

Chromosomes, RAPDs and evolutionary trends of the Neotropical fish *Mimagoniates microlepis* (Teleostei: Characidae: Glandulocaudinae) from coastal and continental regions of the Atlantic forest, Southern Brazil

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Abstract

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Chromosome and random amplified polymorphic DNA (RAPD) markers of samples of *Mimagoniates microlepis* were studied to test the hypothesis that a vicariant event occurred as the result of the orogeny of the coastal mountain range (Serra do Mar; southeastern and southern Brazil). Conventional karyotypes and nucleolar organizer region (Ag-NOR) phenotypes of two samples of *M. microlepis* from the headwaters of the Iguaçu River (southern Brazil) were compared both with each other and with other local populations of the species in the coastal drainage of southeastern Brazil. Additional molecular data (RAPD markers and genetic diversity) were obtained from specimens from coastal and continental regions of southern Brazil. The same diploid number (52 chromosomes), karyotypic formula and Ag-NOR phenotype were found for both analysed samples from the Iguaçu River. A genetic discontinuity was discovered in the comparison of the karyotypical formula of the Iguaçu samples with those from coastal drainages of the region. Polymerase chain reaction-RAPD markers revealed strikingly different molecular profiles between coastal and continental samples and indications of a high degree of genetic variation. Based on these results, we provide some comments on the biogeographical patterns and evolutionary trends for *M. microlepis* from coastal and continental regions of southeastern/southern Brazil.

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Introduction

Karyotype (diploid number/karyotypical formula/nucleolar organizer region type) and molecular markers may yield interesting evolutionary patterns, such as relationships between congeneric species or among populations of a species. Importantly, these genetic characters can provide additional tools for understanding taxonomy, cryptic species

phenomena and population isolation (Torres *et al.* 1998; Ribeiro *et al.* 2003; Torres *et al.* 2004a,b).

The Glandulocaudinae is an inseminating characid group consisting of three genera (*Lophiobrycon*, *Glandulocauda* and *Mimagoniates*) in which their species occur along coastal and continental drainages from Southern Brazil. These species differ from the other sister species (Stervardiinae) by having a hypertrophied caudal-fin gland derived from modified

'club cells' (for details see Weitzman *et al.* 2005). A new genus and species (*Lophiobrycon weitzmani*) was recently described from the headwaters of the Rio Grande watershed drainage (southeastern Brazil) and was proposed to be the sister-group of the monophyletic unit comprising *Mimagoniates* and *Glandulocauda* (Castro *et al.* 2003).

The genus *Mimagoniates* is composed of six species: *M. inequalis*, *M. barberi*, *M. lateralis*, *M. sylvicola*, *M. rheocharis* and *M. microlepis*, the last of which is the most derived species within the genus (Menezes and Weitzman 1990). Despite its geographical distribution being controversial, the genus also presents a wide geographical distribution occurring more frequently in coastal streams from the Atlantic rain forest.

In the state of Paraná (Southern Brazil) *M. microlepis* is frequently found beyond the coastal plain, in the upper Iguaçú and Tibagi rivers (Santanna *et al.* 2006). The distribution of the species seems to be associated with plant cover and some intrinsic environmental conditions such as the accessibility of food and shelter (Mazzoni and Iglesias-Rios 2002).

Genetic studies in the subfamily Glandulocaudinae began with Guimarães *et al.* (1995). These authors studied the karyotype of *M. microlepis* ($2n = 52, 6m+20sm+18st+8a$), *M. lateralis* ($2n = 52, 6m+20sm+16st+10a$) and *Glandulocauda melanogenys* ($2n = 52, 4m+12sm+22st+14a$). The nucleolar organizer regions (NORs) were the variable character among the populations of *M. microlepis* studied, indicating a species-like status for those populations. Sass (2003) described the karyotype of the rare and endangered glandulocaudine *Glandulocauda melanopleura* as $2n = 54 (8m+18sm+14st+14a)$.

The NOR phenotypes are often assumed to be markers for a species-like level in the Neotropical ichthyofauna (Galetti *et al.* 1984; Amemiya and Gold 1988; Venere & Galetti 1989; Torres *et al.* 1998; Torres *et al.* 2004a). It has been proposed that *M. microlepis* represents different species because of the NOR variation between studied populations in coastal streams of southeastern Brazil (Guimarães *et al.* 1995). In the state of Paraná (southern Brazil), the populations of *M. microlepis* in the mid and upper courses of the Iguaçú River are isolated from coastal populations by a coastal mountain range (Serra do Mar), which limits or blocks gene flow between these populations.

Considering that the genetic and evolutionary characteristics of *M. microlepis* are a poorly known, and based on previous evidence regarding a possible process of diversification in the species associated with its isolation, we have tested for a vicariant event caused by the mountain range using genetic-based analyses.

Materials and Methods

The species *M. microlepis* (Fig. 2B) was studied in the headwaters of two isolated streams, both of which feed the Iguaçú River basin (Fig. 1B). Twelve specimens (five male and seven

female) from the Piraquara River (Piraquara, Paraná; 25°29'59"S; 49°02'58"W) and 28 specimens (14 male and 14 female) from the Pequeno River (São José dos Pinhais, Paraná; 25°33'32"S; 49°07'41"W) were examined. Voucher specimens were deposited in the fish collection from the Museu de História Natural Capão da Imbuia (MHNCI), Curitiba, PR, Brazil.

Mitotic-metaphase chromosomes were obtained following the methods of Bertollo *et al.* (1978) and Foresti *et al.* (1993). The NORs were examined following the methods of Howell and Black (1980) and karyotype arrays were determined based on their arm ratios, as described previously (Levan *et al.* 1964). Chromosomes were classified as metacentrics (M), submetacentrics (SM), subtelocentrics (ST) and acrocentrics (A).

DNA was extracted from the muscle using the procedure described by Almeida *et al.* (2001). Muscle samples were 1 cm³ and were triturated into very small fragments, then placed in a microcentrifuge tube containing 1 mL extraction buffer [Tris-HCl 500 mM, ethylenediaminetetraacetic acid (EDTA) 20 mM, NaCl 10 mM pH 8.0 and 1% sodium dodecyl sulphate] to which proteinase K (5 µL; 20 mg/mL) was added. DNA was purified by successive extraction with phenol chlorophane (1 phenol : 1 chlorophyll) and chlorophyll (24 chloroform : 1 isoamyl alcohol) and precipitated with 10% of 3 M NaCl and two volumes of absolute ethanol. The DNA precipitate was washed with 70% ethanol and, after drying, was re-suspended in 150 mL TE (Tris-HCl 3 mM, EDTA 0.2 mM, pH 8.0).

For the random amplified polymorphic DNA (RAPD) analyses, 10 random specimens were selected from coastal and continental locations, with two specimens taken from each location, as follows: Várzea Creek (continental; city of Lapa; L1 and L2; 25°52'57"S; 49°43'26"W), Piraquara River (continental; city of Piraquara; P4 and P5; 25°29'59"S; 49°02'58"W), Mergulhão Creek (coastal; city of Antonina; M4 and M5; 25°17'58"S; 48°44'39"W), Caerana Creek (continental; city of Piraquara; C7 and C8; 25°26'59"S; 49°02'31"W) and Acaraí river system (coastal; city of São Francisco do Sul; SF5 and SF10; 26°17'33"S; 48°35'20"W). This type of specimen selection was carried out to detect specific molecular profiles to support the karyotypical discontinuity observed between coastal and continental samples of *M. microlepis*. Thus, a single 10-mer random primer was used with the sequence of 5'-GTTAGTGC GG-3'. RAPD amplifications were carried out in 15 µL containing 10 ng template DNA, 2.5 µM primer, 3 mM MgCl₂, 2.5 mM dNTPs, 1 unit *Taq* polymerase in the reaction buffer supplied (100 mM Tris-HCl pH 8.3; 500 mM KCl). Polymerase chain reactions (PCR) were performed in a PTC100 (MJ Research, San Francisco, CA, USA) thermocycler with an initial step of 4 min at 92 °C plus 40 cycles of 40 s at 92 °C, 90 s at 40 °C and 120 s at 72 °C with a final extension step of 5 min at 72 °C.

The RAPD products were resolved by electrophoresis in 1.4% agarose gels run with TBE buffer (0.89 M Tris-HCl,

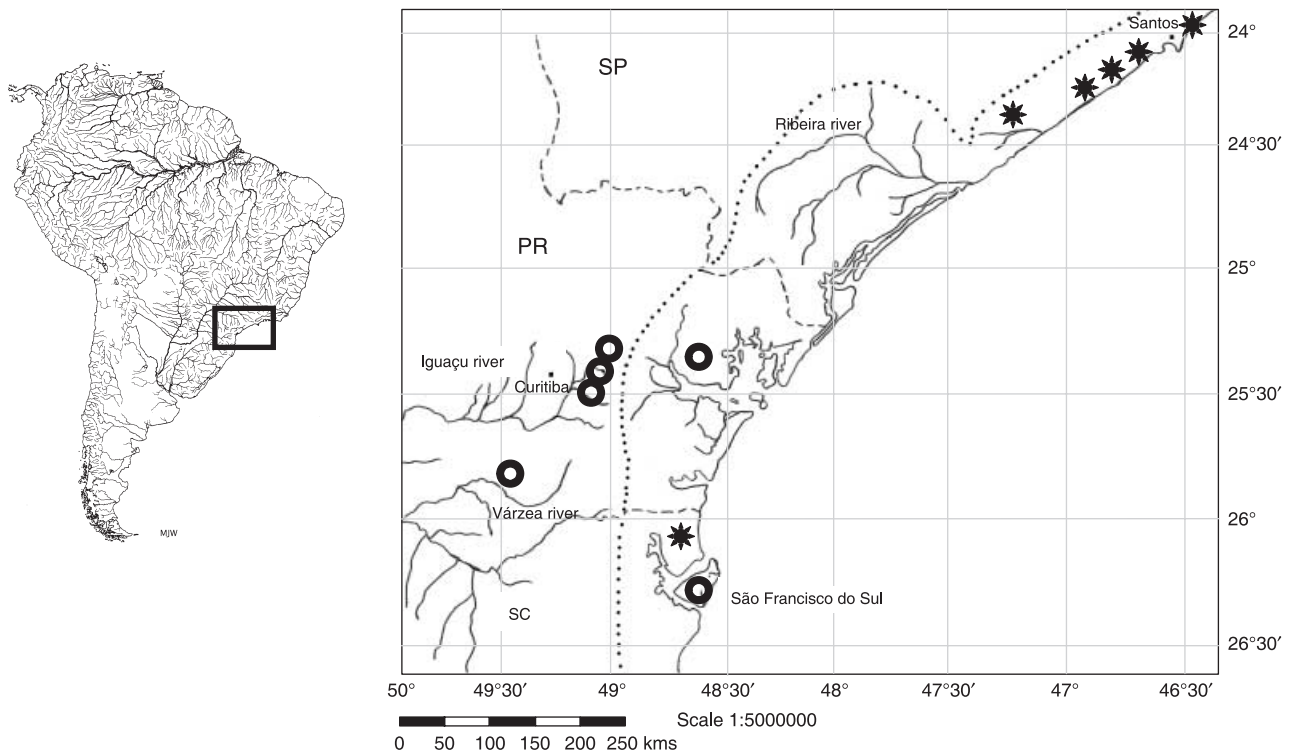


Fig. 1—The geographical distribution of *Mimagoniates microlepis* in southeastern and southern Brazil. SP = State of São Paulo; PR = State of Paraná; SC = State of Santa Catarina. Dotted line shows the mountain chain (Serra do Mar) along the states. The dark stars at right from Serra do Mar (coastal) show the sampling localities of Guimarães *et al.* (1995). The dark circles indicate the sampling localities of this study (chromosome and molecular data).

0.89 M boric acid and 0.08 M EDTA, pH 8.3). Electrophoresis was conducted at 3 V/cm. Gels were stained with ethidium bromide and the image was captured using the digital system KODAK EDAS290.

A tentative estimate concerning the genetic variability of the samples was carried out based on the ratio between the total number of loci and the number of polymorphic loci.

Results

Totals of 109 metaphases from the Piraquara River samples and 158 metaphases from the Pequeno River samples were analysed. For both populations the occurrence of $2n = 52$ chromosomes and identical chromosome formulae, composed of $12m+18sm+14st+8a$, were verified (Fig. 2A,B; Table 1). In addition, the same NOR-bearing chromosome pairs were also identified for both samples. The ribosomal cistrons are located along the whole short arm in the 5th chromosome pair in the group of metacentrics (Fig. 2A,B within the box).

RAPD procedures (Fig. 3) on a single primer resulted in 12 loci in which two homomorphic loci are found (Fig. 3; horizontal arrows) and which correspond to fragments larger than 2072 base pairs. Also, genetic variation was found in

both continental and coastal samples, corresponding to fragments from 1000 to 2072 base pairs (Fig. 3). Molecular, distinguishable taxonomic markers were identified in both the Mergulhão Creek and Acaraí river system populations (Fig. 3; M4 and M5/A5 and A10). In addition, a preliminary estimate concerning the genetic variability among samples was determined at 83%.

Discussion

Genetic studies in the subfamily Glandulocaudinae are very scarce. A single study found the same diploid number ($2n = 52$) for *M. microlepis*, *M. lateralis* and *G. melanogenys* from coastal populations in the south of southeastern Brazil (Table 1). Subtle variation in this character ($2n = 54$) was found in the rare and endangered species *G. melanopleura* from the headwaters of the Iguaçu River (Sass 2003). In spite of this diploid number variation, we reinforce that $2n = 52$ chromosomes seem to be most common in the subfamily.

Mimagoniates lateralis is genetically characterized by having the chromosome formula $6m+20sm+16st+10a$ (Guimarães *et al.* 1995). It has been postulated that narrow karyotypical similarities would be most frequently observed among

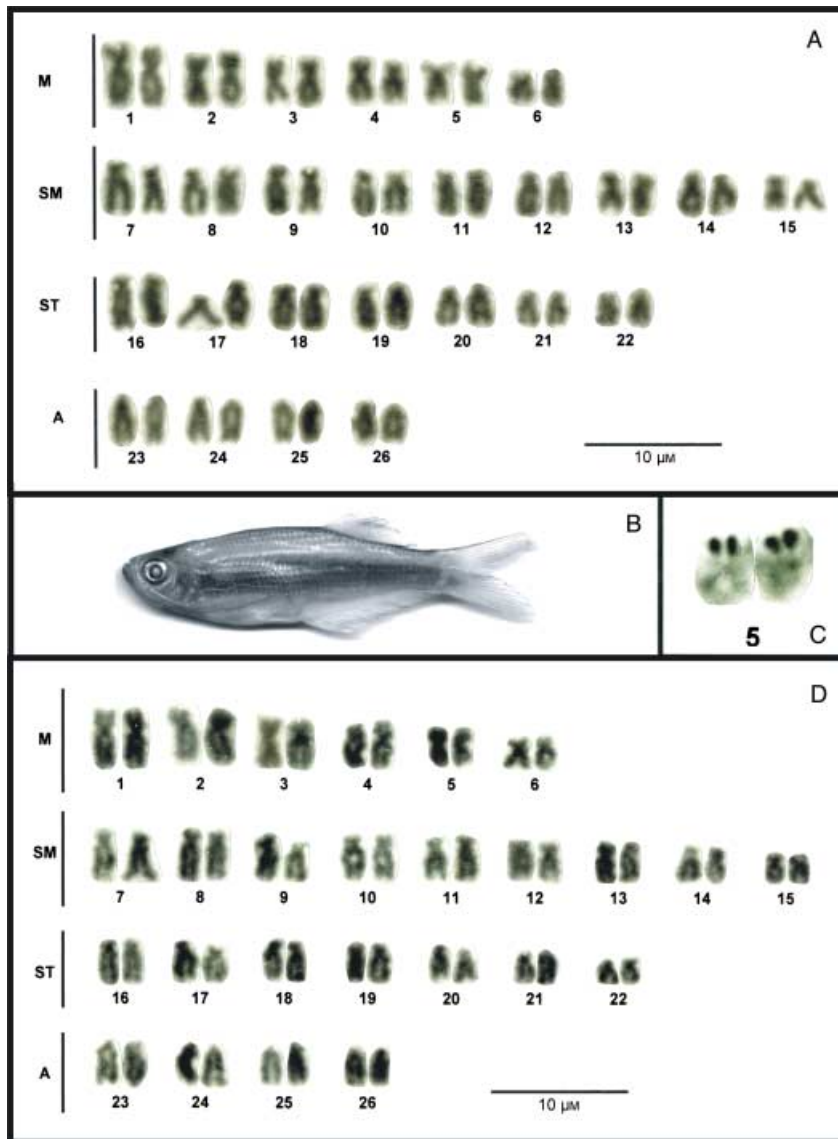


Fig. 2—Composite plate showing part of the genetic data obtained in the study. —**A.** Karyotype of *Mimagoniates microlepis* from São José dos Pinhais, PR, southern Brazil; —**B.** *M. microlepis* 7 cm in length as example to show the species studied; —**C.** The NOR-bearing chromosome pair for both samples; —**D.** Karyotype of *M. microlepis* from Piraquara, PR, southern Brazil.

Mimagoniates spp. occupying geographically related areas (Guimarães *et al.* 1995). Therefore, the striking karyotypical differences observed between *M. lateralis* and *M. microlepis* studied herein (Table 1) could suggest no biogeographical relationships between those areas. On the contrary, a close relationship between those areas seems to be a true hypothesis given the occurrence of the 16 freshwater fish species in drainages from both sides of the Serra do Mar. A long period of isolation of those *Mimagoniates* spp. could be considered an alternative hypothesis to explain the fixation of their remarkable karyotypical differences.

Regarding genetic heterogeneity based on the biogeographical relationships, additional support arises when comparing the karyotypical formulae of *M. microlepis* from the coastal plain (Guimarães *et al.* 1995) and those from the

first plateau of the state of Paraná (Fig. 3A,B; Table 1). The six coastal populations have karyotypical formulae $6m+20sm+18st+8a$, compared with $12m+18sm+14st+8a$ in this study. This karyotypic discontinuity suggests a direct association with the vicariant event of the orogeny of the Serra do Mar Mountains, with subsequent isolation and disruption of gene flow. Variable karyotypes associated with vicariant processes and putative speciation have been described for other Neotropical fish groups (Torres *et al.* 1998, 2004a; Jorge and Moreira-Filho 2000; Centofante *et al.* 2003; Bertollo *et al.* 2004). On the other hand, by occurring in contiguous streams for a single drainage (Iguaçu River) the comparison between the samples studied here suggests that they could form a single population of *M. microlepis* given their identical karyotypical macrostructures.

Table 1 Summary of chromosome data available for Glandulocaudinae species

Species	Locality	Region	2n	Karyotypic formula	Reference
<i>Mimagoniates microlepis</i>	Bertioga, SP, Brazil	Co	52	6m+20sm+18st+8a	Guimarães <i>et al.</i> (1995)
<i>Mimagoniates microlepis</i>	Mongaguá, SP, Brazil	Co	52	6m+20sm+18st+8a	Guimarães <i>et al.</i> (1995)
<i>Mimagoniates microlepis</i>	Itanhaém, SP, Brazil	Co	52	6m+20sm+18st+8a	Guimarães <i>et al.</i> (1995)
<i>Mimagoniates microlepis</i>	Peruíbe, SP, Brazil	Co	52	6m+20sm+18st+8 A	Guimarães <i>et al.</i> (1995)
<i>Mimagoniates microlepis</i>	Juquiá, SP, Brazil	Co	52	6m+20sm+18st+8a	Guimarães <i>et al.</i> (1995)
<i>Mimagoniates microlepis</i>	Pirabeiraba, SC, Brazil	Co	52	6m+20sm+18st+8a	Guimarães <i>et al.</i> (1995)
<i>Mimagoniates microlepis</i>	Piraquara, PR, Brazil	Con	52	12m+18sm+14st+8a	Present study
<i>Mimagoniates microlepis</i>	São José dos Pinhais, PR, Brazil	Con	52	12m+18sm+14st+8a	Present study
<i>Mimagoniates lateralis</i>	Itanhaém, SP, Brazil	Co	52	6m+20sm+16st+10a	Guimarães <i>et al.</i> (1995)
<i>Glandulocauda melanogenys</i>	Paranapiacaba, SP, Brazil	Con	52	4m+12sm+22st+14a	Guimarães <i>et al.</i> (1995)
<i>Glandulocauda melanopleura</i>	Quatro Barras, PR, Brazil	Con	54	8m+18sm+14st+14a	Sass (2003)

SP = state of São Paulo; SC = state of Santa Catarina; PR = state of Paraná; Co = Coastal; Con = Continental; M = metacentrics; SM = submetacentrics; ST = subtelocentrics; A = acrocentrics.
Revised from Guimarães *et al.* (1995).

The silver staining (Ag-) NOR phenotype has been considered a powerful chromosome marker at the species level (Galetti *et al.* 1984; Amemiya and Gold 1988; Vêneré & Galetti 1989). In *G. melanogenys*, *M. lateralis* and *M. microlepis* (Guimarães *et al.* 1995) multiple Ag-NORs (chromosomes bearing two to six) are common, with extreme variability in number and position in each of the species. Ag-NORs in a single chromosome pair seem to be a primitive condition for this character (Almeida-Toledo and Foresti 1985; Klinkhardt 1998). Therefore, our Ag-NOR data support the idea that the samples from the headwaters of the Iguaçú River might be evolutionarily basal to those from the coastal plain (because of the single Ag-NORs found in both samples studied here). However, the presence of shared Ag-NORs between both studied samples of *M. microlepis* reinforces that they form a single population by maintaining gene flow along the main course of the Iguaçú River. This last consideration is supported by the occurrence of *M. microlepis* in the main section of the Iguaçú River, 250 km downstream (Ingenito *et al.* 2004).

Mimagoniates microlepis is remarkably distributed along the coastal plain from the state of Espírito Santo (southeastern Brazil), to the state of Rio Grande do Sul (southern Brazil) occurring also in tributaries from the upper Ribeira and Iguaçú Rivers (Menezes and Weitzman 1990). Thus, we might suggest a narrow biogeographical relationship between the upper Ribeira and Iguaçú Rivers with the coastal drainages from southern and southeastern Brazil. This suggestion is consistent with evidence of 16 fish species occurring in both regions (upper Iguaçú River and the coastal drainages; Abilhoa 2005) and by the fact that several headwaters of the eastern margin of the Paraná Basin have been captured and become direct Atlantic tributaries (Ribeiro 2006).

Considering that geographically related areas would host close genetic stocks (Guimarães *et al.* 1995), we expected

close genetic homogeneity between samples of *M. microlepis* from the upper Iguaçú River and those from coastal drainages. Despite the evidence pointing to the close geographical relationship between the areas analysed, striking differences were found between those karyotypical macrostructures. These not fitted evidences might be explained by a long period of isolation occurred between samples of *M. microlepis* (upper Iguaçú × coastal drainage). Curiously, the remarkable karyotypical differences detected between *M. microlepis* and *M. lateralis* (upper Iguaçú × coastal drainage), and between samples of *M. microlepis* (upper Iguaçú × coastal drainage) seem to be equally a result of the vicariant pressure imposed by the Serra do Mar orogeny. Indeed, additional support for this hypothesis comes from the remarkable molecular genetic differentiation between those areas (upper Iguaçú × coastal drainage; Fig. 3). Such data suggest a taxonomic and phylogenetic revision in the species because it may actually represent different species. In addition, similar molecular taxonomic analyses were successful for species of groupers (*Epinephelus* spp.) from India (Govindaraju and Jayasankar 2004). Also, two morphologically similar species of clown fishes (*Amphiprion sebae* and *Amphiprion chrysoogaster*) were differentiated by different RAPD profiles (Jayasankar 2004). In Neotropical freshwaters, *Astyanax altiparanae* (endemic species from the upper Paraná river basin) was shown to occur in the Iguaçú River by a RAPD-based taxonomy (Prioli *et al.* 2002). With this reasoning the distribution of two *Oligosarchus* species (*O. hepsetus* and *O. longirostris*) might constitute supporting evidence for the hypothesis of different *Mimagoniates* spp. occurring in both sides of the Serra do Mar mountains. *Oligosarchus hepsetus* is distributed from Rio de Janeiro state (SE) to Santa Catarina state (S) and it is possibly the sister species of *O. longirostris*, an endemic species from the Iguaçú River (Menezes 1987; A. C. Ribeiro, personal communication). Therefore, it would

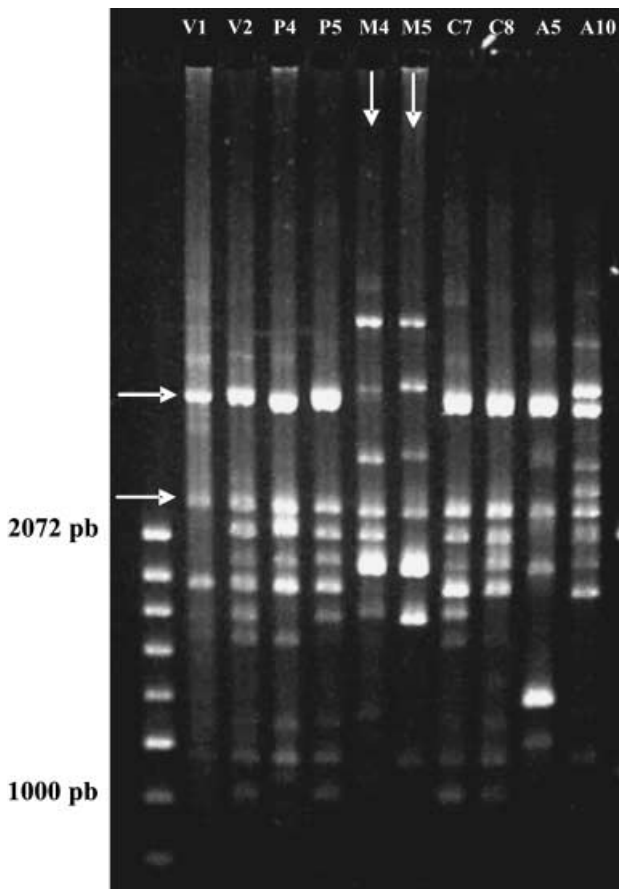


Fig. 3—RAPD-based profiles obtained for some continental and coastal specimens of *Mimagoniates microlepis*; bp, base pairs. V = Várzea Creek (continental), city of Lapa, state of Paraná, southern Brazil; P = Piraquara River (Iguaçu headwaters; continental), city of Piraquara, state of Paraná, southern Brazil; M = Mergulhão Creek (coastal) city of Antonina, state of Paraná, southern Brazil; C = Caerana Creek (Iguaçu headwaters; continental), city of Piraquara, state of Paraná, southern Brazil; A = Acaraí River system (coastal), city of São Francisco do Sul, state of Santa Catarina, southern Brazil. Note the striking genetic differentiation occurring in the specimens from Mergulhão Creek (vertical arrows). Horizontal arrows show the homomorphic markers for the sampled specimens.

seem that the Serra do Mar Mountains have divided an ancestor lineage of the living *O. hepsetus* and *O. longirostris*. The vicariant phenomenon associated with the biodiversity in the genus *Oligosarchus* in the region might be putatively responsible for the diversity observed in *M. microlepis*.

Evidence for the narrow relationship between the upper Iguaçu River and the coastal drainages as the result of events of stream capture exists (Almeida and Carneiro 1998; Angulo 2004). Indeed, such events could have started from the capture of Iguaçu headwaters by coastal rivers (Ribeiro 2006). This may explain the distribution of *M. microlepis* throughout the upper and medium sections of the Iguaçu

River (Weitzman *et al.* 1988; Abilhoa 2005). While these suggestions are possible, our data seem to point to an interesting and different hypothesis concerning the processes of hydrological transformation as well as the impact on the ecology and evolution of the fish assemblage from the upper Iguaçu River and coastal drainages. Hence it seems equally likely that a dispersion hotspot for *M. microlepis* around the upper Ribeira and Iguaçu Rivers might be possible, based on twofold evidence: the distribution of the species throughout those regions and the occurrence of plesiomorphic-stated chromosome characters (NOR phenotype) in the samples from Iguaçu headwaters.

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