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USE OF UNIVERSAL mtDNA PRIMER FOR IDENTIFYING SUCCESS: HOW GOOD ARE THESE FOR INDIAN DEER SPECIES?

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ABSTRACT
Universal primers for mtDNA regions have been developed that can amplify the fragment of mitochondrial gene across large number of taxa across different gross in polymerase chain reaction (PCR) and use the nucleotide sequences to identity biological material of any unknown animal origin and in understanding phylogenetic relationships. Before using these primers, there is a need to access their applicability for targeted taxa, as such primers have been reported a poor PCR amplification in a few taxa. Therefore, we examine reported universial primers of cyt b, 16s rRNA, 12s rRNA and control region for six species of family cervadie. Result indicates that use of universal primers has high homologies between deer species, such as 98% between sambar and hog deer in cytb gene. In 16S rRNA there is highest similarity (98%) between chital *vs* hog deer, samber vs brow antlered deer and in 12s rRNA gene, chital vs samber, chital *vs* hog deer and chital *vs* brow antlered deer has 97% similarity. In control region all the six species has highly variable in nature and sequence similarity was found 55 to 93%. Sometimes result shows very high homology with number of species that give false result in species identification. For avoid this type of problem either go for design of species specific primer or restriction fragment length polymorphism.

KEY WORDS: PCR amplification, chytochrome b, 16s rRNA, Phylogenetic, Taxa.

INTRODUCTION
Genetic variation is an essential characteristic of all organisms that allow them to adapt to changing environmental conditions and has been fixed in species during evolution time scale except a changes due to genetic drift or mutation caused by modification in habitat. This can be directly observed through genetically controlled markers that involve assessment of variation directly at DNA level or through phenotypic expression. The genetic markers detect inter and intra-specific differences (Sharma *et al*., 2008). The species specific diagnostic markers profiles find varied application that include resolving taxonomic conflicts, detecting inadvertent hybridization as well as introgression as forensic tool etc (Underhill *et al*., 2007). Interaspecific differences reveal genetic relatedness within a species and identify evolutionary significant units or genetic stocks. The population genetics conclusions drawn from molecular marker data can provide insights about genetic bottlenecks and strategies of resources management for sustainable utilization. Molecular markers are also put to use in markers assisted selection and planning breeding programmes to achieve genetic improvement in domesticated species. Mitochondrial DNA sequencing analysis is being used recently in forensic investigation, population analysis and phylogenetic surveys of organisms (Meyer *et al*., 1993). Studies of vertebrate species generally have shown that sequences divergence accumulates more rapidly in mitochondrial than in nuclear DNA (Carrera *et al*., 1999). This has been attributed to a faster mutation rate in mtDNA that my result from a lack of repair mechanisms during replication (Guha *et al*., 2007) and small effective population size due to the strict maternal inheritance of the haploid mitochondrial genome (Birky *et al*., 1998). Due to its rapid rate of evolution, mtDNA analysis has proven useful in understanding relationships among closely related species. Different parts of mtDNA are known to evolve at different rates (Mitchell et al., 1993). Almost the entire molecules are transcribed except for the approximately 1KB control region (D loop), where replication and transcription of the molecule is initiated. In general non-coding segments like control region exhibit elevated levels of variation relative to coding sequences such as cytochrome b gene (Brown *et al*., 1993), presumably due to reduced functional constraints and relaxed selection pressure. The 16s rRNA gene in the mitochondrial genome is one of the slowest evolving gene (Mitchell *et al*., 1993), where as rapidly evolving regions are control region. Due to non-mandelian mode of inheritance, the mtDNA molecule is considered a single locus (Avise, 1994). In addition because mtDNA is maternally inherited the phylogenies and population structure derived from mtDNA data may not reflect those of the nuclear genome if gender biased migration or relation or introgression exists (Liu *et al*., 2004). Analyses of mtDNA markers have been extensively to investigate population structure and identification of species. The DNA sequences of sample genes, in part or whole, comprise the most common type of sequences sample used in molecular wildlife forensic. In PCR amplification, primers are the most important components of process and the success of PCR largely depends on the primers.

Primers are short, single stranded DNA molecules used to bind to specific sequences on nucleic acids to serve as a starting point for building a complementary nucleic acid strand. Primers are generally made in pairs called forward and Reverse. These primers are complimentary to regions on the DNA molecule such that they can be extended toward one another with DNA polymerase, forming new DNA molecule. The most important property of a primer is its sequence specificity. This determines what nucleic acid sequences it can anneal to, how well it will bind, how well it will serve as a site for extension of a new nucleic acid molecules. Generally a specific primer is designed to target a DNA sequence by alignment of DNA sequences of different taxa and select regions which are highly conserved across groups. This does not mean that might across different species. In view of genetic diversity of animals, cross priming across species can be a useful strategy to save cost and time. The sequences flanking the mtDNA are conserved accross species and developed in one group to characterize loci can be used in other group (Zordia). This type of primer is called universal primer. A universal primer is a pair for amplifying a fragment of gene of an animal species in a polymerase chain reaction (PCR) or determining the identity of the biological material of an animal of unknown origin at species and sub-species level and use for phylogenetic process (Verma *et al*., 2003) . Now a day's scientist use universal primer very frequently in forensic science for the species identification and population structure due to minimize cost and time (Guha *et al*., 2007). There are number of universal primer present for different gene like cytochrome b, 16srRNA, control region and 16s rRNA. In the present study we document six deer species which are more illegal poaching and trying to see how efficient, use of universal primer developed for identification of gene of mtDNA.

METHODS

We have selected universal primer for each gene of cytochrome b, 16s rRNA, 12s rRNA and control region which are generally used in species identification. For cytb gene the universal primer: is 5'-CCATCCAACAT CTCAGCATGATGAAA-3' and 5'-GCCCCTCAGAATG ATATTTGTCCTCA-3' (Meyer, 1993) for 16s rRNA 5'-CGCCTGTTTATCAAAAACAT-3' and 5'-CTCCGGT TTGAACTCAGATC-3' (Mitchell *et al*., 1993), for control region 5'-TGAATTGGAGGACAACCAGT-3' and 5'-CCTGAAGTAGGAACCAGATG-3'and for 12s rRNA 5'-TTTCATGTTTCCTTGCGGTAC-3' and 5'-AAAGCA CGGCACTGAAGATGC -3'. For control region in NCBI we do not find sequences for that primer pair so that we amplified the sequences for the chital, hogdeer, Swamp deer and Brow-antlered deer. Isolation of DNA from the meat and skin was done for the chital, hogdeer, swamp deer and brow-antlered deer. Due to non DNA viability of control region sequences in NCBI or related species, DNA was used as a template to amplify control region genes using the universal primer: Polymerase chain reaction (PCR) was performed in (M J Research PTC 200 Peltier Thermo Cycler) a final volume of 25 µl reaction volume containing 1x PCR Buffer (10mM Tris-HCl, pH 8.3 at 25^{0}C; 50mM KCl, SIGMA, USA); 5mM $MgCl_2$ (SIGMA, USA); 10 mM dNTPs (SIGMA, USA); 5pmol of each primer (Sigma-Genosys Ltd., USA); 1U Taq polymerase (MBI Fermentas) and varying quantities (50-100ng) of DNA to ensure that the optimum amount for amplification would be present. Amplification conditions were 94^{0}C for 2min followed by 30 cycles at 94^{0}C for 1min, annealing temperature (Ta) 53^{0}C for 1min and 72^{0}C for 1min, with final extension of 72^{0}C for 10 min. Amplified PCR products were purified with a QIAquick spin purification kit (QIAGEN, Germany). Cycle sequencing PCR was performed for these purified PCR products with their respective primers following the suggested composition of master mixture from Applied Biosystems 3130 Genetic Analyzer protocol. Cycle sequencing PCR products were cleaned up by using QIAGEN Dye Spin kit. These products obtained were sequenced (Applied Biosytems 3130 Genetic Analyzer) on both strands and edited. Since we did not found sequences for control region for Indian deer species, so we developed these using universal primer.

Analysis

Similarity matrix was calculated with their respective primers with the sequences downloaded from NCBI and some sequences generated by us that are submitted in NCBI. Table 1 is showing the calculation of similarity matrix with the universal primers. Sequences were aligned and similarity matrix was calculated by ClustalW (htt://www.ebi.ac.uk/clustalw)

RESULTS & DISCUSSION

In present study we have analysed partial sequences of 4 different mitochondrial genes in six Indian species of order cervidae. Very limited data base is present in the NCBI (Table 3) for all of the objected species. We have selected the product of universal primers which are mostly used for the species identification. All the species are most similar nucleotide variable conserved within species. Our reference species along with the mtDNA sequences obtained from NCBI used in its species diversity studies. Accession number of mtDNA cytochrome b, 16s rRNA, control region and 12s rRNA (Table 2) gene sequences examined to estimate interspecies sequences diversity.

TABLE 1: Similarity Matrix of Six deer species of Cytochrome b, 16s rRNA, 12s rRNA and Control region. Family-Cervidae

Species	Chital (%)	Sambar (%)	Barking (%) deer	Hog deer (%)	Swamp deer (%)	Brow-antlered deer (%)
Cytochrome b						
Chital (*Axis axis*)	-	87	86	87	89	88
Sambar (*Cervus unicolor*)	87	-	87	98	90	90
Barking deer (*Muntiacus muntjak*)	86	87	-	87	86	86
Hog deer (*Hyelaphus porcinus*)	87	98	87	-	89	90
Swamp deer (*Cervus duvaucelii)*	89	90	86	86	-	89
Brow-antlered deer (*Cervus eldii* eldii)	88	90	90	90	89	-
16s rRNA						
Chital (*Axis axis*)	-	97	95	98	97	96
Sambar (*Cervus unicolor*)	97	-	95	97	97	98
Barking deer (*Muntiacus muntjak*)	95	95	-	94	96	95
Hog deer (*Hyelaphus porcinus*)	97	97	94	-	97	97
Swamp deer (*Cervus duvaucelii)*	97	97	96	97	-	97
Brow-antlered deer (*Cervus eldii* eldii)	96	98	95	97	97	-
12s rRNA						
Chital (*Axis axis*)	-	97	94	97	95	97
Sambar (*Cervus unicolor*)	97	-	95	96	95	98
Barking deer (*Muntiacus muntjak*)	94	95	-	94	96	94
Hog deer (*Hyelaphus porcinus*)	97	96	94	-	96	95
Swamp deer (*Cervus duvaucelii)*	95	95	93	96	-	94
Brow-antlered deer (*Cervus eldii* eldii)	97	98	94	95	94	-
Control region						
Chital (*Axis axis*)	-	56	88	93	90	89
Sambar (*Cervus unicolor*)	56	-	56	57	55	58
Barking deer (*Muntiacus muntjak*)	88	56	-	88	89	89
Hog deer (*Hyelaphus porcinus*)	93	57	88	-	92	91
Swamp deer (*Cervus duvaucelii)*	90	55	89	92	-	90
Brow-antlered deer (*Cervus eldii* eldii)	89	58	89	91	90	-

TABLE 2: Accession number used in the study

Species	Accession number			
	Cyt b	16s rRNA	12s rRNA	Control region
Chital	AY540851	AY391766	DQ017832	EU870593
Samber	AY456907	DQ832273	AY184434	NC_008414
Hog deer	AY540848	AY391768	AY184435	EU870592
Barking deer	AY225986	AF108039	AM778453	NC_004563
Swamp deer	DQ459338	DQ989296	EU084669	EU921907
Brow- antlered deer	AY607037	AY391772	AY184432	EU870590

TABLE 3: Available Data in NCBI:

	Cyt b	16s rRNA	12s rRNA	Control region
Chital	71	36	32	1
Sambar	66	49	8	57
Hog deer	37	9	11	2
Barking deer	31	8	2	8
Swamp deer	11	6	5	0
Brow- antlered deer	23	10	2	132

The sequences of all the gene fragment of 6 studied species were aligned using CLUSTAL W. Similarity index of cytochrome b gene show the 86-98% similarity among Indian deer species. Sequence similarity was much higher of 98% between sambar and hog deer. 87% between sambar *vs* chital, Sambar vs barking deer, barking deer *vs* hog deer and Hog deer *vs* chital. Relative higher sequences divergence of 90% similarity was observed between swamp deer and sambar, brow-antlered deer and sambar and brow- antlered deer *vs* hog deer. As widely reported that of 16s rRNA and 12s rRNA genes are highly conserved and sequences were 95-98% similar. In 16s rRNA, highest divergence 98% similarity was observed between chital *vs* hog deer, samber *vs* brow antlered deer and 97% similarity with chital vs swamp deer, Sambar *vs* hog deer, hog deer *vs* brow-antlered deer and sambar *vs* swamp deer. In 12s rRNA gene, chital vs samber, chital *vs* hog deer and chital *vs* brow antlered deer has 97% similarity. Control region all the six species has highly variable in nature and sequence similarity was found 55 to 93%. Data indicated that sambar is highly diverged from chital and barking deer as sequence similarity was only 56%. Moderate divergence of brow antler deer *vs* chital, in brow antler deer *vs* barking deer and swamp deer *vs* barking deer has 89% similarity and barking deer as chital, hog deer as barking is 88%. The variability among different gene for six Indian deer species was control region>cytochrome b>12s rRNA >16s rRNA. In mitochondrial DNA, different genes involve at different rate. Some genes change very slowly and can be used to study relationships among groups of organisms (Ex. cytochrome b). Other regions of DNA change at such a rapid rate that every individual in a population is distinct. (*ex*. Control region). Some DNA show intermediate levels of variability and useful for studies of variation within and between populations of a species (16s and 12s rRNA) (Wan *et al*., 2004). So that to see the genetic variation between closely related species or within the species, control region is the better gene for differentiation. In case of cervadie control region has high variability as compare to other gene. A large number of studies in evolutionary biology utilize phylogenetic information obtained from mitochondrial cytochrome b, 16s rRNA, 12s rRNA and control gene (James *et al*., 2008). It has been identified a potent molecule to distinguish the phylogenetic depth of different lineages to family, genus and species in molecular taxonomy (Wilson *et al*., 1885). A vast database of the sequences of cytochrome b, 16s rRNA, and 12s rRNA gene of different animal species has accumulated in public databases (Panneerchelvam *et al*., 2003) such as GenBank, NCBI (http://www.ncbi.nlm.nih.gov) *etc*. We have utilized this capacity of different gene in establishing the identity of the origin of animal parts and product to its family, genus and species sources. The technique developed is based on a pair of universal primer that can amplify a small fragment of these genes from a vast range of animal species (Kocher *et al*., 1989; Hsieh *et al*., 2001: Carrera, 1999; Mitchell *et al*., 1993). But sometimes we have seen that universal primer has high homologies of some closely related species. Then there is problem of origin of species. It could be seen in similarity matrix that few genes show high similarity with number of species. This could be due to use of universal primer and some time can be face false result for species identification in forensic investigation. People are using universal primer for quick result that can give some time different species. So for avoid this type of problem either you design species specific primers or you go for restriction fragment length polymorphism (RFLP) (Wen *et al*., 2007).

Restriction fragment length polymorphism (RFLP) is an extremely sensitive technique for DNA fingerprinting to establish variations within samples based on up to a single base difference (when they occur in a restriction site) (Panneerchelvam *et al*., 2003: Brown, 1980). Though DNA sequencing techniques can characterize DNA very thoroughly, RFLP analysis was developed first and was cheap enough to see wide application (Wen *et al*., 2007). Analysis of RFLP variation was an important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing.

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