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Individual identification and paternity determination in Asian elephant by using microsatellite markers

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ABSTRACT

The objective of this study was to set up a panel of highly polymorphic microsatellite markers suitable for individual identification and parentage verification in Asian elephant. A random sample of elephants was typed for 18 microsatellite loci known to be polymorphic either in African or in Asian elephant. The number of alleles varied from 3 to 18 with an average of 9.9 alleles per locus. The expected heterozygosity *(He***)** values of the markers ranged from 0.568 to 0.927 and PIC values from 0.499 to 0.911. The locus specific mean paternity exclusion (PE) probabilities ranged from 0.299 to 0.852 with an average of 0.632. The cumulative mean exclusion probabilities for the 2 loci with the highest exclusion probabilities was 97.5, for the best 3 was 99.5, and for the best 5 exceeded 99.8. The cumulative individualization potential (P_{ID}) for the best 5 loci was 3.18×10^{-8}, which was equivalent to a probability of 3.15×10^{-7} for selecting 2 identical genotypes in a reference population.

Key words: Asian elephant, Individual identification, Microsatellite markers, Paternity determination

The Asian elephant *(Elephas maximus)* is an endangered species. The current Asian elephant population in the world is estimated as between 30 000 and 50 000 (Dublin *et al.* 2006). However, there has been no sincere attempt in predicting the gradual decrease in numbers over several decades. Poaching of elephants for ivory is the primary cause for the decline of the species in some parts of its range. Asian elephant ivory is highly favoured by many ivory traders and elephant bones and body parts used in the Asian medicinal trade are commodities, much in demand by the global marketplace. Therefore, individualization of elephants from seized evidence becomes an important issue for forensic detection necessitating the development of individual DNA profiling techniques for wildlife forensic investigation. It is absolutely essential to establish standardized identification and parentage test for tracing the origin of individual animals and thereby providing proof of illegal poaching and capture.

Poaching for ivory selectively removes male elephants, resulting in skewed sex ratios and causing deleterious demographic consequences such as reduced genetic variation and lower fecundity (Sukumar 2003). Juvenile mortality is high among inbred populations as compared to non-inbred ones. The survival of isolated and small elephant populations depends on intensive conservation and management efforts, which require an in-depth knowledge of particular populations (Fernando 1993). Molecular genetic techniques can be an effective means of obtaining data critical for their management and conservation (Fernando and Lande 2000). However, this requires a standardized set of highly informative genetic markers. Polymorphic microsatellites are useful for assessing population-level genetic diversity and can be used to identify populations at risk of extinction (Gottelli *et al*. 1994). Microsatellite markers are also useful in detecting inbreeding among the wild populations, which is most likely to happen as a consequence of declining male populations due to poaching and the small size of the populations. These markers are also useful for evaluation of genetic resources, parentage determination or identification of animals (DeNise *et al.* 2004, Xu *et al.* 2005).

The present study was aimed to set up a panel of highly polymorphic microsatellite markers suitable for individual identification, parentage verification and detection of inbreeding in Asian elephant.

MATERIALS AND METHODS

Genomic DNA preparation: Blood samples were collected from a random sample of 337 captive Asian elephants from Kerala, Tamil Nadu, Karnataka and Andaman and Nicobar islands. White blood cells were pelleted from 5 ml of blood following lysis of erythrocytes and washed twice in Tris-

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buffered saline (Montgomery and Sise 1990). The pellet was resuspended in 5 ml of SE buffer (75 mM NaCl, 25 mM EDTA, pH 8.0) and 25 µl of proteinase K (20 mg/ml in distilled water) and 0.5 ml of 10% sodium dodecyl sulphate (SDS) were added. The tubes were incubated at 50 ºC with occasional shaking for 3 h. The protein contaminants were removed by repeated extraction with phenol: chloroform: isoamyl alcohol. DNA was recovered by ethanol precipitation and resuspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Primers: Microsatellite typing was carried out at 21 loci known to be polymorphic in Asian or African elephants. The primers were custom synthesized commercially and obtained in lyophilized form. They were reconstituted in sterile triple distilled water to make a stock solution of 200 p*M*/µl concentration. The solutions were incubated at room temperature for 1 h and then stored at –20°C.

Microsatellite analysis: The PCR amplification was performed on a thermal cycler. Each 10 µl PCR reaction contained 50 ng template DNA, 5 pmol each primer, 200 µmol each of dNTP, 0.3 U of Taq DNA polymerase and 1× PCR buffer (containing 10 mM Tris-HCl (pH 8.8) 50 mM KCl and 0.1% Triton X–100) and 0.9 to 1.5 mM of $MgCl_2$. For direct radiolabelling of the PCR product the forward primer was end labelled with [□–^{32}P]-ATP using polynucleotide kinase. The temperature cycle for PCR consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 1 min at the annealing temperature and 72°C for 1 min. The final cycle was followed by an extension at 72°C for 7 min. The amplification was checked on 2% agarose gels. For analysis, 3 µl of the amplified products were resolved on 6% urea polyacrylamide-sequencing gel. The samples were mixed with formamide loading buffer (deionised formamide containing 0.1% w/v xylene cyanol and bromophenol blue and 2% v/v 0.5 M EDTA) and denatured at 95°C for 5 min and electrophoresed at 30 W for 3–4 h in a sequencing electrophoresis system. After electrophoresis the gels were transferred onto 3a filter paper, wrapped them in Saran Wrap, vacuum-dried for 1.5 to 2 h and put for autoradiography in a tight cassette and the X-ray films were developed after 2–3 days of exposure. To determine the exact size of the alleles, m13 DNA sequencing ladder was co-electrophoresed along with the samples on the sequencing gel. For this, The m13 DNA was sequenced manually using the Sequenase Version 2.0 Sequencing kit as per manufacturers instruction, using □–^{32}PdATP as the radiolabel.

Statistical analyses: Allele frequencies were determined by direct counting. The observed heterozygosity *(H_O)* was estimated as the proportion of observed heterozygotes at a locus to the total number of genotypes and the mean heterozygosity by averaging over all loci. The expected heterozygosity *(H_E)* was calculated using the formula:

$$H_E = 1 - \sum_{i=1} Pi^2$$

where, *pi* is the frequency of ith allele (Ott 1992).

The polymorphic information content (PIC) was calculated in the total number of individuals according to Botstein *et al*. (1980) using the formula:

$$PIC = 1 - [\sum_{i=1}^{k} p_i^2] - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2p_i^2 p_j^2$$

where, *Pi* and *Pj* are the frequency of the ith and jth alleles. The locus-specific mean parentage exclusion (PE) probabilities were computed according to Jamieson (1965) using the formula:

$$P_i = \sum_{j=1}^{n} p_j (1 - p_j)^2 - \sum_{j-1}^{n} \sum_{k>j}^{n} (p_j p_k)^2 [4 - 3(p_j + p_k)]$$

where, *Pi* is the probability of paternity exclusion at the *i*-th locus in the case that all alleles are codominant, *Pj* and *Pk* are the frequencies of the *j*-th and *k*-th alleles, respectively, and *n* is the number of alleles at the locus. The cumulative probability of paternity exclusion was calculated using the formula, CPE = 1– (1–PE_1) (1–PE_2)…… (1–PEn) where PE is the probability of exclusion using each independent locus.

The locus-specific individualization potential *(P_{ID})* was estimated as the sum of the squared genotypic frequencies at a locus and combined them over loci by multiplying the single–locus P_{ID}'s across loci (Sensabaugh 1982).

RESULTS AND DISCUSSION

Of the 21 microsatellites tested, all the loci except Lat13 and Lat18 were amplified well in elephants. One locus, namely Lat07, was monomorphic whereas all the other loci were polymorphic in Asian elephants tested. For the remaining 18 loci, typing was carried out for varying numbers of samples and the polymorphic pattern was recorded. The total number of alleles at a locus (2N), number of different alleles, number of different genotypes, allele size range, observed *(Ho)* and expected *(He)* heterozygosities, polymorphic information content, exclusion probabilities and individualization potential *(P_{ID})* estimated for each locus under investigation are presented in Table 1.

Heterozygosity is a measure of usefulness of the marker. Markers with higher heterozygosity values are more useful for linkage studies, parentage verification and individual identification. In the present study, the observed heterozygosity *(Ho)* varied from 0.763 to 1.000 and the expected heterozygosity *(He)* values of the markers ranged from 0.568 (ILSTS030 locus) to 0.927 (LaT08 locus). The PIC values for different loci under the present study varied from 0.499 (for ILSTS030 locus) to 0.911 (for LaT 08 locus). The extremely high levels of polymorphism exhibited at most of these loci make them ideal for parentage testing. The locus specific mean PE probabilities ranged from 0.299 to 0.852 with an average of 0.632. The cumulative mean exclusion probabilities for the 2 loci with the highest exclusion

Table 1. Number of alleles, genotypes, allele size range, observed and expected heterozygosities, PIC, exclusion probabilities and individualization potential for the different microsatellite loci tested in Asian elephants

Locus	2N	Alleles	Geno-types	Size range (bp)	*Ho*	*He*	PIC	PE	P_{ID}
LaT08	250	18	77	291–439	0.848	0.927	0.911	0.852	0.016
LaT24	148	18	50	298–390	0.946	0.915	0.909	0.833	0.027
LaT26	272	12	52	292–364	0.912	0.889	0.879	0.775	0.034
LA2	160	12	29	211–233	0.925	0.894	0.884	0.787	0.046
FH94	140	11	33	203–225	0.814	0.873	0.860	0.743	0.047
Laf MS03	130	12	30	130–152	0.985	0.879	0.864	0.718	0.067
FH60	142	11	8	118–140	1.000	0.862	0.847	0.723	0.072
FH102	150	13	23	158–192	0.813	0.869	0.857	0.743	0.078
LaT05	48	10	13	369–437	0.958	0.848	0.832	0.705	0.094
LafMS02	292	8	21	123–139	0.979	0.850	0.833	0.702	0.095
LaT25	206	10	30	304–336	0.814	0.827	0.806	0.663	0.059
LA4	284	6	13	108–118	0.993	0.824	0.799	0.645	0.111
EMX1	264	7	14	126–150	0.894	0.784	0.756	0.591	0.110
FH71	78	14	16	228–266	0.944	0.755	0.730	0.571	0.198
FH48	80	6	8	147–161	0.900	0.629	0.574	0.382	0.376
LaT16	80	4	6	328–340	1.000	0.619	0.543	0.338	0.552
LafMS04	80	4	3	135–149	1.000	0.584	0.499	0.299	0.665
ILSTS030	76	3	5	184–210	0.763	0.568	0.499	0.299	0.309
Mean (SE)	159.7	9.9	23.9		0.9	0.8	0.777	0.632	0.164
	(19.3)	(1.0))	(4.6)		(0.02)	(0.03)	(0.03)	(0.04)	(0.05)

2N, Total number of alleles at a locus; *Ho*, observed heterozygosity; *He*, expected heterozygosity; PE, exclusion probability; P_{ID}, individualization potential.

probabilities was 97.5, for the best three 99.5, and for the best 5 exceeded 99.8.

The P_{ID} measures the probability of selecting 2 identical genotypes at a locus in a reference population. The estimated P_{ID} for the most informative locus, Lat08 is 0.016, which is equivalent to a probability of 1 out of 62 of observing a second, identical genotype selected at random from the population. Though this probability is not very useful if considered alone, when P_{ID} is compounded across many loci using the product rule, the probability of observing a particular multi-locus genotype combination becomes extremely small. For the best 2 loci, the combined P_{ID} is equivalent to a probability of 1 out of 2315 ($P_{ID} = 0.000432$) and for the best 5 loci, the figure is equivalent to a probability 3.15×10^{-7} ($P_{ID} = 3.18 \times 10^{-8}$), which could uniquely identify over 630 times the total world population of approximately 50 000 Asian elephants (Table 1).

The Asian elephant is an endangered species. Their future survival depends on intensive conservation and management, based on in-depth knowledge of particular populations. Molecular genetic methods, especially microsatellite analysis provides an effective means of obtaining such information. Our results provide the basic data on the extent of genetic variability present in Asian elephant. This data could be exploited for estimating the level of inbreeding among the wild elephant populations using a non-invasive DNA sampling and subsequent microsatellite analysis based on the panel of polymorphic markers. Any significantly reduced genetic variation and heterozygosity in wild populations could be attributed to an increase in inbreeding in such populations. Thus the data generated from this study could be utilized for the formulation of an accurate and well-organized conservation programme for Asian elephant.

The panel of polymorphic microsatellite markers developed could be used for various uses such as individualization of elephants from seized evidence for forensic detection, for medico-legal and insurance applications, verification of parentage and to trace the origin of individual animals to provide proof of illegal poaching and capture. It can also be used for census of wild elephant populations through a non-invasive approach (DNA isolated from dung samples), as the panel of markers identified is capable of individual identification.

REFERENCES

Botstein D, White R L, Skolnick M and Davis R W. 1980. Construction of a genetic linkage map in human using restriction fragment length polymorphisms. *American Journal of Human Genetics* **32**: 314–31.

DeNise S, Johnston E, Hawerson J, Marshall K, Rosenfeld D, Mekanna S, Sharp T and Edwards J. 2004. Power of exclusion for parentage verification and probability of match for identity in American Kennel club breeds using 17 canine microsatellite markers. *Animal Genetics* **25**: 14–19.

Dublin H, Desai A A, Hedges S, Vie J C, Bambaradeniya C and Lopez A. 2006. *Elephant Range States Meeting*. 24–26 January 2006. Kuala Lumpur, Malaysia.

Fernando P. 1993. Implications of socioecology and genetics on the conservation and management of the Sri Lankan elephant. *A week with elephants. Proceedings of the International Seminar on Asian Elephants*. pp. 298–313. (Eds) Danial J C and Datye H. Bombay Natural History Society, Oxford University Press, Bombay.

Fernando P and Lande R. 2000. Molecular genetic and behavioral analysis of social organization in the Asian elephant *(Elephas maximus*). *Behavioural. Ecology and Sociobiology* **48**: 84–91.

Gotelli D, Sillero-Zubiri C and Applebaum G D.1994. Molecular genetics of the most endangered canid: the Ethiopian wolf *Canis simensis*. *Molecular Ecology* **3**: 301–12.

Jamieson A. 1965. The genetics of transferrins in cattle. *Heredity* **20**: 419–441.

Montgomery G M and Sise J A. 1990. Extraction of DNA from sheep white blood cells. *New Zealand Journal of Agricultural Research* **33**: 437–41.

Ott J. 1992. Strategies for characterizing highly polymorphic markers in human gene mapping. *American Journal of Human Genetics* **51**: 283–90.

Sensabaugh G F. 1982. Biochemical markers for individuality. *Forensic Science Handbook*. pp.338–415. (Ed.) Saferstein R. Prentice-Hall, Eaglewood Cliffs, N J.

Sukumar R. 2003. Asian elephants in zoos: A response to Rees. *Oryx* **37**: 23–24.

Xu YC, Li B, Li W S, Bai S Y, Jin Y, Li X P, Gu M B, Jing SY and Zhang W. 2005. Individualization of tiger using microsatellites. *Forensic Science International* **151**: 45–51.