BIOCHEMICAL GENETIC AND MORPHOLOGICAL DIVERGENCE AMONG THREE SPECIES OF THREAD HERRING (*OPISTHONEMA*) IN NORTHWEST MEXICO

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ABSTRACT

Three sympatric species of thread herring (*Opisthonema*) important in Mexican fisheries have been described solely on the basis of differences in numbers of gill rakers, taking standard length and geographic variation into account. Because the validity of these species has recently been questioned, we sought to reevaluate their taxonomic status. A combined electrophoretic and multivariate morphometric study of *Opisthonema* from northwest Mexico verifies the diagnostic accuracy of gill-raker number, confirms the status of the three species on biochemical genetic evidence, and suggests that external morphological characters might also be used to discriminate these species in the fishery. We infer from biochemical genetic data that *O. bulleri* diverged from the line leading to *O. medirastre* and *O. libertate* much before the separation of these last two species. How these three morphologically and genetically very similar, sympatric species of thread herring evolved in the eastern Pacific is problematic, particularly in light of evidence that *O. bulleri* and *O. medirastre* occasionally hybridize in nature.

RESUMEN

Las tres especies simpátricas de la sardina crinuda (*Opisthonema*), importantes en la pesquería mexicana, han sido descritas basandose sólo en el número de branquiespinas, pero tomando en cuenta la longitud patrón y el sitio geográfico de su captura. Debido a dudas recientes en cuanto a la validez de estas especies, hemos reevaluado su posición taxonómica. Una combinación de estudios electroforéticos, morfológicos multivariados de *Opisthonema*, provenientes de la región noroeste de México, verificó la precisión diagnóstica del número de branquiespinas y confirmó la validez taxonómica de las tres especies. Este análisis permitió además medir la divergencia evolutiva relativa entre estas especies y sugirió que características morfológicas externas podrían servir para distinguirlas. En base a los datos bioquímicos de la genética de estas tres especies, se infiere que *O. bulleri* se separó de la línea evolutiva de *O. medirastre* y *O. libertate* mucho antes de que estas últimas se separaran. La evolución de estas tres especies de sardina crinuda, simpátricas en el Pacífico Este, y muy semejantes en su genética y morfología es confusa, particularmente en vista de evidencia que *O. bulleri* y *O. medirastre* son ocasionalmente capaces de formar híbridos en la naturaleza.

INTRODUCTION

Five morphologically similar species of thread herring (*Opisthonema*) are recognized on the basis of their geographical distributions and numbers of gill rakers in relation to standard length (Berry and Barrett 1963). One species, *O. oglinum*, occurs in the western Atlantic and Caribbean; another, *O. berlangai*, is restricted to the Galápagos Islands; and the remaining three species, *O. bulleri, O. medirastre*, and *O. libertate*, are sympatric in the eastern Pacific from the Gulf of California to Peru. The sympatric species may be separated using Berry and Barrett's (1963) tabular key, which requires information on location of capture, standard length, and the number of ceratobranchial gill rakers on the first gill arch. For specimens captured in northern Mexico and exceeding 130 mm standard length, for example, ranges in numbers of gill rakers are 25–32 for *O. bulleri*, 40–69 for *O. medirastre*, and 59–109 for *O. libertate*. Individuals of the last two species having the same number of gill rakers have very different lengths. Berry and Barrett (1963) also examined eight meristic and six morphometric traits, but concluded that appreciable overlap in the ranges of these characters for species precluded their use in taxonomic separations. They did note, however, that ". . . some females tended to have slightly deeper bodies . . . than males."

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Before the development of purse seining, thread herring had economic significance as live bait in the tuna fishery. They regained value after 1971, when a large sardine fishery was initiated in the Gulf of California (Arvizu-Martinez 1987). Today, this sardine fishery contributes 27% of Mexico's total fisheries landings (M. G. Hammann, pers. comm., calculated from FAO 1986). Landings of thread herring in the northwest region of Mexico are usually much less than those of the Pacific sardine (Cisneros-Mata et al. 1987), but *Opisthonema* dominates landings in El Niño years and tends to be more abundant in the Mazatlán area in most years (Lluch-Belda et al. 1986). Because of the growing economic importance of this sardine fishery, scientific interest in *Opisthonema* has revived. Recently, the validity of the three Pacific coastal species of *Opisthonema* has been questioned (Rodriguez-Sanchez 1985; Lopez-Lemus 1986), although Rodriguez-Dominguez (1987) supports the classification of Berry and Barrett (1963).

In order to settle this taxonomic controversy and to contribute new information on speciation in the genus *Opisthonema*, we applied the techniques of biochemical genetics (allozyme electrophoresis) and multivariate morphometry—tools not available to Berry and Barrett in 1963—to the systematics of thread herring in northwest Mexico. The results we present here fully support the taxonomy of Berry and Barrett. We verify the diagnostic accuracy of gill-raker counts on the basis of congruent, diagnostic, biochemical genetic differences and suggest further that the three species may have distinctive body shapes. Discriminant functions based on a few traits may provide a more practical means than gill-raker counts for species identifications by fisheries biologists working in the field. Finally, from biochemical genetic data, we estimate times of divergence among these closely related species. *O. bulleri* apparently diverged from the line leading to *O. medirastre* and *O. libertate* about 3.2 million years ago, nearly 2 million years earlier than these last two species separated from each other.

MATERIALS AND METHODS

Collections and Samples

Thread herring were obtained from Bahía Magdalena, Baja California Sur (9 collected from a boat at the dock in San Carlos in July 1985); Guaymas, Sonora (21 taken by panga, March 3, 1987); and Mazatlán, Sinaloa (48, 44, and 45 taken in separate trawls by the vessel *Calafia* on the night of March 3, 1987; 44 taken by the vessel *Hapemsa III* on March 27, 1987).

Samples from Bahía Magdalena were frozen and returned whole to Bodega Marine Laboratory (BML), where they were stored in an ultracold freezer at −65° to −70°C until May 1987. The Gulf of California samples were returned on ice to Centro de Investigaciones Biologicas, La Paz, where their first gill arches were removed for gill-raker counts; a numbered label was attached to a gill cover on each specimen in order to maintain identity; and the fish were then stored in an ultracold freezer until transport to the BML in May 1987.

Subsequently, 200 of these fish were classified to species using the gill raker-standard length tabular key of Berry and Barrett (1963) for northern Mexico. Gill-raker counts were not obtained for 11 fish. Of these, 10 were assigned to species on the basis of allozyme phenotypes found to be congruent with the gill-raker diagnoses of the first 200 fish (see Results); one fish was inferred to be a hybrid between *O. bulleri* and *O. medirastre* (see Results). The species compositions of the six collections were as follows: Bahía Magdalena (7 *O. medirastre*, 2 *O. libertate*); Guaymas (all *O. libertate*); *Calafia* 1 (4 *O. bulleri*, 6 *O. medirastre*, 37 *O. libertate*, and 1 hybrid); *Calafia* 2 (6 *O. bulleri*, 5 *O. medirastre*, 33 *O. libertate*); *Calafia* 3 (2 *O. bulleri*, 7 *O. medirastre*, 36 *O. libertate*); and *Hapemsa III* (all *O. bulleri*). Summing the collections, 56 *O. bulleri*, 25 *O. medirastre*, 129 *O. libertate*, and 1 hybrid were obtained.

Electrophoresis

Samples of eye, heart, liver, and epaxial muscle tissues were dissected from each specimen after morphometric and wet-weight measurements were taken (see below). Tissue samples were placed in labeled plastic well trays with equal volumes of 0.5 M Tris-HCl pH 7.1 buffer; trays were covered and frozen in an ultracold freezer at −60°C. Sample trays were removed from the freezer the day before their first use and placed on trays of ice so that tissues thawed slowly. Homogenization of thawed tissue samples was done by hand-held ground-glass pestle. Trays with homogenized samples were refrozen overnight in the ultracold freezer.

Methods for horizontal starch-gel electrophoresis, protein assays, and genetic interpretation of zymograms were substantially the same as those described previously (Ayala et al. 1973; Tracey et al. 1975; Utter et al. 1987). The protocol used to separate and resolve 19 enzymes and proteins is

TABLE 1
Starch-Gel Electrophoretic Protocol Used To Reveal Variation among Thread Herring (*Opisthonema*)

Enzyme or protein (abbr.)	E.C. no.	Tissue[a]	Buffer[b]	No. of loci
Aspartate aminotransferase (AAT)	2.6.1.1	E,L	B	1
Alcohol dehydrogenase (ADH)	1.1.1.1	E	F	1
Esterase (EST)	(nonspecific)	L	E	1
Fumarate hydratase (FUM)	4.2.1.2	M	C,D	1
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	M	B	1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	1.2.1.12	E,L,M	C,D	1
Isocitrate dehydrogenase (IDH)	1.1.1.42	L	D	1
Lactate dehydrogenase (LDH)	1.1.1.27	E+L+M	B	3
Malate dehydrogenase (MDH)	1.1.1.37	M	F	1
Malic enzyme (ME)	1.1.1.40	H	F	1
Di- and tri-peptidases				
(GL) L-gly-leu	3.4.13.11	E	B	1
(LGG) L-leu-gly-gly	3.4.13.11	E	B	1
(LV) L-leu-val + L-leu-tyr	3.4.13.11	E	B	2
(PP) L-phenylalanyl-proline	3.4.13.9	E	B	1
Phosphoglucomutase (PGM)	2.7.5.1	M	A	1
6-phosphogluconate dehydrogenase (6PGDH)	1.1.1.44	E,L	C,D	1
General proteins (PROT)	(nonspecific)	E+M	A	8
Superoxide dismutase (SOD)	1.15.1.1	L	B	1
Triosephosphate isomerase (TPI)	5.3.1.1	M	B,F	1
Totals 19 proteins				29 loci

[a]E, eye; H, heart; L, liver; M, muscle.
[b]Buffers A, B, C, and D as described by Tracey et al. (1975); buffer E is the lithium borate discontinuous buffer system 2 of Selander et al. (1971); and buffer F is the Tris-maleate-EDTA buffer XVIII of Shaw and Prasad (1970).

summarized in Table 1. Proteins are referred to by capitalized abbreviations (Table 1), loci by these same abbreviations italicized in upper and lower case. The isozymes of LDH are distinguished by suffixes A, B, C (Shaklee et al. 1973). Numerical suffixes distinguish among isozymes or multiple proteins in order of increasing anodal migration. Alleles are symbolized by italicized numerals that express millimeter differences in electrophoretic separation of variants from the most common electromorphs observed for each protein. Alleles encoding common electromorphs are arbitrarily designated *100*. Specimens from different population samples were included in every electrophoretic run so that repeated comparisons of the relative mobilities of their allozymes could be made.

For statistical analyses, individuals were grouped into the following nine population samples:

(1) 12 *O. bulleri* pooled from the *Calafia* samples
(2) 44 *O. bulleri* from the *Hapemsa III* sample
(3) 7 *O. medirastre* from Bahía Magdalena
(4) 18 *O. medirastre* pooled from the *Calafia* samples
(5) 21 *O. libertate* from Guaymas
(6) 2 *O. libertate* from Bahía Magdalena
(7), (8), and (9) 37, 33 and 36 *O. libertate* from *Calafia* samples 1, 2, and 3, respectively.

There was no evidence for genetic heterogeneity among the small collections pooled to obtain populations (1) and (4). After testing for heterogeneity of allelic frequencies, conspecific samples were pooled for calculation of interspecific genetic similarities and distances.

Single-individual genotypes were recoded as alphabetical characters, and entered into the BIOSYS-1 program of Swofford and Selander (1981) for calculations of allelic frequencies; average proportions of heterozygous individuals per locus (observed by direct count, H_o, and expected, H_e, according to Nei's [1978] unbiased estimate); proportions of loci polymorphic per population (P, where a locus is considered polymorphic if the frequency of the most common allele does not exceed 0.99); goodness-of-fit tests to Hardy-Weinberg-Castle (H-W-C) equilibrium phenotypic proportions using Levene's (1949) correction for small sample size; and Nei's (1978) unbiased measures of genetic similarity (I) and distance (D). Log-likelihood ratio (G) tests of differences in allelic frequencies among population samples of *O. libertate* were calculated from absolute frequencies after appropriate pooling of rare alleles. The unweighted pair-group method with arithmetic averaging (UPGMA) in the CLUSTER subroutine of BIOSYS was used to estimate genetic distances for the linkages among species.

Morphometrics and Meristics

Measurements were made with vernier calipers on the left side of each frozen fish after its surface had thawed. Sixteen segments of a box truss network (Bookstein et al. 1985) were defined by eight anatomical landmarks: tip of snout, posterior edge of the supraoccipital at the midline, origin of the dorsal fin, anteroventral edge of the preopercular, origin of the pectoral fin, origin of the pelvic fin, origin of the anal fin, and the end of the medial hypurals (Figure 1). Measurements between landmarks are denoted by syntheses of landmark abbreviations; e.g., the distance from the origin of the dorsal fin to the origin of the pectoral fin is called DORFPELV. In addition, measurements were made from tip of snout to the anterior edge of the orbit (SNTORBIT), of standard length (tip of snout to end of the medial hypurals: STANDARD), and of interorbital width at posterodorsal orbit margins (INT). We also recorded whole body and gonad wet weights, sex, and a subjective index of reproductive condition, ranging from 0 (completely undifferentiated) to 4 (ripe).

Statistical analyses of these data were performed with the BMDP statistical software package (Dixon 1981). Natural logarithms of the 19 length measurements were entered into programs 4M, Principal Components Analysis (PCA), with no rotation of factors and extraction of eigenvalues greater than 0.5 specified, and 7M, Step-Wise Discriminant Analysis (SDA), with F-to enter = 4.0 and F-to remove = 3.999. Bivariate plots and one- and two-way ANOVA routines were also used to examine the relationships of single traits with PCA factors and to test the effect of sex and sexual condition on body-depth measures that appeared to be important in species discrimination.

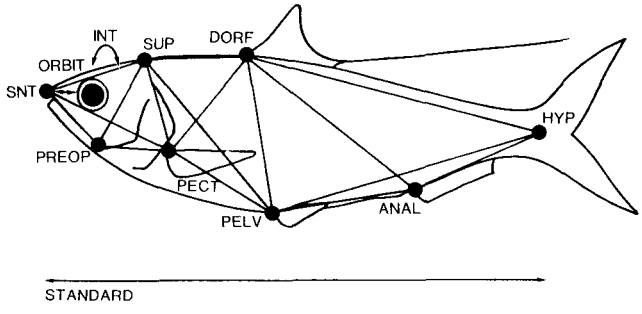

Figure 1. Morphometric measure of *Opisthonema*. Dots mark eight anatomical landmarks defining a three-box truss network of 16 measured chords, each labeled by two end points: ANAL, anal fin origin; DORF, dorsal fin origin; HYP, posterior margin of medial hypurals; PECT, pectoral fin origin; PELV, pelvic fin origin; PREOP, anteroventral edge of preopercular bone; SNT, tip of snout; SUP, posterior edge of supraoccipital bone at the midline. Arrowheads denote ends of three additional measurements: INT, interorbital width at posterodorsal margin of orbit; SNTORBIT, tip of snout to anterior orbit margin; STANDARD, standard length (SNT-HYP).

The numbers of ceratobranchial gill rakers for the Gulf of California specimens were counted by Mr. Ruben Rodriguez-Sanchez (CICIMAR, La Paz, B.C.S.); gill rakers for the Bahía Magdalena fish were counted by L. G. L.-L. Morphometric measurements were done by K. N.; dissection, tissue preparation, electrophoresis, and enzyme assays primarily by L. G. L.-L.; zymogram interpretation and genotype scoring by L. G. L.-L. and D. H. jointly; and statistical analyses of genetic and morphometric data by D. H.

RESULTS

Gill-Raker Counts

Ceratobranchial gill-raker counts for 199 of the 211 specimens in our study fall clearly into three groups as previously described by Berry and Barrett (1963) for *O. bulleri, O. medirastre,* and *O. libertate*. One individual from Mazatlán has a count of 46 (and a standard length of 162 mm), exactly intermediate between the *O. bulleri* and *O. medirastre* means (Figure 2).

Allozyme Variation within and between Species

Starch-gel electrophoresis resolves 29 proteins, each inferred to be encoded by a single locus. A summary of the genetic variability found in our survey of *Opisthonema* is given in Table 2. There are an average of 1.2–1.6 alleles per locus, and 17.2%–37.9% of loci are polymorphic in the nine population samples. On average, individuals are observed to be heterozygous at 5.1%–10.8% of the loci examined; in no case is there a significant

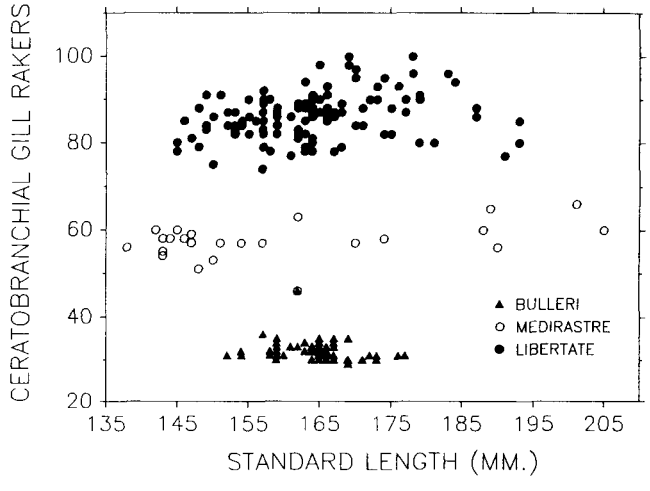

Figure 2. Numbers of gill rakers on the ceratobranchial segment of the first gill arch in relation to standard length for 200 specimens of *Opisthonema* from northwest Mexico. Species identified according to Berry and Barrett (1963), except for an apparent hybrid between *O. bulleri* and *O. medirastre*, which is labeled with the symbols for both species.

TABLE 2

Genetic Variability in Nine Population Samples and in Three Species of Thread Herring (*Opisthonema*) from Northwest Mexico

Species and population	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosities Direct count	H-W-C expected
O. bulleri	50.1	1.4	20.7	0.054	0.055
	(1.5)	(0.1)		(0.025)	(0.026)
Mazatlán, *Calafia*	10.6	1.2	17.2	0.051	0.057
	(0.4)	(0.1)		(0.027)	(0.027)
Mazatlán, *Hapemsa III*	39.6	1.4	31.0	0.054	0.055
	(1.2)	(0.1)		(0.025)	(0.026)
O. medirastre	20.2	1.3	27.6	0.085	0.077
	(1.1)	(0.1)		(0.035)	(0.030)
Bahía Magdalena	4.8	1.2	17.2	0.108	0.076
	(0.5)	(0.1)		(0.049)	(0.033)
Mazatlán, *Calafia*	15.7	1.3	27.6	0.076	0.078
	(0.7)	(0.1)		(0.032)	(0.030)
O. libertate	106.0	1.6	31.0	0.063	0.067
	(5.6)	(0.2)		(0.025)	(0.026)
Guaymas	15.0	1.4	27.6	0.072	0.070
	(1.6)	(0.1)		(0.028)	(0.027)
Bahía Magdalena	1.6	1.2	17.2	0.069	0.109
	(0.1)	(0.1)		(0.041)	(0.046)
Mazatlán, *Calafia 1*	26.4	1.4	31.0	0.061	0.067
	(2.8)	(0.1)		(0.028)	(0.028)
Mazatlán, *Calafia 2*	29.9	1.4	37.9	0.065	0.070
	(1.5)	(0.1)		(0.026)	(0.026)
Mazatlán, *Calafia 3*	34.0	1.3	27.6	0.055	0.056
	(1.1)	(0.1)		(0.022)	(0.023)

Standard errors are shown in parentheses. Sample sizes per locus are numbers of individuals; percentage of loci polymorphic, and heterozygosities are defined in text.

difference between observed and expected heterozygosity (H_e ranging from 5.5% to 10.9%), suggesting that mating is random within populations. The species harbor similar levels of genetic variability, although our small samples of *O. medirastre* apparently tend to have higher heterozygosity than the other two species.

Differentiation among populations within species is slight, although small sample sizes preclude valid statistical tests for most loci. Log-likelihood ratio (G) tests for independence in $r \times c$ tables of allelic frequencies by localities are nonsignificant for four loci tested in *O. bulleri* and in *O. medirastre*. Except for the two fish from Bahía Magdalena, which we initially wanted to keep separate from Gulf of California samples, sample sizes for *O. libertate* populations are large enough to permit tests of independence for six polymorphic loci. These G-tests are all nonsignificant, although the Guaymas and *Calafia* 1 samples appear to have more *103* alleles and fewer *96* alleles at the *Lv-1* locus than the other two *Calafia* samples (tests for the three most heterogeneous loci are shown in Table 3).

Because allelic frequencies appear to be homogeneous among conspecific *Opisthonema* populations sampled in northwest Mexico, we pool conspecific individuals to examine biochemical genetic divergence among species. Frequencies of alleles at 14 loci that are either polymorphic in at least one of the species or have fixed differences between species are given in Table 4. The remaining 15 loci, which are defined as monomorphic within species, share the same allele (at frequencies greater than 0.99) in all three species.

The species are genetically quite similar at the 29 loci studied: Nei's I statistics are 0.89 for *O. bulleri* × *O. medirastre*, 0.84 for *O. bulleri* × *O. libertate*, and 0.93 for *O. medirastre* × *O. libertate* (Table 5). Nei's distance statistics, D—estimates of the number of amino acid substitutions per locus between species—are 0.164, 0.173, and 0.069 for these same three pairwise species comparisons (Table 5).

Between *O. bulleri* and the other two species, fixed allelic differences are found for the *Aat* and *Est* loci, together with marked differences in allelic frequencies for *Fum, Gpi, Ldh-A, Me, 6Pgdh*, and *Sod* (Table 4). No fixed differences distinguish *O. medirastre* from *O. libertate*, but markedly different allelic frequencies occur between these two species for *Fum, Gpi, Mdh-M, Lv-1*, and *Sod*.

Allozymes can be used for taxonomic diagnosis whenever the distributions of phenotypic frequen-

TABLE 3
Log-Likelihood Ratio Tests of the Independence of Allelic Frequencies for Three Loci and Sites of Collection for Four Population Samples of *Opisthonema libertate*

Locus	Alleles	Population samples				Totals	r × c G
		Guaymas	*Calafia* 1	*Calafia* 2	*Calafia* 3		
Gpi	*95*	8	11	14	10	43	
	100	26	43	39	53	161	
	105	8	20	11	9	48	
	Totals	42	74	64	72	252	G = 7.25 6 df, ns
Lv-1	*96*	1	0	5	4	10	
	100	35	63	61	66	225	
	103	6	11	0	2	19	
	Totals	42	74	66	72	254	G = 27.62[a]
Sod	*87*	35	64	61	68	26	
	100	7	10	5	4	228	
	Totals	42	74	66	72	254	G = 4.99 3 df, ns

[a]Significance test not valid because value of first cell (frequency of allele *96* in the Guaymas sample) is expected to be only 1.65 from marginal totals. Pooling frequencies of alleles 96 and 103 in all samples yields G = 3.64, 3 df, ns.

cies are sufficiently nonoverlapping between taxa (Ayala and Powell 1972). Based on our samples of *Opisthonema*, for example, thread herring having the allozyme phenotypes AAT-97 or EST-101 may be unambiguously assigned to *O. bulleri* because these phenotypes do not occur in the other two Pacific coastal species. Two specimens for which we did not obtain gill-raker counts were assigned to *O. bulleri* on the basis of AAT phenotype. In the absence of fixed differences between *O. medirastre* and *O. libertate*, we use the probabilities of multilocus allozyme phenotypes to classify individuals. These probabilities are calculated from the allelic frequencies in Table 4, assuming that phenotypic frequencies are determined independently for each locus (linkage equilibrium) by the Hardy-Weinberg-Castle binomial expansion. For expansion, the composite phenotype {FUM 100, GPI 100, MDH-M 100, SOD 100}, i.e., an individual homozygous for the common allele at each of these four allozyme-coding loci, is expected to occur in 21% of the *O. libertate* population but in only 0.00005% of the *O. medirastre* population. Four specimens missing gill-raker counts but having this composite allozyme phenotype were so assigned to *O. libertate*. The remaining five individuals missing gill-raker counts were also assigned to *O. libertate* based on similar disparities in their expected frequencies of occurrence in the two species. Two, for example, were heterozygous for the Gpi^{95} allele, which does not occur in *O. medirastre*.

The single fish with the exceptional gill-raker count of 46 had an AAT 97/100 phenotype, consistent with its being an interspecific hybrid between *O. bulleri* and one of the other two species. Its composite phenotype for four other allozymes {FUM 104, GPI 100, LV-1 96/100 and SOD 87/100} suggests that this individual most likely resulted from hybridization between *O. bulleri* and *O. medirastre*. This individual was a sexually mature, ripe male. We cannot tell whether it represents an F_1 or a backcross progeny, although the intermediate gill-raker number (Figure 2) suggests the first possibility.

Morphometric Variation and Species Discrimination

A principal components analysis (PCA) of 19 log-transformed morphometric measurements (Figure 1) on each of 206 specimens of *Opisthonema* (3 fish had missing values) produces three factors that account for 88.9% of the variation in data space. Factor I, accounting for 85% of variance in factor space, is correlated strongly and positively with all 19 traits (0.644 to 0.962) and is probably, thus, a reflection of morphometric variation owing to variation in body size (Bookstein et al. 1985). Factor II, accounting for 11.1% of variance in factor space, is substantially, positively correlated with six traits (PECTPELV [0.266], DORFPECT [0.288], STANDARD [0.298], PELVHYP [0.350], ANALHYP [0.522] and SUPDORF [0.645]) and correlated negatively with four others (SUPPREOP [−0.255], DORFPELV [−0.416], SUPPECT [−0.424] and SNTPREOP [−0.426]). Factor III accounts for only 3.9% of

TABLE 4

Allelic Frequencies for 14 Loci That Are Either Polymorphic in at Least One Species or Have Fixed Differences between Species

Locus	Sample size alleles	Species		
		O. bulleri	*O. medirastre*	*O. libertate*
	N	55	25	120
Aat	*97*	1.0	0.0	0.0
	100	0.0	1.0	1.0
	N	28	4	19
Est	*100*	0.0	1.0	1.0
	101	1.0	0.0	0.0
	N	56	25	129
Fum	*100*	0.0	0.42	0.953
	104	1.0	0.58	0.047
	N	56	25	128
Gpi	*95*	0.0	0.0	0.172
	100	0.973	0.16	0.633
	105	0.027	0.84	0.191
	N	56	25	129
Ldh-A	*93*	0.464	0.0	0.0
	100	0.536	1.0	1.0
	N	56	25	129
Ldh-B	*100*	0.98	1.0	1.0
	N	55	19	122
Ldh-C	*100*	1.0	0.974	1.0
	102	0.0	0.026	0.0
	N	55	25	128
Mdh-M	*100*	0.055	0.04	0.848
	103	0.945	0.96	0.152
	N	39	12	69
Me	*100*	0.385	1.0	0.964
	102	0.615	0.0	0.036
	N	56	23	127
Pp	*95*	0.0	0.022	0.0
	100	1.0	0.978	0.972
	102	0.0	0.0	0.028
	N	56	24	129
Lv-1	*96*	0.196	0.375	0.039
	100	0.786	0.604	0.884
	103	0.018	0.021	0.078
	N	56	25	129
Pgm	*100*	0.991	1.0	0.984
	N	54	24	108
6Pgdh	*100*	0.991	0.708	0.620
	103	0.009	0.292	0.375
	N	56	25	129
Sod	*87*	0.0	0.76	0.105
	100	1.0	0.24	0.895

Alleles with frequencies less than 0.01 in all species omitted.

variance in factor space and is correlated substantially only with PELVANAL (−0.265), DORFPELV (−0.261), and SNTORBIT (0.444).

A bivariate plot of Factors I and II, in which individuals are classified to species by gill-raker count or allozyme phenotype (Figure 3), shows remarkable coherence of conspecific individuals. *O. medirastre*, befitting its specific name, lies in a cleft between *O. bulleri* and *O. libertate*. In the upper right corner are Bahía Magdalena specimens of *O. medirastre*, which are substantially larger than specimens from the Gulf of California on the left. The hybrid is centrally located along both axes, and five *O. libertate* and one *O. bulleri* also appear to lie in the cleft between the points for these two species.

Stepwise discriminant analysis (SDA) on the same log-transformed morphometric data set produces, in 12 steps, discriminant functions utilizing 10 variables and yielding a posteriori correct classifications in 96%–100% of cases (Table 6A). Inspection of the order of entry for traits and of trait correlations with Factor II led us to select three traits (SUPPECT, DORFPECT, and DORFPELV) for a second SDA, which produces correct assignments in 84%–95% of cases (Table 6B). Plots of canonical variate scores (linear combinations of the original variables that best discriminate

TABLE 5
Genetic similarity[a] (above Diagonal) and Distance[b] (below Diagonal) among Three Species of Thread Herring (*Opisthonema*)

Species	*O. bulleri*	*O. medirastre*	*O. libertate*
O. bulleri	—	0.894	0.841
O. medirastre	0.164	—	0.934
O. libertate	0.173	0.069	—

[a]Nei's (1978) unbiased *I* statistics
[b]Nei's (1978) unbiased *D* statistics

among groups) for individuals identified to species by either gill-raker count or allozyme phenotype illustrate the discrimination achieved by these two SDA's (Figure 4).

Neither sex nor gonadal condition affects size-corrected body-depth measures. To test the effects of these factors on body depth, which appears to play a big role in the discriminant functions, we constructed two indices:

$$\text{DEPTH (body-depth index)} = \log_e(\text{DORFPECT} + \text{DORFPELV} + \text{DORFANAL}) - \log_e(\text{PECTPELV} + \text{PELVANAL})$$

$$\text{GONDEX (gonad condition index)} = \log_e(\text{GONADWWT}) - \log_e(\text{WETWGTH}).$$

First we performed a one-way ANCOVA on *O. libertate* only, grouping males ($N = 87$) and females ($N = 39$) separately. Slopes of the regressions of DEPTH on GONDEX within sexes are equal ($F = 0.826$, 1/122 d.f., $p = 0.365$) and are not significantly different from zero ($F = 2.913$, 1/123 d.f., $p = 0.090$; indeed, the slopes tend to be negative: -0.006 and -0.018, for males and females, respectively). Mean DEPTHs for the two sexes, adjusted by regression on GONDEX, are not significantly different (0.652 for males vs. 0.647 for females, $F = 0.326$, 1/123 d.f., $p = 0.57$). Small

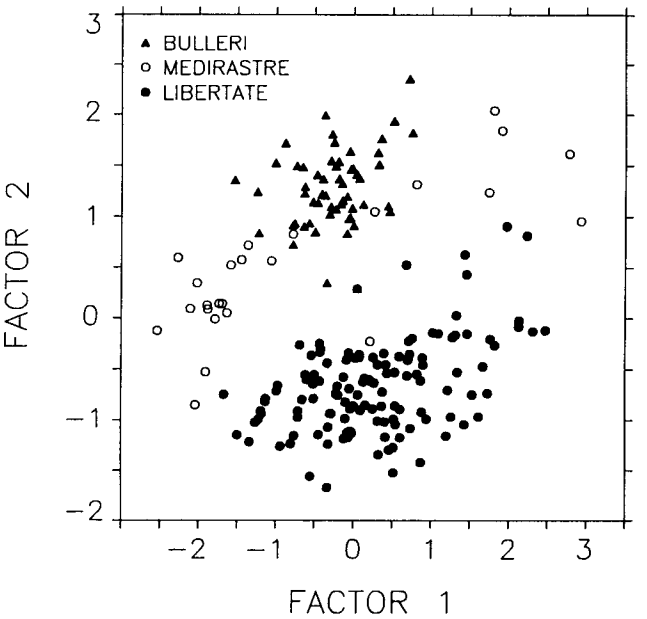

Figure 3. Bivariate plot of scores on the first two principal components extracted from data on 19 morphometric traits measured on 208 *Opisthonema* from northwest Mexico. Species identified either by gill-raker count or allozyme phenotype. An apparent hybrid between *O. bulleri* and *O. medirastre* is labeled with the symbols for both species.

sample sizes preclude a similar analysis for the other two species.

Next, we made a two-way ANOVA of body-depth indices for 207 thread herring, grouping cases by species and by sex. Species contributes almost 95% of total variance ($F = 55.47$, 2/201 d.f., $p < 0.005$), while sex and interaction of species by sex contribute nonsignificant, 2.5% and 1.3% portions of total variance ($F = 1.47$, 1/201 d.f., $p = 0.23$ and $F = 0.77$, 2/201 d.f., $p = 0.46$, respectively). Histograms of the index of body depth for males and females of the three species are presented in Figure 5.

TABLE 6
A Posteriori Classifications by Stepwise Discriminant Analyses Using Morphometric Traits of 207 Thread Herring (*Opisthonema*)

Species*	No. of cases	Percent correct	Number of cases classified into group		
			B	M	L
A. SDA using ten traits					
B	56	98.2	55	1	0
M	25	96.0	0	24	1
L	126	100.0	0	0	126
	207	99.0	55	25	127
B. SDA using three traits					
B	56	92.9	52	4	0
M	25	84.0	1	21	3
L	126	95.2	0	6	120
	207	93.2	53	31	123

*B = *O. bulleri*, M = *O. medirastre*, L = *O. libertate*.

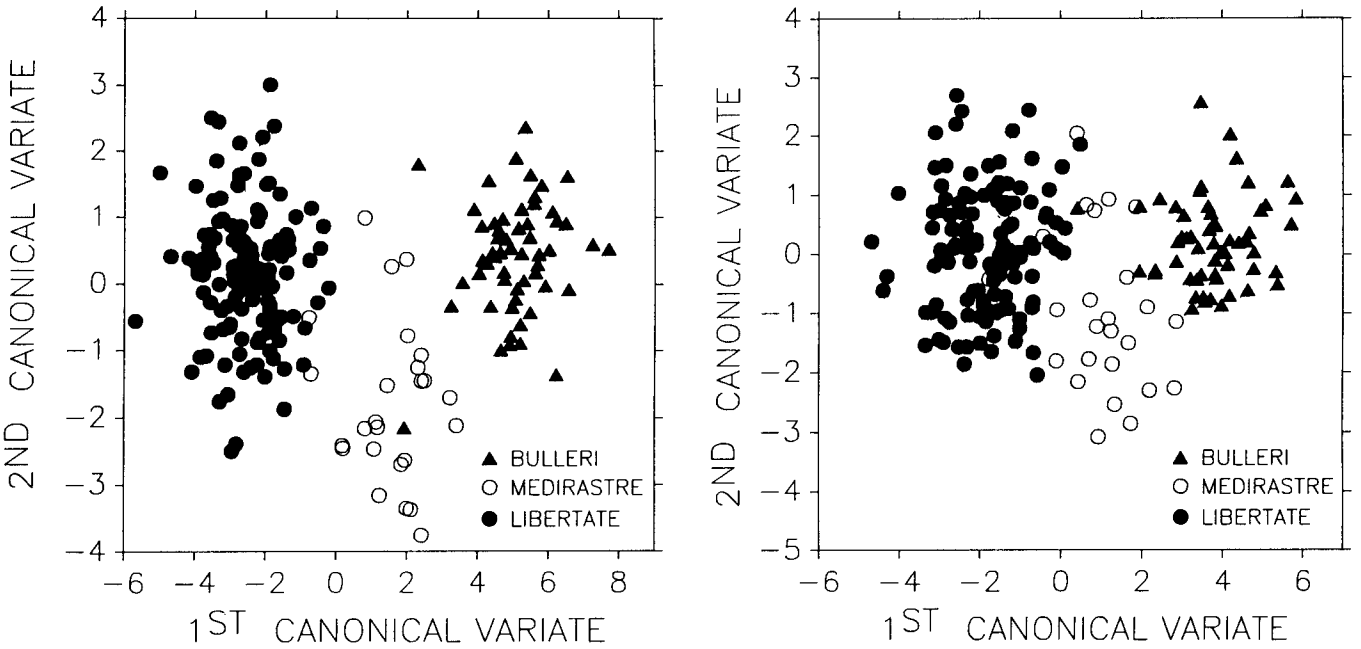

Figure 4. Bivariate plot of scores on the first two canonical variates (orthogonal linear combinations of morphometric traits providing the greatest statistical separation of species) obtained from stepwise discriminant analyses of (*left*) 19 morphometric traits and (*right*) three of these traits—SUPPECT, DORFPECT, and DORFPELV—measured on 207 *Opisthonema* from northwest Mexico. Species identified either by gill-raker count or allozyme phenotype. Hybrid individual omitted.

DISCUSSION

The Three Coastal E. Pacific Species of Opisthonema *Are Valid*

The results of our study fully support the taxonomy of coastal Pacific *Opisthonema* put forward by Berry and Barrett (1963). The three species that they described from the coastal eastern tropical and subtropical Pacific Ocean—*O. bulleri, O. medirastre*, and *O. libertate*—are still best separated by the number of gill rakers on the ceratobranchial segment of the first gill arch, taking standard length into account (Figure 2). These species are genetically distinct, as evidenced by fixed allozyme differences between *O. bulleri* and the other two species and by marked differences among the three

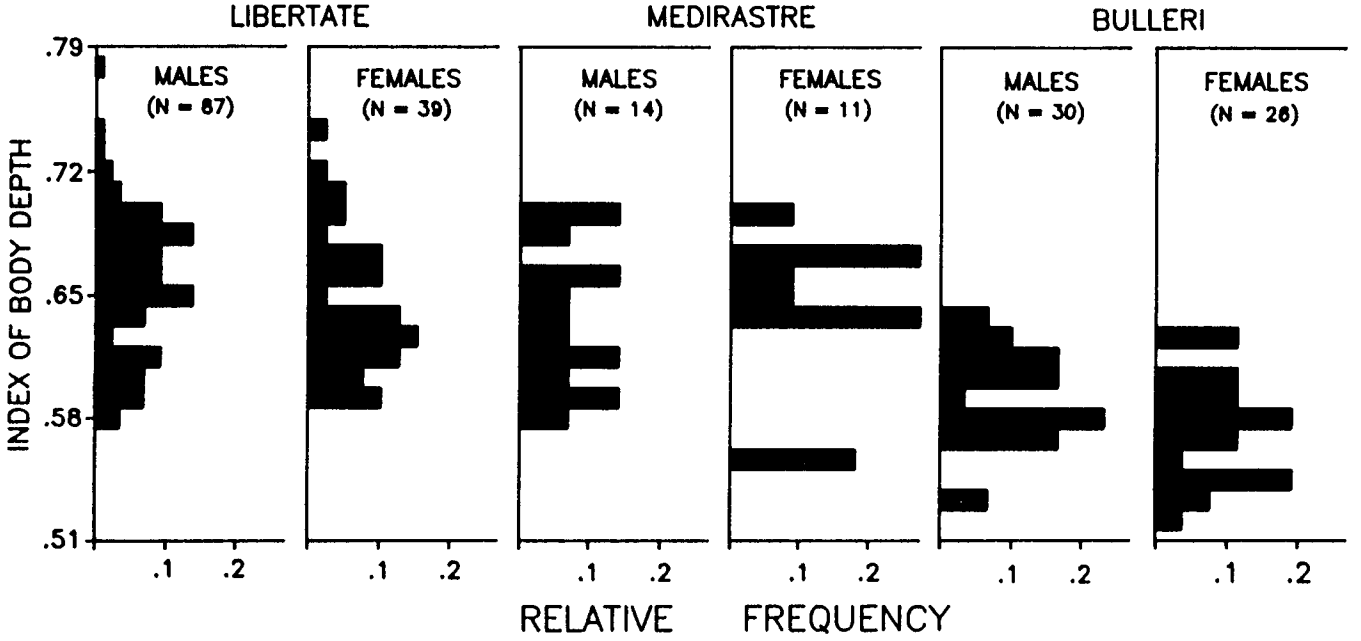

Figure 5. Histograms of an index of body depth for males and females of three species of *Opisthonema* from northwest Mexico.

species in allelic frequencies at several other allozyme-coding loci (Table 4). Indeed, allozymes can be reliably used to classify individuals to species in the absence of data on numbers of gill rakers. Doubts about the taxonomic validity of these species (Rodriguez-Sanchez 1985; Lopez-Lemus 1986) can clearly be laid to rest.

In addition to confirming the status of the three coastal Pacific species of *Opisthonema*, we have shown what Berry and Barrett could not demonstrate using univariate statistics—that these species may be reliably discriminated by multivariate statistical analysis of morphometric traits, particularly by measures of body depth. Although confounding of size and shape variation in morphometrical data generally presents serious difficulties for drawing taxonomic conclusions (Bookstein et al. 1985), a comparison of the results of PCA and SDA suggests that discrimination among *Opisthonema* species depends more on differences in body shapes than on differences in size. It is apparent—both from the large, positive correlations of all morphometric traits with PCA Factor I and from comparison of Figures 2 and 3—that Factor I represents morphometric variation related to size variation. Variance along the orthogonal Factor II axis, then, probably represents mostly variation in shape. Thus the primary use made by SDA of traits that correlate strongly, both positively and negatively, with Factor II suggests that morphometric species discrimination is achieved primarily because of differences in body shape, not size.

In contrast to the remark by Berry and Barrett (1963) that "some females tended to have slightly deeper bodies . . . than males," we have shown that body depth of thread herring is statistically not related to sex or to gonad condition. This further supports our contention that external morphometry, particularly size-corrected measures of body depth, can be used effectively for species discrimination. It may even prove possible to use as few as two or three easily measured traits to identify species in the field or in fishery catches. For example, three traits—SUPPECT, DORFPECT, and DORFPELV—selected because of their behavior in entering the SDA and their strong correlations with Factor II, yield correct classifications ranging from 84% to 95% in a posteriori tests (Table 6B). Other two- and three-character combinations may prove even more accurate, but larger sample sizes from more localities will have to be examined in order to specify optimum least-character discriminant functions.

Geographic Variation in Gene Frequencies and Morphometry

We have examined population samples of *Opisthonema* from only the northern limits of their vast geographical ranges in the eastern Pacific. Three species of *Opisthonema* are clearly distinct in northwest Mexico, but amounts and patterns of intraspecific geographical variation in allozyme frequencies and body shapes must still be considered. Geographical variation is a potential source of error in separating species and also a possible source of insight into the process of speciation in the genus.

Allozyme data give little evidence of geographical variation. No deficiencies of heterozygotes with respect to H-W-C phenotypic proportions result from pooling the *Calafia* samples for *O. bulleri* and *O. medirastre*, but sample sizes are too small to detect much deficiency owing to mixtures of individuals from populations having different allelic frequencies (Chakraborty and Leimar 1987). Genetic differences on such a small geographic scale would be surprising anyway, although we have observed such heterogeneity in proximate samples of northern anchovy, *Engraulis mordax* (Hedgecock and Li 1983; Hedgecock 1986). More rigorous tests of the independence of allelic frequencies over localities are possible for *O. libertate*, but in no case is the null hypothesis rejected. At the *Lv-1* locus, the frequencies of two rare alleles in the *Calafia* 1 sample do resemble those in Guaymas sample, but significance cannot be tested owing to expected frequencies less than 3.0 in two cells (Table 3).

Geographical variation in body size and in body shape, independent of size, occurs in Pacific sardine (*Sardinops sagax*) in the absence of spatial variation in allozyme frequencies (Hedgecock et al. 1985). The anomalous positions of several individuals on the plot of PCA factors (Figure 3) are perhaps similarly explained by differences in the geographic provenance of these particular fish, although we have no evidence for this. The one *O. bulleri* and five *O. libertate* that seem to lie in the cleft occupied by *O. medirastre* in Figure 3 were examined in a series of scatter plots of individual traits against PCA factors. We cannot attribute the positions of these fish to anomalies in particular traits; rather, their differences are multivariate. Interestingly, these individuals are, for the most part, not the same ones misclassified by a posteriori discriminant function assignments. The anomalous *O. bulleri* is misclassified by the SDA done with three traits, but not by the SDA done on all traits. The anomalous *O. libertate*, which, incidentally,

were collected over all localities, are never misclassified. Study of fish from widespread locations might help resolve the causes of intraspecific heterogeneity in morphometry.

Divergence and Speciation in Opisthonema

A correspondence between allozyme differences among species and time since speciation has been observed in many organisms (Nei 1987). Grant (1987) reviews the application of molecular clocks to estimating divergence times for fish, pointing out the large errors associated with these estimates but also their apparent congruence for well-dated divergences below the generic level. We use Grant's (1987) calibration of 19 million years per unit of Nei's D to estimate divergence times for the species of *Opisthonema*. This rate is supported by a substantial data base on the divergence of closely related fishes isolated by the Isthmus of Panama (Gorman et al. 1976; Gorman and Kim 1977; Vawter et al. 1980), a barrier that finally separated the Atlantic and Pacific oceans 3.0–3.5 million years ago (Keigwin 1982). For 12 comparisons reported by these authors, average genetic distance between pairs of species or conspecific populations separated by the Isthmus of Panama range from 0.131 to 0.418, with an average of 0.242.

UPGMA clustering produces an estimate of $D = 0.168$, or 3.2 million years, for separation of the line leading to *O. bulleri* from the line leading to *O. medirastre* and *O. libertate*. Compared to previous estimates of teleost divergence across the Isthmus of Panama, our results suggest that the first divergence of *Opisthonema* in the Pacific took place around the time that separation of the Atlantic and Pacific oceans was completed. Examination of the genetic distances between coastal Pacific *Opisthonema* species and the Atlantic species *O. oglinum* should reveal whether *O. bulleri* is most closely related to *O. oglinum*, as hypothesized by Berry and Barrett (1963), or whether the divergence of Caribbean and Pacific species was essentially trichotomous. For the separation of *O. medirastre* and *O. libertate*, we estimate $D = 0.069$, or 1.3 million years, almost 2 million years after the first split of *Opisthonema* in the Pacific. We know of no geological or major oceanographic changes that might be associated with this time of divergence. It would also be interesting to examine the genetic relatedness of these three Pacific species to the one confined to the Galápagos Islands, *O. berlangai*.

How these three morphologically and genetically very similar sympatric species of thread herring developed reproductive isolation is problematic. That number of gill rakers is the best morphological character separating all five species of *Opisthonema* implies that dietary specialization on different size classes and types of plankton was important in the speciation of this genus. Stomach analyses indicate that *O. libertate* feeds primarily on phytoplankton or small zooplankton, whereas *O. bulleri* and *O. medirastre* feed predominantly on larger zooplankton (Berry and Barrett 1963; Rodriquez-Dominguez 1987). Of course the evolution of alternative feeding morphologies in fishes need not be accompanied by speciation, as shown by Sage and Selander (1975) for *Cichlosoma*. Nevertheless, coastal Pacific *Opisthonema* proves not to be a single species polymorphic for alternative feeding types, contrary to Lopez-Lemus's (1986) preliminary conclusion. Differences in the distributions of these species in the Gulf of California, in their seasonal abundances, and in their reproductive cycles have also been described (Rodriguez-Dominguez 1987); these are the ecological differences that will be important in assessing the separate contributions of the three species to the sardine fishery.

Our discovery of a sexually mature interspecific hybrid between *O. bulleri* and *O. medirastre* presents another problem in understanding speciation in the genus *Opisthonema*. The distinctive gill-raker number (Figure 2) and morphometry (Figure 3) of the *bulleri* × *medirastre* hybrid raises the possibility that, rather than representing geographical variants, those *Opisthonema* occupying anomalous positions in the plot of principal components of morphometric variation (Figure 3) may have resulted from introgression. It will be important in the future to quantify how often interspecific hybridization occurs in nature. If hybridization between the more closely related species *O. medirastre* and *O. libertate* also occurs, it may be very difficult to detect by allozymes because of their great similarity and lack of fixed differences. Analysis of restriction fragment-length polymorphisms of mitochondrial DNA might provide crucial evidence for introgression (Ferris and Berg 1987).

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