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Biogeography of the Neotropical freshwater stingrays (Myliobatiformes: Potamotrygoninae) reveals effects of continent-scale paleogeographic change and drainage evolution

Running title: Biogeography of the Potamotrygoninae

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ABSTRACT

1 *Aim*: Paleogeographic changes have had profound effects on the evolution and diversity of the

2 Neotropical biota. However, the influence of marine incursions on the origin, diversification and

3 distribution of fishes is still incompletely understood. We investigate the biogeographical and

4 chronological patterns of diversification for the marine-derived Neotropical freshwater stingrays

5 (subfamily Potamotrygoninae) at a continental scale.

6 *Location:* Neotropics, South America.

7 *Taxa:* Neotropical freshwater stingrays. Subfamily Potamotrygoninae (Myliobatiformes:

8 Chondrichthyes).

9 *Methods:* We generated a time-calibrated phylogeny for 35 of the 38 valid species of Neotropical

10 freshwater stingrays, from most of the major river basins of South America, using four genes.

11 We used BEAST2 to determine the chronology of population and species divergence events, and

12 'BioGeoBEARS' to infer historical biogeographic patterns.

13 *Results:* The Potamotrygoninae originated during the early/middle Miocene in the upper Amazon

14 region. We recover clades associated with particular geographic areas and detect a recurrent

15 pattern of upper Amazon clades sister to clades in adjacent basins. The timing of dispersals from

16 the upper Amazon to adjacent areas corresponds with the end of the Pebas wetlands. Lower

17 Amazon and Shield associated taxa are relatively young.

18 *Main conclusions*: We propose that the origin of the Neotropical freshwater stingrays is related

19 to marine incursions that occurred during the Oligocene/Miocene. Subsequent diversification of

20 Potamotrygoninae occurred in the Pebas wetland system in the upper Amazon with colonization

21 of adjacent basins. These movements were generally unidirectional, with few lineages returning

22 to the upper Amazon, and we speculate that ecological factors drove this pattern. We observed a

23 burst of potamotrygonine diversification 5 Ma that appears to be related to the modern

24 channelization of the Amazon Basin.

25 *Keywords*: Amazon, Guiana Shield, Marine incursions, Orinoco, Paleogeography, Parana-

26 Paraguay, Pebas Wetlands, South America.

27

1. INTRODUCTION

The Neotropics are home to the greatest diversity of freshwater fishes in the world (Reis et al. 2016). While dominated by fishes that have an ancient association with freshwater, such as the catfishes, characiforms, and weakly electric fishes, the Neotropical ichthyofauna also includes an intriguing component derived from marine-derived lineages. These are lineages that are endemic to continental freshwater habitats but are most closely related to clades that are predominantly distributed in marine environments, including taxa such as anchovies, flatfishes, pufferfishes, drum, needlefishes, and stingrays (Lovejoy et al. 2006; Bloom and Lovejoy, 2017). This biogeographic pattern is mirrored by South American non-fish groups, such as dolphins, manatees, sponges, crustaceans, and mollusks (Nuttall, 1990, Cassens et al. 2000; Hamilton et al. 2001; Vermeij and Wesselingh, 2002; Cavalcanti et al., 2019). Many of the South American marine-derived lineages have colonized and diversified in freshwater environments, and are widely distributed across the continent, suggesting considerable age.

The timing and mechanisms involved in the origin of marine-derived lineages into South America are topics of ongoing investigation. Earlier explanations for the origin of marine lineages evoked the invasion of the South American fluvial system via estuaries and river mouths (Roberts, 1972). Our present understanding is that freshwater invasions might have been associated with paleogeographical events such as changes in sea level and marine incursions (Lovejoy et al. 1998; Wesselingh and Hoorn, 2011; Bloom and Lovejoy, 2011, 2017). Replication of phylogenetic patterns and congruence of age estimates for cladogenetic events leading to the origin of marine-derived lineages provide important clues regarding these hypotheses. Invasions via river mouths could occur at any time, involve any large river system, and, as dispersal events depend on intrinsic properties of lineages – we should not expect replication of phylogenetic patterns and age across lineages. South America has experienced intermittent marine incursion events dating as far as the upper Cretaceous, with peaks of marine influence during the Paleocene, Eocene, Miocene and Pleistocene (Shephard et al. 2010; Wesselingh and Hoorn, 2011; Louterbach et al., 2014). Biogeographic studies propose incursions during the Eocene (the Pozo incursions) and most importantly incursions related to the Pebas Mega-Wetland System of the Miocene as events that facilitated invasions of marine-lineages into

1 Neotropical freshwaters (Lovejoy et al. 2010; Wesselingh and Hoorn, 2011; Bloom and Lovejoy,

2 2011, 2017).

3   The Pebas Mega-Wetland System (hereafter Pebas wetlands or system) was an

4 epicontinental marine/freshwater system covering more than one million km², spanning from the

5 Caribbean to southern South America (Fig. 1) (Hoorn, Wesselingh, Ter Steege, et al. 2010;

6 Hoorn, Wesselingh, Hovikoski, et al. 2010; Shephard et al. 2010; Wesselingh and Hoorn, 2011;

7 Bernal et al. 2019). This system covered the western Amazon region during most of the Miocene

8 (23-10 Ma) (Hoorn, Wesselingh, Hovikoski, et al. 2010; Bernal et al. 2019), but its southernmost

9 boundary is uncertain. Some authors have suggested it extended as far as southern Argentina,

10 connecting to the Paranaense Sea (Aleman and Ramos, 2000; Brea and Zucol, 2011), while

11 others argue that it extended only as far as present-day Bolivia (Wesselingh and Hoorn, 2011;

12 Bernal et al. 2019). The giant wetlands of the Pebas experienced oscillating levels of marine

13 influence, as evidenced by sediments characteristic of both freshwater and tidal conditions

14 (Hovikoski et al. 2010) and a rich fossil record of freshwater to marine-associated lineages,

15 ranging from invertebrates (Wesselingh et al. 2006; Wesselingh and Ramos, 2010) and plants

16 (Hoorn, 2006; Bernal et al. 2019), to crocodylians (Salas-Gismondi et al. 2015). Complex

17 salinity gradients would have offered an ideal setting for the evolution of marine to freshwater

18 transitions, and the connection of the Pebas system to the Caribbean region would have offered

19 proto marine-derived lineages a portal to the upper Amazon (Nuttal, 1990; Lovejoy et al. 2006;

20 Bloom and Lovejoy, 2017; Bernal et al. 2019).

21   Aside from the effects of the Pebas system on the origin of marine-derived lineages in South

22 America, this vast wetland also likely affected biogeographic patterns of any aquatic lineages

23 that occurred there. The Pebas System was fed by rivers flowing from the Andes and older shield

24 drainages of the western Amazon, with periodic connections to the Caribbean Sea, until the

25 establishment of the transcontinental Amazon River in the late Miocene (Figueiredo et al. 2009;

26 Shephard et al. 2010; Wesselingh and Hoorn, 2011; Hoorn et al. 2017). The Pebas was thus a

27 likely dispersal corridor for aquatic organisms throughout the proto-Amazon region, allowing

28 movements between continental drainages that were previously and subsequently isolated

29 (Bernal et al. 2019). However, while studies have hypothesized the role of the Pebas system in

1   isolating faunas (e.g., Hubert and Renno, 2006; Solomon et al. 2008); its influence on the

2   distribution and potential dispersal of freshwater fish species has been less explored.

3   The Neotropical freshwater stingrays (subfamily Potamotrygoninae) represent the only

4   extant, obligate freshwater clade of elasmobranchs (Carvalho et al. 2016). Composed of 38

5   species in four genera (Silva and Loboda, 2019, Loboda et al. In press) potamotrygonines are

6   found in almost every major river basin in South America (Carvalho et al. 2003), with maximum

7   species diversity in the Amazon (28 species). The Potamotrygoninae exhibit considerable

8   ecological diversity, ranging from piscivores to mollusk and insect feeders (Kolmann et al. 2016,

9   Fontenelle et al. 2017; Rutledge et al., 2019), and ranging in size from the >1m *Potamotrygon*

10   *brachyura* to the <25cm Wallace's stingray, or arraia-cururu (*Potamotrygon wallacei*) (Castello

11   et al. 2016; Rosa et al. 2016) (Fig. 2). The beautiful dorsal color patterns of many species make

12   them desirable aquarium specimens in the international ornamental fish trade (Fontenelle and

13   Carvalho, 2016). The sister taxon of the potamotrygonines is composed of two marine species of

14   *Styracura*, from the eastern Pacific and western Atlantic (Lovejoy, 1996; Lovejoy et al., 1998;

15   Carvalho et al. 2016, Fontenelle et al. 2017). The Potamotrygoninae are hypothesized to have

16   originated because of marine incursions into the upper Amazon and Pebas system associated

17   regions during the Miocene (Carvalho et al. 2004, Lovejoy et al. 1998, 2006), and subsequently

18   spread to other river systems in South America. However, other authors argue for an older origin,

19   based on fossil evidence from the Eocene that is attributed to potamotrygonines (Frailey, 1986;

20   Adnet et al. 2014), molecular estimates that show a mid-late Eocene origin for potamotrygonines

21   (Bloom and Lovejoy, 2017), and a morphology-based phylogeny that includes fossil stingrays

22   and places the age of potamotrygonines in the Eocene (Carvalho et al. 2004).

23   The broad geographic distribution and diversity of the Potamotrygoninae, combined with

24   their potential to reveal insights regarding the origins of marine-derived lineages and patterns of

25   faunal connectivity throughout South America, make them an excellent candidate for

26   biogeographic investigation. We used a comprehensive dated molecular phylogeny for the

27   subfamily Potamotrygoninae, including 35 of the 38 species spanning their entire known

28   distribution, to investigate the historical biogeography of the group. We investigated the

29   hypothesis that Neotropical freshwater stingrays originated and diversified in conjunction with

30   marine incursions into the Pebas wetland system in the upper Amazon, and then dispersed to

adjacent basins. We tested whether potamotrygonines originated during the Miocene and whether the ancestral distribution of the group overlapped with the area of the Pebas wetlands (upper Amazon). Additionally, we investigated patterns of biogeographic connectivity in relation to the distribution of potamotrygonines in other river systems, including the rivers of the Guiana and Brazilian shields, the lower Amazon, and the Parana-Paraguay basins.

2. MATERIAL AND METHODS

2.1 Taxon sampling, DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

We analyzed a total of 350 specimens representing 35 out of 38 currently recognized species of the Potamotrygoninae with extensive geographic coverage of the subfamily distribution. We also included the two marine species of the genus *Styracura* (Styracurinae), the marine sister group of Potamotrygoninae, thus sampling 37 out of 40 species in Potamotrygonidae. As outgroups, we included *Urobatis halleri* from the eastern Pacific, *U. jamaicensis*, from the western Atlantic, and *Taeniura lymma,* from the Indo-Pacific, based on previous phylogenetic hypotheses (Lovejoy et al. 1998; Marques, 2001; Aschliman et al. 2012). A complete list of species and localities is provided in Table S1 in Supporting Information.

We extracted genomic DNA and PCR amplified fragments of three mitochondrially encoded (mtDNA) genes: ATP synthase membrane subunit 6 and 8 (*atpase*), cytochrome b (*cytb*) and cytochrome c oxidase I (*coI*); and one nuclear ribosomal internal transcribed spacer 1 (*its-1*). Details regarding molecular protocols are provided in Table S2.

2.2 Phylogenetic analysis and dating inference

We used BEAST2 (v2.4.7, Bouckeart et al. 2014) to estimate phylogeny and divergence times under an uncorrelated log-normal relaxed clock (Drummond et al. 2006), allowing rates to vary among branches. Genes were unlinked and set to a GTR model with a gamma distributed rate of heterogeneity for each gene individually, as determined by jModelTest 2 (Darriba et al.

1   2012). We used a Yule Model prior for rates of cladogenesis and ran four independent analyses

2   for 100 million generations, sampling every 10000 generations. We used Tracer (v1.6,

3   Drummond and Rambaut, 2007) to evaluate convergence and to verify if effective sample size

4   (ESS) was at least 200 for all parameters. We discarded 25% of the initial sample as burn-in. The

5   runs were combined using LogCombiner (v2.4.7, Bouckeart et al. 2014) and the maximum

6   credibility tree was generated in TreeAnnotator (v2.4.7, Bouckeart et al. 2014).

7   We used four different dating priors in the BEAST2 analysis. We used the earliest-known

8   proposed fossil for the potamotrygonine subfamily, *Potamotrygon ucayalensis* (Adnet et al.

9   2014) from the middle Eocene (∼41 Ma), and the fossils of *P. contamanensis, P. canaanorum*

10 and *P. rajachloeae* (Chabain et al. 2017) from the late Oligocene - early Miocene (∼30-20 Ma).

11 We assigned a log-normal distributed prior for the origin of the subfamily Potamotrygoninae

12 using these fossils, and to accommodate date uncertainty, set this prior to have an offset of 20.0,

13 standard deviation (S) of 0.75 and mean (M) of 12.0. We also used the closure of the

14 Panamanian Isthmus as a splitting point between *Urobatis halleri* and *U. jamaicensi*s and

15 between *Styracura schmardae* and *S. pacifica.* We used the start (∼15 Ma) and end (∼3 Ma) of

16 the isolation process between the Pacific and the Atlantic (Lundberg et al. 1998; Leigh and

17 Vermeij, 2014), as a log-normal prior, with offset of 2.5, S of 0.7 and M of 2.0. We used the

18 uplift of the eastern Cordillera in Colombia to date the split between *Potamotrygon magdalenae*

19 (from the Magdalena River basin) and *P. yepezi* (from the Maracaibo Lake basin). We set this

20 log-normal prior based on the start (∼12 Ma) and end (∼5-3 Ma) of the uplift of this mountain

21 range (Hoorn et al. 1995; Gregory-Wodzicki, 2000), with an offset of 2.5, S of 0.75 and M of

22 3.0. Finally, we used the uplift of the Merida Andes as a splitting point between *P. magdalenae +*

23 *P. yepez*i and the rest of the subfamily. The Merida Andes uplift interrupted the connectivity

24 between the rivers draining from Colombia and Venezuela to the rest of the Amazon Basin

25 around 10-8 Ma (Lundberg et al. 1998; Hoorn, Wesselingh, Hovikoski, et al. 2010). We set this

26 prior as a log-normal distribution, with offset of 7.0, S of 0.5 and M of 3.5. We used log-normal

27 distributions for the geological priors because, even without hard bounds, we wanted to shape a

28 higher distribution of probability around the "end point" of each isolation event but not ignore

29 the possibility of earlier divergence (Ho and Phillips, 2009).

30

1 **2.3 Biogeography and diversification**

2   To infer biogeographical history, a probabilistic model of geographical range evolution was

3 performed using the R package 'BioGeoBEARS' (Matzke, 2013). This package uses both

4 Maximum Likelihood and Bayesian approaches to estimate ancestral range probabilities for the

5 nodes of a dated phylogeny based on distributions of extant species. We used 13 freshwater

6 biogeographical areas, as well as a "Marine" area for the outgroups (Fig. 3), which were

7 determined based on areas of endemism proposed by Lundberg et al. (1998), Albert et al. (2011)

8 and Dagosta and de Pinna (2017). We pruned our time-calibrated Bayesian phylogeny by

9 selecting a single individual to represent monophyletic groups within each area, resulting in a

10 tree with 150 terminals that was used for the 'BioGeoBEARS' analyses.

11   We performed biogeographical inference using six different models: DEC, DEC+J;

12 DIVAlike; DIVAlike+J; BAYAREAlike; and BAYAREAlike+J. The likelihood-based

13 Dispersal-Extinction Cladogenesis (DEC – Ree and Smith, 2008; Matzke, 2013) model assumes

14 that derived lineages after cladogenesis will inherit a single-range area, which can be a subset of

15 the ancestor's range; DIVAlike, a likelihood version of the DIVA model (Ronquist and

16 Sanmartin, 2011), allows for derived lineages to inherit more than one area as their range, but

17 does not allow for the area of these lineages to be a subset of the ancestor's range;

18 BAYAREAlike is a likelihood version of the BAYAREA (Landis et al. 2013) and assumed that

19 no range evolution occurs at cladogenesis, and the derived lineages inherit the same range of the

20 ancestral state. The parameter "J" adds founder-event speciation to each of the presented models

21 (see Table 1 and Matzke, 2013, 2014 for additional details). We set the parameter

22 *max_range_size* to five, and we included the *null range* parameter which allows ranges to consist

23 of zero areas, a state of transition in the matrix of DEC models. We used 'BioGeoBEARS' to

24 calculate the log likelihood (lnL) and the corrected Akaike Information Criterion (AICc) to

25 choose the best fitting biogeographical model.

26

27 **3. RESULTS**

28 **3.1 Origin and biogeography of the Neotropical freshwater stingrays**

1   The phylogenetic analysis (Figs. 3, S1, S2) recovered Neotropical freshwater stingrays

2   (Potamotrygoninae) as a monophyletic group that is most closely related to the marine subfamily

3   Styracurinae (composed of two species that occur in coastal waters on either side of the Isthmus

4   of Panama) (Fig. 3). The date of divergence between the freshwater Potamotrygoninae and the

5   marine Styracurinae was estimated at 26.4 Ma (ranging 32.1-20.6 Ma), dating the origin of the

6   Neotropical freshwater stingrays to the late Oligocene to early Miocene, coincident with the start

7   of the Pebas wetlands period (Figs. 3, S2). The best fitting model for was determined to be

8   DEC+J (Table 1), and we recover the most probable ancestral range for the family

9   Potamotrygonidae as marine (Fig. S3), while the most probable ancestral range for the

10   Potamotrygoninae is the upper Amazon (Figs. 3, S3).

11

12   **3.2 Diversification and biogeographic patterns within the Neotropical freshwater stingrays**

13   Within Potamotrygoninae, the first divergence is between the clades

14   *Heliotrygon+Paratrygon* (5 species) and *Plesiotrygon+Potamotrygon* (33 species), with a date

15   of 22.45 (20.6/25.1) Ma (early Miocene) (Fig. 3). Both clades are reconstructed to have most

16   likely ancestrally occupied the upper Amazon (Fig. 3), and both contain lineages that occur in

17   most of the major South American river drainages.

18   In the *Heliotrygon+Paratrygon* clade*, Heliotrygon* occurs only in the upper Amazon region

19   (Peru). Its sister lineage *Paratrygon* is much more widespread, occurring across multiple basins

20   of the Orinoco and Amazon, and exhibits distinct, well-defined clades corresponding to major

21   river basins (Figs. 3, S3). In *Plesiotrygon+Potamotrygon*, the genus *Plesiotrygon* is restricted to

22   the Amazon Basin, occurring from the upper Amazon (Peru) to the lower portions of the main

23   channel of the Amazon River. *Potamotrygon* is the most diverse and widely distributed genus,

24   with a range that encompasses the whole distribution of the subfamily (Figs. 3, S3).

25   According to our biogeographic analysis, the two major lineages of Potamotrygoninae

26   originated in the upper Amazon region and then expanded their distributions to adjacent

27   drainages during the Miocene Pebas wetlands period. Our analysis suggests that freshwater

28   stingray lineages first occupied the Negro/Branco, the trans-Andean region, the Parana-

29   Paraguay, and the Brazilian Shield (Tocantins) prior to 10 Ma (Figs. 3, S3).

1   The late Miocene, after the end of the Pebas period (~10 Ma), roughly corresponds to the

2   time of origin of the modern trans-continental route of the Amazon River. The biogeographical

3   reconstruction provides evidence of the first colonization of the Orinoco Basin and several

4   Brazilian Shield drainages during this period (Figs. 3, S3). We also find evidence that

5   colonization of areas that had already been occupied by freshwater stingray lineages took place

6   at this time.

7   These reconstructions show that during and after the Pliocene, potamotrygonine lineages

8   colonized the lower portion of the main channel of the Amazon River (lower Amazon),

9   drainages associated with the Essequibo and Eastern Guyana, and the Xingu, Tapajós and

10   Parnaíba basins for the first time, and there was ongoing exchange of lineages to and from

11   drainages that had been colonized earlier. Our results show evidence that the lineages in the

12   Essequibo and Eastern Guyana originated from the upper Amazon and Orinoco (Figs. 3, S3d).

13   Lineages in the lower portion of the main channel of the Amazon River and Brazilian Shield

14   drainages originated from the upper Amazon and Negro/Branco ranges. These recent lineages

15   form groups based on locality and/or biogeographical areas, comprising specimens belonging to

16   different species but that are from the same area (Fig. S3). We find evidence for Pliocene

17   colonization of the Parana-Paraguay lineages by lineages associated with upper Amazon (Peru)

18   drainages and Brazilian Shield drainages.

19

20   **4. DISCUSSION**

21   **4.1 Origins of the Potamotrygoninae coincides with the Pebas wetlands.**

22   The origins and diversification of marine-derived lineages in South American freshwaters

23   have been attributed to both invasions of river mouths (Roberts, 1972) and marine incursion

24   events that occurred intermittently since the Cretaceous (Frailey, 1986; Carvalho et al. 2004;

25   Lovejoy et al. 2006; Bloom and Lovejoy, 2011). Several authors have linked marine incursions

26   during the Miocene to origins of marine derived taxa in South American freshwaters, including

27   fishes, mollusks, crustaceans, and mammals (Nuttall, 1990; Cassens et al. 2000; Hamilton et al.

28   2001; Vermeij and Wesselingh, 2001; Bloom and Lovejoy, 2017; Cavalcanti et al., 2019). Bloom

29   and Lovejoy (2017) estimated the ages of 10 Neotropical marine-derived fish lineages and found

1   that while most transitioned to freshwater during the Oligocene, a few origins were estimated as

2   Miocene. Our analysis dates the age of the split of the Neotropical freshwater stingrays from

3   their marine sister group to 26 Ma, with error estimates between 32 and 21 Ma, roughly the

4   boundary between the Oligocene and Miocene. At that time, the proto-Amazon was draining into

5   the Caribbean region, before the closure of the Panama Isthmus, and intense marine intrusions

6   related to the Pebas system, which dominated the western Amazon region, allowed for biotic

7   interchange of faunas between marine and freshwater environments (Hoorn, Wesselingh,

8   Hovikoski, et al. 2010; Leigh et al. 2014). The results place the origin of Potamotrygoninae to a

9   time when the western Amazon was dominated by the Pebas wetlands, corroborating our initial

10   hypothesis that this lineage originated in conjunction with marine incursions into the Pebas

11   wetlands.

12   Our results also indicate that initial diversification of potamotrygonines took place in the

13   Pebas wetlands. The origin of two major lineages of Potamotrygoninae

14   (*Heliotrygon+Paratrygon* and *Plesiotrygon+Potamotrygon*), as well as the origin of the

15   individual potamotrygonine genera, date to the Miocene. The estimated ancestral ranges for

16   Potamotrygoninae, *Heliotrygon+Paratrygon* and *Plesiotrygon+Potamotrygon* are all recovered

17   as upper Amazon (Figs. 3, S3). Indeed, until approximately 10 Ma, the proposed end of the

18   dominance of the Pebas wetlands, all ancestral ranges are estimated to be either upper Amazon

19   or Negro/Branco (Fig. S3), matching the range of paleo-floodplains in the Sub-Andean foreland

20   (Figueiredo et al. 2009; Wesselingh and Hoorn, 2011; Lima and Ribeiro, 2011). Thus, our results

21   suggest that initial diversification and distribution of potamotrygonines is associated with Pebas

22   wetland habitats. We suggest that the initial burst of potamotrygonine diversification may have

23   been associated with the dynamics of the Pebas. Salinity fluctuations and their deleterious effects

24   on incumbent Neotropical freshwater fish lineages, referred to as a competition trough (Vermeij

25   and Wesselingh, 2002; Lovejoy et al., 2006; Bloom and Lovejoy, 2017), may have opened new

26   niche space for salinity-tolerant lineages in these communities, facilitating their diversification.

27   The special ecological circumstances of the Pebas are thought to have also spurred

28   diversification in crocodylians (Salas-Gismondi et al. 2015) and mollusks (Nuttall, 1990).

29   Supporting our arguments, Chabain et al. (2017) show that proposed potamotrygonid fossil teeth

30   recovered from the Oligocene- late Miocene from the Contamana region in Peru show

31   morphological disparities that relate to ecological adaptations and diversification.

1   The estimate for the age of origin of Potamotrygoninae (26 Ma) is older than the 23 Ma

2   proposed by Lovejoy et al. (1998), but younger than the 38.2 (30.1 – 47.7) Ma proposed by

3   Bloom and Lovejoy (2017). However, all three of these estimates roughly coincide with the age

4   of Miocene marine incursions and the Pebas wetlands, and the error estimate for the age

5   proposed here (21 to 32 Ma) overlaps with these earlier estimates. Our age estimates for the

6   Potamotrygoninae origin contrasts with older ages proposed by some authors based on fossil

7   evidence. Based on the phylogenetic position of non-potamotrygonine fossil stingrays from the

8   Green River and Monte Bolca formations, Carvalho et al. (2004) suggest that the origin of the

9   potamotrygonines was older than the Oligocene-Miocene. Fossil evidence for Neotropical

10   freshwater stingrays is scarce, mostly consisting of disarticulated teeth (Adnet et al. 2014;

11   Chabain et al. 2017). Adnet et al. (2014) studied stingray teeth from freshwater Eocene deposits

12   from Contamana in the Peruvian Amazon (CTA-27) that they attributed to potamotrygonines;

13   however, these teeth do not exhibit phylogenetically diagnostic characters for potamotrygonines

14   and their classification depends on their freshwater provenance. Using ecological setting to

15   determine the phylogenetic position of fossils is weakened by the fact that different lineages of

16   stingrays have invaded freshwater habitats on multiple occasions. More recently, Chabain et al.

17   (2017) describe additional fossil stingray teeth from Oligocene-Miocene deposits also from

18   Contamana (CTA-43) that they ascribe to potamotrygonines, and the age of these fossils matches

19   the molecular results presented here. Ideally, fossils with potamotrygonine synapomorphies will

20   eventually be found, enabling an independent test of the age estimates proposed here.

21

22   **4.2 Dispersal of stingrays from the upper Amazon**

23   The dynamics of the Pebas wetlands during the Miocene caused physical reorganizations of

24   river basins and likely created connections between drainages that made fish faunal exchanges

25   possible (Albert et al. 2018; Dagosta and Pinna, 2017, 2019). During this period, the extension

26   and contraction of headwater boundaries played an important role in connecting river basins

27   (Hoorn, Wesselingh, Hovikoski, et al. 2010; Wesselingh and Hoorn, 2011; Carvalho and Albert,

28   2011). The Pebas system occupied most of the current upper Amazon region, extending to the

29   Caribbean to the north, the Orinoco Basin to the northeast, the middle Amazon (Purus arch

1 region) to the east, the Andes to the west, and possibly as far as Bolivia to the south (Albert et al.

2 2018, figure 4; Bernal et al. 2019, figures 2, 3).

3   We propose that the extensive area and connectivity of the Pebas wetlands made it possible

4 for freshwater fishes to disperse from the western Amazon to adjacent basins. Our results show

5 evidence of these dispersals in stingrays: 'BioGeoBears' estimates the ancestral ranges for early

6 nodes (Early/Middle Miocene) of the subfamily Potamotrygoninae as upper Amazon, with

7 dispersals to adjacent drainages in the Negro/Branco, the Parana-Paraguay, and the trans-Andean

8 basins (Figs. 3, 3a). We propose that during the Miocene, the northeastern reaches of the Pebas,

9 marginal to the Purus arch and Guiana Shield (Hoorn, Wesselingh, Ter Steege, et al. 2010),

10 allowed connections between the upper Amazon and Negro/Branco, and the southern-most

11 reaches of the Pebas allowed connections between the Amazon and the Parana-Paraguay.

12

13 **4.3 Colonization of the Brazilian Shield and lower Amazon**

14   Geological evidence associates the modern channelization of the Amazon River with the

15 formation of the Amazon Fan, when Andean sediments were deposited at the Atlantic mouth of

16 the Amazon River (Figueiredo et al. 2009; Hoorn at al. 2017). Ongoing uplift of the Andes

17 during the middle and late Miocene (around 10 Ma), combined with weathering of the Purus

18 arch, caused drastic changes in Amazon Basin physiognomy, allowing rivers that once drained to

19 the Pebas system to overcome paleogeographic barriers such as the Purus and Gurupá arches and

20 begin draining to the Atlantic (Figueiredo et al. 2009; Hoorn, Wesselingh, Ter Steege, et al.

21 2010; Shephard et al. 2010; Wesselingh and Hoorn, 2011; Hoorn et al. 2017). Before this point,

22 the upper and middle Amazon regions were geographically separated from what is now the lower

23 Amazon, and aquatic faunas in these two areas were likely isolated from one another. The

24 modern west to east transcontinental drainage of the Amazon, and the establishment of the lower

25 course of the Amazon, coincides with the end of the Pebas wetlands around 10 Ma (Hoorn et al.

26 2017; Albert et al. 2018). Our study provides evidence that the lower Amazon main channel was

27 colonized during the late Miocene/Pliocene after the connections between these areas and the

28 rest of the Amazon were established. We do not recover any lower Amazon lineages older than 5

29 Ma, and most Brazilian Shield associated lineages (Xingu, Tapajós areas) are no older than 7 Ma

1 (Figs. 3, 4b, 4c, S3). We recover dispersal from the upper Amazon and Orinoco to the Brazilian

2 Shield regions, and from the upper Amazon to lower Amazon, corroborating the idea that the

3 lower Amazon and Brazilian Shield regions were colonized by lineages moving from sources in

4 central and western Amazon once the modern Amazon was established (Figs. 3, 4d). The lack of

5 freshwater stingray lineages in the lower Amazon before 10 Ma provides additional evidence

6 that the marine-freshwater transition for this group was not a result of invasion from the western

7 Atlantic via the mouth of the Amazon.

8

9 **4.4 Paleogeographic connections across the South American landscape**

10   Biogeographic patterns in Potamotrygoninae are directly related to paleogeographic changes

11 in the connections and structure of South American river drainages. Below, we show how our

12 analyses corroborate other biogeographic patterns and paleogeographic events in South

13 American freshwaters.

14   The distribution of closely related taxa across cis/trans-Andean basins is a repeated pattern

15 among many groups of Neotropical fishes (Albert et al. 2006; 2018). The uplift of the Andes

16 isolated western draining (trans-Andean) basins and their associated biotas from eastern draining

17 (cis-Andean) basins (Albert et al., 2006, 2018; Rodríguez-Olarte et al., 2011). Specifically, the

18 uplift of the Eastern Cordillera in Colombia separated the trans-Andean Magdalena Basin from

19 cis-Andean rivers approximately 12-10 Ma, and the uplift of the Merida Andes isolated the trans-

20 Andean Maracaibo Basin from cis-Andean rivers around 8 Ma (Hoorn et al. 1995; Lundberg et

21 al. 1998; Albert et al. 2006, 2018; Hoorn, Wesselingh, Ter Steege, et al. 2010). Our data indicate

22 that the divergence of the trans-Andean potamotrygonine lineage (~ 14 Ma) occurred slightly

23 before the uplift of the Eastern Cordillera. However, while paleogeographic reconstruction

24 predicts a ((Maracaibo, cis-Andean), Magdalena) relationship, we instead find that Maracaibo

25 and Magdalena lineages are sister taxa. This is a pattern observed in many other fish clades

26 (Albert et al., 2006), and could be explained by extinction of ancestral cis-Andean lineages

27 and/or invasion of the Maracaibo from the Magdalena through coastal stream captures (Lundberg

28 et al. 1998, Albert et al., 2006). The findings of this study add to growing evidence for the mixed

29 biogeographical origins of the Maracaibo fauna.

1   Our analyses provide insight regarding the relationship between the Amazon and Orinoco

2 drainages. Prior to the late Miocene (10 - 8 Ma), a north-flowing proto-Amazon/Orinoco

3 connected the current Amazon and Orinoco regions before these basins assumed their modern

4 arrangement (Hoorn et al. 1995; Gregory-Wodnicki 2000; Mora et al. 2010). Subsequent

5 orogeny in the Andean region resulted in sediment accumulation in parts of the sub-Andean

6 foreland and the uplift of the Vaupes arch, which ultimately disrupted the connection between

7 the western Amazon and the Orinoco basins (Hoorn et al. 1995; Mora et al. 2010; Winemiller

8 and Willis, 2011; Albert et al. 2018). These drainages may then have been isolated until a later

9 connection, the Casiquiare, was formed, possibly as late the Pleistocene (Lujan and Armbruster,

10 2011). For freshwater stingrays, our analyses date the earliest entry to the Orinoco to 8 Ma (from

11 the Negro/Branco; Figs. 3, S3), which suggests a role of the proto-Amazon/Orinoco in this

12 distribution. After that, the next reconstructed dispersals from the Amazon (Negro/Branco) to

13 Orinoco occurred at 2 and 1.5 Ma. These more recent dispersals are likely related to the

14 formation of the Casiquiare River, and our findings thus contribute additional evidence for the

15 timing of this important connection between the Amazon and Orinoco.

16   Our results also shed light on the colonization of the Essequibo and other Atlantic-draining

17 Guiana Shield drainages. We found that stingray lineages in the Essequibo and other Atlantic-

18 draining Guiana Shield rivers are relatively young, originating from both the Orinoco and

19 Negro/Branco during the late Pliocene. The close relationships between Branco, Essequibo and

20 Guiana Shield taxa recovered for the Pliocene and Pleistocene can be explained by intermittent

21 river connections between the lower portions of these drainages (as discussed in Lima, 2017);

22 alternatively, these patterns could be related to headwater capture events between the Branco and

23 the proto-Berbice (a large drainage system from the central Guiana Shield that drained most of

24 the Guyana and parts of Brazil, Venezuela and Surinam from the Cenozoic to the Pleistocene –

25 see Sinha, 1968, Schaefer and do Vale, 1997; Lujan and Armbruster, 2011). Pleistocene and

26 younger interactions between the Branco and Essequibo basins can be attributed to the Rupununi

27 Portal, a flooded savanna area that allows for seasonal faunal interchange between these basins

28 (Lovejoy and Araújo, 2000; Hubert and Renno, 2006; De Souza et al. 2012; 2020).

29   We recover three independent interchange events between the Amazon and the Parana-

30 Paraguay basins - two during the Miocene and one during the Pliocene/Pleistocene. The oldest

recovered Parana-Paraguay lineage (*Po. brachyura)* dates to ~12 Ma and resulted from a

colonization from the upper Amazon region (Figs. 3, 4a, S3a). A second independent

colonization event from the Tapajós to the Parana-Paraguay occurred ~8 Ma (Fig. S3f). Finally,

we find evidence that the upper Amazon species *Po. tatianae* is the product of an invasion from

the Parana-Paraguay ~5 Ma (Fig. S3h). We propose that connections between the Amazon and

Parana-Paraguay basins resulted from headwater captures between upper Amazon tributaries

(e.g., the Madeira and the Madre de Díos) and the Bolivian Chaco. A link between these basins

during the Miocene has been supported both by geological and fossil evidence (Lundberg et al.

1998, Uba et al. 2006; Latrubesse et al. 2007, Albert et al. 2018), and has been observed in other

fish taxa (Montoya-Burgos, 2003; Carvalho and Albert, 2011). Similarly, more recent

associations between the Parana-Paraguay and upper portions of Brazilian Shield drainages have

been documented in other fishes (Lima et al. 2007; Lima and Ribeiro, 2011; Ribeiro et al. 2013).

We speculate that a series of Pliocene or younger river captures and reorganizations might have

allowed for exchanges between these areas.

4.5 Upper Amazon as source of diversity and dispersal bias to adjacent basins

As the upper Amazon is the region of origin of Neotropical freshwater stingrays, it is

expected that it would host a high diversity of species. The Pebas system, in its complexity of

habitats and environmental gradients, would have provided ideal conditions for stingray

diversification, and the upper Amazon would have inherited this product (Lundberg et al. 1998;

Vermeij and Wesselingh, 2002). In fact, 14 of 38 potamotrygonine species occur in the upper

Amazon (Table S3). After 10 Ma, a direct connection between the upper and lower Amazon

resulted in the modern trans-continental Amazon River basin. This connection between upper

and lower Amazon likely contributed to the high diversity of potamotrygonine species observed

in the lower Amazon (nine in total). Compared to this main axis of diversity, adjacent basins

(e.g., the Orinoco, Atlantic-draining rivers of the Guianas, and the Parana-Paraguay) have fewer

species (Table S3). The observed differences in levels of diversity among basins is likely related

to evolutionary time (Rabosky, 2009; Gehrke and Linder, 2011): areas that were inhabited earlier

are occupied by older lineages that have had more time to speciate and diversify, resulting in

high species richness, while areas farther from the western Amazon are less species rich and composed of younger lineages (Figs. 3, 4, S1).

We also find evidence for a bias in the directionality of dispersal-- lineages often disperse from the upper Amazon to peripheral basins but not the converse. For example, although both Orinoco and Guiana Shield drainages have been invaded by Amazonian lineages several times, none of the Orinoco or Guyana endemics have re-invaded Amazonian waters. What causes this directionality of dispersal? One possible explanation is evolutionary priority effects, where lineages that are already present in an area prevent the subsequent establishment of competing lineages, ultimately affecting the dispersal success of the latter (Belyea and Lancaster, 1999; Leopold et al. 2015). Potamotrygonine lineages that successfully colonized peripheral basins would have faced competition upon return to the Amazon, as ecological niches in this species-rich area would already be occupied by incumbent species. In this way, macroecological effects could produce dispersal bias that correlates with comparative levels of species richness among the areas involved.

Regardless of age, both lower Amazon lineages and lineages from the Guianas, Orinoco, and Parana-Paraguay are often the sister groups to upper Amazon lineages. Considering the patterns described above, this suggests that the upper Amazon may act as a species pump for adjacent basins and eastern lowlands. Areas classified as species pumps are diversity rich, due to higher speciation rates and persistence of older lineages, and contribute lineage diversity to adjacent, more species-poor regions (Stebbins, 1974; Jetz et al. 2004; Rangel et al. 2018). Another proposed species pump in South America is the Andes, a region of high diversification that contributed to the diversity of adjacent lowland habitats (e.g., Antonelli and Sanmartín, 2011; Ledo and Colli, 2017; Rangel et al. 2018). We propose that, for freshwater stingrays and possibly other fishes, the upper Amazon has acted as a species pump, injecting diversity to adjacent basins over the past 10 million years.

The diversification process is regulated by many different biotic and abiotic factors. Here, we provide phylogenetic evidence for the influence of time, biogeography, and ecology on diversification and distribution patterns in a diverse group of Neotropical freshwater fishes. A combination of lineage age, biogeographical connectivity, and evolutionary priority effects likely

1 play a role in the observed patterns of endemism, distribution, and diversity, highlighting the

2 macroevolutionary complexity of the Neotropics.

3

4

5 **Table**

6 TABLE 1: Models estimated in 'BioGeoBEARS' for ancestral range estimation of

7 potamotrygonine stingrays. For each model, we provide the literature reference, log-likelihood

8 value (lnL), number of parameters (numparams), rate of range expansion (d), rate of range

9 contraction (e), relative weight of jump dispersal at cladogenesis (j), and corrected Akaike's

10 information criteria (AICc). Models are ordered from best to worst fit based on AICc values.

Model	Reference	lnL	numparams	d	e	j	AICc
DEC+J	Matzke, 2014	-260.7868	3	1.00E-12	1.00E-12	0.0229	527.7381
DIVALIKE+J	Matzke, 2014	-262.1620	3	1.00E-12	1.00E-12	0.0233	530.4883
BAYAREALIKE+J	Matzke, 2014	-262.2082	3	1.00E-07	1.00E-07	0.0230	530.5809
DEC	Ree and Smith, 2008	-358.4968	2	9.90E-02	0	0	721.0752
DIVALIKE	Ronquist and Sanmartín, 2011	-473.9102	2	1.20E-01	8.10E-01	0	951.9020
BAYAREALIKE	Yu et al. 2013	-473.9102	2	1.20E-01	8.10E-01	0	951.9020

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14 **Figures**

1
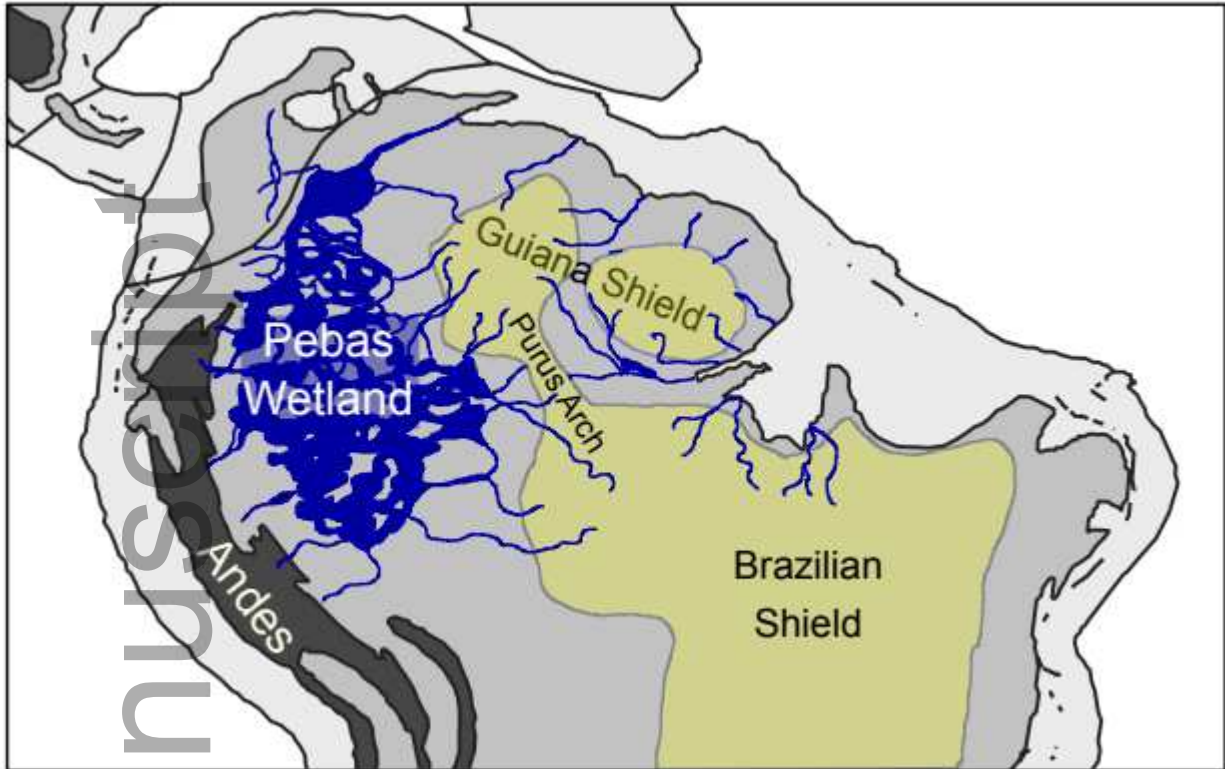
FIGURE 1: Map of South America during the middle Miocene showing position of the Pebas
Wetlands (adapted from Hoorn et al. 2010. using shapefiles from Cao et al. 2017). Shield
formations indicated in yellow, lowlands in gray, continental shelf in light gray, and the Andes in
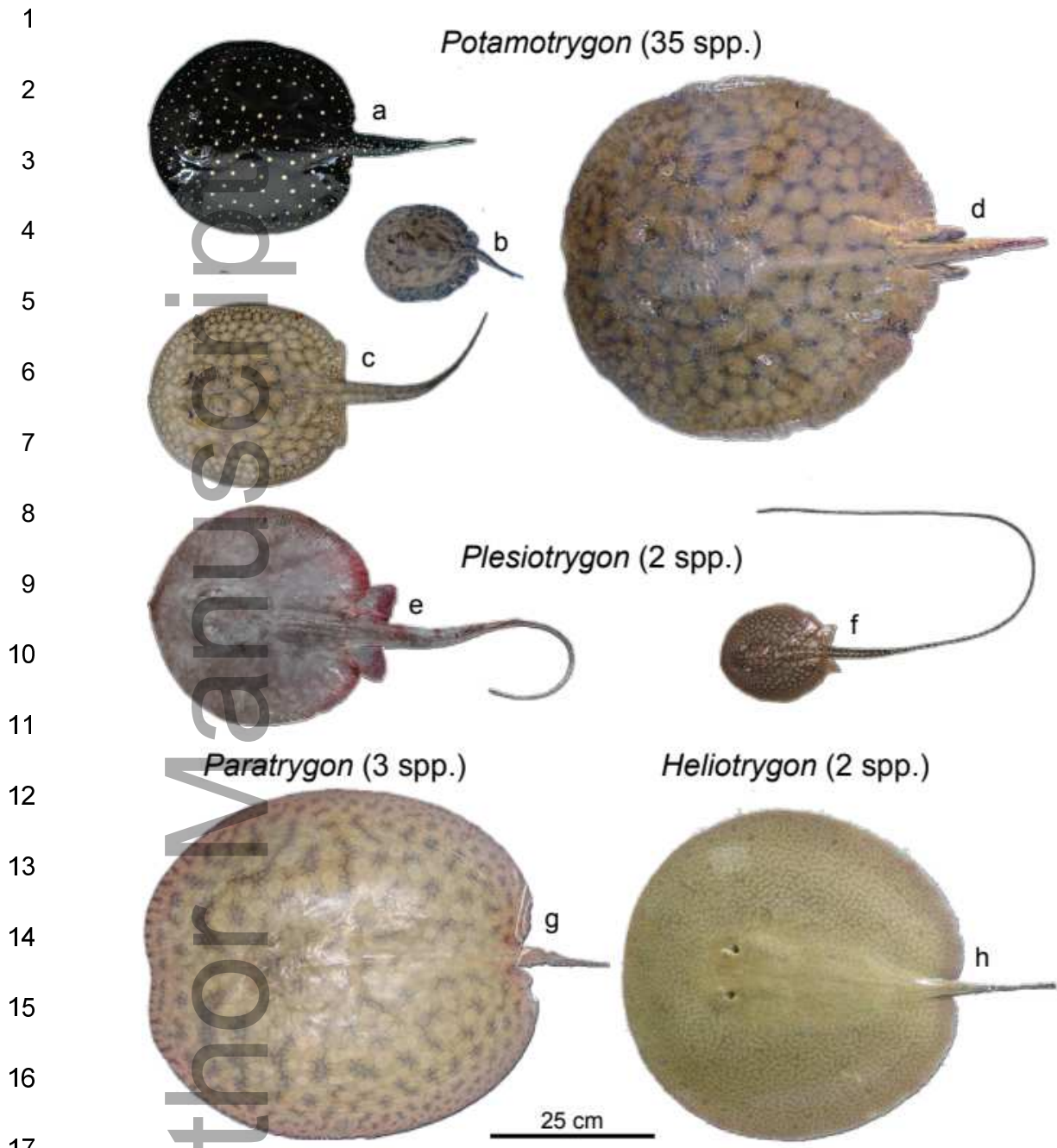dark gray. Map in EPSG:4326 (WGS84) projection.

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FIGURE 2: Representative size and morphological diversity of the four genera of Potamotrygoninae, with currently recognized numbers of species for each genus. **a**: *Potamotrygon albimaculata;* **b**: *Potamotrygon wallacei; **c**: Potamotrygon orbignyi; **d**:*

1　*Potamotrygon brachyura; **e**: Plesiotrygon iwamae; **f**: Plesiotrygon nana; **g**: Paratrygon aiereba;*

2　***h**: Heliotrygon rosai*. Photos by Fernando Marques.

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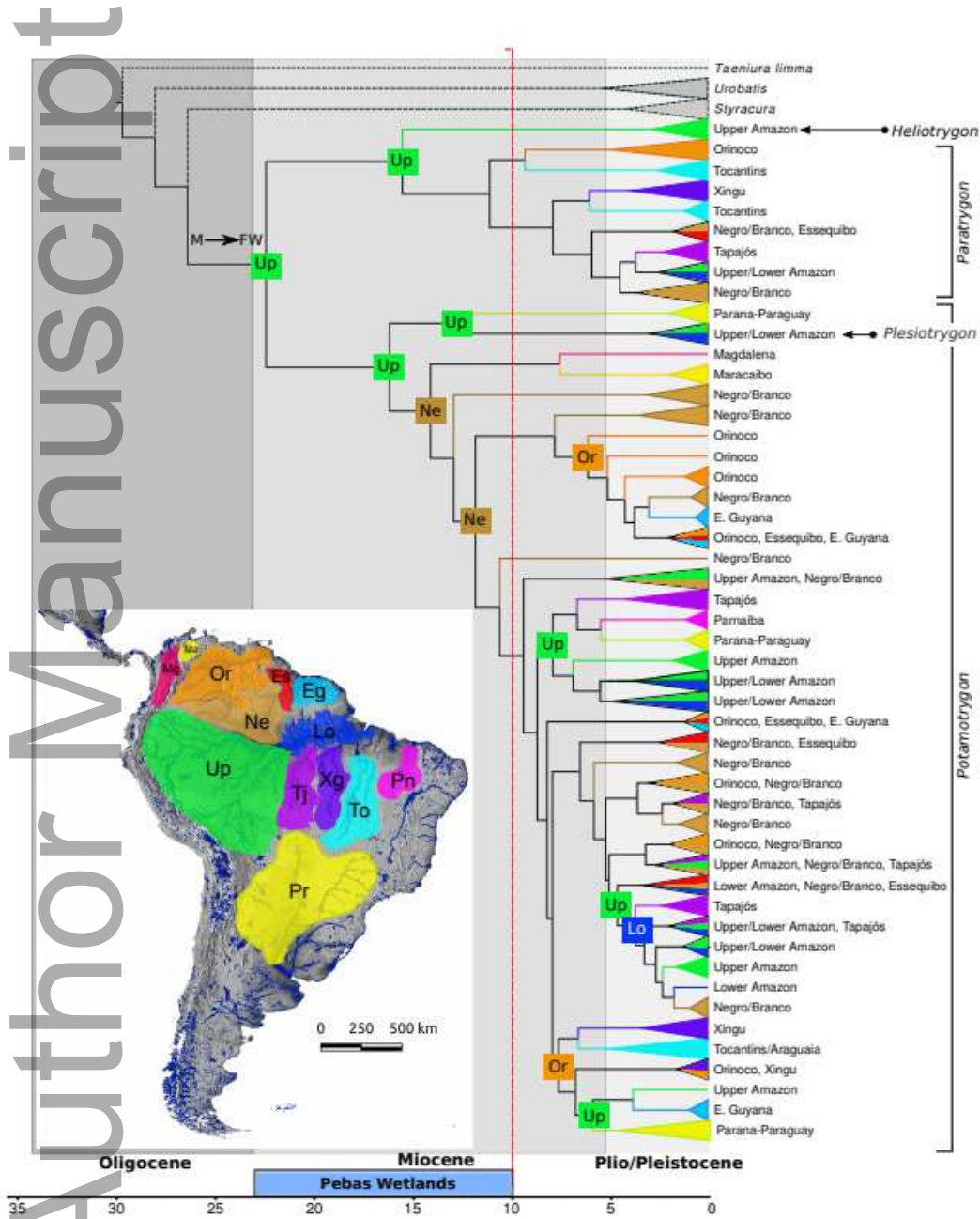
27　study. Terminals indicate biogeographical areas occupied. Selected ancestral area reconstructions

28　are shown (most probable estimated ancestral ranges according to 'BioGeoBEARS'), and

29　reconstructed position of marine to freshwater transition is indicated. Approximate time of Pebas

30　Wetlands is shown, with vertical line showing conclusion. Abbreviations as follows: Eg: Eastern

Guiana; Es: Essequibo; Lo: lower Amazon; Ma: Maracaibo; Mg: Magdalena; Nb: Negro-Branco;

Or: Orinoco; Pn: Parnaíba; Pr: Parana-Paraguay; Tj: Tapajós; Xg: Xingu; To: Tocantins-

Araguaia; Up: upper Amazon. Map in EPSG:4326 (WGS84) projection

1
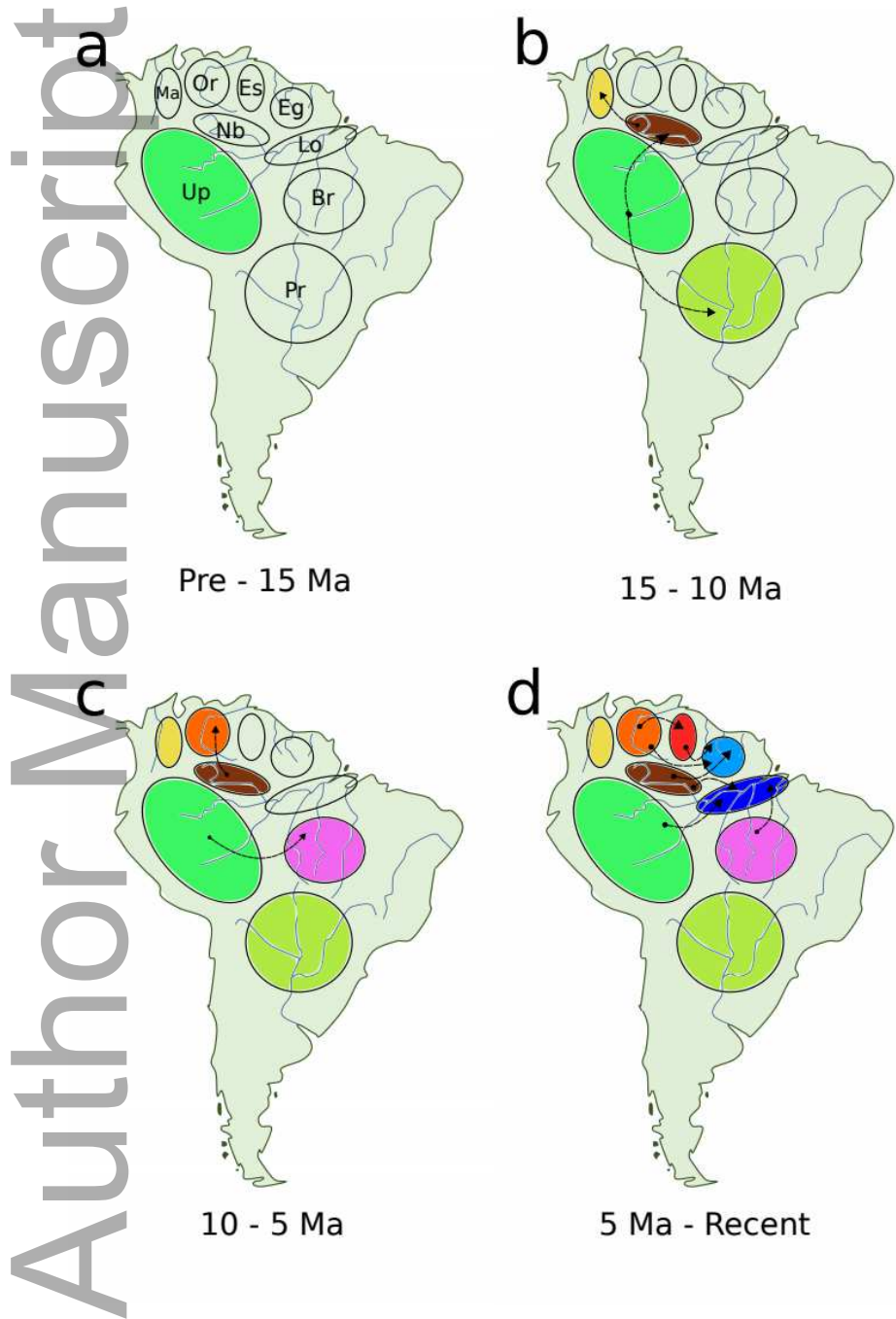
FIGURE 4: Graphical summary of overall changes in the distribution of Potamotrygoninae in South America over time, based on 'BioGeoBEARS' analysis. Only the first colonization(s) of each new area is shown, with arrows indicating the direction of colonization. Abbreviations (shown in panel a) as follows: Br: Brazilian Shield; Eg: Eastern Guiana; Es: Essequibo; Lo: lower Amazon; Ma: Maracaibo; Nb: Negro-Branco; Or: Orinoco; Pr: Parana-Paraguay; Up: upper Amazon. **a**. Before 15 Ma; **b**. Early / middle Miocene (Approx. 15 –10 Ma); **c**. Late Miocene/Pliocene (Approx. 10 – 5 Ma); **d**. Pliocene/ Recent (Approx. 5 Ma -Recent). Map in EPSG:4326 (WGS84) projection.

DATA AVAILABILITY STATEMENT:

Sequences used in this study are available on GenBank under accession codes MW475721 – MW476061 for *coI*, MW480896 – MW481205 for *its1*, MW481727 – MW482078 for *cytb* and MW482079 - MW482433 for *atpase*.

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24 242.

25

26 **BIOSKETCH**

27 João Pedro Fontenelle is interested in the biogeography of the Neotropical region. This work is a

28 component of his PhD work at UTSC on the evolution and diversification of the stingrays of the

1   subfamily Potamotrygoninae. He and the other authors collaborate on questions pertaining

2   freshwater fish diversity and evolution.

3   Author contributions: JPF and NRL conceived the ideas; JPF, NRL, MAK and FPLM all

4   conducted field work and collected the data with additional material from museum collections;

5   JPF, MAK and FPLM analyzed the data; JPF led the writing with assistance from NRL, MAK

6   and FPLM.
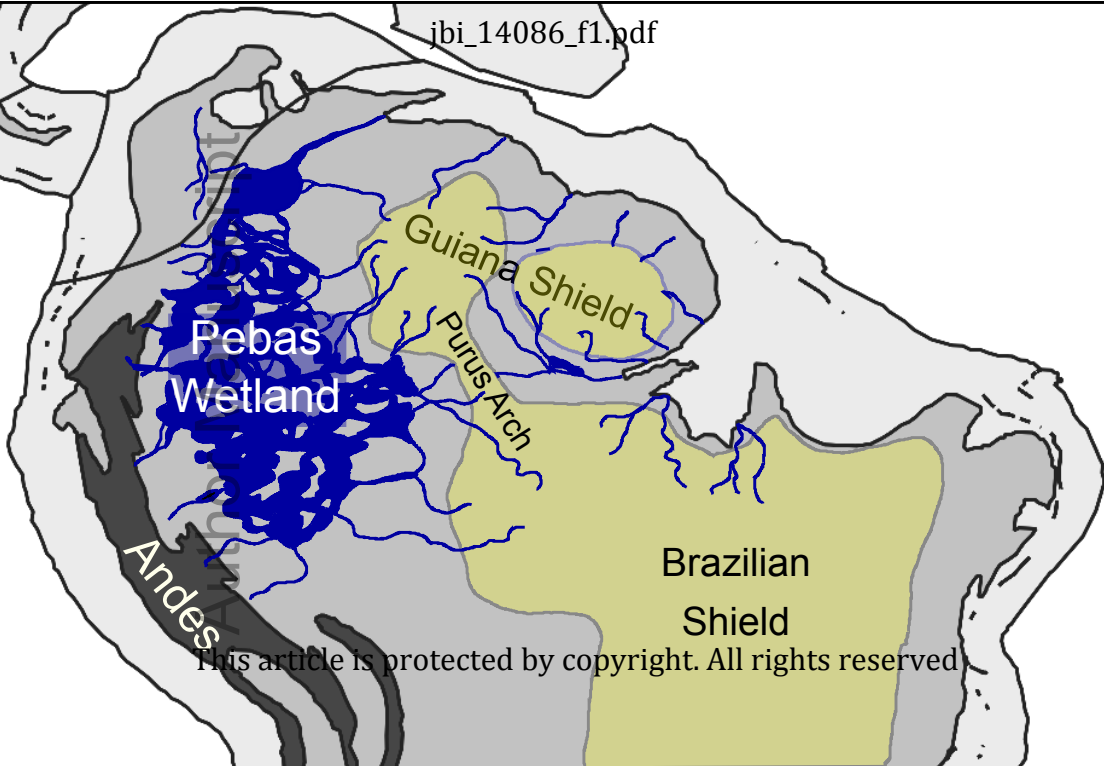
Guiana Shield

Pebas
Wetland

Purus Arch

Brazilian

Shield

Andes

Potamotrygon (35 spp.)

a

d

b

c

Plesiotrygon (2 spp.)

e

f

Paratrygon (3 spp.)

Heliotrygon (2 spp.)

g

h

25 cm
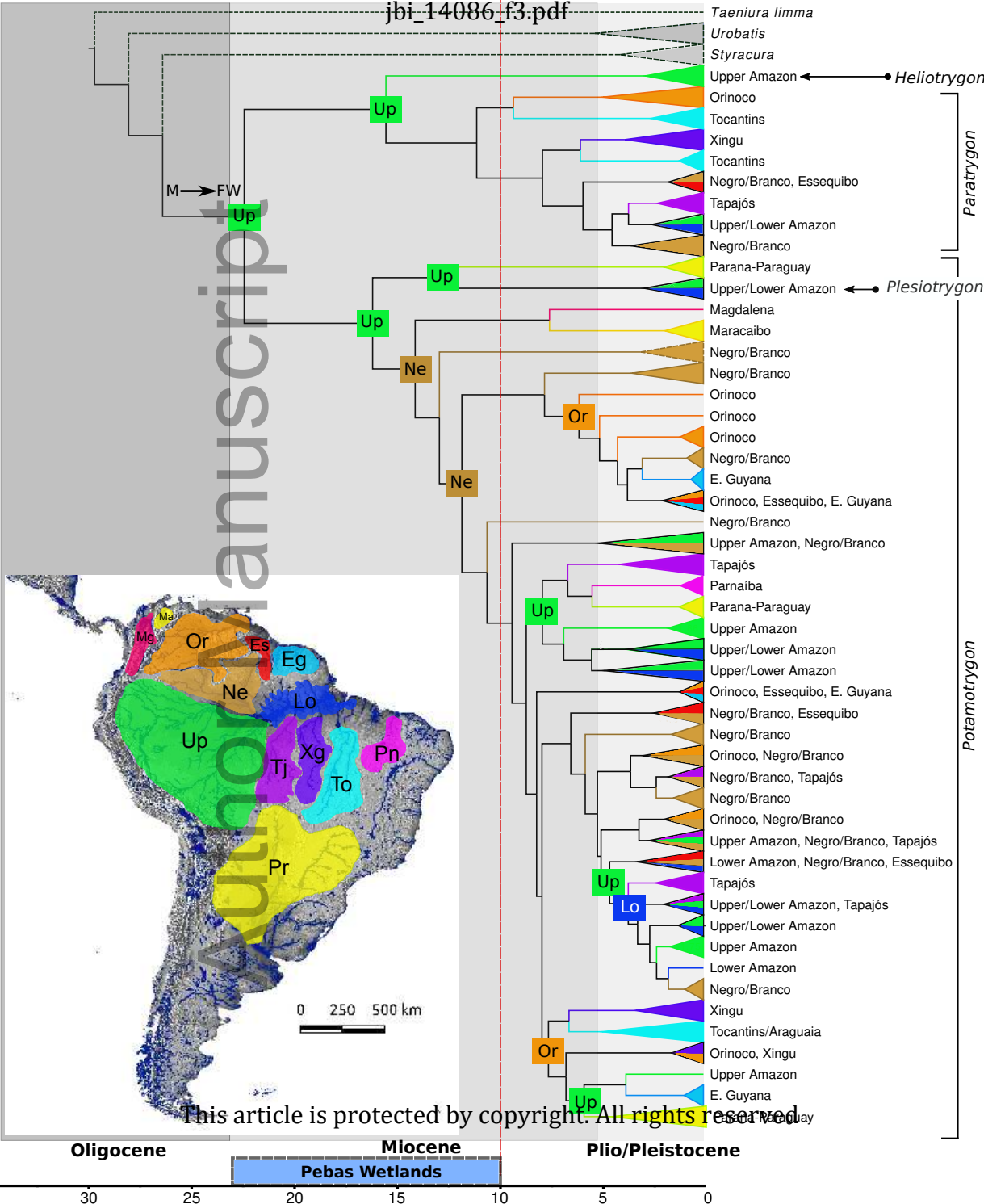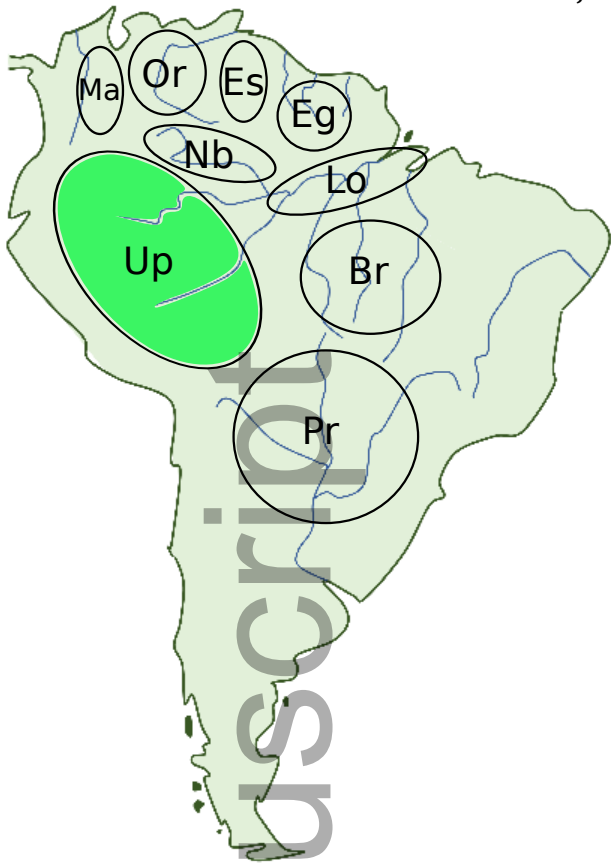
a Pre - 15 Ma
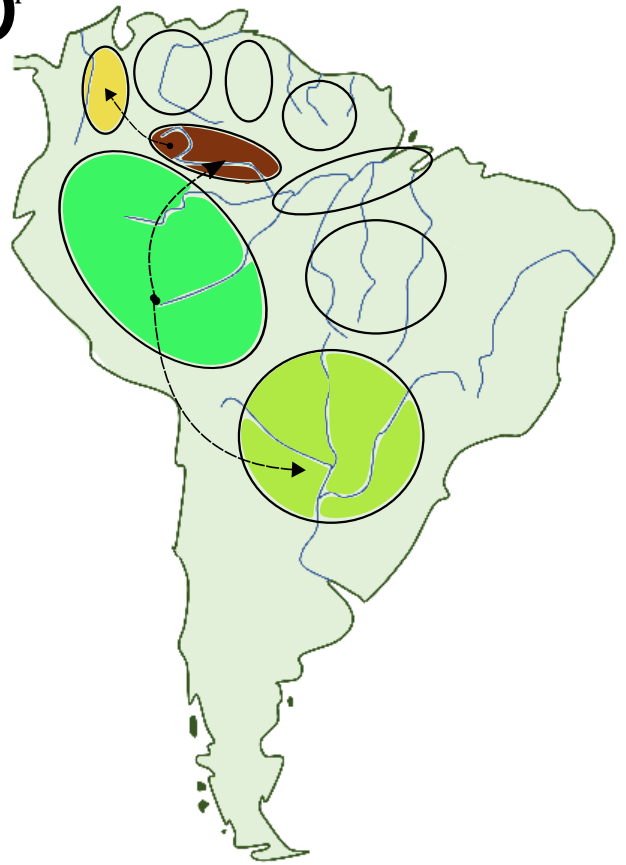
b 15 - 10 Ma

c 10 - 5 Ma

d 5 Ma - Recent