A Coding Variant in the Gene Bardet-Biedl Syndrome 4 (BBS4) Is Associated with a Novel Form of Canine Progressive Retinal Atrophy

Tracy Chew,*,1 Bianca Haase,† Roslyn Bathgate,† Cali E. Willet,§ Maria K. Kaukonen,¶,**,†† Lisa J. Mascord,* Hannes T. Lohi,*§*,**,†† and Claire M. Wade*,*

*School of Life and Environmental Sciences, †Sydney School of Veterinary Science, Faculty of Science, and ‡Sydney Informatics Hub, Core Research Facilities, University of Sydney, 2006, Australia, §Department of Veterinary Biosciences, ¶Research Programs Unit, Molecular Neurology, and ††Folkhälsoinstitet of Genetics, University of Helsinki, 00014, Finland

ORCID IDs: 0000-0001-9529-7705 (T.C.); 0000-0001-8449-1502 (C.E.W.); 0000-0003-1087-5532 (H.T.L.); 0000-0003-3413-4771 (C.M.W.)

ABSTRACT Progressive retinal atrophy is a common cause of blindness in the dog and affects >100 breeds. It is characterized by gradual vision loss that occurs due to the degeneration of photoreceptor cells in the retina. Similar to the human counterpart retinitis pigmentosa, the canine disorder is clinically and genetically heterogeneous and the underlying cause remains unknown for many cases. We use a positional candidate gene approach to identify putative variants in the Hungarian Puli breed using genotyping data of 14 family-based samples (CanineHD BeadChip array, Illumina) and whole-genome sequencing data of two proband and two parental samples (Illumina HiSeq 2000). A single nonsense SNP in exon 2 of BBS4 (c.58A>T, p.Lys20*) was identified following filtering of high quality variants. This allele is highly associated (P_{\text{CHISQ}} = 3.425e^{-14}, n = 103) and segregates perfectly with progressive retinal atrophy in the Hungarian Puli. In humans, BBS4 is known to cause Bardet–Biedl syndrome which includes a retinitis pigmentosa phenotype. From the observed coding change we expect that no functional BBS4 can be produced in the affected dogs. We identified canine phenotypes comparable with Bbs4-null mice including obesity and spermatozoa flagella defects. Knockout mice fail to form spermatozoa flagella. In the affected Hungarian Puli spermatozoa flagella are present, however a large proportion of sperm are morphologically abnormal and <5% are motile. This suggests that BBS4 contributes to flagella motility but not formation in the dog. Our results suggest a promising opportunity for studying Bardet–Biedl syndrome in a large animal model.

KEYWORDS Hungarian Puli whole-genome sequencing blindness obesity infertility

Progressive retinal atrophy (PRA) (OMIA #000830-9615) is the most common cause of hereditary blindness in the domestic dog (Canis lupus familiaris), affecting >100 pure breeds (Whitley et al. 1995). It is clinically and genetically heterogeneous and encompasses several forms of disease which vary by etiology, rate of progression, and age of onset (Downs et al. 2014a). The typical characteristics are gradual night, followed by day vision loss due to the degeneration of rod and cone photoreceptors, and this degeneration continues until the affected animal is completely blind (Parry 1953). Ophthalmic features that become apparent as the retina deteriorates include tapetal hyperreflectivity, vascular attenuation, pigmentary changes, andatrophy of the optic nerve head (Parry 1953; Clements et al. 1996; Petersen-Jones 1998).

PRA is recognized as the veterinary equivalent of retinitis pigmentosa (RP) in humans due to the clinical and genetic similarities between the disorders (Petersen-Jones 1998; Cideciyan et al. 2005; Zangerl et al. 2006; Downs et al. 2011). RP is a common cause of blindness in humans and affects ~1 in 4000 people (Hamel 2006). There are very limited treatment options for both PRA and RP at present (Hamel 2006). For this reason, the dog has become a valuable large animal
model for retinal degeneration, in particular, for testing the efficacy of novel therapeutics such as gene therapy (Pearce-Kelling et al. 2001; Acland et al. 2001; Narfström et al. 2003; Cideciyan et al. 2005; Beltran et al. 2012; Pichard et al. 2016). As of 2016, 256 retinal disease-associated genes were identified for humans (https://sph.uth.edu/retnet/). Some of these genes cause nonsyndromic RP, while others contribute to syndromic disorders such as Bardet–Biedl syndrome (BBS) (Hamel 2006).

Currently, retinal dystrophies in 58 domestic dog breeds have been linked to at least 25 mutations in 19 different genes (Miyadera et al. 2012; Downs et al. 2014b). Canine PRA is typically inherited in an autosomal recessive pattern, although two forms that are X-linked (Vilboux et al. 2008) and one that has dominant inheritance have been reported (Kijas et al. 2002, 2003). Many of these discoveries in the canine were made using candidate gene studies, linkage mapping and genome-wide association studies (GWAS) followed with fine mapping (Acland et al. 1999; Goldstein et al. 2006; Kukekova et al. 2009; Downs et al. 2014b). This success has been facilitated by the unique breeding structure of dogs. Intense artificial selection, genetic drift, and strong founder effects have resulted in stretches of linkage disequilibrium (LD) that can persist for several Mb within breeds, but only tens of kb across breeds (Lindblad-Toh et al. 2005). This species population structure has allowed for the successful mapping of Mendelian traits with fewer markers and subjects compared to human gene mapping studies: as few as 10 unrelated cases and 10 controls (Karlsson et al. 2007; Frischknecht et al. 2013; Jagannathan et al. 2013; Willet et al. 2015; Gerber et al. 2015; Wolf et al. 2015). Such methods are accepted to work extremely well for mapping monogenic traits that segregate within a single breed.

Despite this achievement, there are still many forms of PRA in several breeds of dog that have yet to be genetically characterized. Traits with underlying genetic heterogeneity and a late onset are notoriously difficult to map using linkage or GWAS methods (Hirschhorn and Daly 2005; Korte and Farlow 2013). Although PRA is collectively common, individually, specific forms are relatively rare and it may take many generations until an adequately sized cohort of unrelated case samples are collected. The genetic heterogeneity of PRA can complicate the results of linkage mapping and GWAS, as different causative variants and genes can be responsible for an identical phenotype. In addition, both linkage and GWAS rely on markers to be in LD and segregate with the disease gene, making it difficult to detect rare or de novo variants (Hirschhorn and Daly 2005).

Since the advent of whole-genome sequencing (WGS) and whole exome sequencing technologies, the discovery of causal variants for rare or genetically heterogeneous diseases has become more rapid with fewer case samples necessary for success. One study design of note that has been used in human and more recently in canine studies is the sequencing of parent-proband trios (Zhu et al. 2015; Sayyab et al. 2016). As this method provides the chance for earlier diagnosis than previously possible, this gives patients the opportunity to access more personalized treatment options (Farwell et al. 2015; Zhu et al. 2015; Sawyer et al. 2016).

In a preliminary study, extensive screening of 53 genes associated with autosomal recessive PRA or RP revealed no putative variants that could be associated with PRA in the Hungarian Puli breed (Chew et al. 2017). Here, we combine genotyping array data and WGS data of a parent-proband trio with an additional half-sibling case to identify a potentially novel canine PRA gene. We successfully identify a highly associated mutation in exon 2 of BBS4 (c.58A > T, P\textsubscript{meta} = 3.425e\textsuperscript{-14}, n = 103) that segregates perfectly with the disease phenotype. This mutation encodes a premature stop codon which is expected to result in complete loss of function of the BBS4 protein. The association of BBS4 with canine PRA is a novel finding and presents the first description of an associated variant for PRA in the Hungarian Puli.

**MATERIALS AND METHODS**

**Samples**

This study involved 255 dogs (C. lupus familiaris) that comprised 103 Hungarian Puli and 152 Hungarian Pumi samples. This sample cohort included 14 Hungarian Puli segregating PRA in an autosomal recessive pattern from a previous study (Chew et al. 2017). Three affected Hungarian Pulis (USCF516, USCF519, and USCF5311) were diagnosed with PRA at the age of 2 yr by registered specialists in veterinary ophthalmology. Diagnosis was based on observed ophthalmologic changes including vascular attenuation, hyper-reflectivity, and reduced myelination in the optic nerve head. The parents (USCF347, USCF524, and USCF525) were similarly tested and confirmed as PRA clear. The remaining dogs were >3 yr of age and had normal vision as reported by their owners or veterinarians. Hungarian Pumis are a very closely related breed to the Hungarian Pulis and have been considered as a unique breed only since the 1920s, so were considered as a compatible cohort for this study.

Biological samples from the 255 dogs were collected either as EDTA-stabilized whole blood or buccal cells using noninvasive swabs (DNA Genotek) or indicating Whatman FTA Cards (GE Healthcare). Genomic DNA was isolated from whole blood using the illustra Nucleon BACC2 kit (GE Healthcare) or from buccal cells on swabs using the Performagene Kit. For samples collected on an FTA card, DNA on discs was purified according to the manufacturer’s guidelines.

We ensured that recommendations from the Australian Code for the Care and Use of Animals for Scientific Purposes were strictly followed throughout the study. Animal ethics approval was granted to conduct this research by the Animal Ethics Committee at the University of Sydney (approval number N00/9–2009/3/5109, September 24, 2009) and the State Provincial Office of Southern Finland (ESAVI/6054/04.10.03/2012).

**Genotyping array data**

Genotyping array data of 14 Hungarian Puli and WGS data of a parent-proband trio and one additional half-sibling case (USCF347, USCF516, USCF519, and USCF525) were obtained from the preliminary study (Chew et al. 2017). Genotyping was performed on the CanineHD BeadChip array (Illumina, San Diego, CA) by GeneSeek (Lincoln, NE). WGS was performed as 101 bp, paired-end reads on the Illumina HiSeq 2000 by the Ramaciotti Centre, University of New South Wales, Kensington. The Illumina TruSeq DNA polymerase chain reaction (PCR)-free kit was used to prepare the libraries. The four samples were barcoded and sequenced on two lanes of the sequencing machine. For additional information on sample and data collection, refer to the supplementary information in Chew et al. (2017). Sample information for this study can be found in Supplemental Material, File S1.

**Candidate gene selection**

Comprehensive screening of 53 PRA loci in the Hungarian Puli family revealed no obvious functional variants for the phenotype of interest (Chew et al. 2017). To identify novel candidates, regions concordant with a recessive inheritance pattern were identified using two case (USCF516 and USCF519) and 12 control dogs that were genotyped at 172,938 SNP markers on the CanineHD array. The control dogs included three PRA-clear parents (USCF347, USCF524, and USCF525). Only markers that were genotyped as homozygous for the minor allele in cases,
heterozygous in the parents, and heterozygous or homozygous for the reference allele in the remaining nine control dogs were regarded as target loci (Microsoft Excel 2010).

Candidate genes were selected from the region with the highest frequency and density of concordant SNPs. LD in purebred dogs can span several Mb long (Lindblad-Toh et al. 2005), thus we considered markers within 5 Mb to be in a single haplotype block. Using the corresponding syntenic positional region in the mouse reference genome (mouse genome assembly GRChm38, January 2012 build, the Genome Reference Consortium), we restricted our analysis to genes with a known phenotypic connection to vision using the Mouse Genome Browser (http://jbrowse.informatics.jax.org/). Any genes within the identified regions that were not already assessed in the preliminary PRA gene screening study (Chew et al. 2017) were chosen as positional candidate genes and considered for further analysis.

Whole-genome sequence processing and putative mutation detection

Next-generation sequencing data from two cases (USCF316 and USCF519) and two parental controls (USCF437 and USCF525) were aligned to CanFam 3.1 (Hoeppner et al. 2014). Reads were aligned as pairs using the Burrows-Wheeler Alignment tool with default parameters (Li and Durbin 2009). PCR duplicates were marked using Picard (http://broadinstitute.github.io/picard/). Local realignment around insertion-deletions (indels) was performed using the Genome Analysis Tool Kit (GATK) (McKenna et al. 2010; DePristo et al. 2011).

High quality variants were called for all four individuals simultaneously over 12 candidate genes that were selected from the locus with the highest density of SNPs concordant with autosomal recessive inheritance. Raw variants were first called using HaplotypeCaller provided by GATK (Van der Auwer et al. 2013; McKenna et al. 2010). SNPs were then removed if Quality Depth <2.0, Fisher Strand >60.0, Mapping Quality <40.0, HaplotypeScore >13.0, MappingQualityRankSum < −12.5, and ReadPosRankSum < −8.0. Indels were removed if Quality Depth <2.0, Fisher Strand >200.00, and ReadPosRankSum < −20.0.

The remaining high quality SNPs and indels were annotated using Variant Effect Predictor provided by Ensembl (McLaren et al. 2010). Known population variants obtained from publically available data were not considered as candidates (Lindblad-Toh et al. 2005; Vayse et al. 2011; Axelsson et al. 2013). Exonic variants were manually evaluated for genotype quality and conformation to the expected inheritance pattern using SAMtools tview (Li et al. 2009) and the UCSC Genome Browser. Remaining variants which were predicted by SIFT (Sim et al. 2012) to be deleterious (<0.05) were then considered for genotype validation and segregation analysis in the wider population by Sanger sequencing.

Variant validation and segregation analysis

The pedigree relationships among the 14 array-genotyped individuals for which registered (Australian National Kennel Council) pedigree data were available were tested through identity-by-descent proportions calculated using PLINK (Purcell et al. 2007).

To confirm that the identified mutation was not a sequencing error and that the variant was concordant with the Mendelian expectation of the disorder phenotype, we genotyped 103 Hungarian Puli and 152 Hungarian Pumi for the candidate causative mutation c.58A > T in BBS4 using PCR and Sanger sequencing.

Forward (‘GTTAGCAAGATACATGTTGC-3’) and reverse (‘GACTATTACGCTTTCCAAAA-3’) primers were designed with Primer3 (Rozen and Skaletsky 2000) to amplify a 225 bp product flanking the candidate mutation. PCR was carried out using the AmpliTaq Gold 360 Master Mix (Applied Biosystems) in a 20 µl reaction volume. Following denaturation at 95°C for 15 min, samples underwent amplification for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, followed by a final elongation step at 72°C for 10 min. For the purification of each sample, 7 µl of PCR product was dispensed into 3 µl of master mix containing 10× shrimp alkaline phosphatase (SAP) buffer, 1 U SAP, 1 U Exo I, and water. Enzymatic activity was enabled for 30 min at 37°C and was then deactivated during 15 min at 80°C. Sanger sequencing of purified PCR products was carried out by the Australian Genome Research Facility at Westmead in accordance with the vendor’s instructions.
Assessment of Bbs4 null mouse phenotypes in the dog

In addition to retinal degeneration, previous studies with Bbs4-null mice demonstrated that the protein is implicated in obesity and infertility caused by a failure to form spermatozoa flagella (Mykytyn et al. 2004; Aksanov et al. 2014). Veterinarians who assessed the three affected Hungarian Puli anecdotally described these individuals as obese. A fertility assessment was performed for the sole intact male (USCF519; USCF347 is female and USCF1311 was desexed) by an animal reproduction specialist at the University of Sydney. Semen characteristics were compared with previously reported data for healthy dogs as a breed-matched control was not able to be obtained (Schaer 2009). The sperm-rich fraction of semen was collected by digital stimulation into a polypropylene test tube. Semen volume and color were noted immediately. Spermatozoa were observed using phase contrast microscopy at 100× magnification, and motility was subjectively determined. An aliquot of semen was smeared onto a slide for morphology assessment under oil at 1000× magnification, using previously described criteria (Feldman and Nelson 1987). Sperm count was determined by use of a hemocytometer.

### Assessment of Bbs4 /− mouse phenotypes in the dog

In addition to retinal degeneration, previous studies with Bbs4-null mice demonstrated that the protein is implicated in obesity and infertility caused by a failure to form spermatozoa flagella (Mykytyn et al. 2004; Aksanov et al. 2014). Veterinarians who assessed the three affected Hungarian Puli anecdotally described these individuals as obese. A fertility assessment was performed for the sole intact male (USCF519; USCF347 is female and USCF1311 was desexed) by an animal reproduction specialist at the University of Sydney. Semen characteristics were compared with previously reported data for healthy dogs as a breed-matched control was not able to be obtained (Schaer 2009). The sperm-rich fraction of semen was collected by digital stimulation into a polypropylene test tube.

### RESULTS

#### Target loci and candidate genes

Of the 172,938 SNP markers that were genotyped on the CanineHD BeadChip array for two cases and 12 controls, 363 markers segregated with PRA. Chromosome 30 (chr30) position 25.3–40.0 Mb demonstrated the highest density of concordant SNPs with 103 markers (Figure 1). This region is syntenic to mouse chromosome 9, 55.5–96.3 Mb (GRCm38/mm10 Assembly). The mouse phenome browser indicated that in this region 13 genes involved in vision have been identified, of which 12 are not currently known to be implicated in canine PRA. Chr4 position 0.5–10.5 Mb had the second most (n = 61) number of concordant markers. This region is syntenic to mouse chr13, 9.5–14.5 Mb, and chromosome 8, 122.7–127.7 Mb. Followed by this region is chr20 position 9.5–20.3 Mb.

#### Table 1: Number of SNP and indel variants detected after applying standard hard filtering criteria

| Filtering Criteria                                      | SNP   | Indel |
|--------------------------------------------------------|-------|-------|
| High quality variants in candidate regions              | 2726  | 912   |
| Not a common in canine population                       | 1918  | 900   |
| Exonic variants (total)                                 | 44    | 4     |
| Synonymous                                              | 27    | —     |
| Missense                                                | 16    | —     |
| Nonsense                                                | 1     | —     |
| In-frame insertion                                      | —     | 1     |
| In-frame deletion                                       | —     | 2     |
| Multiple nucleotide polymorphism                        | —     | 1     |
| Manual check for quality and inheritance pattern       | 3     | 0     |
| Predicted as deleterious by SIFT (<0.05)                | 1     | 0     |

---

**Figure 2** BBS4 protein sequence alignment of affected dogs containing the c.58A>T SNP and of the wild-type protein. The SNP in affected dogs results in a premature stop codon (p.Lys20*). Hyphens (-) refer to missing amino acids in the affected dogs relative to the wild-type protein.
with 60 concordant markers. This is syntenic to mouse chr6, 100.0–110.0 Mb. The mouse phenome browser revealed three candidate genes on each of the chr4 and chr20 regions. A total of 18 genes were selected as positional candidates in the current study (Table S1).

**WGS and variant detection**

Sequencing on the Illumina HiSeq 2000 produced an average of 171 million raw reads per dog. Of these reads, 99.3% were successfully mapped to the CanFam 3.1 reference genome, resulting in an average mapped coverage of 6.9× per individual.

In the 18 selected candidate genes, 2726 high quality SNPs were detected, 1918 of which are not currently known population variants (Table 1; Lindblad-Toh et al. 2005; Vaysse et al. 2011; Axelsson et al. 2013). Of the 44 exonic SNPs, there were 27 synonymous, 16 missense, and one nonsense SNP. Two of the missense SNPs, one at MEGF11, chr30:30,251,670 and the other at STRA6, chr30:37,344,538, followed the expected inheritance pattern. Both were predicted by SIFT to be tolerated (P = 1) and therefore were not considered for further analysis. The single nonsense SNP detected occurred at BBS4, chr30:36,063,748 and followed the expected inheritance pattern. This was predicted to be deleterious and was considered for validation and segregation analysis.

A total of 912 indels were detected, 900 of which are not currently known population variants (Table 1). Four of these were exonic, and by manual inspection none of these followed the expected inheritance pattern and so were not considered for further analysis.

**Validation and segregation of putative nonsense variant in BBS4**

A single, putative functional coding variant that passed all hard filtering criteria was identified. The variant results in a stop-gained mutation in BBS4 and is predicted to be deleterious. We manually completed the annotation of BBS4 in the CanFam 3.1 reference genome as exon 1 was evidently missing (refer to File S2 for a full description of the methods used). The complete canine BBS4 protein can be accessed through Genbank (accession KX290494). In the complete BBS4 gene, the putative mutation results in a premature stop codon (p.Lys20*) as a result of c.58A>T SNP in exon 2 (Figure 2).

The 103 Hungarian Puli included the three affected animals and 14 others with normal vision from the same kennel (Figure 3). Pedigree relationships for the 14 individuals for which genotyping array data were available were confirmed through identity-by-descent estimations (Table S2). Through Sanger sequencing, we observed that all three affected dogs (USCF516, USCF519, and USCF1311) were homozygous for the variant allele (T/T), all three obligate carrier parents were heterozygous (A/T), and the remaining unaffected Hungarian Puli were either heterozygous or homozygous for the wild-type allele (A/A, Figure 4). All Hungarian Pumi were homozygous for the wild-type allele. Genotypes for each individual in the study can be found in File S1.

An association of PCHISQ = 3.425e−14 between the c.58A>T SNP in BBS4 to the disease phenotype was found for all validated Hungarian Puli genotypes (n = 103). When including validated Hungarian Pumi genotypes, the association is PCHISQ = 3.252e−14 (n = 255). The genotypes are perfectly consistent with an autosomal recessive pattern of inheritance for the 17 Hungarian Puli individuals with pedigree information, which supports the expected segregation pattern for PRA in this breed (Figure 3).

**Assessment of Bbs4−/− mouse phenotypes in the dog**

The intact affected male Hungarian Puli (n = 1) was found to be subfertile. Semen analysis indicated normal sperm concentration but...
a low total sperm count (13.65\(\times\)10^6). A high proportion (78%) of sperm had abnormal morphology, predominantly as a consequence of spermatozoa tail defects (74%, Table 2). There were <5% of sperm with normal motility.

**DISCUSSION**

In this study, we identify a putative functional variant that is highly associated with the PRA disease phenotype in the Hungarian Puli breed. The variant occurs in a novel canine PRA gene. A preliminary study suggested that it was likely that a novel gene was causing disease in this family because no obvious functional variants were identified in the exons or promoters of any of 53 previously described PRA genes (Chew et al. 2017). Using genotyping array data and WGS data of a parent-proband trio (USCF525, USCF347, and USCF516) and an additional half sibling case (USCF519), we identify a nonsense SNP (p.Lys20*) in exon 2 of BBS4 that is significantly associated with disease (\(P_{CHISQ} = 3.425\times\)10^{-14}, \(n = 103\)). The associated SNP perfectly segregates in an autosomal recessive mode of inheritance. The mutation results in truncation at the N-terminal of the translated BBS4 protein, reducing a 520 amino acid protein down to a 19 amino acid peptide. We predict that nonsense-mediated decay of BBS4 messenger RNA would hinder the expression of functional BBS4 protein (Popp and Maquat 2013). In humans and mice, BBS4 is associated with the syndromic disease, BBS. We also provide some evidence that this form of PRA in the dog is part of a syndromic disease. There are now two BBS genes implied in canine PRA (BBS4 and TTC8; Downs et al. 2014b). As BBS has not been previously reported in the dog, future PRA cases should be monitored for BBS phenotypes and gene mutations as they may provide a potential canine model for human disease.

**BBS4** is one of eight evolutionarily conserved proteins that together form a multi-protein complex referred to as the BBSome (Nachury et al. 2007; Loktev et al. 2008). This complex localizes to primary cilia, a small hair-like organelle that is present on almost all vertebrate cells. Cilia play a vital role in many developmental pathways that occur during vertebrate embryogenesis enabling correct organ differentiation and spatial organization within the body. Primary cilia mediate multiple cell signaling activities in nondividing cells, responding to both mechanical and chemosensory stimuli in multiple body systems as they contain tissue specific sensory receptors (Singla and Reiter 2006; Goetz and Anderson 2010). The ubiquity of primary cilia and the concurrent differences in characteristics that they possess depending on their residing cell type give ciliopathies their clinical heterogeneity. Presumably, a dysfunctional protein that normally localizes to cilia of only one cell type will result in nonsyndromic disease, while proteins essential to cilia on multiple cell types such as those involved in its maintenance will result in syndromic disease.

The formation and maintenance of cilia are highly dependent on the bidirectional (anterograde and retrograde) movement of nonmembrane-bound particles between the cell body and the tip of the cilia via its axonemal microtubules. The mechanism for this is referred to as intraflagellar transport (IFT) (Rosenbaum and Witman 2002). While the BBSome is not directly required for cilia formation, it is essential for the trafficking and organization of IFT complexes and hence has an indirect role in ciliary maintenance (Wei et al. 2012). Disruption in any of the BBSome genes (among 11 others that are not part of the BBSome) can cause failure of this mechanism, resulting in the rare ciliopathy, BBS (Suspitsin and Imyanitov 2016). The degree of importance of each BBS protein and their effect on the ability for the BBSome to carry out its ciliary functions within the various cell types remains elusive.

Studies of human BBS type 4 (OMIM #615982) and Bbs4-null mice show that functions of the canine BBS4 protein are consistent with these theories. Structurally normal primary and motile cilia were observed in knockout mice, suggesting that BBS4 is not required for the formation of cilia (Mykytyn et al. 2004). All affected individuals including the dogs used in this study experience retinal degeneration, despite having normal vision at a very young age (Iannaccone et al. 1999, 2005; Rüse et al. 2002; Mykytyn et al. 2004; Li et al. 2014). This suggests that cilia are correctly formed, however IFT of newly synthesized proteins in the inner segment to the outer segment of photoreceptor cells is compromised, as the only route between the two is through connecting cilia (Marszalek et al. 2000; Mykytyn et al. 2004). These proteins are essential to photoreceptor maintenance and without these, the photoreceptor cells undergo apoptosis.

**Table 2 Semen analysis report of affected Hungarian Puli**

|                | Normal Dog (Schaer 2009) | Affected Hungarian Puli |
|----------------|-------------------------|-------------------------|
| Normal morphology (%) | ≥80                     | 22                      |
| Abnormal morphology (%) | —                      | —                       |
| Head defects         | —                       | 18                      |
| Midpiece defects     | —                       | 2                       |
| Tail defects         | —                       | 74                      |
| Normal motility (%)  | ≥70                     | <5                      |
| Concentration (number per ml) | 4–4000e^6 | 6.5e^6                   |
| Total sperm         | 100–30000e^6           | 13.65e^6                |
| Volume (ml)         | 0.4–40                 | 2.1                     |
| Color               | Cloudy white           | Transparent             |

Normal canine semen characteristics were obtained from Schaer 2009.
BBS is recognized as a syndromic disease, however in the dog, the disease may appear as nonsyndromic PRA. Like canine PRA, BBS is typically inherited in an autosomal recessive manner, except for one report of triallelic inheritance (Katsanis et al. 2001; Forsythe and Beales 2013). In human BBS type 4, symptoms that are observed in addition to RP include obesity, hypogonitalism, polydactyly, mental retardation, renal anomalies, and decreased olfaction (Iannaccone et al. 1999, 2003; Rüse et al. 2002; Li et al. 2014; Aksanov et al. 2014). The severity and frequency of occurrence of each of these symptoms is variable like for all types of BBS, and clinical diagnosis is based on the presence of three to four primary and two secondary symptoms (Forsythe and Beales 2013). The difference in the underlying genetic mutation for reports of BBS type 4 is likely to contribute to this heterogeneity.

The affected Hungarian Puli in this study were predicted to have no functional BBS4, so we compared their phenotypes to those observed in Bbs4–null mice. In these mice, obesity and a complete lack of spermatozoa flagella were observed in addition to retinal degeneration (Mykytyn et al. 2004). In the dog, we observed all of these phenotypes but found that canine spermatozoa flagella were not as severely affected as those in the mouse. We observed 22% of sperm with normal morphology in the dog; however, a large proportion of abnormal sperm had defective flagella (74%) and a very small proportion were motile (<5%; Table 2). This suggests that BBS4 is only of moderate importance to flagella formation but is necessary for providing motility in the dog. More canine samples are required to confirm this.

The difficulty with differentiating nonsyndromic and syndromic disease in companion animals such as the dog is that many of the concurrent symptoms may not be diagnosed or recognized. Obesity is common with 26–43% of pure- and mixed-breed dogs classed as overweight in an Australian survey (McGreevy et al. 2005). As it is widely recognized as a nutritional disease, many people would underestimate the genetic component of this phenotype. Further, in Australia many companion animals are desexed prior to maturity, limiting the opportunity to recognize fertility deficits. Other symptoms such as learning or developmental delay and decreased olfaction may be difficult to assess in animals. For these reasons, we recommend that all human BBS genes might be considered as potential candidate genes for cases of canine PRA with unknown genetic causation. Further studies are required to confirm that BBS4 causes syndromic disease in the dog and this should be monitored as it may potentially be a useful large animal model for human BBS.

ACKNOWLEDGMENTS

We thank the owners and their pets for providing these samples and the veterinarians who phenotyped the Hungarian Puli. We acknowledge Vidhya Jagnanathan and Ranja Eklund with great appreciation for their technical assistance in providing control sample data. We also thank the Sydney Informatics Hub for providing access to the Artemis High Performance Computing system at the University of Sydney.

Author contributions: T.C., B.H., and C.M.W. conceived and designed the experiments. Sample collection and preparation was done by T.C., B.H., M.K.K., H.T.L., and C.M.W. R.B. performed the fertility assessment. T.C. and C.M.W. performed the whole-genome genotyping and resequencing analysis. T.C. and C.E.W. performed bioinformatic analysis of whole-genome sequence data. L.J.M. provided intellectual insight into cilia biology and development of the discussion. T.C. wrote the article with the input and approval of all coauthors.

LITERATURE CITED

Acland, G. M., K. G. Key, C. S. Mellersh, A. A. Langston, J. Rine et al., 1999 A novel retinal degeneration locus identified by linkage and comparative mapping of canine early retinal degeneration. Genomics 59: 134–142.
Acland, G. M., G. D. Aguirre, J. R. Ray, Q. Zhang, T. S. Aleman et al., 2001 Gene therapy restores vision in a canine model of childhood blindness. Nat. Genet. 28: 92–95.
Aksanov, O., P. Green, and R. Z. Birk, 2014 BBS4 directly affects proliferation and differentiation of adipoocytes. Cell. Mol. Life Sci. 71: 3381–3392.
Axelsson, E., A. Ratnakumar, M. L. Arendt, K. Maqbool, M. T. Webster et al., 2013 The genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature 495: 360–364.
Beltran, W. A., A. V. Cideciyan, A. S. Lewin, S. Iwabe, H. Khanna et al., 2012 Gene therapy rescues photoreceptor blindness in dogs and paves the way for treating human X-linked retinitis pigmentosa. Proc. Natl. Acad. Sci. USA 109: 2132–2137.
Chew, T., B. Haase, C. E. Willet, and C. M. Wade, 2017 Exclusion of known progressive retinal atrophy genes for blindness in the Hungarian Puli. Anim. Genet. DOI: 10.1111/age.12553
Cideciyan, A. V., S. G. Jacobson, T. S. Aleman, D. Gu, S. E. Pearce-Kelling et al., 2005 In vivo dynamics of retinal injury and repair in the rhodopsin mutants. Advan. Genet. 50: 253–258.
Clements, P. J., D. R. Sargan, D. J. Gould, and S. M. Petersen-Jones, 1996 Recent advances in understanding the spectrum of canine generalised progressive retinal atrophy. J. Small Anim. Pract. 37: 155–162.
DePristo, M. A., E. Banks, R. Poplin, K. V. Garimella, J. R. Maguire et al., 2011 A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat. Genet. 43: 491–498.
Downs, L. M., B. Wallin-Håkansson, M. Boursnell, S. Marklund, Å. Hedhammar et al., 2011 A frameshift mutation in golden retriever dogs with progressive retinal atrophy endorses SLC4A3 as a candidate gene for human retinal degenerations. PLoS One 6: e21452.
Downs, L. M., R. Hitti, S. Pregnolato, and C. S. Mellersh, 2014a Genetic screening for PRA-associated mutations in multiple dog breeds shows that PRA is heterogeneous within and between breeds. Vet. Ophthalmol. 17: 126–130.
Downs, L. M., B. Wallin-Håkansson, T. Bergström, and C. S. Mellersh, 2014b A novel mutation in TTC8 is associated with progressive retinal atrophy in the golden retriever. Canine Genet. Epidemiol. 1: 4.
Farwell, K. D., L. Shahmirzadi, D. El-Khechen, Z. Powis, E. C. Chao et al., 2015 Enhanced utility of family-centered diagnostic exome sequencing with inheritance model-based analysis: results from 500 unselected families with undiagnosed genetic conditions. Genet. Med. 17: 578–586.
Feldman, E. C., and R. W. Nelson, 1987 Canine and Feline Endocrinology and Reproduction. W. B. Saunders, Philadelphia, PA.
Forsythe, E., and P. L. Beales, 2013 Bardet-Biedl syndrome. Eur. J. Hum. Genet. 21: 8–13.
Frischknecht, M., H. Niehof-Oellers, V. Jagnanathan, M. Owczarek-Lipska, C. Drögemüller et al., 2013 A COL11A2 mutation in Labrador Retrievers with mild disproportionate dwarfism. PLoS One 8: e60149.
Gerber, M., A. Fischer, V. Jagnanathan, M. Drögemüller, C. Drögemüller et al., 2015 A deletion in the VLDLR gene in Eursar dogs with cerebellar hypoplasia resembling a Dandy-Walker-like malformation (DWM/L). PLoS One 10: e0108917.
Goetz, S. C., and K. V. Anderson, 2010 The primary cilium: a signalling centre during vertebrate development. Nat. Rev. Genet. 11: 331–344.
Goldstein, O., B. Zangerl, S. Pearce-Kelling, D. J. Sidjianin, J. W. Kijas et al., 2006 Linkage disequilibrium mapping in domestic dog breeds narrows the progressive rod-cone degeneration interval and identifies ancestral disease-transmitting chromosome. Genomics 88: 541–550.
Hamel, C., 2006 Retinitis pigmentosa. Orphanet J. Rare Dis. 1: 40.
Hirschhorn, J. N., and M. J. Daly, 2005 Genome-wide association studies for common diseases and complex traits. Nat. Rev. Genet. 6: 95–108.
Hoepnner, M. P., A. Lundquist, M. Pirun, J. R. Meadows, N. Zamani et al., 2014 An improved canine genome and a comprehensive catalogue of coding genes and non-coding transcripts. PLoS One 9: e91172.
Iannaccone, A., B. Falsini, N. Haider, G. Del Porto, E. M. Stone et al., 1999 Phenotypic characteristics associated with the BBS4 locus, pp. 187–199 in Retinal Degenerative Diseases and Experimental Therapy, edited by Hollyfield, J. G., M. M. La Vail, and R. E. Anderson. Plenum Press, New York.

Iannaccone, A., K. Myktyyn, A. M. Persico, C. C. Searby, A. Baldi et al., 2005 Clinical evidence of decreased olfaction in Bardet-Biedl syndrome caused by a deletion in the BBS4 gene. Am. J. Med. Genet. A. 132A: 343–346.

Jagannathan, V., J. Bannoehr, P. Plattet, R. Hauswirth, C. Drögemüller et al., 2002 A mutation in the SUZ39H2 gene in Labrador Retrievers with hereditary nasal parakeratosis (HNPK) provides insights into the epigenetics of keratinocyte differentiation. PLoS Genet. 9: e1003848.

Karlsson, E. K., I. Baranowska, C. M. Wade, N. H. Salmon Hillbertz, M. C. Zody et al., 2007 Efficient mapping of mendelian traits in dogs through genome-wide association. Nat. Genet. 39: 1321–1328.

Katsanis, N., S. J. Amley, J. L. Badano, E. R. Eichers, R. A. Lewis et al., 2001 Triallelic inheritance in Bardet-Biedl syndrome, a Mendelian recessive disorder. Science 293: 2256–2259.

Kijas, J. W., A. V. Cideciyan, T. S. Aleman, M. J. Pianta, S. E. Pearce-Kelling and G. M. Acland, 2003 Canine models of ocular disease: outcross breedings define a dominant disorder present in the English mastiff and bull mastiff dog breeds. J. Hered. 94: 27–30.

Korte, A., and A. Farlow, 2013 The advantages and limitations of trait analysis with GWAS: a review. Plant Methods 9: 29.

Kukekova, A. V., O. Goldstein, J. L. Johnson, M. A. Richardson, S. E. Pearce-Kelling et al., 2009 Canine RD3 mutation establishes rod-cone dysplasia type 2 (rcd2) as ortholog of human and murine rmd3. Mamm. Genome 20: 109–123.

Li, H., and R. Durbin, 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754–1760.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan et al., 2009 The sequence alignment/map format and SAMtools. Bioinformatics 25: 2078–2079.

Li, Q., Y. Zhang, L. Jia, and X. Peng, 2014 A novel nonsense mutation in BBS4 gene identified in a Chinese family with Bardet-Biedl syndrome. Chin. Med. J. (Engl.) 127: 4190–4196.

Lindblad-Toh, K., C. M. Wade, T. S. Mikkelsen, E. K. Karlsson, D. B. Jaffe et al., 2005 Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438: 803–819.

Loktev, A. V., Q. Zhang, J. S. Beck, C. C. Searby, T. E. Scheetz et al., 2009 The BBSome subunit links cilogenesis, microtubule stability, and acetylation. Dev. Cell 15: 854–865.

Marszalek, J. R., X. Liu, E. A. Roberts, D. Chui, J. D. Marth et al., 2000 Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors. Cell 102: 175–187.

McGreavy, P. D., P. C. Thomson, C. Pride, A. Fawcett, T. Grassi et al., 2005 Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. Vet. Rec. 156: 695–702.

McKenna, A., M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis et al., 2010 The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20: 1297–1303.

McLaren, W., B. Pritchard, D. Bios, Y. Chen, P. Flicek et al., 2010 Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. Bioinformatics 26: 2069–2070.

Miyadera, K., G. M. Acland, and G. D. Aguirre, 2012 Genetic and phenotypic variations of inherited retinal diseases in dogs: the power of within- and across-breed studies. Mamm. Genome 23: 40–61.

Myktyyn, K., R. F. Mullins, M. Andrews, A. P. Chiang, R. E. Swiderski et al., 2004 Bardet-Biedl syndrome type 4 (BBS4)-null mice implicate Bbs4 in flagella formation but not global cilia assembly. Proc. Natl. Acad. Sci. USA 101: 8664–8669.

Nachury, M. V., A. V. Loktev, Q. Zhang, C. J. Westlake, J. Peränen et al., 2007 A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell 129: 1210–1213.

Narfström, K., M. L. Katz, R. Bragadottir, M. Seeliger, A. Boulanger et al., 2003 Functional and structural recovery of the retina after gene therapy in the PDE6B null mutation dog. Invest. Ophthalmol. Vis. Sci. 44: 1663–1672.

Parry, H. B., 1953 Degenerations of the dog retina. II. Generalized progressive atrophy of hereditary origin. Br. J. Ophthalmol. 37: 487–502.

Pearce-Kelling, S. E., T. S. Aleman, A. Nickle, A. M. Latties, and G. D. Aguirre, 2001 Calcium channel blocker D-cis-diltiazem does not slow retinal degeneration in the PDE6B mutant rcd1 canine model of retinitis pigmentosa. Mol. Vis. 7: 42–47.

Petersen-Jones, S. M., 1998 A review of research to elucidate the causes of the generalized progressive retinal atrophies. Vet. J. 155: 5–18.

Pichard, V., N. Provost, A. Mendes-Madeira, L. Libeau, P. Hulin et al., 2016 AAV-mediated gene therapy halts retinal degeneration in PDE6B-deficient dogs. Mol. Ther. 24: 867–876.

Popp, M. W., and L. E. Maquat, 2013 Organizing principles of mammalian nonsense-mediated mRNA decay. Annu. Rev. Genet. 47: 139–165.

Purcell, S., B. Neale, K. Todd-Brown, L. Thomas, M. A. Ferreira et al., 2007 PLINK: a tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81: 559–575.

Riise, K., T. Kornqvist, A. F. Wright, K. Mykytyn, and V. C. Sheffield, 2002 The phenotype in Norwegian patients with Bardet-Biedl syndrome with mutations in the BBS4 gene. Arch. Ophthalmol. 120: 1364–1367.

Rosenbaum, J. L., and G. B. Witman, 2002 Intraflagellar transport. Nat. Rev. Mol. Cell Biol. 3: 813–825.

Rozen, S., and H. Skaltsky, 2000 Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132: 365–386.

Sawyer, S. L., T. Hartley, D. A. Dyment, C. L. Beaulieu, J. Schwartzentruber et al., 2016 Utility of whole-exome sequencing for those near the end of the diagnostic odyssey: time to address gaps in care. Clin. Genet. 89: 275–284.

Sayyab, S., A. Viluma, K. Bergvall, E. Brunberg, V. Jagannathan et al., 2016 Whole-genome sequencing of a canine family trio reveals a FAM83G variant associated with hereditary footpad hyperkeratosis. G3 (Bethesda) 6: 521–527.

Schaer, M., 2009 Clinical Medicine of the Dog and Cat. CRC Press, Boca Raton, Fl.

Sim, N.L., P. Kumar, J. H. Su, H. Henikoff, G. Schneider et al., 2012 SIFT web server: predicting effects of amino acid substitutions on proteins. Nucleic Acids Res. 40: W452–W457.

Singla, V., and J. F. Reiter, 2006 The primary cilium as the cell’s antenna: signaling at a sensory organelle. Science 313: 629–633.

Susshitpin, E. N., and E. N. Imyanitov, 2016 Bardet-Biedl syndrome. Mol. Syndromol. 7: 62–71.

Van der Auwer, G. A., M. O. Carneiro, C. Hartl, R. Poplin, G. Del Angel et al., 2013 From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. Curr. Protoc. Bioinformatics 11: 11.10.1–11.10.33.

Vayss, A., A. Ratnakumar, T. Derrien, E. Axelson, G. Rosengren Pielberg et al., 2011 Identification of genomic regions associated with phenotypic variation between dog breeds using selection mapping. PLoS Genet. 7: e1002316.

Vilboux, T., G. Chaudieu, P. Jeanin, D. Delattre, B. Hedan et al., 2008 Progressive retinal atrophy in the Border Collie: a new XLTPRA. BMC Vet. Res. 4: 10.

Wei, Q., Y. Zhang, Y. Li, Q. Zhang, K. Ling et al., 2012 The BBSome controls IFT assembly and turnaround in cilia. Nat. Cell Biol. 14: 950–957.

Whitley, R. D., S. A. McLaughlin, and B. C. Gilger, 1995 Update on eye disorders among purebred dogs. Vet. Med. 90: 574–592.
Willet, C. E., M. Makara, G. Reppas, G. Tsoukalas, R. Malik et al., 2015 Canine disorder mirrors human disease: exonic deletion in HES7 causes autosomal recessive spondylocostal dysostosis in miniature Schnauzer dogs. PLoS One 10: e0117055.

Wolf, Z. T., H. A. Brand, J. R. Shaffer, E. J. Leslie, B. Arzi et al., 2015 Genome-wide association studies in dogs and humans identify ADAMTS20 as a risk variant for cleft lip and palate. PLoS Genet. 11: e1005059.

Zangerl, B., O. Goldstein, A. R. Philp, S. J. Lindauer, S. E. Pearce-Kelling et al., 2006 Identical mutation in a novel retinal gene causes progressive rod-cone degeneration in dogs and retinitis pigmentosa in humans. Genomics 88: 551–563.

Zhu, X., S. Petrovski, P. Xie, E. K. Ruzzo, Y. F. Lu et al., 2015 Whole-exome sequencing in undiagnosed genetic diseases: interpreting 119 trios. Genet. Med. 17: 774–781.

Communicating editor: D. L. Bannasch