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SHORT COMMUNICATION

Minimum population size, genetic diversity, and social structure of the Asian elephant in Cat Tien National Park and its adjoining areas, Vietnam, based on molecular genetic analyses

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Abstract Vietnam's elephant population that has suffered severe declines during the past three decades is now believed to number 60–80 individuals in the wild. Cat Tien National Park is thought to be one of the key areas for the recovery of Vietnam's elephants. We carried out a molecular genetic study of elephants in Cat Tien National Park and its adjoining areas with the objectives of estimating minimum population size, assessing genetic diversity, and obtaining insights into social organization. We obtained a minimum population size of 11 elephants based on a combination of unique nuclear microsatellite genotypes and mitochondrial haplotypes. While mitochondrial diversity based on a 600-base pair segment was high in this small sample of individuals, the six microsatellite loci examined showed low diversity and the signature of a recent population bottleneck. Along with nuclear genetic depauperation of Cat Tien's elephants, we also report disruption of normal social organization, with different matrilines having coalesced into a single social group because of anthropogenic disturbance. The results emphasize the critical condition of this elephant population and the need for urgent conservation measures if this population is to be saved.

Keywords Asian elephant · Genetic diversity · Small population · Social organization · Vietnam

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Introduction

Managing small populations of endangered animals is a growing challenge for wildlife managers today. As populations contract and fragment, they become progressively susceptible to environmental, demographic, and genetic stochasticity, thus facing increasing risks of extinction (Shaffer 1987). An understanding of various attributes of small populations, including demography and genetic variability, is essential for mitigating these risks. A primary difficulty in studying small populations of forest-dwelling or elusive animals is ironically the small population size itself, as it renders detection of animals arduous and population size estimates imprecise, with large statistical confidence limits due to small sample sizes (Barnes 2002). Thus, indirect methods have to be employed to circumvent some of these problems (for example, see Taberlet et al. 1997, Barnes 2002, Payne et al. 2003), and genetic techniques have proved to be useful in studying such populations and assisting decisions related to conservation and management (for example, Hedrick 1995, Madsen et al. 1999, Eggert et al. 2003).

The Asian elephant (*Elephas maximus*) is among the many endangered species whose populations have

become fragmented at an alarming rate due to anthropogenic causes (Santiapillai and Jackson 1990). There are few Asian elephant populations of over a thousand elephants outside of India, and the problem is severe in southeast Asia, with Vietnam harbouring among the smallest and most fragmented populations. Vietnam affords about 3,000 km^2 of elephant habitat, which was home to a high density of elephants in the 1970s (Olivier 1978). However, by the 1990s, destruction of nearly 50% of the country's forest cover during and after the American War in Vietnam (see Kemf 1986) had caused the elephant population in the country to dwindle to 300–600 individuals (Dawson 1996), and at present only an estimated 59–81 individuals remain (Heffernan and Cuong 2004), distributed along the western boundaries with Laos and Cambodia (Sukumar and Santiapillai 1996) and in a few other fragments in the Central Highlands and southern Vietnam (Tuoc and Santiapillai 1991). In addition, these 59–81 elephants are in the form of isolated herds of fewer than 30 individuals each (Duckworth and Hedges 1998, Heffernan and Cuong 2004).

One of the remnant populations that has been thought suitable for targeting long-term conservation of elephant in Vietnam is Cat Tien National Park located in southern Vietnam, in the provinces of Dong Nai, Lam Dong, and Binh Phuoc. The dense habitat and low density of elephant in the park make direct observation extremely difficult. Surveys in Cat Tien National Park estimated the number of elephants at ~21 in the late Nineties (Polet and Khanh 1999). In another study using indirect signs of elephant and information from villagers and park staff we estimated the population at 11–17 individuals in 2003 (Varma et al. in press). This also provided us the opportunity to carry out sampling of fresh dung for genetic analyses, allowing for an independent method of estimating the minimum population size. Thus, in the present study, we employ molecular markers in concert with noninvasive sampling to estimate the minimum number of animals in Cat Tien, assess genetic diversity, and obtain preliminary information on social organization based on genetic data.

Methods

Field sampling

Cat Tien National Park, covering approximately 740 km^2, is one of the few remaining tracts of lowland evergreen forest in Vietnam. The park receives a high annual rainfall of 2175–2975 mm and experiences distinct dry (December to April) and wet (May to November) seasons. Besides evergreen forest, deciduous forest and secondary forest with bamboo and rattan are also characteristic of this area. Cat Tien National Park complex consists of Cat Loc, where elephants are absent, and Cat Tien, and hence our field sampling was carried out in Cat Tien and its adjoining areas, the La Nga State Forestry Enterprise (c. 200 km^2), and the Vinh An State Forestry Enterprise (c. 200 km^2) (Fig. 1) Based on latitude and longitude grids, we surveyed 27 blocks (Fig. 1), each of about 6 km^2, for elephant dung during February–April and November–December 2001. Dung that was less than a few days old was sampled, and the outermost layer of dung, which is expected to contain the least degraded DNA, collected into 95% ethanol. The noninvasive sampling technique used thus overcomes the logistic problems associated with obtaining tissue or blood from a free-ranging large mammal like the elephant. Relatively fresh dung was observed only in blocks 18 and 19, and 23 samples were collected that were less than a few days old. We additionally obtained four dung samples from Saigon Zoo and Botanical Garden, of animals that were captured from Vietnam (from Binh Chau Phuoc Buu Nature Reserve, which is about 50 km away from Cat Tien National Park).

Genetic analyses

Extraction involved digestion of 0.5 g of dung with digestion buffer and Proteinase K followed by extraction with phenol/chloroform/isoamyl alcohol and purification using QIAGEN gel purification columns (Fernando et al. 2003a, Vidya et al. 2005). Polymerase chain reactions were carried out using the primers MDL3 and MDL5 (Fernando and Lande 2000) to amplify a 600-bp segment of mtDNA containing the C-terminal of cytochrome b and part of the control region. PCR products were sequenced using the primers MDLseq-1 and MDLseq-2 (see Vidya et al. 2005) in BigDye (Applied Biosystems, Inc.) terminated cycle sequencing reactions, and purified sequencing products were electrophoresed in an ABI Prism 377 DNA Sequencer. Sequences were aligned and edited using SEQUENCHER v.3.1.1 (Gene Codes Corporation 1999) and sequences that differed by at least a single nucleotide were considered different haplotypes. New haplotypes were confirmed by repeating the extraction and PCR.

Six microsatellite loci, the tri- and tetra- nucleotide loci EMX-1, EMX-2, EMX-3, and EMX-4, developed from an Asian elephant (Fernando et al. 2001), and the

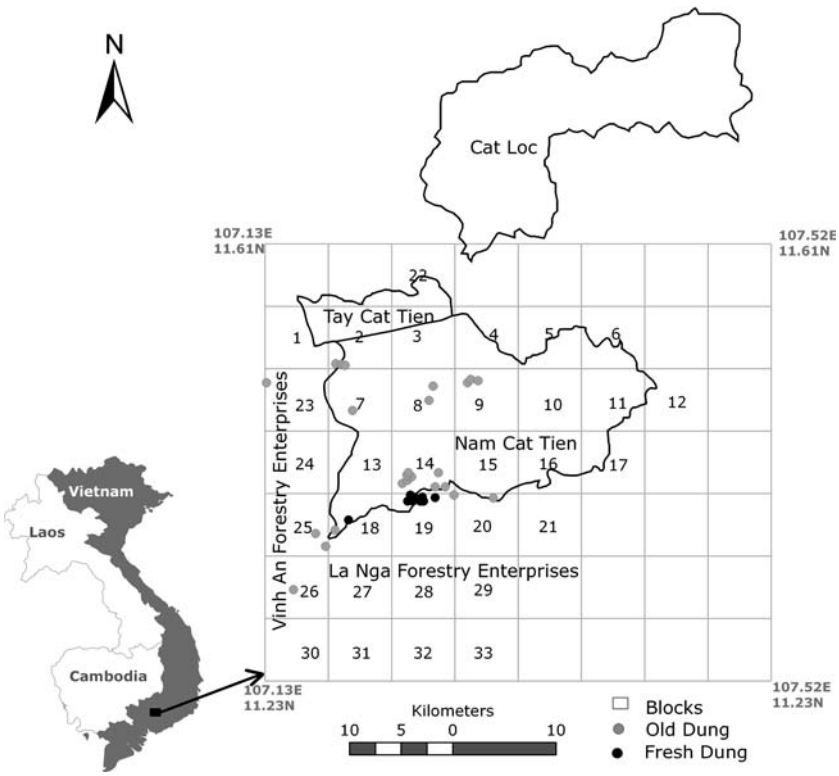

Fig. 1 Map of Cat Tien National Park and its adjoining areas, blocks sampled, and locations of fresh (less than a few days old, and the only samples used for genetic analyses) and old dung found. Cat Tien National Park consists of Cat Loc, which does not harbour elephants, and Cat Tien. Cat Tien is further divided into Tay Cat Tien and Nam Cat Tien. The adjoining areas sampled were Vinh An Forestry Enterprises and La Nga Forestry Enterprises. Inset: Location of Cat Tien in Vietnam

dinucleotide loci LafMS02 and LafMS03, developed from African elephants (Nyakaana and Arctander 1998), were amplified using PCRs, the products electrophoresed on polyacrylamide gels in a DNA Sequencer, and scored using the ABI Gene Scan analysis software v.3.1.2 (Applied Biosystems, Inc.). As dung samples are sub-optimal sources of DNA, care was taken to minimise genotyping errors. Separate areas and dedicated instruments were used for samples with low copy number DNA and barrier tips were used in pipettes always (see Fernando et al. 2003a, Vidya et al. 2005). In addition, genotyping was repeated once more to confirm the genotype. We did not experience a problem with either allelic dropout or PCR inhibition.

Individuals were molecular sexed using a polymorphism in the ZFX and ZFY genes, which are on the X and Y chromosomes, respectively. The portion of the ZFX and ZFY genes containing the polymorphism was PCR amplified with three primers, ZF79F 5′-AAATG CACAAGTGTAAATTCT-3′, ZF324R 5′-GA-ATGGCATCTCTTTACTATG-3′, and ZFY161R 5′-T ACTGGGGAGAAACCCA-3′ (Fernando unpublished). The primer ZFY161R selectively binds to the polymorphic region, such that single bands are obtained in the case of females and double bands in the case of males on electrophoresis of PCR products (see Vidya 2004). The method was standardized using blood samples of captive elephants and, subsequently, dung samples from the four animals from Saigon Zoo and Botanical Garden were used as positive controls to ensure that the PCRs worked correctly.

Genetic data analyses

Haplotype diversity ($\hat{H}$) (pp.180, Nei 1987) and nucleotide diversity (pp.257, Nei 1987) were calculated using Arlequin ver.2.000 (Schneider et al. 2000), and allele frequencies and heterozygosity using C programs (written by TNCV, available on request). Linkage disequilibrium tests between pairs of loci, with the null hypothesis of random association of genotypes at these loci, and the Hardy-Weinberg equilibrium test, with the null hypothesis of random union of gametes at each locus, were performed using Genepop v.3.1 (Raymond and Rousset 1995). Type I errors were corrected for by applying the sequential Bonferroni test *a posteriori* (see Rice 1989). Evidence for a recent population bottleneck was assessed using a test for heterozygosity excess (Cornuet and Luikart 1996) and a graphical test to detect mode-shifts in allele frequency distributions (Luikart et al. 1998) in the program BOTTLENECK v.1.2.02 (Piry et al. 1997). The graphical test is based on the observation that rare alleles (with allele frequencies of 0.001–0.01) show the highest frequency in a frequency distribution of allele frequencies in a natural population, but are easily lost during a population

bottleneck, resulting in a shift in the mode of the distribution towards the intermediate frequency alleles (frequencies 0.101–0.900) in recently bottlenecked populations (Luikart et al. 1998). Genetic relatedness between individuals was calculated using the Program *Relatedness* 5.0 (Queller and Goodnight 1989; Goodnight and Queller 1999), and standard error obtained by a jackknifing procedure across loci. The six loci were known to follow Mendelian inheritance and the average relatedness (±95% confidence interval) between adult females and their offspring in a southern Indian population was 0.437 ± 0.051 (Vidya and Sukumar 2005), validating the use of these loci in calculations of relatedness. A Mantel test (Mantel 1967) was used to test for correlation between nuclear genetic and mitochondrial-based relatedness (C program written by TNCV, available on request). The probability of identity (P_{ID}), which is the probability that two individuals picked at random show identical genotypes at multiple loci, was also calculated. The lower the P_{ID}, the greater the probability that two different individuals are not wrongly scored as identical by the loci used. The expected P_{ID} after correcting for sample size was calculated according to Paetkau et al. (1998) using a C program.

Results

Of the 23 samples collected, genetic data could be obtained from 17 samples, while 6 samples that were over a day old did not yield any PCR product. Amplification was obtained from all four zoo elephants.

Genetic diversity

Mitochondrial diversity was high, with three mitochondrial haplotypes within Cat Tien, haplotype AB (corresponding to haplotype B of Fernando et al. 2000 and AB of Fernando et al. 2003b) and two previously unreported haplotypes, AJ (GenBank Accession No. AY589515) and AK (GenBank Accession No. AY589516). Haplotype diversity was 0.699 ± 0.049 and nucleotide diversity 0.0016 ± 0.0013. Three haplotypes were obtained from the four zoo animals, AB, AD (corresponding to haplotype D of Fernando et al. 2000 and AD of Fernando et al. 2003b), and BO (corresponding to haplotype O of Fernando et al. 2000 and BO of Fernando et al. 2003b). Nuclear microsatellite diversity was low, with mostly just two alleles at the loci examined. The allele frequencies of alleles at the six loci are shown in Table 1. Observed heterozygosity ranged from 0.154 to 0.556, the lowest and highest values both being those at dinucleotide-repeat loci.

Table 1 Heterozygosity and allele frequencies at the six loci in elephants sampled at Cat Tien National Park

Locus	Expected heterozygosity	Observed heterozygosity	Allele	Frequency
EMX-1	0.477	0.357	135	0.393
			144	0.607
EMX-2	0.483	0.273	219	0.409
			225	0.591
EMX-3	0.397	0.364	238	0.727
			254	0.273
EMX-4	0.580	0.444	351	0.400
			375	0.100
			387	0.500
LafMS02	0.142	0.154	133	0.077
			135	0.923
LafMS03	0.545	0.556	137	0.450
			139	0.500
			151	0.050

Minimum population size and tests for detecting population bottlenecks

Using a combination of mtDNA haplotypes and the six microsatellite markers, the 17 usable dung samples could be identified as a minimum of 11 unique animals. The expected P_{ID} was 0.002, indicating that only two in a thousand animals are expected to be wrongly identified as the same individual. Therefore, of the 17 samples, six samples were almost definitely obtained by repeat sampling of the same individuals. Molecular sexing revealed one male in the sample. Based on the bolus diameter of dung (see Vidya et al. 2003), this was classified as an adult.

None of the microsatellite loci showed significant deviation from Hardy–Weinberg equilibrium or linkage equilibrium after Bonferroni corrections were applied, and were thus suitable for use in tests of population bottlenecks. Statistically significant heterozygosity excess was detected using the Infinite Allele Model (Wilcoxon matched-pairs test, $P = 0.016$), while heterozygosity was not significantly higher than expected using the Two Phase Mutation Model ($P = 0.055$) or the Stepwise Mutation Model ($P = 0.055$). A mode shift in allele frequencies was observed with fewer rare alleles than alleles of intermediate frequency (Fig. 2).

Social organization

Nine of the 10 unique females that were genotyped are likely to represent a single social group as dung samples from these were obtained within a span of 5 days

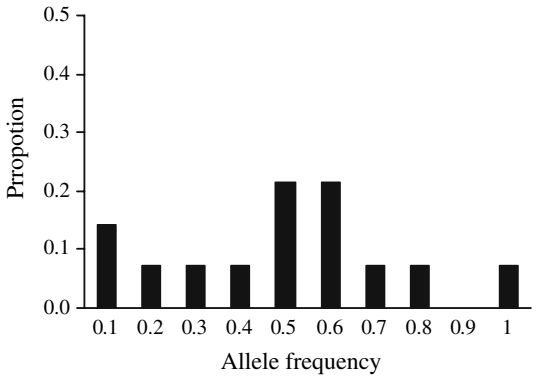

Fig. 2 Proportions of alleles of different allele frequencies in the Cat Tien population

from a small area of ~5 km^2. There were also reports of sightings of a group of about eight elephants by park staff during this time (Varma et al. in press). Thus, three maternal lineages, corresponding to the three mtDNA haplotypes, AB, AJ, and AK, exist within this group. Smaller subsets of animals sampled on a single day also showed more than one haplotype; for instance, three females that were sampled in the Cashew Plantation (part of Block 19) on 6th March 2001 had haplotypes AB, AJ, and AK, and two females sampled on 10th March 2001 in Block 19C (another part of Block 19) showed haplotypes AB and AJ. When nuclear genetic relatedness was examined between individuals of the same mitochondrial haplotype (15 pairwise comparisons), an average relatedness of 0.285 (95% CI –0.053 to 0.622) was found. Average nuclear genetic relatedness between individuals having different mitochondrial haplotypes (39 pairwise comparisons) was significantly lower at –0.344 (95% CI –0.504 to –0.185) (non-overlapping confidence intervals, significant correlation between matrices of genetic relatedness and mitochondrial relatedness using a Mantel test, observed $Z = -12.114$, $P < 0.05$).

Discussion

Our minimum estimate of elephants based on genetic markers matches that obtained by a field survey carried out by us. Varma et al. (in press) estimated a minimum of 11 and an upper limit of 15–17 elephants in the park based on dung counts carried out during the same period as sampling for genetic analysis. While ours is a minimum estimate, repeated sampling can generate genetic data on individuals that can perhaps be used in a mark-recapture analysis to estimate the total population size (Eggert et al. 2003). We obtained only one male in our sample, while two subadult/adult males had been sighted during Varma et al.'s (in press) survey, implying that the minimum number of elephants in the park at the time of sampling must have been 12.

A surprisingly high mitochondrial diversity was observed with three haplotypes being found in elephants from the same area. In contrast, only one haplotype has been found in the world's largest Asian elephant population (of over 9,000 elephants) in the Nilgiris, southern India (Vidya et al. 2005). However, nuclear diversity was overall low in Cat Tien, particularly at the dinucleotide loci. Heterozygosity in Cat Tien was average to low, resulting in the expected P_{ID} being an order of magnitude lower than that in the Nilgiris ($P_{ID} = 0.0004$). Despite the low nuclear diversity, the six loci used were sufficient for individual identification with negligible error in Cat Tien because of the considerably smaller population it harbours. Heterozygosity excess based on the infinite allele model (and also with the two phase mutation model and stepwise mutation model if a slightly lower level of stringency were used) and mode-shift in allele frequency indicated a recent population bottleneck in the Cat Tien population, which is almost certainly anthropogenic. The presence of additional haplotypes in the zoo animals also indicates a possible recent loss of diversity. The low diversity and evidence of a recent bottleneck in this tiny population are cause for concern as inbreeding depression is likely to ensue even if the population survives demographic and environmental stochasticity.

The occurrence of high mitochondrial diversity in concert with low nuclear diversity in Cat Tien is contrary to the absence of mitochondrial diversity and normal nuclear diversity observed in the Nilgiris, southern India. Higher mitochondrial than nuclear diversity could have arisen either due to initial high mitochondrial diversity or due to high population subdivision. High population subdivision is expected to increase mitochondrial diversity to a greater extent than nuclear diversity (Birky et al. 1989) and, if the population in this region had shown high genetic structuring in the past, a population crash could still have left the population with higher mitochondrial than nuclear diversity, the pattern we see today. Opposing patterns of diversity may thus be informative about the population's history or about female social organization but are also a reminder that mitochondrial DNA does not accurately reflect the diversity and viability of a population, nuclear DNA determining evolutionary potential to a large extent. If the study in the Nilgiris and Cat Tien were carried out based exclusively on mitochondrial DNA, and if the difference in population sizes were not so overwhelming, it may have wrongly

been concluded that the Cat Tien population was genetically more viable than the Nilgiri population.

The presence of three different mitochondrial haplotypes and animals that are not genetically related to each other in a single group of elephants deviates from previous observations that all females of a ''family'' group share the same haplotype (Fernando and Lande 2000) and are closely related to one another (Vidya and Sukumar 2005). While a ''social group'' does not necessarily correspond to a ''family group'', based on the number of fresh dung samples collected and the number of elephants directly sighted, there could not have been more than 10 female elephants in the area, and therefore, these elephants would correspond to either a family group or a kinship group under normal circumstances. Further, the low mutation rate of the segment of mitochondrial DNA examined (3% per million years, Fleischer et al. 2001) would lead to shared mitochondrial haplotypes between members of family groups, kinship groups, and even associations of kinship groups in an undisturbed population. Only about 30 haplotypes (at this particular mitochondrial segment examined) have been identified from across the range of the Asian elephant (Fernando et al. 2003b). Thus, the finding of three haplotypes from a social group of nine female elephants is extremely unusual and indicates a coming together of unrelated family groups.

A congregation of free-ranging elephants of different family groups may be envisioned either under conditions of abundant resources or under conditions of stress or disturbance. Large congregations of different family herds, but of the same mtDNA haplotype (Vidya et al. 2005), are an annual phenomenon in the Kabini area of Nagarahole National Park, southern India, during summer when abundant grass and water are available. However, while a higher carrying capacity than the present population size has been estimated for Cat Tien (Sukumar et al. 2002, Varma et al. in press), forage availability is generally thought to be poor in rainforests, and forage abundance is not likely to be the reason in this case for different social groups converging together. Rather, the present scenario in Cat Tien is indicative of remnants of different groups joining together to form a single social unit in the wake of disturbance. Integration of social groups is not surprising given the intelligence, extreme adaptability, and social nature of elephants. For instance, more than one mtDNA haplotype was found in three of nine family units sampled in Queen Elizabeth National Park (Uganda) elephant population (Nyakaana et al. 2001), which had suffered severe poaching during the mid-1970s (Eltringham and Malpas 1980). Visual observation on family groups had also indicated a breakdown in social structure of the surviving family groups, leading to a coalescence of separate matrilines (Eltringham and Malpas 1980; Abe 1994), possibly as a collective defense strategy (Douglas-Hamilton 1973; Laws 1974). Similarly, it is thought that the presence of more than one haplotype within clans in Sengwa, northern Zimbabwe, could also possibly be an outcome of the repeated culling, albeit of entire family groups, there (Charif et al. 2005). It would be interesting to examine cooperation and competition in social groups with animals of more than one matriline.

In conclusion, the small population size, low genetic diversity, and disruption of social organization point to a bleak future for Cat Tien's elephants. Urgent conservation measures are required if this population is to survive (see Sukumar et al. 2002, Varma et al. in press). In addition, continuous monitoring of the population would be required since demographic processes are crucial in small populations (Lande 1988). Examining individuals for possible physical signs of inbreeding depression is also recommended during this monitoring. As the carrying capacity of the park is higher than the number of elephants present (Sukumar et al. 2002, Varma et al. in press), translocation of other small isolated elephant herds from within Vietnam to this area may be considered if the integrity of the habitat can be maintained and illegal killing checked.

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