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Genetic conservation of South African wattled cranes

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ABSTRACT

The wattled crane (*Grus carunculatus*), a species highly dependent on wetlands, is the largest and rarest of the six African crane species. The once vast range of the wattled crane now consists of only three disjunct populations. The South African population has shown dramatic declines and supplementation of this population using eggs from south-central Africa has been proposed. The objectives of this study were to compare levels of genetic variation in South African and south-central African populations to determine if such supplementation is needed, and if so, whether the south-central African populations represent a genetically similar source for supplementation. We surveyed genetic variation in samples from South Africa, Zimbabwe, and Botswana using 12 microsatellite DNA loci and a 400-bp fragment of the mitochondrial D-loop. Samples from Zimbabwe and Botswana were deemed genetically similar and pooled to increase sample size. Subsequent analyses indicate that the pooled south-central and South African populations show differentiation in microsatellite DNA genotypes, as well as mitochondrial DNA. As the results from both genetic markers indicate genetic isolation, these populations should be managed as separate entities. As no indication was seen from either microsatellite or mtDNA data that significant loss of genetic diversity has occurred within South African wattled cranes, supplementation from outside populations may not be necessary at this time.

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1. Introduction

The crane family (*Gruidae*) is among the most threatened groups of birds in the world. Of the 15 extant species, 10 are considered globally threatened (Baillie et al., 2004). Among the many factors threatening cranes, habitat loss, poisoning, and disturbance are predominant in their decline (Meine and Archibald, 1996). The wattled crane (*Grus carunculatus*), the largest and rarest of the six crane species occurring in Africa (Meine and Archibald, 1996), is currently listed as vulnerable by the IUCN (Baillie et al., 2004). This species is highly dependant on wetland habitat for foraging and breeding (Konrad, 1981). Its dependence on wetland habitat makes this species particularly vulnerable to human impacts (McCann et al., 2000). Tarboton et al. (1987) indicates that if a wetland is repeatedly disturbed, these birds will completely abandon a breeding area. Because of the high sensitivity of wattled cranes to human impacts and disturbances, its presence provides a

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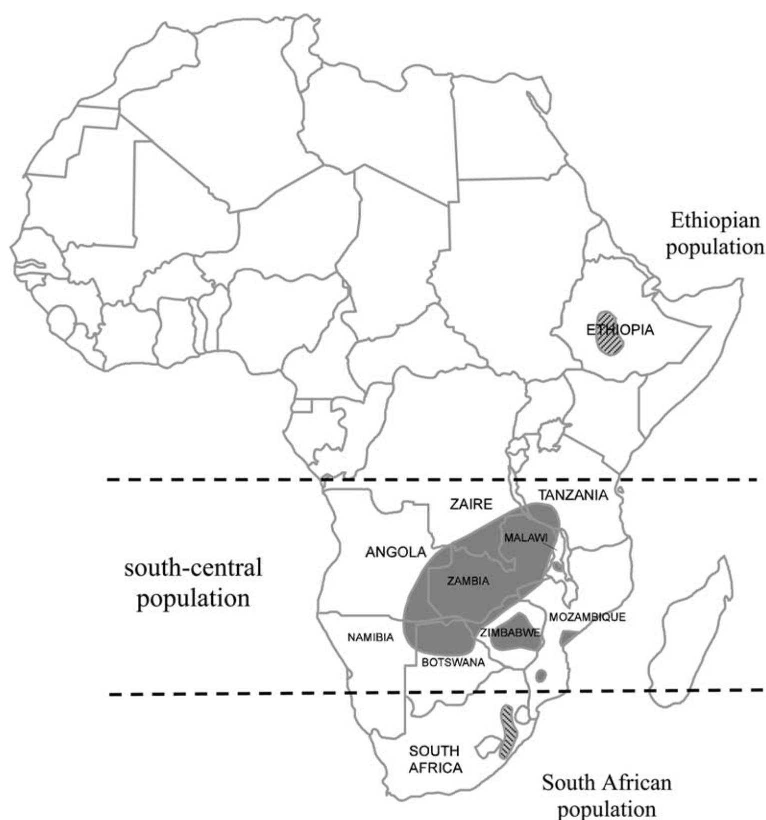

Fig. 1 – Geographical distribution of the wattled crane [adapted from Meine and Archibald (1996)]. The global population consists of three disjunct populations: Ethiopian, south-central, and South African.

positive statement of the health of that habitat (McCann et al., 2000).

The former range of the wattled crane now consists of only three disjunct populations (Fig. 1). Of the estimated 6000–8000 birds remaining, all but a few hundred birds occur in south-central Africa (Angola, Botswana, Congo, Malawi, Mozambique, Namibia, Tanzania, Zaire, Zambia, and Zimbabwe, Baillie et al., 2004, R. Beilfuss personal communication). Within the south-central region, Konrad (1981) suggests that there is strong evidence of movements of birds between subpopulations. The remaining birds reside in two populations that are geographically isolated from the south-central population and each other. These populations include several hundred birds in the highlands of Ethiopia (Meine and Archibald, 1996) and approximately 250 birds in South Africa (McCann et al., 2000). No morphologic differences between the populations have been described and there has not yet been a genetic assessment of wattled crane intra-specific differentiation (Meine and Archibald, 1996).

With a noted reduction in numbers across the range, organized breeding plans were implemented in 1989 to aid in the preservation of wattled cranes (Meine and Archibald, 1996). The international breeding program was initiated primarily with cranes from south-central Africa being exported to many captive rearing facilities within and outside of Africa. Because the South African population, in particular, was declining at an alarming rate (McCann et al., 2000), an additional captive breeding and supplementation program was initiated in South Africa to meet the special needs of that population (Burke et al., 2001). The South African captive population was comprised of birds collected in South Africa and augmented by collections of second eggs from wild South African nests. Due to the success of their captive effort, the first experimental release took place in 1996 (Meine and Archibald, 1996). Along with releases of South African birds reared from collected second eggs, supplementation of the South African population has been proposed using eggs that were laid by captive birds of south-central African ancestry. If the remnant South African population has suffered a loss of genetic variation, an influx of new breeders could serve to replenish variation that could be important for long term persistence (Mills and Smouse, 1994; Frankham, 1995, 1996). However, if the translocation crosses natural breeding barriers, outbreeding depression could result if the populations are presently on differential evolutionary tracks and have evolved adaptations to local environments (Storfer, 1999). Thus, translocation plans should consider the origin and diversity of translocated individuals and their impact on the target populations (Moritz, 1999). Before translocation of south-central African birds into the South African population occurs, molecular genetic data are needed to assess the current genetic status of the South African population. To this end, we assessed and compared the levels of genetic variation found in both the South African and south-central African populations to determine if the loss of genetic variation within the South African population is sufficient to warrant

supplementation, and if so to verify if the south-central African population is of the proper genetic ancestry for supplementation to the South African population. To achieve these goals, we surveyed genetic variation in samples from South Africa, Zimbabwe, and Botswana using mitochondrial and microsatellite DNA loci.

2. Methods

2.1. DNA Sampling

DNA samples were collected from wattled cranes in South Africa (12), Zimbabwe (11), and Botswana (3). For these 26 birds, whole blood was drawn from captive birds of known origin and placed into a storage/lysis buffer [0.1 M Tris, 0.1 M EDTA, 5% SDS, 0.01 M NaCl, (Longmire et al., 1991)] for later extraction. Isolation of DNA from the lysate was accomplished using the Promega Wizard Genomic Purification Kit (Promega Corp., Madison, WI).

2.2. Molecular genetic analysis

Each sample was genotyped at 12 microsatellite loci (Gamμ1, Gamμ2, Gamμ3, Gamμ4, Gamμ5, Gamμ6, Gamμ7, Gamμ9, Gamμ12, Gamμ15, Gamμ101, Gamμ102, see Glenn et al., 1997; Jones et al., 2002) and one 400 base pair sequence of the mitochondrial D-loop using Glenn et al.'s (1999) L-17 and H-393 primers. All microsatellite and mitochondrial DNA markers used were originally designed for whooping cranes (*Grus americana*) (Glenn, 1997), a closely related species to wattled cranes (Krajewski and Fetzner, 1994). Microsatellite amplification followed procedures previously published for crane species (Jones et al., 2002; Jones, 2003). We performed the microsatellite genotyping on a MJ BaseStation automated DNA sequencer [MJ Research, San Francisco, CA]. The fluorescently labeled PCR products were run together with an internal size standard (Promega CXR (red) 60–400 bp ladder) and analyzed with CARTOGRAPHER software (MJ Research, San Francisco, CA).

Mitochondrial D-loop amplicons were sequenced using methods described for the Dynamic ET Kit (Amersham Biosciences Corp., Piscataway, NJ) and run on the MJ BaseStation automated sequencer. Sequences, exported from CARTOGRAPHER software, were aligned using CLUSTAL-X (Thompson et al., 1997) to elucidate areas of polymorphic sequence.

2.3. Microsatellite DNA analyses

Tests for Hardy–Weinberg equilibrium and linkage disequilibrium were performed with GENEPOP (Raymond and Rousset, 1995), and a sequential Bonferroni test (Rice, 1989) was used to compensate for multiple comparisons. Calculations of proportion of polymorphic loci, mean number of alleles per locus, mean number of alleles per polymorphic locus, and observed (H_o) and expected heterozygosity (H_e) were performed using GENETIC DATA ANALYSIS (GDA, Lewis and Zaykin, 2000). Allelic richness, an estimate of allelic diversity that compensates for unequal sample sizes (El Mousadik and Petit, 1996; Petit et al., 1998), was calculated and averaged across loci using FSTAT (Goudet, 2001).

Population differentiation was examined using several approaches. Using Weir and Cockerham's (1984) estimator theta (θ), overall and pairwise analyses of F_{ST} were conducted using GDA and tested for significance by bootstrapping. Overall and pairwise values for the number of migrants per generation between populations (N_m) were calculated using Slatkin's private allele method (Slatkin, 1985; Barton and Slatkin, 1986). An analysis of molecular variance (AMOVA, Excoffier et al., 1992) was performed using ARLEQUIN (Schneider et al., 2000) and tested for significance by permutation. A principal components analysis was used to investigate the correlation of allele frequencies and subsequent genotypes between all individuals sampled using PCAGEN, a program written by Jérôme Goudet (Institute of Ecology, University of Lausanne, Lausanne, Switzerland). Unlike the previous tests, the PCA was performed on an individual basis. Performing the analysis in the absence of population information allows unbiased testing of population structure. From the PCA results, a two dimensional canonical plot showing the first two principal components was produced.

To supplement F_{ST} analyses, we used the Bayesian clustering method of Pritchard et al. (2000) to identify cryptic genetic structure using only genotypic data. Based on Hardy–Weinberg expectations, Pritchard et al.'s (2000) program STRUCTURE iteratively assigns individuals to a user-defined number of anonymous genetic clusters using only information from their genotypes. To reveal genetic structure that may not be apparent otherwise, we ran three independent STRUCTURE analyses using simulations of 100,000 burn-in iterations and 500,000 data iterations to classify individuals into one, two, and three genetic clusters (K), respectively. The probability of the number of K ($P(K/X)$) was determined using the methods of Pritchard et al. (2000). After determination of relevant values for K, individual admixture proportions (i.e., the proportion of an individual's genetics assigned to each genetic cluster) were sorted and displayed by population.

2.4. Mitochondrial DNA analysis

The aligned D-loop sequences were subjected to analyses of haplotypic divergence and gene flow at the population level. First, the individual variability within each of the mitochondrial polymorphic sites was used to define sequence divergence between all haplotypes, where haplotypic divergence was calculated as the number of mutational differences between haplotype sequences. As this is a test of mutational differences between sequences, duplicated sequences were removed prior to analysis. The resulting matrix of pairwise differences between haplotypes was then used to produce a minimum spanning network using MINSPNET written by Laurent Excoffier (Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva). Secondly, gene flow (N_m) and genetic differentiation (F_{ST}) between the south-central and South African populations was estimated from the sequence data using DNASP (Rozas and Rozas, 1997). Estimates of N_m and F_{ST} were derived using calculations from Hudson et al. (1992).

3. Results

3.1. Microsatellite DNA analyses

As the Botswana and Zimbabwe samples are assumed to be in the same genetic population (Konrad, 1981), a preliminary test was done to see if combining these samples into one population would be appropriate. The preliminary analysis of population structure shows that significant F_{ST} values ($p < 0.05$) were found between South Africa and the Botswana and Zimbabwe samples, respectively, and a non-significant F_{ST} value between Zimbabwe and Botswana (Table 1). Similarly, estimates of the number of migrants per generation (N_m) based on the private allele method show higher rates of gene flow between Zimbabwe and Botswana than to South Africa (Table 1). Additionally, Table 2a shows that there were many unique (i.e., private) alleles found in both the South African and Zimbabwe populations. Botswana, however, had only one unique allele. A subsequent analysis, which combined Botswana and Zimbabwe samples, revealed four alleles shared between Zimbabwe and Botswana samples that were not observed in the South African samples (Table 2b). Using Bayesian approaches to determine the number of population subdivisions within these three populations, STRUCTURE suggests that only two genetic clusters exist: South Africa and Zimbabwe/Botswana. As no analyses show differentiation between Botswana and Zimbabwe, we combined these samples together into a single south-central population sample that was used for subsequent analyses.

Within the new pooled data set, the microsatellite genotypes were consistent with Hardy–Weinberg expectations. No test was significant after correction for multiple comparisons. Although the South African population shows a slight reduction in all diversity values, the two populations are not significantly different in gene diversity (H_e), heterozygosity (H_o), or allelic richness (Table 3). The slightly higher gene diversity and allelic richness in the south-central samples is consistent with the finding that more private alleles occur in this population (Table 2b).

After combining Zimbabwe and Botswana samples, the estimates of F_{ST} (0.14, $p < 0.05$) and N_m (0.50) between South Africa and the south-central populations indicate a significant divergence between these populations. The analysis of molecular variance (AMOVA) indicates that although most of the genetic variability lies within individuals (87%, $p < 0.0001$), a significant amount of the variation (13%, $p < 0.0001$) is due to the differences seen between South African and the south-central population. The principal components analysis shows a similar pattern as the more traditional F_{ST} tests. In this analysis where no geographic provenance was used, 35% of the variation can be plotted in one graph (Fig. 2). The resulting canonical plot of individuals illustrates that although there is not strong clustering into populations, the genotypic variation exhibits clear structure. With the exception of individual Z7, Zimbabwe and South African populations are spatially separated. Similarly, we sorted and graphed STRUCTURE's individual admixture proportions by population (Fig. 3). Where two genetic clusters were specified, individuals generally fell within there respective South African and Zimbabwe/Botswana groups. Identical to the results of the PCA, Z7 was the only bird not correctly assigned to its population of origin.

Table 1 – Overall and pair-wise estimates of F_{ST} (lower) and N_m (upper) based on microsatellite DNA genotypes

	South Africa	Zimbabwe	Botswana
South Africa	–	0.47	0.34
Zimbabwe	0.10*	–	0.91
Botswana	0.18*	0.00 NS	–
Overall F_{ST}	0.10*		
Overall N_m	0.55		

Asterisks represent comparisons that are significant at $p < 0.05$.

Table 2 – Alleles found in only one population (i.e., private alleles)

Locus	Allele	Frequency	Found in
(a)			
Gamµ6	126	0.75	South Africa
Gamµ102	124	0.08	South Africa
Gamµ3	115	0.04	South Africa
Gamµ5	178	0.14	Zimbabwe
Gamµ101	204	0.14	Zimbabwe
Gamµ7	145	0.09	Zimbabwe
Gamµ101	186	0.05	Zimbabwe
Gamµ9	193	0.05	Zimbabwe
Gamµ3	107	0.05	Zimbabwe
Gamµ101	201	0.25	Botswana
(b)			
Gamµ6	126	0.75	South Africa
Gamµ102	124	0.08	South Africa
Gamµ3	115	0.04	South Africa
Gamµ101	201	0.25	Zim/Bots
Gamµ6	**120**	**0.25**	**Zim/Bots**
Gamµ1	**134**	**0.15**	**Zim/Bots**
Gamµ7	**133**	**0.14**	**Zim/Bots**
Gamµ5	178	0.14	Zim/Bots
Gamµ101	204	0.14	Zim/Bots
Gamµ7	145	0.09	Zim/Bots
Gamµ2	**177**	**0.07**	**Zim/Bots**
Gamµ101	186	0.05	Zim/Bots
Gamµ9	193	0.05	Zim/Bots
Gamµ3	107	0.05	Zim/Bots

The first table (a) shows the private alleles when all three populations are compared. An additional table was completed (b), showing the private alleles when Botswana and Zimbabwe samples were combined. Combining these samples elucidated four alleles (bold) shared between Zimbabwe and Botswana that were not observed in South Africa.

3.2. Mitochondrial DNA analysis

For the mitochondrial D-loop sequences, 15 sites out of 400 were polymorphic. The minimum spanning network produced by MINSPNET (Fig. 4) illustrates the relationship between the resulting 10 haplotypes. Nine of the 10 haplotypes were unique to the population where they were found; and only haplotype 5 occurred in both Zimbabwe

Table 3 – Descriptive statistics for each population derived from multilocus microsatellite DNA genotypes

Population	Number of samples genotyped	Proportion of polymorphic loci	Alleles per locus	Alleles per polymorphic locus	Allelic richness	Expected heterozygosity (H_e)	Observed heterozygosity (H_o)	Inbreeding within subpopulations (F_{IS})
South-central	14	0.75	2.75	3.33	2.70	0.32	0.29	0.11
South Africa	12	0.67	2.08	2.62	2.08	0.28	0.26	0.05

and South Africa. To further investigate whether one of these two populations gained this common haplotype via a migratory event, we examined microsatellite DNA similarity between individuals carrying the common haplotype. In both the PCA and STRUCTURE diagrams (Figs. 2 and 3), haplotype 5 occurs in birds containing distinct South African and south-central nuclear backgrounds, respectively.

The analyses for matrilineal gene flow and differentiation using mitochondrial DNA variation indicate a significant lack of female gene flow between the South African and south-central populations. Low rates of female migration ($N_m = 0.30$) and high maternal population differentiation ($F_{ST} = 0.45$, $p < 0.05$) provide insight into the structured geographical distribution of the sequences seen in the minimum spanning network (Fig. 4).

4. Discussion

Measures of genetic variation, such as allelic richness and gene diversity (H_e), in all crane species assessed to date are generally low compared to many bird species (Hasegawa et al., 2000; Neff and Gross, 2001; Frankham et al., 2002; Jones et al., 2002, 2005a,b). Wattled cranes are the rarest of the African crane species and show levels of variation only slightly higher than the critically endangered whooping crane (Jones et al., 2002). However our results show that with an average of 2.62 alleles per polymorphic microsatellite DNA locus and six mitochondrial D-loop haplotypes, the South African wattled crane population is not statistically different than the south-central population (3.33 alleles per polymorphic locus and five mitochondrial haplotypes) with respect to levels of molecular genetic variation. Thus, the reduced South African population has retained comparable levels of genetic variation to that of the larger south-central population. Additionally, as mtDNA is more sensitive to bottlenecks than are nuclear loci (Birky et al., 1983; Ashley and Wills, 1987), the retention of six mitochondrial haplotypes within the 12 South African samples indicates that this population does not currently represent a genetically bottlenecked population. With regard to allelic differentiation between populations, there were microsatellite alleles unique to each population. Most notably the South African's private allele at 75% frequency and the south-central population's five alleles between 25% and 14% frequency indicate a lack of gene flow between South Africa and the south-central population. This conclusion is also supported by the F_{ST}, N_m, and AMOVA analyses of the microsatellite DNA data, where significant differences are seen between south-central and South African populations.

In addition to the tests of divergence based on known population assumptions, the principal components and STRUCTURE analyses provided the opportunity to view genetic differentiation in the absence of a priori assumptions of population structure. Although divergence was shown between the Zimbabwe and South African populations, one Zimbabwean individual (Z7) was placed among the South African individuals. Because of the commonality of many microsatellite alleles across all populations, it is possible to obtain composite genotypes that are shared by both South African and Zimbabwe populations. As this bird has a mitochondrial

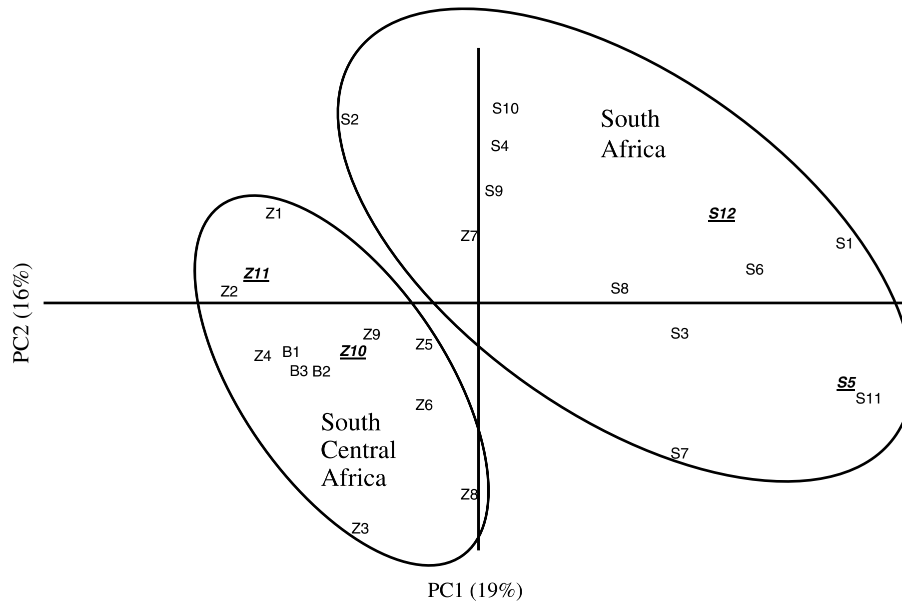

Fig. 2 – Canonical plot of the first two principal components. Ellipses were drawn to illustrate the relative positions of the individuals from each population. Individuals sharing the mitochondrial haplotype 5 are underlined.

DNA haplotype found only in other Zimbabwe birds, the genetic similarity of this bird to South African birds is likely due to chance sharing of common alleles rather than close genetic relatedness.

As with the microsatellite DNA data, the mitochondrial DNA sequence data gives an indication of subdivision between these populations. With only a single haplotype being shared across the south-central and South African populations, the data show a significant lack of matrilineal gene flow across the region. Because the four birds with mitochondrial haplotype 5 do not exhibit microsatellite similarity (Figs. 2 and 3), the common haplotype is likely shared across the populations due to evolutionary ancestry rather than recent migration. This hypothesis is supported by the minimum spanning network (Fig. 4) which shows that this shared haplotype may be the ancestral haplotype. In this study, each population had private mitochondrial haplotypes indicating low inter-populational female mediated gene flow, further illustrating that the south-central and South African populations belong to separate evolutionary lineages.

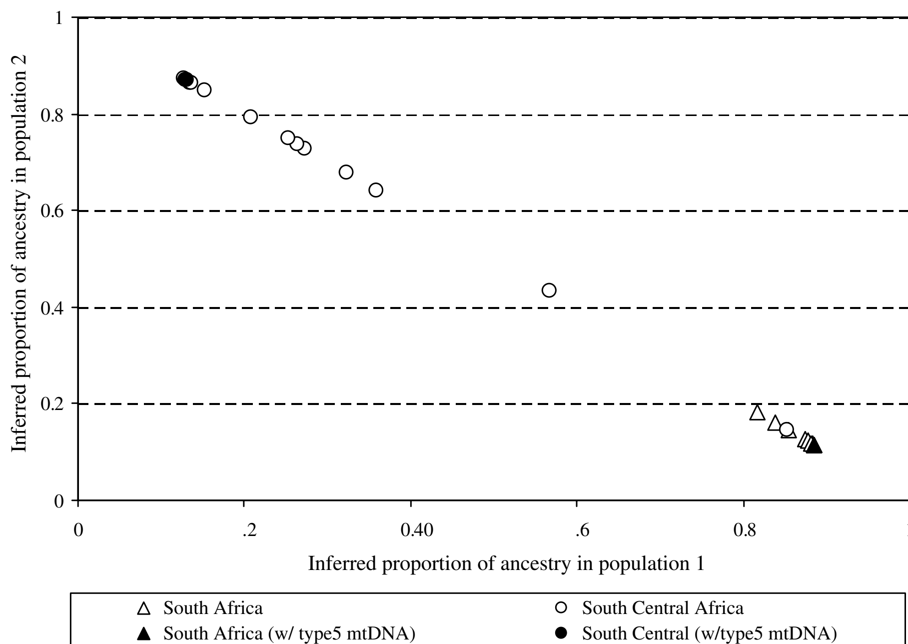

Fig. 3 – Bayesian analysis of the microsatellite DNA genetic structure. The genetic proportions assigned to each cluster (i.e., the admixture proportion) were plotted for all individuals when $K = 2$. Closed symbols indicate individuals sharing the mitochondrial haplotype 5.

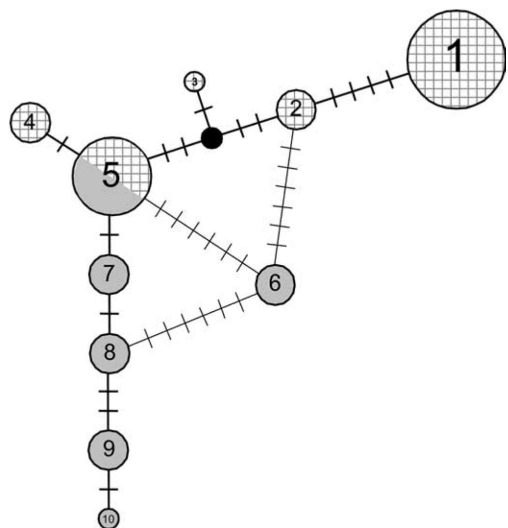

Fig. 4 – Mitochondrial D-loop minimum spanning network. The network was derived from pairwise comparisons of the number of mutational changes between haplotypes using the program MINSPNET. The haplotypes had the following geographical distribution: south-central (haplotypes 1–5); South Africa (haplotypes 5–10). The size of the haplotypic circles represents frequency of occurrence.

Despite small sample sizes, the results of this study support a hypothesis of limited historical gene flow between South Africa and the south-central population. Thus, the South African population is genetically distinct. Genetic drift after recovery from a severe population bottleneck in South Africa could produce such a pattern of differentiation, yet the high levels of mtDNA diversity combined with the relatively equal levels of nuclear variability across the range argue against a genetic bottleneck in that population. In this case, historically limited gene flow is a more plausible explanation. Although once a part of an ancestral population, the current DNA results suggest that the South African population has been isolated from the south-central population for many generations. Given the evidence for genetic isolation presented here, the South African population should be managed separately from the south-central population, at least until additional analyses using larger sample sizes are available. These preliminary findings of genetic distinctiveness of the southern population suggest that translocations between South Africa and the south-central population are not currently advisable.

It is interesting to note that the differentiation seen between these study populations occurred across an area of approximately 600 km, a one-day flight for some cranes (e.g., Melvin and Temple (1982) show that daily migration distances for sandhill cranes can be as much as 740 km). A possible explanation for these findings is that the Kalahari Desert may be providing a geographic barrier to gene flow. Although usually described for non-avian species, the arid regions between South Africa and Botswana/Zimbabwe has been implicated in genetic isolation of several species (van Hooft et al., 2000, and citations within). For the wattled crane in particular, the lack of abundant food resources (e.g., tubers and rhizomes of Cyperus and Eleocharis sedges and Nymphaea waterlilies, Douthwaite, 1974; Konrad, 1981) appears to provide a sufficient deterrent for range expansion across the arid Kalahari region. This conclusion has implications for other crane species as well. It is a common perception that this family of very large birds capable of sustained flights of hundreds of kilometers are likely to intermix if populations are very close, but our results show that we cannot assume population interchange.

Although we can say that gene flow has been limited across the Kalahari, we do not have adequate samples to test gene flow from the south-central population via coastal Mozambique. Within the last century, two historical wattled crane sightings intermediate to the current Zambezi Delta and South African populations have been noted (West, 1976). There is a possibility that gene flow occurred to and from South Africa through the eastern extent of the south-central population rather than at the southwestern border that bounds the Kalahari Desert. If gene flow occurred from Zimbabwe to Mozambique, and Mozambique to South Africa, clinal variation could explain the genetic pattern seen in this study. Additional sampling from the eastern sections of the south-central range will be necessary to explore the possibility that gene flow existed via the coastal Mozambique population.

The significant differences seen between these study populations that are only a few hundred kilometers apart suggests the possibility that other wattled crane populations may have also been historically isolated. A high possibility exists that Ethiopian wattled cranes, far removed from the others by the equatorial zone, represent a distinct subspecies. Current projects are on-going to analyze evolutionary differences across the full range of this species.

The microsatellite and mtDNA analyses for wattled cranes presented here have direct management implications for this species and have more general applicability for conservation of threatened species. The use of two distinct marker systems and several different analytical tools allowed robust inferences regarding demographic history and historical gene flow patterns, despite small sample sizes. The increasing availability of DNA data and new analytical methods for conservation genetics provide new types of information with improved accuracy while relying on fewer assumptions (Pearse and Crandall, 2004). When carefully applied, DNA analyses can supplement more traditional morphometric and geographic surveys to help define units of conservation management by evaluating current and evolutionary gene flow between populations. Such analyses can determine, for example, whether a population is a unique and isolated remnant population or is simply a sink for a nearby source population. Conservation efforts guided by the best genetic tools currently available will allow limited conservation resources to be better allocated and improve prospects for successful recovery of threatened species.

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