MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2010) **10**, 744–747 doi: 10.1111/j.1755-0998.2010.02834.x

MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

A novel multiplex PCR assay for the identification of Indian crocodiles

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Abstract

Illegal hunting has been a major threat for the survival of wildlife fauna, including the three crocodile species that India harbours: *Crocodylus palustris*, *Crocodylus porosus* and *Gavialis gangeticus*. Although law prevents trade on these species, illicit hunting for trade continues to threaten the survival of these endangered species; conservation strategies therefore require a rapid molecular identification technique for Indian crocodiles. A multiplex polymerase chain reaction (PCR) assay with species-specific primers, considered as one of the most effective molecular techniques, is described herein. The primers were designed to yield species-specific sized amplicons. The assay discriminates the three Indian crocodile species unambiguously within a short time period using only simple agarose gel electrophoresis. We recommend this multiplex PCR assay to be used in the identification of Indian crocodile species.

Keywords: conservation, cytochrome *b*, Indian crocodiles, molecular identification, multiplex PCR, species-specific primers

Received 5 November 2009; revision received 21 December 2009; accepted 2 January 2010

The wildlife species of India are facing serious threat through the increasing demands for their lucrative products in the international market (Madhusudan & Karanth 2002; Somanathan 2007). This overexploitation, in addition to the bushmeat crisis, has adversely affected the three Indian crocodile species, mugger (*Crocodylus palustris*), saltwater crocodile (*Crocodylus porosus*) and gharial (*Gavialis gangeticus*), which has led to their inclusion in Appendix I of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) along with highest protection in the Indian Wildlife Protection Act, 1972. To conserve these ancient species, many conservation programmes have also been implemented (Rao 1992; Thorbjarnarson 1992), but hunting for illicit trade as well as for wild meat is still posing a major threat to their existence (Whitaker & Members of the GMTF 2007; Gad 2008; Martin 2008; The Hindu 2009; The Times of India 2009) and calls for effective measures to control illegal hunting. Accurate species identification from the confiscated materials is necessary for proper law enforcement, allowing molecular ecologists to assist in monitoring illegitimate trade (Baker 2008; Eaton *et al.* 2009). Unfortunately, the expropriated samples are often highly degraded or limited in quantity, reducing the effectiveness of conventional methods to reveal species identity (Hsieh *et al.* 2001; Ferri *et al.* 2009). In such cases, molecular techniques have proven to be highly efficient (Holland & Parsons 1999; Teletchea 2009).

The molecular techniques currently in use, such as Sequence Characterized Amplified Region (SCAR) analysis (Yau *et al.* 2002), polymerase chain reaction (PCR)-restriction fragment length polymorphism (Meganathan *et al.* 2009a) and DNA sequencing using novel primers (Meganathan *et al.* 2009b), prove to be time consuming and expensive, whereas the species-specific multiplex PCR assay is an easy, efficient and cost-effective technique (Tobe & Linacre 2008), which performs accurately within the shortest time period without compromising test utility (Gil 2007; Marshall *et al.* 2007; Tobe & Linacre 2008; Dubey *et al.* 2009). Thus, a multiplex PCR assay with species-specific primers, spanning partial cytochrome *b* (cyt *b*) gene, is developed herein.

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We have selected cyt *b* gene, because its efficacy for species identification has been validated (Parson *et al.* 2000; Branicki *et al.* 2003). The multiplex PCR described here produces species-specific sized amplicons, which easily differentiates the three Indian crocodile species using only agarose gel electrophoresis.

Total genomic DNA was extracted from *C. palustris*, *C. porosus*, *Crocodylus niloticus*, *Crocodylus johnstoni*, *Crocodylus siamensis*, *G. gangeticus* and *Caiman crocodilus* as well as from highly putrefied test samples of dead *G. gangeticus*. The whole cyt *b* gene sequences were generated from all seven crocodile species as described previously (Meganathan *et al.* 2009b) and were deposited in GenBank under the accession nos: FJ173276–FJ173286 and GU331894–GU331906. These sequences were aligned with the cyt *b* gene sequence of other crocodile species obtained from GenBank (Table S1, Supporting information) using MEGA 3.1 software (Kumar *et al.* 2004). Based on the alignment, unique forward primers (MUG for *C. palustris*, SAL for *C. porosus* and GHA for *G. gangeticus*) and a common reverse primer (UNI) were constructed (Fig. S1, Supporting information). The primers (Table 1) were carefully designed to amplify a specific sized amplicon for each species.

PCR amplification was first optimized for all the three primers separately in 25.0-μL reaction volume consists of 2.5 μL 10× PCR buffer, 0.2 μM appropriate species-specific forward primer (Table 1), 0.2 μM UNI, 0.25 mM dNTPs, 3.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Invitrogen Life Technologies, Brazil) and 10 ng of specific DNA template. The resulting amplicons were sequenced using BigDye® Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using both forward and reverse primers on a 3100 Avant genetic analyzer to verify species identity. The sequences were deposited in Genbank under the accession nos: GU331907–GU331924. To develop a multiplex PCR assay, the three species-specific primers (0.2 μM each) and the common reverse primer (0.4 μM) were pooled together to amplify DNA from all the three species separately as well as in combination. All the reactions were performed on GeneAmp® 9700 thermal cycler (Applied Biosystems) under the following cycling conditions: Initial denaturation at 94 °C for 5 min followed by 35 cycles of: denaturation at 94 °C for 1 min; annealing at 63 °C for 30 s; and extension at 72 °C for 30 s. Amplification ended with a 7-min final extension step followed by a 4 °C hold. The PCR products were checked in 2.5% agarose gel with 0.5 μg/mL of ethidium bromide stain. The influence of intraspecific variation on this assay was tested with samples of *C. palustris*, *C. porosus* and *G. gangeticus*, collected from a wide geographical range. The specificity of this assay was checked by including DNA samples of other crocodiles (*C. niloticus*, *C. siamensis*, *C. johnstoni* and *Caiman crocodilus*), turtle (*Kachuga tecta*), snakes (*Xenochrophis piscator* and *Ptyas mucosus*) and house lizard (*Hemidactylus flaviviridis*).

We obtained the complete cyt *b* gene sequences (~1156 bp) of crocodile species; these were aligned with the cyt *b* gene sequence of other crocodile species retrieved from GenBank. Based on the alignment, three species-specific forward primers were designed to have high specificity to their respective species by utilizing the interspecific variations, and one common reverse primer was designed from a highly conserved region in the alignment (Fig. S1, Supporting information). The position of primers was selected to produce small-sized amplicons for each species, keeping in mind the case of DNA degradation that may preclude amplification of the complete gene sequence (Wiegand & Kleiber 2001; Butler *et al.* 2003; Mukherjee *et al.* 2007; Alaeddini *et al.* 2009). The primer pairs MUG and UNI resulted into the amplification of ~373-bp fragment for *C. palustris*, whereas the primers SAL and UNI produced ~578-bp amplicon for *C. porosus*, and a ~486-bp product was obtained using the primers GHA and UNI for *G. gangeticus*. The PCR containing a particular species-specific forward primer and the common reverse primer did not result in any amplification for rest of the two species including the negative controls used. A 100% similarity was observed between sequences obtained from these species-specific amplicons and the partial cyt *b* gene sequence of appropriate species.

All the primers were combined in a single reaction to develop a multiplex PCR system, which produced three species-specific amplicons, i.e. 373, 486 and 578 bp separately as well as from the mixed DNA samples in a single reaction (Fig. 1). The primers produced species-specific

Table 1 Details of primers used in the multiplex polymerase chain reaction

Primer	Primer sequence (5′–3′)	Amplicon size (bp)	Primer specific to
MUG	TACGTGGGAAACTCAATCGTGG	373	*Crocodylus palustris*
GHA	TCATCCTGCTCCTCTTATTAATAGCG	486	*Gavialis gangeticus*
SAL	AGCTTCCCTATTCTTCCTATGCACA	578	*Crocodylus porosus*
UNI	GTGTAGGCGAATAGGAAGTATCATTC		Common

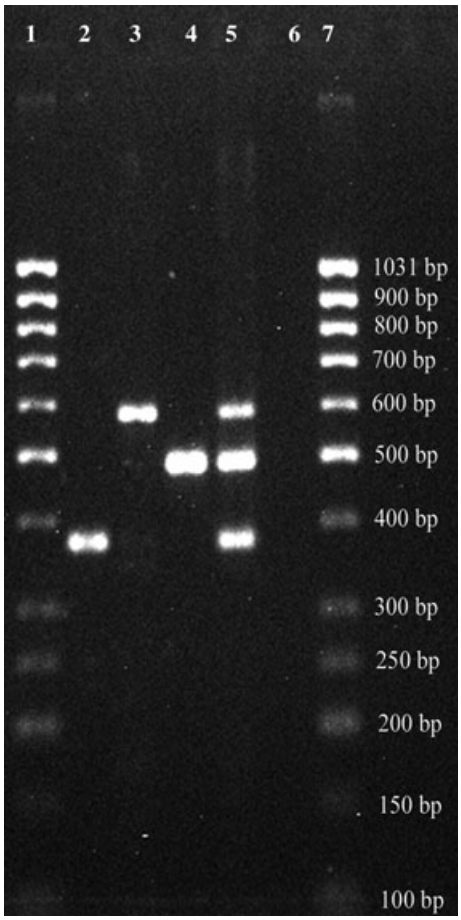

Fig. 1 Multiplex polymerase chain reaction amplification for three Indian crocodile species. Lane 2: 373 bp for *Crocodylus palustris*; lane 3: 578 bp for *Crocodylus porosus*; lane 4: 486 bp for *Gavialis gangeticus*; lane 5: multiplex result for three species; lane 6: negative control; lanes 1 and 7: molecular size markers.

sized amplicons; these enabled easy differentiation of the three species in simple agarose gel electrophoresis without the need of further laboratory analysis. Thus, this technique identifies the three Indian crocodile species within a short time span (~2 h 30 min after DNA isolation). The study included samples collected from various geographical ranges to examine the influence of intraspecific variation on PCR amplification. However, the assay amplified all appropriate fresh samples, as well as the highly putrefied tissue samples of *G. gangeticus*. The specificity of this method was demonstrated by the absence of any amplification in the nontarget species.

A fundamental requirement of conservation genetics is the accurate identification of the species in question. The multiplex PCR method developed herein differentiated three Indian crocodile species unambiguously in simple agarose gel electrophoresis within a matter of hours. The primers also produced consistent results from highly degraded samples. Hence, we recommend the use of this multiplex PCR assay for the rapid molecular identification of Indian crocodile species for the proper implementation of conservation strategies and various ecological studies.

Acknowledgements

The authors are thankful to Madras Crocodile Bank Trust, Mamallapuram, Tamil Nadu, National Chambal Sanctuary Project, Etawah, Uttar Pradesh, and Snake Transit House, Jabalpur, Madhya Pradesh, for providing biological samples for this study. This study was funded by Directorate of Forensic Sciences, Ministry of Home affairs, Government of India, New Delhi.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sequence alignment showing interspecific variations and primer positions.

Table S1 List of crocodile species and their accession nos for cytochrome *b* gene

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