Application of 12S rRNA Gene for the Identification of Animal-Derived Drugs

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ABSTRACT - Purpose. Animal-derived drugs are the major source of biological products and traditional medicine, but they are often difficult to identify, causing confusion in the clinical application. Among these medicinal animals, a number of animal species are endangered, leading to the destruction of biodiversity. The identification of animal-derived drugs and their alternatives would be a first step toward biodiversity conservation and safe medication. Until now, no effective method for identifying animal-derived drugs has been demonstrated; DNA-based species identification presents a brand-new technique. **Methods.** We designed primers to amplify a 523-bp fragment of 12S rRNA and generated sequences for 13 individuals within six medicinal animal species. We examined the efficiency of species recognition based on this sequence, and we also tested the taxonomic affiliations against the GenBank database. **Results.** All the tested drugs were identified successfully, and a visible gap was found between the inter-specific and intra-specific variation. We further demonstrated the importance of data exploration in DNA-based species identification practice by examining the sequence characteristics of relative genera in GenBank. This region of the 12S rRNA gene had a 100% success rate of species recognition within the six medicinal animal species. **Conclusions.** We propose that the 12S rRNA locus might be universal for identifying animal-derived drugs and their adulterants. The development of 12S rRNA for indentifying animal-derived drugs that share a common gene target would contribute significantly to the clinical application of animal-derived drugs and the conservation of medicinal animal species.

INTRODUCTION

Animal-derived drugs refer to a class of drugs that are derived from animals. As we know, animals are the main source of biological products in both modern and traditional medicine, and they are used extensively around the world. More recently, the identification and use of animal-derived drugs has become a focus for many zoologists and ecologists (1-3). Medicinal animal horns and shells (MAHS) drugs, which are considered dermatic derivatives, are one of the most important groups of animal-derived drugs. All the MAHS have two common characteristics of (i) animals' horns or shells are taken for medicinal use; (ii) items are composed mainly of horny cells. For example, deer horn (*Cornu Cervi*) and cornu antelopis (*Cornu Saigae Tataricae*) (from the horns of *Cervus elaphus* and *Saiga tatarica*) are included in the Chinese Pharmacopeia (2010 edition) and Japanese Pharmacopeia (fifteenth edition) (4-5). These drugs are reported to possess anti-coagulatory, anti-fever and anti-inflammatory properties (6-8).

However, with the increased demand for animal-derived drugs, the wild medicinal animal sources are decreasing drastically, and there has been a concomitant increase in the prevalence of adulterated or impostor animal-derived drugs (9-10). Meanwhile, the clinical applications of animal-derived drugs are often non-specific, which leads to the confusion. Thus, to conserve biodiversity and ensure the safety and efficacy of clinical applications, it is urgent that medicinal animal sources be standardized and collected appropriately. The first step of this standardization will be the accurate identification of animal-derived drugs.

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In contrast to fresh specimen of plants, animal-derived drugs are usually processed after they are derived from animals, and are always stored long-term. As a result, their DNA may be partly degraded, which presents additional technical difficulties in species identification. Traditional taxonomic tools and molecular biology techniques, such as RFLP and RAPD, show different shortcomings in identifying animal-derived drugs, so a universal identification method is urgently required (11-13).

DNA-based species identification, with its unique reproducibility, sequence versatility and comparability among different specie, offers a new way to identify animal-derived drugs (14-15). Since *Paul Hebert* originally proposed the concept of DNA barcoding in 2003 (16-18), a growing portion of the scientific community has embraced DNA barcoding as a practical tool for biodiversity studies, i.e., to facilitate inventories of very diverse but taxonomically poorly known regions (19-22). DNA barcoding is a diagnostic technique for species identification, using a short, standardized region. By establishing DNA barcoding systems, we can identify species fast and accurately (23). DNA barcoding, using the mitochondrial *coxI* gene (COI), is now well established for animals. However, the sequence divergence of COI for some animal species, such as *cnidarians* (13) and the West Palaearctic *Pandasyopthalmus* taxa (24), is relatively low, and even invariant.

In this study, we attempted to identify animal-derived drugs by investigating the intra- and interspecific genetic variations of 12S rRNA gene sequenced from MAHS. Intra- and interspecific genetic divergences were assessed by using pairwise calculations. Statistical tests were used to compare divergences, and phylogenetic analyses were performed to look for species monophyly. Genetic clustering algorithms were applied to test whether the coalescent process in the 12S rRNA gene matched species delimitation. Finally, we demonstrated the importance of data exploration in DNA-based species identification practice by examining the sequence characteristics of related genera in GenBank, so as to accurately indentify the MAHS and their confused drugs.

MATERIALS AND METHODS

Taxon sampling

A total of 13 individuals within six species of MAHS, collected from 5 locations in China, were included. Some of the drugs that derived from protected animals were provided by the Animal Research Institute of Chinese Academy of Sciences and China's Forestry Sciences (Table 1). Among these drugs, velvet antler (*Cornu Cervi Pantotrichum*), deer horn (*Cornu Cervi*), antelopis horn (*Cornu Saigae Tataricae*) and buffalo horn (*Cornu Bubali*) are included in the Chinese and Japanese Pharmacopeia. Velvet antler and deer horn are both isolated from *Cervus elaphus* or *Cervus nippon*. Cornu antelopis, cornu bubali originate from *Saiga tatarica* and *Bubalus bubalis*, respectively. The horns of *Ovis aries* and *Capra hircus* are confused with *Cornu Saigae Tataricae*. We also analyzed 38 individuals sequenced from six related species from GenBank. The accession numbers for sequences included in the cladogram are AF091707, AF231028, AF363777, AF401508, AJ457159, AJ490504, AJ846850, AJ849535, AJ885199–AJ885206, AJ885206, AY184433, AY670663, DQ191145–DQ191150, EF490482–EF490491, EU851890–FJ851903, FJ828576– FJ828581, GQ926969– GQ926970 and GU19955–GU19956.

DNA extraction, PCR amplification and sequencing

MAHS were shattered at low temperature, and subsequently grinded in a DNA extraction beveller (Retsch MM400, Germany) for 1 min at 1800 r/min. After that, total DNA was extracted with modified DNAout kits. PCR primers were designed from a ClustalW alignment of 12S rRNA sequences available from GenBank for the 6 species within *Paraxonia*. The forward primer (12S rRNA-LF 5'-CTGGGATTAGATACCCCACTA-3') was anchored at site 417 (numbering relative to the complete 12S rRNA sequence from *Paraxonia*), and the reverse primer (12S rRNA-LR 5'-CAGTATGCTTACCTTGTTACGA-3') was anchored at site 938. We tested the performance of 12S rRNA-LF and 12S rRNA-LR in PCR amplifications using genomic DNA isolated from six species of *Paraxonia*, named *Cervus elaphus*, *Cervus nippon*, *Saiga tatarica*, *Ovis aries*, *Bubalus bubalis* and *Capra hircus*. Preliminary PCR amplifications using 12S rRNA-LF and 12S rRNA-LR had a 100% success rate.

PCRs were performed in 25 μl volumes containing 9.8 μl of PCR-grade water, 4.0 μl of 10×PCR buffer, 4.0 μl of MgCl2 (1.5 mM), 1.0 μl of each primer (0.5 μM), 4.0 μl of dNTPs (0.2 mM), 0.2 μl of Taq polymerase (2.5 units/μl), and 1.0 μl

Table 1. Samples for testing DNA-based species identification

Samples No.	Sources of MAHS	Sampling location	Genbank accessions
01	*Ovis aries*	Sichuan China	HM623875
02	*Bubalus bubalis*	Shandong China	HM623876
03	*Capra hircus*	Qinghai China	HM623877
04	*Cervus nippon*	Jilin China	HM623878
05	*Cervus nippon*	Jilin China	HM623878
06	*Cervus elaphus*	Jilin China	HM623879
07	*Cervus elaphus*	Jilin China	HM623879
08	*Capra hircus*	Qinghai China	HM623880
09	*Ovis aries*	Sichuan China	HM623881
10	*Bubalus bubalis*	Shandong China	HM623882
11	*Capra hircus*	Qinghai China	HM623883
12	*Saiga tatarica*	Xizang China	HM623884
13	*Saiga tatarica*	Xizang China	HM623885

(~50 ng)）of DNA extract. The cycling conditions were an initial step of 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 50 s at 72 °C, followed by 7 min at 72 °C.

The primers 12S rRNA-LF and 12S rRNA-LR were used to amplify a 523-bp fragment of 12S rRNA for all samples. PCR products were electrophoresed in 1.0% TBE agarose gels, stained with ethidium bromide, and visualized under UV light.

PCR products were directly sequenced with the same primers in 20-µl reactions containing 2.0 µl of 5×sequencing buffer, 1.0 µl of each primer (2.5 µM), 13.3 µl of PCR-grade water, 0.2 µl of BigDye (Applied Biosystems), and 1.0 µl of PCR product. The cycling conditions used an initial step of 2 min at 95 °C, followed by 30 cycles of 15 s at 96 °C, 15 s at 52 °C, and 4 min at 60 °C. Sequencing reactions were performed in both directions by using the PCR primers. Sequencing products were purified with Sephadex G-50 (Sigma) columns in multiscreen HV filter plates (Millipore) and then run on an Applied Biosystems ABI 3730XL DNA analyzer (Applied Biosystems Co., USA). The resultant sequences were assembled, edited, and aligned in Seq-Scape V.3.0 (Applied Biosystems, America) before being uploaded to the Barcode of Life Data System.

Data analysis

We downloaded all complete 12S rRNA genes (primarily from complete mitochondrial genomes) for 6 related species of *Paraxonia* that were available in GenBank (November 2010). The sequences were cropped to the 523-bp region with the primer pair 12S rRNA-LF and 12S rRNA-LR. The taxonomic sample and GenBank accessions are available in the public project ‘‘GenBank (12S rRNA)’’ in the Barcode of Life Data System (www.barcodinglife.org).

The sequences were checked and merged using CodonCode Aligner V 3.61 (CodonCode Co., USA), and examined by ClustalX V 2.0 (Higgins D.G.). Polymorphic sites and haplotype diversity were calculated with dnaSP (Universitat de Barcelona, Spain). Pairwise distances were calculated with MEGA V 4.0.1 (25). Wilcoxon signed rank tests were performed to compare intra- and interspecific variability for each pair of 12S rRNA gene following Kress and Erickson (26). The distribution frequency of inter- and intraspecific genetic divergences was calculated with the computer language Perl. We evaluated gaps by comparing the distribution of intra- versus interspecific divergences (27). To study how the presence of MAHS might influence the inferences from 12S gene, we performed a neighbor-joining (NJ) analysis under the K2P model and calculated the sequence divergence among haplotypes in each dataset in MEGA V 4.0.1 (25). The evolutional paths and step lengths of the haplotypes were calculated with Network 4.1.5 (Fluxus Technology

LTD., UK) to evaluate the distance of the organic evolution. BLAST1, which was implemented using the BLAST program (Version 2.2.17), was used to search for the reference database for each query sequence (28).

RESULTS

Sequence information, PCR amplification capability and sequencing success rate

PCR amplification success and sequencing success are important indices for evaluating DNA-based species identification. In this study, both PCR amplifications and sequencing had a 100% success rate. The PCR amplification electropherogram obtained by agarose gel electrophoresis is shown in Fig. 1. A total of 13 sequences were analyzed with dnaSP (Universitat de Barcelona, Spain) and 523 sites (i.e., base pairs) were obtained, including 516 sites excluding gaps/missing data. Of these, 83 sites (15.9%) were polymorphic; the nucleotide diversity (Pi) was 6.9%. The average nucleotide composition of all sequences was calculated in MEGA: T=22.7%, C=22.8%, A=37.2%, G=17.3% for an average AT-richness of 59.9%.

Haplotype analysis

The sequences of the 6 species were analyzed with dnaSP (Universitat de Barcelona, Spain). A total of 11 haplotypes were obtained, and the haplotype diversity was 0.9744. This high haplotype diversity reflects conspicuous variance in the 6 medicinal animal species tested.

Intra- and interspecific genetic divergences analysis

Indeed, for successful DNA-based species identification, sequence variation must be high enough between species so that they can be identified from one another; however, it must be low enough within species that a clear threshold between intra- and interspecific genetic variations can be defined. In this study, the mean intra- and interspecific genetic variations are 0.48% and 8.64%, respectively. All the intraspecific variations measured were smaller than 2.8%, and all the interspecific genetic variations were larger than 2.8%. Importantly, each MAHS could be successfully discriminated from its frequently confused drugs: antelopis horn and sheep horn (*Cornu Ovis Aries*) have a genetic variation of 8.22%; antelopis horn and goat horn (*Cornu Capra Hircus*) have a genetic variation of 7.84%. From the above analysis, we could identify each unique MAHS species according to DNA barcoding standards (3% or higher sequence divergence).

Gap test

Ideal DNA-based species identification should have a conspicuous spacer region delineating intra- and interspecific variation (29-30). In this study, only a small portion of the MAHS in question had overlapping intra- and interspecific variation. A visible gap could be found (Fig. 2), indicating that all the MAHS were able to be identified.

Phylogenetic analysis

The cladogram was clustered based on neighbor-joining (NJ) analysis, and it demonstrates that all MAHS haplotypes strongly grouped with their orthologous mtDNA. The sequences were assembled to six groups in the dendrogram (Fig. 3): Group I comprised of all 12S rRNA sequences from GenBank by searching using the keywords" *Cervus elaphus*" and samples-06, 07; Group II comprised of sequences of "*Cervus nippon*" from GenBank and Samples-04, 05; Group III comprised of sequences of "*Bubalus bubalis*" from GenBank and Samples-02, 10; Group IV comprised of sequences of "*Saiga tatarica*" from GenBank and Samples-12, 13; Group V comprised of sequences of "*Capra hircus*" from GenBank and Samples-03, 08, 11; and Group VI comprised of sequences of "*Ovis aries*" from GenBank and Samples-01, 09. We deduced from the cladogram that this region of 12S rRNA gene was prospective to be investigated for the discrimination of the MAHS and taxonomic taxa.

Haplotype network analysis

We included haplotype network analysis because these not only show the grouping of different species but also provide the evolutional paths and step lengths (31-34). In this study, a total of 11 haplotypes were analyzed with Network 4.1.5 (Fluxus Technology LTD., UK). The network showed that *Antilopinae* (H_10–H_11) was genetically more distant from *Bovinae* (H_1–H_5) than from *Cervinae* (H_6–H_7) in contrast with their morphologic variances (Fig. 4.). As we know, *Antilopinae* and *Bovinae* belong to *Bovidae* morphologically, and therefore it was expected that they should have a shorter genetic distance; however, the 12S rRNA data indicated a relatively long evolutional time between the two.

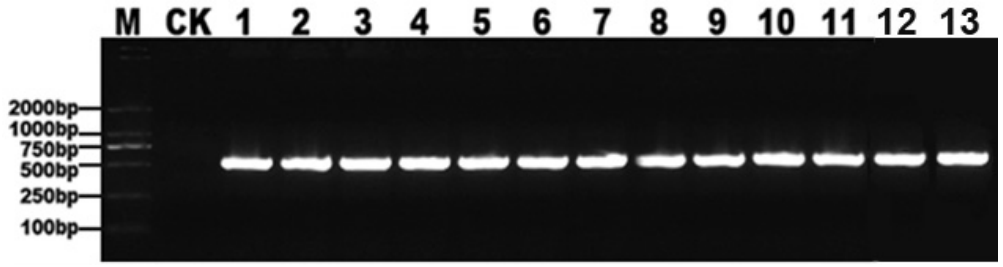

Figure 1. PCR amplification results of 12s rRNA. M: Marker (DL2000), CK: Control, 1–13 refers to the samples 01–13 in Table 1.

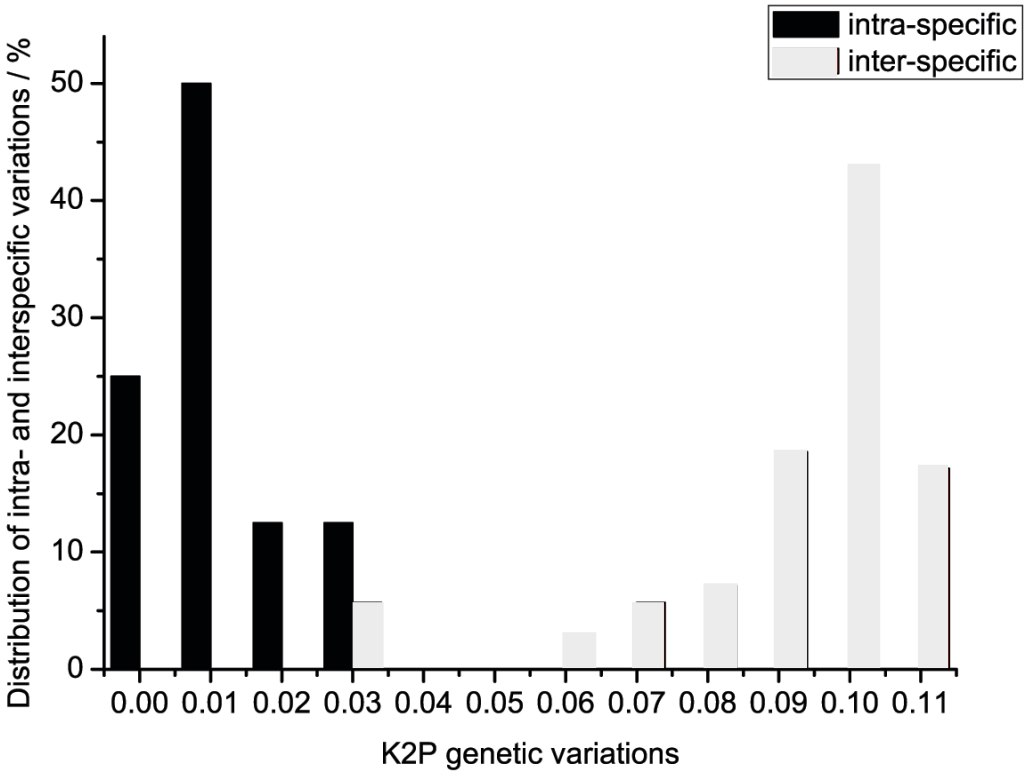

Figure 2. Relative distribution of inter-specific and intra-specific variations.

We deduced from these haplotype network analysis that the molecular variances were much larger than morphological variances among the species within *Bovidae* and *Capridae*.

Evaluation of identification capability

The comparison of species identification capability relative to other genetic fragments is one of the criterions for evaluating the quality of different sequences. In this study, the taxonomic affiliation of each sequence (including 13 test samples and 38 individuals from GenBank) was determined by BLAST1 against the GenBank database. The result indicated that 100% of the mitochondrial sequences were successfully identified.

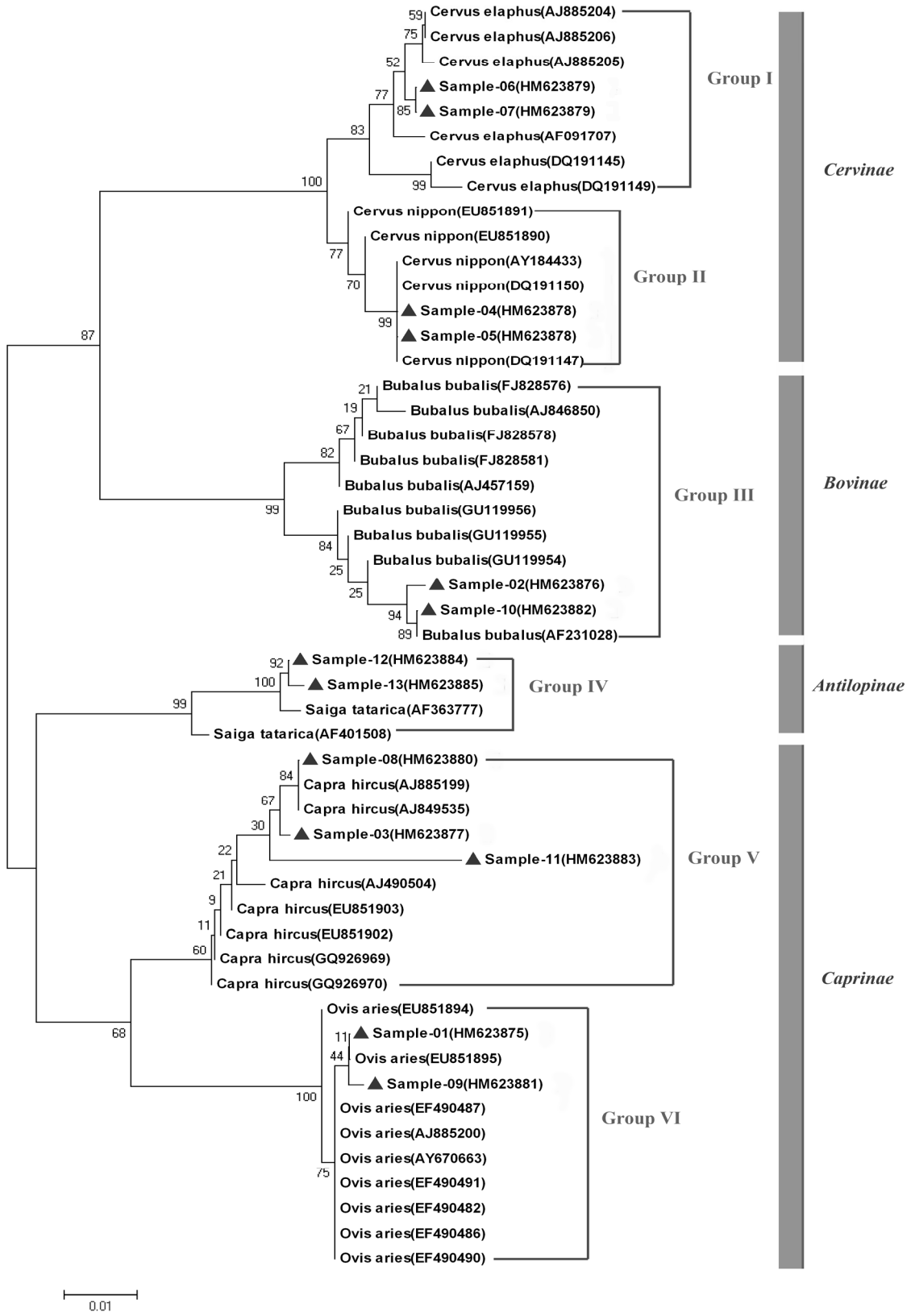

Figure 3. Neighbor-Joining tree based on Kimura-2-parameter distances for 51 sequences of 12S rRNA genes. ▲ represent the experimental individuals.

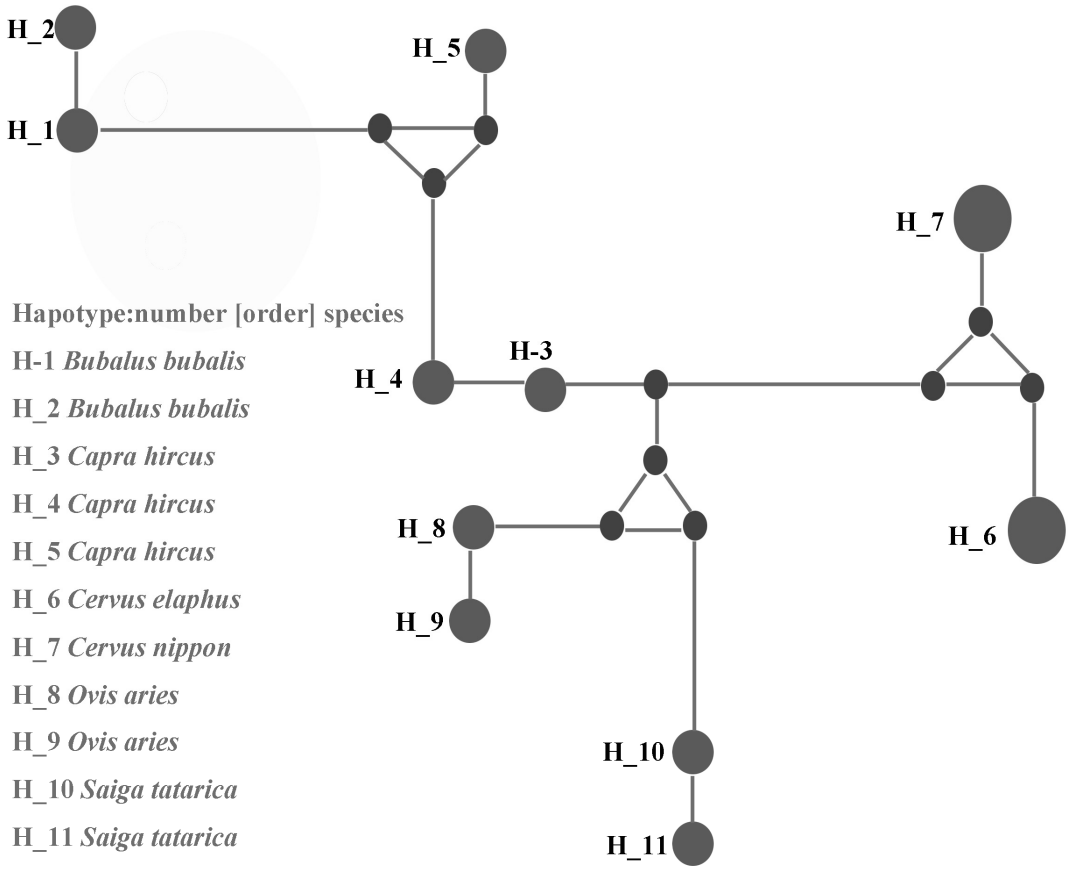

Figure 4. Haplotype network for MAHS ● represent the amount of each haplotype sample, ● represent the evolutional pathway, and the lengths of the lines represent evolutional step length.

DISCUSSION

Conservation of medicinal animal species is an urgent but long-term work, and it is the taxonomists and ecologists of the world who are tasked with identifying the priority species and spaces to ensure that we carry out it efficiently. Unfortunately, there is, as yet, no effective method for the identification of animal-derived drugs, which leads to the confusion of clinical application of animal-derived drugs.

Distinguished from the living plant or fresh tissues of animals, animal-derived drugs are often processed specially after their isolation from animals, e.g., by air drying or dehydration, so morphology is not always a suitable criterion for identifying animal-derived drugs. Meanwhile, their chemical components are often complex and may include many biomacromolecules, which makes traditional taxonomic tools and chemical identification techniques inefficient. In contrast, DNA-based species identification has a low threshold and high efficiency, which makes it a sensible method for the identification of animal-derived drugs.

In this study, the mean interspecies variances are much larger than that the mean intraspecies variances. Because a high interspecies variance is the premise of identifying species (35-36), we conclude that this fragment of the 12S rRNA gene is efficient for the discrimination of the species of MAHS species, allowing us to easily distinguish spurious breeds and frequently confused drugs. The results also indicate that this fragment of the 12S rRNA gene may probably not only be suitable for the exploration of phyletic evolution relationship among the species within MAHS but also offer a valuable reference for the amplification of DNA-based species identification of related animal species.

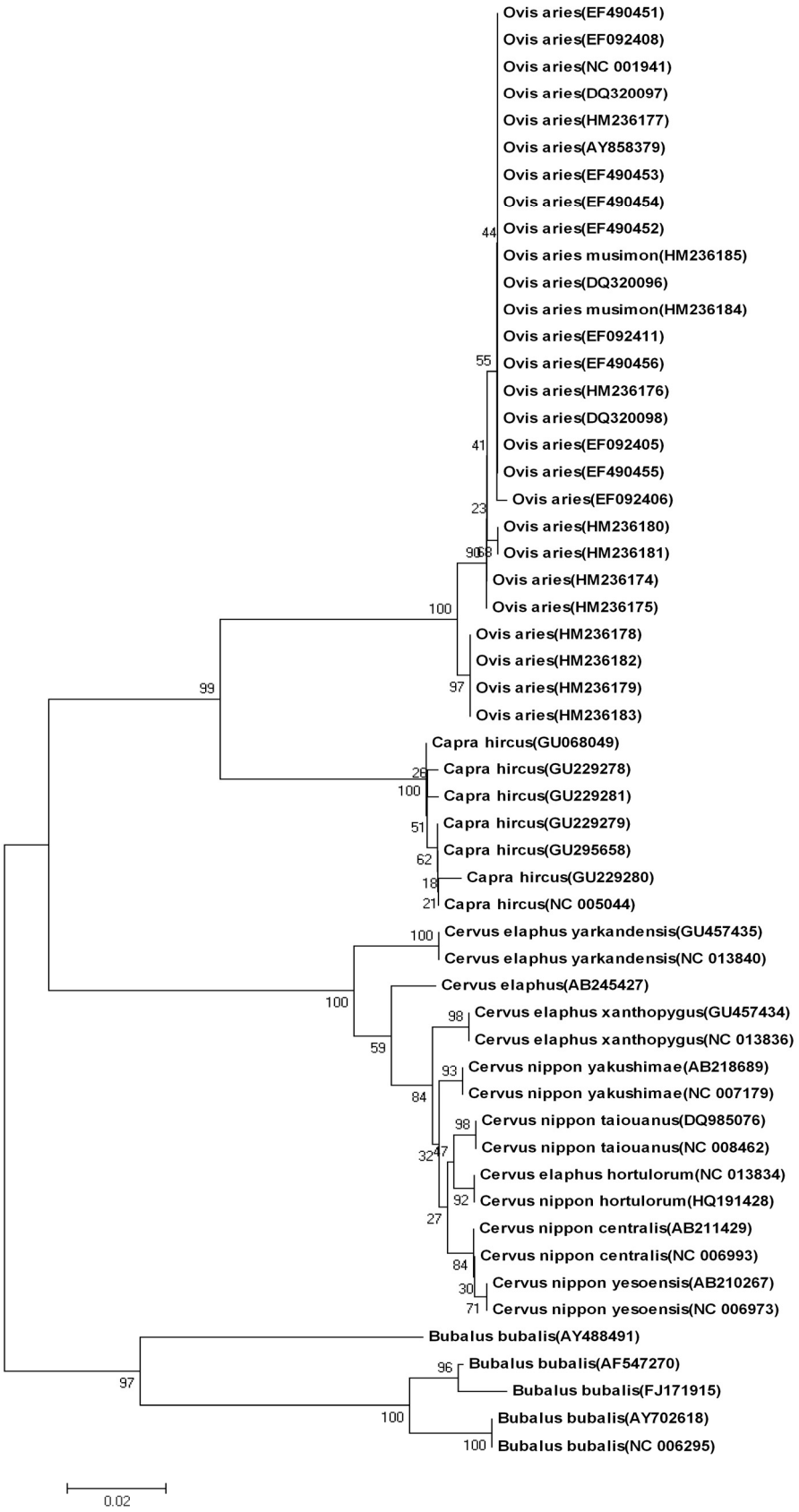

Figure 5 Neighbor-Joining tree based on Kimura-2-parameter distances for 54 sequences of COI gene.

PCR primers were primarily designed according to previous reports (37) and the database available from GenBank. We designed primers 12S rRNA-LF and 12S rRNA-LR, which worked universally for *Paraxonia*. We further suggest that these primers can be applied for 12S rRNA gene within *Paraxonia* in further research.

MAHS present several technical challenges that fresh tissues do not. For example, MAHS are cuticularized and thus very solid. Furthermore, some MAHS samples may have been stored for a long time, and the DNA may be partly degraded. To ensure high DNA extraction efficiency, we investigated several kits. Finally, modified DNAout kits were chosen for this study, and silicon was selected as the substrate to remove the specified proteins and organic compounds that may interfere with the DNA extraction. Moreover, we improved the extract process by increasing the amount of sample used to increase the total amount of DNA extracted. All these procedures ensured high extraction efficiency. The OD ratios (260/280) of al the samples in our study exceeded 1.7, indicating their purity.

As we know, mitochondrial coxI gene (COI) is one of the core barcodes for animals and is now well established for animals, so the utility of COI was tested in this study. The total of 54 COI sequences that were closely related to the species of MAHS was downloaded from GenBank (July 2011). The genetic divergences analysis, barcoding gap test, phylogenetic analysis and the identification capability were carried out following the procedure of 12S rRNA. The results showed that except for the missing data of the species '*Saiga tatarica*', all the MAHS haplotypes grouped with their orthologous mtDNA, the sequences were assembled to four groups in the dendrogram (Fig. 5). The taxonomic affiliation of the 54 sequences was determined by BLAST1 against the GenBank database, indicating that all the mitochondrial sequences were successfully identified. By comparing with the result of 12S rRNA, the interspecies variance between *Cervus nippon* and *Cervus elaphus* was less significant, which indicated that 12S rRNA gene was more efficient within these species.

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