

Phylogeography of the piranha genera *Serrasalmus* and *Pygocentrus*: implications for the diversification of the Neotropical ichthyofauna

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Abstract

The phylogenetic relationships within the piranhas were assessed using mitochondrial sequences with the aim of testing several hypotheses proposed to explain the origin of Neotropical diversity (palaeogeography, hydrogeology and museum hypotheses). Sequences of the ribosomal 16S gene (510 bp) and control region (980 bp) were obtained from 15 localities throughout the main South American rivers for 21 of the 28 extant piranha species. The results indicate that the genus *Serrasalmus* is monophyletic and comprises three major clades. The phylogeographical analyses of these clades allowed the identification of five vicariant events, extensive dispersal and four lineage duplications suggesting the occurrence of sympatric speciation. Biogeographical patterns are consistent with the prediction made by the museum hypothesis that lineages from the Precambrian shields are older than those from the lowlands of the Amazon. The vicariant events inferred here match the distribution of the palaeoarches and several postdispersal speciation events are identified, thereby matching the predictions of the palaeogeography and hydrogeology hypotheses, respectively. Molecular clock calibration of the control region sequences indicates that the main lineages differentiated from their most recent common ancestor at 9 million years ago in the proto Amazon-Orinoco and the present rate of diversification is the highest reported to date for large carnivorous Characiformes. The present results emphasize that an interaction among geology, sea-level changes, and hydrography created opportunities for cladogenesis in the piranhas at different temporal and geographical scales.

Keywords: Amazon, biogeography, Brooks parsimony analysis, molecular clock, mtDNA, phylogeny, piranha

Received 3 August 2006; revision received 20 November 2006; accepted 11 December 2006

Introduction

The Amazon basin hosts the world's highest concentration of both terrestrial and aquatic biodiversity (Myers *et al.* 2000; Reis *et al.* 2004) and its history has thus long fascinated evolutionary biologists. The search for speciation mechanisms

explaining the origin of this diversity has led to the discovery of great congruencies in faunal distributions in a wide array of organisms (Cracraft & Prum 1988; Bates *et al.* 1998; Ron 2000; Hall & Harvey 2002; Racheli & Racheli 2004). Moreover, several hypotheses differing in their emphasis on either geological or ecological processes have been developed to explain the origin and organization of Neotropical diversity (Haffer 1997; Hubert & Renno 2006). However, despite the increasing amount of valuable information

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Table 1 Summary of the major palaeoecological events hypothesized during the last 10 million years ago (Ma), hypotheses of diversification in South American biotas and related predictions for freshwater fishes tested in this study

Palaeoecological events	Dates (Ma)	Hypothesis of diversification	Predictions	References
Raising of palaeoarches due to the Andean foreland dynamic	Last 8	Palaeogeography	(i) Sister clades (intraspecific lineages or species groups) will occur among rivers with contiguous headwaters recently separated by palaeoarches; (ii) differentiation of the lineages from the Orinoco and the Amazon will precede the establishment of the lineages within the Amazon	Räsänen <i>et al.</i> (1987, 1990) Hoorn <i>et al.</i> (1995) Irion <i>et al.</i> (1995) Lundberg <i>et al.</i> (1998) Da Silva & Patton (1998)
Miocene marine incursion (sea-level highstand of 100 m)	5–4	Museum	(i) Basal clades in the phylogeny and phylogeography of the widespread species will come from the Brazilian and Guyana Shields; (ii) species and intraspecific lineages from the lowlands (less than 100 m) will be estimated to establish during the last 4 Myr	Haq <i>et al.</i> (1987) Hoorn (1993) Fjeldså (1994) Nores (1999, 2004) Hall & Harvey (2002) Aleixo (2004) Hubert & Renno (2006)
Headwater capture events between major drainages	Last 10	Hydrogeology	(i) Clades (intraspecific lineages or species groups) will not be monophyletic for a given drainage; (ii) lineages in a given watershed will be nested within the phylogeny of a larger clade located in a different watershed with contiguous headwater	Lundberg <i>et al.</i> (1998) Sivasundar <i>et al.</i> (2001) Montoya-Burgos (2003) Hubert & Renno (2006)

from a broad array of biogeographical studies, the origin of the South American biotas is still largely debated (Bush 1994; Haffer 1997; Tuomisto & Ruokolainen 1997).

Freshwater fishes offer a unique opportunity for biogeographical studies and hypothesis testing. Dispersal of fishes depends directly on temporary connections and boundary displacements between coalescing rivers, and given that watershed history reflects the underlying geology, the chronology of river connections may be traced back in time (e.g. Bermingham & Martin 1998; Montoya-Burgos 2003). The final establishment of the modern South American rivers occurred during the final stage of Andean uplift beginning at 15 million years ago (Ma) (Fig. 1), and culminated during the last 10 million years (Myr) (Lundberg *et al.* 1998). Morphologically based studies of phylogenetic biogeography of South American freshwater fishes support an important diversification predating the Pleistocene climatic fluctuations (Weitzman & Weitzman 1982; Vari 1988; Vari & Weitzman 1990). Recently, evidence based on molecular data has confirmed that an important diversification stage occurred between 10 Ma and 3 Ma (Bermingham & Martin 1998; Lovejoy & De Araújo 2000; Sivasundar *et al.* 2001; Montoya-Burgos 2003), and thereby challenged the Pleistocene perturbations as a potential explanation for the origin of the South American aquatic biotas. These results point to the occurrence of speciation through founder events and allopatric differentiation related

to multiple headwater capture events and temporary connections (hydrogeological hypothesis; Lundberg *et al.* 1998; Montoya-Burgos 2003). However, previous studies of Neotropical fishes have paid little attention to the relative impact of others hypotheses of diversification such as the palaeogeography hypothesis (raising of arches in the Amazonian lowlands during the Tertiary responsible of allopatric speciation among populations separated on each side of these arches; Räsänen *et al.* 1990, 1992; Da Silva & Patton 1993, 1998; Hoorn *et al.* 1995) and the museum hypothesis (species originated by allopatric speciation in the emerged highlands during Miocene marine highstands and later accumulated in the lowlands which act as 'museums'; Fjeldså 1994; Roy *et al.* 1997; Nores 1999). Both hypotheses are amenable to testing by phylogenetic methods (Table 1). The palaeogeography hypothesis predicts that lineages would harbour sister clades among rivers separated by the raising of the palaeoarches in the lowlands (Da Silva & Patton 1993, 1998). By contrast, the hydrogeology hypothesis predicts that lineages from populations and species are nested within a larger clade located in a distinct watershed due to dispersal (Lundberg *et al.* 1998; Montoya-Burgos 2003). Although, both the palaeogeography and hydrogeology hypotheses rely on geomorphological changes that affected the river courses and caused allopatric speciation, the palaeogeography hypothesis predicts allopatric differentiation among populations isolated throughout the species'

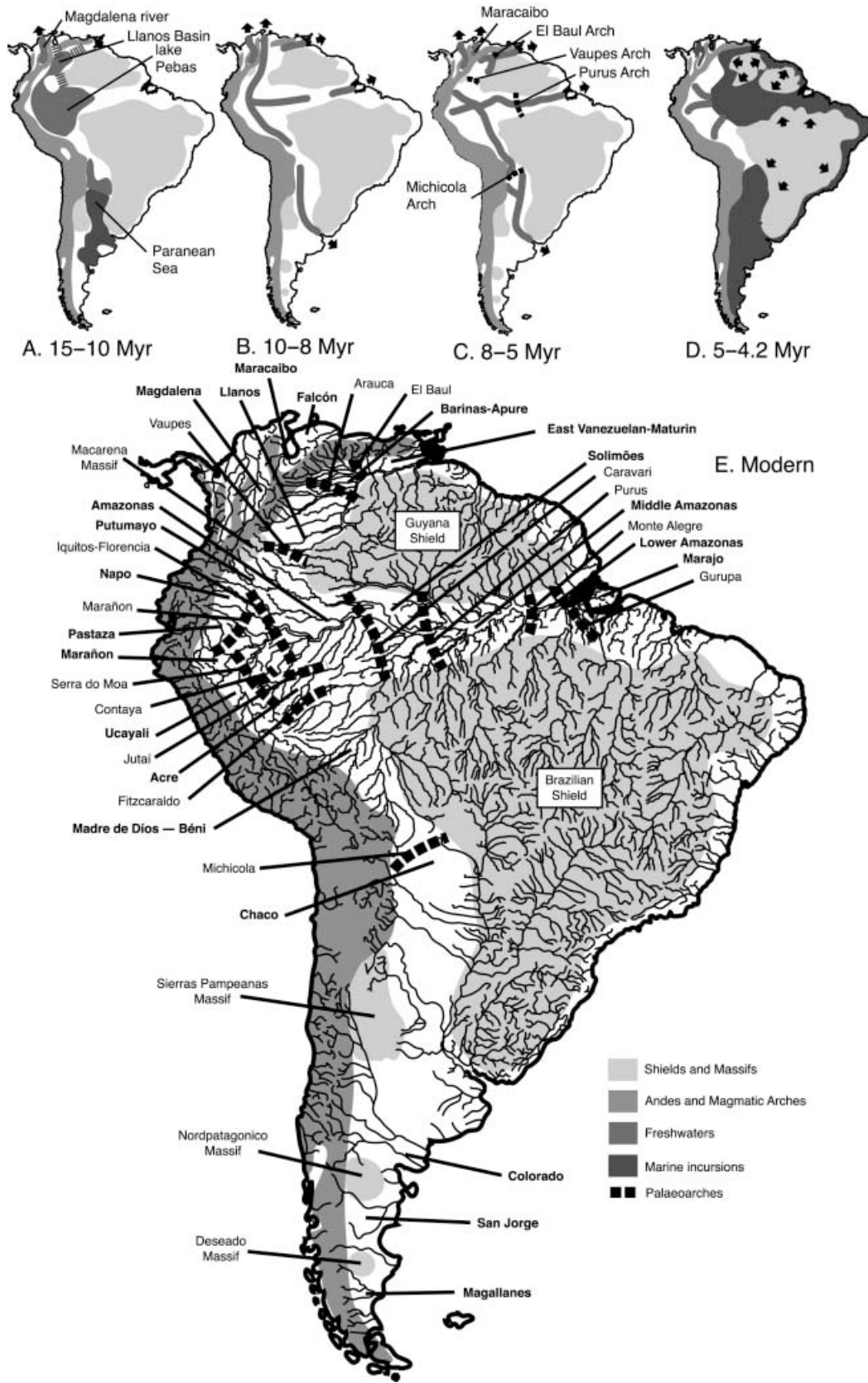


Fig. 1 Evolution of South American river systems during the last 15 Myr (modified from Hubert & Renno 2006). A. Marine incursions and continental lakes from 15 to 10 Myr. B. Marine regressions and continental rivers from 10 to 8 Myr. C. Final establishment of the Orinoco and Amazon Rivers from 8 to 5 Myr. D. Marine incursions from 5 to 4.2 Myr. E. Modern South American geomorphology and hydrologic systems. Sedimentary basins are in bold characters.

range distribution while the hydrogeology hypothesis predicts that species increased their distribution ranges by dispersal, with dispersive populations being subsequently isolated. Although the palaeogeography hypothesis invokes the raising of orogenic arches as a major source of increasing diversity, the hydrogeology hypothesis predicts that headwater capture events and temporary dispersal routes were more important in shaping extant aquatic diversity (Hubert & Renno 2006).

Phylogenetic and phylogeographical studies of closely related species based on mitochondrial DNA (mtDNA) may be expected to provide insights into the history of a region. When mtDNA substitution rates are known, the timing of speciation events may be estimated from genetic distances and compared to known geological events. Likewise, when species distribution ranges are known, phylogenetic biogeography can provide a comprehensive picture of the evolutionary history of a region (Brooks 1981; Nelson & Platnick 1981). Unfortunately, large-scale biogeographical studies are still scarce for Neotropical fishes owing to the paucity of robust phylogenetic hypotheses or detailed knowledge of geographical distribution. The piranha belongs to the Characidae subfamily of Serrasalminae (Buckup 1998). Currently including 28 species ranging from 130 to 420 mm standard length (Jégu 2003), the piranha genera *Serrasalmus* and *Pygocentrus* constitute the most speciose group of large carnivorous Characiformes. These genera represent well-suited candidates for hypothesis testing among Neotropical fishes as: (i) several molecular systematic studies have been recently conducted (Porto *et al.* 1992; Nakayama *et al.* 2002; Hubert *et al.* 2006); (ii) they constitute a clade of freshwater fishes endemic to South America (Orti *et al.* 1996); (iii) species distribution ranges are well documented (Hubert & Renno 2006); (iv) several widely distributed groups of closely related species have been previously described (Fink 1993; Fink & Machado-Allison 2001).

This study focuses on two main issues. The first is to assess the timing of diversification of the genera *Serrasalmus* and *Pygocentrus*, and the second is to use the piranha as a model system to investigate phylogenetically testable hypotheses of diversification in the Neotropics (Table 1; Fig. 1).

Materials and methods

Analytical procedure

We tested the predictions associated with the palaeogeography, museum and hydrogeology hypotheses using the following procedure (Fig. 2). We first checked the support for particular phylogenetic and phylogeographical patterns within piranha species (steps 1a, 1b; Fig. 2). Then, we checked for homogeneity of evolutionary rates through the identified clades and used a well-documented geological event for the calibration of a molecular clock

and the estimation of the cladogenetic event ages (step 2; Fig. 2). We further checked the support for a particular area cladogram using the Brooks parsimony analysis (BPA) procedure. Once detected, cladogenetic events and their ages were used as a landmark for the estimation of the timing of South American river establishment (step 3; Fig. 2). Finally, present patterns were compared with those obtained for other taxa and compared with the predictions drawn from the palaeogeography, hydrogeology and museum hypotheses (Table 1; step 4; Fig. 2).

Taxonomic sampling and DNA sequencing

First, phylogenetic relationships were addressed by constructing an mtDNA phylogeny using the control region and 16S gene among 21 of the 28 species of *Pygocentrus* and *Serrasalmus*. Second, phylogeographical patterns were addressed using mtDNA control region sequences of *Serrasalmus rhombeus*, *S. spilopleura* and *Pygocentrus nattereri* (Fig. 3; Appendix I). Sequences of the mtDNA control region and 16S available in GenBank for the genera *Pristobrycon*, *Pygopristis*, *Catoprion* and *Metynnis* were added as outgroups (Appendix I). Specimens from the Bolivian Amazon were collected by the French 'Institut de Recherche pour le Développement' (IRD) between September 2002 and June 2003 in the Upper Madeira watershed. Specimens from the Brazilian Amazon, Orinoco, São Francisco and Paraná-Paraguay were obtained from a specialized Amazonian fish trader (Iguarapé, Paris).

Genomic DNA was isolated from ethanol-preserved tissues with the DNeasy Tissue Kit (QIAGEN). The mtDNA control region was amplified using the primers CR22U: 5'-TGGTTTAGTACATATTATGCAT (present study) and F-12R: 5'-GTCAGGACCATGCCTTTGTG (Sivasundar *et al.* 2001). These primers amplify a fragment of 980 bp beginning in the position 100 of *Colossoma macropomum* control region (Accession no. AF283963) and including the 3' flanking tRNA genes (tRNA Thr and tRNA Pro). A 510-bp fragment of the 16S gene was amplified using the primers 16Sar-L: 5'-ACGCCTGTTTATCAAAAACAT, and 16Sbr-H: 5'-CCGGTCTGAACTCAGATCACGT (Palumbi *et al.* 1991). Polymerase chain reactions (PCRs) were performed in 50 μ L volumes including 13.5 μ L of template DNA (approximately 1 ng), 3 U of *Taq* DNA polymerase, 5 μ L of *Taq* 10x buffer, 3 μ L of MgCl₂ (25 mM), 4 μ L of dNTP (5 mM) and 3 μ L of each primer (10 μ M). For the control region, PCR conditions were as follows: 94 $^{\circ}$ C (5 min), 10 cycles of 94 $^{\circ}$ C (1 min), 66 to 56 $^{\circ}$ C decreasing 1 $^{\circ}$ C per cycle (1 min 30 s), 72 $^{\circ}$ C (2 min), 25 cycles of 94 $^{\circ}$ C (1 min), 56 $^{\circ}$ C (1 min 30 s), 72 $^{\circ}$ C (2 min), followed by 72 $^{\circ}$ C (5 min). Amplifications of the 16S fragment were performed as follows: 94 $^{\circ}$ C (5 min), 30 cycles of 94 $^{\circ}$ C (1 min), 46 $^{\circ}$ C (1 min), 72 $^{\circ}$ C (1 min), followed by 72 $^{\circ}$ C (5 min). PCR products were sequenced in both directions at Macrogen

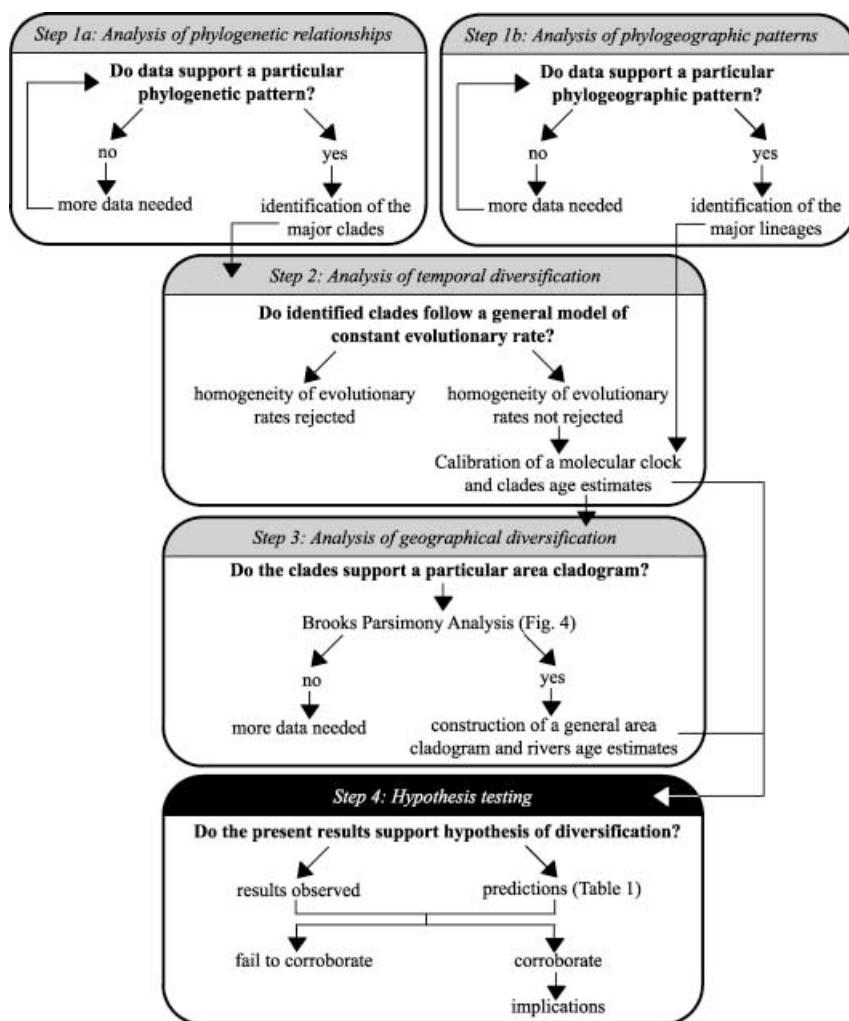


Fig. 2 Analytical procedure developed in the present study for testing the hypotheses of diversification.

(Korea) and consensus sequences were deposited in GenBank. Vouchers have been deposited in the Muséum National d'Histoire Naturelle, Paris (Appendix I).

Step 1a: analysis of phylogenetic relationships

Dealing with alignment ambiguities due to the occurrence of homoplastic changes is an important problem in phylogenetic reconstruction and several methods have been proposed to account for alignment ambiguous sites (e.g. Gatesy *et al.* 1994; Lee 2001). Among them, an efficient strategy consists in performing multiple alignments of a given data set and concatenating the alignments obtained into a single large alignment for subsequent phylogenetic analyses (Wheeler *et al.* 1995; Hubert *et al.* 2005). This method, called Elision, provides several advantages: (i) phylogenetic content of the alignment ambiguous sites is fully included; (ii) alignment uncertainty is treated without a priori; (iii) if nucleotide assignment is randomised,

phylogenetic signal become additive when random noise is averaged. Hence, we performed multiple alignments using CLUSTAL W (Thompson *et al.* 1993) and sequences were aligned with three opening-extending cost ratios: 5–4; 15–6; 20–8 (Hubert *et al.* 2005). Insertions–deletions were coded independently of their size following the method described by Barriel (1994) and implemented in BARCOD (Labarre *et al.* 1998). We compared the results for each of the multiple alignments and Elision in maximum parsimony (MP) and neighbour joining (NJ), as implemented in PAUP*4.0b10 (Swofford 2002). Heuristic MP searches started with stepwise addition of taxa replicated 100 times using tree-bisection–reconnection (TBR) branch swapping, and NJ analyses were performed using the BIONJ algorithm (Gascuel 1997). The number of variable and informative characters, the number of trees and tree length in MP, consistency index (CI; Kluge & Farris 1969) and number of nodes supported by bootstrap proportion using 1000 replicates (BP; Felsenstein 1985) were recorded as indices of alignment consistency.

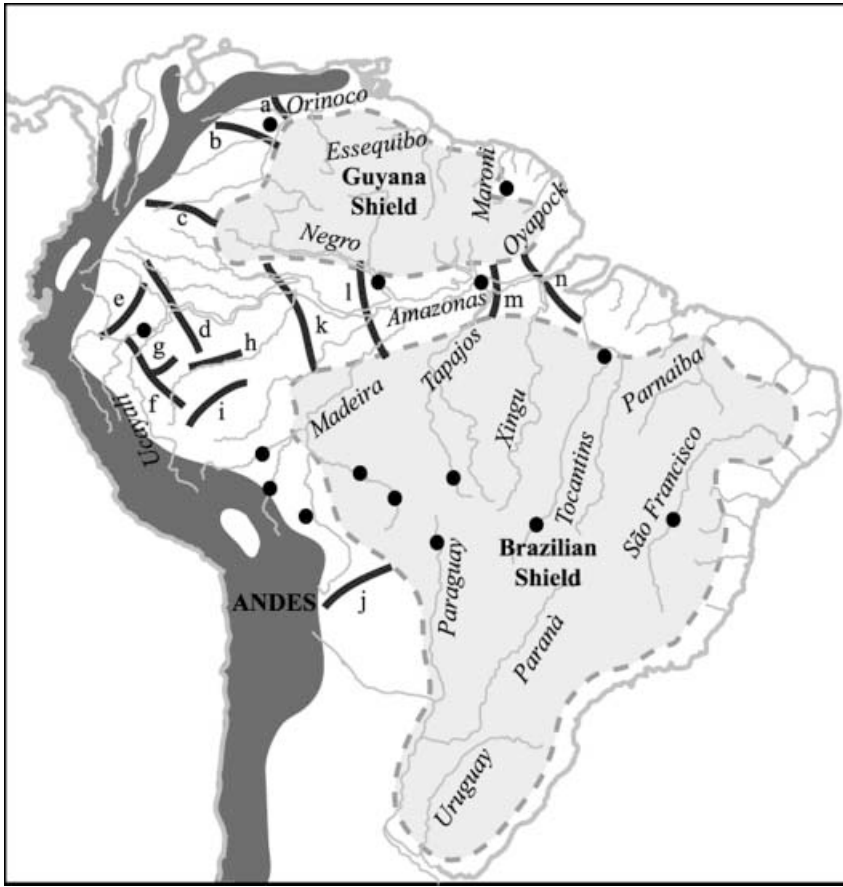


Fig. 3 Map of the South American hydrologic systems and collection localities of the 91 *Serrasalmus* and *Pygocentrus* specimens. Dashed lines delimit the Precambrian shields and bold lines indicate the palaeoarches (a, Barinas-Apuré; b, Arauca; c, Vaupes; d, Iquitos; e, Maraón; f, Serra do Moa; g, Contaya; h, Jutai; i, Fitzcaraldo; j, Michicola; k, Caravari; l, Purus; m, Monte Alegre; n, Gurupa). One point may represent more than one locality.

Step 1b: analysis of phylogeographical patterns

Haplotype phylogenies were constructed using maximum likelihood (ML) as implemented in PHYLML (<http://atgc.lirmm.fr/phylml>) following the algorithm developed by Guindon & Gascuel (2003). The Akaike information criterion (AIC) identified the optimal model as implemented in MODELTEST 3.7 (Posada & Crandall 1998), and was further used for tree searches and bootstrap analyses based on 1000 replicates in PHYLML.

Step 2: analysis of temporal diversification

We checked for substitution rate homogeneity in the mtDNA control region sequences by performing a relative-rate test (RRT) as implemented in RRTREE version 1.1 (Robinson-Rechavi & Huchon 2000) and searched for sister-species harbouring allopatric distributions related with a dated palaeogeographical event in order to calibrate a molecular clock for the control region. Divergence estimates were based on node to tip distances computed using the ML procedure mentioned in step 1b.

Step 3: analysis of geographical diversification

We investigated the geographical diversification using the Brooks parsimony analysis (BPA; Brooks 1981, 1990; Brooks *et al.* 2001), which aims to identify the area cladogram supported by the phylogeny of multiple codistributed clades. Vicariance is the only process affecting all the species similarly, and the area cladogram shared by multiple clades is attributed to vicariance while departures are attributed to dispersal and lineage duplication. In its original formulation, nested clades through the phylogeny are coded as present or absent and the area cladogram is constructed using the parsimony criterion (Brooks 1981). If species range distributions are shaped by vicariance only, the area cladogram mirrors the species phylogeny (Fig. 4A). The search for the most parsimonious area cladogram is not needed as replacing the species by the areas led to the corresponding area cladogram. However, taxa that increased or reduced their range by dispersal or extinction might support different area relationships and the solution may not be unique.

The second formulation of the BPA aimed to identify exceptions to the general area cladogram (Brooks 1990;

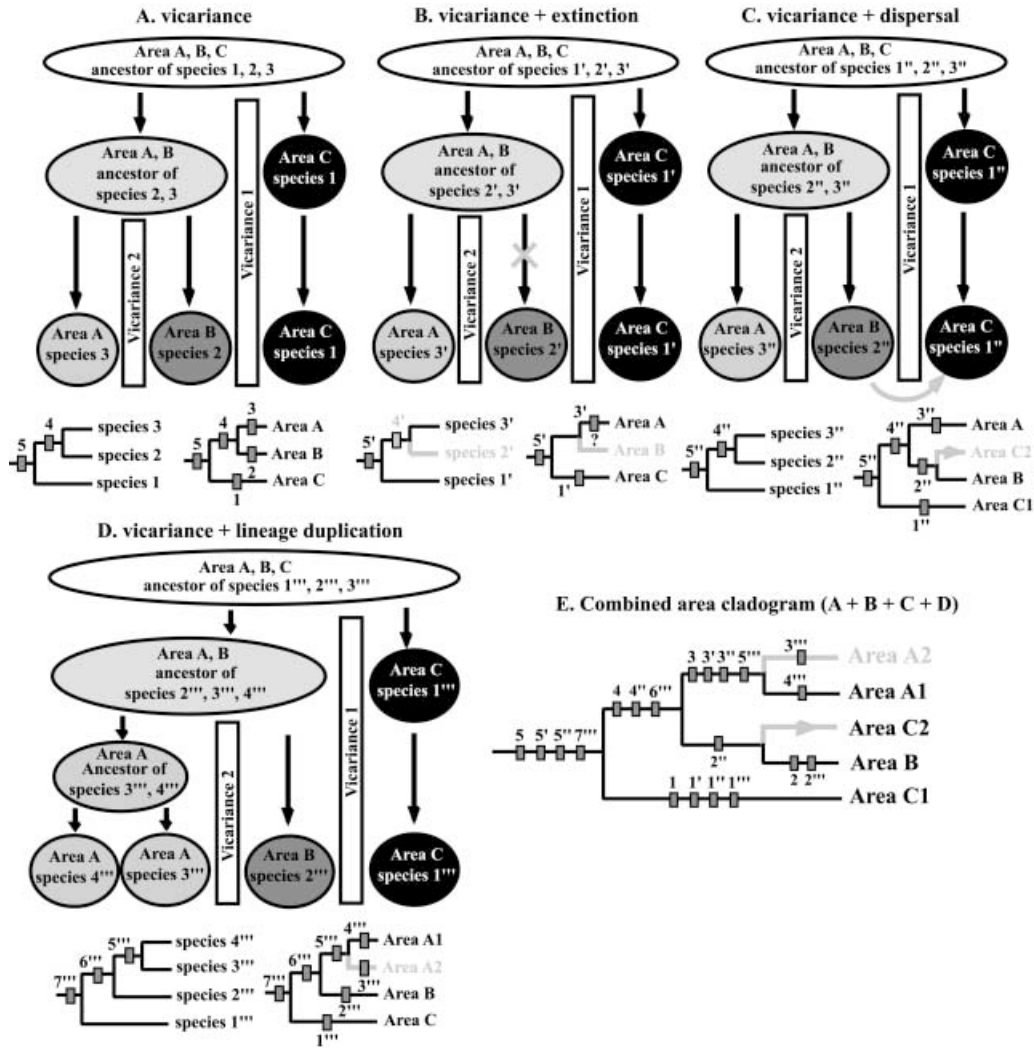


Fig. 4 Area cladogram construction following the analytical procedure of the Brooks parsimony analysis (BPA). A. Area cladogram from species range distribution shaped by vicariance only; B. Area cladogram from species range distribution shaped by vicariance and extinction (cross and dashed lines). C. Area cladogram from species range distribution shaped by vicariance and dispersal (arrow). Area duplications are performed as implemented in the secondary BPA. D. Area cladogram from species range distribution shaped by vicariant and lineage duplication in the same area A. Area duplications (A1 and A2) are performed as implemented in the secondary BPA. E. Combined area cladogram obtained using A, B, C, D area cladogram. Black branches identify the general area cladogram depicting the chronology of the vicariant events and grey branches identify exceptions to the general area cladogram due to extinction, dispersal or duplication events).

Brooks *et al.* 2001). Extinction corresponds to the loss of information and the absence of a taxon in an area is treated as missing data (Fig. 4B; Wiley 1988). In order to circumvent the problem of co-occurring species, the areas, hosting more than one species, are duplicated to maintain the correspondence between the species phylogeny and the area cladogram (secondary BPA; Fig. 4C). Likewise, when two sister species are endemic to a given area, the area is duplicated in order to account for the cladogenetic event (Fig. 4D). However, patterns found in nature often result from a mixed occurrence of vicariance, dispersal and line-

age duplication. The area cladograms from independent clades are needed to construct a general area cladogram (Fig. 4E). Since the absence of a taxon is coded as missing data and the amount of changes in the cladogram is not optimized, the combined area cladogram can be manually constructed.

We applied this procedure to the phylogeny of the piranha genera *Serrasalmus* and *Pygocentrus*. An area cladogram was manually constructed for each of the main clades identified, the repeated pattern of area relationships were identified and the general area cladogram was finally deduced (Appendix II).

Table 2 Summary results of maximum-parsimony and neighbour-joining analyses based on several alignments of piranha mtDNA using different alignment parameters and Elision. Statistics presented include alignment size, informative characters, length and number of inferred trees, consistency index to evaluate the homoplasy levels and statistical support

Alignment	Size*	No. of characters		Tree length†	No. of trees‡	Consistency index†	No. of nodes†§	
		Variable	Informative				MP	NJ
57-sequences control region								
I: Clustal 15–6 + gap	1046	487	304	899	8	0.475	39	44
II: Clustal 5–4 + gap	1171	544	314	938	35	0.451	35	37
III: Clustal 20–8 + gap	1207	564	331	1088	19	0.405	36	42
IV: Elision + gap	3424	1595	947	2942	8	0.439	45	51
54-sequences 16S								
V: Clustal 15–6 + gap¶	536	80	58	104	> 10000	0.673	13	18
57-taxon combined								
VI: I + V	1165	549	362	1023	44	0.491	40	42
VII: IV + V	3960	1595	1007	3065	1	0.446	45	49

*Total number of base pair positions in aligned sequences.

†Excluding uninformative characters.

‡Most parsimonious trees.

§Number of nodes supported by bootstrap values > 50%.

¶The three alignment parameters sets provided the same alignment.

Results

Step 1a: analysis of phylogenetic relationships

We obtained three alignments of the control region sequences that differed in the number of variable and parsimony informative sites due to the occurrence of several alignment ambiguous sites (Table 2). The 15–6 alignment provided the shortest alignment and trees, the greatest CI value and the best-supported scheme of relationships, relative to the two others (20–8 and 5–8 alignments), in both MP and NJ analyses. The majority-rule consensus of the eight most-parsimonious trees obtained with the 15–6 alignment consisted in a basal polytomy. Multiple alignments of the 16S data set converged to the same alignment including 536 sites (80 variable and 58 parsimony informative) but the MP analysis yielded numerous poorly supported trees (Table 2). Considering the low amount of homoplastic changes (CI = 0.673), this was explained by a lack of informative characters due to a rapid accumulation of clades rather than character conflict.

Elision of the mtDNA control region including gap characters provided the eight most-parsimonious trees found with the 15–6 alignment but these trees were better supported with 45 and 51 nodes supported by BP > 50 in MP and NJ, respectively (Table 2). The combined analysis of the mtDNA control region 15–6 alignment and 16S alignment provided 44 most-parsimonious trees leading to a majority-rule consensus consisting in a basal polytomy. By contrast, combined analysis of the control region Elision including gap character and 16S yielded a single well-

supported tree (Table 2). This result argued for an increased congruence among characters when using Elision and emphasized that the present data set supported a particular scheme of phylogenetic relationships (step 1a; Fig. 2).

As previously reported (Ortí *et al.* 1996), *Pygopristis denticulata*, *Catoprion mento* and *Pristobrycon striolatus* constituted a highly supported monophyletic group, placed as the sister group of the *Serrasalmus* and *Pygocentrus* clade, supported by BP = 100 in both MP and NJ trees (Fig. 5). *Pristobrycon* was polyphyletic since *P. calmoni* was nested within *Serrasalmus* in a highly supported sister-relationship with *S. serrulatus*. This result suggests that *P. calmoni* is more closely related to *Serrasalmus* species than *P. striolatus*, in agreement with previous morphological evidences (Jégu 2004). We followed Jégu (2004) and considered *P. calmoni* within *Serrasalmus* in the rest of this section. Both *Serrasalmus* (*sensu* Jégu 2004) and *Pygocentrus* (B4; Fig. 5) were monophyletic and three main lineages were identified within the former (B1, B2, B3; Fig. 5). The NJ tree was resolved in the scheme (((B2, B3), B1), B4) but without statistical support. Although, log-likelihood scores of alternative topologies involving either ((B1, B4) (B2, B3)) or ((B3, B4) (B1, B2)) or ((B2, B4) (B1, B3)) were lower than the original tree, differences were not significant (Shimodaira & Hasegawa 1999; data not shown). However, several morphological synapomorphies support the monophyly of both *Serrasalmus* (Jégu 2004) and *Pygocentrus* (Fink 1993).

Within *Serrasalmus*, the monophyly of the clades B2 and B3 was supported with statistical confidence (Fig. 5). The clade B2 was further divided into three main lineages, B2-1, B2-2 and B2-3 showing high BP values, while the clade B3

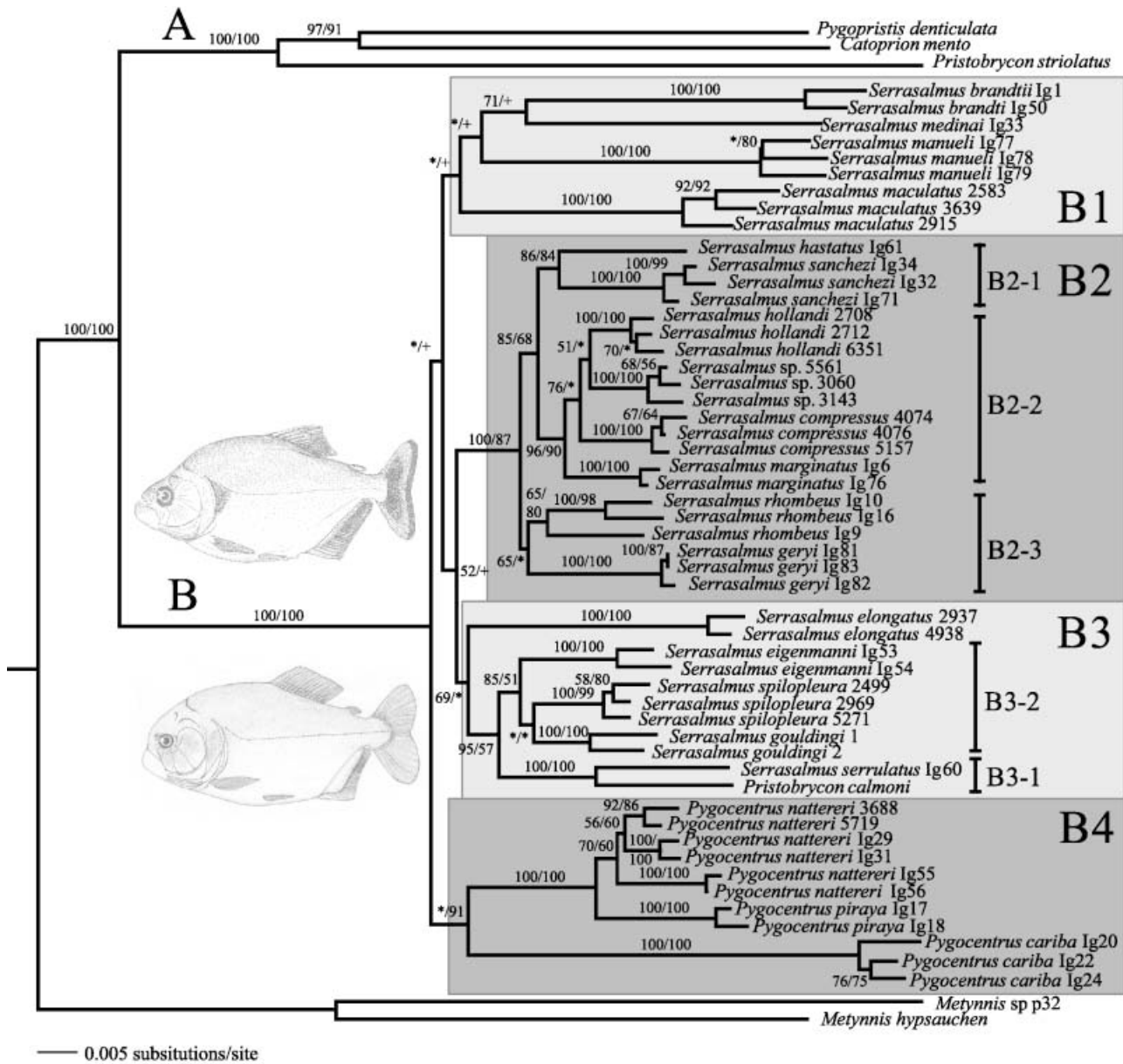


Fig. 5 Neighbour-joining phylogram among the 57 samples of *Pygocentrus*, *Pristobrycon*, *Pygopristis*, *Catoprion* and *Serrasalmus* species obtained with the combined data set including the Elision alignment of the control region and the 16S alignment (alignment VII, Table 2), and rooted with *Metynnis* species. Gap characters are coded following Barriol (1994). Numbers above branches are bootstrap proportion for NJ and MP and using informative characters only. *, nodes observed but not supported by BP > 50; +, nodes not observed in MP; B1 to B4, the four major clades detected among the genera *Serrasalmus* and *Pygocentrus*.

was subdivided into two well-supported clades (B3-1 and B3-2), with *S. elongatus* as the sister-group. Most of the discrepancies found between the MP and NJ trees were related to the clade B1 and the position of *S. maculatus* and *S. medinai*, poorly supported in the NJ tree and nested with other species in the MP tree (Fig. 5). However, the clade B1 was the most poorly sampled from the present phylogeny and more data are needed to resolve its phylogenetic affinities.

Step 1b: analysis of phylogeographical pattern

The AIC indicated that the GTR + I model fitted the control region of *S. rhombeus* and *P. nattereri* better than others while the GTR + I + Γ better fitted the *S. spilopleura* data set and were used for ML searches (Fig. 6). Within rivers, haplotype divergences ranged between 0.2 and 2.3% in *S. rhombeus*, 0.1 and 0.8% in *P. nattereri* and, 0.1 and 0.5% in

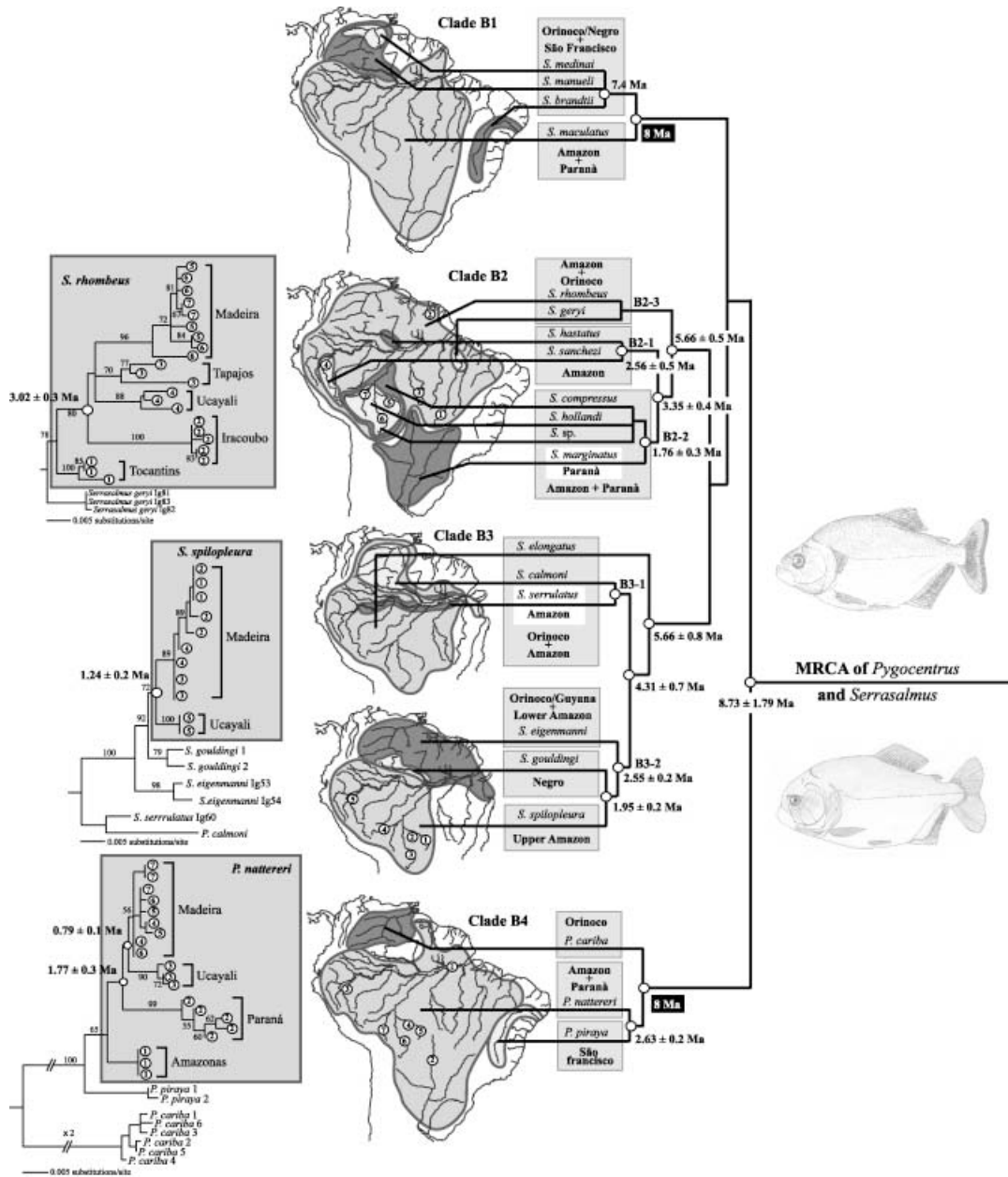


Fig. 6 Historical biogeographical scheme among the clades B1, B2, B3 and B4 following the hypothesized scheme of relationships of the combined data set (Fig. 5), and phylogeography of *Serrasalmus rhombeus* (23 samples), *Serrasalmus spilopleura* (11 samples) and *Pygocentrus nattereri* (20 samples) control region haplotypes. Haplotype phylogeography inferred with ML with the GTR + I model for *Serrasalmus rhombeus*, *P. nattereri* and the GTR + I + Γ model in *S. spilopleura* haplotypes. The model GTR + I was used with the following parameters for *S. rhombeus*: base frequencies A = 0.32, G = 0.22, C = 0.16, T = 0.30, mutation rates A-C = 3.55, A-G = 34.67, A-T = 3.04, C-G = 0.01, C-T = 31.92, G-T = 1.00, proportion of invariable sites = 0.87. The model GTR + I was used with the following parameters for *P. nattereri*: base frequencies A = 0.31, G = 0.23, C = 0.16, T = 0.30, mutation rates A-C = 1.84, A-G = 21.54, A-T = 1.87, C-G = 0.00, C-T = 21.55, G-T = 1.00, proportion of invariable sites = 0.81. The model GTR + I + Γ was used with the following parameters for *S. spilopleura*: base frequencies A = 0.31, G = 0.22, C = 0.17, T = 0.28, mutation rates A-C = 1011.55, A-G = 9550.96, A-T = 9550.96, C-G = 808.06, C-T = 0.00, G-T = 1.00, proportion of invariable sites = 0.76, gamma distribution shape parameter = 0.69. Divergence date estimates in the species phylogeny were computed using the control region sequence divergences obtained in ML with the GTR + I + Γ model. The model GTR + I + Γ was used with the following parameters for the calibration of the molecular clock within the *Serrasalmus* and *Pygocentrus* data set (57 samples data set): base frequencies A = 0.32, G = 0.23, C = 0.15, T = 0.30, mutation rates A-C = 4.58, A-G = 47.20, A-T = 5.11, C-G = 2.07, C-T = 47.20, G-T = 1.00, proportion of invariable sites = 0.51, gamma distribution shape parameter = 0.48. Numbers above branches identify bootstrap proportions (> 50% only). MRCA, most recent common ancestor.

S. spilopleura. Among rivers, haplotype divergences ranged between 2.1 and 4.2% in *S. rhombeus*, 1.2 and 3.0% in *P. nattereri* and, 1.1 and 1.7% in *S. spilopleura*. The three phylogeographical patterns obtained suggested that the splitting events that originated the populations were old enough to allow the establishment of reciprocal monophyly in the mtDNA lineages within each river and further supported the presence of structured lineages (Fig. 3; step 1b).

Within *S. rhombeus*, the tributaries from the Upper Amazon (Ucayali, Madeira) and the Tapajos were resolved in an unsupported polytomy, with the Iracoubo as the sister group ($BP = 80$), while the Tocantins was found in basal position with good statistical confidence (Fig. 6). Interestingly, the mtDNA control region sequences of *S. rhombeus* supported the scenario of drainage establishment (Tocantins (Iracoubo (Tapajos, Ucayali, Madeira))) in contrast with geological results supporting (Iracoubo (Tocantins (Tapajos (Ucayali, Madeira)))) (Hoorn *et al.* 1995; Irion *et al.* 1995; Lundberg *et al.* 1998).

The phylogeography of *S. spilopleura* provided new insights into its geographical distribution. The taxonomy of *S. spilopleura* and *S. eigenmanni* has been complex due to the lack of confident geographical records and diagnostic characters (Jégu & Dos Santos 2001). Hubert *et al.* (2006) recently provided evidence that the citation of *S. eigenmanni* in the Madeira was erroneous and originated from confusion with *S. spilopleura*. Ortega & Vari (1986) tentatively placed specimens morphologically very similar to *S. spilopleura* from the Ucayali within *S. humeralis*, another closely related species (Jégu & Dos Santos 2001). The results of the present study suggest that the specimens from the Ucayali analysed here currently belong to *S. spilopleura*. Furthermore, the divergence level observed between the lineages of *S. spilopleura* from the Madeira and Ucayali Rivers falls within the range observed among populations of *S. rhombeus* and *P. nattereri*. Thus, the phylogeography of the species revealed an extended distribution throughout the headwaters of the Madeira and Ucayali drainages (Fig. 6; Appendix II).

The phylogeography of *P. nattereri* revealed a pattern very similar to *S. rhombeus*. The lineages from the Ucayali and Madeira were placed in a poorly supported sister relationship while the eastern lineage from the Amazon was in a basal and well-supported position (Fig. 6). However, this phylogeographical pattern differed from the other species by the position of the Paraná lineage, nested within the clade of the Amazon lineages in a sister relationship with the Upper Amazon clade. Thus, *P. nattereri* phylogeography supports the scenario of drainage establishment (Amazonas (Paraná (Ucayali, Madeira))) by contrast with the geological evidences supporting (Paraná (Amazonas, Ucayali, Madeira)).

Table 3 Relative-rate test

Lineages	Control region		
	dK	SD	P
B2 vs. B3	0.001	0.003	0.77
B2 + B3 vs. B1	0.006	0.005	0.26
B2 + B3 vs. B4	-0.012	0.007	0.09
B1 vs. B4	-0.006	0.007	0.44

Step 2: analysis of temporal diversification

The RRT performed on mtDNA control region sequences of the three *Serrasalmus* lineages and *Pygocentrus* indicated homogeneous substitution rates throughout lineages B1, B2, B3 and B4 (Table 3). Hence, we calibrated a molecular clock using the sequence divergences inferred in ML with the control region data set (step 2; Fig. 2). The 15–6 alignment provided the less homoplastic scheme of nucleotide assignment and was used for the divergence estimates. The AIC indicated that the GTR + I + Γ model fitted the control region sequences significantly better, and sequence divergences using this model ranged from 1.5 to 10% within *Serrasalmus*, from 3.9 to 10% within *Pygocentrus*, and were of $10.2 \pm 2\%$ on average between *Serrasalmus* and *Pygocentrus* lineages.

The final establishment of the Orinoco has been previously estimated to occur at 8 Ma (Lundberg *et al.* 1998). The node-to-tip distance taken between *S. maculatus* (Amazon and Paraná) and *S. manueli* (Orinoco and Negro) is $4.66 \pm 0.3\%$ (for an age estimate of 8 Myr), that is $0.58 \pm 0.4\%$ per Myr. Likewise, the node-to-tip distance between *P. cariba* (Orinoco) and *P. nattereri* (Amazonas and Paraná) or *P. piraya* (São Francisco) is $4.63 \pm 0.3\%$, that is $0.58 \pm 0.4\%$ per Myr. This rate of sequence divergence was 1.4–1.6 times less than previously reported for other Neotropical fishes since node-to-tip sequence divergences provided an estimated substitution rate of 0.84% per Myr in the genus *Prochilodus* (Sivasundar *et al.* 2001) and 0.93% per Myr in the genus *Hypostomus* (Montoya-Burgos 2003). This difference can be explained by both the exclusion from our analyses of the 5' hypervariable region of the control region and the inclusion of the more conserved 3' flanking tRNA (Sivasundar *et al.* 2001).

The splitting of the *Serrasalmus* and *Pygocentrus* lineages from their most recent common ancestor (MRCA) was hypothesized here to have occurred at 8.73 ± 1.79 Ma, followed by a rapid differentiation of the *Serrasalmus* lineages B1, B2 and B3 achieved between 8 and 5.66 ± 0.8 Ma (Fig. 6). Further splitting of the clade B2 in the subclades B2-1 and B2-2 and the clade B3 in B3-1 and B3-2 were estimated between 3.35 ± 0.4 Ma and 4.31 ± 0.7 Ma, respectively. Most of the contemporary species in clades

B2 and B3, and the speciation event leading to *P. nattereri* and *P. piraya* occurred during the last 3 Myr.

Within the clade B1, the shared origin of *S. brandtii* with *S. medinai* and *S. manueli* suggested an old dispersal event dated around 7.4 Ma. Within the clade B2, *S. rhombeus* and *S. geryi* were in basal position. The phylogeography of *S. rhombeus* supported the presence of an old mtDNA lineage in the Tocantins drainage while the colonization of the upper course of the Amazon and the Guyana Shield was dated around 3.02 ± 0.3 Ma (Fig. 6). These results suggested a shared geographical origin between these sister species dated around 5.66 ± 0.5 Ma. The differentiation of the subclades B2-1 and B2-2 was dated around 3.35 ± 0.4 Ma, the splitting of *S. sanchezi* from the Ucayali and *S. hastatus* from the Negro occurring at nearly 2.56 ± 0.5 Ma and the splitting of *S. marginatus* and *S. compressus*, *S. hollandi* and *S. sp.* at 1.76 ± 0.3 Ma (Fig. 6). Within the clade B3, the cladogenetic event leading to *S. elongatus* was dated at 5.66 ± 0.8 Ma, and the division into B3-1 and B3-2 occurred at 4.31 ± 0.8 Ma (Fig. 6). The subclade B3-2 further subdivided into *S. eigenmanni* in the east and *S. spilopleura* + *S. gouldingi* in the west at around 2.55 ± 0.2 Ma, with *S. gouldingi* separating in the Negro at 1.95 ± 0.2 Ma. The differentiation of the Ucayali and Madeira lineages within *S. spilopleura* was dated at 1.24 ± 0.2 Ma. Within the clade B4, the cladogenetic event leading to *P. nattereri* and *P. piraya* was dated around 2.63 ± 0.2 Ma, while the colonization of the Paraná by *P. nattereri* was dated around 1.77 ± 0.3 Ma and the differentiation of the lineages from the Upper Amazon (Ucayali and Madeira) around 0.79 ± 0.1 Ma (Fig. 6).

Step 3: Analysis of geographical diversification

We used the phylogenetic relationships inferred within the clades B1, B2, B3 and B4 to construct four area cladograms following the BPA procedure (Fig. 7). The area cladograms obtained harboured a single common pattern (Fig. 2; step 3) supporting the biogeographical scheme (Orinoco (Tocantins (Amazonas (Negro (Ucayali, Madeira)))))) (Fig. 7). The presence of a common pattern supported the fact that the palaeoecological events creating the present pattern affected and shaped the species range distributions from clades B1, B2, B3 and B4 in a similar manner. This scenario was consistent with the geological evidence supporting an isolation of the Orinoco prior to the establishment of the Amazon tributaries (Lundberg *et al.* 1998) and supported that the oldest cladogenetic events were found in the eastern part of the Amazon (Fig. 7).

Within the Amazon, the Tocantins boundary displacement was related to the differentiation of clade 22 (21 vs. 20) and may be associated with the raising of the Gurupa Arch, in the lower course of the Tocantins (Fig. 7). Actually, clade 33 (23 vs. 32) may be associated either with the establishment

of the Orinoco and followed by large-scale dispersal across the Amazon tributaries or associated with the establishment of the Tocantins followed by subsequent extinction in this river and dispersal in the Orinoco. However, neither the large distribution pattern of *S. elongatus* across the Amazon River, very similar to *S. rhombeus*, nor its divergence age estimates (5.66 ± 0.8 Ma) supported an origin located in the Orinoco. The diversification of clade 33 better fit the pattern observed elsewhere in clade 22 and consistent with an early differentiation of the Tocantins within the Amazon tributaries.

The differentiation of clade 32 (31 vs. 30) was related to the boundary displacement of the Amazonas and our age estimate of the final establishment of the Amazonas (4.31 ± 0.7 Ma) was consistent with the raising of the Purus Arch, previously hypothesized to have occurred during the last 5 Myr (Hoorn *et al.* 1995; Irion *et al.* 1995). The boundary displacement of the Negro, was related to differentiation of clade 20 (19 vs. 37) and 30 (26 vs. 29) estimated to occur between 3.35 ± 0.4 Ma and 2.55 ± 0.2 Ma. This was consistent with the raising of the Caravari Arch during the last 5 Myr (Hoorn *et al.* 1995; Irion *et al.* 1995). The establishment of the Ucayali and Madeira Rivers, hypothesized here to occur between 1.24 ± 0.2 Ma and 0.8 ± 0.1 Ma, was supported by the phylogeography of species 28 and 36 (*S. spilopleura* and *P. nattereri*) and may be associated with the raising of the Iquitos Arch, responsible for the boundary displacement of the Ucayali (Lundberg *et al.* 1998). This was consistent with the presence of extended Pleistocene deposits in the Ucayali (Räsänen *et al.* 1990).

Several exceptions to the general area cladogram were detected due to five duplication events (Fig. 7). The location of the speciation events leading to *S. manueli* (species 2) and *S. medinai* (species 3); *S. geryi* (species 9) and *S. rhombeus* (species 8); *S. serrulatus* (species 24) and *S. calmoni* (species 25); *S. compressus* (species 13), *S. hollandi* (species 14) and *S. sp.* (species 15) were consistent with a sympatric origin in the Orinoco, Tocantins, Amazonas and Madeira, respectively (Fig. 7). Likewise, several dispersal events in the clades B1, B2, B3 and B4 were observed during the last 8 Myr (Fig. 7). Two dispersal events were inferred from the Orinoco to the Negro (species 2) and the São Francisco (species 5), the latter being associated with the speciation event leading to *S. brandtii* around 7.4 Ma. The colonization of the Upper Amazon tributaries and the Orinoco by *S. rhombeus* (species 8) was inferred as a consequence of dispersal events from the Tocantins dated around 3.02 ± 0.3 Ma. Likewise, the colonization of the Ucayali by *S. serrulatus* (species 24) and the colonization of the Tocantins, Negro and Orinoco by *S. calmoni* (species 25) were inferred to result from dispersal originating in the Amazonas (Fig. 7). The differentiation of *S. sanchezi* (species 11) in the Ucayali originated by dispersal from the Negro at 2.56 ± 0.5 Ma. Similarly, the differentiation of *S. gouldingi* (species 27)

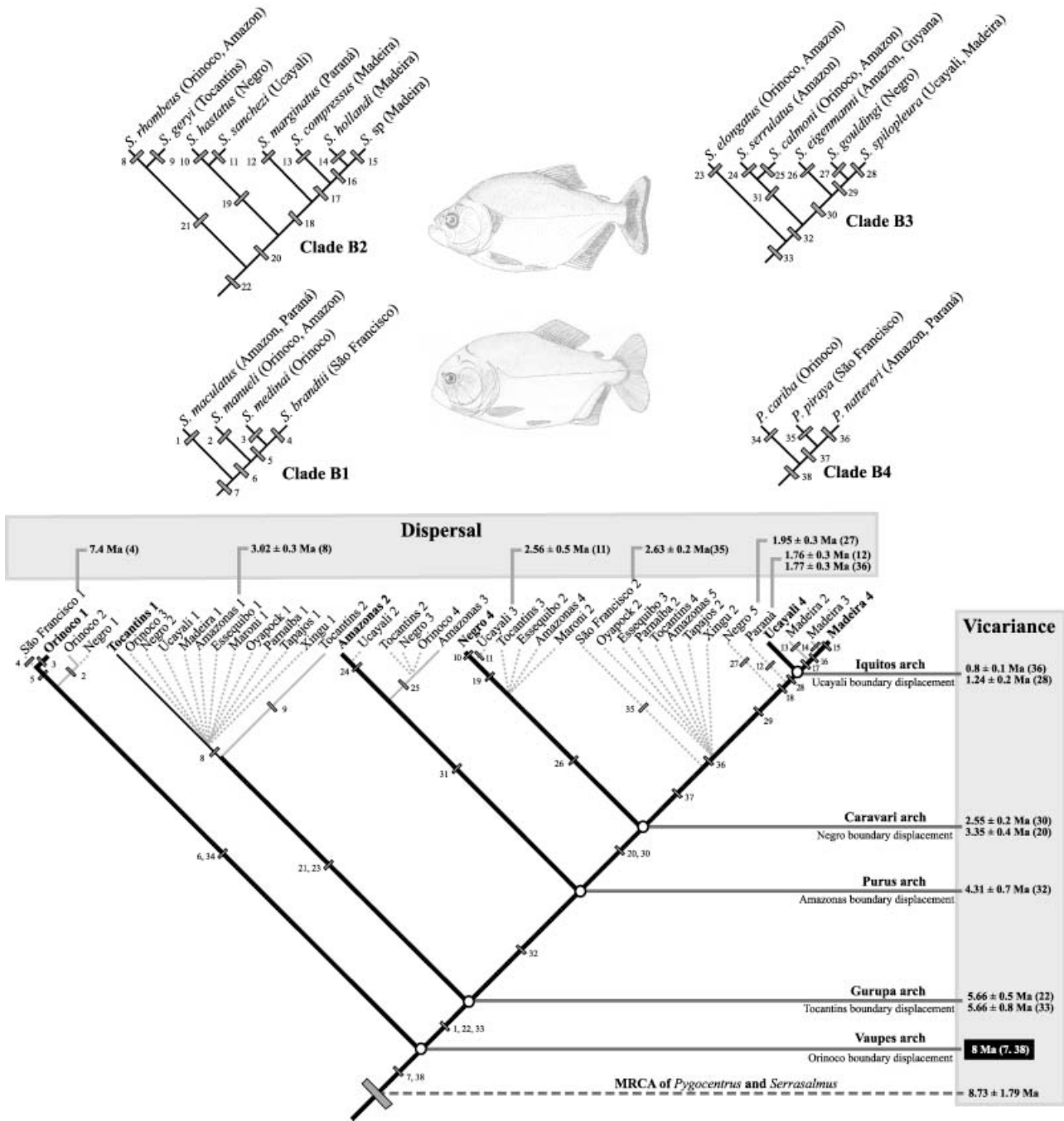


Fig. 7 Cladograms and branching order among the clades B1 to B4, and secondary BPA area cladogram including the Amazon tributaries and the Orinoco, Paraná, Guyana drainages, São Francisco and Parnaíba. Dispersal and vicariance age estimates following Fig. 6. Black branches and grey branches in the tree correspond to the general area cladogram and individual exceptions, respectively. Bold lines and white circles represent vicariant events whereas thin lines and dashed lines correspond to lineage duplications and dispersal, respectively. Boxes on terminal and internal branches represent extant species and ancestors, respectively. Dashed lines associated with boxes correspond to postdispersal speciation events.

in the Negro, *S. marginatus* in the Paraná (species 12) and *P. piraya* (species 35) in the São Francisco originated by dispersal from the Upper Amazon, before the final establishment of the Ucayali, around 1.95 ± 0.2 Ma, 1.76 ± 0.2 Ma

and 2.63 ± 0.2 Ma, respectively (Fig. 7). Concomitantly, the area cladogram suggested that *P. nattereri* colonized the Paraná by dispersal from the Amazon around 1.77 ± 0.3 Ma (Fig. 7).

Discussion

Phylogenetic inferences and the timing of the piranha radiation

The timing of diversification of the genera *Serrasalmus* and *Pygocentrus* is consistent with a rapid differentiation. When compared with other carnivorous families of the order, fossils of the family Erythrinidae, which encompass 14 species distributed throughout South America (Oyakawa 2003), are reported up to the Late Cretaceous (Gayet & Brito 1989; Gayet & Meunier 1998). The family Cynodontidae includes 14 species (Toledo-Pizza *et al.* 1999; Toledo-Pizza 2000, 2003), whose fossils are reported up to the middle Miocene around 13–14 Ma (Lundberg 1997; Lundberg *et al.* 1998). The family Acestrorhynchidae includes 15 species largely distributed in South America (Menezes 2003), and is hypothesized to have originated during the Middle Miocene (Lundberg *et al.* 1998). Although, fossils records of *Serrasalmus* are still debated (Lundberg 1997; Gayet & Meunier 1998; Lundberg *et al.* 1998), palaeontological evidence substantiates a Cretaceous origin of the subfamily Serrasalminae as fossils from the Serrasalmin genera *Colossoma*, *Piaractus* and *Mylossoma* harbour fossil representatives up to the late Cretaceous (Gayet & Meunier 1998). By contrast, the present study suggests a much more recent contribution of *Serrasalmus* and *Pygocentrus* to the Neotropical ichthyofauna. Our molecular calibration suggests that the extant species originate from a hypothetical MRCA around 9 Ma.

The timing of diversification of these genera seems consistent with a rapid differentiation during the Late Miocene, a process that was almost complete at the Pliocene–Pleistocene transition. It is worth noting that estimated divergence rates for mitochondrial ribosomal genes based on fossil records among Serrasalmins range between 0.21 and 0.26% per Myr (Lundberg *et al.* 1998). According to the present rate of diversification, the lack of informative characters in the 16S data set in the early branching of the piranha tree, despite a low level of homoplasy, further support a rapid differentiation of the piranha lineages as previously stated (Ortí *et al.* 1996). Although, the Paraná boundary displacement is complex and may originate more than 20 Ma (Lundberg *et al.* 1998), our results support a more recent colonization of its drainages by dispersal from the Upper Amazon about 2 Ma. On the other hand, the final establishment of the Orinoco is 8 Ma but the estimated age of the *Serrasalmus* and *Pygocentrus* MRCA is consistent with an origin prior to this event, in the proto-Amazon/Orinoco system.

Historical biogeography and congruence with other taxa

The area cladogram of the Amazon tributaries suggests that the history of *Serrasalmus* and *Pygocentrus* has been

apparently quite complex, resulting from a mixed occurrence of vicariant and dispersal events. A similar pattern has been previously reported for several lowland curimatids (e.g. *Steindachnerina* Vari 1989; Vari 1991) and lorcarids (Montoya-Burgos 2003). The present phylogeny is consistent with the geological evidence of the Vaupes Arch uplift at 8 Ma that prompted the final establishment of the Orinoco and its separation from the Amazon. For example, our results support the Vaupes to have caused the first splitting events within the clades B1 and B4, which is also consistent with the great differentiation of the mtDNA lineages previously reported between the migratory species of the genus *Prochilodus* from the two drainages (Sivasundar *et al.* 2001).

The chronology of the vicariant events within the Amazon supports an eastern–western establishment of the Amazon tributaries. This result was congruent with the geological data, which previously showed that the south-western part of the Amazon began to take its current shape only in the last 3 Myr (Hooen *et al.* 1995) while the Brazilian and Guyana Shields have been stable during at least the last 10 Myr (Irion *et al.* 1995; Lundberg *et al.* 1998). The phylogeny of the curimatid genus *Steindachnerina* previously supported that an old splitting event gave rise to the species from the Tocantins (Vari 1991). Similar results were reported for the terrestrial mammal genus *Proechimys* (Da Silva & Patton 1993, 1998) and the Neotropical birds *Xiphorhynchus* (Aleixo 2004), which agrees with the hypothesis that the arches from the Upper Amazon were the latest to uplift.

As recently emphasized (Montoya-Burgos 2003), extensive dispersal occurred among all the major Neotropical drainages. The occurrence of *S. manueli* in the Negro is inferred here to be a consequence of recent dispersal from the Orinoco and a similar distribution pattern is found among several other characiform fishes such as *Hydrolycus wallacei* (Toledo-Pizza *et al.* 1999), *Boulengerella laterstrigata* (Vari 1995), *Curimatopsis evelynae* (Vari 1982), *Cyphocharax multilineatus* and *C. gangamon* (Vari 1992b), *Caenotropus mestomorgmatos* (Vari *et al.* (1995), *Creagrutus runa* and *C. zephyrus* (Vari & Harold, 2001). Although dispersal from the Orinoco to the Amazon was previously reported among needlefishes (Lovejoy & De Araújo 2000), mtDNA lineages of the genus *Potamorhaphis* support a connection that occurred through the Guyana drainages and the Rupununi. Likewise, dispersal events from the Amazon to the Orinoco were revealed for *S. rhombeus*, *S. elongatus* and the clade including *S. serrulatus* and *Pristobrycon calmoni* (B3-1), which correspond to largely distributed taxa across the Amazon. In turn, several species currently distributed across both systems corroborate this result, such as *Cynodon septenarius* (Toledo-Pizza 2000), *Boulengerella cuvieri* and *B. maculata* (Vari 1995), *Curimatella immaculata* (Vari 1992a), *Curimatopsis macrolepis* (Vari 1982) and *Potamorhina altamazonica* (Vari 1984).

The dispersal event between the Orinoco and São Francisco that gave rise to *S. brantii*, may represent an example of coastal dispersal around 7.4 Ma as previously stated for *Hypostomus* between 10 and 5 Ma (Montoya-burgos 2003), *Potamorhaphis* (Lovejoy & De Araújo 2000) and other Characiformes (Hubert & Renno 2006). Compatible species distribution ranges are found in *Hydrolicus tatauaia* (Toledo-Pizza *et al.* 1999), *Poptella longipinnis* (Reis 1989), *Curimata cyprinoides* (Vari 1989), *Prochilodus rubrotaeniatus* (Vari 2004) or *S. eigenmanni* (present study). The phylogeography of Central American fishes also support extensive coastal dispersal occurring near 7 Ma (Bermingham & Martin 1998). The occurrence of dispersal between 10 and 5 Ma was previously attributed to dramatically low sea levels and hypothesized to enhance coastal dispersal through coalescing river mouths (Bermingham & Martin 1998; Montoya-Burgos 2003).

The boundary displacement of the Paraná system is complex and several headwater-capture events have been documented previously (Lundberg *et al.* 1998; Hubert & Renno 2006), the most recent during the last 10 Myr. Dispersal between the Upper Amazon and Paraná was detected for the family Callichthyidae (Reis 1998) and the genus *Hypostomus* with an age estimate of 10–12 Ma (Montoya-Burgos 2003). Our results suggest that the phylogeography of *Pygocentrus nattereri* is consistent with a dispersal event from the Upper Amazon to the Upper Paraguay around 1.8 Ma. Dispersal events between these two systems are further exemplified by the species distribution ranges of *Psectrogaster curviventris* (Vari 1989), *Hemiodus orthonops* (Langeani 2003), *Acestrorhynchus altus* and *A. pantaneiro* (Menezes 2003) and *Pyrrhulina australis* (Weitzman & Weitzman 2003).

Finally, dispersal events were detected between 3 Ma and 1.5 Ma within the Amazon, and may be attributed to slight sea-level increases that happened during the Late Pliocene (Irion *et al.* 1997). The increase of freshwater availability during interglacial times was hypothesized to impound the lower course of the Amazon with freshwater (Irion *et al.* 1995), and a large freshwater lake, with a length of about 2000 km and a width not exceeding 100 km, has been previously suggested from the lower course of the Amazon during the Late Pliocene (Irion *et al.* 1995, 1997; Lundberg *et al.* 1998). The detection here of an increased rate of dispersal during this period is consistent with these observations.

Implications for the diversification of Neotropical fishes

The results of the present study emphasize that the establishment of the Orinoco and the Amazon deeply imprinted the evolution of the piranha lineages and the present molecular calibration provides timing for the raising of the palaeoarches congruent with previous

geological and phylogeographical results. The Vaupes Arch caused several cladogenetic events within the clades B1 and B4 as the Gurupa Arch does within clade B3 or the Purus, Caravari and Iquitos Arches in *Serrasalmus* and *Pygocentrus*. These results are congruent with previous phylogeographical studies in terrestrial mammals arguing that several palaeoarches promoted allopatric differentiation among populations isolated throughout the species' range distribution (Da Silva & Patton 1993, 1998).

Similarly, dispersal events between distinct drainages were hypothesized to promote postdispersal allopatric speciation once the connections ceased. Such temporary connections allowing dispersal might be related to multiple headwater-capture events between the Amazon and either the Paraná (Lundberg *et al.* 1998) or the Orinoco (Hubert & Renno 2006). The inference of dispersal routes at the margin of the Brazilian and Guyana Shields previously suggested that landslides on the periphery of the Shields might be the most common process responsible for dispersal between distinct drainages (Hubert & Renno 2006). Although phylogeographical studies of Neotropical freshwater fishes are scarce, this scenario was corroborated by the occurrence of multiple dispersal events among headwaters of the major drainages in the genus *Hypostomus* (Montoya-Burgos 2003). The present results reveals that several species and intraspecific clades originated from a larger clade located in a distinct watershed due to dispersal, thereby matching the predictions of the hydrogeology hypothesis.

Previous work has emphasized a relationship between endemism levels for freshwater fishes and the location of the emerged lands during the Late Tertiary marine highstand of 100 m above current sea level (Hubert & Renno 2006). Following observations on species ages and emerged lands, the evolution of terrestrial biotas has been postulated to originate from a dynamic process of local differentiation in stable mountain areas and latter accumulation in the lowlands (museum hypothesis; Nores 1999). Hence, this hypothesis predicts that the oldest clades and intraspecific lineages are found in the land higher than 100 m above sea level, while species and populations in the lowlands (less than 100 m above sea level) originated during the last 4 Myr. The area cladogram presented here reveals that the establishment of the rivers from the emerged land of the Guyana and Brazilian Shields is related to the oldest cladogenetic events within *Serrasalmus* and *Pygocentrus*, while the species from the lowlands of the Western Amazon appeared only during the last 4 Myr. Thus, the present results are consistent with the expectations of the museum hypothesis.

Finally, several lineage duplications corresponding to within-drainage speciation events are detected in *Serrasalmus* as exemplified by the three sister-species from the Madeira (*S. compressus*, *S. hollandi* and *S. sp.*) that

originated during the last 2 Myr. This result may represent an example of sympatric speciation (Schluter 2001; Via 2001) and may be related to the great habitat heterogeneity found in the upper watershed of the Madeira (Guyot 1993; Navarro & Maldonado 2002). Genetic structure in relation to different habitat use has been previously reported in populations of *S. maculatus* from the central Amazon (Centofante *et al.* 2002) and high levels of resource partitioning have been observed between closely related *Serrasalmus* species from the Venezuelan llanos (Winemiller 1989). However, alternative processes including allopatric differentiation followed by secondary contact and local extirpations may account for the present patterns and more data are therefore needed to confirm the possible occurrence of sympatric speciation in South American rivers.

Conclusion

Neotropical freshwater species diversity is the result of million years of evolution and most of the extant species actually predate the climatic fluctuations of the Pleistocene (Weitzman & Weitzman 1982; Lundberg 1998; Sivasundar *et al.* 2001; Montoya-Burgos 2003). The historical biogeography of *Serrasalmus* and *Pygocentrus* argues that several documented hydrological and palaeoecological changes deeply influenced the cladogenetic events observed in the phylogeny of these genera. However, and in agreement with previous authors (e.g. Bush 1994), complex interactions between orogenic events, sea level fluctuations and hydrography are emphasized here to concomitantly or alternatively shape the Neotropical freshwater fish diversity during the late Tertiary. Previously, the overwhelming process that had been invoked for explaining the origin of the Neotropical diversity was allopatric speciation (Haffer 1997). However, the genus *Serrasalmus* suggests that diversification within the same drainage may occur and that sympatric speciation may be a non-negligible cause of increasing diversity in the Amazon. The ecology of fishes can have profound effects on the geographical distribution of genetic variation (Lovejoy & De Araújo 2000) and ongoing phylogenetic and population genetic studies of speciation will provide significant improvements of our knowledge about the evolution of the South American rivers and their biotas. This question, however, still constitutes a considerable challenge.

Acknowledgements

This research was supported by Institut de Recherche pour le Développement (IRD, France); Instituto de Biología Molecular y Biotecnología, La Paz (IBM y B, Bolivia), Instituto de Limnología, La Paz (Bolivia), Instituto de Investigaciones de la Amazonia Peruana, Iquitos (Perú); the laboratory GPIA, Montpellier (France). We thank Adrien Leroy (Iguarapé) for providing valuable samples, F. Bonhomme, N. Bierne, B. Guinand and E. Lambert from the GPIA laboratory; G. Rodrigo, N. Mamani, V. Iñiguez

and J.P. Torrico from the IBMB for laboratory supports and facilities. We wish to thank F. Carvajal, A. Parada, L. Torres, T. Yunoki for their help during field sampling, J. Pinto, R. Marin, M. Baudoin, J.D. Durand, J. Mavárez for their interest and M. Legendre for his support.

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-
- Nicolas Hubert is interested in the biogeography and the evolution of the Characiform fishes. This work was part of his PhD on the evolution of the piranha. Jesus Nuñez and Fabrice Duponchelle are eco-physiologists, specializing on the interactions between habitat and life histories in fishes. Didier Paugy is an ecologist of the fish communities from the MNHN (Paris) and Jean-François Renno is a population genetist specializing on the Amazonian fishes. Didier and Jean-François were also Nicolas's PhD advisors.
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Appendix I

Summary of the samples used in the present study

Species	Sequences	Voucher	GenBank Accession no.		
			16S	Control region	River
<i>Catoprius mento</i>	Orti, G. & Sivasundar, A. (unpublished)		AF283932	AF284462	Unknown
<i>Metynnis hypsauchen</i>	Orti, G. & Sivasundar, A. (unpublished)		AF283934	AF283957	Unknown
<i>Metynnis</i> sp. p32	Orti, G. & Sivasundar, A. (unpublished)		AF283933	AF283956	Unknown
<i>Pristobrycon calmoni</i>	Orti, G. & Sivasundar, A. (unpublished)			AF283947	Unknown
<i>Pristobrycon striolatus</i>	Orti, G. & Sivasundar, A. (unpublished)		U33596	AF284463	Unknown
<i>Pygocentrus cariba</i> Ig20	Present study	MNHN 2006–1271	DQ384709	DQ384758	Orinoco
<i>Pygocentrus cariba</i> Ig21	Present study	MNHN 2006–1272		DQ384759	Orinoco
<i>Pygocentrus cariba</i> Ig22	Present study	MNHN 2006–1273	DQ384710	DQ384760	Orinoco
<i>Pygocentrus cariba</i> Ig23	Present study	MNHN 2006–1274		DQ384761	Orinoco
<i>Pygocentrus cariba</i> Ig24	Present study	MNHN 2006–1275	DQ384711	DQ384762	Orinoco
<i>Pygocentrus cariba</i> Ig26	Present study	MNHN 2006–1276		DQ384763	Orinoco
<i>Pygocentrus nattereri</i> 3688	Present study			DQ384764	Madeira
<i>Pygocentrus nattereri</i> 3689	Present study		DQ384712	DQ384765	Madeira
<i>Pygocentrus nattereri</i> 3691	Present study		DQ384713	DQ384766	Madeira
<i>Pygocentrus nattereri</i> 20119	Present study			DQ384767	Madeira
<i>Pygocentrus nattereri</i> 5409	Present study			DQ384768	Madeira
<i>Pygocentrus nattereri</i> 1993	Present study			DQ384769	Madeira
<i>Pygocentrus nattereri</i> 5719	Present study			DQ384770	Madeira
<i>Pygocentrus nattereri</i> 4269	Present study	MNHN 2006–1261		DQ384771	Madeira
<i>Pygocentrus nattereri</i> 20300	Present study			DQ384772	Madeira
<i>Pygocentrus nattereri</i> Ig27	Present study	MNHN 2006–1277		DQ384773	Ucayali
<i>Pygocentrus nattereri</i> Ig29	Present study	MNHN 2006–1278	DQ384714	DQ384774	Ucayali
<i>Pygocentrus nattereri</i> Ig31	Present study	MNHN 2006–1279	DQ384715	DQ384775	Ucayali
<i>Pygocentrus nattereri</i> Ig8	Present study			DQ384776	Paraná
<i>Pygocentrus nattereri</i> Ig85	Present study			DQ384777	Paraná
<i>Pygocentrus nattereri</i> Ig86	Present study			DQ384778	Paraná
<i>Pygocentrus nattereri</i> Ig87	Present study	MNHN 2006–1292		DQ384779	Paraná
<i>Pygocentrus nattereri</i> Ig88	Present study			DQ384780	Paraná
<i>Pygocentrus nattereri</i> Ig55	Present study	MNHN 2006–1285	DQ384716	DQ384781	Amazon
<i>Pygocentrus nattereri</i> Ig56	Present study	MNHN 2006–1286	DQ384717	DQ384782	Amazon
<i>Pygocentrus nattereri</i> Ig57	Present study	MNHN 2006–1287		DQ384783	Amazon
<i>Pygocentrus piraya</i> Ig17	Present study	MNHN 2006–1269	DQ384718	DQ384784	São Francisco
<i>Pygocentrus piraya</i> Ig18	Present study	MNHN 2006–1270	DQ384719	DQ384785	São Francisco
<i>Pygopristis denticulata</i>	Orti, G. & Sivasundar, A. (unpublished)			AF284464	unknown
<i>Serrasalmus brandti</i> Ig1	Present study		DQ384720	DQ384786	São Francisco
<i>Serrasalmus brandti</i> Ig50	Present study	MNHN 2006–1280	DQ384721	DQ384787	São Francisco
<i>Serrasalmus compressus</i> 4074	Present study	MNHN 2005–2166	DQ384722	DQ384788	Madeira
<i>Serrasalmus compressus</i> 4076	Present study	MNHN 2005–2168	DQ384723	DQ384789	Madeira
<i>Serrasalmus compressus</i> 5157	Present study	MNHN 2005–2172	DQ384724	DQ384790	Madeira
<i>Serrasalmus eigenmanni</i> Ig53	Present study	MNHN 2006–1283	DQ384725	DQ384791	Amazon
<i>Serrasalmus eigenmanni</i> Ig54	Present study	MNHN 2006–1284	DQ384726	DQ384792	Amazon
<i>Serrasalmus elongatus</i> 2937	Present study	MNHN 2006–1248	DQ384727	DQ384793	Madeira
<i>Serrasalmus elongatus</i> 4938	Present study	MNHN 2006–1257	DQ384728	DQ384794	Madeira
<i>Serrasalmus geryi</i> Ig81	Present study		DQ384729	DQ384795	Tocantins
<i>Serrasalmus geryi</i> Ig82	Present study		DQ384730	DQ384796	Tocantins
<i>Serrasalmus geryi</i> Ig83	Present study	MNHN 2006–1291	DQ384731	DQ384797	Tocantins
<i>Serrasalmus gouldingi</i> 1	Orti, G. & Sivasundar, A. (unpublished)			AF283944	Unknown
<i>Serrasalmus gouldingi</i> 2	Orti, G. & Sivasundar, A. (unpublished)			AF283945	Unknown
<i>Serrasalmus hastatus</i> Ig61	Present study		DQ384732	DQ384798	Negro
<i>Serrasalmus hollandi</i> 2708	Present study	MNHN 2005–2251	DQ384733	DQ384799	Madeira
<i>Serrasalmus hollandi</i> 2712	Present study	MNHN 2005–2255	DQ384734	DQ384800	Madeira
<i>Serrasalmus hollandi</i> 6351	Present study	MNHN 2005–2267	DQ384735	DQ384801	Madeira
<i>Serrasalmus maculatus</i> 2583	Present study	MNHN 2006–1245	DQ384736	DQ384802	Madeira

Appendix I Continued

Species	Sequences	Voucher	GenBank Accession no.		
			16S	Control region	River
<i>Serrasalmus maculatus</i> 2915	Present study	MNHN 2006–1246	DQ384737	DQ384803	Madeira
<i>Serrasalmus maculatus</i> 3639	Present study	MNHN 2006–1253	DQ384738	DQ384804	Madeira
<i>Serrasalmus manueli</i> Ig77	Present study		DQ384739	DQ384805	Negro
<i>Serrasalmus manueli</i> Ig78	Present study		DQ384740	DQ384806	Negro
<i>Serrasalmus manueli</i> Ig79	Present study	MNHN 2006–1290	DQ384741	DQ384807	Negro
<i>Serrasalmus marginatus</i> Ig6	Present study		DQ384742	DQ384808	Paraná
<i>Serrasalmus marginatus</i> Ig76	Present study		DQ384743	DQ384809	Paraná
<i>Serrasalmus medinai</i> Ig33	Present study		DQ384744	DQ384810	Orinoco
<i>Serrasalmus rhombeus</i> Ig10	Present study	MNHN 2006–1266	DQ384746	DQ384811	Ucayali
<i>Serrasalmus rhombeus</i> Ig16	Present study	MNHN 2006–1268	DQ384747	DQ384812	Ucayali
<i>Serrasalmus rhombeus</i> Ig62	Present study			DQ384813	Ucayali
<i>Serrasalmus rhombeus</i> Ig9	Present study		DQ384745	DQ384814	Tapajos
<i>Serrasalmus rhombeus</i> Ig52	Present study	MNHN 2006–1282		DQ384815	Tapajos
<i>Serrasalmus rhombeus</i> Ig64	Present study			DQ384816	Tapajos
<i>Serrasalmus rhombeus</i> 2494	Present study			DQ384817	Madeira
<i>Serrasalmus rhombeus</i> 20445	Present study	MNHN 2005–2241		DQ384818	Madeira
<i>Serrasalmus rhombeus</i> 6380	Present study	MNHN 2006–1264		DQ384819	Madeira
<i>Serrasalmus rhombeus</i> 3923	Present study	MNHN 2006–1256		DQ384820	Madeira
<i>Serrasalmus rhombeus</i> 3871	Present study	MNHN 2006–1254		DQ384821	Madeira
<i>Serrasalmus rhombeus</i> 3403	Present study	MNHN 2006–1252		DQ384822	Madeira
<i>Serrasalmus rhombeus</i> 3244	Present study	MNHN 2006–1251		DQ384823	Madeira
<i>Serrasalmus rhombeus</i> 6259	Present study			DQ384824	Madeira
<i>Serrasalmus rhombeus</i> 20447	Present study	MNHN 2005–2243		DQ384825	Madeira
<i>Serrasalmus rhombeus</i> Ig95	Present study			DQ384826	Iracoubo
<i>Serrasalmus rhombeus</i> Ig96	Present study			DQ384827	Iracoubo
<i>Serrasalmus rhombeus</i> Ig97	Present study			DQ384828	Iracoubo
<i>Serrasalmus rhombeus</i> Ig98	Present study			DQ384829	Iracoubo
<i>Serrasalmus rhombeus</i> Ig99	Present study			DQ384830	Iracoubo
<i>Serrasalmus rhombeus</i> Ig51	Present study	MNHN 2006–1281		DQ384831	Tocantins
<i>Serrasalmus rhombeus</i> Ig58	Present study	MNHN 2006–1288		DQ384832	Tocantins
<i>Serrasalmus rhombeus</i> Ig63	Present study			DQ384833	Tocantins
<i>Serrasalmus sanchezi</i> Ig32	Present study		DQ384748	DQ384834	Ucayali
<i>Serrasalmus sanchezi</i> Ig34	Present study		DQ384749	DQ384835	Ucayali
<i>Serrasalmus sanchezi</i> Ig71	Present study	MNHN 2006–1265	DQ384750	DQ384836	Ucayali
<i>Serrasalmus serrulatus</i> Ig60	Present study	MNHN 2006–1289	DQ384751	DQ384837	Amazon
<i>Serrasalmus</i> sp. 3060	Present study	MNHN 2005–2187	DQ384752	DQ384838	Madeira
<i>Serrasalmus</i> sp. 3143	Present study	MNHN 2005–2191	DQ384753	DQ384839	Madeira
<i>Serrasalmus</i> sp. 5561	Present study	MNHN 2005–2209	DQ384754	DQ384840	Madeira
<i>Serrasalmus spilopleura</i> 2499	Present study	MNHN 2006–1244	DQ384755	DQ384841	Madeira
<i>Serrasalmus spilopleura</i> 2969	Present study	MNHN 2006–1249	DQ384756	DQ384842	Madeira
<i>Serrasalmus spilopleura</i> 5271	Present study	MNHN 2006–1258	DQ384757	DQ384843	Madeira
<i>Serrasalmus spilopleura</i> 5556	Present study	MNHN 2006–1260		DQ384844	Madeira
<i>Serrasalmus spilopleura</i> 5554	Present study	MNHN 2006–1259		DQ384845	Madeira
<i>Serrasalmus spilopleura</i> 2931	Present study	MNHN 2006–1247		DQ384846	Madeira
<i>Serrasalmus spilopleura</i> 3045	Present study	MNHN 2006–1250		DQ384847	Madeira
<i>Serrasalmus spilopleura</i> 3888	Present study	MNHN 2006–1255		DQ384848	Madeira
<i>Serrasalmus spilopleura</i> 3801	Present study			DQ384849	Madeira
<i>Serrasalmus spilopleura</i> 6354	Present study	MNHN 2006–1263		DQ384850	Madeira
<i>Serrasalmus spilopleura</i> 6328	Present study	MNHN 2006–1262		DQ384851	Madeira
<i>Serrasalmus spilopleura</i> Ig11	Present study	MNHN 2006–1267		DQ384852	Ucayali
<i>Serrasalmus spilopleura</i> Ig37	Present study			DQ384853	Ucayali

Appendix II

Summary of species range distribution in the present study according to Hubert & Renno (2006)

Species	Ori	Ess	Mar	Oya	Parn	SF	Par	Toc	Xin	Tap	Mad	Uca	Ama	Neg
<i>Pristobrycon calmoni</i>	1	0	0	0	0	0	0	1	0	0	0	0	1	1
<i>Pygocentrus cariba</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Pygocentrus nattereri</i>	0	1	0	1	1	0	1	1	0	0	1	1	1	1
<i>Pygocentrus piraya</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Serrasalmus brandti</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>Serrasalmus compressus</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	0
<i>Serrasalmus eigenmanni</i>	1	1	1	1	0	0	0	1	0	0	0*	0	0	1
<i>Serrasalmus elongatus</i>	1	0	0	0	0	0	0	0	0	0	1	1	1	1
<i>Serrasalmus geryi</i>	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>Serrasalmus gouldingi</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Serrasalmus hastatus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Serrasalmus hollandi</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	0
<i>Serrasalmus maculatus</i>	0	0	0	0	0	0	1	1	0	0	1	0	1	1
<i>Serrasalmus manuei</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Serrasalmus marginatus</i>	0	0	0	0	0	0	1	0	0	0	0	0	0	0
<i>Serrasalmus medinai</i>	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Serrasalmus rhombeus</i>	1	1	1	0	1	0	0	1	1	1	1	1	1	1
<i>Serrasalmus sanchezi</i>	0	0	0	0	0	0	0	0	0	0	0	1	0	0
<i>Serrasalmus serrulatus</i>	0	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Serrasalmus spilopleura</i>	0	0	0	0	0	0	0	0	0	0	1	1*	0	0

Ori, Orinoco; Ess, Essequibo; Mar, Maroni; Oya, Oyapock; Parn, Parna'ba; SF, São Francisco; Par, Paraná; Toc, Tocantins; Xin, Xingu; Tap, Tapajos; Mad, Madeira; Uca, Ucayali; Ama, Amazonas; Neg, Negro.

*modified following results of the present study.