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Association of a P2RX7 gene missense variant with brachycephalic dog breeds

Abstract

Stichting International Foundation for Animal Genetics Missense variants are associated with various phenotypic traits and disorders in dogs. The canine P2RX7 gene, coding the ATP-gated P2X7 receptor ion channel, contains four known missense variants. The current study aimed to examine the presence of these variants in a random sample of pedigree and mixed-pedigree dogs. Exons 3, 8, 11 and 13 of the P2RX7 gene, encoding these four respective variants, in 65 dogs were assessed by Sanger sequencing and combined with existing sequencing data from another 69 dogs. The distribution of these variants was then evaluated in all 134 dogs combined and separately within individual breeds including 35 different pure breeds. The rs23314713 (p.Phe103Leu) and rs23315462 (p.Pro452Ser) variants were present in 47 and 40% of all dogs studied respectively, with the rs23314713 variant associated with brachycephalic breeds. Among pedigree dogs, the rs23314713 and rs23315462 variants were associated with brachycephalic and non-brachycephalic breeds respectively. The rs851148233 (p.Arg270Cys) and rs850760787 (p.Arg365Gln) variants were present only in dogs of Cocker Spaniel and Labrador Retriever pedigrees respectively. No other missense variants were found in exons 3, 8, 11 and 13 of the P2RX7 gene within the dogs. In conclusion, the rs23314713 and rs23315462 missense variants of the P2RX7 gene are present in a large proportion of dogs, with the rs23314713 variant associated with a number of brachycephalic breeds. However, the association of this variant with dogs of bulldog ancestry, not brachycephaly per se, cannot be excluded.

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Association of a *P2RX7* gene missense variant with brachycephalic dog breeds

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Running head: Canine *P2RX7* gene missense variants

Summary

Missense variants are associated with various phenotypic traits and disorders in dogs. The canine *P2RX7* gene, coding the ATP-gated P2X7 receptor ion channel, contains four known missense variants. The current study aimed to examine the presence of these variants in a random sample of pedigree and mixed-pedigree dogs. Exons 3, 8, 11 and 13 of the *P2RX7* gene, encoding these four respective variants, in 65 dogs were assessed by Sanger sequencing and combined with existing sequencing data from another 69 dogs. The distribution of these variants was then evaluated in all 134 dogs combined and separately within individual breeds including 35 different pure breeds. The rs23314713 (p.Phe103Leu) and rs23315462 (p.Pro452Ser) variants were present in 47% and 40% of all dogs studied, respectively, with the rs23314713 variant associated with brachycephalic breeds. Among pedigree dogs, the rs23314713 and rs23315462 variants were associated with brachycephalic and non-brachycephalic breeds, respectively. The rs851148233 (p.Arg270Cys) and rs850760787 (p.Arg365Gln) variants were present only in dogs of Cocker Spaniel and Labrador Retriever pedigree, respectively. No other missense variants were found in exons 3, 8, 11 and 13 of the *P2RX7* gene within the dogs. In conclusion, the rs23314713 and rs23315462 missense variants of the *P2RX7* gene are present in a large proportion of dogs, with the rs23314713 variant associated with a number of brachycephalic breeds. However, the association of this variant with dogs of bulldog ancestry, not brachycephaly *per se*, cannot be excluded.

Keywords P2X7 receptor, purinergic receptor, single nucleotide polymorphism, DH82 canine macrophages, MDCK cells, canine, dog

The P2X7 receptor is a trimeric ATP-gated cation channel encoded by the *P2RX7* gene (Sluyter 2017). The P2X7 receptor has important roles in inflammation, immunity and cancer (Di Virgilio *et al.* 2017). In dogs, the P2X7 receptor is present on monocytes, lymphocytes, erythrocytes (Sluyter *et al.* 2007; Stevenson *et al.* 2009), kidney epithelial cells (Jalilian *et al.* 2012b; Zuccarini *et al.* 2017) and neurons of the myenteric plexus (Schafer *et al.* 2018). This receptor is also present in normal brain tissue including the cerebrum (Lee *et al.* 2005; Truve *et al.* 2016), and in glioblastomas, oligodendrogliomas and astrocytomas (Truve *et al.* 2016) from dogs. Canine P2X7 receptor activation results in interleukin-1β release from monocytes (Jalilian *et al.* 2012a) and in whole blood (Roman *et al.* 2009; Spildrejorde *et al.* 2014b; Bartlett *et al.* 2017). Moreover, P2X7 receptor activation results in phosphatidylserine exposure and hemolysis in canine erythrocytes (Sluyter *et al.* 2007; Faulks *et al.* 2016). Other cellular functions of the canine P2X7 receptor are yet to be reported, but its activation is likely to mediate events similar to those shown in humans, where it is an emerging therapeutic target in various disorders such as inflammatory bowel disease, osteoporosis, inflammatory pain and cancer (De Marchi *et al.* 2016).

In a cohort of 69 dogs including 68 blood samples and MDCK cells (derived from a Cocker Spaniel), we previously identified four missense variants in the canine *P2RX7* gene: rs23314713 (p.Phe103Leu), rs851148233 (p.Arg270Cys), rs850760787 (p.Arg365Gln) and rs23315462 (p.Pro452Ser) (Spildrejorde *et al.* 2014a). Another group subsequently found that the rs23314713 variant is associated with susceptibility to glioma (Truve *et al.* 2016), a disease more frequent in several brachycephalic breeds (Hayes *et al.* 1975; Snyder *et al.* 2006; Song *et al.* 2013). Brachycephaly in dogs results in the shortening of the muzzle, flat facial conformation and widening of the skull, and includes breeds such as the Bulldog, Mastiff and Staffordshire Bull Terrier (Bannasch *et al.* 2010; Schoenebeck *et al.* 2012; Packer *et al.* 2015; Marchant *et al.* 2017). To extend these findings, the presence of the four previously described missense variants was evaluated in an additional 65 dogs. This data was then combined with existing data (Spildrejorde *et*

al. 2014a) to determine the distribution of these variants in a random sample of the canine population.

Dog samples were collected in accordance with the Animal and Human Ethics Committees (University of Wollongong). Peripheral blood, drawn into VACUETTE lithium heparin tubes (Greiner Bio-One), was collected from 64 dogs presented at local veterinary clinics with informed consent from pet owners. DH82 cells (derived from a Golden Retriever) were obtained from the European Collection of Cell Cultures. These 65 samples including DH82 cells combined with our previously published cohort of 69 dogs (Spildrejorde *et al.* 2014a) represent 97 pedigree dogs (comprising 35 different pure breeds) and 37 mixed-pedigree dogs (Table S1). The veterinarians in consultation with pet owners assigned the breed of each dog. Pedigree dogs were classified as brachycephalic as reported in other studies (Bannasch *et al.* 2010; Schoenebeck *et al.* 2012; Packer *et al.* 2015; Marchant *et al.* 2017). Mixed-pedigree dogs were assigned as brachycephalic based on known ancestry and/or display of brachycephalic characteristics (shortening of the muzzle, flat facial conformation and widening of the skull). No dogs suffered from glioma at the time of presentation and were not followed up for this cancer post sampling.

DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Primer pairs (Table S2) were used to amplify and sequence exons 3, 8 and 11 and the first half of exon 13 of the canine *P2RX7* gene by PCR as described (Spildrejorde *et al.* 2014a). No other exons of the *P2RX7* gene were sequenced. Resulting sequences were compared to the NCBI Reference Sequence NM_001113456.1, which is from a non-disclosed pedigree. Notably, the alleles in the reference sequence, for each of the four missense variants, are conserved across various mammalian species (Jiang *et al.* 2013). For some dogs, not all exons could be amplified or sequenced despite repeated attempts and successfully achieving this with other exons from the same DNA sample (Table S1). This resulted in different dog numbers being reported below for each exon studied. Differences in heterozygosity, homozygosity, variant

prevalence and mutant allele frequency between groups were compared by the Fisher's Exact test using Prism 5 for Mac OS X (GraphPad Software, San Diego, CA, USA) with $P < 0.05$ considered significant. *P*-value calculations for prevalence include heterozygous and homozygous dog numbers combined. *P*-value calculations for allele frequency include total allele numbers. Analyses did not correct for relatedness of breeds.

Sequence analysis of genomic DNA isolated from 65 new dogs combined with existing sequence data from another 69 dogs (Spildrejorde *et al.* 2014a) revealed (in decreasing order) a variant prevalence of 47%, 40%, 4% and 3% and a mutant allele frequency of 0.38, 0.26, 0.02 and 0.02 for rs23314713 (p.Phe103Leu), rs23315462 (p.Pro452Ser), rs850760787 (p.Arg365Gln) and rs851148233 (p.Arg270Cys), respectively (Table 1; Table S1). A similar prevalence and allele frequency for each variant were also observed among pedigree dogs only (Table 1; Table S1). Linkage disequilibrium analysis (https://www.broadinstitute.org/haploview/haploview) revealed that none of the alleles were in strong linkage disequilibrium (*results not shown*), with the strongest association observed between rs23314713 and rs23315462 ($D' = 0.605$ and $r^2 = 0.088$).

The combined data from pedigree and mixed-pedigree dogs revealed the presence of the rs23314713 (p.Phe103Leu) variant in 76% of brachycephalic dogs and 31% of non-brachycephalic dogs (Table 2), which differed significantly between these two groups ($P < 0.0001$). The allele frequencies of the rs23314713 mutant allele were 0.65 and 0.23 in brachycephalic and non-brachycephalic dogs, respectively (Table 2), and also differed significantly between the two groups ($P < 0.0001$). Among pedigree dogs only, the prevalence ($P < 0.0001$) and allele frequency ($P < 0.0001$) for this variant were also significantly greater in brachycephalic dogs compared to non-brachycephalic dogs (Table 2). Moreover, there was a significantly higher proportion of brachycephalic dogs heterozygous for the rs23314713 variant compared to non-brachycephalic dog among all dogs ($P = 0.0136$) and pedigree dogs only ($P = 0.0323$). Likewise, there was a

significantly higher proportion of brachycephalic dogs homozygous for this variant compared to non-brachycephalic dog among all dogs ($P < 0.0001$) and pedigree dogs only ($P < 0.0001$).

Collectively, the data above indicates that the rs23314713 variant is associated with dogs of brachycephalic pedigree. However, a second possibility is that this variant is associated with dogs of known bulldog ancestry including the Australian bulldog, American Staffordshire terrier and the Staffordshire terrier (Ostrander *et al.* 2017) not brachycephaly *per se*. In support of this, the three bull terriers studied, defined as non-brachycephalic dogs and which have bulldog ancestry (vonHoldt *et al.* 2010), were all homozygous for the rs23314713 variant. Whilst, four of the five Maltese Shih Tzu studied, defined as brachycephalic dogs and which do not have bulldog ancestry (vonHoldt *et al.* 2010), were homozygous for the reference allele at this position. Thus, it remains to be determined if the rs23314713 variant contributes to brachycephaly, but observations in mice do not support this. Despite well-established roles for P2X7 activity in bone formation and homeostasis (Agrawal & Gartland 2015), strains of mice with a loss-of-function *P2RX7* missense variant (Syberg *et al.* 2012) or global ablation of the *P2RX7* gene (Ke *et al.* 2003) do not display facial or skull differences, akin to brachycephaly, compared to mice with normal P2X7 activity.

It should also be noted that our study, compared to others, is relatively small and represents limited breeds. A study of 576 dogs (representing 62 breeds) identified a missense variant (p.Phe452Leu) in *BMP3*, which codes for bone morphogenetic protein 3, is associated with cranioskeletal features of brachycephalic dogs (Schoenebeck *et al.* 2012). Whilst another study of 375 dogs (representing 83 breeds) revealed that a retrotransposon-mediated missplicing of *SMOC2*, which encodes SPARC-related modular calcium-binding protein 2 is associated with brachycephaly (Marchant *et al.* 2017). Both of these proteins have potential roles in bone formation, but how the related genetic variations contribute to brachycephaly remains to be determined (Schoenebeck *et al.* 2012; Marchant *et al.* 2017).

The combined data from pedigree and mixed-pedigree dogs revealed the presence of the second most common variant, rs23315462 (p.Pro452Ser), in 28% of brachycephalic dogs and 47% of non-brachycephalic dogs (Table 2), a difference which approached statistical significance (P = 0.0650). The allele frequencies of the rs23315462 mutant allele were 0.19 and 0.31 in brachycephalic and non-brachycephalic dogs, respectively (Table 2), a difference which also approached statistical significance (P = 0.0547). Notably, among pedigree dogs, the prevalence (P = 0.0141) and allele frequency (P = 0.0124) for this variant were significantly greater in non-brachycephalic dogs compared to brachycephalic dogs (Table 2). Collectively, this data revealed that the rs23315462 variant is potentially more prevalent in non-brachycephalic dogs compared to brachycephalic dogs. This difference mirrors the greater prevalence of the rs23314713 variant in brachycephalic dogs, which can be explained by incomplete linkage disequilibrium between these two alleles (Table S1).

Although case numbers were small among all pedigree and mixed-pedigree dogs combined, the current data suggests that the rs851148233 (p.Arg270Cys) and rs850760787 (p.Arg365Gln) variants are limited to dogs of Cocker Spaniel and Labrador Retriever pedigree, respectively (Table S3). The rs851148233 variant was present in four dogs of Cocker Spaniel pedigree (two Cocker Spaniels, one Cavalier King Charles Spaniel and Cocker Spaniel cross, and one Cocker Spaniel and Poodle cross), but absent in three dogs of Cocker Spaniel pedigree (two Cocker Spaniels and one Poodle and Cocker Spaniel cross). The rs851148233 variant was also absent in two Cavalier King Charles Spaniels, one Maltese and Poodle cross and another 111 dogs of non-Cocker Spaniel pedigree. The rs850760787 variant was present in four Labrador Retriever and one Golden Retriever and Labrador Retriever cross. This variant was absent in six other Labrador Retrievers and another 119 dogs of non-Labrador Retriever pedigree including four Golden Retrievers.

The current study did not reveal any new missense variants in the *P2RX7* gene in any of the dogs sequenced (results not shown). This data contrasts the large polymorphic variation observed in

the human *P2RX7* gene (De Marchi *et al.* 2016). Although the possibility remains that other missense variants are present in the remaining exons not sequenced, the limited polymorphic variation of the canine *P2RX7* gene compared to the human *P2RX7* gene is consistent with genome-wide decreases in genetic variation as a result of population bottlenecks associated with domestication, local population history and breed creation (Ostrander *et al.* 2017). Finally, it should be noted that occasional exons could not be amplified or sequenced despite successfully achieving this with other exons from the same DNA sample. This may be due to sequence variations within the PCR primer binding sites, which were largely intronic, negatively impacting annealing or extension.

In conclusion, the main finding of this study is that the rs23314713 (p.Phe103Leu) and rs23315462 (p.Pro452Ser) variants of the *P2RX7* gene are commonly present in dogs, with the rs23314713 variant associated with a number of brachycephalic breeds, however the association of this variant with dogs of bulldog ancestry, not brachycephaly *per se*, cannot be excluded.

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Table 1 Prevalence and allele frequency of *P2RX7* gene missense variants.

Variant	Amino acid change	Dogs *(n)*	Genotype (*n* of dogs)			Prevalence	Allele frequency
			Reference sequence	Heterozygous variant	Homozygous variant		
All dogs							
rs23314713	p.Phe103Leu	117	62	20	35	47%	0.38
rs851148233	p.Arg270Cys	121	117	4	0	3%	0.02
rs850760787	p.Arg365Gln	130	125	5	0	4%	0.02
rs23315462	p.Pro452Ser	106	64	28	14	40%	0.26
Pedigree dogs							
rs23314713	p.Phe103Leu	88	51	9	27	41%	0.36
rs851148233	p.Arg270Cys	89	87	2	0	2%	0.01
rs850760787	p.Arg365Gln	93	89	4	0	4%	0.02
rs23315462	p.Pro452Ser	79	48	21	10	39%	0.26

Table 2 Prevalence and allele frequency of the rs23314713 (p.Phe103Leu) and rs23315462 (p.Pro452Ser) variants in brachycephalic and non-brachycephalic dogs.

Variant	Brachycephalic	Dogs (*n*)	Genotype (*n* of dogs)			Prevalence	Allele frequency
			Reference sequence	Heterozygous variant	Homozygous variant		
rs23314713 (all dogs)	Yes	42	10	9[a]	23[b]	76%[c]	0.65[d]
	No	75	52	11	12	31%	0.23
rs23314713 (pedigree dogs)	Yes	28	6	4[e]	18[b]	79%[c]	0.71[d]
	No	60	46	5	9	23%	0.19
rs23315462 (all dogs)	Yes	40	29	7	4	28%	0.19
	No	66	35	21	10	47%	0.31
rs23315462 (pedigree dogs)	Yes	26	21	3[f]	2	19%[g]	0.13[h]
	No	53	27	18	8	49%	0.32

[a] $P = 0.0136$ compared to proportion of heterozygous non-brachycephalic dogs (excludes homozygous dogs).

[b] $P < 0.0001$ compared to proportion of homozygous non-brachycephalic dogs (excludes heterozygous dogs).

[c] $P < 0.0001$ compared to prevalence in non-brachycephalic dogs.

[d] $P < 0.0001$ compared to allele frequency in non-brachycephalic dogs.

[e] $P = 0.0323$ compared to proportion of heterozygous non-brachycephalic dogs (excludes homozygous dogs).

[f] $P = 0.0269$ compared to proportion of heterozygous non-brachycephalic dogs (excludes homozygous dogs).

[g] $P = 0.0141$ compared to prevalence in non-brachycephalic dogs.

[h] $P = 0.0124$ compared to allele frequency in non-brachycephalic dogs.

All statistical comparisons by Fisher's Exact test.

Supporting information

Table S1 Distribution of *P2RX7* gene missense variants in a random sample of dogs.

Table S2 Primers used to amplify and sequence missense variants of the canine *P2RX7* gene.

Table S3 Prevalence and allele frequency of the rs851148233 (p.Arg270Cys) and rs850760787 (p.Arg365Gln) variants in dogs of Cocker Spaniel and Labrador Retriever pedigree, respectively.

Table S1 Distribution of *P2RX7* gene missense variants in a random sample of dogs.

					Exon 3	Exon 8	Exon 11	Exon 13
					rs23314713	rs851148233	rs850760787	rs23315462
Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	p.Phe103Leu	p.Arg270Cys	p.Arg365Gln	p.Pro452Ser
Alaskan Malamute	Yes	Asian Spitz	No	M	wt	wt	wt	P/S
Alaskan Malamute	Yes	Asian Spitz	No	MN	wt	wt	wt	P/S
Alaskan Malamute and Siberian Husky cross	No		No	M		wt	wt	P/S
American Bulldog cross Bull Arab cross Greyhound	No		Yes	M	wt	wt	wt	wt
American Staffordshire Terrier	Yes	European Mastiff	Yes	M	F/L	wt	wt	P/S
American Staffordshire Terrier	Yes	European Mastiff	Yes	F	F/L	wt	wt	wt
American Staffordshire Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
American Staffordshire Terrier	Yes	European Mastiff	Yes		L/L	wt	wt	wt
American Staffordshire Terrier	Yes	European Mastiff	Yes	F		wt	wt	
Australian Bulldog	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Australian Bulldog	Yes	European Mastiff	Yes	M	L/L	wt	wt	wt
Australian Bulldog	Yes	European Mastiff	Yes	M	wt	wt	wt	
Australian Cattle Dog	Yes	UK Rural	No	M	F/L	wt	wt	P/S
Australian Cattle Dog and Kelpie cross	No		No	M	wt		wt	
Basenji	Yes	Not determined	No	MN	wt	wt	wt	wt
Beagle	Yes	Scent Hound	No	M	wt	wt	wt	S/S
Beagle	Yes	Scent Hound	No	M	wt	wt	wt	wt
Beagle	Yes	Scent Hound	No	F	wt	wt		wt
Beagle	Yes	Scent Hound	No	MN			wt	

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
Border Collie	Yes	UK Rural	No	F	wt	wt	wt	P/S
Border Collie	Yes	UK Rural	No	F	wt	wt	wt	wt
Border Collie	Yes	UK Rural	No	FN	wt	wt	wt	
Border Collie	Yes	UK Rural	No	M	wt		wt	
Border Collie and Australian Cattle Dog cross	No		No	M	wt	wt	wt	wt
Boxer and Bull Terrier cross	No		Yes	M		wt	wt	wt
British Bulldog	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Bull Arab	Yes		No	M	F/L	wt	wt	wt
Bull Terrier	Yes	European Mastiff	No	FN	L/L	wt	wt	wt
Bull Terrier	Yes	European Mastiff	No	M	L/L	wt	wt	wt
Bull Terrier (miniature)	Yes	European Mastiff	No	FN	L/L	wt	wt	wt
Bull Terrier cross	No		No	MN	F/L	wt	wt	wt
Bullmastiff cross	No		Yes	FN	wt	wt	wt	wt
Cavalier King Charles Spaniel	Yes	Spaniel	Yes		L/L	wt	wt	wt
Cavalier King Charles Spaniel	Yes	Spaniel	Yes	M	wt	wt	wt	S/S
Cavalier King Charles Spaniel and Cocker Spaniel cross	No		Yes	M	wt	R/C	wt	S/S
Chihuahua and Dachshund (miniature) cross	No		Yes	M	F/L	wt	wt	wt
Cocker Spaniel	Yes	Spaniel	No		wt	R/C	wt	wt
Cocker Spaniel	Yes	Spaniel	No	F	wt	wt	wt	wt

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
Cocker Spaniel	Yes	Spaniel	No	M	wt	wt	wt	wt
Cocker Spaniel	Yes	Spaniel	No	MN	wt			
Cocker Spaniel	Yes	Spaniel	No	MN			wt	
Cocker Spaniel (MDCK cells)	Yes	Spaniel	No	F	wt	R/C	wt	P/S
Cocker Spaniel and Poodle cross	No		No	M	wt	R/C	wt	S/S
Corgi cross	No		No	FN	F/L	wt	wt	P/S
Dachshund	Yes	Scent Hound	No	FN	wt	wt	wt	wt
Dachshund (miniature)	Yes	Scent Hound	No	FN	L/L	wt	wt	S/S
Dachshund (miniature)	Yes	Scent Hound	No	M	L/L	wt	wt	S/S
Dachshund (miniature)	Yes	Scent Hound	No	FN	wt	wt	wt	S/S
Dachshund (miniature)	Yes	Scent Hound	No	MN	wt	wt	wt	S/S
Dalmatian and Pitbull Terrier cross	No		No	F		wt	wt	
Fox Terrier (miniature)	Yes	American Terrier	No	MN	F/L	wt	wt	P/S
Fox Terrier (miniature)	Yes	American Terrier	No	F	F/L	wt	wt	wt
Fox Terrier (miniature)	Yes	American Terrier	No	F	wt	wt	wt	P/S
German Shepherd Dog	Yes	New World	No	M	L/L	wt	wt	
German Shepherd Dog	Yes	New World	No	FN	wt	wt	wt	wt
German Shepherd Dog	Yes	New World	No	FN	wt	wt	wt	
German Shepherd Dog cross	No		No	F	F/L	wt	wt	

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
German Shorthaired Pointer	Yes	Pointer Setter	No	F	wt	wt	wt	S/S
Golden Retriever	Yes	Retriever	No	F	wt	wt	wt	P/S
Golden Retriever	Yes	Retriever	No	M	wt	wt	wt	
Golden Retriever	Yes	Retriever	No	FN		wt	wt	
Golden Retriever (DH82 cells)	Yes	Retriever	No	M	wt		wt	wt
Golden Retriever and Labrador Retriever cross	No		No	M	L/L	wt	R/Q	wt
Hungarian Vizsla	Yes	Not determined	No	F	wt	wt	wt	wt
Jack Russell Terrier	Yes	Terrier	No	F	L/L	wt	wt	wt
Jack Russell Terrier	Yes	Terrier	No	F	wt	wt	wt	P/S
Jack Russell Terrier cross	No		No	F	L/L	wt	wt	wt
Jack Russell Terrier cross	No		No	MN	L/L	wt	wt	wt
Jack Russell Terrier cross	No		No	F	wt	wt	wt	wt
Kelpie	Yes	UK Rural	No	M	wt	wt	wt	wt
Kelpie	Yes	UK Rural	No	M		wt	wt	P/S
Labrador Retriever	Yes	Retriever	No	F	F/L	wt	wt	wt
Labrador Retriever	Yes	Retriever	No	F	L/L	wt	wt	wt
Labrador Retriever	Yes	Retriever	No	F	L/L	wt	wt	
Labrador Retriever	Yes	Retriever	No	F	wt	wt	R/Q	P/S
Labrador Retriever	Yes	Retriever	No	F	wt	wt	R/Q	P/S

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
Labrador Retriever	Yes	Retriever	No	F	wt	wt	R/Q	wt
Labrador Retriever	Yes	Retriever	No	F	wt	wt	wt	P/S
Labrador Retriever	Yes	Retriever	No	M	wt	wt	wt	P/S
Labrador Retriever	Yes	Retriever	No	M	wt	wt	wt	wt
Labrador Retriever	Yes	Retriever	No	F		wt	R/Q	S/S
Maltese	Yes	Poodle	No	F	wt	wt	wt	wt
Maltese	Yes	Poodle	No	M	wt	wt	wt	wt
Maltese	Yes	Poodle	No	MN	wt	wt	wt	
Maltese	Yes	Poodle	No	FN	wt		wt	wt
Maltese and Fox Terrier cross	No		No	M			wt	
Maltese and Poodle cross	No		No	F	F/L	wt	wt	wt
Maltese cross	No		No	MN	F/L	wt	wt	
Maltese cross	No		No	F			wt	
Maltese cross	No		No	MN			wt	
Maltese Shih Tzu	Yes	Poodle/Asian Toy	Yes	M	F/L			
Maltese Shih Tzu	Yes	Poodle/Asian Toy	Yes	FN	wt	wt	wt	P/S
Maltese Shih Tzu	Yes	Poodle/Asian Toy	Yes	M	wt	wt	wt	P/S
Maltese Shih Tzu	Yes	Poodle/Asian Toy	Yes	F	wt	wt	wt	S/S
Maltese Shih Tzu	Yes	Poodle/Asian Toy	Yes	MN	wt	wt	wt	wt

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
Mastiff and American Staffordshire Bull Terrier cross	No		Yes	M	L/L	wt	wt	wt
Newfoundland	Yes	Retriever	No	MN		wt	wt	wt
Pitbull Terrier	Yes	Not determined	No	F	wt	wt	wt	
Pomeranian	Yes	Small Spitz	No	FN	wt	wt	wt	
Poodle and Cocker Spaniel cross	No		No	M	wt	wt	wt	S/S
Pug and Beagle cross	No		Yes	F	L/L	wt	wt	S/S
Pug and Pomeranian cross	No		Yes	M	F/L	wt	wt	P/S
Rhodesian Ridgeback	Yes	European Mastiff	No	F	wt	wt	wt	P/S
Rhodesian Ridgeback and Australian Cattle Dog cross	No		No	MN	wt	wt	wt	wt
Rottweiler	Yes	Drover	No	F	wt	wt	wt	S/S
Rottweiler cross	No		No	F			wt	
Rottweiler cross	No		Yes	FN		wt	wt	
Schnauzer	Yes	Schnauzer	No	M	wt	wt	wt	wt
Schnauzer (miniature) and Bulldog cross	No		No	FN	F/L	wt	wt	P/S
Shar-Pei	Yes	Asian Spitz	No	M	wt	wt	wt	P/S
Shih Tzu and Pug cross	No		Yes	M	wt	wt	wt	
Siberian Husky	Yes	Asian Spitz	No	F	wt	wt	wt	P/S
Siberian Husky	Yes	Asian Spitz	No	F	wt	wt	wt	P/S
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	M	F/L	wt	wt	wt

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	FN	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	MN	L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes		L/L	wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F	L/L			
Staffordshire Bull Terrier	Yes	European Mastiff	Yes	F		wt	wt	wt
Staffordshire Bull Terrier	Yes	European Mastiff	Yes			wt	wt	
Staffordshire Bull Terrier and Kelpie cross	No		Yes	F	F/L	wt	wt	wt
Staffordshire Bull Terrier cross	No		Yes	F	F/L	wt	wt	P/S
Staffordshire Bull Terrier cross	No		Yes	FN	F/L	wt	wt	P/S
Staffordshire Bull Terrier cross	No		Yes	F	L/L	wt	wt	P/S
Staffordshire Bull Terrier cross	No		Yes	F	L/L	wt	wt	wt

Table S1 cont.

Breed	Pedigree	Clade[a]	Brachycephalic[b]	Sex[c]	Exon 3 rs23314713 p.Phe103Leu	Exon 8 rs851148233 p.Arg270Cys	Exon 11 rs850760787 p.Arg365Gln	Exon 13 rs23315462 p.Pro452Ser
Staffordshire Bull Terrier cross	No		Yes	F	L/L	wt	wt	wt

[a]Pedigree dogs were assigned into clades according to Parker et al. (2017).

[b]Mixed-pedigree dogs were assigned as brachycephalic based on known ancestry and/or display of brachycephalic characteristics (shortening of the muzzle, flat facial conformation and widening of the skull). Only one breed could be assigned in some dogs of mixed-pedigree when the ancestry of a cross was not fully known or could not be ascertained by visual observation.

[c]Sex: F, female; FN, neutered female; M, male; MN, neutered male.

Empty fields indicate sex unknown or exons not sequenced.

Table S2 Primers used to amplify and sequence missense variants of the canine *P2RX7* gene.

Forward primer (5'-3')	Reverse primer (5'-3')	Exon	Variant	Annealing temperature	Amplicon size
AGTCCAAGTTGCTCCCAGAC	GAGGACAGAAGAGGGAAAAGA	3	rs23314713	63.1^{o}C	349 bp
TTTAATCTGCGTTCCCCTCT	AAGCCTTTTGCATTCATCTTG	8	rs851148233	63.1^{o}C	368 bp
CTCACTAACTCGCACTGAT	TGATTTTGGCCTTATTTTTGC	11	rs850760787	62.2^{o}C	262 bp
GAACCTTAGGGGTGGTCACT	CAAGCTCAGGTGCAAACAAA	13[a]	rs23315462	64.1^{o}C	600 bp

[a]Primer pairs sequence the first half of exon 13 only.

Table S3 Prevalence and allele frequency of the rs851148233 (p.Arg270Cys) and rs850760787 (p.Arg365Gln) variants in dogs of Cocker Spaniel and Labrador Retriever pedigree, respectively.

Variant	Pedigree	Dogs (*n*)	Genotype (*n* of dogs)			Prevalence	Allele frequency
			Reference sequence	Heterozygous variant	Homozygous variant		
rs851148233	Cocker Spaniel	7[a]	3	4	0	57%[b]	0.29[b]
	Non-Cocker Spaniel	114[c]	114	0	0	0%	0
rs850760787	Labrador Retriever	11[d]	6	5	0	45%[e]	0.23[e]
	Non-Labrador Retriever	119[f]	119	0	0	0%	0

[a]Includes four Cocker Spaniels, one Cavalier King Charles Spaniel and Cocker Spaniel cross, one Cocker Spaniel and Poodle cross, and one Poodle and Cocker Spaniel cross.

[b]$P < 0.0001$ compared to non-cocker Spaniels.

[c]Includes two Cavalier King Charles Spaniels, and one Maltese and Poodle cross.

[d]Includes 10 Labrador Retrievers, and one Golden Retriever and Labrador Retriever cross.

[e]$P < 0.0001$ compared to non-Labrador Retrievers.

[f]Includes four Golden Retrievers.

All statistical comparisons by Fisher's Exact test.