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## Description of a new octoploid frog species (Anura: Pipidae: *Xenopus*) from the Democratic Republic of the Congo, with a discussion of the biogeography of African clawed frogs in the Albertine Rift

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### Abstract

We describe a new octoploid species of African clawed frog (*Xenopus*) from the Lendu Plateau in the northern Albertine Rift of eastern Democratic Republic of the Congo. This species is the sister taxon of *Xenopus vestitus* (another octoploid), but is distinguished by a unique morphology, vocalization and molecular divergence in mitochondrial and autosomal DNA. Using a comprehensive genetic sample, we provide new information on the species ranges and intra-specific diversity of African clawed frogs from the Albertine Rift, including the details of a small range extension for the critically endangered *Xenopus itombwensis* and previously uncharacterized variation in *Xenopus laevis*. We also detail a new method for generating cytogenetic preparations in the field that can be stored at room temperature for up to 3 weeks. While extending our understanding of the extant diversity in the Albertine Rift, this new species highlights components of species diversity in ancestral African clawed frogs that are not represented by known extant descendants.

### Keywords

allopolyploid evolution; Albertine Rift; whole genome duplication; advertisement calls; DNA barcode

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### Supporting Information

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

**Table S1.** Specimen, locality, and sequence accession information for Albertine Rift samples. There is no voucher for some samples and GPS coordinates (in decimal degree format) of some localities are approximate. Information for other samples analyzed in this study is available in Evans *et al.* (2004).

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## Introduction

African clawed frogs in the genus *Xenopus* have a complex evolutionary history characterized by both bifurcating and reticulating modes of speciation (reviewed in Evans, 2008). Described species include eight tetraploids, six octoploids and two dodecaploids; additional undescribed species have been inferred using molecular markers (Evans *et al.*, 2004; Evans *et al.*, 2005; Evans, 2007). Some polyploid species, such as the octoploids *Xenopus itombwensis* and *Xenopus wittei*, are sister taxa that probably evolved by ‘regular’ bifurcating speciation from an octoploid ancestor. Other species pairs, such as the octoploids *Xenopus andrei* and *Xenopus vestitus*, share recent common ancestry of half of their genomes while the other half is more divergent. This observation is best explained by allopolyploid speciation – speciation by genome duplication that is associated with hybridization among species. This mode of speciation is further suggested by the creation of allopolyploid individuals by hybridizing and backcrossing different species in the laboratory (Kobel & Du Pasquier, 1986; Kobel, 1996).

Phylogenetic relationships among extant African clawed frogs (*Xenopus* and *Silurana*) suggest the previous existence of up to three ancestral diploids and three ancestral tetraploid species that are not represented by a known extant species with the same ploidy level (Evans, 2007, 2008). Even though these species may now be extinct, the genomes of these ancestors persist as a portion of the genomes of extant allopolyploid species. The prediction that these ‘lost’ ancestral species once existed provides motivation for field studies aimed at discovering their equivalent-ploidy-level descendants, if they exist.

In an ongoing effort to understand the evolution and diversity of clawed frogs, we performed field collections and molecular analyses in regions where allopolyploid descendants of the predicted tetraploid ancestors currently occur, including the Albertine Rift of Central Africa. This effort recently resulted in the discovery of the octoploid species *X. itombwensis* (Evans *et al.*, 2008) and subsequent searches identified another new species that is described here. The inferred evolutionary relationships between this new species, which is octoploid, and other extant species further support the notion that tetraploid ‘lost’ ancestors once existed.

## Methods

### Field collection

The new species was first collected by E. G. and C. K. in July 2009 on the Lendu Plateau, northern Albertine Rift, Democratic Republic of the Congo (DRC), and molecular analyses prompted a subsequent expedition by B. J. E. and C.K. to this locality in December 2009, in order to further characterize this species in terms of its morphology, distribution and vocalization, and to provide additional voucher specimens. Other samples in this study were collected by E. G., C. K., B. J. E. and colleagues, or are reported previously (Evans *et al.*, 2004).

**Analysis of male vocalization and morphology**—Male clawed frogs produce species-specific advertisement vocalizations (calls) by opening a pair of cartilaginous disks in the larynx to produce a ‘click’ (Yager, 1992). Calls of some species are composed of only one click, whereas others produce trains of clicks called a trill. In this study, we recorded advertisement calls from four males from the new species and compared these with a previous analysis of calls from *X. itombwensis*, *X. vestitus* and *X. wittei* (Evans *et al.*, 2008) with data from two additional calls from *X. wittei*. Based on analyses discussed below, these species are closely related to the new species. We report vocal parameters from only two individuals from the new species because the intervals between clicks of the other two were not sufficiently distinguishable for individual clicks to be measured.

We measured call duration, the number of clicks per call, the inter-click interval (time between the start of one click and the start of the next click), the intensity modulation (the change in intensity between the softest and the loudest clicks calculated as  $[(\text{max}-\text{min})/\text{min}]$ , the call duty cycle (the proportion of vocalization in a bout of calls), and the two sound frequencies with the most energy (dominant frequencies;  $DF_{\text{low}}$  is the lower and  $DF_{\text{high}}$  is the higher of the two frequencies) and inter-call interval (time between successive calls in a bout of calling). Most of these parameters have a single value for each call. The inter-click interval is a mean value calculated from all inter-click intervals in a call. Call duty cycle and inter-call interval are measured from bouts of advertisement calls. Advertisement calls, or bouts of advertisement calls, were randomly selected and analyzed. Evans *et al.* (2008) ranked the dominant frequencies by amplitude and presented the mean across individuals of the highest and second-highest frequencies. Here, we instead rank the two frequencies with the highest amplitude by frequency and report the mean of these frequencies ( $DF_{\text{low}}$  and  $DF_{\text{high}}$ ). This is more appropriate because the two frequencies vary in relative amplitude from call to call. For almost all of these parameters, multiple calls from at least two individuals were analyzed. To communicate information about inter- and intra-individual variation in the vocal parameters, we report the grand mean and standard deviation across all replicates, and the sum of squares and residuals divided by their respective degrees of freedom, which is the number of individuals minus one for comparisons among individuals or the number of calls minus one for comparisons among calls.

Advertisement calls were evoked by priming males with human chorionic gonadotropin (Sigma, Oakville, ON, Canada; 100 international units). Vocalizations were recorded ~ 6 h after the injection, at the start of the night cycle. An injected male and a sexually unreceptive female were placed in a 75.7L aquarium, two-thirds filled with unchlorinated water at 20 °C. Recordings were made with a hydrophone (HighTech Inc.; Gulfport, MS, USA sensitivity =  $-164.5 \text{ dB re } 1 \text{ VuPa}^{-1}$ ), stored on CDs (Marantz, Mahawah, NJ, USA; model CDR300) and analyzed using SIGNAL software version 5.0 (Engineering Design, Berkeley, CA, USA).

To provide information on comparative morphology, we measured 12 external morphological characteristics following Tinsley (1973) from preserved specimens of the new species and of a sympatric population of *Xenopus laevis*. Additional information on specimens examined is available in the supporting information.

## Cytogenetics

We previously reported a new method to obtain *Xenopus* cytogenetic preparations by directly harvesting and fixing liver tissue (Evans *et al.*, 2008). Here, we adapted this method to allow the cells to be harvested and fixed in the field, and stored at room temperature for up to 3 weeks during transport back to the laboratory. Animals were killed by immersion in an ~1% MS222 (Sigma) solution. The liver was accessed through a lateral incision and an ~8 mm<sup>3</sup> piece was placed on a pre-cleaned glass slide, wetted with three to four drops of 0.04 M KCl and minced with a new razor blade. The resulting tissue/KCl slurry was then transferred to a 15 mL centrifuge tube and 0.04 M KCl solution was added to produce a final volume of 13.5 mL. The mixture was gently inverted/agitated and incubated at room temperature for 1 h with occasional agitation and inversion. During this time, a fresh 3:1 methanol:glacial acetic acid fixative was prepared. After a final agitation, the tissue/KCl mixture was left for 2 min to allow debris to settle to the bottom of the tube. Ten millilitres was drawn from the top and transferred to a new 50 mL tube. The remaining cell suspension and tissue debris were discarded. Two mL of the fixative were added to the 50 mL tube, followed by gentle agitation. An additional 10 mL of fixative was then added, followed by gentle agitation. The tube was then topped off with fixative to a total of 50 mL, capped and gently agitated. At this point, the samples were stored at room temperature for up to 3 weeks until they were transported back to the laboratory.

In the laboratory, 5–12 mL of the field-fixed samples were transferred to a 15 mL tube, topped up to 13 mL with a fresh 3:1 fixative and washed four times by centrifugation at 500 g for 8 min. For each wash, the supernatant was removed by aspiration down to 0.25 mL and resuspended in 13 mL of a fresh fixative by vortexing. In the final resuspension, 0.5–3 mL of fixative was then added to yield a suitable concentration for slide making. Cleaned slides were wetted with a drop of 3:1 fixative and left to almost dry before a drop of cell suspension was allowed to fall on the surface from a height of ~10 cm. After ~7 s, an additional drop of 3:1 fixative was allowed to fall onto the slide before it was moved to a humid environment and allowed to dry completely. Slides were incubated at 90 °C for 1 h, cooled to room temperature, stained in 8% (v/v) Giemsa in Gurr buffer (pH 6.8) for 2–5 min, rinsed three times in milli-Q H<sub>2</sub>O and dried. Using standard bright-field microscopy, metaphases were located on slides and then captured as digital images. The remaining cell suspensions were stored at –20 °C.

### Molecular data, phylogenetic analysis

We estimated the phylogenetic history of the new species using molecular data from mitochondrial DNA (mtDNA) and autosomal DNA (aDNA). The mtDNA data included 819–2924 base pairs (bp) from *12S* and *16S* ribosomal DNA, the intervening transfer RNA<sup>Val</sup> and cytochrome c oxidase subunit I (*COI*) genes from all known species of African clawed frog, including 2920 or 2921 bp from four samples of the new species. *Pipa pipa* was used as an outgroup to African clawed frogs (Roelants & Bossuyt, 2005). Each species in the mtDNA dataset had at least one full-length (> 2900 bp) sequence; shorter sequences were included to characterize species' ranges. The aDNA data included cloned paralogs from 317–3054 bp of paralogs of the *RAG1* from all known species of African clawed frogs, including 1141 bp from three paralogs from one individual of the new species. MtDNA and aDNA were amplified using previously published primers and aDNA was cloned using Invitrogen cloning kits (Burlington, ON, Canada) as described previously (Evans *et al.*, 2004, 2005; Ivanova, deWaard & Hebert, 2006; Evans, 2007). Data are deposited in Genbank (accession numbers are in supporting information Table S1 and Evans *et al.*, 2004, 2008; Evans, 2007).

Phylogenetic relationships among mtDNA sequences were estimated using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001) using a partitioned analysis with a stem-loop (doublet) model for the rDNA genes and a general time-reversible model with a proportion of invariant sites and a gamma-distributed rate heterogeneity parameter (GTR + I +  $\Gamma$ ) for the *COI* sequence, which was selected using the Akaike Information Criterion with MrModeltest (Nylander, 2004). The *RAG1* phylogeny was also estimated using MrBayes with partitioning by codon position and a GTR + I +  $\Gamma$  model for each position, with model selection using Bayes factors described in Evans (2007). Both analyses used Dirichlet priors for the rate matrix. Two independent MCMC runs were performed for 2.6 and 1.8 million generations for mtDNA and *RAG1*, respectively. A burnin of 500 000 and 100 000 generations (mtDNA and *RAG1* datasets, respectively) was discarded before the construction of a consensus topology based on plots of the posterior distribution of tree likelihoods and parameter estimates.

### Species concept

Essentially all species concepts share a common theme of referring to evolutionarily distinct lineages that persist through time, multiple lines of evidence can be used to identify species, and the chronology by which these lines of evidence become diagnostic varies (de Queiroz, 1998). Even at a genetic level, the onset of genetic barriers to reproduction can be a gradual process that occurs at different times in different parts of the genome (Wu, 2001). For these reasons, we adopt a general lineage concept of species, where a species is defined as a

separately evolving lineage of connected subpopulations (metapopulations) through time (de Queiroz, 1998, 2007). We provide information on morphological, behavioral and genetic divergence as evidence for lineage separation.

### Taxonomic account

*Xenopus lenduensis*, new species Lendu Plateau clawed frog

(Fig. 1)

### Holotype

MCZ A-139853 (field number BJE2957), adult male: Democratic Republic of the Congo, Orientale Province, Bunia District, Djugu Territory, Aboro Region, Aboro West Village, 2.0164°N 30.8311°E, 2081 m a.s.l. December 8, 2009, collected by B. J. E. and C. K.

### Paratypes

MCZ A-139855 (field number BJE2945), adult male: Democratic Republic of the Congo, Orientale Province, Bunia District, Djugu Territory, Jiba Region, Goki Lake, near Jiba, 1.8688°N, 30.6959°E, 1835 m a.s.l. December 11, 2009, collected by B. J. E. and C. K.

MCZ A-139856 (field number BJE2951), other information the same as MCZ A-139855 except collection site is at 1.8604°N, 30.6880°E, 1847 m a.s.l.

MCZ A-139854 (field number BJE2958), adult female: Democratic Republic of the Congo, Orientale Province, Bunia District, Djugu Territory, Aboro Region, Ngonseneyu Town 1.9851°N, 30.8118°E, 1979 m a.s.l. December 6, 2009, collected by B. J. E. and C. K.

UTEP20186 (field number EBG2343), adult male: Democratic Republic of the Congo, Orientale Province, Bunia District, Djugu Territory, Aboro Region, 2.0087°N, 30.8352°E, 2065 m a.s.l. July 6, 2009, collected by E. G and C. K.

UTEP20192 (field number EBG2350), adult female: other information the same as UTEP20186.

### Diagnosis

Evolutionary analyses discussed below indicate that *X. lenduensis* is a member of the *vestitus-wittei* subgroup of African clawed frogs (Kobel, Loumont & Tinsley, 1996), which includes *X. itombwensis* (Evans *et al.*, 2008), *X. wittei* (Tinsley, Kobel & Fischberg, 1979) and *X. vestitus* (Laurent, 1972; Tinsley, 1973). While this group is not a clade because it does not include all descendants of the groups' most recent common ancestor, it is nonetheless descriptive because it does include all descendants of one of the inferred tetraploid ancestors – ancestor 'B.' As detailed below, *Xenopus lenduensis* can be distinguished from other members of the *vestitus-wittei* subgroup and other clawed frogs by: (1) its range on the Lendu Plateau; the only other African clawed frog in this region is *X. laevis*; (2) a unique combination of morphological characters including the absence of a metatarsal claw, short toes, variable presence of dorsal and ventral patterning, a small- to medium-sized subocular tentacle and a rounded snout; (3) its male advertisement call, which is shorter than other members of the *vestitus-wittei* group and characterized by several unique spectral characteristics not found in other clawed frog species; (4) divergent mitochondrial and autosomal genes.

**Morphology and variation**—*Xenopus lenduensis* is a medium-sized African clawed frog with the snout–vent length (SVL) of females and males averaging 48 and 40 mm,

respectively (Table 1). This species has three claws, a prominent but small metatarsal tubercle with no claw, and a subocular tentacle that sometimes extends beyond the margin of the head when viewed from above. Snout shape is variable but generally more rounded than triangular, and dorsal coloration of *X. lenduensis* is light green to olive (Figs 1 and 2a–p). Darker olive spots are usually present on the dorsum, variable in size from ~0.5 to 3 mm, sometimes more densely distributed caudally, or instead present as larger dark olive and irregularly shaped patches. Some individuals have dark brown or black spots on the dorsum, dark olive triangular patches sometimes posterior to the eyes and/or a dark brown patch between the nostrils and the eyes (Fig. 2d–h). Some individuals have a light band connecting the eyes. One individual was observed with a dark olive hood over the dorsal surface of the head, similar to the commonly observed condition in *X. vestitus* (Tinsley, 1973). Some individuals of the new species have a light olive hood similar to that variably reported in *X. wittei* (Tinsley *et al.*, 1979). Nuptial pads of *X. lenduensis* (when present, males only) extend from the base of the arm to the tips of the fingers.

The ventral surface of the legs of *X. lenduensis* is orange or yellow, with this color sometimes extending anteriorly over the ventral surface of the body and ventral surfaces of the arms and head, or fading to a dark creamy color over the ventral surface of the body and head, or with a fairly abrupt transition from orange to cream in the pelvic region (Fig. 2o). The legs and ventral surface sometimes have a few light spots (Fig. 2i) but more commonly with lesser pigmented patches or spots, which are sometimes pink or purple, and sometimes less or more dense on the medial portion of the legs and belly (Fig. 2j–p). Like *X. vestitus* (Tinsley, 1973), the digits are relatively short (Table 1).

**Comparison of morphology with other species**—*Xenopus lenduensis* can be distinguished from species in the genus *Silurana*, and from *Xenopus amieti*, *X. andrei*, *Xenopus boumbaensis*, *Xenopus clivii*, *Xenopus fraseri*, *Xenopus pygmaeus* and *Xenopus ruwenzoriensis* by the absence of a claw on the metatarsal tubercle in *X. lenduensis*. *Xenopus lenduensis* can be distinguished from *Xenopus gilli* and *Xenopus largeni* by the presence of a subocular tentacle in *X. lenduensis*, and by unique dorsal longitudinal blotches present in *X. gilli* but absent in other species of African clawed frog, including *X. lenduensis*. *Xenopus lenduensis* can be distinguished from *X. laevis*, *Xenopus borealis*, *Xenopus muelleri* and *X. new tetraploid 1* (Evans *et al.*, 2004) by the size of the eyes (Tinsley *et al.*, 1979), which are much smaller in the new species. *Xenopus lenduensis* can be distinguished from *Xenopus longipes* by the comparatively short digits of *X. lenduensis* – for example, the fifth toe is shorter than the tibia length in *X. lenduensis* (Table 1), but longer than the tibia in *X. longipes* (Kobel *et al.*, 1996).

Distinguishing *X. lenduensis* from other species in the *vestitus-wittei* subgroup based on morphology is best achieved with a combination of other data on provenance, vocalization and/or molecular variation. The size of *X. lenduensis* is similar to *X. wittei* and *X. vestitus*, which have average adult female SVL typically 46 and 47 mm, respectively, and a male SVL of 37 and 38 mm, respectively (Tinsley *et al.*, 1979; Kobel *et al.*, 1996). *Xenopus itombwensis* is slightly smaller than these species, with female SVL averaging 35 mm and male SVL around 30 mm, although this may be an underestimate of the typical size of adults (Evans *et al.*, 2008). Dorsal coloration of *X. vestitus* is a marbled pattern of light silver-golden to bronze chromatophores over a brown background, sometimes with a dark transverse collar at the base of the head, a light post-orbital area or a light cephalic hood (Tinsley, 1973; Tinsley *et al.*, 1979; Kobel *et al.*, 1996), a pattern that is different from that generally observed in *X. lenduensis*. The dorsal surface of *X. lenduensis* sometimes has dark brown spots, such as in the holotype (Fig. 1a), whereas the dorsal surface of *X. vestitus* lacks dark spots because flecks of darker pigments are finely intermingled with flecks of lighter pigments, imparting a bronze to silvery bronze luster (Tinsley, 1973; Channing & Howell,

2006). Dorsal coloration of *X. wittei* has been reported to be a uniform gray–green, yellow–green or olive without prominent markings or spots (Tinsley *et al.*, 1979; Channing & Howell, 2006), which would be readily distinguished from the usually mottled dorsal pattern of *X. lenduensis*. However, we collected and sequenced *X. wittei* individuals from Kahuzi-Biega National Park (eastern DRC) that had some dorsal markings that are similar to *X. lenduensis*. For this reason, it is difficult to distinguish Kahuzi-Biega populations of *X. wittei* from *X. lenduensis* based on dorsal coloration alone. *Xenopus lenduensis* does not have a dark dorsal band situated caudally with respect to the eyes that is variably observed in *X. itombwensis* (Evans *et al.*, 2008). *Xenopus lenduensis* occasionally has ventral patterning of very dense spots (Fig. 2l) that is variably observed in *X. vestitus* (Tinsley, 1973) and *X. wittei* (Tinsley *et al.*, 1979), although the ventrum of *X. lenduensis* usually has sparse spots, such as on the holotype (Fig. 1c). A key difference between *X. vestitus* and *X. wittei* is the distance between the eyes (Tinsley *et al.*, 1979); the eyes of *X. vestitus* are relatively large and usually more than half of the inter-ocular distance (Tinsley, 1975), whereas the eyes of *X. wittei* are comparatively smaller and the inter-ocular distance is about 2.2 times the eye diameter (Tinsley *et al.*, 1979). Surprisingly, the spacing and size of the eyes of *X. lenduensis* are more similar to *X. wittei* than to its sister taxon *X. vestitus*; the inter-ocular distance averages 2.5 (females) or 2.6 (males) times the eye diameter (Table 1). Another (related) difference is the shape of the snout, which tends to be more pointed in *X. vestitus* than in *X. itombwensis* and *X. wittei* (Tinsley *et al.*, 1979; Evans *et al.*, 2008). The snout of the new species tends to be rounded (Fig. 2).

*Xenopus lenduensis* is readily distinguished by morphology from *X. laevis*, a species with which it is often found (in the same ponds). *Xenopus laevis* is significantly larger in size than *X. lenduensis* ( $P < 0.0001$  for comparisons with each sex, Student's *t*-test, Table 1) and has significantly larger eyes, even after dividing by body size ( $P < 0.001$ ,  $P < 0.0001$  for comparisons between females and males, respectively, Student's *t*-test). *Xenopus laevis* is also slimmer in the hand than *X. lenduensis*, possibly due to more viscous and plentiful skin secretions and/or its larger size.

**Size dimorphism**—Females are significantly larger than males ( $P < 0.0001$ , Student's *t*-test, Table 1), which is consistent with other species of African clawed frog (Kobel *et al.*, 1996).

**Vocalization**—The male advertisement call of *X. lenduensis* is a brief, rapid, trill with short intervals between calls (Table 2, Fig. 3). The interval between clicks is so short (~12 milliseconds) that individual clicks are often difficult to distinguish. The inter-call interval is much shorter in *X. lenduensis* than *X. vestitus* and *X. wittei*. Another major difference between the vocalization of the new species and its closest relatives is the much shorter duration of the *X. lenduensis* call. The dominant frequencies of *X. lenduensis* are very similar to *X. vestitus*, but not *X. itombwensis* or *X. wittei* (Table 2). The call of *X. lenduensis* lacks two distinct phases that characterize the call of *X. itombwensis* and some other species such as *X. laevis* (Kobel *et al.*, 1996). A recording of the call of *X. lenduensis* has been submitted to Amphibiaweb (<http://www.amphibiaweb.org>).

### Description of the holotype and color of the holotype in life

Holotype an adult male, small subocular tentacle present, claws present on toes I–III, metatarsal tubercle prominent but without a claw, webbing complete. Nuptial pads visible on the ventral surface of arms extending from the base of arms over the humeral area and forearm. Dorsum olive, with a dark brown spot on the dorsal surface, ~2 mm in diameter and positioned caudally with respect to the right eye and anterior to the arms (Fig. 1a). Smaller lighter brown spots variably present, becoming denser caudally and on the dorsal

surface of the toes, less dense on the legs. Ventral surface bright orange with dense purplish grey stipples under the head and belly, with a clear orange band in the center and with medium-sized purple spots on the flanking region, especially in a ring around the insertion point of each arm. Inguinal region and ventral surface of legs and feet orange with larger purple spots on the margins of the dorsal surfaces of the legs and feet. Morphological measurements of the holotype are listed in Table 1.

### Color of the holotype in preservative

Dorsum dark olive with brown spots over the back and the dorsal surface of the hind legs, transitioning laterally on flanks to a cream-colored venter; dorsal surface of head dark olive; dorsal surface of limbs olive with dark olive spots with a high density of spots on toes; ventral side of the head speckled with small dark olive spots; venter cream over a central band with fairly dense brown spots on lateral sides of venter; ventral surface of the hind feet cream with some brown spots near the margin with the dorsal surface on upper legs.

### Ploidy

*Xenopus lenduensis* is octoploid and has 72 chromosomes (Fig. 4). The ploidy level revealed by cytogenetic analysis was consistent with ploidy inferences based on phylogenetic analysis of divergent paralogs of the recombination activating gene 1 (*RAG1*).

### *Xenopus lenduensis* barcode

The Genbank accession number of the holotype barcode based on the sequence from the *COI* gene (Hebert *et al.*, 2003) is HQ225707.

### Ecology and distribution

*Xenopus lenduensis* was collected from multiple locations on the Lendu Plateau, northern Albertine Rift, DRC (supporting information Table S1; Fig. 5). *Xenopus lenduensis* was present in small populations of <40 individuals in small bodies of standing water that were occasionally encountered in disturbed agricultural areas. When the habitat was highly disturbed or cleared of vegetation, for example in areas used for water supply by people, this species was not observed, although *X. laevis* was present. *Xenopus lenduensis* therefore seemed to be detrimentally impacted by habitat modification. The Lendu Plateau was probably once a savannah forest mosaic, with forests occurring in patches above 1500 m (Fishpool & Collar, 2006). In the areas where we collected the new species, the forest has been completely destroyed; the plateau is now essentially entirely grassland with a few isolated trees, many of which are introduced. We did not see any primary or secondary forest during our surveys; dense weedy vegetation was sometimes observed in small, highly disturbed valleys.

### Evolutionary relationships with other African clawed frogs

Independent analyses of unlinked genomic regions suggest with strong support that *X. lenduensis* and *X. vestitus* are sister species derived from the same allo-octoploid ancestor. mtDNA of these species places them together in a strongly supported clade (Fig. 5). Using the full mtDNA dataset, the uncorrected *p*-distance between *X. lenduensis* and *X. vestitus* is 0.04048. The uncorrected *p*-distance between these species including only the *COI* 'barcode' region is 0.09386. The uncorrected *p*-distance between these species including only the *I6S* fragment proposed as a barcode fragment for amphibians minus 78 bp on the 3' end (Vences *et al.*, 2005) is 0.03766. This mtDNA relationship supports the previous existence of a maternal (i.e. mtDNA-contributing) tetraploid ancestor of this pair of octoploid species. In Figs 5 and 6, this tetraploid ancestor is indicated by lineages labeled 'B.'



We cloned alleles from three of the four expected *RAG1* paralogs from *X. lenduensis*, and each has a close relationship with an orthologous paralog of *X. vestitus* (Fig. 6). Data were not obtained from *RAG1* paralog  $\beta 2$  from *X. lenduensis*. This is consistent with an absence of data from closely related paralogs of *X. amieti*, *X. andrei*, *X. longipes*, *X. ruwenzoriensis* and *X. cf. boumbaensis* (Evans, 2007), and could be a result of deletion in an ancestor. Data from *RAG1* paralog  $\beta 3$  of *X. cf. boumbaensis* were not obtained, which could be due to deletion or poor amplification (Evans, 2007). Relationships based on aDNA are consistent with previous inferences that at least three tetraploid species contributed their genomes to extant allo-octoploid species such as *X. lenduensis*, but are not represented by a known extant representative with the same ploidy level (Evans, 2007, 2008). In Fig. 6, these inferred tetraploid ancestors are labeled 'A', 'B' and 'C.' By focusing on nodes with at least 95% posterior probabilities in the mtDNA and aDNA trees to infer ancient instances of allopolyploidization, at least three independent allooctoploidization events are suggested. These events include the following pairs of tetraploid ancestors (with the maternal tetraploid ancestor listed first): B+A gave rise to an ancestor of *X. lenduensis* and *X. vestitus*, C+A gave rise to an ancestor of *X. amieti*, *X. andrei*, *X. boumbaensis*, *X. longipes*, *X. ruwenzoriensis* and *X. cf. boumbaensis* and C + B gave rise to an ancestor of *X. itombwensis* and *X. wittei* (Fig. 6). This inference is consistent with those of Evans (2007), which did not include data from *X. lenduensis*.

Interestingly, *X. lenduensis* *RAG1* paralog  $\beta 1$  has three frameshift mutations, whereas *RAG1* paralog  $\alpha 1$  and *RAG1* paralog  $\alpha 2$  appear to be functional at the coding level. This provides additional support for the finding of Evans (2007) that pseudogenization of *RAG1* paralogs occurred in a phylogenetically biased fashion, such that *RAG1* paralogs inherited from one diploid ancestor (the ' $\beta$ ' ancestor) became pseudogenes more frequently than paralogs inherited from the other diploid ancestor (the ' $\alpha$ ' ancestor).

## Etymology

The new species is named after the plateau where it occurs – the Lendu Plateau of the Albertine Rift in Orientale Province, DRC, which lies just west of Lake Albert. This region is also known as the Blue Mountains.

## African clawed frog mtDNA phylogeography in the Albertine Rift

With the exception of *X. laevis*, no species we sampled had substantial levels of intra-specific mtDNA divergence in the Albertine Rift. Our analysis slightly extends the known range of the critically endangered *X. itombwensis* to proximate localities on the Itombwe Plateau. This species was formerly known only from its type locality, whose GPS coordinates were incorrectly reported in the species description, but are correctly reported here (supporting information Table S1; Evans *et al.*, 2008). Because the range extension here is so small, this does not warrant changing the conservation status of this species. The Itombwe Plateau has the most threatened species of amphibians and the highest number of endemic amphibians in the Albertine Rift; this is also the case for birds (Plumtre *et al.*, 2007).

Our analysis indicates that *X. ruwenzoriensis* also occurs in the DRC (Fig. 5; Greenbaum & Kusamba, 2010); this species was formerly only known from the type locality near Bundibugyo in the Semliki Valley, western Uganda (Fischberg & Kobel, 1978), and from the Budongo Forest, east of Lake Albert, also in western Uganda (IUCN, 2010).

We identified three diverged mtDNA lineages in *X. laevis* in the Albertine Rift (Fig. 5). One is closely related to mtDNA lineages sampled from *X. laevis* populations in East Africa (Uganda, Rwanda, Tanzania) and another was closely related to mtDNA lineages sampled in

*X. laevis* populations in portions of west and south-central Africa (Cameroon, Nigeria, Zambia, Botswana). The third *X. laevis* mtDNA lineage from the Albertine Rift is only known from the Lendu Plateau, and to our knowledge, has not been reported previously.

Other inferences of species ranges are generally consistent with previous species distributions that were based on morphology (Tinsley, Loumont & Kobel, 1996). *Xenopus muelleri* occurs over much of east and south-east Africa, extending up Lake Tanganika to the xeric area south of Lake Kivu (near Bukavu). The distribution of *X. pygmaeus* over much of Central Africa reaches its easternmost limit at the region west of lakes Kivu (data not shown) and Albert. The distribution of *X. wittei* was confirmed to include portions of southwestern Uganda, western Rwanda and the Kahuzi-Biega and Lubero Highlands in DRC (Tinsley *et al.*, 1979; Kobel *et al.*, 1996). *Xenopus wittei* mtDNA sequences from the Kahuzi-Biega and Lubero highlands are slightly diverged from those of the type locality in south-western Uganda (Fig. 5). We do not have additional samples of *X. vestitus* beyond the one used in Evans *et al.* (2004); other studies report a broader distribution for this species, but not north of Lake Edward (Tinsley, 1973; Tinsley, 1975).

## Discussion

### Distinctiveness of *X. lenduensis*

We distinguish a new species of African clawed frog, *Xenopus lenduensis*, from other species based on morphology, vocalization and molecular divergence – characteristics that together identify *X. lenduensis* under the general lineage concept of a species. The most straightforward way to narrow down the identity of the new species is its provenance on the Lendu Plateau, DRC, which is the northern extent of the Albertine Rift. The only other African clawed frog that shares this habitat with *X. lenduensis* is *X. laevis*. *Xenopus lenduensis* is easily distinguished from *X. laevis* by the much larger size and the larger eyes of *X. laevis*. Within the *vestitus-wittei* subgroup, the new species is similar in size to its sister taxon *X. vestitus* and to *X. wittei*, but larger than *X. itombwensis*. Dorsal and ventral coloration and morphology distinguish *X. lenduensis* from its sister taxon *X. vestitus*, the former species having an olive dorsum with variable darker olive spots, a usually rounder snout and more widely spaced eyes than *X. vestitus*. Distinguishing *X. lenduensis* from some populations of *X. wittei* that have variable spots on the dorsum could be challenging, although other *X. wittei* populations lack dorsal spots. The fundamental characteristics of the vocalization of the new species, including call duration and inter-call interval, are distinct from other species of African clawed frog, including *X. vestitus* and other members of the ‘*vestitus-wittei*’ subgroup (Table 2; Fig. 2). On a molecular level, mtDNA and nuclear DNA divergence between *X. lenduensis* and *X. vestitus* is substantial and comparable to that seen between other closely related species of African clawed frog such as *X. fraseri* and *X. pygmaeus* or *X. amieti* and *X. longipes* (Figs 5 and 6).

### Allopolyploid speciation and the search for descendants of ‘lost ancestors’

Building on previous molecular phylogenetic studies of African clawed frogs (Carr, Brothers & Wilson, 1987; Kobel, Barandun & Thiebaud, 1998; Measey & Channing, 2003; Evans *et al.*, 2004; Evans *et al.*, 2005; Evans, 2007), the evolutionary relationships between *X. lenduensis* and other species of clawed frog are evaluated here using mtDNA and sequences from cloned paralogs of the *RAG1* gene. Although some paralogs of *RAG1* were not sequenced, possibly because they have been deleted, together, these markers illustrate a sister relationship between *X. lenduensis* and *X. vestitus*. We speculate that *X. lenduensis* and *X. vestitus* are derived from a recent common octoploid ancestor generated from the fusion of two tetraploid genomes indicated by lineages labeled ‘B’ and ‘A’ in Figs 5 and 6. However, we cannot rule out the less parsimonious possibility that each species was formed

from simultaneous, but independent allopolyploidization events involving the same combination of maternal and paternal tetraploid ancestors. In any case, *X. lenduensis* and *X. vestitus* share recent common ancestry of one half of their genome with *X. itombwensis* and *X. wittei*, and the other half with *X. amieti*, *X. andrei*, *X. boumbaensis*, *X. longipes*, *X. ruwenzoriensis* and *X. cf. boumbaensis*.

The discovery of *X. lenduensis* supports previous work that inferred the prior existence of ancestral tetraploid species that did not leave known extant tetraploid descendants (Evans *et al.*, 2005, 2008; Evans, 2007). In addition to these tetraploid ancestors, one or two ancestral diploid ( $2N = 18$ ) *Xenopus* species are predicted, depending on whether tetraploidization occurred in *Xenopus* via autopolyploidization or allopolyploidization. In *Silurana*, analysis of aDNA suggests that tetraploidization occurred by allopolyploidization; hence, another diploid ( $2N = 20$ ) ancestral species is predicted to have existed in addition to the ancestor of the diploid species *S. tropicalis* (Evans *et al.*, 2005; Evans, 2007).

The discovery of descendants of these predicted species would be an unusual example of unsampled species being predicted by phylogenetic inference. The discovery of diploid descendants of the predicted  $2N = 18$  *Xenopus* diploid ancestors would be particularly useful for studies of gene duplication in tetraploid species such as *X. laevis*. Analysis of these descendants would allow, for example, the partitioning of paralogs and singletons (duplicates where one copy became a pseudogene) into subgenomes derived from each diploid ancestor. Knowledge of this information would allow a large-scale test for evidence of phylogenetically biased pseudogenization in duplicated genetic networks after allopolyploidization. A single-locus example of biased pseudogenization was reported recently in the *RAG1* proteins (Evans, 2007), and this result is further supported here by the observation that *RAG1* paralog  $\beta 1$  is pseudogenized in *X. lenduensis* but *RAG1* paralogs  $\alpha 1$  and  $\alpha 2$  seem functional at the DNA level. Another example of biased pseudogenization in *Xenopus* is reported by Bewick, Anderson & Evans (2010) at the *DMRT1* locus.

The recent discovery of *X. lenduensis* and *X. itombwensis* is also interesting because it identifies two pairs of sister octoploid species (*X. lenduensis* + *X. vestitus*) and (*X. itombwensis* + *X. wittei*) that are distributed over much of the Albertine Rift. The distributions of these pairs of sister taxa overlap in the region between Lakes Kivu and Edward in south-western Uganda and the eastern DRC, which is where *X. wittei* and *X. vestitus* occur (Tinsley, 1973, 1975; Tinsley *et al.*, 1979). *Xenopus itombwensis*, the sister taxon of *X. wittei*, occurs on the Itombwe Plateau to the south of *X. wittei* and west of Lake Tanganika. *Xenopus lenduensis*, the sister taxon of *X. vestitus*, occurs on the Lendu Plateau to the north of *X. vestitus*, on the west side of Lake Albert.

### Phylogeography of *X. laevis*

Here, we use '*X. laevis*' *sensu lato* following Kobel *et al.* (1996). This species is distributed from South Africa to southern Sudan and from Nigeria to Tanzania. Previous studies identified at least six major mtDNA lineages in *X. laevis*. These lineages are mostly allopatric, although populations with multiple lineages do exist in South Africa (Du Preez *et al.*, 2009). One mtDNA lineage occurs in the Cape Region of South Africa south-west and west of the Cape Fold Mountains (Grohovaz, Harley & Fabran, 1996; Evans *et al.*, 1997, 2004; Measey & Channing, 2003; Du Preez *et al.*, 2009). Although subspecies are not recognized here, this lineage corresponds in geographic distribution with a portion of the '*laevis*' subspecies (Kobel *et al.*, 1996). A second lineage occurs in central South Africa between the Cape Fold Mountains and Lesotho (Du Preez *et al.*, 2009); this lineage is not included in the analysis presented in Fig. 5. A third lineage occurs in Malawi and north-western South Africa (Grohovaz, Harley & Fabran, 1996; Measey & Channing, 2003; Evans *et al.*, 2004; Du Preez *et al.*, 2009). A fourth lineage occurs in Congo Brazzaville (Evans *et*

*al.*, 2004). A fifth lineage occurs in north-western and central sub-Saharan Africa in Nigeria, Cameroon, Zambia and the DRC, including the southern portion of the Albertine Rift (this study; Evans *et al.*, 1997, 2004; Measey & Channing, 2003; Du Preez *et al.*, 2009). This lineage corresponds in geographic distribution with the subspecies '*sudanensis*' and '*poweri*' (Kobel *et al.*, 1996). A sixth lineage occurs in East Africa and the central Albertine Rift (this study; Evans *et al.*, 1997, 2004; Du Preez *et al.*, 2009) and corresponds in geographic distribution with the '*victorianus*' subspecies (Kobel *et al.*, 1996). Here, we identify a seventh mtDNA lineage within *X. laevis* that occurs on the Lendu Plateau and proximate areas west of Lake Albert (Fig. 5).

Multiple lines of evidence suggest that *X. laevis* contains substantially differentiated populations that may warrant species status (Measey & Channing, 2003). The genetic divergence between mtDNA lineages within *X. laevis* can be of a magnitude comparable to that observed between other species of African clawed frog. For example, in an ~870 bp region of the 16S gene, the uncorrected *p*-distance between the Cape Region population of *X. laevis* and other populations is about 3.2%, which is almost as large as the divergence over the same region of mtDNA between *X. lenduensis* and *X. vestitus*. Many *X. laevis* populations with divergent mtDNA also have distinct vocalizations (MLT, DBK and BJE unpublished data). It would be interesting to compare these vocalizations with that of *X. laevis* from the Lendu Plateau, but unfortunately, we did not obtain recordings of this population. Morphological variation is also substantial – for example, *X. laevis* from the West Cape Region, south-west of the Cape Fold Mountains, is significantly larger than *X. laevis* from other portions of South Africa (Du Preez *et al.*, 2009). However, analysis of mitochondrial and nuclear markers in a transect of individuals running from southwest to north-east South Africa identified a zone of admixture (Du Preez *et al.*, 2009).

The evolutionary relationships among these lineages have implications for elevation of *X. laevis* populations to species status. According to mtDNA, for example, the *X. laevis* population from the Cape Region of South Africa is equally diverged from the *X. laevis* populations from western Africa, from eastern Africa and from the Lendu Plateau. Additional studies are needed to sort out species from population-level relationships within *X. laevis* (Measey & Channing, 2003). In particular, it would be informative to use genetic data to explicitly compare a demographic hypothesis of isolation by distance within *X. laevis sensu lato* with a scenario involving speciation within this clade.

### Biogeography of the Albertine Rift

The Great Rift Valley is an ~6000 km trench between multiple continental plates. From its northern extent in Lebanon, it runs south, dividing Jordan and Israel, down the east side of the Sinai Peninsula and under the Red Sea. It surfaces in Eritrea and Ethiopia, dividing the Ethiopian highlands, and splits into a Western and an Eastern Rift just north of the equator, around the border between Sudan and Uganda and between Ethiopia and Kenya. The Eastern Rift runs through central Kenya and Tanzania and is associated with Mts. Kenya and Kilimanjaro, the highest mountains in Africa. The Western Rift includes the Albertine Rift highlands, which run between the DRC and the western borders of Uganda, Rwanda, Burundi and western Tanzania (Plumptre *et al.*, 2007). Further south, these rifts converge in Malawi and continue to central Mozambique.

*Xenopus lenduensis* and its closest relatives are endemic to the Albertine Rift. This area has a distinct flora and fauna associated with the montane rainforest highlands that grades into lowland tropical rainforests of the Congo Basin to the west and forest savannah mosaic habitat in the east and south (Burgess *et al.*, 2004). The Albertine Rift is a high priority for biodiversity conservation and is recognized, for example, as a biodiversity hotspot (Mittermeier *et al.*, 2004), a distinct terrestrial ecoregion (Burgess *et al.*, 2004), and an

'Endemic Bird Area' (Stattersfield *et al.*, 1998). At least one bird subspecies of the White-browed Crombec, Chapin's Crombec (*Sylvietta leucophrys chapini*), occurs only on the Lendu Plateau – although this subspecies may be extinct due to habitat destruction (Fishpool & Collar, 2006). The exceptional species diversity in this region is in part a consequence of the varied ecosystems associated with the altitudinal gradient and its location between distinct habitat types. Pleistocene and Pliocene climatic oscillations probably affected patterns of speciation in Central Africa and the Albertine Rift, for example in rodents (Huhndorf, Kerbis Peterhans & Loew, 2007), gorillas (Anthony *et al.*, 2007), birds (Bowie *et al.*, 2006; Voelker, Outlaw & Bowie, 2010) and frogs (Evans *et al.*, 2004; Blackburn, 2008).

The recognition of *X. lenduensis* further characterizes the considerable biological diversity in the Albertine Rift, contributing to our growing understanding of amphibian diversity in this region (Plumptre *et al.*, 2007; Behangana, Kasoma & Luiselli, 2009; Greenbaum & Kusamba, 2010 Roelke *et al.*, in press). Unfortunately, the biological diversity of the Lendu Plateau and Albertine Rift faces severe threats due to a very dense human population (Burgess *et al.*, 2007) and the associated anthropogenic disturbance that has destroyed natural vegetation on the Lendu Plateau (B. J. Evans, E. Greenbaum, C. Kusamba pers. obs., Fishpool & Collar, 2006). Based on our locality records, we estimate the extent of the occurrence of *X. lenduensis* to be < 5000 km<sup>2</sup>. Given the catastrophic deforestation and severe habitat fragmentation on the Lendu Plateau and the expected continuing decline of the extent and quality of this habitat, we recommend that this species be classified as endangered under the IUCN Red List Criteria (IUCN, 2001).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

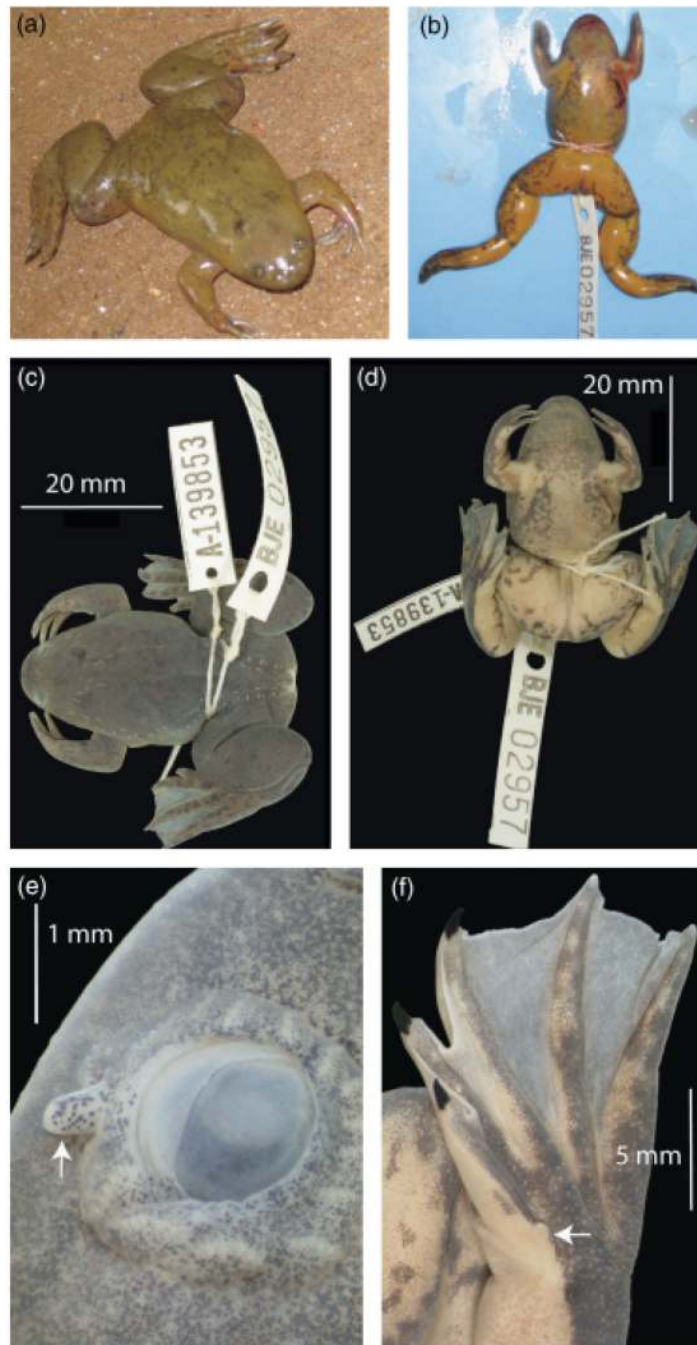
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**Figure 1.**

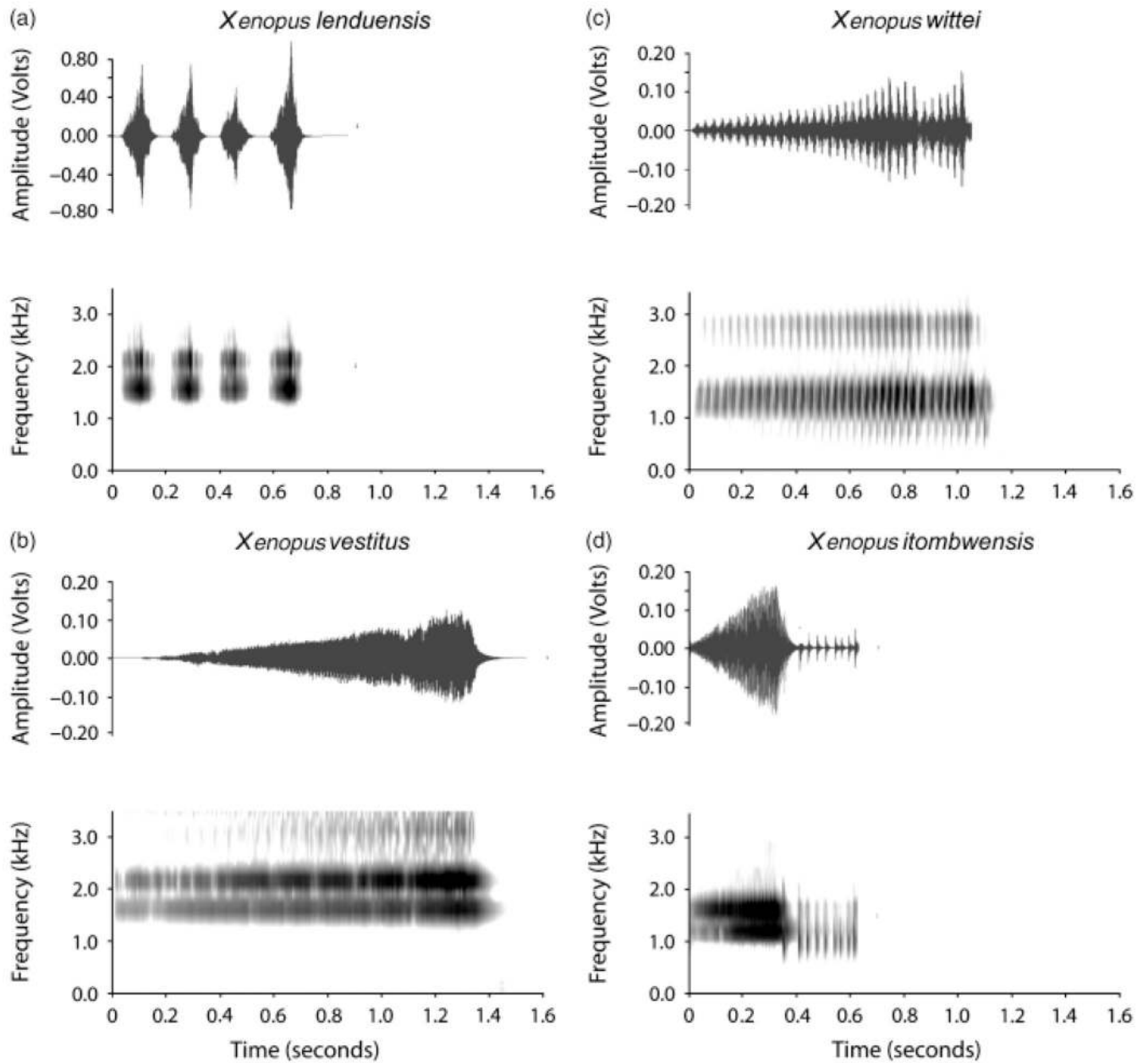
Pictures of holotype MCZ A-139853, (field number BJE2957) including (a) dorsal view in life, (b) ventral view immediately after death, (c) dorsal and (d) ventral view after preservation, and close-ups after preservation indicated by arrows of the (e) subocular tentacle and (f) metatarsal tubercle. (b) Red coloration on the left side, snout and neck of the animal is blood from the incision made for genetic sampling, which appears as a maroon ‘J’ on the left side of the vent just below the attachment point of the arm.





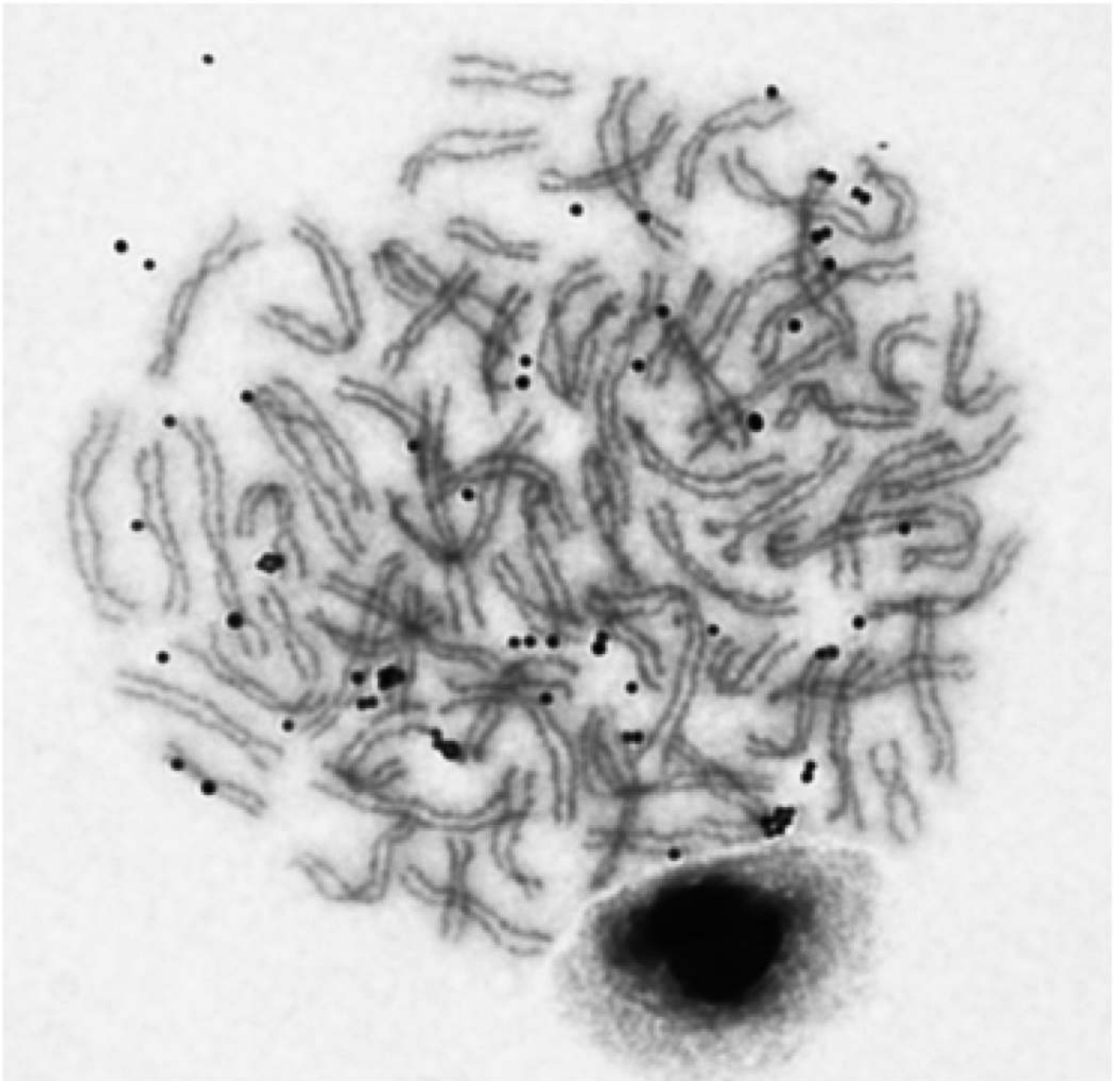
**Figure 2.**

Morphological variation of *Xenopus lenduensis* in life and after preservation. Dorsal views include preserved specimens: (a) paratype MCZ A-139856 (field number BJE2951), (b) paratype MCZ A-139854 (field number BJE2958), (c) paratype MCZ A-139855 (field number BJE2945) and live unvouchered individuals (d–h). Ventral views include preserved specimens: (i) UTEP20198 (field number EBG2356), (j) paratype MCZ A-139856, (k) paratype MCZ A-139855, and (l) paratype MCZ A-139854 and live unvouchered individuals (m–p).

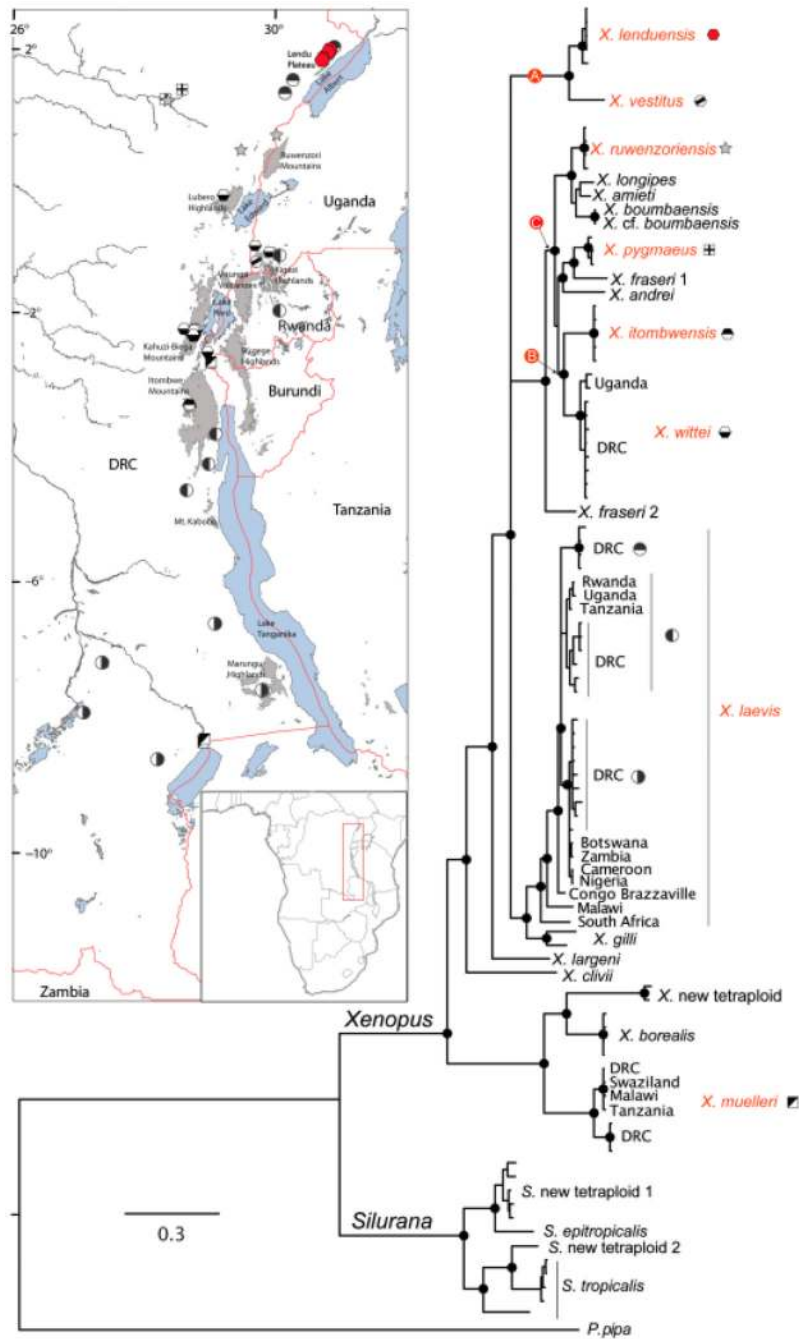


**Figure 3.**

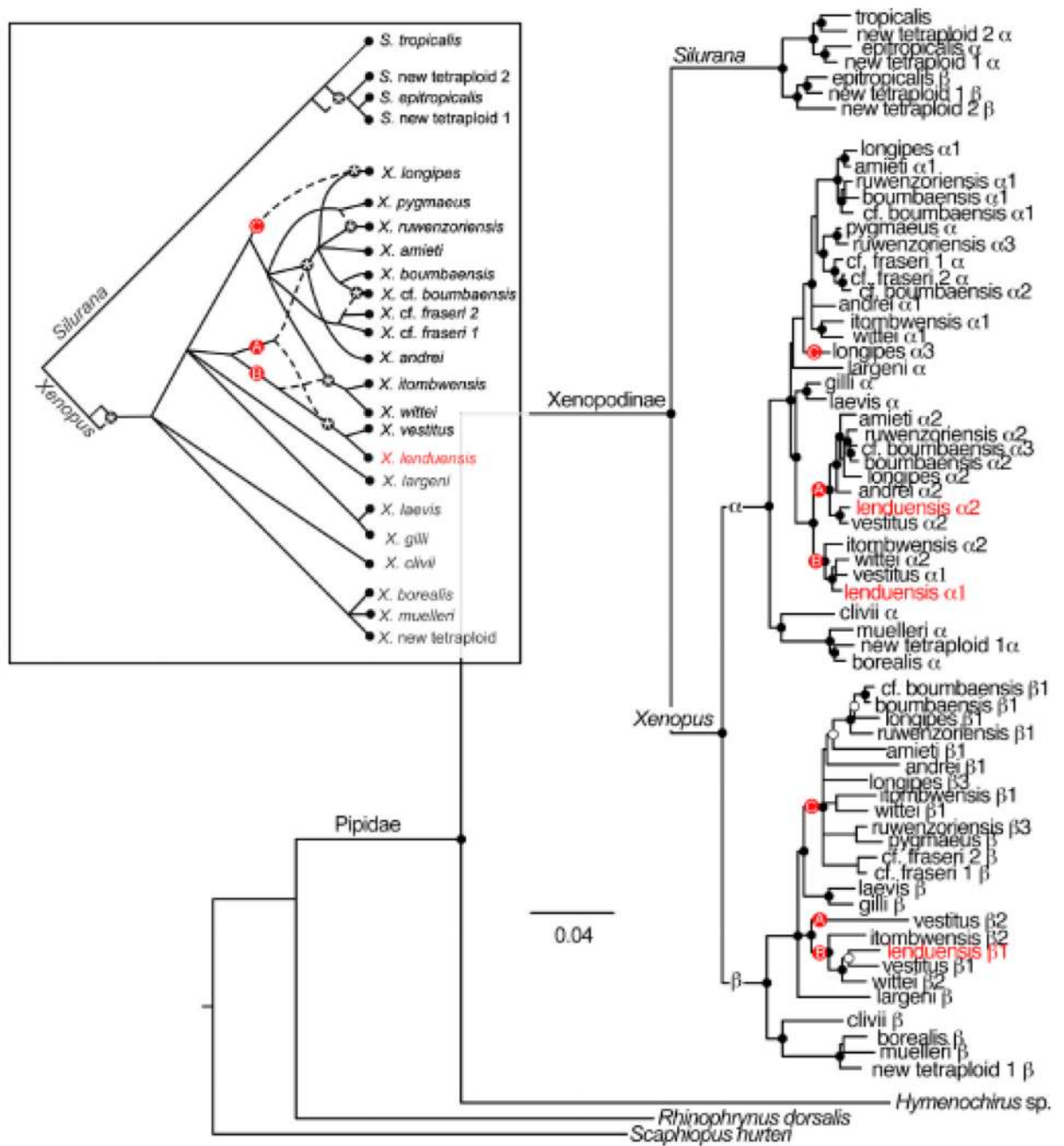
The male vocalization of (a) *Xenopus lenduensis* is distinct from that of closely related species (b) *Xenopus vestitus*, (c) *Xenopus wittei* and (d) *Xenopus itombwensis*. A bout of four advertisement calls is shown for *X. lenduensis*; a single advertisement call is shown for the other species.



**Figure 4.** Giemsa-stained metaphase stage cell from an *Xenopus lenduensis* illustrating octoploidy ( $8n = 72$  chromosomes). This individual was sampled in Ngensengeyu (DRC), MCZ A-140087; fieldcode BJE2929.



**Figure 5.** Mitochondrial DNA phylogeny and location of samples from the Albertine Rift (insert). In the phylogeny, black dots indicate nodes with  $\geq 95\%$  posterior probability. For clarity, some of these nodes are not indicated on terminal branches. At least three tetraploid ancestors are inferred and labeled A, B and C; different combinations of the genomes of these ancestors gave rise to extant octoploid and dodecaploid species (see text). Species with ranges in the Albertine Rift have red names on the phylogeny and symbols indicate their geographic location on the insert. On the insert, regions greater than 2000 m a.s.l are indicated in gray; lakes are blue.



**Figure 6.** *RAG1* phylogeny including *Xenopus lenduensis* supports a sister relationship with the octoploid species *Xenopus vestitus*. Nodes with posterior probabilities equal to 90–95% or >95% are indicated with open and solid dots, respectively. Lettered nodes correspond with each of the two paralogs from the inferred tetraploid ancestors labeled in Fig. 5. Lineages labeled ‘A’ correspond with paralogs from an inferred paternal tetraploid ancestor of *X. lenduensis* and *X. vestitus* and another octoploid ancestral species that gave rise to *Xenopus amieti*, *Xenopus andrei* and *Xenopus boumbaensis*, and the dodecaploid species *Xenopus longipes*, *Xenopus ruwenzoriensis* and *Xenopus cf. boumbaensis*. Lineages labeled ‘B’ correspond with paralogs from an inferred maternal tetraploid ancestor of *X. lenduensis* and

*X. vestitus*, which was also the paternal tetraploid ancestor of *Xenopus wittei* and *Xenopus itombwensis*. Lineages labeled 'C' correspond with paralogs of an inferred maternal tetraploid ancestor of the octoploid ancestral species that gave rise to *X. amieti*, *X. andrei*, *X. boumbaensis* and *X. long-ipes*, *X. ruwenzoriensis* and *X. cf. boumbaensis*. An insert depicts a summary of bifurcating and reticulating relationships in clawed frogs with paternally inherited lineages represented by dashed lines and biparentally or maternally inherited lineages represented by solid lines. In the insert, 'A', 'B' and 'C' refer to ancestral tetraploid lineages instead of the ancestral paralogs. Nodes with asterisks refer to putative instances of allopolyploidization.

Morphological measurements of African-clawed frogs from the Lendu Plateau and proximal regions, Democratic Republic of the Congo, including the holotype of *Xenopus lenduensis*

Table 1

Measurement	Holotype	<i>Xenopus lenduensis</i>						<i>Xenopus laevis</i>					
		Females; n = 27			Males; n = 15 <sup>a</sup>			Females; n = 9			Males; n = 14		
		Average	StDev	Max	Average	StDev	Max	Average	StDev	Max	Average	StDev	Max
Body length	37.69	48.10	5.21	56.43	40.25	2.76	46.02	63.19	4.41	67.75	49.94	2.67	57.41
Body width	15.39	22.01	4.03	28.26	16.50	1.39	18.60	29.58	4.04	36.28	19.80	1.22	22.42
Head width	8.43	9.50	0.72	11.02	8.42	0.69	10.07	12.34	1.01	13.36	10.55	0.41	11.09
Snout length	2.9	3.63	0.47	4.84	3.10	0.34	3.93	4.87	0.60	5.65	4.42	0.40	5.14
Eye diameter	1.78	1.88	0.18	2.24	1.64	0.22	2.01	2.76	0.42	3.27	2.49	0.19	2.77
Interocular distance	5.22	4.64	0.38	5.39	4.21	0.34	5.22	6.15	0.47	6.67	4.81	0.72	5.59
Nostril diameter	1.62	1.42	0.21	1.91	1.29	0.20	1.62	1.61	0.21	1.98	1.42	0.18	1.85
Internarial distance	1.98	1.91	0.21	2.61	1.67	0.29	2.09	2.50	0.33	3.02	2.31	0.36	3.30
Tibia length	16.61	18.33	1.42	20.48	16.69	1.10	18.42	23.84	2.21	28.05	20.92	0.91	22.33
Fifth Toe length	12.66	15.04	1.68	17.64	13.36	1.62	17.29	22.69	2.39	25.05	19.41	1.12	20.87
Elbow-first finger	14.62	15.23	1.00	16.93	14.57	0.66	16.30	21.48	2.24	24.77	18.54	0.78	19.70
First finger length	4.3	4.63	0.49	5.64	4.35	0.44	5.09	7.96	1.48	9.89	6.40	0.70	7.50

<sup>a</sup>Including the holotype.

**Table 2**

Advertisement call characteristics of *Xenopus lenduensis*, *Xenopus vestitus*, *Xenopus wittei* and *Xenopus itombwensis*

<i>X. lenduensis</i>	CN	Duration	IM	Iclicki	DF (low)	DF (high)	CDC	Icalli
Grand mean	9.84	143.86	13.25	11.97	1608.62	2148.01	0.89	169.71
stdev	0.97	9.70	4.37	1.04	24.20	20.77	0.04	14.86
SSbetween(1)	2.86	1321.23	127.10	16.30	20936.60	15288.70	0.00	4008.80
Residuals(36)	0.89	60.08	16.13	0.66	20.40	18.70	0.00 <sup>a</sup>	115.40
<i>X. vestitus</i>								
Grand mean	89.75	1167.02	35.69	12.72	1595.64	2154.50	0.25	1918.52
stdev	16.92	220.17	23.00	0.36	5.80	9.71	0.04	658.82
SSbetween(1)	132.25	7425.00	72.84	0.10	19.13	5.55	0.00	436065.00
Residuals(2)	363.25	68997.00	756.81	0.14	40.87	138.67	0.00 <sup>b</sup>	433542.00 <sup>b</sup>
<i>X. wittei</i>								
Grand mean	45.20	1397.10	6.74	31.06	1288.78	1521.97	0.64	4995.63
stdev	17.60	486.28	5.24	4.18	33.55	32.44	0.24	4553.92
SSbetween(1)	90.13	8.00	70.62	69.01	4480.40	4043.80	0.00	9567602.00
Residuals(3)	382.89	315293.00	13.03	0.34	7.00	55.40	0.08	21536114.00 <sup>c</sup>
<i>X. itombwensis (fast trill)</i>								
Grand mean	18.82	326.20	4.89	17.57	1284.16	1536.69	0.88	681.08
stdev	3.84	59.08	2.44	2.47	174.21	165.20	0.09	102.02
SSbetween(1)	5.60	713.00	35.57	4.24	56202.00	25232.00	0.01	49067.00
Residuals(9)	15.78	3800.00	2.68	6.33	27477.00	27518.00	0.01 <sup>d</sup>	7186.00 <sup>e</sup>
<i>X. itombwensis (slow trill)</i>								
Grand mean	8.82	326.66	0.91	36.43	1145.73	1644.96 <sup>g</sup>	-	-
stdev	2.40	101.09	0.84	9.55	141.36	11.37	-	-
SSbetween(1)	3.10	9412.00	2.35	9.22	160590.00	-	-	-
Residuals(9)	6.06	10309.00	0.43 <sup>f</sup>	100.39	2406.00 <sup>g</sup>	-	-	-

Mean, standard deviation (stdev), sum of squares between individuals divided by the degrees of freedom (SSbetween), and residuals divided by the degrees of freedom (Residuals) are provided for call duration in milliseconds (duration), number of clicks per call (CN), intensity modulation (IM), interclick interval in milliseconds (ICI), two dominant frequencies in kHz (DFlow and DFhigh), and call duty cycle (CDC). See 'Methods' for more information on these parameters. Measurements of the fast and slow trill portions of the *X. itombwensis* advertisement call are indicated separately. For *X. lenduensis*, results are summarized for two individuals for three calls per individual. One hundred and twenty-three and 251 clicks were analysed, respectively, for each individual. For SSbetween and



Residuals degrees of freedom are indicated in parentheses by row except where noted, or not indicated when data were obtained from only one individual, or not indicated for CDC for the slow trill of *X. imbricatus* because CDC is a parameter calculated from both portions of the call.

<sup>a</sup>Four measurements from one individual.

<sup>b</sup>4 d.f.

<sup>c</sup>14 d.f.

<sup>d</sup>13 d.f.

<sup>e</sup>12 d.f.

<sup>f</sup>6 d.f.

<sup>g</sup>8 d.f.