THE ROYAL SOCIETY

Received 14 May 2001
Accepted 10 August 2001
Published online 10 December 2001

A rapid fish radiation associated with the last sea-level changes in southern Brazil: the silverside *Odontesthes perugiae* complex

Luciano B. Beheregaray[1*], Paul Sunnucks[2] and David A. Briscoe[1]

[1]*Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia*
[2]*Department of Genetics, La Trobe University, Melbourne, VIC 3086, Australia*

Coastal freshwater fishes provide valuable models for studying the role of the last glaciations in promoting speciation. To date, the great majority of studies are of Northern Hemisphere taxa, and reflect the influence of vicariant events during, or prior to, the Pleistocene. Microsatellite markers and mitochondrial DNA sequences were used to investigate patterns of population divergence and evolutionary relationships in a freshwater group of silverside fishes (*Odontesthes perugiae* complex), endemic to the recently formed coastal plain of southern Brazil. Lacustrine morphotypes showed concordant patterns of genetic and morphological divergence consistent with the geographical history of the coastal plain. The results support the proposal of a silverside radiation chronologically shaped by the sea-level changes of the Pleistocene and Holocene. The radiating lineage comprises a minimum of three allopatric and two sympatric lacustrine species. Four species displayed extremely high levels of genetic variation and some of the most rapid speciation rates reported in fishes. These features were related to a marine–estuarine origin of the radiation. To the best of our knowledge, this study represents the first molecular phylogeographic survey of a coastal radiation in South America.

Keywords: phylogeography; radiation; speciation; *Odontesthes*; microsatellites; mitochondrial DNA control region

1. INTRODUCTION

Coastal regions can be viewed as highly dynamic areas from the standpoint of historical geography. Periodic oscillations in climate and habitat availability, especially during the last glacial age, have had profound effects on the distribution and evolutionary histories of many biological groups (Hewitt 1996, 2001; Knowles 2001). The influence of glaciations in promoting population subdivision and genetic divergence is particularly well documented for coastal freshwater fishes (e.g. Bermingham & Avise 1986; Bernatchez & Dodson 1991; Schluter 1996; Taylor *et al.* 1997; Avise (2000) and references therein). Local assemblages of coastal freshwater fishes usually display hierarchical patterns of genetic differentiation and phylogeographic structure that reflect the geographical history of the region (Bermingham & Avise 1986; Avise 2000). These assemblages, therefore, provide valuable models for studying the role of geological history in shaping evolutionary divergence and speciation. Nonetheless, the great majority of available studies are geographically and chronologically restricted to Northern Hemisphere taxa and to vicariant events during, or prior to, the Pleistocene (Hewitt 2001).

We investigated patterns of evolutionary divergence and the vicariant history of a group of *Odontesthes* silverside fishes distributed in a coastal region of southern Brazil formed during the Pleistocene and Holocene. *Odontesthes* is a diverse and widespread genus, with a minimum of 13 species groups distributed in marine, estuarine and freshwater environments of temperate South America (Dyer 1998). The focus of our study is the strictly freshwater *O. perugiae* species complex; a group comprising several allopatric and sympatric morphotypes found in the lakes and rivers of southern Brazil, Uruguay and northern Argentina (Bemvenuti 1997; Dyer 1998). Most morphotypes have uncertain taxonomic status and are endemic to the vast system of lakes of the Coastal Plain of Rio Grande do Sul State (CPRS), southern Brazil (Bemvenuti 1995, 1997; Dyer 1998; Beheregaray 2000). The CPRS is an elongated flat area between 29 °S and 34 °S, formed by large fluctuations in climate and sea level during the Pleistocene and Holocene (Villwock *et al.* 1986). The western CPRS contains an alluvial system that drains a large hydrographic basin. The eastern section is covered by a 'multiple sand-barrier complex' created by a minimum of four successive transgression–regression cycles that deposited four discontinuous barrier islands parallel to the coast (Villwock *et al.* 1986). Each barrier originated at the landward limit of a marine transgression and has been preserved as a result of the regression of the shoreline during glacio-eustatic falls in sea level (Villwock & Tomazelli 1995). The geomorphological history of this area is well characterized and the age of the depositional environments has been estimated by oxygen-isotope curves, radiocarbon dating and by stratigraphic and palaeontological studies (Villwock & Tomazelli 1995; Buchmann *et al.* 1998).

A fundamental issue is whether the *O. perugiae* morphotypes endemic to coastal lakes are the outcome of an evolutionary radiation or merely reflect phenotypic plasticity within a single species. The former hypothesis would be

*Author and address for correspondence: Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06520-8106, USA (luciano.beheregaray@yale.edu).

supported if the lacustrine populations are monophyletic, and if there is evidence of reproductive isolation among morphologically divergent populations. To address this question, we analysed the population genetic structure and evolutionary relationships of lacustrine and riverine morphotypes using microsatellite markers and mitochondrial DNA (mtDNA) sequences. Our findings support the proposal of a very rapid silverside radiation associated with the last sea-level changes in southern Brazil. The results are compared with other information available for coastal silverside fishes, to develop a biogeographical scenario of divergence for the *O. perugiae* complex. To the best of our knowledge, this study represents the first molecular phylogeographic survey of a coastal radiation in South America.

2. MATERIAL AND METHODS

(a) *The* O. perugiae *complex: sampling and current taxonomy*

Coastal lacustrine populations of the *O. perugiae* complex are referred to here simply as *perugiae*, to distinguish the complex as a whole from the taxon *O. perugiae*. A total of 202 individuals were collected from seven lakes and one river in southern Brazil (figure 1). The riverine sample corresponds to *sensu strictu O. perugiae* (Evermann & Kendall 1906). Lacustrine samples represent five coastal *perugiae* morphotypes (table 1) distinguished by multivariate analysis using 31 morphometric and 11 meristic variables (Beheregaray 2000). Two of them, classified as *O. perugiae* (Bemvenuti 1997; Dyer 1998) and the recently described *O. mirinensis* (Bemvenuti 1995), occur sympatrically in the southern lakes of the CPRS (Bemvenuti 1997). Samples from the other three morphotypes correspond to the three new species endemic to the northern lakes of the CPRS described by Malabarba & Dyer (2002), including some of the type specimens used in their descriptions. Muscle tissue was preserved in 95% ethanol and voucher specimens deposited at the Museu de Ciências e Tecnologia da PUC-RS (Porto Alegre, RS, Brazil).

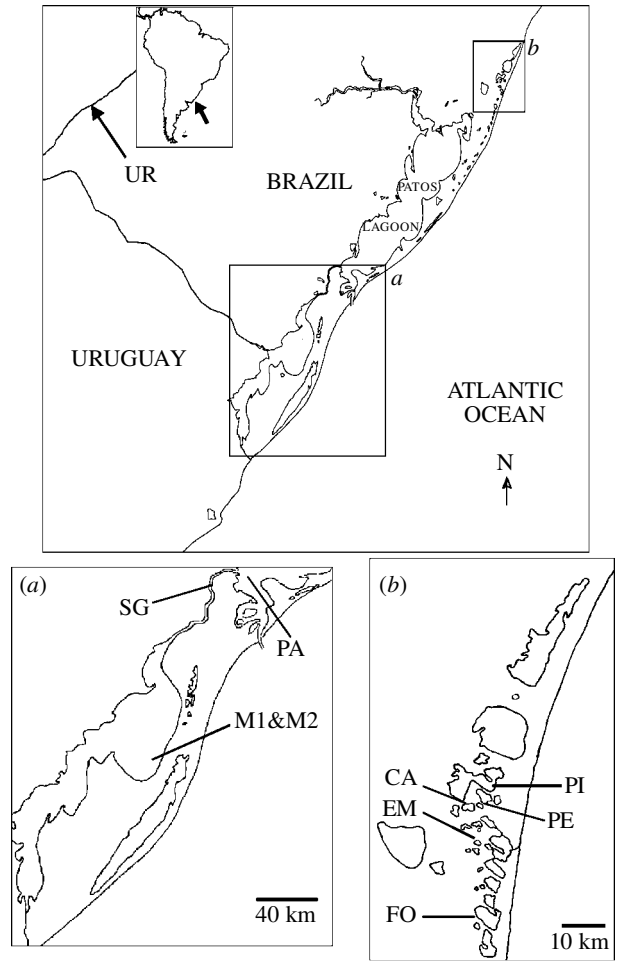

Figure 1. Collection sites in southern Brazil representing nine lacustrine samples and one riverine sample of the *O. perugiae* complex. Map (*a*) shows sites sampled in the southern drainage system: SG, São Gonçalo; PA, Patos Lagoon; M1 and M2, samples of sympatric morphotypes D and E from Mirim Lake 1 and 2, respectively. Map (*b*) shows collection sites in the northern drainage system: CA, Caconde Lake; EM, Emboaba Lake; FO, Fortaleza Lake; PI, Pinguela Lake; PE, Peixoto Lake. UR (top) corresponds to the riverine sample from the Uruguay River.

(b) *Genetic methods*

DNA was extracted using a salting-out method (Sunnucks & Hales 1996). All individuals were screened for variation at 400 bp of the hypervariable section of the mtDNA control region and at eight microsatellite loci. For the polymerase chain reaction (PCR) amplification of the control region fragment, primers of Lee *et al.* (1995) were used under the conditions described in Beheregaray & Sunnucks (2001). Sequence variants were identified using SSCP (single-stranded conformation polymorphism) and sequence analysis following Sunnucks *et al.* (2000). For the microsatellite analysis we PCR-amplified eight dinucleotide loci developed for the taxa undergoing radiation: Odont02, 07, 08, 09, 11, 16, 29 and 39 (Beheregaray & Sunnucks 2000). The reliability of PCR was tested by independently amplifying 10 individuals three times across loci; products from different trials consistently produced the same individual genotype.

(c) *Data analysis*

(i) *Genetic variability, Hardy–Weinberg and linkage tests*

Mitochondrial DNA variability was estimated by computing haplotype and nucleotide diversity in Arlequin 2000 (Schneider *et al.* 2000). For microsatellites, we used Genepop (Raymond & Rousset 1995) to calculate allele frequencies, expected (H_E) and observed (H_O) heterozygosities, and to conduct tests for linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) employing the Markov chain method.

(ii) *Population divergence*

Population divergence was estimated by computing F_{ST} (Weir & Cockerham 1984) using the programs Arlequin for the mtDNA dataset and F-stat (Goudet 1995) for the microsatellites. Arlequin was also used to perform hierarchical analyses of molecular variance (AMOVA) with F_{ST} calculated from the microsatellites and mtDNA. AMOVA was used to test whether the contemporary drainage structure provides a good explanation for the genetic divergence of *perugiae*. Two tests were executed:

(a) all *perugiae* samples assigned as a single group; and (b) samples separated by drainage system (one group of southern versus one of the northern lakes). The significance of F_{ST} and AMOVA comparisons was assessed by permutating the values

Table 1. Summary of genetic variability in one riverine and nine lacustrine samples of the *O. perugiae* species complex based on mtDNA control-region sequences and eight microsatellite loci.
(Letters correspond to distinct morphotypes identified by multivariate morphometric analysis (see text). NH, number of haplotypes; NA, number of alleles at all loci; H_E and H_O, mean expected and observed heterozygosity, respectively. Values in parentheses are standard errors. Tables with the frequency of mtDNA haplotypes and allelic frequency of the microsatellite loci are presented in electronic Appendices A and B available from The Royal Society's Publications Web site. Haplotype sequences were deposited at GenBank (accession nos. AF419859–AF419943).)

			mtDNA			microsatellites		
locality	*n*	morphotype	NH	haplotypic diversity	nucleotide diversity	NA	H_E	H_O
Caconde Lake	20	A	5	0.75 (0.057)	0.022 (0.012)	38	0.44 (0.08)	0.46 (0.08)
Emboaba Lake	10	A	4	0.73 (0.120)	0.018 (0.010)	32	0.49 (0.10)	0.57 (0.11)
Pinguela Lake	18	B	13	0.94 (0.041)	0.018 (0.010)	56	0.62 (0.10)	0.60 (0.11)
Peixoto Lake	10	B	7	0.94 (0.072)	0.021 (0.012)	45	0.62 (0.12)	0.64 (0.13)
Fortaleza Lake	28	C	15	0.93 (0.026)	0.021 (0.011)	64	0.64 (0.11)	0.65 (0.11)
Patos Lagoon	18	D	17	0.99 (0.021)	0.029 (0.015)	67	0.75 (0.07)	0.72 (0.10)
São Gonçalo	24	D	15	0.96 (0.030)	0.024 (0.013)	63	0.68 (0.09)	0.61 (0.07)
Mirim 1	36	D	23	0.97 (0.011)	0.027 (0.014)	77	0.68 (0.10)	0.64 (0.11)
Mirim 2	18	E	11	0.93 (0.040)	0.028 (0.015)	57	0.63 (0.12)	0.65 (0.12)
Uruguay River	20	F	5	0.77 (0.061)	0.007 (0.004)	44	0.56 (0.11)	0.53 (0.10)

2000 times. Significance levels of multiple simultaneous comparisons were corrected with the sequential Bonferroni procedure (Rice 1989).

(iii) *Phylogenetic analysis*

The reconstruction of evolutionary relationships and the establishment of monophyly of *perugiae* represent a challenge because of the presumed small amount of time available for populations to acquire divergent character states. Relationships were investigated using the rapidly evolving microsatellite markers. The chord genetic distance, D_{CE} (Cavalli-Sforza & Edwards 1967), was calculated because it has a superior performance in obtaining the correct tree topology when the divergences are recent (Takezaki & Nei 1996). Trees were reconstructed by the UPGMA method with 2000 bootstrap replications using PHYLIP (Felsenstein 1993). This analysis was carried out after pooling samples identified as genetically homogeneous populations.

A haplotype network was also constructed with the mtDNA control region sequences based on the statistical parsimony of Templeton *et al.* (1992). This method uses a 95% probability criterion to link firstly haplotypes with the smaller number of differences, providing higher resolution than traditional phylogenetic methods for inferring relationships among recently diverged populations (Posada & Crandall 2001). The analysis was performed in TCS (Clement *et al.* 2000); a program that uses predictions from coalescent theory to calculate root probabilities and relative haplotype age.

3. RESULTS

(a) *Genetic variability, linkage and HWE tests*

An extremely high level of mtDNA control region variability was observed in coastal *perugiae*: 80 different haplotypes were detected by SSCP analysis and confirmed by sequencing. By contrast, only five haplotypes were found in the riverine *O. perugiae*. Haplotypic diversity (*h*) of most lacustrine samples was surprisingly high, ranging from 0.93 to 0.99 (table 1). In some localities, nearly every individual was from a different matriline (in Patos Lagoon, 17 out of 18 fishes had unique haplotypes). The exceptions were the samples from Caconde ($h = 0.75$) and Emboaba ($h = 0.73$), which showed similar variation to the riverine sample ($h = 0.77$). Most *perugiae* haplotypes had low sequence divergence (0.25–4.75%), and nucleotide diversity varied little among the populations (table 1). A similar pattern was found in the nuclear DNA: heterozygosity and allele diversity in Caconde and Emboaba were similar to the riverine population and significantly lower than in other lacustrine populations (Wilcoxon signed-rank tests, all $p < 0.05$) (table 1). No significant linkage disequilibrium was found in microsatellite locus pair–population comparisons. Thus, all loci were considered as independent markers. Probability tests detected only five significant deviations from HWE out of 80 comparisons, with three of them in Mirim 1 being due to heterozygote deficiencies. The Wahlund effect appears to be an adequate explanation for the deficiencies as two similar morphotypes were collected in this locality (figure 1). Mirim Lake 1 may contain unrecognized individuals of the E morphotype, or perhaps some individuals with alleles introgressed from that morphotype.

(b) *Genetic divergence*

Analysis of population divergence yielded similar results for both nuclear and mtDNA datasets (table 2). A marked population structure was observed, with the higher differentiation recorded between the riverine and lacustrine samples (table 2). For lacustrine *perugiae*, comparisons among those from the northern drainage (Caconde, Emboaba, Pinguela, Peixoto and Fortaleza) showed much stronger structure than among the southern lakes (Patos, São Gonçalo and Mirim). Substantial divergence was observed between populations represented by different morphotypes: 39 out of 40 comparisons were significant for nuclear and mitochondrial datasets. The only non-significant F_{ST} values between distinct morphotypes (Fortaleza versus Peixoto for mtDNA and Mirim 1 versus Mirim 2 for microsatellites) may be attributable to smaller

Table 2. Population structure measured by F_{ST}.
(Comparisons based on eight microsatellite loci (below diagonal) and mtDNA sequences (above diagonal) between nine lacustrine and one riverine sample of the *O. perugiae* species complex (top matrix), and between samples pooled within species (bottom matrix, see text). All pairwise comparisons were significant ($p < 0.05$) after 2500 permutations, except where indicated by ^{ns}.)

(*a*) all samples	1	2	3	4	5	6	7	8	9	10
1. Caconde Lake	—	0.039^{ns}	0.128	0.141	0.112	0.131	0.147	0.123	0.144	0.237
2. Emboaba Lake	0.049	—	0.110	0.144	0.126	0.126	0.143	0.127	0.156	0.241
3. Pinguela Lake	0.095	0.035	—	0.031^{ns}	0.045	0.027	0.050	0.038	0.062	0.141
4. Peixoto Lake	0.079	0.035	−0.023^{ns}	—	0.041	0.029	0.049	0.039	0.064	0.148
5. Fortaleza Lake	0.078	0.045	0.019	0.008^{ns}	—	0.031	0.054	0.043	0.067	0.141
6. Patos Lagoon	0.171	0.100	0.038	0.039	0.045	—	0.014^{ns}	0.002^{ns}	0.027	0.115
7. São Gonçalo	0.155	0.093	0.033	0.033	0.034	−0.003^{ns}	—	0.025	0.039	0.131
8. Mirim 1	0.150	0.097	0.037	0.036	0.039	0.001^{ns}	−0.001^{ns}	—	0.010^{ns}	0.118
9. Mirim 2	0.133	0.068	0.020	0.022	0.021	0.017	0.014	0.015	—	0.147
10. Uruguay River	0.383	0.343	0.275	0.283	0.268	0.212	0.212	0.221	0.258	—

(*b*) samples pooled as species	morphotype	1	2	3	4	5	6
1. *O.* sp. A (Caconde and Emboaba)	A	—	0.115	0.111	0.118	0.147	0.212
2. *O.* sp. B (Pinguela and Peixoto)	B	0.066	—	0.031	0.026	0.053	0.105
3. *O.* sp. C (Fortaleza)	C	0.063	0.021	—	0.038	0.067	0.118
4. *O. mirinensis* (Patos, São Gonçalo and Mirim 1)	D	0.124	0.040	0.036	—	0.017	0.089
5. *O. perugiae* 'lacustrine' (Mirim 2)	E	0.107	0.026	0.040	0.016	—	0.124
6. *O. perugiae* 'riverine' (Uruguay River)	F	0.368	0.279	0.268	0.206	0.258	—

sample sizes (see pooled samples in table 2*b*). By contrast, a pattern of genetic homogeneity was evident in comparisons of samples with the same morphotype.

The AMOVA results confirmed that a significant proportion of the molecular variance (7.1% for mtDNA and 5.7% for microsatellites, $p < 0.01$) could be explained by differences among the localities. However, AMOVA revealed no significant differentiation when samples were separated by a drainage system ($p > 0.05$ for mtDNA and microsatellites). As a next step, lacustrine samples with no significant structure were pooled, forming five genetically distinct population groups (table 2*b*) that agree with morphotype classification: 'Caconde–Emboaba' (*O.* sp. A), 'Pinguela–Peixoto' (*O.* sp. B), 'Fortaleza' (*O.* sp. C), 'Patos–São Gonçalo–Mirim 1' (*O. mirinensis*) and 'Mirim 2' (lacustrine *O. perugiae*).

(c) *Phylogenetic relationships*

The microsatellite D_{CE} tree provided high bootstrap support for monophyly of the lacustrine *perugiae*, excluding the riverine *O. perugiae* from the coastal group (figure 2*a*). Lacustrine species did not cluster according to drainage structure: the northern *O.* sp. A appeared as basal, while the other two northern species (*O.* sp. B and *O.* sp. C) were derived compared to all *perugiae*. A different genealogical picture was found in mtDNA haplotypes. The mtDNA network revealed a shallow pattern of phylogeographic structure (figure 2*b*): *perugiae* species appear to have recently derived from a population possessing abundant haplotypes '10' (ancestral according to the parsimony criteria) and '13' (the widespread matriline at the centre of network). Most haplotypes did not cluster according to species designation. The close relationship of haplotypes from different species was observed even in the two most diverged phylogroups (represented by haplotypes '2' and '4'). By contrast, as observed with microsatellites, sequences from the riverine *O. perugiae* clustered separately, suggesting that this species is not involved in the diversification of the coastal lineage.

4. DISCUSSION

(a) *The* perugiae *radiation and the last sea-level changes*

For the lacustrine *perugiae* group, both mtDNA and microsatellites showed substantial genetic structure between populations displaying different morphotypes and no significant pattern of structure between populations with the same morphotype. The marked agreement between morphological (Beheregaray 2000; Malabarba & Dyer 2002) and genetic divergence indicates that the diversity of morphotypes is not the product of a single, widespread and phenotypically plastic taxon. This conclusion supports the hypothesis of a silverside radiation along freshwater environments of the CPRS, the first radiation reported for South American silversides. Our results show that *perugiae* is a monophyletic group represented by a minimum of three allopatric (*O.* sp. A, *O.* sp. B and *O.* sp. C) and two sympatric species (*O. mirinensis* and *O. perugiae*). Allopatric *perugiae* are endemic to the small northern drainage of the CPRS—the Tramandaí system (Beheregaray 2000; Malabarba & Dyer 2002). They exhibit significant differences in age and growth parameters (Becker 1995), and a remarkable fine-scale genetic structure: different species are found in lakes separated by less than 5 km. On the other hand, *O. mirinensis* and *O. perug-*

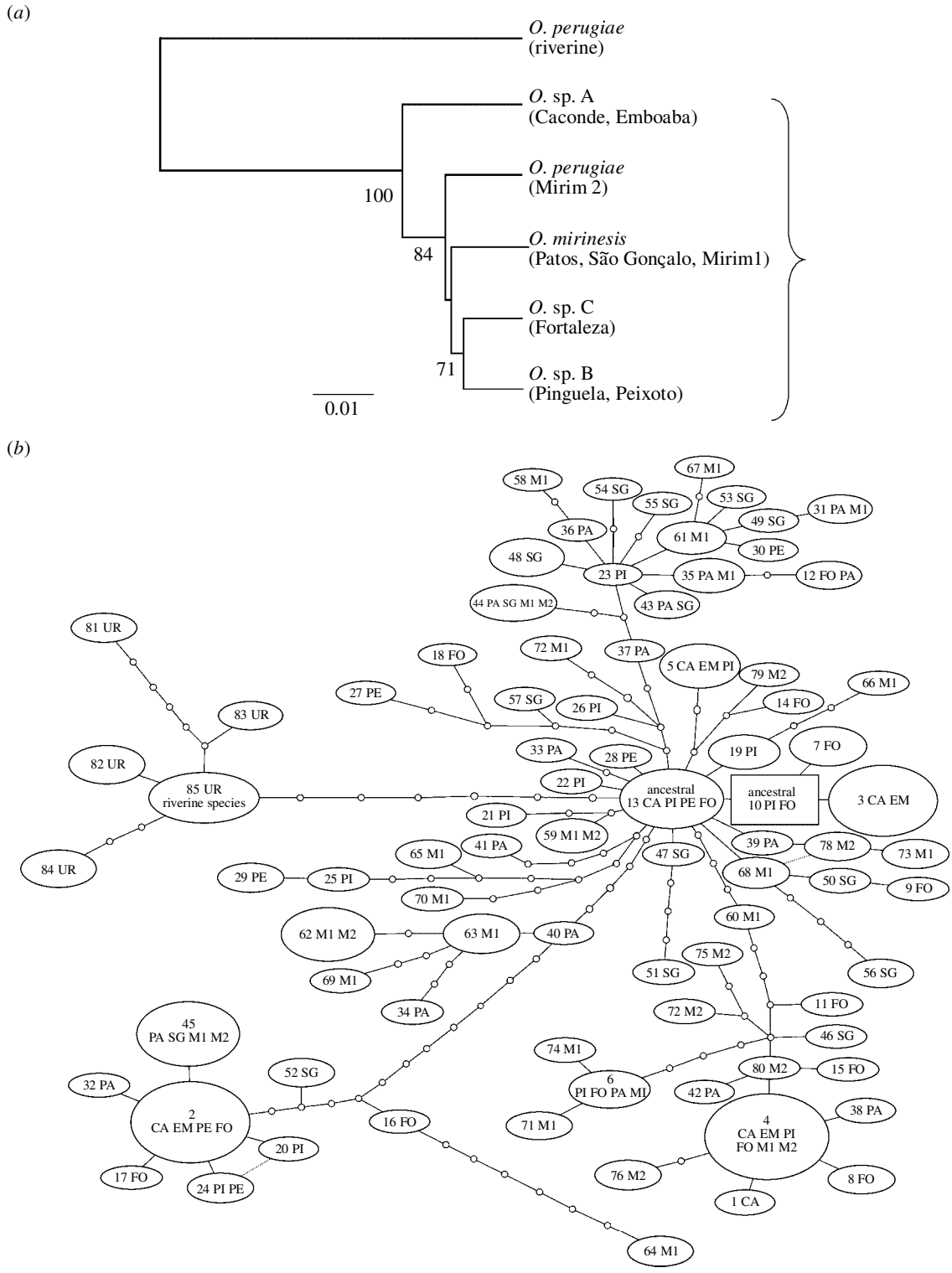

Figure 2. Phylogenetic reconstructions for the *O. perugiae* complex. (*a*) Microsatellite tree generated with the D_{CE} genetic distance and the UPGMA method. Bootstrap values greater than 50% are shown below branches (based on 2000 replicates). (*b*) Mitochondrial DNA network of control region sequences estimated with the method of statistical parsimony of Templeton *et al.* (1992). The numbers are the haplotypes and the letters the collection sites (see legend to figure 1). The size of the ovals is proportional to the haplotype frequency in each population. Each single line indicates one mutation between haplotypes (small circles dividing single lines represent missing haplotypes). Haplotypes '13' and '10' were considered ancestors based on coalescence theory (see text). Homoplasy was low (observed between haplotypes '24' and '20', and '68' and '78').

iae have much wider distributions, occurring sympatrically along the southern drainage—the Patos Lagoon system (Bemvenuti 1995, 1997; Beheregaray 2000).

No significant differences were found using AMOVA when the *perugiae* samples were divided by drainage system. Moreover, F_{ST} analyses and the microsatellite phylogeny showed that the northern *O.* sp. B and *O.* sp. C are more related to the two southern species than to their close neighbour *O.* sp. A. This indicates that contemporary drainage structure does not provide a good explanation

for the diversification of *perugiae*. Instead, we propose a biogeographic scenario of divergence for the radiating lineage chronologically shaped during the last sea-level changes by the deposition of a series of discontinuous sand barriers parallel to the coast. Particularly relevant was the creation around 120 000 years ago (ya) (oxygen-isotope 5e) of the barrier system III, after the third and last Pleistocene marine transgression in the CPRS (Villwock *et al.* 1986; Villwock & Tomazelli 1995). This barrier formed the vast Patos–Mirim lagoonal body in the south and a depositional area in northern CPRS. During the following prolonged regression, lasting between 60 000 and 17 000 years, the northern area evolved as a group of isolated freshwater lakes not reached by subsequent marine transgressions (Schwarzbold & Schäeffer 1984; Villwock & Tomazelli 1995). By contrast, the southern Patos–Mirim is geomorphologically classified as a partially closed lagoon (Calliari 1997). For instance, sedimentary structures and radiocarbon dating of marine fossils revealed the presence of estuarine conditions in Mirim Lake in periods as recent as 4900–4300 ya (Buchmann *et al.* 1998). In our study, the northern 'isolated' Pleistocene lakes are represented by samples from Caconde and Emboaba (*O.* sp. A), which probably correspond to some of the first *perugiae* to be established in permanent freshwater environments. This is consistent with the microsatellite phylogeny where *O.* sp. A is basal to all other *perugiae*, and also with the ancestral condition of this species' haplotypes as illustrated by the mtDNA network.

The last significant marine transgression (maximum peak around 5000 ya) was followed by two regressive movements that formed barrier IV, the most recent barrier system of the CPRS (Villwock *et al.* 1986). This barrier, composed of beach ridges and adjacent dunes, moulded the modern Patos Lagoon estuary (Calliari 1997) and created stable freshwater environments in Mirim Lake by isolating it from the sea (Villwock *et al.* 1986). In the outer edge of the northern CPRS, two elongated groups of interconnected lakes were formed in the gap between barriers III and IV (Schwarzbold & Schäeffer 1984; Villwock *et al.* 1986). These two recent groups are represented here by *perugiae* from Lakes Pinguela and Peixoto (*O.* sp. B), and Fortaleza (*O.* sp. C), respectively. Based on this scenario, *in situ* differentiation of southern *O. mirinensis* and *O. perugiae* and northern *O.* sp. B and *O.* sp. C occurred during the Holocene. This agrees with the microsatellite reconstruction, where *O.* sp. B and *O.* sp. C are the most recently derived *perugiae*. Furthermore, especially for southern species, most mtDNA haplotypes seem to have originated in a very recent past: they are rare, lie in the periphery of the network, and are connected independently to the most common ones via short branches (Avise 2000). The evident association between evolutionary relationships and patterns of endemism of *perugiae* with the geological history of the CPRS contributes to the view that speciation can be promoted by Pleistocene climatic fluctuations (Hewitt 1996; Knowles 2001). Additionally, the four species that possibly speciated less than 5000 ya represent an unprecedented example of a rapid, mostly allopatric, fish radiation driven by Holocene sea-level changes. This conclusion stands out against increasing evidence that speciation in fishes can occur very rapidly under sympatric conditions, especially in recent monophyletic species flocks (e.g. Schluter 1996; Schliewen *et al.* 2001). It also contrasts with studies reporting ecological speciation between estuarine and marine populations of the closely related *Odontesthes argentinensis* (discussed later) (Beheregaray & Levy 2000; Beheregaray & Sunnucks 2001). Due to our reduced number of replicate samples, we cannot explore the evolutionary origin of southern sympatric species. However, the apparent absence of sexual selection and lack of noticeable differences in ecological niches in *perugiae* help to dismiss the idea that the radiation has diversified in a sympatric fashion.

The scenario of chronological diversification described above also fits with two major aspects involved in biological speciation: genetic and morphological divergence. The Pleistocene *O.* sp. A emerged as a highly differentiated *perugiae* based on mtDNA and microsatellites (table 2*b*) and on multivariate morphometric analysis (Beheregaray 2000; Malabarba & Dyer 2002). Accordingly, lower genetic and phenotypic divergence was observed among Holocene species, particularly between sympatric species. Despite such a concordant pattern, the mtDNA genealogy showed that *perugiae* haplotypes do not cluster according to species designation. It appears that the recent age of the radiation has not provided sufficient time for monophyletic sorting of haplotypes within reproductively isolated populations. This is consistent with theoretical and empirical studies in which recently separated populations display paraphyletic patterns for both genes and species trees and low phylogeographical structure (e.g. Powell 1991; Avise 2000; Posada & Crandall 2001). Our study also illustrates the importance of using multigenic phylogenies to obtain information at different time-scales: unlike mtDNA, microsatellites revealed relationships among *perugiae* that generally agree with the Pleistocene–Holocene history of the CPRS. Microsatellite markers can potentially disclose phylogenetic relationships over very short time-intervals (Takezaki & Nei 1996), such as those observed in recent radiations (e.g. Kornfield & Parker 1997; Petren *et al.* 1999), while mtDNA is expected to inform at relatively deeper levels of evolutionary divergence (Sunnucks 2000).

The pronounced concordance between genetic and morphological divergence with geographical history qualifies the different morphotypes as good species based on definitions that do not necessarily rely on the criterion of reciprocal monophyly (e.g. Avise 2000; Moritz *et al.* 2000). The close genealogical relationships of *perugiae* and their endemism to the circumscribed CPRS, classifies this speciose group as a 'species flock' (see Echelle & Kornfield 1984). This validates the description of three new *perugiae* species for the northern drainage (Malabarba & Dyer 2002). Our results confirm that *O. mirinensis* (Bemvenuti 1995) is a valid species for the southern lakes, but disagree with the proposal of Bemvenuti (1997) that *O. mirinensis* and *O. perugiae* are distributed along both the southern and northern drainages of the CPRS. It was also shown that the *O. perugiae* morphotype from the Uruguay River is a genetically divergent taxon that is not phylogenetically associated with the coastal radiation. Given that its type locality is in Argentina (Evermann & Kendall 1906), we suggest that the coastal lacustrine morphotype currently classified as *O. perugiae* (Bemvenuti 1997; Dyer 1998)

represents an undescribed species endemic to the southern lakes of the CPRS.

(b) *Estuarine ancestry and rapid divergence of extremely variable populations*

Phylogenetic reconstructions using mtDNA control region sequences of 440 individuals representing all recognized *Odontesthes* species strongly indicate that coastal *perugiae* are part of a tight and recent genealogical assemblage that includes the much larger and obligatory marine–estuarine *O. argentinensis* (Beheregaray 2000; Sunnucks *et al.* 2000; Beheregaray & Sunnucks 2001). The *O. argentinensis* group consists of widespread marine populations and a few incipient ecological species resident in estuarine environments of the CPRS and Uruguay (Beheregaray & Levy 2000; Beheregaray & Sunnucks 2001). Interestingly, the phylogenetic reconstructions show that several populations of *perugiae* and *O. argentinensis* have, at the centre of their network, the abundant haplotype '13'—the ancestral matriline of these two groups. By contrast, haplotypes from all other *Odontesthes* (including three freshwater species sampled from Mirim Lake) represented more ancient mtDNA lineages unrelated to haplotype '13'. Based on analyses of the demographic and phylogeographic history of coastal *Odontesthes*, it has been proposed that *perugiae* originated from an ancestral marine–estuarine lineage currently represented by *O. argentinensis* (Beheregaray 2000; L. B. Beheregaray, unpublished data).

The evidence pointing to a marine–estuarine ancestry in *perugiae* might help clarify two intriguing features of this lineage. The first relates to the extremely high genetic variability found in the four *perugiae* that presumably diverged during the Holocene (table 1). Their microsatellite variability is at the top end of the range found for freshwater fishes in the review of DeWoody & Avise (2000). Levels of mtDNA diversities were even less anticipated ($h = 0.98$ in *O. mirinensis*), and may represent the highest ever reported for freshwater fishes. Marine fishes typically show higher genetic variability than freshwater species, a difference interpreted as a result of the more stable and larger evolutionary population sizes of marine fishes (Ward *et al.* 1994; DeWoody & Avise 2000). The abundant *O. argentinensis* displays high microsatellite and mtDNA variability, similar to the four *perugiae* (Beheregaray & Sunnucks 2000, 2001). We suggest that recent landlocking of large population segments of a genetically variable marine–estuarine lineage may account for the high variability of Holocene species. On the other hand, the morphotype endemic to the small and isolated group of Pleistocene lakes (*O.* sp. A) had significantly lower variability than other *perugiae*. This could be a result of the long-term effects of genetic drift and population bottlenecks acting on isolated populations. Accordingly, divergence in other *perugiae* is not apparently associated with founder events, and may have occurred without depression of genetic variation because they are distributed in more recent and larger systems of interconnected lakes. However, given that most mtDNA diversity of *O. argentinensis* and *perugiae* is represented by recent haplotypes, we could also speculate that landlocking of *O.* sp. A occurred before demographic expansion and genetic strengthening of the ancestral lineage.

The other interesting feature of *perugiae* is their exceptionally fast rates of speciation. The maximum divergence time based on geology for two of the northern *perugiae* is no more than 5000 years. This equals the most rapid speciations reported for adaptive radiations of fishes (McCune 1997). It is proposed, based on the model of speciation of silverside fishes of Bamber & Henderson (1988), that the rapid divergence of *perugiae* could also be related to its marine–estuarine origin. This model, supported by the reproductive biology and presumed evolutionary patterns of silversides, predicts that physically variable environments, such as estuaries and coastal brackish lagoons, pre-adapt silverside populations to invade, colonize and rapidly speciate into vacant niches in freshwater (Bamber & Henderson 1988). Incipient lakes can provide special conditions for radiations because of their remote access and low or absent competition from endemic lineages specialized in particular resources (Givnish 1997). Due to the recognized plasticity of *O. argentinensis* (e.g. Phonlor & Sampaio 1992; Sampaio & Phonlor 1992), it is expected that habitat conditions in the newly formed lacustrine environments did not provide obstacles to population colonization. Very probably, the landlocked ancestors of *perugiae* had high levels of genetic variability and had been recently pre-adapted for environmental instability in estuarine waters.

The authors gratefully acknowledge B. Dyer and L. Malabarba for advice on the distribution and identification of morphotypes and assistance during fieldwork. They also thank M. Bemvenuti, R. Robaldo and F. Becker for their help with sample collection. L. Bernatchez and C. Moritz provided helpful suggestions to this work. This study was partially sponsored by CAPES (Brazilian Ministry of Education), which supported the field trips to Brazil and a PhD scholarship to L.B.B. This is Key Centre for Biodiversity & Bioresources publication no. 365.

REFERENCES

Avise, J. C. 2000 *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press.

Bamber, R. N. & Henderson, P. A. 1988 Pre-adaptative plasticity in atherinids and the estuarine seat of teleost evolution. *J. Fish Biol.* **33**, 17–23.

Becker, F. G. 1995 Dinâmica de reprodução, alimentação e crescimento de três espécies de peixe-rei (gen. *Odontesthes*), em lagoas costeiras do litoral norte do Rio Grande do Sol (Pisces, Atheriniformes). MSc thesis, Universidade Federal do Rio Grande do Sol, Porto Alegre.

Beheregaray, L. B. 2000 Molecular evolution, biogeography and speciation of the Neotropical silverside genus *Odontesthes*. PhD thesis, Macquarie University, Sydney.

Beheregaray, L. B. & Levy, J. A. 2000 Population genetics of the silverside *Odontesthes argentinensis* (Teleostei, Atherinopsidae): evidence for speciation in an estuary of southern Brazil. *Copeia* **2000**, 441–447.

Beheregaray, L. B. & Sunnucks, P. 2000 Microsatellite loci isolated from *Odontesthes argentinensis* and the *O. perugiae* species group and their use in other South American silverside fish. *Mol. Ecol.* **9**, 629–631.

Beheregaray, L. B. & Sunnucks, P. 2001 Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside *Odontesthes argentinensis*. *Mol. Ecol.* **10**, 2849–2866.

Bemvenuti, M. A. 1995 *Odontesthes mirinensis* sp. n., um novo peixe-rei (Pisces, Atherinidae, Atherinopsinae) para o extremo sul do Brasil. *Revist. Brasil. Zool.* **12**, 881–903.

Bemvenuti, M. A. 1997 Relações morfológicas e osteológicas entre *Odontesthes perugiae* e *O. mirinensis* (Teleostei: Atherinidae, Atherinopsinae). *Atlântica* **19**, 113–131.

Bermingham, E. & Avise, J. C. 1986 Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* **113**, 939–965.

Bernatchez, L. & Dodson, J. J. 1991 Phylogeographic structure in mitochondrial DNA of the lake whitefish (*Coregonus clupeaformis*) and its relation to Pleistocene glaciations. *Evolution* **45**, 1016–1035.

Buchmann, F. S. C., Barbosa, V. P. & Villwock, J. A. 1998 Sedimentologia e paleoecologia durante o máximo transgressivo holôcenico na Lagoa Mirim, RS, Brasil. *Acta Geol. Leopoldensia* **21**, 21–26.

Calliari, L. J. 1997 Environment and biota of the Patos Lagoon estuary: geological setting. In *Subtropical convergence environments: the coast and sea in southwestern Atlantic* (ed. U. Seeliger, C. Odebrecht & J. P. Castello), pp. 13–17. Berlin: Springer.

Cavalli-Sforza, L. L. & Edwards, A. W. F. 1967 Phylogenetic analysis: models and estimation procedures. *Am. J. Hum. Genet.* **19**, 233–257.

Clement, M., Posada, D. & Crandall, K. A. 2000 TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* **9**, 1657–1659.

DeWoody, J. A. & Avise, J. C. 2000 Microsatellite variation in marine, freshwater and anadromus fishes compared with other animals. *J. Fish Biol.* **56**, 461–473.

Dyer, B. S. 1998 Phylogenetic systematics and historical biogeography of the Neotropical silverside family Atherinopsidae (Teleostei, Atheriniformes). In *Phylogeny and classification of neotropical fishes* (ed. L. R. Malabarba, R. E. Reis, R. Vari, Z. M. S. Lucena & C. A. S. Lucena), pp. 519–536. Porto Alegre, Brazil: EDIPUCRS.

Echelle, A. A. & Kornfield, I. 1984 *Evolution of fish species flocks*. University of Maine Press.

Evermann, B. W. & Kendall, W. C. 1906 Notes on a collection of fishes from Argentina, with descriptions of three new species. *Proc. US Natl Mus.* **14**, 121–165.

Felsenstein, J. 1993 PHYLIP *v.3.5* (*phylogeny inference package*), v. 3.5c. Seattle, WA: University of Washington.

Givnish, T. J. 1997 Adaptive radiation and molecular systematics: issues and approaches. In *Molecular evolution and adaptive radiation* (ed. T. J. Givnish & K. J. Systma), pp. 1–54. Cambridge University Press.

Goudet, J. 1995 FSTAT (v. 1.2)—a computer program to calculate *F*-statistics. *J. Heredity* **86**, 485–486.

Hewitt, G. M. 1996 Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* **58**, 247–276.

Hewitt, G. M. 2001 Speciation, hybrid zones and phylogeography—or seeing genes in space and time. *Mol. Ecol.* **10**, 537–549.

Knowles, L. L. 2001 Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. *Mol. Ecol.* **10**, 691–701.

Kornfield, I. & Parker, A. 1997 Molecular systematics of a rapidly evolving species flock: the *mbuna* of Lake Malawi and the search for phylogenetic signal. In *Molecular systematics of fishes* (ed. T. D. Kocher & C. A. Stepien), pp. 25–37. San Diego, CA: Academic Press.

Lee, W., Conroy, J., Howell, W. H. & Kocher, T. D. 1995 Structure and evolution of teleost mitochondrial control region. *J. Mol. Evol.* **41**, 54–66.

McCune, A. R. 1997 How fast is speciation? Molecular, geological, and phylogenetic evidence from adaptive radiations of fishes. In *Molecular evolution and adaptive radiation* (ed. T. J. Givnish & K. J. Systma), pp. 585–610. Cambridge University Press.

Malabarba, L. R. & Dyer, B. S. 2002 Description of three new species of the genus *Odontesthes*, Evermann & Kendall, from the rio Tramandaí drainage, Brazil (Atheriniformes: Atherinopsidae). *Ichthyol. Explorat. Freshwater.* (In the press.)

Moritz, C., Patton, J. C., Schneider, C. J. & Smith, T. B. 2000 Diversification of rainforest faunas: an integrated molecular approach. *A. Rev. Ecol. Syst.* **31**, 533–563.

Petren, K., Grant, B. R. & Grant, P. R. 1999 A phylogeny of Darwin's finches based on microsatellite DNA length variation. *Proc. R. Soc. Lond.* B **266**, 321–329.

Phonlor, G. & Sampaio, L. A. 1992 Effect of salinity on growth and survival of *Odontesthes argentinensis* larvae. *Arq. Biol. Tecnol.* **35**, 138–153.

Posada, D. & Crandall, K. A. 2001 Intraspecific gene genealogies: trees grafting into networks. *Trends Ecol. Evol.* **16**, 37–45.

Powell, J. R. 1991 Monophyly/paraphyly/polyphyly and gene/species trees: an example from *Drosophila*. *Mol. Biol. Evol.* **8**, 892–896.

Raymond, M. & Rousset, F. 1995 Population genetics software for exact tests and ecumenicism. *J. Heredity* **86**, 248–249.

Rice, W. R. 1989 Analysing tables of statistical tests. *Evolution* **43**, 223–225.

Sampaio, L. A. & Phonlor, G. 1992 Freshwater tolerance of marine and estuarine metamorphic larvae of *Odontesthes argentinensis* (Pisces: Atherinidae). *Arq. Biol. Tecnol.* **35**, 85–90.

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T. & Tautz, D. 2001 Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Mol. Ecol.* **10**, 1471–1488.

Schluter, D. 1996 Ecological speciation in post-glacial fishes. *Phil. Trans. R. Soc. Lond.* B **351**, 807–814.

Schneider, S., Kueffer, J., Roessli, D. & Excoffier, L. 2000 ARLEQUIN *v. 2.000: a software for population genetic data analysis*. Geneva: Genetics and Biometry Laboratory, University of Geneva.

Schwarzbold, A. & Schäeffer, A. 1984 Gênese e morfologia das lagoas costeiras do Rio Grande do Sul, Brasil. *Amazoniana* **9**, 81–104.

Sunnucks, P. 2000 Efficient genetic markers for population biology. *Trends Ecol. Evol.* **15**, 199–203.

Sunnucks, P. & Hales, D. F. 1996 Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Mol. Biol. Evol.* **13**, 510–523.

Sunnucks, P., Wilson, A. C. C., Beheregaray, L. B., Zenger, K., French, J. & Taylor, A. C. 2000 SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol. Ecol.* **9**, 1699–1710.

Takezaki, N. & Nei, M. 1996 Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**, 389–399.

Taylor, E. B., Harvey, S., Pollard, S. & Volpe, J. 1997 Postglacial genetic differentiation of reproductive ecotypes of kokanee *Oncorynchus nerka* in Okanagan Lake, British Columbia. *Mol. Ecol.* **6**, 503–517.

Templeton, A. R., Crandall, K. A. & Sing, C. F. 1992 A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619–633.

Villwock, J. A. & Tomazelli, L. J. 1995 Geologia costeira do Rio Grande do Sul. *Notas Técnicas* **8**, 1–45.

Villwock, J. A., Tomazelli, L. J., Loss, E. L., Dehnhardt, E. A., Horn, N. O., Bachi, F. A. & Dehnhardt, B. A. 1986 Geology of the Rio Grande do Sul coastal province. In *Quaternary of South America and Antarctic Peninsula* (ed. J. Rabassa), pp. 79–97. Rotterdam: Balkema.

Ward, R. D., Woodwark, M. & Skibinski, D. O. F. 1994 A comparison of genetic diversity in marine, freshwater, and anadromus fishes. *J. Fish Biol.* **44**, 213–232.

Weir, B. S. & Cockerham, C. C. 1984 Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.

Visit http://www.pubs.royalsoc.ac.uk to see electronic appendices to this paper.