Parasitol Res (2012) 111:263–269
DOI 10.1007/s00436-012-2829-6

ORIGINAL PAPER

Phylogenetic characterization of a microsporidium (*Nosema* sp. MPr) isolated from the *Pieris rapae*

Darui Chen · Zhongyuan Shen · Feng Zhu · Rui Guan · Jiange Hou · Jiao Zhang · Xiaofang Xu · Xudong Tang · Li Xu

Received: 31 October 2011 / Accepted: 13 January 2012 / Published online: 7 February 2012

Abstract Cabbage butterfly (*Pieris rapae*), included in the Lepidoptera genus, *Pieris* family, is the main pest that damages Cruciferae. In this paper, we reported a microsporidian isolate of *Nosema* species which was isolated from *P. rapae* in Zhenjiang City, Jiangsu Province, China. The mature spore of this microsporidium is long oval in shape and 3.8±0.3 × 2.0± 0.2 μm in size. Research results showed that the novel microsporidium cannot infect the BmN cell in vitro and silkworm larvae. The organization of rRNA gene was 5′-SSU rRNA-ITS-LSU rRNA-3′. Phylogenetic trees based on SSU rRNA and LSU rRNA gene sequences were constructed by MEGA 4.0 software. The topology showed that this microsporidium was on the same second branch of *Nosema* clade, and had close relationships to other *Nosema* species. Consequently, this microsporidium was confirmed to be a member of *Nosema* genus, and named as *Nosema* sp. MPr.

Introduction

Microsporidia are obligate intracellular parasites that can infect almost all animals from invertebrates to vertebrates, and are pathogen of insects, fishes, rodents, and human (Baker et al. 1995; Hatakeyama et al. 1997; Keeling et al. 2000; Rao et al. 2005; Rao et al. 2004; Tsai et al. 2003; Vávra et al. 2011; Waters et al. 2004). Currently considered to be basal fungi (Adl et al. 2005; James et al. 2006), more than 1,300 microsporidian species belonging to 160 genera have been reported (Corradi and Keeling 2009), some of which cause great damage to sericulture, apiculture, and aquaculture (Fries et al. 1984; Gresoviac et al. 2000).

There are other microsporidia besides *Nosema bombycis* that infect silkworm, *Bombyx mori*, which is generally thought to be the result of cross infection of microsporidia in silkworm and other insects (Kishore et al. 1994; Bhat et al. 2009). Cabbage butterfly (*Pieris rapae*), included in Lepidoptera genus, *Pieris* family, is the main pest that damages Cruciferae, such as cabbage, rape, cauliflower, and so on. The rate of natural infection of microsporidia in *P. rapae* is high, and there is a great variety of pathogenic microsporidia in *P. rapae*.

Traditional taxonomic studies and species classification of the microsporidia were mainly based on biological characters, and DNA-based molecular markers, have, however, become increasingly important in microsporidian taxonomy (Franzen and Müller 1999; Weiss and Vossbrinck 1999), and the ribosomal RNA (rRNA) gene sequences are currently used in the classification of microsporidia (Hibbett et al. 2007; Ku et al. 2007). In this paper, we report a strain of *Nosema* species isolated from cabbage butterflies (*P. rapae*) in Zhenjiang City, Jiangsu Province, China. We present the sequences of small subunit (SSU) rRNA, internal transcribed spacer (ITS), the large subunit (LSU) rRNA, and their arrangement of the rRNA gene. We also determined its phylogenetic relationship by comparing rRNA sequences, length, and G+C content with that of other microsporidia.

D. Chen · Z. Shen · J. Hou · J. Zhang · X. Xu · X. Tang · L. Xu
Jiangsu University of Science and Technology,
Zhenjiang 212018, China

Z. Shen · F. Zhu · R. Guan · X. Tang · L. Xu
Sericultural Research Institute,
Chinese Academy of Agricultural Sciences,
Zhenjiang, Jiangsu Province 212018, China

Z. Shen (✉)
The Key Laboratory of Genetic Improvement of Silkworm and Mulberry of Agricultural Ministry,
Zhenjiang 212018, China
e-mail: szysri@yahoo.com.cn

Materials and methods

Isolation and purification of *Nosema* sp. MPr

Microsporidian spores of *Nosema* sp. MPr were isolated from the cabbage butterflies (*P. rapae*) collected from Zhenjiang City, Jiangsu Province, China and kept in the Sericulture Research Institute, Chinese Academy of Agricultural Sciences. The homogenates of adult were filtered through four layers of cheesecloth and centrifuged at 3,000×*g* for 15 min. The pellets were resuspended in sterile water and then purified by 90% Percoll gradient centrifugation at 15,000×*g* for 40 min (Undeen and Vavra 1997). The spore band was collected and washed several times with sterile water (Johny et al. 2006; Tsai et al. 2003). The puried spores were stored at 4°C for further use.

Nosema sp. MPr inoculated in BmN cell and silkworm larvae

The purified *Nosema* sp. MPr suspended in 0.2 M KOH solution were preheated at 27°C for 2 h, then incubated at 27°C for 40 min, subsequently mixed with 1×10^6 ml^{-1} BmN cell suspension (BmN cell population/spore population=1:10) completely. The mixed solution was injected into a cell culture capsule after 5 min and sealed up at 27°C for 1 h. When adherent cells grow up completely, the cell culture medium was removed by a Pasteur pipette, and fresh complete medium was added again to seal up and cultivate at 27°C. Samples were observed at regular time according to experiment requirements (Hayasaka et al. 1993). *Nosema* sp. MPr was inoculated to the healthy molted second instar larvae of silkworm, *B. mori*, and the dead were investigated until the period of the moth.

Preparation of *Nosema* sp. MPr genomic DNA

Nosema sp. MPr genomic DNA was extracted by the TEK method (Dong et al. 2010; Xu et al. 2011) with slight improvement. For DNA extraction, a 400-μL suspension of purified *Nosema* sp. MPr (10^{10} spores/mL) was mixed with 40 μL KOH (2 mol/L) in a 1.5-mL Eppendorf tube and incubated at 27°C for 1 h, and equal volumes of TEK buffer (1 mmol/L Tris–HCl, 10 mmol/L EDTA, 0.17 mol/L KCl, pH 8.0) were added and continued to be incubated at 27°C for 1 h. The pH was adjusted to 8.0 with 1 mol/L HCl, 10% SDS was added in order to attain 0.5% in the mixture and kept in an ice bath for 15 min. Proteinase K was added in order to attain 0.5 mg/mL in the mixture and incubated at 50°C for 4 h. Subsequently, isovolumetric tris–phenol extraction was performed twice and washed with chloroform/isoamyl alcohol (24:1 *v*/*v*). NaOAC (10%) was added to the recovered aqueous phase after centrifuging (10,000 r/min, 5 min), and then, the DNA was precipitated by 2.5 times volume of cold ethanol at −20°C for 30 min before centrifuging (12,000 r/min, 10 min). In addition, the precipitated DNA was rinsed twice with 500 μL cold 70% ethanol before drying at 37°C for 5–10 min. Then, the extracted DNA was stored at −20°C after dissolution in 50 μL TE buffer at 65°C for 10 min.

PCR amplification of rRNA core gene of *Nosema* sp. MPr

The primer sets used for *Nosema* sp. MPr rRNA gene amplification and the expected sizes of the amplicons are shown in Table 1 (Huang et al. 2008; Zhu et al. 2010). The 25-μL PCR amplification reaction system are as follows: *Nosema* sp. MPr genomic DNA 30 ng, forward and reverse primers 0.05 μL, respectively, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 10× PCR buffer 2.5 μL, and 1 U Taq DNA polymerase.

The amplification was performed under the following conditions: after initial denaturation of DNA at 94°C for 8 min, 30 cycles were run: 94°C for 30 s, annealing temperatures (43–55°C) for 30 s, and 72°C for 1 min 30 s with a 10-min 72°C extension, and finally store at 4°C.

Cloning and sequencing of rRNA gene of *Nosema* sp. MPr

The amplified products were subjected to 1.0% agarose gel electrophoresis, and the DNA bands were excised and purified. The purified PCR fragments were ligated overnight into PGEM-T Easy vector in the presence of T4 DNA ligase at 4°C. The plasmids were transfected in competent cell *Escherichia coli* DH10B and plated on ampicilin/IPTG/X-Gal agar plates. White colonies were selected, and plasmids were isolated from the cells. After PCR and enzyme digestion identification, the glycerine bacterial liquid was sequenced.

Table 1 Primers used for the amplification of the core sequences of *Nosema* sp. MPr rRNA

Primer	Sequence	Amplicon size (bp)
SSU rRNA		1,245
18f	CACCAGGTTGATTCTGCC	
1537r	TTATGATCCTGCTAATGGTTC	
ITS		220
S1129f	TGAATGTGTCCCTGTTCTTTG	
LS228R	GTTAGTTTCTTTTCCTCC	
5’ LSU		929
L1328f	CACATGGGATCAATAGGATACC	
LSR	TTCCATAACAACCGCCCTACTG	
LSU rRNA		1,553
LSF	GACAGTAGGGCGGTTGTTATG	
MPr-R2	CCTCAAAATGTCGGCATACA	

Construction of phylogenetic tree and analysis of rRNA gene core sequences

The species analyzed together with *Nosema* sp. MPr are given in Table 2. The SSU and LSU rRNA gene sequence of *Nosema* sp. MPr rRNA was aligned with homologous rRNA gene sequences of 25 or 13 microsporidia by the CLUSTALX 1.83 program (Thompson et al. 1997), respectively, using *Amblyospora californica* or *Schizosaccharomyces japonicus* as an outgroup. Phylogenetic trees were constructed with nucleotide sequences by using the MEGA version 4 software (Tamura et al. 2007) and neighbor-joining (NJ) method. The NJ tree was linearized assuming equal evolutionary rates in all lineages. The tree is drawn to scale with branch lengths calculated by using the average pathway method, and were in the units of the number of changes over the whole sequence. All alignment gaps were treated as missing data (complete deletion option). Genetic distances were calculated using the Kimura two-parameter method, and 1,000 bootstrap replications were performed to test the robustness of the estimated phylogenetic trees. Subsequently, the length and G+C content of the microsporidia were analyzed with the EditSeq program of DNAstar software.

Results

Morphological characteristics

The micrograph of the egg, larva, pupa, and adult of *P. rapae* is shown in Fig. 1. Spores were smeared on slides and observed under light microscopy with an ocular micrometer, and the fresh spores were photographed using a microscope USB camera. Light microscopy revealed that fresh *Nosema* sp. MPr spores were long oval in shape and 3.8±0.3× 2.0±0.2 μm in size (mean±standard error, n=50). The refractivity of the spores was a little poorer than that of *N. bombycis*.

Table 2 Microsporidian species and its host used for phylogenetic analysis

Organism name	Host	GenBank accession no.
Amblyospora californica	*Culex tarsalis*	ACU68473
Endoreticulatus sp. CHW-2008 Austria	*Thaumetopoea processionea*	EU260046
Endoreticulatus sp. CHW-2004 Taiwan	*Ocinara lida*	AY502944
Endoreticulatus bombycis	*Bombyx mori*	AY009115
Endoreticulatus schubergi	*Lymantria dispar*	L39109
Encephalitozoon cuniculi	*Homo sapiens*	Z19563
Heterosporis anguillarum	*Anguilla japonica*	AF387331
Microsporidium sp. 57864	*Unknown*	U90885
Nosema carpocapsae	*Cydia pomonella*	AF426104
Nosema oulemae	*Oulema melanopus*	U27359
Nosema thomsoni	*Choristoneura conflictana*	EU219086
Nosema ceranae	*Apis mellifera*	DQ486027
Nosema apis	*Apis mellifera*	U97150
Nosema antheraeae	*Antheraea pernyi*	DQ073396
Nosema sp. SC	*Samia cynthia ricini*	FJ767862
Nosema bombycis	*Helicoverpa armigera*	AY259631
Nosema plutellae	*Plutella xylostella*	AY960987
Nosema sp. C01	*Pieris rapae*	AY383655
Nosema spodopterae	*Spodoptera litura*	AY747307
Pleistophora ovariae	*Notemigonus crysoleucas*	AJ252955
Pleistophora typicalis	*Myoxocephalus scorpius*	AJ252956
Polydispyrenia simuli	*Simulium sp.*	AJ252960
uncultured Nosema	*Eurema blanda arsakia*	EU338534
Vairimorpha sp. CHW-2008a	*Ocinara lida*	EU487251
Vairimorpha sp. C21	*Unknown*	AY311592
Vairimorpha necatrix	*Pseudaletia unipuncta*	EU544672
Nosema sp. PX1	*Plutella xylostella*	AY960986
Encephalitozoon cuniculi	*Oryctolagus cuniculus*	AJ005581
Endoreticulatus sp.CHW-2004 Taiwan	*Ocinara lida*	AY960111

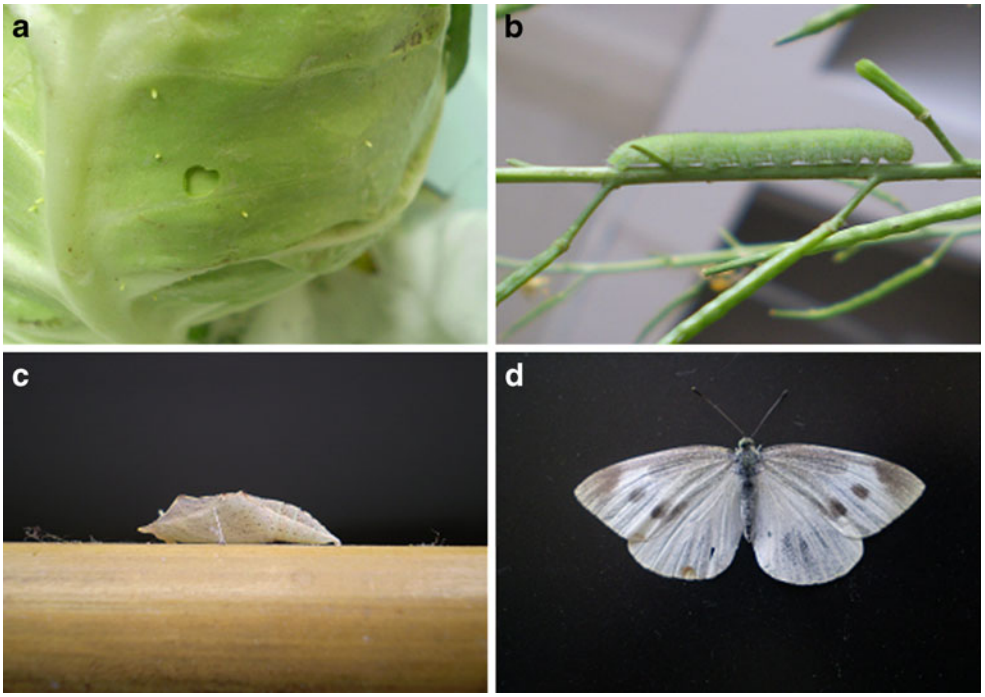

Fig. 1 Micrograph of the eggs, larva, pupa, and adult of *P. rapae*

Infection capability of *Nosema* sp. MPr in BmN cell and silkworm larvae

When the *Nosema* sp. MPr was inoculated to BmN cell after alkaline treatment, the spore refraction was decreased after 1 h, and introcession was discovered obviously in some spores, which indicated that the spores began to germinate under alkaline condition. However, infection of *Nosema* sp. MPr in BmN cell was not observed under an inverted microscope throughout the whole culture phase. Also, this newly isolated microsporidium cannot infect the silkworm larvae.

Analysis of the core sequence of *Nosema* sp. MPr rRNA

The core sequence of *Nosema* sp. MPr rRNA obtained in the present study contains 3,750 bp and was submitted to GenBank (accession number: HQ399665). From the 5′ end, it included the SSU rRNA gene (1,245 bp), the ITS region (37 bp), and the LSU rRNA gene (2,468 bp). The base composition of the *Nosema* sp. MPr rRNA core sequence (the SSU rRNA gene, the ITS region, and the LSU rRNA gene) is 34.51% G+C. The organization of the rRNA genes of *Nosema* sp. MPr is 5′-SSU-ITS-LSU-3′ (Fig. 2), a pattern similar to most microsporidian rRNA regions (Gatehouse and Malone 1998; Huang et al. 2007; Müller et al. 2000; Tsai et al. 2002; Zhu et al. 1994). The G+C content of SSU rRNA is 37.1%, which is consistent with the G+C content (33.9–38.6%) of SSU rRNA of *Nosema* genus (Fries et al. 1996), and that of the ITS rRNA and LSU rRNA is 8.1% and 33.5%, respectively, within the variation extent of LSU rRNA of *Nosema* genus (31.8–35.8%) (Huang et al. 2007). The organization of the core rRNA genes of *Nosema* sp. MPr is similar to *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, *Encephalitozoon intestinalis*, *Enterocytozoon bieneusi*, and so on, whose organization is 5′-SSU-ITS-LSU-3′, which is increasingly being reported as a cause of severe, often disseminated infections, mainly in patients with acquired immunodeficiency syndrome (AIDS) (Baker et al. 1995; Desser et al. 1992; Kahler and Thurston-Enriquez 2007; Saková et al. 2006).

Molecular phylogenetic analysis

Based on the SSU rRNA sequences and NJ methods (Fig. 3a), *Nosema* sp. MPr was clustered into the same clade with *Nosema* genus. The percent of SSU rRNA sequence identity revealed that *Nosema* sp. MPr shared 100% identity with *Nosema carpocapsae*, 98% identity with *Nosema oulemae*, *Nosema thomsoni*, *Nosema ceranae*, *Microsporidium* sp. 57864, *Vairimorpha* sp. CHW-2008a, and *Vairimorpha* sp. C21, and 84% identity with *Nosema antheraeae*, *N. bombycis*, *Nosema plutellae*, and *Nosema spodopterae*.

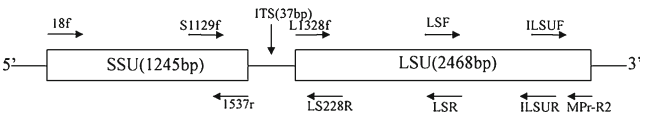

Fig. 2 A schematic diagram of the rRNA gene cluster of *Nosema* sp. MPr rRNA. Gene domains are *boxed* and indicated by *hollow arrows*. The direction of *arrow* indicates the direction of transcription

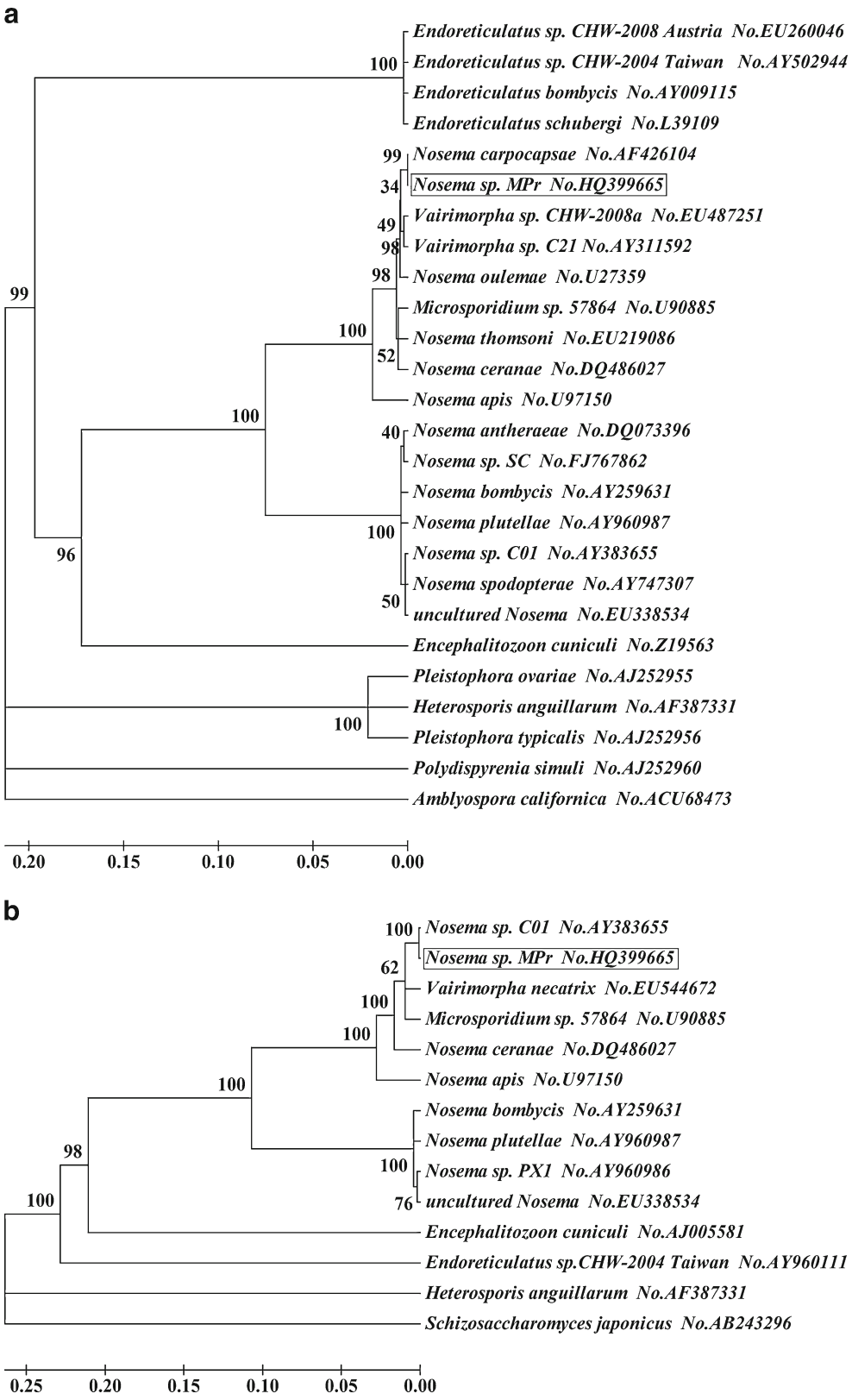

Fig. 3 Neighbor-joining trees generated using the Kimura two-parameter method of MEGA4. Bootstrap values for 1,000 replicates are shown. The branch lengths are arbitrary. *A. californica* and *S. japonicus* were used as the outgroup, respectively. **a** Phylogenetic tree of SSU rRNA. **b** Phylogenetic tree of LSU rRNA

Parallel comparison of the SSU rRNA gene sequences of *Nosema* sp. MPr and *Nosema* sp. C01 showed a sequence identity of 86.4%, though they had the same host—*P. rapae*.

The phylogenetic tree based on the LSU rRNA sequences constructed using NJ (Fig. 3b) indicated that the sequence identity between *Nosema* sp. MPr and *Nosema* sp. C01 was

the highest, 99.8%. *Nosema* sp. MPr shared more than 96% identity with *Vairimorpha necatrix*, *Microsporidium* sp. 57864, and *N. ceranae*. Parallel comparison of the LSU rRNA gene sequences of *Nosema* sp. MPr and *N. bombycis*, *N. plutellae*, *Nosema* sp. PX1, and uncultured *Nosema* showed a sequence identity of 80%.

With the help of MegAlign software, we make a conclusion that *Nosema* sp. MPr shared 78.6% identity with *E. cuniculi* and *E. hellem* on the SSU rRNA sequences. Meanwhile, *Nosema* sp. MPr shared 74.7% identity with *E. hellem* on the LSU rRNA sequences. The other microsporidia which can infect AIDS in human are not compared with *Nosema* sp. MPr because we can only search too short partial rRNA sequences in NCBI, which are not precise. In conclusion, based on the evidence from our phylogenetic analyses and the organization of the rRNA gene region, *Nosema* sp. MPr is included in *Nosema* group, and not closely related to *N. bombycis*.

Discussion

Microsporidia parasitizing in *P. rapae* were various. A novel *Nosema* species (*Nosema* sp. MPr) isolated from *P. rapae* was studied from the perspective of morphology, infection capability, and phylogenesis in this paper. The fresh *Nosema* sp. MPr spores were generally long oval shaped, varied in size, with a mean length and mean width of 3.8 μm (SD, 0.3) and 2.0 μm (SD, 0.2; $n=50$), respectively. This microsporidian isolate also had a lower refractivity than that of *N. bombycis*. All these results showed that the *Nosema* sp. MPr is a unique microsporidium.

Sequence analysis of *Nosema* sp. MPr indicated that the arrangement of rRNA gene was SSU rRNA-ITS-LSU rRNA, consistent with the traditional arrangement of microsporidian rRNA gene (Peyretaillade et al. 1998; Franzen et al. 1998; Zhu et al. 1994; Nilsen et al. 1998; Terry et al. 2003). From the 5′ end, it included the SSU rRNA gene (1,245 bp), the ITS region (37 bp), and the LSU rRNA gene (2,468 bp). Phylogenetic analysis of SSU rRNA and LSU rRNA suggested that this microsporidium and *Nosema* genus were in the same clade; thus, it belonged to *Nosema* genus, and was named as *Nosema* sp. MPr temporarily.

Since many microsporidia are considered to have a wide host range (Tanada and Kaya 1993), the cross infection of microsporidia often occurs between hosts; sometimes, microsporidia can infect new hosts through the transition of intermediate host. Even if various microsporidia were isolated from the same host, they may have different infectivities to the silkworm. A microsporidium isolated from the *P. rapae*, *Nosema* sp. MPr, which can infect the silkworm, has been reported (Dong et al. 2010). Otherwise, another *Nosema* microsporidium isolated from the *P. rapae* cannot infect BmN cell and silkworm larva in this paper, which indicated that the *P. rapae* is probably an intermediate host of microsporidia in nature.

Acknowledgments This work was supported by the earmarked fund for the Modern Agro-industry Technology Research System. We are grateful to all who provided the means for us to access free software, which we have used and cited in this article. We thanked all partners and lab members for their kind help and criticism.

References

Adl SM, Simpson AG, Farmer MA, Andersen RA, Anderson OR, Barta JR, Bowser SS, Brugerolle G, Fensome RA, Fredericq S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn DH, Mann DG, McCourt RM, Mendoza L, Moestrup O, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor MF (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol 52:399–451

Baker MD, Vossbrinck CR, Didier ES, Maddox JV, Shadduck JA (1995) Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. J Eukaryot Microbiol 42:564–570

Bhat SA, Bashir I, Kamili AS (2009) Microsporidiosis of silkworm, *Bombyx mori* L. (Lepidoptera-Bombycidae): a review. Afr J Agric Res 4:1519–1523

Corradi N, Keeling PJ (2009) Microsporidia: a journey through radical taxonomical revisions. Fungal Biol Rev 23:1–8

Desser SS, Hong H et al (1992) Ultrastructure of the development of a species of *Encephalitozoon* cultured from the eye of an AIDS patient. Parasitol Res 78(8):677–683

Dong SN, Shen ZY, Xu L, Zhu F (2010) Sequence and phylogenetic analysis of SSU rRNA gene of five microsporidia. Curr Microbiol 60:30–37

Franzen C, Müller A (1999) Molecular techniques for detection, species differentiation, and phylogenetic analysis of microsporidia. Clin Microbiol Rev 12:243–285

Franzen C, Müller P, Hartmann P, Hegener M, Schrappe V (1998) Polymerase chain reaction for diagnosis and species differentiation of microsporidia. Folia Parasitol (Prague) 45:140–148

Fries I, Ekbohm G, Villumstad E (1984) *Nosema apis*, sampling techniques and honey yield. J Apicult Res 23:102–105

Fries I, Feng F, Silva AD, Slemenda SB, Pieniazek NJ (1996) *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur J Protistol 32:356–365

Gatehouse HS, Malone LA (1998) The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. J Invertebr Pathol 71:97–105

Gresoviac JS, Khattra SJ, Nadler AS, Kent LM, Devlin HR, Vivares PC, Fuente DLE, Hedrick PR (2000) Comparison of small subunit ribosomal RNA gene and internal transcribed spacer sequences among isolates of the intranuclear microsporidian *Nucleospora salmonis*. J Eukaryot Microbiol 47:379–387

Hatakeyama Y, Kawakami Y, Iwano H, Inoue T, Ishihara R (1997) Analysis and taxonomic inference of small subunit ribosomal RNA sequences of five microsporidia pathogenic to the silkworm, *Bombyx mori*. J Seric Sci Jpn 66:242–252

Hayasaka S, Sato T, Inoue H (1993) Infection and proliferation of microsporidians pathogenic to the silkworm, *Bombyx mori* L. and Chinese oak silkworm, *Antheraea pernyi* in Lepidopteran cell line. J Bull Natl Inst Seric Entomol Sci 7:47–63

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, CrousPW DYC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, MozleyStandridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüssler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007) A higher-level phylogenetic classification of the fungi. Mycol Res 111:509–547

Huang WF, Jiang JH, Chen YW, Wang CH (2007) A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie 38:30–37

Huang WF, Bocquet M, Lee KC et al (2008) The comparison of rDNA spacer regions of *Nosema ceranae* isolates from different hosts and locations. J Invertebr Pathol 97:9–13

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, Lumbsch HT, Rauhut A, Reeb V, Arnold AE, Amtoft A, Stajich JE, Hosaka K, Sung GH, Johnson D, O'Rourke B, Crockett M, Binder M, Curtis JM, Slot JC, Wang Z, Wilson AW, Schüssler A, Longcore JE, O'Donnell K, Mozley-Standridge S, Porter D, Letcher PM, Powell MJ, Taylor JW, White MM, Griffith GW, Davies DR, Humber RA, Morton JB, Sugiyama J, Rossman AY, Rogers JD, Pfister DH, Hewitt D, Hansen K, Hambleton S, Shoemaker RA, Kohlmeyer J, Volkmann-Kohlmeyer B, Spotts RA, Serdani M, Crous PW, Hughes KW, Matsuura K, Langer E, Langer G, Untereiner WA, Lücking R, Büdel B, Geiser DM, Aptroot A, Diederich P, Schmitt I, Schultz M, Yah R, Hibbett DS, Lutzoni F, McLaughlin DJ, Spatafora JW, Vilgalys R (2006) Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443:818–822

Johny S, Kanginakudru S, Muralirangan MC, Nagaraju J (2006) Morphological and molecular characterization of a new microsporidian (Protozoa: Microsporidia) isolated from *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). Parasitology 132:803–814

Kahler A, Thurston-Enriquez J (2007) Human pathogenic microsporidia detection in agricultural samples: method development and assessment. Parasitol Res 100(3):529–538

Keeling PJ, Luker MA, Palmer JD (2000) Evidence from beta-tubulin phylogeny that microsporidia evolved from within the fungi. Mol Biol Evol 17:23–31

Kishore S, Baig M, Nataraju B, Balavenkatasubbaiah M, Sivaprasad V, Iyengar MNS, Datta RK (1994) Cross infectivity microsporidians isolated from wild Lepidopterous insects to silkworm, *Bombyx mori L*. Indian J Seric 33:126–130

Ku CT, Wang CY, Tsai YC, Tzeng CC, Wang CH (2007) Phylogenetic analysis of two putative *Nosema* isolates from cruciferous lepidopteran pests in Taiwan. J Invertebr Pathol 95:71–76

Müller A, Trammer T, Ghioralia G, Seitz HM, Diehl V, Franzen E (2000) Ribosomal RNA of *Nosema* algerae and phylogenetic relationship to other microsporidia. Parasitol Res 86:18–23

Nilsen F, Endresen C, Hordvik I (1998) Molecular phylogeny of microsporidians with particular reference to species that infect the muscle of fish. J Eukaryot Microbiol 45:535–543

Peyretaillade E, Biderre P, Peyret F, Duffieux G, Metenier M (1998) Microsporidian *Encephalitozoon cuniculi*, a unicellular eukaryote, with an unusual chromosomal dispersion of ribosomal genes and a LSU rRNA reduced to the universal core. Nucleic Acids Res 26:3513–3520

Rao SN, Muthulakshmi M, Kanginakudru S, Nagaraju J (2004) Phylogenetic relationships of three new microsporidian isolates from the silkworm, *Bombyx mori*. J Invertebr Pathol 86:87–95

Rao SN, Nath BS, Saratchandra B (2005) Characterization and phylogenetic relationships among microsporidia infecting silkworm, *Bombyx mori*, using inter simple sequence repeat (ISSR) and small subunit rRNA (SSU-rRNA) sequence analysis. Genome 48:355–366

Saková K, Sak B et al (2006) Humoral response of chicken infected with the microsporidium *Encephalitozoon hellem*. Parasitol Res 98(5):488–492

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tanada Y, Kaya HK (1993) Protozoan infections: Apicomplexa, microspora. In: Insect pathology. Academic Press, San Diego, pp 414–458

Terry RS, MacNeil C, Dick JTA, Smith JE, Dunn AM (2003) Resolution of a taxonomic conundrum: an ultrastructural and molecular description of the life cycle of *Pleistophora mulleri* (Pfeiffer 1895; Georgevitch 1929). J Eukaryot Microbiol 50:266–273

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL-X windows interference: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tsai SJ, Kou GH, Lo CF, Wang CH (2002) Complete sequence and structure of ribosomal RNA gene of *Heterosporis anguillarum*. Dis Aquat Org 49:199–206

Tsai SJ, Yamane S, Lo CF, Wang CH (2003) The characterization of microsporidian isolates (Nosematidae: *Nosema*) from five important lepidopteran pests in Taiwan. J Invertebr Pathol 83:51–59

Undeen AH, Vavra J (1997) Research methods for entomopathogenic protozoa. In: Lacey LA (ed) Manual of techniques in insect pathology. Academic Press, San Diego, pp 117–151

Vávra J, Kamler M et al (2011) Opportunistic nature of the mammalian microsporidia: experimental transmission of *Trachipleistophora extenrec* (Fungi: Microsporidia) between mammalian and insect hosts. Parasitol Res 108(6):1565–1573

Waters PF, Snowden KF et al (2004) A comparison of homologous genes encoding aminopeptidases among bird and human *Encephalitozoon hellem* isolates and a rabbit *E. cuniculi* isolate. Parasitol Res 93(5):410–418

Weiss LM, Vossbrinck CR (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. In: Wittner M, Weiss LM (eds.) The microsporidia and microsporidiosis. ASM Press, Washington, DC, 4:129–171

Xu XF, Shen ZY et al (2011) Phylogenetic characterization of a microsporidium (*Endoreticulatus* sp. Zhenjiang) isolated from the silkworm, *Bombyx mori*. Parasitol Res. doi:10.1007/s00436-011-2560-8

Zhu X, Wittner M, Tanowitz HB, Cali A, Weiss LM (1994) Ribosomal RNA sequences of *Enterocytozoon bieneusi, Septata intestinalis* and *Ameson michaelis*: phylogenetic construction and structural correspondence. J Eukaryot Microbiol 41:204–209

Zhu F, Shen ZY, Xu XF, Tao HP, Dong SN, Tang XD, Xu L (2010) Phylogenetic analysis of complete rRNA gene sequence of *Nosema philosamiae* isolated from the Lepidopteran *Philosamia cynthia ricini*. J Eukaryot Microbiol 57:294–296