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Population genetics and phylogeographic study of raccoon dog (*Nyctereutes procyonoides*)

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홍 윤 지

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Population genetics and phylogeographic study of raccoon dog (*Nyctereutes procyonoides*)

Advised by Professor Hang Lee

By Yoon Jee Hong

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Population genetics and phylogeographic study of raccoon dog (*Nyctereutes procyonoides*)

Yoon Jee Hong

Department of Veterinary Medicine

The Graduate School

Seoul National University

ABSTRACT

Raccoon dog (*Nyctereutes procyonoides*), originated in East Asia, is classified as six subspecies according to geographical

distribution including introduced population into Europe. Raccoon dog is a true omnivore that plays an important role in maintaining the food web balance in ecosystems and is an effective scavenger. They are also vectors for numerous contagious and zoonotic diseases such as rabies or canine distemper, and parasites. There is public interest on raccoon dog populations due to their influence on ecosystems. Moreover, they quickly colonized European countries, where their rapid population growth has raised concerns about disturbances to ecosystems and the spread of zoonotic diseases. In addition, most studies considering the genetic structure of raccoon dogs focused on populations at a regional scale and thus a population genetic study spanning the broad geographic range of the species is still lacking. Understanding the degree of population structure and genetic differentiation within a species can inform on decisions to conserve the genetic diversity. Information obtained by analyzing microsatellite loci can be used to and determine the genetic structure and recent gene flow between natural populations and serves as a basis for establishing management units (MUs). Lately, opinions that Japanese raccoon dogs should be distinct as a different species were asserted by several studies; karyotypes, morphometric characters, mtDNA and microsatellites analysis.

However, no data of nuclear DNA and Y chromosome was conducted. Evolutionary history from different genes is necessary in estimating relationship between closely related groups.

In this study, as a first step, we identified and characterized 12 microsatellite loci using Korean raccoon dog sample, and applied to other raccoon dog populations. We investigated genetic variation at 16 microsatellite loci (12 developed loci in the present study and four canine loci) for raccoon dogs from seven locations across South Korea, China, southeastern Russia, Finland (introduced from southeastern Russia), Vietnam, and Japan (Honshu and Hokkaido) to compare the genetic diversity and structure among endemic populations, as well as between source and introduced populations. Further, Korean and Japanese populations were analyzed separately to establish long-term conservation and management strategies for raccoon dogs in East Asia. Finally, we investigated nDNA and Y chromosome in this study to define the relationship 1. among continental raccoon dog populations, 2. between original and introduced groups, and 3. between continental and Japanese groups.

First, Polymorphic 12 microsatellite markers (*Nyct* 1 -12) showed no linkage disequilibrium and adequate to apply other raccoon dog populations. Second, our data using 16 microsatellite

loci (12 developed loci in this study and four canine microsatellite loci) showed that the raccoon dog population is divided into two major genetic clusters: continent (South Korean, Chinese, Russian, Finnish, and Vietnamese populations) and island (Japanese populations), which are significantly genetically different (F_{ST}=0.236). There are three subpopulations within the continental raccoon dog population: South Korean/Vietnamese, Chinese and Russian, and Finnish, closely related to the Chinese and Russian populations. The Japanese population consisted of two distinct subpopulations, Honshu and Hokkaido. The genetic diversity and geographic structure of raccoon dogs in East Asia was influenced by natural barriers to gene flow between distinct populations and revealed a typical central–marginal trend in genetic diversity for most central and peripheral populations (continent vs. island, central vs. peripheral in continent, source vs. introduced in continent). The differences between continental and island populations agreed with previous studies that recommended that these populations be considered different species. Third, raccoon dog populations in Korea and Japan showed significant genetic differentiation (F_{ST}=0.247). Substructure was further detected within both Korean and Japanese populations. We proposed

potential management units (MUs) of raccoon dogs based on genetic structuring and gene-flow barrier data obtained in this study. Four MUs were suggested for both Korean (northern, central, southwestern, and southeastern) and Japanese (Hokkaido, Honshu_Kanagawa, Honshu_Wakayama, and Shikoku) raccoon dog populations. Population structure determined in this study and the proposed MUs will be helpful to establish practical strategies for managing Korean and Japanese raccoon dog populations and for preventing infectious diseases for which they are the major vector species. Finally, analysis of four nuclear (CHRNA1, VTN, TRSP, WT1) and ZFY genes showed no genetic differentiation among continental populations, however, significant separation between continental and Japanese raccoon dogs as a species level. According to our study, we suggested Japanese raccoon dog population should be considered as different species from continental populations.

Keywords: Raccoon dog, *Nyctereutes procyonoides*, Population genetics, Genetic differentiation, Microsatellite loci, Management Unit, Nuclear DNA, Y chromosome

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General Introduction

The raccoon dog (*Nyctereutes procyonoides*) is endemic to East Asia, but it was introduced in Europe during the early 20th century (Helle and Kauhala 1991; Pitra et al., 2010). It is classified into six subspecies (Ellerman and Morrison-Scott, 1951; Ward and Wurster-Hill, 1990, Kauhala and Saeki, 2016): *N. p. ussuriensis* (Russia, northeastern China and Eurasia), *N. p. procyonides* (Vietnam and southern China), *N. p. orestes* (Yunnan in China), and *N. p. koreensis* (Korean Peninsula), *N. p. albus* (Hokkaido), and *N. p. viverrinuss* (Japan except Hokkaido). Two subspecies of them inhabit Japan and there have been many researches and studies that Japanese raccoon dogs are sufficiently differentiated from continental populations at the level of a distinct species. This issue has been debated until recently and Kim et al. (2015) suggested that Japanese raccoon dogs (*N. p. viverrinus* and *N. p. albus*) should be separated as *Nyctereutes viverrinus*, independent from continental raccoon dogs, *Nyctereutes procyonoides* renaming two Japanese species as *N. v. viverrinus* and *N. v. albus*.

This species easily adapts to different climates and habitats, including forests, wetlands, damp meadows, agricultural land, and urban areas (Kauhala and Saeki, 2004; Pitra et al., 2010; Kauhala and Kowalczyk, 2011). Its high adaptability to various environments and high reproductive rate has enabled the raccoon dog to quickly

increase the size of its populations and spread across the regions of Europe to which it has been introduced in a short amount of time (Helle and Kauhala 1995; Kauhala and Saeki, 2004; Pitra et al., 2010; Sutor et al., 2010; Kauhala and Kowalczyk, 2011; Sutor et al., 2014; unpublished data). According to Kauhala (1996), the raccoon dog has a high reproductive capacity for a medium-sized carnivore species (mean litter size=8-10), both in its native and introduced ranges (Helle and Kauhala, 1995; Kauhala and Kowalczyk, 2011). Raccoon dogs generally reach sexual maturity at the age of 10 months, but 2- to 3-year-old females produce the highest numbers of pups (Helle and Kauhala, 1995). Raccoon dogs are monogamous, and the male participates in pup-rearing. The female spends time foraging for food for the large litter, with the male guarding the pups when the female is doing so. Raccoon dogs hibernate during the winter in habitats with cold climates. Their hibernation is unique among canids and may have contributed to their spread to northern Europe, where it is very cold (Mustonen et al., 2007). Raccoon dogs settle in burrows, which protect them against cold and predation (Kauhala and Kowalczyk, 2011). In Poland, raccoon dogs prefer to move into active badger shelters, and these can also be used as dens by them (Kauhala and Kowalczyk, 2011).

The geographic distribution range of native raccoon dog populations is rather restricted compared with other Canidae species such as

the red fox (*Vulpes vulpes*) and gray wolf (*Canis lupus*). Red foxes and gray wolves are widely distributed in Eurasia and North America, covering almost the entire northern hemisphere, whereas the native habitat of raccoon dogs is Eastern Asia. This relatively restricted distribution range suggests that it might be restricted by environmental factors. Adverse weather conditions such as very low or high temperature and humidity may be important factors limiting the expansion of raccoon dogs north, west, and south of East Asia. Generally, extreme cold climate and thick snow cover determine the northern range limit for raccoon dogs (mean annual temperature below 0 ℃ and snow depth > 35 cm) (Kauhala and Kowalczyk, 2011), and high altitude (above 800m) and mountains limit its dispersal (Nowak, 1993; Hong et al., accepted in April, 2018). A comparatively smaller home range of raccoon dogs compared to that of other Canidae species might also contribute to their restricted distribution. For example, while the home range of raccoon dogs is reported to be up to 9.5 km^2, that of red fox is up to 358 km^2 and that of grey wolf is 460.5 km^2 (Kim et al., 2008; Karamanlidis et al., 2017; Walton et al., 2017).

Raccoon dog is a true omnivore (Sutor et al., 2010; Kauhala and Kowalczyk, 2011) that plays an important role in maintaining the food web balance in ecosystems (Ågren et al., 2012) and is an effective scavenger (Melis et al., 2007). They are also vectors for numerous contagious and zoonotic diseases such as rabies or canine

distemper, and parasites (Botvinkin et al., 1981; Cherkasskiy, 1998; Hyun et al., 2005; Kauhala and Kowalczyk, 2011; Oh et al., 2012; Sutor et al., 2014). Its high adaptability to various environments and reduction of competitors and predators may enable it to increase its population size in South Korea (Jo, 2015). The growth of Korean raccoon dog population has increased public concerns over their potential role as disease and parasite vectors, especially for contagious zoonotic diseases (Oh et al., 2012; Hong et al., 2013). If population size increase, ecological behaviors are induced, such as sharing a toilet, periodically moving to a new burrow that was used by other animals during hibernation, and living in pairs, which can be negative factors for the population that promote the spread of diseases. Furthermore, some pathogens and parasites can be passed between dogs and wild canids, and also from rodents to foxes, raccoon dogs, dogs, and even to humans (Kauhala and Kowalczyk, 2011).

There is public interest on raccoon dog populations due to their influence on ecosystems and to their role in the spread of diseases in both native and the habitats into which they have been introduced (Hong et al., 2013; Kauhala and Kowalczyk, 2011). In South Korea, rabies has been mostly reported in wild and domestic animals and in humans in the northern part of Seoul/Gyeonggi and Gangwon provinces, and preventive measures against rabies are mainly

focused in these areas (Yang et al., 2011). Japan is a rabies-free country and has been making efforts to prevent future outbreaks; however, raccoon dogs still act as vectors for canine distemper and sarcoptic mange in Japan (Kauhala and Kowalczyk, 2011). Even though the raccoon dog is still colonizing new areas in Europe, as it does so it could disturb the native fauna as an invasive species. Its rapid and successful expansion probably caused the decline of amphibian populations in certain regions due to predation (Jansson, 2010; Kauhala and Kowalczyk, 2011). Hunting has been permitted to control the introduced raccoon dog population in some countries (Kauhala and Kowalczyk, 2011). The Swedish raccoon dog project was initiated in 2008 to remove existing individuals in order to prevent the establishment of a raccoon dog population (Jansson, 2010). The prevention and control of wildlife-related infectious diseases are becoming increasingly important issues. Hence, to establish proper disease risk management strategies it is essential to understand the population structure and connectivity among the populations of animals of epidemiological interest (Drygala et al., 2016). In Russia, raccoon dogs serve as reservoirs of canine distemper, and can affect the endangered Amur tiger population (Seimon et al., 2013). Raccoon dogs are still persecuted in both their native and introduced ranges as a vector of various infectious diseases. However, factors such as epidemics, road kills, predation by feral

dogs, and extreme habitat loss may actually cause raccoon dog populations to decrease (Kauhala and Kowalczyk, 2011).

Wada and Imai (1991) and Wada et al. (1991) reported that a different number of chromosomes for mainland Asia/eastern Europe populations (2n=54) and Japanese subspecies (Hokkaido and Honshu_Kyushu, 2n=38).

External morphological difference such as fur color between mainland and Japanese populations have been reported (Korhonen et al., 1991; Won et al., 2004). Kauhala et al. (1998) observed that the skull size of Finnish raccoon dogs (*N. p. ussuriensis*) was larger than that of Japanese raccoon dogs (*N. p. viverrinus*), and Kim et al. (2015) confirmed that Japanese raccoon dog (*N. p. viverrinus* and *N. p. albus*) have relatively smaller skulls, mandibles and carnassial teeth than Russian, Chinese and Korean raccoon dogs of mainland (*N. p. ussuriensis* and *N. p. koreensis*). According to Ansorge et al. (2009), epigenetic differentiation was detected between original Far East Russian and introduced European raccoon dog populations, even within European populations.

However, studies using genetic methods to assess raccoon dog population structure have had different results from those focused on morphology. Although two haplogroups were found to exist in Europe as the result of multiple introductions from different locations, no geographical structuring within the eastern and northern European populations was detected until now (Pitra *et al.*,

2010; Palauskas *et al.*, 2016). Molecular phylogeographic studies using mtDNA analyses found no differentiation between the original (native) and Finnish (introduced from southeastern Russia) populations (Kim, 2011).

A phylogenetic study using complete cytochrome *b* mtDNA (Kim et al., 2013) grouped South Korean, Chinese, Vietnamese, and Russian raccoon dog populations into a single clade (the continental group), which was distinct from the Japanese clade (the island group) with high genetic separation (Φ_{ST}=0.76). Phylogenetic trees divided the continental group into two sub-clades, which consisted of the South Korean and Vietnamese haplotypes, respectively. However, both sub-clades also contained haplotypes from China. This study suggested that Korean raccoon dogs represent a unique population that has adapted to the northeast Asian environment and are different from all other continental and Japanese raccoon dog populations. Therefore, this suggests that the Korean raccoon dog population should be considered as a valuable biological resource, requiring proper management and conservation strategies.

Recently, the population genetic structure of the raccoon dog in Europe was investigated using microsatellite markers by Griciuvienė et al. (2016), and they reported that high gene flow among four raccoon dog subpopulations contributed to the lack of population structure they observed in Lithuania. Drygala et al. (2016) identified three genetic clusters in Europe.

Although plentiful ecological studies and some phylogenetic research with mitochondrial DNA on raccoon dog have been conducted, there were no previous data on the population structure using microsatellite markers (Kahula and Saeki, 2004). Moreover, canine-derived microsatellite markers applied in raccoon dog (*N. p. procyonides*) (Rogalska-Niznik et al., 2003; Szczerbal et al., 2003) were not profitable to apply to our raccoon dog samples. In this study, we report the isolation and characterization of 12 novel polymorphic loci from *N. p. koreensis*, and their use in determining the genetic structure of raccoon dog populations in South Korea. In addition, most studies considering the genetic structure of raccoon dogs focused on populations at a regional scale and thus a population genetic study spanning the broad geographic range of the species is still lacking. In this study, patterns of genetic diversity were compared among populations and the natural and geographic features leading to the differentiation of raccoon dog populations were examined, particularly regarding the central-marginal trends in the genetic diversity of the raccoon dog populations. 1) We compared continental (South Korea, China, Russia, and Vietnam; the Finnish population was excluded because it was artificially introduced) vs. island (Japan) populations. 2) Within continental populations, we compared central (China and Russia) vs. marginal (South Korea and Vietnam) populations. 3) Within continental populations, we compared original (Russia) vs. introduced (Finland)

populations. 4) Finally, we examined the effect of geographic barrier to gene flow between raccoon dog populations on two isolated islands in Japan, Honshu and Hokkaido.

Understanding the degree of population structure and genetic differentiation within a species can inform on decisions to conserve the genetic diversity or manage/control the species (Gibbs et al., 2008). Moreover, knowledge regarding the dispersal and movement of individuals is needed to understand the epidemiology of zoonotic and animal diseases and pathogens (Cohen et al., 2013; Mullins et al., 2014). In this study, information obtained by analyzing microsatellite loci was used to determine the genetic structure and recent gene flow between natural populations from South Korea and Japan and serves as a basis for establishing management and conservation strategies for populations, e.g., for defining conservation or management units (MUs) (Palsbøll et al., 2007). The genetic structure of wild animal populations is formed due to limited dispersal among some groups of individuals due to artificial or natural barriers. Therefore, investigations of the genetic structure of populations help to provide information for the proper management of wild animal populations and their diseases. This information may be critical for establishing appropriate strategies to prevent and manage the infectious diseases transmitted by raccoon dogs.

Despite there having been several recent genetic studies done

on this species, which used maternally inherited mtDNA and biparentally inherited microsatellites, genetic information using paternally inherited markers is still absent. Moreover, nuclear genes, which are often used to estimate phylogenetic relationships, can provide crucial information to accurately reveal the relationships among East Asian raccoon dog populations. Evolutionary history estimates for diverse genes with different modes of inheritance is necessary when estimating the relationships among closely related groups (Bardeleben et al., 2005; Wahlberg et al., 2009). To better resolve this issue, we analyzed four nuclear genes and those on the Y chromosome.

Microsatellite markers have been widely employed to study aspects of population genetics, such as genetic variation, gene flow, population structuring, and genetic differentiation of various wildlife species (Beaumont and Bruford, 1998; Polziehn et al., 2000; Hu et al., 2007; Lee et al., 2011; Park et al., 2011; Choi et al., 2014; Lee et al., 2015). 16 microsatellite loci were examined to determine the genetic diversity and geographic structure of raccoon dog populations of East Asia and to establish MU for Korean and Japanese raccoon dogs.

Despite the high level of differentiation previously observed among karyotypes, mtDNA, and microsatellite markers, we may fail to detect the existence of differentiation between continental and Japanese populations based on nuclear and Y chromosome genes

because these genes have slower evolutionary rates than mtDNA. However, because slower substitution rates reduce homoplasy and the non-coding regions of nuclear markers accumulate more indels, these genes should still 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, while mitochondrial genes offer only one genealogy. Therefore, we selected four nuclear genes for our study as follows: CHRNA1, VTN, TRSP, and WT1. These have been used for phylogenetic studies of mammalian species, including species of Canindae (Venta et al., 1996; Bardeleben et al. 2005; Koepfli et al., 2006). The ZFY gene demonstrates male-driven evolution (Nakagome et al., 2008). The short interspersed nuclear elements (SINEs) in the *ZFY* gene provide especially useful information on evolutionary history for use in a phylogenetic study because they are not eliminated once inserted into specific sites (Shedlock and Okada, 2000; Slattery et al., 2000; Chen et al., 2014; Tsubouchi et al., 2012).

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3			4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
1	745	KORSG	Seoul/Gyeonggi, Korea	O		O	MH209078	MH209114	MH209150	MH209186	
2	800	KORSG	Seoul/Gyeonggi, Korea			O					
3	1564	KORSG	Seoul/Gyeonggi, Korea	O		O					
4	2852	KORSG	Seoul/Gyeonggi, Korea			O					
5	3261	KORSG	Seoul/Gyeonggi, Korea	O		O					
6	3262	KORSG	Seoul/Gyeonggi, Korea			O					
7	3468	KORSG	Seoul/Gyeonggi, Korea	O		O					
8	4529	KORSG	Seoul/Gyeonggi, Korea			O					MH209222
9	5310	KORSG	Seoul/Gyeonggi, Korea			O					
10	5314	KORSG	Seoul/Gyeonggi, Korea	O		O					
11	5323	KORSG	Seoul/Gyeonggi, Korea	O		O					
12	5325	KORSG	Seoul/Gyeonggi, Korea			O					
13	5388	KORSG	Seoul/Gyeonggi, Korea			O					
14	6326	KORSG	Seoul/Gyeonggi, Korea	O		O					
15	7710	KORSG	Seoul/Gyeonggi, Korea			O					
16	9743	KORSG	Seoul/Gyeonggi, Korea	O		O					
17	9744	KORSG	Seoul/Gyeonggi, Korea			O					
18	9920	KORSG	Seoul/Gyeonggi, Korea	O							
19	9924	KORSG	Seoul/Gyeonggi, Korea			O					
20	9929	KORSG	Seoul/Gyeonggi, Korea	O		O					
21	6284	KORSG	Seoul/Gyeonggi, Korea			O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
22	8451	KORSG	Seoul/Gyeonggi, Korea	O		O					
23	8454	KORSG	Seoul/Gyeonggi, Korea	O		O					
24	8490	KORSG	Seoul/Gyeonggi, Korea			O					
25	9781	KORSG	Seoul/Gyeonggi, Korea	O		O					
26	10344	KORSG	Seoul/Gyeonggi, Korea			O					
27	10345	KORSG	Seoul/Gyeonggi, Korea	O		O					
28	10698	KORSG	Seoul/Gyeonggi, Korea			O					
29	10699	KORSG	Seoul/Gyeonggi, Korea	O		O					
30	10800	KORSG	Seoul/Gyeonggi, Korea			O					
31	11023	KORSG	Seoul/Gyeonggi, Korea			O					
32	354	KORWG	Western Gangwon, Korea	O	O	O					
33	666	KORWG	Western Gangwon, Korea	O	O	O					
34	667	KORWG	Western Gangwon, Korea		O	O					
35	673	KORWG	Western Gangwon, Korea		O	O					
36	727	KORWG	Western Gangwon, Korea		O	O					
37	741	KORWG	Western Gangwon, Korea		O	O					
38	742	KORWG	Western Gangwon, Korea		O	O					
39	743	KORWG	Western Gangwon, Korea		O	O	MH209079	MH209115	MH209151	MH209187	
40	744	KORWG	Western Gangwon, Korea		O	O					
41	1056	KORWG	Western Gangwon, Korea	O	O	O					
42	1566	KORWG	Western Gangwon, Korea		O	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
43	2334	KORWG	Western Gangwon, Korea	O	O	O					
44	3550	KORWG	Western Gangwon, Korea		O	O					
45	3649	KORWG	Western Gangwon, Korea		O	O					
46	3854	KORWG	Western Gangwon, Korea		O	O					
47	3855	KORWG	Western Gangwon, Korea	O							
48	3857	KORWG	Western Gangwon, Korea		O	O					
49	4024	KORWG	Western Gangwon, Korea		O	O					
50	4025	KORWG	Western Gangwon, Korea		O	O					
51	4040	KORWG	Western Gangwon, Korea	O	O	O					
52	4838	KORWG	Western Gangwon, Korea		O	O					
53	7696	KORWG	Western Gangwon, Korea	O	O	O					
54	7697	KORWG	Western Gangwon, Korea		O	O					
55	7698	KORWG	Western Gangwon, Korea		O	O					
56	7699	KORWG	Western Gangwon, Korea		O	O					
57	7712	KORWG	Western Gangwon, Korea		O	O					
58	7713	KORWG	Western Gangwon, Korea		O	O					
59	7714	KORWG	Western Gangwon, Korea		O	O					
60	7715	KORWG	Western Gangwon, Korea		O	O					
61	1057	KORWG	Western Gangwon, Korea		O	O					
62	3189	KORWG	Western Gangwon, Korea		O	O					
63	14256	KOREG	Eastern Gangwon, Korea			O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
64	14257	KOREG	Eastern Gangwon, Korea			O					
65	14258	KOREG	Eastern Gangwon, Korea			O					
66	14259	KOREG	Eastern Gangwon, Korea	O		O					
67	14260	KOREG	Eastern Gangwon, Korea	O		O					
68	14261	KOREG	Eastern Gangwon, Korea	O		O					
69	14262	KOREG	Eastern Gangwon, Korea	O		O					
70	14263	KOREG	Eastern Gangwon, Korea			O					
71	14264	KOREG	Eastern Gangwon, Korea	O		O					
72	14265	KOREG	Eastern Gangwon, Korea	O		O					
73	14266	KOREG	Eastern Gangwon, Korea	O		O					
74	14267	KOREG	Eastern Gangwon, Korea	O		O					
75	14268	KOREG	Eastern Gangwon, Korea	O		O					
76	14269	KOREG	Eastern Gangwon, Korea	O		O					
77	14270	KOREG	Eastern Gangwon, Korea	O		O					
78	14271	KOREG	Eastern Gangwon, Korea	O		O					
79	14272	KOREG	Eastern Gangwon, Korea	O		O					
80	14273	KOREG	Eastern Gangwon, Korea	O		O					
81	14274	KOREG	Eastern Gangwon, Korea	O		O					
82	14275	KOREG	Eastern Gangwon, Korea	O		O					
83	14276	KOREG	Eastern Gangwon, Korea	O		O					
84	14277	KOREG	Eastern Gangwon, Korea			O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3			4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
85	14278	KOREG	Eastern Gangwon, Korea	O		O					
86	14279	KOREG	Eastern Gangwon, Korea	O		O					
87	14280	KOREG	Eastern Gangwon, Korea	O		O					
88	14281	KOREG	Eastern Gangwon, Korea	O		O					
89	14282	KOREG	Eastern Gangwon, Korea	O		O					
90	476	KORCC	Chungcheong, Korea	O		O	MH209080	MH209116	MH209152	MH209188	
91	1074	KORCC	Chungcheong, Korea	O		O					
92	1076	KORCC	Chungcheong, Korea	O		O					
93	3484	KORCC	Chungcheong, Korea	O		O					
94	4636	KORCC	Chungcheong, Korea	O		O	MH209081	MH209117	MH209153	MH209189	
95	6149	KORCC	Chungcheong, Korea	O		O					
96	6151	KORCC	Chungcheong, Korea			O					
97	6152	KORCC	Chungcheong, Korea	O		O					
98	6978	KORCC	Chungcheong, Korea	O		O					
99	7148	KORCC	Chungcheong, Korea	O		O					
100	9536	KORCC	Chungcheong, Korea	O		O					MH209223
101	13443	KORCC	Chungcheong, Korea	O							
102	13457	KORCC	Chungcheong, Korea	O		O					
103	13458	KORCC	Chungcheong, Korea	O		O					
104	13561	KORCC	Chungcheong, Korea			O					
105	13562	KORCC	Chungcheong, Korea			O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3			4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
106	13563	KORCC	Chungcheong, Korea	O		O					
107	13564	KORCC	Chungcheong, Korea			O					
108	13565	KORCC	Chungcheong, Korea	O		O					
109	1075	KORCC	Chungcheong, Korea	O		O					
110	14163	KORJB	Jeonbuk, Korea			O					
111	14164	KORJB	Jeonbuk, Korea	O		O					
112	14165	KORJB	Jeonbuk, Korea			O					
113	14166	KORJB	Jeonbuk, Korea			O					
114	14167	KORJB	Jeonbuk, Korea	O		O					
115	14168	KORJB	Jeonbuk, Korea	O		O					
116	14169	KORJB	Jeonbuk, Korea	O		O					
117	14170	KORJB	Jeonbuk, Korea			O					
118	14171	KORJB	Jeonbuk, Korea	O		O					
119	14172	KORJB	Jeonbuk, Korea	O		O	MH209082	MH209118	MH209154	MH209190	
120	14173	KORJB	Jeonbuk, Korea	O		O					
121	14174	KORJB	Jeonbuk, Korea	O		O					
122	14175	KORJB	Jeonbuk, Korea			O					
123	14176	KORJB	Jeonbuk, Korea	O		O					
124	14177	KORJB	Jeonbuk, Korea	O		O					
125	14178	KORJB	Jeonbuk, Korea			O					
126	14179	KORJB	Jeonbuk, Korea	O		O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3	4				
				Microsatellites	CHRNA1	TRSP	VTN	WT1	ZFY
127	14180	KORJB	Jeonbuk, Korea	O					
128	14181	KORJB	Jeonbuk, Korea	O					
129	14182	KORJB	Jeonbuk, Korea	O					
130	14183	KORJB	Jeonbuk, Korea	O					
131	14184	KORJB	Jeonbuk, Korea	O					
132	14185	KORJB	Jeonbuk, Korea	O					
133	14186	KORJB	Jeonbuk, Korea	O					
134	14187	KORJB	Jeonbuk, Korea	O					
135	14188	KORJB	Jeonbuk, Korea	O					
136	14189	KORJB	Jeonbuk, Korea	O					
137	14190	KORJB	Jeonbuk, Korea	O					
138	14191	KORJB	Jeonbuk, Korea	O					
139	179	KORJN	Jeonam, Korea	O	MH209083	MH209119	MH209155	MH209191	
140	1289	KORJN	Jeonam, Korea	O					
141	1330	KORJN	Jeonam, Korea	O					
142	1784	KORJN	Jeonam, Korea	O					
143	1913	KORJN	Jeonam, Korea	O					MH209224
144	1918	KORJN	Jeonam, Korea	O					
145	1945	KORJN	Jeonam, Korea	O					
146	2147	KORJN	Jeonam, Korea	O					
147	2202	KORJN	Jeonam, Korea	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3	4				
				Microsatellites	CHRNA1	TRSP	VTN	WT1	ZFY
148	2748	KORJN	Jeonam, Korea	O					
149	2757	KORJN	Jeonam, Korea	O					
150	2994	KORJN	Jeonam, Korea	O					
151	2995	KORJN	Jeonam, Korea	O					
152	3069	KORJN	Jeonam, Korea	O					
153	3226	KORJN	Jeonam, Korea	O					
154	3384	KORJN	Jeonam, Korea	O					
155	3413	KORJN	Jeonam, Korea	O					
156	3705	KORJN	Jeonam, Korea	O					
157	3709	KORJN	Jeonam, Korea	O					
158	3720	KORJN	Jeonam, Korea	O					
159	4196	KORJN	Jeonam, Korea	O O					
160	4197	KORJN	Jeonam, Korea	O O					
161	7003	KORJN	Jeonam, Korea	O O					
162	7006	KORJN	Jeonam, Korea	O O					
163	7012	KORJN	Jeonam, Korea	O O					
164	7014	KORJN	Jeonam, Korea	O O					
165	7016	KORJN	Jeonam, Korea	O O					
166	2744	KORJN	Jeonam, Korea	O O					
167	4554	KORJN	Jeonam, Korea	O O					
168	4555	KORJN	Jeonam, Korea	O O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3			4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
169	5297	KORJN	Jeonam, Korea	O		O					
170	679	KORGS	Gyeongsang, Korea			O	MH209084	MH209120	MH209156	MH209192	
171	3678	KORGS	Gyeongsang, Korea			O					
172	13912	KORGS	Gyeongsang, Korea			O					
173	13913	KORGS	Gyeongsang, Korea			O					
174	5278	KORGS	Gyeongsang, Korea			O					
175	6556	KORGS	Gyeongsang, Korea			O					
176	7075	KORGS	Gyeongsang, Korea			O					
177	8271	KORGS	Gyeongsang, Korea			O					
178	8272	KORGS	Gyeongsang, Korea			O					
179	8527	KORGS	Gyeongsang, Korea			O	MH209085	MH209121	MH209157	MH209193	MH209225
180	10249	KORGS	Gyeongsang, Korea			O					
181	10555	KORGS	Gyeongsang, Korea			O					
182	10559	KORGS	Gyeongsang, Korea			O					
183	10752	KORGS	Gyeongsang, Korea			O					
184	10753	KORGS	Gyeongsang, Korea			O					
185	10776	KORGS	Gyeongsang, Korea			O					
186	11010	KORGS	Gyeongsang, Korea			O					
187	11477	KORGS	Gyeongsang, Korea			O					
188	11481	KORGS	Gyeongsang, Korea			O					
189	11482	KORGS	Gyeongsang, Korea			O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
190	11468	KORGS	Gyeongsang, Korea			O					
191	14283	KORGS	Gyeongsang, Korea			O					
192	14284	KORGS	Gyeongsang, Korea			O					
193	14285	KORGS	Gyeongsang, Korea			O					
194	14286	KORGS	Gyeongsang, Korea			O					
195	14288	KORGS	Gyeongsang, Korea			O					
196	14289	KORGS	Gyeongsang, Korea			O					
197	14290	KORGS	Gyeongsang, Korea			O					
198	14291	KORGS	Gyeongsang, Korea			O					
199	14292	KORGS	Gyeongsang, Korea			O					
200	3180	CHN	China		O						
201	3181	CHN	North western, China		O		MH209086	MH209122	MH209158	MH209194	
202	3183	CHN	North eastern, China		O						
203	13756	CHN	North western, China		O						
204	13757	CHN	North western, China		O		MH209087	MH209123	MH209159	MH209195	
205	13758	CHN	North western, China		O		MH209088	MH209124	MH209160	MH209196	
206	13760	CHN	China		O						
207	13762	CHN	China		O						
208	13763	CHN	China		O		MH209089	MH209125	MH209161	MH209197	
209	13764	CHN	China		O		MH209090	MH209126	MH209162	MH209198	
210	13936	CHN	Heilongjiang, China		O						

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3	4				
				Microsatellites	CHRNA1	TRSP	VTN	WT1	ZFY
211	500	RUS	Primorsky, Russia	O					
212	507	RUS	Primorsky, Russia	O					
213	5220	RUS	Primorsky, Russia	O					MH209226
214	5221	RUS	Primorsky, Russia	O					
215	5222	RUS	Primorsky, Russia	O	MH209091	MH209127	MH209163	MH209199	
216	5223	RUS	Primorsky, Russia	O	MH209092	MH209128	MH209164	MH209200	
217	5225	RUS	Primorsky, Russia	O					MH209227
218	5226	RUS	Primorsky, Russia	O					
219	5227	RUS	Primorsky, Russia	O					
220	9853	RUS	Primorsky, Russia	O					
221	11047	RUS	Primorsky, Russia	O					
222	13584	RUS	Primorsky, Russia	O					
223	13585	RUS	Primorsky, Russia	O					
224	13586	RUS	Primorsky, Russia	O					
225	12409	FIN	Finland	O					MH209228
226	12410	FIN	Finland	O					MH209229
227	12411	FIN	Finland	O					
228	12412	FIN	Finland	O					MH209230
229	12413	FIN	Finland	O					
245	12414	FIN	Finland	O	MH209096	MH209132	MH209168	MH209204	

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3	4				
				Microsatellites	CHRNA1	TRSP	VTN	WT1	ZFY
246	12415	FIN	Finland	O	MH209097	MH209133	MH209169	MH209205	
247	12416	FIN	Finland	O					
248	12417	FIN	Finland	O	MH209098	MH209134	MH209170	MH209206	
249	12418	FIN	Finland	O					
250	12419	FIN	Finland	O	MH209099	MH209135	MH209171	MH209207	MH209231
251	12420	FIN	Finland	O					
252	12421	FIN	Finland	O					
253	12422	FIN	Finland	O					
254	12423	FIN	Finland	O					
255	12424	FIN	Finland	O					
256	12425	FIN	Finland	O					
257	12426	FIN	Finland	O					
258	12427	FIN	Finland	O					
259	12428	FIN	Finland	O					
260	11655	FIN	Finland	O					
261	11656	FIN	Finland	O					
262	11657	FIN	Finland	O					
263	11658	FIN	Finland	O	MH209100	MH209136	MH209172	MH209208	
264	11659	FIN	Finland	O					
265	11660	FIN	Finland	O					
266	11661	FIN	Finland	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
267	12519	VNM	Langson, Vietnam		O						MH209232
268	12520	VNM	Langson, Vietnam		O						MH209233
269	12521	VNM	Langson, Vietnam		O		MH209101	MH209137	MH209173	MH209209	MH209234
270	12522	VNM	Langson, Vietnam		O		MH209102	MH209138	MH209174	MH209210	
271	12523	VNM	Langson, Vietnam		O		MH209103	MH209139	MH209175	MH209211	
272	12524	VNM	Langson, Vietnam		O		MH209104	MH209140	MH209176	MH209212	MH209235
273	12525	VNM	Langson, Vietnam		O		MH209105	MH209141	MH209177	MH209213	
274	12526	VNM	Langson, Vietnam		O						MH209236
230	13753	VNM	Langson, Vietnam		O						
231	13754	VNM	Langson, Vietnam		O						
232	13755	VNM	Langson, Vietnam		O						
233	13935	VNM	Langson, Vietnam		O						
234	10367	JPNK	Kanagawa, Japan		O	O	MH209106	MH209142	MH209178	MH209214	
235	10368	JPNK	Kanagawa, Japan		O	O					
236	10370	JPNK	Kanagawa, Japan		O	O					
237	10371	JPNK	Kanagawa, Japan		O	O					
238	10372	JPNK	Kanagawa, Japan		O	O					
239	10373	JPNK	Kanagawa, Japan		O	O					
240	10374	JPNK	Kanagawa, Japan		O	O					
241	10378	JPNK	Kanagawa, Japan		O	O					
242	10379	JPNK	Kanagawa, Japan		O	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
243	10381	JPNK	Kanagawa, Japan		O	O					
244	10383	JPNK	Kanagawa, Japan		O	O					
275	10384	JPNK	Kanagawa, Japan		O	O					
276	10385	JPNK	Kanagawa, Japan		O	O					
277	10389	JPNK	Kanagawa, Japan		O	O					
278	10392	JPNK	Kanagawa, Japan		O	O					
279	10396	JPNK	Kanagawa, Japan		O	O					
280	10398	JPNK	Kanagawa, Japan		O	O					
281	10399	JPNK	Kanagawa, Japan		O	O					
282	10400	JPNK	Kanagawa, Japan		O	O					
283	10401	JPNK	Kanagawa, Japan		O	O					
284	10353	JPNK	Kanagawa, Japan		O	O	MH209107	MH209143	MH209179	MH209215	
285	10355	JPNK	Kanagawa, Japan		O	O					
286	10356	JPNK	Kanagawa, Japan		O	O					MH209237
287	10357	JPNK	Kanagawa, Japan		O	O					
288	10358	JPNK	Kanagawa, Japan		O	O					
289	10359	JPNK	Kanagawa, Japan		O	O					
290	10361	JPNK	Kanagawa, Japan		O	O					
291	10363	JPNK	Kanagawa, Japan		O	O					
292	10364	JPNK	Kanagawa, Japan		O	O					
293	10365	JPNK	Kanagawa, Japan		O	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3	4			
				Microsatellites	CHRNA1	TRSP	VTN	WT1	ZFY
294	14070	JPNW	Wakayama, Japan	O					
295	14071	JPNW	Wakayama, Japan	O					
296	14073	JPNW	Wakayama, Japan	O					
297	14075	JPNW	Wakayama, Japan	O					
298	14076	JPNW	Wakayama, Japan	O					
299	14077	JPNW	Wakayama, Japan	O					
300	14078	JPNW	Wakayama, Japan	O					
301	14080	JPNW	Wakayama, Japan	O					
302	14081	JPNW	Wakayama, Japan	O					
303	14082	JPNW	Wakayama, Japan	O					
304	14084	JPNW	Wakayama, Japan	O					
305	14085	JPNW	Wakayama, Japan	O					
306	14086	JPNW	Wakayama, Japan	O					
307	14087	JPNW	Wakayama, Japan	O	MH209108	MH209144	MH209180	MH209216	
308	14088	JPNW	Wakayama, Japan	O	MH209109	MH209145	MH209181	MH209217	
309	14094	JPNW	Wakayama, Japan	O					
310	14095	JPNW	Wakayama, Japan	O					
311	14098	JPNW	Wakayama, Japan	O					
312	14103	JPNW	Wakayama, Japan	O					
313	14116	JPNW	Wakayama, Japan	O					
314	14118	JPNW	Wakayama, Japan	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1 2 3	4			
				Microsatellites	CHRNA1	TRSP	VTN	WT1	ZFY
315	14127	JPNW	Wakayama, Japan	O					
316	14132	JPNW	Wakayama, Japan	O					
317	14142	JPNW	Wakayama, Japan	O					
318	14144	JPNW	Wakayama, Japan	O					
319	14153	JPNW	Wakayama, Japan	O					
320	14155	JPNW	Wakayama, Japan	O					
321	14157	JPNW	Wakayama, Japan	O					
322	14158	JPNW	Wakayama, Japan	O					
323	14159	JPNW	Wakayama, Japan	O					
324	13413	JPNS	Shikoku, Japan	O	MH209110	MH209146	MH209182	MH209218	
325	13414	JPNS	Shikoku, Japan	O					
326	13415	JPNS	Shikoku, Japan	O					
327	13416	JPNS	Shikoku, Japan	O					
328	13417	JPNS	Shikoku, Japan	O	MH209111	MH209147	MH209183	MH209219	
329	13419	JPNS	Shikoku, Japan	O					
330	13420	JPNS	Shikoku, Japan	O					
331	13421	JPNS	Shikoku, Japan	O					
332	13422	JPNS	Shikoku, Japan	O					
333	13423	JPNS	Shikoku, Japan	O					
334	13425	JPNS	Shikoku, Japan	O					
335	13426	JPNS	Shikoku, Japan	O					

Table1. Information of samples, genes and NCBI accession numbers in each chapters of this study.

No.	CGRB ID	Local Code	Locality	1	2	3	4				
				Microsatellites			CHRNA1	TRSP	VTN	WT1	ZFY
336	13427	JPNS	Shikoku, Japan			O					
337	13428	JPNS	Shikoku, Japan			O					
338	13429	JPNS	Shikoku, Japan			O					
339	13035	JPNH	Hokkaido, Japan		O	O	MH209112	MH209148	MH209184	MH209220	MH209238
340	13036	JPNH	Hokkaido, Japan		O	O	MH209113	MH209149	MH209185	MH209221	
341	13037	JPNH	Hokkaido, Japan		O	O					MH209239
342	13038	JPNH	Hokkaido, Japan		O	O					MH209240
343	13039	JPNH	Hokkaido, Japan		O	O					
344	13040	JPNH	Hokkaido, Japan		O	O					
345	13041	JPNH	Hokkaido, Japan		O	O					
346	13042	JPNH	Hokkaido, Japan		O	O					
347	13043	JPNH	Hokkaido, Japan		O	O					
348	13044	JPNH	Hokkaido, Japan		O	O					
349	13045	JPNH	Hokkaido, Japan		O	O					
350	13046	JPNH	Hokkaido, Japan		O	O					
351	13047	JPNH	Hokkaido, Japan		O	O					

Chapter 1. Population genetic study of raccoon dog (*Nyctereutes procyonoides*) in South Korea using newly developed 12 microsatellite markers

Introduction

The raccoon dog (*Nyctereutes procyonoides,* family Canidae) is endemic to East Asia, but was introduced to Europe in the early 20th century (Pitra et al., 2010). *N. procyonoides* generally inhabits forests, farmlands and even urban areas. *N. procyonoides* is classified into six subspecies (Ellerman and Morrison-Scott, 1951; Ward and Wurster-Hill, 1990): *N. p. ussuriensis* (Russia, northeastern China and Eurasia), *N. p. procyonides* (Vietnam and southern China), *N. p. albus* (Hokkaido in Japan), *N. p. viverrinuss* (Japan except Hokkaido), *N. p. orestes* (Yunnan in China), and *N. p. koreensis* (Korean Peninsula).

N. p. koreensis is an endemic subspecies and is abundant throughout South Korea. In Korea, reduction of its predators and competitors as well as high adaptability to diverse environments, have led to rapid raccoon dog population growth (Hyun et al., 2005).

Consequently, the raccoon dog has become a top predator in Korea despite its modest size. The role of predator in the ecosystem is very crucial, moderating prey densities and contributing to biodiversity (Estes et al., 2001; Ripple and Beschta, 2004; Roemer et al., 2009). In addition, this population increase has raised public health concerns due to the potential for zoonotic transfer of various contagious diseases (Botvinkin et al., 1981; Cherkasskiy, 1998; Hyun et al., 2005; Oh et al., 2012). In several cases, raccoon dogs have been the core species spreading rabies and canine distemper between domestic dog and wild Canidae species in Korea (Hyun et al., 2005; Han et al., 2010; Yang et al., 2011; Oh et al., 2012). After a long break, a rabies outbreak has been ongoing since 1993 in the provinces of Seoul/ Gyeonggi and Gangwon in northern Korea (Hyun et al., 2005; Yang et al., 2011; Oh et al., 2012). To prevent spreading rabies to the other parts of Korea and also control other contagious diseases, it is important to understand the population structure of the raccoon dog in Korea. Studies of genetic structure provide useful information on dispersal patterns or range expansion of the population (Gillespie, 1975). However, there are currently no data on the structure or demographical trends of *N. p. koreensis* (Kahula and Saeki, 2004). To investigate genetic diversity and population structure, we analyzed raccoon dog populations in South

Korea using polymorphic genetic markers.

Microsatellite markers have been widely used to study individual identification, phylogeographic relationships, and genetic variation, as well as population structuring and genetic differentiation of various wildlife species (Beaumont and Bruford, 1998; Polziehn et al., 2000; Hu et al., 2007; Lee et al., 2011; Park et al., 2011). Although plentiful ecological studies and some phylogenetic research with mitochondrial DNA on raccoon dog have been conducted, there are no previous data on the population structure using microsatellite markers (Kahula and Saeki, 2004). Moreover, canine-derived microsatellite markers applied in raccoon dog (*N. p. procyonides*) (Rogalska-Niznik et al., 2003; Szczerbal et al., 2003) were not profitable to apply to our raccoon dog samples as some markers failed for amplification or the other amplified products were monomorphic. In this study, we report the isolation and characterization of 12 novel polymorphic loci from *N. p. koreensis*, and their use in determining the genetic structure of five raccoon dog populations in South Korea.

Materials and Methods

Microsatellite marker development

Twelve raccoon dog microsatellite loci were isolated and characterized using microsatellite biotin-enrichment methods (Ronald et al., 2000; Kim and Sappington, 2004). Genomic DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA). Extracted genomic DNA was digested with *Nde*II and fragments in the size range of 250-700 bp were isolated by electrophoresis on a 1.2 % TBE agarose gel. Products were purified using a DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA, USA).

*Nde*II linkers (1 μg) EP-1 (5'-CCCCCACCTCCTGCCCATCA TAAAAAATC-3') and EP-2 (5'-GATCGATTTTTTATGATGGG CAGGAGGTGGGGG- 3', 5'-phosphorylated, for *Nde*II), described in Ronald et al., (2000), were ligated to the purified DNA fragments using 20 μL T4 DNA ligase (Promega, Fitchburg, WI, USA). These attached linkers provide the priming site EP-3 (5'-CCCCCACCTCCTGCCATCAT-3') for the initial polymerase chain reaction (PCR) amplification. Reaction mixtures contained the following: 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 1.6 μM

EP-3 primer, 1.5 U i-Star *Taq* DNA polymerase (iNtRON Inc, Seoul, Korea), and 20 ng genomic DNA template in a 30 μL final volume. PCR was performed with an initial denaturation of 2 min at 94 ℃ followed by 30 cycles of 1 min at 65 ℃, 30 sec at 72 ℃, 2 min at 72 ℃, and a final extension of 5 min at 72 ℃. Unattached residual linkers of <100 bp were removed from the reactions using Microcon 100 filters (Millipore Corporation, Billerica, MA, USA).

Aliquots (1 μL) of biotinylated capture probe (5' biotin $(CA)_{15}$, 5' biotin $(CT)_{15}$, and 5' biotin $(AGC)_7$) were annealed to 10 μL volumes of linker-ligated DNA in 89 μL of 5× SSC. Reactions were then heated at 95 ℃ for 10 min, cooled on ice for 30 sec, then incubated for 5 min at room temperature. One hundred microliters of washed magnetic beads (1 mg/mL) were added to the DNA and the reactions were incubated for 15 min at room temperature to attract biotinylated probes. Residual unattached fragments were removed by washing three times with 200 μL 2 × SSC at room temperature, and three times with 200 μL of 1 × SSC at optimized temperature for 3 min (65 ℃ for $(CA)_{15}$, 61 ℃ for $(CT)_{15}$, and 67 ℃ for $(AGC)_7$). The DNA was eluted from the beads into 50 μL of water by incubation for 5 min at 95 ℃. The DNA containing repeat sequences was amplified with the EP-3 single primer. The PCR products were ligated to pGEM-T Vector (50 ng/μL) and

transformed into *Escherichia coli* JM109 competent cells (Promega, USA). Positive clones were identified by screening with M13 forward and reverse primers using the method described in Schuelke (2000). The presumptive microsatellite marker amplification was conducted in 10 μL reaction volumes containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM each primer (M13 forward, M13 reverse, each internal repeat primer; $(CA)_{12}$, $(CT)_{12}$, $(AGC)_6$), 0.25 U i-Star *Taq* DNA polymerase (iNtRON Inc), and 10-50 ng of template. The PCR was performed under the following conditions: initial denaturation for 5 min at 96 ℃ followed by 35 cycles of 96 ℃ for 1 min, 50 ℃ for 1 min, and 72 ℃ for 1 min; a final extension at 72 ℃ for 1 min was performed. Subsequent agarose gel electrophoresis enabled selection of repeated sequence inserts, seen as a smeared band pattern, which were sequenced in both directions using M13 primers. Primers for microsatellite amplification were designed using Primer 3 (Rozen and Skaletsky, 2000).

DNA samples and genotyping

We analyzed tissue samples from 104 raccoon dogs from five provinces of South Korea (Seoul/Gyeonggi, n= 22; Gangwon, n= 22; Chungcheong, n=16; Gyeongsang, n=22; Jeolla, n= 22, Fig. 1),

collected by the Conservation Genome Resource Bank for Korean Wildlife (CGRB) associated with rescue centers throughout the South Korea. Samples of each region were collected from scattered points in a region. All genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen). DNA was amplified with specifically designed genotyping primers (Table 2) using a touchdown profile for PCR amplification: initial denaturation for 3 min at 94 ℃, followed by 20 cycles of 94 ℃ for 1 min, annealing temperature of 60−50 ℃ decreased by 0.5 ℃ per cycle, 72 ℃ for 1 min, followed by 20 cycles of 94 ℃ for 1 min, 50 ℃ for 1 min, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. The PCR reaction mixture contained 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 U i−Star *Taq* DNA polymerase (iNtRON Inc), 0.3 μM each of the fluorescently labeled forward primer and unlabeled reverse primer, and 10–50 ng template DNA. Alleles were genotyped using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using a GENESCAN−500 (Rox) size standard, and analyzed with GeneMarker version 1.9 software (SoftGenetics, State College, PA, USA

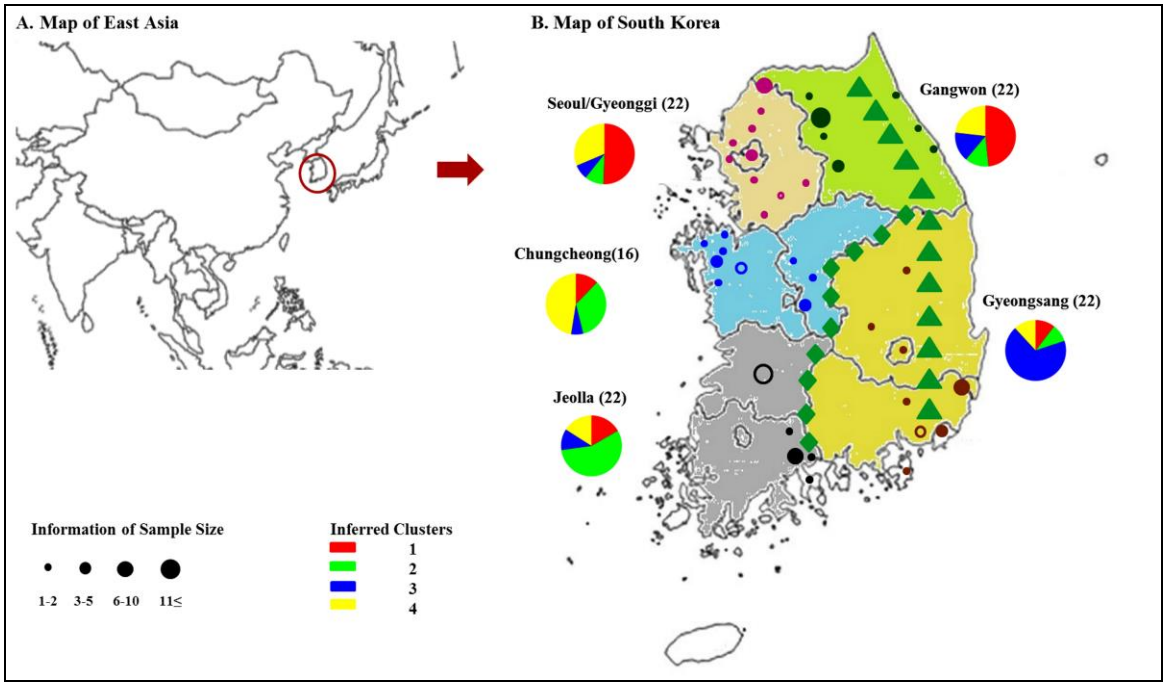

Figure 1. Map of study area and sampling information. A. Map of East Asia, study area is circled; B. map of South Korea, each colored area indicates each population region and each field circle shows each sampling locality. Individuals without specific local information were described with empty circle and pie charts to show proportion of inferred clusters within population. Green triangles: The Taebaek Mountains; Green diamonds: The Sobaek Mountains.

Table 2. Characteristics of the 12 microsatellite loci developed and optimized for *Nyctereutes procyonoides* (Representative Seoul/Gyeonggi population, n=22). Locus name, primer sequences, repeat motif, size range of PCR products, GenBank accession numbers, polymorphism, information of null alleles and neutrality are reported.

Locus	Primer sequence	Fluorescent label (5')	Repeat Motif	Size (bp)	Accession no.	Polymorphism[a]	Null alleles[b]		Selective neutrality[c]	
							Presence	Brookfield frequency	Neutrality	Slatkin's *p* value
Nyct1	F:CTGCTCCAACACCACCATTT R:CCAATTGCGTAAGTCCCAGT	6FAM	$GT_{(13)}$	183-193	JQ663848	Moderate	No	0.023	Yes	0.085
Nyct2	F:ATCTCCCATCCCTATGGTCC R:GAAAAGATTTCAGGTGAACTATCC	6HEX	$CA_{(16)}$	207-223	JQ663849	Moderate	Yes	0.108	Yes	0.277
Nyct3	F:TGGACAAGGTCACACAGGAA R:ACCCTCCAAGTGTTCACGAC	6HEX	$TG_{(17)}$	240-252	JQ663850	Moderate	No	0	Yes	0.362
Nyct4	F:TGCTTCTGTCTCCCCTGTCT R:AGTTCAGCCGGGTTGTAATG	6HEX	$TG_{(14)}$	223-239	JQ663851	Moderate	No	0.003	Yes	0.086
Nyct5	F:CAGGGTTTTGAGGTGGAGAG R:CACAGTGCGTTAGGCATGA	6FAM	$GT_{(13)}$	164-178	JQ663852	Moderate	No	0	Yes	0.759
Nyct6	F:GATCCAGCTGTCACTGCTTT R:GTCTGCTTCTCCCTCTCCCT	6FAM	$GA_{(18)}$	141-171	JQ663853	High	No	0.054	Yes	0.078
Nyct7	F:CTAGCCTCCCCCTACCTTTC R:AACACGAGGTTCACTCCAGG	6FAM	$CT_{(17)}$	121-139	JQ663854	Moderate	No	0.086	Yes	0.545
Nyct8	F:CTGCTACTCCTCCTGCCTGT R:CATTGGAGGCTGTCAGTGAA	6FAM	$TC_{(18)}$	111-127	JQ663855	Moderate	No	0.066	Yes	0.688
Nyct9	F:CCCTCAATGGTCTTATCCCC R:ACGACCCCTTCATCTGACTG	6FAM	$CTG_{(10)}$	168-192	JQ663856	Moderate	No	0	Yes	0.263

Locus	Primer sequence	Fluorescent label (5')	Repeat Motif	Size (bp)	Accession no.	Polymorphism[a]	Null alleles[b]		Selective neutrality[c]	
							Presence	Brookfield frequency	Neutrality	Slatkin's *p* value
Nyct10	F:CTTGCTGCAAATCTCCCATT R:CAAGGAGAGGAGCTGTTTGC	6HEX	$GCT_{(8)}$	178-187	JQ663857	Moderate	No	0.055	Yes	0.538
Nyct11	F:CCAGTCATCCTGCCTTTGTT R:GTGCCCTTGTGGGTTTCTTA	6FAM	$TGC_{(8)}$	104-119	JQ663858	Moderate	No	0	Yes	0.763
Nyct12	F:TAAAGCTCCACAGGGGTCTG R:TCATTCCACCATTCTTCTACCA	6FAM	$GCA_{(8)}$	162-171	JQ663859	Moderate	No	0.0033	Yes	0.323

[a] Based on the expected heterozygosity ($H_{E,}$ shown in Table 3); 0 < low < 0.4, 0.4 ≤ moderate < 0.8, 0.8 ≤ high < 0.9

[b] Presence of null allele discovered based on Brookfield frequency

[c] 'Yes' denotes the null hypothesis of selective neutrality against the presence of selection was not rejected for that locus at P=0.05, and 'No' denotes the null allele hypothesis of neutrality was rejected for that locus

Data analysis

Tests for deviation from Hardy–Weinberg equilibrium (HWE) and linkage equilibrium between loci were conducted with GENEPOP version 4.0 (Raymond and Rousset, 1995). A Bonferroni correction was applied for significance levels for multiple tests (Rice, 1989). The number of alleles (A) and the probability (P) that the difference between expected (H_E) and observed (H_O) heterozygosities was due to chance were obtained using CERVUS version 3.0 (Kalinowski et al., 2007). Allelic diversity was calculated using GenAlEx v6.1 (Peakall and Smouse, 2006) to characterize the polymorphism of each marker. The plausible occurrence of null alleles was tested using Brookfield method in MICROCHECKER (Oosterhout et al., 2004), and Arlequin 3.11 (Excoffier et al., 2005) was employed to test selective neutrality of each locus using the Ewens–Watterson–Slatkin exact test of allele frequency distribution (Slatkin, 1996).

To analyze population structure, FSTAT 2.9.3.2 (Goudet, 1995) was used to calculate pairwise F_{ST} (Weir and Cockerham, 1984). We then investigated genetic structure using the Bayesian clustering function of STRUCTURE 2.2 (Pritchard et al., 2000). Assuming that the data were represented by K separate clusters,

the log posterior probability of the data for a given K, ln Pr(X/K), was generated for each of the 20 STRUCTURE runs at K values of 1–8 for the raccoon dog sample locations. The initial burn−in period was 10,000 followed by 10,000 replications after burn−in (Evanno et al., 2005). Admixture and allele frequency correlated models were chosen for the analysis and ΔK, index which is based on the rate of change in the ln likelihood of the data between successive K (1−8), was also calculated to estimate K (Evanno et al., 2005). The membership coefficients were estimated from 100 replicate runs at K=4 with permutation analysis using CLUMPP version 1.1.2 (Jakobsson and Resenberg, 2007) and the output of genetic clustering was visualized using software DISTRUCT version 1.1 (Rosenberg, 2004).

Results

We screened a total of 384 colonies that were presumed to contain repeat units, and of these, 301 were sequenced using the M13 forward primer. Eighty-two unique sequences (24 of CA repeat, 39 of CT repeat, and 19 of AGC repeat) with more than eight repeats for each sequence unit were selected and used to design primers for PCR amplification. Using these primers, 13 of CA repeat, 14 of CT repeat, and 15 of AGC repeat targeted primers resulted in positive PCR amplification from four raccoon dog DNA samples. Forward primers from each primer set were labeled with two different fluorescent dyes, 6-FAM and 6-HEX, for multiplex PCR applications. Finally, 12 polymorphic and consistently discernible microsatellite markers for Korean raccoon dog populations were obtained.

Characterization of each marker for the 22 Seoul/Gyeonggi raccoon dog samples is presented in Table 2. No significant linkage disequilibrium was found among any of the loci pairs (not shown) and all 12 loci did not show deviation from HWE (Adjusted α=0.004, Table 3). Although MICROCHECKER showed that *Nyct 2* revealed evidence of the presence of null alleles, the Brookfield

frequency indicates the heterozygote deficiency is very low (0.108), so we conclude *Nyct 2* has adequate resolution to serve as a marker as well (Dakin and Avise, 2004). We applied these 12 developed markers to other four populations of South Korea and analyzed genetic structure of them as well. A total of 104 alleles were observed in 104 raccoon dog samples from five provinces for the 12 polymorphic microsatellite markers, with 77 alleles from Seoul/Gyeonggi samples, 64 alleles observed in samples from Gangwon, 73 alleles from Chungcheong samples, 65 alleles from Gyeongsang samples and 66 alleles from Jeolla samples. Descriptive statistics of raccoon dog samples from the five regions are shown in Table 3. All 12 microsatellite markers were polymorphic at all locations, with the number of alleles per locus ranging from 2–11 (mean=8.7). The mean value of expected and observed heterozygosities were 0.723 (0.333–0.864) and 0.619 (0.227–0.733), respectively. Some loci were not in HWE after Bonferroni correction (*Nyct 6* in Gangwon, *Nyct 3* and *5* in Chungcheong, *Nyct 10* in Gyeongsang), however, we confirmed that exclusion of theses markers did not affect the result of STURCTURE analysis.

Table 3. Descriptive statistics for 12 microsatellite loci from samples of Korean raccoon dogs across five locations: Seoul/Gyeonggi, Gangwon, Chungcheong, Gyeongsang and Jeolla provinces.

		Nyct1	*Nyct2*	*Nyct3*	*Nyct4*	*Nyct5*	*Nyct6*	*Nyct7*	*Nyct8*	*Nyct9*	*Nyct10*	*Nyct11*	*Nyct12*	**Mean**
Seoul/ Gyeonggi	***N***	22	22	22	22	22	22	22	22	22	22	22	22	22
	A	7	6	7	6	8	9	8	8	6	3	5	4	6.4
	H_E	0.786	0.646	0.672	0.796	0.76	0.845	0.758	0.72	0.724	0.502	0.556	0.563	0.694
	H_O	0.727	0.455	0.773	0.773	0.818	0.727	0.591	0.591	0.727	0.409	0.591	0.545	0.644
	P	0.138	0.107	0.662	0.885	0.915	0.12	0.105	0.292	0.571	0.612	0.83	0.007	-
Gangwon	***N***	22	21	22	19	21	21	22	22	21	22	18	21	21
	A	5	6	7	8	5	5	7	6	5	2	4	4	5.3
	H_E	0.679	0.692	0.655	0.831	0.609	0.721	0.803	0.699	0.758	0.507	0.643	0.617	0.685
	H_O	0.682	0.714	0.773	0.842	0.714	0.429	0.909	0.636	0.619	0.545	0.667	0.667	0.683
	P	0.992	0.524	0.035	0.117	0.485	0.002*	0.478	0.347	0.231	1	0.589	0.372	-
Chungcheong	***N***	16	13	16	16	16	14	15	14	15	16	16	16	15.3
	A	8	6	8	5	8	7	9	7	6	2	5	2	6.1
	H_E	0.829	0.815	0.752	0.728	0.835	0.828	0.864	0.693	0.816	0.387	0.677	0.516	0.728
	H_O	0.813	0.538	0.375	0.688	0.25	0.786	0.733	0.571	0.667	0.25	0.625	0.25	0.546
	P	0.123	0.061	0.001*	0.341	0.000*	0.3	0.093	0.037	0.026	0.2	0.622	0.054	-
Gyeongsang	***N***	22	22	22	22	22	22	22	22	22	22	22	22	22
	A	7	6	5	5	4	7	8	6	6	3	4	4	5.4
	H_E	0.733	0.747	0.576	0.681	0.757	0.666	0.801	0.738	0.759	0.571	0.747	0.597	0.698
	H_O	0.591	0.545	0.682	0.455	0.682	0.455	0.818	0.636	0.727	0.227	0.636	0.591	0.587

		Nyct1	*Nyct2*	*Nyct3*	*Nyct4*	*Nyct5*	*Nyct6*	*Nyct7*	*Nyct8*	*Nyct9*	*Nyct10*	*Nyct11*	*Nyct12*	**Mean**
	P	0.204	0.01	0.775	0.053	0.7	0.129	0.233	0.055	0.935	0.000*	0.171	1	-
	N	22	22	22	22	22	22	22	22	22	22	22	22	22
	A	6	6	5	6	6	7	9	6	7	2	4	2	5.5
Jeolla	H_E	0.702	0.624	0.739	0.76	0.815	0.765	0.775	0.541	0.817	0.426	0.707	0.333	0.667
	H_O	0.682	0.455	0.818	0.545	0.864	0.773	0.773	0.545	0.591	0.409	0.727	0.227	0.617
	P	0.728	0.022	0.434	0.027	0.769	0.77	0.448	1	0.049	1	0.204	0.179	-
	N	104	100	104	101	103	101	103	102	102	104	100	103	102.3
	A	10	9	13	8	10	10	12	9	9	4	6	4	8.7
Total	H_E	0.783	0.717	0.689	0.776	0.787	0.807	0.804	0.739	0.793	0.515	0.696	0.574	0.723
	H_O	0.692	0.54	0.702	0.653	0.689	0.624	0.767	0.598	0.667	0.375	0.65	0.466	0.619
	P	0.005	0.000*	0.000*	0.008	0.000*	0.000*	0.001*	0.004	0.002*	0.002*	0.426	0.011	-

*Significant deviation from Hardy−Weinberg equilibrium at $\alpha = 0.004$

N : number of samples; A : number of allele; H_E : expected heterozygosity; H_O : observed heterozygosity; P : P−value for Hardy−Weinberg Equilibrium

According to the STRUCTURE analysis, the ΔK value was the highest when K was set at 4, implying that raccoon dog species in the five areas in Korea consist of four distinct populations: 1. Seoul/Gyeonggi and Gangwon, 2. Chungcheong, 3. Gyeongsang and 4. Jeolla (Fig. 2).

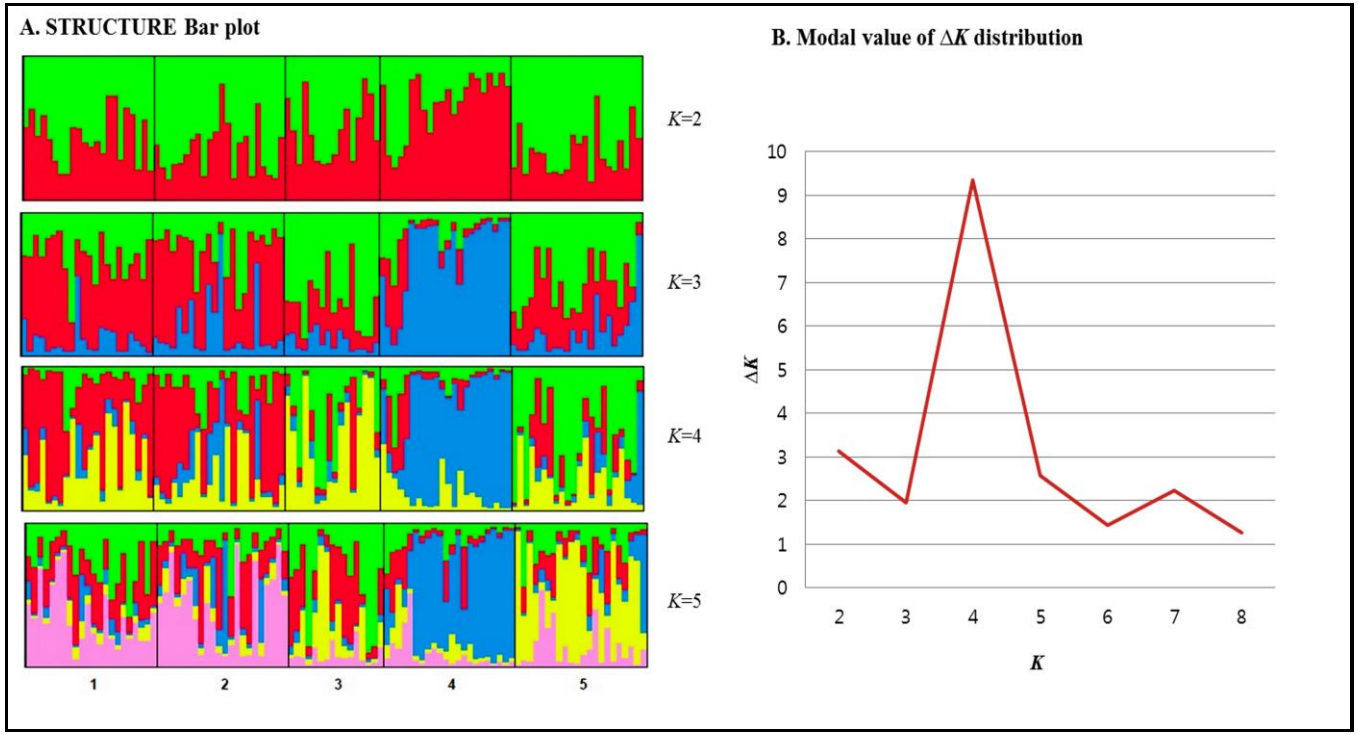

Figure 2. Bar plot of genetic proportion of Korean raccoon dog populations from five regions identified by STRUCTURE analysis (K=4). 1: Seoul/Gyeonggi, 2: Gangwon, 3: Chungcheong, 4: Gyeongsang, 5: Jeolla. The modal value of ΔK distribution is highest at K=4.

Pie chart showing proportion of the STRUCTURE clusters (Fig. 1) and F_{ST} also showed that the raccoon dogs collected from the five locations represent four groups. Furthermore, Nei's genetic distance between locations suggested significant genetic differences among populations (Table 4). According to the analysis, only Seoul/Gyeonggi and Gangwon populations showed a relatively close relationship. The highest level of genetic differentiation was revealed in the pairs involving Chungcheong and this population was significantly differentiated from all the other populations with similar level of F_{ST} value (0.034−0.054). There was higher differentiation between 1. Seoul/ Gyeonggi and Gyeongsang, 2. Seoul/Gyeonggi and Jeolla, and 3. Gyeongsang and Jeolla.

Table 4. Pairwise F_{ST} (below diagonal) and Nei's D_A genetic distance (above diagonal) between Korean raccoon dogs sampled from five locations.

Population	Seoul/Gyeonggi	Gangwon	Chungcheong	Gyeongsang	Jeolla
Seoul/Gyeonggi		0.109	0.229	0.254	0.227
Gangwon	0. 024		0.162	0.170	0.155
Chungcheong	0.054*	0.034*		0.202	0.191
Gyeongsang	0.0712*	0.046*	0.043*		0.226
Jeolla	0.0701*	0.046*	0.047*	0.069*	

*Significant after pairwise F_{ST} test, with pairwise comparisons with $p < 0.005$.

Discussion

Twelve novel microsatellite markers were developed in this study to investigate the genetic structure and diversity of Korean raccoon dogs from five sample locations. A F_{ST}, Nei's genetic distance, and structure analysis implied that Seoul/Gyeonggi (north-western) and Gangwon (north-eastern) populations share genetic characters enough to suggest migration between them, resulting in similar genetic profiles (Fig. 1). However, both populations showed significant differentiation from Chungcheong (mid), Jeolla (south-western) and Gyeongsang (mid and south-estern) populations. Moreover, Seoul/Gyeonggi showed higher differentiation from southern populations, Gyeongsang and Jeolla. Geographical distance is thought to be the main reason for this differentiation. Chungcheong population is located in the center of Korea and moderately differentiated from all the other populations, and its central location could lead to higher genetic diversity than in the other locations. Although, Jeolla and Gyeongsang populations are located next to each other, they showed little evidence of gene exchange with high F_{ST} and distance values (Table 4). The Sobaek Mountains can explain this big difference between two populations.

The Sobaek Mountains split off from the Taebaek Mountains along the boundary between Gangwon and Gyeongsang, trending southwest across the center of the Korean Peninsula to Mt. Jiri, the highest peak (over 2,000m) in the range (Fig. 1). They mark the geographical border between the Jeolla and Gyeongsang regions (Kim et al., 2009). Meanwhile, the Sobaek Mountains separate Gyeongsang from the Chungcheong and Gangwon areas as well. However, the lower altitude of the mountain range (under 1,500m) and several man-made roads for trading (Park et al., 2003) might be the main causes preventing more pronounced differentiation between Gyeonsang and Chungcheong /Gangwon. This theory is in accord with Figure 2. Genetic composition of first four Gyeongsang individuals (Pop 4) from northern part of Gyeongsang is more similar to Chungcheong/Gangwon.

We found five individuals, two from Gangwon and three from Jeolla, respectively, whose origins were questionable (Fig. 2). Over 50% of genetic composition of two Gangwon and one Jeolla individuals was of Gyeongsang origin. According to the collection record, the sampling location of the Jeolla individual is close to the border of Gyeongsang province. Therefore, this individual might have migrated from Gyeongsang area. However, in the case of the

two Gangwon individuals, unless they were transported by humans, it is hard to explain what affected their genetic profiles so greatly, and more intensive study will be necessary. The other two Jeolla individuals also showed over 50% of northern part (Seoul/ Gyeonggi and Gangwon) origin. They are sure from northern part of Jeolla though, specific local information is lacking. Then, we can assume they might come from Seoul/ Gyeonggi or Gangwon province by transportation. Or southern part of Chungcheong near the Jeolla may be the origin of them. Since, Chungcheong has more admixed genetic composition as we mentioned earlier in Figure 1.

Because our data indicate Korean raccoon dogs represent four distinct groups, we should take this into consideration when formulating plans to manage this species. Preventing the spread of rabies is a case in point. Up to now, the outbreak of rabies has been limited to Seoul/ Gyeonggi and Gangwon, and our data confirm that raccoon dogs from these areas essentially represent a single population. Moreover, we could suspect that shared genetic structure facilitated transfer of rabies within this population of raccoon dogs.

Therefore, we suggest that Seoul/Gyeonggi and Gangwon should be treated as a single management unit. Likewise, the other

three populations should be treated as three different management units. Finally, to protect the other three populations from the spread of rabies for the sake of public health, genetic information and structure of the raccoon dog populations should be considered.

The microsatellite markers developed in this study are firstly described specifically for *N. procyonoides koreensis*. Even though a null allele was presented in *Nyct 2*, we used it for the analysis because a very low value of Brookfield frequency of heterozygote deficiency (<0.2, Table 2) causes an unnecessary exclusion for analysis (Dakin and Avise, 2004). Finally, all 12 microsatellite markers can be successfully applied to population genetics studies. We confirmed their utility for application in other raccoon dog subspecies (data not shown). To develop conservation management strategies and to understand the evolutionary history of *N. procyonoides*, studies of phylogenetic relationships, genetic diversity and population genetics using individuals from more geographical locations are required. The markers developed here should facilitate future studies of population genetics of raccoon dog species in Korea, as well as populations in other areas. The genetic information gathered in these studies will contribute to management strategies for Korean raccoon dog species.

Chapter 2. Genetic diversity and population structure of East Asian raccoon dog (*Nyctereutes procyonoides*): genetic features in central and marginal populations

Introduction

The raccoon dog (*Nyctereutes procyonoides*) is endemic to East Asia, but it was introduced in Europe during the early 20th century (Helle and Kauhala 1991; Pitra et al., 2010). It is classified into six subspecies (Ellerman and Morrison-Scott, 1951; Ward and Wurster-Hill, 1990; Kauhala and Saeki, 2016): *N. p. ussuriensis* (Russia, northeastern China and Europe), *N. p. koreensis* (Korean Peninsula), *N. p. procyonides* (Vietnam and southern China), *N. p. orestes* (Yunnan in China), *N. p. viverrinus* (Japan except Hokkaido), and *N. p. albus* (Hokkaido). This species easily adapts to different climates and habitats, including forests, wetlands, damp meadows, agricultural land, and urban areas (Kauhala and Saeki, 2004; Pitra et al., 2010; Kauhala and Kowalczyk, 2011). Raccoon

dogs have a high reproductive rate (Helle and Kauhala 1995; Kauhala and Kowalczyk, 2011) and, after being introduced in several European countries, rapidly expanded their range and increased their populations (Kauhala and Kowalczyk, 2011, Sutor et al., 2014). Raccoon dogs are true omnivores (Sutor et al., 2010; Kauhala and Kowalczyk, 2011) that play an important role in maintaining the food web balance in ecosystems (Ågren et al., 2012). They are also vectors for numerous contagious and zoonotic diseases and parasites (Botvinkin et al., 1981; Cherkasskiy, 1998; Hyun et al., 2005; Kauhala and Kowalczyk, 2011; Oh et al., 2012; Sutor et al., 2014). There is public interest on raccoon dog populations due to their influence on ecosystems and to their role in the spread of diseases in both native and the habitats into which they have been introduced.

The geographic distribution range of native raccoon dog populations is rather restricted compared with other Canidae species such as the red fox (*Vulpes vulpes*) and gray wolf (*Canis lupus*). Red foxes and gray wolves are widely distributed in Eurasia and North America, covering almost the entire northern hemisphere, whereas the native habitat of raccoon dogs is Eastern Asia. This relatively restricted distribution range suggests that it might be

restricted by environmental factors. Adverse weather conditions such as very low or high temperature and humidity may be important factors limiting the expansion of raccoon dogs north, west, and south of East Asia. A comparatively smaller home range of raccoon dogs compared to that of other Canidae species might also contribute to their restricted distribution. For example, while the home range of raccoon dogs is reported to be up to 9.5 km^2, that of red fox is up to 358 km^2 and that of grey wolf is 460.5 km^2 (Kim et al., 2008; Karamanlidis et al., 2017; Walton et al., 2017). Generally, extreme cold climate and thick snow cover determine the northern range limit for raccoon dogs (mean annual temperature below 0 ℃ and snow depth > 35 cm) (Kauhala and Kowalczyk, 2011), and high altitude and mountains limit its dispersal (Nowak, 1993; Hong et al., unpublished data).

Chromosomal (Wada and Imai, 1991; Wada et al., 1991), morphological (Kauhala et al., 1998; Kim et al., 2015), and phylogenetic studies based on mitochondrial DNA (mtDNA) (Pitra et al., 2010, Kim et al., 2013; Paulauskas et al., 2016) have been conducted for the raccoon dog. Wada and Imai (1991) and Wada et al. (1991) reported that a different number of chromosomes for mainland Asia/eastern Europe populations (2n=54) and Japanese

subspecies (Hokkaido and Honshu_Kyushu, 2n=38). Kauhala et al. (1998) observed that the skull size of Finnish raccoon dogs was larger than that of Japanese raccoon dogs, and Kim et al. (2015) reported that Japanese raccoon dogs have relatively smaller skulls, mandibles, and carnassial teeth than mainland (Russia, China, and South Korea) individuals. A phylogenetic study using mtDNA (Kim et al., 2013) grouped South Korean, Chinese, Vietnamese, and Russian raccoon dogs into a single clade (the continental group), which was separated from the Japanese clade (the island group). In Europe, two haplogroups were defined; one clustered with the original continental group. This high genetic divergence of mtDNA within introduced populations might have resulted from multiple introductions from different locations (Pitra et al., 2010; Paulauskas et al., 2016). Recently, the population genetic structure of the raccoon dog in Europe has been investigated using microsatellite markers. Griciuvienė et al. (2016) reported that high gene flow among four raccoon dog subpopulations contributed to the lack of population structure in Lithuania. Drygala et al. (2016) identified three genetic clusters (Finland, Central Europe from western Russian to Germany, and Denmark) in Europe. Hong et al. (2013) developed microsatellite markers for South Korean raccoon dogs

and identified three genetic groups in South Korea, and management units for the species were proposed based on the results (Hong et al., unpublished data).

Although various studies have considered the genetic structure of the raccoon dog, most focused on populations at a regional scale and thus a population genetic study spanning the broad geographic range of the species is still lacking. Understanding the degree of population subdivision and genetic differentiation within a species can inform on decisions to conserve the genetic diversity or manage/control the species (Gibbs et al., 2008). Moreover, knowledge regarding the dispersal and movement of individuals is needed to understand the epidemiology of zoonotic and animal diseases and pathogens (Cohen et al., 2013; Mullins et al., 2014). Microsatellite markers have been widely employed to study aspects of population genetics, such as genetic variation, gene flow, population structuring, and genetic differentiation of various wildlife species (Beaumont and Bruford, 1998; Polziehn et al., 2000; Hu et al., 2007; Lee et al., 2011; Park et al., 2011; Choi et al., 2014; Lee et al., 2015). In the present study, the genetic diversity and geographic structure of raccoon dog populations within six countries was examined using 16 microsatellite loci.

Patterns of genetic diversity were compared among populations, and the natural geographic features, as well as artificial factors, leading to the differentiation of raccoon dog populations were examined. The patterns and magnitudes of potential genetic differentiation among East Asian raccoon dog populations was examined, particularly regarding the central–marginal trends in the genetic diversity of the raccoon dog populations. 1) We compared continental (South Korea, China, Russia, and Vietnam; the Finnish population was excluded because it was artificially introduced) vs. island (Japan) populations. We expected lower genetic variation in the isolated island populations than in their central counterparts due to genetic drift and bottlenecks (Eckert et al., 2008; Guo, 2012). 2) Within continental populations, we compared central (China and Russia) vs. marginal (South Korea and Vietnam) populations. Central populations are larger, more primitive, and more stable than marginal ones (Guo, 2014). 3) Within continental populations, we compared original (Russia) vs. introduced (Finland) populations. We hypothesized that introduced populations had lower levels of genetic diversity than their original source population, unless there was recurrent gene influx from their source population or multiple introductions (Schrey et al., 2011; Guo, 2012). 4) Finally, we

examined the effect of geographic barrier to gene flow between raccoon dog populations on two isolated islands in Japan, Honshu and Hokkaido. These populations are classified as different subspecies.

Materials and Methods

DNA samples and genotyping

Genetic samples from 137 raccoon dogs from seven provinces in six countries were obtained from the Conservation Genome Resource Bank for Korean Wildlife. We complied with the guidelines of the Seoul National University Institutional Animal Care and Use Committee regarding animal samples. Details on sampling sites and sample sizes are shown in Figure 3 and Table 5. There was little information was available regarding the origin of 11 samples from China, except that four were from the northwestern region and two from the northeastern region. The five samples for which locality information was lacking. These samples were combined into one population, as their genetic composition was quite homogenous. Samples from island populations were collected in Honshu and Hokkaido. The Hokkaido raccoon dog has been classified into a different subspecies from the raccoon dogs living in other parts of Japan (*N. p. albus* vs. *N. p. viverrinus*, respectively). We used 16 microsatellite markers for genotype determination: *ZuBeCa 15*, *ZuBeCa 17*, *ZuBeCa 18*, *ZuBeCa* 26, and *Nyct1-Nyct12* (Schläpfer et al., 1999; Schelling et al., 2000; Hong et al., 2013).

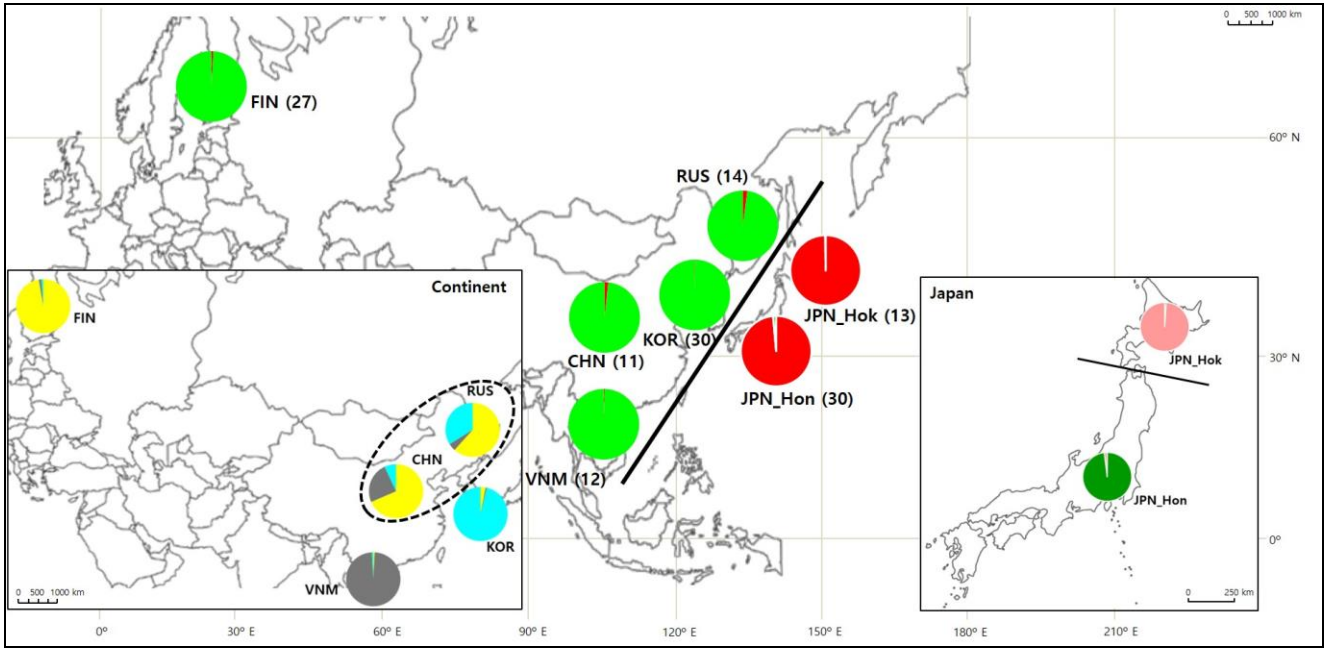

Figure 3. Studying area and sampling information of raccon dog. Pie charts show proportions of the STRUCTURE clusters for all populations (K= 2, center map), continental population (K= 3, lower left inlet map), and Japanese population (K= 2, lower right inlet map). Solid and dotted lines indicate the genetic barriers from BARRIER program using Monmornier alogorithm. KOR: Korea (Cheorwon), CHN: China (Northeastern, Northwestern and unknown parts), RUS: Russia (Primorsky Krai), FIN: Finland (Southern part), VNM: Vietnam (Lang Son), JPN_Hon: Japan_Honshu (Kanaagawa), JPN_Hok: Japan_Hokkaido.

Table 5. Genetic diversity estimates for raccoon dogs.

Location	N	No. of alleles	Allelic diversity	Allelic richness	H_E	H_O	HWE *p-value*	Number and loci with null allele	Subspecies
KOR	30	95	5.9	4.157	0.689	0.693	0.017	-	*N. p. koreensis*
CHN	11	77	5.2	4.205	0.541	0.463	0	3 (*Nyct1, Nyct10, Nyct11*)	*N. p. ussuriensis, procyonides, orestes*
RUS	14	91	3.8	4.263	0.492	0.436	0.009	2 (*Zubeca18, Nyct2*)	*N. p. ussuriensis*
FIN	27	81	4.1	3.666	0.475	0.471	0.006	1 (*Nyct6*)	*N. p. ussuriensis*
VNM	12	70	2.1	3.801	0.197	0.221	0	2 (*Zubeca26, Nyct6*)	*N. p. procyonides*
JPN_Hon	30	83	4.8	3.443	0.643	0.569	0	4 (*Zubeca18, Nyct4, Nyct6, Nyct8*)	*N. p. viverrinus*
JPN_Hok	13	34	5.1	1.804	0.609	0.601	0.388	-	*N. p. albus*

N: number of individual, No. of alleles: number of alleles, Allelic diversity: mean number of alleles, H_O: observed heterozygosity, H_E: expected heterozygosity, HWE *p−value*: The probability of Hardy−Weinberg equilibrium ($p < 0.05$: significant departure from Hardy−Weinberg equilibrium), Loci with null allele: Number of null alleles. (KOR: Korea, CHN:China, RUS: Russia, FIN: Finland, VNM: Vietnam, JPN_Hon: Japan_Honshu (Kanagawa), JPN_Hok: Japan_Hokkaido).

All genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen). The PCR reactions contained 10–50 ng template DNA, 0.3 μM each fluorescent forward primer and nonfluorescent reverse primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.5 U i-Star *Taq* DNA polymerase (iNtRON Inc.). The PCR cycle followed a touchdown method: initial denaturation at 94 ℃ for 3 min; 20 cycles of 94 ℃ for 1 min, reduction of temperature from 60 ℃ to 50 ℃ (decreased by 0.5 ℃ per cycle) for 1 min, 72 ℃ for 1 min; followed by 20 cycles of 94 ℃ for 1 min, 50 ℃ for 1 min, 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min. The ABI Prism 3730 XL DNA Analyzer (Applied Biosystems) was used for genotyping alleles with a GeneScan-500 ROX size standard (Applied Biosystems). GeneMarker v 1.9 software (SoftGenetics) was used to estimate the size of the alleles.

Data analysis

Genetic diversity estimates, including the mean number of alleles, and observed (H_O) and expected (H_E) heterozygosity under the Hardy-Weinberg assumptions were obtained in GenAlEx v6.1 (Peakall and Smouse, 2006), and allelic richness was estimated using FSTAT v. 2.9.3.2 (Goudet, 1995). Each population was tested

for deviations from Hardy–Weinberg equilibrium using the Fisher's method, as implemented in the GENEPOP v3.3 (Guo, 1992; Raymond and Rousset, 1995). The presence and frequency of null alleles for each locus and population were tested using MICROCHECKER v. 2.2.3 (van Oosterhout et al., 2004). To evaluate genetic relationships among populations, pairwise F_{ST} (Weir and Cockerham, 1984) was obtained using FSTAT v. 2.9.3.2 (Goudet, 1995) and Nei's genetic distance using GenAlEx v. 6.1 (Peakall and Smouse, 2006). We applied a Bonferroni correction when testing significance in multiple comparisons (Rice, 1989). GenAlEx v. 6.1 (Peakall and Smouse, 2006) was further used to conduct principal coordinate analysis (PCoA) to visualize geometric relationships among populations based on genotypic differences.

We employed STRUCTURE v. 2.2 (Pritchard et al., 2000) to determine the genetic structure of populations using a hierarchical method based on Bayesian clustering. The number of separate clusters (K) and the log posterior probability for a given K, ln Pr (X/K), were obtained for each of the 30 iterations at K values (from 1 to 7). The initial burn–in period was set to 100,000 replications and followed by 200,000 replications of Markov chain Monte Carlo steps. Then, ΔK was estimated based on the method described by

Evanno et al. (2005). We examined isolation by distance (IBD) (Wright, 1931) by testing the regression of genetic distance (F_{ST}/(1−F_{ST})) on geographic distance (Ln (dis): Km) between each population pair. The Mantel test within GenAlEx v 6.1 (Peakall and Smouse, 2006) was carried out with 999 permutations for correlations. Analysis between two of the Japanese populations were considered meaningless and therefore skipped. BARRIER v. 2.2 (Manni et al., 2004) was carried out to detect genetic barriers to gene flow among populations using Monmonier's maximum difference algorithm with 1000 bootstrapped pairwise F_{ST}. To obtain Delaunay triangulation connecting locations, we applied geographical coordinates for each population. Analysis of molecular variance (AMOVA) and F−statistics (F_{RT}, F_{SR}, F_{ST}, F_{IS}, and F_{IT}) were estimated using GenAlEx v. 6.1 (Peakall and Smouse, 2006). MIGRATE v. 3.2.17 (Beerli and Felsenstein, 2001) was used to calculate the unidirectional migration rate among geographically connected populations and to determine the effective population size of each population. We assumed there was no migration of individuals between continent and Japan, and thus the estimation of migration rate between these populations was omitted. The Finnish population was separated from the remaining continental

populations by a long geographic distance, so it was also excluded.

We conducted the Wilcoxon sign rank test to determine heterozygosity excess (Luikart and Cornuet, 1998) and a mode-shift in allelic frequency distribution (Luikart et al., 1998) for detecting recent genetic bottlenecks in BOTTLENECK v. 1.2.02 (Piry et al., 1999). In the Wilcoxon sign rank test, 1000 simulations of the two-phase mutation model (TPM) employing 10% multiple-step mutations and 90% single-step mutations were performed. Evidence of long-term historical reduction of population sizes was examined using Garza and Williamson's M ratio as implemented in the AGARst version v. 3.3 (Harley, 2003).

Results

Genetic characteristics and diversity

Using the 16 microsatellite markers, 156 alleles were observed in the 137 raccoon dog samples from seven provinces within six countries, ranging from 34 alleles in Hokkaido, Japan (JPN_Hok) to 95 alleles in South Korea (KOR) (Table 5). Of these, 15 were private alleles found only in a single population, nine had a frequency below 5%, and six had a frequency above 10%: one in Finland (12%), three in Vietnam (one allele, 16.7%; the other two alleles, 20.8%), and two in Honshu, Japan (one allele, 10%; the other allele, 23.3%). The mean number of alleles across loci ranged from 2.1 (Vietnam) to 5.9 (South Korea), and the mean values of expected and observed heterozygosity were 0.607 (0.197–0.689) and 0.564 (0.221–0.693), respectively (Table 5). The highest levels of genetic diversity were detected in raccoon dogs from South Korea, whereas the lowest was found in the Vietnamese population (Table 5). Chinese and Russian populations showed relatively higher allelic richness than other populations. Null alleles were detected at more than one locus per population, excluding South Korea and Hokkaido (Table 5). Most loci showed null allele

frequencies higher than 0.10, and two alleles in Chinese populations and one in Vietnamese population showed null allele frequencies higher than 0.20. All loci were included in further analyses because loci with null alleles were present only in a subset of the populations in which heterozygote deficiency possibly due to the Wahlund effect might be responsible for the detection of null alleles. In most of the populations, except the one from Hokkaido, observed heterozygosity significantly from expected heterozygosity under Hardy–Weinberg equilibrium (Table 5) toward heterozygote deficiency.

Genetic relationships and gene flow

Pairwise F_{ST} and gene flow (Nm) among populations are shown in Table 6. The values of F_{ST} (and their derived Nm) ranged from 0.023 (6.489) in China vs. Russia to 0.530 (0.404) in Vietnam vs. Hokkaido, Japan. The South Korean population was slightly differentiated from the Chinese and Russian populations. The raccoon dog populations in China, Russia, and Finland showed low F_{ST} (0.023–0.088). Finnish and Russian populations showed the lowest level of genetic differentiation (F_{ST}=0.064) but distinct genetic differentiation levels from the South Korean (F_{ST}=0.129)

and Vietnamese (F_{ST}=0.237) populations. In addition, the genetic composition of the Vietnamese population clearly differed from that of the other continental populations. The highest level of genetic differentiation was found between Japanese and continental populations (South Korea, China, Russia, Finland, and Vietnam), as evidenced by the highest F_{ST} value (up to 0.530) and by an *Nm* value of 1.419; moreover, all F_{ST} values were significant (P < 0.003). Within Japan, raccoon dogs from Honshu and Hokkaido populations were highly differentiated, with F_{ST}=0.228, supporting the classification of these populations into separate subspecies.

Table 6. Pairwise F_{ST} (below the diagonal) and gene flow (*Nm*, above the diagonal) between raccoon dog populations.

	1	2	3	4	5	6	7
1. KOR (30)		3.472	4.946	2.818	2.037	1.419	0.642
2. CHN (11)	0.089*		6.489	3.898	1.841	1.228	0.612
3. RUS(14)	0.063*	0.023ns		5.568	2.075	1.356	0.645
4. FIN(27)	0.129*	0.088*	0.064*		1.374	1.253	0.570
5. VNM (12)	0.173*	0.165ns	0.173*	0.237*		0.878	0.404
6. JPN_Hon (30)	0.249*	0.274*	0.244*	0.274*	0.336*		1.574
7. JPN_Hok(13)	0.380*	0.457*	0.406*	0.420*	0.530*	0.228*	

*Significant after Bonferroni correction (P<0.003); ns Not significant; Indirect indicator of gene flow (*Nm*) was calculated among geographic populations using the equation, $Nm=1/4\{(1-F_{ST})/F_{ST}$

The separation between Japanese and continental populations was evidenced in the PCoA (Fig. 4A). Among continental populations, Vietnamese raccoon dogs were separated from the other populations (Figs. 4A and 4B), whereas South Korean population clustered with Chinese, Russian, and Finnish populations (Fig. 4A). However, South Korean population was slightly differentiated from the others and the Finnish population was also slightly separated from both Chinese and Russian populations (Fig. 4B). In Japan, the Hokkaido population was independent from the Honshu population (Fig. 4A).

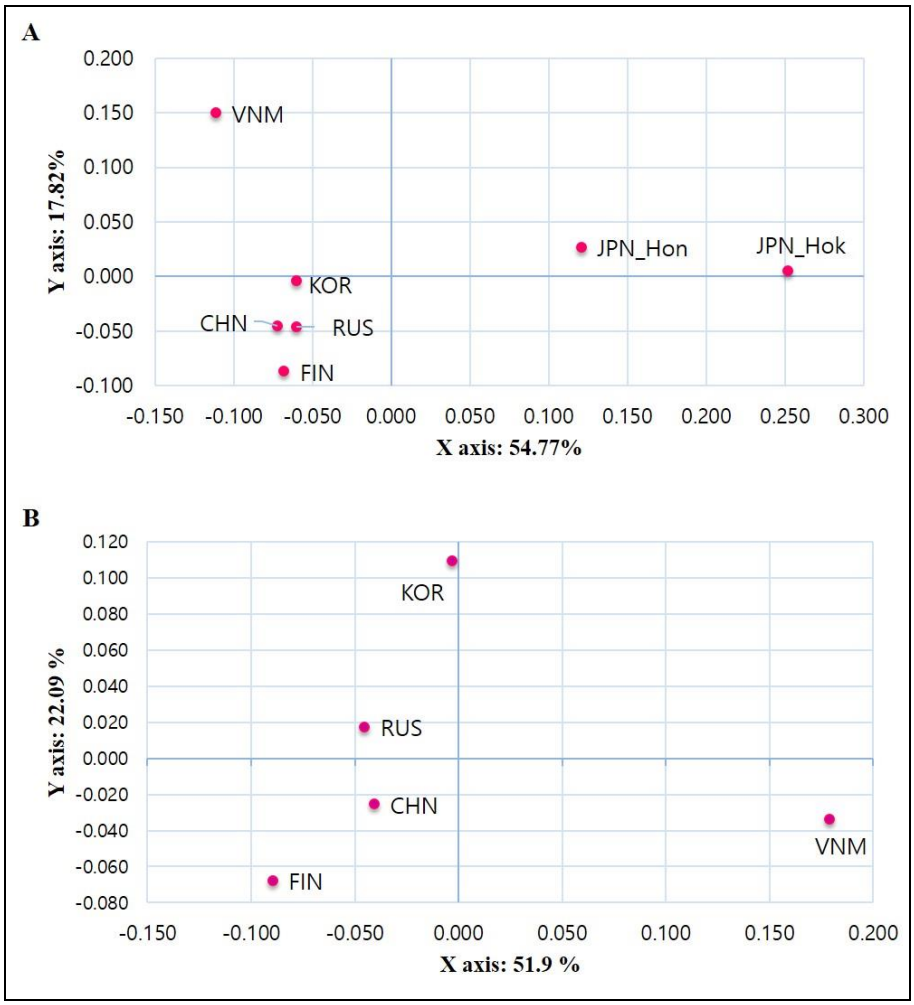

Figure 4. Scatter diagram from a principal coordinate analysis of geographic locations. A: All populations, B: Continental population excluding Japanese population.

Genetic structure

The highest ΔK was observed when K=2 in STRUCTURE analysis, classifying the raccoon dogs from the seven areas into distinct two distinct populations: 1) the continental population (South Korea, China, Russia, Finland, and Vietnam), and the 2) Japanese population (Fig. 5A). The continental population was further divided into three subpopulations: 1) South Korea, 2) China, Russia, and Finland, and 3) Vietnam (Fig. 5B). Within continental subpopulation 2), the Chinese and Russian groups showed more admixed genetic composition than the Finnish group. The Japanese population also comprised two subpopulations: 1) Honshu and 2) Hokkaido (Fig. 5C). The pie chart constructed for all subpopulations based on the proportion of the clusters resulting from STRUCTURE analysis showed the distinct genetic composition of each subpopulation (Fig. 3).

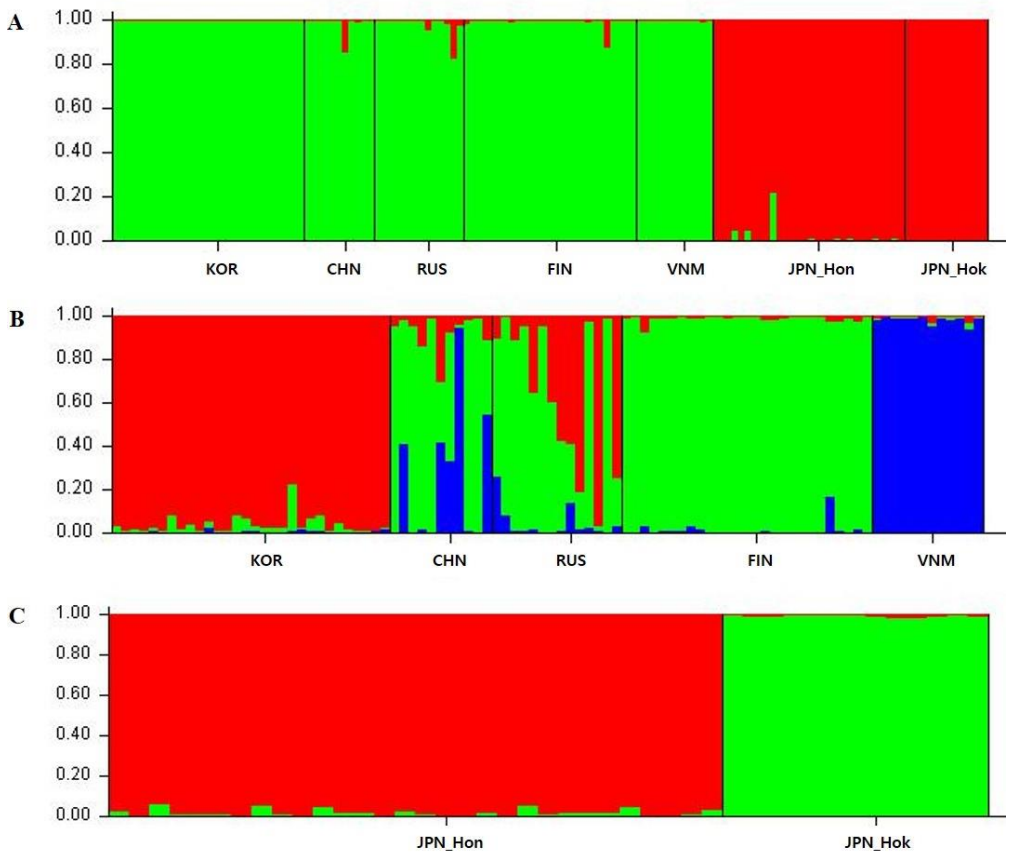

Figure 5. Bar plots of raccoon dog population identified by STRUCTURE analysis. A. All raccoon dog populations from seven regions (K= 2), B. Continental population from five regions (K=4), C. Japanese population from two regions (K=2).

Barrier analysis, based on 1000 replications of pairwise F_{STs}, revealed a strong barrier between the continent and Japan and a barrier between Honshu and Hokkaido in Japan (Fig. 6A). The analysis also supported the separation of Vietnamese and Finnish populations from the other continental populations (Figs. 6A and B).

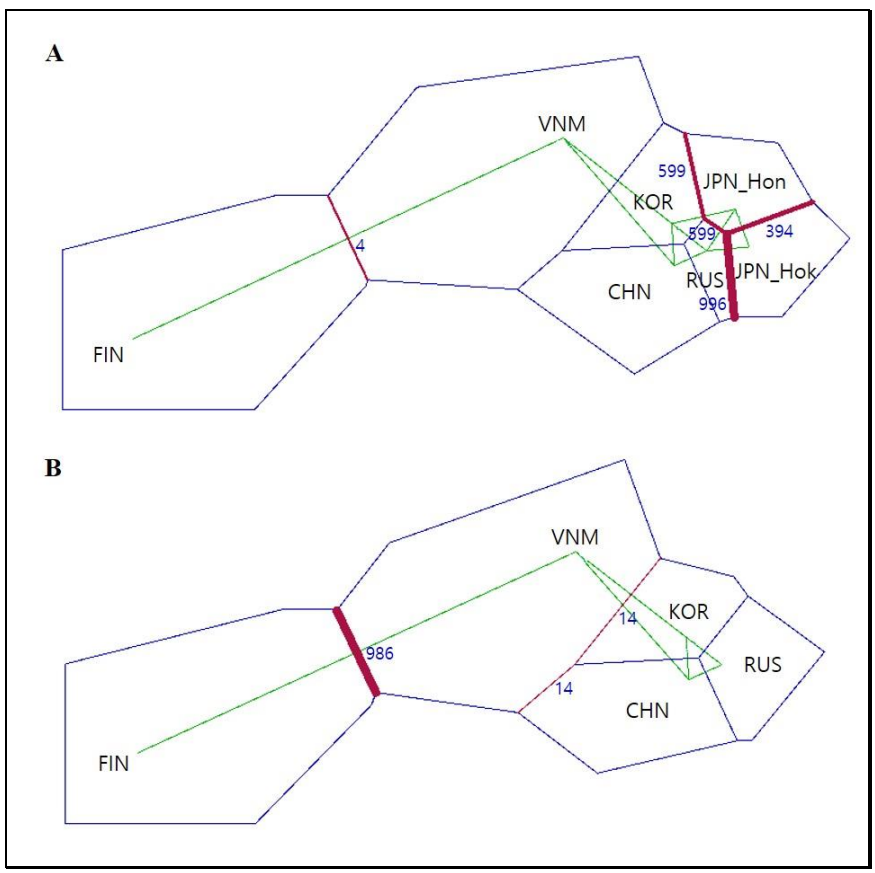

Figure 6. Geographic barriers of limited gene flow determined by BARRIER using Monmonier's algorithm. The red bold lines showing genetic boundaries are proportional to the strength of the barriers with bootstrap values. A: All populations, B: Continental populations excluding Japanese population.

The regression performed in IBD analysis for all populations revealed no significant correlation between genetic and geographic distances ($R^{2=}$0.008; P=0.287). When considering continental populations alone, no significant correlation was revealed ($R^{2=}$0.241; P=0.233), but, when the introduced Finnish population was excluded, the correlation became significant ($R^{2=}$0.966; P=0.014, Fig. 7), indicating geographic distance is one of main factors leading to the genetic differentiation among native continental raccoon dog populations. On the contrary, Japanese and Finnish raccoon dogs, which were isolated by the ocean (physical barrier) or due to long-distance artificial introduction, did not show significant IBD.

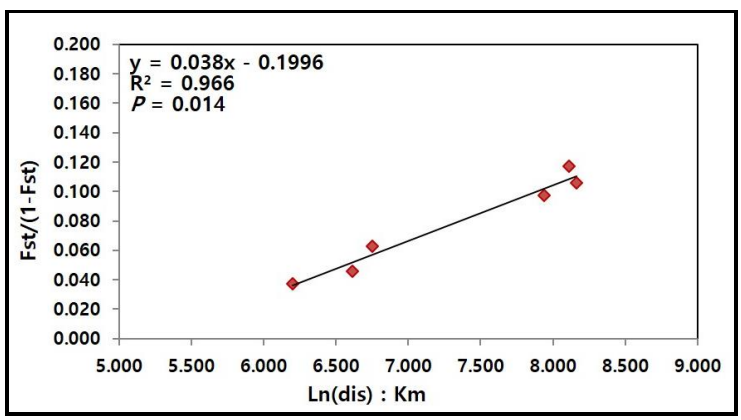

Figure 7. Regression of genetic isolation by geographic distance (IBD) for continental raccoon dog populations excluding Finnish population. Mantel test was carried out with 999 permutations for correlations.

The hierarchical AMOVA revealed that the genetic differentiation among regions (F_{RT}) was higher than that among populations within regions (F_{SR}). Scenario A estimated the differentiation between two regions including six populations (the Finnish population was excluded because it was introduced): 1) Continent (South Korea, China, Russia, and Vietnam) and 2) Japan (Honshu and Hokkaido). The high F_{RT} between continental and Japanese regions (0.180, P=0.001) indicated genetic differentiation between the two regions (Table 7A). Scenario B confirmed the separation of the three continental regions, i.e., 1) South Korea, 2) China, Russia, and Finland, and 3) Vietnam, with F_{RT}=0.081 (P=0.001) (Table 7B).

Table 7. Analysis of molecular variance (AMOVA) of raccoon dog populations based on various genetic groupings.

A								
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**	***F*-Statistics**	**Value**	**P-Value**
Among regions	1	174.371	174.371	1.244	18%	F_{RT}	0.18	0.001
Among populations	4	130.277	32.569	0.835	12%	F_{SR}	0.147	0.001
Among individuals	104	573.856	5.518	0.677	10%	F_{ST}	0.3	0.001
Within individuals	110	458	4.164	4.164	60%	F_{IS}	0.14	0.001
Total	219	1336.505	-	6.919	100%	F_{IT}	0.398	0.001
B								
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**	***F*-Statistics**	**Value**	**P-Value**
Among regions	2	96.851	48.425	0.516	8%	F_{RT}	0.081	0.001
Among populations	2	35.304	17.652	0.357	6%	F_{SR}	0.061	0.001
Among individuals	89	557.239	6.261	0.748	12%	F_{ST}	0.137	0.001
Within individuals	94	448	4.766	4.766	75%	F_{IS}	0.136	0.001
Total	187	1137.394	-	6.387	100%	F_{IT}	0.254	0.001

A. Two regions: 1. Continent (KOR, CHN, RUS, VNM), 2. JPN (Hon, Hok)
B. Three continental regions: 1. KOR, 2. (CHN, RUS, FIN), 3. VNM

The effective size of each population and the unidirectional migration rate among the four continental populations, (excluding the Finnish population), and between the two Japanese populations are shown in Table 8. Gene flow between China and Vietnam was high, and almost twice as high from China to Korea, Russia, and Vietnam as it was in the opposite direction. As presumed for a geographical center with a higher efflux of migrants, the largest population was that from China. In Japan, the largest population was that from Honshu, a gene flow was much higher from Honshu to Hokkaido than in the opposite direction. The Hokkaido population was the smallest among all populations.

Table 8. Effective numbers of population (*Ne*) and migration rate (*m*) among raccoon dog populations.

To / From	*Ne*	*m*						
		KOR	**CHN**	**RUS**	**VNM**	**FIN**	**JPN_Hon**	**JPN_Hok**
KOR	**18,744**	-	1.32	1.27	1.26	-	-	-
CHN	**51,542**	0.48	-	0.85	0.97	-	-	-
RUS	**29,046**	1.00	1.01	-	1.41	-	-	-
VNM	**17,194**	0.97	1.57	0.76	-	-	-	-
FIN	**19,344**	-	-	-	-	-	-	-
JPN_Hon	**22,016**	-	-	-	-	-	-	0.63
JPN_Hok	**5,006**	-	-	-	-	-	1.13	-

−: no estimation between geographically isolated populations (FIN: isolated by long geographic distance from their source populations, JPN_Hon and JPN_Hok: separated by the ocean from the continental populations).

No bottleneck event was detected under the TPM model at P=0.05, and the distribution of allele frequencies revealed a normal L-shaped distribution, indicating that all populations remained stable for a small number of generations (Table 9). In addition, Garza and Williamson's M value ranged from 0.722 to 0.822, implying that all raccoon dog populations maintained a stable size over 100 generations (Table 9).

Table 9. Analysis to detect a recent population bottleneck or past population reduction event within populations.

Locality	Wilcoxon Test (T.P.M) H excess probability	Mode-Shift	Garza & Williamson's M-ratio
KOR	0.46994	normal L-shaped distribution	0.799
CHN	0.31609	normal L-shaped distribution	0.751
RUS	0.53006	normal L-shaped distribution	0.822
FIN	0.70171	normal L-shaped distribution	0.745
VNM	0.21660	normal L-shaped distribution	0.722
JPN_Hon	0.99451	normal L-shaped distribution	0.818
JPN_Hok	0.98145	normal L-shaped distribution	0.757

One-tail probability for an excess of observed heterozygosity relative to the expected equilibrium heterozygosity, computed from the observed no. of alleles under mutation-drift equilibrium; TPM: two-phase model of mutation, M-ratio=Mean ratio of the no. of allele size.

Discussion

The genetic diversity and structure of raccoon dog populations were examined across most of the species' geographic range. Analyses revealed that population structure of raccoon dogs in East Asia and Europe generally agrees with the currently recognized intraspecific taxonomy (Figs. 4 and 5): *N. p. ussuriensis*_CHN, RUS, FIN; *N. p. koreensis*_KOR; *N. p. procyonoide*s_VNM; *N. p. viverrinus*_JPN_Hon, and *N. p. albus*_JPN_Hok. Although three subspecies of raccoon dog, i.e., *N. p. ussuriensis* (Russia, northeastern China and Europe), *N. p. orestes* (Yunnan, China), and *N. p. procyonoides* (Vietnam and southern China), inhabit China, we could not evaluate the position of *N. p. orestes* within the genetic structure presented here due to the lack of samples. However, the structure bar plot in Fig 3B suggests that some individuals from southern China, close to Vietnam, might belong to *N. p. procyonoides*, whereas the others probably belong to *N. p. ussuriensis* in northeastern China (Figs. 4 and 5B). These findings evidence the agreement among genotype distributions provided by microsatellite markers, phylogenetic analysis based on mtDNA, and morphologic characters. In addition, special attention was devoted

to investigate central–marginal trends in genetic diversity. Considering the geographic distribution of raccoon dogs, Chinese and Russian populations might be regarded as central and South Korean and Vietnamese populations as marginal due to their eastern and southern peripheral locations within the East Asian continent. The Japanese population is categorized as an isolated island population, occupying the raccoon dog's peripheral range. The Finnish raccoon dog population should be considered separately as an introduced population. Our main findings are summarized below.

1) Genetic diversity in continental vs. island populations

The continental population, especially Chinese and Russian subpopulations, had higher genetic diversity and larger effective size (Table 8) than the island population. The low genetic diversity and high genetic differentiation found in isolated Japanese subpopulations suggest that the species retains central–marginal trends in genetic diversity, even resulting in the allopatric speciation of isolated island populations. Strong genetic differentiation (F_{ST}=0.236) and lack of gene flow between continental and Japanese raccoon dogs are consistent with the findings of several other studies, which suggested that the Japanese

raccoon dog should be classified as a separate species due to distinct morphological characteristics (Kauhala et al., 1998; Kim et al., 2015), different number of chromosomes (Wada and Imai, 1991; Wada et al., 1991), and geographic isolation. A recent phylogeographic study using mtDNA also showed high genetic separation (Φ_{ST}=0.76, pairwise differences between populations) between continental and Japanese populations (Kim et al., 2013). Kim et al. (2013) also suggested that raccoon dogs migrated to Japan around 0.59–0.67 million years ago, during the middle Pleistocene. After migration, the raccoon dog population experienced isolation after the Japan Sea opened and a bottleneck event resulted in the reduction of genetic diversity.

2) Genetic diversity in central vs. marginal continental populations

Chinese and Russian populations revealed higher genetic diversity than the Vietnamese population, but similar levels to that of the Korean population. Both central populations showed lower genetic diversity but slightly higher allelic richness than the marginal South Korean population. Chinese and Russian raccoon dogs were grouped into a single genetic population, as evidenced by the active gene flow between them (Fig. 5B, Table 6). Indeed, they appeared

differentiated from South Korean and Vietnamese populations, which supported by mtDNA analysis (Kim et al., 2013). The Chinese population is genetically more admixed with the Vietnamese than with the South Korean population (Fig. 3B). This finding is unsurprising, considering that China is located between Russia and Vietnam, and that the Chinese population is geographically close to the Vietnamese population. Although three subspecies of raccoon dog—*N. p. ussuriensis*, *N. p. orestes,* and *N. p. procyonoides*— inhabit China, the level of genetic structure and differentiation among these subspecies has not been studied so far. Therefore, further analyses with sufficient geographical coverage should be carried out to clarify the genetic structure of raccoon dogs within China and their relationships with other populations.

In East Asia, the northern limits of the raccoon dog's distribution are the Chita and Amur regions of Russia (Kauhala and Kowalczyk, 2011), but Russian samples used in the present study were from the southern part of the Primorski region, far south from the northern limit. Therefore, the Russian population used in the present study does not represent a northern marginal population of raccoon dogs; rather, it should be regarded as a central population along with the Chinese population, an approach that was supported

by the STRUCTURE and PCoA analysis (Figs. 3, 4B, and 5B). Therefore, the Chinese and Russian populations examined here should be regarded as central source populations contributing to the marginal continental populations. Furthermore, these central populations might help maintaining the genetic diversity of East Asian continental raccoon dogs. To understand the marginal–central effect of raccoon dogs in northern continental populations, samples from the Chita and Amur regions must be collected and analyzed.

Although the Korean Peninsula is located east of China and south of Russia, and is marginal to the East Asian continent, the genetic diversity of the South Korean population was similar to that of the central populations and presented a moderate level of genetic differentiation from them (Figs. 3, 4B, and 5B). The Korean Peninsula might have been a refugium for raccoon dogs and other mammals (Kim et al., 2013; Lee et al., 2016) during the glacial period, allowing maintaining the gene pool of raccoon dogs in the eastern marginal population while it was effectively differentiating itself from other continental populations (Kim et al., 2013). In addition, most of the North Korean landscape is covered by high mountains, at a general elevation of about 1000 m above sea level

and the Baekdu and Changbai mountain range located along the boundary between China and North Korea ranges from 1100 to 2500 m above sea level (Kim et al., 2004). Because high altitude areas are known to be an unfavorable habitat for raccoon dogs (Hong et al., unpublished data), the high mountains of North Korea could function as a partial dispersal barrier for raccoon dog population in South Korea, contributing to its differentiation from other continental populations. Moreover, the demilitarized zone between South and North Korea established after the Korean War (1950–1953) might be acting as an additional and recent barrier that isolates the South Korean raccoon dog population from the others within the continent.

The Vietnamese raccoon dog population showed the lowest level of genetic diversity (Table 5) and the highest level of genetic differentiation (Table 6, Figs. 3, 4B, and 5B) among East Asian continental populations. These levels are probably due to the peripheral isolation of the population that inhabits the southern limit of the species' range. According to Sterling et al. (2006), raccoon dogs are among the mammal species that are only distributed in the northern part of Vietnam. As a temperate forest–adapted mammal (Kauhala and Saeki, 2016), the raccoon dog range in Vietnam and

Southern China might be composed of marginal, suboptimal habitats. For example, the high temperature and humidity characteristics of the middle and southern part of Vietnam might prevent the raccoon dog population from expanding further into those areas of Vietnam. Compared with the hot, humid, monsoonal, and tropical weather of the middle and southern areas of Vietnam, northern Vietnam is much cooler and seasonal (Sterling et al., 2006). Therefore, global climate change and regional environmental changes are expected to negatively affect the health of the Vietnamese raccoon dog population in a near future. A continuous and extensive monitoring of the Vietnamese raccoon dog population along its southern peripheral range should be adopted to properly manage and conserve this population with unique genetic and ecological adaptations.

Moreover, very different climate condition might be another barrier for structuring raccoon dog population and resulting in recent distribution of subspecies. After introduction to Europe, raccoon dogs migrated west side but not to the east side. China and Vietnam were also western marginal area of raccoon dog species and no spread to the western region. Deserts and steppe with very dry plateaus between Europe and China_Vietnam might be hard

factors for raccoon dogs to spread.

3) Genetic diversity in source vs. introduced continental populations

The Finnish population showed values of genetic diversity similar to the Russian population (Table 5), and shared genetic features with raccoon dogs within the species' native range in Russian Far East and China (Figs. 3, 4B, and 5B). These findings pointed out that the Finnish population originated from the Russian population, but was clearly distinguished from the other continental populations (South Korea and Vietnam). However, despite being included within the same genetic population as Chinese and Russian subpopulations, the Finnish raccoon dog subpopulation had a slightly genetically different composition (Figs. 4, 5B). Three hypotheses might explain this slight differentiation. The first is that the introduced population might have experienced genetic bottlenecks and drift effects such as founder effect while adapting to the European environment after translocation. According to Ansorge et al. (2009), several introduced populations were morphologically distinct from the original samples, and epigenetic variability was detected among migrant populations. The second hypothesis is that, after introduction, the northern Finish subpopulation underwent local

adaptation and differentiation from the main, central European population. This is supported by a recent study using microsatellite markers in which the Finnish population was distinguished from central European and Danish populations, whereas central European raccoon dog populations showed a homogeneous structure due to their recent and rapid expansion (Drygala et al., 2016). However, it should be noted that there was no genetic differentiation between north and central European populations according to mtDNA analysis (Pitra et al., 2010). The third hypothesis is that the native population from which the introduced Finnish population originated was already differentiated from the southern, main population of Russian Far East. Because the introduced European raccoon dog population was originally translocated from the northern part of its range in Russian Far East, and our Russian samples were collected from the southern part of the species range in Russian Far East, the slight differentiation between the Finnish population and the Russian Far East population detected in the present study already existed. To actually examine the genetic effect of the artificial introduction, it will be necessary to collect samples from the northern part of Russian Far East and from central Europe covering the entire range of original and new habitats.

Preadaptation is thought to account for the successful introduction of the raccoon dog into northeastern Europe. Raccoon dogs that were native to Far Eastern Siberia were preadapted to endure the long winter in northeastern Europe due to their thick fur and ability to hibernate (Kauhala and Kowalzyck, 2011). The general environment of Finland is similar to that of the original range of raccoon dogs in Russia, so they might have adapted easily to the new environment in northern Europe.

According to the coancestry bar plots in Figure 3B, the Chinese population shared most genetic characters with the Vietnamese population, and the Russian population shared most genetic characters with the South Korean population. In contrast, the Finnish population had little genetic exchange with other populations. Genetic exchange between the Finnish population and the Chinese or original Russian Far East populations is not possible due to the long geographical distance separating them. For management and conservation purpose, raccoon dog populations in Europe, China/Russia, South Korea, and Vietnam should be regarded as independent subpopulations within the Eurasian continent.

As mentioned earlier, our sampling could not cover all subpopulations within the East Asian continental population and

European introduced populations. In addition, we could not consider subpopulations' density when collecting samples, as this information was not available for most of the range. Thus, caution is necessary to interpret current data, and future studies with extensive, systematic sampling are required to define fine-scale subpopulation structure of raccoon dogs in native and introduced ranges.

4) Genetic characteristics of two isolated island populations in Japan

The Hokkaido population, inhabiting an isolated and northern peripheral region, showed low genetic diversity, particularly allelic richness, and was the smallest population examined here. The population also showed strong and significant genetic differentiation from the Honshu population (F_{ST}=0.228, Table 6), which was similar to the differentiation between continental and island populations (F_{ST}=0.236).

Japan consists of several islands of different sizes, as well as straits among the four main islands, Hokkaido, Honshu, Shikoku, and Kyushu. The Hokkaido population of raccoon dogs was genetically distinct from the Honshu and Shikoku populations based on mtDNA (Kim et al., 2013) and microsatellites (Hong et al., accepted in April,

2018), although the Kyushu population was not included in the analyses. This finding implies that the Tsugaru Strait, between Honshu and Hokkaido (19.5 km), is a strong barrier to gene flow, whereas the much narrower and shallower strait between Honshu and Shikoku (around 3 km wide and surrounded many islands) is not a barrier to raccoon dog migration. Hong et al. (accepted in April, 2018) suggested that the Blakiston's line, situated in the Tsugaru Strait between Hokkaido and Honshu, might be the main factor accounting for the genetic divergence between raccoon dog populations from the two main islands. Tsugaru Strait acts as a virtual geographical boundary for many other mammal species, such as brown bear (*Ursus arctos*), siberian chipmunk (*Tamias sibiricus*), and flying squirrel (*Pteromys volan*s), only inhabiting Hokkaido (Dobson, 1994; Hirata et al., 2013; Japan Wildlife Research Center, 2015), and Japanese mole (*Mogera wogura*), Japanese serow (*Capricornis crispus*), small Japanese mole (*Mogera imaizumii*), and asiatic black bear (*Ursus thibetanus*) inhabiting the areas below the Blakiston's line (Maruyama et al., 1997; Tsuchiya et al., 2000; Yasukochi et al., 2012). The strait is also a boundary for intraspecific differentiation in red fox (*Vulpes vulpes*), least weasel (*Mustela nivalis*), and raccoon dog (Kurose et al., 1999; Inoue et al.,

2007). Therefore, results of the present study, based on microsatellite markers, agreed with that of previous studies based on mtDNA and skull morphology indicating that raccoon dog in Hokkaido is a different subspecies (Kim et al., 2013, 2015).

In summary, factors such as climate, ocean, mountains, and geographic distance were considered as the main barriers separating subpopulations of raccoon dog in East Asia. There are no major artificial barriers to their gene flow in East Asia. Raccoon dogs in the Eurasian continent showed a central–marginal trend in genetic diversity and consisted of three populations: China/Russia, South Korea, and Vietnam. The South Korean population showed a high level of genetic diversity and moderate level of genetic differentiation from the Chinese and Russian populations, suggesting that the Korean Peninsula might have served as an independent refugium for raccoon dog populations in East Asia during the last glacial maximum. Although the introduced raccoon dog population in Finland shared the genetic composition of its original source population, it might have differentiated from it since the introduction. The high genetic differentiation between Hokkaido and Honshu populations in Japan agreed with previous studies reporting that

these populations are separate subspecies. The distinct genetic structure of raccoon dog in continental and island populations supported previous morphological and phylogenetic studies suggesting that continental and Japanese raccoon dog populations should be considered different species. Future studies with extensive sampling will be helpful for understanding the detailed structure of raccoon dog populations in Eurasia.

Chapter 3. Population structure of raccoon dog (*Nyctereutes procyonoides*) in South Korea and Japan using microsatellite loci analysis: implications for disease management

Introduction

The raccoon dog is a true omnivore (Kauhala and Kowalczyk, 2011), which maintains food web stability in ecosystems (Ågren et al., 2102), and also function as an effective scavenger (Melis et al., 2007). Thus, raccoon dogs play an important role as one of the medium-sized predators or scavengers because of the reduction or extinction of their competitors and other major terrestrial predators, helping to maintain ecological balance in the Korean Peninsula (Hong et al., 2013). A phylogeographic study using complete mtDNA cytochrome *b* sequences suggested that Korean raccoon dogs are a unique population that have adapted to the particular environment of Korean Peninsula in northeast Asia and are different from other continental and Japanese raccoon dog

populations (Kim et al., 2013). This study showed that the Korean raccoon dog population should be considered as a valuable biological resource requiring proper management and conservation strategies.

However, wild raccoon dogs (*Nyctereutes procyonoides*) play another role as main host of various infectious diseases such as, rabies, canine distemper, and parasites, which can be transmitted among domestic dogs and wild Canidae species (Kauhala and Kowalczyk, 2011, Yang et al., 2011, Shao et al., 2015). Its high adaptability to various environments and reduction of competitors and predators may enable it to increase its population size in South Korea (Jo, 2015). The growth of Korean raccoon dog population has increased public concerns over their potential role as disease and parasite vectors, especially for contagious zoonotic diseases (Oh et al., 2012; Hong et al., 2013). If population size increase, ecological behaviors such as sharing a toilet, periodic moving to new burrow which was used by other animals during hibernation and living with pair can be negative factors to promote spreading diseases. Furthermore, some pathogens and parasites spread between dogs and wild canids, and from

rodents to foxes, raccoon dogs, dogs, and even to humans (Kauhala and Kowalczyk, 2011). Issues regarding prevention and control of wildlife-related infectious diseases are becoming increasingly important. Hence, as part of effort to establish proper disease risk management strategies, it is essential to understand the population structure and connectivity among the animal populations of epidemiological interest (Drygala et al., 2016). Lack of information on the movement of individuals in the vector and/or host population makes it difficult to control and prevent against disease outbreaks and spread. In South Korea, rabies has been mostly reported in wild and domestic animals and in humans in the northern part of Seoul/Gyeonggi and Gangwon provinces of South Korea, and preventive measures against rabies are focused only on these areas (Yang et al., 2011). However, only limited scientific data are available on the dispersal or movement pattern of raccoon dogs, which is a main wild rabies host in South Korea, with respect to geographical features such as rivers and mountain ranges in South Korea. Japan is a rabies-free country and has been making efforts to prevent future outbreaks; however, raccoon dogs still act as

vectors for canine distemper and sarcoptic mange in Japan (Kauhala and Kowalczyk, 2011).

Estimating gene flow among populations is a representative method to measure the migration of individuals among populations and finally build population structure. Movement of individuals among populations is very important information to estimate disease spread. Because disease can spread by migration even though gene flow was not occurred by mating. However, if gene flow was occurred between populations, it means there was migration between them. Therefore, high gene flow rate reflects high migration rate and high disease spreading rate.

The genetic structure of wild animal population is formed by limited dispersal due to artificial or natural barriers. Therefore, investigation on the genetic structure of populations will be greatly helpful to provide information for the proper management of wild animal populations and their diseases.

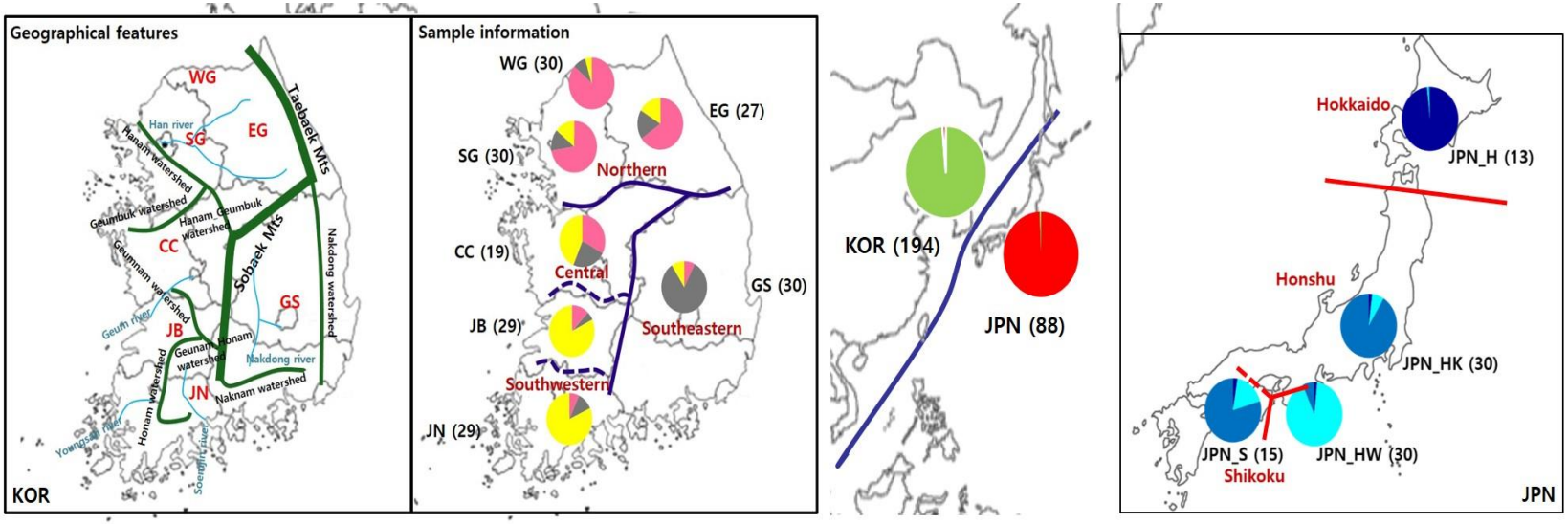

Figure 8. Study areas including geographical features and sampling information of raccoon dogs. Thick and thin solid lines in green indicate representative mountains and watersheds, respectively. Solid lines in blue mean rivers in KOR. Pie charts show proportions of the STRUCTURE clusters of each subdivided groups in Figure 3. Solid (strong) and dotted (weak) lines indicate the genetic barriers from BARRIER program using Monmornier alogorithm. SG: Seoul/Gyeonggi, WG: Western Gangwon, EG: Eastern Gangwon, CC: Chungcheong, JB: Jeonbuk, JN: Jeonnam, GS: Gyeongsang, JPN_HK: Japan_Honshu_Kanagawa, JPN_HW: Japan_Honshu_Wakayama, JPN_S: Japan_Shikoku, JPN_H: Japan_Hokkaido.

Microsatellite markers are widely used to identify individuals and to study phylogeographic relationships, genetic variation, population structuring, and genetic differentiation in various wildlife species (Hong et al., 2013, Lee et al., 2015). Information obtained by analyzing microsatellite loci is used to determine the genetic structure and recent gene flow between natural populations and serves as a basis for establishing management and conservation strategies for population, e.g., for defining conservation or management units (MUs) (Palsbøll et al., 2007). To help establish long-term strategies of population and disease management for raccoon dogs in South Korea and Japan we examined the genetic structure of seven regional populations, by focusing on their genetic diversity and the potential barriers of gene flow among the populations. The findings would provide a valuable resource that may be applied to other mammalian species and related infectious diseases for better management strategies.

Materials and Methods

DNA samples and genotyping

We analyzed tissue samples of 282 raccoon dogs from seven provinces of South Kora (SG: Seoul/Gyeonggi, WG: Western Gangwon, EG: Estern Gangwon, CC: Chungcheong, JB: Jeonbuk, JN: Jeonnam, and GS: Gyeongsang) and four provinces of Japan (HK: Honshu_Kanagawa, HW: Honshu_Wakayama, S: Shikoku, H: Hokkaido) (Fig. 8). All the experimental materials were collected legally and provided by the Conservation Genome Resource Bank for Korean Wildlife (CGRB) to use in this study. Guidelines for the procedures involving animal samples were provided by Seoul National University Institutional Animal Care and Use Committee (SNU-IACUC). Details of sampling locations and sample sizes are shown in Table 10. In the case of Gangwon, the western area is very close to Seoul/Gyeonggi; therefore, these individuals were analyzed separately from the eastern individuals of Gangwon. Sixteen microsatellite loci were used to determine genotypes. Four microsatellite loci (*ZuBeCa 15*, *ZuBeCa 17*, *ZuBeCa 18,* and *ZuBeCa 26*) were originally

developed for the dog, *Canis lupus* (Schelling et al., 2000; Schläpfer et al., 1999). The other 12 microsatellite loci (*Nyct1* to *Nyct12*) were developed for the Korean raccoon dog, *Nyctereutes procyonoides koreensis* (Hong et al., 2013).

Table 10. Genetic diversity estimates for Raccoon dogs.

Location	N	No. of alleles	Allelic diversity	Allelic richness	H_E	H_O	HWE *p-value*	Number and loci with null allele
KOR_SG	30	104	5.8	4.457	0.711	0.622	0.001	2 (*Nyct2, Nyct6*)
KOR_WG	30	95	5.9	4.157	0.689	0.693	0.017	none
KOR_EG	27	95	6.4	4.248	0.720	0.556	0.002	2 (*Nyct6, Nyct9*)
KOR_CC	19	103	6.5	4.875	0.708	0.681	0.000	3 (*Nyct2, Nyct3, Nyct5*)
KOR_JB	29	86	5.4	3.940	0.663	0.609	0.051	3 (*Nyct2,Nyct4, Nyct9*)
KOR_JN	29	88	5.5	4.001	0.658	0.651	0.357	none
KOR_GS	30	93	5.9	4.220	0.683	0.646	0.000	4 (*Nyct2, Nyct4, Nyct6, Nyct10*)
JPN_HK	30	83	4.8	3.443	0.643	0.569	0.000	4 (*Zubeca18, Nyct4, Nyct6, Nyct8*)
JPN_S	15	61	5.7	3.160	0.684	0.688	0.015	1 (*Nyct1*)
JPN_HW	30	66	4.4	3.049	0.632	0.617	0.007	1 (*Nyct9*)
JPN_H	13	34	5.1	1.804	0.609	0.601	0.388	none

N: Number of individual, No. of alleles: Number of alleles, Allelic diversity: Mean number of alleles, H_O: Observed heterozygosity, H_E: Expected heterozygosity, HWE *p−value*: The probability of Hardy−Weinberg equilibrium ($p < 0.05$: significant departure from Hardy−Weinberg equilibrium), Loci with null allele: Number of null alleles

All genomic DNA was extracted using a DNeasy Tissue Kit (QIAGEN) and amplified using the following touchdown profile for PCR amplification: initial denaturation for 3 min at 94° C; followed by 20 cycles of 94 ℃ for 1 min, 60–50 ℃ (decreased by 0.5 ℃ per cycle) for 1 min, 72 ℃ for 1 min; followed by 20 cycles of 94 ℃ for 1 min, 50 ℃ for 1 min, 72 ℃ for 1 min; and a final extension at 72 ℃ for 10 min. The reaction mixture for PCR contained 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 U i-Star *Taq* DNA polymerase (iNtRON Inc), 0.3 μM each of the fluorescent forward primer and nonfluorescent reverse primers, and 10–50 ng template DNA. Alleles were genotyped using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, USA) and a GeneScan-500 ROX size standard (Applied Biosystems, USA), and subsequently analyzed with GeneMarker v1.9 software (SoftGenetics, USA).

Data analysis

Genetic diversity measurements, including mean number of alleles, and observed (H_O) and expected (H_E) heterozygosity under the Hardy-Weinberg assumptions, were obtained using

GenAlEx v6.1 (Peakall and Smouse, 2006). Allelic richness, an important measure of genetic diversity, was determined using the program FSTAT 2.9.3.2 (Goudet, 1995). Deviations from Hardy–Weinberg equilibrium for each population were estimated using the exact probability test with Fisher's method ((Guo and Thompson, 1992) implemented in the GENEPOP v3.3 (Raymond and Rousset, 1995). Null alleles for each locus and population were checked using MICROCHECKER 2.2.3 (van Oosterhout et al., 2004). To analyze genetic relationships among populations, pairwise F_{ST} (Weir and Cockerham, 1984) and Nei's genetic distance were calculated using FSTAT 2.9.3.2 (Goudet, 1995) and GenAlEx v6.1 (Peakall and Smouse, 2006), respectively. We applied a Bonferroni correction to significance levels to account for multiple tests (Rice, 1989).

GenAlEx v6.1 (Peakall and Smouse, 2006) was further used to conduct principal coordinate analysis (PCoA) for the construction of a scatter diagram to visualize geometric relationships among populations. We investigated the genetic structure using a hierarchical method based on the Bayesian clustering function of STRUCTURE 2.2 (Pritchard et al., 2000).

Data were represented by K separate clusters and the log posterior probability for a given K, ln Pr(X/K), was generated for each of the 30 STRUCTURE runs at K values of 1–7 for the locations of the raccoon dog samples. The initial burn–in period was 100,000 replications, followed by another 200,000 replications. Two models, the admixture and the correlated allele frequencies, were chosen for analysis. The ΔK was also calculated using the method described by Evanno et al. (2005).

Isolation–by–distance (IBD) (Wright, 1931) was obtained by regression of genetic distance (F_{ST}/ (1–F_{ST})) on geographic distance (Ln(dis):Km) between pairs of populations. Mantel test was carried out with 999 permutations for correlations. We applied Monmonier's algorithm to detect genetic barriers among populations using BARRIER 2.2 (Manni et al., 2004), and geographical coordinates were used for each population to obtain Delaunay triangulation connecting locations. F–statistics (F_{RT}, F_{SR}, F_{ST}, F_{IS}, and F_{IT}) were estimated via an analysis of molecular variance (AMOVA) among populations and regions, using GenAlEx v6.1 (Peakall and Smouse, 2006).

Lastly, to detect evidence of a recent genetic bottleneck, we performed Wilcoxon sign rank test to determine heterozygosity excess (Luikart and Cornuet, 1998) and a mode-shift in allelic frequency distribution (Luikart et al., 1998), using BOTTLENECK version 1.2.02 (Piry et al., 1999). In the Wilcoxon sign rank test, we chose a two-phase mutation model (TPM) employing 10% multiple-step mutations and 90% single-step mutations with 1,000 simulations. AGARst version 3.3 (Harley, 2003) was used to calculate the Garza and Williamson's M ratio of the number of alleles to the range of allele size, to detect reductions in both recent and historical population sizes.

Results

Genetic characteristics and diversity

In total, 163 alleles were detected in 282 raccoon dog samples from 11 populations using 16 microsatellite markers, ranging from 34 (Japan_Hokkaido) to 104 alleles (Korea_Seoul/Gyeonggi) shown in Figure 8 and Table 10. Twenty-five private alleles were found to be unique to single populations. Most of these alleles had a frequency of <5%. However, four alleles had a frequency of >10% (Korea _Gyeongsang population [one allele, 15%], Japan_Honshu _Kanagawa population [one allele, 10%; another allele, 23.3%], and Japan_ Honshu_Wakayama population [one allele, 16.7%]). Mean values of expected and observed heterozygosity were 0.673 (0.609-0.720) and 0.630 (0.556-0.693), respectively. Mean number of alleles across all the loci ranged from 4.4 (Japan_Honshu_Wakayama population) to 6.5 (Korea_Chungcheong population). High allelic richness was observed in populations from Korea, especially in Korea_Chungcheong region, whereas slightly lower genetic diversity was observed among Japanese populations (Table

10). Null alleles were detected at more than one loci for each population, except three populations (Korea_Western Gangwon, Jeonnam, and Japan_Hokkaido populations; Table 10). Most populations showed low frequencies (approximately, 0.10) of null alleles. However, some loci in Chungcheong and Gyeongsang populations showed null allele frequencies higher than 0.10. All the loci were included in further analyses because loci with null alleles were present only in a subset of populations and because some missing data for each locus and population might have affected the detection of null alleles. Most populations, except Korea_Jeonnam and Japan_Hokkaido populations, showed heterozygosity deviating significantly from the expected heterozygosity under the Hardy−Weinberg equilibrium (Table 10). Wahlund effect or presence of null alleles accounted for significant deviations in the Hardy−Weinberg equilibrium.

Genetic differentiation and gene flow

Pairwise F_{ST}, a measure of genetic differentiation, and gene flow (Nm) between populations are shown in Table 11. Values of F_{ST} and Nm ranged from 0.006/21.987

(Korea_Seoul/Gyeonggi−Korea_Western Gangwon) to 0.436 /0.506 (Korea_Jeonnam−Japan_Hokkaido). Negligible genetic differentiation was observed among Korean raccoon dog populations, with mean F_{ST} being 0.042 (Nm=8.613). F_{ST} between Korea_Jeonbuk and Korea_Gyeongsang populations was the highest (0.076), and the level of differentiation among Korea_Seoul/Gyeonggi, Korea_Western Gangwon and Korea_eastern Gangwon populations was relatively low (F_{ST}=0.006-0.015/Nm=14.288-21.987). Non−significant F_{ST} Value between Chungcheong and Jeonbuk in Korean raccon dogs was detected. This might be reflected by high variation among individuals between two regions, since chungcheong was the highest genetic diverse genetic group. In Japanese populations, three populations from Honshu and Shikokou islands, namely, Japan_Honshu_Kanagawa, Japan_ Honshu_Wakayama, and Japan_Shikoku populations, were moderately differentiated (F_{ST}=0.068-0.112). However, all these population were highly differentiated from Japan_Hokkaido population (F_{ST}=0.228-0.324).

Table 11. Pairwise F_{ST} below the diagonal and gene flow (Nm, above the diagonal) between Raccoon dog populations.

F_{ST} \ Nm	1	2	3	4	5	6	7	8	9	10	11
1. KOR_SG (30)		21.987	17.863	8.355	8.005	5.992	7.261	1.449	1.148	1.172	0.656
2. KOR_WG (30)	0.006*		14.288	6.045	5.454	4.856	6.183	1.419	1.118	1.112	0.642
3. KOR_EG (27)	0.009*	0.015*		8.723	8.446	6.464	7.056	1.351	1.104	1.074	0.610
4. KOR_CC (19)	0.027*	0.047*	0.025*		10.969	6.158	6.166	1.278	1.089	1.034	0.583
5. KOR_JB (29)	0.038*	0.065*	0.036*	0.016ns		10.225	4.902	1.168	0.948	0.970	0.529
6. KOR_JN(29)	0.056*	0.072*	0.051*	0.049*	0.029*		5.467	1.051	0.859	0.856	0.506
7. KOR_GS (30)	0.048*	0.059*	0.049*	0.050*	0.076*	0.066*		1.108	0.983	0.971	0.544
8. JPN_HK (30)	0.242*	0.249*	0.252*	0.253*	0.278*	0.297*	0.289*		4.334	3.424	1.574
9. JPN_ S (15)	0.270*	0.279*	0.277*	0.270*	0.308*	0.325*	0.296*	0.068*		3.663	1.032
10. JPN_ HW (30)	0.286*	0.299*	0.302*	0.303*	0.320*	0.341*	0.319*	0.112*	0.101*		1.145
11. JPN_H(13)	0.373*	0.380*	0.396*	0.416*	0.425*	0.436*	0.407*	0.228*	0.324*	0.304*	

*Significant after Bonferroni correction (P<0.003); ns Not significant; Indirect indicator of gene flow (Nm) was calculated among geographic populations using the equation, $Nm=1/4\{(1-F_{ST})/F_{ST}\}$.

PCoA showed clear separation of Japanese populations from Korean populations (Fig. 9A). Moreover, Korean populations appeared to have three genetic clusters, i.e., populations from (1) Seoul/Gyeonggi and western and eastern Gangwon; (2) Chungcheong, Jeonbuk, and Jeonnam; and (3) Gyeongsang (Fig. 9B). PCoA for the Japanese populations showed that Hokkaido was genetically distinct from the other three populations (Honshu_Kanagawa, Honshu_Wakayama, and Shikoku populations; Fig. 9C). Moreover, an additional subdivision of the Honshu_Wakayama population from Honshu_Kanagawa and Shikoku populations was evident.

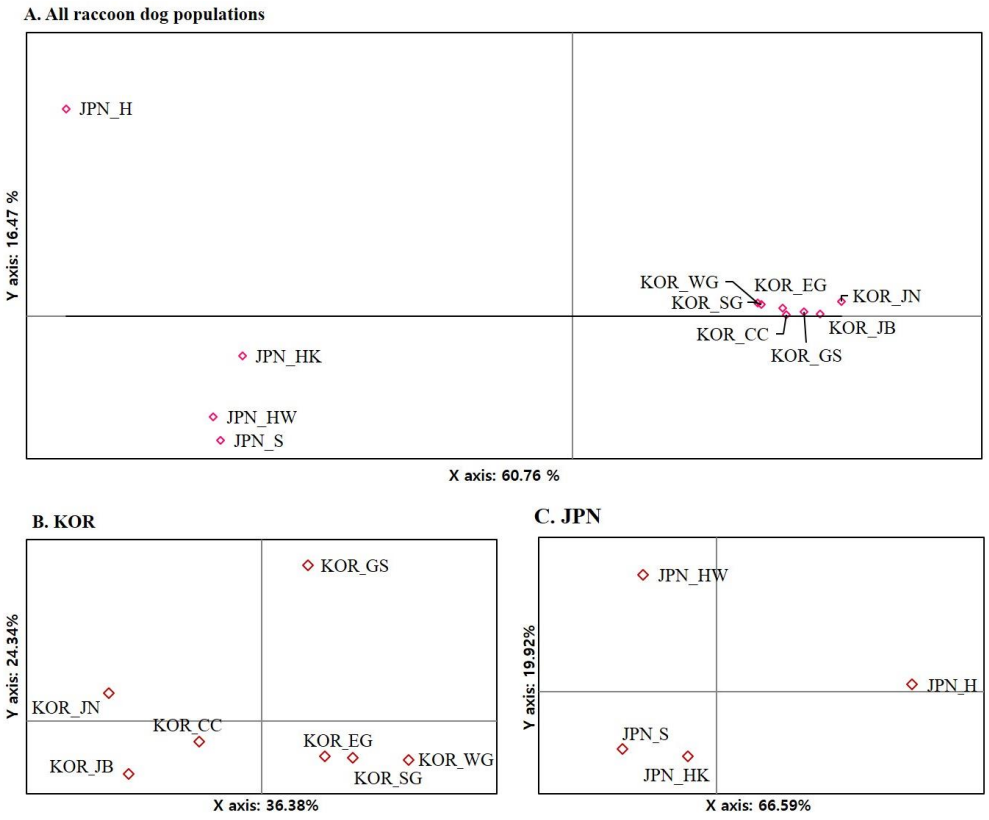

Figure 9. Scatter diagram from a principal coordinate analysis of geographic locations. A: All raccoon dog populations analysis, B: Korean population analysis, C: Japanese population analysis.

Genetic structure

STRUCTURE analysis showed that ΔK value was the highest when K was set to 2, implying that raccoon dogs in 11 areas mainly had two distinct populations, i.e., Korean and Japanese (Figs. 10A and Fig. 11A). Additional substructures within each Korean and Japanese populations were detected and we further carried out structure analysis for both populations, respectively.

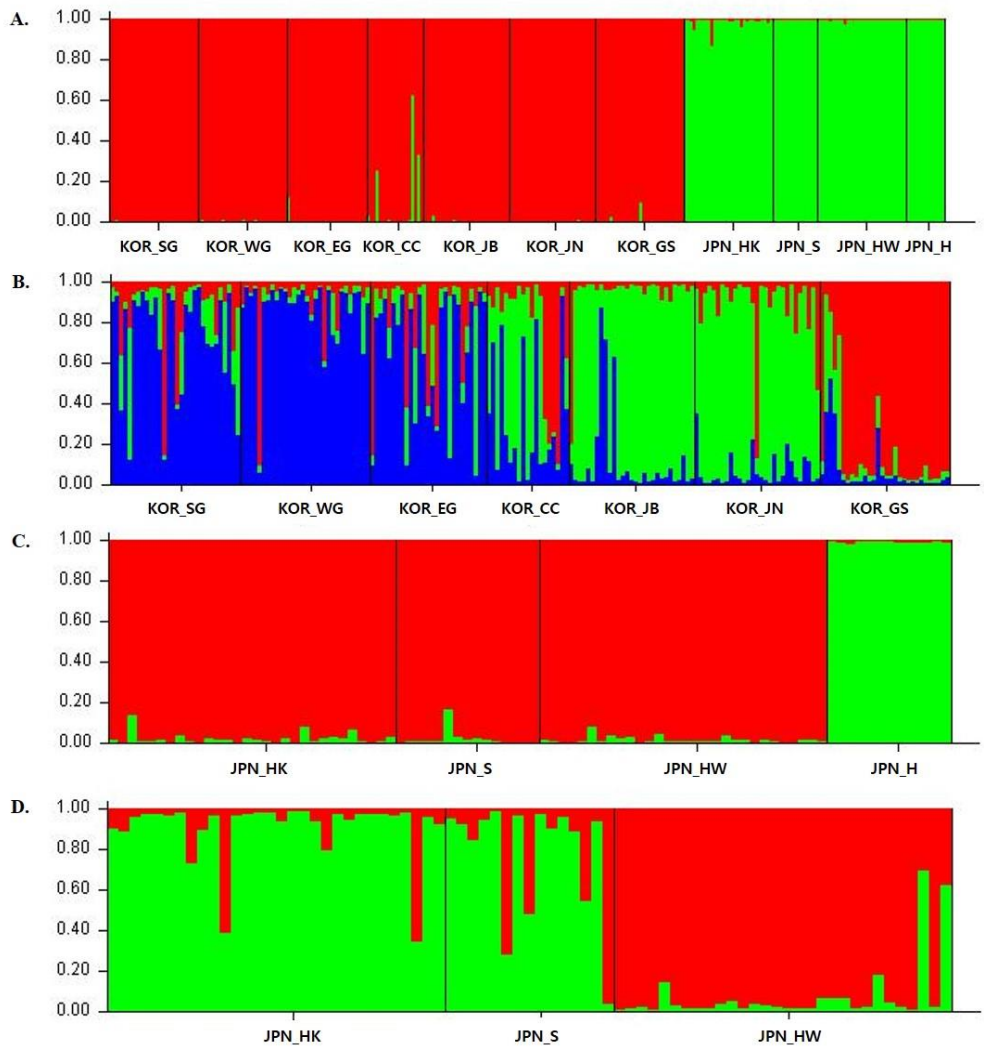

Figure 10. Bar plots of raccoon dog population identified by STRUCTURE analysis. A. All raccoon dog populations (K=2), B. Korean population (K=3), C. Japanese population (K=2), D. Japanese Honshu and Shikoku populations (K=2).

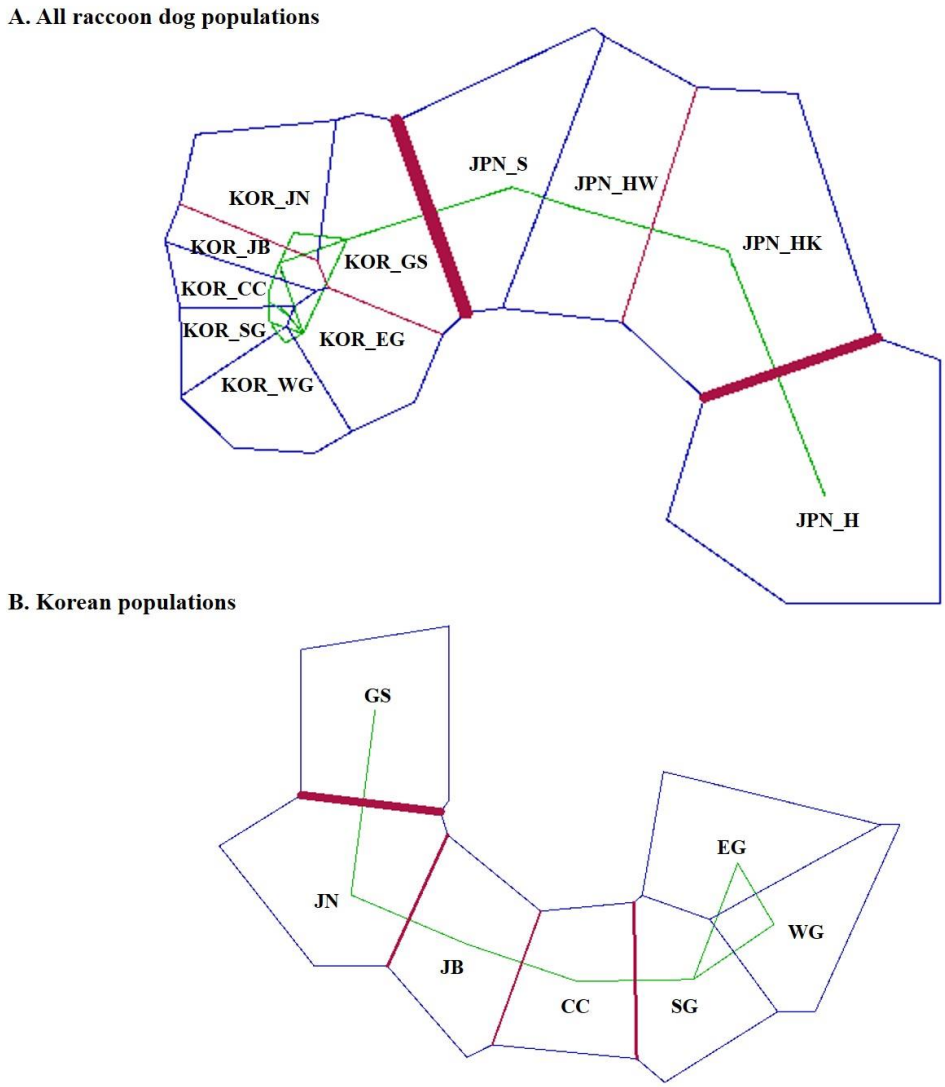

Figure 11. Geographic barriers of limited gene flow determined by BARRIER using Monmonier algorithm. The bold lines showing genetic boundaries are proportional to the strength of the barriers. The thicker lines, the stronger barriers.

The Korean population included three distinct subpopulations (Fig. 10B) even though all the Korean populations showed relatively low F_{ST} (Table 11). Except the Chungcheong population, other Korean subpopulations were mainly divided based on their geographical location, i.e., (1) northern region (Seoul/Gyeonggi and western and eastern Gangwon), (2) southwestern region (Jeonbuk and Jeonnam), and (3) southeastern region (Gyeongsang). The Chungcheong region, which is located in the center of Korea and which is grouped with the southwestern region (Jeonbuk and Jeonnam), shared its genetic structure with all the three sub-structuring areas. Although Jeolla (Jeonbuk and Jeonnam) and Gyeongsang regions occupied the neighboring areas, they showed the highest genetic differentiation (mean F_{ST}=0.071; Table 11).

A strong barrier existed between the southeastern region (Gyeongsang) and other regions, including northern (Seoul/Gyeonggi and eastern and western Gangwon), central, and southwestern (Chungcheong, Jeonbuk, and Jeonnam) regions, in Korea (Figs. 8 and 11B). Weak barriers were detected between the central and southwestern regions and

between the two southwestern regions. However, no barrier was detected among the northern regions (Fig. 11B).

The Japanese population showed the highest ΔK value at K=2 ([1] Honshu_Kanagawa, Honshu_Wakayama, and Shikoku and [2] Hokkaido; Fig. 10C). In addition, Honshu and Shikoku populations appeared to include two subpopulations, namely, (1) Honshu _Kanagawa and Shikoku and (2) Honshu_Wakayama populations (Fig. 10D). A strong barrier existed between the Hokkaido population and other three populations (Honshu_Kanagawa, Honshu_Wakayama, and Shikoku), whereas weak barriers existed among subpopulations in the Honshu and Shikoku populations (Fig. 11A).

For all subdivided subpopulations, a pie chart based on the proportion of coancestry coefficients of STRUCTURE analysis clearly showed the distinct genetic composition of each subpopulation (Fig. 8).

Regression of the genetic isolation by geographic distance (IBD) revealed significant correlation only in Korea (Fig. 12).

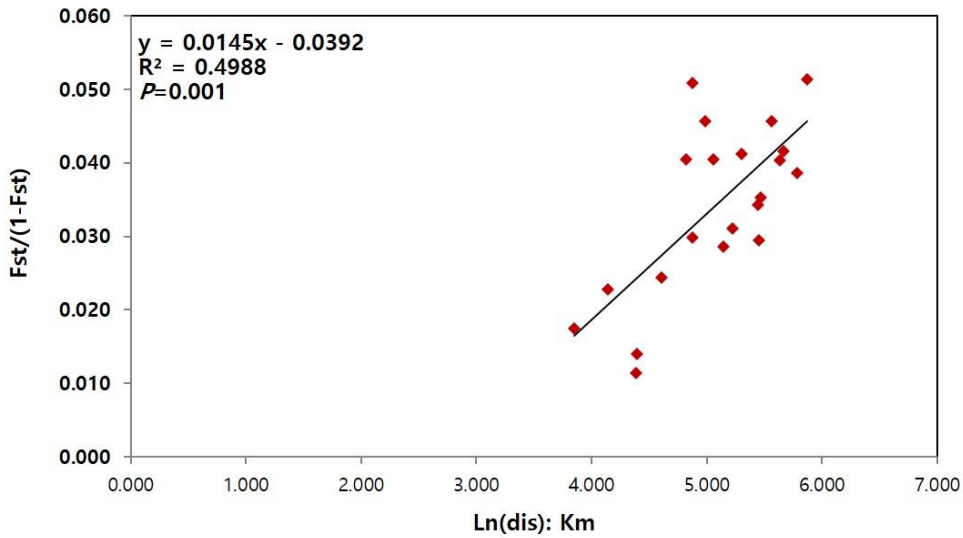

Figure 12. Regression of genetic isolation by geographic distance (IBD) for raccoon dog populations in Korea. Mantel test was carried out with 999 permutations for correlations.

Results of genetic clustering (obtained from STRUCTURE analysis) suggested four scenarios for analyzing molecular variance (AMOVA) among populations and regions: (A) two regions ([1] Korea and [2] Japan), (B) three Korean regions ([1] northern, [2] central and southwestern, and [3] southeastern regions), (C) two Japanese regions ([1] Honshu _Kanagawa, Honshu_Wakayama, and Shikoku and [2] Hokkaido), and (D) two regions in Honshu and Shikoku ([1] Honshu_Kanagawa and Shikoku and [2] Honshu_Wakayama regions). Hierarchical AMOVA showed that genetic differentiation was higher among regions (F_{RT}) than among populations within regions (F_{SR}), as observed for all the hierarchical scenarios, except D (Table 12). In scenario D, higher differentiation was observed among populations within regions (F_{SR}) than among regions.

Table 12. Analysis of molecular variance (AMOVA) of raccoon dog populations based on various five genetic groupings.

A								
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**	***F*-Statistics**	**Value**	**P-Value**
Among regions	1	419.881	419.881	1.624	23%	F_{RT}	0.227	0.001
Among populations	9	235.747	26.194	0.401	6%	F_{SR}	0.072	0.001
Among individuals	271	1573.482	5.806	0.672	9%	F_{ST}	0.283	0.001
Within individuals	282	1258.500	4.463	4.463	62%	F_{IS}	0.131	0.001
Total	563	3487.610		7.159	100%	F_{IT}	0.377	0.001
B								
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**	***F*-Statistics**	**Value**	**P-Value**
Among regions	2	73.071	36.535	0.183	3%	F_{RT}	0.030	0.001
Among populations	4	56.546	14.137	0.141	2%	F_{SR}	0.024	0.001
Among individuals	187	1209.976	6.470	0.753	12%	F_{ST}	0.054	0.001
Within individuals	194	963.000	4.964	4.964	82%	F_{IS}	0.132	0.001
Total	387	2302.593		6.042	100%	F_{IT}	0.178	0.001
C								
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**	***F*-Statistics**	**Value**	**P-Value**
Among regions	1	53.013	53.013	0.784	15%	F_{RT}	0.154	0.001
Among populations	2	53.117	26.558	0.463	9%	F_{SR}	0.108	0.001
Among individuals	84	363.506	4.327	0.485	10%	F_{ST}	0.245	0.001
Within individuals	88	295.500	3.358	3.358	66%	F_{IS}	0.126	0.001
Total	175	765.136		5.089	100%	F_{IT}	0.340	0.001

D								
Source of variation	**df**	**SS**	**MS**	**Est. Var.**	**%**	***F*-Statistics**	**Value**	**P-Value**
Among regions	1	34.956	34.956	0.159	3%	F_{RT}	0.034	0.001
Among populations	1	18.161	18.161	0.334	7%	F_{SR}	0.073	0.001
Among individuals	72	345.583	4.800	0.583	12%	F_{ST}	0.105	0.001
Within individuals	75	272.500	3.633	3.633	77%	F_{IS}	0.138	0.001
Total	149	671.200		4.710	100%	F_{IT}	0.229	0.001

A: Two regions_ 1. KOR (GS, WG, EG, CC, JB, JN and GS) and 2. JPN (HK, S, HW and H)

B: Three Korean regions_1. KOR_SG, KOR_EG, KOR_WG (Northern) 2. KOR_CC, KOR_JB, KOR_JN (Central and South-western) 4. KOR_GS (South-eastern)

C: Two Japanese regions_1. JPN_HK, HW (Honshu) and JPN_S (Shikoku) 2. JPN_H (Hokkaido)

D: Two regions of Honshu and Shikoku_1. JPN_HK (Honshu), JPN_S (Shikoku) and 2. JPN_HW (Honshu)

Bottleneck analysis did not detect any bottleneck event under TPM model at P=0.05, and distribution of allele frequencies showed a normal L−shaped distribution in all the populations (Table 13). Garza and Williamson's M value ranged from 0.757 to 0.886, implying that all the raccoon dog populations maintained a stable size over 100 generations (Table 13).

Table 13. Analysis to detect a recent population bottleneck or past population reduction event within populations.

Locality	Wilcoxon Test (TPM) H excess probability*	Mode-Shift	Garza & Williamson's M-ratio†
KOR_SG	0.46994	normal L-shaped distribution	0.799
KOR_WG	0.48997	normal L-shaped distribution	0.795
KOR_EG	0.39098	normal L-shaped distribution	0.792
KOR_CC	0.16125	normal L-shaped distribution	0.803
KOR_JB	0.12611	normal L-shaped distribution	0.810
KOR_JN	0.31609	normal L-shaped distribution	0.827
KOR_GS	0.29829	normal L-shaped distribution	0.763
JPN_HK	0.99451	normal L-shaped distribution	0.818
JPN_S	0.86157	normal L-shaped distribution	0.886
JPN_HW	0.91232	normal L-shaped distribution	0.883
JPN_H	0.98145	normal L-shaped distribution	0.757

*One-tail probability for an excess of observed heterozygosity relative to the expected equilibrium heterozygosity, computed from the observed number of alleles under mutation-drift equilibrium; TPM: two-phase model of mutation.

†M-ratio=Mean ratio of the number of allele size.

Discussion

We investigated the genetic diversity and structure of the raccoon dog population in South Korea and Japan. Population genetic data obtained in the present study showed that both Korean and Japanese raccoon dog populations were subdivided into several subpopulations. Based on the results of the present study, we proposed four potential MUs for both Korean and Japanese raccoon dog populations.

The raccoon dog population in South Korea appeared to have three subpopulations, namely, northern (Seoul/Gyeonggi, western Gangwon and eastern Gangwon), southwestern (Chungcheong, Jeonbuk and Jeonnam) and southeastern (Gyeongsang) subpopulations. The Chungcheong sub population showed more active genetic exchange with adjacent areas due to the geographic location of Chungcheong, which is the center of South Korea. A previous genetic study involving Korean raccoon dogs and 12 microsatellite markers also demonstrated that the Chungcheong raccoon dog subpopulation formed an intermediate group and that the

southeastern subpopulation (Gyeongsang) was differentiated from other subpopulations. This study asserted that gene flow was limited by the Sobaek mountains (Hong et al., 2013), whereby the high altitude of the Sobaek mountains between the southeastern and southwestern subpopulations led to a high differentiation between these subpopulations. The results of the present study also support the hypothesis that mountains are the main factors responsible for genetic structuring of this species. Melis et al. (2007) observed that raccoon dogs prefer open landscapes, agricultural lands, lakeshores, and regions with elevations lower than 300 m, but can occasionally live at up to 800 m. Two watersheds, Hanam and Geumbuk, above 400–600 m high existing between the northern and central subpopulations (Kim et al., 2004). Although their altitudes are not high for raccoon dog to limit migration, double blocking effects by two watersheds might be acted. Moreover, high Hanam Geumbuk watershed (492–1058 m) which is a joint watershed of two, must be acted as an effective barrier. The Geunam watershed (average 624 m) existing between the central and southwestern subpopulations could function as partial barrier to the migration of raccoon

dogs. On the other hand, there is the Honam watershed (average 627 m) between Jeonbuk and Jeonam; it does not seem to be a barrier for them (Kim et al., 2004). Moreover, unlike home range of raccoon dogs in rural area of Japan (<2.78 km^2), that in rural area of South Korea is quite (<0.8 km^2) (Kim et al., 2008), implying that geographical distance is another important cause of genetic differentiation among raccoon dog subpopulations. A significant correlation between the regression of genetic distance (F_{ST}) and geographical distance also supports this assumption (Fig. 12). Instead, river may not function as a geographic barrier to raccoon dog populations. We tested whether river affects migration of raccoon dogs or not with samples in SG, and locations of samples from other provinces were not adequate for testing river's impact on raccoon dog population. The F_{ST} (0.016) and STRUCTURE (K=1) analyses revealed that the Han river, which runs between the north and south of Seoul (Fig. 8), does not seem to act as a barrier (Appendix S1). Many artificial bridges and temporary connection by ice during winter time might affect to geneflow between them.

We propose four tentative MUs in the South Korean

raccoon dog population based on genetic analyses, namely, northern, central, southwestern, and southeastern subpopulations. Although the central (Chungcheong) subpopulation was grouped with the southwestern (Jeonbuk and Jeonnam) subpopulation, we suggest that the Chungcheong subpopulation is considered as another potential MU. Since the subpopulation in Chungcheong has a diverse genetic composition resulting from active interaction with raccoon dogs in other regions, it might function as a critical zone to regulate the spread of diseases by the animals. A previous study showed that a MU can be smaller than a "subpopulation" by reflecting spatial differences (Yannic et al., 2016), and the Chungcheong region might support this. For instance, the caribou in Eastern Canada is grouped into six genetic groups; however, eight MUs were eventually distinguished, which is consistent with ecological criteria (Yannic et al., 2016). Moreover, potential threats, including disease outbreaks, need to be considered to establish MUs (Yannic et a.l, 2016).

These tentative MUs might help to establish a national strategy for preventing and controlling infectious diseases

and zoonoses such as rabies, which are mainly transmitted by wild raccoon dog population. In South Korea, rabies has been reported in wild and domestic animals and in humans mostly in the northern part of Seoul/Gyeonggi and Gangwon provinces, therefore, preventive measures against rabies have been focused mainly on these areas. However, our results indicated that they were grouped as a single population with active genetic exchanges within the subpopulation of the entire Seoul/Gyeonggi and Gangwon provinces. Therefore, it would be desirable to expand the present bait vaccination program to target all the regions in the northern subpopulation. Then, the geographical barrier between the tentative northern and central MUs might be used as the first line to prevent disease dissemination to the central MU, which might further function as the second buffer zone for the southern MUs. Therefore, the status of infectious diseases in central and northern raccoon dog subpopulations needs to be closely monitored with a concern.

The Hokkaido population, which is within an isolated northern peripheral region, showed the lowest allelic richness,

small effective population size, and low gene exchange with the Honshu and Shikoku populations in Japan. This result is supported by the fact that the Hokkaido raccoon dog has been classified as a different subspecies (*N. p. albus*) from subspecies *N. p. viverrinus* in Honshu_Shikoku_Kyushu region (Kim et al., 2013). Moreover, genetic divergence between Hokkaido and Honshu_Shikoku_Kyushu regions is observed not only in raccoon dogs but also in other mammalian species of similar size, such as red fox (*Vulpes vulpes*) (Inoue et al., 2007). Several studies have reported a large difference in species composition between Honshu_Shikoku_Kyushu and Hokkaido. For example, the Japanese mole (*Mogera wogura*) and Japanese serow (*Capricornis crispus*) inhabit Honshu, Shikoku, and Kyushu. Moreover, the small Japanese mole (*Mogera imaizumii*) and Asiatic black bear (*Ursus thibetanus japonicus*) inhabit Honshu and Shikoku but not Hokkaido, whereas the Brown bear (*Ursus arctos yesoensis*) inhabits only Hokkaido (Maruyama et al., 1997; Tsuchiya et al., 2000; Yasukochi et al., 2012; Hirata et al., 2013).

PCoA, STRUCTURE clustering and regression of IBD analysis also showed that Honshu_Wakayama was

independently separated from Honshu_Kanagawa and Shikoku, despite their geographical locations. Several studies have reported genetic similarities among populations of other small mammalian species inhabiting the eastern Honshu and Shikoku regions. Two mole species, namely, *M. imaizumii* and *M. wogura*, in Shikoku showed close genetic affinity (mtDNA, cyt *b*) with populations in the northern and eastern regions of Honshu, respectively (Tsuchiya et al., 2000). Phylo geographic study of the Japanese macaque (*Macaca fuscata*) by using mtDNA (D-loop) also showed genetic similarity between populations in Shikoku and the eastern part of Honshu (Kauhala and Kowalczyk, 2011). Results of these studies suggest that the substantial differentiation of Kii peninsula in Wakayama prefecture from eastern Honshu formed a refugia in the last glacial age of the Pleistocene period and that this refugia played an important role in the geographical distribution of the abovementioned species. Suzuki et al. (1997) reported that the Kii Peninsula in Wakayama prefecture comprises borders discriminating genetic differentiation in moles and voles. Notably, Imaizumi's red-backed vole (*Myodes imaizumii*) is endemic to the

southern part of the Kii Peninsula (Musser and Carleton, 2005). This suggests that the history of raccoon dogs is similar to that of other species. Our results suggested three distinct genetic populations, namely, Hokkaido, Honshu_ Kanagawa, and Honshu_ Wakayama and Shikoku populations. However, Shikoku is an island and because of the large distance between Shikoku and Honshu_Kanagawa (approximately 600 km), we propose that populations in these two regions should be considered as separate MUs despite their genetic similarity.

Therefore, we suggest that Japanese raccoon dogs have four tentative MUs, namely, (1) Hokkaido (*N. p. albus*), (2) Honshu_Kanagawa, (3) Honshu_Wakayama, and (4) Shikoku (*N. p. viverrinus*), unless otherwise indicated by the results of more extensive and fine-scale sampling and analyses.

Our study suggested potential MUs for managing raccoon dog population in South Korea based on the observed population structure. However, the proposed MUs should be confirmed by additional studies using more samples from wider a range of areas not covered in the present study. In

South Korea, we deduced that high mountain ranges, watersheds, and geographical distances are responsible for the observed population structure, such that they limit gene flow and migration and the dispersal of individuals among subpopulations. Therefore, further studies should be performed with fine-scaled sample collection designed sample collection from potential subpopulations on both sides of tentative gene-flow barriers such as mountains, rivers, or highways. For example, genetic samples of raccoon dog subpopulations in the eastern and western regions of Baekdudaegan, the most prominent mountain range that runs north-south along the eastern side of the Korean peninsula, in Gangwon province can be analyzed to determine whether this mountain range acts as a gene-flow barrier. Because most of raccoon dog samples were from the western region of Baekdudaegan except five samples in Gangwon province. Samples from eastern region did not show any geographical differentiation from western region. Therefore, the function of Baekdudaegan as a gene-flow barrier could not be assessed in our study. In addition, the role of rivers and artificial landscape structures such as express highways,

railroads, and industrial or agricultural regions in forming the population structure should be further investigated.

The demilitarized zone (DMZ) between South and North Korea is one such example. Information on the movement pattern of wild animals across the DMZ is important to establish holistic management plans for wild animal populations in the Korean Peninsula. Especially, appearance of rabies only in the northern part of South Korea casts suspicion on the origin of rabies; it might originate from wild animal populations in the DMZ or North Korea. Although wild animal populations in the DMZ and North Korea are thought to have been separated from those in South Korea by heavy fences for the last over 60 years, some small- and medium-sized mammals are suspected to have crossed these fences. However, this possibility has not been scientifically evaluated to date. Therefore, studies on the fine-scale population structure and genetic diversity analyses may identify cryptic barriers of dispersal, cryptic or isolated subpopulations, or subpopulations with genetic deterioration.

In Japan, sea is another important factor associated with

the structure of Japanese raccoon dog population. It is evident that the sea between the Korean Peninsula and Japanese islands and the Tsugaru Strait between Hokkaido and Honshu are most significant barriers of gene flow among raccoon dog populations in Korea and Japan. However, channel between Shikoku and Honshu seems to affect the population structure less than Tsugaru Strait in these islands. This may be explained by the narrow width of the channels and many small islands located between Shikoku and Honshu and by the swimming ability of raccoon dogs. However, a fine-scale population structure study should be performed in Japanese islands to better understand the dispersal and movement patterns of raccoon dogs in Japan. Detailed information on population structure and gene flow will help in the long-term management and conservation of raccoon dog populations in Japan. Although Japan is a rabies-free country at present, the MUs proposed in the present study and results of subsequent studies will help in developing strategies for preventing and controlling disease dissemination in case there is an unexpected outbreak of rabies or other important diseases spread by raccoon dogs that affect wildlife, livestock,

and/or human health.

In conclusion, our study determined population genetic structure, gene-flow patterns, and genetic diversity of raccoon dog population in South Korea by genetic analysis of microsatellite markers. We proposed tentative MUs based on the population structure and geographical information to properly manage and conserve raccoon dog population and to develop strategies for preventing and controlling diseases spread by raccoon dogs.

Chapter 4. Phylogenetic relationships of raccoon dog (*Nyctereutes procyonoides*) based on four nuclear and Y genes

Introduction

Raccoon dog (*Nyctereutes procyonoides*) is classified as six subspecies according to 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) in Figure 13. Raccoon dog originated in East Asia was introduced to Europe in the early 20th century (Hong et al., 2013; Kauhala and Saeki, 2004; Pitra et al., 2010). Its high adaptability to various environments enables raccoon dog to increase the population size and spread fast in a short time in the introduced regions of Europe (Kauhala and Kowalczyk, 2011; Kauhala and Saeki, 2004; Pitra et al., 2010; Sutor et al., 2014).

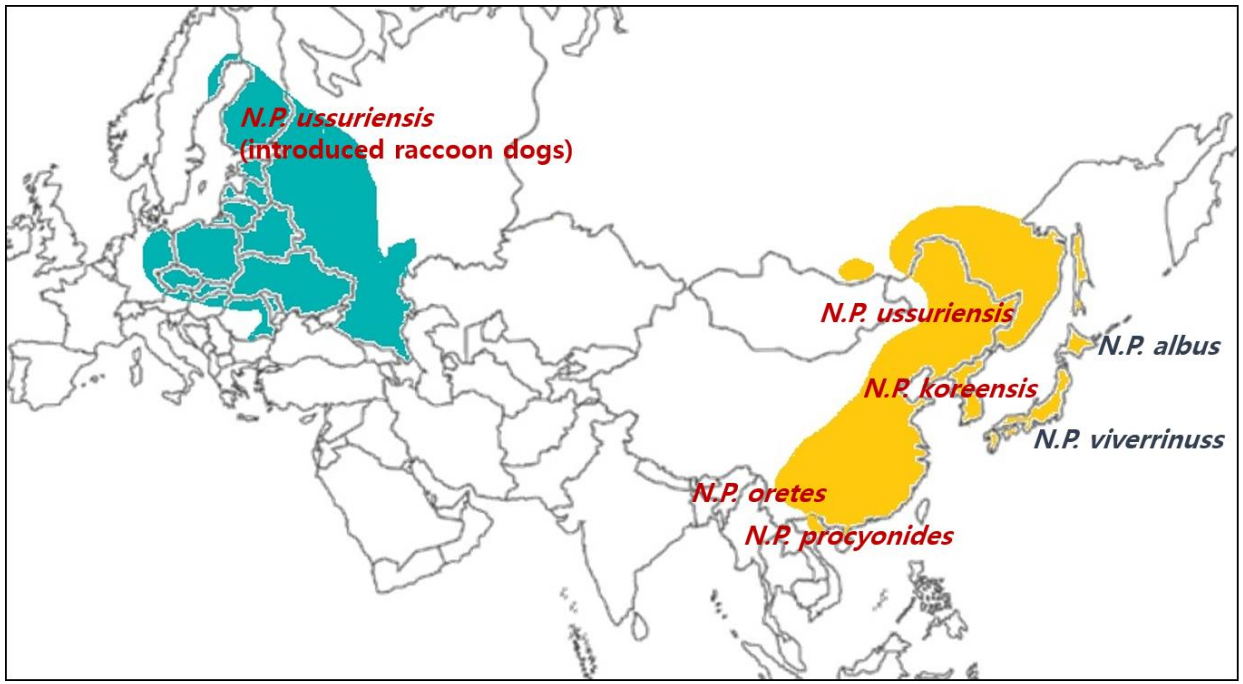

Figure 13. Geographic distribution of Raccon dog, *Nytereutes procyonoides* and Studying area and sampling information. Orange covered areas: origianal ranges, blue covered areas: introduced ranges from south-eastern Russia. (*N. p. ussuriensis*: Russia, China_eastern region, and Europe, *N. p. koreensis*: Korea, *N. p. procyonoides*: China and Vietnam_northern region, *N. p. orates*: China_Yunnan, *N. p. viverrinus*: Japan_Honshu, Shikoku, and Kyushu, *N. p. albus*: Japan_Hokkaido.

According to Ansorge et al. (2009), epigenetic differentiation was detected between original Far East Russian and introduced European raccoon dog populations, even within European populations. However, researches using genetic materials showed different results from morphological study. Although two haplogroups existed in Europe, no geographical structuring within eastern and northern European populations was detected until now (Paulauskas et al., 2015; Pitra et al., 2010). Molecular phylogeographic studies using mtDNA analysis showed no differentiation between original and Finnish (introduced from south-eastern Russia) populations (Kim, 2011). Paulauskas et al. (2015) and Hong et al. (accepted, 2018) also reported that original raccoon dogs still share the genetic composition with populations with both mtDNA and microsatellite analysis. However, information to understand the overall relationships of all raccoon dog populations in the world is still lacking. Therefore, further studies on phylogeographic and population structures of raccoon dogs not only in both East Asia and Europe but also between original and introduced groups are necessary. The most noteworthy group is Japanese raccoon dog population. Two subspecies of them inhabit Japan and there have been many researches and studies that

Japanese raccoon dogs are sufficiently differentiated from continental populations at the level of a distinct species. This issue has been debated until recently and Kim et al. (2015) suggested that Japanese raccoon dogs (*N. p. viverrinus* and *N. p. albus*) should be separated as *Nyctereutes viverrinus*, independent from continental raccoon dogs, *Nyctereutes procyonoides* renaming two Japanese species as *N. v. viverrinus* and *N. v. albus*.

External morphological difference such as fur color between mainland and Japanese populations have been reported (Korhonen et al., 1991; Won et al., 2004). Kauhala et al. (1998) found out skull size of Finnish raccoon dog (*N. p. ussuriensis*) is larger than Japanese raccoon dog (*N. p. viverrinus*) to cause the shape difference and larger tooth size in Finnish population as well. Kim et al. (2015) confirmed that Japanese raccoon dog (*N. p. viverrinus* and *N. p. albus*) have relatively smaller skulls, mandibles and carnassial teeth than Russian, Chinese and Korean raccoon dogs of mainland (*N. p. ussuriensis* and *N. p. koreensis*). According to Wada and Imai (1991), Wada et al. (1991), and Won et al. (2004), number of chromosome between mainland Asia, eastern Europe (2n= 54) and Japanese subspecies (2n=38) was different. Recent phylogeographic study using mtDNA sequences also showed high

genetic separation (Φ_{ST}=0.76) between continental and Japanese population (Kim et al., 2013). In addition, population genetic study using 16 microsatellite markers (Hong, accepted in 2018) indicated that Japanese population was differentiated from Continental (South Korean, Chinese, south-eastern Russian, Finnish and Vietnamese) populations strongly and significantly with F_{ST}=0.228.

Despite of several recent genetic researches using maternally inherited mtDNA and biparentally inherited microsatellites, genetic information using paternally inherited markers was still absent. Moreover, nuclear genes, used for phylogenetic relationship, can provide crucial information to reveal accurate relationship of East Asian raccoon dog species. Evolutionary history from diversity of genes with different mode of inheritance is necessary in estimating relationship between closely related groups (Bardeleben et al., 2005; Wahlberg et al., 2009). To better resolve this issue, we analyzed four nuclear genes and Y chromosome. Despite the high level of differentiation of karyotype, mtDNA, and microsatellite markers, we may fail to detect the existence of differentiation between continental and Japanese populations in nuclear and Y chromosome genes, because of their slower evolutionary rates than mtDNA. However, slower substitution rate reducing homoplasy and

non-coding regions more accumulating indels of nuclear markers provide important phylogenetic information (Bardeleben et al., 2005; Rokas and Holland, 2000). Moreover, sampling of multiple unlinked regions of genome is possible with nuclear markers, while mitochondrial genes offer one genealogy.

Therefore, we selected four nuclear genes: CHRNA1 (Cholinergic receptor, nicotinic, α polypeptide 1 precursor), VTN (Vitronectin), TRSP (Selenocysteine tRNA), and WT1 (Wilms tumor 1). They have been used for phylogenetic study of mammalian species including Canindae species (Bardeleben et al., 2005; Koepfli et al., 2006; Venta et al., 1996). ZFY (Zinc-finger-containing gene located on the Y chromosome) gene shows male-driven evolution (Nakagome et al., 2008), especially, SINEs (Short interspersed nuclear elements) in the ZFY gene provide useful evolutionary history for phylogenetic study (Chen and Yang, 2014; Pecon Slattery et al., 2000; Shedlock and Okada, 2000; Tsubouchi et al., 2012), because they are not eliminated once inserted to specific sites (Shedlock and Okada, 2000; Tsubouchi et al., 2012). The aims of this study are to define the relationship among raccoon dog populations including relationship between original and introduced groups, and between continental and Japanese groups.

Materials and Methods

Samples and DNA extraction

We analyzed 33 individuals for nuclear genes and 19 individuals for ZFY gene, respectively from six countries (Table 14). DNA was extracted 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) and the primer information was listed in Table 14. Each 30 μl PCR reaction contained ~50 ng of DNA, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 1 U i-star *Taq* polymerase (iNtRON Biotechnology, Korea). PCR started with an initial denaturation at 94° C for 5 min; followed by 20 cycles of 30 sec at 94 ℃ for denaturation, 30 sec at 60 ℃ for annealing (decreasing 0.5 ℃ per cycle to 50 ℃), and 30 sec at 72 ℃ for extension; 15 cycles of 30 sec at 94 ℃ for denaturation, 30 sec at 50 ℃ for annealing, and 30 sec at 72 ℃ for extension; and a final extension of 5 min at 72 ℃. PCR products were sequenced both directions.

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) in Table 14. PCR reactions were performed in 30 μl of mixture containing ~50 ng of DNA, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 1 U i-star *Taq* polymerase (iNtRON Biotechnology, Korea). PCR started with initial denaturation at 94 ℃ for 10 min; followed by 10 cycles of 45 sec at 94 ℃ for denaturation, 45 sec at 55 ℃ for annealing (decreasing 1 ℃ per cycle to 45 ℃), and 60 sec at 72 ℃ for extension; 25 cycles of 45 sec at 94 ℃ for denaturation, 45 sec at 45 ℃ for annealing, and 60 sec at 72 ℃ for extension; and a final extension of 10 min at 72 ℃. PCR products were sequenced in both directions using C-ZFY_F/C-ZFY_R primers (Tsubouchi et al., 2012).

Table14. Samples and markers information used in the study.

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

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.

Characteristics analyses of sequence

All the sequences were aligned using Geneious v4.7.6 (Drummond et al., 2009) and Clustal X (Jeanmougin et al., 1998). Each four nuclear genes were independently analyzed and then combined data was also used for analysis. We used PHASE (Stephens et al., 2001) to reconstruct haplotypes because of multiple existence of heterogeneous single nucleotide polymorphisms (SNPs) in many individuals. Individuals of homogeneous and single heterogeneous SNPs obtained one and two haplotypes, respectively, and samples containing multiple existence of heterogeneous SNP obtained more than two haplotypes according to the numbers of heterogeneous SNPs. Then, were generated genetic characteristics and diversity using DnaSP 5 (Librado and Rozas, 2009) and MEGA 6 (Tamura et al., 2013) was used to genetic distance by Kimura-2 parameters. Reconstructed haplotypes were used only for obtaining genetic characteristics, diversity and distance. For ZFY gene, DnaSP 5 (Librado and Rozas, 2009) was used to conduct haplotype diversity (*H*d), nucleotide diversity (π) and genetic distance was obtained by Kimura-2 parameters using MEGA 6 (Tamura et al., 2013). We did not combine nuclear and ZFY sequences for the analysis because of different individuals of samples.

Phylogenetic analyses

jModelTest using AIC criterion (Posada, 2008) was used for determine the model of nucleotide substitution and parameters to carry out both Maximum Likelihood (ML) and Bayesian Inference (BI). Selected models for each gene and combined data set were shown in Table 15. RaxML was used in the CIPRES (Miller et al., 2010) with a rapid bootstrapping and 1000 replicates for constructing ML phylogenetic trees of each gene and Partitioned model was selected for combined data set of four genes. Markov chain Monte Carlo (MCMC) phylogenetic tree was also conducted using Mr. Bayes (Ronquist et al., 2012). We carried out four MCMC runs and 100 thousand generations each, sampling every 100^{th} generation with the discard of first 25% of burn-in. Red fox (*Vulpes vulpes*) sequences belonging to Canidae same as raccoon dog were used as outgroup (Table 14) and the trees were visualized using Figtree v1.3.1 (Rambaut and Drummond, 2009).

Estimation of divergence time

ZFY tree and genetic distance showed the grouping with 100% of individual separation of Japanese population from continental

raccoon dogs, then, divergence time between two populations was estimated using molecular clock methods with uncorrelated and strict models of BEAST v1.8.3 (Drummond and Rambaut, 2007). GTR model for ZFY were used as nucleotide substitution model. 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), *Ailuropda/Ursus* split (7.5 Mya), and Canidae/Ursidae (40 Mya) as a root height according to Perini et al. (2010). Sequences for time priors were obtained from GenBank: *C. mesomelas* (AB622144), *C. latrans* (AB622146), *C. lupus* (AB622147), *V. vulpes* (AB622140), *V. lagopus* (AB622141), *A. melanoleuca* (AB261814), and *U. americanus* (AB261809). In MCMC, 10,000,000 generations of burn-in was used for length of chain. The chain was sampled 1,000 times every 1,000 cycles.

Results

Sequence characteristics of nuclear and ZFY genes

We determined partial sequences of each of four nuclear autosomal genes ranging from 285 to 461 bp including indels (Accession No.: MH209078 – MH209221, Table 1), and all nuclear genes contained variable sites and different numbers of haplotypes by reconstruction process (Table 15). Individuals from two in CHRNA1, 8 in TRSP, and 13 in combined data obtained more than two haplotypes. Nucleotide (π) and haplotype diversity (Hd) of four genes ranged from 0.0011 to 0.058, and 0.415 to 0.927, respectively.

TRSP gene showed nine variable sites, the highest nucleotide substitution rate (2.7%), and VTN was the most conservative gene with 0.5% of substitution rate, and four indels were observed only in WT1 gene. About 48% of the variable sites were parsimony informative, ranged from 37.5 % in WT1 to 100% in CHRNA1. The combined four nuclear genes, 1,493 nucleotides of data set consist of 13% of coding region and 87% of non-coding region. Most of variable and parsimony informative sites and indels were shown in the non-coding regions except for one singleton site occurred at

the coding region in VTN gene of one Japanese individual. This single mutation, C to Y (C or T), in flanking region of VTN was nonsynonymous mutation which is changing arginine to cysteine. Moreover, the other nucleotide substitution in VTN also occurred within Japanese individuals.

Table 15. 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	*Hd*
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	2/33	0.0003	0.351

Var. sites: Variable sites, PI sites: Parsimony informative sites, Hap No: Haplotype number; Seq No: Sequence number, π: Nucleotide diversity, *Hd*: Haplotype diversity.

Genetic difference of combined data among all populations from six regions (Korea, China, Russia, Finland, Vietnam, and Japan) ranged from 0.002 to 0.004 (Table 16) and no significant differentiation was detected among populations. Mean genetic distance within continental population (d=0.003) was higher than that within Japanese population (d=0.001), Finnish population showed the highest mean genetic distance (d=0.003). Genetic distance between Russian and Finnish raccoon dogs was 0.003, especially highest with d=0.007 in TRSP gene (Table 17b). Moreover, in TRSP gene, Finnish population showed relatively higher genetic difference than other populations. Genetic distance between continental and Japanese populations in combined data was 0.003 without significant structure and each gene also revealed no differentiation and sharing haplotypes among populations except VTN gene. Each continental and Japanese populations have their own haplotypes respectively, indeed, no genetic difference (d=0) was revealed within continental five populations. However, distinct separation with d=0.002 between continental and Japanese populations was detected. Complete ZFY gene (936 bp) had a single mutation site among samples (Accession No.: MH209222 – MH209240, Table 1), then, two haplotypes were obtained (Table

15). Values of nucleotide diversity (π) and haplotype diversity (*H*d) were 0.0003 and 0.351, respectively. One nucleotide substitution of SINE I in SFY gene led separation between continental groups (Korea, China, Russia, Finland and Vietnam) and Japanese group with small degree (d=0.001, Table 16) implying that all 15 continental individuals shared the same nucleotide composition, and only one haplotype was obtained from five Japanese raccoon dogs.

Table 16. Kimura-2-Parameters of four nuclear combined data (below diagonal) and ZFY gene (above diagonal) among raccoon dog populations.

	KOR	**CHN**	**RUS**	**FIN**	**VNM**	**JPN**
KOR		0.000	0.000	0.000	0.000	0.001
CHN	0.002		0.000	0.000	0.000	0.001
RUS	0.002	0.002		0.000	0.000	0.001
FIN	0.003	0.003	0.003		0.000	0.001
VNM	0.002	0.002	0.002	0.003		0.001
JPN	0.003	0.004	0.003	0.004	0.002	

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

Table 17. Kimura-2-Parameters of each four nuclear genes among raccoon dog populations.

a. CHRNA1	KOR	CHN	RUS	FIN	VNM	JPN
KOR						
CHN	0.000					
RUS	0.000	0.000				
FIN	0.000	0.000	0.000			
VNM	0.000	0.000	0.000	0.000		
JPN	0.000	0.000	0.000	0.000	0.000	

b. TRSP	KOR	CHN	RUS	FIN	VNM	JPN
KOR						
CHN	0.004					
RUS	0.007	0.002				
FIN	0.004	0.005	0.003			
VNM	0.005	0.004	0.001	0.004		
JPN	0.003	0.005	0.004	0.004	0.006	

c. VTN	KOR	CHN	RUS	FIN	VNM	JPN
KOR						
CHN	0.000					
RUS	0.000	0.000				
FIN	0.000	0.000	0.000			
VNM	0.000	0.000	0.000	0.000		
JPN	0.002	0.002	0.002	0.002	0.002	

d. WT1	KOR	CHN	RUS	FIN	VNM	JPN
KOR						
CHN	0.002					
RUS	0.004	0.004				
FIN	0.002	0.003	0.002			
VNM	0.003	0.002	0.003	0.004		
JPN	0.003	0.002	0.002	0.004	0.001	

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

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 very low enough to ignore them. ML tree of four genes combined was shown representatively (Fig. 14).

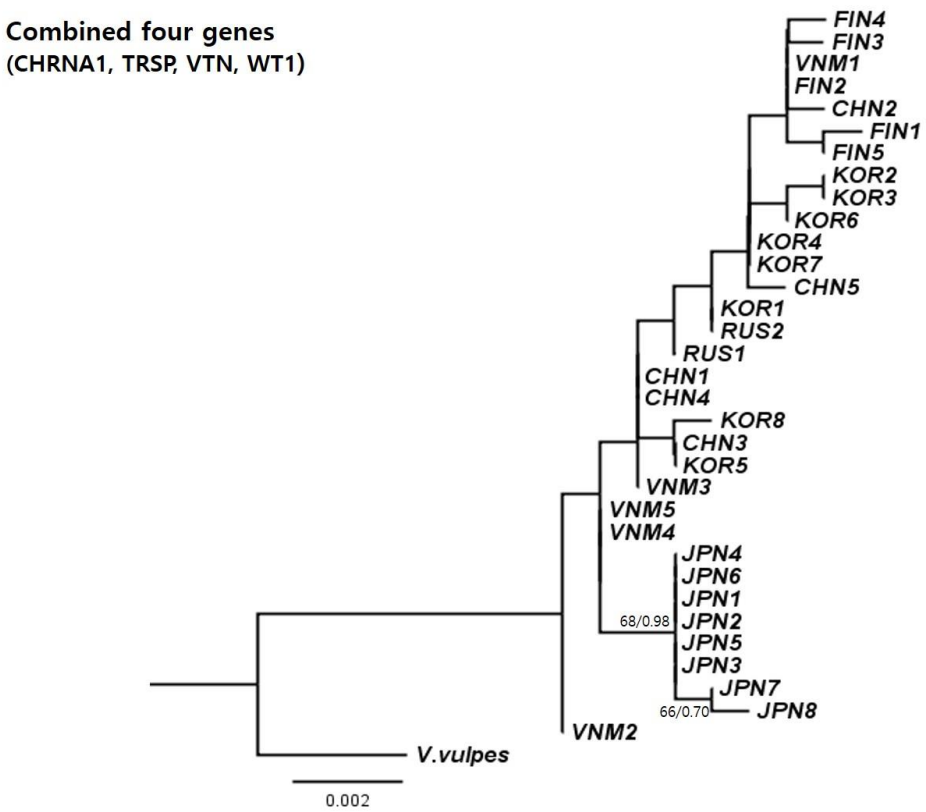

Figure 14. Phylogenetic trees of the four nuclear combined data. Bootstrap for ML and Bayesian posterior probability are shown for branches with over 65% support. Sequence from the red fox (*Vulpes vulpes*) was used as an outgroup.

All phylogenetic trees of each genes and combined data revealed no genetic divergence among continental Korean, Chinese, Russian, and Vietnamese raccoon dogs, nor between original Russian and introduced Finnish individuals (Figs. 14 and 15). However, slight but on going proceeding of separation between continental and Japanese groups was shown, especially, definite differentiation of Japanese group from continental groups was detected in VTN gene (Fig. 15c).

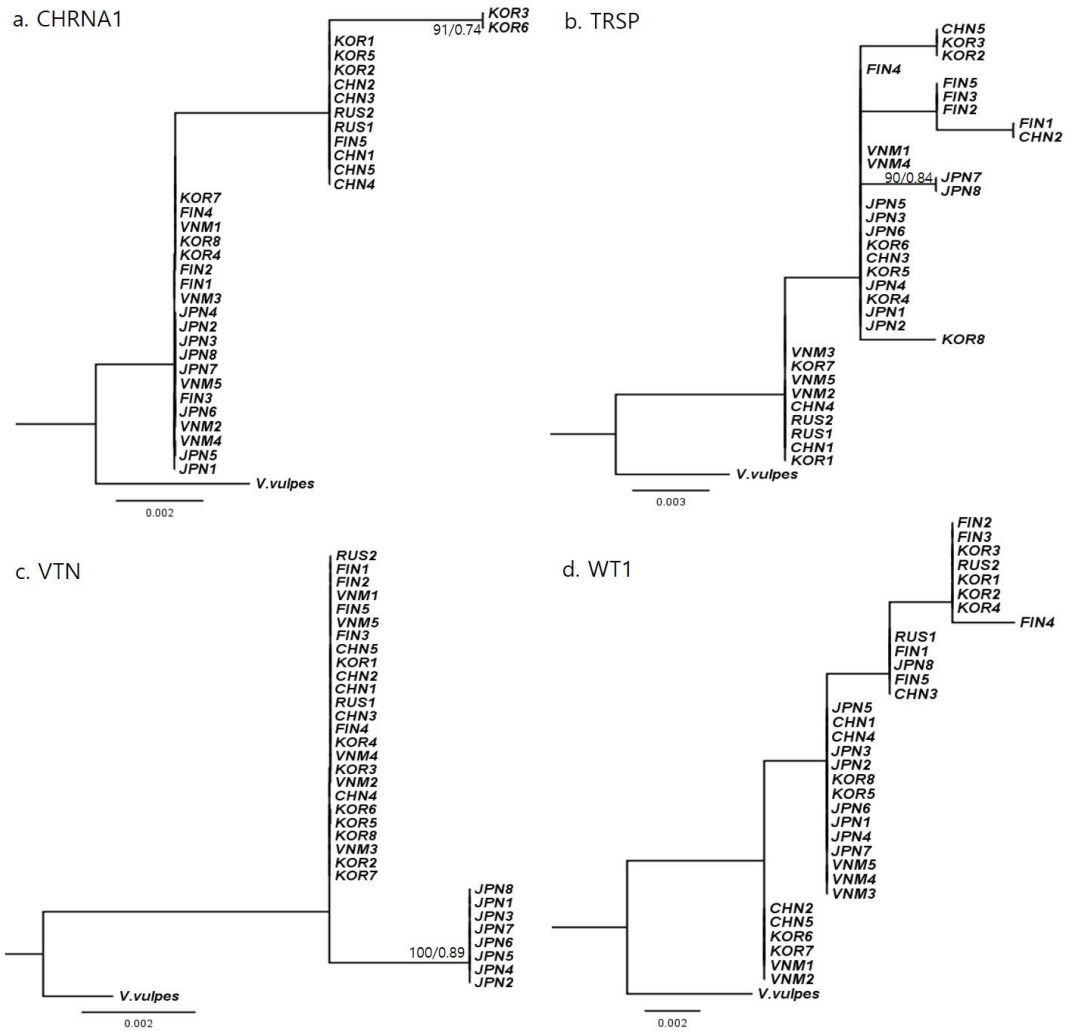

Figure 15. Phylogenetic trees of the four nuclear genes. Bootstrap for ML and Bayesian posterior probability are shown for branches with over 70% support. Sequence from the red fox (*Vulpes vulpes*) was used as an outgroup. a: CHRNA1, b: TRSP, c:VTN, d: WT1.

ML and BI method conducted same phylogenetic trees of ZFY gene. Representative ML tree in Figure 16 indicated that Japanese population constituted separate clade from 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.

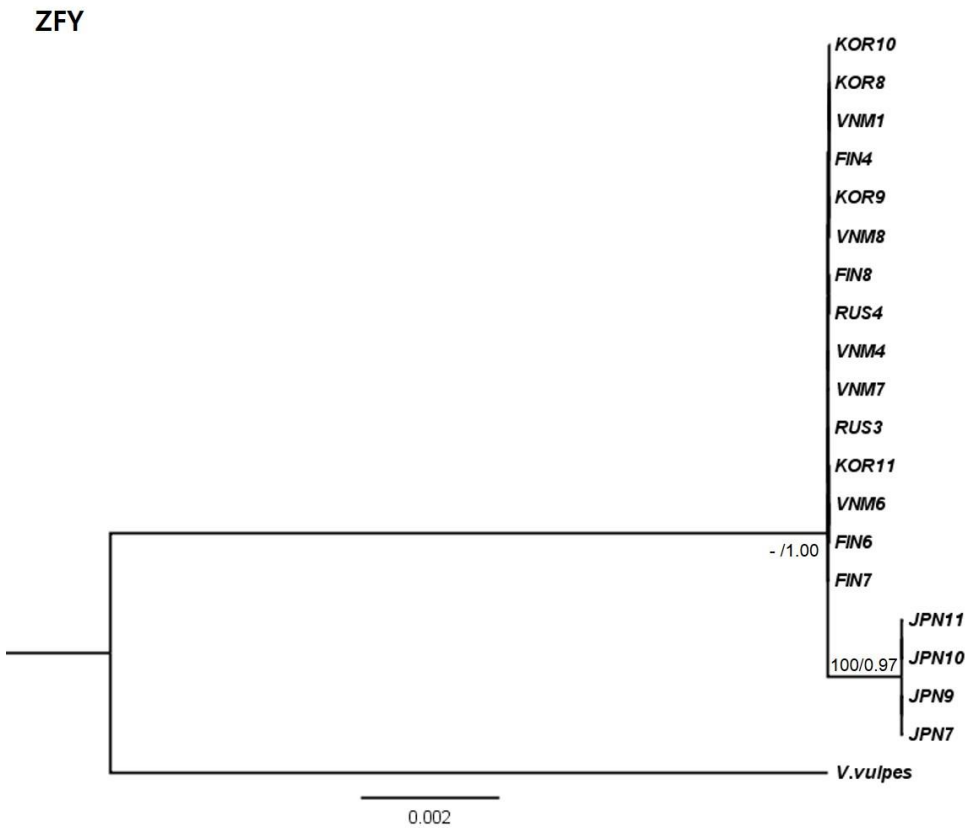

Figure 16. Phylogenetic trees of the ZFY gene. Bootstrap for ML and Bayesian posterior probability are shown for branches with over 90% support. Sequence from the red fox (*Vulpes vulpes*) was used as an outgroup.

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 ZFY gene was about 0.19 Mya, more recent than by mtDNA (0.59–0.67 Mya; Kim (2011)).

Discussion

No geographical grouping was detected not only among continental populations but also between continental and Japanese populations from CHRNA1, TRSP, WT1 and combined data. In general, nDNA has relatively slower rate of nucleotide substitution than mtDNA and sex-linked gene like ZFY (Bardeleben et al., 2005; Erlandsson et al., 2000; Feng et al., 2001; Song et al., 2016; Trujillo et al., 2009). Our study showed that haplotype diversity was high though, nucleotide diversity was low, which indicate only sequence small differences between haplotypes, supporting slow mutation rate. Moreover, Wang et al. (2008) emphasized that nuclear markers might not fully reflect recent speciation because of insufficient time for fixed molecular variation, even though other selective genetic markers or morphological differentiation have occurred, already.

However, two genetic clades existed in East Asia in VTN and ZFY genes; Japanese and continental clades; strengthening the separation of Japanese raccoon dog from continental group. Although mutation rate of VTN was the lowest with 0.5, VTN might be the strong marker to detect evolutionary history in raccoon dogs.

One nuclear nonsynonymous mutation from arginine into cysteine in VTN was occurred at 5' Flanking region which contains the promotor to regulate transcription and gene expression. Even though this mutation was not occurred in the transcriptional binding sites, mutation in flanking region has high possibility to change in transcription, regulation or its function (D'Souza et al., 2004; Fields and Gainer, 2015; Hayashi et al., 1991), thus it finally might affect the phylogenetic history. VTN, expressed in blood, is a gene associated with diseases involving platelet disorders and immune response (Schvartz et al., 1999). It also regulates proteolysis (Schvartz et al., 1999; Singh et al., 2010). Therefore, genetic mutation by VTN related diseases might be occurred. 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 to mice which reflects a possibility not to detect occurrence of genetic mutation in VTN virtually due to lack of visible sign.

Despite genetic difference in VTN gene was very low, 0.002 between continental and Japanese populations is the value to divide them into different genetic groups. Comparison of interspecific

variation within Canidae supports this separation is significant. Genetic distance among four *Canis* species (*Canis mesomelas*, AY885411; *Canis lupus*, AY885410; *Canis latrans*, AY885409; *Canis aureus*, AY885407; (Bardeleben et al. 2005)) ranged d=0 – 0.004, particularly, three of them except *Canis mesomelas* showed 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 0.002 – 0.004. Therefore, Japanese raccoon dogs seem to be distinct as a different species from continental raccoon dog.

In 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 (Chen and Yang, 2014; Pecon Slattery et al., 2000; Shedlock and Okada, 2000; Tsubouchi et al., 2012). Besides, the genetic distance between continental and Japanese populations was 0.001, this much of differentiation was observed in other Canidae species, between *Canis lupus* / *Canis familliaris* and *Canis latrans*, respectively in 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 are considered as independent species.

We estimated split time of Japanese raccoon dogs from continental counterparts as 0.19 Mya in ZFY. Divergence of ZFY gene seemed to occur diverge during the last glacial period of Pleistocene. According to the previous analyses using mtDNA, (Pitra et al. 2010) suggested the migration of raccoon dog groups from continent to Japan during 0.48 – 1.37 Mya, mean= 0.87 Mya) and Kim (2011) calculated the divergence time approximately 0.59 – 0.67 Mya. Both of time estimations correspond to middle of Pleistocene, especially, Kim et al. (2013) showed that Japanese raccoon dog migrated from continent before the last glacial period of Pleistocene and adapted to the new environment. Therefore, divergence of ZFY gene must occurred more recently after migration.

In conclusion, we confirmed that there exist only one phylogenetic clade among continental raccoon dog populations, including Finnish populations originated from eastern Russia by analysis of four autosomal nuclear genes and ZFY gene. However, Japanese raccoon dogs were separated from main continental group

through the analysis of nuclear DNAs and ZFY gene. After migration, Japanese population has differentiated by adapting to different environment. Different chromosomal numbers (Wada and Imai, 1991; Wada et al., 1991) and morphological characters (Kauhala et al., 1998; Kim et al., 2015; Korhonen et al., 1991; Won et al., 2004) and restricted gene flow in mtDNA (Kim et al., 2013) also support the speciation between them. In recent, reproduction between two populations are limited by geographical isolation. Therefore, Japanese raccoon dog is suggested to be classified as an independent species from continental *Nytereutes procyonoides.*

General Discussion

The microsatellite markers developed in this study are firstly described specifically for *N. procyonoides koreensis*. Finally, all 12 microsatellite markers can be successfully applied to population genetics studies. We confirmed their utility for application in other raccoon dog subspecies. The markers developed here contributed to population genetic studies of raccoon dog species in Korea, as well as populations in other areas.

The genetic diversity and structure of raccoon dog populations were examined across most of the species' geographic range. Analyses revealed that population structure of raccoon dogs in East Asia and Europe generally agrees with the currently recognized intraspecific taxonomy. These findings evidence the agreement among genotype distributions provided by microsatellite markers, phylogenetic analysis based on mtDNA, and morphologic characters. In addition, higher in central and relatively lower in marginal populations were shown in genetic diversity.

The continental population, especially Chinese and Russian

subpopulations, had higher genetic diversity and larger effective size than the island population. The low genetic diversity and high genetic differentiation found in isolated Japanese subpopulations suggest that the species retains central–marginal trends in genetic diversity, even resulting in the allopatric speciation of isolated island populations. Strong genetic differentiation and lack of gene flow between continental and Japanese raccoon dogs are consistent with the findings of several other studies, which suggested that the Japanese raccoon dog should be classified as a separate species.

Chinese and Russian populations revealed higher genetic diversity than the Vietnamese population, but similar levels to that of the Korean population. Both central populations showed lower genetic diversity but slightly higher allelic richness than the marginal South Korean population. In East Asia, the Chinese and Russian populations as central populations might help maintaining the genetic diversity. Although the Korean Peninsula is marginal to the East Asian continent, the genetic diversity of the South Korean population was similar to that of the central populations and presented a moderate level of genetic differentiation from them. The Korean Peninsula might have been a refugium for raccoon dogs to maintain the gene pool and differentiate itself from other

continental populations (Kim et al., 2013). The lowest levels of Vietnamese raccoon dogs are probably due to the peripheral isolation of the population that inhabits the southern limit of the species' range.

The Finnish population showed values of genetic diversity similar to the Russian population, and shared genetic features with raccoon dogs within the species' native range in Russian Far East and China. However, despite being included within the same genetic population as Chinese and Russian subpopulations, the Finnish raccoon dog subpopulation had a slightly genetically different composition.

The Hokkaido population, inhabiting an isolated and northern peripheral region, showed the lowest genetic diversity and significant genetic differentiation from the Honshu and Shikoku population, which was similar to the differentiation between continental and island populations. This finding implies that the Tsugaru Strait, between Honshu and Hokkaido (19.5 km), is a strong barrier to raccoon dog migration. Therefore, results of the present study agreed with that of previous studies indicating that raccoon dog in Hokkaido is a different subspecies (Kim et al., 2013, 2015).

To implicate for management of raccoon dog mediated diseases in South Korea and Japan, the present study showed that both Korean and Japanese raccoon dog populations were subdivided into four subpopulations, respectively. Based on the results of the present study and geographical information to properly manage and conserve raccoon dog population and to develop strategies for preventing and controlling diseases spread by raccoon dogs, we proposed potential MUs for both Korean and Japanese raccoon dog populations.

In South Korea, we deduced that high mountain ranges, watersheds, and geographical distances are responsible for the observed population structure. Therefore, further studies should be performed with fine-scaled sample collection designed sample collection from potential subpopulations on both sides of tentative gene-flow barriers such as mountains, rivers, or highways.

In Japan, sea is an important factor associated with the structure of Japanese raccoon dog population. Although Japan is a rabies-free country at present, the MUs proposed in the present study and results of subsequent studies will help in developing strategies for preventing an unexpected outbreak of rabies or other

important diseases spread by raccoon dogs.

According to the result of phylogenetic study using nuclear and Y genes, no geographical grouping was detected not only among continental populations but also between continental and Japanese populations from CHRNA1, TRSP, WT1 and combined data. However, two genetic clades existed in East Asia in VTN and ZFY genes; Japanese and continental clades; strengthening the separation of Japanese raccoon dog from continental group. In conclusion, we confirmed that Japanese raccoon dogs were separated from main continental group through the analysis of nuclear DNAs and ZFY gene.

After migration, Japanese population has differentiated by adapting to different environment. Different chromosomal numbers (Wada and Imai, 1991; Wada et al., 1991) and morphological characters (Kauhala et al., 1998; Kim et al., 2015; Korhonen et al., 1991; Won et al., 2004) and restricted gene flow in mtDNA (Kim et al., 2013) also support the speciation between them. In the present study, we also found out same results from microsatellites, nuclear DNAs, and ZFY genes analysis. In recent, reproduction between

two populations are limited by geographical isolation. Therefore, Japanese raccoon dog is suggested to be classified as an independent species from continental *Nytereutes procyonoides.*

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Appendix

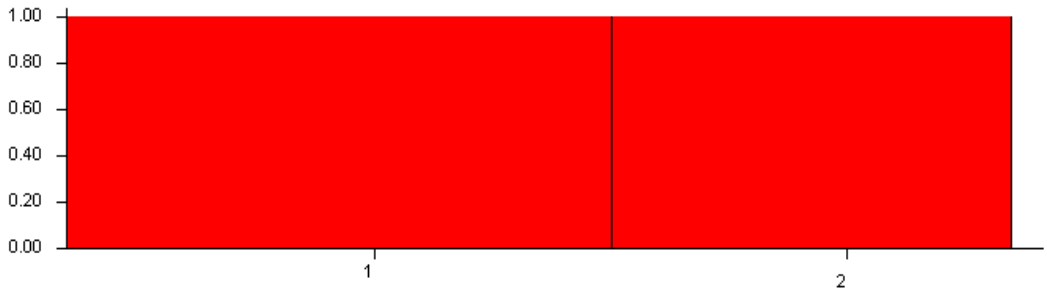

Figure S1. Bar plots of Seoul/Gyeonggi raccoon dog population identified by STRUCTURE analysis (*K*= 1). 1: KOR_SG_Northern part from Han River, 2: KOR_SG_Southern part from Han River.

너구리의 집단유전학 및 계통지리 연구

서울대학교 대학원

수의학과 수의생화학 전공

홍 윤 지

지도교수 이 항

국문 초록

너구리 (*Nyctereutes procyonoides*)는 동아시아 토착종이지만

유럽지역에 인위적으로 도입되어 서식하고 있어 전세계적으로 동아시아와 유럽에 걸쳐 분포하고 있으며, 현재 6개 아종으로 분류되어 있다. 너구리는 잡식성으로 생태계에서 먹이사슬 균형을 유지하는 중요한 역할을 하고 있으며, 또한 훌륭한 청소동물(scavenger) 역할을 수행한다. 너구리는 새로운 환경에 뛰어난 적응력을 갖고 있고 이것은 사람에 의해 변화된 여러 환경에서 그 개체수가 빠르게 증가하는 데 큰 역할을 했다. 이러한 변화된 환경에서의 너구리의 빠른 개체수 증가로 인해 각종 질병 및 기생충, 특히 한국에서는 인수공통전염병의 주요 매개인자로서의 역할에 대한 문제가 대두되고 있다. 너구리는 유럽(러시아 서부)으로 도입된 너구리 개체들은 유럽의 다른 여러 나라들로 빠르게 확산되면서 개체수를 늘려나갔다. 이는 유럽 지역의 생태계 교란과 새로운 매개체에 의한 인수공통 전염병 확산에 대한 문제를 야기해왔다. 또한, 원래의 서식지와 도입된 지역에 서식하는 너구리의 계통유전학적 관계와 집단유전학적 연구들이 근래에 시작되고 있다. 그러나, 그들의 관계와 집단구조를 이해하기 위해서는 더 심도 있는 연구가 필요한 시점이다. 지금까지 너구리에 관한 유전적 연구는 일부 특정 지역 개체군에 국한되어 있었기 때문에, 세계적인 너구리 종의 유전적 다양성 및 구조에 대한 연구가 필요하다. 이러한 너구리 집단구조에 대한 이해는 너구리 개체군 관리와 더불어, 너구리를 매개로 전파되는 질병관리를 위한 관리 단위 설정에 있어서도 유용한 정보를 제공할 것이다. 더불어, 최근에는 일본에 서식하는 너구리 집단은

대륙에 서식하는 집단과는 다른 종으로 구분되어져야 한다는 주장이 선행연구들 (핵형, 형태, 미토콘드리아 DNA 및 microsatellite 분석)을 통해 제기되었다. 일반적으로 근접한 그룹 사이의 유전적 관계를 보기 위해서는 여러 다른 유전자들의 진화적 흐름을 비교하는 것이 필요하지만, 너구리의 핵 DNA와 Y유전자를 통한 종 분류를 위한 계통적 증거는 아직 알려진 바 없다.

너구리 집단의 유전적 다양성 및 구조적 관계를 이해하기 위해 1) 한국너구리의 유전자 시료를 이용하여 12개의 종 특이적 microsatellite marker를 개발하였고, 기 개발된 4개의 microsatellite marker를 함께 사용하여 너구리 집단의 유전적 다양성을 확인하였다. 2) 한국, 중국, 남동 러시아(우수리지역), 핀란드(극동 러시아에서 도입됨), 베트남, 일본(혼슈와 홋카이도) 지역의 너구리 집단 시료를 이용하여 원서식지 내 집단 간, 그리고 원서식지와 도입된 서식지의 집단 간의 유전적 다양성 및 구조를 비교하였다. 또한, 3) 동아시아 너구리의 장기적인 보전 및 관리 방안을 위해 한국과 일본 집단은 추가적인 집단분석을 진행하였다. 마지막으로, 4) 핵DNA와 Y유전자 분석을 통해 대륙 내 너구리 집단 간, 원서식지와 도입서식지 간의 계통관계 및 대륙과 일본 간 너구리의 계통학적 관계를 살펴보았다.

새로운 종특이적 마커개발 결과, linkage disequilibrium과 null allele이 없는 12개의 polymorphic microsatellite marker (*Nyct 1 – 12*)를 성공적으로 개발하였으며, 한국 집단 및 다른 집단 연구에도

확대, 적용시킬 수 있는 유용한 결과를 얻었다. 두 번째, 본 연구에서 개발된 12개의 종특이적 마커와 개과에서 기개발된 4개의 마커 등 총 16개의 마커를 사용하여 분석한 집단 연구에서 너구리는 2개의 큰 유전적 집단 (대륙_한국, 중국, 러시아, 핀란드, 베트남; 섬_일본)으로 나뉘어졌으며, 유전적 분화 정도는 F_{ST} = 0.236으로 유의미한 결과를 나타냈다. 두 개의 큰 그룹 중 대륙의 너구리 내에서는 한국, 중국_러시아, 베트남, 3개의 집단이 확인됐으며, 중국_러시아 집단은 도입 집단인 핀란드 집단과 근연관계에 있음을 보여 주었다. 일본 너구리의 경우도 뚜렷하게 2개의 집단(혼슈, 홋카이도)으로 나뉘는 것을 볼 수 있었다 . 이러한 동아시아 너구리의 유전적 다양성과 적 구조는 자연적인 지리적 장벽에 의한 영향을 받은 것으로 유추할 수 있고, 집단의 유전적 다양성의 경우, 전형적인 중심부_주변부 양상을 따르고 있음을 보였다 (대륙 vs. 섬, 대륙 내 중심부 vs. 주변부, 대륙 내 공급 vs. 도입). 특히, 대륙과 섬 너구리 집단 간의 높은 유전적 분화도는 이들이 종 수준으로 분화가 되었음을 고려해야 한다는 선행 연구들의 결과와 일치하였다. 세 번째, 동아시아 너구리의 관리 방안을 위한 한국 및 일본 너구리 집단 분석을 통해서는, 한국과 일본집단 내에서 각각의 아집단 구조가 형성되어 있음을 알 수 있었다. 이러한 유전적 구조와 유전자 흐름을 바탕으로 잠정적인 너구리의 관리단위(management units, MUs)를 제안하였다. 한국 너구리 집단은 (1. 북부, 2. 중부, 3. 남서부, 4. 남동부)집단으로 일본 너구리 집단은 (1. 홋카이도, 2.

혼슈_가나가와, 3. 혼슈_와카야마, 4. 시코쿠)집단으로 각각 4개의 관리단위로 설정하는 것을 제안하였다. 본 연구를 통해 너구리 집단 구조와 관리단위는 향후 한국과 일본의 너구리 집단을 관리하는 실질적인 전략을 세우는 데 기여할 것으로 보이며, 특히 광견병 등 인수공통전염병의 주요 매개체인 너구리를 통해 확산될 수 있는 전염병 예방 정책에 크게 기여할 것으로 기대된다. 마지막으로, 4개의 핵DNA (CHRNA1, VTN, TRSP, WT1)와 Y유전자 (ZFY)를 통한 계통분류 분석에서는 도입지역의 너구리 집단을 포함해서 대륙 내 너구리 집단 간에는 유전적 분화가 일어나지 않은 것으로 나타났다. 반면, 대륙과 일본 너구리 집단 간에는 뚜렷한 유전적 분화를 확인할 수 있었으며, 기존에 연구된 유전자들에 비해 상대적으로 진화속도가 느린 핵DNA와 Y유전자에서도 일본 너구리 집단이 대륙의 집단과는 상당한 분화가 진행되었음을 시사한다. 따라서, 일본의 너구리는 대륙의 너구리와는 다른 종으로 분류되어야 함을 제안한다.

주요어: 너구리, *Nyctereutes procyonooides*, 유전적 집단구조, 유전적 분화, Microsatellite마커, 관리단위, 핵DNA, Y유전자

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The Impossible Dream (이룰 수 없는 꿈)

그 꿈, 이룰 수 없어도
싸움, 이길 수 없어도
슬픔, 견딜 수 없다 해도
길은, 험하고 험해도

정의를 위해 싸우리라
사랑을 믿고 따르리라
잡을 수 없는 별일지라도
힘껏 팔을 뻗으리라

이게 나의 가는 길이요
희망조차 없고 또 멀지라도
멈추지 않고 돌아보지 않고
오직 나에게 주어진 이 길을 따르리라

내가 영광의 이 길을 진실로 따라가면
죽음이 나를 덮쳐와도 평화롭게 되리
세상은 밝게 빛나리라
이 한 몸 찢기고 상해도
마지막 힘이 다할 때까지

가네
저 별을 향하여

맨 오브 라만차 (The man of Lamancha) 중