Mitochondrial 12S rRNA sequence analysis in wildlife forensics

S. Prakash*, M. S. Patole*, S. V. Ghumatkar†, S. K. Nandode†, B. M. Shinde# and Yogesh S. Shouche*,‡

*National Centre for Cell Science, Pune University Campus, Ganeshkhind, Pune 411 007, India
†Regional Forensic Science Laboratory, Ganeshkhind, Pune 411 007, India
#Forensic Science Laboratory Directorate, Mumbai 400 098, India

Wildlife forensics has not received as much technological attention as human forensics. In this paper we report successful application of mitochondrial 12S rRNA sequence analysis for this purpose. A dried and processed skin suspected to be from tiger was brought to the laboratory for analysis. After DNA extraction, a fragment of mitochondrial 12S rRNA gene was amplified using universal primers. Sequencing of this fragment unambiguously proved that the skin was not from a tiger but was bovine. The method has potential to be used for the diverse samples that are tested in forensic laboratories.

POACHING of animals is a criminal act. Wildlife forensic science has not received technological attention compared to human forensic science, thus few tools are available for species identification and authentication of various biological materials received at forensic laboratories[1]. In this article, we report the successful application of mitochondrial 12S rRNA sequence analysis in an unambiguous species identification from a small amount of dried and processed skin.

Most common methods use immunological probes but the utility of these is restricted by the cross species reactivity of the antisera. There have been few attempts to develop DNA-based technologies for this purpose[1]. Direct sequencing of hypervariable region HV1 has been used for the pedigree analysis of dogs[2] and a 79 bp PCR amplified fragment from the mitochondrial control region was used in the case of suspected wolf poaching[3]. Analysis of 12S mitochondrial rRNA gene has been extensively used in molecular phylogeny[4]. Recently, mitochondrial D loop and cytochrome *b* sequences have been used to assess hybridization and introgresssion of African lion genes in Asiatic lions and the subspecies characterization of Indian and Siberian tigers[5]. In this study, we have used a sequence of mitochondrial 12S rRNA fragment amplified using universal primers[6] for the authentication of a skin sample suspected to be that of a tiger.

The sample from a suspected poached tiger was received at the Pune laboratory. It was in the form of an alkali treated, salt solution boiled, dried skin. Studies based on hair morphology, species specific antisera and total protein pattern were unable to give definitive identification of the source of the skin. Chemical analysis revealed the presence of sulpha dyes indicating that it was painted to look like tiger skin. We attempted to identify its species origin by an analysis of the mitochondrial 12S rRNA sequence.

A 2 mm piece was taken and the DNA was extracted by the procedure used earlier for mosquito larvae[7]. A 450 bp fragment of mitochondrial 12S rRNA was amplified using universal primers (Figure 1). This PCR product was cloned in pGEMT Easy vector (Promega). Complete sequencing of the clone was done with M13 universal forward and reverse primers. The homology search of the sequence with available mitochondrial small subunit rRNA sequences at RDPII database[8] gave complete homology with *Bos taurus* mitochondrial 12S rRNA gene (GeneBank Accession No. V00654). Corresponding sequences from *Panthera leo* (Accession No. Y08505) and *Panthera tigris* (Accession No. 08504) were retrieved and analysed using Clusatl W software (Figure 2). The sequence was very different from any of the two tiger species; there were 39 nucleotide differ-

1 2

Figure 1. PCR amplification of mitochondrial 12S rRNA gene fragment from the skin sample. The universal primers were 12SUniL 1091 – AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT and 12SUniH 1478 – TGACTGCAGAGGGTGACGGGCGGTGTGT. Lane 1, PCR product; Lane 2, molecular weight marker, 100 bp ladder (MBI Fermentas).

‡For correspondence. (e-mail: yogesh@nccs.res.in)

```
B.tau: CAAACTGGGATTAGATACCCCACTATGCTTAGCCCTAAACACAGATAATTACAT
Samp : CAAACTGGGATTAGATACCCCACTATGCTTAGCCCTAAACACAGATAATTACAT
P.tig: ----------------------------------------------------
P.leo: ----------------------------------------------------

B.tau: AAACAAAATTATTCGCCAGAGTACTACTAGCAACAGCTTAAAACTCAAAGGACT
Samp : AAACAAAATTATTCGCCAGAGTACTACTAGCAACAGCTTAAAACTCAAAGGACT
P.tig: -------ACTATCCGCCAGAGAACTACTAGCAACAGCTTAAAACTCAAAGGACT
P.leo: ----AAAACTATCCGCCAGAGAACTACTAGCAACAGCTTAAAACTCAAAGGACT

B.tau: TGGCGGTGCTTTATATCCTTCTAGAGGAGCCTGTTCTATAATCGATAAACCCCG
Samp : TGGCGGTGCTTTATATCCTTCTAGAGGAGCCTGTTCTATAATCGATAAACCCCG
P.tig: TGGCGGTGCTTTATATCCCTCTAGAGGAGCCTGTTCCATAATCGATAAACCCCG
P.leo: TGGCGGTGCTTTACATCCCTCTAGAGGAGCCTGTTCTATAATCGATAAACCCCG

B.tau: ATAAACCTCACCAATTCTTGCTAATACAGTCTATATACCGCCATCTTCAGCAAA
Samp : ATAAACCTCACCAATTCTTGCTAATACAGTCTATATACCGCCATCTTCAGCAAA
P.tig: ATAAACCTCACCATCTCTTGCTAATTCAGCCTATATACCGCCATCTTCAGCAAA
P.leo: ATAAACCTCACCATCTCTTGCTAATTCAGCCTATATACCGCCATCTTCAGCAAA

B.tau: CCCTAAAAAGGAAAAAAAGTAAGCGTAATTATGATA-CATAAAAACGTTAGGTC
Samp : CCCTAAAAAGGAAAAAAAGTAAGCGTAATTATGATA-CATAAAAACGTTAGGTC
P.tig: CCCTAAAAAGGAAGAAAAGTAAGCACAAGTATCTTAACATAAAAAAGTTAGGTC
P.leo: CCCTAAAAAGGAAGAAAAGTAAGCACAAGTGTCTTAACACAAAAAAGTTAGGTC

B.tau: AAGGTGTAACCTATGAAATGGG-AAGAAATGGGCTACATTCTCTACACCAAGAG
Samp : AAGGTGTAACCTATGAAATGGG-AAGAAATGGGCTACATTCTCTACACTAAGAG
P.tig: AAGGTGTAGCCCATGAGATGGGGAAGTAATGGGCTACATTTTCTACAACTAGAA
P.leo: AAGGTGTAGCCTATGAGATGGG-AAGCAATGGGCTACATTTTCTACAATTAGAA

B.tau: AATCAAGCACGAAAGTTATTATGAAACCAATAACCAAAGGAGGATTTAGCAGTA
Samp : AATCAAGCACGAAAGTTATTATGAAACCAATAACCAAAGGAGGATTTAGCAGTA
P.tig: CATC---CACGAAAATCCTTATGAAATTAAGTATTAAAGGAGGATTTAGTAGTA
P.leo: CACC---CACGAAAATCCTTATGAAACTAAGCATTCAAGGAGGATTTAGCAGTA

B.tau: AACTA-AGAATAGAGTGCTTAGTTGAATTAGGCCATGAAGCACGCACACACCGC
Samp : AACTA-AGAATAGAGTGCTTAGTTGAATTAGGCCATGAAGCACGCACACACCGC
P.tig: AATTCGAGAATAGAGAGCTCGATTGAATCGGGCCATGAAGCACGCACACACCGC
P.leo: AATTTGAGAATAGAGAGCTCAATTGAATCGGGCCATGAAGCACGCACACACCGC

B.tau: CCGTCACCCT
Samp : CCGTCACCCT
P.tig: CCGTCACCCT
P.leo: CCGTCACCCT
```

Figure 2. Aligned mitochondrial 12S rRNA sequences from skin sample (samp), *Bos taurus* (B. tau), *Panthera leo* (P. leo) and *Panthera tigris* (P. tig). Positions from the sample sequence that are different from *Panther* sequence are shown in bold; those that are different within two *Panther* species are shown in italics. The primer sequences have been underlined.

ences and 6 alignment gaps. This gave unequivocal evidence that the skin was not from a tiger but was imitation derived from cattle skin. The presence of sulpha dyes supported this observation.

This is the first report of the use of mitochondrial 12S rRNA sequence for unequivoval conformation in wildlife forensics. The primers used in this study are universal and are able to amplify the corresponding region from a wide variety of organisms, including birds and insects. This region gives phylogenetically valuable information[9]. Availability of mitochondrial rRNA sequences in databases enables instant sequence comparisons[10]. The successful use of very little amount of dried, processed skin suggests that preserved, fixed, partially decomposed samples can also be used without difficulty. Although the PCR product was cloned here

before sequencing, identical results were obtained when it was sequenced directly. This method has great potential to give conclusive results for the diverse samples being tested in forensic laboratories.

1. Blackett, R. S. and Kleim, P., *J. Forensic Sci.*, 1992, **37**, 590–596.
2. Savolainen, P., Rosen, B., Holberg, A., Leiter, T., Uhlen, M. and Lundberg, J., *J. Forensci Sci.*, 1999, **42**, 593–600.
3. Savolainen, P. and Lundberg, J., *J. Forensic Sci.*, 1999, **44**, 77–81.
4. Ledje, C. and Arnason, U., *J. Mol. Evol.*, 1996, **43**, 641–649.
5. Shankarnarayanan, P. and Singh, L., *Curr. Sci.*, 1998, **75**, 919–923.
6. Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablanca, F. X. and Wilson, A. C., *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 6196–6200.
7. Kshirsagar, S. G., Patole, M. S. and Shouche, Y., *Anal. Biochem.*, 1997, **253**, 65–69.
8. Maidak, B. L., Cole, J. R., Parker, Jr. C. T., Garrity, G. M., Larsen, N., Bing Li, Lilburn, T. G., McCaughey, M. J., Olsen, G. J., Overbeek, R., Pramanik, S., Schmidt, T. M., Tiedje, J. M. and Woese, C. R., *Nucleic Acids Res.*, 1999, **27**, 171–173.
9. Masuda, R., Lopez, J. V., Slattery, J. P., Yukki, N. and O'Brien, S. J., *Mol. Phylogenet. Evol.*, 1996, **6**, 351–365.
10. Van de Peer, Y., Robbrecht, E., de Hoog, S., Caers, A., De Rijk, P. and De Wachter, R., *Nucleic Acids Res.*, 1999, **27**, 179–183.

ACKNOWLEDGEMENTS. We thank Dr Padma Shastry for a critical reading of the manuscript and Prof. S. K. Brahmachari, Director, CBT, for permission to use the sequencing facility.

Received 11 January 2000; revised accepted 25 February 2000

Fusarium – A new threat to fish population in reservoirs of Kumaun, India

Deepa Bisht*, G. S. Bisht,† and R. D. Khulbe**

Botany Department, Kumaun University, Nainital 263 002, India
*Present address: GBPIHED, Kosi-Katarmal, Almora 263 643, India
**Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora 263 601, India

***Fusarium moniliforme* and *F. udum* (Moniliales: Deuteromycetes) were found to be natural pathogens of freshwater fish in reservoirs, causing mycosis and high mortality in *Barbus rana*, *Channa punctatus*, *Labeo rohita*, *Mastaceamblus armatus*, *Mystus tengra*, *Puntius sophore* and *Wallago attu*. Both the species produced clinical symptoms similar to natural infection in *C. punctatus* and *P. sophore* and caused 40–80% mortality under artificial inoculation. Though 9 other species of extra aquatic fungi belonging to 8 genera of hyphomycetes were also associated with the diseased fish in the reservoir, they were unable to infect the test fish. *Fusarium* species parasitize fish more commonly during summer through rainy season. A temperature above 25°C, coupled with relatively low pH (7.1–7.7) and DO (8.3–9.5 mg l^{-1}) encouraged association and infection of these fungi, whereas low temperature during winter (< 20°C) adversely affected their colonization on the fish. Notably mycosis due to watermolds is prevalent during winter–spring, while extra-aquatic fungi dominate during summer through the rainy season, thus posing a continual threat to fish in the reservoirs. This necessitates an integrated approach to combat mycosis in reservoirs. Besides, prophylatic measures to protect fingerlings before being introduced into the reservoirs, intensive research on biological control of fish diseases caused by *Fusarium* and other fungal species is warranted, not only to increase production in reservoirs but also to conserve several rare fish species.**

RESERVOIR fishery is an important component of inland fisheries in south and south-east Asia. Indian reservoirs exceeding 3 million ha in area are recognized as an invaluable resource for inland fish production[1]. Owing to the existence of both lentic and lotic components, reservoirs not only harbour a wide diversity of fish but also diverse micro-organisms, including fungi pathogenic to fish[2]. Watermolds are a constant and ubiquitous component of aquatic environments and a continuous source of challenge to fish[3,5]. However, geofungi, the extra-aquatic fungi, constitute an integral component of mycoflora in the freshwater ecosystem.

A high prevalence of mycosis in fish species, characterized by descaling, dermal necroses and haemorrhage, which resulted in mass mortality was observed during 1991 in Nanaksagar, a huge man-made reservoir and a well-recognized fish production centre of the Govt. of UP in India. The grievous situation has been implicated due to the infection of watermolds; several species of *Achlya*, *Aphanomyces* and *Saprolegnia* were detected from the symptomatic fish during 1991–1994 and their pathogenicity has been demonstrated[6–8]. Nevertheless, in spite of rigorous isolation, associated pathogens could not be detected from a large number of fish exhibiting mycotic lesions, particularly during the warm period. Since the studies were concentrated on watermolds pathogenic to fish, specific baiting techniques were employed for the isolation of associated fungi and involvement of other fungi was overlooked. We presumed that extra-aquatic fungi (hyphomycetes), which are known to cause diseases in marine as well as freshwater fish in different parts of the world[9–14], are also involved. Consequently, the involvement of these fungi in fish mycosis was investigated during 1994–1996 at Nanaksagar.

A large number of individuals of major contributing fish species was randomly inspected at the fish catching site at Nanaksagar (29°55′N and 79°40′E; 200 m amsl), an artificial reservoir spread over 4662 ha in Kumaun division, India, which harbours a wide variety of fish fauna[15]. The reservoir was built in 1962 primarily to facilitate irrigation in the nearly 40,000 ha downstream areas, however, it has also made a substantial contribution to the state economy through fish production. Im-

†For correspondence.