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Research Article

Allele and genotype frequencies for primary hereditary cataract, multifocal retinopathy 1, and degenerative myelopathy in Pyrenean Mountain dog from Italy

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ABSTRACT

Pyrenean Mountain Dog (PMD) is an ancient dog breed firstly described in XIV century in the Pyrenees Region and nowadays diffused both in Europe and in the US. Hereditary Cataract (HC), defined as the inherited opacity of the lens, involves clinical signs ranging from reduced vision to glaucoma. A molecular basis of HC was firstly described in Staffordshire Bull Terriers and then reported in multiple canine breeds. The HC-associated variation is a single nucleotide deletion in *HSF4* gene that introduces a premature stop codon (c.962del, p.Ala321*). Multifocal Retinopathy 1 (MR) is an ocular disorder characterized by multiple areas of retinal degeneration, caused in various dog breeds (including PMD) by a single nucleotide variant (SNV) in *BEST1* gene that generates a premature stop codon (c.73G>A, p.Arg25*). Degenerative Myelopathy (DM) is an adult-onset, progressive neurodegenerative disease and it is associated to a SNV in *SOD1* gene causing a change in aminoacidic sequence of the protein (c.118G>A, p.Glu40Lys). This causative variant has been described in various dog breeds, including PMD. Aim of this study was to determine the allele frequencies for the abovementioned three genetic diseases in the Italian breeding PMD population. The survey found no dogs carrying the allele (deletion) associated with HC, while three dogs (6 %) were heterozygous (G/A) for the MR-associated variant, and seven dogs (13 %) were heterozygous (G/A) for the DM-associated alteration, indicating that the variant alleles frequency were 0 %, 3 %, and 7 %, respectively. Appropriate mating management is suggested for the prevention of genetic diseases spreading in the PMD population.

Introduction

The Pyrenean Mountain Dog (PMD), also commonly called Great Pyrenees (in the US) or *Patous* (in France), is a traditional breed from the French side of the Pyrenees Mountains. It is an ancient breed, widely used in the French Alps and Pyrenees as herd protectors from wolves and bears and claimed to be derived from white livestock guardian dogs imported from Asia by ancient Romans. After a great demand for this breed in French nobility during the 17th century (due to the fact that the son of King Louis XIV brought one of these dogs to the royal court of his father), the popularity of the breed declined bringing PMD to the verge of extinction by the beginning of the 20th century[1]. This decline was hence stopped with the registration of PMD breed to the British Kennel Club (1920s), to the French *Réunion des Amateurs de Chiens Pyrénées* (1923), and to the American Kennel Club (1935), along with the definition of the breed standard. Recent studies on genomic data grouped this breed in the same clade as the Pharaoh hound, the Cirneco dell'Etna, and the Ibizan hound dog breeds[2]. Few studies are available at this moment in scientific literature exploring the genetic bases of different PMD hereditary diseases and their prevalence, even though these diseases have been diagnosed in the breed. The three following genetic diseases were selected for the present study from the Online Mendelian Inheritance in Animals (OMIA) catalogue[3]: Primary Hereditary Cataract, Multifocal Retinopathy 1, and Degenerative Myelopathy. An overview of the three selected genetic diseases with their corresponding gene, chromosome number, genomic location, variants, effect, inheritance, and OMIA number are summarized in Table 1.

Primary Hereditary Cataract (HC) is a progressive opacity of the lens (appearing at a few weeks to months in age and progressing to total by two-to-three years of age) with clinical features that include altered or

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reduced vision, and changes in eye color and pupil size (OMIA 001758-9615). Barnett[4] firstly described the HC mode of inheritance as single-locus autosomal recessive. Homozygous individuals typically result in complete blindness and, in severe cases, glaucoma development has been observed[5]. This disease occurs in Australian Shepherd, Boston Terrier, and Staffordshire Bull Terrier dog breeds. One of the most common HC-associated variation is a single nucleotide deletion in *HSF4* gene that introduces a premature stop codon (rs1152388410, c.962del, p.Ala321*). Multifocal Retinopathy 1 (MR) is an eye disorder characterized by multiple areas of retinal degeneration developing around three months of age and progressing as the animal ages to focal areas of retinal degeneration and retinal pigment epithelial hypertrophy[6]. Similar to HC, the mode of inheritance is single-locus autosomal recessive (OMIA 001444-9615). This disease occurs in multiple dog breeds derived from the Mastiff line, including PMD, English Mastiff, and Bullmastiff[7]. In these breeds the causative alteration is a single nucleotide variant (SNV) in *BEST1* gene that generates a premature stop codon (c.73G>A, p.Arg25*) firstly described by Guziewicz et al.[8]. Lastly, Degenerative Myelopathy (DM) is an adult-onset (older than 8 years of age), chronic, progressive neurodegenerative disease with an autosomal recessive with incomplete penetrance mode of inheritance (OMIA 000263-9615), occurring in multiple canine breeds including German Shepherd Dog, Boxer, Collie, and Bernese Mountain Dog[9]. Clinical signs of DM include progressive, asymmetric paraparesis of the pelvic limbs ultimately leading to paraplegia and dyspnea[10]. As a late-onset disease, homozygous dogs for the altered allele are fertile and likely to produce offspring, leading to a spreading of this allele[11]. Currently, no treatment for DM has been reported in scientific literature. Insoluble inclusions of misfolded protein can be found in motor neurons cytoplasm in the spinal cord sections, a scenario similar to the human amyotrophic lateral sclerosis[12]. The most common DM-associated mutation is a single nucleotide substitution that causes an amino acid substitution (rs853026434, c.118G>A, p.Glu40Lys) in the *SOD1* gene. Aim of this study was to determine the allele frequency of the variants involved in the risk assessment of HC, MR, and DM in the Italian breeding population of PMD.

Materials and methods

Sample collection

A total of 52 breeding animals (31 studs and 21 bitches) among the animals participating to Italian national dog shows were selected in this study based on the sampling permission accorded by the owner of each dog. Informed oral consent was obtained from the participating owners. Saliva from each dog was collected by buccal swab in accordance with the general recommendations of respect for animal welfare. All efforts were made to minimize animal suffering. The mean age of the sampled animal was 3.29 ± 2.33 years (mean ± SD), ranging from a minimum of 0.27 to a maximum of 8.64 years of age. Mean age for studs and bitches were 3.13 ± 2.48 years (mean ± SD) and 3.52 ± 2.15 years (mean ± SD), respectively.

DNA extraction, amplification, and sequencing of HSF4, BEST1, and SOD1 genes

DNA was extracted from buccal swabs samples using a commercial kit (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The extracted DNA was amplified by conventional PCR using the following primers: HC_F (5'-ATGTGGGTACTGGTTGGGTT-3'), HC_R (5'-CTATCTGCAAAAGTGGCCCA-3'), MR_F (5'-TTCCAAGACCCAGCTGTACC-3'), MR_R (5'-CCTCATCCCATTCCCAGTGT-3'), DM_F (5'-TTGACTGAAGGGAAGTGGGC-3'), and DM_R (5'-ACTAGACCAACACAACACCCA-3'). All the primers were designed using Primer3web software version 4.1.0[13]. The PCR were performed in a thermal cycler (Applied Biosystem 2720 Thermal Cycler) in a total volume of 50 μL using the HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany). The same PCR protocol was applied to the amplification of the three gene fragments. The amplification occurred with a denaturation step at 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. The amplified samples and controls (positive and negative) underwent 1.5 % agarose gel electrophoresis, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA), and visualized in an ultraviolet transilluminator. The expected result consisted in three amplicons of 604, 398, and 417 base pairs (bp), for HC, MR, and DM, respectively. Pre-sequencing PCR product clean-up was performed enzymatically using ExoSAP-IT Express PCR Product Cleanup Reagent (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Standard capillary sequencing was then performed using the SeqStudio Genetic Analyzer (Applied Biosystems by ThermoFisher Scientific, Waltham, MA, USA). The obtained DNA sequences were subsequently aligned to the reference ones for each disease using the software BioEdit version 7.2.5[14].

Statistical and population genetic analysis

Pedigree data of each animal was collected aiming at the inclusion of at least four generations of ancestors (when available) and a pedigree-based Coefficient of Inbreeding (COI) and Ancestor Loss Coefficient (AVK) were calculated using four generations of ancestors[15]. Both COI and AVK were subsequently averaged by year of birth of the dog in order to observe the trend across the years.

Hardy–Weinberg equilibrium (HWE) was tested on the allele frequencies obtained in this study using the fisher test. Fisher's test was preferred to the chi-squared one since the smallest expected frequency in the contingency table was lower than five[16]. A threshold of $p < 0.05$ was regarded as statistically significant for the deviations between measured and expected values, indicating that data at $p \geq 0.05$ is in HWE. Statistical analyses were performed using R software[17].

Results

Pedigree-based analyses

Pedigree data was available for 48 animals (92.3 % of the total sampled animals). After removing the four dogs without known

Table 1
An overview of three genetic diseases with their corresponding gene, chromosome number, genomic location, variant, effect, OMIA variant ID, and inheritance

Disease	Gene	Chr	Genomic location (CanFam3.1)	Variant	Effect	OMIA Variant ID	Inherit
HC	*HSF4*	5	g.82198114_ 82198115insG	c.971_ 972insC	p.L325Tfs*28	568	AR
MR	*BEST1*	18	g.54478586G>A	c.73C>T	p.R25*	275	AR
DM[1]	*SOD1*	31	g.26540342G>A	c.118G>A	p.E40K	36	AR

HC, Hereditary cataract; MR, Multifocal retinopathy; DM, Degenerative myelopathy; Chr, Chromosome; AR, Autosomal recessive.
[1]DM mode of inheritance involves incomplete penetrance. The SOD1 variant is an established genetic risk factor, but not all dogs with the homozygous genotype for the variant allele will develop DM.

ancestors, the reduced dataset consisted of 28 studs and 20 bitches, with an average age of 3.22 ± 2.29 years (mean ± SD), ranging from a minimum of 0.27 to a maximum of 8.64 years of age. The animals in the dataset were born between 2013 and 2021. Based on the pedigree information, COI was calculated on the reduced dataset and 19 out of 48 animals (39.6 %) were identified as inbred (i.e., COI > 0 %), while 29 (60.4 %) had COI = 0 %. The average COI of the total inbred animals was 9.91 % (values ranging from 1.56 to 23.44 %). Differences in average COI in studs and bitches were not statistically significant (t-test, t = -0.41, df = 44.3, p-value = 0.68). Average COI of inbred animals was plotted as a yearly time series plot to visualize its trend across the years of birth of the analyzed animals (Fig. 1). A statistically significant difference (t-test, t = -4.86, df = 12.92, p-value < 0.001) was observed between average COI of animals born before and after 2015 (2.86 and 11.2 %, respectively). Although a trend toward a lower average COI can be observed in Fig. 1, the difference between the means of the two groups (i.e., born between 2015 and 2018 and born after 2018) is not statistically significant.

On the same reduced dataset, AVK was calculated and 14 out of 48 animals (29.2 %) had AVK = 100 % (i.e., each ancestor in the four previous generations was present only once in the pedigree), while 34 (70.8 %) had AVK < 100 %, meaning the presence of repeated ancestors in the pedigree of the animal. The average AVK of animals with repeated ancestors was 89.23 % (values ranging from 66.67 to 96.88 %).

Allelic and genotypic frequencies

The genotyping of the sampled dogs revealed that all the 52 dogs (100 %) were homozygous for the wild type allele (C/C) for HC. Differently, 3 dogs (6 %) were heterozygous (G/A) for the MR related alteration and 7 dogs (13 %) were heterozygous (G/A) for the DM related alteration. No dogs were found homozygous for the mutant allele of each studied disease. The frequency of the mutant allele for MR was 3 %, while the DM one was 7 %. The Hardy-Weinberg equilibrium for MR and DM was determined and the results are reported in Table 2. No departures from HWE were observed.

Discussion

The study presented here is a molecular epidemiological screening of three genetic diseases (namely hereditary cataract, multifocal retinopathy 1, and degenerative myelopathy) in PMD bred in Italy. As per our knowledge, although both MR and DM were previously described in PMD, no molecular screening have been performed before, differently from, for example, the situation in German Shepherd dog, where DM genetic surveys are available in scientific literature from different countries[9]. This scarce investigation in scientific literature is possibly due to a reduced numerosity of this dog breed in comparison to more diffused breeds. Although there is no official information of the number of PMD bred, the Italian kennel club (ENCI) reports an average of 130 dogs/year registered in their database in the last decade. According to the expected PMD lifespan of 10-12 years, an overall number of around 1300-1400 registered animals on the Italian territory was estimated by the authors. This number includes, however, both unneutered private dogs not used for breeding and neutered ones. Only 25 animals are available in the Italian national registry of genetically tested breeding studs and bitches[18], of which ten dogs were included in the sample for this study. The remaining sampled dogs, although not registered as genetically tested breeding dogs, were selected among the dogs participating in national dog shows which often are, therefore, the first choice for breeders' selection. Thus, even if numerically low, the sample of this survey should be representative of the actual Italian breeding PMD population.

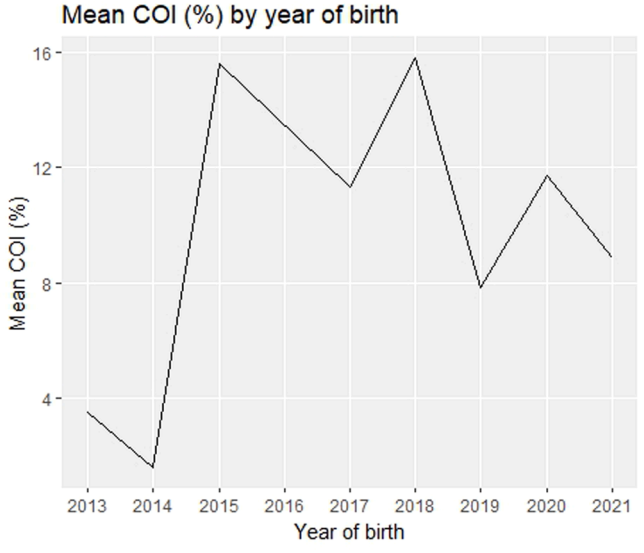

Fig.1. Mean Coefficient of Inbreeding (COI) by year of birth of the 48 Pyrenean Mountain Dog in the study.

Regarding HC, different studies performed on other dog breeds showed variable levels of variant allele frequency, ranging from 7 % to 17 %[19]. Nevertheless, the association of this alteration with the disease is still debated, as different authors in scientific literature reported discordant results on this topic[5,20,21], as well as other changes in different loci have been suggested more recently[22]. In our study, no variant allele for HC was found in the sampled breeding PMD. Regarding MR, a small number of variant alleles were found in the sampled breeding PMD. The presence of the disease in PMD breed has been confirmed since the end of the XXth century[6], however, no breed specific molecular surveys have been reported for MR in PMD. In scientific literature a wide range of allele frequency was reported for the MR-associated variant in different dog breeds. For example, in a study from Majchrakova et al.[19], an allele frequency of 0. 5 % was found in Australian Shepherd dogs, while, a higher allele frequency of 13 % was found by Donner et al.[23] in Brazilian Terrier dogs. Similar to what found in the study with Australian Shepherd dogs, no homozygous dogs for the disease-associated allele were found in our survey. Lastly, regarding DM, a main comparison of results can be done with German Shepherd dogs (GSD), where the genetic disease is well documented and surveyed across the world. Specifically, different studies showed a mutant allele frequency in GSD ranging from 12 to 38 % (respectively in Brazilian and English GSD), according to the country of origin of the sampled dogs[24,25]. The results in GSD are on average higher than what observed in this survey. In other breeds, frequency of DM mutant allele ranges from 12 % in Australian Shepherd[19] to 17 % in Belgian Malinois[26]. In a recent study from Donner et al.[27], over one million dogs (both purebred and mixed breed dogs) were genotyped for 250 disease-associated variants, including those related to MR and DM. In purebred dogs, they found an allele frequency of 1 and 9 % for the MR and DM related disease-associated variants, respectively, which are close to the results obtained in our study (i.e., 3 and 7 % for MR and DM, respectively). Although the study from Donner et al.[27] did not include PMD in the sampled dogs, the large sample number yield a plausible estimate of the true allele frequency for variants that segregate in many different breeds just like the studied DM-related variant.

The results obtained from this survey showed a low frequency of two of the three studied genetic diseases mutant allele in Italian PMD when compared to molecular surveys conducted in other dog breeds. Excluding HC, genetic testing for these variants could be useful in both MR and DM management. In the former, since the frequency is still relatively low, identifying the few heterozygous dogs present in the population could help breeders to exclude them from breeding schemes without risking an inbreeding level increase. In the latter, since the frequency of the DM-associated allele in the population is slightly higher

Table 2
Hardy-Weinberg equilibrium test results.

Disease	Observed genotype frequency (%)			Expected genotype frequency (%)			Mutant allele frequency (%)	Chi-squared test analysis
	Homo wt	Het	Homo mut	Homo wt	Het	Homo mut		
HC	100	0	0	100	0	0	0	-
MR	94.23	5.77	0	94.32	5.60	0.08	2.88	$p = 0.98$
DM	86.54	13.46	0	86.99	12.56	0.45	6.73	$p = 0.88$

HC, Hereditary cataract; MR, Multifocal retinopathy; DM, Degenerative myelopathy; Homo wt, Homozygote wild-type; Het, Heterozygote; Homo mut, Homozygote mutant.

than MR, excluding all the heterozygous dogs from breeding schemes could result in an extreme increase in the coefficient of inbreeding. Therefore, genetic screening in this last scenario could assist breeders in matching their breeding animals in order to avoid the birth of homozygous puppies.

Conclusions

The mutant alleles associated with MR and DM in Italian PMD were found in the sampled population in this survey and their frequency was 3 and 7 %, respectively. On the other hand, no mutant allele associated with HC was found. Genetic testing for these alterations associated with diseases currently lacking an efficient treatment is an important prophylaxis tool to avoid spreading the disease within dog populations.

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CRediT authorship contribution statement

R. Moretti: Formal analysis, Writing – original draft. **G. Massimello:** Investigation, Resources, Writing – review & editing. **S. Chessa:** Writing – review & editing. **S. Sartore:** Writing – review & editing. **A. Tranchero:** Investigation, Writing – review & editing. **M. Profiti:** Investigation, Writing – review & editing. **P. Sacchi:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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