



팔딱이 지렁이(*Perionyx excavatus*) *DDX3* 유전자의 동정 및 특성

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Identification and characteristics of *DDX3* gene in the earthworm, *Perionyx excavatus*

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ABSTRACT

Helicases are known to be a proteins that use the chemical energy of NTP binding and hydrolyze to separate the complementary strands of double-stranded nucleic acids to single-stranded nucleic acids. They participate in various cellular metabolism in many organisms. DEAD-box proteins are ATP-dependent RNA helicase that participate in all biochemical steps involving RNA. DEAD-box3 (*DDX3*) gene is belonging to the DEAD-box family and plays an important role in germ cell development in many organisms including not only vertebrate, but also invertebrate during asexual and sexual reproduction and participates in stem cell differentiation during regeneration.

In this study, in order to identify and characterize *DDX3* gene in the earthworm, *Perionyx excavatus* having a powerful regeneration capacity, total RNA was isolated from adult head containing clitellum. Full length of *DDX3* gene from *P. excavatus*, *Pe-DDX3*, was identified by RT-PCR using the total RNA from head as a template.

Pe-DDX3 encoded a putative protein of 607 amino acids and it also has the nine conserved motifs of DEAD-box family, which is characteristic of DEAD-box protein family. It was confirmed that *Pe-DDX3* has the nine conserved motifs by the comparison of entire amino acids sequence of *Pe-DDX3* with other species of different taxa. Phylogenetic analysis revealed that *Pe-DDX3* belongs to a *DDX3* (PL10) subgroup of DEAD-box protein family. And it displayed a

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high homology with PL10a, b from *P. dumerilii*.

Keywords : earthworm, *Peionyx excavatus*, DEAD-box, conserved motif

초 록

Helicase는 NTP 결합의 화학적 에너지를 이용하여 이중가닥의 DNA와 RNA를 단일가닥으로 분해하여 다양한 생체반응에 기여하는 단백질로 알려져 있으며, 이 중 DEAD-box의 단백질은 주로 RNA와 관련된 대부분의 생화학적 반응에 작용하는 ATP 의존성 helicase로 알려져 있다. 또한 이 단백질 부류에 속하는 DEAD-box3 (DDX3) gene은 척추동물뿐만 아니라 무척추동물에서의 유성 생식과 무성 생식에서 생식세포 발달 및 재생과정 중 줄기세포 분화에 중요한 역할을 하는 인자로 알려져 있다.

이에 본 연구는 강한 재생능력을 가진 것으로 알려져 있는 팔딱이 지렁이(*Perionyx excavatus*)에서 DDX3 gene을 동정하고 그 발현양상을 알아보고자 환대를 포함하는 성체 지렁이의 두부를 절단하여 total RNA를 추출하고, 이를 주형으로 RT-PCR을 수행하여 full length의 DDX3 gene인 Pe-DDX3를 검출하였다.

Pe-DDX3는 607개 아미노산 서열로 이루어져 있으며, DEAD-box 단백질 그룹 내에서 특이적으로 보존되어 있는 9개의 motif가 존재하고 있다. 다른 분류군에 속하는 동물들과의 multiple alignment를 통해 서열 내에 보존되어 있는 아미노산 서열을 확인할 수 있었으며, 아미노산 차원에서의 계통수 분석을 통해 DDX3 (PL10) 하부그룹에 속하는 것을 알 수 있었으며, 또한, 같은 그룹에 속하는 동물 중 *P. dumerilii*의 PL10a, b 단백질과 가장 가까운 유연관계를 확인 할 수 있었다.

핵심용어: 지렁이, 팔딱이, DEAD-box, 보존부위

1. Introduction

Understanding the cellular metabolism underlying growth, reproduction, regeneration and evolution have been studied in various and complex field of researches. Especially, these studies have been revealed the importance of helicases involved in soma and germ cell production during past 30 years.

Helicase protein was first discovered in *Escherichia coli* in 1976¹⁾. Since then RNA and DNA helicases with diverse functions have been found in all organisms.

RNA helicases are enzymes that can separate double-stranded nucleic acids in a NTP-dependent manner and are essential in all aspects of RNA metabolism. Especially, they participate in all biochemical steps involving RNA including transcription, splicing, transport, translation, decay and ribosome biogenesis²⁾.

In 1989, Gorbalenya *et al.*³⁾ defined a group of NTPases and showed that they had several common sequence elements. Based on this analysis, RNA helicases can divide in to some subfamilies, DEAD-box and DExD/H box. DEAD and DExD/H represent the one letter code of the amino acids constituting motif II,

the signature motif, of the helicase domain. These very closely-related families can be distinguished by variations within their conserved motifs.

DEAD-box proteins are defined through the conservation of multiple different motifs including the D-E-A-D (Asp-Glu-Ala-Asp) motif for which was named^{4),5)}. Other subfamilies, DExD/H helicases, share eight conserved sequence motifs, whereas the DEAD-box helicase subgroup has an additional ninth conserved sequence motif.

DDX3, the highly conserved subfamily of the DEAD-box RNA helicase family, plays important roles in RNA metabolism. DDX3X and DDX3Y, the two human paralogous genes of this subfamily of proteins, have orthologous candidates in a diverse range of eukaryotes, from yeast and plants to animals. While DDX3Y, which is essential for normal spermatogenesis, is translated only in the testes, DDX3X protein is ubiquitously expressed, involved in RNA transcription, RNA splicing, mRNA transport, translation initiation and cell cycle regulation. Studies of recent years have revealed that DDX3X participates in HIV and hepatitis C viral infections, and in hepatocellular carcinoma, a complication of hepatitis B and hepatitis C infections⁶⁾. In the urochordates (*i. e.*, *Botryllus schlosseri*) and in diverse invertebrate phyla (represented by model organisms such as: *Drosophila*, *Hydra*, *Planaria*), DDX3 proteins (termed also PL10) seem to be the ancestral forms of both mammalian DDX3X and DDX3Y proteins and are involved in developmental pathways, highly expressed in adult undifferentiated soma and germ cells and in some adult and embryo's differentiating

tissues⁷⁾. But the mechanistic and functional knowledge of DDX3 proteins is limited⁸⁾.

There are many identified DDX3 protein in organisms. But in earthworm, no DDX3 proteins has been reported. In this study, the *DDX3* gene from the earthworm, *P. excavatus*, was cloned and characterized by PCR and base pair sequencing.

2. Materials and Methods

2.1. Cultivation

Perionyx excavatus, which is used for this study, is a member of phylum Annelid, superclass Clitellata, class Oligochaeta, order Opisthopora, suborder Lumbricina, superfamily Megascolecoidea, family Megascolecidae.

They were reared in plastic culture box which the temperature was controlled at 23±2°C, and distilled water was supplied daily to maintain relative humidity of 80%. Culture soil for earthworm was mixed with compost and peat moss (5:1, v/v). Before use, every earthworm were cultivated on filter paper moistened with distilled water for at least 48h to induce purging of the intestine.

2.2. Total RNA extraction

Total RNA of each regenerated tissues was extracted using TRI-reagent (Sigma-Aldrich, USA). The sample of 100 mg with 1 ml TRI-reagent was homogenized by Glass-Taflon homogenizer (Wheaton, USA). Homogenized sample was incubated at room temperature for 5 min, then 200 µl of chloroform was added and mixed well by vortex. It was incubated at room temperature for 7 min followed by centrifuged at 12,000 g, 4°C for 15

min. Upper phase, which contains RNA, was transferred to a new diethyl pyrocarbonate (DEPC)-treated microtube and same volume of isopropanol was added to the sample. It was incubated at -20°C for 10 min, and centrifuged at 12,000 g, 4°C for 10 min. After centrifugation, supernatant was discarded and the pellet washed with 1 ml of DEPC-treated 75% ethanol. The pellet sample (RNA) was air-dried for few min to remove ethanol completely. Dried RNA was solubilized in DEPC-treated water and was heated for 10 min at 55°C. The amount of RNA samples were verified by spectrophotometric analysis at A₂₆₀ and stored at -80°C before use.

2.3. Identification of the full *DDX3* gene from *P. excavatus*.

2.3.1. Primer design for intact *DDX3* gene.

The earthworm *DDX3* sequence was revealed at the onset of the study by Kim⁹⁾, but the sequence was reported as *Vasa*-like gene. To clarify and cloned the full coding region of *DDX3* gene, two set of primers (*DDX3* full-5' and *DDX3* full3' / *DDX3* in-f and *DDX3* in-r) was designed based on the

previously reported sequence [Table 1].

2.3.2. cDNA synthesis for Reverse transcription-Polymerase Chain Reaction

In order to identify *DDX3* that expressed in head regeneration of earthworm, first strand cDNA was synthesized with total RNA from each regenerated samples. Total RNA (3 µg/µl) was combined with 0.5 µg of oligo d(T)15 primer (Promega, USA) and 1µl of 0.1% DEPC-treated TDW incubated at 70°C for 5 min to remove the secondary structure of RNA. Then it was directly placed on ice to prevent re-formation of secondary structure. After 2 min, 3 µl of M-MLV 5×reaction buffer, 1 µl (50 units) of RNase inhibitor, 4 µl of dNTP mix (2.5 mM each), 1 µl (200 units) of M-MLV reverse transcriptase and 3 µl of 0.1% DEPC-treated TDW were added. The mixture incubated at 37°C for 90 min and diluted with 25 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Finally it was incubated at 72°C for 7 min to enzyme inactivation. The amount of resultant cDNA were verified by spectrophotometric analysis at A₂₆₀ and stored at -20°C before use.

[Table 1] List of primer sets for polymerase chain reactions and DNA sequencing

Primer names	Sequences
DDX3 full-5'	5' - ATGGGATTGACAAGCAGATTTGGATGG - 3'
DDX3 full-3'	5' - TTAGACTGGTGAGAACCACCATAAGAG - 3'
DDX3 in-f	5' - AGACATCCCTGTTGAAGCAA - 3'
DDX3 in-r	5' - CATTGAGCAGATCTAGAAGGA - 3'
DDX3 RT-f	5' - CCC CAT CAA GGT GGT AGG AA - 3'
DDX3 RT-r	5' - ACT GCA ACT CGC CCT CTT TC - 3'
β-actin-f	5' - GTTGTCCAGAGGCATTGTTCCAG - 3'
β-actin-r	5' - CACATCTGTTGGAAGGTTGAGAGC - 3'
T7	5' - TAATACGACTCACTATAGGG - 3'
SP6	5' - ATTTAGGTGACACTATAG - 3'

2.3.3. Polymerase Chain Reaction

PCR was performed in a final volume of 50 μ l containing 5 units of ExTaq polymerase (TAKARA, Japan) and its buffer (containing 1.5 mM MgCl₂), 4 μ l of dNTP mix (2.5 mM each), 2 μ l of cDNA template, and 100 pmole of amplification primer pair (*DDX3* full-5' and *DDX3* full-3') which defining a 1,821-bp product. The PCR amplification was carried out for 1 cycle with denaturing at 94°C for 5 min, and subsequently for 33 cycles with denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 90 sec, and a final extension at 72°C for 10 min. The PCR products were stored at 4°C until use.

2.4. Bioinformatics analysis

2.4.1. Protein properties prediction

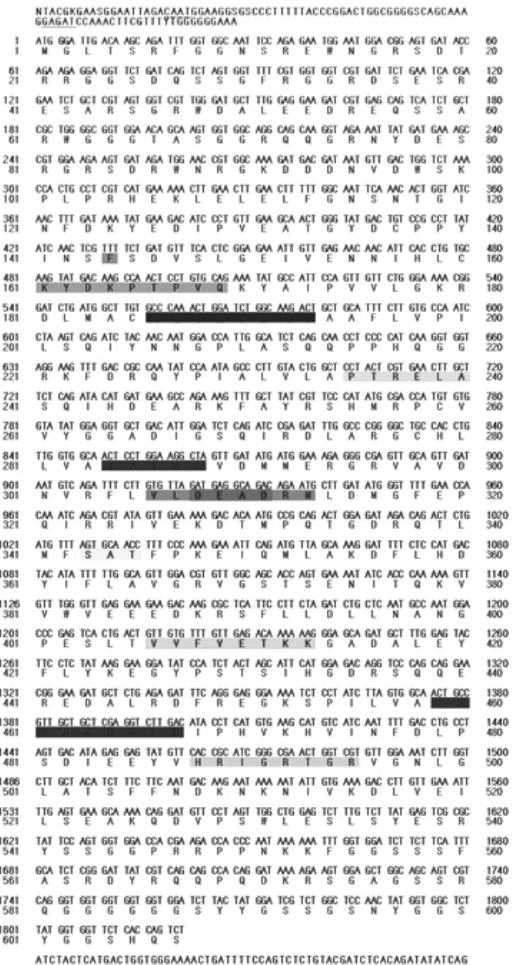
The deduced amino acid sequences of *PeDDX3* was compared with other MMP proteins using BLASTP supported from the National Center for Biotechnology Information (NCBI). The open reading frame (ORF) of *PeDDX3* gene was searched using ORF Finder¹⁰⁾, and the catalytic domain of the deduced protein, *PeDDX3* was identified by Pfam¹¹⁾. A signal peptide was predicted using CBS Prediction Servers SignalP¹²⁾. To predict other protein properties, ExPASy Proteomics Server¹³⁾ was used. Three dimensional (3D) structure of *PeDDX3* was predicted by using PDBsum¹⁴⁾, Swiss-Model¹⁵⁾ and Swiss-PdbViewer programme.

2.4.2. Multiple sequence alignments and phylogenetic profiling

PeDDX3 was compared with the GenBank

Database using BLASTP from NCBI. Multiple alignments of *DDX* proteins were carried out by using the MEGA 4 programme. In order to analyze the evolutionary relationship of

[Fig 1] Nucleotide and deduced amino acid sequences of the *Pe-D DDX3*.



The cloned *Pe-DDX3* cDNA (1,821 bp) encodes the 607 amino acids. The underlined sequence indicates putative signal peptide CAAT and GAGAT boxes. The nine highlighted sequences are the conserved motif of the DEAD-box superfamily, especially the red box means DEAD (Asp-Glu-Ala-Asp) amino acid sequence.

the DDX3 from *P. excavatus*, phylogenetic analysis was conducted with the MEGA4 programme by using Neighbour-Joining (NJ), Minimum-Evolution (ME), and Maximum-Parsimony (MP) methods. Bootstrap analysis was performed with 1,000 replications.

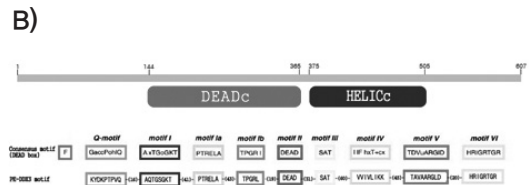
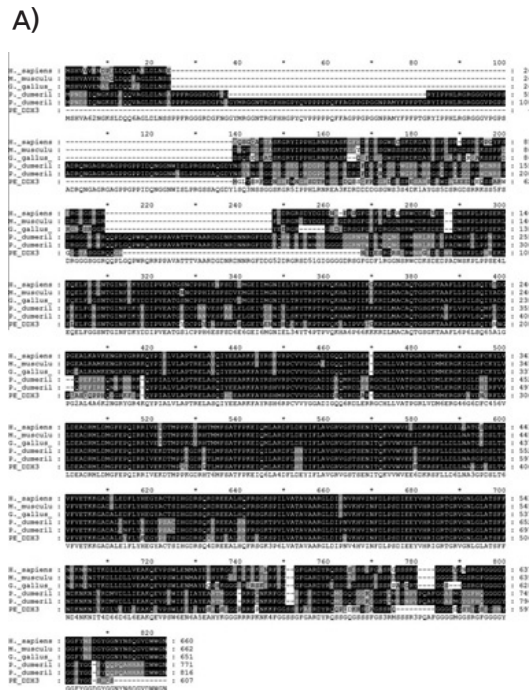
3. Results

3.1. Identification and characterization of DDX3 gene in *P. excavatus*.

The RNA helicases play a fundamental roles in many cellular metabolism. Especially, the DDX3 subfamily is known to be an important factor for soma and germ cell development in both invertebrates and vertebrates. This gene also has been identified from yeasts to various species of animals including molluscs, nematodes and mammals. The earthworm is taxonomically close to molluscs, which has the DDX3 gene, so it was estimated that earthworm also has the DDX3 gene. Therefore, in previous study, the one set of primer was designed to amplify the highly conserved region of the DDX3 genes based on the aligned DEAD domain fragments. Using this fragments, RACE PCR was performed to obtain full sequence of DDX3 gene⁹⁾.

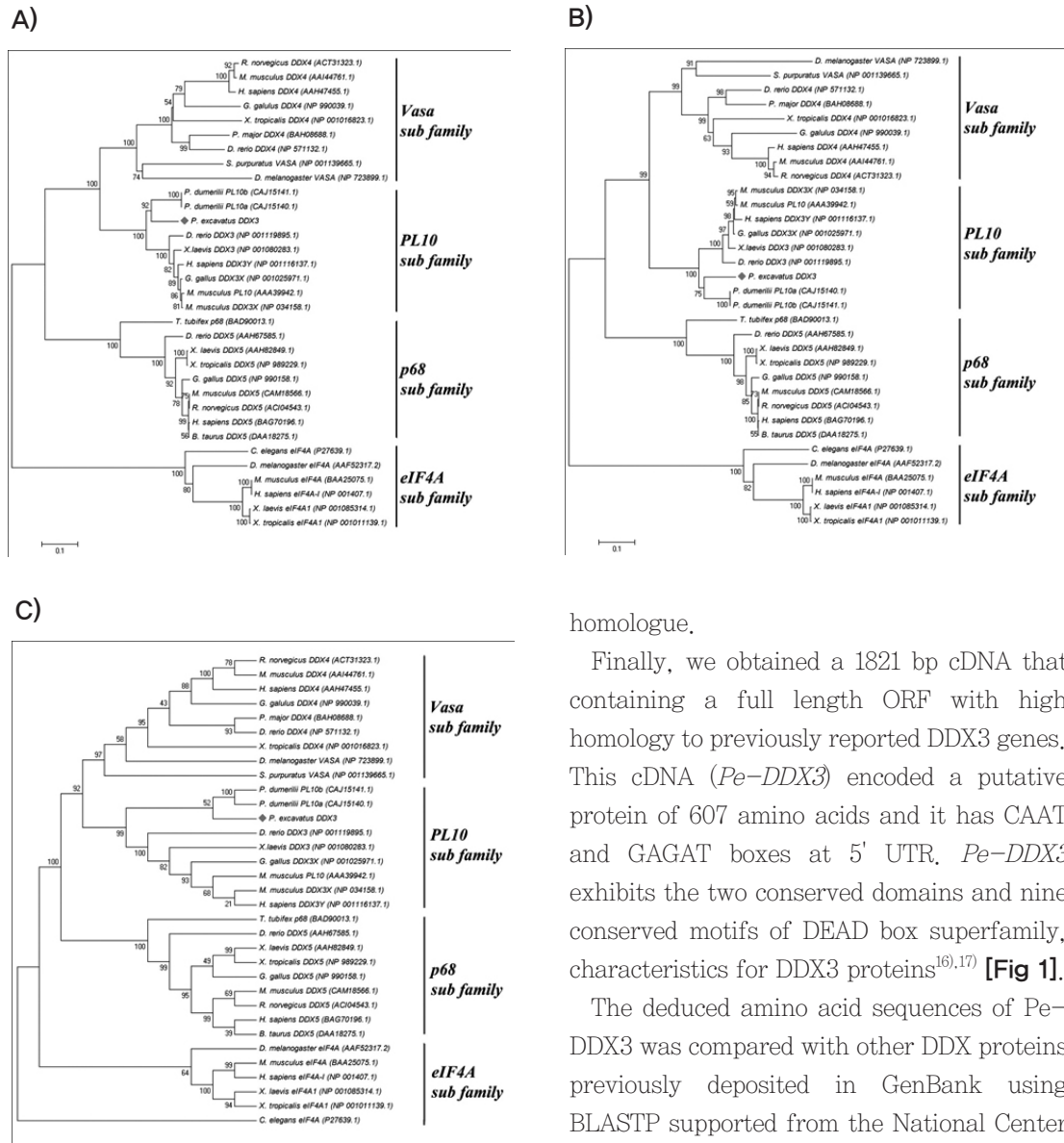
Based on this previous study, one set of primers DDX3 full-5' and DDX3 full-3' was designed to amplify a full sequence of DDX3 gene from the earthworm. The total RNA isolated from sexually matured earthworm head region containing the sex organs was used as a template for RT-PCR. The PCR product was about 1800 bp, and it was cloned for sequencing analysis. After that, the sequence was aligned with DDX3 genes using other species and it was confirmed as DDX3

[Fig 2] Multiple alignment of Pe-DDX3 with other different species DDX3s.



A) The alignment of Pe-DDX3 protein sequence from *P. excavatus* with the other DEAD box family proteins included in the phylogenetic tree [Fig 3]. Black and gray backgrounds indicate identical and similar residues, respectively. Gaps are indicated with dashes. The numbers refer to the amino acid residues in the corresponding protein. Pe-DDX3 showed 67% similarity to clam worm PL10a and PL10b (CAJ15140, CAJ15141), 64% similarity to chicken DDX3X (NP_001025971) and mouse DDX3X (NP_034158), 63% similarity to human DDX3Y (NP_001116137) (B) Diagrammatic representation of the conserved domains and crucial motifs.

[Fig 3] Phylogenetic profiling of the DDX3 with other DDX subfamilies.



These analyses were described for DDX subfamilies (eIF4A, PL10, Vasa and P68) based on the coding sequences of their protein sequences by using A) Neighbour-Joining method; B) Minimum-Evolution method; C) Maximum-Parsimony method. The numbers correspond to bootstrap values for branches based on 1,000 replicates.

homologue. Finally, we obtained a 1821 bp cDNA that containing a full length ORF with high homology to previously reported DDX3 genes. This cDNA (*Pe-DDX3*) encoded a putative protein of 607 amino acids and it has CAAT and GAGAT boxes at 5' UTR. *Pe-DDX3* exhibits the two conserved domains and nine conserved motifs of DEAD box superfamily, characteristics for DDX3 proteins^{16,17} [Fig 1].

The deduced amino acid sequences of *Pe-DDX3* was compared with other DDX proteins previously deposited in GenBank using BLASTP supported from the National Center for Biotechnology Information (NCBI). It is demonstrated that *Pe-DDX3* has DEADc and HELICc conserved domain at the position of 144-385 and 375-505. And it showed 67% similarity to *P. dumerilii* PL10a, b¹⁸ and 64% similarity to *M. musculus* DDX3X¹⁹ and *G. gallus* DDX3X²⁰. Multiple alignment of *Pe-*

DDX3 with other DDX proteins from other species was performed based on the results of BLASTP. The proteins that used in the multiple alignment are described below:

PL10a, b from *P. dumerilii* (CAJ15140, CAJ15141), DDX3Y from *H. sapiens* (NP_001116137), PL10 from *M. musculus* (NP_034158), DDX3X from *G. gallus* (NP_001025971).

Multiple protein alignments showed that *Pe-DDX3* has high sequence identity to the RNA helicase DEAD domain [Fig 2], and it is confirmed again by this alignment that *Pe-DDX3* has 9 conserved motifs of DEAD box family.

There are several subfamilies of DEAD box RNA helicases, containing DDX2 (eIF4A), PL10 (DDX3), Vasa (DDX4), p68 (DDX5), all of which contain a highly conserved DEAD motif. In order to determine which subfamily of DDX include *Pe-DDX3*, phylogenetic analysis of the *Pe-DDX3* was performed using the amino acid sequence. Three type of phylogenetic analysis were conducted using Neighbor Joining (NJ) method, Minimum-Evolution method (ME) and Maximum Parsimony (MP) method. All of the phylogenetic trees of the *Pe-DDX3* indicated that *Pe-DDX3* classified as a member of DDX3 of the DEAD box protein subgroup. And *Pe-DDX3* displayed a high homology with PL10a, b from *P. dumerilii* and DDX3 from vertebrates [Fig 3].

4. Discussion

Before the genome project had been started, many scientists have researched the initial identification of the new gene. These study of sequence identity may provide not only the evolutionary relationship but also the

information about their putative functional characteristics.

In 1988, the incubation of globin mRNA with the translation initiation factor eIF4A and ATP changed the susceptibility of the mRNA to nucleases²¹. Thus, eIF4A altered the structure of the mRNA in such a way, that the RNase digestion pattern changed. Therefore eIF4A could be considered as a helicases that melts secondary structures and makes the RNA accessible to nucleases. Since then, many RNA helicases have been defined and showed several common sequence elements. This analysis, together with the description of a number of proteins involved in RNA metabolism (p68, SrmB, MSS116, vasa, PL10, mammalian eIF4A, yeast eIF4A) resulted, based on the sequence of eIF4A, in the birth of the DEAD-box protein family^{3),4)}.

These RNA helicases function as regulators of differentiation and are deregulated in various forms of cancer like neuroblastoma, retinoblastoma and melanoma^{22),23)}. Concomitant with their diverse functions, RNA helicases form a large family of proteins and are divided into many subfamilies according to their sequences and functions. Among them, the DDX3 are present in a wide range of organisms and are characterized by a core region of 290–360 amino acids that show a high sequence homology to the eIF-4A known to exhibit ATP dependent RNA helicases activity²⁴⁾.

The DDX3 are involved in regulatory processes of RNA metabolism and differentiation of germ and soma tissues²⁵⁾. In some animal models, DDX3 subfamily members are related to not only sexual reproduction but

also asexual reproduction and regeneration, especially in invertebrates and urochordates such as *B. schlosseri*⁷⁾, *P. polygenea*^{26),27)}, *D. japonica*^{28),29)} and *hydra*³⁰⁾. These proteins are detected mainly in differentiating germ cells, soma cells and stem cells.

In previous study by Kim⁹⁾, it had shown for the first time that earthworm also has at least one *DEAD-box* gene. In order to clarify the sequence of this gene, RT-PCR was performed with primers including start codon and stop codon, respectively. And then, a pair-wise sequence comparison was conducted including the predicted amino acid sequences of all clones and corresponding fragments of other ATP-dependent RNA helicases from different organisms. Based on this alignment, it was shown a high similarity of the obtained sequences to other DEAD box proteins. Using this strategy, DDX3 orthologue in *P. excavatus*, called *Pe-DDX3* (1,821 bp) was isolated and confirmed. *Pe-DDX3* is a member of the DEAD-box protein family. All member of this family have two conserved domains and nine conserved motifs. And the identified *Pe-DDX3* also has these conserved features. [Fig 1].

Up to date, the alignment of all annotated sequences from all species in SwissProt reveal that nine conserved sequence motifs have a very little variation. [Fig 2]. The common presence of these motifs is a criterion for clustering within the family and the function of each motif has been studied. Motif II (or Walker B motif) has the amino acids D-E-A-D (Asp-Glu-Ala-Asp), which gave name to the family. This motif is required for ATP binding and hydrolysis with motif I (or Walker A motif), Q-motif and motif VI^{31),32),33)}. Motif

Ia and Ib do not seem to participate directly in ATP binding or hydrolysis. They are necessary for RNA binding in association with motifs IV and V34). Motif III is proposed to participate in linking ATPase and helicase activities and plays an important role in nucleic acid unwinding^{35),36)}. All of these nine motifs were identified in *Pe-DDX3*.

When *Pe-DDX3* gene was first identified by Kim⁹⁾, it was considered that this gene is *Vasa*-related gene, because of different amino acid residues in hydra, planarian and yeast PL10 homolog. To clarify this situation and understand the origin of this gene, we conducted further phylogenetic analysis of the DEAD-box proteins from eIF4A (DDX2), PL10 (DDX3), VASA (DDX4) and p68 (DDX5) groups identified from 32 different taxa [Fig 3]. These analyses were based on NJ, MP and ME approaches.


The sequence comparison and phylogenetic analysis revealed that *Pe-DDX3* most closely related PL10. In fact, 67% amino acid identity with PL10a and PL10b from *P. dumerilii*⁸⁾. Consequently, it was ensured that this gene coded for putative ATP-dependent RNA helicase of the DEAD-box protein³ family [Fig 3].

5. References

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