Syst. Biol. 71(1):78–92, 2022

DOI:10.1093/sysbio/syab040
Advance Access publication June 7, 2021

Accelerated Diversification Explains the Exceptional Species Richness of Tropical Characoid Fishes

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Received 17 February 2021; reviews returned 1 June 2021; accepted 4 June 2021
Associate Editor: Vincent Savolainen

Abstract.—The Neotropics harbor the most species-rich freshwater fish fauna on the planet, but the timing of that exceptional diversification remains unclear. Did the Neotropics accumulate species steadily throughout their long history, or attain their remarkable diversity recently? Biologists have long debated the relative support for these museum and cradle hypotheses, but few phylogenies of megadiverse tropical clades have included sufficient taxa to distinguish between them. We used 1288 ultraconserved element loci spanning 293 species, 211 genera, and 21 families of characoid fishes to reconstruct a new, fossil-calibrated phylogeny and infer the most likely diversification scenario for a clade that includes a third of Neotropical fish diversity. This phylogeny implies paraphyly of the traditional delimitation of Characiformes because it resolves the largely Neotropical Characoidei as the sister lineage of Siluriformes (catfishes), rather than the African Citharinodei. Time-calibrated phylogenies indicate an ancient origin of major characoid lineages and reveal a much more recent emergence of most characoid species. Diversification rate analyses infer increased speciation and decreased extinction rates during the Oligocene at around 30 Ma during a period of mega-wetland formation in the proto-Orinoco-Amazonas. Three species-rich and ecomorphologically diverse lineages (Anostomidae, Serrasalmidae, and Characidae) that originated more than 60 Ma in the Paleocene experienced particularly notable bursts of Oligocene diversification and now account collectively for 68% of the approximately 2150 species of Characoidei. In addition to paleogeographic changes, we discuss potential accelerants of diversification in these three lineages. While the Neotropics accumulated a museum of ecomorphologically diverse characoid lineages long ago, this geologically dynamic region also cradled a much more recent birth of remarkable species-level diversity. [Biodiversity; Characiformes; macroevolution; Neotropics; phylogenomics; ultraconserved elements.]

With more than 6200 known living species, the Neotropics harbor the most species-rich freshwater fish fauna on the planet (Lundberg et al. 2000; Reis et al. 2003, 2016; Albert et al. 2020). The radiations of three large otophysan lineages account for 77% of Neotropical species diversity: Siluriformes (catfishes), Gymnotiformes (South American knifefishes), and Characoidei (characins, tetras, piranhas) (Albert et al. 2011). Scientists regularly discover new species in all three clades, and the ~100 new Neotropical fish species described each year exceed the rate of discovery in any other area on Earth (Reis et al. 2016; Birindelli and Sidlauskas 2018; Fricke et al. 2020). This remarkable fauna originated long ago, with molecular estimates calibrated from the fossil record placing the most recent common ancestor (MRCA) of otophysan fishes in the Jurassic (~150 Ma) and the initial diversification of siluriforms and characoids in the middle Cretaceous (~120 Ma) (Near et al. 2012; Dai et al. 2018; Burns and Sidlauskas 2019). Despite the well-established ancient origin of this fauna, its sparse fossil record has impeded reconstruction of its subsequent trajectory of diversification. Understanding whether Neotropical fishes achieved remarkable species richness early or late in their evolutionary history helps reveal the processes underlying their diversification, providing an important perspective on the rich aquatic biodiversity of South America.

Two macroevolutionary models are commonly posited as alternatives that could have generated tropical biodiversity. The classic museum model posits that the high species diversity of the tropics accumulated steadily over a 100 myr or more under relatively low and constant rates of speciation and extinction (Stebbins 1974), while the alternative cradle model hypothesizes that recent bursts of speciation

produced most tropical diversity (Richardson et al. 2001). Empirical studies in other groups have supported both hypotheses. For example, tropical regions have long accumulated bird species under low extinction rates (Gaston and Blackburn 1996), while Neotropical insect groups such as ants (Moreau and Bell 2013) and beetles (McKenna and Farrell 2006) experienced pulses of speciation interspersed with periods of slower diversification throughout the Cenozoic. Collectively, these studies demonstrate that not all clades exhibit the same tempo of diversification, and the Neotropics can act simultaneously as a museum of some taxa and a cradle of diversity for others (McKenna and Farrell 2006).

Many previous studies have concluded that Neotropical fish diversification aligns with the museum hypothesis, with interpretation of the fossil record indicating the assembly of the genera and families of the modern Neotropical freshwater fish fauna over more than 60 myr (Lundberg et al. 1986, 1998; Lundberg 1997; Hoorn et al. 2010) at a relatively constant rate of lineage accumulation (Albert et al. 2020). The major lineages delimited as modern taxonomic families originated by the late Cretaceous or early Paleogene (Hoorn et al. 2010; Burns and Sidlauskas 2019; Albert et al. 2020). The available fossils of Miocene age are easily assignable to those families (Lundberg et al. 1998), suggesting that turnover was low and extinction of major lineages was rare throughout the Cenozoic. There is also a notable lack of paleontological evidence that the Neotropics experienced extinctions driven by climatic cooling and aridification, as reported in Africa (Hugueny 1989; Stewart 2001; Cohen et al. 2007). A recent comparative analysis based on an expansive molecular phylogeny spanning most ray-finned fish families concluded that the Neotropics likely owe their exceptional freshwater fish diversity to the ancient age of the lineages inhabiting South America rather than to accelerated speciation (Miller and Román-Palacios 2021). Thus, both molecular and paleontological evidence have been used to argue that Neotropical fishes achieved their great species diversity by the Paleogene and that the Neotropics represent a museum of ancient fish diversity more than they do a cradle.

Previous studies on the evolutionary history of Neotropical freshwater fishes have typically drawn their conclusions from a relatively scant fossil record (Hoorn et al. 2010), or from megaphylogenies that synthesize previous data rather than expanding the species-level representation within diverse clades (Rabosky et al. 2018; Miller and Román-Palacios 2021). Recent studies presenting time-calibrated phylogenies with dense taxon sampling for various clades of Neotropical freshwater fishes contradict the museum scenario by reconstructing substantial diversification over the last 30 myr (Mariguela et al. 2016; Melo et al. 2016; Ochoa et al. 2017; Roxo et al. 2019; Kolmann et al. 2020; Fontenelle et al. 2021) following the initial uplift of the Andes (~35–10 Ma) in the proto-Orinoco-Amazonas mega-wetland basin of northwestern South America (Lundberg et al. 1998; Hoorn et al. 2010; Albert et al. 2018). These time-calibrated phylogenies indicate recent origins of many Neotropical fish species even as they confirm that the deepest roots of their enclosing families extend into the Paleogene or Late Cretaceous. Mounting evidence suggests that much of Neotropical ichthyological diversification may have incubated in a recent cradle, albeit one placed within a museum of diversity.

Any test of macroevolutionary hypotheses at the scale of the entire Neotropical ichthyofauna will require time-calibrated phylogenies of the most species-rich lineages. With high species richness and an ancient origin, characoids (Ostariophysi: Characiformes: Characoidei) are an exemplary clade with which to explore the dynamics and drivers of lineage diversification among Neotropical freshwater fishes. Characoidei comprises approximately 2150 known species (Fricke et al. 2020), which represents more than 34% of all Neotropical freshwater fishes (Reis et al. 2003, 2016; Albert et al. 2020). With major lineages in South America and Africa, characoids originated prior to the fragmentation of Gondwana and the opening of the Southern Atlantic Ocean in the Early/Late Cretaceous (Lundberg 1993; Arroyave et al. 2013; Granot and Dyment 2015; Burns and Sidlauskas 2019). Members of this ecologically and morphologically disparate radiation possess specialized oral anatomies and diverse body shapes that adapt them to a variety of trophic niches throughout the aquatic ecosystems of Africa and the Neotropics (Roberts 1972; Burns and Sidlauskas 2019). Characoids count among their number voracious predators (Lewis 1974; Nico et al. 2018), migratory detritivores acting as engineers of carbon flow (Taylor et al. 2006; Melo and Sidlauskas 2017), herbivores (Santos 1981), specialized scale eaters (Sazima 1983; Kolmann et al. 2018), and more than 1200 species of diminutive tetras (Géry 1977; Lima et al. 2003). Characoidei is substantially more species-rich than the African Citharinoidei, which contains 113 species in two families (Citharinidae and Distichodontidae) and was long classified with Characoidei in Characiformes based on morphological traits (Vari 1979; Fink and Fink 1981; Buckup 1998). Previous molecular phylogenetic studies have tested the monophyly of Characiformes by inferring relationships among the major otophysan lineages (Nakatani et al. 2011; Chen et al. 2013; Arcila et al. 2017; Chakrabarty et al. 2017; Betancur-R et al. 2018; Dai et al. 2018); these have obtained conflicting results. Other studies aimed to resolve relationships within specific characoid subclades (Arroyave and Stiassny 2011; Oliveira et al. 2011; Mariguela et al. 2013; Abe et al. 2014; Melo et al. 2014; Thompson et al. 2014; Thomaz et al. 2015; Melo et al. 2016; Melo et al. 2018; Mateussi et al. 2020b). No previous study, however, has investigated the tempo of diversification of characoids to understand the evolutionary origin of the high species richness of Neotropical freshwater fishes.

We linked DNA sequences from more than a thousand nuclear loci to fossil information to generate and calibrate a densely-sampled phylogeny for Characoidei using a phylogenomic approach based on ultraconserved elements (UCEs) and their hypervariable flanking regions (Faircloth et al. 2012, 2020) and test the monophyly of Characiformes, which was originally delimited with morphological characters and is inconsistently resolved as a clade in molecular phylogenetic analyses (Nakatani et al. 2011; Chen et al. 2013; Arcila et al. 2017; Chakrabarty et al. 2017; Betancur-R et al. 2018; Dai et al. 2018; Faircloth et al. 2020). Using this densely sampled phylogenomic tree, we estimate the tempo of lineage diversification in Characoidei, allowing us to determine if the exceptional richness of a clade comprising more than one-third of all Neotropical freshwater fishes resulted from a steady accumulation of species consistent with a museum model of ancient diversification, or from recent bursts of speciation in a cradle of biodiversity.

Materials and Methods

Taxon Sampling and DNA Sequencing

Taxon sampling included 325 specimens of 293 characoid species representing approximately 13.6% of characoid species and 211 characoid genera (79.0%) known to exist in June of 2020 (Froese and Pauly 2019; Fricke et al. 2020). All characoid families are represented except the recently discovered Tarumaniidae, a rare Neotropical lineage now recognized as the sister to Erythrinidae (De Pinna et al. 2018; Arcila et al. 2018). Considering the magnitude of characoid species diversity (approximately 2150 species described to date) and the general consensus that characoid genera represent distinct lineages within characoid families, we designed the study to include as many genera as possible. Type species of each sampled genus and specimens from near type localities were included whenever possible. Outgroup taxa included 31 specimens from the two families of Citharinoidei (Citharinidae and Distichodontidae), eight families of Siluriformes (Astroblepidae, Callichthyidae, Heptapteridae, Loricariidae, Pimelodidae, Pseudopimelodidae, Scoloplacidae, and Trichomycteridae), and one each of Gymnotiformes (*Steatogenys elegans*) and Cypriniformes (*Cyprinus carpio*). The cypriniform taxon was used to root the phylogenies. Voucher specimens were fixed in 96% ethanol or 10% formalin and then transferred to 70% ethanol for permanent storage. All procedures were carried out in accordance with the rules of our animal ethical committee (CEEAA permit 3245 IBB/UNESP). Supplementary Table S1 available on Dryad at http://dx.doi.org/10.5061/dryad.hqbzkh1fm summarizes voucher information with institutional acronyms following Sabaj (2016). Raw read data are archived in the NCBI Sequence Read Archive under Bioproject PRJNA563917 and information about reads for each species appears in Supplementary Table S2 available on Dryad. Details of laboratory methods, data assembly, topology estimations, and calibrations are provided in Supplementary Material available on Dryad.

Concatenated and Coalescent-Based Analyses

Sequence data matrices used in the maximum likelihood and Bayesian inference analyses were partitioned following the Partition-UCE pipeline (Tagliacollo and Lanfear 2018) with models chosen by PartitionFinder v2.1.1 (Lanfear et al. 2012) and the best-fit models assessed for all four edge-trimmed matrices. Three concatenated matrices (50%, 75%, and 90% complete) were used to run five alternative searches with the same parameters to find the maximum likelihood tree for each matrix using RAxML v8 (Stamatakis 2014) with the GTRGAMMA model on a 2×10 CPU, 256 GB *Zungaro* server at IBB/UNESP. We also generated bootstrap support values for each node using RAxML v8 (Stamatakis 2014) and used autoMRE (Pattengale et al. 2009) to test for convergence of bootstrap replicates. Following the best tree search and the bootstrap replicates, we combined the maximum likelihood tree with the bootstrap replicates for each of the three matrices using RAxML v8.

Bayesian inference for each of the three concatenated data sets (50%, 75%, and 90%) was performed with ExaBayes (Aberer et al. 2014) by running two independent Markov chain Monte Carlo (MCMC) simulations, each with one cold and one heated chain, under the default parameters for 10^6 generations (burn-in: 25%; thinning: 500) on 256×3104 CPUs, 4096 GB server at GRID/UNESP. We visualized the parameters for convergence using Tracer v1.6 (Rambaut et al. 2014), accessing the log files from independent runs to ensure stationary and sufficient mixing of parameters [effective sample size (ESS) > 200], and the potential scale reduction factor for estimated parameters was approximately 1.0. We generated the most credible set of trees from the posterior distribution of possible topologies using the consensus algorithm in ExaBayes.

To account for coalescent stochasticity among individual UCE loci and to address the potential problem of a concatenated analysis returning a highly supported but incorrect tree (Mirarab et al. 2014), we inferred a coalescent-based analysis from individual gene trees using a two-step process. First, we used PHYLUCE (Faircloth 2015) to resample the 50%, 75%, and 90% complete matrices by locus and to infer a maximum likelihood tree from every locus in each matrix. Then, we used ASTRAL-II (Mirarab and Warnow 2015) to infer species trees from the best gene trees and generated a majority-rule consensus tree of the results (minimum clade frequency $= 0.7$). Though ASTRAL-II is not strictly a coalescent method, it is statistically consistent with the multispecies coalescent model and

scales well with thousands of loci (Mirarab et al. 2014; Mirarab and Warnow 2015). We used the function "distinct.edges" in the R package "distory" (Chakerian and Holmes 2012) to compare the pairwise edge IDs between phylogenetic trees from the concatenated maximum likelihood and Bayesian analyses and the coalescent ASTRAL-II species tree.

Alternative Topology Tests

The gene-genealogy interrogation (GGI) approach (Arcila et al. 2017) was implemented to interrogate 1141 UCE loci separately and select the most likely match from a subset of possible *a priori* topologies using a combination of model-based approaches and topology tests. This approach allows a direct comparison between our reconstruction of the earliest splits of Otophysi to those recovered in previous studies. We identified four previously proposed arrangements of the two characiform suborders (Characoidei and Citharinoidei) relative to Siluriformes (catfishes), and Gymnotiformes (electric knifefishes): (1) Characoidei sister to Siluriformes rendering Characiformes paraphyletic (Nakatani et al. 2011; Chen et al. 2013; Chakrabarty et al. 2017; our unconstrained topology); (2) Characiformes monophyletic and sister to Siluriformes (Betancur-R et al. 2013); (3) Characiformes monophyletic and sister to Gymnotiformes (Dimmick and Larson 1996); and (4) Characiformes monophyletic and sister to Siluriformes+Gymnotiformes (Fink and Fink 1981; Arcila et al. 2017). We used RAxML to compare the likelihoods for the unconstrained tree (which matches H1 above) with the best phylogeny constrained to each of the other *a priori* hypotheses. Details of the GGI pipeline are available in Supplementary Material available on Dryad.

We used a second approach, the Bayesian concordance analysis (BUCKy) that reconstructs the primary concordance tree from clades supported by the largest proportions of genes (Ané et al. 2006; Larget et al. 2010). Because BUCKy can only run on data sets with 20 or fewer taxa, we needed to reduce taxon sampling substantially before implementing it. We used a balanced subset with 17 taxa spanning the major lineages of otophysans: eight characoids encompassing eight families (*Bryconalestes longipinnis, Chalceus macrolepidotus, Characidium fasciatum, Colossoma macropomum, Curimatopsis cryptica, Lepidocharax burnsi, Pyrrhulina filamentosa*, and *Thoracocharax stellatus*), three citharinoids (*Citharinus congicus, Distichodus decemmaculatus*, and *Xenocharax spilurus*), two siluriforms (*Hypostomus flaveolus* and *Pimelodella* sp.), two gymnotiforms (*Electrophorus electricus* and *Sternopygus macrurus*), and two cypriniforms (*Cyprinus carpio* and *Danio rerio*). In order to include at least two taxa from each major otophysan group in this analysis, sequences from the electric eel *Electrophorus electricus* (SRX4081262) and the zebrafish *Danio rerio* (GCA_000002035.2) were obtained from NCBI. The BUCKy subassembly contained 709 UCE loci represented by at least 75% of the taxa. First, we used MrBayes v3.2.6 (Ronquist and Huelsenbeck 2003) to infer gene trees for each UCE locus. Rather than performing substitution model selection for information-poor UCE loci, we applied a simple HKY+G nucleotide substitution model for each locus. For each locus, we performed two replicate metropolis coupled MCMC simulations with four chains each, 10 million generations in length sampled every 10,000 generations with the first 25% discarded as burn-in. To assess mixing and convergence of replicate runs, we checked that the standard deviation of split frequencies was less than 0.01. We used "mbsum" v1.4.4 to summarize the posterior gene tree distributions and then performed BUCKy concordance factor (CF) estimation using alpha $=1$ for the prior on gene tree discordance (Larget et al. 2010).

Divergence Time Estimation

We estimated a time-scaled phylogeny in BEAST v1.8.2 (Drummond et al. 2012) under an uncorrelated relaxed molecular clock. Because BEAST v1.8.2 does not support large input files due to memory restrictions, none of the matrices used in the prior analyses were compatible. Thus, we used a more stringent cutoff for missing data (using only loci available for 92% of the taxa) to assemble a sufficiently small input matrix for BEAST. This 92% complete edge-trimmed alignment matrix comprised 57 UCE loci (17,400 bp) for the same 356 terminals represented in the concatenated and coalescent analyses. We used the maximum likelihood tree from the 75% complete matrix as a fixed topology in the Bayesian analysis in order to estimate only node ages. A constraint on the root and six fossils spanning the entire characoid radiation were used as calibration points: undetermined alestid teeth (de la Peña Zarzuelo 1996), the anostomid †*Leporinus scalabrinii* (Bogan et al. 2012), the curimatid †*Cyphocharax mosesi* (Travassos and Santos 1955; Malabarba 1996), the bryconid †*Brycon avus* (Woodward 1898), the characids †*Megacheirodon unicus* (Bührnheim et al. 2008), and †*Paleotetra entrecorregos* (Weiss et al. 2012) (see Supplementary Material available on Dryad for details of calibrations). Each fossil estimated the age of the crown group of the clade to which it could be assigned on the basis of previous morphology- and molecular-based phylogenetic evidence (Malabarba 1996; Zink 2014; Bührnheim et al. 2008; Bogan et al. 2012; Weiss et al. 2012; Abe et al. 2014; Ramirez et al. 2017; Melo et al. 2018). These calibrations used log-normally distributed priors (mean $=5.0$ Ma; standard deviation $=1.0$ Ma) to model the uncertainty associated with these node age estimations due to the fragmentary and incomplete nature of the fossil record.

The BEAST analyses used a birth–death model for prior distributions and ran for 200 million generations with a sampling frequency of 10,000 generations. We

verified stationarity and sufficient mixing of parameters (ESS > 200) using Tracer v1.6 (Rambaut et al. 2014). We visually checked the distributions of priors with ESS values below 200 and compared the marginal distributions of each of those priors with the marginal distribution of the data exclusively (Supplementary Material available on Dryad). We processed the 20,001 trees to generate a maximum clade credibility tree using TreeAnnotator v1.8.2. All clade-age estimations are presented as the mean plus 95% highest posterior density values (95% HPD).

Diversification Rates Analyses

We estimated rates of speciation and extinction across the characoid phylogeny and tested for clade-specific shifts using BAMM (Rabosky et al. 2014). We used the consensus tree from the posterior distribution of BEAST as the input file for BAMM and accounted for missing taxa by specifying the number of missing species in each subclade following Eschmeyer's Catalog of Fishes (Fricke et al. 2020). Then we ran two simultaneous chains for a total of 5 million generations, sampling tree space every 1000th generation. We used a burn-in value of 0.5 and checked for MCMC convergence using the "BAMMtools" package (Rabosky et al. 2014) in R (R Development Core Team 2013) to plot log-likelihood values. To account for effects of phylogenetic uncertainty, we conducted BAMM analyses of species diversification across 2500 trees sampled from the posterior distribution. We also used "BAMMtools" to visualize the evolutionary rate dynamics from the BAMM output.

As an alternate measure of lineage diversification, we computed a model-free tip-specific speciation rate metric called the DR statistic (Jetz et al. 2012). As the DR statistic requires a fully sampled phylogeny, we used a stochastic polytomy resolver that accounts for incompletely sampled data, TACT (Chang et al. 2019), to generate a pseudoposterior distribution of completely sampled phylogenies with unsampled taxa obtained from FishBase (Froese and Pauly 2019). Because the DR statistic can be sensitive to small variation in terminal branch lengths (Title and Rabosky 2018), we summarized the DR statistic by family across 100 replicate trees.

We also employed the CoMET model within the R package "TESS" (Höhna et al. 2015) to identify lineage-wide diversification rate shifts and the timing of those events for Characoidei, Characoidea, Erythrinoidea, Alestoidea, Crenuchoidea, and Citharinoidea *sensu* Betancur-R et al. (2018). For each CoMET analysis, we corrected for incomplete sampling and ran the reversible jump MCMC chain until the ESS reached 700 or greater with a burn-in of 30,000 iterations. We generated three independent MCMC simulations for each clade and assessed whether rates sampled from the posterior distributions converged within and between runs (Supplementary Material available on Dryad). Through TESS, we also evaluated the fit of 15 branching-process models to the observed phylogeny for Characoidei. Stepping-stone simulations run for 10,000 generations with 1000 power posteriors estimated the marginal likelihoods of these candidate models, which we used to calculate Bayes Factors and compare their relative fits to the observed characoid phylogeny (Supplementary Table S3 available on Dryad). We also performed posterior-predictive simulations to assess the adequacy of each branching-process model in representing diversification in Characoidei (Supplementary Material available on Dryad).

Results and Discussion

Phylogenetic Relationships and the Paraphyly of Characiformes

The phylogenomic data set consists of 1288 UCE loci (345,179 bp) sequenced from 356 specimens representing 293 characoid species, 79% of all recognized characoid genera, and 31 otophysan outgroups. We assembled and analyzed four UCE matrices that differed in their inclusion of loci with varying amounts of missing data: a 50% complete matrix (1769 loci; 521,671 bp), a 75% complete matrix (1288 loci; 345,179 bp), a 90% complete matrix (147 loci; 45,464 bp), and a 92% complete matrix (57 loci; 17,400 bp). The first three matrices were used to infer phylogenetic relationships by maximum likelihood using RAxML v8 (Stamatakis 2014) (Supplementary Figs. S1–S3 available on Dryad), Bayesian analyses using ExaBayes (Aberer et al. 2014), and coalescent-based analyses using ASTRAL-II (Mirarab and Warnow 2015), whereas the stringent 92% complete matrix was used in the fossil-calibrated relaxed molecular clock analyses in BEAST v1.8.2 (Drummond et al. 2012) (Figs. 1 and 2a, Supplementary Figs. S4–S6 and Supplementary Material available on Dryad).

Phylogenetic analyses of the concatenated UCE data set and the coalescent-based analyses resolve the long-recognized taxonomic group Characiformes as paraphyletic (Figs. 1 and 2a; Supplementary Figs. S1–S6 and Supplementary Material available on Dryad). The geographically widespread Characoidei and Siluriformes (catfishes) resolve as sister lineages with the African Citharinoidei as the sister of that clade (Figs. 1 and 2a). Essentially all previous molecular phylogenetic analyses have also resolved the traditionally delimited Characiformes as paraphyletic; however, these studies differ in the inferred relationships among Characoidei, Citharinoidei, Siluriformes, and Gymnotiformes (Ortí and Meyer 1997; Li et al. 2008; Poulsen et al. 2009; Nakatani et al. 2011; Chakrabarty et al. 2017; Mirande 2017; Dai et al. 2018; Faircloth et al. 2020). The only molecular analyses to support characiform monophyly are three studies using the same set of ~1100 exons (Arcila et al. 2017; Betancur-R et al. 2018; Hughes et al. 2018). In these studies, characiform monophyly results from analyses using a constrained topology analysis (Simion et al. 2020). However, standard

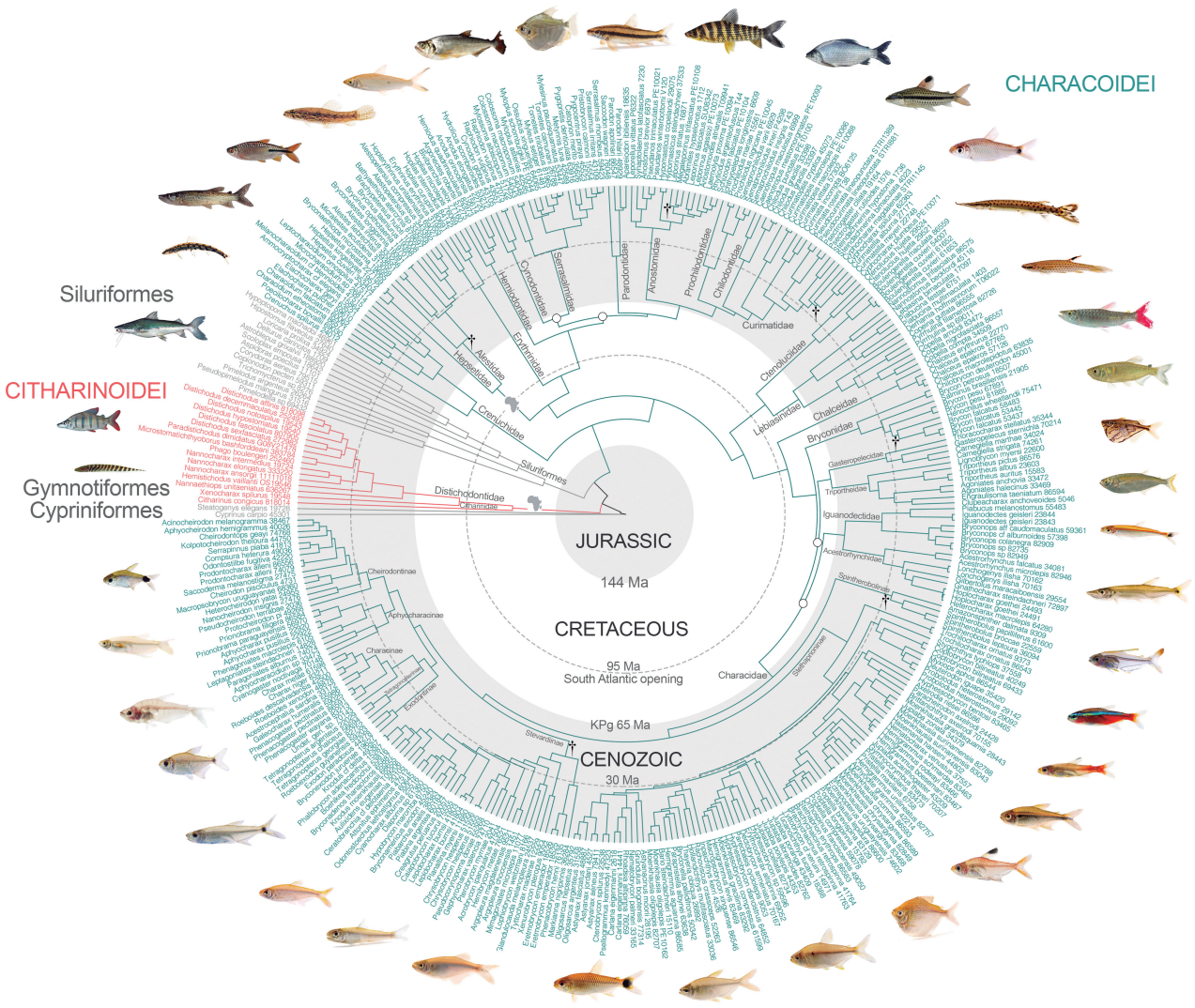

FIGURE 1. Time-calibrated phylogeny of Characoidei and related otophysan taxa including 325 characoids (293 species and 211 genera) based on the best maximum likelihood tree of the 75% complete matrix of ultraconserved elements (1288 loci; 345,179 bp). Node ages were obtained from Bayesian analysis of the 92% complete matrix (57 loci; 17,400 bp) and six fossil calibrations (†). White circles represent nodes with lower than 100% bootstrap support at the family level. African clades denoted in grey silhouettes of the continent. Fish photographs by B. Melo, C. Robertson, J. García-Melo, J. Sullivan, L. García-Melo, M. Sabaj, M. Taylor, R. Schmidt, and Proyeto CaVFish Colombia.

maximum likelihood analyses of concatenated data and coalescent-based analyses reported in these same studies result in characiform paraphyly [Arcila et al. (2017) their Figure 1; Hughes et al. (2018) their Supplementary Figs. S2, S4, and S5 available on Dryad].

Why does constrained topology analysis applied to that particular data set result in characiform monophyly, while all other molecular analyses yield paraphyly? According to Simion et al. (2020), there are problems with the data and analyses in Arcila et al. (2017). Importantly, the tree inference software used by Arcila et al. (2017) contained a software bug (Simion et al. 2020). Reanalysis of their data showed that only two of 1051 loci contain a signal sufficient to discriminate among the 15 *a priori* phylogenetic hypotheses (Simion et al. 2020), rather than 394 of 1051 loci as originally reported. Simion et al. (2020) also concluded that 12.3% (20,000 out of 162,255) of the sequences in Arcila et al. (2017) are contaminated. Taken together, these re-analyses show that the exon-based data set of Arcila et al. (2017) does not contain substantial signal informing resolution of the deepest nodes in the phylogeny of ostariophysans, and constrained topology analysis of those data does not provide strong support for characiform monophyly.

Does our own UCE-based data set contain sufficient power to discriminate among phylogenetic hypotheses such as characiform monophyly or paraphyly and to determine overall genomic concordance with differing phylogenetic trees? To find out, we applied two constrained topology strategies: gene genealogy interrogation (GGI) (Arcila et al. 2017), and BUCKy (Ané et al. 2006; Larget et al. 2010). Results indicate

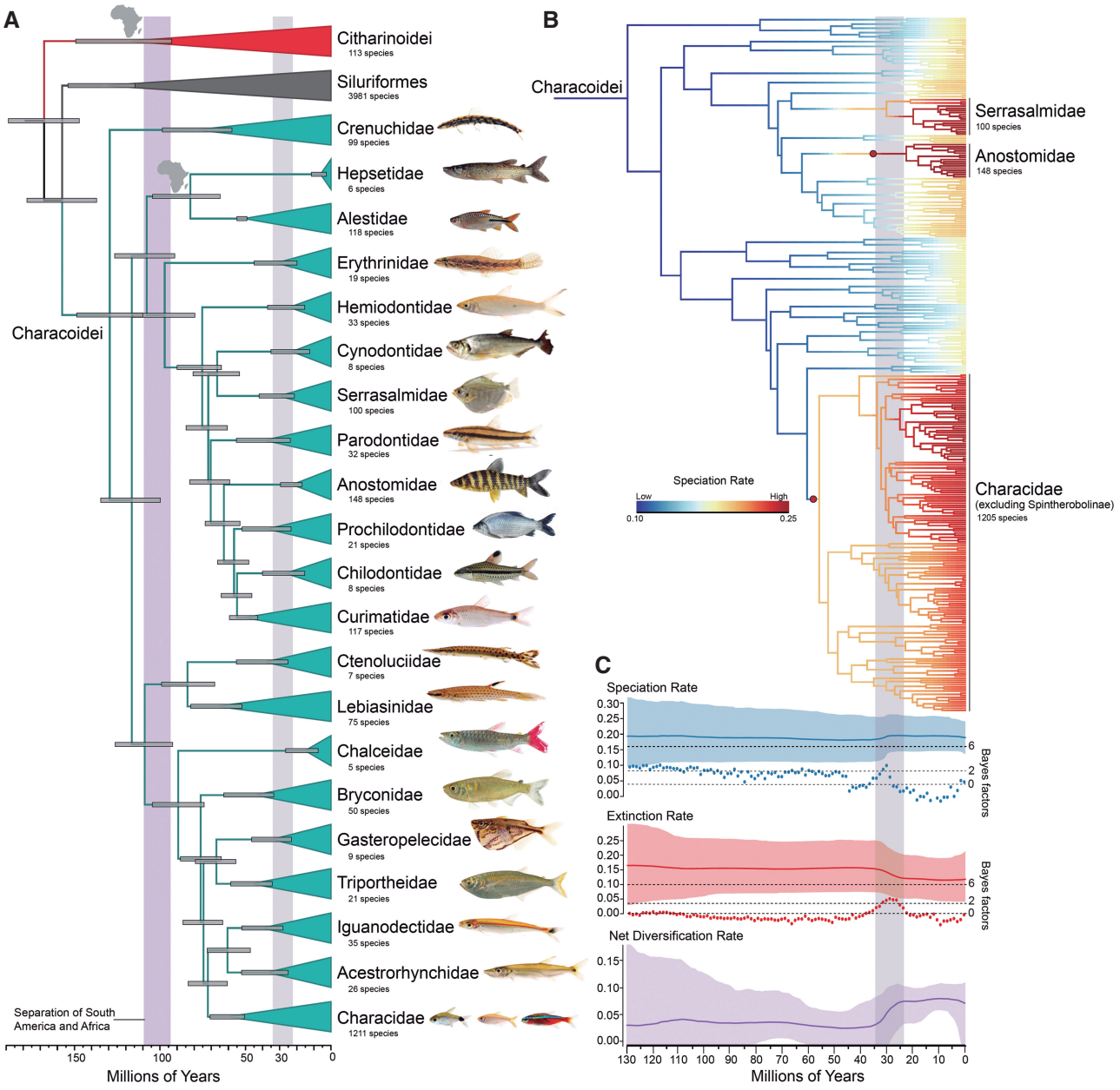

FIGURE 2. a) Time-calibrated phylogeny of characoid fishes and species diversity for each family; horizontal gray bars at nodes are 95% highest posterior density (HPD) intervals; African clades denoted in gray silhouettes of the continent. b) Results of BAMM analysis showing accelerated rates of speciation (warm colors) in three clades: Anostomidae, Serrasalmidae, and Characidae (except Spintherobolinae); shifts in diversification rate (red circles) occur only in Anostomidae and Characidae excluding Spintherobolinae. c) Summary results of the TESS-CoMET analysis for Characoidei, indicating moderate support for a tree-wide increase in speciation rates and decrease in extinction rates at around 30 Ma; rate estimates are represented by solid lines enveloped by a shaded 95% confidence interval; points illustrate statistical support for a shift in rates at a given time interval [in 2 ln (Bayes Factors)]. Vertical gray bars highlight the time period during which characoid diversification is estimated to have increased.

that the UCE data set does contain relevant signal and that no analysis of the UCE data supports characiform monophyly. Using 1141 UCE loci, GGI identified 686 UCE loci that support characiform paraphyly (292 of these loci $p \geq 0.95$) and 454 loci that support one of three topologies that include a monophyletic Characiformes. Among these, 207 loci support Characiformes and Siluriformes as sister lineages, 107 support Characiformes and Gymnotiformes as sister lineages, and 93 support Characiformes as sister to a clade containing Siluriformes and Gymnotiformes. Importantly, no locus supports a topology with characiform monophyly with confidence greater than or equal to 95% (Supplementary Fig. S7 and Supplementary Material available on Dryad). Similarly, CF estimates from the BUCKy analysis using the 75%

complete matrix (709 UCE loci) resulted in a primary concordance tree with a paraphyletic Characiformes (Supplementary Fig. S8 available on Dryad). BUCKy estimates that ~28 UCE loci support a clade containing Gymnotiformes and Citharinoidei (CF = 0.04; 95% CI = 0.002–0.05), while ~21 UCE loci support a clade containing Gymnotiformes, Citharinoidei, and Siluriformes (CF = 0.03; 95% CI = 0.02–0.04). Either of these scenarios renders Characiformes paraphyletic by resolving the African Citharinoidei as sister to a clade other than Characoidei. In contrast with the ~49 loci supporting characiform paraphyly, only ~14 loci support characiform monophyly (CF = 0.02; 95% CI = 0.008–0.03). The remainder are uninformative at this level.

While the most prevalent signal in the UCE data set involves characiform paraphyly, we acknowledge that these data are limited in their power to reject alternative phylogenies. All possible relationships among Characoidei, Citharinoidei, Siluriformes, and Gymnotiformes receive some support, and the BUCKy analysis revealed that most individual loci are uninformative about relationships at the deepest levels of the phylogeny. The lack of a definitive resolution of these earliest branching events may be the product of relatively short internodes, leading to incomplete lineage sorting of gene trees (Alda et al. 2018), or may reflect the theoretical expectation that single short genes will often lack the phylogenetic signal needed to resolve the deepest nodes in the otophysan phylogeny (Chakrabarty et al. 2017; Simion et al. 2020). The limited sample size of non-characoid ostariophysans may also diminish the ability of these analyses to resolve their placement. Despite these limitations, many of the recovered loci do inform the central question. While it may not be possible to definitively reject characiform monophyly using the UCE data, those data best support characiform paraphyly. Other efforts have not convincingly supported characiform monophyly.

Given that several molecular analyses have now challenged characiform monophyly, a re-evaluation of the putative morphological synapomorphies of Characiformes (Fink and Fink 1981) is in order. Fink and Fink (1981) based their original proposal of monophyly on an anatomical investigation based on limited taxon sampling, particularly within Citharinoidei, and recognized that Citharinoidei differed considerably from Characoidei (see also Vari 1979). As such, the distribution of character states across the diversity of Characiformes and the evolutionary history of these morphologies has yet to be fully resolved. One of the putative characiform synapomorphies (the presence of a dorsomedial opening into the posttemporal fossa) varies among characiforms and has a homoplastic reconstruction on our phylogeny. That character may represent a synapomorphy of Characoidei that reversed in Gasteropelecidae and evolved in parallel in the citharinoid family Distichodontidae, rather than being a characiform synapomorphy. This example illustrates that a re-analysis of anatomical variation among characoids and citharinoids using a much broader taxon sampling may clarify the degree of morphological support for characiform monophyly versus the alternative hypothesis of paraphyly.

The UCE phylogeny resolves relationships within Characoidei that generally agree with other molecular analyses (Arcila et al. 2017; Betancur-R et al. 2018; Burns and Sidlauskas 2019). Importantly, the UCE-inferred phylogeny confirms the monophyly of Characidae as defined by Oliveira et al. (2011), which is the most species-rich family of Characoidei. Bootstrap values throughout the tree are generally high, with only four nodes involving relationships among characoid families that are lower than 75% (Supplementary Figs. S1–S3 available on Dryad). All of these weakly supported nodes were also ambiguous in previous molecular studies (Oliveira et al. 2011; Arcila et al. 2017; Betancur-R et al. 2018), reflecting general uncertainty in the phylogenetic resolution of Hemiodontidae, Cynodontidae, Bryconidae, and Characidae. The UCE phylogenies differ from previous work at those weakly supported nodes in resolving Cynodontidae and Serrasalmidae as sister lineages and supporting Characidae as the sister lineage of a clade containing Acestrorhynchidae and Iguanodectidae (Figs. 1 and 2a). The Supplementary Material available on Dryad details additional phylogenetic results.

Ancient Origins of Major Characoid Lineages

The fossil-calibrated relaxed molecular clock analysis (Figs. 1 and 2a, Supplementary Figs. S4–S6 available on Dryad) results in an Early Cretaceous age estimate for the MRCA of Characoidei (129 Ma: 95% HPD, 148–110 Ma) (Supplementary Material available on Dryad). This estimate is older than a previous molecular estimate of ~90 Ma using Sanger-sequenced loci (Burns and Sidlauskas 2019), but broadly congruent with the known biogeography and fossil record of the clade. The age of the characoid MRCA predates the earliest records of isolated characiform teeth from the Late Cretaceous (100–94 Ma) of Sudan and Morocco (Werner 1994; Dutheil 1999) and the Late Cretaceous (72–66 Ma) Neotropical characoid fossil † *Tiupampichthys intermedius* (Gayet et al. 2003).

The time-calibrated UCE phylogeny also indicates that many of the earliest cladogenetic events in Characoidei occurred prior to the separation of South America and Africa in the breakup of western Gondwana that occurred between 110 and 95 Ma (Lundberg 1993; Granot and Dyment 2015) (Figs. 1 and 2a). The MRCA of the African lineages Hepsetidae and Alestidae and their species-rich sister lineage of South American characoids dates to 107 Ma (95% HPD, 126–91 Ma), which is coincident with the early stages of separation between South America and Africa and provides a clear example of Gondwanan fragmentation driving the diversification of freshwater fishes. All

of the lineages currently recognized as characoid families originated by 60 Ma, with most stem ages extending back 75 Ma or more. These results confirm the antiquity of the ecomorphological radiation of characoids into the distinct bodyplans that characterize each family (Burns and Sidlauskas 2019). They also corroborate the paleontological conclusion that by the Eocene, the higher-level taxonomic composition of the Neotropical fish fauna resembled its modern configuration (Lundberg et al. 1998; Albert and Reis 2011). Thus, Neotropical characoids have retained the legacy of early ecomorphological innovation over a vast stretch of time, as would be expected under a museum model of diversification.

Recent Origins of Characoid Species Richness

Three different analytical methods (BAMM: Rabosky et al. 2014; TACT: Jetz et al. 2012; Chang et al. 2019; CoMET: Höhna et al. 2015) support the cradle model of characoid diversification by identifying recent shifts to higher speciation rates at approximately 30 Ma (Fig. 2b,c; Supplementary Figs. S9 and S10 available on Dryad). The fact that diverse methods with varied assumptions all identified accelerated diversification ∼30 Ma increases confidence that the central result is not a statistical artifact. The TESS-CoMET results support the cradle diversification scenario by providing moderate support for a tree-wide rate shift at approximately 30 Ma (2ln Bayes Factor > 2), specifying that characoid extinction rates decelerated, and speciation rates accelerated at around that time (Fig. 2c). That shift aligns precisely with the accelerations identified using BAMM along the stem lineages of the ecomorphologically diverse Anostomidae (headstanders: 148 species), and Serrasalmidae (pacus and piranhas: 100 species) and coincides with a cluster of speciation events within Characidae (tetras: 1211 species), exclusive of the six species in Spintherobolinae. Within Characidae, the early diversification of Stethaprioninae (657 species) aligns particularly closely with the 30 Ma horizon.

The Bayesian pairwise comparisons of diversification models in TESS also support an episodic birth-death model with an overall rate shift at 30 Ma when assuming a diversified sampling strategy (Supplementary Table S3 available on Dryad). Although this study used a diversified sampling method, the test of relative model fit (Supplementary Table S3 available on Dryad), as well as tests of absolute fit (Supplementary Fig. S11 available on Dryad), equally favor a constant rate model when random ("uniform") taxon sampling is assumed. However, the CoMET model's identification of a shift at 30 Ma is robust to corrections for different sampling methods, with indiscernible results from simulations assuming uniform or diversified sampling (Supplementary Fig. S12 available on Dryad). It is well-known that corrections for incomplete and uneven sampling in analyses can reduce power to detect shifts in diversification rates and bias results (Brock et al. 2011; Chang et al. 2019). Our sampling strategy, however, achieved roughly equal representation of genus and family-level lineages throughout the characoid tree. The equally thorough sampling of regions of the characoid phylogeny in which rate shifts were and were not detected reduces the likelihood that sampling bias drove the diversification rate inference.

The detected shift in the general tempo of characoid diversification ∼30 Ma and the particularly prominent acceleration at that time in Anostomidae, Serrasalmidae, and Characidae explain why those three families collectively contain 68% of modern characoid species diversity and represent ∼30% of all Amazonian fishes (Dagosta and de Pinna 2019; Fricke et al. 2020). The remarkable bursts of speciation detected in diversification rate analyses using the UCE time-calibrated phylogeny began approximately 100 myr after the origin of Characoidei, indicating that elevated characoid species richness is a relatively recent phenomenon. The Oligocene shifts also occur 30 myr or more after the separation of each of the three characoid clades from their sister lineages. This conclusion of recent diversification after a long evolutionary fuse runs contrary to a recent diversification analysis that inferred Neotropical fish diversity as the result of steady and constant accumulation of species over vast swaths of time (Miller and Román-Palacios 2021). Instead, the pattern of lineage diversification in the characoid phylogeny reconstructed herein supports the hypothesis that ∼30 Ma the Neotropics cradled the origin of a substantial proportion of species diversity in the most diverse freshwater fish fauna on Earth.

What paleogeographic events in the history of South America might have driven the shifts in diversification rates that account for much of the living species diversity within Characoidei? Significant orogenic activity in the Central Cordillera from central Chile to Colombia drastically altered the landscape of northwestern South America (Lundberg et al. 1998; Hoorn et al. 2010; Evenstar et al. 2015). This high tectonic activity during the Late Oligocene and Early Miocene (∼30–20 Ma) resulted in the subsequent formation of the sub-Andean foreland basin and mega-wetlands (e.g., Pebas, Acre) in the proto-Orinoco-Amazonas system (Lundberg et al. 1998; Hoorn et al. 2010; Albert et al. 2018a). These geological processes redirected, fractured, and recombined watersheds during mega river captures (Tagliacollo et al. 2015; Albert et al. 2018a, 2018b), providing ample opportunities for allopatric speciation, the assembly of novel fish communities (Lundberg et al. 1998, Albert and Reis 2011), and colonization of upland riverine habitats (Silva et al. 2016; Machado et al. 2018). A growing body of evidence suggests that such rearrangements of connectivity within fractal riverine networks drives substantial speciation during continental fish radiations (Melo et al. 2018; Roxo et al. 2019; Albert et al. 2020; Ochoa et al. 2020; Fontenelle et al. 2021) as opposed to the explosive sympatric adaptive radiations that have yielded megadiversity in some lake

ecosystems (Fryer and Iles 1972; Goto et al. 2015; Ronco et al. 2021). The major Oligocene reconfiguration of paleowatersheds that formed the modern Amazon and Orinoco basins potentially accelerated the proliferation of characoids and the increased density of cladogenetic events reconstructed by CoMET and TESS likely reflects the signature of geographic dynamism in that region.

Clade-Specific Factors as Potential Accelerants of Diversification

Despite the compelling coincidence of major shifts in characoid lineage diversification with watershed rearrangement and wetland formation in the proto-Orinoco-Amazonas, geographic events alone do not explain the hyperdiversity of characoids. While three characoid clades accelerated their speciation, many co-occurring lineages did not. Why then were Anostomidae, Serrasalmidae, and Characidae able to generate many species during this period of geographic dynamism, while the majority of characoid families continued to diversify at their background rates? Did some traits specific to those clades allow them to exploit more fully the opportunities for speciation provided by paleogeographic changes to their freshwater landscape?

Numerous traits can render a clade particularly prone to speciation (see Seehausen and Wagner 2014; Albert et al. 2020). One class includes traits that limit gene flow, reduce vagility, and fragment species into isolated populations (Gavrilets et al. 2000; Czekanski-Moir and Rundell 2019), such as small body size, small range, and restricted habitat preferences (Jablonski and Roy 2003; Dagosta and de Pinna 2019; Albert et al. 2020). Such fragmentation can produce complexes of geographically isolated species with or without morphological divergence (Lande 1980; Zink 2014; Czekanski-Moir and Rundell 2019). Alternatively, a propensity for rapid evolution of mate preferences can lead to accelerated speciation via sexual selection with associated divergence in signaling morphologies such as coloration or ornamentation (Panhuis et al. 2001; Ritchie 2007; Kraaijeveld et al. 2011; Maia et al. 2013). A third class of accelerants increase the ability to evolve and speciate in response to ecological opportunity. Traits in this third class include high modularity (Rogers et al. 2013), genome duplication (Santini et al. 2009), and key innovations that allow the exploitation of underutilized resources or habitats (Liem 1973; Schluter 2000). Speciation via an increased propensity to respond to ecological opportunity tends to lead to exceptional ecomorphological disparity, either in a classic lacustrine adaptive radiation (Schluter 2000; Ronco et al. 2021) or during a continental radiation (Arbour and López-Fernández 2016; Silva et al. 2016; Burns and Sidlauskas 2019).

The key to the increased speciation rate of Serrasalmidae most likely lies within ecological opportunity, given that the family's ecomorphological variation spans almost every niche known for characoids (Burns and Sidlauskas 2019; Kolmann et al. 2020). In particular, the family displays extreme disparities in tooth morphologies that adapt them to diverse trophic ecologies such as omnivory, planktivory, herbivory, carnivory, and lepidophagy (Machado-Allison 1983; Nico et al. 2018; Huie et al. 2019; Kolmann et al. 2020; Mateussi et al. 2020a). Species within Anostomidae occupy omnivorous or herbivorous niches, display varied mouth positions and dentition (Sidlauskas and Vari 2008; Ramirez et al. 2017; Lofeu et al. 2021) and feature a bewildering diversity of coloration with various patterns of horizontal stripes, vertical bands, or lateral spots (Géry 1977; Sidlauskas and Vari 2012; Birindelli and Britski 2013; Sidlauskas and Birindelli 2018). The differences in color pattern, mouth position, and tooth morphology that separate anostomids suggests a role for both visually-determined sexual selection and an intrinsic capacity for ecomorphological evolution in the acceleration of speciation. Indeed, a recent experimental study determined that developmental plasticity may underlie this family's propensity for morphological evolution (Lofeu et al. 2021). Lastly, Characidae bears hallmarks of all three classes of potential accelerants: small size, diverse coloration, and high ecomorphological variation (Géry 1977; Weitzman and Vari 1988; Mirande 2010, 2019; Lima et al. 2003; Toledo-Piza et al. 2014). That small average body size predicts a greater propensity toward population fragmentation and allopatric isolation than in the other two families, with many species being endemic to single river systems, and in some cases only a single locality (Lima et al. 2013; Castro et al. 2003; Malabarba et al. 2004). Given the simultaneous action of population fragmentation via low vagility, genetic isolation via sexual selection, and an intrinsic propensity for ecomorphological evolution, it is perhaps unsurprising that Characidae has become the most species-rich group of Characoidei, spinning out more than 1200 species over the last 50 myr, with most species originating in just the last 30 Ma (Fig. 1).

Overall, the three characoid clades with accelerated speciation rates Anostomidae, Serrasalmidae, and Characidae demonstrate remarkable ecomorphological variation, suggesting that an intrinsic propensity for anatomical diversification during speciation in these lineages is highly plausible (Sidlauskas and Vari 2008; Mirande 2010; Ramirez et al. 2017; Burns and Sidlauskas 2019; Kolmann et al. 2020; Lofeu et al. 2021). Anostomidae and Characidae additionally display diverse color patterns (Géry 1977; Lima et al. 2013) that may serve as species recognition signals and increase the likelihood of genetic divergence via the evolution of mating preference. The most species-rich of the three clades, Characidae, adds small body size and thus potentially reduced vagility as a likely factor augmenting speciation. Yet, the possession of these factors by the three clades does not determine which actually catalyzed the rapid diversification of the rich Neotropical freshwater fish fauna. Future studies that explicitly reconstruct the

evolution of vagility, modularity, ecomorphology, or color pattern on a phylogeny spanning Characoidei and the other components of the Neotropical fish fauna may be able to uncover a correlation between some of these factors and net diversification rates. At present, we suggest that all three classes of clade-specific factors plausibly led to the proliferation of characoid diversity since the Oligocene, and that different combinations of these factors may have held primacy in each of the three most rapidly speciating clades of Characoidei.

Conclusions

We reconstructed the history of characoid diversification and revealed how bursts of lineage diversification ~30 Ma in three ecomorphologically diverse ancient lineages contributed to the extraordinary modern species richness of Neotropical freshwater fishes. These results characterize the Neotropics as a cradle of characoid species diversity and strengthen the case that the dynamic paleogeographic history of Greater Amazonia played an important role in the assembly of the richest freshwater fish fauna on Earth. By aligning primarily with the cradle scenario of diversification, our results lead to conclusions that differ from recent studies favoring steady lineage accumulation throughout the long history of the Neotropics (Albert et al. 2020; Miller and Román-Palacios 2021). Ultimately, testing the generality of these cradle and museum hypotheses will require time-calibrated, species-rich reconstructions for all major clades of Neotropical fishes. Remaining questions include whether cichlids, catfishes, killifishes, and electric knifefishes also accelerated diversification during the dynamism of the proto-Orinoco-Amazonas region, or whether those clades adopted a slower path of species accumulation throughout their long histories. Additionally, which clade-specific factors held greatest importance in catalyzing speciation? Only a continued drive to resolve the relationships of the thousands of Neotropical freshwater fish species already known and hundreds more described each year can provide the framework needed to answer those questions.

Fortunately, the goal of a comprehensive phylogeny for all Neotropical fishes is within sight. Concerted international efforts have surveyed great swaths of South America and deposited a wealth of voucher specimens and associated tissues in the world's museums (Page et al. 2015; Birindelli and Sidlauskas 2018). Methodological advances now allow the reliable extraction of genome-scale data from such specimens (Faircloth et al. 2012; Lemmon et al. 2012), computational advances allow the inference of ever larger topologies (e.g., Rabosky et al. 2018), and increased collaborations throughout the biological community recognize the power of working together and synthesizing data to solve grand problems (Sidlauskas et al. 2010; Padilla et al. 2014; Hinchliff et al. 2015). The publication of a characoid phylogeny greatly exceeding this one in diversity and the linking of that phylogeny to similar inference from other fish groups will surely result from the combined efforts of hundreds of scientists from diverse cultures and identities, working in different regions, and studying disparate taxa. By joining skills, data and knowledge, we will harness our own human diversity to understand the origins of the world's most remarkably diverse assemblage of freshwater fishes.

Supplementary Material

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.hqbzkh1fm.

Acknowledgments

The authors are thankful to many colleagues who helped with species identifications and discussions about the evolution of characoids: A. Esguícero, A.L. Netto-Ferreira, C.C. Conde-Saldaña, C.A.M. Oliveira, C. Silva-Oliveira, C.S. Sousa, F.R. Carvalho, F.C.P. Dagosta, F.C. Jerep, F. Langeani, F.C.T. Lima, F. Melo, G.S.C. Silva, G.M.T. Mattox, I. Soares, J.G. Ayala, J.G. Lundberg, J.L.O. Birindelli, L. Reia, M. Marinho, N.T.B. Mateussi, N.A. Menezes, R.P. Ota, and V.A. Bertaco. The authors also thank curators and staff of institutions who generously provided important samples: B. Brown and T. Vigliotta (AMNH), M. Arce H and M.H. Sabaj (ANSP), D. Werneke and J. Armbruster (AUM), J. Zuanon, R. Ribeiro, and L. Rapp Py-Daniel (INPA), R. Covain (MHNG), A. Datovo and M. Gianeti (MZUSP), D.C. Carvalho (PUC), H. López-Fernandez (ROM), R. Reina (STRI), and M. Nirchio (Univ. Machala). Portions of analyses were supported by the Center for Scientific Computing (GRID/UNESP) and *Brycon/Zungaro* servers of the São Paulo State University. Special thanks to photographers of fishes in Figures 1 and 2: John P. Sullivan (Distichodontidae), Ray Schmidt (Alestidae, Hepsetidae), Clinton Robertson (Cynodontidae), Mark H. Sabaj (Siluriformes, Anostomidae, Chilodontidae), Jorge E. Garcia-Melo, Luís J. García-Melo, and Proyeto CaVFish Colombia (Chalceidae, Bryconidae, Triportheidae), Bruno F. Melo (Prochilodontidae), and Martin I. Taylor (all remaining pictures: Crenuchidae, Erythrinidae, Hemiodontidae, Serrasalmidae, Parodontidae, Curimatidae, Ctenoluciidae, Lebiasinidae, Gasteropelecidae, Iguanodectidae, Acestrorhynchidae, Characidae).

Funding

Authors were independently funded by Fundação de Amparo à Pesquisa do Estado de São Paulo: grants #16/11313-8 and #18/24040-5 (to B.F.M.), grants #14/05051-5 and #15/00691-9 (to F.F.R.), grants #14/06853-8 and #18/23883-9 (to L.E.O.), and grant #14/26508-3 (to C.O.); the Conselho Nacional de

Desenvolvimento Científico e Tecnológico, grant #404991/2018-1 and #200159/2020-8 (to B.F.M.), grant #307975/2019-3 (to R.C.B.), grant #306054/2006-0 (to C.O.); the National Science Foundation, grant DEB-1257898 (to B.L.S.), grant DEB-165594 (to M.L.J.S.), grant DEB-1601830 (to J.C.), grant DEB-1242267 (to B.C.F.); the Bingham Oceanographic Fund maintained by the Peabody Museum of Natural History, Yale University (to T.J.N.), the National Institute of Health Predoctoral Training Program in Genetics T32 GM 007499 (to A.G.), the AMNH Axelrod Research Curatorship (to M.L.J.S.), the Edward W. Rose Postdoctoral Fellowship from the Cornell Lab of Ornithology (to M.D.B.), and the Programa de Pós-Graduação em Biologia Comparada da Universidade de São Paulo (to R.M.C.C.).

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