Molecular Cloning and Phylogenetic Analysis of Two Plant-Parasitic Nematode *14-3-3* Genes

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Received: January 4, 2011 Accepted: January 21, 2011 Published: December 1, 2011
doi:10.5539/jas.v3n4p86 URL: http://dx.doi.org/10.5539/jas.v3n4p86

This work is supported by Special Fund for Agroscientific Research in the Public Interest (200903030) and the earmarked fund for Modern Agro-industry Technology Research System, China.

Abstract

Full-length cDNA sequences of highly conserved ubiquitous 14-3-3 proteins were cloned from plant parasitic *Heterodera glycines* and *Meloidogyne incognita* using RT-PCR and RACE methods. The two genes were named as *Hgly2* and *Minc3*, respectively. *Hgly2* consisted of nucleotide sequence of 1027bp and *Minc3* of 1525 bp. And the open reading frames (ORF) encode peptide of 251 and 261 amino acids separately. Homology analysis showed that the deduced amino acid sequences shared the high homology with different nematode species. The phylogenetic analysis indicated that the proteins from plant parasitic nematode were more similar to insect proteins than plant and other animal proteins reported by previous research.

Keywords: *Heterodera glycines*, *Meloidogyne incognita*, 14-3-3 proteins gene, Race, Phylogenetic analysis

1. Introduction

A highly conserved family of regulatory proteins formed by 14-3-3 proteins is seemed to be specific to eukaryotic organisms (Fu *et al*., 2000; Jaubert *et al*., 2004). The 14-3-3 monomers have a molecular weight of approximately 30 kDa and an isoelectric point of about 5, but functional 14-3-3 exists as a dimmer (Wang and Shakes, 1996). First identified in a survey of mammalian brain proteins, 14-3-3 proteins were named on the basis of their separation properties in two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis (Moore and Perez, 1967). 14-3-3 proteins putatively participate in many vital movements. For example, they may be central to integrating the regulation of biosynthetic metabolism, cell proliferation, survival, and other processes in human cells (Rubio *et al*., 2004). *Drosophila* 14-3-3 proteins have been shown to function in RAS/MAP kinase pathways that influence the differentiation of the adult eye and the embryo even regulation the entry into mitosis in the undisturbed cell cycle. These proteins also function in normal cell cycle progression, in addition to checkpoint regulation (Su *et al*., 2001). Moreover in *C. elegans*, 14-3-3 proteins were reported as interacting proteins of a major life span regulator. They could regulate life span and possibly provide the missing link to connect two well known signaling pathways that control longevity: insulin/IGF-1 and caloric restriction (Wang *et al*., 2006; Araiz and Château, 2008). Then another research indicated that 14-3-3 proteins binds to and regulates DAF-16 by sequestering it in the cytoplasm (DAF-16 was another important life span regulator in the insulin/IGF-1 signaling pathway). And the mechanism was similar to the regulation has been reported in mammalian cells (Li *et al*., 2007). 14-3-3 gene even plays a crucial role in the early events leading to polarization of the *C. elegans* zygote (Morton *et al*., 2002).

Study in the plant-nematode interaction, 14-3-3 proteins are thought to be pathogenicity factors involved in the invasion of the root tissue and in the induction and maintenance of feeding cells (Klink *et al*., 2009). A 14-3-3 protein was isolated through direct qualitative analysis of proteins secreted from *M. incognita* J2 (Jaubert *et al*., 2002). Next year, 14-3-3 protein was found in the oesophageal glands of second stage juveniles (Abad *et al*., 2003). In 2008, 486 proteins were identified from *M. incognita* secretome include 14-3-3 (Bellafiore *et al*., 2008). In addition 14-3-3 proteins can bind to and affect a wide variety of plant proteins, such as chaperones that

prevent proteolysis; adaptors for mediating interactions between proteins; regulators of intracellular protein distribution and transcriptional regulators (Davis *et al*., 2009). Hassan *et al*. revealed that 14-3-3 proteins may have a key role in co-ordination of mitosis, metabolism, stress response and organelle trafficking as the feeding site develops (Hassan *et al*., 2010).

The interaction between the nematode and its host plant has been concerned for several decades, especially in damage severely plant-parasite nematode. Here we researched two plant parasite nematode 14-3-3 genes, would lead to a better understanding of the molecular events and regulatory mechanisms involved in plant parasitism and allow the development of target-specific strategies to limit crop damage by these pathogens.

2. Materials and Methods

2.1 Collection of nematode

The nematodes were reared by their host plants in sunlight greenhouse. Cysts of *H. glycines* and oocysts of *M. incognita* incubated at 25 ℃ in the laboratory as described by Nitao *et al*. (1999). Suspension (including J2 hatched in 24 h) was collected into Eppendorf tubes, and then centrifuged at 12000 rpm, 15 min. Repetition until the weight up to 100mg, immersed in liquid nitrogen immediately. J2 were stored at -75°C until further use.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from the frozen materials by RNAiso Plus (TaKaRa, China) following the manufacturer's instructions. The RNA was treated by an RNase-free DNase I for eliminating the residual genomic DNA present in our preparation. The quality of RNA was tested by 1% (w/v) agarose gel electrophoresis. The first-strand cDNA for reverse transcriptional PCR (RT-PCR) was synthesized by following 3′-Full RACE Core Set Ver.2.0 (TaKaRa, China) with 3.5 μL of total RNA as the template.

2.3 Internal amplification

2 pairs of Gene-specific primer (GSP) for amplification *H. glycines* and *M. incognita* 14-3-3 proteins gene were designed by primer 5.0.

Hgly2-1: 5′-GCGTTCGATGATGCGATTGCTG-3′,

Hgly2-2: 5′-ATTTACCCAATCAAAAGTGCC-3′.

Minc3-1: 5′-TCTTGGCGTGTTCTTTCGTCTAT-3′,

Minc3-2: 5′-AAAAGACTGCTTAGCCAACTGAC-3′.

Both reactions were performed with 50 μL reaction mixture containing 2 μL single-stranded cDNA, 8 μL 1× cDNA Dilution Buffer II, 2 μL of each primer (10 uM), 25 μL 2× GC Buffer, 0.5 μL Tag polymerase (5U/μL), 10.5 μL dH_2O. The condition for amplification of cDNA segments were 94°C for 3 min, 30 cycles at 94°C for 30 s, 55°C for 30 s , 72°C for 30 s then 72°C for 10 min.

2.4 RACE

In order to obtain the full-length transcript, a rapid amplification of cDNA ends (RACE) procedure was employed to amplify the 5′ and 3′ end of the coding region according to the instructions (5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0, TaKaRa, China). The full-length cDNA sequences from *H. glycines* and *M. incognita* were named as *Hgly2* and *Minc3*. In *H. glycines*, 5′RACE GSP primers *Hgly2*-3: 5′-TCGGATGTCCAAAGCGTCAAG-3′was applied in outer PCR and *Hgly2*-4: 5′-GTCCAGCTCAGCAATCGCATC-3′ in inner PCR. 3′RACE GSP primers were *Hgly2*-1 (outer PCR): 5′-GCGTTCGATGATGCGATTGCTG-3′ and *Hgly2*-5 (inner PCR): 5′-CTGGCGATGAGCAGGAGG-3′. In *M. incognita*, the GSP primers of 5′RACE were *Minc3*- 3 (outer PCR): 5′-AACACGCCAAGAAGAACGAC-3′ and *Minc3*-4(inner PCR): 5′-TCAGCCTGTTCGGCGAGTTTGG-3′. *Minc3*-5: 5′-ATCGTTATTTGGCTGAGGTTTC-3′used with outer primer and *Minc3*-6: 5′-CACAACAGAGTTACCAAGAGGC-3′ used with inner primer in 3′RACE. The PCR products were electrophoresed on 1% (w/v) agarose gel.

A blast search was completed in NCBIBLAST network server (http://blast.ncbi.nlm.nih.gov/ BLAST.cgi). Deduced amino acid sequences were aligned using DNAstar software. Subcellular localization by SignalP 3.0 Servere (www.cbs.etu.dk/services/signalp) and proteins analysis in ExPASy: ScanProsite tool (http://www.expasy.ch/tools/scanprosite). Alignment and phylogenetic analysis were performed using the software MEGA 4.1 and Clustal X.

3. Results

3.1 Sequence analysis

The cDNA fragments of 338 bp from *H. glycines* and 411 bp from *M. incognita* were attained by RT-PCR approach. The RACE procedure was further employed to obtain full-length sequences of the two genes. And results showed that cDNA clone encoding *Hgly2* was 1027 bp with an open reading frame for a polypeptide of 251 amino acids (Fig.1) and a molecular mass of 28.47 kD. The cDNA clone encoding *Minc3* was 1525 bp with 261 amino (Fig.2) acids and a molecular mass of 29.41 kD. The isoelectric point (*p*I) of *Hgly2* was 4.48 and *Minc3* was 4.36. Both *Hgly2* and *Minc3* have 14-3-3 gene family conserved regions (Fig.3). In addition no signal peptide and transmembrane regions were found.

Further, Subcellular localization and ScanProsite revealed that *Hgly2* and *Minc3* possibility be found in cytoplasmic, nuclear, cytoskeletal, mitochondrial, golgi, endoplasmic and plasma membrane. *Hgly2* was predicted possession 4 protein kinase C phosphrylation sites, 2 cAMP-and cGMP-dependent protein kinase phosphorylation sites, 5 N-myristoylation sites, 7 casein kinase Ⅱ phosporylation sites, 1 Tyrosine kinase phosphorylation site and 2 N-glycosylation sites. *Minc3* potentially hold 9 casein kinase Ⅱ phosporylation sites, 3 protein kinase C phosphrylation sites, 2 cAMP-and cGMP-dependent protein kinase phosphorylation sites, 4 N-myristoylation sites, 1 Amidation site, 1 Tyrosine kinase phosphorylation site and 1N-glycosylation site.

3.2 Homology analysis

The full amino acid sequences we got were aligned with other species (Fig.4). We found that *Hgly2* share 82.46% identity with *Minc3*; 94.76% with *Bursaphelenchus xylophilus* (GU130158.1); 93.59% with *Caenorhabditis brenneri* (EU726795.1); 93.59% with *Caenorhabditis remanei* (XM_003109679.1); 87.45% with *Culex pipiens* (GU227357.1); 88.09% with *Drosophila melanogaster* (NM_165740.2); 70.69% with *Rattus norvegicus* (BC089860.1) and 81.97% with *Homo sapiens* (NM_003404.3).

While *Minc3* identity to *B. xylophilus* was 82.74%; *C. brenneri*, 82.89%; *C. remanei*, 82.89%; *C. pipiens*, 87.45%; *D. melanogaster*, 88.09%; *R. norvegicus*, 70.69% and *Homo sapiens*, 81.97%.

Comparison of protein sequences was also revealed that the N terminal 10 amino acids and the C terminal 20 amino acids showed more varariations than the other parts, indicating that these amino acids may be responsible for the isoform specificities.

3.3 Phylogenetic analysis

Based on the deduced amino acid sequences, a phylogenetic tree was constructed (Fig.5). All the sequences used in our analysis got from NCBI but 14-3-3 proteins from *Entamoeba histolytic* were approved distant related (Wang and Shakes, 1996) and other protozoa not employed. 14-3-3 proteins from 24 species could be divided into 4 groups through the comparison. Nematode proteins except *Trichinella spiralis* were clustered together and appeared to be more closely to insect, distantly to animal. *T. spiralis* was a human parasite more similar to insect than plant parasite nematode. The plant lineage formed a distinct grouping in N-J trees, suggested an early divergence from the other species. Invertebrate proteins group including nematode and insect with the nonepsil on mammalian and the result basically consisted with former research (Wang and Shakes, 1996).

4. Discussion

Protein 14-3-3 was also named tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. Since first reported about 14-3-3 proteins, the researchers have done a lot of work in animal and human parasite (Siles-Lucas *et al*., 2003; Joshua *et al*., 2009) but on the contrary for plant parasite nematodes. We know *H. glycines* and *M. incognita* were two important pathogens for crops and vegetables in the world. As early as 1987, *Meloidogyne* spp. was reported responsible for a large part of the annual 100 billion $ losses attributed to nematode damage in US (Sasser *et al*., 1987). Soybean cyst nematode suppressed US soybean yield more than any other disease during 2003 to 2005, soybean yield suppression was 2.9 million ton in 2003 and 1.9 million ton in 2005 (Wrather and Koenning, 2006). Unfortunately we hadn't high efficiency and environmental friendly control methods presented due to nematodes body was extremely small, physiology and metabolism almost incomprehension for us.

Protein 14-3-3 was speculated to participate in the process nematode infect host plant. And two14-3-3 isoforms isolated from *M. incognita* infective larvae (Jaubert *et al*., 2004; Curtis, 2007; Dubreuil *et al*., 2007) maybe involve in infection. Expression 14-3-3 gene in *H. schachtii* has also been localized within genital primordia of infective J2 (De *et al*., 2001). And we could obtain the partial sequences of *H. glycines* 14-3-3 gene from NCBI (article unpublished) and the full long still not reported. In our study we cloned the full cDNA sequence of

14-3-3 gene from *H. glycines* and *M. incognita* further alignment amino acid sequences, the results showed that the sequence of *H. glycines* 14-3-3 gene was 100% homology with the fragment in GenBank (GenBank: AF402309.1) and *M. incognita* 14-3-3 gene was 97% similar to that previously reported (GenBank: AF070225.1).

The ubiquity of 14-3-3 gene was validated again by our subcellular localization analysis. Multiple sequence alignment and phylogenetic analysis showed 14-3-3 genes were the highly conserved this result was consistent with former research. We supposed the functions of these proteins be conserved between plants and nematodes. The important function was phosphatases. The 14-3-3 proteins bound to a wide array of target proteins and then modulation many proteins involved in phosphatases and protein kinases (Yaffe *et al*., 1997; Masters *et al*., 1999; Sehnke *et al*., 2002). Other functions of 14-3-3 proteins also reported. For example, cell regulatory pathways, including signal transduction, apoptosis, stress response and transformation, could be targeted by one or several 14-3-3 gene isoform products in organisms ranging from yeast to human (Finnie *et al*., 1999; Van Hemert *et al*., 2001).

Therefore studies on these highly conserved proteins may allow novel strategies of plant-parasite nematode control. Maybe a potential strategy by interfering with the expression of 14-3-3 genes would be used to control the two nematodes.

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1    ggtttaattacccaagtttgaggagaattgaaattttatcttttgaaaatcgtctagacctatttaatttttcgaaaa
79   ATG TCT GAC AAC AAA GAA GAA CTC GTT CAG CGG GCT AAG TTG GCT GAG CAA GCT GAG CGC
     M   S   D   N   K   E   E   L   V   Q   R   A   K   L   A   E   Q   A   E   R
139  TAC GAT GAC ATG GCT CAG TCG ATG AAA AAG GTG ACG GAA TTG GGT GCG GAG TTG AGT AAT
     Y   D   D   M   A   Q   S   M   K   K   V   T   E   L   G   A   E   L   S   N
199  GAG GAG CGC AAC CTG TTG TCG GTT GCA TAC AAA AAT GTG GTC GGT GCG CGT CGT TCC TCG
     E   E   R   N   L   L   S   V   A   Y   K   N   V   V   G   A   R   R   S   S
259  TGG CGC GTT ATC TCG TCC ATT GAA CAG AAG ACT GAG GGC TCG GAG AAG AAG CAG CAG ATG
     W   R   V   I   S   S   I   E   Q   K   T   E   G   S   E   K   K   Q   Q   M
319  GCC AAG GAG TAT CGG GAG AAG GTG GAG CAG GAG TTG CGA GAG ATC TGT CAC GAC GTG CTG
     A   K   E   Y   R   E   K   V   E   Q   E   L   R   E   I   C   H   D   V   L
379  GAT TTG CTC GAC AAA TAT CTG ATC CCG AAG GCT GGC AAC CCG GAA TCA AAG GTG TTC TAC
     D   L   L   D   K   Y   L   I   P   K   A   G   N   P   E   S   K   V   F   Y
439  CTG AAG ATG AAG GGT GAC TAC TAC CGC TAC CTG GCT GAG GTT GCC ACC GGT GAC GAC CGA
     L   K   M   K   G   D   Y   Y   R   Y   L   A   E   V   A   T   G   D   D   R
499  AAC GCT GTT GTC GAG AAG TCG CAG CAG TCG TAC CAG GAA GCG TTT GAC ATT GCC AAG GAC
     N   A   V   V   E   K   S   Q   Q   S   Y   Q   E   A   F   D   I   A   K   D
559  AAA ATG CAG CCC ACC CAT CCC ATT CGT CTT GGT CTG GCG CTG AAC TTC TCT GTG TTC TAC
     K   M   Q   P   T   H   P   I   R   L   G   L   A   L   N   F   S   V   F   Y
619  TAT GAA ATT CTG AAC TCA CCG GAC AAG GCG TGC CAA CTC GCC AAA CAG GCG TTC GAT GAT
     Y   E   I   L   N   S   P   D   K   A   C   Q   L   A   K   Q   A   F   D   D
679  GCG ATT GCT GAG CTG GAC ACG CTC AAC GAG GAC TCG TAC AAG GAC TCC ACT CTG ATC ATG
     A   I   A   E   L   D   T   L   N   E   D   S   Y   K   D   S   T   L   I   M
739  CAA CTG TTG CGT GAC AAC TTG ACG CTT TGG ACA TCC GAC ACG GCT GGC GAT GAG CAG GAG
     Q   L   L   R   D   N   L   T   L   W   T   S   D   T   A   G   D   E   Q   E
799  GCT GGT GGT GAG GCG GGC GAA GCT GGT GGC AAC TGA
     A   G   G   E   A   G   E   A   G   G   N   *
836  tcgggcggacagacgttgaagaccaagaaaatgccccacgaaatttttccaccgaatcatttaatgacaattatgctt
914  ttctcaatatggcactttgattgggtaaatttttttgaaaaatcgttcaattaaaattgtcgtttaccagtcactctg
992  aacattaaaaattggcgtttctcaaaaaaaaaaaa
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Figure 1. cDNA sequence and predicted amino acids sequence of *Hgly2*

The lower-case characters indicate noncoding regions; the stop codon is indicated with an asterisk; the shadow areas indicates poly (A); underline sequences were proteins motif.

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1                                                    aatttttttgtcatttttagatcttacgattacatattat
40   ttttctccccatgtttttaatttttttaatttattgttagtatacaactgattatagtccgtagggtctagtgttatttt
119  ttaaattgatttttttctgtatttaagcttgacgagaagtatataatcattatcattcttctttttgggtttgaactttg
198  tgatttttatttgtgtccgaaatgtcccacatgtccgaaatgtcttcccttcttccgcctctcacaacgattaagaaaa
277  ggaaaagacaccttagttgttcactagacattatagatcaagctttacctcatgtcgataagtacacagctttacgcat
356  tagaacctattctacgcggcatgctcgtatatgccaaacatttgtggacgtgggccgataaacgcatagttccaacttc
435  ttccctataggtacctattatcctttctagaggattttctttgtgtttcaattctttttttattataagtttttacatca
514  attccccttataattatcaatttctttttatttcagtattacatattatcttcttacgcttaattttctttcaacaata
593  ATG ACG GAG ACA ATG TTG GAT TGC TCT GAC AAG GTG ACT GAG TCC AAG GAA GAA CTT GTT
     M   T   E   T   M   L   D   C   S   D   K   V   T   E   S   K   E   E   L   V
653  CAG CTT GCC AAA CTC GCC GAA CAG GCT GAG CGT TAT GAT GAC ATG GCA GAG TCA ATG AAG
     Q   L   A   K   L   A   E   Q   A   E   R   Y   D   D   M   A   E   S   M   K
713  AAG GTT ACC GAA TTT GGA GAC GAA CTG TCT AAT GAG GAG CGC AAT CTT CTC TCG GTT GCT
     K   V   T   E   F   G   D   E   L   S   N   E   E   R   N   L   L   S   V   A
773  TAT AAG AAT GTT GTT GGG GCT CGT CGT TCT TCT TGG CGT GTT CTT TCG TCT ATT GAG CAA
     Y   K   N   V   V   G   A   R   R   S   S   W   R   V   L   S   S   I   E   Q
833  AAG ACC GAA GGG GGG AAG AAG ACG ATG ACT AAG GAA TAC CGT GAG AAG ATY GAR KGT GAA
     K   T   E   G   G   K   K   T   M   T   K   E   Y   R   E   K   I   E   X   E
893  TTG CGT GAC ATT TGC AAA GGK GTT ATG AAT CTT CTG GAC AAA TTT CTT ATT CCG AAA GCT
     L   R   D   I   C   K   G   V   M   N   L   L   D   K   F   L   I   P   K   A
953  GGA ACT CCT GAT TCT AAA GTG TTC TAC CTT AAG ATG AAG GGC GAC TAC TAT CGT TAT TTG
     G   T   P   D   S   K   V   F   Y   L   K   M   K   G   D   Y   Y   R   Y   L
1013 GCT GAG GTT TCT TCT GGT GAT GAG TTG ACT GAT GTT ATC GAC AAA TCA CAA CAG AGT TAC
     A   E   V   S   S   G   D   E   L   T   D   V   I   D   K   S   Q   Q   S   Y
1073 CAA GAG GCT TTC GAT ATT GCT AAG GAC AAA ATG CCG CCA ACT CAT CCA ATT CGT CTT GGG
     Q   E   A   F   D   I   A   K   D   K   M   P   P   T   H   P   I   R   L   G
1133 CTG GCT CTC AAC TTT TCG GTC TTC TAC TTT GAG ATT CTC GAC AAT AAG GAC AAG GCT TGT
     L   A   L   N   F   S   V   F   Y   F   E   I   L   D   N   K   D   K   A   C
1193 CAG TTG GCT AAG CAG TCT TTT GAT GAG GCT GTT GCT GAG TTG GAC ACT CTT GAC GAG AAT
     Q   L   A   K   Q   S   F   D   E   A   V   A   E   L   D   T   L   D   E   N
1253 TTG TAC AAG GAC TCG ACT CTC ATT ATG CAA CTT CTT CGC GAC AAT TTG ACT CTG TGG ACT
     L   Y   K   D   S   T   L   I   M   Q   L   L   R   D   N   L   T   L   W   T
1313 TCC GAT GGC GGC GCT GAG GAA CCG GAG GCG GCT CCA ATT GCT GCT GAT GCC GAA GGC ACA
     S   D   G   G   A   E   E   P   E   A   A   P   I   A   A   D   A   E   G   T
1373 AAT TAA
     N   *
1379 gtgaaggaaagtggagtaaataattaaatggtaaagaagggaaaatataaggaaagacaatttttaatttattcgagt
1458 atttgtttgtttatgattatatttttgtctttcgaccatctataaaaaaaagttggcaaaaaaaaaaaa
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Figure 2. cDNA sequence and predicted amino acids sequence of *Minc3*

The lower-case characters indicate noncoding regions; the stop codon is indicated with an asterisk; the shadow areas indicates poly (A); underline sequences were proteins motif.

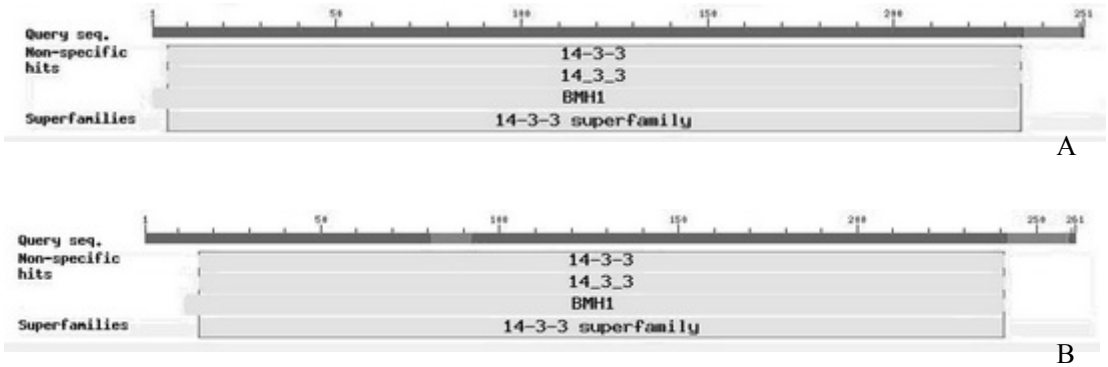

Figure 3. Conserved domains of amino acid sequences of *Hgly2* (A) and *Minc3* (B)

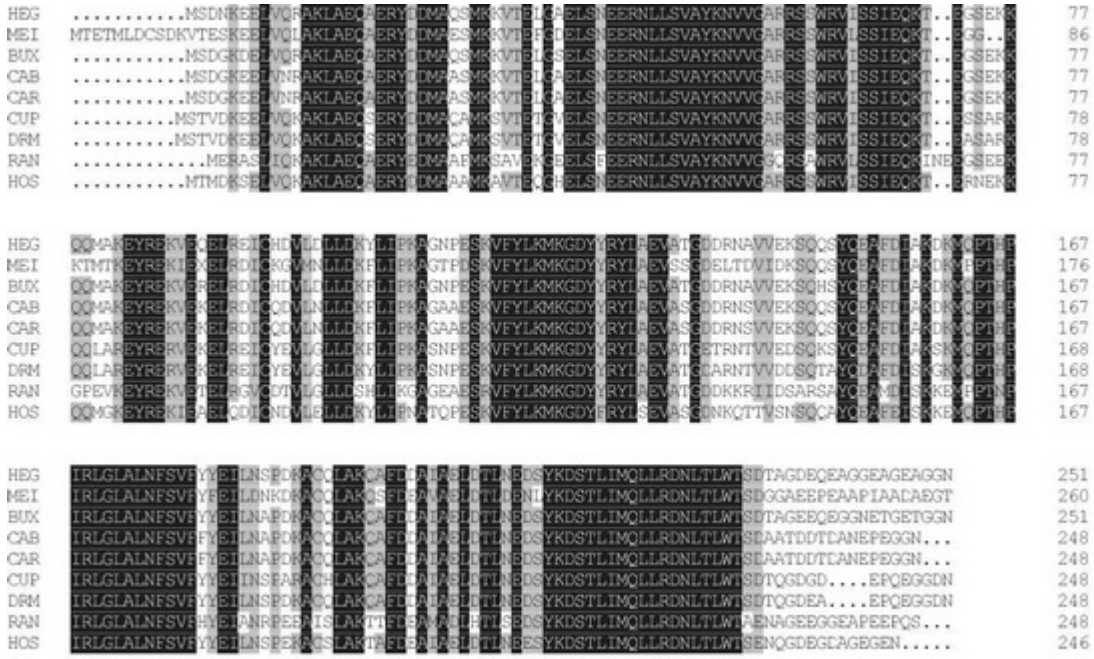

Figure 4. Alignment of deduced amino acid sequences of *Hgly2* and *Minc3* with other species

HEG, MEI, BUX, CAB, CAR, CUP, DRM, RAN, HOS denote14-3-3 protein from *H. glycines*, *M. incognita*, *B. xylophilus*, *C. brenneri*, *C. remanei*, *C. pipiens*, *D. melanogaster*, *R. norvegicus*, *Homo sapiens* respectively; Dots indicate gaps introducing to facilitate the alignment; Identical and similar amino acid residues are shaded in black and gray.

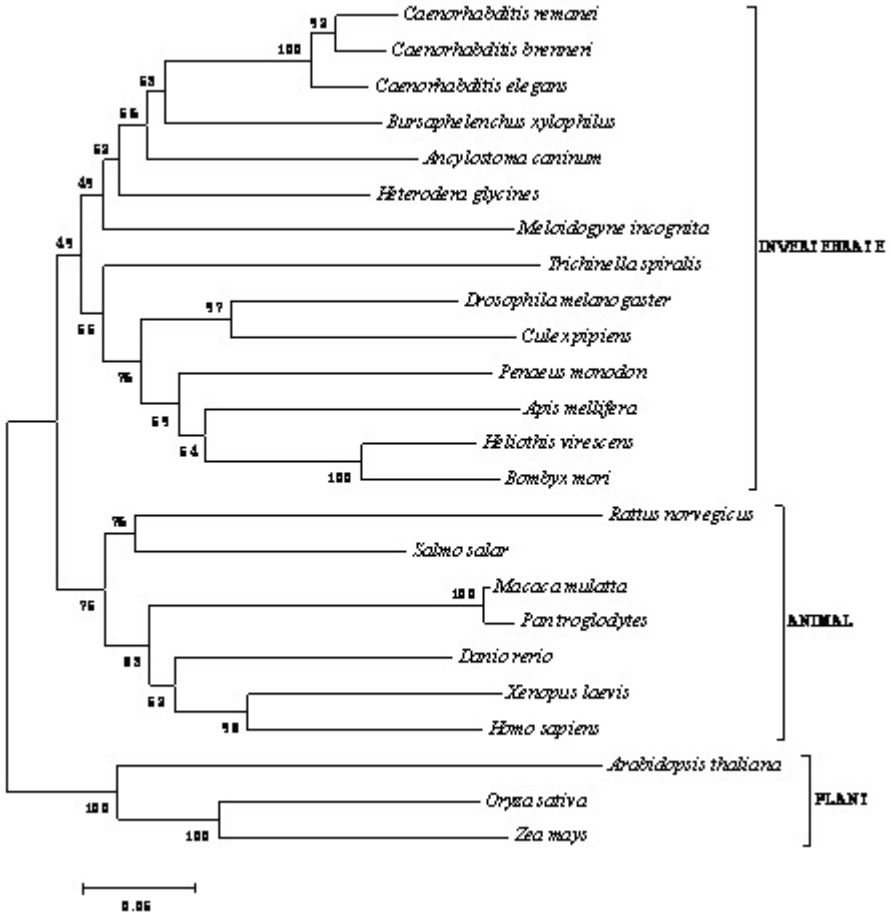

Figure 5. A phylogenetic tree analysis with *Hgly2*, *Minc3* and 22 species 14-3-3 proteins using Neighbor-Joining method