

On the origins of marine-derived freshwater fishes in South America

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ABSTRACT

Aim The South American fish fauna is renowned for its extraordinary diversity. The majority of this diversity is restricted to few major clades that have ancient associations to freshwater habitats. However, at a higher taxonomic level, the South American ichthyofauna is enriched by an extraordinary number of marine derived lineages – lineages that are endemic to freshwaters, but derived from marine ancestors. Here, we test palaeogeographical hypotheses that attempt to explain the origins and exceptional diversity of marine derived fishes in rivers of South America.

Location South America.

Methods We analysed time-calibrated molecular phylogenies, ancestral reconstructions and biogeographical patterns for multiple independent marine-derived lineages.

Results Five of the ten marine-derived lineages in our analysis have biogeographical patterns and stem ages consistent with invasion from the Atlantic Ocean during the Oligocene or Eocene. Drums and pufferfishes reveal patterns and ages that were consistent with the Miocene marine incursion hypothesis. The Amazonian halfbeak is the only lineage younger than the Miocene and invaded Amazonian freshwaters less than a million years ago.

Main Conclusion Our results suggest Miocene marine incursions and the Pebas Mega-Wetland may not explain the high diversity of marine derived lineages in South America. Instead, the Pebas Mega-Wetland may have created a fertile opportunity for diversification of some, but not all marine-derived lineages.

Keywords

Amazon, freshwater fish, habitat transitions, marine-derived lineages, marine incursion, Pebas mega-wetland

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INTRODUCTION

The South American freshwater fish fauna is renowned for its ecological diversity and species richness (Vari & Malabarba, 1998; Winemiller *et al.*, 2008). With > 5000 fish species, the rivers, lakes and swamps of South America are home to the greatest diversity of freshwater fishes in the world, totalling c. 25% of all fish species (Malabarba *et al.*, 1998; Lundberg *et al.*, 2000; Reis *et al.*, 2003, 2016). Yet, more than 97% of all Neotropical fishes are members of three clades: Ostariophysi (catfishes, tetras and electric fishes) Cichlinae (cichlids), and Cyprinodontiformes (killifishes)

(Lovejoy *et al.*, 2010). These clades have an ancient association with continental (fresh) waters and have dominated the Neotropical fish community since the formation the South America continent (Lundberg *et al.*, 2010; Albert & Carvalho, 2011). However, at a higher taxonomic level, the South American ichthyofauna is enriched by an extraordinary number of marine derived lineages (MDLs) – lineages that are endemic to continental freshwaters, but are derived from clades that are predominantly and ancestrally distributed in marine environments – including stingrays, needlefishes, anchovies, herring, pufferfishes and drums (croakers), among others. There are also many non-fish MDLs in South

America, including iniid dolphins (Cassens *et al.*, 2000; Hamilton *et al.*, 2001), manatees (Domning, 1982), shrimps, crabs, sponges and mollusks (Nuttall, 1990; Wesselingh *et al.*, 2002; Lovejoy *et al.*, 2006).

Marine and freshwater habitats are generally considered independent zoogeographical areas (Darlington, 1957; Hutchinson, 1957; Lee & Bell, 1999; Blaber, 2000). Biotic interchanges between marine and freshwater ecosystems are evolutionarily rare events due to the strong biotic and abiotic barriers in place between these habitats (Vermeij & Dudley, 2000; Procheş, 2001; Vermeij & Wesselingh, 2002). In general, biotic interchange between areas is predictable and asymmetrical, with the fauna that is larger, more species rich, more competitive and having a high reproductive ability acting as the donor fauna (Vermeij, 2005). Given the ancient history of the species rich and ecologically diverse incumbent freshwater fish fauna in South America, the biotic barriers in the form of competition and predation are expected to be particularly robust (Vermeij & Dudley, 2000; Vermeij & Wesselingh, 2002; Betancur-R *et al.*, 2012; Bloom & Lovejoy, 2012). The remarkable number and diversity of marine-derived lineages in South America is unparalleled on other continents (Fig. 1). This is paradoxical since the likelihood of competition with and predation by incumbents, in addition to the abiotic barrier posed by physicochemical differences between marine and freshwaters, would suggest a low probability for successful invasions by marine lineages.

Several studies have suggested the rich diversity of MDLs in South American is linked to various marine incursion events (Nuttall, 1990; Hoorn, 1996; Lovejoy *et al.*, 1998, 2006; Cassens *et al.*, 2000; Hamilton *et al.*, 2001; Bloom & Lovejoy, 2011; Cooke *et al.*, 2012). Marine incursions are the inundation of continental land by oceanic waters, resulting from the rise of sea levels, regional tectonics (or other geological activity), or both. Marine incursions have been implicated as pathways for habitat transitions in marine derived lineages for a wide range of taxa across many continents (Lovejoy *et al.*, 1998, 2006; Wesselingh & Salo, 2006; Wilson *et al.*, 2008; Hoorn *et al.*, 2010; Yang *et al.*, 2013). Although South America has experienced numerous marine incursions following its separation from Africa (Lundberg *et al.*, 1998), previous authors identified several specific incursion events

as possible palaeogeographical conduits for marine to freshwater transitions in fishes.

Miocene marine incursions into the Pebas mega-wetland system (Lovejoy *et al.*, 1998, 2006; Monsch, 1998; Boeger & Kritsky, 2003; Bloom & Lovejoy, 2011; Cooke *et al.*, 2012) are the most recent marine incursions to be implicated as a possible driver of marine to freshwater transitions in South America. The Pebas mega-wetland was comprised of a variety of habitat types including lakes, swamps, deltas and estuaries that occurred during the Early to Middle Miocene (24–11 Ma) (Hoorn *et al.*, 2010). The Pebas system occupied more than 1 million km², centred in what is the present-day upper Amazon, and stretched from the Andes Mountains to the Guiana and Brazilian shields, and from Bolivia to the Llanos basin in Colombia and Venezuela where it drained into the Caribbean Sea. The Amazon River had not assumed its current form and orientation at this point, and would not develop its current course and size until around 11 Ma (Figueiredo *et al.*, 2009), at which point, the Pebas Mega-Wetland had ceased to exist. Geological evidence from trace fossils and pollen cores indicate there were repeated marine incursion events from the Caribbean into the Pebas system between 24–11 Ma (Hoorn, 1993; Hovikoski *et al.*, 2005, 2007; Hoorn *et al.*, 2010) and there was likely regional tidal influence, mimicking near-shore marine and estuarine habitats. Thus, the ecological and biogeographical context of the Pebas system is thought to have offered ideal conditions for invasions by marine lineages—this is referred to as the Miocene marine incursion hypothesis (Lovejoy *et al.*, 2006; Bloom & Lovejoy, 2011). Given the palaeogeography and geological timing, a Pebas origin for MDLs predicts that: (1) the age of MDLs should be between 24–11 Ma, (2) the sister lineage to MDLs should include taxa distributed in the Atlantic (Caribbean) and may include the Pacific, and (3) there should be biogeographical congruence among multiple unrelated taxa (Table 1).

Wesselingh & Hoorn (2011) suggested that some MDLs may have originated during Eocene marine incursion events. Evidence from pollen cores indicates Eocene marine incursions occurred in the Andean foreland basin, which today forms the Colombian Llanos and Putumayo, Ecuadorian Oriente, Peruvian Marañon and Ucayali basins (Lundberg *et al.*, 1998; Santos *et al.*, 2008). During the Late Eocene, the



Figure 1 Continental distributions of marine derived freshwater fish lineages, quantified by taxonomic order from Berra (2001). South America harbours the highest diversity of marine derived fish lineages.

Table 1 Hypotheses and predictions for the origins of marine-derived lineages (MDLs) in South American freshwaters. ATL = Atlantic; PAC = Pacific.

Hypotheses	Prediction: age of MDLs	Prediction: distribution of marine sister group of MDLs	References
Late cretaceous marine incursions	70–65 Ma	ATL and/or PAC	Carvalho <i>et al.</i> , 2004; Brito & Deynat, 2004;
Late Palaeocene to early Eocene marine incursions	58–43 Ma	ATL and/or PAC	Carvalho <i>et al.</i> , 2004; Brito & Deynat, 2004;
Eocene marine incursions	34–43 Ma	ATL and/or PAC	Wesselingh & Hoorn, 2011;
Miocene marine incursions (Lago Pebas)	11–24 Ma	ATL and/or PAC	Lovejoy <i>et al.</i> , 1998; Lovejoy <i>et al.</i> , 2006;
Dispersal unrelated to palaeogeographical event	Any; no congruent patterns	Any; no congruent patterns	Roberts, 1972

Peruvian and Ecuadorian areas were inundated by a series of lakes under marine influence known as Lago Pozo (Lundberg *et al.*, 1998). These palaeoenvironments were intermittently connected to the Pacific through a low-lying area in the proto Andes and to the Caribbean through present-day Colombia. An Eocene marine incursion hypothesis for the origin of MDLs predicts that: (1) the age of MDLs should be between 43–34 Ma, (2) the sister lineage to MDLs should include Atlantic and/or Pacific taxa (the strongest evidence would include only a Pacific–Amazon relationship), and (3) there should be biogeographical congruence among multiple unrelated taxa (Table 1).

The oldest marine incursions proposed as candidates for facilitating MDL invasions into South American freshwaters occurred during the Late Cretaceous to Early Palaeogene. These early marine incursions have been linked to the origin of South American freshwater stingrays (Potamotrygonidae), based on fossils for this group (Bruto & Deynat, 2004) and myliobatiform relatives (de Carvalho *et al.*, 2004). The geological history of Amazonia is poorly known during the Late Cretaceous to Early Palaeogene boundary (Wesselingh & Hoorn, 2011), and Amazonia may have experienced marine influence during these time periods (Lundberg, 1998). Despite the paucity of geological data from these time periods, a Cretaceous/Palaeogene marine incursion hypothesis for the origin of MDLs predicts that: (1) the age of MDLs should fall between 70–65 Ma, or 58–43 Ma, (2) the sister lineage to MDLs should include Atlantic and/or Pacific taxa, and (3) there should be biogeographical congruence among multiple unrelated taxa (Table 1).

The previous hypotheses for the origins of MDLs are linked to specific palaeogeographical events that, because of their shared effects on multiple unrelated groups, would be expected to produce congruent biogeographical patterns. The biogeographical null hypothesis is that the origins of MDLs in South America are not linked to specific geological events, but rather a result of random invasions that have occurred over the vast span of time that South American rivers have been open to the oceans. Roberts (1972) argued that South America was ‘open’ to invasion because the vast majority of the major river drainages (Amazon, Orinoco, Parana) are

relatively low-lying with few major barriers (e.g. waterfalls) compared to major river drainages on other continents such as the Congo River in Africa. It is difficult to test for random invasions, but this hypothesis would be supported by lack of congruence of phylogenetic, biogeographical, and temporal patterns among independent clades (Table 1).

While the evolutionary and palaeogeographical processes that explain the high diversity of MDLs in South America have been discussed previously (Roberts, 1972; Goulding, 1980; Lovejoy, 1996; Lovejoy *et al.*, 1998, 2006; Boeger & Kritsky, 2003; Bloom & Lovejoy, 2011, 2012), few empirical studies have used time-calibrated phylogenies to test for congruent patterns across multiple MDLs. Thus, the marine derived fish lineages of South America remain an enigmatic component of Neotropical aquatic diversity. Here, we integrate geological, palaeontological and neontological data to reconstruct a synthetic history (Sidlauskas *et al.*, 2010) of MDLs in South America. We use time-calibrated phylogenies for multiple clades to link phylogenetic pattern with palaeogeographical events, and test biogeographical hypotheses for the origin of MDLs in South America.

MATERIALS AND METHODS

Data

Our strategy was to assemble the most comprehensive phylogenies for as many South American fish MDLs as possible. To be included in our analysis, taxon sampling must include both MDL(s) and a dense representation of possible marine relatives, in order to infer the marine sister lineage to each MDL and their divergence times. We used newly generated and published time-calibrated phylogenies for stingrays, anchovies, pristigasterids, herring, needlefishes, halfbeaks, pufferfish and drum. Below we briefly describe each data set by major taxonomic group.

Stingrays

For the stingray data set (Appendix S2) we used the Aschliman *et al.* (2012) data set as a backbone, but included only

Myliobatiformes and added additional sequences of Potamotrygonidae (South American freshwater stingrays) from Lovejoy *et al.* (1998), de Carvalho & Lovejoy (2011), and Toffoli *et al.* (2008). To reduce the amount of missing data and maximize overlapping gene sampling, we discarded all mitochondrial genes except *Cytb* and *CO1*, but included both *Rag1* and *SCFD2* nuclear genes from the Aschliman data set. The final data set included four genes and 4680bp. Our taxon sampling included 12 of the 28 described species of Potamotrygonidae, a group that is well supported as monophyletic (Lovejoy, 1996; Lovejoy *et al.*, 1998). The stingray data set also includes 16 species of marine myliobatids that represent all major lineages putatively related to Potamotrygonids (Lovejoy, 1996; Lovejoy *et al.*, 1998; Dunn *et al.*, 2003; de Carvalho *et al.*, 2004; Aschliman *et al.*, 2012).

Anchovies, Herring, and Pristigasterids

The anchovies (Engraulidae), herring (Clupeidae) and Pristigasterids (Pristigasteridae) are all members of Clupeiformes. Bloom & Lovejoy (2014) recently presented the most comprehensive molecular phylogeny of Clupeiformes to date, and this chronogram is used herein. The Clupeiformes data set includes 159 species of clupeiformes, with representatives from 64 of the 84 genera and was comprised of four genes (*16s*, *Cytb*, *Rag1*, *Rag2*) and 5211bp. The taxon sampling of freshwater anchovies includes all six genera that have freshwater members and comprehensive species level sampling, including several undescribed species (Bloom & Lovejoy, 2012). Missing South American freshwater anchovies include *Anchoviella vaillanti* from the Rio Sao Francisco and the recently described *Anchoviella juruasanga* from the lower Amazon river (Loeb, 2012). A recent study (Bloom & Lovejoy, 2012) demonstrated that South American freshwater anchovies are the result of a single invasion, and thus we expect these missing taxa are members of the South American clade. There are two species of freshwater herring found in South America, *Rhinosardina amazonica* and *R. bahiensis*; both of these species were included in the clupeiform data set. The pristigasterid taxon sampling included all five freshwater South American species: *Ilisha amazonica*, *Pellona castelnaeana*, *Pellona flavipinnis*, *Pristigaster cayana* and *Pristigaster whiteheadi*. The clupeiform data set also included a broad sampling of marine anchovies, herring and pristigasterids, both from along the coast of South America and elsewhere, which are putatively closely related to each of these respective freshwater lineages (Grande & Nelson, 1985; Lavoué *et al.*, 2007, 2010, 2013; Li & Orti, 2007; Bloom & Lovejoy, 2014).

Needlefishes and Halfbeaks

Needlefishes and halfbeaks are members of Beloniformes, a group that also includes flyingfish, sauries and ricefish. Lovejoy *et al.* (in prep) presented the largest beloniform phylogeny to date (Appendix S3). This data set is a four-gene

matrix (*Cytb*, *Rag1*, *Rag2*, *Tmo4c4*) totalling 3318bp and includes 104 species of beloniforms. Taxon sampling is near complete for needlefishes with 29 of the 34 recognized species represented in the data set, including all seven described species of South American freshwater needlefishes and their marine relatives (Lovejoy & Collette, 2001; Lovejoy *et al.*, 2004). The only South American freshwater halfbeak, *Hyporhamphus brederi*, is also included in the Beloniformes data set, along with 10 marine species of *Hyporhamphus*, and the monotypic marine genera *Arrhamphus*, *Chriodorus* and *Melapedalion*, which are all members of the 'Hyporhamphus clade' (Lovejoy *et al.*, 2004); putative marine sister species occurring along the coasts of South America are well represented in the data set (Lovejoy *et al.*, 2004).

Drums

There are 287 species of drums world-wide, most of which inhabit the coastal areas of the Atlantic, Pacific, and Indian oceans. There are c. 20 species of freshwater drums in South America, which are members of the genera *Plagioscion*, *Pachypops*, *Pachyurus*, and *Pelitipinnis*. We use the data set from Lo *et al.* (2015), who recently investigated relationships among sciaenids, including South American drums and putative marine relatives. The Lo *et al.* data set included 93 species and 52 genera and sequence data from six genes (*Cytb*, *CO1*, *Rag1*, *RH*, *EGR1*, and *EGR2*) for a total of 6619 bp.

Pufferfishes

Pufferfishes (Tetraodontidae) are represented by a single freshwater species in South America, *Colomesus asellus*. The phylogenetic relationships and evolutionary habitat transitions of pufferfishes were investigated by Santini *et al.* (2013) and Yamanoue *et al.* (2011). These studies yielded largely congruent results regarding the age and phylogenetic relationships of *C. asellus*. Here, we include the results from Santini *et al.* (2013), which included 106 species of pufferfishes and their allies and sequence data from two mitochondrial (*CO1* and *Cytb*) and six nuclear genes (*EGRI*, *MLL*, *IRBP*, *Rag1* and *Rh*) for a total of 7281 bp.

Divergence time estimation

Phylogenetic relationships and divergence times were jointly estimated for all MDLs using Bayesian methods (Drummond *et al.*, 2006) in the program BEAST 1.7.2 and 1.8.1 (Drummond *et al.*, 2012). For our BEAST analyses, we used a log-normal tree prior that allows rates to vary across branches, and a birth-death prior for rates of cladogenesis. Data sets were analysed using partitioned analyses, with a partitioning scheme by gene or based on codon position, with each partition implementing a general time reversible model with a gamma distributed rate heterogeneity (GTR + G). Parameter estimates were compiled in TRACER 1.5 (Drummond & Rambaut, 2007) to assess convergence and mixing of runs and to

verify that effective sample sizes were > 200 for all parameters. The first 10% of each run was discarded as burn-in as determined in TRACER. Independent runs were combined using LOGCOMBINER v1.6.1 (Drummond & Rambaut, 2007) and a maximum credibility tree was generated in TREEANNOTATOR v1.6.1 (Drummond & Rambaut, 2007). Absolute ages were determined for each group using multiple fossil and a few select cases, biogeographical calibrations. Specific details for the time calibration of each group are included in the (see Appendix S1 in Supplementary Information).

Ancestral reconstructions

We determined the phylogenetic pattern of South American freshwater lineages using ancestral character reconstruction (ACR). The data sets (detailed above and in S1) for each major fish group were analysed independently. For each species, we coded habitat (marine/freshwater) as a binary, unordered character. We chose ACR over parametric biogeographical methods (e.g. DIVA and LAGRANGE) because MDLs do not occur in both marine and freshwater habitats (i.e. multiple areas) and we were not attempting to invoke the mechanism (dispersal versus vicariance) of divergence. We used the chronograms for each data set to conduct maximum parsimony (MP) and maximum likelihood (ML) ancestral character reconstruction in MESQUITE v2.6 (Maddison & Maddison, 2011). Maximum likelihood reconstructions were conducted using the Mk model (Pagel, 1999).

RESULTS

Phylogenetic and Biogeographical patterns

The time-calibrated phylogenies and distribution patterns for MDLs and their sister lineages are shown in Table 2. Our analyses revealed ten independent MDLs. We found a common biogeographical pattern between MDLs and their sister taxa; all MDLs have a marine sister group that includes a taxon distributed along the northern Atlantic (Caribbean) coast of South America (Fig. 2). Several MDLs also include sister taxa from Pacific coasts of South America.

South American freshwater stingrays (Potamotrygonidae) are the product of a single invasion into South American freshwaters. The potamotrygonids are the sister group to *Himantura pacifica* from the eastern Pacific and *Himantura schmardae* from the western Atlantic (Lovejoy, 1996; Lovejoy *et al.*, 1998; Dunn *et al.*, 2003; Aschliman *et al.*, 2012).

The clade of South American freshwater anchovies is the sister group to *Anchoa filifera*, a marine species from the western Atlantic, distributed along the coasts of northern South America and Central America. Bloom & Lovejoy (2012) found that the South American freshwater anchovy clade is sister to a large clade that includes nearly all marine New World anchovies from both the western Atlantic and eastern Pacific. In this alternative topology the large marine clade included *A. filifera*. Both studies recover *Engraulis*

ringens and *Engraulis anchoita*, which are distributed along the Pacific and Atlantic coasts of South America, as the sister group to all other New World anchovies. Thus, the closest relative to South American anchovies was either widespread across both the Atlantic and Pacific (Bloom & Lovejoy, 2012), or restricted to the western Atlantic (Bloom & Lovejoy, 2014).

There were two independent transitions into South American freshwaters by pristigasterids. The first freshwater pristigasterid lineage (pristigasterids A) includes *Ilisha amazonica*, *Pellona castelnaeana* and *Pellona flavipinnis*, and is the sister group to *Pellona harroweri*, which has a western Atlantic distribution and is the only marine species of *Pellona* in the New World. The second freshwater pristigasterid lineage (pristigasterids B) includes *Pristigaster whiteheadi* and *Pristigaster cayana*. The marine sister lineage to pristigasterids B is a clade that includes *Odontagnathus mucronatus* from the western Atlantic and Indo-Pacific species of *Ilisha*. There are also a number of marine pristigasterids along the coasts of South America from both the Atlantic and Pacific (e.g. *Neopisthopterus*, *Pliosteostoma*, *Opisthopterus*) that have not been included in any phylogenetic study, but are morphologically similar to *Odontagnathus* (Whitehead *et al.*, 1988). In addition, *Ilisha furthii* from the eastern Pacific is the only New World marine *Ilisha*, but was not included in the Clupeiform phylogeny. These taxa remain candidate marine sister lineages to freshwater South America pristigasterids.

The South American herring, *Rhinosardinia*, is the sister to *Lile stolifera* from the eastern Pacific, suggesting a different biogeographical pattern than seen in other taxa. However, *Lile piquitinga* from the Western Atlantic is likely the sister to *L. stolifera* (Whitehead *et al.*, 1988), but *L. piquitinga* was not included in the Clupeiform phylogeny.

Our analysis of beloniformes, which include needlefishes, halfbeaks and flying fishes revealed two separate marine invasions by needlefishes, and one invasion by halfbeaks. The first South American needlefish lineage, *Potamorhaphis/Belonion*, is the sister group to a clade that includes western Atlantic and eastern Pacific marine species. The second needlefish MDL, *Pseudotyllosurus*, is the sister to a pair of trans-Atlantic species. Needlefish relationships have previously been discussed in more detail (Lovejoy & Collette, 2001; Lovejoy *et al.*, 2006). The halfbeak *Hyporhamphus brederi* is the sister to *Hyporhamphus roberti*, a widespread western Atlantic species.

Lo *et al.* (2015) found that South American freshwater drums were monophyletic, indicating a single invasion from marine waters (but *Petilipinnis* was not included in the study). The freshwater drum clade was the sister group to *Paralichthys brasiliensis*, a marine species from the western Atlantic.

Amazonian pufferfish (*Colomesus asellus*) and the closely related estuarine species *C. psittacus* comprise the sister group to the genus *Sphoeroides*, which is comprised of 49 marine and estuarine species with a distribution that includes most major Ocean basins. We follow Santini *et al.* (2013) in

Table 2 Estimated times of divergences between marine derived lineages and their marine sister lineage and time of the first diversification event in South American freshwaters. The highest posterior density for each node age is provided in parentheses. We also indicate the number of freshwater (FW) species included in each data set, and the total number of FW species for each lineage. For the distribution of the sister lineage to MDLs, W. Atl = Western Atlantic and Caribbean, E. Pac = Eastern Pacific. Monotypic lineages do not have crown ages.

	Marine derived lineages	FW species in study/total	Stem age	Crown age	Distribution of sister lineage
Potamotrygonidae (stingrays)	<i>Potamotrygon</i> , <i>Paratrygon</i> , <i>Heliotrygon</i> , <i>Plesiopygion</i>	12/28	38.2 (30.1, 47.7)	25.9 (19.6, 33.3)	W. Atl/E. Pac
Engraulidae (anchovies)	<i>Anchoiella</i> , <i>Anchovia</i> , <i>Lycengraulis</i> , <i>Pterengraulis</i> , <i>Amazonsprattus</i> , <i>Jurengraulis</i>	~14/20	33.3 (26.1, 40.7)	29.4 (23.2, 36.1)	W. Atl/E. Pac
Pristigasteridae A (pristigasterids)	<i>Pellona</i> / <i>Ilisha</i>	3/3	30.2 (16.1, 47.2)	8.0 (3.3, 13.7)	W. Atl/other
Pristigasteridae B	<i>Pristigaster</i>	2/2	45.6 (31.0, 59.8)	6.8 (2.2, 12.4)	W. Atl
Clupeidae (herring)	<i>Rhinosardina</i>	2/2	32.0 (20.1, 43.4)	17.0 (8.1, 26.8)	W. Atl/E. Pac
Belonidae A (needlefishes)	<i>Potamorhaphis</i> , <i>Belonion</i>	5/5	29.9 (19.7, 41.6)	22.34 (13.4, 31.8)	E. Atl. & W. Atl
Belonidae B	<i>Pseudotylusurus</i>	2/2	31.4 (20.4, 44.4)	17.7 (7.9, 28.0)	E. Atl & W. Atl
Hemirhamphidae (halfbeaks)	<i>Hyporhamphus brederi</i>	1/1	0.95 (0.18, 2.0)	–	W. Atl
Sciaenidae (drums)	<i>Plagioscion</i> , <i>Pachypops</i> , <i>Pachyurus</i> , <i>Petiliopsis</i> (not included)	6/22	23.5 (20.3, 27.0)	21 (18.1, 24.1)	W. Atl
Tetraodontidae (pufferfishes)	<i>Colomesus asellus</i> , <i>C. psittacus</i>	1/1	12.9 (9.4–16.4)	–	W. Atl

concluding the transition to freshwater occurred following the split between *Colomesus* and *Sphoeroides*, and *C. psittacus* is euryhaline species that re-invaded coastal habitats.

Age of MDLs

The most conservative estimate for the origins of MDLs is bracketed by the time of the divergence from the closest marine relative (stem age) and the first diversification event of the freshwater clade (crown age) because the transition from marine to freshwater could have occurred anytime along this branch. Therefore, we report the ages and 95% highest posterior density for both stem and crown ages for each MDL (Fig. 2 and Table 2). However, species limits have not been investigated for most MDLs so the crown age may be biased to a younger age. In some cases, (e.g. halfbeaks) MDLs are monospecific, so crown ages cannot be determined. Furthermore, if the event (i.e. dispersal or vicariance) that resulted in the split between the MDL and the marine ancestor was tied to the invasion of freshwater habitat (which seems likely), then the stem age is the best age estimate of the invasion of South American rivers, while the crown age is the first diversification event within South America.

Our diversification time estimates recovered stem age estimates from Eocene to Middle Miocene origin (~45–13 Ma)

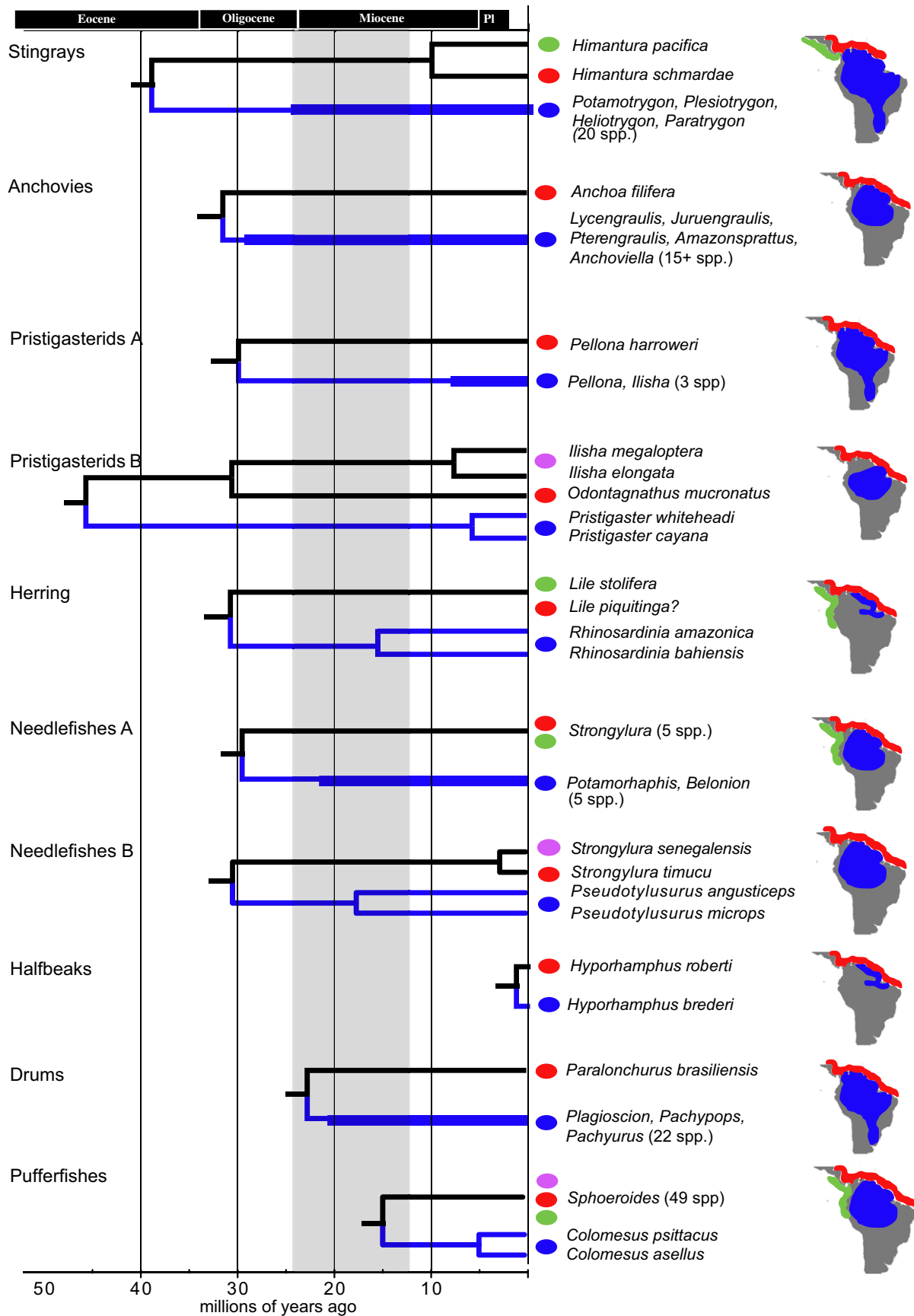
for all MDLs except halfbeaks (Table 2). Seven of the ten MDLs pre-date the Miocene, and of these seven lineages, all but two post-date hypothesized Palaeocene and Eocene incursions. Stingrays have a stem age that dated to the late Eocene (38.2 Ma) and Pristigasteridae B dated to the middle Eocene (45.6 Ma); however, both have a 95% HPDs that include the Oligocene. We recovered largely synchronous stem ages for anchovies (33.3 Ma), Pristigasteridae A (30.2 Ma), herring (32.0 Ma), Belonidae A (29.9 Ma) and Belonidae B (31.4 Ma) which all date to a roughly 3 million-year span during the Oligocene. Drums (23.5 Ma) and pufferfishes (12.9 Ma) both have stem ages that dated to the Miocene. Drums are the only group with both a stem and crown (21 Ma) age during the Miocene. The halfbeak, *Hyporhamphus brederi*, with a stem age of 0.95 Ma, is the only MDL with an estimated age younger than the Miocene.

DISCUSSION

Origins of marine derived lineages in South America

South America has rich freshwater fish diversity with origins dating at least to the Cretaceous (Albert & Reis, 2011). The origins of the remarkable diversity of MDLs in South America, which at higher taxonomic levels may comprise > 65% of clade diversity (Albert *et al.* 2011), has

Figure 2 Summary of ages and distribution patterns for ten South American marine derived lineages (MDLs). In the phylogenies, the marine lineages are indicated in black, and freshwater in blue based on our ancestral character reconstructions. Thickened branches indicate the first diversification event (crown clade) for MDLs with more than two species. The gray shaded time interval shows the timing of the Miocene (24–11 Ma) marine incursions and Pebas Mega-Wetland. The approximate distribution of MDLs (blue) and their marine sister group (Pacific = green, Atlantic = red, elsewhere = purple) is shown on the maps of South America. *Lile piquitinga* was not included in the analysis, but is putatively sister to *Lile stolifera* (Whitehead, 1988).



long perplexed and fascinated biologists (Roberts, 1972; Goulding, 1980). Hypotheses to explain the diversity of MDLs in South America have ranged from Cretaceous vicariance to random invasions (Lovejoy *et al.*, 2006; Bloom & Lovejoy, 2011). Our analysis of ten independent South American MDLs reveals biogeographical patterns and age estimates that implicate an Oligocene origin in five of the 10 lineages, which represents the first evidence for Palaeogene origins of MDLs (Wesselingh & Hoorn, 2011). Our analyses reject hypotheses for earlier origins of MDLs, including Cretaceous, and Late Palaeocene/early Eocene scenarios (Table 1). We also find Miocene marine incursions may explain the origins of few, rather than many marine derived lineages of fishes.

The Miocene marine incursion hypothesis has garnered the most support for explaining the origins of MDLs in South America (Lovejoy *et al.*, 1998, 2006; Bloom & Lovejoy, 2011, 2012; Cooke *et al.*, 2012). The Miocene marine incursion hypothesis predicts that the marine sister group of MDLs should include the Caribbean and the age of MDLs should date to 24–11 Ma. The biogeographical distribution patterns of all MDLs analysed are consistent with a Caribbean/Atlantic origin along the northern coast of South America, which explains the repeated pattern of a marine sister group that includes the western Atlantic. However, only two lineages, drums and pufferfishes, reveal stem ages that are consistent with freshwater origins during Miocene marine incursions (Fig. 3). Instead, most MDLs have stem ages that pre-date the Miocene, suggesting that Miocene marine incursions may not be the primary palaeogeographical event that facilitated the colonization of South American freshwaters by marine ancestors. The robustness of these conclusions varies among groups. For example, both stem and crown age estimates for anchovies and stingrays pre-date the Miocene, providing strong evidence these lineages invaded prior to Miocene marine incursions. Freshwater needlefishes and herring have crown ages that date to 24–11 M and stem age 95% HPD that include the Miocene. Although we argue stem age is the best estimate of colonization time, we cannot rule out a Miocene invasion in these lineages with a crown age that dates to the Miocene. We acknowledge that divergence time analyses often include large error estimates (Drummond *et al.*, 2006),

including those in our study, which make it difficult to reject hypotheses based on clade age alone. Nonetheless, our results suggest that while Miocene marine incursions likely explain the origins of some lineages (i.e. drums and pufferfishes), several lineages likely had earlier origins.

Our analysis is the first to find evidence for the early Oligocene origin for marine derived lineages. During the Oligocene there was a small marine incursion in the Llanos region of northern South America that created an estuarine environment that later formed part of the northern portal that connected the Pebas Mega-wetland to the Caribbean (Bayona *et al.*, 2008; Hoorn *et al.*, 2010; Roddaz *et al.*, 2010). We argue the synchrony of ages (stem ages within a three million year window) and congruent biogeographical pattern for five MDLs (Belonidae A and B, herring, Pristigasteridae A, and anchovies) indicates a common response to a specific palaeogeographical event – in this case, invasion during Oligocene marine incursions. Under this scenario, these five lineages invaded via the northern Llanos, and later penetrated deep into Amazonia and subsequently diversified across the continent, likely during the Pebas mega-wetland period (see below). Intriguingly, the stem age of South American ‘river dolphins’ also may have pre-dated Miocene marine incursions (Xiong *et al.*, 2009; Slater *et al.*, 2010), which supports pre-Miocene origins of numerous marine derived lineages in South America.

The Eocene marine incursion hypothesis predicts that the marine sister group to MDLs could include either the Caribbean or the Pacific and the origin of MDLs would date to 34–43 Ma. South American stingrays were previously thought to have originated during the Miocene (Lovejoy *et al.*, 1998; but see de Carvalho *et al.*, 2004; Lovejoy *et al.*, 2006; Bloom & Lovejoy, 2011), although molecular estimates did not rule out an earlier origin for stingrays (Lovejoy *et al.*, 1998, 2006). Our results indicate an Eocene origin for stingrays, which is consistent with invading during Eocene marine incursions (Table 1). Wesselingh & Hoorn (2011) reported widespread marine influence during the late Eocene in the Andean foreland basin, which likely had both Caribbean and Pacific connections. The marine influence receded by the early Oligocene, which may explain why stingrays had a relatively long waiting period before undergoing diversification (Fig. 2).

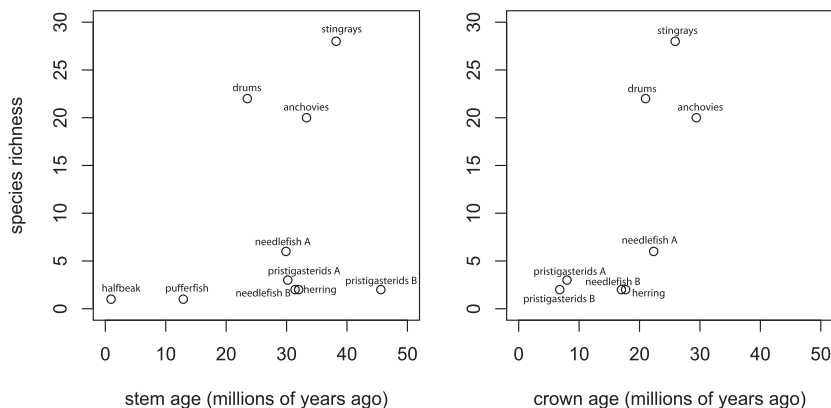


Figure 3 Species richness versus crown [Fig 3(a)] and stem [Fig 3(b)] age of South American fish marine derived lineages (MDLs). Stingrays, anchovies and drum have each diversified geographically and taxonomically, while most MDLs remain species poor.

Freshwater invasions and the role of Pebas Mega-Wetland in MDL diversification

The Pebas Mega-Wetland and Miocene marine incursions have long been thought to have facilitated the invasion and establishment of marine taxa (Nuttall, 1990; Hoorn, 1993; Hoorn *et al.*, 1995; Lovejoy *et al.*, 1998). Our study suggests that many MDLs were present prior to the formation of the Pebas Mega-wetland and Miocene marine influence. We propose that rather than a conduit for many lineages to invade South American rivers, the Pebas Mega-Wetland was a fertile environment for diversification of marine derived lineages. Many MDLs show limited diversification until the Early to Middle Miocene (Figs 2 & 3). In some cases, MDLs became more species rich than their respective marine sister groups, despite being the same age (e.g. anchovies, stingrays and possibly drums), which indicates a shift in diversification rate following invasion of freshwater rivers. For example, anchovies, drums and stingrays each diversified into more than 20 species following invasion of South American rivers (Fig. 3), and the closest marine relatives of these lineages include one (anchovies and drums) or two species (stingrays). In addition to marine-derived fishes, South American 'river dolphins' may have diversified during the Miocene (Slater *et al.*, 2010) and marine derived bivalves appeared and proliferated until the end of the Pebas system (Wesselingh & Macsotay, 2006; Hoorn *et al.*, 2010). The dynamic nature of the Pebas Mega-Wetland and rapidly changing salinity levels may have generated favourable conditions for diversification in lineages with marine ancestry levels by reducing competition with incumbent freshwater fishes (Vermeij & Wesselingh, 2002; Lovejoy *et al.*, 2006; Betancur-R, 2010; Whitehead, 2010; Betancur-R *et al.*, 2012; Bloom & Lovejoy, 2012; Bloom *et al.*, 2013), particularly because the dominant fish clades in South America (Siluriformes, Characiformes, Gymnotiformes, Cichlidae) are unable to tolerate increases in salinity (Myers, 1949).

The Pebas mega-wetland did not promote diversification in all MDLs; some MDLs are represented by one or two species. Lineage diversity is generally expected to increase with clade age under a simple birth-death model (Nee, 2006). Herring, needlefishes and both pristigasterid lineages have five or fewer described species despite being present in South American freshwaters for millions of years, including a time span that encompass the Pebas Mega-wetland. Taken together, the Pebas Mega-wetland promoted diversification in some MDLs, while others have experienced minimal diversification, possibly due to ecological limits or extinction imposed by the rich ichthyofauna of South America (Rabosky, 2009a,b).

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SUPPORTING INFORMATION

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

Appendix S1 Description of time calibration analyses.

Appendix S2 Myliobatiformes Sampling and Genbank numbers.

Appendix S3 Beloniformes Sampling and Genbank numbers.

BIOSKETCHES

Devin Bloom is an ichthyologist and evolutionary ecologist interested in the biogeography, evolution and diversification of fishes, with a particular fondness for anchovies. Nathan Lovejoy is an evolutionary biologist and ichthyologist interested in the biogeography of South American fishes.

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