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GENETIC DIVERSITY ASSESSMENT OF CAPTIVE ASIAN ELEPHANT (ELEPHAS MAXIMUS) POPULATION AT GURUVAYUR ELEPHANT CAMP USING MICROSATELLITE DNA MARKERS

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ABSTRACT OF PROJECT PROPOSAL

1. Title : Genetic diversity assessment of captive Asian elephant (*Elephas maximus*) population at Guruvayur elephant camp using microsatellite DNA markers

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4. Objectives :

   i. To assess the genetic diversity of the captive elephant population at Guruvayur using microsatellite markers.

   ii. To extrapolate the diversity of the population to that of the natural elephant populations to which the elephants originally belonged.

   iii. To develop breeding strategies to maintain the genetic diversity in the captive elephant population of Guruvayur

5. Duration : 2 years

6. Funding agency : Plan Fund

CONTENTS

ABSTRACT

DNA fingerprinting using microsatellite markers and genetic characterization of 43 captive Asian elephants (*Elephas maximus*) of Guruvayur temple was carried out after standardizing a non-invasive method of DNA extraction from dung samples. Population genetics studies on wildlife community structure using DNA markers have been constrained in the past due to difficulties in obtaining tissue or blood samples for DNA extraction. The congregation of 65 elephants of Guruvayur temple at Punnathur Ana Kotta was utilized for the present study. These elephants of various geographic origin offered to the temple by devotees were purchased from different regions of India. Dung samples collected from elephants within six hours after defecation gave complete success in DNA extraction, while 88 – 96 per cent success was obtained from three-day-old dung. Pure DNA was extracted using QIAamp DNA stool mini kit. Polymerase chain reaction was carried out for amplifying three microsatellite loci, namely EMX 1, EMX 2 and EMX 3 using specific primers reported by Fernando for Asian elephants of Sri Lanka. Amplification products were electrophoresed on 6 per cent denatured polyacrylamide gel and data were analyzed using the population genetics analysis software POPGENE.

Though DNA was obtained from dung samples of 62 elephants, we could amplify all the three microsatellite markers from 43 animals only. Five alleles were recorded for EMX 1 and EMX 2, while EMX 3 showed only two alleles. The genotypes of the 43 elephants were assigned as homozygous dominant, heterozygous and homozygous recessive. The geographic origins of only 27 animals were known. The 43 animals were grouped as populations of South India, Assam, Bihar and of unknown origin, and those brought from Andaman and Nicobar Islands. The genetic distance between the five populations ranged from 0.0044 to 0.3595. UPGMA dendrogram constructed using genetic similarity showed that Bihar, Assam and unknown origin populations clustered into one group conforming to the geographic proximity of Bihar and Assam populations. The elephant populations from Andaman and Nicobar Islands and Assam origin showed the highest genetic distance while the elephants from Assam and those grouped as unknown origin showed the lowest genetic distance. South Indian population joined with the cluster comprising Assam,

Bihar and unknown origin elephants. The Andaman population stood out as a separate group. The elephant population of Andamans was reported to be a mixture of various geographic origins. The mean observed heterozygosity is almost half that of expected heterozygosity indicating very high occurrence of inbreeding. Since the temple elephant population is not a natural population and the elephant identity is not based on documentary evidence, detailed analysis of the result has not been done.

1. INTRODUCTION

The elephant is the largest land animal that lives in Asia (*Elephas maximus*) and Africa (*Loxodonta africana*). The Asian elephants live in India, Malaysia, Sumatra, Sri Lanka, Nepal, Bhutan, Bangladesh, Burma, Thailand, Cambodia, Laos, Vietnam, China, Indonesia and Borneo. Asian elephants live in many different habitats including open grasslands, marshes, savannas and forests. The Asian elephant specialist group of the Species Survival Commission of IUCN estimates that there are approximately 38,000 to 51,000 wild Asian elephants. Asian elephants are among the largest herbivores feeding on grasses, leaves, saplings and shrubs. Their life span is up to 70 years.

There are four sub-species of Asian elephants:

- Indian elephant (*E. maximus indicus*)
- Sri Lankan elephant (*E. maximus maximus*)
- Sumatran elephant (*E. maximus sumatrensis*)
- Borneo elephant (*E. maximus borneensis*)

E. maximus indicus survives in separate ranges in Southern India, the Himalayan foothills and North-East India. It is also found in Southern China, Burma, Thailand, Cambodia and the Malaysian Peninsula. Most males of these sub-species have tusks (Shoshani and Eisenberg, 1982).

E. maximus maximus is only found in Sri Lanka. It has a larger skull relative to body size, and commonly has a decolorized area of skin on the forehead and the front of the upper trunk. It is rare to find males with tusks. Males can reach a height of 3.5 metres at the shoulder.

E. maximus sumatrensis is only found in Sumatra. It is the second smallest sub-species, between 1.7 to 2.6 metres at the shoulder. It is sometimes called the pocket elephant because of its size (Medway, 1977).

E. maximus borneensis is found in North Borneo. It is smaller than all the other sub-species. It has larger ears, a longer tail and straighter tusks. Genetic tests found that its ancestors were separated from the mainland population about 3,00,000 years ago (Deraniyagala, 1950,1955; MacKinnon *et al.*, 1996; Fernando *et al.*, 2003a,b).

The extinct Chinese population is sometimes separated as *E. maximus rubridens* (Pink tusked Elephant); it disappeared after the 14th century BC. The Syrian Elephant (*E. maximus asurus*), one of the largest sub-species of the Asian Elephant, went extinct by around 100 BC. This latter population and the Indian elephants were considered the best war elephants in antiquity, and found superior to the smaller North African elephant used by the armies of Carthage.

The Asian elephant is one of the species listed as endangered in IUCN's Red Data List. India is home to approximately 22,700 to 32,400 free ranging elephants (Bist, 2002; Sukumar, 2003; unpublished data from the Asian Elephant Research and Conservation Centre), constituting over half the world's total estimated free ranging Asian Elephant population (Sukumar, 2003). Elephants are distributed across the North-Western and North-Eastern Himalayan foothills, central and southern India, with the population sizes of 1000 – 1500, 9000 – 10000, 1500-2500 and 12500-14500, respectively (Sukumar and Santia Pillai, 1996; Bist, 2002; Sukumar, 2003). The largest Asian elephant population remains in India (Vidya *et al.*, 2005).

In India, as elsewhere in world, elephant numbers have declined substantially over the last few millennia due to habitat loss and fragmentation, historical capture in large numbers for domestication and more recently, poaching of males for ivory. Government of India and various state governments have initiated a large number of schemes for study of ecology, social behaviour, carrying capacity of habitats, pattern of migration and above all conservation of elephant population. Linking population genetics to wildlife population and community structure is an emerging field of molecular genetics. Molecular biology is the new tool to acquire data for conservation, management, planning and prevention of wildlife crimes. But, due to peculiarities of

their habitats and their aggressive response to man, the study of wild elephant population through invasive methods is impractical in the forest.

1.1. GENOTYPING WILD ANIMALS

Recent advances in Molecular Genetics have led to a multitude of studies applying genetic analysis to diverse fields such as development, ecology, evolution, behavior, and conservation. Molecular studies of free ranging populations of large mammals have been constrained in the past by the difficulties in obtaining tissue or blood samples from which DNA can be easily extracted. Samples of dung, shed hair and feathers, sloughed skin, and discarded food wastes were a source of DNA for genetic analysis. Such noninvasive sampling could overcome many sampling constraints (Kohn and Wayne, 1997). Todate, dung, and to a lesser extent, hair has been the most widely employed noninvasive sources of DNA (Fernando *et al.*, 2003a; Vidya and Sukumar, 2005; Vidya et al., 2005). Dung is of particular interest as all animals defecate regularly, and for many species, finding dung is comparatively simple and collection, storage and transport require little technology or expense. Asian elephants usually inhabit poor visibility habitat and the ubiquity of human – elephant conflict over most of their range leads to their developing avoidance or aggressive responses towards humans. Consequently, they are difficult to study by observational methods alone and molecular genetic techniques can be an effective means of obtaining data critical for their management and conservation. In species that are rare, sensitive to risk of extinction or under intensive behavioral study, noninvasive genotyping is preferred because it avoids disturbing the animals under observation. Techniques for genotyping samples with low DNA quantity have become widely employed in ecological studies in the last decade, particularly in analysis of samples obtained noninvasively (Broquet and Petit, 2004; Okello *et al.*, 2005).

1.2. PROBLEMS WITH NONINVASIVE SAMPLES

A number of problems are associated with noninvasive samples, such as copurifying contaminants (Litvaitis and Litvaitis, 1996), low amounts of DNA (Frantzen *et al.*, 1998) and DNA degradation leading to non-amplification, false alleles, sporadic contamination and allelic dropout (Gagneux *et al.*,1997, Kohn and Wayne, 1997; Taberlet *et al.*,1999). Fernando *et al.* (2003a) worked extensively on this topic and concluded that non-invasive genotyping of elephants using DNA from dung is a reality. However, the technique needs standardization before its application to the study of wild animals in the forests.

1.3. GURUVAYOOR ELEPHANT CAMP

Before initiating non-invasive genotyping of wild animals, we decided to standardize the technique in our laboratory through genotyping domesticated elephants. The Asian elephant camp at Guruvayur, associated with the temple has a population of 65 elephants. These elephants were purchased by the devotees from different elephant markets in India and offered to the temple. So, the population comprised of elephants that originated from different states of India and the genetics of the population will be highly diverse than that of other wild population existing in a region or state. In genetic diversity point of view, this sort of diverse captive population is highly significant, especially in a scenario where the genome of elephant population in the country is expected to be more and more drifted towards homozygosity, an adverse impact of forest fragmentation. The restricted animal movement due to shrinking forest boundary and reduction of males due to illegal poaching for extracting tusks resulted in inbreeding. This study has been undertaken to evaluate the diversity of the captive elephant population at Guruvayur through non-invasive methods.

1.4. MICROSATELLITES

Microsatellite DNA marker analysis is a powerful tool in the genetic study of free ranging organisms. Microsatellites are defined as loci where short sequences of DNA

are repeated in tandem arrays. These markers are also known as simple sequence repeats (SSR); in particular the di-nucleotide repeats CA or GA are abundant in most eukaryotic nuclear genomes and are distributed throughout these genomes in dispersed locations. These micro- satellite repeats are often flanked by unique sequences.

Microsatellites are useful because they are highly polymorphic markers as there are often many alleles at a microsatellite locus, each allele having a different number of tandem repeats. This occurs because there is a high mutation rate at microsatellite loci as DNA replicating enzymes make mistakes in the number of repeats at relatively frequent rate. Larger Microsatellites, containing more repeats, tend to be more polymorphic.

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 (Vidya *et al.*, 2005).

Tetra-nucleotide microsatellites are more reliable markers than dinucleotide repeats for noninvasive genotyping because the di-nucleotide repeats are vulnerable to polymerase slippage during PCR especially where DNA quality and quantity are low (Fernando *et al.*, 2001). So, here we consider tetra-nucleotide microsatellites for genetic diversity assessment of captive Asian elephant population at Guruvayur elephant camp.

1.5. OBJECTIVES OF THE PRESENT STUDY

- To assess the genetic diversity of the captive elephant population at Guruvayur using microsatellite markers.
- To extrapolate the diversity of the population to that of the natural elephant population to which the elephants originally belonged.

2. MATERIALS AND METHODS

2.1. SAMPLE COLLECTION

We collected 65 dung samples from the elephant camp at Guruvayur. Dung samples were collected from individuals within six hours after defecation. Though, attempt was made to obtain documentary evidence for the geographic origin of the elephants, it was not possible to obtain reliable data. Hence, based on the available records in the office of the Guruvayur Elephant Camp and information collected from Veterinary Doctor of the Guruvayur Devaswom Board and staff of the Elephant Camp, origin of 27 elephants were ascertained (Table 1). Samples were collected from the outer most layer of dung, which would have endothelial cells sloughed off from the gut lining during the gut passage of the dung bolus. As the surface of the dung bolus is the portion in contact with the gut lining and the first to dry upon deposition, it could contain the least degraded DNA. Therefore, samples were collected from the surface of dung boli in 95 per cent ethanol by scrapping off the crust. The collection bottles with dung samples were stored in -20^{0}C deep freezer.

Table 1. List of elephants included in the study

Sl.No	Name	Place of origin
1	Aadhithyan	Andaman and Nicobar
2	Chenthamarakshan	Andaman and Nicobar
3	Devdas	Assam
4	Vinayakan	Assam
5	Achuthan	Bihar
6	Appu	Bihar
7	Gajendran	Bihar
8	Indrasen	Bihar
9	Junior Madhavankutty	Bihar
10	Kuttyshankaran	Bihar
11	Rajashekharan	Bihar

12	Ramu	Bihar
13	Sathyanarayanan	Bihar
14	Sreedharan	Bihar
15	Valiya kesavan	Bihar
16	Nandan	South India
17	Ravikrishnan	South India
18	Shankaranarayanan	South India
19	Nandini	South India
20	Narayanankutty	South India
21	Padmanabhan	South India
22	Radhakrishnan	South India
23	Ramachandran	South India
24	Ramankutty	South India
25	Sidharthan	South India
26	Thara	South India
27	Umadevi	South India
28	Gokul	Unknown
29	Krishnan	Unknown
30	Kesavan Kutty	Unknown
31	Murali	Unknown
32	Balakrishnan	Unknown
33	Rashmi	Unknown
34	Lakshmi Krishnan	Unknown
35	Gopalakrishnan	Unknown
36	Mukundan	Unknown
37	Kannan	Unknown
38	Peethambaran	Unknown
39	Devi	Unknown
40	Lakshminarayanan	Unknown
41	Sr. Vishnu	Unknown
42	Kuttikrishnan	Unknown
43	Prakasan	Unknown

2.2. DNA EXTRACTION AND POLYMERASE CHAIN REACTION

DNA was extracted using QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's protocols. Polymerase chain reaction was carried out using the following primers viz. EMX1, EMX2, EMX3 (Fernando *et al.,* 2001). DNA was amplified using Hot Star Hi-fidelity Polymerase kit (100U) (Quiagen, Germany) in 25µl reaction mixture using appropriate quantities of template DNA, 5 µl Taq buffer with 0.5 mm dNTPs and 1.5 mm $MgSO_4$, 2.5U of Taq polymerase, 25 ppm/1µm of primer. The incubation mixture was subjected to 40 cycles of amplification in PTC – 100 Thermal Cycler (MJ Research Inc, USA). For all loci, a 95^0 C initial denaturation for 5 min, 94^0 C denaturation for 15 S and a 72^0 C extension for 1 min were employed, followed by a final extension at 72^0 C for 10 min after the completion of all 40 cycles.

2.3. DNA FRAGMENT SEPARATION AND VISUALIZATION

The Polymerase chain reaction amplified-products were electrophoresed in 1.5 per cent agarose gel in TBE buffer (45 mM Tris-borate, 1mM EDTA, pH 8.0). The gel, after the completion of electrophoresis was stained with ethidium bromide and DNA bands were compared with a 100bp DNA ladder (Genei). The gels were documented using VILBER LOURMAT Doc-Print II (France).

Amplification products were further electrophoresed on 6 per cent denatured polyacrylamide gels along with a 100bp DNA ladder (Genei) and pUC19 DNA/MSP1 Digest ladder (Genei). Allele sizes were identified using Kodak Digital Science 1D Image Analysis Software.

2.4. DATA ANALYSIS

The allele sizes were estimated comparing with DNA size markers run on the same gel and the data scored for analysis. The bands were scored '1' for their presence and '0'

for absence in each DNA sample. The elephants were grouped into five groups based on their origin namely, South India, Bihar, Assam, Andaman and Nicobar Islands and those of unknown origin. The data matrices were entered into POPGENE version 1.32 computer package. Heterozygosity statistics for all loci, per cent of polymorphic loci and genetic distance coefficients (Nei, 1978) between the groups were estimated using the software. A UPGMA dendrogram was constructed based on the pair-wise genetic distance between the groups.

3. RESULTS AND DISCUSSION

3.1. PCR AMPLIFICATION OF LOCI

Amplification success of microsatellites varied across loci according to age of dung (Table 2). All the three loci (EMX 1, EMX 2 and EMX 3) successfully amplified in more than 87 per cent of the elephants when fresh DNA extracts (within three days after extraction) were used (Fig.1). Amplification success decreased when the dung samples collected were not fresh. The percentage of amplification success was approximately 43 per cent when older DNA extracts (20 days after collection) were used. All the 3 loci from four animals out of 65 animals did not amplify. The number of samples that did not amplify one or two loci was nine.

Table 2. Number of animals showing amplification of the three loci

Preservative used	Days of dung under preservation	Total number of elephants from which dung samples were used for DNA extraction	Locus amplified, number of elephants and the percentage of amplification success (in parenthesis).		
			Loci		
			EMX1	EMX2	EMX3
Alcohol	1-3 days old	49	47 (95.9%)	43 (87.7%)	43 (87.8%)
Alcohol	12-20 days	16	12 (75%)	9 (75 %)	7(43.8%)

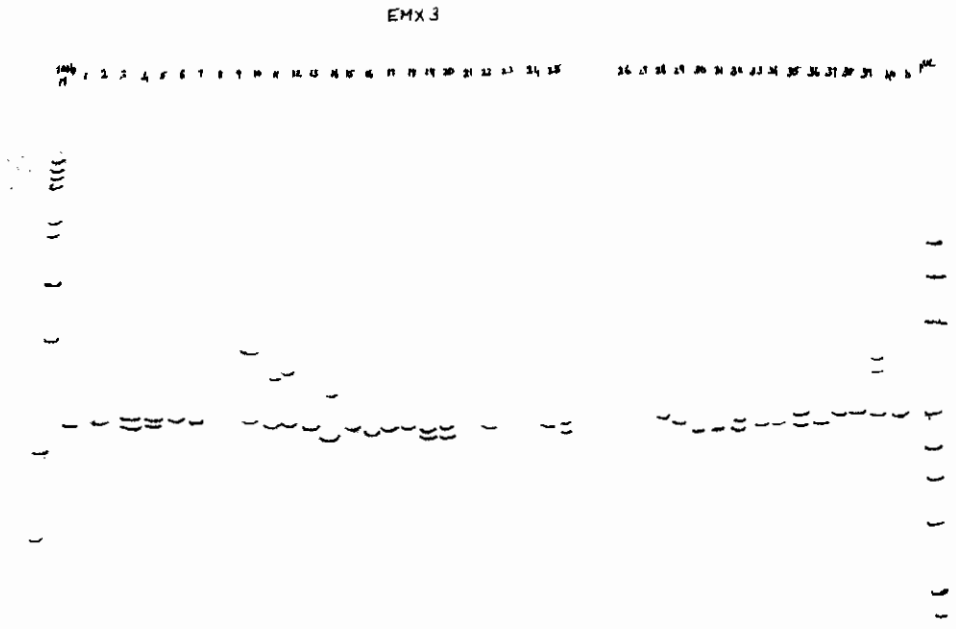

Fig. 1. Sketch of silver stained PAGE glass plate of the locus EMX 3 traced on polythene sheet and photographed

The data from 43 elephants were used for population genetics parameter analysis. The three microsatellite markers namely, EMX 1, EMX 2 and EMX 3 were amplified for 43 elephants and their genotype assigned as homozygous dominant, heterozygous and homozygous recessive. These animals included those originated from South India (Kerala, Karnataka and Tamil Nadu), Assam, Bihar, Andaman and Nicobar Islands, and animals of unknown origin (Table 1) (PLATE 1-7).

Since, Karnataka and Kerala populations in the north of Palghat gap of the Western Ghats cross the borders of the three states of Kerala, Karnataka and Tamil Nadu, these populations were considered as South Indian populations for analysis. Hence, the 27 elephants were considered to have originated from four regions namely, Andaman and Nicobar Islands, Assam, Bihar and South India. The rest of the 16 were

grouped as of unknown origin. The genetic distance coefficients between the paired populations varied from 0.0044 to 0.3595 (Table 3). The UPGMA dendrogram was constructed to represent the genetic similarity between the five populations using Nei's genetic distance coefficients (Fig. 2). The elephant populations of Andaman and Nicobar Islands and Assam origin showed the highest genetic distance while the elephants from Assam and those grouped as unknown origin showed the lowest genetic distance. The genotypes of Assam and unknown origin populations were almost identical probably because many of the unknown origin elephants might have originated from Assam. Animals from Bihar and Assam, and animals of unknown origin were grouped into one cluster. South Indian population joined with the cluster comprising Assam, Bihar and unknown origin elephants. The grouping of elephant populations from Assam and Bihar into one cluster conform to the geographic proximity of the two states. The Andaman population stands out as a separate group. The elephant population of Andamans has been reported to be a mixture of various origins. The elephants currently seen in Andamans originated in the mainland and they were taken to the island for using them in forest coupes probably a few centuries ago. Since, documentary evidence on the exact origin of elephants was unavailable, further analysis on this aspect was not done.

Table 3. Nei's unbiased measure of genetic distance (Nei, 1978)

Elephant Population	1. Andaman & Nicobar Islands (ANI)	2. Assam	3. Bihar	4. South India (SI)	5. Unknown
1. ANI	*****				
2. Assam	0.3595	****			
3. Bihar	0.2904	0.0346	****		
4. SI	0.3287	0.0353	0.2214	****	
5. Unknown	0.1082	0.0044	0.0616	0.1062	****

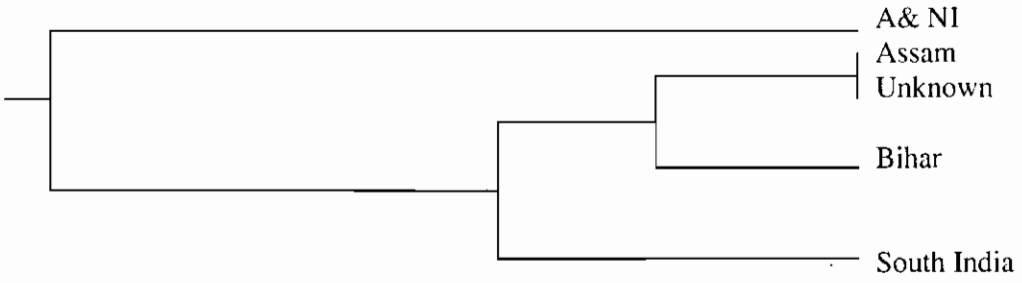

Fig. 2. UPGMA dendrogram showing the genetic similarity of elephant Populations

Heterozygosity statistics for all loci were calculated using Popgene package (Table 4). The observed heterozygosity is lower than the expected heterozygosity in respect of all the loci. The mean observed heterozygosity is almost half that of expected heterozygosity indicating very high occurrence of inbreeding. Since the temple elephant population is not a natural population and the elephant identity is not based on documentary evidence, detailed analysis of the result has not been done.

Table 4. Summary of heterozygosity statistics for all loci

Loucs	Sample size	Observed heterozygosity	Nei's expected heterozygosity	Average Heterozygocity
EMX 1	86	0.4651	0.7623	0.6754
EMX2	86	0.3488	0.7696	0.6768
EMX3	86	0.1395	0.4543	0.3750
Mean	86	0.3178	0.6621	0.5757
S.D.		± 0.1650	± 0.1800	± 0.1739

Only very few studies have been done on the genetic diversity of Indian elephants using non-invasive methods. Vidya and Sukumar (2005) and Vidya *et al.* (2005) studied the population differentiation within and among South Indian elephants.

namely, Nilgiris, Anamalai and Periyar elephant reserves. They could amplify 86 to 97 per cent of the samples using EMX primers. Normal microsatellite diversity was reported by them for the elephant populations of Southern India. Though Nilgiri population is the world's single largest elephant population, it showed lower diversity than other populations of South India. No microsatellite differentiation among localities within Nilgiris was also reported suggesting extensive gene flow. Anamalai and Periyar were not genetically differentiated, but Nilgiri population was genetically distinct from Analmalai and Periyar, probably because of the 25 km wide stretch of Palghat gap. AMOVA of the 3 populations indicated that 94.8 per cent of the total genetic variation was within populations and the rest among populations. Observed heterozygosity was lower in Periyar compared to the other two. Periyar population showed significant inbreeding coefficient (Fis) of 1.00 for EMX 3 locus.

4. CONCLUSIONS

The present study demonstrates the standardization of non-invasive technique of extracting elephant DNA from dung samples and PCR amplification of elephant specific microsatellite loci using the DNA as the template. The protocols developed can be adopted for the study of genetic variability, population structure, movement pattern, mating behaviour and crop raiding of wild elephants through DNA finger printing of wild elephants extracting DNA from dung samples from the forest. The technique can be further adopted for other wild animals such as tiger, panther, leopard, etc.

5. ACKNOWLEDGEMENT

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PLATE I

ASSAM

Devdas

Vinayakan

ANDAMAN

Aadithyan

Chenthamarakshan

Sheshadri

PLATE II
BIHAR

Kesavan

Indrasen

Appu

Kuttyshankaran

Rajashekharan

Ramu

Achuthan

Sathyanarayanan

Sreedharan

PLATE III

KERALA AND KARNATAKA (1)

Nandan

Nandini

Narayanan Kutty

Padmanabhan

Radhakrishnan

Ramachandran

PLATE IV

KERALA AND KARNATAKA (2)

Ramankutty

Ravikrishnan

Shankaranarayanan

Sidharthan

Thara

Umadevi

PLATE V
ORIGIN UNKNOWN (1)

Akshayakrishnan

Balakrishnan

Balu

Chandrashekharan

Damodardas

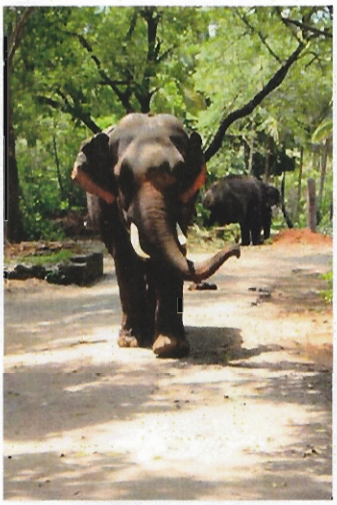
Devi

Gopalakrishnan

Gopikannan

Gopikrishnan

PLATE VI
ORIGIN UNKNOWN (2)



Jr Achuthan

Jr Vishnu

Jr Lakshmanan

Keerthi

Jr Keshavan

Krishna

Krishnan

Kesavankutty

Krishnanarayanan

PLATE VII
ORIGIN UNKNOWN (3)

Kuttikrishnan

Lakshminarayanan

Madhavankutty

Mukundan

Peethambaran

Prakashan

Rashmi

Unnikrishnan

Vineeth