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Phylogenetic Analysis of Complete *rRNA* Gene Sequence of *Endoreticulatus* sp. Shengzhou from the Silkworm, *Bombyx mori* in Zhejiang of China

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Abstract: Researchers employed a sample method directly using purified spores suspension instead of genomic DNA as PCR templete for amplifying complete *rRNA* genes of *Endoreticulatus* sp. Shengzhou, a novel microsporidian isolate from silkworm *Bombyx mori* collected in Shengzhou of China and studied its phyletic evolution of the isolate. The complete *rRNA* gene sequences of the isolate was 4,431 bp long and the arrangement of the *rRNA* genes was reversed as 5'-LSU-ITS-SSU-IGS-5S-3'. Morphological character and phylogenetic analysis based on the SSU *rRNA* gene sequence and ITS sequence indicated that *Endoreticulatus* sp. Shengzhou is closely related to *Endoreticulatus* genus.

Key words: Microsporidia, *Endoreticulatus* sp., parasite, phylogeny, Shengzhou, China

INTRODUCTION

Microsporidia which are unicellular eukaryotes and obligate intracellular parasites have long been recongnized pathogenic agents in sericulture, apiculture and fisheries (Wittner, 1999). Microsporidia were previously divided into primitive eukaryotes however, more and more molecular evidences have demonstrated that these organisms are phylogenetically related to the fungi with remnant mitochondrial organelles (Hirt *et al*., 1999; Keeling *et al*., 2000; Thomarat *et al*., 2004; James *et al*., 2006; Goldberg *et al*., 2008; Lee *et al*., 2008). It is reported that microsporidia consist of approximately 160 genera and 1300 described species (Corradi *et al*., 2008). Several microsporidian genera such as *Nosema bombycis*, *Pleistophora*, *Thelohania*, *Vairimorpha* and *Endoreticulatus* have been found to infect the silkworm *Bombyx mori* (Lu and Jin, 2000; Bhat *et al*., 2009). Besides, *N. bombycis*, the genus *Endoreticulatus* has drawn increased attention because of its widely distribution and interference on inspection of Pebrine (Eric *et al*., 1998; Wang *et al*., 2005; Xu *et al*., 2011). The research on the morphology, spore surface proteins and infectivity had been conducted on *Endoreticulatus* sp. Shengzhou (Huang *et al*., 2004a, b). However, no molecular biological work has been carried out on this organism and its taxonomic position had not been completely ascertained. Here, researchers employed a simple method directly using purified spores suspension instead of genomic DNA as PCR templete for amplifying complete *rRNA* genes of the isolate. By comparing sequences of the Small Subunit (SSU) rRNA and Internal Transcribed Spacer (ITS) of *Endoreticulatus* sp. Shengzhou with the corresponding sequences of the other microsporidia, researchers provide molecular biological evidence confirming the taxonomic position of the new isolate.

MATERIALS AND METHODS

Purification of spores: *Endoreticulatus* sp. Shengzhou was originally isolated from infected silkworms collected from Shengzhou Sericultural Experimental Station, Zhejiang province, China. Spores were propagated and purified as previously described (Hung *et al*., 1998; Gatehouse and Malone, 1998; Tsai *et al*., 2003; Johny *et al*., 2006). Following quantified with a hemocytometer purified spores were stored in deionized water supplemented with antibiotics (Sigma, 100 mg mL^{-1} streptomycin, 100 U mL^{-1} penicillin) at 4°C for later use (Zhang *et al*., 2007).

Morphological observation of the microsporidia isolate: The purified spores of the microsporidia isolate were observed under phase-contrast microscopy (Nikon eclipse Ti) and photographed using the digital sight DS-U2 camera system (Nikon).

Ultrathin sections were cut with a diamond knife mounted on the Reichert-Jung ULTRACUT E ultramicrotome stained in methanolic uranyl acetate and then in lead citrate. The stained grids were observed by using the JEM-1230 (JEOL Ltd.) transmission electron microscope operated at 100 kV.

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Table 1: Primers used to sequence the *rRNA* gene of *Endoreticulatus* sp. Shengzhou

Primers	Sequences	Renaturation temperature (°C)	Amplicon size (bp)
5' end of LSU rRNA			600
LSUF	5'-ACTCTCCTCTTTGCCTCAATCAATC-3'	58.01	
HGR	5'-CTCCTTGGTCCGTGTTTCA-3'	57.80	
LSU rRNA			1,757
HGF	5'-GAAACACGGACCAAGGAGATTAC-3'	57.80	
ILSUR	5'-ACCTGTCTCACGACGGTCTAAAC-3'	61.95	
3' end of LSU rRNA and ITS			499
ILSUF	5'-TGGGTTTAGACCGTCGTGAG-3'	62.00	
S33R	5'-ATAGCGTCTACGTCAGGCAG-3'	62.00	
SSU rRNA			1,254
18f	5'-CACCAGGTTGATTCTGCC -3'	57.30	
1537r	5'-TTATGATCCTGCTAATGGTTC-3'	54.11	
IGS and 5S rRNA			419
ISSUF	5'-GAACCATTAGCAGGATCAT-3'	54.11	
5SR	5'-TACAGCACCCAACGTTCCCAAG-3'	61.94	

Table 2: SSU rRNA sequences of microsporidia used for produce the phylogenetic tree

Microsporidia name	Host name	Accession No.
Endoreticulatus sp. Shengzhou	*Bombyx mori*	JN688870
Endoreticulatus bombycis	*Bombyx mori*	AY009115
Endoreticulatus sp. Zhenjiang	*Bombyx mori*	FJ772431
Endoreticulatus sp. Taiwan	*Ocinara lida*	AY502944
Endoreticulatus sp. Bulgaria	*Lymantria dispar*	AY502945
Endoreticulatus schubergi	*Lymantria dispar*	L39109
Endoreticulatus sp. Austria	*Thaumetopoea processionea*	EU260046
Pleistophora sp. (ATCC 50040)	*Agrotis exclamationis*	U10342
Pleistophora sp. (Sd-Nu-IW8201)	*Spodopter depravata*	D85500
Vittaforma corneum	*Homo sapiens*	L39112
Cystosporogenes legeri	*Lobesia botrana*	AY233131
Cystosporogenes operophterae	*Operophtera brumata*	AJ302320
Vittaforma corneae	*Nosema corneum*	U11046
Nosema plutellae	*Plutella xyllostella*	AY960987
Nosema ceranae	*Apis mellifera*	DQ486027
Nosema spodopterae	*Spodotera litura*	AY747307
Nosema bombycis	*Bombyx mori*	AY259631
Encephalitozoon cuniculi	*Oryctolagus cuniculus*	L39107

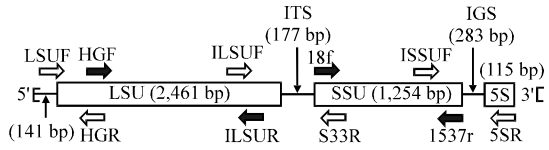

Fig. 1: Schematic diagram of the *Endoreticulatus* sp. Shengzhou *rRNA* gene region. The mature ribosomal RNA (rRNA) domains are indicated by boxes. The black arrows represent primers used to amplify the major coding regions of rRNA while the white arrows represent primers for the 5'-end of the Large Subunit (LSU), the Internal Transcribed Spacer (ITS), the Intergenic Spacer (IGS) and 5SrRNA

Amplification and sequencing of *rRNA* genes: A serial of primers used to amplify *rRNA* genes are from the report of Xu *et al.* (2011), all primers and the expected sizes of the amplicons are shown in Table 1 and each set of primer was amplified in two duplication. The strategy of amplification and sequence is shown in Fig. 1. ExTaq DNA polymerase (Takara) was utilized to amplify *rRNA* genes according to the manufacturer's instructions. About 1 μL purified spores suspension (10^9 spores mL^{-1}) instead of genomic DNA were used as PCR template in 50 μL reaction. Two controls without DNA and with DNA from silkworm were carried out in parallel in the same Veriti 96 well Thermal Cycler (Applied Biosystems, Foster city, CA, USA), respectively. Initial denaturation temperature parameters were 94°C for 8 min, other parameters were selected according to standard PCR condition. PCR products were electrophoresed through 1.0% agarose gel and visualised by ethidium bromide staining.

The products consistent with expected size were purified using DNA Extraction and Purification kit and cloned into pMD20-T Vector (Takara) and the resultant positive clones with the correct size were sequenced with three duplications by Invitrogen Company in Shanghai, China.

Phylogenetic analysis of the microsporidia isolate: The SSUrRNA sequence of the microsporidia isolate was imported into NCBI (http://www.ncbi.nlm.nih.gov/blast/) and blasted with the Blastn program in nucleotide collection (nr/nt) database to obtain other *rRNA* gene sequences with higher homology microsporidian (Table 2). All sequences were aligned by using the ClustalX 1.83 program. Both the neighbor-joining

algorithm (Saitou and Nei, 1987) and the maximum parsimony method (Kolaczkowski and Thornton, 2004) implemented in MEGA 5.0 Software (Tamura *et al*., 2011) was employed to reconstruct phylogenetic trees and Bootstrap support was evaluated based on 1,000 replicates. The SSUrRNA gene sequence of *Encephalitozoon cuniculi* was used as outgroup.

RESULTS AND DISCUSSION

Morphological character of the microsporidia isolate: Light microscopy revealed that the spores of the mocrosporidia isolated were ovate in shap and they had an average size of 2.69±0.12×1.66±0.12 μm (mean±standard error, n = 50) under phase-contrast microscope (Fig. 2a). The isolated was considerably smaller than *Nosema bombycis* (Fig. 2b). Electron micrography of a longitudinal section of a mature spore revealed that spore consisted of an endospore and an exospore with a wavy outline. The coiled region of the polar tube comprised 9 turns and the monokaryotic nuclei was encapsulated in sporoplasm which was enclosed by a plasma membrane. At the posterior end of the spore was a membrane-bound vacuole with amorphous content (Fig. 3). All the above-mentioned features correspond to the principle characteristics of the genus *Endoreticulatus* (Brooks *et al*., 1988).

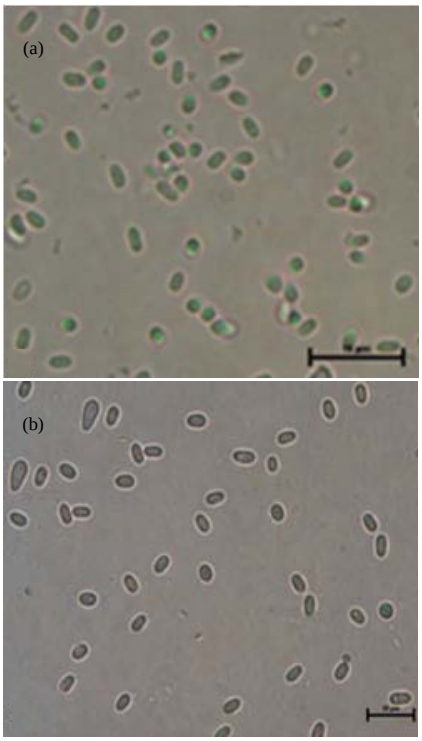

Fig. 2: Light micrographs of; a) *Endoreticulatus* sp. Shengzhou and b) *Nosema bombycis* and (x1,000). Scale bar = 10 μm

The amplication of complete *rRNA* genes of *Endoreticulatus* sp. Shengzhou: Using purified spores instead of genomic DNA as PCR template, complete *rRNA* genes of the *Endoreticulatus* sp. Shengzhou were successfully amplified. The PCR results with various combinations of the primers shown in Table 1 and in Fig. 4 and the resultant amplicons were of the predicted length (Lane 1-10). Conversely, none of the PCR reactions using DNA from silkworm with either the primer set 18f /1537r or the other primer sets produced any amplicon (Lane 11) indicating that PCR products are really origined from microsporidian and there was no interference between the two genomes. For extraction of genomic DNA from microsporidia, traditional method is to get enough cytoplasm released from spores which were stimulated with physical and chemical conditions to germinate (Higes *et al*., 2007) or broken with glass beads (Undeen and Cockburn, 1989; Rao *et al*., 2007). The procedure is complex and time consuming. Ombrouck *et al*. (1997) reported that boiling of spores suspension appeared to be most effective for DNA extraction. However, the PCR results show that purified spores suspension of the *Endoreticulatus* sp. Shengzhou may be directly used as template to amplify *rRNA* genes and cumbersome extraction of genomic DNA can be

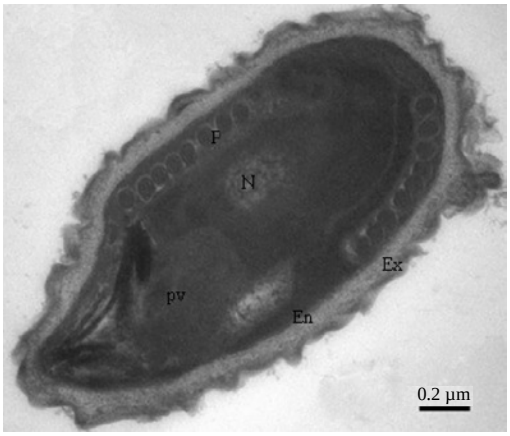

Fig. 3: Transmission electron micrographs of *Endoreticulatus* sp. Shengzhou. The extremity of longitudinal section of spore was in a tip shape rather than regular ovate showing extrusion of polar tube with a attempt to penetrate spore wall. En: Endospore; Ex: Exospores; N: Monokaryotic; P: Polar tube and pv: posterior vacuole

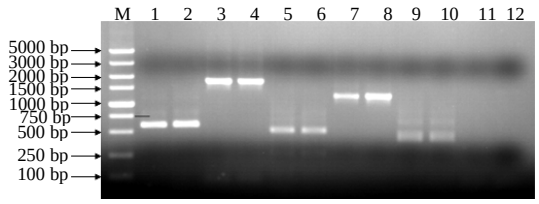

Fig. 4: About 1.0% agarose gel electrophoresis of PCR products. Lane 1 and 2: 5' region of LSUrRNA (primer set LSUF/HGR; 600 bp amplicon), lane 3 and 4: main part of LSUrRNA (primer set HGF/ILSUR; 1757 bp amplicon), lane 5 and 6: 3' end of LSUrRNA-ITS-5' end of SSUrRNA (primer set ILSUF/S33R; 509 bp amplicon), lane 7 and 8: SSUrRNA (primer set 18f /1537r; 1254 bp amplicon), lane 9 and 10: 3' end of SSUrRNA-IGS-5S (primer set, ISSUF/5SR; 319 bp amplicon), lane 11: masculine control with DNA from silkworm (primer set 18f /1537r), lane 12: negative control without DNA (primer set 18f /1537r), lane1, 3, 5 and 7: the PCR templete comes from purified spore suspension, lane 2, 4, 6 and 8: the PCR templete comes from genomic DNA and M: 5,00 bp DNA ladder (Takara)

avoided. So, researchers suggest that purified spores suspension can directly be used as PCR template to amplify some genes of microsporidia.

The sequence and organization of rRNA from the isolated: The complete DNA sequence of *Endoreticulatus* sp. Shengzhou *rRNA* gene obtained in the present study contains of 4,431 bp. The Large Subunit gene (LSU *rRNA*) contains 2,461 bp with 43.48% G+C; Internal Transcribed Spacer (ITS) contains 177 bp with 22.09% G+C; the Small Subunit gene (SSU rRNA) with the accession number JN688870 contains 1,254 bp with 50.96% G+C; Intergenic Spacer (IGS) contains 283 bp with 27.21% G+C and 5SrRNA region contains 115 bp with 45.22% G+C (Fig. 1).

The typical *rRNA* gene order of microsporidia is SSU-ITS-LSU and conforms with the general arrangement of the rRNA operon where the SSU precedes the LSU from the 5'-3' direction. Different from the typical order, the *Endoreticulatus* sp. Shengzhou has a reversed arrangement as 5'-LSU-ITS-SSU-IGS-5S-3' (Fig. 1). The reversed organization also has been found in several species of the microsporidian genus *Nosema* and regarded as a specific characteristic for identifying *Nosema* species (Huang *et al*., 2004a, b; Tsai *et al*., 2005; Ku *et al*., 2007; Zhu *et al*., 2011). Moreover, the similar

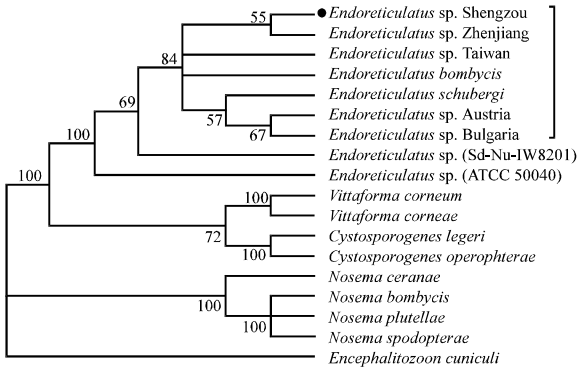

Fig. 5: Phylogenetic analysis of *Endoreticulatus* sp. Shengzhou based on Smaller Subunit (SSU) *rRNA* gene. Phylogenetic tree constructed by the Neighbor Joining algorithm (NJ) Method based on the SSU *rRNA* gene sequences of *Endoreticulatus* sp. Shengzhou and 17 microsporidian species. The tree is rooted using *Encephalitozoon cuniculi* as an outgroup. The numbers of the nodes indicate the level of bootstrap support (%) based on 1,000 resampled date sets

rearrangement of the *rRNA* genes has occurred in both *Glugoides intestinalis* (Refardt and Mouton, 2007) and *Endoreticulatus* sp. Zhenjiang (Xu *et al*., 2011). We provide another evidence that this rearrangement happened independently of the rearrangement in the *Nosema* species and it is not sufficient to identify *Nosema* species by the the arrangement of rRNA subunits.

Molecular phylogenetic analysis: Sequences and arrangement of rRNA are extensively used as important molecular parameters for identifying microsporidia at present (Vossbrinck and Debrunner-Vossbrinck, 2005; Hibbett *et al*., 2007). Here, researchers aligned the *SSUrRNA* gene sequence of *Endoreticulatus* sp. Shengzhou with those of other known microsporidia that infect lepidopteran as well as nonlepidopteran hosts. Phylogenetic tree was constructed with the neighbor-joining analysis and the maximum parsimony analysis based on the SSUrRNA (Fig. 5). In the analysis based on SSUrRNA, *Endoreticulatus* sp. Shengzhou and *Endoreticulatus* sp. Zhenjiang (GenBank accession no. FJ772431.1) fall into a same clade as other *Endoreticulatus* species including *Endoreticulatus* sp. CHW-2004 Taiwan (GenBank Accession No. AY502944.1), *Endoreticulatus bombycis* (GenBank Accession No. AY009115.1), *Endoreticulatus* schubergi (GenBank Accession No. L39109.1), *Endoreticulatus* sp. CHW-2008 Austria (GenBank Accession No. EU260046.1) and

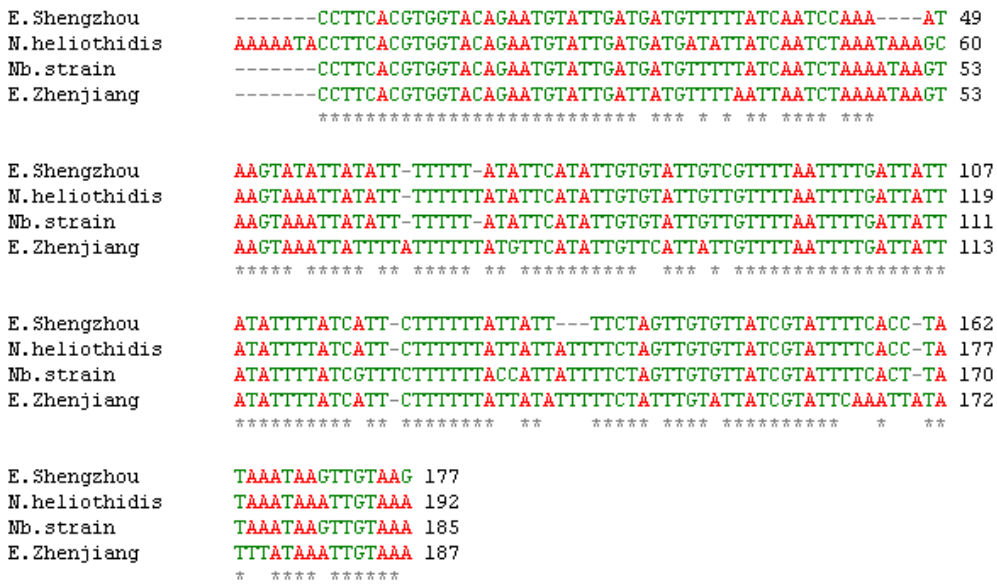

Fig. 6: Alignment of the ITS sequences of *Endoreticulatus* sp. Shengzhou (E. Shengzhou), *Nosema heliothidis*, *Nosema bombycis* strain GD 5 and *Endoreticulatus* sp. Zhenjiang. E. Shengzhou: *Endoreticulatus* sp. Shengzhou; *N. heliothidis*; *Nosema heliothidis*; Nb. strain: *Nosema bombycis* strain GD 5; E. Zhenjiang: *Endoreticulatus* sp. Zhenjiang

Endoreticulatus sp. CHW-2004 Bulgaria (GenBank Accession No. AY502945.1). That the seven microsporidia within the same clade suggests the genus *Endoreticulatus* widely distribute in the nature and have a variety of host. On the basis of the results of analyses, *Endoreticulatus* sp. Shengzhou was assigned to the genus *Endoreticulatus*. A molecular biological proof was offered to ascertain the taxonomic position of *Endoreticulatus* sp. Shengzhou.

Internal Transcribed Spacer (ITS) of *rRNA* gene is located in the region between LSUrRNA and SSUrRNA. The sequences of ITS are hypervariable in base composition among different species, since ITS has no mature ribosome, suffers less selection pressure and keep a rapid speed of evolution. Thus, ITS is also used for species identification (Garcia-Martinez *et al*., 1999; James *et al*., 1996). ITS of *Endoreticulatus* sp. Shengzhou is different from that of *Endoreticulatus* sp. Zhenjiang, though the two organisms are located in the same cluster on the phylogenetic tree based on the SSUrRNA. The former contains 177 bp with 22.09% G+C but the latter is composed of 187 bp with 17.11% G+C. Between two ITS, there are ten deletions and 24 bp different sites, thirten transition sites and eleven transversion sites and the ratio of transition and transversion was approximately 1.18. The ITS sequences of *Endoreticulatus* sp. Shengzhou, *Endoreticulatus* sp. Zhenjiang, *Nosema heliothidis* (L28965.1) and *Nosema bombycis* strain GD 5 (JF443623.1) were analyzed by multi-sequence alignment and these sequences are shown in Fig. 6.

Compared with ITS sequences of other three microsporidia, the ITS sequence of *Endoreticulatus* sp. Shengzhou also occurres some bases deletions, transitions and transversions. The variation of ITS sequences may be caused by different regions and hosts which result in extensive influence on the development and evolution of microsporidia. So, we presume that *SSUrRNA* gene sequences combined with ITS sequences can give a reasonable classification for microsporidia once enough ITS sequences are available and that *Endoreticulatus* sp. Shengzhou is a new species.

CONCLUSION

This study introduces a simple method for ampilifying some genes of microsporidia without extracting genomic DNA. Spores suspension can be directly used as PCR template to qucikly ampilify gene of microsporidia. Besides morphological character, the study also provides a molecular biological evidence based on *SSUrRNA* gene sequences and ITS sequences in support of the argument that *Endoreticulatus* sp. Shengzhou isolated from the silkworm *Bombyx mori* belongs to the genus *Endoreticulatus*.

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