JOURNAL OF FORENSIC SCIENCES

*J Forensic Sci,* September 2011, Vol. 56, No. 5
doi: 10.1111/j.1556-4029.2011.01812.x
Available online at: onlinelibrary.wiley.com

**TECHNICAL NOTE**

**CRIMINALISTICS**

*Poorlin Ramakodi Meganathan,*[1] *M.Sc.; Bhawna Dubey,*[1] *M.Sc.; Kothakota Naga Jogayya,*[1] *M.Sc.; and Ikramul Haque,*[1] *Ph.D.*

# Validation of a Multiplex PCR Assay for the Forensic Identification of Indian Crocodiles*

**ABSTRACT:** A dependable and efficient wildlife species identification system is essential for swift dispensation of the justice linking wildlife crimes. Development of molecular techniques is befitting the need of the time. The forensic laboratories often receive highly ill-treated samples for identification purposes, and thus, validation of any novel methodology is necessary for forensic usage. We validate a novel multiplex polymerase chain reaction assay, developed at this laboratory for the forensic identification of three Indian crocodiles, *Crocodylus palustris*, *Crocodylus porosus*, and *Gavialis gangeticus*, following the guidelines of Scientific Working Group on DNA Analysis Methods. The multiplex PCR was tested for its specificity, reproducibility, sensitivity, and stability. This study also includes the samples treated with various chemical substances and exposed to various environmental regimes. The result of this validation study promises this technique to be an efficient identification tool for Indian crocodiles and therefore is recommended for forensic purposes.

**KEYWORDS:** forensic science, multiplex polymerase chain reaction, Indian crocodiles, species identification, validation, conservation

All the three Indian crocodile species, *Crocodylus palustris* (mugger), *Crocodylus porosus* (saltwater or estuarine crocodile), and *Gavialis gangeticus* (gharial), are under serious threat of extinction (1–3). Although law prevents any illegal activity against these species, illicit trade continues to date and wildlife forensics require effective molecular techniques for flawless identification of confiscated materials to prosecute the criminals. The existing molecular techniques like DNA sequencing (4), sequence characterized amplified region analysis (5), and polymerase chain reaction (PCR)–restriction fragment length polymorphism method (6) are time-consuming and may not be efficient in case of mixed samples. Therefore, a simple multiplex PCR assay was developed based on cytochrome *b* (cyt *b*) gene, in which the species-specific forward primers: MUG for *C. palustris*, SAL for *C. porosus*, GHA for *G. gangeticus* and a universal reverse primer, UNI were designed to yield specific-sized PCR products for each species. This novel multiplex PCR aids in quick and unambiguous identification of Indian crocodile species (7). However, judicious use of this technique in forensic cases requires a validation study to be completed before it can be implemented in the real forensic scenario (8). The validation of the projected technique is essential for ensuring the reliability and reproducibility of the results obtained. Therefore, to establish the utility of this method in forensic examinations, we present the validation results of our multiplex PCR assay, according to the guidelines of Scientific Working Group on DNA Analysis Methods (SWGDAM). In addition, this study also identifies an unknown specimen using this multiplex PCR assay.

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

*Funded by Directorate of Forensic Sciences, Ministry of Home Affairs, Government of India, New Delhi.

Received 15 Mar. 2010; and in revised form 5 July 2010; accepted 15 Aug. 2010.

## Materials and Methods

### *Sample Collection and DNA Isolation*

The authenticated blood samples of *C. palustris*, *C. porosus*, *C. niloticus*, *C. johnstoni*, *C. siamensis*, *G. gangeticus*, and *Caiman crocodilus* and fresh tissue as well as highly putrefied test samples of dead *G. gangeticus* were included in this study. Genomic DNA extraction from blood samples was carried out by standard phenol/chloroform procedure (9) followed by purification 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.

### *Multiplex PCR Amplification*

The multiplex reactions were carried out in 25.0-μL reaction volumes in duplicates on GeneAmp® 9700 thermal cycler (Applied Biosystems, Foster City, CA). The PCR ingredients and the cycling conditions are described in the study by Meganathan et al. (7), and all the ingredients and PCR conditions were kept the same for all the reactions unless otherwise mentioned. The PCR products were checked in 2.5% agarose gel with 0.5 μg/mL of ethidium bromide stain. The electrophoresis was performed at 5 V/cm for 1 h.

### *DNA Sequencing*

To check the reliability of PCR products, the amplicons were sequenced using BigDye® Terminator v 3.1 cycle sequencing kit (Applied Biosystems) using both forward and reverse primers on 3100 Avant genetic analyzer. The sequences were aligned using MEGA 3.1 software (10) to verify the species identity.

*Validation of Multiplex PCR Assay*

The multiplex PCR was carried out for all the samples using the procedure described in the study by Meganathan et al. (7). All the reactions contain 10 ng of DNA templates unless otherwise mentioned. Positive and negative controls were included simultaneously in all the amplifications.

*Species Specificity*—The specificity of this technique for Indian crocodiles was checked by including the DNA samples of *C. palustris* ($N = 20$), *C. porosus* ($N = 20$), and *G. gangeticus* ($N = 25$), collected across the wide geographical range. Further cross-species amplification was checked with other crocodile species (*C. niloticus*, *C. siamensis*, *C. johnstoni*, and *C. crocodilus*), turtle (*Kachuga tecta*), snakes (*Xenochrophis piscator* and *Ptyas mucosus*), and house lizard (*Hemidactylus flaviviridis*).

*Tissue Specificity*—The forensic laboratories often receive the hide or muscle of crocodile species for identification purposes. However, it is highly imperative to check the ability of this assay with various tissue samples as in few cases different tissue samples may be provided for species identification. Thus, the DNA extracted from various tissue samples like hide, muscle, liver, spleen, lung, kidney, heart, and brain of two individual dead gharials was included in the validation study.

*Sensitivity*—The sensitivity of this method was evaluated by performing the multiplex PCR reaction using a range of DNA concentrations (10, 5, and 1 ng; 100, 50, and 10 pg) from the three species. The degraded DNA from bone and highly putrefied tissue samples of dead gharials was also included in the study to check the efficacy of the technique for forensic usage.

*Stability*—Aliquots of 5.0 μL of blood samples of *C. palustris* and *C. porosus* were deposited on filter paper, cotton cloth, wood, and dried leaf. The samples were kept at ambient temperature (24°C [min.] and 36°C [max.] with average 70% RH (during the month of May in Kolkata) for 5 days, and the DNA was extracted for validation.

*Effect of Chemicals on Samples*—Five microliters each of 0.1 M NaOH, 0.1 M HCl, 5% glacial acetic acid, 5 M NaCl, 0.2% SDS was taken on Whatman filter paper (1 cm) separately and 5.0 μL of liquid blood sample of *C. palustris* and *C. porosus* was deposited on the filter papers, whereas 50 mg of fresh tissue sample of *G. gangeticus* was transferred to the microcentrifuge tube containing 1.0 mL of each of the chemicals. All the tubes were kept at ambient temperature for 5 days, and DNA was extracted for multiplex PCR analysis.

*Effect of Heat on Samples*—A 5-μL liquid blood sample of *C. porosus* and *C. palustris* was taken on Whatman filter paper (1 cm), and 50-mg fresh tissue sample of *G. gangeticus* was taken in microcentrifuge tubes. The samples were incubated at different ranges of temperature and time period: (i) ambient temperature for 1, 5, 10, 20, and 30 days, (ii) 56°C for 8, 16, 24, 36, and 48 h, and (iii) 70°C for 1, 2, and 3 h. After incubation, the samples were kept at −80°C until DNA extraction.

*Effect of UV Light on Samples*—The blood stain samples of *C. porosus* and *C. palustris* were prepared on Whatman filter paper as mentioned earlier. The samples were placed at a distance of 15 cm from UV light (source of 280–360 nm wave length; SANKYO DENKI, Hiratsuka, Japan) and directly exposed to the radiation for 1, 3, 5, 8, and 12 h. The DNA was extracted for multiplex PCR assay.

*Effect of $MgCl_2$ Concentration and Thermo-Cycling Conditions on Multiplex PCR*—Concentration of $MgCl_2$ varied from 1.0 to 5.0 mM, and the thermocycling parameters were changed from those previously optimized by varying the annealing temperature ±3°C and cycling number by ±2.

*Identification of Unknown Sample*

An unknown dried hide sample with least identifiable morphological features was obtained from Snake Transit House, Jabalpur, Madhya Pradesh. DNA was isolated, and multiplex PCR was performed with positive as well as negative controls. The amplicons were examined in 2.5% agarose gel having 0.5 μg/mL of ethidium bromide. The utility of this technique was further confirmed by sequencing the resulting amplicons and aligned with the sequences of three Indian crocodile species.

## Results and Discussion

The conservation management of Indian crocodiles requires development of novel molecular techniques for the identification of these species in forensic analyses. In our previous study, we have developed a multiplex PCR assay for the identification of Indian crocodile species. This multiplex yields species-specific amplicons for each species, i.e., 373 bp for *C. palustris*, 486 bp for *G. gangeticus*, and 578 bp for *C. porosus* and differentiates the three species in a simple agarose gel electrophoresis without the need of further sequencing technique. Although this technique resulted in unambiguous species identification, validation studies are mandatory to use this method in forensic analyses.

*Multiplex PCR Validation*

The DNA isolated from all the samples was included in the multiplex validation study. The primers were tested with the DNA samples of three Indian crocodile species, collected in the wide geographical range in India, as the individuals that exist in different environments may possess intra-specific variations in the genome (11–14), and the presence of these nucleotide variations in the primer annealing site contort the results. However, the reaction yielded the expected sized PCR products from all the DNA samples of three Indian crocodile species and did not amplify the DNA of other reptile species used in this experiment. Moreover, the primers were also tested in the electronic PCR (e-PCR) with the cyt *b* gene sequences of other crocodiles available in GenBank and did not produce any products, thus proving the high specificity and reliability of this method for the identification of Indian crocodiles. Although DNA isolated from putrefied tissue and bone samples of *G. gangeticus* yielded highly degraded DNA, the amplification produced a positive result (*c.* 486 bp) for these samples as was obtained for the DNA isolated from different tissue samples of *G. gangeticus*. Furthermore, the primers amplified the expected species-specific fragments: *c.* 373 bp for *C. palustris* and 486 bp for *G. gangeticus* in all DNA dilutions (10, 5, 1 ng, 100, 50, and 10 pg), whereas the primers yielded *c.* 578-bp PCR product specific to *C. porosus* in all DNA dilutions, except 10-pg dilution of DNA. These results substantiate the use of this technique on any vestigial biological materials. The stability of this assay was tested by including the samples deposited on various substrates: filter

paper, cotton cloth, wood, and dried leaf. The amplification was observed in all samples, except for the blood sample deposited on wood and even when these DNA samples were serially diluted (1:2, 1:4, 1:8). This finding was consistent with the validation study carried out by Branicki et al. (15) where the samples deposited on wood did not yield any result.

The samples available for forensic analysis are often exposed to different environmental conditions resulting in poor quality DNA (16,17), which in turn requires a sensitive method to yield consistent results. Therefore, in the validation study, we have subjected the samples to more or less similar conditions of adverse temperature regimes and various kinds of chemical agents. The samples treated with various chemicals and exposed to different heat conditions yielded highly degraded DNA except a few (samples incubated at ambient temperature for 1 day; samples treated with SDS). The DNA extracted from those samples treated with 0.1 M HCl, 0.1 M NaOH, and 5% glacial acetic acid was observed as a complete shearing below 1 kb (in 1% agarose gel electrophoresis). Nevertheless, this multiplex PCR assay amplified the expected products and proved efficient even for these degraded samples (Figs 1 and 2). Moreover, in this validation study, the samples were exposed to direct UV radiation to check the influence of radiation on the samples. The UV radiation is known to cause damage and/or alter the nucleotide sequence in the DNA template (18,19), which may fail to amplify. But this method amplified the expected sized amplicons from DNA extracted from all the samples exposed to UV irradiation. According to SWGDAM, a developmental validation study reporting a PCR-based procedure must appropriately demonstrate the effect of $MgCl_2$ and other thermocycling parameters. Hence, the effect of these parameters was assessed, and we found that the PCR resulted in nonspecific amplifications below 3.5 mM concentration of $MgCl_2$ whereas no amplification resulted above this concentration. The variation in annealing temperature below the optimized temperature (63°C) resulted in nonspecific amplifications, whereas an increase of 1°C above this yielded weak PCR products and no amplification at all was noticed beyond this temperature range. However, there was no effect of cycling conditions on the PCR. All the amplicons were sequenced and aligned with positive controls (the DNA sequences of *C. palustris*, *C. porosus*, and *G. gangeticus*), and 100% similarity was obtained in the alignment results with the sequences of appropriate species. The validation study provides substantial evidence for the effectiveness of the primers to amplify forensic samples and also describes the advantages of this methodology in forensic analyses.

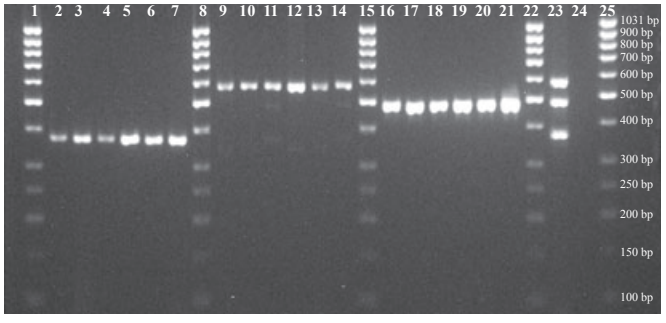


FIG. 1—*Validation results showing positive amplification for samples treated with various chemicals. Lanes 2, 9, and 16: 0.1 M NaOH; lanes 3, 10, and 17: 0.1 M HCl; lanes 4, 11, and 18: 5% glacial acetic acid; lanes 5, 12, and 19: 5 M NaCl; lanes 6, 13, and 20: 0.2% SDS; lanes: 2–7:* Crocodylus palustris*; lanes 9–14:* Crocodylus porosus*: lanes 16–21:* Gavialis gangeticus*; lanes 7, 14, 21, and 23: positive control; lane 24: negative control; lanes 1, 8, 15, 22, and 25: molecular size markers.*

*Identification of Unknown Sample*

The ultimate aim of any technique is to reveal the exact species identity and this criterion should be met along with the validation of the technique. Therefore, the multiplex PCR was performed for the DNA isolated from an unknown sample along with the positive and negative controls. The amplification resulted in *c*. 373-bp product, which commensurates to the position of gene fragment resulting from *C. palustris* (Fig. 3). This is further confirmed by

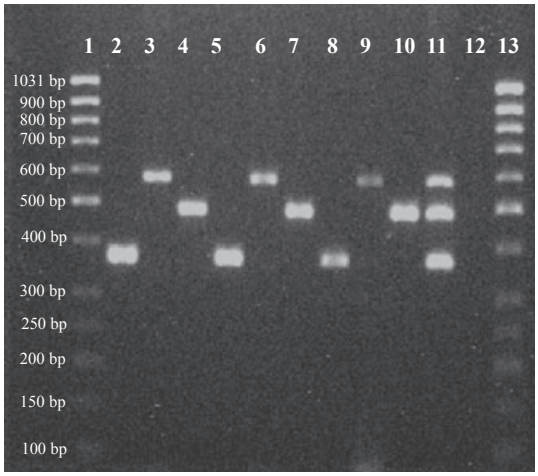


FIG. 2—*Agarose gel electrophoresis for samples exposed to various temperatures in the validation study. Lanes: 2–4: ambient temperature for 30 days; lanes 5–7: 56°C for 48 h; lanes 8–10: 70°C for 3 h; lanes 2, 5, and 8:* Crocodylus palustris*; lanes 3, 6, and 9:* Crocodylus porosus*; 4, 7, and 10:* Gavialis gangeticus*; lane 11: positive control; lane 12: negative control; lanes: 1 and 13: molecular size markers.*

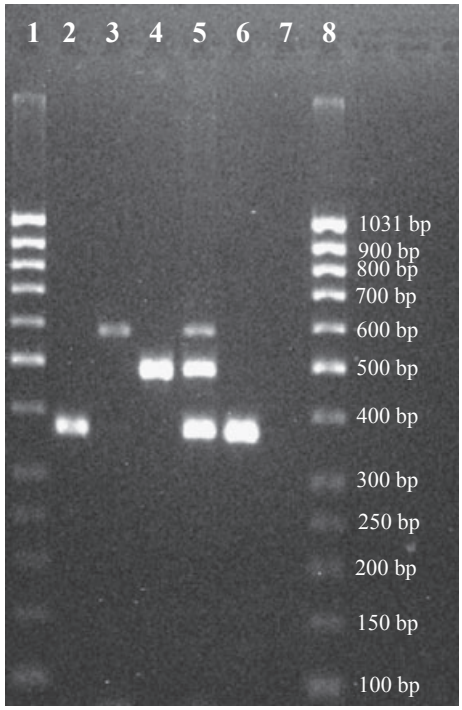


FIG. 3—*Agarose gel electrophoresis result showing 373 bp amplification from unknown sample which corresponds to* Crocodylus palustris. *Lane 2:* C. palustris*; lane 3:* Crocodylus porosus*; lane 4:* Gavialis gangeticus*; lane 5: all three species; lane 6: unknown sample; lane 7: negative control; lanes: 1 and 8: molecular size markers.*

sequencing the amplicon, which resulted in 100% similarity to the cyt *b* sequence of *C. palustris*. Therefore, the species was successfully identified as *C. palustris* using this multiplex PCR assay.

## Conclusion

The multiplex PCR technique for the identification of Indian crocodiles was validated according to the guidelines of SWGDAM and proved efficacious in forensic analyses. This method requires very low quantity of DNA template, efficiently amplifies the product from samples that have been exposed to various conditions, and produces the expected sized fragments from highly degraded tissue as well as bone samples. Moreover, the novel technique revealed the identity of an unknown hide sample as *C. palustris* within a shortest time period in simple agarose gel electrophoresis. Therefore, we suggest utilizing this multiplex PCR assay in the forensic identification of Indian crocodiles for proper enforcement of laws thereby aiding in conservation of these endangered species.

*Acknowledgments*

The authors are thankful to Madras Crocodile Bank Trust, Mamallapuram, Tamil Nadu, Nehru Zoological Park, Hyderabad, Andhra Pradesh, National Chambal Sanctuary Project, Etawah, Uttar Pradesh, and Snake Transit House, Jabalpur, Madhya Pradesh, for providing biological samples for the present study.

## References

1. Manel S, Berthier P, Luikart G. Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. Conserv Biol 2002;16:650–9.
2. Gad SD. Indian Gharial (*Gavialis gangeticus*) on the verge of extinction. Curr Sci 2008;94:1549.
3. Whitaker R. The Gharial: going extinct again. Iguana 2007;14:25–33.
4. Meganathan PR, Dubey B, Haque I. Molecular identification of crocodile species using novel primers for forensic analysis. Conserv Genet 2009;10:767–70.
5. Yau FCF, Wong KL, Wnag J, But PPH, Shaw PC. Generation of a sequence characterized amplification region probe for authentication of *Crocodilian* species. J Exp Zool 2002;294:382–6.
6. Meganathan PR, Dubey B, Haque I. Molecular identification of Indian crocodile species: PCR-RFLP method for forensic authentication. J Forensic Sci 2009;54:1042–5.
7. Meganathan PR, Dubey B, Jogayya KN, Whitaker N, Haque I. A novel multiplex PCR assay for the identification of Indian crocodiles. Mol Ecol Resour 2010;10:744–7.
8. Budowle B, Garofano P, Hellman A, Ketchum M, Kanthaswamy S, Parson W, et al. Recommendations for animal DNA forensic and identity testing. Int J Legal Med 2005;119:295–302.
9. Sambrook J, Russel DW. Molecular cloning, a laboratory manual, 3rd edn. New York, NY: Cold Spring Harbor Press, 2001.
10. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5:150–63.
11. Cedeno-Vazquez JR, Rodriguez D, Calme S, Ross JP, Densmore LD III, Thorbjarnarson JB. Hybridization between *Crocodylus acutus* and *Crocodylus moreletii* in the Yucatan Peninsula: I. Evidence from mitochondrial DNA and morphology. J Exp Zool A Ecol Genet Physiol 2008;309A:661–73.
12. Rodriguez D, Cedeno-Vazquez JR, Forstner MRJ, Densmore LD III. Hybridization between *Crocodylus acutus* and *Crocodylus moreletii* in the Yucatan Peninsula: II. Evidence from microsatellites. J Exp Zool A Ecol Genet Physiol 2008;309A:674–86.
13. Weaver JP, Rodriguez D, Venegas-Anaya M, Cedeno-Vazquez JR, Forstner MRJ, Densmore LD III. Genetic characterization of captive Cuban crocodiles (*Crocodylus rhombifer*) and evidence of hybridization with American crocodile (*Crocodylus acutus*). J Exp Zool A Ecol Genet Physiol 2008;309A:649–60.
14. Hekkala ER, Amato G, DeSalle R, Blum MJ. Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (Crocodylus niloticus) populations. Conserv Genet 2010;11:1435–43.
15. Branicki W, Kupiec T, Pawlowski R. Validation of cytochrome *b* sequence analysis as a method of species identification. J Forensic Sci 2003;48:83–7.
16. Butler JM, Shen Y, McCord BR. The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 2003;48:1054–64.
17. Bär W, Kratzer A, Mächler M, Schmid W. Postmortem stability of DNA. Forensic Sci Int 1998;39:59–70.
18. Friedberg EC. DNA damage and repair. Nature 2003;421:436–40.
19. Ravanat JL, Douki T, Cadet J. Direct and indirect effects of UV radiation on DNA and its components. J Photochem Photobiol B 2001;63:88–102.

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