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CREATING UNIVERSAL PSMD2 (RPN1) PRIMERS FOR A RANGE OF MODEL ORGANISMS AND *HOMO SAPIENS*

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Abstract: Protein degradation plays a key role in many cellular processes, degrading misfolded or abnormal proteins and thereby controlling cell proliferation, DNA repair, and stress response. For this reason, the ways of eliminating such proteins in the context of various human diseases are being actively investigated. The list of human diseases that involve protein degradation pathways includes a wide range of cancers. *PSMD2* or *Rpn1* (26S proteasome subunit, non-ATPase 2) is actively involved in the functioning of the ubiquitin-proteasome system. Expression of *PSMD2* has been found to be upregulated in cancer cells and now it is actively studied as a potential therapeutic target and prognostic marker for several types of cancer. To explore a drug target, it is often necessary to use several model organisms at different stages of research. The goal of our study is to search for conserved *PSMD2* sequences and create oligonucleotide sequences (primers) that would be suitable for conducting polymerase chain reaction (PCR) in a wide range of model organisms and humans. Gene sequences were obtained from the *NCBI Nucleotide database*. Next, we designed oligonucleotide sequences and optimized their parameters. The following candidate sequences were found: 5'-CTGGACATCATGGAGCCCAA-3' and 5'-CCACCATCCACATCCCACAG-3' for sense and antisense strands of *PSMD2*. The next step was to perform *in silico PCR* using an *Online primer designing tool*, which revealed that the PCR product was 266 nucleotides size in five organisms, including human. The PCR product was aligned and phylogenetic analysis was performed. Among organisms examined, the nucleotide sequence of golden hamster *Mesocricetus auratus* was found to be evolutionarily closest to the *PSMD2* sequence in humans, and the sequence of *Drosophila melanogaster* was the most distant. The results of the study can be used for the screening of *PSMD2* expression in different organisms. The approach of finding primers, which match genes of several organisms, can be applied for other genes that encode proteins with neighboring methionine, tryptophan, aspartic and glutamic acids, asparagine, glutamine, cysteine, histidine, lysine, tyrosine, and phenylalanine residues in their amino acid sequences.

Keywords: *PSMD2*, *Rpn1*, primer, sequence, oncogene, protein, nucleotide, model organism, conserved regions.

1. INTRODUCTION

The ubiquitin-proteasome system (UPS) plays a key role in protein degradation in cells. Ubiquitin is a small protein that covalently binds to lysine residues of a target protein. Ubiquitin serves a tag, which is recognized by the proteasome system (Johnson, 2015; Ikeda & Dikic, 2008). The proteasome system performs proteolytic degradation of the ubiquitinated protein. Mammalian cells contain 26S proteasome. It belongs to multisubunit proteases and is localized to cytosol and nucleus (Marshall & Vierstra, 2019).

Proteasomes have been found to be conserved structures. The proteasome-like heat shock protein (hslV protease) was found in bacteria (Valas & Bourne). Archaea (Gille et al., 2003),

budding yeast (Wehmer et al., 2017), protists (Zou et al., 2000), plants (Brukhin et all, 2005), worms (Papaevgeniou & Chondrogianni, 2014), molluscs (Castellanos et al., 2014), insects (Hölzl et al., 2000) and vertebrates (Luker et al., 2003; Gogliettino et al., 2017) contain conserved 20S proteasome core particle.

The 26S proteasome is functionally divided into three parts: two 19S regulatory particles (RP) and one 20S core particle (CP) (Fig. 1). The RP is responsible for recognition of ubiquitin tag and preparation of target proteins for degradation, whereas the CP carries out proteolysis (Marshall & Vierstra, 2019). The RP is composed of base and lid subcomplexes (Fig. 1). The complex also includes α- and β-rings, which are parts of CP. Each of them consists of 7 subunits responsible for proteasome formation (Liu et al., 2023).

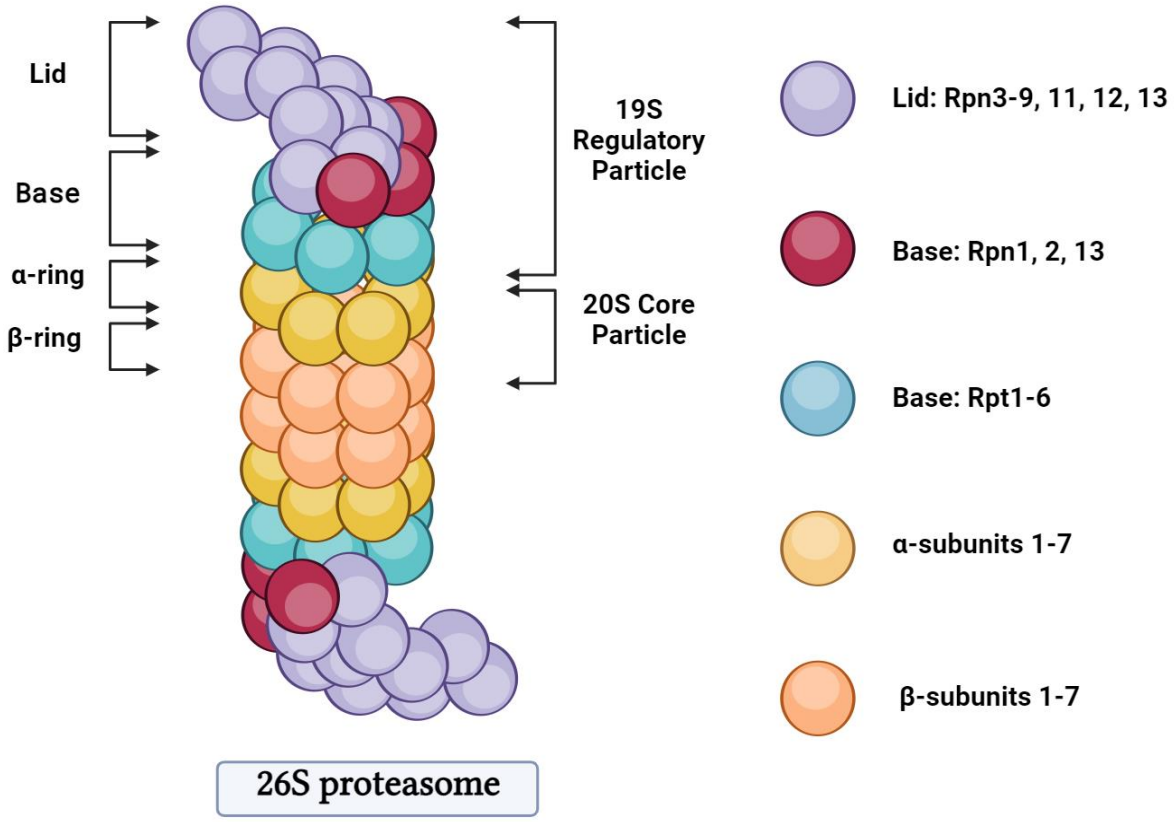

Fig. 1. Schematic representation of 26S proteasome structure. The diagram shows the location of the RP, CP, their parts and subunits. The image created in Biorender.

The base includes three non-ATPase subunits−*Rpn1*, *Rpn2*, and *Rpn13*. They are involved in site-specific binding of proteasome to ubiquitin residues of ubiquitinated proteins (Bard et al., 2018).

The researchers are interested in the *Psmd2* (*Rpn1*) subunit because it is capable of recognizing polyubiquitinated proteins (Mao, 2021). Also, *Rpn1* is necessary for proteasome assembly (Boughton et al., 2021).

It has been established that UPS is responsible for regulating cell apoptosis and cell proliferation. (Almond & Cohen, 2002; Shi et al., 2016). Nowadays, 26S proteasome is also considered to be a target for anti-cancer drugs. It was found that overexpression of *PSMD2* promotes growth of cancer cells (Liu et al., 2023). A number of oncogenic mutations is associated with genes responsible for the ubiquitination and proteolytic degradation of proteins that regulate cell cycle. Efficacy of proteasome inhibitors, such as bortezomib, in suppression of some types of

cancer is evidence of the key role of protein degradation in oncogenesis (Mani & Gelmann, 2005). It was found that knockdown of *PSMD2* led to inhibition of cancer cell growth (Matsuyama et al., 2011). This proves that *PSMD2* can be used to screen for cancer and that research on this gene is relevant today. Moreover, homologs of *PSMD2* are present in many organisms, including those that are widely used as models to study molecular mechanisms of organism's development and cell division (Bertile, 2023; Ankeny & Leonelli, 2011).

For this reason, we set the goal of searching conserved regions in *PSMD2* sequence and identifying a set of primers that allowed us to identify *PSMD2* and synthesize a polymerase chain reaction (PCR) product of the same length in a wide range of organisms. The selected primers would reduce the time required to create new primers for each model animal and, in turn, reduce the cost of PCR analysis.

2. Materials and Methods

2.2. Primers creating and design

To search for specific nucleotide and amino acid sequences of different organisms, we used BLAST (Basic Local Alignment Search Tool), an online tool from the *National Center for Biotechnology Information* (*NCBI*) at the National Institutes of Health (Bethesda, MD, USA) (Altschul et al., 1990; Sayers et al., 2022). To find and design primers we used the *Primer-BLAST tool* (Ye et al., 2012). The search was performed on the tool's website https://www.ncbi.nlm.nih.gov/tools/primer-blast/ using complementary DNA data. The physical parameters of the obtained primers were checked using *Primer3Plus* online tool (https://www.primer3plus.com/index.html).

2.3. Alignment and visualization

Messenger RNA (mRNA) of *PSMD2* gene and amino acid sequence of *Psmd2* (*Rpn1*) protein, and sequences of PCR primers were examined and aligned in bioinformatics software *Geneious Prime* (Biomatters Ltd., Auckland, New Zealand). We have used following parameters: alignment type – global alignment with free end gaps, cost matrix – 65% similarity (5.0-4.0).

2.4. *Phylogenetic Comparative Analysis*

To perform phylogenetic analysis of *PSMD2* mRNA sequences we used the *Geneious Prime* software, using following parameters: genetic distance model – a DNA substitution Hasegawa-Kishino-Yano model, and the neighbor-joining method for creating clusters and building the phylogenetic tree.

3. Results and Discussion

3.1. Primer design

Despite amino acid sequences of homologous proteins of many organisms contain identical regions, it does not mean that the same identity will be found between the corresponding nucleotide sequences. This is caused by redundancy of the genetic code. In other words, many amino acids can be coded by the several types of codons. However, those conservative regions of amino acid sequence that contain methionine and tryptophan residues (encoded by the unique triplets AUG and UGG), or, to a lesser extent, aspartic and glutamic acids, asparagine, glutamine, histidine, lysine, cysteine, tyrosine, and phenylalanine (encoded by two triplet combinations), may be encoded by identical nucleotide sequences. This enables designing 'universal' PCR primers that would allow identifying the nucleotide sequences of homologous genes in taxonomically distant organisms.

We have aligned nucleotide sequences of complementary DNA that encode *Psmd2* in different model organisms - fruit fly *Drosophila melanogaster*, domestic mouse *Mus musculus*, and human *Homo sapiens* (Fig. 1). We have found identical regions in *PSMD2* cDNA in all these organisms. We have used these regions as potential places where PCR primers could be located. The characteristics of these regions and a possibility to identify correct nucleotide sequence, using these regions for PCR priming, where checked by Primer-BLAST. We have identified two primers that are suitable for our purpose: 5'-CTGGACATCATGGAGCCCAA-3' (Forward *PSMD2*) and 5'-CCACCATCCACATCCCACAG-3' (Reverse *PSMD2*).

To perform a high-quality PCR reaction, it is necessary to check the properties of the oligonucleotide sequence obtained. For oligonucleotide sequences used for PCR, it is desirable to have size of 16 to 28 nucleotides. Shorter nucleotide sequences may anneal to non-specific regions in the template, whereas long sequences may form secondary structures and would also be more expensive. The desirable melting point of the PCR oligonucleotide sequences should be between 50 and 62 °C. Too low melting temperatures favor weak attachment of primers to the template at the extension stage, which is usually performed at 60-72 °C, the optimum for thermostable DNA polymerase. Another important parameter is the ratio of GC pairs, which also affects melting temperature and, thus, attachment of primers to the template. The optimal percentage is 45-55%. ΔG is the Gibbs free energy, which is calculated as the maximum value for the last five nucleotides of the 3'-end of the primer. This is the energy that should be spend for breaking hydrogen bonds at the 3'-end of a primer. The higher the value of ΔG, the smaller probability of mispriming. Primer's 3'-end is considered stable when ΔG is at least 2 kcal/mol (Anigbogu, 2020; Yanget al., 2006). One can also need to take into account the difference between annealing temperatures of sense and antisense primers: it is desirable to not exceed 2 °C (Sharma, 2021).

Our structures are 20 bp in size, thermodynamically stable, and have an appropriate percentage of GC pairs in the sequences for primers (Table 1).

Table 1

Parameters of forward (sense) and reverse (antisense) PSMD2 PCR primers

Primer direction	Primer sequencing	Length	Tm:	GC:	3' Stability (ΔG):
Forward	*CTGGACATCATGGAGCCCAA*	20 bp	59.7 °C	55.0 %	4.1 kcal/mol
Reverse	*CCACCATCCACATCCCACAG*	20 bp	60.4 °C	60.0 %	3.7 kcal/mol

We have identified 99 organisms, in whom *PSMD2* could be identified using above PCR primers. Among them, four model organisms were identified that can potentially be used to study expression of *PSMD2* in different conditions (Table 2).

Table 2

Model organisms for which the obtained PSMD2 primers are suitable

Phylum	Species	Common name	NCBI identifier	Name of gene	Isoform of gene
Arthropoda	*Drosophila melanogaster*	Fruit fly	NM_140901.3	*Rpn1*	-
Chordata	*Mesocricetus auratus*	Golden hamster or Syrian hamster	XM_040751066.1	*PSMD2*	-
	Rattus norvegicus/rattus	Black/Brown rat	NM_001031639.1	*PSMD2*	-
	Mus musculus	House mouse	NM_134101.2, XM_030249039.1	*PSMD2*	Transcript variant 1 and X1
	Homo sapiens	Human	NM_002808.5, NM_001278708.2, NM_001278709.2	*PSMD2*	Transcript variant 1, 2, 3

Studies of *PSMD2* in the established organisms can potentially be used in such fields as immunology, pathology, drug discovery, and so forth, and a wide range of models could be interesting for various studies.

The mRNA sequence, which corresponds to our forward *PSMD2* primer, and the amino acid sequence encoded by this sequence is shown in *Fig 2*.

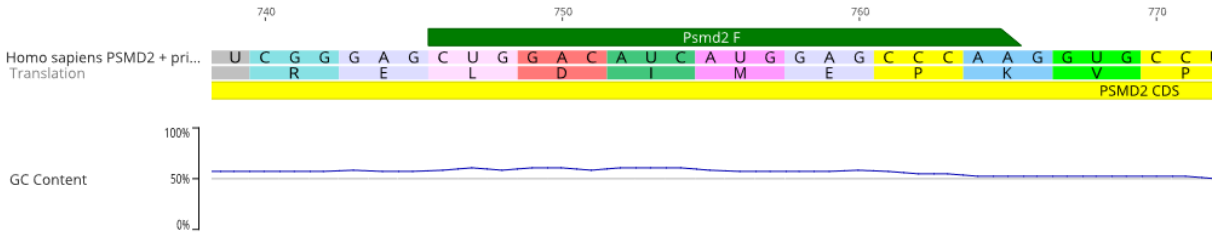

Fig. 2. Nucleotide sequence of mRNA, which is covered by the forward PSMD2 primer, and corresponding amino acid sequence. Location of the primer is shown in green. Percentage of GC pairs is shown as rolling average (the blue line). Yellow line designates the coding region of the gene. Numbers show nucleotide count in the PSMD2 gene of H. sapiens.

As we mentioned above, the identical nucleotide sequences should correspond to amino acid sequences that contain either of the following amino acids: encoded by unique codons – methionine (M) and tryptophan (W), or encoded by two codons – aspartic acid (D), glutamic acid (E), asparagine (N), glutamine (Q), histidine (H), lysine (K), cysteine (C), tyrosine (Y), and phenylalanine (P). Indeed, we can find these amino acids in the amino acid sequence, which is encoded by the nucleotide sequence covered by the forward *PSMD2* primer. In particular, 11 out of 20 nucleotides (55%) encode D, M, E, and K (Fig. 2). However, it is surprising that a part of the nucleotide sequence, covered by the forward primer, codes for the redundantly coded amino acids – leucine, isoleucine, and proline.

Testing the propensity of the primers to form loops showed that the forward *PSMD2* primer can form several possible structures. This is due to the self-complementary regions in positions 2-4 (TGG) and 17-19 (CCA) (Fig. 3A). However, the loop begins to gradually change its shape and position with temperature (Fig. 3B) and already at 35-37 °C the noticed secondary structure is broken (Fig. 3C). Since PCR includes such stages as denaturation (94-96 °C), annealing (45-60 °C), and extension (60-72 °C), the formed loop would be unwound the annealing temperature (Shahzad et al., 2020). This means that during PCR, the primer will fully perform its function and have correct annealing.

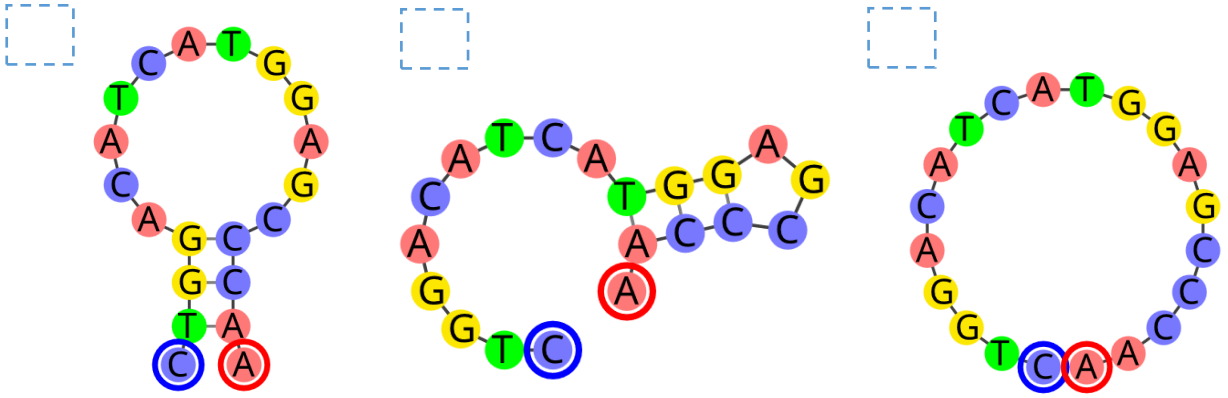

Fig. 3. Possible secondary structures that can be formed by the forward PSMD2 primer at A) 20 °C, B) 30 °C, and C) 35-37°C and above. Blue and red frames indicate the first and last nucleotide of the sequence, respectively.

The probability of forming secondary structures, such as loops, by the reverse primer was found to be negligible. The characteristics of the primer are shown on the *Fig. 4*.

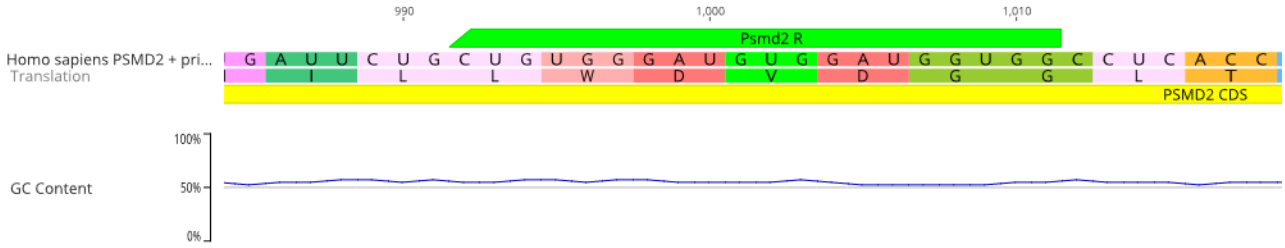

Fig. 4. Nucleotide sequence of mRNA, which is covered by the reverse PSMD2 primer, and corresponding amino acid sequence. Annotations as in the Fig 1.

Similar to the sequence covered by the forward primer, the nucleotide sequence, covered by the reverse primer, codes for the uniquely encoded amino acids, such as tryptophan (Fig. 4). It also codes for two residues of aspartic acid. However, the largest part of the nucleotide sequence, covered by the reverse primer, codes for the amino acids that could be coded by three or more codons, namely valine, leucine, and glycineThe resulting PCR product has a size of 266 nucleotides when tested by *PCR in silico* (Fig. 5). They correspond to approximately 89 amino acids. The length of the product sequence was the same in all organisms tested.

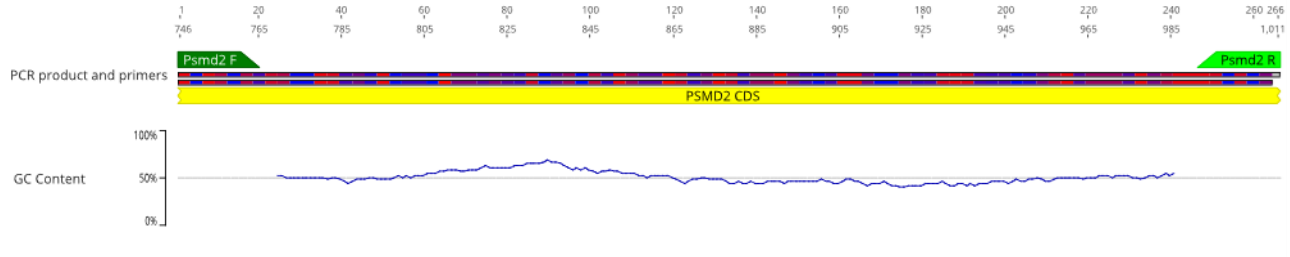

Fig. 5. In silico PCR product of Homo sapiens (variant 3) with selected primers (green). First line denotes nucleotide sequence, the second one denotes amino acid sequence and the yellow line designates the coding region of the gene. Blue, red, purple, and maroon sections indicate nucleotide triplets and amino acids according to their hydrophilicity. The bottom panel shows a diagram of the GC pair content (percentage) in the PCR product.

The position of the amplifiable fragment was different, depending on the organism: *Homo sapiens* – from nucleotide 1035 to 1300 (variant 1), from 757 to 1022 (variant 2), from 746 to 1011 (variant 3); *Mus Musculus* – from 1438 to 1703 (variant X1), from 1024 to 1339 (variant 1); *Mesocricetus auratus* – from 1050 to 1315, *Rattus norvegicus* – from 1014 to 1279, *Rattus rattus* – from 1054 to 1319, and *Drosophila melanogaster* – from 1137 to 1402.

3.2. Alignment PSDM2 (Rpn 1)

We have aligned putative products that could be obtained in PCR that uses DNA or mRNA from different organisms and the pair of 'universal' *PSMD2* primers (Fig. 6).

Fig. 6. Multiple alignment of conservative parts of PSMD2 (Rpn1) PCR products that could be amplified by the designed primers in Homo sapiens (human), Rattus norvegicus (black rat) and Rattus rattus (brown rat), Mesocricetus auratus (golden hamster), and Drosophila melanogaster (fruit fly).

3.3. Evolutionary relatedness of PSDM2 (Rpn 1) in model organisms

To build the phylogenetic tree, we used cDNA sequences of *PSMD2* belonged to various organisms. The list of the organisms includes *Danio rerio* (zebrafish), *Drosophila melanogaster* (fruit

fly), *Gallus gallus* (chicken), *Xenopus laevis* (African clawed frog), *Mesocricetus auratus* (golden hamster), *Mus musculus* (domestic mouse), *Rattus norvegicus* (black rat), *Rattus rattus* (brown rat), *Heterocephalus glaber* (naked mole-rat), and *Oryctolagus cuniculus* (rabbit) (*Fig. 7*).

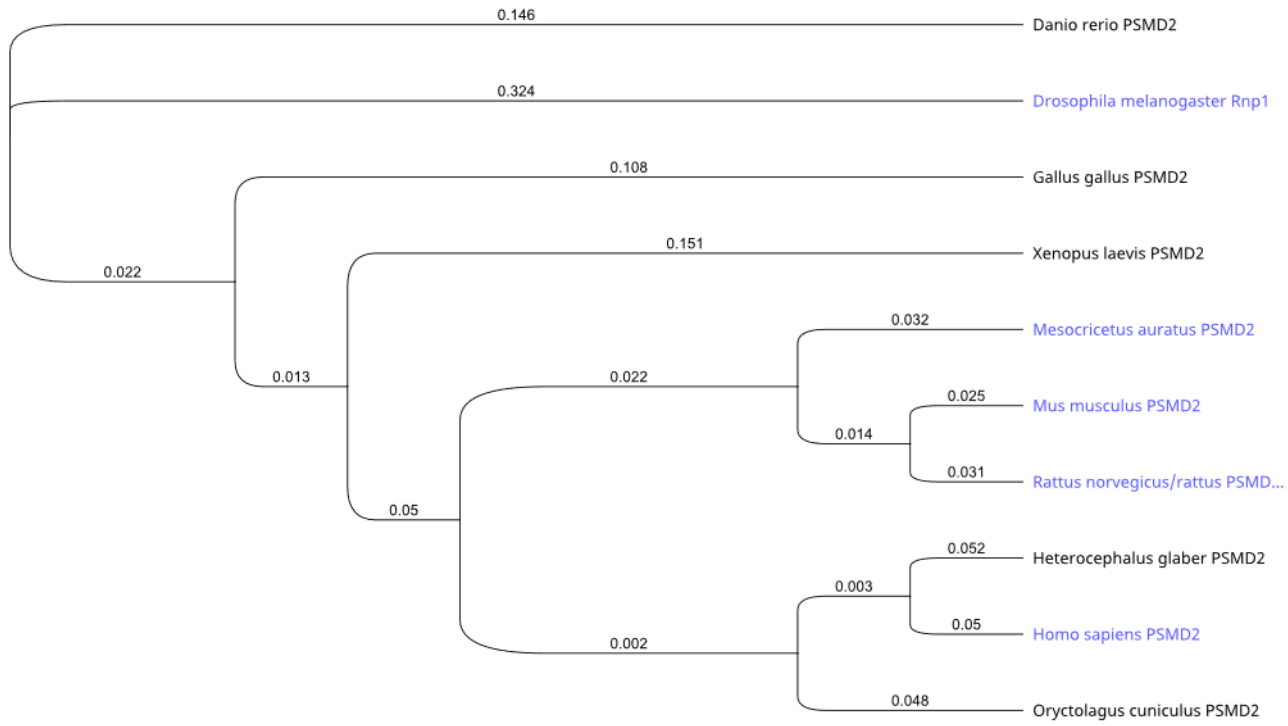

Fig. 7. Phylogenetic tree of the PSMD2 (Rpn1) gene. Species whom PSMD2 could be detected in by the 'universal' primers are highlighted in blue.

The results of the analysis show that *Homo sapiens* and *Heterocephalus glaber* lie within the same node and there is the smallest evolutionary distance between them that indicates the greatest sequence similarity between these two species. Rabbit (*Oryctolagus cuniculus*) represents a separate branch. However, the naked mole-rat and the rabbit are not among the organisms in whom *PSMD2* could be detected by the 'universal' primers.

The cluster that includes *Mesocricetus auratus*, *Mus musculus*, and *Rattus norvegicus, Rattus rattus* is also close to humans. This is because all the species, which are listed in this cluster, are mammals. *PSMD2* sequence of golden hamster is closer to that of human compared to *PSMD2* sequences of rats and the mouse. *PSMD2* sequences of *Xenopus laevis* and *Gallus gallus* are more evolutionary distant from the human's sequences than the sequences from mammals. However, all vertebrates belong to the same branch.

The phylogenetic tree also has two separate branches−*Danio rerio* and *Drosophila melanogaster*. They have more pronounced nucleotide differences compared to humans, as evidenced by the alignment of the fruit fly's *Rpn1* above (Fig. 6). This is explained by the fact that the fruit fly belongs to arthropods, and zebrafish belongs to fish. However, fish belong to vertebrates, it is evolutionarily closer to humans compared to *Drosophila melanogaster*.

4. Conclusions

This study confirms that the *Psmd2* (*Rpn1*) protein has conserved regions, as well as nucleotide sequence that encodes this protein, and that the same primers can be designed for different model organisms and human. This makes designed *PSMD2* primers universal tools that allow

simplifying and reducing the cost of PCR, as well as conducting studies on several models depending on the specificity of the research.

The regions of the nucleotide sequence that are covered by the 'universal' primers correspond to the amino acid sequence, which contains at least one amino acid encoded by a unique triplet. Such amino acid sequence also contains amino acids that could be encoded by two variants of triplets. However, our case also shows that identical regions of nucleotide sequence can code for the amino acids, which correspond to three or more variants of triplets in the genetic code. Likely, the identity of such regions also depends on the phylogenetic relationships between different organisms, including codon usage preference. Nevertheless, the same approach could be used for the search of 'universal' primers for the genes that encode other important proteins, especially those, rich in methionine and tryptophan residues, and, to a lesser extent, in cysteine, aspartic and glutamic acids, asparagine, glutamine, lysine, histidine, tyrosine, and phenylalanine. Nevertheless, codon usage preference should be taken into account.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Додон Д., Господарьов *Д.* Створення універсальних праймерів *PSMD2* (*Rpn1*) для ряду модельних організмів та *Homo sapiens*. *Журнал Прикарпатського університету імені Василя Стефаника*, 10 (2023), 34-44.

Деградація білків відіграє ключову роль у багатьох клітинних процесах, руйнуючи неправильно згорнуті або аномальні білки і таким чином контролюючи проліферацію клітин, репарацію ДНК та реакцію на стрес. З цієї причини активно досліджуються способи елімінації таких білків у контексті різних захворювань людини. До переліку захворювань людини, в яких задіяні шляхи деградації білків, входить широкий спектр онкологічних захворювань. Psmd2 або Rpn1 (26S субодиниця протеасоми, не-АТФаза 2) бере активну участь у функціонуванні убіквітин-протеасомної системи. Було виявлено, що експресія *PSMD2* регулюється в ракових клітинах, і зараз він активно вивчається як потенційна терапевтична мішень і прогностичний маркер для декількох типів раку. Для вивчення мішені ліків часто необхідно використовувати кілька модельних організмів на різних етапах дослідження. Метою нашого дослідження є пошук консервативних послідовностей *PSMD2* та створення олігонуклеотидних послідовностей (праймерів), придатних для проведення полімеразної ланцюгової реакції (ПЛР) у широкому спектрі модельних організмів та людини. Послідовності генів були отримані з бази даних *NCBI Nucleotide*. Далі ми сконструювали олігонуклеотидні послідовності та оптимізували їхні параметри. Було знайдено такі послідовності-кандидати: 5'-CTGGACATCATGGAGCCCAA-3' та 5'-CCACCATCCACATCCCACAG-3' для смислового та антисмислового ланцюгів PSMD2. Наступним кроком було проведення *ПЛР in silico* з використанням *Online primer designing tool*, який показав, що розмір продукту ПЛР у п'яти організмів, включаючи людину, становить 266 нуклеотидів. ПЛР-продукт було вирівняно та проведено філогенетичний аналіз. Серед досліджених організмів виявилося, що нуклеотидна послідовність золотистого хом'яка *Mesocricetus auratus* еволюційно найближча до послідовності *PSMD2* у людини, а послідовність *Drosophila melanogaster*−найвіддаленіша. Результати дослідження можуть бути використані для скринінгу експресії *PSMD2* в різних організмах. Підхід пошуку праймерів, які відповідають генам кількох організмів, може бути застосований і для інших генів, які кодують білки, що мають у своїй амінокислотній послідовності сусідні залишки метіоніну, триптофану, аспарагінової та глутамінової кислот, аспарагіну, глутаміну, цистеїну, гістидину, лізину, тирозину та фенілаланіну.

Ключові слова: *PSMD2*, *Rpn1*, праймер, послідовність, онкоген, білок, нуклеотид, модельний організм, консервативні ділянки.