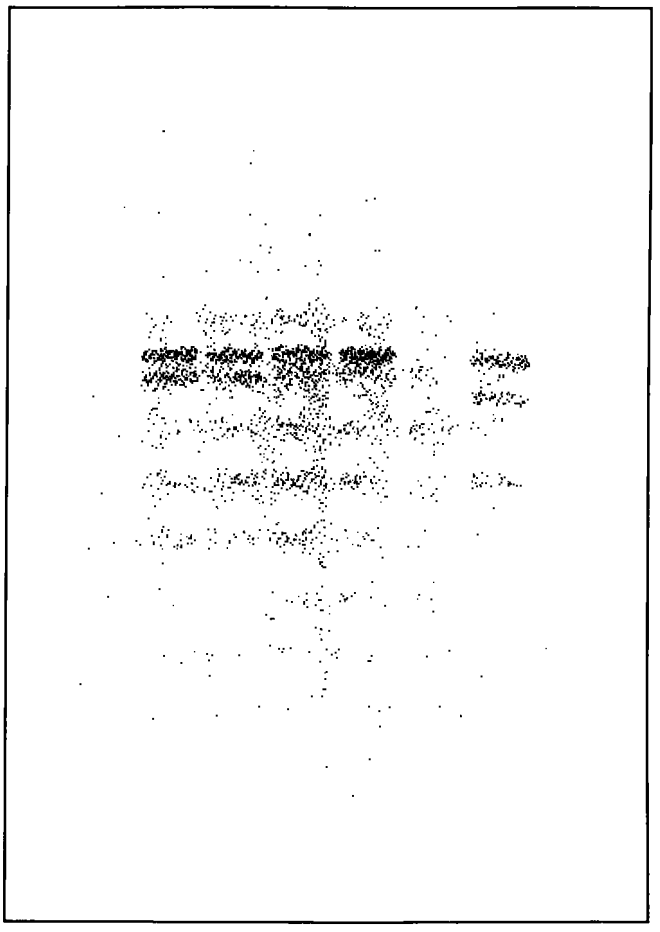

Figure 2
A Southern blot of *Hind*III digests of genomic DNA isolated from individual *Paralabrax* eggs and probed with biotinylated *Paralabrax* mtDNA. The eggs can easily be identified on the basis of the fragment patterns by comparison with those in Figure 1. One preparation (lane 5) did not contain sufficient DNA for identification. From left to right: *P. maculatofasciatus* (2), *P. nebulifer* (2), and *P. clathratus* (1).

because there was not sufficient mtDNA for visualization of the restriction fragments. However, samples as small as 0.6 mg (one *Paralabrax* egg) could be identified after Southern transfer and hybridization with a biotinylated probe (Fig. 2). Although greater sensitivity could have been obtained with a radioactively labeled probe, the results obtained with the biotinylated probe were sufficient to identify an individual fresh or ethanol preserved egg, and avoided the cost and problems of probe half-life and waste disposal associated with radioactively labeled probes.

Discussion

The results of the electrophoretic and mtDNA analyses are similar in several respects. First, the absolute level of genetic differentiation between the *Paralabrax* species is relatively small and is consistent with values typically found between other closely related organisms (Avise and Aquadro 1982, Ferris and Berg 1987). Second, the comparison of *P. maculatofasciatus* and *P. nebulifer* yields the smallest genetic distance of the three pairwise comparisons.

A major focus of the electrophoretic and mtDNA analyses was to find a biochemical character which would unambiguously differentiate the early-life-history stages of the three *Paralabrax* species found in southern California coastal waters. The electrophoretic investigation revealed several apparently fixed allelic differences between any two of the three species and demonstrated that a screening of a minimum of two electrophoretic loci will result in a positive specific identification. This methodology can be used to identify late larvae, juveniles, or adults in which there is sufficient tissue (or enzyme activity) to survey two or more loci. However, it was not always possible to score the diagnostic loci in *Paralabrax* eggs and larvae smaller than 10 mm total length. Although it may have been possible to improve the resolution of the electrophoretic techniques, we chose to rely upon mtDNA restriction fragment differences to identify *Paralabrax* eggs and early larvae.

The restriction endonuclease analysis of *Paralabrax* mtDNA, like the electrophoretic survey, demonstrated considerable genetic differentiation among the three species. Several restriction endonucleases were each capable of distinguishing the three species of basses. Furthermore, the mtDNA analysis not only worked with relatively large adult tissue samples, but with quantities as small as an individual egg. Consequently, the mtDNA analysis is the method of choice for the identification of *Paralabrax* early-life-history stages.

Several ecological and assessment studies involving *Paralabrax* eggs and early larvae have not been possible because of an inability to specifically identify the early-life-history forms. The mtDNA isolation and analysis techniques presented in this paper will facilitate such studies. Furthermore, because the methods work with ethanol-preserved as well as fresh specimens, there is no need to separate *Paralabrax* eggs and larvae from a fresh plankton tow while at sea.

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