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The Frequency Variations of the Oxytocin Receptor Gene Polymorphisms among Dog Breeds

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Abstract: The domestic dog (*Canis familiaris*) has been diverged from wolves 15,000–100,000 years ago and certain genetic changes may have enabled them to adapt to the human social niche. In this study, we investigated the polymorphisms of the oxytocin receptor gene in dogs and wolves in order to investigate the possibility of the genetic change in the oxytocin system during dog domestication processes. Genotypes of the oxytocin receptor gene polymorphisms were determined in dogs and wolves. The single nucleotide polymorphisms (SNP; rs22927829, rs8679682, rs22896457) were observed in the sampled dogs. On the other hand, for the SNPs rs22927823 and rs22927826, only homozygous GG genotypes were observed (n=25). The frequencies of the SNPs (rs8679682 and rs22896457) were significantly different among wolves and dogs, and also among dog breed groups ($p<0.05$, chi-square test). The frequency profile of these SNPs among dog breed groups supports the hypothesis that there were selections on the oxytocin receptor gene during the dog domestication processes.
Key words: dogs, wolves, domestication, oxytocin receptor gene, single nucleotide polymorphisms.

Introduction

The domestic dog (*Canis familiaris*) belongs to the Canidae family which contains 34 closely related species which diverged within the last 10 million years[1]. Recent phylogenetic analysis of 30 out of 34 living wild canids had revealed that the grey wolf is the most closely related living relative of the domestic dog[2]. At present, there are more than 700 breeds in the world, 194 of which are recognized by the Japan Kennel Club (JKC) (http://www.jkc.or.jp/modules/worlddogs/). Recent studies on dog genes have successively created genetic cladograms of dog breeds[3, 4]. Especially, in the cladogram suggested by vonHoldt (2010), there was a surprising correspondence between genetic and phenotypic/functional breed groupings[4]. The grey wolf and the domestic dog are estimated to be diverged 14.9 thousand years ago[5], however the process of dog domestication is still poorly understood.

The process of domestication involves selection of specific phenotypes. Some researchers believe that domestication started when a population of ancient wolves started to exploit human-related food sources[6]. Certain genetic changes may have enabled them to adapt to the human social niche during an early phase of the domestication process. Recent genome-wide research have revealed some signals of selection in the dog genome,

including the signals near genes which relate to memory formation, behavioral sensitization, and/or the Williams –Beuren syndrome in humans characterized by social traits such as exceptional gregariousness[7]. Later research also has revealed some signals of selection in the genes related to the reproduction, digestion and metabolism, and neurological process[8]. The genes related to the neurological process may be especially strong candidates for one of the genes selected for in the early stages of dog domestication, since the domestication process often involves behavioral and neurological traits, such as the reduction of aggression, which allows for complex interactions with humans[9]. Indeed, recent research had revealed the association of dopamine and serotonin related genes with aggression in domestic dogs[10].

The neuropeptide oxytocin is known to be involved in social behavior, including social bonding and social cognition[11-14]. Oxytocin increases trust and more frequently directs the gaze to the eye region of faces in humans[11, 12]. Oxytocin knockout (OTKO) mice are also more aggressive than wild-type mice[13]. Remarkably, OTKO mice are deficit of recognizing familiar conspecifics after repeated social encounters, although olfactory and non-social memory functions are intact[13]. Oxytocin is further suggested to be critical for the formation of a partner preference in the female prairie vole[14]. Some studies have also shown that some oxytocin receptor polymorphisms are related to stress response and attachment behaviors[15, 16]. A study in humans has shown that genetic variation of the oxytocin receptor modulates the effectiveness of social buffering[15]. Another study in infants has shown that an oxytocin receptor polymorphism is associated with attachment security[16]. Since oxytocin system has been shown to be widely related to the formation and function of social groups, we have investigated the polymorphisms of the oxytocin receptor in dogs and wolves, which is located on chromosome 20 in dogs, in order to understand the contribution of the changes in the oxytocin system to the processes of dog domestication.

Materials and Methods

Animals and DNA isolation

Blood samples of 228 dogs were collected from voluntary recruited dogs in Japan. Blood samples of three wolves (2 *Canis lupus lupus* and 1 *Canis lupus arctos*) were collected from privately owned wolves in Japan. Each of them was owned by different owners and none of the wolf samples were related by blood. Two DNA samples of wolves (*Canis lupus subsp.*) were also kindly shared from the Wildlife Research Center of Kyoto University. Genomic DNA was isolated using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). The number of dogs in each breed is shown in Table 1.

Data Base Search for the Polymorphism

The polymorphisms were searched by using the Ensembl and National Center for Biotechnology Information (NCBI) data base. Two missense variants and three synonymous variants in the exon were selected for further analyses (rs22927823, rs22927826, rs22927829, rs8679682, rs22896457).

PCR amplification

Three fragments including the five selected polymorphisms of the oxytocin receptor gene were amplified by PCR using primer pairs shown in Table 2. The PCR reactions were performed in a total volume of 50 μl containing 20-50 ng genomic DNA, 0.2 μM of each primer, 0.4 mM of dNTP, 1 U KOD-FX Neo DNA polymerase (TOYOBO, Osaka, Japan) and 25 μl 2×PCR buffer in a PCR Thermal Cycler Dice Touch (Takara, Shiga, Japan). Samples were initially denatured at 94°C for 2 min, followed by 40 cycles of 98°C for 10 s, 65°C for 30 s, and a final extension at 72°C for 7 min.

Genotyping and sequencing

The PCR products were purified using Nucleo Spin® Gel and PCR Clean-up Kit (Takara) after confirmed by 1.5% agarose gel electrophoresis. The concentration of the purified-PCR products were calculated using 1.5% agarose gel electrophoresis. The 30 ng purified-PCR products are

Table 1. The number of dogs in each breeds and the genetically clustered breed groups used for the analysis.

Ancient and Spitz					Toy				
		n					n		
Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457	Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457
Akita	0	0	1	1	Pomeranian	1	1	2	2
Shiba	11	29	53	54	Chihuahua	0	0	6	6
Samoyed	0	0	1	1	Shih Tzu	1	3	23	23
Spaniels, Scent hounds, and Poodles					Working				
		n					n		
Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457	Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457
American Cocker Spaniel	0	1	7	7	German shepherd	0	0	3	3
English Cocker Spaniel	0	0	3	3					
Cavalier King Charles Spaniel	0	0	2	2					
Beagle	1	2	4	4					
Basset Hound	0	0	3	3					
Toy Poodle	0	0	18	18					
Standard Poodle	1	3	11	11					
Sight hounds and Herding					Retrievers				
		n					n		
Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457	Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457
Borzoi	0	2	2	2	Golden Retriever	2	2	8	8
Welsh Corgi Pembroke	0	0	2	2	Labrador Retriever	5	6	13	13
Collie	0	1	2	2	Barnese Mountain Dog	0	0	1	1
Austrlian Shepherd	0	0	1	1					
Shetland Sheepdog	0	2	4	4					
Border Collie	1	2	8	8					
Small terriers					Mastiff-like				
		n					n		
Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457	Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457
Yorkshire Terrier	0	0	5	5	Jack Russel Terrier	0	0	4	4
Cairn Terrier	0	0	1	1	Boxer	1	1	1	1
					Other Breeds and Mix				
		n					n		
Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457	Breeds	rs22927823 rs22927826	rs22927829	rs8679682	rs22896457
Kai	0	1	2	2	Maltese	0	1	1	1
Shikoku	0	0	2	2	Great Pyrenees	0	0	1	1
Kishu	0	0	1	1	Welsh Terrier	0	0	1	1
Spitz	0	0	1	1	Bedlington Terrier	0	0	1	1
Miniature Schnauzer	1	5	6	6	Norfolk Terrier	0	0	1	1
Miniature Dachshund	0	0	8	8	Wire-haired Fox Terrier	0	0	4	4
Boronese	0	0	1	1	Mix	0	1	8	8

Blood samples were collected and the oxytocin receptor gene polymorphisms were investigated in dogs. The dog breeds were separated into eight breed groups: 1) ancient and spitz breeds, 2) toy dogs, 3) spaniels, scent hounds, and poodles, 4) working dogs, 5) small terriers, 6) sight hounds and herding dogs, 7) retrievers, and 8) mastiff-like dogs following cladograms of vonHoldt and Parker for analysis.

mixed with 7.5 pmoles primer in a total volume of 15 μl. The samples were shipped to the Takara CDM Center (Mie, Japan) and subjected to the Premix Sequence Analysis (Takara).

Statistical analysis

The dog breeds were separated into eight breed groups: 1) ancient and spitz breeds, 2) toy dogs, 3) spaniels, scent hounds, and poodles, 4) working dogs, 5) small terriers, 6) sight hounds and herding dogs, 7) retrievers, and 8) mastiff-like dogs following vonHoldt's (2010) cladogram[4] (Table 1). Since the Shiba Inu breed was not included in vonHoldt's cladogram, we classified them into the ancient and spitz breed group according to the cladogram suggested by

Table 2. Primers used for PCR amplification to genotype the dog oxytocin receptor gene polymorphisms.

	Primer	Sequence (5'-3')
rs22927823 and rs22927826	Forward	CCTCCTCCGAAGGGCTTG
	Reverse	CGCAGCGAGAAGATGTGC
rs22927829	Forward	ACCTACCTGCTGCTGCTCAT
	Reverse	GCTCCCTCCTCCGAGGTC
rs8679682 and rs22896457	Forward	ACCCCTACCCTCCCTTCAG
	Reverse	AAGCCCTTGGAGGCAGTC

Parker (2004)[3]. Other dog breeds not shown in vonHoldt' s cladogram were eliminated from further analyses. The working dogs breed group was eliminated from further analyses since there were only three individuals. The dog breeds in each breed group are shown in Table 1.

The frequencies of each single nucleotide polymorphisms (SNP) were assessed for deviation from Hardy–Weinberg equilibrium and were compared between dogs and wolves and between dog breed groups using chi-square test. $P<0.05$ was considered significant.

Results

For the SNPs, rs22927823 and rs22927826, only homozygous GG genotypes were observed (n=25). For the SNP, rs22927829, 97% (n=61) of the sampled dogs (n=63) owned homozygous TT genotypes. Heterozygous AT genotype was observed in one Boxer and homozygous AA genotype was observed in one Miniature Schnauzer. For the SNP, rs8679682, 53% (n=120) owned homozygous TT genotypes, 34% (n=78) owned heterozygous CT genotypes and 13% (n=29) owned homozygous CC genotypes. For the SNP, rs22896457, 96% (n=220) owned homozygous TT genotypes, 3% (n=7) owned heterozygous CT genotypes and homozygous CC genotype was observed in only one Boxer. The SNPs, rs8679682 and rs22896457, were genotyped in five wolf samples. For the SNP, rs8679682, homozygous TT genotype was observed in only one out of five wolves, and homozygous CC genotypes were observed in four of five wolves. For the SNP, rs22896457, two out of five wolves owned homozygous TT genotypes, other two out of five wolves owned heterozygous CT genotypes and one out of five wolves owned homozygous CC genotype.

As shown in Table 3 and Fig. 1, there were significant differences of oxytocin receptor variant (rs8679682 and rs22896457) frequencies among wolves and dogs ($p<0.05$, chi-square test). For the SNP, rs8679682, C variant allele was more common in wolves (80%) than in dogs (30%), whilst the T variant allele was less common in wolves (20%) than in dogs (70%). For the SNP, rs22896457, C variant allele was more common in wolves (40%) than in dogs (2%), whilst the T variant allele was less common in wolves (60%) than in dogs (98%). The frequencies of each SNPs were not in Hardy–Weinberg Equilibrium ($p<0.05$) except for the rs22896457 SNP in wolf population.

As shown in Table 3 and Fig. 1, there were significant differences of oxytocin receptor variant (rs8679682 and rs22896457) frequencies among dog breed groups ($p<0.05$, chi-square test). The frequencies of each SNPs were in Hardy–Weinberg Equilibrium ($p>0.05$) except for the rs8679682 SNP in the ancient and spitz breed group and for the rs22896457 SNP in the mastiff-like dog group. The frequency of C variant allele of the rs8679682 SNP was more common in the mastiff-like dog group (80%), which is very close to the frequency in wolves (80%), compared to the toy dogs, the spaniels, scent hounds, and poodles breed group, small terriers, the sight hounds and herding dogs bred group, and the retrievers (32-42%). The frequency of C variant allele of the rs8679682 SNP was less common in the ancient and spitz breed group (5%) than in the toy dogs, the spaniels, scent hounds, and poodles breed group, small terriers, the sight hounds and herding dogs bred group, and the retrievers (32-42%). The frequency of C variant allele of the rs22896457 SNP

Table 3. Frequency of oxytocin receptor single nucleotide polymorphisms (SNPs) and combined genotypes in wolves and dogs.

SNPs	Wolf or Dog or Dog Breed Group	Genotype Frequency			HWE	Allele Frequency				
rs8679682		CC	CT	TT		C	T			
	Wolf (n=5)	0.80	0.00	0.20	p=0.025	0.80	0.20			
	Dog (n=227)	0.13	0.34	0.53	p=0.006	0.30	0.70			
	Ancient and Spitz (n=55)	0.02	0.05	0.93	p=0.006	0.05	0.95			
	Toy (n=31)	0.06	0.52	0.42	p=0.314	0.32	0.68			
	Spaniels, Scent hounds, and Poodles (n=48)	0.19	0.40	0.42	p=0.255	0.39	0.61			
	Sight hounds and Herding (n=19)	0.11	0.47	0.42	p=0.820	0.34	0.66			
	Retrievers (n=22)	0.09	0.55	0.36	p=0.402	0.36	0.64			
	Small terriers (n=6)	0.33	0.17	0.50	p=0.108	0.42	0.58			
	Mastiff-like (n=5)	0.60	0.40	0.00	p=0.576	0.80	0.20			
rs22896457		CC	CT	TT		C	T			
	Wolf (n=5)	0.20	0.40	0.40	p=0.709	0.40	0.60			
	Dog (n=228)	0.00	0.03	0.96	p=0.002	0.02	0.98			
	Ancient and Spitz (n=56)	0.00	0.02	0.98	p=0.946	0.01	0.99			
	Toy (n=31)	0.00	0.00	1.00	p=0.946	0.00	1.00			
	Spaniels, Scent hounds, and Poodles (n=48)	0.00	0.02	0.98	p=0.942	0.01	0.99			
	Sight hounds and Herding (n=19)	0.00	0.00	1.00	p=0.942	0.00	1.00			
	Retrievers (n=22)	0.00	0.05	0.95	p=0.913	0.02	0.98			
	Small terriers (n=6)	0.00	0.33	0.67	p=0.624	0.17	0.83			
	Mastiff-like (n=5)	0.20	0.00	0.80	p=0.025	0.20	0.80			
Combined genotype rs8679682-rs22896457		CC-CC	CC-CT	CC-TT	CT-CC	CT-CT	CT-TT	TT-CC	TT-CT	TT-TT
	Wolf (n=5)	0.20	0.40	0.20	0.00	0.00	0.00	0.00	0.00	0.20
	Dog (n=227)	0.00	0.01	0.11	0.00	0.01	0.33	0.00	0.00	0.52
	Ancient and Spitz (n=55)	0.00	0.02	0.00	0.00	0.00	0.05	0.00	0.00	0.93
	Toy (n=31)	0.00	0.00	0.06	0.00	0.00	0.52	0.00	0.00	0.42
	Spaniels, Scent hounds, and Poodles (n=48)	0.00	0.00	0.19	0.00	0.02	0.38	0.00	0.00	0.42
	Sight hounds and Herding (n=19)	0.00	0.00	0.11	0.00	0.00	0.47	0.00	0.00	0.42
	Retrievers (n=22)	0.00	0.00	0.09	0.00	0.00	0.55	0.00	0.05	0.32
	Small terriers (n=6)	0.00	0.17	0.17	0.00	0.17	0.00	0.00	0.00	0.50
	Mastiff-like (n=5)	0.20	0.00	0.40	0.00	0.00	0.40	0.00	0.00	0.00

The proportion of each genotype, allele and combined genotype are provided for wolves, dogs and seven dog breed groups.
Statistical tests for Hardy-Weinberg Equilibrium (HWE) are also provided.

was more common in the mastiff-like dog group (20%) and in the small terriers (17%), which is about the half of the frequency in wolves (40%), than in the toy dogs, the spaniels, scent hounds, and poodles breed group, the sight hounds and herding dogs bred group, and the retrievers (0-2%).

Discussion

The oxytocin system is widely related to the behaviors and cognition in many mammals as shown in mice[13], prairie voles[14] and humans[11, 12, 15, 16]. In order to investigate the possibility of the genetic change in the oxytocin system during dog domestication processes, we investigated the polymorphisms of the oxytocin receptor gene in dogs and wolves. Genome-wide studies on dog genes had revealed that there are at least two domestication bottlenecks[2, 5]. The first would have been related to the divergence of the domestic dog from wolves 15,000–100,000 years ago[2]. The recent one would be related to the breed creation and the artificial selection conducted within the past few hundred years[5]. Breeds seem to be different in several aspects of their behavior due to the effects of these artificial selections[17-19], and we hypothesized that there would be variations in the oxytocin receptor genes among dog breeds.

As predicted, there were frequency variations in the oxytocin receptor genes among dog breeds. For the rs8679682 SNP, the ancient and spitz breed group had the least frequency of C allele among seven breed groups. This is surprising since even though the ancient and spitz breed group is genetically related most closely to wolves,

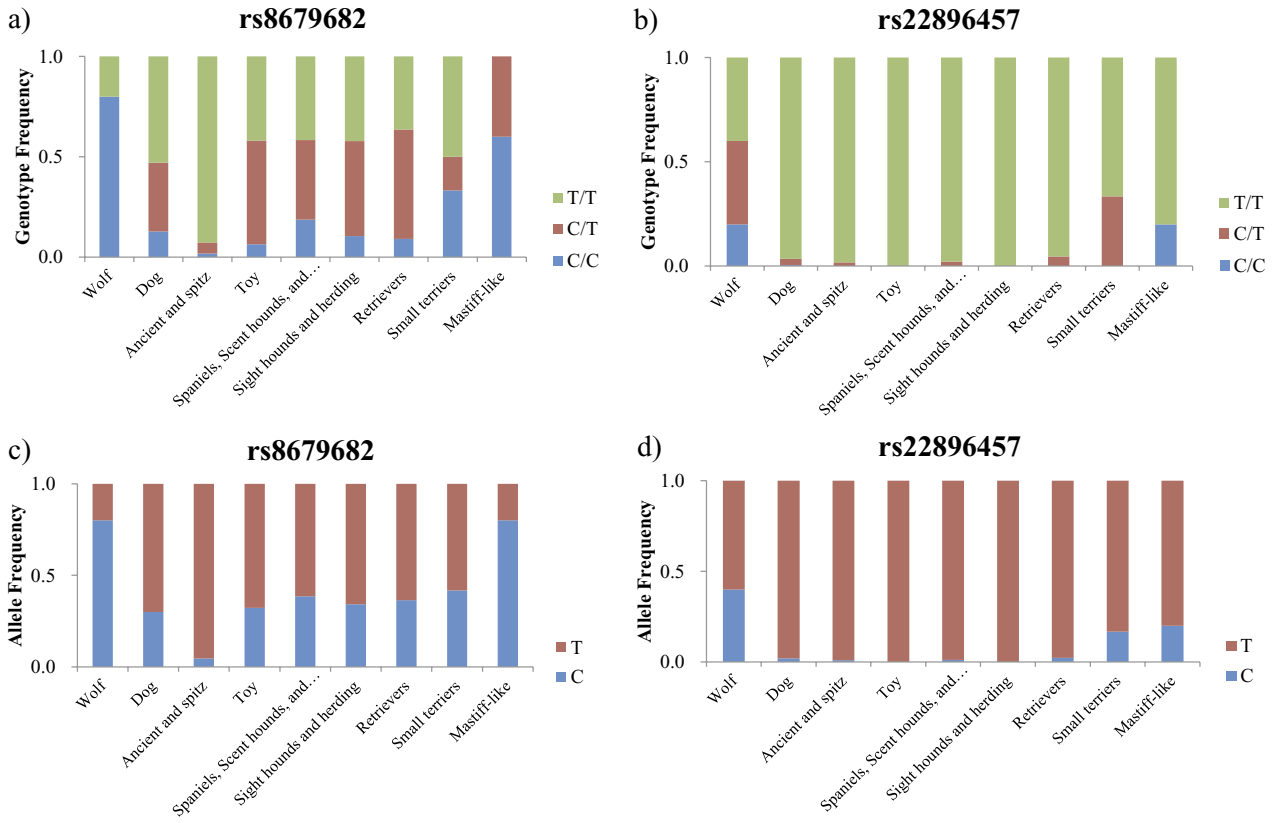

Fig. 1 Genotype frequency and Allele frequency for the SNPs, rs8679682 and rs22896457, in wolves and dogs.
Genotype frequency and allele frequency for the SNPs rs8679682 and rs22896457, in wolves and dogs and in dog breed groups (Ancient and spitz, Toy, Spaniels, Scent hounds, and Poodles, Sight hounds and herding, Retrievers, Small terriers and Mastiff-like) were compared. The frequencies were significantly different for both SNPs between wolves and dogs and also among dog breed groups ($p<0.05$, chi-square test). (a) Genotype frequency of rs8679682 (b) Genotype frequency of rs22896457 (c) Allele frequency of rs8679682 (d) Allele frequency of rs22896457

rs8679682 SNP profile was most different from the profile in wolves. On the other hand, the mastiff-like dog group, which is genetically furthest from wolves, had the most frequency of C allele, which is at almost the same level as in wolves. This can be interpreted as during the early domestication processes, there was a selection or genetic drift related to this SNP and the frequency of the C allele dropped dramatically in the domestic dogs. As the artificial selection began and humans started to control the breeding, C allele gradually increased. Considering the artificial selection creating the mastiff-like dogs, the C allele frequency increased to reach almost the same level in the mastiff-like dogs as the wolves. The rs22896457 SNP also shows the same tendency as the rs8679682 SNP. The C allele frequency, which was almost 40% in wolves is almost 0% in the ancient and spitz breed group, stays at almost 0% for the toy dogs, spaniels, scent hounds, and poodles, working dogs, sight hounds and herding dogs, retrievers, is about 17% for the small terriers, and in the mastiff-like dogs which is believed to be the furthest away genetically show almost 20%.

Recently, three oxytocin receptor gene polymorphisms associated with human directed social behavior in dogs have been reported[20]. This study suggested that oxytocin receptor gene polymorphisms have an impact on proximity seeking tendency towards an unfamiliar person and their owner, as well as friendliness of the dogs toward strangers, although the mediating molecular regulatory mechanisms are yet unknown[20]. Although the direct influence on the behaviors would be hard to interpret since the SNPs in the present study, rs22896457 and rs8679682, are synonymous variants, the frequency profile of these SNPs among dog breed groups supports the hypothesis that there were selections on oxytocin receptor gene during the

dog domestication processes. Further studies on oxytocin receptor gene polymorphisms and their associations with dog behaviors are required.

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