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Comparative Molecular Phylogeny and Evolution of Sex Chromosome DNA Sequences in the Family Canidae (Mammalia: Carnivora)

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To investigate the molecular phylogeny and evolution of the family Canidae, nucleotide sequences of the zinc-finger-protein gene on the Y chromosome (ZFY, 924–1146 bp) and its homologous gene on the X chromosome (ZFX, 834–839 bp) for twelve canid species were determined. The phylogenetic relationships among species reconstructed by the paternal ZFY sequences closely agreed with those by mtDNA and autosomal DNA trees in previous reports, and strongly supported the phylogenetic affinity between the wolf-like canids clade and the South American canids clade. However, the branching order of some species differed between phylogenies of ZFY and ZFX genes: *Cuon alpinus* and *Canis mesomelas* were included in the wolf-like canid clades in the ZFY tree, whereas both species were clustered in a group of *Chrysocyon brachyurus* and *Speothos venaticus* in the ZFX tree. The topology difference between ZFY and ZFX trees may have resulted from the two-times higher substitution rate of the former than the latter, which was clarified in the present study. In addition, two types of transposable element sequence (SINE-I and SINE-II) were found to occur in the ZFY final intron of the twelve canid species examined. Because the SINE-I sequences were shared by all the species, they may have been inserted into the ZFY of the common ancestor before species radiation in Canidae. By contrast, SINE-II found in only *Canis aureus* could have been inserted into ZFY independently after the speciation. The molecular diversity of SINE sequences of Canidae reflects evolutionary history of the species radiation.

Key words: Canidae, molecular evolution, SINE, X chromosomal gene, Y chromosomal gene

INTRODUCTION

The family Canidae (Mammalia, Carnivora) comprises 35 species in 13 genera (Wilson and Reeder, 2005), and the members of this family are widespread through the world excluding the Antarctic. To date, many researchers have investigated the evolutionary history of Canidae. Phylogenetic studies of Canidae have been done on the basis of morphological data (Tedford et al., 1995), mitochondrial

DNA (mtDNA) (Geffen et al., 1992; Wayne et al., 1997) and autosomal DNA (Bardleben et al., 2005; Wayne and Ostrander, 2007). In addition, Zrzavý and Řičáňková (2004) showed the phylogenetic relationships within Canidae based on morphological and molecular datasets. Consequently, three clades (red fox-like canids, wolf-like canids, and South American canids) have been defined by molecular phylogenies (Wayne et al., 1997; Bardleben et al., 2005). However, several phylogenetic issues remain unresolved on the above clades and the branching order among genera that are not contained the three clades. Whereas numerous studies based on mtDNA and autosomal DNA have been reported, there have been few comparative studies based on sex chromosomal genes to date.

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Molecular phylogeny in the order Carnivora based on sex chromosomal DNA has been examined in Felidae (Pecon-Slaterry and O'Brien, 1998), Ursidae (Nakagome et al., 2008) and Mustelidae (Yamada and Masuda, 2010), using genetic data of the *ZFY* gene (zinc-finger-containing gene located on the Y chromosome) and its homologous *ZFX* gene on the X chromosome. The *ZFY* and *ZFX* genes have been found in most of placental mammals (Page et al., 1987). Both of the two genes are located outside the pseudoautosomal region (PAR) of sex chromosomes (Page et al., 1987), and genes outside PAR of the Y chromosome do not recombine with those of the X chromosome in male meiosis and are inherited as haploidy. In addition, as Nakagome et al. (2008) reported, genes outside of PAR, including *ZFY*, may be under male-driven evolution or undergo degradation due to accumulation of deleterious mutations through a Muller's ratchet effect (Charlesworth and Charlesworth, 1997), genetic hitchhiking (Charlesworth, 1996), background selection (Charlesworth, 1996) and retroposable elements' insertion (Charlesworth, 1991).

Insertion of small interspersed number elements (SINEs) into the *ZFY* genes has been reported in many mammals, such as Ursidae (Nakagome et al., 2008), Mustelidae (Yamada and Masuda, 2010), and Felidae (Pecon-Slaterry et al., 2000b, 2004). In addition, Pecon-Slaterry et al. (2000a) reported the SINE insertion into the *ZFY* gene of *Canis familiaris* (Canidae). However, it is unknown whether such SINEs are found in the *ZFY* gene of other species in Canidae. The SINE sequences are dispersed in nuclear genome via transfer RNAs (Alberts et al., 2001), and more than 104 copies of SINEs are located in eukaryotic genome (Shedlock and Okada, 2000). The SINEs are thought to be useful for phylogenetic studies because of they are not eliminated after insertion to specific genomic sites (Shedlock and Okada, 2000).

In the present study, nucleotide sequences of both *ZFY* and *ZFX* genes were determined for twelve canid species, which can be classified into all three canids clades distributed in Eurasia, Africa, and North and South America. Based on the sequence data, the phylogenetic relationships among the species in Canidae and molecular evolution were

investigated, compared with the previously reported data on other carnivores. In addition, molecular characteristics of SINEs newly identified from the *ZFY* gene of Canidae are discussed.

MATERIALS AND METHODS

Samples and DNA extraction

Samples of bloods, muscles, feces, or hair were obtained from males of twelve species in the family Canidae: *Alopex lagopus* (arctic fox), *Fennecus zerda* (fennec fox), *Vulpes vulpes* (red fox), *Canis aureus* (golden jackal), *Canis mesomelas* (black-backed jackal), *Canis latrans* (coyote), *Canis lupus* (gray wolf), *Canis familiaris* (domestic dog), *Cuon alpinus* (dhole), *Speothos venaticus* (bush dog), *Chrysocyon brachyurus* (maned wolf), and *Nyctereutes procyonoides* (raccoon dog) (Table 1).

Whole blood samples were stored at -20°C ; separated white blood cells were stored in 99% ethanol at 4°C until use. Muscle and fecal samples were preserved in 99% ethanol at 4°C . Hair samples with roots were preserved at 4°C . Total DNA was extracted from whole blood (100 μl each) and pellets of white blood cells and muscle samples (about $3 \times 3 \times 3$ mm) using the DNeasy Blood and Tissue Kit (Qiagen), from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen), and from hair samples (20–30 hairs) with the QIAamp DNA Micro Kit (Qiagen). The DNA extracts in 100–200 μl of TE buffer were preserved at 4°C until use.

PCR amplification of the *ZFX* and *ZFY* genes

The final introns of the *ZFX* and *ZFY* genes were amplified using polymerase chain reaction (PCR) primers (U-ZF-2F/U-ZF-2R) reported by Nakagome et al. (2008) (Fig. 1). PCR amplifications were performed in 20 μl of a reaction mixture containing 1 μl of DNA extract, 0.1 μl of rTaq polymerase (5 units/ μl , Takara), 2 μl of 10 \times buffer (Takara), 1.6 μl of dNTPs (2.5 mM, Takara), and 0.2 μl of each (25 pmol/ μl) of two primers. For fecal samples, 0.4 μl of bovin serum albumin (20 mg/ml, Boehringer) was added to the above reaction mixture to eliminate some effects of PCR inhibitors.

Touchdown PCR cycling conditions were as follows: 94°C for 10 min; 10 cycles of 94°C for 1 min, 54°C for 30 sec, decreasing 1°C at every cycle, and 72°C for 2 min; 25–35 cycles of 94°C for 1 min, 44°C for 30 sec, and 72°C for 2 min; and 72°C for 10 min.

Cloning of PCR products and nucleotide sequencing

The PCR products were cloned by using the TA Cloning Kit (Invitrogen), following the manufacturer's instructions. Positive clones containing insert DNAs of the expected molecular sizes were

Table 1. Profiles of samples used in the present study.

Scientific name	Common name	Natural distribution*	Origin if known/Supplier	Accession numbers of analyzed gene**	
				ZFY	ZFX
<i>Vulpes vulpes</i>	Red fox	Old and New World	Hokkaido/ Hokkaido Institute of Public Health, Japan	AB622140	AB622129
<i>Alopex lagopus</i>	Arctic fox	Holarctic	Asahiyama Zoo, Japan	AB622141	AB622130
<i>Fennecus zerda</i>	Fennec fox	Sahara	Inokashira Park Zoo, Japan	AB622142	AB622131
<i>Cuon alpinus</i>	Dhole	Asia	Yokohama Zoorasia, Japan	AB622143	AB622132
<i>Canis mesomelas</i>	Black-backed jackal	Subsaharan Africa	Tennoji Zoological Gardens, Japan	AB622144	AB622133
<i>Canis aureus</i>	Golden jackal	Old World	Bulgaria/ Trakia University, Bulgaria	AB622145	AB622134
<i>Canis latrans</i>	Coyote	North America	Tennoji Zoological Gardens, Japan	AB622146	AB622135
<i>Canis lupus</i>	Gray wolf	Holarctic	Asahiyama Zoo, Japan	AB622147	AB622136
<i>Canis familiaris</i>	Dog	Domestic	Domestic/ Asahiyama Zoo, Japan	AB622147	AB622136
<i>Chrysocyon brachyurus</i>	Maned wolf	Northeast South America	Ueno Zoological Gardens, Japan	AB622148	AB622137
<i>Speothos venaticus</i>	Bush dog	Northeast South America	Yokohama Zoorasia, Japan	AB622149	AB622138
<i>Nyctereutes procyonoides</i>	Raccoon dog	Japan and East Asia	Japan/ Morioka Zoological Park, Japan	AB622150	AB622139

*Cited from Wayne (1993).

**Sequence data will appear at the DDBJ/GenBank/EMBL nucleotide sequence database with the accession numbers.

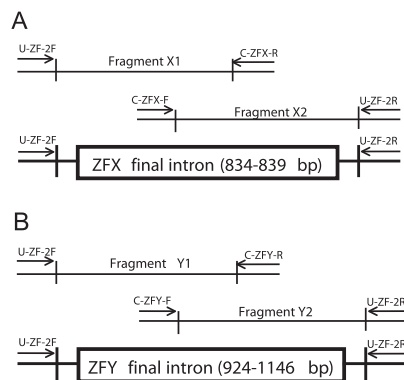


Fig. 1. Positions and directions of PCR and sequencing primers for the *ZFX* final intron (**A**) and the *ZFY* final intron (**B**) of Canidae.

incubated in 2 ml of LB broth (Invitrogen) with shaking at 37°C overnight. Plasmid DNA was then isolated with the QIAprep Miniprep Kit (Qiagen) according to manufacturer's instructions, dissolved in 50 µl of TE buffer, and stored at 4°C.

Ten to fourteen clones were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), and applied to an automated sequencer (ABI3730, Applied Biosystems). Based on an alignment of obtained sequences, PCR primers specific to the *ZFX* final intron (C-ZFX-F: 5'-GTTCCCTTAAGGCAGGCATA-3' and C-ZFX-R: 5'-AAAGCTTAAATCCACCTATGAAA-3') and the *ZFY* final intron (C-ZFY-F: 5'-CAAGTTAGCATAAATTTGGTTTG-3' and C-ZFY-R: 5'-TGTCTCTGCCTCTCTGTGTCTC-3') of Canidae were newly designed in the present study (Fig. 1).

Direct sequencing

PCR amplifications using newly designed primers (Fig. 1) for *Ca. latrans* and *Ch. brachyurus* were performed in 50 µl of a reaction mixture containing 10 µl of the DNA extract, 0.1 µl of rTaq polymerase (5 units/µl, Takara), 5 µl of 10× buffer (Takara), 4 µl of dNTPs (2.5 mM, Takara), 0.5 µl of each primer (25 pmol/µl), and 1 µl of bovin serum albumin (20 mg/ml, Boehringer). The touchdown PCR conditions for fragments X1 and X2 (Fig. 1A) were as follows: 94°C for 10 min; 35 cycles of 94°C for 2 min, 56°C for 30 sec, and 72°C for 2 min; and 72°C for 10 min. The touchdown PCR conditions for fragment Y1 (Fig. 1B) were as follows: 94°C for 10 min; 10 cycles of 94°C for 1 min, 65°C for 30 sec, decreasing 0.5°C every cycle, and 72°C for 2 min; 25–35 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 2 min; and 72°C for 10 min. The touchdown PCR conditions for fragment Y2 (Fig. 1B) were as follows: 94°C for 10 min; 10 cycles of 94°C for 1 min, 60°C for 30 sec, decreasing 1°C every cycle, and 72°C for 2 min; 25–35 cycles of 94°C for 1 min, 50°C for 30 sec and 72°C for 2 min; and 72°C for 10 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). Sequencing was carried out using newly designed primers, as mentioned above.

PCR amplifications using U-ZF-2F/U-ZF-2R primers (Nakagome et al., 2008) for *A. lagopus* were performed in 50 µl of a reaction mixture containing 2 µl of the DNA extract, 0.1 µl of rTaq polymerase (5 units/µl, Takara), 5 µl of 10× buffer (Takara), 4 µl of dNTPs (2.5 mM, Takara), and 0.5 µl of each primer. The touchdown PCR cycling conditions were as follows: 94°C for 10 min; 10 cycles of 94°C for 1 min, 54°C for 30 sec, decreasing 1°C at every cycle, and 72°C for 2 min; 25–35 cycles of 94°C for 1 min, 44°C for 30 sec, and 72°C for 2 min; and 72°C for 10 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). Sequencing was carried out using newly designed primers, as mentioned above.

Data analysis

The *ZFY* (accession no. AB261807) and *ZFX* (AB261815) final intron sequences of *Ursus arctos* (brown bear) as an outgroup were cited from Nakagome et al. (2008). In addition, the *ZFY* (AB491592) and *ZFX* (AB491601) final intron sequences of *Mustela itatsi* (Japanese weasel) were cited from Yamada and Masuda (2010).

The sequence alignment and construction of neighbor-joining (NJ) trees using Kimura's (1980) two-parameter distance model were performed with MEGA 4 (Tamura et al., 2007). Phylogenetic trees based on maximum parsimony (MP) and maximum likelihood (ML) methods were constructed with PAUP* version 4.0b10 (Swofford, 2002). Insertion or deletion (indel) sites were eliminated from calculation. Specific search conditions for the MP analysis included starting trees obtained by stepwise addition of sequences with 1000 replicates, general heuristic search and the tree-bisection-reconnection (TBR) branch swapping algorithm. All other settings were set by default. For the ML analysis, the program Modeltest 3.06 (Posada and Crandall, 1998) was used to select the most appropriate model of molecular evolution through a hierarchical likelihood ratio test. The model selected for sequence data for the *ZFY* genes was Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) following a gamma distribution shape parameter (HKY-G model), and that for the *ZFX* gene data was Kimura unequal base frequencies (K81uf) model (Kimura, 1981). Bootstrap values (Felsenstein, 1985) were calculated from 1000 replicates for NJ with MEGA 4, and 100 for ML and 1000 for MP with PAUP* version 4.0b10. In addition, a haplotype network was constructed by using software TCS (Clement et al., 2000), where a gap was counted as one nucleotide substitution.

Pairwise differences were calculated for sequences of *ZFY* and *ZFX* final introns, using Kimura's (1980) two parameter distance model in MEGA4. The *ZFY/ZFX* ratios were calculated for each *ZFY* matrix element with the corresponding element from *ZFX*, using Kimura's (1980) two parameter distance matrices. Mean *ZFY/ZFX* values were computed from all pairwise estimates ($n = 66$).

RESULTS

Phylogenetic relationships among the *ZFY* final intron sequences of Canidae

The *ZFY* final intron sequences (924–1146 base-pairs, bp) were obtained from all twelve canid species examined (Appendix). In addition, a sequence alignment showed that SINEs and other insertional sequences were found in the *ZFY* final intron, as mentioned below.

Of three equally parsimonious trees yielded by MP analysis, one tree, of which topology was the most similar to those generated by ML and NJ methods, was selected for indicating bootstrap values: this MP tree was almost identical to the ML tree, where *F. zerda*, *A. lagopus* and *V. vulpes* were grouped into one cluster. The ML tree is shown in Fig. 2 as a representative of three methods with bootstrap values for ML, MP and NJ above each branch.

Three fox species, *V. vulpes*, *A. lagopus*, and *F. zerda*, formed a monophyletic group, supported by 64% (ML), 63% (MP) and 63% (NJ) bootstrap values (Fig. 2). They were included in "the red fox-like canids clade" by previous molecular phylogenetic studies (Wayne et al., 1997; Bardeleben et al., 2005). Five species of *Canis*, *Ca. mesomelas*, *Ca. aureus*, *Ca. latrans*, *Ca. lupus*, and *Ca. familiaris*, and *Cu. alpinus* were clustered with 73/63/64 bootstrap values (Fig. 2). This monophyletic group was congruent with "the wolf-like canids clade" defined in previous molecular phylogenetic studies (Wayne et al., 1997; Bardeleben et al., 2005). Within the wolf-like canids clade, the sequence of *Ca.*

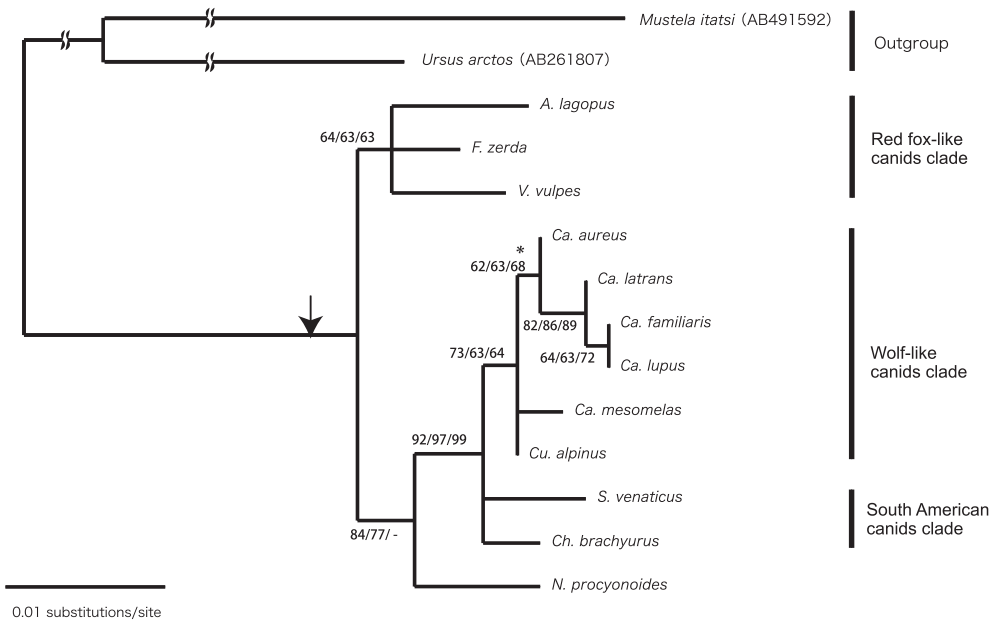


Fig. 2. A maximum-likelihood (ML) tree as a representative phylogenetic tree of the Canidae ZFY sequences (924–1146 bp). The scale indicates genetic distances. Numbers near the internal branches are bootstrap values for ML (100 replications), MP (1000 replications) and NJ (1000 replications) in order. Because when bootstrap values are less than 50% for all three methods, the branching is bifurcated. An arrow indicates event of an insertion of SINE-I before species radiation of Canidae. An asterisk shows an independent insertion event of SINE-II into the ZFY final intron of *Ca. aureus*.

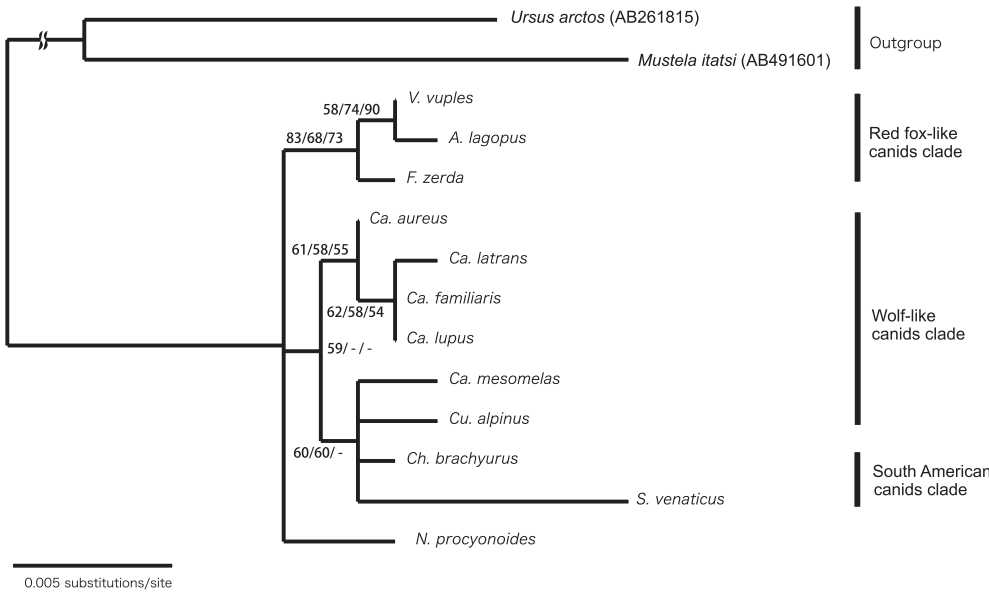


Fig. 3. A maximum-likelihood (ML) tree as a representative phylogenetic tree of the Canidae ZFX sequences (834–839 bp). The scale indicates genetic distances. Numbers near the internal branches are bootstrap values for ML (100 replications), MP (1000 replications) and NJ (1000 replications) in order, where less than 50% were shown by dashes.

familiaris (domestic dog) was identical with that of *Ca. lupus* (gray wolf). *Chrysocyon brachyurus* and *S. venaticus*, both of which were included in “the South American canids clade” (Wayne et al., 1997; Bardeleben et al., 2005), were clustered with “the wolf-like canids clade”, supported by 92/97/99 bootstrap values (Fig. 2).

The position of *N. procyonoides* was different between the

NJ tree and the other trees: *Nyctereutes procyonoides* was split from the other species in the NJ tree, whereas it was a sister taxon to the wolf-like and South American canids clades in the ML and MP trees.

In addition to two types of SINEs (mentioned below), five types of shorter insertional fragments were found in the ZFY final intron of Canidae (Appendix): 7-bp fragment (nucleotide sites, ns 91–97) in *N. procyonoides* and the three fox species; 9-bp fragment (ns 200–208) in *Ca. aureus*; 54-bp fragment (ns 518–572) in *Cu. alpinus*; 8-bp fragment (ns 644–651) in *S. venaticus*; and 4-bp fragment (ns 1182–1185) in *N. procyonoides*. Although homology searches using the DNA databases were made for the above 54-bp fragment, no resultant homologues were found. Features of the other small fragments were not clarified by the homology search.

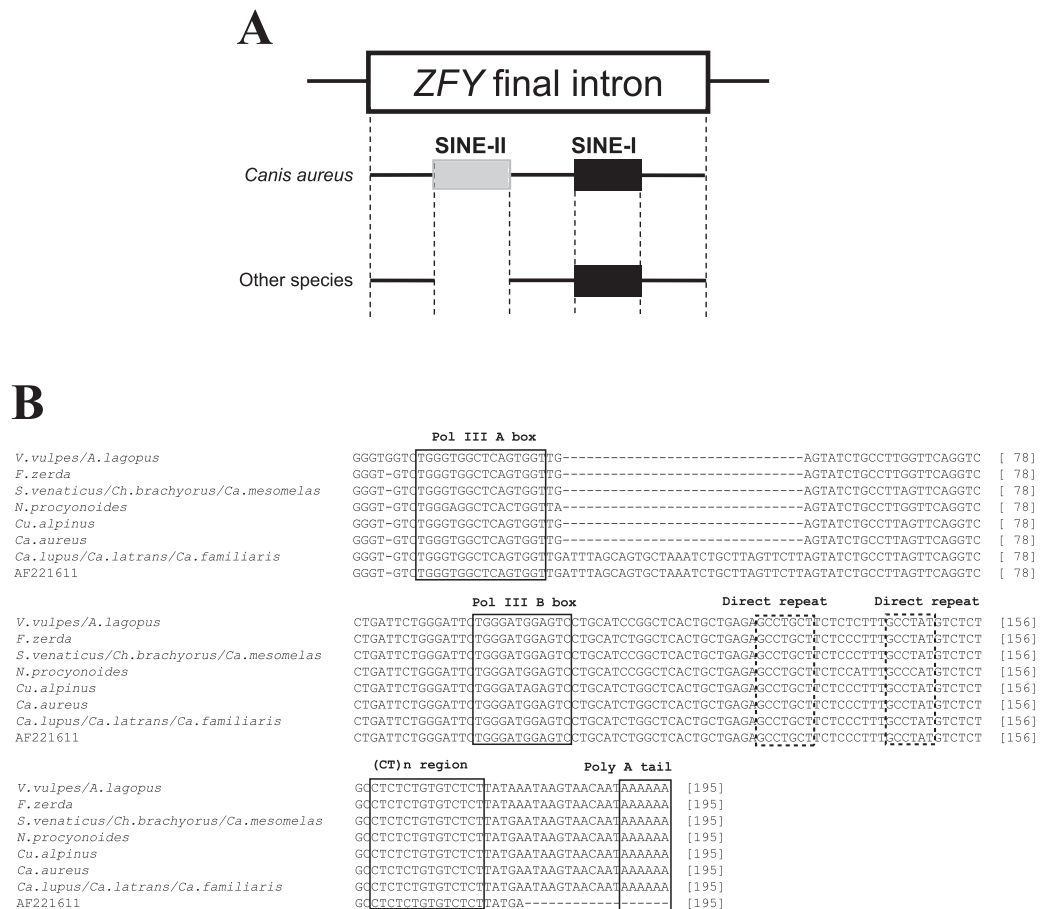
Phylogenetic relationships among the ZFX final intron sequences of Canidae

The ZFX final intron sequences (834–839 bp) were obtained from all the twelve Canidae species (Table 1). Of the two equally parsimonious trees that resulted from MP analysis, one tree, of which topology was more similar to those of ML and NJ trees, was selected for calculating bootstrap values. Because ML, MP and NJ trees indicated the similar topologies to each other, the ML tree is shown in Fig. 3 as a representative of the three methods with bootstrap values for ML, MP

and NJ, indicated in this order above each branch. The three foxes, *A. lagopus*, *F. zerda*, and *V. vulpes* (red fox-like canids clade), were clustered with 83/68/73 bootstrap values (Fig. 3), in agreement with the ZFY tree (Fig. 2). Four species of *Canis*, *Ca. aureus*, *Ca. familiaris*, *Ca. latrans*, and *Ca. lupus*, formed a monophyletic group with 61/58/55 bootstrap values (Fig. 3), in congruence with the ZFY tree (Fig.

Table 2. Pairwise differences (Kimura's two parameter distances) of the ZFY (upper) and ZFX (below) final intron sequences among the twelve species of Canidae.

ZFX/ZFY	<i>V. vulpes</i>	<i>A. lagopus</i>	<i>F. zerda</i>	<i>Ca. aureus</i>	<i>Ca. mesomelas</i>	<i>Cu. alpinus</i>	<i>Ca. latrans</i>	<i>Ca. lupus</i>	<i>Ca. familiaris</i>	<i>Ch. brachyurus</i>	<i>S. venaticus</i>	<i>N. procyonoides</i>
<i>V. vulpes</i>		0.011	0.011	0.019	0.019	0.019	0.021	0.022	0.022	0.018	0.020	0.022
<i>A. lagopus</i>	0.001		0.012	0.019	0.019	0.019	0.021	0.022	0.022	0.018	0.020	0.023
<i>F. zerda</i>	0.002	0.004		0.014	0.017	0.014	0.017	0.018	0.018	0.015	0.018	0.020
<i>Ca. aureus</i>	0.006	0.007	0.006		0.004	0.002	0.002	0.003	0.003	0.005	0.008	0.019
<i>Ca. mesomelas</i>	0.008	0.010	0.008	0.005		0.004	0.007	0.008	0.008	0.005	0.008	0.019
<i>Cu. alpinus</i>	0.008	0.010	0.008	0.005	0.005		0.004	0.005	0.005	0.005	0.008	0.019
<i>Ca. latrans</i>	0.008	0.010	0.006	0.002	0.007	0.007		0.001	0.001	0.008	0.010	0.021
<i>Ca. lupus</i>	0.007	0.008	0.007	0.001	0.006	0.006	0.001		0.000	0.009	0.011	0.022
<i>Ca. familiaris</i>	0.007	0.008	0.007	0.001	0.006	0.006	0.001	0.000		0.009	0.011	0.022
<i>Ch. brachyurus</i>	0.007	0.008	0.007	0.004	0.004	0.004	0.006	0.005	0.005		0.007	0.018
<i>S. venaticus</i>	0.015	0.016	0.015	0.011	0.011	0.011	0.013	0.012	0.012	0.010		0.020
<i>N. procyonoides</i>	0.007	0.008	0.007	0.006	0.008	0.006	0.008	0.007	0.007	0.007	0.015	

**Fig. 4.** (A) Insertional positions of SINE sequences in the ZFY final intron of Canidae. Solid lines show homologous sequences among the species, and broken lines indicate the positional relationships of sequences among the species. (B) An alignment of SINE-I sequences identified from the homologous sites of the twelve Canidae species. The SINE sequence of the domestic dog (*Canis familiaris*, AF221611) (Pعون-Slatery et al., 2000a) is shown as a reference. (C) The SINE-II sequence (210 bp) found in *Ca. aureus*. RNA polymerase III boxes A and B and terminator, and (CT)n region are enclosed by solids lines. Direct repeat motifs are enclosed by broken lines. The CAN-SINE specific insertion sequence is underlined.

2). Among those, the sequence of *Ca. familiaris* was identical with that of *Ca. lupus*. *Canis mesomelas* and *Cu. alpinus* clustered with *Ch. brachyurus* and *S. venaticus* with 60/60/-bootstrap values (Fig. 3). The difference between the NJ tree and the other trees (ML and MP) was the position of *Cu. alpinus*; the ML and MP trees showed that *Cu. alpinus* clustered with the group consisting of *Ca. mesomelas*, *Ch. brachyurus*, and *S. venaticus*, whereas the NJ tree indicated that *Cu. alpinus* was a sister taxon of the latter group. *Nyctereutes procyonoides* was remote from the other species (Fig. 3).

In contrast with the *ZFY* sequences, no insertional fragments were found in the *ZFX* final intron sequences.

Estimation of *ZFY/ZFX* mutation ratio

In order to evaluate the evolutionary difference between *ZFY* and *ZFX* genes, matrices composed of Kimura's (1980) two parameter genetic distances among all pairs of taxa were made separately for the two genes (Table 2). The *ZFY/ZFX* values were then calculated at each pair of species. The mean pairwise *ZFY/ZFX* value was 2.00, showing a more rapid mutation rate in the *ZFY* gene than the *ZFX* gene.

Identification of SINE sequences within the *ZFY* final introns of Canidae

Sequence homology searches revealed that 165–195-bp fragments (ns 810–1004) (Appendix) within the *ZFY* final introns of all the twelve canid species were most homologous (from 96% [117/121 bp] to 100% [176/176 bp]) to a SINE sequence (accession no. AF221611) of *Ca. familiaris*, which is reported to possess structural features, such as the 5' region containing the split block promoter for RNA polymerase III common to tRNA genes, followed by a unique sequence and a CT repeat, and terminated by a 3' poly A tail (Pecon-Slatery et al., 2000a). Therefore, we refer to the 166–195-bp fragments identified in the present study as SINE-I (Fig. 4A, B). The length difference among SINE-I sequences resulted from 30-bp insertions in the three *Canis* species (*Ca. familiaris*, *Ca. latrans*, and *Ca. lupus*) (Fig. 4B).

In addition, another insertional fragment (ns 243–453) was found in only the *ZFY* final intron of *Ca. aureus* (Fig. 4A, C; Appendix). Because this sequence also possessed the general SINE features as well as the Pol III terminator (Fig.

4C), we name this SINE-II. By contrast, no SINEs were found in the *ZFX* final introns of any canid species examined in the present study.

Of the determined SINE-I sequences, 41 nucleotide sites were polymorphic, including 30-bp indels (Fig. 4B). Figure 5 shows a parsimonious network of SINE-I sequences. Three species of *Canis* (*Ca. familiaris*, *Ca. latrans*, and *Ca. lupus*) having the 30-bp insertional sequence were separated from the other nine species. The SINE-I sequences of *Ca. mesomelas*, *Ch. brachyurus*, and *S. venaticus* were identical to each other. Two foxes (*A. lagopus* and *V. vulpes*) shared identical SINE-I sequences and were closely related to the other fox, *F. zerda*, forming the red fox-like canids clade (Fig. 5). The phylogenetic position of *N. procyonoides* was remote from the other species (Fig. 5), in agreement with the *ZFY* (Fig. 2) and *ZFX* (Fig. 3) trees.

DISCUSSION

Phylogenetic relationships among Canidae species based on sex chromosomal DNA sequences

The present study investigated the phylogenetic relationships among canid species inferred from the sex chromosomal DNA sequences. The molecular phylogenetic trees obtained from Y chromosomal DNA (*ZFY* sequences) and X chromosomal DNA (*ZFX* sequences) commonly showed that the canid species examined were divided into two main clades: the red fox-like canids and wolf-like canids clades, consistent with the previous molecular phylogenetic studies (Wayne et al., 1997; Bardeleben et al., 2005). Both Y chromosomal and X chromosomal DNA data always supported the *A. lagopus*/*F. zerda*/*V. vulpes* cluster (red fox like-canids clade), in agreement with the previous studies based on mtDNA (Wayne et al., 1997) and autosomal DNA (Bardeleben et al., 2005; Wayne and Ostrander, 2007). In addition, the phylogenies of both sex chromosomal DNA data showed that four *Canis* species (*Ca. aureus*, *Ca. familiaris*, *Ca. latrans*, and *Ca. lupus*) clustered together, supporting the wolf-like canids clade previously reported on the basis of mtDNA (Wayne et al., 1997) and autosomal DNA (Bardeleben et al., 2005; Wayne and Ostrander, 2007).

On the other hand, the phylogenetic positions of *Cu. alpinus*, *Ca. mesomelas*, *Ch. brachyurus*, *S. venaticus*, and *N. procyonoides* were different between the *ZFY* and *ZFX* trees. *Cuon alpinus* and *Ca. mesomelas* clustered with other *Canis* species of the wolf-like canids clades in the *ZFY* tree (Fig. 2), whereas the former two species were grouped with *Ch. brachyurus* and *S. venaticus* in the *ZFX* tree (Fig. 3). Molecular phylogenies of mtDNA (Wayne et al., 1997) and autosomal DNA (Bardeleben et al., 2005; Wayne and Ostrander, 2007) commonly showed that the two species (*Cu. alpinus* and *Ca. mesomelas*) were clustered with the wolf-like canids clade. In addition, Zrzavý and Říčanová (2004) using the morphological and molecular data sets also revealed that both species were grouped into the wolf-like canids clade. This is not incongruent with the phylogeny shown in Fig. 2.

Diploid chromosome numbers of *Cu. alpinus* ($n = 78$) and *Ca. mesomelas* ($n = 78$) are identical with those of the other *Canis* species ($n = 78$) forming the wolf-like canids clade, but not with *Ch. brachyurus* ($n = 76$) and *S. venaticus* ($n = 74$) (Wayne, 1993). The present study indicates that the

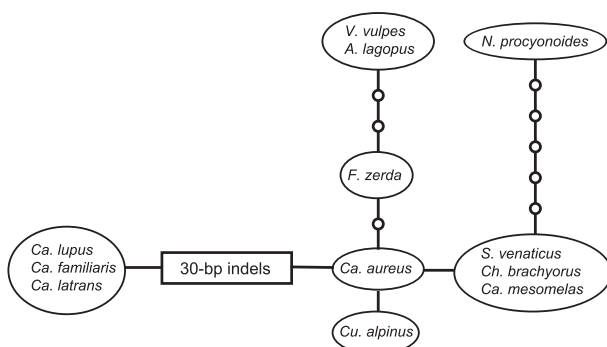


Fig. 5. A parsimonious network of SINE-I sequences. An indel was counted as one nucleotide substitution. Open circles show presumed sequence types. One bar between circles indicates one nucleotide substitution.

two species of South America cluster with the wolf-like canids clade, showing higher bootstrap values (more than 92%, see Fig. 2). Wayne and Ostrander (2007) reviewed the molecular phylogeny of Canidae using a large data set, and showed the earlier split of the fox-like canids clade from the others and the subsequent separation from the others into the South American canids clade and the wolf-like canids clade. The present study strongly supports the phylogenetic affinity between the latter two clades.

The phylogenetic position of *N. procyonoides* was different in the *ZFY* and *ZFX* trees. Molecular phylogenetic studies of mtDNA showed that *N. procyonoides* is not closely related to the fox-like, wolf-like, and South American canids clades (Wayne et al., 1997). On the other hand, autosomal DNA phylogenies showed that *N. procyonoides* was a sister taxon to the red fox-like canids clade (Bardeleben et al., 2005; Wayne and Ostrander, 2007). A morphological study indicated that *N. procyonoides* is included in the South American canids clade (Tedford et al., 1995). Both morphological and molecular data sets of Zrzavý and Řiřáňková (2004) showed that *N. procyonoides* was basal to the wolf-like and South American canids clades. Meanwhile, the 7-bp fragment (ns 91–97) (Appendix) was found to have been inserted into the *ZFY* final intron of *N. procyonoides* and the three fox species. Because such an insertion is thought to be cladistically informative, the common insertion of the 7-bp fragment indicates the close relatedness among *N. procyonoides* and the three fox species *F. zerda*, *A. logopus*, and *V. vulpes*, supporting the phylogenetic relationship reported by Wayne and Ostrander (2007).

The present study revealed that the mutation rate of *ZFY* sequences in Canidae was higher than that of *ZFX*, as the mean pairwise *ZFY/ZFX* was about 2.00. Such a greater mutation rate for the Y chromosomal genes than the X is similar to those reported in other carnivoran families such as Felidae (Pecon-Slattery et al., 1998), Ursidae (Nakagome et al., 2008) and Mustelidae (Yamada and Masuda, 2010), in concordance with male-driven evolution, reported by Haldane (1947), Miyata et al. (1987), Shimmin et al. (1993), Makova and Li (2002) and Goetting-Minesky and Makova (2006).

Notably, both the *ZFY* and *ZFX* sequences of *Ca. familiaris* (domestic dog) were identical to those of *Ca. lupus* (gray wolf). This sequence identity is not incongruent with that dogs were domesticated from gray wolves: Vila et al. (1997) reported that the domestication time is at least 14,000 years ago. Tsuda et al. (1997) also reported that domestic dogs have maintained a large degree of mtDNA polymorphisms introduced from their ancestral wolf populations.

SINE insertions in the *ZFY* final introns

Carnivore-specific SINE (CAN-SINE) sequences have been identified from all carnivore families (Vassetzky and Kramerov, 2002). The SINE insertions within the *ZFY* final intron in Carnivora were reported in Felidae (7/34 species: Pecon-Slattery et al., 2000b, 2004) and Ursidae (8/8 species: Nakagome et al., 2008). The present study reports for the first time that the SINE-I in the *ZFY* final introns is shared by all the twelve canid species examined. In addition, the SINE-II found in the present study possessed characteristics

specific to CAN-SINEs (Vassetzky and Kramerov, 2002): the SINE-II sequence contained two direct repeats (GAGC-CTGC and GCCTGT) and insertion (TGGCCCGGGGCG) between Pol III A Box and B Box (Fig. 4B, C). From this, the SINE-II identified in the present study can be classified into a member of CAN-SINEs.

Yamada and Masuda (2010) reported an insertion of one copy of CAN-SINE in the *ZFY* final intron, which is specific to only *Meles anakuma* (Japanese badger) and *Mustela erminea* (ermine) among nine mustelid species investigated. We then re-examined those mustelid *ZFY* sequences of Yamada and Masuda (2010), and found a different insertion of another copy of SINE, which is common to all the nine mustelid species, in addition to the CAN-SINE specific to *Meles anakuma* and *Mustela erminea*. Thus, the insertional pattern of SINE-I and -II in canids identified in the present study is similar to that of the mustelid *ZFY* sequences of Yamada and Masuda (2010).

Canis latrans, *Ca. familiaris* and *Ca. lupus* shared the 30-bp insertion between Pol III A Box and Pol III B Box of SINE-I, compared with the other canid species. This indicates that the three *Canis* species had a common ancestor even after original insertion of SINE-I in canids. Similarly, among CAN-SINEs specific in the *ZFY* final intron of *Meles* spp. (Eurasian badgers), 12-bp deletions were common to *Meles anakuma* (Japanese badger) compared with other continental *Meles* species (Tashima et al., 2011). In addition to SINEs in the *ZFY* final intron, 4–54-bp insertions were found in single species and one lineage including multiple species in Canidae. Because the *ZFY* gene is located in the nonrecombining region on the Y chromosome (Pecon-Slattery et al., 2000b), it seems reasonable that more insertions are distributed in the *ZFY* gene than in the *ZFX* gene. Although the origins and functions of the small insertional sequences found in the present study are unknown yet, such insertions can be cladistically informative markers for phylogenetic analyses.

Because the SINE-I sequences occur commonly in all the twelve species examined, they could have been inserted into the *ZFY* final introns before species radiation in Canidae. By contrast, SINE-II could have been inserted into the *ZFY* final intron of *Ca. aureus* lineage-independently after the speciation in Canidae. The studies on molecular diversity of SINEs as well as other small insertional sequences found in the *ZFY* gene provide a potential to further understanding the evolutionary history on diversification of Canidae.

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<i>A. lagopus</i>	GTGAGGGTGCACAAGTTCATAGTACAGTGTGCTCTGCAAGCTCAGAGATGTAATAGTATGTATCCACAGAGGTGTTAAATAGTTTAGCTACTAGAC	[99]
<i>F. zerda</i>	[99]
<i>V. vulpes</i> T..... C.....	[99]
<i>Ca. aureus</i> A..... TC.....	[99]
<i>Ca. mesomelas</i> A..... T.....	[99]
<i>Cu. alpinus</i> A..... T.....	[99]
<i>Ca. latrans</i> A..... TC.....	[99]
<i>Ca. familiaris</i> A..... TC.....	[99]
<i>Ca. lupus</i> A..... TC.....	[99]
<i>S. venaticus</i> C..... A..... T.....	[99]
<i>Ch. brachyurus</i> A..... T.....	[99]
<i>N. procyonoides</i> T.....	[99]
<i>A. lagopus</i>	CATATGTAGCTTTTATGTGTATTAATAAAAAA-TAATTATAAGAATTCAAGTATTCATGAATGATTTCTGGAATAAAAAACAAATAGGCTTTGAGTCAG	[198]
<i>F. zerda</i> -..... T..... G.....	[198]
<i>V. vulpes</i> -..... T.....	[198]
<i>Ca. aureus</i> A..... T.....	[198]
<i>Ca. mesomelas</i> -..... T..... A.....	[198]
<i>Cu. alpinus</i> -..... T.....	[198]
<i>Ca. latrans</i> -..... T.....	[198]
<i>Ca. familiaris</i> -..... T.....	[198]
<i>Ca. lupus</i> -..... T.....	[198]
<i>S. venaticus</i> -..... T..... A.....	[198]
<i>Ch. brachyurus</i>	T..... -..... T..... G.....	[198]
<i>N. procyonoides</i> C..... -..... T.....	[198]
<i>A. lagopus</i>	A-----CATTGAGGACAGAGTGAAGTCTCAGGATATTTTA-----	[297]
<i>F. zerda</i>	-----	[297]
<i>V. vulpes</i>	-----	[297]
<i>Ca. aureus</i>	.GTAAATAGG..... A..... ATTTTTTTTTTTTTTTTAAAGATTTTATTTATTTATTCATGATAGTCACACAGA	[297]
<i>Ca. mesomelas</i> A.....	[297]
<i>Cu. alpinus</i> A.....	[297]
<i>Ca. latrans</i> A.....	[297]
<i>Ca. familiaris</i> A.....	[297]
<i>Ca. lupus</i> A.....	[297]
<i>S. venaticus</i> A.....	[297]
<i>Ch. brachyurus</i> A.....	[297]
<i>N. procyonoides</i> A.....	[297]
<i>A. lagopus</i>	-----	[396]
<i>F. zerda</i>	-----	[396]
<i>V. vulpes</i>	-----	[396]
<i>Ca. aureus</i>	GAGAGACAGAGAGGCAGAGACACAGGCAGAGGAGAGAAGCAGGCTCCATGCAGGGAGCCCGACGTGGGATTTCGATCCCGGGTCTCCAGGATCACGCCCGG	[396]
<i>Ca. mesomelas</i>	-----	[396]
<i>Cu. alpinus</i>	-----	[396]
<i>Ca. latrans</i>	-----	[396]
<i>Ca. familiaris</i>	-----	[396]
<i>Ca. lupus</i>	-----	[396]
<i>S. venaticus</i>	-----	[396]
<i>Ch. brachyurus</i>	-----	[396]
<i>N. procyonoides</i>	-----	[396]
<i>A. lagopus</i>	-----GTGGAAATTAATAATCCCTAAATTAATGTTATTTTCATC	[495]
<i>F. zerda</i>	-----	[495]
<i>V. vulpes</i>	-----	[495]
<i>Ca. aureus</i>	GGCCAGAGGCAGGCGCCAAACCGCTGCGCCACCCAGGATCCCATCAGGATATTTTA	[495]
<i>Ca. mesomelas</i>	-----	[495]
<i>Cu. alpinus</i>	-----	[495]
<i>Ca. latrans</i>	-----	[495]
<i>Ca. familiaris</i>	-----	[495]
<i>Ca. lupus</i>	-----	[495]
<i>S. venaticus</i>	-----	[495]
<i>Ch. brachyurus</i>	-----	[495]
<i>N. procyonoides</i>	-----	[495]

Appendix. An alignment of the ZFY final intron sequences. Dots show identical nucleotides with those of *A. lagopus*. Dashes indicate gaps.

<i>A. lagopus</i>	TGAGTGAAGAAATTTAACTTTT	TTGTCAGCTTTATGCTATAAAG	[594]
<i>F. zerda</i>			[594]
<i>V. vulpes</i>			[594]
<i>Ca. aureus</i>			[594]
<i>Ca. mesomelas</i>		C	[594]
<i>Cu. alpinus</i>	AACTTTAAAGTTAAAGTTAAAGTTTAAAGTTAAATTTAAATTTAACTTTT		[594]
<i>Ca. latrans</i>			[594]
<i>Ca. familiaris</i>			[594]
<i>Ca. lupus</i>			[594]
<i>S. venaticus</i>			[594]
<i>Ch. brachyurus</i>			[594]
<i>N. procyonoides</i>		T	[594]
<i>A. lagopus</i>	CAGGCATTATTTATAGAAGCAGAGGACAAATATATCAGTTGCCATTGG	ATAATTCTTTCCAGTATAATCTTGATGTTGTAATAATTTT	[693]
<i>F. zerda</i>	T		[693]
<i>V. vulpes</i>			[693]
<i>Ca. aureus</i>			[693]
<i>Ca. mesomelas</i>			[693]
<i>Cu. alpinus</i>			[693]
<i>Ca. latrans</i>			[693]
<i>Ca. familiaris</i>			[693]
<i>Ca. lupus</i>			[693]
<i>S. venaticus</i>	TCCATTGG		[693]
<i>Ch. brachyurus</i>			[693]
<i>N. procyonoides</i>		A	[693]
<i>A. lagopus</i>	CAGCAGGTATGTTGAATTTCAAGTTAGCATAAATTTGGTTGCAGTAATGAAACTGAAAGGTTTTATGAATTGTTTTGTGGATTATTTCTATAGAT		[792]
<i>F. zerda</i>	T . T A	A	[792]
<i>V. vulpes</i>	T . T A T	A	[792]
<i>Ca. aureus</i>	T . T C	A . A	[792]
<i>Ca. mesomelas</i>	T . T C	A . A	[792]
<i>Cu. alpinus</i>	T . T C	A . A	[792]
<i>Ca. latrans</i>	T . T C	A . A	[792]
<i>Ca. familiaris</i>	T . T C	A . A	[792]
<i>Ca. lupus</i>	T . T C	A . A	[792]
<i>S. venaticus</i>	T . T C . G	A . A	[792]
<i>Ch. brachyurus</i>	T . T C	A . A	[792]
<i>N. procyonoides</i>	T . T A A T	A . A	[792]
<i>A. lagopus</i>	TCCCAAGTATATATGGCTTTTTATTGTTACTTATTTATAAGAGACACAGAGAGGCAGAGACATAGGCAAAGAGAGAAGCAGGCTCTCAGCAGTGAGCC		[891]
<i>F. zerda</i>		G	[891]
<i>V. vulpes</i>			[891]
<i>Ca. aureus</i>	C	G	[891]
<i>Ca. mesomelas</i>	C	G	[891]
<i>Cu. alpinus</i>	C	G	[891]
<i>Ca. latrans</i>	C	G	[891]
<i>Ca. familiaris</i>	C	G	[891]
<i>Ca. lupus</i>	C	G	[891]
<i>S. venaticus</i>	C	G	[891]
<i>Ch. brachyurus</i>	C	G	[891]
<i>N. procyonoides</i>	C G TG		[891]
<i>A. lagopus</i>	GGATGCAGGACTCCATCCAGAATCCAGAATCAGGACCTGAACCAAGGCAGATACT	CAACCACTGAGC	[990]
<i>F. zerda</i>	A		[990]
<i>V. vulpes</i>			[990]
<i>Ca. aureus</i>	A T		[990]
<i>Ca. mesomelas</i>		T	[990]
<i>Cu. alpinus</i>	A T		[990]
<i>Ca. latrans</i>	A T	AAGAACTAAGCAGATTTAGCACTGCTAAAT	[990]
<i>Ca. familiaris</i>	A T	AAGAACTAAGCAGATTTAGCACTGCTAAAT	[990]
<i>Ca. lupus</i>	A T	AAGAACTAAGCAGATTTAGCACTGCTAAAT	[990]
<i>S. venaticus</i>		T	[990]
<i>Ch. brachyurus</i>		T	[990]
<i>N. procyonoides</i>		T G	[990]

Appendix. Continued.

<i>A. lagopus</i>	CACCCAGACCACCCCTAT-TATGGCTTTAAATGTCCTTTAAACTGGAAATGAGATTGTAGTTACATTGAATGAAGTTTCTGAACAAGATTTCTTCAT	[1089]
<i>F. zerda</i>-.....-.....	[1089]
<i>V. vulpes</i>-.....	[1089]
<i>Ca. aureus</i>-.....A.....C.....	[1089]
<i>Ca. mesomelas</i>-.....A.....C.....	[1089]
<i>Cu. alpinus</i>-.....A.....C.....	[1089]
<i>Ca. latrans</i>-.....A.....C.....	[1089]
<i>Ca. familiaris</i>-.....A.....C. G.....	[1089]
<i>Ca. lupus</i>-.....A.....C. G.....	[1089]
<i>S. venaticus</i>-.....A.....—.....	[1089]
<i>Ch. brachyurus</i>-.....A.....	[1089]
<i>N. procyonoides</i>	.T.....-.....A.....	[1089]
<i>A. lagopus</i>	TACTGGGTTTTCTTTTACCTCTTTTGTGAAAAACAAAAGAATATTAGCTTTTAGGAGTACTTTAGCACTATTTTCAGAGCACACACTTTA—AGT	[1188]
<i>F. zerda</i>A.....C.....—.....	[1188]
<i>V. vulpes</i>G.....—.....	[1188]
<i>Ca. aureus</i>A.....—.....	[1188]
<i>Ca. mesomelas</i>A.....—.....	[1188]
<i>Cu. alpinus</i>A.....—.....	[1188]
<i>Ca. latrans</i>A.—.....G.....—.....	[1188]
<i>Ca. familiaris</i>A.—.....G.....—.....	[1188]
<i>Ca. lupus</i>A.—.....G.....—.....	[1188]
<i>S. venaticus</i>A.....—.....	[1188]
<i>Ch. brachyurus</i>A.....—.....	[1188]
<i>N. procyonoides</i>A.....C.....TTTA.....	[1188]
<i>A. lagopus</i>	GGTTGAAGAACCAAAGCAGAACTTGGTTTGATCACTCATGTTCTTTTCTTTCTTTCTTAG	[1250]
<i>F. zerda</i>	[1250]
<i>V. vulpes</i>G.....	[1250]
<i>Ca. aureus</i>C.....	[1250]
<i>Ca. mesomelas</i>C.....	[1250]
<i>Cu. alpinus</i>C.....	[1250]
<i>Ca. latrans</i>C.....	[1250]
<i>Ca. familiaris</i>C.....	[1250]
<i>Ca. lupus</i>C.....	[1250]
<i>S. venaticus</i>G.....C.....	[1250]
<i>Ch. brachyurus</i>-.....C.....	[1250]
<i>N. procyonoides</i>-.....	[1250]

Appendix. Continued.