

Tracking the history of an invasion: the freshwater croakers (Teleostei: Sciaenidae) in South America

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Submitted: 5 September 2014
Accepted: 22 November 2014
doi:10.1111/zsc.12098

Boeger, W.A., Marteleto, F.M., Zagonel, L., Braga, M.P. (2014). Tracking the history of an invasion: the freshwater croakers (Teleostei: Sciaenidae) in South America. —*Zoologica Scripta*, 00, 000–000.

In this study, the competing hypotheses of single vs. double colonisation events for freshwater Pachyurinae (Sciaenidae) in South America is tested and the historical biogeography of the expansion of this clade within the continent is reconstructed based on phylogenetic analysis. Parsimony and Bayesian inference (BI) for 19 marine and freshwater species assigned to Sciaenidae, Haemulidae and Polypteridae were determined based on partial sequences of the mitochondrial *16S* and *cytochrome b* genes and fragments of the nuclear *Tmo-4C4* and *rhodopsin* genes. A parsimonious ancestral character reconstruction of euryhalinity was performed on a clade of families of closely related fishes to evaluate the role of ecological fitting in the colonisation of freshwater by a marine sciaenid. The parsimony and BI phylogenetic hypotheses for the concatenated sequences supported the monophyly of the freshwater Sciaenidae. Divergence of the two freshwater clades of Sciaenidae, Pachyurinae and *Plagioscion*, occurred within the Amazon Basin. Within Pachyurinae, two clades were recovered: one composed of species from the Amazon and the Paraná Basin and a second with representatives from the São Francisco and south-eastern Atlantic basins. The results were compatible with the hypothesis of a single colonisation event of South American freshwater habitats by a marine lineage. The hypothesis of gradual adaptation to freshwater was rejected in favour of the hypothesis of ecological fitting. Sciaenidae, or a subordinate lineage within the family, is ancestrally capable of withstanding exposure to low-salinity habitats, which putatively facilitated the colonisation of freshwater habitats. The subsequent diversification and expansion of Pachyurinae across South America followed this colonisation and replicated the general pattern of the area relationships of South American river basins for several other fish groups.

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Introduction

Sciaenidae (Percomorpharia) includes fishes commonly known as croakers or drums, consisting of nearly 270 species across 70 genera. The family is widely distributed in tropical and subtropical regions worldwide (Nelson 2006). Although most sciaenids are marine, species from six genera are restricted to freshwater environments on different continents; four of these genera comprise species endemic to South America. Sasaki (1989) indicated that the South American sciaenids comprise two putatively independent marine-derived lineages (MDLs, Bloom & Lovejoy, 2011). One MDL is composed of *Plagioscion* species, and the other consists of the Pachyurinae (Sasaki 1989; Casatti 2000). The Pachyurinae include *Pachyurus* Agassiz in Spix & Agassiz, 1831, *Pachypops* Gill, 1861 and the recently erected *Petilipinnis* Casatti 2000 (Sasaki 1989; Casatti 2000).

The two putative South American MDLs of Sciaenidae are often considered phylogenetically distant, sister groups to marine species (Chao 1978; Sasaki 1989; Casatti 2000; Santos *et al.* 2013). This relationship suggests that the origins of *Plagioscion* and Pachyurinae are associated with independent colonisation events of freshwater environments in South America (Casatti 2000; Boeger & Kritsky 2003; Lovejoy *et al.* 2006; Cooke *et al.* 2012) facilitated by marine transgressions that occurred during the Late Oligocene and the early to mid-Miocene (Boeger *et al.* 2003; Cooke *et al.* 2012). Most recently, Cooke *et al.* (2012) suggested that both South American MDL clades of Sciaenidae may have originated from a common ancestor through a single dispersal into freshwater but their analysis included only two marine species of Sciaenidae and did not consider most previously proposed sister groups for the included lineages of freshwater MDLs, such as those suggested by Boeger *et al.* (2003), Chao (1978), Santos *et al.* (2013) and Sasaki (1989).

Therefore, this study explores the relationships of both South American sciaenid MDLs by reconstructing a phylogenetic hypothesis based on two nuclear and two mitochondrial fragments from representatives of *Plagioscion*, Pachyurinae and several marine species from selected genera, many putative sister groups of the freshwater lineages. Based on the present and available phylogenetic hypotheses, molecular dating, and fossil records, the scenario of freshwater colonisation is re-evaluated, and a hypothesis regarding the biogeographic history of Pachyurinae in continental South America is presented. The analysis also tests the evolutionary status of the genera of Pachyurinae.

Furthermore, the process involved in the colonisation of freshwater habitats by MDLs is typically associated with marine transgression and the gradual adaptation to freshwater in estuarine-like environments (Lovejoy *et al.*

2006). An alternative hypothesis to this gradual adaptation suggests that animal lineages subjected to landlock (Schultz & McCormick 2013) were predisposed to survive the new environmental freshwater conditions. Thus, we present an analysis to test the idea that ecological fitting (Janzen 1985; Agosta & Klemens 2008) represented a central process in the colonisation of freshwater habitats by the marine Sciaenidae.

Materials and methods

Tissue samples were obtained from four different South American river basins, and representative samples of marine sciaenid species were also captured in Barra Velha (state of Santa Catarina, Brazil) (Table 1). Fish specimens were identified by specialists (see Acknowledgments) or with the help of specialised literature. Available voucher specimens (tissue and fish specimens) are deposited in the Museu de História Natural Capão da Imbuia (MHNCI) and the Laboratório de Ictiologia of the Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul (MCP) (Table 1). Additionally, representative specimens are available at the collections of the Laboratório de Ictiologia Sistemática da Universidade Federal do Tocantins (UNT) and of the Museu de Zoologia de São Paulo (MZUSP) (Table 1). Representative specimens are specimens used as reference for the identification of the fish sampled in this and other similar studies. Fin, liver or muscle tissue was collected from fresh specimens, then fixed in absolute ethanol and stored in EDTA–DMSO buffer (Seutin *et al.* 1991).

DNA extraction was performed using an EZ-DNA kit following the manufacturer's instructions (Biosystems, Brazil). Fragments of two mitochondrial (*16S rDNA* and *cytochrome b*) and two nuclear genes (*Tmo-4C4* and *rhodopsin*) were obtained from nine freshwater croaker species, eight marine sciaenids, *Polydactylus oligodon* (Polynemidae) and *Conodon* sp. (Haemulidae) (Table 1). The 16S rRNA fragment was amplified in a 25- μ L reaction with the following final concentrations of components: 2 μ M each primer, 3 mM dNTPs, 6 mM MgCl₂, 0.1 U of *Taq* DNA polymerase, 1 \times buffer and 2.4 ng/ μ L of template DNA. The cytochrome b fragment was amplified in a 25- μ L reaction with the following final concentrations of components: 2 μ M each primer, 0.2 mM dNTPs, 3 mM MgCl₂, 0.1 U of *Taq* DNA polymerase, 1 \times buffer and 2.4 ng/ μ L of template DNA. Both nuclear fragments (*Tmo-4C4* and *rhodopsin*) were amplified in a 50- μ L reaction with the following final concentrations of components: 0.4 μ M each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.02 U of *Taq* DNA polymerase, 1 \times buffer and 1.2 ng/ μ L of template DNA. The primers used in these reactions for the *16S*,

Table 1 The fish species included in this study with their respective distributions, collection localities, specimens and samples deposited, and GenBank numbers produced sequences

Species name	Distribution	Collection locality (coordinates)	Voucher/Reference specimen/Tissue sample	GenBank
<i>Pachypops fourcroyi</i> (La Cepède, 1802)	Amazon and Orinoco Basins, and rivers of the Guianas.	Rio Almas, state of Tocantins 12°58'09.58"S	UNT 5973**	1. KP020034 2. KP020053 3. KP020072 4. KP020091
<i>Petitipinnis grunniens</i> (Jardine, 1843)	Amazon, Cuyuni and Essequibo River Basins.	48°32'34.38"W Rio Almas, state of Tocantins 12°58'09.58"S 48°32'34.38"W	UNT 3686** MCP 48371***	1. KP020041 2. KP020060 3. KP020079 4. KP020098
<i>Pachyurus adpersus</i> Steindachner, 1879	South-eastern Atlantic Basin, including the Mucuri, Doce and Paraíba do Sul River Basins.	Poço da Barra, Rio Paraíba do Sul, state of Rio de Janeiro 22°28'49.17"S 43°53'22.78"W	MHNCI 12642* MCP48382* MCP48382***	1. KP020035 2. KP020054 3. KP020073 4. KP020092
<i>Pachyurus bonariensis</i> Steindachner, 1879	Uruguay and Paraguay Basins and lower parts of the Paraná River.	Porto Xavier, Rio Uruguai, state of Rio Grande do Sul 27°53'53.64"S	MCP48378***	1. KP020036 2. KP020055 3. KP020074 4. KP020093
<i>Pachyurus francisci</i> (Cuvier, 1830)	São Francisco River Basin in Brazil.	55°16'06.88"W Rio São Francisco, state of Minas Gerais	MZUSP 014678* MCP48379***	1. KP020037 2. KP020056 3. KP020075 4. KP020094
<i>Pachyurus junki</i> Soares & Casatti 2000;	Amazon River Basin in Brazil.	18°18'39.30"S 45°14'28.35"W Rio Tocantins, Ipuéiras, state of Tocantins 11°14'14.73"S 48°28'13.74"W	UNT 4358** MCP48380***	1. KP020038 2. KP020057 3. KP020076 4. KP020095
<i>Pachyurus squampinnis</i> Agassiz, 1831	São Francisco River Basin in Brazil.	Rio São Francisco, state of Minas Gerais 18°18'39.30"S 45°14'28.35"W	MZUSP 014679 MCP48381***	1. KP020039 2. KP020058 3. KP020077 4. KP020096
<i>Plagioscion squamosissimus</i> (Heckel, 1840)	Amazon and Orinoco River Basins, and rivers of the Guianas.	Rio Tocantins, state of Tocantins 4°11'14.80"S 49°44'27.10"W	MHNCI 12643*	1. KP020042 2. KP020061 3. KP020080 4. KP020099
<i>Plagioscion</i> sp.	Paraná River Basin in Brazil	Rio Paraguay, state of Mato Grosso do Sul 19°35'58.97"S 57°26'54.55"W	–	1. KP020043 2. KP020062 3. KP020081 4. KP020100

Table 1 Continued

Species name	Distribution	Collection locality (coordinates)	Voucher/Reference specimen/tissue sample	GenBank
<i>Cynoscion jamaicensis</i> (Vaillant & Bocourt, 1883)	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12647*	1. KP020028
		26°38'01.75"S	MCP48370***	2. KP020047
		48°40'45.75"W		3. KP020066
				4. KP020085
<i>Isopisthus parvipinnis</i> (Cuvier, 1830)	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12649*	1. KP020029
		26°38'01.75"S	MCP48372***	2. KP020048
		48°40'45.75"W		3. KP020067
				4. KP020086
<i>Paralichthys brasiliensis</i> (Steindachner, 1875)	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12648*	1. KP020040
		26°38'01.75"S	MCP48373***	2. KP020059
		48°40'45.75"W		3. KP020078
				4. KP020097
<i>Micropogonias furnieri</i> (Desmarest, 1823)	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12644*	1. KP020033
		26°38'01.75"S	MCP48374***	2. KP020052
		48°40'45.75"W		3. KP020071
				4. KP020090
<i>Latimus breviceps</i> Cuvier, 1830	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12646*	1. KP020031
		26°38'01.75"S	MCP48375***	2. KP020050
		48°40'45.75"W		3. KP020069
				4. KP020088
<i>Menticirrhus americanus</i> (Linnaeus, 1758)	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12645*	1. KP020032
		26°38'01.75"S	MCP48376***	2. KP020051
		48°40'45.75"W		3. KP020070
				4. KP020089
<i>Stellifer rastrifer</i> (Jordan, 1889)	Atlantic Ocean	Barra Velha, state of Santa Catarina	MHNCI 12650*	1. KP020045
		26°38'01.75"S	MCP48377***	2. KP020064
		48°40'45.75"W		3. KP020083
				4. KP020102
<i>Polydactylus oligodon</i> (Günther, 1860)	Atlantic Ocean	26°38'01.75"S		1. KP020044
		48°40'45.75"W		2. KP020063
		Fish Market of Aracaju, state of Sergipe	MCP48369***	3. KP020082
		10°56'49.71"S		4. KP020101
<i>Conodon</i> sp.	Atlantic Ocean	37°04'22.78"W		1. KP020027
		Fish Market of Aracaju, state of Sergipe	MCP48368***	2. KP020046
		10°56'49.71"S		3. KP020065
		37°04'22.78"W		4. KP020084

1 = 16S; 2 = Cytb; 3 = Rhod; 4 = TMO. Collection acronyms: Museu de História Natural Capão da Imbuia (MHNCI); Laboratório de Ictiologia Sistemática da Universidade Federal do Tocantins (UNIT); Laboratório de Ictiologia of the Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul (MCP). * = voucher; ** = reference specimen; *** = tissue voucher.

cytochrome b, *Tmo-4C4* and *rhodopsin* genes were 16SAr (5' CGCCTGTTT ATCA AAAACAT 3') and 16SBr (5' CCGGTCTGAACTCAGATCACGT 3') (Palumbi *et al.* 1991); L14725L (5' CGAAACTAATGACTTGAAAAAC-CACCGTTG 3') and HMVZ16 (5' AAATAGGAARTATCAYTCTGGTTTRAT 3') (Santos *et al.* 2013); *Tmo-fl-5'* (5' CCTCCGGCC TTCCTAAAACCTCTC 3') and *Tmo-rl-3'* (5' CAT-CGTGCTCCTGGGTGACAAAGT 3') (Streelman & Karl 1997); and RHPco1F (5' CATCGTCC GGAGTCCTTATG 3') and RH1039R (5' TGCTT GTTCATG CAGATGTAGA 3') (Chen *et al.* 2003), respectively. The thermocycling conditions included an initial denaturation period of 95 °C for 4 min, followed by either 30 (16S) or 35 cycles of denaturation at 92–95 °C for 30–40 s, annealing at 50–57 °C for 30–60 s, and extension at 72 °C for 30–90 s, with a final extension period at 72 °C for 5 min.

The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV light. The successfully amplified products were purified using either the MinElute PCR Purification kit (Qiagen Inc., Valencia, CA, USA) or the Montage® PCR Cleanup kit (Milipore, Billerica, MA, USA) following the manufacturer's instructions. Cycle sequencing was conducted with the same primers using Big Dye v. 3 (Applied Biosystems®, Foster City, CA, USA), with purification using Sephadex™ G-50 medium (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), on an ABI3130 sequencer. Both strands of each fragment were sequenced to verify accuracy. The obtained sequences could be unambiguously aligned, except for 61 bp of the 16S gene, which were omitted from the remaining analyses. All sequences were deposited in the GenBank database (Table 1).

The phylogenetic relationships among the studied species were inferred via parsimony and Bayesian inference (BI). The hypothesis based on parsimony was constructed using PAUP* v. 4.0b10 (Swofford 2003). All characters were unordered and equally weighted, and the search was heuristic, employing the tree bisection–reconnection (TBR) algorithm, random addition and 1000 repetitions. Bootstrap value (Bv) for the parsimony criterion was calculated for 1000 repetitions. Bayesian inference was implemented in BEST v. 2.3 (Liu 2008) and BEAST (v. 1.7.2; Drummond *et al.* 2012). The species used in the analyses are presented in Table 1. The species of marine Sciaenidae included in the analysis are all neotropical and include putative sister groups to both Sciaenidae MDLs analysed (Chao 1978; Sasaki 1989; Boeger *et al.* 2003; Santos *et al.* 2013), among others. Marine Sciaenidae from the Old World were not included in the analysis since, according to Xu *et al.* (2014), these lineages originated elsewhere, long before

(about 120 Ma) the approximate date of colonisation of the freshwater habitats in South America (Oligoce/Miocene). The outgroups consisted of one species each from Polynemidae (*Polydactylus oligodon* Günther, 1860) and Haemulidae (*Conodon* sp.), as suggested by Johnson (1993), Chao (1978) and Betancur *et al.* (2013), thus representing phylogenetically distant and closely related families to Sciaenidae.

The applied nucleotide substitution models were those suggested by the BIC criterion in MRMODELTEST (v. 2.3 in <http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html>), which resulted in TIM2+G for the *TMO* gene, HKY+I+G for *rhodopsin*, GTR+I+G for *cytochrome b* and TIM2+G for 16S. The analyses were performed in BEST for 30 000 000 generations, with the sampling frequency set at 10 000. Burn-in was set at 30 000, so that 1000 trees were accepted for each run after the likelihood values had plateaued. In BEAST, the number of generations was set at 200 million, with a sampling frequency of 10 000 and a burn-in of 200 000. The resulting trees were summarised using TreeAnnotator in the BEAST package.

The divergence times of the Sciaenidae lineages were assessed through concatenated alignment and BI within BEAST 1.7 (Drummond *et al.* 2012) using the uncorrelated relaxed clock (Drummond *et al.* 2006). Time calibration of the reconstruction was based on the oldest fossil records observed for each clade of Neotropical Sciaenidae. The distribution of the priors for each respective TMRCA (time to the most recent common ancestor) was defined as log-normal (offset = 0.75; prior mean = 1.0) in the following manner: for the clade composed of *Plagioscion* species, the oldest available fossil dates from approximately 14 Ma (Monsch 1998), and the minimum age of Pachyurinae was determined to be 21 Ma based on the dating of a fossil identified as *Pachypops furcroid* (Monsch 1998). The MCMC analysis was set at 60 000 000 generations, with sampling every 6000 generations and a burn-in of 10%. The ESS was examined in TRACER 1.6. All analyses were performed independently three times for validation.

Because of the absence of more comprehensive experimental data evaluating the tolerance of marine fish lineages to freshwater, the percentage of marine species that can withstand reduced salinity (i.e. species that inhabit brackish water environments) among all marine, brackish and anadromous species was considered as a measure of the inherited euryhalinity of each fish family included in the analysis. The number of species associated with each type of environmental salinity was obtained from FishBase (Froese & Pauly 2014), aided by the *rfishbase* package (Boettiger *et al.* 2012). The percentage of brackish-to-marine species and the percentage of freshwater species among the total species in each family closely related to

the Sciaenidae, according to Betancur *et al.* (2013), were optimised onto a portion of the phylogenetic hypothesis of Betancur *et al.* (2013) to trace the history of this character using the parsimony criterion within Mesquite (Maddison & Maddison 2011).

Results

Nineteen taxa were sequenced (Table 1): nine freshwater sciaenids, eight marine sciaenids and two outgroups, which resulted in a set of four concatenated sequences of 2562 bp, among which 514 were parsimony informative. The following fragment lengths were observed: 511 bp for *Tmo-4C4*, 670 bp for *rhodopsin*, 740 bp for *cytochrome b* and 641 bp for *16S rRNA*.

The analyses performed with both the parsimony (Fig. 1) and BI (Fig. 2) criteria indicated a monophyletic clade composed of all freshwater Sciaenidae, with high bootstrap (Bv) and posterior probability (PP) values (94 and 100%, respectively). The parsimony analysis resulted in two equally parsimonious trees (EPTs) [tree length = 2068; consistency index (CI) = 0.52; retention index (RI) = 0.45]. The phylogenetic relationships of marine sciaenids recovered from the parsimony analysis were inconsistent between the EPTs, resulting in mostly unresolved relationships with low bootstrap values (Fig. 1). The Bayesian analyses conducted using both software programs

(BEST and BEAST) were considered adequate and resulted in estimated sample sizes (ESS) above 2000.

In all phylogenetic hypotheses, *Plagioscion* spp. and Pachyurinae were reciprocally monophyletic sister groups with prevalent high bootstrap values (Bv = 4 and 60%, respectively) and posterior probability (PP = 100% for both clades) (Figs 1 and 2). All subordinate branches also presented equally high Bv and PP values.

Both analyses recovered two clades within Pachyurinae (Figs 1 and 2). One was composed of *Pa. adspersus*, *Pa. squamipennis* and *Pa. francisci*, with *Pa. adspersus* as the basal species. The other clade was composed of *Pe. grunniens*, *Pa. junki*, *Pa. bonariensis* and *Pp. fourcroi*, with *Pe. grunniens* as the basal species.

According to the BI hypothesis (Fig. 2), the sister species (among those included in the analysis) of the neotropical freshwater Sciaenidae is *Pl. brasiliensis*, with fairly high PP (90%) (Fig. 2). *Micropogonias furnieri*, *C. jamaicensis* and the two species of *Isopisthus* constitute a monophyletic group (PP = 100%) that is basal to *Pl. brasiliensis* and the freshwater species (PP = 96%).

Molecular dating indicated that the marine-to-freshwater transition occurred between 30.58 Ma, when the ancestor of the neotropical freshwater species of Sciaenidae and its putative sister group (*Pl. brasiliensis*) diverged, and 25.35 Ma, when the basal lineages of neotropical freshwater

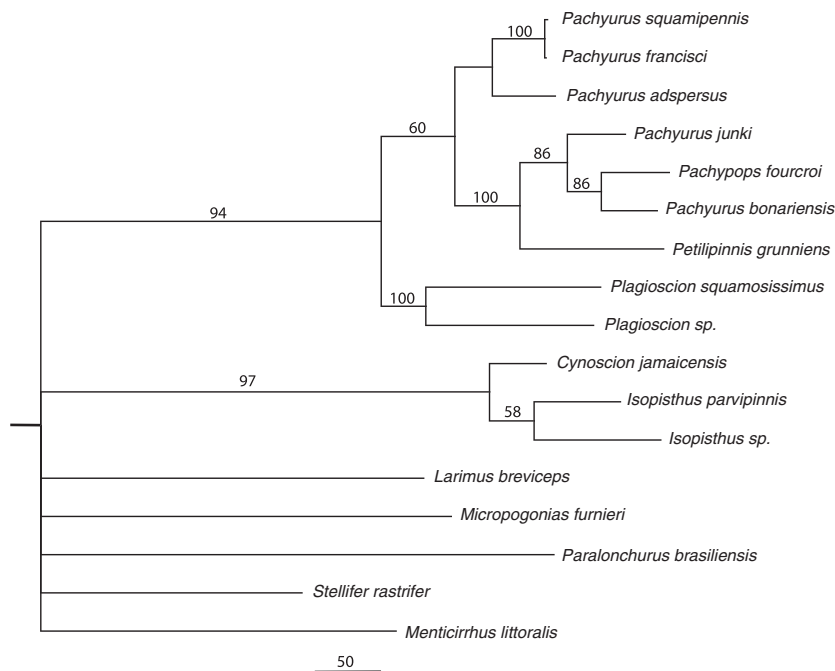


Fig. 1 A strict consensus cladogram of two equally parsimonious trees depicting the phylogenetic relationships of the freshwater neotropical Sciaenidae and selected marine species. The bootstrap value is presented above the branches. Branches with bootstrap values lower than 50% were collapsed.

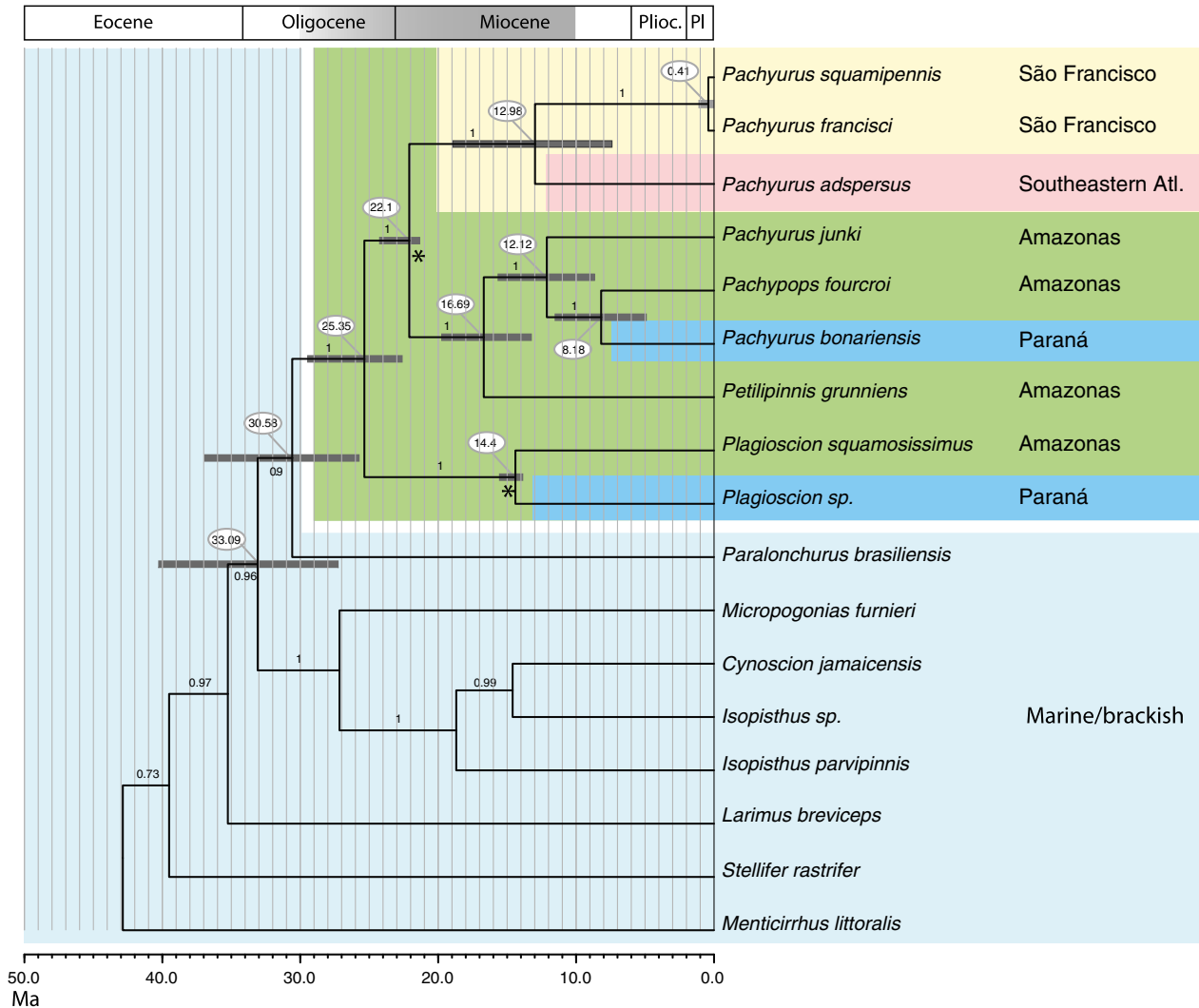


Fig. 2 Ultrametric tree constructed with BEAST depicting the predicted mean divergence dates for neotropical freshwater Sciaenidae (within callouts) and the respective 95% HPD (bars). The posterior probability values are presented above each branch. Asteriscus indicate dates of fossil records used in calibration. The reconstruction of the ancestral areas of each clade is indicated by the colour and the name of the river basin to the right of the taxon names.

Sciaenidae first diversified. The initial diversification of the Pachyurinae was dated to 22.1 Ma (24.25–21.34 Ma), whereas the divergence of *Plagioscion sp.* and *Pg. squamosissimus* was dated to 14.4 Ma (15.55–13.87 Ma).

According to FishBase (Froese & Pauly 2014), the percentage of species of extant marine Sciaenidae that can withstand brackish water conditions (approximately 47%) is significantly greater than that calculated for the combined marine Actinopterygii (approximately 18%) (Fig. 3, Table S1). Among the families/clades most closely related to Sciaenidae, the optimisation of the percentage of brackish species among the total number of species associated with saline environments (except the exclusively

freshwater+freshwater and brackish species) indicated a high percentage of euryhaline species (Fig. 3). However, among these closely related families, only three include species that colonised freshwater habitats: Moronidae, Tetraodontidae (representing the Tetraodontiformes) and Sciaenidae. In summary, all of the families with representatives in freshwater environments presented elevated euryhalinity (>47%).

Discussion

The monophyly of the neotropical freshwater Sciaenidae, represented by Pachyurinae and *Plagioscion* species, is strongly supported by the phylogenetic hypothesis resulting

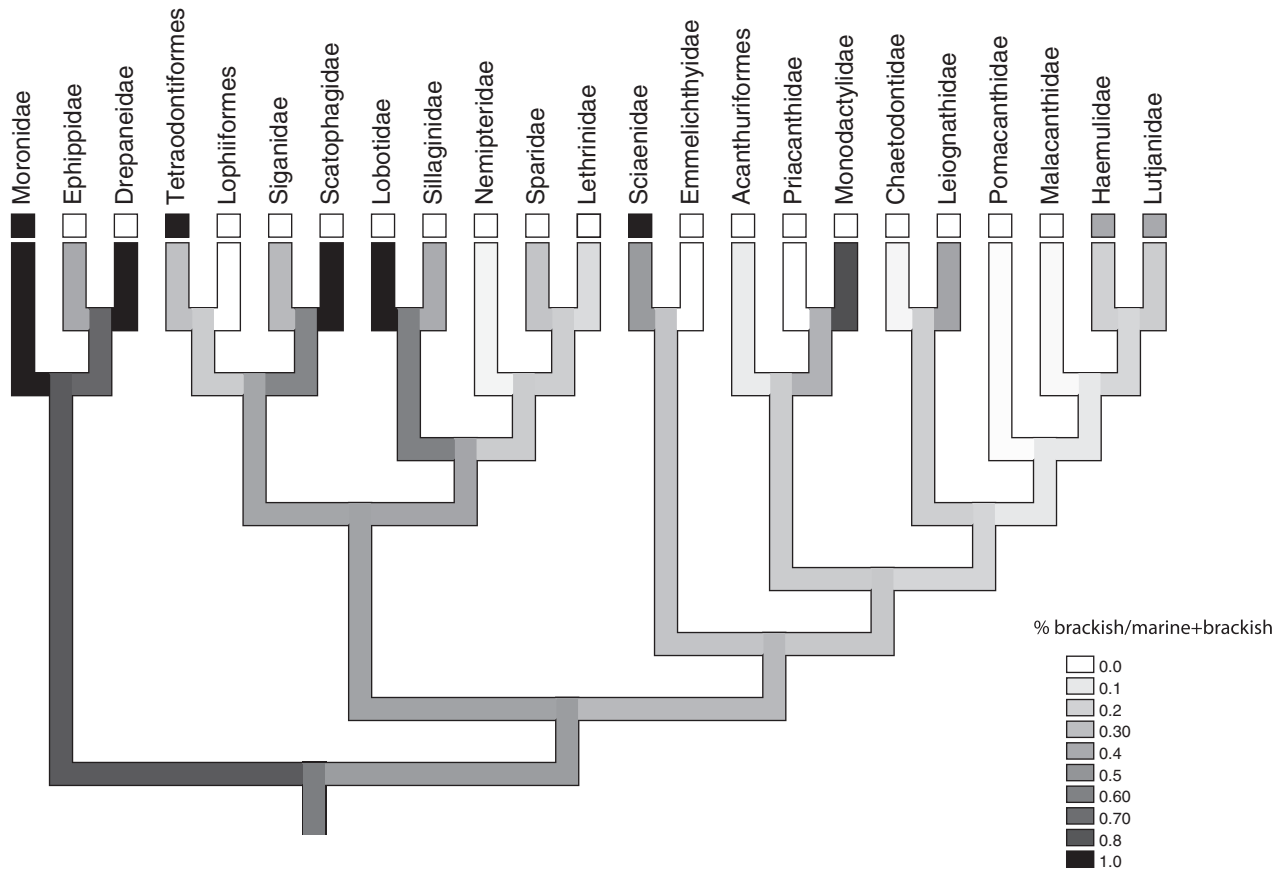


Fig. 3 Parsimony reconstruction (least squares) of the percentage of marine species that are also observed in global estuarine environments for distinct families closely related to Sciaenidae according to the phylogenetic hypothesis of Betancur *et al.* (2013) as a measure of euryhalinity. The black square below the family name denotes families with exclusively freshwater species. The white square denotes families that do not have exclusively freshwater representatives. The grey squares denote families with brackish/freshwater representatives.

from all criteria examined. All of the remaining sciaenids in the analysis are marine species, and optimisation of the environment type onto the phylogeny (Fig. 2) indicates that the freshwater lineage was secondarily derived. These results are fully compatible with the hypothesis of a single colonisation of the freshwater habitats of South America from the marine environment and contradict hypotheses of an independent origin for the freshwater neotropical Sciaenidae (Casatti 2000; Boeger & Kritsky 2003; Santos *et al.* 2013). The most recent proposal (Santos *et al.* 2013) supporting the previous hypothesis of independent freshwater habitats colonisation by the Sciaenidae MDLs is based on branches presenting lower bootstrap values and posterior probabilities, which most likely reflect the inadequacy of the combination of fragments used in the reconstruction.

The hypothesis that neotropical freshwater habitat was colonised by sciaenids in the northern region of South America, as suggested by Boeger & Kritsky (2003), is supported by the results of this study. Optimisation of

hydrological basins onto the present phylogeny (Fig. 2) and that of Cooke *et al.* (2012) results in the Amazon Basin as the ancestral distribution area for the basal lineage of the sciaenid MDL.

With high values of PP, the sister group relationships recovered from the BI suggest that species of *Paralonchurus* are the closest relatives of the neotropical Sciaenidae MDL. An identical sister relationship is suggested by Boeger & Kritsky (2003) based solely on parasitic Monogenea (Platyhelminthes). This result confirms the expected pattern for MDLs that colonised South America from the north and putatively preceded the elevation of the Isthmus of Panamá during the Pleistocene (Lovejoy *et al.* 2006). These MDLs are sister groups of the extant amphio-American lineages (Brooks *et al.*, 1981; Hoberg *et al.* 1998; Lovejoy *et al.* 2006).

Molecular dating indicates that the transition of the ancestral freshwater sciaenid lineage (*Pachyurinae* + *Plagioscion*) into freshwater occurred sometime between 30.58 and

25.35 Ma. This timing is older than that previously proposed based on fossils, parasites and/or molecular dating (Boeger & Kritsky 2003; Cooke *et al.* 2012). Considering the corresponding lower and upper HPD for the above limits, the transition could have occurred between 36.95 and 22.61 Ma. Thus, the putative origin of freshwater drums in South America most likely dates to the Oligocene, thus preceding the extensive marine transgressions that occurred during the Miocene (Lundberg *et al.* 1998). However, minor marine transgressions also occurred between 30 and 20 Ma (late Oligocene–early Miocene) in the region of Mar del Plata, the eastern Amazon, Patagonia and the eastern Orinoco (Lundberg *et al.* 1998), and prior to this period, from 43 to 30 Ma, a large lake-like estuarine environment with variable salinity reached the region of present day Peru and Ecuador (Lundberg *et al.* 1998).

Some researchers (e.g. Lovejoy *et al.* 2006; Cooke *et al.* 2012) assume that extensive estuarine-like environments allowed a gradual ‘adaptation’ of the ancestors of many MDLs to freshwater and facilitated the slow invasion of continental freshwater habitat. Although this assumption may be potentially realistic for some lineages, for Sciaenidae and other MDLs, such as needlefishes (Lovejoy *et al.* 2001), the scenario may be reconstructed differently.

The Sciaenidae are mostly associated with coastal environments and are abundant in estuaries, particularly in large rivers throughout the world (Sasaki 1989). There are approximately 17 species of Sciaenidae endemic to freshwater worldwide. In addition to the South American MDL, Sciaenidae are also present in North America, represented by a single species, *Aplodinotus grunniens* Rafinesque, 1819, while *Boesemania microlepis* (Bleeker, 1858) is reported from both freshwater and brackish habitats in Asia. The optimisation of the relative number of brackish species on the cladogram of Betancur *et al.* (2013) (Fig. 3) indicates that although several closely related families present similar or higher percentages of euryhaline species compared with Sciaenidae, only the relatively distant Moronidae and Tetraodontidae (representing the Tetraodontiformes) include endemic freshwater species on distinct continents. The lack of endemic freshwater species in lineages with high apomorphic or plesiomorphic high euryhalinity most likely reflects the absence of historical opportunities to colonise freshwater. Correspondingly, other marine clades that were simultaneously subjected to ‘facilitating’ or landlock events (Schultz & McCormick 2013), such as the marine transgressions in South America (Monsch 1998), did not successfully colonise freshwater continental habitats. If the opportunities were similar, the difference in the success of freshwater colonisation must reside in the inherent ability of sciaenids and other MDLs to withstand the challenges of the transition (as opposed to gradually adapt to new conditions).

Accordingly, Xu *et al.* (2014) recently presented supporting evidence for Sasaki’s (1989) hypothesis that Sciaenidae originated in marine habitats of the Americas. Their molecular clock indicates that the family originated in the early Cretaceous (145–113 Ma). During this period, variations in the levels of the oceans and in the tectonics of large mountain ranges, such as the Andes (Lundberg *et al.* 1998), promoted the formation of extensive seaways during cyclic events of transgressions and regressions (Lundberg *et al.* 1998). This palaeohydrological scenario was most likely accompanied by the formation of many estuarine areas, with numerous hydrological basins discharging freshwater into these internal seaways (Lundberg *et al.* 1998).

Under this setting, the ancestor and/or early lineages of Sciaenidae, were expectedly exposed to selective pressures associated with variable environmental salinity. The reconstruction of ancestral character states onto the phylogeny of Betancur *et al.* (2013) also supports this scenario (Fig. 3). The increasing euryhalinity in Sciaenidae is apomorphic, thus supporting the acquisition of this ability within the family.

This ancestral ability to withstand low habitat salinity was most likely retained by all or many of the descendant species – at least by the ancestors of the Sciaenidae MDLs. Lovejoy *et al.* (2001) suggested a similar scenario for freshwater needlefish and Potamotrygonidae rays. This tolerance to variable salinity may have predisposed these ancestral Sciaenidae to withstand extensive salinity changes, even when landlocked during cycles of marine transgressions.

This scenario is consistent with the concept of ecological fitting as proposed by Janzen (1985) and subsequently expanded by Brooks & McLennan (2002). According to Agosta & Klemens (2008), ‘ecological fitting is the process whereby organisms colonise and persist in novel environments, use novel resources or form novel associations with other species as a result of the suites of traits that they carry at the time they encounter the novel condition’. Phylogenetic conservatism is one of the proposed mechanisms underlying ecological fitting (Agosta & Klemens 2008).

The retention of ancestral abilities (i.e. those abilities that may have evolved under the same ancestral selective pressure, in this case, the ability to withstand low salinity) is a process that is observable in both experimental (Otwiński & Nemenman 2013; Leiby & Marx 2014) and deducible studies. For instance, species of Cichlidae mainly inhabit freshwater habitats and their present distribution, particularly on continents in the Southern Hemisphere, supports the hypothesis that their ancestors dispersed via marine environments to their present day distribution areas (see review in Pariselle *et al.* 2011). Molecular dating and fossil records (Friedman *et al.* 2013) support this scenario. The ability of freshwater Cichlidae species to withstand

long exposure to marine salinity most likely represents the retention of an ancestral aptitude (Suresh & Lin 1992; Freire *et al.* 2008) and is widespread within the family (Langston *et al.* 2010).

We suggest that this general scenario also explains why not all fish clades putatively subjected to identical processes of transgressions and regressions of the ocean became MDLs in South America. There is fossil evidence indicating that a diverse marine fauna was present in the marine/estuarine habitats associated with marine transgressions (Monsch 1998). Thus, it is apparent that it was mainly (or only) lineages that already presented tolerance to freshwater that were able to survive the process of landlock or the rapid transition from marine to freshwater. Further testing of this hypothesis for MDLs present in all regions of the world would provide additional testing of the concept of ecological fitting by phylogenetic conservatism (Agosta & Klemens 2008).

Geographical expansion followed the introduction of Sciaenidae to the freshwater habitat of South America. Although limited in the number of representative species considered, the resulting phylogenetic analysis and distributional data provided insights into the expansion and diversification of the clade on the continent (Fig. 2). At approximately 25 Ma, the clades represented by Pachyurinae and *Plagioscion* spp. diverged, expanded their distribution and diversified. While the natural distribution of *Plagioscion* spp. includes the Magdalena, Amazon, Orinoco and Paraná basins and the rivers of Guyana, pachyurines are present in all of these regions, except in the Magdalena Basin. However, species of Pachyurinae also occur in the rivers Uruguay and Paraguay (lower and upper Paraná Basin, respectively) and the Rio São Francisco and south-eastern Atlantic basins (specifically the Rio Doce, Rio Mucuri and Rio Paraíba do Sul). Possible explanations for these distinct distributional patterns of the two clades were explored by Boeger & Kritsky (2003), but the present results provide an additional opportunity to evaluate alternative hypotheses based on a more comprehensive phylogenetic hypothesis.

For instance, Boeger & Kritsky (2003) accept the non-monophyly of the freshwater Sciaenidae and suggest that the difference in the geographic distributions of Pachyurinae and *Plagioscion* could be explained by differences in the timing of expansion and diversification. According to these authors, Pachyurinae could have reached the small coastal south-eastern Atlantic basin through the putative connection with the Paraná Basin that closed approximately 20 Ma (Malabarba 1998). While the first diversification event detected for Pachyurinae has been dated to approximately 19 Ma (Cooke *et al.* 2012) and 22 Ma (this study), the initial diversification of *Plagioscion* occurred at a later date, estimated at approximately 17.8 (Cooke *et al.* 2012)

and 14.4 Ma (this study). Thus, the available evidence supports the earlier diversification of Pachyurinae, as suggested by Boeger & Kritsky (2003). However, because of differences in the phylogenetic relationships recovered herein compared with those presented by Boeger & Kritsky (2003), asynchronous diversification of the clades no longer represents a reasonable explanation for the disparity in the geographical distributions of the two lineages of freshwater Sciaenidae in South America. The present phylogenetic analysis indicates that the Pachyurinae found in the south-eastern Atlantic basins (e.g. Rio Paraíba do Sul) are most closely related to species occurring in the São Francisco Basin, and the hypothesis of Boeger & Kritsky (2003) is therefore rejected in this work.

The reconstruction of the ancestral distribution onto the phylogeny (Fig. 2) suggests independent colonisation of Pachyurinae into the Paraná River, São Francisco and south-eastern Atlantic basins from Amazonian lineages. This pattern of biogeographical distribution is compatible with the area cladogram for fishes in tropical South America proposed by Albert & Carvalho (2011), with basal lineages in the Amazon Basin, followed by independent colonisations of the Rio São Francisco, south-eastern Atlantic (but see Discussion below) and Rio Paraná basins.

Pachyrus francisci and *Pa. squamipennis* are endemic sister species in the São Francisco Basin. The divergence of these two species putatively occurred recently within this basin, at approximately 0.41 Ma (Fig. 2). Although the fish fauna of the São Francisco Basin present many other pairs of congeneric species, most do not represent lineages that originated within the basin but rather distinct lineages of the same genus that colonised this river system independently, for example *Brycon* spp. (Abe *et al.* 2014) and *Acestrorhynchus* spp. (Toledo Piza 2007). However, it is unclear whether the divergence of *Pa. squamipennis* and *Pa. francisci* was associated with sympatric or allopatric speciation within the basin followed by secondary contact.

The phylogenetic proximity of the sciaenid clade from the São Francisco Basin to *Pa. adpersus* from the Paraíba do Sul and other south-eastern Atlantic basins (Rio Doce and Rio Mucuri) reveals the common history of part of the ichthyofauna of these river systems. The separation of these lineages was calculated to have occurred approximately 13 Ma (Fig. 2), but their ancestral distribution and scenario of diversification are not evident. Ribeiro (2006) suggested that these basins have been repeatedly connected over evolutionary time, as indicated by the occurrence of several sister groups between the coastal drainage and adjacent upland crystalline shield rivers. Considering the putative original distribution of the ancestor to the pachyurines (Fig. 2), we postulate that the São Francisco Basin was colonised by an Amazonian lineage of Sciaenidae, based simply on the

proximity of these two basins. The hypothesised period of the colonisation of the coastal Atlantic rivers of the south-eastern Atlantic basins (with its respective 95% HPD) by a lineage derived from the São Francisco Basin overlaps with reports of several headwater capture events of other fish groups occurring between the systems of the Rio Tietê, Rio Paraíba do Sul, Rio São Francisco and Rio Ribeira do Iguaçu between 15 and 28 Ma (Ribeiro 2006). Regardless of which coastal river basin was initially colonised, the spread of *Pa. adspersus* among the south-eastern Atlantic basins was likely associated with dispersion through wetland and delta connections during marine regressions (Albert & Reis, 2011), as has been observed in several other fish groups (Pereira *et al.* 2012; Roxo *et al.* 2012).

In addition to Sciaenidae, other fish groups suggest similar biogeographic relationships between the Amazon and São Francisco basins. For instance, two species of piranhas from the São Francisco are considered to represent introductions from the Amazon Basin with subsequent speciation [*Pygocentrus piraya* (Cuvier, 1819) and *Serrasamus brandtii* Lütken, 1875 (Hubert *et al.* 2007)]. The calculated dates of the colonisation events of the São Francisco system by ancestors of these species (Hubert *et al.* 2007) are younger (approximately 2.63 Ma for *Py. piraya* and 7.4 Ma for *S. brandtii*) than those calculated for the clade of Pachyurinae (approximately 22.1–12.93 Ma on average). These differences in the dates of the introductions of the different fish lineages to the São Francisco Basin appear to reflect multiple or continuous opportunities for colonisation by fishes from the neighbouring Amazon Basin, particularly during the Miocene and Pliocene.

Eastern tributaries of the Tocantins River and western tributaries of the São Francisco River (the largest tributaries of this basin) originate at higher elevations on the Central Brazilian Plateau (Lima & Caires 2011). This region is highly erosional, which most likely facilitates transfer between these two river systems either via headwater capture events or simply through dispersion using shared permanent or temporary wetlands. The proximity of the headwaters of the tributaries of the Tocantins and São Francisco rivers is notable. In some cases, the tributaries are located <2 km from one another. Periods of heavy pluviosity, common during the Miocene–Pliocene (Albert & Reis, 2011), most likely facilitated the exchange of fish fauna. The region known as the ‘águas emendadas’ (connected waters) of the Sapão (São Francisco River Basin) and Galheiros Rivers (Tocantins River Basin) reflects the proximity of their respective headwaters and is considered to be a connection that allows limited exchange between the ichthyofauna of the two basins (Lima & Caires 2011).

The origin of *Pa. bonariensis*, a member of a clade composed of mostly Amazonian species (*Pe. grunniens*,

Pp. fourcroy and *Pa. junki*), strongly supports the putative north-to-south expansion of the western lineage, possibly associated with the northward shift of the boundary between the Amazon and Paraná systems at approximately 11.8–10 Ma in the late Miocene (Lundberg *et al.* 1998). Thus, through headwaters captured from the Palaeo-Amazon–Orinoco, the Paraná system incorporated a number of Amazonian fish fauna (Lundberg *et al.* 1998) by vicariance. The only extant native representatives of the Sciaenidae found in the Paraná River Basin are *Pl. tenertzi* and *Pa. bonariensis*.

Concluding, although we can associate some speciation events observed for the Sciaenidae MDL with vicariance, much diversification could also be easily explained by geographic expansion followed by isolation and, perhaps, by sympatric speciation in at least one case (i.e. *Pa. squamipennis* and *Pa. francisci*).

Finally, the taxonomic status of Pachyurinae has been marked by constant transfers among genera. For instance, *Pp. fourcroy* was initially proposed as *Pa. nattereri* Steindachner, 1863, and *Pa. adspersus* was described as *Pp. adspersus* Steindachner, 1879 (Casatti 2002). The phylogenetic relationships among the studied lineages indicated that two Pachyurinae genera – *Petilipinnis* and *Pachypops* – should be re-evaluated. The species presently allocated within these two genera will most likely be transferred to *Pachyurus* if a natural classification is desired. In particular, according to the phylogenetic relationships recovered in the present study, *Petilipinnis*, recently proposed by Casatti (2001), is most likely a member of *Pachyurus*. The single diagnostic character of this genus is the presence of one spine on the anal fin instead of the two spines observed in *Pachypops* and *Pachyurus* species. This character could easily represent an autapomorphy for the single species from *Petilipinnis* and be of minimal value as support for a new genus.

Acknowledgements

Yoshimi Sato, Osvaldo Takeshi Oyakawa, Paulo de Tarso Chaves, Jorge Dergan and Elineide Marques provided support with identification of fishes. Jorge Dergan, Elineide Marques, Marília Carvalho Brasil-Sato, Raphael O. Ribeiro, Marcus Vinicius Domingues, Karin H. Fehlauer and Diogo Hungria helped with the collection of freshwater Sciaenidae. Nathan Lovejoy, Márcio Pie and W-J Chen provided assistance in laboratorial procedures. WAB is fellow of the Conselho Nacional de Pesquisas e Desenvolvimento Científico e Tecnológico (CNPq). This study was funded by CNPq.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Number of species for families closely related to Sciaenidae (according to Betancur *et al.* 2013) occurring in habitat presenting distinct salinities and the ratio between brackish species to marine and brackish species as a measure of euryhalinity.