Comparative morphology, phylogeny and classification of African seasonal killifishes of the tribe Nothobranchiini (Cyprinodontiformes: Aplocheilidae)

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A comparative morphological study of 42 species of the African tribe Nothobranchiini provided 138 characters that, combined with available DNA sequences (3327 bp), highly supported monophyly of the tribe, making it possible to generate the first unambiguous diagnoses for included genera (*Fundulosoma*, *Pronothobranchius* and *Nothobranchius*). The analyses corroborate *Fundulosoma* as the sister group to a clade containing all other Nothobranchiini. This study first reports a broad diversification of jaw and branchial structures in *Nothobranchius*, the most diverse aplocheiloid genus, traditionally divided into subgenera. Six subgenera are recognized (*Adiniops*, **Cynobranchius** **subgen. nov.**, *Nothobranchius*, *Paranothobranchius*, **Plesiobranchius** **subgen. nov.** and *Zononothobranchius*). *Cynobranchius* is erected for a clade of deep-bodied, zooplankton-feeding species, diagnosed by 16 morphological synapomorphies, of which nine are probably related to their specialized feeding habits. The clade containing *Cynobranchius* and the monotypic subgenus *Plesiobranchius* is supported as the sister group to a clade containing all other subgenera of *Nothobranchius*. *Paranothobranchius*, comprising the largest aplocheiloid species, is diagnosed by 17 apomorphic conditions, most of them related to specialized piscivorous habits. However, this study indicates that predator jaw morphology arose independently three times in *Nothobranchius*. *Aphyobranchius*, formerly including specialized surface-dwelling species, is considered to be a synonym of *Adiniops*.

ADDITIONAL KEYWORDS: African savannas – *Fundulosoma* – morphological diversification – *Nothobranchius* – osteology – *Pronothobranchius* – seasonal life cycle.

INTRODUCTION

Killifishes of the aplocheiloid subfamily Nothobranchiinae comprise a diverse clade endemic to Africa, occurring in most tropical and subtropical biomes of the continent (e.g. Costa, 2008). The gorgeous colour patterns and elaborate fin morphology have made them popular aquarium fishes, often being bred by aquarium associations. Members of these associations have also frequently studied their taxonomy, ecology and behaviour, making efforts to explore most parts of their area of occurrence. As a consequence, however, the great majority of publications concerning Nothobranchiinae taxonomy in the last six decades have appeared in aquarium magazines, and representative specimens are still rare in scientific institution collections (Costa, 2015, 2017a, b). In contrast, specimens of several species kept by those aquarium associations under controlled conditions have been consistently used in molecular analyses, making Nothobranchiinae relationships relatively well known (e.g. Murphy & Collier, 1999; Collier *et al.*, 2009; Dorn *et al.*, 2014). However, most Nothobranchiinae genera are poorly diagnosed by morphological characters (Costa, 2015), and their skeletal structures have been reported in only a few studies (e.g. Parenti, 1981; Costa, 2009a, 2015). Among these genera are *Fundulosoma* Ahl, 1924, *Nothobranchius* Peters, 1868, and *Pronothobranchius* Radda, 1969, which together form the tribe Nothobranchiini (e.g. Costa, 2008).

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Fundulosoma and *Pronothobranchius*, usually considered monotypic genera (e.g. Wildekamp, Romand & Scheel, 1986), are endemic to western Africa, whereas the diverse genus *Nothobranchius*, with >70 nominal species, occurs in central and eastern Africa (e.g. Costa, 2008). Remarkably, *Nothobranchius* exhibits a broader range of adaptive radiation than any other African aplocheiloid, comparable only to the South American aplocheiloid genus *Austrolebias* Costa, 1998 (Costa, 2006a, 2009b) and the Andean cyprinodontoid genus *Orestias* Valenciennes, 1839 (Parenti, 1984). For example, *Nothobranchius* includes some small species barely reaching 35 mm total length (i.e. species of the *Nothobranchius korthausae* group; Costa, 2009a) and large predatory species reaching ~180 mm of total length (i.e. *Nothobranchius ocellatus* (Seegers, 1985); Wildekamp & Haas, 1992), as well as bottom-dwelling zooplankton feeders (i.e. species of the *Nothobranchius microlepis* complex; Wildekamp & Haas, 1992) and surface-dwelling species with typical insectivorous dentition (i.e. *Nothobranchius janpapi* Wildekamp, 1977).

All species of the three Nothobranchiini genera are uniquely found in seasonal pools of savannas and seasonally dry forests of sub-Saharan Africa, and their life cycles are associated with the local climate (e.g. Wildekamp, 2004; Costa, 2008). These seasonal pools disappear during dry seasons, but resistant eggs in the diapause embryonic stage survive buried in the bottom substrate until the next wet season, when eggs hatch and a new generation quickly develops (e.g. Wourms, 1972). Local climates and their respective rainy seasons are variable along the huge geographical range of the Nothobranchiini, but usually there are two annual rainy seasons in equatorial areas under the influence of the Intertropical Convergence Zone and a single rainy period in higher latitudes (Goudie, 1996). Consequently, two different generations of nothobranchiine species arise every year in populations situated in equatorial areas, where the geographical range of most species is concentrated (e.g. Costa, 2017a). Similar seasonal life cycles are also exhibited by South American aplocheiloid killifishes, formerly called annual fishes because of the equivocal past assumption of having only a single generation each year (Myers, 1942). Today, after more intensive field studies, they have become known as seasonal killifishes, a term that may be applied to any species exhibiting this specialized life cycle mode (e.g. Costa, 2002).

Seasonal killifishes are kept by aquarium fish associations and laboratories for several generations as dry eggs are easily stored for months. In addition, as a result of being short-lived species that have the shortest lifespan among vertebrates, seasonal killifishes have became important model organisms for gerontological studies (Genade *et al.*, 2005; Lucas-Sánchez *et al.*, 2014). However, Nothobranchiini taxonomy is still unsatisfactory. At present, no formal diagnoses based on

derived character states are available for *Fundulosoma* and *Pronothobranchius*, and *Nothobranchius* and its included subgenera are still subjectively diagnosed (e.g. Wildekamp, 2004). The objectives of the present study are as follows: to conduct the first broad morphological comparative analysis for the Nothobranchiini, mostly based on skeletal structures, searching for phylogenetically informative characters; to perform the first phylogenetic analysis combining morphological characters with available DNA sequences for a large sample of Nothobranchiini taxa representing all the main lineages delimited in previous morphological and molecular studies (e.g. Wildekamp, 2004; Dorn *et al.*, 2014); and to provide consistent generic and subgeneric diagnoses for the Nothobranchiini.

MATERIAL AND METHODS

SPECIMENS

Specimens used in morphological studies are deposited in the ichthyological collections of the Royal Museum for Central Africa (MRAC), Tervuren and the Institute of Biology, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil. Type specimens of most species of the Nothobranchiini were also examined for external morphology in the following institutions: Natural History Museum, formerly British Museum, Natural History (BMNH), London; Muséum National d'Histoire Naturelle (MNHN), Paris; Senckenberg-Museum, Frankfurt (SMF); Zoologisches Forschungsmuseum und Museum Alexander Koenig (ZFMK), Bonn; Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin (ZMB), Berlin; Zoologisches Institut und Zoologisches Museum, Hamburg (ZMH); and Zoologische Staatssammlung, Munich (ZSM). A list of material analysed appears in the Supporting Information (Appendix S1).

COMPARATIVE MORPHOLOGY

The comparative morphological analysis primarily included 39 species of Nothobranchiini available for osteological preparations, representing all the tribe lineages. Outgroups included three taxa representing different lineages of Nothobranchiinae [*Chromaphyosemion bitaeniatum* Ahl, 1924, *Epiplatys annulatus* (Boulenger, 1915) and *Fundulopanchax gardneri* (Boulenger, 1911)] and one species of Aplocheilinae [*Pachypanchax playfairii* (Günther, 1866)]. Taxon selection was directed to taxa having DNA sequences available in GenBank. The character analysis primarily focused on bone morphology, which has been responsible for most informative characters in analyses focusing on other aplocheiloid

groups (Costa, 1998a, 2011a, 2015). Osteological features were examined in specimens prepared following Taylor & Van Dyke (1985). The analysis also included external morphology of specimens preserved in 70% ethanol, mainly focusing on the cephalic laterosensory system, contact organs, fin morphology and squamation. In diagnoses and figures, numbers within square brackets are according to the list of characters in the Supporting Information (Appendix S2). Terminology for osteological structures is according to Costa (2006b) and, for cephalic neuromast series, Costa (2001).

PHYLOGENETIC ANALYSIS

The list of morphological characters, following Sereno (2007) for character statement formulation, appears in Supporting Information Appendix S2, and the data matrix showing the distribution of character states among terminal taxa appears in Supporting Information Table S1. Morphological characters were analysed using maximum parsimony (MP) methods performed with TNT 1.1 (Goloboff, Farris & Nixon, 2008). The phylogenetic analysis was rooted in the aplocheiline *P. playfairii*, which is a member of a clade sister to a clade containing all African aplocheiloids (e.g. Costa, Amorim & Mattos, 2017). The search for most parsimonious trees was conducted using the 'traditional' search algorithm and setting random taxon-addition replicates to ten, tree bisection–reconnection branch swapping, multitrees in effect, collapsing branches of zero length, characters equally weighted, and a maximum of 1000 trees saved in each replicate. Character states were treated as unordered. Autapomorphies were included in order of to support diagnoses of monotypic genera and subgenera. Branch support was assessed by bootstrap analysis, using a heuristic search with 1000 replicates and the same settings used in the MP search.

Morphological characters were also combined with a molecular database, total of 3327 bp, comprising partial sequences of the following nuclear genes: glycin transporter 1 (*GLYT1*), myosin heavy chain 6 (*MYH6*), SH3 and PX domain containing 3 (*SH3PX3*), G-protein coupled receptor 85 (*SREB2*) and zic family member 1 (*ZIC1*). These sequences, obtained from GenBank, were first published by Near *et al.* (2012), Dorn *et al.* (2014), Pollux *et al.* (2014) and Pohl *et al.* (2015). The combined dataset was analysed using MP as described above, with all morphological characters treated as unordered, and genes analysed giving equal weight to all sites. This same combined dataset was also analysed using a Bayesian approach (BA) with the MrBayes 3.2 software (Ronquist *et al.*, 2012); data were partitioned, using models of nucleotide substitution for each locus indicated by jModeltest 2.1.7 (Darriba *et al.*, 2012): GTR + I + G for *MYH6*, HKY + I + G for *GLYT1*, HKY + I for

ZIC1, HKY + G for *SREB2*, and K80 + I + G for *SH3PX3*; for the morphological data partition, the model JC69 + G was used following Lewis (2001). The number of generations was set to 5 000 000, with sampling of every 100 generations. Other parameters were set by default. The convergence of the Markov chain Monte Carlo (MCMC) chains were graphically assessed by evaluating the stationary phase of the chains using Tracer v. 1.5 (Rambaut *et al.*, 2013). Consensus topology and posterior probabilities were obtained after applying a burn-in of the first 25% of the generated trees. The combined database comprised all taxa used in the morphological analysis, except two species of *Nothobranchius* (*Nothobranchius geminus* Wildekamp, Watters & Sainthouse, 2002 and *Nothobranchius luekei* Seegers, 1984), for which DNA sequences were not available. It additionally included seven other species of *Nothobranchius* (*Nothobranchius bojiensis* Wildekamp & Haas, 1992, *Nothobranchius boklundi* Valdesalici, 2010, *Nothobranchius furzeri* Jubb, 1971, *Nothobranchius kadleci* Reichard, 2010, *Nothobranchius kilomberoensis* Wildekamp, Watters & Sainthouse, 2002, *Nothobranchius krammeri* Valdesalici & Hengstler, 2008 and *Nothobranchius niassa* Valdesalici, Bills, Dorn, Reichwald & Cellerino, 2012), for which DNA sequences were available but material for osteological preparations was not; for these taxa, only characters taken from the external morphology of their type series were used.

RESULTS

The comparative morphological analysis generated 138 characters (see Supporting Information Appendix S2 for list of characters and Table S1 for distribution of character states among terminal taxa), of which 92 were new and 46 already described in previous studies (Parenti, 1981; Costa, 1998b, 2009a, 2011b, 2012). The MP analysis of morphological characters generated >100 equally most parsimonious trees (see resulting strict consensus tree in Fig. 1). Although a low resolution was found in several tree zones, this analysis supports with high bootstrap values a clade comprising all Nothobranchiini, a clade comprising all Nothobranchiini except *Fundulosoma*, and a clade comprising all species of *Nothobranchius*. Relationships among *Nothobranchius* lineages were not well resolved, but some included clades had high support.

The two analyses (MP and BA) combining morphological and molecular data resulted in identical robust trees. In BA, support was nearly maximal for most clades (i.e. posterior probability 1.00; Fig. 2), but a tendency of BA to artificially inflate high branch supports has been well documented (e.g. Simmons, Pickett & Miya, 2004). More significantly, high bootstrap values
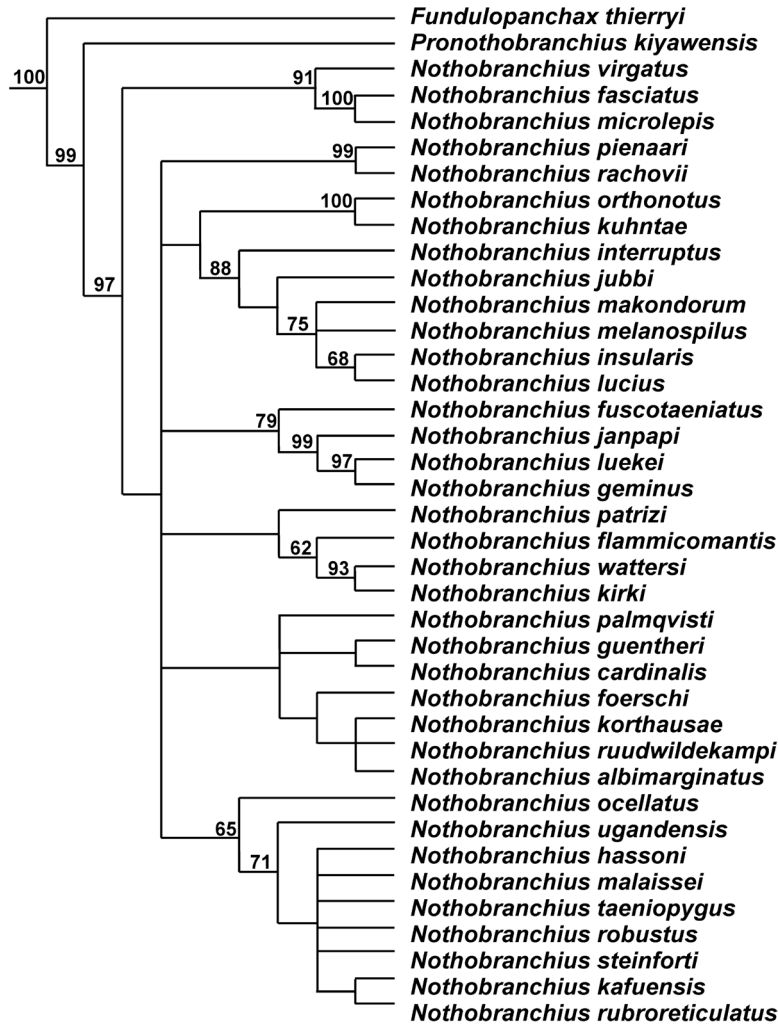
Figure 1. Strict consensus tree of 100 most-parsimonious trees from the analysis of 138 morphological characters for 39 species of Nothobranchiini (outgroups not depicted). Numbers above nodes are bootstrap percentages >50%.

were found for the great majority of the most inclusive clades (i.e. >90%; Fig. 2). This resulting tree topology supports the new generic classification proposed in the next section, where the tribe Nothobranchiini and included clades are formally recognized as genera, and subgenera are primarily diagnosed on the basis of apomorphic character states, numbered in square brackets according to Supporting Information Appendix S2.

TAXONOMIC ACCOUNTS

TRIBE NOTHOBRANCHIINI GARMAN, 1895

Diagnosis: Presence of robust, anteriorly directed ventral process on dentary [15.1] (vs. process absent); dorsal margin of opercle angular [35.1] (vs. slightly curved); anterior margin of third

pharyngobranchial narrow and pointed in dorsal view [61.1] (vs. broad and rounded); first and second hypobranchials rounded [71.1] (vs. angular); neural spine of first vertebra deeper than wide [78.1] (vs. wider than deep); hypurals forming single plate, without median gap [81.1] (vs. separated by median gap); prezygapophyses of preural vertebrae 2–3 absent [85.1] (vs. prezygapophyses present); median radials of anal fin superficially or not ossified [86.1] (vs. well ossified); dorsal portion of cleithrum deeper than wide [90.1] (vs. wider than deep); lateral process of pelvic bone absent [98.1] (vs. present); anterior section of supraorbital neuromast series separated in two subsections by interspace [106.1] (vs. continuously arranged); lachrymal and dermosphenotic canals open [110.1] (vs. closed); presence of thickened epidermal tissue over scales of anterior-most part of frontal region [111.1] (vs.
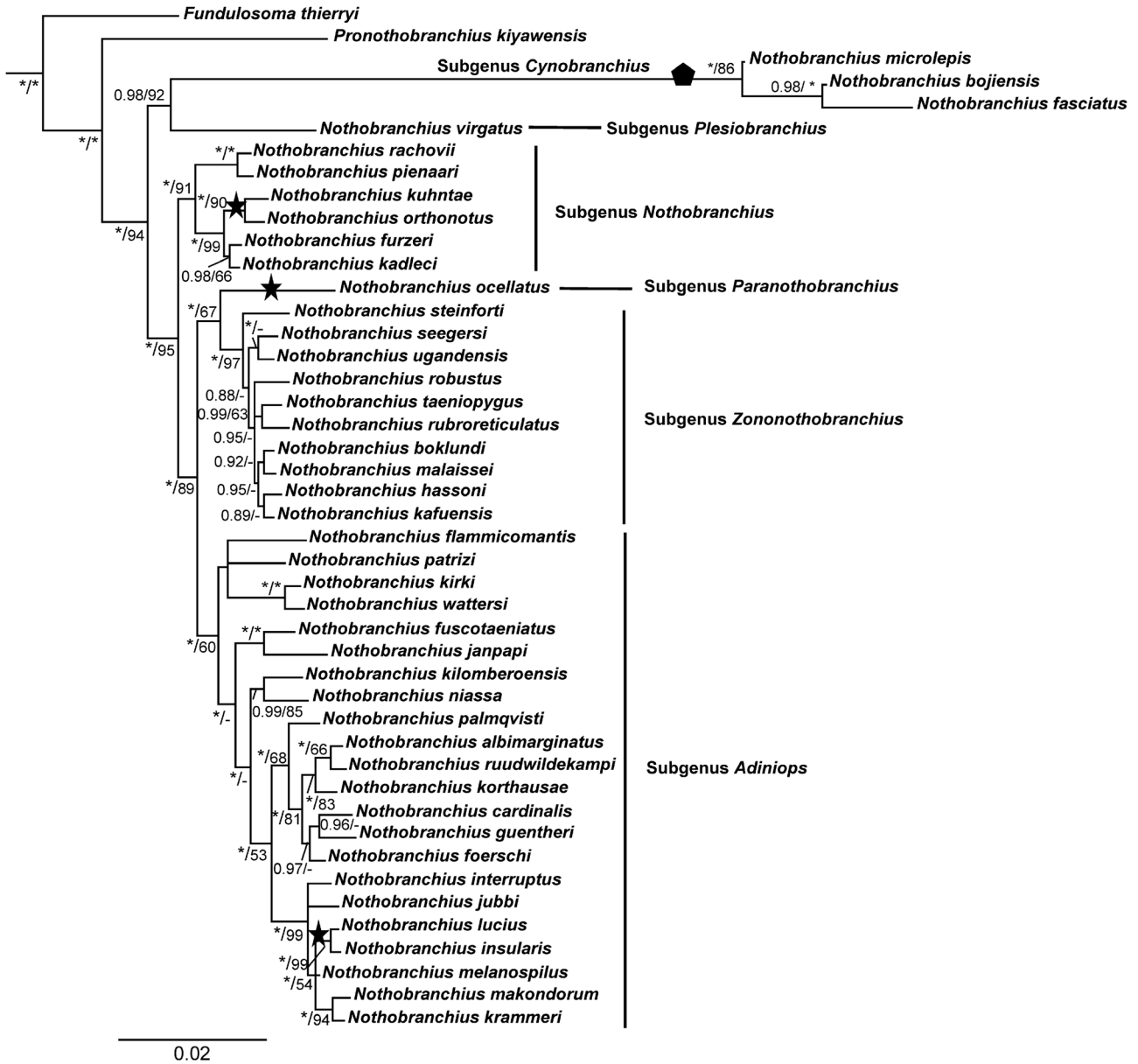
Figure 2. Phylogenetic relationships among 45 species of Nothobranchiini (outgroups not illustrated), inferred by using 138 morphological characters and partial sequences of the nuclear-encoded genes *GLYT1*, *MYH6*, *SH3PX3*, *SREB2* and *ZIC1*, total of 3327 bp. Numbers near branches indicate posterior probability values derived from the Bayesian analysis (BA), followed by bootstrap support values >50% taken from the maximum parsimony (MP) analysis. *Maximum support values; – indicates bootstrap values below 50%. Stars indicate lineages exhibiting predator-like jaw morphology; pentagon indicates lineage with branchial zooplankton-feeder specializations.

absent); urogenital papilla, in females, with aperture surrounded by expanded membrane to form pocket-shaped structure overlapping anterior portion of anal fin [116.1] (vs. simple aperture, without expansions); anal fin, in females, long, spatula shaped [122.1] (vs. nearly fan shaped). Also differs from all other African aplocheiloids by: posterior portion of ascending

process of premaxilla not distinctively shaped [4.0] (vs. tapered to form slightly concavity on posterior portion of mesial edge); a reversal.

Included genera: Fundulosoma Ahl, 1924, *Nothobranchius* Peters, 1868 and *Pronothobranchius* Radda, 1969.

GENUS *FUNDULOSOMA* AHL, 1924

Synonyms: None.

Type species: Fundulosoma thierryi Ahl, 1924. By monotypy.

Diagnosis: Prominent anterior triangular flap on sub-proximal region of maxilla [13.1] (vs. absence). Also differs from other Nothobranchiini genera by seven plesiomorphic morphological conditions: posterior projection of premaxillary alveolar arm well developed [9.0] (vs. rudimentary); absence of lateral groove on distal region of maxilla [14.0] (vs. deep groove present); anterior margin of autopalatine slightly convex in lateral view [20.0] (vs. approximately straight); absence of spine-like contact organs on dorsal and anal fins of males [103.0] (vs. present at least on distal portion of those fin-rays); branchiostegal membrane of males short, not visible externally [118.0] (vs. long, visible externally); absence of filaments on distal margin of dorsal and anal fins of males [119.0] (vs. presence); and anal fin of males nearly rectangular [120.0] (vs. primarily fan shaped). *Fundulosoma* also differs from all other Nothobranchiini, except *N. ocellatus*, by: ventral extremity of cleithrum projecting forwards much beyond ventral extremity of scapula [89.0] (vs. both extremities in close proximity).

Included species and distribution: A single species, *Fundulosoma thierryi* Ahl, 1924, has been recorded from a wide geographical area in West Africa, including main river basins of Niger, Benin, Burkina Faso, Togo, Ghana, Mali, Senegal and Gambia. The colour pattern variability recorded for different populations within this broad geographical range suggests that different species may have been referred to *F. thierryi*.

GENUS *PRONOTHOBRANCHIUS* RADDA, 1969

Synonyms: None.

Type species: Nothobranchius kiyawensis Ahl, 1928. By original designation.

Diagnosis: Posterior margin of premaxillary ascending process perpendicular to its main axis, resulting in sub-rectangular process [5.1] (vs. oblique, forming subtrapezoidal process); anterior and posterior ceratohyals separated by broad cartilaginous interspace [47.1] (vs. narrow interspace); proximal extremity of first epibranchial wide, about seven times distal extremity [49.1] (vs. narrow, about four times or less); interarcual cartilage long, longer than first epibranchial [50.2] (vs. shorter); articulatory cartilaginous head of proximal process of third epibranchial broad, about twice as broad as other third epibranchial cartilaginous heads [53.1] (vs. nearly equal or slightly broader); anterior condyle of second pharyngobranchial forming angle >90° [57.2] (vs. < or ~90°); neural spine of second vertebra broad, about four times wider than neural spine of third vertebra [79.1] (vs. about twice wider); dorsal and ventral margins of hypural plate forming angle >90° [82.1] (vs. <90°); and presence of pointed expanded flap on anterior portion of cleithrum [92.1] (vs. flap absent).

Included species and distribution: Pronothobranchius kiyawensis (Ahl, 1928), first described from northern Nigeria, has been considered as the single valid species (e.g. Wildekamp *et al.*, 1986), including in its synonymy two nominal species, *Pronothobranchius gambiensis* (Svensson, 1933) from the Gambia river basin, and *Pronothobranchius seymouri* (Loiselle & Blair, 1971) from the Accra plains in Ghana. In a recent aquarium journal publication (Valdesalici, 2013), the three nominal species appear as valid, and a fourth species, *Pronothobranchius chirioi* Valdesalici, 2013 from the Niger river basin, is described. As those nominal species cannot be distinguished on the basis of available data, only *P. kiyawensis* (Ahl, 1928) is considered here as valid, although it probably constitutes a species complex in need of a taxonomic revision. *Pronothobranchius* occurs in a vast area of West Africa, including the main rivers basins of Niger, Nigeria, Burkina Faso, Ghana, Senegal and Gambia.

GENUS *NOTHOBRANCHIUS* PETERS, 1868

Synonyms: Adiniops Myers, 1924: 6 (type species: *Fundulus guentheri* Pfeffer, 1893; original designation; here considered as subgenus); *Zononothobranchius* Radda, 1969: 163 (type species: *Nothobranchius rubroreticulatus* Blache & Milton, 1960; original designation; here considered as subgenus); *Aphyobranchius* Wildekamp, 1977: 326 (type species: *N. janpapi* Wildekamp, 1977; original designation); *Paranothobranchius* Seegers, 1985 (type species: *Paranothobranchius ocellatus* Seegers, 1985; original designation; here considered as subgenus).

Type species: Cyprinodon orthonotus Peters, 1844; By original designation.

Diagnosis: The genus *Nothobranchius* is distinguished from all other genera of the Nothobranchiini by all

included species sharing three apomorphic conditions: lachrymal canal length about two or three times the bone width [76.1] (vs. nearly equal); presence of long and pointed posterior processes on the basal portion of middle rays of the anal fin [87.1] (vs. absence); and preopercular canal open [109.1] (vs. completely or partly closed).

Included taxa: About 70 valid species, in six subgenera: *Adiniops* Myers, 1924, *Cynobranchius* subgen. nov. *Nothobranchius* Peters, 1868, *Paranothobranchius* Seegers, 1985, *Plesiobranchius* subgen. nov. and *Zononothobranchius* Radda, 1969.

SUBGENUS *CYNOBRANCHIUS* SUBGEN. NOV.

urn:lsid:zoobank.org:act:D998F949-2024-4D4E-8679-D93B66345DF7

Type species: Fundulus microlepis Vinciguerra, 1897.

Diagnosis: Deep oblique notch on dorsal surface of premaxillary ascending process [6.1] (vs. absence); teeth short and conical on outer row of dentary [16.1] (vs. fang shaped); basihyal triangular, broad [38.1] (vs. primarily subtriangular, narrow, sometimes pentagonal or rectangular); anterior extremity of ventrolateral process of urohyal much posteriorly placed to dorsal process [42.1] (vs. in close proximity); anterior portion of anterior ceratohyal distinctively directed upwards [44.1] (vs. approximately horizontal to slightly directed upwards); presence of three branchiostegal rays attached to anterior portion of anterior ceratohyal [46.1] (vs. two); proximal process of third epibranchial distinctively longer than distal process [51.1] (vs. nearly equal); second pharyngobranchial wider than long in dorsal view [56.1] (vs. longer than wide); third pharyngobranchial teeth minute [62.2] (vs. well developed); dentigerous plate of fourth pharyngobranchial sub-rectangular, extending above third epibranchial [63.1] (vs. rounded, limited to area between third pharyngobranchial and fourth epibranchial); numerous gill-rakers on first branchial arch, six or seven on dorsal portion, 18–20 on ventral [65.2] (vs. one to five + 8–15); vomer teeth absent [72.1] (vs. present); presence, in males, of widened structure on lateral portion of genital region, suggesting pumping mechanism [115.1] (vs. absence of similar structures); anal fin, in females, long, its length measured between middle base middle distal margin about twice anal-fin base length [123.1] (vs. equal or slightly longer); pelvic fin, in males, long, reaching anterior portion of anal fin [126.1] (vs. short, not or barely touching anal fin); and presence, in males, of dark grey bar on suborbital

region [132.1] (vs. absence). Also distinguished from other Nothobranchiini, except species of subgenus *Nothobranchius* by: anterior process of opercle long, rod shaped [37.1] (vs. short, rectangular).

Etymology: The name *Cynobranchius* is an allusion to the superficial resemblance between species included in this new subgenus of *Nothobranchius* and species of the South American aplocheiloid genus *Cynolebias* Steindachner, 1876 (from the Greek *cyno* = dog, taken from the name *Cynolebias*, and *branchius* = gills, taken from the name *Nothobranchius*). Gender masculine.

Included species: Three species have been recognized as valid: *N. bojiensis* Wildekamp & Haas, 1992, *Nothobranchius fasciatus* Wildekamp & Haas, 1992 and *N. microlepis* (Vicinguerra, 1897). However, their limits are still unclear, because morphological characters used to diagnose them overlap greatly among the proposed species (Wildekamp & Haas, 1992), indicating that a taxonomic revision of this subgenus is needed.

Distribution: Coastal basins of southern Somalia and Kenya.

SUBGENUS *PLESIOBRANCHIUS* SUBGEN. NOV.

urn:lsid:zoobank.org:act:60E816B8-7280-4866-BEC5-181827A47B4D

Type species: Nothobranchius virgatus Chambers, 1984.

Diagnosis: lateral portion of second pharyngobranchial truncate in dorsal view [60.1] (vs. pointed); and fifth ceratobranchial broad, triangular in dorsal view [69.1] (vs. narrow, boomerang shaped). Also distinguished from all other Nothobranchiini, except *Adiniops*, by: expansion on sub-distal portion of anterior margin of autopalatine [21.1] (vs. expansion absent). *Plesiobranchius* is similar to *Cynobranchius* and distinguished from all other Nothobranchiini by five unique apomorphic conditions: posterior process of quadrate short, its length distinctively shorter than basal portion of bone without process [27.1] (vs. about equal or slightly longer); osseous flap between distal and posterior processes of third epibranchial extending beyond line between processes [54.1] (vs. not surpassing line); first post-cleithrum absent [93.1] (vs. present); pelvic bone broad, wider than long [97.1] (vs. longer than wide); pelvic fin with seven well-developed rays [128.1] (vs. five or six well-developed rays, sometimes plus one rudimentary).

Etymology: The name *Plesiobranchius* refers to the superficial resemblance between *N. virgatus*, the type species of this new subgenus of *Nothobranchius*, and some species of the South American aplocheiloid genus *Plesiolebias* Costa, 1989 (from the Greek *plesios* = primitive, taken from the name *Plesiolebias*, and *branchius* = gills, taken from the name *Nothobranchius*). Gender masculine.

Included species and distribution: A single species, *N. virgatus* Chambers, 1984, endemic to the middle White Nile River drainage, Nile River basin, South Sudan and Ethiopia.

SUBGENUS *NOTHOBRANCHIUS* PETERS, 1868

Synonyms: None.

Type species: Cyprinodon orthonotus Peters, 1844. By original designation.

Diagnosis: Well-developed lateral flap on proximal region of fourth ceratobranchial [68.0] (vs. flap rudimentary or absent).

Included taxa: The subgenus *Nothobranchius* includes the following species: *N. furzeri* Jubb, 1971, *N. kadleci* Reichard, 2010, *Nothobranchius krysanovi* Shidlovskiy, Watters & Wildekamp, 2010, *Nothobranchius kuhntae* Ahl, 1926, *Nothobranchius mayeri* Ahl, 1935, *Nothobranchius mkuziensis* (Fowler, 1934), *Nothobranchius orthonotus* (Peters, 1844), *Nothobranchius pienaari* Shidlovskiy, Watters & Wildekamp, 2010 and *Nothobranchius rachovii* Ahl, 1926. The poorly known *N. mkuziensis* is probably a valid species closely related to *N. rachovii*. *Nothobranchius kuhntae* has been considered a synonym of *N. orthonotus* (e.g. Wildekamp, 2004; Vrtilek & Reichard, 2016). The exact type locality of *N. kuhntae* is unknown, and the present examination of type material of *N. orthonotus* and *N. kuhntae* did not provide any morphological evidence that they are different species. However, further investigation involving a detailed morphological analysis is necessary to confirm this synonymy. On the other hand, *N. mayeri* Ahl, 1935 also considered as a synonym of *N. orthonotus* (e.g. Wildekamp *et al.*, 1986), probably is a valid species, differing from the latter species by a different dentition, as revealed through examination of type specimens.

Distribution: River basins of central and southern Mozambique, and adjacent areas in Malawi, South Africa and Zimbabwe.

SUBGENUS *PARANOTHOBRANCHIUS* SEEGERS, 1985

Synonyms: None.

Type species: Paranothobranchius ocellatus Seegers, 1985. By original designation.

Diagnosis: Deep concavity on medial portion of premaxilla, close to symphysis [1.1] (vs. concavity absent); anterior process of premaxillary alveolar arm rudimentary [7.1] (vs. well developed); teeth of inner row of premaxilla about four times greater than teeth of outer row [10.2] (vs. smaller or slightly greater); jaws long, lower jaw about 1.5 times longer than distance between anterior tip of quadrate and posterior margin of preopercle [19.1] (vs. shorter to slightly longer); apical region of autopalatine thick, dorso-ventrally flattened [23.1] (vs. thin, laterally flattened); antero-dorsal condyle of hyomandibula distinctly longer than postero-dorsal condyle [32.1] (vs. about equal); presence of small denticles on gill-rakers of first ceratobranchial [66.1] (vs. absence); posterior process of parasphenoid robust and long, reaching posterior portion of basioccipital [74.1] (vs. thin, reaching middle portion of basioccipital); presence of pointed anterior expansion on lateral process of sphenotic [75.1] (vs. absence); 36–38 vertebrae [77.1] (vs. 26–32); ventral extremity of cleithrum projecting ventrally beyond scapula [89.0] (vs. both extremities in close proximity); six or seven neuromasts in posterior section of supraorbital neuromast series [108.2] (vs. three or four); scales irregularly arranged on flank [113.1] (vs. regularly); scales extending on ~70% of caudal-fin base in males [114.1] (vs. ~25–30%); orbital membrane free except on its dorsal region [117.1] (vs. completely attached); pelvic-fin bases separated by interspace [127.0] (vs. in contact or united); presence of ocellate spot on middle part of basal portion of caudal fin in females [137.1] (vs. absence).

Included species and distribution: A single species, *N. ocellatus* (Seegers, 1985), occurring in the coastal river basins of eastern Tanzania, between Ruvu and Matandu river basins.

SUBGENUS *ZONONOTHOBRANCHIUS* RADDA, 1969

Synonyms: None.

Type species: Nothobranchius rubroreticulatus Blache & Milton, 1960. By original designation.

Diagnosis: Anterior process of alveolar arm of premaxilla triangular [8.1] (vs. trapezoidal or

sub-rectangular); presence of pointed expansions on anterior margin of hyomandibula [33.1] (vs. pointed expansions absent); and basihyal pentagonal in dorsal view [38.2] (vs. subtriangular, triangular or rectangular). *Zononothobranchius* shares two unique morphological synapomorphies with *Paranothobranchius*: teeth broadly distributed on middle portion of vomer [73.1] (vs. restricted to its antero-middle portion); and osseous membrane of pelvic bone poorly developed [96.1] (vs. well developed).

Included species: N. boklundi Valdesalici, 2010, *Nothobranchius brieni* Poll, 1938, *Nothobranchius capriviensis* Watters, Wildekamp & Shidlovskiy, 2015, *Nothobranchius chochamandai* Nagy, 2014, *Nothobranchius flagrans* Nagy, 2014, *Nothobranchius hassoni* Valdesalici & Wildekamp, 2004, *Nothobranchius ivanovae* Valdesalaci, 2012, *Nothobranchius kafuensis* Wildekamp & Rosenstock, 1989, *Nothobranchius kardashevi* Valdesalaci, 2012, *Nothobranchius malaissei* Wildekamp, 1978, *Nothobranchius milvertzi* Nagy, 2014, *Nothobranchius neumanni* (Hilgendorf, 1905), *Nothobranchius nubaensis* Valdesalici, Bellemans, Kardashev & Golubtsov, 2009, *Nothobranchius oestergaardi* Valdesalici & Amato, 2011, *Nothobranchius polli* Wildekamp, 1978, *Nothobranchius robustus* Ahl, 1935, *Nothobranchius rosenstocki* Valdesalici & Wildekamp, 2005, *N. rubroreticulatus* Blache & Miton, 1960, *Nothobranchius sainthouse* Nagy, Cotterill & Bellstedt, 2016, *Nothobranchius seegersi* Valdesalici & Kardashev, 2011, *Nothobranchius steinforti* Wildekamp, 1977, *Nothobranchius streltsovi* Valdesalici, 2016, *Nothobranchius symoensi* Wildekamp, 1978, *Nothobranchius taeniopygus* Hilgendorf, 1891, *Nothobranchius ugandensis* Wildekamp, 1994.

Distribution: Central African highlands, including the White Nile, upper Congo, upper Zambesi, upper Great Ruaha, upper Wami and Malagarasi river basins and the Tchad, Victoria, Albert, Eyasi and Tanganyika lake drainages, in Chad, Cameroon, Democratic Republic of Congo, Ethiopia, Kenya, South Sudan, Tanzania, Uganda and Zambia.

SUBGENUS *ADINIOPS* MYERS, 1924

Synonyms: Aphyobranchius Wildekamp, 1977b: 326 (type species: *N. janpapi* Wildekamp, 1977; original designation).

Type species: Fundulus guentheri Pfeffer, 1893. By original designation.

Diagnosis: Morphologically differs from other subgenera by: absence of deep oblique notch on dorsal surface of ascending process of premaxilla [6.0] (vs. presence in *Cynobranchius*); fang-like teeth on outer row of dentary [16.0] (vs. short and conical in *Cynobranchius*); presence of two branchiostegal rays attached to anterior portion of anterior ceratohyal [46.0] (vs. three in *Cynobranchius*); third pharyngobranchial teeth well developed [62.0] (vs. minute in *Cynobranchius*); fifth ceratobranchial narrow, boomerang shaped in dorsal view [69.0] (vs. broad, triangular in *Plesiobranchius*); first post-cleithrum present [93.0] (vs. absent in *Cynobranchius* and *Plesiobranchius*); pelvic bone narrow, longer than wide [97.0] (vs. wider than long in *Cynobranchius* and *Plesiobranchius*); lateral flap on proximal region of fourth ceratobranchial rudimentary [68.1] (vs. well developed in subgenus *Nothobranchius*); absence of deep concavity on medial portion of premaxilla close to the symphysis [1.0] (vs. presence of deep concavity, in *Paranothobranchius*); anterior process of alveolar arm of premaxilla well developed [7.0] (vs. rudimentary in *Paranothobranchius*); apical region of autopalatine thin, laterally flattened [23.0] (vs. thick, dorso-ventrally flattened in *Paranothobranchius*); absence of small denticles on gill-rakers of first ceratobranchial [66.0] (vs. presence in *Paranothobranchius*); absence of pointed anterior expansion on lateral process of sphenotic [75.0] (vs. presence in *Paranothobranchius*); ventral extremity of cleithrum not projecting ventrally beyond scapula [89.0] (vs. projecting in *Paranothobranchius*); scales regularly arranged on flank [113.0] (vs. irregularly in *Paranothobranchius*); anterior process of alveolar arm of premaxilla trapezoidal or sub-rectangular [8.0] (vs. triangular in *Zononothobranchius*); and without pointed expansions on anterior margin of hyomandibula [33.0] (vs. pointed expansions present in *Zononothobranchius*).

Included species: Nothobranchius albimarginatus Watters, Wildekamp & Cooper, 1998, *Nothobranchius annectens* Watters, Wildekamp & Cooper, 1998, *Nothobranchius cardinalis* Watters, Cooper & Sainthouse, 2007, *Nothobranchius eggersi* Seegers, 1982, *Nothobranchius elongatus* Wildekamp, 1982, *Nothobranchius flammicomantis* Wildekamp, Watters & Sainthouse, 1998, *Nothobranchius foerschi* Wildekamp & Berkenkamp, 1979, *Nothobranchius fuscotaeniatus* Seegers, 1997, *Nothobranchius geminus* Wildekamp, Watters & Sainthouse, 2002, *Nothobranchius guentheri* (Pfeffer, 1893), *Nothobranchius hengstleri* Valdesalici, 2007, *Nothobranchius insularis* Costa, 2017, *Nothobranchius interruptus* Wildekamp & Berkenkamp, 1979, *N. janpapi* Wildekamp, 1977, *Nothobranchius jubbi* Wildekamp & Berkenkamp,

1979, *Nothobranchius kilomberoensis* Wildekamp, Watters & Sainthouse, 2002, *Nothobranchius kirki* Jubb, 1969, *Nothobranchius korthausae* Meinken, 1973, *Nothobranchius krammeri* Valdesalici & Hengstler, 2008, *Nothobranchius lourensi* Wildekamp, 1977, *Nothobranchius lucius* Wildekamp, Shidlovskiy & Watters, 2009, *Nothobranchius luekei* Seegers, 1984, *Nothobranchius makondorum* Wildekamp, Shidlovskiy & Watters, 2009, *Nothobranchius melanospilus* (Pfeffer, 1896), *Nothobranchius niassa* Valdesalici, Bills, Dorn, Reichwald & Cellerino, 2012, *Nothobranchius palmqvisti* (Lönnberg, 1907), *Nothobranchius patrizii* (Vinciguerra, 1927), *Nothobranchius rubripinnis* Seegers, 1986, *Nothobranchius ruudwildekampi* Costa, 2009, *Nothobranchius vosseleri* Ahl, 1924, *Nothobranchius wattersi* Ng'oma, Valdesalici, Reichwald & Cellerino, 2013 and *Nothobranchius willerti* Wildekamp, 1992.

Distribution: Coastal river basins of southern Somalia, Kenya, Tanzania and northern Mozambique, besides Lake Malawi drainage.

DISCUSSION

### MONOPHYLY OF THE NOTHOBRANCHIINI

Parenti (1981) first proposed monophyly of the group today classified as tribe Nothobranchiini, but considering a single valid genus, *Nothobranchius*. This clade was then diagnosed by an open preopercular canal, but as herein observed, this condition is not applicable to *Fundulosoma* and *Pronothobranchius*, being useful only to diagnose *Nothobranchius* in the sense of the present study. In fact, the preopercular canal is closed or partly closed in well-preserved adult specimens of the genera Fundulosoma and Pronothobranchius, and therefore is not diagnostic of Nothobranchiini. Apomorphic open lachrymal and dermosphenotic canals are consistently present in all species of the tribe.

On the basis of the examination of specimens cleared and stained for osteological study belonging to six species of Nothobranchiini, Parenti (1981) also diagnosed this group by the supposed apomorphic condition of the interarcual cartilage, which would be directly attached to the cartilaginous head of the second pharyngobranchial. However, the present study, based on a large sample of cleared and stained specimens, showed that the interarcual cartilage attachment in the Nothobranchiini does not differ from other aplocheiloids. Finally, based on aquarium literature data (i.e. Scheel, 1968), Parenti (1981) diagnosed Nothobranchiini as having oval eggs, instead of spherical. This character, which requires keeping and breeding individuals in aquaria to obtain the data, was not analysed in the present

study. However, recent studies on egg morphology in *Nothobranchius* (e.g. Kwon, Jung & Kim, 2015) indicate that eggs are spherical, thus not differing from other aplocheiloids.

Costa (2009a) found several apomorphic character states shared by all nine species of *Nothobranchius* examined in that study, but species of the genera *Fundulosoma* and *Pronothobranchius* were not then available. Seven of those character states are herein supported as diagnostic synapomorphies of the Nothobranchiini: there is a robust, anteriorly directed ventral process on the dentary (Fig. 3B–J), in contrast to the absence of any process as in other aplocheiloids (Fig. 3A); the dorsal margin of the opercle is angular (Fig. 4B), instead of being slightly curved (Fig. 4A); the first and second hypobranchials are rounded (Fig. 5B–G), instead of being angular (Fig. 5A); the neural spine of the first vertebra is long, deeper than wide (Fig. 6D), instead of wider than deep (Costa, 2015: fig. 1A, B); the hypurals are ankylosed to form a single plate (Fig. 6B, C), instead of having a median gap (Fig. 6A); there is a thickened epidermal tissue covering the scales of the anterior-most part of the frontal region; and, in females, the urogenital papilla aperture is surrounded by an expanded membrane, forming a pocket-shaped structure overlapping the anterior portion of the anal fin, instead of being a simple aperture, without expansions.

Another eight derived character states are listed herein to support monophyly of the Nothobranchiini: the anterior margin of the second pharyngobranchial is narrow and pointed in dorsal view (Fig. 7B), instead of being broad and rounded (Fig. 7A); there is no well-developed prezygapophysis on the preural vertebrae 2 and 3 (Fig. 6B), instead of the presence of prominent prezygapophyses (Costa, 2015: fig. F, G); the median radials of the anal fin are superficially or not ossified (Fig. 6O), instead of being well ossified (Costa, 2009c: fig. 5D); the dorsal portion of the cleithrum is deeper than wide (Fig. 6F–J), instead of wider than deep (Fig. 6E); there is no triangular lateral process on the pelvic bone (Fig. 6K–N) as in most other African aplocheiloids (Costa, 1998b: fig. 15B), although a different truncate process is present in *N. janpapi* and closely related species (Fig. 6L; see discussion below) and a lateral projection may be present in *N. microlepis* (Fig. 6K); the anterior section of the supraorbital neuromast series is separated in two subsections by an interspace, instead of being continuously arranged; the lachrymal and dermosphenotic canals are open, instead of closed; and, in females, the anal fin is long and spatula shaped, instead of being nearly fan shaped. The present comparative analysis also indicated that the posterior portion of the ascending process of the premaxilla is not tapered to form a slightly concavity on its mesial edge (Fig. 3N–Q),
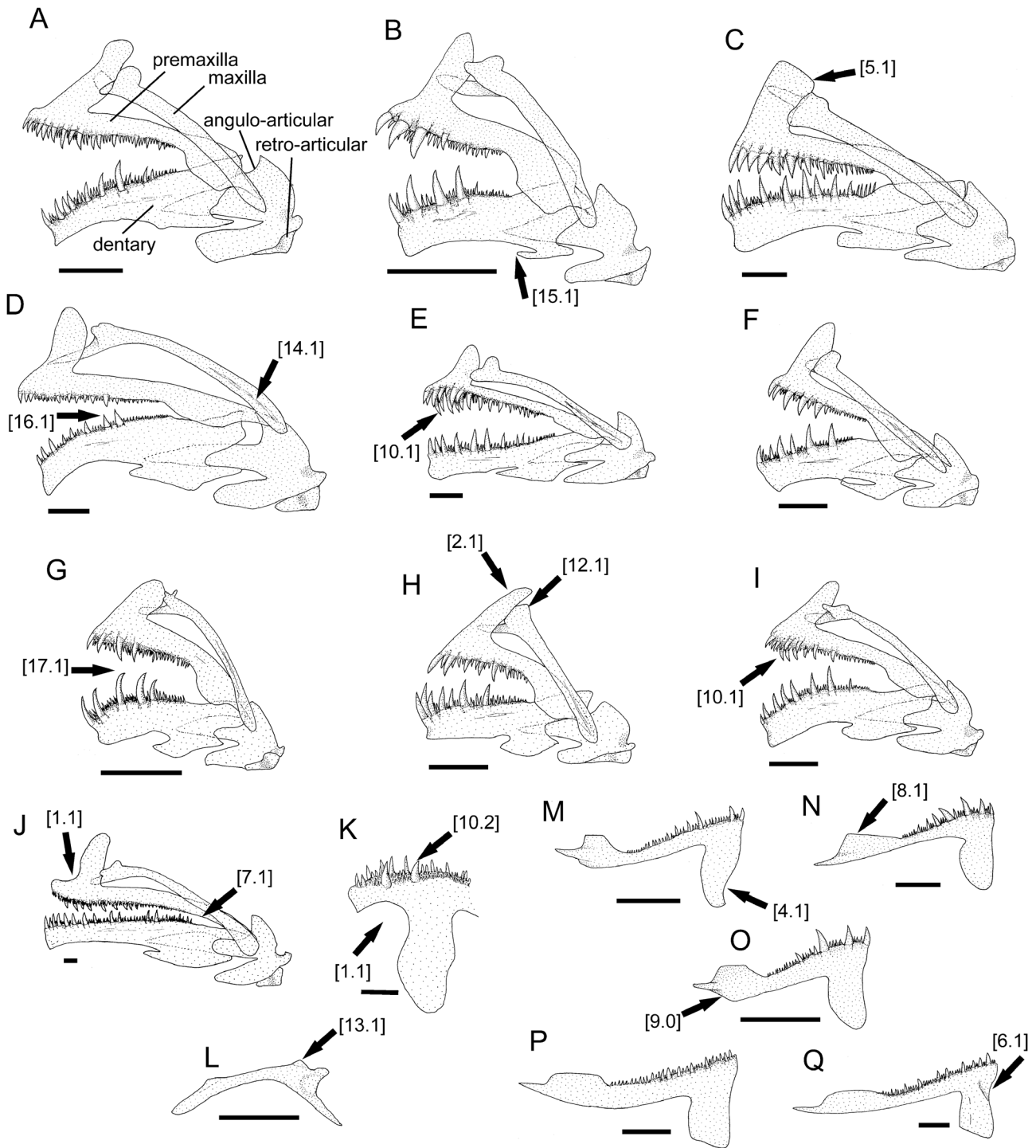
Figure 3. Jaw morphology. A–J, left jaws, left lateral view (cartilages not illustrated), of: *Fundulopanchax sjoestedti* (A), *Fundulosoma thierryi* (B), *Pronothobranchius kiyawensis* (C), *Nothobranchius microlepis* (D), *Nothobranchius orthonotus* (E), *Nothobranchius ugandensis* (F), *Nothobranchius janpapi* (G), *Nothobranchius geminus* (H), *Nothobranchius lucius* (I) and *Nothobranchius ocellatus* (J). K, left premaxilla, proximal portion, ventral view, of *N. ocellatus*. L, left maxilla, dorsoposterior view, of *F. thierryi*. M–Q, left premaxilla, dorsolateral view, of: *Fundulopanchax gardneri* (M), *N. ugandensis* (N), *F. thierryi* (O), *P. kiyawensis* (P) and *N. microlepis* (Q). Numbers in square brackets are character states numbered according to Supporting Information Appendix S2. Scale bars = 1 mm.
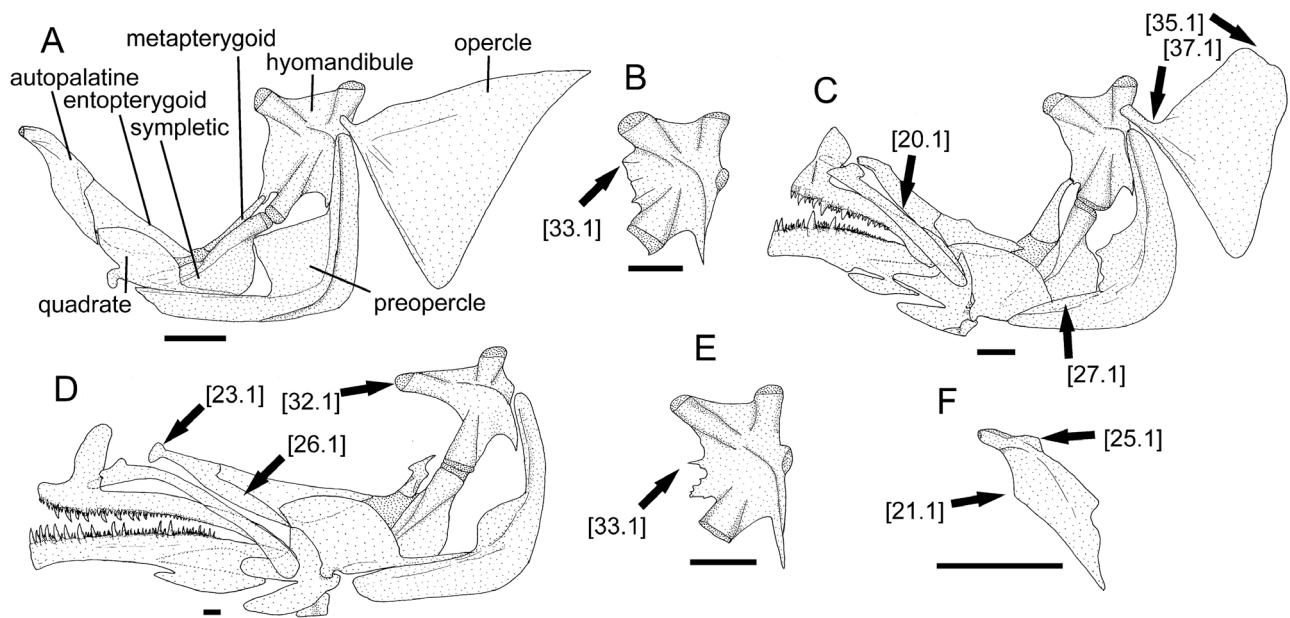
Figure 4. Jaw suspensorium morphology (all bones in left lateral view). A, left jaw suspensorium and part of opercular series of *Fundulopanchax sjoestedti*. B, left hyomandibule of *Pronothobranchius kiyawensis*. C, D, left jaws, jaw suspensorium and part of opercular series of *Nothobranchius fasciatus* (C) and *Nothobranchius ocellatus* (D). E, left hyomandibule of *Nothobranchius ugandensis*. F, left autopalatine of *Nothobranchius janpapi*. Numbers in square brackets are character states numbered according to Supporting Information Appendix S2. Larger stippling indicates cartilage. Scale bars = 1 mm.

as occurs in all other African aplocheiloids (Parenti, 1981; Fig. 3M), here considered as a reversal.

### PHYLOGENETIC POSITION OF *FUNDULOSOMA* AND *PRONOTHOBRANCHIUS*

Ahl (1924a) described *Fundulosoma* as a monotypic genus for *F. thierryi*, and four years later described *Nothobranchius kiyawensis* Ahl (1928), which was later designated as the type species of *Pronothobranchius*, then proposed as a subgenus of *Nothobranchius* (Radda, 1969). Both taxa were poorly known during the decades following Ahl's description. Scheel (1968) considered *Fundulosoma* as an intermediate genus between *Nothobranchius* and *Fundulopanchax*, as well as *N. kiyawensis* as probably being more closely related to *Fundulosoma* or *Aphyosemion* than to *Nothobranchius*. Parenti (1981), however, found evidence supporting *Fundulosoma* as a member of a clade containing species of *Nothobranchius*, including *N. kiyawensis*, which justified the placement of both *Fundulosoma* and *Pronothobranchius* in the synonymy of *Nothobranchius*.

Most subsequent workers did not follow Parenti's synonymy proposal, considering *Fundulosoma* and *Pronothobranchius* as valid genera (e.g. Wildekamp *et al.*, 1986; Radda & Pürzl, 1987; Seegers, 1997; Murphy & Collier, 1999; Dorn *et al.* 2014). *Fundulosoma* was considered to be more closely related to *Fundulopanchax* than to *Nothobranchius* by some authors (e.g. Radda &

Pürzl, 1987; Wildekamp, 1996), but no derived condition shared by these two genera was described to justify the proposed relationships. Likewise, Radda & Pürzl (1987) considered *Pronothobranchius* more closely related to *Fundulopanchax* than to *Nothobranchius*. In contrast, molecular data supported *Fundulosoma* in a clade also comprising *Nothobranchius* and *Pronothobranchius*, but its sister group relationships were ambiguous (Murphy & Collier, 1999). A more recent molecular study supported *Pronothobranchius* as the sister group of *Nothobranchius* (Dorn *et al.*, 2014), a hypothesis herein corroborated.

Parenti (1981) considered *F. thierryi* as the sister group of a clade comprising all other Nothobranchiini by the ventral process of the post-temporal being primitively present in *F. thierryi* and supposedly lost in other Nothobranchiini. The present comparative analysis shows that a well-developed ventral process of the post-temporal is present in several Nothobranchiini (see distribution of character state 88.1 in Table S1), making the character ambiguous, but the phylogenetic analyses highly support *F. thierryi* as the sister taxon of the clade comprising all other Nothobranchiini (Figs 1 and 2).

The clade containing *Pronothobranchius* and *Nothobranchius* is here corroborated by seven unambiguous morphological synapomorphic conditions: the alveolar arm of the premaxilla bears a rudimentary posterior projection (Fig. 3N, P–Q), contrasting with
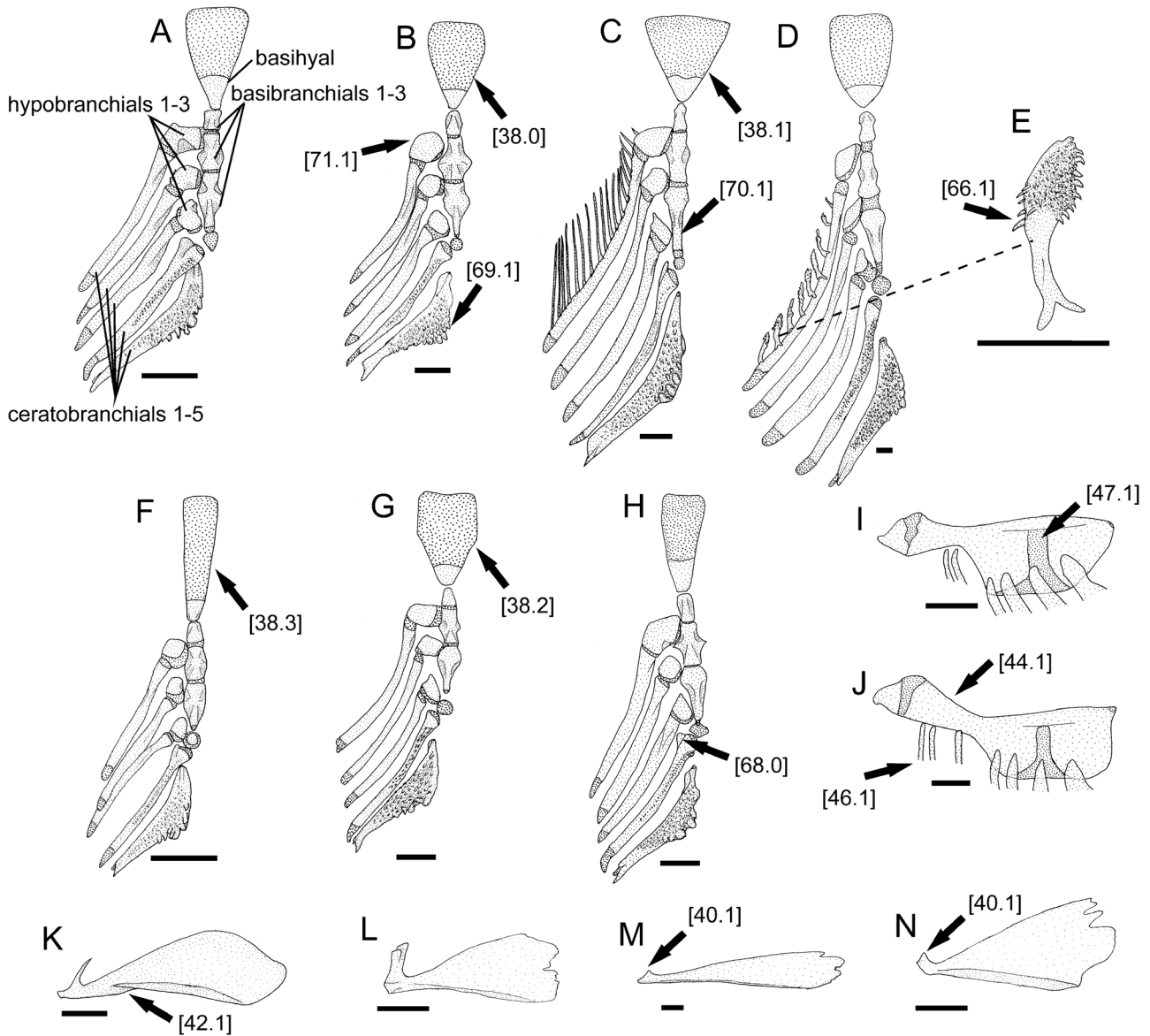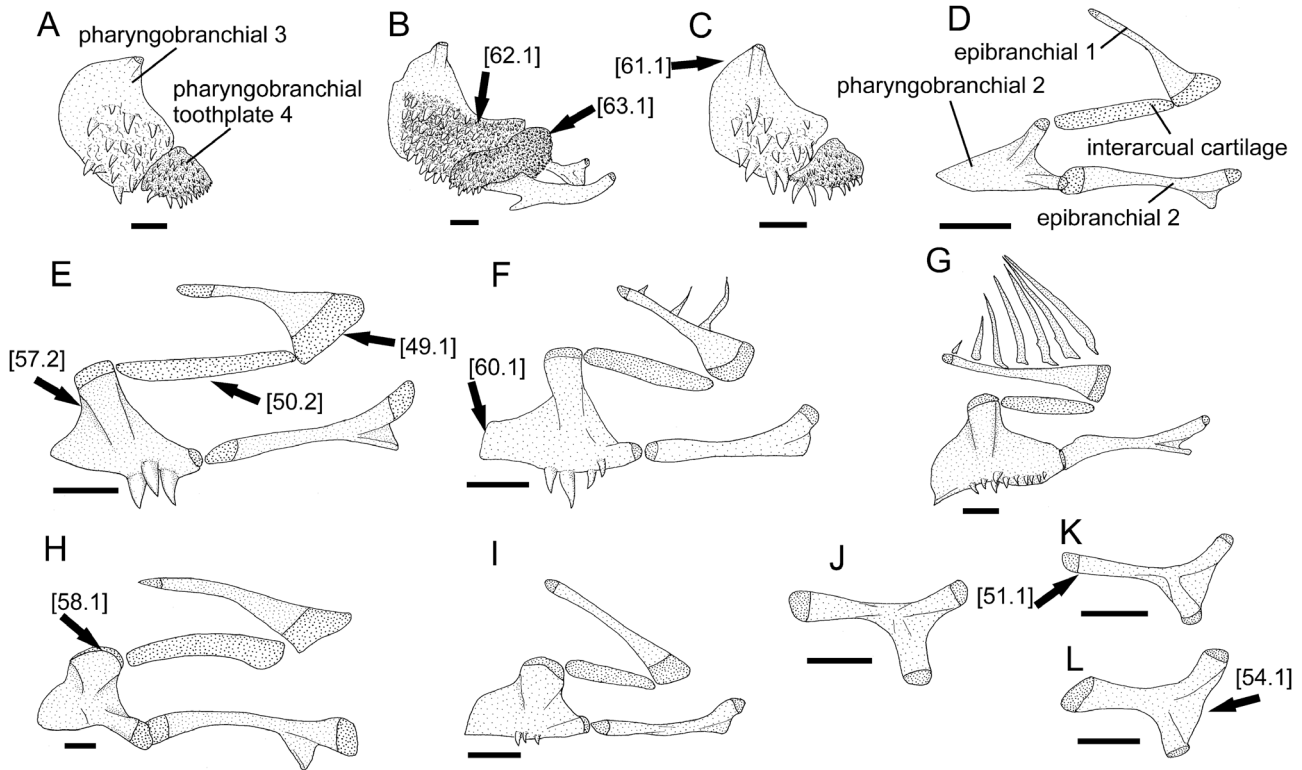
Figure 5. Hyoid and ventral branchial arch morphology. A–H, basihyal and left and middle portion of ventral branchial arches, dorsal view, of *Fundulopanchax sjoestedti* (A), *Nothobranchius virgatus* (B), *Nothobranchius fasciatus* (C) and *Nothobranchius ocellatus* (D), with detailed view of gill-raker (E), *Nothobranchius luekei* (F), *Nothobranchius ugandensis* (G) and *Nothobranchius piennari* (H) (gill-rakers illustrated only in C and D). I, J, left ceratohyals, left lateral view, of *Pronothobranchius kiyawensis* (I) and *Nothobranchius microlepis* (J). K–N, urohyal, left lateral view, of *Nothobranchius microlepis* (K), *P. kiyawensis* (L), *N. ocellatus* (M) and *N. ugandensis* (N). Numbers in square brackets are character states numbered according to Supporting Information Appendix S2. Larger stippling indicates cartilage. Scale bars = 1 mm.

the well-developed projection in *Fundulosoma* (Fig. 3O) and other African aplocheiloids (Fig. 3M); there is a deep lateral groove on the distal portion of the maxilla (Fig. 3C–J), whereas a lateral groove is absent in all other aplocheiloids (Fig. 3A, B); the dorsal and anal fins in males have spine-like contact organs on the distal portion of fin rays, which terminate in short filaments along fin margin, instead of spine-like contact organs

and filaments being absent as in *Fundulosoma*; the branchiostegal membrane is long in males, easily visible externally in both live and preserved specimens (vs. short, not visible externally as in *Fundulosoma*); and the anal fin in males is primarily fan-shaped, sometimes sub-trapezoidal, but never rectangular like most other aplocheiloids. In addition, in all species of the genera *Nothobranchius* and *Pronothobranchius*, except
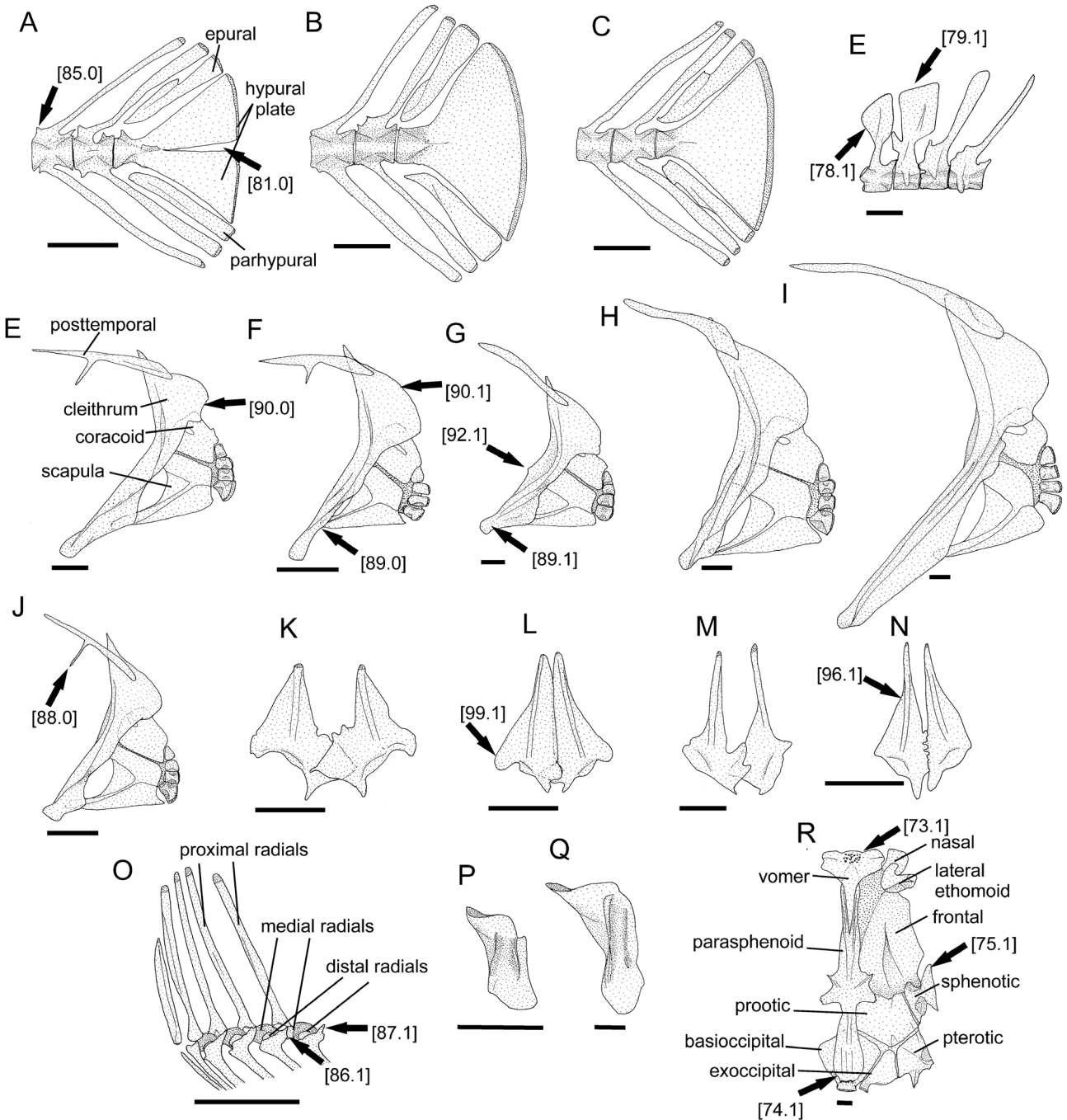
Figure 6. Vertebrae, fin support and neurocranium morphology. A–C, caudal skeleton, left lateral view, of *Fundulopanchax sjoestedti* (A), *Pronothobranchius kiyawensis* (B) and *Nothobranchius orthonotus* (C). D, first four vertebrae, left lateral view, of *P. kiyawensis*. E–J, left shoulder girdle (postcheitra not illustrated), left lateral view, of *F. sjoestedti* (E), *Fundulosoma thierryi* (F), *P. kiyawensis* (G), *Nothobranchius virgatus* (H), *Nothobranchius ocellatus* (I) and *Nothobranchius janpapi* (J). K–N, pelvic bones, ventral view, of *Nothobranchius microlepis* (K), *Nothobranchius geminus* (L), *N. ocellatus* (M) and *Nothobranchius taeniopygus* (N). O, anterior portion of anal-fin support, left lateral view, of *Nothobranchius korthausae*. P, Q, lachrymal of *F. thierryi* (P) and *Nothobranchius wattersi* (Q). R, neurocranium, middle and left portion, ventral view, of *N. ocellatus*. Numbers in square brackets are character states numbered according to Supporting Information Appendix S2. Larger stippling indicates cartilage. Scale bars = 1 mm.

Figure 7. Dorsal branchial arch morphology. A–C, middle tooth-plates, ventral view, of *Fundulopanchax sjoestedti* (A), *Nothobranchius microlepis* (B) and *Nothobranchius ugandensis* (C). D–I, anterior bones, ventral view, of *F. sjoestedti* (D), *Pronothobranchius kiyawensis* (E), *Nothobranchius virgatus* (F), *N. microlepis* (G), *Nothobranchius ocellatus* (H) and *N. ugandensis* (I). J–L, third epibranchial, ventro-posterior view, of: *P. kiyawensis* (J), *N. microlepis* (K) and *N. virgatus* (L). Numbers in square brackets are character states numbered according to Supporting Information Appendix S2. Larger stippling indicates cartilage. Scale bars = 0.5 mm.

N. ocellatus, the anterior margin of the autopalatine is approximately straight in lateral view (Fig. 4B), instead of being slightly convex as in other aplocheiloids; and the ventral extremities of the cleithrum and scapula are situated in close proximity (Fig. 6G–K), vs. ventral extremity of the cleithrum projecting forwards much beyond the ventral extremity of the scapula (Fig. 6E, F).

Uniquely in *Fundulosoma*, there is a prominent anterior triangular flap on the sub-proximal region of the maxilla (Fig. 3L). *Fundulosoma thierryi*, the only species of the genus, also differs from all other species of the Nothobranchiini by possessing a lyre-shaped caudal fin, with posterior fin extensions on the dorsal and ventral tips of the fin with variable length in different populations.

Pronothobranchius is here diagnosed by nine unique apomorphic morphological conditions: the posterior margin of the premaxillary ascending process is perpendicular to the main axis of the process, resulting in a sub-rectangular process (Fig. 3C), instead of oblique, forming a subtrapezoidal process as in other Nothobranchiini (Fig. 3B, D–J); the anterior and posterior ceratohyals are separated by a broad cartilaginous interspace (Fig. 5I), vs. a narrow interspace (Fig. 5J); the proximal extremity of the first epibranchial is wide, about seven times wider than the distal extremity (Fig. 7E), thus differing from the relatively narrow epibranchial extremity of other Nothobranchiini (Fig. 7F–I); the interarcual cartilage is long, longer than the first epibranchial (Fig. 7E), vs. shorter (Fig. 7F–I); the articulatory cartilaginous head of the proximal process of the third epibranchial is broad, about twice as broad as other third epibranchial cartilaginous heads (Fig. 7J), vs. nearly equal or slightly broader (Fig. 7K–L); the anterior condyle of the second pharyngobranchial forms an angle >90° (Fig. 7E), vs. < or ~90° (Fig. 7F–I); the neural spine of the second vertebra is distinctively broad, reaching about four times the width of the neural spine of the third vertebra (Fig. 6D), vs. about twice as wide (Costa, 2015: fig. 5B); the posterior bones of the caudal skeleton are distinctively widened, with the dorsal and ventral margins of the hypural plate forming an angle >90° (Fig. 6B), vs. <90° (Fig. 6A, C); and the shoulder girdle is broad and compact, exhibiting a pointed expanded flap on the anterior portion of the

cleithrum (Fig. 6G), vs. a similar flap absent (Figs. 6E, F, H, J). Another derived morphological character state present in *Pronothobranchius* but similarly occurring in the *Nothobranchius* subgenus *Zononothobranchius* is the presence of pointed expansions on the anterior margin of the hyomandibula (Fig. 4B; vs. expansions absent, Fig. 4A, B).

MONOPHYLY OF *NOTHOBRANCHIUS* AND INCLUDED SUBGENERA

Nothobranchius was described by Peters (1868) to include a single species, *Cyprinodon orthonotus* Peters, 1844, but this genus was consistently considered as valid only after 1924. Ahl (1924b) resurrected *Nothobranchius*, but Myers (1924) considered *Nothobranchius* as a monotypic genus, besides describing *Adiniops* as a subgenus of *Aphyosemion*, including other species today placed in *Nothobranchius*. Nine years later, Myers (1933) recognized *Adiniops* as a synonym of *Nothobranchius*, establishing its limits as it is at present. In this broadly used sense, *Nothobranchius* comprises ~70 valid species with high morphological diversity. Its monophyly is supported here, but only three apomorphic morphological character states may unambiguously diagnose *Nothobranchius*: the lachrymal canal is long, its longitudinal length being about two or three times the bone width (Fig. 6Q), instead of being compact, with length about equal or slightly longer than bone width (Fig. 6Q); the basal portion of the middle radials of the anal fin terminates in a long and pointed posterior processes, connected by ligaments to the adjacent median radial (Fig. 6O), vs. process absent in other taxa (Costa, 2009c: fig. 5A); and the preopercular canal is opened, instead of being completely or partly closed in *Fundulosoma* and *Pronothobranchius*. Another derived condition, consisting of a constriction on the posterior portion of the third basibranchial (Fig. 5B–D), is here interpreted as synapomorphic for *Nothobranchius*, but reversed in the *N. janpapi* group (Fig. 5F); in other aplocheiloids, that constriction is absent (Fig. 5A).

Seegers (1985) described the monotypic genus *Paranothobranchius* for the large piscivorous *P. ocellatus*, diagnosing it by external morphological characters, including large size, pointed snout, slender body, numerous scales on the longitudinal series and posteriorly placed dorsal fin. Wildekamp & Haas (1992) considered *Paranothobranchius* as a subgenus of *Nothobranchius*, containing *N. ocellatus*, endemic to the coastal rivers of eastern Tanzania, and three species from the river basins of southern Somalia and Kenya: *N. bojiensis*, *N. fasciatus* and *N. microlepis*. Wildekamp & Haas (1992) also used characters of external morphology

with great plasticity among aplocheiloid killifishes to diagnose *Paranothobranchius*: numerous irregularly arranged scales on the longitudinal series, large mouth with strong dentition, long head, male hump, and posteriorly placed dorsal and anal fins. However, the present study indicates that *Paranothobranchius* is polyphyletic. The clade comprising *N. bojiensis*, *N. fasciatus* and *N. microlepis* is part of a basal lineage of *Nothobranchius* that also includes *N. virgatus*, and which is supported by five unique morphological synapomorphic conditions: the posterior process of the quadrate is short, its length being distinctively shorter than the basal portion of the bone without process (Fig. 4C), thus differing from other taxa, in which the process is about equal or slightly longer than the basal portion of the quadrate (Fig. 4A, B, D); the osseous flap situated between the distal and posterior processes of the third epibranchial is expanded, extending beyond the line between these processes (Fig. 7K–L), instead of not surpassing that line (Fig. 7H); the first post-cleithrum is absent, vs. present in other taxa; the pelvic bone is broad, slightly wider than long (Fig. 6K), instead of being longer than wide (Fig. 6L); and there are seven well-developed pelvic-fin rays, instead of five or six well-developed rays, sometimes with a seventh rudimentary ray. This clade was formerly supported by molecular data, but with low branch support (Dorn *et al.*, 2014). Both the morphological and the combined datasets generate trees corroborating this clade with high support values (Figs 1 and 2).

The clade containing *N. bojiensis*, *N. fasciatus* and *N. microlepis* is a morphologically distinctive group of relatively deep-bodied species, here recognized as a new subgenus, *Cynobranchius*, diagnosed by 16 unambiguous morphological synapomorphies. Nine of these synapomorphies are concentrated in the branchial and hyoid arches and are probably related to their specialized feeding habits that mainly include minute components of the zooplankton (Wildekamp & Haas, 1992). The most obvious is the presence of long and numerous gill-rakers on the first branchial arch (Fig. 5C), a condition frequently found in zooplankton feeders (compare with the short gill-rakers in *N. ocellatus*, Fig. 5D). Although most species of *Nothobranchius* have relatively long gill-rakers on the first branchial arch (Costa, 2009a), in *Cynobranchius* they are exceptionally numerous, with six or seven gill-rakers on the dorsal portion and 18 to 20 on the ventral, in contrast to one to five + 8–15 as in other Nothobranchiini. Other character states involving branchial and hyoid arches are: the basihyal is triangular and broad (Fig. 5C), a shape never occurring in other Nothobranchiini taxa (Fig. 5B, D, F–G); the anterior extremity of the ventrolateral process of the urohyal is much more posteriorly placed than the urohyal dorsal process (Fig. 5K),

in contrast to being positioned nearby (Fig. 5L); the anterior portion of the anterior ceratohyal is steeply directed upwards (Fig. 5J), instead of approximately horizontal or slightly directed upwards (Fig. 5I); there are three branchiostegal rays attached to the anterior portion of the anterior ceratohyal (Fig. 5J), vs. two in other Nothobranchiini (Fig. 5I); the proximal process of the third epibranchial is distinctively longer than the distal process (Fig. 7K), thus contrasting with nearly equal process occurring in other taxa (Fig. 7L); the second pharyngobranchial is wider than long in dorsal view (Fig. 7B), vs. longer than wide (Fig. 7C); the teeth of the third pharyngobranchial are minute (Fig. 7B), never well developed as in other taxa (Fig. 7C); and the dentigerous plate of the fourth pharyngobranchial is lengthened, acquiring a sub-rectangular shape and extending above the third epibranchial (Fig. 7B), thus differing from the relatively shorter bone, rounded, limited to the area between the third pharyngobranchial and the fourth epibranchial (Fig. 7C). In addition, three unique apomorphic conditions shared by species of *Cynobranchius* are related to dentition and jaw morphology: there is a deep oblique notch on the dorsal surface of the ascending process of the premaxilla (Fig. 3D), whereas in other members of the Nothobranchiini a shallow depression is present in that position (Fig. 3E); the teeth of the outer row of the dentary are short and conical (Fig. 3D), instead of being fang shaped (Fig. 3B, C); and there is no tooth on the vomer, in contrast to teeth being present in other Nothobranchiini.

Species of *Cynobranchius* also differ from other Nothobranchiini by having pale colours in males, remarkably lacking the red pigmentation occurring in other Nothobranchiini. This condition is similar to that exhibited by the South American aplocheiloid subgenus *Cynolebias*, in which male coloration is as pale as in females and red pigmentation, when rarely present, is restricted to the anal fin (Costa, 2001). Species of the latter group, instead of using visual performances during courtship behaviour as in other aplocheiloids, use sounds (Belote & Costa, 2003; Costa *et al.*, 2010) that are produced by the specialized pharyngobranchial apparatus (Costa, 2009d). Reports of reproductive behaviour indicate that courtship in *Cynobranchius* differs greatly from other Nothobranchiini (i.e. male approaching female from below, vs. laterally; Wildekamp & Haas, 1992), but studies directed to detect sounds are not available for this fish group. However, two unique character states shared by species of *Cynobranchius* are possibly related to specialized reproductive behaviour: in males, there is a widened structure on the lateral portion of the genital region, suggesting a pumping mechanism for fertilization, and in females, the anal fin is proportionally longer than in

any other Nothobranchiini, with a length about twice the fin width.

Other unique morphological features shared by species of *Cynobranchius* are: the pelvic fin is long in males, reaching the anterior portion of the anal fin, thus contrasting with other Nothobranchiini that have shorter pelvic fins, not or barely touching the anterior margin of the anal fin; and the presence, in males, of a dark grey bar on the suborbital region, a colour pattern never occurring in other Nothobranchiini. Another derived character state shared by all species of *Cynobranchius*, but homoplastically occurring in species of the *N. rachovii* group, subgenus *Nothobranchius*, is the long, rod-shaped anterior process of the opercle (Fig. 4C), differing from the short and rectangular process in other taxa (Fig. 4B).

The new monotypic subgenus *Plesiobranchius*, the sister group of *Cynobranchius*, is endemic to the White Nile River drainage. It is diagnosed by two unique apomorphic features: the lateral portion of the second pharyngobranchial is truncate in dorsal view (Fig. 7F), instead of pointed (Fig. 7E); and the fifth ceratobranchial is broad and triangular in dorsal view (Fig. 5B), instead of narrow and boomerang shaped (Fig. 5C) as in all other congeners, except in *N. luekei*, in which this bone is short and compact (Fig. 5F).

Paranothobranchius as here delimited contains a single large pike-like species, *N. ocellatus* (Fig. 10B, C), and is diagnosed by 17 apomorphic conditions. Four of these diagnostic character states are concentrated in the highly modified jaw structure. The huge jaws, formed by long bones with numerous, relatively small teeth (Fig. 3J), are unique among the Nothobranchiinae. The lower jaw is ~1.5 times longer than the distance between the anterior tip of the quadrate to the posterior margin of the preopercle (Fig. 4D), thus differing from other taxa, in which the lower jaw is shorter or only slightly longer than that distance (Fig. 4B). The premaxilla is remarkable by exhibiting a deep concavity on the its medial portion, close to the symphysis, and by having the anterior process of the alveolar arm of the premaxilla rudimentary (Fig. 3J), besides bearing hypertrophied teeth in the inner row of the premaxilla that are about four times larger than the teeth of the outer row (Fig. 3K), conditions not occurring in other Nothobranchiini (Fig. 3B–I). Three other diagnostic derived character states comprise modifications of the jaw suspensorium and the neurocranium in the area of articulation with the jaw suspensorium: the apical region of the autopalatine is distinctively thick and dorso-ventrally flattened (Fig. 4D), vs. thin, laterally flattened (Fig. 4C); the antero-dorsal condyle of the hyomandibula is distinctively longer than the posterodorsal condyle (Fig. 4D), vs. about equal in other taxa (Fig. 4C); and there is a pointed anterior expansion on the lateral process

of the sphenotic (Fig. 6R), vs. a similar process never present (Costa, 2011b: fig. 3C).

The specialized traits involving the modified structure of the jaws and jaw suspensorium in *N. ocellatus* have been hypothesized to be related to its piscivorous habits and in part have arisen in parallel in the South American piscivore aplocheiloid killifish *Austrolebias prognathus* Amato, 1986 (Costa, 2011b). Another three species of *Nothobranchius*, *N. kuhntae*, *N. lucius* and *N. orthonotus*, to a lesser extent have specialized predator jaws, including the presence of an inner row of large teeth directed inside the mouth (Fig. 3E, I). In a preliminary morphological analysis involving fewer taxa and fewer characters (Costa, 2011b), *N. ocellatus* was considered to be the sister species of *N. orthonotus*, a hypothesis herein refuted in both the morphological and combined analyses. The distribution of character states in the present tree topology indicates that predator jaw morphology (character state 10.1) arose three times independently in the genus *Nothobranchius*, once in the subgenus *Nothobranchius* (clade *N. kuhntae* plus *N. orthonotus*), once in *Paranothobranchius* (*N. ocellatus*) and once in *Adiniops* (*N. lucius*) (Figs 1 and 2). Another autapomorphic condition of *Paranothobranchius* tentatively interpreted as functionally associated with the piscivorous habits is the presence of small denticles on the gill-rakers of the first ceratobranchial (Fig. 5D, E), which would act to avoid prey escaping through the branchial opening, a condition not found elsewhere among African aplocheiloids. In all other African aplocheiloids, small denticles are not present on gill-rakers of the first branchial arch (Fig. 5C), but a similar condition is also found in South American predator aplocheiloids of the *Cynolebias* zeta-clade (Costa, 2001).

Other unique features found unequivocally to diagnose *Paranothobranchius* are: the posterior process of the parasphenoid is robust and long, reaching the posterior portion of the basioccipital (Fig. 6R), vs. thin, reaching the middle portion of the basioccipital (Costa, 2011b: Fig. 3C); the total number of vertebrae is 36–38, vs. 26–32 in all other taxa; the ventral extremity of the cleithrum projects ventrally much beyond the scapula as a result of a lengthened central process of the cleithrum (Fig. 6I), vs. the extremities of cleithrum and scapula being placed in close proximity (Fig. 6E–H); there are six or seven neuromasts in the posterior section of the supraorbital neuromast series, vs. three or four; scales are irregularly arranged on the flank, vs. regularly; scales extend at ~70% of the caudal-fin base in males, vs. about at only 25–30%; the orbital membrane is free except on its dorsal region, vs. completely attached; the pelvic-fin bases are separated by interspace, vs. in contact or united; and there is an ocellate spot on the middle part of the basal

portion of the caudal fin in females (vs. ocellate spot always absent).

As previously supported by molecular evidence (Dorn *et al.*, 2014), the present study indicates that *Paranothobranchius* is not closely related to *Cynobranchius* as proposed by Wildekamp & Haas (1992). However, it supports *Paranothobranchius* as the sister group to a clade here formally recognized as subgenus *Zononothobranchius*, which both share two unambiguous synapomorphies: teeth are broadly distributed on the middle portion of the vomer (Fig. 6R), instead of being restricted to its antero-middle portion (Costa, 2011b: fig. 3C); and the osseous membrane of the pelvic bone is poorly developed (Fig. 6M, N), in contrast to being well developed (Fig. 6K, L). Other derived character states that corroborate *Paranothobranchius* and *Zononothobranchius* relationships are the presence of a rudimentary dorsal process of the urohyal (Fig. 5M, N; vs. well developed, Fig. 5K, L), with reversals in two species of the latter subgenus (*N. robustus* and *N. steinforti*); and a broad anterior condyle of the second pharyngobranchial that is more than twice as wide as the medial condyle (Fig. 7H, I; vs. narrow, Fig. 7E–G), independently acquired in *N. orthonothus* and *N. kuhntae*, and reversed in *N. robustus*.

Zononothobranchius, as here delimited, includes *N. rubroreticulatus* from Chad lake drainage, its type species (Radda, 1969), and several other species occurring in the uplands of central Africa. Monophyly of *Zononothobranchius* is supported by two unique morphological synapomorphies: the anterior process of the alveolar arm of the premaxilla is triangular (Fig. 3F), a condition never occurring in other Nothobranchiini (Fig. 3B–E), and the basihyal is nearly trapezoidal in dorsal view (Fig. 5G), thus differing from the subtriangular, triangular or rectangular basihyal of other members of the Nothobranchiini (Fig. 5B–F). Species of *Zononothobranchius* also share a derived morphology of the hyomandibula, in which there are pointed expansions on its anterior margin (Fig. 4E; vs. pointed expansions absent, Fig. 4C), but a similar derived condition is also present in *Pronothobranchius* (see Discussion about *Pronothobranchius* above).

The subgenus *Nothobranchius* as here delineated comprises a clade endemic to a southern African area, including river basins of central and southern Mozambique and adjacent areas. This clade, well supported by molecular data (Shidlovskiy *et al.*, 2010; Dorn *et al.*, 2014), is morphologically heterogeneous, including a subclade of small species, the *N. rachovii* species group (Shidlovskiy *et al.*, 2010), usually reaching ~35 mm SL, and a clade of larger species reaching between ~45 and 60 mm SL, among which is *N. orthonotus*, with specialized predator-like dentition (Fig. 3E), which often preys on small vertebrates (Polacik &

Reichard, 2010). *Nothobranchius* is morphologically diagnosed by a single character state, consisting of a well-developed lateral flap on the proximal region of the fourth ceratobranchial (Fig. 5H; vs. flap absent, Fig. 5F, G). The presence of a lateral flap on the fourth cera-tobranchial is also found in most non-Nothobranchiini Aplocheiloidei (Fig. 5A), which is a primitive condition for the Nothobranchiinae (e.g. Costa, 2015). The condition occurring in the subgenus *Nothobranchius* is therefore interpreted as a reversal, because in all other Nothobranchiini that flap is rudimentary or absent (Fig. 5B–G). In addition, the second basibranchial in species of the subgenus *Nothobranchius* has a sharp pointed lateral process (Fig. 5H), but this condition is not assigned as synapomorphic because of the great morphological variability of this bone among the Nothobranchiini (e.g. Fig. 5B, C, E–H), making delimitation of character states subjective.

Adiniops is here recognized as a valid subgenus for a diversified clade, highly supported by molecular data (Dorn *et al.*, 2014). However, no unique synapomorphic condition was found to corroborate it, although the clade is well supported in the combined analysis presented here (Fig. 2). The analysis indicates that the presence of an anterior expansion on the sub-distal portion of the autopalatine (Fig. 4F; vs. expansion absent, Fig. 4C) that occurs in the two basal-most lineages of *Adiniops* is synapomorphic for this subgenus, but reversed in a clade containing all other species.

The morphological comparative analysis revealed some character states uniquely shared by *N. janpapi*, *N. geminus* and *N. luekei*: the basihyal is narrow, and approximately rectangular in dorsal view (Fig. 5F), a shape not occurring in any other congener (Fig. 5B–D, G, H); the third basibranchial is not constricted in its posterior portion (Fig. 5F), considered as a reversal because a derived constriction is present in all other congeners (Fig. 5B–D); the interarcual cartilage is minute, reaching less than half first epibranchial length (vs. two-thirds or more, Fig. 7E–I); and the caudal fin bears a middle projection, a morphological condition often present in the Aplocheilinae and in basal lineages of the Nothobranchiinae, but never in other Nothobranchiini. However, the analyses consistently indicated that the clade comprising *N. janpapi*, the type species of *Aphyobranchius*, *N. geminus* and *N. luekei*, is inside a more inclusive clade containing species of *Adiniops* (Figs 1 and 2). The segregation of *N. janpapi*, *N. geminus* and *N. luekei*, or even the closely related *N. fuscotaeniatus*, into a separate subgenus would result in a paraphyletic *Adiniops*. Therefore, *Aphyobranchius* is placed here in the synonymy of *Adiniops*.

Nothobranchius janpapi, *N. geminus* and *N. luekei* also differ greatly from other congeners by living close to the water surface (personal observation), contrasting with other Nothobranchiini that live at mid-water or close to the bottom. As typical surface-dwelling taxa, these species are relatively more slender than other congeners, a condition resulting in a narrower dorso-ventral constitution of several bones, including cleithrum (Fig. 6J), hypural plate and first vertebrae (Costa, 2009a). However, *N. janpapi* differs from *N. geminus* and *N. luekei* by some morphological jaw structures. In *N. janpapi*, the teeth of the outer series of the dentary are curved near the tip, there is a distinctive small process on the medial portion of the maxilla, and the ascending process of the premaxilla is short (Fig. 3G). Uniquely in *N. geminus* and *N. luekei*, the ascending process is robust and long, but teeth are not curved as in most other congeners (Fig. 3H).

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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:

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 Nothobranchiini.
Table S1. Data matrix of distribution of character states of 138 morphological characters among 43 terminal taxa.