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Leopard tortoises in southern Africa have greater genetic diversity in the north than in the south (Testudinidae)

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Abstract

In contrast to mammals, little is known about the phylogeographic structuring of widely distributed African reptile species. With the present study, we contribute data for the leopard tortoise (*Stigmochelys pardalis*). It ranges from the Horn of Africa southward to South Africa and westwards to southern Angola. However, its natural occurrence is disputed for some southern regions. To clarify the situation, we used mtDNA sequences and 14 microsatellite loci from 204 individuals mainly from southern Africa. Our results retrieved five mitochondrial clades; one in the south and two in the north-west and north-east of southern Africa, respectively, plus two distributed further north. Using microsatellites, the southern clade matched with a well-defined southern nuclear cluster, whilst the two northern clades from southern Africa corresponded to another nuclear cluster with three subclusters. One subcluster had a western and central distribution, another occurred mostly in the north-east, and the third in a small eastern region (Maputaland), which forms part of a biodiversity hotspot. Genetic diversity was low in the south and high in the north of our study region, particularly in the north-east. Our results refuted that translocations influenced the genetic structure of leopard tortoises substantially. We propose that Pleistocene climatic fluctuations caused leopard tortoises to retract to distinct refugia in southern and northern regions and ascribe the high genetic diversity in the north of southern Africa to genetic structuring caused by the survival in three refuges and subsequent admixture, whereas tortoises in the south seem to have survived in only one continuous coastal refuge.

KEYWORDS

Bergmann's rule, conservation, phylogeography, population structuring, *Stigmochelys pardalis*

1 | INTRODUCTION

In contrast to mammals, little is known about the phylogeographic structuring of widely distributed African reptile species (Barlow et al., 2013), and some putatively widely distributed species proved to be species complexes (*Agama* spp., Leaché, Wagner, et al., 2014; *Hemidactylus fasciatus* group, Leaché, Fujita, Minin, & Bouckaert, 2014; Wagner, Leaché, & Fujita, 2014; *Naja melanoleuca* complex, Wüster et al., 2018; *Pelomedusa* spp., Petzold et al., 2014; Vargas-Ramírez et al., 2010; *Trachylepis varia* complex, Weinell & Bauer, 2018), underlining the need of more research. Land tortoises (Testudinidae) are in this context of special interest because all species are considered to be imperilled. In sub-Saharan Africa, exclusive of Madagascar and smaller islands of the Indian Ocean, eight tortoise genera with 20 species occur. This is worldwide the region with the highest tortoise diversity. The family Testudinidae is distributed over all continents except Antarctica and Australia and comprises 18 extant genera and 65 species (TTWG, 2017), which qualifies

sub-Saharan Africa as a global biodiversity hotspot for tortoises. Only few sub-Saharan taxa are widely distributed, and the species with the largest distribution range is the leopard tortoise (*Stigmochelys pardalis*). It occurs from the Horn of Africa through East Africa to South Africa and ranges from there inland and north-westwards to Namibia, southern Angola, Botswana, and southern Zambia. The species is listed by the IUCN in the threat category Least Concern (TTWG, 2017), even though it has become rare in some regions outside of reserves and fenced farmland due to human consumption, trade, frequent fires and electric fences (Baker, Kabigumila, Leuteritz, Hofmeyr, & Ngwava, 2015; Evans, 1988).

A nearly range-wide mitochondrial phylogeography for *S. pardalis* showed that the highest genetic diversity is found in southern Africa, where five out of seven mitochondrial lineages were identified (Fritz et al., 2010). This study did not support the distinctiveness of the long-recognized subspecies *S. p. pardalis* and *S. p. babcocki*, and the two taxa were synonymized. As far as known, morphological and genetic variation is decoupled, and considerable body size differences follow a poorly understood geographic pattern. The largest tortoises that may exceed 75 cm or even 85 cm straight shell length occur in the very north and south of the range, whilst geographically intermediate populations harbour much smaller individuals of less than 30 or 40 cm shell length (Fritz et al., 2010).

The distribution range of *S. pardalis* excludes large portions of western and eastern South Africa but appears to be nearly continuous in the south (Baker et al., 2015). The species' natural occurrence in the south-west of South Africa, however, is controversial. Greig and Burdett (1976) proposed that humans exterminated leopard tortoises in the south-western coastal regions but Branch, Benn, and Lombard (1995) countered this hypothesis with evidence that this species does not occur at archaeological sites in the south-western Cape, even though remains of another tortoise, *Chersina angulata*, are common there. Consequently, a recent South African distribution map of the species indicated occurrences of *S. pardalis* in the extreme south-western part South Africa as being introduced (Hofmeyr, Boycott, & Baard, 2014). The reason why leopard tortoises appear to be nearly absent in the west of South Africa may be a consequence of low population densities due to summer aridity. Branch et al. (1995) speculated that the absence of *S. pardalis* in parts of eastern South Africa may be because they never occurred there, as tortoise bones in general have low occurrences at archaeological sites in that region.

Another complication in assessing the natural distribution of *S. pardalis* is that leopard tortoises are favourite pets, which do well in captivity (Greig & Burdett, 1976). People frequently collect the colourful hatchlings and juveniles along roads to take home, which may be far from the tortoise's origin. These captives often escape or are placed in the care of zoos, reptile parks or conservation agencies when they become large and unwanted. In the past, conservation agencies released captive leopard tortoises into nature reserves in the Western Cape (Greig & Burdett, 1976) and areas within their assumed natural range in KwaZulu-Natal (Wimberger, Armstrong, & Downs, 2009), contributing to confusion of the species' natural range. If introduced tortoises established in these regions, mismatched phylogeographic patterns and signatures of genetic admixture would be expected. In this respect, the sampling used by Fritz et al. (2010) was too patchy for reliable conclusions. In addition, this investigation was restricted to mitochondrial DNA (mtDNA), which is inherited only in the maternal line and provides no direct information about gene flow and admixture. Thus, denser sampling and biparentally inherited markers are required to improve understanding of the phylogeographic and population genetic patterns of *S. pardalis*.

In the present study, we combined mtDNA sequences with information from 14 microsatellite loci to elucidate the following questions: (a) Does the differentiation pattern of the biparentally inherited microsatellite loci match that of the maternally inherited mtDNA? (b) Does genetic differentiation of leopard tortoises in southern Africa follow general phylogeographic paradigms? (c) Does genetic evidence support the assumption that leopard tortoises from parts of their range in South Africa are introduced?

2 | MATERIAL AND METHODS

2.1 | Sampling

Blood, saliva or tissue samples of 204 leopard tortoises were examined (Supporting Information Table S1), including data for 43 tortoises from a previous study (Fritz et al., 2010). Of the latter, microsatellite data were generated, if samples were still available. One sample from Springbok, South Africa, used by Fritz et al. (2010) turned out to be erroneously associated with another mtDNA sequence. This sample was resequenced, and the correct cyt *b* sequence was combined with its microsatellite data. Most samples were stored in ethanol at −80°C until processing. Some blood samples were preserved on FTA classic cards (Whatman, GE Healthcare, Munich, Germany) at room temperature.

Fieldwork and sampling in South Africa were permitted by the Limpopo Provincial Government (permit ZA/Lp/80202), Ezemvelo KwaZulu-Natal Wildlife (permit OP 139/2017), CapeNature (permit AAA007-00212-0056), the Department of Environmental Affairs, Eastern Cape (permit CRO117/13C & CRO 118/13CR), and Biodiversity Northern Cape Province (permit 245/2015). Fieldwork and sampling in Namibia were permitted by the Ministry of Environment and Tourism (permit 1910/2014). Tortoises were hand-collected, and blood and tissue samples (scale clippings) were taken as approved by the Ethics Committee of the University of the Western Cape under ethical clearance number

ScRiRC2008/39. Tortoises were released at the capture sites after sampling and taking measurements.

2.2 | DNA extraction, PCR and sequencing

Total DNA was isolated using the InnuPrep DNA Mini Kit for tissues and the InnuPrep Blood DNA Mini Kit for blood samples (both kits: Analytik Jena AG, Jena, Germany). DNA from blood on FTA cards was extracted using the illustra tissue & cells genomicPrep Mini Spin Kit (GE Healthcare) following the protocol for genomic DNA from animal tissue.

Since no specific primers for microsatellite loci for *S. pardalis* have been characterized yet, 54 primers developed for other turtle species were tested (Supporting Information Table S2). PCRs were run using the cycling conditions from Supporting Information Table S3 and a reaction volume of 10 µl containing 10–20 ng of total DNA, 0.5 units of *Taq* polymerase (Bioron GmbH, Ludwigshafen, Germany), the buffer recommended by the supplier (without $MgCl_2$, 10×), 1.5 mM $MgCl_2$ (Bioron), 0.2 mM of each dNTP (Roth, Karlsruhe, Germany), 2 µg Bovine Serum Albumin (BSA; Thermo Fisher Scientific Inc., Washington, MA, USA) and 0.3 µM of each primer. In case of amplification, the presence of the microsatellites was confirmed by sequencing the PCR products with unlabelled primers in both directions. Then, three multiplex PCRs and seven single-locus PCRs were performed using a reaction volume of 10 µl containing 10–20 ng of total DNA, 0.5 units of *Taq* polymerase (Bioron), the buffer recommended by the supplier (without $MgCl_2$, 10×), 2.0 mM $MgCl_2$ (Bioron), 0.2 mM of each dNTP (Roth) and 2 µg BSA. Each multiplex PCR comprised a specific set of primers at a specific concentration (Supporting Information Table S4). Thermocycling conditions are summarized in Supporting Information Table S5. Fragment lengths were determined using an ABI 3730 DNA Analyzer. For this purpose, 1 µl of the PCR product was diluted in 99 µl of double-distilled water; 1 µl of this dilution was mixed with 8.5 µl Hi-Di Formamide (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA), 0.25 µl double-distilled water and 0.25 µl GeneScan-600 LIZ Size Standard (Applied Biosystems). The software PEAK SCANNER 1.0 (Life Technologies) was used for scoring fragment lengths. Null allele frequencies for the microsatellite loci were examined using MICRO-CHECKER 2.2.3 (van Oosterhout, Hutchinson, Wills, & Shipley, 2004). Furthermore, the presence of Hardy–Weinberg equilibrium and linkage equilibrium was examined using ARLEQUIN 3.5.2.1 (Excoffier & Lischer, 2010), resulting in the selection of 14 loci at which 177 samples could be genotyped.

In addition, the mitochondrial cytochrome *b* (cyt *b*) gene, known to be phylogeographically informative for *S. pardalis* (Fritz et al., 2010), was amplified using the specific primers for *S. pardalis* from Fritz et al. (2010). PCR conditions were initial denaturation at 95°C for 5 min, 35 cycles with denaturation at 95°C for 45 s, annealing for 45 s at 58°C and extension at 72°C for 90 s, with final extension at 72°C for 10 min. A total reaction volume of 25 µl contained 1.25 units *Taq* polymerase (Bioron), the recommended reaction buffer (complete 10×) containing $MgCl_2$, 0.2 mM of each dNTP (Roth), 0.4 µM of each primer, 2 µg BSA and 20–40 ng of DNA. PCR products were purified using the ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems; 1:20 dilution; modified protocol: 30 min at 37°C, 15 min at 80°C). For sequencing the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the primers mt-c-For2/mt-E-Rev2 (Fritz et al., 2006) was used. After an initial denaturation at 96°C for 1 min, 25 cycles were run with denaturing at 96°C for 10 s, annealing at 50°C for 5 s and elongation at 60°C for 75 s. Cycle sequencing reaction products were purified using the Performa DTR V3 96-Well Short Plate (Edge Biosystems, Gaithersburg, MD, USA), with each well filled with 400 µl Sephadex™ (GE Healthcare; 1:20 solution). Sequences were resolved on an ABI 3730 DNA Analyzer (Applied Biosystems). In total, 152 cyt *b* sequences of 1,136 bp length were obtained.

2.3 | Data processing

2.3.1 | Microsatellites

Microsatellite data of the 177 leopard tortoises were analysed using the unsupervised Bayesian clustering approach of STRUCTURE 2.3.4 (Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard, Stephens, & Donnelly, 2000), applying the admixture model and correlated allele frequencies. STRUCTURE searches in the data set for partitions which are, as far as possible, in Hardy–Weinberg equilibrium and linkage equilibrium. Unsupervised analyses were chosen because this approach clusters samples strictly according to their genetic information, without any presumptions about population structuring (e.g., geographic distances, sampling sites). All calculations were run for $K = 1–10$, and the most likely number of clusters (K) was determined using the ΔK method (Evanno, Regnaut, & Goudet, 2005) as implemented in the software STRUCTURE HARVESTER (Earl & vonHoldt, 2012) and mean Ln probabilities. Calculations were repeated 10 times for each K using a MCMC chain of 750,000 generations for each run, including a burn-in of 250,000 generations. Population structuring and individual admixture were then visualized using DISTRUCT 1.1 (Rosenberg, 2004). Individuals with a membership proportion below 80% were treated as having admixed ancestries (Barilani et al., 2007; Randi, 2008). STRUCTURE is known to be prone to bias from uneven sample sizes (Puechmaille, 2016) and to detect typically only the uppermost hierarchical level of population differentiation (Evanno et al., 2005). Therefore, STRUCTURE was also run for subsamples corresponding to clusters identified in the run for the entire data set, but with admixed

individuals (Supporting Information Table S1) excluded. In addition, Principal Component Analyses (PCAs) were run to examine population structuring using microsatellite data and the R package ADEGENET (Jombart, 2008) for R 3.2.3. The advantage of PCAs is that they are less sensitive to sample size bias and not dependent on population genetic assumptions (Puechmaille, 2016). Four distinct PCAs were executed. In the first analysis, all 177 individuals were included, and symbols were coloured after STRUCTURE clusters. The second PCA used only those 168 individuals for which mitochondrial haplotypes were known; symbols were coloured according to mitochondrial clades. The third PCA included only the tortoises from the northern STRUCTURE cluster (80 individuals), with symbols coloured according to STRUCTURE clusters. The fourth PCA comprised only those 74 individuals from the northern STRUCTURE cluster with mtDNA information available; symbols were coloured according to the mitochondrial clades (Supporting Information Table S1).

2.3.2 | Mitochondrial DNA

The 152 new cyt *b* sequences were aligned with 43 previously published sequences (Fritz et al., 2010; Supporting Information Table S1) using BIOEDIT 7.0.9.0 (Hall, 1999). For phylogenetic analyses, individual sequences were collapsed into haplotypes using POPART (https://popart.otago.ac.nz). *Psammobates tentorius* (GenBank accession number DQ497318), *Chersina angulata* (DQ497292), *Chersobius boulengeri* (DQ497308) and *Chersobius signatus* (DQ497309) served as the outgroups. Evolutionary relationships were inferred using Maximum Likelihood and Bayesian approaches. PARTITIONFINDER 2 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016), and the implemented Bayesian Information Criterion were used to determine the best partitioning scheme and the model of sequence evolution. According to the results of PARTITIONFINDER 2, Maximum Likelihood calculations were conducted with RAxML 7.2.8 (Stamatakis, 2006) using the GTR+G model and an unpartitioned data set. Five independent searches were performed using different starting conditions and the fast bootstrap algorithm to explore the robustness of the results by comparing the best trees. Then, 1,000 non-parametric thorough bootstrap replicates were calculated and plotted against the best tree. Bayesian analysis was performed using MRBAYES 3.2.1 (Ronquist et al., 2012) with two parallel runs (each with four chains) and default parameters using the same substitution model and an unpartitioned alignment. The chains ran for 10 million generations with every 100th generation sampled. Calculation parameters were analysed using the software TRACER 1.6 (Rambaut, Suchard, Xie, & Drummond, 2014) and a burn-in of 2.5 million generations to assure that both runs converged. Subsequently, only the plateau of the most likely trees was sampled using the same burn-in, and a 50% majority rule consensus tree was generated. In addition, a parsimony network was calculated using POPART (https://popart.otago.ac.nz). For accession numbers of newly identified haplotypes, see Supporting Information Table S1.

2.3.3 | Diversity within and divergence among population clusters

Diversity and divergence parameters were estimated for population clusters revealed by STRUCTURE analyses. For doing so, a frequency table for microsatellite alleles was produced in CONVERT 1.31 (Glaubitz, 2004). ARLEQUIN 3.5.2.1 was used to infer locus-specific observed (H_O) and expected heterozygosities (H_E) and for performing AMOVAs (10,000 permutations) for microsatellite and mtDNA data of clusters identified by STRUCTURE analyses and PCAs. FSTAT 2.9.3.2 (Goudet, 1995) was used for computing values for locus-specific allelic richness.

3 | RESULTS

3.1 | Population structuring

STRUCTURE analyses using all samples ($n = 177$) revealed two clusters, one corresponding to southern collection sites and the other to more northerly sites. In a few, mainly geographically intermediate sites, admixed tortoises were recorded as well as tortoises of the two clusters together or in close proximity (Figure 1). The southern cluster (Figure 1a, red) corresponded largely to one mitochondrial lineage (Figure 1a, red), whilst the northern cluster comprised tortoises harbouring mainly haplotypes of two other mitochondrial lineages (Figure 1a, blue and orange).

The mitochondrial lineages red, blue and orange represented distinct clades in phylogenetic analyses (Supporting Information Figure S1) and distinct haplotype clusters in parsimony network calculations (Supporting Information Figure S2). These lineages were already described by Fritz et al. (2010) under other names. The red lineage corresponded to clade III, the blue lineage to clades VI and VII, and the orange lineage to clades IV and V of Fritz et al. (2010). Clades VI and VII, as well as clades IV and V, differed only from one another when additional mitochondrial genes were used for calculations.

Additional STRUCTURE runs for each of the two clusters with admixed individuals excluded revealed $K = 1$ as best solution for the southern cluster and $K = 3$ for the northern cluster (Figure 2 and Supporting Information Figure S3). For the northern subset, one of the clusters (Figure 2a: N1) corresponded to samples from Namibia and the Northern Cape; a few samples assigned to this cluster came also from the Free State and KwaZulu-Natal (Figure 2b). The other two clusters were represented by samples from the centre and north-east of South Africa and adjacent Mozambique (Figure 2b: N2 and N3). About one-third of the tortoises in these regions had admixed ancestries, and the clusters were not fully consistent with mitochondrial lineages

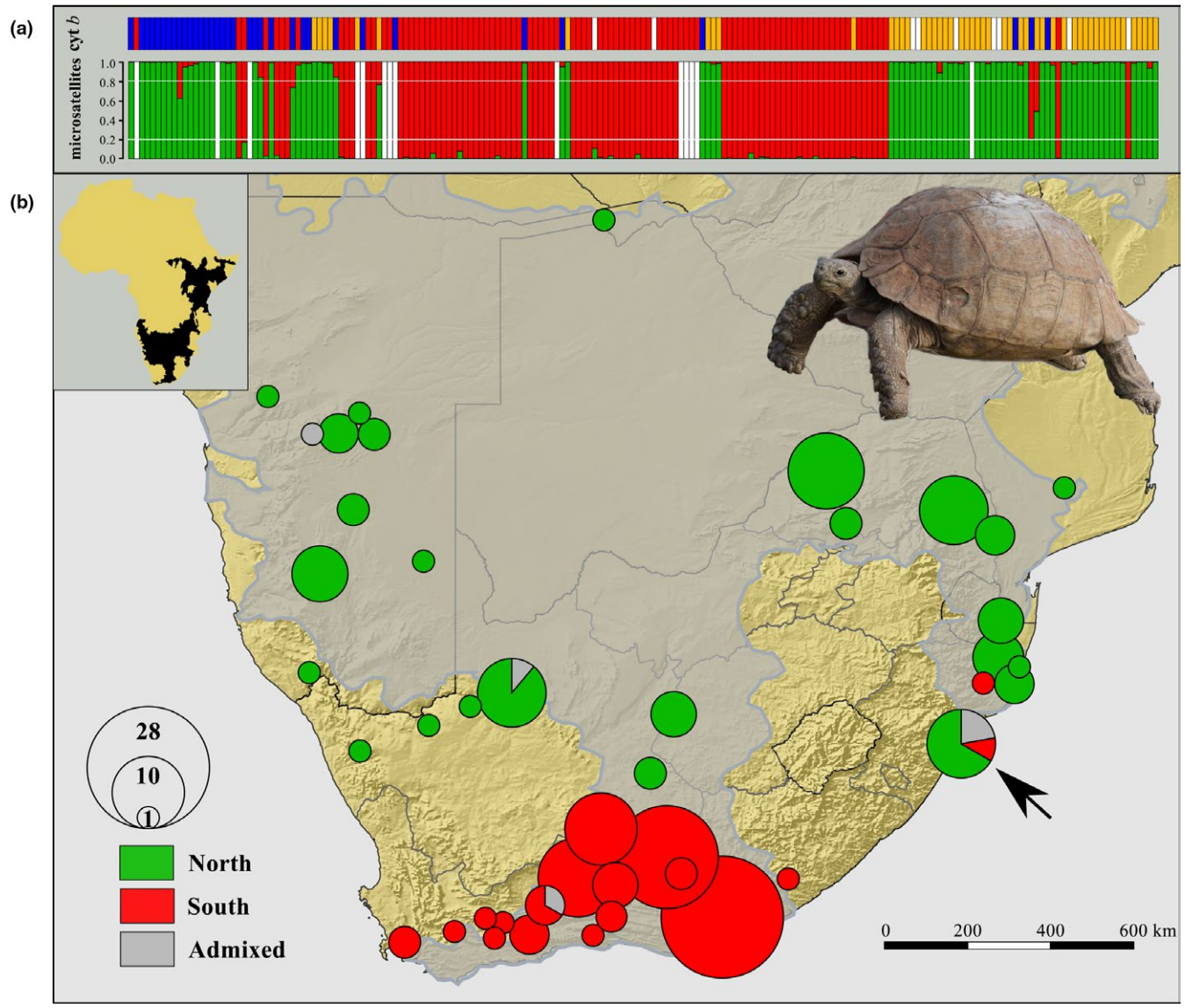

FIGURE 1 (a) Genotypic structuring of 177 leopard tortoises (*Stigmochelys pardalis*) from 49 sites as inferred by STRUCTURE using 14 microsatellite loci (run with best probability value shown). Individuals are arranged from west to east (Supporting Information Table S1). Distinct clusters are colour-coded; white = missing data. Within each cluster, an individual is represented by a vertical segment that reflects its ancestry. Mixed ancestries are indicated by differently coloured sectors corresponding to inferred genetic percentages of the respective cluster. The mitochondrial clade of each sample is shown above the STRUCTURE diagrams (red, orange, blue; white = missing data). (b) Distribution range of *S. pardalis* (according to Baker et al., 2015; shaded in grey) and lumped sampling sites. Inset shows whole range (black). Colours of sampling sites correspond to STRUCTURE clusters. Symbol sizes indicate sample sizes; slices (percentages) represent turtles with mixed ancestries or conflicting cluster assignment. Individual sampling sites are arbitrarily spread to show percentages; for original locations see Supporting Information Table S1. The arrow highlights Ballito Game Reserve, South Africa [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 2a). Whilst cluster N1, with considerable admixture mainly with cluster N2, matched quite well with the "blue mitochondrial lineage," most tortoises of clusters N2 and N3 shared the same mitochondrial lineage ("orange"). In addition, a few individuals showed mismatched mitochondrial haplotypes.

Principal Component Analyses (Figure 3) supported the results of the STRUCTURE runs and the near absence of nuclear genomic admixture between northern and southern leopard tortoises, but indicated massive admixture among the three clusters of the northern subset. In addition, as when mitochondrial haplotypes were compared to STRUCTURE results (Figures 1 and 2), mismatches between mitochondrial lineages and nuclear genomic clusters were evident mainly for the northern subset (Figure 3).

3.2 | Diversity within and divergence between STRUCTURE clusters

In agreement with its nuclear genomic and mitochondrial substructuring (Figures 1 and 2), genetic diversity was higher

in the northern than in the southern cluster (Table 1). With a comparable sample size, the northern cluster had significantly more mitochondrial haplotypes and higher haplotype and nucleotide diversities. For microsatellites, the average number of alleles per locus ($n_{\bar{A}} = 15.3$) was almost twice as high as for the southern cluster ($n_{\bar{A}} = 8.3$). The northern

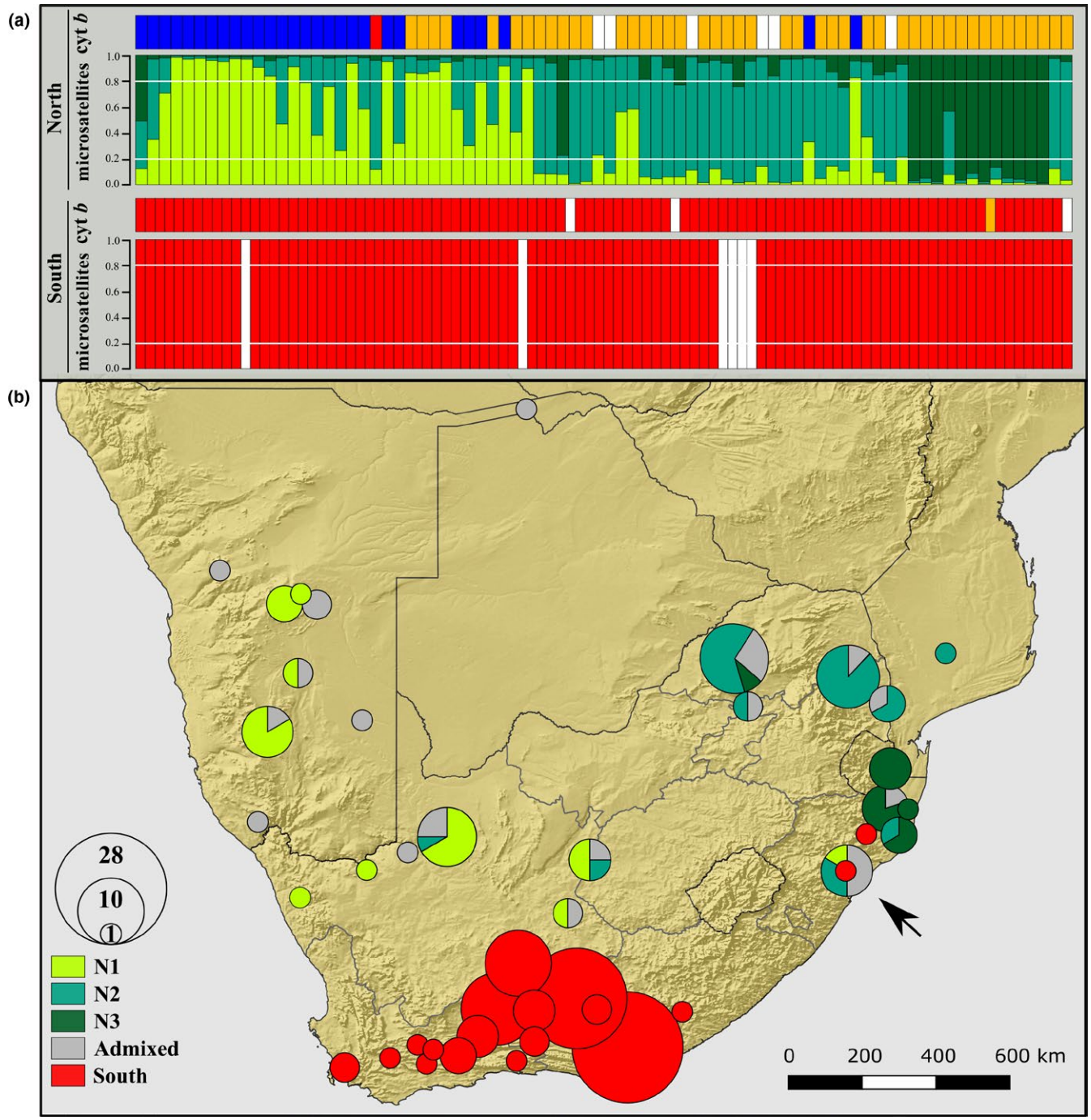

FIGURE 2 (a) Genotypic structuring of the two subsets (northern and southern clusters; admixed individuals excluded) as inferred by STRUCTURE**.** The runs with the best probability values are shown. Individuals are arranged from west to east (Supporting Information Table S1). Distinct clusters are colour-coded; white = missing data. Within each cluster, an individual is represented by a vertical segment that reflects its ancestry. Mixed ancestries are indicated by differently coloured sectors corresponding to inferred genetic percentages of the respective cluster. The mitochondrial clade of each sample is shown above the STRUCTURE diagrams (red, orange, blue; white = missing data). Colours of lumped sampling sites in the map (b) correspond to the southern cluster and the three northern subclusters. Symbol sizes indicate sample sizes, slices, percentages of turtles with mixed ancestries or conflicting cluster assignment. Individual sampling sites are arbitrarily spread to show percentages; for original locations see Supporting Information Table S1. The arrow highlights Ballito Game Reserve, South Africa. The superimposed red symbol indicates that this sample was used in another STRUCTURE run (for the southern cluster) [Colour figure can be viewed at wileyonlinelibrary.com]

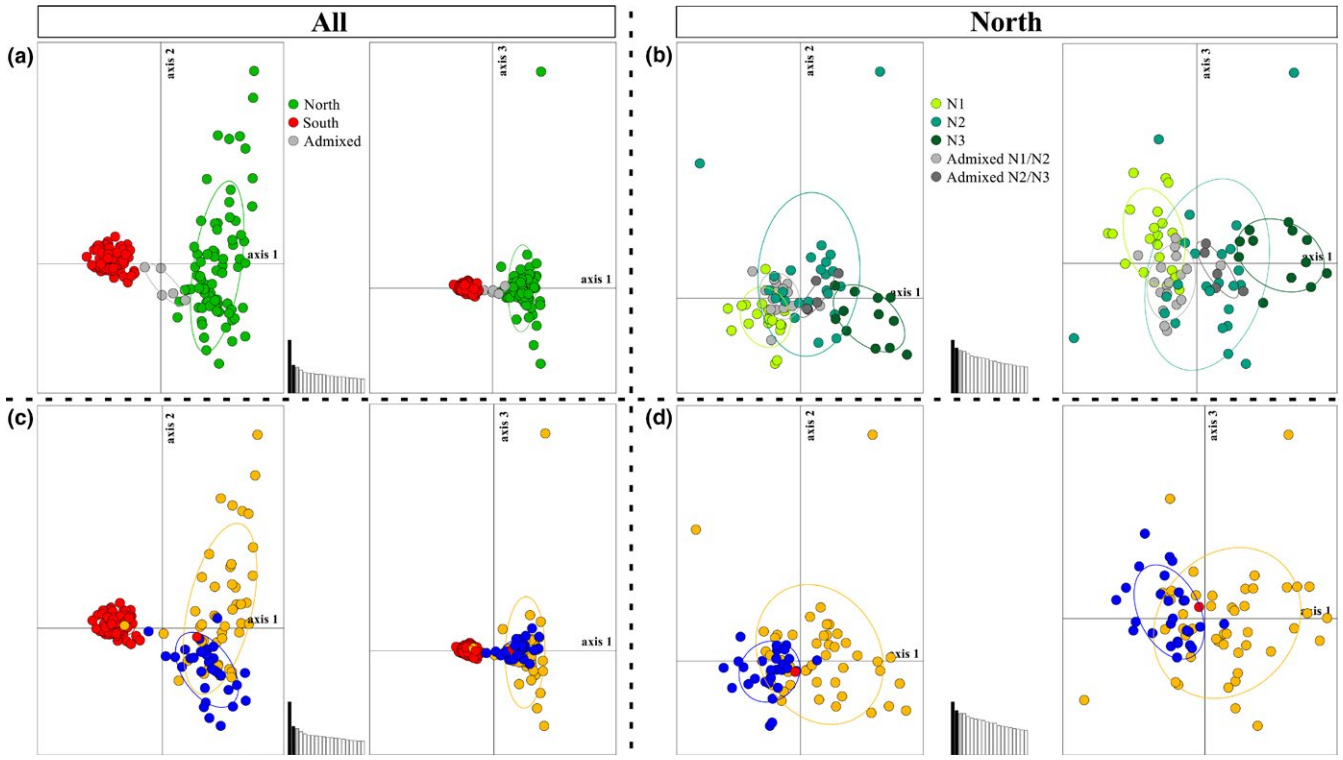

FIGURE 3 Top: Principal Component Analyses (PCAs) using microsatellite data of (a) 177 leopard tortoises (all samples) and (b) 80 tortoises of the northern cluster coloured according to STRUCTURE results. Bottom: PCAs using microsatellite data of (c) 168 tortoises (all samples with mtDNA data) and (d) 74 individuals of the northern cluster coloured according to mitochondrial clades (red, orange and blue). For (a), axes 1–3 explain 5.23%, 2.76% and 2.54%; for (b), 3.79%, 3.22% and 3.05%; for (c), 5.42%, 2.94% and 2.70%; and for (d), 4.09%, 3.44% and 3.22% of variance, respectively. Ovals indicate 95% confidence intervals [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Genetic diversity of STRUCTURE clusters with admixed individuals excluded

	Microsatellites							mtDNA				
Cluster	***n***	n_A	$n_{\bar{A}}$	n_P	***AR***	H_O	H_E	***n***	n_{HT}	n_{pHT}	***h***	π
North	80	214	15.3	109	15.20	0.718	0.797	74	29	27	0.907	9.782
South	92	116	8.3	11	8.07	0.549	0.611	89	14	12	0.688	2.269
N1	21	117	8.4	18	7.20	0.701	0.777	21	9	8	0.876	8.371
N2	24	170	12.1	55	9.43	0.737	0.806	19	13	10	0.930	4.164
N3	12	95	6.8	10	6.79	0.685	0.721	12	5	2	0.576	0.667

Note. *AR*, allelic richness; *h*, haplotype diversity; H_E, average expected heterozygosity; H_O, average observed heterozygosity; *n*, number of individuals; $n_{\bar{A}}$, average number of alleles per locus; n_A, number of alleles; n_{HT}, number of haplotypes per cluster; n_P, number of private alleles; n_{pHT}, number of private haplotypes per cluster; π, nucleotide diversity.

cluster had nearly 10x more private alleles ($n_P = 109$) than the southern cluster ($n_P = 11$). The same general pattern was also reflected by the observed heterozygosity, with $H_O = 0.718$ in the northern cluster and $H_O = 0.549$ in the southern cluster.

Sample sizes of two clusters from the northern subset (Table 1; N1 and N2) were similar. Even though cluster N3 was represented by only approximately half the number of individuals, its diversity resembled for microsatellites cluster N1. The highest genetic diversity was found for N2. With an average of 12.1 alleles per locus, it had almost double the number of alleles per locus compared to cluster N3 ($n_{\bar{A}} = 6.8$). N2 also had the highest number of private alleles ($n_P = 55$) compared to N1 ($n_P = 18$) and N3 ($n_P = 10$).

The northern and southern clusters differed by an F_{ST} value of 0.176 for microsatellites ($p < 0.05$), whilst subclusters N1–N3 within the northern cluster differed by substantially lower F_{ST} values, ranging from 0.034 to 0.096 (Table 2). Accordingly, an AMOVA for the northern and southern

clusters for microsatellites revealed that 17.59% of the molecular variance occurred between the clusters and 82.41% within the clusters. For mtDNA a much stronger divergence was revealed for the clusters, with 72.92% molecular variance between and 27.08% within the clusters. For microsatellite data of the three northern subclusters (N1–N3), 5.22% of the molecular variance occurred between the subclusters and 94.78% within the subclusters. For mtDNA, the respective values were 53.80% (between subclusters) and 46.20% (within subclusters).

4 | DISCUSSION

Using microsatellite and mitochondrial markers combined, we revealed a pronounced north–south differentiation in southern African leopard tortoises (Figure 1), with a clear substructure in the north of the study area, but not in the south (Figure 2). When the two marker systems are compared, a largely concordant pattern is found for the southern cluster, with one mitochondrial lineage (red) corresponding to the southern microsatellite cluster (Figures 1–3). In contrast, the northern microsatellite cluster embraces two distinct mitochondrial lineages (orange, blue), with some admixed tortoises, or tortoises with mismatched mitochondrial haplotypes, or conflicting genotypic identity. This indicates either natural dispersal and gene flow or translocated tortoises. The latter is most likely true for the Ballito Game Reserve in KwaZulu-Natal, close to the city of Durban. We recorded there, besides leopard tortoises with unexpected genetic identity (southern cluster, blue and red mtDNA lineages; Figures 1 and 2, Supporting Information Figure S2), also one evidently non-native tortoise species (*Chersina angulata*) that must have been released there. Apart from Ballito Game Reserve, translocation cannot be excluded for some other records of tortoises of mismatched genetic identity. However, in general, our data do not suggest that the genetic structure of *S. pardalis* has been much altered by human activity, particularly in the southern and western populations. Moreover, we found no evidence for populations introduced to regions where no leopard tortoises occurred before. In such non-native populations, a high degree of nuclear genomic admixture and conflicting syntopic mitochondrial lineages would be expected, given that the founders originated from different source populations. Also, mismatched genetic identities compared to neighbouring native populations can indicate introduction. However, such disturbances were rather rare and largely restricted to single individuals. In general, and contrary to expectations, we found a geographically consistent pattern that makes sense in light of the biology of leopard tortoises and matches the phylogeographic patterns of at least some other species.

TABLE 2 Fixation indices (F_{ST} values) for the subclusters of the northern cluster. Values above the diagonal are for mtDNA; below diagonal, microsatellite data

	N1	N2	N3
N1	—	0.062*	0.184*
N2	0.034*	—	0.067
N3	0.096*	0.044*	—

*$p < 0.05$.

Several studies have shown that leopard tortoises move long distances, facilitating exchange among distinct populations. Leopard tortoises generally have large home ranges, especially in arid environments (up to 205 ha, Nama Karoo, McMaster & Downs, 2006), whereas home ranges in more mesic environments are smaller (13.5–160 ha, Bertram, 1979; Hailey & Coulson, 1996; Monadjem, McCleery, & Collier, 2013). It seems that females move larger distances than males, at least in some seasons (McMaster & Downs, 2006), irrespective of humidity. Hence, gene flow should be more female mediated with a somewhat wider spread of mitochondrial lineages compared to nuclear genomic differentiation patterns.

Accordingly, we expected that microsatellite analyses should reveal a more intricate differentiation pattern than those for maternally inherited mtDNA. This is the case in the northern part of our study region, where microsatellites identify three weakly differentiated subclusters (N1–N3; Figures 2 and 3) corresponding to only two mitochondrial lineages. However, this conclusion must be treated with caution because the two mitochondrial lineages diverge into four when additional genes are used for phylogenetic and network calculations (Fritz et al., 2010). Nevertheless, one of the three nuclear subclusters (N3) is confined to a small area at the border of South Africa (KwaZulu-Natal) and Mozambique. The same region also has an endemic snake species (*Xenocalamus transvaalensis*, Portillo et al., 2018), several endemic dwarf chameleon species (*Bradypodion* spp., Tilbury & Tolley, 2009) and an endemic mitochondrial clade of the helmeted terrapin (*Pelomedusa galeata* subclade Ib, Vamberger, Hofmeyr, Ihlow, & Fritz, 2018), suggesting high biogeographic differentiation. In fact, this region (Maputaland) together with two more southern regions constitutes the Maputaland-Pondoland-Albany hotspot, characterized by high plant and vertebrate endemism (Perera, Ratnayake-Perera, & Procheş, 2011). Since Maputaland is relatively mesic compared to other parts of the range of *S. pardalis*, tortoises probably move shorter distances, which likely supports some divergence.

The weakest differentiation was found in the southern part of our study area, which comprises relatively mesic (Fynbos, Albany Thicket) and semi-arid habitats (Succulent and Nama Karoo). The tortoises from south-western South Africa also belong to this cluster, indicating that they are either native,

expanded their ranges recently, or have been introduced from nearby southern sites. Both the second and third possibilities are feasible, because this species does not occur at archaeological sites of the region (Branch et al., 1995).

It is likely that past climatic fluctuations contributed to current genetic diversity of *S. pardalis*. Barlow et al. (2013) and Vamberger et al. (2018) have shown that ranges and genetic differentiation of two co-distributed species, the puff adder (*Bitis arietans*) and the helmeted terrapin (*Pelomedusa galeata*), have been strongly influenced by Pleistocene climate fluctuations. For both species inland regions of South Africa became temporarily inhospitable, whilst coastal and more northerly areas remained habitable, supporting the divergence of northern and southern lineages. This matches well with the pattern in leopard tortoises and suggests the survival of leopard tortoises in three refugial patches whilst in the south the tortoises survived most likely in a continuous coastal refuge, explaining the greater diversity and subsequent admixture in the north. In this context, the occurrence of genetic lineages in the Maputaland refuge that represent different stages of divergence, from conspecific lineages to distinct species, is not surprising. It is a commonplace that lineages of different age co-occur in many regions of the world. When such communities are forced to retreat into the same refugia, some lineages will be lost but the remaining will necessarily reflect a subset of the different scales of differentiation present before, and for some previously widely distributed lineages the divergence process will start only in the new allopatric situation. A suchlike multifaceted pattern is, for instance, also known for the classical glacial refugia of the Western Palearctic, where old genetic lineages survived that existed already long before the Ice Age and where young endemic lineages of different age evolved during the individual Pleistocene glaciations (Hewitt, 2000; Joger et al., 2007).

The genetic complexity of northern leopard tortoises compared to a relatively uniform southern lineage resembles the phylogeographic pattern of the rock skink *Trachylepis sulcata*, for which a southwards directed range expansion was inferred (Portik, Bauer, & Jackman, 2011). Even when the underlying causes for the observed similarities may differ, this situation underlines that more studies on the phylogeography of southern African reptiles are needed for revealing general paradigms. Further research is also needed to understand how the genetic clusters of *S. pardalis* correlate with morphology. The southern cluster corresponds to the largest leopard tortoises in our study area, which may reach up to 75 cm straight carapace length (Fritz et al., 2010), whilst the northern cluster comprises medium-sized tortoises of <40 cm (based on data for 13 adult individuals from Lapalala and Mookgophong, Limpopo, South Africa, M. Vamberger unpubl., and six adult tortoises from Congella farm, Namibia, Fritz et al., 2010). Interestingly, Fritz et al. (2012) found in the South American Chaco tortoise (*Chelonoidis chilensis*) a similar pattern of genetically relatively poor and large tortoises in the south. As in *S. pardalis*, genetically more diverse, small Chaco tortoises occur in the north of the range of this species. Fritz et al. (2012) suggested that the genetic pattern in *C. chilensis* matches, but is inverse to the phylogeographic paradigm of "southern genetic richness" of the northern hemisphere (Hewitt, 2000). Without correlating the observed size variation with genetic patterns, Werner, Koroloker, Sion, and Göçmen (2016) described similar size variation also for the western Palaearctic spur-thighed tortoise (*Testudo graeca*). Werner et al. (2016) concluded that this, and most likely other, tortoise species follow Bergmann's rule in that size increases with latitude, in line with the assumption that geographic size variation of chelonians follows generally Bergmann's rule (Ashton & Feldman, 2003). Yet, attributing size variation of leopard tortoises to Bergmann's rule alone is too simplistic. In Somalia and Ethiopia, close to the equator, gigantic leopard tortoises occur that may even exceed the large-sized South African *S. pardalis* (Fritz et al., 2010), contradicting a size increase with latitude. Also, the results of Joos et al. (2017) for size variation in the widely distributed European pond turtle (*Emys orbicularis*) question the role of Bergmann's rule for chelonians.

With respect to genetic diversity, we lack sufficient data from northern central South Africa and beyond, in particular from more northerly regions. Thus, we currently cannot draw any conclusion how genetic diversity might correlate there with size variation. Future research should focus on disentangling the impact of genetic and environmental factors, such as aridity, seasonality and food availability, that are likely to have shaped together both morphological and genetic variation in leopard tortoises.

5 | CONCLUSIONS

Our study has provided evidence that on a large scale the differentiation patterns of mitochondrial and nuclear genomic markers (mtDNA, microsatellites) are consistent for leopard tortoises and reflect a natural pattern with little human disturbance. Phylogeographic differentiation seems to follow general paradigms, with distinct southern and northern population clusters, a pattern emerging also in other species (*Bitis arietans*, Barlow et al., 2013; *Pelomedusa galeata*, Vamberger et al., 2018; *Trachylepis sulcata*, Portik et al., 2011). Furthermore, the distribution of leopard tortoises of one northern subcluster (N3) matches the distribution ranges of an endemic snake species (*Xenocalamus transvaalensis*, Portillo et al., 2018), several endemic dwarf chameleon species (*Bradypodion* spp., Tilbury & Tolley, 2009) and a genetic lineage of the helmeted terrapin (*P. galeata*, Vamberger et al., 2018). With an increasing number of species studied genetically, more cases are expected to be reported. The conservation status of subcluster

N3 needs further investigation because its range (northern KwaZulu-Natal; most likely eastern Swaziland) shows excessive and continuous degradation over the past 20–30 years (Jewitt, Goodman, Erasmus, O'Connor, & Witkowski, 2015; Schoeman, Newby, Thompson, & Berg, 2013). Further research is also warranted for a better understanding of the correlations of body size variation and genetic structuring. For nature conservation, our microsatellite data offer the opportunity to assign abandoned *S. pardalis* to source regions, which will help avoiding genetic pollution by releasing tortoises of mismatched genetic identity.

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REFERENCES

Ashton, K. G., & Feldman, C. R. (2003). Bergmann's rule in nonavian reptiles: Turtles follow it, lizards and snakes reverse it. *Evolution*, *57*, 1151–1163. https://doi.org/10.1111/j.0014-3820.2003.tb00324.x

Baker, P. J., Kabigumila, J., Leuteritz, T., Hofmeyr, M., & Ngwava, J. M. (2015). *Stigmochelys pardalis. The IUCN Red List of Threatened Species, 2015*, e.T163449A1009442. https://doi.org/10.2305/iucn.uk.2015-4.rlts.t163449a1009442.en

Barilani, M., Sfougaris, A., Giannakopoulos, A., Mucci, N., Tabarroni, C., & Randi, E. (2007). Detecting introgressive hybridisation in rock partridge populations (*Alectoris graeca*) in Greece through Bayesian admixture analyses of multilocus genotypes. *Conservation Genetics*, *8*, 343–354. https://doi.org/10.1007/s10592-006-9174-1

Barlow, A., Baker, K., Hendry, C. R., Peppin, L., Phelps, T., Tolley, K. A., … Wüster, W. (2013). Phylogeography of the widespread African puff adder (*Bitis arietans*) reveals multiple Pleistocene refugia in southern Africa. *Molecular Ecology*, *22*, 1134–1157. https://doi.org/10.1111/mec.12157

Bertram, B. (1979). Homing by a leopard tortoise in the Serengeti. *African Journal of Ecology*, *17*, 245–247. https://doi.org/10.1111/j.1365-2028.1979.tb00261.x

Branch, W. R., Benn, G. A., & Lombard, A. T. (1995). The tortoises (Testudinidae) and terrapins (Pelomedusidae) of southern Africa: Their diversity, distribution and conservation. *South African Journal of Zoology*, *30*, 91–102. https://doi.org/10.1080/02541858.1995.11448377

Earl, D. A., & vonHoldt, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, *4*, 359–361. https://doi.org/10.1007/s12686-011-9548-7

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, *14*, 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Evans, D. (1988). Leopard tortoise – *Geochelone pardalis* (Bell, 1828). In R. Luxmoore, B. Groombridge, & S. Broad (Eds.), *Significant trade in wildlife: A review of selected species in CITES Appendix II. Volume 2: Reptiles and invertebrates* (pp. 5–11). Cambridge, UK: IUCN.

Excoffier, L., & Lischer, H. E. L. (2010). ARLEQUIN suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*, 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Fritz, U., Alcalde, L., Vargas-Ramírez, M., Goode, E. V., Fabius-Turoblin, D. U., & Praschag, P. (2012). Northern genetic richness and southern purity, but just one species in the *Chelonoidis chilensis* complex. *Zoologica Scripta*, *41*, 220–232. https://doi.org/10.1111/j.1463-6409.2012.00533.x

Fritz, U., Auer, M., Bertolero, A., Cheylan, M., Fattizzo, T., Hundsdörfer, A. K., … Wink, M. (2006). A rangewide phylogeography of Hermann's tortoise, *Testudo hermanni* (Reptilia: Testudines: Testudinidae): Implications for taxonomy. *Zoologica Scripta*, *35*, 531–543. https://doi.org/10.1111/j.1463-6409.2006.00242.x

Fritz, U., Daniels, S. R., Hofmeyr, M. D., González, J., Barrio-Amorós, C. L., Široký, P., … Stuckas, H. (2010). Mitochondrial phylogeography and subspecies of the wide-ranging sub-Saharan leopard tortoise *Stigmochelys pardalis* (Testudines: Testudinidae) – a case study for the pitfalls of pseudogenes and GenBank sequences.

Journal of Zoological Systematics and Evolutionary Research, *48*, 348–359. https://doi.org/10.1111/j.1439-0469.2010.00565.x

Glaubitz, J. C. (2004). CONVERT: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. *Molecular Ecology Notes*, *4*, 309–310. https://doi.org/10.1111/j.1471-8286.2004.00597.x

Goudet, J. (1995). FSTAT (Version 1.2): A computer program to calculate *F*-statistics. *Journal of Heredity*, *86*, 485–486. https://doi.org/10.1093/oxfordjournals.jhered.a111627

Greig, J. C., & Burdett, P. D. (1976). Patterns in the distribution of southern African terrestrial tortoises (Cryptodira: Testudinidae). *Zoologica Africana*, *11*, 249–273. https://doi.org/10.1080/00445096.1976.11447532

Hailey, A., & Coulson, I. M. (1996). Differential scaling of home-range area to daily movement distance in two African tortoises. *Canadian Journal of Zoology*, *74*, 97–102. https://doi.org/10.1139/z96-013

Hall, T. A. (1999). BIOEDIT: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hewitt, G. (2000). The genetic legacy of the Quaternary ice ages. *Nature*, *405*, 907–913. https://doi.org/10.1038/35016000

Hofmeyr, M. D., Boycott, R. C., & Baard, E. H. W. (2014). Family Testudinidae. In M. F. Bates, W. R. Branch, A. M. Bauer, M. Burger, J. Marais, G. J. Alexander, & M. S. de Villiers (Eds.), *Atlas and red list of the reptiles of South Africa, Lesotho and Swaziland* (pp. 70–85). Pretoria, South Africa: South African Biodiversity Institute.

Hubisz, M. J., Falush, D., Stephens, M., & Pritchard, J. K. (2009). Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, *9*, 1322–1332. https://doi.org/10.1111/j.1755-0998.2009.02591.x

Jewitt, D., Goodman, P. S., Erasmus, B. F. N., O'Connor, T. G., & Witkowski, E. T. F. (2015). Systematic land-cover change in KwaZulu-Natal, South Africa: Implications for biodiversity. *South African Journal of Science*, *111*, 1–9. https://doi.org/10.17159/sajs.2015/20150019

Joger, U., Fritz, U., Guicking, D., Kalyabina-Hauf, S., Nagy, Z. T., & Wink, M. (2007). Phylogeography of western Palaearctic reptiles – Spatial and temporal speciation patterns. *Zoologischer Anzeiger*, *246*, 293–313. https://doi.org/10.1016/j.jcz.2007.09.002

Jombart, T. (2008). ADEGENET: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*, 1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Joos, J., Kirchner, M., Vamberger, M., Kaviani, M., Rahimibashar, M. R., Fritz, U., & Müller, J. (2017). Climate and patterns of body size variation in the European pond turtle, *Emys orbicularis*. *Biological Journal of the Linnean Society*, *122*, 351–365. https://doi.org/10.1093/biolinnean/blx056

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2016). PARTITIONFINDER 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, *34*, 772–773. https://doi.org/10.1093/molbev/msw260

Leaché, A. D., Fujita, M. K., Minin, V. N., & Bouckaert, R. R. (2014). Species delimitation using genome-wide SNP data. *Systematic Biology*, *63*, 534–542. https://doi.org/10.1093/sysbio/syu018

Leaché, A. D., Wagner, P., Linkem, C. W., Böhme, W., Papenfuss, T. J., Chong, R. A., … Burbrink, F. T. (2014). A hybrid phylogenetic–phylogenomic approach for species tree estimation in African *Agama* lizards with applications to biogeography, character evolution, and diversification. *Molecular Phylogenetics and Evolution*, *79*, 215–230. https://doi.org/10.1016/j.ympev.2014.06.013

McMaster, M., & Downs, C. T. (2006). Do seasonal and behavioral differences in the use of refuges by the leopard tortoise (*Geochelone pardalis*) favor passive thermoregulation? *Herpetologica*, *62*, 37–46. https://doi.org/10.1655/04-16.1

Monadjem, A., McCleery, R. A., & Collier, B. A. (2013). Activity and movement patterns of the tortoise *Stigmochelys pardalis* in a subtropical savanna. *Journal of Herpetology*, *47*, 237–242. https://doi.org/10.1670/12-070

Perera, S. J., Ratnayake-Perera, D., & Procheş, Ş. (2011). Vertebrate distributions indicate a greater Maputaland-Pondoland-Albany region of endemism. *South African Journal of Science*, *107*, 52–66. https://doi.org/10.4102/sajs.v107i7/8.462

Petzold, A., Vargas-Ramírez, M., Kehlmaier, C., Vamberger, M., Branch, W. R., Du Preez, L., … Fritz, U. (2014). A revision of African helmeted terrapins (Testudines: Pelomedusidae: *Pelomedusa*), with descriptions of six new species. *Zootaxa*, *3795*, 523–548. https://doi.org/10.11646/zootaxa.3795.5.2

Portik, D. M., Bauer, A. M., & Jackman, T. R. (2011). Bridging the gap: Western rock skinks (*Trachylepis sulcata*) have a short history in South Africa. *Molecular Ecology*, *20*, 1744–1758. https://doi.org/10.1111/j.1365-294X.2011.05047.x

Portillo, F., Branch, W. R., Conradie, W., Rödel, M.-O., Penner, J., Barej, M. F., … Zassi-Boulou, A.-G. (2018). Phylogeny and biogeography of the African burrowing snake subfamily Aparallactinae (Squamata: Lamprophiidae). *Molecular Phylogenetics and Evolution*, *127*, 288–303. https://doi.org/10.1016/j.ympev.2018.03.019

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*, 945–959.

Puechmaille, S. J. (2016). The program STRUCTURE does not reliably recover the correct population structure when sampling is uneven: Subsampling and new estimators alleviate the problem. *Molecular Ecology Resources*, *16*, 608–627. https://doi.org/10.1111/1755-0998.12512

Rambaut, A., Suchard, M. A., Xie, D., & Drummond, A. J. (2014). *TRACER v1.6*. Retrieved from https://beast.bio.ed.ac.uk/Tracer

Randi, E. (2008). Detecting hybridization between wild species and their domesticated relatives. *Molecular Ecology*, *17*, 285–293. https://doi.org/10.1111/j.1365-294X.2007.03417.x

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MRBAYES 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*, 539–542. https://doi.org/10.1093/sysbio/sys029

Rosenberg, N. A. (2004). DISTRUCT: A program for the graphical display of population structure. *Molecular Ecology Notes*, *4*, 137–138. https://doi.org/10.1046/j.1471-8286.2003.00566.x

Schoeman, F., Newby, T. S., Thompson, M. W., & van den Berg, E. C. (2013). South African national land-cover change map. *South African Journal of Geomatics*, *2*, 94–105.

Stamatakis, A. (2006). RAxML-VI-HPC: Maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*, 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Tilbury, C. R., & Tolley, K. A. (2009). A new species of dwarf chameleon (Sauria; Chamaeleonidae, *Bradypodion* Fitzinger) from KwaZulu Natal South Africa with notes on recent climatic shifts and their influence on speciation in the genus. *Zootaxa*, *2226*, 43–57.

TTWG [Turtle Taxonomy Working Group] (2017). *Turtles of the world. Annotated checklist and atlas of taxonomy, synonymy, distribution, and conservation status* (8th ed.). Lunenburg, MA: Chelonian Research Foundation and Turtle Conservancy (Chelonian Research Monographs 7).

Vamberger, M., Hofmeyr, M. D., Ihlow, F., & Fritz, U. (2018). In quest of contact: phylogeography of helmeted terrapins (*Pelomedusa galeata*, *P. subrufa* sensu stricto). *PeerJ*, *6*, e4901. https://doi.org/10.7717/peerj.4901

van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., & Shipley, P. (2004). MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, *4*, 535–538. https://doi.org/10.1111/j.1471-8286.2004.00684.x

Vargas-Ramírez, M., Vences, M., Branch, W. R., Daniels, S. R., Glaw, F., Hofmeyr, M. D., … Fritz, U. (2010). Deep genealogical lineages in the widely distributed African helmeted terrapin: Evidence from mitochondrial and nuclear DNA (Testudines: Pelomedusidae: *Pelomedusa subrufa*). *Molecular Phylogenetics and Evolution*, *56*, 428–440. https://doi.org/10.1016/j.ympev.2010.03.019

Wagner, P., Leaché, A. D., & Fujita, M. K. (2014). Description of four new West African forest geckos of the *Hemidactylus fasciatus* Gray, 1842 complex, revealed by coalescent species delimitation. *Bonn Zoological Bulletin*, *63*, 1–14.

Weinell, J. L., & Bauer, A. M. (2018). Systematics and phylogeography of the widely distributed African skink *Trachylepis varia* species complex. *Molecular Phylogenetics and Evolution*, *120*, 103–117. https://doi.org/10.1016/j.ympev.2017.11.014

Werner, Y. L., Koroloker, N., Sion, G., & Göçmen, B. (2016). Bergmann's and Rensch's rules and the spur-thighed tortoise (*Testudo graeca*). *Biological Journal of the Linnean Society*, *117*, 796–811. https://doi.org/10.1111/bij.12717

Wimberger, K., Armstrong, A. J., & Downs, C. T. (2009). Can rehabilitated leopard tortoises, *Stigmochelys pardalis*, be successfully released into the wild? *Chelonian Conservation and Biology*, *8*, 173–184. https://doi.org/10.2744/ccb-0773.1

Wüster, W., Chirio, L., Trape, J.-F., Ineich, I., Jackson, K., Greenbaum, E., … Broadley, D. G. (2018). Integration of nuclear and mitochondrial gene sequences and morphology reveals unexpected diversity in the forest cobra (*Naja melanoleuca)* species complex in Central and West Africa (Serpentes: Elapidae). *Zootaxa*, *4455*, 68–98. https://doi.org/10.11646/zootaxa.4455.1.3

SUPPORTING INFORMATION

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