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Population Genetics of the Silverside *Odontesthes argentinensis* (Teleostei, Atherinopsidae): Evidence for Speciation in an Estuary of Southern Brazil

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Allozyme electrophoresis was used to investigate genetic population structure among 245 individuals of the silverside *Odontesthes argentinensis* sampled from localities in southern Brazil and Uruguay. Striking differences in allozyme frequencies and genetic variability were detected among localities. Analysis of these differences indicated two distinct populations: one distributed in a wide marine coastal zone; and one in the peripheral environment of the Patos Lagoon estuary in southern Brazil. The population in Patos Lagoon appears to have originated from an ancestral marine population and been established in the estuary by a founder effect. The extent and pattern of genetic divergence found in our study, the existence of diverse reproductive strategies in the estuarine silversides, and differences in morphology of eggs and larvae, indicate that speciation is taking place in Patos Lagoon. It is suggested that the two populations should be considered separately when formulating management and conservation strategies.

SILVERSIDES are atheriniform fishes comprised of two monophyletic groups: Atherinopsidae, the New World silversides, and Atherinidae, the Old World silversides (Saeed et al., 1994; Dyer and Chernoff, 1996). Silversides are distributed in marine, estuarine, and freshwater environments of tropical and temperate regions around the world. They generally display an overall uniformity of morphology and life-history strategy, commonly living in isolated and semi-isolated populations in estuaries and coastal lagoons (Bamber and Henderson, 1988; Creech, 1991).

In Brazil, silversides occur mainly in coastal lagoons and marine waters of the southern region, with the genus *Odontesthes* comprising at least five freshwater and two marine species (Dyer, 1993). One of the marine species, *Odontesthes argentinensis*, has a wide distribution in coastal and estuarine environments from the southeast coast of Brazil (25°S) to Chubut Province, in Argentina (43°S; Dyer, 1993). It is an economically important resource for local fisheries in southern Brazil, Uruguay, and northern Argentina (Chao et al., 1986; de Buen, 1953), and an appropriate organism for bioassay studies (Phonlor and Cousin, 1997).

In southern Brazil, there is a form of *O. argentinensis* that is an annual resident of estuarine waters of the Patos Lagoon (Chao et al., 1985). In comparison with marine silversides distributed in the adjacent coastal zone, estuarine silversides display different life-history strategies concerning spawning period, spawning site selection, and habitat preference (Bemvenuti, 1987; Phonlor and Vinagre, 1989). Additionally, estuarine silverside eggs are significantly smaller, with a thin chorion and three to six filaments attached to the surface of the egg at one end (Phonlor and Cousin, 1997; L. Beheregaray and G. Phonlor, unpubl.). Marine silverside eggs have a very thick chorion, with approximately 18 looped filaments attached at both ends and three filaments attached to the surface of the egg at only one end (Phonlor and Cousin, 1997; L. Beheregaray and G. Phonlor, unpubl.). The existence of distinct chorion morphologies is very unusual, considering that this character is generally constant and useful in teleost systematics (Ivankov and Kurdyayeva, 1973). The number and arrangement of chorionic filaments have been reported to be species-specific characters in atheriniform fishes (White et al., 1983). Differences in life history and egg morphology have confounded the taxonomy of *O. argentinensis* in the Patos Lagoon estuary (Bemvenuti, 1987), although, in a multivariate analysis of morphometric and meristic characters conducted by Bemvenuti (1993), the estuarine and marine forms of *O. argentinensis* could not be separated unequivocally. Nevertheless, as these forms are distinguished by characters usually suggested to confer ecological and perhaps reproductive isolation, it is of interest to examine their genetic structure and to assess genetic divergence between the estuarine and marine forms.

Allozyme electrophoresis has proven a powerful approach to measure genetic variation (Utter, 1991) and validate existence of cryptic,

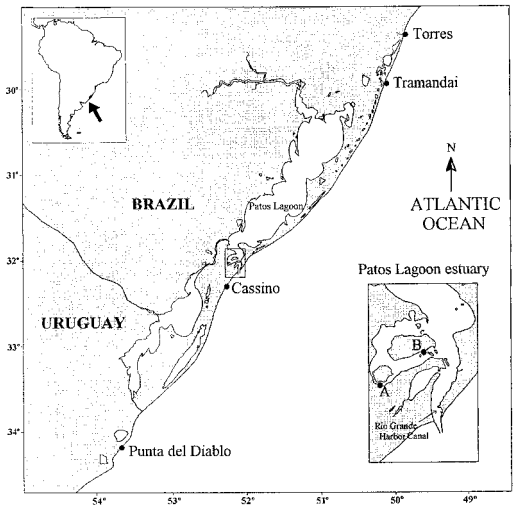

Fig. 1. Collection sites of *Odontesthes argentinensis* in Brazil (Torres, Tramandai, Patos Lagoon estuary A and B, and Cassino) and Uruguay (Punta del Diablo).

sympatric, or parapatric species (Shaklee et al., 1982; Buth, 1984; Harrison, 1991). This approach potentially could be useful for resolving nomenclatural problems in silversides, because silverside taxonomy based on anatomy is usually not definitive because of a high degree of morphological similarity among species (Prodohl and Levy, 1989; Creech, 1991; Ivantsoff and Crowley, 1991). To date, several allozyme studies have distinguished closely related populations and species of silversides in North America (Johnson, 1975; Echelle and Echelle, 1984), South America (Prodohl and Levy, 1989), Europe (Creech, 1991), and Australia (Crowley and Ivantsoff, 1990; Ivantsoff and Crowley, 1991).

The purpose of this study is to examine the population genetic structure of *O. argentinensis* by using allozyme electrophoresis of samples collected from Patos Lagoon estuary and from marine environments. Through the results of this investigation, we expect to provide a better understanding of the relationship between estuarine and marine forms.

Materials and Methods

Samples of *O. argentinensis* were obtained by gill nets from the Atlantic Ocean at Torres, Tramandai, and Cassino, in southern Brazil, at Punta del Diablo, in Uruguay, and from two sites in the Patos Lagoon estuary, in southern Brazil (Fig. 1). Collections were made during spawning at each locality, and egg morphology was used to differentiate between marine and estuarine spawners. A total of 245 adult individuals was collected, placed on ice prior to freezing, and then transported to the laboratory. Skeletal muscle and liver samples were dissected, then homogeneized with a glass rod; the resultant supernatant was stored at −80 C.

Horizontal starch-gel electrophoresis was carried out for 20 enzyme systems according to procedures outlined in Shaw and Prasad (1970) and Richardson et al. (1986), with some modifications. Gels were prepared as 14% suspensions of hydrolyzed starch (Penetrose 30, Refimações de Milho Brasil). Buffer systems employed were tris-citric EDTA pH 7.0 (Shaw and Prasad, 1970), tris-citric EDTA pH 8.0 (Siciliano and Shaw, 1976), tris-maleate pH 7.8 (Harris and Hopkinson, 1977), and discontinuous tris-citric pH 6.0-5.1 (Guiñez and Galleguillos, 1986). Electrophoretic conditions used for each buffer are presented in the Appendix. Identification of alleles among populations was confirmed by running samples from different sites on the same gel. The minimum number of specimens assayed per polyallelic locus per site was 24. Enzyme nomenclature follows recommendations of IUBMBNC (1992), and alleles were labeled by sequential alphabetical code according to their decreasing anodal mobility.

Statistical analyses were performed with BIOSYS-1 (Swofford and Selander, 1981). Genetic variability was calculated as percentage of polyallelic loci, P (using the 0.95 criterion for the commonest allele), and the mean heterozygosity per locus, H (calculated as the mean of individual locus heterozygosities). Observed and expected proportions of heterozygous genotypes at each locus were averaged over loci to obtain means (H_O and H_E, respectively). Agreement of genotypic frequencies with Hardy-Weinberg expectations was verified with an exact test with Levene's (1949) correction for small sample sizes. To estimate population divergence, we used Wright *F*-statistics, Nei's (1978) unbiased genetic distance (D), and a χ^2 contingency table analysis; *P*-values where adjusted with the sequential Bonferroni technique using $\alpha = 0.05$ (Rice, 1989). A dendrogram was constructed from Nei's (1978) genetic distance, using an unweighted pair group method of analysis with arithmetic averaging (UPGMA).

Results

A total of 22 gene loci, encoding 31 putative alleles, were recorded for the 20 enzyme systems assayed. Five of the enzymes were polyallelic for one locus at the 95% level: *G3pdh-A, Gpi-A, Hk-A, Ak,* and *mMdhp-A*. Banding patterns observed

TABLE 1. GENOTYPIC DISTRIBUTIONS OF POLYALLELIC LOCI IN *Odontesthes argentinensis* FROM SOUTHERN BRAZIL AND URUGUAY (H_E IS MEAN EXPECTED AND H_O MEAN OBSERVED HETEROZYGOSITY ± SE).

Locus	Torres	Tramandai	Patos Lagoon-A	Patos Lagoon-B	Cassino	Punta del Diablo
Ak	bc (3)	bb (1)	ab (2)	ab (2)	bb (4)	bb (2)
	bd (6)	bc (2)	bb (1)	ad (1)	bc (2)	bd (8)
	dd (15)	bd (6)	bd (1)	bb (1)	bd (21)	cd (1)
		cd (1)	cc (1)	cc (2)	cd (5)	dd (16)
		dd (15)	cd (10)	dc (8)	dd (28)	
			dd (24)	dd (23)		
Gpi-A	bb (27)	ab (1)	bb (36)	ab (2)	ab (3)	ab (1)
	bc (3)	bb (22)	bc (3)	bb (36)	bb (50)	bb (24)
		bc (2)		bc (2)	bc (2)	bc (5)
G3pdh-A	bb (28)	ab (1)	bb (39)	bb (38)	bb (74)	bb (22)
	bc (2)	bb (23)	bc (1)	bc (2)	bc (6)	bc (3)
		bc (1)				
Hk-A	aa (15)	aa (17)	aa (12)	aa (18)	aa (60)	aa (23)
	ab (9)	ab (8)	ab (17)	ab (15)	ab (18)	ab (6)
			bb (11)	bb (7)	bb (2)	bb (1)
mMdhp-A	aa (3)	aa (5)	aa (40)	aa (40)	aa (16)	aa (1)
	ab (12)	ab (12)			ab (36)	ab (10)
	bb (9)	bb (8)			bb (8)	bb (19)
H_E	0.061 ± 0.03	0.060 ± 0.03	0.041 ± 0.03	0.043 ± 0.03	0.061 ± 0.03	0.054 ± 0.02
H_O	0.062 ± 0.03	0.059 ± 0.03	0.037 ± 0.02	0.036 ± 0.02	0.063 ± 0.03	0.052 ± 0.02

were in accordance with recognized subunit structures of these enzymes in vertebrates: *Hk-A* and *Ak* (monomeric pattern), *G3pdh-A* and *Gpi-A* (dimeric pattern), and *mMdhp-A* (tetrameric pattern). The 17 monoallelic loci provided no information about population heterogeneity but were scored for computing average heterozygosities and genetic distances. Genotypic distributions of the individuals from all six localities were in Hardy-Weinberg equilibrium (*P* varied between 0.113 and 1.0). The proportion of polyallelic loci ranged from 0.17 in the samples from the Patos Lagoon estuary to 0.22 in the remaining four samples. Observed mean heterozygosities (H_O) ranged among localities from 0.036 to 0.063 (Table 1), with a mean (± SE) for all samples of 0.051 ± 0.024.

Allele frequencies differed significantly among locations at three of five polyallelic loci: *Hk-A*, *Ak*, and *mMdhp-A* (Table 2). At the diallelic locus *mMdhp-A*, there was a decrease in frequency of the allele "b" from the north (63% in Torres) and from the south (80% in Punta del Diablo) in the direction to the Patos Lagoon estuary, where this allele was not found (Table 1). Other examples that allele heterogeneity is associated geographically are demonstrated by the distribution of alleles at *Ak* and *Hk-A* (Fig. 2).

Genetic heterogeneity over all samples indicated a substantial degree of population subdivision (F_{ST} = 0.162). Exclusion of samples from Patos Lagoon from the dataset resulted in reduced heterogeneity (F_{ST} = 0.029). Nei's (1978) mean genetic distances were low or nil in comparisons among marine sites (*D* ranged from 0.000 to 0.006) and in the comparison between

TABLE 2. χ^2 CONTINGENCY TABLE ANALYSIS OF ALLELE FREQUENCIES AT POLYALLELIC LOCI ACROSS ALL LOCALITIES SAMPLED.

Locus	Allele number	χ^2	df	*P*
Ak	4	37.977	15	0.0009
Gpi-A	3	8.568	10	0.571
G3pdh-A	3	11.417	10	0.326
Hk-A	2	48.805	5	0.000
mMdhp-A	2	166.380	5	0.000
Totals		273.146	45	0.000

df = degrees of freedom; *P* = probability.

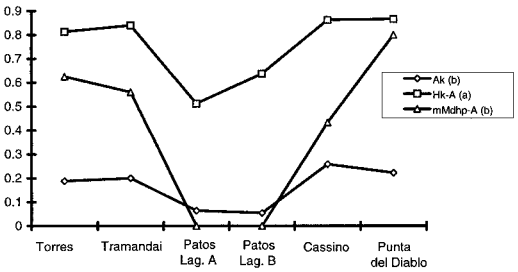

Fig. 2. Distribution of alleles at *Hk-A*, *Ak*, and *mMdhp-A* among localities.

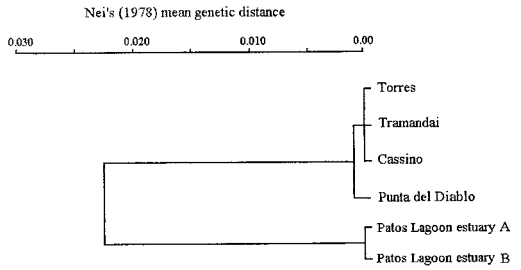

Fig. 3. UPGMA dendrogram summarizing Nei's (1978) genetic distance among localities.

estuarine sites (D = 0.000). However, considerable genetic divergence (D ranged from 0.012 to 0.036) was found between marine and estuarine samples. The UPGMA dendrogram of Nei's genetic distance (Fig. 3) confirmed the divergence between the four marine and the two estuarine locations.

Discussion

Low genetic divergence among marine samples of *O. argentinensis* supports the hypothesis of a marine population in southern Brazil and Uruguay, with high levels of gene flow. High levels of gene flow in organisms with varied life-history strategies have been reported in several vertebrate (Maggioni et al., 1994) and invertebrate marine species (Weber et al., 1993; Marins et al., 1995; Delevedove, 1996) distributed over considerable distances along the western South Atlantic ocean, particularly in coastal waters of the southern Brazilian shelf. The general form of the circulation in the southern Brazilian shelf is characterized by a combination of processes (wind forcing, water mass circulation, and mesoscale variability of the Brazil Current) that produces a complex and highly dynamic hydrographic system, with a high degree of seasonal variation in both direction of flow and water mass composition (Lima et al., 1996). The apparent genetic homogeneity of many species in this marine coastal area may be a result of the dynamics of a hydrographic system that promotes dispersal among geographic populations during reproductive or larval periods.

In contrast, there is significant genetic divergence between estuarine and marine samples of *O. argentinensis*. Observed heterozygosities differed considerably between estuarine (H_O = 0.036–0.037) and marine (H_O = 0.052–0.063) samples, and the two differed significantly in allele distributions at three of five polyallelic loci. This substantial genetic divergence occurs on a very small geographic scale (less than 20 Km), and there are no known geographical barriers separating marine from estuarine silversides. These results indicate strongly that very little to no gene flow occurs between marine and estuarine silversides.

The Patos Lagoon estuary is part of the Patos-Mirim Lagoon system, the formation and evolution of which was governed by development of a multiple sand barrier complex due to eustatic sea-level changes during the Quaternary (Villwock, 1978). The original estuarine conditions were established when geomorphological events formed barrier III during the late Pleistocene, some 120,000 years ago (Calliari, 1997). This barrier effectively isolated the system from the sea, although a channel (represented in the present by the Rio Grande Harbor Canal) drained waters from the extensive basin to the ocean (Calliari, 1997). Assuming a divergence rate for silverside allozyme loci of 1D = 5 million years (Nei, 1972), the mean value of genetic distance between estuarine and marine populations (D = 0.23) suggests a lineage separation about 115,000 years ago. Even considering that differences in rate of molecular evolution used in molecular clocks can result in very different estimates (Avise, 1994), and that local and group specific clocks are more useful than universal ones (Stepien and Kocher, 1997), it is still interesting to note that time of divergence estimated here is consistent with the geological calculation of formation of the Patos Lagoon estuary. Very likely, the estuarine population of *O. argentinensis* was founded from an ancestral marine population that was trapped inside the estuary as it was formed.

Natural selection also may have influenced life-history traits and consequently favored divergence of the estuarine population. Evidence to support this hypothesis comes from comparison of the functional significance of the reproductive strategies of the estuarine and marine populations. In Cassino Beach, the marine population lays eggs in a dynamic surf zone where spawning substrate is scarce (Phonlor and Vinagre, 1989), whereas estuarine silversides spawn on submerged vegetation in marshes of the Patos Lagoon estuary (Bemvenuti, 1987). Estuarine eggs are very slender and have a frail chorion (Phonlor and Cousin, 1997; L. Beheregaray and G. Phonlor, unpubl.), typical of species that spawn in protected and calm environments (Ivankov and Kurdyayeva, 1973). They also have a reduced number of adhesive filaments (Phonlor and Cousin, 1997; L. Beheregaray and G. Phonlor, unpubl.) and are deposited on sites where spawning substrate is not a limiting factor. We suggest that eggs with a frail chorion

and decreased adhesive capacity could represent an adaptation to the estuarine environment. A similar example is found in *Fundulus heteroclitus* where northern and southern populations from the east coast of North America differ in several traits, including egg morphology, allozymes, and mitochondrial and nuclear DNA (Cashon et al., 1981; Morin and Able, 1983; Bernardi et al., 1993).

The extent and pattern of divergence between estuarine and marine populations of *O. argentinensis* indicate that speciation is occurring in the Patos Lagoon estuary. Bottleneck or founder events likely induced divergence of the estuarine silversides, a suggestion consistent with findings in several other fish species (Echelle and Echelle, 1984; Ovenden and White, 1990; Moran and Kornfield, 1995). Our study also provides an example of speciation associated with significant behavioral and ecological divergence. In spite of their general similarity in adult morphology (Bemvenuti, 1993), estuarine and marine populations of *O. argentinensis* are distinguishable genetically and appear to behave as different species. Therefore, each population should be considered separately when formulating management and conservation policies. Results presented here emphasize the need for morphological analysis to identify significant anatomical differences between estuarine and marine forms. Further studies, using nuclear and mitochondrial DNA markers, could better clarify the extent of genetic divergence of estuarine silversides and thus enhance understanding of mechanisms of speciation of coastal fishes.

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APPENDIX. ENZYMES, ENZYME COMMISSION NUMBER, LOCUS ABBREVIATIONS, TISSUES, AND BUFFER SYSTEMS EMPLOYED.

Enzyme	EC No.	Locus	Tissue	Buffer*
Acid phosphatase	3.1.3.2	*Acp-1*	L	I
Adenylate kinase	2.7.4.3	*Ak*	L	I
Aspartate aminotransferase	2.6.1.1	*mAat-A*	L	IV
		mAat-B	L	
Creatine kinase	2.7.3.2	*Ck-A*	M	II
Fructose-biphoshate aldolase	4.1.2.13	*Fbald-A*	M	III
Glutamate dehydrogenase	1.4.1.2	*Gtdh-A*	L	IV
Glucose dehydrogenase	1.1.1.47	*Gldh*	L	IV
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6pdh*	L	II
Glucose-6-phosphate isomerase	5.3.1.9	*Gpi-A*	L	I
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3pdh-A*	M	III
Hexokinase	2.7.1.1	*Hk-A*	L	I
''Unidentified dehydrogenase''	—	*''Udh''*	L	II
Isocitrate dehydrogenase	1.1.1.42	*sIdhp-A*	L	II
Lactate dehydrogenase	1.1.1.27	*Ldh-A*	M	III
Malate dehydrogenase	1.1.1.37	*mMdh-A*	M	III
		sMdh-A		
$NADP^+$-dependent Malate dehydrogenase	1.1.1.40	*mMdhp-A*	L	IV
Phosphoglucomutase	5.4.2.2	*Pgm-1*	L	I
Phosphogluconate dehydrogenase	1.1.1.44	*Pgdh-1*	L	II
Superoxide OD	1.15.1.1	*sSod-A*	L	II
Xantine dehydrogenase	1.2.3.27	*Xdh-1*	L	IV

L, liver; M, muscle. *Buffer systems: I-disc. tris-citric pH 6.0–5.1 (Guiñez and Galleguillos, 1986) 11W/7h; II-tris-citric EDTA pH 7.0 (Shaw and Prasad, 1970) 5.2W/6h; III-tris-citric EDTA pH 8.0 (Siciliano and Shaw, 1976) 7.4W/4h; IV-tris-maleate pH 7.8 (Harris and Hopkinson, 1977) 8.5W/7h.