International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 14 (3), pp. 001-006, March, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Author(s) retain the copyright of this article.

Full Length Research Paper

Development of a PCR-based method for detection of *Nosema pernyi*

Yi-Ren Jiang[1,2], Zhen-Hua Deng[2], Sheng-Lin Shi[2], Rui-Sheng Yang[2], Yan-Zhuo Li[2], Yu-Xi Duan[1*] and Li Qin[2*]

[1]College of Plant Protection, Shenyang Agriculture University, Shenyang 110866, China.
[2]College of BioScience and Biotechnology, Shenyang Agriculture University, Shenyang 110866, China.

Accepted 10 October, 2019

***Nosema pernyi* is the lethal pathogen of pebrine disease in *Antheraea pernyi*. We have developed a PCR-based method for detection of *N. pernyi* using specific primers. The primers were designed by the reported conserved regions of microsporidian SSU rRNA. When the genomic DNA of *N. pernyi* was used as the DNA template, the specific DNA sequences were amplified by PCR. It was observed that PCR diagnosis of *N. pernyi* using the three sets of primers provided increased specificity and sensitivity when compared with light microscopy**.

Key words: *Nosema pernyi*, *Antheraea pernyi*, pebrine disease, spores, PCR, detection.

INTRODUCTION

Microsporidia are unicellular parasites commonly found in invertebrates. The infective stage of these parasites is the spore, which possesses a rigid cell wall that protects the parasite outside its host (Lujian et al., 1998). The pathogenesis of pebrine disease is that the hosts are infected by microsporidia. Nowadays, the loss of sericulture production caused by pebrine disease is increasingly serious, but the common technique of inspection on pebrine disease is still the conventional light microscopy detection, the accuracy and efficiency of which are hard to control. It is difficult to diagnose pebrine disease without using laboratory equipment, so numerous methods have been developed for the diagnosis of microsporidia in hosts, such as electron microscopy (Liu et al., 1973; Cheung et al., 1995), staining (Weber et al., 1992; Liu et al., 2007), serology (Knell et al.,1978; Guo et al., 1994; Shamim et al., 1997; Tang et al., 1999), *in situ* hybridization (Velasquez et al., 1999; Wei et al., 2005), etc. However, these methods are not feasible for routine diagnosis due to their expensiveness and the fact that they are time consuming.

With the development of molecular biology, molecular method became an alternative method for the laboratory diagnosis of microsporidiosis. The small subunit rRNAs of microsporidia are highly conserved (Vossbrinck et al., 1987; Weiss et al., 1999; Huang et al., 2004; Liyama et al., 2004), so molecular techniques based on PCR and the amplification of ribosomal RNA gene fragments, seem to be superior because of their high specificity and sensitivity as well as their ability to detect all microsporidian developmental stages (Kawakami et al., 2001; Hester et al., 2002; Klee et al., 2006).

Nosema pernyi is the lethal pathogen of pebrine disease in *Antheraea pernyi*. Pebrine disease has specific negative effects on *A. pernyi*, there are much serious economic loss in the process of breeding *A. pernyi* in China every year. Now, the spread of this disease alarmed the sericulture, thus studies on the disease became the focus of everyone's attention.

Due to much growth in the inspection of microsporidian spores in recent years, molecular methods were developed to improve accurate diagnosis of *N. pernyi* in the laboratory. Here, we present a rapid, specific, and sensitive method for the detection of *N. pernyi* in *A. pernyi* by PCR amplification of partial rRNA gene

*Corresponding author. E-mail: duanyx6407@163.com, qinli1963@163.com. Tel: +86-24-88497163.

fragments.

MATERIALS AND METHODS

Preparation of spores of *N. pernyi*

We isolated and purified the spores of *N. pernyi* using the combined methods of differential centrifugation and percoll density gradient centrifugation. Percoll is an effective medium for cleaning microsporidian (Jouvenaz, 1981), so we used percoll as the medium, and compared the effect of percoll purification between single density gradient and discontinuous density gradient. We selected 30, 50 and 70% percoll as the single density gradient, and 25, 50, 75% and 25, 50, 75, 100% percoll as the discontinuous density gradient.

Tubes containing the spore-percoll mixture were centrifuged at -4 at 15,000 rpm for 30 min. The pellet, which contained purified spores, was washed with sterile NANO pure water, centrifuged again at 1500 rpm for 3 min, and resuspended in sterile NANO pure water. A hemacytometer (Wolk et al., 2000) was used to count the spores. The spore concentration was adjusted to 10^9 spores•mL^{-1} of water and the spores were recounted to ensure that the spore density was correct and stored at 4°C until use.

DNA preparation

DNA was prepared from the frozen tissue specimens of the infected *N. pernyi* and healthy by incubation at 55°C for 4 h in TE buffer (10 mmol•L^{-1} Tris-HCl, pH 8.0, 10 mmol•L^{-1}EDTA, pH 8.0) containing 100 mmol•L^{-1} NaCl, 2% SDS, 0.039 mol•L^{-1} DTT and proteinase K (100 µg•mL^{-1}) followed by phenol-chloroform extraction involving RNase (100 µg•mL^{-1}) and ethanol precipitation. DNA prepared by ethanol precipitation was resuspended in TE buffer, and extracted samples were kept at -20°C until use.

PCR amplification

Three sets of primers were used: (1) P_1,5'- CACCA GGTTG ATTCT GCCTG AC -3' and P_2, 5'- GCAAC CATGT TACGA CTTAT ATCAG A -3'; (2) N_1, 5'- GTTGA TTCTG CCTGA GGTAG AC -3' and N_2, 5'- CAATG GTATC TAATC ACCTT CG- 3'. (3) P_{11}, 5'- CACCA GGTTG ATATT GCCTG AC- 3' and P_{22}, 5'- GCAAC CATTA TACGA CTTAT AT- 3'. Primer P_1/P_2 was designed from the sequence of a gene of small subunit rRNA of *Vairimorpha necatrix* (Vossbrinck *et al.*, 1987). Primer N_1/N_2 was designed from a partial sequence of SSU rRNA gene of *Nosema* sp. (Wang et al., 2001). Primer P_{11}/P_{22} was design from a partial sequence of SSU rRNA gene of microsporidia in this study.

25 µL PCR mixture that contained 0.2 µM of primers, 0.2 mM of dNTPs, 1×buffer, 2 mM $MgCl_2$, 1 units Taq DNA polymerase (Takara, Dalian China), appropriately diluted template DNA (approximately 50 ng) was used. The PCR procedure started for an initial 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, followed by a final extension of 5 min at 72°C.

Analysis of PCR products by agarose gel electrophoresis

Amplified PCR products were electrophoresed through 1.0% agarose gel with DNA marker (DL2000), stained with ethidium bromide, and visualized using UV illumination. A digital image of each gel was analyzed using a software program by Bio-Rad.

RESULTS AND DISCUSSION

Purification of spores of *N. pernyi*

As shown in Table 1, the purification effect of percoll discontinuous density gradient centrifugation is better than that of single density gradient centrifugation. Purification effect of discontinuous density gradient 25, 50, 75, 100% is better than that of 30, 50, 75%. The results of discontinuous density gradient 25, 50, 75, 100% tended to higher purity and more numbers of spores of *N. pernyi*. Two purification methods showed a similar trend that the layer closer to the tube bottom is purer and more, the middle layer less and the first layer least or not even. The purity of the strip layer near the tube bottom is from 91.8 to 95%.

Primer specificity

As shown in Figure 1, DNA from spores of *N. pernyi* was well extracted. The DNA band is clear, bright, good in integrity, and no tailing phenomenon. As shown in Figure 2, primer (P_1/P_2) could amplify only DNA of the partial small subunit rRNA of *N. pernyi*. Moreover, it never gave an amplification product when DNA from *A. pernyi* uninfected by *N. pernyi* was used as template (Lane 1-3). This primer pair seems to be specific for *N. pernyi*.

As expected, *N. pernyi*, *A. pernyi* and moth infected by *N. pernyi*, and mixture of *N. pernyi* and *A. pernyi* infected by *N. pernyi* gave clear amplicons of the size (600 bp) when primer pair N_1/N_2 was used (Lane 4-8). Primers P_{11} and P_{22} gave an amplification product from the DNAs of *A. pernyi* infected by *N. pernyi*, purified *N. pernyi*, moth infected by *N. Pernyi* (Lane 9, 11 and 12). The length of the products was estimated to be 1500 bp. Meanwhile, DNA sample from *A. pernyi* uninfected by *N. pernyi* and no template DNA gave any PCR products (Lane 10, 13).

Primer sensitivity

In recent years, the sensitivity of detection of microsporidia using molecular techniques based on PCR has been studied and reported (Qin et al., 2002; Pan et al., 2007). We selected primer pair N_1/N_2 to investigate the sensitivity of detection of *N. pernyi*. Different contents of DNA from *N. pernyi* spores were diluted with ddH_2O from 960 ng down to 0.47 ng experimentally. Figure 3 shows that primer N_1/N_2 generated an amplicon of 600 bp when the content of DNA from *N. pernyi* spores was at least 0.47ng in the reaction volume of 25 µL. However, there is no PCR products in *A. pernyi* uninfected by *N. pernyi* even if its content of DNA was up to 1310 ng.

Molecular method is an alternative method for the laboratory diagnosis of microsporidiosis. Molecular techniques based on PCR seem to be superior because of their high specificity, so in recent years many

Table 1. Purification results of the spores of *N. pernyi.*

Percoll gradient	Percoll concentration (%)	Mix with spores	Samping sites	Average of spores	Average of impurity	Purity (%)
Single density	30	No	First layer	5.6	11.0	33.7
			Second layer	8.4	7.8	52.0
	50	No	First layer	8.8	11.0	44.4
			Second layer	13.0	6.4	67.0
	70	No	First layer	17.4	11.6	60.0
			Second layer	10.2	1.0	91.1
	50	Yes	First layer	2.8	3.0	48.3
			Second layer	6.8	4.4	60.7
	70	Yes	First layer	3.4	2.6	56.6
			Second layer	7.4	4.0	65.0
Discontinuous density	30, 50, 70	—	First layer	2	8	20.0
		—	Second layer	4	5	44.4
		—	Third layer	112	10	91.8
Discontinuous density	25, 50, 75, 100	—	First layer	0	9	0
		—	Second layer	50	24	67.6
		—	Third layer	135	10	93.1
		—	Fourth layer	228	12	95.0

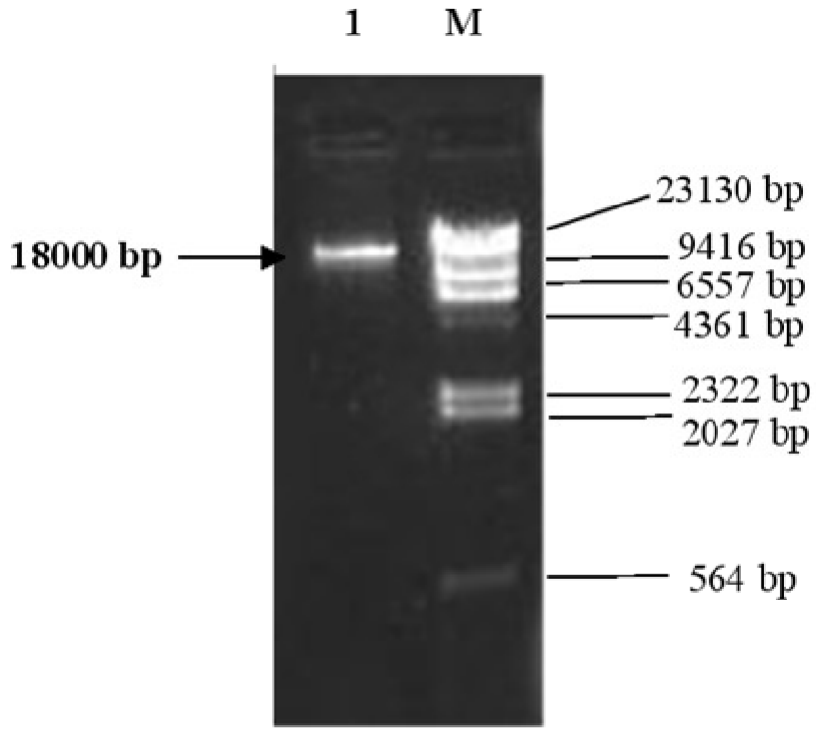

Figure 1. Agarose gel electrophoresis result of Genomic DNA of *N. pernyi* (Lane M, marker (DL 2000); Lane 1, purified *N. pernyi*).

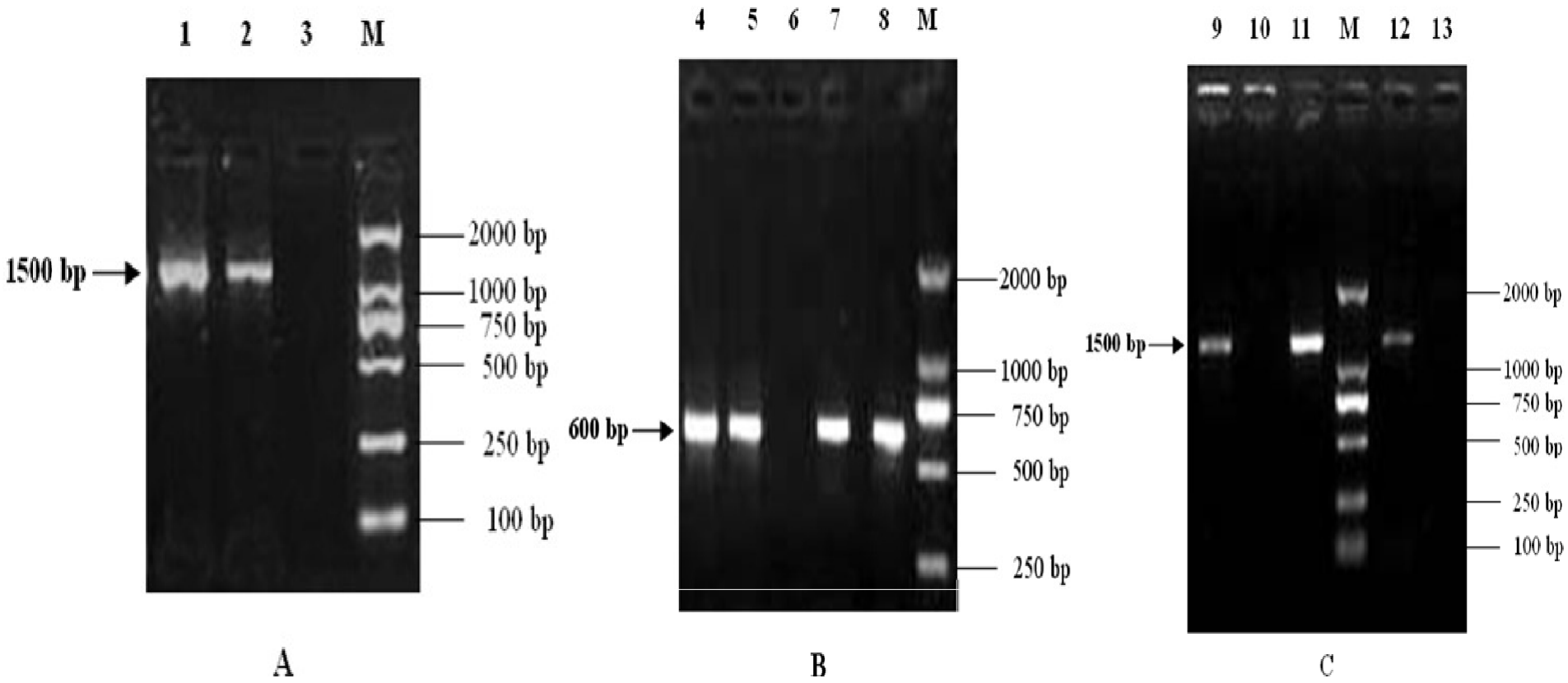

Figure 2. PCR amplicons of *N. pernyi*, *A. pernyi* infected by *N. pernyi*, and *A. pernyi* uninfected by *N. pernyi* generated with primers. Figure 2A, Primer P_1/P_2; Figure 2B, Primer N_1/N_2; Figure 2C, Primer P_{11}/P_{22}. (Lane M, marker (DL 2000) ; Lane 1, purified *N. pernyi*; Lane 2, *A. pernyi* infected by *N. pernyi*; Lane 3, *A. pernyi* uninfected by *N. pernyi*; Lane 4, purified *N. pernyi*; Lane 5 mixture of purified *N. pernyi* and *A. pernyi* uninfected; Lane 6, *A. pernyi* uninfected by *N. pernyi*; Lane 7, *A. pernyi* infected by *N. pernyi*; Lane 8, tussah moth infected by *N. pernyi*; Lane 9, *A. pernyi* infected by *N. pernyi*; Lane 10, *A. pernyi* uninfected by *N. pernyi*; Lane 11, purified *N. pernyi*; Lane 12, tussah moth infected by *N. pernyi*; Lane 13, no template DNA)

inspection methods based PCR were reported in microsporidia which infects the hosts, such as *Bombyx mori*, bumble bees and other economic insects (Kawakami et al., 2001; Liu et al., 2004; Klee et al., 2006).

In this paper, all three primer pairs gave amplification products when DNAs from purified spores of *N. pernyi* and infected host tissue were used as template, and never gave PCR products from DNAs of *A. pernyi* uninfected by *N. pernyi*. Thus, the three sets of primers are expected to show high specificity to *N. pernyi*. The diagnosis technique of pebrine disease of *A. pernyi* based PCR will have broader utilization prospects in sericulture. Its application could focus on the early detection of inspection.

ACKNOWLEDGEMENT

This work was supported by grants from the National Modern Agriculture Industry Technology System Construction Project (Silkworm and Mulberry) and the Scientific Research Project for Education Department of Liaoning Province (L2010512)

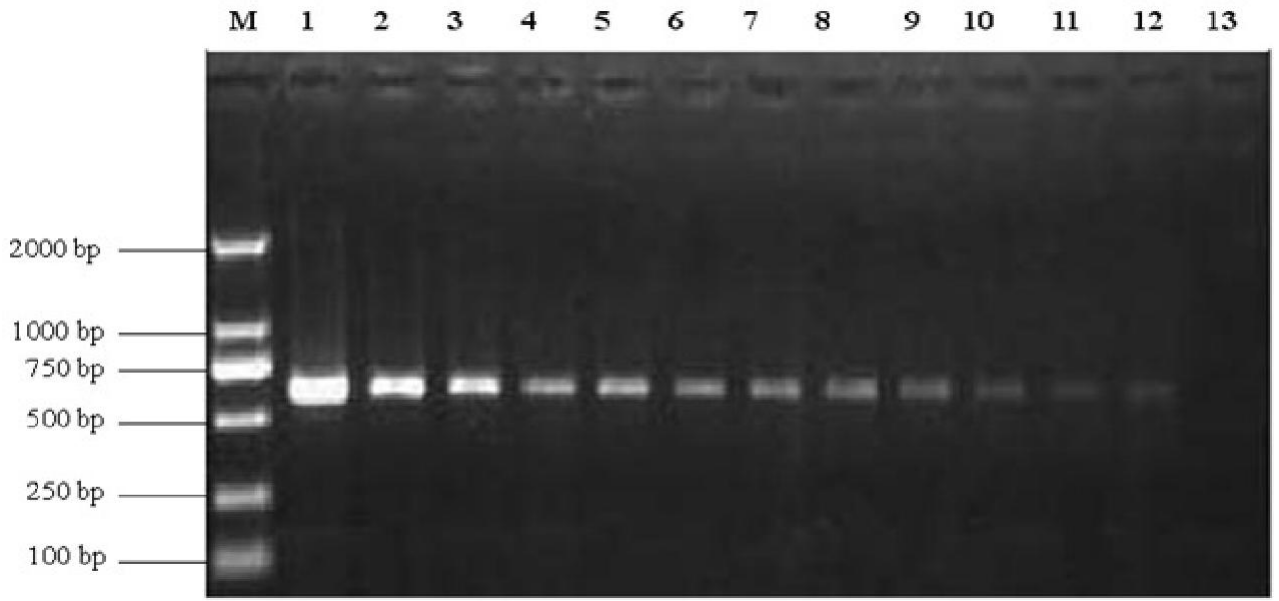

Figure 3. PCR products of a fixed number of DNA from *N. pernyi* spores (from Lane 1 to Lane 12: 960 ng, 480 ng, 240 ng, 120 ng, 60 ng, 30 ng, 15 ng, 7.5 ng, 3.75 ng, 1.88 ng, 0.94 ng, 0.47 ng, respectively), amplified with the primer pairs N_1/N_2 (expected amplicon length 600 bp). Spores were diluted with ddH_2O. Lane M, marker (DL 2000); Lane 13, *A. pernyi* uninfected by *N. pernyi* (1310 ng).

REFERENCES

Cheung WWK, Wang JB (1995). Electron microscopic studies on *Nosema mesnili* Paillot (Microsporidia: Nosematidae) infecting the Malpighian tubules of *Pieris canidia* larva. Protoplasma, 186:142-148.

Guo XJ, Huang KW, Shen ZY (1994). Comparative studies on the serological relationships between Microsporidian spores from different places. Acta Sericologica Sinica, 20:154-157 .

Hester JD, Varma M, Bobst AM, Ware MW, Lindquist HDA, Schaefer FW (2002). Species-specific detection of three human-pathogenic microsporidial species from the genus *Encephalitozoon* via fluorogenic 5' nuclease PCR assays. Mol. Cell. Probes, 16: 435-444.

Huang WF, Tsai SJ, Lo CF, Yamane S, Wang CH (2004). The novel organization and complete sequence of the ribosomal RNA gene of Nosema bombycis. Fungal Gene. Biol., 41: 473-481.

Jouvenaz DP (1981). Percoll: an effective medium for cleaning microsporidian spores. J. Invertebr. Pathol., 37: 319.

Kawakami Y, Iwano H, Hatakeyama Y, Inoue T, Canning EU, Ishihara R (2001). Use of PCR with the specific primers for discrimination of *Nosema bombycis*. J. Sericult. Sci. Japan, 70: 43-48.

Klee J, Tay WT, Paxton RJ (2006). Specific and sensitive detection of *Nosema bombi* (Microsporidia: Nosematidae) in bumble bees (*Bombus spp.*; Hymenoptera: Apidae) by PCR of partial rRNA gene sequences. J. Invertebr. Pathol., 91: 98-104.

Knell JD, Zam SG (1978). A serological comparison of some species of microsporida. J. Invertebr. Pathol., 31:280-288.

Liu HJ, Liu TP (1973). Scanning electron microscope observations on the spore of *Nosema apis* Zander. Tissue and Cell, 5:581-584.

Liu JP, Cao Y, Smith JE, Xu XY (2004). Studies on the application of PCR molecular diagnosis to silkworms with simulated pebrine disease. Scientia Agriculture Sinica, 37: 1925-1931.

Liu JP, Zeng L (2007). Staining and discrimination of *Nosema bombycis* spores with Calcofluor White M2R. Acta Entomologica Sinica, 50:1185-1186.

Liyama K, Chieda Y, Yasunaga-Aoki C, Hayasaka S, Shimizu S (2004). Analyses of the ribosomal DNA region in *Nosema bombycis* NIS 001. J. Eukaryot Microbiol., 51: 598-604.

Lujan, HD, Conrad JT, Clark CG, Touz MC, Delbac F, Vivares CP, Nash TE (1998). Detection of microsporidia spore-specific antigens by monoclonal antibodies. Hybridoma, 17: 237-243.

Pan ZH, Gong CL, Zheng XJ (2007). Study on the detection sensitivity of PCR method for silkworm *nosema bombycis* DNA. J. Changshu Insti. Technol., 21: 51-54.

Qin QL, Miao L, Song XJ, Ren L, Li X, Zhang Q, Ding C (2002). Detect the Pebrine of insects rapidly and effectively Proceedings of Academy of the Entomological Society of China (ESC),. Beijing, China. pp. 177-181.

Shamim M, Debjani Ghosh, Baig M, Nataraju B, Datta RK, Gupta SK (1997). Production of Monoclonal Antibodies

Against *Nosema bombycis* and their Utility for Detection of Pebrine Infection in *Bombyx mori* L. J. Immunoassay Immunochem*.,* 18: 357-370.

Tang XC, Huang ZR, Ning B, Zhang CY (1999). Detection of the Spores of *Nosema antheraea* by Monoclonal Antibody Technique. Acta Sericologica Sinica*,* 5: 221-224.

Velasquez JN, Carnevale S, Labbe JH, Chertcoff A, Cabrera MG, Oelemann W (1999). In situ hybridization: a molecular approach for the diagnosis of the microsporidian parasite *Enterocytozoon bieneusi*. Hum. Pathol, 30: 54-58.

Vossbrinck CR, Maddox JV, Friedman S, Debrunner-Vossbrinck BA, Woese CR (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature, 326: 411-414.

Weber R, Bryan RT, Owen RL, Wilcox CM, Gorelkin L, Visvesvara GS (1992). Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. The Enteric Opportunistic Infections Working Group. N. Engl. J. Med., 326:161-166.

Wei YD, Zhang GZ, Lu CD (2005). Studies on Detection of *Nosema bombycis* by Hybridization *in situ*. Acta Sericologica Sinica, 31: 64-68

Weiss LM, Vossbrinck CR (1999). Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. In: Wittner M and Weiss LM, Editors. The Microsporidia and Microsporidiosis, ASM Press, Washington, DC, pp 129–171.

Wolk DM, Johnson CH, Rice EW, Marshall MM, Grahn KF, Plummer CB, Sterling CR (2000). A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon syn. Septata intestinalis*. Appl. Environ. Microbiol., 66: 1266-1273

.