DEVELOPMENT OF SPECIES SPECIFIC DNA MARKER AS BARCODE SEQUENCE OF GREATER INDIAN RHINOCEROS [*RHINOCEROS UNICORNIS*] FROM NORTHEAST INDIA

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ABSTRACT

Northeast India is a reservoir of rich biodiversity, especially Greater one-horned rhinoceros (*Rhinoceros unicornis)* which is facing extinction. DNA barcoding being an exciting tool for accurate documention of biodiversity with a gene sequence, in the present article DNA barcode of *R. unicornis* is determined for the first time by amplifying mitochondrial COI gene region using cross species primer designing and other bioinformatics analysis (Accession No. JN41700). Phylogeny analysis of obtained sequence with other related species distinctly maintains the assigned taxonomic position.

Key words: *DNA barcoding, mitochondrial DNA, COI-5', Rhinoceros unicornis*

INTRODUCTION

Northeast of India, a reservoir of rich biodiversity for much of India's flora and fauna, is important for conservation of several species of diverse phyla (Reddy and University, 2008) including Greater Indian Rhinoceros (*Rhinoceros unicornis)*. There is a considerable depletion in biodiversity of Northeast India, especially the Greater one-horned rhinoceros or Indian rhinoceros which is characterized by a snout with one horn (Order: Perissodactyla, Family: Rhinoceretidae). Once rhinocaros ranged throughout the entire stretch of the Indo-Gangetic Plain (Foose and van Strien, 1997). It is now marginalised to its last strongholds in Northeast India and in protected areas at Terai of Nepal due to threats imposed from poaching and destruction of habitat (Poudyal *et al*., 2009). The alarming rate in reduction of 90% population of this species over the past 20 years brought it to verge of extinction. Identification of *Rhinoceros* species using classical taxonomy is difficult and almost impossible to its traded products. DNA barcoding region of cytochrome oxidase subunit 1 (COI) provides strong species-level resolution for varied animals groups and may serve as the primary key for retrieval of such information (Steinke *et al*., 2009). The successful species specific PCR amplification of CooI region requires *in silico*

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testing of robust cross species primers where no direct species-specific sequence information is available (Lucio *et al*., 2011).

In the present study we selected cross species primer by checking the specificity and feasibility of different DNA barcoding primer available with us to amplify the targeted barcode sequence of *R. unicornis* to develop species-specific DNA marker as barcode sequence of this species of rhinoceros of Northeast India. The selected primer pair successfully amplified the COI region and the sequence was submitted to global database (Accession No. JN41700). The subsequent phylogeny analysis of generated sequences using bioinformatics with other sequences within and among the species of genus *Rhinoceros* along with other related genus was also performed.

MATERIALS AND METHODS

Sample collections and DNA isolation :

Rhinoceros unicornis hairs were collected from State Zoo, Guwahati wherein this species was brought from Kaziranga National Park (26° 31'N 93° 58'E to 26° 21'N 92° 41'E) at Assam, India. The samples were preserved in 100% (absolute) ethanol in micro centrifuge tubes and brought to the laboratory for our study. About 50-100 hair roots were cut aseptically and DNA was isolated by phenol-chloroform-isoamyl alcohol (25:24:1) extraction method (Sambrook and Russell, 2001)

In silico analysis and selection of primer :

Different combinations of primer pairs available in our laboratory were tested by *in silico* PCR (ePCR, www.bioinformatics.org/sms2/product.html) amplification analysis with complete mtDNA sequence (Accession No. NC_001779) of *R. unicornis*. The best combinations were : FOLMER L CO1 2198 R 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' and AUS FISH F(22bp) 5' CCA ACC ACA AAG ACA TTG GCA C 3' and selected for *in vitro* amplification.

PCR amplification :

Polymerage Chain Reaction (PCR) amplification of COI region was carried out using 50 ng of genomic DNA, 0.2mM of each dNTP, 10pmol of each selected primer, 0.5 units of high fidelity Taq polymerase enzyme, 1X PCR buffer and 1.5mM $MgCl_2$ in a 25 µl final reaction volume. The PCR conditions were as follows: 94°C for 3 min, 40 cycles at 94°C for 1min, 45°C for 2 min, 72°C for 3 min, and a final extension at 72°C for 10 min. The sequencing of purified amplicon was sequenced using AB 13700 automated DNA sequencer.

Phylogeny analysis :

The open Reading Frame (ORF) of obtained sequence checked for correct amino acid sequences using online software ORF prediction (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) are submitted to the National Center for Biotechnology Information (NCBI) database. Related sequences are obtained from global database and analysed using the ClustalX program (Thompson *et al*., 1997). Pair-wise nucleotide intra and interspecies sequence divergences were calculated using the Kimura-2-parameter (K2P) model and the neighbour-joining (NJ) analysis in MEGA 4.2 (Tamura *et al*., 2007) to examine relationship among taxa. A total of 1000 bootstrap replicates were calculated for the NJ tree construction.

RESULTS AND DISCUSSION

The primer sequences are derived from 5 sets of available primers from our laboratory used to generate DNA barcode sequence of birds, economic animals, invertebrates, fish and farm animals employing the concept of cross species primer, often used to amplify part of a gene or genome from where no species specific sequence information is available. A complete mitochondrial DNA sequence of *R. unicornis* are taken from GenBank (Accession No. NC_001779) and the different forward and reverse primers were checked *in silico* for their specificity and feasibility with respect to this sequence and bird (BIRD COX F) and fish (AUS FISH F) DNA barcoding primers most suitably amplify *R. unicornis* yielding 650-700 bp PCR product of COI region. The *in vitro* PCR amplification resulted 681 bp COI region and nucleotide BLAST confirmed our sequence similarity with *R. unicornis* in both NCBI and BOLD (Biology of Life Database) confirmed our success and submitted in GenBank (Accession No. JN41700). We for the first time submitted DNA barcode sequence of Indian rhinoceros in global database. The phylogeny analysis showed the proper clustering of our sequence with other *Rhinoceros* species (Fig. 1). The other related species and genus diverge distinctly following their correct taxonomic order and the DNA barcoding technique could be effectively employed for species identification and conservation of endangered Indian rhinoceros (Dalton and Kotze, 2011).

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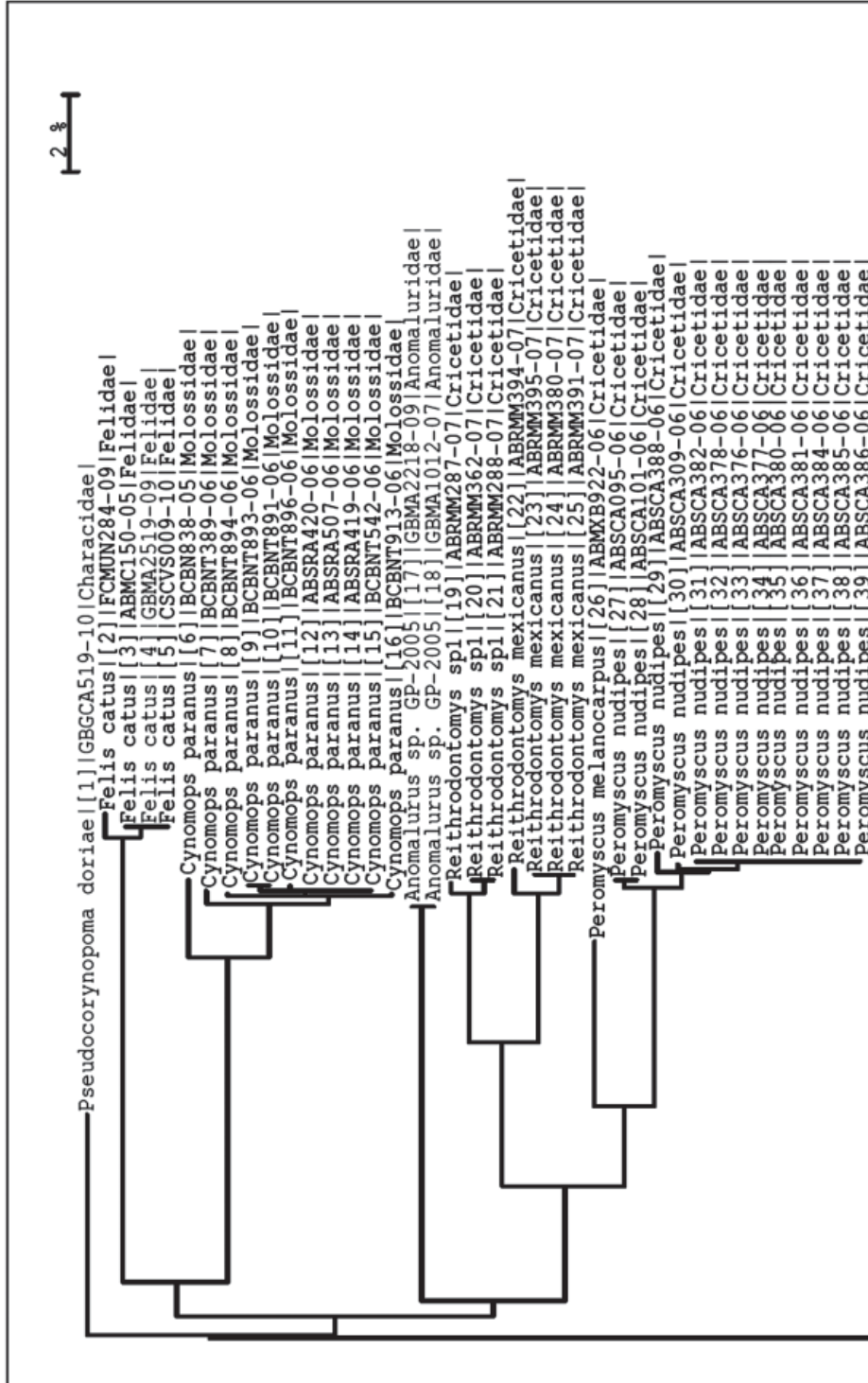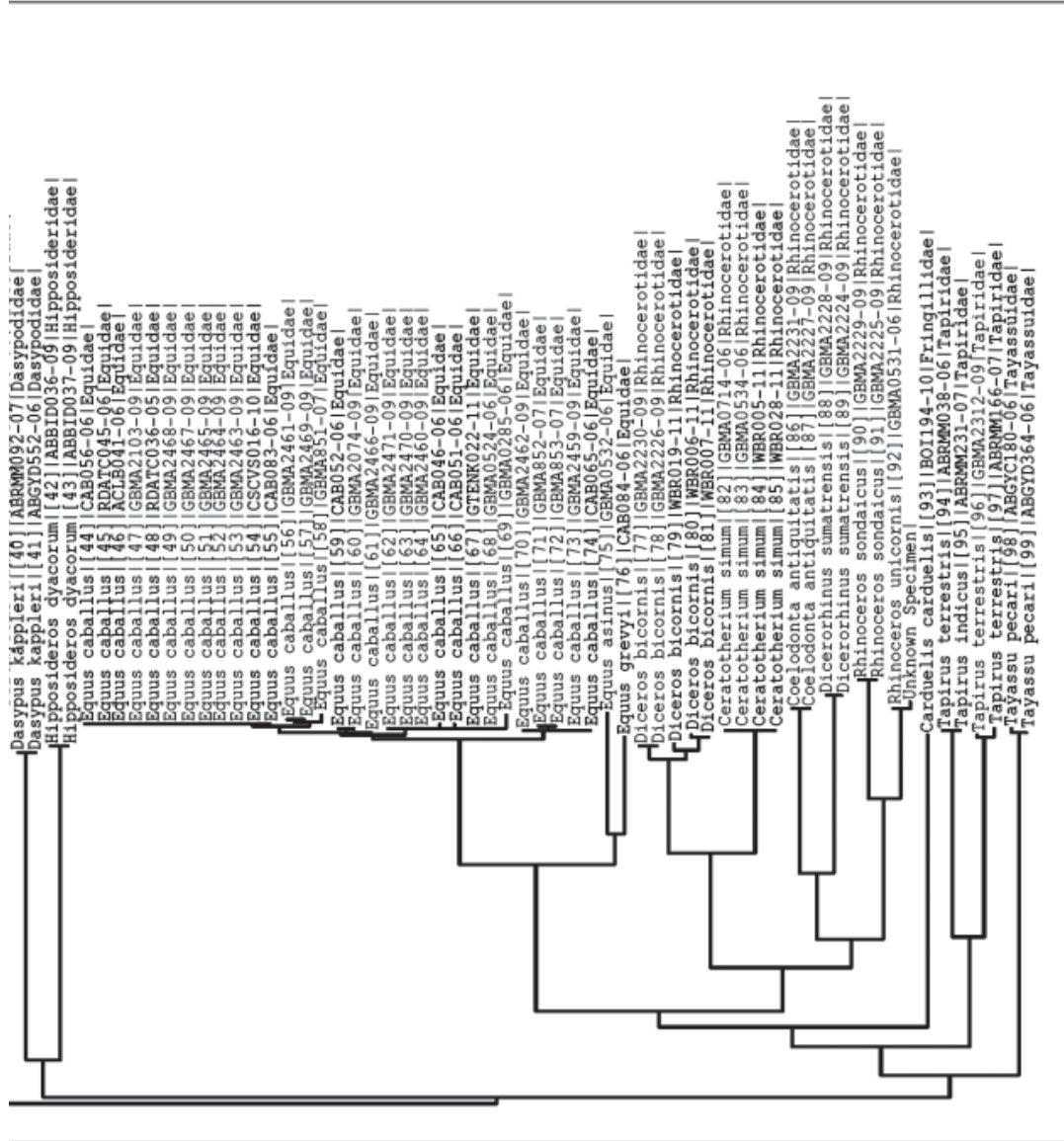
Fig. 1(a)

Fig. 1(b)

Fig.1 a&b The BOLD Identification System based identification of *R. unicornis*. The tree is auto generated as per the sample identification domain of the database. The sequence clustered with *R. unicornis* of the database sequences

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