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

Stefano Valdesalici*, Roger Bills, Alexander Dorn***, Kathrin Reichwald**** and Alessandro Cellerino***, *******

Nothobranchius niassa, new species, is described based on specimens collected in pools within seasonal streams in upper catchments of the Rovuma River, Niassa Reserve, northern Mozambique. It differs from its congeners by a unique combination of characters: dorsal fin rays 15–18, anal fin rays 15–17, light blue iris, light blue dorsal and anal fins with curved red stripes, and red pectoral fin. According to analysis of sequence variation within the cytochrome oxidase I locus, *N. niassa* is a well-distinct taxon related neither to the *N. melanospilus* species group nor to *N. kirki*, but clusters with very high support with a clade including *N. guentheri*, *N. albimarginatus*, *N. korthausae*, *N. foerschi*, *N. cardinalis* and *N. kilomberoensis* (subgenus *Adiniops*). Within this clade, the closest related species is *N. kilomberoensis*, known from Kilombero River floodplain, Tanzania, although with moderate support.

Introduction

The annual killifish genus *Nothobranchius* includes about fifty species in the sub-tropical and tropical part of eastern Africa (Seegers, 1997; Wildekamp, 2004). Three *Nothobranchius* species are currently known from Northern Mozambique ("East Coast" province sensu Skelton, 1994). All belong to the *Nothobranchius melanospilus* species group (Valdesalici, 2007; Wildekamp et al., 2009): *N. hengstleri*, which is only known from an isolated pool near the Nassoro village, *N. krammeri*, from the Meronvi River basin, both distribution areas are within the Cabo Delgado province (Valdesalici, 2007; Valdesalici & Hengstler, 2008), and *N. makondorum*, with a large distribution within the basins of the eastward-flowing coastal lowland rivers Mbwemkuru, Lukuledi, and Rovuma in south-

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eastern Tanzania, and in northeastern Mozambique south to the Melela River (Wildekamp et al., 2009).

The Niassa Reserve in northern Mozambique has a surface area of 42 000 km² and is the country's largest protected area/nature reserve. The reserve is bordered by the Rovuma River in the north (forming the border with Tanzania), the Lugenda River in the southeast, the Luatize River in the southwest, and the Lucheringo River in the west. Numerous small tributaries feed into these larger rivers and there are many small seasonal streams, dambos, and swamps in the upper catchment regions. During fish surveys within this reserve in August 2003 and August 2004, the second author collected *Nothobranchius* specimens in such seasonal pools with standing water (Bills, 2004). Here we show that these specimens belong to the subgenus *Adiniops* (Huber, 2000), are genetically related to *N. kilomberoensis* known from the Kilombero River floodplain in Tanzania (Wildekamp et al., 2002) and we describe them as a new species, *Nothobranchius niassa*.

Materials and methods

Morphology. Measurements and counts were taken as described in Amiet (1987), Huber (1992) and Valdesalici (2010). Measurements were made with a digital calliper, partly under a dissecting microscope, and rounded to the nearest 0.1 mm. All measurements are presented as percentage of standard length (SL), except for eye diameter and snout length, which are given as a percentage of head length (HL). Terminology for the cephalic neuromast series follows Scheel (1968), for the frontal squamation as described in Hoedeman (1958). Osteology was studied on cleared and stained (c&s) specimens (Taylor & Van Dyke, 1985), which were only stained for bones. Data used also for comparisons are from Wildekamp et al. (2002, 2009). Figures in brackets after counts indicate the number of specimens examined with that particular condition.

Types and additional material are deposited in the South African Institute for Aquatic Biodiversity, Grahamstown (SAIAB). Comparative material is deposited in the Museo Civico di Storia Naturale "Giacomo Doria", Genova (MSNG).

Molecular analysis. Total DNA was extracted from fin clip, which were incubated overnight in 600 µl of digestion buffer (10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5 % SDS) with addition of 20 µl Proteinase K at 55 °C. After digestion, 600 µl of 1:1 phenol-chloroform was added and centrifuged at 12 100 rpm for 10 min. The clear aqueous phase was transferred to a new tube containing 1 ml 99 % ethanol, centrifuged at 12 100 rpm for 10 min and decanted. The pellet was washed with 500 µl of 70 % ethanol and centrifuged at 12 100 rpm for 5 min followed by decantation and air-drying. The pellet was finally re-suspended in 50 µl TE-Buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Several sets of primers had to be designed for mitochondrial COI gene, their position along the COI sequences is shown in Table 1. The first set of primers was designed by aligning the complete sequence of COI from *N. furzeri* (NC_011814.1) with that of two other Cyprinodontiformes, *Kryptolebias marmoratus* (NC_003290.1) and *Cyprinodon rubrofluvitalis* (EF442803.2), in order to identify conserved regions. As these primers often failed to amplify COI from species other than *N. furzeri* (*N. rachovii* in particular), a second set of primers was designed by aligning the COI sequences of

Table 1. *COI*- primers used in this study

name	sequence	position	reference
22f	AATCAYAAAGATATCGGCAC	41–61	NC_011814.1
20f	ACAGGCTGRACAGTCTATCC	380–400	NC_011814.1
132f	GACCCAGCTGGWGGAGGAGA	555–577	NC_011814.1
23r	ACWGAAAGWACTTCNCGTTT	1466–1447	NC_011814.1
3r	CATGAAGCGTGTAGCCTGAA	1231–1212	NC_011814.1
136r	CCTGCTAAGCCTAGGAAGTG	1198–1179	NC_011814.1
19f	TTTCACARTATCAAACCCC	537–559	NC_011814.1
21r	AATGCTTCTCAGAYAATAAA	1439–1420	NC_011814.1

N. furzeri, *N. rachovii*, and *Fundulosoma thierryi* obtained from the previous set of primers in order to obtain primers which give rise to robust amplification in *Nothobranchius*. The longest sequence in the allignement is 1296b long an it is from *F. gardneri*; the sequences from *N. niassa* are 625b long.

Amplification reactions were performed at 25 µl final volume, each with 2.5 µl 10 × PCR buffer; 1.5 µl 25 mM $MgCl_2$; 0.5 µl each of 10 mM dNTP mix, 10 µM of each forward and reverse primer, 0.25 µl 5 U/µl Taq Polymerase (Qiagen); and 1 µl (100–150 ng) of genomic DNA. The PCR-program for the primer combination 22f–23r and 20f–3r comprised a denaturation step at 94 °C for 120s, followed by 10 cycles with a touchdown (94 °C for 30s, touchdown from 55 °C to 50 °C, 0.5 °C decrease at every step) followed by 30 cycles (94 °C for 30s, 50 °C for 30s, 72 °C for 90s), and a final elongation step for 180s at 72 °C. For the primer combination 19f–21r, the same program was used except that the touchdown was performed from 51 °C to 46 °C and the annealing temperature was 46 °C. For the primer combination 132f and 136r, the following cycling-program was used: denaturation step at 94 °C for 120s, 35 cycles (94 °C for 30s, 57 °C for 30s and 72 °C for 30s) and an elongation step of 72 °C for 60s.

Sequencing reaction was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (ABI; Weiterstadt, Germany), followed by fragment separation and detection on an ABI 3730xl capillary sequencer. After quality clipping, sequences were assembled using the GAP4 module of the Staden Sequence Analysis Package. Visual inspection and manual editing of sequences was undertaken using GAP4.

Sequences were aligned using ClustalW (Thompson et al., 1994) implemented in MEGA 4 (Tamura et al., 2007) and checked manually. Phylogenetic Analyses weres performed by Bayesian inference using MrBayes (Ronquist & Huelsenbeck, 2003). The dataset was partioned into one portion for each codon position and a general time reversible model with gamma distribution and invariable sites (GTRΓ+I) was selected for each partition. Number of generations was set to 1 000 000 with sampling of every 100 generations. Default settings of priors and temperature were used and two chains were run in parallel. The first 25 % generations were discarded as burnin. Convergence was checked by standard deviations of splits and the simulation was stopped when standard deviation of splits was < 0.01.

Nothobranchius niassa, new species
(Figs. 1–2)

Nothobranchius kirki: Bills, 2004: 7

Holotype. SAIAB 73777, 1 male, 27.7 mm SL; Mozambique: Niassa Reserve, Lukombe River drainage: first river crossing the Mbstamila–Mantondovela road, pools in dry river bed; 12°08'17"S 37°32'03"E; R. Bills, 14 Aug 2003.

Paratypes. SAIAB 73777, 1 male, 22.6 mm c&s, 1 female, 21.5 mm; same data as holotype. –SAIAB 73902, 2 males, 28.0 & 29.1 mm SL, 1 female, 26,7 mm SL; Mozambique: Niassa Reserve, Lugenda River drainage: tributary stream of Incalau River, Mbatamila–Mussoma road; 12°23'55"S 37°40'21"E; R. Bills, 17 Aug 2004. – SAIAB 73817, 1 male, 26.5 mm SL, 1 female, 25.4 mm SL; Mozambique: Niassa Reserve: Mbatamila–Matondovela road; 12°07'49"S 37°26'10"E; R. Bills, 14 Aug 2004. – SAIAB 74017, 1 male, 27.2 mm SL, 1 female, 25.2 mm SL; Mozambique: Niassa Resereve: Mbatamila–Mussoma Road, Lucombe stream pools, Nyati Road; 12°05'15"S 37°33'38"E; R. Bills, 22 Aug 2003. – SAIAB 73905, 2 males, 26.8 & 28.9 mm SL, 1 female, 21.9 mm SL; Mozambique: Niassa Resereve, Lugenda River drainage: Mbatamila–Mussoma road, Gomesh stream near Macula; 12°11'29"S 37°38'12"E; R. Bills, 17 Aug 2004. – SAIAB 73806, 1 male, 20.6 mm SL; Mozambique: Niassa Resereve: Mbatamila–Matondovela Road; 12°08'05"S 37°24'18"E; R. Bills, 18 Aug 2003. – SAIAB 73791, 1 male, 26.2 mm SL; Mozambique: Niassa Resereve: Nakajambo stream, pools in stream bed, Mbatamila–Matondovela road; 12°07'45"S 37°21'41"E; R. Bills, 14 Aug 2003.

Material examined (non types)**.** SAIAB 74019, 7, 20.2–23.8 mm SL; Mozambique: Niassa Reserve: Mbatamila–Mussoma Road, Lucombe stream pools, Nyati Road; 12°05'15"S 37°33'38"E; R. Bills, 22 Aug 2003.

Diagnosis. *Nothobranchius niassa* belongs to the subgenus *Adiniops*, which has a preopercular red pattern, wide chevrons on side and a deep red caudal fin without any dark margin in males. It is distinguished from all other species of this

subgenus by the unique combination of the following characters in males: dorsal fin rays 15–18, anal fin rays 15–17, light blue iris, light blue dorsal and anal fins with curved red stripes, and red pectoral fin.

Description. Measurements and counts were taken from 10 males and 5 females (Table 2), habitus and colouration is illustrated in Figures 1–2. Somewhat elongate *Nothobranchius*, maximum observed length in males 29.1 mm SL. Dorsal profile nearly straight on head, convex from nape to end of dorsal-fin base. Ventral profile convex, slightly concave to nearly straight on caudal peduncle posterior to dorsal and anal fin. Snout slightly pointed, mouth directed upwards, lower jaw longer than upper, posterior end of rictus at same level as or slightly above centre of eye. Branchiostegal membrane projecting posteriorly from opercle.

Dorsal and anal fins posterior to mid-body, rounded, fin tips with short filamentous rays. Tip of dorsal fin reaching caudal fin. Dorsal and anal fins covered with an opaque mucus film and with papillate contact organs along fin rays, denser on anal fin. Pectoral fin approximately triangular, tip reaching pelvic fin. Pelvic fin long, tip reaching anal-fin origin. Caudal fin subtruncate. Dorsal-fin rays 15–18; anal-fin rays 15–17; caudal-fin rays 26; pectoral-fin rays 16; pelvic-fin rays 6.

Scales cycloid, body and head entirely scaled, except for ventral surface of head. Scales in median lateral series 27–30 + 3–4 on caudal-fin base. Cephalic squamation pattern irregular G-type. Anterior neuromast series of 'open' type. Central supraorbital series in shallow groove with 2–3 neuromasts. Posterior cephalic neuromast series curved with 2–3, rarely 4, neuromasts. Preopercular neuromast system in open groove, distal ridge slightly overlaps opercle. One neuromast on each scale of median longitudinal series.

Basihyal bone sub-triangular, basihyal cartilage spatulate. Six branchiostegal rays. Vomerine teeth separate in three patches. Lateral process of post-temporal short or rudimentary, single antero-dorsal process of urohyal. Second pharyngobranchial with 3 teeth. Total number of vertebrae 32. Premaxilla and dentary with many irregularly distributed unicuspid, slightly curved teeth of different size, a small number of larger ones on the outer row of upper and lower jaw.

Female. Maximum observed lenght 26.7 mm SL. Anal fin triangular with rounded tip. Pelvic fin short, tip reaching anus. Anal fin positioned more posterior (59.1–62.1 % SL vs. 54.4–58.9) and caudal peduncle less deep (10.1–10.9 % SL vs. 11.0–13.2) than in males. Branchiostegal membrane not projecting from opercle.

Coloration. Male (Fig. 1). Body and head scales light blue with a dark red margin, creating a reticulated pattern on body and head, posterior scales margin width on anterior part of body. Posterior part of caudal peduncle, lips, snout, frontal, and dorsal portion of head red. Branchiostegal membrane red with light blue rim. Dorsal

Table 2. Morphometric data of the type series of *Nothobranchius niassa*.

	holotype	males (n = 10)	females (n = 5)
Standard length (mm)	27.7	20.6–29.1	21.1–26.7
Percentage of standard length			
Depth at pelvic fin	25.9	22.8–30.3	21.8–24.6
Predorsal length	61.0	56.4–61.7	57.5–61.0
Length of dorsal-fin base	23.4	21.8–31.6	20.8–27.4
Preanal length	58.8	54.4–58.9	59.1–62.1
Length of anal-fin base	21.2	17.7–22.8	14.6–18.2
Prepelvic length	48.3	43.9–51.0	46.4–50.7
Length of caudal peduncle	18.7	18.7–24.2	21.0–25.1
Depth of caudal peduncle	11.5	11.0–13.2	10.1–10.9
Head length	30.6	30.1–35.0	29.9–32.7
Percentage of head length			
Snout length	21.1	21.1–30.7	23.2–26.8
Eye diameter	31.7	27.5–33.8	29.0–32.8

Fig. 1. *Nothobranchius niassa*, topotype, male, about 20 mm SL; Mozambique: Niassa Reserve, Lukombe River drainage.

Fig. 2. *Nothobranchius niassa*, topotype, female, about 20 mm SL; Mozambique: Niassa Reserve, Lukombe River drainage.

and anal fins red with a pattern of light blue spots, forming irregular stripes. Pelvic fin red with a pattern of rounded light blue spots. Pectoral fin red with light blue margin. Caudal fin red. Iris light blue, with faint black vertical bar through centre of eye.

Female (Fig. 2). Body and head scales light brown, scales on sides with light blue to silvery centers, ventrally whitish. Opercular region silvery to whitish. Paired and unpaired fins hyaline. Iris silvery, with faint black vertical bar through centre of eye.

Distribution. *Nothobranchius niassa* is currently only known from seasonal pools and streams in the Rovuma River drainage in the Niassa Reserve (Fig. 3). The *Nothobranchius* specimens collected on the swampy pools near the Mielele River banks (SAIAB 73948) and those collected into the Naruvale stream (SAIAB 73930) cannot be attributed to any known species, because only females were collected, which does not allow a species identification. Probably the sample SAIAB 73930 belongs to *N. niassa* because it is within the species distribution range and shows no differences in morphology.

Habitat notes. The type locality was at the time of collection a pool within the bed of a seasonal stream, about 3 m wide and roughly circular, less than 50 cm deep, no vegetation, leaf litter and gravel as bottom substrate (Fig. 4). The water was light clay gray, slightly turbid with low conductivity (about 100 µS/cm), the water temperature was about 20 °C. Other fish species collected at the type locality were *Barbus atkinsoni*, *B.* cf. *bifrenatus*, *B.* cf. *lineomaculatus*, *B. radiatus*, *B. toppini*, *Clarias gariepinus*, and *Petrocephalus catostoma*. Others known habitats were similar to type locality.

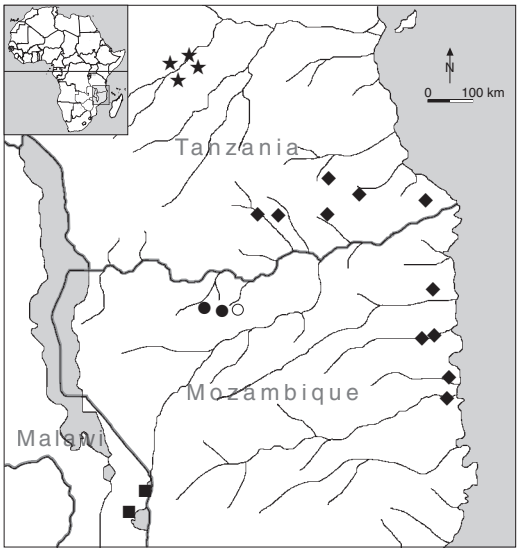

Fig. 3. Distribution of: *Nothobranchius niassa* (●,○: type locality); *N. makondorum* (◆); *N. kilomberoensis* (★); *N. kirki* (■).

Etymology. Name given in reference to the Niassa Reserve, where this new species occurs. A noun in apposition.

DNA analyses. To investigate the preliminary phylogenetic relationship of *N. niassa* within the genus, we sequenced a part of the mitochondrial cytochrome oxidase I (COI) locus of 29 specimens from 19 species found in Kenya, Tanzania, and Mozambique (Table 3). This locus was already used to study the phylogeny of *Nothobranchius* (Terzibasi et al., 2008; Dorn et al., 2011) and is widely-used for DNA barcoding (Ratnasingham & Hebert, 2007). *Fundulopanchax gardneri* served as outgroup.

The analysis clearly shows that *N. niassa* is a well-separated taxon without any close similarity to any of the species sampled. Further, the analysed *Nothobranchius* species form three monophyletic clades (Fig. 5): Clade C comprises the *N. melanospilus* group (Wildekamp et al., 2009) and related species: *N. jubbi*, *N. elongatus*, *N. melanospilus*, *N. krammeri*, *N. makondorum*, *N. lucius*. This clade is moderately supported by a posterior probability of 0.80. Clade B comprises *N. furzeri* and *N. kuhntae* and is robustly supported by posterior probability of 0.99; and Clade A is formed by some members of the subgenus *Adiniops*: *N. cardinalis*, *N. foerschi*, *N. guentheri*, *N. korthausae*, *N. albimarginatus*, *N. kilomberoensis* and *N. niassa*. This clade has a very strong statistical support (posterior probability 0.99). Within this clade, *N. niassa* and *N. kilomeroensis* appear to form a monophyletic unit, although the posterior probability is only 0.62.

This phylogenic analysis excludes any close relationship between *N. niassa* and *N. kirki*. *Nothobranchius kirki* indeed appears as an isolated species, which cannot be placed in any of the three described clades.

Discussion

Nothobranchius niassa was first identified as *N. kirki* (Bills, 2004), which is known from Malawi in the catchments of Lakes Chiuta and Chilwa (Jubb, 1969; Wildekamp, 2004). Morphologically, *N. niassa* and *N. kirki* are indeed similar, but the two species differ osteologically by the number of vertebrae (32 vs. 28–29), by the lateral process of the posttemporal (rudimentary vs. well developed), and on the basis of male coloration. Males *N. niassa* differ from males *N. kirki* by iris coloration (light blue vs. golden), pectoral fin coloration (red vs. hyaline), dorsal fin pattern (striped vs. somehow spotted with light blue margin), anal fin pattern and coloration (light blue with red stripes vs. orange with a proximal light blue stripe and black margin), caudal fin coloration (red vs. orange with black margin).

The other geographically close species is *N. makondorum* known from southeastern Tanzania, and northeastern Mozambique. Morphologically *N. niassa* and *N. makondorum* are similar, but differ by relatively fewer scales on median lateral series (27–30 vs. 29–34). Additionally, males *N. niassa* are similar to males *N. makondorum* of the blue phenotype in having a light blue iris and a red caudal fin, but they clearly differ by pectoral fin coloration (red vs. hyaline), the anal fin coloration and pattern (light blue with curved red stripes vs. light blue-green with few proximal red elongated spots over fin rays), and the caudal fin margin (absent vs. grey to black rim).

Molecular phylogeny also excludes a close relationship between *N. niassa* and *N. makondorum* or *N. kirki*, the two species with the closest distribution range. Aim of the present analysis was simply to show that *N. niassa* specimens cluster together and are genetically separated from any other *Nothobranchius* to support the morphologi-

cal analysis, which remains the foundation of a species description. For this limited aim, the use of mitochondrial sequences alone is perfectly justified. In addition, due to the high variability of cox1, 600 bp show numerous informative sites and this locus proved to be very informative in distinguishing closely-related *Nothobranchius* species as demonstrated by two published papers (Terzibasi et al., 2008; Dorn et al., 2011) and was selected for this reason. A complete discussion of the relationship between these clades and their regional distribution is beyond the scope of this paper. A multilocus analysis for the entire genus *Nothobranchius* including both mitochondrial and nuclear *loci* is in its finals stages (A. Dorn, K. Reichwald, A. Cellerino; in preparation). This analysis will also reveal the exact placing of *N. kirki* within the genus, which remains unresolved when only the COI locus is analyzed.

According to the analyses of mitocondrial DNA sequence variation, *N. niassa* is clearly part of a strongly supported clade which comprises several species of the subgenus *Adinops*. Molecular phylogeny suggests that within this clade, *N. niassa* is more closely related to *N. kilomberoensis*. This relationship is not supported by very high posterior probability and would need to be confirmed by analysis of other (nuclear) loci. Morphological analysis also suggests a close similarity between *N. niassa* and *N. kilomberoensis*. They show overlapping meristics and measurement ranges, apart from pre-anal length (54.4–58.9 vs.59.3–60.3) and caudal peduncle depth (11.0–13.2 vs.14.3–14.4) in males (measured in 10 and 2 specimens, respectively). Both species also present the same pattern of cephalic squalation and neuromasts; additionally, males are similar in having a light blue iris, a light blue dorsal and anal fins with red curved stripes, and a red caudal fin. They do, however, differ by dorsal- and anal-fin pattern (completely striped vs. stripes limited at posterior proximal portion), pectoral fin coloration (red vs. hyaline), snout pattern (upper and lower jaw plain red vs. spotted). Additionally the

Table 3. List of samples used in the DNA study. AG-code – lab code, species, collection point, primers used for *COI* analysis and GenBank accession numbers for respective species. Sequence of *F. gardneri* from Dorn et al. (2011).

AG-code	species	population	primers	*COI*
AG005	*Nothobranchius kilomberoensis*	Ifakara	132 & 136	JQ310170
AG131	*N. flammicomantis*	Kisaki	19 & 21	JQ310163
AG146	*N. albimarginatus*	Kiparanganda	132 & 136	JQ310161
AG157	*N. korthausae*	Aquarium strain	132 & 136	JQ310172
AG168	*N. korthausae*	Mafia Island	132 & 136	JQ310173
AG169	*N. krammeri*	Cabo Delgado	132 & 136	JQ310174
AG202	*N. melanospilus*	Lukwale River	132 & 136	JQ310178
AG203	*N. lucius*	Kinungamkele	132 & 136	JQ310175
AG206	*N. interruptus*	Aquarium strain	132 & 136	JQ310166
AG219	*N. lucius*	Kiziko	132 & 136	JQ310176
AG222	*N. makondorum*	Messalo River	132 & 136	JQ310159
AG224	*N. makondorum*	N´tessa	132 & 136	JQ310177
AG228	*N. makondorum*	Nakapanya	132 & 136	JQ310160
AG231	*N. foerschi*	Kiforu	132 & 136	JQ310164
AG232	*N. guentheri*	Zanzibar	132 & 136	JQ310165
AG234	*N. cardinalis*	Lisinjiri River	132 & 136	JQ310162
AG240	*N. kirki*	Chilwa	132 & 136	JQ310171
AG244	*N. jubbi*	Mnanzini	132 & 136	JQ310168
AG245	*N. jubbi*	Mnanzini	132 & 136	JQ310169
AG246	*N. jubbi*	Witu	132 & 136	JQ310167
AG247	*N. jubbi*	Ewaso Ng´iro	132 & 136	JQ310158
AG250	*N. niassa*	Niassa Reserve (SAIAB 74019)	132 & 136	JQ310179
AG251	*N. niassa*	Niassa Reserve (SAIAB 73806)	132 &136	JQ310180
AG252	*N. niassa*	Niassa Reserve (SAIAB 73817)	132 & 136	JQ310181
AG253	*N. niassa*	Niassa Reserve (SAIAB 73777)	132 & 136	JQ310182
AG254	*N. niassa*	Niassa Reserve (SAIAB 73902)	132 & 136	JQ310183
AG239	*Fundulopanchax gardneri*	Akure	22 & 23	JN021562

Fig. 4. Type locality of *Nothobranchius niassa*: Mozambique: Niassa Reserve, Lukombe River drainage: pools in dry river bed, first river crossing the Mbstamila–Mantondovela road.

females differ by body pattern (absence of dark brown to black spots vs. presence).

Wildekamp et al. (2002) included *N. kilomberoensis* in the *N. korthausae* species group based on male coloration and pattern. According to the DNA sequence analyses, *N. niassa* and *N. kilomberoensis* do not show close affinity with *N. korthause*. However, all three species clearly belong to the subgenus *Adiniops*.

A connection between Kilombero and Rovuma River during Paleocene is likely (Stankiewicz & De Wit, 2006). *Nothobranchius niassa* is the first species of the subgenus *Adiniops* found in Mozambique and appears in geographic discontinuity with respect to all other species of the subgenus. It will be, therefore, interesting to research the area between the Kilombero and Rovuma Rivers close to Lake Malawi, where additional species of this subgenus may be present.

Comparative material. *Nothobranchius* sp.: SAIAB 73948, 1 female 43,0 mm SL; Mozambique: Mielele River margins. – SAIAB 73930, 1 female, 24.3 mm SL; Mozambique: Naruvale stream.

N. kilomberoensis: MSNG 56050A, 1 female, 35.0 mm SL; MSNG 56050B, 1 female, 30.0 mm SL c&s; Tanzania: Kilombero River drainage. – Stefano Valdesalici private collection CSV1001, 1 male 23.6 mm SL; Tanzania: Kilombero River drainage.

N. kirki: MRAC B1-14-P-91-97, 6 males, 34.8–39.2 mm SL, 1 female 39.1 mm SL; Malawi: Kachulu. – SAIAB 98797, 10 males, 27.0–31.5 mm SL; Malawi: Ntaja. – Stefano Valdesalici private collection CSV1002, 1 male, 39.0 mm SL c&s; Malawi: Lake Chilwa drainage.

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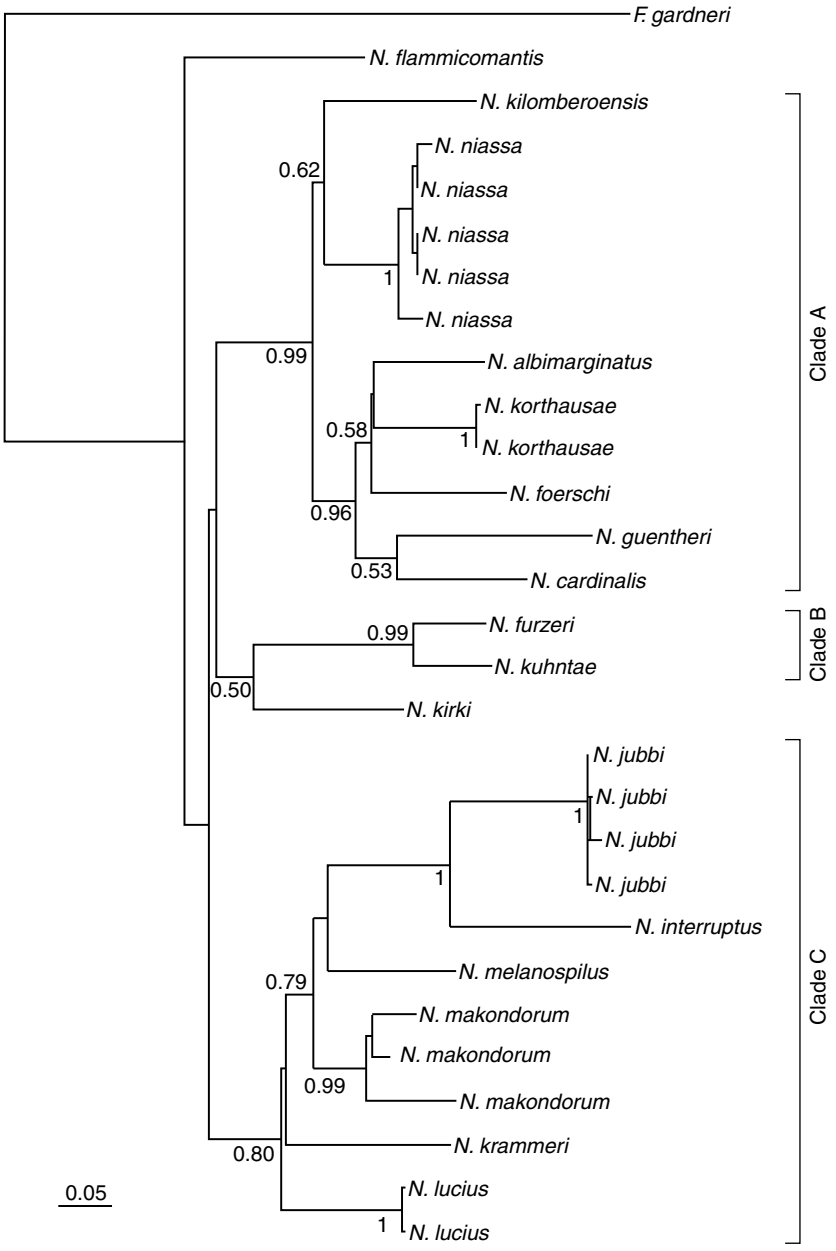

Fig. 5. Phylogenetic tree based on the mitochondrial locus COI and Bayesian analysis. Support values are posterior probabilities. Tree obtained with 1 000 000 generations using partitioned data set (one partition for each position of the codon) and GTR+Γ+I substitution model. Scale bar indicates substitutions per site.

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