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RESEARCH

# Molecular Characterization and Phylogenetic Analysis of the *Eukaryotic Translation Initiation Factor 4A* Gene in *Antheraea pernyi* (Lipdoptera: Saturniidae)

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**ABSTRACT.** Eukaryotic initiation factor 4A (eIF-4A) is an essential component for protein translation in eukaryotes. The *eIF-4A* gene (*ApeIF-4A*) was isolated and characterized from *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae). The obtained cDNA sequence was 1,435-bp long with an open reading frame of 1,266 bp encoding 421 amino acids. The predicted amino acid sequence shared several conserved features as found in known eIF-4As and revealed 74 and 78% identities with eIF-4As of *Homo sapiens* L. and *Drosophila melanogaster* (Meigen), respectively. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that *ApeIF-4A* was transcribed at four developmental stages and in all tissues tested, suggesting that it plays an important role in development of *A. pernyi*. Homologous alignment suggested that eIF-4As are highly conserved throughout evolution of eukaryote organisms. Phylogenetic trees based on the amino acid and nucleotide sequences of *eIF-4A* demonstrated a similar topology with the classical systematics, suggesting that it has the potential value in phylogenetic inference of eukaryotes.

**Key Words**: *Antheraea pernyi*, eukaryotic initiation factor 4A, expression pattern, phylogenetic inference

Eukaryotic initiation factor 4A (eIF-4A) is an essential component for protein translation in eukaryotes. As an important part of the initiation factor complex elF-4F, eIF-4A is necessary for ribosome loading onto mRNA (Svitkin et al. 2001, Schütz et al. 2008). eIF-4A is a member of the DEAD-box family of RNA helicases (Rogers et al. 2002). The ATPase and helicase activity of free eIF-4A is activated when eIF-4A becomes a part of cap-binding complex (Grifo et al. 1984, Pause et al. 1993). It has been suggested that eIF-4A plays a critical role in the cell growth and development (Surakasi and Kim 2010).

For phylogenetic analysis, the protein-coding gene shows a number of favorable properties (Wiegmann et al. 2000). The protein-coding genes evolve more slowly and are less prone to base-composition bias than mitochondrial markers (Lin and Danforth 2004), and they typically present fewer alignment issues than ribosomal genes (Danforth et al. 2006). Recently, many nuclear protein-coding genes have been evaluated for phylogenetic utility in eukaryotes (Wild and Maddison 2008, Regier et al. 2009, Liu et al. 2010b, Li et al. 2010, Liu and Qin 2011, Sun et al. 2011). The *eIF-4A* gene has been isolated from various kinds of eukaryotes organisms. However, its potential value in phylogenetic inference has not been assessed.

Chinese oak silkworm, *Antheraea pernyi* (Guérin-Méneville) (Lipdoptera: Saturniidae), is an important economic insect used for silk production and insect food source (Liu et al. 2010a). In this study, based on Expressed Sequence Tag (EST) sequencing strategy, the *eIF-4A* gene from *A. pernyi* (*ApeIF-4A*) was isolated and characterized. In addition, the sequences of this gene from various eukaryote organisms were used to evaluate the potential value in phylogenetic study.

## Materials and Methods

**Silkworms and Tissues.** The *A. pernyi* strain (*Shenhuang No. 1*) was used in this study. The larvae were reared routinely on oak trees (*Quercus liaotungensis Koidz*). Ten tissues including hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes (♂), ovaries (♀), brain, and muscle were dissected from the larvae at day 10 of the fifth instar. They were frozen immediately in liquid nitrogen and stored at −80°C. Eggs at day 5, larvae of the fifth stage, pupae, and moths were also frozen in liquid nitrogen and stored at −80°C.

**Cloning of the *ApeIF-4A* Gene and Sequence Analysis.** From an *A. pernyi* pupal full-length cDNA library (Li et al. 2009), based on SMART (switching mechanism at 5′-end of RNA transcript) method, one EST encoding eIF-4A homolog was isolated by random sequencing. So, the cDNA clone (No. Ap0249) was used to complete the *ApeIF-4A* full-length cDNA sequence. This cDNA sequence has been deposited in GenBank under accession no. KC481238.

The open reading frame (ORF) finder tool of National Center of Biotechnology Information (NCBI) was used to identify the ORF at the website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The BLAST search was performed at the site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The isoelectric point (pI) and molecular weight (MW) of the deduced amino acid sequences were predicted using the Compute pI/MW Tool at the Expert Protein Analysis System site (http://web.expasy.org/compute_pi/).

**RNA Isolation and First-Strand cDNA Synthesis.** RNAprep Pure Tissue Kit (TIANGEN Biotech, Beijing, China) was used to extract total RNA in accordance with the manufacturer's instructions. DNAase I was used to remove the genomic DNA. The quality and quantity of the extracted RNA were determined by the ratio of $OD_{260}/OD_{280}$ with ultraviolet spectrophotometer. The integrity of the RNA was analyzed by 1.2% (W/V) agarose gel electrophoresis. The first-strand cDNA was generated with 2 µg of total RNA per sample by TIANScript RT Kit (TIANGEN Biotech, Beijing, China) following the manufacturer's instructions. Then the first-strand cDNA was stored at −20°C for later use.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses.** For amplification of the entire ORF of *ApeIF-4A*, the primer pair eIF4A-F (5′-ATGTC TCATT CATCT GAAAG AA-3′) and

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eIF4A-R (5′-TCAGA TGAGG TTGGC CACAT CAC-3′) was used, which generated a product of 1,266 bp. For RT-PCR analysis of *ApeIF-4A*, the primer pair LYQ205 (5′-TCCAT CGCTC AGGCT GTTAT-3′) and LYQ206 (5′-GTGCT CGTCT GTCAC TTTCA-3′) was designed, which produced a product of 340 bp. The *actin* gene was used as an internal control (Wu et al. 2010), with the gene-specific primer pair LYQ85 (5′-CCAAA GGCCA ACAGA GAGAA GA-3′) and LYQ86 (5′-CAAGA ATGAG GGCTG GAAGA GA-3′), which generated a 468-bp fragment. The PCR reactions were performed on a BIO-RAD S1000 Thermal Cycler (Bio-Rad Laboratories, Inc.). The PCR amplification was carried out in a reaction volume of 25 µl containing 0.5 µl of cDNA sample, 10 pM of each primer, 2 mM $MgCl_2$, 2 mM of dNTP, 2.5 µl of 10× buffer, and 1 U Taq DNA polymerase (TIANGEN Biotech, Beijing, China). The cycling parameters were an initial denaturation at 95°C for 3 min, followed by 27 cycles each comprising denaturation at 95°C for 30 s, annealing temperature of 56°C for 30 s, and extension temperature of 72°C for 30 s. The amplification products were analyzed by 1.5% (W/V) agarose gel electrophoresis. Each of the total RNA samples was used as PCR template in negative control of RT-PCR reaction to ensure no contamination of the genomic DNA. The PCR products were purified from the gel and sequenced to ensure the specificity.

**Phylogenetic Analysis.** For phylogenetic analysis, 37 representative eIF-4A amino acid sequences and their corresponding nucleotide sequences were used. These eIF-4A sequences included 18 invertebrates (*A. pernyi* [AGK45314], *Bombyx mori* L. [ABF51379], *Plutella xylostella* L. [ABY66383], *Papilio xuthus* L. [BAM17712], *Danaus plexippus* L. [EHJ75348], *Drosophila melanogaster* (Meign) [NP_476595], *Glossina morsitans Westwood* [ADD19944], *Culex quinquefasciatus Say* [XP_001843031], *Aedes aegypti* L. [XP_001656674], *Pediculus humanus* L. [XP_002424727], *Acyrthosiphon pisum* (Harris) [XP_001952053], *Tribolium castaneum* (Herbst) [NP_001177648], *Phaedon cochleariae* F. [CCJ09440], *Megachile rotundata* F. [XP_003705530], *Bombus impatiens Cresson* [XP_003492849], *Apis mellifera* L. [XP_623285], *Branchiostoma floridae Hubbs* [XP_001843031], and *Saccoglossus kowalevskii Agassiz* [XP_002742284]), 9 vertebrates (*Homo sapiens* L. [NP_001407], *Rattus norvegicus* (Berkenhout) [NP_955404], *Mus musculus* L. [BAE40541], *Sus scrofa* L. [NP_001093666], *Xenopus tropicalis Gray* [AAH84468], *Xenopus laevis* (Daudin) [NP_001085314], *Oreochromis niloticus* L. [XP_003458967], *Ictalurus punctatus Rafinesque* [ADO28856], and *Danio rerio Hamilton* [NP_938180]), 7 plants (*Chlamydomonas reinhardtii P. A. Dang.* [XP_001691452], *Oryza sativa* L. [NP_001058481], *Ricinus communis* L. [XP_002521376], *Arabidopsis thaliana* L. [NP_566469], *Glycine max* L. [XP_003522265], *Vitis vinifera* L. [XP_002278119], and *Populus trichocarpa Torr. & A. Gray* [XP_002308545]), and 3 fungi (*Penicillium digitatum* (Pers.) Sacc. [EKV16508], *Aspergillus niger Tiegh.* [XP_001400296], and *Saccharomyces cerevisiae Meyen ex E. C. Hansen* [NP_012985]). The amino acid and nucleotide sequence alignment were performed by MEGA 5.0 (Tamura et al. 2011). Amino acid and nucleotide sequences of the *eIF-4A* gene were used to reconstruct the phylogenetic relationships based on the maximum likelihood method, respectively. The maximum-likelihood tree was generated with 1,000 bootstrap replicates using MEGA 5.0 (Tamura et al. 2011). Before undertaking phylogenetic search, a preferred substitution model was selected based on the lowest Bayesian Information Criterion scores using MEGA 5.0. The JJT + G model was selected for the amino acid sequence dataset, and the TN93 + G + I model was selected for the nucleotide sequence dataset, respectively.

## Results and Discussion

**Sequence Analysis of the *A. pernyi eIF-4A* Gene.** In this study, the *eIF-4A* gene was isolated and identified from the *A. pernyi* pupal full-length cDNA library (Li et al. 2009). An expected specific amplification product was recovered from the cDNA sample of *A. pernyi* by using the primer pair eIF4A-F and eIF4A-R that span the ORF (data not shown). The subsequent sequencing of the amplification product conformed the assembled sequence was valid. However, no PCR amplification was obtained when the genomic DNA was used as template, indicating that the genomic DNA of the *ApeIF-4A* gene has introns. It has been known that the genomic DNAs of the *eIF-4A* genes from close relatives, *B. mori* and *P. xylostella,* contain four and five introns, respectively.

The cDNA sequence and deduced amino acid sequence of *ApeIF-4A* are shown in Figure 1A. The obtained 1,435-bp cDNA sequence contains a 5′-untranslated region (UTR) of 10 bp, a 3′-UTR of 159 bp with a poly (A) tail, and an ORF of 1,266 bp that encodes a polypeptide of 421 amino acids. A canonical polyadenylation signal sequence AATAAA is also observed at the position of 1,391 bp. The deduced amino acid sequence has a predicted MW of 47.53 kDa and an isoelectric point of 5.04. Blastp analysis revealed that the predicted protein sequence of this cDNA shares 74 and 78% identities with those of known eukaryotic translation initiation factor 4A of *H. sapiens* (NP_001407, Kim et al. 1993) and *Dr. melanogaster* (NP_476595, Hernández et al. 2004), respectively.

As shown in Figure 1B, the conserved domains prediction revealed that the deduced amino acid sequence contains the conserved features as found in known eIF-4As, including the ATP-binding site, the $Mg^{++}$-binding site (DEAD box), the nucleotide-binding region, and the motif III that is involved in coupling ATP hydrolysis to RNA binding. This result also revealed that this protein belonged to the P-loop_NTPase superfamily, a diverse family of proteins involved in ATP-dependent RNA unwinding that is needed in a variety of cellular processes including splicing, ribosome biogenesis, and RNA degradation (Svitkin et al. 2001, Rogers et al. 2002, Schütz et al. 2008). Therefore, we referred to the protein characterized in this study as *A. pernyi* eukaryotic translation initiation factor 4A. Two conserved regions named as DEADc and HELICc (helicase_c) were positioned at 50–250 and 26–372 in ApeIF-4A, respectively.

**Expression Patterns.** In this study, we used RT-PCR approach to measure the expression pattern of *ApeIF-4A*. The negative controls exhibited no amplification products (data not shown). By sequencing, we confirmed that the positive RT-PCR products were amplified from the *ApeIF-4A* sequence. As shown in Figure 2A, *ApeIF-4A* mRNA was found to be expressed at four development stages, including egg, larva, pupa, and moth. *ApeIF-4A* mRNA was also found to be present in all tested tissues of the fifth stage larvae, including hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes, ovaries, brain, and muscle (Fig. 2B).

The lepidopteran model insect *B. mori* is a closely relative of *A. pernyi*. Sequence comparison revealed that *A. pernyi* eIF-4A shared 94% identity to *B. mori* eIF-4A (ABF51379). Analysis of approximate expression patterns inferred from EST sources, available in GenBank, showed that *B. mori eIF-4A* mRNA was expressed during four development stages including egg, larva, pupa, and moth. Analysis of the microarray information, available in SilkDB (Duan et al. 2010), revealed that *B. mori eIF-4A* mRNA (sw22934) was expressed in all tissues tested, including testes, ovaries, head, fat body, integument, midgut, hemolymph, Malpighian tubules, and silk glands. In another lepidopteran species, *P. xylostella*, its eIF-4A shares 92% identity to *A. pernyi* eIF-4A. Surakasi and Kim (2010) have showed that the *P. xylostella eIF-4A* mRNA was expressed in all four development stages (egg, larva, pupa, and moth) and in all tissues tested (hemolymph, fat body, gut, and epidermis). Our RT-PCR results regarding *eIF-4A* in *A. pernyi* are consistent with those observed in *B. mori* by in silico expression analysis and in *P. xylostella* by RT-PCR analysis (Duan et al. 2010, Surakasi and Kim 2010).

Our findings showed that *ApeIF-4A* was expressed during four development stages and in various tissues. Expression of *eIF-4A* has been well documented in other insects, such as *B. mori* and *P. xylostella* (Surakasi and Kim 2010, Tanaka et al. 2010). Moreover, in *B. mori*, *eIF-4A* shows constitutive expression in environmental stresses (Wang et al. 2008, Tanaka et al. 2010). Even in plants, it has been

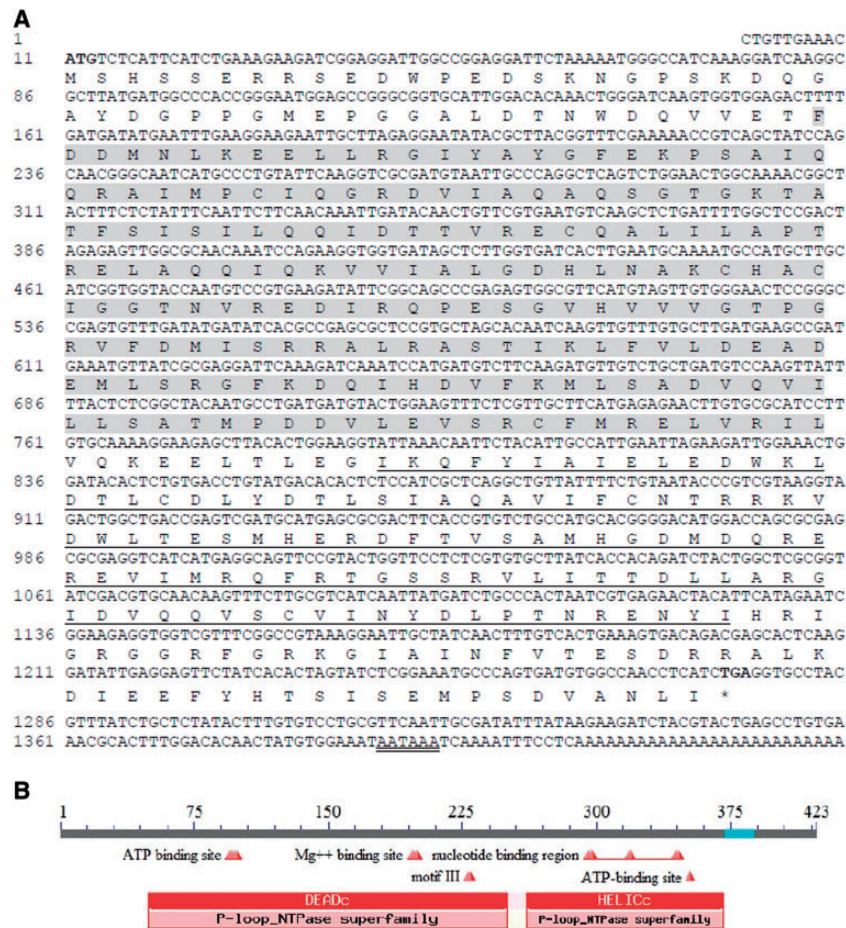


**Fig. 1.** The complete nucleotide and deduced amino acid sequence of the *A. pernyi eIF-4A* gene. (A) cDNA sequence and deduced amino acid sequence of *A. pernyi eIF-4A* gene. The initiation codon ATG is bolded and the termination codon TAG is bolded and marked with an asterisk. The conserved region DEADc positioned at 50-250 is shaded and HELICc positioned at 261-372 is underlined. (B) The predicted conserved features of *A. pernyi* eIF-4A, including the ATP-binding site, the $Mg^{++}$-binding site (DEAD box), the nucleotide binding region, and the motif III.

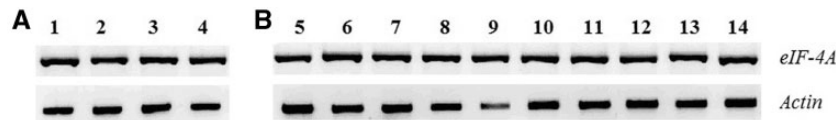


**Fig. 2.** Expression patterns of the *A. pernyi eIF-4A* gene (A) during the four developmental stages and (B) in various tissues of the fifth stage larvae; 1–4 represents egg, larva, pupa and moth, respectively, and 5–14 represents hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes, ovaries, brain and muscle, respectively.

shown that *eIF-4A* is an ideal constitutively expressed control gene that can be introduced into transgenic plants as a reporter gene (Mandel et al. 1995).

**Homologous Alignment.** From a database search by using ApeIF-4A sequence as a query, the homologs were found to be present in several kinds of eukaryote organisms, including invertebrates, vertebrates, plants, and fungi. Of these eIF-4A sequences available to date, only five are from lepdopteran species including *A. pernyi*, *B. mori*, *P. xylostella*, *Pa. xuthus*, and *D. plexippus,* although the order Lepidoptera is the second largest insect order and includes the most damaging agricultural pests and beneficial insects.

The relatedness of these homologs from various eukaryote organisms was assessed by calculating the identities from 37 representative eIF-4A sequences. Multiple sequence alignments revealed that, among these eIF-4A sequences, *A. pernyi* eIF-4A had sequence identity of 94% to *B. mori* eIF-4A (ABF51379) and 63% to *S. cerevisiae* eIF-4A (NP_012985). By multiple sequence alignments, we found that *A. pernyi* eIF-4A had 92–94% sequence identity to four known eIF-4As of lepidopteran species, 75–80% sequence identity to eIF-4As of other insects, 72–73% sequence identity to eIF-4As of vertebrates, 64–65% sequence identity to eIF-4As of plants, and 63–70% sequence identity to eIF-4As of fungi. In Figure 3, eight eIF-4A sequences are aligned, including three insect species (*A. pernyi* [AGK45314], *B. mori* [ABF51379], *Dr. melanogaster* [NP_476595]), two vertebrates (*H. sapiens* [NP_001407] and *Da. rerio* [NP_938180]), two plants (*Ar. thaliana* [NP_566469] and *O. sativa* [NP_001058481]), and one fungus (*S. cerevisiae* [NP_012985]). More than 60% amino acid sequence identity among these proteins suggested that they were highly conserved during the evolution of eukaryote organisms after they diverged from a common ancestor.

Furthermore, sequence alignment revealed that the amino acid sequences for the conserved features of ApeIF-4A including the ATP-binding site, the $Mg^{++}$-binding site, the nucleotide binding region, and the motif III were highly identical to those of known eIF-4As. The observation preliminarily confirms the active role of ApeIF-4A in formation of translation initiation complex like other DEAD box proteins.

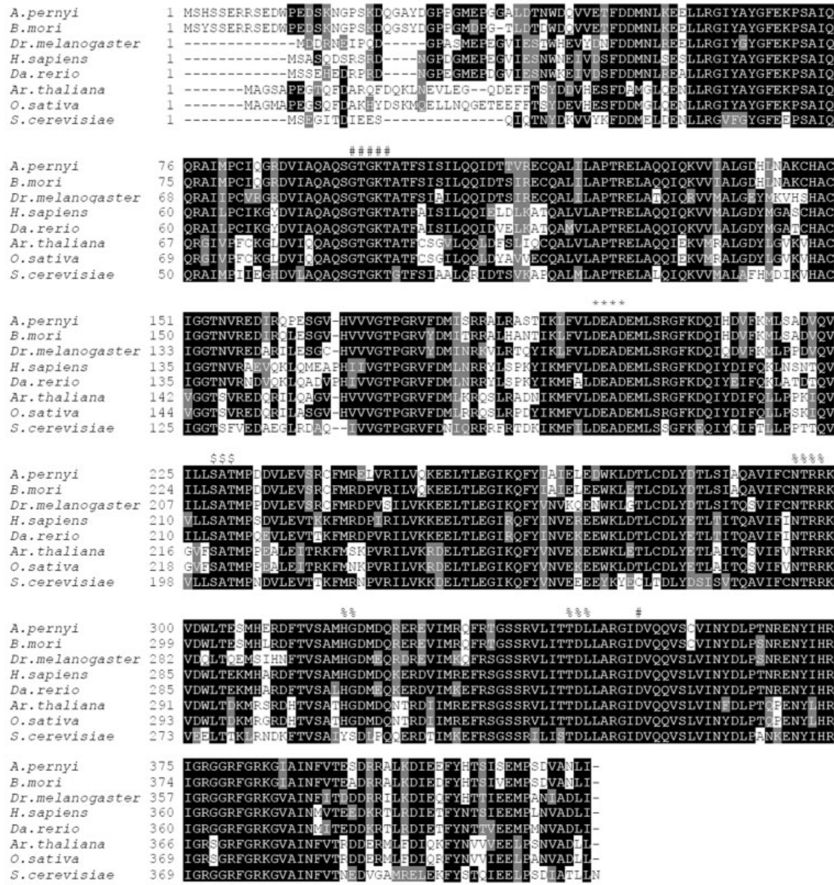

**Fig. 3.** Sequence comparisons of eIF-4As. The eIF-4A sequences from *A. pernyi* (AGK45314), *B. mori* (ABF51379), *Dr. melanogaster* (NP_476595), *H. sapiens* (NP_001407), *Da. rerio* (NP_938180), *Ar. thaliana* (NP_566469), *O. sativa* (NP_001058481), and *S. cerevisiae* are aligned. The sign # shows the position of the ATP-binding site, sign * shows the position of the $Mg^{++}$-binding site, sign % shows the position of the nucleotide binding region, and sign $ shows the position of the motif III that is involved in coupling ATP hydrolysis to RNA binding.

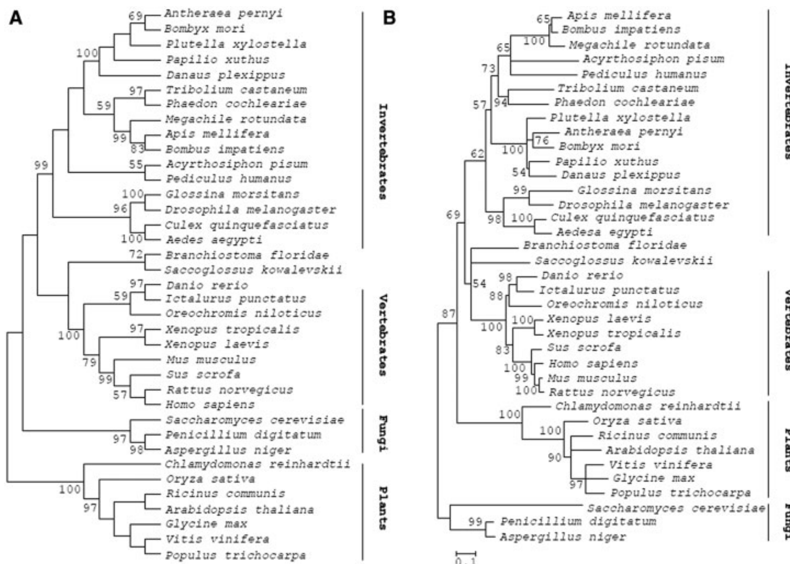


**Fig. 4.** Maximum-likelihood trees based on the amino acid (A) and nucleotide (B) sequence comparisons of the *eIF-4A* genes. Numbers at nodes are bootstrap *P* values.

**Phylogenetic Analysis.** The amino acid and nucleotide sequences of *eIF-4A* from 37 eukaryote organisms were used to reconstruct their phylogenetic relationships, respectively (Fig. 4). Both phylogenetic analyses based on the maximum likelihood method revealed that *A. pernyi* was closely related to *B. mori*. In the phylogenetic trees, the used sequences were well divided into four groups with high bootstrap support, corresponding to invertebrates, vertebrates, plants, and fungi. In vertebrate group, nine used sequences were divided into three

subgroups, corresponding to Neopterygii, Amphibia, and Mammalia. Within insect group, they were obviously divided into four subgroups with high bootstrap support, corresponding to Lepidoptera, Diptera, Coleoptera, and Hymenoptera. *Br. floridae* and *Sa. kowalevskii* were positioned between vertebrates and invertebrates. Overall, the obtained phylogenetic trees followed the classical evolutionary trend.

It is common that ribosomal RNA sequences from all living organisms can be aligned and has become a major tool in establishing phylogenetic relationships (Hou et al. 2008). In addition, some ribosomal proteins such as RPL25 are also highly conserved (Metzenberg et al. 1993). Recently, many nuclear protein-coding genes have been used to reconstruct the phylogenetic relationships in eukaryotes, such as *enolase* I (Wild and Maddison 2008, Regier et al. 2009, Liu et al. 2010b), *topoisomerase* and *arginine kinase* (Wild and Maddison 2008), *will die slowly* (Li et al. 2010), *selenophosphate synthetase* (Sun et al. 2011), *lysyl-tRNA synthetase* (Liu and Qin, 2011), and *doba decarboxylase* (Regier et al. 2008). In this study, the phylogenetic trees based on the amino acid and nucleotide sequences of *eIF-4A* demonstrated a similar topology with the classical systematics, suggesting that it has the potential value in phylogenetic inference of eukaryotes.

In summary, we have isolated the *eIF-4A* gene from *A. pernyi*. The *eIF-4A* gene is expressed throughout four developmental stages and in all tested tissues, suggesting that it plays an important role in development of *A. pernyi*. Homologous alignment suggested that eIF-4As are highly conserved throughout evolution of eukaryote organisms.

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