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Molecular Phylogeny Based on the κ-Casein and Cytochrome b Sequences in the Mammalian Suborder Ruminantia

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Abstract. Nucleotide sequences for the κ-casein precursor proteins have been determined from the genomic DNAs or hair roots of the Ruminantia. The coding regions, exons 2, 3, and 4, were amplified separately via the three kinds of PCRs and then directly sequenced. The primers were designed from the sequence of bovine κ-casein gene; they were applicable for the amplification of the κ-casein genes from the 13 species in the Ruminantia except exon 2 of the lesser mouse deer. These results permitted an easy phylogenetic analysis based on the sequences of an autosomal gene. A phylogenetic tree was constructed from the mature κ-casein sequences and compared with the tree of the cytochrome b genes which were sequenced from the same individuals. The Cervidae (sika deer, *Cervus nippon*) were separated from the branch of the Bovidae on the tree of κ-casein genes with a relatively high confidence level of the bootstrap analysis, but included in the branch of the Bovidae on the tree of cytochrome b genes. The κ-casein tree indicated a monophyly of the subfamily Caprinae, although the internal branches were uncertain in the Caprinae. The tree based on the nucleotide sequences of cytochrome b genes clearly showed the relationships of the closely related species in the genus *Capricornis* consisting of serow (*C. smatorensis*), Japanese serow (*C. crispus*), and Formosan serow (*C. swinhoei*). These results would be explained by the difference of resolving power between the κ-casein and the cytochrome b sequences.

Key words: Evolution—κ-Casein—Cytochrome b—Artiodactyla — Ruminantia — Caprinae — *Capricornis*

Introduction

The Artiodactyla appeared in the fossil record near the Paleocene–Eocene boundary and rapidly radiated (Novacek 1982). Although the Bovidae are a main family in the suborder Ruminantia, the monophyly of the Bovidae has been poorly established from morphological evidence (Kraus and Miyamoto 1991). Molecular phylogenetic analysis is a powerful method for resolving evolutionary relationships. However, the molecular data from the Ruminantia also led to confusion. Protein sequences of the fibrinopeptide (Goodman 1981) and the ribonuclease (Beintema et al. 1986) supported the monophyly of the Bovidae and of the Cervidae. Partial sequences of the mitochondrial 12S and 16S ribosomal genes suggested the paraphyly of the Bovidae (Gatesy et al. 1992). The 2.7 kb of the complete 12S and 16S genes and three adjacent tRNA genes indicated monophyly of the Bovidae, though the separate analysis for the 16S genes was

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Table 1. Taxa examined in this study

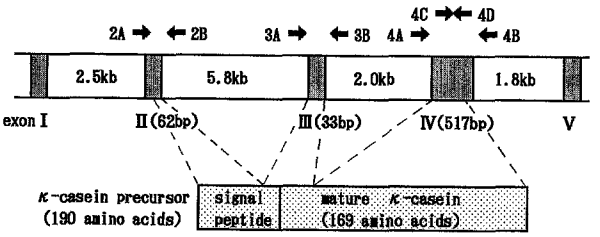
Family	Bovid tribe	Species	Common name
Bovidae	Bovini	*Bos taurus*	Cattle
		Bubalus bubalis	Water buffalo
	Saigini	*Saiga tatarica*	Saiga
	Caprini	*Capra hircus*	Goat
		Ovis aies	Sheep
	Rupicaprini	*Rupicapra rupicapra*	Chamois
		Oreamnos americanus	Rocky Mountain goat
		Nemorhaedus goral	Goral
		Capricornis sumatrensis	Serow
		Capricornis crispus	Japanese serow
		Capricornis swinhoei	Formosan serow
Cervidae		*Cervus nippon*	Sika deer
Tragulidae		*Tragulus javanicus*	Lesser mouse deer
Suidae		*Sus scrofa*	Pig

not consistent with the conclusion from the 2.7-kb sequence (Allard et al. 1992). The 1,140 bp of the complete cytochrome b genes did not show a monophyly of the Bovidae; the Cervidae were included in the branch of the Bovidae (Irwin et al. 1991).

The substitution rate in mammalian mtDNA was five to ten times higher than that in chromosomal genes. Therefore, saturation of changes occurred in a relatively short period (Brown et al. 1979; Nei 1987; Irwin et al. 1991). Brown et al. (1979) showed that the estimate of the substitutions in mtDNA was most accurate in the last 5 million years and then became increasingly less accurate for greater divergence times. The radiation of the pecoran families may have occurred about 23–28 million years ago (Kraus and Miyamoto 1991). Thus, the difficulty of the phylogeny in the Ruminantia might be due to the limit of the resolving power of mtDNA sequences (Irwin et al. 1991).

Another explanation is an introgressive hybridization between the ancestral species of Bovidae and Cervidae. Mitochondrial genes have been inherited from only the maternal lineage. Thus, a phylogenetic tree of a mitochondrial gene reveals the relationships of the maternal lineage. The data of Carr and Hughes (1993) implied a mtDNA flow from mule deer (*Odocoileus hemionus*) to white-tailed deer (*Odocoileus virginianus*) by comparison of the cytochrome b sequences. The phylogenetic trees from mtDNA of extant species might be muddled by introgression.

An alternative way to confirm the phylogeny is to construct the phylogenetic trees of autosomal genes. We have determined the sequences of the κ-casein gene for phylogenetic analysis. Kappa-casein is a milk protein that forms the casein micells along with the α- and



2A : 5'-CTAATTGTTTAATTTAATTTAGGTGCA-3' (2578-2604)
2B : 5'-CAAAACTGATTAGATGAAATTAACTC-3' (2664-2689)
3A : 5'-TCTTGTTTCTGATTCTTTCTCTGAATCTC-3' (8451-8479)
3B : 5'-GGATTCATATCTCTACTGGAAACAAACTTA-3' (8519-8548)
4A : 5'-TAATACCATTCTGCATAATTTATTTTTTTACAG-3' (10496-10528)
4B : 5'-CTGCGTTGTCTTCTTTGATGTCTC-3' (11022-11045)
4C : 5'-CCCACATTTATCATTTATGGCCATTCC-3' (10801-10827)
4D : 5'-GATTTCTGTTTTATCCTGATTTTTCTTTGG-3' (10829-10858)

Fig. 1. Structure of the bovine κ-casein gene and the primers for amplification of the artiodactyl κ-casein genes. *Numbers in parentheses* are the positions according to the bovine κ-casein gene (Alexander et al. 1988).

β-caseins. These casein genes are clustered on an autosome and are homologous except for the κ-casein gene (Mercier et al. 1990). The κ-casein gene has a quite different structure from the other casein genes and is evolutionarily related to the fibrinogen gene family (Alexander et al. 1988). Consequently, there probably has been no crossing over between the κ- and the other casein genes since the divergence of the mammals. Because κ-casein plays an essential function in the mammalian milk and its evolutionary rate of the mature proteins is rapid (Mercier et al. 1990), the sequences of the κ-casein genes will provide a useful phylogenetic tree based on an autosomal gene. In this study, we sequenced the κ-casein and the cytochrome b genes from Ruminantia to compare the phylogenetic trees of the two genes.

Materials and Methods

DNA Sources (Table 1). DNA samples were prepared from the muscle of cattle (*Bos taurus*), sheep (*Ovis aies*), goat (*Capra hircus*), pig (*Sus scrofa*), Japanese serow (*Capricornis crispus*), sika deer (*Cervus nippon*), and lesser mouse deer (*Tragulus javanicus*). The DNA of water buffalo (*Bubalus bubalis*) was extracted from a blood sample as described by Sambrook et al. (1989). The hair roots from the Japanese serow, Formosan serow (*Capricornis swinhoei*), serow (*Capricornis sumatrensis*), goral (*Nemorhaedus goral*), chamois (*Rupicapra rupicapra*), Rocky Mountain goat (*Oreamnos americanus*), and saiga (*Saiga tatarica*) were used as templates for the PCR amplifications (Saiki et al. 1985).

κ-Casein Sequences. Sequencing was performed as described in the previous paper (Chikuni et al. 1994b). Primers used for PCR amplifications are shown in Fig. 1. The primers were designed from the sequence of the bovine κ-casein gene (Alexander et al. 1988). The exons 2, 3, and 4 were amplified via PCRs using the primers 2A/2B, 3A/3B, and 4A/4B, respectively (Fig. 1). The sequencing for exon 4 was performed with the two segments that were amplified via the two kinds of second PCRs using the primers 4A/4D and 4C/4B. The PCRs were run as follows: each cycle of denaturation for 1 min at 93°C,

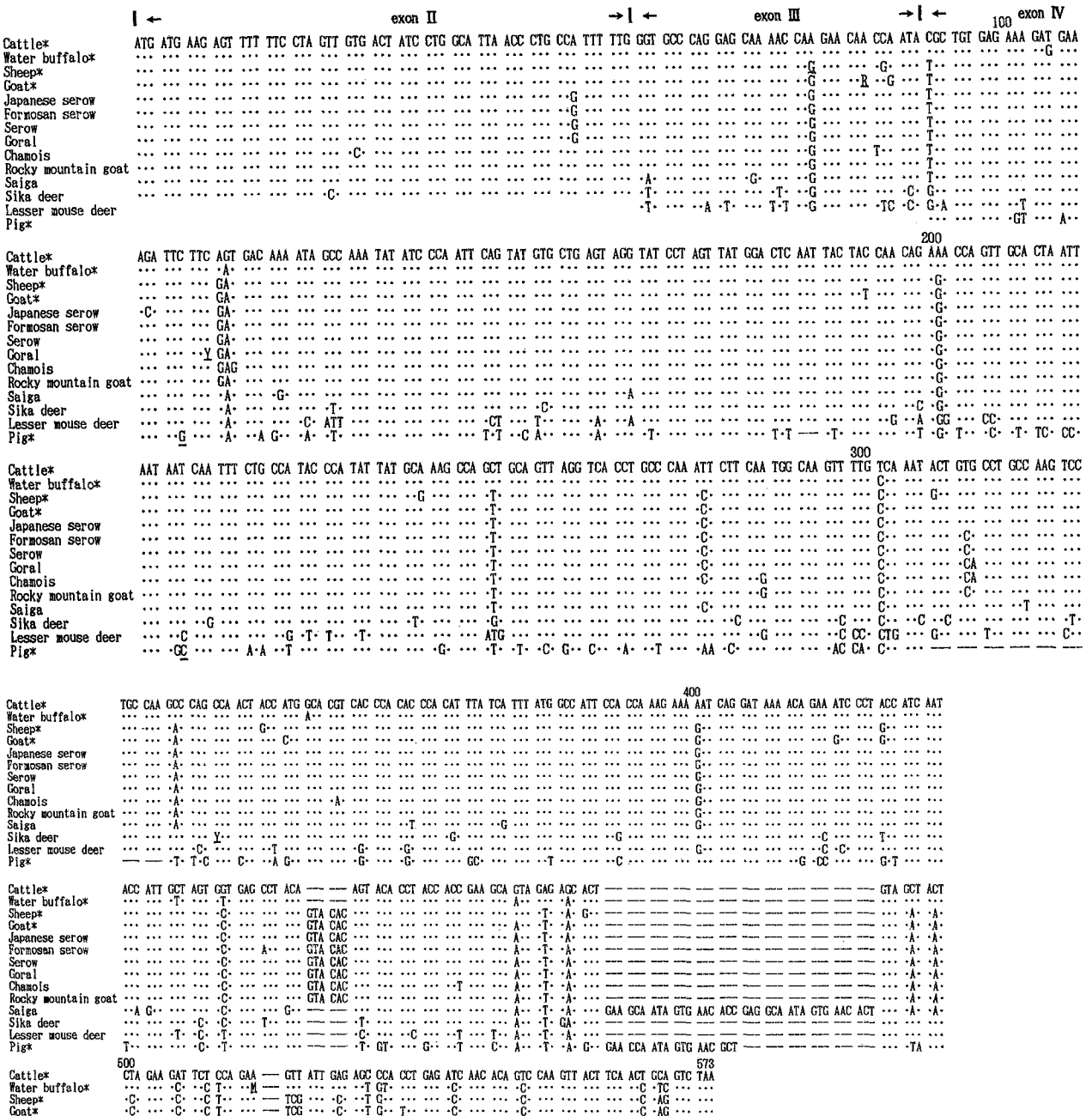


Fig. 2. Nucleotide sequences for κ-casein precursor proteins from the 14 species in the Artiodactyla. *Dots* indicate nucleotides that are identical to the bovine A-type gene. Nucleotides 1–57, 58–90, and 91–3′ terminal are coded in exon 2, 3, and 4, respectively. Exon 2 of the lesser mouse deer and pig and exon 3 of the pig have not been sequenced because these regions were not amplified by the PCR. *The nucleotide sequences for the bovine, water buffalo, sheep, goat, and pig κ-casein precursors were reported in the previous papers (Alexander et al. 1988; Chikuni et al. 1994b; Furet et al. 1990; Coll et al. 1993; Levine et al. 1992). Polymorphic sites and the different nucleotides from the published sequences are *underlined.* Polymorphisms: R, A/G: Y, C/T: M, A/C.

annealing for 1 min at 55°C for exon 2 or 60°C for exons 3 and 4, and extension for 1 min at 72°C for 35 cycles. The PCR product was purified by electrophoresis on a 4% NuSieve GTG agarose gel (FMC Bio Products, USA) and then used as a template for the cycle sequencing with a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., USA).

The hair-root sample was extracted as described in Higuchi et al. (1988) with a slight modification. The genomic DNA was extracted from one or two hair roots using a proteinase K digestion and then amplified via PCR in one tube. The PCR product from the hair-root sample was purified by electrophoresis and then used as a template for the second PCR using the same primer set. The pure DNA for sequenc-

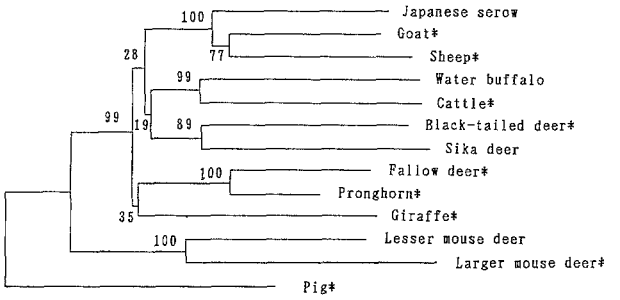


Fig. 3. A phylogenetic tree based on the complete sequences of cytochrome b genes from the 13 species in the Artiodactyla. *The cytochrome b genes of cattle, sheep, goat, black-tailed deer, fallow deer, pronghorn, giraffe, and large mouse deer were reported in the previous papers (Anderson et al. 1982; Irwin et al. 1991).

ing was obtained from the second PCR product using the same procedures as the first PCR.

Cytochrome b Sequences. The entire cytochrome b genes were determined from the DNAs of the Japanese serow, water buffalo, sika deer, and lesser mouse deer. The sequence was directly determined from the PCR product which was amplified by using the forward primers MI03 (5′-GACTAATGATATGAAAAACCATCGTTG-3′), MI01 (5′-CAAATCCTCACAGGCCTATTCCTAGC-3′), and MI05 (5′-TCCACGAAACAGGATCCAACAACCC-3′), and the reverse primers MI02 (5′-TAGGCGAATAGGAAATATCATTCGGGTTTGAT-3′) and MI04 (5′-TTGTTCTTCATCTCTGGTTTACAAGAC-3′). The primers, corresponding bovine mtDNA numbers L14465–14491 (MI03), L14643–14668 (MI01), L15112–15136 (MI05), H15315–15346 (MI02), and H15683–15709 (MI04), were designed from the sequences of the bovine, human, and chicken mtDNAs (Anderson et al. 1981, 1982; Desjardin and Morais 1990). The PCRs were run as follows; each cycle consisting of denaturation for 1 min at 93°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C for 35 cycles.

The 646 bp of the partial sequences of cytochrome b genes were determined from the hair roots of the seven Caprinae. This region was amplified via PCR using the primers MI01/M/I02.

Data Analysis. Phylogenetic analyses were conducted with the ODEN program, version 1.1.1, developed by Y. Ina in the National Institute of Genetics. The number of nucleotide substitutions per site was estimated by the six-parameter method (Gojobori et al. 1982). The number of amino acid replacements per site was estimated by Kimura's (1983) method. The phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei 1987) using the pig sequences as an outgroup. Bootstrap analyses (Felsenstein 1985) based on 100 resampling events were conducted to estimate the stability of the phylogenetic trees.

Results and Discussion

Direct Sequencing for κ-Casein Genes

The nucleotide sequences for κ-casein precursor proteins were determined by the combination of sequencing of exons 2, 3, and 4. This sequencing strategy was based on the hypothesis that artiodactyl κ-casein genes are homologous. The order Artiodactyla diverged about 64–68 million years ago, and the subfamily Bovinae and the subfamily Caprinae diverged about 20 million years ago (Novacek 1982). The published sequence of the sheep κ-casein cDNA (Furet et al. 1990) was 92.7% identical with the bovine sequences in the amino acid coding region. The sequence of the water buffalo κ-casein gene, which was determined by the same procedure as described in this paper, was 96.7% identical with the bovine sequences (Chikuni et al. 1994b). When the primer regions were highly similar among artiodactyl κ-casein genes, target regions were amplified by using the bovine primers as well as using the conserved primers in cytochrome b sequencing (Kocher et al. 1989; Kocher and White 1989). The length of the primers, 24–33 nucleotides, was enough to prevent an unexpected annealing. If the primer set annealed to the other genes, the length of the PCR products would not be equal to the fragments of the κ-casein gene. Alexander et al. (1988) showed that bovine κ-casein gene consisted of five exons with the amino acid sequence encoded in exons 2, 3, and 4. We designed the PCR primers near the amino acid coding regions of the bovine gene to determine the entire sequences of the κ-casein amino acid coding regions (Fig. 1).

The bovine, water buffalo, sheep, goat, Japanese serow, Formosan serow, serow, goral, chamois, Rocky Mountain goat, saiga, lesser mouse deer, and pig DNAs were amplified via the PCRs and then directly sequenced except for exon 2 from the lesser mouse deer and pig and exon 3 from the pig. The sequences are shown in Fig. 2. The gene sequences of the sheep and goat were identical to the cDNA sequences (Furet et al. 1990; Coll et al. 1993) except for a synonymous substitution at position 78 of the sheep gene and a synonymous polymorphism at position 84 of the goat gene. The pig gene differed slightly from the cDNA sequence (Levine et al. 1992) at positions 114 and 222. A substitution at position 114 caused an amino acid change from Phe to Leu on the deduced sequence. Another one at position 222 was synonymous.

Pigs, the species in the suborder Suina, diverged early from the Ruminantia in artiodactyl history (Novacek 1982). Therefore, it was speculated that the similarity between pig and bovine genes would be lower than that among the genes of the Ruminantia. Compared with the bovine κ-casein sequence, the published pig cDNA was more different than the sheep and goat cDNAs; a 3-bp deletion, a 24-bp deletion, an 18-bp insertion, and a 3-bp insertion were detected (Levine et al. 1992). The determined sequence of the pig κ-casein exon 4 was in agreement with the published cDNA sequence. The deduced amino acid sequences from exons 2 and 3 of the other species were specific for the signal peptide of κ-casein. Thus, we concluded that the nucleotide sequences determined in this study were the coding regions for the κ-casein precursor proteins.

The signal peptide of the bovine κ-casein was en-

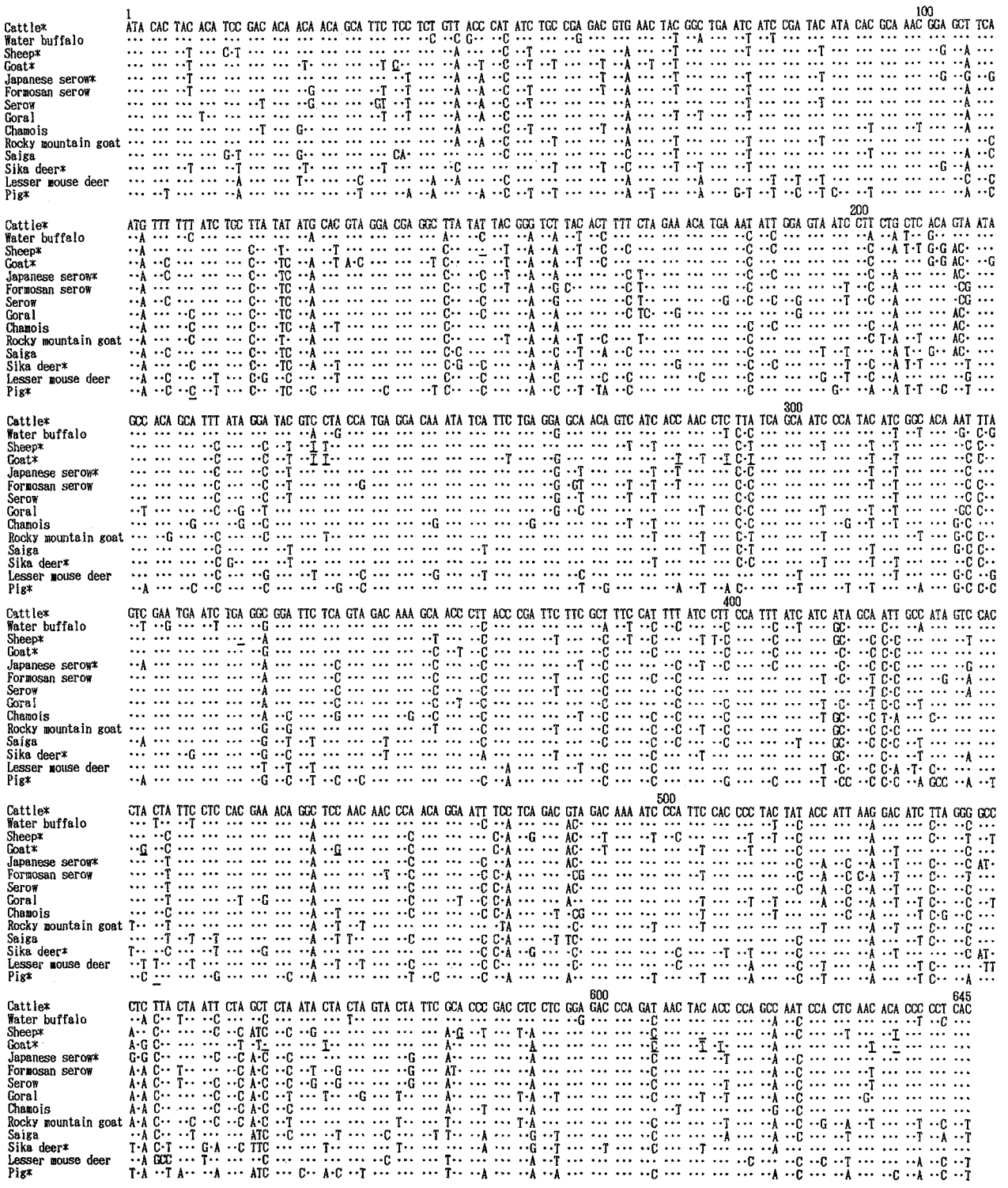


Fig. 4. Partial sequences of the cytochrome b genes from 14 species in the Artiodactyla. *The sequences of cattle, sheep, goat, pig, Japanese serow, and sika deer were reported in the previous papers (Anderson et al. 1982; Irwin et al. 1991; Chikuni et al. 1994a). The different nucleotides from the published sequences are *underlined.*

coded in the 57 nucleotides of exon 2 and the six nucleotides of exon 3 (Alexander et al. 1988). Nucleotide substitutions in this region were synonymous in the Japanese serow, Formosan serow, serow, and Goral genes. The nonsynonymous substitutions in the chamois, saiga, sika deer, and lesser mouse deer genes resulted in conservative amino acid replacements (Fig. 2). As the mature κ-casein region was more variable than the signal peptide region (Mercier et al. 1990), and exon 2 of the lesser mouse deer was not determined, we constructed

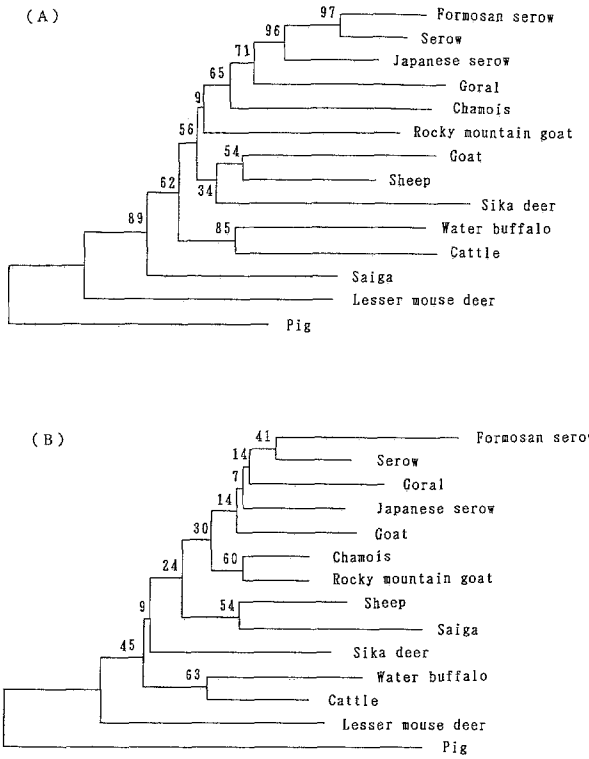


Fig. 5. Phylogenetic trees of cytochrome b based on **(A)** the 645-bp nucleotide sequences and **(B)** the deduced amino acid sequences. The *number* at each branch indicates the numbers of times the branch was found in 100 bootstrap replicates.

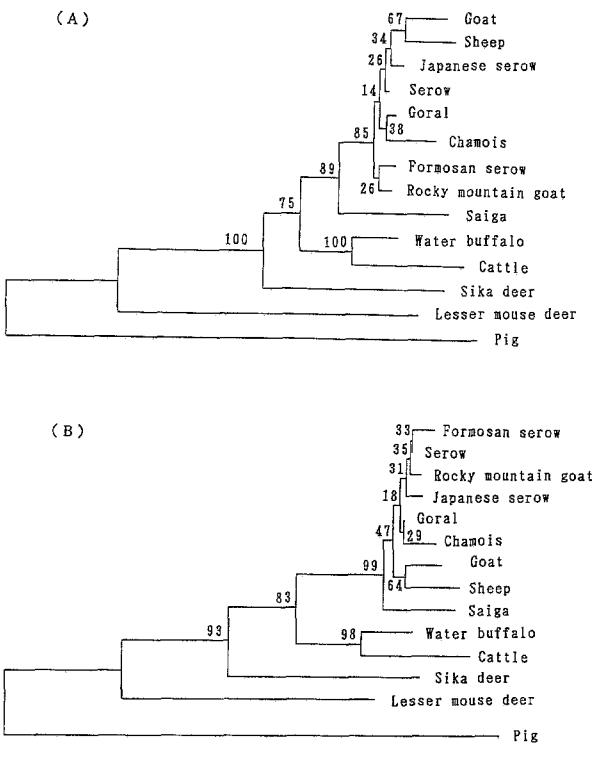


Fig. 6. Phylogenetic trees of the mature κ-caseins based on **(A)** nucleotide sequences and **(B)** the deduced amino acid sequences. The *number* at each branch indicates the numbers of times the branch was found in 100 bootstrap replicates.

the phylogenetic trees from the mature κ-casein sequences (Figs. 2, 6).

Phylogenetic Analysis

Irwin et al. (1991) constructed phylogenetic trees from the complete sequences of cytochrome b genes of mammals. In their trees, the divergence between the Bovidae and the Cervidae was uncertain; the cow, sheep, goat, pronghorn fallow deer, giraffe, and black-tailed deer belonged in one group. In order to confirm the placements of our species, especially sika deer, we sequenced the complete cytochrome b genes from the lesser mouse deer, sika deer, water buffalo, and Japanese serow and then constructed a phylogenetic tree from these four species together with the eight species of the previous reports (Irwin et al. 1991; Anderson et al. 1982). Figure 3 shows that the placement of the Cervidae including the sika deer was uncertain in the neighbor-joining tree of the complete cytochrome b genes as in the parsimony tree of Irwin et al. (1991). The giraffe, pronghorn, and fallow deer were separated from the Bovidae, but the sika deer and black-tailed deer were included in the branch of the Bovidae. The confidence levels of the bootstrap analysis were low at the branches of the Bovidae and the Cervidae.

For the phylogenetic analysis of the 14 species, we used the partial sequences of cytochrome b genes because this region was easily sequenced from the first PCR product (Fig. 4). Figure 3 indicates that ambiguity of the placement of sika deer in Fig. 5 has not resulted from the difference of the sequence length. Figure 5 shows the neighbor-joining trees based on the 645 nucleotide sequences (Fig. 5A) and the deduced 215 amino acid sequences (Fig. 5B).

Because of the rapid evolution in synonymous sites and the biased transition/transversion ratio in mtDNA, the saturation of nucleotide substitutions affects the evolutionary analyses. Transversions were used for the phylogenetic analyses to avoid the saturation effect (Irwin et al. 1991; Kraus and Miyamoto 1991; Allard et al. 1992). Adachi et al. (1993) stated that the amino acid sequence was more useful than the nucleotide sequence in studying deeper branches in evolutionary trees. However, the sika deer branch was not separated from the branch of the Bovidae in the tree based on the amino acid of the cytochrome b sequences (Fig. 5B).

Figure 6 shows the phylogenetic trees based on the mature κ-casein sequences. The trees of both the nucleotide sequences and the amino acid sequences indicated monophyly of the Bovidae, and a divergence of the Cervidae between the Tragulidae and the Bovidae branches. These trees indicated also monophyly of the subfamily Caprinae with a high confidence level. In the Caprinae, the branching pattern differed between the trees of the nucleotides and the amino acids, because the numbers of substitutions were less than 16 nucleotides

Table 2. Rates of substitutions for mature κ-casein (κ-CN) and cytochrome b (Cyt.b) ($\times 10^{-9}$/site/year)

	Divergence time (Myr)[a]	Nucleotides		Amino acids	
		κ-CN	Cyt.b	κ-CN	Cyt.b
Cattle/pig	60	2.0(1.7)	1.8 (1.6)	5.6(3.8)	0.83 (0.78)
Cattle/sheep, goat	20	1.9(1.8)	4.1 (3.8)	4.6(4.1)	1.5 (1.5)
Sheep/goat	5	2.6(2.5)	11.3 (11.0)	4.9(4.7)	3.9 (3.8)

[a] Most probable year from fossil records (Irwin et al. 1991) Data are corrected by the six-parameter method (Gojobori et al. 1982) for nucleotide substitutions and Kimura's (1983) method for amino acid replacements. Numbers in parentheses are the noncorrected rates

and less than 10 amino acids in the mature κ-casein sequences of the Caprinae excepting the saiga.

The subfamily Caprinae is composed of four tribes—Saigini, Ovibonini, Caprini, and Rupicaprini. The trees based on the nucleotide sequences of the cytochrome b and the κ-casein genes indicated that the saiga is a distant species from the Caprini and the Rupicaprini (Figs. 5A, 6A). The tree of the κ-casein was constructed from the shared sequences without the regions of deletion and insertion. The Caprini (sheep and goat) and the Rupicaprini (Japanese serow, Formosan serow, serow, goral, chamois, and Rocky Mountain goat) had the insertion of a GTACAC, whereas the saiga had no insertion in this region as the other Artiodactyla. This result was consistent with the genetic distances among the nucleotide sequences which suggested the early divergence of the saiga in the Caprinae history.

Mitochondrial DNA is favorable to examine the closely related species because of its fast evolution rate (Nei 1987). Figure 5A shows the relationships among the species in the tribe Rupicaprini, especially in the genus *Capricornis* with the high confidence level of the bootstrap analysis. Alternatively, the tree of the κ-casein genes did not indicate the relationships among the species in the *Capricornis* (Fig. 6). The substitutions in mtDNA were approximately linear with evolutionary time in the last 10 million years (Nei 1987). Brown et al. (1979) estimated the initial rate of substitutions for mtDNA as 1×10^{-8}/site/year from the RFLP analyses. The rates of substitutions for the cytochrome b and κ-casein genes were calculated using the divergence times in Table 2. The rate of the cytochrome b gene at the divergence time between the sheep and goats was consistent with the rate in Brown et al. (1979). The *Capricornis* would have radiated after the divergence of the sheep and goats (Fig. 5A). Thus, the cytochrome b genes evolved about four times faster than the κ-casein gene in the *Capricornis* (Table 2). This rapid evolution of the cytochrome b genes resulted in the higher resolving power for the *Capricornis*.

The genetic distance for the phylogenetic trees was corrected by the six-parameter method (Gojobori et al. 1982) for the nucleotide sequences or Kimura's (1983) method for the amino acid sequences. Nevertheless, the saturation effect was observed on the cytochrome b sequences (Table 2). The rates of the nucleotide substitutions at the cattle/pig and the cattle/sheep, goat were 16 and 36% of the initial rate of the sheep/goat, respectively. In the cytochrome b genes of 14 species, 79.1% of the substitutions occurred at third positions of codons (Fig. 4). The change of rate would result from multiple substitutions at third positions. The rapid radiation of the suborder Ruminantia may have occurred about 23–28 million years ago (Kraus and Miyamoto 1991). In this short period, the substitution rate of the cytochrome b genes was changing drastically by way of the saturation effect. If the evolutionary rate was biased in the Bovidae and the Cervidae, the rapid radiation and the saturation effect would result in an obscure pattern for the phylogenetic trees.

The pattern of substitutions in the κ-casein genes differed from that in the cytochrome b genes. Substitutions at the third positions of codons were 33.5% in the κ-casein (Fig. 2) and 79.1% in the cytochrome b genes. Synonymous substitutions were 26.4% in the κ-casein and 82.8% in the cytochrome b genes. This low ratio of the synonymous substitutions in κ-casein genes resulted in the high rate of amino acid replacements in the mature κ-caseins.

The saturation effect was not seen in the κ-casein genes. The rate of nucleotide substitutions in the κ-casein genes was stable throughout the period from 5 to 60 million years ago (Table 2). Because the Cervidae diverged in this period and the numbers of the substitutions were adequate for differentiation, the tree of the κ-casein indicated the placement of the sika deer with high bootstrap confidence.

The best strategy for circumventing the incongruence between gene trees and species trees is to obtain sequence data from several independently evolving loci (Honeycutt and Adkins 1993). The nucleotide sequences coding for amino acids are generally determined from mRNAs that are extracted from fresh tissues. However, it is difficult to obtain the sequence of a chromosomal gene because of the complexity of cloning procedures and necessity of the fresh tissue. We determined the nucleotide sequences for κ-casein precursor proteins without cloning procedures. The target regions were amplified via PCRs and then directly sequenced from the hair roots of animals. These procedures will facilitate the phylogenetic analysis based on the sequences of a chromosomal gene.

The nucleotide sequences reported in this paper have been submitted to the DDBJ with accession numbers D14368–14381, D32171–32189, and D32191–32199.

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