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A new, narrowly distributed, and critically endangered species of spiny-throated reed frog (Anura: Hyperoliidae) from a highly threatened coastal forest reserve in Tanzania

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Amphibians are in decline globally due to increasing anthropogenic changes, and many species are at risk of extinction even before they are formally recognised. The Coastal Forests of Eastern Africa is a hotspot of amphibian diversity but is threatened by recent land use changes. Based on specimens collected in 2001 we identify a new species from the coastal forests of Tanzania. The new species belongs to the spiny-throated reed frog complex that comprises a number of morphologically similar species with highly fragmented populations across the Eastern Afromontane Region, an adjacent biodiversity hotspot comprising of numerous isolated montane forests. The new species is the first coastal forest member of this otherwise montane clade. We formally describe this species, assess its distribution and conservation threat, and provide a revised key to species of the spiny-throated reed frog complex. We highlight the most important characters distinguishing the new species from the other similar reed frog species. Recent surveys at the type locality and also more broadly across the region failed to find this new species. The conservation threat of this species is critical as the only known locality (Ruvu South Forest Reserve) is currently subjected to devastating land use changes.

Key words: Coastal Forests of Eastern Africa, conservation, habitat destruction, *Hyperolius ruvuensis* sp. n., *Hyperolius spinigularis,* Tanzania
Ruvu South Forest Reserve

INTRODUCTION

Amphibians are threatened by extinction across the globe (Stuart et al., 2004), with extinction rates exceeding those of other vertebrate groups (Hof et al., 2011). Adding to the increased threat to amphibians, the distribution of areas with the highest species richness often corresponds with areas impacted disproportionately by multiple threat factors, such as climate change, land use changes, and chytridiomycosis (Hof et al., 2011). The current challenge faced by biologists, particularly those in tropical countries where biodiversity is most concentrated, is the race to describe species before they go extinct.

The Coastal Forests of Eastern Africa represent an area of high amphibian species richness (e.g., Poynton et al*.,* 2007) but many of the small remaining forest patches are relatively poorly known (Barratt et al*.,* 2014). The area has long been identified as an area of importance for biodiversity (Burgess et al*.,* 1992; 1998; Burgess & Clarke, 2000) but has suffered major land use changes (Burgess et al*.,* 1992, Tanzania Forest Conservation Group, 2012; Godoy et al., 2011). The long-term survival potential of species in these forests therefore remains uncertain given current trends in anthropogenic threats. Given the rapid rate of change, efforts in highlighting the biodiversity of this region, identifying key areas for conservation, and monitoring the health of populations are of the utmost importance.

In 2001 four specimens of a hyperoliid frog species were collected from a coastal forest in Tanzania identifiable to a clade of spiny-throated reed frogs. This clade exhibits a distinctive gular flap, with almost all species having asperities on the gular, and some with spines on the chest, and/or groin (*Hyperolius burgessi* Loader et al*.,* 2015*, H. davenporti* Loader et al*.,* 2015, *H. minutissimus* Schiøtz and Westergaard 2000*, H. spinigularis* Stevens 1971, *H. tanneri* Schiøtz 1982*, H. ukwiva* Loader et al*.,*

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2015). The spiny-throated reed frogs comprise a clade of several morphologically similar species that are found on isolated mountains across the Eastern Afromontane (EAM hereafter) region adjacent to the Coastal Forests of Eastern Africa (Lawson et al., 2015, Loader et al., 2015). These coastal specimens were not taxonomically evaluated and remained in the herpetology collection of the Natural History Museum in London. Recent fieldwork in the coastal forests of Tanzania has failed to secure any further individuals assigned to this coastal population.

In this study we assess the population of *H.* cf. *spinigularis* collected from Ruvu South Forest Reserve, a coastal forest patch less than 45 km from the major city of Dar es Salaam. Given that this material is the only non-montane record of the spiny-throated reed frogs, rare and apparently not recently collected, we review its taxonomic placement, biogeographic significance and conservation risk.

MATERIAL AND METHODS

Molecular Data

Specimens were collected by Frontier Tanzania (stored at the Natural History Museum, London, see collecting details in type description) and stored in 70% ethanol. Samples of muscle and/or liver were taken from representative individuals and preserved in 95% ethanol. Specimens included in this study are listed in Table 1, with expected occurrence data per species and Genbank numbers (KX455694-KX455723). Phylogenetic relationships of *H.* cf. *spinigularis* from Ruvu South Forest Reserve and all other known spiny-throated reed frogs (based on Loader et al., 2015, Lawson et al., 2015) were estimated between all individuals using a previously published gene dataset including one mitochondrial (ND2) and three nuclear (c-Myc, POMC, RAG1) genes, with *Hyperolius mitchelli* used as an outgroup. In addition we included from a smaller sampling of individuals one mitochondrial partial gene (16SrRNA). To reconstruct relationships, sequences were aligned using MUSCLE (Edgar, 2004), excluding poorly aligned regions of all genes using GBlocks (Castresana, 2000). The optimal model of molecular evolution for our gene partition (GTR+G) was found using PartitionFinder v.1.1.1 (Lanfear et al., 2012). For the complete concatenated alignment, intra- and inter-clade distances were calculated using the Species Delimitation plugin v1.04 for Geneious Pro (Masters et al., 2011). Molecular phylogenies were constructed using Bayesian and maximum likelihood (ML) approaches in BEAST (v.2.1.3) and RAxML v.8.0.0 (Ronquist et al., 2012, Stamatakis, 2014). To examine species boundaries across the reconstructed phylogeny we applied a Bayesian implementation of the General Mixed Yule-Coalescent model ("bGMYC" package v.1.0.2 for R, Reid & Carstens, 2012) using trees from the BEAST analysis. In BEAST, the first 10% of generations were discarded as burnin for both convergence and tree estimates. Convergence was investigated using Tracer v.1.6 (Rambaut et al., 2014) through a visual inspection of adequate mixing and ESS estimates >200. The maximum clade credibility tree was calculated for BEAST using TreeAnnotator. ML node support in RAxML was evaluated by non-parametric bootstrapping with 1000 replicates. BEAST analysis was run with a coalescent, constant size tree-prior and a strict molecular clock (as recommended for recent population-level analyses). Each locus was rate scaled to reflect the faster evolution times in mtDNA using rates outlined in Lawson et al. (2015). To address alternative phylogenetic hypotheses, we enforced topological constraints on our RAxML trees and performed AU, KH and SH topology tests in CONSEL v.0.20 (Shimodaira & Hasegawa, 2001).

Morphology

Material was examined from the Natural History Museum, London (BMNH) in addition to material previously documented in Loader et al. (2015). Morphological measurements were taken to the nearest 0.1 mm using Mitutoyo Absolute Digimatic Calipers (CD-6"C) with the aid of a Leica MZ8 stereo microscope (Leica Microsystems GmbH, Wetzlar, Germany). Sex was determined by the presence or absence of the gular flap in adult specimens. Measurements in this analysis match those in Loader et al. (2015) and include: Snout-Urostyle Length (SUL), Head Width (HW), Head Length Diagonal from corner of mouth (HLD), Head Length Diagonal from jawbone end (HLDJ), Nostril-Snout (NS), Inter-narial (IN), Eye to Nostril (EN), Eye Distance (EE), Inter-orbital (IO), Tibiafibula Length (TL), Thigh Length (THL), Tibiale Fibulare Length (TFL), Foot Length (FL), Forelimb Length (FLL), Hand Length (HL), Width of Gular Flap (WGF), and Height of Gular Flap (HGF). Qualitative characters were further investigated: gular shape, proportions and spinosity to assess differences from congeneric species. In order to assess the overall pattern of morphometric variation in these species (see Table 1 for specimen list) we also conducted a principal component analysis on log-transformed data using the Ggbiplot package in R (R Development Core Team, 2014; Wickham, 2009; Venables & Ripley, 2002).

Coastal forest surveys and remote sensing analysis of habitats

The geographic distribution of *H.* cf. *spinigularis* from Ruvu South Forest Reserve was mapped using the original collection records made by Frontier Tanzania in 2001. Additionally, we constructed a map of the points where major surveys have been conducted in other parts of the coastal forests of Tanzania using our own data and other published literature (Fig. 4). The data were accumulated on the basis of major collections in the region including Arthur Loveridge (Loveridge, 1942), Frontier Tanzania and Kim Howell (Appendix 7 in Burgess & Clarke, 2000), Frontier Tanzania (2001), and Barratt (unpublished data) (see Online Appendix 1).

We conducted an analysis of the habitat change in Ruvu South Forest Reserve since 1998 (Fig. 5A, Online Appendix 2). The land-cover change analysis covers Ruvu South Forest Reserve and two other nearby forest reserves (Pugu and Kazimzumbwi), which historically contained similar forest types. The most recent image used in the analysis was a Landsat 8 image dated 13 June 2014, chosen as the most recent cloud free image

Table 1. Details of specimens included in this study for molecular and morphological analyses (Modified from Table 3 in Lawson et al*.,* 2015). Genbank accession numbers of new sequences generated for this study (KX455694-KX455723) are shown per gene, for all other sequences please see Lawson et al. (2015).

Species	Altitudinal range occurence	Habitat	Expected Occurrence	Voucher numbers	GenBank accession numbers of new sequences				
					16s	ND2	C Myc	POMC	RAG1
H. burgessi	East Usambara:	Submontane forest	14,774 km^2	FMNH18989	KX455710				
	900–1100 m			FMNH 274258	KX455709				
	Nguru: 900–1000 m			FMNH 274259					
	Uluguru: 980 m			FMNH 274310					
				FMNH 274311					
				FMNH 274312					
				FMNH 274313					
				FMNH 274314					
				FMNH 274321					
				FMNH 274322					
				FMNH 274323					
				FMNH 274324	KX455706				
				FMNH 274482					
				FMNH 274483					
				FMNH 274484					
				FMNH 274944	KX455705				
				MTSN 8238					
				MTSN 8240					
				MTSN 8259					
				MTSN 8260					
				MTSN 8267					
				MTSN 8273					
H. davenporti	Livingstone: 2010 m	Montane forest edge	28 km^2	MTSN 7453	KX455703				
				MTSN 7464	KX455695	KX455714	KX455722	KX455719	
				MTSN 7465	KX455694	KX455715	KX455723	KX455718	
				MTSN 7467		KX455716		KX455717	
H. minutissimus	Njombe: 2010 m	Montane forest edge and grassland	14,904 km^2	FMNH 274290					
				MUSE 11023					
				MUSE 11024					
				MUSE 11026					
				MUSE 11028					
				MCZ DK R771421	KX455702				
				MCZ DK R771422	KX455701				
				MCZ DK R771423	KX455700				
				MCZ DK R771424	KX455699				
				MCZ DK R771426					
				MCZ DK R771432	KX455698				
H. ruvuensis sp. n.	lowland Tanzania: 230m	Coastal forest thicket, swamp	2 km^2	BMNH 2002.410					
				BMNH 2002.411	KX455696	KX455712		KX455721	
				BMNH 2002.412					
				BMNH 2002.413	KX455697	KX455713		KX455720	

Table 1. *Continued.*

Species	Altitudinal range occurence	Habitat	Expected Occurrence	Voucher numbers	GenBank accession numbers of new sequences				
					16s	ND2	C Myc	POMC	RAG1
H. spinigularis	Malawi: 690 m	Submontane forest and forest edge	5,488 km^2	FMNH 274894					
				FMNH 274943					
	Mozambique: 1250 m			FMNH 274945					
				FMNH 274947					
				FMNH 274949					
				FMNH 274950					
				MVZ 266050					
H. tanneri	West Usambara:	Submontane forest and forest edge	4 km^2	FMNH274287					
				FMNH274288	KX455708				
				FMNH274289	KX455707				
				FMNH 18804	KX455711				
H. ukwiva	Rubeho: 1660 m	Montane forest edge	1,179 km^2	KMH36053					

of the area. The historical image used in the analysis was a Landsat 5 image dated 16 May 1998, selected as the most cloud free Landsat image covering the three forest reserves near the year that the specimens were collected (2001).

As separate training data were chosen for each scene, no prepossessing was performed on the Landsat imagery, with the exception of improving the georeferencing of the 1998 Landsat 5 scene using the georeferencer plugin in QGIS. In 1998, the three different forest types that dominated Ruvu South Forest Reserve were East African coastal dry forest, East African coastal scrub forest, and degraded variants of each (Burgess & Clarke, 2000). There were also large portions of wooded grassland with a mixture of larger trees and bushes. Training data for the classifications was based on expert knowledge of the area and high-resolution imagery on Google Earth ranging in dates from 2004 to 2014. For the 1998 scene, 232 training polygons were drawn, while for the 2014 scene, 154 training polygons were drawn.

To make better use of limited training data, the Landsat images were segmented using mean-shift segmentation from the Orfeo Toolbox. The spatial radius was set to 5 pixels, the range radius was set to 2 pixels, and the minimum object size to 5 pixels. These settings were chosen after visual experimentation to arrive at a segmentation that did not appear to lump different land-cover types into the same segments. The segment size, mean and variance were then calculated for bands corresponding to Landsat 5 bands 1–5, and 7, 1-arc SRTM elevation data, slope, and a hillshade image corresponding to the particular Landsat scene. The segments were classified in R (R Development Core Team, 2013) using Random Forest and output as TIFF images. Several classifications were generated for each Landsat scene and compared with high-resolution imagery until there was good visual agreement. The classifications for the two years were then compared using raster algebra in R to arrive at a map of land-cover change. To remove small areas of change due to georeferencing disagreements and speckle, a 5 pixel orthogonal sieve was applied to arrive at the final land-cover change map.

RESULTS

Phylogeny

Bayesian and Maximum Likelihood methods both agreed on previously published evolutionary relationships within the spiny-throated clade (see Fig. 1, Loader et al., 2015, Lawson et al., 2015). The smaller sampling dataset of 16S mtDNA also agreed on the topology recovered with our multilocus dataset, though support and pairwise distances between species were consistently lower. Intra- and inter-clade distances using the Species Delimitation tool are given in Table 4 for the larger gene and individual sampled dataset. Analysis using the Species Delimitation plugin in Geneious Pro support previous taxonomic units (as in Loader et al*.,* 2015) in addition to the new species here described. As in Loader et al., (2015) it seems that *H. burgessi* and *H. minutissimus* might consist of more than one species (See Table 4). Comparing all individual gene trees, our phylogenetic results appear largely reliant upon the fully resolved mtDNA relationships within this lineage, as many of the nuclear loci appear to retain ancestral polymorphisms, particularly in more recently divergent species.

Topology tests using likelihood scores (Table 2) indicated a significant difference between our optimal tree (*H. ruvuensis* sp. n. as sister taxon to a clade containing *H. spinigularis, H. burgessi* and *H. davenporti*) and alternative topologies, thus refuting the grouping of *H. ruvuensis* sp. n. with either *H. davenporti* or *H.*

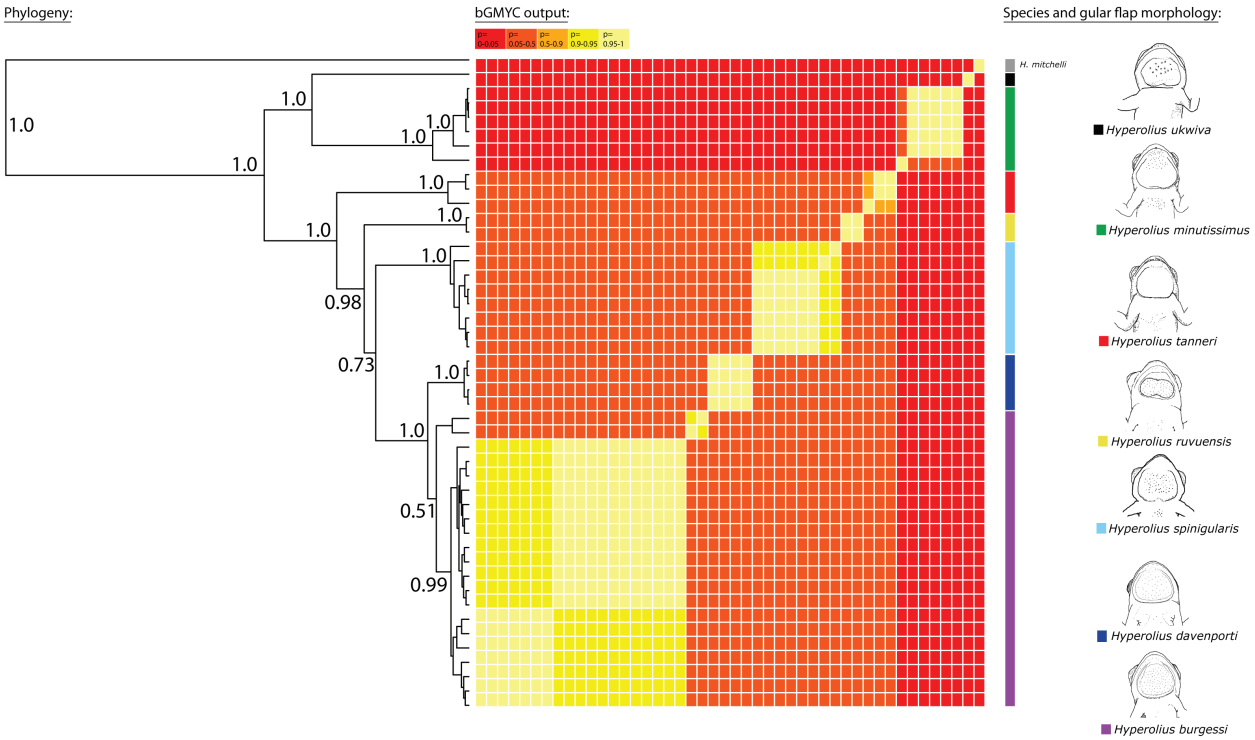

Fig. 1. Bayesian phylogeny of the spiny-throated reed frog species complex including *Hyperolius ruvuensis* sp. n. Support for clades is shown on nodes as well as bGMYC species delimitation results shown in colour coding and male gular flap morphology.

burgessi (Table 2). Topology test scores from trees pairing *H. ruvuensis* sp. n. with *H. spinigularis* from Mozambique and Malawi were consistently lower than our optimal tree but not significantly different.

Morphology

Measurements for specimens examined are given in Table 3. Principal component analysis of *H. ruvuensis* sp. n. males and females separately including morphological data from Loader et al. 2015 shows largely overlapping results (Fig. 2), and does not distinguish *H. ruvuensis* sp. n. based on morphometric measures as unique from the rest of the *H. spinigularis* complex. The main trait to distinguish *Hyperolius ruvuensis* sp. n. from the other members of the spiny-throated reed frog complex is the distinctive bilobed shape, disc-like platform, and spinosity of the gular flap, which is evident in both male specimens included in this study, and the relatively large snout-urostyle length in females (See Loader et al., 2015).

Systematics

***Hyperolius ruvuensis* sp. n.** Barratt, Lawson and Loader
Ruvu spiny reed frog
Figs. 3A, B

Holotype.— BMNH 2002.410 (male, field tag KMH 23565, held at the Natural History Museum, London) collected on 18 May 2001 in Ruvu South Forest Reserve (07° 02' 21.1" S; 38° 54' 58.3" E, 230 m a.s.l) by Frontier-Tanzania.
Paratypes.— Male: BMNH 2002.412 (field tag KMH 23567). Females: BMNH 2002.411 (field tag KMH 23566), BMNH 2002.413 (field tag KMH 23569) collected at same locality and date as of the Holotype.
Diagnosis.— Horizontal pupil with distinctive gular flap in males. As with most other members of the spiny-throated clade (*H. burgessi, H. davenporti, H. minutissimus, H. spinigularis, H. ukwiva*), *H. ruvuensis* sp. n. also has the presence of dermal asperities (including on the body and chin region) on the ventrum, unique

Table 2. Results from topology tests of alternative relationships. Most likely tree topologies displayed in rank order from top to bottom. Key: 1 – optimal tree (as in Fig. 1), 2 – *H. ruvuensis* sp. n. and *H. spinigularis* constraint, 3 – *H. ruvuensis* sp. n. and *H. burgessi* constraint, 4 – *H. ruvuensis* sp. n. and *H. davenporti* constraint, obs – the observed log-likelihood difference, bp – bootstrap probability, np – bootstrap probability calculated from multiscale bootstrap, pp=Bayesian posterior probability. AU – Approximately Unbiased test, KH, Kishino-Hasegawa test, SH – Shimodaira-Hasegawa test, WKH – Weighted Kishino-Hasegawa test, WSH – Weighted Shimodaira-Hasegawa test. *significantly different than optimal tree.

	obs	bp	np	pp	AU	KH	SH	WKH	WSH
1	-1.6	0.656	0.656	0.837	0.687	0.671	0.823	0.671	0.864
2	1.6	0.344	0.344	0.163	0.317	0.329	0.626	0.329	0.547
3*	48.5	0	1e-04	7e-22	3e-04	0.001	0.001	0.001	0.001
4*	48.5	0	1e-04	7e-22	3e-04	0.001	0.001	0.001	0.001

Table 3. *Hyperolius ruvuensis* sp. n. morphology. All measurements in to the nearest 0.1 millimetres, with * indicating the holotype.

Genus	*Hyperolius*	*Hyperolius*	*Hyperolius*	*Hyperolius*
Species	*ruvuensis* sp. n.	*ruvuensis* sp. n.	*ruvuensis* sp. n.	*ruvuensis* sp. n.
Museum Number	BMNH 2002.410*	BMNH 2002.411	BMNH 2002.412	BMNH 2002.413
Country	Tanzania	Tanzania	Tanzania	Tanzania
Sex	M	F	M	F
SUL	16.8	25.4	18.7	24.2
Head Width (HW)	6.1	9.2	6.2	8.3
Head Length Diagonal (HLD) corner of mouth	5.3	7.2	5.4	6.9
Head Length Diagonal (HLD) from jawbone end	6.3	8.3	6.4	8.1
Nostril-Snout (NS)	1.0	1.3	1.1	1.2
Inter-narial (IN)	2.1	2.5	2.1	2.3
Eye to Nostril (EN)	1.9	2.3	1.9	2.2
Eye Distance (EE)	3.6	4.0	3.7	4.0
Inter-orbital (IO)	3.1	4.8	2.7	4.5
Tibiafibula Length (TL)	8.7	12.3	9.1	11.8
Thigh Length (THL)	8.5	11.8	9.2	11.4
Tibiale Fibulare Length (TFL)	5.3	7.3	5.8	7.1
Foot Length (FL)	7.4	10.3	7.6	10.1
Forelimb Length (FLL)	4.2	5.3	4.8	5.2
Hand Length (HL)	4.9	6.7	4.8	6.6
Gular Flap: Width	4.8	-	5.1	-
Gular Flap Height	3.2	-	3.6	-

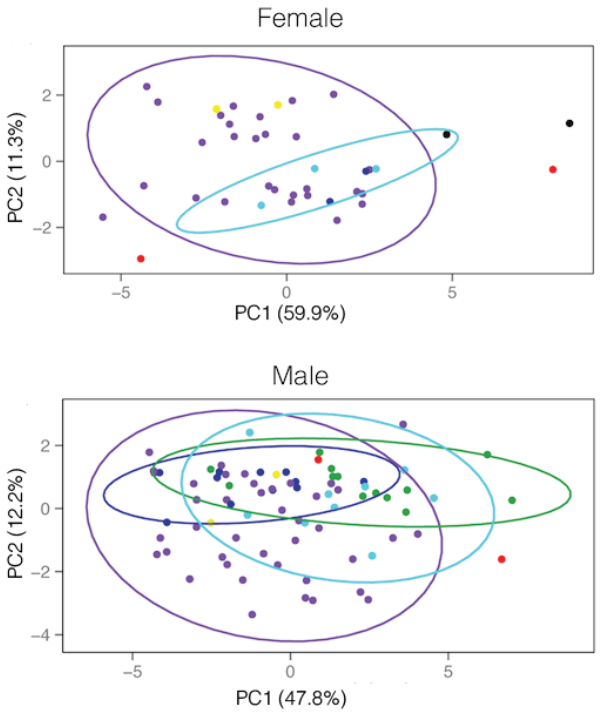

Fig. 2. Principal Component Analysis of morphological divergence between species. Raw morphological measurements for *H. ruvuensis* sp. n. shown in Table 3. For the other species in the complex we used data from Additional file 2 in Loader et al. 2015.

amongst hyperoliids. The presence of asperities on the gular flap diagnoses this species from *H. tanneri,* for which they are absent. The distribution of dermal asperities in two distinct circular patches differs from the anteriorly positioned distribution of asperities in *H. minutissimus* and *H. ukwiva,* and the evenly distributed asperities on the gular flap in *H. burgessi, H. davenporti* and *H. spinigularis*. Furthermore, *H. ruvuensis* sp. n. males have a bilobed and rounded gular flap - distinctive from the rounded gular flap of *H. burgessi, H. davenporti* and *H. minutissimus* (see Fig. 1). The bilobed gular flap in *H. ruvuensis* sp. n. is similar to that seen in *H. spinigularis* from Malawi and *H. ukwiva* from Rubeho, although in *H. ruvuensis* sp. n. it is much more pronounced and raised, forming a disc-like structure on the gular flap (see Figs. 1, 3B). This raised disc like gular flap is a diagnostic character for males of *H. ruvuensis* sp. n. Based on molecular data the species is genetically distinct from close relatives, and is the sister taxon to a clade comprising of *H. davenporti, H. burgessi* and *H. spinigularis*, being minimally 5.9% pairwise divergent from its closest relative (*H. davenporti*) based on ND2, C Myc, POMC and RAG1 genes. *Hyperolius ruvuensis* sp. n. further has an allopatric distribution with respect to other species in the complex and is the only member found at low elevation within the coastal forest belt of Eastern Africa (Fig. 4).

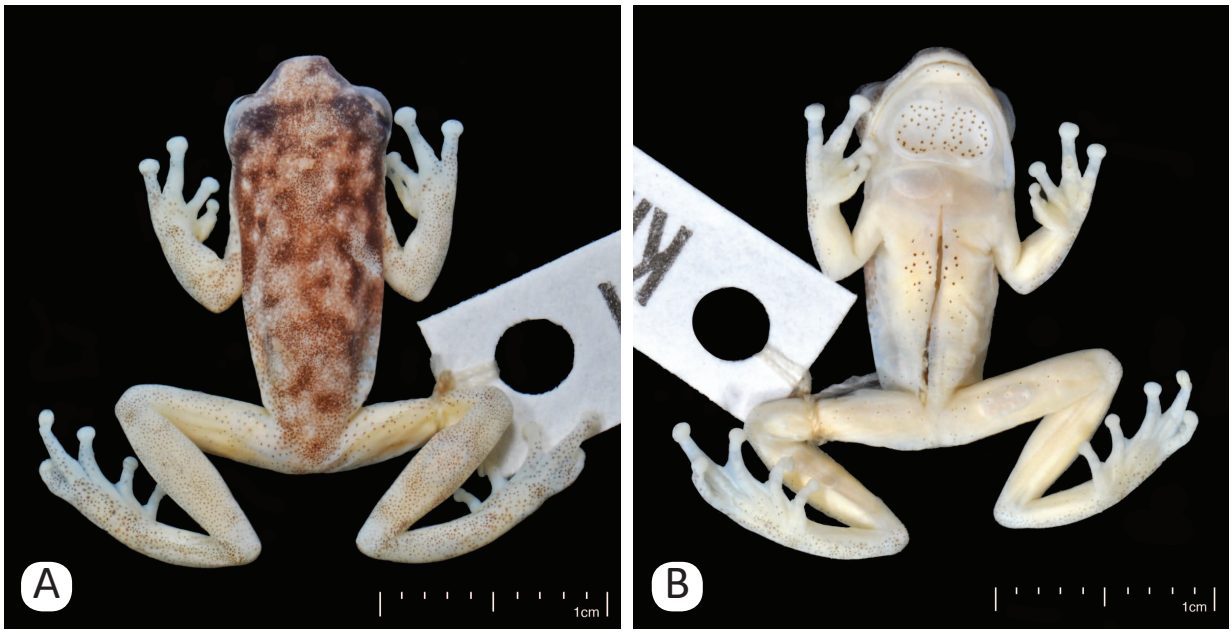

Fig. 3. Dorsal (A) and ventral (B) views of the holotype of *H. ruvuensis* sp. n. BMNH 2002.410. Scale bar = 1cm.

Description of holotype.— Small to moderate sized hyperoliid. Pupil horizontal. Snout blunt and slightly rounded. Canthus rostralis angular, being slightly convex on the horizontal plane and slightly concave on the vertical plane. Distance between eyes is 3.6 mm and interorbital distance is 3.1 mm. The inter-narial distance is 2.1 mm, greater than narial distance to the eye (1.9 mm). The nostril to snout is 1.0 mm. The width of head (6.1 mm) equals 0.36 of the body length (16.8 mm). The gular flap width is more than (4.8 mm) the height (3.2 mm). The gular flap is raised and bilobed, anteriorly narrowing. It is marked by black asperities (ca. 65) distributed across the gular flap in two distinct patches on each lobe. Asperities are evenly distributed on each lobe, though a small patch without asperities is present towards the lower central part of each lobe. Tibio-tarsal articulation of the adpressed hind limb reaching the eye. Tibio-tarsal (8.7 mm) is almost equal to thigh length (8.5 mm). The tibiale fibulare length is 5.3 mm. Toes have expanded fleshy discs with the foot being 7.4 mm. Webbing is extensive almost reaching the base of the fleshy discs on all toes apart from the first toe where it only reaches the first tubercle. The forelimb length is 3.3 mm, less than the hand length (4.8 mm). The hands have expanded, rounded fleshy discs. Webbing just reaching distal subarticular tubercle of the outer finger and slightly reduced on all other fingers. Dorsal skin surface granular with a single minute black asperity surmounting many of the granules. Ventral skin surface strongly granular with black asperities on the mentum (ca. 8), gular flap (ca. 60), abdomen (ca. 40) and undersurfaces of the femur (ca. 20 on each femur). Ventral asperities much more prominent than those of the dorsum.

Paratypes.— Head and body proportions in close agreement with those of the holotype (Fig. 3, Table 3). The distribution of the asperities of the male paratype is in close agreement with that of the holotype. As with other *H. spinigularis* group species the proportions of the gular flap in males, diagnostic for the species, shows slight variation which means care needs to be taken in applying this character, though the unique shape and spinosity of *H. ruvuensis* sp. n. should enable clear differentiation from other members of the complex (Figs. 1, 3B). Webbing of all the material conforms to that of the holotype.

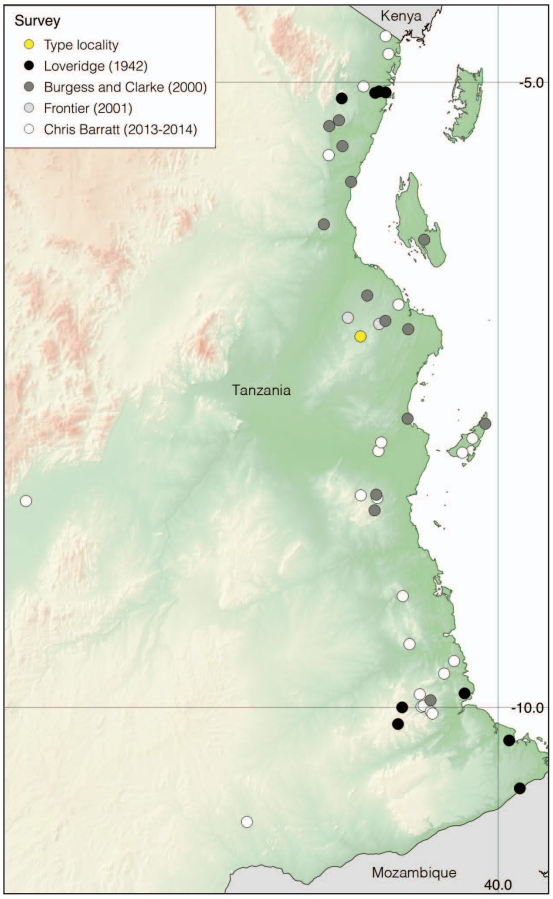

Fig. 4. Map of coastal Tanzania showing the type locality of *H. ruvuensis* sp. n. (yellow circle) and additional coastal forest localities that have been surveyed but did not yield members of the spiny-throated reed frog complex. See Online Appendix 1 for locality data and sources.

Table 4. Species delimitation results for the spiny-throated reed frog complex using the Species Delimitation Plugin for Genious Pro (Masters et al., 2011) with our Bayesian phylogeny from Figure 1. Delimitation results show all taxa are monophyletic, and show the closest relative for each species. Intra-dist shows intra-specific genetic distance between samples within each species (values of 0 indicate a single representative per species), Inter-dist shows inter-specific genetic distance to the closest relative.

Species	Closest relative	Monophyletic?	Intra-dist	Inter-dist
H. mitchelli	*H. ukwiva*	yes	0	0.164
H. ukwiva	*H. minutissimus*	yes	0	0.06
H. minutissimus	*H. ukwiva*	yes	0.01	0.06
H. tanneri	*H. davenporti*	yes	0.007	0.049
H. ruvuensis sp. n.	*H. davenporti*	yes	0.003	0.059
H. spinigularis	*H. davenporti*	yes	0.007	0.041
H. davenporti	*H. burgessi*	yes	0.003	0.018
H. burgessi	*H. davenporti*	yes	0.01	0.018

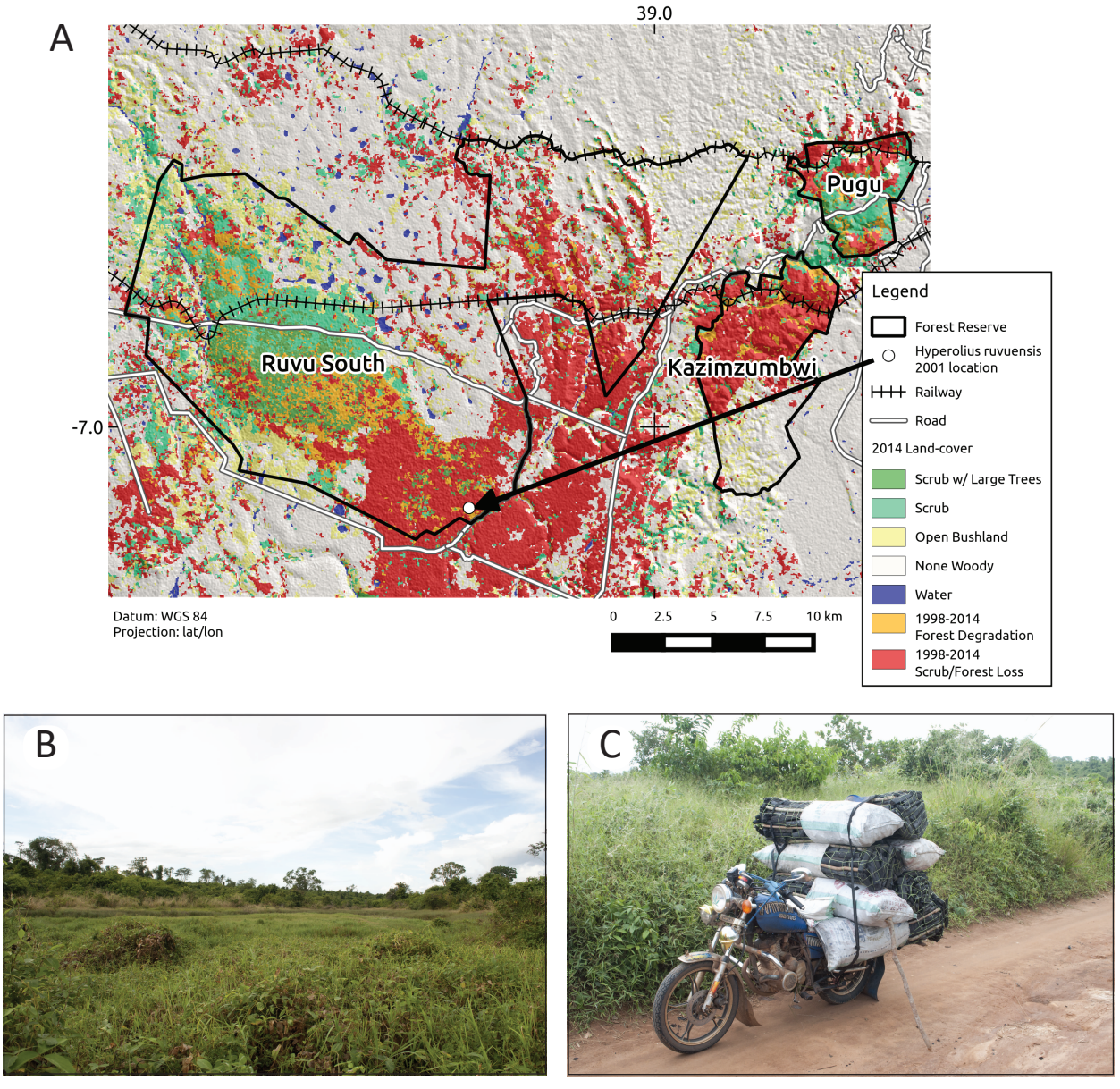

Fig. 5. Habitat change and photographs of Ruvu South Forest Reserve in April 2015. (A) Habitat change from 1998–2014, see also Online Appendix 2. (B) Grassland swamp area of type locality based on original GPS co-ordinates, (C) Charcoal being transported by motorbike illegally from Ruvu South Forest Reserve, a common sight in the coastal forests.

Colour patterning of adults.— *In life:* Head and dorsum is brown with a creamy white mottling on back, in some individuals the mottling extends along the side of the animal from the anterior end. The ventrum side is generally white with the exception of the asperities in males which are dark brown/black. Forelimbs and hindlimbs are mottled creamy white colour matching the dorsum, with flashes of orange on the thighs and feet and faded white heel spots. *In preservative:* The holotype (BMNH 2002.410) is a creamy colour, with the asperities and pigmentation of chromatophores on the dorsum resulting in a brown mottled appearance. Forelimbs, hindlimbs and feet are cream coloured with scattered brown chromatophores on the dorsal side. The ventral side is cream coloured with the exception of the asperities on the abdomen, gular flap and mentum. The male paratype (BMNH 2002.412) resembles the holotype in basic patterning but the colour is largely absent from the dorsum and head. The female paratypes also exhibit colour and pattern variation, BMNH 2002.411 is cream coloured with several small patches of brown on the dorsum, legs and forelimbs, and BMNH 2002.413 is cream with more subtle brown patches. Both female specimens have scattered patches of cream colour where the brown pigment is reduced. All specimens had lateral dark edged white stripes (either thin or irregular in size and outline) ending anteriorly in a narrow stripe meeting at the snout.

Sexual dimorphism.— Females attain a much larger size than the males (Table 3). Asperities of the dorsum are slightly weaker in the females and completely absent from the ventral side. Males are easily distinguished from the females by their characteristic bilobed and raised gular flap (Fig. 3B).

Advertisement Call.— No advertisement call is known.

Etymology.— The species is named after Ruvu South Forest Reserve where the specimens were collected and is the current extent of the species occurrence.

Distribution and habitat.— The species is likely endemic to Ruvu South Forest Reserve in Tanzania (See Figs. 4, 5A). Specimens were collected by Mr. David Emmett who provided valuable information on the habitat of the type locality. Specimens were found on reeds and bushes in a swampy open grassland area beside a permanent pond on a lowland plain (230 m a.s.l). Adjacent to the grassland was some sparse forest cover which kept the type locality partially shaded during the day (canopy height of <10 m, ground vegetation layer >50 % cover and shrub layer <10 % cover). One of the authors of the paper (SL) was able to revisit Ruvu South Forest Reserve in April 2015, where a rapid survey was conducted. The survey failed to discover any individuals of this species with a one day survey conducted at the type locality and two night surveys in varied habitats (swamp and forest) located in the northern part of the Forest Reserve. Figure 5A shows habitat classifications in Ruvu South Forest Reserve with the location of the type locality. Estimates of forest loss and severe habitat degradation are also given and show severe habitat degradation around the type locality – as also evidenced by ground truthing (see also Figs. 5B–D). Furthermore, on a broader scale, surveys across the coastal region in Tanzania failed to find any specimens referable to this species (Fig. 4). The apparent restriction of *Hyperolius ruvuensis* sp. n. solely to Ruvu South Forest Reserve seems plausible and not due to sampling deficiencies across the region.

IUCN red listing.— Because the area of occupancy is probably less than 10 km^2, all individuals are in a single sub-population and the extent of its habitat and possibly the number of reproductively active individuals are declining, we recommend the species to be listed as Critically Endangered based on the IUCN red list criteria (IUCN, 2012). The species cannot be classified as Extinct due to the lack of exhaustive surveys in known and expected habitat.

Key to the East African Spiny-throated Reed Frogs

As in Loader et al. (2015) we present a key that should identify adult male specimens of all presently described species.

1a	Gular flap with black dotted asperities, species not found in West Usambara Mountains	2
1b	Gular flap lacking any asperities, species found in West Usambara Mountains	*H. tanneri*
2a	Black dotted asperities evenly distributed across the gular flap	3
2b	Black dotted asperities distributed on anterior and mid region of the gular flap	6
3a	Gular flap bilobed	4
3b	Gular flap not bilobed	5
4a	Gular flap strongly bilobed, with asperities distributed into two discernable circular raised platforms, demarcating the area, species found in coastal forests of Tanzania.	*H. ruvuensis* sp. n.
4b	Gular flap bilobed, with asperities distributed regularly across the gular flap. Species found in Malawi and Mozambique	*H. spinigularis*
5a	Gular flap rounded with posterior and anterior ends more equal. The gular flap is usually either equal or wider than height, species found in Southern Highlands of Tanzania.	*H. davenporti*
5b	Gular flap narrowly tapering anteriorly and usually equal or greater in height, species found in East Usambara, Nguru, and Uluguru Mountains	*H. burgessi*
6a	Gular flap not bilobed and found in Udzungwa Mountains. Females reach a moderate size 18–24mm	*H. minutissimus*
6b	Gular flap bilobed, and found in Rubeho Mountains. Females reach a large size >25mm	*H. ukwiva*

DISCUSSION

Biogeography

Our phylogenetic reconstruction of the spiny-throated reed frog clade is consistent with the multi-locus gene tree of Lawson et al. (2015), showing generally high divergence between species. Our analyses places *H.*

ruvuensis sp. n. as sister taxon to a clade containing *H. burgessi, H. davenporti* and *H. spinigularis* (see Fig. 1) though the relationships between the latter clade are not well resolved. Topology tests on alternative relationships suggest most are significantly worse, however, an alternative topology with *H. ruvuensis* sp. n. forming a clade with *H. spinigularis* in Malawi and Mozambique was not significantly worse. The lack of resolution prevents robust biogeographic conclusions but we can speculate upon a potential scenario given the best topology and known distribution of species. The position of *H. ruvuensis* sp. n. and *H. tanneri* – two geographically widely separated populations, relative to the *H. spinigularis, H. davenporti* and *H. burgessi* clade – lends support to a formerly relatively widespread coastal and montane ancestor that became increasingly fragmented and restricted to both montane and coastal regions. Such a scenario has been previously speculated upon in other groups (Kingdon, 1989; Burgess et al., 1998) with a number of examples of sister group relationships among coastal and montane regions embedded in montane or coastal clades. This has been specifically shown in birds (e.g. Roy et al., 1997) and plants (e.g. Dimitrov et al., 2012) occurring in both montane and lowland rainforest habitats.

Climate fluctuations have been important in expanding and contracting forest habitats in East Africa (Burgess & Clarke, 2000), and such fluctuations were likely important in speciation processes that produced the current extant species in the *H. spinigularis* complex with their currently restricted distributions. Such changes in species ranges were documented in Lawson et al. (2015) potentially producing peripatric populations (e.g. *H. tanneri*, *H. davenporti*) and the new species documented here could comprise another example – particularly given the potential niche shift to coastal forest, open woodland type habitat. Furthermore, *H. ruvuensis* sp. n. restricted to the coastal forests and a relatively divergent species (based on molecular differences) provides a piece of evidence that might suggest the relative longevity of coastal forests. Burgess and Clarke (2000) argued that endemism in coastal forests was likely in part attributed to the old age, or non-inundated habitats in the region). This was also shown in African violets (*Saintpaulia* spp.), in a study by Dimitrov et al. (2012) who suggested the presence of micro-endemic species in their analysis supports the existence of lowland refugia even during glacial maxima.

Conservation

The coastal forests are an important ecosystem for conservation in Africa due to its rich biodiversity (Myers et al., 2000, Azeria et al., 2007). However, many coastal forests have either disappeared completely or have been reduced to extremely small patches less than 20 km^2 in size (Burgess et al., 1998). Across the Coastal Forests of Eastern Africa, there are over 1750 endemic plant and 100 endemic vertebrate species respectively, which are in many cases present in several forests (Conservation International, 2015), however similarly to *H. ruvuensis* sp. n. there are also micro-endemics likely restricted to single sites (e.g. several millipede and amphibian species see Burgess et al., 1998). The type locality and only known location of *H. ruvuensis* sp. n., Ruvu South Forest Reserve, is one of the few remaining areas of coastal forest near to Dar es Salaam, and in recent years has undergone severe deforestation for fuelwood, timber and biofuel production (Gwegime et al., 2013; see Figs. 5A–C). Our analysis of the forest reserve shows habitat change over the last 16 years (1998–2014) with particularly high rates of deforestation in areas formerly covered in coastal forest relative to the areas that are dominated by coastal thicket. Particularly worrying is the level of habitat change in the southern parts of the reserve, which includes the precise type locality of *H. ruvuensis* sp. n.

Assessing the impact of habitat change has on amphibian assemblage in Ruvu South Forest Reserve is currently not possible given the lack of data on the spatial distribution of species and population numbers. Gross habitat changes (Fig. 5A, Online Appendix 2), as recorded for this area, however are likely to impact amphibian assemblage but it is unclear how this might specifically impact the new species here described, only recorded once from grassland swamps that adjoin forest in 2001. Our rapid surveys in both the type locality and northern parts of the reserve with historically similar habitat in 2015 failed to find the species, though more extensive survey efforts are necessary to better validate its potential absence. Today the adjoining areas are heavily degraded woodland with evidence of extensive charcoal burning, which could have had an impact on the species but this remains speculative and requires a monitoring program to better understand whether the species is indeed absent or declining.

All members of the spiny-throated reed frog complex have small distributions, with the consequence that all species are classified as threatened in recently compiled IUCN red list assessments. *Hyperolius ruvuensis* sp. n. in particular is of high conservation concern due to the high rates of deforestation in Ruvu South Forest Reserve, and its extremely small extent of occurrence (Table 1, Fig. 4). For all East African spiny-throated reed frogs, it will be important to establish the full extent of their distributions, with future sampling of the Eastern Afromontane and Coastal Forests of Eastern Africa, as these data have important conservation implications.

Beyond the conservation of this newly described species – Ruvu South Forest Reserve and, more broadly, the coastal forests of Tanzania are highly threatened habitats that require further conservation attention. These habitats provide important sustainable resources for human populations (Burgess et al., 1992; Tanzania Forest Conservation Group, 2012) but forest resources are currently being used unsustainably and in many cases, illegally, depleted. The region is currently being subjected to an unprecedented level of human induced habitat change, and without drastic intervention the forests will be entirely lost in the coming years.

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