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ORIGINAL PAPER

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IDENTIFICATION OF MUTATIONS OF SELECTED GENES CAUSING RETINAL DEGENERATION IN THE DOMESTIC CAT (*FELIS CATUS*) – *IN SILICO* ANALYSIS

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ABSTRACT

Retinal degenerations are a series of genetically inherited diseases resulting in significant visual impairment and blindness. Among domestic cat breeds, there are degenerations of different courses associated with mutations in *CEP290*, *CRX*, *AIPL1* and *KIF3B* genes. The aim of this study was to design diagnostic tests to identify the mutated alleles. The primers for PCR and restriction enzymes for PCR-RFLP were designed to detect mutations in genes. Mutation in the nucleotide sequence encoding AIPL1 protein causes a change in the protein structure, where a monomer is formed instead of a homodimer. Interactions of CEP290, CRX, AIPL1 and KIF3B proteins with other proteins that play a role in the proper functioning of the retina were observed. The occurring interactions between some of these proteins suggest a possible link between diseases caused by mutations of genes encoding these proteins. In other animal species, co-expression of the analyzed genes with other genes affecting retinal functions was noted.

Key words: domestic cat, retinal atrophy, *CEP290*, *CRX*, *AIPL1*, *KIF3B*

INTRODUCTION

Over the years, a number of retinal disorders occurring in the domestic cat have been distinguished. Retinal abnormalities in a several number of species have been grouped together under the name of Progressive Retinal Atrophy (PRA). The term refers to conditions that lead to blindness. The causes of degeneration (PRA) are mainly genetic. The course of retinal degeneration varies in individuals of different breeds of the same species and even within the same breed.

The autosomal recessively inherited disease is progressive retinal atrophy of late onset (rdAc) in Abyssinian cats [Narfström 1985]. Menotti-Raymond et al. [2007] found that a mutation in the nucleotide sequence of the *CEP290* gene (encoding a 290 kDa centrosomal protein) is responsible for the development of rdAc in the domestic cat. The protein encoded by this gene plays an important role in Intraflagellar Transport (IFT). Functional IFT is crucial for maintaining photoreceptors that in a state of continuous regeneration [Pazour and Rosenbaum 2002, Badano et al. 2006]. In the domestic cat, the *CEP290* gene is located in chromosome B4. A single nucleotide polymorphism (SNP) occurs in intron 50: IVS50 + 9T>G. This results in the formation of a canonically strong cut site and the consecutive insertion of four base pairs. Insertion of additional base pairs induces a change in the reading frame in the mRNA and the formation of a stop codon leading to premature termination of the protein [Menotti-Raymond et al. 2007].

Early onset cone and rod dysplasia (Rdy) in Abyssinian cats is inherited in an autosomal dominant manner. Menotti-Raymond et al. [2010] described a mutation in the Cone-Rod Homeobox (*CRX*) gene that was cosegregated with Rdy. The *CRX* gene reacts with numerous transcriptional regulators that are specific to photoreceptors. Non-functional *CRX* causes impaired differentiation of photoreceptor cells and successively leads to

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Table 1. Primers obtained with Primer3web software

Gene	Primer forward (F)	Primer reverse (R)	Tm [°C]	GC [%]	PCR product size [bp]
CEP290	AGCAACAGAGAGGGAGCAAA	CAGTTGCCCCAAGAGTTCAT	F: 59.23 R:58.08	50 (F/R)	152
CRX	GGGCATCTCAGACTCCTACA	AAATAGGAGCTCGGAGACCC	F: 58.22 R: 58.58	55 (F/R)	240
AIPL1	AGACGTGGAGCCTGAGTAAC	AAATAGGAGCTCGGAGACCC	F: 59.11 R: 59.02	55 (F/R)	210
KIF3B	AATGTCATCTCTGCCCTGGT	GGCATCTTTAGGGTCCTCGT	F: 58.71 R: 59.17	50 (F) 55 (R)	231

[https://primer3.org]

Table 2. Restriction enzymes obtained with NEBcutter

Gene	Restriction enzyme	Recognized sequence and cutting site	Wild type gene		Mutated gene		Heterozygote
			Number of cutting sites	Predicted number and size of bands in 6% polyacrylamide gel	Number of cutting sites	Predicted number and size of bands in 6% polyacrylamide gel	Predicted number and size of bands in 6% polyacrylamide gel
CEP290	*Mluc*I	/AATT	1	2 – 91 bp and 61 bp	0	1 – 152 bp	3 – 152 bp, 91 bp and 61 bp
CRX	*BsiE*I	CGRY/CG	1	2 – 141 bp and 99 bp	0	1 – 240 bp	3 – 240 bp, 141 bp and 99 bp
AIPL1	*Sau96*I	G/GNCC	1	2 – 130 bp and 80 bp	0	1 – 210 bp	3 – 210 bp, 130 bp and 80 bp
KIF3B	*Mwo*I	GCNNNNN/NNGC	2	3 – 112 bp, 66 bp and 53 bp	1	2 – 165 bp and 66 bp	4 – 165 bp, 112 bp, 66 bp (two fragments seen as one band) and 53 bp

[https://nc3.neb.com/NEBcutter]

their degeneration. In the domestic cat, the *CRX* gene is located in chromosome E2. The researchers noticed a deletion of 546th cytosine in exon 4 of this gene. The mutation produces a STOP codon too early, resulting in the protein's premature termination.

In Persian cats the disease has an early onset. Lyons et al. [2016] conducted analyses focusing on a gene encoding Aryl-Hydrocarbon Interacting Protein-Like 1 (*AIPL1*), which has been shown to be associated in the domestic cat with retinal degeneration. Researchers identified a mutation in the coding sequence (CDS) in this gene – substitution of 577th cytosine to thymine, resulting in a change of arginine to a STOP codon and loss of nearly 40% of the protein. *AIPL1* is essential for the proper functioning of the enzyme phosphodiesterase 6 (PDE6), which is crucial in the normal function of the cones and rods. The degeneration caused by a mutation in this gene is referred to as Leber congenital amaurosis (LCA) [Lyons et al. 2016].

The progressive retinal degeneration seen in Bengal cats is a disease of early onset. Cogné et al. [2020] conducted an association study of the genome of Bengal cats and identified a gene associated with PRA. This gene was the gene encoding Kinesin Family Member 3B (*KIF3B*) protein located in the domestic cat on chromosome A3. *KIF3B* segregates with disease, and in 8-week-old individuals with the *KIF3B*-/- mutation. Incorrect localization of rhodopsin in the inner segments of the photoreceptors and consequent functional and structural degeneration of the photoreceptors was evident. The mutation in the sequence of this gene is a point mutation, where guanine is converted to adenine. This results in a change of alanine to threonine due to a shift in the reading frame [Cogné et al. 2020].

The aim of the study was to identify *in silico* mutations within selected genes determining retinal degeneration in the domestic cat (*Felis catus*), to identify cat breeds at risk, and to propose breed-appropriate molecular diagnostic tests.

MATERIAL AND METHODS

Nucleotide sequences of genes: *CEP290*, *CRX*, *AIPL1* and *KIF3B* were obtained from the Ensembl V105 database [www.ensembl.org] as: ENSFCAG000028019, ENSFCAG000046705, ENSFCAG00000023174 and ENSFCAG000031881, respectively. Amino acid sequences were downloaded from the GenBank V245 database [www.ncbi.nlm.nih.gov/genbank]. The numbers of the collected amino acid sequences of the proteins were: CEP290 – NP_001095128.1, XP_023112011.2, XP_023112012.2, XP_023112013.2; CRX – XP_044901347.1; AIPL1 – XP_044900551.1, XP_044900552.1, XP_023099626.1, XP_023099627.2,

XP_044900553.1, XP_044900554.1; KIF3B – XP_006929918.1.

To achieve the objectives the following methodological steps have been taken:

1. Creation of mutant nucleotide sequences and amino acid sequences altered by mutations in the genes encoding the amino acid sequences

   Based on the previously mentioned publications, mutant nucleotide sequences and altered protein sequence as a consequence of mutations in the genes were created from extracted wild-type sequences.
2. Comparison of the nucleotide sequences of the genes

   The wild-type sequences of the genes were compared with the created mutant sequences using BLAST program [blast.ncbi.nlm.nih.gov]. Protein sequences encoded by wild nucleotide sequences were compared with amino acid sequences encoded by genes with mutation by COBALT program [www.ncbi.nlm.nih.gov/tools/cobalt].
3. Primer design

   Using the Primer3web V4.1.0. program [Untergasser et al. 2012, Koressaar and Remm 2007, primer3.org] primers were designed. For this purpose, nucleotide sequences of exon 50 and intron 50 of the *CEP290* gene, exon 4 of the *CRX* gene, CDS of the *AIPL1* gene, and exon 1 of the *KIF3B* gene were used. In each case, the site where the mutation may occur was marked according to the instructions provided on the program website. This guaranteed that primers yielding a PCR product with a possible mutation site were obtained.
4. Restriction enzyme selection and gel design

   The selection of suitable restriction enzymes for PCR-RFLP (Restriction Fragment Length Polymorphism) was done using the NEBcutter V3.0.15 program [Vincze et al. 2003; nc3.neb.com/NEBcutter]. Specific restriction enzymes were searched in the program, recognizing a specific sequence and cutting the nucleotide sequence of the PCR product obtained from previously designed primers. Restriction enzymes were selected for each PCR product to distinguish the wild-type sequence from the sequence with the mutation.
5. Spatial models of proteins

   An attempt was made to build spatial models of CEP290, CRX, AIPL1, and KIF3B proteins using the SWISS-MODEL program [Waterhouse et al. 2018, swissmodel.expasy.org] and to compare possible models obtained from amino acid sequences encoded by wild-type nucleotide sequences with models obtained from amino acid sequences formed from mutated nt sequences in FATCAT V2.0 program [fatcat.godziklab.org].
6. Protein interaction analysis

   Functional associations and interactions of CEP290, CRX, AIPL1, and KIF3B proteins with other proteins were examined using the STRING V11.5 database [string-db.org]. This program reports known and predicted linkages between proteins.

RESULTS AND DISCUSSION

Table 1. contains primers obtained with Primer3web software. Table 2. lists the restriction enzymes selected with the NEBcutter program, as well as the number of cutting sites and the predicted number and size of the bands in a 6% polyacryloamide gel.

Spatial models

All templates for CEP290 matched by the SWISS-MODEL program [swissmodel.expasy.org] were characterized by proteins other than CEP290. The highest sequence similarity, at 30.19%, had a model based on template 6I5j.2.A. The model was built on the basis of the amino acid sequence (XP_023112011.2.) encoded by a wild-type nucleotide sequence.

The amino acid sequence of CRX (XP_044901347.1) encoded by the wild-type nucleotide sequence and the aa sequence encoded by the mutated nucleotide sequence were matched by the SWISS-MODEL program [swissmodel.expasy.org] to the 2dms.1.A template, the homeotic protein OTX2. Comparison of the models in the FATCAT program [fatcat.godziklab.org] showed no differences between the protein model derived from the nucleotide sequence of wild-type and mutant.

For AIPL1 sequences numbered XP_044900551.1, XP_044900552.1, XP_023099627.2, and XP_04490055-4.1, the SWISS-MODEL program [swissmodel.expasy.org] matched the same template for aa sequences encoded by nt wild-type sequences as for aa sequences encoded by nt mutant sequences. Whereas for sequences numbered XP_023099626.1 and XP_044900553.1 the templates matched by the program differed. For the aa sequences encoded by nt wild-type sequences, the matched template was 5v35.1.A with 89.38% sequence similarity and with 91.43% sequence similarity, respectively. The program predicted these protein isoforms as homodimers (Fig. 1 and 2). The 5u9k.1.A template was matched to the aa sequences encoded by the mutant nucleotide sequence, with sequence similarity of 89.38% and 91.43%, respectively. The protein isoforms encoded by the mutated nucleotide sequence were predicted to be monomers (Fig. 1 and 2).

The SWISS-MODEL program matched the KIF3B protein sequence (XP_006929918.1) encoded by the nt wild-type sequence and the KIF3B protein sequence (XP_006929918.1) encoded by the nt mutated sequence

Fig. 1. Models of the AIPL1 protein, sequence no. XP_023099626.1. (A) model of the protein formed from the wild-type nucleotide sequence; (B) model of the protein formed from the mutated nucleotide sequence [https://swissmodel.expasy.org/]

Fig. 2. Models of the AIPL1 protein, sequence no. XP_044900553.1. (A) model of the protein formed from the wild-type nucleotide sequence; (B) model of the protein formed from the mutated nucleotide sequence [https://swissmodel.expasy.org/]

to the 3b6u.1.A template, the structure of the KIF3B protein. The sequence similarity for the protein encoded by the wild nucleotide sequence was 99.44%, and for the protein encoded by the nt sequence containing the mutation was 99.15%. Comparison of the obtained models using the FATCAT program showed no major differences except for the difference of one amino acid (Fig. 3).

Protein interactions

One of the predicted proteins linked to the CEP290 protein is the Retinitis Pigmentosa GTPase Regulator (RPGR) (score: 0.997) (Fig. 4). Mutation in the protein's coding sequence is the most common cause of X-linked retinitis pigmentosa. Rachel et al. [2012] used mouse models for the interaction of CEP290 and RPGR and retinal degeneration was observed in all of them. Interactions with the Calmodulin Binding Motif Containing Protein 1 (IQCB1) (score: 0.949) were also predicted (Fig. 4). This protein also forms cilia [Sang et al. 2011]. A mutation in the *IQCB1* gene has been linked to early-onset retinal degeneration in the black-footed cat (*Felis nigripes*). It was also predicted to be associated with Kinesin Family Member 3A (KIF3A) protein (Fig. 4). The prediction score was 0.771. This protein enters into a heterotrimeric complex together with KIF3B or KIF3C and one non-motor unit. A mutation in the *KIF3B* subunit has been linked to retinal degeneration in Bengal cats [Cogné et al. 2020].

Among others, the CRX protein was predicted to be associated with Retina And Anterior Neural Fold Homeobox 2 (RAX2) (Fig. 5). The prediction of this association was 0.980. Mutations in the *RAX2* gene have been linked to autosomal recessive retinitis pigmentosa in humans [Van de Sompele et al. 2019]. Other proteins associated with CRX are rhodopsin (RHO) and photoreceptor-specific nuclear receptor (NR2E3) subfamily 2, group E, member 3 (Fig. 5). The predicted binding to NR2E3 was estimated at 0.785, and to RHO at 0.744. Rhodopsin is located in the rods and is involved in reactions to convert photons into nerve impulses [Hargrave 2001]. The NR2E3, on the other hand, is crucial in the development of photoreceptors [Haider et al. 2009]. This protein was also predicted to be associ-

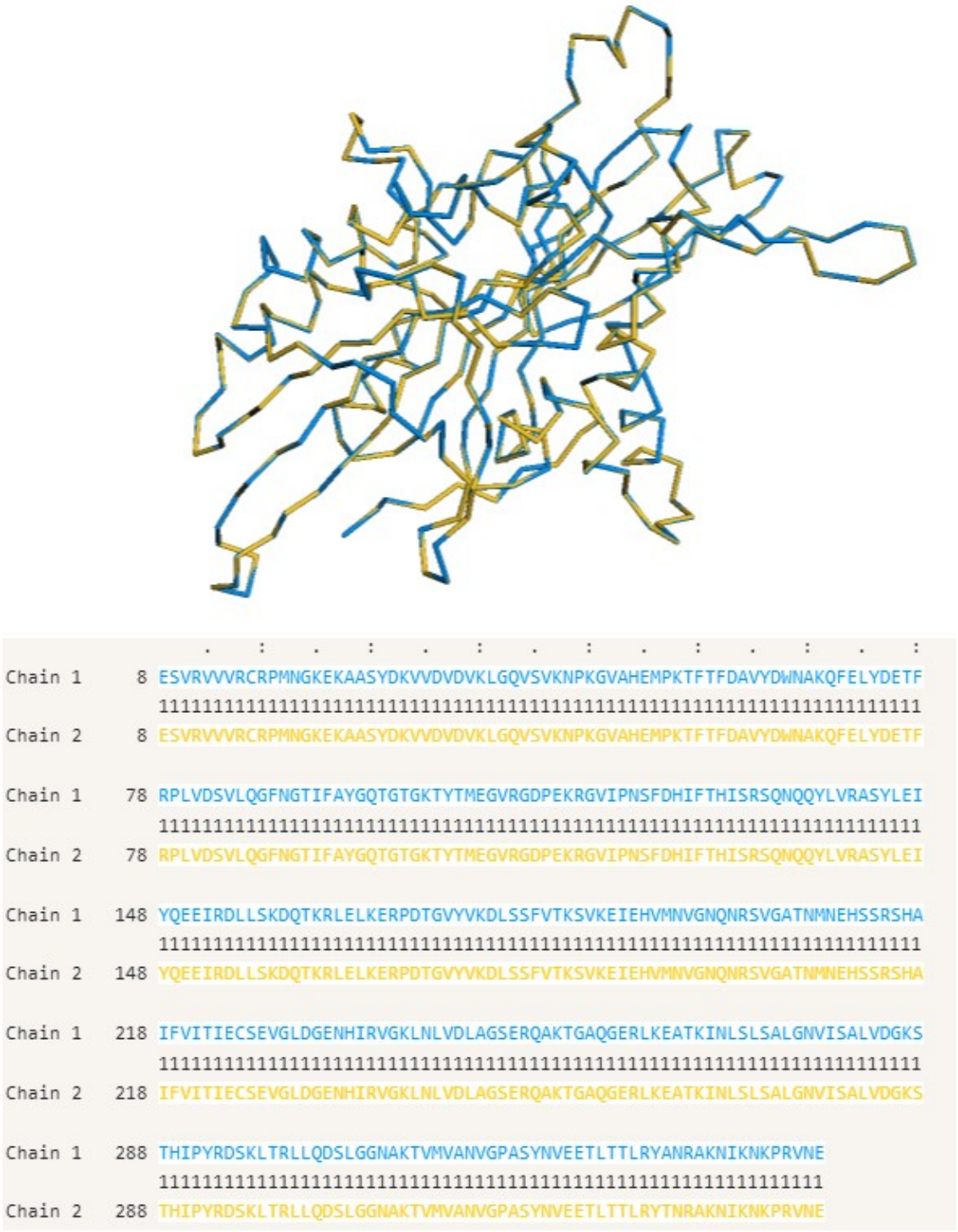

Fig. 3. Comparison of spatial models of the KIF3B protein encoded by the wild-type nucleotide sequence (blue) with the protein encoded by the mutated nucleotide sequence (yellow)

ated with the AIPL1 (score: 0.712) and CEP290 (score: 0.677) (Fig. 5).

One of the proteins linked to AIPL1 was the Negative regulator of ubiquitin-like proteins 1 (NUB1) (Fig. 6). The prediction score was 0.916. The NUB1 expression was observed in developing cones and rods, and in the mature retina it is localized in rods [Kanaya et al. 2004]. The AIPL1 protein was also predicted to be linked to the homeotic protein of the cones and rods (CRX) (Fig. 6).

Mutation in *CRX* results in cone and rod dysplasia (Rdy). The predictive score of this association was 0.712.

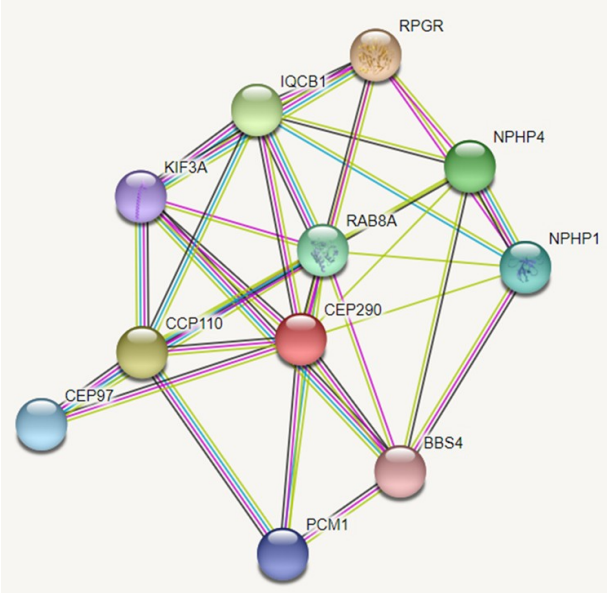

Fig. 4. Predictive analysis of the strength of the association of the CEP290 protein with other proteins (performed with the STRING v. 11.5)

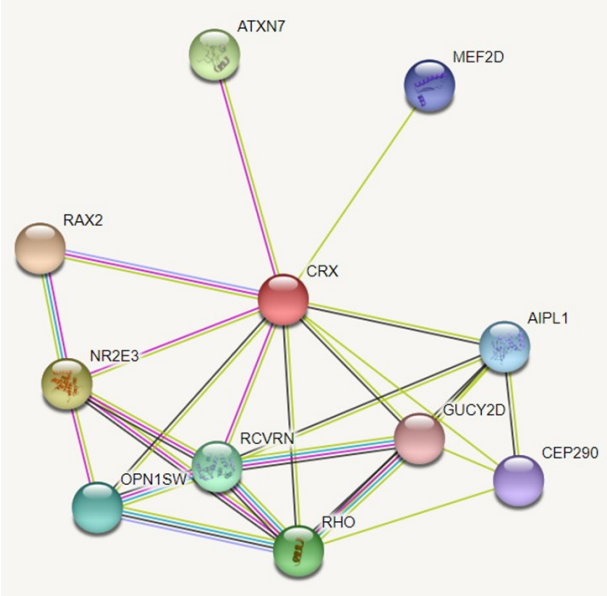

Fig. 5. Predictive analysis of the strength of the association of the CRX protein with other proteins (performed with the STRING v. 11.5)

KIF3B was predicted to be associated with KIF3A and Kinesin Associated Protein 3 (KIFAP3) (Fig. 7). These predictions were scored at 0.992 for KIFAP3 and 0.975 for KIF3A. The KIF3A and KIF3B proteins, together with a non-motile subunit, namely KIFAP3, form a heterotrimeric complex that drives kinesin-2 [Cogné et al. 2020]. Linkage was also observed with the Intraflagellar Transport Protein (IFT) (Fig. 7). It has been shown that mutations in genes encoding IFT proteins can cause retinal degeneration in mice [Mykytyn et al. 2004, Nishimura et al. 2004]. The association KIF3B was noted additionally with the RPGR protein (Fig. 7), whose association was also seen with the CEP290 protein (Fig. 4).

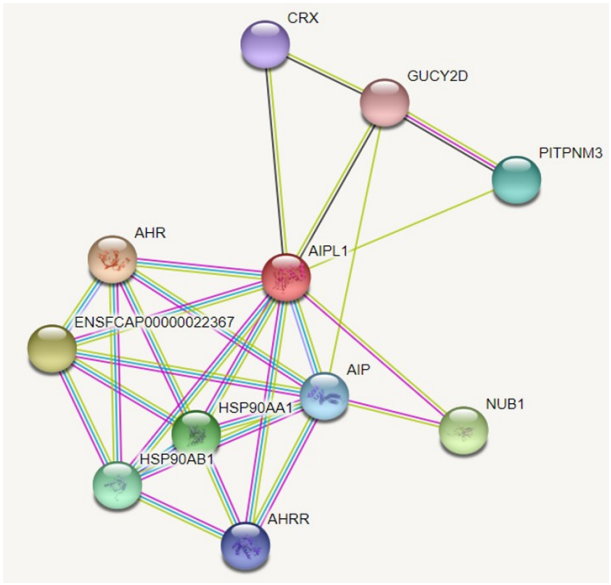

Fig. 6. Predictive analysis of the strength of the association of the AIPL1 protein with other proteins (performed with the STRING v. 11.5)

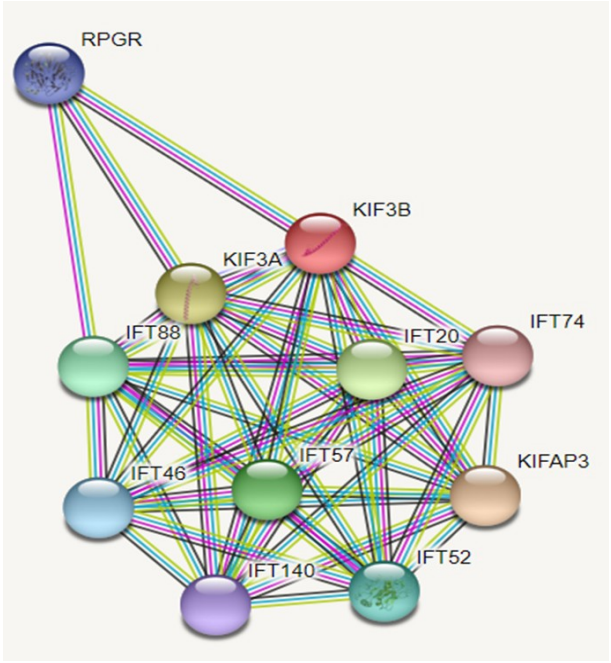

Fig. 7. Predictive analysis of the strength of the association of the KIF3B protein with other proteins (performed with the STRING v. 11.5)

No co-expression of *CEP290*, *CRX*, *KIF3B* and *AIPL1* genes with other genes was observed in the domestic cat. However, co-expression of these genes with other genes has been observed in other species, such as humans, the brown rat and the zebrafish.

CONCLUSIONS

Designed primers for amplification in the PCR reaction of fragments of selected genes and selected, depending on the mutation in a given gene, restriction enzymes can be used in molecular diagnostic tests PCR-RFLP. With such tests, it will be possible to quickly obtain information about the genotype of a particular individual and undertake appropriate treatment. Information about the disease or carrier of a particular individual will enable breeders to make the right breeding decision. It is recommended to carry out molecular diagnostic tests, especially in the case of such domestic cat breeds as Abyssinian, Somali, Siamese, Persian, and Bengal cats, due to the disease-causing gene mutations detected in their genotypes. It was found that all the analyzed proteins, CEP290, CRX, AIPL1, and KIF3B, have connections with other proteins whose functions are related to the proper functioning of the retina. Mutation and consequent malfunction of one protein can impair related proteins' function. Co-expression of *CEP290*, *CRX*, *AIPL1* and *KIF3B* genes has been observed in many other species, which provides a rationale for further co-expression studies in the domestic cat and may suggest more mutations in the genes underlying a particular degeneration.

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IDENTYFIKACJA MUTACJI WYBRANYCH GENÓW POWODUJĄCYCH ZWYRODNIENIE SIATKÓWKI U KOTA DOMOWEGO (*FELIS CATUS*) – ANALIZA *IN SILICO*

STRESZCZENIE

Zwyrodnienia siatkówki to szereg chorób dziedziczonych genetycznie skutkujących znacznym upośledzeniem wzroku i ślepotą. Wśród ras kota domowego wyróżnia się zwyrodnienia o różnym przebiegu związane z mutacjami genów: *CEP290*, *CRX*, *AIPL1* oraz *KIF3B*. Celem pracy było zaprojektowanie testów diagnostycznych pozwalających na identyfikację nosicieli zmutowanych alleli. Startery do PCR i enzymy restrykcyjne do PCR-RFLP zostały zaprojektowane w celu wykrycia mutacji w genach. Mutacja w sekwencji nukleotydowej kodującej białko AIPL1 powoduje zmianę w strukturze białka, gdzie zamiast homodimeru powstaje monomer. Zaobserwowano interakcje białek CEP290, CRX, AIPL1 i KIF3B z innymi białkami odgrywającymi rolę w prawidłowym funkcjonowaniu siatkówki. Występujące interakcje pomiędzy niektórymi z tych białek sugerują możliwy związek pomiędzy chorobami wywołanymi mutacjami genów kodujących te białka. U innych gatunków zwierząt odnotowano koekspresję analizowanych genów z innymi genami wpływającymi na funkcjonowanie siatkówki.

Słowa kluczowe: kot domowy, zwyrodnienie siatkówki, *CEP290*, *CRX*, *AIPL1*, *KIF3B*

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