# Genetic evidence for spatial structuring in a continuous African elephant (*Loxodonta africana*) population

**Alida de Flamingh, Catherine L. Sole, Rudi J. van Aarde**

**Abstract** Earlier studies on savannah elephants (*Loxodonta africana*) investigated the genetic structure of fragmented or isolated populations. Contrastingly, this study aimed to determine if there was genetic evidence for spatial structuring in a continuous elephant population in the Kavango-Zambezi Transfrontier Conservation Area (KAZA-TFCA). We sequenced one mtDNA gene region for 88 individuals and genotyped 100 individuals for ten nuclear microsatellite loci. Bayesian Clustering Algorithms in Geneland identified groups of genetically similar individuals. An analysis of molecular variance determined if these groups (sub-populations) were significantly differentiated. We identified geographic areas with high genetic divergence (genetic barriers) between samples using a GIS landscape genetic toolbox. There were three significantly differentiated mtDNA sub-populations ($F_{st} = 0.787$) and two nDNA sub-populations that were not significantly differentiated (Fst $= -0.02$; Rst $= -0.045$), implying obstructed mtDNA, but high nDNA gene flow across the study region. The KAZA-TFCA population has a genetic diversity (mtDNA pairwise number of differences ($\pi$) $= 2.59$; nDNA mean alleles/locus and $H_e = 7.5, 0.71$) higher than other southern African populations, and inter-population movements may be responsible for maintaining this genetic diversity. We discount anthropogenic and geographic barriers as the primary drivers of genetic structuring in the KAZA-TFCA population and suggest that future studies should consider the influence of intrinsic factors (resource dependencies and social variables that limit movement) when investigating the genetic structure of elephant populations. We recommend continued support for conservation initiatives that aim at maintaining and restoring connectivity between populations, which in so doing may ensure inter-population gene flow and uphold the current genetic state of the KAZA-TFCA population.

**Keywords** Genetic structure · Continuous population · Kavango-Zambezi · Transfrontier conservation area · Degraded DNA · Faecal DNA · *Loxodonta africana*

**Data Accesibitlity**: DNA sequences: Genbank accession numbers KC179715–KC179727. Step-based extraction protocol—Supporting Information.

A. de Flamingh (✉) · R. J. van Aarde (✉)
Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Hatfield, Private Bag X20, Pretoria 0028, South Africa
e-mail: adeflamingh@gmail.com

R. J. van Aarde
e-mail: rjvaarde@zoology.up.ac.za

C. L. Sole
Scarab Research Group, Department of Zoology and Entomology, University of Pretoria, Hatfield, Private Bag X20, Pretoria 0028, South Africa

## Introduction

The fragmentation of populations into discontinuous and isolated entities is a global reality, often driven by anthropogenic landscape transformation, habitat loss and over-exploitation (e.g. in amphibians (Dixo et al. 2009), birds (Segelbacher et al. 2003), mammals (Proctor et al. 2005) and reptiles (Gibbon et al. 2000)). The African savannah elephant (*L. africana*) typifies this scenario in that the over-exploitation (e.g. Whitehouse and Harley 2001; Okello et al. 2008a) and the compression of remnant

populations into protected areas (Laws 1970; Melton 1985; van Aarde and Jackson 2007) have fragmented a once continuous population (e.g. Barnes 1999; Georgiadis et al. 1994) into isolated units. Landscape transformation (Hoare and du Toit 1999; Osborn and Parker 2003; Graham et al. 2009) and the obstruction of dispersal opportunities (e.g. van Aarde and Jackson 2007; Loarie et al. 2009) further enhanced isolation and fragmentation, and may have contributed to the decline in elephant numbers and their distributional ranges. These are some of the factors that have contributed to the listing of the African elephant as an Appendix I species in all countries but Botswana, Namibia, Zimbabwe and South Africa, where is it listed as Appendix II by the Convention for the International Trade of Endangered Species (CITES) and as a vulnerable species in the red data list of the International Union for the Conservation of Nature (IUCN).

Developing landscape linkages to negate the negative consequences of fragmentation and isolation [e.g. through the establishment of Transfrontier Conservation Areas (TFCAs)] makes conservation sense only if these landscape linkages would improve dispersal opportunities and gene flow. Published information on gene flow and the genetic structure of elephant populations is mostly available for relatively isolated remnants of previously continuous populations (e.g. isolated populations within countries: Archie et al. (2006, 2008); fragmented populations within countries: Nyakaana and Arctander (1999), Okello et al. (2008a, b), and Epps et al. (2013); and fragmented populations among countries: (Georgiadis et al. (1994), Comstock et al. (2002), Eggert et al. (2002), Nyakaana et al. (2002), and Ishida et al. (2013)). Most of these earlier studies (e.g. Comstock et al. 2002; Nyakaana et al. 2002; Okello et al. 2008b; Ahlering et al. 2012a, b) assume a priori populations or sub-populations, where the genetic characteristics of pre-defined populations and sub-populations are compared. Consequently, the findings of these studies may not hold for elephants that form part of a continuous population where their distributional range precludes the a priori delineation of sub-populations.

The elephants that we studied in the Kavango-Zambesi Transfrontier Conservation Area (KAZA-TFCA) form part of such a widespread population (Chase and Griffin 2006; Chase 2007). The elephant populations in the five countries that form part of the KAZA-TFCA include approximately 130,000 elephants in northern Botswana, 16,000 in north-eastern Namibia, and 50,000 in north-western Zimbabwe (inferred from Chase and Griffin 2005, Chase 2011, and The Elephant Database 2011). The KAZA-TFCA includes 36 protected areas (van Aarde and Jackson 2007) that form part of a larger conservation network spanning across $\sim$278,000 km$^2$ (Metcalfe and Kepe 2008). The elephant population in this region is one of the few that has not been spatially fragmented, where anthropogenic disturbances may have altered some local elephant movements (Chase and Griffin 2009; Roever et al. 2013a) but apparently do not limit their movements across international borders (Chase and Griffin 2006; Roever et al. 2013b). Additionally, these elephants have not been exposed to management interferences such as culling. Although elephant populations on the periphery of the KAZA-TFCA have been subjected to intensive poaching [as suggested by the largest ivory seizure on record in 2002 which shows illegal ivory originating in Kafue National Park (Wasser et al. 2007)], the elephant population in the central core area of the KAZA-TFCA has not faced the intensities of poaching experienced by elephant populations elsewhere in Africa (Burn et al. 2010). The KAZA-TFCA elephant population may therefore be considered as a population structured by natural forces compared to the apparent artificial structure of elephant populations that live in fragmented and isolated landscapes. We expect that genetic structuring and gene flow in this population would be the outcomes of natural rather than anthropogenic forces. Determining the genetic structure and the gene flow across the KAZA-TFCA could inform us on the benefits that might be accrued through efforts that aim at restoring historical distributional ranges. This may be especially important for more isolated populations that are structured in response to human induced isolation through the confinement of distributional ranges, where future conservation initiatives may aim at linking landscapes to restore historical distributional ranges.

We used molecular techniques to determine if elephants in the KAZA-TFCA are structured into genetically distinct sub-populations, and if so, whether these sub-populations had gender specific genetic structures by comparing mitochondrial DNA (maternally inherited) and nuclear DNA (biparentally inherited). We hypothesised that (i) there would be high mtDNA and nDNA gene flow across the entire region due to the lack of dispersal barriers, and (ii) the patterns of structuring implied by mtDNA and nDNA will differ as a consequence of gender differences in spatial use (Stokke and du Toit 2002; Shannon et al. 2006) and social behaviour (e.g. Archie et al. 2006, 2008) of male and female elephants.

## Materials and methods

### DNA collection and extraction

Fresh elephant faecal samples were collected from 120 locations across the KAZA-TFCA in October 2010 (Fig. 1). To minimise the possibility of collecting more than one sample from a single individual, samples were once off collected at least 5 km apart along a unidirectional

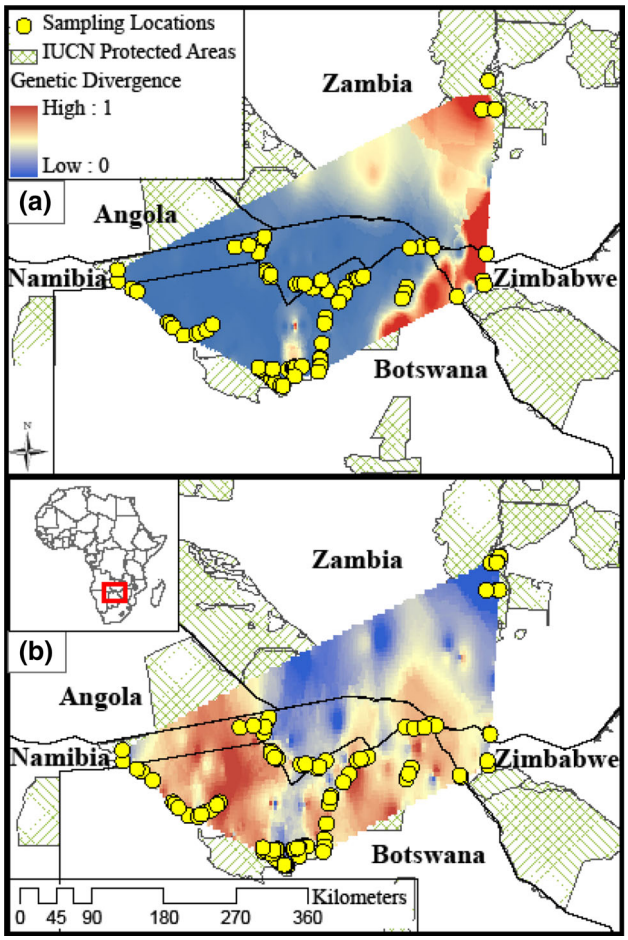


**Fig. 1** The mtDNA genetic divergence map **a** showed definite genetic discontinuities across the landscape (indicated in *red*), with samples in the north and east of the study area being separated from each other and from the rest of the study region, while the nDNA genetic divergence map **b** showed little evidence for any explicit genetic discontinuities. The *yellow circles* represent sampling locations for mtDNA (n = 88) and nDNA (n = 100), and the green cross-hatched areas show IUCN category I-VI Protected Areas. (Color figure online)

transect. We minimised possible cross-sample and environmental contamination in field by collecting from only one dung pile at a specific location, using a new set of sterile latex gloves for each collection, immediately sealing the collection jar with a piece of parafilm after collection, and placing the collection jar in two zip-locked bags after collection. To increase our chances of collecting viable, relatively intact/amplifiable DNA, we ensured that each faecal sample was fresh to the extent that it still had wet mucus from which to collect a small sample (2–3 cm diameter/golf ball size). Samples were stored at room temperature in plastic jars containing absolute ethanol (see Fernando et al. (2003) and Vidya and Sukumar (2005)).

Approximately 2 ml of the homogenised dung and ethanol mixture was centrifuged for 30 min, after which the supernatant was discarded. This process was repeated three times, and the remaining pellet was used as starting material for DNA extraction. We used the Qiagen DNA Stool Mini Kit (Southern Cross Biotechnology, Cape Town, SA) to extract DNA following a modified protocol for isolation of DNA from stools for human DNA analysis. Modifications to the protocol included increasing the vortex, centrifuge and incubation times, and decreasing the final elution buffer to 100 µl to maximise the eluted DNA concentration (see Supplementary Data for the modified protocol). We minimised cross-sample contamination during the DNA extraction procedure by extracting DNA from a maximum of four samples at a time and working in a specific low-copy extraction laboratory. It should also be noted that, at the time, there were no other researchers working with mammalian samples in the DNA extraction laboratory.

## DNA amplification and genotyping

We amplified a 509 nucleotide base pair (bp) mitochondrial fragment of the D-Loop control region with primers LafCR1 and LafCR2 (Nyakaana and Arctander 1999) and genotyped individuals using ten fluorescently labelled nuclear microsatellite loci (Table 1). Polymerase chain reactions (PCRs) for both mtDNA amplification and nDNA microsatellite amplification were done using the Qiagen Multiplex PCR kit (Southern Cross Biotechnology, Cape Town, SA) and the Veriti 96 well Thermal Cycler from Applied Biosystems (Foster City, CA). We minimized the possibility of contamination by working beneath a laminar flow system (Vivid Air Flow Bench—4,006 V) with an ultra violet light that is used to decontaminate the workbench before each PCR setup, in a dedicated no-DNA PCR-setup laboratory using only filtered pipette tips. DNA was added to the PCR reagents in a separate laboratory. The DNA-loaded samples were transferred to a separate facility for PCR cycling and amplification and all PCR and fragment analyses included a no-template control to test for contamination. The cycling conditions for mtDNA and microsatellite amplification followed the Standard Multiplex PCR and Microsatellite/Short Amplicons PCR protocols of the Qiagen Multiplex PCR kit respectively. Successfully amplified samples were identified through gel electrophoresis where PCR products were combined with a loading buffer (10 mM Tris–HCl (pH 7.6), 0.03 % bromophenol blue, 0.3 % xylene cyanol FF, 60 % glycerol and 60 mM ECTA) and run alongside an sizing agent (GeneRuler$^{TM}$100 bp Plus DNA Ladder). The mtDNA products were run for 25 min at 100 Volts on a 1.5 % Agarose gel and the nDNA products were run for 40 min at 50 V on a 2.5 % Agarose gel, using a GelXLultra (Labnet international inc.) electrophoresis machine and viewed using a Viber Lourmat ultra violet viewer in the freeware computer program DScaler (version 4.1.15 © 2005).

**Table 1** Marker characteristics of 10 nuclear DNA microsatellite markers used to genotype 100 individuals

| Marker name | Repeat motif | Source | Size (bp) | Annealing temperature (°C) | No. of alleles | HO | HE | HWE deviations ($p < 0.01$) |
|---|---|---|---|---|---|---|---|---|
| LaT08 | (TAGA)16 | Archie et al. (2003) | 166–234 | 56 | 10 | 0.828 | 0.851 | 0.460 |
| Lat13 | (CATC)21 | Archie et al. (2003) | 234–262 | 56 | 7 | 0.559 | 0.699 | <0.001* |
| Lat17 | (GGAT)15… (GGAT)10 | Archie et al. (2003) | 323–355 | 56 | 8 | 0.780 | 0.835 | 0.732 |
| Lat24 | (GGAT)22 | Archie et al. (2003) | 211–231 | 56 | 9 | 0.778 | 0.858 | 0.208 |
| FH1 | (CA)12 | Comstock et al. (2000) | 81 | 55 | 6 | 0.545 | 0.569 | 0.381 |
| LA3 | (CA)10 | Eggert et al. (2000) | 165–171 | 55 | 3 | 0.857 | 0.503 | <0.001* |
| FH39 | (CA)18 | Comstock et al. (2000) | 242 | 60 | 10 | 0.632 | 0.779 | 0.011 |
| FH102 | (CT)11(CA)14 | Comstock et al. (2000) | 179 | 60 | 9 | 0.546 | 0.559 | 0.252 |
| LA5 | (CA)13 | Eggert et al. (2000) | 130–154 | 52 | 7 | 0.526 | 0.618 | 0.130 |
| Lat25 | (CCAT)15 | Archie et al. (2003) | 298–318 | 52 | 6 | 0.628 | 0.779 | 0.002* |

*loci were observed heterozygosity (HO) and expected heterozygosity (HE) deviated significantly from Hardy–Weinberg Equilibrium (HWE)

Mitochondrial DNA was purified using the NucleoSpin®-Extract ll purification protocol (Machery-Nagel, GmbH and Co, KG, Düren), cycle sequenced using the Big Dye Terminator ver. 3.1 (Applied Biosystems, Foster City, CA) and subjected to Sodium-Acetate precipitation. Mitochondrial DNA sequencing and nDNA fragment analysis were done using Applied Biosystems 3130xl and 3500xl automated sequencers (Life Technologies, Carlsbad, US) at the DNA Sequencing facility of the University of Pretoria, South Africa. Genescan fragment analysis included the DS 33 Filter set G5 using the 500 bp size standard GeneScan™—LIZ500™ (Applied Biosystems—manufactured in Warrington WA1 4SR, United Kingdom) at a 14:1000 ratio of Formamide. Genescan fragment analysis results were viewed and manually scored using the computer program GeneMapper ® Software Version 3.7 (Applied Biosystems, Foster City, USA). We corrected for nDNA microsatellite genotyping errors by repeating the genotyping process (PCR and fragment analysis) for samples that amplified as homozygote genotypes, samples that did not amplify clearly (marker profiles with stutter and unclear allele peaks) and samples where alleles where unique. Only heterozygotes with clear marker profiles and easily identifiable peaks where scored and recorded on the first amplification round, and all new/unique alleles or unclear heterozygotes were verified by at least one, but up to four, additional rounds of genotyping. Samples that amplified as homozygotes for a specific allele two or more times were accepted as true homozygotes. Samples were repeated up to four times per locus, after which they were scored as missing data if the genotypes were still unclear. We used the program Arlequin (Excoffier and Lischer 2010) to calculate per locus deviations from Hardy–Weinberg Equilibrium (HWE) based on 100 000 Markov chain steps and 1,000 dememorisation steps, and the program GENEPOP version 4.3 (Rousset 2008) to test for gametic disequilibrium (Linkage Disequilibrium) based on Markov Chain Parameters that included 100,000 dememorisation steps, 1,000 number of batches, and 50,000 iterations per batch tested. We also employed the program MICROCHECKER© version 2.2.3 (Van Oosterhout et al. 2004) to identify loci-specific genotype errors (such as large- allele drop out and null allele amplification). The MICROCHECKER© analysis was based on a 95 % Confidence Interval (CI) for 10,000 permutations per analysis. The probabilities for the observed number of homozygotes per allele are calculated using a cumulative binomial distribution (Weir 1996) and the p values of all homozygote size classes were calculated by ranking these observed values to randomized genotypes (expected values). These significance values are then combined to calculate Fisher's probability test to identify genotype errors.

## Haplotype analysis (mtDNA)

Sequence alignment and data formatting were respectively done using Webprank (Goldman Group Software, European Bioinformatics Institute 2011) and ClustalW (Thompson et al. 1994). We identified mtDNA haplotypes using DnaSP 5.10.01 (Librado and Rozas 2009) and analysed them by drawing a midpoint-rooted Neighbour Joining tree in MEGA 5 (Tamura et al. 2011), a Median-Joining network in Network 4.6.1.0 (Fluxux Technology Limited) and by mapping the geographic distribution of the haplotypes using the program ArcMap (©ESRI 2011, ArcGIS Desktop: Release 10, Redlands, CA: Environmental Systems Research Institute).

## Population structure analysis (mtDNA and nDNA)

Bayesian Clustering Algorithms incorporated in the program Geneland (Version 4.0.0) (Guillot et al. 2008) were used to

identify groups of genetically similar individuals. Sequence data (mtDNA data) were converted to haploid data based on binary code, where all the polymorphic sites (n = 25) were considered as individual loci, with the possibility of four alleles (ACTG) at each locus. Geneland run parameters included a spatial uncertainty coordinate of 0.01, the possibility of having 1–10 populations, 100,000 Markov-Chain Monte Carlo (MCMC) iterations with a thinning factor of 100, and incorporated the Correlated Allele Frequency model and the Spatial model. The results included geographic maps of the probability of population membership for mtDNA- and nDNA-based genetically distinct groups.

An analysis of molecular variance (AMOVA) determined if these groups (henceforth referred to as sub-populations) were significantly differentiated ($F_{st}$). We incorporated a step-wise mutational model to account for different microsatellite allele lengths ($R_{st}$). The AMOVA for the mtDNA was done in Arlequin (Excoffier and Lischer 2010), while the AMOVA for the nDNA was done using GenAlEx (Peakall and Smouse 2006). We then calculated the mtDNA diversity as the average pairwise differences ($\pi$), the nDNA diversity as the average number of alleles per locus and the Expected Heterozygosity averaged across all loci (He) in Arlequin (Excoffier and Lischer 2010).

We used a Mantel test using the ade4 package (Chessel et al. 2004) in R 2.13.1 (© 2011 The R Foundation for Statistical Computing) to test for Isolation By Distance (IBD) to establish if geographic distance (measured from the centroids of each sub-population) could explain genetic differentiation (a pairwise genetic distance matrix). The centroid distance matrix was calculated using ArcMap (©ESRI 2011, ArcGIS Desktop: Release 10, Redlands, CA: Environmental Systems Research Institute) and the Geospatial Modelling Environment—0.7.1.0 (© Hawthorne L. Beyer 2009–2012), and the genetic distance matrix was calculated in Arlequin (Excoffier and Lischer 2010).

To locate genetic barriers in the landscape, we identified areas with high genetic divergence between individual samples by using a Geographic Information System (GIS) landscape genetic toolbox [the Single Species Genetic Divergence tool (Vandergast et al. 2011)]. The genetic divergence landscapes showed areas with high genetic continuity indicated in blue (suggesting high gene flow) and areas with high genetic discontinuities indicated in red (suggesting obstructed gene glow) when mapped in ArcMap 10 (©ESRI 2011, ArcGIS Desktop: Release 10, Redlands, CA: Environmental Systems Research Institute).

## Results

Ultimately, out of the 120 samples collected, we successfully sequenced 88 individuals (73 %) for the mtDNA fragment (Genbank Accession Numbers for haplotypes are KC179715–KC179727) and genotyped 100 individuals (83 %) for the ten nuclear loci. All 100 individuals had unique genotypes, therefore indicating that none of the samples were from the same individual. Genotypes differed by at least four loci between all pairs of samples. Three of the microsatellite markers showed significant deviations from HWE (Table 1—Lat13 and Lat25 showed excessive homozygotes, while LA3 showed excessive heterozygotes), but none of the markers showed significant ($p < 0.01$) Linkage Disequilibrium between any of the pairs of loci. Based on MICROCHECKER genotype error estimations, three alleles showed evidence of an excess of homozygotes at one size class (allele), however, it is possible that excess homozygotes may naturally be present in a population (due to factors like inbreeding, the Wahlund effect, or sex-linkage (Allendorf et al. 2013)), and considering that the same dataset showed significant deviations from HWE, it may be that excessive homozygotes at some loci are actually present in our study population, and not an artefact of genotyping errors. We do, however, concede the possibility of retaining false homozygotes due to genotyping errors. No locus showed any evidence of scoring error due to stuttering or large allele dropout. It is important to note that the initial screening as described by de Flamingh et al. (2014) included 12 microsatellite markers, however, two were removed from our dataset due to the presence of multiple genotype errors (excessive homozygotes at multiple size classes, as compared to loci in this study that showed excess homozygotes at only one size class).

### Haplotypes (mtDNA)

Seven of the 13 haplotypes (Genbank Accession Numbers KC179715–KC179727) occurred only once, while one haplotype featured in 65 % of the samples. Seven of the haplotypes (Haplotype 1, 2, 3, 4, 6, 12, and 13) were unique to the genetic sequence database GenBank (National Centre for Biotechnology Information), four haplotypes (Haplotype 5, 7, 9 and 10) were previously recorded by Ishida et al. (2013), and two haplotypes (Haplotype 8 and 10) were previously recorded as *L. cyclotis* (African forest elephant) mtDNA sequences by Debruyne et al. (2003) and Debruyne (2005). Most haplotypes were geographically shared (Fig. 2), with only a few haplotypes occurring at discrete locations (e.g. Haplotype 1, 2 and 3 were closely grouped within Zimbabwe, and Haplotype 13 occurred only once at the extreme northern range of the sampling region in Zambia). Most of the haplotypes were grouped together in the Median-Joining Network (Fig. 3) except for Haplotypes 1, 2, 3 and 13, which were grouped distinctly from the rest of the

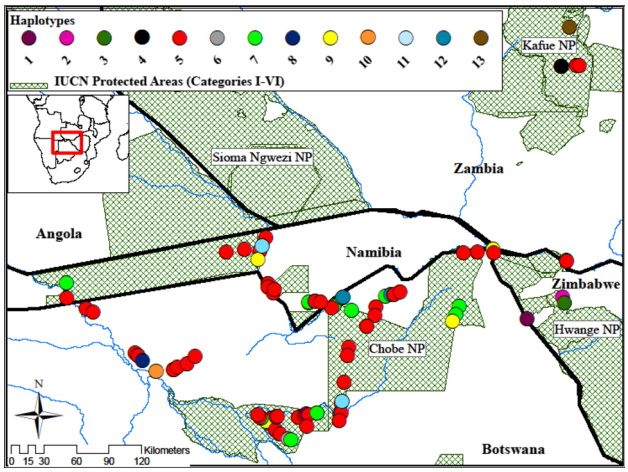


**Fig. 2** A geographical representation of the mtDNA haplotypes in the KAZA region. *Colours* annotate different haplotypes, and *green* cross-hatched areas show IUCN Category I-VI Protected Areas, including four National Parks (NPs) that fall within the greater KAZA region. (Color figure online)

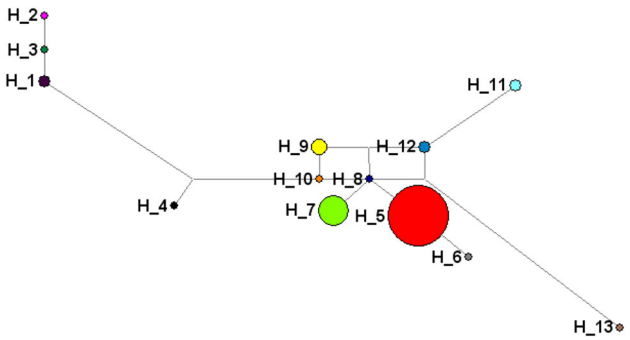


**Fig. 3** A median-joining haplotype network with haplotype symbol colours corresponding to the assigned haplotype *colours* in Fig. 1. The size of the *circles* corresponds to the number of individuals with that haplotype, and the length of the branches approximates the number of mutational events that separate the haplotypes. (Color figure online)

haplotypes. These distinct haplotypes differ from all other haplotypes by at least six base-pairs (six mutational events), while Haplotype 1, 2 and 3 differ from each other by only one base-pair (one mutational event). The Neighbor-Joining Tree (Fig. 4) supported both the geographic distribution of the haplotypes and the Median-Joining Network, with high support for the clustering of Haplotypes 1, 2 and 3 as a distinct clade, but little support for inter-haplotypic structuring throughout the rest of the haplotype clades.

## Population structure (mtDNA and nDNA)

The Geneland Bayesian Clustering Algorithms (BCAs) identified three genetically distinct mtDNA sub-populations

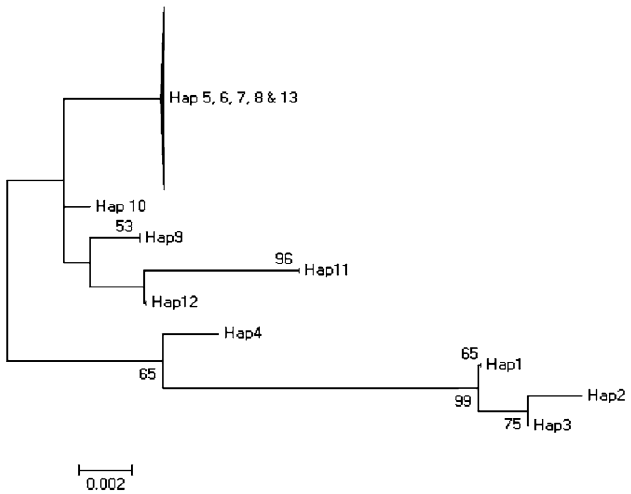


**Fig. 4** A midpoint-rooted neighbour-joining tree showing the clade groupings (bootstrap support calculated for 1,000 bootstrap repeats) of all 13 mtDNA haplotypes

(Fig. 5 b–d) and two genetically distinct nDNA sub-populations (Fig. 5 e, f). None of these populations were Geneland "ghost" populations (see Guillot et al. 2005), as all of the identified populations were the model population of multiple individuals. Mitochondrial DNA sub-population 1 (Fig. 5b) spanned almost the entire study area and included 81 individuals with haplotypes 1, 5, 7, 8, 9, 10, 11, and 12. Mitochondrial DNA sub-population 2 (Fig. 5c) suggested linkage between the extreme north and the central part of the study area and included three individuals with haplotypes 1, 2 and 3. Mitochondrial DNA sub-population 3 (Fig. 5d) suggested linkage between the extreme east and the central part of the study area and included four individuals with haplotypes 4, 5 and 13. However, Geneland did not indicate linkage between mtDNA sub-population 2 in the north and sub-population 3 in the east of the study area.

The nDNA data suggested that there were only two distinct sub-populations, with nDNA sub-population 1 (Fig. 5e) spanning the study area while nDNA sub-population 2 (Fig. 5f) was limited to the extreme northern parts of the study area.

Genetic distances varied independently of geographic distances for mtDNA sub-populations ($r = -0.76$; $p > 0.5$). Because we identified only two nDNA sub-populations, we did not test for isolation by distance for nDNA sub-populations.

Most of the mtDNA based genetic variation could be attributed to variation among sub-populations (AMOVA: $F_{st} = 0.787$, df = 2, $p < 0.05$, Table 2), while the nDNA based genetic variation was ascribed to differences between individuals within sub-populations (AMOVA: $F_{st} = -0.02$; $R_{st} = -0.045$, df = 1, $p > 0.05$, Table 3). The mtDNA sub-populations differed significantly, while

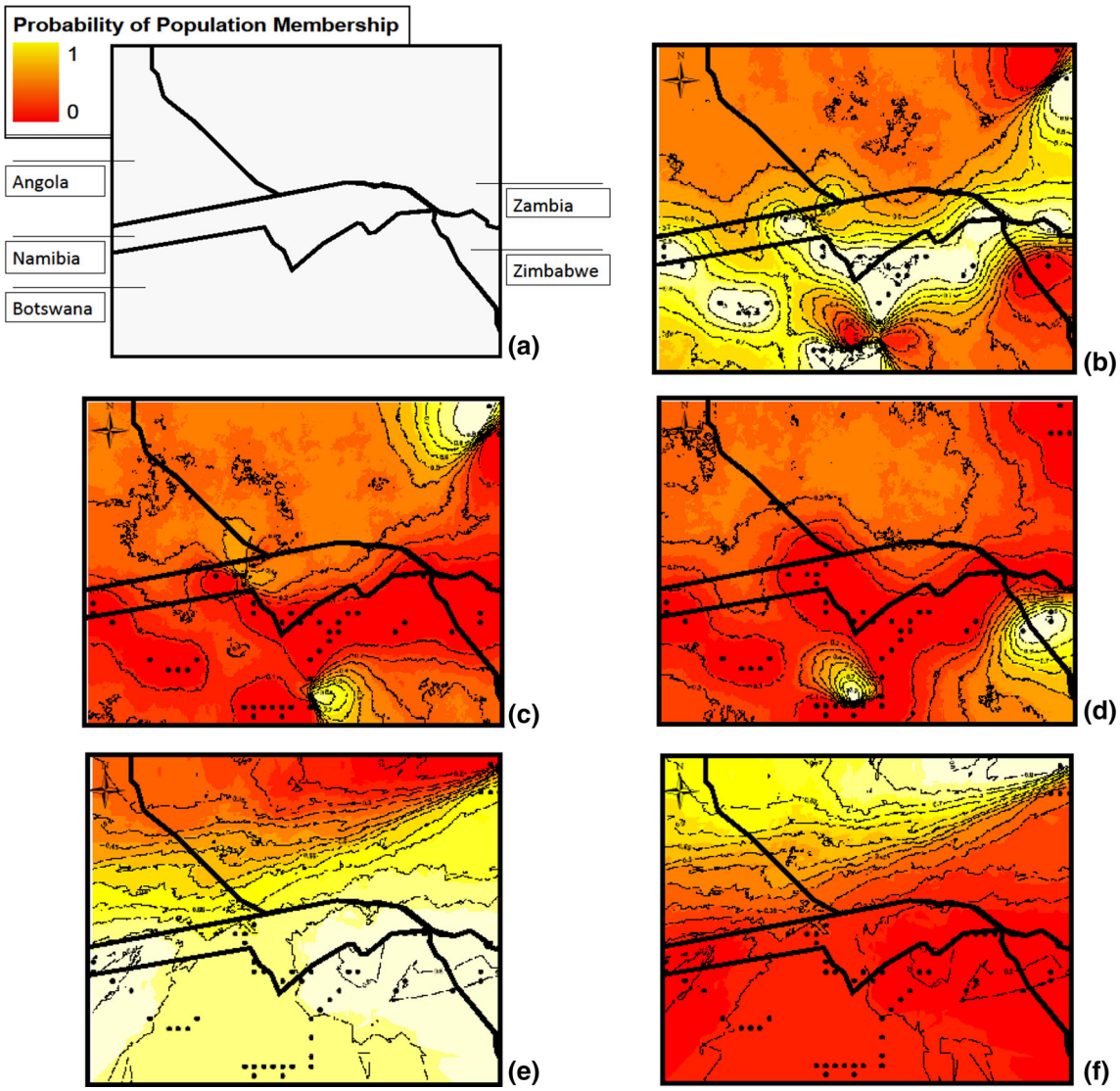


**Fig. 5** **a–f** Maps of the posterior probability to belong to mtDNA sub-populations (**b**, **c** and **d**) or nDNA sub-populations (**e** and **f**) calculated in Geneland for the KAZA region (**a**). The *black dots* indicate the sampling locations (mtDNA = 88 and nDNA = 100)

nDNA sub-populations did not differ significantly for both measures of variation ($F_{st}$ and $R_{st}$). The average pairwise differences ($\pi$) calculated for mtDNA was 2.59, and the average number of alleles per locus and the Expected Heterozygosity averaged across all loci (He) calculated for nDNA was 7.5 and 0.71 respectively.

The mtDNA divergence map (Fig. 1a) identified areas with high genetic discontinuities (indicated in red) that separated both the northern (mtDNA sub-population 2) and the eastern sub-population (mtDNA sub-population 3) from each other and from the central sub-population (mtDNA sub-population 1). Conversely, the microsatellite nDNA divergence map (Fig. 1b) showed no explicit genetic discontinuities in the landscape, indicating that gene flow was high across the total study region.

## Discussion

We showed that the KAZA-TFCA's elephant population, like populations elsewhere in Africa (Nyakaana and Arctander 1999; Okello et al. 2008b; Ahlering et al. 2012a, b; Ishida et al. 2013), was structured into genetically distinct mtDNA sub-populations. Although the regionally shared mtDNA haplotypes (e.g. Haplotype 5, Fig. 2) suggested some mtDNA gene flow, the significant genetic differentiation between the three mtDNA sub-populations suggests that gene flow between sub-populations was restricted, most probably as a consequence of the well recorded philopatry among female elephants that spend most of their lives in breeding herds (e.g. Archie et al. 2006, 2008; Okello et al. 2008b). Our findings furthermore suggest high

**Table 2** An analysis of molecular variation (AMOVA) indicated that most of the genetic variation found in the mtDNA data could be attributed to significant inter-population genetic differences ($p < 0.01$ for both the variation among populations (Va) and the fixation index)

| Source of variation | d.f. | Sum of squares | Variance components | Variation (%) |
|---|---|---|---|---|
| Among populations | 2 | 39.2 | 3.29 | 78.73 |
| Within populations | 85 | 75.48 | 0.89 | 21.27 |
| Total | 87 | 114.68 | 4.17 | 100 |
| Fixations index | $F_{st}$ | 0.787 | | |

**Table 3** An analysis of molecular variation (AMOVA) indicated that most of the genetic variation found in the nDNA data could be attributed to the genetic variation found within individuals, where the populations do not show significant inter-population differentiation ($F_{st}$) ($p > 0.5$), even when applying the step-wise mutation model ($R_{st}$)

| Source of variation | d.f. | Sum of squares | Variance components | Variation (%) |
|---|---|---|---|---|
| Among populations | 1 | 3.15 | 0.000 | 0 |
| Within populations | 98 | 417.71 | 0.566 | 15 |
| Within individuals | 100 | 313 | 3.130 | 85 |
| Total | 199 | 733.87 | 3.696 | 100 |
| Fixation Index | $F_{st}$ | −0.02 | | |
| | $R_{st}$ | −0.045 | | |

mtDNA gene flow within each of the mtDNA sub-populations, moderate levels of gene flow between the central sub-population (Botswana) and the northern (Zambia) and eastern (Zimbabwe) sub-populations, but limited gene flow between the Zambia and Zimbabwe sub-populations. Nuclear DNA genetic differentiation between the two sub-populations, however, was not significant, thus supporting the notion of sustained gene flow across the entire study region. This is not surprising given the male biased dispersal known for elephants (Nyakaana and Arctander 1999; Archie et al. 2008; Okello et al. 2008b; Ahlering et al. 2012a).

Genetic discontinuities across the KAZA-TFCA landscape appeared gender specific. For instance, the mtDNA sub-populations were clearly separated by areas of high genetic discontinuity, with high gene flow within sub-populations but obstructed gene flow between sub-populations, whereas there were no explicit areas that appeared to obstruct gene flow for nDNA. Others (e.g. Nyakaana and Arctander 1999; Nyakaana et al. 2002; Okello et al. 2008b; Ishida et al. 2013) ascribed this gender difference to differences in male and female social behaviour and spatial utilisation patterns, more specifically to the restricted roaming behaviour of core social groups (Archie et al. 2006, 2008; Fishlock and Lee 2012) where most females limit their land use to home ranges smaller than those of males that generally roam across the ranges of several breeding herds (Hall-Martin 1987; Jackson and Erasmus 2005). Gene-dispersion, therefore, is predominantly male-mediated (Nyakaana and Arctander 1999; Archie et al. 2008; Okello et al. 2008b).

Elephant populations on the periphery of the KAZA-TFCA (for example, in Hwange and Kafue National Parks) might have been subjected to intensive poaching (Wasser et al. 2007). Poaching in the core part of our study, however, was less than what has been observed elsewhere (Burn et al. 2010), and we therefore assumed that the structuring of the KAZA-TFCA elephant population was not due to poaching. We do, however, acknowledge that persistent poaching may change the local structuring of elephant populations, especially where entire matrilineal lines may be removed (personal communication—S. Wasser 2013).

Natural and intrinsically driven genetic structuring in the absence of anthropogenic and extrinsic barriers distinguishes our study from those of others (Eggert et al. 2002; Nyakaana et al. 2002; Ahlering et al. 2012c; Epps et al. 2013). We discounted man-made or landscape barriers and geographic distance as the primary drivers of genetic structuring in the KAZA region and suggest that future studies should consider the influence of intrinsic factors [resource dependencies and social variables that limit movement, such as the distribution of water as a key resource (de Beer and van Aarde 2008; Loarie et al. 2009; Young et al. 2009) and restricted roaming behaviour of core social groups (Archie et al. 2006, 2008; Fishlock and Lee 2012)] when investigating the genetic structure of elephant populations.

Given the realities of limited resources for conservation, it may be important to identify priority areas for conservation, for instance, by identifying historical or existing landscape linkages between populations that may restore or maintain population connectivity. This is especially important for TFCAs that strive to increase connectivity between isolated protected areas to create larger ecological networks. Our results provide evidence for such a functional landscape linkage. The high nDNA gene flow in conjunction with movement routes with suitable habitat (based on resource selection modelling) identified by Roever et al. (2013b) suggest a functional linkage between Chobe (Botswana) and Kafue (Zambia) National Parks. This linkage may be direct or indirect through an intermediate population/populations. The differentiated mtDNA genetic structure compared to the high nDNA gene flow suggests that it is mainly bulls that roam between these national parks, a finding that is in agreement with dispersal

patterns identified elsewhere [e.g. where male elephants disperse into new areas before females (Druce et al. 2008) and where males dominate newly established populations (Ahlering et al. 2012a)].

Our study encourages conservation initiatives that strive to counter the effects of population fragmentation and isolation by restoring or maintaining landscape linkages between populations [e.g. through the creation of "mega-parks" (van Aarde and Jackson 2007) and TFCAs (Hanks 2001, 2003)]. Such initiatives may benefit spatially fragmented elephant populations by maintaining or restoring linkages that increase the chance of persistence, enlarge gene pools, increase genetic diversity, and offset the impacts of locally high elephant populations.

Landscape linkages may also ensure the maintenance of inter-population movements, and in so doing maintain the current genetic state of the KAZA-TFCA elephant population. The KAZA elephant population is genetically more diverse than other southern African populations (Essop et al. 1996; Whitehouse and Harley 2001), suggesting that elephants in this region do not suffer inbreeding depression, genetic drift, or other genetically detrimental factors often associated with populations below a genetically viable population size (Fairbanks and Andersen 1999).

This study provides a baseline for future studies that seek to determine the genetic structure of naturally structured elephant populations and/or elephant populations that occur in continuous landscapes. Furthermore, by integrating a similar genetic approach with other studies that investigate elephant spatial ecology (e.g. Roever et al. 2013b), future research may identify ecologically sensible linkages between elephant populations in other southern African countries. Identifying such landscape linkages may be crucial to the success of conservation initiatives considering the current anthropocene era in which space for conservation is becoming an increasingly limited resource.

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**SUPPLEMENTARY DATA**

A modified protocol for nuclear and mitochondrial DNA isolation from degraded elephant faecal DNA. The asterisks indicate the steps of the original protocol (Qiagen DNA Stool Mini Kit - Southern Cross Biotechnology, Cape Town, SA) that were modified.

| Step | Description |
|---|---|
| **1*** | Mix sample bottle contents till relatively homogenized. Centrifuge 2 ml of this mixture for 30 minutes. Discard supernatant. Repeat the process three times and use the remaining pellet as the starting material for DNA extraction. |
| **2*** | Add 1.7ml Buffer ASL to each sample and vortex continuously for 15 minutes. |
| **3*** | Centrifuge sample at full speed for 30 minutes to pellet stool particles |
| **4** | Pipet 1.4 ml of the supernatant into a new 2ml microcentrifuge tube and discard the pellet. |
| **5*** | Add 1 InhibitEX tablet to each sample and vortex immediately and continuously for 5 minutes. Incubate suspension for 5 minutes at room temperature to allow inhibitors to adsorb to InhibitEX matrix. |
| **6*** | Centrifuge sample at full speed to 6 minutes to pellet stool particles and inhibitors bound to InhibitEX matrix |
| **7** | Immediately after centrifuge stops, pipet all of the supernatant into a new 1.5ml microcentrifuge tube and discard the pellet. Centrifuge at full speed for 3 minutes. |
| **8** | Pipet 25 µl proteinase K into a new 2 ml microcentrifuge tube. |
| **9** | Pipet 600 µl supernatant from step 7 to the 2 ml microcentrifuge tube containing proteinase K. |
| **10** | Add 600 µl Buffer AL and vortex for 15 seconds. |
| **11*** | Incubate at 70°C for 30 minutes. |
| **12** | Add 600 µl of ethanol to the lysate ad mix by vortexing. |
| **13** | Label the lid of a new QIAamp spin column in a 2ml collection tube. Carefully apply 600 µl lysate from step 12 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 minute. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate. |
| **14** | Carefully open the QIAamp spin column, apply a second aliquot of 600 µl lysate and centrifuge at full speed for 1 minute. Place the QiaAmp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate. |
| **15** | Repeat step 14 to load the third aliquot of the lysate onto the spin column. |
| **16** | Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 minute. Place the QIAamp spin column in a new 2 |

| | |
|---|---|
| | ml collection tube, and discard the collection tube containing the filtrate. |
| **17** | Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 minutes. Discard the collection tube containing the filtrate. |
| **18** | Place the QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute. |
| **19*** | Transfer the QIAamp spin column into a new, labelled 1.5 ml microcentrifuge tube. Carefully open the QIAamp spin column and pipet 100 µl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 10 minutes at room temperature, then centrifuge at full speed for 10 minutes to elute DNA. |