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RESEARCH ARTICLE

Phylogenetic relationships between different raccoon dog (*Nyctereutes procyonoides*) populations based on four nuclear and Y genes

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Abstract

Background The raccoon dog (*Nyctereutes procyonoides*), endemic to East Asia, is classified as six subspecies according to their geographical distribution including a population introduced to Europe. Studies on phylogenetic relationship or population genetics in both native and introduced areas have been carried out recently. Lately, opinions that Japanese raccoon dogs should be classified as a different species were asserted based on several studies using karyotypes, morphometric characters, mtDNA, and microsatellites analysis. However, no data pertaining to the nuclear DNA (nDNA) or Y chromosome are available.

Objective To estimate the relationship among the species using different genes is necessary in understanding of the history of this species.

Method Therefore, we investigated nDNA and Y chromosomes in our study to define relationships: (1) between continental raccoon dog populations, (2) between original and introduced groups, and (3) between continental and Japanese groups.

Results The analysis of four nuclear (*CHRNA1*, *VTN*, *TRSP*, *WT1*) and *ZFY* genes indicated that there had been no genetic differentiation among the continental populations. However, significant differences were observed between continental and Japanese raccoon dogs in *VTN* and *ZFY* genes implying genetic differentiation has been going between them.

Conclusion To better understand the phylogenetic relationship among raccoon dog populations, further study will be necessary.

Keywords Raccoon dog · Molecular phylogeny · Nuclear DNA · Y chromosome · Evolutionary history

Introduction

The raccoon dog (*Nyctereutes procyonoides*) is classified into six subspecies according to their geographical distribution and morphology: *N. p. ussuriensis, N. p. koreensis, N. p. procyonoides, N. p. oretes, N. p. viverrinus,* and *N. p. albus* (Ellerman and Morrison-Scott 1966) (Fig. 1). Raccoon dogs, which are endemic to East Asia, were introduced into

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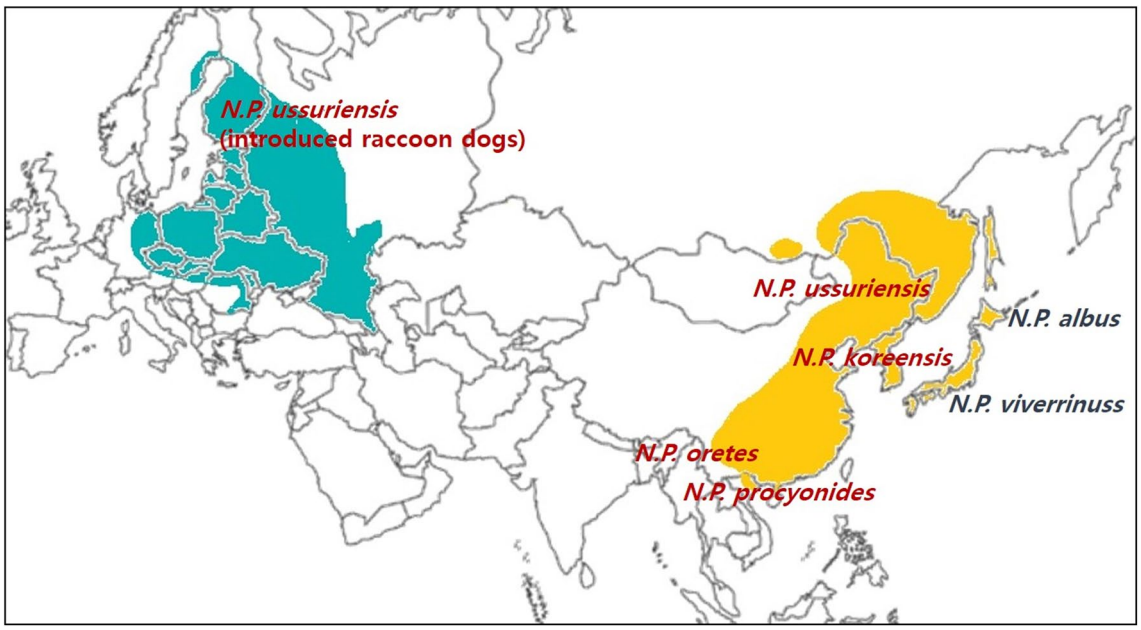

Fig. 1 Geographic distribution of Raccon dog, *Nytereutes procyonoides* and studying area and sampling information. Orange covered areas: origianal ranges, blue covered (color figure online)

Europe in the early twentieth century (Kauhala and Saeki 2004; Pitra et al. 2010; Hong et al. 2013). Its high adaptability to various environments enables the raccoon dog to increase its population size and proliferate in a short time in the introduced regions of Europe (Kauhala and Saeki 2004; Pitra et al. 2010; Kauhala and Kowalczyk 2011; Sutor et al. 2014). According to the epigenetic analysis using non-metric skeletal characters (Ansorge et al. 2009), differentiation was detected between original Far East Russian and introduced European raccoon dog populations, and even within European populations. However, genetic studies showed different results from the morphological study. Although two haplogroups existed in Europe, no geographical structuring within eastern and northern European populations was detected until now (Pitra et al. 2010; Paulauskas et al. 2016). Molecular phylogeographic studies using mtDNA analyses showed no differences between original and Finnish (introduced from south-eastern Russia) populations (Kim 2011). Hong et al. (2018) also confirmed using both mtDNA and microsatellite analysis that original raccoon dogs still share their genetic composition with the Finnish population. However, there is still a lack of information necessary to reveal the genetic relationship among all populations of the raccoon dog.

The most noteworthy group is the Japanese raccoon dog population. Two subspecies inhabit Japan (*N. p. viverrinus* and *N. p. albus*), and there are numerous studies suggesting that Japanese raccoon dogs are sufficiently differentiated from continental populations at species level. Kim et al. (2015) suggested that Japanese raccoon dogs should be classified as a separate species *Nyctereutes viverrinus* with two subspecies as *N. v. viverrinus* and *N. v. albus*. External morphological differences in fur color between continental and Japanese populations have been reported (Korhonen et al. 1991; Won et al. 2004). Kauhala et al. (1998) reported that the skull size of the Finnish raccoon dog (*N. p. ussuriensis*) is larger than that of the Japanese raccoon dog (*N. p. viverrinus*). Kim et al. (2015) confirmed that skulls, mandibles, and carnassial teeth of Japanese raccoon dogs (*N. p. viverrinus* and *N. p. albus*) were smaller than those of continental raccoon dogs (*N. p. ussuriensis* and *N. p. koreensis*) in Russia, China, and Korea.

According to Wada and Imai (1991), Wada et al. (1991), and Won et al. (2004), the number of chromosomes varied between continental (2n = 54) and Japanese (2n = 38) populations of the raccoon dog. A recent phylogeographic study using mtDNA sequences also showed a high genetic differentiation between the two populations (Kim et al. 2013). In addition, a population genetic study using 16 microsatellite markers indicated that the Japanese population was greatly differentiated from the continental populations (Hong et al. 2018).

Although several studies on the phylogenetics of the raccoon dog have dealt with maternally inherited mtDNA and biparentally inherited microsatellites, there has been no studies estimating phylogenetic relationships based on paternally inherited genes. Moreover, nuclear genes, used to analyze phylogenetic relationships, can provide crucial

information to accurately reveal relationships between East Asian raccoon dog subspecies. An understanding of the evolutionary history based on a diversity of genes is necessary to estimate the relationship between closely-related groups (Bardeleben et al. 2005; Wahlberg et al. 2009).

Despite the high level of differentiation in karyotype, mtDNA, and microsatellite markers, we may be unable to detect the existence of differentiation in nuclear and Y chromosome genes between the continental and Japanese populations, because of their slower evolutionary rates compared to mtDNA. However, a slower substitution rate reduces homoplasy, and non-coding regions of nuclear markers accumulate indels so that these characters can provide important phylogenetic information (Rokas and Holland 2000; Bardeleben et al. 2005). Moreover, sampling of multiple unlinked regions of the genome is possible with nuclear markers, whereas mitochondrial genes offer only one genealogy.

To better resolve this issue, we analyzed nuclear genes and the Y chromosome. We selected four nuclear genes: *CHRNA1* (Cholinergic receptor, nicotinic, α polypeptide 1 precursor), *VTN* (Vitronectin), *TRSP* (Selenocysteine tRNA), and *WT1* (Wilms tumor 1) to compare genetic variation among different raccoon dog populations. They have been used for the phylogenetic study of mammals including Canidae (Venta et al. 1996; Bardeleben et al. 2005; Koepfli et al. 2006). A zinc-finger-containing gene located on the Y chromosome (*ZFY*) shows male-driven evolution (Nakagome et al. 2008). Specifically, short interspersed nuclear elements (SINEs) in the *ZFY* gene provide useful evolutionary history for phylogenetic study (Pecon Slattery et al. 2000; Shedlock and Okada 2000; Tsubouchi et al. 2012; Chen and Yang 2014), because they are not eliminated once inserted into specific sites (Shedlock and Okada 2000; Tsubouchi et al. 2012). Therefore, the aim of this study was to reveal the genetic relationships among raccoon dog populations in eastern Eurasian continent, Japan, and the introduced region using four nuclear gene and *ZFY* gene markers.

Materials and methods

Samples and DNA extraction

We analyzed 33 raccoon dogs for nuclear genes and 19 raccoon dogs for the *ZFY* gene from six countries (Table 1 and S1). All the samples were collected by Conservation Genome Research Bank for Korean Wildlife (CGRB). Samples were obtained from carcasses by road-kill or hunting. All procedures followed the guidelines of Seoul National University Institutional Animal Care and Use Committee (SNU-IACUC). DNA was extracted from tissue using DNeasy® Tissue and Blood Kits (Qiagen, Valencia, CA, USA).

PCR and sequencing of four nuclear genes

Four nuclear genes, *CHRNA1*, *VTN*, *TRSP*, and *WT1*, were amplified by polymerase chain reaction (PCR) with primers listed in Table 1. Each 30 µl reaction mixture contained ~ 50 ng of DNA, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 1 U i-star *Taq* polymerase (iNtRON Biotechnology, Seongnam, Korea). PCR started with an initial denaturation at 94 °C for 5 min; followed by 20 cycles of 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing (decreasing 0.5 °C per cycle to 50 °C), and 30 s at 72 °C for extension; 15 cycles of 30 s at 94 °C for denaturation, 30 s at 50 °C for annealing, and 30 s at 72 °C for extension; and a final extension of 5 min at 72 °C. PCR products were purified and sequenced by using using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA) and an ABI Prism™ 3730 XL automated sequencer (Applied Biosystems Inc, CA, USA), respectively.

PCR and sequencing of *ZFY* gene

The final intron of the *ZFY* gene was amplified by PCR using U-ZF-2F/ U-ZF-2R primers (Nakagome et al. 2008) (Table 1). Each 30 µl reaction mixture contained ~ 50 ng of DNA, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 1 U i-star *Taq* polymerase (iNtRON Biotechnology, Seongnam, Korea). PCR started with an initial denaturation at 94 °C for 10 min; followed by ten cycles of 45 s at 94 °C for denaturation, 45 s at 55 °C for annealing (decreasing 1 °C per cycle to 45 °C), and 60 s at 72 °C for extension; 25 cycles of 45 s at 94 °C for denaturation, 45 s at 45 °C for annealing, and 60 s at 72 °C for extension; and a final extension of 10 min at 72 °C. A primer pair C-ZFY_F/C-ZFY_R (Tsubouchi et al. 2012) was used for sequencing. Purification and sequencing were performed in the same way as those for the four nuclear genes.

Characteristic analyses of sequences

All the sequences were aligned using Geneious v4.7.6 (Drummond et al. 2009) and Clustal X (Jeanmougin et al. 1998). Each of the four nuclear genes were independently analyzed and combined sequences of four genes were also used for analyses. We used PHASE (Stephens et al. 2001) to reconstruct haplotypes because of the existence of multiple heterozygous single nucleotide polymorphisms (SNPs) in most of the individuals. Individuals with homoozygous and single heterozygous SNPs exhibited one and two haplotypes, respectively, and individuals with multiple heterozygous SNPs exhibited more than two haplotypes in accordance with the number of heterozygous SNPs. Subsequently, we

Table 1 Samples and markers information used in the study

Gene	Region	Primers (5′→3′)	References	Sample location and size						Outgroup
				KOR	CHN	RUS	FIN	VNM	JPN	
CHRNA1	Intron 8p	F: GACCATGAAGTCAGACCAGGA G R: GGA GTA TGT GGTCCATCACCAT	Bardeleben et al. (2005)	8	5	2	5	5	8	AY885330
TRSP	5′ flanking region 34/87 p	F: GGGCTTCTGAAAGCCGACTT R: CCGCCCGAAAGGTGGAATTG	Bardeleben et al. (2005)	8	5	2	5	5	8	AY609122
VTN	Exon 4p, Intron 4, Exon 5p	F: AGTGAGGCCTGGGTACCC R: GAAGAAGTAGACCCGCTCCC	Bardeleben et al. (2005)	8	5	2	5	5	8	AY885425
WT1	Intron 8 p/ Exon 9p	F: GAGAAACCATACCAGTGTGA R: GTTTTACCTGTATGAGTCCT	Koepfli et al. (2006)	8	5	2	5	5	8	AY928758
ZFY	Final Intron	U-ZF-2F: GACCTGATTCCAAACAGTAC U-ZF-2R: GCCACAAATCATGCAAGG	Nakagome et al. (2008)	4	–	2	4	5	4	AB622140
		C-ZFY-F: CAAGTTAGCATAAATTTGGTTTG C-ZFY-R: TGTCTCTGCCTCTCTGTGTCTC	Tsubouchi et al. (2012)							

Gene names are CHRNA1_Cholinergic receptor, nicotinic, α polypeptide 1 precursor; VTN_Vitronectin; TRSP_Selenocysteine tRNA; WT1_Wilms tumor 1; ZFY_ zinc-finger-containing gene located on the Y chromosome. Red fox (*Vulpes vulpes*) sequences were used as Outgroup for all the genes

KOR South Korea, *CHN* China, *RUS* south-eastern Russia, *FIN* Finland, *VNM* Vietnam, *JPN* Japan

assessed genetic characteristics and diversity using DnaSP 5 (Librado and Rozas 2009), and MEGA 6 (Tamura et al. 2013) was used to determine genetic distance by Kimura-2 parameters. Reconstructed haplotypes were used only to obtain genetic characteristics, diversity, and distance. For the *ZFY* gene, DnaSP 5 (Librado and Rozas 2009) was used to assess haplotype diversity (*H*d), nucleotide diversity (π) using MEGA 6 (Tamura et al. 2013). Genetic distance were determined by Nei's genetic distance for nuclear genes using GenAlEx 6.5 (Peakall and Smouse 2012) and Kimura-2 parameters for *ZFY* gene using MEGA 6 (Tamura et al. 2013).

Phylogenetic analyses

We used International Union of Pure and Applied Chemistry (IUPAC) code so that each individuals has only one haplotypes for constructing phylogenetic trees. Models for Maximum Likelihood (ML) and Bayesian Inference (BI) trees were determined according to Akaike information Criterion (AIC) (Akaike 1974) in jModelTest v0.1.1 (Posada 2008). Selected models for each gene and combined data set are shown in Table 2. RaxML function in CIPRES (Miller et al. 2010) was used with a rapid bootstrapping, and 1000 replicates for constructing ML phylogenetic trees of each gene and Partitioned model were selected for combined data set of the four genes. Additionally, Markov chain Monte Carlo (MCMC) phylogenetic trees were constructed by using Mr. Bayes v3.2.1 (Ronquist et al. 2012). MCMC was set to run 1 million generations and every 100 generations were sampled. The first 25% of generations was discarded as burn-in. Sequences of red fox (*Vulpes vulpes*) belonging to the same family Canidae were used as outgroup (Table 1). Constructed rees were visualized by using Figtree v1.3.1 (Rambaut and Drummond 2009).

Estimation of divergence time

Divergence time between the two populations was estimated using molecular clock methods with uncorrelated and strict models of BEAST v1.8.3 (Drummond and Rambaut 2007). A TPM2uf for *ZFY* was selected as a nucleotide substitution model by jModelTest. However, BEAUti does not accept TPM2uf model, general time reversible (GTR) model was replaced by according to Lecocq et al. (2013). Yule process was used as the tree prior and normal distribution was chosen for priors of model parameters. Normal distribution was selected for calibrating divergence time. We used three time priors, Canini/Vulpini split (8 Mya), Ailuropoda/Ursus split (7.5 Mya), and Canidae/Ursidae (40 Mya) as a root height in accordance with Perini et al. (2010). Sequences for time priors were obtained from GenBank: *Canis mesomelas* (AB622144), *C. latrans* (AB622146), *C. lupus* (AB622147), *Vulpes vulpes* (AB622140), *V. lagopus* (AB622141), *A. melanoleuca* (AB261814), and *Uursus americanus* (AB261809). MCMC chain werew run for 10,000,000 generations, and every 1000 generations were sampled.

Results

Sequence characteristics of nuclear and *ZFY* genes

We determined partial sequences for each of the four nuclear autosomal genes ranging from 285 to 461 bp including indels (MH209078–MH209221), and all nuclear genes contained variable sites and different number of haplotypes by reconstruction process (Table 2). Two individuals in *CHRNA1*, eight individuals in *TRSP*, and 13 individuals in combined four nuclear data exhibited more than two haplotypes. Nucleotide (π) and haplotype diversity (*H*d) of four genes ranged from 0.001 to 0.058, and 0.415 to 0.927, respectively.

The *TRSP* gene showed the most variable sites (nine sites: 2.7% of total), and *VTN* contained the fewest variable

Table 2 Sequence characteristics, genetic diversity, and models of substitution for each four nuclear and combined data and ZFY gene

	Nucleotides bp	Var. sites	PI sites	Singleton	Indel	Models	Hap no /seq no	π	*H*d
CHRNA1	285	2	2	0	0	K80	4/48	0.0028	0.648
TRSP	339	9	6	3	0	TVM	29/74	0.0058	0.927
VTN	408	2	1	1	0	HKY	3/34	0.0011	0.415
WT1	461	7	2	1	4	HKY + G	5/40	0.0023	0.729
Combined nuclear genes	1493	20	14	2	4	TVM + G	96/127	0.0027	0.994
ZFY	936	1	1	0	0	TPM2uf	2/19	0.0003	0.351

Var. sites variable sites, *PI sites* parsimony informative sites, *Hap No* number of haplotypes, *Seq No* number of sequences, π nucleotide diversity, *Hd* haplotype diversity

sites with 0.5% of total sites. Four indels were observed in the *WT1* gene. About 48% of the variable sites were parsimony informative and ranged from 37.5% in *WT1* to 100% in *CHRNA1*. The combined dataset of the four nuclear genes comprised 1493 nucleotides with 13% of coding region and 87% of non-coding region. Most of the variable and parsimony-informative sites and indels were observed in the non-coding regions except for the one singleton site that was observed in the coding region in the *VTN* gene of one Japanese raccoon dog. This single mutation, C to Y (C or T), in the flanking region of *VTN* was a nonsynonymous mutation, with a change from arginine to cysteine. Moreover, the other nucleotide substitution in *VTN* also occurred within Japanese individuals.

Average pairwise genetic distance of combined data among all populations from six regions (Korea, China, Russia, Finland, Vietnam, and Japan) ranged from 0.028 to 0.202 (Table 3) and no significant differentiation was detected among continental populations. However, two genetic clades existed in East Asia; continental and Japanese populations, respectively. Genetic distance between continental and Japanese populations in the combined data was d = 0.166. Mean genetic distance within the continental population was higher than that within the Japanese population (data not shown), and the Finnish population showed the highest mean genetic distance (data not shown) in four combined nuclear genes. *CHRNA1* gene showed relatively higher distance value among populations than the genetic distance, shown in *TRSP* and *WT1* genes (Table S2a). Both *TRSP* and *WT1* genes revealed no differentiation and shared haplotypes among populations (Table S2b and d). In *VTN* gene, each of the continental and the Japanese populations have their own haplotypes, and no genetic difference (d = 0) was revealed within the five continental populations. However, distinct separation with d = 0.727 between continental and Japanese populations was detected in the *VTN* gene (Table S2c). The complete *ZFY* gene (936 bp) had a single mutation site among all the samples (MH209222–MH209240), and two haplotypes were exhibited (Table 2). Values of nucleotide diversity (π) and haplotype diversity (*H*d) were 0.0003 and 0.351, respectively. One nucleotide substitution of SINE I in the *ZFY* gene led to a small degree (d = 0.001, Table 3) separation between the continental groups (Korea, China, Russia, Finland, and Vietnam) and the Japanese group, implying that all 15 continental individuals shared the same nucleotide composition, and only one haplotype was observed among four Japanese raccoon dogs.

Phylogenetic analysis of each/ combined nuclear genes and *ZFY* gene

The phylogenetic trees of nuclear genes estimated by ML and BI showed similar results with a few minor discrepancies of grouping among individuals, but the bootstrap values were low enough to ignore them. ML tree of four genes combined is shown in Fig. 2. All phylogenetic trees of each gene and combined data revealed no genetic divergence among continental Korean, Chinese, Russian, and Vietnamese raccoon dogs, nor between original Russian and introduced Finnish individuals (Fig. 2 and Fig. S1). However, minor differentiation between continental and Japanese groups was observed. There was definite differentiation of the Japanese group from continental groups detected in the *VTN* gene (Fig. S1c). According to Fig. S1, VNM2 was always divided into the same branches as KOR7 in the trees based on the four nuclear genes: *CHRNA1, TRSF, VTN*, and *WT1*. However, it was separated from KOR7 in combined tree shown in Fig. 2. Respective two polymorphic sites were detected between KOR7 and VNM2 both in *TRSP* and *WT1* genes. These polymorphism did not show up remarkable differentiation in each phylogenetic trees. However, prominent differentiation between two individuals was shown in the combined tree.

Both the ML and BI methods constructed the same phylogenetic tree of the *ZFY* gene. The representative ML tree in Fig. 3 indicates that the Japanese population was grouped as a separate clade from the continental populations with 100% of bootstrap value and 0.95 of posterior probability. No differentiation was detected among continental raccoon dog populations including introduced Finnish ones with a single haplotype.

Table 3 Ne's genetic distance of four nuclear combined data (below diagonal) and Kimura-2 parameters ZFY gene (above diagonal) among raccoon dog populations

	KOR	CHN	RUS	FIN	VNM	JPN
KOR		0	0	0	0	0.001
CHN	0.035		0	0	0	0.001
RUS	0.028	0.034		0	0	0.001
FIN	0.073	0.069	0.045		0	0.001
VNM	0.069	0.085	0.043	0.109		0.001
JPN	0.179	0.202	0.128	0.199	0.125	

KOR South Korea, *CHN* China, *RUS* south-eastern Russia, *FIN* Finland, *VNM* Vietnam, *JPN* Japan

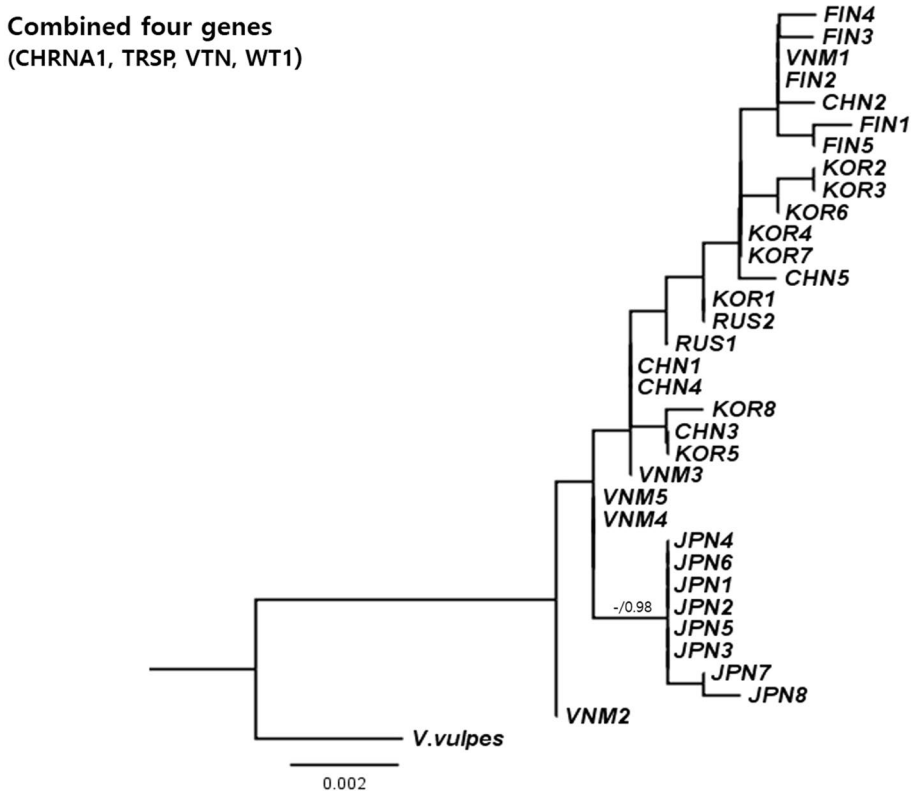

Fig. 2 Maximum likelihood phylogenetic tree of the four nuclear combined data. Bootstrap for ML and Bayesian posterior probability are shown for branches with over

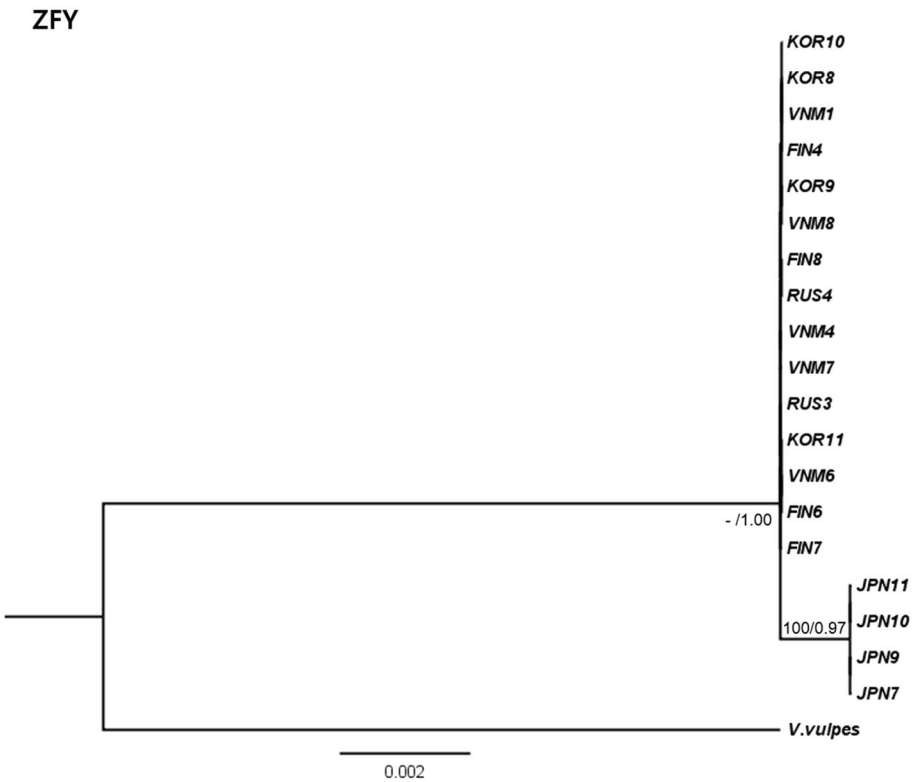

Fig. 3 Maximum likelihood phylogenetic tree of the ZFY gene. Bootstrap for ML and Bayesian posterior probability are shown for branches with over 85% support

The *ZFY* gene tree and genetic distance showed 100% individual separation of the Japanese population from continental raccoon dogs

Divergence time

We estimated divergence time between continental and Japanese raccoon dog populations according to the phylogenetic grouping of *ZFY* trees. Split time of Japanese raccoon dog from the continental populations by the *ZFY* gene was about 0.19–0.46 Mya, more recent than mtDNA [[0.59–0.67 Mya; Kim (2011)].

Discussion

No geographical grouping was detected among continental populations from *CHRNA1*, *TRSP*, and *WT1* genes on phylogenetic analysis. In general, nDNA has a relatively slower rate of nucleotide substitution than mtDNA and Y-linked gene like *ZFY* (Erlandsson et al. 2000; Feng et al. 2001; Kirkpatrick and Hall 2004; Bardeleben et al. 2005; Trujillo et al. 2009; Song et al. 2016). Moreover, Wang et al. (2008) emphasized that nuclear markers might not fully reflect recent speciation due to an insufficient amount of time for fixed molecular variation to occur, even though other selective genetic markers or morphological differentiation have already occurred, as shown in the study testing species–level taxonomy of the finless porpoise (*Neophocaena phocaenoides*).

However, two genetic clades existed in East Asia in the *VTN* and combined data of four nuclear genes as well as *ZFY* gene of the Japanese and continental clades, strengthening the separation of the Japanese raccoon dog from the continental group. *VTN* might be a potential marker to elucidate evolutionary history in raccoon dogs. One nuclear nonsynonymous mutation from arginine to cysteine in *VTN* occurred at the 5′ flanking region, which contains the promotor to regulate transcription and gene expression. Even though this mutation did not occur in the transcriptional binding sites, mutation in the flanking region has a high possibility to result in a change in transcription, regulation, or its function (Hayashi et al. 1991; D'Souza et al. 2004; Fields and Gainer 2015); thus, it might affect phylogenetic history as shown in the combined data of four genes (Fig. 2).

VTN, expressed in blood, is a gene associated with diseases involving platelet disorders and immune response (Schvartz et al. 1999). There have been reports that *VTN* also regulates the proteolysis involved in metastatic cancers of humans and rodents (Schvartz et al. 1999; Singh et al. 2010). Therefore, genetic mutation in *VTN* might result in the occurrence of related diseases. However, Schvartz et al. (1999) reported that *VTN* deficiency in mice did not affect their survival, fertility, and development, which suggests its dispensability or substitutability by alternative components. Raccoon dog might have a similar mechanism as seen in mice. Although we detected the occurrence of genetic mutation in *VTN,* there might lack visible signs of the diseases.

To assess our result that *VTN* gene in Japanese raccoon dogs seemed to be differentiated from continental population, we compared the genetic distance values among interspecific sequences of Canidae using Kimura-2 parameters. Although the genetic difference in the *VTN* gene was low, a genetic distance of d = 0.002 between the continental and Japanese raccoon dogs is sufficient to divide them into different genetic groups. Comparison of interspecific variation within Canidae corroborates that this difference is significant. Genetic distance among four *Canis* species [*Canis mesomelas*, AY885411; *Canis lupus*, AY885410; *Canis latrans*, AY885409; *Canis aureus*, AY885407; (Bardeleben et al. 2005)] was in the range of d = 0–0.004; particularly three of them except *Canis mesomelas* showed the same haplotype (d = 0). Genetic distance among four *Vulpes* species [*Vulpes vulpes*, AY885425; *Vulpes macrotis*, AY885424; *Vulpes corsac*, AY885423; *Vulpes zerda*, AY885417; (Bardeleben et al. 2005)] ranged from 0.002 to 0.004. Therefore, *VTN* gene in Japanese raccoon dogs might be adapted and evolved differently from continental raccoon dogs and further study of this gene is necessary.

In the *ZFY* gene, only one substitution occurred in SINE I and this mutation was a definite identification factor for the divergent clustering between continental and Japanese raccoon dogs. SINEs are considered as powerful evolutionary markers for molecular phylogenetic studies (Pecon Slattery et al. 2000; Shedlock and Okada 2000; Tsubouchi et al. 2012; Chen and Yang 2014). Besides, the genetic distance between continental and Japanese populations was 0.001, and similar differentiation was observed in other Canidae species, such as between *Canis lupus*/*Canis familiaris*, and *Canis latrans* in the *ZFY* gene comparison (Tsubouchi et al. 2012). Therefore, we concluded that differentiation between continental and Japanese raccoon dog groups in *ZFY* warrants that the two groups need to be considered as independent species.

We estimated the split time of divergence of *ZFY* in Japanese raccoon dogs from continental counterparts as 0.19–0.46 Mya. Divergence of the *ZFY* gene seemed to have occurred during the middle of Pleistocene. According to the previous analyses using mtDNA, (Pitra et al. 2010) it is suggested that the migration of raccoon dog groups from continental regions to Japan occurred around 0.48–1.37 Mya (mean = 0.87 Mya) and Kim (2011) calculated the divergence time approximately to be 0.59–0.67 Mya. Such difference reflects the different evolutionary history of the two genes. Chan et al. (2012) suggested that the difference in divergence time between mtDNA

and the Y chromosome might be due to sex-biased dispersal, reduced male effective population size, or unequal generation length. Raccoon dogs are monogamous (Kleiman 1977) and no dispersal difference between males and females has been reported. However, there is no information in the literature pertaining to the demographic process or dispersal patterns while for raccoon dogs migrating from the continent to the Japanese islands. Moreover, it is known that males mate with more than one female in captivity (Heptner et al. 1998). According to the study in shorebirds (Jackson et al. 2017), polygamous relationships have higher gene flow and slow down population divergence. Therefore, similar event might be occurred during the raccoon dog's migration. Finally, estimates of divergence time between continental and Japanese raccoon dogs using mtDNA and Y chromosome correspond to the middle of the Pleistocene period, and Kim et al. (2013) showed that Japanese raccoon dogs migrated from continental regions before the last glacial period of the Pleistocene and adapted to the new environment. Therefore, divergence of the *ZFY* gene might occur during the Mid-Pleistocene is the cases in mtDNA. This may imply that long-term geographical isolation could be an essential factor in the differentiation observed between continental and Japanese raccoon dogs.

We confirmed that there exists only one phylogenetic clade among continental raccoon dog populations, including Finnish populations originating from eastern Russia through the analysis of four autosomal nuclear genes and *ZFY* gene. However, Japanese raccoon dogs were separated from the main continental group based on the analysis of nuclear DNAs and *ZFY* gene. After migration, Japanese population has differentiated by adapting to a different environment. Different chromosomal numbers (Wada and Imai 1991; Wada et al. 1991), morphological characteristics (Korhonen et al. 1991; Kauhala et al. 1998; Won et al. 2004; Kim et al. 2015), and restricted gene flow in mtDNA (Kim et al. 2013) also support the speciation between them. Moreover, microsatellite analysis indicated that much differentiation has occurred between the continental and Japanese raccoon dogs at the species level (Hong et al. 2018). In recent times, genetic exchange between the two populations would be limited due to geographical isolation, and it has been suggested that the Japanese raccoon dog should be considered as an independent species from the continental *Nyctereutes procyonoides*. Differentiation in *VTN* and *ZFY* genes between continental and Japanese raccoon dog populations in our study cannot be excluded the result of incomplete lineage sorting. However, diverse clues have been reported to support that *VTN* and *ZFY* genes could be informative markers for raccoon dog phylogenetic analysis. To better understand the phylogenetic relationship among raccoon dog populations, especially between continental and Japanese populations, comparison of longer sequences and more diverse genes will be necessary.

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Author contributions YJH designed the experiments, carried out the molecular experiments, data analysis and wrote the manuscript. HL discussed the results, helped to draft the manuscript, and advised for discussion. KSK and MSM conceived of the study, discussed the results and reviewed the manuscript. All authors approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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