Comparative morphology, phylogeny, and classification of West African callopanchacine killifishes (Teleostei: Cyprinodontiformes: Nothobranchiidae)

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A phylogenetic analysis combining 63 morphological characters and DNA sequences (3296 bp), comprising segments of the mitochondrial genes *16S* and *ND2*, and the nuclear gene *28S*, for 19 taxa of the West African killifish tribe Callopanchacini and 11 out-group taxa, highly supported the monophyly of the tribe, and made it possible to provide the first unambiguous diagnoses for the included genera (*Archiaphyosemion*, *Callopanchax*, *Nimbapanchax*, and *Scriptaphyosemion*). The monophyly of the Callopanchacini is supported by six morphological synapomorphies: posterior portion of the mandibular channel consisting of a single open groove; basihyal pentagonal, as a result of a nearly rectangular basihyal cartilage and a triangular bony support; dorsal process of the urohyal usually absent, sometimes rudimentary; presence of a wide bony flap adjacent to the proximal portion of the fourth ceratobranchial; a broad bony flap adjacent to the proximal portion of the fifth ceratobranchial; and haemal prezygapophysis of the pre-ural vertebra 2 ventrally directed. The analysis indicates that the medially continuous rostral neuromast channel, commonly used to diagnose the tribe, is plesiomorphic. This study also indicates that, among African aplocheiloids, the annual life cycle style developed once in *Callopanchax*, and then again independently in the clade containing *Fundulopanchax* and *Nothobranchius*.

INTRODUCTION

The tribe Callopanchacini is a killifish group endemic to West Africa, between Senegal and Ghana, in an area nearly coincident with the West African Forest, which is among the most significant biodiversity hot spots in the world (Myers *et al.*, 2000). Presently, the Callopanchacini comprises four genera: *Archiaphyosemion* Radda, 1977, *Callopanchax* Myers, 1933, *Nimbapanchax* Sonnenberg & Busch, 2009, and *Scriptaphyosemion* Radda & Pürzl, 1987 (Murphy, Nguyen & Collier, 1999; Sonnenberg & Busch, 2009). Callopanchacines are usually found in shallow streams and marginal areas of perennial swamp pools, except for species of *Callopanchax* that are often found in seasonal pools (e.g. Scheel, 1990). The taxonomy of callopanchacines has been problematic, however: often established by superficial descriptions, and with a great volume of taxonomical data about genera and species being restricted to publications in the aquarium fish literature. In addition, specimens are often rare and poorly preserved when deposited in scientific collections. The number of valid species varies from about 18 to 23, depending on the author (e.g. Sonnenberg & Busch, 2009, 2010, 2012), and genera are still poorly diagnosed by morphological characters, making a thorough assessment of the Callopanchacini necessary.

The history of the systematics of the Callopanchacini begins with the description of *Haplochilus petersi*

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Sauvage, 1882, followed by the description of *Haplochilus liberiensis* Boulenger, 1908 36 years later. Generic allocation in *Haplochilus*, a name derived from an unjustified emendment of *Aplocheilus* McClelland, 1839 by Agassiz (1846), resulted from the weak and inconsistent generic diagnoses for cyprinodontiform fishes available at that time (e.g. Garman, 1895), with genera encompassing unrelated species from different and distant parts of the world. This poor generic classificatory scheme was changed by a series of studies developed by George Myers during the first half of the 20th century. Firstly, Myers (1924) described *Aphyosemion* Myers, 1924, which included the present callopanchacines and many other nothobranchiids from Central–West Africa. Thus, between 1924 and 1966, all species belonging to the present Callopanchacini were placed in *Aphyosemion* (e.g. Poll, 1941; Daget, 1954; Lambert, 1958).

Subsequently, Myers (1933) described *Callopanchax* Myers, 1933 as a subgenus of *Aphyosemion*, distinguished from all other subgenera by the presence of a dorsal fin with 17–19 rays, anteriorly placed on the body. The only taxon included in *Callopanchax* was a species from Sierra Leone then misidentified as *Aphyosemion sjoestetdi*, keeping the remaining known callopanchacines in the subgenus *Aphyosemion*. That misidentification was corrected only 33 years later, when Clausen (1966) compared the type species of *Callopanchax*, then a popular aquarium fish, with the type of *Fundulus sjoestedti* Lönnberg, 1895 from Cameroon. Clausen (1966) concluded that the type of *Callopanchax* was an unnamed species, describing it as *Aphyosemion occidentale* Clausen, 1966. Curiously, in the same paper, Clausen (1966) described a new genus, *Roloffia* Clausen, 1966, with *A. occidentale* as the type species. *Roloffia* included all species of the present-day tribe Callopanchacini. This generic classification was followed by all authors that described new callopanchacines between 1971 and 1982 (e.g. Romand, 1981), although this generic name had already been considered invalid by the International Commission of Zoological Nomenclature (ICZN, 1974; for a complete history about this ICZN decision, see Scheel, 1990). Between 1982 and 1995, however, callopanchacine taxa were again placed in *Aphyosemion* (Romand, 1982; Romand & Ozouf-Costaz, 1995).

Radda (1977) did not recognize callopanchacines as members of a single genus or subgenus. He proposed a new subgeneric classification for *Aphyosemion*, following Myers (1933) in restricting the subgenus *Callopanchax* to *A. occidentale*, but proposing a new subgenus, *Archiaphyosemion* Radda, 1977, for all other callopanchacine species. *Aphyosemion guineense* Daget, 1954 was designated as the type species of *Archiaphyosemion*. In a published phylogenetic tree (Radda, 1977: 215), the subgenera *Callopanchax* and

Archiaphyosemion appeared close to the tree base, but not as closely related terminals.

Subsequently, Parenti (1981) also rejected the monophyly of the callopanchacines, considering *Callopanchax* as a subgenus of *Fundulopanchax*, and *Archiaphyosemion* as a subgenus of *Aphyosemion*. In Parenti's (1981: 390) phylogenetic tree, *Fundulopanchax* is the sister group of the genus *Nothobranchius* Peters, 1868, whereas *Aphyosemion* is the sister group of the clade comprising *Fundulopanchax* and *Nothobranchius*.

Parenti's (1981) generic classification of African aplocheiloids was not followed by most subsequent workers (e.g. Wildekamp, Romand & Scheel, 1986; Amiet, 1987; Radda & Pürzl, 1987; Scheel, 1990; Romand, 1992; Romand & Ozouf-Costaz, 1995); however, criticisms were subjective and lacked evidence to support a contrary classification. An exception was Amiet (1987), who found inconsistencies in all Parenti's characters distinguishing *Aphyosemion* and *Fundulopanchax*, proposing that *Fundulopanchax* was in fact a subgenus of *Aphyosemion*.

Radda & Pürzl (1987) kept *Archiaphyosemion*, *Callopanchax*, and *Fundulopanchax* as subgenera of *Aphyosemion*, and created a new subgenus, *Scriptaphyosemion*, for some species previously placed by Radda (1977) in *Archiaphyosemion*. Species then placed in *Scriptaphyosemion* share a combination of features, including red colour pattern with red or yellow margins on unpaired fins, small size (below 40 mm SL), and haploid chromosome numbers of 19–21. *Aphyosemion geryi* Lambert, 1958 was designated as the type species of *Scriptaphyosemion*, a subgenus then considered to be more closely related to the *Aphyosemion calliurum* species group of the subgenus *Mesoaphyosemion* Radda, 1977.

In contrast to Parenti (1981) and Radda & Pürzl (1987), Romand (1986) and Romand & Ozouf-Costaz (1995) followed Clausen's (1966) hypothesis of callopanchacine monophyly, considering *Callopanchax*, *Archiaphyosemion*, and *Scriptaphyosemion* as closely related subgenera of *Aphyosemion*. The hypothesis of callopanchacine monophyly was subsequently corroborated in the first molecular analysis of aplocheiloid killifishes by Murphy & Collier (1997). On the basis of mitochondrial DNA (mtDNA) sequences for 23 aplocheiloid killifishes, they found consistent support for monophyly of three callopanchacine species, then placed in 'Roloffia': 'Roloffia' occidentale, the type species of *Callopanchax*, 'Roloffia' geryi, the type species of *Scriptaphyosemion*, and 'Roloffia' maeseni. More unexpectedly, the callopanchacine clade was hypothesised to be more closely related to *Epiplatys* Gill, 1862 than to *Aphyosemion* and *Fundulopanchax*, justifying a provisional inclusion of callopanchacines in 'Roloffia'. This phylogenetic hypothesis was again supported in a new mtDNA phylogeny of African

aplocheiloids by Murphy *et al.* (1999), now including 18 nominal and one undescribed callopanchacine species, but with out-groups limited to three species of *Epiplatys*. The monophyly of the subgenera *Callopanchax* and *Scriptaphyosemion* were highly supported, whereas the monophyly of *Archiaphyosemion* was supported by the maximum-likelihood (ML) and neighbour-joining (NJ) analyses, but not by the maximum-parsimony (MP) analysis, which indicated that *A. guineense* was more closely related to species of *Callopanchax* and *Scriptaphyosemion* than to the other species placed in *Archiaphyosemion* by Radda (1977). The three subgenera were then proposed as full genera, but no morphological character was provided to diagnose them. Based on this study, Huber (2000) formally named the tribe Callopanchacini to include those three genera.

In a phylogenetic context, Costa (2009a) diagnosed *Callopanchax* based on apomorphic osteological characters, and found evidence indicating paraphyly of *Archiaphyosemion*, as reported by Murphy *et al.* (1999). Sonnenberg & Busch (2009) re-analysed callopanchacine relationships on the basis of a combination of mitochondrial and nuclear DNA sequences for eight callopanchacines and a single out-group. They found the same results reported by Murphy *et al.* (1999), including *Archiaphyosemion* being paraphyletic. As a consequence, *Archiaphyosemion* was restricted to its type species, *A. guineense*, and a new genus, *Nimbapanchax* Sonnenberg & Busch, 2009, was created to place the remaining species. More recently, intrageneric molecular phylogenies were provided for the genera *Callopanchax* (Sonnenberg & Busch, 2010) and *Scriptaphyosemion* (Sonnenberg & Busch, 2012).

The above historical overview shows that although substantial recent efforts have been made to establish well-supported hypotheses of relationships among callopanchacines (Murphy *et al.*, 1999; Sonnenberg & Busch, 2009, 2010, 2012), callopanchacine genera are still poorly diagnosed by morphological characters and their skeletal structures have been documented in just a few studies (Parenti, 1981; Costa, 2009a). The objectives of this study are: to conduct a comparative analysis of the laterosensory system and skeletal structures of callopanchacines, searching for new informative characters; to perform a first phylogenetic analysis integrating morphological characters with mitochondrial and nuclear gene sequences for a broad sample of callopanchacines and out-groups taken from different previous studies; and to provide more consistent generic diagnoses for callopanchacine genera.

MATERIAL AND METHODS

COMPARATIVE MORPHOLOGY

Morphological studies were developed in specimens deposited in the ichthyological collection of the Institute of Biology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (UFRJ), mostly obtained from donation or exchange with European museums in the past. A list of material analysed appears in Appendix S1. For the focal taxon, the tribe Callopanchacini, the comparative analysis included 11 species available for morphological preparations in the UFRJ collection, which represent all of the tribe lineages as delimitated in previous phylogenetic studies (Sonnenberg & Busch, 2009, 2010, 2012). Out-group selection was directed to taxa representing different lineages of the three aplocheiloid families, according to most recent studies, and at the same time having DNA sequences available in GenBank compatible with those available for callopanchacines (see below). As out-groups, the comparative analysis comprised seven species representing the main lineages of the Nothobranchiidae (Parenti, 1981; Murphy & Collier, 1999; Murphy *et al.*, 1999), including species of the genus *Epiplatys*, the putative sister group of the Callopanchacini (Murphy & Collier, 1997), and the genera *Aphyosemion*, *Fundulopanchax*, and *Nothobranchius*, which together have been considered to be the sister group of the clade comprising *Epiplatys* plus the Callopanchacini (Murphy & Collier, 1997; Murphy *et al.*, 1999). As the primary objective of this paper is to check informative morphological variability within the Callopanchacini, an extensive phylogenetic analysis of the Nothobranchiidae involving all of the still-controversial numerous subgenera of *Aphyosemion*, *Epiplatys*, and *Fundulopanchax* (*sensu* Murphy & Collier, 1999; Collier, Murphy & Spinoza, 2007) is beyond the scope of this study. Out-groups also included representatives of the two other aplocheiloid families, the Rivulidae and Aplocheilidae (Parenti, 1981; Murphy & Collier, 1997; Costa, 2004). After the phylogenetic analyses, morphological comparisons were performed for the other 38 nothobranchiid species listed in Costa (2012) to check for variability in the morphological characters described herein. The character analysis focused on the cephalic laterosensory system of specimens preserved in 70% ethanol and osteological features of specimens after preparation, following Taylor & Van Dyke (1985). In the character analysis below, the numbers within brackets match the list of characters in Appendix S2. The terminology for osteological structures followed Costa (2006), for frontal squamation, Hoedeman (1958), and for the cephalic neuromast series, Costa (2001).

PHYLOGENETIC ANALYSIS

The list of morphological characters, following Sereno (2007) for character statement formulation, is given in Appendix S2. The distribution of their character states were checked among the 21 terminal taxa indicated

in 'Comparative morphology' above; the data matrix showing the distribution of character states among terminal taxa appears in Table S1. Morphological characters were analysed using MP methods performed with TNT 1.1 (Goloboff, Farris & Nixon, 2008). The phylogenetic analysis was primarily rooted in the aplocheilid taxon *Aplocheilus panchax* (Hamilton-Buchanan, 1822), as the Aplocheilidae have been considered to be the sister group of the clade comprising all other aplocheiloids (Murphy & Collier, 1997; Costa, 2004). Alternatively, the trees were rooted in the rivulid taxa, following a more traditional view of aplocheiloid relationships (Parenti, 1981; Costa, 1998a), but results concerning callopanchacine relationships were always the same, and thus are not discussed further here. The search for most-parsimonious trees was conducted using the 'traditional' method: setting random taxon-addition replicates to ten tree bisection and reconnection branch swapping multi-trees, in effect collapsing branches of zero length, with characters equally weighted, and with a maximum of 1000 trees saved in each replicate. Character states were treated as unordered. Autapomorphies were included in order to support the diagnoses of monotypic genera. Branch support was assessed by bootstrap analysis, using a heuristic search with 1000 replicates and with the same settings used in the MP search.

Morphological characters were also combined with a molecular database, of 3296 bp, comprising segments of the mitochondrial gene encoding ribosomal RNA *16S* (508 bp) and NADH dehydrogenase subunit II (*ND2*; 1556 bp), and nuclear ribosomal RNA *28S* (1169 bp), using the same phylogenetic methods described above, with all morphological characters treated as unordered and genes analysed giving equal weight to all sites. The combined data set, with partitioned data, was further submitted to a Bayesian approach, using MR BAYES 3.2 (Ronquist *et al.*, 2012). Models of nucleotide substitution for each molecular data set were chosen using JMODELTEST 2.1.6 (Darriba *et al.*, 2012): GTR + I + G for *16S*, TrN + I + G for *ND2*, and GTR + I + G for *28S*. For the morphological data partition, the model JC69 + G was used, following Lewis (2001). The number of generations was set to 1 000 000, with a sampling of every 1000 generations. Other parameters were set by default. After the first 25% of the sampled trees were discarded as burn-in, the standard deviation of split frequencies was below 0.01.

Besides comprising all taxa used in the morphological analysis, the molecular database included an additional nine callopanchacines and one nothobranchiid out-group; a complete list of terminal taxa appears in Table S2. Sequences were obtained from GenBank, first published in Murphy & Collier (1997), Hrbek & Larson (1999), Vermeulen & Hrbek (2005), Sonnenberg, Nolte & Tautz (2007), Sonnenberg & Busch (2009, 2010, 2012),

Wildekamp, Shidlovskiy & Watterns (2009), and Shidlovskiy, Watters & Wildekamp (2010), except for *16S* and *28S* sequences of *Kryptolebias brasiliensis* (Valenciennes, 1821), which were obtained using the following procedures. Specimens (UFRJ 8807, 5; Brazil, Rio de Janeiro, Caxias) were fixed in absolute alcohol just after collection and later preserved in the same solution. Total genomic DNA was extracted from muscle tissue of the right side of the caudal peduncle using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions. DNA fragments of *K. brasiliensis* were amplified using the primers 16sar-L (5′-CGCCTGTTTAYCAAAAACAT-3′) and 16sbr-H (5′-CCGGTCTGAACTCAGATCACGT-3′), specifically for the gene *16S* (Palumbi *et al.*, 2002), and LSU D1,D2 fw1 (5′-AGCGGAGGAAAAGAAACTA-3′) and LSU D1, D2 rev1 (5′-TACTAGAAGGTTCGATTAGTC-3′) (Sonnenberg *et al.*, 2007), specifically for the fragment of the D1–D2 region of *28S*. A polymerase chain reaction (PCR) was performed in 30-µL reaction mixtures containing 5× Green GoTaq Reaction Buffer (Promega), 3.3 mM MgCl$_2$, 1 mM of each primer, 75 ng of total genomic DNA, 0.21 mM of each dNTP and 1 U of Taq polymerase, with the following thermocycling profile: (1) one cycle of 4 min at 94 °C; (2) 35 cycles of 1 min at 92 °C, 1 min at 56 °C, and 1 min at 72 °C; and (3) one cycle of 4 min at 72 °C. In all PCR reactions, negative controls without DNA were used to check for contamination. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 10-µL reaction volumes containing 1 µL BigDye 2.5, 1.55 µL 5× sequencing buffer (Applied Biosystems), 2 µL of the amplified products (10–40 ng), and 2 µL primer, using the following thermocycling profile: 35 cycles of 10 s at 96 °C, 5 s at 54 °C, and 4 min at 60 °C. The sequencing reactions were purified and denatured, and the samples were run on an ABI 3130 Genetic Analyzer. Sequences were edited using MEGA 5 (Tamura *et al.*, 2011), aligned using CLUSTALW (Chenna *et al.*, 2003), and subsequently optimized manually. A list of GenBank accession numbers is given in Table S2. In order of to assess the impact of morphological characters, molecular data were also analysed separately. Unambiguous diagnostic characters for callopanchacine genera were obtained by character state optimization of the combined analysis tree using TNT 1.1.

RESULTS

The comparative study generated 63 morphological characters (for the list of characters, see Appendix S2; for character analysis, see Appendix S3; and for the

distribution of character states among terminal taxa, see Table S1), of which 24 were new and 39 had already been described in previous studies (Parenti, 1981; Costa, 1990, 1998a, 2005, 2009a, b, 2011, 2012). The MP analysis of morphological characters alone generated six equally most-parsimonious trees, with the resulting strict consensus tree depicted in Figure 6.

The callopanchacine clade and all of the callopanchacine genera and suprageneric clades proposed in previous phylogenetic studies (Murphy *et al.*, 1999; Sonnenberg & Busch, 2009; Costa, 2009a) were corroborated, although branch support is relatively weak for *Nimbapanchax* and the clade comprising all of the callopanchacines except *Nimbapanchax* (bootstrap values 66 and 58%, respectively). Reanalysis of DNA sequences combining molecular data taken from different studies (e.g. Sonnenberg & Busch, 2009, 2010, 2012) provided 24 equally most-parsimonious trees, for which the strict consensus tree also supported those same clades (Fig. 7A). A similar result was obtained when these morphological and molecular data were combined into a single MP analysis, in which the strict consensus tree for the two equally most-parsimonious trees found showed bootstrap values higher than in the other analyses (Fig. 7B). A similar tree (not depicted) was also generated using a Bayesian approach. Some clades supported by low bootstrap values in the MP analysis (< 75%), including the *Fundulopanchax* and *Aphyosemion* clades, the clade comprising *Archiaphyosemion*, *Callopanchax*, and *Scriptaphyosemion*, and the clade comprising all species of *Scriptaphyosemion* except *Scriptaphyosemion schmitti* (Romand, 1979) and *Scriptaphyosemion cauveti* (Romand & Ozouf-Costaz, 1995), had high posterior probabilities (> 0.98) in the Bayesian analysis (Fig. 7). In addition, a clade comprising *Scriptaphyosemion wieseae* Sonnenberg & Busch, 2012, *Scriptaphyosemion bertholdi* (Roloff, 1965), *Scriptaphyosemion guignardi* (Romand, 1981), and *Scriptaphyosemion chaytori* (Roloff, 1971), not recovered in the MP analysis (Fig. 7), received high support (posterior probability 0.95) in the Bayesian analysis. The highly supported tree combining morphological characters and nuclear and mitochondrial DNA sequences provided the basis for the generic diagnoses proposed below.

TAXONOMIC ACCOUNTS
TRIBE CALLOPANCHACINI HUBER, 2000

Diagnosis
Members of the tribe Callopanchacini differ from other nothobranchiids by six synapomorphies: posterior portion of the mandibular channel consisting of a single open groove (6.1; Fig. 1C), versus two separated grooves; basihyal pentagonal as a result of a nearly rectangular basihyal cartilage and a triangular bony support (27.1; Fig. 3A,B), versus subtriangular; dorsal process of the urohyal usually absent, sometimes rudimentary (29.1; Fig. 3C,D), versus always well developed; presence of a wide bony flap adjacent to the proximal portion of the fourth ceratobranchial (37.1; Fig. 3A,B), versus flap short or absent; a broad bony flap adjacent to the proximal portion of the fifth ceratobranchial (39.1; Fig. 3A,B), versus flap short; haemal prezygapophysis of the pre-ural vertebra 2 ventrally directed (57.1; Fig. 5A–G), versus anteroventrally directed.

Included genera
Archiaphyosemion Radda, 1977, *Callopanchax* Myers, 1933, *Nimbapanchax* Sonnenberg & Busch, 2009, and *Scriptaphyosemion* Radda & Pürzl, 1987.

NIMBAPANCHAX SONNENBERG & BUSCH, 2009
Nimbapanchax Sonnenberg & Busch, 2009: 8–9 (type species: *Nimbapanchax leucopterygius* Sonnenberg & Busch, 2009; type by original designation).

Diagnosis
Distinguished from other callopanchacines by one synapomorphy: second vertebra with a fan-shaped neural process (49.1; Fig. 5A), versus rectangular (Fig. 5B).

Included taxa
Five species, according to Sonnenberg & Busch, 2009: *Nimbapanchax jeanpoli* (Berkenkamp & Etzel, 1979); *Nimbapanchax leucopterygius* Sonnenberg & Busch, 2009; *Nimbapanchax melanopterygius* Sonnenberg & Busch, 2009; *Nimbapanchax petersi* (Sauvage, 1882); and *Nimbapanchax viride* (Ladiges & Roloff, 1973).

CLADE *ARCHIAPHYOSEMION* + *CALLOPANCHAX* + *SCRIPTAPHYOSEMION*

Diagnosis
Species of this group are distinguished from other members of its tribe by having an abrupt widening preceded by a narrow constriction of the posterior portion of the parasphenoid (45.1; Fig. 4B), versus a gradual widening posteriorly (Fig. 4A).

ARCHIAPHYOSEMION RADDA, 1977
Archiaphyosemion Radda, 1977: 214 (proposed as a subgenus of *Aphyosemion* Myers, 1924; type species *Aphyosemion guineense* Daget, 1954; type by original designation).

Diagnosis
Distinguished from all other genera of the Callopanchacini by nine autapomorphies: fontal squamation E-patterned (11.1), versus G-patterned; dorsal
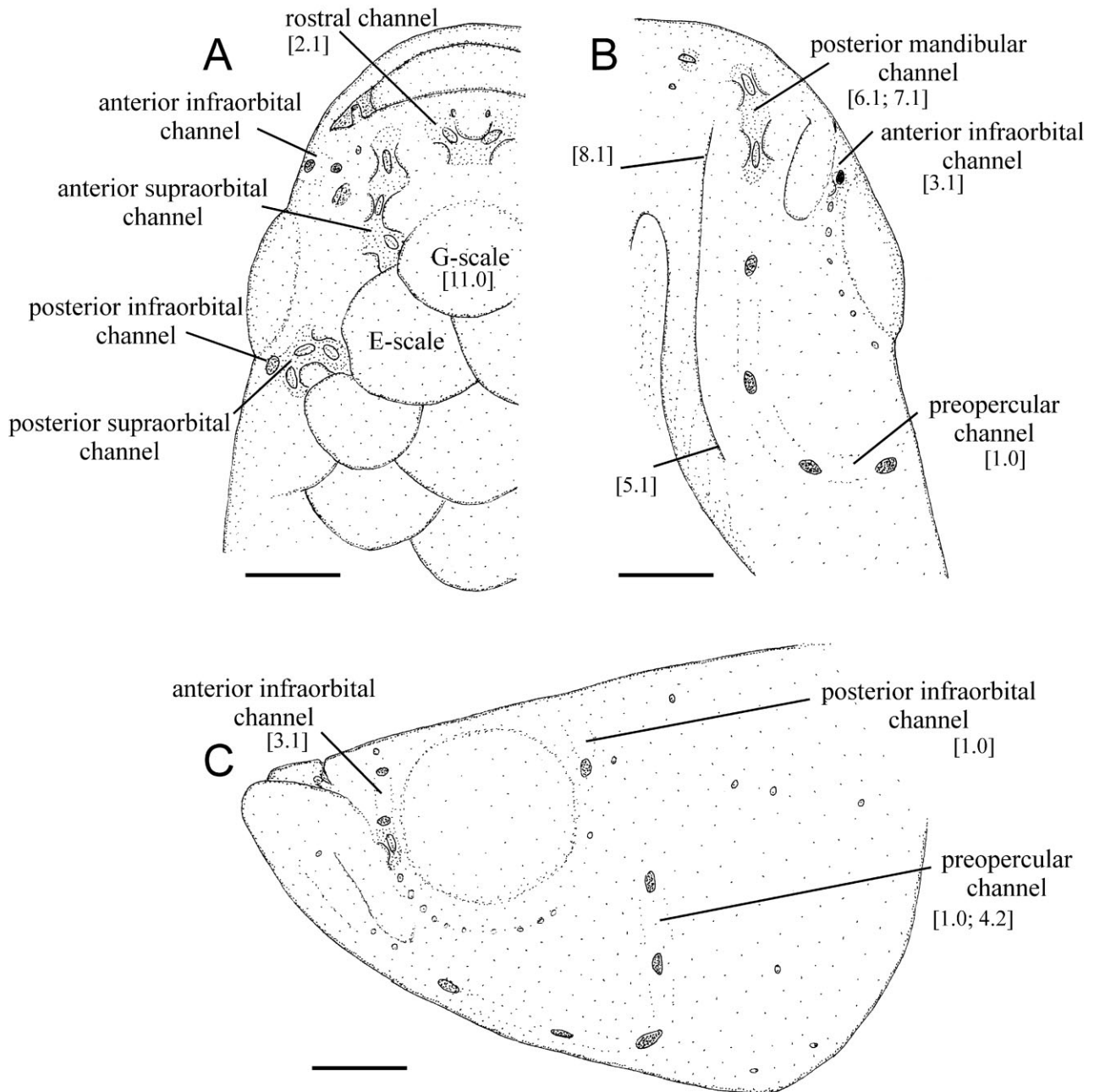
Figure 1. Cephalic laterosensory system of *Nimbapanchax leucopterygius*: A, left side, dorsal view; B, left side, ventral view; and C, left lateral view. Numbers in brackets are character states numbered according to Appendix S2. Scale bars: 1 mm.

process of the maxilla broad (18.1; Fig. 2A), versus narrow (Fig. 2B); dorsal process of the autopalatine prominent (22.1; Fig. 2A), versus minute (Fig. 2B); anterior dorsal condyle of the hyomandibula distinctively longer than posterior dorsal condyle (24.1; Fig. 2A), versus approximately equal (Fig. 2B); cartilaginous portion of the basihyal about equal in length to osseous portion length (26.1; Fig. 3A), versus longer (Fig. 3B); bifid proximal portion of the first epibranchial (30.1;

Fig. 3A), versus not bifid (Fig. 3B); second and third epibranchials longer than half the length of adjacent ceratobranchials (31.1; Fig. 3A), versus shorter (Fig. 3B); bony flap adjacent to the articular head of the fourth ceratobranchial placed in a subproximal position (38.1; Fig. 3A), versus proximal (Fig. 3B); and a short fifth ceratobranchial (40.1), with a row of broad teeth on the medial portion of the bone (Fig. 3A), versus fifth ceratobranchial long, with narrow, irregularly
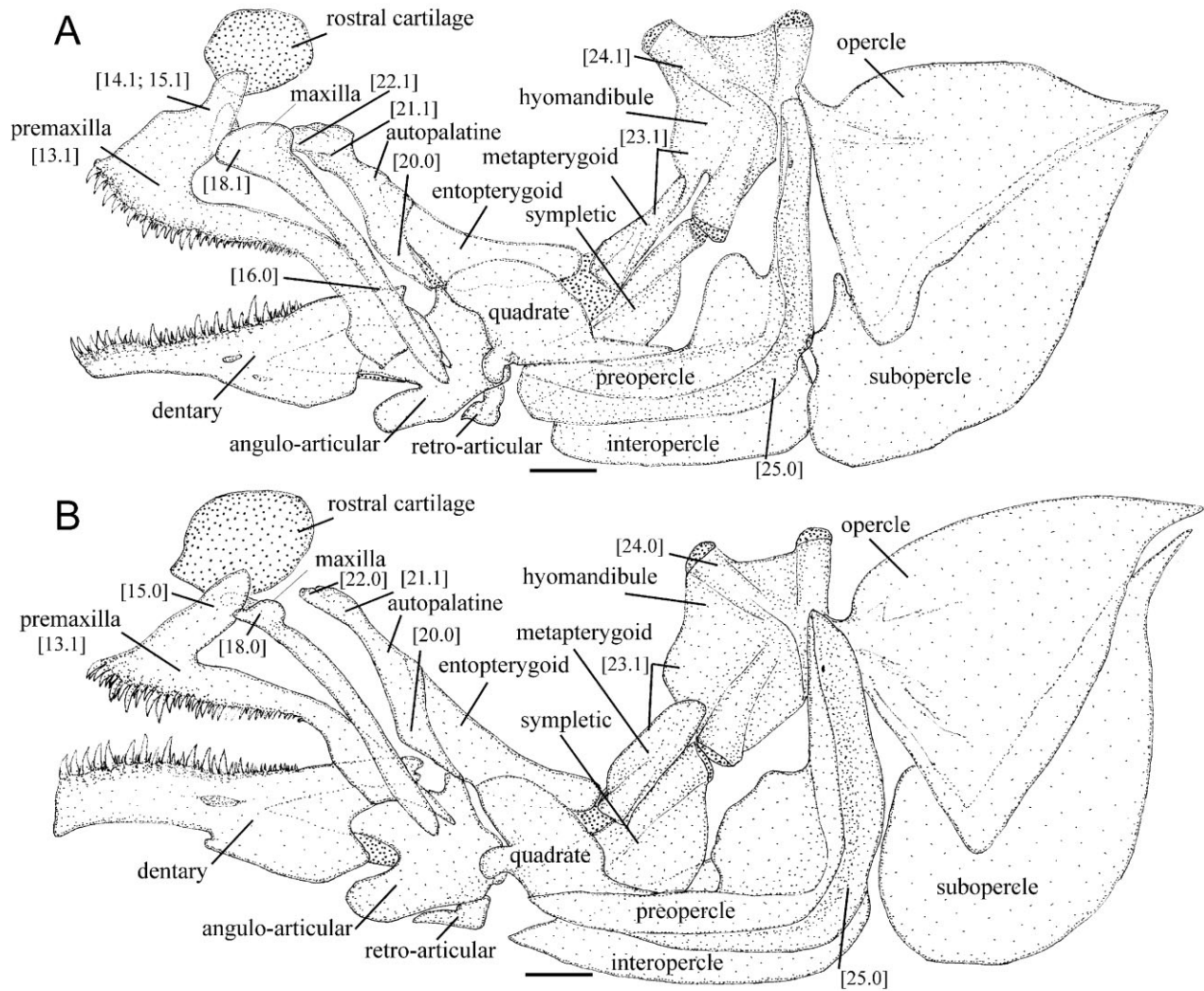
Figure 2. Jaws, jaw suspensorium, and opercular series, left lateral view, of *Archiaphyosemion guineense* (A), and *Callopanchax monroviae* (B). Numbers in brackets are character states numbered according to Appendix S2. Larger stippling indicates cartilage. Scale bars = 1 mm.

arranged teeth (Fig. 3B). *Archiaphyosemion* is similar to *Callopanchax* as species of both genera have long interarcual cartilage, longer than the first epibranchial (33.1; Fig. 3A), versus shorter than the first epibranchial.

Included taxa
A single species: *A. guineense* (Daget, 1954).

CLADE *CALLOPANCHAX* + *SCRIPTAPHYOSEMION*

Diagnosis
Species of the genera *Callopanchax* and *Scriptaphyosemion* share four osteological synapomorphies, which distinguish them from other callopanchacines: urohyal drop-shaped, becoming strongly deep poste-

riorly (28.1; Fig. 3D), versus slender (Fig. 3C); second pharyngobranchial teeth absent (34.1; Fig. 3B), versus present (Fig. 3A); parietal short and elliptical (41.1; Fig. 4D), versus long and rectangular (Fig. 4C); first vertebra with an anteromedian laminar process on the neural process (48.1; Fig. 5B), versus laminar process absent (Fig. 5A).

GENUS *CALLOPANCHAX* MYERS, 1933

Callopanchax Myers, 1933: 184 [proposed as a subgenus of *Aphyosemion* Myers, 1924; type species *Aphyosemion sjoestedti* non *A. sjoestedti* (Lönnberg, 1895) (misidentification of *Aphyosemion occidentale* Clausen, 1966); type by original designation].
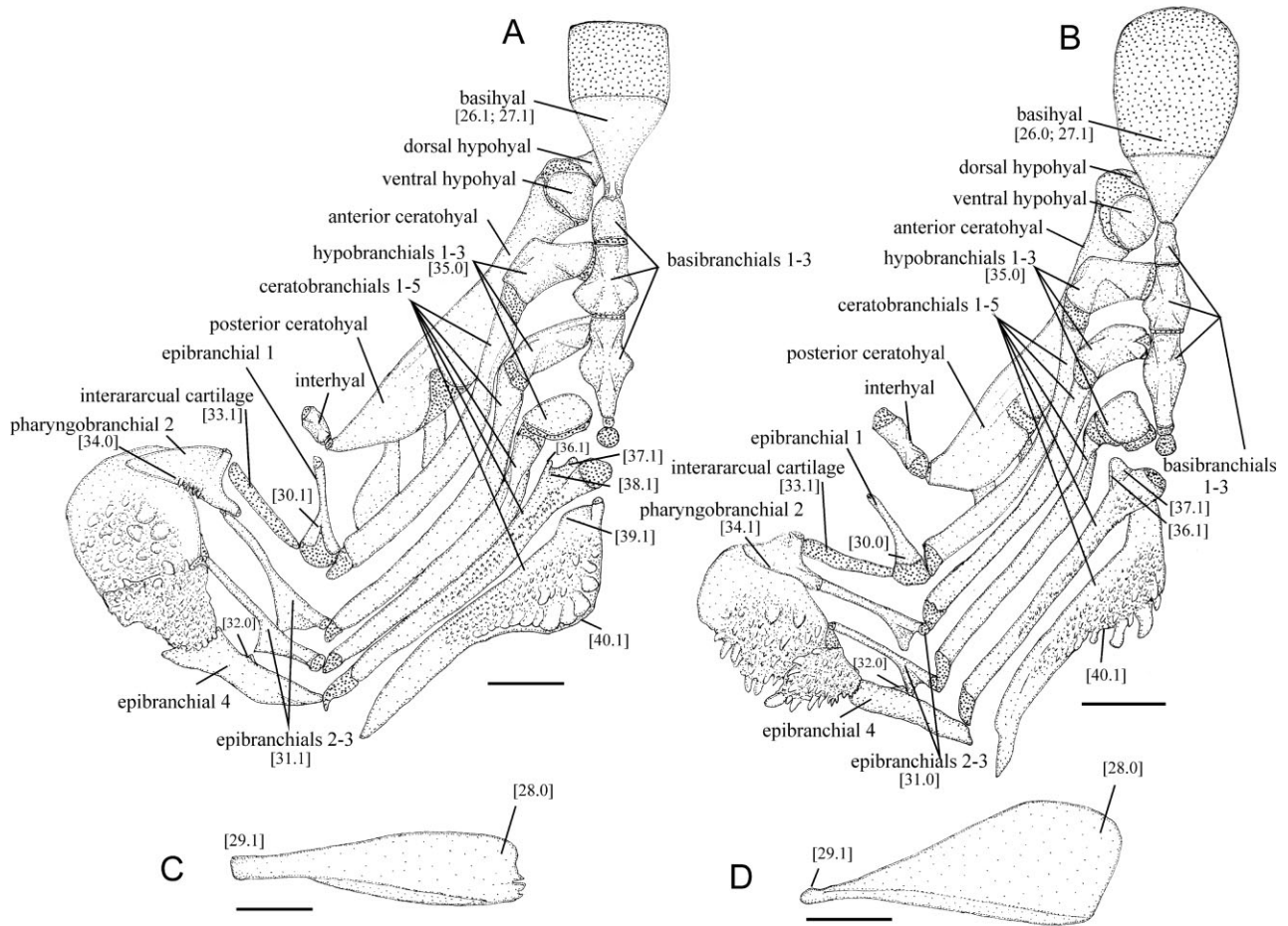
Figure 3. Hyoid and branchial arches: ventral portion in dorsal view and dorsal portion in ventral view, of *Archiaphyosemion guineense* (A) and *Callopanchax monroviae* (B); urohyal, left lateral view, of *A. guineense* (C), and *C. monroviae* (D). Numbers in brackets are character states numbered according to Appendix S2. Larger stippling indicates cartilage. Scale bars: 1 mm.

Roloffia Clausen, 1966: 338 (type species *Aphyosemion occidentale* Clausen, 1966; type by original designation).

Diagnosis
Species of the genus *Callopanchax* differ from species of all other genera of the Callopanchacini by four osteological synapomorphies: dorsal portion of the autopalatine twisted and narrow as a result of a internal torsion (21.1; Fig. 2B), versus not twisted and broad (Fig. 2A); anteroventral margin of the hyomandibula convex as a result of an anterior expansion of the ventral portion of the hyomandibula and adjacent metapterygoid (23.1; Fig. 2B), versus hyomandibula and adjacent metapterygoid not expanded (Fig. 2A); neural processes of the first vertebra medially fused (47.1; Fig. 5B), versus not fused (Fig. 5A); neural prezygapophyses of the anterior caudal vertebrae long (52.1; Fig. 5D), versus short (Fig. 5E).

Included taxa
Four valid species, according to Sonnenberg & Busch (2010: *Callopanchax monroviae* (Roloff & Ladiges, 1972); *Callopanchax occidentale* (Clausen, 1966); *Callopanchax sidibei* Sonnenberg & Busch, 2010; and *Callopanchax toddi* (Clausen, 1966).

SCRIPTAPHYOSEMION RADDA & PÜRZL, 1987
Scriptaphyosemion Radda & Pürzl, 1987: 8 (proposed as a subgenus of *Aphyosemion* Myers, 1924; type species *Aphyosemion guineense geryi* Lambert, 1958; type by original designation).

Diagnosis
Species of *Scriptaphyosemion* are distinguished from all other callopanchacines by two synapomorphies: vomerine teeth absent (42.1), versus present; and neural
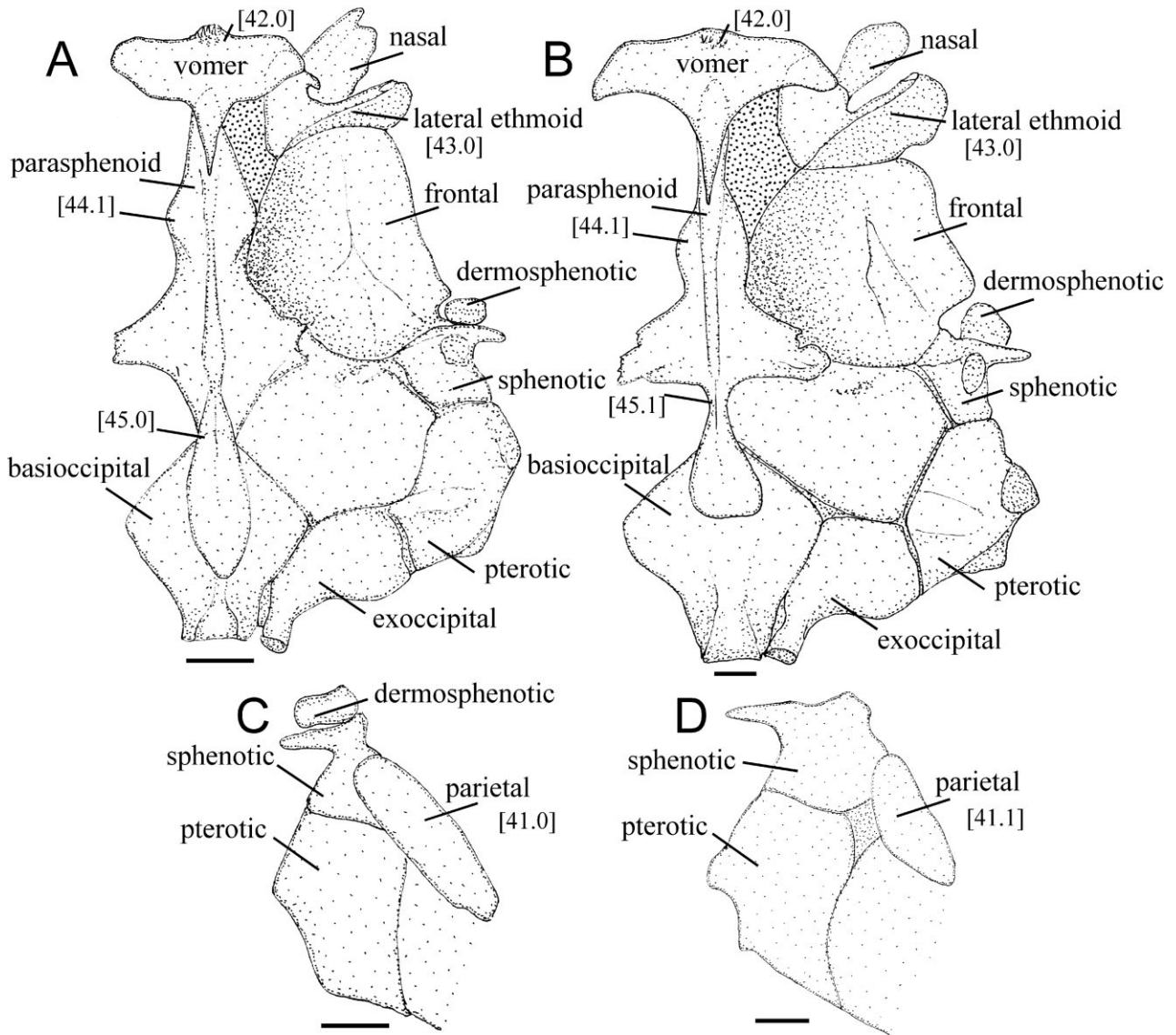
Figure 4. Neurocranium: ventral middle and left portion, ventral view, of *Nimbapanchax leucopterygius* (A) and *Callopanchax monroviae* (B); lateroposterior portion, dorsal view, of *Nimbapanchax leucopterygius* (C) and *Callopanchax monroviae* (D). Larger stippling indicates cartilage. Scale bars: 0.5 mm.

and haemal spines of the pre-ural vertebra 2 distinctively curved (56.1; Fig. 5F), versus approximately straight (Fig. 5G).

Included taxa
Thirteen species, according to Sonnenberg & Busch (2012): *Scriptaphyosemion banforense* (Seegers, 1982); *Scriptaphyosemion bertholdi* (Roloff, 1965); *Scriptaphyosemion brueningi* (Roloff, 1971); *Scriptaphyosemion cauveti* (Romand & Ozouf-Costaz, 1995); *Scriptaphyosemion chaytori* (Roloff, 1971); *Scriptaphyosemion fredrodi* (Vandermissen, Etzel & Berkenkamp, 1980); *Scriptaphyosemion geryi* (Lambert, 1958); *Scriptaphyosemion guignardi* (Romand, 1981); *Scriptaphyosemion liberiense* (Boulenger, 1908); *Scriptaphyosemion nigrifluvi* (Romand, 1982); *Scriptaphyosemion roloffi* (Roloff, 1936); *Scriptaphyosemion schmitti* (Romand, 1979); and *Scriptaphyosemion wieseae* Sonnenberg & Busch, 2012.
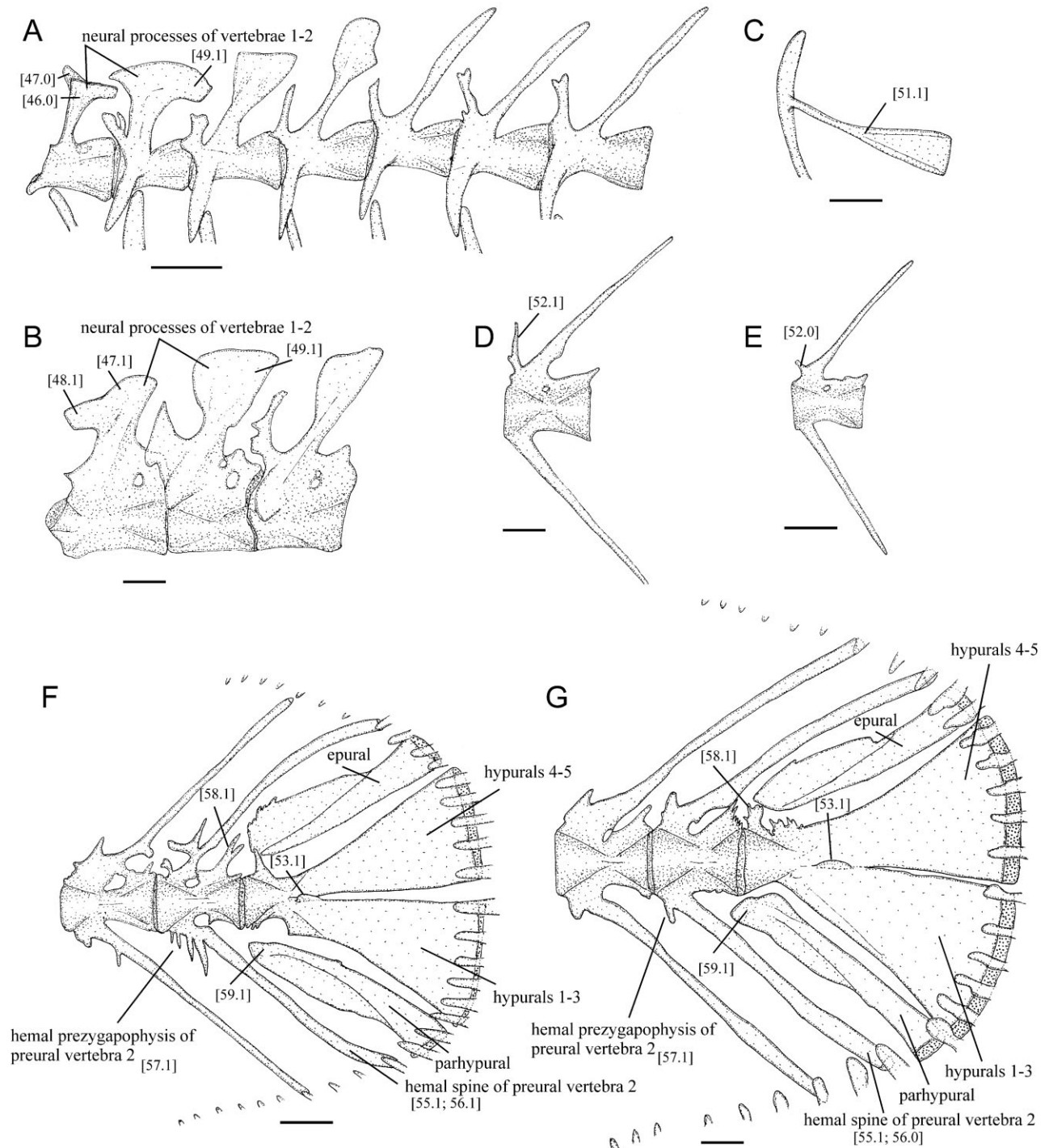
Figure 5. Vertebrae and caudal skeleton (all in left lateral view except A, in laterodorsal view): first vertebrae of *Nimbapanchax leucopterygius* (A) and *Callopanchax monroviae* (B); epipleural rib of second precaudal vertebra of *N. leucopterygius* (C); fourth caudal vertebra of *C. monroviae* (D) and *N. leucopterygius* (E); caudal skeleton of *Scriptaphyosemion guignardi* (F) and *Callopanchax occidentalis* (G). Larger stippling indicates cartilage. Scale bars: 0.5 mm.

DISCUSSION

SIGNIFICANCE OF MORPHOLOGICAL CHARACTERS FOR THE PHYLOGENY OF THE CALLOPANCHACINI

The monophyly of the group first recognized under the name 'Rollofia' (Clausen, 1966), presently constituting the tribe Callopanchacini, is highly supported both by the analysis of morphological characters alone and when morphological and molecular data are integrated into a single analysis (Figs 6, 7). The present analyses also corroborate hypotheses of phylogenetic relationships among callopanchacine genera based only on molecular data (Murphy *et al.*, 1999; Sonnenberg & Busch, 2010), improving the support of callopanchacine clades (Fig. 7). Most characters found to be phylogenetically informative, and thus decisively contributing to explicit diagnoses of the Callopanchacini and included genera, were concentrated in the external morphology of the cephalic laterosensory system and bone morphology of jaw suspensorium, hyoid and branchial arches, neurocranium, vertebrae, and caudal skeleton (see also Appendix S3). This comparative morphological analysis greatly differs from other morphological studies that used only details of the rostral neuromast series (Clausen, 1966) or osteological characters directed to more inclusive aplocheiloid groups (Parenti, 1981).

The comparative analysis of the cephalic laterosensory system provided a new character supporting the monophyly of the Callopanchacini, consisting of the posterior portion of the mandibular channel forming a single open groove (Fig. 1C), instead of two separated grooves, as in other nothobranchiids. Curiously, the only other well-established diagnostic character of the cephalic laterosensory system to delimit callopanchacines used by Clausen (1966) and most subsequent authors, the morphology of the neuromast series of the laterosensory open channel present on the rostral region, cannot be considered as evidence of Callopanchacini monophyly. This series was first named as 'frontal' neuromasts by Clausen (1966), but then subsequently named as rostral neuromast series by Costa (2001), as this channel is situated immediately posterior to the snout, on the rostral region, instead of coinciding in position with the frontal bone, as already noted by Clausen (1967: 20). This channel segment was also equivocally considered as part of the supraorbital channel by Scheel (1968), but as that channel segment does not correspond in position with the supraorbital channel of other teleosts (e.g. Nelson, 1972), they cannot be considered homologous. In callopanchacines, the rostral neuromast channel is medially continuous (trianguloid pattern, according to Clausen, 1966, 1967; closed pattern, according to Scheel, 1968), differing from other
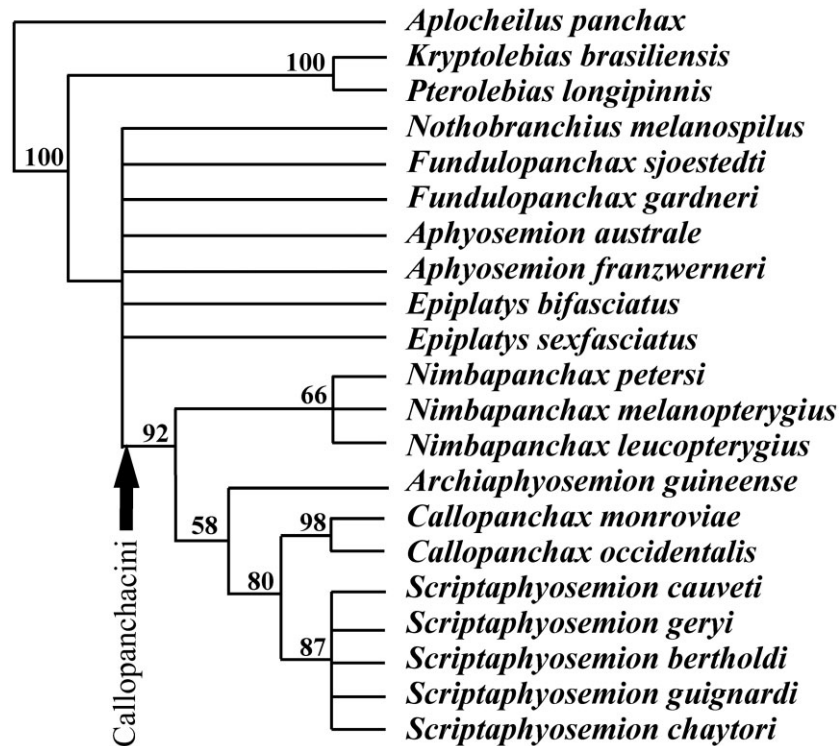
Figure 6. Strict consensus tree of the two most-parsimonious trees from the analysis of 63 morphological characters for 21 aplocheiloid killifish taxa. Numbers above the node are bootstrap percentages higher than 50%.
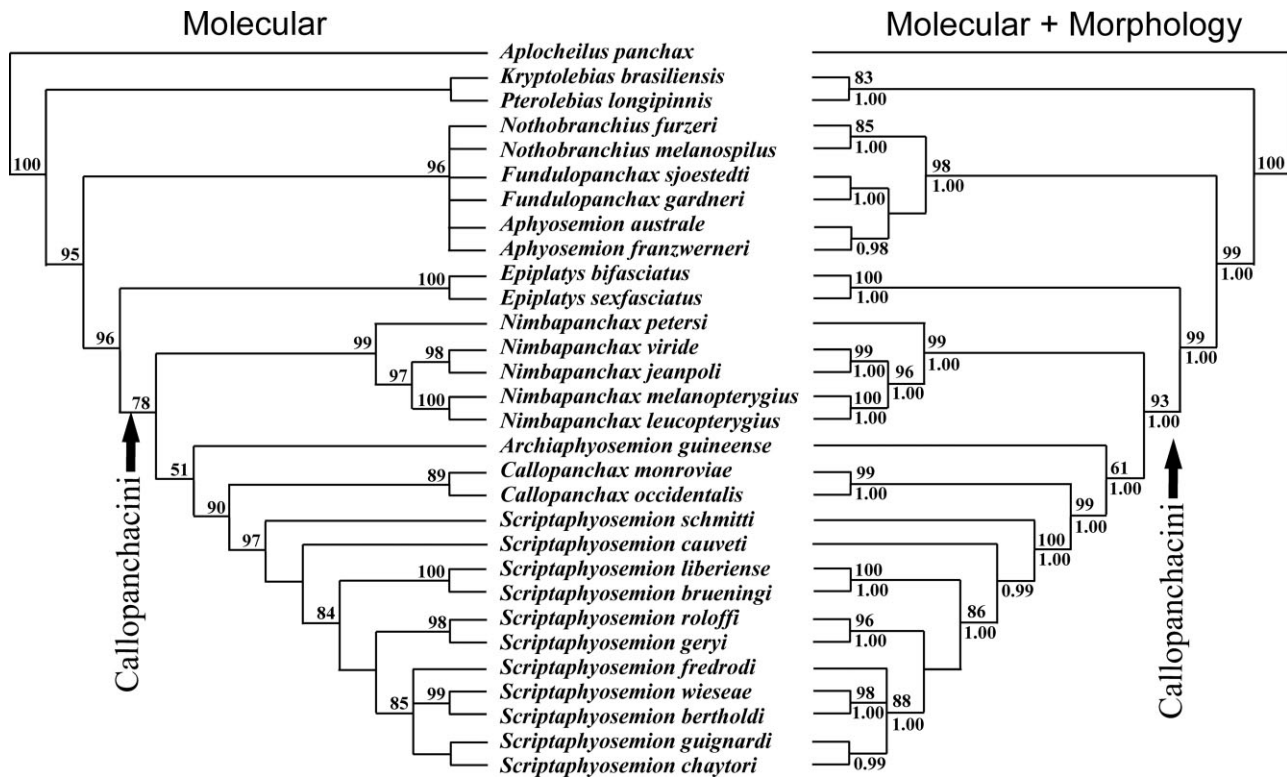
Figure 7. Phylogenetic relationships among 19 taxa of the Callopanchacini and 11 out-group taxa: left, strict consensus tree of the 24 most-parsimonious trees from the analysis of molecular data (3296 bp), comprising segments of the mitochondrial genes *16S* and *ND2*, and the nuclear gene *28S*; right, strict consensus tree of the two most-parsimonious trees from the combined analysis of the same molecular data set and 63 morphological characters. Numbers above the node are bootstrap percentages higher than 50%, below are posterior probabilities of the Bayesian analysis higher than 0.95.

nothobranchiids except for species of *Epiplatys*, in which the channel is medially separated (trapezoid pattern, according to Clausen, 1966, 1967; open pattern, according to Scheel, 1968). As the former condition also occurs in *Aplocheilus* it is considered plesiomorphic for nothobranchiids, and thus does not corroborate the monophyly of callopanchacines, although it is useful in distinguishing them from some other nothobranchiid genera (e.g. *Aphyosemion*, *Fundulopanchax*, and *Nothobranchius*). Clausen (1966) also diagnosed 'Rollofia' by the absence of 'bristle-shaped' filamentous contact organs on body scales, which are present in several species of *Fundulopanchax* and *Nothobranchius*, but are absent in most other nothobranchiids, and are thus not diagnostically informative for callopanchacines.

Particularly interesting is the high information content of osteological features for the study of callopanchacine phylogenetic relationships, providing a total of 21 characters that are useful in unambiguously diagnosing the Callopanchacini and included clades (see the taxonomic accounts above), all first described here or only briefly recorded in a former preliminary analysis (Costa, 2009a). Previous tentative generic morphological diagnoses were mostly restricted to fin-ray counts, rela-

tive position of unpaired fins, and details of the colour pattern (e.g. Myers, 1933; Radda & Pürzl, 1987), but these conditions are highly variable among different lineages of aplocheiloid killifishes, being effectively diagnostic for closely related species only. In addition, the evolution of certain character states, such as dorsal fin with numerous rays, and its origin being anteriorly placed in the body, is often correlated with the acquisition of an annual life cycle, as discussed by Costa (1998a), occurring homoplastically in several aplocheiloid lineages.

More recently, Sonnenberg & Busch (2009) formally diagnosed *Nimbapanchax*, when it was succinctly distinguished from *Archiaphyosemion* by frontal squamation usually G-patterned (versus E-patterned), mean values of overlapped number of scales and vertebrae, adults reaching 60 mm SL (standard length, the distance between snout and caudal-fin base) (versus 65 mm SL), and details of the colour pattern of the caudal fin in males. Excepting frontal squamation E-patterned, confirmed in the present study as being autapomorphic for *Archiaphyosemion*, these diagnostic features do not support the monophyly of any genus nor provide a practical tool for recognizing members of each genus. In

contrast, the use of bone morphology provided a total of nine unambiguous character states to distinguish *Archiaphyosemion* and *Nimbapanchax*: second vertebra with a fan-shaped neural process (49.1; Fig. 5A) in *Nimbapanchax*, versus rectangular in *Archiaphyosemion*; posterior portion of the parasphenoid with abrupt posterior widening preceded by a narrow constriction in *Archiaphyosemion* (45.1; Fig. 4B), versus gradual posterior widening in *Nimbapanchax*; dorsal process of the autopalatine prominent in *Archiaphyosemion* (22.1; Fig. 2A), versus minute in *Nimbapanchax*; anterior dorsal condyle of the hyomandibula distinctively longer than posterior dorsal condyle in *Archiaphyosemion* (24.1; Fig. 2A), versus approximately equal in *Nimbapanchax*; cartilaginous portion of the basihyal about equal in length to the osseous portion length in *Archiaphyosemion* (26.1; Fig. 3A), versus longer in *Nimbapanchax*; bifid proximal portion of the first epibranchial in *Archiaphyosemion* (30.1; Fig. 3A), versus not bifid in *Nimbapanchax*; second and third epibranchials longer than half the length of adjacent ceratobranchials in *Archiaphyosemion* (31.1; Fig. 3A), versus shorter in *Nimbapanchax*; bony flap adjacent to the articular head of the fourth ceratobranchial placed in a subproximal position in *Archiaphyosemion* (38.1; Fig. 3A), versus proximal in *Nimbapanchax*; a short fifth ceratobranchial (40.1), with a row of broad teeth on the medial portion of the bone in *Archiaphyosemion* (Fig. 3A), versus fifth ceratobranchial long, with narrow, irregularly arranged teeth in *Nimbapanchax*; and a long interarcual cartilage, longer than the first epibranchial in *Archiaphyosemion* (33.1; Fig. 3A), versus shorter in *Nimbapanchax*. These data demonstrate the high performance of osteological features for diagnosing callopanchacines and other nothobranchiids, although these are often omitted in taxonomic and phylogenetic studies.

BIOGEOGRAPHICAL AND ECOLOGICAL CONSIDERATIONS

The Callopanchacini is geographically confined to the forests of the West African region between Senegal and Ghana, a distribution pattern highly contrasting with that of the morphologically similar clade comprising *Aphyosemion* and *Fundulopanchax*, mainly concentrated in the region between Togo and the Congo River mouth (e.g. Murphy & Collier, 1997, 1999; Murphy *et al.*, 1999). This putative disjunctive pattern involving the two clades, although not constituting sister groups (e.g. Murphy & Collier, 1997; the present study), was interpreted by Murphy & Collier (1997) and Murphy *et al.* (1999) as a consequence of the trans-Saharan marine transgressions that separated those African regions between the Late Cretaceous and the Early Palaeogene. Mainly in the Cenomanian (96–91 Mya) and in the Early Eocene (*c.* 53 Mya), the sea level reached a maximum

of over 200 m above the present level, separating West Africa from the remaining continental Africa by an epicontinental sea (Haq, Hardenbol & Vail, 1987; Guiraud *et al.*, 2005; Otero, 2010). More recently, Collier *et al.* (2007) analysed the geographically widespread genus *Epiplatys*, the sister group of the Callopanchacini, according to Murphy & Collier (1997), considering the uplands of West Africa as the centre of origin of the genus, and again claiming a Late Eocene origin for the clade comprising *Epiplatys* and the Callopanchacini. The results of the present study, in which the monophyly of the Callopanchacini and the sister-group relationships between *Epiplatys* and the Callopanchacini are highly supported by the morphological data alone, and by the combined analysis, may be regarded as further evidence of past isolation of the West African area. As fossils records for aplocheiloid killifishes are still unknown, however, direct inferences about the timing of diversification of nothobranchiids cannot yet support accurate estimates. Recently, Dorn *et al.* (2014) performed a time-calibrated phylogeny using calibration points based on the fossil record of non-cyprinodontiform teleosts, estimating a maximum age of 10.7 Myr for the East African genus *Nothobranchius*, which suggests a more recent age for nothobranchiids.

Among callopanchacines, species of the genus *Callopanchax* have been reported to be annual killifishes (e.g. Scheel, 1968; Sonnenberg & Busch, 2010), living in seasonal pools and ditches instead of permanent streams like other representatives of the tribe. This kind of life cycle, in which resistant eggs undergo an embryonic diapause stage during dry seasons (Wourms, 1972), is also known to occur in other aplocheiloid killifishes, including the Central–West and East African genera *Fundulopanchax* and *Nothobranchius* (e.g. Bailey, 1972), and several South American rivulid genera (Costa, 1998b, 2011). Parenti (1981) considered all genera of African annual killifishes to comprise a single evolutionary lineage, but more recent studies have indicated that the annual life cycle style developed once in *Callopanchax* and independently in the remaining nothobranchiids (Murphy *et al.*, 1999; Costa, 2009a). The present study strongly supports this latter view of the independent acquisition of the annual life cycle style in *Callopanchax*, as the non-annual condition occurs in all members of the successive sister-group lineages to *Callopanchax*, comprising all the remaining callopanchacine genera and *Epiplatys*, thus placing *Callopanchax* distantly related to the other annual killifish clade containing *Fundulopanchax* and *Nothobranchius* (Fig. 7).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Data matrix of the distribution of character states of 63 morphological characters among 21 terminal taxa.

Table S2. List of species used in the molecular phylogeny and respective GenBank accession numbers.

Appendix S1. List of material examined for the analysis of morphological characters.

Appendix S2. List of character statements used to reconstruct the phylogenetic hypothesis amongst the Callopanchacini.

Appendix S3. Character analysis.