



Development of Cost-Effective PCR-RFLP Methods for Screening Mendelian Disorders in Chihuahua Dogs

Mehmet Cevat TEMİZKAN^{1, a}

¹ Yozgat Bozok University,
Faculty of Veterinary
Medicine,
Department of Genetics,
Yozgat, TÜRKİYE

^a ORCID: 0000-0002-4353-6759

Selective breeding practices have impacted the intra-breed genetic diversity of Chihuahuas, increasing homozygosity for deleterious alleles and predisposing the breed to specific hereditary conditions. Although DNA sequencing represents the golden standard for genetic diagnosis, its cost and technical requirements limit its widespread use in routine veterinary practice. This study developed comprehensive and cost-effective Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) methods to screen for Mendelian genetic disorders in Chihuahuas. Using the Online Mendelian Inheritance in Animals (OMIA) database and reference genomic sequences, specific primer-enzyme sets were designed for nine genetic variants associated with eight disorders: ichthyosis (*ALOXE3* and *SDR9C7*), epidermolytic hyperkeratosis (*KRT10*), Ehlers-Danlos syndrome (*COL5A2*), verrucous epidermal keratinocytic nevi (*NSDHL*), methaemoglobinaemia (*CYB5R3*), Alexander disease (*GFAP*), progressive rod-cone degeneration (*PRCD*), and spinal dysraphism (*NKX2-8*). Predicted electrophoresis profiles confirmed that the selected enzymes generate distinct digestion patterns, enabling the clear differentiation of homozygous wild-type, heterozygous, and homozygous mutant genotypes. These protocols offer a robust, rapid, and economical alternative to sequencing for identifying carriers and affected individuals. The integration of these methods into clinical diagnostics and breeding programs provides a practical tool for facilitating informed breeding decisions.

Key Words: Chihuahua, Dog, hereditary diseases, polymerase chain reaction, restriction fragment length polymorphism

Chihuahua Irkı Köpeklerde Mendelyen Bozuklukların Taranması için Ekonomik PCR-RFLP Analizlerinin Geliştirilmesi

İrk içi ıslah uygulamaları, Chihuahua ırkının genetik çeşitliliğini etkilemekte, genetik hastalık taşıyıcısı alellerin homozigotluğunu artırmakta ve bu ırkı belirli kalıtsal hastalıklara yatkın hale getirmektedir. Genetik hastalıkların tespitinde altın standart olarak kabul edilen DNA dizileme teknikleri, maliyeti ve teknik gereksinimleri nedeniyle rutin veteriner hekimliği uygulamalarında yaygın kullanım imkanı bulamamaktadır. Bu çalışmada, Chihuahua ırkında Mendelyen genetik bozuklukların taranması amacıyla kapsamlı, ucuz ve uygulanabilir Polimeraz Zincir Reaksiyonu-Restriksiyon Parça Uzunluk Polimorfizmi (PCR-RFLP) tarama setleri geliştirilmiştir. Online Mendelian Inheritance in Animals (OMIA) veritabanı ve referans genomik diziler kullanılarak; iktiyozis (*ALOXE3* ve *SDR9C7*), epidermolitik hiperkeratoz (*KRT10*), Ehlers-Danlos sendromu (*COL5A2*), verrüköz epidermal keratinositik nevus (*NSDHL*), methemoglobinemi (*CYB5R3*), Alexander hastalığı (*GFAP*), progresif rod-koni dejenerasyonu (*PRCD*) ve spinal disrafizm (*NKX2-8*) olmak üzere sekiz genetik bozuklukla ilişkili dokuz genetik varyant için spesifik primer-enzim setleri tasarlanmıştır. Öngörülen elektroforez profilleri, seçilen enzimlerin; homozigot yabancıl tip (wild-type), heterozigot ve homozigot mutant genotiplerin net bir şekilde ayırt edilmesine olanak tanıyan belirgin kesim bantları oluşturduğunu doğrulamıştır. Bu protokoller, taşıyıcıların ve hastalıktan etkilenen bireylerin tanımlanmasında, dizileme yöntemine kıyasla güçlü, hızlı ve ekonomik bir alternatif sunmaktadır. Bu testlerin klinik tanı ve yetiştirme programlarına entegrasyonu, bilinçli ıslah kararlarının alınmasını kolaylaştırmak adına pratik bir araç sağlamaktadır.

Anahtar Kelimeler: Chihuahua, köpek, kalıtsal hastalıklar, polimeraz zincir reaksiyonu, restriksiyon parça uzunluk polimorfizmi

Received : 03.12.2025
Accepted : 06.01.2026

Correspondence

Mehmet Cevat TEMİZKAN
Yozgat Bozok University,
Faculty of Veterinary
Medicine,
Department of Genetics
Yozgat – TÜRKİYE

drctemizkan@gmail.com

Introduction

The domestic dog (*Canis lupus familiaris*) exhibits remarkable phenotypic diversity resulting from centuries of selective breeding (1, 2). However, selection to maintain breed standards can compromise intra-breed genetic diversity and increase the homozygosity of deleterious alleles (3, 4). This genetic bottleneck predisposes various breeds to specific hereditary conditions (3, 5). Recent advances in canine genomics have significantly facilitated the identification of such disorders (6, 7). Detecting carriers and excluding them from breeding programs are primary steps toward eliminating these disorders (8). Like many dog breeds, the Chihuahua is predisposed to several genetic disorders (5-7).

Research into Mendelian genetic disorders in Chihuahuas has accelerated recently. Prominent dermatological conditions include ichthyosis caused by variants in

ALOXE3 (arachidonate lipoxygenase 3) and *SDR9C7* (short chain dehydrogenase/reductase family 9C member 7) (9, 10); hyperkeratosis, associated with *KRT10* (keratin 10) (7, 11-13); and verrucous epidermal keratinocytic nevi, linked to *NSDHL* (NAD(P) dependent steroid dehydrogenase-like) (14). Chihuahuas are also prone to connective tissue genetic disorders like Ehlers-Danlos syndrome (associated with *COL5A2* gene: collagen type V alpha 2 chain) (15) and metabolic defects such as methaemoglobinemia (associated with *CYB5R3* gene: cytochrome b5 reductase 3) (16), and neurological conditions include Alexander disease (associated with *GFAP* gene: glial fibrillary acidic protein) (17-19) and spinal dysraphism (associated with *NKX2-8* gene: NK2 homeobox 8) (20, 21). Additionally, ocular disorders like progressive rod-cone degeneration (associated with the *PRCD* gene: photoreceptor disc component) have been documented (22).

Accurate diagnosis is critical for veterinary practice and breeding management (6). While DNA sequencing (Sanger or Next-Generation Sequencing) remains the "gold standard", its cost and equipment requirements limit accessibility in routine practice (23-25). Conversely, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) offers a robust, cost-effective, and rapid alternative (23), allowing discrimination between wild-type and mutant alleles via specific enzymatic digestion profiles (26-28). Although positive results may require sequencing confirmation, using PCR-RFLP to exclude negative samples significantly reduces costs in large-scale analyses (23, 29).

This study aimed to develop a comprehensive PCR-RFLP primer-enzyme set for detecting Mendelian genetic disorders in Chihuahuas. We designed methods specific to nine genetic variants associated with eight hereditary disorders. This protocol provides veterinarians and breeders with a practical, economic tool for screening and informed breeding decisions.

Materials and Methods

Research and Publication Ethics: This study was based on the design of molecular diagnostic protocols using publicly available genomic data. As no biological samples were collected from live animals, ethics committee approval was not required.

Gene and Variation Selection: The "Online Mendelian Inheritance in Animals" (OMIA) database was used to identify genetic disorders and causal mutations (30). Up-to-date literature and database searches revealed 19 recorded genetic disorders or traits in the Chihuahua breed, with key mutations identified for 15. Although pyruvate kinase deficiency has been reported in Chihuahuas, it was excluded as the breed-specific key mutation remains undefined (31).

Following the exclusion of coat color variants, 13 variations corresponding to 11 distinct genetic disorders were evaluated. Primer-enzyme sets could not be designed for four disorders (*GH1*-associated dwarfism,

NSDHL-missense mutation-associated verrucous epidermal keratinocytic nevi, *NHLRC1*-associated Lafora disease, and *MFSD8*-associated neuronal ceroid lipofuscinosis) due to technical limitations, such as the absence of suitable restriction sites or nucleotide differences altering enzyme binding between wild-type and mutant alleles. Consequently, the study focused on detecting nine variants including *ALOXE3*, *SDR9C7*, *KRT10*, *COL5A2*, *NSDHL* (deletion mutation associated), *CYB5R3*, *GFAP*, *PRCD*, and *NKX2-8* genes (Table 1). Reference sequences were retrieved and cross-verified from the National Center for Biotechnology Information (NCBI) GenBank and Ensembl databases.

Design of PCR Primers and Restriction Analysis: Restriction maps for the identified variant regions were analyzed using *NebCutter V3.0.20* (32). Enzymes were selected based on their ability to generate distinct DNA fragment sizes distinguishable on agarose gel, contingent on the presence or absence of the variation.

Specific primer pairs for target amplification were designed using *PerIPrimer* (33). Off-target binding was assessed using the NCBI Primer-BLAST tool (34). Primer binding sites and restriction recognition sequences were screened with the Ensembl database to exclude potential interfering SNPs, ensuring selection from sequences conserved across all transcripts. All primers were designed to preclude the generation of non-specific amplicons smaller than 1,000 bp, thereby facilitating optimization via PCR annealing conditions. Although a potential off-target product of 938 bp was identified solely for the *CYB5R3* method, it is not anticipated to compromise PCR efficiency given the significantly smaller size of the target amplicon (170 bp). Primer parameters, including melting temperatures (T_m), GC content, and potential secondary structures, were calculated using *PerIPrimer*. Theoretical primer sequences, T_m values, and GC percentages for each primer, selected restriction enzymes, amplicon, and fragment sizes are provided in Table 2. However, as PCR efficiency depends on laboratory conditions and reagents, experimental optimization of the annealing temperature is required.

Prediction of Electrophoretic Profiles: To validate the theoretical accuracy of the methods, post-digestion banding profiles were simulated using *NebCutter V3.0.20* (32). Simulation parameters were set to 1.4% agarose gel (TBE Buffer), 100 bp DNA ladder, 180 mm run length, and a 6 V/cm electric field.

Results

We designed comprehensive PCR-RFLP protocols for diagnosing eight genetic disorders resulting from nine distinct variations in the Chihuahua breed. The analyses indicated that the 11 selected restriction enzymes clearly distinguished between homozygous wild-type, heterozygous, and homozygous mutant genotypes across all targeted gene regions (Figure 1).

Hereditary Skin Disorders: Chihuahuas are predisposed to three genetically distinct dermatological conditions. Ichthyosis has been identified in association with variations in the *ALOXE3* and *SDR9C7* genes (9, 10). For the *ALOXE3* mutation, digestion with PpuMI cleaves the mutant allele into 393 bp and 194 bp fragments (Figure 1A), while the wild-type allele remains undigested (557 bp). Conversely, for *SDR9C7*-associated ichthyosis, digestion with either Aval or BsoBI yields 603 bp and 233 bp fragments for the wild-type allele, whereas the mutant allele remains intact at 836 bp (Figure 1B).

For hyperkeratosis, digestion of the *KRT10* gene with Acil produces diagnostic fragments of 131 bp and 69 bp for the wild-type allele. The mutant allele lacks the restriction site, appearing as a single 200 bp band (Figure 1C).

Verrucous epidermal keratinocytic nevi can arise from two distinct *NSDHL* variations: a missense mutation and a deletion (14, 35). As detailed in the Methods, an RFLP method could not be designed for the missense mutation. However, the deletion variant is detectable using PstI; the enzyme cleaves the wild-type sequence into 360 bp and 157 bp fragments, while the mutant allele remains undigested (Figure 1E).

Systemic Hereditary Disorders: In the diagnosis of *COL5A2*-associated Ehlers-Danlos syndrome, the BsaI enzyme digests the wild-type amplicon into 429 bp and 352 bp fragments. The mutant allele is not digested; however, due to the characteristic 27-bp deletion (15), the resulting single band appears at 754 bp rather than the theoretical 781 bp (Figure 1D).

For *CYB5R3*-associated methaemoglobinaemia, the BaeGI enzyme produces 92 bp and 78 bp bands in mutant individuals, whereas non-carriers (wild-type) exhibit a single 170 bp band (Figure 1F).

In *GFAP*-associated Alexander disease, BsiWI digestion yields two bands (356 bp and 201 bp) for the wild-type allele, while the mutant allele appears as a single 557 bp band (Figure 1G).

For *NKX2-8*-associated spinal dysraphism, the BtgZI enzyme cleaves the wild-type allele into 449 bp and 57 bp fragments, leaving the mutant allele undigested at 507 bp.

Eye-Related Hereditary Disorders: Progressive rod-cone degeneration associated with the *PRCD* gene can be identified using two alternative enzymes: ApaLI and BsrGI. Digestion occurs in the wild-type allele when using ApaLI, and in the mutant allele when using BsrGI. Both enzymes generate fragments of similar sizes (approx. 257 bp/222 bp) (Figure 1H).

Table 1. Summary of the evaluated genetic disorders, key mutations, and reference sequences

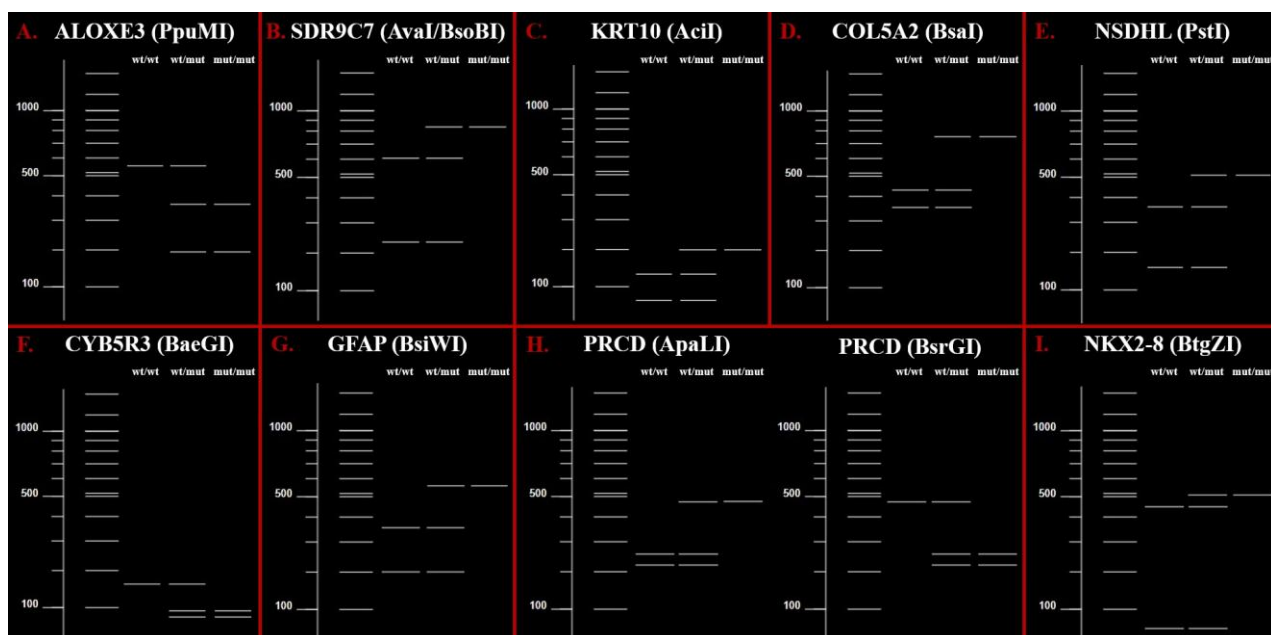
OMIA Disorder / Variant ID	Disorder	Year of Key Mutation Detection	Gene	Accession Numbers (Ensembl and NCBI) and Variants	Mode of Inheritance
003011-9615 / 1834	Ichthyosis	2025	<i>ALOXE3</i>	ENSCAFT00845048710.1 / XM_038665511.1:c.1379G>A	Autosomal Recessive
002659-9615 / 1540	Ichthyosis, non-epidermolytic	2023	<i>SDR9C7</i>	ENSCAFT00845016366.1 / XM_038678828.1:c.454C>T	Autosomal Recessive
001415-9615 / 1579	Hyperkeratosis	2023	<i>KRT10</i>	ENSCAFT00030031038.1 / XM_038547368.1:c.437G>A	Autosomal Dominant
002295-9615 / 1460	classical Ehlers-Danlos syndrome	2022	<i>COL5A2</i>	ENSCAFT00000023545.5 / XM_005640393.4:c.3388_3414del	Autosomal Dominant
002117-9615 / 1268	Verrucous epidermal keratinocytic nevi	2020	<i>NSDHL</i>	ENSCAFT00000030435.5 / XM_014111859.3:c.718_722delGAACA	X-linked Dominant
002131-9615 / 967	Methaemoglobinaemia	2020	<i>CYB5R3</i>	ENSCAFT00000099594.1 / NM_001048084.1:c.580A>C	Autosomal Recessive
001208-9615 / 114	Alexander disease	2016	<i>GFAP</i>	ENSCAFT00000063838.2 / XM_022422887.1:c.719G>A	Autosomal Dominant
001298-9615 / 76	Progressive rod-cone degeneration	2015	<i>PRCD</i>	ENSCAFT00000049113.3 / NM_001097560.2:c.5G>A	Autosomal Recessive
000938-9615 / 622	Spinal dysraphism	2013	<i>NKX2-8</i>	ENSCAFT00845004712.1 / XM_038672956.1:c.449delinsTT	Autosomal Recessive

Table 2. Details of the designed PCR-RFLP methods: primers, restriction enzymes, and predicted digestion products

OMIA Disorder / Variant ID	Gene	Primer Pairs	Tm (°C)	GC Ratio (%)	Restriction Enzymes**	Product Size (bp)
003011-9615 / 1834	<i>ALOXE3</i>	F: CTTTGTCCATCCAGTCTACAAGGTC	61.38	45	PpuMI (RG/GWCCY)	Undigested: 557 Digested: 393/194
		R: GGAGGAAATGCTTGGGTATTTGG	62.28	47		
002659-9615 / 1540	<i>SDR9C7</i>	F: GTGTGAATGGATGAACTGCT	58.65	45	AvaI / BsoBI (C/YCGRG)	Undigested: 836 Digested: 603/233
		R: CTATTGTGTTAGCTCCCTCCCT	61.48	50		
001415-9615 / 1579	<i>KRT10</i>	F: GGTGGATATGGAGGAGATGG	59.20	55	AciI (C/CGC)	Undigested: 200 Digested: 131/69
		R: TATTGCTGTAGTCACGAGG	57.51	45		
002295-9615 / 1460	<i>COL5A2</i>	F: GTCATTACCTCGCTTCATCTC	61.56	50	BsaI (GGTCTCN/N ₄)	Undigested: 754 Digested: 429/352
		R: GATTTCTTCAAGTCACCCATCTC	59.56	43		
002117-9615 / 1268	<i>NSDHL</i>	F: GCCCTTACATATCCCAGAGCC	62.58	57	PstI (CTGCA/G)	Undigested: 512 Digested: 360/157
		R: GAATAAATGAGTAGCACGCACCC	62.35	47		
002131-9615 / 967	<i>CYB5R3</i>	F: ATACAGCAGGTGCTCACTCAG	62.35	52	BaeGI (GKGC/M/C)	Undigested: 170 Digested: 92/78
		R: AACAGCCCAGTTTGCATCAC	61.95	50		
001208-9615 / 114	<i>GFAP</i>	F: CTCAGTTGCTCTCCATAAGAACAC	61.68	45	BsiWI (C/GTACG)	Undigested: 557 Digested: 356/201
		R: CCCTCATTCTCTGGTGAAGGT	62.22	50		
001298-9615 / 76	<i>PRCD</i>	F: CAGGTCCTAGTCACATCTGTATCC	62.06	50	ApaLI (G/TGCAC)	Undigested: 477 Digested: 257/221
		R: CGTGCTCTGATGGGAAACCT	62.68	55		
000938-9615 / 622	<i>NKX2-8</i>	F: GCTACAAGCTGAAGCG	55.41	56	BtgZI (GCGATGN10/)	Undigested: 507 Digested: 449/57
		R: GATATCCTGGCTAAAGTCCCT	59.53	45		

*The fragment sizes presented are theoretical values based on restriction mapping analysis.

**Nucleotides shown in bold within the restriction enzyme sequences indicate the variation nucleotides.

**Figure 1.** Predicted agarose gel electrophoresis patterns of PCR-RFLP methods for nine genetic variants.

* wt/wt: homozygous wild-type; wt/mut: heterozygous; mut/mut: homozygous mutant

Discussion

The containment and eradication of genetic disorders represent primary objectives in modern dog breeding. Despite its global popularity, the Chihuahua breed is predisposed to several hereditary conditions

due to a constricted gene pool (5-7). In this study, we developed cost-effective PCR-RFLP primer-enzyme sets for the detection of eight Mendelian disorders that affect Chihuahuas and are often challenging to diagnose phenotypically. These sets are designed for easy implementation in veterinary clinics and diagnostic

laboratories. However, the primer and enzyme combinations in this study were selected based on strict thermodynamic criteria and restriction mapping to ensure high specificity, minimizing the risk of off-target amplification or incomplete digestion in clinical applications.

While DNA sequencing technologies offer high sensitivity, they can be cost-prohibitive and time-consuming for routine screening, particularly in developing countries or resource-limited settings (36, 37). In these contexts, PCR-RFLP remains a reliable, rapid, and economical method for genotyping (23, 29). As highlighted in recent studies, PCR-based enzymatic digestion methods continue to offer flexible solutions requiring minimal instrumentation (36, 37). Such flexibility is crucial when screening for disorders with diverse inheritance patterns. Certain genetic disorders may cause embryonic lethality in homozygous recessive genotypes or hemizygous males (potentially associated with *NSDHL*). Other conditions may exhibit a dominant inheritance (*COL5A2*, *GFAP*). Regardless of the mode of inheritance, the PCR-RFLP method enables the detection of the defective allele. Moreover, this method remains feasible for analyzing samples collected from post-mortem specimens or embryos.

The hereditary skin disorders addressed in this study often share overlapping phenotypic presentations. Notably, ichthyosis has recently been shown to arise from distinct mutations in the *ALOXE3* and *SDR9C7* genes (9, 10). The primer sets designed here, compatible with PpuMI and Aval/BsoBI digestion, allow for the molecular differentiation of these variants. Similarly, the differential diagnosis of *KRT10*-associated hyperkeratosis and *NSDHL*-associated verrucous epidermal nevi is critical for determining appropriate treatment protocols (13, 14). However, it must be noted that PCR-RFLP may not be applicable for all mutation types, as observed with the *NSDHL* missense mutation, where the single-nucleotide change did not alter a restriction site.

Beyond dermatological conditions, the early diagnosis of life-threatening systemic and neurological disorders is vital to prevent the breeding of carriers. Spinal dysraphism caused by *NKX2-8* variation leads to severe neurological defects in offspring (20). In the BtgZI restriction method, the resulting short 57 bp fragment in carriers (wt/mut) may be indistinguishable from primer dimers. However, the clear distinction between the 507

bp mutant and 449 bp wild-type bands is sufficient to identify heterozygotes. To achieve optimal resolution and differentiate these bands clearly, the use of higher concentration agarose gels (2% to 4%) is advisable rather than the simulated 1.4% gel (Figure 11). Furthermore, the ability to detect rare but severe conditions such as *COL5A2*-associated Ehlers-Danlos syndrome and *GFAP*-associated Alexander disease provides breeders with a comprehensive health screening tool. Additionally, screening for *CYB5R3*-associated methaemoglobinaemia, a metabolic defect, provides clinicians with crucial data for pre-anesthetic risk assessment (16). However, the enzymatic digestion of the mutant *CYB5R3* allele generates relatively small fragments (92 bp and 78 bp). In the absence of wet-lab validation, there is a potential risk that primer dimers could mimic these low-molecular-weight bands, leading to a misinterpretation of wild-type samples as heterozygotes. Therefore, optimization of PCR conditions to minimize dimer formation and the use of high-percentage agarose gels are recommended for accurate interpretation of this primer-enzyme combination.

While PCR-RFLP sets for progressive rod-cone degeneration detection have been previously described (22, 26-28), including the use of ApaLI in Labrador Retrievers (38), the inclusion of BsrGI in this study provides laboratories with greater flexibility regarding enzyme availability. Notably, the single primer pair designed herein permits *PRCD* detection using either ApaLI or BsrGI. Moreover, these new sets can serve as a confirmatory mechanism to validate results from existing methods, thereby enhancing diagnostic reliability.

Predicted electrophoresis profiles confirmed that all designed primer-enzyme pairs clearly distinguish between homozygous wild-type, heterozygous, and homozygous mutant genotypes. Notwithstanding these design considerations, experimental validation of the proposed sets is recommended prior to clinical implementation.

In conclusion, this protocol set offers a cost-effective, rapid, and robust method for screening Mendelian genetic disorders in the Chihuahua breed. The integration of this method into routine veterinary practice will significantly contribute to early diagnosis and the breeding of healthy generations by identifying carriers.

References

- Ostrander EA, Wayne RK. The canine genome. *Genome Res* 2005; 15: 1706-1716.
- Parker HG, Dreger DL, Rimbault M, et al. Genomic analyses reveal the influence of geographic origin, migration, and hybridization on modern dog breed development. *Cell Rep* 2017; 19: 697-708.
- Calboli FC, Sampson J, Fretwell N, Balding DJ. Population structure and inbreeding from pedigree analysis of purebred dogs. *Genetics* 2008; 179: 593-601.
- Leroy G. Genetic diversity, inbreeding and breeding practices in dogs: results from pedigree analyses. *Vet J* 2011; 189: 177-182.
- Asher L, Diesel G, Summers JF, McGreevy PD, Collins LM. Inherited defects in pedigree dogs. Part 1: Disorders related to breed standards. *Vet J* 2009; 182: 402-411.
- Donner J, Anderson H, Davison S, et al. Frequency and distribution of 152 genetic disease variants in over 100,000 mixed breed and purebred dogs. *PLoS Genet* 2018; 14: e1007361.

7. Meadows JR, Kidd JM, Wang GD, et al. Genome sequencing of 2000 canids by the Dog10K consortium advances the understanding of demography, genome function and architecture. *Genome Biol* 2023; 24: 187.
8. Farrell LL, Schoenebeck JJ, Wiener P, Clements DN, Summers KM. The challenges of pedigree dog health: Approaches to combating inherited disease. *Canine Genet Epidemiol* 2015; 2: 3.
9. Vinberg C, Rietmann SJ, Soto S, et al. ALOXE3 missense variant in a Chihuahua with autosomal recessive ichthyosis. *Anim Genet* 2025; 56: e70055.
10. Kiener S, Castilla E, Jagannathan V, Welle M, Leeb T. SDR9C7 missense variant in a Chihuahua with non-epidermolytic ichthyosis. *Anim Genet* 2023; 54: 562-565.
11. Kiener S, Åhman S, Jagannathan V, Soto S, Leeb T. Heterozygous KRT10 missense variant in a Chihuahua with severe epidermolytic ichthyosis. *Anim Genet* 2023; 54: 766-768.
12. Leeb T, Roosje P, Welle M. Genetics of inherited skin disorders in dogs. *Vet J* 2022; 279: 105782.
13. Marin Garcia PJ, Llobat L. Inheritance of monogenic hereditary skin disease and related canine breeds. *Vet Sci* 2022; 9: 433.
14. Christen M, Austel M, Banovic F, Jagannathan V, Leeb T. NSDHL frameshift deletion in a mixed breed dog with progressive epidermal nevi. *Genes* 2020; 11: 1297.
15. Kiener S, Chevallier L, Jagannathan V, et al. A COL5A2 in-frame deletion in a chihuahua with Ehlers-Danlos syndrome. *Genes* 2022; 13: 934.
16. Jaffey JA, Reading NS, Abdulmalik O, et al. Clinical, metabolic, and molecular genetic characterization of hereditary methemoglobinemia caused by cytochrome b5 reductase deficiency in 30 dogs. *Sci Rep* 2020; 10: 21399.
17. Gruber A, Pakozdy A, Leschnik M, Mai S, Weissenböck H. Morbus alexander - 4 fälle bei hunden in österreich. *Wien Tierarztl Monatsschr* 2010; 97: 298.
18. Messing A, Brenner M, Feany MB, Nedergaard M, Goldman JE. Alexander disease. *J Neurosci* 2012; 32: 5017-5023.
19. Van Poucke M, Martlé V, Van Brantegem L, et al. A canine orthologue of the human GFAP c. 716G> A (p. Arg239His) variant causes Alexander disease in a Labrador retriever. *Eur J Hum Genet* 2016; 24: 852-856.
20. Safra N, Bassuk AG, Ferguson PJ, et al. Genome-wide association mapping in dogs enables identification of the homeobox gene, NKX2-8, as a genetic component of neural tube defects in humans. *PLoS Genet* 2013; 9: e1003646.
21. Wilson JW, Kurtz HJ, Leipold HW, Lees GE. Spina bifida in the dog. *Vet Pathol* 1979; 16: 165-179.
22. Kohyama M, Tada N, Mitsui H, et al. Real-time PCR genotyping assay for canine progressive rod-cone degeneration and mutant allele frequency in Toy Poodles, Chihuahuas and Miniature Dachshunds in Japan. *J Vet Med Sci* 2016; 78: 481-484.
23. Fong WY, Ho CC, Poon WT. Comparison of direct sequencing, real-time PCR-high resolution melt (PCR-HRM) and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis for genotyping of common Thiopurine intolerant variant alleles NUDT15 c. 415C> T and TPMT c. 719A> G (TPMT* 3C). *Diagnostics* 2017; 7: 27.
24. Heather JM, Chain B. The sequence of sequencers: The history of sequencing DNA. *Genomics* 2016; 107: 1-8.
25. Slatko BE, Gardner AF, Ausubel FM. Overview of next-generation sequencing technologies. *Curr Protoc Mol Biol* 2018; 122: e59.
26. Cooper AM. Developing A Canine Genetic Testing Program at the Atlantic Veterinary College: Pilot Study on Disease Susceptibility Loci in Labrador Retrievers. Doctoral dissertation, Prince Edward Island: University of Prince Edward Island, 2020.
27. Dostal J, Hrdlicova A, Horak P. Progressive rod-cone degeneration (PRCD) in selected dog breeds and variability in its phenotypic expression. *Vet Med Czech* 2011; 56: 243-247.
28. Ray K, Acland GM, Aguirre GD. Nonallelism of erd and prcd and exclusion of the canine RDS/peripherin gene as a candidate for both retinal degeneration loci. *Invest Ophthalmol Vis Sci* 1996; 37: 783-794.
29. Andree KB, Loi B, Vallainc D, et al. Investigation of the utility of PCR-RFLP as a rapid alternative to DNA sequencing for interrogation of the genetic sex of Mugil cephalus. *Anim Reprod Sci* 2024; 270: 107614.
30. Nicholas FW, Tammen I, Sydney Informatics Hub. "Online Mendelian Inheritance in Animals (OMIA)". <https://omia.org/> 2025.
31. Gultekin G, Raj K, Foureman P, et al. Erythrocytic pyruvate kinase mutations causing hemolytic anemia, osteosclerosis, and secondary hemochromatosis in dogs. *J Vet Intern Med* 2012; 26: 935-944.
32. Vincze T, Posfai J, Roberts RJ. NEBcutter: A program to cleave DNA with restriction enzymes. *Nucleic Acids Res* 2003; 31: 3688-3691.
33. Marshall OJ. PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* 2004; 20: 2471-2472.
34. Ye J, Coulouris G, Zaretskaya I, et al. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 2012; 13: 134.
35. Leuthard F, Lehner G, Jagannathan V, Leeb T, Welle M. A missense variant in the NSDHL gene in a Chihuahua with a congenital cornification disorder resembling inflammatory linear verrucous epidermal nevi. *Anim Genet* 2019; 50: 768-771.
36. Temizkan MC, Sonmez G, Sayar E. Detection of arachnomelia syndrome in Simmental cattle in Türkiye. *Vet Med Sci* 2025; 11: e70442.
37. Ayadi W, Smaoui F, Gargouri S, et al. Use of allele-specific qPCR and PCR-RFLP analysis for rapid detection of the SARS-CoV-2 variants in Tunisia: A cheap flexible approach adapted for developing countries. *PLoS One* 2025; 20: e0321581.
38. Takanosu M. Different allelic frequency of progressive rod-cone degeneration in two populations of Labrador Retrievers in Japan. *J Vet Med Sci* 2017; 79: 1746-1748.