

Original Research Article

A New Spore Wall Protein 9 Gene Cloned from *Nosema pernyi* (Microsporidia) Isolated from Chinese Oak Silkworm, *Antheraea pernyi*

ABSTRACT

Aims: China has plenty of oak trees, is the cradle of tussah (*Antheraea pernyi*) industry. Pebrine as a serious disease along with tussah rearing is difficult to solve. This pathogen named *Nosema pernyi*, which can infect the Chinese oak silkworm. The spores of *N. pernyi* have thick spore wall constructed by exospore and endospore. Spore wall proteins contact with host cells are related to microsporidia infection.

Methodology: In this study, we use the percoll gradient centrifugation method to purify spores of *N. pernyi*. Electron microscopy was used to detect the spore wall structure. A spore wall protein 9 gene was cloned and analyzed.

Results: A spore wall protein gene was cloned including an open reading frame (ORF) of 954 bp coding for a theoretical 317 amino acids protein. BLASTp showed a high amino acid sequence homology with spore wall protein in other microsporidia species. We named this gene NpSWP9, and a prokaryotic expression vector was constructed. Recombinant plasmid of NpSWP9-E1 Vector was transferred into Transetta (DE3). According to SDS-PAGE result, the molecular weight of the target protein was 38 kDa under the condition of IPTG for 5 h.

Conclusion: A new spore wall protein gene was identified in *N. pernyi* from *A. pernyi*. This research provided a good basis for further studies on cellular localization and immunodetection of NpSWP9 in *N. pernyi*.

Keywords: *Antheraea pernyi*; *Nosema pernyi*; spore wall protein

1. INTRODUCTION

Microsporidia are obligate, fungi-like, intracellular eukaryotic parasites. The infection-species ranged from invertebrates to vertebrates, such as humans, fish, silkworm, bees and other insects^[1]. Microsporidia has a close evolutionary relationship with Opisthokonta, and they belong to few species which have complete maturation and reproductive period in host cells. Most of the previous studies have indicated that they have a common ancestor with the fungus, but it is difficult to determine the exact nature of systematic development. Whole genome structure has proved that there are various similarities between microsporidia and zygomycete^[2]. More than 1500 species of microsporidia are found at present, about 14 genus can infect human^[3], especially the species *Eenterocytozoon* and *Encyphalitozoon*. The immune system of a healthy people can exclude the microporidia from body^[4,5]. Invertebrates, such as insects, they lack the acquired immunity, and have become the main hosts of microsporia^[3,6]. The genus *Nosema* is one of most representative species in the pebrine disease of silkworm. *Nosema bombycis*, parasitize in *Bombyx mori*, has ever caused the destruction of sericultural industry in France. Even today, the microsporidia remains a threat to the silk rearing, and it was the only quarantine pathogen during the egg production^[7]. They can cause a considerable loss in the silk production.

The main structure of the spherical microsporidia contains: electron-dense spore wall, protoplasts, nucleus and polar filament. Observing under an electron microscope, the spore wall consists of endospore wall with a very thin cytomembrane and exospore wall^[8]. The thick exospore wall can help them defense the innate immunity or adverse period in vitro. At present, there are two infecting ways founded: one is polar filament ejection, and another is swallowed by the hosts^[9]. When spore wall proteins destroyed, the infect ability of microsporidia has a significant decrease^[10]. Spore wall protein seems related to microsporidia infection. *N. bombycis* as the earliest discovered microsporidia, several spore wall proteins have been found and localized: SWP5, SWP7 and SWP9^[10]. SWP9 takes part in the form process of spore wall, and SWP7 is a scaffolding protein, which helps the SWP9 compete its function during the spore wall forming process, both were located in the exospore^[11]. Ultrathin immunofluorescence microscopy results showed SWP8, SWP12, SWP25, SWP26, SWP30 and SWP32 are spore wall proteins in *N. bombycis*^[9,12,13]. Research on the genus *Encephalitozoon* infect with human, several spore wall proteins have been reported in *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis*. Five exospore wall proteins were found, such as the SWP1 and SWP2 of *E. intestinalis*^[14], ExP1 of *E. cuniculi*^[15], EhSWP1a of *E. cuniculi* and EnSWP1b of *Encephalitozoon hellem*^[16]. Six endospore wall proteins were reported, EnP1 of *E. cuniculi* and *E. intestinalis* respectively^[17,18]; EnP2 and SWP3 of *E. cuniculi*, EcCDA of *E. cuniculi* and *E. intestinalis*^[19]. *Nosema pernyi* is a lethal pathogen that causes pebrine in Chinese oak silkworm, *Antheraea pernyi*^[20]. Using boiling method and SDS method, spore wall proteins were extracted from *N. pernyi*. Three different proteins numbered P35, P32 and P29 were preliminarily identified to be spore wall proteins by mass spectrometry. Peptide finger printing predicted that the primary structure of P32 was similar to nuclear pore proteins, suggesting its function related to selective permeability of spore wall, and P35 was similar to VASA2n, suggesting its involvement in spore propagation^[21]. Using the mass spectrometry analyze the 30 KDa protein of *N. pernyi*, one protein homologous to *N. bombycis* spore wall protein 8 was identified, and named NpSWP8. They have 90% identical and both of them had the typical heparin-binding motif^[22]. Another protein, SWP1 of *N. pernyi* was cloned and expressed in prokaryotic system in the later study^[23]. In this study, we cloned a new spore wall protein gene from *N. pernyi*, and a prokaryotic expression vector NpSWP9-E1 was constructed. This study on the spore wall protein will be a basis for the further studies on the microsporidia.

2. MATERIAL AND METHODS

2.1 REARING OF TUSSAH SILKWORM

The Chinese oak silkworm, *Antheraea pernyi* (Lepidoptera: Saturniidae), were reared in outdoor under normal condition on a diet of fresh *Quercus liaotungensis* plantation, at the oak tree garden of Shenyang Agricultural University (Shenyang, China) at 22-35°C and 30%-50% R.H and 13L+11D photoperiodic condition during autumn. The microsporidia infected larvae were collected by observation of black spots on surface.

2.2 MICROSPORIDIA SPORES PURIFICATION

N. pernyi were purified from the infected larvae using Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) density gradient centrifugation ultracentrifuge (Hitachi, Tokyo, Japan), as described previously^[24]. The purified spores were at the bottom layer, and washed by repeated centrifugation in PBS.

2.3 MORPHOLOGICAL OBSERVATION

N. pernyi were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After dehydration in an ethanol series and propylene oxide, the fragments were embedded in an Epon resin and sectioned using a Reichert-Jung ultramicrotome. Sections were stained with uranyl acetate and lead citrate and observed under a Transmission electron microscopy (JEOL JEM-1200EX, Tokyo, Japan) at an accelerating voltage of 600 kV.

2.4 GENE CLONING AND PROKARYOTIC EXPRESSION VECTOR CONSTRUCTION

Total RNA was extracted from *N. pernyi* using Trizol method (RNAiso plus, Takara, Japan). Reverse transcription was performed accord to the instruction of the PrimeScript® 1st Strand

86 cDNA Synthesis Kit (Takara, Japan). Primers for SWP9 gene were designed according to
87 EST data of *N. pernyi* constructed in our laboratory and other sequence of homologous
88 genes. The forward primer was SWP9-F: 5'-GGTTA TTCAT TTCAA CACTCG-3', and the
89 reverse primer was SWP9-R: 5'-TTTAA TTAGA TTAGG ATATA ACGTC-3'. The PCR
90 reaction mix comprised of 1 µl cDNA, 17.3 µl H₂O, 2.5 µl 10×PCR buffer, 2 µl 5 mM dNTP
91 mix, 1 µl of each 10 mM primer, and 0.2 µl DNA polymerase (Takara, Shiga, Japan).
92 Amplification was performed in a T100 Cyclor (Bio-Rad, California, USA) under the following
93 conditions: 94°C for 3 min, 32 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed
94 by a final extension at 72°C for 10 min. All amplification products were separated on a 1.2%
95 agarose gel, then the right size were ligated into the pMD18-T vector (Takara, Shiga,
96 Japan), and transformed into *E. coli* DH5α competent cells. Positive colonies were identified
97 by PCR and sequenced by the Sangon Biotech Co., Ltd (Shanghai, China). Open reading
98 frame (ORF) and deduce amino acid sequence were performed through the website
99 (<http://www.ncbi.nlm.nih.gov/>). Putative signal peptide was predicted using SignalP 4.1
100 Server (www.cbs.dtu.dk/services/SignalP/). The theoretical PI (isoelectric point) and MW
101 (molecular weight) of the mature protein were computed using Compute pI/MW
102 (http://www.expasy.org/tools/pi_tool.html). Homology analysis of the deduced amino acid
103 sequence was performed using the BLAST tool in GenBank (Blastp). Multiple sequence
104 alignments were performed using ClustalX software^[25], and the unrooted tree was generated
105 with TreeView Version 1.6.6^[26].

106 According to the sequence result, expression vector primer was designed: SWP9-EX -F: 5'-
107 ATGTC GTACA GAGTC AACTC TGC -3', SWP9-EX-R: 5'- TTAAT TAGAT GAGGA TAATA
108 ACGTC TTG -3'. Using the PrimeSTAR® HS DNA Polymerase (Takara, Japan), the PCR
109 product was amplified and linked to pEASY®-Blunt E1 Expression Vector (Transgene,
110 Beijing).

111 2.5 SDS-PAGE DETECTING NPSWP9 EXPRESSION

112 Recombinant plasmid was extracted and transferred into BL21. Cultivating the positive cells
113 to OD₆₀₀=0.5, adding IPTG (200 mg/ml) to induce the protein expression at 120 r·min⁻¹ after
114 5 h in 37°C. SDS-PAGE was performed observing the protein expression.

116 3. RESULTS AND DISCUSSION

117 3.1 MORPHOLOGICAL OBSERVATION OF SPORE WALL

118 Pebrine is a disease caused by microsporidia, which can cause larvae slow growth,
119 undersized body and poor appetite^[27]. The symptoms of pebrine silkworm appear tiny black
120 spots all over the surface of the insect body (Fig. 1). This disease can cause economic loss
121 in the tussah industry, always account for 20%-30% every year. In some years, serious
122 disease can lead to 90% reduction^[28]. This pebrine disease is due to parasitic microsporidia,
123 *N. pernyi*, belonging to the Nosema Genus. It is a lethal pathogen which is the only
124 quarantine disease in sericultural production because of horizontal and vertical
125 transmission^[7]. Longitudinal sections and cross-sections of spores revealed that the spore
126 wall consisted of an electron-dense exospore (EX) and electron-lucent endospore (EN) layer
127 by transmission electron microscopy. It seems thicker in the two polar of the spore, and the
128 EX is not smooth but full of wrinkles. The sporoplasm was enclosed by a plasma membrane
129 (Fig. 2).

130 Results should be clearly described in a concise manner. Results for different parameters
131 should be described under subheadings or in separate paragraph. Table or figure numbers
132 should be mentioned in parentheses for better understanding.

133 3.2 CDNA CLONING AND SEQUENCE CHARACTERIZATION OF NPSWP9

134 An open reading frame (ORF) of 954 bp cDNA sequence we named *NpSWP9* (GenBank
135 accession No. KJ210790) was cloned (Fig. 3). The deduced amino acid sequence has a
136 molecular weight of 37.16 KDa (MW) and an isoelectric point (pI) of 5.75. Bioinformatic
137 analysis revealed that the gene has no signal peptide. Blastp analysis indicated that this
138 protein is similar with other SWP9.

139 NpSWP9 from *N. pernyi*, other homologous proteins from *Nosema bombycis*, *Nosema apis*,
140 and 4 species of *Encephalitozoon* genus were performed using the Clustal X program.
141 Sequence alignment showed that the amino acid sequence of NpSWP9 is most similar to
142 NbSWP9 from *N. bombycis* with 78.98% identity. To assess the phylogenetic relationship
143 between NpSWP9 and other microsporidia spore wall protein 9 or similar proteins. The
144 unrooted phylogenetic tree was generated with TreeView (Version 1.6.6), and it is shown in
145 Fig. 4. The result indicated that different spore wall proteins were assigned to corresponding
146 groups related with different host.

147 3.3 EXPRESSION OF NPSWP9 FUSION PROTEIN

148 We constructed the recombinant expression plasmid NpSWP9-E1. *NpSWP9* gene was
149 linked with pEASY®-Blunt E1 Expression Vector (Fig. 5A). The recombinant vectors were
150 transformed into the *E. coli* transsetta (DE3) strains. After IPTG inducing, the positive clones
151 can express about 38 kDa theoretic proteins, which is in agreement with the size calculated
152 from the sequence (Fig. 5B). The result demonstrated that the spore wall proteins from
153 microsporidia can expressed in *E. coli* prokaryotic system.

154 Microsporidia as a group of eukaryotic intracellular parasites, they can infect most
155 vertebrates and invertebrates^[29]. They have a distinctive mechanism for infecting host cells.
156 There are two hypotheses about the invasion: long coiled polar tube can extrude from the
157 spores and penetrate the host cell membranes; another study showing phagocytosis
158 happened through spore wall protein recognition^[30]. Spore wall proteins (especially the
159 proteins in the exospore), as composition of rigid spore wall, they connect with the host cells
160 directly. They play an important role in the microsporidian invasion, but little is known about
161 the proteins' function. Anti-exospore antibody can reduce the microsporidia infection^[31]. We
162 list the majority spore wall proteins found in microsporidia from NCBI, most of them are
163 extract from silkworm (Table 1). SWP1 and SWP2 in *E. intestinalis* have cysteine-rich
164 polypeptides with similar N-terminal domains and with repeating amino acid units at C-
165 terminus^[14]. EnP1 and EnP2, as another two proteins found in *E. cuniculi*,
166 immunolocalisation data indicate that they are associated with the chitin-rich layer^[16]. But in
167 the later study, EnP1 protein is not limited to the endospore but it was also found on the
168 exospore surface and anchoring disk complex^[18]. SWP5 in *N. bombycis* is a spore wall
169 localized to the spore wall and interacts with the polar tube^[10]. This protein was identified in
170 *N. bombycis* using 2D-PAGE, spore phagocytosis assays indicated that NbSWP5 can
171 protect spores from phagocytic uptake by cultured insect cells. It may be involved in
172 structural integrity and modulating invasion^[32]. Other two proteins, SWP7 and SWP9, both
173 are found in the exospore, endospore and polar tube^[11]. These studies indicated that some
174 spore wall proteins may not only are the component spore wall, but also have other function
175 in the inner of spores.

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192 **Table 1. Reported spore wall protein genes in some microsporidia species**

Species	Name	location	Length (bp)	GeneBank accession number	Number of amino acids	MW (KDa)	pI	reference
<i>Encephalitozoon intestinalis</i>	SWP1	exospore	1370	AF355750.1	388	50	4.89	Hayman <i>et al.</i> , 2001
<i>Encephalitozoon intestinalis</i>	SWP2	exospore	3212	AF355749.1	1002	150	3.82	Hayman <i>et al.</i> , 2001
<i>Encephalitozoon cuniculi</i>	EnP1	endospore	1071	ECU01_0820	357	40.5	9.14	Peuvel <i>et al.</i> , 2001
<i>Encephalitozoon cuniculi</i>	EnP2	endospore	663	ECU01_1270	221	22.5	8.74	Peuvel <i>et al.</i> , 2001
<i>Nosema bombycis</i>	SWP5	exospore	561	EF683105.1	186	20.3	4.54	Li <i>et al.</i> , 2012
<i>Nosema bombycis</i>	SWP7	exospore & endospore	876	EF683107.1	291	32.8	4.50	Yang <i>et al.</i> , 2015
<i>Nosema bombycis</i>	SWP9	exospore & endospore	996	EF683109.1	331	42.8	6.19	Yang <i>et al.</i> , 2015
<i>Nosema bombycis</i>	SWP12	Not listed	687	ABV48900.1	228	26.6	6.49	Chen <i>et al.</i> , 2013
<i>Nosema bombycis</i>	SWP16	exospore	1152	EOB14338.1	383	44	9.17	Wang <i>et al.</i> , 2015
<i>Nosema bombycis</i>	SWP25	endospore	807	EF683102.1	268	30	8.08	Wu <i>et al.</i> , 2009
<i>Nosema bombycis</i>	SWP26	exospore	672	EU677842.1	223	51	5.55	Zhu <i>et al.</i> , 2013
<i>Nosema bombycis</i>	SWP30	endospore	837	EF683101.1	278	32	8.11	Wu <i>et al.</i> , 2008
<i>Nosema bombycis</i>	SWP32	exospore	951	EF683103.1	316	37.4	7.34	Wu <i>et al.</i> , 2007
<i>Nosema pernyi</i>	SWP1	endospore	869	KJ573111.1	278	32	7.02	Wen <i>et al.</i> , 2014
<i>Nosema antheraeae</i>	SWP8	Not listed	486	HQ170610.1	161	18.4	4.91	Zhang <i>et al.</i> , 2010
<i>Nosema antheraeae</i>	SWP12	Not listed	687	KC193258.1	228	26.5	6.49	Chen <i>et al.</i> , 2012

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194 **Fig. 1. The tiny black spots at integument of pebrine disease silkworm**

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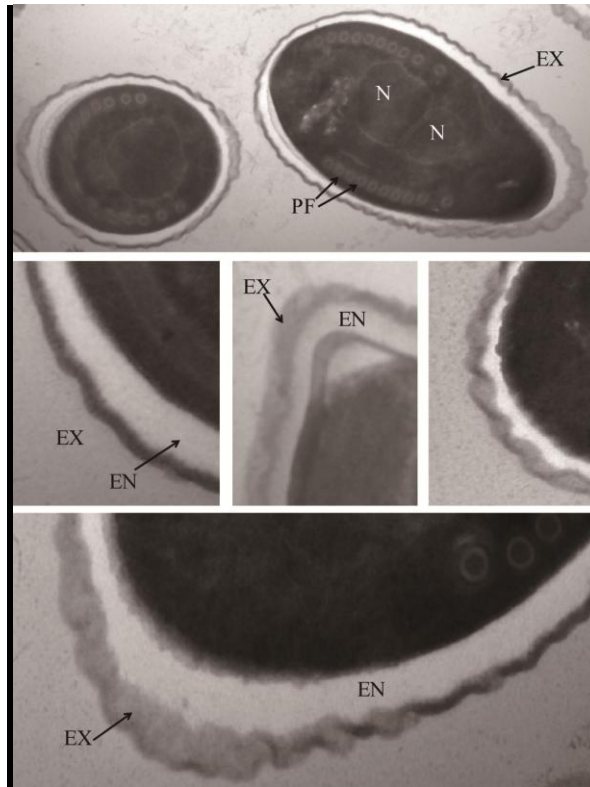
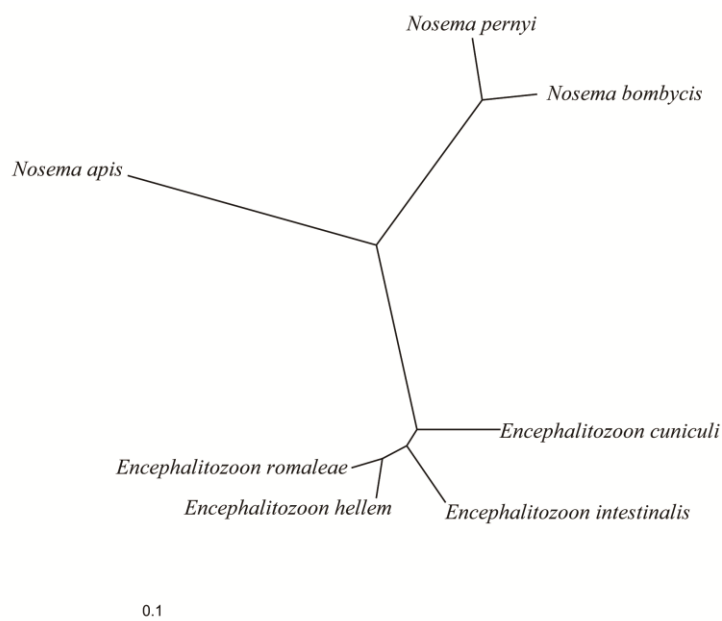


Fig. 2. The spore wall micrographs of *Nosema pernyi* under TEM.
Longitudinal section and cross-section of *N. pernyi* spores, showing the exospore (EX), endospore (EN), nucleus (N), polar filament (PF).

1 **ATG**TCGTACAGAGTCAACTCTGCTGATTCTGTTTATGATTAACGGAAGATACAAGAAGATTAATCCAAAAGCCTATCCAACTAGTGATGAA
M S Y R V N S A D S F M I N G R Y K K I N P K A Y P T S D E
91 AGAAATAAGTTCAATAAAATTTGCTCAAGAGGGAGATAAAGCTGTTCTTACTGCATATCCTTATCCAGAACATTTTTCTTTGGAACCAGAA
R N K F N K I A Q E G D K A V L T A Y P Y P E H F S L E P E
181 TGGCATTTTACACTTTGCGATAATGTTTATTTAACTTATGGAAAATAAGAGTCAATGTTTGTGAGTATTACAAGAAAAACAAGCAAGAT
W H F T L C D N V Y F N L W K I R V N V C E Y Y K K N K Q D
271 GACATGGTGGGTCTTGTACGCCACAGATGGAGCTAAAGTTGATCGTTTCTTTAGGCAAGTTCCTAACATGTCTGATGATGATTACAAG
D M V G L V T P P D G A K V D R F F R Q V P N M S D D D Y K
361 AAATTATTTTTTGATACTGATGAAAAACGGTAAAGGTTGGGCTTCAGCTGCCACTAAGTTTATTGATATTGTTCTTATGCTTATTGATAAA
K L F F D T D E N G K G W A S A A T K F I D I V L M L I D K
451 CCAGAAGATGCAAAATTAAGACGGTAAGCTTCAGAAAAATTCAAAAAGATCTTGAATCTTTGTTCTCAGTTGGGCTATTTCGGAT
P E E C K L K D G K L P E K F Q K D L E S F V S Q L G Y S D
541 GAAGATATGAAAAACATTGCTGATGAAATTCCTAATTTCTTTATTCAATTCGGAAGCTTCCCTACTGTCAATTCATTCTACTATCTAC
E D M K N I A D E I P N F F I Q F G K A F P T V I H S T I Y
631 TCCAGATTTTATTATTTCTTCTTGTCTTGACTATCAACGGAAATTTTGATTTTTCAGAAATCGAGAAATCTGAAGGAATGAAACTTGAG
S R F Y Y F F L F L T I N G N F D F S E I E K S E G M K L E
721 GAATTTAACAGACAAACCATGAAGGTTATGGCTACGGTATTGCTCAAATTTTCTCTAAAGTTTACGAAGAGAATTATACTACGAAAAT
E F N R Q T M K V M A T V F A Q I F S K V Y E E N Y N Y E N
811 AAGGATGCAGGTTTTATGGATAAAGTACTTTCTTATTTTCAAGTGTGAGTATAATATTGATAATGTTAGGGATCAATCCAAGAATATTGAG
K D A G F M D K V L S Y F S V S D N I D N V R D Q S K N I E
901 ACTGTGAAAAAGTTTTAACTCATAACAAGACGTTATTATCTCTATCTAAT**TAA**ATTTAGTTATTATTCAAAAAA
T V K K V L T H N K T L L S S S N *

Fig. 3. cDNA sequence and predicted amino acid sequence of *NpSWP9*.
The initiation codon ATG is bold, the termination codon TAA is bolded and marked with an asterisk.

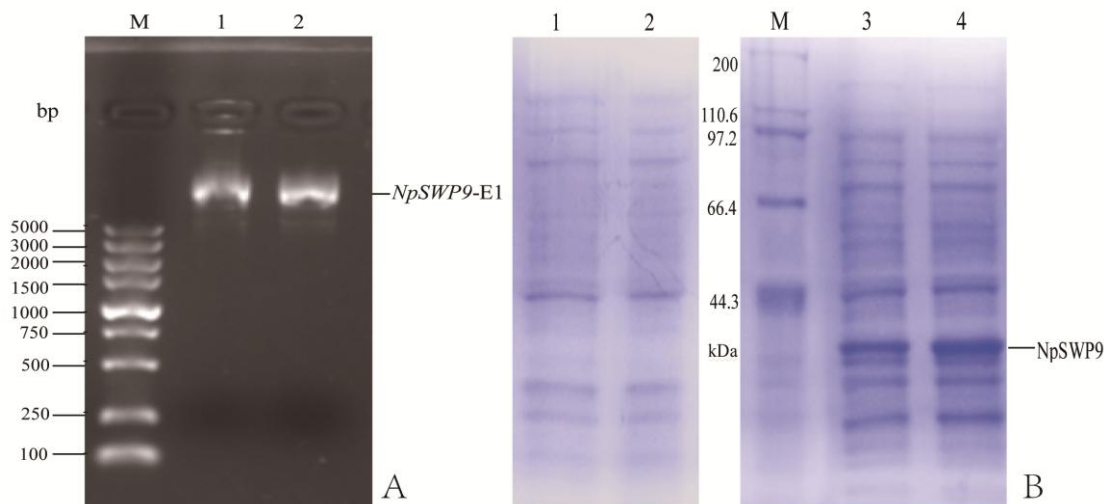
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209 **Fig. 4. Phylogenetic analysis of the NpSWP9 from *N. pernyi* and other microsporidia**
 210 **by using TreeView (version 1.6.6).**

211 The GenBank accession numbers of the homology proteins: *Encephalitozoon hellem*
 212 (XP_003887190.1), *Encephalitozoon romaleae* (XP_009264796.1), *Encephalitozoon intestinalis*
 213 (XP_003073171.1), *Encephalitozoon cuniculi* (AGE96659.1), *Nosema pernyi* (AJA32518.1),
 214 *Nosema apis* (EQB59678.1), *Nosema bombycis* (ABV48897.1).



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217 **Fig. 5. Recombinant NpSWP9-E1 Expression Vector (A) and SDS-PAGE detect**

218 **NpSWP9 protein expression (B).**

219 A: M, 5000 bp DNA marker; 1 and 2, recombinant NpSWP9 vector. B: 1 and 2, proteins of
 220 NpSWP9-E1 bacteria without induction; M, 200 kDa weight standard protein marker; 3 and 4,
 221 proteins of NpSWP9-E1 bacteria after induction.
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4. CONCLUSION

In this study, we have cloned a new spore wall protein named as NpSWP9 in Chinese oak silkworm parasite *N. pernyi*. Transmission electron microscopy was used to detect the shape of exospore and endospore. A recombinant expression plasmid of NpSWP9-E1 has successful constructed, and the recombinant protein can be expressed in *E. coli* prokaryotic system. This research provided a basis for further studies on cellular localization and immunodetection of NpSWP9 in *N. pernyi*. Research on the microsporidia (*N. pernyi*) will beneficial for us to control the pebrine disease in sericulture.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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