

Amplification success and feasibility of using microsatellite loci amplified from dung to population genetic studies of the Asian elephant (*Elephas maximus*)

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The use of microsatellite DNA markers in large mammal conservation genetics has been limited by the logistic difficulty in obtaining optimal sources of DNA from free-ranging animals. Here, we show high amplification success of microsatellite DNA from elephant dung samples collected under field conditions. Amplification success depended on how fresh the sample was when collected, and on the sensitivity of primers at individual loci. No significant allelic dropout was observed. We also examined the loci for selective neutrality, independent inheritance of loci, and the probability of identity of individuals to confirm their utility in studies of population genetic structure.

MOLECULAR studies of free-ranging populations of large mammals have been constrained in the past by the logistic difficulties in obtaining tissue or blood samples from which DNA can be easily extracted. Subsequently, the advent of polymerase chain reaction (PCR) made it feasible to amplify DNA from suboptimal sources like faeces, feather and hair. Several studies of large mammals followed, amplifying mitochondrial DNA—which has a high copy number in cells—from faeces^{1–4}. However, studies of nuclear microsatellite DNA from faeces are limited. Microsatellites or simple sequence repeats (SSRs) or short tandem repeats (STRs) are repeat units of 1–5 nucleotides arranged in a tandem fashion with usually less than 30 such repeats at a locus⁵. Being codominant, biparentally inherited and often highly polymorphic, microsatellites are useful in studies of genetic variation, population genetic structure (in which populations are examined for similarities and differentiation in genetic composition), gene flow, relatedness and paternity. Previous studies amplifying microsatellite DNA from faeces include those by Kohn *et al.*² on brown bears, Constable *et al.*⁶ on baboons, Gerloff *et al.*⁷ on bonobos, Taberlet *et al.*⁸ on brown bears, Reed *et al.*⁹ on seals, Kohn *et al.*¹⁰ on coyotes, and Eggert *et al.*¹¹ on African elephants. However, such non-invasive sampling has not been widely applied since the low quality and quantity of DNA in dung have often led to low amplification success rates, genotyping errors, inhibition and allelic dropout¹².

Here we describe the feasibility of microsatellite amplification from dung of free-ranging Asian elephant (*Elephas*

maximus), and the suitability of six microsatellite loci we have amplified for population genetic studies. With a free-ranging population of only 41,400–52,300 individuals¹³, the Asian elephant is listed as threatened in the IUCN Red List. Non-invasive techniques are thus appropriate for the study of this species. Previous molecular studies on the Asian elephant have focused on examining mitochondrial sequences^{14–17}. Fernando *et al.*¹⁸ demonstrated a comparable level of genotyping errors while amplifying microsatellite DNA from fresh dung and blood samples of captive elephants, but there has been no detailed report of microsatellite amplification success from a large set of non-invasively collected field samples. Unlike samples from captive animals, those from free-ranging elephants cannot be collected immediately upon defecation and this may affect amplification success. We thus examine a few factors that may affect amplification success, like the time period between defecation and collection of the sample, the collection preservative, total DNA concentration, and the sensitivity of the primers. We also examine some properties of these loci such as selective neutrality, linkage disequilibrium, and the probability of identity, to ensure that they are useful in studies of population genetic structure and gene flow.

We collected a total of 321 dung samples from various field sites in southern, east-central, northern and northeastern India. Samples were collected from the outermost layer of dung, which would have sloughed-off endothelial cells, usually into 95% ethanol, though 14 samples were collected in storage buffer (100 mM NaCl, 10 mM Tris pH 8.4, 25 mM EDTA pH 8, 1% SDS, 20 mM guanidine thiocyanate). Extraction followed Fernando *et al.*¹⁸ and involved digestion of 0.5 g (wet weight) of dung with 1 ml digestion buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA, 2% SDS) and 20 µl Proteinase K (20 mg/ml), followed by extraction with phenol/chloroform/isoamyl alcohol, and purification using QIAGEN gel purification columns (and manufacturer's protocol). Six microsatellite loci were amplified: EMX-1, EMX-2, EMX-3 and EMX-4 characterized from an Asian elephant¹⁹, and LafMS02 and LafMS03 characterized from the African elephant genome²⁰.

PCR was carried out in 12.5 µl volumes, using 2 µl DNA, 0.25 µl of each fluorescently labelled primer (Biosynthesis, 10 pmol), 0.1 µl Taq DNA polymerase (Perkin Elmer Ampli Taq DNA polymerase), 0.491 µl 10 mM dNTP mix, 0.163 µl 100 mg/ml BSA, 0.019 µl of 1M MgCl₂, 0.154 µl 4M KCl, 0.123 µl 1M Tris pH 8.4, and 8.95 µl water. The annealing temperatures used for the six loci were as follows: EMX-1, EMX-2, EMX-3: 67°C for 1 min, EMX-4 and LafMS03: 60°C for 1 min, and LafMS02: 68°C for 1 min. For all loci, a 92°C denaturation for 1 min, and a 72°C extension for 1 min were employed, followed by an extension at 72°C for 15 min after the completion of all (35–45) cycles. PCR products were electrophoresed on 5.6% polyacrylamide gels (Figure 1), along with the internal size standard Tamra 350 or 500

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(Applied Biosystems, Inc.). Allele sizes were identified using the ABI Gene Scan analysis software v.3.1.2 (Applied Biosystems, Inc.), and alleles scored following the guidelines in Fernando *et al.*¹⁸. We also carried out a dilution experiment using the loci EMX-1, EMX-2 and EMX-3. Ten extracts of total DNA concentration 9–190 ng/μl (average 49.6, median 39.5), as measured with a fluorometer, were diluted 1/10, 1/20, 1/50 and 1/100 times, and PCR carried out using these extracts. We examined if heterozygotes showed non-amplification of one allele ('allelic dropout') when diluted due to insufficient DNA.

The test for Hardy–Weinberg equilibrium for selective neutrality of loci, and the linkage disequilibrium test between pairs of loci for independent inheritance of alleles at these loci, were performed²¹ for each population using Genepop v.3.1. Type I errors were corrected for by applying the sequential Bonferroni test *a posteriori*²². We also calculated the probability of identity using these microsatellites for the largest Asian elephant population in the Nilgiris–Eastern Ghats. The probability of identity (P_{ID}) is the

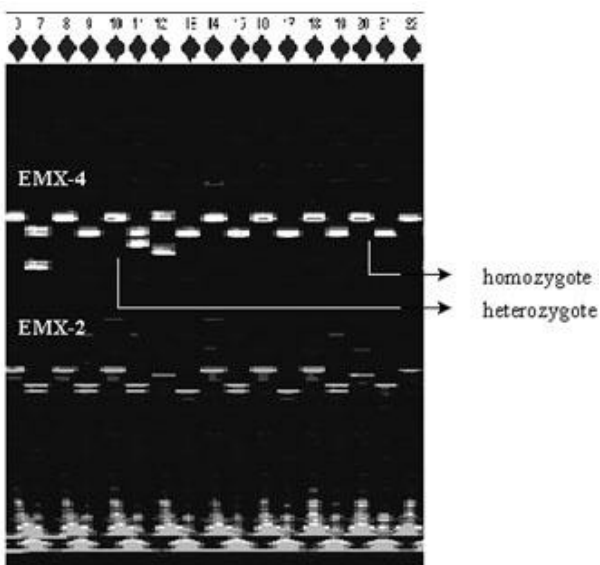


Figure 1. Portion of a Gene Scan image (transformed from colour to greyscale) showing amplification of alleles at two loci. Each lane corresponds to an individual. Even lanes were loaded 5 min after the odd lanes so that leakage across lanes would not be falsely identified as an allele. (Upper row) Locus EMX-4 with genotypes 387/387, 351/387 and 375/387 in lanes 6, 7 and 11 respectively. (Lower row) Locus EMX-2 with genotypes 224/224 and 218/224 in lanes 6 and 7, respectively.

probability that two individuals picked at random show identical genotypes at multiple loci. Thus, lower the P_{ID} , greater the probability that two different individuals are not wrongly scored as identical by the loci used. A P_{ID} of 0.01 or lower is required for population genetic studies and for population size estimation using mark–recapture methods²³, while a P_{ID} of 0.001–0.0001 would be required for individual identification and forensic applications²⁴. The theoretical expected P_{ID} after correcting for sample size was calculated according to Paetkau *et al.*²⁵ and the expected P_{ID} among sibs according to Waits *et al.*²⁴. When loci are inherited independent of each other, the total P_{ID} is a product of individual P_{ID} s of the loci. Theoretical P_{ID} s may often not reflect the actual P_{ID} in a population due to population substructure²⁴, and it becomes necessary to also calculate the observed P_{ID} , which was computed as the proportion of all possible pairs of individuals that had identical genotypes. This was done for the locus with the highest heterozygosity first, followed by the addition of loci in decreasing order of heterozygosity, and recalculating the number of identical genotypes. All the P_{ID} calculations were carried out using C programs.

Amplification success of microsatellites was high and varied across loci. The percentage of amplification at four loci (EMX-1, EMX-2, EMX-3 and LafMS03) was greater than 94, while this was lower at the other two loci (EMX-4 and LafMS02) (Table 1). However, the lower amplification at LafMS02 was possibly due to the fact that amplification of this locus from 123 extracts was carried out when the DNA extracts were two years old, and therefore there could have been some degradation of DNA. Amplification success at this locus using fresh extracts was 95.6% ($n = 184$; amplification success 80.5% with 123 old extracts). Samples collected in buffer did not amplify satisfactorily, possibly due to insufficient buffer volume to sample volume ratio. Many of the samples that did not amplify could be traced to decay of samples in the field. Amplification success decreased when the dung samples collected were not fresh (collected from the field when they were about 1–5 days old) compared to those that were a few to several hours old (Wilcoxon matched pairs test, $P = 0.028$). However, this differential decrease in amplification varied across loci, possibly due to differential sensitivities of primers (Table 1). In addition, the number of samples that did not amplify at all six loci was only 4 out of 321, corresponding to 1 of the 307 samples collected in alcohol, while the number of samples in alcohol that

Table 1. Percentage of amplification from fresh and 1–5 day(s)-old samples collected in 95% alcohol/buffer that amplified at the loci used

Preservative	Age of dung	<i>N</i>	EMX-1	EMX-2	EMX-3	EMX-4	LafMS02	LafMS03
Both	All ages	321	95.0	94.4	94.1	82.2	87.9	94.1
Alcohol	Fresh (few hours)	261	97.7	98.1	97.3	90.4	90.4	96.6
Alcohol	~1–5 days	46	89.1	91.3	91.3	47.8	84.8	87.0
Buffer	Fresh (few hours)	14	64.3	35.7	42.9	42.9	50.0	71.4

did not amplify at any two loci was 10, and at any one locus was 57 (73% of the total non-amplifications). This suggests that the success of amplification is dependent on the sensitivity of the loci, in addition to template DNA¹⁸.

The proportions of alleles that amplified were significantly ($P < 0.001$), but not strongly correlated to total DNA concentration, with Spearman's R values of 0.65, 0.62, and 0.51 for loci EMX-1, EMX-2, and EMX-3 respectively. On the other hand, a similar analysis on a set of blood samples using EMX-3 gave a Spearman's R of 0.83 ($P < 0.001$). Although not directly comparable, a weaker correlation can be expected from dung samples as non-elephant DNA is also present in high proportions^{18,26}.

When sets of different dilutions were examined pairwise using a Wilcoxon matched-pairs test, the following results were obtained. At locus EMX-1 and EMX-2, the 1/100 dilution set showed significantly lower ($P < 0.05$) amplification than the other sets, except for the 1/50 dilution. The 1/50 dilution showed significantly lower amplification than the less-diluted samples, but there was no difference in the proportion of alleles amplified between the undiluted sample and the sample diluted up to 1/20 times. At EMX-3, there was no difference in amplification success between the undiluted sample and the 1/10 dilution. The 1/20, 1/50 and 1/100 dilutions showed significantly lower amplification than the former, but no differences amongst themselves. Thus, there appeared to be sufficient target DNA in our DNA extracts as to make allelic dropout an unlikely source of error.

Polymorphism was low at the tri- and tetranucleotide loci, with 4, 2, 2 and 3 alleles at EMX-1, EMX-2, EMX-3, and EMX-4 respectively, and moderately high at LafMS02 and LafMS03, with 6 and 7 alleles respectively. While there are no published population-level studies of microsatellite variation in Asian elephant populations for comparison, the allelic richness at the dinucleotide loci was comparable to those in published studies of the African savannah elephant^{11,27}. The Hardy-Weinberg equilibrium test showed significant departure from equilibrium only at loci EMX-3 and LafMS02 in Periyar ($P < 0.001$, first significant P for the sequential Bonferroni correction in each population = 0.008) and EMX-3 in Central India ($P = 0.006$). None of the other 45 comparisons was significant, indicating that these significant differences were probably due to non-random mating within these populations, rather than selection on the loci. No significant linkage disequilibrium was observed between any pair of loci in any population ($P > 0.01$; first significant P for the sequential Bonferroni correction in each population = 0.003). The P_{ID} curves are shown in Figure 2. The expected $P_{ID(ubiased)}$ was 0.0004 and the observed P_{ID} was 0.0018, thus low enough to use these loci in studies of population genetic structure. However, the theoretical $P_{ID(sib)}$ was 0.0286, which implies that about 3% of siblings that are sampled may be wrongly identified as the same individual. Therefore, additional loci would be required in studies of kinship and paternity.

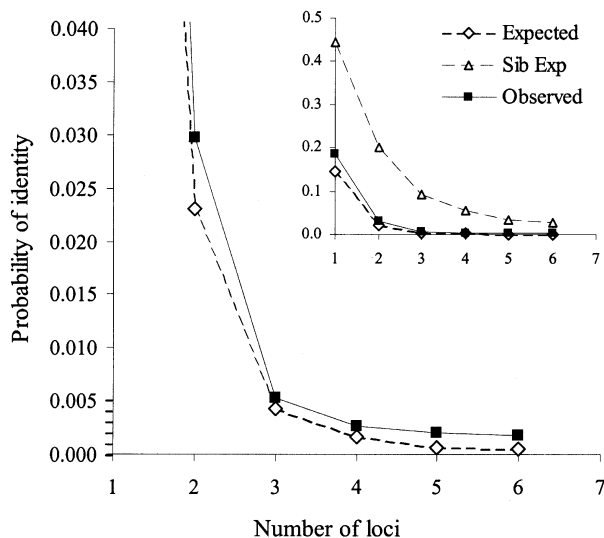


Figure 2. Theoretical and observed P_{ID} s based on the Nilgiris-Eastern Ghats population ($n = 127$). Loci were added in decreasing order of heterozygosity. (Inset) Curve for $P_{ID(sib)}$ also, while the same figure has been enlarged on a different Y-axis scale to show the difference between the expected and observed curves.

This study demonstrates the applicability of using microsatellite DNA by non-invasive sampling. We used protocols optimized for sample collection, extraction of DNA, and PCR amplification. We found the age of the dung samples to be crucial to successful amplification, though the sensitivity of primers at individual loci also played a role. This field study demonstrates a high success rate of amplification of microsatellite DNA from dung. This high amplification success, along with the simplicity of sample collection procedures, make non-invasive sampling a promising tool for conservation genetic analyses of the Asian elephant. The loci we used showed levels of polymorphism that seem to be typical of elephants and sufficiently low P_{ID} s as to make them appropriate markers for various genetic studies. The use of these microsatellite loci in examining population genetic structure and gene flow is ongoing.

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Factors affecting the occurrence of *Porphyra vietnamensis* Tanaka & Pham-Hoang Ho along the Goa coast

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***Porphyra vietnamensis* Tanaka & Pham-Hoang Ho occurs in the intertidal region of the Goa coast, during monsoon months from June to September. The growth and distribution patterns of *P. vietnamensis* populations along the Goa coast were studied quantitatively. Data on algal distribution, abundance, biomass and hydrological parameters were collected at three sites (Dona Paula, Reis Magos estuarine sites and Vagator open coast), every fortnight for a period of 4 months (June–September 2002). Highest biomass was recorded at Vagator (1.3 kg m⁻²) followed by Dona Paula (0.9 kg m⁻²) and Reis Magos (0.5 kg m⁻²). The study reflected that nutrients (nitrate, nitrite and phosphate) and temperature were the factors responsible for the growth of this alga. Among them, phosphate was the main growth-promoting factor at Vagator; this was supported by its positive and significant correlation with the algal biomass. Low temperature was found to be conducive for algal growth, since it exhibited negative and significant correlation with algal biomass. Morphological observation of this alga indicated that thalli growth was relatively larger and well developed at Vagator compared to the other two sites.**

THE marine red alga *Porphyra* (Rhodophyta, Bangiales), popularly known as ‘Nori’ in Japan, has an annual value of over US \$18 billion, and occurs widely on temperate and tropical rocky shores throughout the world^{1,2}. Nearly 133

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