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Integrative taxonomic analysis of new collections from the central Angolan highlands resolves the taxonomy of African pipistrelloid bats on a continental scale

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Ten years ago, the genus-level and species-level taxonomy of African pipistrelloid bats was in a state of flux. In spite of advances in the past decade, gaps in collecting from species-rich regions like Angola have hampered efforts to revise this group. We report on new collections of pipistrelle-like bats from the poorly sampled central highlands of Angola (1000–1500 m a.s.l.) as well as comparative material from lower-lying areas of Eswatini and South Africa. Specimens identified as *Neoromicia anchietae*, collected 400–700 km east of the holotype locality in the western highlands of Angola, were genetically and morphologically distinctive from *N. anchietae s.l.* from South Africa and Eswatini. We describe herein this latter lineage as a distinct species from low-lying areas of south-eastern Africa, distinct from *N. anchietae s.s.*, which is therefore restricted to the central and western Angolan highlands. We also identified shallow to deep genetic divergence between different African regions in other recognized pipistrelloid species, such as conspecificity between the long-eared species *Laephotis angolensis* from Angola and *Laephotis botswanae* from northern Botswana, northern Namibia and south-western Zambia. Our phylogeny supports a recently proposed generic classification of African pipistrelloid bats.

ADDITIONAL KEYWORDS: Africa – Mammalia – mitochondrial DNA – morphometrics – *Neoromicia* – taxonomy – Vespertilionidae.

INTRODUCTION

Up until 10 years ago, the genus-level and species-level taxonomy of small-sized pipistrelloid (pipistrelle-like) bats of the family Vespertilionidae from Africa

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was largely unresolved. Reflecting this general lack of consensus at the genus level, 28 African species previously assigned to *Eptesicus* Rafinesque, 1820, *Hypsugo* Kolenati, 1846, *Neoromicia* Roberts, 1926 and *Pipistrellus* Kaup, 1829 were all subsumed under the genus *Pipistrellus* by Van Cakenberghe & Happold (2013). Species are notoriously difficult to identify in the field, complicating the species-level taxonomy of the group. Recent revisions of South, West and East African, and Malagasy taxa, have gone a long way to resolve the thorny taxonomic issues at the genus level and provide a clear framework in which to define new species. Using integrative taxonomic approaches, recent taxonomic reviews of pipistrelloids have been undertaken in Madagascar (Bates *et al.*, 2006; Goodman *et al.*, 2012, 2015), West Africa (Koubinová *et al.*, 2013; Monadjem *et al.*, 2013, 2021a), southern Africa (Kearney *et al.*, 2002; Kearney, 2005) and Africa generally (Monadjem *et al.*, 2021b). Based on detailed phylogenetic analysis, the last-mentioned study proposed a new generic classification for African pipistrelloid bats. Nevertheless, lack of sampling in biodiverse regions of Africa such as Angola represent important missing pieces in the puzzle of African pipistrelloid taxonomy at the genus and species level (Taylor *et al.*, 2018; Beja *et al.*, 2019). This is illustrated by the examples given below of species described from Angola, which concern new collections reported in this study in a broader African context, which we propose brings clarity to the generic and specific taxonomy of African pipistrelloids in general and indicates future gaps where broader taxonomic and geographic sampling may identify further undescribed species.

Based on distinctive characters of the baculum (penis bone) and karyotype, the species *Vesperugo anchietae* Seabra, 1900 described originally from Cahata, Angola, was historically placed alone in Africa in the genus *Hypsugo* distinct from all other African pipistrelloid genera such as *Eptesicus*, *Pipistrellus* and *Neoromicia* (Hill & Harrison, 1987; Kearney *et al.*, 2002; Kearney, 2005; Bates *et al.*, 2006; Goodman *et al.*, 2015). Although, based on morphological grounds, the species was considered to occur in Madagascar as well as southern Africa by Bates *et al.* (2006), on molecular grounds, Goodman *et al.* (2015) showed it to be restricted to the African continent, with a sister species, *Hypsugo bemainty* Goodman *et al.*, 2015 occurring on Madagascar. Based on a molecular phylogeny of pipistrelle-like bats across the African continent, Monadjem *et al.* (2021b) showed that *Hypsugo anchietae* (Seabra, 1900) is best included in the genus *Neoromicia*, as it is neither related to the tropical African group of species previously included in *Hypsugo* nor to *Hypsugo s.s.* from Eurasia. On external and cranial characters, *N. anchietae* is difficult to distinguish from *Pipistrellus hesperidus* (Temminck, 1840) and *Pipistrellus rusticus* (Tomes, 1861), making chromosomal, bacular and molecular characters critical to distinguish these species (Kearney *et al.*, 2002; Monadjem *et al.*, 2020, 2021a, b). However, a recent examination of penis size and shape suggests that the males of these taxa at least can be distinguished relatively easily (Fasel *et al.*, 2020).

Traditionally, the genus *Laephotis* Thomas, 1901 is comprised of four species of long-eared African pipistrelloid bats, one of which was described from 15 km west of Dala in Angola, *Laephotis angolensis* Monard, 1935. However, phylogenetic analysis by Monadjem *et al.* (2021b) showed that this genus also comprises several short-eared species. Nevertheless, the taxonomy of the four long-eared species is complicated by morphological similarity and the absence of clear-cut characters between them (Hill, 1974; Stanley & Kock, 2004; Kearney & Seamark, 2005). However, according to some authors *L. angolensis* overlaps morphologically and may be conspecific with *Laephotis botswanae* Setzer, 1971 (Van Cakenberghe & Seamark, 2020). Based on new collections of Angolan long-eared bats, *L. angolensis* from Angola, we review the phylogeny of this long-eared group based on molecular characters and show that the four long-eared species form a well-supported monophyletic clade within *Laephotis*, but that *L. botswanae* and *L. angolensis* are indeed genetically similar and probably conspecific.

Montane regions of Africa are associated with increased rates of speciation, leading to high levels of micro-endemism and diversity in a range of plant and animal taxa, including bats (Taylor *et al.*, 2012, 2019; Schoeman *et al.*, 2013; Herkt *et al.*, 2016). With the advent of integrated taxonomic approaches and the accelerated availability of DNA sequence data, the last three decades has seen an 18% increase in the number of recognized Afro-Malagasy bat species (Taylor *et al.*, 2019), including several montane-associated species (e.g. Taylor *et al.*, 2012; Monadjem *et al.*, 2019, 2020). The bat fauna of many regions of Africa remains unexplored or poorly sampled, and this is also true of Angola, where historical records and recent acoustic studies recognize a species richness of at least 73 species (Taylor *et al.*, 2018; Beja *et al.*, 2019; Weier *et al.*, 2020). New biological explorations in the central Angolan highlands by the National Geographic Okavango Wilderness Project (NGOWP) in recent years have greatly expanded our knowledge of the plants, invertebrates and vertebrates of this region (e.g. Goyder *et al.*, 2018; Taylor *et al.*, 2018; Midgley & Engelbrecht, 2019; Conradie *et al.*, 2020; 2021; De Moor & Ferreira, 2020; Hallermann *et al.*, 2020; Nielsen *et al.*, 2020; Weier *et al.*, 2020; Krásová *et al.*, 2021; Skelton *et al.*, 2021), as well as the high ecological value

of unique ecosystems such as peatlands (Lourenco *et al.*, 2022). The importance of the study area as a speciation hotspot is clearly highlighted by recent new species descriptions, e.g. baboon spiders (*Ceratogyrus* Pocock, 1897; Midgley & Engelbrecht, 2019), bubble-nesting fish (*Microctenopoma* S.M. Norris, 1995; Skelton *et al.*, 2021) and rain frogs (*Breviceps* Merrem, 1820; Nielsen *et al.*, 2020) as well as the description of a highly genetically divergent lineage of striped mouse (*Rhabdomys* Thomas, 1916; Krásová *et al.*, 2021). In other groups, such as aquatic invertebrates of the order Trichoptera, several unnamed species await formal description (De Moor & Ferreira, 2020).

These NGOWP expeditions between 2016 and 2019 also yielded new collections of bats including 91 DNA-barcoded specimens of pipistrelle-like bats. In this paper, we undertake an integrative taxonomic study of southern African pipistrelloids based on this new collection of short-eared and long-eared pipistrelloids from Angola, in relation to 25 positively identified (by baculum or DNA) pipistrelle-like bats from South Africa and Eswatini. Based on deep genetic divergence and morphological (craniodental and bacular) distinctness, we describe a new species from low-lying savanna and coastal forest habitats as *N. anchietae s.l.*, (nearly) restricting *N. anchietae s.s.* to the central and western Angolan highlands. Importantly, this study also resoundingly confirms a recent generic classification for African pipistrelloids (Monadjem *et al.*, 2021b), and it also supports earlier studies demonstrating the critical importance of African highland regions as centres of pipistrelloid and vespertilionid diversity and micro-endemism (e.g. Monadjem *et al.*, 2013, 2019). We also identify further genetic and biogeographic lineages that await formal description.

MATERIAL AND METHODS

Collection of bats and comparative museum collections

Using mistnets (Ecotone, Poland) of 6, 9 and 12 m lengths, a two-bank harp trap (Faunatech, Australia) and searches of roosts under the flaking bark of trees, 91 bats were collected between early 2016 and late 2019 from seven localities in the central highlands of Angola, for comparison with comparative collections from Eswatini, South Africa, Mozambique and Botswana (Supporting Information, Table S1; Fig. 1a, b). All formalin-fixed voucher specimens were deposited in the Durban Natural Science Museum, South Africa, under collection and exportation permits from the Angolan government (34/INBAC.MINAMB/2016, 002/GGPTBOK/18, 151/INBAC.MINAMB/2019) and import permits from Ezemvelo KwaZulu-Natal Nature Conservation (OP 4364/2018, OP 995/2021). Comparative material was examined from the Ditsong National Museum of Natural History, the Durban Natural Science Museum and the collection of the University of Eswatini (Supporting Information, Table S1; Fig. 1a).

Molecular analyses

DNA extraction, amplification and sequencing

DNA was extracted from pectoral muscle tissues using the Zymogen Quick-DNA Miniprep Plus Kit (Irvine, CA, USA). Genetic confirmation of species identification was performed by amplifying the cytochrome oxidase subunit one (*CO1*), cytochrome *b* (*cytb*) and 12S ribosomal RNA (12S) mitochondrial genes and determining the DNA sequence.

For the *cytb* gene amplification, we prepared 1 × DreamTaq buffer (10×, ThermoFisher Scientific, Waltham, MA, USA), 2 µL *Cytb*-LGL 765 forward primer (10 mM, Metabion International AG, Planegg, Germany) (5'-GAA AAA CCA YCG TTG TWA TTC AAC T-3'), 2 µL *Cytb*-LGL 766 reverse primer (5'-GTT TAA TTA GAA TYT YAG CTT TGG G-3') (10 mM, Metabion International) (Bickham *et al.*, 1995), 1 µL dNTPs mix (10 mM, Thermo Scientific), 2 µL $MgCl_2$ (25 mM, Thermo Scientific), 0.25 µL DreamTaq polymerase (5 U/µL, Thermo Scientific) and nuclease-free water (Ambion, Foster City, CA, USA) to a final volume of 45 µL. A volume of 5 µL extracted DNA was added to the reaction and incubated in a SimpliAmp automated thermal cycler (Thermofisher Scientific) at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min 30 s; and 72 °C for 10 min. Amplification of the partial *CO1* gene was done using the Folmer-LCO1490 forward (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and Folmer-HCO2198 reverse (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (10 mM, Integrated DNA Technologies, USA) (Folmer *et al.*, 1994) primers as described for the *cytb* gene at 94 °C for 2 min; 45 cycles of 94 °C for 30 s, 48 °C for 50 s and 72 °C for 90 s; and 72 °C for 10 min. The 12S gene was amplified using the 12S-L2226M1 forward (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and 12S-U1230M2-CH reverse (5'-GCA CTG AAA ATG CYT AGA TG-3') (10 mM, Integrated DNA Technologies, USA) (Hassanin *et al.*, 2012) primers as described for the *cytb* gene at 94 °C for 4 min; five cycles of 94 °C for 45 s, 60 °C for 60 s and 72 °C for 60 s; 30 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 60 s; and 72 °C for 10 min.

Following PCR amplification, reactions were analysed on a 1.5% agarose gel (Lonza, Basel, Switzerland) and amplicons were gel-purified using the Zymoclean Gel DNA Recovery Kit (Zymogen Research, USA). All amplicons were subjected to Sanger sequencing

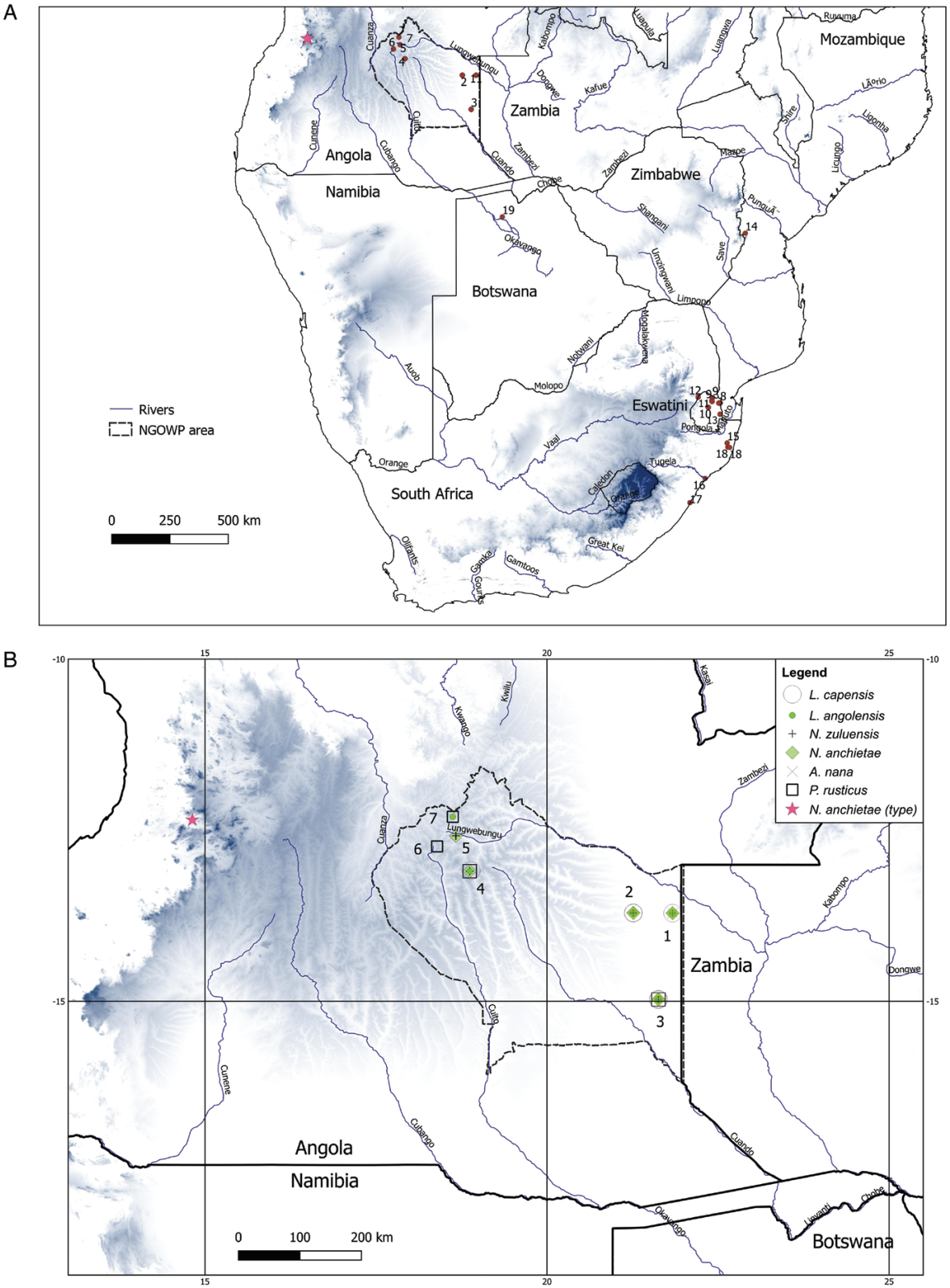

Figure 1. Maps showing A, 19 localities in southern Africa of all pipistrelloid bat specimens examined by this study (numbers correspond with locality numbers given in Supporting Information, Table S1; duplicate numbers represent closely

for both the forward and reverse reactions on an ABI 3100 DNA sequencer (AE Applied Biosystems, USA) at the sequencing facility of the University of Pretoria. Host gene sequences were subsequently compared to bat sequences available in the public domain (on the NCBI GenBank and BOLD databases), and results were interpreted and compared with the respective morphological field identifications.

Phylogenetic analysis

Sequences were edited and ClustalW multiple alignments were generated using the BIOEDIT sequence alignment editor software v.7.2.5 (Hall, 1999). The CIPRES Science Gateway v.3.1 (Miller *et al.*, 2010) was used to determine the best DNA substitution model for nucleotide sequence analysis using jMODELTEST2 (Darriba *et al.*, 2012) and for constructing Bayesian phylogenies using BEAST v.1.10.4 software (Drummond & Rambaut, 2007). Bayesian Markov Chain Monte Carlo (MCMC) chains were set to 20 million iterations, sampling every 2000 steps for optimal ESS scores. Output files were visually inspected to check for convergence using TRACER v.1.7 software (Drummond & Rambaut, 2007). The final phylogenies were constructed in TreeAnnotator with a burn-in value of 10%. For visualization and manipulation of the phylogenetic trees, FIGTREE v.1.4.2 software was used.

MORPHOMETRIC ANALYSES

We measured intact crania and mandibles from 68 genetically (DNA barcode) identified short-eared pipistrelloid specimens from Angola, and an additional 25 positively identified (DNA barcode or baculum data) specimens from Eswatini and South Africa, using 12 craniodental variables (see definitions below). Standard external measurements for mass (g) and forearm length (mm) were obtained from the field notes of P.J.T., M.K., S.M.W., F.P.D.C. and D.C.T., and from museum records. Following Monadjem *et al.* (2021b), the following 12 cranial and dental variables were taken to the nearest 0.01 mm using either Mitutoyo or Tesa digital callipers with an accuracy of 0.01 mm: (1) greatest length of skull measured dorsally from occiput to anterior point of skull (GSKL); (2) condyloincisive length from occipital condyles to front of incisors (CIL); (3) condylocanine length from occipital condyles to front of canines (CCL); (4) zygomatic width, the greatest distance across the zygoma (ZYGO); (5) greatest braincase width taken in the frontal plane above the zygomatic arches (GWB); (6) greatest skull height, taken from the lowest point of the basioccipital to the highest point of the cranium (GSH); (7) postorbital width, narrowest dorsal width posterior to the postorbital at the constriction of the cranium (POB); (8) greatest breadth of cranium at mastoid processes (MAST); (9) greatest mandible length, taken from the posteriormost point of the condyles to the anteriormost point of the incisors (MAND); (10) width across the third upper molars, taken across the outermost point of the alveoli of the third molars (M3–M3); (11) complete upper canine–molar tooth row, taken from the anteriormost point of the alveolus of the canine to the posteriormost point of the alveolus of the third molar (C–M3); and (12) width across upper canines (C–C), taken across the outermost points of the alveoli of the canines.

Juvenile bats having unossified epiphyses of the finger bones were excluded from further analysis. Since all variables were normally distributed based on the Wilks' lamba test, *t*-tests for sexual dimorphism were conducted for mass, forearm length and 12 cranial variables in the three species having the largest sample sizes, *L. capensis*, *N. anchietae s.s.* (Angola) and *N. anchietae s.l.* (South Africa and Eswatini combined) (results not shown but available on request from P.J.T.). In all cranial variables, males and females did not differ significantly, hence the sexes were combined for later multivariate analysis. In *N. anchietae s.s.* (Angola), females were significantly larger than males in forearm length (31.7 and 30.3 mm, respectively, $t = 3.147$, 12° of freedom, $P < 0.01$) and mass (4.14 and 3.38 g, $t = 4.867$, 11° of freedom, $P < 0.01$). In *N. anchietae s.l.*, females were significantly larger in forearm length (31.6 and 29.9 mm, respectively, $t = 6.5549$, six degrees of freedom, $P < 0.01$). Summary statistics were obtained for each cranial variable and forearm length and mass for each taxon in the study. Multivariate analysis (principal component analysis, PCA) was conducted on 12 log-transformed cranial variables using the programme PAST v.3.2 (Hammer *et al.*, 2001).

BACULAR MORPHOLOGY

Ten bacula were prepared following the methods indicated in Taylor *et al.* (2018). The following eight measurements were recorded from digital images of

spaced GPS points classified as the same locality) and B, seven localities of six species of pipistrelloid bats collected in central Angola by the National Geographic Okavango Wilderness project (in the area demarcated by the dashed lines) between 2016 and 2019. Species identifications based on *CO1*, 12S RNA and *cytb* genes. Shaded regions represent elevations of 1200 m a.s.l. and greater. The star represents the holotype locality of *N. anchietae* in the western Angolan highlands.

the ventral surface of bacula using the measurement tool of GNU IMAGE MANIPULATION v.2.10.30 software program (Kimball *et al.*, 2021): total length (anterior most point of tip to posterior most point of basal projection), basal width (greatest width across basal projections), basal height (height of longest basal projection), basal notch height (height of notch in base of baculum), tip width (greatest with across tip projections), tip length (length of longest tip projection), tip notch depth (height of notch taken from anterior most tip point to base of tip notch), greatest constriction (width of baculum shaft taken at point of greatest constriction). All the data mentioned above are available on request from PJT.

RESULTS

MOLECULAR PHYLOGENETICS

Similar tree topologies were obtained for all three mitochondrial genes (*cytb*, *CO1* and 12S) and for maximum likelihood, maximum parsimony, neighbour-joining and Bayesian trees, and thus only Bayesian trees are presented (Fig. 2; Supporting Information, Figs S1, S2). All trees supported (with 1.0 Bayesian prior probability support, BPP) the monophyly of four genus-level groups for southern African pipistrelle-like taxa as suggested by Monadjem *et al.* (2021b): *Afronycteris* Monadjem *et al.*, 2020 for *A. nana* (Peters, 1852); *Pipistrellus* comprising *P. hesperidus* and *P. rusticus*; *Neoromicia* for *N. anchietae*, *N. somalica* (Thomas, 1901) and *N. zuluensis* (Roberts, 1924); and *Laephotis* for the short-eared *Laephotis capensis* (A. Smith, 1829) and the long-eared *L. angolensis*, *L. botswanae*, *Laephotis namibensis* Setzer, 1971 and *Laephotis wintoni* Thomas, 1901. In the last-mentioned genus, in all three gene trees two well-supported (BPP of 1.0) subclades represented the short-eared and long-eared representatives mentioned above.

At the species level, from *cytb* sequences, six species groups were recognized from central Angola, *L. capensis*, *L. angolensis*, *A. nana*, *N. anchietae*, *N. zuluensis* and *P. rusticus*. The same six species identified from *cytb* sequences (Fig. 2; Table 1) were recognized by trees based on *CO1* (Supporting Information, Fig. S1; Table S2) and 12S RNA (Supporting Information, Fig. S2; Table S3).

Angolan *N. anchietae s.s.* was distinct from the closest-matching *N. anchietae s.l.* from South Africa and Eswatini (11.0 and 4.5% in *cytb* and 12S RNA, respectively; Table 1; Supporting Information, Table S3), described below as a new species, as well as from *N. bemainty* from Madagascar (4.3% in 12S RNA) and from East African sequences allocated to *N. somalica* (11.6% in *cytb*, 11.9% in *CO1* and 4.6% in 12S RNA; Table 1; Supporting Information, Tables S2, S3). In *cytb*, *N. anchietae s.l.* from Angola and South Africa/Eswatini are sister groups although this clade is not well supported (BPP < 0.7). In the 12S RNA gene, *N. anchietae s.l.* from South Africa/Eswatini form a well-supported sister group (BPP 0.9) with *N. zuluensis*, *N. bemainty* and *N. somalica*, with Angolan *N. anchietae s.s.* as sister to this group (BPP 1).

In the *cytb* gene, *P. rusticus* from Angola grouped in a clade with Namibian and Eswatini sequences of this species (the type locality of *P. rusticus* is from Ovamboland, Namibia), but with high intragroup divergence (P = 4.5%; Table 1). In the *CO1* gene, Angolan *P. rusticus* was genetically distinct from South African and Eswatini sequences of this species (P = 8.9%; Supporting Information, Table S2). In the 12S RNA gene, Angolan *P. rusticus* was 3.9% distinct from *P. rusticus* from Eswatini and West Africa (Supporting Information, Table S3).

We also found sequence divergence within other recognized species groups. For example, deep *cytb* sequence divergence separated *N. zuluensis* from southern Africa from *N.* cf. *zuluensis* from East Africa (P = 9.1%; Table 1). Although well-supported clades of *N. zuluensis* from Angola and South Africa/Eswatini were revealed in the 12S RNA tree, mean intraspecific divergences were low (P = 0.4%; Supporting Information, Table S2). Two well-supported clades of *L. capensis* from southern Africa (including Angola) and East Africa differed in *cytb* sequences by 3.3% (Fig. 2; Table 1). However, in the 12S RNA Bayesian tree, southern and East African clades of *L. capensis* were not well supported and were genetically similar (P = 1.4%; Supporting Information, Table S3). A well-supported *A. nana* clade comprised four separate well-supported subclades (Fig. 2), which varied from each other by 4.2 to 5.9% (*cytb*; Table 1). These four subclades are geographically defined, representing a widespread East African and south-central African clade and three distinct regionally defined clades in South Africa, East Africa and West Africa (Fig. 2). On the other hand, in the 12S RNA tree, three clades of *A. nana* from West and East Africa and Angola were not well supported and varied from 2.0 to 2.4% (Supporting Information, Fig. S3; Table S3). A moderate divergence (P = 3.1%, *cytb*; Table 1) distinguished two well-supported clades of *P. hesperidus* from (i) South African temperate forests (GenBank sequences reported in Moir *et al.*, 2020) and the higher-elevation (1500 m a.s.l.) Bulembu Forest in western Eswatini, and (ii) Jilobi Forest in lower-lying eastern Eswatini together with a number of low-lying localities from Mozambique, Central-East Africa and Madagascar (Fig. 2). In spite of the lower numbers of

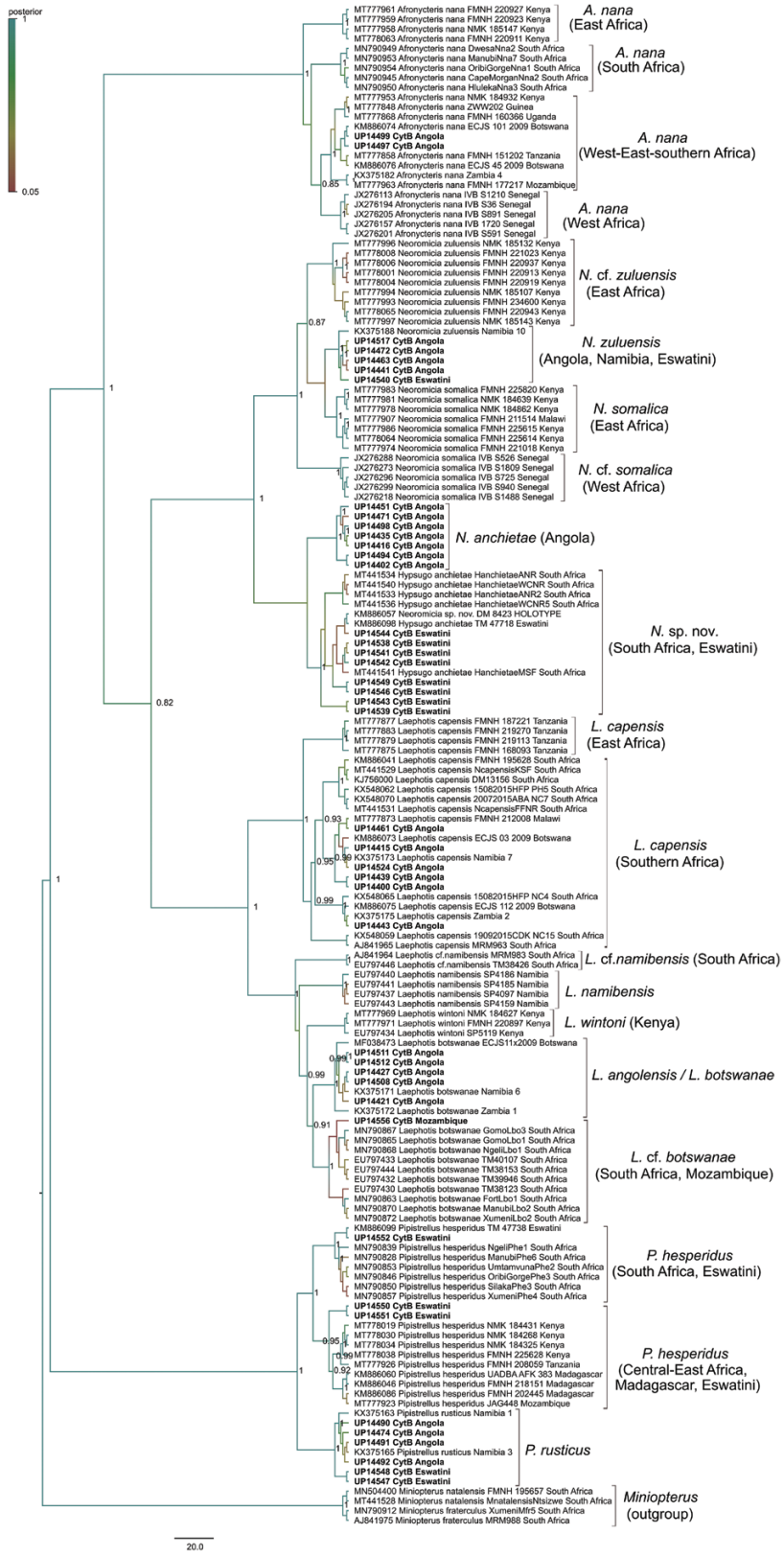

Figure 2. Bayesian phylogeny of mitochondrial cytochrome *b* sequences (708 nucleotides) of pipistrelle-like Vespertilionidae from the genera *Afronycteris*, *Neoromicia*, *Laephotis* and *Pipistrellus* sampled from Angola, South Africa and Eswatini,

sequences available, this distinction was less obvious in the *CO1* (Supporting Information, Fig. S1; Table S2) and 12S RNA (Supporting Information, Fig. S2; Table S3) sequences, where subclades associated with specimens from the Bulembu and Jilobi Forests in Eswatini only differed by 1.2 and 0.7%, respectively.

A well-supported clade of long-eared *Laephotis* species (comprising *L. angolensis*, *L. botswanae*, *L. namibensis* and *L. wintoni*) was recovered in the *cytb* tree (BPP 1.0; Fig. 2). Similarly, *L. angolensis* and *L.* cf. *botswanae* formed a well-supported clade in *CO1* sequences (Supporting Information, Fig. S1) and *L. angolensis*/ *L.* cf. *botswanae* and *L. namibensis* formed a well-supported clade in 12S RNA (Supporting Information, Fig. S2). The type localities of the holotypes of these four species are located in Angola (Tyihumba), northern Botswana (Shakawe), Namibia (Gobabeb, Namib Desert) and Kenya (Kitui), respectively (Monadjem *et al.*, 2020). Sequences referable to both *L. angolensis* (Angola; this study) and *L. botswanae* from northern Botswana (ECJS11/2009; GenBank MF038473; Pierce *et al.*, 2011; Hassanin *et al.*, 2017), northern Namibia (GenBank KX375171; Benda *et al.*, 2016) and south-western Zambia (GenBank KX375172; Benda *et al.*, 2016) were combined into one well-supported *cytb* clade (Fig. 2), indicating conspecificity of these two taxa. Similarly, in the 12S RNA gene tree, the same specimen mentioned above from northern Botswana (WCJS11/2009; GenBank MF038673; Pierce *et al.*, 2011; Hassanin *et al.*, 2017) was grouped into one clade with specimens from Angola and Mozambique (this study; Supporting Information, Table S1). In the *cytb* tree, the above-mentioned *L. angolensis*/ *L. botswanae* clade was grouped (BPP 0.91) with a clade identified as *L.* cf. *botswanae* from South Africa and Mozambique. These two *cytb* clades differed by 2.8% uncorrected sequence divergence (Table 1). In *CO1* sequences, the *L.* cf. *botswanae* specimen from Mozambique referred to above differed by 1.4% from an Angolan clade of *L. angolensis* (Supporting Information, Fig. S1).

Paraphyly was identified within *L. namibensis s.l.* in *cytb* sequences, with Namibian *L. namibensis s.s.* being distinct from *L. namibensis s.l.* from the Western Cape Province of South Africa (P = 3.8%; Table 1). The latter, South African-endemic clade forms a sister group (Bayesian support of 1) to the other four species in the group (*L. angolensis*, *L. botswanae*, *L. namibensis* and *L. wintoni*).

External and craniodental morphology of Angolan pipistrelloids

Univariate summaries of external and cranial measurements of Angolan short-eared pipistrelloids and comparative specimens are provided in Table 2 and described below. Molecular sequencing of museum voucher specimens allowed morphological and morphometric comparisons on unambiguously identified taxa. A clear morphological distinction in the Angolan sample was apparent between specimens typically having a small anterior upper premolar (*P. rusticus*, *A. nana* and *N. anchietae*) and those without a small anterior upper premolar (*L. capensis*, *L. angolensis* and *N. zuluensis*), an important character used in previous classifications of pipistrelloid bats. In rare cases, *N. zuluensis* may possess anterior premolars resulting in misidentification as *N. anchietae s.l.*, for example, in IYSIS_M_20 (lab number UP14540), a specimen from Eswatini in this study (Fig. 2; Supporting Information, Figs S1, S2; A.M., pers. comm.) and a specimen from Marloth Park, Mpumalanga Province in South Africa [FMNH (Field Museum of Natural History) catalogue number 195631 (GenBank 12S sequence number JQ039236)] (Goodman *et al.*, 2012; T.K., pers. comm.). Nevertheless, apart from the exceptions above, this character was consistent in our recent collections as verified by DNA evidence, justifying separate morphometric analysis of the two groups defined by this character. Morphometric analysis of all Angolan pipistrelloid specimens combined (i.e. with and without the anterior premolar) resulted in considerable overlaps among species (results not shown but available from P.J.T.).

Among the above species, *L. angolensis* can be easily distinguished from all others in its relatively long ear length, and this species was not considered in further craniometric analyses. Additionally, *A. nana* is distinctly smaller in cranial size than *N. rusticus* and *N. anchietae* and has a distinctly domed forehead, hence this species was not included in further craniometric analyses. Principal component analysis of 12 log-transformed cranial and dental variables was conducted on the remaining Angolan pipistrelloid specimens with (Fig. 3) and without (Fig. 4) the

and the outgroup *Miniopterus* spp. (Miniopteridae) sampled from South Africa and Eswatini. Bayesian phylogenetic analysis was performed using the Hasegawa–Kishino–Yano model incorporating invariant sites and a gamma distribution (HKY+I+G). Posterior probabilities of > 0.7 are indicated at internal nodes. Samples obtained in this study are indicated in bold. Numbers in brackets at the end of annotations for species clades represent the number of reference sequences used (GenBank accession numbers are available in Supporting Information, Table S1). Branch colours indicate posterior probabilities at each node.

Table 1. Uncorrected P-distances (number of base differences per site from averaging over all sequence pairs) obtained between (bottom left) and within (bold face; on diagonals) groups of pipistrelloid bats from new collections in Angola, South Africa and Eswatini in relation to GenBank sequences of comparable African species for the mitochondrial cytochrome *b* gene. See Supporting Information, Table S1 for details of specimens used in the study. This analysis involved 156 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 708 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). Abbreviations for groups are as follows: Anan(EA) = *Afronycteris nana* (East Africa), Anan(SA) = *A. nana* (South Africa), Anan(WA) = *A. nana* (West Africa), Anan(WESA) = *A. nana* (West-East-southern Africa), Lang = *Laephotis angolensis/ L. botswanae* (Angola, Namibia, Botswana, Zambia), Lbot(SA) = *L.* cf. *botswanae* (South Africa, Mozambique), Lcap(SA) = *L. capensis*, Lcap(EA) = *L. capensis* (East Africa), Lcf.nam = *L.* cf. *namibensis* (South Africa), Lnam = *L. namibensis* (Namibia), Lwin = *L. wintoni* (Kenya), Nanc = *Neoromicia anchietae s.s.* (Angola), Ncf.som(WA) = *N.* cf. *somalica* (West Africa), Ncf.zul(EA) = *N.* cf. *zuluensis* (East Africa), Nsom(EA) = *N. somalica* (East Africa), Nsp.nov = *N.* sp. nov. (= *N. hlandzeni* from South Africa and Eswatini), Nzul(SA) = *N. zuluensis* (southern Africa), Phesp(SA) = *Pipistrellus hesperidus* (southern Africa), Phesp (SEAM) = *P. hesperidus* (southern and eastern Africa and Madagascar), Prust = *P. rusticus*, Outgroup = outgroup (*Miniopterus* spp.)

	Anan (EA)	Anan (SA)	Anan (WA)	Anan (WESA)	Lang	Lbot (SA)	Lcap (EA)	Lcap (SA)	Lcf.nam	Lnam	Lwin	Nanc	Ncf.som (WA)	Ncf.zul (EA)	Nsom (EA)	Nsp.nov	Nzul	Phesp (SA)	Phesp (SEAM)	Prust	Outgroup
Anan(EA)	**0.0178**																				
Anan(SA)	0.052	**0.0065**																			
Anan(WA)	0.055	0.046	**0.0046**																		
Anan(WESA)	0.059	0.050	0.042	**0.0086**																	
Lang	0.166	0.158	0.166	0.165	**0.0077**																
Lbot(SA)	0.151	0.147	0.155	0.153	0.028	**0.0033**															
Lcap(EA)	0.172	0.164	0.165	0.167	0.099	0.089	**0.0057**														
Lcap(SA)	0.173	0.169	0.162	0.164	0.097	0.085	0.033	**0.0129**													
Lcf.nam	0.150	0.148	0.153	0.156	0.052	0.044	0.101	0.102	**0.0046**												
Lnam	0.147	0.145	0.151	0.152	0.040	0.032	0.098	0.097	0.038	**0.00**											
Lwin	0.153	0.153	0.158	0.159	0.035	0.031	0.096	0.097	0.053	0.030	**0.0072**										
Nanc	0.174	0.179	0.174	0.179	0.152	0.141	0.152	0.153	0.151	0.148	0.152	**0.0204**									
Ncf.som(WA)	0.170	0.177	0.164	0.171	0.155	0.145	0.155	0.146	0.146	0.146	0.151	0.118	**0.0056**								
Ncf.zul(EA)	0.179	0.184	0.170	0.182	0.144	0.144	0.159	0.152	0.149	0.145	0.152	0.124	0.107	**0.0095**							
Nsom(EA)	0.171	0.170	0.163	0.175	0.150	0.145	0.155	0.146	0.143	0.138	0.152	0.116	0.097	0.085	**0.0307**						
Nsp.nov	0.168	0.162	0.163	0.170	0.157	0.144	0.162	0.157	0.154	0.146	0.149	0.110	0.128	0.116	0.119	**0.0058**					
Nzul(SA)	0.177	0.172	0.162	0.175	0.161	0.159	0.160	0.155	0.158	0.148	0.158	0.122	0.104	0.091	0.087	0.127	**0.0155**				
Phesp(SA)	0.207	0.201	0.213	0.212	0.191	0.188	0.198	0.199	0.209	0.199	0.198	0.181	0.181	0.197	0.186	0.207	0.203	**0.0072**			
Phesp(SEAM)	0.213	0.199	0.215	0.213	0.194	0.191	0.197	0.199	0.208	0.202	0.202	0.183	0.181	0.199	0.186	0.205	0.198	0.031	**0.0175**		
Prust	0.202	0.190	0.197	0.195	0.207	0.197	0.197	0.199	0.204	0.210	0.210	0.198	0.205	0.215	0.198	0.204	0.208	0.124	0.124	**0.0449**	
Outgroup	0.230	0.229	0.229	0.229	0.245	0.238	0.213	0.212	0.236	0.241	0.246	0.224	0.217	0.225	0.216	0.214	0.229	0.249	0.252	0.239	**0.0735**

Table 2. Summary statistics for mass, forearm, and 12 craniodental variables for voucher specimens of DNA barcoded taxa identified in this study (Angola) and comparative positively identified (from barcoding or baculum characters) specimens from South Africa and Eswatini. The country of origin of measured specimens from each species is indicated below and full details of specimens included in the morphometric study are included in Supporting Information, Table S1

Variable	*A. nana*	*L. capensis*	*N. anchietae s.s.*	*N. hlandzeni*	*N. zuluensis*	*P. hesperidus*	*P. rusticus*
Counties represented	Angola	Angola	Angola	South Africa, Eswatini	Angola	Eswatini	Eswatini, Angola
Mass							
N	2	26	19	9	11	5	11
Mean	3.40	5.08	3.82	4.39	3.95	5.20	4.68
SD	0.85	0.93	0.50	0.60	0.65	0.84	0.41
Min.	2.80	3.00	3.00	4.00	3.00	4.00	4.00
Max.	4.00	6.50	4.80	5.50	5.00	6.00	5.10
Forearm							
N	2	26	19	9	11	5	11
Mean	29.25	31.62	31.09	31.07	30.53	30.70	29.21
SD	3.18	1.52	1.51	0.96	0.90	2.34	0.95
Min.	27.00	28.50	27.50	29.50	29.00	28.00	27.80
Max.	31.50	34.00	33.50	32.00	32.00	33.20	30.20
GSKL							
N	2	20	20	17	10	6	11
Mean	10.88	12.87	12.11	13.12	11.99	12.66	11.75
SD	0.98	0.69	0.46	0.23	0.57	0.34	0.32
Min.	10.18	11.11	11.22	12.69	10.84	12.24	11.30
Max.	11.57	13.81	12.90	13.51	12.70	13.12	12.28
CIL							
N	2	23	20	17	10	6	11
Mean	10.23	12.41	11.47	12.50	11.39	12.12	11.23
SD	0.57	0.72	0.50	0.15	0.63	0.33	0.23
Min.	9.82	10.27	10.33	12.07	10.04	11.59	10.80
Max.	10.63	13.39	12.24	12.69	12.24	12.55	11.50
CCL							
N	2	23	20	17	10	6	11
Mean	9.79	12.10	11.16	12.05	11.09	11.76	10.92
SD	0.69	0.73	0.47	0.20	0.58	0.31	0.21
Min.	9.30	9.74	10.08	11.57	9.83	11.30	10.61
Max.	10.28	13.21	11.90	12.48	11.94	12.09	11.19
ZYGO							
N	2	20	19	17	11	6	11
Mean	6.62	7.93	7.27	8.03	7.28	8.37	7.43
SD	0.29	0.71	0.40	0.31	0.48	0.33	0.21
Min.	6.41	6.22	6.53	7.43	6.35	7.82	7.04
Max.	6.82	9.14	7.78	8.56	8.10	8.70	7.78
GWB							
N	2	22	20	17	11	6	11
Mean	5.85	6.72	6.41	6.70	6.29	7.03	6.34
SD	0.23	0.42	0.23	0.14	0.32	0.27	0.34
Min.	5.68	5.65	5.87	6.45	5.65	6.62	5.42
Max.	6.01	7.44	6.84	7.04	6.79	7.37	6.61
GSH							
N	2	19	20	17	10	6	11
Mean	4.67	5.32	5.30	4.69	5.19	4.62	5.24
SD	0.59	0.29	0.27	0.26	0.32	0.40	0.18
Min.	4.25	4.58	4.43	4.11	4.62	4.07	4.92

Table 2. Continued

Variable	*A. nana*	*L. capensis*	*N. anchietae s.s.*	*N. hlandzeni*	*N. zuluensis*	*P. hesperidus*	*P. rusticus*
Counties represented	Angola	Angola	Angola	South Africa, Eswatini	Angola	Eswatini	Eswatini, Angola
Max.	5.09	5.77	5.62	5.08	5.55	5.05	5.47
POB							
N	2	24	20	17	11	6	11
Mean	3.18	3.39	3.43	3.57	3.32	3.63	3.56
SD	0.15	0.16	0.13	0.08	0.17	0.24	0.10
Min.	3.07	3.11	3.13	3.36	2.98	3.25	3.35
Max.	3.28	3.70	3.61	3.68	3.59	3.88	3.72
MAST							
N	2	18	20	17	10	6	11
Mean	6.33	7.13	6.77	7.10	6.64	7.35	6.75
SD	0.28	0.44	0.29	0.18	0.38	0.39	0.24
Min.	6.13	6.33	6.07	6.73	5.84	6.75	6.25
Max.	6.52	7.82	7.20	7.35	7.16	7.78	7.08
MAND							
N	2	24	20	17	11	6	11
Mean	7.34	9.37	8.61	9.37	8.33	9.35	8.66
SD	0.28	0.66	0.35	0.20	0.58	0.09	0.28
Min.	7.14	7.49	7.82	9.07	7.16	9.21	8.17
Max.	7.53	10.60	9.17	9.76	9.36	9.47	9.15
M3–M3							
N	2	24	20	17	11	6	11
Mean	4.39	5.39	4.87	5.27	4.89	5.57	5.20
SD	0.23	0.39	0.25	0.15	0.45	0.24	0.17
Min.	4.23	4.20	4.29	4.92	4.05	5.29	4.96
Max.	4.55	5.91	5.27	5.46	5.56	5.96	5.40
CM–3							
N	2	24	20	17	11	6	11
Mean	3.56	4.63	4.25	4.63	4.20	4.53	4.14
SD	0.14	0.28	0.21	0.13	0.26	0.14	0.12
Min.	3.46	3.70	3.70	4.32	3.75	4.36	3.97
Max.	3.66	4.97	4.55	4.89	4.63	4.71	4.39
C–C							
N	2	24	20	17	11	6	11
Mean	3.01	3.88	3.43	3.93	3.44	4.08	3.70
SD	0.49	0.44	0.26	0.22	0.34	0.15	0.13
Min.	2.66	2.53	2.91	3.65	2.88	3.88	3.40
Max.	3.36	4.52	3.88	4.61	3.95	4.21	3.81

anterior upper premolar, and in the first case, due to the large genetic divergence observed in *N. anchietae s.l.*, positively identified specimens of *N. anchietae s.l.* (*N* = 15) from South Africa and Eswatini were added for comparison. Positively identified specimens of the morphometrically similar *P. hesperidus* (*N* = 6) were also added for comparison, although this species was not identified during our study in central Angola. In Figure 3, the first PC (positive loadings for all measurements except for skull height which has a high negative loading; Table 3) clearly distinguishes (without overlap) smaller sized *N. anchietae s.s.* and *P. rusticus* having proportionately deeper crania from larger sized *N. anchietae s.l.* (described below as a new species) and *P. hesperidus* having proportionately shallower crania. Supporting the distinct differences between *N. anchietae s.s.* from Angola and *N. anchietae s.l.* from South Africa and Eswatini (*N. hlandzeni*, described below), condylo-incisive skull length (CIL) varies from 10.3 to 12.2 mm (mean 11.5 mm) in Angolan *N. anchietae* to 12.1 to 12.7 mm (mean 12.5 mm) in *N. hlandzeni* while skull height (GSH) varies from 4.43

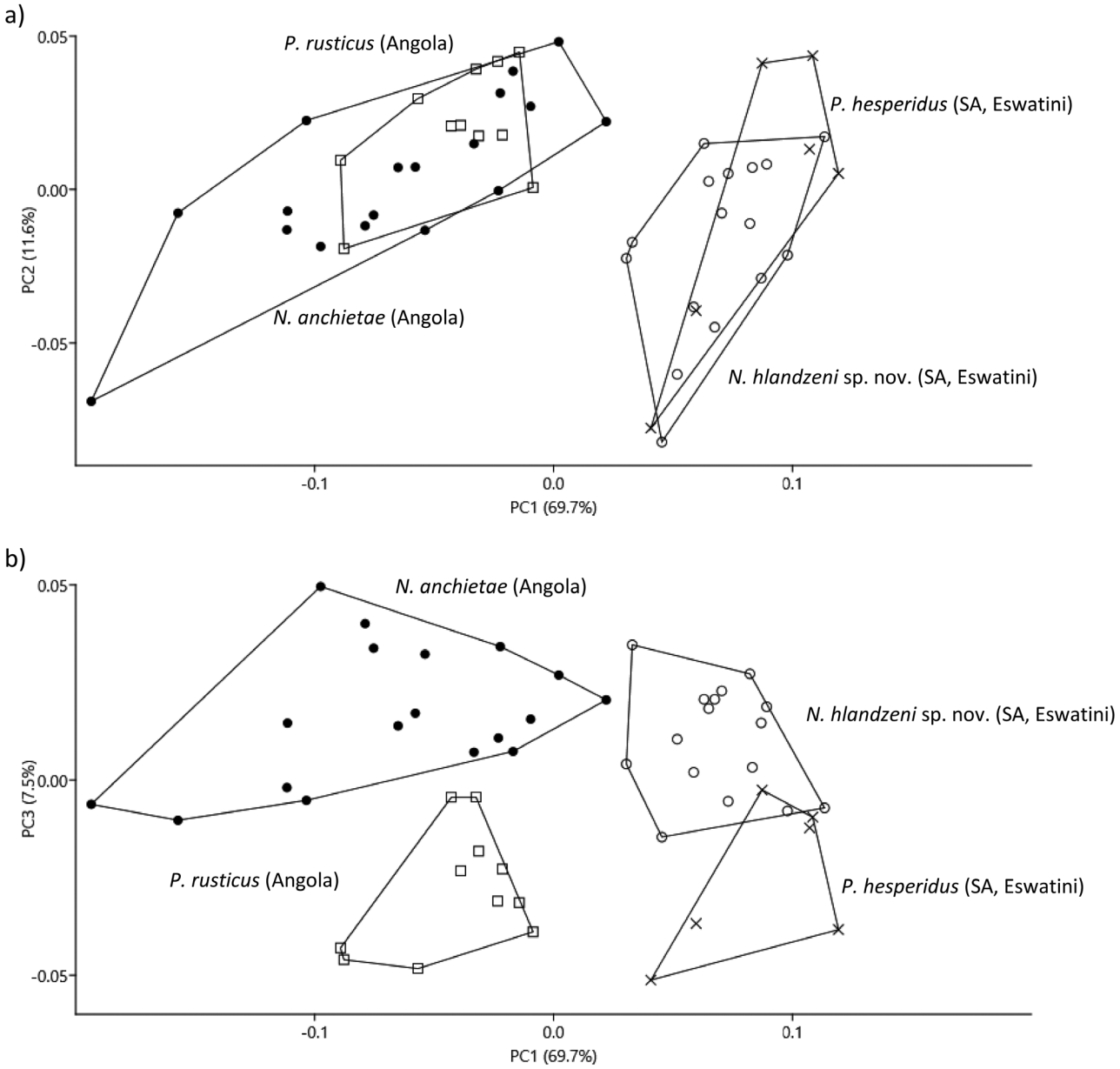

Figure 3. PCA of components 1 and 2 (a) and components 1 and 3 (b) from 12 log-transformed cranial and dental variables in DNA barcoded Angolan small vespertilionid bats with anterior upper premolar present, in comparison with positively identified samples from South Africa and Eswatini. Species identification based on DNA barcoding of the *cytb*, *CO1* and 12S genes for *P. rusticus* (open squares) and *N. anchietae* (dots) from Angola and on either sequences or baculum photographs for *N. hlandzeni* (open circles) from South Africa and Eswatini and *P. hesperidus* (crosses) from Eswatini and South Africa.

to 5.62 mm (mean 5.30 mm) in Angolan *N. anchietae* and 4.11 to 5.08 mm (mean 4.69 mm) in *N. hlandzeni* (Table 2). Individuals of *N. anchietae* from Angola plot at high positive scores of PC3 [relatively short (C–M3) and broad (M3–M3, C–C) palatal region, Table 3] relative to *P. rusticus* (relatively long and narrow palatal region). The same character separates the new species (broader, shorter palate) from *P. hesperidus* (longer, narrower palate) in samples from South Africa and Eswatini (Fig. 3; Table 3). A discriminant analysis of the four species shown in Figure 3, using jack-knifed scores, indicated a 94% correct classification of these four species based on the 12 cranial variables: 94% of the 17 specimens of *N. anchietae* were correctly allocated (one specimen was misclassified as *P. rusticus*), 100% of the 12 *P. rusticus* were correctly allocated, 94% of the 16 *N. anchietae s.l.*,

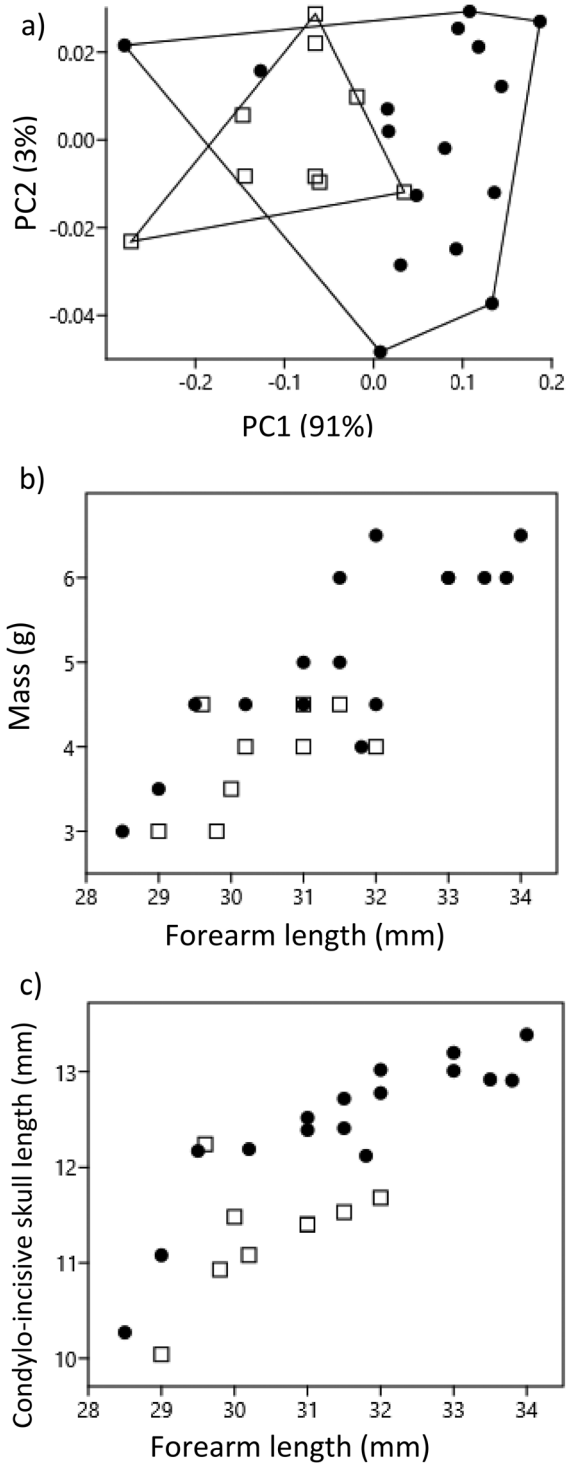

Figure 4. PCA of 12 log-transformed cranial and dental length variables (a) and plots of forearm length and mass (b) and forearm and condylobasal skull length (c) in Angolan small vespertilionid bats with anterior premolar absent (open squares: *N. zuluensis*; dots: *L. capensis*). Species identification based on DNA barcoding of the *cytb*, *CO1* and 12S genes.

Table 3. PCA variable loadings for 12 craniodental variables in genetically barcoded short-eared pipistrelloid bat specimens from Angola, Eswatini and South Africa possessing anterior upper premolars: *N. anchietae s.s.* from Angola (*N* = 17), *N. hlandzeni* from South Africa and Eswatini (*N* = 16), *P. rusticus* from Angola (*N* = 12) and *P. hesperidus* from Eswatini and South African (*N* = 6)

	PC1	PC2	PC3
GSKL	0.24427	-0.10215	0.40404
CIL	0.27694	-0.07532	0.32374
CCL	0.26364	-0.03847	0.31620
ZYGO	0.36351	0.17078	-0.10772
GWB	0.23195	0.11006	0.12113
GSH	-0.25496	0.86768	0.32438
POB	0.13756	0.22746	-0.02530
MAST	0.23650	0.17605	0.12925
MAND	0.27137	-0.10191	0.11045
M3–M3	0.28678	0.19899	-0.44798
C–M3	0.30096	-0.06482	0.30492
C–C	0.47103	0.22661	-0.42101

Table 4. PCA variable loadings for 12 craniodental variables in genetically barcoded short-eared pipistrelloid bat specimens from Angola lacking anterior upper premolars: *L. capensis* (*N* = 16) and *N. zuluensis* (*N* = 9)

	PC1	PC2	PC3
GSKL	0.22855	0.007503	0.23680
CIL	0.26735	-0.01189	0.17244
CCL	0.27622	-0.03975	0.14358
ZYGO	0.31662	0.33356	-0.06756
GWB	0.24247	0.10841	0.068873
GSH	0.13915	0.30755	-0.62454
POB	0.12688	0.62175	-0.01392
MAST	0.22733	0.35681	0.26510
MAND	0.34443	-0.00584	0.39690
M3–M3	0.33864	-0.11966	-0.29697
C–M3	0.28000	-0.32343	0.16896
C–C	0.49203	-0.38442	-0.38662

described below, were correctly allocated (one specimen was misclassified as *P. hesperidus*) and 83% of the six *P. hesperidus* were correctly allocated (one specimen as misclassified as *N. anchietae s.l.*, described below).

Concerning individuals of the two Angolan pipistrelloid taxa lacking the anterior premolar, PCA reveals some overlap in skull size, but *L. capensis* has a larger-sized cranium and mandible than *N. zuluensis* (Fig. 4a). The two species can also be distinguished by an occipital 'helmet' found in *L. capensis* but not *N. zuluensis* (Monadjem *et al.*, 2020). The variable loadings from the PCA (Table 4) reveal that PC1 reflects overall skull size and

that PC1 dominates craniometric variation between the two species (PC1 explained 91% of the total variance, compared to only 3% in PC2). Simple plots of forearm length, mass and condylobasal skull length also show the difference in size between the two species with some overlap (Fig. 4b, c). Larger individuals > 5 g in mass, 32 mm in forearm length and 12 mm in skull length were identified on molecular grounds as *L. capensis*, but two smaller *L. capensis* specimens (which were possibly subadult) overlapped in size with *N. zuluensis*.

Baculum morphology

Bacula of southern Africa *N. anchietae s.l.* (Angola, Zambia, Botswana, South Africa and Eswatini) were mostly similar in morphology (Fig. 5), with bilobed bases (with the exception of Botswana) and tips, and measuring > 1.0 mm in total length (Table 5). Individuals from Eswatini, the northern KwaZulu-Natal region of South Africa and Angola had longer bacula than other sampled taxa. The Angolan specimen (*N. anchietae s.s.*) was differentiated from other *N. anchietae s.l.* southern African individuals based on well-defined lateral projections of the baculum tip. These lateral projections were present in a Zambian specimen of *N. anchietae s.l.* illustrated in Hill & Harrison (1987). Angolan, Eswatinian and South African bacula featured asymmetric bases, with one lobe (position variable) noticeably more distended than the other. The baculum of the Botswana specimen did not bear a bilobed base and was distinctly shorter than other southern African samples. It is likely that this individual is a subadult (as evidenced by minimal toothwear), with a baculum that was still developing.

Taxonomy

Order Chiroptera

Family Vespertilionidae Gray, 1821

Neoromicia Roberts, 1926

***Neoromicia hlandzeni* sp. nov.**

Lowveld serotine

Pipistrellus (Hypsugo) anchietae Hill & Harrison (1987) (In part): Zambia?

Pipistrellus (Hypsugo) anchietae Koopman (1993) (In part): South Africa.

Hypsugo anchietae Cotterill (1996): Zimbabwe.

Hypsugo anchietae Kearney *et al.* (2002): KwaZulu-Natal Province, South Africa.

Hypsugo anchietae Kearney (2005): Zimbabwe, Limpopo and KwaZulu-Natal provinces, South Africa.

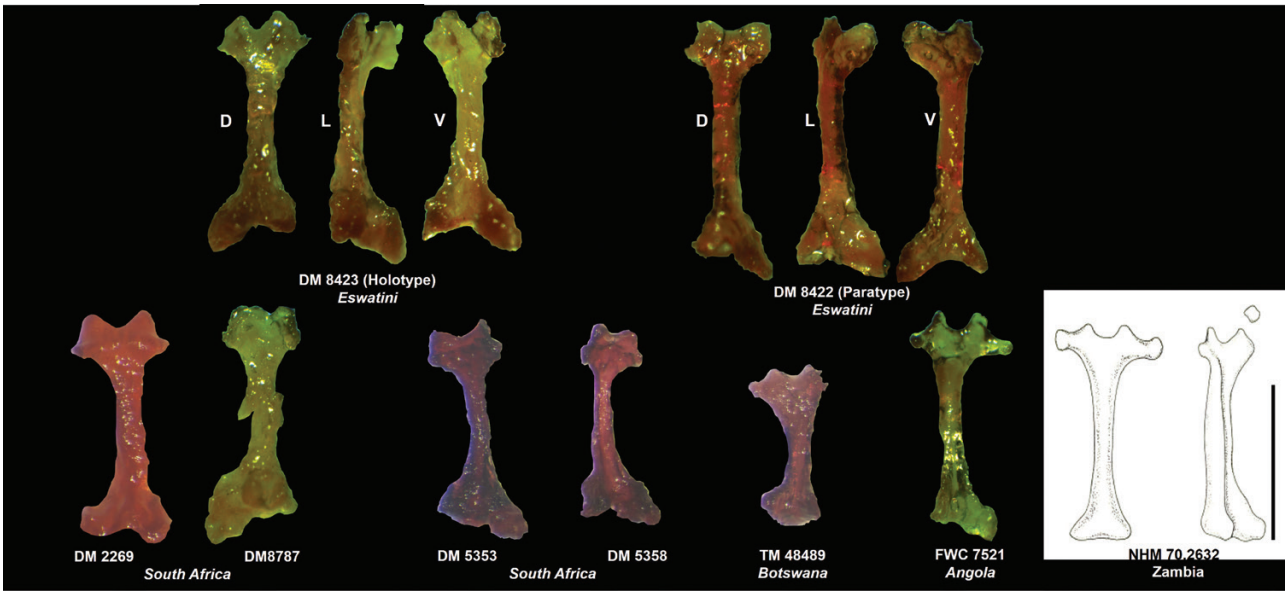

Figure 5. Photographs and drawings of bacula of *N. hlandzeni* sp. nov. from Eswatini (DM 8423, 8422), KwaZulu-Natal Province, South Africa (DM 2269, 8787, 5353, 5358), together with *N. anchietae* from Botswana (TM 48489), Angola (FWC 7521), and Zambia (NHM 70.2632; from Hill & Harrison, 1987). In the top row, D=dorsal, L=lateral and V=ventral view. Images in the lower row are of the ventral view, except for the far right image which is of the lateral view. Abbreviations of museums as follows: DM = Durban Natural Science Museum; TM = Ditsong National Museum of Natural History; NHM = The Natural History Museum, London. FWC indicates the field number of Fenton (Woody) Cotterill. The vertical scale bar on the bottom right indicates 1 mm.

Table 5. Bacular measurements of 10 *Neoromicia anchietae s.l.* individuals from southern Africa. Museum abbreviations: DM = Durban Natural Science Museum; TM = Ditsong National Museum of Natural History

Catalogue number	Locality	Total length	Basal width	Basal height	Basal notch height	Tip width	Tip length	Tip notch depth	Greatest constriction
DM 8423 (Holotype)	Eswatini: Mlawula Nature Reserve	1.91	0.79	0.63	0.25	0.64	0.49	0.13	0.20
DM 8422 (Paratype)	Eswatini: Mlawula Nature Reserve	1.73	0.44	0.40	0.25	0.60	0.45	0.10	0.17
TM 47718 (Paratype)	Eswatini: Mlawula Nature Reserve	1.74	0.62	-	-	0.55	0.41	0.10	0.13
DM 8512	Eswatini: Mbuluzi Nature Reserve	1.70	0.60	0.41	0.19	0.56	0.58	0.07	0.17
DM 2269	South Africa: KwaZulu-Natal, iSimangaliso Wetland Park	1.70	0.80	0.52	0.29	0.67	0.35	0.11	0.18
DM 8787	South Africa: KwaZulu-Natal, Phinda Private Game Reserve	1.76	0.62	0.58	-	0.62	0.47	0.10	0.20
DM 5353	South Africa: KwaZulu-Natal, Harold Johnson Nature Reserve	1.62	0.58	0.36	-	0.61	0.42	0.10	0.12
DM 5358	South Africa: KwaZulu-Natal, Umkomaas, Empisini Nature Reserve	1.34	0.49	0.37	0.14	0.41	0.32	0.08	0.11
DM 16054	Angola	1.63	0.47	0.55	0.24	0.72	0.44	0.14	0.14
TM 48489	Botswana	1.09	0.43	0.29	0.12	0.54	0.34	0.09	0.16

Hypsugo anchietae Simmons (2005) (In part): Zimbabwe, South Africa.

Hypsugo anchietae Monadjem *et al.* (2010) (In part): Zimbabwe, Mozambique, Eswatini, South Africa.

Neoromicia anchietae Monadjem *et al.* (2020) (In part): Zimbabwe, Mozambique, Eswatini, South Africa.

Laephotis anchietae Simmons & Cirranello (2022) (In part): Zimbabwe, Mozambique, Eswatini, South Africa.

Holotype: Durban Natural Science Museum (DM) No. 8423. The adult male specimen collected by Ara Monadjem (field number AM 2066) on 5 September 2005, has muscle tissue in 90% ethanol and the body preserved in ethanol, with skull (Fig. 6) and baculum (Fig. 5) extracted and cleaned. *cytb* sequences are available from GenBank (No. KM886057).

Type locality: Eswatini (formerly Swaziland), Mlawula Nature Reserve, near the Siweni train siding in the north-eastern lowveld savanna region of the country (latitude 26.17998°S; longitude 32.04871°E). The specimen was netted in riparian vegetation over a small side channel of the Mbuluzi River at an elevation of 110 m a.s.l.

Paratypes: DM No. 8422; Ditsong National Museum of Natural History (TM) No. 47718. Both adult males, with bacula, from Mlawula Nature Reserve, Eswatini.

Etymology: eHlandzeni means the 'lowveld' or wilderness in the SiSwati language indicating the specific lowveld (low-lying savanna) habitat of the new species which differentiates it from *N. anchietae* known from higher-lying (> 1000 m a.s.l.) elevations. With SiSwati names, the prefix is typically dropped when a noun is turned into a name.

Diagnosis: The species is distinguished genetically (11% divergence in *cytb* sequences) from its sister species, *N. anchietae* from Angola. When compared with craniometric data of positively identified (from either mtDNA sequences or baculum) *N. hlandzeni* from South Africa and Eswatini ($N = 17$; Table 2; Fig. 3), specimens of *N. anchietae s.s.* from Angola ($N = 20$) have distinctly smaller-sized skulls (Fig. 3; Table 2). Greatest skull length varies from 11.2 to 12.9 mm (mean 12.1 mm) in Angolan *N. anchietae* and from 12.7 to 13.5 mm (mean 13.1 mm) in *N. hlandzeni.* Based on material examined in this study, there is a definite and clear dental feature that distinguishes both *N. anchietae* and *N. hlandzeni* from *P. hesperidus*. Unless the teeth are worn, the inner, anterior (larger of two) upper incisor is clearly bifid in *N. anchietae* and *N. hlandzeni*, although sometimes this is only manifested as a 'step' in the tooth. Neither

the step, nor the bifid condition was found in any *P. hesperidus* examined in this study. This condition of the inner upper incisor is confirmed by Van Cakenberghe & Happold (2013). The original description by Seabra (1900) refers to the inner upper incisor as 'tricuspid'. The baculum is > 1.30 mm long and bears a bilobed tip and base, with one of the basal lobes more enlarged than the other. Eswatini and South African individuals of *N. hlandzeni* can be distinguished from Angolan and Zambia samples of *N. anchietae* (also > 1.30 mm long) by their lack of well-defined lateral projections of the tip lobes (Fig. 5).

Description: In pelage coloration and general body size and appearance, the new species is indistinguishable from *N. anchietae* (see description of Seabra, 1900 in Van Cakenberghe & Seamark, 2020; Van Cakenberghe & Happold, 2013). The new bat represents a relatively small-sized vespertilionid species with relatively large ears. The thumb is relatively long and has a distinct white marking at the base, on the thumb pad (Fig. 7). The tragus is about half the length of the ear, with a rounded and convex posterior surface and basal lobe. The antitragus is clearly visible and triangular in shape. The pelage is longish (up to 6 mm in length), bicoloured (hairs darker below), dark brown to yellowish brown dorsally and light brown, cream or white ventrally, darker in the pelvic area.

The skull is relatively small and fragile for an African vespertilionid with a 'notably depressed head', i.e. concave lateral profile (Seabra, 1900; Fig. 6). There are five upper cheek teeth (including a minute anterior premolar), five lower cheek teeth and one canine and two upper and three lower incisors on each side. The anterior (inner) upper incisor is typically bifid or retains a 'step' in individuals with worn teeth. The posterior (outer) incisor is much smaller, about half the length of the anterior incisor or less. In cranial shape, the new species, together with *N. anchietae*, have an upturned end to the skull in the region of the incisors and canines relative to *P. rusticus* and *P. hesperidus* giving a concave lateral profile, whereas the others have a straighter profile. Furthermore, the zygomatic arch is not straight but has a wavy profile in *N. anchietae* and *N. hlandzeni*, whereas in *P. rusticus* and *P. hesperidus* it is straight.

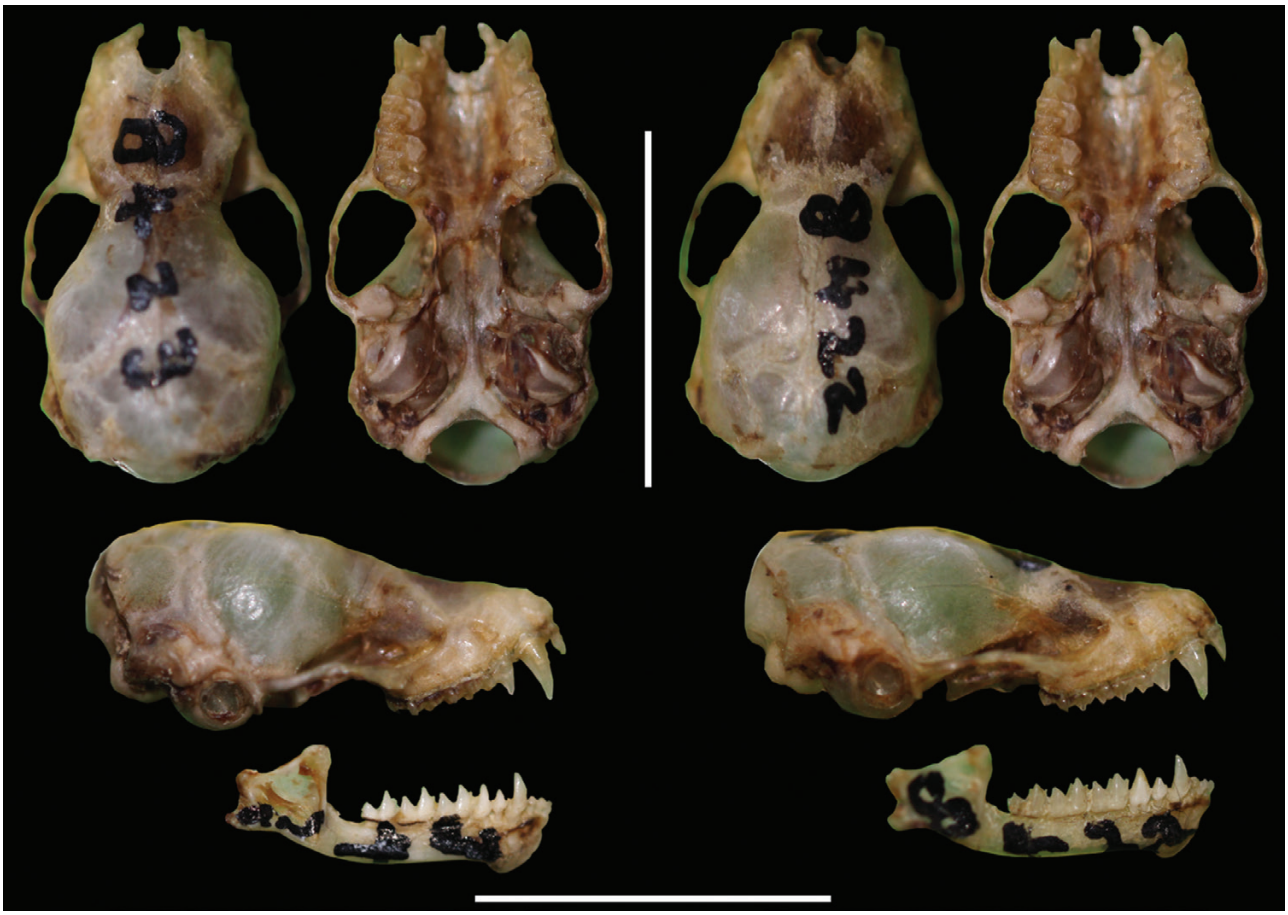

Figure 6. Dorsal, ventral, lateral cranial and lateral mandibular views of DM 8423 (holotype) and DM 8422 (paratype) of *N. hlandzeni* sp. nov. Scale bar represents 1 mm.

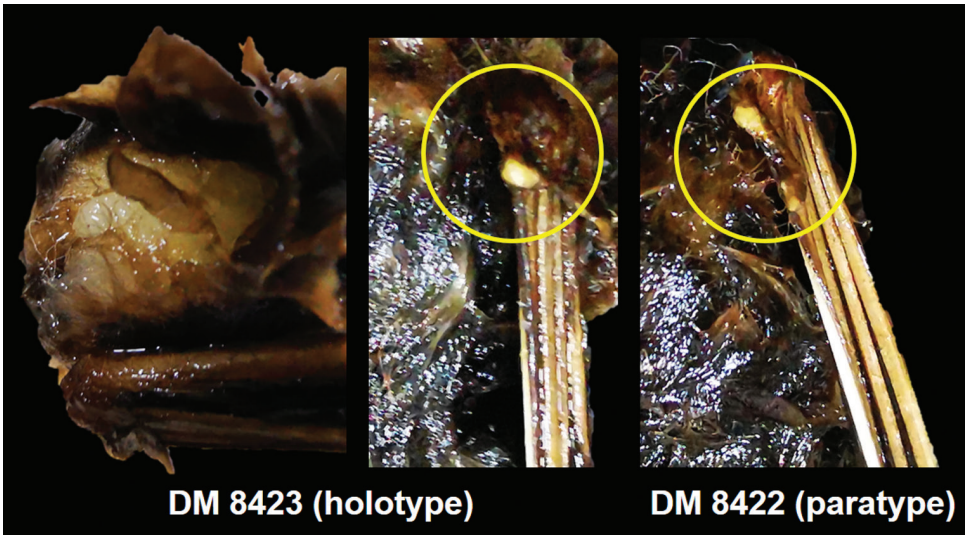

Figure 7. Lateral photograph of the alcohol-preserved head and anterior portion of the forearm and thumb of the holotype of *N. hlandzeni*, DM 8423 (left) and close-up photographs of the thumb and proximal forearm of the holotype, DM 8423 (centre) and paratype, DM 8422 (right) showing a distinct white marking at the base to the thumb. The thumb (encircled) is slightly longer than that of a typical pipistrelloid bat.

Based on eight genotyped and released individuals in the Eastern Cape Province of South Africa (Moir *et al.*, 2020), of which mtDNA sequences of five were included in our molecular study (Fig. 2), the echolocation call parameters of this species are almost indistinguishable from those of *P. hesperidus*: duration = 2.73 ± 0.32 ms; maximum frequency = 87.09 ± 8.74 kHz; minimum frequency = 46.06 ± 1.26 kHz; characteristic frequency = 46.63 ± 1.29 kHz; frequency at knee = 50.5 ± 2.08 kHz.

Biology: The species seems to be associated with coastal and scarp forests in the Eastern Cape and KwaZulu-Natal provinces of South Africa (Moir *et al.*, 2020), the lowveld savanna of Eswatini and well wooded riparian areas in Zimbabwe and Mozambique (Monadjem *et al.*, 2020). These habitats contrast somewhat with the higher-elevation habitat of *N. anchietae* based on the type specimen from Cahata in western Angola (*c.* 1300 m a.s.l.) and the localities in central Angolan represented in the current study (1000–1300 m a.s.l.). Further molecular sampling is required to determine whether previous records from Zambia, Botswana and the Democratic Republic of Congo (DRC) represent *N. anchietae* or *N. hlandzeni*. Preliminary bacular analyses and comparison with published records indicate that Zambian samples represent *N. anchietae s.s.*

DISCUSSION

In terms of phylogenetic implications, for the three mitochondrial genes analysed in our study (*cytb*, *CO1* and 12S RNA), our results concur exactly with the scheme presented by Monadjem *et al.* (2021b) for African pipistrelle-like bats in the tribes Vespertilionini and Pipistrellini, particularly in recognizing the following monophyletic genera: *Laephotis* (for *capensis*, *wintoni*, *namibensis*, *botswanae*, *angolensis*), *Neoromicia* (for *zuluensis*, *somalica*, *anchietae*, *hlandzeni* sp. nov.), *Pipistrellus* (for southern African *rusticus* and *hesperidus*) and *Afronycteris* (*nana*). Although Monadjem *et al.* (2021b) did not include *N. anchietae* in their study, they proposed, based on its sister group relationship with *N. bemainty*, that this species would belong to the genus *Neoromicia* rather than to the genus *Hypsugo* where it was previously assigned. Our study clearly confirms this hypothesis; based on 12S RNA sequences of Angolan *N. anchietae*, they were equidistant (P = 4.3–4.5%) from *N. bemainty* from Madagascar as well as from the newly described *N. hlandzeni* from southern Africa. Although the sister species relationship between *N. anchietae* and *N. hlandzeni* was not strongly supported by *cytb* or 12S RNA (< 0.7 BPP), in both genes, these two species formed a sister clade to a well-supported clade of *N. zuluensis* and *N. somalica*.

Although our mitochondrial DNA sequence data indicate paraphyly and/or deep genetic divergences between southern, eastern and/or western African clades within *A. nana*, *N. somalica*, *N. zuluensis*, *P. rusticus* and *P. hesperidus*, further integrative taxonomic studies are required within these species groups across Africa to formally describe cryptic lineages within these species.

Our description of a new southern African lowland species of pipistrelloid bat identifies *N. anchietae s.s.* as a highland-associated species and thus draws

attention to the importance of Afromontane regions as hotspots of bat speciation, diversity and micro-endemism. Our study focuses on new samples of pipistrelloid bats from the central Angolan highlands comprising the headwaters of the Cuanza, Cuita and Cuanavale rivers of the Okavango catchment ('water tower') and the Lungwebunga River of the Zambezi catchment. Our analysis resulted in the re-definition of the taxonomic and geographic limits of *N. anchietae*, restricting its range to the central and western highlands of Angola based on the studied material. This reclassification is underpinned by robust molecular evidence to reveal that *N. anchietae* is endemic to this ecologically important water tower. Populations previously assigned to this species from South Africa and Eswatini, assigned here to *N. hlandzeni*, are ecologically distinct, known from distinct coastal forest and low-lying ('lowveld') savanna localities, which further support the specific identity of these populations.

Similar to *N. anchietae*, the long-eared *L. angolensis* was commonly recorded in central Angola by our study. The species was thought to be near endemic to the central highlands of Angola, with an outlier known from the highlands of south-eastern DRC (Monadjem *et al.*, 2020). However, our study showed that *cytb* and 12S RNA sequences referable to *L. botswanae* from northern Namibia, northern Botswana and/or south-western Zambia could not be distinguished from those of *L. angolensis* from Angola, strongly supporting the conspecificity of these two taxa. Thus, we recommend synonymizing the more recent name of *L. botswanae* (Setzer, 1971) under the senior name, *L. angolensis* Monard, 1935. Specimens of *L. botswanae s.l.* from South Africa and Mozambique are slightly divergent from the *L. angolensis*/ *L. botswanae* clade (P = 2.8% in *cytb*), but future integrative taxonomic studies of larger samples are required to establish whether this clade in synonymous with *L. angolensis* or not.

Krásová *et al.* (2021) recently described 12 distinct molecular operational taxonomic units (MOTUs) of rodents as being endemic to Angola, almost exclusively to Afromontane forest-grassland and central high-lying mesic miombo savannas. The high species richness of Angolan rodents was attributed to mixed affinities with three distinct biogeographic groups (following Linder *et al.*, 2012), Congolese, East African and southern African. Similar affinities and relatively high species richness were found in our study of central Angolan pipistrelle-like bats, with widespread species such as *A. nana*, *L. capensis*, *P. rusticus* and *N. zuluensis* showing shallow to deeper genetic divergences indicative of Late Pliocene to Early Pleistocene vicariance events between Angolan, East African, West African and southern African populations. Further integrative taxonomic studies may reveal further speciation events associated with these evolutionary pulses.

Apart from their critical importance as a water tower for the region, the headwaters of the Okavango Basin rank as a critically important biodiversity hotspot whose effective conservation is paramount. This is particularly important given the vulnerability of the region to anthropogenic threats such as deforestation for agriculture and settlement, fires, resource overharvesting and unsustainable abstraction of water and the creation of dams (Huntley *et al.*, 2019). In conclusion, our findings highlight the need for further taxonomic studies on African pipistrelloids, particularly in the central highlands of Angola as a speciation hotspot for bats.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Details of specimens included in molecular, morphological (craniodental or bacular) and/or morphometric analysis of pipistrelloid bats from Angola, Eswatini, South Africa, Mozambique and Botswana. Abbreviation of Museums: DM (Durban Natural Science Museum); TM (Ditsong National Museum of Natural History); UNESWA (University of Eswatini). Availability of skull or baculum specimens indicated by 'x'. GenBank numbers are included for specimens having DNA sequences for the three mitochondrial genes, cytochrome *b* (*cytb*), cytochrome oxidase subunit 1 (*CO1*) and 12S ribosomal RNS (12S). Specimens marked with asterisks under 'Skull' indicate those having skulls available but not measured in final morphometric analyses.

Table S2. Uncorrected P-distances (number of base differences per site from averaging over all sequence pairs) from the *CO1* mitochondrial gene are shown, obtained between (bottom left) and within (on diagonals in bold face; 'n/c' indicates sample sizes too small for calculation) groups of pipistrelloid bats from Angola, Eswatini and South Africa in relation to GenBank sequences of comparable African species for the *CO1* mitochondrial gene. See Supporting Information, Table S1 for details of specimens used in the study. This analysis involved 118 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 478 positions in the final dataset. Analyses were conducted in MEGA X (Kumar *et al.*, 2018). Abbreviations for groups are as follows: Anan = *Afronycteris nana*, Lang = *Laephotis angolensis*, Lbot = *L. botswanae*, Lcap(Ang) = *L. capensis* (Angola), Lcap(AngSA) = *L. capensis* (South Africa and Angola), Nanc = *Neoromicia anchietae*, Nsom = *N. somalica*, Nzul = *N. zuluensis*, Phesp = *Pipistrellus hesperidus*, Prust(Ang) = *P. rusticus* (Angola), Prust(SA) = *P. rusticus* (southern Africa), Outgroup = outgroup (*Miniopterus* spp.).

Table S3. Uncorrected P-distances (number of base differences per site from averaging over all sequence pairs) from the 12S RNA mitochondrial gene are shown, obtained between (bottom left) and within (on diagonals in bold face; 'n/c' indicates sample sizes too small for calculation) groups of pipistrelloid bats from Angola, Eswatini and South Africa in relation to GenBank sequences of comparable African species for the 12S RNA mitochondrial gene. See Supporting Information, Table S1 for details of specimens used in the study. This analysis involved 72 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 783 positions in the final dataset. Analyses were conducted in MEGA X (Kumar *et al.*, 2018). Abbreviations for groups are as follows: Anan(Ang) = *Afronycteris nana* (Angola), Anan(EA) = *A. nana* (East Africa), Anan(WA) = *A. nana* (West Africa), Lang = *Laephotis angolensis*/*botswanae* (Angola, South Africa, Mozambique), Lcap(EA) = *L. capensis* (East Africa), Lcap(SA) = *L. capensis* (southern Africa), Lnam = *L. namibensis*, Nanc = *Neoromicia anchietae*, Nbem = *N. bemainty* (Madagascar), Nsom = *N. somalica* (East Africa), Nsp_nov = N. sp. nov. (= *N. hlandzeni* from South Africa and Eswatini), Nzul(Ang) = *N. zuluensis* (Angola), Nzul(SA) = *N. zuluensis* (South Africa, Eswatini), Phesp(SA) = *Pipistrellus hesperidus* (South Africa, Eswatini), Pcf.rust(SWA) = *P.* cf. *rusticus* (West Africa, Eswatini), Prust = *P. rusticus* (Angola), Outgroup = outgroup (*Miniopterus* spp).

Figure S1. Bayesian phylogeny of mitochondrial cytochrome oxidase one sequences (478 nucleotides) of Vespertilionidae genera *Afronycteris*, *Neoromicia*, *Laephotis*, *Pipistrellus* sampled from Angola, South Africa and Eswatini and the outgroup, *Miniopterus* spp. (Miniopteridae). Bayesian phylogenetic analysis was performed using the Hasegawa–Kishino–Yano model incorporating invariant sites and a gamma distribution (HKY+I+G). Posterior probabilities of > 0.7 are indicated at internal nodes. Samples obtained in this study are indicated in bold. Branch colours indicate posterior probabilities at each node.

Figure S2. Bayesian phylogeny of mitochondrial 12S sequences (478 nucleotides) of pipistrelle-like Vespertilionidae genera *Afronycteris*, *Neoromicia*, *Laephotis* and *Pipistrellus* sampled from Angola, South Africa and Eswatini and outgroup *Miniopterus* spp. (Miniopteridae). Bayesian phylogenetic analysis was performed using the Hasegawa–Kishino–Yano model incorporating invariant sites and a gamma distribution (HKY+I+G). Posterior probabilities of > 0.7 are indicated at internal nodes. Samples obtained in this study are indicated in bold. Branch colours indicate posterior probabilities at each node.