

Authentication of DNA multiplex PCR for Indian snake species for forensic purposes

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Abstract

Identification of confiscated animal samples in forensic laboratories is nearly impossible due to processing and degradation. Consequently, DNA-based molecular methods are optimal for fulfilling the necessary criteria. The molecular technique needed to be robust to function with all DNA samples from different environments. We used SWGDAM (Scientific Working Group on DNA Analysis Methods) references to validate a new multiplex PCR technique for forensic identification of 3 vulnerable Indian snake species-Python morulus, *Ptyas mucosus*, and *Naja naja*. The multiplex PCR's specificity, repeatability, stability, and sensitivity were tested on DNA samples exposed to potentially unfavorable conditions such as chemical treatment and environmental regimes. This validation investigation suggests that the forensic technique will identify the above Indian snake species.

Keywords: 16S rRNA, multiplex PCR, wildlife forensics, validation analysis, conservation studies

Introduction

The significance of forensic DNA in investigating crimes involving wildlife has grown. No definitive identifications have been produced by either the morphological or serological investigations. Several DNA-based species identification technologies have been developed in recent years to guarantee the efficient enforcement of animal protection regulations. The annual slaughter of countless snakes in India for their skins adds to the worldwide issue of wildlife trafficking. Historical evidence indicates that India is among the world's top exporters and manufacturers of snakeskin. The skin trade and seizures show that the black market for snake venom is growing rapidly across the country (TRAFFIC International, 2007). According to research conducted by Kim and Shelef in 1986^[16] and Hseih *et al.* in 1998^[12], India's three most endangered snake species are the Indian Rock Python, the Indian cobra, and the rat snake. Illegal trade has continued for these animals despite legislative protections. Consequently, effective molecular methods are required for wildlife forensics in order to identify stolen goods and apprehend those responsible. It can be quite a challenge to cope with mixed samples using contemporary molecular techniques such as DNA sequencing (M. Jerome, 2003)^[17], SCAR analysis (Yau FCF; 2002)^[22] and the RFLP-PCR approach (Dubey *et al.*, 2010)^[7]. In order to achieve this goal, we created UNI and forward primers that are particular to each snake species: Python morulus (PR), *Ptyas mucosus* (PTR) and *Naja naja* (NR). These primers will generate PCR products of the expected sizes for each of these species. According to Dubey *et al.* (2009)^[8], these species of Indian snakes may now be quickly and definitively identified using the new multiplex PCR method. Before this strategy can be employed in a real-life forensic context, though, a validation

study is required (Budowle B; 2005)^[5]. Thorough validation is necessary to determine the reproducibility and limitations of genetic approaches before they can be completely understood. Based on the standards supplied by the Scientific Working Group on DNA Analysis Methods for human subjects, this methodology was used because no published validation guidelines exist for forensic work involving non-human creatures (<http://www.cstl.nist.gov/div831/strbase/validation/SWGDA> M). The document used for validation. In the validation experiments, the following are examined: heteroplasmy and reproducibility; concentration of DNA templates; presence of other forms of DNA; chemical treatments; substrate variation; environmental conditions; and thermocycling parameters. This inquiry also makes use of this multiplex PCR technique to uncover a specimen that had not been recognized before.

Material and Methods

Species selected for this study

Selected as well-studied commercial species, the Indian Rat Snake (*Ptyas mucosa*), Indian Cobra (*Naja naja*), and Rock Python (*Python molurus*) are each supported by distinct sequence data stored in the GenBank and Barcode of Life Databases (BoLD).

Collection of samples and Extraction of DNA

From the Snake Park in Kolkata, West Bengal, India, obtained the natural specimens of the Indian Rat Snake (*Ptyas mucosa*), Indian Cobra (*Naja naja*), and Indian Rock Python (*Python molurus*). According to the manufacturer's instructions, genomic DNA was isolated from tissue samples with a QIAmpTM tissue DNA extraction kit (Qiagen, GmbH Germany). By the usual Phenol-

Chloroform technique, blood DNA was extracted (J. Sambrook, 1989) [15]. DNA was taken out of formalin-fixed tissue and sloughed skin using the techniques described by Shedlock (A.M. Shedlock, 1997) [1] and Fetzner (J.W. Fetzner, 1999) [9]. DNA samples were purified on a Millipore Corporation, MA, Microcon-100 filter column.

Amplification of PCR

Using 16S rRNA Forward and 16S rRNA Reverse primers, a segment 16S rRNA gene was amplified (Palumbi *et al.* 1991) [18]. 20ng of DNA template 5 mM MgCl₂ 1 mM dNTPs, 0.2 M of each primer, 2.5 µl of 10x buffer, and 1.5U of Taq polymerase (Invitrogen Life Technologies, Brazil) (Applied Biosystems) were used in a 25 µl reaction volume using the GeneAmp® PCR system 9700. 35 cycles of denaturation at 940 C for 30 seconds, annealing at 480 C for 1 minute, and extension at 720 C for 30 seconds—with a final extension at 720 C for 5 minutes and a 40 C hold—followed an initial denaturation at 940 C for 4 minutes. On a 2% agarose gel, the amplified fragments were detected with 0.5 g/ml ethidium bromide dye.

DNA Sequence analysis

Amplicons were sequenced with a 3100 Avant genetic analyzer and a Big Dye® Terminator v 3.1 cycle sequencing kit (Applied Biosystems), utilizing both forward and reverse primers to ensure the integrity of the PCR products. MEGA 3.1 (Kumar S., 2004) [21] aligned the sequences and confirmed the species.

Validation studies

The multiplex PCR was performed on all samples following the method reported by Dubey *et al* (Dubey; 2009) [8]. Unless otherwise specified, each reaction includes 10ng of DNA templates. In all amplifications, both positive & negative controls were included simultaneously. Concerning the following aspects, the method was verified:

- a. **Reproducibility:** The amplification of targeted areas of 16S rRNA gene was evaluated by running PCR in triplicate and comparing the resulting sequences.
- b. **Heteroplasmy:** Specific sequences of the 16S rRNA gene were generated by amplifying DNA extracted from tissue, blood, and shed skin of a single individual and compared.
- c. **Stability:** For 10, 20, and 30 days, the blood samples were kept at room temperature and relative humidity between 80% and 90% on filter paper, cotton fabric, metal, dried leaves, and wood.
- d. **Sensitivity:** To investigate how DNA concentration affected PCR, different concentrations of DNA templates (10ng, 5ng, 1ng, 0.5ng, 0.1ng, and 0.01ng/l) were utilized.
- e. **Chemical treatments:** An investigation was conducted to assess the impact of various chemicals on the recovery of DNA and its subsequent amplification using PCR. Tissue samples weighing 25 mg were treated with several solutions, including 0.1M NaOH, 0.1M HCl, 5% acetic acid, 5M NaCl, and 0.2% SDS. These samples were then stored separately in tubes at controlled temperature for a duration of five days.

f. **Temperature treatment:** Both primer sets amplified DNA isolated from tissue samples at varied temperatures (excluding 700C for five h, where primer set I amplified alone).

g. **PCR conditions:** Amplification did not occur when the concentration of MgCl₂ was below 2.0 mM, whereas values over 6.0 mM resulted in non-specific amplification. b) A rise of 30 degrees Celsius in the annealing temperature did not impact the amplification for either primer set, whereas a reduction of 30 degrees Celsius also had no effect. The alterations in cycle number (2) did not adversely affect the PCR results.

Unidentified Sample Analysis

At the Snake Transit House in Jabalpur, Madhya Pradesh, a badly cut up, road-killed snake specimen sample with no distinct characteristics was collected. Multiplex PCR was done with positive and negative standards after DNA was extracted. The amplicons were looked at in a 2.5% agarose gel that had 0.5 µg/mL of ethidium bromide added to it. Sequencing the amplicons that were made and comparing them to sequences from other Indian Snake species showed that this method works.

Results and discussion

Forensic identification of Indian snake species using novel molecular techniques is essential for the conservation management of these species. Our previous research involved creating a multiplex PCR assay to distinguish between different species of snakes in India. The three species can be distinguished from one another in a straightforward agarose gel electrophoresis without resorting to any additional sequencing technique thanks to the multiplex's ability to generate species-specific amplicons (i.e., 380 bp for *Python molurus*, 265 bp for *Ptyas mucosus*, and 130 bp for *Naja naja*) (Figure 1). There was no doubt about the species identified using this method, but validation studies are required before it can be used in forensic investigations.



Python molurus



Naja naja



Ptyas mucosa

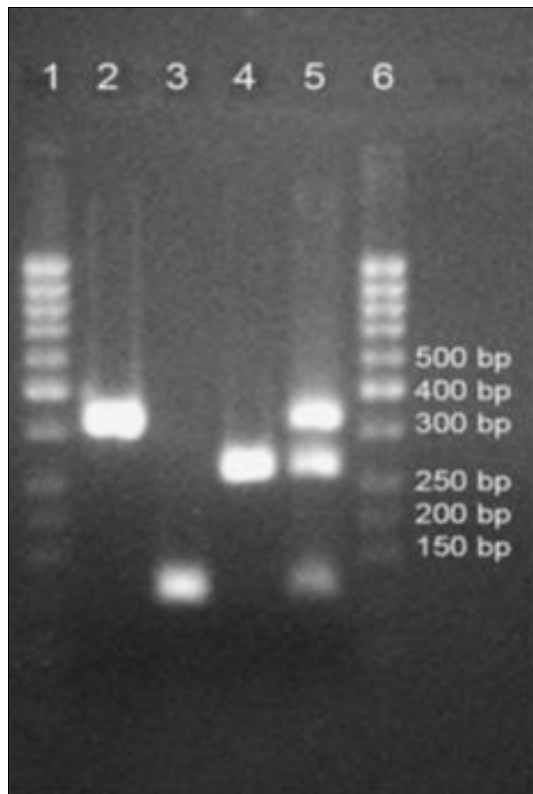


Fig 1: Multiplex PCR amplification using all primers and DNA template from Lane 2, *Python morulus*; Lane 3, *Ptyas mucosus*; Lane 4, *Naja naja*; Lane 5, DNA from all three species. Lane 1 and 6 are molecular size markers.

Multiplex PCR Validation

DNA from every separated sample was used in multiplex validation. Intra-specific changes in the genome may exist in individuals living in different environments; these nucleotide variations in the primer annealing site may distort the results. The primers were evaluated on DNA samples from three distinct snake species found throughout India. Although the reaction did not increase the DNA of any other reptile species utilized in this experiment, it did generate PCR products of the proper size from every DNA sample of three different species of Indian snakes. Experiments conducted on the primers in electronic PCR (e-PCR) using the 16S rRNA gene sequences of several snake species found in GenBank demonstrated that this technology is very specific and reliable for identifying the three Indian snakes in question. Despite the highly degraded state of the

DNA, successful amplification was achieved from decomposed tissue and blood samples of *Python morulus*. The amplification yielded a positive result for these samples (380 bp), similar to the DNA isolated from other *Python morulus* tissue samples. The primers also amplified the expected species-specific fragments at 10 different DNA concentrations (10, 5, 1ng, 100, 50, and 10pg): 130 bp for *Naja naja* and 265 bp for *Ptyas mucosus*. We used filter paper, cotton cloth, wood, and dried leaf to verify this assay's stability. The amplification remained after DNA was diluted (1:2, 1:4, 1:8) in all models except the blood sample dumped on wood. These findings were confirmed by a validation investigation by Branicki *et al.* (Branicki's W; 2003) [2], which revealed that samples placed on wood produced no readings. Reliable results require a sensitive technique since the DNA in the models accessible for forensic examination frequently deteriorates as a result of exposure to different environmental conditions (Butler JM; 2003, Br W; 1998) [3, 4].

The samples were subjected to a variety of temperatures, relative humidity (80%–90%) and chemical agents in the validation research, among other challenges. With the exception of a small number of samples, DNA in the chemically treated and heat-treated samples (samples treated with SDS; samples incubated at room temperature for one day) was substantially damaged. DNA taken from samples treated with 0.1 M HCl, 0.1 M NaOH, and 5% glacial acetic acid fully sheared below 1 kb when 1% agarose gel electrophoresis was run. The target products were effectively amplified by this multiplex PCR assay even in the degraded state of the samples (Figs. 2 and 3). Moreover, to investigate the effects of radiation on the models, direct UV radiation was given to the samples in this validation investigation. Amplification could fail if UV radiation has damaged the DNA template or if its nucleotide sequence has changed (Friedberg EC, 2003; Ravana JL, 2001) [10, 19]. Every UV-exposed sample had its DNA extracted, and this technique amplified amplicons to the predicted size. SWGDAM states that a developmental validation research presenting a PCR-based method should sufficiently show the impact of MgCl₂ and other thermocycling parameters. We thus assessed the influence of these factors and found that, at MgCl₂ concentrations below 3.5 mM, the PCR generated nonspecific amplifications, but not at values above this threshold. When the annealing temperature was dropped below the ideal level (63°C), nonspecific amplification resulted. By contrast, when the temperature was increased by 100°C above this, PCR products weakened and no amplification was seen. Still, various cycle conditions had little effect on the PCR. Following the sequencing of every amplicon, the alignment findings revealed a 95% similarity with the sequences of the relevant species (positive controls included DNA sequences from *Python morulus*, *Ptyas mucosus*, and *Naja naja*). The validation study outlines the advantages of this method to forensic analysis and provides solid proof that the primers work well for amplifying forensic materials.

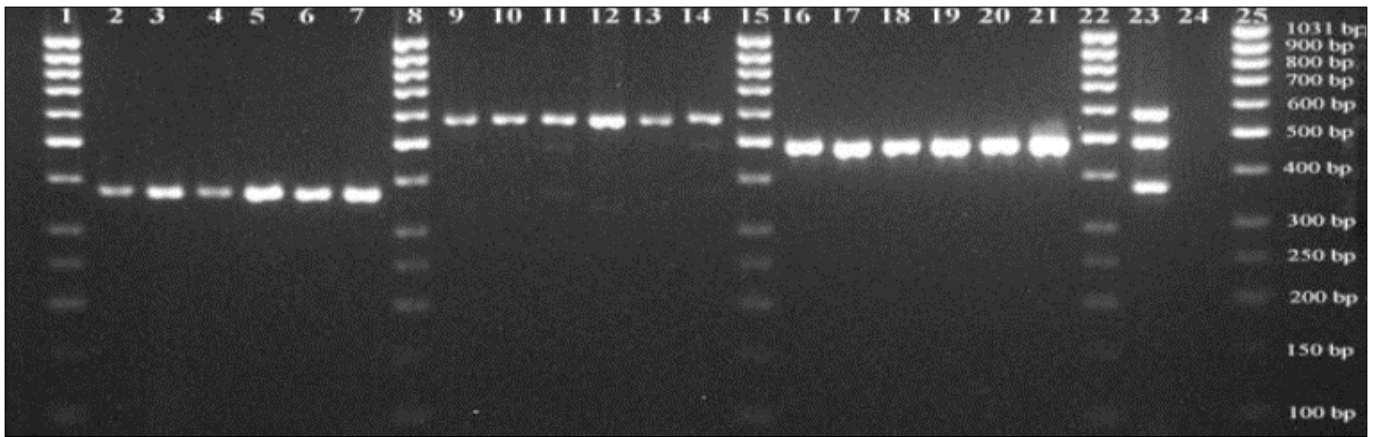


Fig 2: Validation results showing positive amplification for samples treated with various chemicals. Lanes 2, 9 & 16: 0.1 M NaOH; Lanes 3, 10 & 17: 0.1 M HCl; Lanes 4, 11 & 18: 5% Glacial acetic acid; Lanes 5, 12 & 19: 5M NaCl; Lanes 6, 13 & 20: 0.2% SDS; Lanes: 2- 7: *Python molurus*; Lanes 9-14: *Naja naja*; Lanes 16-21: *Ptyas mucosus*; Lanes 7,14, 21 & 23: positive control; Lane 24: negative control; Lanes 1,8,15,22 and 25: molecular size markers.

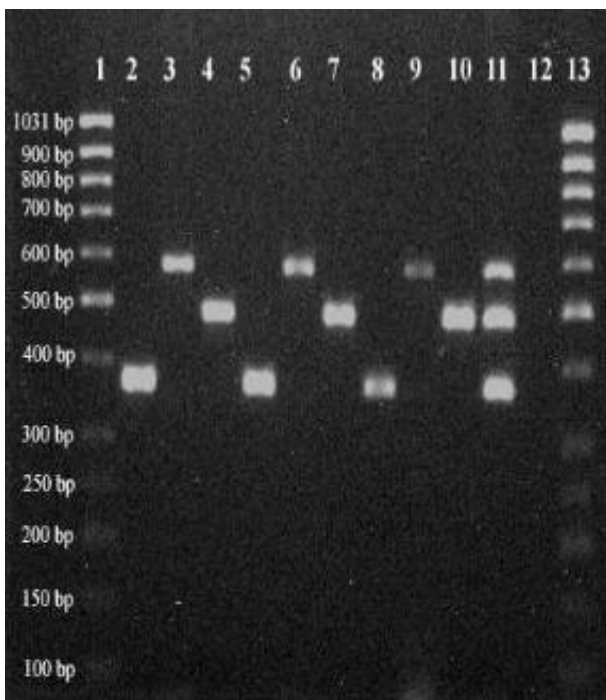


Fig 3: Agarose gel electrophoresis for samples exposed to various temperatures in the validation study. Lanes: 2- 4: ambient temperature for 30 days; Lanes 5- 7: 56°C for 48 hrs; Lanes 8- 10: 70° C for 3 hrs; Lanes 2, 5 & 8: *Python molurus*; Lanes 3, 6 & 9: *Naja naja*; 4, 7 & 10: *Ptyas mucosus*; Lane 11: positive control; Lane 12: negative control; Lanes: 1 & 13: molecular size markers.

Identification of Unknown Sample

Any technique worth its salt should be able to validate itself while also revealing the true identity of the species at hand. Therefore, DNA isolation from a mystery sample and positive and negative controls underwent multiplex PCR. A 380-bp product was obtained from the amplification, which corresponds to the expected location of a gene fragment derived from *Python morulus* (Fig. 4). After sequencing the amplicon, we found that it was identical to the 16S rRNA sequence of *P. morulus*, providing further confirmation. Since this multiplex PCR assay confirmed the species to be *Python morulus*, the process was deemed successful.

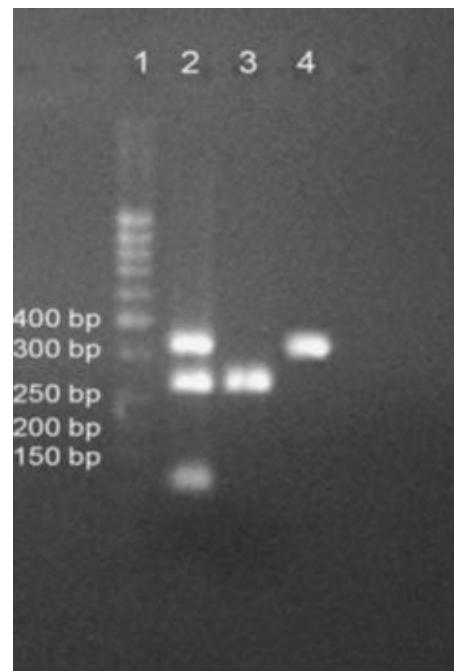


Fig 4: Multiplex PCR assay applied in identifying Lane 3, Unknown road-killed road-killed snake specimen; Lane 4, Shed skin sample of *Python morulus*; Lane 2, Multiplex PCR amplicon standards; Lane 1, Molecular size marker.

Conclusion

We propose using Legal determination of Indian Snake varieties using multiplex PCR to ensure the correct application of laws, which will help to protect these threatened animals. The ultiplex PCR technique was validated in accordance with SWGDAM guidelines for identifying Indian Snake species and was found to be helpful in forensic analyses. This process effectively enhances the result from samples subjected to various circumstances and produces the appropriate sized fragments, even from severely deteriorated tissue and blood samples, using a small amount of DNA template. In addition, the innovative technique quickly recognized the identity of an unidentified hidden samples as *Python morulus* using basic agarose gel electrophoresis.

Acknowledgments

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