#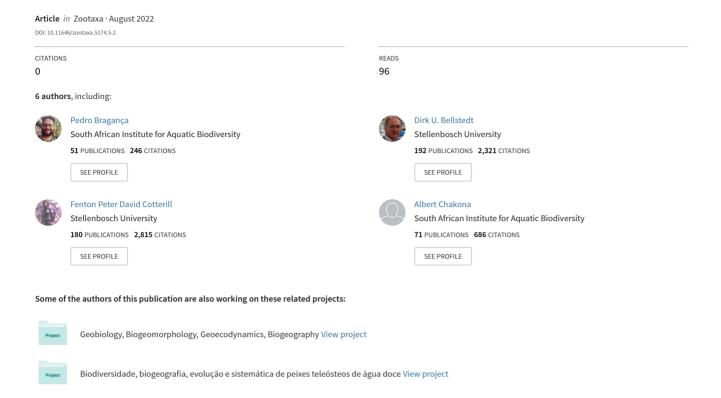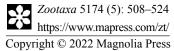 Nothobranchius balamaensis (Cyprinodontiformes: Nothobranchiidae), a new species of annual killifish from northern Mozambique

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Article

Nothobranchius balamaensis (Cyprinodontiformes: Nothobranchiidae), a new species of annual killifish from northern Mozambique

PEDRO H. N. BRAGANÇA[1*], DIRK U. BELLSTEDT[2,6], P. DE WET VAN DER MERWE[2,7], FENTON P.D. COTTERILL[3,8], BRIAN R. WATTERS[5,9] & ALBERT CHAKONA[1,4,10]

[1]*NRF-South African Institute for Aquatic Biodiversity, Makhanda (Grahamstown), South Africa, 6140*
[2]*Department of Biochemistry, Stellenbosch University, South Africa*
[3]*Department of Earth Sciences, Stellenbosch University, South Africa*
[4]*Department of Ichthyology and Fisheries Science, Rhodes University, Makhanda (Grahamstown), South Africa, 6140*
[5]*6141 Parkwood Drive, Nanaimo, British Columbia V9T 6A2, Canada*
[6] *https://orcid.org/0000-0002-6376-4855*
[7] *https://orcid.org/0000-0001-9315-599X*
[8] *https://orcid.org/0000-0003-4561-018X*
[9] *https://orcid.org/0000-0002-7651-6500*
[10] *https://orcid.org/0000-0001-6844-7501*
Corresponding author: ✉ pedrobra88@gmail.com, ✉ p.braganca@saiab.ac.za; ✉ https://orcid.org/0000-0002-8357-7010

Abstract

A new seasonal killifish of the genus *Nothobranchius* is described from the Montepuez River system in northern Mozambique. The new species, *Nothobranchius balamaensis* Bragança & Chakona, is differentiated from congeners by its characteristic colour pattern and molecular data further support its taxonomic distinctiveness. Phylogenetic results based on two mitochondrial and three nuclear genes confirms *N. balamaensis* is closely related to *N. kirki* and *N. wattersi*; all three belonging to the Coastal-Inland Clade. The new species is most similar in colour pattern to *N. kirki* sharing the characteristic of a deep red-orange colouration in the basal, proximal, and medial zones of the caudal and anal fins that grades to orange in the distal zone. This is a key feature that distinguishes these two species from all other *Nothobranchius*. The main distinguishing features between the new species and *N. kirki* is the presence of a light blue to white band or series of irregular markings in the proximal zone of the anal fin in *N. kirki*, versus the absence of such a colour pattern element in *N. balamaensis*, as well as differences in the dorsal fin pattern. When compared to all population groups of *N. wattersi*, the colour pattern of *N. balamaensis* is distinctive. *Nothobranchius balamaensis* is a relatively slender member of the genus, a characteristic that clearly distinguishes it from both *N. kirki* and *N. wattersi*. *Nothobranchius balamaensis* is currently only known from a few specimens from the type locality.

Key words: Cyprinodontiformes, Nothobranchiidae, Neogene rifting, Molecular phylogeny, *Nothobranchius kirki*, *Nothobranchius wattersi*

Introduction

The killifish genus *Nothobranchius* Peters 1868 comprises 95 species that inhabit seasonal pools in sub-Saharan Africa (Van der Merwe *et al.* 2021; Nagy 2021; Nagy *et al.* 2021; Nagy & Watters 2022) with a distribution extending from north-eastern Cameroon and Chad in the north-west to southern Somalia in the east, and southward through Uganda, parts of Kenya, Tanzania, Zambia, Mozambique, south-eastern DRC, south-eastern Zimbabwe, the Caprivi region of Namibia and north-eastern South Africa. This distribution closely matches that of savannah vegetation from central to southern Africa but also extends to the East African Coast where the vegetation consists of Mesic Miombo containing savannah elements in what is described as East African Mosaic (White 1983; Clarke 1998). It is in the latter region that the genus reaches its highest species diversity (Dorn *et al.* 2014; Van der Merwe *et al.* 2021). *Nothobranchius* are commonly referred to as 'annual' or seasonal killifishes, with all species having an annual or semi-annual life cycle, depending on regional rainfall patterns. Their life cycle is adapted to the seasonal drying up of their habitats, with very rapid growth and maturation of the fish. Their eggs, which are laid in the mud substratum

of seasonal pools, undergo repeated phases of dormancy or diapause (with a maximum of three), allowing them to survive the dry season (Peters 1963; Wourms 1965, 1972a, 1972b, 1972c; Furness *et al.* 2015; Pinceel *et al.* 2015). This is a complex process in which some eggs develop relatively quickly allowing them to hatch immediately at the onset of the rainy season, while others may remain dormant until a subsequent rainy season (Pinceel *et al.* 2015). The fishes rapidly develop into reproductively active adults usually within a few weeks (Polačik & Reichard 2011; Blažek *et al.* 2013) and live for short periods, ranging from a few weeks to, at most, a few months (Markofsky & Matias 1977; Watters 2009; Nagy 2015). Their rapid development has facilitated the use of *Nothobranchius* species, including *N. furzeri* Jubb, 1971, the fastest developing and shortest-lived vertebrate, as model species for ageing studies (Terzibasi *et al.* 2007; Cellerino *et al.* 2016; Platzer & Englert 2016).

In the past, the delimitation of species boundaries within *Nothobranchius* has mostly relied on the largely informative colour pattern of males, contrasting with a similar and conserved external morphology characterised by relatively deep body shape, comparable positioning of the dorsal and anal fins, and wide overlaps in scale and fin-ray counts. However, since the early part of this century, the use of molecular approaches facilitated not only the elucidation of *Nothobranchius* relationships (Dorn *et al.* 2014; Van der Merwe *et al.* 2021) but importantly, has also been a contributing factor in the identification and description of a significant number of new species over the past 13 years (e.g. Wildekamp *et al.* 2009; Ng'oma *et al.* 2013; Nagy *et al.* 2017; Watters *et al.* 2020; Nagy *et al.* 2020; Nagy 2021; Nagy *et al.* 2021). This has allowed a much more rigorous approach in which morphological and molecular lines of evidence are compared and taxonomic decisions are based on consilience between the two, which has gained almost universal support not only in fish taxonomy but organismal taxonomy in general.

In this context, Costa (2018) proposed a subgeneric revision of the tribe Nothobranchini based on the examination of osteological and morphological characters combined with the molecular data of Dorn *et al.* (2014). Costa (2018) provided evidence supporting the recognition of six subgenera within *Nothobranchius* of which five: *Cynobranchius* Costa, 2018, *Nothobranchius* Peters, 1868, *Paranothobranchius* Seegers, 1985, *Plesiobranchius* Costa, 2018 and *Zononothobranchius* Radda, 1969, have been confirmed and supported by the comprehensive phylogenetic analysis of Van der Merwe *et al.* (2021). In his study, Costa (2018) corroborated the presence of unique morphological characters defining the subgenus *Aphyobranchius*, but synonymised it into the subgenus *Adiniops*, with members of the Coastal-Inland Clade (*sensu* Van der Merwe *et al.* 2021). Considering the low support values in both Costa (2018) and Van der Merwe *et al.* (2021), further molecular and morphological evidence is therefore required before the two subgenera and the Coastal-Inland Clade can be clearly defined.

Despite northern Mozambique having a predominantly tropical climate and the presence of extensive seasonal swamps and wetlands, which are ideal habitats for *Nothobranchius* killifishes, prior to the discovery of the new species described herein, only five species of the genus were known from that region. These species are: *N. hengstleri* Valdesalici, 2007, the type locality of which is an isolated pool near Nassoro village in the Cabo Delgado Province (Valdesalici 2007); *N. krammeri* Valdesalici & Hengstler, 2008 which is known from the Meronvi River, also in the Cabo Delgado Province (Valdesalici & Hengstler 2008); *N. makondorum* Wildekamp, Shidlovskiy & Watters, 2009 which has a broad distribution range from the Melela River in Mozambique extending into the coastal lowland rivers Mbwemkuru, Lukuledi and Rovuma in south-eastern Tanzania (Wildekamp *et al.* 2009); *N. niassa* Valdesalici, Bills, Dorn, Reichwald & Cellerino, 2012 which is currently known from seasonal pools and streams in the Rovuma River system in the Niassa Province (Valdesalici *et al.* 2012); and *N. kirki* Jubb, 1969 only recently collected from a locality in the southern extremity of the Niassa Province (H. Hengstler, pers. comm.). *Nothobranchius kirki* was previously only known from the drainage basin of lakes Chilwa and Chiuta in Malawi; however, the Mozambique population occurs in the headwaters region of a tributary subsystem of the Lurio River, that drains east-north-eastwards through northern Mozambique to the Indian Ocean. The first four of these aforementioned species belong to the subgenus *Adiniops*, whereas the last-mentioned, *N. kirki*, is a member of the Coastal-Inland Clade (Van der Merwe *et al.* 2021).

An expedition conducted in 2013 during a consultancy assessment by AC, in tributaries of the Montepuez River system close to Balama village in the Cabo Delgado Province, northern Mozambique, resulted in the collection of *Nothobranchius* specimens with a distinctive colour pattern and morphological features that differentiate them from currently described *Nothobranchius* species from this region. In a most recent comprehensive phylogeny including 75 species and five undescribed species of *Nothobranchius*, Van der Merwe *et al.* (2021) provided further evidence for the genetic distinctiveness of these specimens (designated in that study as *Nothobranchius* sp. 'Northern Mozambique'). Here we formally describe this new species as *Nothobranchius balamaensis* Bragança & Chakona.

Materials and methods

Specimen collection. Specimens of the new species were collected in March 2013 using a SAMUS725 Electrofisher. A further 10 specimens of the sympatric *Nothobranchius makondorum* were also collected from the same locality. Captured fishes were euthanised with clove oil following recommended ethics guidelines of the NRF-South African Institute for Aquatic Biodiversity (NRF-SAIAB). A small piece of muscle tissue was taken and preserved in 95% ethanol, and source specimens were fixed in 10% formalin for at least 10 days. Voucher specimens were put through 10% and 50% ethanol to gradually wash out the formalin and eventually transferred to 70% ethanol for long-term storage. The type material has been deposited at National Research Foundation – South African Institute for Aquatic Biodiversity (NRF-SAIAB), Makhanda/Grahamstown, South Africa.

Molecular analysis. The extraction of genomic DNA, PCR amplification of genes used for phylogenetic analyses, their sequencing and alignment are as described in Van der Merwe *et al*. (2021). The sequences used for phylogenetic analysis were from Van der Merwe *et al*. (2021) and of species recently described in Nagy *et al*. (2020), Watters *et al*. (2019) and Watters *et al*. (2020). However, only one representative per species was chosen for this study with the exception of *N. virgatus* (where two specimens were used due to their geographic range), *N. neumanni* (because they spanned distinct drainages) and in closely related taxa (4 specimens of *N.* sp. 'Southern Kenya') including two specimens of *N. balamaensis*.

The aligned sequences of the different genes obtained from the above taxa were combined, and each gene treated as a separate partition. Phylogenetic analyses were performed using maximum likelihood (RaxML 8.0) (Stamatakis 2014) on the CIPRES Science Gateway (Miller *et al*. 2010), as described in Van der Merwe *et al*. (2021). Prior to phylogenetic analyses each gene alignment was assessed for the best-fitting molecular model on the CIPRES Science Gateway using jModelTest2 v2.1.6 (Guindon & Gascuel 2003; Darriba *et al*. 2012). Although new gene sequences were added to the alignment matrices as presented in Van der Merwe *et al*. (2021) the best-fitting molecular model was still found to be GTR+I+G for all gene regions and was implemented in these analyses. Node support was assessed by bootstrap analysis.

Morphological analysis and colour pattern. Morphometric measurements and meristic counts of the three male specimens and one female specimen were taken as described in Nagy (2014) with the following additional counts: caudal-, anal- and pelvic-fin rays. Measurements were made under a dissecting microscope using a digital calliper and rounded to the nearest 0.1 mm. All body measurements are presented as percentages of standard length (SL) and all head measurements are given as percentages of head length (HL). Terminology for the cephalic neuromast series follows Costa (2001) and frontal squamation description follows Hoedeman (1958). Measurements and counts for the new species are presented in Table 1. Comparative information regarding colour pattern and some morphological characters of close congeners belonging to the Coastal-Inland Clade (*sensu* Van der Merwe *et al*. 2021), and other northern Mozambique *Nothobranchius* species, were derived from both the literature (Valdesalici 2007; Valdesalici & Hengstler 2008; Wildekamp *et al*. 2009; Valdesalici *et al*. 2012; Ng'oma *et al*. 2013), as well as from the personal records and photographs of one of the present authors (BRW).

Species concept. The Evolutionary Species Concept (ESC) of Wiley (1978), as encompassed by the Phylogenetic Species Concept (PSC) and additionally the Recognition Species Concept (RSC) (Mayden 1999, 2002), was used to recognise this distinct species. Complementary datasets in the conceptual framework of the ESC (Cotterill *et al*. 2014) were used to achieve a consilient solution to the definition of this species. We did not use the Genetic Species Concept (GSC) (Baker & Bradley 2006) because we are not in agreement with the arbitrary thresholds of genetic distance (ca. 5% cytochrome b divergence) used in attempts to delineate a 'good' species as this confounds attempts to define subspecies according to the Biological Species Concept (BSC) (Cotterill *et al*. 2014).

Results

The results of the molecular analysis, specifically regarding primer specificity, gene amplification, codon positions and clade descriptions are described in greater detail in Van der Merwe *et al*. (2021). Genbank Accession Numbers of the sequences used in this study can be obtained from Van der Merwe *et al*. (2021), Nagy *et al*. (2020), Watters *et al*. (2019) and Watters *et al*. (2020). The taxa referred to *Nothobranchius* sp. 'Madi Opei' and *Nothobranchius* cf. *eggersi* in the phylogeny of Van der Merwe *et al*. (2021) have been recently described as *Nothobranchius elucens* (Nagy 2021) and *Nothobranchius nikiforovi* (Nagy *et al*. 2021) respectively and are indicated in this phylogeny with

these new names. The alignment lengths of the sequenced genes were as follows: COI, 1551 base pairs (bp); ND2, 1045 bp; Glyt, 977 bp; MyH6, 840 bp; and SNX33, 983 bp respectively with a total alignment length of 5396 bp. The most likely phylogenetic tree retrieved in the Maximum Likelihood analysis onto which the bootstrap values have been drawn on the nodes is presented in Figure 1.

Nothobranchius balamaensis, new species, is retrieved as sister to *N. kirki* and *N. wattersi* together with *Nothobranchius* sp. 'Southern Kenya', the sister species to the previous three species, in a group referred to as the Coastal-Inland Clade by Van der Merwe *et al.* (2021). This clade is sister to a larger group consisting of a clade encompassing the subgenus *Aphyobranchius* and members of the subgenus *Adiniops* in what is referred to as the Coastal Clade (Dorn *et al.* 2014; Van der Merwe *et al.* 2021).

As shown in Figure 1, *Nothobranchius balamaensis* is phylogenetically distinct from other species known to occur in northern Mozambique: *N. makondorum*, *N. niassa*, *N. krammeri*, *N. kirki* (marked with stars) and, by inference, *N. hengstleri*. The latter species, although not included in the phylogeny (Figure 1), was placed in the *N. melanospilus* group by Valdesalici (2007), based on colour pattern and morphological characters. That position was later confirmed by the molecular component of a study by Wildekamp *et al.* (2009) in which *N. hengstleri* was retrieved as a member of a well-supported monophyletic group including *N. makondorum*. The close genetic association between these two species suggests, therefore, that *N. balamaensis* is likely also phylogenetically distinct from *N. hengstleri*.

The results of morphometric and meristic determinations for the holotype and paratypes of the new species are presented in Table 1 and, where appropriate, discussed in following sections.

Taxonomy

Nothobranchius balamaensis Bragança & Chakona, new species
Figures 2, 3, 4; Table 1

Nothobranchius sp. "Northern Mozambique" Van der Merwe *et al.* (2021).

Holotype. SAIAB 190261, male, 29.8 mm SL; Muhukwa upstream bridge on second tributary south-east of Balama village, Cabo Delgado Province, Mozambique, 13°25'59.1''S 38°36'41.2''E; Chakona, A; 05 Mar 2013.

Paratypes. SAIAB 190255, 2 males (27.5–28.9 mm SL), 1 female (29.0 mm SL); site and collection data as for holotype.

Diagnosis. *Nothobranchius balamaensis* is distinguished from all other members of the genus, except *N. kirki*, by the following unique combination of characters in males: body colouration bright iridescent light blue, with narrow brown-red to orange scale margins creating an irregular reticulated pattern; anal and caudal fins overall orange, darker in basal and proximal zones, grading through medial zone to lighter orange in distal zone and devoid of distinctive markings or banding; dorsal fin with marbled pattern in proximal region comprising irregular brown-red markings on yellow-orange background, distinct striated pattern parallel to fin rays in medial and distal zones with inter-ray membrane distinctive black between first 4–5 rays; anal fin shape mildly trapezoidal. *Nothobranchius balamaensis* differs from the closely related and most similar *N. kirki* by some elements of male colour pattern: anal fin devoid of markings or banding in proximal zone (vs. narrow light blue to white proximal band or series of irregular markings in *N. kirki*); dorsal fin with marbled proximal zone and prominently striated medial and distal zones (vs. marbled or vermicular pattern throughout proximal and medial zones with only a hint of striation pattern in distal zone of some specimens).

Description. General body features illustrated in Figures 2 and 3, morphometric and meristic characters of holotype and paratypes presented in Table 1. The specimens examined were sexually mature but had probably not reached maximum adult size, although this may be one of the smaller members of the genus, with standard length of 29.8 mm in the largest of the three available males. Dorsal profile concave to nearly straight on head, convex from nape to end of dorsal-fin base; slightly concave to nearly straight on caudal peduncle. Ventral profile convex from lower jaw to end of anal-fin base insertion; slightly concave to nearly straight on caudal peduncle. Body moderately elongate and slender. Greatest body depth at vertical at pelvic-fin origin. Head triangular in lateral view. Snout slightly pointed. Mouth directed upwards, lower jaw slightly projecting past anterior limit of upper jaw. Jaw teeth canine, irregularly arranged, outer teeth greater in number than inner teeth.

TABLE 1. Morphometric and meristic data for holotype and paratypes of *Nothobranchius balamaensis*. Holotype (H) values included in range, mean and standard deviation (SD) of males.

	Males (n = 3)				Female (n = 1)
	H	range	Mean	SD	
Standard length (mm)	29.8	27.5–29.8	28.8	1.0	29.4
Head length (mm)	9.1	8.7–9.1	8.9	0.2	9.3
Percent of standard length					
Total length	120.8	120.5–124.2	121.8	2.1	120.7
Body depth at pelvic fin origin	24.5	23.7–24.6	24.3	0.5	23.5
Head length	30.5	30.5–31.3	31.0	0.4	31.6
Preanal length	62.8	61.5–63.7	62.6	1.1	63.3
Predorsal length	61.7	60.9–61.7	61.4	0.4	61.6
Prepelvic length	50.7	50.7–54.0	52.1	1.7	52.0
Prepectoral length	35.2	35.2–36.3	35.9	0.6	35.0
Caudal peduncle length	17.8	17.8–18.0	17.9	0.1	17.3
Caudal peduncle depth	10.7	10.4–11.2	10.8	0.4	11.9
Dorsal-fin base length	22.5	21.8–22.5	22.1	0.4	21.4
Anal-fin base length	20.5	19.8–20.5	20.2	0.4	17.3
Caudal-fin length	21.8	21.2–22.5	21.8	0.6	21.4
Percent of head length					
Head width	52.7	51.1–54.0	52.6	1.5	51.6
Head depth	51.6	51.6–52.9	52.2	0.6	51.6
Interorbital width	36.3	33.3–36.3	34.3	1.7	31.2
Eye diameter	26.4	25.6–27.6	26.5	1.0	25.8
Snout to eye end length	49.5	48.3–51.1	49.6	1.4	49.5
Snout length	20.9	20.7–21.2	20.9	0.2	17.2
Meristics		range	Mode		
Dorsal-fin rays	16	16	16		17
Anal-fin rays	17	17–18	18		17
Caudal-fin rays	27	25–27	–		27
Pectoral-fin rays	17	16–17	17		17
Pelvic-fin rays	5	5	5		5
Scales mid-longitudinal series	28	27–28	27		27
Scales transverse	9	9	9		9
Scales circumpeduncular	12	12	12		12

Dorsal fin in male sub-rectangular, extremity rounded, with very short rudimentary fin-ray extensions along distal margin, tip reaching posterior portion of caudal peduncle; anal fin mildly trapezoidal with rounded extremity, short filaments along distal margin, tip reaching middle portion of caudal peduncle. In female, dorsal fin rounded, anal fin typically sub-triangular, slightly longer than dorsal fin, tip reaching middle portion of caudal peduncle. Caudal fin subtruncate, posterior margin slightly convex. Pectoral fin sub-elliptical, posterior extremity reaching pelvic-fin base. Pelvic fin small, tip reaching between anus and anal-fin origin in male, reaching anus in female; pelvic-fin bases in close proximity. Dorsal-fin origin on vertical slightly in front of anal-fin origin (mean for males: predorsal length as % of SL 61.4; preanal length 62.6). Dorsal and anal fin bases covered with opaque mucous film and with papillate contact organs along fin rays, denser on anal fin.
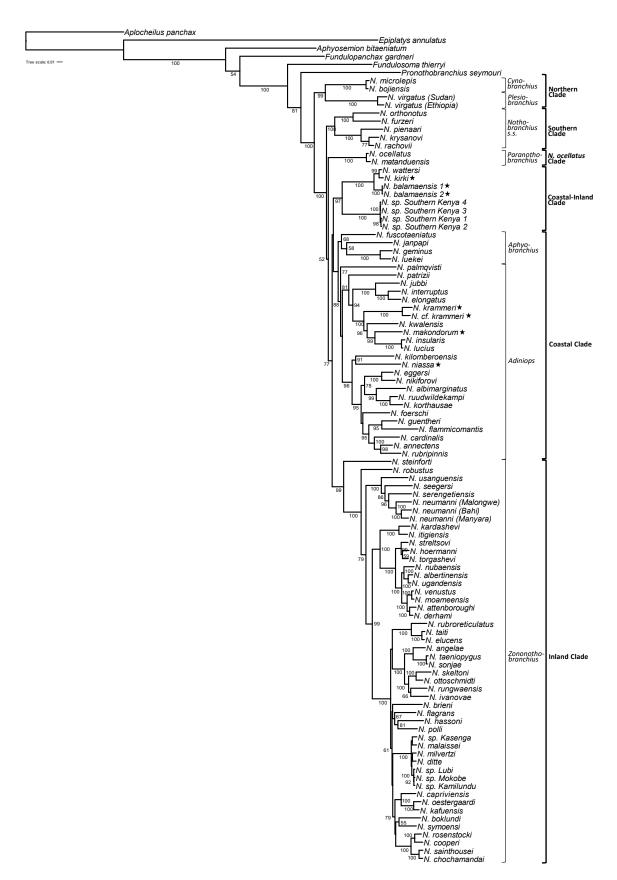
FIGURE 1. The results of the maximum likelihood analysis from the combined data set of five gene fragments. Node support as bootstrap values is indicated at the respective nodes. Only node support greater than 50% is indicated, if less it was omitted. Subgeneric classification and clades are indicated to the right of the species names. Species occurring in northern Mozambique are indicated with black stars following the species names.

Scales cycloid, body and head entirely scaled, except ventral surface of head (Fig. 4). Body squamation extending over 25% of caudal fin; no scales on dorsal and anal fin bases. Cephalic squamation pattern irregular G-type.

Two neuromasts anteriorly positioned, placed in shallow depressions, just before rostrum (Fig. 4). Anterior supraorbital series of neuromasts arranged in two separate sections, each placed in shallow depression, anterior section with one or two neuromasts, posterior section with two; additional minute neuromast may be present between depressions. Posterior supraorbital series with three to four neuromasts in shallow depression. Infraorbital series with 2–3 neuromasts in preorbital region followed by 12–15 minute neuromasts, and by one well-developed neuromast in posterior region; pre-opercular series in open groove with 14–19 neuromasts; mandibular 16–17. One neuromast per scale of lateral line.

FIGURE 2. *Nothobranchius balamaensis*, preserved specimens: **A**, male, holotype, 29.8 mm SL (SAIAB 190261); **B**, female, paratype, 29.0 mm SL (SAIAB 190255).

FIGURE 3. *Nothobranchius balamaensis*, male, holotype, 29.8 SL (SAIAB 190261) showing live colouration.
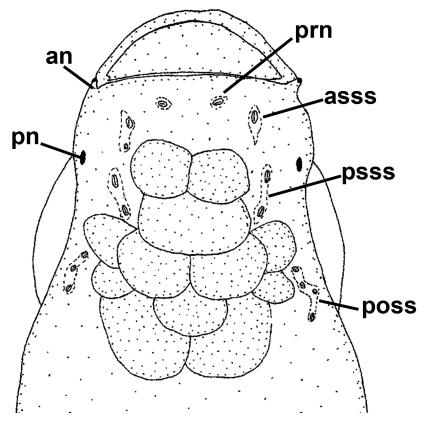
FIGURE 4. Diagrammatic representation of the latero-sensory system and frontal squamation on the dorsal surface of the head in *Nothobranchius balamaensis*. Abbreviations: asss: anterior section of the anterior supraorbital series; an, anterior naris; pn, posterior naris; psss: posterior section of the anterior supraorbital series; poss: posterior supraorbital series; prn: posterior rostral neuromast.

Colouration in alcohol. Male (Fig. 2A). Flank, dorsum and head light brown; body overall yellow-brown, lighter ventrally. Dorsal fin pale grey with chromatophores scattered over entire fin, concentrated close to insertion of fin rays; membrane between anterior rays black. Anal fin pale grey in proximal portion, hyaline in medial and distal portions. Caudal fin pale grey. Pectoral and pelvic fins hyaline, with minute chromatophores scattered over fin membrane.

Female (Fig. 2B). Flank and dorsum pale brown, side of head and ventral region pale yellow, darker in dorso-anterior region, chromatophores forming distinct reticulation pattern. All fins hyaline, with small chromatophores scattered over fin membrane.

Live Colouration. Male (Fig. 3). Scales on flank and head iridescent light blue to turquoise with dark brown-red margins, grading to orange posteriorly and ventrally, creating a reticulation pattern. Scales on the abdomen smaller, pale iridescent light blue with prominent orange margins. Scales on dorsum grade to blue-grey with darker grey margins. Lips, snout, frontal and dorsal portion of head light grey-brown. Ventral portion of head light blue to white. Dorsal fin with marbled pattern in proximal region, comprising blue to yellow-orange background with irregular brown-red marbled markings; medial and distal zones show distinct striated pattern parallel to fin rays which grade from light blue anteriorly through pale orange in the middle part of fin to darker orange posteriorly; inter-ray membrane distinctive black between the first 4–5 anterior rays, grading to dark brown-red and dark orange posteriorly; very short fin-ray extensions appear grey. Anal fin orange, darker in proximal zone, becoming lighter in medial and distal zones which show indistinct striation pattern parallel to the fin rays, in relatively darker and lighter shades of orange; short fin-ray extensions grey. Caudal fin deep orange in basal, proximal and medial zones grading to lighter orange in distal zone with striation pattern parallel to fin rays in relatively light and dark orange; narrow marginal zone grey. Pelvic fin orange. Pectoral fin hyaline. Iris yellow to blue, with indistinct black vertical bar through centre of eye.

Distribution. *Nothobranchius balamaensis* is known only from the type locality, in the seasonal Naconha River, a sub-catchment of the upper Montepuez River system in the Balama region of the Cabo Delgado Province, north-eastern Mozambique (Fig. 5).
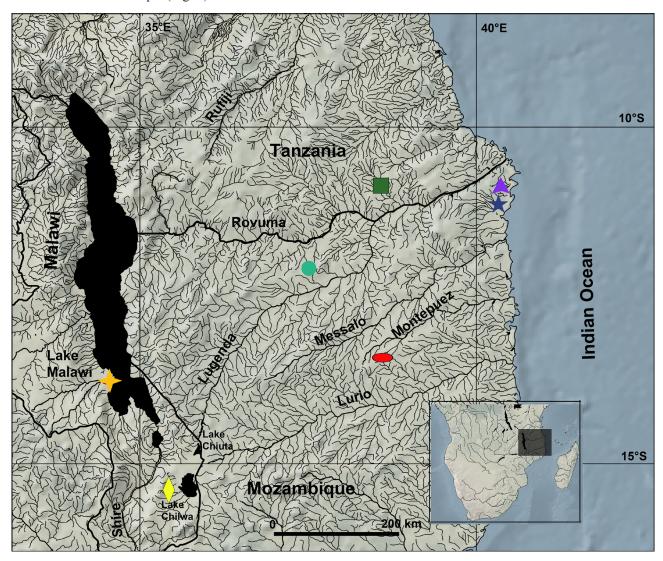

FIGURE 5. Type localities of *Nothobranchius* species occurring in northern Mozambique and neighbouring regions. Red elipse, *N. balamaensis*; yellow lozenge, *N. kirki*; orange four pointed star, *N. wattersi*; light blue dot, *N. niassa*; green square, *N. makondorum*; purple arrow, *N. hengstleri*; blue five pointed star, *N. krammeri*.

Ecology. The habitat comprised a pool in a seasonal stream course (< 1 m in depth) with turbid and slow flowing water (Fig. 6). The substratum was predominantly fine mud interspersed with sections of fine sand. The riverbanks were covered by grass with remnants of Miombo vegetation. *Nothobranchius balamaensis* occurred syntopically with *N. makondorum*, and the cyprinid *Enteromius litamba*.

The documented rainfall pattern affecting north-eastern Mozambique indicates an extended dry season in autumn, winter and spring (May to November) with a single distinct rainfall season from December to April. Total annual rainfall as for example at Mocimboa da Praia (969 mm), Pemba (874 mm) and Nampula (1059 mm) is typical for the region (after WorldClimate website 2016) and a similar rainfall pattern may be expected in the Balama area where *Nothobranchius balamaensis* occurs.

This unimodal rainfall pattern differs from the bimodal pattern seen further north in the central coastal part of Tanzania. Coastal areas to the north as close as Kilwa in Tanzania (approximately 290 km from Balama) show what could be referred to as a moderately bimodal rainfall pattern with peaks in November-December (short rains or "vuli") and March-May (long, more intense rains or "masika") separated by an interval of lesser rainfall.

Etymology. The specific name, *balamaensis*, refers to the Balama region in the Cabo Delgado Province, Mozambique, where the type locality for the species is located.

FIGURE 6. Type locality of *N. balamaensis*, a seasonal pool in the Montepuez River system, Balama region, Cabo Delgado Province, northern Mozambique. Photographed on 5 March, 2013.

Discussion

The rate of discovery and description of new *Nothobranchius* species has increased in recent years, with 43 of the 95 currently recognised species in this genus having been described since 2010 (Fricke *et al.* 2021). The greatest recorded diversity of the genus occurs in the coastal region of East Africa, with almost 40% of the 95 currently recognised species of *Nothobranchius* occurring there in what appears to be a diversity hotspot (Dorn *et al.* 2014; Van der Merwe *et al.* 2021). This is, however, rather attributable to greater accessibility leading to the many expeditions conducted in the coastal areas of Tanzania, whilst relatively few surveys have been undertaken in northern Mozambique. Therefore, considering that several coastal systems in Mozambique and their associated seasonal wetlands remain poorly explored, it is likely that the diversity of seasonal killifishes in northern Mozambique remains largely unknown, with information limited to the species descriptions. Among the challenges that have been identified as major impediments to taxonomic research in southern Africa are: dwindling taxonomic expertise, limited financial resources, and the lack of prioritisation for biodiversity research (Skelton & Swartz 2011). Northern Mozambique in particular remains one of the most poorly explored regions in southern Africa and recent political instability in the area is a serious impediment to biodiversity research. The description of *N. balamaensis* increases the number of species of the genus known from northern Mozambique to six.

The phylogeny of the genus as presented here is the most comprehensive to date and includes 84 of the 95 described species plus five taxa of an affiliation yet to be determined. *Nothobranchius balamaensis* belongs to the Coastal-Inland Clade comprising: *N. kirki* from the endorheic Lake Chilwa and Lake Chiuta in Malawi, and the immediately adjacent area in Mozambique; *N. wattersi* from the Lake Malawi and upper Shire River region; and an undescribed species in southern coastal Kenya (Van der Merwe *et al.* 2021). The comprehensive phylogeny of

Van der Merwe *et al.* (2021) recovered the Coastal-Inland Clade as sister to the so-called Coastal Clade, which comprises a monophyletic clade of species currently placed in the subgenus *Adiniops* and a monophyletic clade that includes species in the subgenus *Aphyobranchius*. However, the relationships between these clades were not well supported and resolved; therefore, Van der Merwe *et al.* (2021) concluded more molecular data are required to resolve their phylogenetic relationships. Costa (2018), following an examination of the molecular data of Dorn *et al.* (2014) and osteological data, had previously placed both *N. kirki* and *N. wattersi* in the subgenus *Adiniops* because they share many synapomorphies; however, that phylogeny was less well sampled, with unresolved nodes. Here our phylogeny includes more taxa, yet these relationships remain unresolved. The subgeneric classification of this new species should, therefore, be deferred pending greater phylogenetic clarity, and a detailed examination of the osteological characters of *N. balamaensis* and the undescribed south-eastern Kenya taxon.

Interspecific variation in colour pattern. The close genetic relationship between *N. balamaensis* and *N. kirki* is clearly reflected in certain shared features of colour pattern unique to these two species: the overall relatively deep red-orange colour of the basal, proximal and medial zones of the caudal and anal fins grading to a prominent orange in the distal zone (Figs.7A–B). In general, the red-orange colour in *N. kirki* tends to a slightly stronger red hue compared to *N. balamaensis* although this character does vary on an inter- and intra-population level in *N. kirki*. Other aspects of colour pattern, and one of fin morphology of males, are also shared by these two species and, while not unique, are in combination sufficiently uncommon among *Nothobranchius* to further emphasize their close relationship: the dorsal fin membrane is black between the first few anterior rays; body colouration iridescent light blue, with narrow brown-red scale margins creating an irregular reticulated pattern; prominent orange to orange-red scale margins on the abdomen (in *N. kirki* this has led to the common name "Red-belly Notho"; Wildekamp 2004); narrow black to grey distal margin on the caudal fin, which may be intermittent; and a mildly trapezoidal shape of the anal fin in some populations. Principal features that distinguish the two species from one another, as presented in more detail in the diagnosis section are, essentially: the presence in *N. kirki* of a light blue to white band in the proximal part of the anal fin, either continuous or irregular and broken, and indistinct in many specimens from the Lake Chiuta area (Fig. 7B) (versus a complete absence of any such banding or other markings in *N. balamaensis*); dorsal fin with marbled or vermicular pattern throughout proximal and medial zones with only a poorly developed striation pattern in the distal zone in some specimens (versus a marbled pattern in proximal region with distinct striated pattern in medial and distal zones in *N. balamaensis*); pale blue or white fin-ray extensions on the caudal fin (versus absent or pale grey in *N. balamaensis*); and, *N. balamaensis* is distinctly more slender than *N. kirki*, as evident from various morphometric parameters.

Nothobranchius wattersi (Figs. 7C–F), a species that also belongs to the Coastal-Inland Clade, having close genetic relationships to *N. kirki* and *N. balamaensis* (Van der Merwe *et al.* 2021), shows significant interpopulation variation in colour pattern across its range of distribution and greater divergence of characteristics when compared to the other two species. While some features are shared by some populations with *N. kirki* and *N. balamaensis*, none of them exhibit the unique colour pattern element of an overall relatively deep red-orange colour in the basal, proximal and medial zones of the caudal and anal fins, that grades to a prominent orange in the distal zone.

Populations of *N. wattersi* in the northern part of its distribution range (from Chia in the north, southwards to Chipoka) (Figs. 7C–D) typically have uniformly deep red caudal and anal fins, the former consistently showing a narrow black or grey marginal band, as does both *N. kirki* and *N. balamaensis*. The anal fin invariably has a narrow pale blue to white proximal band or series of disconnected irregular markings, similar to that seen in *N. kirki*, but absent in *N. balamaensis*. Some populations may show a very mild trapezoidal anal fin shape, also shown by *N. kirki* and *N. balamaensis*. The dorsal fin pattern is generally similar to that of *N. kirki* and, therefore, somewhat different to that of *N. balamaensis*; however, it should be noted that some specimens from the Salima area show a marbled proximal zone and distinctly striated pattern in the medial and distal zones similar in some respects to that of *N. balamaensis*, but also different in that the Salima specimens have brown-red markings on a blue-green background (Fig. 7C). One common feature of the dorsal fin is the presence of a black membrane between the first few anterior rays, although this feature is shown only by *N. wattersi* from the northern area of distribution and is more poorly developed and inconsistent than in the other two species.

Nothobranchius wattersi in the middle part of its range (Fig. 7E), mainly in the southern and southeastern shore region of the Kasankha Bay part of Lake Malawi, most commonly display a uniformly orange-red caudal fin without a marginal black band and lacking the gradational zoning that is so characteristic of *N. kirki* and *N. balamaensis*. The anal fin, similarly, has an orange-red background colour, but overlain by pale blue to white markings that are

irregular and vermicular in the proximal zone and more linear in the medial and distal zones, creating a striated pattern of sorts parallel to the fin rays. Some populations may show a very mild trapezoidal anal fin shape. The dorsal fin pattern is similar to that of *N. kirki*, although lacking the black colouration of the membrane between the anterior rays; it is, therefore, different to that of *N. balamaensis*.

In the southern part of its range, in the upper Shire River area, *N. wattersi* populations (Fig. 7F), have a uniformly deep red caudal fin, with a very narrow, often barely discernible, black marginal band. The anal fin is rounded, and the colour pattern generally comprises red dots and vermicular markings showing some small degree of orientation parallel to fin rays, on a pale blue or yellow background, that is distinct from the anal fin colour patterns displayed by *N. kirki* and *N. balamaensis*.

FIGURE 7. Adult male wild-caught live specimens of *Nothobranchius kirki* and *N. wattersi* from Malawi: **A** and **B.** *N. kirki*, Lake Chiuta area (field code MW 88-9); **C.** *N. wattersi*, type area near Salima, northern population group (field code MW 92-5); **D.** *N. wattersi*, Chia Lagoon area, northern population group (field code MW 92-4); **E.** *N. wattersi*, Golomoti-Chinganji area, middle population group (field code MW 88-13); **F.** *N. wattersi*, Hoba-upper Shire River area, southern population group (field code MZMW 09-6). Photographs by B.R. Watters.

Of note is that the body colouration of *N. wattersi* throughout the range of distribution is, in general, quite similar to that of *N. kirki* and *N. balamaensis*, being bright iridescent light blue, with narrow brown-red to orange scale margins (depending on the population) creating an irregular reticulation pattern on the flanks. However, one significant difference is that in all populations of *N. wattersi* the scale margin colouration is not prominent in the abdominal region with no "red-belly" or "orange-belly" effect as in *N. kirki* and *N. balamaensis*.

In summary, various features of colour pattern shared by *N. kirki*, *N. balamaensis* and *N. wattersi*, especially by the former two species, corroborate the close relationship inferred from the molecular data (Van der Merwe *et al.* 2021; and as presented here). Conversely, there are important differences in colour pattern characteristics that serve to distinguish the newly described *Nothobranchius balamaensis* from its closest relatives.

In general, *Nothobranchius wattersi* and *N. kirki* are deeper-bodied than the moderately slender *N. balamaensis*, which has a range (means in parentheses) for males of body depth as % of SL of 23.7–24.6 (24.3) versus 27.1–36.1 (31.0) in *N. kirki*, and 24.4–34.8 (29.3) in *N. wattersi*; data for *N. kirki* and *N. wattersi* from Ng'oma *et al.* (2013).

In a broader context the slight angularity seen in the anal fin of males of *N. balamaensis* and *N. kirki*, and to a rare extent in some populations of *N. wattersi*, is not commonly found in *Nothobranchius*. Within *Adiniops*, this feature is shared only by *N. flammicomantis* Wildekamp, Watters & Sainthouse, 1998 and rounded anal fins are typical. However, the angularity exhibited by these species is not nearly as prominent as in males in three members of the subgenus *Aphyobranchius*: *N. janpapi* Wildekamp, 1977, *N. luekei* Seegers, 1984 and *N. geminus* Wildekamp, Watters & Sainthouse, 2002, which possess a well-developed trapezoidal shape to the anal fin; this feature is, probably, an adaptation to near surface-dwelling and mid-water spawning habits. These species are also relatively slender, to a greater degree than *N. balamaensis*, and the origin of the dorsal fin is positioned posteriorly to that of the anal fin – probably another adaptation to a surface-dwelling nature. By contrast, *N. balamaensis*, *N. kirki* and *N. wattersi* typically have the origin of the dorsal fin set slightly forward of that of the anal fin. Males of *Nothobranchius fuscotaeniatus* Seegers, 1997 also possesses a well-developed trapezoidal shape to the anal fin but its body is not slender, nor is its dorsal fin set back; and it occupies a mid-water niche in the habitat (Watters 2016).

Biogeography. Biogeographically, the two member species of the Coastal-Inland Clade in Malawi, *N. kirki* and *N. wattersi* are allopatric in their distribution, with the latter being restricted to the southern part of the Lake Malawi drainage system within the southernmost section of the African Rift Valley. *Nothobranchius kirki* mainly occurs in the lakes Chilwa/Chiuta basin (with the exception of the population collected recently by Holger Hengstler; see previous comments) to the south-east of Lake Malawi that formed as a result of the uplift and eastward tilting of the eastern shoulder of the Lake Malawi rift. This formed an effective barrier between the two species and they probably diverged in the Middle Pleistocene [0.44 (0.24–0.68) Mya], for which Van der Merwe *et al.* (2021) constrains the timing for the tectonic events, which isolated Lake Chilwa (first proposed by Watters 1991). Although there are no records of sites hosting *N. kirki* directly associated with Lake Amaramba, it is highly likely that it also occurs in the Lake Amaramba Basin as one of the present authors (BRW) has collected this species from the Lifune stream system that drains into a marsh zone connecting lakes Chiuta and Amaramba.

The Coastal-Inland Clade underscores the evidence for a past connection between the Lake Chilwa and Malawi depressions with the Rovuma River drainage system (Van der Merwe *et al.* 2021). Currently, Lake Chiuta borders Lake Chilwa to the north-east, which is one of the main headwaters of the Lugenda River, a primary tributary of the Rovuma River. Geologic evidence reveals that, prior to late Neogene rifting, the Chilwa and Chiuta region was drained by headwaters flowing north-eastwards to the Indian Ocean through the Rovuma River. The deepening of the Malawi graben, around 800 ka, established the modern-day Lake Malawi outlet through the Shire River, and the draining of a larger palaeo-lake, whose wave action likely formed the sandbar which today partially divides the smaller lakes Chilwa and Chiuta (Lancaster 1981; Ivory *et al.* 2016). This constraint of ~800 ka, as invoked from Ivory *et al.* (2016), as the tectonic trigger of the speciation of *N. balamensis* in the Early to Mid-Pleistocene [1.15 (0.78–1.67)] significantly refines the timing of the past historical connection proposed as the main explanation for the close affinities between the fish fauna of Lake Chilwa and coastal rivers of northern Mozambique (Tweddle, 1979).

Conservation status. Recent IUCN Red List assessments of the *Nothobranchius* species occurring in northern Mozambique rated *N. hengstleri* as Endangered, *N. niassa*, *N. krammeri* and *N. kirki* as Vulnerable, and the widely distributed *N. makondorum* as Least Concern (Nagy & Watters 2019). The main threats to these seasonal killifishes include rapid expansion of agriculture, mining and infrastructure development that alter and degrade their critical and sensitive habitats (Nagy & Watters 2019). Considering that *N. balamaensis* is known solely from the type locality, this underscores the need for further surveys within this region aimed at identifying key threats and assessing its conservation status. This is particularly important, because of the extensive graphite mining activity in the vicinity of Balama. Knowledge of the distribution and status of this new species will help to guide sustainable infrastructure development to promote economic development while safeguarding the biodiversity heritage of the region.

Acknowledgments

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