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Ecological and Phenotypic Diversification after a Continental Invasion in Neotropical Freshwater Stingrays

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Synopsis Habitat transitions are key potential explanations for why some lineages have diversified and others have not—from *Anolis* lizards to Darwin's finches. The ecological ramifications of marine-to-freshwater transitions for fishes suggest evolutionary contingency: some lineages maintain their ancestral niches in novel habitats (niche conservatism), whereas others alter their ecological role. However, few studies have considered phenotypic, ecological, and lineage diversification concurrently to explore this issue. Here, we investigated the macroevolutionary history of the taxonomically and ecologically diverse Neotropical freshwater river rays (subfamily Potamotrygoninae), which invaded and diversified in the Amazon and other South American rivers during the late Oligocene to early Miocene. We generated a time-calibrated, multi-gene phylogeny for Potamotrygoninae and reconstructed evolutionary patterns of diet specialization. We measured functional morphological traits relevant for feeding and used comparative phylogenetic methods to examine how feeding morphology diversified over time. Potamotrygonine trophic and phenotypic diversity are evenly partitioned (non-overlapping) among internal clades for most of their history, until 20–16 mya, when more recent diversification suggests increasing overlap among phenotypes. Specialized piscivores (*Heliotrygon* and *Paratrygon*) evolved early in the history of freshwater stingrays, while later trophic specialization (molluscivory, insectivory, and crustacivory) evolved in the genus *Potamotrygon*. Potamotrygonins demonstrate ecological niche lability in diets and feeding apparatus; however, diversification has mostly been a gradual process through time. We suggest that competition is unlikely to have limited the potamotrygonine invasion and diversification in South America.

Introduction

An important goal of evolutionary biology is to understand why some lineages ecologically diversify, while others do not. In many examples of the former, a habitat transition can offer access to resources not previously available, either because these resources themselves are novel or because competitors or predators are absent (Grant 1981; Losos et al. 1997; Schluter 2000). The ecological opportunity afforded to lineages that transition to new habitats can provide means of breaking phylogenetic niche conservatism (PNC) and generating evolutionary novelty (Wiens and Graham 2005; Martin and Wainwright 2013). In other words, an invading lineage may diversify in ecology and phenotype away from close relatives remaining in ancestral habitats (i.e., niche lability; Losos et al. 2003; Kozak and Wiens 2006; Wiens et al. 2010). Examples of animals diversifying after habitat transitions abound: from cichlids colonizing rift lakes and rivers (Wagner et al. 2012; Lopez-Fernandez et al. 2013), Caribbean *Anolis* lizards and Darwin's finches colonizing offshore islands (Pinto et al. 2008; but see Burns et al. 2002; Huie et al. 2021), to icefishes (notothenioids) and snailfishes (Liparidae) invading Antarctic oceans (Eastman and Clarke 1998;

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Matschiner et al. 2015)—habitat transitions play a recurrent role in prefacing ecological diversification in vertebrates.

However, not all habitat transitions offer opportunity for diversification. For example, some marine lineages, upon entering freshwater, diversify ecologically while others do not (or only diversify in terms of species richness; Vega and Wiens 2012; Bloom et al. 2013). Conversely, invasions in reverse, from freshwater to saltwater, are exceedingly rare (Betancur et al. 2015; Davis et al. 2017). Herring and anchovies, as well as cottoid fishes like sculpins, exhibit patterns of PNC, with freshwater and saltwater lineages occupying similar niches (Bloom and Lovejoy 2012; Buser et al. 2019). These lineages have speciated, sometimes prodigiously, but their ecologies and phenotypes remain like their marine sister taxa. In contrast, some species of freshwater grunters (Terapontidae) and needlefishes (Belonidae) significantly altered their phenotypes and trophic niches upon shifting to freshwater habitats (Davis et al. 2012, 2014; Kolmann et al. 2020). Of these examples, only grunters exhibit increased lineage diversification compared to their marine relatives (Davis et al. 2012, 2014). Repeated invasions of freshwater by different fish lineages therefore offer unique opportunities to investigate how different factors shape evolutionary and ecological outcomes.

South America is host to at least 12 fish lineages that were ancestrally marine but now occupy freshwater systems (Bloom and Lovejoy 2017), from flounders (Pleuronectiformes) and silversides (Atheriniformes) to pipefishes (Syngnathiformes) (Hughes et al. 2020). Several other animal groups (e.g., dolphins, sirenids, decapods, and so on) made similar ecological shifts. One of the most striking examples of marine invaders to South America are the river rays. These stingrays (Potamotrygoninae) originated sometime during the mid-Eocene to early Miocene (i.e., 46–22.5 Mya), when marine stingrays invaded ancient brackish lowlands in South America (Lovejoy et al. 1998, 2006; Fontenelle et al. 2021a). Potamotrygonines include 40+ species in four genera and although they are not the sole freshwater elasmobranchs (sharks and rays), they display the highest species richness of any extant freshwater elasmobranch lineage. They are also the only extant elasmobranch lineage to diversify solely in freshwater, whereas most other freshwater sharks and rays (e.g., *Carcharhinus leucas,* sawfishes, *Himantura*, and *Glyphis* spp.) are technically euryhaline (Thorson and Watson 1975). Notably, there are also insectivorous potamotrygonines, a dietary mode that is unknown among all other sharks and rays (Shibuya et al. 2009; Kolmann et al. 2016). How have ecology and phenotype diverged in these freshwater stingrays, relative to their marine sister lineage?

To reconstruct the evolution of dietary mode and corresponding phenotypic feeding adaptations in potamotrygonines, a robust phylogeny is required. To date, most phylogenies of Potamotryoninae have been limited by reliance on single genes (Lovejoy et al. 2006; Carvalho and Lovejoy 2011), limited sets of genes (i.e., mitochondria; Toffoli et al. 2008; Garcia et al. 2015; Bloom and Lovejoy 2017), limited taxon sampling (Kirchhoff et al. 2017), or issues with alpha taxonomy (but see Fontenelle et al. 2021a, 2021b). A robust, time-calibrated phylogeny is required to trace the evolution of diet and morphology, and to quantitatively test whether potamotrygonines demonstrate patterns of (A) niche lability, as suggested by their ecological diversity; or (B) niche conservatism, as seems common for other marine-invaders of South America (Lovejoy and Collette 2001; Yokoyama and Goto 2005; Betancur 2010; Buser et al. 2019).

Our present study therefore had four primary objectives: (1) generate a robust, time-calibrated molecular phylogeny for Potamotrygonidae, (2) couple the phylogeny with published diet data to determine how often different trophic guilds have evolved, (3) document diversification of feeding phenotypes among potamotrygonines, and (4) explore whether freshwater rays exhibit patterns of niche conservatism or niche lability. We expect that South American river rays have capitalized on novel resources in freshwaters and will exhibit considerable niche lability, as suggested by their pronounced ecological and phenotypic diversity, as well as evidence of trophic specialization (Shibuya et al. 2009; Kolmann et al. 2016; Rutledge et al. 2019). Since foraging is time-consuming and demands high net energy intake and expenditure, study of feeding behavior in a functional context is particularly relevant to ecological diversification (Wainwright et al. 2004).

Materials and methods

Taxon sampling, DNA extraction, amplification, and sequencing

Our dataset includes 21 of 40 described species from all the currently described genera (see Supplementary Materials). We included multiple specimens of some polymorphic and widely distributed species like Paratrygon aiereba (Loboda et al. 2021), and *Potamotrygon motoro*, as well as specimens of an undescribed species, *Potamotrygon* sp. “Demerara” from Guyana. For outgroups, we included seven species, including the sister lineage of Potamotrygoninae (*Styracura schmardae* and *S. pacifica*) and more distantly related dasyatids and urotrygonids (*Pteroplatytrygon violacea, Dasyatis* *geijskesi, D. guttata, Taeniura lymma, Neotrygon kuhlii, Urotrygon simulatrix, and Urobatis halleri*; see Supplementary Table S1). Specimens were obtained from museum collections and when collected personally by the authors were done so according to University of Toronto Scarborough UACC protocol (# 20010982). Muscle or fin tissue was stored in 95% ethanol. A complete outline of DNA extraction, amplification, and sequencing protocols can be found in the Supplementary Materials.

Alignment and phylogenetic analysis

Forward and reverse sequences were used to construct consensus sequences, which were then edited by trimming the distal ends of ambiguous base-pair (bp) calls in GENEIOUS v6 (Kearse et al. 2012). The resulting sequences were aligned in GENEIOUS using the MUSCLE plugin and protein-coding genes were translated to amino acids to confirm an open reading frame. Aligned sequences were then used to generate the following datasets: (1) the three mitochondrial genes (*co1, ATP6* and part of ATP8, *cytb*) combined, (2) *RAG1*, (3) *ENC1*, (4) *SCFD2*, and (5) *ITS 1* and *ITS 2*. We also concatenated all data to form a single matrix of 8270 bp for 38 taxa. We used PartitionFinder (Lanfear et al. 2012) to determine the best-fit model of molecular evolution and partition schema simultaneously for each gene. Models were selected using a Bayesian Information Criterion (BIC), using a “greedy” search scheme. Partitioned maximum likelihood (ML) tree searches were performed with GTR + G models for each partition using the program RAxML (Stamatakis 2006) to create our gene trees. ML bootstrap estimates were based on 1000 replicates using the rapid bootstrapping algorithm in RAxML (Supplementary Appendix II).

We used BEAST (v. 1.8.3; Drummond and Rambaut 2007) to simultaneously estimate phylogeny and diversification times using a Bayesian framework. We partitioned our data according to gene, with unlinked parameters and default priors. We used an uncorrelated lognormal tree prior and a birth–death prior for our expectation of cladogenesis. We ran two separate BEAST analyses for 100 million generations, sampling every 5000 generations, and automatically discarding the first 10% of trees as burn-in. We used Tracer 1.6 (Drummond and Rambaut 2007) to assess convergence and mixing of runs and to verify that effective sample sizes (ESS) were > 200 for all parameters. An additional 20 million generations from the beginning of each run were discarded as burn-in. To determine divergence times, we used two fossil and two geological time calibrations (see Supplementary Appendix II for details). The tree with the highest posterior probability was retained for comparative analyses described below.

Trait evolution, CT-imaging, and functional morphology

Diet data were obtained from the literature and other sources (Table 1). Species were grouped into trophic guilds (e.g., piscivore and insectivore) if > 75% of that species’ diet was reported as being a particular prey type (e.g., crustaceans, insects, and so on). We treated diet as a multistate discrete character and we inferred the evolutionary history of this character across the phylogeny using Bayesian stochastic character mapping (Huelsenbeck et al. 2003; Bollback 2006) to estimate changes in diet states across the branches of the phylogeny, which is useful in estimating rates of transitions between diet states and the relative residency time taxa spent in each state (diet guilds), using the make.simmap function in phytools (Revell 2012).

Morphological traits pertaining to feeding performance were measured from micro-computed tomography scans (X-Tek HMXST-225 Micro-CT, Center for Nanoscale Systems, Harvard University) or 2D radiographic imaging of museum specimens, appropriate for batoids given their flattened profile (ANSP, ROM, MCZ, MZUSP, and CUMV). We used a 1-mm Aluminum filter and scanning parameters varied from 70 from 120 kV and 95 to 400 uA, with exposures standardized to 1000 ms. We anticipate that morphometric differences among species are greater than differences within species.

Functional characters were chosen based on their demonstrable link with performance or behavior as indicated by studies of feeding functional morphology (Dean et al. 2007; Anderson 2009; Arbour and López-Fernández 2013; Balaban et al. 2015; Shibuya et al. (2012); Kolmann et al. 2018, 2016; Rutledge et al. 2019; Feilich and López-Fernández 2019) and described in detail in Supplementary Appendix III. The following phenotypic variables (Fig. 1) were measured, describing the shapes and relationships of the jaw cartilages (upper and lower), the hyomandibular cartilage (linking the jaws to the cranium), and the propterygium (supporting the base of the pectoral fins): (a) mechanical advantage (MA), describing jaw leverage; several variables related to (b)–(d) jaw shape and function: (b) maximum jaw depth (upper and lower), (c) jaw aspect ratio (upper and lower), and (d) symphyseal height (lower jaw); (e) occlusal offset, a measure of the alignment of the occlusal surface and jaw joint; (f) lower jaw dentition length, describing the size of the toothed area; (g) hyomandibular offset, a proxy for jaw protrusility, (h) jaw adductor fossa length, a proxy

Table 1 Summary of sources for potamotrygonine dietary information from the literature

Genus	Species	Diet	Region	Reference
Heliotrygon	*gomesi*	Piscivore	Upper Amazon	Lucanus (pers. comm.)
Paratrygon	*aiereba*	Piscivore	Amazon Basin	Lasso et al. (1996); Barbarino and Lasso (2005); Shibuya et al. (2009)
Plesiotrygon	*iwamae*	Crustacivore	Amazon Basin	Charvet-Almeida (2001); Shibuya et al. (2016)
Plesiotrygon	*nana*	Insectivore	Upper Amazon	Charvet-Almeida (2001); Lasso et al. (2013)
Potamotrygon	*boesemani*	Omnivore, poorly known	Guiana Shield	Rosa et al. (2008); Lucanus (pers. comm.); Kolmann (pers. obs.)
Potamotrygon	*brachyura*	Omnivore	Parana-Paraguay	Achenbach and Achenbach (1976); López-Rodríguez et al. (2019)
Potamotrygon	*falkneri*	Omnivore	Parana-Paraguay	Lonardoni et al. (2006); Silva and Uieda (2007); Pagliarini et al. (2020)
Potamotrygon	*henlei*	Molluscivore	Lower Amazon	Pantano-Neto (2001); Charvet-Almeida (2005)
Potamotrygon	*histrix*	Omnivore	Parana-Paraguay	Achenbach and Achenbach (1976); Lasso et al. (2013); Shibuya et al. (2016)
Potamotrygon	*leopoldi*	Molluscivore	Lower Amazon	Charvet-Almeida (2005); Lasso et al. (2013)
Potamotrygon	*magdalenae*	Insectivore	Orinoco	Ramos-Socha and Grijalba-Bendeck (2011); Márquez-Velásquez et al. (2019)
Potamotrygon	*marinae*	Omnivore, poorly known	Guiana Shield	Deynat (2006); Lucanus (pers. comm.); Kolmann (pers. obs.)
Potamotrygon	*motoro* Ucayali	Crustacivore	Upper Amazon	Shibuya et al. (2009); Almeida et al. (2010); Vasconcelos and Sá-Oliveira (2011)
Potamotrygon	*motoro* Orinoco	Crustacivore	Orinoco	Almeida et al. (2010); Vasconcelos and Sá-Oliveira (2011)
Potamotrygon	*motoro* Xingu	Crustacivore	Lower Amazon	Almeida et al. (2010); Vasconcelos and Sá-Oliveira (2011)
Potamotrygon	*orbignyi*	Insectivore	Amazon Basin	Shibuya et al. (2009); Moro et al. (2011); de Gama & de Souza Rosa 2015 and 2020
Potamotrygon	*schroederi*	Omnivore	Orinoco	Araújo (1998); Lasso et al. (2013)
Potamotrygon	*scobina*	Crustacivore	Amazon Basin	Braganca et al. (2004); de Gama & de Souza Rosa 2015 and 2020
Potamotrygon	*signata*	Insectivore	Parnaiba	Moro et al. (2012)
Potamotrygon	*tigrina*	Omnivore	Upper Amazon	Carvalho & Lovejoy, 2011; Lasso et al. (2013)
Potamotrygon	*wallacei*	Omnivore	Upper Amazon	Shibuya et al. (2009); Shibuya et al. (2016)
Potamotrygon	*yepezi*	Insectivore	Orinoco	Araújo (1998); Lasso et al. (2013); Lasso & Sanchez-Duarte (2011)
Potamotrygon	sp. 'Demerara'	Omnivore	Guiana Shield	Kolmann (personal observation of gut contents)

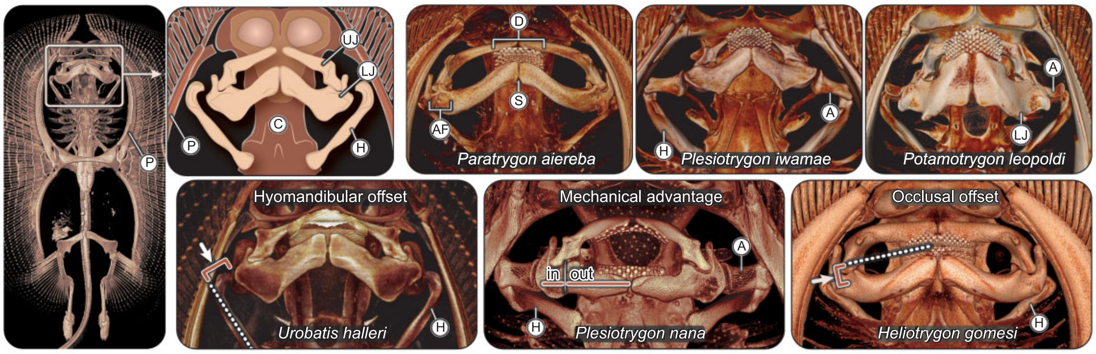

Fig. 1 Skeletal anatomy and measured phenotypic traits in stingray genera. Left: whole skeleton of *U. halleri*, with box drawn around the region of interest for right-side panels. Top right: anatomical schematic of potamotrygonid cranial anatomy and three representative ray species with labeled anatomical characters of interest. Bottom right: measurements of hyomandibular offset, MA, and occlusal offset. "In" and "Out" denote the in-lever and out-lever measurements, respectively. Solid lines in occlusal and hyomandibular offset figures denote the measured value, distances tangent to the dotted line. Anatomical lettering as follows: (A) angular cartilage, (AF) jaw adductor fossa, (C) chondrocranium, (D) dentition, (H) hyomandibula, (LJ) lower jaw (P), pectoral propterygium, (S) mandibular symphysis, and (UJ) upper jaw.

for muscle attachment size; (i) gape width; (j) jaw–hyomandibulae offset, a proxy for joint range of motion; (k) hyomandibular cartilage aspect ratio, a proxy for hyomandibular gracility; and (l) propterygial aspect ratio, a proxy for rigidity of the skeletal elements that anchor the pectoral fins, which are used during both swimming and feeding (Wilga et al. 2012; Kolmann et al. 2016). All distance variables were measured in centimeters; phenotypic measurements were then standardized relative to chondrocranial length, thereby adjusting for body size. Descriptions of measurement approaches and functional connotations of the above measures are described in Supplementary Appendix III.

To visualize the major axes of phenotypic variation in biomechanical attributes across taxa and to characterize species along ecological axes (diet), we performed a phylogenetically explicit Principal Components Analysis (Revell 2009) using the phyl.pca function in the phytools R package (v. 0.6–99; Revell 2012) and projected the phylogeny onto this scatterplot of PC values to form a phylomorphospace (Sidlauskas 2008; Fig. 4). Finally, we used Blomberg's *K* to estimate the phylogenetic signal of individual feeding traits, using the phylosig function and 1000 replicates (phytools; Revell 2012). While phylogenetic signal is not synonymous with PNC (Losos 2008), it can be a prerequisite of diagnosing a pattern of PNC (Crisp and Cook 2012). Values of *K* greater than 1.0 indicate that species traits are more similar than expected under Brownian motion and may suggest a pattern of niche conservatism (Blomberg et al. 2003; Cooper et al. 2010).

Lineage, ecological, and phenotypic diversification analyses

We visualized and then quantified historical changes in species accumulation using lineage-through-time (LTT) plots and the gamma statistic, respectively (Pybus and Harvey 2000). These methods illustrate whether lineage diversification has slowed through time, has accelerated, or holds constant with extinction (but, see Fordyce 2010). However, early bursts in lineage accumulation may be rare or arise from sampling biases, so do not necessarily demonstrate a connection between ecological and evolutionary processes (Ingram et al. 2012; Pennell et al. 2012). We also assessed how phenotypic and ecological (diet) diversity have changed in parallel to lineage diversification using a disparity-through-time (DTT) approach. We estimated diet and phenotypic disparity (the average squared Euclidean distance among all pairs of data points; Harmon et al. 2003; Slater et al. 2010) across our time-calibrated tree, relative to a simulated Brownian motion model of phenotypic evolution iterated over 10,000 generations, following Murrell (2018) (*dtt1* function modified from geiger; Pennell et al. 2014). We then calculated the phenotypic disparity index (MDI) for potamotrygonines, i.e., the difference in area between the simulated Brownian curve and our clade's observed phenotypic disparity (Slater and Harmon 2013). However, since MDI estimations at multiple time points are plagued by a high false-positive rate, we used Murrell (2018)'s two-tailed rank envelope method to test for significance between the Brownian and experimental MDI values.

We also explored whether competition with entrenched freshwater species may limit species richness in freshwater rays, following the method of Betancur et al. (2012). We examined whether stingray species richness across South American basins and in exemplar river basins, negatively or positively correlates with entrenched species diversity in those rivers. We surveyed the primary literature to determine the number of species in each of the 13 major South American drainages (according to Reis et al. 2016) as well as in 15 smaller river basins (Garcia et al. 2015; Dagosta and De Pinna 2017, 2019; Fontenelle et al. 2021a; see Supplementary Appendix IV). We then regressed potamotrygonine species richness against the overall species richness for each particular basin using OLS regression in R (Supplementary Appendix IV).

Results

Molecular data and phylogenetic relationships

The total molecular dataset resulted in a concatenated matrix of 8270 bp. This final matrix includes data for more than 75% of all extant potamotrygonid taxa (including marine *Styracura*) and comprises data from 36 species including outgroups (Supplementary Appendix V). BEAST was run twice with identical results recovered from each run; the resulting tree is shown in Fig. 2. Our analyses supported the monophyly of the family Potamotrygonidae with the amphi-American *Styracura, S. pacifica*, and *S. schmardae* (Styracurinae), recovered as the sister taxon to freshwater potamotrygonines. We also recovered separation of freshwater potamotrygonines into two major clades: [*Plesiotrygon* + *Potamotrygon*] and [*Heliotrygon* + *Paratrygon*]. *Potamotrygon* is paraphyletic since *Plesiotrygon* spp. are nested as sister to *Potamotrygon brachyura*, with these taxa diverging before the split of the remaining *Potamotrygon*. Marine and freshwater potamotrygonids split from one another circa 41.0 – 44.0 Mya (Fig. 2; Supplementary Figures), while the separation of freshwater genera occurred between ca. 34.0 and 16.0 Mya. The divergence between *Paratrygon* + *Heliotrygon* from other potamotrygonines occurred between 34.0 and 23.0 Mya; while the split among *Potamotrygon* and

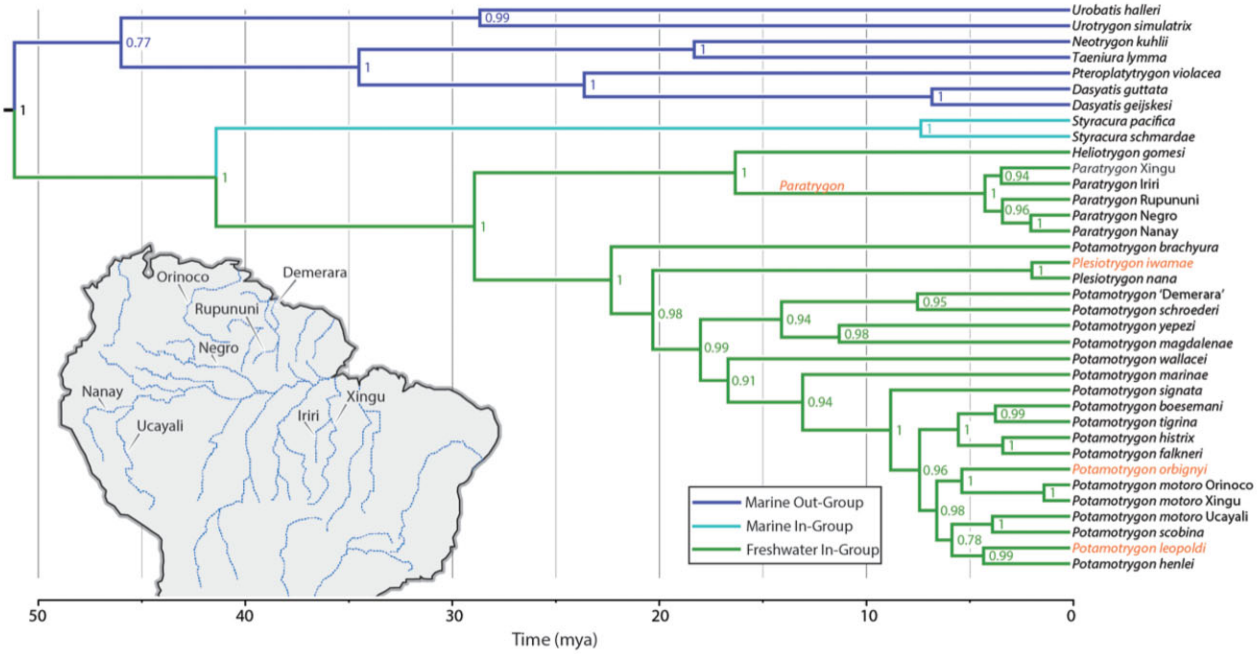

Fig. 2 Time-calibrated Bayesian phylogeny estimated from all nuclear and mitochondrial genes. Numbers above nodes represent posterior probabilities (PP). Dark blue branch colors designate distant marine outgroups (Dasyatoidea), light blue branches designate amphi-American *Styracura* (marine in-group, Styracurinae), while the freshwater in-group (Potamotrygoninae, proper) is colored green. Scale of the *x*-axis is in millions of years from the present. Map inset shows location of rivers from which we sampled polymorphic taxa (e.g., *P. aiereba* and *P. motoro*). Genera in orange font are dietary specialists and correspond to the cranial diagrams we use in Fig. 1.

Plesiotrygon occurred ca. 27.0 and 16.0 Mya (with *P. brachyura* splitting from all other species ca. 27.0 and 17.0 Mya).

Diet evolution

Stochastic character mapping estimates that omnivory is the predominant diet guild for potamotrygonines, with taxa spending three times longer in omnivorous roles than the next most common diet state, piscivory (57.5 vs. 178.7). However, transitions from omnivorous states to other diets were more frequent than the reverse (i.e., shifts from other diets to omnivory), suggesting that omnivory plays a transitory role in freshwater ray diet evolution (Table 2). Transitions from omnivory to crustacivory and from crustacivory to insectivory were the most frequent transitions (1.7 and 1.49, respectively). Transitions from piscivory to either molluscivory or crustacivory were the rarest (0.15 and 0.24, respectively; Table 2); however, these transitions were not actually observed. Novel dietary modes have arisen independently throughout the tree, typically from omnivorous ancestors (Fig. 3). However, molluscivores evolved from crustacivores, as did the insectivorous *Potamotrygon orbignyi*. Insectivory has evolved at least four times: in *Plesiotrygon nana* (although these data are based on anecdotal evidence; Lucanus, pers comm; Table 1); in the lineage composed *of Potamotrygon magdalenae* and *P. yepezi;* in *P. signata;* and in *P. orbignyi*. Other specialized feeding modes have arisen only once: piscivory evolved at the base of the *Paratrygon* + *Heliotrygon* clade and molluscivory evolved the lineage leading to *P. leopoldi* and *P. henlei*. The marine sister clade to potamotrygonines, *Styracura*, feed predominantly on coastal benthic invertebrates, namely decapods and annelids (O'Shea et al. 2021).

Feeding morphospace

The first three axes of the PCA represent 23.7%, 13.8%, and 11.9% of the variance in the data, respectively (Table 3). Species which loaded on the negative end of PC1 had larger gapes, robust jaws (thicker symphyses and lower aspect ratios), high jaw MAs, and stouter pectoral propterygia (Fig. 4). Species on the positive end of PC1 had longer hyomandibulae. Rays which loaded positively on PC2 had higher MA, larger gapes, scissor-like jaw occlusion (indicated by occlusal offset), stouter propterygia, and morphologies suggesting greater jaw protrusion. Species on the negative end of PC2 had higher posterior MA, larger jaw muscle attachment areas (adductor fossa length), more robust jaws, and more robust hyomandibulae (Table 3; Fig. 4).

Figure 4 shows trend in feeding morphology relative to diet. In general, most dietary guilds overlap in the

Table 2 Summary of Bayesian stochastic character mapping analyses of discrete diet traits across the time-scaled phylogeny for Potamotrygonidae

Diet guild	**Paired transitions**				**Mean total time spent per state:**
Omnivores:	Omni → Crust	Omni → Fish	Omni → Insect	Omni → Mollusks	178.7–**54**%
	1.704	1.008	2.705	0.609	
Piscivores:	Fish → Crust	Fish → Insect	Fish → Mollusks	Fish → Omni	57.5–**17**%
	0.242	0.34	0.153	0.604	
Insectivores:	Insect → Crust	Insect → Fish	Insect → Mollusks	Insect → Omni	43.5–**13**%
	0.821	0.312	0.167	0.724	
Crustacivores:	Crust → Fish	Crust → Insect	Crust → Mollusks	Crust → Omni	34.6–**10**%
	0.296	1.492	0.918	0.675	
Molluscivores:	Mollusks → Crust	Mollusks → Fish	Mollusks → Insect	Mollusks → Omni	16.1–**4**%
	0.287	0.265	0.298	0.421	

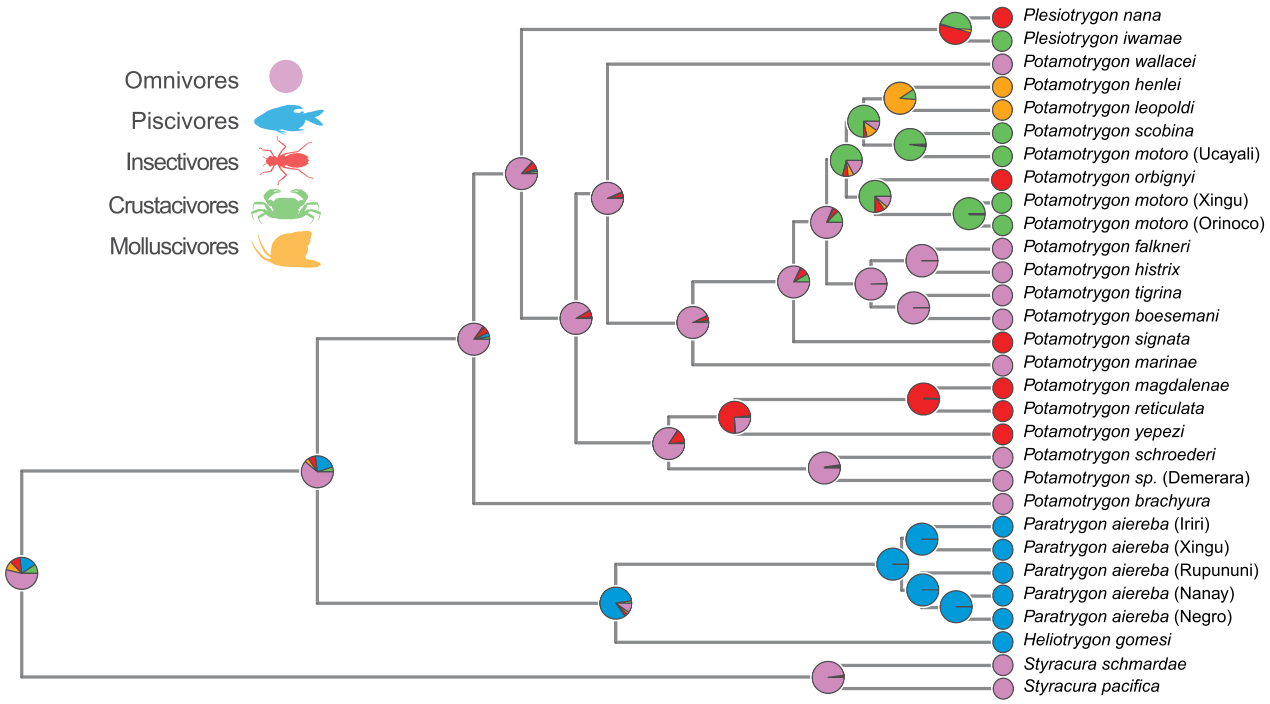

Fig. 3 The evolution of diet specialization in Potamotrygonidae as estimated by stochastic character mapping. Pink are omnivores, blue are piscivores, orange are molluscivores, red are insectivores, and green are crustacivores.

medial region of the morphospace, indicating an "average" morphology can be linked to a diversity of diets. Some piscivorous, molluscivorous and insectivorous taxa, however, were exceptions to this. The piscivorous taxa, *Paratrygon* and *Heliotrygon* (bottom region of PCA), were characterized by large gapes with few teeth, robust pectoral propterygia, and bow-like jaws (Fig. 4). Insectivores partially overlap with crustacivores and omnivores in morphospace, while molluscivores like *P. leopoldi* and *P. henlei* (left side of PCA) ordinate negatively on PC1, with trait loadings corresponding to robust jaws with high jaw leverage (Fig. 4). The obligate insectivore *P. orbignyi* (upper right of PCA; Moro et al. 2011) occurs in a region of morphospace characterized by trait loadings associated with cranial kinesis, having longer hyomandibulae and angular cartilages, as well as gracile jaws with much lower leverage. Conversely, other insectivores like *P. signata* (more centered in the PCA) are indistinguishable in morphospace from most omnivores and crustacivores.

Table 3 PCA variance and loadings for trait values used to generate phylomorphospaces. Trait abbreviations as follows: (a) *antMA/postMA*: anterior and posterior MA (leverage), (b) *LoSymH*, average jaw depth at the jaw symphyses (upper and lower), (c) *UJ/LJAspect*, upper (UJ) and lower (LJ) jaw aspect ratio, (d) *OccOff*, occlusional offset (cm—measure of jaw closing tooth occlusion), (e) *LoDentW*, lower dental row length, (f) *HyoOff*, hyomandibular offset (cm—linear measure of jaw protrusion), (g) *AddFossa*, jaw adductor fossa length (cm), (h) *GapeW*, gape width (cm), (i) *JawHyoGap*, jaw–hyomandibulae offset (joint range of motion between hyomandibulae and jaws), (j) *HyoAspect*, hyomandibular cartilage aspect ratio, and (k) *PropterAspect*, propterygia aspect ratio (as a proxy for rigidity of the skeletal elements that anchor the pectoral fins)

Traits ↓ PC Axes →	**PC1–23.7%**	**PC2–13.8%**	**PC3–11.9%**	**PC4–11.3%**	**PC5–8.0%**	**PC6–7.1%**
LoDentW	– 0.494	– 0.436	0.287	– 0.381	– 0.154	– 0.094
GapeW	– 0.427	0.159	0.032	0.650	0.532	0.006
LoSymH	– 0.778	0.046	– 0.299	0.027	0.193	0.000
JawHyoGap	0.257	0.511	0.144	– 0.368	0.136	– 0.510
HyoOff	0.272	0.480	0.218	– 0.536	0.176	0.125
AddFossa	– 0.567	– 0.160	0.339	– 0.417	– 0.134	0.329
OccOff	– 0.052	– 0.182	– 0.717	– 0.141	– 0.209	0.025
PropterArc	0.177	0.071	– 0.604	– 0.300	0.117	0.528
antMA	– 0.773	0.246	– 0.310	– 0.082	0.203	– 0.100
pstMA	– 0.858	– 0.029	0.007	– 0.374	0.125	– 0.226
UJAspect	– 0.505	0.671	0.037	0.103	– 0.295	0.179
LJAspect	– 0.199	0.605	– 0.124	0.232	– 0.644	– 0.108
HyoAspect	– 0.220	– 0.534	– 0.155	0.151	– 0.285	– 0.307
PropterAspect	– 0.336	– 0.061	0.553	0.274	– 0.105	0.363

Only 2 out of 14 feeding traits exhibited K values greater than one (jaw hyoid gap and propterygial aspect ratio), suggesting the vast majority (85%) of these feeding traits exhibit no or very little phylogenetic structure (average $K = 0.396$). A total of five of these traits (gape width, adductor fossa length, occlusal offset, and upper and lower jaw aspect ratios) had K scores statistically indistinguishable from zero, indicating no phylogenetic signal and practically no support for niche conservatism (Supplementary Appendix VI).

Patterns of lineage accumulation, ecological, and phenotypic diversification

Neither the LTT plots nor the γ-statistic indicate any evidence for a declining rate of lineage accumulation in potamotrygonines ($\gamma = 0.13$; $P = 0.89$; Fig. 5), as might be expected for clades undergoing an early burst of diversification (Harmon et al. 2003). Instead, ecological and phenotypic disparity gradually increase over time in potamotrygonines, beginning below Brownian expectations and suggesting little overlap in phenotype among genera (Harmon et al. 2003; Slater et al. 2010). Little overlap in phenotype among stingray lineages (i.e., low subclade disparity) is observed until ca. 20.0 – 16.0 Mya (overall MDI = 0.0744, rank envelope test: $P = 0.009$; P-interval = 0.0009, 0.0179), when overall disparity increases within clades and exceeds simulated levels, with a sharp upturn occurring from ca. 10.0 to 7.0 Mya (ca. 0.825–0.9 relative time). Changes in ecological disparity precede changes in phenotypic disparity, suggesting a lag between dietary specialization and phenotypic specialization of around ca. 4.0 – 5.0 Mya.

There was a strongly positive relationship between potamotrygonine species diversity and regional ichthyofaunal diversity ($R^2 = 0.943$; $P < 0.001$), while a much weaker positive correlation was found for potamotrygonine species diversity and river-specific ichthyofaunal diversity ($R^2 = 0.004$; $P = 0.803$; Supplementary Appendix IV). The upper Amazon (Rio Nanay) was a conspicuous outlier here, having both a high resident potamotrygonine diversity (12 species) and high diversity of other resident fish taxa (244 species, probably a considerable underestimate).

Discussion

We found, in agreement with others, that potamotrygonids are monophyletic and that freshwater potamotrygonines invaded South American freshwaters once 40 million years ago, then diversified solely in continental systems (Lovejoy et al. 1998; Bloom and Lovejoy 2017; Fontenelle et al. 2021a, 2021b). The first split within extant potamotrygonines occurred circa 28.0 Mya (34.0 – 23.0 95% HPD), between the *Paratrygon* + *Heliotrygon* and *Plesiotrygon* + *Potamotrygon* clades (Fig. 2). All extant genera were present circa 20.0 – 10.0 million years ago, concurrent with the

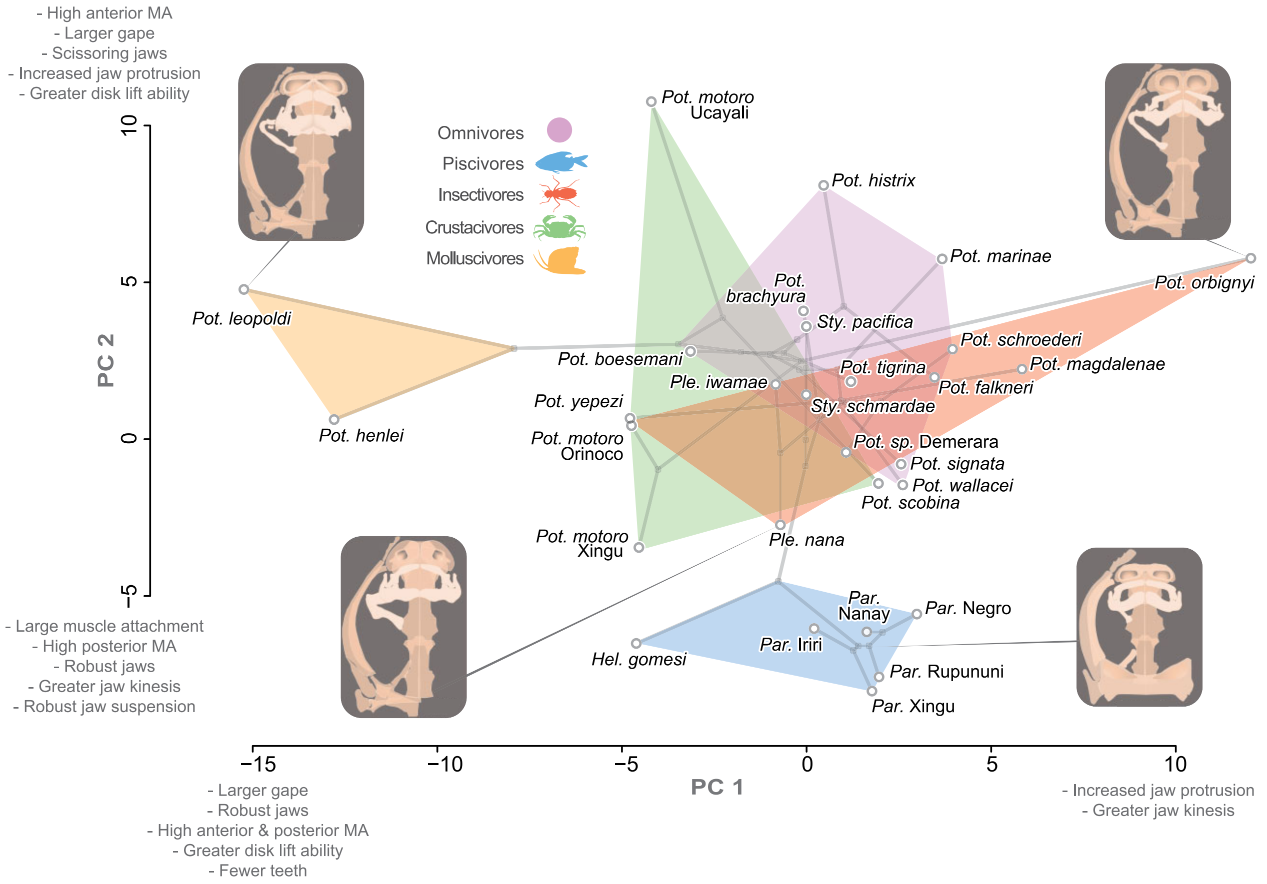

Fig. 4 Freshwater stingrays occupy a greater region of morphospace than their marine relatives (*Styracura*). Scatter plot of principal component scores onto which the phylogeny has been projected, i.e., phylomorphospace. Points represent a species' PC scores; text and arrows show the direction of how certain functional traits load on the PC axes. Convex hulls outline taxa sharing a particular diet guild (omnivores, piscivores, crustacivores, insectivores, and molluscivores). Skulls show differences in morphologies among major lineages and dietary guilds.

Pebas system (Hoorn et al. 2010), the hypothesized cradle for these stingrays and other marine invaders (Bloom and Lovejoy 2017; Fontenelle et al. 2021a). It is only after all extant genera were in place that ancestral state reconstructions suggest strong evidence for dietary specialization (piscivory) arising in the ancestor of *Paratrygon* + *Heliotrygon*. Shortly after (ca. 17.0 – 11.0 Mya), the first instances of insectivory arose in the lineage leading to extant taxa like *P. yepezi* and *P. magdalenae*, and then crustacivores and molluscivores evolved in the ancestors of lower Amazon taxa like *P. leopoldi* (ca. 10.0 – 15.0 Mya; Fig. 6).

Specialization on these resources began in the latter half of potamotrygonine history in freshwater; the predominant ancestral state for most lineages within Potamotrygoninae was omnivory. However, transition rates from omnivory to other diet guilds were asymmetrical, with freshwater rays transitioning *from* omnivory more often than they transitioned *to* omnivory (Table 2). This pattern suggests that omnivory acted as a niche "crossroads" rather than a dead-end (Pos et al. 2019; but see Burin et al. 2016; Egan et al. 2018). Ecological invaders that are also trophic generalists often see more success in novel habitats than dietary specialists (McKnight et al. 2017, and references therein), suggesting that omnivory is only a dead-end for organisms in contest with confamilials. Later instances of dietary specialization, like the evolution of molluscivory, arose from earlier specializations for feeding on decapod crustaceans. Perhaps feeding on relatively stiff-bodied crabs and shrimp (Hepburn et al. 1975) made it possible for these rays to eventually access still-harder prey like gastropods (Kolmann et al. 2015). Unlike crustacivores or omnivores, however, once evolving piscivory, *Paratrygon* and *Heliotrygon* did not explore other diet niches, suggesting that piscivory may be an evolutionary dead-end for these stingrays, as appears to be the case for other piscivorous fishes (Collar et al. 2009).

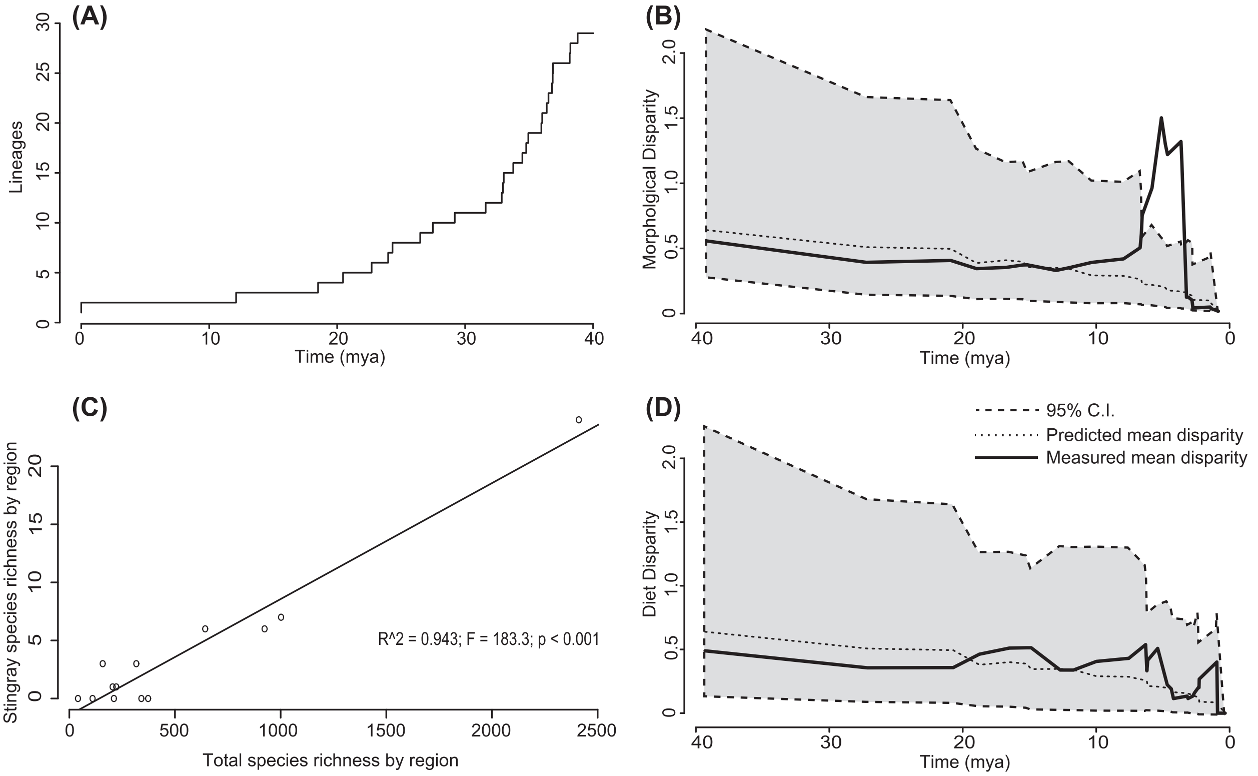

Fig. 5 LTT, DTT, and Species Richness plots for Potamotrygoninae. Top plot (**A**) denotes the actual relationship between lineage accumulation (untransformed) and time since the present. Upper right plot (**B**) is the trait disparity (feeding functional traits) through time. Bottom left plot (**C**) is potamotrygonine regional species richness vs. total fish species richness. The lower right plot (**D**) is the diet disparity through time. The two dashed lines represent the confidence intervals around simulated trait distributions, the dotted line is the Brownian expected disparity, while the solid lines represent the actual measured disparity of feeding traits and diet (respectively).

The diversity of diet (and morphology) in Potamotrygoninae can be appreciated by comparing the subfamily to the two marine coastal species in its sister lineage, *Styracura* (Carvalho et al., 2016). *Styracura schmardae* has a broad distribution through the Caribbean, but is generally found along the eastern coast of Central America, while it is even more poorly known congener, *S. pacifica*, occupies the western coast of Central and South America (Dalmau et al. 2020; O'Shea et al. 2020, 2021; Sales et al. 2020). These marine *Styracura* occupy the center of our phylomorphospace (Fig. 4), and thus more closely resemble the hypothetical ancestor of all potamotrygonids, rather than exploring the boundaries of trait space like their freshwater sister taxa have (Figs. 4 and 5). This suggests a pattern of evolutionary stasis for *Styracura*'s feeding apparatus and trophic niche, despite these species having had equal time to evolve, and having access to similar dietary resources (except insects), as freshwater potamotrygonines. In contrast, many freshwater potamotrygonines appear to have specialized on particular resources, rather than opportunistically feeding on all of them.

The diversity of diets in potamotrygonines is reflected by their diverse phenotypes: some morphologies appear well-suited for dismantling softer-bodied, elusive prey (e.g., fishes), tougher prey (e.g., insect larvae), and even a range of stiffer-bodied prey, from decapod crustaceans to bivalves and gastropods. This latter trophic strategy, molluscivory, is relatively rare among batoid fishes, with the nearest molluscivorous relatives to potamotrygonines (e.g., *Pastinachus* spp.; Devadoss 1978) separated by more than 65 million years of evolution (Aschliman et al. 2012; Rutledge et al. 2019). However, the feeding morphology employed by freshwater molluscivores like *P. leopoldi* and *P. henlei* () echoes that of marine durophages: all have robust jaws, with high MA and broad muscle attachment areas (Figs. 1 and 4). Furthermore, the hypertrophied jaw muscles and closely interdigitating teeth in *P. leopoldi* are additional durophagous hallmarks shared with marine molluscivorous rays (Summers 2000; Underwood et al. 2017; Rutledge et al. 2019). The similarities between molluscivorous potamotrygonines and other durophagous rays, like myliobatids, offer a compelling example of ecomorphological convergence.

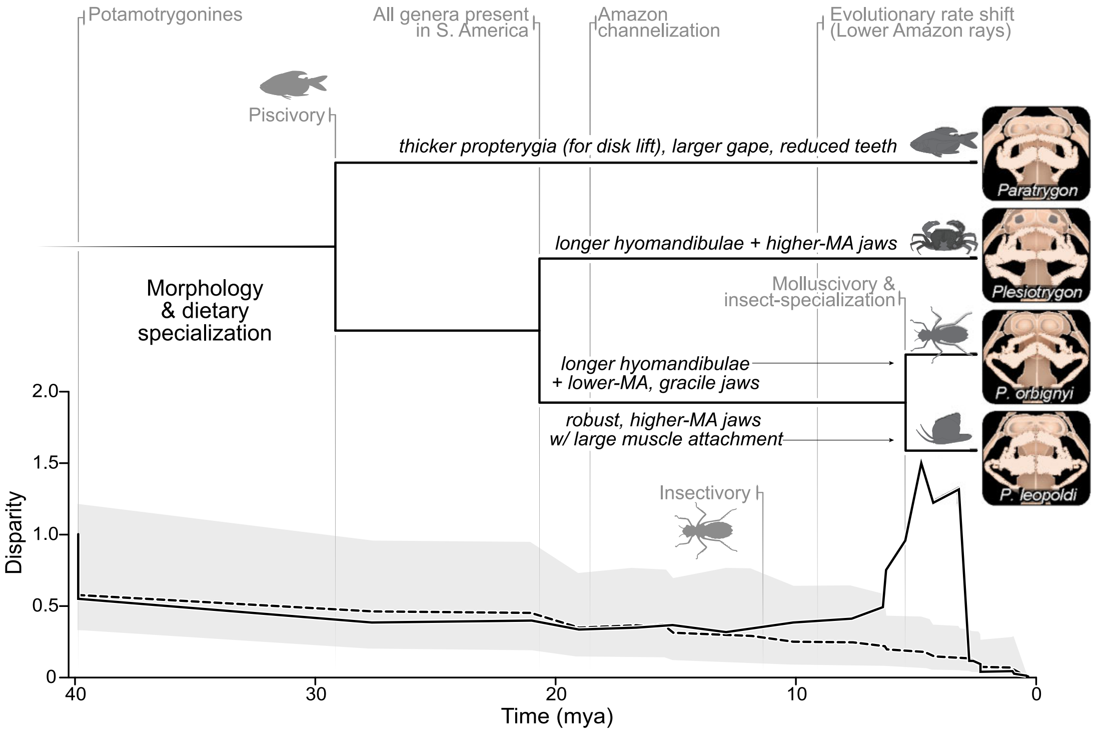

Fig. 6 DTT plot for Potamotrygoninae and a timeline for phenotypic and dietary novelties. The bottom half of the figure shows a DTT plot for Potamotrygoninae; dashed lines represent the median simulated (Brownian motion) subclade disparity across 10,000 simulations. The solid line represents the observed subclade disparity for potamotrygonines. The gray shaded region represents the 95% range of simulated Brownian subclade disparity. Vertical lines represent the relative origin of new dietary modes and geological occurrences of note. Horizontal dendrogram describes the changing phenotypic configurations of the feeding apparatus, with their corresponding dietary specialization.

Similarly, piscivorous freshwater rays share striking resemblance with distantly related, extant marine piscivorous batoids, like the electric ray *Torpedo* and butterfly rays (*Gymnura*), in having wide, gracile jaws with reduced dentitions (Dean et al. 2007). The bauplan of freshwater species like *Paratrygon* are also remarkably like those of extinct marine rays from the Monte Bolca formation—fishes with circular disks, broad pectoral propterygia, and wide mouths—body plans thought to be lost in modern stingrays (Marramà et al. 2019), but found in the modern Amazon River. We propose that the stout pectoral propterygia seen in *Gymnura, Paratrygon*, *Heliotrygon,* and extinct *Lessiniabatis* are multifunctional structures useful for swimming, as well as when these rays ambush evasive fishes, draw these prey beneath their bodies, and confine them with their fins until consumed (see Wilga et al. 2012; Kolmann et al. 2016).

While the above instances document how freshwater ray feeding morphologies are convergent or parallel with those of distantly related marine stingrays, insectivorous potamotrygonines are seemingly unique among myliobatiforms (Kolmann et al. 2016). Insectivory poses considerable mechanical and chemical challenges. Chitin, the primary component of insect cuticle, is particularly tough and therefore robust to processing by predators (Vincent and Wegst 2004). Kinetic jaws give insectivorous rays the ability to roll or shear the upper and lower jaws against one another, which is necessary for “chewing” insect cuticle apart (Kolmann et al. 2016; Laurence-Chasen et al. 2019). Potamotrygonines may have achieved some of this cranial kinesis by decoupling jaw movement even further from the skull and suspensory skeleton (i.e., the hyomandibulae) relative to their dasyatoid relatives, through the evolution of an intermediate skeletal element, the angular cartilage, which intervenes between the jaws and hyomandibulae like an extra link in the kinematic chain (Fontenelle et al. 2017). In batoid fishes, evolutionary increases in jaw kinesis afforded by disassociation or relaxation of jaw linkages (between jaws and cranium, or jaws and hyomandibulae) seems to be a common motif for species feeding on complex prey (Kolmann et al. 2014; Fontenelle et al. 2017), i.e., that the freedom of movement provided by a “looser” jaw suspension facilitates processing of prey items with tough outer coverings (Dean et al. 2007). However, insectivores occupy a large region of the feeding morphospace for potamotrygonines, suggesting that either diverse behaviors or multiple phenotypes facilitate insectivory (Fig. 4; Kolmann et al. 2016).

Despite their biome conservatism (Bloom and Lovejoy 2012), freshwater rays appear to be considerably labile with respect to their feeding ecology, at least as far as the biotic dimensions of niche are concerned (Pearman et al. 2008; but see Peterson 2011). While potamotrygonines appear to have undergone niche diversification as a result of their invasion of freshwater, other marine-derived lineages exhibit a pattern of niche conservatism (e.g., anchovies, pufferfishes, and sculpins; Bloom and Lovejoy 2012; Santini et al. 2013; Buser et al. 2019). What explains the differing outcomes among these marine-derived lineages? One possibility is that rays invading the Amazon did not face much in the way of competition (with other rays or fishes in general), whereas actinopterygian marine invaders competed with incumbent Amazonian ray-finned fishes which occupied potential niches. Support for this possibility comes from the observation that stingray lineages which emigrated from the main Amazon River basin to peripheral basins rarely (if ever) re-invaded natal waters, where competition with other stingrays would presumably be intense (Fontenelle et al. 2021a). But what about competition with teleost fishes? Some evidence suggests that *Potamotrygon* can forage for prey buried far deeper in the sediment, thereby accessing invertebrate prey sequestered from sympatric sediment-sifting bony fishes (Garrone-Neto and Sazima 2009; Garrone-Neto and Carvalho, 2011). We speculate that evolutionary priority effects (Belyea and Lancaster 1999; Leopold et al. 2015), where previously established lineages act as competitors to re-invading ones, shape the ecological establishment of marine-derived fishes in freshwater (Múrria et al. 2018). Once established in a new habitat, competition among confamilials promoted the need for resource partitioning through niche differentiation (Yoder et al. 2010), which may also explain why the appearance of extant potamotrygonine lineages was concurrent with trophic specialization.

Conclusions—the case for a potamotrygonine adaptive ray-diation?

Neotropical freshwater rays underwent a habitat transition, which was followed by profound diversification in terms of dietary ecology and parallel diversification of feeding phenotypes: telltale signs of classic adaptive radiations (Yoder et al. 2010; Gillespie et al. 2020). Our data demonstrate that freshwater rays continued to diversify as time progressed, and failed tests for rapid, early bursts of both lineage and trait diversification. Perhaps an early-burst pattern has been obfuscated by high background levels of extinction (Uhen and Pyenson 2007; Rabosky and Lovette 2008) or recent and continuing hybridization (Fontenelle et al. 2021b). However, if we consider the more traditional aspects of adaptive radiation, divergent selection caused by competition, character displacement among confamilials, and the exploitation and partitioning of new adaptive zones (Givnish 2015), an adaptive radiation is more probable. Only starting with Simpson (1953) is some aspect of "explosive" speciation considered to be a hallmark of adaptive radiation, which would exclude classic radiation examples like Darwin's Finches or Australian marsupials which do not demonstrate particularly rapid rates of speciation or diversification (Givnish 2015). Additionally, some evidence suggests that adaptive radiations in clades shifting from marine to freshwaters may exhibit a substantial lag in lineage diversification after initial colonization (Thacker et al. 2022). The early establishment of stingray clades with non-overlapping feeding motifs suggests ecological partitioning of resources (Harmon et al. 2003; Slater et al. 2010). The degree of trophic specialization across the family (Fig. 3), and the observation that the phenotypically and ecologically disparate ray assemblages are also the most speciose (Supplementary Appendix IV), might suggest character displacement in putative cradles like the upper and lower Amazon (Fontenelle et al. 2021a). A definitive ruling on these issues requires diet studies of greater detail be undertaken for these diverse ray assemblages and assessment of whether trophic specialists occupy novel adaptive optima.

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Supplementary data

Supplementary data available at ICB online.

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