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Development of a DNA marker by minisatellite associated sequence amplification (MASA) from the endangered Indian rhino (*Rhinoceros unicornis*)

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Abstract

Rhinoceroses are highly endangered species and their protection warrants immediate remedial measures. Development of DNA markers is envisaged to complement global efforts of the conservation of these extant animals. Minisatellite associated sequence amplification (MASA) of DNA from Indian rhinoceros (*Rhinoceros unicornis*) and three sub-species of South African black rhinoceros (*Diceros bicornis*) was carried out using a primer based on consensus sequence of the minisatellite repeat locus 33.15. Several bands in the range of 3.0 kilobases (kb) to 650 base pairs (bp) were identified that were useful for successful differentiation of *R. unicornis* from *D. bicornis*. Of these fragments, a 688 bp one, unique to *R. unicornis* was cloned and sequenced (Accession No. AF-296689). The band patterns uncovered by MASA and the species-specific hybridisation of pSG5 may be utilised as a tool for differentiating the *R. unicornis* genome from that of *D. bicornis*. This approach may also be adopted for the development of DNA-based genetic marker(s) useful for identification of other endangered species.

Keywords: *Rhinoceros unicornis*; *Diceros bicornis*; Minisatellite associated sequence amplification; Species-specific amplicon; Dinucleotide repeat-island; Endangered species

1. Introduction

Genetic analysis is an important tool for the management and conservation of endangered animals [1,2]. There are currently five extant rhinoceros species, all of which are listed as highly endangered by Convention on International Trade in Endangered Species (CITES).[1] These are the Javan (*Rhinoceros sondaicus*), Sumatran (*Didermocerus sumatrensis*), Indian (*Rhinoceros unicornis*), black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. Habitat encroachment and poaching are cited as the main reasons for the dramatic decline in the rhino populations.[2] The absence of forest corridors between isolated rhino populations found in Jaldapara (West Bengal) and Kaziranga National Park (Assam), India, has restricted potential gene flow. Furthermore, inbreeding within these small fragmented populations has contributed to their loss of genetic variability [1–3].

Earlier, molecular studies have attempted to differentiate species and sub-species of rhinoceroses [4–6]. Additional markers could be used to determine the origin of a biological sample (e.g. poached horn) from particular animals and be useful for the efficient management of both individuals and populations. Allozyme studies have shown that the African and Indian rhinoceroses diverged approximately 26 million years ago [7]. Subsequent studies on mitochondrial DNA showed and estimated divergence time between the African white rhinoceros and the Indian rhinoceros to be approximately 27 million years [8]. Other methods have also been reported as potential genetic tools for rhino genome analysis [9,10].

In this paper we evaluated minisatellite associated sequence amplification (MASA) as a method to distinguish between *R. unicornis* and *D. bicornis*, as well as three sub

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[1] http://www.cites.org/

[2] http://www.cites.org/eng/resources/species.html

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species of *D. bicornis* from Africa, namely *D. bicornis bicornis*, *D. bicornis minor* and *D. bicornis michaeli*.

2. Materials and methods

2.1. Collection of blood samples and DNA isolation

DNA from the three sub-species of *D. bicornis* from South Africa, which included *D. bicornis bicornis*, *D. bicornis michaeli* and *D. bicornis minor* was prepared following standard methods [5]. Blood collection and DNA isolation from wild *R. unicornis* species from the Jaldapara Wildlife Sanctuary (West Bengal, India) was reported earlier [10]. For additional interspecies comparisons, DNA was extracted from peripheral blood samples of the langur (*Presbytis entellus*), cow (*Bos indicus*), goat (*Capra hircus*), rat (*Rattus norvegicus*), cobra (*Naja naja*) and humans following standard protocols [3,10]. Also, DNA or blood samples of gharial (*Gavialis gangeticus*), garden lizard (*Calotes versicolor*), lion (*Panthera leo persica*) and horse (*Equus caballus*) were received from the sources mentioned in the acknowledgement.

2.2. Minisatellite associated sequence amplification (MASA)

MASA was performed using an oligonucleotide primer 5′ CACCTCTCCACCTGCC 3′ (Rama Biotechnologies, Hyderabad, India) based on the consensus sequence of minisatellite locus 33.15 [11–13]. The details of MASA reactions, agarose gel electrophoresis and methods for size estimation of the resultant amplicons have been reported earlier [10,13].

2.3. Radioactive MASA and polyacrylamide gel electrophoresis

Labelled MASA reactions with genomic DNA from *R. unicornis* and *D. bicornis* were carried out in 25 μl reactions containing approximately 25 ng of template DNA, 20 pmol of primer, 0.25 units of Taq DNA polymerase (Bangalore Genei, India), 2.5 mM $MgCl_2$, 200 μM of dTTP, dCTP, dGTP each, 0.5 μl of [α^{35}S] dATP (specific activity 1250 Ci/mmol, NEN, Boston, USA), 50 mM KCl, 20 mM Tris–HCl (pH 8.3), 0.1% Triton X-100 and an equal volume of mineral oil. PCR amplification was performed on a thermal cycler (Perkin Elmer, Cetus) employing initial denaturation at 96 °C for 2 min followed by 35 cycles comprising subsequent steps of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and primer extension at 72 °C for 1 min. On completion of the cycles, the reaction mixture was incubated further at 72 °C for 5 min. Approximately 5 μl of the amplified products were resolved on a 40 cm long, 3.5% non-denaturing polyacrylamide gel in 1 × TBE (Tris–Borate EDTA) buffer for 8 h at a 400 V constant voltage. After electrophoresis, the gel was dried for 2 h at 80 °C, exposed to X-ray film and autoradiography was conducted following established procedure [13].

2.4. Molecular cloning of the 688 base pair amplicon from R. unicornis

A 688 base pair band unique to *R. unicornis* was identified. Approximately 10 μl of the cold MASA product was electrophoretically separated on a 20 cm long 1.5% agarose gel in 0.5 × TBE buffer. The bands were sized using molecular size marker ϕX174 DNA (Bangalore Genei, India). The 688 bp band was sliced from the agarose gel and processed for subsequent purification, cloning and sequencing following established protocols [10]. The sequences of one of the resultant clone, pSG5, was deposited in GenBank (Accession No. AF296689).

2.5. Southern blot hybridisation

MASA generated amplicons of DNA from *R. unicornis*, the three subspecies of *D. bicornis* and several other animals

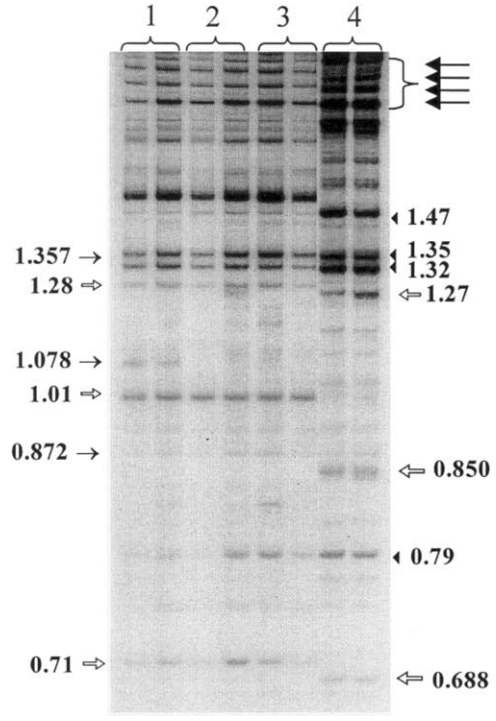

Fig. 1. Minisatellite associated sequence amplification (MASA) profiles of *R. unicornis* and sub-species of *D. bicornis*. The labelled amplicons resolved on the polyacrylamide gel were generated using a 16 base long oligo primer representing consensus sequence (5′ CACCTCTC-CACCTGCC 3′) of minisatellite locus 33.15. Panels on top represent DNA samples from: 1-*D. bicornis bicornis*; 2-*D. bicornis minor*; 3-*D. bicornis michaeli* and 4-*R. unicornis*. The clearly resolved bands of 1270, 850 and 688 bp (⇛) specific to *R. unicornis* and 1.28, 1.01 kb and 710 bp bands specific to *D. bicornis* sub-species are shown. Molecular size marker ϕX174 is given on the left (→).

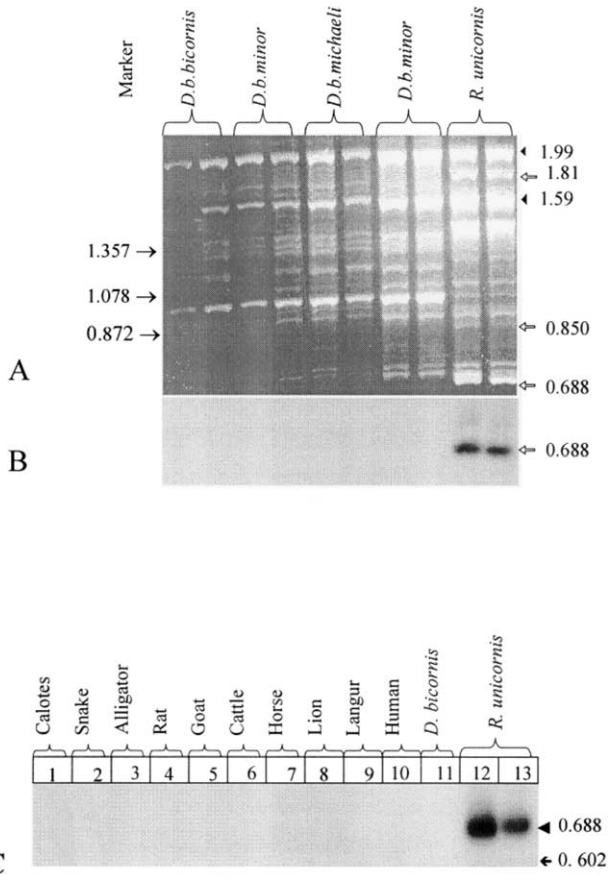

Fig. 2. MASA generated amplicons from different rhino samples using 16 base long oligo primer resolved on the agarose gel (A) the corresponding hybridisation profile of the blot with the labelled pSG5 probe is shown in panel (B) and the same with additional DNA samples, in panel (C) molecular size marker ϕX174 is given on the left and bands, 1.81 kb, 850 and 688 bp, specific to *R. unicornis* are marked (⇒) on the right of the panel. Note the exclusive hybridisation of pSG5 to *R. unicornis* DNA.

species were resolved by agarose gel electrophoresis as described above, transferred to nylon membrane, hybridized with labelled pSG5 insert DNA and autoradiographed [10,12].

3. Results

The MASA generated amplicons from the three sub-species of *D. bicornis* and *R. unicornis* revealed several bands (Fig. 1), which distinguish *R. unicornis* and *D. bicornis*. Four bands, in the range of 2–3 kb, were detected in all the samples (Fig. 1, multiple arrows). In addition, in the low molecular weight region, four other bands (1.47, 1.35, 1.32 and 0.79 kb, indicated by the arrowheads) of varying signal intensities were detected. Three bands of 1.28, 1.01 kb and 710 bp of similar signal intensities were detected in all the sub-species of *D. bicornis* but were absent in *R. unicornis*. Similarly, three other bands of 1.27 kb, 850 and 688 bp were detected exclusively in *R. unicornis*. However, the MASA band pattern could not differentiate between subspecies *D. bicornis*.

When MASA generated amplicons (Fig. 2A) from *R. unicornis* and sub-species of *D. bicornis* hybridized with pSG5, a 688 bp band was only detected in *R. unicornis* (Fig. 2B). No signal was detected with DNA samples from any other species.

4. Discussion

The MASA approach used in the present study identified band profiles specific to the rhinoceros species *R. unicornis* and *D. bicornis*, but failed to detect differences among the three sub-species of *D. bicornis*. Thus, MASA profiles may be used as a reliable tool to distinguish between these species and may be adapted for the development of species-specific markers leading to the identification of a broad range of endangered species.

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