

GENETIC RELATIONSHIPS AMONG THREE SPECIES OF THE GENUS *SARDINELLA* (CLUPEIDAE)

MARIA R. MENEZES

National Institute of Oceanography, Dona Paula, Goa - 403 004, India

ABSTRACT

An electrophoretic analysis of 17 enzyme coding loci was undertaken in three clupeid species, *Sardinella longiceps* Val., *S. albella* Val., and *S. fimbriata* Val., collected from the west coast of India. At MDH-2*, MEP* and PGM* loci the three species shared no alleles. Nei's genetic distance values ranged from 0.31 ± 0.15 to 1.68 ± 0.51 . Four loci were polymorphic in *S. albella*, and three in *S. fimbriata* at $P=0.95$ level. No locus was polymorphic by the 95% criterion in *S. longiceps*. Average heterozygosity ranged from 0.006 ± 0.01 to 0.048 ± 0.093 . The genetic variation in *S. longiceps* was less than 25% of that of *S. albella* and *S. fimbriata*.

Key-words : Allozymes, heterozygosity, genetic distance.

INTRODUCTION

The Clupeidae, with scores of genera and over 300 species is taxonomically most diverse (Parrish, Serra and Grant, 1989). In India, the clupeids as a group constitute about one third of marine fish landings. Sardines form the most important group among clupeids and are represented by 9 species. A few species like *Sardinella longiceps*, *S. fimbriata*, *S. gibbosa* and *S. albella* occur in large shoals along the west and south-east coasts of India (Jhingran, 1975).

An efficient use of biological resources requires a thorough knowledge of the amount and distribution of genetic variability within the species considered (Ryman, 1983). Enzyme electrophoresis provides a relatively simple and rapid method of measuring genetic variation (Menezes, Martins and Naik, 1992). Genetic changes can be detected as a shift in frequency of the common alleles, as loss of rare alleles because of genetic drift, and as reduction in mean heterozygosity through loss of common alleles (Smith and Conroy, 1992). The large biochemical differences between species, so readily resolved by gel electrophoresis, make this also a valuable technique for identifying members of different species (Menezes, 1993). Gel phenotypes can be scored over most stages of the life cycle and their genetics more easily interpreted than the morphological and meristic characters employed in taxonomy (Allendorf and Utter, 1979; McAndrew and Majumdar, 1983).

The present study was conducted on allozymes, to determine the phylogenetic relationships among the three *Sardinella* species, *S. longiceps*, *S. albella* and *S. fimbriata*.

The degree of genetic variation within *S. albella* and *S. fimbriata* was also estimated and compared to that of *S. longiceps* samples studied earlier (Menezes, 1994).

MATERIAL AND METHODS

The specimens were collected from the fish landing centres of the west coast of India. *S. longiceps* samples were collected from Panaji (Goa) in December 1988, October '90, January '91 and February '93 and from Malpe and Mangalore, in January '89. *S. albella* and *S. fimbriata* specimens were obtained from Panaji (Goa) during February '93. The fish were kept frozen at -20° prior to analyses. Liver, heart, eye and skeletal muscle tissues were dissected from each specimen for electrophoretic analyses. Tissue samples were kept in plastic well-trays on ice during dissection, then covered and stored frozen at -20°C overnight. On the day of electrophoresis, samples were allowed to thaw slowly on ice. The cell-lysate obtained by freezing and thawing was directly subjected to electrophoresis (Menezes and Taniguchi, 1988). Horizontal starch gel electrophoresis was used for the separation of allozymes at different enzyme loci. The gel consisted of 12% starch (hydrolysed) and electrophoresis was conducted at 80 mA for 4 hours at 4°C . After electrophoresis the gel was sliced horizontally and each slice was stained for a separate enzyme system. Staining recipes followed are that of Shaw and Prasad (1970). The buffer system used was citric acid – aminopropylmorpholine of pH 6.0 (Clayton and Tretiak, 1972). Detection of isozymes and nomenclature of locus designation followed are that of Shaklee, Allendorf, Morizot and Whitt (1990).

The list of enzymes, tissue specificity and loci are given in Table I. Allelic variants were designated according to their relative mobility, the most common allele being designated 100 and other alleles being given numbers that indicated their mobility relative to that of the common allele. Cathodal systems were designated in a similar way but were given a negative sign. The identity or difference of alleles at the same locus was decided by the position of isozymes on the same gel.

Allele frequencies at each locus in the three species were calculated by a simple gene counting method assuming that a protein with the same mobility was controlled by the same allele even in different species, and that variations within species were controlled by two or more codominant alleles (Nozawa, Shotake, Kawamoto and Tanabe, 1982). From the gene frequency data thus obtained, the genetic similarity (I) and genetic distance (D) were calculated between every pair of the species compared. Nei (1972) measures ($D = -\log_e I$), were used. Nei (1975) proposed a formula for estimating the phylogenetic divergence time (t years) from D; that is: $t = 5 \times 10^6 D$. The above estimations of D and t are based on the assumption that the amino-acid substitution rate is constant among different proteins.

Table I — List of enzymes examined, locus names and tissue specificity observed.

Enzyme	Enzyme number	Locus	Tissue specificity
Aspartateaminotransferase (AAT)	2.6.1.1	AAT*	Skeletal muscle
Creatine kinase (CK)	2.7.3.2	CK*	Skeletal muscle
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	G3PDH-1* G3PDH-2*	Skeletal muscle Liver
Haemoglobin (HB)		HB*	Heart
Isocitrate dehydrogenase (IDHP)	1.1.1.42	IDHP-1* IDHP-2*	Heart Liver
Lactate dehydrogenase (LDH)	1.1.1.27	LDH-A* LDH-B* LDH-C*	Skeletal muscle Skeletal muscle Eye
Malate dehydrogenase (MDH)	1.1.1.37	MDH-1* MDH-2*	Skeletal muscle Skeletal muscle
Malic enzyme (MEP)	1.1.1.40	MEP*	Skeletal muscle
Phosphogluconate dehydrogenase (PGDH)	1.1.1.44	PGDH*	Liver
Phosphoglucomutase (PGM)	5.4.2.2	PGM*	Skeletal muscle
Sarcoplasmic protein (PROT)		PROT-1* PROT-2*	Skeletal muscle Skeletal muscle

The degree of genetic variability was estimated by calculating: (1) % of polymorphic loci where the frequency of the commonest allele is ≤ 0.99 , and ≤ 0.95 . (2) Mean number of alleles locus⁻¹. (3) Mean observed heterozygosity locus⁻¹ individual⁻¹ including monomorphic loci. (4) Mean expected heterozygosity locus⁻¹. Hardy-Weinberg statistical analysis was followed (Hartl, 1980). Average heterozygosity per individual was determined by totalling the number of heterozygotes for each locus, dividing this by the total number of individuals in the sample, and averaging over all loci.

RESULTS

For the nine enzymes, haemoglobin and general proteins studied, 17 loci were detected in *S. albelli* and *S. fimbriata*. In *S. longiceps* samples (Menezes, 1994), 19 loci were detected from ten enzymes, general proteins and haemoglobin. The allozyme variation observed in the three species is given in Table II. No locus was polymorphic by the 95% criterion in *S. longiceps*. However, seven loci were polymorphic at very low degrees of variability. In *S. albelli*, four loci (IDHP-1*, IDHP-2*, PGDH* and PGM*) were polymorphic at P=0.95 level and three (IDHP-1*, IDHP-2* and PGDH*) in *S. fimbriata*. The average observed heterozygosity per locus was 0.006 ± 0.01 (*S. longiceps*),

0.048±0.093 (*S. albella*), and 0.044±0.092 (*S. fimbriata*). Allele frequencies and χ^2 values for the differences between observed and expected Hardy-Weinberg numbers of phenotypes of each polymorphic enzyme are given in Table III. Genotypes for the species conformed to Hardy-Weinberg proportions ($P>0.05$) at all loci, with the exception of *PGM** locus in *S. fimbriata*, which had a deficit of heterozygotes. The reason for this significant result is unknown and needs a further study of samples from other areas.

Table II — Estimates of genetic variability in three clupeid species

	Genetic variability		
	<i>S. longiceps</i>	<i>S. albella</i>	<i>S. fimbriata</i>
No. of loci	19	17	17
No. of P	7	4	6
No. of P*	0	4	3
Proportion of P	0.368	0.235	0.353
Proportion of P*	-	0.235	0.176
Mean no. of alleles	1.37	1.24	1.29
Average heterozygosity (H) ± S.D.			
Observed (H _o)	0.006 ± 0.01	0.048 ± 0.093	0.044 ± 0.092
Expected (H _e)	0.006 ± 0.01	0.045 ± 0.086	0.043 ± 0.083

P*, polymorphic ($P<0.95$); P, polymorphic ($P<0.99$)

The electrophoregrams of isozymes were compared among the three species. *S. longiceps* could be easily differentiated from the other two species since it shared only 18% of the sampled loci, while *S. albella* and *S. fimbriata* shared 71%. At *MDH-2**, *MEP** and *PGM** loci, the three species shared no alleles. In order to estimate the degree of genetic difference between the three species, the genetic distance (D) was calculated between every pair of species using the allele frequencies shown in Table III. Table IV gives the list of genetic distances and identity for each pair of species. The average genetic distance was greatest between *S. longiceps* and *S. fimbriata* ($D = 1.683 \pm 0.507$) and the lowest between *S. albella* and *S. fimbriata* ($D = 0.305 \pm 0.145$) followed by *S. longiceps* and *S. albella* ($D = 1.601 \pm 0.482$).

DISCUSSION

A comparison of genetic variability among the 3 *Sardinella* species in this study shows that the oil sardine *S. longiceps* has less than 25% of the average heterozygosity of *S. fimbriata* and *S. albella*, *S. albella* and *S. fimbriata* appear to have levels of variation more or less similar to those reported in allozyme studies of clupeids (see Hedgecock,

Table III — Allele frequencies at 17 loci in three clupeid species.

Locus	Allele	Species		
		<i>S. longiceps</i>	<i>S. albella</i>	<i>S. fin. briala</i>
AAT*	100	0	1	0.931
	90	0.961	0	0
	60	0	0	0.009
	50	0.009	0	0
	<i>n</i>	54	47	56
	χ^2	0.005	0	0.005
CK*	-100	0	1	1
	-125	1	0	0
	<i>n</i>	94	47	56
G3PDH-1*	-100	0	1	1
	-125	0.993	0	0
	-150	0.007	0	0
	<i>n</i>	134	47	56
	χ^2	0.007	0	0
G3PDH-2*	100	0.996	1	1
	50	0.004	0	0
	<i>n</i>	124	47	56
	χ^2	0.002	0	56
HB*	-100	0	1	1
	-150	1	0	0
	<i>n</i>	80	47	56
IDHP-1*	160	0	0	0.833
	140	0.015	0.875	0.167
	100	0.985	0.125	0
	<i>n</i>	100	28	29
	χ^2	0.023	0.163	0.36
IDHP-2*	140	0	0	0.905
	130	0	0.872	0
	120	0	0	0.095
	100	1	0.128	0
	<i>n</i>	84	39	42
LDH-A*	χ^2	0	0.843	0.465
	-100	0	1	1
	-140	1	0	0
	<i>n</i>	134	47	56
LDH-B*	100	0.960	1	1
	90	0.004	0	0
	<i>n</i>	134	47	56
	χ^2	0.002	0	0

Locus	Allele	Species		
		<i>S. longiceps</i>	<i>S. albella</i>	<i>S. fimbriata</i>
LDH-C*	100	1	1	1
	<i>n</i>	80	47	56
MDH-1*	-100	0	1	1
	-150	1	0	0
	<i>n</i>	134	47	56
MDH-2*	100	1	0	0
	50	0	0	1
	40	0	1	0
	<i>n</i>	134	47	56
MEP*	125	0.005	0	0
	100	0.995	0	0
	90	0	0	1
	70	0	1	0
	<i>n</i>	100	47	56
	χ^2	0.003	0	0
PGDH*	100	0	0.915	0.912
	90	0.986	0.085	0.088
	80	0.014	0	0
	<i>n</i>	104	47	56
	χ^2	0.022	0.407	0.863
PGM*	140	0	0.096	0
	130	0	0.904	0
	120	0	0	0.045
	110	0	0	0.955
	100	1	0	0
	<i>n</i>	54	47	56
	χ^2	0	0.919	7.75*
PROT-1*	-100	0	1	1
	-125	1	0	0
	<i>n</i>	134	47	56
PROT-2*	-100	0	1	1
	-125	1	0	0
	<i>n</i>	134	47	56

Hutchinson, Li, Sly and Nelson, 1989). Average heterozygosities for marine teleosts are 0.055 ± 0.036 , based on 106 species (Smith and Fujio, 1982), 0.058 ± 0.006 for 31 species (Powell, 1975), and 0.051 ± 0.034 for 51 species (Nevo, 1978). High levels of genetic variation are reported in the clupeiformes, $H = 0.074 \pm 0.019$ for 7 species (Smith and Fujio, 1982). In general, high heterozygosities might be a feature of the pelagic clupeids or small fish (Selander and Kaufman, 1973; Valentine, 1976). The Pacific sardines, however, showed little genetic variation both within and between popula-

tions (Hedgecock, Hutchinson, Li, Sly and Nelson, 1989). In a study of 32 allozyme-coding loci the average heterozygosity ranged from 0.5% to 1.6%, results similar to those of the Indian oil sardine, *S. longiceps* (Menezes, 1994). Slightly greater heterozygosities were found in Spanish sardines off Florida and Brazil, ranging between 2.0 and 2.7% (Wilson and Alberdi, 1991). Many factors determining the level of genetic variability (e.g., occurrence of historical bottlenecks, migration rates and effective population size) act simultaneously on the whole genome and the relative levels of genetic variation at different sets of loci are therefore expected to be associated (Ryman, 1983; McAndrew and Majumdar, 1983; Menezes, Naik and Martins, 1993).

Table IV — Estimates of genetic identity and distance between the three clupeid species; values above diagonal are estimates of genetic distance, those below are estimates of genetic similarity.

Species	<i>S. longiceps</i>	<i>S. albella</i>	<i>S. fimbriata</i>
1. <i>S. longiceps</i>	-	1.6017	1.6830
2. <i>S. albella</i>	0.2016	-	0.3055
3. <i>S. fimbriata</i>	0.1858	0.7367	-

The abundance of oil sardine fishery, as revealed by landings, is subject to wide fluctuations. A greatly reduced population size for a single generation can have a drastic effect on genetic variation (Hartl, 1980). The loss of genetic variability may result in a situation where the population is no longer able to exploit its former ecological niche (Ryman, 1981). The low genetic variation in Pacific sardines was attributed to a small number of founders (Hedgecock, Hutchinson, Li, Sly and Nelson, 1989). A slow population increase after a bottleneck, or a repeated number of bottlenecks, may result in heavy loss of genetic variation (Chakraborty and Nei, 1977). A reduction in population size in the past, due to commercial exploitation, might explain the low genetic variation in the Indian oil sardine *Sardinella longiceps* (Menezes, 1994). The great applicability and the strong need for quantitative analyses of the present type in relation to genetic conservation programmes have been discussed by several authors (Allendorf and Phelps, 1981; Ryman, 1991).

The overall levels of genetic divergence between populations or species can be reduced to a single value using any of various published statistical indices of genetic identity or distance. The most commonly used measure is the genetic identity, I , of Nei (1972), which ranges from 1 (populations identical) to 0 (total dissimilarity). Generally, conspecific populations have I values above 0.9 and rarely as low as 0.8, whilst identity levels between congeneric species can be expected to range from 0.25

to 0.85, and species from different, but confamilial, genera usually show I values below 0.3.

For the 3 species studied in the present work, values of genetic identity for all possible pairwise comparisons are given in Table IV. Also shown are values for Nei's (1972) genetic distance, D , which is the converse of his genetic identity and ranges from 0 (no difference) to infinity (no genes in common). A feature of Nei's I and D is that sampling errors for estimates of genetic divergence between species are little affected by the number of individuals used and depend almost entirely on the number of loci analysed (Nei and Roychoudhury, 1974). For the estimation of divergence between species, even sample sizes as small as 1 individual will give fairly robust estimates of I or D (Nei, 1978; Gorman and Renzi, 1979; Thorpe, 1982).

From the genetic identity values in Table IV, the genetic interrelationships of the 3 species studied can be estimated. A high level of similarity is found between *S. albelli* and *S. fimbriata*. The I value (0.737) falls within the range expected for congeneric species. The genetic identities between these species and *S. longiceps* are low ($I=0.20$), and just below the lower end of the range expected for congeneric species. High levels of genetic identity between different species could be related to recent speciation (Avice and Ayala, 1976).

Values of D are considered to increase with time of evolutionary divergence in a manner which may be stochastically linear (Thorpe, 1989). This phenomenon forms the basis of what has come to be known as the "molecular clock hypothesis" (Thorpe, 1982). The average genetic distance between the oil sardine and the other two sardines was 1.64, a value that is typical of genetic distances between well-differentiated genera in the same family (Thorpe, 1982). The D values indicate a long divergence time of about 8 m. yr. for *S. longiceps*, using a conservative calibration factor of $1 D = 5$ m. yr. (Nei, 1975).

Implicit in the search for relationships between genetic variability and life-history characteristics, is the hypothesis that levels of genetic variation reflect intensities of balancing selection, *i.e.*, forms of selection that maintain genetic variation. It is also possible, however, that all or part of the genetic variation evident among fishes is irrelevant to adaptation and hence, is unaffected by natural selection (Mitton and Lewis, 1989). If the genetic variation is predominantly neutral, then its levels would be best explained by (i) time since the last bottleneck (or perhaps since speciation), (ii) the neutral-mutation rate, and (iii) the effective population size. Several surveys of genetic variation have reported relationships between population size and genetic variation (Soule, 1976; Nevo, Beiles and Ben-Shlomo, 1984) When effective population size is very small, drift will erode the genetic variability at virtually all polymorphic loci. The very low levels of heterozygosity found in *S. longiceps* indicate the possibility that

there has not been sufficient time since speciation or since the last severe bottleneck for genetic variation to rise to its equilibrium level.

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