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RESEARCH ARTICLE

NUCLEAR C-MOS GENE FOR FORENSIC IDENTIFICATION OF CROCODILE SPECIES.

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Abstract

A reliable wild animal's recognition system is necessary for quick allowance of the justice linking wildlife crimes. Development of molecular methods is befitting the need of the time, this study reports new primers able to amplifying fragment from nuclear protein coding genes in six crocodile species i.e. oocyte maturation factor (c-mos). These novel primers amplify 490bp of coding region; this region can be used as an efficient tool for forensic discrimination of crocodiles species. In this study mentioned primers likely to be useful in both population analyses as well as for forensic discrimination of selected crocodile species.

Introduction:-

A great need exists to develop the group of nuclear markers available for molecular systematic studies of crocodiles. Fortunately, the current accretion of freely existing expressed sequence tag (EST) from wide range of animal taxa provides an information base that can be used to design strong and potentially widespread DNA primers for nuclear protein coding genes. Primary goal has been to develop new primers for single copy nuclear genes that could be used in population genetic and forensic identification of the crocodilian species.

Crocodilians constitute a small order, Crocodylia, within the class Reptilia. According to current molecular biology systematic, the order Crocodylia consists of 23 species existing within two families i.e. Crocodylidae and Alligatoridae. The family Crocodylidae harbors the genera *Crocodylus, Osteolaemus, Tomistoma* and *Gavialis*. However, the current status of crocodiles describes them as species under serious threat due to their habitat destruction and illegal poaching for their valuable products (10; 12). All crocodilians are listed in Appendix I or II of Convention on International Trade in Endangered Species (CITES) and the 2007 International Union for the Conservation of Nature (IUCN). Red List of Threatened Species declared five species of crocodiles as critically endangered (IUCN/SSC, 2007). The species identification based on morphological characteristics has not proved expedient in cases of processed skin/meat products seized as illegal harvests whereas, molecular techniques have proved to be potential tools to assist the forensic identification. As a matter of fact, the DNA is more stable molecule for all sorts of natural stresses therefore; species identification based on DNA techniques is more promising and reliable (1).

Over the past few years many molecular identification techniques have been proved to be effective tool for species identification (2;4;). However, among them DNA sequencing has proved to be most robust and reliable technique. Mitochondrial DNA sequence analysis is also enormously useful in studies of evolutionary history and extensive sampling of DNA sequences has helped to establish the genetic diversity and allowed researchers to analyze evolutionary relationships in crocodiles(5;9;14), the mitochondrial DNA sequences have demonstrated their forensic

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utility in identification of species constructing phylogenetic tree and dissemination at basal nodes and deeper nodes but some of the nuclear genes have also played very important role in forensic discrimination of wildlife products (20). Currently there are no single-copy nuclear gene sequences that can be used for forensic discrimination of crocodile species and their phylogenetic analysis. Nuclear primers based species identification in forensic studies has the advantage as conclusions can be verified by examining a number of independent primers. This is very useful in cases, where PCR contamination is a possibility, or when hybridization between species is known to occur (18). Here, conserved genes are targeted for amplification with broad-ranged primers which are divergent enough to resolve closely related species. Therefore, the nuclear gene data can be best assessed to rebuild nodes at deepest levels of the reptilian tree for their discrimination (16). In view of their utility, we have selected nuclear gene i.e. oocyte maturation factor (C-mos) for species identification purpose.

The c-mos gene is single-copy, without introns, and is just over 1000 base pairs (bp) (3). It has been found in the genomes of amphibians, birds and mammals. The human c-mos open reading frame is 346 codons (17). There are no repetitive elements in the sequences and few insertions or deletions that would complicate sequences alignment among vertebrates. These characteristics make these gene are very amenable to PCR amplification from genomic DNA and direct sequencing of PCR products.

Materials and Methods:-

Sample collection and DNA extraction:-

Blood samples from *C. Palustris* (N=15), *C. porosus* (N=09), *C. siamensis* (N=06), *C.niloticus* (N=06), *Caiman crocodiles* (N=06) and *G. gangeticus* (N=25) were collected from Madras Crocodile Bank Trust (MCBT), Mammalapuram, Tamilnadu, India. Whereas, the tissue samples from 15 dead gharials (fresh and purified tissue) were collected from National Chambal Sanctuary project, Agra, Uttar Pradesh, India. All the biological samples are maintained in the repository of Central Forensic Science Laboratory, Kolkata, West Bengal, India. Total Genomic DNA extraction was carried out by standard phenol: chloroform procedure (11) followed by cleaning with Microcon 100 filter column (Millipore). DNA extraction from tissue samples was performed using Qia tissue DNA extraction kit (Qiagen, Valencia, CA) as per the manufacturer guidelines.

Primer design and PCR amplification:-

The complete C-mos gene sequences of crocodiles were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) database (Table 1) for primer designing using Primer 3 software. Sequence alignment was done using Clustal W program (15) and MEGA 5 software (13) . The regions with not more than five mismatches with high similarity in 3' ends in all six crocodile sequences were chosen and the primers F- CCC TTG GGC TCT GGG GGC TT; R- TGA GGA GCT CTG GGG CAC GG were designed. PCR reactions were performed using total reaction volume of 20µl containing 2.0µl of 10X buffer (Containing 200mM Tris-HCl, pH 8.4, and 500mM KCl) (Invitrogen Life Technologies), 2.0µl of dNTPs (2.5mM each) (MBI Fermentas, Glen Burnie, MD), 0.6µl of Exprime *Taq* DNA polymerase (5U/ µl) (Genet Bio), 0.2µl each of forward and reverse primers and 1.0µl of genomic DNA (100 pg). Lastly, sterile water was added to make up the final volume to 20 µl. All the PCR reactions were carried out on Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, CA) with PCR conditions: 94^{o}C for 5 min of initial denaturation followed by 35 cycles of: denaturation at 95^{o}C for 30s; annealing at 60^{o}C for 30s; extension at 72^{o}C for 30s and amplification ended with 7 min final extension step followed by a 4^{o}C hold. Amplicons were checked in 2% agarose gel with negative control.

DNA sequencing and data analysis:-

The PCR products were twice purified by ethanol (2.5 volume) and 2M Sodium acetate, pH 5.6 (0.1 volume) using standard procedure (11). Cycle sequencing was performed following the standard protocol of BIG-DYE version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) using forward as well as reverse primers. The products were sequenced using ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). The newly obtained gene sequence was aligned with the available nuclear genome sequences of crocodile species. The resulting sequences were individually subjected to BLAST search and were translated into amino acid sequences to confirm the protein coding genes. The molecular distances were calculated by using MEGA5 (13). The synonymous and non-synonymous substitution rates were calculated using the ka/ks calculator (19).

Table 1:- List of crocodile species and their accession numbers for four nuclear genes as retrieved from NCBI database

Species Name	Accession Number
Tomistoma schlegelii	JF315172.1
Osteolaemus tetraspis	JF315228.1
Alligator mississippiensis	JF315210.1
Alligator sinensis	JF315214.1
Melanosuchus niger	JF315214.1
Paleosuchus palpebrosus	JF315211.1
Paleosuchus trigonatus	JF315213.1

Table 2:- List of the intra-species polymorphic sites at 490 bp C-mos in six species

Species (n in total)	Position	Nucleotide	n
Gavialis gangeticus (25)	210[a]	T/G	13/12
	336	A/G	23/2
	389	T/G	24/1
Crocodylus porosus (09)	187	C/G	7/2
	220	A/C	6/3
	346	T/A	3/6
	458	G/A	8/1
	476	A/G	7/2
Crocodylus palustris (15)	394	C/A	10/5
	405	T/A	13/2
Caiman Crocodilus (06)	21	C/T	2/4
	276	A/C	3/3
	347	T/A	5/1
	468	G/C	1/5
Crocodylus siamensis (06)	331	A/G	5/1
	405	A/G	4/2
Crocodylus niloticus (06)	17	T/C	4/2
	340	A/C	2/4
	478	T/A	3/3

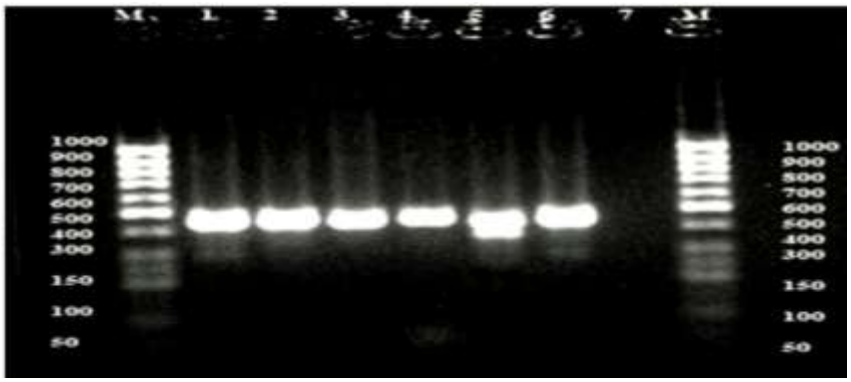
[a] Nucleotide numbering started from the first nucleotide next to the 3´-terminal of forward primer in all species.

Table 3:- Nucleotide sequence diversities in the 490bp of C-mos (below diagonal) and C-mos (above the diagonal)[a] between six crocodile species examined in this study.

	G. gangeticus	***C. porosus***	***C. palustris***	***C. niloticus***	***C. Crocodylus***	***C. Siamensis***
G. gangeticus		0.025	0.007	0.003	0.034	0.027
C. porosus	0.077		0.028	0.003	0.036	0.005
C. palustris	0.083	0.005		0.025	0.041	0.002
C. niloticus	0.082	0.016	0.021		0.038	0.002
C. Crocodylus	0.120	0.109	0.116	0.107		0.039
C. Siamensis	0.075	0.005	0.010	0.021	0.109	

[a] C-mos nucleotide sequence data was collected from NCBI.

Figure 1:- Two percent agarose gel showing PCR products of six crocodile species, Lanes 1-6 represent 490 bp amplicons obtained using primers C-Mos specific primers



Results and discussion:-

DNA forensics is now becoming a key investigative tool to combat wildlife crimes. At the same time, the way in which DNA evidence is generated and presented in the court is also coming under renewed scrutiny. In the present study we have successfully amplified and analyzed partial sequence of 490 bp (Figure 1), c-mos sequence data were collected for 67 individuals of crocodiles in six species i.e. *C. Palustris*, *C.porosus*, *C.siamensis*, *C.niloticus*, *Caiman crocodiles* and *G.gangeticus* and aligned them with sequences of similar regions of other species of crocodiles to find out the most congenial nucleotide variable region, conserved within species. The information on species-specific nucleotide variation would have great relevance for lineage differentiation of desired animal species. Of the 490 sites examined, there were 353 constant sites, 9 parsimony uninformative sites, and 128 parsimony informative sites. Due to high conservation and a lack of introns (often the location of most variation in nuclear genes) produced relatively low genetic distance values within this dataset (Table 2). We calculated the mean sequence distances which ranged from 0.005% to 0.12%, higher than the reference mean sequence distance values ranged from 0.005% to 0.041% based on nuclear sequence distance (Table 3), C-mos having a potentiality to discriminate the crocodile species for their conservation. Hence, we recommend the use of these novel primers in the forensic as well as populations of endangered crocodile species, which will help in the effective implementation of wildlife convention to conservation of these species. The sequence data further we used for construction of phylogeny.

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