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Research Article

Mitochondrial 12S rRNA gene sequence analysis, a tool for species identification

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Abstract

Biodiversity is under threat worldwide. Many species of animals, birds and reptiles have experienced considerable decline in their populations within the past two centuries due to illegal trade, poaching and habitat destruction. The biodiversity in India is under threat due to growing wildlife crime. In this study a segment of mitochondrial 12S rRNA was used to develop an easy and standard protocol to identify a number of species from variety of samples. Polymerase chain reaction (PCR) was done after DNA extraction and quantification. PCR products were then sequenced bi-directionally and aligned to a database non reductant nucleotide sequence. It was then confirmed that the sequences aligned specifically to mitochondrial sequences from expected species of origin. Bioinformatics tools were then applied for phylogenetic and genetic diversity analysis. Result of this study showed that universal primer could amplify partial mitochondrial 12rRNA gene from all studied animals, birds and reptiles. Based on the sequences obtained, a Neighbour joining tree was constructed using K2P model. This molecular technique together with bioinformatics tools provides a reliable and fast method for species identification and taxonomic classification.

Keywords: Species identification,12S rRNA, polymerase chain reaction, bioinformatics.

Introduction

Species identification in veterinary field has gained importance in the recent past. This is because earth is losing wildlife in an alarming rate due to habitat destruction, illegal trade and wild life poaching (Kumar *et al*. 2014). Conventional methods of species identification have limited applications in the case of degraded, fixed and cooked samples, thus making nucleic acid based techniques more reliable, sensitive and specific. So it is significant to develop reliable and accurate methods to screen DNA sequences variation for species identification (Pereira *et al*. 2008). Most commonly used DNA based techniques include RAPD (random amplified polymorphic DNA) (Martinez and Yman 1998), AFLP (amplified fragment length polymorphism) (Vos *et al*. 1995), RFLP (random fragment length polymorphism) (Wolf *et al*. 1999, Veerkar *et al*. 2002), DNA hybridization, restriction enzyme digestion, PCR (polymerase chain reaction) using species specific primers, DNA sequencing (Birstein and de Salle 1998), DNA barcoding, (Friedheim 2016), PCR-RFLP (polymerase chain reaction -restriction fragment length polymorphism) and HRM (high resolution melting) analysis (Panprommin *et al*. 2019).

Reports suggest that among types of DNA, mitochondrial DNA has been used considerably for species identification (Gupta *et al*. 2015).

Species identification based on mitochondrial DNA implies that significant amount of sequence variations could be observed even

within closely related species because of the inter-species and intra-species sequence variations (Irwin *et al*. 1991, Hayashi *et al*. 1985). Mitochondrial DNA can be isolated from small amount of tissues samples containing degraded DNA because of the presence of its multiple copies in cell. Moreover, the high mutation rate due to limited repair ability as compared to nuclear DNA and its conserved regions allow the development of universal primers. These properties makes the mitochondrial DNA more preferable than nuclear DNA for species identification (Yang *et al*. 2014). In the present study we selected a universal primer pair to target 12S rRNA sequence and thus amplify homologous mitochondrial 12S rRNAsequences from wide variety of samples.

Material and methods

Sample collection and DNA isolation

DNA was extracted from skin, feathers, hair, decayed and post-mortem samples. Exfoliated skin of king cobra (*Ophiophagus hannah*), feathers of house crow (HC) (*Corvus splendens*), albino crow (AC) (*Crovus splendens*), rose ringed parakeet (*Psittacula krameri*), hair, post-mortem and decayed samples of spotted deer (*Axis axis*), sambar deer (*Rusa unicolor*), hog deer (*Axis porcinus*), Indian crested porcupine (*Hystrix indica*), toddy cat (*Paradoxurus hermaphroditus*) and sloth bear (*Melursus ursinus*) were collected from Thrissur, state museum and zoo. Hair samples of lion tailed macaque (*Macaca silenus*), bonnet macaque (*Macaca radiata*), tiger (*Panthera tigris*) were supplied by the authority of Thiruvananthapuram museum and zoo. DNA was extracted from above samples by using QIAmp tissue extraction kit (QIAGEN, GmbH, Hilden, Germany) according to manufacturer's instructions. Integrity of the isolated DNA was checked by running agarose (0.8%) gel electrophoresis in tris- acetate EDTA buffer (TAE buffer) (40 mM tris-acetate, 2mM EDTA, pH 8.0) (Sambrook and Russel 2001). Concentration of DNA in the extracted samples were quantified by the measurement of absorbance at 260nm. Measurements of absorbance at 260nm were recorded by using Nanodrop with 1µl of sample.

PCR amplification and sequencing

Mitochondrial 12S rRNA gene having approximately 450 bp was amplified using universal primers (forward primer: 5'-CAAACTGGGATTAGATACCCCACTAT-3', reverse primer:5'-GAGGGTGACGGGCGGTGTGT-3') (Kocher *et al*. 1989). The PCR amplifications were performed in 50µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol each forward and reverse primers, 100ng of template DNA and 1U Taq DNA polymerase (GeNei, Bangalore) and nuclease free water to make up to 50 µl volume. Polymerase chain reaction was carried out in a thermal cycler (BIO-RAD, USA) with following thermal cyclic conditions: initial denaturation step of 95 ^{o}C for 5 min., followed by cycles consists of denaturation at 95 ^{o}C, 30 sec, annealing at 60.4 ^{o}C for 1 min., extension at 72 ^{o}C for 1 min. and the final extension step was at 72 ^{o}C for 5 min. The PCR products were then subjected to electrophoresis on a 1% agarose gel. During the whole procedure PCR blanks were incorporated to check contamination. The amplified products were then purified using Qiagen ® QIA quick PCR purification kit and amplicons were sequenced in both directions. Sequencing was carried out through commercially available sequencing facility (Agrigenome, Kakkanad, Kochi). Sequences obtained were then submitted as individual entries for BLAST analysis.

Sequence analysis

Sequences obtained were checked with chromatogram, after aligning the forward and

reverse sequences. The best sequences were selected to cross verify the obtained data with sequence submitted in NCBI, USA. (National centre for biotechnology information). Homology search was carried out using the nucleotide BLAST (BLASTn) search tool implemented by NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The first BLAST hit represented the highest degree of identity. Apart from this multiple sequence alignment was carried out using CLUSTAL W algorithm implemented in BioEdit 7.0.9.0 software (Hall 1999) and MEGA 6 software (Tamura *et al*. 2013). Multiple sequence alignment results was confirmed by comparing output data with phylogenetic analysis conducted using MEGA 6 using neighbour joining method (Saitou and Nei, 1987) and phylogenetic tree was constructed with boot strap value of 1000 replicates (Felsenstein 1985). As recommended by the consortium for barcode of life (BOL; http://www.barcoding.si.edu/protocols.html) the intra species and inter species distances were calculated by using Kimura-2 parameter model (Kimura 1980) using MEGA 6.0 software.

Results

Concentration of amplified sequences ranged from 14.86 ng/µl to 50 ng/µl. On BLAST analysis all hits occurred in the mitochondrial gene, rather than nuclear genome. Results of BLAST analysis showed that sequence length varied among species. Size of the amplicon varied between 350 to 450 bp. All sequences obtained in the study was submitted to GenBank and accession numbers were obtained. The albino crow 12S rRNA showed maximum sequence length of 444 bp. Percentage identity varied among species from 90-100% with query coverage of 100% for all the sequences. Mitochondrial 12S rRNA sequence of albino crow showed maximum identity score with mitochondrial 12S rRNA sequence of house crow (*Corvus splendens*) with 99% identity and 100% query coverage are shown in Table 1.

Average nucleotide frequencies in the sequences were 22.5(T/U), 25.0(C), 34.2 (A), 18.3 (G). Maximum nucleotide frequencies were showed by nucleotide 'A'. Aligned 12SrRNA sequences of all 13 samples contained 202 conserved region (C), 258 variable region (V), 172 prisomy informative sites (Pi) and 85 singleton regions (S) showed in Table 2.

Neighbour joining tree was constructed (Kimura 1980) and found that except *Macaca radiata* and *Macaca silensus* , all other members of class mammalia clustered as one clade with 66% node support. *Panthera tigris* and *Paradoxurus hermaphroditus* formed a single group with node distance of 95%. Members under class birds clustered as a common clade with node support of 100%. *Macaca radiata* and *Macaca silenus* clustered together with 100% node support. *Ophiophagus hannah* seen as an out group on phylogenetic analysis (Fig.1).

Discussion

Mitochondrial 12SrRNA is highly conserved and has been used as molecular marker for the species identification. It is reported that amplification of conserved region of mitochondrial 12S rRNA using universal primers could identify not only different species but also its molecular phylogeny (Arif *et al*. 2011).

The primer pair used in this study could amplify specific region from wide variety of organisms like mammals, birds, reptiles, fishes and insects (Zhang and Ryder 1994, Dratch *et al*. 1996, Girish *et al*. 2004, Nakamura *et al*. 2002, Gupta *et al*. 2008, Barbar *et al*. 2015). Most of the researchers have used species specific mitochondrial 12S rRNA primers to identify species. Studies using universal primer are less when compared to species specific primer. Primers which we used in this study could amplify partial mitochondrial 12S rRNA gene from wide variety of samples (Reptiles, birds

and animals) with specificity.

Table 1. Details of species used in the present study, NCBI GenBank BLAST search results based on 12SrRNA sequences and accession numbers obtained for the sequence in the present study.

Species	GenBank submitted species with which closest match	Size of the amplicon	Similarity (%)	Query coverage (%)	Accession number obtained
Ophiophagus hannah	*Ophiophagus hannah* (JN 687930.1)	426bp	86	100	MH532954
Corvus splendens (AC)	*Corvus splendens* (KY050718.1)	444bp	98	100	MH532955
Corvus splendens (HC)	*Corvus splendens* (KY050718.1)	438bp	99	100	MH532956
Psittacula krameri	*Psittacula krameri* (EU197058.1)	391bp	100	100	MH532957
Hystrix brachyura	*Hystrix brachyura* (AY012117.1)	437bp	97	100	MH538943
Axis axis	*Axis axis* (MF435989.1)	429bp	99	100	MH538944
Rusa unicolor	*Rusa unicolor* (MF177027.1)	396bp	99	100	MH538945
Axis porcinus	*Axis porcinus* (JN632600.1)	428bp	99	100	MH538946
Melursus ursinus	*Melursus ursinus* (FM177763.1)	358bp	100	100	MH538947
Paradoxurus hermaphroditus	*Paradoxurus hermaphrodites* (HQ634961.1)	434bp	99	100	MH538948
Panthera tigris	*Panthera tigris* (KJ508413.2)	442bp	100	100	MH538949
Macaca silenus	*Macaca silenus* (AY224263.1)	441bp	99	100	MH538950
Macaca radiata	*Macaca radiata* (LC225392.1)	441bp	94	100	MH538951

Table 2. Nucleotide frequencies in the 12SrRNA sequences among animals, birds and reptiles under study. All the frequencies are represented in percentage.

Common name	Scientific name	T(U)	C	A	G	Total
King cobra	*Ophiophagus hannah*	23.9	25.4	35.9	14.8	426.0
Albino crow (AC)	*Crovus splendens*	21.6	29.5	26.6	22.3	444.0
House crow (HC)	*Corvus splendens*	21.9	29.5	27.2	21.5	438.0
Parakeet	*Psittacula krameri*	16.9	30.2	33.0	19.9	391.0
Porcupine	*Hystrix brachyura*	22.7	22.9	35.9	18.5	437.0
Spotted deer	*Axis axis*	23.5	22.6	36.8	17.0	429.0
Sambar deer	*Rusa unicolor*	23.2	21.7	37.6	17.4	396.0
Hog deer	*Axis porcinus*	24.1	21.0	37.9	17.1	428.0
Bear	*Melursus ursinus*	25.1	20.1	36.0	18.7	358.0
Toddy cat	*Paradoxurus hermaphroditus*	24.9	21.9	35.5	17.7	434.0
Tiger	*Panthera tigris*	22.4	24.2	35.5	17.9	442.0
Bonnet macaque	*Macaca radiata*	21.5	27.7	34.7	16.1	441.0
Lion tailed macaque	*Macaca silenus*	20.4	27.9	33.3	18.4	441.0
Average		22.5	25.0	34.2	18.3	423.5

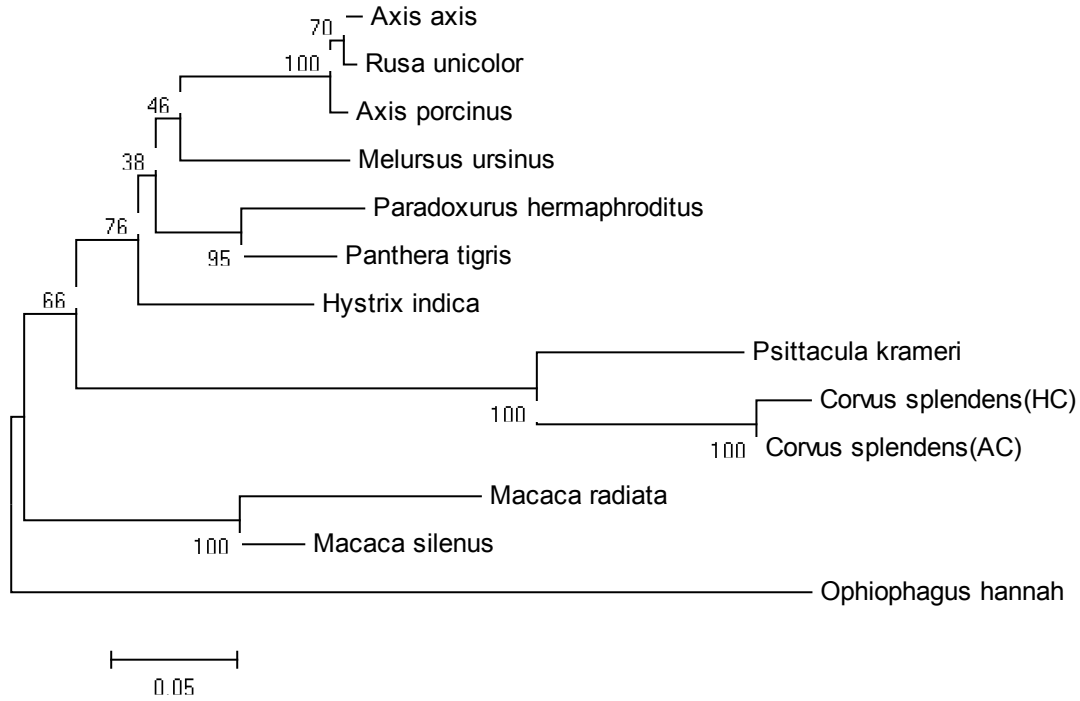

Figure 1. Neighbour joining tree constructed based on 12S rRNA sequence of different species which includes animals, birds and reptiles. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed based on the Kimura2 parameter method. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 6.

In the present study universal primer could amplify partial mitochondrial 12S rRNA gene having comparatively larger size (approximately 450 bp) from all animals, birds and reptiles under study. Mitochondrial 12S rRNA gene sequence of sloth bear was comparatively smaller with 358bp (Zhang and Ryder, 1994).This larger sized amplicons will avoid the amplification of homologous nuclear DNA. Reported smaller amplicons (approximately 100bp) of mitochondrial 12S rRNA gene might hinder specificity because of the presence of multiple similar sequences in different species and homologous nuclear DNA sequences (Melton and Holland, 2007). Sequence analysis and BLAST search results demonstrated that these primers were truly universal which can be used to amplify 12S rRNA region of various species with no misidentification. The results of multiple sequence alignment with our sequences showed the presence of conserved motifs among species which is an indicative of mitochondrial DNA, is an agreement with the previous studies (Yang *et al*. 2014).

Sequence analysis of partial mitochondrial 12S rRNA gene obtained in this study using bioinformatics tool showed multiple polymorphic sites that helps in intra-species and inter-species identification (Barbara *et al*. 2015). Thus the primer pair specifically amplified mitochondrial 12S rRNA gene by avoiding the amplification of nuclear homologues sequences. Species identification by using mitochondrial DNA is sensitive due to the presence of multiple copy number and its circular conformation (Yang *et al*. 2014). High copy number of mitochondrial DNA made it possible to isolate DNA and species identification from wide varieties of samples like hair shafts, feathers and decayed samples (Galtier *et al*. 2009). Circular conformation of mitochondrial DNA allows more efficient amplification compared to nuclear DNA (Panday *et al*. 2014). This is the first study of this kind in India.

Conclusion

In India wild life related crime is a growing problem. It includes live animal trafficking and illegal poaching. Meat industry in India is facing serious issue of fraudulent mislabelling of game meat products due to large profit margin. Legal actions could take only with solid evidences which help in identification of these seized samples. Molecular biology based techniques can answer this issue by providing sufficient data. Mitochondrial gene study provides a promising tool in wild life forensics for species identification because of its special characteristics. This study could provide a standard and an easy identification method based on mitochondrial 12S rRNA gene, to identify different species from wide variety of samples at its genus level. Here we are trying to give genetic 12S rRNA gene database of different species for further study on wild life forensics, population and phylogenetic studies.

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Reference

Arif I.A., Khan H.A. 2009. Molecular markers for biodiversity analysis of wildlife animals: a brief review, Animal Biodiversity Conservation 32: 9-17.

Barbar M.E., Hussain T., Wajid A., Nawaz A., Nadeem A., Sha S.A., Shahid M.A., Ahmad N., Javed K., Abdullah M. 2015. Mitochondrial cytochrome b and d-loop sequence based genetic diversity in Mareecha and Bareela camel breeds of Pakistan. Journal of Animal and Plant Sciences 25: 591-594.

Birstein V.J. and de Salle R. 1998. Molecular phylogeny of Acipenserinae. Molecular Phylogenetic Evolution 9: 141–155 .

Dratch P., Shafer J., Hoesch R. and Espinoza E. 1996. Comparison of electrophoretic and chromatographic methods for analysis of deer haemoglobins. International Society for Animal Genetics. 27: 17–42.

Felsenstein, J .1985. Confidence limit on phylogenies: an approach using the boot strap. Evolution. 39: 783-791.

Friedheim, S. (2016). Comparison of Species Identification Methods, DNA Barcoding versus Morphological Taxonomy. Biology, 301(54): 74-86.

Galtier, N., Nabholz, B., Glémin, S., & Hurst, G. D. D. (2009). Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Molecular Ecology, 18(22), 4541–4550. doi:10.1111/j.1365-294x.2009.04380.x

Girish P.S., Anjaneyulu A.S.R., Viswas K.N., Anand M., Rajkumar N., Shivkumar B.M., Bhaskar S. 2004. Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat Science, 66: 551–556.

Gupta A, Bhardwaj A, Supriya, Sharma P, Pal Y, Mamta., and Kumar S. 2015. Mitochondrial DNA- a Tool for Phylogenetic and Biodiversity Search in Equines. Journal of Biodiversity & Endangered Species S1:006.

Gupta A.R., Patra R.C., Das D.K., Gupta P.K., Swarup D., Saini M.2008. Sequence characterization and polymerase chain reaction, restriction fragment length polymorphism of the mitochondrial DNA 12S rRNA gene provides a method for species identification of Indian deer. Mitochondrial DNA. 19: 394–400.

Hall T.A .1999. BioEdit: a user-friendly biological sequence alignment editor and analysis programme for windows 95/98/NT. Nucleic Acids Symposium

Series, 41: 95-98.

Hayashi J.I., Tagoshira Y., Yoshida M.C. 1985. Absence of extensive recombination between inter and intra species mtDNA in mammalian cells. Experimental Cell Research, 160: 387-395.

Irwin D.M., Kocher T.D., Wilson A.C. 1991. Evolution of the cytochrome b gene of mammals Journal of Molecular Evolution, 32: 123-144.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16: 111-120.

Kocher T.D., Thomas W.K., Meyer A., Edwards S.V., Paabo, S., Villablaned F.X., Wilson A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences of the United States of America. 86, 6196-6200.

Kumar V. P., Kumar D., Goyal S.P. 2014. Wildlife DNA forensic in curbing illegal wildlife trade: specie identification from seizures. International Journal of Forensic Science & Pathology, 2: 38–42.

Martinez I., Yman I.M. 1998. Species identification in meat products by RAPD analysis. Food Research International, 31: 459-466.

Melton T., Holland C. 2007. Routine forensic use of the mitochondrial 12S ribosomal RNA gene for species identification. Journal of Forensic Sciences, 52: 1305-1307.

Nakamura, M., Ishibashi, Y., & Syuiti, A. (2002). Novel primer sets for species-specific amplification of the mitochondrial 12S rRNA genes in four Japanese woodpeckers (Picidae, Piciformes). Molecular Ecology Notes, 2(4), 419–421.

Panday R., Jha D.K., Thapa N., Pokharel B.R., Aryal N.K. 2014. Forensic Wildlife Parts and their Product Identification and Individualization using DNA Barcoding. The Open Forensic Science Journal, 7:6-13.

Panprommin D., Soontornprasit, K., Pangeson, T. 2019. Comparison of three molecular methods for species identification of the family Cichlidae in Kwan Phayao, Thailand. Mitochondrial DNA Part A, 30(1): 184-190

Pereira F., Carneiro J., Amorim A. 2008. Identification of species with DNA- based technology: current progress and challenges. Recent Patents on DNA & Gene Sequenc.و 2:187-199.

Saitou N., Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425

Sambrook J., Russel D.W. 2001. Molecular cloning: A laboratory manual. 3rd ed., Cold spring harbor laboratory press, New York, pp. 2000.

Tamura K., Slecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

Veerkar E.L.C., Nijman L.J., Boutaga K., Lenstra J.A. 2002. Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat Science, 60: 365-369.

Vos P., Hogers R., Bleeker M., Reijans M., Lee T., Hornes M. 1995. AFLP a new technique for DNA finger printing. Nucleic Acids Research. 23: 4407-4414.

Wolf C., Rentsch J., Hubner P. 1999. PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. Journal of Agricultural and Food Chemistry, 47: 1350-1355.

Yang L., Tan Z., Wang D., Xue L., Guan M.,

Huang T., Li R. 2014. Species identification through mitochondrial rRNA genetic analysis. Scientific Reports. 4: 4089.

Zhang Y.P., Ryder O.A. 1994. Phylogenetic relationships of bears (the Ursidae) inferred from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution, 3: 351-359.