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Cheju horses and their origin

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1. Introduction

One of the oldest records (Chosun history in Chinese Han Records cited by Nam, 1996) on Korean horses says that one of the princes of Wee-man Chosun dynasty sent 5000 horses and grain for military use to the King of the Chinese Moo dynasty in B.C. 109. However, no descriptive records are found about the horses raised during the period

Cheju horses are small (120 - 130 cm in mature height), hardy horses inhabiting the island of Cheju in Korea. The historical record indicates that Mongols introduced 160 horses into the island in 1276, producing horses for the next 100 years (Nam 1969). Therefore, Cheju horses are generally assumed to be of Mongolian origin. However, archeological studies as well as historical records suggest that horses were present on the island prior to the Mongolian introduction; horse bones were identified in a local excavation along with tools considered to be about 2,500 years old (Shin et al. 1992) and Cheju horses were known to be exported as early as in 1073. (Jeung-bo-moon-hun-bee-go cited by Nam, 1996).

2. History of horse production on Cheju Island

Cheju has been called many different names including Tamna, Mora, Tammora, Tamboora, Sup-ra and Do-ee, and the present name, Cheju originated from Cheju Mok (one of the counties belonging to Cholla Do) assigned by Choong-Ryul of Koryu Dynasty in 1295. The natural environment of Cheju has been considered good for horse production since prehistoric era. Domesticated horses may have been raised since the Bronze age and through the Three-Kingdom period. During

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the Koryu dynasty, horses were sent to the central government. In the last period of the Koryu dynasty (1276), Mongols introduced Mongolian horses onto the island and set up Mongolian-style horse farms, which opened a new era in the Cheju horse production history, improving horse breeds and thus producing high-performance horses and exporting them to Mongolia and Chinese Myung. Demands for horses rapidly increased in Lee dynasty, and as a result, the number of large-scale horse farms were established on the Cheju island, which became famous in Asia for its horse production.

Horses produced on Cheju were called Tamna horses, Cheju horses, Chorang horses, Toh horses and National horses. There have been studies on the origin of Cheju horses using paleontological and biological methods as well as historical records. However, none of the studies showed their nativeness or their relationship to ponies that used to inhabit the mainland. However, one recent study (main part of this presentation, Kim et al., 1999), which was done with mtDNA D-loop polymorphism, showed that Cheju horses consist of variety of horses including Mongolian horses and suggested that horses had inhabited the island before Mongolian introduction.

3. Morphological chracteristics of extant Cheju horses

Physical measurements of Cheju horses at different growing stage are shown in Table 1. The average mature height of male and female horses is about 127 and 123 cm, respectively. Although no records have shown physical characteristics of ancestors of current Cheju horses, records on their export to the central government and China for military or royal use suggest that the stature of those horses might have been much larger than that of the extant horses. Extensive mobilization of large horses for exportation, and inbreeding over hundreds of years may have resulted in poor stock population, leading to the current population with small stature.

4. Determination of the origin of Cheju horses

Molecular techniques have been widely used to analyze phylogenetic

relationships among various animal groups. The popularity of these techniques, especially DNA sequence analyses, is due mainly to the evolutionary information that can be drawn from sequence data. By comparing DNA sequences one can derive evolutionary relationships, levels of variability and geographic substructuring within and between groups of animals (Avise et al. 1987; Harrison 1989)..

The mitochondrial DNA (mtDNA) of most animals is about 16 kilobases (kb) of circular, supercoiled DNA that is maternally inherited. Two features of mtDNA make it particularly valuable for phylogenetic studies. First, evolution of mtDNA occurs primarily as single base pair substitutions, with only infrequent major sequence rearrangements (Wolstenholme 1992). Second, the rate of mtDNA evolution appears to be as much as 10 times faster than that of nuclear DNA (Brown et al. 1979). These features facilitate the use of mtDNA as a tool for determining relationships among individuals within species and among closely related species with recent times of divergence (Avise et al. 1979; Brown et al. 1979). The D-loop region of mtDNA is known to be more variable in sequence than other regions (Cann et al. 1984) and thus has been frequently used by molecular geneticists for phylogenetic analyses of closely related groups (e.g., to determine intraspecific phylogenies).

Phylogenetic relationships between horse breeds have been determined using restriction fragment length polymorphism (RFLP) of mtDNA (Georgy and Ryder 1986; Wang et al. 1994; Ishida et al. 1996). However, results of these studies provide only limited information because the number of polymorphic sites determined by RFLP

The main goal of this study was to determine the historical origin of Cheju horses using nucleotide sequence polymorphism of the mtDNA D-loop region. Phylogenetic relationships among Cheju, Mongolian, Yunnan (a Chinese local breed) and Przewalskii horses were determined to evaluate if Cheju horses are either of Mongolian or mixed origin. In addition, relationships to other breeds such as Thoroughbred and Swedish horses were also determined using previously published sequences. If the Cheju horses are a locally adapted breed originating from Mongolian horses, we would expect them to cluster together in a phylogenetic analysis. On the other hand, if Cheju horses constitute a breed of mixed origin we would predict that individual Cheju horses will cluster with horses from distinct groups that are related to the potential founder lines of the Cheju horses.

Materials and methods

Preparation of DNA and PCR amplification of mtDNA D-loop

Total DNA (genomic and mtDNA) was extracted from blood samples of 21 Cheju, 11 Mongolian, 1 Przewalskii (P1, studbook number 319) and 2 Chinese Yunnan (designated Y1 and Y2) horses by modification of the method reported by Miller et al. (1988). Cheju and Mongolian samples were preselected for sequencing by PCR-RFLP analysis of mtDNA D-loop to include a range of genetically distinct individuals. PCR-RFLP patterns generated by MboI and HincII digestions of mtDNA showed seven different RFLP genotypes for the Cheju samples analyzed (C1-C7), whereas only three genotypes were detected in the Mongolian samples (M1-M4) with M3 and M4 having the same RFLP genotype. The contribution of the MboI and HincII restriction sites was relatively small compared to the overall polymorphism later detected by sequencing.

The D-loop region was amplified by PCR using the following primers: L-strand, 5'ACACCAGTCTTGTAAACCAG3' (sequence in position 15909-15928 of the human mtDNA) and H-strand, 5'TCATCTAGGCATTTTCAGTG3' (position 607-626) (Anderson et al. 1981). Polymerase chain reaction was performed in 50 l volumes, each containing 500 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM MgCl2, 0.2 M of each primer, 100 M of each dNTP, and one unit of Taq polymerase. Reaction profiles included a 4-min denaturation step at 94C followed by 30 cycles, each consisting of 1 min denaturation at 94C, 1 min annealing at 60C, 1.5 min of extension at 72C, and then a final 4-min extension step at 72C.

Cloning the PCR products and sequencing

PCR products were gel-purified and cloned using TA cloning kits according to the manufacturer's instructions (Invitrogen, San Diego, CA, USA). DNA inserts were sequenced by single-strand PCR using the ABI PRISMTM Dye Terminater Cycle Sequencing kit (Applied Biosystems Division, Perkin-Elmer Cetus, Emeryville, CA, USA). Given the length of the DNA region amplified (more than 1102 base-pairs each) clones were sequenced in both directions using two universal vector primers and two primers internal to the insert (L-5'GGGTTTGGCAAGATTGTGT3', H-5'TGCATACCCCATCCAAGTCAA3').

Sequences were determined using an Applied Biosystems 373A DNA sequencer and analyzed with SeqEd software (Perkin-Elmer, Applied Biosystems). Full sequences of the D-loop region were assembled by overlapping forward and reverse sequencing products. Ambiguous sequences were confirmed by re-sequencing the region in question using the same clone or different clones from the same individual. Unique substitutions were confirmed by sequencing clones from a second independent PCR product.

Data analyses

Sequences of the mtDNA D-loop region were aligned using CLUSTAL-V multiple alignment software (Higgins et al. 1992). The tandem repeat motif "CACCTGTG" was not included in the analysis because the number of repeats was variable within individuals, indicating a high degree of heteroplasmy (as also shown by Xu and nason 1994).

Phylogenetic analysis of mtDNA haplotypes was performed using PAUP software version 4.0.0d6d for Macintosh (Swofford 1997). Genetic distances were estimated using both the absolute number of nucleotide differences in the D-loop sequence and the Tamura-Nei distance (Tamura and Nei 1993) calculated on the basis of an equal substitution rate per site. Phylogenetic trees of seven Cheju, four Mongolian, two Yunnan, one Przewalskii, one Swedish (S1, unknown breed; Xu and Árnason 1994) and three Thoroughbred (T1 - T3) horses (Ishida et al. 1994) were constructed using both maximum parsimony (Fitch 1977) and neighbor-joining (Saitou and Nei 1987) methods. The donkey (E. asinus) sequence (D1; Xu et al. 1996) was used as an outgroup. The statistical confidence of each node in the maximum parsimony analysis was estimated by 1,000 random bootstrap resamplings of the data (50% majority-rule consensus plus other groups compatible with this tree) using the heuristic search option (Felsenstein 1985). Tamura-Nei distances were used for the neighbor-joining method.

Results

Sequence variation in the mtDNA D-loop region

Nucleotide substitutions and insertions/ deletions in the mtDNA D-loop region

(including tRNAPro and parts of tRNAThr and tRNAPhe) of seven Cheju and four Mongolian, two Yunnan, one Przewalskii, one Swedish and three Thoroughbred horses are shown in Fig 1. Analysis of the mtDNA D-loop sequences (excluding the tandem repeat region) showed 66 polymorphic sites, representing 6% of the total DNA sequence analyzed (1102 bp). The number of repeats in the tandem repeat region varied both among and within individual horses from 7 to 35. Nine of the 66 variable positions represented insertion/deletion of single base pairs. The remaining 57 variable positions were single nucleotide substitutions, only two of which were transversions, indicating a strong transitional bias that is common in mammalian mitochondrial evolution (Vigilant et al. 1991). The average percentage of polymorphic sites was 6.0% for the entire 1102 bp region analyzed and 6.4% for the D-loop exclusively (excluding the tRNA genes). Fourteen percent of the total number of polymorphic sites were found in a highly variable region between positions 201 and 300 .

The total number of polymorphic sites in the 1102 bp region was 41, 26, 15 and 3 for seven Cheju, four Mongolian, three Thoroughbred and two Yunnan horses, representing 5.9, 6.5, 5.0 and 1.5 polymorphic sites/individual, respectively. Mongolian horses appeared to be the most heterogeneous, followed by Cheju, Thoroughbred and Yunnan horses, respectively. However, the levels of variation detected were sample size-dependent. Thus, caution should be observed in drawing specific conclusions on genetic heterogeneity within and among breeds.

Phylogenetic Analysis

Pairwise genetic distances (Table 2) showed a large variation among individuals within and among groups. As expected, the average interbreed Tamura-Nei distance (=0.0182, SE=0.0006) was greater than the average intrabreed distance (=0.0145, SE=0.0009).

Maximum parsimony and neighbor-joining trees produced similar patterns. The maximum parsimony tree showed that, although three Cheju samples (C1, C2 and C5) were significantly clustered with Mongolian samples, others were grouped with distantly related breeds (Fig 2A). Cheju sample C1 was grouped with a Mongolian sample (M1) with a bootstrap probability of 81% while samples C2 and C5 clustered with M2, M3 and M4 with a 77% bootstrap probability. On the other hand, Cheju samples C3, C4, C6 and C7 were distantly clustered with

Yunnan, Thoroughbred, Swedish and Przewalskii horses. Yunnan samples Y1 and Y2 were further clustered into one clade (89%), while Thoroughbred sample T1 and T3 cluster together (88%) but independently from T2. The neighbour-joining tree constructed from Tamura-Nei distances showed a similar pattern, dividing the analyzed samples into three distinct groups: 1) C1, M1, C6, P1, C4 and S1; 2) C3, C7, Y1, Y2, T1, T2 and T3; and 3) C2, M2, C5, M3 and M4 (Fig 2B). Both maximum parsimony and neighbor-joining trees showed that Cheju horses clustered with Mongolian horses as well as with horses from other distantly related breeds.

Discussion

On the basis of historical records Cheju horses have been assumed to be of Mongolian origin (Nam 1969). Although characteristics such as height, body length and head length show similarities to traits typical of Mongolian breeds, morphological features of animals may vary considerably with environmental conditions. For example, height, which has been a common measure used for distinguishing Cheju horses from modern breeds, increases with improved feeding regimes. In the present study we used a morphology-independent method based on mtDNA sequence variation to more precisely define the origin of Cheju horses. Using the sequence polymorphism of the mtDNA D-loop region we found that some extant horses on Cheju Island are very closely related to Mongolian horses in their maternal lineage but others are not (Table 2 and Fig 2). However, at this time we cannot entirely rule out the possibility that all Cheju horses are exclusively of Mongolian origin in their matrilines because the number of Mongolian horses sampled was small and the Mongolian breed is itself heterogeneous (e.g., five distinct morphological types have been identified among the extant breeds). Our analysis indicates that Thoroughbred horses may also be of mixed origin in their maternal lineage. On both phylogenetic trees one of the Thoroughbred horses (T2) did not cluster with the other two, suggesting that either an extensive differentiation has occurred in this recently developed breed, or more than one maternal lineage has been involved in the formation of the breed.

Ishida et al. (1995) previously established phylogenetic relationships among horse breeds using a 271 bp region of the mtDNA (between tRNAPro and a large

conserved sequence block). When we used this 271 bp region to study the relationship of Cheju horses to other horse breeds (data not shown), the result was different from that found using the entire D-loop region. This may not be surprising given that the 1102 bp region analyzed is four times larger than the 271 bp region and, therefore, contains a higher number of phylogenetically informative sites.

Levels of D-loop variation within and among the studied breeds are consistent with those reported for other domestic species. For example, Loftus et al. (1994) consistently found lower intrabreed than interbreed genetic variation in cattle. In the present study, average Tamura-Nei distance between individuals from different breeds was 1.8%, with individual pairwise values as large as 3.3% (e.g., between samples M2 and T2). Consistent with other studies (Ishida et al. 1994, 1995) these values suggest that domestic horse breeds are relatively ancient. In addition, the large variation in the Tamura-Nei distances observed among Mongolian samples is consistent with the morphological heterogeneity observed in this breed.

All horse samples tested showed similar distances from the donkey outgroup (0.098 - 0.106), indicating internal rate consistency, as also shown in cattle breeds that had similar distances from Bison (Loftus et al. 1994). Under the assumption of the molecular clock we can estimate the rate of nucleotide substitution (λ) based on the equation $d=2\lambda t$ where d is the number of nucleotide substitutions between a pair of sequences and t is the divergence time (Ishida et al. 1995). Assuming that the divergence of the ancestral Equus species occurred about 3.0 million years ago (George and Ryder 1986, Lindsay et al. 1980), and using the mean genetic distance between the studied horse breeds and the donkey (d=0.10), we can estimate an evolutionary rate ($\lambda = d/2t$) of 1.7 x 10-8 substitutions per nucleotide site per year. The divergence time of the domestic horse breeds can then be estimated using the observed average genetic distance among the studied breeds (d=0.018). Assuming a constant molecular clock we estimate that domestic horse breeds diverged about 0.5 million years ago (=0.018/[2 x 1.7 x 10-8]), which is within the time range estimated by Ishida et al. (1995).

We provide the first molecular evidence supporting the historical record that Mongols introduced horses onto Cheju island in 1276 (Nam 1969), showing that some extant Cheju horses are descendants of Mongolian matrilines. However, phylogenetically distinct lineages of some Cheju horses suggest that other horse

breeds contributed to the extant population. Therefore, we speculate that Cheju horses are of mixed origin in their maternal lineage, and that horses may have existed on the island prior to the introduction of Mongolian horses in 1276. In addition, our results support previous studies showing that domestic horse breeds have a relatively ancient origin (Ishida et al. 1994, 1995). Studies of mtDNA variation in other potential contributors to the Cheju horse gene pool, including equine fossils from Cheju Island (i.e., ancient DNA analysis) would provide a better understanding of the origin of this Korean horse breed.

Accession numbers

GenBank accession numbers for the mtDNA D-loop of C1 - C7, M1 - M4, P1, Y1 and Y2 are AF014405, AF014406, AF014407, AF014408, AF014410, AF014411, AF014412, AF014413, AF014414, AF014415, AF056071, AF014409, AF014416 and AF014417, respectively.

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Table 1. Body weight, wither height and body length of male and female horses at different growing stages.

Age(mo)	Body weight(kg)		wither height(cm)		Body length(cm)	
	Male	Female	Male	Female	Male	Female
1	52.9	48.2	82.4	83.0	74.7	73.8
2	66.7	63.4	85.9	86.7	79.8	79.4
6	113.4	114.2	97.0	98.5	96.0	96.9
12	161.9	165.0	107.6	109.1	109.9	111.7
18	191.2	193.4	113.1	114.1	115.8	117.7
24	208.6	208.9	115.9	116.1	117.4	119.0
30	221.4	220.6	117.7	116.8	221.4	220.6
36	237.0	237.7	119.5	117.6	118.7	119.1
42			121.8	119.0	121.1	120.9
48			124.5	121.0	124.9	123.9
54			127.0	122.9	128.8	126.8
60			127.9	123.3	130.5	127.2

Adapted from Yang et al. 1996.

Tabe 2. Pairwise distances betwen taxa*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	C1	-	159	111	111	169	102	139	37	207	168	169	177	158	120	158	233	148	130	1014
2	C2	17	-	168	187	102	158	196	168	65	102	140	273	234	177	245	301	235	205	1005
3	C3	12	18	-	111	196	83	65	130	216	197	216	102	120	121	139	250	130	111	983
4	C4	12	20	12	-	216	102	102	111	235	216	216	187	177	102	139	234	130	92	1017
5	C5	18	11	21	23	-	187	206	159	55	18	37	253	224	187	234	281	225	177	995
6	C6	11	17	9	11	20	-	130	121	206	168	187	158	146	92	187	243	177	158	1017
7	C7	14	20	6	10	21	13	-	139	225	206	225	167	168	111	93	205	83	102	993
8	M1	4	18	14	12	17	13	14	-	197	158	178	215	196	102	158	272	149	130	1028
9	M2	22	7	23	25	6	22	23	21	-	74	93	293	263	206	274	330	265	215	1018
10	M3	18	11	21	23	2	18	21	17	8	-	35	263	224	187	235	291	226	196	1018
11	M4	18	15	23	23	4	20	23	19	10	6	-	244	205	206	234	291	225	196	1006
12	Y1	19	29	11	20	27	17	17	23	31	28	26	-	28	196	206	158	197	187	1050
13	Y2	17	25	13	19	24	16	17	21	28	24	22	3	-	186	206	139	196	177	1051
14	P1	13	19	13	11	20	10	11	11	22	20	22	21	20	-	149	262	139	120	1063
15	T1	17	26	15	15	25	20	9	17	29	25	25	22	22	16	-	139	9	139	1029
16	T2	25	32	22	25	30	26	21	29	35	31	31	17	15	28	15	-	129	252	1059
17	T3	16	25	14	14	24	19	8	16	28	24	24	21	21	15	1	14	-	129	1028
18	S1	14	22	12	10	19	17	10	14	23	21	21	20	19	13	15	27	14	-	1006
19	D1	100	99	97	100	98	100	97	101	100	100	99	103	103	104	101	104	101	99	-

* Figures above the diagonal are Tamura-Nei distances x 104 calculated on the basis of an equal substitution rate per site. Those below the diagonal are absoluted distances calculated based on the total number of nucleotide differences in the mtDNA D-loop region, excluding the tandem repeat region, of Cheju (C1-C7), Mongolian(M1-M4), Yunnan(Y1-Y2), Przewalskii(P1), Thoroughbred(T1-T3), Swedish(S1), horses and a donky(D1).

Fig. 1. Nucleotide substitutions and gaps mtDNA D-loop, tRNAPro, and parts of tRNAThr and tRNAPhe of seven Cheju (C1-C7), four Mongolian (M1-M43), two Chinese Yunnan (Y1-Y2), one Przewalskii (P1), one Swedish (S1) and three Thoroughbred (T1-T3) horses. Data for the last two breed sere adapted from Xu and Åmason (1994), and Ishida et al. (1994), respectively. Dots indicate matching with sequence of C1. Nucleotide position numbers on the top of the figure begin with the 42nd base of tRNAThr sequence as position 1. The heteroplasmic repeat region was exculded in the labelling of the positions. * and + are MboI and HincII restriction sites, respectively.

Fig 1 Nucleotide substitutions and gaps

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                                                                   +
                              *+                              *   11
     111111122222222222222223333334444444445555666666777777777888899990 0
   23422266771122233378889934567700044450008077790344556680011567845
   0916784604077894566012895129122391236913497012838686756225785860 38
C1 -GATCACCGCTAAAATTGTTAAGATTATAACTAAAC-GTCCAT-AA-TCGGGACACAACATTC-AT
C2 ......C.A.........A...G..CC...G..G..T.A....C.GGC...AAG.......A...G.
C3 ......-.A.................C...........A.TT.C......AA.GTG........-..C
C4 ........A..G...C.....GA..C.....C..G...............A.GTG.......-...
C5 ........A..G..G..A.....GCC......GG...A...GC..GC....AG...C.-.A...G.
C6 ........A.........C..GA..C............A.T.G........A.G.........-..C
C7 ........A..G..............C.C..TC.....A.T.........AA.GTG..G........
M1 ........A..G..G......G.....................C.........................
M2 ........A..GG.G..A......CC...G..G..T.A...GC.GGC...AAG...C...A.-.G.
M3 ........A..G..G..AC....GCC......GG...A...GC..GC....AG.....-.A...G.
M4 ........A..G..G..A..G..GCC......GG...A...G....C....AG...C.-.A...G.
Y1 ...C.G.TA.......C...G....C............A.TTGC....CTA..GTG...-....-...
Y2 ...C.G.TA.......C...G....C............A.TTG.....CT...GTG...-......G.
P1 ........AT.GG.........GA..C.....C.....ACT..C..........G..........-...
T1 .AG.....A.CG.G............C.C..TC..G.TA.T..CT.....AA.GTG....C..-...
T2 .AGC...TA.......CA.G.....C.CT.TC..G.TA.TT..T.....AA.GTG....C.C-CG.
T3 .AG.....A..G.G............C.C..TC..G.TA.T.GCT.....AA.GTG....C..-...
S1 T...T...A..G...C..........CG....C.....A....C..........GTGTC..C..-...
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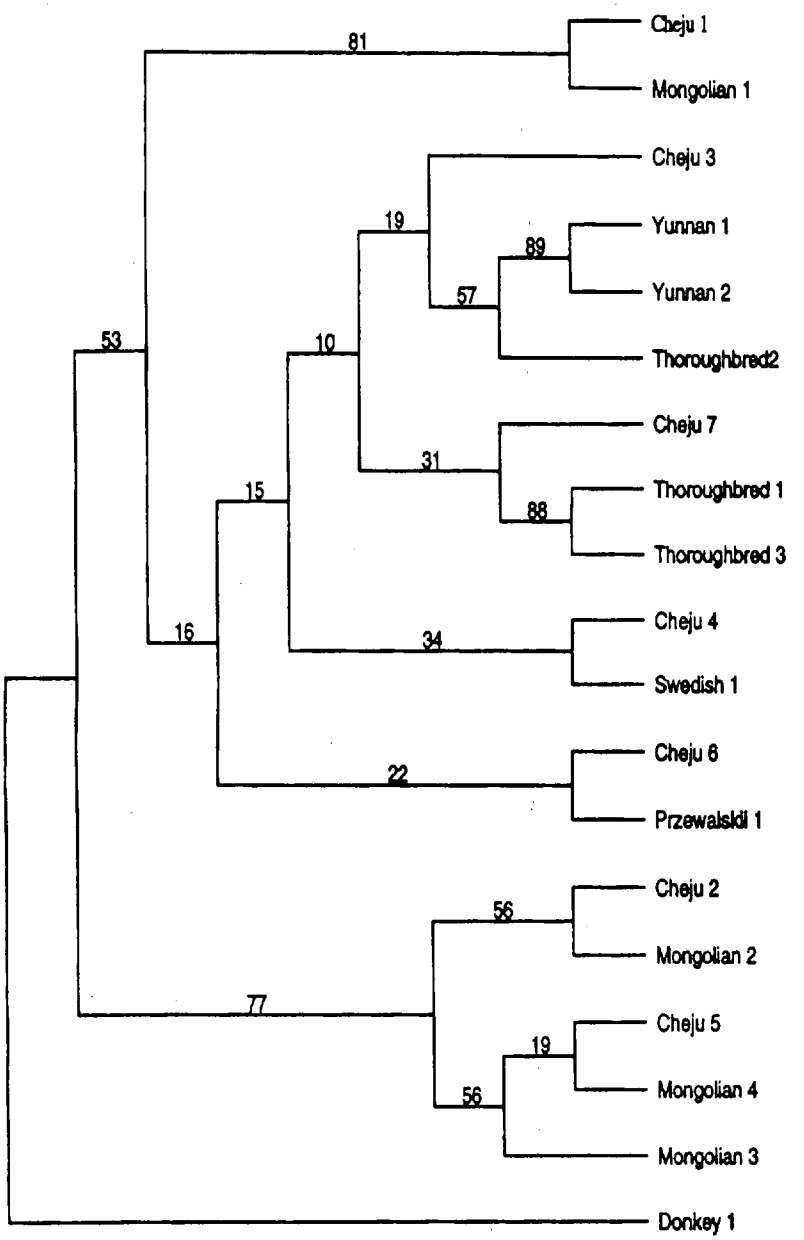
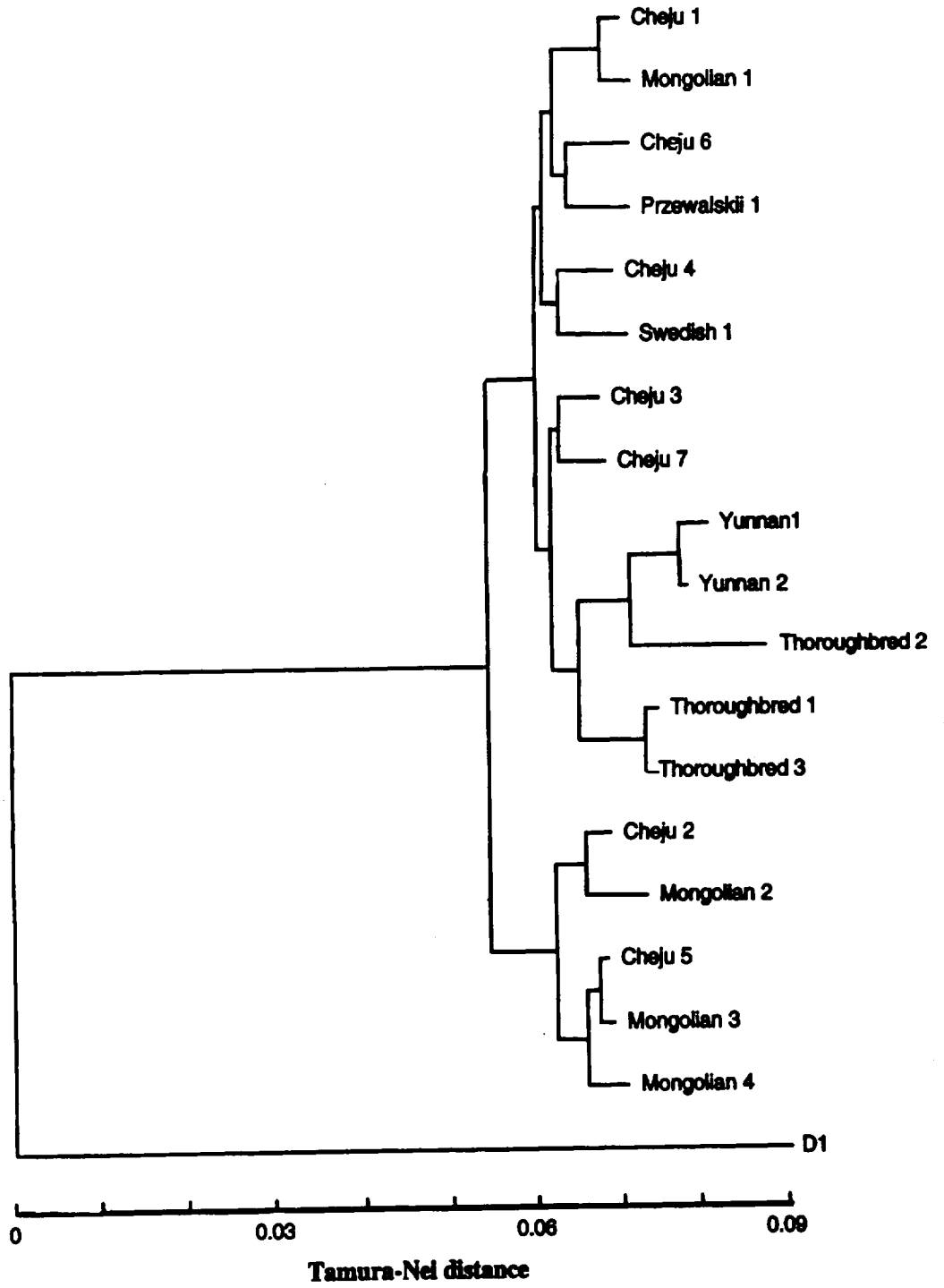
Fig. 2. (A) Phylogenetic tree of 18 horse including Cheju, Mongolian, Chinese Yunnan, Przewalskii, Swedish and Thoroughbred along with a donkey as an outgroup. The tree was drawn using the maximum parsimony method(heuristic search). Figures on internodes are bootstrap probabilities(in %) based on 1,000 bootstrapped maximum parsimony trees. (B) Neighbor-joining tree based on the Tamura-Nei distances reported in Table 2.




Cheju 1
Mongolian 1
Cheju 6
Przewalskii 1
Cheju 4
Swedish 1
Cheju 3
Cheju 7
Yunnan1
Yunnan 2
Thoroughbred 2
Thoroughbred 1
Thoroughbred 3
Cheju 2
Mongolian 2
Cheju 5
Mongolian 3
Mongolian 4
D1
0
0.03
0.06
0.09
Tamura-Nei distance