RESEARCH ARTICLE

Genetic Panel Screening of Nearly 100 Mutations Reveals New Insights into the Breed Distribution of Risk Variants for Canine Hereditary Disorders

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Abstract

Background
The growing number of identified genetic disease risk variants across dog breeds challenges the current state-of-the-art of population screening, veterinary molecular diagnostics, and genetic counseling. Multiplex screening of such variants is now technologically feasible, but its practical potential as a supportive tool for canine breeding, disease diagnostics, pet care, and genetics research is still unexplored.

Results
To demonstrate the utility of comprehensive genetic panel screening, we tested nearly 7000 dogs representing around 230 breeds for 93 disease-associated variants using a custom-designed genotyping microarray (the MyDogDNA panel test). In addition to known breed disease-associated mutations, we discovered 15 risk variants in a total of 34 breeds in which their presence was previously undocumented. We followed up on seven of these genetic findings to demonstrate their clinical relevance. We report additional breeds harboring variants causing factor VII deficiency, hyperuricosuria, lens luxation, von Willebrand’s disease, multifocal retinopathy, multidrug resistance, and rod-cone dysplasia. Moreover, we provide plausible molecular explanations for chondrodysplasia in the Chinook, cerebellar ataxia in the Norrbottenspitz, and familial nephropathy in the Welsh Springer Spaniel.

Conclusions
These practical examples illustrate how genetic panel screening represents a comprehensive, efficient and powerful diagnostic and research discovery tool with a range of
applications in veterinary care, disease research, and breeding. We conclude that several known disease alleles are more widespread across different breeds than previously recognized. However, careful follow up studies of any unexpected discoveries are essential to establish genotype-phenotype correlations, as is readiness to provide genetic counseling on their implications for the dog and its breed.

Introduction

Novel mutations that underlie canine Mendelian disorders and traits are described at an accelerating pace, with 199 such variants currently described in the OMIA database [1]. Accordingly, the number of hereditary diseases and genetic predispositions that a dog could potentially be tested for is steadily increasing. For some breeds, up to ten breed-relevant DNA tests are commercially available through different laboratories [2,3], posing a burden on dog owners, breeders, and clinicians seeking information and pursuing comprehensive health screening. Therefore, there is a need for accurate and comprehensive, cost-effective and easy to use DNA screening solutions that provide breeders with results for several breed relevant disorders with one sampling, analysis and reporting effort. Furthermore, such screening holds great potential to serve as a discovery and diagnostic tool for researchers and veterinary clinicians.

Several canine genetic disorders such as progressive retinal atrophies (PRAs; [4]), pyruvate kinase deficiency [5], cystinurias [6] and spinocerebellar ataxias [7,8] are genetically heterogeneous, which means that multiple simultaneous genetic tests of variants in the same gene, or in different genes, are needed to understand their background across and within pure breeds. It is also nearly impossible to know in advance which DNA tests apply to a mixed breed dog. Genetic panel screening solutions that facilitate comprehensive molecular diagnostics and help exclude known disease-causing mutations are therefore expected to support pet health care and research. Testing for other mutations, besides the known breed-relevant ones, also has the potential to advance knowledge on breed prevalence of disease-linked variants in dogs. Initial research discovery studies are often limited to one or a few breeds, leaving the true breed and geographic distribution of a new disease-associated variant incompletely explored. Although many mutations are considered breed specific [3], others such as the ones underlying degenerative myelopathy (DM; [9]), hyperuricosuria (HUU; [10]) or factor VII (FVII) deficiency [11,12] are thought to be ancient in their origins and therefore potentially widespread across breeds. Shared breed ancestry is also reflected in the disease heritage of breed groups, with examples including primary lens luxation (PLL; [13]) in the terrier breed group, and collie eye anomaly (CEA; [14]) in collie-like herding breeds. Inadvertent or planned crossbreeding may further complicate the distribution pattern of disease-causing variants.

We report here on the experience gained by an initial DNA panel screening feasibility evaluation, testing for 93 variants underlying canine inherited disorders in nearly 7000 dogs representing more than 230 breeds. Our findings illustrate the power of such a comprehensive genetic approach as a diagnostic platform and research discovery tool providing insights into the distribution of disease alleles and inherited disorders across canine breeds. We further demonstrate the clinical presentation and relevance of several of the novel discoveries for the newly affected breeds. More broadly, our study highlights general issues related to high fidelity multiplex testing, such as the need for careful genetic and clinicopathological follow up of unexpected findings, maintenance of close ties with the research and dog community, and appropriate genetic counseling.
Materials and Methods

Animals and samples

All canine buccal swab or EDTA blood samples used for multiplex screening (N = 6788) were originally submitted for commercial DNA testing at Genoscoper Laboratories (Genoscoper Laboratories Oy, Helsinki, Finland) during 2013 to 2015. The majority of the study sample consisted of Finnish dogs (69.5%), while samples from Russia (5.5%), the Netherlands (4.8%), Germany (3.5%), the United States (2.7%), Great Britain (2.0%), France (1.9%) and Norway (1.0%) represented other major subgroups. There were 233 different dog breeds represented among the studied dogs, with 156 breeds accounted for with ≥5 individuals (S1 Table). The majority of the examined dogs (N = 4668; 68.8%) represented breeds with at least one prior known breed-relevant DNA test included among the tested genetic variants. The breed of a dog was reported by its owner with accompanying registration information confirming the dog as being registered under Fédération Cynologique Internationale (FCI) or American Kennel Club (AKC)- standards. This study was ethically approved by the Animal Ethics Committee of the State Provincial Office of Southern Finland, Hämeenlinna, Finland (permit number: ESAVI/6054/04.10.03/2012). All dog owners provided written consent for the use of their dog’s DNA sample for research purposes.

Follow up samples of 233 Brazilian Terriers genotyped for the canine multifocal retinopathy (cmr1) variant [15] only, and 41 Norrbottenspitzes genotyped for the Finnish Hound cerebellar ataxia variant [16] only, were selected from the biobank of the canine genetics research group at the University of Helsinki.

Multiplex panel design and validation

A custom genotyping microarray for selected canine disease-associated variants (S2 Table) was developed based on the robust and widely utilized Illumina Infinium HD Ultra platform (Illumina, Inc., San Diego, CA, USA), and validated as the MyDogDNA® test through Genoscoper Laboratories Oy, Helsinki, Finland [17]. In brief, a comprehensive literature and online database review [1,2] was first performed to identify and compile template sequence information on target mutations underlying canine inherited disorders. Second, genotyping assays were designed according to the manufacturer’s guidelines (Illumina, Inc.) to directly target known point mutations, insertions and deletions. Each disease marker was replicated three times in the array design. Finally, the accuracy and reproducibility of the panel test results was validated with a set of experiments [17] addressing source sample type, internal replicate concordance, test-retest reproducibility, and with evaluation by an independent DNA testing laboratory (Antagene, France) submitting a selection of samples with known disease genotypes for blind testing. Specific disease assays were validated by 1) genotyping of samples with genotypes known a priori; 2) sequencing validation of identified disease genotypes a posteriori; 3) synthetic oligonucleotides for rare conditions where no control samples were available.

Genotyping

Microarray analyses were carried out following manufacturer-recommended standard protocols (Illumina, Inc., San Diego, CA, USA). All genotype data from samples with call rates below 95% of the analyzed markers was discarded to ensure high quality data, and all genotype calls were manually evaluated.

All mutant allele findings in unexpected breeds were further confirmed by standard capillary sequencing on an ABI3730xl DNA Analyzer instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at the Sequencing Unit of the Finnish Institute of Molecular Medicine (FIMM).
Sequencing primers for each investigated disease variant are listed in S3 Table. PCR reactions were performed on ~20 ng of genomic DNA using AmpliTaq Gold Master Mix (Applied Biosystems, Waltham, MA, USA) with reactions initiated at 95°C for 5 min, followed by 34 cycles of 30 s at 95°C, 30 s at 64°C and 45 s at 72°C, and finally 10 min at 72°C. Pre-sequencing PCR product clean-up was performed enzymatically using Exonuclease I (Exo1) and FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) according to the manufacturer’s protocol. Additional separate genotyping for cmr1 in Brazilian Terriers took place using KASP genotyping chemistry according to the manufacturer’s assay design (LGC Ltd., Middlesex, UK). Additional separate genotyping for Finnish Hound cerebellar ataxia in the Norrbottenspitz was carried out on a LightCycler 480 II instrument (Roche Diagnostics, Basel, Switzerland) using standard probe hydrolysis detection chemistry with LC480 Probe Master Mix (Roche Diagnostics), and the following primers: 5’-CGTAGACTACGAGACTGCATTTATTCA-3’ (forward) and 5’-GATTA AACATAGCTTGTGCACTGTGT-3’ (reverse); and probes: 5’-TGCTGCTCAGATGCTA-3’ (VIC) and 5’-TGCTGCTCAGGTGCTA-3’ (FAM).

Coagulation factor VII deficiency measurements

We collected citrated blood samples for coagulation analysis from a seven-year-old Welsh Springer Spaniel female and her litter of six dogs, approximately two and a half years old at the time of this study. The carrier female and her litter represented all genotypes (Nnormal = 1, Ncarrier = 3, Naffected = 3) of a variant (F7 c.407G>A) predisposing to factor VII deficiency. Routine coagulation screening tests carried out included measurement of prothrombin and partial thromboplastin times (Movet Oy, Kuopio, Finland) as well as specific coagulation factor activity analyses using factor deficient plasma as previously described [11].

Urine uric acid measurements

We evaluated manifestation of hyperuricosuria in fourteen Lagotto Romagnolo dogs representing all genotypes (Nnormal = 7, Ncarrier = 3, Naffected = 4) of a predisposing variant (SLC2A9 c.616G>T). The sample consisted of eight females and six males with a median age of one year and one month at the time of the study (range ten months to seven years). Hyperuricosuria was evaluated based on a previously described urine test [18], evaluating urine uric acid: creatinine concentration ratios. Fresh midstream catch urine samples were used for the determination. Uric acid was analyzed by an enzymatic method, and creatinine by a kinetic picric acid method, using the reagents and adaptations of an automatic chemistry analyzer (Konelab 30i, Thermo Fisher Scientific Oy, Vantaa, Finland).

Ophthalmology examinations

Ophthalmology examinations in Brazilian Terriers were performed by an ECVO (European College of Veterinary Ophthalmologists) panelist or an ECVO diplomate. The examinations included ophthalmoscopy, biomicroscopy and funduscopy, using tropicamide as the mydriatic. An Optical Coherence Tomography (OCT) examination of one dog homozygous for the cmr1 mutation (BEST1 c.73C>T) was carried out at the HUS Eye and Ear Hospital (Helsinki, Finland) using an OCT Plus SPECTRALIS device (Heidelberg Engineering GmbH, Heidelberg, Germany). No mydriatic was used for the OCT examination.

Statistical and pedigree analyses

The statistical significance of observed differences in phenotype values between groups of dogs was evaluated by standard unpaired t-tests and one-way analysis of variance (ANOVA).
Pedigrees were drawn using GenoPro® genealogy software [19], based on information retrieved from the Finnish Kennel Club registry database [20].

Results

Microarray-based panel testing yields high fidelity results

We developed a custom-designed Illumina Infinium technology-based bead chip SNP genotyping microarray to screen dogs for 93 different genetic variants underlying a wide variety of canine hereditary disorders and genetic predispositions (S2 Table). The performance of the microarray was extensively validated as also described elsewhere [17]. Blood and buccal swab samples performed equally well on the array (median marker call rates of 98.1% vs 98.2%, respectively). Internal replicate and test-retest (reproducibility) concordances were 99.8%. Only markers reaching 100% sensitivity and specificity upon testing with control genotypes, or validated by an experiment with a synthetic control oligonucleotide, were considered for reporting.

High overall carrier frequency of known mutations across breeds

For this study, we screened samples from 6788 dogs representing 233 breeds between 2013 and 2015. Mutant alleles of well-known hereditary disease traits were encountered in breeds as expected based on current literature and database information. Carrier frequencies for known breed-relevant disorders are listed in S1 Table for breeds represented by at least 30 dogs. Based on the examined set of 93 disease variants, we observed that 17.8% (N = 1208) of the tested dogs carried at least one of the tested variants, while 2.5% (N = 170) were genetically affected for a disease condition. The maximum number of disorders carried by any individual dog was three (observed in three dogs). Two disorder variants were carried by 1.0% (N = 66), and one disorder variant by 16.8% (N = 1139) of the tested dogs.

The genetic panel screening approach revealed 15 known disease-linked variants in an additional 34 breeds in which they have not previously been documented in the peer-reviewed literature (Table 1). Each mutant allele finding in an additional breed was confirmed by a second genetic technology, Sanger sequencing. After confirming the genetic presence of a mutation in an additional breed, it is crucial to establish a genotype-phenotype correlation by exploring whether the risk variant phenotypically manifests on a different genetic background. In this study, clinical validation was initiated with owner interviews regarding clinical manifestations, and where possible, continued by recruitment of genetically affected dogs for relevant clinico-pathological examinations as described in the following sections.

Factor VII (FVII) deficiency is genetically widespread across breeds

Factor VII deficiency is a recessively inherited mild coagulopathy that may typically go completely unnoticed until revealed by routine coagulation screening tests or episodes of excessive bleeding due to trauma or surgery [11,12]. An F7 missense mutation underlying canine FVII deficiency was originally characterized in research colonies of Beagles, but later also found in pet Beagles [11], Alaskan Klee Kais [12], and Scottish Deerhounds (U. Giger, unpublished). We report here ten additional breeds of varying ancestry in which the presence of the FVII deficiency-associated variant (F7 c.407G>A) had not been previously reported (Table 1). In an effort to investigate whether the presence of the mutant allele also leads to a bleeding tendency in these additional breeds, we evaluated in vitro the coagulation in heterozygous and homozygous Welsh Springer Spaniels, and in a homozygous Finnish Hound. The three homozygous mutant Welsh Springer Spaniels dogs recruited for this study had no prior history of
| Disorder                          | Gene and mutation | Previously reported breeds | Additional breed(s) with N observed carriers |
|----------------------------------|-------------------|-----------------------------|---------------------------------------------|
| Catalase Deficiency              | CAT c.979G>A (p.Ala327Thr) | Beagle | American Foxhound: 1/1 (100%) |
| Chondrodysplasia (Dwarfish)      | ITGA10 c.2083C>T (p.Arg695*) | • Norwegian Elkhound, grey  
|                                  |                   | • Karelian Bear Dog | Chinook: 5/47 (10.6%) |
| Catrio-mandibular Osteopathy (CMO) | SLC37A2 c.1332C>T (p.Asp444Asp) | • Cairn Terrier  
|                                  |                   | • Scottish Terrier  
|                                  |                   | • West Highland White Terrier | • Australian Shepherd: 1/140 (0.7%)  
|                                  |                   |                          | • Lanscahirre Heeler: 1/40 (2.5%) |
| Factor VII (FVII) Deficiency     | F7 c.407G>A (p.Gly136Glu) | • Alaskan Klee Kai  
|                                  |                   | • Airedale Terrier  
|                                  |                   | • Beagle  
|                                  |                   | • Giant Schnauzer  
|                                  |                   | • Scottish Deerhound | • American Foxhound: 1/1 (100%)  
|                                  |                   |                          | • Finnish Hound: 54/237 (22.8%)  
|                                  |                   |                          | • German Wirehaired Pointer: 1/3 (33.3%)  
|                                  |                   |                          | • Irish Water Spaniel: 1/3 (33.3%)  
|                                  |                   |                          | • Japanese Spitz: 1/6 (16.7%)  
|                                  |                   |                          | • Miniature Schnauzer: 1/7 (14.3%)  
|                                  |                   |                          | • Papillon/Phalene: 5/41 (12.2%)  
|                                  |                   |                          | • Sealyham Terrier: 1/1 (100%)  
|                                  |                   |                          | • Welsh Springer Spaniel: 18/76 (23.7%) |
| Familial Nephropathy (FN)        | COL4A4 c.115A>T (p.Lys40*) | • American Cocker Spaniel  
|                                  |                   | • English Cocker Spaniel | Welsh Springer Spaniel: 10/76 (13.2%) |
| Hyperuricosuria (HUU)            | SLC2A9 c.616G>T (p.Cys188Phe) | • Black Russian Terrier  
|                                  |                   | • Dalmatian  
|                                  |                   | • English Bulldog  
|                                  |                   | • >10 other breeds | • Danish-Swedish Farmdog: 13/168 (7.7%)  
|                                  |                   |                          | • German Hunting Terrier: 3/22 (13.6%)  
|                                  |                   |                          | • Kromfohrländer: 1/160 (0.6%)  
|                                  |                   |                          | • Lagotto Romagnolo: 52/637 (8.2%)  
|                                  |                   |                          | • Spaniel de Pont-Audemer: 1/4 (25%)  
|                                  |                   |                          | • Swedish Vallhund: 3/71 (4.2%) |
| Multi-drug resistance 1 (MDR1)   | ABCB1 c.295_298delAGAT (p.Asp75fs) | • Australian Shepherd  
|                                  |                   | • Collie  
|                                  |                   | • Shetland Sheepdog  
|                                  |                   | • >10 other breeds | • Chinook: 7/47 (14.9%)  
|                                  |                   |                          | • Danish-Swedish Farmdog: 2/168 (1.2%) |
| Multifocal Retinopathy 1 (cmr1)  | BEST1 c.73C>T (p.Arg25*) | • Australian Shepherd  
|                                  |                   | • Bulldog  
|                                  |                   | • Great Pyrenees  
|                                  |                   | • Mastiff  
|                                  |                   | • >10 other breeds | Brazilian Terrier: 25/107 (23.4%) |
| Multifocal Retinopathy 3 (cmr3)  | BEST1 c.1388delIC (p.Pro463fs) | Lapponian Herder | Finnish Lapphund: 3/218 (1.4%) |
| Myotonia Congenita               | CLCN1 c.2665insA (p.Arg889fs) | Australian Cattle Dog | Border Collie: 2/29 (6.9%) |
| Primary Lens Luxation (PLL)      | ADAMTS17 c.1473+1G>A | • American Hairless Terrier  
|                                  |                   | • Chinese Crested  
|                                  |                   | • Jack Russell Terrier  
|                                  |                   | • >20 other breeds | Danish-Swedish Farmdog: 37/168 (22.0%) |
| Progressive Early-Onset Cerebellar Ataxia | SEL1L c.1972T>C (p.Ser658Pro) | Finnish Hound | Norbottenspitz: 13/103 (12.6%) |
| Rod-Cone Dysplasia 3 (rcd3)      | PDE6A c.1940delA (p.Asn616fs) | • Welsh Corgi Cardigan  
|                                  |                   | • Welsh Corgi Pembroke | • Chinese Crested: 1/48 (2.1%)  
|                                  |                   |                          | • Pomeranian: 1/8 (12.5%) |
| von Willebrand's disease, type I | VWF c.7437G>A (p.Ser2479Ser) | • Bernese Mountain Dog  
|                                  |                   | • Doberman  
|                                  |                   | • Manchester Terrier  
|                                  |                   | • >10 other breeds | • Barbet: 2/39 (5.1%)  
|                                  |                   |                          | • Brazilian Terrier: 1/107 (0.9%)  
|                                  |                   |                          | • Dutch Shepherd Dog—Longhaired: 1/16 (6.3%)  
|                                  |                   |                          | • Kromfohrländer: 13/160 (8.1%) |

(Continued)
experiencing clinically excessive hemorrhage, but one of them exhibited marked bleeding following routine phlebotomy performed for this study. However, the mutant homozygotes had significantly prolonged prothrombin times (PT) compared to adult littermates and laboratory reference values (Fig 1A), and normal activated partial thromboplastin times (aPTT; data not shown), which is indicative of FVII deficiency. The diagnosis was further confirmed by specific FVII activity measurements on blood plasma from the same dogs (Fig 1B).

We were subsequently able to recruit one Finnish Hound homozygous for the FVII mutation for this study. Its clinical history was unremarkable, but its PT was also prolonged (72 s) compared to the reported laboratory reference values (10–20 s), indicating FVII deficiency.

Predisposition to urate urolithiasis due to the common hyperuricosuria (HUU) mutation in Lagotto Romagnolo

Another variant known to be common across several breeds, like the Dalmatian and Black Russian Terrier, is the SLC2A9 gene mutation (c.616G>T) predisposing to HUU and uric acid calculi formation [10,18,21]. We confirmed the genetic presence of this risk factor in six
additional breeds (Table 1) that consequently may be predisposed to complications caused by uric acid crystal formation in the urinary tract. We therefore further examined uric acid concentrations in urine samples from four Lagotto Romagnolo dogs homozygous for the risk allele, and found that all of them showed a marked increase (average 30-fold) in urine uric acid/creatinine ratio (Fig 2). One of the affected Lagotti had experienced what was described as an episode of stranguria.

Prevalent manifestations of primary lens luxation (PLL) in Danish-Swedish Farmdogs

The ADAMTS17 gene variant (c.1473+1G>A) associated with the development of PLL (displacement of the ocular lenses due to degeneration of zonular fibers) is prevalent in many

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**Fig 2. Hyperuricosuria in the Lagotto Romagnolo.** Elevated urine uric acid/creatinine ratios (F(2,11) = 85.9, p = 1.93x10^{-7}) in Lagotti homozygous for the SLC2A9 gene c.616G>T variant indicate metabolic derangement and predisposition to urate stone formation. (**P < 0.001; n.s. = not significant)

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terrier breeds [13,22]. Although some terrier ancestry is likely present in the Danish-Swedish Farmdog, no active testing of the PLL mutation had been pursued prior to the panel screening of this study. We show here that the previously reported ADAMTS17 mutation is prevalent in the breed with a 21.9% carrier frequency (Table 1). We identified five adult dogs (2.5–4.5 years old) homozygous for the risk variant of which four were confirmed by an examining veterinarian as exhibiting clinical lens luxation in at least one eye requiring surgical treatment. The remaining homozygous dog was two and a half years old and not yet manifesting any ocular signs, but is now being closely monitored for PLL.

Progressive early-onset cerebellar ataxia transferred between Nordic breeds

A rapidly progressing severe generalized cerebellar ataxia with tremors, and failure to thrive was characterized in Finnish Hound puppies [16]. Since the responsible mutation (c.1972T>C) in the SEL1L gene was discovered, breeders have successfully used genetic screening to eradicate the lethal disorder from the breed, and affected dogs are no longer encountered in practice. The disease variant was considered breed-specific to the Finnish Hound; yet, our panel test found carriers from another Nordic breed, the Norrbottenspitz (also known as the Nordic Spitz). The Finnish breed club describes the Norrbottenspitz population as being fairly heterogeneous with open stud books. We explored the relatedness of all thirteen confirmed carriers using the Finnish Kennel Club registry and observed that they shared ancestry to the same dogs registered into the breed in the 1990’s (Fig 3). We subsequently screened an additional separate 41 Norrbottenspitzes, all representing different unrelated litters, and observed no further carriers of the mutant allele.

Importantly, a mating of two carrier dogs was observed in the registry pedigree of the Norrbottenspitz, and one puppy from the resulting litter had been euthanized at the age of four months due to progressive neurological signs first observed at the age of nine weeks. The veterinary medical records indicated that the puppy had suffered from generalized ataxia with hypermetria, tremor, and bilateral absence of blink reflexes. A diagnosis of cerebellar cortical abiotrophy with enlarged cerebrospinal fluid compartment and sulci was confirmed by magnetic resonance imaging (MRI). No changes to the cerebrum or brainstem were detected. Although no DNA sample was available to confirm the genotype of the euthanized puppy, these clinical findings strikingly resemble those reported in Finnish Hounds with cerebellar ataxia [16].

ITGA10 gene mutation causes dwarfism in Chinooks

The Chinook is a rare sled dog breed native to New England, tracing back to a male husky/mastiff-type dog with the same name born in 1917. Breeders first documented chondrodysplastic Chinooks in the 1990’s. All known cases in the breed can be traced back to a brother-sister mating producing the first known case. No conclusive information on the ancestry of these dogs exists. Intriguingly, we present an unexpected molecular explanation for one type of disproportionate dwarfism in the Chinook breed: an ITGA10 variant (c.2083C>T) which was already known to cause disproportionate short stature dwarfism of varying severity in the Norwegian Elkhound and Karelian Bear Dog breeds [23]. This incidental finding by DNA panel screening led us to cooperate with breeders aiding in the identification of at least one chondrodysplastic Chinook dog we confirmed to be homozygous for the ITGA10 variant, and several other carriers that are all related to the original cases from the 1990’s (Fig 4A). The homozygous mutant dog has a veterinary clinician-confirmed dwarf phenotype (Fig 4B), and early-onset hip problems and osteoarthritis were also reported.
Familial nephropathy (FN) due to a COL4A4 mutation in the Welsh Springer Spaniel

Presence of a hereditary renal condition resembling human Alport syndrome and known by the names familial nephropathy (FN) or autosomal recessive hereditary nephropathy (ARHN) has long been known in the English Cocker Spaniel breed [24]. Since identification of the responsible mutation (c.115A>T) in the type IV collagen gene COL4A4, controlled breeding and exclusions in registering offspring of known carriers have contributed to the elimination of the condition from the gene pool of the English Cocker Spaniel breed. Through DNA panel screening, we report here that the same mutation is also genetically present in several lines of the Welsh Springer Spaniel breed. Incidentally, this finding was made possible by breeders setting out to actively explore the prevalence of a far milder condition discovered by us in the breed, FVII deficiency described above. By the analysis of the pedigree registry of the Finnish Springer Spaniel breed, we were able to trace the origin of the mutation to a single individual.
Kennel Club, we confirmed that a mating between two carrier dogs had taken place, producing a puppy that had been euthanized at the age of 10 months due to chronic renal insufficiency (classified as International Renal Interest Society stage 3 of 4).

Fig 4. Chondrodysplasia in the Chinook. (A) Inheritance of chondrodysplasia in a pedigree of Chinooks. All carriers of the responsible variant (ITGA10 c.2083C>T), and a genetically affected dog (squared) confirmed to have a dwarf phenotype are related to the first dwarves documented in the 1990’s as a result of a brother-sister mating (marked with an x). The mutation was likely propagated in the population through the sire of that mating (marked with an arrow), who was further bred to several other females. Gender symbols of dogs with unconfirmed genotypes are shaded gray. (B) A Chinook displaying the dwarfism phenotype (left; and squared in the pedigree) with crooked and shortened legs is shown in comparison to a normal Chinook (right).

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Canine multifocal retinopathy (cmr)- associated variants in the Brazilian Terrier and Finnish Lapphund

Different mutations in the BEST1 gene are known causes of canine multifocal retinopathy (cmr) in Mastiff-related breeds, Coton de Tulear, and Lapponian Herder [15,25]. In this study, we report the first encounter of the cmr type 1 (cmr1) variant (BEST1 c.73C>T) in a terrier breed, the Brazilian Terrier. Moreover, a few carriers of the cmr3 variant (BEST1 c.1388delC) originally found in Lapponian Herder were also found in the closely related Finnish Lapphund breed.

We further explored the prevalence of cmr1 in the Brazilian Terrier by separate genotyping in a randomly selected sample population of 233 dogs. Five mutant allele homozygotes (2.1%) and 52 carriers (22.3%) were identified, closely corresponding to the carrier frequency observed during the initial panel screening (23.4%). Of the five mutant homozygotes, three had previously undergone ophthalmology examinations or were re-examined for this study, and two were not available for clinical evaluation. We did not observe any apparent retinal changes, such as multifocal areas of retinal elevations with subretinal accumulation of serous pink-tan fluid, in the examined Brazilian Terriers homozygous for the cmr1 variant (Table 2).

During sequencing validation of the cmr1 nonsense variant (BEST1 c.73C>T; p.Arg25*), we incidentally also discovered another synonymous (silent) and therefore likely benign base substitution at the same position (c.73C>A [p.Arg25Arg]) in the several breeds (Cirneco dell’Etna, Parson Russell Terrier, Rhodesian Ridgeback, Schipperke, and St Bernard).

Other disease variant findings in additional breeds

Beyond the findings pursued in more detail above, we found other known mutations in additional breeds as well (Table 1). Further studies will be crucial in addressing if these known variants cause a similar clinical phenotype in the additional breeds discovered. The well-known MDR1 (multi-drug resistance 1) mutation c.295_298delAGAT [26] was observed in two previously undocumented breeds, the Chinook and Danish-Swedish Farmdog. Notably, the vWF gene variant c.4937A>G previously associated with von Willebrand’s disease (vWD) type II [27] was present in many more breeds than currently acknowledged. A variant identified as a risk factor for craniomandibular osteopathy (CMO) in terriers [28] was also found at low frequency in other breeds. Based on the genetic findings of this study, examination of the rod-cone dysplasia 3 (rcd3; [29]) variant PDE6A c.1940delA as a factor explaining retinal signs in the Chinese Crested and Pomeranian breeds is warranted. The discovery of a variant (CLCN1 c.2665insA) known to cause myotonia congenita in the Australian Cattle Dog [30] in another breed popular in Australia, the Border Collie, calls for an investigation of whether its presence is limited to a single line of dogs or is more widespread in the breed. Acatalasemia, the deficiency of catalase enzyme activity associated with a CAT gene variant (c.979G>A), was

Table 2. Summary of ophthalmological examinations in the Brazilian Terrier.

| Dog | Year of birth | Sex | Year of ophthalmology examination (s) | Findings |
|-----|---------------|-----|--------------------------------------|----------|
| Dog 1 | 2006 | Female | 2008, 2014 | No abnormalities |
| Dog 2 | 2007 | Female | 2011, 2013 | No abnormalities |
| Dog 3 | 2004 | Female | 2008, 2014, 2015 | • 2008: Distichiasis and suspected vitreous degeneration  
• 2014: Cortical and posterior polar cataract and vitreous degeneration  
• 2015: No abnormalities in the ocular fundus detected by Optical Coherence Tomography |

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originally characterized in research colonies of Beagles \[31,32\]. In this study, we observed the variant also in a carrier American Foxhound. To our knowledge, presence and manifestation of acatalasemia due to the aforementioned CAT variant has not been previously documented in the pet Beagle population. We therefore note that we identified pet Beagle carriers, and a ten-month-old mutant homozygous Beagle through panel screening. During this investigation, the genetically affected Beagle developed gangrene of the oral cavity leading to the removal of three teeth at the age of eighteen months, which could be a manifestation of acatalasemia \[31\].

**Discussion**

Through the application of a novel DNA panel screening test for nearly 100 disorders on a cohort of almost 7000 dogs, we found a high overall frequency of mutation carriers (17.8\%) while discovering a sixth (15/93) of the tested disease-causing variants in additional breeds. Our findings demonstrate the power of multiplex screening as an efficient genetic testing and discovery platform for research, diagnostics and breeding. Our pioneering examples further highlight several general implications related to the approach.

First, panel screening yields new insights into the prevalence and significance of genetic risk variants across breeds. We show that several mutations are more widespread across breeds than reported in original studies with a limited sample size or breed focus, and they often--but not always--cause the same condition in the additional breeds. Therefore, there is a need for careful follow up of any unexpected discoveries. We encourage publication of such findings made during routine DNA testing, but preferably in combination with appropriate clinical evidence supporting genetic counseling and demonstrating relevance of the discovery for breeding selections and veterinary care. Within the scope of this study, we provide strong evidence supporting the clinical relevance of six known inherited conditions in additional breeds, while setting the groundwork for several future investigations.

Second, panel screening is particularly efficient when examining the breed distribution of widespread mutations likely ancient in origin. Accordingly, we present several discoveries on putative ancient variants in additional breeds (e.g., FVII, HUU, PLL, MDR1, and vWD). These disorders also represent recessively inherited conditions with clinical signs that may only be evident upon exposure to a trigger, or not primarily attributed to a specific genetic defect. One could expect such ancient variants to be readily retained in the population. We hypothesize that many more breeds harboring known ancient (and other) mutations will be discovered during future DNA screening as the numbers of tested disease variants, breeds, and dogs of each breed increase.

Third, open-minded panel screening even when no gene tests are known to be relevant for a breed \textit{a priori} may lead to surprising discoveries that are relevant for breed health. This is exemplified by our discoveries of “Finnish Hound” cerebellar ataxia in the Norrbottenspitz, and “Norwegian Elkhound” dwarfism in the Chinook. Such findings also illustrate how inadvertent more recent transfer of mutations between breeds breaks perceived patterns of breed-specificity of genetic disease variants. Pedigree databases alone are unlikely to account fully for the molecular genetic disease heritage historically introduced into a breed. Several breed clubs are engaged in cross-breeding projects to expand the gene pool and improve the health of their breed. While these efforts are commendable, we postulate that they may further benefit from comprehensive genetic screening to ensure that known disease variants are not inadvertently introduced and subsequently enriched in the breed.

More specifically, we established presence of several disorders in additional breeds. Based on the combination of molecular, clinicopathologic and pedigree evidence presented herein, breeders and veterinarians should be aware of these conditions in their breeds and patients,
respectively. FVII deficiency should be considered in Welsh Springer Spaniels and Finnish Hounds, which could exhibit bleeding tendency upon trauma or surgery. Lack of spontaneous bleeding signs in homozygous dogs highlights the mild nature of this coagulopathy, but raises concerns when combined with additional impairments of hemostasis such as hepatic disease, poisoning, and certain drug therapy. The awareness of HUU in additional breeds such as the Lagotto Romagnolo is helpful in guiding veterinarians to appropriate dietary selection, drug therapy and regular follow up for dogs at risk. Similarly, our discovery of PLL in the Danish-Swedish Farmdog has already proven crucial for proper monitoring and care of this potentially painful and severe ocular condition in dogs at risk. Swift availability of a molecular explanation for this phenotype and a validated DNA test is a relief to breeders. Based on documenting the shared ancestry of all identified carriers, we find it likely that the ataxia mutation originally found in the Finnish Hound was introduced into the Norrbottenspitz breed by registration uptake of dogs of unconfirmed heritage. Our fortunate discovery of the variant transfer has since led to active screening of the breed, and thereby prevention of the birth of further affected puppies while keeping healthy carriers in the gene pool. We further provide a molecular genetic cause for one form of dwarfism segregating in the Chinook breed, simultaneously providing an intriguing ancestral link to the Norwegian Elkhound breed.

Finally, we also established the presence of a variant known to cause FN in the English Cocker Spaniel in Welsh Springer Spaniels. Homozygosity for this variant provides a plausible retrospective explanation for renal failure in at least one puppy. Although the genotype of the puppy could not be confirmed, protein truncation by the COL4A4 mutation, likely causing inability to combine with other collagen chains [24], makes it plausible that the pathological consequences are similar as in the original discovery breed. Truncation of the P-glycoprotein drug transporter (ABCB1 gene) due to the well-known MDR1 mutation [26] is likely to pose a risk for severe neurotoxic side effects in any dog being administered drugs normally transported by the protein. We therefore alert veterinary clinicians to the presence of MDR1 also in the Chinook and Danish-Swedish Farmdog.

Our examination of cmr1 eye disease serves as an example of the practical importance of two hallmarks of high fidelity panel screening: 1) confirmation of any novel finding with a second genetic technology; and 2) clinical evaluation of the relevance of the genetic finding for the breed before breeding advice is given. First, sequencing confirmation of cmr1 findings revealed the triallelic nature of position c.73C in the BEST1 gene. While some breeds carry the actual c.73C>T mutation, others harbor a likely harmless synonymous c.73C>A change. Awareness of this triallelic site in routine DNA testing is important, as it may lead to erroneous interpretations with some genotyping technologies, particularly if applied to mixed breed dogs. It remains to be determined whether all three alleles at this specific position are present in some breeds. Second, the clinical validation of a variant in an additional breed may end in lack of evidence for a pathological phenotype. Based on the evidence at hand, cmr1 is unlikely to be a primary health concern in Brazilian Terriers warranting DNA testing, and carriers should not be eliminated from breeding programs. In general, the lack of a disease phenotype in additional breeds may necessitate re-evaluation of the initially reported disease-association in the original breed.

Taken together, our findings illustrate the broad potential of panel screening to: 1) advance breed health and genetics research through information on the breed distribution of mutations; 2) further pet care through preventive medicine on conditions with actionable treatment (e.g., awareness of bleeding propensity before surgery, guidance of dietary selection and monitoring in HUU, prophylactic medication and surgery in PLL, selection of safe medication based on pharmacogenetics such as MDR1 genotype); 3) support veterinary molecular diagnostics (e.g., by provision of a plausible genetic explanation for clinical signs related to nephropathy in the
Welsh Springer Spaniel); 4) prevent an emerging but still hidden severe breed health issue from spreading (e.g., ataxia in the Norrbottenspitz); and 5) effectively explain the molecular background of a breed health-related phenotype not being the primary research focus of any research group (e.g., dwarfism in the Chinook).

This is the first large scale report of DNA panel screening across purebred dogs to date, and as such we note that the investigation should be expanded to include additional disease variants and breeds in the future. Moreover, our study sample was biased towards specific breeds with active DNA testing routines and interests. We also expect some of the disease carrier frequencies reported herein to be higher than would be observed in randomly selected samples of the respective breeds due to bias generated by testing of relatives of carrier and affected dogs. The vast majority of the studied dogs represented breeds with at least one known breed-relevant test among the screened variants, and thus at least a minimum return from the testing. We believe that comprehensive DNA testing also on the remaining ~1/3 of dogs (including breeds with health concerns lacking molecular explanations, breeds lacking an active culture of DNA testing, and more rare understudied breeds) is best justified by the practical examples of novel findings in more than 30 breeds recounted in this report. The Danish-Swedish Farmdog also provides an extreme example of a breed entering the study with no known relevant DNA tests, and leaving it with four potential new conditions to consider in veterinary care and breeding. Although multiplex screening is a powerful diagnostics and research tool from the perspective of a veterinary clinician or researcher, a breeder or dog owner should not be blinded by the number of tests performed. Rather, one should think of panel screening as an accurate, convenient and cost-efficient method to obtain as many breed-relevant test results as possible with one analysis while simultaneously contributing to breed health- and canine genetics research in a novel way. Genetic counseling based on panel screening results should make it clear that the purpose of the test is not to provide a universal health certificate or give a verdict on which dog can be bred. The testing simply provides information on the specific mutations being assayed, leaving other unknown genetic and environmental health risks unexplored. Conveying these messages appropriately is particularly important when screening dogs with no prior known breed-relevant tests. Taken together, despite the need for comprehensive tools to manage the growing number of known disease mutations in breeding, the best long term solution that prevents enrichment and emergence of recessive disorders is informed breeding selections regarding known deleterious variants in combination with maintenance of genetic diversity guided by pedigree-based statistics and modern molecular genetic measurements, as previously discussed [33].

Conclusions

We conclude that DNA multiplex screening for canine hereditary disorders represents a reliable and powerful discovery and utility tool for applications in veterinary diagnostics, genetic research, and sustainable breeding. We demonstrate that several disease-causing mutations are likely affecting and more widespread than recognized during original discovery studies due to shared breed ancestry or more recent cross-breeding. Careful genetic and clinicopathological verification to establish genotype-phenotype correlations should be part of a high quality panel screening service. Importantly, comprehensive disease screening needs to be carried out, interpreted and reported responsibly, maintaining close ties to high quality genetics research, caring veterinarians, breeders, breed health committees and kennel clubs. Panel screening, as any single DNA test, should only be viewed as one part of a breeding decision and strategy. Holistic approaches working towards overall breeding goals that are beneficial not only for the immediate health of the offspring, but also for the sustainability of the breed as a whole, are recommended.
Supporting Information

S1 Table. Number of studied dogs by breed. (XLS)

S2 Table. Disease variants screened in the study. (XLS)

S3 Table. Sequencing primers. (XLS)

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Author Contributions

Conceived and designed the experiments: JD HL UG.

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