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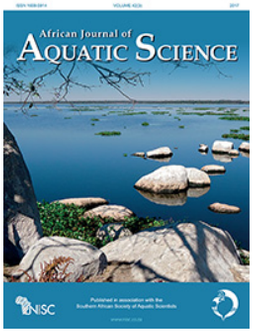

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Nothobranchius cooperi (Teleostei: Cyprinodontiformes): a new species of annual killifish from the Luapula River drainage, northern Zambia

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Nothobranchius cooperi, **Nagy, Watters and Bellstedt, new species, is described from seasonal streams and ephemeral pools associated with the upper Mansa River system in the middle Luapula drainage and systems draining into the low-lying area marginal to the southwestern part of Lake Bangweulu, in the Luapula province of northern Zambia. It belongs to the *N. brieni* species group. Males of *Nothobranchius cooperi* are distinguished from congeners by the following unique combination of characters: body scales with broad orange posterior margin, forming a highly irregular cross-barred pattern; anal fin fairly uniform orange-red with irregular to regular, light blue-green zone close to the base; caudal peduncle length 1.2–1.3 times its depth; prepelvic length 48.8–51.9% SL; and head depth 75–77% of head length. Genetic divergence of the mitochondrial COI and ND2 genes and nuclear S7 gene support the distinction of the new species from its closest known relative, *N. rosenstocki* and confirms its position in the *N. brieni* species group.**

http://zoobank.org/urn:lsid:zoobank.org:pub:532BCF55-947F-4D96-BF70-04F2D1A11B2D

Keywords: Mansa River, mtDNA analyses, nDNA analyses, Nothobranchiidae, *Nothobranchius rosenstocki*, taxonomy

Introduction

The genus *Nothobranchius* Peters, 1868 currently includes about 75 valid species that occur mainly in river drainages of eastern and south-eastern Africa that are subject to seasonal rainfall (Seegers 1997; Watters 2009). It is the most species-rich and geographically widespread genus of seasonal nothobranchiid killifishes (Wildekamp 2004). All known species have an annual or semi-annual life cycle and reproduce in the seasonally arid savannah biome. They inhabit temporary pools and swamps during the rainy season (Skelton 2001), depositing eggs in the substratum. The habitats of all species are characterised by the presence of a vertisol-type substratum that, critically, includes swelling clay minerals of the smectite group, most commonly montmorillonite. Such clay minerals will acquire interlayer water during the wet season, which is then slowly and progressively released during the dry season. This creates favourable conditions in the substratum by preventing total desiccation of the eggs as they develop, interspersed with phases of diapause through the dry season (Watters 2009). *Nothobranchius* species are highly sexually dimorphic and dichromatic. The typically robust and colourful males contrast to the smaller, dull females (Jubb 1981; Wildekamp 2004).

The diversification of *Nothobranchius* exhibits interesting aspects across south-central Africa to the west of the principal arms of the East African Rift. Here, the south-eastern upper drainage of the Congo River basin has been identified as a region with particularly complex phylogeographic patterns in aquatic organisms (Lévêque 1997), reflecting active speciation and a high level of endemism (Balon and Stewart 1983; Jackson 1986; Malaisse 1997; Cotterill 2005; Snoeks et al. 2011). This subcatchment of the Congo River drains the Katanga province, previously called Shaba, of the Democratic Republic of the Congo and north-western Zambia. Several ichthyological studies have focused on the oviparous cyprinodontiform fishes of the region (Poll 1963, 1976; Tait 1965; Wildekamp 1978; Valdesalici and Wildekamp 2004, 2005; Valdesalici and Amato 2011; Nagy 2014a, 2014b, 2014c; Nagy et al. 2016).

The landscapes of the upper Congo basin are characterised by extensive gently sloping pediments, whose steep escarpments bound wide valleys with impeded drainage, characterised by seasonally inundated shallow wetlands and floodplains (Flügel et al. 2015; Guillocheau et al. 2015). The Luapula River forms part of the Bangweulu-Mweru ecoregion (Thieme et al. 2005; Abell et al. 2008). The Bangweulu basin is a vast seasonal wetland fed by several rivers, the largest being the Chambeshi, which flows out of Lake Bangweulu into the Luapula, which in turn flows into Lake Mweru. The Mumbatuta and Mambilima Falls are prominent knickpoints that divide the 480 km channel of the Luapula into three main sections

(Flügel et al. 2015). Both falls are effective barriers to fish dispersal (Worthington 1933; Lavoué 2012; Van Steenberge et al. 2014).

Five *Nothobranchius* species have previously been identified within the catchment of the Luapula (Figure 1): *N. chochamandai* Nagy, 2014, which is known from the Lufutishi system in the middle Luapula drainage; *N. malaissei* Wildekamp, 1978, found on the lower Luapula plain and in associated temporary rivers, downstream of Mambilima Falls; *N. sainthousei* Nagy, Cotterill and Bellstedt, 2016, from the Chimembe River near the Luono-Luapula confluence; *N. symoensi* Wildekamp, 1978, known only from a relatively restricted part of the upper Luapula drainage, above the Mumbatuta Falls; and *N. rosenstocki* Valdesalici and Wildekamp, 2005, which, as recognised in the original description, included populations associated with several smaller river systems within the upper Luapula drainage above the Mumbatuta Falls, including the type locality, as well as a second group of populations in the headwaters region of the Mansa River, a right-bank tributary of the Luapula between the Mambilima and Mumbatuta Falls (Rosenstock 1991; Schmidt 1999, 2008; Wood 2001; Valdesalici and Wildekamp 2005; Nagy 2014b; Nagy et al. 2016). Males of the five species are characterised by a colour pattern having an irregular reticulation on the sides that may form irregular cross-bars; spotted red-brown patterning of the caudal fin; and the presence of a light blue margin to the caudal fin.

The above-mentioned species, together with all other *Nothobranchius* species from other parts of the upper Congo and Zambezi drainages in Katanga and Zambia, have been assigned to the *N. brieni* species group (Valdesalici 2010; Nagy et al. 2016). All known members of this species group have allopatric distributions (Nagy 2014a, 2014b, 2014c; Nagy et al. 2016). The nine currently known additional species of the *N. brieni* species group are: *N. boklundi* Valdesalici, 2010, from the Luangwa Valley in eastern Zambia; *N. brieni* Poll, 1938, from the middle Lualaba drainage in Katanga; *N. capriviensis* Watters, Wildekamp and Shidlovskiy, 2015, with a localised range within the upper Zambezi drainage, in the Zambezi Region (formerly Caprivi Strip) of Namibia; *N. flagrans* Nagy, 2014, from the lower Lufira drainage in Katanga; *N. hassoni* Valdesalici and Wildekamp, 2004, from the lower Lufira drainage in Katanga; *N. kafuensis* Wildekamp and Rosenstock, 1989, from the Kafue and upper Zambezi drainages in southern Zambia; *N. milvertzi* Nagy, 2014, from the Lake Mweru basin in northern Zambia; *N. oestergaardi* Valdesalici and Amato, 2011, from the Lake Mweru Wantipa basin in northern Zambia and *N. polli* Wildekamp, 1978, from the upper Lufira drainage in Katanga. These members of the *N. brieni* species group represent the southwestern limit of the range of the genus in southern Africa (Skelton 1994; Watters et al. 2015).

During the course of a detailed study of the diversity and distribution of *Nothobranchius* species in the Luapula drainage, including molecular analyses of representative sets of samples, the current authors have concluded that the two principal groups of populations previously regarded collectively as *N. rosenstocki*, show distinctively different characters. This indicates that the Mansa River populations and those associated with the low-lying area marginal to the southwestern part of Lake Bangweulu near Samfya, represent a distinct species, herein described as *N. cooperi* Nagy, Watters and Bellstedt, new species.

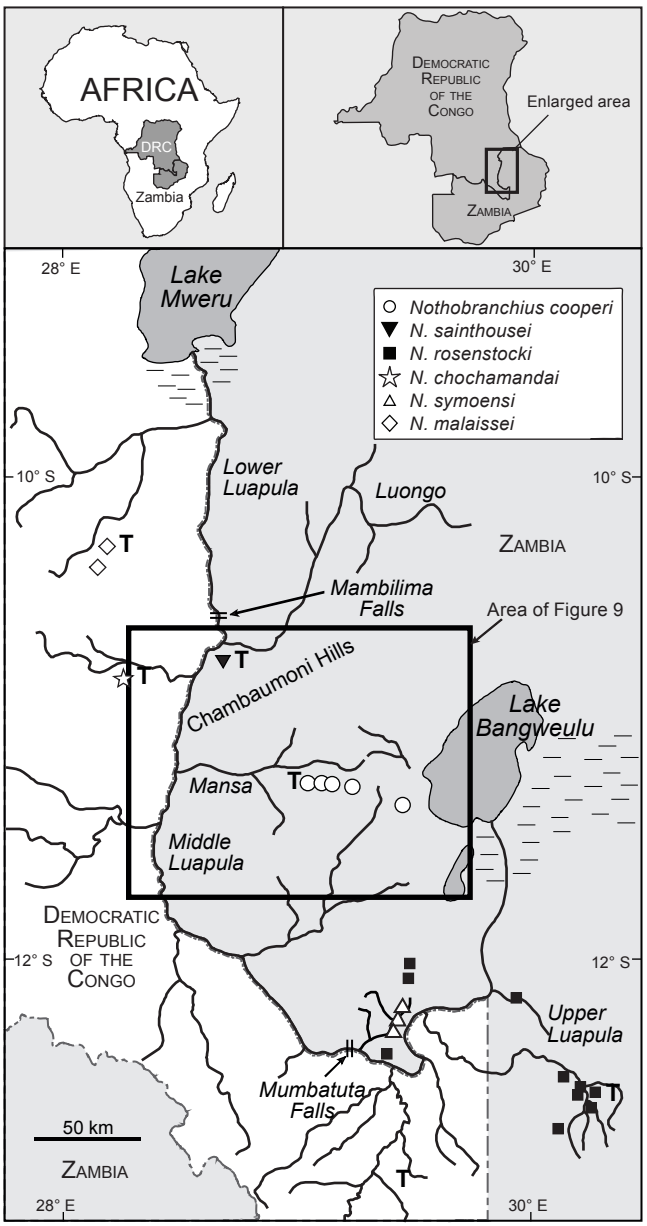

Figure 1: Map of the Luapula River drainage showing distribution of *Nothobranchius* populations used for morphological and molecular comparisons in the current study and currently known localities of the respective species. *Nothobranchius cooperi* (open circle), *N. sainthousei* (solid inverted triangles), *N. chochamandai* (open stars), *N. malaissei* (open diamonds), *N. rosenstocki* (solid squares), *N. symoensi* (open triangles). T, type locality of respective species. Individual symbols may represent multiple sites where occurrences are in close proximity to one another. Box across the Middle Luapula to Lake Bangweulu region represents the area shown in more detail in Figure 9

Materials and methods

Specimens used for morphometric and meristic determinations were initially fixed in the field in approximately 7% formalin, then transferred to 70% ethanol for long-term preservation. Fin clips of representative voucher specimens were stored in 99% ethanol for molecular analysis. Selected male and female specimens were retained live and maintained in aquaria to observe colouration, behaviour, breeding biology and potential maximum size. Type series and comparative specimens, all being wild-collected specimens, were deposited at the MRAC, Royal Museum for Central Africa, Tervuren.

Morphological analysis

Conventional measurements of bilaterally symmetrical features were taken on the left side of specimens using digital vernier callipers, partly under a dissecting microscope, to a precision of 0.1 mm. Twenty-three measurements and five counts were taken, as described in Nagy (2014a). Furthermore, the postorbital length is defined as the greatest horizontal distance between the posterior bony margin of the orbit and that of the operculum, whereas the suborbital depth is defined as the greatest vertical distance between the inferior bony margin of the orbit and the ventral surface of the head. Standard length (SL) is presented in mm; all other measurements are expressed as percentages of standard length, except subunits of the head, which are presented as proportions of head length (HL). Fin-ray counts include all visible elements. Scale count in the mid-longitudinal series includes all scales between the upper attachment of the opercular membrane and the base of the caudal fin. Excluded are the small scales posterior to the hypural junction. The nomenclature for frontal squamation follows Hoedeman (1958). Terminology for the cephalic sensitive system is after Gosline (1949) and Stenholt Clausen (1967). Osteological observations are based on three specimens cleared and stained, for bones only, according to the procedure of Taylor and Van Dyke (1985).

Uni- and multivariate biostatistical analyses of the morphometric and meristic variables were performed using Minitab 16, from Minitab, Inc. Proportions of measurements were calculated in order to remove size effects from variation in body shape. Scatter plot graphs, with the proportions plotted against standard length or head length, were generated to examine allometric growth effects individually. Morphometric data were log-transformed with base-10 log, whereas meristic data were square root transformed before the biostatistical tests were carried out in order to meet standards for statistical and hypothesis testing (Sokal and Rohlf 1995, 2009; Zuur et al. 2007; McDonald 2008; Zar 2010). Because of pronounced sexual dimorphism, data for males and females were analysed separately.

Non-parametric Mann–Whitney U-tests were used for univariate comparisons between species pairs, whereas non-parametric Kruskal–Wallis tests were used to identify morphological variations among multiple populations. Hypotheses of statistical significance were two-tailed, generated to compare differences of mean ranks among morphometric variables (Sokal and Rohlf 1995, 2009; Zar 2010; Dytham 2011). The significance level was set a priori at $p < 0.05$ and $p < 0.01$ was considered as a highly significant result. A sequential Bonferroni test was applied to correct for multiple pairwise comparisons (Rice 1989).

Best subsets regression was employed to identify the smallest subset of the most distinctive predictors of morphometric variables for each new species. *R*-squared values were compared for models of the same size, whereas Adjusted *R*-squared was used to compare models with different numbers of predictors. The multivariate technique of principal component analysis (PCA) was employed on the correlation matrix of the best subsets of morphometric variables in order to visualise differences in morphometric characters between species. PCA is used as a distribution-free ordination method to display graphically uncorrelated linear combinations of the original variables in a multivariate dataset (James and McCulloch 1990; Zuur et al. 2007). The correlation matrix was selected to standardise variables to zero mean and ignore differences between different scales and units (Quinn and Keough 2002).

Molecular analysis

Genomic DNA was extracted either from fin clips or muscle tissue using the Qiagen DNeasy kit following the manufacturer's instructions. Sequences of the complete protein-coding ND2 mitochondrial gene (±1 200 base pairs), the complete mitochondrial cytochrome oxidase subunit I (COI) gene (± 1 600 base pairs) and the nuclear S7 ribosomal protein gene, intron 1 intron (±700 base pairs) were amplified using primers specifically designed for this purpose based on the whole mitochondrial and nuclear genome sequences of *Nothobranchius furzeri* (Genbank accession numbers, NC_011814.1 and CCSH00000000.1, respectively). The forward and reverse primer sequences of the ND2 gene were ND2_4060F (CTA ATA TAA GCT TTT GGG CCC ATA CC) and ND2_5218R (TGC ATG CAG AAG ATG TGG GT), of the COI genes, TRNYF1 (AGG GAG TTA CAA TCC ACC ACT ATT T) and TRNSR1 (ATG GGG GTT CAA TTC CTT CCT TT) and of the nuclear S7 gene S7_F1 (TCT TCC TTG GCC GTC GTT AAC) and S7_R1 (CTT CAC TAT TTT GGC GCT GGT AC), respectively. Polymerase chain reactions (PCR) were performed in 25 µl volumes. PCR mixtures consisted of 15.9 µl ddH_2O; 2.5 µl $MgCl_2$ (25 mM); 2.5 µl 10X JMR-455 PCR buffer (Southern Cross Biotechnology, Cape Town, RSA); 0.1 µl Supertherm Taq (5 U $µl^{-1}$, Southern Cross Biotechnology, Cape Town, RSA); 1 µl of a 5 mM dNTP solution (KAPA Biosystems, Cape Town, RSA) 0.25 µl of each primer (20 µM), 0.25 µl BSA (4 µg $µl^{-1}$); 1 µl of template DNA solution (50–100 ng $µl^{-1}$) and DMSO (1.25 µl). The thermal profile used for amplification of the ND2 and COI genes was 5 minutes at 80 °C, followed by 35 cycles of 1 minute at 95 °C, 1 minute at 58 °C and 3 minutes at 72 °C and finally 5 minutes at 72 °C. The thermal profile used for amplification of the S7 gene was 5 minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 50 seconds at 55 °C and 1 minute at 72 °C and finally 7 minutes at 72 °C. PCR products were visualised on 2% agarose gels. Purified PCR products were cycle sequenced at the Central Analytical Facility, University of Stellenbosch, in both forward and reverse directions using the BigDye Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. For ND2 sequencing, forward primers ND2_4060F and ND2NtFint (TAC ACT CNT GAC TCC CRG ATR TCA TAC ARG G) and the reverse primer ND2_5218R were used. For COI sequencing, forward primers TRNYF1 and COI852F (CTT TAT TGT TTG AGC CCA CCA CA) and reverse primers COI874R (TGT GGT GGG CTC AAA CAA TAA AG) and TRNSR1 were used. For S7 sequencing, the S7_F1 and S7_R1 primers were used. Sequence chromatograms were analysed using ChromasPro 2.1.4. (Technelysium, Tewantin, Australia). Sequence alignment was performed using Geneious 9 and 10 (Biomatters Ltd., Auckland, New Zealand) and Bioedit 7.2.5 (Hall 1999). The sequence alignment was partitioned for phylogenetic analysis. Phylogenetic analyses were performed using maximum likelihood (ML) analyses using RAxML on CIPRES (Stamatakis 2006). Phylogenetic analyses were rooted on three outgroup taxa; the West African *Pronothobranchius seymouri*, representing the sister genus of *Nothobranchius* and *N. bojiensis* and *N. virgatus* from the northern distributional range of this African genus. Specimens included in the molecular analyses with sampling localities in drainage systems and GenBank accession numbers are summarised in Table 1.

Results

Nothobranchius cooperi was found to belong to the *Nothobranchius brieni* species group, which includes another 14 species. Within this species group, *N. rosenstocki*, *N. sainthousei* and *N. symoensi* were regarded as most similar to *N. cooperi*, based on close geographical distributions and their colour patterns. The distinctiveness of *N. cooperi* was confirmed by morphometric and molecular evidence, which is elaborated on below.

Morphology

Morphometric and meristic characters of *Nothobranchius cooperi* are listed in Table 2, whereas measurements of *N. rosenstocki*, *N. sainthousei* and *N. symoensi* are listed

Table 1: *Nothobranchius* specimens used for molecular analysis, with collection locality, known distribution in drainage systems and GenBank accession numbers

Species	Country	Locality	Population	Drainage system	COI	ND2	S7	
N. bojiensis 1	Kenya	Habaswein	KEN 10-2	Ewaso Ng'iro drainage	MF069549	MF069574		MF069513
N. bojiensis 2	Kenya	Ewaso Ng'iro	KEN 10-1	Ewaso Ng'iro drainage	MF069548	MF069573	Allele a	MF069511
							Allele b	MF069512
N. brieni	DRC	Bukama	CD 13-4	middle Lualaba drainage	MF069550	MF069575		MF069514
N. chochamandai 1	DRC	Kasomeno	CD 13-11	middle Luapula drainage	MF069554	MF069579		MF069521
N. chochamandai 2	DRC	Kasomeno	CD 13-11	middle Luapula drainage	MF069553	MF069578	Allele a	MF069519
							Allele b	MF069520
N. cooperi 1	Zambia	Mansa	ZAM 07-8	middle Luapula drainage	MF069565	MF069590		MF069538
N. cooperi 2	Zambia	Mansa	ZAM 07-8	middle Luapula drainage	MF069524	MF069592		MF069541
N. cooperi 3	Zambia	Mansa	ZAM 07-8	middle Luapula drainage	MF069566	MF069591	Allele a	MF069539
							Allele b	MF069540
N. flagrans	DRC	Kabungu River	CD 13-7	middle Lufira drainage	MF069552	MF069577	Allele a	MF069517
							Allele b	MF069518
N. hassoni	DRC	Bunkeya	CD 13-8	middle Lufira drainage	MF069551	MF069576	Allele a	MF069515
							Allele b	MF069516
N. malaissei	DRC	Sange	DRCH 2006-06	lower Luapula drainage	MF069557	MF069582	Allele a	MF069526
							Allele b	MF069527
N. milvertzi 1	Zambia	Chienge	ZM 12-20	Lake Mweru basin	MF069556	MF069581	Allele a	MF069524
								MF069525
N. milvertzi 2	Zambia	Chienge	ZM 12-20	Lake Mweru basin	MF069555	MF069580	Allele a	MF069522
							Allele b	MF069523
N. oestergaardi 1	Zambia	Mweru Wantipa	ZAM 10-4	Lake Mweru Wantipa basin	MF069561	MF069586		MF069532
N. oestergaardi 2	Zambia	Mweru Wantipa	ZAM 10-4	Lake Mweru Wantipa basin	MF069560	MF069585	Allele a	MF069530
							Allele b	MF069531
N. polli 1	DRC	Kyembe	DRCH 2006-02	upper Lufira drainage	MF069562	MF069587		MF069533
N. polli 2	DRC	Kyembe	CD 16-10	upper Lufira drainage	MF069547	MF069572	Allele a	MF069509
							Allele b	MF069510
N. rosenstocki 1	Zambia	Kasanka N. P.	ZAM 07-10	upper Luapula drainage	MF069568	MF069593		MF069542
N. rosenstocki 2	Zambia	Luapula River	ZAM 07-3	upper Luapula drainage	MF069563	MF069588	Allele a	MF069534
							Allele b	MF069535
N. rosenstocki 3	Zambia	Luapula River	ZAM 07-7	upper Luapula drainage	MF069564	MF069589	Allele a	MF069536
							Allele b	MF069537
N. sainthousei 1	Zambia	Mweshi	ZM 12-19	middle Luapula drainage	MF069559	MF069584		MF069529
N. sainthousei 2	Zambia	Mweshi	ZM 12-19	middle Luapula drainage	MF069558	MF069583		MF069528
N. symoensi	Zambia	Luapula River	ZAM-07-4	upper Luapula drainage	MF069569	MF069594		MF069543
N. virgatus	Sudan	Fula Azarga	SD 10-3	Wadi Al Ghallah system	MF069570	MF069595		MF069544
P. seymouri	Ghana	Kasseh	GHALOZ 03-16	Volta River drainage	MF069571	MF069596	Allele a	MF069545
							Allele b	MF069546

in Table 3. Highly significant divergence of morphological characters between males of *N. rosenstocki* populations from the upper Luapula and *N. cooperi* was found (non-parametric Kruskal–Wallis test, $p < 0.01$ with sequential Bonferroni correction, data not shown). Notably significant morphological differences included prepelvic length, caudal peduncle length and depth and head depth. All three populations of *N. rosenstocki,* from the upper Luapula drainage, exhibited similar morphological traits, with no significant divergence (Kruskal–Wallis test). Furthermore, differences of morphological characters between males of *N. cooperi* and those of *N. rosenstocki*, *N. sainthousei* and *N. symoensi* were significantly divergent (Mann–Whitney *U*-test with sequential Bonferroni correction, Table 3). In summary, highly significant differences were observed in respective suites of mensural characters in *N. cooperi* compared to *N. rosenstocki*, *N. sainthousei* and *N. symoensi*. Overall, *N. cooperi* was found to exhibit highly significant differences from all three species in caudal peduncle length, caudal peduncle depth and anal-fin ray count.

Graphical comparison of biometric differences using PCA resolved the correlation matrix of seven distinctive morphometric characters of all male specimens examined in the analysis (8.6:1 subject to item ratio). The first two principal components were retained, supported by the eigenvalue-one criterion and proportion of the components in total variance. The first principal component (PC1) explained 34.3% of the variation among specimens in the multivariate dataset, whereas PC2 represented 29.8%. PC1 explained much of the variation in interorbital width, suborbital depth and eye diameter, whereas PC2 was associated mainly with variation in preanal length and caudal peduncle length (Table 4). Significantly, *N. cooperi* grouped separately on the score plot of PC1 vs PC2 (Figure 2). *Nothobranchius cooperi* was confined entirely within the negative domain of the first PC axis, without overlap with *N. sainthousei*, which was situated entirely in the negative part of the first axis; there was no overlap in this PCA plot with *N. symoensi*, which was situated mainly in the positive domain of the first axis. *Nothobranchius cooperi* was situated entirely in the positive domain of the second axis. There was no overlap with *N. rosenstocki* and *N. sainthousei*, which were situated mainly in the negative domain of the second axis. The discrete position of *N. cooperi* recovered in the PCA corroborates the hypothesis that it can be separated from the most similar species using morphological characters.

Table 2: Morphometric and meristic data of holotype and paratypes of *Nothobranchius cooperi*. Standard length in mm, all other measurements as percentages of standard length, head length, or in ratios. Holotype values included in range, mean and SD. H = holotype; SD = standard deviation

		Males			Females		
	H	(*n* = 8)			(*n* = 5)		
		Range	Mean	SD	Range	Mean	SD
Standard length	25.8	20.9–25.8			18.4–19.3		
Percentage of standard length							
Total length	122.1	121.7–123.6	122.6	0.7	122.8–124.5	123.5	0.9
Body depth at pelvic-fin origin	27.9	27.9–31.4	30.3	1.1	26.7–29.8	27.9	1.4
Head length	32.9	32.5–36.2	33.8	1.3	33.2–36.4	34.7	1.2
Preanal length	60.1	58.9–62.9	60.5	1.3	62.7–67.2	65.5	1.8
Predorsal length	56.6	55.0–59.0	57.5	1.4	61.0–63.3	62.1	1.0
Prepelvic length	49.2	48.8–51.9	50.1	1.1	53.3–59.8	55.8	3.1
Prepectoral length	32.9	32.5–36.2	33.5	1.2	31.4–36.4	34.0	1.9
Caudal peduncle length	18.2	17.1–18.9	18.1	0.6	18.5–20.2	19.3	0.6
Caudal peduncle depth	14.3	13.8–15.4	14.5	0.5	10.9–11.9	11.3	0.4
Dorsal-fin base length	26.0	26.0–30.1	27.5	1.3	21.8–25.7	23.7	1.6
Anal-fin base length	22.1	20.5–25.8	21.9	1.7	14.3–18.1	16.1	1.5
Caudal fin length	22.1	21.7–23.6	22.6	0.7	22.8–24.5	23.5	0.9
Percentage of head length							
Head width	55	53–59	55.0	1.8	48–55	51.0	2.5
Head depth	75	75–77	75.8	0.8	66–72	69.0	2.4
Interorbital width	33	33–37	34.2	1.6	30–34	31.6	1.8
Postorbital length	53	50–55	52.5	1.9	46–53	49.7	2.6
Suborbital depth	21	19–24	20.5	1.5	14–16	14.7	1.0
Eye diameter	25	21–25	23.7	1.7	25–30	27.0	1.6
Snout to eye end length	47	45–50	47.5	1.9	47–54	50.3	2.6
Snout length	21	21–25	22.2	1.4	19–22	20.8	1.5
Ratios							
Head width in % of its depth	73	70–77	72.5	2.0	72–76	73.8	1.8
Caudal peduncle length in % of its depth	127	120–129	125.1	3.3	170–176	171.7	2.6
Meristics		Range	Mode		Range	Mode	
Dorsal-fin rays	14	14–16	15		15–16	16	
Anal-fin rays	15	14–15	14		15–16	16	
Scales mid-longitudinal series	25	25–28	26		26–27	26	
Scales transverse	11	10–11	11		10–11	11	
Scales circumpeduncular	12	12	12		10–12	12	

Table 3: Morphometric and meristic data of males of the comparative material of *Nothobranchius rosenstocki*, *N. sainthousei* and *N. symoensi*. Results of Mann–Whitney *U*-tests two-tailed statistical significance after Bonferroni correction are marked with * for significant result ($p < 0.05$) and ** for highly significant result ($p < 0.01$). Standard length in mm; all other measurements as percentages of standard length, head length, or in ratios. SD = standard deviation

	N. rosenstocki				*N. sainthousei*				*N. symoensi*			
	males (n = 19)				males (n = 8)				males (n = 8)			
	range	mean	SD	Mann–Whitney (p)	range	mean	SD	Mann–Whitney (p)	range	mean	SD	Mann–Whitney (p)
Standard length	21.5–34.9				24.1–40.8				25.6–39.3			
Percentage of standard length												
Total length	120.1–124.6	122.1	1.5	0.5592	117.6–123.7	121.2	2.6	0.7929	119.4–123.4	121.0	1.3	0.0074
Body depth at pelvic-fin origin	26.0–32.6	29.3	1.7	0.1442	26.1–32.2	29.2	2.5	0.5635	29.6–34.6	31.5	1.6	0.1563
Head length	29.9–38.7	33.0	2.4	0.0384*	25.1–29.3	27.1	1.2	0.0009**	30.3–33.0	31.3	1.1	0.0054**
Preanal length	55.7–62.1	59.1	1.6	0.0117*	58.0–61.5	60.2	1.2	0.9581	59.2–61.3	60.4	0.7	0.9164
Predorsal length	52.0–63.4	55.2	2.7	0.0001**	51.9–58.5	56.1	2.2	0.2271	55.1–60.1	57.9	1.5	0.5635
Prepelvic length	45.1–49.0	47.7	1.2	0.0001**	48.9–54.4	50.5	1.9	0.7929	48.2–51.4	49.7	1.0	0.5635
Prepectoral length	29.8–36.3	32.5	1.9	0.0944	25.1–29.4	27.9	1.5	0.0009**	29.3–34.8	31.4	1.9	0.0181
Caudal peduncle length	19.0–22.0	20.1	0.9	0.0001**	18.0–20.1	18.6	0.6	0.1893	18.7–21.2	20.1	0.7	0.0019**
Caudal peduncle depth	11.9–13.4	12.5	0.5	0.0001**	12.5–14.0	13.0	0.5	0.0014**	11.8–13.4	12.9	0.5	0.0009**
Dorsal-fin base length	20.1–32.2	29.0	3.0	0.0594	26.5–29.2	28.0	1.0	0.3184	25.8–29.6	28.0	1.5	0.5635
Anal-fin base length	17.5–24.8	21.8	1.7	0.5069	19.5–23.1	22.0	1.4	0.5635	18.6–22.0	20.3	1.1	0.0406
Caudal fin length	20.1–27.1	22.2	1.8	0.5592	17.9–24.1	21.4	2.7	0.9581	18.4–23.4	20.6	1.5	0.0074
Percentage of head length												
Head width	53–60	56.3	2.1	0.1302	71–79	74.6	2.4	0.0009**	55–60	57.4	1.9	0.0239
Head depth	78–84	79.6	2.1	0.0001**	88–94	90.4	2.3	0.0009**	74–78	76.5	1.6	0.4008
Interorbital width	31–35	32.8	1.2	0.0710	40–48	44.7	3.0	0.0009**	32–40	37.5	2.6	0.0239
Postorbital length	49–54	51.3	1.5	0.1053	49–55	50.8	2.1	0.0831	49–55	52.5	2.3	0.9581
Suborbital depth	19–24	20.9	1.5	0.3956	19–26	22.5	2.4	0.1278	21–26	22.4	1.4	0.0136
Eye diameter	23–29	25.9	1.6	0.0058**	26–32	29.2	2.1	0.0009**	23–26	24.6	1.2	0.3720
Snout to eye end length	46–51	48.7	1.5	0.1172	45–52	49.1	2.0	0.1036	44–51	47.1	2.6	0.7929
Snout length	24–28	24.8	1.3	0.0019**	18–21	19.5	1.0	0.0009**	20–25	21.8	1.8	0.4948
Ratios												
Head width in % of its depth	68–78	70.7	2.2	0.0594	79–86	82.5	2.7	0.0009**	73–79	75.0	2.6	0.0136
Caudal peduncle length in % of its depth	158–164	160.7	2.1	0.0001**	140–147	143.3	2.5	0.0009**	152–159	156.1	2.7	0.0009**
Meristics	range	mode			range	mode			range	mode		
Dorsal-fin rays	14–17	15		0.7300	15–19	16		0.0209*	16–18	17		0.0016**
Anal-fin rays	14–16	15		0.0045**	15–19	16		0.0028**	17–18	17		0.0009**
Scales mid-longitudinal series	25–30	28		0.0169*	29–31	29		0.0009**	27–31	29		0.0023**
Scales transverse	10–11	11		0.6517	10–11	11		0.7132	10–12	10		0.7132
Scales circumpeduncular	10–12	12		–	10–12	12		–	12	12		–

Table 4: Factor loadings for the first two principal component axes of five distinctive morphometric characters and proportions of variance explained by the selected principal components, in males of *Nothobranchius cooperi*, *N. rosenstocki*, *N. sainthousei* and *N. symoensi*

Morphometric characters	PC1	PC2
Preanal length	−0.182	0.627
Interorbital width	0.552	0.428
Suborbital depth	0.497	-0.067
Eye diameter	0.626	0.016
Caudal peduncle length in % of its depth	0.152	−0.647
Eigenanalysis of the correlation matrix		
Eigenvalue	1.7162	1.4912
Explained variance (% of total variance)	34.3	29.8
Cumulative variance (%)		64.1

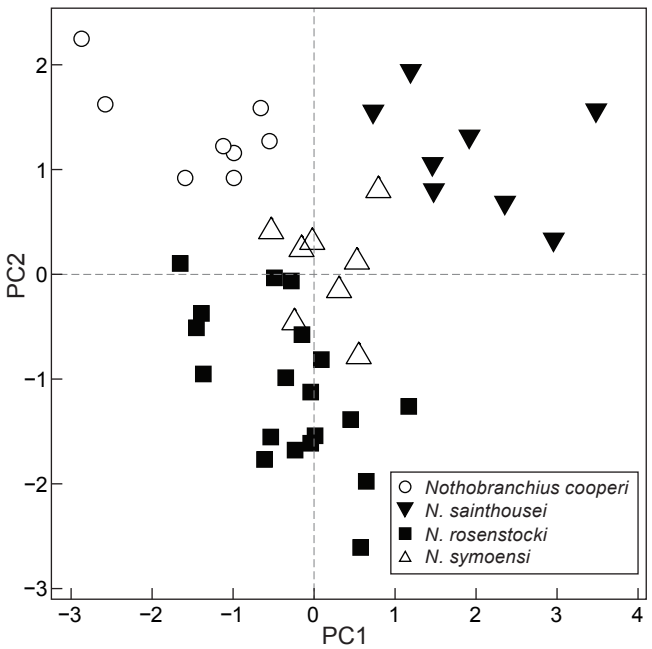

Figure 2: Comparative morphometry in males of *Nothobranchius cooperi* (open circles), *N. rosenstocki* (solid squares), *N. sainthousei* (solid inverted triangles) and *N. symoensi* (open triangles): score plot of principal component analysis on morphometric characters, first component vs second component

Molecular analysis

In order to assess whether DNA sequence data would support the morphological and colour pattern differences, the mitochondrial ND2 and COI, as well as the nuclear S7 sequences, were compared and phylogenetic analyses were performed on a combined sequence matrix. The sequences of the genes of 32 specimens, representing 12 species in the target study region of northern Zambia and Katanga province of DRC, as well as six specimens of three species used as outgroups, were aligned and compared. In order to allow a comparison with our earlier studies (Nagy et al. 2016), we included the same ingroup species in the current analysis. The earlier study was based on partial ND2 sequences alone; in the current study complete ND2, COI and S7 sequences were used to provide more sequence data. Therefore, we included all currently known species from the entire Luapula drainage, as well as from the rest of the entire Congo drainage, namely the nearby Lake Mweru and Lake Mweru Wantipa basins and the neighbouring drainage of the Lufira and the Lualaba drainage (listed in Table 1).

Base calling in the chromatograms of the haploid mitochondrial COI and ND2 sequences was uncomplicated. The nuclear S7 sequence chromatograms revealed that some individuals possessed homozygous alleles where base calling was again uncomplicated. However, other individuals often showed heterozygote sequences in chromatograms. In heterozygotes, single nucleotide polymorphisms could be assigned to their respective alleles by comparison to the sequences of homozygotes of the same species. In many heterozygotes it was additionally found that the two alleles of one individual differed by distinctive indels. Where indels occurred, the chromatograms showed clear base calling up to the insertion or deletion, whereafter the chromatograms showed hybrid peaks. Sequences of the respective alleles were read from the chromatograms by comparing individual peaks with those of homozygotes, which allowed assignment of one base to the homozygote sequence, i.e. the first allele, whereas the second base could be unequivocally assigned to the second allele. Both of the S7 sequences of heterozygotes were included and were referred to as 'a' and 'b' alleles of each specimen, respectively. A sequence matrix of each of these S7 sequences and their respective mitochondrial N2 and COI sequences was generated, i.e. the mitochondrial N2 and COI sequences were duplicated for each S7 allele of heterozygotes and then used for phylogenetic analysis. The phylogeny generated on this sequence matrix is presented in Figure 3. Phylogenetic analysis always retrieved heterozygote alleles of the same species sister to each other indicating the robustness of this approach.

The phylogenetic analysis recovered a fully resolved and well-supported phylogeny. This retrieved all the species included in our earlier analyses based on partial ND2 sequences only (Nagy et al. 2016). All specimens of *N. cooperi* comprised a well-supported and distinct group sister to *N. rosenstocki sensu stricto*, its closest known relative and further confirms the affinities of *N. cooperi* in the *N. brieni* species group.

Comparative material

Nothobranchius rosenstocki: MRAC 97-089-P-0011, holotype, male, 33.5 mm SL; MRAC 97-089-P-0012, paratype, male, 31.1 mm SL; Zambia: upper Luapula drainage, 12°30′ S, 30°08′ E; collected by P Goudswaart, 8 Aug 1997. — MRAC B3-028-P-0018–0022, 5 males, 21.5–26.4 mm SL; Zambia: upper Luapula drainage, 12°09.93′ S, 29°55.87′ E; collected by B Watters et al., 20 March 2007. — MRAC B3-028-P-0040–0041, 2 males, 26.8–31.5 mm SL; Zambia: upper Luapula drainage, 12°09.73′ S, 29°55.64′ E; collected by B Nagy and F Milvertz, 4 April 2012. — MRAC B3-028-P-0042–0053, 5 males, 25.6–34.9 mm SL; 7 females, 21.7–24.8 mm SL; Zambia: upper Luapula drainage, 12°23.06′ S, 29°23.18′ E; collected by B Nagy and F Milvertz, 5 April 2012. — MRAC B3-028-P-0036–0039, 4 females, 24.2–27.7 mm SL;

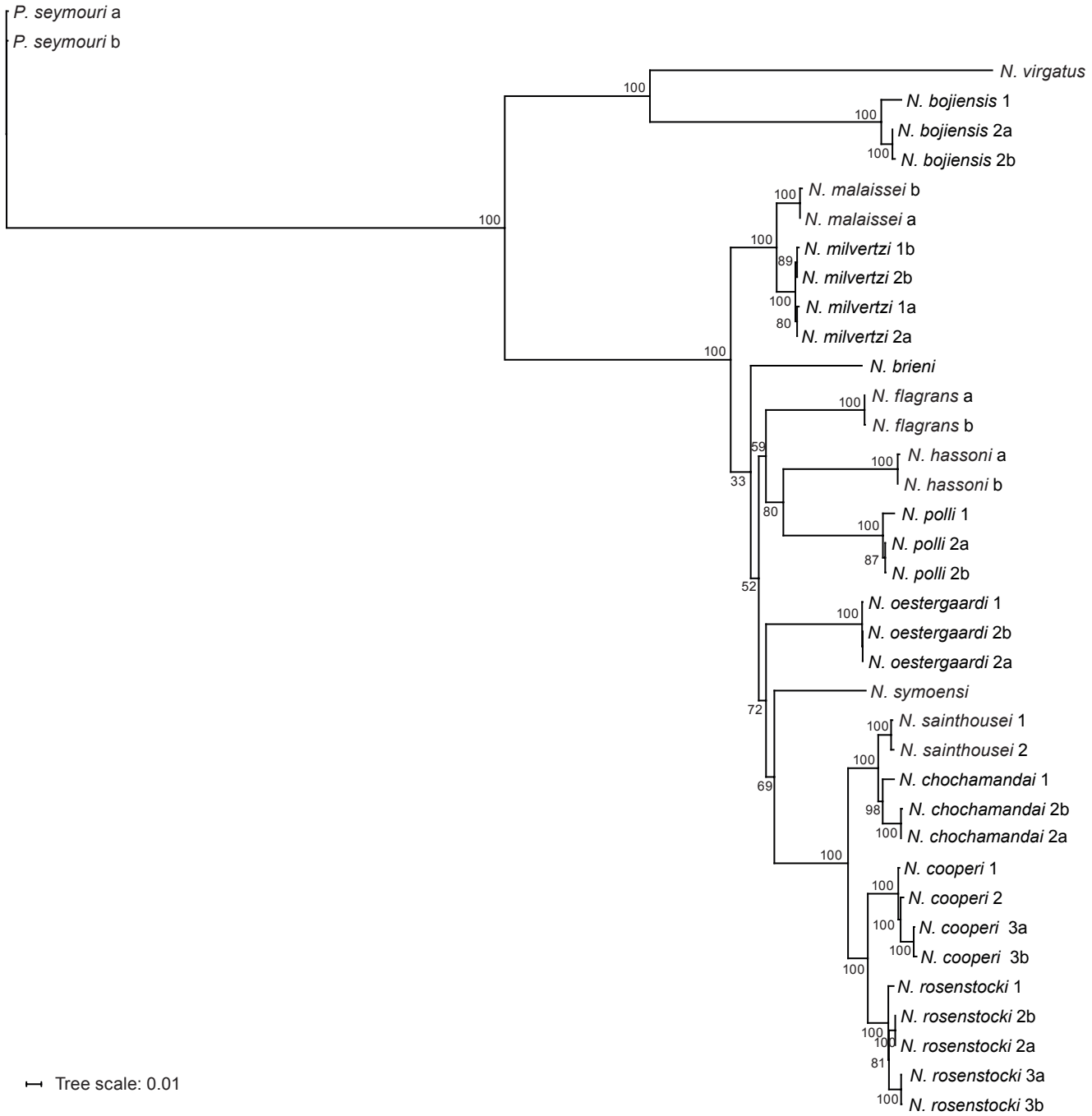

Figure 3: Phylogenetic tree based on sequences of the mitochondrial COI and ND2 genes and nuclear S7 gene and maximum likelihood analysis. Branch support is indicated in bootstrap percentages and the tree is shown with proportional branch lengths

Zambia: upper Luapula drainage, 12°33.13′ S, 30°12.58′ E; collected by B Watters et al., 26 March 2007. — MRAC B3-028-P-0054–0059, 5 males, 23.3–33.2 mm SL; female, 25.9 mm SL, Zambia: upper Luapula drainage, 12°36.29′ S, 30°15.88′ E; collected by B Nagy and F Milvertz, 11 April 2012.

Nothobranchius sainthousei: MRAC B5-027-P-0001, holotype, male, 32.9 mm SL; MRAC B5-027-P-0005–0011, 5 males, 24.1–36.1 mm SL and 2 females, 25.4–27.5 mm SL; MRAC B5-027-P-0002–0004, 2 males, 40.0–40.8 mm SL and 1 female, 29.9 mm SL; Zambia: Luapula Province: Luapula drainage: 10°43.51′ S, 28°38.22′ E; collected by B Nagy and F Milvertz, 6 April 2012.

Nothobranchius symoensi: MRAC B3-028-P-0011-2, male, 31.5 mm SL; female, 28.8 mm SL; MRAC B4-008-P-0013, male, 39.3 mm SL; Zambia: upper Luapula drainage, 12°14.45′ S, 29°25.78′ E; collected by B Nagy and F Milvertz, 5 April 2012. — MRAC B3-028-P-0005, male, 32.1 mm SL; Zambia: upper Luapula drainage, 12°18.90′ S, 29°24.98′ E; collected by B Watters et al., 21 March 2007. — MRAC B3-028-P-0006–0010, 3 males,

25.6–32.1 mm SL; 2 females, 23.9–25.7 mm SL; MRAC B4-008-P-0012, male, 34.6 mm SL; Zambia: upper Luapula drainage, 12°18.89′ S, 29°24.48′ E; collected by B Nagy and F Milvertz, 5 April 2012. — MRAC 73-25-P-1108–1109; holotype and paratype, male and female, 21.5–30.5 mm SL; DR Congo: Katanga province: Malinde River drainage, 12°56′ S, 29°22′ E; collected by JJ Symoens, 23 April 1962.

***Nothobranchius cooperi* Nagy, Watters and Bellstedt, new species**
(Figures 4–8)

Holotype
MRAC B3-028-P-0035, male, 25.8 mm SL; Zambia: Luapula province; middle Luapula drainage; seasonal streams and associated seasonal pools (11°15.85′ S, 29°02.57′ E) in the upper Mansa River system, 20 km east of Mansa town, on the road to Samfya; BR Watters, BJ Cooper, O Schmidt and W Bishopp, 23 March 2007; preserved in the field.

Paratypes
MRAC B3-028-P-0028–0034, 7 males, 20.9–24.6 mm SL; — MRAC B3-028-P-0023–0027, 5 females, 18.4–19.3 mm SL; collected with the holotype; preserved in the field.

Diagnosis
Nothobranchius cooperi belongs to the *Nothobranchius brieni* species group, sensu Nagy et al. (2016). With the exception of *N. rosenstocki* and *N. sainthousei*, it is distinguished from all other species of that group by having broad orange (vs red-brown) posterior scale margins on the trunk and anal fin, with a uniform orange-red margin (vs light blue, yellow, red-brown or black margin). It is distinguished from *N. sainthousei* by having an anal fin that is uniform orange-red with an irregular to regular, light blue-green zone close to the base (vs orange-brown spots and orange-brown margin) and a wider, more prominent light blue marginal band to

Figure 4: *Nothobranchius cooperi*, MRAC B3-028-P-0035, holotype male, 25.8 mm SL; Zambia: Luapula Province, middle Luapula River drainage, Mansa River system

Figure 5: *Nothobranchius cooperi*, wild-caught male, not preserved. Location: type locality, about 20 km east of the town of Mansa, upper Mansa River system Luapula Province, middle Luapula River drainage

Figure 6: *Nothobranchius cooperi*, wild-caught male, not preserved. From the easternmost known location, about 11 km west of Samfya, in a drainage system flowing into the low-lying area marginal to the southwestern part of Lake Bangweulu

Figure 7: *Nothobranchius cooperi*, wild-caught male, not preserved. Location: type locality, about 20 km east of the town of Mansa, upper Mansa River system Luapula Province, middle Luapula River drainage

Figure 8: *Nothobranchius cooperi*, wild-caught female, not preserved. Location: type locality, about 20 km east of the town of Mansa, upper Mansa River system Luapula Province, middle Luapula River drainage

the caudal fin; head length 32.5–36.2% SL (vs 25.1–29.3); prepectoral length 32.5–36.2% SL (vs 25.1–29.4); and head width 70–77 in % of its depth (vs 79–86). It is distinguished from *N. rosenstocki* by having a prepelvic length 48.8–51.9% SL (vs 45.1–49.0); and a head depth 75–77% HL (vs 78–84). Furthermore, the species is characterised by a caudal peduncle length 1.2–1.3 times its depth, compared to 1.4–1.5 times in *N. sainthousei*; and 1.6 times in *N. rosenstocki.*

Description

General body features are illustrated in Figures 4–8. Morphometric and meristic characters of holotype and paratypes are summarised in Table 2. A relatively small *Nothobranchius* species, maximum observed length in males 25.8 mm SL. General body shape robust, laterally compressed and deep. Greatest body depth in front of pelvic fin origin: 27.9–31.4% SL. Greatest body width at pectoral-fin base with body progressively narrowing towards caudal fin base. Dorsal profile convex from tip of snout to base of last dorsal fin ray, straight to slightly concave on caudal peduncle. Ventral profile convex from lower jaw to base of last anal fin ray, straight to slightly concave on caudal peduncle. Caudal peduncle shallow, length 1.2–1.3 times its depth. Anus situated directly in front of anal fin origin.

Head short, laterally compressed, deeper than wide. Head width 70–77% of its depth. Snout slightly pointed, about the size of eye diameter. Mouth supraterminal, slightly oblique in profile. Jaws subequal, lower jaw longer than upper, posterior end of rictus at same level or slightly ventral to centre of eye. Premaxilla and dentary with many irregularly distributed conical, slightly curved teeth at outer row of lower and upper jaws. Orbit relatively small, 21–25% of HL, in anterior half of head, in dorsal portion of head side. Branchiostegal membrane projecting posteriorly from opercle.

Dorsal fin origin anterior to anal fin origin, both fins originating posterior to mid-length of body. Extremity of dorsal and anal fins rounded, with small contact organs in form of papillae on fin rays and distal margin with short filamentous rays. Posterior extremity of dorsal fin reaching caudal fin base. Dorsal fin 14–16 rays; anal fin 14–15 rays. Dorsal fin origin between neural spines of vertebrae 10 and 12. Anal fin origin between pleural ribs of vertebrae 10 and 12. Pectoral fin subtriangular, insertion slightly posterior to margin of opercular opening, base slightly oblique, upper fin rays placed slightly anteriorly to lower fin rays, tip reaching or slightly overlapping base of pelvic fin. Pelvic fin subabdominal, origin at about mid-length of body, short, bases medially separated, tip reaching urogenital papilla. Caudal fin subtruncate, with 14–16 branched rays, plus 3 to 4 unbranched smaller rays at dorsal and ventral origins.

Scales cycloid, body and head entirely scaled, except for ventral surface of head. No scales on dorsal and anal fins. Scales in mid-longitudinal series 25–28 plus two or three small scales on caudal-fin base. Transverse rows of scales in front of dorsal-fin origin 10–11; scale rows around caudal peduncle 12.

Cephalic squamation pattern variable, holotype presenting E-type, with E-scales overlapping each other at median lateral margin (Hoedeman 1958). Nostril in front of orbit, with single oblique aperture. Frontal neuromasts separate in two rows of shallow grooves, one neuromast in each groove. Cephalic sensory system at supraorbital level in two discontinuous shallow grooves, with one and two exposed neuromasts, whereas at supratemporal level in a curved groove, with four exposed neuromasts. Preorbital canal in shallow groove with three exposed neuromasts; postorbital canal in a very short groove with one exposed neuromast; infra-orbital series with about a dozen neuromasts at ventral and posterior margin of orbit, plus one neuromast just posteriorly from postorbital canal. Preopercular canal in an open groove with around a dozen exposed neuromasts. Mandibular canal in shallow groove with about ten very small neuromasts. One neuromast on each scale along trunk mid-longitudinal series. Total vertebrae 26–27.

Females smaller than males, maximum observed size 19.3 mm SL. Body and head somewhat less laterally compressed and more slender than in males (head depth 66–72% HL vs 75–77, caudal peduncle depth 10.9–11.9% SL vs 13.8–15.4). Dorsal and caudal fins rounded. Anal fin subtriangular, tip rounded, central rays longer and more rigid. Dorsal and anal fins positioned more posteriorly than in male (61.0–63.3% SL vs 55.0–59.0 and 62.7–67.2% SL vs 58.9–62.9, respectively). Anal-fin and dorsal-fin base lengths smaller than in male (14.3–18.1% SL vs 20.5–25.8 and 21.8–25.7% SL vs 26.0–30.1, respectively). Pelvic fin short, tip reaching anus. Branchiostegal membrane not projecting posteriorly from opercle. No papillae or epidermal tissue present on dorsal and anal fins.

Colouration

Live male (Figures 5–7): Scales on trunk and head light iridescent blue with broad orange-red posterior margins, forming an irregular reticulated and coarse, highly irregular cross-bar pattern. Scales on abdomen faint blue to silver, most with narrow orange-red margins. Snout, frontal and dorsal portions of head orange-red; throat pale blue to yellow. Exposed part of branchiostegal membrane orange to orange-red. Iris golden, with a poorly developed black vertical bar through centre of eye. Background colour of dorsal fin light blue-green with a golden hue, grading to grey overlain by iridescent blue-green in distal zone. Irregular orange-red spots present, larger and more distinct at base of fin, grading into a striped pattern, parallel to fin rays, towards distal edge. Some irregular dark grey spots occasionally present in submarginal zone. Prominent black markings present on membrane between first 3–4 anterior fin rays. A thin blue margin, usually discontinuous, occasionally present on dorsal fin (Figure 6); whereas in other specimens either absent or very sparsely developed (Figure 7). Anal fin fairly uniform orange-red with an irregular to regular, light blue-green zone close to base. Some specimens with a second, poorly developed, light blue-green band, usually represented only by a row of spots, extending across central part of anal fin (Figures 5–6). A very narrow blue edging occasionally present on anal fin (Figure 5); whereas in some populations either absent or very sparsely developed (Figure 7). Specimens showing second band and blue edging to anal fin most commonly showing also a blue margin to dorsal fin. Base colour of

bulk of caudal fin grey with iridescent blue-green on membrane between rays, overlain by orange-red patches concentrated near fin base and extending distally as orange-red streaks. Grey base colour of caudal fin intensified in distal parts of fin forming a vague subdistal band, replaced distally by an iridescent blue marginal band. Pelvic fins orange-red. Pectoral fins dominantly hyaline, with faint orange at base and light blue margins.

Live female (Figure 8): Scales on trunk and head pale grey-brown, darker on dorsum and lighter to silver on venter. Vague reticulation on posteroventral portions of flank, due to some scales having relatively dark grey margins. Scales immediately above mid-longitudinal line, over abdominal region, have a blue iridescence, extending across upper part of operculum to eye. Iris golden. All fins hyaline.

Distribution

Nothobranchius cooperi is currently known from five sites situated alongside, or close to, the road between the towns of Mansa and Samfya (Figure 9). A main grouping of four sites occurs between about 20 and 36 km east of Mansa town, the type locality being the westernmost site. A fifth site occurs 11 km west of Samfya. The distance covered by this narrow range of distribution is about 45.5 km. All sites represent seasonal streams and seasonal pools associated with the streams, situated in broad, shallow valleys (Figures 10 and 11). The streams associated with the western group of localities drain in a generally northward direction into the upper reaches of the Mansa River, which flows westwards and represents a right-bank tributary of the Luapula River.

Although the *Nothobranchius* species of the Luapula River system show close evolutionary relationships and appear to have had a common founder species, the modern regional drainage network preserves striking evidence of several major reorganizations (Moore and Larkin 2001; Goudie 2005; Moore et al. 2007, 2012), exemplified in significantly different histories of the precursors comprising the modern Luapula system. These changes in geomorphology and hydrology are invoked as primary drivers of allopatric speciation evident in the regional biogeographical patterns (Cotterill 2006; Cotterill and de Wit 2011).

The Mansa River joins the middle reaches of the Luapula between two significant natural barriers: upstream, the

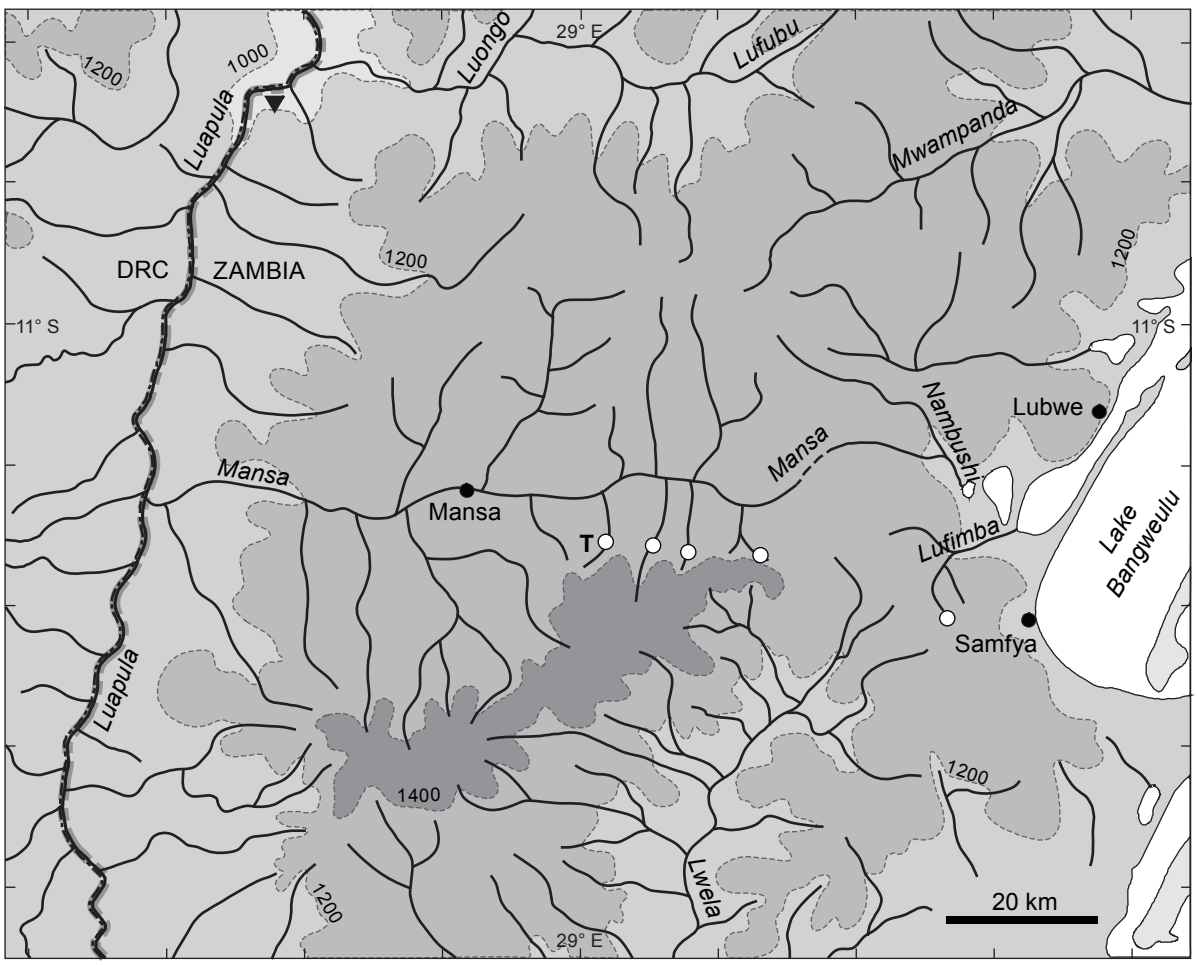

Figure 9: Map showing the topography and drainage in the Mansa-Samfya area of the Luapula Province of Zambia and the distribution of known occurrences of *Nothobranchius cooperi* (open circles) and *N. sainthousei* (solid inverted triangles). T, type locality of *N. cooperi*. Grey shades indicate 200 m contour intervals. The regional setting of the area covered by this map is indicated on Figure 1

Figure 10: Type locality for *Nothobranchius cooperi*. Situated about 20 km east of Mansa, in the upper Mansa River system, Luapula Province, middle Luapula River drainage. Habitat comprised a densely vegetated seasonal stream and associated roadside ditches and pools within a broad shallow valley. Photograph taken 28 March, 1997

Figure 11: Easternmost location for *Nothobranchius cooperi*, near Samfya, within the drainage of the seasonal Lufimba system flowing into a low-lying area marginal to the southwestern part of Lake Bangweulu. Habitat comprised very shallow remnant pools among dense grass in a broad shallow valley. Photograph taken 28 March, 1997

Mumbatuta Falls isolates the river section from the upper Luapula and the currently known occurrences of *N. rosenstocki* and *N. symoensi*; downstream, the Mambilima Falls contains the lower part of the Luapula drainage and Lake Mweru (Figure 1), effectively isolating *N. malaissei* from other Luapula species.

All localities of *N. cooperi* occur to the north of a NE-SW-trending topographic high that forms the watershed separating the Mansa River from streams draining into the low-lying Samfya area near Lake Bangweulu (Figure 9). The latter streams flow south-westwards via the Lwela River system, on into the Luapula River downstream of the Mumbatuta Falls, which constitutes the barrier isolating *N. cooperi* from *N. rosenstocki* and *N. symoensi* in the upper Luapula system.

The easternmost occurrence of *N. cooperi*, near Samfya, lies within the seasonal Lufimba system flowing into a low-lying floodplain along the southwestern shore of Lake Bangweulu, which includes the satellite lakes: Lake Kasongole and the southwestern segment of Lake Chifunabuli. Also draining into this section of floodplain is the Nambushi River, of which the headwaters, as indicated by satellite imagery, appear to have seasonal connections with the Mansa River. Therefore, although the *N. cooperi* sites are associated with two different local drainage systems, there is evidence of connections between the two. Links between the easternmost habitat and the Upper Luapula River could also, potentially, exist via Lake Bangweulu; however, for a relatively poor-swimming fish such as *Nothobranchius*, the lake itself would probably constitute a significant barrier to migration.

As currently known, the range of *Nothobranchius cooperi* is separated from that of *N. sainthousei* by the watershed of the Chambaumoni Hills (Figure 1). Furthermore, any recent links between *N. cooperi* and the occurrences of *N. sainthousei* and *N. chochamandai* via the Luapula River itself are considered unlikely, given the physiological characteristics and ecological preferences of *Nothobranchius* fishes in general. In this regard, it should be noted that major rivers can also constitute significant barriers to gene flow in *Nothobranchius* (Bartáková et al. 2015; Watters et al. 2015).

Ecology

The middle Luapula drainage receives a mean annual rainfall of 1 020–1 120 mm concentrated between December and May (Symoens 1987; Hughes and Hughes 1992), which maintains high water levels, peaking from March to May. Wetlands are at their lowest levels between September and January, when seasonal streams and most dambos are dry. The town of Mansa, close to the main group of *N. cooperi* occurrences, receives a mean annual rainfall of 1 105 mm (Archer 1971). Across the range of *N. cooperi*, annual rainfall generally increases from west to east, because of the influence of Lake Bangweulu (Davies 1971). Samfya, near the easternmost locality, receives a mean annual rainfall of 1 538 mm.

The type locality consists of a stream system flowing via multiple narrow channels through dense grasses with associated roadside ditches and residual pools, also thickly vegetated (Figure 10). Maximum water depth was ~1 m in the vicinity of a culvert through which the water was flowing. The *Nothobranchius* were present in high density, generally favouring the still or relatively slow-flowing, more thickly vegetated parts of the system. During the course of three visits to the type locality during mid- to late afternoon in late March to early April over a fifteen- year period in 1997, 2007 and 2012, the water quality was: pH 6.18–6.35, total dissolved solids <10–16, temperature 24–26 °C. The water was only mildly turbid. Other sites where water quality was measured showed values within the same ranges, except for a slightly higher temperature of 28 °C in small shallow isolated pools at the easternmost locality (Figure 11) where the water was not flowing and was therefore more susceptible to the effects of solar warming.

Nothobranchius cooperi was the only *Nothobranchius* species observed at all the sites. The accompanying fauna at some locations consisted of non-annual species, including *Enteromius* and *Ctenopoma* species, as well as *Micropanchax* cf. *hutereaui* and a second unidentified *Micropanchax* species.

Conservation status

Based on the limited knowledge of its geographic distribution, the conservation status of *N. cooperi* is uncertain. However, knowledge indicates that the currently known extent of occurrence (EOO) of <20 000 km² and the area of occupancy (AOO) are in continuing decline, as a result of the expansion of agriculture. Using IUCN (2012) criteria, the species appears to qualify as Vulnerable (B1bii). Additional collecting efforts targeting suitable habitats should be conducted, in particular within the drainage of the upper Mansa River, north of the main group of known sites and in the drainage system of streams flowing into the low-lying area marginal to the southwestern part of Lake Bangweulu, in order to understand the geographic distribution of this species better.

Biology

Aquarium maintenance of selected individuals was undertaken for the observation of breeding behaviour and biology. *Nothobranchius cooperi* has an annual mode of reproduction, typical of the genus. Under captive conditions, peat moss was used successfully as an artificial spawning substrate. An embryonic development period of two to four months was observed at about 24 °C. As with the related species *N. sainthousei* and *N. rosenstocki*, it is a micropredator, feeding on small aquatic crustaceans, worms, insect larvae and other zooplankton.

Etymology

The specific epithet is given in honour of Barry J Cooper, renowned collector and breeder of killifish, for his significant contributions to the field study of *Nothobranchius* and to the killifish hobby in general. A noun in genitive.

Recommendation 50A of the ICZN (1999) indicates that, when a name is proposed in a multi-authored work, but only some of the authors are directly responsible for the name and satisfying the criteria that make the name available, then the authors directly responsible should be identified explicitly. In accordance with this Recommendation, we wish to motivate the authorship of the new species as Nagy, Watters and Bellstedt. Nagy and Watters were responsible for the collecting and identification of the species and for designing the manuscript. Bellstedt's phylogenetic research corroborated this new species. Cotterill and van der Merwe performed the

DNA sequencing and phylogenetic analysis and their contribution is acknowledged through co-authorship of this publication.

Discussion

Comparative morphometrics and colour pattern

Males of *N. cooperi* are distinguished from the most similar species in the Luapula drainage by the following characters (Figures 12a–d and Tables 2–3):

Males of *N. cooperi* differ from those of *N. rosenstocki* (represented in colour on page 179 in Nagy 2014b) by having a relatively greater prepelvic length (48.8–51.9% SL vs 45.1–49.0, $p < 0.001$, Mann–Whitney *U*-test, two-tailed); smaller caudal peduncle length (17.1–18.9% SL vs 19.0–22.0, $p < 0.001$; and 120–129 in % of its depth vs 158–164, $p < 0.001$); greater caudal peduncle depth (13.8–15.4% SL vs 11.9–13.4, $p < 0.001$); and smaller head depth (75–77% HL vs 78–84, $p < 0.001$).

Males of *N. cooperi* differ from those of *N. sainthousei* (represented in colour in Nagy et al. 2016, p 213) by having the anal fin with an irregular to regular, light blue-green zone close to the base (vs orange-brown spots and

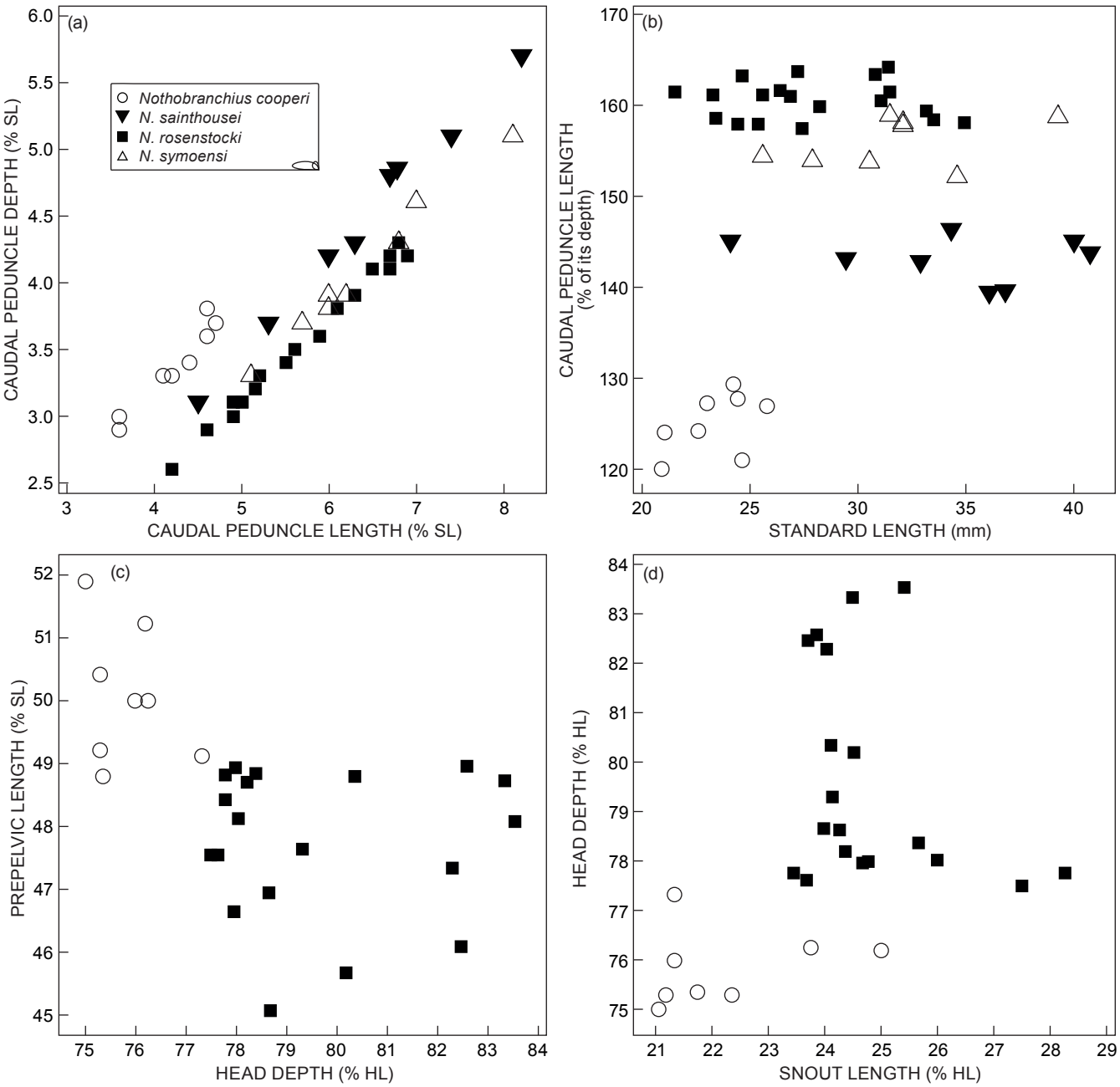

Figure 12: Comparisons of morphometric characters in males for *Nothobranchius cooperi* (open circles), *N. rosenstocki* (solid squares), *N. sainthousei* (solid inverted triangles) and *N. symoensi* (open triangles): a) caudal peduncle depth in % SL plotted against caudal peduncle length in % SL; b) caudal peduncle length in % of its depth plotted against standard length in mm; c) prepelvic length in % SL plotted against head depth in % HL; d) head depth in % HL plotted against snout length in % HL

orange-brown margin) and a wider, more prominent light blue marginal band to the caudal fin; a relatively greater head length (32.5–36.2% SL vs 25.1–29.3, $p < 0.001$); greater prepectoral length (32.5–36.2% SL vs 25.1–29.4, $p < 0.001$); smaller head width (53–59% HL vs 71–79, $p < 0.001$; and 70–77 in % of its depth vs 79–86, $p < 0.001$); smaller head depth (75–77% HL vs 88–94, $p < 0.001$); smaller interorbital width (33–37% HL vs 40–48, $p < 0.001$); smaller eye diameter (21–25% HL vs 26–32, $p < 0.001$); greater snout length (21–25% HL vs 18–21, $p < 0.001$); smaller caudal peduncle length (120–129 in % of its depth vs 140–147, $p < 0.001$); and less number of scales in mid-longitudinal series (25–28 vs 29–31, $p < 0.001$).

Males of *N. cooperi* differ from those of *N. symoensi* (represented in colour in Nagy 2014b, p. 179) by having the dorsal fin without distinct margin (vs light blue distinct margin); anal fin uniform orange-red with an irregular to regular, light blue-green zone close to the base (vs a spotted to vermiform pattern across the entire fin, with a prominent light blue margin); caudal fin grey overlain by orange-red spots and streaks with irregular, relatively narrow light blue margin (vs irregular, spotted red-brown pattern and with distinct light blue margin); relatively greater caudal peduncle depth (13.8–15.4% SL vs 11.8–13.4, $p < 0.001$); smaller caudal peduncle length (120–129 in % of its depth vs 152–159, $p < 0.001$); and less anal-fin rays (14–15 vs 17–18, $p < 0.001$).

Morphometric comparisons with the most closely related species reveal that males of *N. cooperi* exhibit a smaller caudal peduncle length in % of its depth (Figures 12a–b) and are characterised against the most closely related *N. rosenstocki* by smaller head depth and relatively smaller prepelvic length (Figures 12c–d).

Furthermore, *N. cooperi* differs from the other members of *N. brieni* species group by the following characters: anal fin uniform orange-red with an irregular to regular, light blue-green zone close to the base, without subdistal band (vs anal fin medial and distal portions yellow in *N. boklundi* and *N. polli*; anal fin with yellow subdistal band and red-brown distal margin in *N. brieni*; with narrow light blue distal margin in *N. chochamandai*, anal fin with orange-red subdistal band and dark grey distal margin in *N. flagrans*; anal fin with orange subdistal band and red-brown distal margin in *N. hassoni, N. malaissei* and *N. milvertzi*; anal fin with cream to light blue subdistal band and black distal margin in *N. capriviensis* and *N. kafuensis*; anal fin with cream to light blue subdistal band and black distal margin in *N. oestergaardi*); caudal fin base colour grey with overlaid faint orange-red spots proximally, grading distally into streaks, without subdistal band and with relatively narrow, irregular light blue margin (vs light blue or orange with red-brown spots and narrow black distal margin in *N. boklundi*; light blue to yellow with red-brown spots, without subdistal band and with distinct light blue distal margin in *N. brieni* and *N. malaissei*; red-brown with light blue spots and orange-red subdistal band and distinct dark grey distal margin in *N. flagrans*; light blue with red-brown markings, with orange subdistal band and distinct dark red-brown distal margin in *N. hassoni*; light blue or plain orange without distinct markings, with cream to light blue subdistal band and distinct black distal margin in *N. capriviensis* and *N. kafuensis*; red with orange subdistal band and distinct dark red-brown distal margin in *N. milvertzi*; pale red or pale blue with cream to light blue subdistal band and red-brown distal margin in *N. oestergaardi*; light blue with red-brown irregular markings, with light blue subdistal band and narrow black distal margin in *N. polli*).

Molecular analysis and phylogenetic affinities

Three of the species known from the Luapula drainage, *N. malaissei*, *N. symoensi* and *N. rosenstocki*, were first assigned by Valdesalici and Wildekamp (2005) to the *N. malaissei* species group, based on male colour pattern. These, together with all other *Nothobranchius* species from other parts of the upper Congo and Zambezi drainages in Katanga and Zambia, are now known to comprise the *N. brieni* species group (Nagy 2014b, 2014c; Nagy et al. 2016)

Nothobranchius cooperi also belongs to the *N. brieni* species group, united by several diagnostic features shared by its members (Nagy et al. 2016). Based on the colour pattern, *N. cooperi* is most similar to *N. rosenstocki, N. sainthousei, N. chochamandai*, *N. malaissei* and *N. symoensi*, representing all species of the genus known from the same drainage system (Figure 1). Males of these taxa are characterised by having an irregular reticulated pattern on the sides of the body that may form irregular cross-bars, a red-brown or orange-red spotted or streaked pattern on the caudal fin with, usually, a light blue to blue-green distal margin.

Building on two earlier studies (Dorn et al. 2014; Watters et al. 2015), the molecular evidence confirms this taxonomy. The molecular analyses corroborate the morphological evidence for (1) the distinctiveness of all individual *Nothobranchius* species and (2) a monophyletic *N. brieni* clade that encompasses all south-central African killifishes. The phylogenetic analyses by Dorn et al. (2014) of one mitochondrial (partial COI) and five nuclear gene sequences included 46 recognised and four putative *Nothobranchius* species. They revealed *Nothobranchius* to comprise four speciose clades that have diversified in their respective geographical regions. The taxa sampled from south central Africa (the target study area of the upper Congo drainage and environs) included: *N. boklundi*, *N. hassoni*, *N. kafuensis*, *N. malaissei*, *N. oestergaardi* and the putative species *N.* 'species Lubumbashi'. These were all retrieved as a monophyletic and well-supported clade corresponding to the *N. brieni* species group. Using partial sequences of the ND2 and 16S rDNA mitochondrial genes, Watters et al. (2015) also retrieved this well-supported monophyletic *N. brieni* clade. They compared *N. capriviensis*, *N. kafuensis*, *N. hassoni*, *N. malaissei*, *N. polli*, *N. rosenstocki* (*sensu lato*) and *N. symoensi*. Our earlier molecular analysis (Nagy et al. 2016), based only on partial ND2 sequences, included additional species from Katanga, as well as *N. brieni* for the first time and further supported the monophyly of the *N. brieni* species group. The current study is supported by a larger sequence matrix of mitochondrial ND2 and COI and nuclear S7 sequences and includes all valid species analysed in the above studies. Our expanded taxon sampling provides further robust support for the *N. brieni* species group. Furthermore, it confirms the evolutionary affinities of all known species of the genus occurring in the study region and the species distinctiveness of *N. cooperi*.

Biogeographical implications

Until quite recently, information on the *Nothobranchius* fauna of the south-eastern upper drainage of the Congo River basin has been fragmentary (Sainthouse 1985). Together with *N. chochamandai* and *N. sainthousei, Nothobranchius cooperi* is the third species to be recognised from the middle Luapula drainage between the Mumbatuta and Mambilima Falls. Current knowledge reveals that all the allopatric species of *Nothobranchius* are restricted to floodplains within their respective river systems (Figure 1). Considering the diverse terrain of the Katanga-Luapula region, with its rich ichthyofauna (Lévêque et al. 2008), the potential exists for discoveries of additional species in this relatively unexplored area. For example, the Lwela River drainage has not been extensively investigated and the upper reaches of the system especially (Figure 9), may host populations of either *N. cooperi* or distinct species. However, political instability and/or sparse communications still render much of the region inaccessible (Nagy 2014a, 2014b, 2014c).

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