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Divergence times of the *Rhoadsia* clade (Characiformes: Characidae)

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The family Characidae is the most diverse group of fishes in the Neotropics with challenging systematics. The three genera *Carlana*, *Parastremma*, and *Rhoadsia*, formerly considered the subfamily Rhoadsiinae, are now included in the subfamily Stethaprioninae. Previous phylogenetic analyses did not include all genera of Rhoadsiinae, specifically *Parastremma*. Here, we estimated the phylogenetic relationships and divergence times of the genera of Rhoadsiinae (the *Rhoadsia* clade) relative to the most representative genera of the Characidae. We used six molecular markers from the mitochondrial and nuclear genome to estimate the phylogeny and divergence times. We confirmed the monophyly of the *Rhoadsia* clade. Furthermore, we estimated that the Central American genus *Carlana* and the western Colombian genus *Parastremma* diverged approximately 13 Mya (95% HPD 8.36–18.11), consistent with the early-closure estimates of the Isthmus of Panama (~15 Mya). The genus *Rhoadsia*, endemic to Western Ecuador and Northern Peru, was estimated to originate at around 20 Mya (95% HPD 14.35–25.43), consistent with the Andean uplift (~20 Mya).

Keywords: Biogeography, Freshwater fishes, Phylogeny, Stethaprioninae, Systematics.

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La familia Characidae es el grupo más diverso de peces en el Neotrópico con una sistemática compleja. Los tres géneros *Carlana*, *Parastremma* y *Rhoadsia*, antes considerados en la subfamilia Rhoadsiinae, ahora se consideran dentro de la subfamilia Stethaprioninae. Los análisis filogenéticos publicados no incluyen todos los géneros de Rhoadsiinae, específicamente *Parastremma*. Aquí, estimamos las relaciones filogenéticas y los tiempos de divergencia de los géneros de Rhoadsiinae (el clado *Rhoadsia*) en relación con los géneros más representativos de Characidae. Utilizamos seis marcadores moleculares del genoma mitocondrial y nuclear para estimar la filogenia y el tiempo de divergencia. Confirmamos la monofilia del clado *Rhoadsia*. Además, estimamos que el género centroamericano *Carlana* y el género colombiano occidental *Parastremma* divergieron aproximadamente hace 13 millones de años (95% HPD 8.36–18.11), lo que es consistente con recientes estimaciones del cierre del Istmo de Panamá (~15 millones de años). Se estimó que el género *Rhoadsia*, endémico del oeste de Ecuador y el norte de Perú, se originó hace alrededor de 20 millones de años (95% HPD 14.35–25.43), consistente con el levantamiento de los Andes (~20 millones de años).

Palabras clave: Biogeografía, Filogenia, Peces de agua dulce, Sistemática, Stethaprioninae.

INTRODUCTION

The Family Characidae is the most diverse family of fishes in the Neotropics (Albert, Reis, 2011; Fricke *et al*., 2022). Due to its great diversity, species of this large group are classified into various subfamilies. However, classifying the species into subfamilies is still challenging and constantly changing as new information becomes available. The use of molecular markers in combination with morphology has helped clarify a lot of the uncertainty within Characidae. That is the case of the former subfamily Rhoadsiinae with the three genera *Rhoadsia* Fowler, 1911, *Parastremma* Eigenmann, 1912, and *Carlana* Strand, 1928 (Cardoso, 2003) (here also referred as the *Rhoadsia* clade). However, more recent total-evidence phylogenetic analysis prompted the reclassification of this group into a larger subfamily Stethaprioninae (Mirande, 2019), which is consistent with phylogenomic evidence (Betancur-R. *et al*., 2019).

The subfamilial recognition and membership of the *Rhoadsia* clade have shifted over time. For example, the *Rhoadsia* clade includes the genus *Rhoadsia* with two species recognized, *R. minor* Eigenmann & Henn, 1914 and *R. altipinna* Fowler, 1911, the genus *Carlana* with its only species *C. eigenmanni* (Meek, 1912), and the genus *Parastremma* with three species, *P. album* Dahl, 1960, *P. pulchrum* Dahl, 1960, and *P. sadina* Eigenmann, 1912. Cardoso (2003) defined the group as having a single series of teeth in the premaxilla when young and a two series when reaching adulthood (except for *Carlana* which does not develop an outer series). Adult specimens have two conical teeth in their outer series and five multicuspid teeth in their inner series. Mirande (2009, 2010) proposed the inclusion of *Nematocharax* in the subfamily Rhoadsiinae *sensu* Cardoso (2003), based on morphological phylogenetic analyses. The characters that unified the four genera were the form of teeth of inner premaxillary row with cusps aligned in straight series, without anterior concavity and five or more cusps of anterior maxillary teeth (Mirande, 2010). By contrast, a more recent phylogeny based on combined morphological and molecular data showed that these four genera are not monophyletic. That hypothesis recovered *Nematocharax* Weitzman, Menezes & Britski, 1986 closer to the polyphyletic genus *Moenkhausia* Eigenmann, 1903 (Mariguela *et al*., 2013; Mirande, 2019), a group of medium size fish (~12 cm) widely distributed in the Amazon basin and adjacent drainages, which were classified as part of a tribe Stethaprionini. On the other hand, the genera *Rhoadsia* and *Carlana* were closer to species like *Pseudochalceus kyburzi* Schultz, 1966 and *Nematobrycon palmeri* Eigenmann, 1911 (Mirande, 2019) mainly found in the northwestern South America; together, these were classified as members of a tribe Rhoadsiini. Other members of this Rhoadsiini tribe can also be found in the Amazon basin and Southeast Brazil. Consequently, these genera are now within the much larger subfamily Stethaprioninae (Mirande, 2019). Although *Parastremma* was generally assumed to be within the *Rhoadsia* clade with *Rhoadsia* and *Carlana*, *Parastremma* has not been formally included in a phylogeny until recently, where it was used as an outgroup of populations of *Rhoadsia* sp. along with *Carlana* (Cucalón *et al*., 2022). However, Cucalón *et al.* (2022) did not sample other characid taxa to test the monophyly of the *Rhoadsia* clade, nor did they perform a fossil-calibrated divergence time analysis to estimate when members of the group diverged from each other.

In this study, we investigated the phylogenetic relationships and divergence time of the *Rhoadsia* clade (Rhoadsiinae *sensu* Cardoso, 2003) relative to other members of the family Characidae, with the intension to provide a better understanding of the evolutionary history of the *Rhoadsia* clade within the Characidae.

MATERIAL AND METHODS

Data collection. Sequences for representative taxa within families of Characoidei (*sans* Crenuchoidea) were retrieved from GenBank using phylogenies estimated in Mirande (2019) and Terán *et al*. (2020) as guides to maximize phylogenetic diversity. Most extensive sampling was done within the family Characidae to achieve representative sampling of most clades within the subfamilies. In addition, clades outside of Characidae were represented with up to 6 species per family. We retrieved sequences representing three mitochondrial markers, 16S Ribosomal RNA (*16S*) (~600 bp), Cytochrome Oxidase I (*COI*) (~600 bp), and Cytochrome b (*Cytb*) (~600 bp), and up to three nuclear markers, Myosin Heavy Chain 6 (*Myh6)* (~1000 bp), Recombination Activating 1 (*RAG1*) (~1200 bp), and Recombination Activating 2 (*RAG2*) (~1200 bp), when available from GenBank. These markers were chosen since they are commonly used for phylogenetic reconstructions and were the most frequently available across taxa. Genes available from different individuals were chosen arbitrarily for each species, however whenever possible genes coming from the same individual were selected to reduce the likelihood of contamination (see Tab. **S1**). Mitochondrial genes were obtained from complete mitogenomes when available using a custom script (available from: https://doi.org/10.6084/m9.figshare.21367089.v1). The mitochondrial genes *Cytb* and *COI* of species of Rhoadsiinae *sensu* Cardoso (2003) were retrieved from Cucalón *et al.* (2022), including *Rhoadsia altipinna*, *R. minor*, *Parastremma sadina*, and *Carlana eigenmanni*. Other genes like *16S*, *RAG1* and *RAG2* were amplified in the laboratory. We used primers reported from others studies for *16S* (Palumbi, 1996), *RAG1* (López *et al*., 2004; Li, Ortí, 2007; Oliveira *et al*., 2011), and *RAG2* (Oliveira *et al*., 2011; this study) (see Tab. **S2**). The polymerase chain reaction (PCR) was carried out using the following volumes: For a 15 µl reaction we used 7.5 µl of GoTaq® master mix (www.promega.com), 0.3 µl of each primer at 10 µM (Tab. **S2**), 2 µl of DNA template, and complemented the reaction with molecular grade water. We performed a nested PCR for *RAG1*, and *RAG2*, and a regular PCR for the *16S*, with the following thermocycler protocol. For the first PCR nested and the regular PCR, one cycle of denaturation for 1 min at 95°C, 30 cycles of denaturation at 95°C for 1 min, annealing (50–52°C) (Tab. **S2**), extension at 72°C for 2 min, and one cycle of final extension at 72°C for 10 min. The second PCR nested was similar as the first PCR except it ran for 35 cycles.

Alignment. Each gene was aligned independently using MAFFT version 7.453 (Katoh, Standley, 2013) using the option –auto recommended when unsure which alignment strategy to use based on data size. Then, the aligned genes were concatenated and converted to NEXUS format for further analyses using the tool AMAS (Alignment Manipulation And Summary) (Borowiec, 2016).

Phylogenetic analysis. We used Maximum Likelihood (ML) to reconstruct the phylogeny of the *Rhoadsia* clade relative to the family Characidae. Species from the other families within Characoidei (*sans* Crenuchoidea) were used as outgroup taxa. The ML analysis was carried out using IQ-TREE2 v. 2.0.6 (Minh *et al*., 2020), using a partitioned model (Chernomor *et al*., 2016), with 1000 iterations of ultrafast bootstrap (Hoang *et al*., 2017). To determine the best partitioned substitution model for phylogenetic analysis, we implemented ModelFinder (Kalyaanamoorthy *et al*., 2017) to simultaneously estimate each gene's best-fit substitution model and best-fit alignment partitioning model scheme (option --merge). ModelFinder selects the best-fit model that minimizes the Bayesian Information Criterion (BIC) score. We enforced the relationship of some of the deep nodes based on phylogenomic results from Betancur-R *et al.* (2019) as followed: Chalceidae + Characidae, sister to Alestoidea and Erythrinoidea + Curimatoidea. The ML tree was edited for visualization using the R packages phytools v1.0-1 (Revell, 2012) and ggtree v3.2.1 (Yu *et al*., 2017).

Divergence time estimation. Divergence times among clades were estimated using BEAST version 2.6.4 (Bouckaert *et al*., 2019). We constrained the analysis using a starting tree with estimated divergence time based on the penalized likelihood (PL) method (Cole *et al*., 2014) implemented in the software treePL (Smith, O'meara, 2012) following Maurin (2020). We used the rooted ML tree generated from IQ-TREE as the input tree for treePL and calibrated the nodes using 13 calibration nodes used in Kolmann *et al.* (2021). See Tab. **S3** for detailed information about the node calibration including, taxa calibrated, mean age, minimum and maximum age, fossil species name, and tips (*i.e*., species in the tree) used to inform treePL which node to calibrate. Most parameters were set through the program BEAUti version 2.6.4 (Bouckaert *et al*., 2019) included in the BEAST2 package. The parameters were as followed: We assumed that all genes had the same molecular clock and tree topology by linking the "Clock" and "Tree" model for all partitions. The "Site model" was left unliked across partitions to use the best-fit substitution model. The substitution model was estimated using ModelFinder (Kalyaanamoorthy *et al*., 2017) from IQTREE2 v. 2.0.6 (Minh *et al*., 2020) using the option "TESTONLY" to exclude testing of the free rate model (assumed by default), since this is not currently implemented in BEAST2. The option "--merge" was also set to estimate the best substitution model scheme to optimize the number of parameters used during the analysis. The molecular clock model was set as an uncorrelated relaxed clock log-normal to allow for rate heterogeneity across branches. We used the birth-death (Gernhard, 2008) model tree prior and calibrated the nodes of the phylogeny using prior settings following the 13 calibration fossils used in Kolmann *et al*. (2021) (Tab. **S3**). For details on fossil calibration and rationale see materials and methods described in Kolmann *et al*. (2021). The prior distribution for each calibration was set as exponential to account for increasing uncertainty at further points in the past, except for the root node that was uniform distribution, following Kolmann *et al*. (2021). To fix the tree topology during the Markov Chain Monte Carlo (MCMC) chain, we modified the XML file by removing the lines that contained the operators "wide-exchange," "narrow-exchange," "subtree-slide," and "Wilson-balding" following the instruction from the BEAST2 website (www.beast2.org). We ran the analysis with 100 million MCMC iterations, sampling every 5000 generations. The log file was inspected in Tracer (Rambaut *et al*., 2014) for convergence of the MCMC. We summarized the maximum clade credibility (MCC) tree discarding 10% burn-in in TreeAnnotator from the BEAST package version 2.6.3 (Bouckaert *et al*., 2019). The time calibrated (*i.e.*, MCC tree) and ML trees were visualized in FigTree (Rambaut, 2016). We used the R package MCMCtreeR (Puttick, 2019) to visualize the posterior ages distribution of the MCC tree.

RESULTS

Sampling and phylogenetic analysis. Sequences for a total of 211 species of the Characoidei group were obtained for the phylogenetic reconstruction. The total length of the concatenated sequence alignment after trimming was 8276 bp. Accession number of the gene sequences used including the ones obtained from this study for the *Rhoadsia* clade can be found in Tab. **S1**. The best scheme substitution models selected by ModelFinder for the ML method allowing the incorporation of the free rate model were GTR+F+R5 (*16S*), GTR+F+I+G4 (*COI*), GTR+F+I+G4 (*Cytb*), and TIM2e+R5 (*Myh6*, *RAG1*, *RAG2*). For the Bayesian divergence time analysis (without allowing for free rate model), the best substitution model scheme was GTR+F+I+G4 (*16S*), GTR+F+I+G4 (*COI*), GTR+F+I+G4 (*Cytb*), and TIM2e+I+G4 (*Myh6*, *RAG1*, *RAG2*). The MCMC run reached stationarity with ESS greater than 200.

Phylogenetic relationships of the *Rhoadsia* clade. The ML tree placed the *Rhoadsia* clade (ultrafast bootstrap [BS]: 100) within the subfamily Stethaprioninae (Fig. 1). *Rhoadsia* was reconstructed as monophyletic (BS: 100), and this clade was sister to a highly supported clade formed by *Carlana eigenmanni* and *Parastremma sadina* (BS: 100).

The closest relative to the *Rhoadsia* clade was the species *Pseudochalceus kyburzi* (BS: 100). That clade was sister of *Nematobrycon palmeri* (BS: 99) and followed by *Inpaichthys kerri* Géry & Junk, 1977 (BS: 83). Refer to Fig. **S4** for full ML tree and BS support values for all nodes.

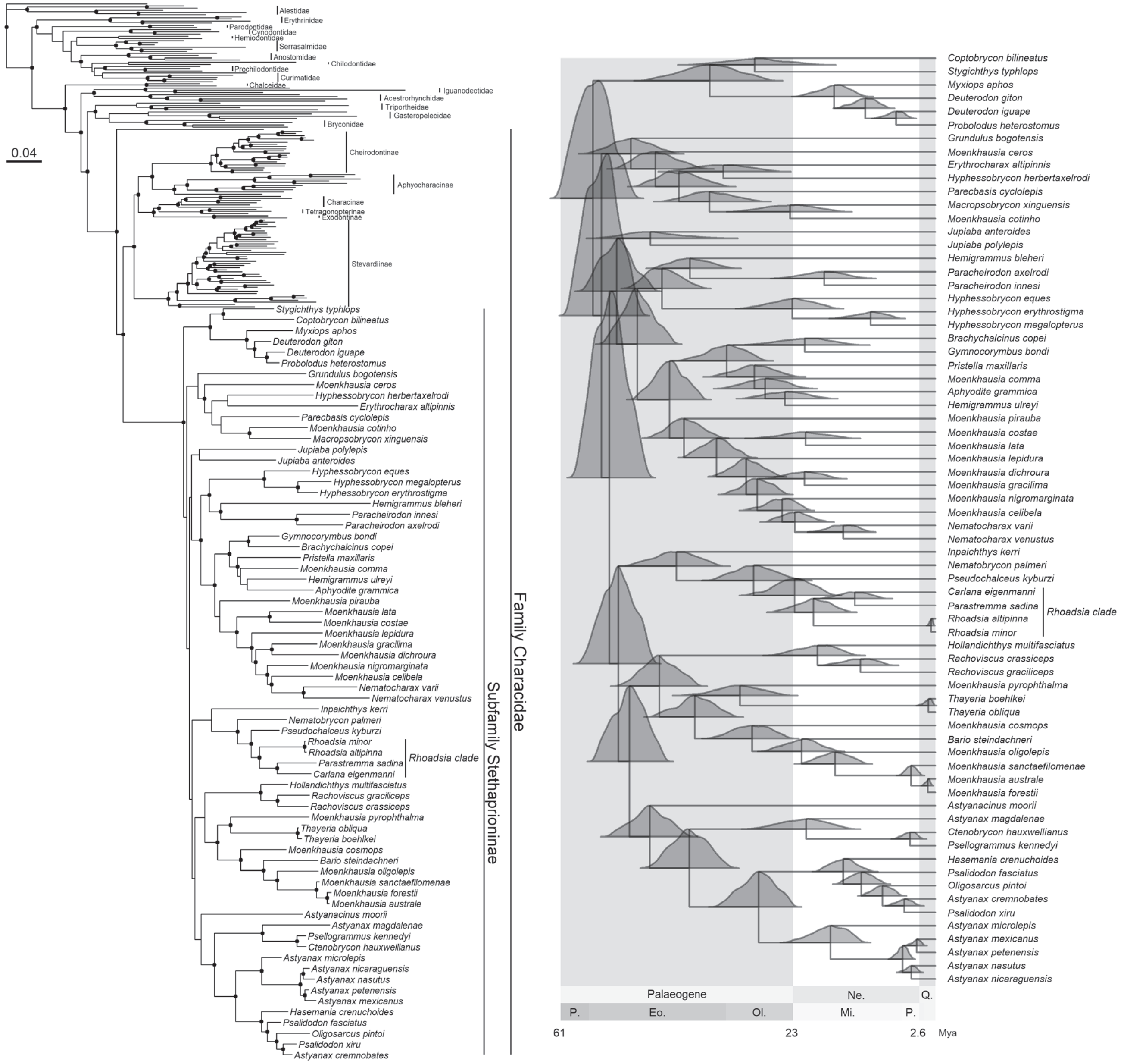

FIGURE 1 | Maximum likelihood phylogeny (left) of representative members of family Characidae and Bayesian chronogram (right) of the subfamily Stethaprioninae. Only species names of the subfamily Stethaprioninae are displayed in both trees (full trees in Figs. **S4** and **S5**). Nodes with ultrafast bootstrap support ≥95 are shown with a black dot (left). The nodes on the time tree depict the posterior distribution of the age estimates (right). Abbreviations of geological ages left to right, top to bottom: Ne = Neogene, Q = Quaternary, P = Paleocene, Eo = Eocene, Ol = Oligocene, Mi = Miocene, P = Pliocene.

The divergence time of members of the *Rhoadsia* clade. Based on the Bayesian estimation, the genus *Rhoadsia* diverged from its sister clade, including the Central American *Carlana* and Colombian *Parastremma*, about 19.72 Mya (95% HPD 14.35–25.43). The genera *Carlana* and *Parastremma* diverged about 13.02 Mya (95% HPD 8.36–18.11). Refer to Fig. **S5** for full maximum clade credibility tree with 95% HPD for all nodes.

DISCUSSION

We herein inferred the phylogenetic relationships and divergence times of the *Rhoadsia* clade (Rhoadsiinae *sensu* Cardoso, 2003), based on six molecular markers from the mitochondrial and nuclear genome. The phylogenetic relationships of *Rhoadsia* and *Carlana* to other characids were consistent with previous reports (Mirande, 2019; Terán *et al*., 2020). The three genera of the *Rhoadsia* clade — *Rhoadsia*, *Carlana*, and *Parastremma* — showed a monophyletic relationship (Fig. 1) within the subfamily Stethaprioninae. Of this group, the divergence time of the genus *Rhoadsia* was estimated to be 19.72 Mya (95% HPD 14.35–25.43), while *Carlana* and *Parastremma* were estimated at about 13.02 Mya (95% HPD 8.36–18.11).

Relationships of the *Rhoadsia* clade and closest relative within the subfamily Stethaprioninae. The phylogenetic relationships of the *Rhoadsia* clade were consistent with the most recent phylogeny of the group based on morphological and molecular data (Mirande, 2019; Terán *et al*., 2020). In this study, we added *Parastremma sadina* to the analysis. The genus *Parastremma* with three valid species, *P. album*, *P. pulchrum*, and *P. sadina* (the latter analyzed here) has been previously designated within Rhoadsiinae *sensu* Cardoso (2003) based on morphology but without formal phylogenetic analysis (Cardoso, 2003; Mirande, 2010; Jimenez-Prado *et al*., 2015). The genus *Parastremma* is endemic to the Chocó-Darien ecoregion in Colombia (DoNascimiento *et al*., 2017). The phylogeny presented here shows *Parastremma sadina* sister to the monotypic *Carlana* from Central America, corroborating its place within the *Rhoadsia* clade (Fig. 1). The genus *Carlana* with its only species *Carlana eigenmanni* has been regarded previously as a junior synonym of *Rhoadsia* by some authors (Eigenmann, Myers, 1921; Géry, 1977) while other authors associated *Carlana* with the subfamily Cheirodontinae after observing that *Carlana* was the only member of the *Rhoadsia* clade keeping a single tooth series in the premaxilla in adult fish as opposed to a double series (Fink, Weitzman, 1974). However, this trait appears to be a homoplasy.

The genus *Rhoadsia* contains two recognized species, *R. minor*, and *R. altipinna.* This genus is endemic to drainages from the Pacific slope of the Andean mountains from northern Ecuador to northern Peru, an area known for being highly threatened (Aguirre *et al*., 2021). The most distinctive characteristic of *Rhoadsia* is a dark spot located on the side of the body below the insertion of the dorsal fin (Jimenez-Prado *et al*., 2015). Although their taxonomic status as two species have being questioned by some authors (Géry, 1977), recent studies based on molecular markers showed the two species appear to differ genetically and are allopatrically distributed (Aguirre *et al*., 2016; Cucalón *et al*., 2022), although their body form varies similarly along the altitudinal gradient (Malato *et* *al*., 2017; Aguirre *et al*., 2019). Nevertheless, the taxonomic distinction within the genus *Rhoadsia* might still require further study using a more integrative approach.

The genera *Rhoadsia*, *Carlana*, and *Parastremma* analyzed here formed a monophyletic *Rhoadsia* clade nested within a broader clade including *Pseudochalceus kyburzi*, *Nematobrycon palmeri*, and *Inpaichthys kerri* with high support (BS: 83) (Fig. 1). This clade is also recovered in Mirande (2019) as one of the subclades within the tribe Rhoadsiini *sensu* Mirande (2019). A similar clade is observed in Terán *et al*. (2020), but including *Grundulus bogotensis* (Humboldt, 1821). However, the relationships of the *Rhoadsia* clade to other genera, such as *Pseudochalceus* Kner, 1863, *Nematobrycon* Eigenmann, 1911, and *Inpaichthys* Géry & Junk, 1977, are incongruent and weakly supported in our study (Fig. 1), Mirande (2019), and Terán *et al.* (2020). Both Mirande (2019) and Terán *et al.* (2020) recover the aforementioned clade sister to a clade including *Hollandichthys* Eigenmann, 1909, *Rachoviscus* Myers, 1926, *Thayeria* Eigenmann, 1908, and *Bario* Myers, 1940 (among other species). We found the latter genus sister to a clade including mostly *Astyanax* Baird & Girard, 1854 (BS: 48), and this sister to the clade formed by the *Rhoadsia* clade, *Pseudochalceus*, *Nematobrycon*, and *Inpaichthys* (BS: 57). Also, Betancur-R *et al.* (2019) inferred a phylogeny for Characoidei based on genomic data, where *Rhoadsia* cf. *altipinna* and *Inpaichthys kerri* (the only members of this clade they included) did not form a clade; rather they recovered *Rhoadsia* cf. *altipinna* sister to *Grundulus bogotensis* while *Inpaichthys kerri* in a clade closer to *Hollandichthys multifasciatus* (Eigenmann & Norris, 1900), *Rachoviscus crassiceps* Myers, 1926, *Hemigrammus ocellifer* (Steindachner, 1882), *Bario steindachn eri* (Eigenmann, 1893), and *Thayeria obliqua* Eigenmann, 1908. Hence, the relative placements of *Grundulus bogotensis* and *Inpaichthys kerri* were inconsistent with Mirande (2019), Terán *et al*. (2020), and our phylogeny.

It is worth noting that *Grundulus bogotensis*, which appears sister to *Inpaichthys kerri* in Terán *et al*. (2020) and sister to *Rhoadsia* cf. *altipinna* in Betancur-R *et al.* (2019), in this study, is placed in a different clade in congruence with Mirande (2019), sister to other members of the tribe Grundulini *sensu* Mirande (2019), although with relatively low ultrafast bootstrap support (BS = 87) (Fig. 1; Fig. **S4**). Other studies based on morphology have placed *Grundulus* Valenciennes, 1846 closest to *Spintherobolus* (Román-Valencia *et al*., 2010), although this hypothesis seems less likely. Recent studies, including molecular data, place *Spintherobolus* Eigenmann, 1911 close to the base of Characidae in a separate subfamily Spintherobolinae (Oliveira *et al*., 2011; Betancur-R *et al*., 2019; Mirande, 2019; Terán *et al*., 2020; this study - Fig. **S4**) and hardly ever sister to *Grundulus*. The subfamilial placement of some clades of the Characidae still seems to represent a challenge based on the discrepancies observed across studies which are often accompanied with low support.

The genus *Nematocharax*, a Brazilian freshwater fish that was previously thought to be closely related to the *Rhoadsia* clade (Weitzman *et al*., 1986; Mirande, 2009, 2010), has shown to be problematic in regards to its systematics due to morphological similarities with various characid genera (Weitzman *et al*., 1986). Weitzman *et al.* (1986) suggested the potential relationship of *Nematocharax* with the *Rhoadsia* clade due to the compressed, relatively deep body, long dorsal fin, and fully toothed maxilla, which are characteristics found in the *Rhoadsia* clade. The phylogenetic relationship was then supported in Mirande (2010), uniting *Nematocharax* with members of the *Rhoadsia* clade by the form of the teeth of the inner premaxillary tooth row with cusps aligned in straight series and without anterior concavity and five or more cusps in the anterior maxillary teeth. However, the designation of *Nematocharax* as a member of the *Rhoadsia* clade was challenged after the inclusion of the nuclear and mitochondrial markers into the phylogeny, placing *Nematocharax* sister to species of the polyphyletic genus *Moenkhausia* (Mariguela *et al*., 2013; Mirande, 2019; this study).

Major historical processes associated with the divergence of the *Rhoadsia* clade. The high levels of endemism found in Western Ecuador have been attributed to species isolation due to the uplift of the Andes estimated at 20 Mya (Schaefer, 2011). In addition, the effect of the uplift of the Andes has been investigated on the diversification of birds (Benham, Witt, 2016; Hazzi *et al*., 2018) and plants (Luebert, Weigend, 2014). More recently, it has been associated with the high diversity of the freshwater fish family Characidae in South America (Melo *et al*., 2022) and South American freshwater fishes in general (Cassemiro *et al*., 2021). Freshwater fishes are especially prone to diversify after such geological events due to their limitation to move outside their river systems. The subfamily Stethaprioninae has being previously estimated to have diversified close to 30 Mya (Melo *et al*., 2022). Other species-rich families like Trichomycteridae and Loricariidae (Siluriformes) are associated with major geological events in South America including multiple marine transgressions and regressions as well as mountain formations between 55–10 Mya (Cassemiro *et al*., 2021). The origin of the genus *Rhoadsia* seems consistent with these estimations, showing a time of divergence from its closest relative (14–25 Mya) (Fig. 1), within the range of the formation of the Andes (Schaefer, 2011).

The genera of the *Rhoadsia* clade each inhabit adjacent, non-overlapping regions (Western Ecuador, Colombia, and Central America). The genus *Carlana* is found only in Central America from Panama to Nicaragua (Cardoso, 2003). The genus *Parastremma* inhabits Colombia's freshwater rivers on both the Caribbean slope and Pacific slope, and is the closest geographically to *Rhoadsia* from Western Ecuador (Eigenmann, 1912; Cardoso, 2003; DoNascimiento *et al*., 2017). Both *Parastremma* and *Rhoadsia* are part of the Tumbes-Chocó-Magdalena ecoregion spanning from Southeastern Panama to Northwestern Peru, but their species ranges do not overlap. Fish faunas from Western Ecuador and Western Colombia are known to differ, potentially indicating independent evolutionary histories (Eigenmann, 1921; Aguirre *et al*., 2017). Eigenmann (1921) suggested that *Rhoadsia* and *Parastremma* independently dispersed from the east of the Andes, although other hypotheses appear equally likely (*i.e*., north-south or south-north origin). It is noteworthy that species like *Hoplias microlepis* (Günther, 1864), which inhabit Western Ecuador like *Rhoadsia*, are also found in the Chagres River, Panama, while absent in Colombia, presenting a disjoint distribution (Eigenmann, 1921; Mattox *et al*., 2014). This may indicate that the subdivision in fish composition observed between Western Ecuador, Western Colombia, and Central America does not seem universal for all fishes of this region. However, genetic analysis is still needed to determine the level of divergence between the two disjunct populations of *Hoplias* Gill, 1903.

The genus *Parastremma* and the Central American genus *Carlana* diverged ~13 Mya (95% HPD 8.36–18.11). This is consistent with recent estimates of an ancient closure of the Isthmus of Panama (~15 Mya) and migration patterns of other land and freshwater organisms into Central America (Hurt *et al*., 2009; Bacon *et al*., 2015a; Thacker, 2017), rather than the traditionally accepted closure at 3.5 Mya (reviewed in Coates and

Stallard, 2013). Furthermore, dispersal events prior to the full closure of the Isthmus of Panama appear to be commonly inferred across taxa (Bermingham, Martin, 1998; Thacker, 2017), and the divergence between *Carlana* and *Parastremma* seems to be more consistent with an early closure of the Isthmus as well (Coates, Stallard, 2013; Bacon *et al*., 2015a; Montes *et al*., 2015). We also observed a similar divergence time between the species *Astyanax microlepis* Eigenmann, 1913 found exclusively in Colombia and its sister clade that includes *A. mexicanus* (De Filippi, 1853), *A. petenensis* (Günther, 1864), *A. nasutus* Meek, 1907, and *A. nicaraguensis* Eigenmann & Ogle, 1907 from North and Central America (~20 Mya, 95% HPD 11.79–23.17) (Fig. 1). The timing of the closure of the Isthmus of Panama is still an ongoing debate (Bacon *et al*., 2015a,b; Hoorn, Flantua, 2015; Montes *et al*., 2015; O'Dea *et al*., 2016; Jaramillo *et al*., 2017; Molnar, 2017) and we cannot discard the possibility of the divergence between *Carlana* and *Parastremma* happening in South America first followed by the dispersal of an ancestral population of *Carlana* into Central America, followed by extinction. Given the Gondwanan origin of characiforms (Arroyave *et al*., 2013), it is safe to assume that the genus *Carlana* should have expanded up into Central America not earlier than 18 Mya at the beginning of the Miocene. It has been hypothesized that later geological events like the Pliocene high sea stand and the rise of the Central Cordillera might have contributed to the extirpation of some species in Central America, allowing others to occupy some new niches favoring allopatric speciation and the high endemism found in the lower Mesoamerican region (Smith, Bermingham, 2005).

In this study, we include a member of the genus *Parastremma* in a densely-sampled molecular phylogeny of the Characidae for the first time, supporting a relationship sister to *Carlana* and both closely related to *Rhoadsia* as a clade, supporting the monophyly of the *Rhoadsia* clade. We also estimated the divergence times of the group, with the Ecuadorian genus *Rhoadsia* diverging from its closest relative between 14–25 Mya, consistent with the uplift of the Andean Mountains. The Central American *Carlana* and Colombian *Parastremma* diverged between 8–18 Mya, consistent with the early closure hypothesis of the Isthmus of Panama. This study adds knowledge regarding the evolutionary history and biogeography of the *Rhoadsia* clade.

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AUTHORS’ CONTRIBUTION

Roberto V. Cucalón: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing-original draft, Writing-review and editing.

Milton Tan: Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing-review and editing.

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The authors declare no competing interests.

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