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

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YW and LQ designed the study. Authors YM and PL performed the experiment. Authors DW and WZ did some statistical analysis. Author YW wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: China has plenty of oak trees that form the cradle of tussah (*Antheraea pernyi*) industry. Pebrine is a serious disease along with tussah rearing and 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 used the percoll gradient centrifugation method to purify spores of *N. pernyi*. Electron microscopy was used to detect the spore wall structure. Recombinant prokaryotic expression vector was constructed and induced in *Escherichia coli*. SDS-PAGE and Western blot (WB) was performed to detect the protein expression.

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Results: A 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 (Isopropyl β-D-Thiogalactoside) for 5 h. According to the WB result, about 40 kDa band was detected by anti-HIS tag antibody.
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 invertebrate (humans, wild and domestic animals) to vertebrate (silkworm, bees and other insects) hosts [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 1300 species of microsporidia are found at present, about 17 species in 9 genera can infect human [3], especially the species *Enterocytozoon* and *Encephalitozoon*. The human microsporidiosis can be asymptomatic or cause a selflimiting diarrhea, mainly in immunocompetent persons. However, it may cause persistent diarrhea, weight loss and malabsorption in immunecompromised patients [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*, parasitizes to *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 (the spore) contains: electron-dense spore wall, polaroplast, nucleus, posterior vacuole and polar filament. Observing under an electronmicroscope, the spore wall consists of endospore wall with a very thin cytomembrane and exospore wall [8]. The thick exospore wall can help them protect against to the innate and adaptive immunity or adverse period in vitro (especially when the insect died). At present, there are two infecting ways: one is polar filament ejection, and another is phagocytosed by the host cell [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 (sporogony phase), and SWP7 is a scaffolding protein, which helps the SWP9 compete its function during the spore wall forming process, both were located in the exospores [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 exospores wall proteins were found, such as the SWP1 and SWP2 of *E. intestinalis* [14], ExP1 of *E. cuniculi* [15], EhSWP1a 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 for *E. cuniculi* and *E. intestinalis* [19].

Nosema pernyi is a lethal pathogen that causes pebrine in Chinese oak silkworm, *Antheraea pernyi* [20]. In a study 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 prokayotic 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. MATERIALS 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 by Transmission Electron Microscopy (TEM)

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 TEM (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 cDNA Synthesis Kit (Takara, Japan). Primers for SWP9 gene were designed according to EST (Expressed Sequence Tag) data of *N. pernyi* constructed in our laboratory and other sequence of homologous genes. The forward primer was SWP9-F: 5'-GGTTA TTCAT TTCAA CACTCG-3', and the reverse primer was SWP9-R: 5'-TTTAA TTAGA TTAGG ATATA ACGTC-3'. The PCR reaction mix comprised of 1 µl cDNA, 17.3 µl H_2O, 2.5 µl PCR buffer (10×), 2 µl 5 mM dNTPs, 1 µl of each primer (10 mM), and 0.2 µl DNA polymerase (Takara, Shiga, Japan). Amplification was performed in a T100 Cycler (Bio-Rad, California, USA) under the following 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 by a final extension at 72°C for 10 min. All amplification products were separated on a 1.2% agarose gel, then the right size were ligated into the pMD18-T vector (Takara, Shiga, Japan), and transformed into *E. coli* DH5α competent cells. Positive colonies were identified by PCR and sequenced by the Sangon Biotech Co., Ltd (Shanghai, China). Open reading frame (ORF) and deduce amino acid sequence were performed through the website (http://www.ncbi.nlm.nih.gov/). Putative signal peptide was predicted using SignalP 4.1 Server (www.cbs.dtu.dk/services/SignalP/). The theoretical PI (isoelectric point) and MW (molecular weight) of the mature protein were computed using Compute pI/MW (http://www.expasy.org/tools/pi_tool.html). Homology analysis of the deduced amino acid sequence was performed using the BLAST tool in GenBank (Blastp). Multiple sequence alignments were performed using ClustalX software [25], and the unrooted tree was generated with TreeView Version 1.6.6 [26].

According to the sequence result, expression vector primer was designed: SWP9-EX -F: 5'-ATGTC GTACA GAGTC AACTC TGC -3', SWP9-EX-R: 5'- TTAAT TAGAT GAGGA

TAATA ACGTC TTG -3'. Using the PrimeSTAR® HS DNA Polymerase (Takara, Japan), the PCR product was amplified and linked to pEASY®-Blunt E1 Expression Vector (Transgene, Beijing).

2.5 SDS-PAGE Detecting NPSWP9 Expression and Western Blot Analyses

Recombinant plasmid was extracted and transferred into BL21 (competent *E. coli* cells). Cultivating the positive cells to OD_{600}=0.5, adding IPTG (inducer of protein expression to 200 mg/ml) to induce the protein expression at 120 $r \cdot min^{-1}$ after 5 h in 37°C. SDS-PAGE was performed observing the protein expression. For western blot (WB), proteins were transferred to a PVDF membrane (0.45 μm pore size, GE Helthcare, USA). Anti-His mouse monoclonal antibody (1:10000) were used (Sangon, Shanghai, China). The blot was washed 3 times in PBST, then incubated for 2 h at room temperature with an Goat anti-mouse HRP conjugated (1:10000) secondary antibody (Sangon, Shanghai, China). The blot was revealed using the ECL system (Sangon, Shanghai, China) under ChemiDoc XRS+ (Bio-rad, CA, USA) [27].

3. RESULTS AND DISCUSSION

3.1 Morphological Observation of Spore Wall

The symptoms of pebrine silkworm appear tiny black spots all over the surface of the insect body (Fig. 1). Longitudinal sections and cross-sections of spores revealed that the spore wall consisted of an electron-dense exospore (EX) and electron-lucent endospore (EN) layer by TEM. It seems thicker in the two polar of the spore, and the EX is not smooth but full of wrinkles. The sporoplasm was enclosed by a plasma membrane (Fig. 2). Pebrine is a disease caused by microsporidia, which can cause larvae slow growth, undersized body and poor appetite [28]. This disease can cause economic loss in the tussah industry, always account for 20%-30% every year. In some years, serious disease can lead to 90% reduction [29]. This pebrine disease is due to parasitic microsporidia, *N. pernyi*, belonging to the *Nosema* Genus. It is a lethal pathogen which is the only quarantine disease in sericultural production because of horizontal and vertical transmission [7].

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

3.2 CDNA Cloning and Sequence Characterization of *NpSWP9*

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

NpSWP9 from *N. pernyi*, other homologous proteins from *Nosema bombycis*, *Nosema apis*, and 4 species of *Encephalitozoon* genus were performed using the Clustal X program. Sequence alignment showed that the amino acid sequence of NpSWP9 is most similar to NbSWP9 from *N. bombycis* with 78.98% identity. To assess the phylogenetic relationship between NpSWP9 and other microsporidia spore wall protein 9 or similar proteins. The unrooted phylogenetic tree was generated with TreeView (Version 1.6.6), and it is shown in Fig. 4. The result indicated that different spore wall proteins were assigned to corresponding groups related with different host. The *Encephalitozoon* always infected with human or mammal, they all in one clade, and the host of *Nosema* genus are insect, they form another clade.

3.3 Expression of NpSWP9 Fusion Protein

We constructed the recombinant expression plasmid NpSWP9-E1. *NpSWP9* gene was linked with pEASY®-Blunt E1 Expression Vector (Fig. 5A). The recombinant vectors were transformed into the *E. coli* transetta (DE3) strains. After IPTG inducing, the positive clones can express about 38 kDa theoretic proteins, which is in agreement with the size calculated

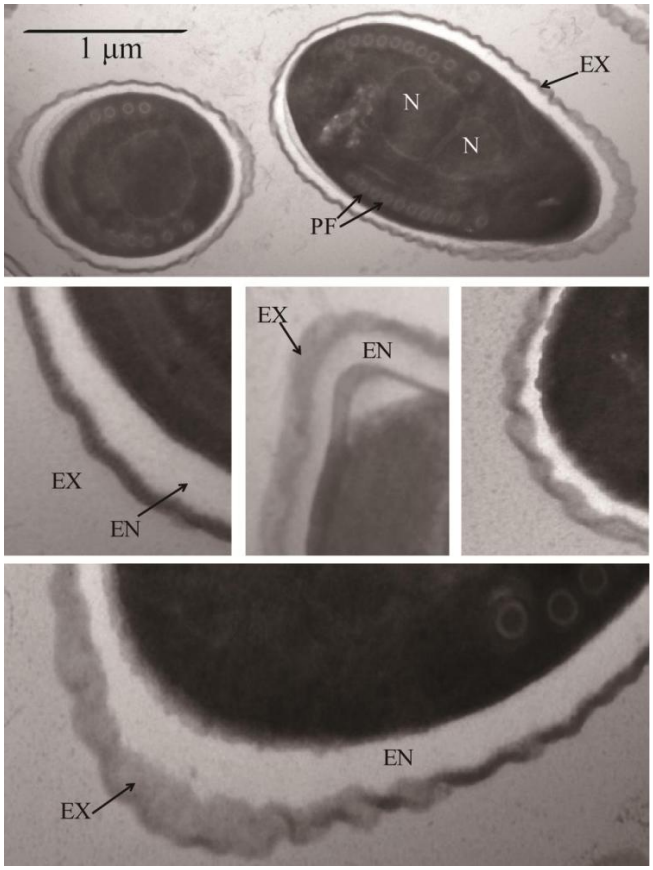

Fig. 2. The spore wall micrographs of *Nosema pernyi* under TEM
Longitudinal section and cross-section of N. pernyi spores, showing the exospore (EX), endospore (EN), nucleus (N), polar filament (PF).

from the sequence (Fig. 5B). According to the WB result, the recombinant bacteria could yield a target fusion protein with predicted molecular weight of about 40 kDa detected by anti-HIS tag antibody (Fig. 5C). The result demonstrated that the spore wall proteins from microsporidia can expressed in *E. coli* prokaryotic system.

Microsporidia as a group of eukaryotic intracellular parasites, they can infect most vertebrate and invertebrate hosts [30]. They have a distinctive mechanism for infecting host cells. There are two hypotheses about the invasion: long coiled polar tube can extrude from the spores and penetrate the host cell membranes; another study showing phagocytosis happened through spore wall protein recognition [31]. Spore wall proteins (especially the proteins in the exospore), as composition of rigid spore wall, they connect with the host cells directly. They play an important role in the microsporidian invasion, but little is known about the proteins' function. Some research has indicated that anti-exospore antibody can reduce the microsporidia infection [32]. We list the majority spore wall proteins found in microsporidia from NCBI, most of them are extract from silkworm (Table 1). SWP1 and SWP2 in *E. intestinalis* have cysteine-rich polypeptides with similar N-terminal domains and with repeating amino acid units at C-terminus [14]. EnP1 and EnP2, as another two proteins found in *E. cuniculi*, immunolocalisation data indicate that they are associated with the chitin-rich layer [16]. But in the later study, EnP1 protein is not limited to the endospore but it was also found on the exospore surface and anchoring disk complex [18]. SWP5 in *N. bombycis* is a spore wall protein localized to the spore wall and interacts with the polar tube [10]. This protein was identified in *N. bombycis* using 2D-PAGE, spore phagocytosis assays indicated that NbSWP5 can protect spores from phagocytic uptake by cultured insect cells. It

may be involved in structural integrity and modulating invasion [33]. Other two proteins from *N. bombycis*, are SWP7 and SWP9, both found in the exospore, endospore and polar tube [11]. These studies indicated that some spore wall proteins may not only are the component spore wall, but also have other function in the inner of spores.

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1   ATGTCGTACAGAGTCAACTCTGCTGATTCGTTTATGATTAACGGAAGATACAAGAAGATTAATCCAAAAGCCTATCCAACTAGTGATGAA
    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  AGAAATAAGTTCAATAAAATTGCTCAAGAGGGAGATAAAGCTGTTCTTACTGCATATCCTTATCCAGAACATTTTTCTTTGGAACCAGAA
    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 TGGCATTTTACACTTTGCGATAATGTTTATTTTAACTTATGGAAAATAAGAGTCAATGTTTGTGAGTATTACAAGAAAAACAAGCAAGAT
    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 GACATGGTGGGTCTTGTTACGCCACCAGATGGAGCTAAAGTTGATCGTTTCTTTAGGCAAGTTCCTAACATGTCTGATGATGATTACAAG
    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 AAATTATTTTTTGATACTGATGAAAACGGTAAAGGTTGGGCTTCAGCTGCCACTAAGTTTATTGATATTGTTCTTATGCTTATTGATAAA
    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 CCAGAAGAATGCAAATTAAAAGACGGTAAGCTTCCAGAAAAATTCCAAAAAGATCTTGAATCTTTTGTTTCTCAGTTGGGCTATTCGGAT
    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 GAAGATATGAAAAACATTGCTGATGAAATTCCCAATTTCTTTATTCAATTCGGAAAAGCTTTCCCTACTGTCATTCATTCTACTATCTAC
    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 TCCAGATTTTATTATTTCTTCTTGTTCTTGACTATCAACGGAAATTTTGATTTTTCAGAAATCGAGAAATCTGAAGGAATGAAACTTGAG
    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 GAATTTAACAGACAAACCATGAAGGTTATGGCTACGGTATTTGCTCAAATTTTCTCTAAAGTTTACGAAGAGAATTATAACTACGAAAAT
    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 AAGGATGCAGGTTTTATGGATAAAGTACTTTCTTATTTCAGTGTCAGTGATAATATTGATAATGTTAGGGATCAATCCAAGAATATTGAG
    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 ACTGTGAAAAAAGTTTTAACTCATAACAAGACGTTATTATCCTCATCTAATTAAATTTAGTTATTATTCAAAAAAAAAAAAAAAAAAA
    T  V  K  K  V  L  T  H  N  K  T  L  L  S  S  S  N  *
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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.

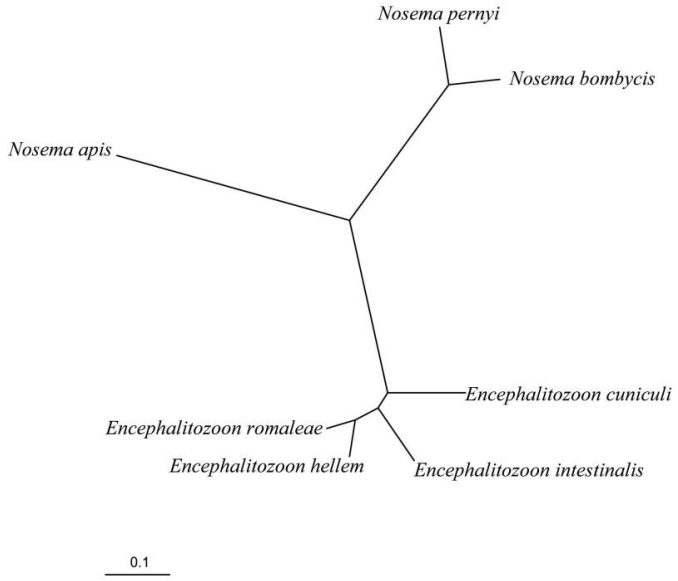

Fig. 4. Phylogenetic analysis of the NpSWP9 from *N. pernyi* and other microsporidia by using TreeView (version 1.6.6)
The GenBank accession numbers of the homology proteins: Encephalitozoon hellem (XP_003887190.1), Encephalitozoon romaleae (XP_009264796.1), Encephalitozoon intestinalis (XP_003073171.1), Encephalitozoon cuniculi (AGE96659.1), Nosema pernyi (AJA32518.1), Nosema apis (EQB59678.1), Nosema bombycis (ABV48897.1).

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

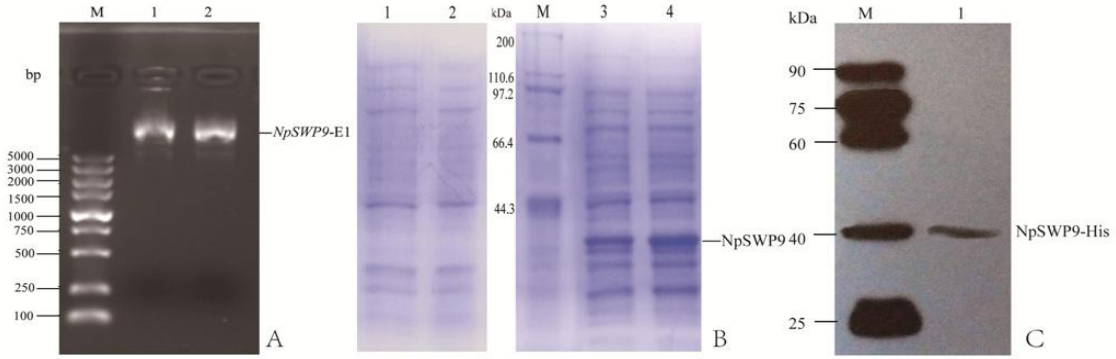

Fig. 5. Recombinant NpSWP9-E1 Expression Vector (A), SDS-PAGE detect NpSWP9 protein expression (B) and WB analysis using HIS-tag antibody

A: M, 5000 bp DNA marker; 1 and 2, recombinant NpSWP9 vector. B: 1 and 2, proteins of NpSWP9-E1 bacteria without induction; M, 200 kDa weight standard protein marker; 3 and 4, proteins of NpSWP9-E1 bacteria after induction. C: 90 kDa weight western marker; 1, western blot analysis of NpSWP9.

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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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