IDENTIFICATION OF BIG CAT SPECIES BY DNA BASED PCR-RAPD TECHNIQUE

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Characterization of genetic variation at the DNA level has generated significant advances in species identification of various animals for forensic purpose. Random Amplified Polymorphic DNA (RAPD) is the simplest molecular method effectively used to develop species specific DNA fingerprinting patterns. PCR-RAPD technique was adapted in the present study to produce species specific fingerprinting pattern in the three wild big cats namely tiger, lion and leopard. Out of the ten random primers used, three primers viz., M13, D1 and U3 produced polymorphic banding patterns. Where, primer U3 was effectively used to differentiate tiger and lion tissue samples, primer M13 differentiated tiger and leopard species and primer D1 was ideal for comparing all the three species of big cats. Overall, the simplicity of RAPD-PCR patterns could make this technique suitable for forensic identification of various tissue samples among big cats.

Key word: Polymerase Chain reaction, Species identification, DNA fingerprinting, big cats.

Wild cats, viz., Asiatic lion (Panthera leo persica), Indian or Bengal tiger (Panthera tigris tigris) and leopard (Panthera pardus) are some of the important wild animals which have become endangered and facing the risk of extinction in India (Parij et al., 2001). These wild cat populations, particularly those outside protected reserves, are fragmented, suffer from intense poaching pressure, dwindling prey base and over used habitat. A major problem in the battle against wild animals poaching is identification of seized parts and products of poached animals, after species-specific characteristics such as antlers, claws, skin or hide have been removed. Earlier, identification of wildcat’s tissues involves use of traditional methods based on morphology, anatomy and also by using electrophoretic and immunological techniques. But these methods are accurate only up to the family levels, and hence are not specific up to species level. As a result, allegations of wild animals poaching cannot be often proven because vital evidence linking killed animals and poachers are inadequate. So there is a need to develop an effective method to identify the seized parts of different wild animals. In the last two decades, several DNA based technique have been developed to characterize the living creatures. Random Amplified Polymorphic DNA (RAPD) is one such technique which is based on amplification of genomic DNA by Polymerase Chain reaction (PCR) using random sequence oligonucleotide primers (Williams et al., 1990). The main advantage of this technique is that, it can identify and
type a large number of genetic markers quickly and is cost effective.

In the present work, PCR fingerprinting technique based on the use of arbitrary primers (RAPD-PCR) have been developed and compared for their ability to generate “fingerprint” patterns characteristics of three different big wild cat species. Their potential application as reliable and simple methods for determination of the different species is discussed.

MATERIALS AND METHODS

Sample collection
A total of 50 big cats, comprising of 20 tigers, 25 lions and 5 leopards housed in Bennerghatta National park, Bangalore, were sampled in this study. Blood samples were collected from tail or femoral vein by immobilizing the animal in squeeze cages. In addition to blood samples, some confiscated skin samples sent for forensic identification to Institute of Animal Health and Veterinary Biologicals, Bangalore are also used for the present study.

Isolation of DNA
High molecular weight genomic DNA was extracted from blood and skin samples by using procedure described by Miller et al. (1988) and Huges and Gorosope, (1991), respectively with some modifications. The quality and quantity of DNA were determined by spectrophotometer and agarose gel electrophoresis and the total DNA was dissolved in 250-500µl of Tris EDTA. A ratio between 1.70 and 1.90 at 260 and 280 nm in a spectrophotometer was considered as pure DNA sample. If the purity ratio was below 1.70, then the DNA samples were further purified by Phenol: Chloroform method.

RAPD primers
A total of ten arbitrary oligonucleotide primers were designed for amplification of genomic DNA which was obtained from Microsynth laboratory, Switzerland. The sequence and length of the primers used are presented in Table 1. The selected primers were reconstituted in low Tris EDTA buffer with a concentration of 100 P.mol/µl as stock solution and 20 P.mol/µl as working solution.

PCR amplification
The amplification reaction was carried out in programmable thermal cycler (Ependroff). Each 20 µl reaction mix comprised of 100 ng of template DNA, one µl of primer (20 p.mol/µl), 100mM of dNTP mix, 0.33 unit of Taq DNA polymerase, 25mM Magnesium chloride and 10x PCR buffer (Bangalore Genei). A PCR programme with an initial denaturation 94°C for two minutes, second denaturation for 94°C for one minute, annealing at 35°C for one minute, and extension duration of one minute at 72°C was done for 35 cycles. One final extension at 72°C for ten minutes was included in the programme. The PCR products were electrophoresed at 50V in 2% agarose gel along with a 100bp DNA molecular size marker. RAPD fingerprints were visualized and documented using Gel documentation system.

Statistical analysis
sharing was calculated as an expression of similarity of RAPD bands of animals from either the same or different species or breed (Gwakisa et al., 1994, Kantanen, wt al., 1995, Smith et al., 1996) using the following formula.

\[ BS = \frac{2 \times (B_a B_b)}{(B_a + B_b)} \]

Where, Bab – The number of bands shared by individuals a and b,

Ba – The total number of bands for individuals a and,

Bb – The total number of bands for individual b.

Mean average difference (MAPD): was calculated as an expression of inter species dissimilarities. This value was calculated on
Table 1: Primer Specific RAPD Patterns in Different Wildcats.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence 5’- 3’</th>
<th>Size range of amplified bands(bp)</th>
<th>Number of species-specific RAPD fragments characteristic for;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tigers</td>
</tr>
<tr>
<td>U3</td>
<td>GCATGCATGT</td>
<td>350-800bp</td>
<td>2</td>
</tr>
<tr>
<td>M13</td>
<td>GTTGTAAAACGACGGCCCA</td>
<td>290-670bp</td>
<td>3</td>
</tr>
<tr>
<td>D1</td>
<td>CAGCTATGACCAG</td>
<td>200-800bp</td>
<td>4</td>
</tr>
</tbody>
</table>

RAPD bands obtained with the primers using the following three formulae (Gilbert et al., 1990).

1. Percentage difference (PD) = \( \frac{N_{ab}}{(N_a + N_b)} \times 100 \)
2. Average percentage difference (APD) = \( \frac{1}{C \sum P_{Di}} \)
3. Mean average percentage difference (MAPD) = \( \frac{1}{R \sum R_{i}} \sum APD \)

Where

\( N_{ab} \) - The number of fragments that differed between two individuals for a single primer
\( N_a \) - The number of fragments resolved in individual a
\( N_b \) - The number of fragments resolved in individual b
\( C \) - The number of interbreed pair-wise comparisons and
\( R \) - The number of random primers used.

RESULTS

About 350-400 µg of pure genomic DNA could be isolated from 10-15 ml blood from each animal without using phenol. Purity of most of the DNA sample extracted was good as indicated by the OD 260/280 ratio which ranged between 1.7 to 1.9.

Out of ten random primers, only six could amplify genomic DNA and of these, only three primers (U3, M13, and D1) produced low to high polymorphic finger prints in DNA samples of different wildcats, which were then tried to explore the possibility of using them to differentiate the species of wildcats. Each of these primers produced different banding patterns.

Individual RAPD primers were separately used for studying variation in DNA fingerprint patterns. Species wise DNA samples were studied to compare the variation between the species. On finding species-specific band/s, individual samples constituting each species were separately amplified with the same primers to examine if the species-specific bands are present in all the individuals constituting that particular species.

![Figure II: Electrophoretic band pattern of the amplified product of wild big cats DNA from blood sample with random primers U3, M13 and D1.](image-url)
These three random primers generated a total of 19 scorable bands in tigers, 24 bands in lion and 16 in leopard, ranging in size from approximately 200 to 1,100 base pairs (bp). All the three random primers yielded amplified fragments that were consistently polymorphic and specific between the three species. Primer U3 produced tiger specific fragments of sizes 745bp and 440bp. In case of lions a 296bp fragment was uniquely observed. However, no leopard specific band could be identified by this primer. Hence primer U3 can be effectively used to differentiate tiger and lion tissue samples. The primer M13 produced high polymorphic bands in all the three wildcats. It produced two bands of size 221bp and 785bp in case of tigers. Lower band of 290bp and 670bp size were specifically amplified in case of leopards. This primer can be effectively employed to differentiate the tiger and leopard species. Primer D1 produced more polymorphic bands than the other primers. It amplified specific fragments of size 464, 572, 707 and 806bp in all the individual DNA samples of tigers. In lions, it produced specific fragments of sizes 557 and 688bp size. It also produced fragments of sizes 451, 557, 670 and 785bp specifically in case of leopards. This primer seems to be ideal for comparing all the three species of wildcats viz., tiger, lion and leopard. The same set of primers also used for DNA samples which are extracted from various confiscated skin samples. Among ten suspected skin samples studied two were found to be of tiger, and one from leopard.

**Band sharing analysis**

The average band sharing between species of wild cats for primers U3, M13 and D1 were 0.52, 0.62 and 0.62, respectively, while, the overall band sharing ranged from 0.4 (primer U3) to 0.714 (primer M13 ). The average band sharing within the wildcats for primers U3, M13 and D1 were 0.94, 0.96 and 0.95, respectively. Dissimilarity between RAPD patterns of different wildcat species was expressed in terms of Average Percentage Difference (APD) for each primer and Mean Average Percentage Difference (MAPD) for all the primers combined together. The MAPD between tiger and lion, lion and leopard and tiger and leopard were 23.16±1.03, 20±2.356 and 25.42±3.10, respectively. There was a significant difference between the MAPD values between the species of wildcats.

**DISCUSSION**

Most of the forensic cases in India involving big cats are either of tigers or leopards. The Wildlife Protection Society of India (WPSI) has documented the killing of 61 tigers and 188 leopards, since January 2002. The actual number is estimated to be at least seven times higher, since a majority of wildlife crimes go unreported (9). This RAPD-PCR method can provide material evidence to wildlife enforcement for judicial organizations. It is well established that RAPD technique can be successfully used to identifying species specific DNA fingerprint patterns in various animals (Singh et al., 2004, Bardakeri et al., 1994 and Rossetto et al., 1995). The present study aimed at developing a technique to identify different wildcats namely tiger, lion and leopards. Primers used in the present study were designed and utilized for the first time. Hence there are no earlier reports for comparison. However, these primers are found to be quite useful in differentiating the wild big cat species. Primer U3 produced tiger specific fragments of sizes 745bp and 440bp. In case of lions, a 296bp fragment was uniquely observed. However, no leopard specific band could be identified by this primer. Hence primer U3 can be effectively used to differentiate tiger and lion tissue samples. The primer M13 produced high polymorphic bands in all the three wildcats. It produced two bands of size 221bp and 785bp in case of tigers. Lower band of 290bp and 670bp size were specifically amplified in case of leopards. This primer can be effectively employed to differentiate the tiger and leopard species. Primer D1 produced more polymorphic bands than the other primers. It amplified
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Based on pair wise similarity index (band sharing), the magnitude of the intraspecies RAPD variation in different wildcat species was found to be lower compared to interspecies variation. Both within and between species, primer D1 showed highest polymorphism, whereas primer U3 produces least variation.

CONCLUSION
The potential use of the RAPD-PCR technique, by using primer U3, M13 and D1, to differentiate three main wild big cats has been effectively demonstrated. RAPD-PCR profile generated by the selected primers resulted in relatively simple DNA fingerprints from which the species of wild big cats can visually inferred, making this technique especially suitable for routine identification of tissue samples from different big cats for forensic purpose.

REFERENCES