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Research article

Genetic diversity and relationship of D-loop sequences among animals in Cervidae and their application for preliminary screening of sambar deer origin in unknown meat

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Abstract

The D-loop sequences of seven Cervidae species—sambar deer (*Rusa unicolor*), rusa deer (*Rusa timorensis*), sika deer (*Cervus nippon*), Eld's deer (*Rucervus eldii*), muntjac (*Muntiacus muntjak*), chital deer (*Axis axis*) and hog deer (*Axis porcinus*)—were analyzed for their genetic diversity and relationships. D-loop amplifications of DNA extracted from blood samples (five specimens/species) showed product sizing in the range 1,000–1,100 bp. These amplicons were sequenced and analyzed for genetic diversity within species. Genetic diversity was highest in hog deer (haplotype diversity (h) = 0.9, nucleotide diversity (π) = 0.02796) and lowest in muntjac (h = 0.4, = 0.00879). The phylogenetic relationship of the D-loop sequence (701 bp) was analyzed. Most samples were in the same subclade as their original species, indicating the possibility of using the D-loop as target DNA for cervid species identification. This was tested by examining three meat samples of unknown species origin. The D-loop amplification of the unknown meats showed two different sizes of amplicons of 1,000 bp (Unknown1) and 1,500 bp (Unknown2, Unknown3). These D-loop sequences (281 bp) were compared with those of cervid references. The two unknown meats presenting 1,500 bp amplicons were an outgroup and one was in the same subclade as muntjac. The results were confirmed using BLAST analysis from which the two outgroup samples were similar to domestic pig (99.74%) and dog (97.82%), respectively, while the third sample had greatest similarity to muntjac (97.81%). The method presented here could be used with meat samples for preliminary screening to prove whether or not the sample was from sambar deer (a protected species in Thailand), which could be observed by either the size of the amplicons or the phylogenetic relationship of the D-loop sequence.

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Introduction

Species identification of seized evidence is essential for prosecution in cases involving illegal trade in endangered animals. For this purpose, techniques based on analytical DNA analysis are preferable to more subjective morphological analysis because the nucleotide sequences showing inter-species variation can be targeted in the DNA analysis. These can be either gene sequences such as *Cyt b* (Singh et al., 2012), *16S rRNA* (Sarri et al., 2014) or *ND2* (O'Bryhim et al., 2017) or non-gene sequences such as D-loop (Gupta et al., 2011). In such cases, the target DNA is primarily amplified, followed by examination of the amplicons to determine the species either by the amplicon profile (Martinez and Yman, 1998; Sumathi et al., 2015) or the amplicon size (Geng, 2015; Safdar and Junejo, 2015).

In Thailand, the sambar deer (*Rusa unicolor*) is a protected animal listed under the Wildlife Preservation and Protection Act, B.E. 2562 (Wildlife Preservation and Protection Act, 2019). Nowadays, sambar deer meat has become popular among consumers and while currently, the rearing of sambar deer for meat production is allowed, legal permission for farming must be first approved. Consequently, the smuggling of sambar deer meat has been reported as well as cases recorded of other meats sold in markets as sambar deer meat (Vorajinda et al., 2019). In both cases, identification of sambar deer meat is important for successful prosecution. The study of deer meat identification was first reported by Matsunaga et al. (1998) who differentiated red deer from sika deer using polymerase chain reaction (PCR)-restriction fragment length polymorphism of the *Cyt b* gene. However, since large amplicons are required to carry out enzymatic restriction, this technique may not be suitable for degraded samples such as processed meat and PCR amplification with species-specific primer has been introduced with PCR amplification on *12S rRNA* for species identification of red deer, fallow deer and roe deer meats being documented (Fajardo et al., 2007). Nonetheless, a species-specific primer cannot differentiate samples of different subspecies.

Another technique, called forensically informative nucleotide sequencing (FINS), has become a widely used method for species identification, where a species-specific sequence is amplified and sequenced to identify species. For example, the ingredients in food products have been successfully identified using FINS (Armani et al., 2013; Huang et al., 2014; Santaclara et al., 2014). Vorajinda et al. (2019) showed that a partial sequence of *Cyt b* had species identification capability in the Cervidae using FINS analysis but unfortunately, sambar deer and rusa deer could not be differentiated as they are very closely-related species. Therefore, another DNA region, the D-loop, is of interest because this nucleotide variation has been reported for its ability to classify subspecies (Wu et al., 2004; Feng et al., 2008). Additionally, the nucleotide comparison of D-loop sequences has successfully been used for Chinese sika deer identification of unknown animal skins (Wu et al., 2005).

The current study aimed to examine the genetic diversity of D-loop sequences in seven species of animals in the Cervidae and to determine the phylogenetic relationships among them. From these results, the possibility of identifying sambar deer species using the FINS method was investigated. Meat samples of unknown origin were analyzed to determine the likelihood they were sambar deer. The results of the study should be useful for wildlife forensic investigation.

Materials and Methods

Samples

The study used seven species of Cervidae—sambar deer (*R. unicolor*), rusa deer (*R. timorensis*), sika deer (*C. nippon*), Eld's deer (*R. eldii*), chital deer (*A. axis*), hog deer (*A. pocinus*) and muntjac (*M. muntjak*)—sourced from Khon Kaen Zoo, Thailand. Blood samples (each 5 mL) were collected from five animals of each species. The blood samples were kept in blood collection tubes containing 500 µL of 0.5 M ethylene-diamine-tetraacetic acid at 4°C until use. This study was carried out with permission from the Animal Ethics Committee (ACUC-KKU-43/61), Khon Kaen University, Khon Kaen, Thailand. Three samples of unknown meat were retrieved from local restaurants located in Kaeng Krachan district, Phetchaburi (Unknown1, Unknown2) and Tha Uten district, Nakhon Phanom (Unknown3), Thailand. They were all advertised as sambar deer meat by the restaurants. The meat samples were kept in cleaned plastic containers at 4°C until used.

Molecular analysis of D-loop sequences

The genomic DNA was extracted from blood (50 µL) and meat (25 mg) samples using DNeasy blood & tissue (QIAGEN) according to the manufacturer's instructions. The quantity and quality of extracted DNA was determined using a NanoDrop spectrophotometer. All the PCR amplifications of D-loop sequences were performed with the following thermal profile: 94°C for 2 min followed by 30 cycles of

denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The primers used were designed from the conserved region of tRNA-Pro and tRNA-Phe sequences on the mitochondrial DNA of cervids (AF291884.1), namely DL1 5'-TCAACACCCAAAGCTGAAGTT-3' and DL2 5'-TTTCAGTGCCTTGCTTTGCTG-3', respectively. The PCR reaction, comprising 0.2 ng DNA, 0.4 µM of each primer and 1xPCR Master Mix (Vivantis), was set up and then, deionized water was added to bring the reaction volume up to 25 µL. PCR products were determined using agarose gel electrophoresis. For D-loop sequence analysis, the PCR products were sent to Macrogen, South Korea. Sequences were verified, assembled and aligned using the BioEdit 7.0 program (Hall, 1999).

Analyses of genetic diversity and relationship

The nucleotide diversity (π), the number of haplotypes (n) and haplotype diversity (h) within and among individuals were analyzed using the DnaSP 5.0 software package (Librado and Rozas, 2009). The phylogenetic analysis of D-loop sequences (overlapping sequences sized 701 bp) were examined using the MEGA version X program (Kumar et al., 2018). Using this program, the genetic distance analysis of 35 taxa was performed based on the K2-parameter (substitution framework with a free parameter for both transitions and transversions, accounting for the higher substitution rate of transitions in mitochondrial DNA). Then, the phylogenetic tree was constructed using neighbor-joining. The reliability of the branches of the tree was examined using 1,000 bootstrap re-sampling.

Species identification of unknown meats

DNA extractions from the unknown meat samples were performed, followed by PCR amplifications of the D-loop region, as described earlier. The DNA sequences of amplicons were analyzed based on comparison with D-loop reference sequences of known cervids (38 taxa in total) using the MEGA version X program with genetic distance analysis (K2-parameter). Then, neighbor-joining with 1,000 bootstrap re-sampling was applied for phylogenetic tree construction. As the D-loop sequences of the unknown samples were shorter than those of the cervid references, the overlapping sequence that could be compared was 281 bp.

Results

Polymerase chain reaction amplification of D-loop

The D-loop regions of the DNA samples were amplified using the designed primer pair DL1 and DL2 that had been designed from the conserved sequences flanking the D-loop region of cervids, namely tRNA-Pro and tRNA-Phe. Amplicons with sizes of 1,000 bp (Eld's deer, muntjac, chital deer, hog deer), 1,100 bp (rusa deer, sika deer) and 1,150 bp (sambar deer) were produced, indicating the success of D-loop amplification (Fig.1). The D-loop amplifications of 35 DNA samples carried out in this study identified PCR products with the sizes in the range 1,000–1,150 bp, depending on the species. The nucleotide sequences of amplicons were deposited in GenBank with the Accession numbers: Sambar1-5 (MZ501700–501704), Rusa1-5 (MZ501705–MZ501709), Sika1-5 (MZ501710–MZ501714), Elds1-5 (MZ501715–MZ501719), Muntjac1-5 (MZ501720–MZ501724), Chital1-5 (MZ501725–MZ501729) and Hog deer1-5 (MZ501730–MZ501734).

Genetic diversity and relationship

The partial nucleotide sequences (701 bp) of D-loop amplicons (1,000 bp) were analyzed for the number of haplotypes, haplotype diversity and nucleotide diversity.

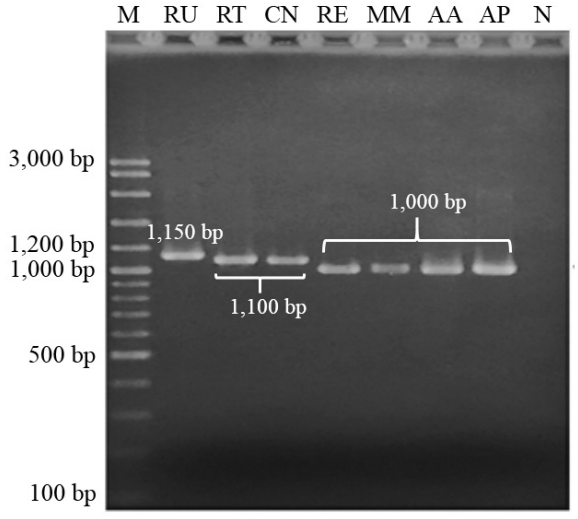

Fig. 1 Polymerase chain reaction (PCR) amplification of D-loop from DNA samples of seven species of cervids: sambar deer (RU), rusa deer (RT), sika deer (CN), Eld's deer (RE), muntjac (MM), chital deer (AA) and hog deer (AP), where sizes of PCR products are compared with 100 bp DNA ladder (M) and N represents negative control.

The molecular diversity statistics for all samples and for each species are summarized in Table 1. The *h* values of each species were in the range 0.400–0.900. The nucleotide diversity was greatest in hog deer (π = 0.02796) and lowest in muntjac (π = 0.00879). The genetic relationships of the D-loop sequences (701 bp) among the 35 samples of cervids are shown in Fig. 2. The D-loop sequences were classified into two clades, with sambar deer, rusa deer, sika deer and Eld's deer in clade 1 and muntjak, hog deer and chital deer in clade 2. Notably, most sequences were clustered together in the same subclade with their original species except for hog deer5 and rusa3, which were presented in the subclade of chital deer and sambar deer, respectively. From these results, it was clearly possible to use the D-loop sequence as the target DNA for the classification of animals in the Cervidae.

Species identification

The D-loop sequences of the unknown meats (Unk1–Unk3) were amplified using the primers DL1 and DL2. PCR products were identified in all samples (Fig.3) with sizes of approximately 1,000 bp (Unk1) and 1,500 bp (Unk2, Unk3). The DNA sequences of these D-loop sequences were analyzed and phylogenetically compared with those of referenced species based on the overlapping sequences of the D-loop (281 bp). The results and the pairwise distances are shown in Fig.4 and Table 2, respectively. From the phylogenetic analysis, Unknown1 was clustered in the same clade as muntjak. In contrast, Unknown2 and Unkown3 were positioned in an outgroup of the phylogenetic tree indicating the possibility of their non-cervid origin. The variable nucleotide positions of D-loop sequences from sambar deer and Unk1-Unk3 are presented in Fig. 5.

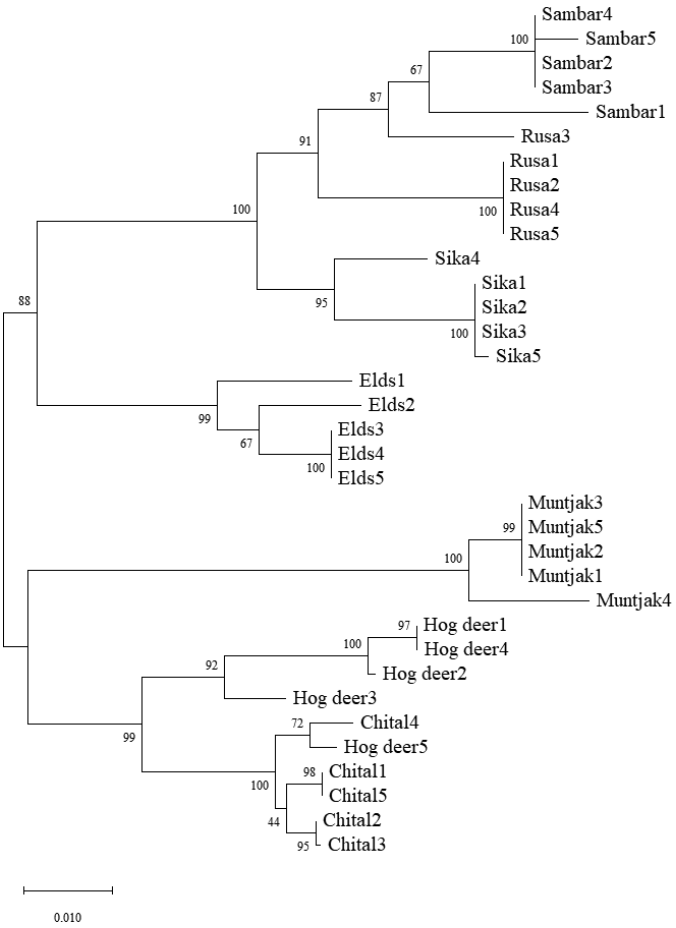

Fig. 2 A neighbor-joining tree of D-loop sequences (701 bp) among cervid samples, where bootstrap values are indicated at each node.

Table 1 Number of individual samples (N), number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (π) in D-loop (701 bp) of each cervid

Cervid	N	Number of haplotypes	h	π
Sambar deer (*R. unicolor*)	5	3	0.700±0.218	0.01484±0.00957
Rusa deer (*R. timorensis*)	5	2	0.400±0.237	0.01977±0.01256
Sika deer (*C. nippon*)	5	3	0.700±0.218	0.01343±0.00872
Eld's deer (*R. eldii*)	5	3	0.700±0.218	0.01830±0.01171
Muntjac (*M. muntjak*)	5	2	0.400±0.237	0.00879±0.00594
Chital deer (*A. axis*)	5	3	0.800±0.164	0.00902±0.00609
Hog deer (*A. porcinus*)	5	4	0.900±0.161	0.02796±0.01757
Overall	35			

Values are shown as mean ± SE

Fig. 3 Polymerase chain reaction amplification of D-loop from DNA samples of unknown meats (Unk1–Unk3), where M indicates size marker and N is negative control.

Fig. 4 A neighbor joining tree of D-loop (281 bp) among the unknown meats (Unk1–Unk3) and referenced sequences of cervids, where bootstrap values are exhibited on nodes.

Table 2 Pairwise distances (distance measure = Kimura-2 parameter) among reference cervids and unknown samples (below diagonal) and values of corresponding standard error computed using bootstrapping (above diagonal)

	Sambar_h1	Sambar_h2	Sambar_h3	Rusa_h1	Rusa_h2	Sika_h1	Sika_h2	Sika_h3	Elds_h1	Elds_h2	Elds_h3	Muntjac_h1	Muntjac_h2	Chital_h1	Chital_h2	Chital_h3	Hog_h1	Hog_h2	Hog_h3	Hog_h4	Unk1	Unk2	Unk3
Sambar_h1		0.012	0.012	0.013	0.014	0.016	0.014	0.016	0.021	0.018	0.017	0.025	0.026	0.022	0.022	0.023	0.025	0.022	0.019	0.023	0.025	0.034	0.054
Sambar_h2	0.036		0.000	0.017	0.014	0.017	0.015	0.017	0.021	0.020	0.019	0.024	0.025	0.020	0.020	0.021	0.022	0.021	0.018	0.020	0.022	0.035	0.054
Sambar_h3	0.036	0.000		0.017	0.014	0.017	0.015	0.017	0.021	0.020	0.019	0.024	0.025	0.020	0.020	0.021	0.022	0.021	0.018	0.020	0.022	0.035	0.054
Rusa_h1	0.040	0.065	0.065		0.013	0.012	0.015	0.012	0.018	0.017	0.014	0.022	0.023	0.018	0.018	0.017	0.019	0.017	0.015	0.018	0.024	0.033	0.052
Rusa_h2	0.044	0.048	0.048	0.040		0.017	0.017	0.017	0.020	0.018	0.018	0.026	0.026	0.019	0.021	0.022	0.022	0.022	0.018	0.020	0.023	0.034	0.055
Sika_h1	0.052	0.070	0.070	0.036	0.061		0.012	0.000	0.021	0.020	0.017	0.026	0.026	0.022	0.023	0.022	0.022	0.022	0.019	0.017	0.023	0.026	0.036	0.057
Sika_h2	0.040	0.057	0.057	0.048	0.065	0.036		0.012	0.020	0.018	0.019	0.027	0.025	0.021	0.022	0.022	0.021	0.019	0.018	0.021	0.025	0.037	0.056
Sika_h3	0.052	0.070	0.070	0.036	0.061	0.000	0.036		0.021	0.020	0.017	0.026	0.026	0.022	0.023	0.022	0.022	0.019	0.017	0.023	0.026	0.036	0.057
Elds_h1	0.091	0.096	0.096	0.074	0.087	0.092	0.087	0.092		0.012	0.013	0.025	0.025	0.019	0.021	0.020	0.018	0.017	0.017	0.020	0.026	0.035	0.053
Elds_h2	0.078	0.091	0.091	0.069	0.073	0.087	0.074	0.087	0.036		0.012	0.023	0.024	0.017	0.020	0.019	0.019	0.018	0.017	0.018	0.023	0.034	0.053
Elds_h3	0.065	0.078	0.078	0.048	0.069	0.065	0.078	0.065	0.040	0.036		0.024	0.024	0.018	0.018	0.017	0.018	0.017	0.016	0.017	0.024	0.033	0.052
Muntjac_h1	0.127	0.127	0.127	0.109	0.127	0.137	0.142	0.137	0.123	0.114	0.114		0.012	0.022	0.023	0.025	0.025	0.024	0.022	0.025	0.014	0.035	0.061
Muntjac_h2	0.137	0.137	0.137	0.118	0.137	0.137	0.132	0.137	0.123	0.123	0.123	0.036		0.024	0.025	0.027	0.026	0.025	0.021	0.027	0.015	0.036	0.059
Chital_h1	0.101	0.083	0.083	0.074	0.083	0.106	0.092	0.106	0.082	0.069	0.073	0.108	0.127		0.008	0.011	0.016	0.015	0.013	0.009	0.023	0.033	0.055
Chital_h2	0.101	0.083	0.083	0.074	0.083	0.106	0.092	0.106	0.091	0.087	0.073	0.113	0.132	0.016		0.011	0.016	0.015	0.015	0.011	0.023	0.032	0.056
Chital_h3	0.106	0.097	0.097	0.070	0.097	0.102	0.097	0.102	0.082	0.078	0.065	0.127	0.147	0.028	0.028		0.016	0.015	0.015	0.006	0.025	0.032	0.055
Hog_h1	0.119	0.101	0.101	0.083	0.101	0.101	0.096	0.101	0.069	0.073	0.069	0.123	0.133	0.061	0.061	0.061		0.008	0.014	0.016	0.025	0.033	0.054
Hog_h2	0.101	0.091	0.091	0.065	0.101	0.083	0.078	0.083	0.069	0.073	0.060	0.123	0.133	0.052	0.052	0.052	0.016		0.013	0.015	0.024	0.032	0.054
Hog_h3	0.082	0.073	0.073	0.056	0.073	0.074	0.078	0.074	0.065	0.069	0.056	0.100	0.100	0.044	0.052	0.052	0.044	0.036		0.015	0.023	0.032	0.053
Hog_h4	0.106	0.087	0.087	0.079	0.087	0.111	0.097	0.111	0.087	0.073	0.069	0.127	0.147	0.020	0.028	0.008	0.061	0.052	0.052		0.025	0.032	0.056
Unk1	0.127	0.108	0.108	0.118	0.118	0.137	0.132	0.137	0.133	0.114	0.114	0.044	0.057	0.118	0.113	0.127	0.133	0.123	0.109	0.127		0.036	0.060
Unk2	0.245	0.250	0.250	0.223	0.234	0.255	0.267	0.255	0.238	0.228	0.222	0.249	0.260	0.223	0.223	0.217	0.223	0.212	0.217	0.217	0.260		0.051
Unk3	0.473	0.480	0.480	0.451	0.480	0.504	0.496	0.504	0.459	0.451	0.444	0.529	0.513	0.473	0.488	0.473	0.466	0.466	0.466	0.480	0.521	0.437	

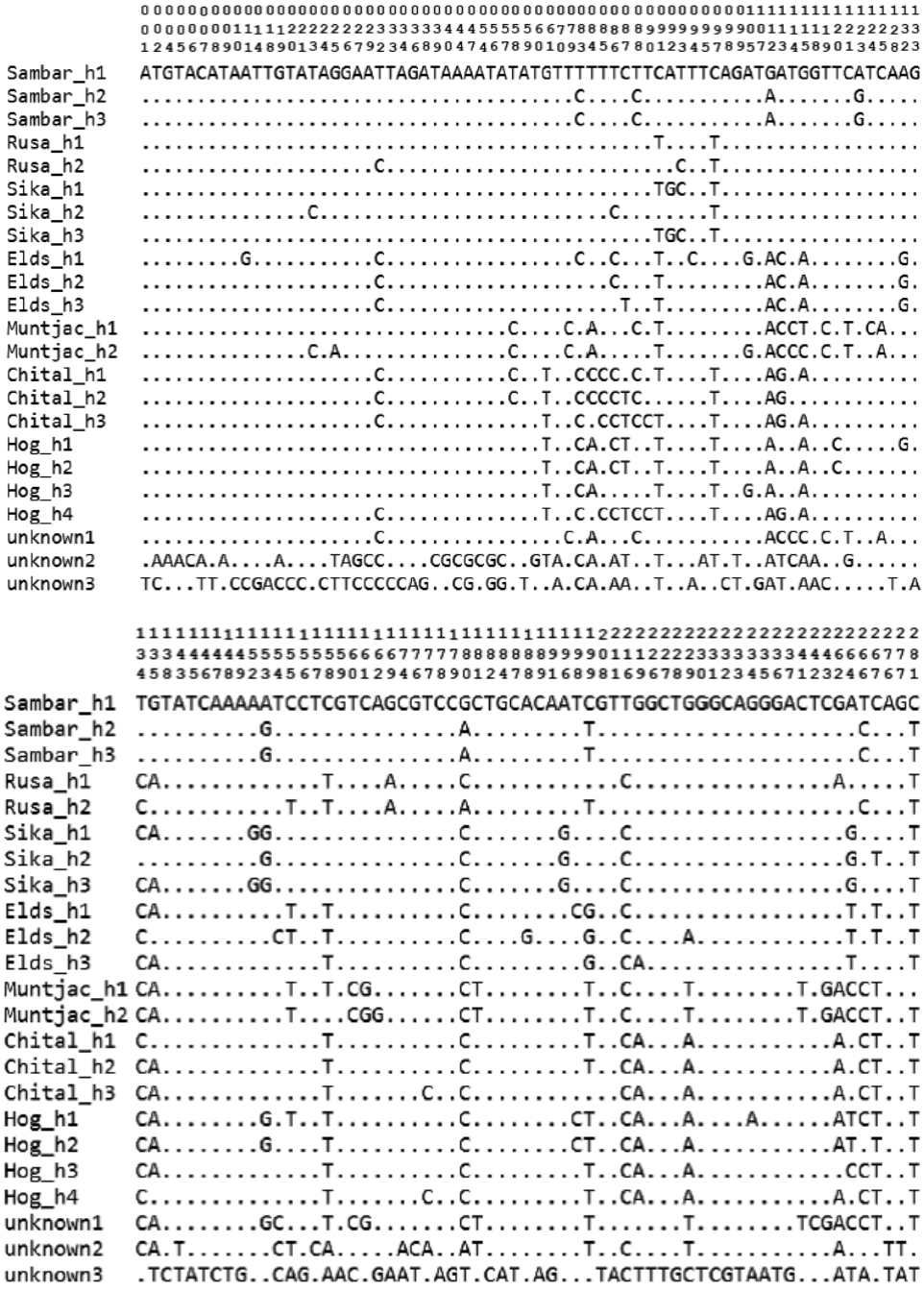

Fig. 5 Polymorphic sites in partial sequences of D-loop (281 bp) from seven reference cervids species and three unknown meats (Unk1–Unk3), where three-digit numeric header values indicate position of variable nucleotide and "." represents identical nucleotides

Discussion

The current study investigated species identification of cervids animals, particularly sambar deer as a protected animal in Thailand. Comparisons of DNA sequences using the FINS technique was used as a molecular tool. The D-loop sequence was targeted because it has been proven as a successful molecular marker for species (Hsieh et al., 2011; Gu et al., 2016) and subspecies (Zhang et al., 2006) identification. However, before the identification of sambar deer could be carried out, it was necessary to examine the nucleotide sequences of D-loop from cervids. PCR amplifications of D-loop sequences were investigated from seven cervids (5 samples each) of sambar deer, rusa

deer, sika deer, Eld's deer, muntjac, chital deer and hog deer. The sizes of amplicons ranged from 1,000–1,150 bp, consisting of the D-loop sequence plus adjacent tRNA-Pro (65 bp) and tRNA-Phe (68 bp). The nucleotide sequences of these amplicons were analyzed and from the overlapping sequences of the D-loop (701 bp), the nucleotide variations within species were highest in hog deer and lowest in rusa deer and muntjac (Table 1). These results differed from Hill et al. (2019) in that the current study did not identify any genetic variations on the D-loop within the hog deer species. This may be explained by the fact that current samples originated from the same species but from different populations, perhaps resulting in dissimilar genetic variations (Wu and Fang, 2005). Even though samples from the same population were used in the current study, sequence analysis of different DNA targets also gave dissimilar genetic variations, as muntjac in the Khon Kaen Zoo had the highest genetic variations among *Cyt b* sequences (Vorajinda et al., 2019).

The sambar deer species were identified using analysis of the phylogenetic relationships of D-loop sequences (701 bp). Most animals were grouped in the same subclade as their originating species except for rusa3 and hog deer 5, which were in the same subclade as sambar deer and chital deer, respectively (Fig.2). This was also reported in other studies in which close proximity between rusa deer and sambar deer (Martins et al., 2018) and hog deer and chital deer (Hill et al., 2019) were elucidated. Nonetheless, it was evident that the nucleotide sequence of the D-loop (701 bp) could be used for cervid identification. Therefore, unknown meats from local restaurants were identified as being of having cervid origins. The D-loop regions were amplified using the DL1 and DL2 primers and amplicons were produced from all three unknown meats with sizes of 1,000 bp (Unknown1) and 1,500 bp (Unknown2 and Unknown3). Subsequently, the D-loop sequences of the unknown meats were compared with those of D-loop references, though only the overlapping sequences of 281 bp could be compared. From the phylogenetic analysis, Unknown1 was grouped in the same subclade as muntjak (Fig. 4). Notably, Unknown2 and Unknown3 were in the outgroup. To confirm these results, BLAST analysis was conducted of the original sizes of the D-loop nucleotide sequences from the unknown meats—868 bp (Unknown1), 383 bp (Unknown2) and 972 bp (Unknown3). From the BLAST results, Unknown1 was close to northern red muntjac (*Muntiacus muntjak vaginalis*) with 97.81% similarity. For the two unknown meats in the outgroup, the BLAST analysis indicated that Unknown2 and Unknown3 were domestic pig (*Sus scrofa domesticus*) and dog (*Canis lupus familiaris*) with 99.74% and 97.82%, respectively.

This study showed that identification of uncertain cervid origin could be performed based on FINS analysis of a partial sequence of the D-loop (281 bp). Previously, FINS analysis of a partial sequence of the D-loop (137 bp) had been performed to identify an ivory figurine as being from an Asian elephant, with the results also confirmed by BLAST analysis from which the D-loop sequence was similar to Asian elephant with 99% similarity (Gupta et al., 2011). Species identification that can be confirmed by a partial sequence will prove very useful for wildlife forensic investigation, which often deals with degraded DNA samples (Spencer et al., 2010). Although, the current study should have used mtDNA reference sequences from GenBank for reference clades in the phylogenetic analysis, samples that are not of cervid origin can be excluded from consideration using the current method. In addition, non-cervid origin could be preliminarily observed from the size of the amplicons (as Unknown2 and Unknown3 had a product size of 1,500 bp). This finding might be helpful for laboratories without access to DNA sequencing. In summary, the current study demonstrated a method for sample examination of cervid origin based on FINS analysis of a partial sequence analysis of the D-loop. This can be used as a preliminary screening technique for sambar deer identification in wildlife forensic science. For practical use, PCR amplification using the DL1 and DL2 primer is recommended as the first step for screening for sambar deer origin. If a PCR product of size 1,000 bp results from the amplification, then the sample might be sambar deer. The DNA sequencing of the amplicon should be carried out in the second step. Lastly, to confirm sambar deer origin, the DNA sequence from the meat sample should be analyzed to identify any genetic relationships with cervid reference sequences.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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