



INSTITUTO DE BIOCÊNCIAS
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**DIVERSIFICAÇÃO DOS CARACTERES SEXUAIS SECUNDÁRIOS E SUAS
IMPLICAÇÕES FILOGENÉTICAS EM APHYOCHARACINAE (CHARACIDAE:
CHARACIFORMES)**

PORTO ALEGRE

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Orientador: Prof. Dr. Luiz Roberto Malabarba

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SUMÁRIO

RESUMO	2
ABSTRACT	3
EVOLUTION OF SECONDARY SEXUAL CHARACTERS: DESCRIPTION OF SEXUALLY DIMORPHIC STRUCTURES AND PHYLOGENETIC IMPLICATIONS IN THE SUBFAMILY APHYOCHARACINAE (CHARACIFORMES: CHARACIDAE)	4
INTRODUCTION	4
MATERIAL AND METHODS	9
Ingroup and outgroup Sampling	9
Morphological data.....	10
Secondary sexual characters.....	11
Molecular data	12
Phylogenetic analysis	13
Molecular based data.....	13
Morphology based data	14
Ancestral character state reconstruction (ACSR).....	15
REFERENCES	16

LISTA DE TABELAS

Table 1 – Characidae’s diversity across lineages (Fricke et al., 2020)..... 7

Table 2 – Sequences of primers..... 13

RESUMO

Os peixes exibem amplos padrões de diversificação. Entre os Ostariophysi, uma das maiores radiações em vertebrados, Characiformes representa um de seus grupos mais diversos, onde as espécies de água doce estão amplamente distribuídas na região Neotropical. Várias formas de estruturas sexualmente dimórficas são conhecidas como caracteres sexuais secundários, que estão intimamente relacionadas aos ciclos reprodutivos dos peixes. Características sexualmente dimórficas foram relatadas em muitas espécies de Characidae e podem estar associadas a uma variedade de estruturas modificadas de machos adultos e, às vezes, fêmeas. Os atributos do sistema sexual e as variações morfológicas desses caracteres foram aqui implementados como fontes de novos dados. Modificações do sistema sexual secundário foram o foco deste estudo, que correlacionou a evolução dos caracteres sexuais secundários à diversificação filogenética na subfamília Aphyocharacinae. Através da análise da reconstrução de estados de caráter ancestral com base em uma hipótese filogenética das relações evolutivas dos afiocaracíneos, propusemos novos conjuntos de caracteres envolvendo: a modificação de estruturas corporais em espécimes adultos, e a presença/ausência na ocorrência de ganchos ósseos nos raios das nadadeiras pélvicas e anal em machos sexualmente dimórficos. As informações disponíveis sobre os estados de caráter dos caracteres sexuais secundários revelaram possível reconstruir os eventos históricos da evolução dos táxons de Aphyocharacinae ao longo do tempo. As histórias evolutivas dos caracteres recuperadas aqui corroboraram hipóteses de relações filogenéticas dentre os Aphyocharacinae. A presença ancestral de ganchos ósseos nos raios das nadadeiras pélvicas e anal foi recuperada na maioria das espécies da subfamília, onde a ausência dessas estruturas foi considerada uma novidade evolutiva que suporta a monofilia de um clado composto por *Leptagoniates*, *Paragoniates*, *Phenagoniates* e *Xenagoniates*; características sexualmente dimórficas evoluíram uma vez no ancestral comum do *Prionobrama*, e sustentam a monofilia e o diagnóstico do gênero; a distribuição dos ganchos ósseos ao longo dos raios da nadadeira pélvica recupera as relações filogenéticas entre as espécies de *Aphyocharax*. Nossas principais conclusões suportam o grande potencial das informações associadas aos caracteres do sistema sexual, onde investigações adicionais, incluindo novos conjuntos de caracteres morfológicos, devem ser consideradas em futuras análises filogenéticas.

Palavras-chave: Peixes de água doce; Dimorfismo sexual; Métodos comparativos filogenéticos; Evolução de caracteres; Biologia evolutiva.

ABSTRACT

Fishes exhibit broad patterns of diversification. Among the Ostariophysi, one of the greater radiations in vertebrates, Characiformes represents one of its most diverse groups where the freshwater species are extensively distributed across Neotropical region. Vast arrays of sexually dimorphic structures are known as secondary sexual characteristics that are closely related to the reproductive cycles of fishes. Sexually dimorphic characteristics have been reported in many species of Characidae, and can be associated to a variety of modified structures of adult males, and sometimes females. Attributes of the sexual system and morphological variations of these characters were implemented herein as sources of new data. Modifications of the secondary sexual system were the focus of this study, which correlated the evolution of secondary sexual characters to the phylogenetic diversification within the subfamily Aphyocharacinae. Through the analyses of reconstruction of ancestral character states based on a phylogenetic hypothesis of evolutionary relationships of the aphyocharacins, we proposed new suites of characters involving: the modification of body structures in adult specimens, and the presence/absence on the occurrence of bony hooks on pelvic and anal-fin rays in sexually dimorphic males. Available information on the character states of secondary sexual traits revealed possible to reconstruct the historical events of the evolution of Aphyocharacinae taxa over time. Evolutionary character histories recovered here corroborated hypotheses of phylogenetic relationships within the Aphyocharacinae. The ancestral presence of bony hooks on pelvic and anal-fin rays was recovered in most species of the subfamily, where the absence of these structures were considered an evolutionary novelty supporting the monophyly of a clade composed by *Leptagoniates*, *Paragoniates*, *Phenagoniates* and *Xenagoniates*; sexually dimorphic features have evolved once in the common ancestor of *Prionobrama*, and further supports the monophyly and the diagnosis of the genus; the distribution of bony hooks along pelvic-fin rays recover the phylogenetic relationships among species of *Aphyocharax*. Our main conclusions supports the great potential of information associated with characters the sexual system, where further investigations by including new sets of morphological characters should be considered in future phylogenetic analyses.

Key-words: Freshwater fishes; Sexual dimorphism; Phylogenetic comparative methods; Character evolution; Evolutionary biology.

EVOLUTION OF SECONDARY SEXUAL CHARACTERS: DESCRIPTION OF SEXUALLY DIMORPHIC STRUCTURES AND PHYLOGENETIC IMPLICATIONS IN THE SUBFAMILY APHYOCHARACINAE (CHARACIFORMES: CHARACIDAE)

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INTRODUCTION

Fishes account for more than one-half of the world's living vertebrates (~60,000 species) with more than 32,000 species. They also exhibit the most diverse patterns of morphology, habitat use, behavior and overall biology (Nelson *et al.*, 2016) in vertebrates. It is estimated that about a third of all freshwater fishes worldwide are distributed in approximately 12% of the total continental surface area, i.e. South America, of the planet (Reis *et al.*, 2016). Within Actinopterygii, teleosts are the most species-rich and diverse group, and among them the superorder Ostariophysi accounts for about 68% of the known freshwater species (Nelson *et al.*, 2016; Betancur-R *et al.*, 2017); and is one of the exceptional radiations in vertebrates (Alfaro *et al.*, 2009). Easily recognized by endemism, the Neotropical fishes are mostly comprised by ostariophysans; representing around 70% of its diversity (Albert *et al.*, 2011).

The order Characiformes represents one of the most diverse groups of Ostariophysi and occurs in Africa, southern North America, and is most diverse in Central and South America. Characiformes are currently comprised of more than 2200 valid species (Nelson *et al.*, 2016; Fricke *et al.*, 2020). The family Characidae comprises approximately 50% of species belonging to neotropical characiforms (Fricke *et al.*, 2020, Table 1), with a wide spectrum of morphological and biological variations, including the prevalence of small sized fishes (Azevedo, 2010).

This amazing diversity can also be observed in the strategies and reproductive tactics utilized by characids (Wootton, 1984; Winemiller, 1989; Taphorn, 1992). Among species of this lineage, the most common mode of reproduction are the externally fertilizing species with minimal evidence of external sexual dimorphism (Weitzman & Malabarba, 1998). Others, however, exhibit a vast array of sexually dimorphic structures that make genders distinguishable, and display elaborate anatomical modifications possibly associated with complex reproductive courtship behaviors (*e.g.* Burns *et al.*, 1995, 1997; Bushmann *et al.*, 2002; Malabarba, 1998; Malabarba & Weitzman, 1999, 2000, 2003; Malabarba *et al.*, 2004; Oliveira *et al.*, 2012; Weitzman *et al.*, 2005; Abrahão *et al.*, 2019). Secondary sexual characteristics may be directly or indirectly related to the reproductive act (Vazzoler, 1996) and are predominantly found in adult males (Wiley & Collette, 1970; Burns & Weitzman, 1996; Malabarba & Weitzman, 2003; Oliveira *et al.*, 2012). Dimorphic sexual characteristics have been reported in many species of Characidae, and include variation in structures such as fin shape, size, coloration, bony hooks on fin rays, and hypertrophied tissues (*e.g.* Burns & Weitzman, 1996; Burns *et al.*, 1997, 1998; Malabarba, 1998; Malabarba & Weitzman, 1999, 2003; Bushmann *et al.*, 2002; Malabarba *et al.*, 2004; Lima & Sousa, 2009; Menezes & Weitzman, 2009; Lucena & Malabarba, 2010; Miquelarena & López, 2010; Zanata & Camelier, 2010; Jerep & Malabarba, 2011; Oliveira *et al.*, 2012; Dala-Corte & Fialho, 2014; Camelier & Zanata, 2014; Vieira *et al.*, 2016; Longoni *et al.*, 2018). Modifications of the secondary sexual system are a data rich source of characters for phylogenetic studies for those characids that bear such modifications; an observation that is equally true and important whether they utilize insemination or external fertilization strategies (Weitzman & Malabarba, 1998; Malabarba, 1998; Menezes & Weitzman, 2009).

The analysis of ancestral character states based on explicitly measured characteristics allows the estimation of the history of examined characters when based on a phylogenetic hypothesis of evolutionary relationships (Joy *et al.*, 2016). Available information on the character states of contemporary species combined with a phylogeny makes it possible to reconstruct the historical events of the evolution of taxa over time, thereby providing a way to test hypotheses about evolution and adaptation (Pagel, 1999). These analyses depend on the application of evolutionary models that are sufficiently realistic to recover ancestral states, and are generally applied based on a phylogenetic hypothesis inferred from the same data (Joy *et al.*, 2016).

Due to the incredibly large number of species and the variety of forms, the phylogeny of Characidae continues to be investigated (*e.g.* Mirande, 2009, 2010; Oliveira *et al.*, 2011). Over the past decade, the characids have had the highest number of described species of all characiforms (Oliveira *et al.*, 2011; Fricke *et al.*, 2020). Recent morphological (Malabarba & Weitzman, 2003; Mirande, 2009, 2010) and molecular studies (Calcagnotto *et al.*, 2005; Javonillo *et al.*, 2010; Oliveira *et al.*, 2011), including a total evidence approach (Mirande, 2019), have been conducted to clarify the phylogenetic relationships among species of Characidae. Results from these studies on the species composition and phylogenetic relationships among the subfamilies and genera of Characidae, and its relationship with other families of the order, unfortunately remains unresolved (Oliveira *et al.*, 2011). To date, only a few genera and subfamilies of characids have been recovered as monophyletic. The limitations for a better understanding of phylogenetic relationships of characids is likely having an adequately focused sampling of internal (characids) and external (characiforms) taxa. Further, using attributes of the sexual system (Weitzman & Malabarba, 1998) and morphological modifications of this system (Malabarba, 1998; Lima & Sousa, 2009) as sources of character data may provide new information and perspectives that will likely aid our understanding for the phylogeny of complex or little-known groups (Weitzman & Malabarba, 1998; Lima & Sousa, 2009).

Javonillo *et al.* (2010) proposed “Clade B” characids as the sister lineage to Stevardiinae (*sensu* Thomaz *et al.*, 2015). Clade B includes species of *Exodon*, Cheirodontinae, Aphyocharacinae, Tetragonopterinae and Characinae (excluding *Gnathocharax*). This clade has been recovered in studies asking different phylogenetic questions using morphological (Mirande, 2009, 2010) and molecular data (Oliveira *et al.*, 2011), as well as a total evidence analysis (Mirande, 2019), and presently “Clade B” includes the subfamilies Exodontinae, Tetragonopterinae, Characinae, Aphyocharacinae and Cheirodontinae. The Exodontinae was first proposed by Fowler (1958), and was recently resurrected as a subfamily of Characidae comprised as monophyletic by *Roeboexodon* Géry, 1959; *Exodon* Müller & Troschel, 1844 and *Bryconexodon* Géry, 1980 (Mirande, 2019; Fricke *et al.*, 2020). Tetragonopterinae has been recognized for a long time as a large assemblage of fishes and is currently recognized as a monophyletic group comprised only by the genus *Tetragonopterus* Cuvier, 1816 (Melo *et al.*, 2016; Fricke *et al.*, 2020). Characinae holds the type species of the family and order, currently recognized as a monophyletic group of nine genera (*Acanthocharax* Eigenmann, 1912; *Acestrocephalus* Eigenmann, 1910; *Charax* Scopoli, 1777; *Cynopotamus* Valenciennes, 1849;

Galeocharax Fowler, 1910; *Microschemobrycon* Eigenmann, 1915; *Phenacogaster* Eigenmann, 1907; *Priocharax* Weitzman & Vari, 1987; *Roeboides* Günther, 1864) (Mattox & Toledo-Piza, 2012; Mirande, 2019; Fricke *et al.*, 2020). Aphyocharacinae has been recently revised with *Axelrodia lindeae* plus seven genera currently assigned to the subfamily (*Aphyocharacidium* Géry, 1960; *Aphyocharax* Günther, 1868; *Leptagoniates* Boulenger, 1887; *Paragoniates* Steindachner, 1876; *Phenagoniates* Eigenmann & Wilson, 1914; *Prionobrama* Fowler, 1913; *Xenagoniates* Myers, 1942) (Tagliacollo *et al.*, 2012; Mirande, 2019). Cheirodontinae is the most diverse group of “Clade B” and to date is one of the most extensively studied subfamilies of characids, with 16 genera currently assigned (*Acinocheirodon* Malabarba & Weitzman, 1999; *Aphyocheirodon* Eigenmann, 1915; *Cheirodon* Girard, 1855; *Cheirodontops* Schultz, 1944; *Compsura* Eigenmann, 1915; *Ctenocheirodon* Malabarba & Jerep, 2011; *Heterocheirodon* Malabarba, 1998; *Kolpotocheirodon* Malabarba & Weitzman, 2000; *Macropsobrycon* Eigenmann, 1915; *Nanocheirodon* Malabarba, 1998; *Odontostilbe* Cope, 1870; *Prodontocharax* Pearson, 1924; *Protocheirodon* Vari, Melo & Oliveira, 2016; *Pseudocheirodon* Meek & Hildebrand, 1916; *Saccoderma* Schultz, 1944; *Serrapinnus* Malabarba, 1998) (Mirande, 2019; Fricke *et al.*, 2020), but there is still controversy concerning hypotheses of internal phylogenetic relationships (Malabarba, 1998; Bührnheim *et al.*, 2008; Mirande, 2009, 2010, 2019; Oliveira *et al.*, 2011; Mariguela *et al.*, 2013).

Table 1 – Characidae’s diversity across lineages (Fricke *et al.*, 2020).

Subfamily	Number of valid species	Last 10-year descriptions*
Stethaprioninae	610	122
Characids <i>incertae sedis</i>	37	-
Aphyoditeinae	10	3
Spintherobolinae	6	-
Exodontinae	4	-
Tetragonopterinae	13	10
Characinae	96	9
Aphyocharacinae	23	1

Cheirodontinae	65	10
Stevardiinae	347	51

*2011–2020

First proposed by Eigenmann (1909), the subfamily Aphiocharacinae (*sic*), included fishes with a single series of well-developed teeth on the premaxilla, mandible and maxilla, large parietal and frontal fontanelles, gill-membranes free from the isthmus and each other, and presence of an adipose fin. Eigenmann (1910) assigned the genera *Coelurichthys* Miranda Ribeiro, 1908 (= *Mimagoniates* Regan, 1907), *Odontostilbe*, *Holoshesthes* Eigenmann, 1903 (= *Odontostilbe*), *Cheirodon*, *Aphyocharax* and *Holoprion* Eigenmann, 1903 (= *Aphyocharax*), later including *Aphyodite* (Eigenmann, 1912) and *Prionobrama* (Fowler, 1913) to the subfamily. Eigenmann (1915) later classified Aphiocharacinae as members of the subfamily Cheirodontinae, and Weitzman (1962), based on the lack of strong evidence on differences in cranial anatomy, included them as part of Characinae. More recently Aphiocharacinae was revalidated as a subfamily of Characidae (Géry, 1972, 1977) composed only by *Aphyocharax* and subsequent studies at taxonomic level (Souza-Lima, 2004) and about relationships were carried out by cladistic methods (Mirande, 2009, 2010). Although not phylogenetically tested, a close relationship between genera of Aphiocharacinae and Paragoniinae was mentioned by Géry (1977), and further investigated by Quevedo (2006) with members of Paragoniinae and a few representatives of Characidae (including three species of *Aphyocharax*). The latter reserved definitive conclusions of interrelationships between the subfamilies in lieu of more information. Based on morphological data, Mirande (2009, 2010) recovered a monophyletic Aphiocharacinae, composed of *Aphyocharax* and members of Paragoniinae (*Leptagoniates*, *Paragoniates*, *Phenagoniates*, *Prionobrama*, *Rachoviscus* Myers, 1926 and *Xenagoniates*) plus *Inpaichthys* Géry & Junk, 1977. In a total evidence analysis, Tagliacollo *et al.* (2012) proposed a new hypothesis of relationships for the Aphiocharacinae, removing *Inpaichthys* and *Rachoviscus*, and proposed adding *Aphyocharacidium*. Following the total-evidence approach by combining the available information of morphology and molecular-based studies, Mirande (2019) presented new relationships of species of Characidae, including the addition of *Axelrodia lindeae* in Aphiocharacinae.

Regarding secondary sexual characters and patterns of reproduction, numerous questions remain unanswered for many species of Characidae. Yet, there is unprecedented potential for understanding the inter- and intrafamilial relationships from characteristics of the

sexual systems among groups of Characidae (Weitzman & Malabarba, 1998). Their utility has been demonstrated as essential for corroboration of hypotheses of phylogenetic relationships in monophyletic groups of Characidae, *e.g.* Xenobryconini (Weitzman & Fink, 1985), Cheirodontinae (Malabarba, 1998), and Glandulocaudinae (Menezes & Weitzman, 2009). Within the groups of “Clade B”, the Cheirodontinae is the most well-documented in the literature, with several studies concerning their reproductive biology and dimorphic structures (*e.g.* Fink & Weitzman, 1974; Burns *et al.*, 1997; Malabarba, 1998; Malabarba *et al.*, 2004; Gonçalves *et al.*, 2005; Azevedo *et al.*, 2010; Oliveira *et al.*, 2010, 2012; Malabarba & Jerep, 2014). Discussions focused on the secondary sexual characteristics for members of Aphyoditeinae, Tetragonopterinae, Characinae and Aphyocharacinae are scarce, and more commonly found in species descriptions or generic revisions.

This study is designed to analyze morphological and molecular characters for members of Aphyocharacinae. One primary goal is characterizing the occurrence of secondary sexual characters and diagnosing the patterns of evolution of these features among this highly diverse group of fishes.

MATERIAL AND METHODS

Datasets include morphological features and DNA sequences from five genes (two mitochondrial and three nuclear) of characid species were generated for this study. Phylogenetic analyses utilizing Bayesian Inference (*Beast2.6; *RevBayes1.0) were conducted to investigate the subfamily Aphyocharacinae and to recover and propose hypotheses for the internal relationships of constituent taxa. Based on the interrelationships of aphyocharacins, the morphological features and the hypothesis framework were used to conduct ancestral reconstruction analyses to explore the patterns of evolution of secondary sexual characters within Aphyocharacinae through time.

Ingroup and outgroup Sampling

The ingroup used to test the relationships of members from Aphyocharacinae includes eight species of *Aphyocharax* (*Ap. alburnus*, *Ap. anisitsi*, *Ap. avary*, *Ap. colifax*, *Ap. dentatus*, *Ap. erythrurus*, *Ap. gracilis*, *Ap. nattereri*, *Ap. pusillus*, *Ap. rathbuni*, *Ap. yekwanae*), *Aphyocharacidium bolivianum*, *Aphyocharacidium* n. sp., *Axelrodia lindeae*, and species of the genera *Leptagoniates*, *Paragoniates*, *Phenagoniates*, *Prionobrama*, *Xenagoniates*. Those taxa

follow the most recent phylogenetic hypotheses for the subfamily, based on morphological and molecular evidence (Oliveira *et al.*, 2011; Tagliacollo *et al.*, 2012; Mirande, 2019).

Outgroup taxa were selected based on recent phylogenies proposed by Oliveira *et al.* (2011) and Tagliacollo *et al.* (2012). According to their hypotheses, Characidae is a strongly supported clade comprised by four monophyletic units. Species from the subfamilies Exodontinae (*Exodon paradoxus* Müller & Troschel, 1844; *Roeboexodon guyanensis* (Puyo, 1948)), Tetragonopterinae (*Tetragonopterus argenteus* Cuvier, 1816; *Tetragonopterus chalceus* Spix & Agassiz, 1829), Characinae (*Charax leticiae* Lucena, 1987; *Cynopotamus kincaidi* (Schultz, 1950); *Microschemobrycon casiquiare* Böhlke, 1953) and Cheirodontinae (*Cheirodon ibicuhiensis* Eigenmann, 1915; *Compsura heterura* Eigenmann, 1915; *Heterocheirodon yatai* (Casciotta, Miquelarena & Protogino, 1992); *Kolpotocheirodon theloura* Malabarba & Weitzman, 2000; *Macropsobrycon uruguayanae* Eigenmann, 1915; *Odontostilbe fugitiva* Cope, 1870; *Serrapinnus calliurus* (Boulenger, 1900); *Serrapinnus piaba* (Lütken, 1875)) were selected as outgroups and, in addition, members from Spintherobolinae (*Spintherobolus ankoseion* Weitzman & Malabarba, 1999; *Spintherobolus broccae* Myers, 1925; *Spintherobolus leptoura* Weitzman & Malabarba, 1999) were included as additional outgroups. The molecular sequence data are based on a novel set of 58 sequences from five genes of 34 Characidae taxa (Supporting Information 1).

Morphological data

Counts and measurements followed Fink & Weitzman (1974), with the addition and modifications of the following: anal-fin base length measured from the anal-fin origin to the last anal-fin ray; horizontal scale rows below lateral line counted to pelvic-fin insertion. Morphometric data were acquired point-to-point with a digital caliper (0.01 mm). Measurements and counts were taken on the left side of specimens, except when measurements and/or counts would be skewed by an abnormal or damaged specimen. In a case like this, the data were obtained from the right side of the specimens. Morphometric data are included in Support Information 5.

Osteological nomenclature follows Weitzman (1962), following modifications from Zanata & Vari (2005). Counts of supraneurals, teeth, fin hooks, vertebrae, procurrent caudal-fin rays were taken from x-ray images on Faxitron X-ray LX60, and cleared and stained (c&s) specimens were prepared according to the protocol of Taylor & Van Dyke (1985). Vertebral counts include the Weberian apparatus as four elements, and the fused PU1+U1 of the caudal

region as a single element (Weitzman, 1954). Precaudal vertebrae and caudal vertebrae definitions follow Weitzman (1962).

Definitions concerning the explicit localization of primary and secondary branching of pelvic-fin and anal-fin rays in sexually dimorphic taxa are proposed herein for the first time in characids (Figure 1). Primary branching is considered here as the first split of a fin ray, which consequently differentiates each ramification: medial and lateral primary ramifications on pelvic-fin rays; anterior and posterior primary ramifications on anal-fin rays. Secondary branching corresponds to all subsequent splits and derived ramifications of pelvic and anal-fin rays.

Cleared and stained (c&s) and whole specimens were imaged on a Nikon Multizoom AZ100.

Examined material (Supporting Information 2) came from the following Institutions: Academy of Natural Sciences of Philadelphia, Philadelphia (ANSP); Auburn University Museum of Natural History, Auburn (AUM); California Academy of Sciences, San Francisco (CAS); Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, Maringá (NUP); Cornell University Museum of Vertebrates, Ithaca (CUMV); Field Museum of Natural History, Chicago (FMNH); Illinois Natural History Survey, University of Illinois at Urbana-Champaign, Champaign (INHS); Laboratório de Biologia e Genética de Peixes, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Botucatu (LBP); Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (MCP); Museum of Comparative Zoology, Harvard University, Cambridge (MCZ); Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima (MUSM); Museu de Zoologia, Universidade de São Paulo, São Paulo (MZUSP); Naturhistoriska riksmuseet, Departments of Vertebrate Zoology and Palaeozoology, Stockholm (NRM); Royal Ontario Museum, Department of Natural History, Toronto (ROM); Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS). Institutional acronyms follow Sabaj (2019).

Secondary sexual characters

Observations for the analyses of secondary sexual characteristics consisted of viewing specimens under a stereomicroscope to detect and describe the occurrence of external morphological features related to sexual dimorphism, primarily focused on the characterization

of bony hooks on fin rays. Sexes of adult specimens were checked from previously granted authorization for dissection from Examined material.

Morphological descriptions of the bony hooks on the fin rays provide detailed descriptions across species about the number, form (tip and base shape), distribution (concentration of bony hooks on fin ray regions), development and orientation (insertion along the fin rays) of these characters. The terminology used to describe the bony hooks follows Weitzman & Fink (1985), Reis (1989), Malabarba (1998), Malabarba & Weitzman (2000), and Vieira *et al.* (2016).

Molecular data

Sequences of the mitochondrial genes 16S rRNA (16S), cytochrome b (*cyt b*), and nuclear genes myosin heavy chain 6 gene (*Myh6*), recombination activating gene 1 (RAG 1) and recombination activating gene 2 (RAG 2) from the studies of Oliveira *et al.* (2011) and Tagliacollo *et al.* (2012) are available on GenBank database (Benson *et al.*, 2012), and were retrieved for this study. Additional sequences of the mitochondrial gene 16S were added for 16 specimens of *Aphyocharax alburnus* (n=2), *Aphyocharax avary* (n=2), *Aphyocharax erythrurus* (n=5), *Aphyocharax pusillus* (n=1), *Leptagoniates steindachneri* (n=2) and *Paragoniates alburnus* (n=4), with the remaining genes assigned as missing data. DNA sequences were deposited in GenBank database (Accession N°. XXXX). Tissue samples and sequence information are listed in Supporting Information 1.

DNA extraction was completed with the QIAGEN DNeasy Blood & Tissue Kit by Qiagen© per the manufacturers instructions, and all work was carried out at the Nancy and Larry Fuller Lab of Evolutionary Biology (Cornell University Museum of Vertebrates – Lab of Ornithology, Ithaca – EUA).

Sequences of the mitochondrial gene 16S rRNA (16S) were amplified by polymerase chain reaction (PCR) (Table 2). PCR amplifications were performed in 25µl reactions using Phusion® High-Fidelity PCR Master Mix with HF Buffer by New England BioLabs® Inc., consisting of: 12.5µl reaction buffer (with correction of 1.25µl MgCl₂ in some samples), 1.25µl of each primer at 10µM, 1–2µl DNA, and 6.75–9µl of double-distilled water. Amplification of 16S sequences were retrieved using one complete round of PCR with cycles of amplification as follows: (1) 30 seconds at 98°C (initial denaturation), (2) 10 s at 98°C, (3) 30 s at 54°C, (4) 30 s at 72°C, and 5 min at 72°C (final elongation), with steps (2) through (4) repeated 35 times. PCR products were checked by electrophoresis in an agarose gel and sequenced in both

directions at Biotechnology Resource Center – BRC facility (Institute of Biotechnology, Cornell University, Ithaca – EUA).

Table 2 – Sequences of primers.

Gene	Primer name	Primer sequence (5'–3')	Source
16S rRNA	16Sar	ACGCCTGTTTATCAAAAACAT	[1–2]
	16Sbr	CCGGTCTGAACTCAGATCACGT	[1–2]

^[1]Palumbi, 1996; ^[2]Palumbi *et al.*, 2002.

Phylogenetic analysis

Molecular based data

Each gene sequence was independently aligned using the MUSCLE algorithm (Edgar, 2004) with default parameters in MEGA7 software (Kumar *et al.*, 2016), subsequently, alignments were inspected by eye for any possible misalignment.

The species tree was estimated using BEAST v2.6.0 (Bouckaert *et al.*, 2014) and utilizing the StarBeast template on XSEDE v2.6.1 (Drummond & Rambaut, 2007; Suchard & Rambaut, 2009) through CIPRES Science Gateway (Miller *et al.*, 2010). DNA alignments from each gene were considered as partitions, and models of molecular evolution and gene trees were unlinked. Substitution models for DNA alignments were estimated with the Bayesian Information Criterion (BIC) method (Schwartz, 1978) implemented by PartitionFinder v1.1.1 (Lanfear *et al.*, 2012) under default parameters, generating the information necessary on Site Model settings for priors on substitution models for the partitions. The multi-species Coalescent was set for Population function as “constant”. Specifications for priors in the Species Tree was set to Birth-Death Model, and Gamma distributions were determined for partitions Clock rates. The species tree was estimated in two runs, and each run was performed with 400000000 MCMC generations with trees stored every 20000 iteration and pre burn-in at 0 (zero) with 20,000 trees retained. Evolution of log likelihood scores and checks for the stationary of all model parameters of each run via ESS values were visualized and completed using Tracer v1.7.1 (Rambaut *et al.*, 2018), with 10% of the initial states discarded as burn-in. The tool TreeAnnotator on XSEDE (Drummond & Rambaut, 2007) was used on CIPRES to summarize the trees post 10% burn-in.

The concatenated aligned DNA sequence data matrix of 58 specimens (34 species) based on a multilocus dataset (16S, CytB, Myh6, RAG1, RAG2) (Table 3) resulted in a matrix with 4,303 aligned base pairs (bp) with taxa from the five subfamilies that are currently known as “Clade B” of Characidae (Exodontinae, Tetragonopterinae, Characinae, Cheirodontinae and Aphyocharacinae) plus Spintherobolinae.

Mean completeness of character are ~70% for most taxa, The influence of missing data on phylogenetic analyses have been thoroughly considered, e.g. Wiens & Morrill (2011), wherein no evidence has been found for inaccuracy of Bayesian estimates and phylogenetic placement when >90% of missing data are present.

Morphology based data

A morphological data matrix of the subfamily Aphyocharacinae and other subfamilies within Characidae was assembled using Mesquite version 3.31 (Maddison & Maddison, 2017). Bayesian Inference of the character matrix was conducted in RevBayes v1.0.13 software (Höhna *et al.*, 2016). The Mk model proposed by Lewis (2001) was implemented for estimating phylogenetic trees from discrete morphological data. This model is a generalization of the Jukes-Cantor substitution model (Jukes & Cantor, 1969) assuming a Markov process for character change, allowing for multiple character-state symmetrical changes along a single branch (Wright & Hillis, 2014). “Relaxed-clock models” were implemented including a constant-rate birth-death branching process by applying a Birth-Death model as a prior on the distribution of tree topologies and node ages (Höhna *et al.*, 2015), where diversification, turnover, speciation and extinction rates were estimated. Rho was included to model incomplete taxon sampling, considered as the probability of sampled taxa (this study) and known described species of Characiformes (numbers from Fricke *et al.*, 2020).

Considering the computational efforts needed for such large-scale data analyses, the estimation of BI was performed using clusters at the Centro Nacional de Supercomputação (CESUP) of Universidade Federal do Rio Grande do Sul (UFRGS).

The morphology-based analysis was performed using an extended matrix of Mirande (2019). Herein, the addition of nine taxa currently assigned to the subfamily Aphyocharacinae (*Aphyocharacidium* n. sp., *Aphyocharax alburnus*, *Ap. avary*, *Ap. colifax*, *Ap. erythrurus*, *Ap. gracilis*, *Ap. pusillus*, *Ap. rathbuni*, *Ap. yekwanae*) and twenty three new characters were added to the matrix previously published by Mirande (2019). This resulted in a morphological character matrix with 543 characters for 54 taxa (Supporting Information 3). The new characters

were coded in all of the species noted above and left as missing data for the remaining characidae that were selected from the original matrix. The codification of characters 34, 45, 90, 91, 92, 123, 124, 125, 143, 189, 190, 203, 324, 325, 326, 384, 385, 391, 392, 393, 394, 395, 408, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 432, 433, 434, 441, 442, 446, 447, 448, 449, 450, 451, 452, 453, 512 and 513 were checked for members of Aphyocharacinae available from Mirande (2019), and the differences observed are highlighted in the Results. Characters 92 and 450 in Mirande (2019) were necessarily modified and are also addressed in the Results. The above listed characters were also coded for the newly added taxa.

Though the addition of new characters are assigned as missing data for the remaining taxa of Mirande's (2019) original matrix, increasing data set size can improve phylogenetic estimation methods (Wright & Hillis, 2014). The influence of missing data on morphological super matrix was tested by Dillman *et al.* (2016), and results show that matrices composed by more than 60% of missing data are still viable to reconstruct strongly supported and highly resolved hypotheses of relationships under Parsimony analysis.

The tree was estimated using two independent runs, each run performed with 400000 MCMC generations with trees stored every 100 iteration and pre burnin at 10% with 8000 trees retained. The distribution of log likelihood scores was examined to determine stationarity for each run and achieve convergence through ESS values using Tracer v1.7.1 (Rambaut *et al.*, 2018), with 10% of the initial states discarded as burn-in. The Bayesian inference hypothesis based on morphological data was obtained as the Maximum Clade Credibility tree (MCC).

Ancestral character state reconstruction (ACSR)

From the Bayesian phylogenetic hypotheses generated with the data collected during this study, an analyses of ancestral character reconstruction was performed through the application of scripts using the R packages: ape (Paradis *et al.*, 2004), phytools (Revell, 2012), parallel (R Core Team, 2017), geiger (Harmon *et al.*, 2008). The ACSR analyses were conducted under the following models of discrete character evolution: "ER" – equal rates for all permitted transitions; "ARD" – all-rates-different for permitted transitions; "SYM" – symmetric backward and forward rates for all permitted transitions (Harmon *et al.*, 2020). When comparing models fitted by maximum likelihood, the best model was assessed under the Akaike Information Criterion method (AIC) with a correction to small sample size (AICc; Burnham & Anderson, 2002). AICc values were calculated for each applied fitted model. Akaike weights were retrieved from AICc values, and were used for selection of best fitted model (see Results).

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