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Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Patterns of morphological variation among populations of the widespread annual killifish *Nothobranchius orthonotus* are independent of genetic divergence and biogeography

Milan Vrtílek and Martin Reichard

Abstract

Populations of annual killifish of the genus *Nothobranchius* occur in patchily distributed temporary pools in the East African savannah. Their fragmented distribution and low dispersal ability result in highly structured genetic clustering of their populations. In this study, we examined body shape variation in a widely distributed species, *Nothobranchius orthonotus* with known phylogeographic structure. We tested whether genetic divergence of major mitochondrial lineages forming two candidate species is congruent with phenotypic diversification, using linear and geometric morphometry analyses of body shape in 23 wild populations. We also conducted a common-garden experiment with two wild-derived populations to control for the effect of local environmental conditions on body shape. We identified different allometric trajectories for different mitochondrial lineages and candidate species in both sexes. However, in a principal components analysis of population-level body shape, the separation among mitochondrial lineages was incomplete. Higher similarity of mitochondrial lineages belonging to different candidate species than that of same candidate species prevented distinction of the two candidate species on the basis of body shape. Analysis at the individual level demonstrated that *N. orthonotus* express high intrapopulation variability, with major overlap among individuals from all populations. In conclusion, we suggest that *N. orthonotus* be considered as a single species with an extensive geographic range, strong population genetic structure and high morphological variability.

Key words: Geometric morphometry – body shape – species delimitation – annual killifish – *Nothobranchius*

Introduction

A common aspect of contemporary species concepts is the perception of a species as a separately evolving metapopulation lineage (de Queiroz 2007). In this context, even a single property could support the separation of an organism into two or more species, although it does not guarantee it (de Queiroz 2007). Hence, multiple lines of evidence are required for rigorous species delimitation ('unified species concept'), rather than focusing on a single criterion suggested by rival species concepts (Dayrat 2005; Hey 2006; de Queiroz 2007). Here, we analyse morphological variation in a freshwater fish species with wide distribution, where separation into two species has been suggested by molecular phylogenetic data using both mitochondrial and nuclear markers (Bartáková et al. 2015). We ask whether genetic distinctiveness among metapopulations is driven by historical separation and ensuing phylogeographic structure without morphological diversification, or whether there is morphological evidence for the existence of separate species. In essence, our methodology follows the 'morphological species concept' to test congruence in species delimitation between alternative species concepts.

Annual killifishes of the genus *Nothobranchius* Peters, 1868, are patchily distributed across the East African savannah and comprise species that vary greatly in the extent of their distribution (Wildekamp 2004). They inhabit regularly desiccating water bodies where local populations survive the dry periods through desiccation-resistant eggs encased in dry mud. When pools form after seasonal precipitation, fish hatch synchronously (Polačik et al. 2011), grow fast and soon reach sexual maturity (Blažek et al. 2013). The life cycle is rapid and completed before the pool disappears within only few months (Terzibasi Tozzini et al. 2013). This adaptation to temporary pools has resulted in considerable fragmentation in the distribution of *Nothobranchius* populations (Bartáková et al. 2013). Additionally, large rivers constitute major barriers to gene flow, often isolating populations from opposite banks (Bartáková et al. 2015). Contemporary dispersal appears limited to temporary connections between pools due major flooding after intensive precipitation (Bartáková et al. 2013, 2015). Range fragmentation and limited dispersal ability contribute to pronounced genetic differentiation of *Nothobranchius* populations (Dorn et al. 2011; Bartáková et al. 2013, 2015), similar to that observed in South American annual killifishes (García et al. 2012).

Nothobranchius orthonotus (Peters, 1844) is a species of *Nothobranchius* with one of the most extensive ranges. It is found in temporary pools from KwaZulu-Natal province in South Africa in the south to the Zambezi drainage in Malawi in the north (i.e. approx. 1300 km along a latitudinal axis), with most populations inhabiting Mozambique (Wildekamp 2004; Neumann 2008). The type locality of *N. orthonotus* is in the proximity of Quelimane city in central Mozambique (Wildekamp 2004). Throughout Mozambique, *N. orthonotus* co-occurs syntopically with two other *Nothobranchius* lineages (Reichard et al. 2009): the R-complex composed of three species – *Nothobranchius rachovii* Ahl, 1926, *Nothobranchius pienaari* Shidlovskiy et al. 2010; *Nothobranchius krysanovi* Shidlovskiy et al. 2010; and the F-complex formed by *Nothobranchius furzeri* Jubb, 1971 and *Nothobranchius kadleci* Reichard 2010 (Dorn et al. 2011). Each complex is formed by vicariant sister species that are considered to be ecologically similar (Polačik et al. 2014). Among the co-occurring species, *N. orthonotus* is readily recognizable by larger body size, a large supra-terminal mouth, straight dorsal profile and male colouration (white margins on the dorsal and anal fins). Female *N. orthonotus* body shape (e.g. the position of the mouth and dorsal head profile) and the presence of dark spots on an otherwise non-pigmented body enable their clear distinction from females of the co-occurring species.

Interpopulation morphological differences in *N. orthonotus* consist primarily of variation in body shape and male colour pattern (Fig. 1). However, the intensity of male colouration is affected by water turbidity in the pool with males being pale-coloured when turbidity is high or when stressed. In particular,

Corresponding author: Milan Vrtílek (vrtilek@ivb.cz)
Contributing authors: Martin Reichard (reichard@ivb.cz)

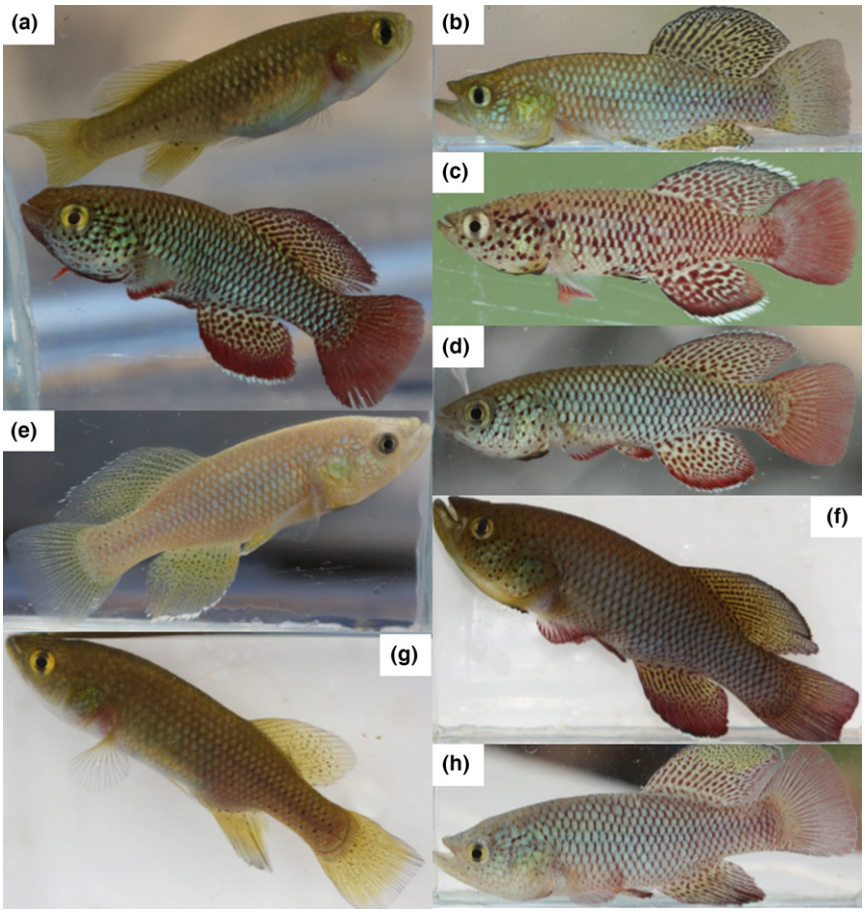

Fig. 1. Colour variation in wild-caught *N. orthonotus*. Specimens (a) – (d) belong to North Coast, (e) – (g) Central and (h) Limpopo-Chefu mitochondrial lineages. The specimens in (a) show marked sexual dimorphism. Specimen (g) is a *N. orthonotus* female from the same pool as male specimen (f). Photos by M. Reichard and R. Blažek

the environmental conditions are highly variable among even closely located populations (Reichard et al. 2009, 2014). Notably, the pattern of body shape variation among *N. orthonotus* populations is so far only anecdotally known and has not been thoroughly studied. Due to the extensive range of *N. orthonotus* and its great degree of morphological variation, the taxonomic status of several populations is disputed. A number of species originally described as separate were later synonymized with *N. orthonotus* (Wildekamp 2004), while species from other *Nothobranchius* lineages, co-occurring with *N. orthonotus*, are split into well-supported taxa (Reichard 2010; Shidlovskiy et al. 2010; Dorn et al. 2011; Bartáková et al. 2013, 2015).

On a large geographic scale, five major mitochondrial lineages were identified on *cytochrome b* data in *N. orthonotus* being predominantly isolated (Bartáková et al. 2015). These are concordant with earlier pilot work on the three Mozambican *Nothobranchius* complexes by Dorn et al. (2011), using *cytochrome oxidase I* sequences. While phylogenetic relationships among the mitochondrial lineages still await reliable resolution, a strict barrier to gene flow between these lineages was identified not only on mitochondrial, but also on nuclear (microsatellite) markers (Bartáková et al. 2015). Thus, the mitochondrial haplogroups can be divided into northern and southern groups (delimited by the Buzi River), that may represent candidate species as they are genetically diagnosable (Bartáková et al. 2015).

Here, we analyse morphological variation in a species with wide distribution, where separation into two species has been suggested by both mitochondrial and nuclear genetic markers (Bartáková et al. 2015). We employ a method of geometric morphometry complemented with traditional linear morphometry to examine body shape variation among wild populations of African annual fish, *Nothobranchius orthonotus*. We also conducted a common-garden experiment with two wild-derived populations to examine the genetic basis of the body shape differences. Our objective was to test the effect of genetic divergence on body shape diversification in populations of widely distributed species, *N. orthonotus*. Specifically, we tested the differences between large mitochondrial lineages *sensu* Bartáková et al. (2015), and by implication between two candidate species separated by the Buzi River. Our null hypothesis was that body shape in *N. orthonotus* is population-specific and independent of affiliation to a mitochondrial lineage. In this case, we would predict populations to be similar in body shape even among different lineages, thereby supporting *N. orthonotus* being a single, widely distributed species. Alternatively, body shape variation may correspond to mitochondrial lineage variation. Thus, if two or more clusters can be recognized, the taxonomic status of *N. orthonotus* should be revisited.

Methods

Field sampling

Specimens of *N. orthonotus* were sampled from central and southern Mozambique (Fig. 2) during five expeditions between 2010 and 2013 (data on the populations sampled are given in Table S1). We considered fish from separate temporary pools as 'populations'. Fish were collected using a seine net (2.7 × 0.7 m with mesh size 5 mm) and triangular dip-net

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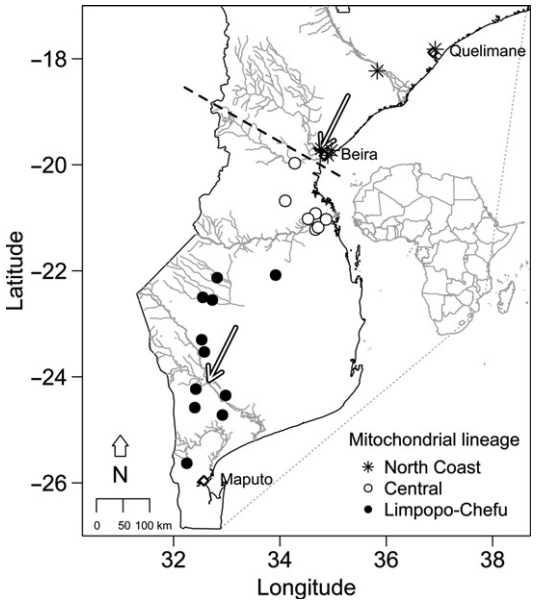

Fig. 2. Map of southern and central Mozambique with sampled populations of *N. orthonotus*. Different symbols denote population affiliation to mitochondrial lineage. The two candidate species, that is two clusters of haplogroups with no introgression, are delimited with dashed line. Origin of wild-derived populations used in common-garden experiment is indicated by arrows. Additional information for each population is given in Table S1.

(45 cm side and 4-mm mesh size). The target sample size was 15–20 fish of each sex per population, but lower sample sizes were collected in some populations. We sampled a total of 22 populations of males and 23 of females, with 268 and 351 specimens, respectively (Table S2).

The grouping of populations into lineages and the terminology followed the results of a phylogeographic study of *N. orthonotus* (Bartáková et al. 2015). However, only three lineages are present in the current study: Limpopo-Chefu, Central and North Coast (Fig. 1) which correspond geographically to the Southern, Central and *kuhntae* mitochondrial lineages, respectively, in Dorn et al. (2011). We included a single Incomati lineage population in the Limpopo-Chefu lineage as they are sister lineages (Bartáková et al. 2015). According to Bartáková et al. (2015), we consider the North Coast lineage to be a representative of a candidate Northern species, and Limpopo-Chefu and Central lineage to constitute a candidate Southern species.

Upon collection, fish were killed with an overdose of anaesthetic (clove oil) and stored in 10% formaldehyde solution before transport to the laboratory for processing (collection permits: DPPM/084/7.10/10, DPPM/069/7.10/11, DPPM/089/7.10/12, DPPM/167/7.10/12, DPPM/069/7.10/13). In the laboratory, samples were bathed in tap water for 24 h, and each specimen was pinned to a dark background using a needle to standardize the position among individual specimens during photographing. For geometric morphometry, we took a picture of the left side of each fish with a digital camera (SONY Cyber-Shot DSC-TX10). In few cases (partial specimen damage), the right side was photographed and the picture was flipped in the image software prior to further analyses. After photographing, each fish was measured for linear morphometry using digital callipers with 0.01 mm precision.

Linear morphometry

We employed traditional linear morphometry as a comparative and complementary body-shape analysis to standard geometric morphometry. Linear morphometry also allows us to account for variation that is not captured using two-dimensional configuration of landmarks, for example length of fins or width of the body. We defined 20 linear characters (Fig. 3a) that were log-transformed to linearize the allometric relationships, and the values were projected in principal components analysis (PCA) using the function 'prcomp' in R software ver. 3.1.3 (R Core Team 2015). At least one principal component (PC) axis was expected to account for body size variation and describe allometric scaling (Jolicoeur 1963). We then tested whether this size-correlated shape axis projected against body size (log-transformed Standard length) differed among lineages in slope, elevation (intercept) or shift (Fig. 1 in Warton et al. (2006)) on the standardised major axis (SMA) using the functions 'slope.com' and 'elev.com' in the 'smatr' package ver. 3.4-3 in R (Warton et al. 2006). This method allows for the comparison of allometric trajectories even among groups with different body sizes (e.g. Sidlauskas et al. 2011). As intrapopulation variation was relatively high compared to interpopulation variation, we also calculated the mean values of linear characters for each population and used the means in a subsequent PCA to further evaluate interpopulation differences.

Geometric morphometry

For the geometric morphometry, we defined a set of 13 landmarks (Fig. 3b) that were digitized using TPSDIG2 software ver. 2.16 (Rohlf 2010). We followed general recommendations for their definition and repeatability in choosing their position (Zelditch et al. 2004). We then performed generalised procrustes analysis (GPA) on the *x*,*y*-coordinates of landmark configurations in the 'geomorph' package ver. 2.1.5 for R software (Adams and Otárola-Castillo 2013). During the GPA, landmark configuration was transformed to remove the non-shape variability (position, rotation and scale), and Centroid size was computed for each specimen. We used Centroid size – the square root of the sum of squared distances of landmarks from the configuration centroid – as an estimate of body size. We also visually checked outlying individuals using the 'plotOutliers' function.

We used superimposed landmark configurations for the PCA and non-parametric multivariate (procrustes) analysis of variance (np-MANOVA). In the analysis of interlineage differences, we performed np- MANOVA based on an iterative residual randomization permutation procedure (Collyer et al. 2014). This approach is equivalent to distance-based ANOVA designs (Anderson 2001). We tested the differences among mitochondrial lineages with individual configurations taking Centroid size as a covariate, either Lineage or Species as a grouping factor and a nested factor of Population with the 'advanced.procD.lm' function. As with the linear morphometry PCA, intrapopulation variation was relatively high in the PCA on individual landmark configurations. We removed intrapopulation body shape variation by calculating the mean body shapes for each population separately to concentrate on population differences (Mitteroecker and Bookstein 2011). We used these mean configurations in an exploratory PCA. This enabled more straightforward interpretation of the PCA results as we reduced the number of influential PCs (Mitteroecker and Bookstein 2011).

Common-garden experiment: fish maintenance and sample collection

We conducted a common-garden experiment with two wild-derived populations from different mitochondrial lineages, Limpopo-Chefu and North Coast (Southern and Northern candidate species, respectively), to examine the differences in body shape variation during ontogeny under common environmental

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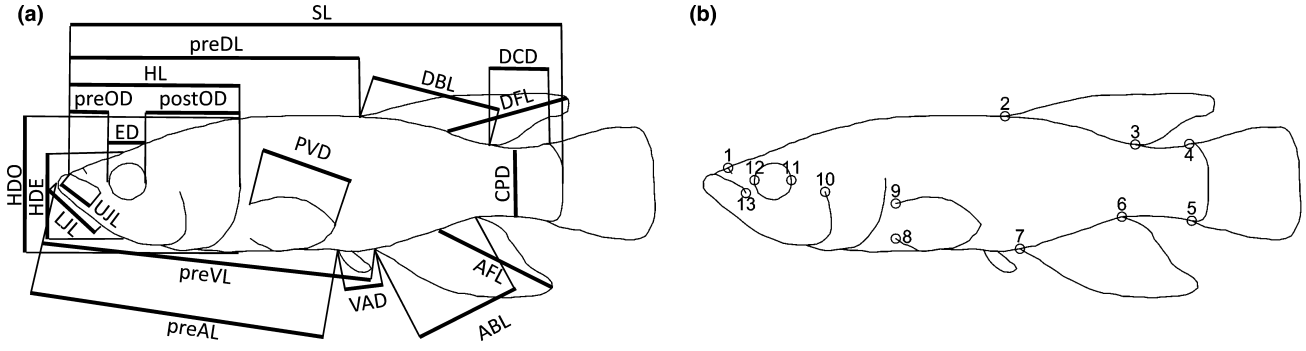

Fig. 3. Characters used in linear morphometry (a) and position of landmarks used in geometric morphometry (b). Measured linear characters in (a) were: *Standard Length* (SL), *Head Length* (HL), *pre-Orbital Distance* (preOD), *post-Orbital Distance* (postOD), *Eye Diameter* (ED), *Lower Jaw Length* (LJL), *Upper Jaw Length* (UJL), *Head Depth at Eyes* (HDE), *Head Depth at Operculum end* (HDO), *pre-Dorsal fin Body Length* (preDL), *pre-Anal fin Body Length* (preAL), *pre-Ventral fin Body Length* (preVL), *Pectoral to Ventral fin Distance* (PVD), *Ventral to Anal fin Distance* (VAD), *Dorsal to Caudal fin Distance* (DCD), *Anal fin Base Length* (ABL), *Dorsal fin Base Length* (DBL), *Caudal Peduncle Depth* (CPD), *Dorsal fin Length* (DFL), *Anal fin Length* (AFL). Note that *Inter-Orbital Distance* (IOD) is not shown. *Inter-Orbital Distance* is the distance between orbits measured from above the head. The landmarks shown in (b) were: 1 – snout groove, 2 – frontal insertion of dorsal fin, 3 – distal insertion of dorsal fin, 4 – dorsal insertion of caudal fin, 5 – ventral insertion of caudal fin, 6 – distal insertion of anal fin, 7 – frontal insertion of anal fin, 8 – ventral insertion of pectoral fin, 9 – dorsal insertion of pectoral fin, 10 – dorsal end of pre-opercular groove, 11 – distal end of orbit, 12 – frontal end of orbit, 13 – distal end of mouth

conditions. The source fish stocks were collected in Mozambique in 2012 (Limpopo-Chefu, collection code MZCS2012 NO002: S 24°3′49″, E 32°43′56″ and North Coast, collection code MZCS2012 NO528: S 19°41′51″, E 34°46′59″, Fig. 2) and transported to facilities in the Institute of Vertebrate Biology ASCR in Brno, Czech Republic (export permit: 117MP/2012). A total of six males and 22 females, and 16 males and 26 females were imported for Limpopo-Chefu and North Coast population respectively, and a mating design that maximized outbreeding was used.

The experimental fish were the F1 generation of the wild-caught fish. Fish were hatched from the eggs following a standard protocol (Vrtílek and Reichard 2015) on 23 May 2013. Hatched fish were kept in 4 l aquaria for the first 5 days and then transferred into 28 l aquaria with approximately 60 individuals per aquarium. Fish density was maintained equal among the aquaria, and fish were transferred to larger aquaria as they grew. Their density further declined through the removal of individuals due to sampling and natural mortality. Juvenile fish were fed with live *Artemia* spp. *nauplii* three times a day and weaned onto live and frozen chironomid larvae at 14 days (fed once a day). The aquaria were equipped with air-driven filtration and one-third of the water volume was exchanged every 4 days. The temperature was kept at 26 ± 1°C, and photoperiod was set to 14/10 h (light/dark). We terminated the experiment when the period of intensive growth ceased after 138 days, on 7 October 2013 (i.e. at age of 19 weeks). We sampled the captive populations at the age of 19 days – 'juveniles' (21 individuals from Limpopo-Chefu (L-C) and 20 from North Coast (NC) population); 26 and 33 days – pooled into 'young adults' (19 males from both populations and 12 L-C and 19 NC females) when fish can be reliably sexed, and at 138 days – 'old adults' (12 males from both populations and 15 L-C and 19 NC females). At each sampling, fish were sacrificed with an overdose of clove oil and preserved in 10% formaldehyde solution.

Analysis of the experimental fish was performed using geometric morphometry only, following the same procedures and analyses as for the wild specimens. We did not use linear morphometry because the size of juvenile fish prevented precise measurement with a standardized method.

The fish (both wild and laboratory-bred) used in this study have been deposited in the collection of the Institute of Vertebrate Biology of the Czech Academy of Sciences in Brno, Czech Republic, under individual codes. A subset (237 of 784 specimens) was dissected for another study and is no longer available (Table S2).

Results

Body shape variability in wild populations – linear morphometry

In the PCA on linear characters of wild individuals, the first three PCs explained over 95% of variation in both sexes. The first principal component (PC1) explained most of this variability (93.4% and 92.5% in males and females, respectively). As we expected, PC1 was mainly related to the body size expressed as log-Standard length (log-SL) (Pearson correlation, $r = -0.992$ and $r = -0.993$, for males and females, respectively) and described correlated changes between body shape and body size (Table 1). Thus, PC1 constitutes an allometric vector (Jolicoeur 1963), and the PC1 score of individual characters represents their allometric relationship with body size (Table 1). Most characters deviated from isometry marginally, but clear negative allometry was recorded between body size (log-SL) and *Eye Diameter* in both sexes and *Anal fin Length* in females (Table 1). This suggests a general trend of allometry in these fish where the eye (in both sexes) and the anal fin (in females) grow at a slower rate than body size.

The linear morphometry PCA was largely confounded by the effect of the body size differences that were loaded onto PC1. A comparison of the relationship between PC1 and body size (log-SL) among lineages in males revealed that there were different allometry slopes among lineages and among candidate species (standardised major axis (SMA), 'Lineage × log-SL' interaction, df = 2, *Likelihood Ratio (LR)* = 16.16, $p < 0.001$; 'Species × log-SL' interaction, df = 1, *LR* = 4.96, p = 0.026). In females, allometry slopes only tended to vary among lineages and species (SMA, 'Lineage × log-SL' interaction, df = 2, *LR* = 5.06, p = 0.080; 'Species × log-SL' interaction, df = 1, *LR* = 2.42, p = 0.120), but they differed in the elevation (i.e. intercept) of the allometry trend lines (SMA, 'Lineage' effect, df = 2, *Wald statistic* = 47.53, $p < 0.001$; 'Species' effect, df = 1, *Wald statistic* = 13.81, $p < 0.001$; Table 2). Subsequent pairwise comparison among lineages (Table 2) indicated steeper relative eye growth in Limpopo-Chefu lineage males than in Central or North Coast lineage males. In females, Central lineage fish had consistently relatively smaller eyes and anal fin length than the two other lineages (Table 2).

After the removal of the effect of different body size by the PC1, lineages or populations were mixed in the multivariate space of individual-based PCA. The overlap among populations

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Table 1. Score of linear characters on the first three principal components of PCA on the wild individuals. PC1 represents an allometric growth vector. The characters deviating from isometry (square root of 1/20 = 0.224) on PC1 exhibit either negative (< 0.224) or positive (> 0.224) allometry. The most important characters on a particular axis, that is deviating more than 0.05 from the isometric coefficient (0.224) on PC1 and 0.4 from zero on PC2 and PC3, are in bold.

		Males			Females		
	Abbreviation	PC1	PC2	PC3	PC1	PC2	PC3
Eigenvalue		1.272	0.153	0.141	1.228	0.160	0.141
% of explained variance		93.417	1.343	1.143	92.504	1.568	1.217
Head Length	HL	0.212	−0.038	0.111	0.211	0.100	−0.019
Pre-Orbital Distance	preOD	0.247	−0.067	0.254	0.239	0.218	−0.070
Post-Orbital Distance	postOD	0.225	−0.019	0.096	0.229	0.013	−0.008
Eye Diameter	ED	**0.137**	0.005	0.053	**0.149**	−0.075	−0.013
Inter-Orbital Distance	IOD	0.246	0.067	0.166	0.233	0.035	−0.225
Lower Jaw Length	LJL	0.248	0.066	0.017	0.253	0.043	−0.063
Upper Jaw Length	UJL	0.263	−0.006	**0.403**	0.267	0.041	−0.060
Head Depth at Eyes	HDE	0.251	0.129	0.191	0.239	0.135	−0.137
Head Depth at Operculum end	HDO	0.249	0.054	0.029	0.250	−0.114	−0.145
Pre-Dorsal fin Body Length	preDL	0.211	−0.026	−0.020	0.224	−0.125	−0.007
Pre-Anal fin Body Length	preAL	0.219	−0.014	−0.006	0.229	−0.145	−0.005
Pre-Ventral fin Body Length	preVL	0.222	0.010	0.007	0.232	−0.219	−0.054
Pectoral to Ventral fin Distance	PVD	0.229	−0.102	0.006	0.252	**−0.632**	−0.097
Ventral to Anal fin Distance	VAD	0.224	−0.202	0.039	0.235	0.147	0.119
Dorsal to Caudal fin Distance	DCD	0.196	**−0.825**	−0.389	0.200	−0.212	**0.881**
Anal fin Base Length	ABL	0.198	0.133	−0.105	0.184	0.154	−0.125
Dorsal fin Base Length	DBL	0.200	0.184	−0.044	0.212	−0.031	−0.126
Caudal Peduncle Depth	CPD	0.231	−0.022	−0.014	0.222	0.033	−0.007
Dorsal fin Length	DFL	0.233	0.325	**−0.532**	0.221	**0.443**	0.166
Anal fin Length	AFL	0.196	0.273	**−0.484**	**0.148**	0.357	0.189

and lineages was substantial in both sexes as apparent on the plot of PC2 and PC3 (Fig. S1) (1.3% and 1.6% of variation explained in males, 1.1% and 1.2% in females, respectively).

We then used the population means of the linear characters to remove intrapopulation variation while enhancing the interpopulation differences. In the PCA, PC1 explained > 96.9% of interpopulation variability in both males and females and again represented the effect of body size (mean log-SL for population) variation (Pearson correlation, $r = -0.995$, in both sexes). The population means from different lineages were again largely mixed in the PCA space. However, populations from the Central lineage separated from the North Coast lineage on PC3 (0.6% of explained variation in males and 0.5% in females) (Fig. 4). Central lineage females further tended to separate from the Limpopo-Chefu lineage populations (Fig. 4b). In males, populations from the Central lineage had on average relatively larger *Dorsal to Caudal fin Distance* (i.e. longer caudal peduncle) and smaller *Head Depth at Eyes* (lower frontal head profile) compared to the North Coast lineage (Fig. 4a). Central lineage populations of females had on average relatively larger *Dorsal to Caudal fin Distance* (longer caudal peduncle) (Fig. 4b) than the North Coast and Limpopo-Chefu lineages. Thus, a longer caudal peduncle seems to be a specific feature of fish from the Central lineage populations at least when compared to the North Coast lineage.

Body shape variability in wild populations – geometric morphometry

Principal components analysis of landmark configurations of wild individuals resulted in first three PCs explaining 56.5% of variation (30.0, 15.1 and 11.4%, PC1, PC2 and PC3, respectively) in males and 52.8% (25.0, 17.1, 10.7%) in females. However, there was no pattern consistent with structuring into populations, lineages or into the two candidate species along any PC of the individual data (see Fig. S2 for PC1 and PC2 plot).

First, body shape differences were tested among lineages and then between candidate species. The relationship between body size (Centroid size – CS) and body shape defined by landmark configuration was lineage-specific in both sexes (np-MANOVA, Table 3). While the allometry was strictly lineage-specific in males, strong differences were found only between Central lineage females and the other two lineages (Table 4). When grouping was done with respect to candidate species instead of mitochondrial lineages, the outcome was qualitatively identical in males with species-specific allometry slopes ('CS × Species' interaction, $F_{1,267} = 2.32$, Z-score = 2.28, $p = 0.010$). In females, however, the species grouping supported the existence of parallel allometric trajectories (i.e. with a different intercept: 'Species' effect, $F_{1,350} = 18.283$, Z-score = 11.510, $p < 0.001$)

Table 2. Analysis of allometry among mitochondrial lineages based on linear morphometry of wild individuals. Results of standardised major axis (SMA) used on relationship between allometric vector (PC1) and body size (log-Standard length). Coefficients of slope for males and elevation (=intercept) for females (± 95% confidence interval) and pair-wise comparison between lineages (top-right for males and bottom-left for females) are given.

			Significance of differences		
	Slope Males	Elevation Females	Limpopo-Chefu	Central	North coast
Limpopo-Chefu	4.907 ± 0.092	−16.452 ± 0.252	-	**	**
Central	4.577 ± 0.167	−16.563 ± 0.252	***	-	NS
North coast	4.671 ± 0.107	−16.420 ± 0.228	NS	***	-

NS = non-significant, * p = 0.05–0.01, ** p = 0.01–0.001, *** p < 0.001.

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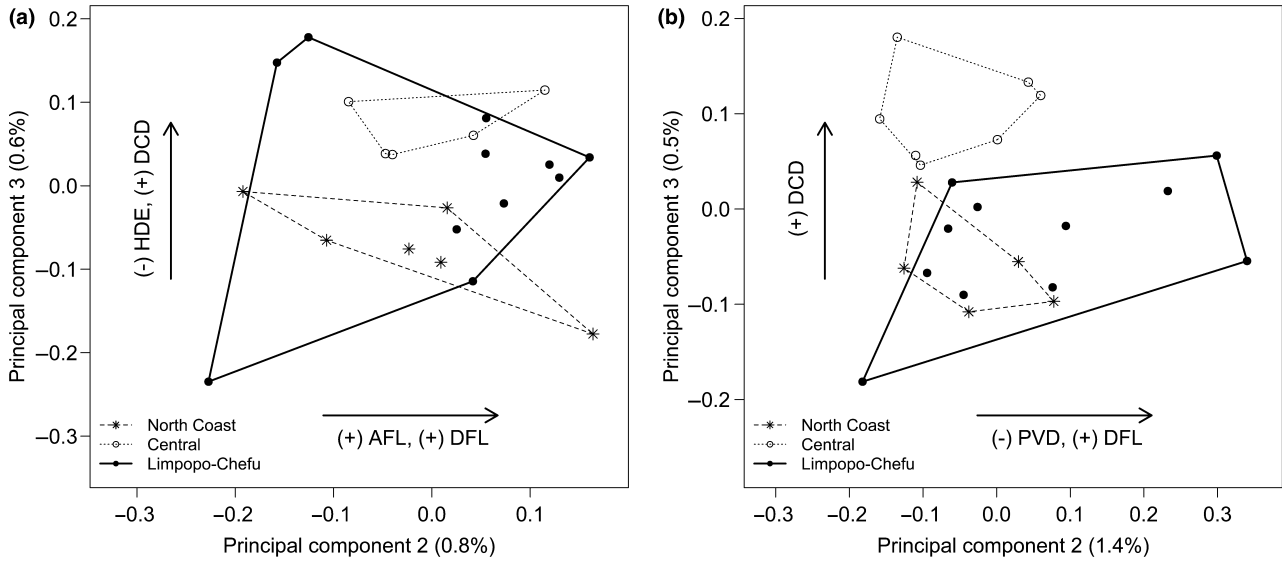

Fig. 4. PCA of variation in linear characters of wild populations. Distribution of population means for males (a) and females (b) along the PC2 and PC3 axes that account for the largest variability portion described after effect of body size was removed (PC1). For PCA on individuals, see Fig. S1.

as the allometry slopes were similar among species ('CS × Species' interaction, $F_{1,350} = 0.650$, Z-score = 0.636, p = 0.717).

In the PCA on landmark configurations averaged per population (population mean shapes), the first three PCs accounted for 74.0% of variation in males and 71.7% in females. In males, almost half of the variation was explained by PC1 (47.9%), and in females the major part was accounted for by PC1 (37.2%) and PC2 (24.7%). For the population mean shapes, there was generally no pattern consistent with structuring into either lineages or the two candidate species. The exceptions are PC2 (15.6%) in males, where Central lineage populations tended to separate from the North Coast lineage (Fig. 5a) and PC4 (7.9%) in females where Central lineage populations almost separated from both the Limpopo-Chefu and North Coast lineages (Fig. 5b). The position of Central lineage populations in male PC2 indicates they have a relatively streamlined body with a longer caudal peduncle and smaller mouth positioned closer to the eye (Fig. 5a). Along female PC4, Central lineage populations were characterized by pectoral fins shifted frontally, the distal end of the anal fin shifted frontally (i.e. anal fin base shortened in favour of caudal peduncle length) and the anterior insertion of the dorsal fin shifted distally (i.e. dorsal fin shortened in favour of extension of the rear part of the body) (Fig. 5b). The differences captured by these two axes (PC2 in males and PC4 in females) were uncorrelated with body size (CS) (Pearson correlation, PC2: $r = 0.156$, PC4: $r = -0.177$).

The geometric morphometry analyses were congruent with linear morphometry and demonstrated differences primarily between Central and North Coast lineage populations with the North Coast lineage typically being similar to the Limpopo-Chefu lineage. The candidate species grouping was not confirmed by body shape variation. The length of the caudal peduncle was the diagnostic trait for discrimination of Central versus North Coast lineage, identified by both morphometry methods and in both sexes.

Ontogenetic change of body shape in two wild-derived populations

In the PCA on landmark configurations for wild-derived captive fish, first three PCs explained 59.6% and 61.9% of variation in males and females, respectively. In males, the two populations tended to separate along PC1 (31.1%). The combination of PC1 (31.1%) and PC2 (20.1%) partly separated females into the two populations (data not shown). Other PCs explained relatively a small amount of variation (< 17.2% in males and < 9.8% in females per each PC) and did not cluster individuals into the two populations.

The ontogenetic trajectories of body shape variation were population-specific in both sexes ('log-CS × Population' interaction, $F_{1,102} = 2.51$, $Z = 2.28$, p = 0.011, for males; $F_{1,105} = 3.72$, $Z = 3.33$, p = 0.002, for females; Table 5). The populations did not follow a linear pattern in the relationship between PC1 and

Table 3. Analysis of allometry among mitochondrial lineages based on geometric morphometry of wild individuals. Results of np-MANOVA for males and females with body size (Centroid size) as a covariate. Calculation of posterior probabilities is based on randomized residual permutation procedure with 9999 iterations (Collyer et al. 2014).

	Males					Females				
Parameter	df	R^2	F	Z-score	p	df	R^2	F	Z-score	p
CS	1	0.101	43.565	23.241	< 0.001	1	0.087	47.499	26.418	< 0.001
Lineage	2	0.070	15.006	9.700	< 0.001	2	0.052	14.305	9.365	< 0.001
Lineage × Population	19	0.256	5.801	4.313	< 0.001	20	0.259	7.095	5.224	< 0.001
CS × Lineage	2	0.009	1.958	2.001	0.007	2	0.009	2.441	2473	0.005

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Table 4. Correlation coefficients of lineage slopes for males (top-right) and females (bottom-left).

Lineage	Central	Limpopo-Chefu	North coast
Central	–	0.625***	0.722*
Limpopo-Chefu	0.876*	–	0.814*
North coast	0.763.	0.786.	–

p = 0.06–0.05, * p = 0.05–0.01, ** p = 0.01–0.001, *** p < 0.001.

body size (log-CS) except for North Coast females (Fig. 6). In males, the largest difference was recorded between the 'young adults', converging as the males grew (Fig. 6a). Females diverged during the period from 'juveniles' to 'young adults' and then exhibited parallel trends during adulthood (Fig. 6b). In both sexes, PC1 links a relatively deep body, mouth shifted superiorly and short caudal peduncle (Fig. 6). These changes were more pronounced in the North Coast population (Fig. 6).

Discussion

Our analyses showed considerable intra- and interpopulation variation in the morphology of wild *N. orthonotus*. Fish also showed population-specific body shape changes during growth under standardised conditions in the common-garden experiment. Overall, the outcomes were congruent for male and female data sets. While standardised major axis (linear characters) and np-MANOVA (landmark configurations) yielded an outcome that qualified the separation of mitochondrial lineages and candidate species into valid morphological species, PCAs on both linear and geometric morphometry data demonstrated that there was no consistent separation. In wild populations, body shape variation showed an extensive overlap among populations and mitochondrial lineages. Moreover, the wild population means were rather randomly distributed than clustered into mitochondrial lineages in the multivariate body shape space (PCA). Only the Central lineage separated from the North Coast lineage in population-average comparisons, although not in the individual-based data set. In conclusion, tangible delineation based on body shape was not possible for mitochondrial lineages nor the candidate species of *N. orthonotus* despite the fact that they differed significantly in allometric trajectories.

Currently, *N. orthonotus* is considered as either single polytypic species (Wildekamp 2004; Neumann 2008) or a species complex consisting of at least two species (Jubb 1975a,b; Seegers 1997; Dorn et al. 2011, 2014; Terzibasi Tozzini et al. 2013). The latter hypothesis stems from the assumed existence of *N. kuhntae* (Ahl, 1926), a vicariant species putatively morphologically distinct from *N. orthonotus sensu stricto* (Seegers 1997). The type locality of *N. kuhntae* lies in the vicinity of Beira harbour in central Mozambique (Jubb 1975b; Wildekamp 2004). However, such a distinct lineage was neither supported by mitochondrial nor nuclear markers in the phylogeographic analysis of *N. orthonotus* (Bartáková et al. 2015). Notably, the geographic distribution of the mitochondrial lineage containing populations of the putative *N. kuhntae* – North Coast (cf. *kuhntae*) in Bartáková et al. (2015) or *kuhntae* in Dorn et al. (2011) – includes the type localities of both *N. kuhntae* and *N. orthonotus*. Therefore, due to the nomenclature rule of priority, this mitochondrial lineage, if considered a separate species, should be named *Nothobranchius orthonotus* (Peters, 1844). In this context, the name *Nothobranchius kuhntae* must be further considered a younger synonym for *N. orthonotus*.

So far, the presence of a small-scale endemic *kuhntae*-morphotype in *N. orthonotus* has not been excluded due to the absence of morphological analysis. An isolated population or populations may be markedly phenotypically distinct as, for example in the case of Vancouver Island marmot, *Marmota vancouverensis* (Swarth, 1911) (Cardini et al. 2009). This island species shows very low genetic divergence from its continental sister species as the colonization event of the island underwent relatively recently (Cardini 2003), yet it is morphologically distinct to a degree qualifying its designation as a separate species (Cardini et al. 2009). However, our data demonstrated that the populations of *N. orthonotus* in the vicinity of Beira do not exhibit specific body shape. Thus, our data do not support the existence of a *kuhntae*-morphotype within *N. orthonotus*.

Nothobranchius orthonotus is one of the most widespread species of the genus (Wildekamp 2004; Neumann 2008) with a known distribution spanning over more than a thousand kilometres (Bartáková et al. 2015). Generally, phenotypic variation is assumed to be high in geographically widespread species. This is true even in morphologically homogenous groups as evidenced by, for example, the contact zone in the ring species complex of *Ensatina* Gray, 1850 salamanders (Moritz et al. 1992). The fragmented area of distribution and limited dispersal among populations may additionally promote phenotypic diversification among more distant populations (Coyne and Orr 2004). However, a purely geographic delimitation of morphologically similar populations into separate species may not represent evolutionary relationships and can be misleading (Harrington and Near 2012).

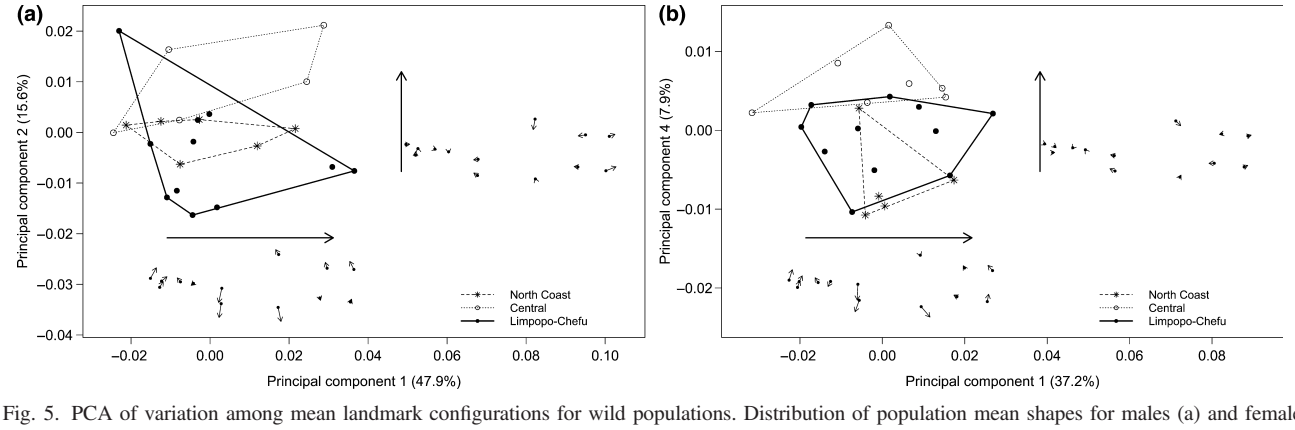

Fig. 5. PCA of variation among mean landmark configurations for wild populations. Distribution of population mean shapes for males (a) and females (b) in the space defined by two selected PCs. For the body shape change vectors, the PC minimum was used as a reference and arrows indicate the magnitude and direction of change along the axis to maximum. The vectors of body shape change were twofold magnified. For PCA on individuals, see Fig. S2.

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Table 5. Analysis of allometry between two wild-derived populations raised under common-garden conditions based on geometric morphometry. Results of np-MANOVA for males and females with body size (log-Centroid size) as a covariate. Calculation of posterior probabilities is based on randomized residual permutation procedure with 9999 iterations (Collyer et al. 2014). Male ($N = 62$) and juvenile ($N = 41$) superimposed landmark configurations and females ($N = 65$) with the same set of juveniles as in males ($N = 41$) were tested in two separate analyses.

	Males					Females				
Parameter	df	R^2	F	Z-score	p	df	R^2	F	Z-score	p
log-CS	1	0.066	8.456	5.903	< 0.001	1	0.179	27.231	16.434	< 0.001
Population	1	0.137	17.397	13.066	< 0.001	1	0.126	19.175	13.958	< 0.001
log-CS × Population	1	0.020	2.574	2.305	0.011	1	0.024	3.717	3.330	0.001

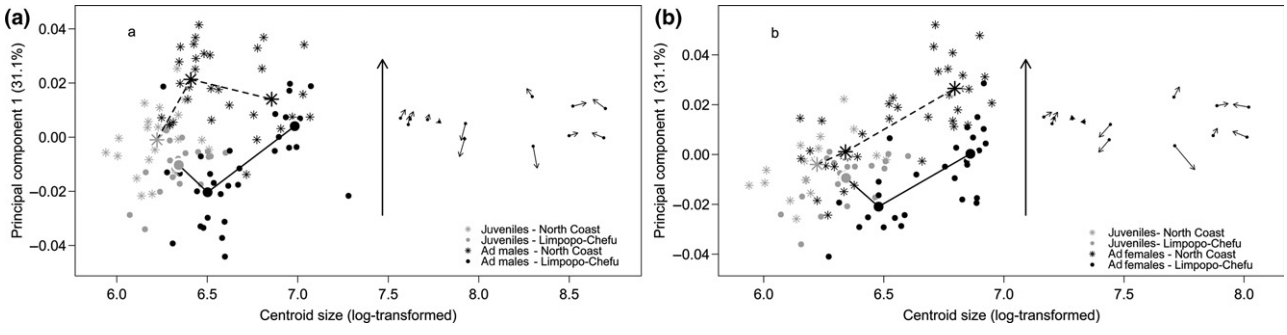

Fig. 6. Ontogenetic changes in body shape in two wild-derived populations under common-garden experimental conditions. Relationship between PC1 and body size (log-Centroid size) in males and juveniles (a) and in females and juveniles (b). For the body shape change vectors, the PC minimum was used as a reference and arrows indicate the magnitude and direction of change along the axis to maximum.

Universally, a multifaceted approach is necessary to properly test species delimitation hypotheses (for example, reptiles: Wiens and Penkrot 2002; Leaché et al. 2009; ants: Schlick-Steiner et al. 2006). Molecular phylogeny provides a platform for further analysis of phenotypic variation and other traits such as reproductive isolation to support eventual long-term evolutionary separation, in a so-called 'congruent integrative taxonomical approach' (Padial et al. 2010). For example, the species complex of the ecologically specialized trapdoor spider, *Aptostichus atomarius* Simon, 1891, with highly fragmented population distribution and limited dispersal could be identified as 20 geographically and genetically isolated species (Bond and Stockman 2008). However, based on the results of combined data on morphometry, meristics, mitochondrial DNA analysis and ecological modelling, considering genetic exchangeability and ecological interchangeability, Bond and Stockman (2008) conclude that the complex is composed of five 'cohesion species' containing deep divergences in mitochondrial DNA. This interpretation of the thorough analysis uncovers cryptic diversity while preventing unnecessary and potentially harmful taxonomic inflation (Isaac et al. 2004; Zachos 2014).

The morphological overlap in species of the South American annual killifish genus *Austrolebias* Costa, 1998 (D'Anatro and Loureiro 2005; García et al. 2009, 2012) suggests that the morphological evolution of divergences in allopatric populations of annual killifish is rather slow. Even the diagnostic traits being used in *Austrolebias*, as relative pre-dorsal and preanal lengths, were shown to profoundly overlap among designed species (García et al. 2009, 2012). The age of *N. orthonotus* as the time of its separation from the *N. furzeri/N. kadleci* clade, estimated to be 2–4 Mya (Dorn et al. 2011, 2014), is comparable to 3 Mya in the *A. adloffi* (Ahl, 1922) complex (García et al. 2009) or 7–13 Mya in the *A. bellottii* (Steindacher, 1881) complex (García et al. 2012). Given that there is a secondary contact between the Limpopo-Chefu and Central lineages but no contact or introgression between the spatially adjacent Central and North Coast lineages (Bartáková et al. 2015), any trait that would be specific for one of the *N. orthonotus* candidate species delimited by the Buzi River (see Fig. 2) can be used for their diagnosis (Padial and de la Riva 2006; de Queiroz 2007). After eliminating intrapopulation variation using population mean values, the longer caudal peduncle appeared to be specific for Central lineage populations compared to both Limpopo-Chefu and North Coast lineage populations, irrespective of sex. However, no such diagnostic trait was identified in individual-based data set.

Morphological distinctiveness is traditionally the first cue to describe a new species despite being potentially misleading. For example, solely colour-based descriptions represent a myopic and premature approach to biodiversity classification (Sánchez Herrera et al. 2015) and may lead to harmful taxonomic inflation (Isaac et al. 2004). In *Nothobranchius* species complexes from Mozambique, the geographic structure of male colouration has driven the formal split of the widespread *N. rachovii* complex (Shidlovskiy et al. 2010), largely co-occurring with *N. orthonotus*. The three species in *N. rachovii* complex differ also in karyotypes, having different chromosome numbers, which leads to their full reproductive isolation (Shidlovskiy et al. 2010; Bartáková et al. 2015). In another Mozambican species complex, *N. kadleci* is a distinctly coloured vicariant sister species of *N. furzeri* (Reichard 2010), without genetic introgression (Bartáková et al. 2015). Therefore, the pattern of male colour variation in combination with geographic distribution may in fact be a useful secondary trait for distinguishing among well-supported evolutionary lineages in *Nothobranchius* fish (Shidlovskiy et al. 2010; Dorn et al. 2011; Bartáková et al. 2015). This is true also for other killifish genera, such as African forest-dwelling *Chromaphyosemion* Radda, 1971 (Agnèse et al. 2006), South American *Austrolebias* (García et al. 2009) or Caribbean *Rivulus* Poey, 1860 (Ponce de León et al. 2014). However, in *N. orthonotus,* colouration is a problematic trait for species diagnosis being not

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only site-specific (Fig. 1) but also highly affected by current environmental conditions (e.g. turbidity).

Our aim was to test body shape differences among large phylogenetic lineages of *N. orthonotus*. We examined a complex phenotypic trait (body shape) in several populations covering most of the known *N. orthonotus* distribution including localities with putative *N. kuhntae*. The approach we used differs from the approach of some researchers who neglect natural interpopulation variation and describe new species on the basis of a single or few neighbouring localities. Our results show subtle but consistent differences between mitochondrial lineages of *N. orthonotus*. However, we reject separation of the candidate species due to incongruence between the results for mitochondrial data and candidate species. Moreover, we did not identify any morphologically distinct group that could be readily diagnosed within *N. orthonotus*. With current knowledge of phylogenetic relationships, low diagnosability in the candidate species and considering high interpopulation variation in body shape and male colouration, we propose to accept *N. orthonotus* as a single polymorphic species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCA of variation in linear characters in individual wild fish.

Figure S2. PCA of variation among individual landmark configurations of wild fish.

Table S1. List of characteristics of sampled populations of *N. orthonotus*.

Table S2. List of specimens used for body shape analysis in this study.

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