

Original Article

Evolutionary history, biogeography, and a new species of *Spherooides* (Tetraodontiformes: Tetraodontidae): how the major biogeographic barriers of the Atlantic Ocean shaped the evolution of a pufferfish genus

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ABSTRACT

Tetraodontidae is the most speciose family of Tetraodontiformes and is represented by fish popularly known as pufferfishes. They are characterized by modified jaws with four dental plates and the ability to inflate their bodies. Tetraodontids are distributed throughout the world and have a wide range of habitat use. One of its genera, *Spherooides*, shows a biogeographical pattern, with 19 of its 21 species restricted to coastal regions of the Americas. Although represented in large-scale phylogenies, the evolutionary history and biogeography of the genus have not been explored in detail. The present study aims to understand the historical and biogeographic processes that shaped the evolutionary history of *Spherooides*. Including samples from all biogeographic regions of its occurrence, we reconstruct a phylogenetic/biogeographic history hypothesis for the genus. Our results show that *Spherooides* is a paraphyletic group comprising *Colomesus*; indicate a central role of the biogeographic barriers of the Atlantic Ocean in the diversification of the genus; and identified a cryptic species in Brazilian waters, formally known as *S. spengleri*, described here through integrative taxonomy. We also propose nomenclatural changes given the position of *Colomesus* deeply nested within *Spherooides*.

Keywords: Amazon basin; *Colomesus*; ecological speciation; Isthmus of Panama; marine incursions; reef fish

INTRODUCTION

Tetraodontidae, commonly known as pufferfish, are the most diverse family of the order Tetraodontiformes, with 192 valid species in 28 genera (Fricke et al. 2023). Pufferfishes occur throughout the world and have a wide range of habitats, living in shallow tropical and warm temperate waters, open pelagic environments, coral reefs, mangroves, and even freshwater (Alfaro et al. 2007). In the many habitats pufferfishes inhabit, they have important trophic roles as consumers, with most species being zoobenthivorous (Ferreira et al. 2004). They are widely known for their ability to inflate their bodies with water or air when stressed or disturbed (Brainerd 1994, Wainwright and Turingan 1997). Some species are highly poisonous due to the presence of the strong alkaloid neurotoxin tetrodotoxin in their tissues (Nelson 2006, Katikou et al. 2022).

Sphoeroides Anonimus [Lacépède], 1798 depicts the plasticity of ecological attributes of the family, given the significant range of habitat use among the 21 currently valid species of the genus (Fricke et al. 2023). This ecological variability includes the use of shallow marine waters, brackish, incursions into freshwater, and species living in deep marine habitats (Shipp 2003). *Sphoeroides* has a marked biogeographic pattern, restricted to the Pacific and Atlantic coasts of the Americas, except for one species in the Eastern Atlantic and one with a circumglobal range (Fricke et al. 2023).

Morphologically, *Sphoeroides* is characterized by an oblong body with a short dorsal fin and anal fin rounded to somewhat falcate with 6–8 rays; pectoral fins with 13 to 17 rays and one rudimentary dorsalmost ray; caudal fin truncate to rounded, rarely slightly concave; skin prickles and lappets present or absent; interorbital space broad to narrow; single nasal tube on each side of head, varying from short and wide to high and elongate, with two openings near tip; upper portion of lateral line system well defined, reaching caudal-fin base (Jordan and Evermann 1898, Shipp 1974, Cervigón 1996).

Despite the inclusion of *Sphoeroides* species in previous phylogenetic hypotheses, none of these proposals focused on the evolutionary history of the genus, nor included species from all biogeographic regions where *Sphoeroides* occurs (Yamanoue et al. 2011, Amaral et al. 2013, Santini et al. 2013). Usually found in these phylogenetic reconstructions are the sister-group relationship between *Sphoeroides* and *Lagocephalus* Swainson, 1839, and the paraphyly of *Sphoeroides* due to the position of *Colomesus* Gill, 1884 nested within *Sphoeroides*. However, the relationship between *Sphoeroides* and *Colomesus* was not addressed in detail in any of the studies.

Investigating the evolutionary and biogeographic history of *Sphoeroides* can provide clues about the role of the main biogeographic barriers of the Atlantic Ocean in the diversification of its reef fauna. In this area, five main biogeographic barriers are recognized as relevant in shaping the diversity of reef fishes: the Mid-Atlantic Barrier (the ~3500 km of open deep water between the Western and Eastern Atlantic); the land bridge between the Mediterranean and the Red seas; the Amazon-Orinoco Barrier in northern South America; the Isthmus of Panama, the land bridge between the Atlantic and the Eastern Pacific; and the cold Benguela current off south-west Africa (Briggs 1995, Bellwood and Wainwright 2002, Floeter et al. 2008).

In this sense, the present study aims to understand the historical and biogeographic processes that shaped the evolutionary history of *Sphoeroides*. For this purpose, we used sequences of the mitochondrial gene cytochrome oxidase I (*COI*) to reconstruct a hypothesis of the phylogenetic and biogeographic history of the genus, which we will discuss first. We then describe a new species of *Sphoeroides* from Brazilian waters that was identified in the process, through integrative taxonomy.

MATERIALS AND METHODS

Molecular data

DNA extraction, PCR amplification, and sequencing

Total genomic DNA of 24 samples of *Sphoeroides* sp. and 14 samples of *Sphoeroides marmoratus* Lowe, 1838 were extracted from muscle tissue preserved in 98% ethanol using a saline protocol (Miller et al. 1988). A fragment of the mitochondrial cytochrome oxidase I (*COI*) gene was amplified using the primers FishF1 and FishR1 (Ward et al. 2005). PCR reactions were carried out with an initial denaturation step at 95 °C for 2 minutes (min), followed by 35 cycles of 94 °C for 30 seconds (s), 52 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The PCR products were sequenced in both directions using the same primers at the Fundação Oswaldo Cruz (FIOCRUZ).

Data analyses

The electropherograms obtained were checked, edited, and assembled in the SEQMAN program (DNASTar Inc.). Consensus sequences were visually inspected to reduce possible errors. The generated sequences were deposited in GenBank (Supporting Information, Table S1). Sequences of 15 species of *Sphoeroides* ($N = 81$) and three species of *Colomesus* ($N = 17$) were obtained from GenBank and the Barcode of Life Data (BOLD) System (Supporting Information, Table S1). We checked and collated information from all sequences obtained in the databases to maximize identification reliability. Sequences with questionable identification were either excluded or identified in the Supporting Information.

All sequences were aligned in MEGA 7.0 (Kumar et al. 2015) using the Clustal W algorithm (Thompson et al. 1994). The substitution model was obtained using jModelTest 2.1 (Darriba et al. 2012) based on the Bayesian Information Criterion (BIC). Inter- and intraspecific genetic divergence was performed in the R package ‘ape’ (Paradis and Schliep 2019) based on the nucleotide substitution model TN93 (Tamura and Nei 1993). The plot was generated using the R software package (R Core Team 2020).

To investigate the phylogenetic relationships and estimate the divergence time between the *Sphoeroides* species, we conducted two Bayesian analyses. The first one was executed in BEAST 2.5 (Bouckaert et al. 2019), conducted with TN93 model, a strict clock and the calibrated Yule Model; employing secondary calibration points following the results obtained in Santini et al. (2013)—*Lagocephalus* + *Sphoeroides*/*Colomesus* node at approximately 31.4 million years old (Myr) in age. We ran two independent analyses, with 50 million generations sampled every 5000 generations, with the first 20% of trees removed

as burn-in. The second method was implemented in MrBayes 3.2.6 (Ronquist *et al.* 2012), with two independent runs of four concomitant Markov Chain Monte Carlo (MCMC) runs for 15 million generations and sampling parameters every 1000 generations. Tracer 1.7 software (Rambaut *et al.* 2018) was used to check the results of the runs. *Lagocephalus guentheri* Miranda Ribeiro, 1915 was used as the outgroup.

A haplotype network was produced using the method of Templeton *et al.* (1992) (TCS method, Clement *et al.* 2000) implemented in PopArt 1.4 (Leigh and Bryant 2015). We also conducted two species delimitation tests, the Multi-rate Poisson Tree Processes (mPTP) (Kapli *et al.* 2017) and the Generalized Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough 2013).

To estimate the ancestral geographic range and biogeographic history of *Spherooides*, we used the phylogeny obtained in BEAST to perform an analysis using the R package 'BioGeoBears' (Matzke 2013a, b). BioGeoBears uses its version of three different models to access the biogeographical history: dispersal-vicariance analysis (DIVA—DIVALIKE; Ronquist 1997); Bayesian analysis of Biogeography (BAYAREALIKE model; Landis *et al.* 2013); and dispersal-extinction-cladogenesis (DEC model; Ree and Smith 2008). None of these models includes founder-event speciation (jump-dispersal parameter 'j'), which is performed separately for the different models (Matzke 2014). We performed six non-stratified analyses using each model (i.e. DEC, DIVA, and BAYAREA), including the +j parameter. We used two sequences of each *Spherooides* species and sequences of *Colomesus*, including one brackish and two freshwater species, *Colomesus psittacus* Bloch and Schneider, 1801, *Colomesus asellus* Müller and Troschel, 1849, and *Colomesus tocantinensis* Amaral, Brito, Silva & Carvalho 2013, respectively. *Spherooides pachygaster* Müller and Troschel, 1848 was omitted from the biogeographic reconstruction due to the considerable sampling gap in the Atlantic that could affect the accuracy of the analysis.

The distribution of *Spherooides* and *Colomesus* species were checked on the IUCN Red List website (<https://www.iucnredlist.org>). In addition, six relevant biogeographic regions (modified from Spalding *et al.* 2007, Briggs and Bowen 2012) were designated for the analyses: (A) Amazon Basin, (EA) Eastern Atlantic, (EP) Eastern Pacific, (C) Greater Caribbean, (N) North-western Atlantic, and (S) South-western Atlantic.

Sampling and morphological identification

Ninety-nine specimens of *Spherooides* sp. from the Brazilian coast were examined (Supporting Information, Fig. S1) in the AZUSC, MCZ, MNRJ, MZUSP, NPM, UF, USNM, and ZUEC collections [institutional abbreviations following Sabaj (2020)]. Moreover, 16 specimens of *Spherooides marmoratus* were collected in São Tomé and Príncipe and on Terceira Island, Azores, Portugal (Supporting Information, Fig. S1). We examined 61 specimens of *Spherooides spengleri* (Bloch 1785) from UF, nine from USNM, three from MZUSP, and one from AZUSC (Supporting Information, Fig. S1). We also examined five specimens of *S. marmoratus* from UF (Supporting Information, Fig. S1). The list of specimens examined in this study and the sample sites is detailed in the Supporting Information, Comparative Material, Fig. S1. Counts and measurements of morphological

and meristic traits follow Shipp (1974). Measurements were taken with electronic callipers to the nearest tenth of a millimetre (mm).

RESULTS

Molecular analyses

Our phylogenetic analyses are the most representative of the genus to date, with 15 of the 21 valid species of *Spherooides* and samples from all biogeographic regions where it occurs, including the virtually cosmopolitan *Spherooides pachygaster*, plus the three valid species of *Colomesus*. Our analyses retrieved five major clades (Fig. 1): clade A, containing *S. pachygaster*, the only species that occurs outside the Atlantic/Eastern Pacific system, the sister group of all species in the genus. According to the time-tree, this species could have diverged from the other species at about 24.8 Mya (19.7–29.6 Mya, 95% highest posterior density, HPD). Clade B is composed of *Spherooides yergeri* Shipp, 1972, *Spherooides annulatus* Jenyns, 1842, *Spherooides lispus* Walker, 1996, and *Spherooides testudineus* Linnaeus, 1758. The estimated divergence of its separation from the other clades is around 19.0 Mya (14.9–23.6, 95% HPD). Clade C is composed of *S. spengleri*, *S. marmoratus*, and *Spherooides* sp., and the divergence between this clade and clades D and E was estimated at 16.6 Mya (12.6–20.5, 95% HPD). Clade D, sister of clade E, includes *Spherooides greeleyi* Gilbert, 1900, and the three species of *Colomesus*. The estimated divergence time between these clades is about 15.4 Mya (11.7–19.1, 95% HPD). Finally, clade E is formed by *Spherooides dorsalis* Longley, 1934 + *Spherooides parvus* Shipp & Yerger, 1969, *Spherooides lobatus* Steindachner, 1870, *Spherooides tyleri* Shipp, 1972, and *Spherooides maculatus* Bloch & Schneider, 1801 + *Spherooides nephelus* Goode & Bean, 1882. These five major groups were strongly supported in both analyses, with a minimum value of posterior probability of 99%.

Phylogenetic relationships among lineages from the major groups are notable. In clade A, the sequences of *S. pachygaster* from Uruguay form a monophyletic and distinct group from the other *S. pachygaster* samples included here (north-east Atlantic and Indo-Pacific). In clade B, the sequences identified as *S. lispus* settled with the sequences of *S. annulatus*. The topology of clade C strongly suggests the monophyly of *Spherooides* sp., notably more related to *S. marmoratus* than to *S. spengleri* (as identified in Brazil hitherto). In particular, *Spherooides* was recovered as paraphyletic due to the position of *Colomesus* deeply nested within clade D. In this same clade, two lineages of *C. asellus* were recovered, henceforth *C. asellus* 1 and *C. asellus* 2. In clade E, reciprocal monophyly was not observed between *S. maculatus* and *S. nephelus*. There is a significant genetic distance between the Brazilian sequence and the north-west Atlantic sequences of *S. dorsalis*.

The results of the lineages delimitation tests were mainly similar, differing only in the definition of few lineages. The GMYC pointed out 21 species, while the mPTP retrieved only 16 species. The similarities between the two tests were as follows: *S. pachygaster* distinguished into two putative lineages (Uruguay sequences separated from the others); *S. yergeri*, *S. spengleri*, *S. marmoratus*, *Spherooides* sp., *S. greeleyi*, *S. lobatus*, *S. tyleri*, *S. parvus*, *S. maculatus* + *S. nephelus*, *C. psittacus*, and *C. asellus* 1 as

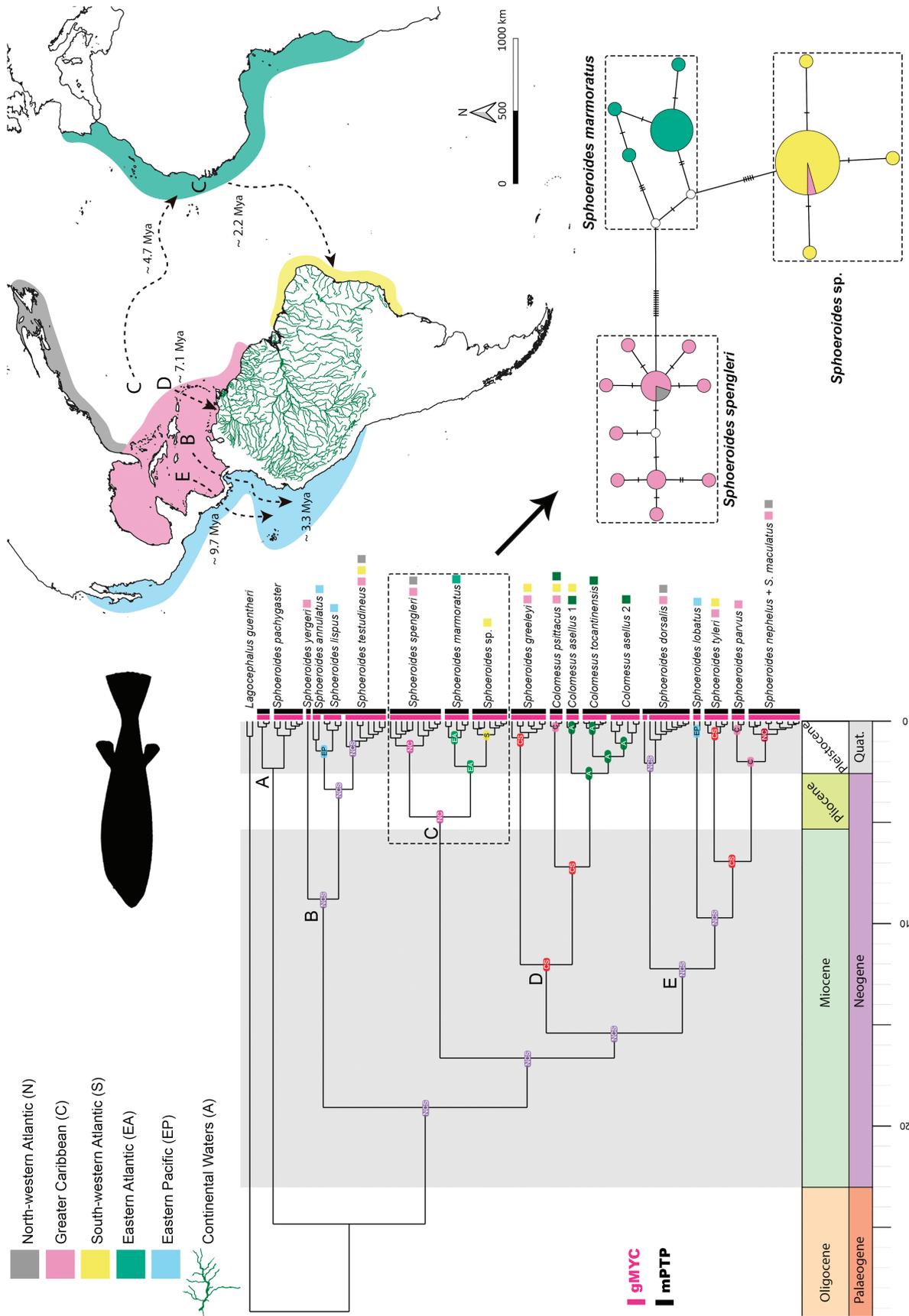


Figure 1 . Time-tree phylogeny and biogeographic estimation of the genus *Spherooides* based on the mitochondrial gene cytochrome oxidase I (*COI*). Node states represent the ancestral range estimation, according to the best-fitting model recovered by the biogeographic estimation (BAYAREALIKE+). Vertical bars show the results of the lineages delimitation tests: pink vertical bars, gMYC; black vertical bars, mPTP. The

well-defined lineages (Fig. 1). The reduced number of lineages in mPTP include clade B (*S. annulatus*, *S. lispus*, and *S. testudineus* recognized as a single lineage), clade D (*C. tocaninensis* and *C. asellus 2* as a single lineage) and clade E (*S. dorsalis* as a single lineage in opposition to the GMYC result showing a putative unrecognized lineage represented by the South-western Atlantic sequence).

Regarding the cases with apparent intraspecific divergence, the genetic distance between the *S. pachygaster* sequences from Uruguay and the Indo-Pacific was 1.8%; between *Sphoeroides* sp. and *S. spengleri* 3.8%; between Brazilian *S. dorsalis* and *S. dorsalis* from the north-west Atlantic, 1.6%. Meanwhile, where the topology revealed the absence of interspecific divergence, the genetic distance between *S. annulatus* and *S. lispus* was 0.6%, and between *S. maculatus* and *S. nephelus* was 0.1% (Fig. 2).

Geographic ancestral range: BAYAREALIKE+J was the best-fitting model (Supporting Information, Table S2), based on the LnL and AICc values. Our analysis suggests that the genus probably originated in the Western Atlantic in the Early Miocene. In clade B, the ancestral range was recovered in the Caribbean, with a subsequent dispersion to the Eastern Pacific of *S. annulatus* + *S. lispus*. In clade C, the ancestral range was estimated to be in the Caribbean/North-western Atlantic; furthermore, the ancestral node of *S. marmoratus* + *Sphoeroides* sp. was present in the Eastern Atlantic and was later dispersed to Brazil. In clade D, the crown node was estimated only in the Caribbean; however, the ancestor of the clade containing *C. asellus 1*, *C. asellus 2*, and *C. tocaninensis* was recovered only in the Amazon Basin. The common ancestor of clade E was recovered in the Western Atlantic, i.e. the same distribution as the original lineage of the genus; followed by dispersal and speciation to the Eastern Pacific of the ancestor of *S. lobatus* and the permanence of a lineage in the Atlantic corresponding to the clade *S. tyleri* (*S. nephelus* + *S. maculatus*).

SYSTEMATICS

A new Western Atlantic pufferfish is hereby described, based on molecular and morphological characters, as follows:

Sphoeroides camila Carvalho-Filho, Rotundo, Pitassy & Sazima sp. nov.

Figs 3–4; Tables 1–2

Zoobank registration: [urn:lsid:zoobank.org:act:A7CE6DBB-CFB0-4006-B9F8-F80FEB5E4A01](https://zoobank.org/urn:lsid:zoobank.org:act:A7CE6DBB-CFB0-4006-B9F8-F80FEB5E4A01).

English proposed common name: Southern bandtail puffer.

Portuguese (Brazil) proposed common name: Baiacu-pinima.

Synonymy (in part): *Tetraodon marmoratus*: Ranzani 1839.

***Sphoeroides spengleri*:** Jordan and Evermann 1898, Miranda Ribeiro 1915, Carvalho-Filho 1999, Rocha and Rosa 2001

Menezes 2003, Sampaio and Nottingham 2008, Garcia *et al.* 2015, Pinheiro *et al.* 2015, 2018, Nóbrega *et al.* 2015, Gasparini 2017, Guimarães-Costa *et al.* 2020, Rotundo *et al.* 2021.

Holotype: MZUSP 126947 (Field number: ACF82020001). 92.2 mm Standard Length (SL), Arraial do Cabo, Rio de Janeiro, Brazil. 22°52'S, 42°01'W, collected by A. Carvalho-Filho, February 1982 (Fig. 3).

Paratypes (29 specimens): AZUSC 6924 (6 specimens, 79.0–97.4 mm SL), Praia do Forno ou Praia Grande, Arraial do Cabo, Rio de Janeiro, Brazil, 22°57'S, 42°1'W, between the surface and 5 m, col. Pedro Hollanda Carvalho and Carlos E.L. Ferreira, 21 Mar. 2015; MCZ 12129, 36.5 mm SL, Vigia, Baía de Marajó, Pará, Brazil, 0°50'N 48°7'W, col. Thayer Expedition to Brazil: Louis Agassiz and party, 28 Feb. 1866; MNRJ 7717, 97.2 mm SL, Praia Santa Helena, Vitória, Espírito Santo, Brazil, 20°18'S, 40°17'W, col. V. Carvalho, 25 Jan. 1945; MNRJ 53329 (2 specimens, 103.5–115 mm SL), same data as AZUSC 6924; MZUSP 45607, 85 mm SL, Arquipélago dos Alcatrazes, São Paulo, Brazil, 24°06'S, 45°42'W, 6 m, col. R.L. Moura, 1 Nov. 1992; MZUSP 46633 (2 specimens, 31.1–68.8 mm SL), Porto Belo, Santa Catarina, Brazil, 27°9'S, 48°33'W, 5 m, col. A. Carvalho-Filho, Jan. 1988; MZUSP 49079, 99 mm SL, Ilha dos Frades, Vila de Paramana, Salvador, Bahia, Brazil, 12°58'S, 38°30'W, 28 Jul. 1993; MZUSP 81099 (2, 82.3–91.9 mm SL), Arraial do Cabo, Rio de Janeiro, col. A. Carvalho-Filho, Feb. 1982; MZUSP 126949 (2, 79.6–103.9 mm SL), same data as AZUSC 6924; NPM 7080 (2, 71.0–79.0 mm SL), same data as AZUSC 6924; UF 19203 (4, 56.4–79.2 mm SL), Praia de Itapoã, Salvador, Bahia, Brazil, 12°57'S, 38°22'W, col. Virginia Almeida, 7 Jul. 1970; USNM 83162 (2, 71.8–104 mm SL), Rio de Janeiro, Brazil, col. C. Wilkes, 1838–1842 (Fig. 3); USNM 104232, 90.4 mm SL, Recife, Pernambuco, Brazil, R. Von Ihering, 1932; ZUEC 4957, 84.9 mm SL, Praia da Fortaleza, Ubatuba, São Paulo, Brazil, 23°25'S, 45°4'W, 0.5 m, col. I. Sazima, 14 Jun. 2000; ZUEC 5665 78.6 mm SL, Ilhote, Ilha Bela, São Paulo, Brazil, 23°46'S, 45°20'W, col. C. Sazima and I. Sazima, 27 Mar. 2002.

Diagnosis: *Sphoeroides camila* sp. nov. is distinguished from its congeners by the following combination of characters: pectoral-fin rays 13–14 (rarely 11, 12, 15, or 16), not including a rudimentary one on upper fin sheath; dorsal-fin rays 7–8 (rarely 6); anal-fin rays usually 6–8, rarely 5 (unbranched rays 0–2, branched rays 4–6); dorsum with two pale lappets before opercle, none black; 24–34 lappets on left sagittal section of body; 1–8 lappets on head before opercle; adults without prickles on dorsum or sides of body (rarely a small patch after interorbital space), prickles occasionally present in juveniles from interorbital to middle back; very distinct row of black spots on ventrolateral body from chin to end of caudal-fin peduncle, the mid-ones vertically oblong, those on extremities rounded and considerably smaller; no black rounded spots above ventrolateral row (Fig. 4A, B); caudal fin with a dark bar at base and a

haplotype network is from clade C. Scale bar in Myr. Biogeographic regions were symbolized by coloured squares. Dotted arrows indicate the estimation of ancestor dispersion routes for each clade (B-E). Numbers indicate the age of the dispersion routes in Myr.

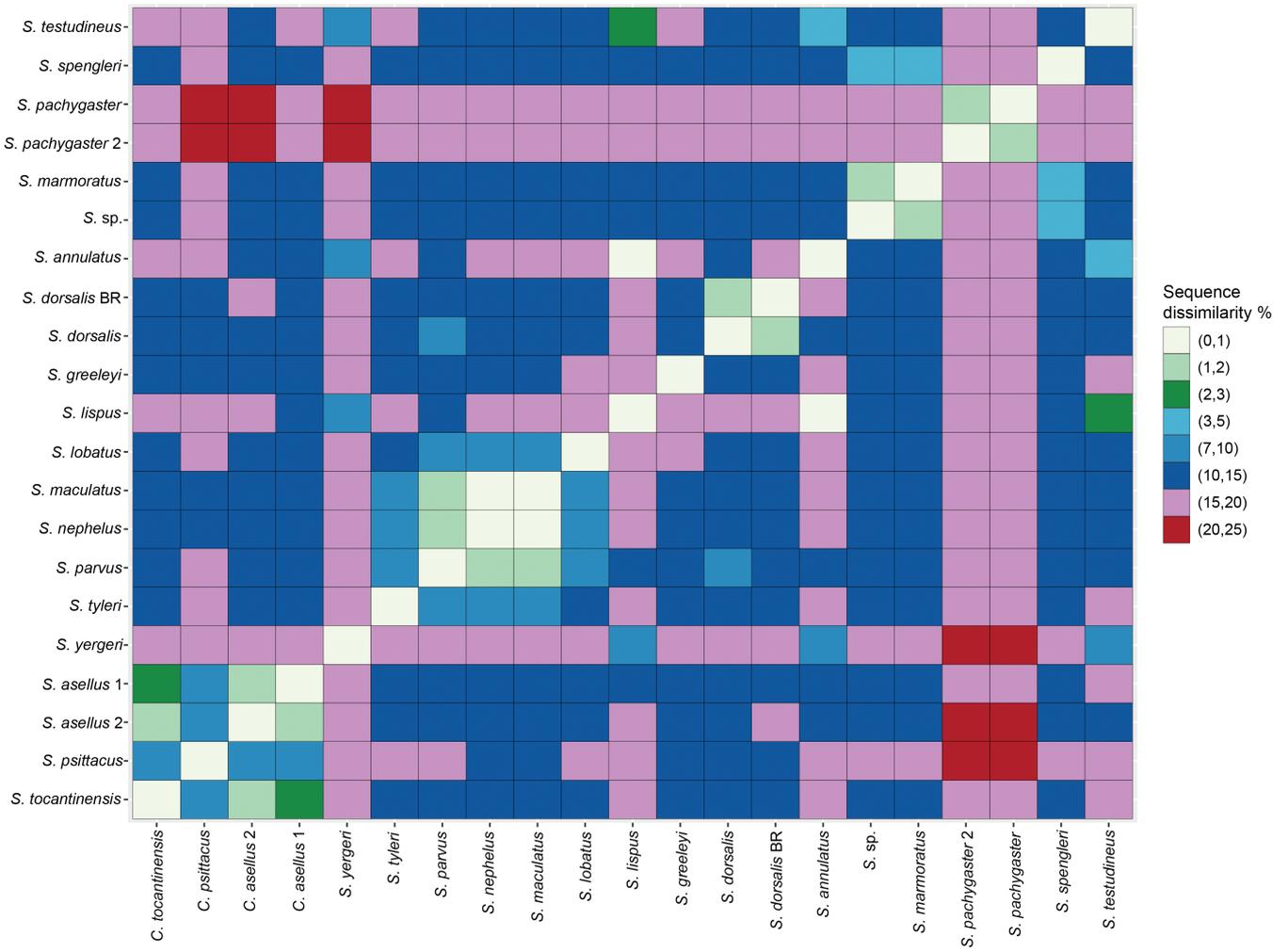


Figure 2. Genetic divergence matrix based on the nucleotide substitution model TN93 of *Spherooides* species. The colours represent the mean percentage of genetic dissimilarity between species.



Figure 3. (A) *Spherooides camila* holotype (MZUSP 126947), 92.2 mm SL, collected by A. Carvalho-Filho at Arraial do Cabo, Rio de Janeiro, Brazil. (B) Paratype and oldest known specimen of *S. camila* (USNM 83162), 104 mm SL, collected by C. Wilkes between 1838 and 1842.

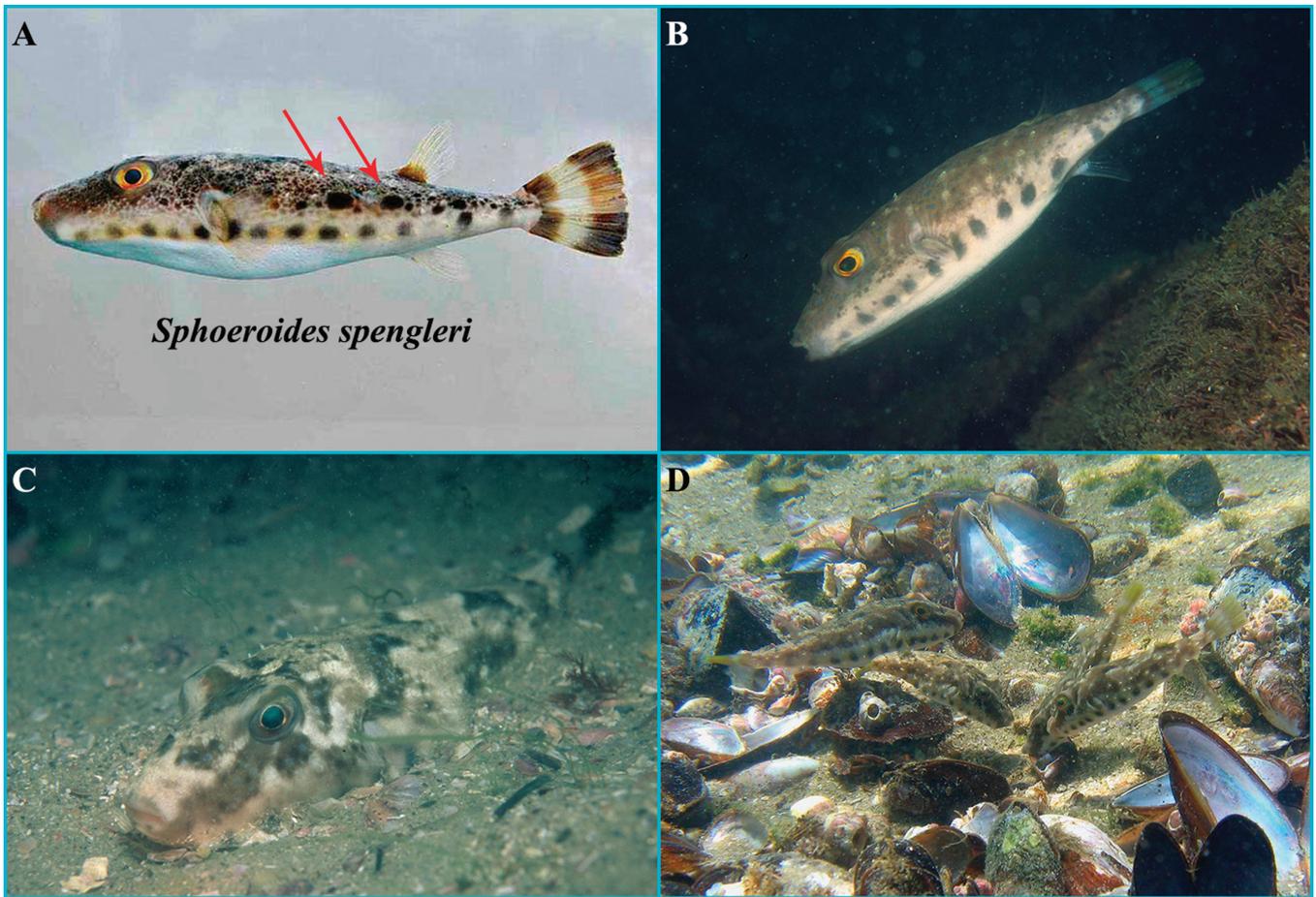


Figure 4. Lateral views of (A) *S. spengleri* and (B) *S. camila* specimens; red arrows indicate a pair of black blotches on the dorsum of *S. spengleri*, absent in *S. camila*. (C) *Sphoeroides camila* individual partly buried in sandy bottom. (D) *Sphoeroides camila* individuals eating mussels opened by a diver, an opportunistic foraging behaviour elicited by human interference. Figure (A) by Robertson & Van Tassell (2019). Figure (C) by Peter Wirtz.

very wide yellow-greenish bar posteriorly. Size up to about 14 cm SL, usually 10 cm SL.

Description: Based on holotype and 29 paratypes. Morphometrics and meristic counts are shown in Table 1.

Body oblong; head large, 33.3–38.4% of SL, longer in specimens up to 50 mm SL; mouth terminal, upper jaw projects slightly beyond lower; snout conical, long; nostrils paired, nares at the end of the moderate tube; eye moderate, 15–30% on Head Length (HL), (longer in specimens smaller than 50 mm SL); interorbital moderate, 8–14% of HL, concave (more evident in specimens up to 50 mm SL) to somewhat flat; gill slit arch-shaped, slightly shorter than the pectoral-fin base. Dorsal-fin rays 7–8 (rarely 6); anal-fin rays usually 6–8, rarely 5 (unbranched rays 0–2, branched rays 4–6); dorsal and anal fins nearly opposite, base lengths similar; pectoral fin rounded, moderate, 32–41% of HL, with 13–14 (rarely 11, 12, 15, or 16) rays, not including a rudimentary one on upper fin sheath; caudal fin truncate to slightly rounded with 11 rays, one upper and two lower unbranched. Lappets usually in two rows from the dorsum to the anterior dorsal-fin area, one or two pairs before pectoral-fin insertion; often one lappet between each of the black blotches of the ventral row; 1–8 lappets on head before opercle; 24–34 lappets on left sagittal section of body. Prickles

on dorsum and ventral areas, more evident in specimens up to 50 mm SL, those of the dorsum often absent in adults. Vertebrae 17 [base on X-rays of seven specimens (Fig. 5) and 18 dissected and discarded specimens].

Coloration in life: dorsolateral head and body light reddish to greyish or olivaceous brown, sides and belly whitish. Upper snout pale with one dark irregular area in front of nares; a conspicuous lateral dark band from lower maxilla to eye; usually peppered with black spots and blue ocelli; interorbital dark blotch, often with anterior and posterior prolongations, resulting in an almost cross-shaped mark; dorsum with a pair of black blotches, often peppered with white spots and iridescent blue circles and/or ocelli (Figs. 4B, 7). A conspicuous lateral row of 11–14, usually 13, black blotches, rounded from chin to pectoral fin, followed by tear-shaped oblong marks from pectoral fin to end of caudal peduncle, the largest blotch at middle body. A light horizontal band is often present between dark dorsum and ventral row of blotches. Pectoral and dorsal fins light yellowish to greenish, their bases dark; anal fin usually whitish to yellowish; caudal fin with distinct dark brown to dusky black bar on its base, followed by a relatively thin pale one and a wide, yellowish-green distal bar, usually at least 40% of fin-length. Lappets usually pale, conspicuous, occasionally with sparse dark pigment; one type specimen with dark lappets on upper part of body posteriorly

Table 1. Morphometric data of the holotype and 29 paratypes of *S. camila*, and examined specimens of *S. spengleri*. Standard length in mm, the other measurements in percentage of standard length

	Holotype (MZUSP 126947)	Paratypes (N = 29)	<i>S. spengleri</i> (N = 26)
Standard length	92.2	31.1–115.0	19.4–154.0
<i>Percentage of standard length</i>			
Head length	33.3	34.5–38.4	36.4–38.7
Eye	7.9	6.5–9.2	7.3–12.2
Interorbital space	4.8	4.0–5.2	4.4–7.9
Snout	19.1	17.9–20.8	18.7–22.3
Post-orbital length	11.7	10.2–14.2	10.0–11.7
Dorsal-fin base	7.7	6.4–9.6	6.9–9.5
Anal-fin base	7.5	5.9–7.6	5.9–8.0
Pectoral-fin length	15.3	12.7–15.3	12.6–14.6
Caudal-fin length	20.5	18.5–24.1	19.7–23.8
Pre-dorsal length	72.4	70.4–77.3	71.4–74.9
Pre-anal length	72.3	71.9–78.5	72.7–76.7
<i>Number of fin-rays</i>			
Dorsal-fin rays	7	7–8	7
Anal-fin rays	6	6–7	6
Pectoral-fin rays	14	13–15	13
<i>Lappets</i>			
Dorsum before opercle	2	2–4	None
Left sagittal section (LSS)	28	24–34	10–18
LSS before opercle	4	3–11	None

Table 2. Selected characters of the *S. marmoratus* complex

Characters	<i>S. marmoratus</i>	<i>S. spengleri</i>	<i>S. camila</i>
Lappets on dorsum	One pair, black	Usually none	At least one pale pair
Black spots beneath eye	Present	Absent	Absent
Blue spots/circles on dorsum	Absent	Usually absent	Present
Lateral-ventral row of spots	10–12, usually rounded spots	11–14, usually 13 rounded spots	11–14, usually 13 tear-shaped spots
Black spots above lateral-ventral row	0–2, diffuse	2–3, conspicuous	None
Distal bar of caudal fin	Dark and wide	Black and thin	Greenish-yellow and wide

to dorsal-fin base. Short row of black spots above ventral row lacking. Eyes yellow. Preserved specimens with dark dorsum, all dark blotches prominent over whitish to tan ground coloration, blue circles/ocelli absent (Fig. 3). Occasionally a few with sparse to overall dark pigment after dorsal-fin base. Caudal-fin distal bar dark, grayish, never black.

Etymology: A noun in apposition. The specific name honours Camila Carvalho, one of the daughters of the first author of the new species. We do not use the genitive *camilae*; this name was inadvertently used in a presentation at an ichthyological meeting.

Distribution: *Sphoeroides camila* is currently recorded from Northern to South-eastern Brazil; vagrants may occur in the Southern Caribbean.

Natural history: *Sphoeroides camila* is common in shallow, clear reef waters, up to 20 m deep along the Brazilian coast, occasionally to 70 m depth (AZUSC 6925). It is usually observed alone,

in pairs or small groups, and often several individuals are found in a small area, hovering over the substrate. It is rarely found in seagrass beds, a behavioural difference between *S. camila* and *S. spengleri*, which is typically observed in seagrass plains. It is a generalist zoobenthivore (Fig. 4), but frequently consumes zooplankton and drift material in the water column or at water surface. Diet is composed of crustaceans, molluscs, worms, echinoderms, and zooplankton larvae, which agree with findings for its congener *S. spengleri* in Florida (Targett, 1978). Several individuals of *Sphoeroides camila* were attracted to mussels opened by a diver (Fig. 4D), which indicates opportunistic foraging, a feature observed for several other pufferfish species (e.g. Duncan and Szelistowski 1998, Kalogirou 2013, Sazima 2019). An individual was observed following a hunting gold spotted eel *Myrichthys ocellatus* Lesueur, 1825 and snatching two small crabs flushed by the eel, which again demonstrates its opportunistic foraging. *Sphoeroides camila* is diurnal, spending the night in reef crevices or partially buried on the adjacent sand/gravel bottom, with the eyes and upper dorsum exposed (Fig. 4C).

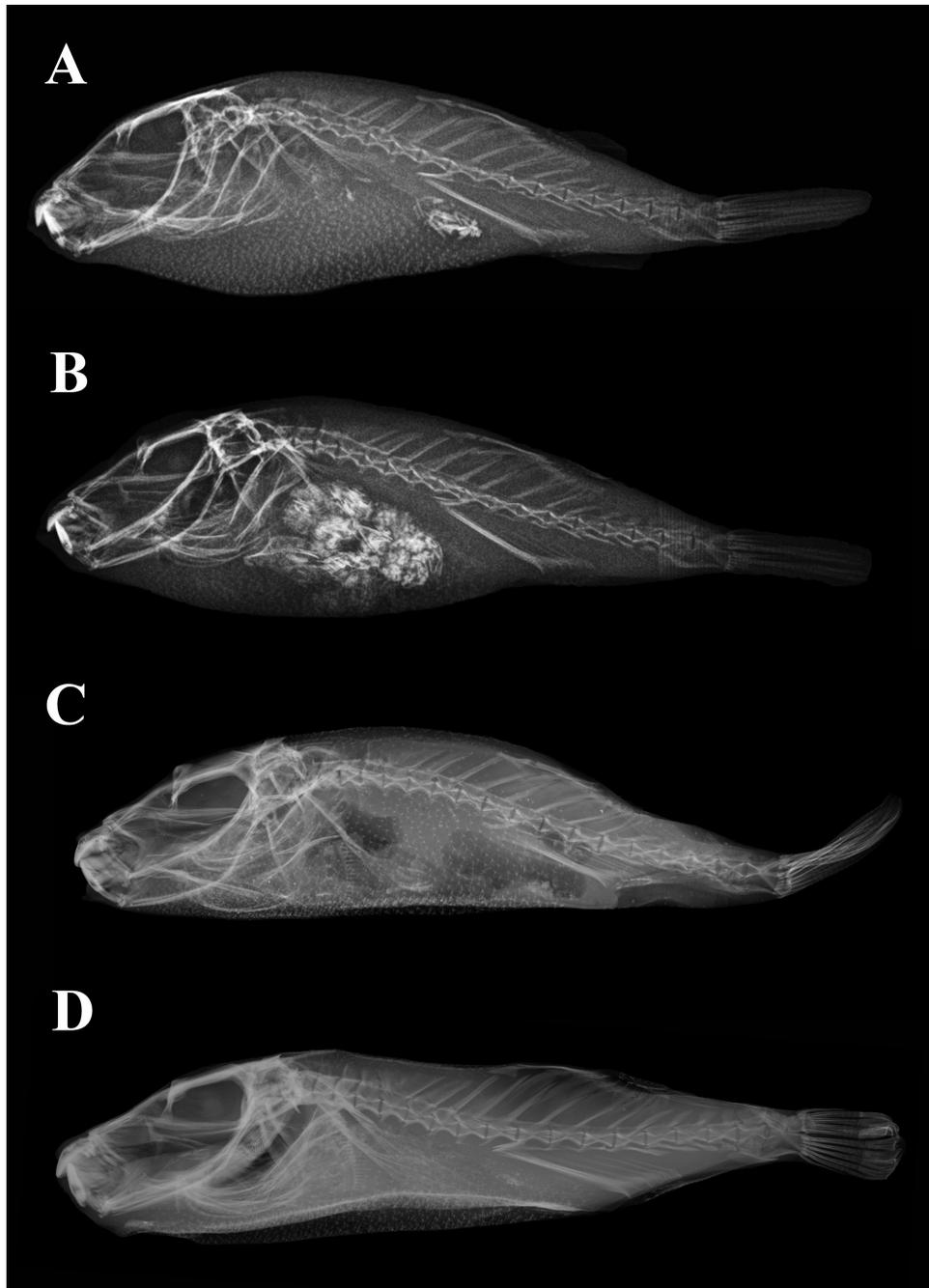


Figure 5. X-rays of selected specimens of *S. camila* and *S. spengleri*. (A) *Sphoeroides camila* (AZUSC 6924). (B) *Sphoeroides camila* (AZUSC 6925). (C) *Sphoeroides camila* (USNM 83162). (D) *Sphoeroides spengleri* (USNM 104232).

Remarks

For more than a hundred years, *S. camila* was identified as *S. spengleri*. Both are morphologically very similar and, with the Eastern Atlantic *S. marmoratus*, compose a complex of similar species characterized by a conspicuous lateral row of black marks. These three species have a common ancestor and as a result of geographic isolation evolved into different species. *Sphoeroides marmoratus*, given an ocean of distance, differs unmistakably from both American species by the presence of a pair of black lappets on the dorsum and a row of distinct spots beneath the eye, characters lacking in its Americas congeners. Additionally, *S. marmoratus* has 10–12 blotches (usually 11) in the lateral-ventral

row vs. 11–14 (usually 12) in American congeners. On the other hand, *S. camila* differs from *S. spengleri*, other than in molecular loci, by several characters: the new species presents at least one pair of pale, well-developed lappets on dorsum (usually absent in *S. spengleri*); 24–34 lappets on left sagittal section of body (10–18 in *S. spengleri*); oblong blotches of the lateral-ventral row vertically tear-shaped at midbody (all spots of *S. spengleri* rounded, seldom horizontally elongated, Fig. 4A, B); distal bar of caudal fin yellowish-green and covers at least 40% of fin-length (black and covering not more than 30% of fin-length in *S. spengleri*). Preferred habitats also differ between the two American species: *S. camila* prefers hard substrates such as rocky and coralline reefs,



Figure 6. *Sphoeroides asellus*, an exclusively freshwater species within the genus that was recovered as a sister group (together with *Sphoeroides tocantinensis*) of *Sphoeroides psittacus*, a brackish and coastal species.

whereas *S. spengleri* selects seagrass beds. Number of vertebrae in both species, 17 (this paper and Tyler 1980). Specimen MZUSP 7695, examined by Shipp in 1969 and identified by him as *S. spengleri*, actually is the new species described here: presence of two pale lappets on dorsum before the pectoral-fin base; vertically oblong black spots on ventrolateral row before and after pectoral-fin insertion; absence of rounded black spots above the ventrolateral row; a wide bar on posterior margin of caudal fin, not black and paler than the caudal-fin base bar, undoubtedly characterise a specimen of *S. camila*.

Discussion

Phylogenetic and biogeographic analyses

Our results validate findings from previous studies and enrich the knowledge about the diversity, evolution, and biogeography of *Sphoeroides*. We built the most representative phylogenetic hypothesis of the genus to date, which allowed us to infer the crucial role of geological events that occurred in the Atlantic/Eastern Pacific system in the past ~25 Myr in the diversification of the genus. We also describe a new species of *Sphoeroides* from Brazilian waters supported by different lines of evidence through integrative taxonomy, and propose nomenclatural changes based on the position of *Colomesus* deeply nested within *Sphoeroides*.

In his book on Atlantic Ocean Tetraodontidae, Shipp (1974) speculated on zoogeographic and evolutionary patterns among some Tetraodontidae species based on morphological and geographical data. Broadly speaking, he postulated a close relationship between *Sphoeroides*, *Lagocephalus*, and *Colomesus* and proposed *S. pachygaster* (in his own words, ‘the most divergent and atypical species of *Sphoeroides*’) as a sister group to the other *Sphoeroides*. Both relationships were recovered in the most recent phylogenies, including representatives of *Sphoeroides* (Yamanoue et al. 2011, Amaral et al. 2013, Santini et al. 2013), always indicating *Lagocephalus* as the sister group of *Sphoeroides* + *Colomesus*, and *S. pachygaster* as the sister of the remaining *Sphoeroides* + *Colomesus*. However, the position of *Colomesus* in relation to *Sphoeroides* in the analyses was controversial. Shipp (1974) considered *Colomesus* the sister group of *Sphoeroides*, whereas in Yamanoue et al. (2011), Amaral et al. (2013), and Santini et al. (2013) *Colomesus* is nested within *Sphoeroides* (despite the different relative positions in each of the

analyses). In Yamanoue et al. (2011) and Santini et al. (2013), *Colomesus* was recovered as a sister group of *Sphoeroides* (except *S. pachygaster*), whereas in Amaral et al. (2013) *Colomesus* is the sister of a group including *S. nephelus*, *S. tyleri*, and *S. greeleyi* in the neighbour-joining analyses and sister of only *S. greeleyi* in the Maximum Likelihood analyses. Here, we have similar results, that is, *Colomesus* nested within *Sphoeroides* (and the consequent non-monophyly of the latter) and *S. pachygaster* as a sister group to the remaining *Sphoeroides*. We also recovered *S. greeleyi* as the sister group of *Colomesus*, forming clade D, with maximum support values (Fig. 1).

With the insertion of more species, including the newly generated sequences of *Sphoeroides camila* (formerly *S. spengleri* from Brazil) and *S. marmoratus*, we were able to glimpse a broader picture of the diversification of the genus. The first point to be emphasized is the new species described here. All individuals from Brazil previously identified as *S. spengleri* were recovered as a distinct clade, with deep genetic divergence (~4%) from *S. spengleri* sequences from the Greater Caribbean. When combined, tree topology, clear biogeographic split, deep genetic distance between the sequences, lineages delimitation tests, and the noticeable morphological differences between individuals from the Caribbean and Brazil (see the Systematics section) strongly support the proposition of the new species from Brazilian waters. Surprisingly, *S. camila* was recovered as more related to *S. marmoratus* from the Eastern Atlantic than to *S. spengleri* from the Western Atlantic (Fig. 1). Although uncommon, the closer relationship between Brazil and the Eastern Atlantic, rather than Brazil and the Greater Caribbean, has already been found in other reef fish groups (e.g. *Clepticus*, *Scartella*, and *Hippocampus*; Beldade et al. 2009, Araujo et al. 2020, Li et al. 2021, respectively).

The biogeographical estimation suggests two subsequent crossings of the Atlantic as the explanatory scenario for the above-mentioned relationship (Fig. 1). First, a dispersal to the Eastern Atlantic from the Caribbean, followed by a new crossing to the South-western Atlantic. The Mid-Atlantic barrier, the Amazon-Orinoco Barrier, and the Isthmus of Panama are possibly relevant agents in this diversification scenario. The closure of the Isthmus of Panama triggered changes in Atlantic Ocean circulation patterns, including the strengthening, around 3.6 Mya, of the Gulf Stream and the Equatorial Undercurrents

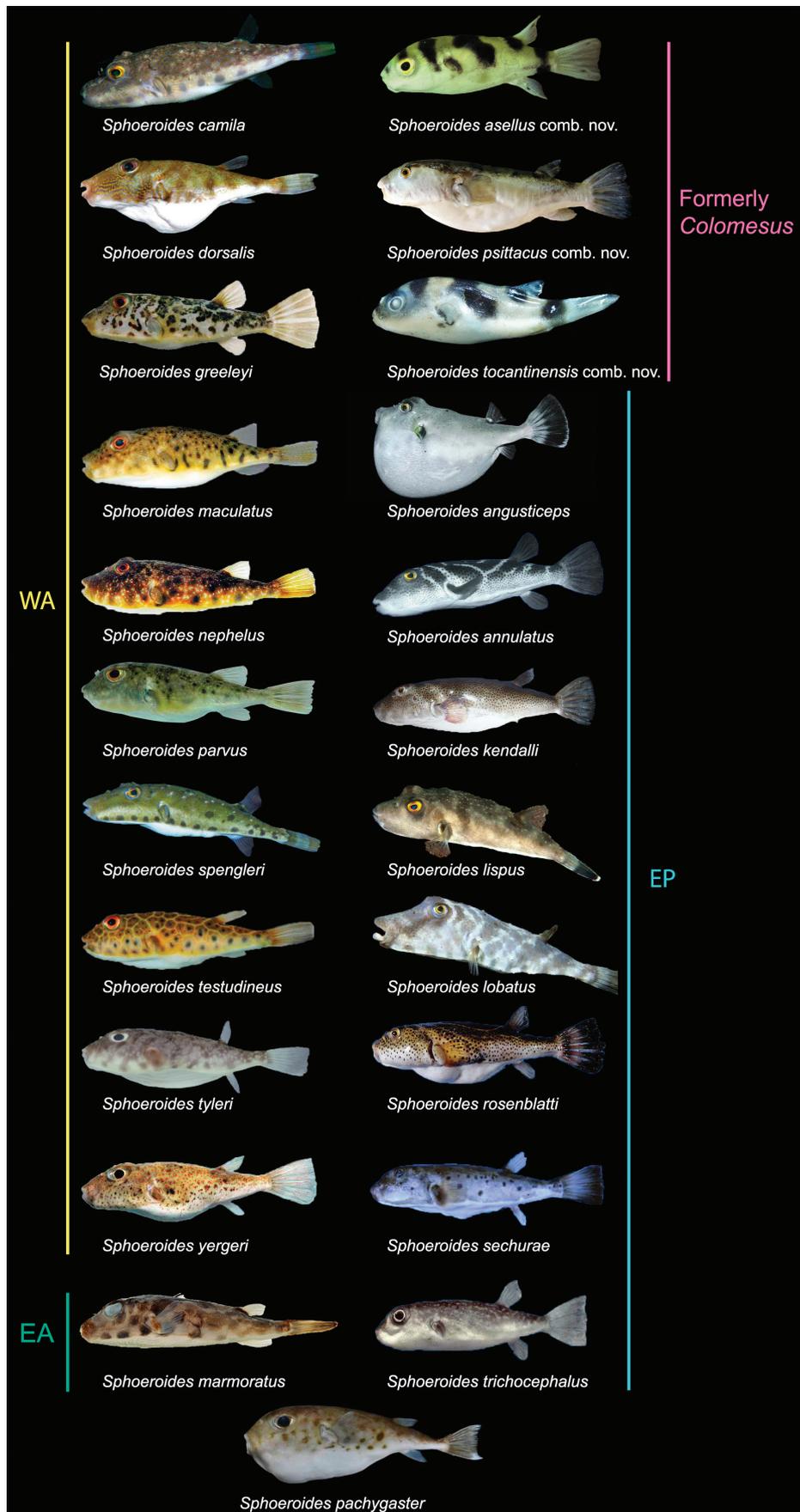


Figure 7. Diversity of the genus *Sphoeroides*. On the left, yellow line represents *Sphoeroides* species from the Western Atlantic (WA); green line represents the only species from Eastern Atlantic (EA), *S. marmoratus*. On the right, pink line represents species of the former *Colomesus* genus; blue line represents species of the Eastern Pacific. Lower centre, *S. pachygaster*.

(Berggren and Hollister 1974, Burton *et al.* 1997), two currents that carry water from the Western to the Eastern Atlantic. Furthermore, during the beginning of the Pleistocene, about 2.4 Mya, there was a substantial increase in the sedimentation rates of the Amazon River and a higher frequency of sea-level fluctuations, which together reduced the permeability of the Amazon-Orinoco Barrier (Rocha 2003, Robertson *et al.* 2006, Araujo *et al.* 2022). We hypothesize that these enhanced eastward currents were the possible migration route of the common ancestor of *S. marmoratus* and *S. camila* to colonize the Eastern Atlantic; simultaneously, the less permeable Amazon-Orinoco barrier isolated the North and South Atlantic lineages. Later, the South Equatorial Current may have brought larvae and/or rafting individuals from the Eastern Atlantic to Brazil. The eventual interruption of gene flow caused by the Mid-Atlantic Barrier would have resulted in *S. marmoratus* and *S. camila* isolated on each side of the Atlantic. These cladogenetic events were estimated at 4.7 and 2.2 Mya (Fig. 1), respectively, and are consistent with our proposed hypothesis. It should be noted that a haplotype of *S. camila* was sampled at Isla Mujeres, Quintana Roo, Mexico (Fig. 1). Due to the deep genetic distance between *S. spengleri* and *S. camila*, the presence of a haplotype of the latter species in the Caribbean may be a case of secondary contact after range expansion to the Southern Caribbean. Several species originally from Brazil have managed to cross the Amazon-Orinoco Barrier, being carried to the Caribbean by the North Brazil Current, using the Great Amazon Reef System below the Amazon-Orinoco plume as a faunistic corridor (Peterson and Stramma 1991, Rocha 2003, Francini-Filho *et al.* 2018, Araujo *et al.* 2022). Indeed, specimens of *S. camila* were sampled (with molecular confirmation) near the mouth of the Amazon River (3°30'N, 49°46'W) at 73 m depth, below the plume. Another possibility is the existence of a real population of *Spherooides camila* in the Caribbean, less abundant than *S. spengleri*. Sampling efforts in the South Caribbean may shed light on this puzzle.

The Eastern Pacific species included in the analyses were accommodated in very different places on the tree (Fig. 1). *Spherooides annulatus* + *S. lispus* are sisters of *S. testudineus* in clade B (estimated divergence date = 3.4 Mya), and *S. lobatus* is sister of *S. nephelus* + *S. maculatus* and *S. tyleri* in clade E (estimated divergence date = 9.7 Mya). The results of the phylogenetic and historical biogeographic analysis evidence two independent split events between the Western Atlantic and Eastern Pacific lineages, with an approximate difference of 6.3 Myr (Fig. 1). Ecological differences between lineages associated with a long and complex process of closing the Isthmus of Panama may explain this scenario. The lifting of the Panama land bridge begins with the underwater collision of the Panama Arc with South America at around 24 Mya. It was a gradual process that allowed extensive interoceanic water exchange until about 9.2 Mya, when the deep-water passages were closed. However, the inter-ocean exchange continued in shallow waters through straits, canals, and mangroves, until the complete closure of the Isthmus, about 3.2 Mya (Lessios 2008, O'Dea *et al.* 2016). In clade E, *S. dorsalis*, which was recovered as a sister group of the other species, preferentially inhabits deep waters (Shipp 1974). Additionally, *S. tyleri* and *S. lobatus* can be found at depths of up to 80 m and 107 m, respectively (Figueiredo and Menezes 2000, Nielsen *et al.* 2010a). Therefore, the preference for deeper

water habitats may have been the ancestral condition of clade E, inducing an early split promoted by the closure of deep-water interoceanic communication. This hypothesis is supported by the temporal coincidence between the closure of deep-water seaways (9.2 Mya) and the estimated separation of species from the Western Atlantic and Eastern Pacific (9.7 Mya). In contrast, the estimated separation in clade B (3.4 Mya) indicates that gene flow through the isthmus was only interrupted at its very final closure. Before then, the genetic connection was probably maintained through narrow mangrove-like remnant channels (Lessios 2008). Therefore, the use of shallow water and mangrove-like habitats should be essential to maintain population connectivity between the Western Atlantic and Eastern Pacific until the full emergence of the Isthmus of Panama. These attributes are found in species of clade B: *S. lispus* and *S. testudineus*, separated by 3.4 Mya, inhabit shallow waters up to 20 m (Nielsen *et al.* 2010b, Shao *et al.* 2014). This latter species is also one of the most euryhaline of the genus (excluding *Colomesus*, see above), tolerating very low salinity levels (Shipp 2003). This seems to explain the late split between the Western Atlantic and Eastern Pacific in clade B.

The internal relationships of clade D (*S. greeleyi* + *Colomesus*) unequivocally indicate a gradual tolerance to freshwater in the evolutionary history of the clade. *Spherooides greeleyi* is a known inhabitant of the lower reaches of estuaries and mangrove-estuary-reef ecotones (Contente *et al.* 2011, Xavier *et al.* 2012). *Colomesus psittacus*, which occurs in brackish and coastal waters in northern South America, was recovered as a sister group of exclusively freshwater species, *C. asellus* and *C. tocantinensis*, suggesting a transition from brackish to freshwater in the diversification of the clade, and not a secondary marine invasion by *C. psittacus*, as proposed by Santini *et al.* (2013).

There is a vast body of evidence that indicates marine incursions from the Caribbean into the Western Amazon during the Miocene (20.4–10 Mya, Hoorn 1993). At that time there was a large wetland system in the Upper Amazon, Lake Pebas, connected to the Caribbean and containing a series of salinity gradients (Lundberg *et al.* 1998, Bayona *et al.* 2007). Some authors suggest more recent incursions (Nores 1999, Hubert and Renno 2006, Hubert *et al.* 2007); however, there is no geological evidence of a marine influence in Amazonia after 7 Mya (Hovikoski *et al.* 2010, Bloom and Lovejoy 2011, Wesselingh and Hoorn 2011). The divergence between *C. psittacus* and its strictly freshwater relatives was estimated at 7.2 Mya (5.0–9.6 Mya, HPD), consistent with the previous estimate of Santini *et al.* (2013) (2.5–6.5 Mya) and Yamanoue *et al.* (2011) (4.1–17.8 Mya). Despite the chronological amplitude of the estimates, possibly due to the different calibration strategies (Santini *et al.* 2013) and the molecular markers used, it is conceivable that *Colomesus* entered the Amazon Basin through these marine incursions (Fig. 1). When these corridors closed, gene flow stopped and *Colomesus* radiated into the Amazon Basin. Tetraodontidae fossils from the Miocene in the north-west Amazon Basin and the phylogeographic evidence of a west-to-east diversification direction of *C. asellus* in the Amazon Basin are consistent with the hypotheses of initial colonization from marine corridors in the Western Amazon (Monsch 1998, Cooke *et al.* 2012).

Colomesus is one of approximately 30 Tetraodontidae lineages that have successfully colonized freshwater, including species

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