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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 throughout 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 (Bardeleben et al., 2005; Wayne and Ostrander, 2007). In addition, Zrzavý and Řičánková (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; Bardeleben 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-Slattery 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 haploid. 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-Slattery et al., 2000b, 2004). In addition, Pecon-Slattery 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 × 3 × 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 (U1-ZF/U1-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× 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; and 72°C for 2 min. 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 sequenced.

**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 sequenced.

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

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Common name</th>
<th>Natural distribution*</th>
<th>Origin if known/Supplier</th>
<th>Accession numbers of analyzed gene**</th>
</tr>
</thead>
<tbody>
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<td>Vulpes vulpes</td>
<td>Red fox</td>
<td>Old and New World</td>
<td>Hokkaido/ Hokkaido Institute of Public Health, Japan</td>
<td>AB622140 AB622129</td>
</tr>
<tr>
<td>Alopex lagopus</td>
<td>Arctic fox</td>
<td>Holarctic</td>
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<td>AB622141 AB622130</td>
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<tr>
<td>Fennecus zerda</td>
<td>Fennec fox</td>
<td>Sahara</td>
<td>Inokashira Park Zoo, Japan</td>
<td>AB622142 AB622131</td>
</tr>
<tr>
<td>Cuon alpinus</td>
<td>Dhole</td>
<td>Asia</td>
<td>Yokohama Zoora, Japan</td>
<td>AB622143 AB622132</td>
</tr>
<tr>
<td>Canis aureus</td>
<td>Golden jackal</td>
<td>Subsaharan Africa</td>
<td>Tennso Zoogical Gardens, Japan</td>
<td>AB622144 AB622133</td>
</tr>
<tr>
<td>Canis latrans</td>
<td>Coyote</td>
<td>North America</td>
<td>Tennso Zoogical Gardens, Japan</td>
<td>AB622146 AB622135</td>
</tr>
<tr>
<td>Canis lupus</td>
<td>Gray wolf</td>
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</tr>
<tr>
<td>Canis familiaris</td>
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<td>Domestic/ Asahiyama Zoo, Japan</td>
<td>AB622147 AB622136</td>
</tr>
<tr>
<td>Chrysocyon brachyurus</td>
<td>Maned wolf</td>
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<td>Ueno Zoological Gardens, Japan</td>
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<td>Speothos venaticus</td>
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<td>Yokohama Zoora, Japan</td>
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<tr>
<td>Nyctereutes procyonoides</td>
<td>Raccoon dog</td>
<td>Japan and East Asia</td>
<td>Japan/ Morioka Zoological Park, Japan</td>
<td>AB622150 AB622139</td>
</tr>
</tbody>
</table>

*Cited from Wayne (1993).  
**Sequence data will appear at the DDBJ/GenBank/EMBL nucleotide sequence database with the accession numbers.
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-R: 5′-CAAGTTGAGCTAAATTTGGTTTG-3′ and C-ZFY-F: 5′-TGTCTCTGCCTCTCTGTGTCTC-3′) and the ZFY final intron (C-ZFY-F: 5′-CAAGTTAGCATAAATTTGGTTTG-3′ and C-ZFY-R: 5′-TGTCCTGCGCTCTGCGTCTC-3′) of Canidae were newly designed in the present study (Fig. 1).

**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 gene 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 seed genomes 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.

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 most 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” defined in 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.
A. lagopus was identical with that of Ca. lupus. 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: Nyceretes 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. 2).
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.

<table>
<thead>
<tr>
<th></th>
<th>V. vulpes</th>
<th>A. lagopus</th>
<th>F. zerda</th>
<th>Ca. aureus</th>
<th>Ca. mesomelas</th>
<th>Cu. alpinus</th>
<th>Ca. latrans</th>
<th>Ca. lupus</th>
<th>Ca. familiaris</th>
<th>Ch. brachyurus</th>
<th>S. venaticus</th>
<th>N. procyonoides</th>
</tr>
</thead>
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<tr>
<td>V. vulpes</td>
<td>0.011</td>
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<td>0.019</td>
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</table>

**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) (Pecon-Slattery 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.
Among those, the sequence of Canis familiaris was identical with that of Canis lupus. Canis mesomelas and Canis alpinus clustered with Ch. brachyurus and S. venaticus with 60/60% bootstrap values (Fig. 3). The difference between the NL tree and the other trees (ML and MP) was the position of Canis alpinus; the ML and MP trees showed that Canis alpinus clustered with the group consisting of Canis mesomelas, Ch. rachyurus, and S. venaticus, whereas the NJ tree indicated that Canis alpinus was a sister taxon of the latter group. Nycereutes 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 Canis 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-Slattery 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 results from 30-bp insertions in the three Canis species (Canis familiaris, Canis latrans, and Canis lupus) (Fig. 4B).

In addition, another insertional fragment (ns 243–453) was found in only the ZFY final intron of Canis 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 (Canis familiaris, Canis latrans, and Canis lupus) having the 30-bp insertional sequence were separated from the other nine species. The SINE-I sequences of Canis 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 (Canis aureus, Canis familiaris, Canis latrans, and Canis 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 Canis alpinus, Canis mesomelas, Ch. brachyurus, S. venaticus, and N. procyonoides were different between the ZFY and ZFX trees. Cuon alpinus and Canis mesomelas clustered with other Canis species of the wolf-like canis 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 Canis mesomelas) were clustered with the wolf-like canids clade. In addition, Zrzavy and Říčánková (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 Canis 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
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 Řičánková (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 (Vassetzkzy 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 (Vassetzkzy and Kramerov, 2002): the SINE-II sequence contained two direct repeats (GAGCCTGCC and GCTGCT) and insertion (TGAGGGGGAGG). 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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**REFERENCES**


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### Appendix

An alignment of the ZFY final intron sequences. Dots show identical nucleotides with those of *A. lagopus*. Dashes indicate gaps.
A. lagopus  
F. zarda  
V. vulpes  
Ca. auritus  
Ca. mesomelas  
Cu. alpinus  
Ca. latrans  
Ca. familiaris  
Ca. lupus  
S. venaticus  
Ch. brachypus  
N. procyonoides

A. lagopus  
F. zarda  
V. vulpes  
Ca. auritus  
Ca. mesomelas  
Cu. alpinus  
Ca. latrans  
Ca. familiaris  
Ca. lupus  
S. venaticus  
Ch. brachypus  
N. procyonoides

A. lagopus  
F. zarda  
V. vulpes  
Ca. auritus  
Ca. mesomelas  
Cu. alpinus  
Ca. latrans  
Ca. familiaris  
Ca. lupus  
S. venaticus  
Ch. brachypus  
N. procyonoides

A. lagopus  
F. zarda  
V. vulpes  
Ca. auritus  
Ca. mesomelas  
Cu. alpinus  
Ca. latrans  
Ca. familiaris  
Ca. lupus  
S. venaticus  
Ch. brachypus  
N. procyonoides

A. lagopus  
F. zarda  
V. vulpes  
Ca. auritus  
Ca. mesomelas  
Cu. alpinus  
Ca. latrans  
Ca. familiaris  
Ca. lupus  
S. venaticus  
Ch. brachypus  
N. procyonoides

Appendix. Continued.
### Sex Chromosome DNA of Canidae

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<th>Species</th>
<th>Sequence</th>
<th>Length</th>
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<td><em>A. lagopus</em></td>
<td>TACTGGGTTTTTCTTTTACCTTTTGGAAACAAAGAAATTTAGCTTTTAAAGGTACCTTTCTTTTTCCTTTCTTACAG</td>
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</tr>
<tr>
<td><em>F. zerda</em></td>
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<td>1089</td>
</tr>
<tr>
<td><em>V. vulpes</em></td>
<td>G</td>
<td>1089</td>
</tr>
<tr>
<td><em>C. aureus</em></td>
<td>A</td>
<td>1089</td>
</tr>
<tr>
<td><em>C. mesomelas</em></td>
<td>A</td>
<td>1089</td>
</tr>
<tr>
<td><em>O. alpinus</em></td>
<td>A</td>
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</tr>
<tr>
<td><em>C. latrans</em></td>
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<td>1089</td>
</tr>
<tr>
<td><em>C. familiaris</em></td>
<td>A</td>
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</tr>
<tr>
<td><em>C. lupus</em></td>
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</tr>
<tr>
<td><em>S. venaticus</em></td>
<td>A</td>
<td>1089</td>
</tr>
<tr>
<td><em>C. brachyurus</em></td>
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</tr>
<tr>
<td><em>N. procyonoides</em></td>
<td>T</td>
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</table>

Appendix. Continued.