Genetic diversity and spatial genetic structure of African wild dogs (*Lycaon pictus*) in the Greater Limpopo Transfrontier Conservation Area

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ABSTRACT

The Greater Limpopo Transfrontier Conservation Area (GLTFCA) is one of the last refuges for the endangered African wild dog and hosts roughly one-tenth of the global population. Wild dogs in this area are currently threatened by human encroachment, habitat fragmentation and scarcity of suitable connecting habitat between protected areas. We derived genetic data from mitochondrial and nuclear markers to test the following hypotheses: (i) demographic declines in wild dogs have caused a loss of genetic variation, and (ii) Zimbabwean and South African populations in the GLTFCA have diverged due to the effects of isolation and genetic drift. Genetic patterns among five populations, taken with comparisons to known information, illustrate that allelic richness and heterozygosity have been lost over time, presumably due to effects of inbreeding and genetic drift.

Genetic structuring has occurred due to low dispersal rates, which was most apparent between Kruger National Park and the Zimbabwean Lowveld. Immediate strategies to improve gene flow should focus on increasing the quality of habitat corridors between reserves in the GLTFCA and securing higher wild dog survival rates in unprotected areas, with human-mediated translocation only undertaken as a last resort.

Key words: Carnivore; dispersal; gene flow; habitat fragmentation; phylogeography; Southern Africa; trans-frontier conservation areas.

INTRODUCTION

African wild dogs (*Lycaon pictus*) were previously distributed throughout much of sub-Saharan Africa (Creel and Creel 1998); however, the species has experienced notable population declines (Fanshawe et al. 1997). As a result, the International Union for the Conservation of Nature and Natural Resources (IUCN) listed wild dogs as Endangered (Criteria C2a) in 2004 due to a continuing decline of mature individuals and a population structure with no subpopulation containing more than 250 mature individuals. This status was re-affirmed in 2012 (Woodroffe and Sillero-Zubiri 2012). Fewer than 1,400 mature African wild dogs in 27 countries are thought to remain (Woodroffe and Sillero-Zubiri 2012).

A viable population of wild dogs (roughly one-tenth of the global population; personal observation R. Groom) occurs in the Greater Limpopo Transfrontier Conservation Area (GLTFCA), which encompasses 99,800 km^2 in Zimbabwe, South Africa and Mozambique (Wolmer 2003). The area became fragmented during the Zimbabwean land reform programme in the early 2000s and large tracts of natural habitat have been lost in privately owned areas (Lindsey et al. 2008). Wild dog packs require large home ranges (from 357 to 930 km^2; Mills and Gorman 1997), being mainly limited by prey density, interspecific competition (from lions *Panthera leo* and spotted hyaenas *Crocuta crocuta*), and deliberate and accidental human-caused mortality (Mills and Gorman 1997; Creel and Creel 1998; Bach et al. 2010). Consequently, many natural areas in Zimbabwe are now too small to maintain viable wild dog populations (Lindsey et al. 2004).

When habitat becomes fragmented, wild dogs tend to naturally disperse between suitable habitat patches to find unrelated mates, which maintains a meta-population structure (McNutt 1996). Wild dogs can disperse over hundreds of kilometres and fences seldom prohibit them (Davies-Mostert et al. 2012), but the scarcity of suitable habitat between protected areas can prevent them from reaching far-distanced reserves (Woodroffe et al. 1997). As a result, small populations can lose genetic variation because of the increased impact of inbreeding (i.e. mating amongst relatives; see e.g. Perrin and Mazalov 2000; Keller and Waller 2002; Charlesworth and Willis 2009). Inbreeding eventually leads to increased homozygosity and the expression of deleterious mutations, which in turn reduces survival and reproductive success of individuals (Keller and Waller 2002; Frankham et al. 2004; O'Grady et al. 2006).

Reduced fitness due to genetic depletion has been reported in small populations of other canids (e.g. captive wolves (*Canis lupus*), Laikre and Ryman 1991; grey wolves on Isle Royale, Peterson et al. 1998; Scandinavian wolves, Liberg et al. 2005; Mexican wolves (*Canis lupus baileyi*), Fredrickson et al. 2007). In the case of an isolated and genetically depleted population of grey wolves in Scandinavia, the arrival of one immigrant led to selective outbreeding, which caused an increase in heterozygosity, the introduction of new alleles, and an exponential population growth (Vilà et al. 2003). For African wild dogs, the loss of heterozygosity is likely to be mitigated due to their natural behaviour of avoiding inbreeding within a pack (Girman et al. 1997; Spiering 2011). Both sexes disperse from their natal area to enhance breeding opportunities and avoid inbreeding (Fuller 1992). Nevertheless, inbreeding has been confirmed in a semi-natural meta-population in KwaZulu-Natal, South Africa, with reduced longevity for inbred individuals (Spiering et al. 2011). This would suggest that inbreeding avoidance can be compromised if natural dispersal and outbreeding are restricted (Spiering et al. 2011; Leigh et al. 2012).

Another force that acts on small, isolated populations is genetic drift (Hamilton 2009). A wild dog population in Zambia, for instance, suffered from low allelic richness due to restricted dispersal and genetic drift (Leigh et al. 2012). Allelic richness has also been lost at the major histocompatibility complex (MHC), which comprise adaptive genes related to the immune system (Marsden et al. 2009). This phenomenon is mainly apparent in areas that were recently recolonized and are relatively isolated from other wild dog populations (Marsden et al.

2009). Genetic drift and limited gene flow can also lead to strong genetic structuring between populations; this has been reported for wild dog populations across Africa (Girman et al. 2001; Marsden et al. 2012).

The effective population size will decrease if dispersal between populations is restricted (Hamilton 2009). The effective population size is naturally low in African wild dogs due to their breeding biology where, in most cases, only the monogamous alpha pair breeds (Creel and Creel 1995; McNutt 1996). Hence, only the alpha male and female, or breeding individuals, contribute gametes to the next generation (McNutt 1996). The effective population size is further reduced if subpopulations become too small (Hamilton 2009). Not surprising therefore that the average effective population size of wild dog populations across Africa has declined notably over the past decades (Girman et al. 1997; 2001; Leigh et al. 2012; Marsden et al. 2012).

To this end, it is important to understand connectivity between fragmented natural reserves in the GLTFCA, one of the last bastions for wild dog conservation in southern Africa. It is unknown whether natural dispersal still occurs, or whether restricted dispersal has led to genetic isolation of various wild dog populations in this area. There might also be a barrier to gene flow between Kruger National Park and the Zimbabwean Lowveld, as suggested by Marsden and co-workers (2012). An understanding of the spatial genetic structuring and levels of genetic diversity within populations is essential for understanding population viability (Hamilton 2009; Allendorf et al. 2012) and setting conservation priorities. In this study we assess whether wild dog populations within the Zimbabwean and South African parts of the GLTFCA are genetically diverged, possibly driven by a decline in population size and / or an increase in population fragmentation. We derived genetic data from mitochondrial and nuclear markers to test the following predictions: (i) reported demographic declines have caused a loss of heterozygosity and allelic richness in wild dogs, and (ii) Zimbabwean and South African populations have diverged due to the effects of isolation and / or genetic drift.

To examine genetic structure and dispersal amongst wild dog populations in the GLTFCA, we used nuclear microsatellites and mitochondrial sequences. These neutral markers are frequently used in conservation genetic studies as they provide information on population trends, inbreeding indications, and gene flow patterns (Frankham et al. 2004; Goldstein and Schlotterer 1999; Lopez et al. 1997). Neutral genetic markers cannot be

used as a proxy for adaptive variation, the importance of selection or population health *per se*; however, it does provide some information about the overall levels of genetic diversity, and especially population subdivision (Holderegger et al. 2006).

METHODS

Sample collection and laboratory protocols

Samples were collected between 2008 and 2013 from protected areas within the GLTFCA: (i) Savé Valley Conservancy (SVC); (ii) Bubye Valley Conservancy (BVC); (iii) Gonarezhou National Park (GNP); (iv) Nuanetsi Ranch (NR); in southern Zimbabwe; and (v) Kruger National Park (KNP) in north-eastern South Africa (see Fig. I for more detail). Blood was collected whenever a wild dog was immobilised (usually for collaring or de-snaring) and tissue was obtained from individuals found dead in the field. Fresh faecal samples (1 to 2 days old) were collected to enlarge the sample size. All samples were stored in RNA stabilization solution and kept at -20 ºC. DNA was extracted using the QIAamp DNA Mini Kit for blood and tissue samples and QIAamp DNA Stool Mini Kit for faecal samples (Qiagen), according to manufacturer's instructions.

We conducted sequence-based typing for the mitochondrial control region (primer combination described in Leigh 2005) and cytochrome-*b* gene (primer combination described in Smit et al. 2011); the control region allowed direct comparisons with earlier studies (Girman et al. 2001; Marsden et al. 2012). The PCR reactions contained 1 unit of enzyme (SuperTherm; Southern Cross Biotechnology), 20 pmol of each primer, 1x PCR Buffer, 2.5 mM $MgCl_2$, 200 µM dNTP and ~ 10 ng of DNA product. For faecal samples we varied the concentration of DNA to facilitate amplification (see e.g. Ntie et al. 2010). Standard PCR cycling parameters were used as described in Pitra et al. (2006). Amplification was verified in 1% agarose gels. We cycle sequenced successful amplicons using BigDye chemistry (Life Technologies), and analysed these on an ABI 3730 DNA Analyzer (Life Technologies).

We chose nine physically unlinked polymorphic microsatellite loci based on their previous use in wild dog genetic studies (*cf.* Marsden et al. 2012). The forward primer of each locus was labelled with a fluorescent dye (5HEX, 56-FAM, or 5ATTO550N) and amplified using the Qiagen Multiplex PCR mix. Approximately 1 ng of DNA was

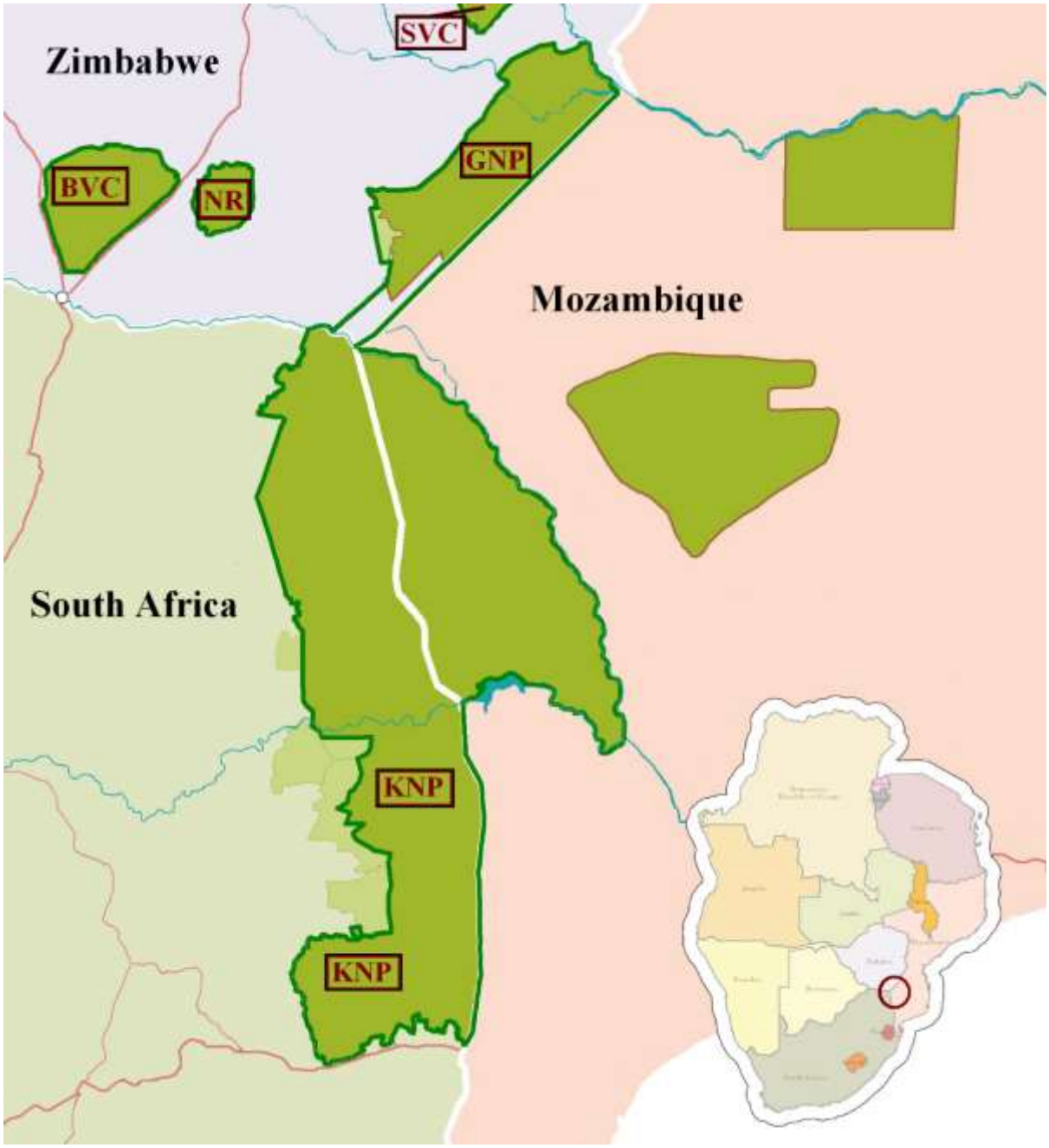

Figure I. A map of the Greater Limpopo Transfrontier Conservation Area; adapted from SANparks website. African wild dog samples were collected from the following protected areas: Savé Valley Conservancy (SVC); Gonarezhou National Park (GNP); Nuanetsi Ranch (NR); Bubye Valley Conservancy (BVC); and Kruger National Park (KNP).

included in the reactions. We amplified and genotyped all blood and tissue samples twice and faecal samples three times, and samples that showed relatively weak but readable results additional times, to check for allele dropout. Samples were analysed on an ABI 3730 DNA Analyzer (Life Technologies).

Genetic analyses

Chromatograms were checked with Geneious 6.1.5 (Biomatters Ltd.), and aligned with the Multiple Geneious Alignment (incorporated into Geneious) with a 65% cost matrix, and a gap extension penalty of 3 (for the control region). DNaSP 5.10.1 (Librado and Rozas 2009) was used to calculate haplotype and nucleotide diversities. Sequence fragments were analysed singly to allow for comparison with the control region data of Marsden et al. (2012) as well as together (control region and cytochrome-*b* fragments generated in the present study). As a first assessment of overall spatial structure, we used NETWORK Software (Bandelt et al. 1999) to create a median-joining network for the control region (published haplotypes were included with our data for the control region) as well as the combined control region and cytochrome-*b* fragments (see Joly et al. 2007 for some discussion on pitfalls of different network approaches). Spatial structure (using analyses of molecular variance; AMOVA) was assessed in Arlequin 3.5 (Excoffier and Lischer 2010) by calculating the overall Φ_{ST} value as well as pairwise Φ_{ST} values between sampling localities.

Microsatellite loci were scored with Geneious 6.1.5 (Biomatters Ltd.) and tested for genotypic linkage disequilibrium (using log-likelihood ratio G-statistics) and deviation from Hardy-Weinberg equilibrium with FSTAT v2.9.3.2 (Goudet 2002). Significance values were adjusted according to the Bonferroni correction. Gene diversity indices were estimated based on observed (H_O) and expected (H_E) heterozygosity using Arlequin 3.5 (Excoffier and Lischer 2010). Allelic richness (R_s) and private alleles were calculated with HP-Rare v1.0 (Kalinowski 2005), using rarefaction to compensate for differences in sample size between populations. To evaluate inbreeding, F_{IS} was calculated in FSTAT using Bonferroni adjustment for multiple comparisons. $N_EE_{STIMATOR}$ v1.3 (Ovenden et al. 2007) was used to calculate contemporary estimates of effective population size (N_e) with the linkage disequilibrium (LD) test (Hill 1981). The LD methods provides reliable estimates when the sample size is the same or larger than the true N_e (England et al. 2006), which is the case in this study. Population structure was investigated using several methods. Firstly, genetic differentiation between populations was assessed using AMOVA applications and F_{ST} values within the program ARLEQUIN v3.5. Pairwise ϕ_{ST} was used as a comparison. STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to test for genetic clustering among the wild dog groups. We chose the admixture model for the ancestry of individuals, and assumed independent allele frequencies. The program was run five times (for K values up to 5) for 1,000,000 MCMC iterations with twenty

replicas per K; the first 100,000 iterations were discarded as burn-in. The most likely value for K (i.e., the most likely number of clusters) was determined by examining the second-order rate change of Ln P(X/Y) following Evanno et al. (2005) as implemented in CLUMPAK (Kopelman et al. 2015). FLOCK 3.1 (Duchesne and Turgeon 2012) was similarly used to determine population structure as an alternative to STRUCTURE and verify results. Furthermore, we used the GENELAND software version 4.05 (Guillot et al. 2005) to provide a better definition of spatial genetic units by integrating spatial coordinates and combining different markers into the calculation. GENELAND was run five times to verify the consistency of the results, with the following parameters: 500,000 MCMC iterations, 500 thinning, maximum rate of Poisson process fixed to 500, uncertainty attached to spatial coordinates fixed to 20 km, and K set up to 5. The number of expected migrants per generation between populations was calculated with BayesAss v3.0 (Wilson and Rannala 2003), which was run 5 times with 10 million MCMC iterations, 1 million iterations discarded as burnin, and 1,000 intervals between samples for MCMC.

RESULTS

Mitochondrial results

Control region (278 bp) and cytochrome-*b* (418 bp) sequences were generated for 168 and 131 wild dogs respectively. We found 8 and 4 haplotypes for the control region and cytochrome-*b* respectively for the wild dogs analysed from the GLTFCA, mostly with single site changes separating these. Haplotype diversity ranged from 0.035 (SVC) to 0.686 (BVC) for the control region, and 0.00 (KNP) to 0.358 (SVC) for cytochrome-*b*. The number of haplotypes (*h*) and nucleotide diversity (π) for each wild dog population is given in Table I, in combination with previous data (Marsden et al. 2012). No obvious loss of gene diversity was observed for the control region between studies.

Control region and cytochrome-*b* sequences were combined for 126 individuals from the GLTFCA for which we had comparable data for both sequencing fragments. The total alignment contained 23 variable (polymorphic) sites and collapsed into 9 haplotypes. The spread of haplotypes among the five sampled areas is illustrated in a median-joining network (Fig. IIA). The most common haplotype was found in 76 wild dogs (60%) and occurred in

Table I. Genetic diversity for mitochondrial DNA (control region) and nuclear DNA (microsatellite) markers of five African wild dog populations in the Zimbabwean and South African part of the GLTFCA. Data from Marsden et al. 2012 is included into the graph to allow for comparison. Effective population size is based on the linkage disequilibrium methods with a 95% confidence interval.

			Control Region		**Microsatellites**		
Data	**Populations**	***n* (mtDNA/nDNA)**	***h***	**π x 10^3**	**R_S**	**H_E**	**N_e**
Current data	SVC	57/61	2	0.4 (± 0.0)	3.06	0.577 (± 0.13)	64.0 (42.8-103.1)
	BVC	21/ 23	6	7.0 (± 1.0)	2.66	0.517 (± 0.21)	58.5 (21.9-infinite)
	GNP	29/ 39	3	11.9 (± 5.0)	2.82	0.514 (± 0.21)	79.1 (41.4-235.9)
	NGR	22/ 25	3	3.1 (± 1.0)	2.95	0.570 (± 0.10)	21.8 (13.5-37.7)
	KNP	39/ 40	2	1.8 (± 0.0)	3.79	0.706 (± 0.10)	30.0 (22.6-40.4)
Previous data	Kruger	94/97	2	1.45 (± 1.4)	4.60	0.714 (± 0.12)	21.8 (11.6-43.5)
	Lowveld	15/14	1	0	4.09	0.698 (± 0.13)	7.8 (6.1-10.5)
	Okavango	90/12	6	22.20 (± 11.6)	4.54	0.703 (± 0.11)	28.3 (13.1-92.7)
	Hwange	47/14	5	11.20 (± 6.4)	5.68	0.789 (± 0.07)	12.4 (9.9-15.9)
	Selous	37/23	3	19.42(± 10.5)	4.90	0.697 (± 0.16)	20.5 (16.2-27.0)
	Masai	32/32	2	6.18 (± 4.0)	4.31	0.646 (± 0.20)	11.5 (9.8-13.6)
	Serengeti	41/20	3	9.32 (± 5.5)	4.97	0.751 (± 0.09)	11.9 (6.8-20.8)
	Laikara	?	2	7.50 (± 2.3)	4.41	0.686 (± 0.14)	21.5 (18.7-24.8)

* Listed are the number of sampled individuals (*n*); number of haplotypes (*h*); nucleotide diversity (π± SD); allelic richness (R_S); expected heterozygosity (H_E ± SD) and effective population size (N_e). Current data originates from this study and previous data from Marsden et al. (2012).

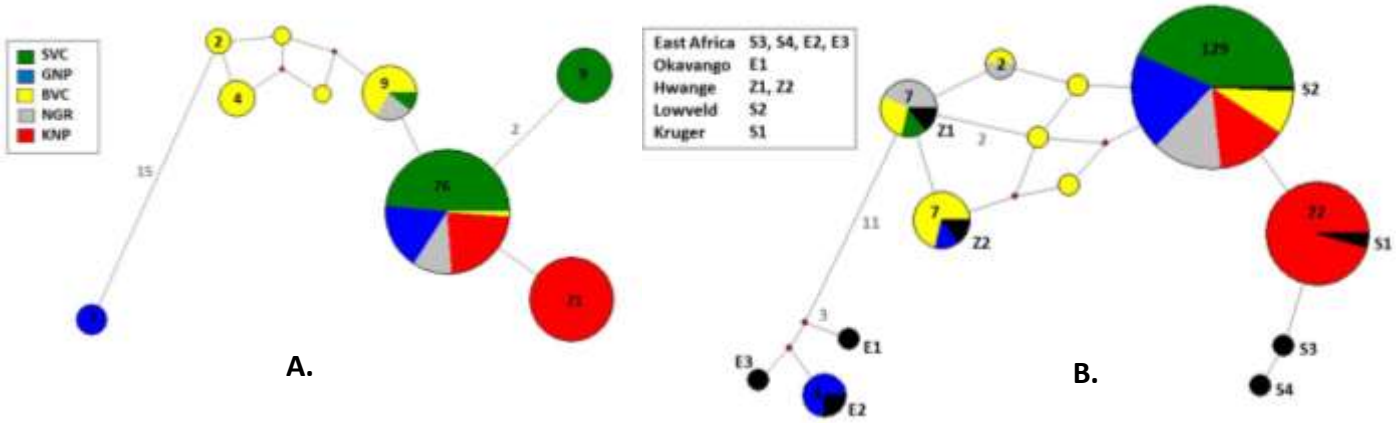

Figure II. A. Median-joining network of haplotypes based on control region and cytochrome-*b* sequences. Five African wild dog populations are included in the network, all located in the Greater Limpopo Transfrontier Conservation Area: Savé Valley Conservancy (SVC); Gonarezhou NP (GNP); Nuanetsi Ranch (NR); Bubye Valley Conservancy (BVC); and Kruger NP (KNP). Haplotype sizes are proportional to the number of individuals (N > 1 are indicated in the circles). Numbers indicated on the branches refer to the number of mutational steps (when higher than 1) separating the haplotypes. B. A comparison of control region sequences retrieved during this study with sequences retrieved by Marsden et al. (2012) (Marsden et al. haplotypes are indicated in black).

every population. Several of the different conservation areas had haplotypes unique to that specific area; for example, the BVC has 4 unique haplotypes while KNP (shared by 21 wild dogs), SVC (shared by 9 wild dogs) and GNP (shared by 3 wild dogs) each has a single unique haplotype. Most of these haplotypes are separated by single site changes with the exception of the GNP specific haplotypes that is separated by 15 mutational steps from its closest neighbour. When we compare our results to haplotypes published by Marsden et al. (2012), it appears that the distinct GNP haplotype originates from Eastern Africa (Fig IIB) (it is identical to the Marsden et al. haplotype E2 and closely related to the Marsden et al. haplotypes E1 and E3).

Genetic diversity was significantly structured across the GLTFCA with 33% of the variation accounted for by among-population differences (Φ_{ST} = 0.331; P<0.00) (see Table II for among population values). Pairwise Φ_{ST} analyses showed that all populations were significantly differentiated from one another (Table II) with the exceptions two comparisons involving NR (SVC and GNP).

Table II. Populations pairwise F_{ST} values for six genotyped African wild dog populations. Left (bottom) side of the table shows the genetic distances based on microsatellite loci (F_{ST}; lower left) and mitochondrial DNA sequences (Φ_{ST}; upper right); only results that were significant are shown.

	SVC	BVC	GNP	NR	KNP
SVC	X	0.566	0.230	-	0.339
BVC	0.051	X	0.215	0.447	0.663
GNP	0.041	0.040	X	-	0.239
NR	0.049	0.038	0.027	X	0.397
KNP	0.130	0.137	0.126	0.108	X

Microsatellite results

A total of 188 wild dogs was genotyped for 9 microsatellite loci. Allele frequencies were tested for departure from the Hardy-Weinberg equilibrium in a locus by locus and population by population manner. Using the Fisher's exact test (as is appropriate for hypervariable markers; see Rousset and Raymond 1995), we observed no significant deviation from Hardy-Weinberg equilibrium in the form of a heterozygosity deficit or excess (Excoffier and Slatkin 1998). To rule out genotyping errors (in the form of null alleles), we calculated the confidence intervals for null allele frequencies. The results indicated an acceptable (low) probability (~ 0.13) across all populations and loci, therefore null alleles are not common enough to a result in false numbers of homozygotes.

Significant linkage disequilibrium was uncommon but irregularly observed across populations and pairs of loci (4%), of which FH3965 was most consistently out of equilibrium. Since wild dogs live in packs of close relatives, and sample collection (mainly for faecal samples) could have led to oversampling of individuals within the same packs, levels of apparent linkage disequilibrium are plausible (Hedrick 2000). Girman et al. (2001) also found linkage disequilibrium across loci for African wild dogs, which was similarly attributed to oversampling of related individuals. Marsden et al. (2012), on the other hand, used the same loci but found lower levels linkage disequilibrium, which can be explained by lower sampling effort per population and a much larger geographical area. To confirm results, we re-analysed the data excluding FH3965, which had no influence on the outcome.

Genetic diversity indices were calculated for all microsatellite loci and across wild dog populations (Table I). KNP had the highest allelic richness (R_S = 3.79) and BVC the lowest (R_S = 2.66), but differences between populations were relatively small. Allelic richness has reduced significantly (t = -6.03; p = 0.000) when compared to data from Marsden et al. (2012), for which wild dog samples were collected in KNP and the Lowveld 5 to 15 years prior to our sample collection (which translates to 1 to 3 generations; Woodroffe and Sillero-Zubiri 2012). The expected heterozygosities (H_E) also differed little amongst populations sampled in the current study, varying from 52% in BVC to 71% in KNP, but showed a reduction over time (t = -4.03; p = 0.002). A reduction of R_S and H_E could have been caused by inbreeding and genetic drift in the studied wild dog populations due to reduced gene flow. Private allelic richness, which is another good indication of gene flow, was highest in KNP (1.01) and second highest in SVC (0.41). This could indicate that connectivity of these populations is prohibited to some extent, as unlimited dispersal between populations would have led to a more homogenised gene pool. The overall effective population size (N_e) is, on average, 51 individuals, which is higher than previous estimates. The lowest N_e has been measured for KNP, which could be the result of a low population size linked with limited connectivity to neighbouring packs (Randall et al. 2010).

Table III. Migration rates and inbreeding coefficient (F_{IS}) between of 5 genotyped African wild dog populations, based on 9 microsatellite loci: Savé Valley Conservancy (SVC); Gonarezhou NP (GNP); Nuanetsi Ranch (NR); Bubye Valley Conservancy (BVC); and Kruger NP (KNP).

Individuals from	F_{IS} (± SD)	Migration to	Individuals per generation (± SD)
SVC	0.0636 (±0.1434)	BVC	0.0170 (± 0.0164)
		NGR	0.0290 (± 0.0263)
		GNP	0.0412 (± 0.0286
		KNP	0.0082 (± 0.0080)
BVC	0.1197 (±0.0877)	SVC	0.0387 (± 0.0156)
		GNP	0.0167 (± 0.0144)
		NGR	0.0121 (± 0.0117)
		KNP	0.0080 (± 0.0079)
GNP	0.0089 (±0.0084)	SVC	0.0992 (± 0.0819)
		BVC	0.2782 (± 0.0256)
		NGR	0.2414 (± 0.0369)
		KNP	0.0681 (± 0.0232)
NGR	0.2023 (±0.1445)	SVC	0.0058 (± 0.0057)
		BVC	0.0127 (± 0.0123)
		GNP	0.0333 (± 0.0184
		KNP	0.0443 (± 0.0316)
KNP	0.0273 (±0.0223)	SVC	0.0052 (± 0.0052)
		BVC	0.0119 (± 0.0114)
		GNP	0.0133 (± 0.014)
		NGR	0.0155 (± 0.0153)

To further test for genetic differentiation among wild dog populations, an analysis of molecular variance (AMOVA) was performed which showed that 14% of the variation is accounted for by between population differences (F_{ST} = 0.137; P < 0.00). Pair-wise F_{ST} comparisons indicated that all populations were significantly differentiated (Table II). KNP in the very south of the GLTFCA was the most distinct population. Migration rates between populations (by means of dispersing individuals per generation) are low, but some migration is apparent (Table III). Migration is lowest from and to KNP, and highest from and to GNP, which is located in the centre of the GLTFCA; these results would suggest an 'isolation-by-distance' model.

To assess the number of differentiated populations, we performed a STRUCTURE analysis for all individuals assuming random mixture and shared ancestry (based on the life history of wild dogs). Allele frequencies and likeliness of genetic ancestry were calculated and two clusters (K = 2) were found to be the best fit for our data (Fig. III). All populations in the Lowveld of Zimbabwe (SVC in the north, BVC and NR in the west, and GNP in the central-east) clustered together, whilst KNP is clearly separated. Alleles are nevertheless shared between both

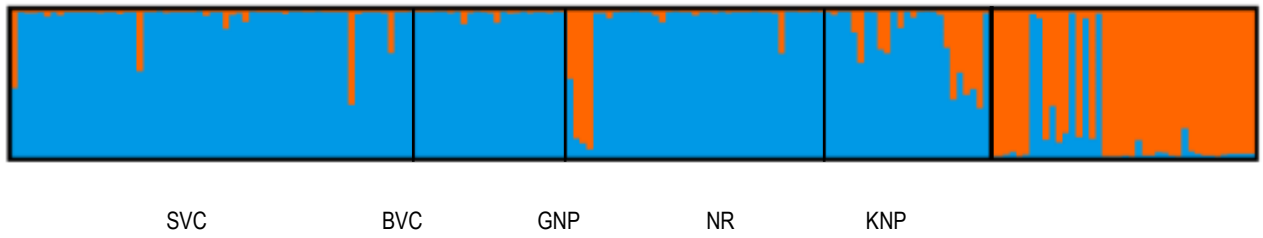

Fig. III. Visualisation of genetic clusters (K= 2) among 188 African wild dogs from the GLFTCA as suggested by CLUMPAK (Kopelman et al. 2015): Savé Valley Conservancy (SVC); Gonarezhou NP (GNP); Nuanetsi Ranch (NR); Bubye Valley Conservancy (BVC); and Kruger NP (KNP). Main clusters are SVC together with adjacent reserves (BVC, GNP and NR), and KNP, although alleles are shared among them.

clusters, which could reveal recent admixture or low levels of migration. To confirm results we also conducted population genetic analyses with Flock, which was designed to reveal genetic structure assuming a substantial proportion of admixed specimens. Flock analysis concluded that a minimum of two genetically differentiated populations can be assumed (K ≥ 2). Highest genetic distances were found between KNP and the populations in the Zimbabwean Lowveld. GENELAND confirms these results and clustered KNP separate from the Lowveld in a spatial structure analysis (Figure IV).

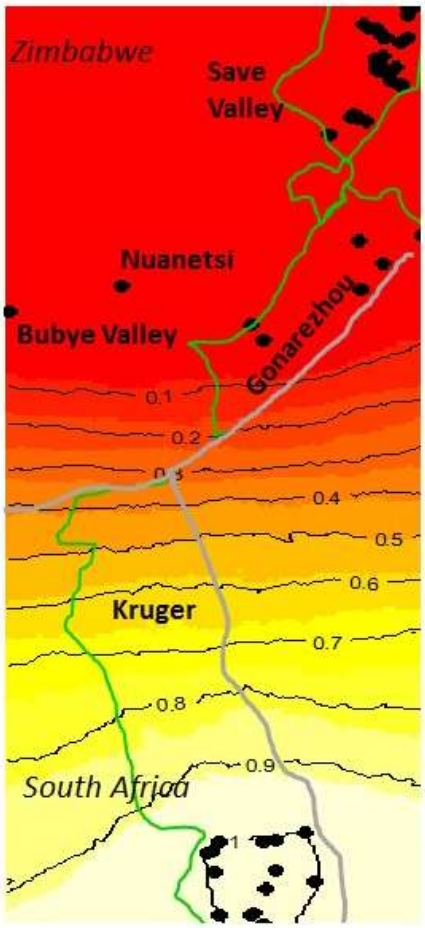

Fig. IV. Visualisation of the two spatial genetics units of African wild dogs in the GLTFCA, integrating spatial coordinates and information from mitochondrial and nuclear markers.

DISCUSSION

Genetic patterns among five geographically isolated African wild dog populations in the GLTFCA illustrate that allelic richness and heterozygosity of selectively neutral genes have been lost over time, presumably due to effects of inbreeding and genetic drift. Furthermore, genetic structuring in the GLTFCA has occurred due limited gene flow, which was most apparent between KNP and the Zimbabwean Lowveld. This raises some concerns about the robustness of natural meta-population dynamics within this important cluster of wild dog populations. These results are discussed in more detail below.

Given that wild dog numbers have substantially decreased over recent years, one would intuitively expect a loss of diversity. Such potential losses of diversity could have negative effects on the long-term survival of this

charismatic carnivore as acceptable levels of genetic diversity ensure high evolutionary potential through the ability to adapt. When data are compared to Marsden et al. (2012), a loss of allelic richness (t = -6.03; p = 0.000) and expected heterozygosity (t = -4.03; p = 0.002) over time is apparent, which could relate to a recent reduction in population size and restricted gene flow. A minimum of one generation has passed between the previous sample collection (2007) and our sample collection (2012). The data we used for comparison from KNP even originates from 1991-1995, which is over three wild dogs generations ago (Woodroffe and Sillero-Zubiri 2012). However, caution must be taken to use this data as representatives of genetic health (in the strict sense of the word) since our study included neutral markers (Holderegger et al. 2006). Nonetheless, previous studies similarly suggest that genetic diversity in adaptive markers also decreased in African wild dogs (Girman et al. 1997; 2001; Spiering 2011; Leigh et al. 2012; Marsden et al. 2012).

More than 50% of the wild dogs included in the present study shared a single mitochondrial haplotype which occurred throughout the study area. Several of the other haplotypes were also shared among populations. These shared mitochondrial haplotypes indicate that GLTFCA reserves historically existed in a meta-population network. When comparing our sequences to haplotypes generated by Marsden et al. (2012), it is clear that there was (or perhaps continues to be) some connectivity with eastern Africa, indicated by three individuals from GNP. The exchange of individuals among the reserves is low enough so as not to completely erode genetic structuring, as seen from unique haplotypes and the number of migrants calculated based on nuclear data. Structure analyses indicated the presence of two main clusters; these correspond to a Lowveld group in Zimbabwe and a Kruger group in South Africa. This division across the Limpopo River might be explained by the absence of settled wild dog packs in northern Kruger (Marnewick et al. 2014), and the large distance (approximately 300 to 400 km) between southern Kruger and the Lowveld of Zimbabwe. Why wild dog packs have not settled permanently in the northern parts of the Kruger remains moot; however, understanding the dynamics of wild dog settlement in specific regions is crucial to optimal management and corridor construction.

Because the geographical size of our study area is relatively small and wild dogs can disperse over far distances (McNutt 1996), a homogenous population might have been predicted for the study area. This somewhat surprising result indicating genetic structuring in the GLFTCA could relate to genetic drift in the absence of

frequent dispersal. The 'one-migrant-per-generation' model by Wright (1931) was originally assumed to be sufficient to overcome drift while allowing for local adaptation. But since many of the model's assumptions are violated in endangered populations, Mills and Allendorf (1996) suggested that up to 10 reproducing migrants per generation are required to maintain a genetically homogenous population. This would support our findings that despite the presence of gene flow in the GLTFCA, the amount of reproducing migrants per generation is too low to overcome genetic drift and avoid genetic structuring. This affect is enhanced in African wild dogs because only few migrating individuals have a chance to reproduce and add genes to the gene pool (Girman et al. 1997; Leigh 2005). The genetic structure found amongst wild dog populations suggests an isolation-by-distance model, which implies that the chance of mating, and sharing alleles, will decrease with increasing geographical distance (Wright 1942). Expectedly, dispersal between neighbouring reserves (mainly GNP) is more prevalent than those between geographically distant wild dog populations (SVC and KNP).

Limited natural dispersal of wild dogs in the GLTFCA does not have to be of major conservation concern, as inbreeding will still be largely prevented when few individuals successfully migrate and reproduce between populations (Lenormand 2002; Frankham et al. 2004). Genetic differences among populations may even be advantageous to increase overall regional genetic diversity (Storfer 1999), so in the current scenario translocations are not a management recommendation. Indeed, wild dogs have never been moved extensively around southern Africa, and to our knowledge never to any of the reserves included in this study. This should, however, not detract from the understanding that connectivity between the few remaining populations of wild dogs is partly constrained as a result of (a) lack of suitable habitat, (b) low survival rates during dispersal (see e.g. Gusset et al. 2008; Lindsey et al. 2005), and / or (c) a high mortality rate in the buffer zone of protected areas (Van der Meer et al. 2013). Unsuitable habitat between reserves, human encroachment and illegal poaching outside protected areas remain a major threat to wild dogs (ZPWMA 2009). This is the issue that should be the main focus in conservation efforts at present.

In conclusion, wild dogs in the GLFTCA have lost neutral genetic diversity over the last one to three generations as might be expected in an endangered species that has suffered recent population declines and a range contraction. Dispersal still occurs between the populations, although at lower frequencies than is optimal to

maintain allelic diversity. The GLTFCA is one of the last important refuges for African wild dogs, and enhancing dispersal and habitat connectivity within this system will contribute to the long-term survival of the species. We suggest trying to increase habitat corridors for natural dispersal between reserves in the GLTFCA together with efforts to secure a higher survival rate in unprotected areas. Identifying least-cost corridors to connect remote populations of endangered species is known to facilitate range-wide conservation (Huck et al. 2010; Rabinowitz and Zeller 2010).

Low costs of dispersal for wild dogs can be achieved by actively encouraging coexistence between wild dogs and people, through campaigns to encourage tolerance of dispersing wild dogs, reducing conflict through livestock protection interventions and minimising snare-related mortality through concerted anti-poaching efforts. Illegal poaching and other unnatural mortalities could be a barrier to dispersal and result in genetic isolation of far-distanced populations (Malviya and Ramesh 2015; Rodiquet et al. 2015). Therefore, reducing human-wildlife conflict is an important conservation tool for many far-dispersing large carnivores. Ongoing genetic monitoring at regular intervals, ideally every five years (based on their generation time; Woodroffe and Sillero-Zubiri 2012), will be necessary to evaluate whether these interventions effectively maintain genetic structure, and may provide insight as to whether human-mediated dispersal through translocation may become necessary in the future.

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