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Research Article

Whole-Genome Sequencing Mapping of Candidate Genes of *Cervus Nippon Hortulorum*, Presumed to be Sika Deer from the Korean Peninsula

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Abstract | The deer known as Sika deer, which has been extinct in Korea since 1920, is known to be a very important biological resource in Korea, but it is very difficult to establish a standard for Korean deer due to indiscriminate introduction. In particular, the genetic diversity and phylogenetic relationships of various subspecies of deer are known to be uncertain, so securing deer genes that can represent Korea is very important. We found a document observing Sika deer near the 38th parallel, and we were confident that this deer would inhabit North Korea and the Russian Maritime Province. Therefore, in this study, in order to explore the phylogenetic relationships of various Sika deer, we collected samples of Sika deer living around the Maritime Province and North Korea that had the morphology suggested in the literature, collected mtDNA of the deer, and analyzed whole-genome sequencing (WGS) to determine whether they could be classified as subspecies of Cervus nippon hortulorum in the Korean Peninsula. 11 Sika deer with similar morphology to those presented in ancient literature were finally selected. Most of the sequence reads of the mtDNA gene sequence were mapped to a 2.8 Gbp region, which is 97.6% of the total region in all reads mapping. The average number of reads was confirmed to be 708,020,765. The experimental flower deer group possessed an average of 19,994,130 SNP mutations along with 996,204 insertions and 1,050,106 deletions, thus exhibiting a clear difference from the previously reported Japanese Cervus nippon hortulorum population and suggesting that it may have been a deer native to the Korean Peninsula.

Keywords | *Cervus nippon*, Whole genome sequencing, Single-nucleotide polymorphism, Sika deer, MtDNA, Korea

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INTRODUCTION

The Sika deer (*Cervus nippon*) is an important member of the native fauna of eastern Asia and has been widely introduced to many other parts of the world (McCullough, 2009). In the early Pleistocene, Sika deer inhabited only northern China and Taiwan; however, from the middle Pleistocene to the Holocene, they expanded to China, Mongolia, Vietnam, Russia, Korea, and Japan (McCullough, 2009; McCullough *et al*., 2009; Saggiomo *et al*., 2020) Skia deer are currently distributed in northeastern Asia from Vietnam in the south and China and Korea to Russia in the north and also on the continental islands of Taiwan and the Japanese Archipelago (McCullough, 2009). Since the early 1990s, Asian sika deer populations have been decimated through overhunting and habitat loss in many areas. Sika deer are extinct from the wild in South Korea. North Korea still possesses wild populations of Sika deer in the northern part of the country along the Chinese and Russian borders. Small numbers cross the borders between North Korea, Russia in the Far East, and northeastern China. Populations from Far East Russia and northeastern China are genetically similar and related to the southern clade present in South Korea (McCullough *et al*., 2009) (Figure 1).

Figure 1: The *Cervus nippon*, which is thought to have been distributed in Korea, China, and Russia, was estimated to be a subspecies of *C. n. hortulorum* (McCullough 2009; McCullough *et al*., 2009; Saggiomo *et al*., 2020).

Historically, there were six Sika deer subspecies in China, including *C. n. hortulorum, C. n. sichuanicus, C. n. kopschi, C. n. grassianus, C. n. mandarinus*, and *C. n. taiouanus*. Only the first three subspecies are present in Mainland China (Saggiomo *et al*., 2020; Dhakal *et al*., 2023). Currently, serious genetic pollution has occurred in many populations of sika deer, particularly in China. Therefore, the status of many subspecies remains unclear.

Perhaps more importantly, despite the commercial value and medical importance of sika deer, few genomic resources exist for this species that could improve our understanding of its genetic diversity and population structure (Prabhu *et al*., 2019; Singh *et al*., 2019). Mitochondrial DNA (mtDNA) (Wang *et al*., 2011; Zhang *et al*., 2011; Zhang *et al*., 2011) is a powerful molecular tool for phylogenetic analysis and genetic diversity assessment of animals (Kitpipit *et al*., 2012; Liu *et al*., 2012; Yoon *et al*., 2017). Advances in WGS technology using mitochondrial DNA now allow us to sequence an individual's entire genome and detect selective sweeps (Ababaikeri *et al*., 2020), which can provide insights into genome biology and mechanisms of adaptation to extreme environments (Snyder *et al*., 2010). Genomic differences can shed light on the genetic basis of adaptation to diverse environments and provide insights into functionally important genetic variation (Andersson and Georges, 2004). Furthermore, combining WGS data with population genomic approaches can characterize adaptive variation in an unprecedented way (Ababaikeri *et al*., 2020; Snyder *et al*., 2010). Therefore, using WGS in our study can be a valuable research method to explore genetic variation across subspecies of Sika deer and environmental factors. In particular, it is very difficult to provide accurate information on the Sika deer of the Korean Peninsula because there is still no accurate indicator for the overall genome variation analysis of the Sika deer of the Korean Peninsula, and the research results analyzing genetic variation to support the research results analyzed in the study of Park *et al*. (2024), are insufficient.

Therefore, in this study, we performed whole genome sequencing (WGS) analysis to restore the deer living on the Korean Peninsula, to confirm whether the Primorsky deer is genetically similar to the Korean deer, and to provide basic data to confirm that it is a subspecies of the Korean deer through genetic diversity among individuals using single nucleotide polymorphisms (SNPs).

MATERIALS AND METHODS

Animal Samples

The samples of individuals for which the genetic analysis was performed were the same as those used in our previous study, and the sample processing and preservation methods were the same as those used by Park *et al*. (2024). The samples were extracted from the tissues of individuals believed to be Korean deer living in the Primorskaya Oblast and neighboring areas of North Korea (Figure 2), and Primorskaya Oblast Agricultural Academy signed a Memorandum of Understanding (November 25, 2014) in a previous study (Jangsu *et al*. No. 82-2014-003). We selected and used 11 deer with external characteristics similar to those of Korean deer suggested in the 1920 Japanese literature and the research literature of (Whitehead, 1993) (Dhakal *et al*., 2023). All animal handling and experimental procedures were approved by the Hankyong National University Animal Experimental Ethics Committee (IACUC approval HK-2021-1).

Figure 2: Photographs of the Sika deer sampling site and Sika deer farm used in this study; **A:** Sika deer sampling site; **B:** Farm where Sika deer are raised.

MtDNA Extraction

Tissue sample preparation for analysis of genetic variation in Sika deer was performed using the method of Park *et al.* (2024). All sika deer tissues were stored in -196°C liquid nitrogen and transported to the laboratory. Upon arrival at the laboratory, according to the manufacturer's instructions, mtDNA was extracted using a mitochondrial DNA isolation kit (#K280-50, Biovision, MA, US). A 20 µl volume of the extracted DNA was eluted using the TE buffer of the extraction kit and then stored at -20 until PCR and sequencing (Table 1).

Table 1: DNA extraction results for native deers in South Korea.

No	Name	Sex	260 / 280	ng/ul	Total amount
1	21108	Female	2.118	111.6	20
2	21103	Female	2.067	135.4	20
3	21101	Female	2.095	327.8	20
4	22110	Female	2.087	185.5	20
5	22118	Female	1.908	433.2	20
6	22112	Female	2.152	24.1	20
7	12101	Male	2.090	88.0	20
8	12109	Male	2.022	181.8	20
9	12106	Male	2.140	111.5	20
10	12110	Male	2.042	217.7	20
11	12104	Male	1.975	551.3	20

PCR and Direct Sequencing using MtDNA

In order to determine the genetic variation and subspecies of Sika deer, we designated the region for analysis in the gene sequence of *Cervus elaphus* (red deer; https://www.ncbi.nlm.nih.gov/genome/10790?genome assembly_id=1656646), which is the primary gene sequence of *Cervus nippon*, and the experimental method for the designated region was applied by the method of Frank *et al.* (2016). To confirm the base sequence by PCR amplification of the D-loop region of mitochondrial DNA, PCR was performed in a total volume of 20 µl using a composition of 20 ng/µl of DNA template, 1 µl of 10 pmol primer pairs (Table 2), and high-quality reagents including Anti-HS taq (cat no. AHS-101, TNT Research, South Korea) and Anti HS Taq premix (2x) (cat no. AHP-201, TNT research, South Korea). The PCR conditions were initial denaturation at 95°C for 12 min, denaturation at 95°C for 30 sec, annealing at 55°C or 60°C for 30 sec, and extension at 72°C for 30 sec for 35 cycles, and the final extension was performed at 72°C for 7 min. The amplified PCR fragment was subjected to electrophoresis on a 1.5% agarose gel to confirm the PCR fragment size, and the PCR product was purified using MagExtractor PCR and Gel clean-up (cat no. F0986K, TOYOBO) according to the user manual. The purified amplified product was sequenced directly using an ABI Prism 3730xl DNA Sequencer (Applied Biosystems).

Table 2: mtDNA D-loop PCR primer pair sequences.

Primer Type	Sequence	GC (%)	Nt	Tm
CST2	TAATATACTG-GTCTTGTAAACC	32	22	56
CST39	GGGTCGGAAGGCT-GGGACCAAACC	67	24	77

Direct Sequence Data Analysis

Phylogenetic analysis of native Korean deer species was performed by identifying sequence variants using the MEGA-X program (Takagi et al., 2023) for the D-loop sequence and calculating allele frequencies and genetic distances using Nei's DA method. Genetic distance information is graphically depicted in a phylogenetic tree Venn diagram to confirm the genetic relationships of each sample.

WGS Analysis and SNP Annotation

To clearly analyze the filtering criteria for SNP calling and alignment to a non-target reference genome (Cervus elaphus), we applied the analysis methods of Park *et al.* (2024) and Frank *et al.* (2016). We performed whole genome resequencing of Cervus eLaphus (red deer) to identify variants and perform gene annotation for useful genes based on database information. The samples identified as *Cervus hotolinum* through mtDNA D-loop analysis were subjected to NGS to confirm genetic mutations. An NGS library was produced using the TrueSeq Nano DNA kit, and 151bp read sequences of the paired-end reads were analyzed for NGS data production. Quality was confirmed to be 98% or higher for the Q20 score and 92% or higher for the Q30 score using FastQC. In the case of Cervus Hotolinum, as the published reference genome was not confirmed, reference mapping was performed using the reference genome

of a similar species, red deer (Cervus elaphus; red deer: 2.88 Gbp) (https://www.ncbi.nlm.nih.gov/genome/10790?genomeassembly_id=1656646). For mapping, alignment to the reference sequence was performed using BWA-MEM. Overlapping reads were removed using Sambamba, variant extraction was performed using SAMTools, and the extracted SNPs were annotated using SnpEff.

RESULTS AND DISCUSSION

Results of Phylogenetic Analysis using mtDNA D-Loop

To identify the domestic native deer species, PCR and direct sequencing were performed using a combination of CST2 and CST39 primers to analyze the base sequence of the D-loop region of mitochondrial DNA based on the results of Park *et al.* (2024). The CST2 primer set confirmed a size difference of 113–700 bp in the amplified product for each sample, and the CST39 primer set confirmed a size distribution of 142–700 bp in the size of the amplified product (Table 3). Using the base sequence information of the secured D-loop primer sets, the mutation pattern appearing in each individual was confirmed, allele frequency was calculated, and the genetic distance between individuals was calculated using the secured genotype frequency value to analyze the phylogenetic tree. For the 11 domestic native deer, the base sequences of 10 samples, excluding sample 12106, were aligned using the CST2 primer for phylogenetic analysis (Figure 4). As a result, the entire group was largely divided into two groups, and the upper six were separated into two groups but were estimated to be one group, as the distance cutoff was low at 23. The base sequences obtained using the CST39 primer set were aligned with those of seven animals while excluding four animals (21103, 22112, 12110, and 12104), and the phylogenetic tree was analyzed. The results confirmed a similar pattern that was not significantly different compared to that obtained for CST2 (Figure 5).

Table 3: mtDNA D-loop sequencing product size of native deers.

No.	Name	Sex	CST2	CST39
1	21108	Female	700	700
2	21103	Female	700	252
3	21101	Female	700	700
4	22110	Female	700	700
5	22118	Female	700	700
6	22112	Female	700	250
7	12101	Male	700	700
8	12109	Male	700	700
9	12106	Male	113	700
10	12110	Male	700	253
11	12104	Male	700	142

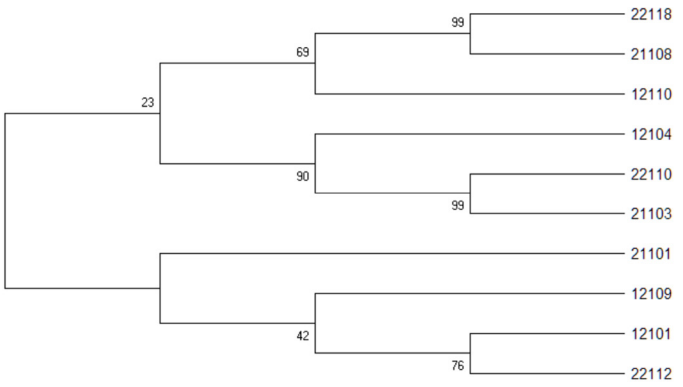

Figure 4: Phylogenetic tree result using CST2 primer set for 10 Korean native deer.

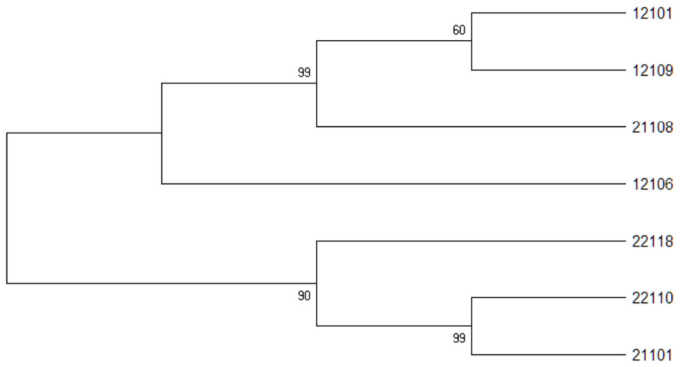

Figure 5: Phylogenetic tree result using CST39 primer set for 7 Korean native deer.

Table 4: NGS data information filtered after generation.

Sample	Total read bases (bp)	Toatl reads	GC (%)	Q20 (%)	Q30 (%)
12101	103,540,353,176	709,542,618	44.47	98.29	94.06
12104	104,791,709,609	714,464,642	44.25	98.50	94.63
12110	102,533,233,686	700,055,034	44.42	98.47	94.59

WGS Mapping and SNP Annotation Results for Three Native Deer

Based on the mtDNA analysis results, three individuals were selected from a cluster with the most similar genetic sequences (Figure 4), and the results of filtering before performing mapping and annotation analysis after producing NGS data for reference sequence mapping for the three native deer in Korea are presented in Table 4. The total number of reads was confirmed to be 708,020,765 on average. By performing whole genome resequencing of Cervus eLaphus (red deer) to identify variants and comparing them with database information, the GC ratio was 44.38%, Q20 had an average of 98.42%, and Q30 had 94.43%, confirming that subsequent mapping and variant extraction was possible (Figure 3). The base pair length of the total read was also confirmed to be 103 Gbp, thus confirming that data of greater than 30x depth were produced, considering a reference sequence length of 2.88 Gbp. The NGS data, a crucial step in our research, underwent rigorous quality

control before being mapped to the *Cervus elaphus* reference sequence (Table 5). This mapping process represented a significant milestone and revealed that a staggering 97.6% of the sequence reads were successfully mapped to the 2.8 Gbp region, with an average of 708,020,765 reads. This high mapping rate confirms the reliability of our data and resulted in a total depth of 33.51x. After mapping, the mapped sequence variants (SNPs, insertions, and deletions) were assessed using SAMTools (Table 6).

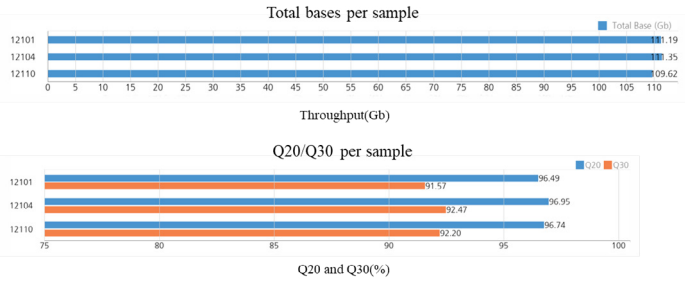

Figure 3: Throughput and Q20/Q30 scores of Raw data.

Table 5: Mapped data Stats.

Library name	Mapped Site	Total Read	Mapped Read	Mean Depth
12101	2,817,812,948 (97.62%)	709,542,618	708,432,176 (99.84%)	33.16
12104	2,886,603,524 (97.64%)	714,464,642	713,337,965 (99.84%)	33.6
12110	2,886,603,524 (97.59%)	700,055,034	698,991,096 (99.85%)	33.77

Table 6: Variant calling results.

Library name	Number of SNPs	Number of Insertions	Number of deletions
12101	19,890,501	992,998	1,046,824
12104	20,096,288	1,002,171	1,053,769
12110	19,995,600	993,443	1,049,726

Variant Annotation

SnpEff was used to analyze the annotation information, such as amino acid changes due to mutations, for the three individuals most genetically similar to the Korean Peninsula Sika deer. The flower deer experimental group was confirmed to possess an average of 19,994,130 SNP mutations along with 996,204 insertions and 1,050,106 deletions. We observed that 90.72% of the total mutations corresponded to SNPs, and the remaining 4.52% and 4.76% were confirmed as insertions and deletions, respectively. This was similar to the general patterns of base and structural mutations observed in mammals. After annotating all acquired mutation information in the gene database using SnpEff, the types of annotations were confirmed as presented in Table 7. The most common region mutation was in the intergenic region (46.45 %), and this was followed by the intron region (38.91 %). Subsequently, it was confirmed that the upstream and downstream gene regions, UTR, and synonymous, missense, and non-coding transcript exon regions that exhibit the potential to affect amino acid formation in the gene region account for approximately 15.13% of the mutations in the entire region (Table 8).

Table 7: Annotation type and average variants count.

Type of annotation	Count	Ratio
intergenic	10,051,938	46.45%
intron	8,418,775	38.91%
upstream gene	2,040,003	9.43%
downstream gene	802,344	3.71%
3' UTR	158,941	0.73%
synonymous	101,759	0.47%
missense	77,407	0.36%
5' UTR	46,018	0.21%
non-coding transcript exon	27,263	0.13%
intragenic	19,073	0.09%

In the 1970s, when many types of livestock, including deer, were introduced to Korea, the original species of Sika deer that had been preserved to some extents were likely crossed with breeds imported from other countries as part of livestock improvement projects, thus causing genetic disruption to Korean Sika deer (McCullough 2009; Park *et al.*, 2024; Ba *et al.*, 2015; Jiang *et al.*, 2016). In particular, the Sika deer is currently reported to be extinct in Korea as of 2017, and no further research is being conducted. However, some people have reported catching the Sika deer on the border between Korea and the United States. Therefore, our study urgently needed basic data analysis to establish marker factors for the Sika deer in Korea, and through this study, we hoped to rekindle interest in conservation research on the Sika deer in Korea. Therefore, based on data from a previous study that classified Korean animals near Russia and the Korean Peninsula, we selected 11 individuals with the most similar external appearances to the Korean Sika deer and analyzed their diversity using mtDNA sequences. As a result, a group that matched Taiwanese *Cervus nippon taiouanus* was formed similar to that in the study by Park *et al.* (2024), and four of these matched the base sequence of *Cervus nippon hortulorum*.

Among them, three individuals connected to one cluster were analyzed by WGS, and the results of the mapping analysis of the *Cervus elaphus* reference sequence demonstrated that an average of 99.84% of the reads were mapped, thus allowing us to obtain reliable data with a total depth of 33.51x. These results were found to be sufficiently reliable to identify the size or variation of the slightly shorter genome compared to the entire genome size of deer (Takagi *et al.*, 2023; Kim *et al.*, 2020). The analyzed data were stored in the NCBI for Biotechnology Information database.

Table 8: Annotation type count.

Library name	Type of annotation	Count	Ratio
12101	intergenic_region	9,999,327	46.14%
	intron_variant	8,379,366	38.66%
	upstream_gene_variant	2,030,368	9.37%
	downstream_gene_variant	800,043	3.69%
	3_prime_UTR_variant	158,536	0.73%
	synonymous_variant	101,642	0.47%
	m1ssense vanant	76,915	0.35%
	5_prime_UTR_variant	46,073	0.21%
	non_coding_transcript_exon_variant	26,982	0.12%
	intragenic_variant	19,269	0.09%
12104	intergenic_region	10,092,233	46.11%
	intron_variant	8,467,568	38.69%
	upstream_gene_variant	2,050,695	9.37%
	downstream_gene_variant	807,575	3.69%
	3_prime_UTR_variant	160,010	0.73%
	synonymous_variant	102,194	0.47%
	m1ssense vanant	78,110	0.36%
	5_prime_UTR_variant	46,202	0.21%
	non_coding_transcript_exon_variant	27,187	0.12%
	intragenic_variant	18,870	0.09%
12110	intergenic_region	10,064,255	46.22%
	intron_variant	8,409,392	38.62%
	upstream_gene_variant	2,038,946	9.36%
	downstream_gene_variant	799,293	3.67%
	3_prime_UTR_variant	158,278	0.73%
	synonymous_variant	101,621	0.47%
	m1ssense vanant	77,196	0.35%
	5_prime_UTR_variant	45,779	0.12%
	non_coding_transcript_exon_variant	27,620	0.13%
	intragenic_variant	19,079	0.09%

SnpEff arranges the effects by putative sorting order considering impact of variants. "most deleterious" one is shown first (transcript 1).

SNP analysis using NCBI indicated that 90.77% of the total variation corresponded to SNPs, and 46.45% of the variation was in the intergenic region. This was followed by 38.91% in the intron region. It was confirmed that the Sika deer exhibited significant genetic differences from populations in other regions. Genetic information is currently registered with the NCBI (Repository: Institute of Animal Developmental Biotechnology of Hankyong National University in Korea; Biobank: www.ncbi.nlm.nih.gov/bioproject/PRJNA831892; Organization: Hankyong National University of Korea). According to our results, it belongs to *Cervus hortulorum* and *Cervus nippon hortulorum* and is different from *Cervus nippon taiouanus* (Yuasa *et al*., 2007; Nagata *et al*., 1999; Takagi *et al*., 2023).

In particular, the haplotypes of *Cervus nippon hortulorum* and *Cervus nippon taiouanus* were grouped into one branch, and it was confirmed that the Sika deer of the Korean Peninsula separated and proliferated as a new subspecies as local indigenousization progressed (Nagata *et al*., 1999; Takagi *et al*., 2023; Lü *et al*., 2006). These results are similar to the results of the research report of Liu *et al*., 2021 based on analysis of mtDNA diversity and various SNP mutations (Tamate *et al*., 2021; Liu *et al*., 2021). We tried to find individuals in Russia that are externally identical to Korean deer, but the most externally similar species were very rare. In particular, due to the national conditions of Russia, there were limitations in collecting samples, so only 11 specimens could be collected. The analysis using a small number of samples was insufficient to obtain statistical results, and it was also difficult to conduct a comprehensive study because the open data genome for comparison was limited to Cervus elaphus.

However, our study results may be insufficient, but they mean that some deer individuals in the Korean Peninsula and neighboring regions of Russia are different from previously known Japanese deer individuals, suggesting that they may be subspecies of the Korean Peninsula-specific Sika deer. In particular, based on this study, it is thought to serve as basic data for confirming the unique clade of the Korean Peninsula-specific Sika deer through continuous genome analysis.

CONCLUSIONS AND RECOMMENDATIONS

Based on the WGS analysis and various mutation results of SNP in the 11 individuals used in our study, we were able to obtain the results that the selected individuals may have different genetic patterns from the Japanese deer, suggesting that they may have been newly separated from a common ancestor due to the natural environment. Therefore, through the study so far, we were able to secure samples of individuals with similar appearance and genetic diversity to deer that have never inhabited the area, and this can at least partially confirm the hypothesis that they were similar to *Cervus nippon hortulorum* but were distributed separately as a new subspecies. In addition, if next-generation sequencing (NGS) analysis is conducted to build a genome map using data obtained through De novo sequencing analysis based on this study, it is expected that biomarkers for the Sika deer on the Korean Peninsula and specific conservation plans for the Sika deer can be proposed.

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NOVELTY STATEMENTS

This study is the first to use a genome analysis method to identify Korean deer and to find Korean deer.

AUTHOR'S CONTRIBUTIONS

Yong Su Park and Sang Hwan Kim: Conceptualization.
Yong Su Park, You Sam Kim, Min Jee Oh and Dong Won Seo: Methodology.
Myung Hum Park and You Sam Kim: Software.
Yong Su Park, Dong Won Seo, Min Jee Oh and Sang Hwan Kim: Validation.
You Sam Kim; investigation, Myung Hum Park: Formal analysis.
Yong Su Park; data curation, Yong Su Park and Dong Won Seo: Resources
Yong Su Park and Dong Won Seo: Writing—original draft preparation.
Sang Hwan Kim: Writing—review and editing.
Yong Su Park and Myung Hum Park: Visualization.
Sang Hwan Kim: Supervision.
Sang Hwan Kim: Project administration.
Sang Hwan Kim: Funding acquisition.

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Conflict of Interest
The authors declare no conflicts of interest.

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