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ORIGINAL ARTICLE

# Characterization of a highly conserved *Antheraea pernyi spermidine synthase* gene

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## Abstract

In the present study, we isolated a *spermidine synthase* gene from *Antheraea pernyi* (*ApSpds*) using expressed sequence tag method. The obtained cDNA sequence of 1483 bp contains an open-reading frame of 864 bp encoding a polypeptide of 287 amino acids. Sequence analysis revealed that ApSpds belonged to class I of AdoMet-MTase family, and exhibited 30% identity to those from bacteria, 45–48% identity to fungi, 36–47% identity to plants, 52–54% identity to vertebrates and 53–80% identity to invertebrates. Phylogenetic analysis found that the used Spds protein sequences were well divided into five groups corresponding to bacteria, fungi, plants, invertebrates and vertebrates, respectively. These results further confirmed that Spds is highly conserved through evolution of life organisms. The *ApSpds* mRNA is expressed during all four developmental stages and is present in all examined tissues with the highest abundance in the muscle, in which the relative mRNA expression level was 1.6 times higher than in the fat body. Although not significant, the mRNA level decreased after high-temperature exposure suggesting that the *Spds* gene may not be involved in temperature stress tolerance in *A. pernyi*. Taken together, our results suggested that *ApSpds* play an important role in development of silkworm.

**Keywords** *Antheraea pernyi* · Spermidine synthase · Expression pattern · Evolution

## Introduction

Spermidine synthase (Spds) also known as *S*-adenosylmethioninamine: putrescine, 3-aminopropyltransferase has been isolated from bacteria (Wang et al. 2016), plants (Imai et al. 2004), animals (Raina et al. 1984; Samejima and Yamanoha 1982) and human (Kajander et al. 1989). Spermidine synthase is a key enzyme in the biosynthesis of spermidine from putrescine (Neily et al. 2011). The enzyme catalyzes the transfer of the propylamine group from *S*-adenosylmethioninamine to putrescine (Patwa et al. 2011). Spermidine is a major polyamine (PA) and ubiquitous polycation that is essential for cell growth and proliferation (Igarashi and Kashiwagi 2000; Pegg 1986). Polyamines are low-molecular weight aliphatic amines that are found in all living cells, and are able to interact with negatively charged macromolecules as DNA and RNA, proteins and phospholipids to activate and stabilize these molecules (Evans and Malmberg 1989). Spermidine synthase is conserved in primary structure between eubacteria, archaea, plants, fungi, and animals (Michael 1999), and used as a target to investigate the polyamine biosynthesis in parasitic protozoa (Gilroy et al. 2011).

In plants, the spermidine biosynthesis has been well studied. High cellular level of polyamines correlates with tolerance to a wide arrays of environmental stress such as temperatures (He et al. 2002; Shen et al. 2000), salinity (Krishnamurthy and Bhagwat 1989; Li et al. 2016), hyperosmosis (Besford et al. 1993), hypoxia (Nada et al. 2004), and oxidative stress (Kurepa et al. 1998). In insect, the changes in spermidine levels have been recorded during Diptera embryogenesis (Callaerts et al. 1992). In *Bombyx mori*, spermidine is especially abundant in silk gland, gonads, mucous gland, and sucking stomach (Hamana et al. 1984). However, the knowledge on insect spermidine biosynthesis remains limited.

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In this study, we have characterized the *Spds* gene isolated from *Antheraea perny* (named as *ApSpds*). *Antheraea perny* belongs to the family Saturniidae of Lepidoptera, is an important economic insect used for silk production and is also a high-quality food resource (Liu et al. 2010). We identified the expression patterns of the *ApSpds* during developmental stages, its tissue distributions, and function under temperature stress. In addition, the Spds protein sequences from various organisms were comparatively analyzed and used to reconstruct their phylogenetic relationship. Our work would provide basic information for understanding the spermidine biosynthesis in insects.

## Materials and methods

### Materials

*Antheraea pernyi* strain Shenhuang No. 1 was used in this study. The eggs at day 5, the five-instar larvae at day 10, pupae and moths were collected as samples. Then hemolymph, fat body, midgut, silk glands, integument, malpighian tubules, testes (♂), ovaries (♀), brain and muscle were dissected from the five-instar larvae at day 10. All samples were frozen immediately in liquid nitrogen and then stored at − 80 °C for further use. To examine the *ApSpds* mRNA expression under temperature stress, silkworm pupae were passed through 46 °C-heat shock treatment for 3 h; another batch of silkworm pupae was passed through 26 °C control treatment. After these treatments, fat body was dissected from silkworm pupae to extract total RNA. Ten pupae samples were pooled for each treatment.

### Isolation of the *ApSpds* gene and sequence analysis

Based on the *A. pernyi* EST resources from a pupal full-length cDNA library constructed in our laboratory (Li et al. 2009), a homolog encoding Spermidine synthase was identified. The full-length cDNA clone was used to complete the full-length cDNA sequence of the *ApSpds* gene. The primer pair LYQ221 (5′-ATGGA TAAAT TACAA AACAA ATG-3′) and LYQ222 (5′-TCAGG CTAGC CTATA CTTCA C-3′) was successfully used for amplification of the entire open reading frame (ORF) of this gene. The cDNA and deduced amino acid sequence analysis were performed using the software DNASTAR (DNASTAR Inc., www.dnastar.com). The isoelectric point and molecular weight of the deduced amino acid sequence were predicted at http://www.expasy.org/tools/pi_tool.html. Conserved domains were predicted at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/.

### Homologous comparison and phylogenetic analysis

The amino acid sequences of the *Spds* genes from various organisms were used for homologous alignment and phylogenetic analysis. The putative amino acid sequence of the *Spds* gene from *B. mori* was obtained from SilkDB, an open-access genomic database for *B. mori* (Duan et al. 2010). Multiple sequence alignments were performed using Clustal X software (Thompson et al. 1997). A phylogenetic analysis was constructed using maximum likelihood (ML) method with bootstrap test of 1000 replications under WAG model by MEGA version 6.0 (Tamura et al. 2011).

### RNA extraction, cDNA synthesis, and RT-PCR analysis

The RNAprep pure Tissue Kit (TIANGEN Biotech Co. Ltd., Beijing, China) was used to extract total RNA. Using 2 μg of total RNA per sample, the first-strand cDNA was generated using TIANScript RT Kit (TIANGEN) with the oligo $(dT)_{15}$ primer. For RT-PCR analysis, the *ApSpds* gene-specific primer pair LYQ118 (5′-CAAAA CAAAT GGTTT AAGGA-3′) and LYQ119 (5′-TGACA ACACA GAGGT AGGAA-3′) was used, which generated a 224 bp fragment. 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. PCR was carried out using the following conditions: 95 °C for 3 min; followed by 25 cycles of 45 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C; and a final extension at 72 °C for 7 min. The PCR products were analyzed on a 1.2% agarose. PCR products were purified from the gel and sequenced.

qRT-PCR was performed using the primer pair FqRT25 (5′-TTTCT TTCCT ACCTC TGT-3′) and RqRT26 (5′-TTCTA TCACC TTCTC ATCTA-3′) in a Roche Light Cycler 480 (Hoffmann-La Roche Ltd., Switzerland), under following condition: initial denaturation at 95 °C for 2 min; followed by 40 cycles of 15 s at 95 °C, 30 s annealing at 60 °C, 30 s extension at 68 °C; followed by a stage of 60–95 °C determine melting curves of the amplified products. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The experiment was carried out 3× for parallel measurements for each cDNA sample. A two-tailed Student's test was used to determine the statistical difference between the groups. All comparisons were considered significant at $p < 0.01$.

## Results

### Sequence analysis of the *ApSpds* gene

The complete cDNA sequence of the *ApSpds* gene was obtained from a pupal full-length cDNA library previously constructed in our laboratory (Li et al. 2009). The cDNA sequence and the deduced amino acid sequence of the *ApSpds* gene are shown in Fig. 1. The obtained cDNA sequence of the *ApSpds* gene is 1483 bp in length, having a 5′-untranslated region (UTR) of 140 bp, a 3′-UTR of 441 bp with a canonical polyadenylation signal sequence AATAAA. We also observed other three polyadenylation signal sequence AATAAA in its 3′-UTR sequence. It contains an ORF of 864 bp that encodes a polypeptide of 287 amino acids, with the predicted molecular weight of 32.32 kDa and isoelectric point of 5.074. Blastp search against NCBI showed that the amino acid sequence of this cDNA had 80% identity to the putative *B. mori* spermidine synthase (PF01564). Thus, we referred to the protein as *A. pernyi* spermidine synthase. The sequence data of the *ApSpds* gene have been deposited in GenBank under accession number KF955551.

S-adenosylmethionine-dependent methyltransferases (SAM or AdoMet-MTase) are enzymes that use S-adenosyl-L-methionine (SAM or AdoMet) as a substrate for methyltransfer, creating the product S-adenosyl-L-homocysteine (AdoHcy). There are at least five structurally distinct families of AdoMet-MTases, and class I is the largest and most diverse. Within class I, enzymes can be classified by different substrate specificities (small molecules, lipids, nucleic

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1                ACCAGTGTGACGACTAGCTTCGAAATACTACAAGTTTTAACCATTACCAGTTTCGCGGCGAACCA
66   TTGAAGTATCCTATTAATTACAACGTGGTCTGGACGTTAAACTTCAGTGTACATTTAAATTTTACTGGCCTATAA
141  ATGGATAAATTACAAAACAAATGGTTTAAGGAGTCATGTGAGATGTGGCCTGGTGCCACTTTTACTTTCGAAGTA
       M  D  K  L  Q  N  K  W  F  K  E  S  C  E  M  W  P  G  A  T  F  T  F  E  V
216  AAGGAAGTGCTCCAGTCAGGAAAATCACAGTACCAAAACATAGATGTGTTTGATACTACAAGCCTCGGTAGAGTT
       K  E  V  L  Q  S  G  K  S  Q  Y  Q  N  I  D  V  F  D  T  T  S  L  G  R  V
291  TTGGTGCTAGATGACATCATACAATGTACGCAGAAAGATGAATTTTCATACCAGGAAATGATTTCTTTCCTACCT
       L  V  L  D  D  I  I  Q  C  T  Q  K  D  E  F  S  Y  Q  E  M  I  S  F  L  P
366  CTGTGTTGTCACAAAAATCCAGAGAAGGTTTTAATAGTGGGCGGAGGAGACGGAGGTGTTGCAAGGGAAGTTGCA
       L  C  C  H  K  N  P  E  K  V  L  I  V  G  G  G  D  G  G  V  A  R  E  V  A
441  AAACATCCGAAAGTTAAAGAAATTGTTCAGGTAGAAATAGATGAGAAGGTGATAGAAGTATCTAAGAAATATTTA
       K  H  P  K  V  K  E  I  V  Q  V  E  I  D  E  K  V  I  E  V  S  K  K  Y  L
526  CAATTCATGGCGGTCGGTTTCGACAGTGAGAAACTCGAACTGCACGTCGGCGATGGCTTCGAATTTATGAAGAAT
       Q  F  M  A  V  G  F  D  S  E  K  L  E  L  H  V  G  D  G  F  E  F  M  K  N
601  CATTCCCAAGAATTTGACGTGATCATTACAGATAGTAGTGACCCCATAGGTCCGGCTGTGAATCTTTTTCTCGAG
       H  S  Q  E  F  D  V  I  I  T  D  S  S  D  P  I  G  P  A  V  N  L  F  L  E
676  AACTATTTTTCGCTGATGAAGAGCGCTCTAAAACCGAACGGAATAGTGTGCTCTCAAGCTGGGACGATATGGAAC
       N  Y  F  S  L  M  K  S  A  L  K  P  N  G  I  V  C  S  Q  A  G  T  I  W  N
751  GACTTGGATCTGGTGACCAGCACATTGGGTTACTGCAGGAACCAGTTCCCAGCGGCTGCATATGCTTACACCACT
       D  L  D  L  V  T  S  T  L  G  Y  C  R  N  Q  F  P  A  A  A  Y  A  Y  T  T
826  GTCCCAGCATACCCTTCAGGGCCCATAGGTTTCGTGATTGGCTCATTGGATAAGAACATAAAATTTGACCAGCCT
       V  P  A  Y  P  S  G  P  I  G  F  V  I  G  S  L  D  K  N  I  K  F  D  Q  P
901  ACATTGGTCTTCTCGCGGGAAGACGAGAAGGCGATGAATCTAAAATACTACAACAGCGATATCCACAAAGCGGCC
       T  L  V  F  S  R  E  D  E  K  A  M  N  L  K  Y  Y  N  S  D  I  H  K  A  A
976  TTTGTTTTACCAACGTTTGTGAAGTATAGGCTAGCCTGAAATTATTAACAACGTACTTAACTCTGCATTTATTGT
       F  V  L  P  T  F  V  K  Y  R  L  A  *
1051 TACGCCTCTAATGTTTTAATGCATAATATTGTTAATATACATATATAATGAAGCTCTGTTTTATGTTTGTTTGTT
1126 GTGGAGCTAATTTTATTTTTGAACGTTTTAAATAATGAAACATATTTTTTAGGTCTAAGTTCTACGAACAAAAGT
1201 ATATTTATGTACCTAGTTAATACAGTCATTTTAAGGATATATCAATAAGCGGTTGTATACTCCAGTGCCTTACAT
1276 TCCGTATCATTCTTAAGAGGTTTTTAAAATAAATTTTAAATATACAATATCAAATAGATGCAATTCACTAAAATC
1351 TCCATTGATTTTGAAATCTTTACTTATTGGATGTGGAACTTAGGCGATTTTAATAAAATTTATTATTTTAAATT
1426 AAATTATACTGTGAAATAAATGTATCTAATAAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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**Fig. 1** The cDNA sequence and deduced amino acid sequence of the *ApSpds* gene. The initiation codon ATG is bold, and the termination codon TAA is bold and marked with an asterisk. The canonical polyadenylation signal sequence AATAAA is doubled underlined

acids, etc.) and different target atoms for methylation (nitrogen, oxygen, carbon, sulfur, etc.). Conserved Domains prediction revealed that the amino acid sequence of the *ApSpds* gene belonged to class I of AdoMet-MTase family.

## Expression profiles of the *ApSpds* gene

We examined the tissue distribution and expression profiles of *ApSpds* during insect development. The sequencing analysis confirmed that a positive RT-PCR product was amplified from the *ApSpds* gene sequence. The *ApSpds* mRNA was expressed during four developmental stages, including the egg, larva, pupa and adult (Fig. 2a), suggesting that *ApSpds* plays an important role in the development of *A. pernyi*.

*ApSpds* mRNA was present in all tissues examined including the hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, spermaries, ovaries, brain and muscle (Fig. 2a). Among these tissues, the mRNA levels in the muscle were the most abundant. The qRT-PCR data further indicated that the relative mRNA expression level in the muscle was 1.658 times higher than in the fat body (significant, $p < 0.01$) (Fig. 2b).

The total RNA extracted from the fat body of *A. pernyi* pupae was further used to detect the levels of *ApSpds* transcript after exposure to high-temperature treatment. Our data showed that although *ApSpds* mRNA expression levels were reduced under high-temperature stress (Fig. 2c), the reduction was not significant suggesting that the *Spds* gene may not be involved in temperature stress tolerance in *A. pernyi*.

## Homologous alignment and phylogenetic analysis

In GenBank database, ApSpds protein homologs were found in bacteria, fungi, plants, invertebrates and vertebrates. In the present study, we chose 41 representative Spds protein sequences from bacteria (3), fungi (3), plants (10), invertebrates (16) and vertebrates (9) to assess their sequence similarity. Multiple sequence alignment revealed that ApSpds had 53–80% identity to Spds sequences from invertebrates including insects, 52–55% identity to vertebrates, 36–47% identity to plants, 45–48% identity to fungi, and 31% identity to bacteria (Fig. 3).

The 41 representative Spds sequences were further used to reconstruct their phylogenetic relationships (Fig. 4). In the phylogenetic tree based on ML method, all Spds sequences selected were divided into five groups corresponding to the known fungi, bacteria, plants, invertebrates and vertebrates. Within the invertebrate group, three lepidopteran Spds sequences from *D. plexippus*, *A. pernyi* and *B. mori* were clustered into a monophyletic subgroup, and the ApSpds sequence was found to be closely related to *B. mori* Spds with 80% confidence support. We also found that the Spds sequences could be clustered into the same branch according to their order, such as Diptera (*Drosophila melanogaster*,

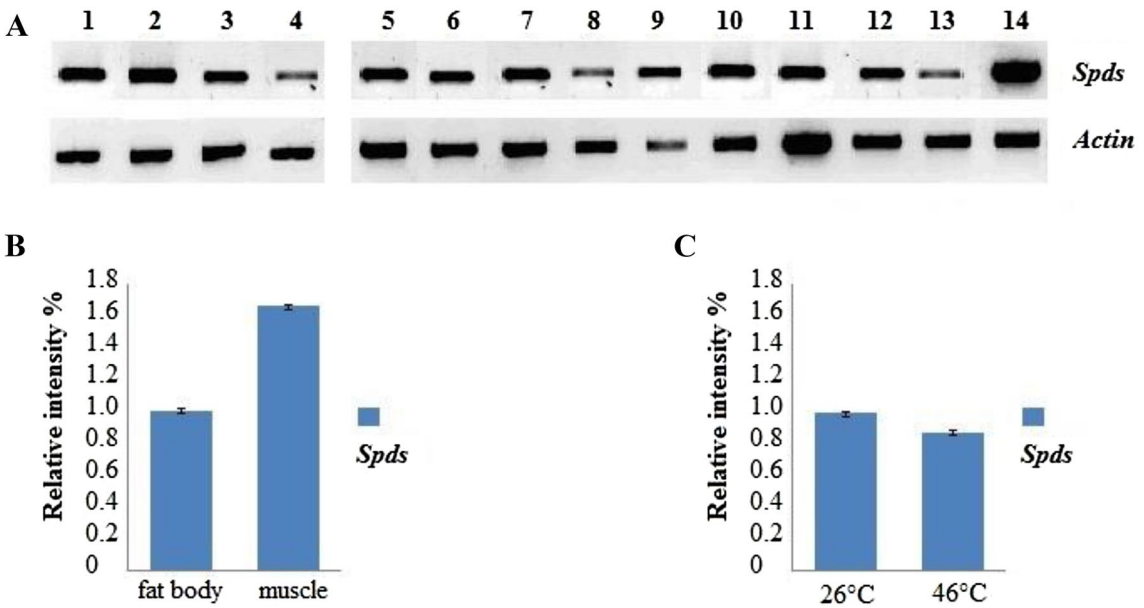


**Fig. 2** Expression patterns of the *ApSpds* gene. **a** The RT-PCR result. **b** The relative intensity in the fat body and muscle determined by qRT-PCR method. **c** Expression change in the fat body after heat shock determined by qRT-PCR method. The *actin* gene was used as an internal control. Lanes 1–4 show eggs at day 5, five-instar larvae, pupae, and adults, respectively. Lanes 5–14 show hemolymph, fat body, midgut, silk glands, integument, Malpighian tubules, testes, ovaries, brain, and muscle, respectively

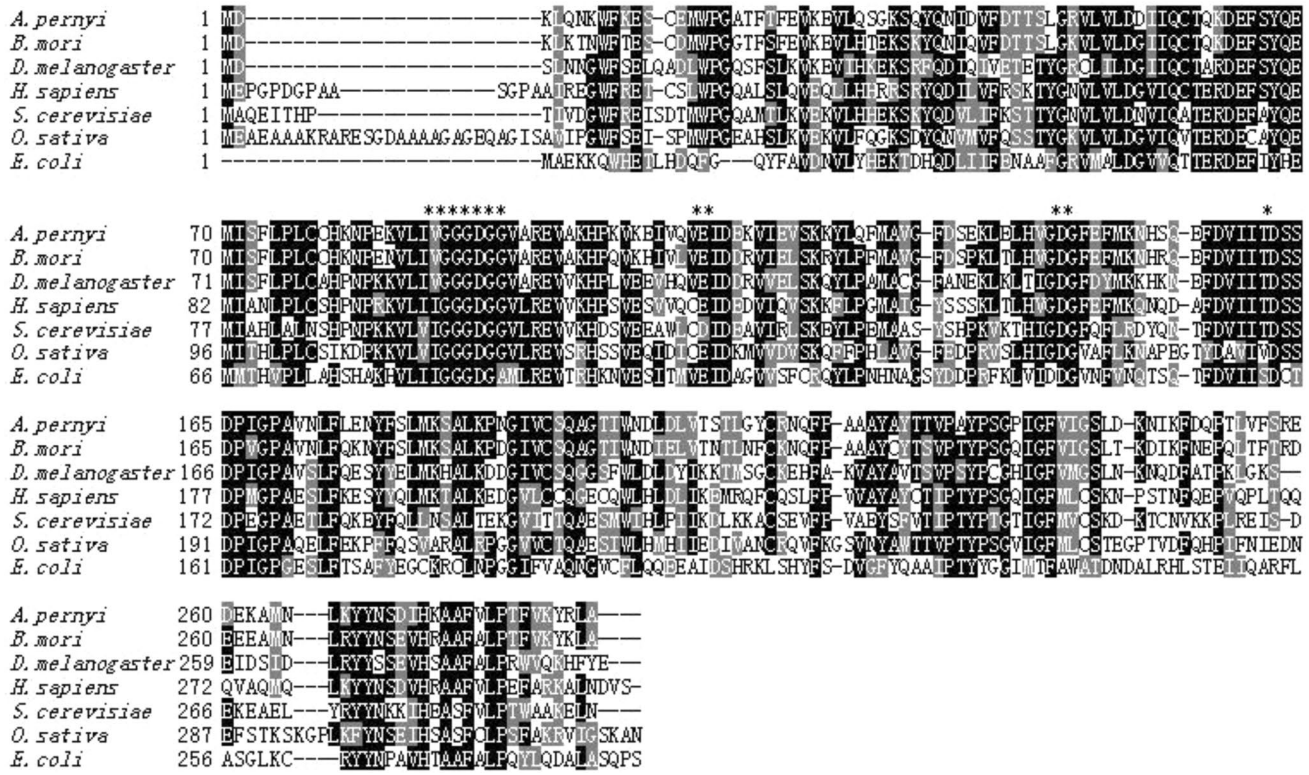


**Fig. 3** Sequence alignment of Spds proteins. The position of the catalytic active sites (asterisk) is marked. Identical amino acids are highlighted in black and positive amino acids are highlighted in gray. The protein sequences from *Antheraea pernyi* (AHK12775), *Bombyx mori* (GIBMGA005897), *Drosophila melanogaster* (AFH06323), *Homo sapiens* (NP_003123), *Saccharomyces cerevisiae* (AAC17191), *Oryza sativa* (EEE67009), and *Escherichia coli* (EFU53996) were aligned

*Aedes aegypti* and *Culex quinquefasciatus*), Hymenoptera (*Bombus impatiens*, *Apis mellifera* and *Megachile rotundata*), supporting the monophyly of Lepidoptera, Diptera, and Hymenoptera.

## Discussion

This is the first report on the characterization of spermidine gene in Lepidopteran insects. We found great similarity in 30–40 kDa molecular weight of ApSpds to known spermidine synthases from *E. coli*, *Thermotoga maritime* and *Nicotiana sylvestris*, which were reported to be around 30–40 kDa (Parvin et al. 2010). We also found a high sequence similarity of Spds in bacteria, fungi, plants, invertebrates and vertebrates suggesting that *Spds* gene is a highly conserved through evolution of life organisms.

Spermidine synthase belongs to the putrescine aminopropyltransferase (PAPT) family, which contains a signature sequence of V-(LA)-(LIV)(2)-G-G-G-X-G-X(2)-(LIV)-X-E (where X is any amino acid; PROSITE entry PS01330) (Hofmann et al. 1999). Sequence alignment of proteins in the aminopropyltransferase family enabled the identification of motifs and amino acid residues that are proposed to be important for catalytic activity (Korolev et al. 2002). The alignment of Spds amino acid sequences revealed that this pattern is also observed in insect Spds sequences.

The lepidopteran model insect *B. mori* is closely related to *A. pernyi*. ApSpds protein sequence shares 80% identity to *B. mori* Spds. Large-scale EST resource and extensive microarray information for this species are available on public domain SilkDB (Duan et al. 2010) and GenBank, respectively. The in silico gene expression analysis based on the available EST resource shows that the *B. mori Spds* gene is expressed in the egg, larva, pupa and adult. The microarray data available in SilkDB indicate that *B. mori Spds* gene (sw18965) is expressed in testis, ovaries, fat body, head, integument, midgut, hemolymph, malpighian tubules and silk glands. These in silico expression analysis of the *B. mori Spds* gene are in line with those observed by RT-PCR in *A. pernyi*. The expression of *Spds* gene during the developmental stages and in all tissues detected suggested that it plays an important role in development of silkworm.

Spermidine functions in stress signaling pathways and helps build up stress tolerance activity under stress conditions. Overexpression of spermidine synthase can

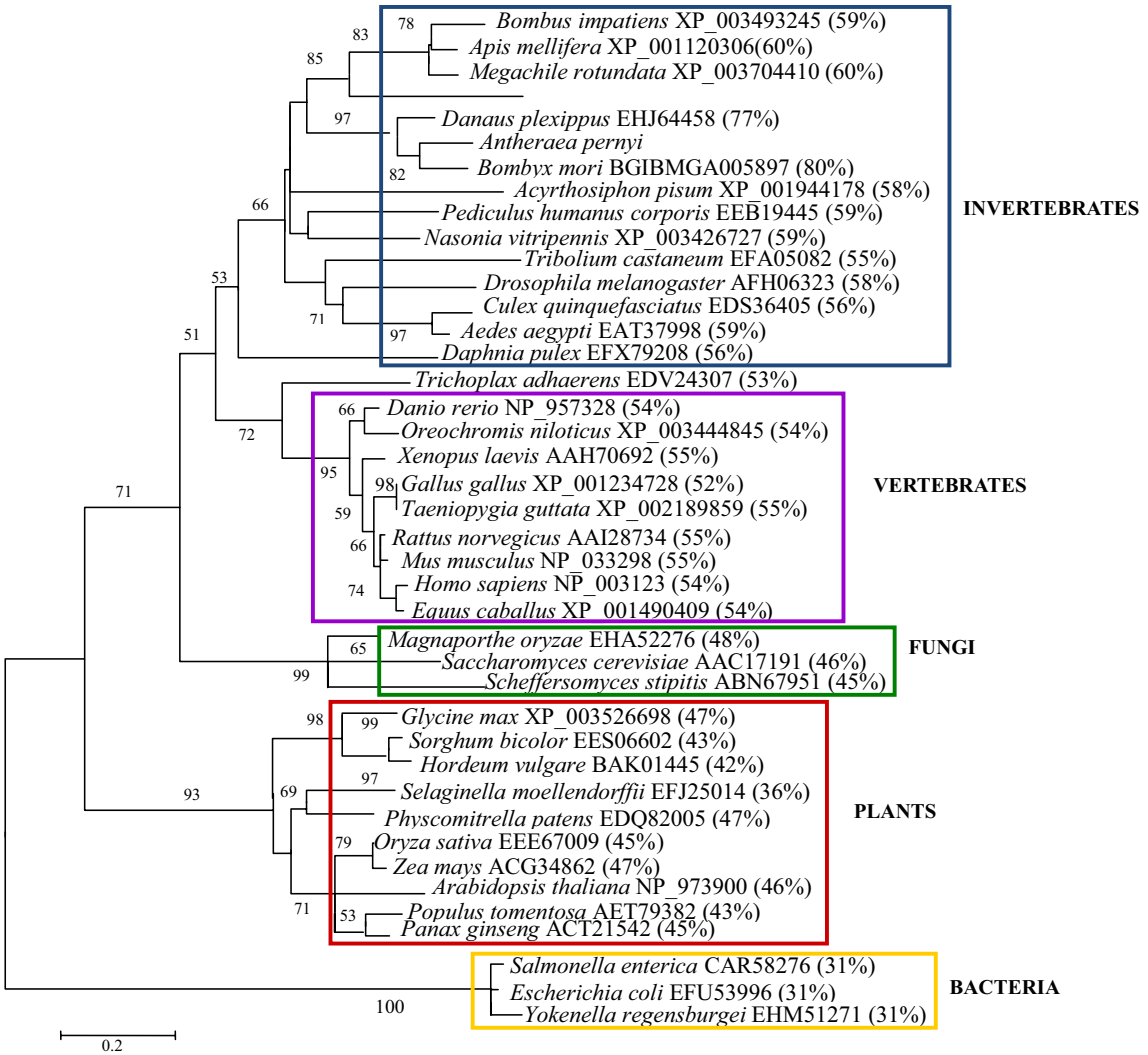


**Fig. 4** Phylogenetic tree based on the amino acid sequence comparisons of Spds proteins. Numbers at nodes represent bootstrap *p* values (> 50%). Public database accession numbers of Spds proteins are shown following the names of organisms. Identity (%) in parentheses following accession number is obtained by pairwise alignment of amino acid sequence of ApSpds with indicated Spds from other organisms

enhance tolerance to multiple environmental stress in some plants, such as *Arabidopsis thaliana*, *Citrus sinensis* and *Camellis sinensis* (Kasukabe et al. 2004; Fu and Liu 2013; Zhu et al. 2015). Analysis of expression profile of the *ApSpds* gene revealed a tissue-specific distribution, with the highest expression in the muscle. The muscles of insect, whose basic unit is the sarcomere, are the motors for powering all the external and internal movements (Perkins and Tanentzapf 2014). The muscular systems support insect body, help maintain insect posture, limbs movement, wings movement, ovipositor and viscera, close spiracles, operate various pumps including cibarial pump and the pumping of the poison glands, and generate heat by 'shivering' (Chapman 1998; Aagaard and Bangsbo 2006). Hence, the high expression of *Spds* in insect's muscles suggests its essential role in *A. pernyi* behavior.

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## Compliance with ethical standards

**Conflict of interest** All the authors declare that they have no conflict of interest.

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