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Species identification of rhinoceros horns using the cytochrome b gene

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Abstract

Material suspected of originating from species of *Rhinoceros* is frequently seized by forensic organizations investigating trade in endangered species. At present identification of the species is possible by DNA sequencing of the material, such as powdered rhinoceros horns. The unambiguous identification of rhino products using a 402 bp fragment of cytochrome b gene was investigated. This DNA sequence may not only assist in the identification of the unknown sample, but can be used to determine the phylogenetic relationships of rhinoceros species. Sequences of suspect rhinoceros horns were compared with the sequences registered in GenBank. The maximum value of genetic distance among white rhinoceros was 0.0176, and 0.0333 among black rhinoceros. In the comparison among rhinoceros species, the greatest genetic distance was between black and Indian rhinoceros (0.1564). The rhinoceros sequences extracted from GenBank and 13 samples in this study were clustered and separated from other mammals. Holstein cow was used as an out-group and was clustered with cattle in the phylogenetic tree. The results of this phylogenetic study also showed that there were four major branches among rhinoceros species from a common origin. The amplification of the 402 bp fragment of the cytochrome b gene was found to be able to detect rhinoceros DNA even in the ratio of 1:19 with Holstein cow DNA. In the initial identification of species from unknown powdered material, all the unknown samples were found to be from rhinoceroses. In phylogenetic analysis, the results supported the morphological hypothesis. The method used in this study can be applied in the identification of processed products of rhinoceros horns, such as sculptures, daggers, powders or even mixture powdered prescriptions.

Keywords: Species identification; Rhinoceros; Cytochrome b gene; Phylogenetic study

1. Introduction

The trade in conservational rhinoceros horns is a problem in many parts of the world and contrary to CITES regulations. The trade in rhino products is a problem especially in parts of Asia where the rhinoceros horns are used traditionally as the materials of sculptures or pulverized as drug products for medical purposes. However, the identification of the processed products and powdered samples of suspected rhinoceros material in the absence of morphological characteristics is problematic.

Rhinoceros are one of the most endangered species of mammal. They belong to the order perissodactyl and the family Rhinocerotidae, in which there are four genera recognised currently, *Rhinoceros*, *Dicerorhinus*, *Diceros* and *Ceratotherium*. *Rhinoceros* and *Dicerorhinus* are found in Asia, and *Diceros* and *Ceratotherium* in Africa. The four genera comprise five living species: the Indian rhinoceros (*Rhinoceros unicornis*), Javan rhinoceros (*Rhinoceros sondaicus*), Sumatran rhinoceros (*Dicerorhinus sumatrensis*), white rhinoceros (*Ceratotherium simum*) and black rhinoceros (*Diceros bicornis*). However, the phylogenetic relationships

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among these species are controversial. The study based on the restriction site mapping of an mtDNA ribosomal region by Morales and Melnick [1] supported the morphological hypothesis, based upon the number of horns, of Simpson [2] and Loose [3]. According to the morphological hypothesis, the African genera (*Ceratotherium* and *Diceros*) and the Asian genus *Dicerorhinus* have been clustered in a two-horned group, the other Asian genus *Rhinoceros* was the only member of one-horned group. The hypothesis of geographic distribution was proposed by Pocock [4] and Groves [5], and supported recently by Tougard et al.'s study [6] based on the analysis of mitochondrial cytochrome b and 12S rRNA gene sequence. According to the hypothesis of geographic distribution, the two African genera (*Ceratotherium* and *Diceros*) belonged to the same group, and the two Asian genera (*Rhinoceros* and *Dicerorhinus*) were another group. Guerin [7], Prothero and Schoch [8] and Cerdeno [9] supported the hypothesis of both morphological and geographic features. According to their hypothesis, the rhinoceroses were separated to three groups, the two Asian genera were separated to two different groups in addition to the African group.

The cytochrome b gene of mitochondrial DNA (mtDNA) has been used in phylogenetic studies [6,10] and species identification [11]. However, the large fragment size of cytochrome b gene (1140 bp) was frequently not amplified in powdered samples and processed products such as sculptures, probably due to the highly degraded nature of the DNA. In our previous study, the conservation animals were successfully identified by the analysis of a partial cytochrome b sequence (402 bp) [12]. DNA from seized rhinoceros horns or the sculptures was isolated, and 402 bp fragments were amplified and sequenced. The 402 bp fragments were investigated in the applications of species identification and phylogenetic relationships of rhinoceroses. To solve the problems of mixed powders from different species or counterfeits for medical uses, the powders of different ratios of rhinoceros and Holstein cow were mixed to determine sensitivity of rhinoceros DNA was evaluated by this system.

2. Materials and methods

2.1. DNA sources

All the samples were all provided by the Council of Agriculture (COA). Six samples from rhinoceros horns were of black rhinoceroses (Black 1–3) and white rhinoceroses (White 1–3), respectively, one was of Indian rhinoceros (Indian 1). Six processed sculptures of suspect rhinoceros horns (Unknown 1–6) were provided. A sample of Holstein cow horn was also provided. Approximately 1–6 and 100 mg of each sculpture and each of the other samples, respectively, were pulverized under lysis buffer or liquid nitrogen in a mortar and pestle. The powder was transferred to a 1.5 ml microcentrifuge tube and DNA was extracted by salt/chloroform method [13], and then purified using the DNA extraction kit (Blood and Tissue Genomic Mini Kit, Viogene, USA). The final volume of each DNA was about 30 μl. The isolated DNA was quantified with ultra-violet detection by spectrophotometer.

2.2. Nested PCR amplification of partial cytochrome b gene and cycle sequencing of PCR products

Nested PCR amplification was adopted in this study due to the highly degraded nature of the DNA. The primers for first PCR amplification were L14696 and H15197, which were designed according to our previous study. The sequences of L14696 and H15197 were 5′-TCTCACATGGACTTCAACCA-3′ and 5′-CCGATATAAGGGATTGCTGA-3′, respectively. The universal primers for second PCR amplification were L14724 and H15149, as described by Kocher et al. [14] and Irwin et al. [11]. Numbering of primers was according to the human mtDNA sequence [15]. The sequences of L14724 and H15149 were 5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′ and 5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′, respectively. Both PCR amplifications were performed in a 50 μl reaction mixture, which contained approximately 1–10 ng of genomic DNA or 1 μl product of first PCR, 100 ng each of primers, 2.5 unit of VioTaq DNA polymerase (Viogene, USA) and reaction buffer (10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% (w/v) gelatin). Amplification was conducted in a 4800 Perkin-Elmer thermal cycler with the following conditions for 35 cycles: 95 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s. Cycle sequencing of PCR products was performed using the primers L14724 or H15149 and the BigDye™ Terminator Kit (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit). The sequencing reaction was also conducted in a 4800 Perkin-Elmer thermal cycler with the following conditions for 25 cycles: 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. The cycle sequencing products were separated with 5% denatured Long Ranger™ gel (FMC BioProducts, Rockland, Maine, USA) and detected using an automatic DNA sequencer (Applied Biosystems 373A DNA sequencer).

2.3. Phylogenetic analysis

Sequences were aligned using the PileUp program of GCG computer package (Wisconsin Package, Version 10.2, Genetics Computer Group (GCG), Madison, WI), and the phylogenetic tree was constructed by the neighbor-joining method [16], generated by Kimura's two-parameter model, of Phylip computer package.

2.4. Preparations of mixture samples, PCR amplifications and cycle sequencing

The powder of rhinoceros and Holstein cow samples were mixed according to the ratios of 1:1, 1:9 and 1:19 separately.

```
           1                                                  50
Consensus  ATGACTAACA TCCGTAAATC CCACCCACTA ATCAAAATTA TCAANCACTC
   Indian  .......... .......... t......... g.t.....c. ....c.....
 Indian 1  .......... .......... t......... g.t.....c. ....c.....
    Javan  .......... .t..c..... t......t.. .......... .t..c..t..
    White  .......... .......... .......... .......... ....c.....
  White 1  .......... .......... .......... .......... ....c.....
  White 2  .......... .......... .......... .......... ....c.....
  White 3  .......... .......... .......... .......... ....c.....
Unknown 3  .......... .......... .......... ........c. ....t.....
Unknown 5  .......... .......... .......... ........c. ....t.....
  Black 3  .......... .......... .......... .......... ....t.....
Unknown 1  .......... .......... .......... .......... ....t.....
Unknown 2  .......... .......... .......... .......... ....t.....
  Black 1  .......... .......... .......... .......... ....t.....
Unknown 4  .......... .......... .......... .......... ....t.....
  Black 2  .......... .......... .......... .......... ....t.....
Unknown 6  .......... .......... .......... .......... ....g.....
    Black  .......... .......... .......... .......... ....t.....
 Sumatran  .....c.... ....c..... .......... .......... ....c.....
 Holstein  .......... .t..a..g.. .......... ..a......g .a..ca.tg.

          51                                                  100
Consensus  ATTCATCGAC CTACCCACCC CATCAAACAT TTCAGCCTGA TGAAATTTTG
   Indian  .......... .....t.... .......... c...t.t... .....c....
 Indian 1  .......... .....t.... .......... c...t.t... .....c....
    Javan  .......... .....t.... .......... ....t.t... .....c...a
    White  .........t ..g....... .......... c........g ..........
  White 1  .........t ..g....... .......... c........g ..........
  White 2  .........t ..g....... .......... c........g ..........
  White 3  .........t ..g....... .......... c........g ..........
Unknown 3  ......t..t ..g....... .......... c......... ..........
Unknown 5  ......t..t ..g....... .......... c......... ..........
  Black 3  .......... .......... .......... .......... ..........
Unknown 1  .......... .......... .......... .......... ..........
Unknown 2  .......... .......... .......... .......... ..........
  Black 1  ...t...... .......... .......... .......... ..........
```

Fig. 1. The partial sequences of cytochrome b gene. Sequences were aligned by the PileUp program of GCG computer package, and the consensus sequences were deduced by the Pretty program. The symbol '•' and 'N' indicate the same base as the consensus sequence and four possible nucleotides, respectively. All the samples tested were 402 bp in size. Indian, Javan, white, black and Sumatran represented the standard samples of Indian rhinoceros, Javan rhinoceros, white rhinoceros, black rhinoceros and Sumatran rhinoceros, respectively. The sequences of these standard samples were extracted from GenBank, accession numbers were X97336, AJ245725, Y07726, X56283 and AJ245723. Holstein represented the sample of Holstein cow.

```
Unknown 4  ...t...... .......... .......... .......... ..........
  Black 2  .......... .......... .......... c......... ..........
Unknown 6  .......... .......... ...g...... .......... ..........
    Black  .......... .......... .......... .......... ..........
 Sumatran  ...t...... ..g..t.... .......... ....t..... .....c....
 Holstein  .......... ..t..ag... .......... ....t.a... ........c.

         101                                                   150
Consensus  GCTCCCTACT AGGAATCTGC CTAATCTTAC AAATCCTAAC CGGACTATTC
   Indian  .......gt. .......... t......... .g........ a.........
 Indian 1  .......gt. .......... t......... .g........ a.........
    Javan  ........t. .......... t......... .g.....g.. a.........
    White  .......g.. .......... t......... ....t..... ..........
  White 1  .......g.. .......... t......... ....t..... ..........
  White 2  .......g.. .......... t......... ....t..... ..........
  White 3  .......g.. .......... t......... ....t..... ..........
Unknown 3  .......g.. .......... t......... ....t..... ..........
Unknown 5  .......g.. .......... t......... ....t..... ..........
  Black 3  ....t..... .......... ......c... .......... .........t
Unknown 1  ....t..... .......... ......c... .......... .........t
Unknown 2  ....t..... .......... ......c... .......... .........t
  Black 1  ....t..... .......... ......c... .......... .........t
Unknown 4  ....t..... .......... ......c... .......... .........t
  Black 2  ....t..... .......... ......c... .......... .........t
Unknown 6  ....t..... .......... ......c... .......... .........t
    Black  ....t..... .......... ......c... .......... .........t
 Sumatran  .......... .......... .......... .......... ..........
 Holstein  .t.....c.. g......... ......c... .......c.. a..c......

         151                                                   200
 Consensus CTTGCCATAC ACTACACACC AGACACAACA ACTGCCTTCT CATCCGTTGC
   Indian  .......... .......c.. .......... ..c.....t. .......ca.
 Indian 1  .......... .......c.. .......... ..c.....t. .......ca.
    Javan  .......... .......c.. .......... ........t. .......a..
    White  .......... .......... ........t. .......... ....t..c..
  White 1  .......... .......... ........t. .......... ....t..c..
  White 2  .......... .......... ........t. .......... ....t..c..
  White 3  .......... .......... ........t. .......... ....t..c..
Unknown 3  .......... .......... .......... .......... ....t..c..
```

Fig. 1. (*Continued*)

```
Unknown 5  .......... .......... .......... .......... ....t..c..
  Black 3  .....t.... .t..t..... .......... .......... ..........
Unknown 1  .....t.... .t..t..... .......... .......... ..........
Unknown 2  .....t.... .t..t..... .......... .......... ..........
  Black 1  .....t.... .t..t..... .......... ..c....... ..........
Unknown 4  .....t.... .t..t..... .......... ..c....... ..........
  Black 2  .....t.... .t..t..... .......... ..c....... ..........
Unknown 6  .....t.... .t..t..... .......... ..c....... ..........
    Black  .....t.... .t..t..... .......... .......... ..........
 Sumatran  ..c..a.... .t........ ...t...... ..c....... .......a..
 Holstein  ..a..a.... ........t. c......... ..a..a.... .c..t...a.

         201                                                   250
Consensus  CCATATCTGT CGAGACGTAA ACTACGGCTG AATTATCCGC TACCTCCATG
   Indian  .........c .......... .t........ ...a..t... ..........
 Indian 1  .........c .......... .t........ ...a..t... ..........
    Javan  .........c .......... .t........ ...a..t... ..........
    White  .......... ........g. .t........ .......... ..t.......
  White 1  .......... ........g. .t........ .......... ..t.......
  White 2  .......... ........g. .t........ .......... ..t.......
  White 3  .......... ........g. .t........ .......... ..t.......
Unknown 3  .......... ........g. .t........ .......... ..t.......
Unknown 5  .......... ........g. .t........ .......... ..t.......
  Black 3  ...c...... .......... .......... .......... .....a..c.
Unknown 1  ...c...... .......... .......... .......... .....a..c.
Unknown 2  ...c...... .......... .......... .......... .....a....
  Black 1  ...c...... .......... .......... .......... .....a..c.
Unknown 4  ...c...... .......... .......... .......... .....a..c.
  Black 2  ...c...... .......... .......... .......... .....a..c.
Unknown 6  ...c...... .......... .......... .......... .....a..c.
    Black  ...c...... .....g.... .......... .......... .....a....
 Sumatran  ...c...... .......... .......t.. .......... ...ac.....
 Holstein  .........c ........g. .......... ...c.....a ...a.a..c.

         251                                                   300
Consensus  CCAACGGAGC ATCCATATTC TTTATCTGCC TATTCATCCA CGTAGGACGC
   Indian  .......... .......... ..c....... ....t..t.. t........a
 Indian 1  .......... .......... ..c....... ....t..t.. t........a
    Javan  .......... .......... ..c....... ....t..t.. t........a
    White  .......... .......... .......... .......... ..........
```

Fig. 1. (*Continued*)

```
  White 1  .......... .......... .......... .......... ..........
  White 2  .......... .......... .......... .......... ..........
  White 3  .......... .......... .......... .......... ..........
Unknown 3  .......... .......... .......... .......... ..........
Unknown 5  .......... .......... .......... .......... ..........
  Black 3  .t........ .........t .......... .......... .a........
Unknown 1  .t........ .........t .......... .......... .a........
Unknown 2  .t........ .........t .......... .......... .a........
  Black 1  .t........ .........t .......... .......... .a........
Unknown 4  .t........ .........t .......... .......... .a........
  Black 2  .t........ .........t .......... .......... .a........
Unknown 6  .t........ .........t .......... .......... .a........
    Black  .a........ .........t .......... .......... .a........
 Sumatran  .......... .......... ..c....... ....t..... .........a
 Holstein  .a........ t..a..g..t .........t ...at..g.. .........a

          301                                                   350
Consensus  GGCCTCTATT ACGGATCCTA CACCTTCCTA GAAACCTGAA ACATCGGAGT
   Indian  .....t..c. .t.....t.. .......... .....t.... ....t...a.
 Indian 1  .....t..c. .t.....t.. .......... .....t.... ....t...a.
    Javan  .....t..c. .......tc. .......... .....t.... ........a.
    White  ..ta...... .......a.. t......... .......... ..........
  White 1  ..ta...... .......a.. t......... .......... ..........
  White 2  ..ta...... .......a.. t......... .......... ..........
  White 3  ..ta...... .......a.. t......... .......... ..........
Unknown 3  ..ta...... .......... t......... .......... ..........
Unknown 5  ..ta...... .......... t......... .......... ..........
  Black 3  .......... .......... .......... .......... ..........
Unknown 1  .......... .......... .......... .......... ..........
Unknown 2  .......... .......... .......... a......... ..........

  Black 1  .......... .......... .......... .......... ..........
Unknown 4  .......... .......... .......... .......... ..........
  Black 2  .......... .......... .......... .......... ..........
Unknown 6  .......... .......... .......... .......... ..........
    Black  .......... .......... .......... a......... ..........
 Sumatran  ........c. .......... .......... .......... .t......ac
 Holstein  ...t.a.... ....g..t.. ...t..t... .....a.... .t..t.....
```

Fig. 1. (*Continued*)

```
          351                                                        400
Consensus  TATCCTACTA TTTACCCTAA TAGCCACTGC ATTCATAGGC TACGTCCTAC
   Indian  .......... .......... .......a.. g........t ..........
 Indian 1  .......... .......... .......a.. g........t ..........
    Javan  c......... c......... .......a.. g......... ..........
    White  ....t..... ..c..t.... .......c.. .......... ..........
  White 1  ....t..... ..c..t.... .......c.. .......... ..........
  White 2  ....t..... ..c..t.... .......c.. .......... ..........
  White 3  ....t..... ..c..t.... .......c.. .......... ..........
Unknown 3  ....t..... ..c..t.... .......... .......... ..........
Unknown 5  ....t..... ..c..t.... .......... .......... ..........
  Black 3  ...t...... .......... .......... .......... ..t.......
Unknown 1  ...t...... .......... .......... .......... ..t.......
Unknown 2  ...t...... .......... .......... .......... ..t.......
  Black 1  .......... .......... .......... .......... ..t.......
Unknown 4  .......... .......... .......... .......... ..t.......
  Black 2  .......... .......... .......... .......... ..t.......
Unknown 6  .......... .......... .......... .......... ..t.......
    Black  ...t...... c.c..ag... .......a.. .......... ..t.......
 Sumatran  c...t.c... c.c..... ....t..a.. .......... ..........
 Holstein  a.....t..g c.c..ag... .......a.. ...t.....a ..........

          401
Consensus  CA
   Indian  ..
 Indian 1  ..
    Javan  ..
    White  ..
  White 1  ..
  White 2  ..
  White 3  ..
Unknown 3  ..
Unknown 5  ..
  Black 3  ..
Unknown 1  ..
Unknown 2  ..
  Black 1  ..
Unknown 4  ..
  Black 2  ..
Unknown 6  ..
    Black  ..
 Sumatran  ..
 Holstein  ..
```

Fig. 1. (*Continued*).

DNA was extracted from the mixture samples by the method mentioned in Section 2.1. PCR amplification and cycling sequencing were performed as those mentioned in Section 2.2.

3. Results and discussion

3.1. Nested PCR amplification of partial cytochrome b gene and cycle sequencing of PCR products

The isolated DNA was highly degraded and could not be visualized by agarose gel electrophoresis, therefore nested PCR amplification was adopted. The primer pairs, L14696/H15197 and L14724/H15149, were used to amplify part of the cytochrome b gene in two sequential reactions. The second set of primers was used to sequence the nested PCR products. The size of PCR products from these samples were all about 486 bp. Excluding the sequences of the primers and $tRNA^{Glu}$ gene, the exact size of partial cytochrome b gene was 402 bp within the 486 bp fragment. DNA sequences were aligned by PileUp program of GCG computer package, and the consensus sequences were deduced by pretty program (Fig. 1). Sequences were also compared with the sequences registered in GenBank by the Fasta program of GCG computer package. The results showed that the most similar sequences were of black rhinoceros (Unknown 1, 2, 4 and 6), or white rhinoceros (Unknown 3 and 5). The sample of Holstein cow was used as the out-group and analyzed following the procedures as rhinoceros samples. The results indicated that the most similar sequence was of cattle (*Bos taurus*), and the similarity was 100.0% over the 402 bp.

Another means of identifying the body parts from which six sculptures where derived was by scanning electron microscopic/energy dispersive X-ray (SEM/EDX) analyses. Based on the investigation of microscopic characteristics and elemental ingredients, they were all made of hair fibers, where the major element detected was sulfur, rather than bones, where the major elements would have been phosphorus and calcium (data not shown).

3.2. Phylogenetic analysis

The diversity of 402 bp sequences for all the examined samples was analyzed. Genetic distances between the examined species are shown in Table 1. The maximum value of genetic distance among white rhinoceros was 0.0176, and 0.0333 among black rhinoceros. In the comparison among rhinoceros species, the greatest genetic distance was between black and Indian rhinoceros, with a value of 0.1564. Among the studied samples, the maximum value was 0.2295 between Unknown 3 (and 5) and Holstein cow. A phylogenetic tree was constructed by the neighbor-joining method (Fig. 2). The rhinoceros sequences (named Javan, Sumatran, Indian, black and white) extracted from GenBank and 13 samples (including the unknown samples) in this study were clustered and separated from other mammals, such as human, macaque, dog, civet, pangolin, tiger, leopard cat, cat, cattle, deer, muntjac, sheep, serow, pig and mouse. The 402 bp sequences of these other mammals have been

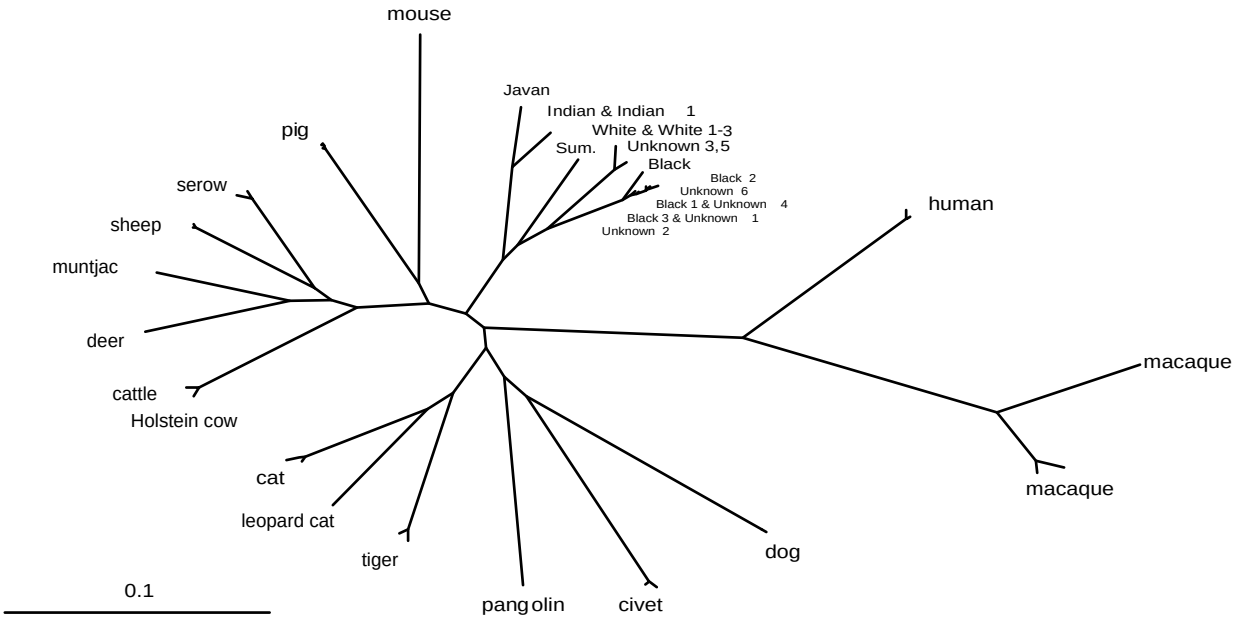


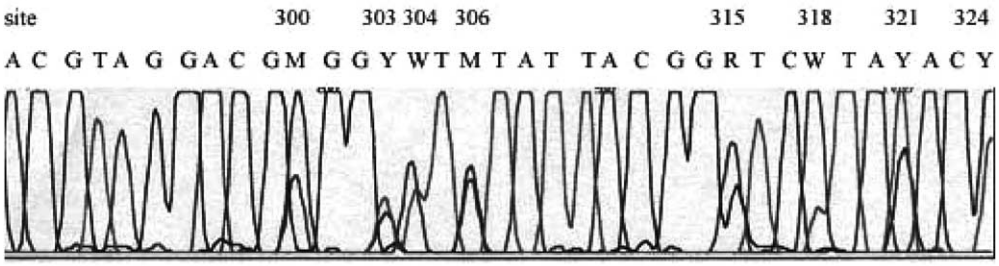
Fig. 2. The radial phylogenetic tree was constructed by the neighbor-joining method. The tree was based on the partial sequence (402 bp) of cytochrome b gene. Sum. represents the Sumatran rhinoceros.

Table 1
Genetic distances between the animals examined in this study[a]

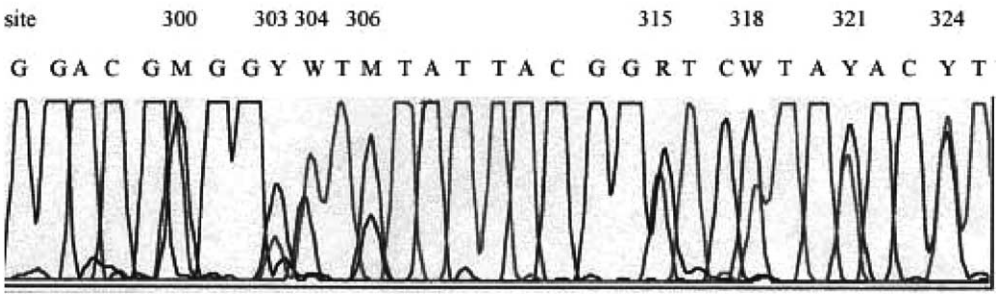
	Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Indian	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	Indian 1	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	Javan	0.0539	0.0539	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	White	0.1266	0.1266	0.1414	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	White 1	0.1266	0.1266	0.1414	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	White 2	0.1266	0.1266	0.1414	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	White 3	0.1266	0.1266	0.1414	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–
8	Unknown 3	0.1235	0.1235	0.1440	0.0176	0.0176	0.0176	0.0176	–	–	–	–	–	–	–	–	–	–	–	–
9	Unknown 5	0.1235	0.1235	0.1440	0.0176	0.0176	0.0176	0.0176	0	–	–	–	–	–	–	–	–	–	–	–
10	Black 3	0.1438	0.1438	0.1440	0.0946	0.0946	0.0946	0.0946	0.0862	0.0862	–	–	–	–	–	–	–	–	–	–
11	Unknown 1	0.1438	0.1438	0.1440	0.0946	0.0946	0.0946	0.0946	0.0862	0.0862	0	–	–	–	–	–	–	–	–	–
12	Unknown 2	0.1438	0.1438	0.1440	0.0946	0.0946	0.0946	0.0946	0.0862	0.0862	0.0050	0.0050	–	–	–	–	–	–	–	–
13	Black 1	0.1409	0.1409	0.1470	0.0974	0.0974	0.0974	0.0974	0.0890	0.089	0.0075	0.0075	0.0125	–	–	–	–	–	–	–
14	Unknown 4	0.1409	0.1409	0.1470	0.0974	0.0974	0.0974	0.0974	0.0890	0.089	0.0075	0.0075	0.0125	0	–	–	–	–	–	–
15	Black 2	0.1350	0.1350	0.1470	0.0919	0.0919	0.0919	0.0919	0.0835	0.0835	0.0075	0.0075	0.0125	0.0050	0.0050	–	–	–	–	–
16	Unknown 6	0.1414	0.1414	0.1475	0.0977	0.0977	0.0977	0.0977	0.0920	0.0920	0.0100	0.0100	0.0151	0.0075	0.0075	0.0075	–	–	–	–
17	Black	0.1564	0.1564	0.1507	0.1010	0.1010	0.1010	0.1010	0.0953	0.0953	0.0228	0.0228	0.0177	0.0306	0.0306	0.0306	0.0333	–	–	–
18	Sumatran	0.1293	0.1293	0.1147	0.1182	0.1182	0.1182	0.1182	0.1180	0.1180	0.1095	0.1095	0.1095	0.1010	0.1010	0.1066	0.1070	0.1100	–	–
19	Holstein	0.2282	0.2282	0.2219	0.2266	0.2266	0.2266	0.2266	0.2295	0.2295	0.2120	0.2120	0.2186	0.2120	0.2120	0.2120	0.2128	0.2010	0.2136	–

[a] The value generated by Kimura two-parameter model represented the evolutionary distance between two sequences.

A



B



C

Site / Sample Name	300	303	304	306	315	318	321	324
Holstein cow	A	C	T	A	G	T	C	T
White rhinoceros	C	T	A	C	A	A	T	C
Mixed samples	M	Y	W	M	R	W	Y	Y

Fig. 3. The partial electropherograms of sequences from mixed DNA (A and B). The mixture samples of white rhinoceros and Holstein cow were mixed according to the ratios of 1:9 (A) and 1:19 (B) separately. The sites of IUPAC-IUB codes represented the different sequences between white rhinoceros and Holstein cow (C). Numbering of the sites was according to A (number 1) of ATG start codon of cytochrome b gene.

analyzed in our previous study [12]. Holstein cow was clustered with cattle in the tree, supporting the validity of the tree's construction. The topology of four major branches from a common origin was constructed. Javan and Indian were clustered in the same branch. Sequences of sample Indian 1 were identical with those of Indian from GenBank. Samples of Unknown 3 and 5 were clustered in the same branch as white rhinoceroses and samples of Unknown 1, 2, 4 and 6 were in the same cluster of black rhinoceroses.

The data obtained in this study was compared to that supporting the three hypotheses of phylogenetic relationships [6]. The results presented in this study support the morphological hypothesis. This conflicts with the results of Tougard et al.'s [6], which is based on the full cytochrome b and 12S rRNA genes. It is interesting that different hypotheses are supported by analysis of either partial fragments or full lengths of the cytochrome b gene; this may explain the phenomenon of controversial phylogeny.

3.3. Analysis of mixture samples

To evaluate the sensitivity of the cytochrome b gene when powders containing more than one species was present, Black 1 or White 1, and Holstein cow powders were mixed according to the ratios of 1:1, 1:9 and 1:19. The mixed DNA was used as a template to amplify part of the cytochrome b gene using the primers described. The PCR products were sequenced and the examples of partial electropherograms are shown in Fig. 3. The sequences of rhinoceros can be detected unambiguously even in the ratio of 1:19 in all the mixed samples. Therefore, the test is sensitive for rhinoceros DNA detection in mixture of different species or counterfeits by this system.

4. Conclusion

We have developed a robust method for the identification of Rhinoceros DNA from highly degraded samples. The

need to unambiguously identify products from any of the extant rhino species is greater than ever, as all the species are on the verge of extinction. We hope that this test can only add to the reduction in trade in these endangered species.

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