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Examining multiple paternity in the raccoon dog (*Nyctereutes procyonoides*) in Japan using microsatellite analysis

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ABSTRACT. We analyzed the genotypes of three pregnant females and their litters to investigate the phenomenon of multiple paternity in wild raccoon dogs (*Nyctereutes procyonoides*) using 17 microsatellite markers. If a female has mated with only one male during estrus, then the maximum number of paternal alleles will not exceed two among littermates with the same father. The results revealed two out of three litters had three or four paternal alleles at one or five microsatellite loci. Therefore, the female had mated with more than one male during estrus. To the best of our knowledge, the present study is the first to report the possibility of multiple paternity in wild raccoon dogs.

KEY WORDS: microsatellite, multiple paternity, raccoon dog

The raccoon dog (*Nyctereutes procyonoides*) is native to Japan and is considered a monogamous carnivore. The home ranges of a male and female pair overlap almost totally [11, 20]. In captivity, estrus, the period when females are willing to mate, has been found to last for 3.9 ± 1.2 days [27]. The gestation period is 61.0 ± 2.0 days [27] and pregnant females give birth to 4–6 offspring between late spring and early summer [10]. A previous study reported that in captive raccoon dogs, females mated with several males and a litter was sired by several males [23]. This phenomenon has been called “multiple paternity”. Multiple paternity has been shown in several wild carnivore species [1, 3, 5, 17, 19, 28, 29] and, in some cases, multiple paternity has been observed in carnivore species originally thought to be monogamous [1, 5, 19]. However, multiple paternity has not been reported in wild raccoon dogs. In foxes, which are also monogamous carnivores, high population density may influence the occurrence of multiple paternity [1, 19]. Although the habitat of raccoon dogs has expanded to urban areas, fragmented natural landscapes result in high population density [15, 16, 21]. Thus, wild raccoon dogs might display multiple paternity under similar conditions.

There have been numerous reports on sarcoptic mange in wild raccoon dogs [12, 21, 22]. There might be increased direct contact among raccoon dogs, if females of wild raccoon dogs mate with several males during one estrus. This phenomenon might lead to epizootic disease risk (i.e. sarcoptic mange) of wild raccoon dogs during mating season. The objective of the present study was to examine the phenomenon of multiple paternity in the wild raccoon dog by analyzing the genotypes of three pregnant females and their litters using microsatellite markers.

Between March 2014 and March 2018, raccoon dog carcasses were collected in the Misato, Miyazawa, and Jumonji areas of Takasaki City, Gunma Prefecture, Japan. These animals were captured as part of pest control measures for the prevention of agricultural damage and they were killed by licensed hunters on behalf of Takasaki City. Among these raccoon dog carcasses, three pregnant females were targeted (Table 1). When the animals were dissected, muscle tissue samples from the mothers and fetuses were taken and the canine teeth of the mothers were collected for age estimation. Collected samples were frozen at −20°C until each subsequent analysis was performed.

Total DNA was extracted from muscle tissue samples using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) and the DNA extracts were stored at 4°C. We used a Canine Genotypes Panel 1.1 Kit (Thermo Fisher Scientific, Waltham, MA, USA) to amplify 18 microsatellite loci using polymerase chain reaction (PCR). PCR was performed with 4.5 μl of Primer Mix, 4.5 μl of Master Mix (Buffer, dNTP, Phusion Hot Start DNA Polymerase), and 1 μl of DNA extract (1 $ng/\mu l$). Thermocycling conditions

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Table 1. Information on each mother

	Date of capture	Estimated age	Litter size
Litter 1	2014/5/2	1	8
Litter 2	2017/4/28	3	3
Litter 3	2017/5/16	1	3

Table 2. Summary of genotypes for litter 1

	Genotype					
	AHTk211	CXX279	REN169018	INU055	REN54P11	INRA21
Mother	95/97	-	159/170	205/207	242/248	73/80
N1	89/97	-	165/170	205/205	242/248	73/80
N2	89/97	-	165/170	205/205	242/248	73/80
N3	89/97	115/115	159/165	201/207	242/242	73/73
N4	89/97	119/119	159/165	205/205	242/242	73/80
N5	89/97	119/119	159/165	205/207	242/242	73/80
N6	91/97	-	159/165	207/207	242/248	73/73
N7	89/97	-	165/170	205/207	242/248	73/73
N8	89/97	-	159/165	205/207	242/242	73/80
	AHT137	REN169D01	AHTk253	INU005	INU030	FH2848
Mother	139/149	205/207	294/294	97/107	141/145	233/235
N1	135/149	205/205	283/294	107/107	145/147	233/237
N2	135/139	205/207	283/294	107/107	145/147	233/235
N3	115/149	205/207	291/294	107/115	141/145	233/233
N4	119/139	205/207	283/294	107/119	141/145	233/235
N5	119/139	205/207	294/298	107/119	145/147	233/235
N6	135/139	205/207	294/298	73/107	141/145	233/235
N7	135/149	205/207	283/294	73/107	145/147	233/237
N8	135/139	205/207	294/298	107/107	145/147	235/235
	AHT121	FH2054	REN162C04	AHT h171	REN247M23	
Mother	79/107	155/159	202/212	226/230	271/277	
N1	107/107	151/159	202/212	224/230	273/277	
N2	107/107	151/159	202/202	226/228	271/273	
N3	107/107	151/155	202/206	224/230	275/277	
N4	107/107	151/155	202/202	224/230	271/273	
N5	107/107	151/155	202/202	228/230	271/273	
N6	107/107	159/159	206/212	224/226	271/273	
N7	107/107	151/159	202/212	228/230	273/277	
N8	107/107	151/155	202/206	228/230	271/273	

The allele not transmitted from the mother is underlined. The locus with more than three paternal alleles is highlighted.

were 98°C for 3 min; followed by 30 cycles of 98°C for 15 sec, 60°C for 75 sec, and 72°C for 30 sec; and finally 72°C for 5 min. PCR fragments were sequenced with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and microsatellite genotypes were determined by Peak Scanner Software v1.0 (Applied Biosystems). PCR was performed several times to confirm genotypes reproducibility. The genotypes among mothers and fetuses were compared.

One (AHTh260) out of 18 loci was removed from the analysis because its genotype was indeterminate. The microsatellite genotype of each individual was determined for 16–17 loci. Comparing the genotype of litter 1 and their mother, the littermates showed more than two non-maternal alleles at INU055, AHT137, AHTk253, INU005, and FH2848, where the numbers of postulated paternally derived alleles were three, three, three, four, and three, respectively (Table 2). Consequently, extra paternity was confirmed in 5 out of 17 marker loci for litter 1. There was no evidence of multiple paternity in litter 2 (data not shown), with all littermates explained assuming a single father. Three paternal alleles were detected at the INU030 locus in litter 3, which suggested multiple paternity among littermates (Table 3).

For litter 1, the genotypes of the fetuses in the 16 loci inherited 1 allele from their mother, excluding 1 locus (CXX279) which was not determined as the genotype of the mother. Considering the other alleles of the fetus, three or four paternal alleles were estimated in 5 (AHT137, INU005, AHTk235, INU055, and FH2848) out of 17 loci (Table 2). In litter 3, three paternal alleles were estimated at one locus (INU030, Table 3). If a female has mated with one male during estrus, then one or two paternal alleles may be shown per locus of the litter. Accordingly, the biological fathers of litters 1 and 3 may have been several males. Microsatellites

Table 3. Summary of genotypes for litter 3

	Genotype					
	AHTk211	CXX279	REN169018	INU055	REN54P11	INRA21
Mother	91/93	115/115	166/166	205/205	243/248	73/80
N1	93/93	115/115	159/166	205/205	243/243	73/73
N2	91/93	115/115	166/166	201/205	243/248	73/80
N3	91/93	115/115	159/166	205/205	243/243	73/73
	AHT137	REN169D01	AHTk253	INU005	INU030	FH2848
Mother	116/116	205/205	291/291	107/111	143/145	235/238
N1	116/116	205/205	291/291	107/115	143/143	235/235
N2	116/116	203/205	291/291	107/111	141/145	235/235
N3	116/116	205/205	291/295	107/115	145/145	235/235
	AHT121	FH2054	REN162C04	AHTh171	REN247M23	
Mother	115/115	138/138	202/205	224/226	273/279	
N1	115/115	138/164	202/202	224/224	273/275	
N2	115/115	138/164	198/202	224/228	273/275	
N3	107/115	138/164	202/202	224/226	275/279	

The allele not transmitted from the mother is underlined. The locus with more than three paternal alleles is highlighted.

have mutation rates ranging from 10^{-3} to 10^{-4} per locus per generation [4, 6], which is higher than other genetic markers, e.g. mitochondrial DNA and single nucleotide polymorphism [6, 7, 14]. However, if mutation rates from 10^{-3} per locus per generation occurred at 1 out of 17 loci, then this probability is less than 0.1%. On the other hand, genotyping problems arising from null alleles, artefacts or allele dropout may have occurred [9]. In particular, multiple paternal alleles were estimated at one locus in litter 3; it could not be denied that mistyping was influenced. However, in litter 1, multiple paternal alleles were observed in five markers in N3 (Table 3). Moreover, genotype reproducibility was confirmed by performing PCR several times. Therefore, there was a high probability of multiple paternity in wild raccoon dogs in Japan.

The mothers of litters 1 and 3 were estimated to be one year old from the cement of their canine teeth. Raccoon dogs start dispersal during autumn in the year of their birth [20] and sexual maturation occurs at 9–11 months of age [8]. Thus, the mothers of litters 1 and 3 were pregnant for the first time. Raccoon dogs are monogamous animals [11], with adults expected to mate with only their breeding pair. However, young raccoon dogs, immediately after dispersal, might not easily make a breeding pair. Therefore, the mothers of litters 1 and 3 might have mated with several males during one estrus because they were still young and had not decided on a breeding pair. On the other hand, previous studies have reported that the multiple paternity of the fox family and raccoons (*Procyon lotor*) is influenced by high population density [1, 5, 17, 19]. By undertaking a camera trapping survey in this study area, the population density of the raccoon dogs in 2014 (when the mother of litter 1 was captured) was higher than the year when the other pregnant females were captured [24, 25]. Thus, multiple paternity in wild raccoon dogs might be related to high population density, similar to that seen in foxes and raccoons. A previous study suggested that high encounter rates among raccoons, due to high population density and a male-biased sex ratio, influenced multiple paternity [17]. Hence, multiple paternity of raccoon dogs might be influenced by high encounter rates in high population density, as well. Moreover, male-biased sex ratio might lead to an increase in encounters by females with males and allow greater choosiness without reducing mating opportunities [13, 17].

Female wild raccoon dogs probably mated with several males during a single estrus. It is suggested that wild raccoon dogs make contact with multiple individuals during a single mating season. A relationship between the transmission of infectious disease in the wild and contact rates has been reported [2, 26]. Contact rates during the mating season may relate with the disease transmission process [18]. Thus, mating season may be an important factor in disease (i.e. sarcoptic mange) transmission in raccoon dogs. To the best of our knowledge, the present study is the first to report the possibility of multiple paternity in wild raccoon dogs. However, this result must be verified with more evidence from future studies.

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