Praca oryginalna *Original paper*

Phylogenetic analysis of steroid 21-hydroxylase (CYP21) of the wolf and selected breeds of dogs in connection with the role of this enzyme in the pathogenesis of many diseases in the Canidae family

BARBARA KOSOWSKA, TOMASZ STRZAŁA, KATARZYNA BRZEZIŃSKA, KRZYSZTOF GRABOWSKI, MAGDALENA MOSKA, TADEUSZ DOBOSZ*, ARLETA LEBIODA*

Workshop of Cell Molecular Biology, Department of Genetics, Wrocław University of Environmental and Life Sciences, ul. Kożuchowska 7, 51-631 Wrocław
*Institute of Molecular Techniques, Department of Forensic Medicine, Wroclaw Medical University, ul. M. Curie-Skłodowskiej 52, 50-369 Wrocław

Kosowska B., Strzała T., Brzezińska K., Grabowski K., Moska M., Dobosz T., Lebioda A.

Phylogenetic analysis of steroid 21-hydroxylase (CYP21) of the wolf and selected breeds of dogs in connection with the role of this enzyme in the pathogenesis of many diseases in the Canidae family

Summary

The aim of this study was to determine the nucleotide sequence of the CYP21 gene in the wolf and representatives of five breeds of dogs (selected according to a classification by Parker et al, 2004) in connection with the key role of the product of this gene in the genesis of many diseases in dogs. Nuclear DNA of dogs was obtained from peripheral blood, and the wolf's DNA was isolated from muscle tissue. The amplification of the 21HS gene was carried out in 10 fragments under standardized PCR conditions. The reaction products were sequenced. The sequence of amino acids in the protein was determined on the basis of the nucleotide sequence. The sequences obtained in our study and those retrieved from the GeneBank database were compared with the Mafft program (15) and subjected to phylogenetic analysis with the MrBayes 3.2 program (35). We detected a total of nine SNP mutations in introns and exons. Furthermore, a deletion of two nucleotides, that differentiates the breeds, was detected in the promoter region. Only two differences between the dogs and the wolf were found in SNP: one in an exon and one in an intron. Genetic distance was determined between the selected breeds of dogs and between the wolf and the dogs of each breed. In addition, we estimated the evolutionary distances between amino acid sequences of the dog/wolf and homologous 21HS sequences of eight different vertebrate species obtained from GeneBank. It was shown that, among mammals, the amino acid sequence of the dog/wolf is the most similar to the sequence of the pig, and the least similar to that of the human. The sequences determined in this study may provide a reference point for the research on the CYP21 gene structure and expression in various tissues of dogs for therapeutic purposes.

Keywords: canis lupus familiaris, 21steroid hydroxylase, pathogenesis, phylogenetics, CYP21

Achievements in genetics have dramatically expanded our understanding of the relationship between genetic variation and the development of pathology, explaining the genesis of many diseases (45). A good example is the 21HS gene, which due to its site of expression and described mutations, may be a factor in the development of both severe systemic metabolic diseases and disorders of local character. Steroid 21-hydroxylase (21HS, CYP21, P450c21) belongs to the cytochrome P450protein family. In vertebrates, 21-hydroxylase catalyses the hydroxylation of the 21st carbon atom in steroid hormones. The 21HS gene converts progesterone and its derivative, 17-hydroxyprogesterone, to metabolites: 11-deoxycortisol and deoxycorticosterone (27). Thus, it performs an essential step in the synthesis of the main adrenal hormones: cortisol, corticosterone, and aldosterone. During adrenal steroidogenesis in humans, the most common disturbance is the step catalyzed by 21HS, usually because of a reduction in enzyme activity (1). The

inactivity of 21HS leads to the inhibition of both mineralocorticoid- and glucocorticoid synthesis. This leads to abnormal functioning of the feedback mechanism between cortisol and the adrenocorticotrophic hormone (ACTH). Low concentrations of cortisol stimulate the pituitary to increase the secretion of ACTH. This results in an increased synthesis of progesterone and 17-hydroxyprogesterone. As a result of 21 hydroxylase deficiency, these compounds cannot be converted into 11-deoxycortisol and deoxycorticosterone. This leads to an alternative pathway of androgen metabolism that results in hyperandrogenism (31). As a result, patients have a wide spectrum of symptoms, usually in the form of congenital adrenal hyperplasia (CAH) (38). The human CYP21 gene was sequenced in the 1980s (13, 44), and the mutations described were linked with particular symptoms of CAH. Determining the relationship between the type of mutation and the nature and severity of 21HS deficiency symptoms, proved very useful in the diagnosis of pre- and neonatal human CAH. Symptoms similar to human congenital adrenal hyperplasia have also been found in dogs. The disease is called by various names, but most commonly it is classified as a CAH-like syndrome or Alopecia X (37). Predispositions to the disease are common in northern dog breeds: Alaskan Malamutes, Elkhounds, and Chow Chows, as well as in Pomeranians and Miniature Poodles. Increased susceptibility of specific dog breeds suggests that a genetic factor is involved in the disease. The symptoms include: symmetric alopecia on the trunk, thighs, neck and tail, and the hyperpigmentation of hairless body areas. The results of diagnostic tests often showed high levels of 17-hydroxyprogesterone, as in the cases of CAH in humans (8, 9). So far, recent studies have not pointed to Cyp21 gene mutations as a potential source of the disease in dogs (40). Owing to the non-specificity of symptoms, these cases are believed to have different etiopathogenesis. This theory is supported by the fact that many therapies are effective mostly in single cases and by claims that many different therapies are in opposition to one another (3, 14, 24, 25, 36).

In mammals, 21 hydroxylase activity was shown not only in the adrenal cortex, but also in skin keratinocytes (34, 48), leukocytes (47), cardiomyocytes (18), the central nervous system (46), kidneys (20) and male gonads (4).

In recent years scientists have described a link between the CYP21 gene and carcinogenesis. De novo mutations and changes in CYP21 gene expression have been observed in many human cancers, including breast, colon, lung, esophageal and ovarian cancers. In humans and dogs, in adrenal adenoma cells, characterized by increased secretion of cortisol, scientists noted changes in the expression of genes encoding enzymes involved in steroidogenesis, including 21-HS. This suggests the occurrence of mutations in the gene (10, 48). The administration of this enzyme in the form of natural or synthetic molecules may have potential benefits in prevention and therapy of cancer (33).

Recent studies have also documented the influence of 21HS on the etiology of rare autoimmune disorders. Many allelic variants were identified as associated with a risk of deregulation of both innate and adaptive immune responses (21). It was shown that the etiology of autoimmune Addison's disease has a strong genetic basis in both humans and dogs (26). Addison's disease occurs more often in dogs than in humans; 70% of cases are young and middle-aged females (19, 23). The breeds most susceptible to the disease are Pitbull Terrier, Amstaff, Chihuahua, Cocker Spaniel, Golden Retriever, Lhasa Apso, Schnauzer and Yorkshire Terrier (19, 29). Some cases of Addison's disease have a genetic basis, others are presumably autoimmune, or the causes have not been identified (19, 36, 39). Full symptoms of an autoimmune disease develop only after many months. During this period, serum autoantibodies are formed against the crucial steroidogenic enzyme: 21HS. Patients show a periodic compensation or asymptomatic preclinical condition characterized by increased levels of ACTH and rennin. Subsequently, the patients develop symptoms of adrenal insufficiency. Mitchell et al (26) postulate that in Addison's disease the local steroidogenic failure causes intolerance to own adrenal antigens, which may be a key factor in disease progression. Despite the loss of tolerance, molecules, involved in both the adaptive and innate immune systems, continue to form immunological synapses and are involved in MHC-antigen-TCR signaling. So far, the genes (other than allelic variants of MHC) responsible for the formation of autoantibodies in Addison's disease have not been identified.

The CYP21 gene sequence in the wolf, the direct ancestor of the dog, has not been described. Furthermore, no studies have been conducted on the genetic diversity of the 21HS locus in dogs of various breeds. There is only one known sequence of the canine CYP21 gene, that of the Beagle dog (22, 40). Morphological breed differences in dogs are enormous, but it is unclear whether they are accompanied by variation in the sequence and structure of the 21HS gene in wolves and dogs of various breeds.

This study was devoted to determining the nucleotide and amino acid sequence of steroid-21-hydroxylase of the wolf and several selected dog breeds. Phylogenetic analyses were performed, showing the basic differences in the gene structure and amino acid sequence of the protein 21HS of wolves and dogs against the background of homologous sequences for other vertebrate species. The alignment of the amino acid sequences of the enzyme derived from representatives of selected species made it possible to describe evolutionary changes in the structure of 21HS and show evolutionary distances between the sequences. These results may be useful in developing new therapies in the future.

Material and methods

Dog breeds were selected on the basis of a classification by Parker et al (32), who analyzed the polymorphism of STRs (Short Tandem Repeats) of 85 dog breeds and differentiated Canis *lupus familiaris* species in 4 clusters. The study used one representative of the breed: Lhasa Apso and Siberian Husky (representing cluster I), Bullterrier (cluster II), Bouvier des Flanders (cluster III) and Weimaraner (cluster IV). Samples derived from the European wolf (*Canis lupus lupus*) were obtained from the Mammal Research Institute of the Polish Academy of Sciences in Białowieża. Dog DNA was extracted from peripheral blood with a „QIAamp DNA Blood Mini Kit" (Qiagen). DNA isolation from the wolf's muscle tissue was performed with a „Sherlock AX" (A&A Biotechnology) kit. Then electrophoresis was performed in 1% agarose gel, for qualitative and quantitative evaluation of DNA isolate. The amplification of the CYP21 gene was carried out in 10 fragments. PCR reaction conditions: 96°C × 2 min, (95°C, 1 min, 59°C – 1 min, 72°C, 1 min) × 30 cycles, 72°C – 10 min. The reaction was carried out in a volume of 25 µl of the following components:

- buffer: 2.5 µl,
- glycerol: 2.5 µl/Q-Solution: 5.0 µl,
- dNTP mix: 0.4 µl,
- primers (concentration 25 pmol/µl) 0.5 µl of each,
- genomic DNA: 1 µl,
- Taq polymerase (5 U/µl): 0.2 µl,
- water: up to 25 µl.

Primer sequences were as follows:

1. F 5'gac gga agg act caa agg ag 3'
   R 5' tga cca tgg ctt cct cga tg 3'
2. F 5' ctg ctg ggt ttg acg cag aa 3'
   R 5' ctc acc tca cag aac tcc tg 3'
3. F 5' tac tcg cag aca agc tgg tg 3'
   R 5' gta cta agg tgt cct cct gc 3'
4. F 5' acc tgc gcc atc atc tgt ca 3'
   R 5' ctg cct cag ctg ctt ctc ta 3'
5. F 5' ttc cct ttc tca ggg tga gg 3'
   R 5' tag tcg gtc atg tcc ctc ca 3'
6. F 5' tag aga agc agc tga ggc ag 3'
   R 5' cct aga aca acc cag tgg ag 3'
7. F 5' gga cct ttt cat tgg cgg ca 3'
   R 5' gct ctt agg aga gtc acc tg 3'
8. F 5' tcc cga atc ccg tac aga ga 3'
   R 5' tag aac cag ggt cca gta gc 3'
9. F 5' cac tta gac gag acg gtc tg 3'
   R 5' acg ggg ttt gta cgg gag aa 3'
10. F 5' ctc agc atg cag cct ttc ca 3'
    R 5' gcc ctt cac gga aat gaa gc 3'

All primers had a length of 20 bp and a melting point of 57C. PCR products were purified with a „GenElute ™ PCR Clean-Up Kit" (Sigma) and sequenced twice in both directions. The sequences obtained were aligned with BioEdit v.7.0.0 (12), and on this basis consensus sequences were determined. To construct a phylogenetic tree, sequences obtained in this study and a sequence of the Beagle dog's 21-HS gene (DQ341429) downloaded from Genebank were used. The rooting of the phylogenetic tree was performed with the CYP21 gene sequence of the domestic cat (DQ341429). Sequences were aligned with the Mafft program (15), and the tree was constructed with the MrBayes program (35), using the HKY substitution model suggested by the MrAIC program (30). Ten million MCMC repeats were made with a burn-in equal to 25% of all trees. On the basis of the known nucleotide sequence of the canine 21-HS, the positions of introns and exons were determined. On this basis, the amino acid sequence in 21 hydroxylase of the wolf was determined. Subsequently, canine amino acid sequences of 21-HS were aligned with other orthologus sequences from GeneBank with the T-Coffee program (28). To determine the phylogenetic status of the canine amino acid sequence of 21HS (identical to the seven breeds of dogs and the wolf), a phylogenetic tree was created with the following species: dog [BAB79541.1], human [AAB59440.1] domestic cattle [AAA83247.1], domestic swine [AAM11646.1], house mouse [AAA37114.1], brown rat [AAD05573.1], Japanese eel [BAC76051.1] and domestic sheep, whose sequence was obtained from the PIR database (http://pir.georgetown.edu/) [A43349]. The tree was created using 10 million MCMC replications and a 25% relative burn-in value.

Results

In this study, the nucleotide sequence of the wolf's (CLL) 21HS locus was determined, as well as homologus sequences of five dog breeds: Weimaraner (WW), Lhasa Apso (LA), Bouvier des Flanders (BO), Husky (H) and Bull Terrier (BU). All alleles of the CYP21 gene derived from the selected dog breeds were homozygous in length and consisted of 2460 bp. The total length of all ten exons of the alleles examined consisted of 1479 bp and contained information about the structure of a peptide of 492 amino acids (aa). In the area of the gene (Table 1) a total of nine SNP substitutions were identified: six in exons and three in introns. In addition, one SNP change was found in the 5'-UTR sequence and in the sequence of the 3' end of the promoter. None of the mutations caused changes in the protein sequence. Only two dog breeds: BU and WW, had homozygous 21 HS gene sequences. The most variable DNA profile of the 21HS locus was found in BO (8 SNPs). The total nucleotide variation between all dog breeds was greater than the differences between the dogs and the wolf. All SNP differences in the structure of the CYP21 gene sequence between the dog breeds examined and the wolf consisted of only two SNPs: in exon 1 and intron 2 (Table 1). In addition, at position -78 of the promoter sequence, in the wolf and dogs from the BO and LA breeds, adenine was observed, whereas at the same position in BU and H dogs guanine was detected. Overall, the nucleotide sequence of the CYP21 gene had the same length in the wolf, the 5 dog breeds examined here and in the Beagle dog, whose sequence was downloaded from GenBank. The SNP mutations identified do not cause changes in the aa sequence of the 21HS protein. Thus, aa sequences of Canis lupus familiaris and Canis lupus lupus proved identical.

Tab. 1. SNP location in the 21HS sequence of the domestic dog and the wolf with allele frequency. Symbols used: pos. – position, aa – amino acid, BO – Bouvier, BULL – Bull Terrier, H – Husky, LA – Lhasa apso, WW – Weimaraner, CLL – wolf, BG – Beagle. Synonymous substitutions were marked in red

SNP localization	pos.	allele	codon number	codon letter	aa	individual	Number of individuals with genotype	Allele frequency
Promoter	–78	G/A G/G	–	–	–	LA, BO, CLL H, BULL, BG	3 3	A – 0.25 G – 0.75
Exon 1	108	T/T C/C	36	TGT TGC	Cys Cys	CLL THE OTHERS	1 6	T – 0.14 C – 0.86
Exon 2	364	A/G G/G A/A	92	CCA/CCG CCG CCA	Pro Pro Pro	H, LA BO, BULL, WW, BG CLL	2 4 1	A – 0.29 G – 0.71 –
Intron 2	438	T/T C/C	–	–	–	CLL THE OTHERS	1 6	T – 0.14 C – 0.86
Exon 4	814	C/T C/C T/T	177	TTC/TTT TTC TTT	Phe Phe Phe	BO BULL, H, LA, WW CLL, BG	1 4 2	T – 0.36 C – 0.64 –
Intron 5	1106	C/C T/T	–			LA THE OTHERS	1 6	C – 0.14 T – 0.86
Intron 7	1630	C/T C/C T/T	–	–	–	BO BULL, H, LA, CLL WW, BG	1 4 2	C – 0.64 T – 0.36
Intron 7	1656	G/A A/A G/G	–	–	–	BO LA THE OTHERS	1 1 5	A – 0.21 G – 0.79
Exon 9	2025	C/A A/A C/C	383	ATC/ATA ATA ATC	Ile Ile Ile	BO BULL, BG H, LA, WW, CLL	1 2 4	C – 0.64 A – 0.36
Exon 10	2238	C/T C/C	419	TTC/TTT TTC	Phe Phe	BO THE OTHERS	1 6	T – 0.07 C – 0.93
Exon 10	2391	C/T C/C	470	AGC/AGT AGC	Ser Ser	BO, LA BULL, H, WW, CLL, BG	2 5	T – 0.14 C – 0.86
Exon 10	2433	G/A A/A G/G	484	GGG/GGA GGA GGG	Gly Gly Gly	BO BULL, WW, BG H, LA, CLL	1 3 3	A – 0.5 G – 0.5
UTR	2513	G/A G/G A/A	–	–	–	H BO, LA, BULL, WW CLL	1 4 1	A – 0.25 G – 0.75

While analyzing the total length of the gene (including the promoter region) in dogs and the wolf, we found that the promoter region of both alleles in the wolf was 2 bp longer than one of the alleles of the promoter sequence in H and BU dogs and the sequence of the BE breed downloaded from GenBank. The cause of these differences in the length of the promoter was the presence of a 2-bp deletion at position -79/-80 in one of the alleles of H and BU dogs. The deletion was not observed in BO and LA.

Table 2 shows the interspecific variation of the 21HS gene of dog and wolf sequences against the background of seven orthologous sequences from other vertebrate species. In all species the gene contains 10 exons and 9 introns. The length of the encoding gene is in the range from 2460 bp (the dog and wolf) to 2922 bp (cattle). Polymorphism in the exon length ranges from 1464 bp in mice to 1494 bp in sheep. Table 2 shows interindividual variation in the 21HS gene length.

Tab. 2. Interspecific variation of the Cyp21 gene length and the total length of exons and introns in different species. The Cyp21 gene sequences of the species marked in red were determined in our study. The other seven sequences were obtained from GenBank

Name of the species	Gene length (bp)	Total exons length (bp)	Total introns length (bp)
dog	2460	1479	981
wolf	2460	1479	981
mouse	2554-2629	1464-1473	1081-1165
rat	2681	1483	1198
sheep	–	1494	–
cattle	2922	1491	1431
swine	2539	1479	1060
human	2706-2712	1485-1488	1221-1226
Japanese eel	–	1572	

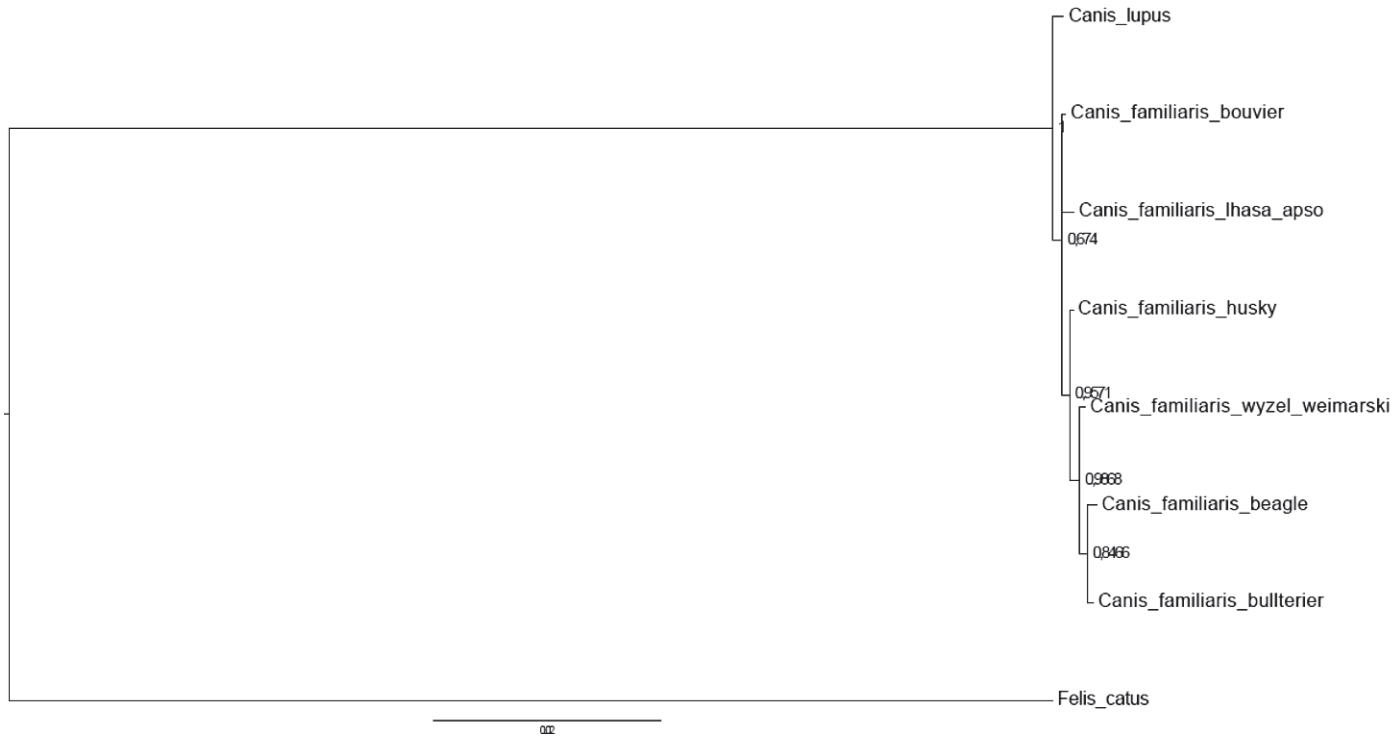

Fig. 1. Phylogenetic tree of seven nucleotide sequences of the 21-hydroxylase steroid gene derived from six breeds of dogs and the wolf. The tree was rooted with the sequence of the domestic cat. Numbers at nodes specify their probability values estimated by the MrBayes program

The phylogenetic tree generated from seven 21HS nucleotide sequences is presented in Fig. 1. The sequences are for the wolf, the 5 dog breeds examined here, and the Beagle (obtained from GenBank). The branches of the tree represent the most likely evolutionary distances estimated on the basis of genetic differences between the gene sequences analyzed in the dog breeds and the wolf. The oldest branch of the evolutionary tree is a clade of the wolf with the node of a significant value. The node closest to the wolf developed in three subclades. Two formed from a sequence of the BO and LA breeds, and the third consists of the nucleotide sequence of the H, WW, BU and BE breeds. In the last subclade, sequences of H and WW are significantly separated from sequences of BU and BE, combined into a single clade, which proved evolutionarily the youngest. This node has no significant value, which means that the layout of the branch may be somewhat different from the one presented.

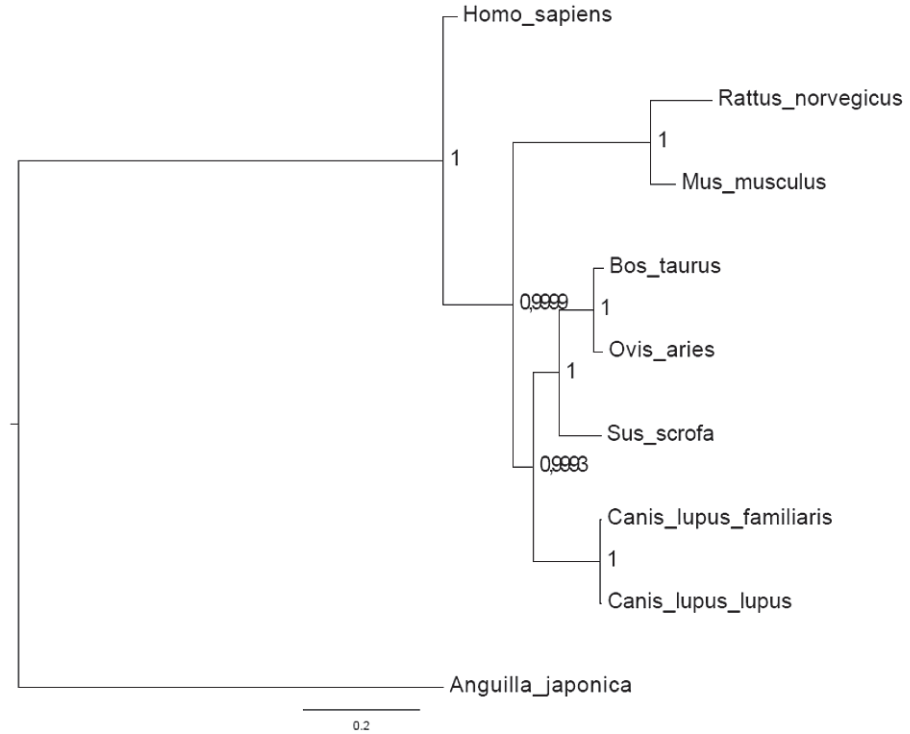

Fig. 2. Phylogenetic tree based on amino acid sequences of the steroid 21-hydroxylase of the wolf/dog and seven species of vertebrates, whose nucleotide sequences are compared in Table 2. Numbers at nodes specify their probability values estimated by the MrBayes program

Fig. 2 shows the phylogenetic tree generated from the 21HS aa sequence of the wolf/dog, and seven species of vertebrates, whose nucleotide sequences are compared in Table 2. The tree topology is compact, and its nodes are highly significant. The tree was rooted with the evolutionarily oldest sequence of the Japanese eel. The aligned 21HS aa sequences of 8 mammalian species and the eel showed significant homology (70-

-85%). Moreover, it was shown that there are absolutely conserved domains for all species tested, including the eel (aa sequence homology between the mammals and the eel was 39-43%). In the clade most different from the other mammals there is an aa sequence of the human. From the significant node closest to the human aa sequence, two clades were developed. The first belongs to rodents. The second was built from a sequence of the pig and, the common clade of cattle and sheep, located nearby. To sum up, the canine 21HS aa sequence is the most similar to the sequence of the pig, and the least similar to the human sequence.

Discussion

In this study nucleotide sequences of the CYP21 gene of dogs and the wolf were determined. The dogs came from four clusters and five breeds: Weimaraner, Lhasa apso, Bouvier, Husky and Bull Terrier. The gene sequences of all the dogs and wolves had the same length of 2460 bp. They differed from each other only by nine synonymous substitutions, a 2-bp deletion in the promoter region of the gene and SNP changes in the sequence of the 3'UTR. The open reading frame in all cases consisted of 1479 bp, and the canine 21HS aa sequence consisted of 492 aa residues. The most significant change detected in the gene sequence was a 2-bp deletion in the promoter region because it highly diversified the breeds in relation to each other and in relation to the wolf. The deletion was detected in H and BU dogs, which proved to be heterozygous, but it was not found either in the wolf or in the LA and BO breeds. This mutation is likely to have been developed later and the wild-type genotype, without the deletion of the promoter, was inherited by the older dog breeds from the wolf.

The canine ancestor split from the common line of Canoidea in the early Eocene, more than 50 million years ago. The divergence of canines began about 10 million years ago (42, 43). The species Canis familiaris is relatively young, having developed about 100 thousand years about (42). The direct and only living ancestor of the domestic dog is the wolf (*Canis lupus*) in its diverse morphological and geographical forms. This is confirmed by phylogenetic analysis performed on the basis of DNA tests, such as cytochrome b gene analysis (43), polymorphism of microsatellite loci (11) and mtDNA (41). The results obtained in this study add to genetic evidence for the thesis that in the history of Canis lupus familiaris speciation has never occurred, and that the wolf and the dog are still one species. Dogs still naturally crossbreed with wolves and give fertile offspring. Differences between dogs and wolves in the 21HS gene sequence were smaller (only two silent SNP changes), than between the representatives of the five most genetically diverse breeds of the domestic dog. Although classified as the same species, they are phenotypically more different than, for example, the wolf and the husky.

Many researchers are particularly interested in the 21HS gene of the dog because of the potential association between mutations detected in the locus of this gene and the canine diseases described in the introduction. In some dog breeds, especially in Pomeranians, symptoms resemble the human form of CAH (37). Researchers have searched for mutations in the gene sequence of the domestic dog that are potentially responsible for the symptoms found in dogs suffering from the disease. Takada et al (40) located several silent SNPs in exons and introns in the sequence of the CYP21 gene of seven dog breeds. Unfortunately, no mutations leading to changes in the 21HS-encoded enzyme were found, so the cause of this disease remains unknown. Interesting in the studies by Takada et al (40) was the hypothesis that guanine at position -78 in the promoter sequence is a component of the AGGTCA motif, probably responsible for the regulation of gene transcription. Takada and his colleagues initially attributed a significant role in the etiology of CAH-LS to a substitution in this position. Eventually, they found no evidence of it. These results seem to exclude the suggestion of Takada et al (40) about the existence of a transcription factor binding site at this position of the described promoter motif. Such motifs are strongly preserved through evolution. Sequence variation within the canine DNA demonstrated in this paper, does not support the suggestion of an important role it would play in the process of transcription. In this study, in the promoter at position -78, only the wolf was homozygous A/A, whereas the dog breeds (LA and BO) were heterozygous A/G. Some species of mammals have 2 copies of the 21 HS gene. These include the human, the gorilla and the chimpanzee (16). The orangutan has 3 copies of the CYP21 gene (17). The rat, an evolutionarily younger relative of the mouse (5), also has two copies of this gene (47). In a duplicated form, the CYP21 gene also occurs in sheep and cattle (2, 6). In all these species, owing to gene duplication, more changes at the DNA level can be expected as a result of sequence rearrangements between the active gene and its own copy, which is either active or inactive, and existing in the form of a pseudogene. The results of the phylogenetic analysis of aa 21HS sequences performed in this study show that canine aa sequence is evolutionarily closest to that of the domestic swine (similarity between the dog and the pig lies in the fact that both species have only one copy of this gene) and the least related to the human sequence. These results suggest that the single canine CYP21 gene sequence should be more stable than those in species with a duplicate of this gene.

The 21HS canine nucleotide sequences determined in this study should be a point of reference for the description of other gene sequences expressed in various tissues of the body. As indicated by the current knowledge, in different tissues the gene product in the form of an amino acid sequence of the CYP21 protein may

have a different structure and properties from those shown in this work, because of the existence of alternative splicing and epigenetic modifications.

References

1. *Barg E., Tokarska M., Wikiera B., Kosowska B.*: Congenital Adrenal Hyperplasia – Advances in Diagnosis. Adv. Clin. Exp. Med. 2003, 12, 507-515.
2. *Cameron P. U., Tabarias H. A., Pulendran B., Robinson W., Dawkins R. L.*: Conservation of the central MHC genome: PFGE mapping and RFLP analysis of complement, HSP70, and TNF genes in the goat. Immunogenetics 1990, 31, 253-264.
3. *Cerundolo R., Lloyd D. H., Vaessen M. M., Mol J. A., Kooistra H. S., Rijnberk A.*: Alopecia in pomeranians and miniature poodles in association with high urinary corticoid: Creatinine ratios and resistance to glucocorticoid feedback. Vet. Record. 2007, 160, 393-397.
4. *Chung B. C., Matteson K. J., Miller W. L.*: Structure of a bovine gene for P-450c21 (steroid 21-hydroxylase) defines a novel cytochrome P-450 gene family. Proc. Natl. Acad. Sci. USA 1986, 4, 211-219.
5. *Corneli P. S.*: Complete Mitochondrial Genomes and Eutherian Evolution. J. Mammal. Evol. 2002, 9, 281-305.
6. *Crawford R. J., Hammond V. E., Connel J. M., Coghlan J. P.*: The structure and activity of two cytochrome P450c21 proteins encoded in the ovine adrenal cortex. J. Biol. Chem. 1992, 267, 16212-16218.
7. *Darriba D., Taboada G. L., Doallo R., Posada D.*: ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 2011, 27, 1164-1165.
8. *Frank L. A.*: Growth hormone-responsive alopecia in dogs. J. Am. Vet. Med. Assoc. 2005, 226, 1494-1497.
9. *Frank L. A., Hnilica K. A., Oliver J. W.*: Adrenal steroid hormone concentrations in dogs with hair cycle arrest (Alopecia X) before and during treatment with melatonin and mitotane. Vet. Dermatol. 2004, 15, 278-284.
10. *Galac S., Kool M. M., Naan E. C., Daminet S., Mol J. A., Kooistra H. S.*: Expression of the ACTH receptor, steroidogenic acute regulatory protein, and steroidogenic enzymes in canine cortisol-secreting adrenocortical tumors. Domest. Anim. Endocrinol. 2010, 39, 259-267.
11. *García-Moreno J., Matocq M. D., Roy M. S., Geffen E., Wayne R. K.*: Relationships and genetic purity of the endangered Mexican wolf based on analysis of microsatellite loci. Conserv. Biol. 1996, 10, 376-389.
12. *Hall T. A.*: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 1999, 41, 95-98.
13. *Higashi Y., Yoshioka H., Yamane M., Gotoh O., Fujii-Kuriyama Y.*: Complete nucleotide sequence of two steroid-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene. Proc. Natl. Acad. Sci. USA 1986, 83, 2841-2845.
14. *Huang H. P., Lien J. H., Chang P. H.*: Effect of Castration on Hair Re-growth in Pomeranians with Hair Cycle Arrest (Alopecia X). JVCS 2009, 1, 17-19.
15. *Katoh K., Toh H.*: Parallelization of the MAFFT multiple sequence alignment program. Bioinformatics 2010, 26, 1899-1900.
16. *Kawaguchi H., Golubic M., Figueroa F., Klein J.*: Organization of the chimpanzee C4-CYP21 region: implications for the evolution of human genes. Eur. J. Immunol. 1990, 20, 739-745.
17. *Kawaguchi H., Klein J.*: Organization of C4 and Cyp21 loci in gorilla and orangutan. Hum. Immunol. 1992, 33, 153-162.
18. *Kayes-Wandover K. M., White P. C.*: Steroidogenic enzyme gene expression in the human heart. J. Clin. Endocrinol. Metab. 2000, 85, 2519-2525.
19. *Klein S. C., Peterson M. E.*: Canine hypoadrenocorticism: part I. Can. Vet. J. 2010, 51, 63-69.
20. *Lajic S., Eidsmo L., Holst M.*: Steroid 21-hydroxylase in the kidney: demonstration of levels of messenger RNA which correlate with the level of activity. J. Steroid Biochem. Mol. Biol. 1995, 52, 181-186.
21. *Lessard C. J., Ice J. A., Adrianto I., Wiley G. B., Kelly J. A., Gaffney P. M., Montgomery C. G., Moser K. L.*: The genomics of autoimmune disease in the era of genome-wide association studies and beyond. Autoimmun. Rev. 2012, 11, 267-275.
22. *Lindblad-Toh K., Wade C. M., Mikkelsen T. S., Karlsson E. K., Jaffe D. B., Kamal M., Clamp M., Chang J. L., Kulbokas E. J. 3rd, Zody M.C.*, et al.: Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 2005, 438, 803-819.
23. *Mausberg E. M., Drögemüller C., Dolf G., Rüfenacht S., Welle M., Leeb T.*: Exclusion of patched homolog 2 (PTCH2) as a candidate gene for alopecia X in Pomeranians and Keeshonden. Vet. Rec. 2008, 163, 121-123.
24. *Mausberg E. M., Drögemüller C., Leeb T., Dolf G., Rüfenacht S., Welle M.*: Evaluation of the CTSL2 gene as a candidate gene for alopecia X in Pomeranians and Keeshonden. Anim. Biotechnol. 2007, 18, 291-296.
25. *Mausberg E. M., Drögemuller C., Rüfenacht S., Welle M., Roosje P., Suter M., Leeb T.*: Inherited alopecia X in Pomeranians. Dtsch. Tierärztl. Wochenschr. 2007, 114, 129-134.
26. *Mitchell A. L., Pearce S. H.*: Autoimmune Addison disease: pathophysiology and genetic complexity. Nat. Rev. Endocrinol. 2012, 8, 306-316.
27. *Nebert D. W., Russell D. W.*: Clinical importance of the cytochromes P450. Lancet 2002, 360, 1155-6112.
28. *Notredame C., Higgins D. G., Heringa J.*: T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 2000, 302, 205-217.
29. Novartis Animal Health.: Addison's Disease: Uncommon or Underdiagnosed? J. Vet. Med. 2003, 50, supplement.
30. *Nylander J.*: MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University 2004.
31. *Pang S., Yang X., Wang M., Tissot R., Nino M., Manaligod J., Bullock L. P., Mason J. I.*: Inherited congenital adrenal hyperplasia in the rabbit: absent cholesterol side-chain cleavage cytochrome P450 gene expression. Endocrinology 1992, 31, 181-186.
32. *Parker H. G., Kim L. V., Sutter N. B., Carlson S., Lorentzen T. D., Malek T. B., Johnson G. S., DeFrance H. B., Ostrander E. A., Kruglyak L.*: Genetic structure of the purebred domestic dog. Science 2004, 304, 1160-1164.
33. *Rajput N., Gautam V., Sahni Y. P., Jain S.*: Cytochrome P450: A Novel Approach In Cancer Therapy. J. Vet. Pharmacol. Tox. 2009, Vol. 8, Issue 1-2/1-8.
34. *Rogoff D., Gomez-Sanchez C. E., Foecking M. F., Wortsman J., Slominski A.*: Steroidogenesis in the human skin: 21-hydroxylation in cultured keratinocytes. J. Steroid. Biochem. Mol. Biol. 2001, 78, 77-81.
35. *Ronquist F., Huelsenbeck J. P.*: MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003, 19, 1572-1574.
36. *Schaer M.*: Acute Adrenocortical Insufficiency. Proc. of 30th World Congress of the World Small Animal Veterinary Association 2005.
37. *Schmeitzel L. P., Lothrop C. D., Rosenkrantz W. S.*: Congenital adrenal hyperplasia-like syndrome. Kirk's Current Veterinary Therapy XII. Philadelphia: Saunders W. B. 1995, 600-604.
38. *Speiser P. W., Dupont B., Rubinstein P., Piazza A., Kastelan A., New M. I.*: High frequency of nonclassical steroid 21-hydroxylase deficiency. Am. J. Hum. Genet. 1985, 37, 650-667.
39. *Stafford D.*: The Great Mimic: Canine Addison's Disease. Vet. Tech. 1999, 20, 490-496.
40. *Takada K., Kitamura H., Takiguchi M., Saito M., Hashimoto A.*: Cloning of canine 21-hydroxylase gene and its polymorphic analysis as a candidate gene for congenital adrenal hyperplasia-like syndrome in Pomeranians. Res. Vet. Sci. 2002, 73, 159-163.
41. *Tsuda K., Kikkawa Y., Yonekawa H., Tanabe Y.*: Extensive interbreeding occurred among multiple matriarchal ancestors during the domestication of dogs: evidence from inter- and intraspecies polymorphisms in the D-loop region of mitochondrial DNA between dogs and wolves. Genes Genet. Syst. 1997, 72, 229-238.
42. *Vila C., Amorim I. R., Leonard J. A., Posada D., Castroviejo J., Petrucci-Fonseca F., Crandall K. A., Ellegren H., Wayne R. K.*: Mitochondrial DNA phylogeography and population history of the grey wolf canis lupus. Mol. Ecol. 1999, 8, 2089-2103.
43. *Wayne R. K.*: Molecular evolution of the dog family. Trends. Genet. 1993, 9, 218-224.
44. *White P. C., New M. I., Dupont B. O.*: Structure of human steroid 21-hydroxylase genes. Proc. Natl. Acad. Sci. USA 1986, 83, 5111-5115.
45. *Wiebolt J., Koeleman B. P., van Haeften T. W.*: Endocrine autoimmune disease: genetics become complex. Eur. J. Clin. Invest. 2010, 40, 1144-1155.
46. *Yu L., Romero D. G., Gomez-Sanchez C. E., Gomez-Sanchez E. P.*: Steroidogenic enzyme gene expression in the human brain. Mol. Cell. Endocrinol. 2002, 190, 9-17.
47. *Zhou M. Y., Vila M. C., Gomez-Sanchez E. P., Gomez-Sanchez C. E.*: Cloning of two alternatively spliced 21-hydroxylase cDNAs from rat adrenal. J. Steroid. Biochem. Mol. Biol. 1997, 62, 277-286.
48. *Ziaja J., Cholewa K., Mazurek U., Cierpka L.*: Molekularne podstawy syntezy aldosteronu i kortyzolu w prawidłowych nadnerczach i w gruczolakach kory nadnerczy. Endokrynol. Pol. 2008, t. 59, nr 4.

Corresponding author: Barbara Kosowska, ul. Kożuchowska 7, 51-631 Wrocław; e-mail: barbara.kosowska@up.wroc.pl