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Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*

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Abstract

The identification of incipient ecological species represents an opportunity to investigate current evolutionary process where adaptive divergence and reproductive isolation are associated. In this study we analysed the genetic structure of marine and estuarine populations of the silverside fish *Odontesthes argentinensis* using nine microsatellite loci and 396 bp of the mitochondrial DNA (mtDNA) control region. Our main objective was to investigate the relationship among estuarine colonization, divergent selection and speciation in silversides. Significant genetic structure was detected among all marine and estuarine populations. Despite the low phylogeographic structure in mtDNA haplotypes, there was clear signal of local radiations of haplotypes in more ancient populations. Divergence among marine populations was interpreted as a combined result of homing behaviour, isolation by distance and drift. On the other hand, ecological shifts due to the colonization of estuarine habitats seem to have promoted rapid adaptive divergence and reproductive isolation in estuarine populations, which were considered as incipient ecological species. This conclusion is supported by the existence of a set of environmental factors required for successful reproduction of estuarine ecotypes. The pattern of genetic structure indicates that phenotypic and reproductive divergence evolved in the face of potential gene flow between populations. We suggest that the 'divergence-with-gene-flow' model of speciation may account for the diversification of estuarine populations. The approach used can potentially identify 'incipient estuarine species', being relevant to the investigation of the evolutionary relationships of silversides in several coastal regions of the world.

Keywords: adaptive divergence, incipient estuarine speciation, microsatellites, mitochondrial DNA, *Odontesthes*, silverside fish

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Introduction

Identification of processes that have created the current patterns of population divergence in a species can be an intricate task, but it is central to understanding the history and evolutionary consequences of speciation events. Inferences about speciation are usually conceived after the event is completed (e.g. by comparing closely related species), and are more likely to provide information about the characteristics of species than about the processes that give rise to species (McPhail 1994). In this context, revealing the existence of incipient ecological species represents an opportunity to investigate ongoing evolutionary processes in scenarios where adaptive divergence and reproductive isolation are associated. This may be done by studying populations that have experienced ecological shifts or invaded novel habitats, situations where natural selection can play an important role in adaptive divergence and speciation (see Orr & Smith 1998 for a review). For example, Lu & Bernatchez (1999) showed that the extent of reproductive isolation

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between sympatric lake whitefish ecotypes appears to be driven by the potential for occupying distinct trophic niches. Rundle *et al*. (2000) presented evidence that reproductive isolation can be promoted in populations of sticklebacks that evolved under different ecological conditions. The comprehension of the role of ecology in the formation of new species requires integration of ecological, evolutionary and behavioural aspects, and a better knowledge of the selective pressures operating in natural populations (Orr & Smith 1998). Nonetheless, several studies that do not necessarily meet all these criteria have shown that different ecotypes of fishes have experienced rapid genetic divergence, and are, in many cases, on the brink of speciation (e.g. Ovenden & White 1990; McPhail 1994; Pigeon *et al*. 1997; Taylor *et al*. 1997; van Oppen *et al*. 1998; Markert *et al*. 1999; Beheregaray & Levy 2000). Analysis of the genetic structure of incipient ecological species can therefore provide insights into the relationship of divergent selection and reproductive isolation, and thus enhance our understanding of mechanisms of speciation.

Silverside fishes (families Atherinidae and Atherinopsidae) can be found in marine, estuarine and freshwater environments of tropical and temperate regions around the world. Marine silversides generally have similar life history strategies, occurring in large numbers in semi-isolated populations in estuaries and coastal lagoons (Potter *et al*. 1986; Bamber & Henderson 1988; Creech 1991; Beheregaray & Levy 2000). In 1988, Bamber & Henderson proposed a general model of speciation of silversides based on observations of their reproductive biology and morphological variability and on speculations about their evolutionary patterns. The model predicts that semi-isolated and physically variable environments, such as estuaries and coastal brackish lagoons, act to select for generalist genotypes that can adjust their morphology, physiology and behaviour to a wide range of conditions. This selected plasticity would pre-adapt estuarine silverside populations to invade, colonize and radiate into vacant niches in freshwater (Bamber & Henderson 1988). This hypothesis is corroborated by evolutionary patterns displayed by silversides in some regions of the world, notably in the Mesa Central, a geologically active area of the Mexican Plateau. In this area, 18 freshwater species of *Chirostoma* are assumed to have evolved from marine ancestor(s) of a coastal species (Barbour 1973; Echelle & Echelle 1984).

In the recently formed coastal plain of southern Brazil, one group of speciose silversides (genus *Odontesthes*) seems to be particularly useful to study the role of ecology and geographical history in promoting speciation. In marine waters, this group is represented by *Odontesthes argentinensis*, which ranges from the southeast coast of Brazil (25° S) to Chubut Province, in Argentina (44° S) (Dyer 1993), exhibiting peripheral populations resident in estuaries and brackish environments (Bemvenuti 1993; Beheregaray & Levy 2000). In Patos Lagoon estuary, southern Brazil, estuarine silversides show different life-history strategies and phenotypic and physiological divergence compared to adjacent marine silversides. Differences have been documented in spawning period, spawning site selection and habitat use (Bemvenuti 1987; Phonlor & Vinagre 1989), egg and larvae morphology (Phonlor & Cousin 1997; Beheregaray & Phonlor unpublished data), and fertilization success under different water salinities (Sampaio 1992). Patterns of allozyme divergence detected between *O. argentinensis* from Patos Lagoon and nearby marine localities led to the proposal that the estuarine population is in the process of speciation (Beheregaray & Levy 2000).

Phylogenetic reconstructions using mitochondrial DNA (mtDNA) sequences of the genus *Odontesthes* indicate that *O. argentinensis* populations compose an assemblage that has recently given rise to the freshwater *O. perugiae* species complex (Beheregaray 2000; Sunnucks *et al*. 2000; Beheregaray *et al*. 2002). The *O. perugiae* complex represents an example of a remarkably rapid radiation shaped during the last sea-level changes by the deposition of discontinuous sand barriers parallel to the coast (Beheregaray 2000; Beheregaray *et al*. 2002). We hypothesize that the diversification of silversides in southern Brazil is a product of the dynamic geographical history of the area, coupled with rapid adaptive divergence displayed by marine populations that invaded and colonized estuarine environments.

In this study we used sequences of the mtDNA control region and information from nine microsatellite loci to test the hypothesis that populations with an estuarine life history show genetic divergence when compared to adjacent and more distant marine silversides. Our main objective is to investigate the relationship among estuarine colonization, divergent selection and speciation in silversides, an approach that potentially allows the identification of what we call 'incipient estuarine species'. This analysis has yet to be performed in silversides and could be relevant to understanding the diversification of these fishes in other coastal regions, such as the east and west coasts of Australia (Potter *et al*. 1986), Mexico (Echelle & Echelle 1984), the southern United States (Johnson 1975), and southern Europe (Henderson & Bamber 1987).

Materials and methods

Sampled localities

A total of 196 individuals was collected using gill nets and hook and line from eight sites throughout most of the distribution range of *Odontesthes argentinensis* (Fig. 1). Collections were made in 1997 and 1998, in contrast to the allozyme study of Beheregaray & Levy (2000) which concentrated in areas from southern Brazil sampled in 1993

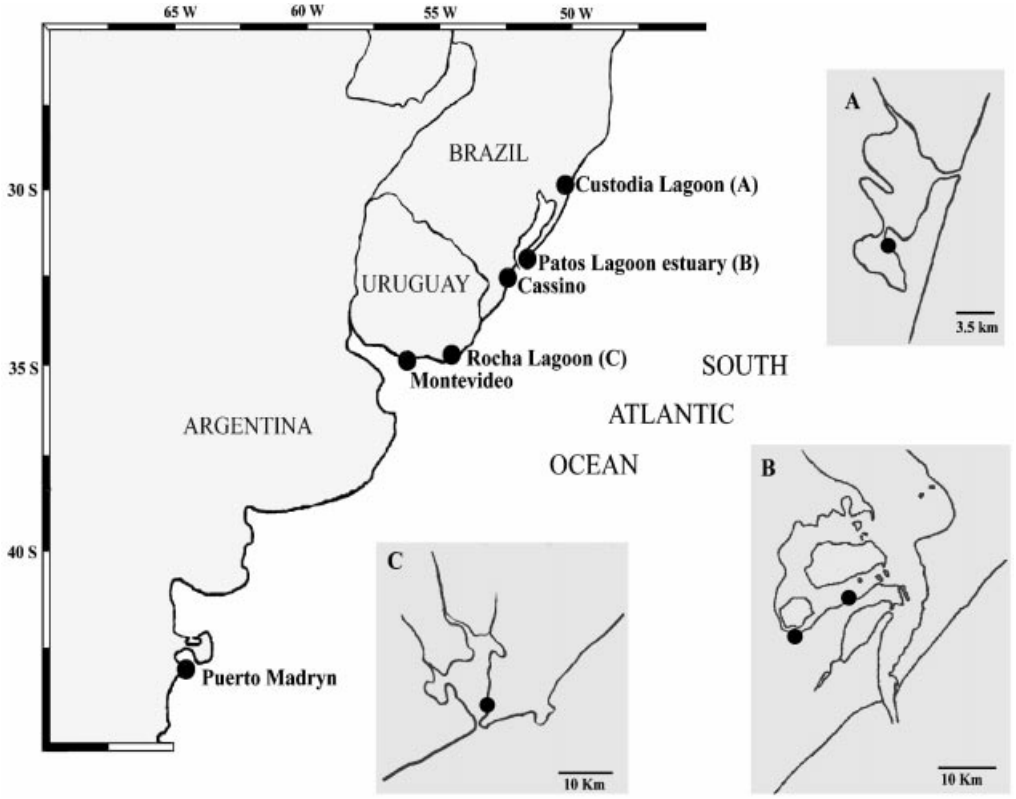

Fig. 1 Marine and estuarine localities of *Odontesthes argentinensis* sampled for this study. Custódia, Patos and Rocha Lagoons are presented in detail (black circles indicate sampled sites).

and 1994. Muscle tissue was excised from the fish and preserved in 80–95% ethanol. Marine samples were obtained from open beaches at two sites in Cassino, southern Brazil (in winter 1997 and in spring 1998), one in Montevideo, Uruguay, and one in Puerto Madryn, Argentina. Estuarine samples were collected from three partially enclosed coastal bodies: Custódia Lagoon and Patos Lagoon estuary (in winter 1997 and in spring 1998) in southern Brazil, and Rocha Lagoon in Uruguay. As partially protected coastal bodies that receive continental and marine water exchange, Rocha and Custódia Lagoons meet important criteria used in a definition of an estuary (Pritchard 1967), and are thus considered as such in the following analyses. The terms 'marine and estuarine ecotypes' will be used to refer to individuals sampled at marine and estuarine localities, respectively.

mtDNA: single-stranded conformation polymorphism (SSCP) and sequencing

Genomic DNA was extracted using a salting-out method (Sunnucks & Hales 1996). Polymerase chain reaction (PCR) amplification of the mtDNA control region was performed with the oligonucleotide primers 'D' and 'E' (Lee *et al*. 1995). Sequence variants in the resulting 400 base pair (bp) fragment were identified using SSCP analysis as described in Sunnucks *et al*. (2000). PCR contents used for the SSCP based on a 10-μL radiolabelled reaction were: 50–100 ng of template DNA, 12 pmol of each primer, 0.5 units of *Taq* DNA polymerase (Promega), 200 μM of dCTP, dGTP, dTTP and dATP, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 0.05 μL [α^{33}P]-dATP at 1000 Ci/mmol overlaid with mineral oil. Amplification was carried out using a 'step-up' PCR programme: 94 °C for 3 min, then five cycles at 94 °C for 30 s, 45 °C for 1 min and 72 °C for 1 min, followed by 34 cycles at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min, and a final extension of 72 °C for 5 min. Fresh PCR product was prepared for between two and seven individuals representing each SSCP phenotype. Product bands were excised from agarose gels, purified with Bresa-clean DNA purification kit (Geneworks) and sequenced in an ABI 377 automated DNA sequencer following the manufacturer's directions.

Microsatellite amplification and screening

Samples from all populations were scored at nine *Odontesthes* microsatellite loci (details of loci and amplification conditions in Beheregaray & Sunnucks 2000). The cloned alleles from all loci used are perfect

dinucleotide repeats. Loci Odont 08 and Odont 16 were assayed in multiplex PCR reactions. Ten selected individuals were independently amplified three times across all loci to test the reliability of PCR: products from different trials consistently produced the same individual genotype.

mtDNA data analysis

Owing to their high similarity, control region sequences could easily be aligned by eye. Population genetic diversity at the haplotype level (h) was calculated from frequency data and, at the nucleotide level estimated by the number of substitutions between sequences (Nei 1987) using the Kimura 2-parameter (K2P) genetic distance (Kimura 1980) with a gamma distribution of 0.52 empirically determined by maximum likelihood in PAUP* (Swofford 2001). This approach is indicated for analyses of the 5′ hypervariable segment of the control region and for data sets with different rates of transitions and transversions (Kumar *et al*. 1993). The extent of genetic divergence between samples was estimated by the *F*-statistic ϕ_{ST} (Excoffier *et al*. 1992), which includes information on haplotype frequency and molecular distance (K2P distance was used). Significance of pairwise population comparisons was assessed by 1000 permutations. We also performed a hierarchical analysis of molecular variance, AMOVA (Excoffier *et al*. 1992) using ϕ_{ST}. For this analysis four tests were executed applying the following criteria to assign populations to groups: (i) all populations as one single group; (ii) all estuarine; and (iii) all marine populations as a single group; and (iv) one group containing estuarine vs. one with marine populations. The significance of the observed variances for each hierarchical comparison was tested by 1000 permutations. All analyses were conducted using the computer package ARLEQUIN 2000 (Schneider *et al*. 2000).

Genealogical relationships among mtDNA sequences were examined by constructing a haplotype network with the parsimony method of Templeton *et al*. (1992). It estimates the maximum number of substitutions to connect parsimoniously two haplotypes with 95% confidence, firstly linking sequences with the smaller number of differences. Thus, it potentially provides high resolution for inferring relationships among genes with low levels of divergence. It also estimates haplotype outgroup probabilities, allowing identification of the most ancient haplotypes in the sample. The analysis was performed with the software TCS vs. 1.06 (Clement *et al*. 2000).

Microsatellite data analysis

The mean number of alleles per locus, allele frequencies, and expected (H_E) and observed (H_O) heterozygosities were estimated with GENEPOP 3.1 (Raymond & Rousset 1995). This program was also used to test for Hardy–Weinberg equilibrium, linkage disequilibrium and heterogeneity in allele frequency distribution for all loci and all pairwise comparisons using the Markov chain method with 1000 iterations. Levels of genetic divergence at microsatellite loci were investigated by computing F_{ST} (Weir & Cockerham 1984) with the program *F*-STAT (Goudet 1995). Significance of F_{ST} for all loci and pairwise population comparisons was assessed by permutating the values 1000 times.

When doing multiple simultaneous comparisons, we used the sequential Bonferroni procedure (Rice 1989) with $\alpha = 0.05$ to adjust the statistical significance levels.

If colonization of estuaries by *O. argentinensis* has occurred in the recent past and founders remained isolated from neighbour populations, we should expect estuarine fish to display lower genetic diversity due to founding events. We analysed the microsatellite data set with the program BOTTLENECK (Cornuet & Luikart 1996) to test for founder effects in *O. argentinensis*. This program estimates the expected distribution of the heterozygosity from the observed number of alleles at each locus and population assuming mutation-drift equilibrium. Bottlenecked populations are predicted to display an excess of heterozygosity compared to the expected values because the number of alleles is more severely affected than the heterozygosity by a population contraction (Cornuet & Luikart 1996). The probability of significant heterozygosity excess was calculated using a Wilcoxon sign-rank test. Computations were based on both stepwise mutation (SMM) and two-phase mutation (TPM) models (Di Rienzo *et al*. 1994).

Correlation of mtDNA and microsatellites with geographical distance

Analyses of correlation between pairwise multilocus fixation indices and geographical distances were carried out using the Mantel permutation test (Mantel 1967) executed in ARLEQUIN. We compared the mtDNA and microsatellite matrices obtained from the fixation indices separately with the geographical distance between samples. This distance was measured in kilometres approximately according to the form of the coastline. Three kinds of tests were conducted for each comparison: (i) with the entire set of eight samples; (ii) using only estuarine samples; and (iii) using only marine samples. Tests were also repeated without the inclusion of the geographically extreme sample collected at Puerto Madryn.

Results

High levels of mtDNA haplotypic variation

A 396-bp portion of the 5′-end of the mtDNA control region was sequenced and confidently aligned for 142 individual

Odontesthes argentinensis. Sequence comparison revealed high levels of control region variability: 66 unique haplotypes (Fig. 2 and Table 1) were found in our sample of 196 individuals screened by SSCP. The SSCP analysis proved to be reliable in unambiguously detecting different haplotypes. It discriminated sequences separated by only one nucleotide substitution in 58 cases. Haplotypic diversity differed little among populations (0.85–0.94). Nucleotide diversity values were also similar (0.014–0.018; Table 2), except for Puerto Madryn (0.009). Sixty-two of the 396 aligned nucleotide positions were variable (Fig. 2), and most haplotypes differed by one to three substitutions (K2P sequence divergence ranged from 0.25 to 4.5%; nucleotide diversity in the total sample was 0.014,

Table 1 Distribution and frequency of the 66 mtDNA control region haplotypes found in six populations of *Odontesthes argentinensis*

Haplotype	Custódia Lagoon	Cassino	Patos Lagoon	Rocha Lagoon	Montevideo	Puerto Madryn
1	—	0.020	—	—	—	—
2	—	0.020	—	—	—	—
3	—	0.040	—	—	—	—
4	—	0.020	—	—	—	—
5	—	0.020	—	—	—	—
6	—	0.020	—	—	—	—
7	—	0.020	—	—	—	—
8	—	0.020	—	—	—	—
9	—	0.020	—	—	—	—
10	—	0.280	0.050	—	0.031	—
11	0.050	0.040	0.025	—	0.062	—
12	—	0.020	—	0.026	—	—
13	—	0.180	0.075	0.158	0.125	0.065
14	—	0.020	—	—	—	—
15	—	0.080	—	—	—	—
16	—	0.040	—	—	—	—
17	—	0.020	—	—	—	—
18	—	0.020	—	—	—	—
19	—	0.080	—	—	—	—
20	—	0.020	—	—	—	—
21	0.250	—	0.025	0.053	—	—
22	0.050	—	—	—	—	—
23	0.150	—	—	—	—	—
24	0.300	—	0.125	—	—	—
25	0.050	—	—	—	—	—
26	0.100	—	—	—	—	—
27	0.05	—	—	—	—	—
28	—	—	—	0.079	—	—
29	—	—	—	0.105	—	—
30	—	—	—	0.079	—	—
31	—	—	—	0.105	0.031	—
32	—	—	—	0.053	—	—
33	—	—	—	0.053	—	—
34	—	—	—	0.131	—	—
35	—	—	—	0.026	—	0.188
36	—	—	—	0.053	—	—
37	—	—	—	0.053	—	—
38	—	—	—	0.026	—	—
39	—	—	—	—	0.094	—
40	—	—	—	—	0.062	—
41	—	—	—	—	0.031	—
42	—	—	—	—	0.125	0.25
43	—	—	—	—	0.094	—
44	—	—	—	—	0.094	—
45	—	—	—	—	0.125	—
46	—	—	0.025	—	0.062	—
47	—	—	—	—	0.031	—

Table 1 *Continued*

Haplotype	Custódia Lagoon	Cassino	Patos Lagoon	Rocha Lagoon	Montevideo	Puerto Madryn
48	—	—	—	—	0.031	—
49	—	—	0.025	—	—	—
50	—	—	0.025	—	—	—
51	—	—	0.125	—	—	—
52	—	—	0.025	—	—	—
53	—	—	0.025	—	—	—
54	—	—	0.050	—	—	—
55	—	—	0.025	—	—	—
56	—	—	0.025	—	—	—
57	—	—	0.025	—	—	—
58	—	—	0.025	—	—	—
59	—	—	0.175	—	—	—
60	—	—	0.025	—	—	—
61	—	—	0.050	—	—	—
62	—	—	0.025	—	—	—
63	—	—	0.025	—	—	—
64	—	—	—	—	—	0.188
65	—	—	—	—	—	0.25
66	—	—	—	—	—	0.0625

Table 2 Summary of genetic variability in *Odontesthes argentinensis* based on mtDNA control region sequences and nine microsatellite loci. The first three populations are estuarine and the others are marine

		Mitochondrial DNA			Microsatellites		
	Sample size	*NH*	Haplotypic diversity	Nucleotide diversity	*NA*/ max.–min.	H_E	H_O
Patos Lagoon	40	21	0.940 (0.020)	0.016 (0.008)	13.8/25–4	0.82 (0.04)	0.82 (0.05)
Custódia Lagoon	20	8	0.847 (0.051)	0.016 (0.009)	10.8/18–4	0.83 (0.05)	0.86 (0.05)
Rocha Lagoon	38	14	0.924 (0.020)	0.016 (0.009)	12.3/22–4	0.80 (0.06)	0.80 (0.04)
Cassino	50	20	0.881 (0.032)	0.018 (0.009)	14.7/26–3	0.81 (0.05)	0.82 (0.06)
Montevideo	32	14	0.939 (0.017)	0.014 (0.007)	12.1/20–4	0.81 (0.04)	0.78 (0.06)
Puerto Madryn	16	6	0.850 (0.045)	0.009 (0.005)	6.8/14–2	0.65 (0.07)	0.61 (0.08)

NH, number of haplotypes; *NA*, mean number of alleles per locus; max.–min., maximum and minimum number of alleles per locus considering all loci; H_E, mean expected heterozygosity; H_O, mean observed heterozygosity. Values in parentheses are standard errors.

SE ± 0.0075). Plotting of pairwise sequence differences vs. the number of transition substitutions showed that transitions increased linearly at all levels of haplotype divergence (results not shown), suggesting that control region sequences are free of saturation. Overall, these results indicate that *O. argentinensis* haplotypes have a recent history of divergence.

Microsatellite variability, Hardy–Weinberg equilibrium and linkage disequilibrium

High levels of allelic diversity (6–30 alleles) and mean heterozygosity (0.61–0.95) were observed across all loci (see Appendix I and Table 2 for population values). Levels of microsatellite variability were similar to those reported in a review of 12 species of marine fishes (DeWoody & Avise 2000). All populations, except that from Puerto Madryn, showed similar variability in allele number and heterozygosity (Table 2). The lower variability of Puerto Madryn samples may be due to small sample size ($n = 16$), and also because it represents the southern end of the distribution of *O. argentinensis* (Dyer 1993), an area sporadically frequented by migrant individuals (A. Gozstonyi personal communication). No significant departures from Hardy–Weinberg equilibrium were observed across populations ($P = 0.08–0.46$), or loci ($P = 0.028–0.87$) after adjusting for multiple comparisons, and there was no indication of excess or deficiency of

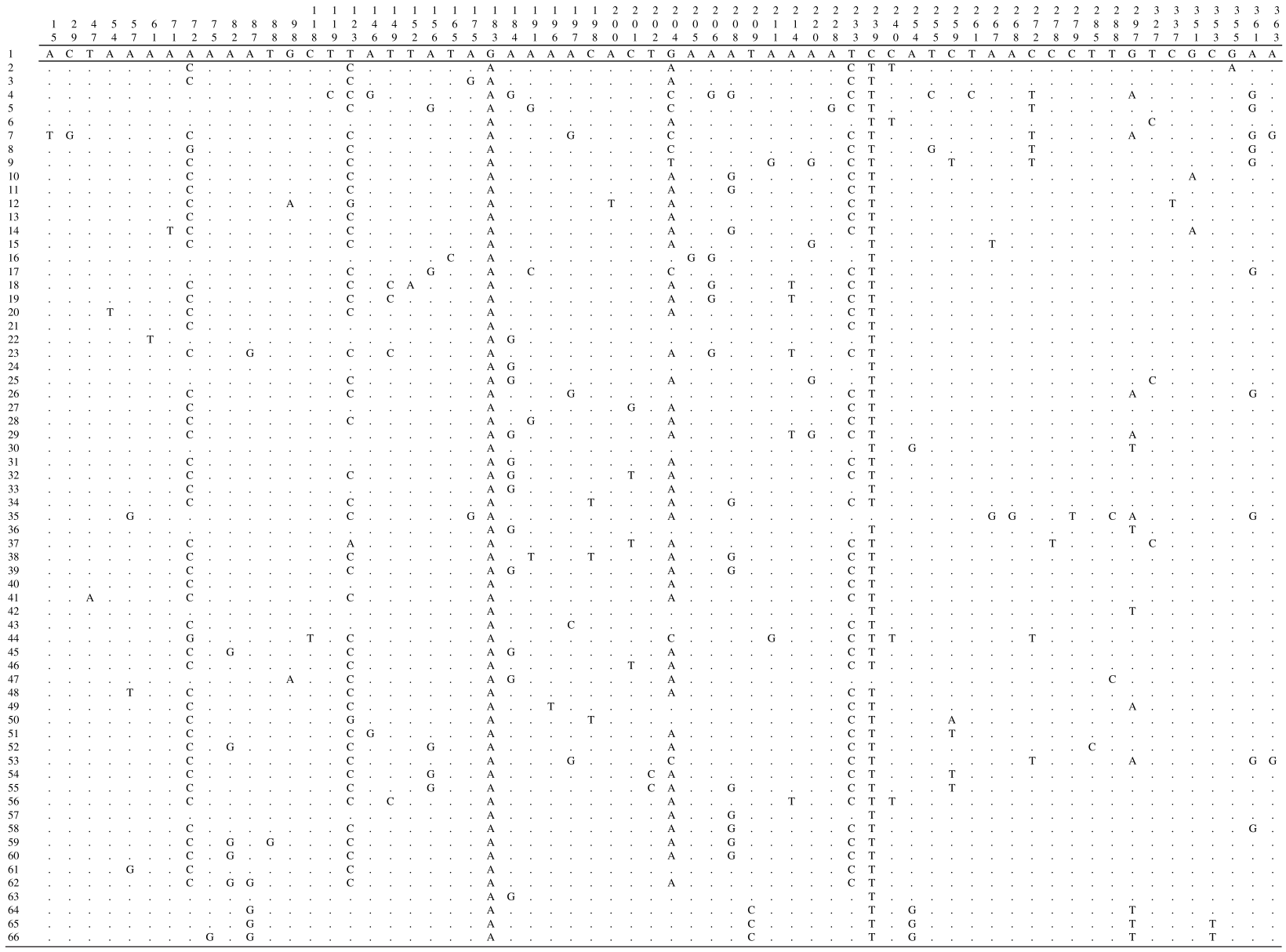

Fig. 2 Variable positions of 66 mitochondrial DNA control region haplotypes of *Odontesthes argentinensis*. Dots represent matches with nucleotides present in haplotype 1. GenBank accession numbers for these sequences are AF431643–AF431708.

	1	2	3	4	5	6
1 Custódia Lagoon		0.133**	0.062**	0.099**	0.102**	0.151**
2 Cassino	0.017*		0.063**	0.068**	0.058**	0.123**
3 Patos Lagoon	0.011*	0.010*		0.054**	0.047**	0.097**
4 Rocha Lagoon	0.019*	0.011*	0.001^{ns}		0.044**	0.100**
5 Montevideo	0.010*	0.007^{ns}	0.004^{ns}	0.003^{ns}		0.076**
6 Puerto Madryn	0.096**	0.061**	0.076**	0.051**	0.072**	

Table 3 Pairwise fixation indices between six *Odontesthes argentinensis* populations based on nine microsatellite loci (F_{ST}, below diagonal) and on mtDNA control region haplotypes (ϕ_{ST}, above diagonal). *P* is the probability that any random value obtained after 1000 permutations is > observed value

*$P < 0.05$; **$P < 0.01$; ns, not significant.

Hierarchical level	% variation among populations	% variation within populations	% variation between groups of populations
All localities	7.6	92.4	
Estuarine localities	7.8	92.2	
Marine localities	7.2	92.8	
Marine vs. estuarine	7.2	92.2	0.6^{ns}

Table 4 Analysis of molecular variance (AMOVA) among *Odontesthes argentinensis* mtDNA control region haplotypes based on ϕ_{ST}

All variances for each hierarchical comparison were significant ($P < 0.01$), unless indicated with ^{ns}.

heterozygotes in the data set. Consequently, the null hypothesis of random mating in the populations was not rejected. Fisher's exact tests for linkage disequilibrium revealed no significant locus-pair/population comparisons at the 5% confidence level.

Lack of genetic divergence between samples from the same locality

For both microsatellite and mtDNA data sets we found no overall significant differences between samples collected in 1997 and 1998 at the two sites in Patos Lagoon estuary, and between the two sites in Cassino. Genetic homogeneity between sites within Patos Lagoon and Cassino was also found in an allozyme analysis conducted in samples collected in 1993 and 1994 (Beheregaray & Levy 2000). These results are consistent with the assumption that these geographical areas support two resident populations of *O. argentinensis*. Therefore, we pooled samples from the two sites within Patos Lagoon and the two sites within Cassino.

Significant genetic divergence among localities

Significant population structure was detected in the distribution of control region mtDNA haplotypes. Of the 66 haplotypes, only 10 were distributed in more than one locality, and none were detected at all localities (Table 1). The large differences in haplotype frequency distribution resulted in high levels of population differentiation: all ϕ_{ST} population pairwise comparisons were significant ($P < 0.01$; Table 3). The 'two-categories' AMOVA tests (comparing all localities, only estuarine, and only marine localities) showed that statistically significant amounts of the molecular variance could be attributed to differences among populations (Table 4). The remaining variance (92.9–94.3%) was distributed within populations, which is expected given the high number of haplotypes found in each population. The AMOVA test comparing estuarine and marine groups did not show significant differences, and the variance was distributed among populations within each group (7.2%) and within populations (92.2%).

Microsatellite data exhibited much less marked genetic structure than did mtDNA (Table 3), but the analysis indicated the existence of genetic subdivision in the data set. Heterogeneity in allele frequency distributions was found for all loci (Fisher's exact test, $P < 0.001$), and significant differences were detected over all loci for F_{ST} ($P < 0.01$). Four of the 11 F_{ST} pairwise comparisons were statistically different from zero (Table 3).

The only positive correlations between genetic differentiation and geographical distance were observed in the comparisons among all marine samples ($Z = 154$, $P < 0.05$ for mtDNA, and $Z = 115$, $P < 0.05$ for microsatellites). High correlation coefficients were obtained when Puerto Madryn was excluded from the analysis, but permutations resulted in no significant *P*-values, possibly because of the small number of sample sites. The concordant results obtained from both classes of markers suggest that in the marine environment, large-scale gene flow in *O. argentinensis* could be related to the distance separating populations.

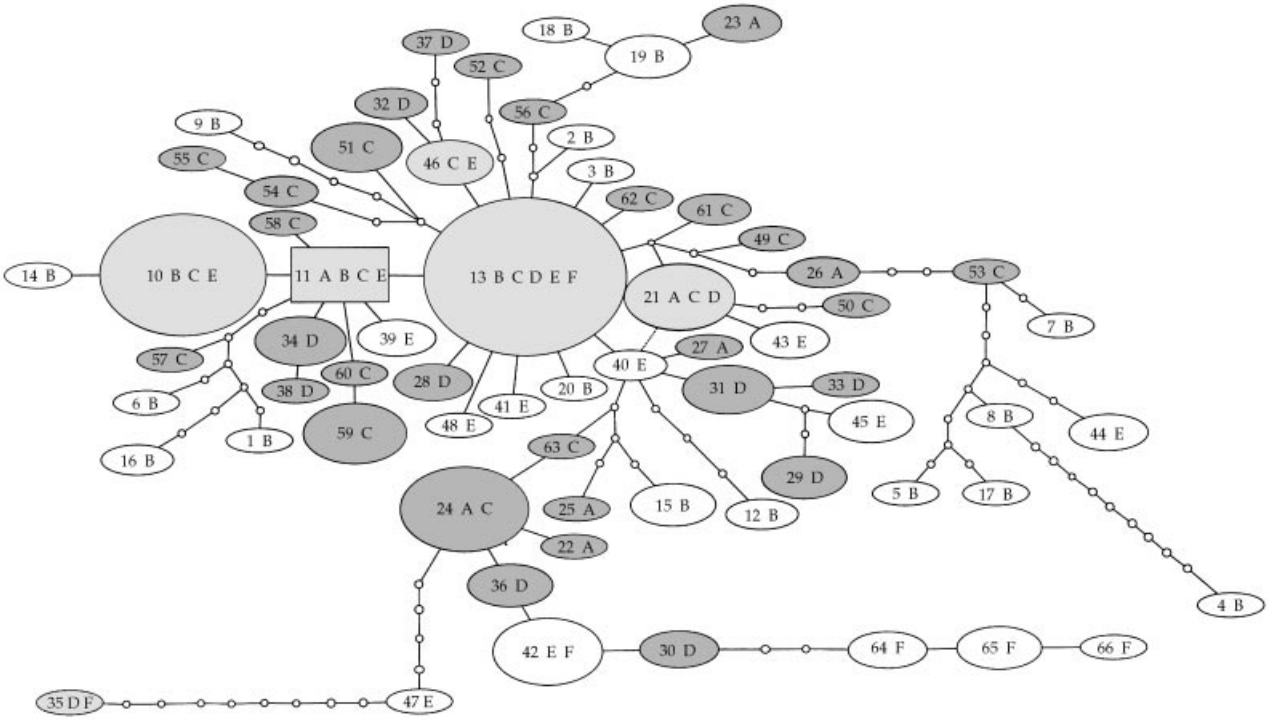

Fig. 3 Network of mtDNA control region haplotypes of *Odontesthes argentinensis*. Genealogical relationships were estimated by the parsimony method of Templeton *et al*. (1992). Numbers correspond to haplotype, and letters to populations: A, Custódia Lagoon; B, Cassino; C, Patos Lagoon; D, Montevideo; E, Rocha Lagoon; F, Puerto Madryn. White ovals represent marine haplotypes; dark grey ovals are estuarine haplotypes; grey ovals are haplotypes shared by both marine and estuarine populations. The size of ovals is proportional to the number of individuals sharing a particular haplotype. Each single line indicates one mutation between haplotypes (small circles dividing single lines represent missing haplotypes). Haplotype 11 (marked as a rectangle) had the highest outgroup probability. The dotted line between haplotypes 21 and 40 represents one alternative connection.

Lack of recent bottlenecks

We found no indication of recent reductions in effective population size in any estuarine or marine population. Tests for heterozygosity excess produced no significant population *P*-values (0.97–0.99 based on SMM; 0.08–0.89 based on TPM). This is consistent with the observation that populations displayed similar levels of variability. It suggests that, if founder effects occurred during estuarine colonization they might have been over-written by subsequent gene flow and mutation.

Haplotype network and the local radiation of mtDNA lineages

The network can be characterized as a star phylogeny with low phylogeographic structure (Fig. 3). Haplotype 11 (represented as a rectangle) had the highest outgroup probability. It is closely related to haplotype 13, the one at the centre of the network. Haplotype 13 has a widespread geographical distribution, occurring at high frequency in southern Brazil and Uruguay, and also being present in the remote population from Argentina. The star phylogeny allows a tentative assignment of polarity to the sequence of haplotype changes, working outwards from the presumed most ancient types at the interior. The resulting information was then used to seek a historical pattern in the network. We adopted a conservative approach in this analysis (i.e. haplotype 51 and 54 from Patos are assumed to have arisen from haplotype 13 in Patos, rather than from other populations that also possess 13). A note of caution, concerning 'missing data', must be introduced into the following interpretation. Some haplotypes may have existed previously in some populations, but have been lost from extant ones. Equally, some haplotypes may still be present in extant populations but have been missed during sampling. This analysis indicates that haplotypes from some populations originated from more ancient sequences present in the same population. For example, in estuarine silversides 23 haplotypes are apparently derived from mtDNA lineages found in the same locality, eight came from marine lineages, and only five from haplotypes found

in other estuarine populations. Equivalent numbers for marine haplotypes are: 22 (same locality), seven (from estuarine populations), and six (from other marine populations).

Discussion

Origin of populations

The mtDNA genealogy shows that *Odontesthes argentinensis* populations consist of a tight assemblage of haplotypes mostly separated by a small number of mutations. The centre of the network is represented by haplotypes 11 and 13. These haplotypes share important features observed in ancestral lineages: they are abundant, geographically widespread and have given rise to a large number of related and rare haplotypes (Crandall & Templeton 1993; Avise 2000). This genealogical pattern is consistent with a scenario of recent population expansions (Avise 2000). It appears that haplotypes 11 and 13 were present in the ancestral lineage and have very recently originated the majority of marine and estuarine matrilines. This suggestion is corroborated by the similar values of nucleotide diversity observed in populations and by a phylogenetic analysis of *Odontesthes* based on ~1300 bp of mtDNA conducted by Beheregaray (2000). In that study, *O. argentinensis* emerged as the most recently derived marine species of the genus, having as ancestor a group of freshwater species that diversified during the Pleistocene. The presumed Pleistocene age of *O. argentinensis* is also consistent with the geological history of formation of estuarine environments in southern Brazil (Calliari 1997) and with its close evolutionary association with the recently radiated *O. perugiae* complex (details in Beheregaray *et al.* 2002).

Despite the low phylogeographic structure, the network contains strong signal of local radiations of haplotypes in Cassino and Patos Lagoon (i.e. haplotypes that could most frequently derive from older sequences in the same population). In Cassino, 15 of 19 haplotype changes probably occurred locally; in Patos Lagoon the corresponding number is 17 of 20. However, this pattern was not clear in other populations (e.g. Custódia), possibly because they were colonized much more recently. For instance, estuarine conditions in Patos Lagoon date from the Pleistocene (Calliari 1997), whereas Custódia Lagoon was formed as a result of a regressive episode that followed the marine transgression of 5000 years ago (Villwock *et al*. 1986). The radiations of haplotypes in Patos Lagoon and Cassino, and the existence of adjacent, closely related freshwater species that show localized endemism (Beheregaray 2000; Beheregaray *et al*. 2002), suggest that the southern end of Brazil's coastal plain represents the source of origin of *O. argentinensis* populations.

Lower structure in neutral markers compared to allozymes: evidence for selection?

Our data set from Patos Lagoon and Cassino populations can also be compared with an allozyme study conducted in these localities by Beheregaray & Levy (2000). Cassino and Patos Lagoon silversides displayed largely overlapping microsatellite allele frequency distributions (Appendix I), and significant but moderate mtDNA divergence (F_{ST} = 0.063). On the other hand, the allozyme locus *mMdhp-A* showed a fixed allele in the estuary and two common alleles in Cassino, producing a locus F_{ST} of 0.29. Despite differences in polymorphism among markers and associated power for disclosing genetic structure (Hedrick 1999), the F_{ST} value of *mMdhp-A* is much higher than those found for other allozyme loci (Beheregaray & Levy 2000), suggesting that this locus may be under selection. A recent paper that compares allozymes and microsatellites in estuarine and marine populations of the European sea bass is particularly relevant here (Lemaire *et al*. 2000). The authors proposed that six (of 28 available allozyme loci) were non-neutral due to higher F_{ST} compared to microsatellites. The stronger differentiation was attributed to the estuarine life cycle of this species, which would promote selection in some loci because of their role in adaptation to salinity changes and resistance to environmental stress (Lemaire *et al*. 2000). The higher degree of differentiation exhibited by *mMdhp-A* in comparison with other allozyme loci and with the presumably neutral mtDNA and microsatellite loci, suggests that diversifying selection could be operating at the *mMdhp-A* locus in the estuary of Patos Lagoon.

Population subdivision among marine localities

Organisms with high dispersal capabilities and large population sizes are anticipated to show high levels of gene flow and low population structure. *O. argentinensis* has a wide geographical distribution (Dyer 1993), occurs in abundant schools, and has a presumed high migration capability during its adult stage (Beheregaray & Levy 2000). Nonetheless, our analysis revealed substantial genetic structure among marine localities. This structure is probably not due to historical isolation in geographically disconnected environments, as indicated by the shallow phylogeographic pattern among haplotypes. In marine animals genetic divergence can occur even in high-dispersal organisms because of the effects of isolation by large distances, selection, genetic drift, or homing behaviour (Palumbi 1992). For both mtDNA and microsatellite data sets, we detected a significant association between genetic differentiation and geographical distance among all marine samples. Thus, it is possible that the distance separating marine populations is affecting large-scale gene

flow. An ecological, fine-scale factor that could be added to geographical distance is larval retention followed by homing. This hypothesis predicts that population structure in marine fishes can be created due to the persistence of the planktonic phase in a particular area followed by the homing of adults to specific spawning sites (reviewed by Sinclair & Iles 1989). This scenario is clearly more likely to arise in taxa with demersal spawning and in species where all early life history phases are found in the same geographical area (Sinclair & Iles 1989). These two prerequisites are satisfied by *O. argentinensis* (Bemvenuti 1987). A combination of genetic drift, isolation-by-distance and homing behaviour may account for the pattern of structure among marine populations of this species.

Genetic and adaptive divergence in 'incipient estuarine species'

Significant genetic divergence was observed between estuarine and nearby marine populations, a finding consistent with allozyme data (Beheregaray & Levy 2000). In contrast to marine localities, genetic divergence among estuarine populations was not correlated with geographical distance. Fine-scale structure was documented between samples from Patos Lagoon and Cassino, less than 20 km apart. This structure was stronger than that observed between Cassino and Montevideo, separated by more than 500 km. Furthermore, given that large variations in salinity are not a limiting factor for growth and survival of *O. argentinensis* (Phonlor & Sampaio 1992), we assume that marine and estuarine parapatric ecotypes can occasionally overlap in distribution. An absence of absolute geographical and physiological barriers to gene flow, coupled with the large sizes of populations and the recent age of estuarine environments, suggest that genetic drift alone cannot account for the observed genetic divergence. Accordingly, we argue that shifts from a marine to an estuarine life history have promoted rapid adaptive divergence and reproductive isolation in estuarine silversides. Estuarine and marine ecotypes display large differences in spawning behaviour and spawning site selection: the former lay eggs on submerged vegetation of sheltered marshes (Bemvenuti 1987), while the latter spawn near-shore in a dynamic surf-zone where spawning substrate is very scarce (Phonlor & Vinagre 1989). The ecotypes also show remarkable differences in egg morphology. Marine eggs have a very thick chorion and approximately 18 adhesive looped filaments attached to the surface of the egg at both ends, and three filaments attached at only one end (Phonlor & Cousin 1997). The resistant chorion protects the embryo from the effects of water dynamics, and the adhesive filaments serve primarily to attach eggs to the substrate (Guandalini *et al*. 1994). Estuarine eggs, on the other hand, have a very thin chorion and only three to six adhesive filaments attached to the surface of the egg at one end (Phonlor & Cousin 1997). This pattern is markedly similar to the eggs of freshwater *Odontesthes* species (e.g. Guandalini *et al*. 1994). Estuarine eggs are deposited in sites where substrate is not a limiting factor, and their delicate chorion is typical of species that spawn in sheltered environments (Ivankov & Kurdyayeva 1973). It appears that eggs with a frail chorion and decreased adhesive capacity represent an adaptation to an estuarine spawning strategy, a suggestion presented by Beheregaray & Levy (2000). In addition, bioassay results indicate that marine and estuarine ecotypes have different fertilization success under varying water salinities: under controlled laboratory conditions and in a salinity of 5 (commonly found in the estuary of Patos Lagoon), marine eggs have a maximum fertilization rate of 53%, while estuarine eggs show fertilization rates up to 97% (Sampaio 1992). Finally, estuarine ecotypes complete all their life cycle inside the estuary (Bemvenuti 1987), which would favour homing and adaptation to a typical estuarine life-history strategy.

The suite of ecological differences observed between marine and estuarine ecotypes indicate that strong divergent selection could be acting on estuarine populations. We have shown that estuarine matrilines tend to display local radiations, at least for the older populations. The apparent absence of founder effects and the less marked structure revealed by microsatellites are signs that the establishment of estuarine populations has proceeded in the presence of nuclear gene flow from nearby populations. Our example adds to a list of recent studies that propose substantial levels of phenotypic and reproductive divergence without complete suppression of gene flow (e.g. Smith *et al*. 1997; Lu & Bernatchez 1999; Schneider *et al*. 1999; Korol *et al*. 2000). The pattern of divergence revealed in this study and in Beheregaray & Levy (2000), including evidence for selection on an allozyme locus, suggests an important role for ecology in shaping the evolution of reproductive isolation in estuarine silversides. A theoretical model that could explain how these incipient estuarine species have evolved is the 'divergence-with-gene-flow model of speciation' (Endler 1977). This model predicts that in parapatric scenarios with partial or no barriers to gene flow, reproductive isolation can gradually evolve when strong, multifarious and divergent selection acts on geographically separated subpopulations (Rice & Hostert 1993). We hypothesize that one isolation mechanism that may be operating in *O. argentinensis* is environment-dependent postzygotic isolation. In this situation, the restricted core range of estuarine silversides would favour localized positive assortative mating. When individuals from divergently selected lines reproduce, they would produce an intermediate phenotype that is selectively inferior under specific environmental circumstances (Rice & Hostert 1993). Environment-dependent factors required

for successful reproduction in estuaries (e.g. modified egg morphology and high fertilization rate in low salinity) are strong candidates to reduce the viability of a nontypical estuarine phenotype.

While we have proposed a favoured candidate speciation model, we acknowledge that the phenomenon of ecological speciation in nature is notoriously difficult to understand, not least because of the difficulty in teasing out the roles of historical factors and geography in promoting speciation (Rice & Hostert 1993; Schluter 1996; Moritz *et al*. 2000). In advance of more definitive testing of the major forces underlying incipient speciation in the present *Odontesthes* lineages (ideally sequence analysis of markers near and within functionally important genes involved in the divergence, R. Butlin personal communication), we consider the 'divergence-with-gene-flow model of speciation' to fit best the present data.

Fisheries management of 'incipient estuarine species'

In spite of the large differences in the chorionic filaments of eggs from Patos Lagoon (which are regarded as species-specific characters in atheriniform fishes; White *et al*. 1983), adult estuarine and marine ecotypes are morphologically indistinguishable (Bemvenuti 1993). Nonetheless, we have shown that estuarine ecotypes, although ignored by current taxonomy, represent truly incipient species with phenotypic attributes that are likely to be of significance in population persistence (see Crandall *et al*. 2000). *O. argentinensis* is an economically important resource for local fisheries in southern Brazil, Uruguay and northern Argentina (De Buen 1953; Chao *et al*. 1986). From a management point of view estuarine and marine populations should be treated as individual stocks when formulating fisheries policies. We emphasize that fishery practices that aim to conserve the genetic integrity of these geographical populations are likely to result in long-term management success.

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This work is part of Luciano B. Beheregaray's PhD thesis on speciation, phylogeny and biogeography of the silverside genus *Odontesthes*. In this project, a range of molecular markers was used to reconstruct recent and historical relationships among silversides from temperate South America. Luciano is currently a Postdoctoral Fellow at the Department of Ecology and Evolutionary Biology, Yale University. He was supervised by Paul Sunnucks, who has interests on evolution and population genetics of several organisms, particularly of invertebrates.

Appendix I Allele frequency of nine microsatellite loci in six populations of *Odontesthes argentinensis* from Brazil, Uruguay and Argentina. Number of genotyped individuals is shown between brackets. Alleles are in base pairs

Locus-allele	Custódia Lagoon	Cassino	Patos Lagoon	Rocha Lagoon	Montevideo	Puerto Madryn
Odont02	**(20)**	**(48)**	**(40)**	**(38)**	**(32)**	**(16)**
129	0	0.01	0	0	0	0
133	0.075	0.042	0.013	0.026	0.047	0
135	0	0.042	0	0.013	0	0.031
137	0.075	0.063	0.112	0.118	0.078	0.281
139	0.1	0.094	0.087	0.092	0.063	0.281
141	0.225	0.031	0.125	0.105	0.078	0
143	0.15	0.052	0.087	0.092	0.078	0.063
145	0.025	0.177	0.038	0.013	0.172	0.063
147	0	0.083	0.025	0.053	0.047	0.063
149	0	0.063	0.013	0.079	0.094	0.031
151	0.075	0.052	0.038	0.039	0.031	0.031
153	0.025	0.052	0.025	0.039	0.031	0.125
155	0.025	0.021	0.05	0.013	0.031	0.031
157	0.075	0.01	0.063	0.066	0.094	0
159	0	0.021	0.05	0.039	0	0
161	0.075	0.01	0.038	0.066	0.047	0
163	0	0.031	0.013	0.026	0.016	0
165	0.025	0.031	0.013	0.013	0	0
167	0	0.01	0.013	0.013	0.016	0
169	0	0.021	0.025	0	0.016	0
171	0.025	0.021	0.038	0	0.016	0
173	0.025	0.01	0.025	0.026	0	0
175	0	0.01	0.05	0.039	0.016	0
177	0	0	0	0	0.016	0
179	0	0	0.013	0	0	0
181	0	0	0.025	0.013	0.016	0
183	0	0.021	0.013	0	0	0
187	0	0.01	0.013	0	0	0
189	0	0.01	0	0	0	0
191	0	0	0	0.013	0	0
Odont07	**(20)**	**(50)**	**(35)**	**(38)**	**(30)**	**(16)**
157	0	0	0	0	0.017	0
159	0	0	0	0	0	0.094
161	0	0.01	0	0.026	0.067	0.031
163	0	0.01	0.029	0.039	0	0.063
165	0.05	0.04	0.071	0.053	0.083	0.031
167	0.175	0.04	0.1	0.026	0.067	0
169	0	0.02	0.029	0.013	0	0
171	0	0.01	0	0.013	0.117	0
173	0.025	0.05	0.071	0.026	0.067	0.313
175	0.1	0.16	0.029	0.092	0.083	0.063
177	0.175	0.09	0.043	0.158	0.117	0.031
179	0.075	0.08	0.086	0.184	0.1	0.031
181	0.075	0.16	0.1	0.145	0.133	0.125
183	0.1	0.11	0.214	0.066	0.05	0.094
185	0.025	0.04	0.086	0.066	0	0
187	0.025	0.05	0.014	0.039	0.017	0.031
189	0	0.04	0	0	0	0.031
191	0	0.02	0	0	0	0.031
193	0.1	0.01	0.014	0.039	0.033	0.031
195	0.025	0	0.043	0	0	0
197	0.025	0.03	0.029	0.013	0.017	0
199	0	0.03	0.014	0	0.033	0
205	0.025	0	0	0	0	0

Appendix I *Continued*

Locus-allele	Custódia Lagoon	Cassino	Patos Lagoon	Rocha Lagoon	Montevideo	Puerto Madryn
209	0	0	0.029	0	0	0
Odont08	**(20)**	**(48)**	**(37)**	**(36)**	**(32)**	**(16)**
126	0	0.01	0	0	0	0
128	0.075	0.042	0.041	0.028	0.031	0
130	0.175	0.24	0.27	0.319	0.297	0.531
132	0.075	0.115	0.054	0.083	0.016	0.063
134	0.025	0.052	0.054	0.139	0.031	0
136	0.025	0.042	0.122	0.042	0.109	0
138	0.05	0.063	0.027	0.014	0.031	0
140	0.025	0.083	0.014	0.014	0.031	0.063
142	0.125	0.094	0.203	0.194	0.25	0.219
144	0.025	0.031	0.041	0.069	0.016	0
146	0.025	0.031	0.014	0.014	0.047	0.063
148	0.025	0.01	0.014	0	0	0
150	0	0	0.014	0.028	0	0
152	0.025	0.01	0.027	0	0	0
154	0.05	0	0.054	0.028	0	0
156	0.05	0.042	0.014	0.014	0.094	0.031
158	0.025	0	0	0	0.016	0.031
160	0.075	0.031	0.014	0	0	0
162	0	0.042	0.014	0.014	0	0
164	0.075	0.01	0	0	0.016	0
166	0.05	0	0	0	0	0
168	0	0.021	0.014	0	0.016	0
170	0	0.01	0	0	0	0
176	0	0.01	0	0	0	0
178	0	0.01	0	0	0	0
Odont09	**(20)**	**(48)**	**(40)**	**(38)**	**(32)**	**(15)**
149	0	0	0	0.013	0	0
153	0.05	0.01	0.013	0	0.016	0
155	0	0	0	0	0.016	0
157	0.05	0.031	0	0.026	0.031	0
159	0.025	0.104	0.038	0.013	0.047	0.433
161	0.175	0.115	0.338	0.316	0.219	0.1
163	0.075	0.167	0.075	0.145	0.078	0.2
165	0.1	0.073	0.15	0.053	0.063	0
167	0.1	0.063	0.1	0.171	0.219	0.267
169	0.175	0.135	0.075	0.118	0.156	0
171	0.075	0.052	0.05	0.053	0.094	0
173	0.05	0.073	0.05	0.053	0.031	0
175	0.025	0.073	0.05	0.026	0.031	0
177	0.075	0.052	0.038	0	0	0
179	0	0.021	0	0	0	0
181	0.025	0.031	0	0	0	0
183	0	0	0.013	0.013	0	0
193	0	0	0.013	0	0	0
Odont11	**(18)**	**(50)**	**(39)**	**(37)**	**(32)**	**(16)**
128	0	0.03	0.013	0	0.016	0
130	0	0	0	0.014	0	0
132	0	0	0.013	0.014	0.031	0
134	0	0.01	0	0	0	0.031
136	0	0	0	0	0.016	0
138	0	0.02	0	0	0	0
140	0	0	0.026	0	0.031	0.063
142	0.389	0.43	0.423	0.446	0.469	0.813

Appendix I *Continued*

Locus-allele	Custódia Lagoon	Cassino	Patos Lagoon	Rocha Lagoon	Montevideo	Puerto Madryn
144	0.083	0.13	0.051	0.081	0.125	0
146	0	0.05	0.026	0.027	0.047	0
148	0.111	0.12	0.154	0.135	0.109	0.031
150	0	0.02	0.026	0.014	0	0
152	0.278	0.12	0.205	0.216	0.125	0.063
154	0.056	0.06	0.064	0.041	0	0
156	0	0	0	0.014	0.031	0
160	0.056	0	0	0	0	0
164	0.028	0.01	0	0	0	0
Odont16	**(19)**	**(49)**	**(39)**	**(38)**	**(29)**	**(16)**
120	0	0	0	0	0.017	0
128	0	0	0.013	0	0	0
130	0.289	0.398	0.372	0.526	0.362	0.219
132	0.605	0.571	0.474	0.395	0.448	0.781
134	0.079	0.031	0.141	0.026	0.155	0
136	0.026	0	0	0.053	0.017	0
Odont23	**(20)**	**(49)**	**(40)**	**(37)**	**(31)**	**(16)**
108	0	0	0	0.014	0	0
112	0.225	0.163	0.125	0.095	0.065	0.25
114	0	0.041	0.075	0.108	0.065	0.156
116	0	0.051	0.038	0.027	0.081	0.094
118	0.05	0.041	0.013	0	0.048	0
120	0	0.01	0.013	0	0	0
122	0.225	0.163	0.087	0.095	0.21	0.063
124	0	0.031	0.038	0	0.016	0
126	0.025	0.082	0.05	0.027	0.048	0
128	0.125	0.092	0.112	0.176	0.097	0.188
130	0	0.02	0.013	0.041	0.065	0.031
132	0.05	0.061	0.063	0.081	0.065	0
134	0.075	0.071	0.2	0.149	0.145	0.188
136	0.025	0.041	0.05	0	0.016	0
138	0	0.031	0	0.027	0	0
140	0	0.01	0.025	0.041	0	0
142	0	0	0	0.014	0	0
144	0	0	0	0.014	0	0
146	0	0.031	0.013	0.027	0.032	0
148	0.125	0.031	0.063	0.041	0.016	0.031
150	0.075	0.031	0.025	0.027	0.032	0
Odont29	**(20)**	**(50)**	**(40)**	**(37)**	**(32)**	**(16)**
118	0	0.02	0.025	0	0	0
122	0.3	0.63	0.488	0.5	0.422	0.656
124	0.55	0.25	0.387	0.405	0.469	0.313
126	0.05	0.09	0.05	0.068	0.063	0.031
128	0.1	0	0.038	0.027	0.047	0
130	0	0.01	0.013	0	0	0
Odont39	**(19)**	**(49)**	**(40)**	**(37)**	**(32)**	**(16)**
163	0.026	0.041	0.05	0.027	0.109	0
165	0.237	0.255	0.225	0.216	0.203	0.438
167	0.132	0.163	0.075	0.149	0.203	0.344
169	0.053	0.194	0.25	0.284	0.219	0
171	0.053	0.01	0	0.027	0.016	0
173	0.026	0.02	0.038	0.027	0	0.031
177	0.026	0.061	0.075	0.095	0.063	0.063
179	0	0.02	0.013	0	0.031	0
181	0.026	0	0	0	0	0.031

Appendix I *Continued*

Locus-allele	Custódia Lagoon	Cassino	Patos Lagoon	Rocha Lagoon	Montevideo	Puerto Madryn
185	0.053	0.031	0.05	0.014	0.031	0
187	0	0	0	0.041	0	0
189	0.026	0.01	0.038	0.054	0.031	0.031
191	0.158	0.112	0.05	0	0.047	0
193	0	0.01	0.063	0.014	0.016	0.031
195	0.158	0.051	0.038	0.054	0.016	0
197	0.026	0.01	0.013	0	0.016	0.031
199	0	0.01	0.025	0	0	0