New insights into the taxonomic status, distribution and natural history of De Witte's Clicking Frog (*Kassinula wittei* Laurent, 1940)

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Abstract

Kassinula is a monotypic genus of small frog in the family Hyperoliidae, only represented by *Kassinula wittei*. This species morphologically resembles both *Kassina* Girard, 1853 and *Afrixalus* Laurent, 1944, and its taxonomic status has been debated for decades. It has previously been subsumed within *Kassina*, and is currently placed as a sister genus to *Afrixalus*, although it has not been included in any phylogenetic studies until now. This species is poorly represented in museum collections and is only known from fewer than 35 specimens from southern Democratic Republic of the Congo and adjacent Zambia. Newly collected material from central Angola, a range extension of 400–800 km west of previously known localities, allowed us to revisit the taxonomic placement of the genus with the aid of phylogenetic analysis and shed light on its geographic distribution, morphology and natural history. Although our phylogenetic analysis is limited to a single mitochondrial gene (*16S*), we place *Kassinula* in the subfamily Hyperoliinae and closely related to *Afrixalus*, with a high degree of confidence. Further phylogenetic studies are needed before formally synonymising *Afrixalus* with *Kassinula*.

Keywords: Amphibia; Angola; miniaturisation; Okavango; Hyperoliinae

Introduction

When Laurent (1940) worked through material that GF de Witte collected for the Congo Museum (now Musée Royal de l'Afrique Centrale, Tervuren, Belgium), he found a very small, unusual frog, which he described as a new genus and species, *Kassinula wittei.* He later concluded that the new genus more closely resembled *Kassina,* with which he synonymised it (Laurent and Combaz 1950). This taxonomic arrangement was followed by subsequent authors (Schmidt and Inger 1959; Liem 1970; Broadley 1971; Dubois 1987),

except for Perret (1985), who also regarded it as related to *Kassina*, but considering it as a subgenus rather than a full genus. Drewes (1984) resurrected the genus *Kassinula*, which has remained the accepted taxonomic arrangement (Tandy and Drewes 1985; Poynton and Broadley 1987; Schiøtz 1999; Channing 2001; Channing et al. 2012; Channing and Rödel 2019). In a subsequent study, Channing (1989) reanalysed Drewes' (1984) morphological characters of the Hyperoliidae and evaluated the taxonomic status of this species as being a sister taxon to *Afrixalus* and not closely related to *Kassina* as previously assumed by other authors. When *Kassinula wittei* was described in 1940, the genus *Afrixalus* did not exist yet. It was only described four years later, in 1944, and this partly explains the early placement of *Kassinula* as more closely related to *Kassina*, and the origin of the subsequent taxonomic confusion and discussions regarding the taxonomic placement of this genus.

Morphologically, some authors consider *Kassinula wittei* as *Afrixalus*-like (Poynton and Broadley 1987), while others refer to it as a small *Kassina*-like frog (Drewes 1984; Channing 2001; Channing and Rödel 2019). In the original description of *Kassinula wittei*, Laurent (1940) described the specimens he examined as not being fully grown, but the diminutive size of this paedomorphic genus has more recently been clarified to have resulted from miniaturisation (Yeh 2002). Although this was in relation to *Kassina* and not *Afrixalus*, which in itself was regarded to have undergone partial miniaturisation in relation to other Hyperoliidae. Adults range between 12.5 and 21.4 mm snout–urostyle length (SUL) (Schmidt and Inger 1959), in contrast with adults of *Afrixalus* and *Kassina*, which can reach a size of 20–40 mm and 32–52 mm SUL respectively (Channing and Rödel 2019). *Kassinula* has not been included in any phylogenetic studies to date (Frost et al. 2006; Portik et al. 2019), because of the absence of fresh tissue samples.

Kassinula can be distinguished from other Hyperoliid frogs based on the absence of vomerine teeth and the fusion of the external metatarsals to the basal phalanges (Laurent 1940). Drewes (1984) performed a detailed analysis of the osteology of Hyperoliidae and found that *Kassinula* possesses a posterolateral process of the hyoid, which is absent in other Hyperoliids, a vocal pouch apparatus that is very complex in structure in comparison to other 'kassinoid' frogs (e.g. *Kassina*, *Phlyctimantis* and *Tornierella = Paracassina*), and they retain cartilaginous intercalary elements in adults, paedomorphic characteristics that are common in amphibian lineages undergoing a miniaturisation process (Wells 2007). He concluded that *Kassinula* is closely related to *Semnodactylus*, which in turn is very similar to *Paracassina*. Reanalysis of available morphological data revealed a similar relationship, except that *Kassinula* is more similar to *Afrixalus* than to *Kassina*, although this study concluded that the relations between these genera are still unresolved (Channing 1989). That study erected four subfamilies within the family Hyperoliidae (Hyperoliinae, Kassininae, Tachyneminae, Leptopelinae), and placed *Kassinula* in the subfamily Hyperoliinae, alongside other genera such as *Afrixalus* and Hyperolius. In contrast, other similar 'kassinoid' looking genera (including *Kassina*, *Opisthothylax*, *Semnodactylus*, *Paracassina* and *Phlyctimantis*) were placed in a separate subfamily Kassininae (Channing 1989). In a recent phylogeny of the family Hyperoliidae, Portik et al. (2019) only recognised two subfamilies within the Hyperoliidae: Hyperoliinae and Kassininae. This partly agrees with the subfamilies proposed by Channing (1989), except for the placement of *Opisthothylax* and *Tachycnemis* within the subfamily Hyperoliinae, rendering the subfamily Trachycneminae invalid. The subfamily Leptopelinae had been transferred to the family Arthroleptidae (Frost et al. 2006). The complexity and somewhat confusing taxonomic history of *Kassinula* therefore remains

unresolved due mostly to the lack of genetic information to complement the current understanding based on existing morphological analyses.

Until recently, *Kassinula wittei* was only known to occur in the bogs and seepages in southern Democratic Republic of the Congo (DRC) and adjacent Zambia (Channing and Rödel 2019). The recent discovery and specimen collections of *Kassinula wittei* in central and eastern Angola (NGOWP 2017) allowed analyses of the taxonomic status of this genus using genetic information, and further improved understanding of the geographic distribution and natural history of this poorly documented species.

Materials and methods

Sampling

During recent expeditions (2016–2019) to the upper catchments of the Okavango, Cuando and Zambezi rivers in south-central Angola, as part of the National Geographic Okavango Wilderness Project (NGOWP), a large series of small Hyperoliid frogs (n = 40) were collected from seepages draining into the wetlands or larger source lakes (Figure 1). These were identified as *Kassinula wittei*, based on the small size, unique colour pattern and limited webbing of the hind feet (Channing 2001; Channing and Rödel 2019). This species was previously only known from northern Zambia and adjacent DRC. Each specimen was collected as a voucher, fixed in 10% formalin and thereafter transferred to 70% ethanol for long-term storage at the Port Elizabeth Museum (PEM). Representative material will be returned to the Instituto Nacional da Biodiversidade e Áreas de Conservação (INBAC) and Instituto Superior de Ciências da Educação da Huíla (ISCED). Prior to formalin fixation, liver or thigh muscle samples were collected and preserved in 99% ethanol for future genetic analyses. Additional specimens were also collected in a flooded grassland near Mona Quimbundo, Lunda-Sul Province and among miombo leaf litter near Congolo River Ranger Camp, Luando Nature Strict Reserve, Malanje Province as part of ad hoc surveys by PVP (2018–2019), and are housed in Luanda at the Kissama Foundation Collection (FKH0150–51 and FKH091, respectively).

Phylogenetic analyses

Genomic DNA was isolated from tissues with a standard salt extraction method (Bruford et al. 1992) using lysis (Buffer ATL; Qiagen) and elution (Buffer AE; Qiagen) buffers. Standard PCR procedures were utilised to amplify one partial ribosomal gene (16S ribosomal RNA [16S rRNA]). PCR amplification was carried out using the primer pair L2510 (5'-CGCCTGTTTATCAAAAACAT-3') and H3080 (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al. 2002). Amplification was carried out using 20–50 ng µl−1 extracted genomic DNA, in a 25 µl PCR reaction, containing 12.5 µl TopTaq Mastermix (Qiagen; containing 10x PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, and 0.75 U Taq polymerase), 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), and 8.5 µl of the genomic DNA and denucleated water combined (1–2 µl DNA and 6.5–7.5 water). The cycling profile for all the genes was as follows: initial denaturing step at 94 °C for 5 min, followed by 35–40 cycles of 94 °C for 30 s, 50–54 °C for 45 s, and 72 °C for 45 s (35 cycles), with a final extension at 72 °C for 8 min. The prepared PCR products were sent to Macrogen Corp. in Amsterdam, Netherlands for sequencing (after purification) with the forward primers only. Newly generated sequences were deposited on Genbank (MT938914–20).

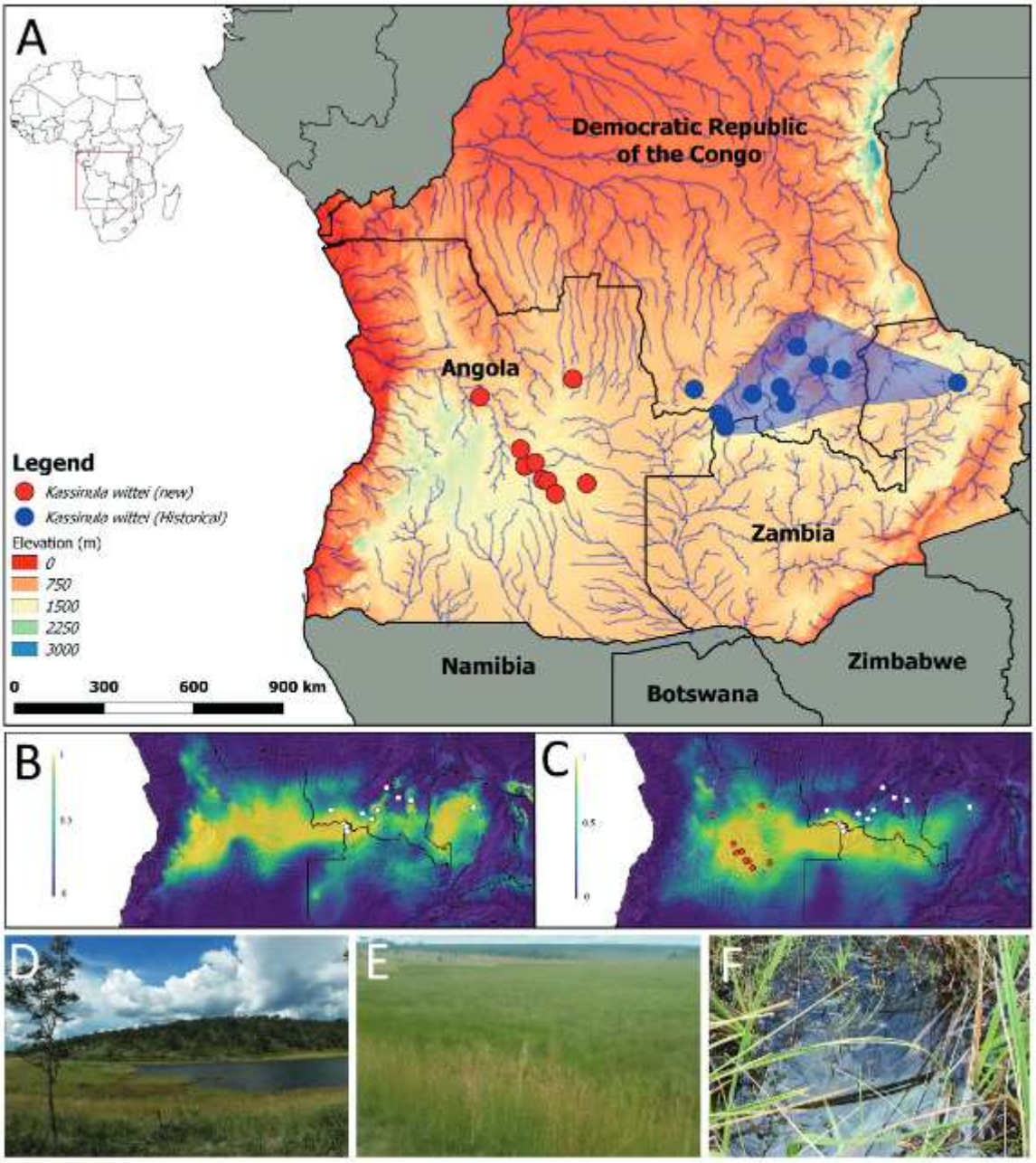

Figure 1: A – updated geographical distribution of *Kassinula wittei* Laurent, 1940. Red dots represent the new records documented in the current study, while blue dots and blue polygon represent the known historical records and interpreted distribution by IUCN (2013) respectively (IUCN interpreted distribution does not encompass all historical records). B – species distribution model for historical records exclusively, C – species distribution model for all records. D, E, F – habitat where species was found at Cuanavale River source lake, Cuando Cubango Province, Angola. Photos: D, E, F – Werner Conradie

The sequence trace files were manually checked for errors using BioEdit Sequence Alignment Editor v.7.2.5 (Hall 1999) and aligned, along with the previously accessioned GenBank sequences, using MUSCLE v.3.7 in CIPRES (Edgar 2004). The dataset was supplemented with sequences from Portik et al. (2019), deposited on Genbank. Two nuclear (RAG1, TYR) and one mitochondrial (16S) gene alignment were obtained from Portik (2018), and were pruned to include members of Hyperoliinae, Kassininae and two species of Arthroleptis (outgroup). Three separate alignments were created in MEGA X v.10.1.7 (Kumar et al. 2018; 16S: 539 bp, RAG1: 895 bp, TYR: 621 bp) and the non-aligning section

of 16S was removed from the alignment resulting in 336 bases being retained for further analysis.

Individual gene trees were constructed in MEGA X using the Maximum Likelihood algorithm, with 100 bootstrap replicates and the GTR+I+G nucleotide substitution model. The Congruence Index (I_{cong}; http://max2.ese.u-psud.fr/icong/index.help.html; de Vienne et al. 2007) was utilised to test for congruence between individual gene trees. All gene-tree combinations were found to be congruent and a concatenated dataset of the three genes was created for additional phylogenetic analyses.

The first, second, and third codon positions of all three alignments were tested individually for saturation using DAMBE v.6.4.67 (Xia 2013), and saturation was found to be absent. The best-fitting models of molecular evolution were selected using Partitionfinder 2 (Lanfear et al. 2016) with the following settings: BIC model selection criterion, rBayes models, linked branches and all partition schemes searched. The best-fitting model scheme selected included 3 partitions: 16S (SYM+I+G); RAG1 1, RAG1 2, TYR 1, TYR 2 (GTR+I+G); and, RAG1 3, TYR 3 (K80+G).

Bayesian inference (BI) analysis was estimated on the CIPRES Science Gateway XSEDE online resource (http://www.phylo.org; Miller et al. 2010; Tamura et al. 2013) using a Bayesian analysis (MrBayes v.3.2.7a; Ronquist et al. 2012) with uniform priors for all parameters, using the best-fit nucleotide substitution models for each gene codon partition (see above). Using the Markov Chain Monte Carlo (MCMC) analysis, two parallel runs of 20 million generations were performed, with trees being sampled every 1 000 generations, using BEAGLE to speed up the process. The number of generations discarded as burn-in was determined using Tracer v.1.6.0. (Rambaut and Drummond 2007). The effective sample size (ESS) was found to be above 200 for all parameters and the runs reached convergence, indicating that a burn-in of 10% was adequate.

Maximum likelihood (ML) analysis was conducted using the GTRGAMMA model in RAXML-HPC v.8.2.12 (Stamakis 2014) on the CIPRES Science Gateway. A random starting tree was used and the ML analysis was assessed using the rapid bootstrap method, a codon partition scheme, and 1 000 bootstrap replicates. Both trees were viewed in Figtree v.1.4.2 (Rambaut 2014). To investigate the phylogenetic relatedness of *Kassinula*, sequence divergence values of 16S were estimated using the uncorrected pairwise distance model in MEGA X, using 500 bootstrap replicates.

Morphology

Specimens were measured to the nearest 0.1 mm using digital callipers under a Nikon SMZ1270 dissecting microscope for the following 15 morphological characters as defined by Watters et al. (2016): snout–urostyle length (SUL, direct-line distance from tip of snout to posterior margin of vent), head width (HW, at the widest point; gonial angle at the jaws), head length (HL, from the posterior edge of the jaws to the tip of the snout), interorbital distance (IOD, the shortest distance between the anterior corners of the orbits), eye diameter (ED, horizontally from the anterior to the posterior corner of the eye), eye–nostril distance (EN, from the anterior corner of the eye to the posterior margin of the nostril), internarial distance (IND, shortest distance between the inner margins of the nostrils), snout length (SL, distance from the tip of the snout to the anterior corner of the eye), tibia length (TL, distance from the outer surface of the flexed knee to the heel/tibiotarsal inflection), foot length (FL, from the base of the inner metatarsal tubercle to the tip of toe IV), thigh length (THL,

distance from the vent to the knee articulation), hand length (HAL, from the base of the outer palmar tubercle to the tip of finger IV), forearm length (FLL, from the flexed elbow to the base of the outer palmar tubercle), upper eyelid width (UEW, greatest width of the upper eyelid margins, measured perpendicular to the anterior-posterior axis), and finger IV disk width (Fin4DW, the widest horizontal diameter of finger IV). All measurements were taken on the right side of the body. Webbing formulae follow the scheme of Rödel (2000).

Call analysis

Advertisement calls of 10 individuals were recorded in the field using a Samsung Galaxy Note 3 mobile phone from the Cuanavale River Source (15, 28 February, 1 March 2016), a Blackview BV90000Pro-F mobile phone from lower Quembo River (28 November 2019) and a Edirol R09 recorder with directional microphone from Mona Quimbundo (12 March 2019). Ambient temperature was not recorded. For each individual, two consecutive calls were selected from field recordings to measure the temporal and spectral characteristics. Following Conradie et al. (2018), temporal characteristics of the recorded advertisement calls were recorded using a cut-off limit of 10% of the peak amplitude using custom software (Verburgt et al. 2011). Spectral characteristics were calculated per call using Fast Fourier Transform with an FFT frame size of 1 024 samples, a 75% overlap, a Hamming window and a frequency resolution of 21.53 Hz. All recordings were initially filtered with a high-pass filter with a limit of 1 000 Hz. The frequency bandwidth of a call was measured at 10 dB below the frequency of maximum energy. Acoustic terminology followed the recommendations for anuran call descriptions described by Köhler et al. (2017) and graphical presentations of calls were produced with the R package Seewave (Sueur et al. 2008). All advertisement calls used for the call analysis have been deposited for curation with Fonoteca Zoológica (FonoZoo; www.fonozoo.com) with the following accession numbers: FZ 1027–36.

Mapping and species distribution modelling

To produce a contemporary geographical distribution map for *Kassinula wittei,* we sourced observation locality data from published datasets (e.g. Laurent 1940; Schmidt and Inger 1959; Broadley 1971; Drewes 1984), museum databases (PEM, AMNH - American Museum of Natural History), and online databases (http://www.vertnet.org). The online GeoNames gazetteer (http://www.geonames.org/) or GEOLocate Web Application (https://www.geo-locate.org/web/WebGeoref.aspx) were used to georeference all historical data. Distribution data were mapped in QGIS v.3.2 (http://qgis.org).

To analyse the bioclimatic features affecting the habitat suitability for K. wittei, we downloaded 19 bioclimatic variables and elevation data from the WorldClim data set (Fick and Hijmans 2017; http://www.worldclim.org/) at a spatial resolution of 30 arc-second (~1 km2). For those variables, we ran a correlation model to eliminate collinearity between variables in the sampled area and within sample points (Candau and Fleming 2005). To describe the bioclimatic envelope, we selected variables that reflect the average and extremes of temperature/precipitation, to capture all the climatic influence over the distribution of the species (Bittencourt-Silva et al. 2016; Alba-Contreras 2018; Lourenço-de-Moraes et al. 2019). This was achieved by using a correlation threshold of 0.7 (e.g. Dor et al. 2013) to discriminate between groups of variables (Judge et al. 1982; Kalnins 2017), resulting in the following predictor variables used for the models: annual mean temperature (BIO1), maximum temperature of the warmest month (BIO5), minimum temperature of the coldest month (BIO6), annual mean precipitation (BIO12), and precipitation of driest quarter

(BIO17).

For the species distribution models, we used all macroclimatic variables selected previously (BIO1, BIO5, BIO6, BIO12, and BIO17), and fitted the models in the maxent R-package, based on point processes (Phillips et al. 2017). The sampling area included a buffer of 400 km North-East-South from the most peripheral observations of K. wittei, while we delimited the western border by the coastline, to avoid bias for the ecological requirements of the species (Carretero and Sillero 2016). We used hinge features only with the regularisation parameter set to 2.5 to produce smoother response curves and reduce overfitting (Briscoe et al. 2016; Enriquez-Urzelai et al. 2019). To evaluate the MaxEnt models we assessed 10-fold cross-validation (Enriquez-Urzelai et al. 2019). For each repetition, we used 70% of the data and computed the area under the ROC curve (AUC) with the remaining 30%. Finally, we averaged the AUCs of the 10 repetitions.

We ran the MaxEnt model with two different input datasets, in order to compare potential geographic distribution between the historical data only and all known observation localities, respectively.

Results

Phylogenetic analyses

The four lowest uncorrected pairwise distances of 4.77 ± 0.74 (standard error), 4.25 ± 0.94, 4.73 ± 1.06 and 4.41 ± 1.07 separated *Kassinula wittei* from *Afrixalus*, *Heterixalus*, *Paracassina* and *Tachycnemis*, respectively (Table 1). The low p-distance values separating *Kassinula wittei* from various taxa across Hyperoliidae supports the placement of this genus within the family and more specifically within the subfamily Hyperoliinae. An intraspecific divergence of ~1% separates the samples indicating relatively low levels of genetic diversity among Angolan *Kassinula wittei*. Both phylogenetic algorithms found congruent topologies (Figure 2, Supplementary Figure), with an increased support for the Bayesian topology. The differing placement of several taxa (i.e. Acanthixalus spp.) and lower support values within the phylogeny, compared with Portik et al. (2019), are likely an artefact of the smaller dataset afforded to this study. Both algorithms placed *Kassinula wittei* within the *Heterixalus* + *Afrixalus* clade, with Bayesian inference further supporting its placement within the *Afrixalus* genus recovering a supported relationship between *Kassinula wittei* and *Afrixalus* cf. laevis.

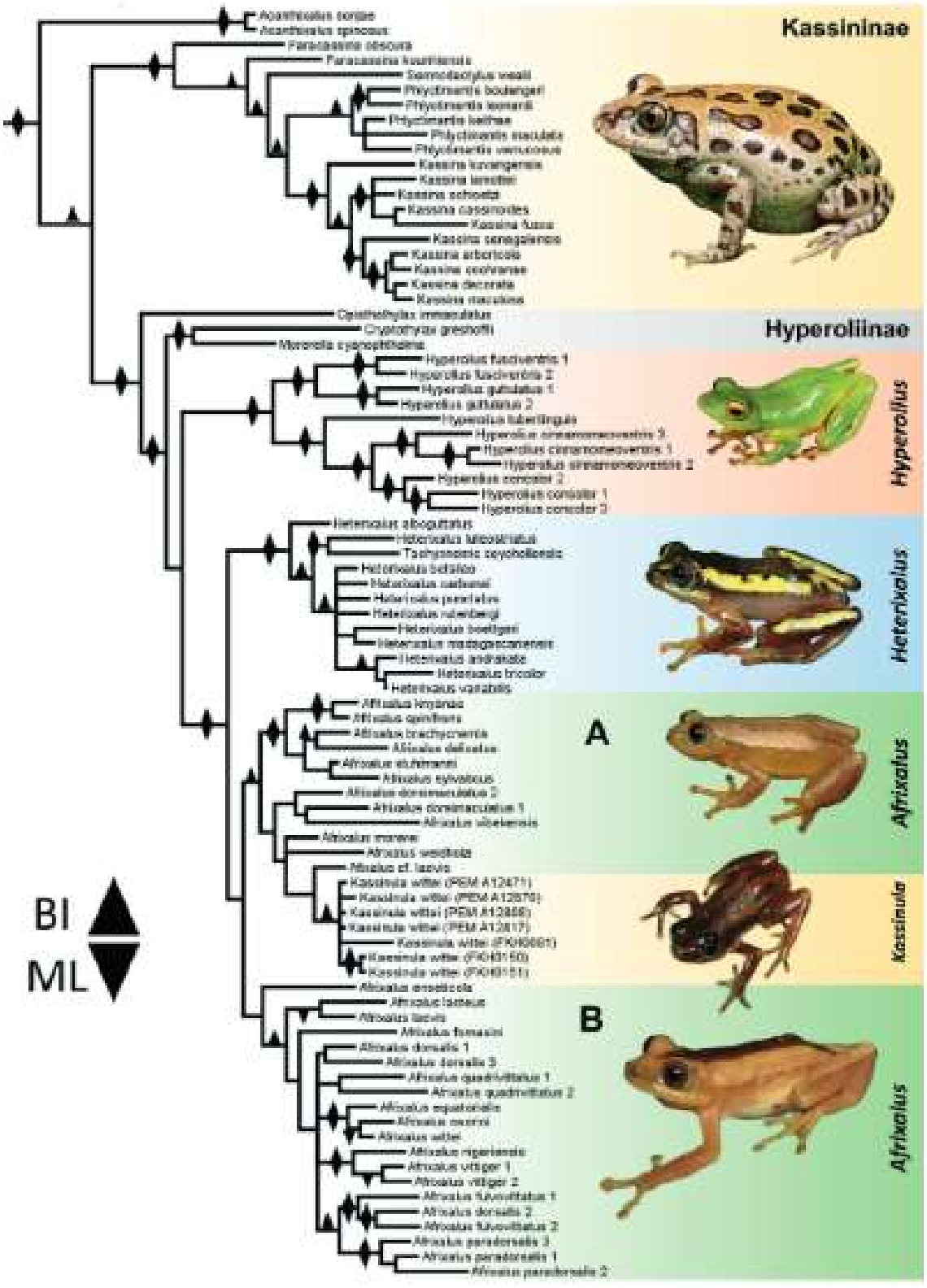

Figure 2: Bayesian Inference (BI) tree with Maximum Likelihood (ML) support overlaid. Black triangles denote significant support at the nodes. ML bootstrap values ≥75% and BI posterior probabilities ≥0.90 were considered supported

Table 1: Sequence divergences (uncorrected pairwise distances) between genera for 16S. The values in bold are mean intraspecific sequences divergences, the values below are mean interspecific sequence divergences values and the values above are standard errors

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Acanthixalus*	**1.00**	1.30	1.82	1.61	1.43	1.35	1.27	1.41	1.27	1.43	1.30	1.22	1.53	1.55
2	*Afrixalus*	8.78	**6.00**	1.47	1.19	0.97	1.25	1.03	0.74	0.98	1.13	0.97	1.02	1.22	1.01
3	*Arthroleptis*	14.29	11.72	**4.00**	1.56	1.58	1.57	1.70	1.53	1.60	1.69	1.75	1.68	1.78	1.60
4	*Cryptothylax*	9.23	8.13	10.97	**na**	1.41	1.29	1.30	1.30	1.34	1.56	1.44	1.35	1.28	1.39
5	*Heterixalus*	8.26	6.68	10.56	7.63	**1.00**	1.44	1.24	0.94	1.23	1.43	1.18	1.17	1.53	0.75
6	*Hyperolius*	11.26	12.11	14.91	10.76	12.14	**9.00**	1.20	1.31	1.27	1.30	1.24	1.18	1.22	1.41
7	*Kassina*	6.73	7.75	12.41	7.15	7.12	10.60	**2.00**	1.07	1.12	1.29	0.78	0.92	0.93	1.21
8	*Kassinula*	7.53	4.77	9.87	6.51	4.25	10.97	5.96	**1.00**	1.11	1.21	1.06	1.04	1.33	1.07
9	*Morerella*	6.25	6.60	10.82	6.55	6.15	10.11	5.55	5.23	**na**	1.28	1.08	1.09	1.36	1.21
10	*Ophistothothylax*	8.04	7.92	11.43	9.82	8.32	10.72	7.06	5.95	6.55	**na**	1.17	1.18	1.43	1.42
11	*Paracassina*	6.14	6.46	11.48	7.44	6.12	9.72	3.26	4.73	4.12	5.13	**1.00**	0.70	1.09	1.26
12	*Phlyctimantis*	6.34	7.44	12.21	7.41	6.73	10.32	4.57	5.24	5.35	6.03	2.70	**3.00**	0.98	1.21
13	*Semnodactylus*	8.04	8.64	13.24	5.95	9.14	10.44	4.07	7.27	6.85	7.74	3.88	4.12	**na**	1.50
14	*Tachycnemis*	9.28	6.52	10.14	7.19	2.55	11.42	6.68	4.41	5.39	7.78	5.81	6.47	8.08	**na**

Species distribution modelling

The species distribution model showed high AUC values (mean AUC = 0.963 ± 0.0172, standard deviation, with 95% confidence intervals: 0.947–0.972) indicating good fit n the model performance. The variable importance tests indicated that minimum temperature of the coldest month (BIO6) alongside annual mean precipitation (BIO12) are the most important variables (mean AUC = 0.986 and 0.257, respectively) influencing the distribution of K. wittei. Climatic suitability decreased drastically when BIO6 increased above ~ 8 °C and BIO12 fell below ~1 000 mm.Models derived from using only the historical records indicated that the Angola plateau potentially supports suitable habitat for *Kassinula* (Figure 1c). This prediction was verified by the new records obtained from south-central Angola. The refined model, which includes all known localities for this species, predicts K. wittei to be restricted to the central and eastern Angolan plateau eastward through to north-western Zambia, with lower suitability extending eastwards to Malawi (Figure 1b).

Systematic Account

Because of the fact that this species is very poorly represented in museum collections and that information is scattered among numerous scientific papers, we present a consolidated systematic account of *Kassinula wittei*, which includes the newly collected material from Angola.

***Kassinula wittei* Laurent, 1940**

De Witte's Clicking Frog (Figure 3)

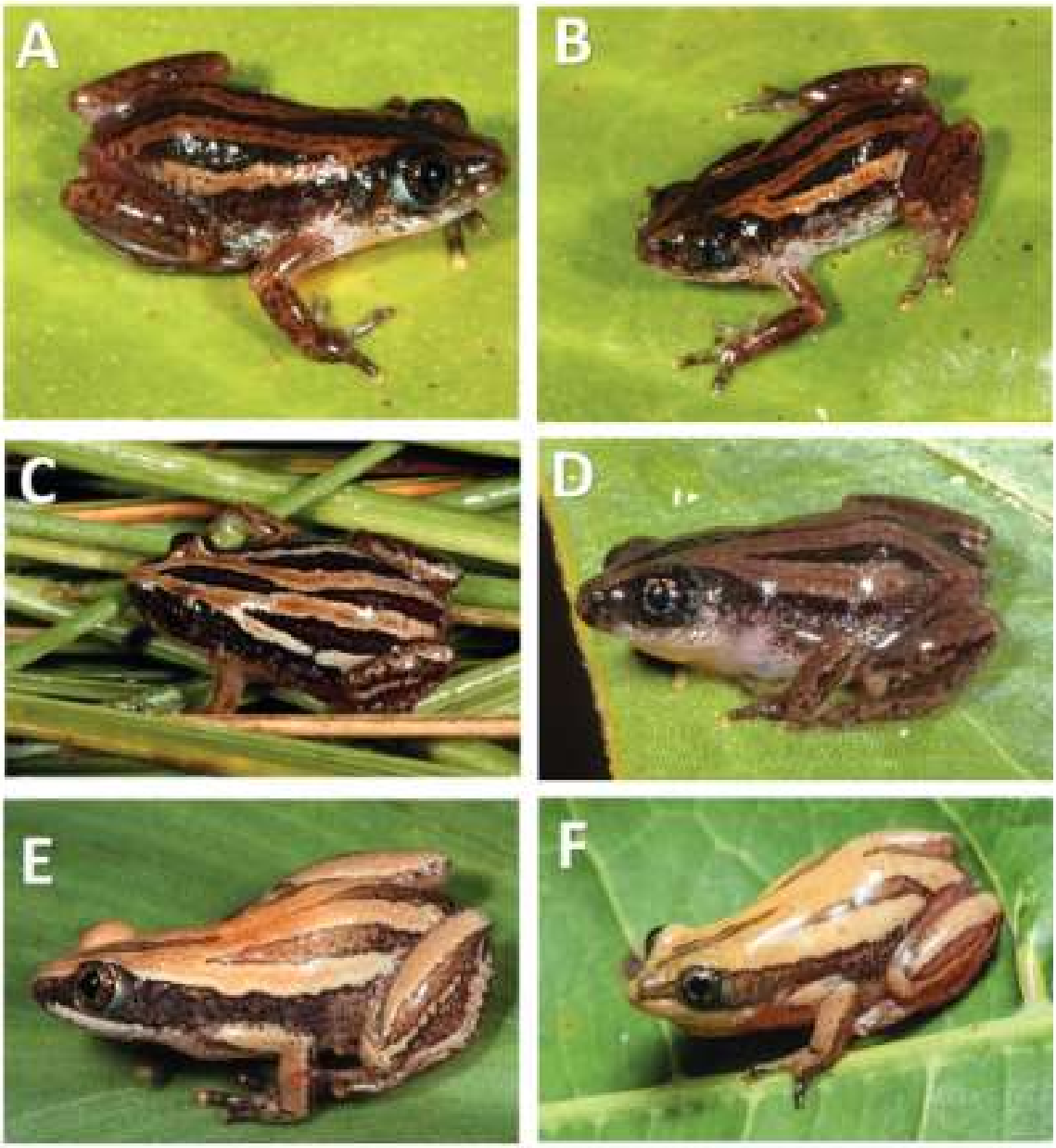

Figure 3: A photograph series of live *Kassinula wittei* from Angola. A, B, C – Cuanavale River Source, D – Mona Quimbundo, E – Congolo River Ranger Camp, Luando Nature Strict Reserve, F – west of Quemba town. Photos: A, B, C – Werner Conradie, D, E – Pedro Vaz Pinto, F – Chad Keates

Chresonymy: Laurent 1940:314; Laurent 1941:106; Laurent and Combaz 1950:273; Schmidt and Inger 1959:186; Liem 1970:10; Broadley 1971:120; Schiotz 1975:64; Drewes 1984:55–56; Drewes 1985:186; Tandy and Drewes 1985:191; Poynton and Broadley 1987:184; Dubois 1987: 37; Channing 2001:185; Channing, Rödel, Channing 2012:239; Channing and Rödel 2019: 146

Holotype: MRAC 21524, collected from Kansenia (approx. −10.31256°, 26.03999°), DRC by GF De Witte on 17 July 1931.

Paratypes (2): MRAC 34511, collected from Kanzenze (approx. −10.52000°, 25.20667°), DRC by G.F. De Witte on 22 August 1931 and MRAC 31431, collected from Kando (near Tenke) (−10.80000°, 26.22000°), DRC by GF De Witte.

Additional material examined (40 specimens): PEM A12468–75, PEM A12792–3, INBAC (2), PEM A14274–5, −13.08537°, 18.89098°, Cuanavale River source lake, Moxico Province, Angola, 28 February 2016; PEM A12870–2, INBAC (1), −13.13624°, 19.04591°, Quembo River source lake, Moxico Province, Angola, 29 October 2016; PEM A12807–12, −12.68727°, 18.36067°, Cuito River source lake, Moxico Province, Angola, 24 November 2016; PEM A12817–8, −12.66925°, 18.35206°, Cuiva River source, Moxico Province, Angola, 25 November 2016; PEM A14271–3, WC-6745 (INBAC), −12.58117°, 18.67106°, Lungué-Bungo (= Lungwebungu) River camp, first oxbow on right side, Moxico Province, Angola, 18 November 2019; PEM A14276–80, WC-6865 (INBAC), −13.51877°, 19.28487°, Quembo River eastern tributary (Micongo River) past village, Moxico Province, Angola, 27 November 2019; PEM A14281–4, −13.20191°, 20.22144°, Luio River camp floodplains, Moxico Province, Angola, 30 November 2019; PEM A14270, PEM A14285, −12.16960° 18.22965°, west of Quemba, Moxico Province, Angola, 17 November and 10 December 2019, respectively.

General Description: Minute stocky frog; pupil vertical, eyes large; tympanum not visible externally; vomerine teeth absent; males with a single large folded circular gular pouch with a free posterior flap (see illustration in Schmidt and Inger [1959], p 186); tips of toes and fingers slightly swollen into small discs; fingers without webbing; webbing reaching less than half-way between tubercles of the third toe; fifth toe has a distinct proximal basal tubercle, with the basal phalanx of fifth toe fused to the fourth toe; inner metatarsal tubercle short, but fairly wide; subarticular tubercles well developed. Outer metatarsal tubercle indiscernible.

Description of newly collected material: Morphological measurements are presented in Table 2. Body slender, widest at midbelly, with a narrow head (HW/SUL 0.3). The head is acutely rounded from above and rounded in profile. Head length moderate (HL/SUL 0.4). Nostrils small, rounded, pointed upwards, positioned closer to the snout than to the eye (EN/SL 0.6). Internarial distance is less than distance between eye and nostril (IND/EN 0.8). Eyes directed anterolaterally, protruding, and barely visible from below, relatively small (ED/HW 0.4; ED/SUL 0.1), nearly equal to snout length (ED/SL 0.9). Pupils vertical. Distance between anterior corners of eyes double the internarial distance (IND/IOD 0.5). The angle of the jaw is situated posteriorly to the posterior edge of the eye. Tympanum not visible, but a slight skin elevation in this area is observed. Jaws without dentition; choanae large, oval in shape, located at anterior margins of roof of mouth; vomer processes and teeth absent; tongue long (up to 5.0 mm), narrow proximally, broad distally (maximum 3.0 mm), slightly bifurcated distally, proximally attached to lower jaw. No median lingual papilla present. The dorsal surfaces of the head, trunk and limbs are smooth, with no glands and skin folds present. Supratympanic fold inconspicuous to absent. Ventral surface smooth.

Table 2: Morphological measurements (mm) of newly collected *Kassinula wittei* specimens (see materials and methods for explanation of abbreviations, mean ± standard deviation)

	Females (n = 1)	Males (n = 39)
SUL	16.7	14.2 ± 1.7
HW	4.6	4.2 ± 0.5
HL	5.3	5.0 ± 0.4
IOD	2.6	2.1 ± 0.3
ED	1.6	1.7 ± 0.3
EN	1.3	1.2 ± 0.1
IND	1.1	1.0 ± 0.1
SL	2.1	2.0 ± 0.3
TL	6.4	5.4 ± 0.7
THL	5.9	6.0 ± 0.7
FL	6.5	5.9 ± 0.7
HAL	3.9	3.6 ± 0.4
FLL	3.4	3.0 ± 0.3
UEW	0.9	0.9 ± 0.1
Fin4DW	0.4	0.4 ± 0.1

The forelimbs are slender, hand small (HAL/SUL 0.3), fingertips slightly rounded into small discs (FIN4DW 0.4). Relative finger lengths I<II<IV<III; subarticular tubercles distinct, rounded, with one on finger I, two on fingers II African Zoology 2020, 55(4): 311–322317 to IV, with the proximal subarticular tubercles small, but distinct. No webbing between fingers. Thenar tubercle small, rounded, partially obscured by nuptial pad that reaches the distal phalanx of the first finger; palmar tubercles and inner metacarpal tubercles absent. No Table 2: Morphological measurements (mm) of newly collected Kassinula wittei specimens (see materials and methods for explanation of abbreviations, mean ± standard deviation) supernumerary tubercles are present on the palm.

Hind limbs short (TL/SUL 0.4; FL/SUL 0.4), foot nearly equal to tibia length (TL/FL 0.9); thighs are moderately developed and equal in length to tibia (TL/THL 0.9); relative toe lengths are I<II<V<III<IV. The toe tips are slightly expanded into small discs; subarticular tubercles: one on toes I and II, two on toes III and V, and three on toe IV. Webbing formula: toe I (0), toe II i/e (0.5/1), toe III i/e (1/1), toe IV i/e (2.75/2.75), toe V (2). Toe V and VI fused up to the distal subarticular tubercle with only a slight trace of webbing between them. Inner metatarsal tubercle conical and prominent, outer metatarsal tubercle absent.

Colouration: Dorsum dark brown with paravertebral and lateral light brown bands that merge behind the eye; a fine dark brown line or series of stipples runs along the centre of each light brown band; dark vertebral band sometimes interrupted midway towards the posterior; dark brown band from snout to groin; tibia dark brown, with irregular light brown spots or transverse band; iris dark brown to golden with a black vertical pupil; venter white; toe disc yellow; throat of males yellow.

Size (SUL): Males = 12.5–22.0 mm; Females = 16.7–21.4 mm (see Table 2). Specimens collected from the peatlands (defined as permanent wetlands containing a minimum of 30 cm peat depth, of which at least a third consists of dead organic material [Grundling and Grootjans 2016]) in central Angola tend to be smaller (males = 12.6–15.6 mm; female = 16.7 mm) than both eastern Zambia and DRC (males 12.5–19.5 mm; females = 18.4–21.4 mm)

and western Angolan specimens (males 19.2–22.0 mm; females = unknown) collected from streams or flooded areas.

Advertisement call: This species calls from grassy bogs, seepages, flooded wetlands or the flooded margins of streams at higher altitudes. The call has been described as a series of brief, high-pitched double metallic clicks, lasting 0.1 s and separated by 0.2 s, with a dominant frequency of 4.0–4.2 kHz (Schiøtz 1975, 1999; Channing 2001; Channing and Rödel 2019). This characterisation of the call does not follow the recommendations of Köhler et al. (2017) and is somewhat different from our findings, most likely because of differences in ambient temperature when recordings were made. *Kassinula wittei* calls recorded in this study (Figure 4) consist of a number of note groups, with each note group comprising of two notes (analogous to the double clicks referred to above) although the last note group of a call may have only a single note or a very soft second note. Each note has two or more distinct pulses, with the first pulse being of high amplitude and the preceding pulses of much lower amplitude (Figure 4). Temporal and spectral parameters of the calls are provided in Table 3, showing that the duration of the note groups (91.77 ± 5.34 ms) and the 'separation' of the note groups (analogous to inter-note group interval; 190.78 ± 10.71 ms) are similar to the 100 ms and 200 ms, respectively, reported by Channing (2001) for these parameters. The maximum call energies recorded in this study (4 760.2 ± 64.36 [4 522–5 109] Hz) were however higher than the range reported by Channing (2001). It is most likely that these differences are because of slightly warmer temperatures for recordings in this study, which would be congruent with quicker repetition rates of notes or note groups, shorter notes and higher maximum call energies (Gerhardt and Huber 2002).

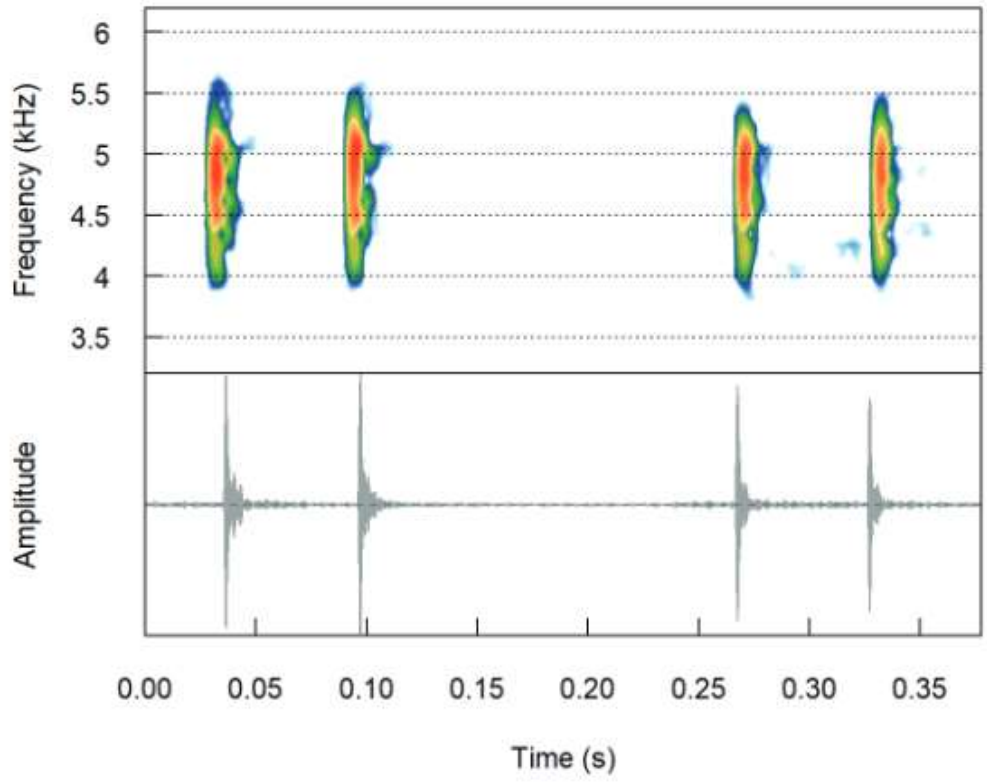

Figure 4: A single advertisement call of a *Kassinula wittei* from the Cuanavale River source, Angola, showing two note groups per call with each note group characteristically consisting of two notes

Table 3: Advertisement call parameters measured for *Kassinula wittei* (n = 10). All temporal and spectral parameters are presented in milliseconds (ms) and Hertz (Hz) respectively. Values are mean ± standard error with the range provided in brackets

Call parameter	
Number of note groups per call	2.3 ± 0.15 [1–4]
Call duration	457.22 ± 52.85 [278.47–753.88]
Call period	4 455.21 ± 308.62 [3 302.66–6 639.25]
Calls repetition rate (per minute)	14 ± 0.87 [9.04–18.17]
Dominant frequency range (Hz)	952.7 ± 130.35 [354–1 573]
Inter-call interval	3 998.15 ± 298.71 [3 005.56–6 187.19]
Inter-note group interval	190.78 ± 10.71 [145.14–233.21]
Maximum call energy (Hz)	4 760.2 ± 64.36 [4 522–5 109]
Note duration	6.5 ± 0.68 [2.32–10.33]
Note group duration	91.77 ± 5.34 [66.06–114.18]
Note group period	283.91 ± 15.84 [212.78–355.83]
Note period	83.73 ± 4.85 [63.02–108.67]

Natural History*:* *Kassinula wittei* breeds in flooded grassy areas, where males call from the base of grass stems, which are partially submerged (Figures 1d-f and Figure 3). We found them calling both low down and high up on stems (~50 cm), similar to other Hyperolius and *Afrixalus*, in February and March, and October through to December. A single female collected in November was gravid. In Angola, it was found sympatrically with, and in the same microhabitat as Hyperolius angolensis, H. bocagei, H. nasutus, H. raymondi, Phrynobatrachus mababiensis, Ptychadena keilingi, P. taenioscelis and P. uzungwensis. Schmidt and Inger (1959) suggest, based on the small size reported by Laurent (1940), that metamorphosis takes place at 12 mm SUL. Reproduction and tadpoles are unknown (Channing et al. 2012), but it presumably takes place in flooded grassland. In frog species undergoing a miniaturisation process, strategies that involve deposition of terrestrial eggs with direct development or hatch into tadpoles that remain in the nest are common (Wells 2007). All Hyperoliids are known to have tadpoles that metamorphose in water, and discovering the reproductive mode of *Kassinula* would help to understand its miniaturisation process. The diet of this species is unknown, but it probably feeds on tiny prey such as ants, termites, mites, and collembolans, items that are common in the diet of miniaturised frogs and scarce in the diets of larger species (Wells 2007).

Habitat: It occurs mainly in moist savanna and miombo woodlands, where it breeds in subtropical or tropical seasonal wetlands or flooded grassland (Figures 1d-f; Channing and Rödel 2019). Also associated with widely spaced freshwater lakes and marshes/seepages, and occurring in grassy bogs on a granite outcrop near the Zambezi Rapids, Mwinilunga District (Poynton and Broadley 1987). At most of the Angolan sites, this species was closely associated with peatbogs on the edges of the source lakes or associated floodplains. It has been found at elevations of 1 181–1 750 m above sea level (Schmidt and Inger 1959; this study).

Distribution: This species is known from southern DRC, western and northern Zambia and eastern and central Angola (Figure 1a). Our species distribution model indicates that it might be much more widely distributed, but that the main distribution is restricted to the central regions of interior Angola and north-western Zambia.

Localities (Figure 1a): Angola: Cuanavale River source lake, −13.08537° 18.89098°; Quembo River source lake, −13.13624° 19.04591°; Cuito River source lake, −12.68727° 18.36067°; Cuiva River source, −12.66935° 18.35206°; Lungwebungu River crossing, −12.58117° 18.67106°; Quembo River eastern tributary (Micongo River), −13.51877° 19.28487°; Luio River floodplains, −12.58117° 18.67106°; west of Quemba, −12.16960° 18.22965°; Mona Quimbundo, −10.06447° 19.81518°, Congolo River Ranger Camp, Luando Nature Strict Reserve, −10.59639° 17.01417°.

Democratic Republic of the Congo: Bwalo, −9.78306° 27.89111°; 'Kando pres de Tenke', 10.83333° 25.73333°; Kansenia, −10.31256° 26.04000°; Kanzenze, −10.52000° 25.20667°; Kasaji, −10.36667, 23.45000; Lufwa, −9.66167° 27.19500°; Mukelengia, left affluent Kalumengongo and right affluent Lualaba, Upemba National Park, −9.08121° 26.54338°.

Zambia: 20 km N (by road) of Ikelenge, Zambezi River, Zambezi Rapids, −11.12540° 24.19115°; Hillwood Farm, Chinkumina Dambo, −11.25445° 24.32695°; Hillwood Farm, Sakeji River,−11.25781° 24.32039°; Mungwi, Kasama, N. Rhodesia, −10.17306, 31.36917; 'Chitanta Plain, 29 km N of Mwinilunga', −11.50490° 24.37945°.

Discussion

Based on the evidence from a single genetic marker (16S), *Kassinula* can be placed in the subfamily Hyperoliinae with a high degree of confidence and is shown to be closely related to the genus *Afrixalus*. This agrees with the placement proposed by Channing (1989), which was based solely on morphology. Both the BI and ML analysis (Figure 2; Supplementary Figure) place *Kassinula* within the genus *Afrixalus*, rendering the latter paraphyletic. The discordance of the two algorithms, in placement of several taxa is likely a product of the limited dataset afforded to this study and may change with increased genetic sampling and addition of additional genes, especially slower evolving nuclear genes. Drewes (1984) justified that the smaller size of *Kassinula*, the retention of paedomorphic features such as cartilaginous intercalary elements in the skeleton as adults, and extensive skeleton features should provide it with generic status. Adding to this, *Kassinula* differs morphologically from *Afrixalus* by the presence of fused outer toes, the near absence of webbing and a very complex vocal apparatus. For these reasons, we refrain from synonymising *Afrixalus* with *Kassinula*, pending additional genetic sampling and improved phylogenetic support. Should further work support the above taxonomic adjustment, then we will see *Afrixalus* being synonymised with *Kassinula*, as the latter takes taxonomic priority (Laurent 1940, 1944). Our results and those of Portik et al. (2019) separate *Afrixalus* species between West and East Africa as well as between forest/forest edges or savanna/grassland species. However, there are a few exceptions to this rule, such as *Afrixalus fornasini*, an East African savanna species that groups with the West Africa forest clade. *Kassinula* is a savanna species that groups with the East Africa savanna/grassland *Afrixalus* clade. These relationships are interesting and warrant further investigation.

We did not have genetic material or sound recordings from the eastern populations of the DRC or Zambia to test the specific status of the newly collected Angolan opulations. We do not however, anticipate any species-level difference between eastern and western material, because we expect these populations to be connected, as shown by the species distribution models (Fig 1b–c). Furthermore, the new material is morphologically very similar to those of DRC/Zambia, except that males collected from eastern Angola around the peatlands are on

average smaller. The populations found in the Cuanza River basin in western Angola and the ones from eastern DRC/Zambia are larger in size, and thus associated with a more terrestrial lifestyle (Drewes 1984). Zambian frogs were found in seepages over rocks, while the western Angolan individuals were found among leaf litter in miombo woodland, a microhabitat typically used by miniaturised frogs (Wells 2007).

The newly collected material from central Angola represents a >400 km range extension to the west from the nearest DRC/Zambian records. The currently known geographical distribution of the species falls entirely within the Angolan Miombo Woodland and the Central Zambezian Miombo Woodland ecoregions (Burgess et al. 2004). This wide area broadly overlaps with Poynton's (1999) Zaire-Zambezi biogeographical region defined for African amphibians, that includes the extensive watershed of the upper Zaire and Zambezi river systems with around 1 750 000 km2. Interestingly, *Kassinula* is the only known endemic genus to the Zaire-Zambezi region, reinforcing its strong association with miombo woodlands. Our species distribution modelling (both historical only and all recorded localities) showed that the minimum temperatures during the coldest month (BIO6), or some other correlate thereof, acts as an important driver shaping the spatial distribution of K. wittei, with a lesser contribution from annual precipitation (BIO12). This is in agreement with Poynton and Broadley (1991) who provide evidence of temperature as one of the most important factors affecting the distribution of African amphibians. Furthermore in the review performed by Bradie and Leung (2017) it was shown that temperature and precipitation are two of the three most important predictor variables for species distribution models performed for the class Amphibia.

Interestingly, the majority of Angolan specimens were found in peatlands. Peatlands are often formed in areas subject to high rainfall and poor drainage, and can be extensive in eastern Angola. They were first referred to in the pioneering work on Angola done by Gossweiler and Mendonça (1939) who recorded the local name of Tenga applied to these permanently waterlogged systems around river sources and margins, present in Moxico and Lunda-Sul provinces. It is possible that most of the Angolan population of *Kassinula* is closely associated with peatlands, which provide a wet grassland environment all year round. Amphibians are very susceptible to water loss, as a result of their physiology (Jørgensen 1997, Wells 2007), and smaller bodied species even more so (Wells 2007, Gouveia and Correia 2016), and are thus very susceptible to the desiccation of the habitats they occupy (Wells 2007). The small size and thin, smooth and permeable skin of *Kassinula wittei*, similar to other small-bodied *Hyperolius bocagei* and *H. nasutus* occurring in the same microhabitat, probably makes this species highly susceptible to desiccation, and may be a determinant in this apparently close association to a permanently flooded habitat such as the peatlands. However, small size can also be an advantage, allowing those species to occupy niches that are unavailable for larger frogs, and to persist in areas where larger vertebrates have been extirpated (Wells 2007).

The amphibian species count for Angola increased recently from approximately 119 (Ceríaco et al. 2018; Marques et al. 2018; Baptista et al. 2019) to 133 species (Ernst et al. 2020). *Kassinula wittei* represents a new addition to the list of amphibian species occurring in the country, which is expected to continue growing as the results of ongoing studies are published.

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