ORIGINAL ARTICLE

Marine incursions, cryptic species and ecological diversification in Amazonia: the biogeographic history of the croaker genus *Plagioscion* (Sciaenidae)

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ABSTRACT

Aim We propose a phylogenetic hypothesis for the marine-derived sciaenid genus *Plagioscion* in the context of geomorphology and adaptation to freshwaters of South America, and assess the extent to which contemporary freshwater hydrochemical gradients influence diversification within a widely distributed *Plagioscion* species, *Plagioscion squamosissimus*.

Location Amazon Basin and South America.

Methods Using nuclear and mitochondrial DNA sequence data, phylogenetic analyses were conducted on the five nominal *Plagioscion* species, together with representatives from *Pachyurus* and *Pachypops*, using character and model-based methods. Genealogical relationships and population genetic structure of 152 *P. squamosissimus* specimens sampled from the five major rivers and three hydrochemical settings/'colours' (i.e. white, black and clear water) of the Amazon Basin were assessed.

Results Phylogenetic analyses support the monophyly of *Plagioscion* in South America and identify two putative cryptic species of *Plagioscion*. Divergence estimates suggest that the *Plagioscion* ancestor invaded South America via a northern route during the late Oligocene to early Miocene. Within *P. squamosissimus* a strong association of haplotype and water colour was observed, together with significant population structure detected between water colours.

Main conclusions Our analyses of *Plagioscion* are consistent with a biogeographic scenario of early Miocene marine incursions into South America. Based on our phylogenetic results, the fossil record, geomorphological history and distributional data of extant *Plagioscion* species, we propose that marine incursions into western Venezuela between the late Oligocene and early Miocene were responsible for the adaptation to freshwaters in *Plagioscion* species. Following the termination of the marine incursions during the late Miocene and the establishment of the modern Amazon River, *Plagioscion* experienced a rapid diversification. *Plagioscion squamosissimus* arose during that time. The formation of the Amazon River probably facilitated population and range expansions for this species. Further, the large-scale hydrochemical gradients within the Amazon Basin appear to be acting as ecological barriers maintaining population discontinuities in *P. squamosissimus* even in the face of gene flow. Our results highlight the importance of divergent natural selection through time in the generation and maintenance of sciaenid diversity in Amazonia.

Keywords

Amazon Basin, divergent natural selection, ecological gradients, marine-derived lineage, phylogeography, *Plagioscion squamosissimus*, South America.

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INTRODUCTION

The Amazon drainage basin is home to the world's richest freshwater fish fauna. Like other tropical systems, the biodiversity of Amazonia has been largely attributed to palaeogeographic and climatic events that occurred during the Tertiary (Moritz *et al.*, 2000; Rull, 2008). Indeed, the majority of Neotropical fishes have been physiologically constrained to freshwater habitats and appear to have a long history of evolution dating back to the final separation of the South American and African continents (e.g. Siluriformes and Characiformes) (Lundberg *et al.*, 1998, 2010; Reis *et al.*, 2003). However, South America also accommodates several species and small clades of freshwater fishes that evolved from predominantly marine lineages. In these cases, the role of ecologically based divergent natural selection across salinity gradients probably facilitated the physiological changes necessary for adaptation to freshwater. Based on taxonomic, phylogenetic, biogeographic and fossil data, it is generally thought that these lineages colonized South America relatively recently, as a product of marine incursions that entered the Amazon during the Oligocene and Miocene (Hoorn, 1993; Webb, 1995; Lovejoy *et al.*, 1998, 2006; Lundberg *et al.*, 1998; Monsch, 1998; Boeger & Kritsky, 2003; Wesselingh & Macsotay, 2006). Marine incursions produced numerous estuarine environments of varying salinity and in concert with significant geological changes may have favoured ecological shifts from saltwater to freshwater. Further, based on fossil data and biogeographic patterns exhibited between marine-derived fish species, it has become apparent that the process of adaptation to Neotropical freshwaters has been repeated many times in parallel by taxonomically diverse species (Lovejoy *et al.*, 2006). Biogeographic reconstructions of invasions by marine-derived lineages can advance our knowledge of spatial and temporal aspects of fish adaptation and speciation and, more broadly, of the biotic consequences of past sea level changes (Lovejoy *et al.*, 1998, 2006; Beheregaray *et al.*, 2002; McCairns & Bernatchez, 2010; Bloom & Lovejoy, 2011).

The Sciaenidae comprises approximately 78 genera and 287 species of fish distributed throughout the warm coastal and estuarine waters of the Atlantic, Indian and Pacific Oceans (Chao, 1978; Casatti & Chao, 2002). In South America, there are some Sciaenidae species that have adapted to freshwaters and become physiologically restricted and speciated in this new environment. These include members of *Plagioscion*, *Pachyurus*, *Petilipinnis* and *Pachypops*. Current phylogenetic hypotheses based on fossil, distributional and palaeohydrological data (Sasaki, 1989; Monsch, 1998; Boeger & Kritsky, 2003) advocate secondary colonization of sciaenids into the continent from marine derivatives during the marine incursions of the Oligocene and Miocene. Further, these data also suggest independent adaptation and colonization events of *Pachyurus* + *Pachypops* and *Plagioscion* clades, respectively, with the *Plagioscion* colonizing later (Sasaki, 1989; Monsch, 1998; Boeger & Kritsky, 2003).

Plagioscion Gill, 1861 is a genus of particular importance to subsistence and commercial fisheries of the Amazon Basin (Santos *et al.*, 1984; Casatti, 2005). Further, it is also a biogeographic indicator of key events relating to the marine incursions into South America. According to Casatti (2005) there are five valid nominal species assigned to this genus (*Plagioscion squamosissimus*, *Plagioscion auratus*, *Plagioscion magdalenae*, *Plagioscion ternetzi* and *Plagioscion montei*). While the phylogenetic placement of *Plagioscion* is unclear (Sasaki, 1989; Casatti, 2005), Boeger & Kritsky (2003) suggest a western Atlantic/eastern Pacific sister genus *Paralonchurus* based on a phylogeny of dactylogyrid parasites of the genus *Euryhaliotrema*. The geographic association of this sister relationship is consistent with the hypothesis that *Plagioscion* evolved freshwater tolerance as a product of marine incursions into northern South America. Indeed, fossil evidence suggests that the ancestral *Plagioscion* species colonized the freshwaters of South America sometime between the early Oligocene and middle Miocene when western Venezuela was predominantly an estuarine bay-like environment (Lundberg, 1998; Monsch, 1998). During that time, a Palaeo-Amazon/Orinoco Basin flowed northward, contributing freshwater into Venezuela, and enabling a major pathway south for marine transgressions into South America (Lundberg, 1998). Today, native *Plagioscion* species occur throughout the Magdalena, Amazon, Orinoco and lower Paraná basins, as well as in rivers of the Guianas, while some have also been introduced to the upper Paraná and São Francisco river basins (Casatti, 2003, 2005).

This study has two main objectives. First, to propose a phylogenetic hypothesis for *Plagioscion* that includes all valid species of the genus. Using this phylogeny we aim to date species origins and assess their biogeographic history in the context of major geological and eustatic events that may have facilitated their adaptive evolution from marine to freshwater habitats. Our second objective is to clarify the intra-specific phylogeographic history of the most widely distributed and commercially important *Plagioscion* species, *P. squamosissimus*. In particular, our aim was to assess the influence of freshwater hydrochemistry on the population structure of *P. squamosissimus* within the Amazon Basin. In the event that population genetic structure is associated with hydrochemisty after controlling for other factors (e.g. riverine distance and geomorphology), ecologically based divergent natural selection may well be a persistent force in the generation of Sciaenidae diversity. Indeed, there is now a mounting body of evidence suggesting that divergent natural selection and ecological speciation might be an important biodiversity driver across multiple taxa and environments (reviewed in Schluter, 2000; Coyne & Orr, 2004; Rundle & Nosil, 2005; Schluter & Conte, 2009), including the tropics (Smith *et al.*, 1997, 2001; Schneider *et al.*, 1999; García-París *et al.*, 2000; Ogden & Thorpe, 2002; López-Fernández *et al.*, 2010).

The Amazon Basin actually provides an ideal setting in which to test for divergent natural selection in *P. squamosissimus* as a result of hydrochemistry. Within the Amazon Basin, there are marked hydrochemical and ecological gradients that

are known to impose physiological constraints upon its aquatic communities (Junk *et al.*, 1983; Henderson & Crampton, 1997; Rodríguez & Lewis, 1997; Saint-Paul *et al.*, 2000; Petry *et al.*, 2003). Differentiated largely by sediment composition, geochemistry and optical characteristics, these aquatic conditions have been divided into three water types or 'colours' (Sioli, 1984): (1) white water which has an Andean origin is turbid in nature and characterized by large amounts of dissolved solids; (2) clear water, which is comparatively transparent, contains a low content of dissolved solids; and (3) black water, which is transparent yet stained by tannins and humic acids leached from vegetation, and which differs most dramatically from clear water by its low pH. Unlike other species of *Plagioscion*, *P. squamosissimus* can be found abundantly in all three water colours across river systems, making it an ideal candidate with which to test for population structure driven by hydrochemistry. Furthermore, research has shown that, despite showing high larval dispersal from spawning sites, *P. squamosissimus* larvae are actually quite sensitive to hydrochemical variables such as temperature, pH and dissolved oxygen (Bialetzki *et al.*, 2004).

Here, using data from both nuclear and mitochondrial genes we reconstruct the phylogenetic relationships of the genus *Plagioscion* and propose a biogeographic scenario for the invasion of freshwaters consistent with the marine incursions of the early Miocene. We also show evidence for the contribution of freshwater hydrochemistry to genetic structure in Amazonian populations of *P. squamosissimus*. In this way, we highlight the importance of divergent natural selection through time in the maintenance and generation of Sciaenidae diversity in the Neotropics.

MATERIALS AND METHODS

Sampling

For phylogenetic analysis, tissue samples from the five nominal *Plagioscion* species (*P. squamosissimus, P. auratus, P. temetzi, P. magdalenae* and *P. montei*) and three representatives of *Pachyurus* and *Pachypops* (*Pachyurus junki, Pachypops fourcroi* and a currently undescribed *Pachypops* 'Tapajós' species that is morphologically similar to *P. fourcroi* but with a gas bladder more typical of *Pachyurus* species) were collected in the field or obtained from commercially harvested animals during January and February of 2005 and 2008 (Table 1a). Based on molecular analysis of four genes across 53 sciaenid taxa, *Pachyurus* appears as sister taxon of *Plagioscion* (W.-J. Chen, National Taiwan University, pers. comm.). For outgroups we included two strictly marine Sciaenidae (*Cynoscion guatacupa* and *Micropogonias furnieri*) (Table 1a). While the systematics of the large family Sciaenidae is far from resolved (see Sasaki, 1989; Boeger & Kritsky, 2003; Casatti, 2003), morphological analyses suggest that the likely marine sister group of *Plagioscion* is *Cynoscion* (Chao, 1978). The second outgroup used in this study is part of a set of four candidate genera (*Micropogonias*, *Nebris*, *Otolothodies*, *Penna*, *Paralonchurus*)

that could also be potentially related to *Plagioscion* (N.L.C., unpublished data).

For the phylogeographic analysis of *P. squamosissimus*, 152 specimens were sampled from 11 sites across a vast area of the Amazon Basin (approximate riverine distance *c.* 2200 km) (Table 1b). Our study transect included five major river systems (Amazon, Madeira, Branco, Negro and Tapajós rivers) and hydrochemical settings (white, black and clear water) of the Amazon Basin (Fig. 1, Table 1b). From each sample site we obtained up to 25 individuals. Fish were caught using a beach seine net; they were euthanized, muscle tissue was dissected from behind the dorsal fin and preserved in 95% ethanol, and geographic coordinates and hydrochemical variables were recorded for each sampling location (Table 1b). Voucher specimens were deposited at the ichthyology collection of the Museu Nacional (UFRJ), Rio de Janeiro, Brazil.

Genetic methods

Genetic data were obtained from both the mitochondrial (mtDNA) and nuclear (nDNA) genomes. DNA was extracted via a modified salting-out protocol (Sunnucks & Hales, 1996). The mtDNA adenosine triphosphatase subunits 6 and 8 (ATPase 6 and 8) were amplified via polymerase chain reaction (PCR) using primers and conditions specified in Corrigan *et al.* (2008) and sequenced for all samples. The single-copy nDNA fragment of the recombination activating gene 1 (*RAG1*) was amplified using a nested PCR for each species, and for putative cryptic *Plagioscion* species identified using mtDNA data (details below). For the first round PCR, *RAG1* was amplified using the primers RAG1-2510F[a] (Li & Orti, 2007) and RAG1-4090R[a] (López *et al.*, 2004). For the second round PCR, *RAG1* was amplified using the primers RAG1-2533F[b] and RAG1-4078R[b] (López *et al.*, 2004). Each 30 µL reaction contained 0.6 µM of each primer, 3 mM MgCl$_2$, 0.6 mM of each dNTP, 5× buffer (Promega, Madison, WI, USA) and 1 U Taq polymerase (Promega). Amplification was carried out using the following programme: 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min.

Using 2% TRIS-acetate-EDTA (TAE) agarose gel electrophoresis, PCR products were separated, excised and purified using an ULTRA CLEAN 15 DNA purification kit (Mo Bio Laboratories, West Carlsbad, CA, USA). Purified DNA was sequenced in an AB3730xl sequencer.

Phylogenetic analyses

All sequence data were aligned using SEQUENCHER 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and submitted to GenBank (accession numbers JN683723–JN683822). To reconstruct phylogenetic relationships we employed character-based and model-based (maximum parsimony, MP, maximum likelihood, ML, and Bayesian inference, BI) methods of analysis for both ATPase 6 and 8 and *RAG1* sequence data. Analyses based on MP were conducted in PAUP* 4.0b10

Table 1 Sciaenidae species and sample locations: (a) species used in the phylogenetic analysis, their collection locality ('upper', 'middle' or 'lower' Amazon Basin, 'Paraná' Basin or 'marine' for samples from the south coast of Brazil) and markers sequenced, and (b) *Plagioscion squamosissimus* samples used in the phylogeographic analysis, including sampling locations, sample size (*n*) and water colours.

(a) Phylogenetic analysis

Species	Collection locality	Markers sequenced
Plagioscion squamosissimus	Upper, middle	ATPase 6 and 8, *RAG1*
Plagioscion auratus	Middle	ATPase 6 and 8, *RAG1*
Plagioscion magdalenae	Middle	ATPase 6 and 8, *RAG1*
Plagioscion ternetzi	Paraná	ATPase 6 and 8, *RAG1*
Plagioscion montei	Middle	ATPase 6 and 8, *RAG1*
Pachypops foucroi	Middle	ATPase 6 and 8, *RAG1*
Pachypops 'Tapajós' (new sp.)	Middle	ATPase 6 and 8, *RAG1*
Pachyurus junki	Middle	ATPase 6 and 8, *RAG1*
Cynoscion guatacupa	Marine	ATPase 6 and 8, *RAG1*
Micropogonias furnieri	Marine	ATPase 6 and 8, *RAG1*

(b) Phylogeographic analysis of *P. squamosissimus*

| River	Site	*n*	Water colour	Latitude	Longitude
Branco	B2	9	Black	1°03′23.00″ S	61°51′29.00″ W
Branco	B1	5	Black	1°15′59.00″ S	61°50′55.00″ W
Negro	N1	22	Black	3° 4′44.00″ S	60°14′44.00″ W
Madeira	M1	20	White	3°28′14.00″ S	58°52′5.00″ W
Amazon	A1	20	White	3°20′40.00″ S	60°7′10.00″ W
Amazon	A2	12	White	3° 6′56.00″ S	59°32′19.00″ W
Amazon	A3	18	White	3°4′39.00″ S	58°13′13.00″ W
Amazon	A4	18	White	2°33′7.00″ S	57°1′59.00″ W
Amazon	A5	5	White	2°10′21.00″ S	54°58′21.00″ W
Amazon	A6	4	White	2°28′10.00″ S	54°30′5.00″ W
Rio Tapajós	T1	14	Clear	2°52′17.00″ S	55°9′38.00″ W
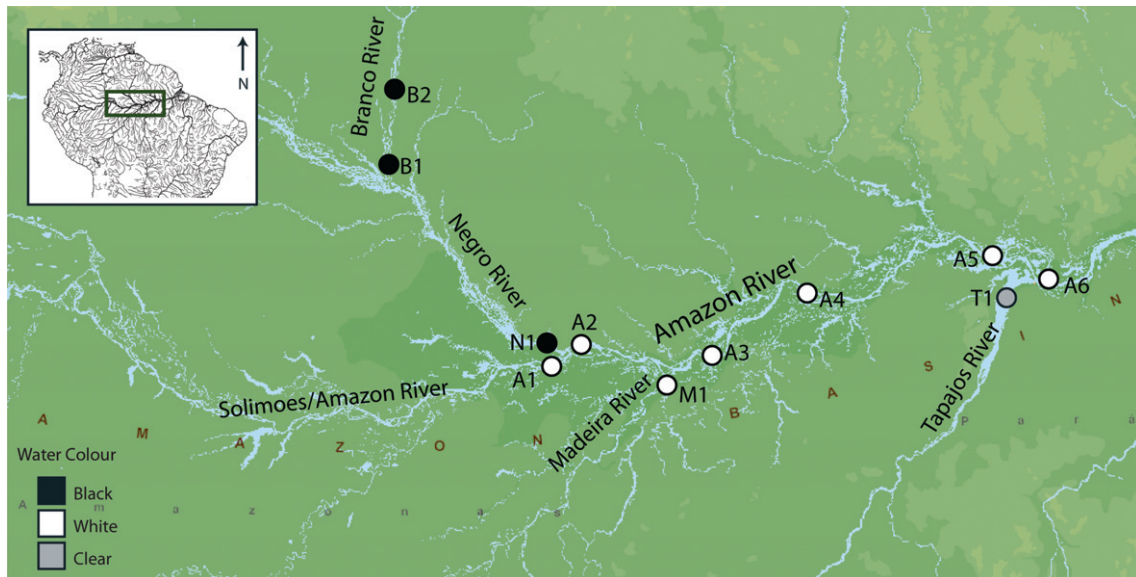
Figure 1 Sampling localities of *Plagioscion squamosissimus* in the Amazon Basin. Each site is distinguished by a site label shaded according to water colour. Black water sites are labelled B2, B1 and N1; white water sites are labelled A1–A6 and M1; and clear water sites are labelled T1. The inset identifies the study area within northern South America.

(Swofford, 2003) using a heuristic search strategy for the most parsimonious tree. All characters were treated as unordered and unweighted. Bootstrap resampling based on 1000 replicates was used to assess support of relationships.

For ML phylogenetic analyses we used MODELTEST 3.06 (Posada & Crandall, 1998) to estimate the most likely model of sequence evolution for our ATPase 6 and 8 and *RAG1* data. Based on the Akaike information criterion (AIC), Tamura–Nei (I+G) was selected as the most likely model of sequence evolution for both ATPase 6 and 8 and *RAG1*. Corrected genetic distances based on 816 bp of ATPase 6 and 8 and 1498 bp of *RAG1* were calculated in PAUP* 4.0b10. ML trees were obtained in PAUP* 4.0b10 using model parameters specified by MODELTEST and also assessed with 1000 bootstrap replicates.

Bayesian inference was conducted using MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001). Here, MRMODELTEST 2 (Nylander, 2004) was employed to determine the best fit model of sequence evolution for both ATPase 6 and 8 and *RAG1*. Based on the AIC, the general time reversible (I+G) model was selected for both datasets. Using Bayesian inference we also constructed a concatenated phylogenetic tree incorporating the appropriate parameters of sequence evolution for each gene. The Bayesian analysis was run using a Metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm from randomly generated starting trees for six million generations with sampling every 1000 generations. Both the standard deviation of splits frequencies and the potential scale reduction factor were used as a convergence diagnostic and the initial 25% of samples were discarded in the burn-in.

Molecular dating of speciation events

Divergence times using the concatenated BI phylogeny were estimated using the MCMC approach implemented in the program BEAST 1.6.1 (Drummond & Rambaut, 2007). There is some uncertainty about the correct marine sister taxon of *Plagioscion*, so date estimates of freshwater invasion should be interpreted with caution. Nonetheless, our tree was calibrated using the oldest known *Plagioscion* and *Pachypops* fossils (Monsch, 1998) and the marine transgressions of the middle Miocene. Furthermore, we calibrated isolation of *Plagioscion magdalenae* with the isolation of the Magdalena Basin (Lundberg *et al.*, 1998). Using these calibrations, we estimated the time to most recent common ancestor (TMRCA) for each species using the relaxed clock method that allows for branch specific variation, drawn from a uncorrelated lognormal distribution (Drummond *et al.*, 2006). Tree priors were modelled according to Yule speciation process, the age at the node of *Plagiocion* and *Pachyurus/Pachypops* was set to 22 ± 6 Ma and the age of the node of *Plagioscion magdalenae* was set to 12 ± 4 Ma. All other priors were set at their default values. The MCMC analyses were run for 60 million generations and sampled every 100th generation with the first 10% of samples discarded as a burn-in. Using TRACER 1.4 (Rambaut & Drummond, 2006), we examined our results and confirmed

that sufficient effective sample sizes had been achieved (> 200) and stationarity was reached. The analysis was run three times independently to validate these results.

Phylogeographic analysis of *Plagioscion squamosissimus*

Genealogical relationships between 152 specimens identified as *P. squamosissimus* were investigated by constructing a haplotype network from mtDNA ATPase 6 and 8 sequence data in TCS 1.21(Templeton *et al.*, 1992; Clement *et al.*, 2000). Summary statistics including nucleotide diversity (π) and mean number of pairwise differences (PWD) were estimated for each population in ARLEQUIN 3.01 (Excoffier *et al.*, 2005). The demographic history of *P. squamosissimus* in the Amazon Basin was assessed by comparing a mismatch distribution of the data with a model of demographic growth (Rodgers & Harpending, 1992; Excoffier *et al.*, 2005). Mismatch analysis calculates the estimator of time to growth (τ) and the mutation parameter (θ) (Schneider & Excoffier, 1999). Using the ATPase 6 and 8 mutation rate (μ) of 1.4% per million years after Bermingham *et al.* (1997), we applied the formula $t = \tau/2\mu$ to estimate the timing of the demographic growth. To further assess the signal of demographic growth, we applied Fu's F_S (1997) test of selective neutrality to dataset. Large negative F_S values are generally observed under demographic growth (Fu, 1997).

To assess levels of genetic differentiation among populations of *P. squamosissimus*, we calculated the fixation index Φ_{ST} in ARLEQUIN. We also used this estimator to test for correlations between population genetic structure and geographic distance (isolation by distance, IBD) (Wright, 1943). The significance of the association was tested using a Mantel permutation test (Mantel, 1967) with 1000 matrix randomizations. The degree of population structure associated with water colour was also explored with an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). AMOVA partitions total variance into covariance components, and tests the significance of the variance components associated with chosen hierarchical levels of genetic structure based on the fixation index, Φ_{ST}. Two AMOVAs were conducted. AMOVA 1 partitioned the dataset into regions defined by water colour: (1) black (B2, B1, N1), (2) white (A1–A6, M1), and (3) clear water (T1) populations. AMOVA 2 tested for population subdivision between two true white water rivers: (1) the Madeira River (M1) and (2) the Amazon River (A1–A5).

RESULTS

Sequence characteristics

We generated 816 bp of the mtDNA ATPase 6 and 8 genes and 1476 bp of the nuclear gene *RAG1* for all *Plagioscion* species, outgroup taxa and major lineages of *P. squamosissimus* identified in the phylogeographic analyses. The mtDNA dataset were characterized by 344 variable characters, of which 284 were parsimony informative. The base frequencies

were 28.5% A, 38.4% C, 9.40% G and 23.7% T. Tamura–Nei (I+G) corrected pairwise differences ranged from 0.048 between *P. squamosissimus* from Meta River, Colombia and the Amazon River, to 0.650 between *Cynoscion guatacupa* and *Pachypops fourcroi*. For the nuclear gene *RAG1*, the base frequencies were 23.3% A, 27.6% C, 23.4% G and 25.7% T. Tamura–Nei (I+G) corrected pairwise differences ranged from 0.00, between *P. squamosissimus* samples from Meta and the Amazon rivers, to 0.042, between *Pachypops* 'Tapajós' and *Micropogonias furnieri*.

Phylogenetic analysis of mtDNA and nuDNA

For the mtDNA ATPase 6 and 8 dataset all methods of phylogenetic analyses resulted in largely concordant tree topologies. They strongly supported the monophyly of *Plagioscion* in which *P. auratus* appears as the basal lineage, as well as the monophyly of *Pachyurus + Pachypops* in which *Pachyurus junki* appears basal. We present only the ML tree with bootstrap values for each method (Fig. 1a and Appendix S1 in Supporting Information). In each case, the *Plagioscion* and *Pachyurus + Pachypops* clades were strongly supported. Inter-specific relationships within the *Pachyurus + Pachypops* clade were well resolved with strong bootstrap support. Within the *Plagioscion* clade, however, two putative cryptic *Plagioscion* species (these were identified as *P. squamosissimus* in the field) were resolved in the phylogeny, both with strong bootstrap support (herein referred to as *Plagioscion* sp. 1 and sp. 2). *Plagioscion* sp. 1 appears sister to the *P. squamosissimus* clade, whereas *Plagioscion* sp. 2 is closely related to *P. montei*. Additionally, *P. squamosissimus* from the Meta River appeared basal to and relatively divergent from *P. squamosissimus* sampled from the Amazon, Madeira, Negro and Tapajós rivers (based on 152 specimens). Within the *Plagioscion* clade the relationship of *P. magdalenae* with *P. montei* and *P. ternetzi* was poorly supported. In contrast, the placement of *P. squamosissimus* as a recently derived lineage was well supported in all analyses.

For the *RAG1* dataset, only the ML and the BI resulted in consistent tree topologies (Fig. 1b, Appendix S1). While all methods of phylogenetic analysis produced well-supported *Plagioscion* and *Pachyurus + Pachypops* clades, inter-specific relationships within *Plagioscion* were not consistent in the MP analysis and the placement of *P. squamosissimus* was poorly resolved. For these reasons we decided to base our discussion on results from the ML and BI analyses. Inter-specific relationships between *Pachyurus* species were consistent with mitochondrial results. However, this was not the case between *Plagioscion* species, which generally showed clades with poor statistical support. This was particularly true regarding the placement of *P. magdalenae*, *P. montei*, *P. ternetzi* and *Plagioscion* sp. 1. Nonetheless, as with mtDNA analyses, *P. auratus* appears basal, *Plagioscion* sp. 2 is closely related to *P. montei* and *P. squamosissimus* is a recently derived linage. As expected for a slowly evolving sequence marker, *P. squamosissimus* for the Meta River could not be distinguished from other *P. squamosissimus* samples by the *RAG* dataset.

The concatenated ATPase 6, 8 and *RAG1* BI tree was fully resolved regarding relationships among species of *Plagioscion* (Fig. 2a). In this analysis, *P. auratus* appears sister to *P. magdalenae*, followed by two sister clades consisting of the remaining *Plagioscion* species. *Plagioscion ternetzi* appears basal to *Plagioscion* cryptic sp. 1 and *P. squamosissimus*, while *P. montei* and *Plagioscion* cryptic sp. 2 form a separate clade.

Divergence estimates

Estimates of TMRCA and their corresponding 95% lower and upper highest probability densities are shown in Fig. 3 and Table 2. Assuming that *Pachyurus + Pachypops* are sister to *Plagioscion*, our results indicate a single freshwater lineage composed of *Pachyurus + Pachypops + Plagioscion* estimated to be 31 Ma (Oligocene). Based on this result, divergence between marine and freshwater lineages probably took place during the early to middle Oligocene. The minimum age estimates of the *Plagioscion* and *Pachyurus + Pachypops* clades suggest that *Pachyurus + Pachypops* may have radiated before *Plagioscion* during the late Oligocene to early Miocene, while radiation within the *Plagioscion* group began towards the end of the Miocene around 15 Ma.

Phylogeography and adaptation in *Plagioscion squamosissimus*

Of the 152 *P. squamosissimus* samples sequenced from 11 sites within the Amazon Basin (excluding the Meta River), we identified 74 unique haplotypes. Within these there were 89 variable characters and 56 were parsimony informative. The haplotype network linked all *P. squamosissimus* samples, except those sampled from the Meta River, based on a criterion of 95% statistical parsimony (Fig. 4). tcs identified a haplotype found only in white water (Amazon and Madeira rivers) as ancestral. According to this network, haplotypes from black or clear water rivers are more derived than the majority of white water haplotypes. Within the network, there appears to be little correlation between haplotype and geographic distance. This is because, in many instances, the clear water haplotypes are more closely related to the black water haplotypes that are over 1000 km away than to neighbouring white water haplotypes. Contrary to this result, the Mantel test revealed a significant positive correlation between genetic differentiation and geographic distance ($r = 0.33$, $P = 0.029$). However, when black and clear water sites were excluded from the Mantel test, the correlation between distance and genetic differentiation increased markedly ($r = 0.62$, $P < 0.000$), suggesting that IBD between white water sites contributed substantially to the overall signal of IBD. Based on the association of haplotype with water colour, rather than with tributary or river system, we suggest a more complex model of diversification than a simple IBD model (discussed further below).
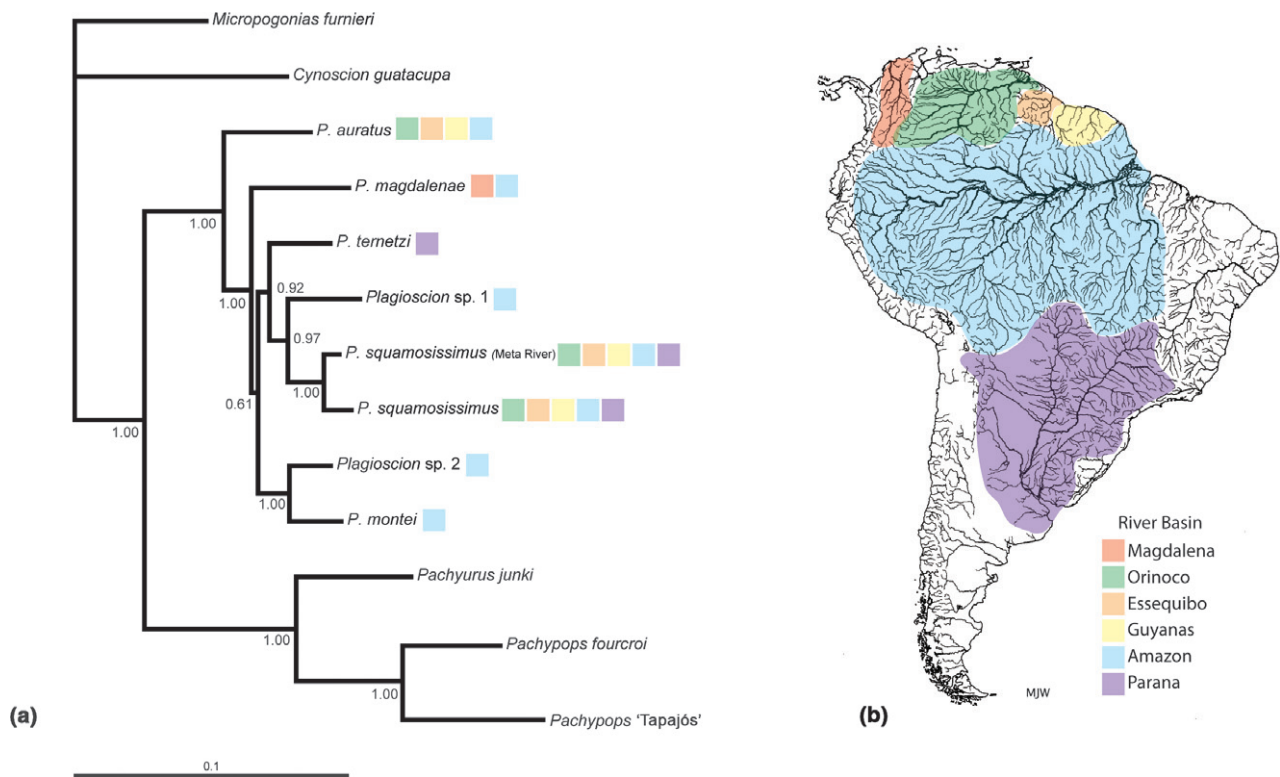
Figure 2 (a) Bayesian inference phylogenetic tree showing the genetic relationships inferred from the concatenated dataset including mitochondrial ATPase 6 and 8 genes and the nuclear *RAG1* gene for *Plagioscion* and *Pachyurus* + *Pachypops* species within South America. Numbers below branches are the posterior probabilities. The coloured boxes beside each *Plagioscion* species represents particular river basins of South America (as in b) which that species is known to inhabit (after Casatti, 2005). (b) The major river basins of South America are shown; each basin is denoted by a particular colour.

Consistent with event(s) of historical population expansion, the haplotype network displays a star-like configuration (excluding Meta River samples). Indeed, based on mismatch analysis, our data did not deviate from a model of demographic expansion (Fig. 5; sum of squared deviations = 0.0046, $P = 0.619$; raggedness index = 0.0072, 0.913), a result corroborated by a large negative F_S value ($F_S = -25.19$, $P < 0.001$). Assuming the ATPase mutation rate of Bermingham *et al.* (1997), estimated τ values suggest that the demographic expansion occurred approximately 2.6 Ma, during the Pleistocene (range = 1–3.6 Ma, $\alpha = 0.05$).

On average, genetic diversity was moderate within each population (pairwise distance = 5.34, $\pi = 0.006$). Significant population structure based on θ_{ST} analysis was detected between populations sampled from black, white and clear water rivers, indicative of reduced gene flow between water colours (Table 3). In contrast, non-significant population structure was detected between samples from white water rivers, including comparisons between different rivers (e.g. Amazon and Madeira rivers). While there was some population structure between the Branco (seasonally black/white) and Negro rivers (black), this was considerably lower than pairwise θ_{ST} comparisons between black and white water populations. In support of this pattern, AMOVA (Table 4) found that

25.07% of the genetic variation within our sample was accounted for by differences between water colours ($P < 0.0001$). On the other hand, there were no detectable differences between river systems of the same water colour (Madeira River versus Amazon River; $P = 0.41$).

DISCUSSION

In this study we propose a phylogenetic hypothesis for the marine-derived sciaenid *Plagioscion* based on mitochondrial and nuclear sequences obtained for all valid species of the genus. Our analyses are consistent with a biogeographic scenario of early Miocene incursions into freshwaters of South America and also disclose two putatively new cryptic species of *Plagioscion*. In addition, we present a phylogeographic analysis of *P. squamosissimus* (a commercially important species in northern South America) using a sizeable sample collected from a vast area in the Amazon Basin. The latter analysis provides support for the theory that large-scale hydrochemical gradients found in Amazonia act as ecological barriers, maintaining abrupt population discontinuities in *P. squamosissimus*. In this way, our study highlights the importance of divergent natural selection through time in the generation and maintenance of sciaenid diversity in the Neotropics.
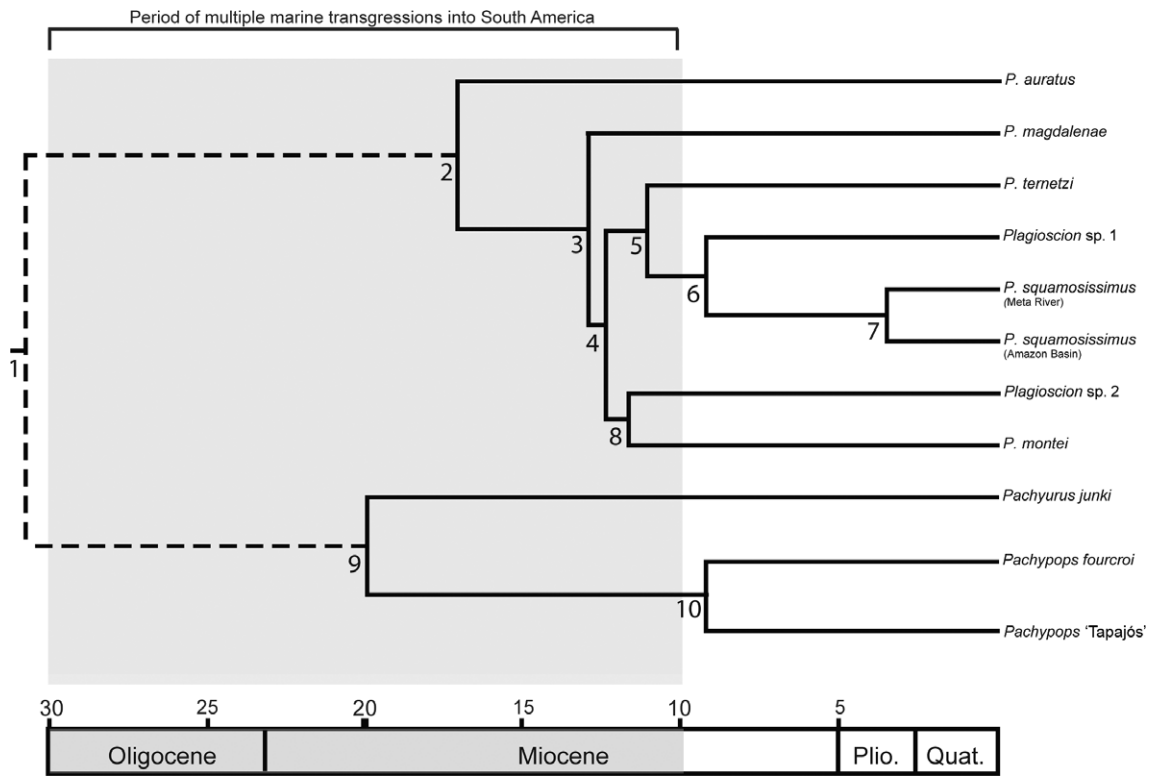
Figure 3 Chronogram showing divergence date estimates for *Plagioscion* and *Pachyurus* + *Pachypops* species within South America. Time to most recent common ancestor (TMRCA) estimates for each node with 95% lower and upper highest probability densities are shown in Table 2. The period of marine transgressions refers to multiple events, as illustrated in Fig. 6.

Table 2 Time to most recent common ancestor (TMRCA) statistics and 95% lower and upper highest probability density (HPD) as calculated in BEAST 1.6.1 using the relaxed clock method that allows for branch specific variation, drawn from a lognormal distribution for *Plagioscion squamosissimus* in the Amazon Basin. Node number is as shown in Fig. 3.

Node number	TMRCA (Ma)	95% HPD lower (Ma)	95% HPD higher (Ma)
1	31.182	43.6856	19.1322
2	17.764	22.9288	12.5772
3	13.445	17.2276	9.6835
4	12.673	16.4343	8.9678
5	11.381	15.1374	7.8365
6	9.216	12.71	5.93
7	3.329	5.547	1.3299
8	7.155	10.8677	3.5247
9	19.48	28.4705	10.8676
10	11.589	18.0703	5.605

Age and trajectory of sciaenid invasions into freshwater

Multidisciplinary studies involving palaeoenvironmental data are providing an ever increasing array of evidence supporting the marine influence in South America, particularly during the Miocene (Lundberg *et al.*, 1998; Hovikoski *et al.*, 2010).

Indeed, it is the Miocene marine incursions that are thought to have contributed most substantially to modern Amazonian biodiversity, especially within the region of focus of this study (e.g. Lovejoy & Collette, 2001; Lovejoy *et al.*, 2006; Bloom & Lovejoy, 2011). Beginning in the late Oligocene (*c.* 30–20 Ma), these marine transgressions permeated the eastern Orinoco and Magdalena basins as well as eastern Amazonia and the region of Mar del Plata and Patagonia (Lundberg *et al.*, 1998) (Fig. 6a). However, 20–11.8 Ma marked the beginning of more extensive marine transgressions (Fig. 6b). During that time the 'Pebas system' in the western Amazon was formed. This relatively long-lived basin consisted of brackish to freshwater salinities that drained northwards towards the Caribbean via the large body of marine water known as the 'Pebasian Sea' in western Venezuela (Lundberg *et al.*, 1998; Wesselingh *et al.*, 2002; Hovikoski *et al.*, 2010) (Fig. 6c). Also during that time, an extensive marine incursion known as the Paranan Sea occurred in the Mar del Plata region in the Paraná estuary reaching as far north as the Chapare buttress in central-eastern Bolivia (Lundberg *et al.*, 1998).

Considering the extent and frequency of marine incursions into South America, one or more of these transgressions and interchanges with freshwater could have facilitated the adaptation of sciaenids from a marine to freshwater environment. While *Plagioscion* and *Pachyurus* + *Pachypops* may or may not have invaded South America separately, our estimates suggest that, regardless, it was probably the less extensive marine
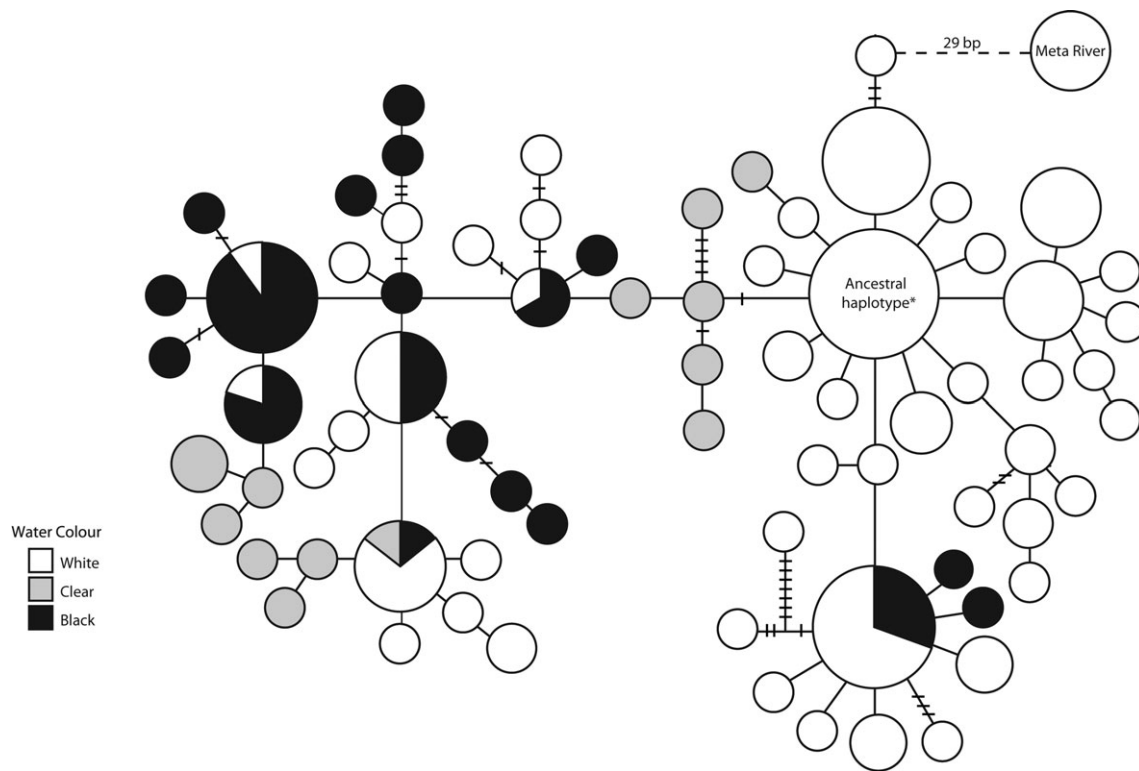
Figure 4 Statistical parsimony network for mitochondrial DNA ATPase 6 and 8 haplotypes for *Plagioscion squamosissimus* sampled in the Amazon Basin. Relationships among haplotypes are estimated using the parsimony method of Templeton *et al.* (1992). Each circle denotes a unique haplotype and the area of the circle is proportional to its frequency. The shade(s) of the circle represents the water colour of the sampling locality, as in Fig. 1.
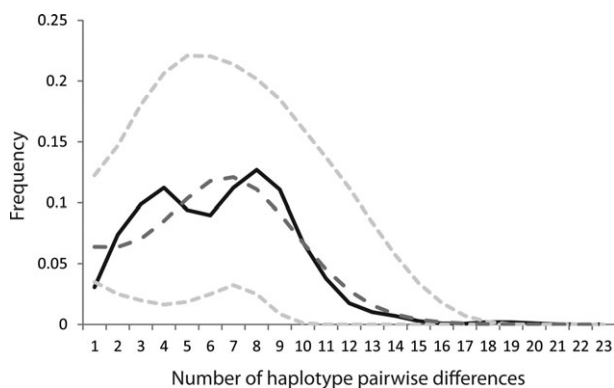

Figure 5 Mismatch distribution of all individuals of *Plagioscion squamosissimus* sampled from the Amazon Basin (*n* = 152). The black solid line represents the observed relative frequencies of nucleotide differences between pairs of individuals, the dark grey dashed line represents the distribution fitted to the data under a model of demographic expansion, and the dashed light grey lines represent the 95% confidence interval values of 1000 simulations.

incursions of the late Oligocene and early Miocene that facilitated the invasion(s) (Fig. 4). While the date of freshwater invasion estimated here might be tentative, its range is nonetheless consistent with marine incursions of that time. Because the distributions of *Plagioscion* and *Pachyurus* +

Pachypops species span both the northern and southern basins of South America, there is debate surrounding the transgression responsible for the freshwater adaptation (i.e. northern or southern). In a northern invasion, the northern-most river basins would be expected to host basal extant lineages. Likewise, in a southern invasion, the most basal lineages should be found in the Paraná Basin. Based on our phylogenetic analysis (Fig. 3), we are able to address both predictions for *Plagioscion* species. Firstly, we can rule out a southern invasion because the two species distributed throughout the Paraná Basin, *P. squamosissimus* and *P. ternetzi*, are both recently derived lineages. In contrast, the most basal extant *Plagioscion* species, *P. auratus*, has a predominantly northeastern distribution throughout the eastern Amazon and Orinoco basins (Casatti, 2005). Additionally, the second most basal extant species, *P. magdalenae*, which actually possesses the least derived gas bladder morphology, is distributed throughout the Magdalena and eastern Amazon basins (Chao, 1978, 2003; Casatti, 2005). Therefore, phylogenetic history and biogeography are consistent with a north to south colonization and radiation of *Plagioscion* species as a product of northern marine incursions during the late Oligocene.

Our results are consistent with both fossil and geomorphological data. In particular, the presence of *P. magdalenae* in the Magdalena Basin supports an early northern colonization as the Magdalena has been isolated from the remaining northern

Table 3 Pairwise θ_{ST} comparisons for the 11 sampled populations of *Plagioscion squamosissimus* in the Amazon Basin. Sample sites have been grouped by geographic proximity and water colour (see Table 1 for more details).

	Black			White							Clear
	B1	B2	N1	S1	M1	A1	A2	A3	A4	A5	T1
Black											
B1	0										
B2	0.141*	0									
N1	0.185*	−0.025	0								
White											
S1	0.520*	0.283*	0.328*	0							
M1	0.460*	0.181*	0.229	0.103	0						
A1	0.558*	0.267*	0.322*	0.022	0.018	0					
A2	0.507*	0.238*	0.294*	0.105	−0.001	0.011	0				
A3	0.427*	0.173*	0.227	0.097	−0.020	0.006	−0.001	0			
A4	0.374*	0.092	0.187	0.101	−0.030	0.033	−0.003	−0.077	0		
A5	0.523*	0.307*	0.447*	0.242*	0.167	0.185	0.095	0.105	−0.042	0	
Clear											
T1	0.277*	0.132*	0.170*	0.328*	0.267*	0.302*	0.324*	0.256*	0.203*	0.398*	0

*$P \leq 0.05$.

Table 4 Analysis of molecular variance (AMOVA) for mitochondrial DNA genes ATPase 6 and 8 in *Plagioscion squamosissimus*. In AMOVA 1, regions include: (1) seasonally black/white (B1), (2) black (N1), (3) white (S1, A1, M1, A2, A3, A4, A5) and (4), clear water populations (T1). In AMOVA 2 regions include: (1) the Rio Madeira, and (2) white water sites A1–A5.

Black versus white versus clear water				White versus white water			
Source of variation	% variation	FI	*P*	Source of variation	% variation	FI	*P*
Between water colours	25.07	Φ_{CT} 0.251	0.000*	Between white water rivers	−2.91	Φ_{CT} −0.029	0.265
Between populations	3.72	Φ_{SC} 0.050	0.010*	Between populations	6.04	Φ_{SC} 0.059	0.012*
Between individuals	71.21	Φ_{ST} 0.288	0.000*	Between individuals	96.87	Φ_{ST} 0.031	0.015*

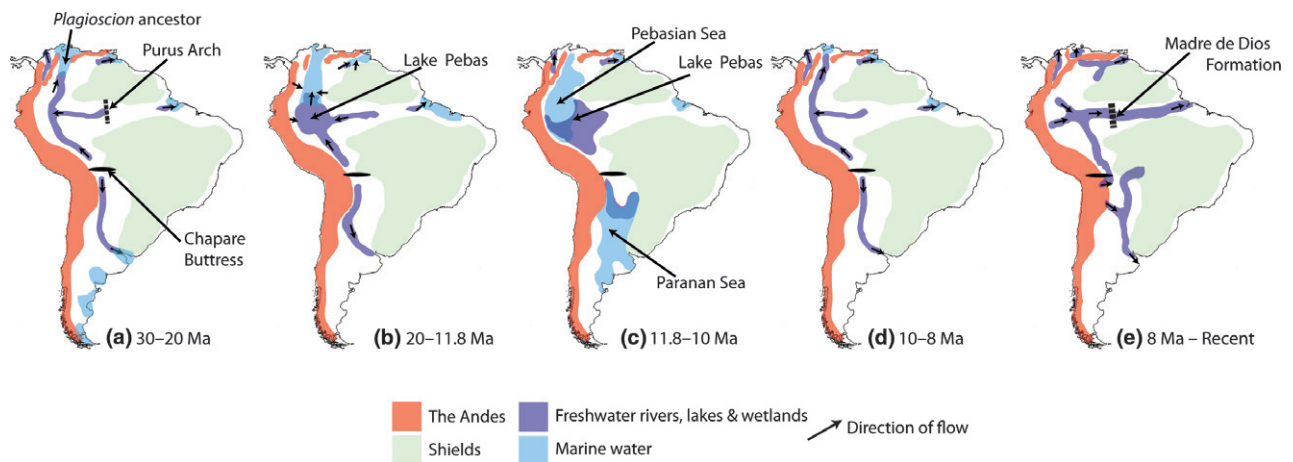FI, fixation index; significant results are indicated by *.

Figure 6 Palaeogeography of South America, depicting the location and frequency of marine transgressions since the middle Tertiary (modified from Lundberg *et al.*, 1998).

basins since the late Miocene (*c.* 11.8 Ma) (Lundberg *et al.*, 1998). In addition, the oldest known *Plagioscion* fossils are from the Pebas Formation in north-western South American

dating to the middle Miocene (*c.* 15 Ma) (Monsch, 1998). These fossils actually pre-date the major marine transgressions that formed the Pebasian and Paranan seas between 11.8 and

10 Ma (Fig. 6c) and indicate that adaptation to freshwaters by *Plagioscion* probably took place earlier (i.e. late Oligocene and early Miocene), as suggested by our Bayesian molecular dating (Fig. 4). Accordingly, it is thought that freshwater stingrays (*Potamotrygon*) (Lovejoy *et al.*, 1998), freshwater anchovies (*Jurengraulis* and *Anchovia*) (Lovejoy *et al.*, 2006) and needle-fish (*Potamorrhaphis*) (Lovejoy & Collette, 2001) share similar age estimates in relation to palaeogeographic events between the late Oligocene to early Miocene.

Considering the fossil record, geomorphological history, distributional data of extant *Plagioscion* species and our phylogenetic results, it is most likely that marine transgressions into western Venezuela between the late Oligocene and early Miocene were responsible for the transition to freshwaters in *Plagioscion* species. All other marine transgressions occurring within northern South America were either small or geographically isolated from Lake Pebas where fossil *Plagioscion* species were reported. The Orinoco transgression, for example, was isolated by the Andean Arc, during which time the eastern Amazon transgression was also isolated from the west by the Purus Arch (Lundberg *et al.*, 1998). Thus, by the early Miocene, *Plagioscion* species had probably adapted to the freshwaters of South America and were establishing a southerly colonization route.

By the middle Miocene both *Plagioscion* and *Pachyurus* + *Pachypops* species were likely to have been well established in the freshwaters of South America. However, it was not until the end of the marine transgressions during the late Miocene that much of the *Plagioscion* radiation begun. It was during this time that the modern Amazon River and its largest tributaries were becoming established, opening up new available habitat and fusing western and eastern biotas (Hoorn *et al.*, 1995; Lundberg *et al.*, 1998) (Fig. 6d). While the full establishment of the Amazon River did not occur until the late Pliocene, the Amazon River had started to form as a compound response to the breaching of the eastern rim of the sedimentary basin known as the Madre de Dios Formation (Içá Formation in Brazil) and erosion of the proto-Amazon River (Hoorn *et al.*, 1995; Lundberg *et al.*, 1998; Rossetti *et al.*, 2005; Campbell *et al.*, 2006) (Fig. 6e). In fact, based on our phylogeny, each *Plagioscion* species (including putative cryptic species) that arose during that time is distributed within the modern Amazonian River and its tributaries east of the Madre de Dios formation. What this suggests is that from about 10–15 Ma until recently, conditions for speciation (via adaptive divergence and/or vicariance) have favoured the evolution of the genus *Plagioscion*. For this reason, we move forward in time and discuss phylogeographic relationships between populations of *P. squamosissimus* within the Amazon River and its major tributaries to identify recent mechanisms that might have influenced *Plagioscion* diversity.

Phylogeography and adaptation of *Plagioscion squamosissimus*

Plagioscion squamosissimus originated within South America sometime between the late Miocene and early Pliocene based on our estimates of TMRCA (Fig. 3, Table 2). During that time, the Amazon River had begun establishing and the marine incursions into South America had terminated (Hoorn *et al.*, 1995; Lundberg *et al.*, 1998). Our results suggest that *P. squamosissimus* arose in north-western South America prior to the full establishment of the modern Amazon River. For instance, *P. squamosissimus* sampled from Meta River in Colombia appear basal to those from the Amazon River (TMRCA *c.* 3.33 Ma). Following the full establishment of the Amazon River system during the late Pliocene to early Pleistocene *c.* 2.5 Ma (Campbell *et al.*, 2006), subsequent colonization and population expansion within this new available habitat would have occurred. Consistent with this inference is the estimated time of demographic expansion within this region for *P. squamosissimus* at approximately 2.6 Ma (Table 3).

The final establishment of the modern Amazon River may not only have facilitated population and range expansion, but it may also have promoted adaptive divergence. Our data suggest that the different mineralogical and geochemical compositions (i.e. water colours) of the Amazon Basin may have also driven divergent natural selection between these environments. For instance, the genealogy of *P. squamosissimus* shows a pronounced association of haplotype with water colour, with black and clear water haplotypes derived from presumably ancestral white water haplotypes. Overall, this genealogy and other summary statistics derived from our phylogeographic dataset (Figs 5 & 6, Table 3) are consistent with Pliocene–Pleistocene population expansion (Avise, 2000) with additional signals of local haplotype radiations within black and white water river systems in particular. However, there are also several haplotypes shared between individuals from different water colours, further indicating that a simple model of allopatric divergence between ecotypes is not sufficient to explain the association of haplotype and water colour. Rather, our data probably represent a model of parapatric divergence, or divergence-with-gene-flow across water colours, in which an impermeable geographic barrier is not necessary to promote reproductive isolation. Instead, gene flow is reduced but not terminated across hydrochemical gradients between populations, a consequence of divergent natural selection that may eventuate in reproductive isolation via ecological speciation (Endler, 1977; Schluter, 2000; Coyne & Orr, 2004).

Despite the wide geographic distribution of *P. squamosissimus* (Casatti, 2005) our frequency-based analysis revealed substantial genetic structure associated with water colour. While we detected a significant association between genetic differentiation and geographic distance, we also found higher levels of genetic differentiation between selective environments than within them (Tables 3 & 4), corroborating our inferences made by genealogy-based analyses. In particular, using AMOVA we found that population differentiation was highly significant between white, black and clear water populations, but not significant between white water populations from different rivers (Amazon and Madeira rivers). This result was

particularly important as it ruled out the possibility of population structure generated by allopatric isolation within a tributary regardless of its water colour.

Our phylogeographic scenario for *P. squamosissimus* is likely to include the dimension of selection in addition to vicariant biogeographic history. Following the final establishment of the Amazon River, white water ecotypes of *P. squamosissimus* would have colonized the newly available habitat in an easterly direction, invading black and clear water habitats and exploiting new niches. Reduced competition in black and clear waters would have facilitated rapid adaptation and population expansion, driving evolution in parapatry with related white water populations. Indeed, it has been shown that ecologically based adaptive evolution can actually be reinforced or facilitated by range expansions (Zayed & Whitfield, 2008; Freedman *et al.*, 2010). While we cannot rule out microallopatric biogeographic events, under this scenario it is likely that divergent natural selection would have been necessary to overcome the homogenizing effects of gene flow between river systems of different water colours.

Despite the fact that most notable examples of divergent natural selection involve freshwater fish (Schluter, 1995; Beheregaray & Sunnucks, 2001; Barluenga & Meyer, 2004; McKinnon *et al.*, 2004; Bernatchez *et al.*, 2010; Elmer *et al.*, 2010), the process of adaptation driven by ecological processes has received little attention in studies of Amazonian speciation. Nonetheless, by identifying loci under selection using genome scans we have detected divergent natural selection between black, white and clear water rivers of the Amazon Basin across taxonomically diverse fishes (G.M.C., N.L.C. & L.B.B., unpublished). Furthermore, results for *P. squamosissimus* presented here are consistent with the overall pattern of reduced gene flow due to hydrochemical gradients observed in our other studies. In the light of this, we propose that the phylogeographic history of *P. squamosissimus* has been influenced by a combination of ecology and geomorphological history.

CONCLUSIONS

Here we have presented phylogenetic and biogeographic hypotheses consistent with geomorphological history and fossil data that account for the marine invasion and diversification of the sciaenid genus *Plagioscion* into freshwaters of South America. *Plagioscion* probably invaded the freshwaters of South America via a northern route during late Oligocene and early Miocene marine incursions, with the subsequent establishment of a southerly colonization route. However, diversification of this genus appears to have largely taken place following the cessation of the marine incursions during the late Miocene and the birth of the Amazon River system. Within *P. squamosissimus*, diversification and population structure also appear to be associated with water colour and ecological gradients. Unexpectedly, our work also revealed two new putative cryptic *Plagioscion* species, suggesting a taxonomic revision of the genus and adding to the ever-growing body of research detecting cryptic Amazonian fish species (e.g. Litt-

mann *et al.*, 2001; Hubert *et al.*, 2007; Sistrom *et al.*, 2009; Piggott *et al.*, 2011). In conclusion, we show that divergent natural selection resulting in the adaptation of sciaenids to freshwaters has persisted through time and probably continues as a diversifying mechanism within contemporary populations of *P. squamosissimus* across freshwater hydrochemical gradients within the Amazon Basin. This result has implications for how we attribute mechanisms of diversification, such as geomorphological events and ecological settings, to the evolution of Amazonian fish diversity.

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SUPPORTING INFORMATION

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

Appendix S1 Maximum likelihood phylogenetic trees showing the genetic relationships inferred from (a) the mitochondrial ATPase 6 and 8 genes and (b) the nuclear *RAG1* gene for *Plagioscion* and *Pachyurus* + *Pachypops* species within South America.

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BIOSKETCHES

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