

# **Testing the efficacy of bat monitoring methods for identification and species surveys in KwaZulu-Natal Province, South Africa**

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## Abstract

Multi-method sampling approaches are becoming increasingly popular for investigating species occurrence at specific sites, as there is a need to accurately monitor species with limited time and resources. In this study, a multi-method comparative approach was used to survey bat species in the foothills of the Drakensberg Mountain range in KwaZulu-Natal Province, South Africa. We used historical museum records and species distribution modelling (SDM) to predict which species would likely occur in our study area. We then compared physical capture (by deploying mist nets) with acoustic surveys (using an Anabat bat detector) to assess the bat species assemblages present. SDM predicted eight bat species to occur from the historical checklist of 28 species recorded in the broader region as no museum records existed for the specific study area. Species detection by acoustic data yielded the highest number of detected species ( $n = 11$ ) while active trapping yielded nine species from 54 individuals, namely, *Laephotis botswanae*, *L. capensis*, *Myotis tricolor*, *Pipistrellus hesperidus*, *Rhinolophus clivosus*, and *Tadarida aegyptiaca* with molecular confirmation required for *Miniopterus fraterculus*, *R. darlingi*, and *R. swinnyi*. These complementary sampling methods may be necessary for accommodating the limitations of each individual method for a more inclusive assessment of bat species richness in a targeted landscape. The present study could be used as a model approach to assess the biodiversity and demographics of other taxa and in other habitats.

**Keywords:** Chiroptera, Maxent, morphometrics, cytochrome b, Anabat, South Africa

## Introduction

Bats (order Chiroptera) present unique challenges for identification and sampling due to their elusive, nocturnal lifestyle, and the presence of cryptic species (Monadjem *et al.* 2010a). There are a range of methods that are used to survey, identify, and map the distribution of bat species, including various physical capture techniques, species distribution modelling (SDMs), and acoustic surveys. It has been suggested that a combination of sampling methods is necessary to counter the

limitations of individual techniques and improve the efficiency of species surveys (Hayes *et al.* 2009; Lintott *et al.* 2013). In addition, current studies are increasingly combining morphological features with molecular sequences for identifying bats (Carstens *et al.* 2002; Monadjem *et al.* 2013). As a result, multi-method sampling approaches, with integrative taxonomy for correct identification, are becoming increasingly popular (Silva and Barnard 2017).

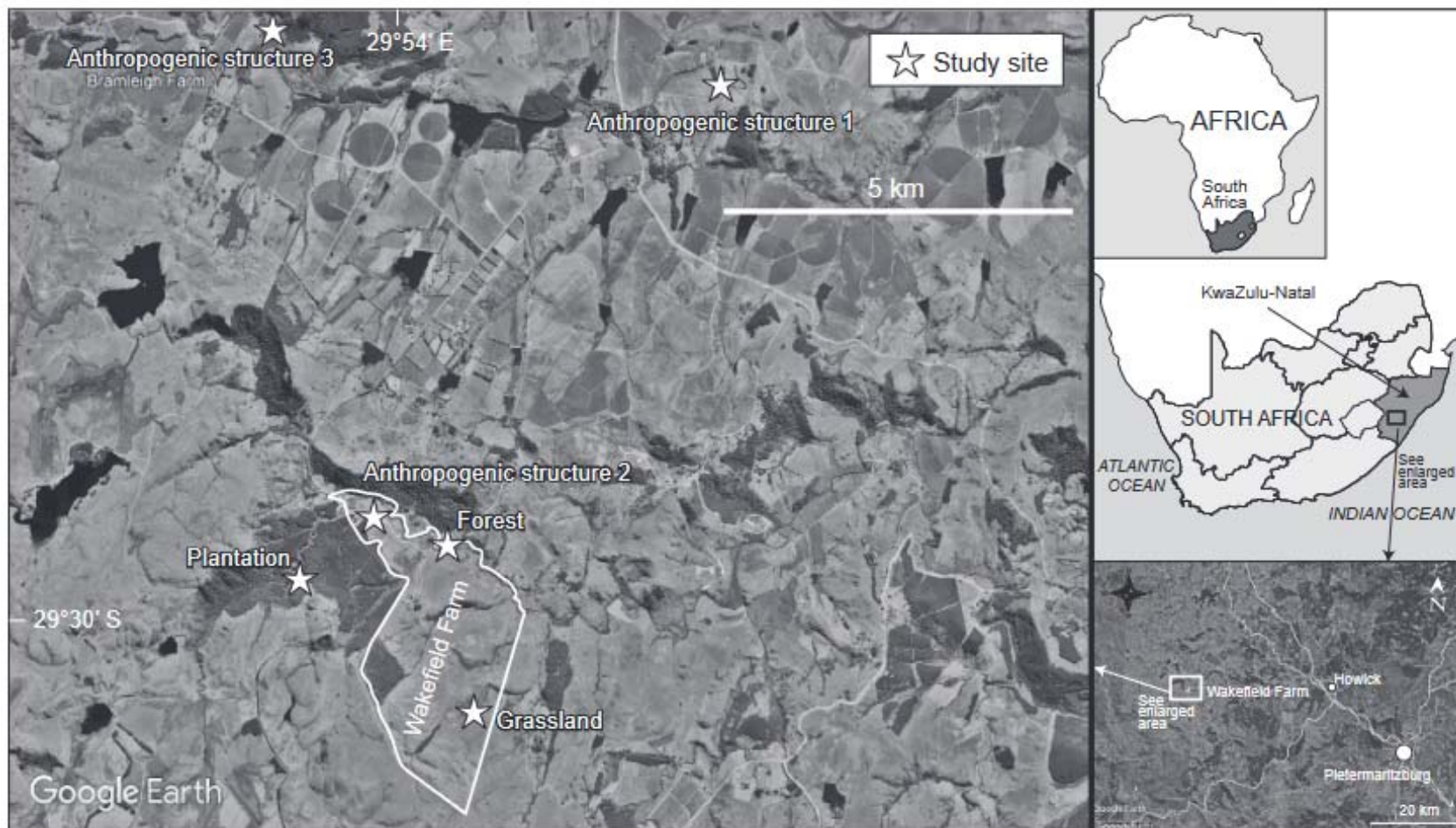
There is extensive knowledge on species identifications and their occurrence in natural history museum collections sampled over the last century (Cotterill 1995, 2002; Graham *et al.* 2004), including the original description of type specimens (Bradley *et al.* 2014). These collections have played an important role in the process of mapping species distributions in the past. There has been a recent revival and increased use of these historic natural history databases for SDMs, which is a widely applied, remote mapping tool that uses occurrence data with environmental variables for predictive modelling of geographic distributions of species (Anderson and Gonzalez 2011; Bradley *et al.* 2014; Monadjem *et al.* 2021a). This remote estimation of species distributions based on natural history museum records may best inform the species predicted to be present at a site, and hence the potential sampling sites to aim for during surveys and monitoring assessments (Smeraldo *et al.* 2018; Monadjem *et al.* 2021a).

Museum specimens and SDMs allow insights into either the past or predicted distribution of species, but only some form of survey can confirm the current presence of a species at a specific site. Traditionally, these surveys involved the physical capture of bats (e.g., from roosts, through shooting, or more usually with the use of mist nets) (Rautenbach *et al.* 1981). In contrast, more recently acoustic detectors have been used to record echolocation calls of bats without having to capture the animals (Gorresen *et al.* 2008; Stathopoulos *et al.* 2018). Hence, ultrasonic bat detectors remotely detect and record the echolocation calls of free-flying bats without observer disturbance (Monadjem *et al.* 2007; Gorresen *et al.* 2008;). This automated detection method has the limitation of species validation, which is dependent on available reference call libraries (Taylor *et al.* 2013; Monadjem *et al.* 2017), as well as the intra-specific variation of calls influenced by habitat (Mutumi *et al.* 2016) and sex (Kearney *et al.* 2019) as reviewed by Russo *et al.* (2018). Despite

these limitations, bat detectors are a cost-effective, non-invasive, and widely applicable surveying method that are, for example, advocated as a standard protocol for environmental impact assessments (MacEwan *et al.* 2020).

Examination of external characters remains the most common form of field identification for bat species (Bader *et al.* 2015; Tsang *et al.* 2016). Although it is possible to identify many bat species using traditional morphological characters such as tragus shape, pelage colour, and some external measurements (e.g. the forearm length), the discovery of cryptic species that are typically indistinguishable on external morphology (see Stoffberg *et al.* (2010), and Monadjem *et al.* (2021b) for examples) has resulted in molecular techniques becoming increasingly important in recent decades (e.g. Carstens *et al.* 2002; Monadjem *et al.* 2013; Zhou *et al.* 2016). As a result, molecular data and their associated phylogenetic analyses are often used to complement morphological identifications (Ratrimomanarivo *et al.* 2007; Vallo *et al.* 2008; Taylor *et al.* 2012; Monadjem *et al.* 2021c).

Although KwaZulu-Natal (KZN) Province in South Africa has received relatively good coverage with respect to bat surveys (Taylor 1998; Monadjem *et al.* 2010a), this coverage has not been uniform. As a result, some important biogeographic regions, such as the foothills of the Drakensberg Mountains, have received little attention from mammalogists (Simelane *et al.* 2018). Yet, this region is predicted to undergo rapid anthropogenic land cover transformation (Jewitt *et al.* 2015). Therefore, the aim of this study was to produce a checklist of bat species for one particular area (Wakefield) within the foothills of the Drakensberg in the KZN Midlands. The objectives of the study were to: 1) compile museum records from the greater KZN region; 2) predict species occurrence at Wakefield using SDMs; 3) use both physical and acoustic surveys to record bat occurrences at Wakefield; and 4) use an integrative taxonomic approach to identifying bat species captured at Wakefield by combining traditional morphological characters and molecular sequences.



**Figure 1.** The six sampling sites (indicated by black dots) on Wakefield Farm and surrounding properties in Nottingham Road, KwaZulu-Natal (KZN) Province, South Africa, with inserts of Africa, KZN Province and the country.

## **Methods**

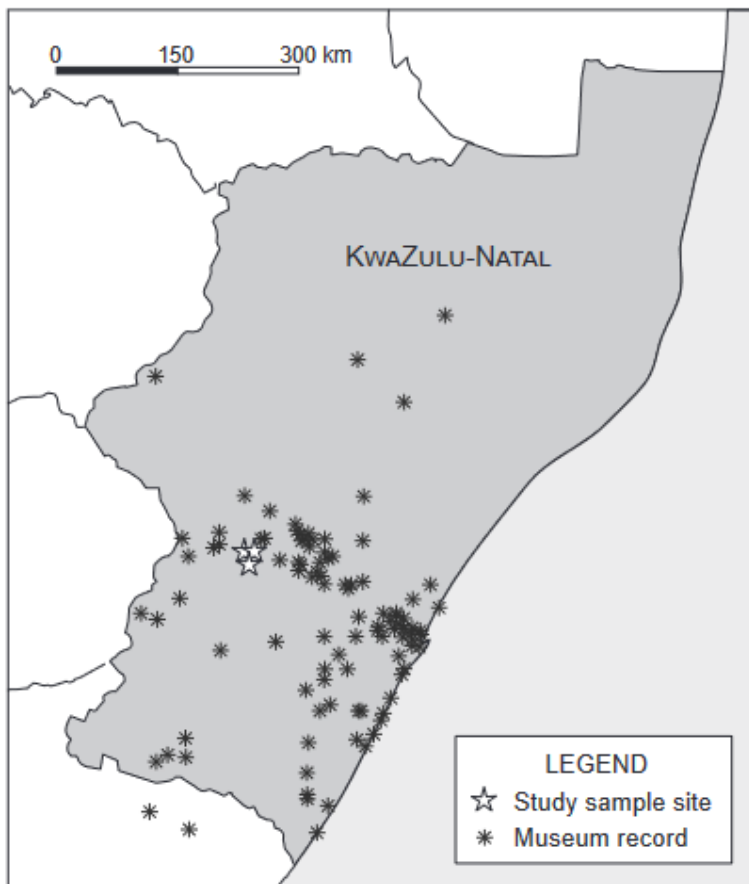
### ***Study area***

The present study was conducted in the mid-altitude landscape of farming properties around Nottingham Road in KZN Province, South Africa (Figure 1). Sampling was conducted across all seasons (from June 2016 to May 2017) and a variety of anthropogenic structures (29°29'12.4"S 29°53'52.7"E), transformed and natural habitats such as mistbelt forest (29°29'24.7"S 29°54'25.4"E), exotic plantations (29°30'04.3"S 29°53'38.1"E), and grasslands (29°30'39.0"S 29°54'30.8"E) for spatial and temporal coverage. The study area falls within the Drakensberg Foothill Moist Grassland vegetation unit of the Sub-escarpment Grassland Bioregion at between 1370 and 1780 metres above sea level (Rutherford *et al.* 2006; Simelane *et al.* 2018). This summer rainfall region experiences a mean annual precipitation of 890 mm with 26 frost days per year and a mean annual temperature of 14.6° C (Mucina *et al.* 2006). Midday average temperatures were 25.2° C during the hottest month of January and 18.8° C during the coldest month of July. A full description of the study area is provided by Simelane *et al.* (2018).

### ***Museum records and SDM***

Records from 265 localities within the one-degree grid cell of 29° South and 30° East (Figure 2), situated in the KZN Midlands were compiled from natural history museum records from the following chiropteran databases in South Africa: 1) the Ditsong Museum of Natural History (Pretoria), Gauteng Province; 2) Durban Natural Science Museum (Durban), KZN Province; and 3) Amathole Museum (King William's Town), Eastern Cape Province. These records included cross-referencing to verify against the record list in the authoritative "*Bats of Central and Southern Africa: A biogeographic and taxonomic synthesis*" by Monadjem *et al.* (2010a) which includes distributional records from museums internationally. Following Pearson *et al.* (2007), chiropteran occurrence data recorded from the broader KZN Midlands region with more than four records were used for SDM based on Maxent version 3.3 (Phillips *et al.* 2006) to predict bat species occurrence in our specific study area. Of the 28 species on record, only 16 species met the modelling criteria of more than four museum records. To avoid pseudo

replication, any repeated locality records for the same species were excluded. The following WorldClim bioclimatic variables as well as altitude were used since the pairwise Pearson's correlations between each of them was less than 0.7 as suggested by Phipps *et al.* (2017): annual mean temperature (BIO1), mean diurnal range (BIO2), isothermality (BIO3), minimum temperature of coldest month (BIO6), annual precipitation (BIO12), precipitation seasonality (BIO15), and precipitation of coldest quarter (BIO19) (Schulze *et al.* 1997; O'Donnell and Ignizio 2012). Equal training sensitivity and specificity was used as the logistic threshold for a species' presence and a 20-kilometre cut-off distance from the study area was set to visualise the species distribution maps in QGIS version 2.18 (<http://qgis.osgeo.org>). The resultant area under curve (AUC) from this procedure (the values of which range from 0 to 1) provide an indication of the model's performance (Phillips *et al.* 2006).



**Figure 2.** A map of bat museum records (\*) used for species distribution modelling (SDM) in the broader region of KwaZulu-Natal (KZN) Province, South Africa. ★ = the specific study area

## ***Field surveys***

A combination of 6 m and 9 m (2.4 m high, 14x14 mm mesh, Ecotone, Gdynia, Poland) mist nets were set across roads, riverine and near roost sites in each of the six sampling sites (anthropogenic structures 1 and 2, agricultural fields, forest, grassland and plantation habitats) and checked every 15 minutes from sunset until bat activity decreased or weather interrupted sampling (Kunz and Kurta 1988). Mist netting was undertaken during all sampling seasons (i.e., winter, spring, late summer, early summer, and autumn). After removal of captured bats from mist nets, the sampled specimens were then placed in a clean bat bag for the subsequent recording of body mass (g) using a 50 g spring balance hanging scale (Pesola® Lightline; Schindellegi, Switzerland) and a 1 kg digital hanging scale (American Weigh Scales; Norcross, U.S.A.) for validation. Standard external measurements that included total, tail, hind foot, ear, and forearm lengths were recorded using a pair of 150 mm digital callipers (Tork Craft; Midrand, South Africa).

Two wing biopsies were taken from the plagiopatagium of each specimen using a sterile 3 mm diameter dermal punch (Kruuse; Langeskov; Denmark) which were then stored in 99% ethanol (Worthington-Wilmer and Barratt 1996). The sampled specimens were then released near the site of capture and sterile collection of feces from the specimens' holding bags were stored in a labelled Eppendorf tube with silica gel before freezing (Herrera *et al.* 2001). An Anabat Express bat detector (Titley Electronics, Balina, Australia) was deployed 1-2 metres above ground facing upwards to optimize capturing echolocation calls, on an alternating schedule with the mist net sampling at the same position at each sampling site. The "Night" sampling mode on the Anabat was set to 12 hours from dusk to dawn with a data division ratio of 8 and sensitivity of 115. The recorded echolocation calls were analysed using AnalookW programme version 0.3.8.13 (C. Corben) by applying anti-noise and species filters as described by Taylor *et al.* (2013) and Monadjem *et al.* (2017) as detailed in Appendix A. Due to the overlapping characteristics of *Myotis tricolor* and *Laephotis botswanae* calls with *L. capensis*, all files were manually checked to avoid misclassification.



Furthermore, *Rhinolophus simulator* and *R. darlingi* are reported together as their echolocation parameters are similar and cannot be confidently separated in the field (Monadjem *et al.* 2007).

### ***Identification of bat species***

The “*Bats of Southern and Central Africa: A biogeographic and taxonomic synthesis*” by Monadjem *et al.* 2010a) was used to identify bats in the field to the species level based on the recorded external morphometric measurements. We augmented this with molecular identification in cases where bats could not be identified with certainty in the field. Each wing biopsy sampled was rehydrated twice before DNA extraction using the Roche High Pure PCR Template Preparation kit (Roche Applied Science, Germany) following manufacturer’s instructions except that samples were left overnight to aid tissue digestion. DNA extracts were amplified using universal mammalian cytochrome b (*Cyt b*) primers (L12745, H15195, Inqaba Biotec Pretoria) to aid species identification and to allow for comparable sequences (Irwin *et al.* 1991). TaKaRa EmeraldAmp GT PCR Master Mix (Inqaba Biotec, Pretoria) was used as Taq polymerase in a 25 uL polymerase chain reaction (PCR) with a range of family-specific thermal cycling conditions. Gel electrophoresis using 3% agarose gel, and the GeneRuler 100bp Plus DNA ladder was used to visualise the amount of amplified DNA extract indicated by the presence and brightness of 1500 bp bands. Instructions of the Roche High Pure PCR Product Purification Kit were followed before bi-directional cycle sequencing using the quarter reaction method and Big Dye terminator cycle-sequencing kit (Perkin-Elmer, Foster City, U.S.A.) at an annealing temperature of 48° C. Precipitation of DNA was performed using the sodium acetate method and then sequenced at the Bioinformatics Centre at the University of Pretoria, Pretoria, South Africa. A total of 52 sample sequences were checked, edited, and aligned in MEGA 5.2 (Tamura *et al.* 2011) before comparison to 70 published reference sequences on GenBank with *Afronycteris nana*, *Hipposideros caffer*, *Otomops martiensseni* and *Scotophilus dinganii* as outgroups. Two unpublished references of sequences had to be used due to lack of any other comparisons for *Laephotis* (Trujillo *et al.* 2008). A p-distance matrix and neighbour-joining (NJ) tree, set with uniform rates among sites, complete deletion of missing

data and the nucleotide substitution model set at 1000 bootstraps, was constructed for each genus to confirm the species identification of each sample and identify discrete genetic variants. Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted using a representative dataset of sample and reference sequence variants in PhyML 3.1 (Guindon and Gascuel 2003) and MrBayes v3.2.6 (Ronquist *et al.* 2012), respectively. FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to visualise the most parsimonious tree configuration with nodal support from 65% and 0.8 reported for bootstrap support and posterior probabilities, respectively. These analyses were based on the best-fit model of sequence evolution selected under the Akaike Information Criterion (AIC) in jModeltest 2.1.10 (Darriba *et al.* 2015).

### **Comparison of methods**

The  $\chi^2$  test (McCullagh and Nelder 1989) was used to assess the total species counts between the survey techniques of mist net captures and acoustic recordings. All statistical analyses were calculated in Microsoft Excel with an expected value of 12 species in total (Wilson 2014).

## **Results**

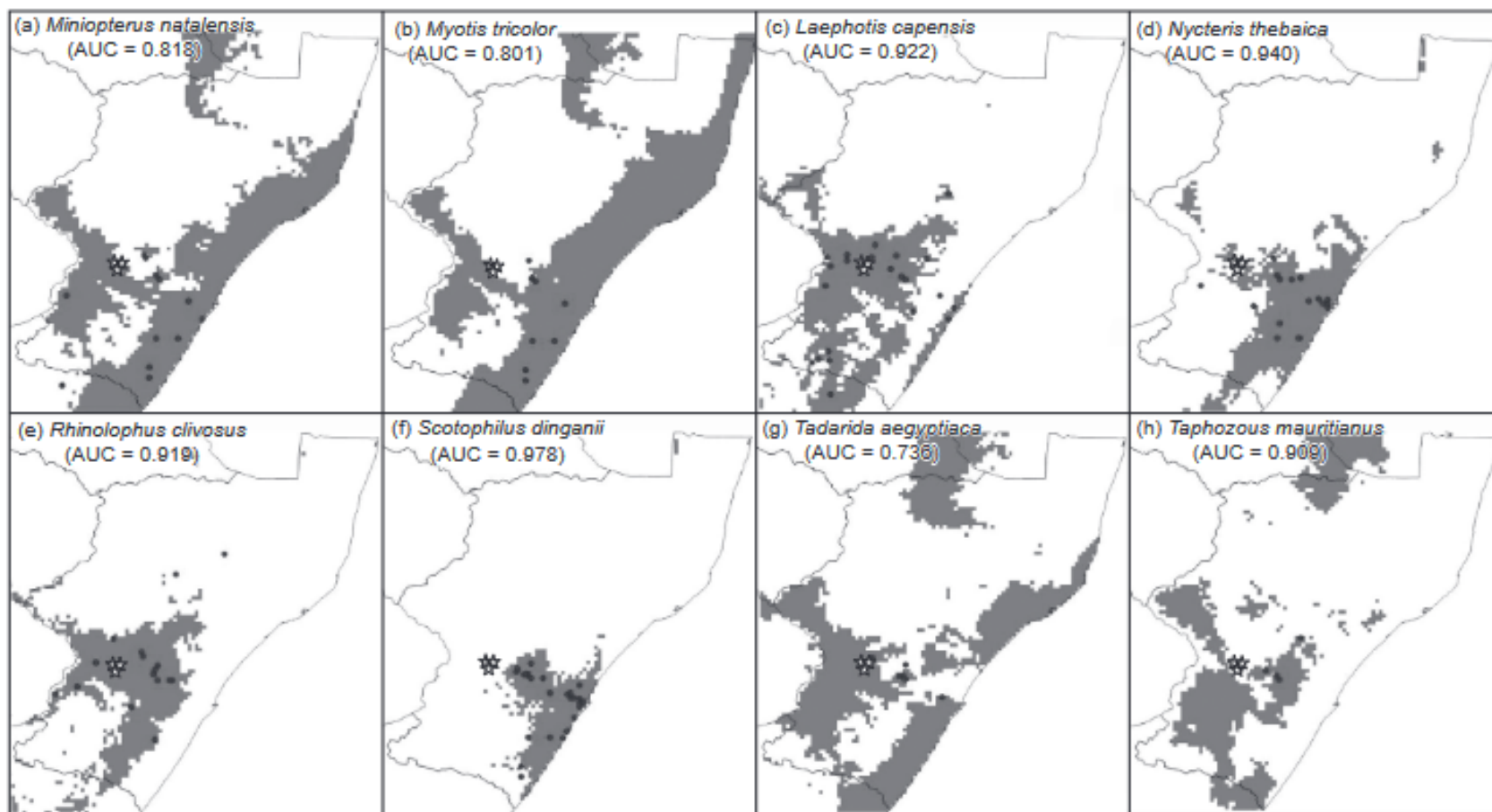
### **Museum records and SDM**

Museum records indicated that 28 species of bats from 20 genera and seven families had previously been recorded in the broader region (Table 1), but none of these were recorded in the sampling area. The closest records were within adjacent quarter degree squares at least 30 km away (African Chiroptera Report 2016; Figure 2). Based on Maxent, the following eight species (in eight genera and six families) were predicted to occur in our study area (Table 1; Figures 2 and 3): *L. capensis*, *Nycteris thebaica*, *Miniopterus natalensis*, *Myotis tricolor*, *R. clivosus*, *Scotophilus dinganii*, *Tadarida aegyptiaca*, and *Taphozous mauritanus* with an AUC range of between 0.736 to 0.978.

**Table 1.** A list of bat species predicted to occur (✓) or not to occur (X) in the study area based on species distribution modelling (SDM) based on Maxent from the list of 28 species obtained from museum records of the broader region in KwaZulu-Natal (KZN) Midlands, KwaZulu-Natal Province, South Africa. N/A indicates species which were not modelled due to insufficient records. Scientific naming convention follows that of the American Society of Mammalogists (ASM) mammal diversity database as of 2021 (Simmons and Cirranello 2022).

Family	Scientific name	Name	Red list status	Species predicted (Y) or not to occur (N) in the study area from the region
Pteropodidae	<i>Epomophorus wahlbergi</i>	Wahlberg's epauletted fruit bat	Least concern (Schoeman et al. 2016)	N
Emballonuridae	<i>Taphozous mauritanus</i>	Mauritian tomb bat	Least concern (Monadjem et al. 2016n)	Y
Hipposideridae	<i>Hipposideros caffer</i>	Sundevall's leaf-nosed bat	Least concern (Monadjem et al. 2016b)	N
Nycteridae	<i>Nycteris thebaica</i>	Egyptian slit-faced bat	Least concern (Monadjem et al. 2016k)	Y
Miniopteridae	<i>Miniopterus fraterculus</i>	Lesser long-fingered bat	Least concern (MacEwan et al. 2016a)	N
	<i>Miniopterus cf. inflatus</i>	Greater long-fingered bat	Near threatened (Richards et al. 2016a)	N/A
	<i>Miniopterus natalensis</i>	Natal long-fingered bat	Least concern (MacEwan et al. 2016b)	Y
Molossidae	<i>Chaerephon pumilus</i>	Little free-tailed bat	Least concern (Monadjem et al. 2016a)	N
	<i>Mops condylurus</i>	Angolan free-tailed bat	Least concern (Monadjem et al. 2016e)	N/A
	<i>Otomops martiensseni</i>	Large-eared giant mastiff bat	Least concern (Richards et al. 2016b)	N
	<i>Tadarida aegyptiaca</i>	Egyptian free-tailed bat	Least concern (MacEwan et al. 2016c)	Y
Rhinolophidae	<i>Rhinolophus clivosus</i>	Geoffrey's horseshoe bat	Least concern (Jacobs et al. 2016a)	Y
	<i>Rhinolophus simulator</i>	Bushveld horseshoe bat	Least concern (Jacobs et al. 2016b)	N
	<i>Rhinolophus swinnyi</i>	Swinny's horseshoe bat	Vulnerable (Jacobs et al. 2016c)	N/A

Vespertilionidae	<i>Afronycteris nana</i>	Banana bat	Least concern (Monadjem et al. 2016j)	N
	<i>Cistugo lesueuri</i>	Leseur's wing-gland bat	Least concern (Avenant et al. 2016a)	N/A
	<i>Eptesicus hottentotus</i>	Long-tailed serotine	Least concern (Watson et al. 2016)	N/A
	<i>Kerivoula argentata</i>	Damara woolly bat	Near threatened (Monadjem et al. 2016d)	N/A
	<i>Laephotis botswanae</i>	Botswana long-eared bat	Least concern (Taylor et al. 2016)	N/A
	<i>Laephotis capensis</i>	Cape serotine	Least concern (Monadjem et al. 2016i)	Y
	<i>Laephotis cf. wintoni</i>	De Winton's long-eared bat	Vulnerable (Avenant et al. 2016b)	N/A
	<i>Myotis bocagii</i>	Rufous myotis	Least concern (Monadjem et al. 2016f)	N/A
	<i>Myotis tricolor</i>	Temminck's myotis	Least concern (Monadjem et al. 2016g)	Y
	<i>Myotis welwitschii</i>	Welwitsch's myotis	Least concern (Monadjem et al. 2016h)	N/A
	<i>Neoromicia anchietae</i>	Anchieta's pipistrelle	Least concern (Monadjem et al. 2016c)	N/A
	<i>Pipistrellus hesperidus</i>	Dusky pipistrelle	Least Concern (Monadjem et al. 2016l)	N
	<i>Scotoecus albobfuscus</i>	Thomas's house bat	Near Threatened (Richards et al. 2016c)	N/A
	<i>Scotophilus dinganii</i>	Yellow-bellied house bat	Least concern (Monadjem et al. 2016m)	Y



**Figure 3.** The distribution of each of the eight species (A – H) predicted to occur in the study area in the KwaZulu-Natal (KZN) Midlands, KwaZulu-Natal Province, South Africa as modelled by species distribution modelling (SDM) based on Maxent (Phillips *et al.* 2006). Each red circle indicates the museum records used by the model to predict the resultant distribution indicated by the grey pixels. ★ = the specific study area.

**Table 2.** A comparison of each species detection method (mist netting and acoustic detection based on an Anabat Express detector) grouped according to whether it recorded the species in the study area (✓) or not (X) or it was unable to be identified by the methods (?) or it was not applicable (NA) because it did not meet the criteria of species distribution modelling (SDM) based on Maxent

Species	Actual and predicted occurrences		Monitoring method		Physical capture determination	
	Museum records	Maxent models	Acoustic capture	Physical capture	Morphological ID	Molecular ID
<i>Afronycteris nana</i>	F	F	S	F	F	F
<i>Epomophorus wahlbergi</i>	F	F	F	F	F	F
<i>Hipposideros caffer</i>	F	F	F	F	F	F
<i>Laephotis botswanae</i>	F	N/A	S	S	S	S
<i>Laephotis capensis</i>	F	S	S	S	S	S
<i>Miniopterus fraterculus</i>	F	F	S	S	?	S
<i>Miniopterus natalensis</i>	F	S	S	F	?	F
<i>Chaerephon pumilus</i>	F	F	F	F	F	F
<i>Myotis tricolor</i>	F	S	S	S	S	S
<i>Nycteris thebaica</i>	F	S	F	F	F	F
<i>Otomops martiensseni</i>	F	F	F	F	F	F
<i>Pipistrellus hesperidus</i>	F	F	S	S	F	S
<i>Rhinolophus clivosus</i>	F	S	S	S	S	S
<i>Rhinolophus darlingi</i>	F	N/A	?	S	F	S
<i>Rhinolophus simulator</i>	F	F	?	F	?	F
<i>Rhinolophus swinnyi</i>	F	N/A	S	S	?	S
<i>Scotophilus dinganii</i>	F	S	S	F	F	F
<i>Tadarida aegyptiaca</i>	F	S	S	S	S	S
<i>Taphozous mauritanus</i>	F	S	F	F	F	F
Total	0	8	11	9	5	9

## **Field surveys**

Nine bat species from four families were recorded from 54 mist netted individuals after 2121.8 mist net hours (see Appendix B), namely, *Laephotis botswanae*, *L. capensis*, *M. tricolor*, *M. fraterculus*, *P. hesperidus*, *R. clivosus*, *R. darlingi*, *R. swinnyi*, and *T. aegyptiaca*. In comparison, a total of 5,173 files consisting of 189,536 calls were recorded from 11 species over 69 nights using the Anabat echolocation detector. The acoustically-identified species included *L. botswanae*, *L. capensis*, *M. fraterculus*, *M. tricolor*, *P. hesperidus*, *R. clivosus*, *R. swinnyi*, and *T. aegyptiaca* with three species only detected by acoustic surveys, namely, *Afronycteris nana*, *M. natalensis* and *S. dinganii*. The results of acoustic and mist net data were 83% similar in species composition across all sampling surveys as *R. darlingi* was the only species detected by mist netting (Table 2).

## **Final identification**

A total of nine species from 54 individuals were sampled during this study as *L. botswanae*, *L. capensis*, *M. tricolor*, *R. clivosus*, *T. aegyptiaca*, with two individuals identified as *M. fraterculus* and *R. swinnyi* based on molecular data. Further genetic comparisons revealed five individuals which were morphometrically misclassified in the field as either *L. capensis* ( $n = 3$ ) or *R. clivosus* ( $n = 2$ ) were genetically identified as *P. hesperidus* and *R. darlingi*, respectively (Appendix C). The use of molecular data increased the number of species detected by physical captures due to the additional recognition of *P. hesperidus*, *R. darlingi* and confirmation of *M. fraterculus*, and *R. swinnyi*.

## **Comparison of methods**

A combined species checklist for the study area consists of 14 bat species utilising all methods with the totals between each comparative method indicated in Table 2. Based on SDMs, only 8 species were predicted to occur in our study area (Table 2), however, we captured three species, *L. botswanae*, *R. darlingi* and *R. swinnyi*, that could not be modelled due to small sample sizes. Even including these three

species, the number of species predicted to occur by SDM ( $n = 8$ ) is one less than the total 12 species we recorded by either physical or acoustic sampling. Although species detection by acoustic data yielded the highest number of detected species ( $n = 11$ ), this however, was not significantly different from mist netting data ( $\chi^2 = 0.361$ ;  $df = 1$ ;  $n = 20$ ;  $P = 0.452$ ).

## Discussion

The relatively low number of historical occurrence records around our study area emphasises the relevance of attempting to better document the bat species richness of this area before further anthropogenic transformation occurs (Jewitt *et al.* 2015). The SDM of the 16 species modelled compare relatively well to published distributional maps despite some limitations, while the regional estimate of 28 species from historical data illustrates the large differences in sampling bias and temporal scale (Monadjem *et al.* 2010a; Brinkley *et al.* 2021). The limitations of presence-only records and sampling area bias inherently associated with historical museum records may have been accentuated by using a minimum of four occurrence records to model the potential distributions (although see Pearson *et al.* 2007). Failure to consider biogeographic and temporal sampling bias and seasonal requirements may underestimate the potential species range as indicated by the active sampling results of *A. nana*, *M. fraterculus*, *P. hesperidus* and *S. dinganii* (Smeraldo *et al.* 2018) while rare species are also excluded which do not meet the modelling criteria (Brinkley *et al.* 2021).

Another possible explanation for the misaligned predictive distributions of some species is the transitional zone of biomes with the increasing factor of altitude in the study area which is known to greatly affect bat species richness (Schoeman *et al.* 2013; Reardon and Schoeman 2017). This study area is not centered within the KZN hotspot of bat species richness yet falls within the grassland biome which is the second richest after the savanna (Gelderblom *et al.* 1995; Cooper-Bohannon *et al.* 2015). Museum records and SDM predicted the presence of *N. thebaica* and *T. mauritanus* which were neither captured nor detected, possibly due to their low-duty cycle and low intensity echolocation calls, absence of preferred roosting



microhabitat or seasonal movements (Monadjem *et al.* 2010a). Except for these two species, all other species predicted by the SDM were detected either physically or acoustically. We also captured three additional species (*L. botswanae*, *R. darling*, *R. swinnyi*) which could not be modelled due to small sample size as well as four species (*A. nana*, *M. fraterculus*, *P. hesperidus*, *S. dinganii*) which were not predicted to occur. This illustrates the potential challenges of relying only on museum records or SDMs (Graham *et al.* 2004; Anderson and Gonzalez 2011).

The only other known bat survey in the study area was undertaken by Kearney and Seamark (2015 unpub.), who using traditional morphology-based identification, detected 5 species from 30 individuals, namely *L. capensis*, *L. botswanae*, *R. clivosus*, *R. swinnyi*, and *T. aegyptiaca*, all of which were captured in the current study in addition to *M. tricolor*, *M. fraterculus*, *P. hesperidus* and *R. darlingi* thus being new records for the study area. Furthermore, *R. darlingi* was recorded in our study area but has previously not been recorded from the broader region, with the closest records being in northern KZN (Monadjem *et al.* 2010a). Despite the relatively short *cyt b* sequences of some samples and the absence of published reference sequences for other species, the combined phylogenetic tree produced is comparable to published phylogenies such as that of Eick *et al.* (2005). We were unable to confirm the presence of *M. fraterculus* in the field without molecular identification due to the overlapping morphometric measurements of our specimens with the respective sister species *M. natalensis*. As our field identification of *R. swinnyi* was ambiguous, molecular sequencing was required for species identification. Furthermore, an additional two species were detected by molecular analysis which belong to the *Rhinolophus* species complex (Stoffberg *et al.* 2010), and the largely cryptic family Vespertilionidae, as *R. darlingi* and *P. hesperidus*, respectively, which we had misidentified based on external morphology as *R. clivosus* and *L. capensis*. Exhibiting this *Rhinolophus* complex, Sample Bat 6 and Bat 34 are hypothesised to more likely be *R. darlingi* than *R. damarensis* due to the locality, climate and biome as outlined by Jacobs *et al.* (2013), despite being associated with the *R. damarensis* reference sequence with low bootstrap support (Appendix C). Although *cyt b* is relatively cheap, easy, and commonly used for molecular identification, it can be less informative than the mitochondrial D-loop and nuclear genes and is especially problematic where species complexes, such as the

genus *Rhinolophus*, are present (Dool *et al.* 2016; Demos *et al.* 2019). DNA barcoding may be considered for future studies to overcome the limitations of *cyt b* (Hajibabae *et al.* 2007; Mayer *et al.* 2007; Clare *et al.* 2011).

Twelve bat species were acoustically-detected during 69 sampling nights despite the high number of failed recording attempts due to microphone overload and battery failure under low temperatures. The limitations of acoustic bat detectors are well-documented due to the high variability, overlap and incorrect classification of species calls due to the complications of flight and harmonics during echolocation (Clement *et al.* 2014b; Russo and Voight 2016; Braun de Torrez *et al.* 2017). Although the bat detector was used at numerous locations for multiple survey nights as recommended by Skalak *et al.* (2012) and Moir *et al.* (2020), longer periods of sampling effort are needed with several detectors to make robust comparisons of community structure and habitat associations. One of the limitations of using one type of acoustic recorder is that echolocation calls cannot be compared to call libraries made with other types of bat detectors while an over-reliance on acoustic sampling may significantly underestimate true species richness (Monadjem *et al.* 2007, 2017; Taylor *et al.* 2013). It is well-known that mist netting is a selective, biased sampling method that is often out-performed by acoustic data and harp traps which unfortunately were not available for use in the present study (Galindo-Gonzalez and Sosa 2003; Flaquer *et al.* 2007; Pech-Canche *et al.* 2011; Tanshi and Kingston 2021).

Mist netting often underestimates bat species richness as high-flying canopy and open-air species are rarely sampled at ground sites that do not accommodate areas of high bat activity, due to access to the required sheltered 'flyways' such as roads and rivers (Cockle *et al.* 1998; Galindo-Gonzalez and Sosa 2003; Gehrt and Chelsvig 2003). This may explain the relative abundant sampling of clutter-edge foragers such as *L. capensis* and *R. clivosus* due to increased funnelling and coverage of mist nets, yet it does not explain the absence of *A. nana*, *M. natalensis* and *S. dinganii* which are all clutter-edge foragers that were only acoustically detected (Monadjem *et al.* 2010a). Some of the detected species however, namely *L. capensis*, *S. dinganii* and *T. aegyptiaca*, are known to be synanthropic and roost in human settlements which explains the sampling of *T. aegyptiaca* despite it being

categorised as an open-air forager (Monadjem *et al.* 2010a; Doty and Martin 2013).

## Conclusion

These results support the notion that neither capture-based nor acoustic surveying techniques alone provide a complete estimate of bat species richness of an area and both methods are required to ensure maximum sampling efficiency (Lintott *et al.* 2013; Taylor *et al.* 2013). It is suggested that historical museum records be collated to model potential species distributions before any active sampling to best adapt assessment and identification methods, selection of sampling sites and seasons to the potential species present (Monadjem *et al.* 2021a). This would enable targeted roost searches which should be included in future studies as they yielded the highest numbers of species detections compared to active and passive sampling by Flaquer *et al.* (2007). Bats are important bioindicators that require a multi-faceted approach for the assessment and identification of species to determine their assemblages within an area. SDM and acoustic surveys are well-established as complementary sampling techniques (Yates and Muzika 2006; Weller and Baldwin 2011; Clement *et al.* 2014b), yet this investigation represents the first study for the area combining a range of assessment and identification techniques in South Africa to accurately determine local bat species assemblages. As global threats to bats increase, further multi-faceted surveys are needed to gain better insights into bat biology that would assist management and conservation strategies.

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