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Multi-Gene mtDNA Primers for Use with Non-invasive Sampling of Asian Elephants

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Abstract. Understanding population genetic structure and phylogeography can be useful in framing conservation strategies for the Asian elephant. Non-invasive genetic sampling allows for such information to be obtained. However, amplifying long fragments from poor-quality faecal DNA is challenging. Therefore, we developed primers to amplify 270–474 bp fragments to cover ≈2 kb of the Asian elephant mtDNA genome across ATP8-ATP6, ND5 and D-loop regions. We obtained a 1978-bp sequence (in six fragments) from up to two-week-old faecal samples with a success rate of 79.8%. These primers would provide fine resolution phylogeography for the Asian elephant, and population-specific SNPs would aid in forensic tracking.

Introduction

The Asian elephant (*Elephas maximus*) has been obliterated from 95% of its historical range, whereas, in India, its geographic distribution has shrunk by 70% since the 1960s (Sukumar 2006). The only surviving Proboscidean species in Asia is enlisted as 'Endangered' by IUCN, is placed on 'Appendix I' of CITES and is a 'Schedule I' species as per the Wildlife (Protection) Act, 1972 of India. Once widespread in India, the species has retreated to four general areas: north-eastern, central, north-western and southern India with a total population of ≈27,000 individuals (PED-MoEFCC 2017).

Thorough information on population genetic structure and the distinctiveness of populations are imperative for management of a threatened animal species (Avise 1995). Genetic tools are useful in delineating population units of conservation importance (Crandall *et al*. 2000; Fraser & Bernatchez 2001; Vidya *et al*. 2005a, b) in addition to understanding behaviour, evolution, and planning conservation strategies for large social animals like elephants (Fernando & Lande 2000; Fernando *et al*. 2003; Vidya & Sukumar 2005).

There has been considerable interest in understanding the phylogeography of the Asian elephant (Fernando *et al*. 2000, 2003; Fleischer *et al*. 2001; Vidya *et al*. 2009) ever since early studies showed differences between mainland and Sri Lankan populations (Nozawa & Shotake 1990; Hartl *et al*. 1995) and the presence of two divergent clades of mitochondrial DNA (Hartl *et al*. 1996). A majority of the studies on the phylogeography or population genetic structure of the Asian elephant focus on using mitochondrial DNA (mtDNA) (Fernando *et al*. 2000; Vidya *et al*. 2009; Zhang *et al*. 2015), although some studies have additionally examined nuclear markers (Fernando *et al*. 2003; Vidya *et al*. 2005a, b; Ahlering *et al*. 2011). Almost all studies based on mtDNA have used the non-coding D-loop region. Exceptions include studies by Hartl *et al*. (1996) and Fleischer *et al*. (2001) that used cytochrome b (Cyt b), and by Vandebona *et al*. (2002) using NADH dehydrogenase subunit 5 (ND5). Most of these studies have been limited to the use of sequences that were 400–700 bp long, covering a single locus whereas improved insight of the Asian elephant phylogeography may be obtained with longer sequences across multiple genes.

Molecular techniques involving non-invasive genetic sampling using dung or faecal matter

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have been used successfully to examine evolutionary radiation, gene flow and sub-structures of elephant populations (Fernando *et al*. 2000; Vidya *et al*. 2005a, b; Ahlering *et al*. 2011; Zhang *et al*. 2015). However, if the dung samples collected are old, the faecal DNA may be sheared and degraded. The most widely used mtDNA primers for Asian elephants amplifying the D-loop region are over 600 bp long (Fernando & Lande 2000) and may be challenging to amplify from poor quality source material. Designing primers with robust annealing to yield amplicons that are 200–500 bp long could facilitate amplification from non-invasive faecal DNA and allow long stretches of mtDNA to be assembled from the shorter fragments. There were a few published primers targeting regions other than the D-loop, but they had been tested only in the African elephants (e.g. Finch *et al*. 2014) or were universal mammalian primers having the risk of human cross-amplification (Vandebona *et al*. 2002). Therefore, we aimed to design and optimize suitable multi-gene primers for amplification of the published polymorphic regions of the ATP synthase, ND5 and D-loop regions of mtDNA from faecal DNA of varying quality for better understanding of evolutionary radiation in Asian elephants.

Material and methods

DNA extraction from faecal samples

We collected elephant faecal samples, ranging from fresh to approximately two weeks old, from the Rajaji Tiger Reserve and the adjoining Forest Divisions in Uttarakhand, India, opportunistically while surveying the area on foot. The samples were placed in sterile 50 ml screw-cap containers with silica gel immediately after collection to prevent moisture-induced degradation. We oven-dried the samples at 50°C at the laboratory before storage at room temperature up to one year before DNA extraction.

We scraped the top layer from the faecal samples containing sloughed-off intestinal epithelial cells using a sterile blade into sterile 2.0 ml polypropylene tubes. We followed the standard DNA extraction protocol suggested by the manufacturer using column-based QIAGEN QIAamp DNA Stool Mini Kit after overnight digestion with stool lysis buffer at 56°C in a water bath. The eluted DNA was stored at -20°C until further use. We used a negative control during all the extraction batches to check for contamination.

Designing the mtDNA primers

Though the Asian and the African elephants diverged approximately 6.8 million years ago (Roca *et al*. 2015), conserved sequences are present and primers are known to cross-amplify. Therefore, we aimed to amplify parts of the mtDNA genome displaying polymorphism in different populations of Asian and African elephants as represented in the public domain database of NCBI GenBank. We found polymorphism at ATP synthase F0 subunits 8 (ATP8) and 6 (ATP6) (Finch *et al*. 2014), ND5 (Vandebona *et al*. 2002), and the D-Loop region (Fernando & Lande 2000; Sulandari & Zein 2012; Vidya *et al*. 2009), which were suitable for further investigation.

Therefore, we decided to redesign one set of ATP8-ATP6 primers having 84 bp overlap with an existing set of primers (Finch *et al*. 2014) using a published Asian elephant complete mtDNA genome (GenBank Accession no. NC_005129.2) as reference. The primers now amplify a fragment including partial COX2, tRNA-Lys, ATP8, and partial ATP6. We also designed two similar sets of primers compatible with an existing study (Vandebona *et al*. 2002) for the ND5 region, with 84 bp overlap to facilitate contig building. The widely used D-loop primers for elephants (MDL5-MDL3; Fernando & Lande 2000) amplify a product of ≈630 bp, including partial Cyt b, tRNA-Thr, tRNA-Pro and partial D-loop, for which we observed a success rate of <30% from degraded faecal DNA (Goyal *et al*. unpublished). Fernando et al. (2003) proposed a set of internal primers (MDLseq-1 and MDLseq-2) for the D-loop region corresponding to the primer set MDL5-MDL3, but these also generate products close to 600 bp. Therefore, we decided to design internal primers with shorter amplicons (ranging from 270 to 474 bp) to use

with the existing D-loop primers, producing two amplicons with 104 bp overlap. Redesigning of the existing primers was done manually by aligning the primers to the complete mtDNA genome of the Asian elephant and modifying the nucleotides as necessary. We used Primer3web v4.1.0 (Koressaar & Remm 2007; Untergasser *et al*. 2012) to design the novel primers.

Standardization of amplification conditions

To optimize the annealing temperature, we subjected all six sets of primers (Table 1) to a broad temperature gradient of 50°C to 64°C. The reactions consisted of Thermo 2X Maxima Hot Start Green PCR Master Mix, 1.5 μg bovine serum albumin, 3 pmoles of each of the forward and reverse primers, 3 μl genomic DNA of variable concentration and RNase free water to bring the reaction volume up to 15 μl. The thermocycler profile was as follows: initial denaturation (95°C for 5 min), followed by 40 cycles of denaturation (95°C for 30 sec), annealing (varying temperature as per experimental gradient for 40 sec) and extension (72°C for 40 sec) followed by a final extension at 72°C for 10 min before the 4°C hold. All PCR reactions contained a negative as well as a positive control. The resulting amplicons were directly loaded into SYBR green stained 2% w/v agarose gels and run using TAE buffer before visualizing in an ultraviolet light-based gel documentation system. A 100 bp DNA ladder was run with each round of electrophoresis for recording the presence/absence of desired bands of predicted length.

Species confirmation from the amplicons

The successful amplicons were purified of excess primers and nucleotides by enzymatic hydrolyzation using Exonuclease I and Shrimp Alkaline Phosphatase. We subjected the resultant products to Sanger sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). We used the ethanol precipitation method to clean up the sequencing products. We then dissolved them in HiDi Formamide (Applied Biosystems) prior to capillary injection in an ABI 3530 XL Genetic Analyser. The data were compared with the sequence repository of NCBI GenBank for species confirmation.

Results and discussion

We observed positive amplification of predicted lengths in all six sets of primers with no bands in negative controls of extractions or PCRs (Fig. 1).

Optimal annealing temperature

The optimal annealing temperature (Ta) for all the primers selected (Table 1) for this study for producing sharp bands varied between 52°C and 58°C (Table 2). We did not observe the presence of any non-specific bands or smearing at the optimized Ta for any of the fragments. The presence of primer-dimers was also minimal.

Amplification success rates

After determining the optimal Ta, we tested the primers with several faecal DNA extracts (n = 11 to 82) to record proportions of positive amplification. The overall success rate with faecal DNA in all the primers varied between 68.8% and 93.9% (mean = 79.8%, Table 2). We observed >90% success rates in amplifying the ND5 fragment using the primer pair EmND5_2F-EmND5_2R and the D-loop fragment amplified by MDL3mF-MDL3. Lowest success (68.8%)

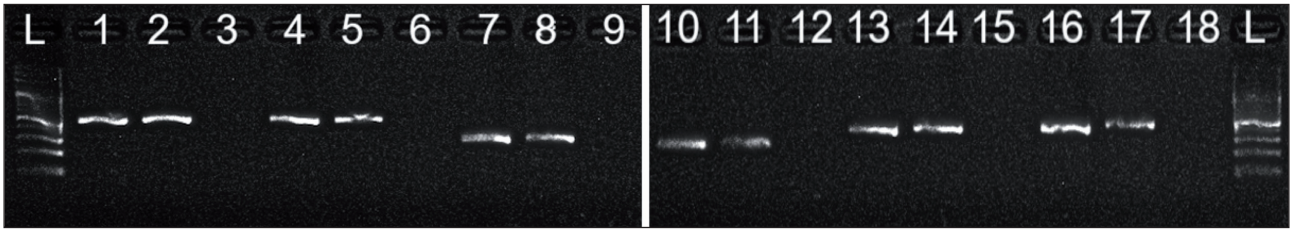

Figure 1. Agarose gel electrophoresis of representative amplicons produced by the primers used. Lane key: L: 100 bp ladder, 1-18: PCR products of MT24mF and MT24mR (1-3), MT25F and MT25R (4-6), EmND5_1F and EmND5_1R (7-9), EmND5_2F and EmND5_2R (10-12), MDL5 and MDL5mR (13-15), MDL3mF and MDL3 (16-18). Lanes 3, 6, 9, 12 and 18 contain the negative PCR controls.

Table 1. Primer sequences.

Primer	Primer sequence (5' – 3')
MT24mF	AGTCTCATCAGAAGATGTTCTCC
MT24mR	GGATAGTTCTTCGTTCACTTCT
MT25F	TCGCCTTCTTTTCCCAATCT
MT25R	GGTGTTCCTTGTGGGAGAA
EmND5_1F	CGTATTGGCGATATAGGCTTC
EmND5_1R	AGTGGGTAAAAGCGGATGAG
EmND5_2F	AGGCCCTACCCCAGTATCAG
EmND5_2R	TGGGTAAAGGCTAGATGTGGTT
MDL5	TTACATGAATTGGCAGCCAACCAG
MDL5mR	GGGTAAATAATGTGATGCACGATT
MDL3mF	CCCTGCAAGTAAACCAATCCGCT ATGT
MDL3	CCCACAATTAATGGGCCCGGAGCG

was observed in the first fragment of the ND5 (EmND5_1F-EmND5_1R).

Species confirmation

All positive amplifications produced high-quality sequences with sharp discernible peaks in the chromatograms and the Q value for each base was >20 with no 'dye blobs'. The comparison of the sequences we generated with the NCBI GenBank data repository confirmed that 100% of the sequences generated during this study belonged to the Asian elephant and the desired mtDNA fragment.

Our results show that it is possible to generate ≈2.0 kb high-quality mtDNA sequence data from non-invasively collected faecal DNA of the Asian elephant with high to moderate success rates from fresh to two-week-old dung samples. The primers described in this study all encompass polymorphic regions of the mtDNA, and hence, screening samples across the geographic range of the species would provide valuable information on the phylogeographic characteristics.

In the recent past, regional studies have shown the presence of new haplotypes in different parts of the mtDNA genome in the Asian elephant (Vandebona *et al*. 2002; Thitaram *et al*. 2015) highlighting the need of a range-wide, multi-gene study. Besides, location-specific single nucleotide polymorphisms (SNPs) fixed at the population level have been used in wildlife forensics (Ogden & Linacre 2015; Kumar *et al*. 2016). Hence, identifying an array of SNPs in Asian elephants could aid forensic researchers in tracking poaching cases to the origin of the samples, thereby contributing to the conservation of Asian elephants. In addition, evidence of positive selection at the genes responsible for the oxidative phosphorylation pathway has been observed in African forest and savannah elephants, probably to adapt to the harsh climatic conditions they face (Finch *et al*. 2014). As the Asian elephant also occupies a variety of temperature regimes, studies on such protein-

Table 2. Details of the mtDNA primers designed and tested to amplify a total of 1978 bp.

Fragment	Size (bp)	Primer	Status	Amplicon		Ta (°C)	Success rate (%)
				Position*	Size (bp)		
ATP8-ATP6	845	MT24mF	Modified	7477 – 7950	474	58	72.7 (n=11)
		MT24mR	Modified				
		MT25F	Finch *et al*. (2014)	7867 – 8321	455	52	70.6 (n=17)
		MT25R	Finch *et al*. (2014)				
ND5	458	EmND5_1F	Designed	12,262 – 12,533	272	56	68.8 (n=16)
		EmND5_1R	Designed				
		EmND5_2F	Designed	12,450 – 12,719	270	58	93.8 (n=16)
		EmND5_2R	Designed				
D-loop	675	MDL5	Fernando & Lande (2000)	15,151 – 15,548	398	56	78.9 (n=19)
		MDL5mR	Designed				
		MDL3mF	Designed	15,418 – 15,825	408	56	93.9 (n=82)
		MDL3	Fernando & Lande (2000)				

* Based on complete mitochondrial genome of *Elephas maximus* (Accession number: NC_005129.2).

coding regions (ATP synthase and NADH dehydrogenase) using our new primers may also reveal interesting spatial variations in such genes.

Conclusion

We aimed to design primers to amplify polymorphic regions (ca. 2.0 kb) of Asian elephant mtDNA based on published literature. This included partial fragments of the ATP8-ATP6 complex (ca. 850 bp), ND5 (ca. 450 bp) and D-loop (ca. 630 bp) regions from non-invasive samples. We designed 8 novel oligos to obtain 270–474 bp-fragments. We were successful in amplifying a total of 1978 bp in three contigs from non-invasive faecal DNA with a mean success rate of 79.8%. Therefore, these primers would be useful in generating data from across the range of the Asian elephant to study multi-gene phylogeography and genetic variation.

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