

# Sequence and Phylogenetic Analysis of SSU rRNA Gene of Five Microsporidia

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Received: 21 May 2009 / Accepted: 24 August 2009 / Published online: 19 September 2009  
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**Abstract** The complete small subunit rRNA (SSU rRNA) gene sequences of five microsporidia including *Nosema heliothidis*, and four novel microsporidia isolated from *Pieris rapae*, *Phyllobrotica armata*, *Hemerophila atrilineata*, and *Bombyx mori*, respectively, were obtained by PCR amplification, cloning, and sequencing. Two phylogenetic trees based on SSU rRNA sequences had been constructed by using Neighbor-Joining of Phylip software and UPGMA of MEGA4.0 software. The taxonomic status of four novel microsporidia was determined by analysis of phylogenetic relationship, length, G+C content, identity, and divergence of the SSU rRNA sequences. The results showed that the microsporidia isolated from *Pieris rapae*, *Phyllobrotica armata*, and *Hemerophila atrilineata* have close phylogenetic relationship with the *Nosema*, while another microsporidium isolated from *Bombyx mori* is closely related to the *Endoreticulatus*. So, we temporarily classify three novel species of microsporidia to genus *Nosema*, as *Nosema* sp. PR, *Nosema* sp. PA, *Nosema* sp. HA. Another is temporarily classified into genus *Endoreticulatus*, as *Endoreticulatus* sp. Zhenjiang. The result indicated as well that it is feasible and valuable

to elucidate phylogenetic relationships and taxonomic status of microsporidian species by analyzing information from SSU rRNA sequences of microsporidia.

## Introduction

Microsporidia are obligate intracellular parasites that infect a broad range of invertebrates and humans, they were recognized as a causative agent for many hosts including insects, fishes, rodents, and primates. In 1857, the first microsporidian species—*N. bombycis* was described by Nageli [1]. Until now, more than 1,200 microsporidia species belonging to ~150 genera have been reported [2, 3].

The adaptation of microsporidia to a parasitic lifestyle precipitates a number of profound changes which lead to a seemingly paradoxical mixture of characteristics [4]: on one hand, parasites may evolve extremely complex and sophisticated mechanisms to invade their host and evade its defenses, while on the other hand, they may also appear more “simple” by dispensing with characteristics they no longer need as they increasingly depend on host metabolism for nutrients and energy. Although microsporidia were considered to be eukaryotes, they lack some typical eukaryotic characteristics including mitochondria, peroxisomes, classical stacked Golgi apparatus, and 80S ribosomes. The microsporidia contain 70S ribosomes, ribosomal subunits (30S and 50S), and rRNAs (16S and 23S) with prokaryotic size, and they have no separate 5.8S rRNA. They also lack flagella, pilus and 9 + 2 microtubular structure [5]. Up to now, the genome size has been determined for numerous microsporidian species, and they range from 19.5 Mbp in *Glugea atherinae* to just 2.3 Mbp in *Encephalitozoon intestinalis* [6].

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For a long time, microsporidia were generally regarded as primitively simple eukaryotes and classified as an early branch from prokaryotes to eukaryotes [7], and they were universally thought to be classified as Protista, Protozoa, Microspora in five kingdom system. This conclusion was supported by early molecular phylogenetic evidence, but more and more recent trees constructed from the conserved protein genes provide further evidence for the argument that microsporidia should properly be classified as fungi. Thus, in 1998 Cavalier-Smith [8] classified the microsporidia into the kingdom Fungi. And in 2002 NCBI concluded a description of microsporidia as Cellular Organisms, Eukaryote, Fungi/Metazoa group, Fungi, Microsporidia. For the Society of Classification has not approved the description of microsporidia, there is not universal agreement on the evolution and classification of microsporidia.

There has been increasing interest in studying large subunit rRNA (LSU rRNA), SSU rRNA, internal transcribed spacers (ITS), translation elongation factors (EF), heat shock protein (HSP), and beta-tubulin of microsporidium at the molecular level, which provided important evidence for the phylogenetic analysis of microsporidia. As a conserved gene, the SSU rRNA universally existed in eukaryotic and prokaryotic organisms, varied with constant rate, and can be used as the molecular clock of evolution. For its relatively highly variable regions provides important information for the relationship among species, the SSU rRNA gene also can be used as the biological identification label. And the molecular characterization of different microsporidia has widely relied on sequencing and analyzing of SSU rRNA genes [9], which also can be used as a molecular marker for estimating relationships among microsporidia [10]. Therefore, the SSU rRNA gene plays an important role in the phylogenetic research.

Traditional taxonomic studies and species classification of the microsporidia are mainly based on biological characters such as morphology and development of spore, microsporidium–host relationships, life cycle, parasitic site as well as transmission way. However, for some basic biological problems, taxonomic determinations based on these criteria alone can sometimes be problematic. With the development of molecular biotechnology, many *Nosema* species were proven to be distant from *N. bombycis* in

molecular phylogeny, and divided into other genus [11, 12]. So, phylogenetic analysis of SSU rRNA gene is an important tool for the classification of microsporidian species, which helps to construct more valuable classification system for the microsporidia.

In this paper, we cloned the complete SSU rRNA sequences of five microsporidia, compared them with the corresponding sequences of other 94 microsporidia for which data were available, and have preliminarily determined the taxonomic status of the four novel microsporidian isolates by constructing and analyzing of phylogenetic tree, and comparing the length and G+C content of SSU rRNA sequences.

## Materials and Methods

### The Origin of Microsporidia

The five species of microsporidia studied in this paper were isolated from diverse geographic regions and different hosts. The names of isolates, host insects, geographic regions, and accession number for them are described in Table 1. The five microsporidian species were isolated by Professor Shen ZhongYuan in 2006–2007 and kept in Pathology Department of Sericulture Research Institute of Chinese Academy of Agricultural Sciences. Additionally, the four novel microsporidian isolates which were separately isolated from *Pieris rapae*, *Phyllobrotica armata*, *Hemerophila atrilineata*, and *Bombyx mori* were unclassified.

### Preparation of Genomic DNA of Microsporidia

To prepare the genomic DNA of microsporidia, 400  $\mu$ l suspension of purified spores ( $10^{10}$  spores/ml) was mixed with 40  $\mu$ l KOH (2 mol/l) in a 1.5 ml Eppendorf tube and incubated at 27°C for 1 h, TEK buffer (1 mmol/l Tris–HCl, 10 mmol/l EDTA, 0.17 mol/l KCl, pH 8.0) was added and continued incubating at 27°C for 1 h. Adjusted pH to 8.0 with 1 mol/l HCl, added 10% SDS in order to attain 0.5% in the mixture and kept it in ice-bath for 15 min. 20 mg/ml proteinase K (TaKaRa Biotechnology Co. LTD) was added

**Table 1** The host insects, geographic regions and accession of five microsporidian species

No.	Microsporidia	Host insects	Geographic regions	Accession
1	<i>Nosema</i> sp. PR	<i>Pieris rapae</i>	Zhenjiang, Jiangsu, China	EU864531
2	<i>Nosema</i> sp. PA	<i>Phyllobrotica armata</i>	Mianyang, Sichuan, China	EU864528
3	<i>N. heliothidis</i>	<i>Helicoverpa armigera</i>	Baoding, Heibei, China	EU864529
4	<i>Nosema</i> sp. HA	<i>Hemerophila atrilineata</i>	Zhenjiang, Jiangsu, China	EU864527
5	<i>Endoreticulatus</i> sp. Zhenjiang	<i>Bombyx mori</i>	Zhenjiang, Jiangsu, China	EU864530

in order to attain 200 µg/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). 10% NaOAC 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 40 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.

#### Amplification, Cloning and Sequencing of SSU rRNA Gene

To amplify the SSU rRNA genes, primer sets were designed as Huang et al. [13] described, forward primer: 5'-CACCAG GTTGATTCTGCC-3'; reverse primer: 5'-TTATGATCCT GCTAATGGTTC-3'. The amplification was performed with following condition: after initial denaturation of DNA at 94°C for 8 min, 28 cycles were run—94°C for 1 min, 43°C for 1 min 40 s, and 72°C for 1 min 40 s—with a 10 min 72°C extension.

The PCR amplified products were cloned into pGEM-T vector and then sequenced. All the sequences were submitted to GenBank, and the accession number were shown in Table 1.

#### Construction of Phylogenetic Tree and Analysis of SSU rRNA Gene Sequences

The SSU rRNA gene sequences were aligned using the “ClustalX 1.83” program. Phylogenetic trees based on the resultant alignments were, respectively, constructed using the Neighbor-Joining algorithm (NJ) of Phylip software with Kimura-2 parameter and UPGMA algorithm of MEGA 4.0 software with Maximum Composite Likelihood, and one thousand replications were generated to test the robustness of the tree. Subsequently, the length and GC content of the complete SSU rRNA sequences of 22 microsporidia were analyzed with the Editseq program of DNASTar software, and the identity and divergence of the sequences of 26 microsporidia were analyzed with the MegAlign program of DNASTar software.

## Results

#### Analysis of Phylogenetic Tree of Microsporidia

The complete SSU rRNA gene sequences of the five microsporidia were aligned with the homologs from other

94 species of various microsporidian genera, such as *Nosema*, *Amblyospora*, *Endoreticulatus*, *Vairimorpha*, *Pleistophora*, *Encephalitozoon*, and *Glugea*, available in public databases (Table 2). And *Beauveria bassiana* was used as an out-group (Table 2).

Both the two phylogenetic trees of SSU rRNA genes (Figs. 1, 2) constructed by Neighbor-Joining and UPGMA revealed that, besides *Nosema* microsporidia, the microsporidia of other genera such as *Amblyospora*, *Endoreticulatus*, *Vairimorpha*, *Pleistophora*, *Encephalitozoon*, *Glugea* clustered in each clades, which is consistent to early published classifications of microsporidia. Consequently, the phylogenetic tree we constructed is valuable.

Three novel microsporidian isolates isolated from *Pieris rapae*, *Phyllobrotica armata*, and *Hemerophila atrilineata* separately, were grouped in the *Nosema* clade, indicating that this three novel microsporidian isolates belong to the genus *Nosema*. Therefore, we temporarily name them as *Nosema* sp. PA, *Nosema* sp. PR and *Nosema* sp. HA, respectively. Another novel microsporidium isolated from *Bombyx mori* got clustered with the *Endoreticulatus* clade. So, we propose to classify it into the genus *Endoreticulatus*, with temporary designation *Endoreticulatus* sp. Zhenjiang. These two phylogenetic trees based on the SSU rRNA sequences suggest that *Nosema* sp. PR is closely related to *N. bombycis*, while *Nosema* sp. PA, *Nosema* sp. PR, and *Nosema* sp. HA have close relationship with each other.

Further analysis of two SSU rRNA phylogenetic trees manifests that *Vairimorpha lymantriae*, *Vairimorpha necatrix*, *Oligosporidium occidentale*, and 20 *Nosema* species form a large cluster which can be classified into two parts. Part one is composed of four *Nosema* species cloned in this experiment and other nine *Nosema* species, while part two is composed of *Vairimorpha lymantriae*, *Vairimorpha necatrix*, *Oligosporidium occidentale*, and seven *Nosema* species. Consequently, we divide 20 *Nosema* species into two groups for further analysis. Group I comprise 13 *Nosema* species and group II comprise other seven *Nosema* species.

#### Analysis of Length and GC Content of SSU rRNA Gene of Microsporidia

We analyzed the complete SSU rRNA sequences of 22 microsporidia and found that the lengths of complete SSU rRNA gene sequences of *Nosema* species ranged from 1231 to 1393 bp, and that of *Endoreticulatus* species ranged from 1252 to 1254 bp (Table 3). For the four *Nosema* species cloned in this experiment, the lengths of complete SSU rRNA gene sequences of *Nosema heliothidis*, *Nosema* sp. PA, *Nosema* sp. PR, and *Nosema* sp. HA are 1232 and 1233 bp, respectively, and that of *Endoreticulatus* sp.

**Table 2** The taxon, accession number, author, and published year of microsporidia and *Beauveria bassiana* which were aligned for homology

No.	Taxon	Accession	No.	Taxon	Accession	No.	Taxon	Accession
1	<i>Amblyospora californica</i>	U68473	33	<i>Hazardia milleri</i>	*AY090067	65	<i>Pleistophora typicalis</i>	AJ252956
2	<i>Amblyospora canadensis</i>	*AY090056	34	<i>Heterosporis anguillarum</i>	AF387331	66	<i>Polydispyrenia simulii</i>	*AJ252960
3	<i>Amblyospora cinerei</i>	*AY090060	35	<i>Hyalinocysta chapmani</i>	*AF483838	67	<i>Pseudoloma neurophilia</i>	*AF322654
4	<i>Amblyospora connecticus</i>	*AF025685	36	<i>Ichthyosporidium</i> sp.	L39110	68	<i>Pseudonosema cristatellae</i>	AF484694
5	<i>Amblyospora crenifera</i>	*AY090061	37	<i>Intrapredatorus barri</i>	*AY013359	69	<i>Schroedera plumatellae</i>	*AY135024
6	<i>Amblyospora excrucii</i>	*AY090044	38	<i>Janacekia debaisieuxi</i>	AJ252950	70	<i>Spraguea lophii</i>	*AF104086
7	<i>Amblyospora ferocious</i>	*AY090062	39	<i>Kabatana takedai</i>	*AF356222	71	<i>Tetramicra brevifilum</i>	*AF364303
8	<i>Amblyospora indicola</i>	*AY090051	40	<i>Larssonsonia obtusa</i>	AF394527	72	<i>Thelohania solenopsae</i>	AF134205
9	<i>Amblyospora khaliulini</i>	*AY090047	41	<i>Loma embiotocia</i>	*AF320310	73	<i>Trachipleistophora hominis</i>	AJ002605
10	<i>Amblyospora opacita</i>	*AY090052	42	<i>Microgemma caulleryi</i>	*AY033054	74	<i>Trichonosema pectinatellae</i>	*AF484695
11	<i>Amblyospora stictici</i>	*AY090049	43	<i>Microsporidium prosopium</i>	*AF151529	75	<i>Vairimorpha necatrix</i>	Y00266
12	<i>Amblyospora stimuli</i>	*AY090050	44	<i>Nadelspora canceri</i>	*AF305706	76	<i>Vairimorpha lymantriae</i>	AF033315
13	<i>Amblyospora weiseri</i>	*AY090048	45	<i>Oligosporidium occidentalis</i>	*AF495379	77	<i>Vavraia culicis</i>	AJ252961
14	<i>Ameson michaelis</i>	L15741	46	<i>Tubulinosema acridophagus</i>	AF024658	78	<i>Vavraia oncooperae</i>	X74112
15	<i>Antonospora scoticae</i>	AF024655	47	<i>Nosema apis</i>	U97150	79	<i>Vittaforma corneae</i>	U11046
16	<i>Bacillidium</i> sp.	*AF104087	48	<i>Nosema bombi</i>	AY008373	80	<i>Weiseria palustris</i>	AF132544
17	<i>Berwaldia schaefermai</i>	*AY090042	49	<i>Nosema carpocapsae</i>	*AF426104	81	<i>Antonospora locustae</i>	AY376351
18	<i>Brachiola algerae</i>	AM422905	50	<i>Nosema ceranae</i>	DQ486027	82	<i>Glugea plecoglossi</i>	*AJ295326
19	<i>Bryonosema plumatellae</i>	*AF484692	51	<i>Nosema furnacalis</i>	U26532	83	<i>Glugea</i> sp. GS1	*AJ295325
20	<i>Culicosporella lunata</i>	AF027683	52	<i>Nosema granulosis</i>	*AJ011833	84	<i>Tubulinosema kingi</i>	*DQ019419
21	<i>Cystosporogenes operophtherae</i>	AJ302320	53	<i>Nosema oulemae</i>	U27359	85	<i>Nosema plutellae</i>	AY960987
22	<i>Encephalitozoon cuniculi</i>	Z19563	54	<i>Nosema portugal</i>	AF033316	86	<i>Nosema</i> sp. C01	AY383655
23	<i>Encephalitozoon lacertae</i>	*AF067144	55	<i>Nosema tyriae</i>	AJ012606	87	<i>Nosema</i> sp. PX1	AY960986
24	<i>Endoreticulatus bombycis</i>	*AY009115	56	<i>Nosema vespula</i>	U11047	88	<i>Nosema spodopterae</i>	AY747307
25	<i>Endoreticulatus schubergi</i>	L39109	57	<i>Ordospora colligata</i>	AF394529	89	<i>Pleistophora mulleri</i>	*EF119339
26	<i>Nucleospora salmonis</i>	*AF186003	58	<i>Orthosomella operophtherae</i>	AJ302317	90	<i>Endoreticulatus</i> sp. CHW-2004 Bulgaria	AY502945
27	<i>Flabelliforma magnivora</i>	AJ302318	59	<i>Ovipleistophora mirandellae</i>	*AF356223	91	<i>Endoreticulatus</i> sp. CHW-2004 Taiwan	AY502944
28	<i>Glugea anomala</i>	AF044391	60	<i>Parathelohania anophelis</i>	AF027682	92	<i>Endoreticulatus</i> sp. CHW-2008 Austria	*EU260046
29	<i>Glugea atherinae</i>	U15987	61	<i>Parathelohania obesa</i>	*AY090065	93	<i>Nosema bombycis</i>	AY259631
30	<i>Gurleya daphniae</i>	*AF439320	62	<i>Pleistophora hippoglossoides</i>	AJ252953	94	<i>Nosema antheraeae</i>	DQ073396
31	<i>Marssoniella elegans</i>	*AY090041	63	<i>Pleistophora mirandellae</i>	AJ252954	95	<i>Beauveria bassiana</i>	*EU334679
32	<i>Gurleya vavrai</i>	AF394526	64	<i>Pleistophora ovariae</i>	AJ252955			

Note: The SSU rRNA gene noted with '\*' is partial sequence

Zhenjiang is 1254 bp. These results provide further molecular information for the four novel microsporidian species in our study.

It is found as well that the GC content of the *Nosema* sp. PR, *Nosema* sp. PA, *N. heliothidis*, and *Nosema* sp. HA varied from 33.98 to 34.25%, and the GC content of

**Fig. 1** Phylogenetic tree constructed by the Neighbor-Joining method for the SSU rRNA sequences of microsporidia. *Beauveria bassiana* is included as an out-group. Neighbor-Joining consensus tree used 1000 bootstrap replicates. The number represents the percentage of bootstrap values

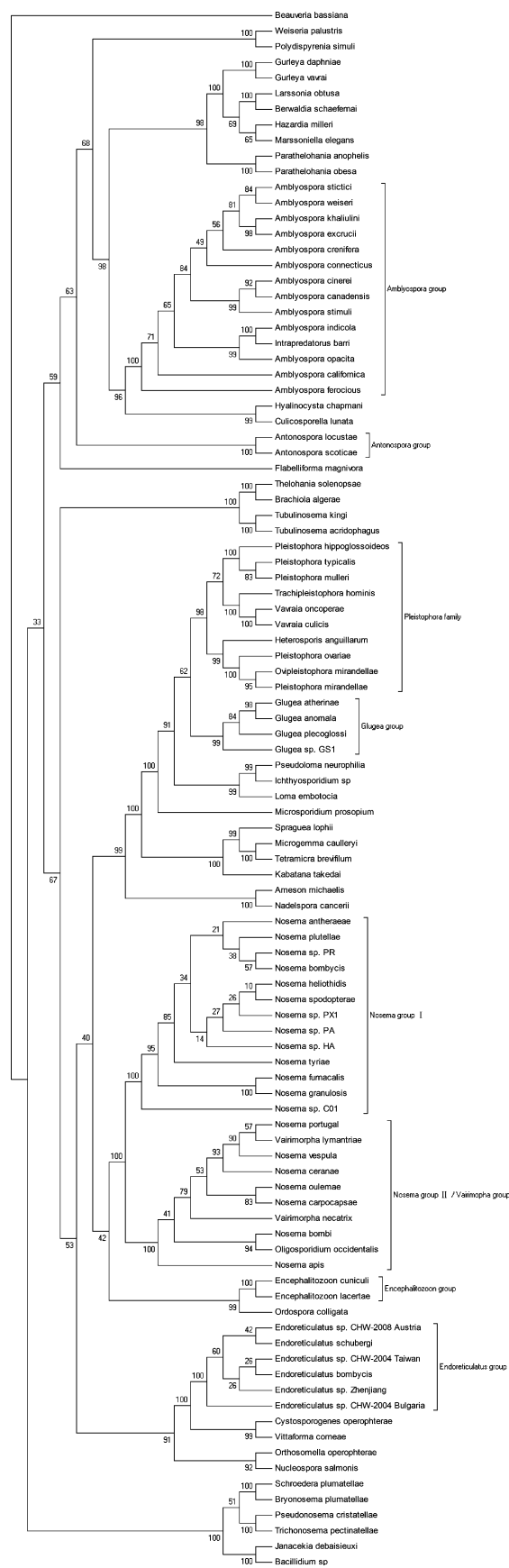
*Endoreticulatus* sp. Zhenjiang is 50.96% (Table 3). The GC content of the SSU rRNA gene in *Nosema* species ranged from 33.93 to 38.73%, and that of *Endoreticulatus* species ranged from 50.96 to 51.12% (Table 3), which is significantly higher than that *Nosema* species. Based on the GC content, the nucleotide acid length and phylogenetic analysis for the SSU rRNA gene, we suggested that the four novel microsporidian species, *Nosema* sp. PA, *Nosema* sp. PR and *Nosema* sp. HA, belong to genus *Nosema*, while *Endoreticulatus* sp. Zhenjiang belongs to the genus *Endoreticulatus*.

Additionally, we analyzed the lengths and GC content of the two *Nosema* groups. *Nosema* group I, which contains 1231–1237 bp with GC content of 33.93–34.93%, is shorter and lower than that of *Nosema* group II, which contains 1242–1393 bp with GC content of 35.75–38.73%.

#### Analysis of Identity and Divergence of the SSU rRNA Sequences of Microsporidia

The identities and divergence of SSU rRNA gene among 20 *Nosema* species varied from 83 to 100%, and 0–0.193, while that among six *Endoreticulatus* species varied from 98.3 to 99.7%, and 0.003–0.017, respectively. The identities and divergence of SSU rRNA gene between *Nosema* and six *Endoreticulatus* varied from 66.0 to 71.0%, and 0.369–0.457. Consequently, the genus *Endoreticulatus* is markedly different from the genus *Nosema*, and the relationship among *Endoreticulatus* species is closer than that of *Nosema* species. Additionally, the identities and divergence of SSU rRNA gene among *Nosema* species in group I varied from 96.3 to 100% and 0–0.038, while that among *Nosema* species in group II varied from 94.8 to 99.8%, and 0.002–0.054, indicating that there was higher degree of similarity of SSU rRNA gene sequence among the group I species than that among group II species in *Nosema*.

It is clear that the SSU rRNA genes of microsporidia have high identities and less divergence among species in same genus and have lower identities and higher divergence between different genera. Consequently, it is limited to measure the close phylogenetic relationships between various species in the genus based on the identities and divergence of the SSU rRNA gene, while the identities and divergence of the SSU rRNA gene are more suitable for the analysis of phylogenetic relationships between different genera and the determination of taxonomical status on the genus level.



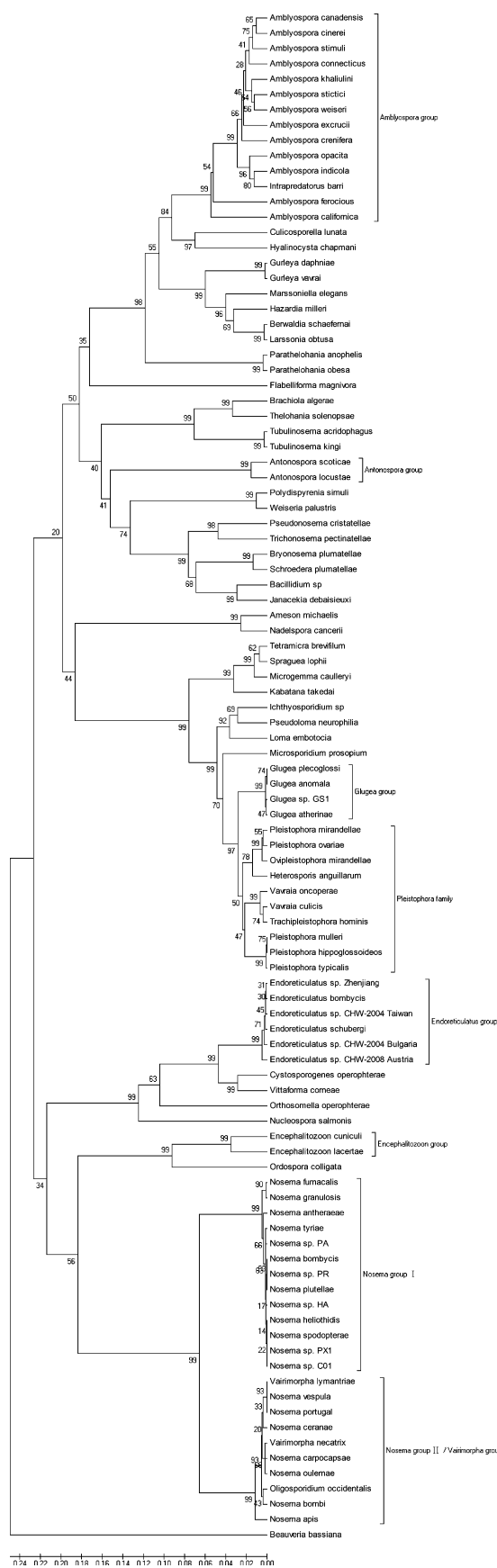
**Fig. 2** Phylogenetic tree constructed by the UPGMA method for the SSU rRNA sequences of microsporidia. *Beauveria bassiana* is included as an out-group. The UPGMA tree used 1000 bootstrap replicates. The number represents the percentage of bootstrap values, and the evolutionary distance was shown below

**Discussion**

Ribosomal gene sequences of microsporidia are frequently used for diagnostic tests, phylogenetic studies, epidemiological investigations of the host range, and the geographic distribution and pathogenetic studies of fish pathogens [14]. Baker et al. [15] analyzed a short fragment of LSU rRNA gene of eight *Nosema* species, and found the *N. bombycis* shared high homologies with other *Nosema* species isolated from Lepidoptera insects, low homologies with *Vairimorpha* species, and very low homologies with *Amblyospora* species. Slamovits [11] proposed transferring *N. locustae* to the genus *Antonospora*, as *Antonospora locustae* n. comb. by constructing a phylogenetic tree based on the SSU rRNA gene and with the ultrastructure of mature *N. locustae* spores.

In this study, three novel microsporidia, isolated from *Pieris rapae*, *Phyllobrotica armita*, and *Hemerophila atrilineata*, were classified into genus *Nosema*, as *Nosema* sp. PR, *Nosema* sp. PA, *Nosema* sp. HA, respectively. And the novel microsporidium isolated from the silkworm in countryside in Zhenjiang, Jiangsu Province, China, was temporarily classified into genus *Endoreticulatus*, as *Endoreticulatus* sp. Zhenjiang. The first report of *Endoreticulatus* species which infected *Bombyx mori* was in Mianyang Silkworm Eggs Production Farm and Santai Silkworm Eggs Production Farm in Sichuan Province, China. Subsequently, the *Endoreticulatus* species were successively found in silkworm eggs production in China. For their rather small size, they were usually called “mini-spore”. In 1995, Wan Yongji classified a “mini-spore” isolated from the silkworm in Sichuan Province into genus *Endoreticulatus* by studying its morphological character, ultrastructure, and life cycle [16].

Further analysis of two SSU rRNA phylogenetic trees manifests that the 20 *Nosema* species can be divided into two groups. Group I comprise 13 *Nosema* species, while group II comprise other seven *Nosema* species. The comparison of the SSU rRNA genes revealed that the phylogenetic relationship among 13 *Nosema* species in group I is closer than that of seven *Nosema* species in group II. For the significant differences between *Nosema* group I and *Nosema* group II, the phylogenetic trees based on SSU rRNA gene also provide further evidence for the argument that the genus *Nosema* should properly be separated into two groups [10, 15, 17, 18]. And SSU rRNA gene can be used to explore the differences among *Nosema* species,



**Table 3** The length and GC content of complete SSU rRNA sequences of 22 microsporidian species

No.	Taxon	Lengths (bp)	GC content (%)	No.	Taxon	Lengths (bp)	GC content (%)
1	<i>Nosema</i> sp. HA	1232	34.09	12	<i>N. tyriae</i>	1233	34.39
2	<i>Nosema</i> sp. PA	1233	33.98	13	<i>N. apis</i>	1242	38.73
3	<i>Nosema</i> sp. PR	1232	34.25	14	<i>N. bombi</i>	1247	38.09
4	<i>N. antheraeae</i>	1237	34.28	15	<i>N. ceranae</i>	1259	36.46
5	<i>N. bombycis</i>	1232	34.17	16	<i>N. oulemae</i>	1251	36.85
6	<i>N. furnacalis</i>	1232	33.93	17	<i>N. portugal</i>	1393	35.75
7	<i>N. heliothidis</i>	1232	34.09	18	<i>N. vespula</i>	1245	36.79
8	<i>N. plutellae</i>	1231	34.93	19	<i>E. schubergi</i>	1252	51.04
9	<i>Nosema</i> sp. C01	1236	34.22	20	<i>Endoreticulatus</i> sp. CHW-2004 Bulgaria	1254	51.04
10	<i>Nosema</i> sp. PX1	1232	34.09	21	<i>Endoreticulatus</i> sp. CHW-2004 Taiwan	1254	51.12
11	<i>N. spodopterae</i>	1232	34.09	22	<i>Endoreticulatus</i> sp. Zhenjiang	1254	50.96

study the microsporidian phylogenetic relationship, and provide more evidence for the classification of microsporidia.

The complete SSU rRNA gene sequence of the *N. heliothidis* had 100% identity with the previously reported homologous sequences of *Nosema* sp. PX1 (isolated from *Plutella xylostella*) and *N. spodopterae* (isolated from *Spodoptera litura*). But the other ribosomal RNA sequences of these three microsporidian species, such as LSU, ITS, IGS and 5S sequences, did not have such high identity (data not shown). So the SSU rRNA sequence may not be a suitable marker for identifying closely related microsporidia [18, 19]. In 2007, Ku [20] constructed phylogenetic trees of the LSU rRNA, SSU rRNA, alpha-tubulin, beta-tubulin, and RPB1 (DNA dependent RNA polymerase II largest subunit) sequences of *Nosema* sp. PX1, *Nosema* sp. PX2, *N. spodopterae* and *N. bombycis* with four kinds of algorithm, found that PX2 was a novel species of microsporidia, and PX1 was closer to *N. bombycis* and probably a sub-species of *N. bombycis*. Recently, the ITS gene of ribosome was frequently used for the identification of parasite species and strain, and the molecular diagnosis for its inter-specific divergence. Consequently, SSU rRNA gene is suitable for the phylogenetic relationship analysis between genera and can be used as the taxonomical status at the genus level, while ITS gene of ribosome is better than SSU rRNA gene in the analysis of phylogenetic relationship between different species in the same genus and can be used for the classification of microsporidium at the species level. Therefore, phylogenetic analysis based on the combination of SSU rRNA gene and ITS gene of ribosome will provide more molecular evidence for the classification and evolution study of microsporidia.

Microsporidia are obligate intracellular parasites. They are different from independent organisms, and they live in the environments controlled by the genomes of the hosts

with coevolution [21]. Since many microsporidia are considered to have a wide host range [22], the cross infection of microsporidia often occurs between hosts, sometimes, microsporidia can infect new hosts through the transition of intermediate host, generate and evolve in this procedure. So, the gene sequences of microsporidia have some variations through the long evolutionary process. Phylogenetic trees constructed from the SSU rRNA sequences of microsporidia indicated that microsporidia formed complicated phylogenetic relationship with each other in the evolutionary process. Therefore, the research of phylogenetic relationship based on microsporidian SSU rRNA gene and the epidemiological investigation of the host range have practical value on the prevention of microsporidiosis.

**Acknowledgments** We thanked all partners and lab members for kindly help and criticism, especially for Professor XingJia Shen and ShunMing Tang.

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