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Phylogenomics of Piranhas and Pacus (Serrasalmidae) Uncovers How Dietary Convergence and Parallelism Obfuscate Traditional Morphological Taxonomy

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Abstract.—The Amazon and neighboring South American river basins harbor the world's most diverse assemblages of freshwater fishes. One of the most prominent South American fish families is the Serrasalmidae (pacus and piranhas), found in nearly every continental basin. Serrasalmids are keystone ecological taxa, being some of the top riverine predators as well as the primary seed dispersers in the flooded forest. Despite their widespread occurrence and notable ecologies, serrasalmid evolutionary history and systematics are controversial. For example, the sister taxon to serrasalmids is contentious, the relationships of major clades within the family are inconsistent across different methodologies, and half of the extant serrasalmid genera are suggested to be non-monophyletic. We analyzed exon capture to reexamine the evolutionary relationships among 63 (of 99) species across all 16 serrasalmid genera and their nearest outgroups, including multiple individuals per species to account for cryptic lineages. To reconstruct the timeline of serrasalmid diversification, we time-calibrated this phylogeny using two different fossil-calibration schemes to account for uncertainty in taxonomy with respect to fossil teeth. Finally, we analyzed diet evolution across the family and comment on associated changes in dentition, highlighting the ecomorphological diversity within serrasalmids. We document widespread non-monophyly of genera within Myleinae, as well as between *Serrasalmus* and *Pristobrycon*, and propose that reliance on traits like teeth to distinguish among genera is confounded by ecological homoplasy, especially among herbivorous and omnivorous taxa. We clarify the relationships among all serrasalmid genera, propose new subfamily affiliations, and support hemiodontids as the sister taxon to Serrasalmidae. [Characiformes; exon capture; ichthyochory; molecular time-calibration; piscivory.]

The family Serrasalmidae, piranhas and pacus (Fig. 1), is a diverse freshwater clade of characiform fishes found throughout tropical and subtropical South America. Ninety-eight extant species are primarily distributed east of the Andes, with just a single species found west in the Maracaibo Basin. The serrasalmid fossil record extends the historical distribution of the family further west into the Magdalena region (Lundberg et al. 2010). While piranhas are generally carnivorous, their sister taxa, the pacus, are herbivores that include some of the primary seed dispersers in flooded forests or varzea (Goulding 1980; Correa et al. 2007). Despite their keystone status and commercial significance throughout Amazonia, the ecologies of piranhas and pacus are poorly understood and often misrepresented. The ferocious reputation of piranhas stems largely from accounts of their feeding on corpses (Sazima and Guimarães 1987) or nipping bathers, the latter attributed to the protective nature of piranhas defending nests (Haddad and Sazima 2003, 2010; but see Kolmann et al. 2020). We have only recently understood the role that pacus play in structuring forests, as large frugivores that can disperse seeds over great distances (ichthyochory) (Correa et al. 2015, 2016). Some rainforest trees appear particularly specialized for ichthyochory, with seeds that have greater germination probability after digestion by fishes (Anderson et al. 2009) or by bearing fleshy fruit with increased buoyancy (Horn et al. 2011; Correa et al. 2018). Large pacus like the iconic tambaqui (*Colossoma*) and pirapitinga (*Piaractus*) are attracted to the sound of fruit falling in the water, even "staking out" fruiting trees for weeks at a time (Goulding 1980).

From fruits and seeds, to fins and flesh, serrasalmids feed on a wide variety of prey (Correa et al. 2007), but the evolutionary ecology of many genera remains understudied. Medium-sized pacus (Myleinae) feed heavily on the leaves, flowers, and stems of riparian and aquatic plants (Correa and Winemiller 2014), with some adapted to scraping river weed (Podostemaceae) off rocks in a manner unique among otophysan fishes (Andrade et al. 2019a; Huie et al. 2019). Contrary to the popular idea of piranhas as exclusively carnivorous, their diets vary greatly across seasons, ontogeny, and species. Some piranhas are facultative frugivores (*Pristobrycon*; Nico and Taphorn 1988; Correa and Winemiller 2014), while others feed on either fish scales (lepidophages, i.e., *Catoprion*; Goulding 1980; Sazima and Machado 1990; Kolmann et al. 2018) or fins (pterygophages, i.e., *Serrasalmus elongatus*; Röpke et al. 2014). Whether some of these specialized diets are convergent or unique to certain clades remains uncertain, despite previous efforts (Correa et al. 2007),

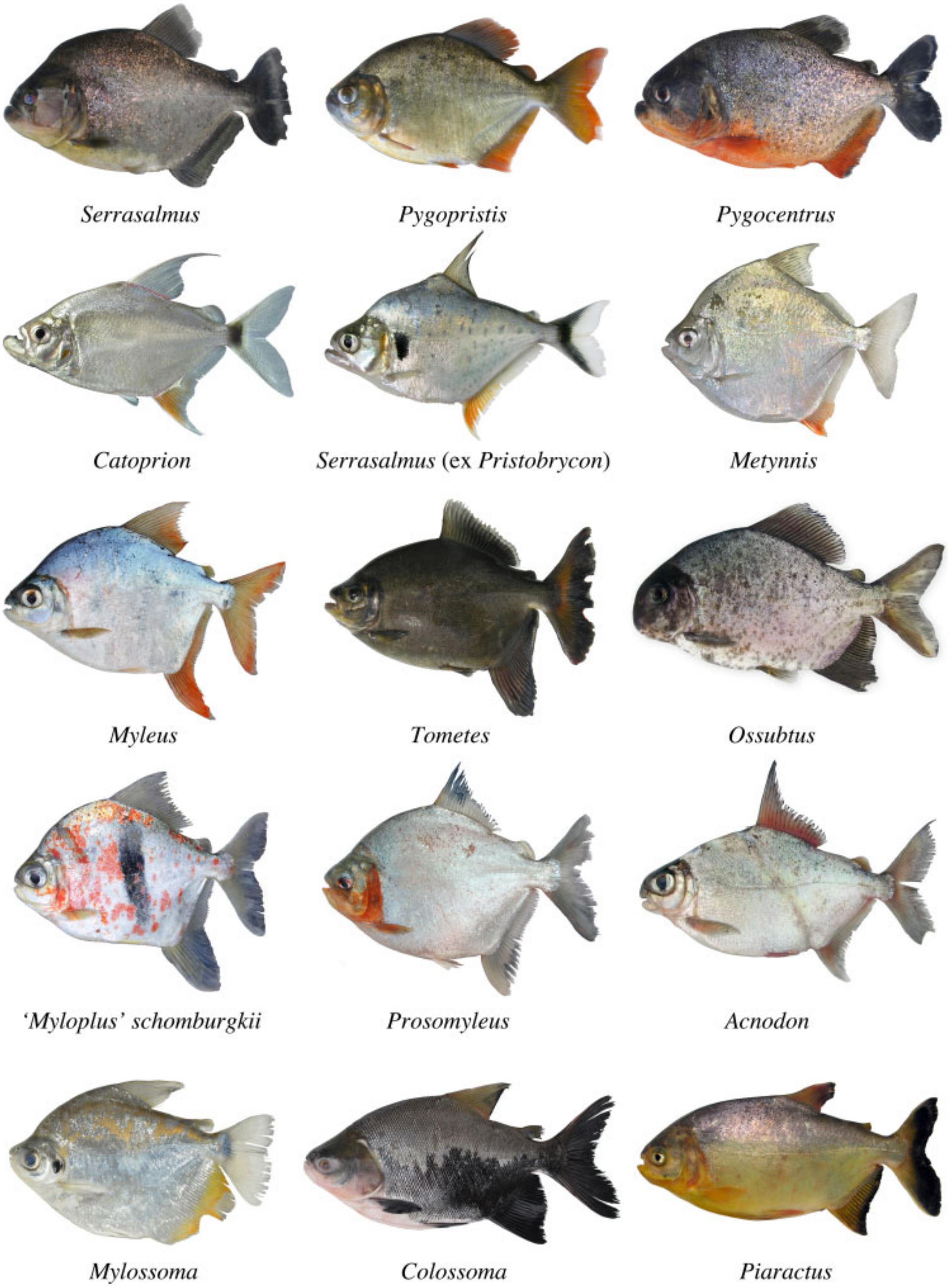

FIGURE 1. Morphological diversity of various serrasalmid species and genera. Piranhas divided from pacus by vertical and horizontal lines. Photo of *Ossubtus* by L. Sousa, others by M. Sabaj.

because of the lack of a well-resolved phylogenetic framework for the family.

Early classifications (Eigenmann 1915; Norman 1929; Gosline 1951; Huelsenbeck et al. 2003) relied on dentition to divide serrasalmids into two major groups, those with two rows of teeth on the upper jaw ("herbivorous" pacus) versus one row ("carnivorous" piranhas). A more comprehensive and cladistic analysis of morphology by Machado-Allison (1982, 1983, 1985) also divided serrasalmids into two major groups, but disagreed on their composition by transferring *Metynnis*, previously classified as a pacu, to the piranha clade. The cladistic analysis of the fossil †*Megapiranha* by Cione et al. (2009) supported the placement of *Metynnis* sister to piranhas and provided the first morphological evidence for the non-monophyly of the remaining pacus, based primarily on tooth morphology. Tooth characteristics and arrangement remain the best characters for distinguishing pacu taxa (Andrade et al. 2013, 2016; Nico and Taphorn 1988), with morphologies that vary along numerous phenotypic axes, from molariform to incisiform, spatulate to crenulate (Huie et al. 2019; Kolmann et al. 2019).

Molecular phylogenies (Ortí et al. 1996, 2008) support the morphological phylogeny of Cione et al. (2009) wherein the piranhas + *Metynnis* clade are sister to medium-sized pacus like *Acnodon* and *Myleus* (Myleinae) and are therefore nested within pacus (Supplementary Material available on Dryad at http://dx.doi.org/10.5061/dryad.6t1g1jww0). However, molecular studies using mitochondrial genes (Ortí et al. 1996, 2008; Hubert et al. 2007; Freeman et al. 2007) revealed problems with generic monophyly and failed to reconcile genus-level relationships. Burns and Sidlauskas (2019), using two mitochondrial and two nuclear genes, resolved piranhas as paraphyletic, with larger piranhas (*Pygocentrus*) sister to *Colossoma*, and with *Serrasalmus* sister to a clade uniting *Myloplus* + *Catoprion*. Thompson et al. (2014) constructed the most rigorous serrasalmid phylogeny to date and found rampant non-monophyly within Myleinae and among *Serrasalmus* and *Pristobrycon* species (Supplementary Material available on Dryad). These molecular analyses had limited taxonomic sampling, including only two of the five *Pristobrycon* species, and lacked *Utiaritichthys*, which recent DNA barcoding suggests is nested within *Myloplus* (Machado et al. 2018). Finally, even the identity of the sister clade to serrasalmids is contentious. The hemiodontids (Oliveira et al. 2011; Betancur-R et al. 2019) and all curimatoids (i.e., "anostomoids" Calcagnotto et al. 2005; Burns and Sidlauskas 2019) have been proposed as the nearest relatives to pacus and piranhas.

Improving our understanding of the timeline of lineage, phenotypic, and ecological diversification in serrasalmids first requires broader species-level sampling. Resolution of recalcitrant relationships or questionable monophyly (e.g., *Serrasalmus*) should benefit from phylogenomic approaches that leverage much larger data sets than previously available. Thompson et al. (2014) dated their serrasalmid phylogeny with two fossil calibrations and found that the divergence between pacus and piranhas (+ *Metynnis*) began during the middle Paleocene (~60 Ma) and dated the onset of diversification for Myleinae to the late Eocene (45 Ma). The age of other lineages within serrasalmids are more uncertain, despite a rich fossil record that could improve our understanding of the timeline of diversification within the family and their nearest characiform relatives (Dahdul 2004, 2007).

We used exon-capture phylogenomic methods to reassess relationships among serrasalmid genera and expanded species-level sampling relative to earlier studies by 26%, to test whether genera are monophyletic. Using multiple fossil calibrations to estimate relative divergence times among lineages, we explored how uncertainty about fossil choice shapes our estimates of the timing of lineage diversification in serrasalmids. Finally, we used ancestral character state estimation to document dietary diversity, novelty, convergence and parallelism across extant serrasalmid lineages, as well as when major diet specializations arose in these fishes. We postulate that dietary homoplasy (convergence or parallelism) may be shaping dental morphology and discuss the effect this phenomenon has on taxonomic character states used to distinguish among serrasalmid genera.

MATERIALS AND METHODS

Taxonomic Sampling, DNA Extraction, and Exon Capture Protocols

Exon sequence data were analyzed for 194 individuals (Supplementary Table S1 available on Dryad) including 44 previously published in Andrade et al. (2017) and Betancur-R et al. (2019). We dropped five taxa due to the low number of sequences assembled (from Arcila et al. 2017 and Betancur-R et al. 2019), and another seven taxa from our latest sequencing efforts for mislabeling or contamination. Outgroups included 59 individuals representing 50 species distributed among 38 genera in 12 families of Characiformes, with particularly dense sampling within Curimatoidea (Betancur-R et al. 2019). Given the poor sampling in previous studies of the putative nearest serrasalmid outgroups, we included every species from Cynodontidae and all but one genus from Hemiodontidae (including the never-before sequenced monotypic genus *Micromischodus*). The ingroup, serrasalmidae, included 135 individuals representing all 16 nominal valid genera, 63 of the 99 nominal valid species, and 16 OTUs which may represent cryptic lineages within phenotypically variable and wide-ranging taxa (e.g., *Myleus cf. setiger, Serrasalmus cf. eigenmanni*) for 63% taxon completeness for the family. A probe set for exon capture previously optimized for Otophysi by Arcila et al. (2017) was used to capture 1051 exons for sequencing.

Genomic DNA was extracted from muscle biopsies or fin clips preserved in 90–99% ethanol using either Qiagen DNEasy kits or the phenol-chloroform protocol in the Autogen platform available at the Laboratory of Analytical Biology at the National Museum of Natural History (Smithsonian Institution) in Washington, D.C. Laboratory protocols for library preparation and probe sets for exon capture were optimized for Otophysi, and followed the procedures described in Arcila et al. (2017). Library preparation and target enrichment was completed at Arbor Biosciences (www.arborbiosci.com). Paired-end sequencing (100 bp) was performed at the University of Chicago Genomics Facility on a HiSeq 4000, with up to 192 enriched libraries were combined to form multiplex pools for sequencing in a single lane.

Data Assembly and Alignment

We used the bioinformatics pipeline optimized by Hughes et al. (2020) to obtain sequence alignments for 951 exon markers from an initial set of 1051 (Supplementary Appendix S1 available on Dryad). Raw FASTQ files were trimmed with Trimmomatic v0.36 (Bolger et al. 2014), to remove low-quality sequences and adapter contamination. Trimmed reads were then mapped with BWA-MEM (Li and Durbin 2009) against a fasta file containing all sequences used for bait design (see Arcila et al. 2017). SAMtools v1.8 was used to remove PCR duplicates and sort the reads that mapped to each of the exons (Li et al. 2009b). Sorted reads (by species) were then assembled individually for each exon using Velvet (Zhang et al. 2018), and the longest contig produced by Velvet was used as the initial reference sequence for input to aTRAM v2.0 (Allen et al. 2017). aTRAM was run with Trinity v2.8.5 as the assembler to extend contigs iteratively. Redundant contigs with 100% identity produced by aTRAM were removed with CD-HIT v4.8.1 using CD-HIT-EST (Li and Godzik 2006; Fu et al. 2012). Open reading frames for remaining contigs were identified with Exonerate (Slater and Birney 2005). Sequences for each exon were aligned with MACSE v2.03 (Ranwez et al. 2018).

Resulting data matrices were combined with previously published exon sequences (Arcila et al. 2017; Betancur-R et al. 2019) (Supplementary Table S1 available on Dryad). For quality control and assessment, estimated gene trees were visually assessed and flagged when Serrasalmidae was not monophyletic. These gene trees were visually inspected to detect and remove putatively paralogous sequences, and samples with extremely long branches relative to conspecifics and congenerics. We excluded 13 loci for having a large number of suspected paralogs, and alignments with fewer than 100 sequences ($n=5$) also were removed from downstream analyses.

Phylogenetic Inference

A species tree was estimated under the multi-species coalescent (MSC) using ASTRAL-III v5.6.3 (Mirarab et al. 2014; Zhang et al. 2018), with individual gene trees estimated under maximum likelihood (ML) using IQTREE v1.6.10 (Nguyen et al. 2014). Each locus was partitioned by codon position, according to automatic model selection parameters obtained from ModelFinder using the “TESTMERGE” option (Kalyaanamoorthy et al. 2017), with 10-independent ML searches for each gene alignment. Concatenated amino acid and nucleotide matrices also were analyzed with IQTREE, with nucleotide sequences partitioned by codon position and the best substitution model was fitted using the “TEST” option of ModelFinder. Protein sequences were translated from nucleotides using AliView v1.0 (Larsson 2014) and the best model across all genes selected using ModelFinder. Ten-independent searches were run for each concatenated analysis. Branch support for the ML analyses of concatenated matrices was assessed with 1000 ultra-fast bootstrap (UFBoot) replicates (Minh et al. 2013) and 1000 SH-like approximate likelihood ratio test (SH-aLRT) replicates (Guindon et al. 2010). Support for the species tree topology obtained with ASTRAL-III was assessed with local posterior probabilities (PP, Sayyari and Mirarab 2016).

Fossil Calibrations and Rationale

The oldest fossils associated with Serrasalmidae are isolated pacu-like teeth from the Bolivian El Molino Formation (~73–60 Ma; Gayet 1991; Gayet and Meunier 1998). Although often used to date the origin of the family, these fossil teeth are unusual for serrasalmids for several reasons: (1) their small size of <0.75–1.0 mm (Gayet 1991; Gayet and Meunier 1998; Gayet et al. 2001) despite most serrasalmid teeth being far larger (>1 cm; Shellis and Berkovitz 1976; Kolmann et al. 2019); (2) pacu teeth, while complex in shape, lack the lingual cusp visible in Gayet et al. (2001, Fig. 7D); (3) these fossil teeth lack any of the interlocking morphologies typical of extant serrasalmid dentitions (Kolmann et al. 2019); and (4) the timeline of Bolivian serrasalmid fossil teeth leaves a 25–32 Ma gap in the fossil record until the first confidently-identified serrasalmid tooth, that is, teeth of the same size and shape as modern taxa (~38 Ma; DeCelles and Morton 2002; Dahdul 2007; Lundberg et al. 2010).

The El Molino fossils could represent juvenile serrasalmid teeth, but this hypothesis seems unlikely due to the absence of larger adult teeth and the well-known taphonomic bias toward larger-sized skeletal elements. Furthermore, these fossil teeth strongly resemble dentitions from distantly related alestids (Alestidae) discovered in various North African deposits (Murray 2003, 2004a,b). It is possible that these Bolivian fossils are stem characoids, or simply indistinguishable at a more circumscribed exclusive taxonomic level (as others have suggested, see Patterson 1993; Otero et al. 2008). This scenario is supported by the non-monophyly of South American characoids with respect to the African Alestoidea (Alestidae + Hepsetidae), and its sister group relation to exclusively Neotropical taxa in Neotropical Erythrinoidea + Curimatoidea (Arcila et al. 2017; Betancur-R et al. 2019). Given this uncertainty, we excluded the putative pacu teeth from the El Molino formation in our first set of calibrations (Scheme 1) but included them in our second set to assess its effect on divergence times within the family (Scheme 2; Supplementary Material available on Dryad). These calibration schemes included 11 outgroup fossils, and three additional serrasalmid calibration points are based on Miocene fossils summarized in Lundberg et al. (2010).

To explore how the ambiguity surrounding these fossils alters our estimates of serrasalmid diversification, we used two different fossil calibration schemes and contrast the timelines produced by these analyses (and by previous studies, e.g., Broughton et al. 2013; Burns and Sidlauskas 2019). We calibrated Scheme 1 with 15 fossil calibrations and Scheme 2 with 14 fossils. We used exponential distributions on each fossil prior except for the root, which used a normal distribution (Chen et al. 2010), in order to account for increasing uncertainty at further points in the past. Mean and standard deviations were estimated based on the calibration setting from other studies (e.g., Broughton et al. 2013; Chen et al. 2010; Thompson et al. 2014; Burns and Sidlauskas 2019) which used the same fossils as calibrations points.

The first 11 fossil calibrations dealt with calibrations external to Serrasalmidae, in other characiform families.

Within Characoidea, we dated the divergence between Characidae and Chalceidae, using fossil *Paleotetra* from the Aiuruoca Tertiary Basin (Weiss et al. 2012, 2014), Minas Gerais State in Eocene-Oligocene sediments (Garcia et al. 2000) (minimum age/offset =23.0 Ma, mean =11.75). Two fossils were used to date within Alestoidea; for dating the base of Alestoidea *sans* Hepsetidae, we used fossil †*Alestoides eocaenicus* from Eocene Dormaal, near Brabant, Belgium (minimum age/offset =48.6 Ma, mean =3.2) (Zanata and Vari 2005; Gaudant and Smith 2008; Chen et al., 2013). We also used fossils of the extant genus *Hydrocynus* to date the divergence between *Hydrocynus* + *Micralestes*, from the middle Eocene Hamada of Méridja deposits, in southwestern Algeria (Hammouda et al. 2016) (minimum age =37.0 Ma/offset, mean =3.85).

We used two fossils pertaining to Erythrinidae; firstly, we used fossils attributed to Erythrinoidea (Gayet et al. 2003) from the Late Cretaceous to Paleocene of Bolivia (Gayet and Brito 1989; Gayet 1991; Gayet and Meunier 1998) to date the root of our phylogeny, that is, the node uniting Characoidea with Curimatoidea + Alestoidea (*sensu* Betancur-R et al. 2019) (minimum age/offset = 58.2 Ma, mean =13.82). To calibrate the node uniting *Hoplerythrinus* + *Hoplias*, we used teeth attributed to †*Paleohoplias assisbrasiliensis* (Gayet et al. 2003) from the late Miocene Solimões Formation of Acre State, Brazil (Latrubesse et al. 1997; Cione et al. 2003; Gross et al. 2011) (minimum age =7.2 Ma/offset, mean =17.0). Finally, we used fossil cynodontid teeth to calibrate the node uniting *Hydrolycus* + [*Rhaphiodon*, *Cynodon*]. These fossils are from middle Miocene sediments associated with the La Venta fauna near Tolima, Colombia (minimum age/offset =7.2 Ma, mean =17.0; Lundberg 1997; Cione and Casciotta 2010).

Three fossils were used to date within anostomoids and one fossil from related Parodontidae; for dating the base of Anostomidae, we used a fossil oral tooth attributed to *Leporinus* sp. from the lower Pozo Formation, Contamana, Peru (Antoine et al. 2016), Middle Eocene sediments (minimum age/offset =35.0 Ma, mean =7.7; Burns and Sidlauskas 2019). We also used fossils of †*Leporinus scalabrinii* (Bogan et al. 2012) to date the divergence between *Abramites hypselonotus* + *Leporinus striatus*, from the late Miocene deposits of the Ituzaingó Formation in Entre Ríos, Argentina (Marshall et al. 1983; Cione et al. 2000, 2009) (minimum age/offset = 6 Ma, mean =9.7). Finally, to calibrate the node uniting *Cyphocharax* + *Psectrogaster* with *Curimata*, we used †*Cyphocharax mosesi* from the Tremembe Formation, Sao Paulo, Brazil in Oligocene sediments (Malabarba 1996) (minimum age =23.0 Ma/offset, mean =11.7). †*Cyphocharax mosesi* was originally proposed as forming a polytomy with the genera *Cyphocharax*, *Curimatella*, and *Steindachnerina* (Malabarba 1996; Burns and Sidlauskas 2019).

We also used fossil teeth attributed to *Parodon* by Roberts (1975) to date the divergence between *Apareiodon* + *Parodon*, from mid-late Miocene deposits of the Loyola Formation near Cuenca, Ecuador (Bristow 1973) (minimum age/offset =11.2 Ma, mean =15.7) (Hungerbühler et al. 2002).

Within Serrasalmidae, four fossil calibrations were used; firstly, for Scheme 2, we used the isolated pacu teeth first described in Gayet (1991), and used by Broughton et al. (2013) and Thompson et al. (2014) to date the divergence of serrasalmids from other non-serrasalmid characiforms (minimum age =61.0 Ma/offset, mean =12.9). Whereas Broughton et al. used this fossil to represent the MRCA for *Pygocentrus* + *Hemiodus*, Thompson et al. used these fossil teeth to calibrate the node uniting *Serrasalmus* + *Piaractus*. For Scheme 1, we removed this calibration and replaced with pacu teeth described by DeCelles and Horton (2003) from the Paleocene-Eocene Santa Luca Formation, Bolivia (minimum age =38.0 Ma/offset, mean =6.75). To calibrate the node uniting *Colossoma* + *Mylossoma*, we used teeth and partially articulated jaws documented by Lundberg et al. (1986) and Dahdul (2004) from the Miocene Castillo Formation, Venezuela (Rincon et al. 2014) (minimum age/offset =17.2 Ma, mean =7.0). The pacu fossils from above predate fossils of *Piaractus* (Sánchez-Villagra and Aguilera 2006) from the Tortonian Urumaco Formation in Falcón State, Venezuela (Dahdul 2004). Next, we used fossil teeth attributed to indeterminate myleines (medium-sized pacus) to calibrate the MRCA of *Acnodon* + *Myloplus* (Roberts 1975; Dahdul 2004) from the mid-late Miocene Loyola Formation near Cuenca, Ecuador (Bristow 1973) (minimum age/offset =11.2 Ma, mean =9.0) (Hungerbühler et al. 2002; Dahdul 2004). Finally, to calibrate the MRCA of all piranha genera, we used the upper Miocene fossil premaxilla described as †*Megapiranha paranensis* discovered in Entre Ríos, Argentina (Cione et al. 2009) (minimum age/offset =6.8 Ma, mean =10.4).

We used BEAST 2 (v2.5.0; Bouckaert et al. 2014) to generate relaxed-clock divergence time estimates (Drummond et al. 2006) on four 50-gene subsets of our data, randomly selected from the 200 most complete genes, and pruned so that only one tip per taxon remained. We transformed the concatenated ML DNA topology into a chronogram under penalized likelihood using the *chronos* function in R (ape v5.3; Paradis et al. 2019). This chronogram was used as a starting tree for BEAST 2 v2.5.0 (Bouckaert et al. 2014; Supplementary S2 available on Dryad) to generate relaxed-clock divergence time estimates. The topology of the resulting trees was also constrained to match the concatenated nucleotide phylogeny. Each subset of 50 genes was run independently twice for 2.0×10^{8} generations. Convergence was assessed in Tracer v1.7.1 (Rambaut et al. 2018) by checking that ESS values were greater than 200 for all parameters. Independent runs from each of the four different subsets were combined in LogCombiner if their 95% highest posterior densities for divergence times overlapped, and a maximum clade credibility tree was generated in TreeAnnotator for each of the two calibration schemes.

All subsets had 103 included taxa, and Subset 1 had 4425 sites, Subset 2 had 5264 sites, Subset 3 had 5288 sites, and Subset 4 had 5096 sites. For each BEAST2 run, we used the GTR + gamma as our site model for each locus. We used a birth–death model tree prior for node time estimation, allowing for both speciation and extinction rates to vary for any given lineage (Drummond et al. 2006). We fixed the topology of our starting tree by turning off the following operators in BEAST2: (1) set "wide-exchange" = "false", (2) set "narrow-exchange" to "false", (3) set "subtree-slide" to 0, and (4) set "Wilson-Balding" to 0. Convergence of each gene subset was assessed individually in Tracer (v. 1.7.1) by checking that ESS values were greater than 200 for all parameters. Independent runs from each of the four different subsets were combined in LogCombiner if their 95% highest posterior densities for divergence times overlapped, and a maximum clade credibility tree was generated in TreeAnnotator for each of the two calibration schemes.

Ancestral State Reconstruction of Diet Evolution in Piranhas and Pacus

We used ancestral state reconstructions on both discrete and continuous data to map the evolution of broad diet guilds and then the degree of piscivory/herbivory across serrasalmids. Serrasalmid taxa were assigned to diet categories based on the results of a clustering algorithm (function *hclust*) with a 60% dissimilarity threshold (Egan et al. 2018; Pos et al. 2019). Starting from a meta-analysis of gut content studies from the literature, we excluded data that reported frequency of occurrence (%FO) and preferentially used volumetric or composite (e.g., index of relative importance) metrics when available (except 4/63 OTUs). For taxa where only qualitative diet data were available (8/63 OTUs), we assumed equal importance of all prey categories. The cluster analysis proposed five discrete diet categories (Supplementary Materials available on Dryad): (1) frugivores (seeds and fruits), (2) planktivores/algivores, (3) omnivores/folivores (flowers, leaves, insects), (4) fin and scale feeders, and (5) piscivores. "Fin and scale-feeders" defines those taxa that have been reported to primarily consume some combination of scales, fin rays, and chunks of fishes (e.g., *Serrasalmus elongatus*, a fin-nipper or *Catoprion*, a scale-feeder; Gonzalez and Vispo 2003; Röpke et al. 2014; Nico and de Morales 1994). We used stochastic character mapping (Huelsenbeck et al. 2003; Bollback 2006) to reconstruct the evolution of these diet modes across the dated phylogeny (Scheme 1) with the *make.simmap* function in the R package phytools v. 0.6–99 (Revell 2012). We used AICc to choose among different transition rate models (ER: equal rates, SYM: symmetrical rates, and ARD: all rates different) and used 1000 simulations. Due to the computational intensity of re-running BEAST to obtain a posterior sample of trees for two dating scenarios (each with four separate runs of 200 loci), for three different methods of tree reconstruction, we opted to only analyze diet evolution on the concatenated nucleotide phylogeny. This phylogeny strongly resembles the topology of previous studies (Thompson et al. 2014) and all three methods are congruent with respect to the relationships among major serrasalmid clades (Fig. 2).

Discrete diet categories, however, do not capture the multidimensionality of trophic niches and diet resources of predators in the wild. To infer whether the ancestors of major serrasalmid clades consumed a greater proportion of plant material or fish flesh, we quantified the amount of either material from the published diet data described above. We summed all reported plant materials (e.g., seeds, fruits, leaves, etc.) and all reported fish materials (e.g., fins, scales, flesh, etc.) into singular categories and averaged values among different studies. We also used this method to explore whether species within the paraphyletic genus *Tometes* (and other phytophagous species) evolved similar diets independently or inherited this trophic mode from a common ancestor. We then plotted each continuous trait (herbivory, piscivory, or phytophagy) on the concatenated nucleotide phylogeny using the *contMap* and *phenogram* functions (Revell 2012).

RESULTS

Relationships Among the Major Serrasalmid Clades and Characiforms in General

In our concatenated nucleotide and amino acid analyses, hemiodontids were strongly resolved as the sister clade to serrasalmids, as suggested by others (Oliveira et al. 2011; Arcila et al. 2017; Betancur-R et al. 2019; Faircloth et al. 2020) (Fig. 2a,b). However, our coalescent analysis could not resolve the relationships among cynodontids, hemiodontids, and serrasalmids, although each clade was strongly resolved as monophyletic by all analyses (Fig. 2, upper left insets). As suggested by others (Burns and Sidlauskas 2019; Betancur-R et al. 2019), all of our analyses confidently supported the monophyly of an "anostomoid" clade and its close relationship to cynodontids, hemiodontids, and serrasalmids (weakly supported in Faircloth et al. 2020). Within the "anostomoid" clade, Anostomidae was sister to a subclade composed of Prochilodontidae and Curimatidae + Chilodontidae. Unlike Burns and Sidlauskas (2019) we resolved Parodontidae as sister to the "anostomoids" clade, whereas they (and Oliveira et al. 2011) found that scrapetooths were sister to serrasalmids, hemiodontids, and "anostomoid" clade combined. Other studies which did not find parodontids sister to the "anostomoid" clade either lack any cynodontids in their sampling (Burns and Sidlauskas 2019; Faircloth et al. 2020) or have skewed taxon sampling (e.g., Calcagnotto et al. 2005); whereas others which include denser taxon sampling (Betancur-R et al. 2019) have resolved parodontids as sister to anostomoids. The position of both cynodontids

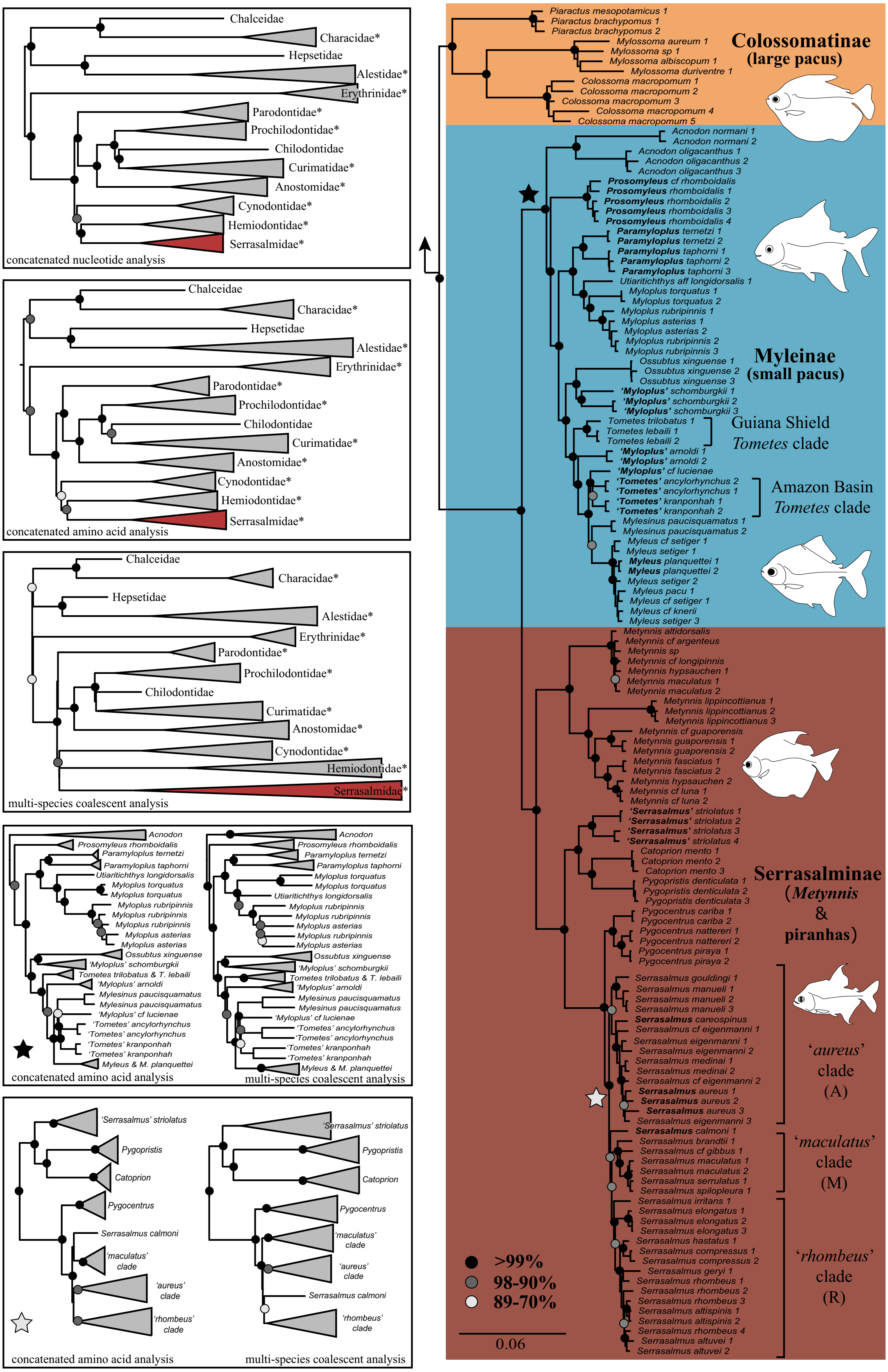

FIGURE 2. Comparison of concatenated nucleotide and amino acid trees and clade support in Serrasalmidae. Black circles represent nodes with > 99% UFBoot and aLRT support. Grey star indicates piranha generic inset, black star indicates the pacu generic inset. Individual OTU names are bolded where their generic affiliation has been reassigned based on our phylogenetic evidence. Resulting phylogenies from (A) concatenated nucleotide and (B) amino acid analyses of family-level relationships. (C) multi-species coalescent phylogeny for family-level relationships relative to Serrasalmidae. (D) Concatenated amino acid and (D) coalescent multi-species phylogenies for Myleinae. (E) Concatenated amino acid and (D) coalescent multi-species phylogenies for Serrasalminae. (F) Phylogeny from concatenated nucleotide analysis.

and parodontids, being long branches, appear critical to resolving the relationships among serrasalmids and their allies, with the superfamily Curimatoidea. The immediate sister taxon to the Curimatoidea, including headstanders, piranhas, pacus, scrapetooths, and halftooths (Betancur-R et al. 2019), are the wolf fishes and aimara (Erythrinidae), with strong-moderate support from concatenated analyses and weak support in our coalescent analysis. Deeper relationships among characiform outgroups follow Betancur-R et al. (2019).

We resolved Serrasalmidae into three major, well-supported clades (*sensu* Ortí et al. 1996; Cione et al. 2009; Thompson et al. 2014 and *contra* Calcagnotto et al. 2005; Burns and Sidlauskas 2019): Myleinae, Serrasalminae (*Metynnis* + piranhas) and Colossomatinae, new subfamily (larger pacus) (Fig. 2e). For the Colossomatinae, *Colossoma*, *Mylossoma*, and *Piaractus* were resolved as reciprocally monophyletic (Fig. 2e, orange box). Myleinae are sister to Serrasalminae (Ortí et al. 1996; Cione et al. 2009; Thompson et al. 2014). Within Myleinae (Fig. 2c,e blue box), *Acnodon* is resolved as sister to all other genera (in agreement with Thompson et al. 2014, but *contra* Ortí et al. 1998; Cione et al. 2009) and *Prosomyleus* (ex. *Myloplus*) *rhomboidalis* as sister to all other myleines (Ortí et al. 1998). Excluding *Pr. rhomboidalis*, other myleines are divided into two large clades: (1) *Myloplus asterias, M. rubripinnis, M. torquatus,* and *Utiaritichthys* are sister to *Paramyloplus* (ex. *Myloplus*) *ternetzi* and the newly described *Pa. taphorni*; (2) the *Ossubtus* and "*Myloplus*" *schomburgkii* clade (Machado et al. 2018) are sister to *Tometes*, *Mylesinus*, *Myleus*, "*Myloplus*". For the Myleinae clade (2): *Myleus setiger* is resolved as polyphyletic with respect to *Myleus knerii, Myleus pacu*, and *Myleus* (ex. *Myloplus*) *planquettei* with *Mylesinus paucisquamatus* resolved as sister to *Myleus* + *M. planquettei* in our concatenated nucleotide analysis (*sensu* Ortí et al. 1998). Also regarding Myleinae clade (2): *Tometes* is paraphyletic (as in Ortí et al. 1998; Thompson et al. 2014), with Amazon basin *Tometes ancylorhynchus* and *T. kranponhah* forming an immediate clade with "*Myloplus*" cf. *lucienae*, and then *Myleus* and *Mylesinus*, while Guiana Shield-dwelling *Tometes lebaili* and *T. trilobatus* are sister to the clade composed of *Myleus, Mylesinus,* "*Myloplus*" *arnoldi,* and other *Tometes* (Fig. 2c,e).

Within Serrasalminae (Fig. 2e maroon box), *Metynnis* is sister to a clade of all piranha genera. Within *Metynnis* are three primary clades: (1) *Metynnis luna* and *M. fasciatus* (and a lone individual of *M. hypsauchen*), which form a clade sister to *Metynnis guaporensis*. These species are in turn sister to (2) *Metynnis lippincottianus*. Clades (1) and (2) are sister to (3) *Metynnis maculatus, M. argenteus, M. longipinnis, M. altidorsalis,* and another individual of *M. hypsauchen*. For piranhas, all analyses find that the *Catoprion* + *Pygopristis* + "*Serrasalmus*" *striolatus* clade is sister to all other piranhas including *Pygocentrus*, *Serrasalmus*, and *Pristobrycon* (Hubert et al. 2007; Thompson et al. 2014; Fig. 2d). *Pristobrycon* is paraphyletic as suggested previously (Thompson et al. 2014), with the type for the genus, *P. calmoni*, nested within *Serrasalmus* (as in Hubert et al. 2007; Cione et al. 2009). *Pygocentrus* is supported as monophyletic (*contra* Ortí et al. 1998; Freeman et al. 2007) and is sister to all *Serrasalmus* (including *Pristobrycon* taxa).There are three primary clades within *Serrasalmus*: (1) the "*aureus*" clade (A), whereby short-snouted taxa like *Serrasalmus gouldingi* and *S. manueli* are sister to other short-snouted *Pristobrycon* taxa like *P. careospinus*, *P. aureus*, as well as *Serrasalmus medinai* and *Serrasalmus eigenmanni* (this last taxon is confusingly placed in either *Serrasalmus* or *Pristobrycon*) (Fig. 2d,e). The second primary *Serrasalmus* clade, the "*maculatus*" (M) group includes *Serrasalmus brandtii*, *S. gibbus*, *S. maculatus*, *S. serrulatus*, and *S. spilopleura*. Finally, the third *Serrasalmus* clade, "*rhombeus*" (R), includes tall-bodied taxa like *Serrasalmus hastatus and S. compressus, sister to S. geryi*, *S. rhombeus*, *S. altispinis*, and *S. altuvei*. As in previous studies, the relationships of these three *Serrasalmus* clades to one another is variable depending on reconstruction method; however, all are resolved with high support and the monophyly of many species are supported, contrary to previous studies (Thompson et al. 2014).

Comparison of Concatenation and Species Tree Methods

Concatenated analyses based on nucleotides or protein sequences placed hemiodontids as sister to serrasalmids with strong support (99/98 and 94/93 UFBoot/SH-aLRT, respectively). In contrast, the MSC approach produced a polytomy among three families, Serrasalmidae, Hemiodontidae, and Cynodontidae, albeit with strong support for this trichotomy (PP >0.95). All other relationships among main characiform lineages were consistent with higher-level phylogenetic studies of the order (Betancur-R et al. 2019; Supplementary Material available on Dryad).

Concatenated and MSC approaches supported three major clades within Serrasalmidae (Fig. 2), with some disagreement on relationships within these clades. Concatenated amino acid and MSC approaches support a different relationship among the three major lineages within the genus *Serrasalmus*, relative to the concatenated nucleotide analysis (Fig. 2). These three major lineages are the "*rhombeus*" clade (R), the short-snouted (brachycephalic) "*aureus*" clade (A), and the "*maculatus*" clade (M). Both the concatenated and MSC approaches applied to the amino acid data set resolved the *Serrasalmus maculatus* clade (M) as sister to the remaining *Serrasalmus* taxa, the ["*rhombeus*"clade (R) + *Pristobrycon calmoni*] and the "*aureus*" clade (A), with high support, but this relationship differs in the concatenated nucleotide analysis (Fig. 2). All analyses resolve *Pygocentrus* as sister to *Serrasalmus* and the associated *Pristobrycon* species nested therein, with high support (Fig. 2) (Supplementary Material available on Dryad).

The concatenated amino acid analysis and the coalescent analysis resolve *Mylesinus paucisquamatus* as

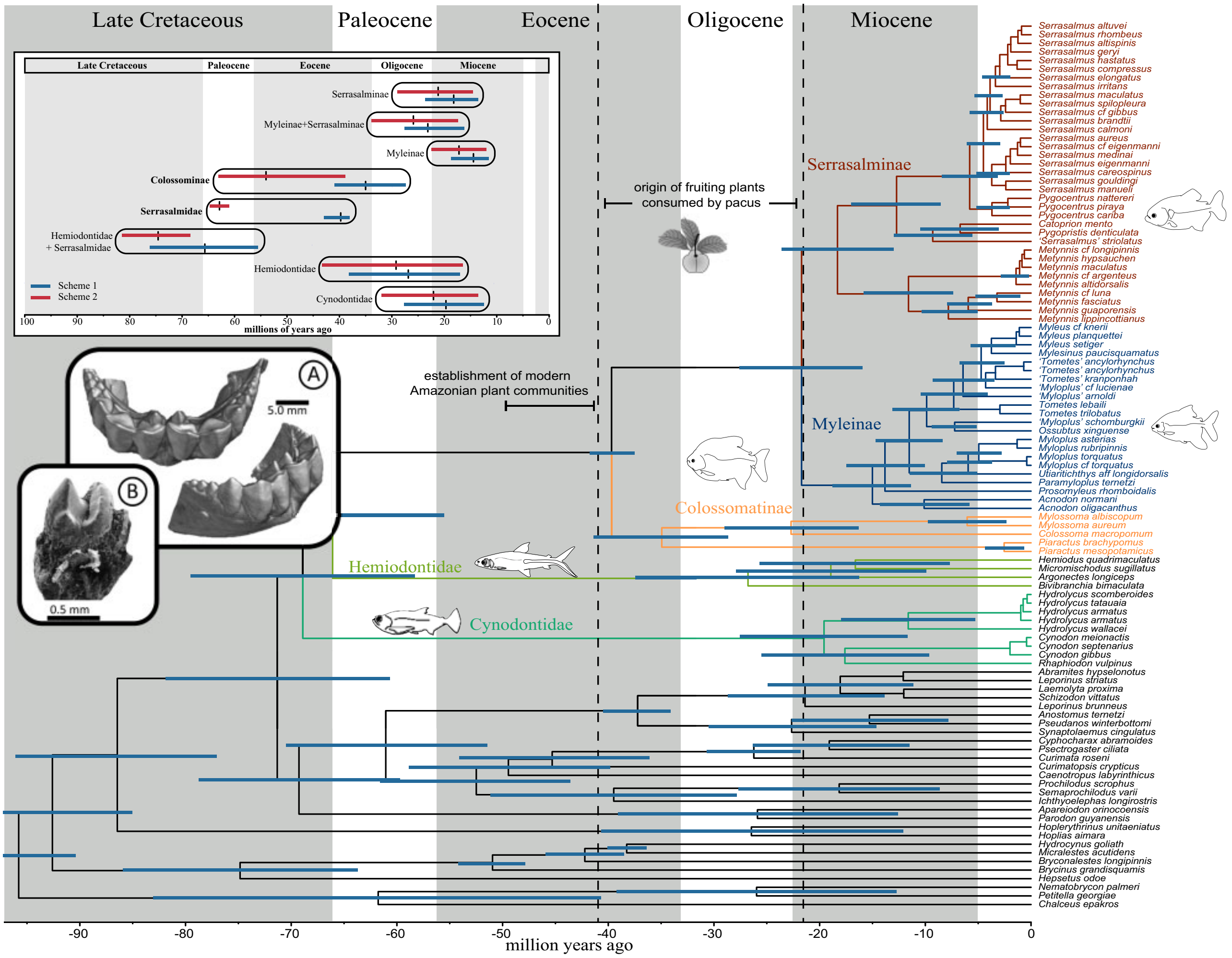

FIGURE 3. Ultrametric tree is the time-calibrated phylogeny using calibration Scheme 1. Inset shows a comparison of age estimates (HPDs) for select taxa based on both time-calibration schemes (blue bars represent HPD range from Scheme 1 and red bars represent HPD ranges from Scheme 2). Inset shows differences in HPD estimates for dating Schemes 1 and 2, ranges are younger for Scheme 1, relative to Scheme 2. Fruit icon is the rubber tree, *Hevea brasiliensis*, a preferred prey item of large-bodied pacus (Goulding 1980). Tooth images from (a) CT scans of *Piaractus orinoquensis* (UMMZ 214745) courtesy M. Kolmann and (b) reproduced from Gayet (2001; Fig. 7d).

sister to the clade comprised of *Tometes ancylorhynchus T. kranponhah* and "*Myloplus*" *cf. lucienae*, albeit with low support in the amino acid data set (Fig. 2). Alternatively, the concatenated nucleotide data set resolves *Mylesinus paucisquamatus* as sister to the *Myleus* clade with moderately high support. The placement of *Utiaritichthys* differed between the concatenation and MSC analyses, though it was always nested within *Myloplus sensu lato* (Fig. 2). For the first time, we can report that the newly described *Paramyloplus* (ex. *Myloplus*) *taphorni* (Andrade et al. 2019), an endemic to the Mazaruni Basin in Guyana, is sister to *Paramyloplus* (ex. *Myloplus*) *ternetzi*, a northern Guiana Shield endemic. Our study also firmly supports *Acnodon* as monophyletic and sister to all remaining members of Myleinae, something that all previous studies found difficult to resolve with certainty (Fig. 2).

Time Calibration

Bayesian analyses in BEAST2 converged on estimates of the posterior distributions, as indicated by ESS values >200 for all parameters. For each fossil calibration scheme, independent runs based on each of the four 50-gene subsets produced very similar mean-age estimates for all nodes. In contrast, we note marked differences between the two fossil calibration schemes with most obvious discrepancies in the estimated age for the MRCA of Serrasalmidae (HPD = 38–42.9 Ma vs. 61–64.6 Ma) and the split between serrasalmids and their hemiodontid sister clade (HPD =55.5–76 Ma vs. 68–81), although the latter estimates overlap considerably (Fig. 3). The ages of more inclusive clades overlap considerably between both dating schemes. Piranhas (Serrasalminae) are Miocene in age, in agreement with Thompson et al. (2014), with the modern radiation of

Serrasalmus (and *Pristobrycon*) stemming from Messinian time periods (5.3–7.2 Ma). The split between *Pygocentrus* and *Serrasalmus* being only slightly older, Tortonian-Messinian (combined HPD = 6–9 Ma; Fig. 3). The split between piranhas and their more herbivorous cousins, *Metynnis* (both Serrasalminae), occurred on a long branch during the mid-Oligocene to mid-Miocene (combined HPD = 14–24 Ma). While the radiation of carnivorous piranhas happened rapidly, the diversification of medium-sized pacus (Myleinae) was more gradual, occurring sometime within the same timeframe as *Metynnis* from piranhas (mid-Oligocene to mid-Miocene) (combined HPD = 11–30 Ma; Fig. 3). Conversely, the largest pacu species (Colossomatinae) diverged from one another (*Piaractus* from *Mylossoma* + *Colossoma*) early on in their history, late Eocene to mid-Oligocene (combined HPD = 27–41 Ma; Fig. 3) according to Scheme 1 or earlier in Scheme 2, from late Paleocene to late Eocene (combined HPD = 39–63; Fig. 3).

Diet Reconstruction

For our stochastic character mapping of diet states, the transition model with the lowest AICc (129.6) was the "SYM" or Symmetrical Rates model, where forward and reverse transitions for each state have the same parameter. We found that shifts between piscivorous diets and fin-/scale-feeding modes were the most common transitions, being 43–47% more frequent than shifts from folivorous and omnivorous feeding modes to frugivory (for example). Transitions between these two carnivorous diets were almost equally probable (Supplementary Table and Fig. S5 available on Dryad). While diet transitions from frugivory to piscivory were rare, transitions from frugivorous diets to fin-/scale-feeding modes were twice as likely; in fact, just as likely as transitions from frugivory to folivory or folivory to planktivory. When continuous diet characteristics were considered, many of the scale- and fin-feeding piranhas consume considerable portions of seeds and fruits as well (Supplementary Table and Fig. S5a available on Dryad). Planktivores and algivores appear to be the least labile feeding modes, with low frequency transitions only to folivorous/omnivorous diets (Fig. 4; Supplementary Table and Fig. S5 available on Dryad). Folivores and omnivores transitioned to both frugivorous and planktivorous/algivorous diets.

In general, there is strong support for the ancestor of all serrasalmids being herbivorous in some manner, most likely either folivorous or frugivorous. Large-bodied pacus (*Colossoma, Piaractus*) are more frugivorous than smaller-bodied pacus (Correa et al. 2007), with more recent frugivores (*M. planquettei, M. asterias*) evident within both Myleinae and Serrasalminae (e.g., *Pygopristis Pristobrycon*). Notable are the multiple instances of specialized "phytophagy" in select taxa like *Tometes Acnodon Ossubtus* and *Utiaritichtys*, which specialize on the flowers, leaves, and stems of particular river weed plants found only in fast-flowing rapids (Pereira and Castro 2014; Andrade et al. 2019) (Fig. 4 inset "A").

Within Serrasalminae, most species of *Metynnis* are partially to entirely planktivorous or algivorous, novelties for serrasalmids and hallmarks for the *Metynnis* clade in general (Canan and Gurgel 2002) (Fig. 4). We also found strong support that the ancestor of all piranhas was frugivorous, while the ancestor of Serrasalminae was folivorous/omnivorous. The advent of piscivory or scale-feeding in piranhas first indicated in the MRCA of the *Pygocentrus* + *Serrasamus* clade (Fig. 4). Evidence also suggests that some piranhas have reverted to a more plant-based diet, for example, *Serrasalmus* (ex. *Pristobrycon*) *aureus*; although, the "*aureus*" clade of piranhas, like *Serrasalmus gouldingi* and *Serrasalmus eigenmanni* are known to feed on fruits, seeds, leaves, insects as well as occasional fishes as adults (Nico and Taphorn 1988; Prudente et al. 2016) (Fig. 4; Supplementary Fig. S5a available on Dryad).

DISCUSSION

Suggestions for Serrasalmid Taxonomy, Moving Forward

Serrasalmid taxonomy and systematics are long "fraught with confusion and instability" (Nico et al. 2018:172). Recent morphological studies have helped distinguish and diagnose a variety of valid genera and species (e.g., Pereira and Castro 2014; Andrade et al. 2016a,b,c, 2017, 2018, 2019; Ota et al. 2016; Mateussi et al. 2018; Nico et al. 2018; Escobar et al. 2019). Likewise, recent molecular studies have helped place those taxa in a phylogenetic framework (Hubert et al. 2007; Ortí et al. 2008; Thompson et al. 2014) and uncovered new species-level diversity (Machado et al. 2018). The current study provides strong molecular support for recognizing three major lineages of Serrasalmidae at the subfamilial rank: Colossomatinae (new subfamily; pacus common to lowland, black water and white water habitats), Myleinae (widely distributed pacus particularly common to upland clear- and black water habitats; Goulding 1980), and Serrasalminae (*Metynnis* and all piranhas, cosmopolitan). Furthermore, our analyses strongly support the sister group relationship between Myleinae and Serrasalminae in congruence with previous phylogenies based on morphological (Cione et al. 2009) and molecular (Ortí et al. 2008; Thompson et al. 2014) data. We establish the new subfamily Colossomatinae to delineate the large-bodied, frugivourous pacus from smaller myleine pacus (see Supplementary available on Dryad for morphological synapomorphies).

Our results provide compelling evidence for other taxonomic changes as well. As suggested by previous studies, we consider *Pristobrycon* Eigenmann 1915 to be a junior synonym of *Serrasalmus* Lacepède 1803. The type species, *Pristobrycon calmoni*, consistently nests within *Serrasalmus* (Fig. 2; Thompson et al. 2014), although its precise placement differs among our analyses of amino acids, nucleotides and MSC. Moreover, *P. calmoni* has

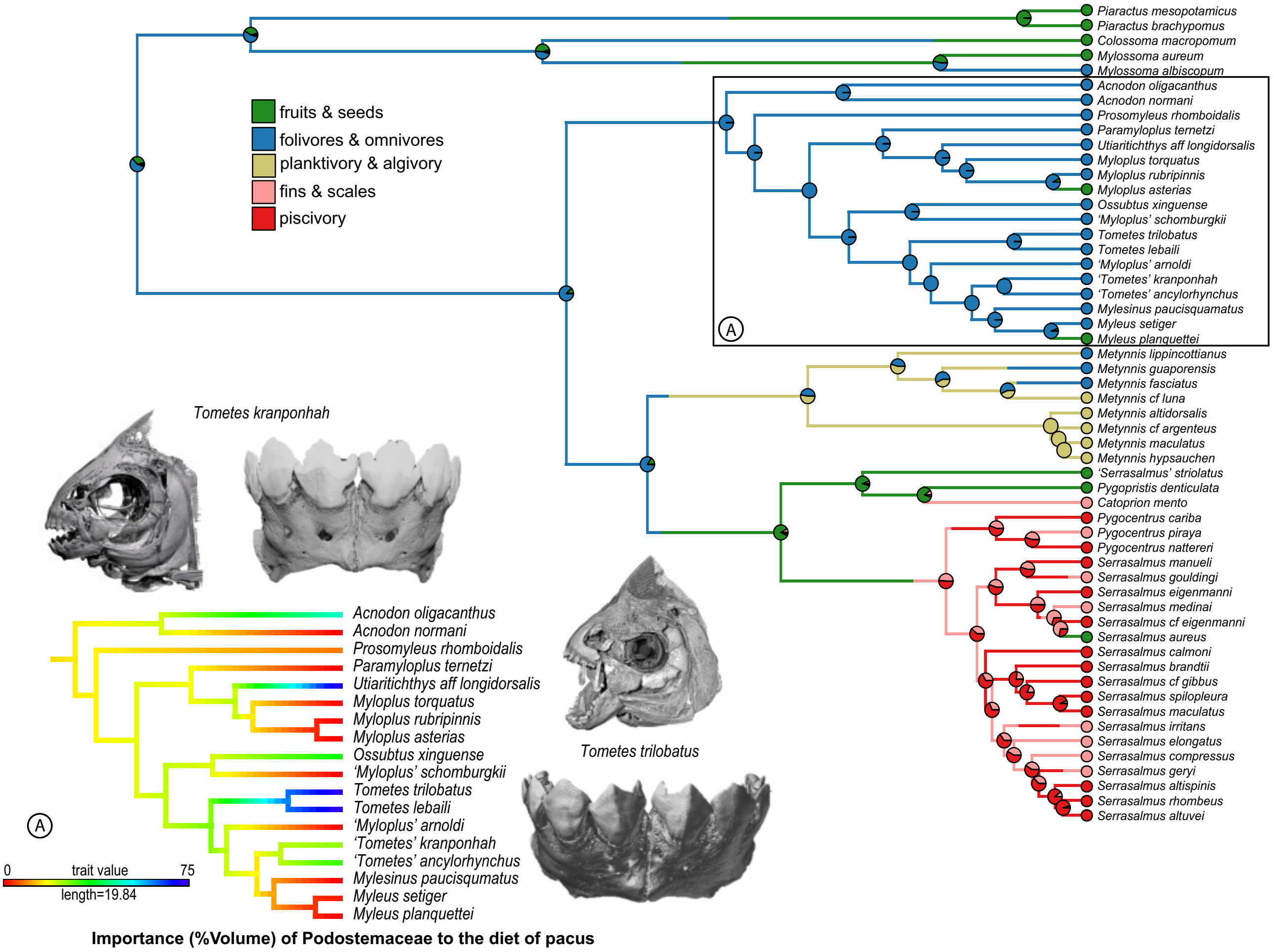

FIGURE 4. Diet state evolution across Serrasalmidae. The common ancestor of all serrasalmids was most likely herbivorous, as were the MRCAs of all three major serrasalmid clades. (1) *Fruits and seeds*: frugivorous and granivorous species, which feed on fruits, seeds, and incidental plant parts. (2) *Folivores and omnivores*: generalist herbivores and those species feeding to a minor extent on aquatic arthropods; (3) *planktivory and algivory*: fishes feeding on plankton, algae, and other plant parts; (1) *fins and scales*: scales, fins, found in diets; (2) *piscivory*: whole or partial fishes found in stomachs. Boxed clade labeled (A) is magnified in the bottom left and a continuous trait heatmap is shown, demonstrating the importance (% volumetric contribution) of riverweed (Podostemaceae) to the diet of different phytophagous pacus like *Tometes, Utiaritichthys*, and *Acnodon oligacanthus*. CT scans of *Tometes* skulls and lower jaws demonstrate similar tooth morphologies in paraphyletic taxa, "*Tometes*" *kranponhah* (upper left) and *Tometes trilobatus* (lower middle).

preanal serrae, a characteristic shared with *Serrasalmus* (Machado-Allison 2002). Our results also support the transfer of *P. aureus* and *P. careospinus* to *Serrasalmus* (and continued placement of *Serrasalmus eigenmanni* in *Serrasalmus*). *Pristobrycon striolatus* and the cryptic *P. scapularis* (see Andrade et al. 2019) are not closely related to *Serrasalmus* and warrant a new genus. Pending formal taxonomic description, we refer those species to "*Serrasalmus*" *striolatus* and "*Serrasalmus*" *scapularis* to highlight their eventual departure from this generic affiliation.

Within Myleinae, our analyses support a clade containing *Myloplus asterias* type species of *Myloplus* Gill 1896, as well as *M. rubripinnis* and *M. torquatus*. Other species traditionally assigned to *Myloplus* are herein transferred to other genus-level groups. Thus, we elevate the nominal subgenus *Prosomyleus* Géry 1972 to genus to contain the type species *Pr. rhomboidalis* and resurrect *Paramyloplus* Norman 1929 to contain *Pa. ternetzi* (type species) and *Pa. taphorni*. *Myloplus planquettei* is transferred to *Myleus* as it nests deeply within the clade containing the type species, *Myleus setiger*. All other species previously placed in *Myloplus* (e.g., *M. arnoldi M. schomburgkii M. lucienae*) are referred to "*Myloplus*" pending formal morphological reassessment of *Myloplus sensu stricto* and morphologically similar taxa not sampled in the current study.

The genus *Tometes* Valenciennes in Cuvier and Valenciennes 1850 is clearly paraphyletic (Fig. 2; Machado et al. 2018). We restrict *Tometes* to the Guiana Shield clade (Fig. 2) containing *T. trilobatus* (type species) and *T. lebaili*. Other species previously assigned to *Tometes* are distributed in rivers draining the Guiana Shield into the Orinoco and Negro (*T. makue*) and Amazon (*T. camunani*), and rivers draining the Brazilian Shield into the Amazon (*T. ancylorhynchus*, *T. kranponhah* and *T. siderocarajensis*) (Arcila et al. 2017). We provisionally refer those species to "*Tometes*".

Finally, our study included one of three valid species currently in *Utiaritichthys* Miranda Ribeiro 1937. Our analyses placed that species sister to *Myloplus sensu stricto*. The type species of *Myloplus*, *M. asterias*, shares an elongate bauplan with *Utiaritichthys*. We refrain from synonymizing *Utiaritichthys* with *Mylopus* until more representatives of the former taxon are analyzed.

The aforementioned taxonomic changes are informed by robust phylogenetic analyses and may guide future reappraisals of the family, particularly at the genus-level. As our study shows, the morphological diagnosis of clades is broadly plagued by homoplasy when evaluated against an independently derived phylogenetic framework. Clearly, Serrasalmidae still requires significant morphological work.

An inconvenient Tooth: Homoplasy, Uncertainty, and Taxonomic Characters

From taxonomic identification to fossil calibrations, our understanding of serrasalmid evolution is substantially influenced by the selective regimes acting on tooth form and function. Authors like Géry (1977) used tooth morphotypes to distinguish between serrasalmid subfamilies and more recent studies have used these same tooth morphologies to make assumptions about diet (Huby et al. 2019), the latter classifying species as either herbivorous or carnivorous depending entirely on whether that taxon has molariform or triangular teeth (respectively). Our findings reinforce the notion that serrasalmid diets are considerably more diverse than commonly believed, although these trends had been well-documented (Goulding 1980; Correa et al. 2007). Similarly, "herbivory" and "carnivory" are not ecological monoliths. For example, silver dollars or pacucitos (*Metynnis*) feed on plankton and algae, whereas other medium-sized pacus consume some insects (e.g., *Mylesinus*; Santos et al. 1997), or even scales (*Acnodon normani*; Leite and Jégu 1990). Similarly, piranhas are not all piscivorous—many have reverted to more herbivorous feeding modes and some lineages (e.g., *Pygopristis*, "*Serrasalmus*" *striolatus*; Nico and Taphorn 1988) are not ancestrally piscivorous. Instead, the evolution of serrasalmid feeding structures and diets is far more diverse and complex than widely appreciated.

Tooth shape is not predictive of diet among most herbivorous and carnivorous piranhas. The specialized scale-feeding wimple piranha, *Catoprion mento*, has teeth unlike any of its relatives (Kolmann et al. 2018, 2019), while its sister taxon *Pygopristis*, the only serrasalmid with pentacuspid teeth, feeds on plants and insects as an adult (Fink 1989; Nico 1991). There appear to be notable patterns of ecomorphological convergence or parallelism, as defined by Stayton 2015, page 2: "*convergence refers to situations where two or more lineages with* ***different*** *initial character states transition to the same character state. In this context, convergence is distinguished from parallelism, in which two or more lineages with* ***the same*** *initial character states both transition to a different shared character state*." In our data set, species assigned to the non-monophyletic genus *Tometes* share remarkably similar sharp, incisiform teeth, which relates to how these species feed on rheophilic river weed (Fig. 4 inset; Andrade et al. 2016; Huie et al. 2019; Kolmann et al. 2019). If phytophagy in upland rapids was present in the common ancestor of *Tometes* and "*Tometes*," and then lost in "*Myloplus*" *arnoldi Mylesinus* and *Myleus*, then similar dentitions in the paraphyletic genus *Tometes* stems from parallelism. Conversely, if phytophagy is novel for both *Tometes* and "*Tometes*," then convergent dentitions are suggested. In either case, morphological adaptations for similar herbivorous diets strongly suggests that dental diagnostic characters (although useful for identification of species in the field) are not suitable as synapomorphies, at least for *Tometes*.

Similarly, all species formerly assigned to *Pristobrycon* are noted for having short, deep skulls whereas most *Serrasalmus* have more elongate faces (Machado-Allison 1985). We suggest that most short-snouted *Serrasalmus* (like *S. manueli* or *S. gouldingi*) and short-snouted *Serrasalmus* (ex. *Pristobrycon*) in the "*aureus*" clade are omnivorous (Supplementary Fig. 5b available on Dryad) and often consume plant material. Shorter jaws are more effective at transferring muscle forces to hard prey like seeds (Goulding 1980; Prudente et al. 2016), so dietary convergence among brachycephalic piranhas may be misleading for morphological taxonomy. These examples highlight the difficulty in translating diagnostic characters useful for identifying species in the field to phylogenetically informative characters that reflect shared evolutionary history. They also highlight how incorporating natural history observations (e.g., diet) and functional considerations can help taxonomists steer clear of homoplasy.

Disagreement Over the Origin (Time) of Serrasalmids, but not their Diversification Timeline

We propose that a dating scheme which casts doubt on the taxonomic affinity of putative pacu teeth from Cretaceous deposits in Bolivia, is the more conservative approach to estimating the origin of serrasalmids. Previous studies using these Bolivian fossils date the origin of Serrasalmidae as either Paleocene (~60 Ma; Burns and Sidlauskas 2019) or late Cretaceous (65+ Ma; Thompson et al. 2014), in contrast to a younger age estimated in this study (38–42.9 Ma) by disregarding such fossils as serrasalmids. However, isolated fossil material, particularly disarticulated teeth, are difficult to assign to specific taxonomic groups. Paleontologists studying elasmobranchs with heterodont dentitions (jaws with differently shaped teeth) have a long-established history of skepticism when designating extinct species or evaluating taxonomic affinity based solely on isolated teeth (Shimada 2005; Whitenack and Gottfried 2010; Marrama and Kriwet 2017). Similarly, convergent and heterodont tooth morphologies are quite prevalent among serrasalmids, as well as many characiforms in general (Murray et al. 2004a; Kolmann et al. 2019), which may lead taxonomic classification of fossils astray.

Given the remarkable teeth of serrasalmids, it is curious that their likely sister taxon, the hemiodontids (Betancur-R et al. 2019; Burns and Sidlauskas 2019; Faircloth et al. 2020; this study), have miniscule teeth or are entirely edentulous (Roberts 1974, 1975). At first glance, serrasalmids would appear to have more in common with their toothier distant relatives, the payaras (Cynodontidae) than hemiodontids (halftooths); however, we note that our estimates for the divergence among these three clades is Paleocene-Eocene (55–81 Ma), enough time for edentulism and near-edentulism to have evolved independently among mammals, in both aardvarks (Afrotheria) and anteaters (Xenartha) (Upham et al. 2019). Burns and Sidlauskas (2019) suggested a late Cretaceous split between halftooths and serrasalmids, with the separation between these sister taxa and the "anostomoid" clade (Anostomidae, Curimatidae, etc.) happening not much earlier. Our estimates place the most recent common ancestor of all these groups within a similar range of ages, as Upper Cretaceous from 61 to 80 Ma (Thompson et al. 2014; Fig. 3). Our estimates for the origin of modern hemiodontid genera are younger than other hypotheses (Burns and Sidlauskas 2019) by some 6–20 my; however, scarce records of halftooths in the fossil record (perhaps because of their edentulism or near-edentulism) make this a difficult clade to age with confidence. We also note that for the first time using molecular data, our data support a sister relationship of *Micromischodus* to *Hemiodus* as suggested previously (Roberts 1971, 1974). Finally, our estimate for the divergence among modern cynodontid genera is 12–27 Ma (Fig. 3), which to our knowledge is the first time-calibrated molecular hypothesis for their origin.

Although pacus have a well-documented fossil record (Lundberg et al. 1998, 2009; Gayet et al. 2001; Dahdul 2007), the assumption that isolated teeth are the product of evolutionary stasis (Lundberg et al. 1986) may be premature without (1) broader consideration of outgroups, (2) evaluations of convergence across the phylogeny, or (3) discovery of more articulated skeletons. Nevertheless, our timeline for serrasalmid diversification is congruent with earlier studies, even studies which used molecular clock estimates based on mutation rates rather than fossil calibrations (Hubert et al. 2007a,b). These studies and ours suggest that the most diverse and iconic piranha genera, *Serrasalmus* and *Pygocentrus*, arose recently with an MRCA arising around 8–10 Ma, although this split was dated slightly older by Thompson and colleagues (2014) (Fig. 3). Most of the extant diversity within *Serrasalmus* is consistently resolved to be around 5–6 million years old (Hubert et al. 2007a, 2007b). Even though myleines and serrasalmines are the same age, these medium-sized pacus (and *Metynnis*) have not seen the same sort of recent radiation as piranhas which appear to have diversified quite rapidly and recently across South American lowland basins (Hubert et al. 2007b).

Observations on the Co-Evolutionary Timelines of Fruiting Plants and Fruit-Eating Pacus

The diversification of serrasalmids, particularly large-bodied, fruit-eating pacus, has been associated in the literature with the coincident diversification of fruiting plants (Correa et al. 2015). This shared co-evolutionary timeline between frugivorous fishes and their prey plants has relied primarily on stem ages of the asterids, rosids, and other plant groups like the spurges (Euphorbiaceae) (Horn et al. 2011; Correa et al. 2015). However, modern Amazonian plant communities are thought to have established themselves roughly ~40–50 Ma during the Eocene, and particularly those plants relying on ichthyochory (fish-based seed dispersal; Jaramillo et al. 2010). For example, the Eocene marks a period of dispersal/cladogenesis within Podostomaceae; although, the myleines did not arise to capitalize on this resource until the late Oligocene/early Miocene (Ruhfel et al. 2016). Our proposed timeline for serrasalmid diversification (Fig. 3) is also Eocene (56–23 Ma) in age, rather than Paleocene (66–56 Ma), and corresponds better with the crown ages of the most recent common ancestors of plant genera consumed by pacus today. *Colossoma macropomum* consumes fruits and seeds from rubber trees (*Hevea spruceana*), tucumã or jauari palms (*Astrocaryum* sp.), pouteria trees, and even the hallucinogenic iporuru plant (*Alchornea* sp.) (Goulding 1980). Similarly, pirapitinga (*Piaractus*) also consume tucumã palm fruit, as well as fava (*Vicia faba*), and even luffa (Cucurbitaceae) (Goulding 1980). The ages of these plant genera are all late Eocene to Oligocene in age (~41–22 Ma; Wojciechowski 2003; Schaefer et al. 2009; Bartish et al. 2011; Roncal et al. 2012), and correspond intriguingly with the diversification timeline of frugivorous, large-bodied pacus (*Mylossoma Colossoma* and *Piaractus*) and most other serrasalmid genera. These estimates are also in line with the Eocene timeline for fruit-eating vertebrates like birds and mammals (Fleming and Kress 2011).

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: **New Subfamily of Serrasalmidae Bleeker 1859:** Colossomatinae Kolmann, Hughes, Hernandez, Arcila, Betancur-R, Sabaj, López-

Fernández & Ortí 2020 **urn:lsid:zoobank.org:act:** 5CFA1B72-E6A4-426F-84C1-0596A7AAA52D **Type Genus:** Colossoma Eigenmann & Kennedy 1903 http://dx.doi.org/10.5061/dryad.6t1g1jww0.

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