The Knysna elephants: a population study conducted using faecal DNA

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Abstract

The elephants of the Knysna region continue to survive, despite fears that there was only a single surviving female. Their range is larger than previously believed, and includes the Afromontane forest and mountain fynbos. The five individuals detected in this study were all females, and share a single mitochondrial DNA control region haplotype with individuals from Addo Elephant National Park. At least two of these elephants appear to be first-order relatives, and the others may be part of a single matrilineal group. The genetic diversity detected is lower than that found in most African savanna populations, but is higher than that found at Addo, where individuals represent the descendents of a severe population size bottleneck. Levels of genetic diversity are more similar to those detected at Kruger National Park, suggesting that the Knysna elephants represent a remnant of the once widespread populations of South Africa.

Key words: faecal DNA, noninvasive sampling, relict population

Résumé

Les éléphants de la région de Knysna ont survécu, même si l'on a craint un moment qu'il ne subsiste qu'une seule femelle. Leur dispersion est plus vaste qu'on ne le croyait et comprend la forêt afromontagnarde, le fynbos de montagne. Les cinq individus repérés lors de cette étude étaient tous des femelles et partagent un seul haplotype dans la région de contrôle de l'ADN mitochondrial avec les individus du Parc National d'Addo. Au moins deux de ces éléphants semblent avoir des liens familiaux de premier ordre et les autres pourraient faire partie d'un même groupe matrilinéaire. La diversité génétique détectée est plus faible que celle qu'on trouve dans la plupart des populations des savanes africaines, mais elle est plus élevée que celle que l'on observe à Addo, où les individus représentent les descendants d'un seul résidu extrêmement petit d'une population antérieure. Le niveau de diversité génétique est plus semblable à celui relevé au Parc National Kruger, ce qui suggère que les éléphants de Knysna sont un résidu des populations autrefois répandues en Afrique du Sud.

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Introduction

In the forests and fynbos of the Knysna region of the southern Cape of South Africa, a small population of elephants continues to survive. These are the southern-most elephants in the world and represent a very small fraction of the estimated 10,000 elephants that inhabited the Cape prior to the arrival of European settlers in 1650 (Hall-Martin, 1992). Lydekker (1907) classified the Knysna elephants as a distinct subspecies, (*Elephas africanus toxotis*), and they were rumoured to be the largest living elephants (Greig, 1982). Although a subsequent genetic study cast doubt on the distinctiveness of the population of elephants at Addo Elephant National Park (Essop, Hall-Martin & Harley, 1996) and this has been extrapolated to include the Kynsna elephants, the Knysna population itself has not been studied genetically.

The decline of the Knysna elephants has occurred over decades, and probably began with the liberal granting of hunting licenses during the 19th century (Domisse, 1951). Beginning in 1860, the elephants of this region were given protection by the Cape government, but by 1876 only an estimated 400–500 elephants remained and these were under heavy pressure by ivory hunters (Phillips, 1925). In 1908, the population was declared to be Royal Game,

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having declined to an estimated 20 individuals (Kinloch, 1968). In 1970, the population was estimated at eleven, and the continued decline was attributed to illegal hunting (Carter, 1970).

In 1994, three young female elephants from Kruger were introduced in an effort to supplement the Knysna population. The smallest one died on release, but the other two were expected to join what was believed to be the only surviving Knysna elephant, an elderly cow, in the montane forest region. However, they only joined her for two short periods in 1994–1995, choosing instead to spend approximately 80% of their time in the more open fynbos habitat (Seydack, Vermeulen & Huisamen, 2000; Milewski, 2002). Disappointed that they ranged well outside the approximately 200 km^2 forest region (Patterson, 2004) and did not team up with the elderly cow, conservation officials moved the young elephants to the Shamwari Game Reserve in the Eastern Cape in 1999. Before their removal; however, the Kruger females revealed a great deal about the ecology of savanna elephants in an Afromontane forest and fynbos habitat.

This study is only one part of a larger investigation of the distribution, diet, range, and history of the Knysna elephants that was conducted by Gareth Patterson. Here, we investigate the number of unique multi-locus genotypes, or individuals, detected in an extensive dung survey, the sexes of those individuals, the relatedness between them and the level of genetic diversity present in this population. The results suggest that the surviving Knysna elephants are closely related to the elephants of Addo Elephant National Park and Kruger National Park and that the level of genetic variability is lower than that found in savanna populations by Comstock *et al.* (2002). Both the allelic diversity and heterozygosity values are higher than those found by Whitehouse & Harley (2001) at Addo. Despite the small population size, the level of genetic diversity is comparable with that found at Kruger NP.

Materials and methods

Thirty-five dung samples were collected opportunistically between November 2002 and February 2003. Estimates of the age of the dung were made during collection and, when possible, the circumference of the dung bolus was measured. Samples were boiled in the field to prevent the transmission of disease, and preserved using a buffer of 20% DMSO, 100 mM Tris and 0.25 M EDTA, saturated with NaCl (Amos *et al.*, 1992). They were stored at room temperature prior to shipment to the laboratory for analysis.

Total genomic DNA was extracted using the guanidine/thiocyanate/silica method described in Eggert, Rasner & Woodruff (2000); Eggert, Maldonado & Fleischer (2005), including control blanks for each reagent. For identification of individuals, samples were screened at nine microsatellite loci previously developed for African elephants (Table 1). Amplifications were performed in an MJ Research PTC-100 thermocycler in 10 μl volumes containing 0.5 U Ampli*Taq* Gold® DNA Polymerase (Applied Biosystems, Inc., Foster City, CA, USA), 1X Ampli*Taq* Buffer II, 0.4 μM fluorescently labelled forward primer, 0.4 μM unlabelled reverse primer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 10X BSA (New England Biolabs, Ipswich, MA, USA), and 1 μl of the DNA extract. The profile consisted of a single denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95° for 45 s, annealing at locus-specific temperatures (Table 1) for 35 s and primer extension at 72°C for 35 s. Amplification products were separated in an ABI 373 or ABI 3100 automated sequencer (Applied Biosystems, Inc.) and allele sizes were scored using GENESCAN 2.7 and GENOTYPER 2.5 (Applied Biosystems, Inc.). An east African savanna elephant sample was included as a control in all reactions, and served to standardize scores between genotyping reactions and DNA sequencing platforms. Samples were amplified at each locus twice for confirmation and all homozygous genotypes were confirmed in a third amplification reaction.

The data was analysed in GENEOP v.3.4 (Raymond & Rousset, 1995) to detect significant departures from

Table 1 Microsatellite loci used to identify individual Knysna elephants

Locus	AT(°C)	No. alleles	H_{obs}	Citation
FH48	57	5	0.800	Comstock *et al.*, 2000
FH60	61	4	0.600	Comstock *et al.*, 2000
FH67	55	2	0.000	Comstock *et al.*, 2000
FH71	52	6	0.800	Comstock *et al.*, 2000
FH103	57	2	0.500	Comstock *et al.*, 2000
LA4	52	2	0.500	Eggert *et al.*, 2000
LafMSO3	50	2	0.500	Nyakaana & Arctander, 1998
LafMSO5	49	5	0.750	Nyakaana & Arctander, 1998

No. of alleles, average (SD) = 3.5 (1.7); H_{obs}, average (SD) = 0.556 (0.261).

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expected values under Hardy–Weinberg equilibrium and significant linkage disequilibrium between loci. To examine the power of the microsatellite loci to differentiate between individuals in the population, the program PROB-id5 was used. This program computes $P(ID)_{random}$, the power to differentiate between randomly chosen individuals, as well as a more conservative measure, $P(ID)_{sib}$, the power to differentiate between siblings (Waits, Luikart & Taberlet, 2001).

Relatedness between individuals was inferred using KINSHIP 1.3.1 (Queller & Goodnight, 1989), a program that is designed to perform pairwise relatedness calculations using data from codominant, single-locus genetic markers such as microsatellites. The program can also test the likelihoods of the values generated by generating a series of pairs at random (using the allele frequencies) and determining the resulting likelihood ratios. The program then finds the ratios needed to exclude errors at significance levels of $P = 0.05$, 0.01 and 0.001.

The sex of the individual was determined using the ZFX/ZFY loci and the methods outlined in Fernando & Melnick (2001). To investigate the relationships between matrilines in Knysna and other African populations, a 593-bp fragment of the mitochondrial DNA control region was amplified using primers MDL3 and MDL5 (Fernando *et al.*, 2000). Amplification products were purified using the QiaQuick PCR Purification Kit (Qiagen, Valencia, CA, USA), and sequences for both strands were determined in ABI 3100 automated sequencer (Applied Biosystems, Inc.). Sequences were aligned and compared in SEQENCHER 4.2.2 (Gene Codes Corp., Ann Arbor, MI, USA).

Results

Twenty-one of the thirty-five samples were estimated to be 1–4 days old. Age of dung was estimated by the presence/absence of fatty sheen, colouration, odour, decay, as well as consideration of climatic conditions and exposure to light. Other evidence of the elephant, such as footprints and feeding signs, also contributed to age estimation. Of these, 18 (86%) were successfully genotyped. Five samples were estimated to be between 5 and 9 days old and genotypes were obtained from four (80%) of these. The remaining nine samples were estimated to be older than 10 days and none was successfully genotyped at more than four loci.

Eight of the nine loci were found to be polymorphic, having between two and six alleles (Table 1). The value of $P(ID)_{random}$, or the power to differentiate between randomly chosen individuals, was estimated at 9.4×10^{-12}, and the more conservative measure, $P(ID)_{sib}$, the power to differentiate between siblings was estimated to be 0.0002. Once it was confirmed that these loci were sufficient to provide the power to differentiate between siblings, genotypes were compared using an Excel macro program and unique genotypes were identified. Those that differed at either one or two loci were re-examined to reduce the probability of individuals being identified based on allele scoring or data entry errors.

Five unique genotypes were detected between two and nine times (Table 2). All loci were found to conform to Hardy–Weinberg expectations, and there was no linkage disequilibrium detected between loci. All genotypes were found to represent female elephants. Bolus circumferences did not differ significantly between individuals (Kruskal–Wallis $_{0.05,4} = 2.62$, $P > 0.50$), which may result from the fact that large differences were observed between measurements for a single individual (Table 2).

The five elephants shared a single mitochondrial DNA control region haplotype, one that is identical to one of the

Table 2 Sex and bolus circumference for individuals detected in this study

Sample	Sex	Sampling date	Duplicate	Dung bolus circumference (cm)
KE7	F	24-Nov-02		40.5
		24-Nov-02	KE8	37.0
		24-Nov-02	KE9	40.0
KE10	F	15-Jan-03		Not measurable
		26-Jan-03	KE15	44.7
		16-Feb-03	KE25	43.5
		22-Feb-03	KE27	37.5
KE14	F	26-Jan-03		40.5
		29-Jan-03	KE16	Not measurable
		12-Feb-03	KE23	38.0
		22-Feb-03	KE28	34.8
		22-Feb-03	KE29	42.0
		22-Feb-03	KE30	47.0
		22-Feb-03	KE31	43.0
		22-Feb-03	KE33	38.9
		22-Feb-03	KE35	39.1
KE11	F	18-Jan-03		40.2
		18-Jan-03	KE12	41.5
		18-Jan-03	KE13	47.4
		2-Feb-03	KE17	39.0
KE32	F	22-Feb-03		39.0
		22-Feb-03	KE34	37.8

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two detected by Eggert *et al.* (2000) at Addo Elephant National Park (haplotype Addo1). This haplotype differs by 1 bp from the first and 3 bp from the second of the two haplotypes detected by Nyakaana & Arctander (1999) at Kruger National Park.

KINSHIP analysis suggests that individuals KE10 and KE14 are very closely related. The relatedness value of 0.54 suggests a first-order, or parent-offspring, relationship but cannot tell us which individual is likely to be the parent. Examination of the dung bolus circumferences might have been helpful, as the mother of a younger individual might have larger boli. However, they are not informative due to large size differences between the different measurements from each individual. Relatedness values between 0.29 and 0.38 were found for these pairs: KE7/KE10, KE11/KE14 and KE11/KE32. All of these relatedness values were found to be significant at $P < 0.001$.

Discussion

The presence of four elephants in the Knysna region had been shown previously. Just prior to this study, a forest guard sighted a young bull, and early in the project author G. Patterson discovered evidence of another two young individuals. The fourth elephant, a young female, was seen by forest guards in 2002. Thus, the fifth elephant found in this study, a female, brings to six the number of probable Knysna elephants, as the male was not detected. The Knysna elephants do not seem to be, as some had predicted, inevitably doomed to extinction.

Since the completion of this study, there is evidence that suggests that a Knysna calf was born. Dung boli circumference was 23 cm, suggesting a calf of approximately 1.6 years old. This, and other evidence gathered by Gareth Patterson, indicates that at the very least, one breeding bull is or has recently been present in this population.

Nevertheless, there are reasons for concern. First, there appear to be few breeding bulls in this population. The young bull was not detected in this study, despite extensive collection efforts. Of the 35 samples collected, only one had a bolus circumference of <35 cm. Thus, our study provides no evidence of young calves, or that the females were reproducing. Other elephant populations in South Africa, most notably the Addo elephants, have survived population size bottlenecks and increased impressively following protection from illegal hunting. At least one study has suggested that the lack of increase in the Knysna elephant population is the result of poor nutrition (Seydack *et al.*, 2000). While our study can make no claims about elephant nutrition, we can suggest that a lack of breeding bulls may be an alternate explanation for the current stagnation of population size.

Secondly, the elephants detected in this study appear to be related. At least one pair may be first order relatives and several others may be half-siblings. Although there is currently more allelic diversity present in these five elephants than in a comparable sample from Addo, the small size of this population means that genetic drift is likely to be a serious problem. Assuming that these females breed, at such a small population size, inbreeding will likely occur once the offspring of these elephants reach breeding age. While the introduction of a single bull might initiate reproduction now, it may be wise to consider introducing a second bull once the female offspring reach approximately 10 years of age, to encourage outbreeding.

The single mtDNA control region haplotype of the Knysna elephants matches one detected previously at Addo Elephant National Park, and is similar to the two haplotypes detected at Kruger National Park by Nyakaana & Arctander (1999). These haplotypes were found to be closely related by Eggert *et al.* (2000) and were joined in a clade of African savanna elephants by two haplotypes from Zimbabwe. While all of the South African haplotypes were found in this clade, the elephants of Zimbabwe, Namibia and Botswana were found in both deeply diverged savanna clades (Eggert *et al.*, 2000). These results likely reflect the retention of ancestral haplotypes in the larger historical population sizes of Zimbabwe, Namibia and Botswana, rather than being evidence of genetic differentiation between the South African elephants and populations in other southern African states.

While some have declared the Knysna elephant population to be almost extinct (Essop *et al.*, 1996), our results indicate that there were at least five elephants present in 2002–2003. A study by Patterson (2004) indicates that the range of these elephants is larger than was previously believed, including the forest, fynbos and plantations of the Knysna region. While we believe our results provide cause for optimism, we caution that if this population is not augmented with bulls, either through natural recruitment from an unknown source or through translocation of bulls from either Addo or Kruger, earlier predictions of the demise of the Knysna elephants will inevitably come true.

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