J Forensic Sci, September 2009, Vol. 54, No. 5
doi: 10.1111/j.1556-4029.2009.01119.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

P. R. Meganathan,[1] *M.Sc.; Bhawna Dubey,*[1] *M.Sc.; and Ikramul Haque,*[1] *Ph.D.*

Molecular Identification of Indian Crocodile Species: PCR-RFLP Method for Forensic Authentication*

ABSTRACT: South East Asian countries are known for illegal poaching and trade of crocodiles clandestinely, to be used in skin, medicinal, and cosmetic industries. Besides crocodiles being listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora, India has its Wildlife Protection Act, 1972 for conservation of crocodile species. Hitherto, lack of any rapid and reliable technique for examinations of crocodile-based crime exhibits such as skin, bones, etc. has been a major problem for an effective promulgation of law on illegal trade. DNA-based identification of species using PCR-RFLP technique for an apt identification of all the three Indian crocodile species namely, *Crocodylus porosus, Crocodylus palustris* and *Gavialis gangeticus* is presented here. A 628 bp segment of cytochrome *b* gene was amplified using novel primers followed by restriction digestion with three enzymes i.e., *Hae*III, *Mbo*I, and *Mwo*I, separately and in combination. The technique has produced a species-specific pattern for identifying the three crocodile species individually, which fulfills the requirement for its forensic application. It is expected that the technique will prove handy in identification of all the three Indian crocodile species and strengthen conservation efforts.

KEYWORDS: forensic science, Indian crocodiles, cytochrome *b,* restriction fragment length polymorphism, species identification, conservation

Crocodilians constitute a small order Crocodilia, represented by 23 species (1) of which three species, mugger (*Crocodylus palustris*), saltwater or estuarine crocodile (*Crocodylus porosus*), and gharial (*Gavialis gangeticus*) are found in India. They have been over exploited in the past for their lucrative products and extirpated from their natural habitat in some parts of the world (2,3). The IUCN Red list of the threatened species regarded mugger as a vulnerable species, estuarine crocodile as a species under lower risk, and gharial as critically endangered (http://www.iucnredlist.org). They have also been included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Many countries have constituted legal policies to conserve the existing crocodile species (4,5). India promulgated the Wildlife Protection Act in 1972, which includes all the three species in Schedule I and forbids illegal hunting in order to conserve these threatened species. In addition to strict conventions, implementations of many conservation programs have improved the status of populations of these species in the wild (5,6). However, illegal trade and poaching continues to exacerbate the existence of these antiquities. Unfortunately the limited data available on these species and lack of simple scientific technique for the identification of confiscated biological materials prove to be major hurdles in effective law enforcement. In this perspective, the use of molecular methods for species identification is inevitable.

Many of the reported molecular techniques involve analysis of mitochondrial DNA (mtDNA). As most of the forensic laboratories receive highly degraded and/or vestigial samples, the mtDNA may be the only source for species determination (7–9). The cytochrome *b* (cyt *b*) gene in mtDNA has been validated as a useful marker for species identification (10) and as an effective tool for phylogenetic studies (11,12). Although the universal or species-specific primers for this gene are well documented (13,14), the DNA sequencing method currently being used for species identification is prohibitively expensive for laboratories with limited facilities (15). A simpler alternative to the DNA sequencing technique is restriction fragment length polymorphism analysis of PCR products (PCR-RFLP). This technique proves to be a simple, rapid, and cost-effective method for species identification (16,17) and has been used in forensic examination to authenticate the confiscated biological materials (18,19).

Herein, we have used cyt *b* primers, designed for previous studies involving authentication of existing crocodile species (P.R. Meganathan, B. Dubey, and I. Haque, unpublished data), to develop a simple PCR-RFLP technique for the forensic identification and differentiation of three Indian crocodile species. The current study holds promising results for effective forensic investigation in order to prevent the encroachment of law and to conserve these endangered species.

Materials and Methods

Sample Collection and DNA Extraction

Authenticated biological samples were obtained from Madras Crocodile Bank Trust (MCBT), Centre for Herpetology, Mamallapuram, Tamilnadu, India and National Chambal Sanctuary Project, Agra, Uttar Pradesh, India under the consent of Ministry of Environment and Forests, Government of India, New Delhi. Whole blood samples from *C. palustris*, *C. porosus*, and *G. gangeticus* and fresh tissue and highly putrefied test samples from dead gharials were included in the present study. All the biological samples are maintained in the repository of Central Forensic Science Laboratory, Kolkata, West Bengal, India. Genomic DNA extraction from

[1]National DNA Analysis Centre, Central Forensic Science Laboratory, 30 Gorachand Road, Kolkata 700 014, West Bengal, India.

*The study is funded by Directorate of Forensic Sciences, Ministry of Home Affairs, Government of India.

Received 24 July 2008; and in revised form 13 Oct. 2008; accepted 25 Oct. 2008.

blood samples was carried out by standard phenol:chloroform procedure (20) and further purified using Microcon 100 centrifugal filter column (Millipore Corporation, Billerica, MA). DNA extraction from tissue samples was performed using Qia tissue DNA extraction kit (Qiagen, Valencia, CA) as per the manufacturer's guidelines.

PCR Amplification

PCR reactions were performed in 25 μL reaction volumes, containing 2.5 μL (0.2 μM) of the following primers: CP14126: 5′-ACC AAG ACT TGA GGC ACG AAA AAC C-3′; CP14860: 5′-AGG ATA AAT GGG AGC AGG AAG TG-3′, 1.25 μL of $MgCl_2$ (2.5 mM) (Invitrogen Life Technologies, Sao Paulo, Brazil), 2.5 μL of dNTPs (2.5 mM each) (MBI Fermentas, Glen Burnie, MD), 2.5 μL of 10× buffer (containing 200 mM Tris–HCl, pH 8.4, and 500 mM KCl) (Invitrogen Life Technologies), 1.0 μL of Taq DNA polymerase (5 U/μL) (Invitrogen Life Technologies), and 3.0 μL of genomic DNA (100 ng), under the following cycling conditions: 94°C for 5 min of initial denaturation followed by 30 cycles of: denaturation at 95°C for 1 min; annealing at 50°C for 30 sec; extension at 72°C for 45 sec. Amplification ended with a 5 min final extension step followed by a 4°C hold. All the reactions were carried out in Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, CA). The amplicons were checked in 2% agarose gel containing 0.5 μg/mL of ethidium bromide stain.

DNA Sequencing

In order to select the appropriate restriction enzymes for PCR-RFLP reaction the amplification products were sequenced. The PCR products were purified twice by precipitation with 100% ethanol (2.5 volume) (Merck, Darmstadt, Germany) and 3 M sodium acetate, pH 5.6 (0.1 volume) (Sigma Aldrich, Taufkirchen, Germany) using standard procedure (20). Cycle sequencing was performed following the standard protocol of BIG-DYE version 3.1 cycle sequencing kit (Applied Biosystems) using both primers (CP14126 and CP14860). The products were sequenced using ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems).

RFLP Analysis of PCR Products

The 628 bp cyt *b* gene sequences of Indian crocodile species were analyzed for restriction enzyme sites using Restriction Mapper Version 3.0 (http://www.restrictionmapper.org) and three restriction enzymes, *Hae*III, *Mbo*I and *Mwo*I (New England Biolabs, Beverley, MA), were selected for further experimental analysis. First the PCR products were digested with 5 units of each restriction enzyme separately and secondly, the PCR products were digested with a mixture of *Hae*III (5 U) and *Mbo*I (2.5 U). All digestion reactions were performed in 10.0 μL reaction volume according to the manufacturer's instruction (New England Biolabs) for 4 hours. The patterns of resulting fragments were observed in 2.5% agarose gel using ethidium bromide stain (0.5 μg/mL).

Results and Discussion

All the three Indian crocodile species are included in Schedule I in the Wildlife Protection Act, 1972 and are prohibited from hunting or any illegal trade. However, the illegal poaching continues to be a major threat for these species (5,21). Conservation strategies demand a simple molecular technique for the investigation of wildlife crimes. Therefore, the current study focuses on the development of an easy and reliable method to identify and differentiate all three Indian crocodile species for forensic authentication. In this analysis we have used our primers for the amplification of partial (628 bp) cyt *b* gene in all three Indian crocodile species (Fig. 1). These primers have already proven their efficiency in amplifying degraded samples (P.R. Meganathan, B. Dubey, and I. Haque, unpublished data). The 628 bp fragment fulfills the requirement of species identification through PCR-RFLP and overcomes the need for DNA sequencing technology (22–24).

The amplified partial cyt *b* gene sequences were mapped to determine the restriction sites to produce a species-specific restriction pattern. Three enzymes, *Hae*III, *Mbo*I, and *Mwo*I were found to generate distinctive restriction pattern in all three crocodile species in electronic RFLP (Table 1). The PCR-RFLP analyses yielded species-specific restriction patterns in the direct digestion of PCR products with each of the restriction enzyme, *Hae*III, *Mbo*I, and *Mwo*I (Fig. 2) separately as well as in combination with *Hae*III and *Mbo*I (Fig. 3). The restriction enzymes *Hae*III and *Mbo*I produced discrete bands in comparison to *Mwo* I for all three crocodile species. Thus the two enzymes, *Hae*III and *Mbo*I were used in combination to digest the partial cyt *b* products to yield a specific restriction pattern. Although a single enzyme was sufficient to identify or to differentiate the species (25), the current study utilizes three different enzymes to produce species-specific patterns that minimize the possibility of incorrect interpretations due to unexpected polymorphisms that may arise or occur in the species present in diverse ecological or geographical locations (18). The presence of mutation in a test sample for a particular restriction enzyme will yield different restriction digestion pattern as

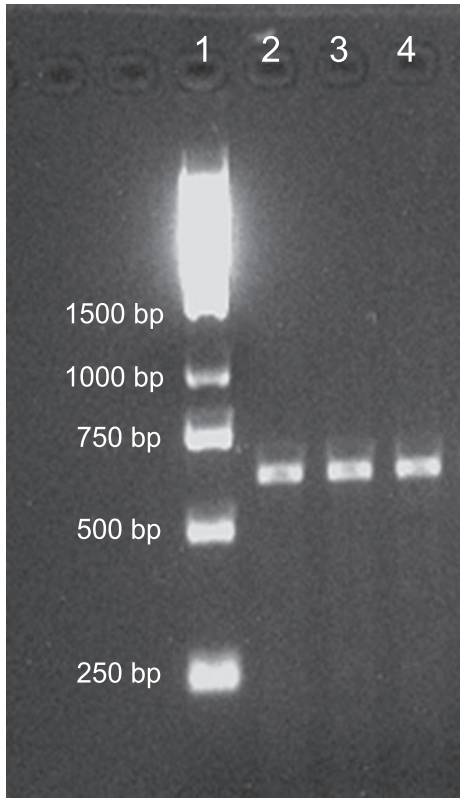

FIG. 1—*2% agarose gel showing 628 bp PCR products from amplification of DNA from three crocodile species. Lane 1: 1 kb molecular size standards; Lane 2:* Crocodylus palustris*; Lane 3:* Crocodylus porosus; *Lane 4:* Gavialis gangeticus.

TABLE 1—*Restriction digestion patterns of amplified cyt* b *gene in three Indian crocodile species obtained from Restriction mapper 3.0.*

Species Name	*Hae*III (Size in bp)	*Mbo*I (Size in bp)	*Mwo*I (Size in bp)	*Hae*III + *Mbo*I (Size in bp)
Crocodylus palustris	382	253	325	253
	253	213	310	169
		169		129
				84
Crocodylus porosus	500	334	325	340
	70	169	293	121
	64	131	16	70
				67
				65
Gavialis gangeticus	316	466	310	316
	183	167	263	99
	68		60	84
	66			68
				66

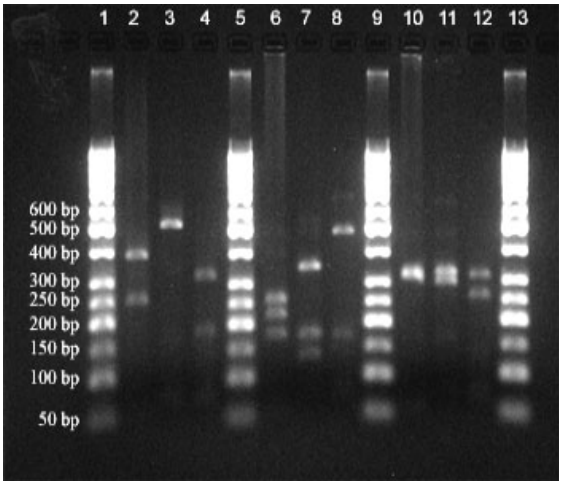

FIG. 2—*The agarose gel (2.5%) showing PCR-RFLP analysis of 628 bp amplified fragment from three crocodile species,* Crocodylus palustris *(2, 6 & 10),* Crocodylus porosus *(3, 7 & 11) and* Gavialis gangeticus *(4, 8 & 12) digested with* Hae*III (2–4),* Mbo*I (6–8), and* Mwo*I (10–12). Lanes1, 5, 9 & 13 are molecular markers.*

compared to the reference sample. In this case the test sample may be digested with multiple restriction enzymes in order to verify a similar observation ensuring the existence of any mutation in the restriction site. Hence in forensic examinations, one enzyme can be used to identify these Indian crocodile species or the enzyme combinations may be utilized to infer the mutational changes in the restriction site.

The different restriction enzymes utilized produced satisfactory results in the differentiation and identification of Indian crocodile species. Our major goal was to develop a simple and rapid method for the authentication of Indian crocodiles, but as most often the forensic laboratories receive highly degraded samples for examination (26), the determination of species identity using these samples becomes a challenging task (27–29). In this regard the current protocol proves efficient even in cases of highly degraded tissue samples as DNA source in forensic identification of species without the further need of sequencing analysis.

Conclusion

The species-specific patterns generated by present PCR-RFLP analyses of cyt *b* gene are helpful in characterization of the Indian

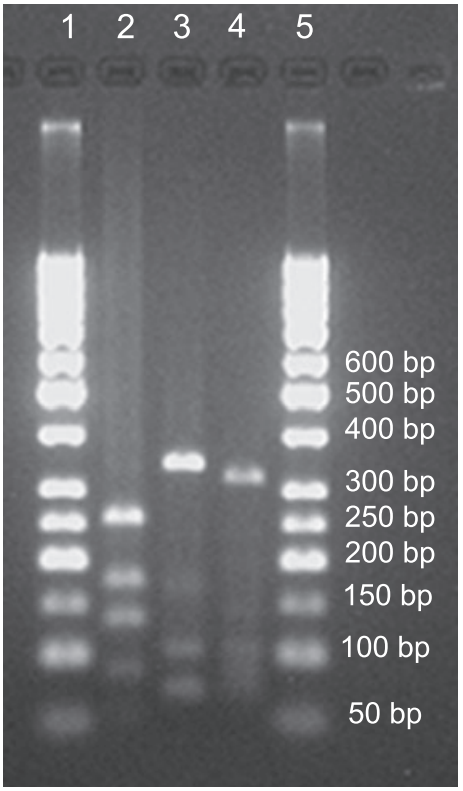

FIG. 3—*2.5% agarose gel showing electrophoretic separation of cyt* b *PCR products digested with* Hae*III and* Mbo*I. Lanes 1 & 5: Molecular marker; Lane 2:* Crocodylus palustris*; Lane 3:* Crocodylus porosus*; Lane 4:* Gavialis gangeticus.

crocodile species. This concise PCR-RFLP protocol with a set of three enzymes allows the discrimination between three different crocodile species, thus helping in rapid evaluation of species status of the sample in question. This technique could be a valuable tool for forensic laboratories and wildlife personnel requiring identification of the confiscated biological materials in cases related to crocodile poaching, thereby contributing to the conservation of these keystone species.

Acknowledgments

The authors are thankful to Madras Crocodile Bank Trust (MCBT), Centre for Herpetology, Mamallapuram, Tamilnadu, India and National Chambal sanctuary project, Agra, Uttar Pradesh, India for providing the biological samples for the study.

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Additional information and reprint requests:
Ikramul Haque, Ph.D.
National DNA Analysis Centre
Central Forensic Science Laboratory
30 Gorachand Road
Kolkata 700 014
West Bengal
India
E-mail: haque_cfslk@yahoo.co.in