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Marked differentiation in a new species of dwarf stonebasher, *Pollimyrus cuandoensis* sp. nov. (Mormyridae: Teleostei), from a contact zone with two sibling species of the Okavango and Zambezi rivers

Bernd Kramer[a]*, Herman van der Bank[b] and Michael Wink[c]

[a]University of Regensburg, Zoological Institute, D-93040 Regensburg, Germany; [b]Department of Zoology, University of Johannesburg, African Centre for DNA Barcoding, Johannesburg, South Africa; [c]Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, D-69120 Heidelberg, Germany

We studied the systematic status of a form of *Pollimyrus* of the lower Kwando River that is flanked by the Okavango River, inhabited by *Pollimyrus castelnaui* (Boulenger, 1911), and the Zambezi River, inhabited by *Pollimyrus marianne* Kramer et al., 2003. In morphology and electric organ discharges (EODs), the Kwando phenotype proved well differentiated from both *P. castelnaui* and *P. marianne*. Sequence analysis of the cyt *b* gene confirmed that the three forms or species form a monophyletic clade, with *P. castelnaui* sister to the other two species. Genomic fingerprinting with ISSR-PCR confirmed differentiation of the Kwando form, that we recognize as a different species, *P. cuandoensis* sp. nov., from its sister species, *P. marianne*. A considerable amount of EOD and morphological variation was revealed among samples of *P. cuandoensis* sp. nov. from four different locations on the lower Kwando River, possibly due to hybrid introgression. This seems an ideal system for testing theories of parapatric speciation.

Keywords: speciation; parapatric; cline; hybridization; electric organ discharge; DNA sequence analysis; genomic fingerprinting

Introduction

The dwarf stonebasher, *Pollimyrus castelnaui* (Boulenger, 1911), is a miniature snout-fish found in parts of southern Africa. It is present in the Cunene, Okavango, upper Zambezi and Kafue rivers, but also in northern parts of Lake Malawi (Skelton 2001). The fish, or a form of it, is probably much more widespread, as it has also been found in the Buzi System (Mozambique) and the Luongo River (Upper Congo system, Zambia; R. Bills, personal communication). A critical comparison among allopatric populations has not been made, with one exception (see Kramer et al. 2003).

The type locality of *P. castelnaui* is the Okavango River and its inland delta, whereas the only recently discovered sibling species, *P. marianne* Kramer et al., 2003 inhabits the Upper Zambezi River (Figure 1). In the Kwando River, a Zambezi tributary with occasional contact to the Okavango, there are populations of dwarf stonebasher whose status is unresolved: some local populations appeared differentiated from *P. marianne* at the species level in terms of morphology and electric organ

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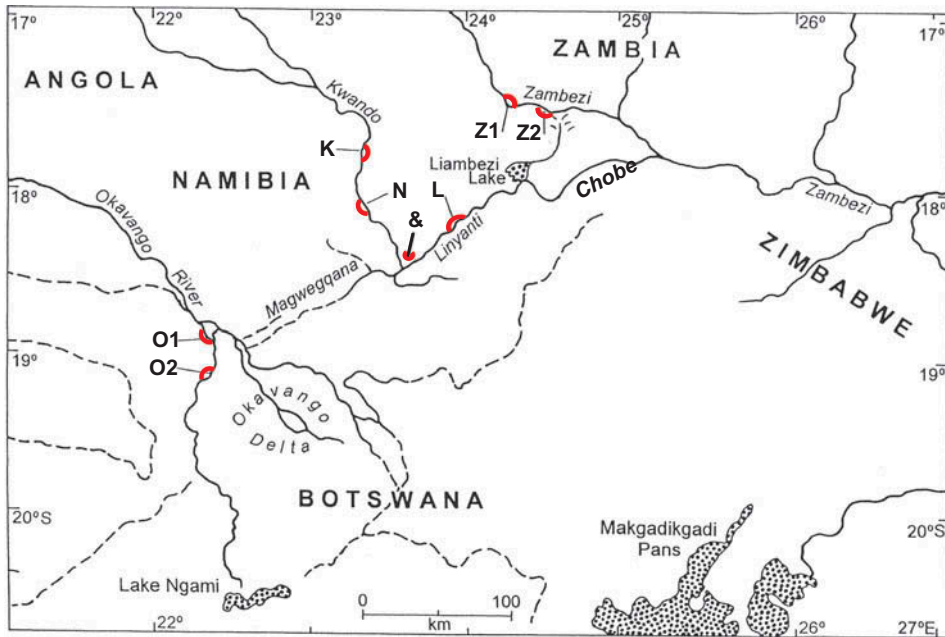*Corresponding author. Email: bernd.kramer@biologie.uni-regensburg.de

Figure 1. Partial map of Upper Zambezi system and Okavango delta geography. Localities where *Pollimyrus* specimens were sampled indicated as: Z1, Zambezi: Katima Mulilo; Z2, Zambezi: Lisikili; L, Kwando system: Linyanti: Sampis; "&" symbol, Kwando system: Nkasa Island; N, Kwando: Nakatwa; K, Kwando: Kongola Bridge; O1, Okavango: Guma Lagoon; O2, Okavango: Makwena.

discharges (EODs), whereas they did not in terms of cytochrome-*b* DNA sequence analysis. In addition, there was also some evidence of a cline in morphology. Specimens that were sampled from terminal Kwando River localities in the south, known to be occasionally flooded by the Zambezi, resembled *P. marianne* specimens more closely than specimens sampled from further upstream with less influence from the Zambezi. Similar studies of local variability in EOD waveform of mormyrids from the Lower Guinea in West-Central Africa were published by Gallant et al. (2011) in relation to electrocyte morphology, and Arnegard et al. (2010) in relation to relatively minor differentiation between body morphology compared to EOD waveform differentiation.

The terminal parts of the Kwando River represent a contact zone between the two major systems on either side, west and east. Whereas EOD waveforms of both *P. castelnaui* and *P. marianne* (from the Zambezi) were characteristically different from each other, stable, and easily identified at the population and individual level, the EOD of Kwando specimens combined characteristics of both species in a manner that was found in neither, resulting in a characteristically different but more variable waveform. EOD playback studies have demonstrated spontaneous preference of like-to-like for *P. marianne* (Zambezi specimens) and *P. castelnaui* (Okavango specimens, Markowski et al. 2008). High variability of a communication signal in the contact zone of two

parapatric phenotypes indicates the possibility of introgression by hybridization (Wirtz 1999; Bronson et al. 2003; Brodin & Haas 2006).

This situation requires research into the morphology, EOD and DNA further to the studies of Kramer et al. (2003). DNA studies have already greatly contributed to resolve the status of mormyrid fish in the past; for example, for more tropical African regions, Agnèse and Bigorne (1992); Lavoué et al. (2000, 2004); Sullivan et al. (2000, 2002, 2004); Arnegard and Hopkins (2003); Lavoué and Sullivan (2004); Arnegard et al. (2006); and Feulner et al. (2006, 2007). For southern Africa, the studies by Van der Bank and Kramer (1996); Kramer and Van der Bank (2000); Kramer et al. (2004, 2007) and Kramer and Swartz (2010) are examples.

In order to ascertain the status of the Kwando dwarf stonebashers more clearly, we increased the number of samples from the two main systems, and several localities on the Kwando River, to strengthen our database for additional DNA, EOD and morphology studies at higher resolution. We also addressed the question of a cline by comparing samples from four Kwando localities.

Material and methods

The two main rivers, the Okavango and the Zambezi, run in parallel in a south-easterly direction over wide ranges of Angola, Zambia, Namibia and Botswana. The Kwando is a smaller river in the middle, at approximately equal distance ($\sim$ 100 km) from both main systems (Figure 1). The terminal (southern) part of the Kwando River turns sharply 90° north-east towards the Upper Zambezi River (from where on the Kwando is called, in turn, Linyanti and Chobe rivers, the latter being inundated by the Zambezi in times of flood; Figure 1). The 90° turning point also marks a sporadic water connection to the Okavango System (Selinda spillway or Magwegqana; Figure 1). The terminal sections of the Kwando River are thus sporadically connected to both major rivers, the Okavango and the Zambezi.

Morphology

Depending on the state of specimen conservation and the focus of the analyses, up to 188 specimens from the Upper Zambezi and Kwando rivers and the Okavango River were compared. Specimen numbers are indicated where appropriate. Usually 15 (at least 12) measurements and usually four (at least three) counts were taken on anatomical characters (Figure 2). The following abbreviations were used: PDL, predorsal length – distance from tip of snout to dorsal fin origin; PAL, distance from tip of snout to anal fin origin; LD, dorsal fin length; LA, anal fin length; LPF, length of pectoral fin; PPF, distance from pectoral fin origin to pelvic fin origin; pD, distance from dorsal fin origin to end of caudal peduncle; CPL, length of caudal peduncle (end of anal fin base to midbase caudal fin); CPD, depth of caudal peduncle – the least vertical distance across the caudal peduncle; LSo, length of snout – distance from tip of snout to posterior orbital rim of eye; OD, eye diameter, measured horizontally from anterior orbital rim to posterior orbital rim; HL, head length – distance from tip of the snout to bony edge of the operculum; Na, distance between the pair of nares of one side (from centre to centre); BD, body depth – the greatest vertical distance across the body; SL, standard length – distance from tip of snout to midbase caudal fin; TL,
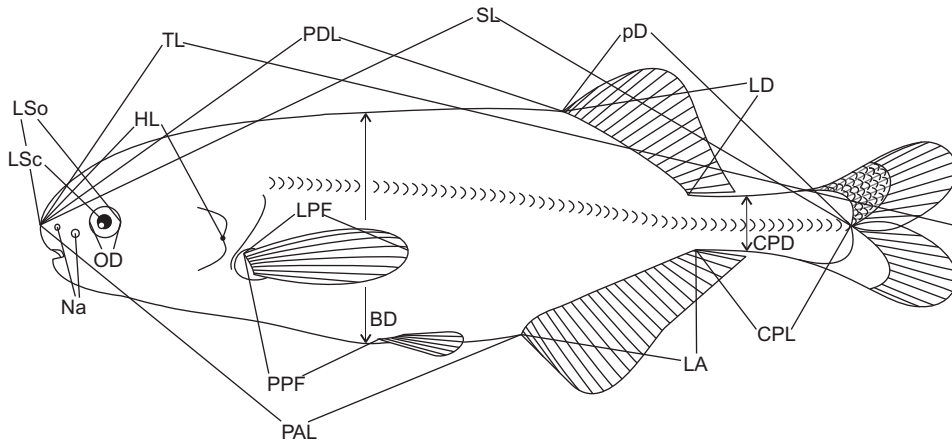
Figure 2. Anatomical characters measured. For abbreviations see Material and methods.

total length – distance from tip of snout to end caudal fin; nD, number of dorsal fin rays; nA, number of anal fin rays; SPc, number of scales around caudal peduncle; SLS, number of scales in linear series along the lateral line row, as detailed in Skelton (2001, p. 67). SLS range of accuracy, ± 2 counts.

Abbreviations used to represent institutions and collections cited follow Leviton et al. (1985) and Fricke and Eschmeyer (2012). Specimens examined were initially identified using dichotomous keys in Bell-Cross and Minshull (1988) and Skelton (2001), which are considered effective for fish populations occurring in southern Africa. The reference for current taxonomic status was Eschmeyer (2012).

Electric organ discharges

EODs were recorded immediately after capture in a 37-l plastic aquarium with fresh river water from the site where the fish were collected. Conductivity changes possibly affecting the EOD were, therefore, excluded.

Water temperature ($\pm 0.1°$C) and conductivity ($\pm 1\,\mu$S cm^{-1}) were constantly monitored using an electronic apparatus (LF318 Wissenschaftlich-Technische Werkstätten, WTW, Germany). Fish were placed between a pair of carbon rod electrodes that were connected to a differential amplifier with variable gain (up to $\times 10$; 0.2 Hz ... 100 kHz; filter slopes, –3 dB per octave; electronics workshop, Biology Department, University of Regensburg). Amplifier output was recorded with a digital storage oscilloscope (100 MHz/9 bit/10 000 points per sweep), and data were numerically transferred onto disk via digital interface. Usually eight traces per fish were recorded. Field equipment was battery-operated.

Custom-designed computer programs were used for analysis of EODs (programmed using a software package for signal analysis, Famos v5-v6 by imc Co., Berlin). When necessary, EOD duration was corrected to 25°C using a Q_{10} value of 1.5 (Kramer & Westby 1985) before data analysis. Definition of waveform variables related to EOD phases P0, N0, P1, N1, P2 (Figure 3): P1, peak amplitude of positive P1 phase (from baseline to peak); P0, P2, positive peak amplitudes of pre- and
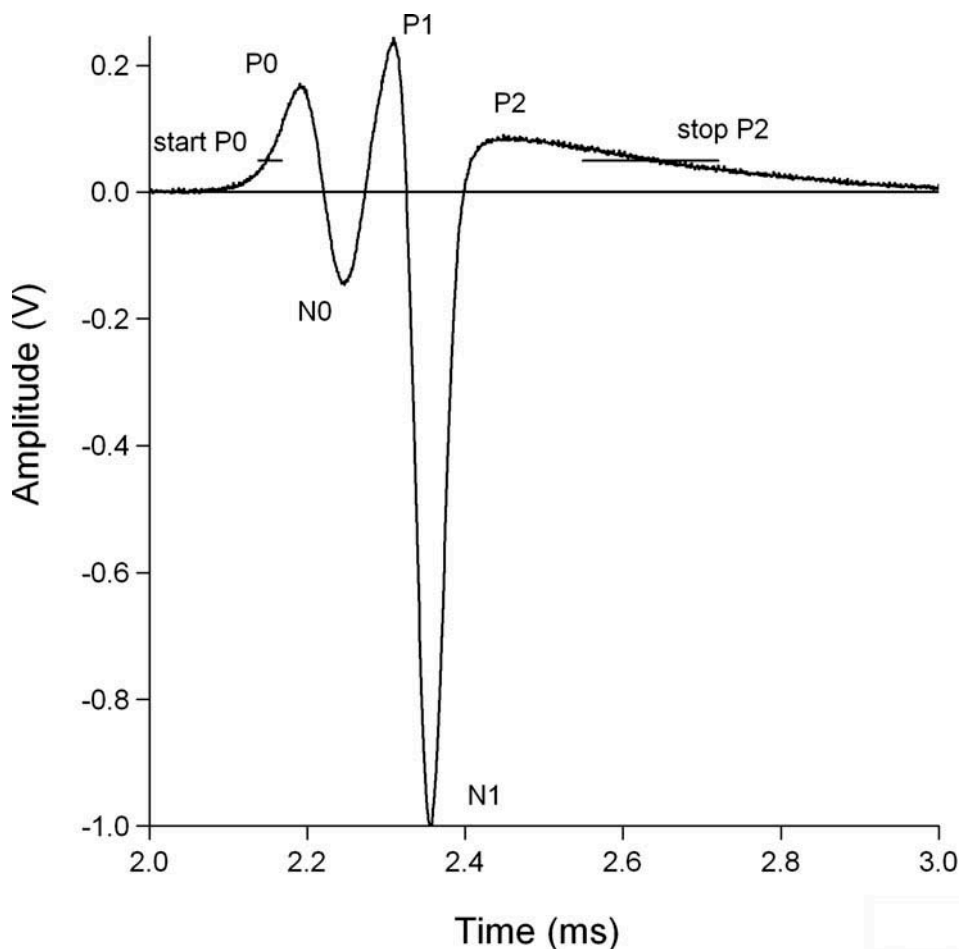
Figure 3. The different phases of the EOD waveform observed in *Pollimyrus cuandoensis* sp. nov., with baseline superimposed. Phases start and stop at zero-crossings, except for first start (start P0) and last stop (stop P2), as indicated. Threshold value is 5% of N1 amplitude. Peak amplitudes are measured from baseline.

post-potentials, respectively; N0, N1, negative peak amplitudes of N0 and N1 phases of EOD; N0dur, P1dur, N1dur, durations of respective phases; P1N1sep, separation (or interval) between the peaks of the P1 and N1 phases; P1area, N1area, areas under the P1 and N1 phases. Durations in microseconds; amplitudes in relative volts (re: N1 phase = 1 V). Area-under-curve measures, dimension ($V \times$ milliseconds). Start and end points of an EOD were determined at a threshold level of 5% of N1amp; other phases start and end between zero-crossings. When in a certain EOD P0- or P2 peaks were smaller than 5% of N1, an EOD started or ended at P0 peak or P2 peak, respectively.

Following EOD recording the fish were killed by an overdose of the anaesthetic 2-phenoxy-ethanol, SL determined using vernier callipers, and fixed in 10% formaldehyde solution for morphological studies.

Statistical analysis

Principal component analyses (PCA) on correlations among anatomical and EOD waveform characters were used to test differences in body shape or EOD waveform among populations because they do not require a priori assumptions about taxonomic groups. Analyses of variance (ANOVA) were performed to test hypotheses of no difference between samples for each character individually. Multivariate analyses of variance (MANOVA) were useful in order not to overestimate differentiation when examining the hypothesis of no morphological difference between fish from different origins by inferential statistics (McGarigal et al. 2000). *P* values are two-tailed unless otherwise stated. For interpreting the principal components in terms of the anatomical characters, we determined the component loadings, i.e. the principal component structure (see McGarigal et al. 2000). For assessing the significance of component loadings we followed Tabachnick and Fidell (2007). These authors recognize five levels of significance: loadings >0.32 or <-0.32 are poor, >0.45 or <-0.45 fair, >0.55 or <-0.55 good, >0.63 or <-0.63 very good, and >0.71 or <-0.71 excellent. These benchmarks account for 10%, 20%, 30%, 40% and 50% of the variance in the component, respectively. We also performed discriminant analyses to find the best separation among samples in multidimensional space. The software used was JMP v. 7 to 9 (SAS Institute, Cary, NC, USA, 2003–2010).

Genetic studies

DNA isolation

DNA was isolated from ethanol preserved tissues or scales using standard procedures as described earlier (Kramer et al. 2007).

Amplification of cytochrome b by PCR and sequencing

The mitochondrial cytochrome *b* gene was amplified by PCR and sequenced as in previous publications (Kramer et al. 2007).

The aligned sequences were analysed by MEGA5 (Tamura et al. 2011). Maximum likelihood was used to reconstruct the phylogeny. Conditions: substitution model: general time reversible (GTR) model; rates among sites: gamma distributed with invariant sites $(G + I)$ and five discrete gamma categories. Tree inference options: ML heuristic method: nearest neighbour interchange (NNI). All codons were included. Phylogeny test: Bootstrap method with 1000 replications.

ISSR genomic fingerprinting

Total DNA of *P. castelnaui*, *P. marianne* and *P. cuandoensis* sp. nov. was amplified using the ISSR primer MW4 $(GACA)_4$ (ISSR, inter-simple-sequence-repeat). The PCR products were separated by high resolution polyacrylamide electrophoresis (PAGE) as described earlier (Kramer et al. 2007).

Results

Anatomy

Because of the unresolved status of the Kwando/Linyanti population in the study of Kramer et al. (2003), we added measurements or counts of four more anatomical

characters to our database of specimens from the Zambezi, Okavango and Kwando systems (Appendix Table 2.1) for finer statistical resolution. The first hypothesis tested was the null hypothesis of all samples from different origins being similar; that is, if true, there would be no statistically significant differences among them. Available for inferential statistical comparisons were specimens from four localities on the Kwando/Linyanti River, and one each from the Upper Zambezi and Okavango rivers. As expected, a MANOVA rejected the assumption of no differences, that is, there is at least one pair of samples that differ in at least one character ($P < 0.0001$; Table 1). Subsequent ANOVAs located significant differences in each character used for the analysis, that is, there is not a single character among the 17 studied that did not contribute to the overall MANOVA result of significant variation among samples (Table 1).

Post-hoc tests identified the pairs of samples that were differentiated, and in which characters. For example, Okavango samples differed from Upper Zambezi samples in 11 characters ($P < 0.01$, plus four at $P < 0.05$; Table 1), confirming previous conclusions of differentiation on the species level. The sample from Kwando-Kongola Bridge differed markedly from the Upper Zambezi sample in 10 (plus three) characters, and from Okavango samples in six characters. Kwando-Nakatwa samples differed from Okavango samples in eight characters, and from Zambezi samples in six (plus five). The Linyanti/Sampis sample is geographically closest to the Zambezi, and the two samples differed by an astounding nine characters, whereas with regard to the more distant Okavango sample there were six. These results confirm differentiation of the Kwando samples from the two established species on either side, the Okavango and Zambezi rivers. There was only one (plus one) difference between the pair of Kwando samples, Kongola and Nakatwa, that were the most remote and upstream from the connecting channels to both main rivers, Okavango and Zambezi. However, the Linyanti sample differed from the neighbouring Nakatwa sample in four and from the Kongola sample (the furthest upstream locality) in two characters, suggesting some differentiation also among Kwando samples.

Principal component analysis on correlations showed that the first three components captured 55% of the variation in the dataset, and that by removing redundancy, dimensionality was successfully reduced. Principal component 1 (PC1) was loaded most strongly and positively by SLS, LA, LD, pD, LPF, nA and SPc, and negatively by PAL, PDL, HL, PPF, OD, CPD and BD (with significance of components decreasing from "excellent" to "fair"; see component loadings, Appendix Table 2.3). PC1 thus represents a gradient of a long rear section, beginning at about the origins of the unpaired fins, that is correlated with a short frontal body (or vice versa). PC2 was loaded positively by BD, PPF ("very good"), and more weakly by a few other characters (SPc, LSo, CPL, CPD, nD, nA; significance for the last two, "poor"). Similarly, for PC3 there was a single character only that was highly significantly loading PC3, CPL ("excellent", negative); the significance of all others was "fair" (CPD) or "poor" and the correlation of positive sign (LD, LA, nA).

In a 3D plot in PC1–PC3 coordinates the clouds of points for Okavango, Upper Zambezi, and the Kwando samples occupy separate spaces. Whereas specimens from the Upper Zambezi tend to have positive scores for PC1, Okavango and Kwando specimens tend to have negative scores. Okavango and Kwando specimens were differentiated on PC2, with Kwando specimens showing a tendency for positive scores,

Table 1. MANOVA on morphological characters of *Pollimyrus castelnaui/marianne* species complex.

	PDL/SL	PAL/SL	LD/SL	LA/SL	LPF/HL	PPF/SL	pD/SL	CPL/SL	CPD/CPL	LS(o)/HL	OD/HL	HL/SL	BD/SL	nD	nA	SPc	SLS
MANOVA	**<10⁻⁴**																
ANOVA	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**<10⁻⁴**	**0.0049**	**0.0003**	**<10⁻⁴**	**<10⁻⁴**
post tests[1]																	
L,N			**<0.01**	**<0.01**							**<0.01**		**<0.01**				
L,K											**<0.01**		**<0.01**				
L,O						**<0.01**				**<0.01**			**<0.01**				
L,Z	**<0.01**	**<0.01**	**<0.01**	**<0.01**		**<0.01**		**<0.01**	**<0.01**	**<0.01**		**<0.01**	**<0.01**			**<0.01**	**<0.01**
N,K			<0.05														
N,O	**<0.01**		**<0.01**	**<0.01**		**<0.01**		**<0.01**	**<0.01**		**<0.01**		**<0.01**			**<0.01**	**<0.01**
N,Z		<0.05	<0.05			**<0.01**	<0.05		<0.05		**<0.01**	<0.05	<0.05				
K,O						**<0.01**			**<0.01**	**<0.01**	**<0.01**		**<0.01**			**<0.01**	**<0.01**
K,Z	**<0.01**	**<0.01**	<0.05		**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**		**<0.01**	**<0.01**	<0.05			**<0.01**	**<0.01**
O,Z	**<0.01**	**<0.01**	<0.05	**<0.01**	**<0.01**		**<0.01**	**<0.01**	<0.05	<0.05	**<0.01**	**<0.01**	<0.05	**<0.01**	**<0.01**	**<0.01**	**<0.01**

Notes: MANOVA P-value, same for Wilk's lambda, Roy's greatest root, Hotelling–Lawley trace and Pillai trace tests; $F_{\geq 17,131} \geq 12.755$. P-values < 0.01, bold. ANOVAs, $F_{4,144} \geq 3.902$.

[1]Games–Howell test. L, Linyanti ($N = 28$ specimens); N, Nakatwa ($N = 11$); O, Okavango ($N = 34$), Z, Upper Zambezi ($N = 61$), K, Kongola ($N = 15$). For means, medians, standard errors, etc., see Appendix 2, Table 2.1; for abbreviations of characters, see Material and methods. Locality "O" refers to O1 and O2; "Z" refers to Z1 and Z2 in map of Figure 1.
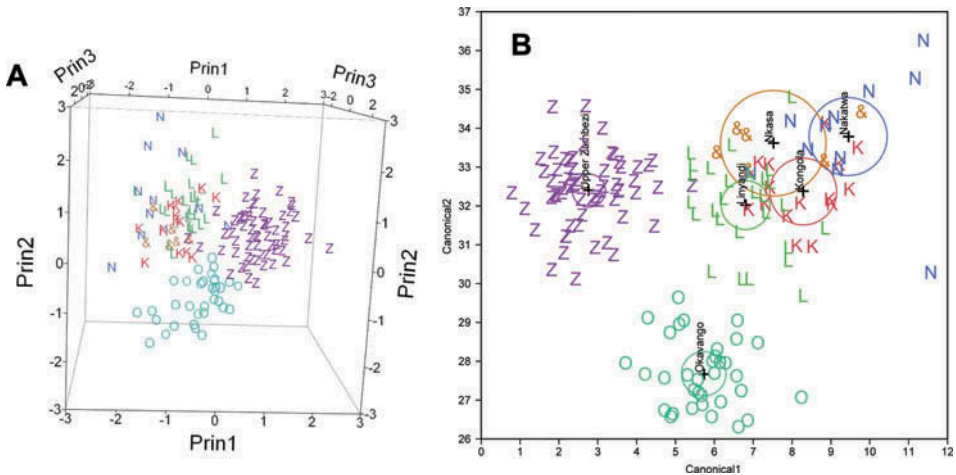
Figure 4. Multivariate analysis on correlations of morphological characters for *Pollimyrus castelnaui/marianne* species complex from Upper Zambezi/Okavango/Kwando system. (A) Principal component analysis, showing the first three components, Prin1–Prin3. Specimens from different localities on Kwando River: red K symbols, Kongola Bridge (*N* = 15); blue N symbols, Nakatwa (*N* = 11); green L symbols, Linyanti (*N* = 28); beige ampersands, Nkasa (*N* = 6). Specimens from main rivers: violet Z symbols, Upper Zambezi River (*N* = 61); blue-green O symbols, Okavango (*N* = 34). (B) as (A), but discriminant analysis. Circles, 95% confidence limits around centroids.

whereas Okavango scores were all negative. A small region of overlap is seen between Upper Zambezi and certain Kwando specimens (Figure 4A, 3D plot).

A discriminant analysis with origin as grouping variable confirms excellent differentiation of Okavango from Upper Zambezi specimens, and of both from Kwando specimens (Figure 4B; *N* = 155). Not a single specimen was misclassified among the three rivers. The seven specimens misclassified were all among the different Kwando samples: one of the 27 Linyanti samples as a Kongola specimen; three of the 11 Nakatwa specimens as a Kongola, a Linyanti and a Nkasa specimen; one of the six Nkasa specimens as a Kongola specimen; and two of the 15 Kongola specimens as a Linyanti and a Nakatwa specimen. Both geographically and in the graph, among the different Kwando samples the Linyanti sample was closest to those from the Okavango and Upper Zambezi, in spite of their strong differentiation in terms of MANOVA/ANOVA. In conclusion, the anatomical results confirm that samples from these origins were differentiated from each other: Okavango, Upper Zambezi and Kwando.

In August 2004 we collected additional specimens for more detailed DNA and EOD comparisons among the three populations, Okavango (*N* = 9), Kwando (Kongola, *N* = 20), and Upper Zambezi (*N* = 23). We established that these specimens were drawn from the same populations that had been sampled at these places previously, by identical medians in SPc counts (Appendix Table 2.2; differences same-same non-significant). As shown previously, samples from different origins differed statistically significantly amongst each other in SPc counts (ANOVA $F_{2,49} = 45$; $P < 0.0001$). The Kwando-Kongola Bridge specimens' counts (of median $14 \pm$ SIQ 0; *N* = 18) were

significantly higher than the Okavango specimens' counts (of median $12 \pm$ SIQ 1; $N = 9$) at $P = 0.0256$, and lower than the Zambezi/Lisikili specimens' counts (of median $16 \pm$ SIQ 0; $N = 23$) at $P < 0.0001$ (Scheffé's post hoc tests, two-sided). The difference between the Okavango and the Zambezi specimens' counts was also significant at $P < 0.0001$.

Electric organ discharges

Figure 5 illustrates the characteristic differences in pulse waveform among samples from the three rivers, Okavango, Kwando and Zambezi. *Pollimyrus marianne*'s EOD is a simple triphasic waveform that goes head-positive first (P1) and is followed by a strong head-negative (N1) and a head-positive terminal phase (P2) of rather long duration and about the same or somewhat weaker amplitude than P1 ("Type 3" discharge of Kramer et al. 2003; Figure 5A). *P. castelnaui*'s EOD (Figure 5C) is a pentaphasic pulse with P2 stronger than P1, whereas the negative N1 again is the strongest phase by far. In addition to these three phases there are two weaker prepotentials not present in *P. marianne*'s EOD, P0 and N0, which are positive and negative, respectively ("Type 1" discharge).

The EODs of Kwando river specimens differ from both ("Type 2" discharge; Figure 5B). They resemble *P. marianne*'s EODs by a P2 not stronger (usually weaker) than P1, even in specimens where P0 is strongest among the positive potentials.
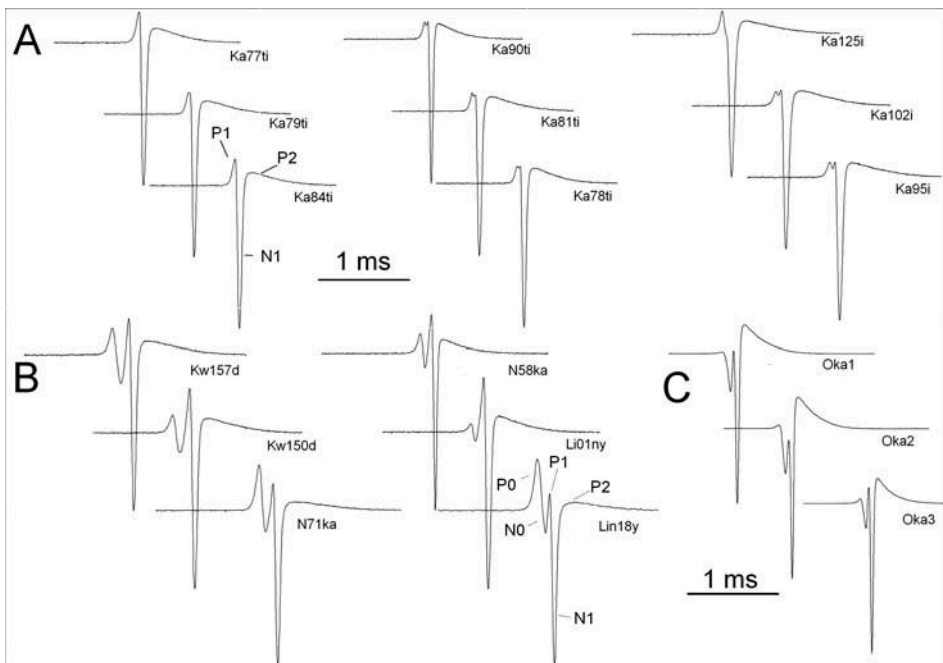

Figure 5. EOD waveforms of *Pollimyrus* species from three rivers. (A) *P. marianne*, Upper Zambezi River; (B) *P. cuandoensis* sp. nov., Kwando River; (C) *P. castelnaui*, Okavango River. From: Kramer et al. (2003).

Kwando specimens' EODs differ from *P. marianne*'s EODs by being pentaphasic rather than triphasic. Kwando specimens' EODs resemble *P. castelnaui*'s EODs by their five phases; they differ, however, in relative amplitudes of phases. P0 is very weak and often barely noticeable in Okavango specimens known so far, and the P2 phase is strongest. In Kwando specimens, rather than P2 it is the P1 or P0 phase that is the strongest positive potential, and the relative amplitudes of the first three phases, P0, N0 and P1, show an amount of variation not found in *P. castelnaui*. A few, rare Kwando specimens even resemble *P. marianne*'s EODs, especially specimens from Linyanti (closest location to the Zambezi). Conversely, *P. marianne* specimens from Zambezi may feature small indentations on P1 of their Type 3 discharge, called "Subtype 3" (the six most right-side EODs on Figure 5A). In conclusion, the EODs recorded from samples from the three rivers, Okavango, Zambezi and Kwando were well differentiated from each other.

The differences between *P. marianne* and *P. castelnaui* EODs have been described by Kramer et al. (2003) and Markowski et al. (2008) but a comparison of specimens originating from different localities within the Kwando system is lacking. For the present study additional samples were collected from the Upper Zambezi, the Kwando, and the Okavango in August 2004. The Upper Zambezi sample of 77 specimens corresponded to that reported in Kramer et al. (2003): all had either a Type 3 discharge or a Subtype 3 discharge (a Subtype 3 discharge differs from a Type 3 discharge by a small indentation of the P1 phase; see Figure 5). Whereas in the Kramer et al. (2003, table 1) study the ratio between Type 3:Subtype 3 waveforms was 1.8 (63:35), it was about 0.4 (23:54) in the 2004 sample originating from the same locality (Lisikili). The 2004 Kwando sample originates from Kongola Bridge, and all but one of 40 specimens showed a Type 2 waveform. The single exception (specimen Kong106) resembled most closely a Type 3 waveform with a preceding, weak P0 potential merging into P1 on the rising slope of P1, leaving no room for an N0 potential, required for a Type 2 discharge. This appears to be a very rare intermediate waveform between Kwando and Zambezi waveforms. Already in the previous 2003 study a single specimen among a sample of 24 from Kongola Bridge had shown a Type 3 waveform. The 13 specimens from the 2004 Okavango: Guma Lagoon sample did not present any differences from previous results: all showed a Type 1 waveform.

A MANOVA on 17 EOD characters revealed significant ($P < 0.0001$) variation among four local populations within the Kwando system (Table 2, based on the measurements summarized in Appendix Table 3.1). Only three characters did not vary significantly among populations; all other characters depended significantly on origin (as shown by subsequent ANOVAs, Table 2). Specimens originating from Kongola Bridge, which is the location the furthest upstream, differed markedly from their closest downstream neighbours, as shown by post-hoc paired comparisons: 11 significant differences (at $P < 0.01$, plus one at $P < 0.05$) between Kongola and Nakatwa specimens. The comparison of Kongola to Linyanti specimens, from the downstream end of the study area, yielded $6 + 1$ significant differences. Nakatwa specimens differed from Linyanti specimens also in $6 + 1$ characters. The small Nkasa sample ($N = 7$) in the centre still differed from the Nakatwa sample in $2 + 2$ characters, and in none from Linyanti specimens.

At the 5%-of-N1amplitude criterion used, EOD duration was about 0.5 ms, except in specimens from Nakatwa whose EODs were about one fifth shorter than in specimens from the other Kwando/Linyanti origins (Appendix Table 3.1; ANOVA

Table 2. MANOVA on EOD waveform characters for local populations of *Pollimyrus cuandoensis* sp. nov. in the Kwando River.

	P0 (V)	N0 (V)	P1 (V)	P2 (V)	P0dur (ms)	N0dur (ms)	P1dur (ms)	N1dur (ms)	P2dur (ms)	P0N0sep (ms)	N0P1sep (ms)	P1N1sep (ms)	P0area (V × ms)	N0area (V × ms)	P1area (V × ms)	N1area (V × ms)	P2area (V × ms)
MANOVA									**<0.0001**								
ANOVA[1]	**0.003**			0.0167	**0.0056**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0008**	**<0.0001**	**<0.0001**	**<0.0001**		**<0.0001**	**0.0005**	**<0.0001**	**0.0027**
post tests[1]																	
K,L	<0.01				<0.05	<0.01				<0.01	<0.01	<0.01		<0.01			
K,N		<0.05				<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01
K,&																	
L,N		<0.01						<0.01	<0.01	<0.05		<0.01				<0.01	<0.01
L,&																	<0.01
N,&								<0.01		<0.05		<0.05				<0.01	

[1]Games-Howell test. *P*-values <0.01, bold.

MANOVA *P*-value, same for Wilk's lambda, Roy's greatest root, Hotelling–Lawley trace and Pillai trace tests ($F_{\geq 17,109} \geq 6.478$). ANOVAs: $F_{3,123} = 3.543$, $P = 0.0167$ for P2, etc. For abbreviations of characters, see Material and methods.

Symbols for localities K, L, N, and "&" refer to Kongola Bridge ($N = 63$ specimens), Linyanti (Sampis, $N = 33$), Nakatwa ($N = 24$), and Nkasa Island ($N = 7$), respectively.

$F_{3,123} = 14.036$, $P < 0.0001$). Accordingly, Nakatwa EODs were significantly shorter than both Kongola and Linyanti EODs ($P < 0.01$, Games–Howell post-hoc test). In a few characters there appeared to be a weak geographical cline, i.e. decreasing P0dur but increasing P2 amplitude when going downstream. In order to reduce the complexity of this description, a principle component analysis was performed. The variable EOD duration (EODdur) was excluded, being only the sum of the four duration measures.

The first three components explained 78.5% of the variation in the dataset, showing that PCA very efficiently reduced dimensionality by removing redundancy; 36.7% of the variation was captured by PC1 alone (Appendix Table 3.2). PC1 was loaded most strongly by P2 peak amplitude, P2 area and P2 duration, and also by P1 duration, P1 area, P1 peak amplitude, N0–P1 separation and N1 area (all "excellent", except P2 duration and N1 area: "very good", and P1 peak amplitude: "good"). PC1 was loaded negatively by P0 peak amplitude ("excellent"), P0 area (very good") and P0 duration ("good"). PC1 thus represents a gradient mainly for the P2 and P0 phases in which Kwando EODs vary most, and also for the N1 and P1 phases. An increase in characteristics of the main discharge, P1, N1 and P2, is linked with a decrease in characteristics of the initial P0 phase, and vice versa. PC2 captured 28.3% of the variation in the dataset, and thus represents an additional important gradient. It was loaded positively by N0 area and N0 duration ("excellent" and "very good", respectively), N1 duration, P0–N0 separation, P0 area (all "very good"), and negatively by N0 peak amplitude ("excellent"). Since N0 is a negative peak, this is a positive loading. PC2 seems a gradient mainly for the N0 phase being strong when its forerunner, P0, was also strong and of long duration. PC3 captured much less of the variation (13.5%) than PC2; the only "good" loading was by P1 duration, and among the remaining loadings only two were "fair" and nine "poor".

A 3D plot in PC1–PC3 coordinates shows the majority of points for Kongola, Nakatwa and Linyanti specimens accumulating in different spaces (Figure 6A). The points of the few Nkasa specimens were widely scattered, with the exception of the space occupied by Nakatwa specimens. Kongola specimens tended to more positive values on PC1 and PC3 than Nakatwa specimens. This is in agreement with the shorter EOD duration of the Nakatwa specimens (Appendix Table 3.1).

The canonical plot of Figure 6B shows the points and multivariate means in the two dimensions that best separate the groups. The means and 95% confidence limits for Kongola, Nakatwa and Linyanti specimens were well separated from one another, but a few points of each either overlapped, or were very close. The few Nkasa specimens were widely scattered and overlapped with all groups except Nakatwa. Discriminant analysis misclassified 19 (15%) of 127 specimens: two, three and seven of 63 Kongola specimens were misclassified as Linyanti, Nakatwa and Nkasa specimens, respectively; one and two of 33 Linyanti specimens were misclassified as Kongola and Nakatwa specimens, respectively; one of the 24 Nakatwa specimens was misclassified as a Kongola specimen; three of the seven Nkasa specimens were misclassified as Linyanti specimens.

Genetic studies

Cytochrome b

The phylogenetic reconstruction of nucleotide sequences of mitochondrial cytochrome *b* with maximum likelihood shows that *P. castelnaui*, *P. marianne* and *P. cuandoensis*
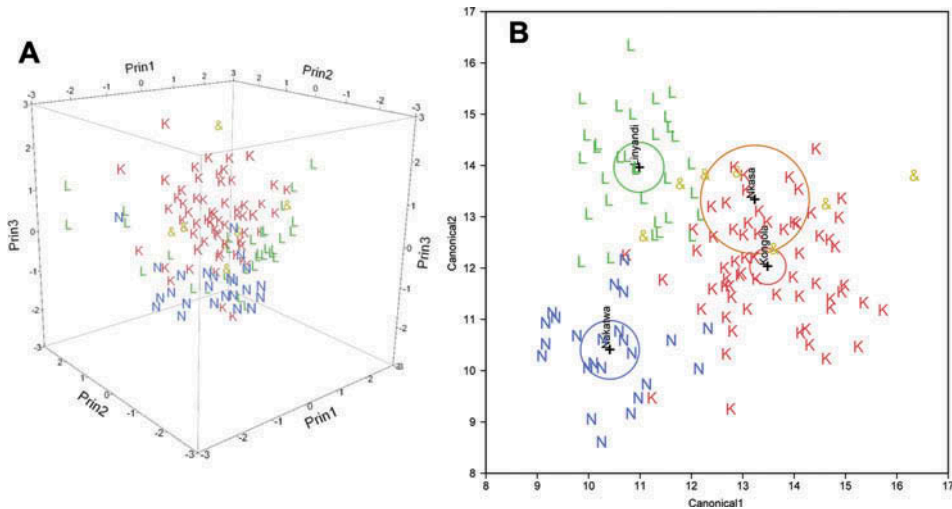
Figure 6. Multivariate analysis on correlations of characters of the electric organ discharge for *Pollimyrus* specimens from various localities on the Kwando/Linyanti system. (A) Principal component analysis on 17 EOD characters ($N = 127$ specimens), showing the first three components, Prin1–Prin3. Specimens from Kongola Bridge ($N = 63$) marked by red K symbols, Nakatwa ($N = 24$), blue N symbols, Nkasa ($N = 7$), beige ampersand symbols, Linyanti/Sampis ($N = 33$), green L symbols. (B) As (A), but discriminant analysis. Circles, 95% confidence limits around centroids.

sp. nov. form a monophyletic species complex (bootstrap support 100%; Figure 7). *Pollimyrus castelnaui* is separated from the *P. marianne* and *P. cuandoensis* sp. nov. complex which form two clades but without a clear species separation even though both taxa show different morphological and electrophysiological profiles and geographical distribution.

Genetic p-distances between *P. castelnaui* and *P. marianne*, and *P. castelnaui* and *P. cuandoensis* sp. nov. are 2.2–4.4% for the former and 2.5–3.6% for the latter. These distances provide an estimated time scale for divergence of 3.8–7.6 or 4.3–6.2 million years ago, respectively, assuming a molecular clock of 0.58% distance equals 1 million years (obtained for freshwater fish; Burridge et al. 2008). Distances between the *P. marianne* and *P. cuandoensis* sp. nov. clades are about 1.2%, indicating a divergence about 2.1 million years ago.

ISSR profiles

Genomic fingerprinting of *P. castelnaui*, *P. marianne* and *P. cuandoensis* sp. nov. by ISSR-PCR produced a complex profile of PCR products (Appendix Table 2.4). Most bands were identical for the three *Pollimyrus* species (such as bands 4 and 10–13). *Pollimyrus cuandoensis* sp. nov. can be distinguished from other *Pollimyrus* species by band 7. Bands 5 and 8 are shared between *P. marianne* and *P. cuandoensis* sp. nov., supporting their sister-group relationship. All other bands vary freely among the three taxa which provide evidence for active hybridization. Because band 7 is unique for

Pollimyrus marianne 44657
61
Pollimyrus marianne 44663
Pollimyrus marianne 44660
Pollimyrus cuandoensis 51442
Pollimyrus marianne 51440
Pollimyrus marianne 51442
Pollimyrus marianne 44668
65 *Pollimyrus marianne* 44659
Pollimyrus marianne 44654
Pollimyrus marianne 44658
Pollimyrus cuandoensis 44677
Pollimyrus marianne 44665
Pollimyrus marianne 44667
Pollimyrus marianne 44656
Pollimyrus marianne 44662
Pollimyrus marianne 44648a
Pollimyrus cuandoensis 44673
Pollimyrus marianne 44655
Pollimyrus marianne 44661
Pollimyrus cuandoensis 44671
Pollimyrus marianne 44652
Pollimyrus marianne 44651
Pollimyrus marianne 44653
Pollimyrus marianne 44647
Pollimyrus marianne 44649
100
69 *Pollimyrus castelnaui* 51444
Pollimyrus castelnaui 51446
Pollimyrus castelnaui 16559
Pollimyrus castelnaui 51443
Pollimyrus castelnaui 16554
Pollimyrus castelnaui 16560
Pollimyrus castelnaui 16561
Pollimyrus castelnaui 16558
Pollimyrus isidori
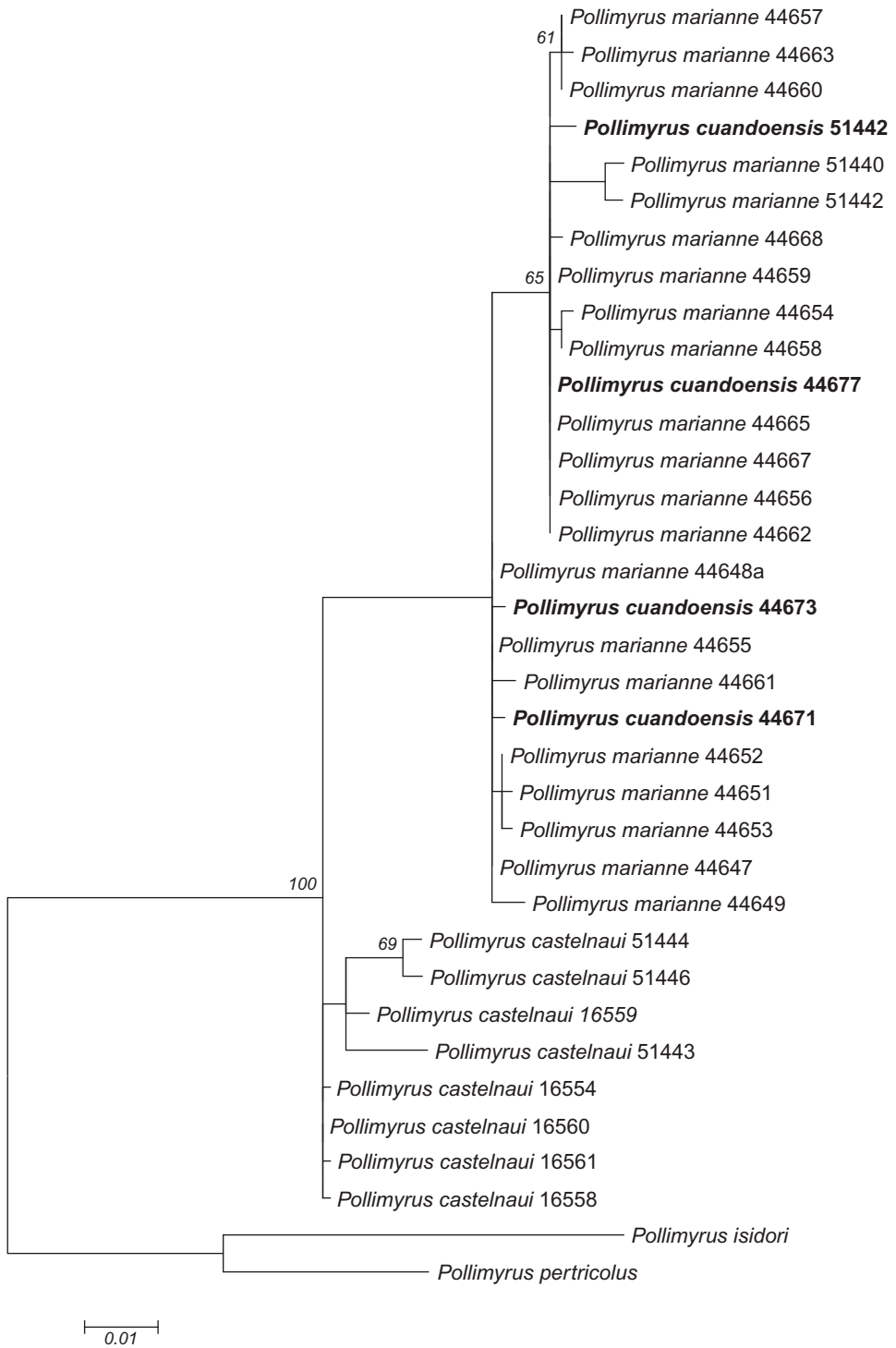*Pollimyrus pertricolus*

0.01

Figure 7. Molecular phylogeny of the *P. castelnaui* species complex. Phylogeny reconstruction by maximum likelihood is illustrated as a phylogram in which branch length is correlated with genetic distance. Outgroup, *Pollimyrus isidori* (Valenciennes, 1846) and *Pollimyrus petricolus* (Daget, 1954).

P. cuandoensis sp. nov. we conclude that the hybridization is not ongoing but had occurred some time ago when the three taxa were in regular contact with one another.

Systematics

Pollimyrus Taverne, 1971

Diagnosis (translated from Taverne 1971, p. 108)

Fairly short to elongate body; snout shorter than the postorbital portion of the skull, with a high upper jaw ("une mâchoire supérieure haute") that is curved backwards and slightly longer rostrally than lower jaw, mouth terminal, inferior or subinferior; caudal peduncle two to five times longer than deep; 15 to 36 dorsal fin rays; 21 to 31 anal fin rays; 9 to 12 pectoral fin rays; 35 to 70 lateral line scales; 7 to 21/8 to 23 scales in a transverse line of the body; 8 to 17/6 to 20 scales in a transverse line between the dorsal and the anal fins; 12 to 20 scales around the caudal peduncle; 5 to 9/6 to 10 bicuspid teeth; lateral ethmoid present but reduced; mesethmoid rather large and curved backwards; six circumorbital bones; anterior orbital and first infraorbital not fused; four hypural bones; 39 to 45 vertebrae.

Type species: *Pollimyrus isidori* (Valenciennes, 1846).

Included species. Valid unless stated otherwise.

adspersus, *Mormyrus* Günther, 1866. Current status: valid as *Pollimyrus adspersus* (Günther, 1866).

brevis, *Marcusenius* Boulenger, 1913. Current status: valid as *Pollimyrus brevis* (Boulenger, 1913).

castelnaui, *Marcusenius* Boulenger, 1911. Current status: valid as *Pollimyrus castelnaui* (Boulenger, 1911).

cuandoensis sp. nov., *Pollimyrus* Kramer, van der Bank and Wink, 2013.

guttatus, *Petrocephalus* Fowler, 1936. Current status: valid as *Pollimyrus guttatus* (Fowler, 1936).

isidori, *Mormyrus* Valenciennes, 1847. Current status: valid as *Pollimyrus isidori* (Valenciennes, 1847).

maculipinnis, *Marcusenius* Nichols and La Monte, 1934. Current status: valid as *Pollimyrus maculipinnis* (Nichols & La Monte, 1934).

marianne, *Pollimyrus* Kramer, van der Bank, Flint, Sauer-Gürth and Wink, 2003.

nigricans, *Marcusenius* Boulenger, 1906. Current status: valid as *Pollimyrus nigricans* (Boulenger, 1906).

nigripinnis, *Marcusenius* Boulenger, 1899. Current status: valid as *Pollimyrus nigripinnis* (Boulenger, 1899).

pedunculatus, *Marcusenius* David and Poll, 1937. Current status: valid as *Pollimyrus pedunculatus* (David and Poll, 1937).

petherici, *Marcusenius* Boulenger,1899. Current status: valid as *Pollimyrus petherici* (Boulenger, 1898).

petricolus, *Marcusenius* Daget, 1954. Current status: valid as *Pollimyrus petricolus* (Daget, 1954).

plagiostoma, *Marcusenius* Boulenger, 1889. Current status: valid as *Pollimyrus plagiostoma* (Boulenger, 1898).

pulverulentus, *Marcusenius* Boulenger, 1899. Current status: valid as *Pollimyrus pulverulentus* (Boulenger, 1899).

schreyeni, *Pollimyrus* Poll, 1972.

stappersi, *Marcusenius* Boulenger, 1915. Current status: valid as *Pollimyrus stappersi* (Boulenger, 1915).

tumifrons, *Marcusenius* Boulenger, 1902. Current status: valid as *Pollimyrus tumifrons* (Boulenger, 1902).

Pollimyrus castelnaui (Boulenger, 1911)

Marcusenius castelnaui Boulenger 1911, p. 402.
Pollimyrus castelnaui Taverne 1971, p. 105.

Type specimens. Syntypes: BMNH 1910.5.31.11-12 (2).

Type locality. Lake Ngami basin, Bechuanaland (Botswana), Africa. However, Boulenger (1911, p. 400) also stated that the name of this locality was used for convenience only, the real origin being "the Okavango River and vast extent of marshes (of which Lake Ngami is a part)", and Bell-Cross and Minshull (1988, p. 110) specify explicitly that the Types were collected "from the Okavango River by R.B. Woosman" although no precise place is given (name spelled "Woosnam" in Boulenger 1911). In August 2006 the area of Lake Ngami as marked on maps was completely dry, no lake or water body to be seen anywhere near townships of Sehithwa and Toteng (B.K. personal observation). Permanent-looking, wide gravel roads passed right through the "lake" in its south-western part. Since then water has returned to Lake Ngami (September 2012; B.K., personal observation).

Pollimyrus cuandoensis sp. nov.
(Figure 1K, L)

Type specimens. Holotype: ZSM 41805, field no. Kon26G, 50 mm SL (live), from Kwando River at Kongola Bridge, 17°47′33″ S, 23°20′33″ E, 25 August 1999, water conductivity and temperature, 236 µS cm^{-1} and 19°C. Paratypes: ZSM 39522, same date and place, specimens Kon02G, Kon16G, Kon17G, Kon18G, Kon19G, Kon21G, Kon22G, Kon23G, Kon25G, Kon27G, Kon28G, Kon29G, Kon30G, Kon31G, Kon39G, Kon40G, size range 32–60 mm SL (measured alive).

Nontypes: From Nkasa Island, 18°26.5′ S, 23°38′ E: ZSM 39515, specimens 1Fish, 2Fish, 7 Sept. 1993; ZSM 39516, specimens 3Fish, 6Fish, 8 Sept. 1993; ZSM 39517, specimen 10Fish; ZSM 39518, specimens 12Fish, 13Fish; size range 33–60 mm.

From Nakatwa, 18°06′ S, 23°23′ E: ZSM 39519 (6), specimens N56ka, N57ka, N58ka, N59ka, N62ka, N63ka, 9 March 1994; ZSM 39520 (12), specimens N64ka, N66ka, N67ka, N68ka, N69ka, N70ka, N71ka, N74ka, N76ka, N77ka, N78ka, N79ka, 10 March 1994; ZSM 39521 (3), specimens N81ka, N96ka, N97ka, 11 March 1994; size range 24–56 mm.

From Kongola Bridge, 17°47′33″ S, 23°20′33″ E: ZSM 39523, specimens Kon01G, Kon18G*, 24 January 2001, size range 48–58 mm SL; ZSM 41773, K89–K92, K97–K112 (20 specimens), 8 August 2004, size range 2.3–3.9 cm.

From Sampis, Linyanti River, 18°04′59.6″ S, 24°02′7″ E: ZSM 39532 (14), specimens Lin01–Lin14, 9 September 1997; ZSM 39533 (15), specimens Lin15–Lin28, and "without no.", 10 September 1997; size range 35–65 mm SL.

Type locality. Kwando (Cuando) River at Kongola Bridge, 17°47′33″ S, 23°20′33″ E.

Diagnosis

SPc, median = 14 (range 12–16; N = 19); PDL, mean = 0.6516 of SL (range 0.6333–0.6852; N = 19); LD, mean = 0.1636 of SL (range 0.14–0.1818); LA, mean = 0.216 of SL (range 0.18–0.2417); PPF, mean = 0.1696 of SL (range 0.1507–0.1873); CPL, mean = 0.2181 of SL (range 0.1898–0.24); CPD, mean = 0.3474 of CPL (range 0.3214–0.4146); LSo, mean = 0.4517 of HL (range 0.4059–0.5); BD, mean = 0.2753 of SL (range 0.2467–0.3051); nD, median = 17 (range 15–18); nA, median = 23 (range 21–24); SLS, median = 50 (range 44–55); PAL, mean = 0.6014 of SL (range 0.5849–0.6343); LPF, mean = 0.8319 of HL (range 0.7639–0.9257); pD, mean = 0.395 of SL (range 0.3796–0.426); OD, mean = 0.2635 of HL (range 0.2376–0.3050); HL, mean = 0.2422 of SL (range 0.2255–0.2633); Na, contained in HL, mean = 9.02 (range 8.31–9.92). Electric organ discharge, EOD, with five phases and last phase P2 not stronger than P1. (See Remarks).

Description

Body oblong shape. Head broadly rounded with a small, terminal mouth; head and body dorsoventrally compressed. Dorsal fin (a) origin situated about two thirds of standard length from snout, (b) obliquely orientated, anteriorly higher and posteriorly lower, (c) first few rays anteriorly longer than those posteriorly, and (d) median number of rays 17 (15–18). Anal fin (a) opposite dorsal but origin more anteriorly and also obliquely orientated, (b) anteriorly lower and posteriorly higher, in male specimens anterior 10 or so rays longer than those posteriorly, (d) margin broadly rounded, (e) and median number of rays 23 (21–24). Scales (a) cycloid with reticulate striae, except in centre, extending anteriorly to operculum, pectoral and pelvic fins. SPc, median = 14 (12–16). Caudal peduncle slender and subcylindrical over the entire length, usually less than a quarter in standard length, SL. Forked tail fin with broadly rounded lobes overlapping at midbase. Electric organ discharge with five phases and of short duration, a mean 0.5 ms (range 0.34–0.64 ms) at 25°C and "5% amplitude-of-N1-phase criterion" (see Material and methods).

Colour in preservation. Mottled brown, throat and belly light beige covered with small brown spots.

Colour in life. Similar to preserved specimens.

Ecology. Prefers to hide in weeds if present, floating or not, often high up in water column, sometimes even at the surface under a water lily leaf in bright sunlight. Also

found on rocky bottom, hiding in crevices and holes; will do so also in aquarium during the day where there is no predation pressure. Will often not struggle in weeds brought to shore, so as not to raise attention. Appears not to be a strong swimmer, in contrast to the *Petrocephalus* species of southern Africa that are only somewhat bigger.

Distribution

At present known only from the lower section of the Kwando (Cuando) River, Caprivi Strip, Namibia.

Remarks

The most useful (quickest) character to distinguish between *P. castelnaui*, *P. marianne* and *P. cuandoensis* sp. nov. is the electric organ discharge, EOD, which is characteristically different in the three species. *P. cuandoensis* sp. nov. can be recognized by its median number of scales around the caudal peduncle which is usually 14 rather than 12 in *P. castelnaui* and 16 in *P. marianne*. *P. cuandoensis* sp. nov. is also distinguished from *P. castelnaui* by a combination of morphological characters, such as lower scores for PDL, PPF, CPD, LSo, OD, and BD in the latter. From *P. marianne*, *P. cuandoensis* sp. nov. is distinguished by higher scores for PPF, CPD, LSo, OD, BD and nD, lower scores for LA and SLS in the latter.

Etymology

The species name *cuandoensis* refers to the Kwando (Cuando) River that passes through the Caprivi Strip, Namibia.

Pollimyrus marianne Kramer et al., 2003

Type specimens. SAIAB 66943. Paratypes: SAIAB 66944 (10). Plus non-type material.

Type locality. Upper Zambezi River, Lisikili backwater (17°29′ S, 24°26′ E), Namibia: East Caprivi.

For material examined, see Appendix 1.

Discussion

The objective of the present paper was to clarify the status of the *Pollimyrus* population inhabiting the semi-isolated Kwando system between the two main river systems, the Okavango in the west and the Zambezi in the east, that are inhabited by *P. castelnaui* (Boulenger, 1911) and *P. marianne* Kramer et al., 2003, respectively. The lower Kwando has tenuous links to both major systems on either side (Figure 1), and the Kwando *Pollimyrus* show aspects of "intermediate" forms. The present paper shows that the Kwando form of *Pollimyrus* is a well-differentiated phenotype in both morphology and electric organ discharges (EODs). DNA sequence analysis of the cytochrome *b* gene demonstrated the Kwando form's close relationship with *P. marianne*, and that both form a sister clade to *P. castelnaui*. Genomic ISSR fingerprinting confirmed the

differentiation of the Kwando form from *P. marianne*, and we recognize the Kwando form as *Pollimyrus cuandoensis* sp. nov. *Pollimyrus cuandoensis* individuals have distinct ISSR profiles. Eight individuals (nos. 44670–5, 4479 and 44682) have band 7, one (no. 44669) has a combination of bands 2, 5 and 9, and three (44676, 44677 and 44679) have combinations of bands 1, 2, 3 and 8, which were not found in any of the *P. castelnaui* or *P. marianne* individuals (Appendix Table 2.4).

With a more extensive anatomical dataset, the present study confirmed the differentiation of *P. marianne* from *P. castelnaui* (Kramer et al., 2003), visualized in the multivariate plots of Figure 4A, B. New is the observation that *P. cuandoensis* sp. nov. proved well differentiated morphologically from both above-mentioned species (Figure 4, Table 1). Among 155 specimens including samples of all three species, discriminant analysis with origin as a grouping variable did not misclassify a single specimen as representing another species (Figure 4B). Most of the morphological variation among the three species was represented by PC1, a gradient for a longer rear section beginning at about the origin of the unpaired fins, that was correlated with a short frontal body (or vice versa). Second (PC2) was a gradient for body depth and width, caudal peduncle depth and length, and length of snout. Third was a gradient for a short and deep caudal peduncle (or vice versa, long and slender). These results seem to show that for *Pollimyrus* different adaptations to a compromise between swimming power versus manoeuvrability play a major role in the environment of the Upper Zambezi–Okavango System that includes the Kwando. Marked seasonal differences in water volume are known both from the Upper Zambezi and the Okavango. The former is known as a reservoir river that in spring does not only inundate huge areas of the savannah but also regularly floods the lower sections of the Kwando, Linyanti and Chobe rivers. With large seasonal variation in water level, the Okavango terminates in a vast expanse of marshes, an inland delta. The Kwando appears stagnant for much of its lower sections for much of the year. These ecological differences may have brought about the morphological differentiation among the three species of *Pollimyrus*.

The differentiation among the three species goes beyond morphology. Besides categorical differences in electric organ discharge (EOD) waveform (Figure 5), certain acoustical characteristics of the courtship songs of male *P. castelnaui* and *P. marianne* were found to differ (Lamml & Kramer 2006). The male of the single Kwando *Pollimyrus* pair that spawned in the laboratory gave courtship songs that combined features of both parapatric neighbour species (Lamml & Kramer 2006). Mutual EOD playback tests with *P. castelnaui* and *P. marianne* demonstrated a spontaneous preference of experimental subjects for EODs of their own species (*P. marianne* or *P. castelnaui*), and a mathematical model (Markowski et al. 2008) succeeded in transforming the EOD waveform of one species into that of the other by only small parameter changes of the physiological variables underlying the mechanism of EOD generation (identified by Bennett 1971, and modelled by Westby 1984). These results suggested an evolutionary key role for the two communication signals, the EOD and the courtship song, in the differentiation of the two species. Their differentiation must have occurred between 3.8 to 7.6 million years ago according to cyt-*b* data of the present study, assuming a molecular clock rate as estimated by Burridge et al. (2008). The differentiation of *P. cuandoensis* sp. nov. must have been more recent, at 2.1 mya. *Pollimyrus cuandoensis* sp. nov. exhibits an EOD waveform that, although more variable, is clearly distinct from EODs of *P. marianne* and *P. castelnaui*. Both in EOD waveform and morphology the Kwando samples close to influx from the two major

river systems, Okavango and Zambezi, seem to show more affinity with the main river species, respectively, than more distant, upstream Kwando samples (samples Nakatwa, Nkasa influenced by the Okavango, Linyanti by the Zambezi, versus Kongola from where the type specimens were chosen). Invasion of the lower Kwando by Zambezi specimens is highly likely because of the seasonal flooding of the Chobe–Linyanti section of the Kwando by the Zambezi. Destabilization of the Kwando form's differentiation by gene flow from the two main river systems is the most likely reason why the Kwando form's EOD waveform is more variable.

Speciation may be the result of sexual selection (Andersson 1994; Andersson & Simmons 2006). Sexual selection on EOD waveform has been demonstrated experimentally in *Marcusenius pongolensis* (Fowler, 1934) and *M. altisambesi* Kramer et al. 2007, both intra- and inter-sexually (Hanika & Kramer 2005, 2008; Machnik & Kramer 2008; Machnik et al. 2010). Communication by EOD clearly plays an important role in courtship and spawning in both dwarf stonebasher species (Baier & Kramer 2007), in *Pollimyrus adspersus* (Günther, 1866), studied by Bratton and Kramer (1989); Crawford (1991); Crawford and Huang (1999), in *P. isidori* (Valenciennes, 1846), studied by Crawford et al. (1997), and in *M. pongolensis* (Werneyer & Kramer, 2005). Whereas in *M. pongolensis*, *M. altisambesi* and *M. macrolepidotus* (Peters, 1852) both sexes vocalize in social context (Lamml & Kramer 2007), in the *Pollimyrus* species studied so far only the males have been observed to do so (e.g. Crawford & Huang 1999, and all other studies mentioned above). Therefore, the males of *Pollimyrus* species may not have experienced sexual selection pressure to develop truly sexually different or dimorphic EOD waveforms, such as observed in the *M. macrolepidotus* species complex (Kramer 1997; Kramer et al. 2007). Members of the genus *Marcusenius* all seem to be characterized by high male variability in EOD waveform, thus facilitating the evolution of true sexual dimorphism (Scheffel & Kramer 1997; Kramer 2013).

Male EOD waveforms in *P. adspersus* differ only statistically from female EODs; there is broad overlap, and the small sexual differences are of no relevance in mate selection as shown by Bratton and Kramer (1989) and Crawford (1991). According to the mate recognition concept by Paterson (1988) one might expect a sexually distinctive EOD ornament in the male sex that is also identifying the species. The lack of such a distinctive ornament in all *Pollimyrus* species studied so far, including the dwarf stonebashers of the present study (Markowski et al. 2007), may be due to the fact that the sex of a *Pollimyrus* male in reproductive condition is already obvious from his intense courtship singing and from his successfully defending a territory with a nest.

The different Kwando samples studied in the present paper show an amazing degree of anatomical and electrical differentiation amongst each other (MANOVA, Tables 1 and 2; Figures 4 and 6). Kongola and Nakatwa specimens were most clearly differentiated morphologically from both established species in the main rivers. Corresponding to geography, the Linyanti population was anatomically less strongly differentiated from the two species in the two major river systems. This reflects the terminal Kwando population being swamped by Zambezi specimens when the Zambezi floods the terminal Kwando sections, such as the Chobe and, to a lesser degree, the Linyanti.

The differences between the EOD waveforms of *P. castelnaui* and *P. marianne* were categorically distinct (i.e. with no transitional forms), making statistical comparison unnecessary. This is in contrast to the quantitative differences among the different

Kwando populations that MANOVA, PCA and discriminant analysis techniques all confirmed to differ significantly from one another. Whereas Kongola specimens scored strongest on PC1, which was loaded most strongly by duration and "area" measures for the second half of the EOD, Nakatwa specimens tended to have higher PC2 scores, loaded most strongly by characters from the first half of the EOD. Linyanti specimens were somewhere in between these two. Compared to the other Kwando samples, Nakatwa specimens exhibited EODs shorter by one fifth, suggesting an influence from the Okavango (gene flow). This shows that starting from Kongola (the locality furthest upstream) there is not just a continuous cline following the Kwando downstream, but a more complicated and diverse pattern. This underscores once more that the Okavango Delta/Upper Zambezi region recently has been, or still is, a hotspot of fish speciation (Joyce et al. 2005).

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Appendix 1. Material examined

1.1. Morphometrics

Pollimyrus castelnaui (Boulenger, 1911)

(1) *Pollimyrus castelnaui* (Boulenger, 1911). Syntypes BMNH 1910.5.31.11-12 (2) from Okavango River, Botswana.

(2) *Pollimyrus castelnaui*. ZSM 39534, 35 specimens from Makwena River Camp, Okavango, 19°03′16.2″ S, 22°22′51.3″ E, field codes Oka01v–Oka03v, Oka05v–Oka07v, Oka13v–Oka17v, AOka18v, BOka18v, Oka19v, AOka20v, BOka20v, Oka22v–Oka31v, AOka32v, BOka32v, Oka33v–Oka38v, Oka40v, 20–21 January 2001, water conductivity, 37 µS/cm, 29.9°C, coll. F.H. Van der Bank, J. Engelbrecht, B. Kramer.

Pollimyrus marianne Kramer et al., 2003

(3) ZSM 39524 (4), ZSM 39525 (3), seven specimens from Katima Mulilo, Upper Zambezi, 17°29′ S, 24°17′ E, 11 September 1993, field codes 33Fish, 39Fish, 41Fish, 42Fish, 53Fish, 56Fish, 57Fish, water conductivity, 81 µS/cm, 21.8°C, coll. F.H. Van der Bank and B. Kramer.

(4) ZSM 39526 (2), ZSM 39527 (2), Four specimens from Lisikili, Upper Zambezi, 17°32′ S, 24°26′ E, 5–7 March 1994, field codes L30isi, L32isi, L47isi, L52isi, water conductivity 56.1 µS/cm, 26.8°C, coll. F.H. Van der Bank and B. Kramer.

(5) ZSM 39528 (4), ZSM 39529 (18), ZSM 39530 (18), ZSM 39531, 53 specimens from Lisikili, Upper Zambezi, 17°32′ S, 24°26′ E, 7 April 1996, field codes Ka72–Ka75, Ka91, Ka92, Ka94, Ka96, Ka98, Ka99, Ka101- Ka104, Ka107, Ka109–Ka126, Ka139- Ka147, Ka149, Ka160–Ka162, Ka164–Ka168, Ka170, Ka172, water conductivity 66 µS/cm, 25.8°C, coll. F.H. Van der Bank and B. Kramer.

(6) Seventy-seven specimens from Upper Zambezi, Lisikili L1, 17°32′31.9″S, 24°26′17.7″ E, field codes Lisi01–Lisi21, Lisi23, Lisi25–Lisi29, Lisi32 Lisi36,

ZSM 41775 (L37–L56), Lisi57–Lisi61, Lisi63–Lisi68, Lisi70–Lisi72, Lisi74–Lisi84, ZSM 41776 (JHB1), water conductivity 91.5 μS/cm, 19.0°C, 5–6 August 2004, coll. F.H. Van der Bank and B. Kramer.

Pollimyrus cuandoensis sp. nov.

(7) ZSM 39515 (2), ZSM 39516 (2), ZSM39517 (1), ZSM 39518 (2), seven specimens from Nkasa Island in Mamili National Park, Kwando/Linyanti, 18°26.5′ S, 23°38′ E, 7–9 September 1993 (field codes, 1Fish, 2Fish, 3Fish, 6Fish, 10Fish, 12Fish, 13Fish). Water conductivity, 108 μS/cm, 18–19°C, coll. F.H. Van der Bank and B. Kramer.

(8) ZSM 39519 (6), ZSM 39520 (12), ZSM 39521 (3), 21 specimens from Nakatwa, Kwando River, Mudumu National Park, 18°16′ S, 23°23′ E, 9–15 March 1994, field codes N56ka–N59ka, N62ka–N71ka, N74ka–N79ka, N81ka, N82ka, water conductivity 130 μS/cm, 24.9°C, coll. F.H. Van der Bank and B. Kramer.

(9) ZSM 39532 (14), ZSM 39533 (15), 29 specimens from Linyanti-Sampis, 18°04′59.6″ S, 24°02′7″ E, 8–10 September 1997, field codes Lin01–Lin28, Ohne_Nr., water conductivity 156.8 μS/cm, 21.3°C, coll. F.H. Van der Bank and B. Kramer.

(10) ZSM 39522, 17 specimens from Kongola Bridge (where the Trans Caprivi Highway B8 crosses the Kwando River), 17°47′33″ S, 23°20′33″ E, 25 August 1999, field codes Kon02G, Kon16G, Kon17G, Kon18G, Kon19G, Kon21G, Kon22G, Kon23G, Kon25G–Kon31G, Kon39G, Kon40G, water conductivity 236 μS/cm, 19°C, coll. F.H. Van der Bank and B. Kramer.

(11) ZSM 39523, two specimens from Kongola Bridge, 24 January 2001, field codes Kon01g, Kon18g*, water conductivity 160 μS/cm, 26.6°C, coll. F.H. Van der Bank, J. Engelbrecht and B. Kramer.

1.2. EOD specimens

Pollimyrus castelnaui (Boulenger, 1911)

(1) All of the above under 1.1 (2), plus:

(2) Four specimens from Guma Lagoon, Okavango Delta, Thoage River, 18°57′46.6″ S, 22°22′25.3″ E, 10 August 2004, field codes Guma (no no.), Guma 132–Guma 134, water conductivity 38 μS/cm, 21.4°C, coll. F.H. Van der Bank and B. Kramer.

(3) Nine specimens from Makwena, Okavango, Thoage River, 19°03′13.8″ S, 22°22′42.6″ E, 11 August 2004, field codes Makwena163, Makwena164, Makwena166, Makwena169, Makwena170, Makwena172–Makwena175, same water and temperature, coll. F.H. Van der Bank and B. Kramer.

Pollimyrus cuandoensis sp. nov.
From Kwando River (total of 63 specimens):

(4) Five specimens from Kongola Bridge, 10 April 1996, field codes Kw150d, Kw154d–Kw158d, SL 4.95–6.07 cm, water conductivity 100 μS/cm, 25–26°C, coll. F.H. Van der Bank and B. Kramer.

(5) Sixteen specimens from Kongola Bridge, 25 August 1999, field codes Kon16G–Kon19G, Kon21G–Kon23G, Kon25G–Kon31G, Kon39G, Kon40G, water conductivity 236 μS/cm, 19°C, coll. F.H. Van der Bank and B. Kramer.

(6) Two specimens from Kongola Bridge, 24 January 2001, field codes Kon01g, Kon18g, water conductivity 160 μS/cm, 26.6°C, coll. F.H. Van der Bank, J. Engelbrecht and B. Kramer.

(7) Forty specimens from Kongola Bridge, 8 August 2004, field codes Kong88–Kong95, Kong97–Kong105, Kong107–Kong129, water conductivity 175–209 μS/cm, 17.9°C, coll. H. Van der Bank and B. Kramer.

(8) Twenty-four specimens from Nakatwa, 9–15 March 1994, N56ka–N59ka, N62ka–N71ka, N74ka–N79ka, N81ka, N82ka, N96ka, N97ka, water conductivity 130 μS/cm, 24.9°C, coll. F.H. Van der Bank and B. Kramer.

(9) Eight specimens from Linyanti-Sampis, 8 April 1996, field codes Li01ny, Li02ny, Li04ny–Li06ny, Li08ny, Li09ny, Li11ny, water conductivity 100 μS/cm, 25–26°C, coll. F.H. Van der Bank and B. Kramer.

(10) ZSM 39532 (14), ZSM 39533 (14), Twenty-eight specimens from Linyanti-Sampis, 8–10 September 1997, field codes Lin01–Lin14, Lin15–Lin28, coll. F.H. Van der Bank and B. Kramer.

(11) Seven specimens from Nkasa Island, 9–10 September 1993, field codes 1Fish, 2Fish, 3Fish, 6Fish 10Fish, 12Fish, 13Fish, water conductivity 108 μS/cm, 18–19°C, coll. F.H. Van der Bank and B. Kramer.

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(12) Seventy-seven specimens from Upper Zambezi, Lisikili L1, 17°32′31.9″ S, 24°26′17.7″ E, field codes Lisi01–Lisi21, Lisi23, Lisi25–Lisi29, Lisi32–Lisi61, Lisi63–Lisi68, Lisi70–Lisi72, Lisi74–Lisi84, water conductivity 91.5 uS/cm, 19.0°C, 5–6 August 2004, coll. F.H. Van der Bank and B. Kramer.

1.3. Material examined for DNA

Pollimyrus castelnaui (Boulenger, 1911)

IPMB nos. 16554, 16555, 16558, 16559, 16560, 16561, Botswana: Okavango River: Makwena River Camp, 19°03′16.2″ S, 22°22′51.3″ E, coll. H. van der Bank.

IPMB nos. 44698, 45377, 45378, Botswana: Okavango River: Makwena Lagoon, 19°03′45.3″ S, 22°23′24.3″ E, coll. H. van der Bank, March 2002.

IPMB nos. 51443, 51444, 51446, second generation fish born in captivity from parent fish from Botswana: Okavango River: Makwena Lagoon, 19°03′45.3″ S, 22°23′24.3″ E, coll. H. van der Bank, March 2002.

Pollimyrus cuandoensis sp. nov.

IPMB nos. 44669, 44670, 44671, 44672, 44673, 44674, 44675, 44676, 44677, 44679, 44682, Namibia: Kwando River: Kongola Bridge, 17°47′26.7″ S, 23°20′40.0″ E, coll. H. van der Bank and B. Kramer, 8 August 2004.

Pollimyrus isidori (Valenciennes, 1846)

GenBank accession no. AF095302 (Sullivan et al. 2000).

Pollimyrus marianne Kramer et al., 2003

IPMB nos. 44647, 44648, 44649, 44651, 44652, 44653, 44654, 44655, 44656, 44657, 44658, 44659, 44660, 44661, 44662, 44663, 44664, 44665, 44666, Namibia: Upper Zambezi: Lisikili, 17°32′31.9″ S, 24°26′17.7″ E, coll. H. van der Bank and B. Kramer, 6 August 2004; IPMB 4467, 44668, as previous samples, but 7 August 2004.

IPMB 51440, Namibia: East Caprivi: Upper Zambezi: upstream from Kalimbeze Fishing Camp, 17°29′ S, 24°26′ E, coll. H. van der Bank and B. Kramer, August 1999; IPMB 51442, second generation fish born in captivity from parent fish from Namibia sampled at same location and date.

Pollimyrus petricolus (Daget, 1954)

GenBank accession no. AF201608 (Sullivan et al. 2000).

Appendix 2. Morphological and genetic differences between samples

Table 2.1. Morphological measures for samples of the *Pollimyrus castelnaui/marianne* species complex from various origins. For abbreviation of morphological characters, see Material and Methods.

	PDL/SL	PAL/SL	LD/SL	LA/SL	LPF/HL	PPF/SL	pD/SL	CPL/SL	CPD/CPL	LS(o)/HL	OD/HL	HL/SL	HL/Na	BD/SL	nD	nA	SPc	SLS	SL (cm)
Zambezi (Z1,Z2)																			
Mean/median	0.6310	0.5764	0.1738	0.2292	0.8990	0.1529	0.4136	0.2285	0.3124	0.4281	0.2023	0.2302	10.867	0.2607	17	23	16	55	6.2
Min	0.6111	0.5497	0.1486	0.1643	0.8053	0.1350	0.2802	0.1973	0.2573	0.3780	0.1677	0.2115	8.8	0.2199	14	21	13	50	3.4
Max	0.6778	0.6206	0.2009	0.2587	1.0137	0.1796	0.4983	0.2585	0.3923	0.4790	0.2246	0.2741	13.083	0.2893	19	26	18	59	7.0
SE/SIQ	0.0016	0.0018	0.0014	0.0018	0.0060	0.0012	0.0034	0.0015	0.0034	0.0028	0.0016	0.0013	0.1144	0.0018	0.5	1	0	1.5	0.09
N	64	64	64	64	64	64	64	64	64	64	64	64	64	64	63	64	62	64	64
Kwando: Kongola (K)																			
Mean/median	0.6516	0.6014	0.1636	0.216	0.8319	0.1696	0.395	0.2181	0.3474	0.4517	0.2635	0.2422	9.0188	0.2753	17	23	14	50	4.6
Min	0.6333	0.5849	0.14	0.18	0.7639	0.1507	0.3796	0.1898	0.3214	0.4059	0.2376	0.2255	8.3125	0.2467	15	21	12	44	3.0
Max	0.6852	0.6343	0.1818	0.2417	0.9257	0.1873	0.426	0.24	0.4146	0.5	0.3050	0.2633	9.9167	0.3051	18	24	16	55	5.7
SE/SIQ	0.0029	0.003	0.0022	0.0031	0.0139	0.002	0.0028	0.003	0.0061	0.0066	0.005	0.0024	0.1204	0.0035	0.5	0.5	0	1.5	0.153
N	19	19	19	19	15	19	19	19	19	19	19	19	19	19	19	19	18	19	19
Kwando: Nakatwa (N)																			
Mean/median	0.6466	0.5924	0.1540	0.2030	0.8976	0.1731	0.3942	0.2331	0.3533	0.4495	0.2590	0.2419	9.1774	0.2762	16	22	14	46	4.2
Min	0.6157	0.5280	0.1352	0.1717	0.8123	0.1516	0.3428	0.2049	0.3136	0.3858	0.2016	0.2120	7.5	0.2563	14	20	12	44	2.4
Max	0.6683	0.6254	0.1762	0.2280	1.0817	0.1898	0.4360	0.2560	0.4125	0.5613	0.3225	0.2792	10.875	0.3073	18	24	16	52	5.6
SE/SIQ	0.0032	0.0051	0.0025	0.0032	0.014	0.0024	0.0044	0.0027	0.0055	0.0113	0.0076	0.0039	0.196	0.0028	1	0.75	1	2.875	0.242
N	20	21	20	21	21	21	20	21	21	21	21	21	21	19	18	21	21	11	21
Kwando: Nkasa (&)																			
Mean/median	0.6575	0.6069	0.1621	0.2109	0.8470	0.1807	0.3927	0.229	0.3183	0.4328	0.2364	0.2498	11.0812	0.2972	15	21	14	48	5
Min	0.6411	0.5955	0.1515	0.2049	0.7612	0.1714	0.3428	0.2103	0.2985	0.3882	0.2055	0.2413	8.5455	0.2743	12	21	12	46	3.3
Max	0.6765	0.6250	0.1727	0.2185	0.8884	0.1861	0.4147	0.2394	0.3462	0.4681	0.2649	0.2576	13.3636	0.3245	17	23	16	50	6
SE/SIQ	0.0059	0.0047	0.0036	0.0025	0.0179	0.0022	0.0039	0.0043	0.0059	0.0114	0.0084	0.0032	0.7159	0.0081	0.375	0.75	0	0.5	0.45
N	6	6	6	6	7	6	6	6	7	7	7	6	7	6	7	7	7	6	6
Kwando: Linyanti (L)																			
Mean/median	0.6513	0.5984	0.1629	0.2134	0.8682	0.1767	0.4023	0.2306	0.3326	0.4501	0.2023	0.2402	8.8352	0.2967	17	23	16	50	5.5

(*Continued*)

Table 2.1. (Continued).

	PDL/ SL	PAL/ SL	LD/ SL	LA/ SL	LPF/ HL	PPF/ SL	pD/SL	CPL/ SL	CPD/ CPL	LS(o)/ HL	OD/ HL	HL/ SL	HL/Na	BD/ SL	nD	nA	SPc	SLS	SL (cm)
Min	0.6280	0.5755	0.1458	0.1849	0.7912	0.1555	0.3257	0.2063	0.2838	0.4085	0.1677	0.2228	7.7222	0.2578	15	20	12	46	3.5
Max	0.6731	0.6295	0.1792	0.2348	0.9643	0.2005	0.4341	0.2565	0.3937	0.4961	0.2379	0.2635	9.5385	0.3277	18	25	16	52	6.5
SE/SIQ	0.0019	0.0021	0.0017	0.0021	0.0095	0.0019	0.0039	0.0027	0.0058	0.0036	0.0033	0.0016	0.086	0.0029	0.5	0.5	1	1.5	0.126
N	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	28	29	29
Okavango (O1,O2)																			
Mean/median	0.6463	0.5954	0.1664	0.2143	0.8577	0.1474	0.3933	0.2123	0.2993	0.4159	0.2049	0.2407	9.0654	0.2504	16	22	12	49	4.4
Min	0.5995	0.5698	0.1391	0.1864	0.7849	0.1296	0.3402	0.1914	0.2596	0.3761	0.1817	0.2213	8.0	0.2171	14	20	12	46	3.4
Max	0.6864	0.6272	0.1871	0.2366	0.9242	0.1629	0.4143	0.238	0.3521	0.4579	0.23	0.2604	10.4444	0.2895	18	24	14	55	5.4
SE/SIQ	0.0026	0.0021	0.0019	0.0019	0.0067	0.0015	0.0028	0.002	0.0035	0.003	0.0022	0.0015	0.1007	0.0027	0.5	0.875	0.375	1.5	0.092
N	35	35	35	35	35	34	35	35	35	35	35	35	35	35	35	35	35	35	35
P. castelnaui syntypes (BMNH 910.5.31.11-12(2), "Lake Ngami"																			
Mean/median	0.6474	0.5974	0.1874	0.2373	0.9093	0.1474	0.3903	0.2101	0.3286	0.4187	0.2353	0.2248	10.7692	0.3033	18	23	12	52.5	6.2
Type 1	0.6473	0.5907	0.1947	0.2463	0.8939	0.1296	0.3877	0.1997	0.3333	0.4361	0.2299	0.2213	10.2308	0.2928	18	23	12	52	6
Type 2	0.6475	0.6040	0.1801	0.2283	0.9248	0.1629	0.3929	0.2205	0.3239	0.4014	0.2406	0.2283	11.3077	0.3137	18	23	12	53	6.4
I_2 range	0.0002	0.0067	0.0073	0.0090	0.0155	0.0167	0.0026	0.0104	0.0047	0.0174	0.0054	0.0035	0.5385	0.0104	0	0	0	0.5	0.215

Notes: SE, standard error. Median and SIQ (semi-interquartiles), for count measures (nD, nA, SPc, SLS). For locality symbols, refer to map of Figure 1.

Table 2.2. Morphological measures for samples of the *Pollimyrus castelnaui/marianne* species complex from various origins, sample collected in 2004 for additional EOD and DNA analyses. For abbreviation of morphological characters, see Material and methods.

	PDL/SL	nD	nA	SPc	SL (cm)
Zambezi (Z1, 2004)					
Mean/median	0.6416	17	24	16	3.4
Min	0.616	17	23	13	2.6
Max	0.6789	19	25	16	4.6
SE/SIQ	0.0033	0.5	0.5	0	0.089
N	23	23	23	23	23
Kwando: Kongola (K, 2004)					
Mean/median	0.6556	16.5	22	14	3.1
Min	0.6374	15	21	12	2.3
Max	0.6971	17	23	16	3.9
SE/SIQ	0.0039	0.5	0.5	0.25	0.101
N	20	20	20	20	20
Okavango (O1, 2004)*					
Mean/median	0.6446	17	23	12	3.1
Min	0.6262	16	21	12	2.6
Max	0.6651	18	24	14	3.8
SE/SIQ	0.0046	0	0.5	1	0.139
N	9	9	9	9	9

Notes: SE, standard error. Median and SIQ (semi-interquartiles), for count measures (nD, nA, SPc). For localities Z1, K, O1 refer to map of Figure 1.
*After EOD recording, four additional specimens were too damaged to take tissue samples, but were good for DNA.

Table 2.3. Principal component analysis on correlations for morphological characters of the *Pollimyrus castelnaui/marianne* species complex. For abbreviations of morphological characters, see Material and methods.

Eigenvalue	5.4323	2.4326	1.5939	1.2067	1.1324	0.8608	0.7524
Percent	31.954	14.31	9.376	7.098	6.661	5.064	4.426
Cum %	31.954	46.264	55.64	62.738	69.4	74.463	78.889
Component loadings							
PDL/SL	−0.7401	0.0695	0.239	0.205	−0.2048	−0.0802	0.159
PAL/SL	−0.8328	0.0845	0.2058	0.071	−0.0814	−0.0361	0.0797
LD/SL	0.6561	−0.0058	0.3642	0.2598	0.1459	0.0144	−0.3464
LA/SL	0.7108	0.0639	0.3863	0.1587	−0.1981	0.0909	0.0092
LPF/HL	0.5304	0.248	0.0095	−0.0916	−0.4416	0.0445	0.0507
PPF/SL	−0.537	0.6505	−0.0172	0.2935	−0.0568	−0.1298	−0.0338
pD/SL	0.6038	0.2217	−0.2482	0.2319	0.3631	0.1691	−0.2982
CPL/SL	0.1693	0.4756	−0.7559	0.1269	0.118	−0.1877	0.0798
CPD/CPL	−0.3864	0.4514	0.4795	−0.0915	0.1863	0.5012	−0.0687
LSo/HL	−0.2065	0.5602	−0.2375	−0.4011	−0.4207	0.0559	−0.2725
OD/HL	−0.4888	0.2267	−0.0656	−0.5734	0.176	0.2088	−0.0293
HL/SL	−0.7224	−0.0945	−0.0074	0.0942	0.5078	−0.0326	0.1967
BD/SL	−0.3604	0.6788	0.2062	0.4038	−0.0915	−0.1777	−0.0761
nD	0.4029	0.3549	0.299	−0.3322	0.3169	−0.4628	−0.1522
nA	0.4849	0.3218	0.3764	−0.3362	0.1298	−0.3201	0.2876
SPc	0.4592	0.6147	−0.1183	0.0666	0.201	0.3194	0.3206
SLS	0.7756	0.1025	0.0294	0.0692	−0.1129	0.113	0.4107

Notes: $N = 155$ specimens from Okavango ($N = 34$), Upper Zambezi ($N = 61$), and Kwando: Kongola Bridge ($N = 15$), Nakatwa ($N = 11$), Nkasa ($N = 6$), Linyanti/Sampis ($N = 28$).

Table 2.4. ISSR profiles of *P. castelnaui*, *P. marianne* and *P. cuandoensis* sp. nov. Shown are the informative bands nos 1 to 13; X = band is present. X symbols of bold and underlined style (in colour online) are highlighting bands characteristic of species.

Taxon	IPMB ID	1	2	3	4, 10, 11, 12, 13	5	6	7	8	9
castelnaui										
	16555		X	X	X		X			X
	16558		X	X	X		X			X
	16561		X	X	X		X			X
	44698	X	X		X					X
	45377	X	X		X		X			X
	45378	X		X	X	X	X			X
marianne										
	44647		X		X	X			X	
	44648		X	X	X		X		X	
	44651	X		X	X	X			X	
	44652	X			X	X				X
	44654		X		X		X			X
	44655	X			X	X				X
	44656	X		X	X	X	X		X	
	44657		X	X	X	X	X			X
	44658	X	X		X	X			X	
	44659	X	X	X	X					X
	44660		X	X	X	X	X		X	
	44661		X	X	X	X	X			X
	44662		X	X	X	X			X	
	44663	X		X	X	X	X		X	
	44664	X			X					X
	44665	X		X	X	X				X
	44666		X	X	X	X				X
	44667		X		X	X			X	
	44668		X	X	X	X				X
cuandoensis sp. nov.										
	44669		X		X	X				X
	44670		X		X	X	X	**X**	X	
	44671	X		X	X		X	**X**	X	
	44672		X	X	X		X	**X**	X	
	44673	X		X	X		X	**X**		X
	44674			X	X		X	**X**		
	44675			X	X			**X**	X	
	44676	X	X	X	X	X			X	
	44677	X	X	X	X	X	X		X	
	44679	X	X	X	X	X	X	**X**	X	
	44682		X	X	X		X	**X**	X	

Appendix 3. Electrical characters for different populations of *P.cuandoensis* sp. nov. from the Kwando River

Table 3.1. EOD waveform characters for local populations of *Pollimyrus cuandoensis* sp. nov. in the Kwando River. For abbreviations, see Material and methods.

Sample origin	P0 (V)	N0 (V)[1]	P1 (V)	P2 (V)	P0dur (ms)	N0dur (ms)	P1dur (ms)	N1dur (ms)	P2dur (ms)	EODdur (ms)	P0N0sep (ms)	N0P1sep (ms)	P1N1sep (ms)	P0area ($V \times ms$)	N0area ($V \times ms$)	P1area ($V \times ms$)	N1area ($V \times ms$)	P2area ($V \times ms$)	SL (cm)
Kongola (K)																			
Mean	0.1273	−1.404	0.2735	0.0957	0.0656	0.066	0.0608	0.0747	0.2374	0.5045	0.0645	0.0782	0.0443	0.062	0.0061	0.0099	0.0395	0.0178	4.37
SE	0.0076	0.0064	0.0047	0.0027	0.0027	0.0018	0.0013	0.0009	0.0077	0.0083	0.0011	0.0016	0.005	0.005	0.0004	0.0003	0.0005	0.0009	0.127
Min	0.0209	−0.0212	0.1495	0.0522	0.0201	0.0223	0.0366	0.0594	0.0859	0.3435	0.0421	0.0576	0.0359	0.0003	0.0003	0.0045	0.0295	0.0037	2.45
Max	0.2827	−0.2756	0.3385	0.1614	0.1127	0.1086	0.0836	0.0939	0.3582	0.6357	0.0926	0.1107	0.054	0.0181	0.015	0.0139	0.0518	0.034	6.1
N	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63	63
Nakatwa (N)																			
Mean	0.1321	−0.1116	0.2683	0.086	0.0568	0.0496	0.0482	0.063	0.1914	0.4090	0.0481	0.063	0.0362	0.0055	0.0037	0.0073	0.0333	0.0131	4.37
SE	0.0122	0.0088	0.009	0.0024	0.0035	0.0019	0.0014	0.0014	0.0085	0.117	0.0018	0.0012	0.0005	0.0007	0.0004	0.0004	0.0007	0.0007	0.232
Min	0.0506	−0.0519	0.1707	0.0571	0.0208	0.0363	0.0353	0.0527	0.1067	0.2976	0.0315	0.056	0.0324	0.0008	0.0012	0.0035	0.0273	0.0048	2.42
Max	0.2934	−0.2052	0.3407	0.1097	0.0874	0.0703	0.0643	0.0816	0.2648	0.5156	0.064	0.0823	0.0412	0.0149	0.008	0.0098	0.0393	0.019	5.58
N	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
Nkasa (&)																			
Mean	0.1015	−0.113	0.2527	0.0992	0.0568	0.0651	0.06	0.0769	0.2394	0.4982	0.0654	0.0736	0.0465	0.0046	0.0047	0.0089	0.0416	0.0184	4.96
SE	0.0218	0.0111	0.013	0.0086	0.0092	0.0048	0.005	0.0027	0.0294	0.0349	0.0042	0.0039	0.0027	0.0014	0.0008	0.0009	0.0015	0.0034	0.39
Min	0.0267	−0.0647	0.2133	0.0711	0.0224	0.0519	0.0466	0.0691	0.1309	0.3989	0.0511	0.0596	0.0406	0.0004	0.0022	0.0059	0.0369	0.0075	3.45
Max	0.2008	−0.1445	0.3001	0.1424	0.0841	0.0897	0.0797	0.0865	0.3781	0.6571	0.0827	0.09	0.0585	0.0104	0.0081	0.0131	0.0466	0.0368	6
N	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Linyanti (L)																			
Mean	0.1019	−0.1023	0.2846	0.1028	0.0479	0.0518	0.0543	0.0757	0.2507	0.4804	0.0558	0.0652	0.0417	0.0045	0.0036	0.009	0.0407	0.0193	5.72
SE	0.0164	0.0097	0.0123	0.0033	0.0049	0.0021	0.0024	0.0013	0.0076	0.0083	0.0019	0.002	0.005	0.0011	0.0004	0.0006	0.0005	0.0009	0.109
Min	0.0147	−0.0089	0.1072	0.0523	0.0173	0.022	0.0257	0.0658	0.1382	0.4097	0.035	0.0405	0.0349	0.0002	0.0001	0.0018	0.036	0.0058	3.53
Max	0.3733	−0.225	0.4107	0.1457	0.1184	0.076	0.0908	0.0981	0.3522	0.6338	0.0736	0.0833	0.05	0.0223	0.0099	0.0146	0.0474	0.0311	6.71
N	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33	33

[1]Negative values for N0: maximum and minimum reversed (that is, taken absolute). For locality symbols in parentheses, refer to map of Figure 1.

Table 3.2. Principal component analysis on correlations for 17 EOD characters of specimens of *Pollimyrus cuandoensis* sp. nov. from various origins of the Kwando/Linyanti system. For abbreviations, see Material and methods.

Eigenvalue	6.2355	4.8092	2.2914	1.4928	0.7926	0.4408	0.3903
Percent	36.68	28.29	13.479	8.781	4.662	2.593	2.296
Cum Percent	36.68	64.969	78.448	87.229	91.892	94.485	96.781
Component loadings							
P0 (V)	−0.714	0.6126	0.1742	0.116	0.2115	−0.0815	0.0325
N0 (V)	0.0681	−0.8348	0.3909	0.2037	−0.2166	0.1045	−0.1358
P1 (V)	0.6023	−0.4413	0.0008	−0.1172	0.5322	0.3364	0.1626
P2 (V)	0.8293	0.0634	−0.4025	0.2016	−0.1325	−0.0526	0.074
P0dur (ms)	−0.6256	0.5879	0.3698	−0.0142	0.0953	−0.0247	0.1228
N0dur (ms)	0.4455	0.7028	−0.2175	−0.4378	−0.069	0.0843	−0.1182
P1dur (ms)	0.7253	−0.2072	0.5629	−0.225	−0.027	−0.2089	−0.0166
N1dur (ms)	0.2181	0.6483	0.5036	0.448	0.0538	0.1232	−0.1536
P2dur (ms)	0.6771	0.4022	−0.3887	0.389	0.0738	−0.0519	−0.0597
P0N0sep (ms)	0.1621	0.6993	0.1419	−0.3913	−0.3639	0.3789	−0.0857
N0P1sep (ms)	0.7236	0.2032	0.3582	−0.4045	0.1029	−0.2808	−0.2016
P1N1sep (ms)	0.5707	0.3267	0.4606	0.1408	−0.3453	−0.0398	0.4442
P0area (V × ms)	−0.6841	0.6286	0.2901	0.1048	0.1482	−0.0167	0.0088
N0area (V × ms)	0.176	0.8298	−0.3626	−0.3016	0.144	−0.0663	0.1129
P1area (V × ms)	0.8076	−0.2415	0.4032	−0.2162	0.2421	0.0365	0.083
N1area (V × ms)	0.6485	0.4429	0.3483	0.4553	0.1165	0.0792	−0.1322
P2area (V × ms)	0.7755	0.314	−0.4252	0.3103	−0.013	−0.0433	−0.0003

Notes: $N = 127$ specimens: Kongola Bridge ($N = 63$), Nakatwa ($N = 24$), Nkasa ($N = 7$), Linyanti/Sampis ($N = 33$).