PCR amplification and sequencing of mitochondrial 12S rRNA gene fragment from *Muntiacus muntjak* (Indian muntjac)

Indian muntjac (*Muntiacus muntjak*) is the smallest deer of the Indian subcontinent, popularly known as barking deer. The animal is listed as endangered species in the Wildlife Protection Act[1]. Wildlife forensics has not received the technological attention that human forensics has, and there is a need to develop newer, faster and reliable tools for the identification and authentication of diverse biological samples received at the laboratories. Indian muntjac has the lowest number of chromosomes in mammals ($2n = 6$ in female and 7 in male) compared to Chinese muntjac (*Muntiacus reevesi*) that has 46 chromosomes. Cell line derived from *M. muntjak* has been used extensively in the studies of chromatin structure and cytogenetics[2–4]. Complete characterization of cell lines is essential for cell banks and for the regulatory requirements in biopharmaceutical production. Cell line characterization procedures classically include analysis of distinguishing markers of the cells. We have previously shown the utility of mitochondrial 12S rRNA gene sequences in wildlife forensics[5] and also in the authentication of cell lines[6,7]. In this paper, we report the cloning and sequencing of mitochondrial 12S rRNA fragment from the fibroblast cell line derived from *M. muntjak*.

Indian muntjak cell line, CCL 157 (ref. 8) was grown in Ham's F-10 medium supplemented with 20% foetal calf serum. DNA extraction and PCR was carried out as described earlier[5]. Briefly, mitochondrial 12S rRNA gene fragment was amplified from total DNA using universal primers[9]. This gave a fragment of 450 bp which was then cloned in pGEM T Easy vector (Promega). After screening the recombinant clones, the one with desired insert size was selected. Plasmid DNA was prepared[10] and sequenced with M13/pUC universal forward and reverse primers on automated DNA sequencer. This gave complete, unambiguous sequence of the insert DNA with flanking vector sequences at either end. The homology search of the sequence was done using BLAST at NCBI site and also Similarity Matrix at RDP II site[11]. Both the searches showed homology to the mitochondrial 12S rRNA sequence of *M. reevesi*[12], GenBank accession no. M35877. Similarity matrix generated at RDP II site showed maximum homology with *M. reevesi* (0.967) followed by *Cervus unicolor* (sambar, 0.957), *Cervus elaphus* (American elk, 0.954) and *Hydropotes inermis* (Chinese water deer, 0.942). Since this was the only other sequence available from genus *Muntiacus* at GenBank, our sequence was aligned with it using CLUSTAL W. Out of the 20 differences, two are addition/deletion, one transversion and the remaining 17 transitions. Mitochondrial gene sequences, especially those of rRNA genes have been proved valuable to trace the evolutionary lineage[13]. Figure 1 shows the phylogenetic tree for the sequences. It would have been ideal to compare this tree with the earlier one generated using mitochondrial DNA RFLPs[14], but sequences from other *Muntiacus* species are not available in the database and we do not have access to those species. The *M. muntjak* sequence has been deposited at GenBank with accession no. AF294731.

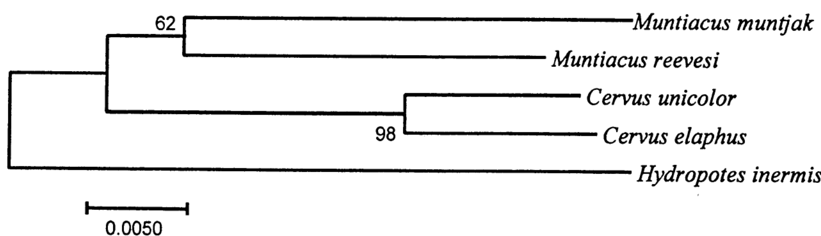

Figure 1. Phylogenetic tree generated using neighbour joining method in MEGA[15]. Kimura 2 parameter distances were used, values at nodes indicate boot strap values after 500 replicates. Scale represents distance.

This is the first report of the mitochondrial rRNA sequence from this organism. Availability of this sequence will be valuable for two important applications. Firstly, the organism being listed as endangered species by Government of India, the sequence will be useful in identification of samples in forensic applications. We have earlier showed the utility of mitochondrial 12S rRNA sequences in wildlife forensics[5]. PCR can be performed on dried, processed, partially degraded materials and availability of sequences in the database facilitates instant sequence comparisons.

Secondly, this information will also be valuable for the authentication of cell line. This cell line is widely used in many cytogenetic analyses. Currently there is no method available for the authentication. Mislabelling and cross-contamination are common problems in cell cultures. HDA and sequence analyses of mitochondrial 12S and 16S rRNA have been developed as a powerful tool for the detection of these[6,7]. The final confirmation for authentication would involve comparison of the sequence with known ones and thus availability of this sequence in the database is valuable for the purpose of authentication of this cell line. Since the technique is based on PCR amplification of the desired sequence, small numbers of cells are required for the analysis. In our laboratory, we have been able to amplify the DNA directly from frozen ampoules of cells without the need to grow them.

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Status of chloroquine efficacy against *Plasmodium falciparum* in pregnant women in a tribal area of central India

The World Health Organization (WHO) recommended that all pregnant women living in malaria-endemic areas should be given initial antimalarial treatment followed by chemoprophylaxis throughout pregnancy, in an effort to prevent the adverse effects of *Plasmodium falciparum*[1]. In India, chloroquine (CQ) is the drug of choice, though the susceptibility status of *P. falciparum* to CQ in pregnant women is not known. A longitudinal study carried out earlier in Mandla district, Madhya Pradesh (MP) revealed malaria as a major health problem in this area and 21% pregnant women were positive for malaria; 36% were *P. vivax* and 64% were *P. falciparum*. There were no instances of cerebral malaria or death, however anaemia was commonly prevalent in 80% of the women[2]. Hospital-based studies showed that pregnant women with falciparum or vivax malaria were significantly more anaemic than non-infected pregnant women or infected non-pregnant women[3]. CQ failed to cure 8% pregnant women with uncomplicated *P. falciparum* infections[2]. To evaluate the efficacy of CQ (25 mg/kg body weight) in the treatment of *P. falciparum* in pregnant women, this study was initiated in a malaria meso-endemic area of central India.

The study was conducted from October to December 1996 in 10 villages (90% Gond ethnic tribe) in Mandla district. The villages are scattered, thinly populated and without any communication. Villagers are mostly illiterate, poor and work mainly in forest nurseries. In this community deliveries occur at home with the assistance of family members or by traditional birth attendants. Malaria transmission is almost perennial. *P. vivax* is the dominant species from February to June and *P. falciparum* is mainly prevalent from July to January.

Pregnant women (4–7 months) with fever, with a history of fever, headache and joint pains were screened for malaria parasite. Information was obtained from each patient about age, parity and antimalarial drug use. Thick and thin blood films were made from finger pricks and stained with Giemsa. The study protocol was approved by the Institutional Review Committee, Malaria Research Centre (Indian Council of Medical Research, New Delhi). Only pregnant women with asexual forms of *P. falciparum* and with no history of antimalarial drug intake in the previous seven days and whose urine samples were negative for CQ by Dill and Glazko method[4] were eligible for this study after obtaining verbal consent. Women with signs of severity were excluded[5]. The patients were given CQ (25 mg/kg body weight) under supervision in 3 divided doses. *In vivo* test was carried out according to Rieckmann's simplified method[6]. The procedure for classifying the response to treatment is presented in Figure 1. The study group (21) was given insecticide-treated bed-nets with the advise to sleep under these bed-nets.

Information was obtained about fever or vomiting after the drug was taken and about the use of bed-nets and their urine samples were tested again for CQ on day 2. Treatment failure was defined by the presence of asexual forms of *P. falciparum* on day 2 or later in the 4 weeks of follow-up. Early failures were treated with Fansidar (1500 mg sulphadoxine and 75 mg pyrimethamine, SP) and followed weekly for 4 weeks. Patients who became parasitaemic at day 14 or later were given CQ again and also followed for 4 weeks. Patients with asexual parasitaemia after second dose of CQ were treated with Fansidar.

Haemoglobin (Hb) was estimated in field by the haematic acid method, originally described by Sahli[7], before treatment and subsequently after treatment during follow-up on day 7 or 14.

Out of 100 pregnant women screened, 28 were found positive with *P. falciparum* and 2 with mixed infections of *P. vivax* and *P. falciparum*. Twenty-one women were eligible for the study (mean age 26.5 ± 3.5 years; mean weight 47.5