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Mitochondrial DNA Polymorphism in Cheju and Tsushima Native Horses Using SSCP Analysis

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ABSTRACT

The mitochondrial DNA (mtDNA) D-loop region was amplified from Cheju (K and Tsushima (Japan) native horses by polymerase chain reaction (PCR). The oligonucleotide primers used to amplify the equine mtDNA D-loop region was designed using tRNA-Thr and tRNA-Phe sequences in mtDNA regions highly conserved in many other animal species. There were 1,127 base pairs (bp) in the D-loop region. The middle of the region contained 20 tandem repeats of an 8-bp equine-specific sequence, TGTGCACC. We designed primers for PCR-mediated single-strand conformation polymorphism (SSCP) analysis that amplified a 322 bp fragment, which contained the most variable region according to our sequence data. SSCP analysis of denatured amplification products was carried out by polyacrylamide (8%) gel electrophoresis followed by silver staining. The SSCP analysis identified four different band patterns (A, B, C, and D) and comparison of these four nucleotide sequences identified 34 base substitutions. The A type (90%) was found in most of the Cheju native horses, while the D type (96%) in nearly all the Tsushima native horses. These results show that SSCP analysis of the D-loop region is useful for detecting polymorphism and verifying the lineage of Cheju and Tsushima native horses.

Key words : D-loop, equine, lineage, polymorphism, SSCP, substitution, tandem repeat.

INTRODUCTION

The order Perissodactyla comprises three extant families: the Equidae (horses), Tapiridae (tapirs), and Rhinocerotidae (rhinoceroses). The order and the three families are well defined, making these taxa attractive for both intra- and interfamilial comparisons at the molecular level (Irwin et al., 1991; Xu and Arnason, 1994; Ishida et al., 1995; Xu et al., 1996a, b).

While it is known that the Cheju (Korea) native horse, which breeds on Cheju Island dispersed from

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the Korean Peninsula, its origin remains unclear. The Mongols introduced 160 horses to Cheju Island in 1276, and bred horses there for the next 100 years (Nam, 1969). Therefore, Cheju native horses are generally assumed to be of Mongolian origin. On the other hand, Hayashida (1958) reported that the native horses of Japan and adjacent areas originated from two ancestral lines (small horses and medium-sized horses), and that Cheju native horses are similar to the small horses found in southern areas of Japan. Nozawa and Kondo (1970) reported that gene frequencies controlling coat color and immunological blood types of Cheju native horses are similar to those of the medium-sized horses, while their body size is consistent with the small-sized ones. However, Nozawa (1971) has called body measurements as a biological indicator for phylogenetic inference into question. In previous studies, we suggested that Cheju native horses were more closely related to *Equss przewalskii* among species of *Equus*, based on restriction fragment length polymorphism (RFLP) of mtDNA (Oh et al., 1994).

Normally, cytoplasmic transmission of maternal mtDNA results in homoplasmic individuals with a single major mtDNA sequence in all tissues. Compared with nuclear DNA, mtDNA has an accelerated nucleotide substitution rate that has been widely utilized as a molecular clock for estimating the time of recent evolutionary events (George and Ryder, 1986). Due to its clonal and maternal inheritance, mtDNA has a low effective population size and can be strongly differentiated geographically (Berky et al., 1983). Partial or complete sequences of mtDNA have been used extensively to estimate inter- and intraspecific differentiation in a wide range of taxa (Xu and Arnason, 1994; Lee et al., 2000).

The non-coding, but functionally important, control region (the D-loop) is known to have rapidly evolving flank regions and a more conserved central domain (Uphole and Dawid, 1977). Two recent reports described sequence data for the equine D-loop, as well as D-loop polymorphism, as revealed by polymerase chain reaction (PCR)-RFLP or single-strand conformation polymorphism (SSCP) analysis (Ishida et al., 1994).

SSCP analysis is a technique that relies on the ability of a single (or multiple) nucleotide change to alter the electrophoretic mobility of a single-stranded DNA molecule under non-denaturing conditions (Orita et al., 1989). Under non-denaturing conditions most single-stranded DNA molecules will assume one or more stable three-dimensional conformations that depend on the nucleotide sequence. In many cases, a single nucleotide change will cause a conformational change that can be detected as a change in the electrophoretic mobility (Tomas et al., 1994; Marklund et al., 1995; Kim et al., 1998).

Consequently, in order to elucidate variation in the mtDNA D-loop region in the Cheju and Tsushima native horses, we sequenced the D-loop region and subsequently carried out PCR-SSCP analysis.

MATERIALS AND METHODS

Sample collection and total DNA extraction

Peripheral blood samples were obtained from 30 Cheju native horses (Chejudo Livestock Promotion Institute) and 25 Tsushima native horses through the kind co-operation of Nagasaki University. Genomic DNA, containing chromosomal DNA and mtDNA, was isolated from blood from these 55 individuals using standard techniques (Sambrook et al., 1989). The DNA concentrations ranged from 20 to 80 ng/μl. Purified DNA was diluted 10 times with distilled water, and 1 μl of diluted DNA (2 ~ 8 ng) was used for amplification reactions in 50 μl volumes.

PCR amplification of mtDNA D-loop region

Total DNA was used as a template to amplify fragments of the D-loop region by PCR. The oligonucleotide primers used to amplify the mtDNA

D-loop region by PCR were designed using tRNA-Thr and tRNA-Phe sequences in highly conserved regions of mtDNA common to many other animal species. The primer sequences were as follows: L-strand prime 5′-AGGACTATCAAAGGAGAAGC-3′, H-strand primer 5′-AACGGGGGAAGAAGGG-TTGA-3′. The PCR mixture contained 30.5 μl distilled sterile water, 5 μl of 10x reaction buffer (10 mM Tris-HCl, pH 9.0 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100; Promega, USA), 2 μl of a dNTP mix (final concentration 200 μM, Promega, USA), 2.5 units of *Taq* DNA polymerase (Promega, USA), 3 μl of each primer, and 4 μl of total genomic DNA. Amplification was performed in a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Cetus, USA) under the following cycle conditions. An initial denaturation step for 3 minutes at 94°C was followed by 30 cycles of 1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C. A final primer extention step for 7 min at 72°C followed completion of the 30 thermal cycles. Two microliters of product were used for electrophoresis on 2% agarose gels, run at 100 V for 1 hr in 1x TBE.

Cloning the PCR products and sequencing

The PCR products were ligated into pT-Adv vector and cloned using the AdvanTAge PCR Cloning Kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. The cloned DNA fragments were sequenced using Cy5-labeled Vector inner primer, M13-40 primer, and M13-reverse primer, and a Cy5 AutoCycle Sequencing Kit on an ALFexpress DNA sequencer (Pharmacia Biotech, USA). Samples were electrophoresed at 1500 V for 700 min on 6% acrylamide-7 M urea gels in 0.6x TBE buffer.

SSCP analysis and sequencing of variable region in the D-loop

Forward (SSCP-F: 5′-ACATAATATCATTTATCT-TAC-3′) and reverse (SSCP-R: 5′-TGTGTGAGCAT-GGGCTGATTA-3′) PCR primers were designed to amplify the most variable region according to our sequence data (Fig. 1).

PCR amplification was performed in 12 μl reaction mixtures containing 10 mM Tris-HCl, 50 mM KCl, 50 ng of genomic DNA, 20 pM of each primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 2 units of *Taq* polymerase. The PCR protocol includes an initial denaturation step at 94℃ for 5 min, followed by 30 cycles (55℃, 2 min; 72℃, 3 min; 94℃, 1 min). The final extension was for 7 min.

For SSCP analysis, the diluted PCR product was added to 5 μl of denaturing buffer (95% formamide, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were then heated to 95℃ for 3 min and immediately placed in an ice-water bath and maintained at the temperature required for subsequent electrophoresis. Electrophoresis was carried out in a Hoefer SE600 unit (180 × 160 × 0.75 mm) at 20℃ in 0.6x TBE buffer for 17 hrs at 200 V. The gels were subsequently silver-stained, as described elsewhere (Caetano-Anolles and Gresshoff, 1994) with modified concentrations of acetic acid (10%), silver nitrate (1.8 g/L), and sodium carbonate (15 g/L). Sequencing SSCP variants was performed using a Cy5 AutoCycle Sequencing Kit, according to the manufacturer's instructions (Phamacia Biotech, USA).

RESULTS

We amplified and cloned 1,203 base pairs (bp) from four Cheju (Korea) and three Tsushima (Japan) native horses to determine the mtDNA D-loop sequence. The total number of bases in the region was 1,127 bp for all clones (Fig. 1; coordinates 74 to 1200). Intra-population nucleotide differences was not found. The tRNA-Pro sequence and the 5′ end of the D-loop region (between nucleotides 250 and 530) in Cheju and Tsushima native horses were highly variable (Fig. 1). Therefore, we designed

Figure 1. Nucleotide alignment of the D-loop containing regions from Cheju and Tsushima native horses mtDNAs. The nucleotide sequences were displayed from 5′ to 3′. Dots indicate that the base is identical to that of CNH. Asterisks below the aligned sequences denote variations of bases. CNH, Cheju native horse; TNH, Tsushima native horse.

PCR-SSCP primers to target a 322 bp fragment in this variable region, which included the most variable sites in the native horses (Fig. 1).

PCR-SSCP analysis of amplified products from 30 Cheju native horses and 25 Tsushima native horses were carried out by 8% polyacrylamide gel electrophoresis followed by silver staining. Four variants were clearly detected, and were reproducible (Fig. 2). The variable band patterns of the SSCP-targeted region were designated A (27 individuals), B (2 individuals), C (1 individual), and D (25 individuals) type (Table 1). Most of the Cheju native horses were the A type (90%, 27/30), with a few type B and D individuals, while almost all the Tsushima native horses were type D (96%, 24/25), with one type C individual.

To determine the relationship between SSCP band pattern and nucleotide substitution, we sequenced this region from the four variable types by cyle sequencing. Each pattern obtained by SSCP analysis corresponded to specific nucleotide substitutions (Fig. 2). Among four types, base substitutions were present at 30 nucleic acid sites within the SSCP-targeted region (indicated with asterisks in Fig. 3). Four nucleotide substitutions, identified as three transitions and one transversion, were found in the 5′ SSCP-targeted region (indicated with asterisks in Fig. 3).

The base composition of these sequences was as follows: A 309-316 (27.4-28%); C 343-351 (30.4-31.1%); G 167-173 (14.8-15.4%); T 294-301 (26.1-26.8%), indicating that the mtDNA D-loop region is A/T rich as in many other mammals (Table 2). In the middle of the D-loop region, there were tandem repeats (20 repeats) of an 8 bp equine-specific sequence TGTGCACC in all four types (Fig. 3). This portion of the sequence was G/C rich and had the following base composition: A 12.5%, C 37.5%, G 25%, and T 25%.

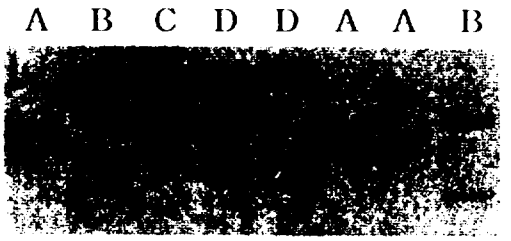

Figure 2. PCR-SSCP analysis of the D-loop polymorphic regions. The PCR-amplified 322 bp DNA fragments were denatured by heating and loaded on to a 8% polyacrylamide gel. The results were detected by silver staining. A, B, C and D indicate the SSCP variant types.

Table 1. Number of Detected SSCP Variants

Type*	Cheju Native Horse		Tsushima Native Horse	
	*n***	freq. (%)	*n*	freq. (%)
A	27	90	0	0
B	2	6.7	0	0
C	0	0	1	4
D	1	3.3	24	96

* Variant types were given in fig. 2.

** *n* = number of individuals

DISCUSSION

Sequence analysis of these native horses shows that a highly variable D-loop region containing nucleotide substitutions, located at the site of heavy-strand replication, is similar to those reported in other species (Horai et al., 1990; Ron et al., 1993; Ishida et al., 1994; Loftus et al., 1994; Takeda et al., 1995). Additionally, the base composition of the

Table 2. Characteristics of mtDNA D-loop Region from the SSCP Variants

Type*	Length (bp)	Base Content (%)			
		A	C	G	T
A	1,127	28.0	30.8	15.0	26.2
B	1,127	27.4	31.1	15.4	26.1
C	1,127	28.0	30.6	15.0	26.4
D	1,127	28.0	30.4	14.8	26.8

* A, B, C and D types were given in fig. 2.

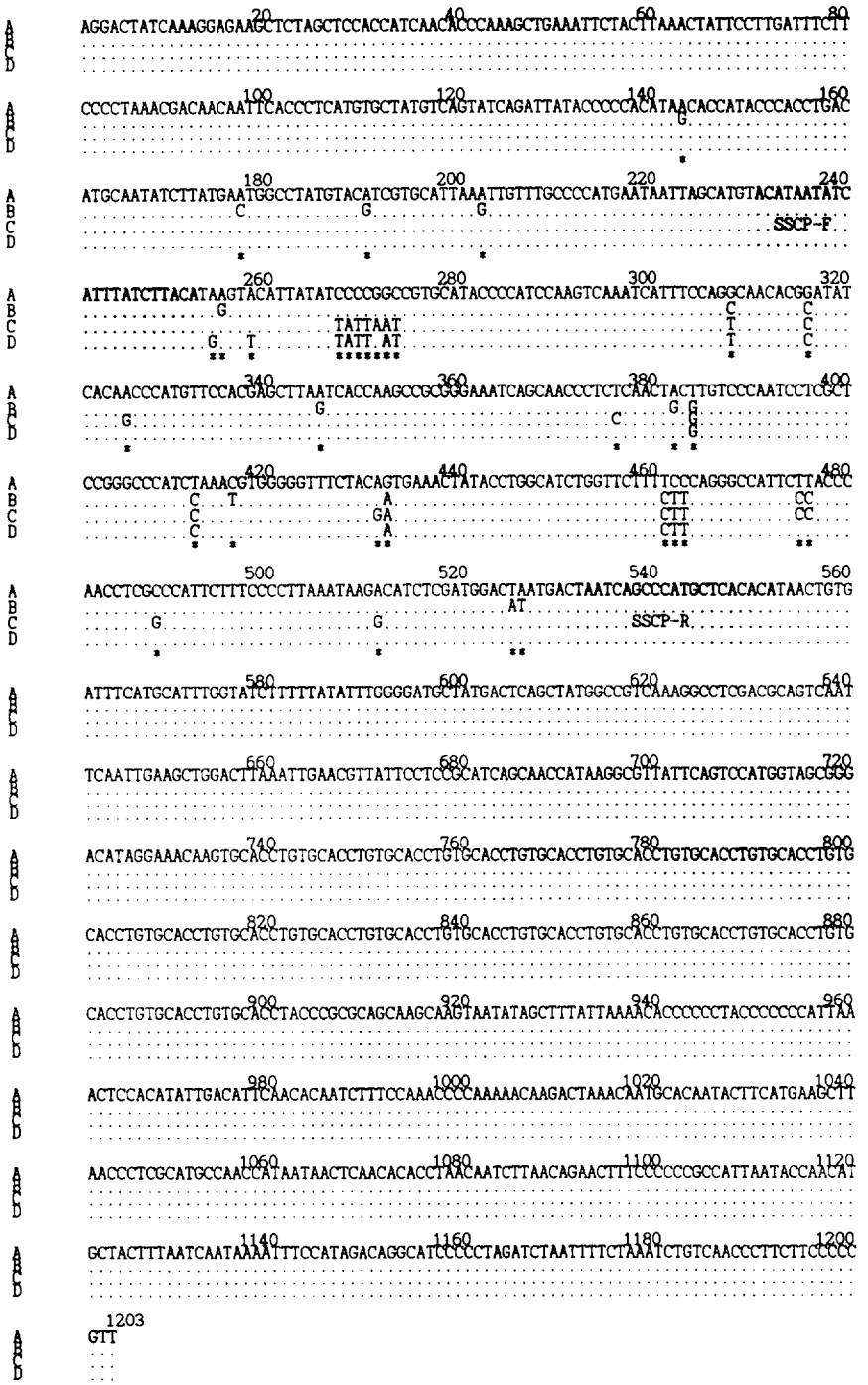

Figure 3. Nucleotide alignment of the D-loop containing regions from 4 SSCP variants mtDNAs. The nucleotide sequences were displayed from 5′ to 3′. SSCP primer regions are boxed and A, B, C and D at the start of the sequence panel were named SSCP variants. Dots indicate that the base is identical to that of A. Asterisks below the aligned sequences denote variations of bases.

D-loop was A/T rich (53.5-54.8%) in the Cheju and Tsushima native horses (Table 2), as in many other mammals.

As shown in Figure 1, there are 20 tandem repeats of an 8 bp equine-specific sequence (TGTGCACC) in the middle of the mtDNA D-loop. Similar tandem repeats within the D-loop have been reported in the green monkey (Karawya and Martin, 1987), rabbit (Mignotte et al., 1990), pig (Ghivizzani et al., 1993; Takeda et al., 1995), harbor seal (Arnason and Rand, 1992), elephant seal (Hoelzel et al., 1993) and thoroughbred horse (Ishida et al., 1994). Pig species have 9 ~ 25 repeats of a 10 bp sequence (CGTGCGTACA) (Takeda et al., 1995), rabbits have 10 tandem repeats of a 20 bp sequence (GCACGTACACCCGTACGCAC) (Mignotte et al., 1990), and horses have 18 and 22 repeats of an 8 bp sequence (TGTGCACC) (Ishida et al., 1994). The number of repeats in the Cheju and Tsushima native horses detected in this study differed from the number reported in thoroughbred horses reported (Ishida et al., 1994). Variation in repeat copy numbers results in mtDNA length variation and heteroplasmy (Mignotte et al., 1990; Ghivizzani et al., 1993; Hoelzel et al., 1993). Madsen et al. (1993) discussed the mechanism of heteroplasmy, which occurs by slipped mispairing between repeated sequences during DNA replication. It would be interesting to investigate the incidence of heteroplasmy in equine mtDNA in relation to other species.

The PCR-SSCP analysis detected four electrophoretic band patterns that distinguished Cheju from Tsushima native horses. Previous studies that analyzed RFLPs of mtDNA in thoroughbred horses, and Cheju and Tsushima native horses using four restriction enzymes, *Bam*HI, *Eco*RI, *Hin*dIII, and *Ava*II (Oh et al., 1994, 1997), found polymorphism in Cheju native horses and thoroughbreds, while the Tsushima native horse population was monomorphic. The results of the PCR-SSCP analysis were consistent with the RFLP data in that The A types (90%, 27/30) was found in most of the Cheju native horses while the D type (96%, 24/25) was found in Tsushima native horses (Table 1).

In conclusion, this study showed that PCR-SSCP analysis of the mtDNA D-loop region is a suitable technique for detecting intraspecific lineages and polymorphism between Cheju and Tsushima native horses.

The nucleotide sequence data for the mtDNA D-loop region for the Tsushima native horses is stored in the GenBank Nucleotide Sequence Databases under accession numbers AF169009 and AF169010.

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