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Tortoise forensics: conservation genetics of the leopard tortoise *Stigmochelys pardalis* in southern Africa

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Abstract. Sub-Saharan Africa harbours an outstanding diversity of tortoises of which the leopard tortoise *Stigmochelys pardalis* is the most widespread. Across its' range the species is impacted by habitat transformation, over-collection for human consumption and the pet trade, road mortality, and electrocution by electric fences. Most leopard tortoises in southern Africa are nowadays restricted to reserves and private farms. So far confiscated tortoises are frequently released into a nearby reserve without knowledge on their area of origin. This is problematic, as it has been demonstrated that the leopard tortoise harbours five distinct mitochondrial lineages, of which three occur in the southern portion of the species' distributional range (South Africa, Namibia, and Botswana). Using 14 microsatellite loci corresponding to 270 samples collected throughout southern Africa, we found a clear substructuring in the north constituting four clusters (western, central, north-eastern, and eastern). Genetic diversity was particularly high in the north-east and decreased towards the south. In addition, we found a significant size difference between the studied populations. Our basic morphological analysis showed that tortoises from the southern cluster tend to grow bigger than tortoises from the north. We established a comprehensive genetic database for South Africa and Namibia that can serve as a conservation management tool for the assignment and potential release of translocated or seized leopard tortoises based on genetic affiliation.

Key words. Conservation management, management units, microsatellites, Namibia, pet trade, sub-Saharan Africa, South Africa, Testudinidae.

Introduction

Tortoises and turtles are among the most imperilled biota on the planet, with approximately 60.4% of 356 species threatened or recently extinct (Lovich et al. 2018, Turtle Conservation Coalition 2018). Of 121 tortoises that existed since the Pleistocene, 69 (57%) already disappeared (Lovich et al. 2018). The impacts that are responsible for this alarming situation include habitat destruction, climate change, unsustainable overexploitation for human consumption as well as for the international pet trade, and the introduction of pathogens (Wimberger et al. 2011, Lovich et al. 2018). These also apply to the leopard tortoise *Stigmochelys pardalis* (Bell, 1828) (Evans 1988). Between 1987 and 1991, *S. pardalis* accounted for 76% of tortoises exported from Africa for the international pet trade and declines in some areas have been attributed to unsustainable collection and trade (Branch 2012). Over the last five years 171,444 living *S. pardalis*, have been exported from Africa for commercial purposes (CITES trade database 2020). As a consequence, the once widespread species is now considered threatened in some regions, while it has already disappeared from few areas in eastern and western South Africa (Hofmeyr & Baard 2014). Natural bush fires and deliberate fires set to promote regrowth and increase forage quality for livestock account for a major proportion of injuries and mortalities (Kabugumila 2001), while severe droughts and road mortality also contribute to population decline (Boycott & Bourquin 2000). Today the vast majority of tortoises, including *S. pardalis*, are found on privately owned fenced farm land and reserves. Unfortunately, their large body size and highly domed carapace make leopard tortoises susceptible to being killed by electric fences (Burger & Branch 1994, Arnot & Molteno 2017, Macray 2017).

Recent molecular genetic analyses revealed the species to constitute five distinct mitochondrial lineages (Spitzweg et al. 2019). One is distributed in the south, a second in the north-west, and a third in the north-east of southern Africa, while two additional lineages are distributed further north (Spitzweg et al. 2019). Using microsatellites Spitzweg et al. (2019) revealed the southern mitochondrial clade to match with a well-defined southern nuclear cluster, whilst the north-western and north-eastern clades of southern Africa

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corresponded to another nuclear cluster with three subclusters. Besides the genetic diversity, the leopard tortoise expresses considerable geographic variation in body size, shell shape, and colouration. Loveridge and Williams (1957) attributed these differences to subspecies variation. However, the distribution of the mitochondrial lineages or the respective nuclear clusters does not match with the recognized subspecies (Fritz et al. 2010, Spitzweg et al. 2019). These findings demonstrate the importance of the application of conservation genetic techniques to preserve the genetic diversity of the species. While previous studies suffered from incomplete sampling in some areas (Spitzweg et al. 2019) our present study adds crucial data from northern South Africa completing these sampling gaps.

We examined the population structure and reveal genetic differentiation of *S. pardalis* using fine scale analyses of nuclear microsatellite markers. We deliver an essential database for future conservation relocation practices that will improve the conservation management and preservation of the genetic diversity of *S. pardalis*.

Material and methods

Sampling, selection of microsatellite loci, and general data evaluation strategy

In total, 270 leopard tortoises from all across South Africa, adjacent Namibia, and southern Mozambique were studied (Supplementary document S1). These include 204 specimens previously sampled (Spitzweg et al. 2019). For genetic analyses, a small amount of blood or tissue was collected from live tortoises in accordance with methods approved by the Ethics Committee of the University of the Western Cape (Ethics Reference Number (AR 19/4/1). All specimens were subsequently released at their respective collection site. Blood samples were preserved on FTA classic cards (Whatman, GE Healthcare, Munich, Germany) and stored at room temperature. For most tortoises sampled in South Africa, the carapace length (CL) was measured to the nearest 1 mm using a measuring tape. Fieldwork and sampling in South Africa was permitted by the Limpopo Provincial Government (ZA/LP/91608), the Department of Environmental Affairs, Biodiversity Northern Cape Province (245/2015), Ezemvelo KZN Wildlife (OP 139/2017), and Biodiversity North West Province (NW 6124/10/2018).

The same 14 nuclear microsatellite loci were targeted as in our previous study on *S. pardalis* (Spitzweg et al. 2019). For DNA extraction, PCR, and genotyping information see Spitzweg et al. 2019.

Data analyses

For conservation genetic purposes microsatellite data of 270 leopard tortoises were analysed using the unsupervised Bayesian clustering approach of STRUCTURE 2.3.4 (Pritchard et al. 2000, Hubisz et al. 2009), applying the admixture model and correlated allele frequencies. STRUCTURE searches for partitions which are, as far as possible, in Hardy-Weinberg equilibrium and linkage equilibrium. Unsupervised analyses were selected because this approach clusters samples strictly according to their genetic information, without making presumptions about population structuring (e.g. geographic distances, sampling sites). All calculations were performed for $K = 1–10$, and the most likely number of clusters (K) was determined using the ΔK method (Evanno et al. 2005) as implemented in the software STRUCTURE HARVESTER (Earl & von Holdt 2012) and mean Ln probabilities. Calculations were repeated 10 times for each K using a MCMC chain of 750,000 generations and a burn-in of 250,000 generations. Population structuring and individual admixture were visualized using DISTRUCT 1.1 (Rosenberg 2004). Individuals with a membership proportion below 80% were considered to have admixed ancestries (Barilani et al. 2007, Randi 2008). STRUCTURE is prone to bias from uneven sample sizes (Puechmaille 2016) and typically detects only the uppermost hierarchical level of population differentiation (Evanno et al. 2005). Therefore, the analysis was repeated for subsamples corresponding to previously identified clusters but excluding admixed individuals (Supplementary document S1). In addition, STRUCTURE was repeated for each of the respective subclusters of the northern cluster.

Further, Principal Component Analyses (PCAs) were performed to examine population structuring using microsatellite data and the package ADEGENET (Jombart 2008) for Cran R 3.2.3 (R Development Core Team 2015). PCAs are less sensitive to sample size and are independent from population genetic assumptions (Puechmaille 2016). Two distinct PCAs were executed. In the first analysis, all 270 individuals were included, and symbols were coloured after STRUCTURE clusters (north, south, admixed). The second PCA included only the tortoises from the northern STRUCTURE cluster (151 individuals), with symbol colours corresponding to STRUCTURE subclusters (N1, N2, N3, N4, Admixed).

Diversity within and divergence between population clusters

Diversity and divergence were estimated for population clusters revealed by STRUCTURE. A frequency table for microsatellite alleles was produced using COVERT 1.31 (Glaubitz 2004). ARLEQUIN 3.5.2.1 (Excoffier & Lischer 2010) was applied to infer locus specific observed (H_O) and expected (H_E) heterozygosity and to perform AMOVAs (10,000 permutations) for microsatellite clusters revealed by STRUCTURE. FSTAT 2.9.3.2 (Goudet 1995) was used to compute values for locus specific allelic richness.

Morphology

Data on carapace length (CL) of 137 adult tortoises was analysed using the packages DPLYR (Wickham et al. 2019),

TIDYR (Wickham & Henry 2019), and MULTCOMP (Hothorn et al. 2008) and visualized using the packages GGPLOT2 (Wickham 2016), GGTHEMES (Arnold 2019), and KNITR (Xie 2019) for Cran R 3.2.3. A two-way ANOVA was applied using main genetic clusters identified by STRUCTURE (south/north) and sex as fixed effects to test for significant difference in carapace length (response variable). Subsequently, differences were identified using a Tukey HSD test (Dubitzky et al. 2013).

Results

Population structuring

Using all samples (N = 270) STRUCTURE revealed two clusters as the best solution (Supplementary documents S2, S3), one corresponding to southern sampling sites and the other to more northern sites within South Africa, adjacent Namibia, and southern Mozambique. Admixed ancestry was only observed in a few sites in the Provinces Western Cape, Northern Cape, and North West (grey coloured section of pie charts; Supplementary document S2). In addition, tortoises from both clusters were found to live in close proximity to each other in several localities (Supplementary document S2).

Additional STRUCTURE runs for each of the two clusters excluding admixed individuals revealed $K = 1$ as the best solution for the southern cluster but suggested further structuring for the northern subset ($K = 4$; Fig. 1, Supplementary document S1). Thus, the northern subset (Fig. 1b: N1) included a cluster corresponding to samples from Namibia and the South African Northern Cape Province. Two additional clusters (Fig. 1b: N2, N4) contained samples from central-southern Africa with a clear north–south separation. The fourth cluster (Fig. 1b: N3) corresponded to a smaller area in the north-east of South Africa, and southern Mozambique. A high number of tortoises from central and north-eastern South Africa showed admixed ancestries (Fig. 1b).

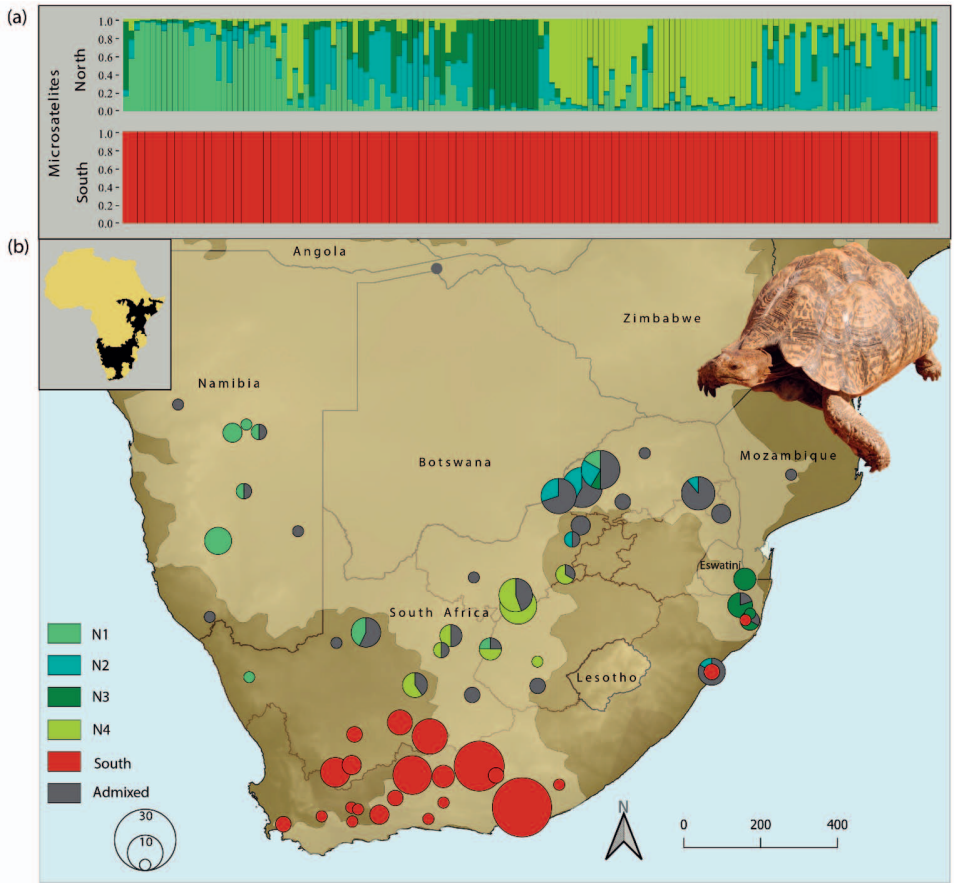


Figure 1. a) Genotypic clustering of the two subsets (northern and southern, excluding admixed individuals) as inferred by STRUCTURE. The runs with the best probability values are shown. Distinct clusters are colour coded corresponding to STRUCTURE inferred clusters (see b). Within each cluster an individual is represented by a vertical bar that reflects its ancestry. Mixed ancestry is indicated by differently coloured segments corresponding to inferred genetic percentages of the respective cluster. b) The distribution range of *S. pardalis* is displayed as shaded area according to Baker et al. 2015 and Spitzweg et al. 2019. Individual sampling sites were lumped for clarity. Symbol sizes refer to the respective sample size. Slices represent percentages of individuals with mixed ancestries or conflicting cluster assignments. For original sites see Supplementary document S1. Inset shows the whole putative distributional range (black) of *Stigmochelys pardalis* according to TTWG (2017).

STRUCTURE runs for each of the four clusters of the northern subset revealed $K = 1$ as the best solution in all four runs (Supplementary document S4). Therefore, no additional substructure could be found in the north.

Principal Component Analyses (PCAs) supported the results of the STRUCTURE runs and the near absence of admixture between tortoises of the northern and southern cluster, but indicated a high level of admixture between the four clusters of the northern subset (Fig. 2).

Diversity within and divergence between STRUCTURE clusters

In agreement with its nuclear genomic substructuring (Supplementary document S1, 2), genetic diversity was higher among tortoises from the northern cluster than it was in the south (Table 1). However, despite similar sample sizes, the northern cluster had a higher haplotype diversity. For microsatellites the average number of alleles per locus ($n_{\hat{A}} = 18.6$) in the north was twice as high as for the southern cluster ($n_{\hat{A}} = 9.3$). The northern cluster had almost twenty times more private alleles ($n_p = 138$) compared to the southern cluster ($n_p = 7$). The observed pattern was also reflected by a higher heterozygosity in the north ($H_O = 0.709$) than in the south ($H_O = 0.549$).

When comparing the four clusters of the northern subset (N1–N4, Fig. 1) the highest genetic diversity was observed within subcluster N2. With an average of 10.8, subcluster N2 had the highest number of alleles per locus, followed by subcluster N4 (9.6), N1 (8.4), and N3 (6.8). In concordance, subcluster N2 also had the highest number of private alleles ($n_p = 28$) while subcluster N3 had the lowest ($n_p = 8$).

The two main clusters (northern and southern, Supplementary document S2) differed by an F_{ST} value of 0.145 ($p < 0.05$) while the northern subclusters N1–N4 (Fig. 1) differed by substantially lower F_{ST} values ranging from 0.039 to 0.107 (Table 2). In accordance, an AMOVA for the northern and southern clusters revealed 14.49% of the molecular variance to occur among and 85.51% within the two clusters. Regarding the northern subclusters N1–N4 (Fig. 1) only 6.24% of the molecular variance was found to occur among, while 93.76% of variance occurred within subclusters.

Table 1. Genetic diversity of STRUCTURE clusters excluding admixed individuals. n – number of individuals; n_A – number of alleles; $n_{\hat{A}}$ –average number of alleles per locus; n_p – number of private alleles; AR – allelic richness; H_O – average observed heterozygosity; H_E – average expected heterozygosity.

Cluster	n	n_A	$n_{\hat{A}}$	n_p	AR	H_O	H_E
North	151	130	18.6	138	17.611	0.709	0.803
South	110	261	9.3	7	9.237	0.549	0.622
N1	19	117	8.4	17	7.245	0.748	0.784
N2	15	151	10.8	28	9.954	0.773	0.841
N3	12	95	6.8	8	6.786	0.685	0.721
N4	28	134	9.6	22	9.297	0.675	0.743

Morphology

A two-way ANOVA was conducted that examined the effect of genetic cluster and sex on body size of 137 *S. pardalis*. The northern cluster was composed of 42 males (mean CL = 34.3 ± 7.9 cm SD) and 28 females (mean CL = 40.8 ± 9.3 cm SD) whereas the southern cluster contained 35 males (mean CL = 45.4 ± 7.3 cm SD) and 32 females (mean CL 42.7 ± 10.9 cm SD). We found a statistically significant difference in carapace length between clusters ($n_{southern} = 67$, $n_{northern} = 70$; two-way ANOVA: $F(1) = 22.79$, $p = > 0.001$) but not in sexes ($n_{males} = 77$, $n_{females} = 60$; two-way ANOVA: $F(1) = 1.50$, $p = 0.236$). A subsequent Tukey HSD post hoc test based on the results of the ANOVA interaction between location and sex ($F(1) = 9.04$, $p = > 0.01$) revealed

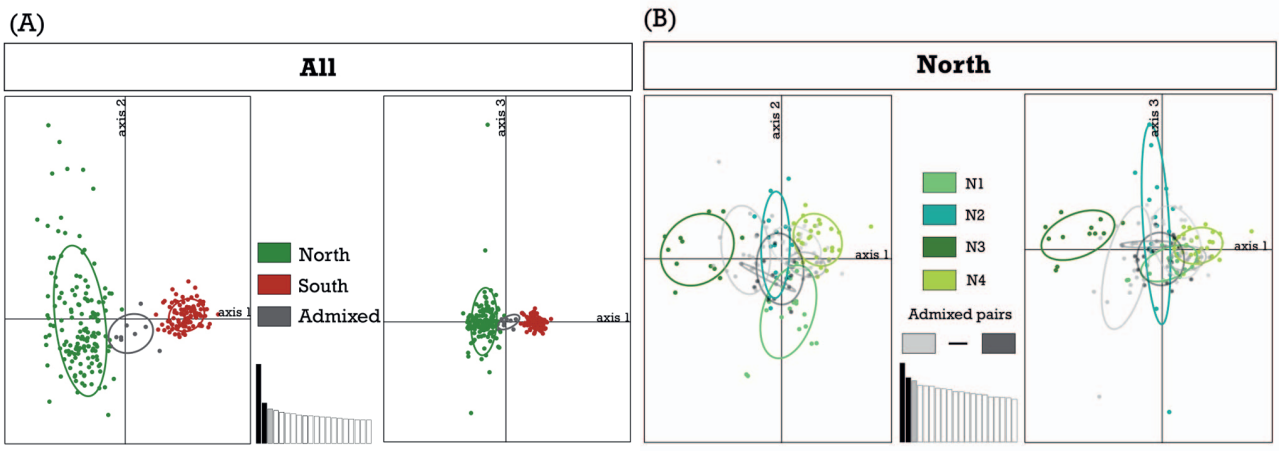


Figure 2. Principal Component Analyses (PCAs) using microsatellite data of A) 270 individuals (all samples) and B) 151 individuals of the northern cluster coloured according to STRUCTURE results. For A) axes 1–3 explain 10.25%, 5.22%, and 4.47% of variance; for B) axes 1–3 explain 6.45%, 5.27% and 5.01%, respectively. Ovals indicate 95% confidence intervals.

Table 2. Fixation indices (F_{ST} values) for microsatellite data of the northern subclusters N1–N4 (Fig. 2). All values are statistically significant ($p < 0.05$).

	N1	N2	N3
N2	0.039	–	
N3	0.093	0.051	–
N4	0.059	0.031	0.107

that male tortoises from the southern cluster were significantly larger (Tukey HSD test, $p < 0.001$) than male tortoises from the northern cluster (Fig. 3). In contrast no significant difference was observed between northern and southern female tortoises (Tukey HSD test $p = 0.84$). Within the northern cluster, females were found to be significantly larger (Tukey HSD test $p = 0.02$) than males while no intersexual difference could be demonstrated between females and males from the southern cluster (Tukey HSD test $p = 0.59$).

Discussion

In recent years wildlife forensics became a key means of enforcing legislation surrounding the illegal trade in protected and threatened species (Mucci et al. 2014, Ogden & Linacre 2015), which also holds true in South Africa. However, the identification of the geographic origin of seized animals is based on our ability to assign a sample to a particular population. This requires the source population to be sufficiently genetically distinct from other populations and is usually reliant on the existence of population data from multiple geographic areas (Ogden & Linacre 2015). Our study provides a comprehensive genetic database for wildlife forensics of translocated or seized leopard tortoises in southern Africa developed in collaboration with the South African National Biodiversity Institute (SANBI) and the Gauteng Department of Agriculture and Rural Development (GDARD). Compared to Spitzweg et al. (2019), our sampling covers the whole distribution range of *S. pardalis* in South Africa, closing previous sampling gaps in central and northern South Africa. Here, we confirm the pronounced north–south genetic differentiation in southern African leopard tortoises reported by Spitzweg et al. (2019) (Supplementary document S2) and demonstrate the distribution of the southern cluster to stretch further to the north than anticipated. According to Baker et al. (2015) there is a major distribution gap for leopard tortoises in the western part of the Northern Cape Province and the northwestern part of the Western Cape Province. However, we confirmed *S. pardalis* from the southern parts of the Northern Cape Province, well within the proposed distributional gap, and reveal these tortoises to belong to the southern cluster (Supplementary document S2). Even though many herpetologists believe that *S. pardalis* has been introduced in western South Africa (Hofmeyr & Baard 2014), our results and field observations (confirming viable population) support a natural occurrence in this region. In addition, we investigated the geographic variation in body size due to the observations of Loveridge and Williams (1957). We demonstrate a pronounced geographic variation in carapace length of male tortoises between the two major microsatellite clusters from the north and south (Fig. 3). Males of the southern cluster reach carapace lengths of up to 65 cm (mean CL = 45.4 ± 7.3 cm SD) while males from the northern cluster stay significantly smaller (max CL = 50 cm, mean = 34.3 ± 7.9 cm SD; Fig. 3). Although *S. pardalis* females generally grow larger than males (Branch 2012) we could not confirm a significant difference in carapace length between southern and northern females. Most probably a combination of environmental factors such as aridity, seasonality, and food availability are responsible for shaping the morphological variation in *S. pardalis*. Similarly, Loehr et al. (2007) reported aridification to be correlated with lower average and maximum body size in females of the world's smallest tortoise, *Chersobius signatus*.

It is important to determine the geographic origin of seized leopard tortoises, as they can be locally adapted to a specific climate and might not survive non-analogous environmental conditions as demonstrated by Wimberger et al. (2009). Our basic morphological analyses show that body size is not necessarily a proxy for geographic origin, even though considerable geographic variation was expected (Loveridge & Williams 1957). Furthermore, releasing animals from different genetic clusters in inappropriate re-

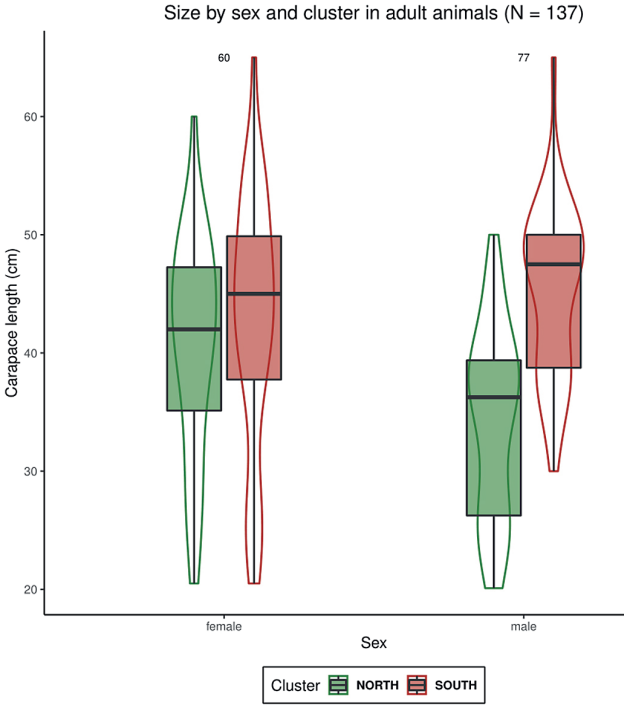


Figure 3. Differences in carapace length of females and males from the major microsatellite clusters (south and north). Boxplots show median, minimum, and maximum. Violin plots show the probability density of data.

gions can result in extinction or genetic pollution of local gene pools (Butler 1994, Fitzpatrick & Shaffer 2007). A similar case was suspected in France where certain populations of the European pond turtle *Emys orbicularis* appear to be polluted with genes from Italian populations (Vamberger et al. 2015), but the impact has not been investigated. While our results are in agreement with Spitzweg et al. (2019) in finding no substructuring in the southern cluster (Fig. 1), our increased sampling revealed the northern cluster to harbour an additional fourth subcluster (N4) instead of three as previously assumed (Spitzweg et al. 2019). The additional subcluster occurs in the eastern part of the Northern Cape, throughout the Free State, and in the southern part of the North West Province of South Africa (Fig. 1). A high number of tortoises sampled in central and northeastern South Africa show admixed ancestries (Fig. 1; grey coloured sections of pie charts). The reason for this may be the close proximity to the O. R. Tambo International Airport in Johannesburg, where tortoises are frequently confiscated and released nearby. The phylogeographic pattern of *S. pardalis* parallels with that of the puff adder *Bitis arietans* (Barlow et al. 2013) and freshwater terrapins of the genus *Pelomedusa* (Vamberger et al. 2018) supporting a natural distribution pattern. The distribution of the southern cluster of *S. pardalis* corresponds well with subclade Ic of *Pelomedusa galeata*, while the northern subclusters N3 and N4 correspond to *Pelomedusa* subclades Ib and Ia (Vamberger et al. 2018). The northern subclusters N1 and N2 mirror the distribution of *Pelomedusa subrufa* (Vamberger et al. 2018). *Stigmochelys pardalis* is one component of the South African biota whose phylogeographic structure was shaped by physiography and climate, as has also been demonstrated for *Pelomedusa* (Vamberger et al. 2018). To preserve the genetic diversity and geographic structure of *S. pardalis*, we propose five management units for southern Africa. These correspond to the two major microsatellite clusters and the northern subclusters N1–N4 (Fig. 1). Using our genetic database, the confiscated tortoises should be genetically tested and assigned to one of the five microsatellite clusters before being considered for reintroduction into the wild. If assignment to a genetic cluster is successful, release in a safe location within the distributional boundaries of the correct genetic cluster can be considered. However, a suitable release protocol should include a thorough pre-release health screening to prevent the introduction of pathogens into wild populations (Wimberger et al. 2011). This study represents the foundation for further conservation management decisions and future conservation actions for leopard tortoises in southern Africa.

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Supplementary data

The following data are available online:

Supplementary document S1. Samples of *Stigmochelys pardalis* used in the present study.

Supplementary document S2. Genotypic clustering of 270 leopard tortoises (*Stigmochelys pardalis*) from 66 sites.

Supplementary document S3. Delta K and Ln P values for STRUCTURE runs for the complete data set and for each of the two clusters, South and North, separately.

Supplementary document S4. Delta K and Ln P values for STRUCTURE runs for each northern subcluster (N1–N4) separately.