Received: 28 April 2020 | Revised: 16 June 2020 | Accepted: 18 June 2020
DOI: 10.1111/jvp.12890

ORIGINAL ARTICLE

Analysis of polymorphisms of canine Cytochrome P 450-CYP2D15

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Abstract

Cytochrome P450 (CYP) proteins constitute a large ancient family of oxidative enzymes essential for the efficient elimination of a wide variety of clinically used drugs. Polymorphic variants of human CYP2D6 are associated with the conversion rate and efficacy of several drugs such as antidepressants. Polymorphisms of the canine orthologue CYP2D15 are of interest because these antidepressants are also used in dogs with behavioral problems and the outcome of the treatment is variable. However, the annotated *CYP2D15* gene is incomplete and inaccurately assembled in CanFam3.1, hampering DNA sequence analysis of the gene in individual dogs. We elucidated the complete exon–intron structure of *CYP2D15* to enable comprehensive genotyping of the gene using genomic DNA. We surveyed variations of the gene in four diverse dog breeds and identified novel polymorphisms in exon 2 in border collies. Further investigation to establish the impact of these canine CYP2D15 polymorphisms on interindividual variability in expression and function of this metabolizing enzyme is now feasible. Further knowledge of CYP pharmacogenetics will help individualize therapy and thereby increase therapeutic efficacy, especially in the use of antidepressants in veterinary behavioral medicine.

KEYWORDS
canine, CYP2D6, cytochrome P 450-CYP2D15, dog, genetic polymorphisms, pharmacogenetics

1 | INTRODUCTION

The cytochrome P450 (CYP) drug-metabolizing enzymes are critical to the efficient elimination of many drugs in clinical practice. Existing CYP genetic polymorphisms explain the inherited genetic differences in drug metabolic pathway which can affect individual response predisposing patients to adverse drug reaction or therapeutic failure (Court, 2013). Much of the available data on the CYPs so far concern the human CYP. In humans, the highly polymorphic drug-metabolizing enzyme CYP2D6 is associated with the rate of metabolizing over 25% of currently marketed drugs in humans, including dysarrhythmics, adrenoceptor antagonists, and antidepressants (Teh & Bertilsson, 2012). Over 100 documented alleles of *CYP2D6* are registered and are often characterized by extensive metabolizer and poor metabolizer phenotypes, altering drug metabolization significantly between individual and ethnicities (https://www.pharmvar.org/gene/CYP2D6).

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Antidepressants, like clomipramine and fluoxetine, are also commonly used in the treatment of behavioral problems in dogs (King et al., 2004) and cats (Seksel & Lindeman, 2001). Although polymorphisms of the canine orthologous gene *CYP2D15* are described, the available research to the functional effect of polymorphisms of *CYP2D15* on enzymatic activity is limited (Roussel et al., 1998; Shou, 2003) . The skipping of exon 3, leading to deletion of 51 amino acids, must be considered as a splice variant, not as a genetic polymorphism (Martinez et al., 2013; Roussel et al., 1998). Protein variants with this deletion have no detectable enzymatic activity (Paulson et al., 1999; Roussel et al., 1998). Various combinations of the amino acid substitutions Gly186Ser, Ile250Phe, and Ile307Val seem to have little or no effect on the conversion rates of the substrates celecoxib and bufuralol (Paulson et al., 1999; Roussel et al., 1998). A variant with the substitutions Ile338Val and Lys407Glu appears to have a 2-fold lower Km and 4-fold higher Vmax for bufuralol than the variant with the reference amino acids (Roussel et al., 1998).

The paucity of knowledge of the variability of canine CYP proteins can be explained by the high level of similarity between the various encoding genes. This similarity makes it difficult to design PCR oligonucleotides for the amplification of genomic DNA fragments that are derived from a single CYP gene. Indeed, the knowledge of polymorphisms of *CYP2D15* is mainly based on the analysis of mRNA sequences obtained from liver biopsies of laboratory dogs (Paulson et al., 1999; Roussel et al., 1998; Sakamoto et al., 1995).

Pappas and Katsiabas identified polymorphisms in a genomic DNA fragment of *CYP2D15* containing exons 5, 6, and 7. It was suggested that their method could be used as a tool for quick assessment of canine *CYP2D15* polymorphism (Pappas & Katsiabas, 2003). However, such an assessment should preferably include the complete gene. Gaps in the DNA sequence of canine *CYP2D15* of the reference genome CanFam3.1 cloud the exon–intron structure of the gene. In addition, we noticed from comparison of *CYP2D15* cDNA and CanFam3.1 that the assembly of the gene in the reference genome is incorrect. These circumstances hamper genotyping of the complete coding DNA sequence of the gene from easily obtainable genomic DNA. Here, we present a comprehensive analysis of the exon–intron structure of canine *CYP2D15*, which enables genotyping of the complete gene in cohorts of privately owned dogs. We surveyed variations of the gene in four diverse dog breeds.

2 | MATERIALS AND METHODS

2.1 | Dogs

DNA of 12 dogs of each of the breeds bull mastiff (mastiff type), English cocker spaniel (modern European type), border collie (herding type) and Rottweiler (mastiff type) was randomly selected from the DNA database of the Department of Clinical Sciences of Utrecht University. The dogs were privately owned and visited the department for various reasons. EDTA blood samples were obtained for DNA isolation with a written informed consent statement from the owners to use the samples for scientific research. The DNA was isolated from the samples using a Chemagic™ MSM I robot (Perkin Elmer).

2.2 | DNA sequence analysis

Oligonucleotides for PCR amplification and DNA sequencing of the exons of canine *CYP2D15* are listed in Table 1. The PCRs for exons 1, 2, 7, and 8–9 were performed with 25 ng genomic DNA, 3 U Platinum Taq DNA polymerase (Thermo Fisher Scientific), 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, 1 M betaine, and 1× Platinum buffer. Temperature cycling conditions were 5 min at 95°C, 35–40 cycles of 30 s at 95°C, 30 s at Tm °C, 30 s at 72°C, and a final elongation step at 72°C for 10 min. The PCRs for exons 3–4 and 5–6 were performed with 25 ng genomic DNA, 0.25 U PFX Taq DNA polymerase (Thermo Fisher Scientific), 1 mM $MgSO_4$, 0.3 mM each dNTP, 0.3 μM each primer, 1x enhancer buffer, and 1× PFX buffer. Temperature cycling conditions were 5 min at 95°C, 35–40 cycles of 15 s at 95°C, 30 s at Tm °C, and 30 s at 68°C. All amplifications were performed on an ABI 9700 Thermal Cycler (Applied Biosystems).

TABLE 1 Oligonucleotides for amplification and DNA sequence analysis of exons of canine *CYP2D15*

Exon	Forward primer[a]	Reverse primer[a]	Tm[b]	*N*[c]
1	TCGCCCTGACATATTGACTC	**GGACATCATCTTCCCATCTCC**	55	35
2	**GCTAAGAAGAGGTCTGATCCAGC**	CCGCCTGGGTCCTCATTC	58	40
3–4	**GCGGGAAGGGTGTCAGAG**	GCCTCGATCCTCTTTCCTGG	58	35
5–6	GGATCGAGGCGGACTTAGG	**GGACAAACCAGGCTCAAGGG**	58	35
7	**GTCCCTTGAGCCTGGTTT**	GCACATTTCAGCCTGTCTTC	58	40
8–9	**AGTCCCTTAGCCCTGCCAT**	AGCCACCAAACTGGTTTATTGTAC	55	35

[a]Primers in boldface were used in DNA sequencing reactions.
[b]Tm = annealing temperature of the PCR in °C.
[c]*N* = number of temperature cycles of the PCR.

The primers were degraded with 1U Exonuclease I (Thermo Fisher Scientific) at 37°C for 45 min. The enzyme was inactivated at 75°C for 15 min. DNA sequencing reactions were performed with the fragments and primers indicated in Table 1 using BigDye® Terminator kit v3.1 (Thermo Fisher Scientific) according to the manufacturer's protocol. The products were purified by gel filtration with Sephadex G50 (Sephadex G-50 Superfine; Amersham) on a multiscreen MAHV N45 plate (Millipore Bedford). The products were analyzed on a 3130XL Genetic Analyzer (Applied Biosystems), and the DNA sequencing results were evaluated with SeqMan pro 14 software of the DNASTAR Lasergene package.

The obtained DNA sequences were compared with the reference *CYP2D15* cDNA NM_001003333 and with the reference genome CanFam3.1 using blastn at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The possible effect of polymorphisms on protein function was predicted with the Polyphen-2 tool (http://genetics.bwh.harvard.edu/pph2/). The homologous variations in the human orthologue *CYP2D6* were analyzed (polyphen accession P10635).

3 | RESULTS AND DISCUSSION

3.1 | Exon–intron structure

A transcript of *CYP2D15* that includes the complete coding sequence is annotated in the reference cDNA accession NM_001003333.1. To bridge a gap in the assembly of the gene in CanFam3.1, we designed PCR primers on both sides of the gap in the region of exon 3. Comparison of the sequence of the PCR product from genomic DNA with the cDNA sequence revealed that the gap contains an intron with bordering exon fragments (Figure 1a,c). The DNA sequence analysis also corrected several errors in the annotated DNA sequence of exon 4 that were due to the assembly of a low-quality read in CanFam3.1. The GenBank accession number of this DNA sequence is MT239388.

Remarkably, the DNA sequence of exons 8 and 9 that we determined diverged across its full length from the reference genome sequence. The level of identity between the DNA sequences was only 79%. In contrast, the identity with the reference cDNA sequence of *CYP2D15* was 100% in the coding DNA sequences of the two exons, indicating that the proper genomic fragment, belonging to *CYP2D15*, had been analyzed. The correct exon 8–intron 8–exon 9 DNA sequence is shown in Figure 1b. The GenBank accession number of this DNA sequence is MT239389.

3.2 | Polymorphisms

We selected four breeds with little genetic relationship (Parker et al., 2004) for a comprehensive analysis of polymorphisms in the coding DNA sequence of the gene. Twelve each of bull mastiffs, English cocker spaniels, Rottweiler and border collies were analyzed for variations in the coding DNA sequences. The observed nonsynonymous polymorphisms are listed in Table 2. The polymorphisms in exons 4, 5, and 6 have been described before (Pappas & Katsiabas, 2003; Paulson et al., 1999; Roussel et al., 1998; Sakamoto et al., 1995). The polymorphisms encoded by exon 2 observed in border collies are novel. These two polymorphisms are in complete linkage disequilibrium in the 12 dogs that were analyzed. Both variations are predicted to be benign for protein function. It should be noted that the human orthologue CYP2D6 has Phe at position 112, which corresponds with the alternative allele of the canine protein at position 115. This lack of evolutionary constraint suggests that the residue has limited impact on metabolic activity of the protein.

3.3 | Impact on drug metabolism

The cytochrome P-450 drug-metabolizing enzymes are critical to the efficient elimination of many drugs in clinical practice. Several human CYPs have been shown to exhibit polymorphic expressions, most notably CYP2D6. Polymorphisms in the gene encoding canine CYP2D15 have the potential to impact the metabolism of drugs (Court, 2013). Given the range of clinically important substrates for human CYP2D6, additional work is needed to understand the potential role of canine CYP2D15 in the clearance of drugs commonly used by veterinarians. Of particular, clinical interest is the known CYP2D15 substrates, clomipramine, fluoxetine, metoclopramide, and tramadol. By completing and correcting the annotation of the canine *CYP2D15* gene, we enable surveys of variations of the gene that may affect the efficacy of canine drugs. However, a polymorphism does not necessarily mean that there is a substantial effect on the pharmacokinetics of the drug. The enzyme may still be functional, or other enzymes may also be capable of metabolizing the drug, and a dysfunctional polymorphism simply shunts the drug's metabolism toward these alternate pathways.

The novel nonsynonymous polymorphisms in exon 2 of *CYP2D15* are predicted to be benign for protein function. However, several poor metabolizing variants have been observed in the homologous region of the human orthologue *CYP2D6*. We therefore think experimental evaluation of the effect of these variations is warranted.

Screening for canine CYP polymorphic enzymes will help individualize therapy and thereby increase therapeutic efficacy in veterinary medicine in general and in case of 2D15 specific in the field of veterinary behavioral medicine where fluoxetine and clomipramine are commonly used to treat behavioral conditions in dogs and cats (Kaur, Voith, & Schmidt, 2016; Landsberg, 2001; Overall, 2013; Simpson & Papich, 2003).

(a)

```
   L  F  L  A  R  Y  G  R  A  W  R  E  Q  R  R  F  S  L  S
GGTTGTTCCTGGCGCGCTACGGGCGCGCGTGGCGCGAGCAGCGGCGCTTCTCGCTGTCCA

T  L  R  N  F  G  L  G  R  K  S  L  E  Q  W  V  T  E  E  A
CCCTGCGCAACTTCGGCCTGGGCAGGAAGTCCCTGGAGCAGTGGGTGACCGAGGAGGCCT exon 3

S  C  L  C  A  A  F  A  E  Q  A
CGTGCCTCTGCGCGGCCTTCGCCGAGCAGGCGGgtgagcggcgcggcgcaggtcccgggg

cgcggggatgcgcgggagggggaggcggaggcgagcgcggacccgcccgctgccccgc intron 3

  G  R  P  F  G  P  G  A  L  L  N  K  A  V  S  N  V  I  S  S
agGCCGCCCCTTCGGCCCCGGCGCGCTGCTGAACAAGGCGGTGAGCAACGTGATCTCGTC

  L  T  Y  G  R  R  F  E  Y  D  D  P  R  L  L  Q  L  L  E  L
GCTCACCTACGGGCGCCGCTTCGAGTACGACGACCCGCGGCTGCTCCAGCTGCTGGAGCT exon 4

  T  Q  Q  A  L  K  Q  D  S  G  F  L  R  E
CACCCAGCAGGCGCTGAAGCAGGACTCCGGCTTCCTGCGTGAG
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(b)

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cttctggggccgaggggttattcaaagggtccaggagtgcgccaggcggagtgtgtgccc intron 7
atgcatgtttggtggcaggggcccgggcatcccgtggcccagaccccacccacacaggca

            G  T  T  L  I  T  N  L  S  S  V  L  K  D  E  K
tctcctgccagGGGACGACACTCATCACCAACCTGTCGTCAGTGCTAAAGGACGAGAAGG

V  W  K  K  P  F  R  F  Y  P  E  H  F  L  D  A  Q  G  H  F
TCTGGAAGAAGCCCTTCCGCTTCTACCCCGAGCACTTCCTGGACGCCCAGGGCCACTTCG exon 8

V  K  H  E  A  F  M  P  F  S  A
TCAAGCATGAGGCCTTCATGCCCTTCTCTGCAGgtgcgcgggtgcccggctcgccgaccc
ctccgagggagtcttggaggctggggcccggcgccgggcttactgggcgctccttcccca intron 8

       G  R  R  V  C  L  G  E  P  L  A  R  M  E  L  F  L  F
cccgcagGCCGCCGCGTCTGCCTCGGGGAGCCCCTGGCCCGCATGGAGCTCTTCCTCTTC

 F  T  C  L  L  Q  R  F  S  F  S  V  P  A  G  Q  P  R  P  S
TTCACCTGCCTCCTGCAGCGCTTCAGCTTTTCAGTGCCTGCGGGGCAGCCCCGGCCCAGC exon 9

 D  H  G  V  F  T  F  L  K  V  P  A  P  F  Q  L  C  V  E  P
GACCACGGGGTCTTCACCTTCCTGAAGGTTCCAGCCCCCTTCCAGCTCTGTGTGGAGCCT

 R  *
CGCTAGGGGCAGGAACCACCACCCCCCGCCGCCCGGCTCCTCAGCAGGGGCCCGA
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(c)

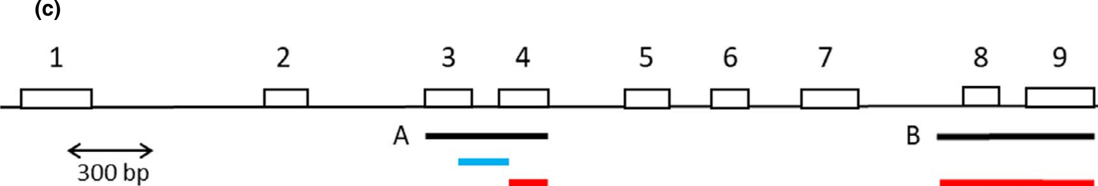

FIGURE 1 Corrections of the annotated canine *CYP2D15* gene. (a) Exon 3, intron 3, and exon 4 DNA sequence of *CYP2D15*. The DNA sequence that is missing from CanFam3.1 is underlined. Discrepancies between exon 4 and the annotated gene are marked with gray. These are probably due to a low-quality trace file (TI 285297625) used for the assembly. The GenBank accession number of this DNA sequence is MT239388. (b) Corrected DNA sequence of the region of exons 8 and 9 of *CYP2D15*. The discrepancy with CanFam3.1 is due to the use of a low-quality trace file (TI 304660157) in the assembly. The GenBank accession number of this DNA sequence is MT239389. The DNA sequences of (a) and (b) are derived from a border collie. The intron sequences are in lower case. The encoded amino acids are placed above the center of the codons. *: stop codon. (c) Structure of the canine *CYP2D15* gene. The position of the DNA sequences given in a) and b) are indicated by the black bars. The blue bar indicates the position of the gap in CanFam3.1 that has been filled and the red bars indicate low quality DNA sequence that have been corrected. The numbered box represent the exons of *CYP2D15* [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Polymorphisms of CYP2D15 in 48 dogs of 4 dog breeds (12 dogs per breed)

Breed	exon	cDNA[a]	Protein[b]	f[c]	dbSNP146
Border collie	2	325A>G	Ile109Val	0.46	rs851583126
Border collie	2	345G>C	Leu115Phe	0.46	rs852145716
Border collie	4	556A>G	Ser186Gly	0.33	–
English cocker spaniel	4	556A>G	Ser186Gly	0.79	–
Rottweiler	4	556A>G	Ser186Gly	0.95	–
Bull mastiff	4	556A>G	Ser186Gly	1	–
All 4 breeds	5	748A>T	Ile250Phe	1	rs852652101
All 4 breeds	6	919A>G	Ile307Val	1	rs851791778

[a]Based on reference cDNA NM_001003333.1, A of startcodon = 1.

[b]Based on reference protein NP_001003333.1.

[c]Frequency of nonreference allele.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

M.A.E.V.H and L.S. conceived of the presented idea. P.L. helped to work out material and methods. L.S. was involved in planning and supervised the work. M.A.M.D. and M.V. sequenced the DNA samples. P.L., M.V., and R.G. aided in interpreting the results. M.A.E.V.H., P.L., and M.A.M.D drafted the manuscript, and P.L. designed the figure and table. All authors discussed the results and commented on the manuscript. All authors have read and approved the final manuscript.

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How to cite this article: van Hagen MAE, Schipper L, Oosterveer-van der Doelen MAM, Vos-Loohuis M, Gehring R, Leegwater PA. Analysis of polymorphisms of canine Cytochrome P 450-CYP2D15. *J vet Pharmacol Therap*. 2020;43:602–607. https://doi.org/10.1111/jvp.12890