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Barcoding and traditional health practitioner perspectives are informative to monitor and conserve frogs and reptiles traded for traditional medicine in urban South Africa

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Abstract

Published literature suggests that indigenous cultural practices, specifically traditional medicine, are commonplace among urban communities contrary to the general conception that such practices are associated to rural societies. We reviewed literature for records of herptiles sold by traditional health practitioners in urban South Africa, then used visual confirmation surveys, DNA barcoding, and folk taxonomy to identify the herptile species that were on sale. Additionally, interviews with 11 SePedi and IsiZulu speaking traditional health practitioners were used to document details of the collection and pricing of herptile specimens along with the practitioners' views of current conservation measures aimed at traditional medicine markets. The herptile specimens sold by traditional health practitioners included endangered and non-native species. The absorbance ratios of DNA extracted from the tissue of herptiles used in traditional medicine were found to be unreliable predictors of whether those extractions would be suitable for downstream applications. From an initial set of 111 tissue samples, 81 sequencing reactions were successful and 55 of the obtained sequences had species level matches to COI reference sequences on the NCBI GenBank and/or BOLD databases. Molecular identification revealed that traditional health practitioners sometimes mislabel the species they use. The mixed methodology employed here is useful for conservation planning as it updates knowledge of animal use in indigenous remedies and can accurately identify species of high conservation priority. Furthermore, the study highlights the possibility of collaborative conservation planning with traditional health practitioners.

Barcoding and traditional health practitioner perspectives are informative to monitor and conserve frogs and reptiles traded for traditional medicine in urban South Africa

Running title: Herptile traditional medicine in South Africa

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Abstract: Published literature suggests that Indigenous cultural practices, specifically traditional medicine, are commonplace among urban communities contrary to the general conception that such practices are associated to rural societies. We reviewed literature for records of herptiles sold by traditional health practitioners in urban South Africa, then used visual confirmation surveys, DNA barcoding, and folk taxonomy to identify the herptile species that were on sale. Additionally, interviews with 11 SePedi and IsiZulu speaking traditional health practitioners were used to document details of the collection and pricing of herptile specimens along with the practitioners' views of current conservation measures aimed at traditional medicine markets. The herptile specimens sold by traditional health practitioners included endangered and non-native species. The absorbance ratios of DNA extracted from the tissue of herptiles used in traditional medicine were found to be unreliable predictors of whether those extractions would be suitable for downstream applications. From an initial set of 111 tissue samples, 81 sequencing reactions were successful and 55 of the obtained sequences had species level matches to COI reference sequences on the NCBI GenBank and/or BOLD databases. Molecular identification revealed that traditional health practitioners sometimes mislabel the species they use. The mixed methodology employed here is useful for conservation planning as it updates knowledge of animal use in Indigenous remedies and can accurately identify species of high conservation priority. Furthermore, the study highlights the possibility of collaborative conservation planning with traditional health practitioners.

Keywords: Bio-cultural diversity, Ethno-herpetology, Indigenous knowledge systems, Mixed-method analyses, Zootherapy

Introduction

Traditional medicine, or the Indigenous knowledge and practices that people of different cultures use for maintenance of physical and mental health, is prevalent across the world (World Health Organization, 2000, 2019). A World Health Organization report stated that 88% of 179 member states that responded to a global

survey acknowledged use of traditional medicine by their citizens (World Health Organization, 2019). South Africa is among the developing countries where use of traditional medicine is common, and this practice also occurs in the country's most urbanised areas such as Johannesburg and Durban (Longmore, 1958; Du Toit, 1980; Ngwenya, 2001; Williams & Whiting, 2016). Traditional cultural practices have also been recorded in urban areas of other countries such as Bolivia (Macía et al., 2005), United Sates of America Balick et al., 2000), and Brazil (Alves & Rosa, 2007). South Africa's urban traditional medicine markets generally trade in illegally acquired wildlife a majority of which are plant species (Williams & Whiting, 2016). Traditional medicine practices involve both lethal and non-lethal use of plants and animals. Non-lethal traditional medicinal use would for example only involve using leaves of some plants. Conversely, lethal use involves killing some plants to get their roots, removing fungi (with their mycelium) from their substrate, or killing animals to use their tissue in Indigenous remedies.

Although traditional medicine mostly relies on plants (Solovan et al., 2004; Nascimento et al., 2016), animal use in Indigenous remedies nonetheless remains important to society as demand for animal-based remedies leads to overexploitation of animal species (Still, 2003; Alves et al., 2013). Investigating this use of animals in traditional medicine is important for updating knowledge of this Indigenous practice, informing collaborative conservation management of animals used in traditional medicine, and exploration of the economic value of animal trade for medicinal purposes (Alves et al., 2013). Research interest on the use of animals in traditional medicine has been low (Solovan et al., 2004). Among animals that are used in Indigenous remedies across the world, there are at least 331 herptile species (47 amphibians and 284 reptiles) and this number of herptile species known to be used for traditional medicine purposes could increase when comprehensive studies of traditional medicine use are conducted (Alves et al., 2013).

Research focused on traditional medicine markets generally has a problem with identification of specimens available at those markets (Veldman et al., 2020). Morphology-based identifications of animal specimens from traditional medicine markets in urban South Africa showed that some specimens could only be identified to genus or higher taxonomic ranks depending on how well the morphological traits are preserved (Simelane & Kerley, 1998; Ngwenya, 2001; Whiting et al., 2011). Species level identifications of traditional medicine market specimens with badly preserved diagnostic traits can be obtained with DNA barcoding (Whiting et al., 2011). Furthermore, the Indigenous names that traditional health practitioners use for species of interest can also be compared to molecular taxonomy with DNA barcoding (Veldman et al., 2020). The term traditional health practitioner refers to people that are deemed capable of incorporating plants, animals, or minerals in healing practices that are based on Indigenous cultural practices (World Health Organization, 1978). DNA barcoding is an effective tool for identifying both known and unknown species by comparing fragments of an individual's DNA with DNA sequences of individuals from several species (Hebert et al., 2003b). Using DNA barcoding to confirm the identity of species in traditional medicine markets helps increase our knowledge on the (number of) species that are being sold at those markets and the related conservation pressures (Veldman et al., 2020), and to also detect substitution of species in Indigenous remedies (Newmaster et al., 2013; Veldman et al., 2020). Substitution of plant species in Indigenous remedies poses a risk to human health if non-toxic plants are substituted with toxic species (Ouarghidi et al., 2012). DNA barcoding of traditional medicine specimens in this instance is vital to identifying human health risks in addition to confirming species' identification. Use of DNA barcoding to confirm the identity of Indigenous medicine specimens hence has promising prospects but its use remains low (Mishra et al., 2016).

Previous studies show that herptile diversity in traditional medicine practices is generally understudied and that South Africa's urban traditional medicine markets have some animal specimens with badly preserved morphological traits (Simelane & Kerley, 1998; Ngwenya, 2001; Whiting et al., 2011). It is thus worth introducing DNA barcoding to solve the identification problems that come with badly preserved morphology as highlighted by previous research on animals in traditional medicine markets. Research focusing on urban areas in developing countries provides opportunity for innovative scientific approaches that can benefit urban sustainability (Nagendra et al., 2018). As growth of cities on the African continent continues to place pressure on their surrounding environment to meet the needs of the urbanised human populations (Grant, 2015), studies that investigate urban utilisation of wildlife contribute to context-specific interventions to mitigate

conservation threats posed by city-dwellers' use of wildlife. Updated understanding of threats to South Africa's anuran amphibians and reptiles, this study's focal taxa, is important as 5% of reptile species and 12% of frog species described from the country are listed on the IUCN Red List of threatened species (IUCN, 2021).

This study aimed to increase understanding of the use of herptiles in traditional medicine and update records of herptile species targeted for South Africa's urban traditional medicine trade. Achieving these aims required tackling the following questions: 1) Can DNA barcoding pinpoint which herptile species are sold for traditional medicine purposes in South African cities? 2) How are the herptiles of Indigenous medicinal value collected and preserved? 3) What is the accuracy of the Indigenous names used for herptiles found in traditional medicine markets? 4) What are the perceptions of traditional health practitioners towards current conservation measures aimed at traditional medicine markets?

Materials and Methods

To achieve the aims of this study, existing literature on South Africa's urban traditional medicine markets was reviewed for records of trading in anuran amphibians and reptiles, traditional health practitioners were interviewed to increase understanding of their practices, and herptiles species were identified through visual confirmation during visits to the traditional medicine markets and with DNA barcoding targeting cytochrome*c* oxidase marker 1 (COI) of herptile specimens from those markets.

Literature review

A search of the keywords: animal + traditional medicine + "South Africa" on Google Scholar (https://scholar.google.com/) returned results of literature whose titles and abstracts were pre-screened for mentions of animal use in South African traditional medicine. Following this initial screening, the suitable articles were studied to find records of herptiles sold specifically in South Africa's urban traditional medicine markets or shops. Subsequently, availability of reference DNA sequences for the herptiles species matching the inclusion criteria of the literature review was verified with searches on the National Center for Biotechnology Information (NCBI) GenBank database (using the search query ((*Species name*) AND (CO1[Gene Name] OR COI[Gene Name])) and the Barcode of Life Data Systems (BOLD) database (using the search query "*Species name* ").

Fieldwork: interviews, tissue sampling and visual observation

Visual confirmation surveys of herptile specimens available for sale at traditional medicine markets/shops by the first author involved visiting a total of six markets/shops in Polokwane, Pretoria, Johannesburg, Pietermaritzburg and Durban from August to December 2020 (Figure 1). Morphology-based identification of species using visual confirmation was based on wildlife guides for reptiles (Alexander & Marais, 2007; Marais, 2008). In accordance with North-West University Health Research Ethics Committee's guidelines, the participation of traditional health practitioners at the markets/shops was sought after the first author explained the purpose of this study in SePedi (language spoken by people of Pedi culture) to practitioners from Limpopo and IsiZulu (language spoken by people of Zulu culture) for Gauteng and KwaZulu-Natal practitioners. SePedi was the preferred language for the Limpopo participants, IsiZulu was the most spoken language at the markets/shops in Gauteng and KwaZulu-Natal. Following explanation of the study, 11 traditional health practitioners consented to participation in this study (two in Limpopo, two in Gauteng and seven in KwaZulu-Natal). An informal conversational interview approach was used to collect data about herptiles of traditional medicine value; their Indigenous names, and the collection and preservation methods used for those herptiles. This interview approach relies on continuous participant observation without predetermined questions (Gall et al., 2003). The approach was chosen due to traditional health practitioners expressing apprehension towards researchers based on what they explained as past unpleasant experiences

with researchers and conservation practitioners. This interview was guided by the first author's conversation with participants and questions were introduced to the conversation when participants were forthcoming with details about their practices. Answers to these questions were written in a field book once the practitioners gave permission for their answers to be recorded in that manner. The reasons for the practitioner's apprehension towards researchers were also noted.

Tissue samples were collected from specimens sold by nine participants at four of the six localities visually surveyed in Gauteng and KwaZulu-Natal markets/shops (Figure 1) as they gave consent for this collection while the two participants in Limpopo said they could not give consent as they did not own the traditional medicine shops. A total of 111 samples were collectively obtained from Gauteng and KwaZulu-Natal (Dataset S1). Practitioners were asked the IsiZulu names for each sampled specimen and notes were made of any morphological features that were still visible on those specimens. Notes about morphology were written as the traditional health practitioners did not allow use of cameras at the markets/shops.

Distinctive morphological traits were not visible on all sampled specimens as sometimes all that remained were ventral scutum, or bones with flesh but no skin. Opting for collection of tissue samples instead of taking entire specimens minimises this study's environmental impact as removal of entire specimens may prompt traditional health practitioners to acquire replacement specimens to satisfy demand from customers or patients.

DNA extraction and absorbance measurements

From the acquired samples, outside layers of tissue that were most likely exposed to contamination were shaven/scraped off and discarded before taking ˜25mg of tissue for DNA extraction. This tissue's genomic DNA was extracted using the standard extraction protocols for animal tissue provided by the manufacturer in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Duren, Germany).

To assess the suitability of the extracted DNA samples for downstream applications, (amplification and sequencing) their purity was determined through measures of absorbance using ultraviolet-visible spectroscopy (UV–visible spectrophotometry) where the peak absorbance of pure nucleic acid is 260 nm (Desjardins & Conklin, 2010; Koetsier & Cantor, 2019). These absorbance measurements were carried out on the NanoDrop One Spectrophotometer (Thermo Scientific) according to manufacturer's instructions. Blank measurements were first performed with 2 µl of the reference solution (elution buffer used during DNA extractions) to minimise this solution's contribution to the absorbance of the extracted DNA. To be able to make inferences about purity of the extracted DNA, spectrophotometry results from this study's sample are compared to typical absorbance of pure nucleic acid for DNA; a 260/280 nm (A260/280) absorbance ratio of ˜1.8 (1.85 – 1.88) and a 260/230 nm (A260/230) absorbance ratio in the range of 1.8 – 2.3 (Desjardins & Conklin, 2010; Koetsier & Cantor, 2019). Samples with 260/280 nm and 260/230 nm absorbance ratios greater or equal to 1.8 are generally considered suitable for downstream applications (Koetsier & Cantor, 2019), but there are likely to be exceptions to these general guidelines for interpreting absorbance ratios. Negative absorbance ratios could be an indication of contamination that is emitting light instead of absorbing it, absorbance ratios that are minor outliers generally give an indication that DNA extraction procedures need to be improved, while major outlier absorbance ratios suggest presence of impurities in the sample (Desjardins and Conklin 2010). Outliers were determined using the interquartile rule where the minor outliers in the absorbance ratios are lower than the first quartile value minus 1.5 times the interquartile range (i.e., Q1 – 1.5(IQR)), the major outliers are higher than the third quartile plus 1.5 times the interquartile range (i.e., Q3 + 1.5(IQR)), and interquartile range is calculated by subtracting the first quartile value from the third quartile value (i.e., IQR = Q3 – Q1).

Following the absorbance measurements, all extracted DNA samples were used to amplify DNA barcode fragments with a polymerase chain reaction (PCR). In addition to PCR being a step towards obtaining DNA barcodes, it also provides an indication of whether the success of this barcoding conforms to absorbance ratio guidelines. This amplification targeted a region of a length of maximum 664 bp of the COI gene with

a primer set from a previous study by Nagy et al., (2012); RepCOI-F (5'-TNT TMT CAA CNA ACC ACA AAG A-3') and RepCOI-R (5'-ACT TCT GGR TGK CCA AAR AAT CA-3'). For DNA barcoding animals, the mitochondrial 5' end of the cytochrome *c* oxidase subunit 1 marker (COI) is proposed as a universal barcode marker (Hebert et al., 2003b). The PCR reactions were performed in total volumes of 25 µl: 12.5 µl Thermo Scientific DreamTaq Green PCR Master Mix (X2) (with DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, at 0.4 mM each and 4 mM $MgCl_2$), 1.25 µl (10 µM) of each of the two RepCOI primers mentioned above, 3 µl of the template DNA elution and 7 µl Thermo Scientific Nuclease-free water (PCR-grade). The reactions were carried out in the Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc) using the following PCR protocol: initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 30s, annealing at 48.5°C for 30s, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes, and subsequent storage of PCR products at 4°C. The PCR products were visualised on a 1% agarose gel under ultraviolet light on the E-BOX CX5 stand-alone gel imaging system (Vilber Lourmat Deutschland GmbH).

Sequencing protocol

Purification and sequencing of PCR products was outsourced to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa). They cleaned PCR products using the ExoSap Protocol: 10 µl amplified PCR product and 2.5 µl ExoSAP master mix (Exonuclease I 20 U/ul and Shrimp Alkaline Phosphatase 1 U/ul) mixed well and incubated at 37°C for 15 minutes then held at 80°C for 15 minutes. The Nimagen, BrilliantDye Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 was used to sequence fragments according to manufacturer's instructions. The cycle sequencing protocol provided by the sequencing company was as follows: 10 µl NEB OneTaq 2X MasterMix with standard buffer, 1 µl genomic DNA (10-30ng/µl), 1 µl of forward and reverse primer each (10µM) (using the same primers, RepCOI-F and –R, as in initial amplification), and 7 µl Nuclease free water. The sequencing PCR profile was 94°C for 5 min, 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 60 seconds, followed by 10 minutes at 68°C and subsequently held at 4°C. Subsequently, products were cleaned with the ZR-96 DNA Sequencing Clean-up Kit and the cleaned products were injected on an Applied Biosystems ABI with a 50cm array (using POP7). Sequence chromatograms were analysed using the FinchTV analysis software.

Sequences obtained from the commercial sequencing company were trimmed with the Decontamination Using Kmers (BBDuk) trimmer, paired, then assembled using De Novo assembly on the Geneious Prime® 2022.0.2 (https://www.geneious.com/prime/) sequence analysis software (Biomatters New Zealand Ltd). The BOLD Identification System (IDS) was used to compare this study's sequences to reference samples on the BOLD database (https://v3.boldsystems.org/index.php/IDS_IdentificationRequest) for verification of the sequence and species identity using neighbour-joining placement (Ratnasingham & Hebert, 2007). Subsequently, The Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) was also used for a second comparison of all of this study's sequences with published sequences on the NCBI Nucleotide collection (nr/nt) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine sequence and species identity using the MegaBlast (Zhang et al., 2000) algorithm for identifying highly similar sequences. A difference of 2% or less between DNA sequences was used as a limit for discriminating between species (Hebert et al., 2003a; Pereira et al., 2013). Morphology of specimens was used to supplement molecular identification. The sequences obtained from study were deposited in the NCBI GenBank database under the accession numbers [GenBank: XXX-XXX] (accession numbers to be added later).

Results

Literature review

The published sources reviewed here recorded a total 34 herptile species (one anuran and 33 reptile species) with other herptiles only being identified to genus or higher taxonomic ranks from the South African urban

traditional medicine markets/shops of Eastern Cape (Simelane & Kerley, 1998), Gauteng (Whiting et al., 2011) and KwaZulu-Natal (Ngwenya, 2001). Eight of those 34 herptile species reported in previous literature did not have COI reference samples available on either BOLD or NCBI GenBank databases at the drafting of this article in June 2022 (Table 1).

From the species recorded in previous literature (Table 1),*Kinixys natalensis* Hewitt, 1935 and *Smaug giganteus*(Smith, 1844) have their conservation status as vulnerable, while*Eretmochelys imbricata* (Linnaeus, 1766) is critically endangered (IUCN, 2022). Furthermore, published literature has records of four species from South Africa's urban traditional markets that are not native to the country: *Cordylus tropidosternum* (Cope, 1869) is endemic to Eastern African countries (Broadley & Branch, 2002),*Kinixys belliana* Gray, 1831 occurs in the north of the Southern African region and beyond (Turtle Taxonomy Working Group, 2021),*Naja melanoleuca* Hallowell, 1857 is from Central and West African countries (Wüster et al., 2018) and *Psammophis phillipsii*(Hallowell, 1844) occurs in West African countries (Leaché et al., 2006).

Fieldwork: interviews, tissue sampling and visual observation

Through visual surveys, 9 of the 34 species identified in published literature were confirmed to be on sale among plants and other animal specimens at traditional medicine shops and open markets (Table 2) in the urban areas of three South African provinces (Gauteng, KwaZulu-Natal, and Limpopo). The traditional health practitioners who provided access to these tissue samples explained their apprehension towards conservation practitioners because conservation law enforcement previously confiscated specimens in their possession instead of seeking to collaborate with them to introduce measures that both adhered to environmental laws and respected Indigenous cultural practices. This collaboration is something they were willing to consider.

Traditional health practitioners from the surveyed traditional medicine markets reported obtaining the herptile specimens they use or sell by either hunting the animals themselves, buying from hunters that regularly go on hunts to supply multiple traditional health practitioners, or taking roadkill and animals that died of natural causes. Practitioners specifically target species that they require at the time of their hunts, while the hunters take orders for specific animals from traditional medicine practitioners and will also opportunistically hunt other species they encounter when hunting to fulfil their list of orders. Traditional health practitioners and the hunters that supply them with herptile species employ the same tissue preservation methods and the specimens are either preserved at home or at the open market. They remove visible body fat and internal organs. The body fat has traditional medicine value and is stored in bottles, while the internal organs are usually discarded. Following removal of fat and internal organs, the carcasses are smothered with ash and/or salt and placed in the sun to dehydrate them.

Dried specimens of herptiles and other animals are placed together on display for customers. According to the traditional health practitioners, people usually buy body parts or small pieces (relative to an animal's size), and it is uncommon for someone to buy an entire carcass. The pricing for each piece of animal that a person wants to buy was noted to be uniform among this study's participants with the exception being *Pseudaspis cana* (Linnaeus, 1758) which was priced at 40% higher than the rest of the herptiles being sold by those participants. The reason provided for this pricing difference was because the participants believed that *P. cana* preyed on other snake species. Due to how they are sold, herptile specimens were sometimes found missing parts of the body or only pieces of bones with muscles and skin remained. Specimens at open traditional medicine markets (in Durban and Johannesburg) were either removed at the end of each business day to be stored together overnight in plastic containers or they were left on the stalls and covered with plastic sheets. All storage of specimens is at ambient temperature; there is no refrigeration.

DNA barcoding of traditional medicine market samples

The absorbance measurements of the extracted DNA suggest that the traditional health practitioners' preservation of herptiles using salt and/or ash can preserve DNA for molecular identification. Based on the

A260/280 absorbance ratio, 20.7% of the 111 extracted DNA samples were not suitable for downstream analysis, while the A260/230 ratio suggested that 29.7% of the samples would be unsuitable due to their absorbance ratios being either negative or outlier values (Figure 2).

The subsequent amplification and sequencing outcomes were partially in line with the interpretation of absorbance ratios as 25 of the extracted DNA samples with negative or outlier absorbance ratios were successfully amplified and sequenced when interpretations of their absorbance suggested they would be unsuitable for downstream applications (Table 3). Conversely, 16 extractions with absorbance ratios that were interpreted as being suitable for downstream applications could not be amplified or sequenced. A total 81.1% (90 of 111) of the extractions were successfully amplified (based on the PCR products visualised using gel electrophoresis) and subsequently sent for sequencing. DNA sequences were successfully obtained from 81 samples (72.9% of original sample) and sequencing reactions failed for the remaining 9 of 90 amplicons. From the 81 DNA sequences, 38 had exact species matches with on the BOLD database with 99.13 - 100% similarity (Table 3). An additional three sequences had species level matches with 99.0% pairwise similarity on the NCBI Genbank database (Ng & Tay, 2004), with e-value of zero suggesting that there is no better match besides that current result (Metzler, 2006). Lists of nearest matches rather than exact species matches were returned for 12 sequences on the BOLD database with 98.12% - 99.05% similarity and one sequence with 98.9% similarity on the NCBI Genbank database (Table 3). The identity of these 13 sequences with lists of nearest species matches were confirmed using morphology as the specimens from which the tissue was obtained had not yet been cut to a point of being difficult to recognise. A 93.8% similarity to *P. phillipsii* which is not native to South Africa was returned as the highest match for one of this study's DNA sequences on the NCBI GenBank database. This match to a non-native species was likely due to the sample being obtained from a native species of the same taxonomic group and the morphological traits observed during visual surveys provide confirmation of a genus (*Psammophis* sp.) level identification (Table 2).

Using molecular identification and observed morphological traits, 55 tissue samples collected during this study were matched to one genus and 12 species of reptiles that had already been recorded in published literature and one additional reptile species that was not recorded in previous literature (Table 2). Twenty-four of the 26 remaining sequences had no matches on the BOLD database, but it could be ascertained that they were DNA fragments of reptiles by comparing them to their NCBI GenBank reference sequence matches of reptiles with a similarity of 81.2 – 86.9%, using 70% similarity to reference sequences as a threshold below which the results would not be meaningful (Baxevanis et al., 2020). Two of the 26 remaining sequences matched with reference sequences from mammal species: *Ictonyx striatus* (Perry, 1810) with 99.2% similarity on the NCBI GenBank database and *Procavia capensis* (Pallas, 1766) with 98.14% similarity on the BOLD database. These mammalian tissues were obtained from pieces of bone and muscle that a traditional health practitioner mislabelled as either uxam or imbulu, IsiZulu names for *Varanus* spp. (Table 3).

Molecular identification of species verified some of the IsiZulu names used by traditional health practitioners to identify reptiles used in Indigenous remedies and also revealed mislabelling of animal tissue with their distinguishing features removed during sale (Table 3). Some IsiZulu names for the specimens were accurate up to species level (e.g.,*Dendroaspis angusticeps* (Smith, 1849), imamba eluhlaza in IsiZulu) while other Indigenous names were only accurate to higher taxonomic ranks, for example specimens named as unwabu (IsiZulu word for members of Chamaeleonidae) were later confirmed to be *Chamaeleo dilepis* with molecular identification. Further examples of DNA barcoding as a tool for verification of folk taxonomy include specimens broadly labelled as snakes and monitor lizards in IsiZulu (inyoka and uxam/imbulu respectively) being confirmed up to species level by DNA barcoding as *P. cana* and *Varanus niloticus* (Linnaeus, 1766) respectively (Table 3). Mislabelling of tissue meant for use in Indigenous remedies involved herptile tissue (e.g., *N. melanoleuca* mislabelled as *Naja mossambica* Peters, 1854, imfezi in IsiZulu), and mammalian bone and muscle which was mislabelled as a *Varanus* sp. (uxam/imbulu in IsiZulu) (Table 3).

Discussion

The current study aimed to combine visual surveys, literature reviews, DNA barcoding and interviews of traditional health practitioners in documenting and updating the knowledge of herptile use in South Africa's urban traditional medicine markets. This study further provided insights into Indigenous tissue preservation methods and the willingness of some traditional health practitioners to collaborate in conservation initiatives aimed at traditional medicine markets.

DNA barcoding Indigenous medicine specimens

Although Indigenous tissue preservation methods can preserve DNA for molecular identification, storage of those specimens increases risk of DNA contamination as they are openly displayed, and multiple species are stored together in one container at ambient temperatures. Furthermore, there is a risk of DNA degradation from daily temperature and humidity fluctuations (Asari et al., 2018), as a result of those storage methods. Spectrophotometry used to measure the absorbance of DNA samples extracted from herptile specimens gave indications of the purity of the extracted DNA in comparison with other coextracted products (DNA versus other molecules), but not in terms of exogenous DNA contamination (endogenous DNA versus DNA contamination). These absorbance measurements were sometimes inconclusive about suitability of extracted DNA samples for downstream applications. Some extractions that were expected to be unsuitable for downstream applications due to their negative or outlier absorbance ratios were amplified, and DNA sequences were obtained from them. Additionally, some extractions with absorbance ratios close to that of pure nucleic acid (1.8) were expected to be suitable for downstream applications but they failed in subsequent reactions.

Since the DNA sequences from 26 of 81 specimens could not be identified to species level, a continuation of this research could be to start with obtaining species level identifications by barcoding the 12S, 16S, ND1, ND2, ND4 or cytochrome *b* genes which have previously been used in molecular identification of reptiles (Vences et al. 2012). It is also possible that some of this study's COI sequences could not be identified due to absence of reference sequences on the BOLD and NCBI GenBank databases. Of the 418 reptile species known to be distributed in South Africa (Uetz et al., 2022) only 86 had COI reference sequences on the NCBI GenBank and/or BOLD databases at the time of drafting this text (in June 2022). The results further showed that from the 34 herptile species recorded in published literature as being offered in the South African urban traditional medicine circuit, eight did not have COI reference sequences on either NCBI GenBank or BOLD databases.

DNA barcoding compared to morphology-based identification

This study's DNA barcoding confirmed 12 species and one genus of reptiles from the 33 reptile species reported in published literature which relied on morphology-based identification. The published literature also reported one anuran species from traditional medicine markets whereas no anurans were found during the survey and sampling phase of this study. *Philothamnus semivariegatus* (Smith, 1840) was identified in this study using DNA barcoding (Table 2), and Whiting et al., (2011) previously recorded a genus level identification (*Philothamnus* sp.) of a morphologically similar species. Inconsistency between molecular identifications and those based on morphology shows a need for additional barcoding studies of Indigenous medicine specimens (Veldman et al., 2020). The hitherto estimated species richness of traditional medicine markets is likely underestimated due to the state of preservation of some specimens making it difficult to obtain morphology-based identification up to species rank (Whiting et al., 2011).

The mislabelling of animal tissue used for traditional cultural purposes shown in this study (Table 3), has also been recorded in other studies (Gombeer et al., 2021). Mislabelling could be intentional when practitioners lie to meet customer expectations (Bitanyi et al., 2012). It is also possible that tissue is deliberately mislabelled so the practitioners can charge higher prices for them, but this was not the case in this study as the mislabelled species were sold at the same price as the correctly labelled species. Substitution of tissue in

Indigenous remedies is said to pose human health risks when toxic plants are the substitute (Ouarghidi et al., 2012). No acute health issues have been associated with ingestion of herptile tissue (Ngwenya, 2001; Du Preez & Cook, 2004; Anthony & Bellinger, 2007). There is however zoonoses risk associated with ingestion of herptile tissue and it is worth investigating it for increased understanding of human health effects of using animals in traditional medicine. Some of the possible zoonotic infections include *Salmonella* spp. associated with eating *Crocodylus* spp. meat and a chance of zoonotic parasitic disease caused by *Gnathostoma* spp. nematodes in the undercooked flesh of frogs and reptiles (Magnino et al., 2009). Furthermore, people can be accidental hosts of some herptile parasites (Pantchev & Tappe, 2011), and herptiles are reservoirs of zoonotic parasites which may be considered a public health concern (Mendoza-Roldan et al., 2020)

Conservation issues

South Africa's urban traditional medicine markets rely more on reptile species than anuran amphibian species (34 reptile species vs 1 anuran species were jointly recorded by this and other studies). This trend of greater dependence on reptiles than amphibians for Indigenous remedies is global (Alves et al., 2013). The underestimation of species richness at some of South Africa's urban traditional medicine markets highlighted by Whiting et al. (2011) makes it difficult to estimate the proportion of herptile species that are considered to have traditional medicine value and the underestimation of endangered species in particular could lead to the traditional medicine markets' conservation impacts being misevaluated. It has previously been difficult to estimate the number of individuals per species harvested for traditional medicine markets and their impact on wildlife populations as traditional health practitioners were reluctant to talk about their practices (Whiting et al., 2011). There is hope for lessening this reluctance as practitioners that participated in this study expressed willingness to collaborate with researchers or conservation practitioners. With such collaboration the species at traditional medicine markets can be comprehensively documented and identified using molecular and morphology-based identifications.

A collaborative approach to managing conservation issues arising from traditional medicine markets would not only be just, but it is also legally required. South Africa's overarching environmental management legislation is supportive of collaborative conservation planning as it states that decisions relating to the natural environment must account for the interest, needs, and values of interested parties and recognise all forms of knowledge including Indigenous knowledge (Republic of South Africa, 1998). Collaboration will of course require additional research resources as efforts to find synergies between Indigenous cultures and modern practices will require extra field days and additional ethics approvals to protect Indigenous knowledge and its custodians from exploitation. Within the context of South African environmental legislation, Indigenous medicinal uses of wildlife (as a form of Indigenous knowledge) should not be dismissed by conservation practitioners. This environmental legislation further states that management of the environment should equitably provide for people's needs and their cultural interests (Republic of South Africa, 1998). Functional collaborations with traditional health practitioners have in the past been demonstrated by modern health professionals both in South Africa (Nkhwashu et al., 2021) and other parts of the African continent (Kayombo et al., 2007) despite disagreement between the two parties, thus providing hope that collaborations with conservation practitioners are achievable. With collaboration the traditional health practitioners can be encouraged to openly substitute endangered species with abundant species of lower conservation priority thus limiting negative impacts of Indigenous remedies. The manner in which practitioners sell animals for traditional remedies can be considered to limit negative conservation impacts (albeit unwittingly); pieces of animal tissue are sold, rather than the entire carcass, thus allowing an individual animal to be used by multiple people.

Traditional health practitioners would make suitable conservation ambassadors due to the respect they have from people that follow Indigenous cultural practices (Simelane & Kerley, 1998), and these practitioners' choices influence which species are collected for traditional medicine markets through their hunts or outsourcing to dedicated hunters. Another prospect for lessening conservation pressure of traditional medicine markets, but not necessarily their accumulation of endangered species, is that traditional health practitioners

are willing to take animals that died due to accidental or natural causes (Whiting et al., 2011). There is perhaps opportunity for collaboration between traditional health practitioners with initiatives that monitor and collect roadkill on busy roads to lessen the hunting pressure on species used in Indigenous remedies.

Studies of this nature that focus on the urban areas of developing countries can contribute to increased understanding of urban sustainability in such countries and can also contribute to policymaking when such research is published in high visibility journals (Nagendra et al., 2018). The study represents the first attempt in South Africa to comprehensively document herptiles in urban traditional medicine markets and combine DNA barcoding with morphology-based identifications and folk taxonomy to identify herptile species at those markets. This study transcends disciplines by combining Indigenous knowledge with DNA barcoding and social science methodology for outcomes that can be used for socially inclusive conservation planning. The wide applicability of the mixed-methods approach employed here is demonstrated by Gombeer et al. (2021) using site visits, DNA-based identification, and focus group discussions to identify bushmeat that was smuggled to Belgium from West African countries and highlight prevalence of Indigenous cultural practices in the urban centre of a developed country. Incorporating molecular identification in the introduction of collaborative monitoring of traditional medicine markets is likely to improve understanding of their species richness and prevent over-exploitation of herptile species while being considerate of Indigenous practices that make use of animals. A collaborative and mixed methods approach is also necessary because in its absence the use of herptiles (and other animals) has continued unmonitored while Indigenous practices and their custodians have continually been excluded from conservation planning.

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Data Accessibility and Benefit-Sharing

Data Accessibility

Sequence data have been deposited on the NCBI GenBank database under the accession numbers (to be added later). Data collected during tissue sampling are available as Supplementary Material S1.

Benefit-Sharing

Benefits Generated: The research conducted provides a first step to collaborative conservation planning for use of herptiles in traditional medicine contrary to current approaches that scarcely consider that South African legislation allows the country's citizens to utilise its natural resources within the confines of laws that provide for Indigenous knowledge systems to be considered in conservation planning.

Benefits Generated: This research provides details about the practices of traditional health practitioners and also their willingness for collaboration is managing the use of herptiles in traditional medicine. Conservation practitioners can utilise these details to make their planning more integrative.

Ethics statement

The Indigenous Healers Organisation in South Africa agreed to its members being approached for participation in a research project. Informed consent was obtained from the participants of this study after the first author explained the purpose of this research and that they could revoke their consent to participate at any point. This explanation was in either SePedi or IsiZulu which were languages preferred by the participants. Ethics approval for this study was obtained from the North-West University Animal Care, Health and Safety Research Ethics Committee (Ethics number: NWU-00185-18-S5) and Hasselt University Social-Societal Ethics Committee (Reference: REC/SMEC/VRAI/189/127). The research conducted complies with the Nagoya Protocol on Access and Benefit-sharing (UID: ABSCH-IRCC-ZA-257320-1)

Author Contributions

Fortunate M. Phaka carried out fieldwork, conducted laboratory analysis and wrote the initial draft of the manuscript; Fortunate M. Phaka, Louis H. du Preez, Maarten P. M. Vanhove, Jean Hugé: designed the project, acquired funding, and revised the initial manuscript; Edward Netherlands, Maarten Van Steenberge provided training and supervision for laboratory analysis, revised the initial manuscript; Erik Verheyen , Gontran Sonet contributed to the project design, and revised the initial manuscript.

Tables and Figures

Table 1: Published literature records of herptile species from South Africa's urban traditional medicine.

Classification
Frogs
Schismaderma carens (Bufonidae) [1]
Reptiles
Acanthocercus atricollis (Agamidae) [1, 2]
Acontias plumbeus (Scincidae) [1]
Bitis arietans (Viperidae) [1, 2, 3]
Chamaeleo dilepis (Chamaeleonidae) [1]
Chersina angulata (Testudinidae) [1]
*Cordylus tropidosternum** (Cordylidae) [1]
Cordylus vittifer (Cordylidae) [1]
Crocodylus niloticus (Cordylidae) [1, 2, 3]
Dendroaspis angusticeps (Elapidae) [1]
Dendroaspis polylepis (Elapidae) [1]
Dispholidus typus (Colubridae) [1]

Eretmochelys imbricata (Cheloniidae) – CR [1]
Gerrhosaurus flavigularis (Gerrhosauridae) [1]
Gerrhosaurus major (Gerrhosauridae) [1]
Hemachatus haemachatus (Elapidae) [1, 2]
*Kinixys belliana** (Testudinidae) [1]
Kinixys natalensis (Testudinidae) – VU [2]
Kinixys speckii (Testudinidae) [1]
Lamprophis aurora (Lamprophiidae) [1]
*Naja melanoleuca** (Elapidae) [2]
Naja annulifera (Elapidae) [2]
Naja mossambica (Elapidae) [1, 2]
*Psammophis phillipsii** (Psammophiidae) [1]
Psammophylax rhombeatus (Psammophiidae) [1]
Psammophylax tritaeniatus (Psammophiidae) [1]
Pseudaspis cana (Pseudaspididae) [1]
Python natalensis (Pythonidae) [2, 3]
Smaug giganteus (Cordylidae) – VU [1]
Smaug warreni (Cordylidae) [1]
Stigmochelys pardalis (Testudinidae) [1, 2]
Thelotornis capensis (Colubridae) [2]
Varanus albigularis (Varanidae) [1, 2, 3]
Varanus niloticus (Varanidae) [1, 2, 3]
CR – Assessed to be Critically Endangered (IUCN, 2022). VU – Assessed to be Vulnerable (IUCN, 2022). *Species not nat

Table 2: Herptiles identified from South Africa's urban traditional medicine markets by visual confirmation and DNA barcoding.

Classification
Reptiles
Acanthocercus atricollis (Agamidae) [1]
Bitis arietans (Viperidae) [1]
Chamaeleo dilepis (Chamaeleonidae) [1]
Crocodylus niloticus (Cordylidae) [1]
Dendroaspis angusticeps (Elapidae) [1]
Hemachatus haemachatus (Elapidae) [1]
Naja melanoleuca (Elapidae) [1]
Naja annulifera (Elapidae) [1]
Naja mossambica (Elapidae) [1]
Philothamnus semivariegatus
Psammophis sp. (Psammophiidae)
Pseudaspis cana (Pseudaspididae) [1]
Python natalensis (Pythonidae) [1]
Stigmochelys pardalis (Testudinidae) [1]
Varanus albigularis (Varanidae) [1]
Varanus niloticus (Varanidae) [1]
Visual = Identified using visual confirmation during surveys of traditional medicine markets /shops (identification based on

Table 3: Identification of herptile species from traditional medicine markets using folk taxonomy and DNA barcoding.

#	**IsiZulu names provided by participants**	**Molecular identification (% match)**	**Absorbance 260/280 nm**	**Absorbance A260/230 nm**
D20	Ibululu (*Bitis arietans*)	*Bitis arietans* (100%)	1.89	17.33
D33	Ibululu (*Bitis arietans*)	*Bitis arietans* (99.82%)	1.53	1.77
J23	Ibululu (*Bitis arietans*)	*Bitis arietans* (99.83%)	1.81	3.89
J03	Imamba (*Dendroaspis* sp.)	*Hemachatus haemachatus* (98.24%)	1.90	3.56
D01	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.92	0.41
D02	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.59	0.9
D03	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.80	-2.21
D04	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	0.38	-0.14
D24	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	0.26	-0.08
D25	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.23	1.67
D44	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	2.94	0.27
D55	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.93	-5.86
D56	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.9	5.62
D57	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.72	1.98
D58	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.79	3.40
J07	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Philothamnus semivariegatus* (99.48%)	1.89	3.8

J15*	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Psammophis* sp. (93.8%)	1.14	-0.23
J20	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	2.72	0.44
J29	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	2.05	0.36
J30	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	4.07	0.27
P06	Imamba eluhlaza (*Dendroaspis angusticeps*)	*Dendroaspis angusticeps* (100%)	1.29	334.9
D13	Imfezi (*Naja mossambica*)	*Naja melanoleuca* (100%)	-7.16	0.16
D26	Imfezi (*Naja mossambica*)	*Naja melanoleuca* (100%)	1.84	2.72
D42	Imfezi (*Naja mossambica*)	*Naja mossambica* (99.79%)	1.26	-11.25
J01	Imfezi (*Naja mossambica*)	*Naja mossambica* (100%)	1.76	6.58
J21	Imfezi (*Naja mossambica*)	*Naja annulifera* (99.8%)	2.03	0.33
J31	Imfezi (*Naja mossambica*)	*Naja annulifera* (99.79%)	1.79	3.02
J32	Imfezi (*Naja mossambica*)	*Naja annulifera* (99.79%)	1.73	-9.71
J04	Inyoka (Serpentes sp.)	*Pseudaspis cana* (100%)	0.38	-0.08
J05*	Inyoka (Serpentes sp.)	*Python natalensis* (100%)	1.77	-2.31
J24*	Inyoka (Serpentes sp.)	*Python natalensis* (100%)	2.48	0.4
J18	Isibankwa (Scincidae sp.)	*Varanus albigularis* (99.09%)	1	-0.48
D22	Unwabu (Chamaeleonidae sp.)	*Chamaeleo dilepis* (99.65%)	1.78	13.58
D23	Unwabu (Chamaeleonidae sp.)	*Chamaeleo dilepis* (99.65%)	2.01	2.98
D39	Unwabu (Chamaeleonidae sp.)	*Chamaeleo dilepis* (99.65%)	-4.29	0.28
D16	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.9%)	1.73	4.85
D41	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.94%)	1.58	-11.29

D46	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.76%)	1.39	0.68
D47	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.93%)	1.70	-4.89
D48	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (99%)	1.73	-1.91
D49	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (99%)	-0.79	0.04
D50	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.94%)	7.51	0.33
D51	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.85%)	9.78	0.27
D52	Uxam/Imbulu (*Varanus* sp.)	*Varanus albigularis* (99.28%)	1.34	-0.17
D53	Uxam/Imbulu (*Varanus* sp.)	*Varanus albigularis* (99.28%)	2.13	0.28
D54	Uxam/Imbulu (*Varanus* sp.)	*Varanus albigularis* (99.28%)	1.82	8.94
J06*	Uxam/Imbulu (*Varanus* sp.)	*Procavia capensis*[a] (98.12%)	1.30	-0.6
J09	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (99.04%)	1.83	3.96
J10	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (99.13%)	0.55	-0.07
J17*	Uxam/Imbulu (*Varanus* sp.)	*Ictonyx striatus* [a] (99.2%)	2.61	0.28
J25	Uxam/Imbulu (*Varanus* sp.)	*Varanus albigularis* (98.38%)	1.87	-1.06
J26	Uxam/Imbulu (*Varanus* sp.)	*Varanus albigularis* (99.03%)	1.75	-2.07
P01*	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (98.7%)	1.80	3.93
P02*	Uxam/Imbulu (*Varanus* sp.)	*Varanus niloticus* (99%)	1.87	13.25
T01*	Uxam/Imbulu (*Varanus* sp.)	*Crocodylus niloticus* (100%)	1.64	1.56
T02*	Uxam/Imbulu (*Varanus* sp.)	*Crocodylus niloticus* (100%)	1.65	1.48

Note: Species arranged alphabetically according to IsiZulu names used by traditional health practitioners. Mislabelled species are highlighted in grey. [a] Not a herptile species. *Bone and muscle tissue that was difficult to identify based on morphology.	Note: Species arranged alphabetically according to IsiZulu names used by traditional health practitioners. Mislabelled species are highlighted in grey. [a] Not a herptile species. *Bone and muscle tissue that was difficult to identify based on morphology.	Note: Species arranged alphabetically according to IsiZulu names used by traditional health practitioners. Mislabelled species are highlighted in grey. [a] Not a herptile species. *Bone and muscle tissue that was difficult to identify based on morphology.	Note: Species arranged alphabetically according to IsiZulu names used by traditional health practitioners. Mislabelled species are highlighted in grey. [a] Not a herptile species. *Bone and muscle tissue that was difficult to identify based on morphology.	Note: Species arranged alphabetically according to IsiZulu names used by traditional health practitioners. Mislabelled species are highlighted in grey. [a] Not a herptile species. *Bone and muscle tissue that was difficult to identify based on morphology.

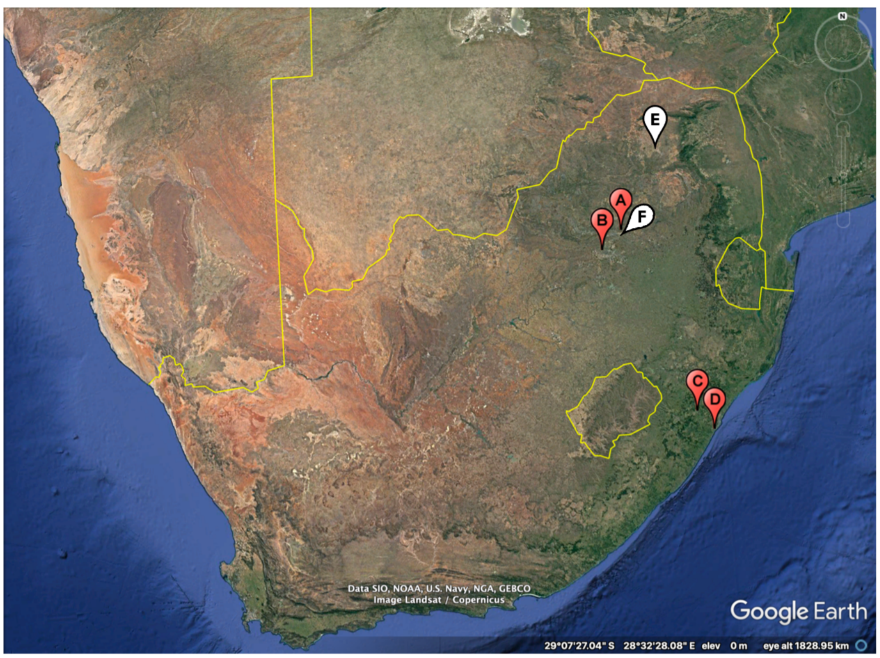

Figure 1: Locations of six traditional medicine markets/shops across five provinces visited during this study (visualised using Google Earth https://earth.google.com). Visuals surveys and tissue sampling carried out at locations marked red: A = Pretoria Muthi Shop (-25.73872°, 28.17838°), B = Faraday Muthi Market (-26.21167°, 28.04538°), C = Pietermaritzburg Muthi Shop (-29.58974°, 30.39069°), D = Warwick Muthi Market (-29.85483°, 31.01055°). Visual surveys only carried out locations marked white: E = Ga-Mokekolwana (-23.89158°, 29.44961°), F = Kwa Mai Mai Traditional Market (-26.20710°, 28.05894°).

Note: Muthi is the IsiZulu word for both modern and traditional medicine. 'Muthi market' is a term generally used by South Africans (regardless of culture) in reference to traditional medicine shops.

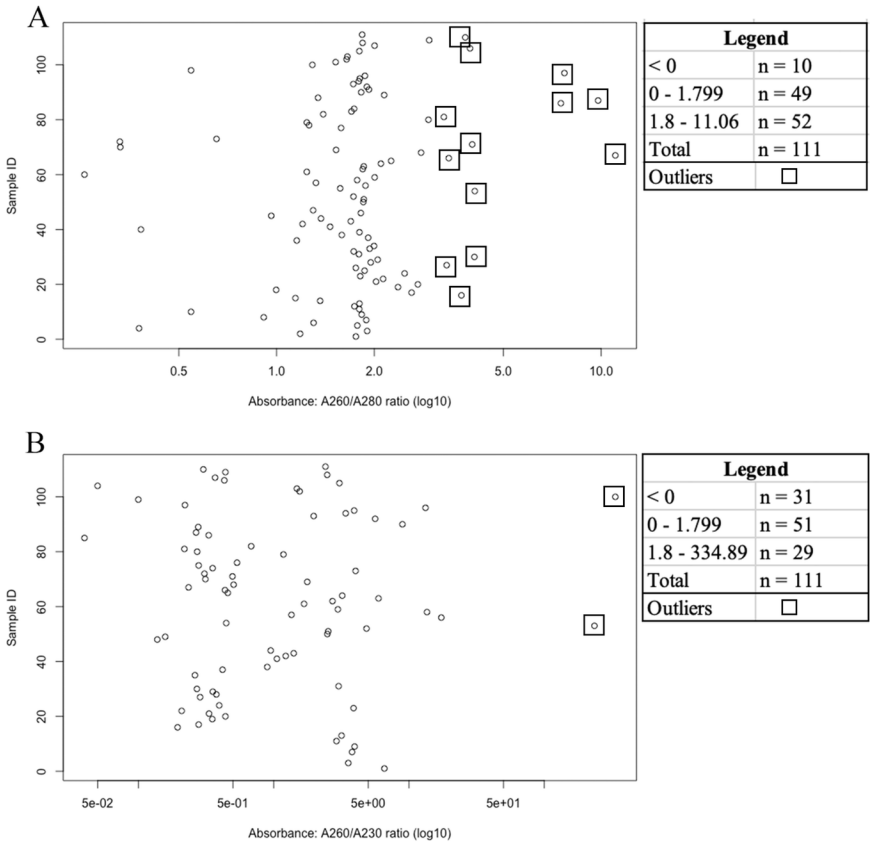

Figure 2: Scatterplots of A260/280 and A260/230 absorbance ratios of DNA extracted from herptile specimens sold at traditional medicine markets. The log transformed (log10) x axes exclude 10 and 31 negative absorbance ratios from plots A and B respectively as logs of negative numbers cannot be calculated (in the legend, the ratios were not log transformed). Samples with negative absorbance ratios suggest the DNA extraction protocols require improvements, while extreme values (with square outlines) on the plots suggest the samples could have contaminants. Extreme values are ratios that are higher than the sum of the upper quartile (Q3) of the absorbance ratios added to the product of the interquartile range (IQR) multiplied by 1.5 (Outliers > Q3 + IQR X 1.5).