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# Comparative phylogenetic analysis of intergenic spacers and small subunit rRNA gene sequences of two microsporidian isolates from *Antheraea myllita*

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**Two microsporidian isolates extracted from infected tasar silkworms (*Antheraea mylitta*) collected from forest area in Deoghar district, Jharkhand, India were subjected to PCR amplification using intergenic spacer (IGS) region and small subunit rRNA (SSU-rRNA) gene specific primers followed by cloning and sequencing. The IGS and SSU-rRNA gene sequences were analysed to derive the identity of the microspoidian isolates and establish their phylogenetic relationships. The phylogenetic analysis of test isolates included assessment of variation in sequences and length of IGS and SSU-rRNA genes with reference to 16 different microsporidian sequences. The results proved that IGS sequences have more variation than SSU-rRNA gene sequences. Analysis of phylogenetic trees reveal that both test isolates have very close relationship with each other as well as with three *Nosema* reference species viz., *N. philosamia* and *N. antheraea* isolated from *Philosamia cynthia ricini* and *Antheraea pernyi* in China respectively, and *N. disstriae* isolated from *Malacosma disstriae* in Canada. The test microsporidian isolates revealed closer relationship with other *Nosema* reference strains compared to *Nosema* sp. (NIK-1s_mys) from India. The study results indicate that the IGS or/and SSU rRNA sequence based analysis is suitable and valuable to ascertain phylogenetic relationships between various microsporidian strains/species.**

**Key words:** Microsporidia, *Antheraea mylitta*, small subunit rRNA, intergenic spacer, phylogenetic relationship.

## INTRODUCTION

*Antheraea mylitta* Drury (Lepidoptera, Saturniidae) a tropical tasar silkworm found in the states of Jharkhand, and Andhra Pradesh in India are reared on forest trees, viz. *Terminalia arjuna* Haines, *Terminalia tomentosa*

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**Table 1.** Details of the microsporidian isolates: Their place of collection, host and morphology.

| S/N | Name of microsporidian isolates | Host | Place of collection (forest area/village), district, latitude/longitude/elevation | Spore size (μm) | |
|---|---|---|---|---|---|
| | | | | Length | Width |
| 1 | MIJ-2pD | *Antheraea mylitta* | Pindari forest area, District: Deoghar, Jharkhand, India. 24˚28'49.88N/86˚42'0.00E/249.8 | 5.10±0.20 | 3.20±0.01 |
| 2 | MIJ-4cD | *Antheraea mylitta* | Chechai forest area, District: Deoghar, Jharkhand, India. 24˚28'49.88N/86˚42'0.00E/249.8 | 4.50±0.12 | 2.56±0.01 |

MIJ, Microsporidia India Jharkhand.

Haines and *Shorea robusta* Roxb. Tasar silkworm rearing generates substantial rural employment in India. However, rearing of *Antheraea mylitta* often causes infectious diseases like microsporidiosis, virosis, bacteriosis and muscardine. Among these, the most devastating is microsporidiosis caused by the microsporidian *Nosema* sp. causing severe cocoon crop loss (Singh, 2011).

Microsporidia are a diverse group of spore-forming obligate intracellular parasites that include more than 1300 described species under 160 genera (Corradi and Keeling, 2009). They infect a wide range of invertebrates and vertebrates including insects, fishes and mammals (Wittner and Weiss, 1999; Weiss, 2001). They have the smallest genomes among eukaryotic organisms and cause a variety of important medical, agricultural, veterinary, sericulture and ecological impacts (Keeling and Fast, 2002). The ultrastructural and phenotype-based classification systems faced several problems that were largely overcome through ribosomal DNA (r-DNA) sequence analysis (Baker et al., 1995; Hung et al., 1998). Several studies have been attempted to classify microsporidian species and strains through amplification and sequencing variable regions of the genome such as the ribosomal Internal Transcribed Spacer region (ITS), Inter-Genic Spacer region (IGS), Large Sub-Unit (LSU) and Small Sub-Unit rRNA (SSU rRNA) gene (Huang et al., 2008; Santin et al., 2009; Dong et al., 2010; Li et al., 2012). In fungi, the noncoding spacer regions of rDNA, which evolve rapidly, have been utilized in inferring phylogeny among more closely related taxa. The IGS region have been examined in the course of evolutionary and taxanomic studies of fungi (Erland et al., 1994; Molin et al., 1993; Aminnejad et al., 2009). Nowadays, the non-coding spacer regions (ITS/IGS) have been found valuable to study and establish relationships among closely related taxa particularly in fungi and other organisms. The ITS / IGS sequence regions can develop variations within genera that distinguish them at intra species level. The taxonomic value of ITS/IGS region is due to their significant heterogeneity in length and nucleotide sequences. This study targeted the SSU-rRNA gene and IGS region located in between the SSU-rRNA and 5S-rRNA gene cluster of the microsporidia. The IGS region that has the most rapidly evolving sequence provides significant data considered phylogenetically useful for delineating relationships within species (Hillis et al., 1991).

The aim of our study was to identify and delineate microsporidian isolates based on IGS and SSU rRNA gene sequences. In order to analyze the IGS and SSU rRNA gene sequences, we had amplified IGS and SSU rRNA gene followed by cloning and sequencing. We defined and analyzed inter and intra-individual variations in the IGS and SSU rRNA gene sequences of test microsporidian isolates. The heterogeneity of r-DNA sequence derives the genetic variations of isolates and elucidates phylogenetic relationship among defined microsporidian species/strains.

## MATERIALS AND METHODS

### Collection, isolation and purification of microsporidian spores

Two strains of microsporidia were extracted from individual infected moths of *A. mylitta* collected from the forests areas of Deoghar district Jharkhand, India by maceration and suspended in 0.85% NaCl followed by filtration through layers of cheese cloth and centrifugation at 3500 r/min for 10 min. The spore pellet obtained was further purified through density gradient ultracentrifugation using Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) described by Undeen (Undeen and Alger, 1971). The details of microsporidian isolates, places of collection, host and size are given in Table 1.

### Spore morphology (length and width)

The morphology of purified fresh spores was observed under phase contrast microscope (Carl Zeiss-AXIO, Humburg, Germany) and measurements were recorded according to the method of Undeen (Undeen and Vavra, 1997). Fresh spores were spread in water agar on glass micro-slides and measured using an ocular micrometer under phase contrast microscope. All the measurements are presented in micrometers as mean values of 12 individual observations.

### DNA extraction and purification

Genomic DNA was extracted from the sporoplasm using the glass bead method (Undeen and Cockburn, 1989). DNA concentration

**Table 2.** Detail of SSU-rDNAs sequences submitted in NCBI of two microsporidian isolates.

| S/N | Microsporidian isolates ID | Sequences details | | | |
|---|---|---|---|---|---|
| | | SSU-rRNA (bp) | Gene bank accession no. | IGS (bp) | Gene bank accession no. |
| 1 | MIJ-2pD | 1232 | KU187949 | 270 | KP151550 |
| 2 | MIJ-4cD | 1232 | KU187950 | 281 | KP151552 |

and quality was determined both by spectrophotometry at 260 and 280 nm and on 0.8% agarose gel,using a known quantity of λ DNA (10 ng/µl) as a standard before use in subsequent PCRs. Any possibility of host DNA contamination was checked using insect mitochondrial primers. A working solution of DNA (10 ng/µl) was prepared in sterile double distilled water and the concentration and purity was determined as per standard protocol.

#### PCR amplification of SSU-rRNA gene

The genomic DNA of both microsporidian isolates were amplified using SSU-rRNA gene primers [Forward-5'-CACCAGGTTGATTCTGCCTGAC-3' and Reverse - 5'-GATATAAGTCGTAACATGGTTGC-3'] as previously described (Wang et al., 2006). The amplified products excised from the agarose gel were eluted using the QIA quick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

#### PCR amplification of IGS region

Genomic DNA from the two microsporidian isolates was amplified with primers (forward primer - 5'-CGTCGTCTATCTAAGATGGTATTATC-3' Reverse primer - 5'-TACAGCACCCAACGTTCCCAAG-3') designed from *Nosema bombycis* SSU-rRNA gene sequences (D85503 and D85504) having product size between 1115 and 1141 bp (26 mer) (Huang et al., 2008). PCR amplification was carried out in 20 µl, using 10 ng DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 1 U of Taq Polymerase (MBI Fermentas, USA). The amplification conditions were: 94°C denaturation for 3 min, followed by 35 cycles of 94°C for 60s, 55°C annealing for 2 min, and extension at 72°C for 30 s, with a final extension of 10 min on a thermal cycler (MJ Reaserch). The primers generated expected fragment size of a range between 510 and 515 bp [117 bp-16s SSU rRNA gene, 279 bp-IGS, and 115 bp-5s rRNA gene]. The amplified products were visualized on 1.2% agrose gel and purified by QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

#### Cloning and sequencing of SSU-rRNA gene and IGS region

The purified DNA fragments were ligated into a pJET1.2 cloning vector in the presence of T4 DNA ligase (CloneJET PCR Cloning Kit, Thermo Scientific) at 22°C. The ligated products were transfected into JM101 competent cells and plated on ampicillin agar plates. White colonies were selected, and plasmids were isolated from the cells using the GeneJET plasmid miniprep kit (Fermentas Life Sciences). After isolating plasmid DNA from transformed *Escherichia coli* cells, the plasmids were digested with restriction enzyme (Bgl II) to check whether they contained the desired insert. Three clones from each of the microsporidian isolates were sequenced using DNA sequencing kit (BDT version 3.1) on a semi-automatic DNA sequencer (ABI Prism 310, Applied Biosystems, Perkin Elmer) with M13 universal primers at Eurofins Genomics India Pvt. Ltd., Bangalore, India. The sequences were cleaned of any vector contamination using a vector screen program, (NCBI, Bethesda, Maryland, USA). The final sequences were deposited in NCBI GeneBank and the details are given in Table 2.

#### Phylogenetic analysis of SSU-rRNA and IGS-Sequences

The analysis of SSU-rRNA and IGS sequence homology was carried out using BLAST search from NCBI database. In contrast, 16 non-redundant microsporidian sequences from *Nosema*/*Vairimorpha* species including an out-group *Glugoides intestinalis* for IGS and *Encepalatazoon hellem* for SSU-rRNA were retrieved from the NCBI database. These sequences were aligned with IGS and SSU-rRNA gene sequences of test microsporidian isolates including an Indian *Nosema* reference strain in CLUSTAL W program (Higgins et al., 1994) (Table 3). The molecular phylogenetic trees were constructed from aligned sequences using maximum likelihood using the branch and bound option (with 500 bootstrap replicates) of the MEGA program (Version 6.0) (Tamura et al., 2013). The cloned and sequenced IGS and SSU-rRNA gene sequences of the test isolates and 16 *Nosema* reference strains were checked for sequence similarity using the Sequence Identity Matrix in BioEdit software (Hall, 1999) (Tables 4 and 5).

## RESULTS

### Morphological characterization

The spore sizes of both the isolates ranged from 4.50 to 5.10 <mu>m in length and 2.56 to 3.20 <mu>m in width. Details are given in Table 1.

### Molecular characterization

#### *PCR amplification, cloning and sequencing of IGS fragment and SSU-rRNA gene*

The PCR amplification for both the microsporidian isolates using targeted IGS primer sets was successfully carried out. The amplified fragment consisted of a partial region of SSU-rRNA gene followed by complete sequences of IGS and 5Sr RNA gene with an expected fragment size of approximately 500 bp Substantial full-length sequences of the IGS cloned gene was obtained

**Table 3.** Taxon, source, host and Gene Bank Acc. No. of microsporidian species used in the phylogenetic analysis.

| Accession number | Organism name | Host | Order | Family |
|---|---|---|---|---|
| KP151550 | MIJ-2pD | *Antheraea mylitta* | Lepidoptera | Saturniidae |
| KP151552 | MIJ-4cD | *Antheraea mylitta* | Lepidoptera | Saturniidae |
| KP177890 | NIK-1s_mys | *Bombyx mori* | Lepidoptera | Bombycidae |
| FJ767862 | *Nosema philosamia ricini* | *Philosamia Cynthia* | Lepidoptera | Saturniidae |
| DQ073396 | *Nosema antheraeae* | *Antheraea pernyi* | Lepidoptera | Saturniidae |
| HQ457431 | *Nosema disstriae* | *Malacosma disstriae* | Lepidoptera | Lasiocampidae |
| AY960987 | *Nosema plutellae* | *Plutella xylostella* | Lepidoptera | Plutellidae |
| HQ457432 | *Nosema fumiferanae femiferane* | *Choristoneura* | Lepidoptera | Tortricidae |
| JF443684 | *Nosema bombycis* | *Bombyx mori* | Lepidoptera | Bombycidae |
| HQ891818 | *Vairimorpha* sp. | *Bombyx mori* | Lepidoptera | Bombycidae |
| AF394525 | *Glugoides intestinalis* | *Daphnia magna* | Cladocera | Daphniidae |
| *EU338534* | *Uncultured Nosema arsakia* | *Eurema blanda* | Lepidoptera | Pieridae |
| FJ969508 | *Nosema* sp. *PA armata Baly* | *Phyllobrotica* | Coleoptera | Chrysomelidae |
| FJ772435 | *Nosema heliothidis armigera* | *Helicoverpa* | Lepidoptera | Noctuidae |
| AY747307 | *Nosema spodopterae* | *Spodoptera litura* | Lepidoptera | Noctuidae |
| JN882299 | *Nosema* sp. *HA atrilinata* | *Hemerophila* | Lepidoptera | Geometridae |
| JN792450 | *Endoreticulatus* sp. | *Bombyx mori* | Lepidoptera | Bombycidae |
| DQ445481 | *Nosema bombycis* | *Bombyx mori* | Lepidoptera | Bombycidae |
| L19070 | Encephalitozoon hellem | *Homo sapience* | -- | Encephalitozoonidea |

for both the isolates. Similarly, SSU-r RNA gene for both microsporidian isolates amplified with an expected fragment size of 1232 bp. Appreciable full-length sequences of the SSU-rRNA cloned gene were obtained and the sequences were successfully submitted to the NCBI-Gen Bank. The individual accession details for the submitted IGS and SSU rRNA gene sequences are given in Table 2.

### *Length and sequence variation in IGS and SSU-rRNA gene sequences*

The IGS and SSU-rRNA gene sequences of 16 different microsporidians species with similar homology downloaded from NCBI were utilized for analysis. The sequence similarity of IGS sequence between test microsporidian isolates was observed to be 86%, while, SSU-rRNA gene sequence similarity was 99%. The average sequence similarity between test isolates and all reference *Nosema* sp. for IGS was 75%, whereas, 97% similarity was found in case of SSU-rRNA gene. Highest (90%) IGS sequence similarity was observed between the test isolates [MIJ-2pD and MIJ-4cD], and a reference strain *Nosema antheraeae*, while, both test isolates had least (34%) similarity with *Varimorpha* reference species (Table 4). On the other hand, SSU-rRNA gene sequence similarity between test and reference microsporidians strains were very high with both test isolates showing about 99% similarity with almost all reference *Nosema* strains except NIK-1s_mys. Accordingly, it was clearly observed that test isolates had substantial low similarity for IGS sequences compared to SSU-rRNA gene sequences (Tables 4 to 5). As expected, a very low level of similarity was observed between both IGS and SSU-rRNA gene sequences of test isolates and out-group species: *Glugoides intestinalis* for IGS and *Encepalatazoon hellem* for SSU-rRNA (Tables 4 to 5).

### *Phylogenetic analysis based on IGS and SSU-rRNA gene sequences*

Two dendrograms were constructed based on IGS or SSU-rRNA gene sequences. In both cases, test isolates separated from reference microsporidia species with above 50% bootstrap value (Figures 1 and 2). The analysis of both phylogenetic trees manifested that the test isolates and 16 *Nosema* reference strains including an out-group separated into two major clades. In the IGS phylogenetic tree, the test microsporidian isolates and three *Nosema* reference species [*N. philosamia* (FJ767862.1), *N. antheraeae* (DQ073396.1) and *N. disstriae* (HQ457431)] grouped in a single clade, while in SSU-rRNA gene phylogenetic tree one additional uncultured *Nosema* sp. (EU338534) joined the test isolate group. The other eleven *Nosema* reference species including Indian *Nosema* sp. (NIK-1s_mys)

**Table 4.** IGS sequences similarity matrix of 18 different microsporidians strain including two experimental isolates.

| Sequence | MIJ-2pD (KP153550) | MIJ-4cD (KP153552) | NIK_1s_mys (KP177890) | Nosema sp. (FJ767862) | Nosema antheraeae (DQ073396) | Nosema disstriae (HQ457431) | Nosema plutellae (AY960987) | Nosema fumiferanae (HQ457432) | Nosema bombycis (JF443684) | Vairimorpha sp. (HQ891818) | Glugoides intestinalis (AF394525) | Uncultured Nosema (EU338534) | Nosema sp.PA (FJ969508) | Nosema heliothidis (FJ772435) | Nosema spodopterae (AY747307) | Nosema sp.HA (JN882299) | Endoreticulatus sp. (JN792450) | Nosema bombycis (DQ445481) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MIJ-2pD (KP153550)* | ID | 0.86 | 0.64 | 0.86 | 0.88 | 0.81 | 0.71 | 0.68 | 0.66 | 0.34 | 0.34 | 0.65 | 0.64 | 0.65 | 0.62 | 0.66 | 0.65 | 0.65 |
| *MIJ-4cD (KP153552)* | 0.86 | ID | 0.69 | 0.90 | 0.90 | 0.85 | 0.75 | 0.71 | 0.69 | 0.37 | 0.36 | 0.69 | 0.69 | 0.67 | 0.66 | 0.68 | 0.69 | 0.69 |
| *NIK_1s_mys (KP177890)* | 0.64 | 0.69 | ID | 0.69 | 0.67 | 0.68 | 0.69 | 0.66 | 0.81 | 0.38 | 0.36 | 0.80 | 0.78 | 0.76 | 0.76 | 0.78 | 0.73 | 0.73 |
| *Nosema sp. (FJ767862)* | 0.86 | 0.90 | 0.69 | ID | 0.90 | 0.87 | 0.74 | 0.70 | 0.69 | 0.37 | 0.37 | 0.70 | 0.70 | 0.66 | 0.66 | 0.69 | 0.69 | 0.69 |
| *Nosema antheraeae DQ073396)* | 0.88 | 0.90 | 0.67 | 0.90 | ID | 0.84 | 0.73 | 0.69 | 0.68 | 0.38 | 0.34 | 0.69 | 0.67 | 0.65 | 0.64 | 0.68 | 0.68 | 0.68 |
| *Nosema disstriae (HQ457431)* | 0.81 | 0.85 | 0.68 | 0.87 | 0.84 | ID | 0.73 | 0.67 | 0.69 | 0.39 | 0.36 | 0.70 | 0.69 | 0.66 | 0.64 | 0.69 | 0.69 | 0.69 |
| *Nosema plutellae (AY960987)* | 0.71 | 0.75 | 0.69 | 0.74 | 0.73 | 0.73 | ID | 0.87 | 0.71 | 0.40 | 0.35 | 0.71 | 0.68 | 0.67 | 0.67 | 0.68 | 0.66 | 0.66 |
| *Nosema fumiferanae (HQ457432)* | 0.68 | 0.71 | 0.66 | 0.70 | 0.69 | 0.67 | 0.87 | ID | 0.67 | 0.39 | 0.34 | 0.67 | 0.67 | 0.65 | 0.65 | 0.68 | 0.65 | 0.65 |
| *Nosema bombycis (JF443684)* | 0.66 | 0.69 | 0.81 | 0.69 | 0.68 | 0.69 | 0.71 | 0.67 | ID | 0.39 | 0.38 | 0.86 | 0.78 | 0.84 | 0.82 | 0.84 | 0.74 | 0.74 |
| *Vairimorpha sp. (HQ891818)* | 0.34 | 0.37 | 0.38 | 0.37 | 0.38 | 0.39 | 0.40 | 0.39 | 0.39 | ID | 0.31 | 0.38 | 0.39 | 0.39 | 0.37 | 0.39 | 0.37 | 0.37 |
| *Glugoides intestinalis (AF394525)* | 0.34 | 0.36 | 0.36 | 0.37 | 0.34 | 0.36 | 0.35 | 0.34 | 0.38 | 0.31 | ID | 0.35 | 0.35 | 0.36 | 0.35 | 0.40 | 0.34 | 0.34 |
| *Uncultured Nosema (EU338534)* | 0.65 | 0.69 | 0.80 | 0.70 | 0.69 | 0.70 | 0.71 | 0.67 | 0.86 | 0.38 | 0.35 | ID | 0.78 | 0.79 | 0.84 | 0.82 | 0.75 | 0.75 |
| *Nosema sp.PA (FJ969508)* | 0.64 | 0.69 | 0.78 | 0.70 | 0.67 | 0.69 | 0.68 | 0.67 | 0.78 | 0.39 | 0.35 | 0.78 | ID | 0.75 | 0.76 | 0.79 | 0.73 | 0.73 |
| *Nosema heliothidis (FJ772435)* | 0.65 | 0.67 | 0.76 | 0.66 | 0.65 | 0.66 | 0.67 | 0.65 | 0.84 | 0.39 | 0.36 | 0.79 | 0.75 | ID | 0.80 | 0.84 | 0.74 | 0.74 |
| *Nosema spodopterae (AY747307)* | 0.62 | 0.66 | 0.76 | 0.66 | 0.64 | 0.64 | 0.67 | 0.65 | 0.82 | 0.37 | 0.35 | 0.84 | 0.76 | 0.80 | ID | 0.83 | 0.77 | 0.77 |
| *Nosema sp.HA (JN882299)* | 0.66 | 0.68 | 0.78 | 0.69 | 0.68 | 0.69 | 0.68 | 0.68 | 0.84 | 0.39 | 0.40 | 0.82 | 0.79 | 0.84 | 0.83 | ID | 0.75 | 0.75 |
| *Endoreticulatus sp. (JN792450)* | 0.65 | 0.69 | 0.73 | 0.69 | 0.68 | 0.69 | 0.66 | 0.65 | 0.74 | 0.37 | 0.34 | 0.75 | 0.73 | 0.74 | 0.77 | 0.75 | ID | 1.00 |
| *Nosema bombycis (DQ445481)* | 0.65 | 0.69 | 0.73 | 0.69 | 0.68 | 0.69 | 0.66 | 0.65 | 0.74 | 0.37 | 0.34 | 0.75 | 0.73 | 0.74 | 0.77 | 0.75 | 1.00 | ID |

clustered separately with test isolates in three small sub groups in both cases (Figures 1 and 2). In addition, the reference *Vairimorpha* species separated alone in the same clade with all reference *Nosema* sp. and test isolates in both generated dendrograms. As expected, *G. intestinalis* (AF394525) and *E. hellem* (L19070) used as an out-group in IGS and SSU-rRNA based dendrograms, respectively got separated from both major clades in the constructed trees (Figures 1 and 2).

## DISCUSSION

The present study aimed at characterization of two strains of microsporidia isolated from the tasar silkworm *A. mylitta* based on morphology and phylogenetic analysis of variations in IGS and SSU-rRNA gene sequences.

### Morphological characterization

Morphological studies did not reveal any major variations in spore width and length of the isolates. Since spore size for a given species may vary

**Table 5.** SSU-rRNA gene sequences similarity matrix of 18 different microsporidians strain including two experimental isolates.

| Sequence | MIJ-2pD (KU187949) | Nosema heliothidis (FJ772435) | Nosema sp.SC (FJ767862) | Nosema spodopterae (AY747307) | Nosema bombycis (JF443577) | MIJ-4cD (KU187950) | Nosema sp.PA (FJ969508) | Nosema antheraeae(DQ073396) | Nosema sp.HA(JN882299) | Nosema disstriae(HQ457431) | Nosema bombycis(DQ445481) | Nosema fumiferanae(HQ457432) | Nosema plutellae (AY960987) | Endoreticulatus sp.(AF240355) | Uncultured Nosema(EU338534) | Vairimorpha sp. Sd (HQ891818) | Nosema sp. NIK-1s (AY713309) | Encephalitozoon hellem (L19070) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *MIJ-2pD (KU187949)* | ID | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.97 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema heliothidis (FJ772435)* | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.97 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema sp.SC (FJ767862)* | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.97 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema spodopterae (AY747307)* | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.97 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema bombycis (JF443577)* | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.97 | 0.98 | 0.82 | 0.47 | 0.42 |
| *MIJ-4cD (KU187950)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.96 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema sp.PA (FJ969508)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.97 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema antheraeae(DQ073396)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.99 | 0.98 | 0.96 | 0.98 | 0.82 | 0.46 | 0.42 |
| *Nosema sp.HA(JN882299)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.99 | 0.98 | 0.96 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema disstriae(HQ457431)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.99 | 0.98 | 0.96 | 0.98 | 0.82 | 0.47 | 0.42 |
| *Nosema bombycis(DQ445481)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.99 | 0.98 | 0.97 | 0.97 | 0.82 | 0.47 | 0.42 |
| *Nosema fumiferanae(HQ457432)* | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | ID | 0.98 | 0.97 | 0.97 | 0.82 | 0.47 | 0.42 |
| *Nosema plutellae (AY960987)* | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | ID | 0.96 | 0.97 | 0.81 | 0.47 | 0.42 |
| *Endoreticulatus sp.(AF240355)* | 0.97 | 0.97 | 0.97 | 0.97 | 0.97 | 0.96 | 0.97 | 0.96 | 0.96 | 0.96 | 0.97 | 0.97 | 0.96 | ID | 0.95 | 0.80 | 0.48 | 0.40 |
| *Uncultured Nosema(EU338534)* | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.98 | 0.97 | 0.97 | 0.97 | 0.95 | ID | 0.81 | 0.46 | 0.42 |
| *Vairimorpha sp. sd(HQ891818)* | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.81 | 0.80 | 0.81 | ID | 0.39 | 0.43 |
| *Nosema sp. NIK-1s (AY713309)* | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 | 0.46 | 0.47 | 0.47 | 0.47 | 0.47 | 0.47 | 0.48 | 0.46 | 0.39 | ID | 0.53 |
| *Encephalitozoon hellem (L19070)* | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.42 | 0.40 | 0.42 | 0.43 | 0.53 | ID |

with respect to the host, the morphological characteristics are not considered for delineating identity of microsporidian isolates (Brooks et al., 1972).

## Molecular characterization

Genotyping studies based on the ribosomal RNA small subunit gene (SSU-rRNA) sequence analysis are a promising tool for organism identification (Zhu et al., 2010; Ku et al., 2007). The ribosomal DNA comprises of highly conserved SSU gene sequences that occur in multiple copies within the eukaryotic genome and has been widely exploited since many years, for deriving phylogeny inference (Liu et al., 2012). The phylogenetic relationships of several microsporidian genera including *Vairimorpha* and *Nosema* were studied based on their SSU-rDNA sequence similarity (Baker et al., 1994; Dong et al., 2010). Three different microsporidia species showed 100% identity of SSU r-RNA gene sequences while their ITS, IGS and 5S sequences varied (Dong et al., 2010). Molecular variations have been established among *Trichosporon* isolates from close geographical locations based

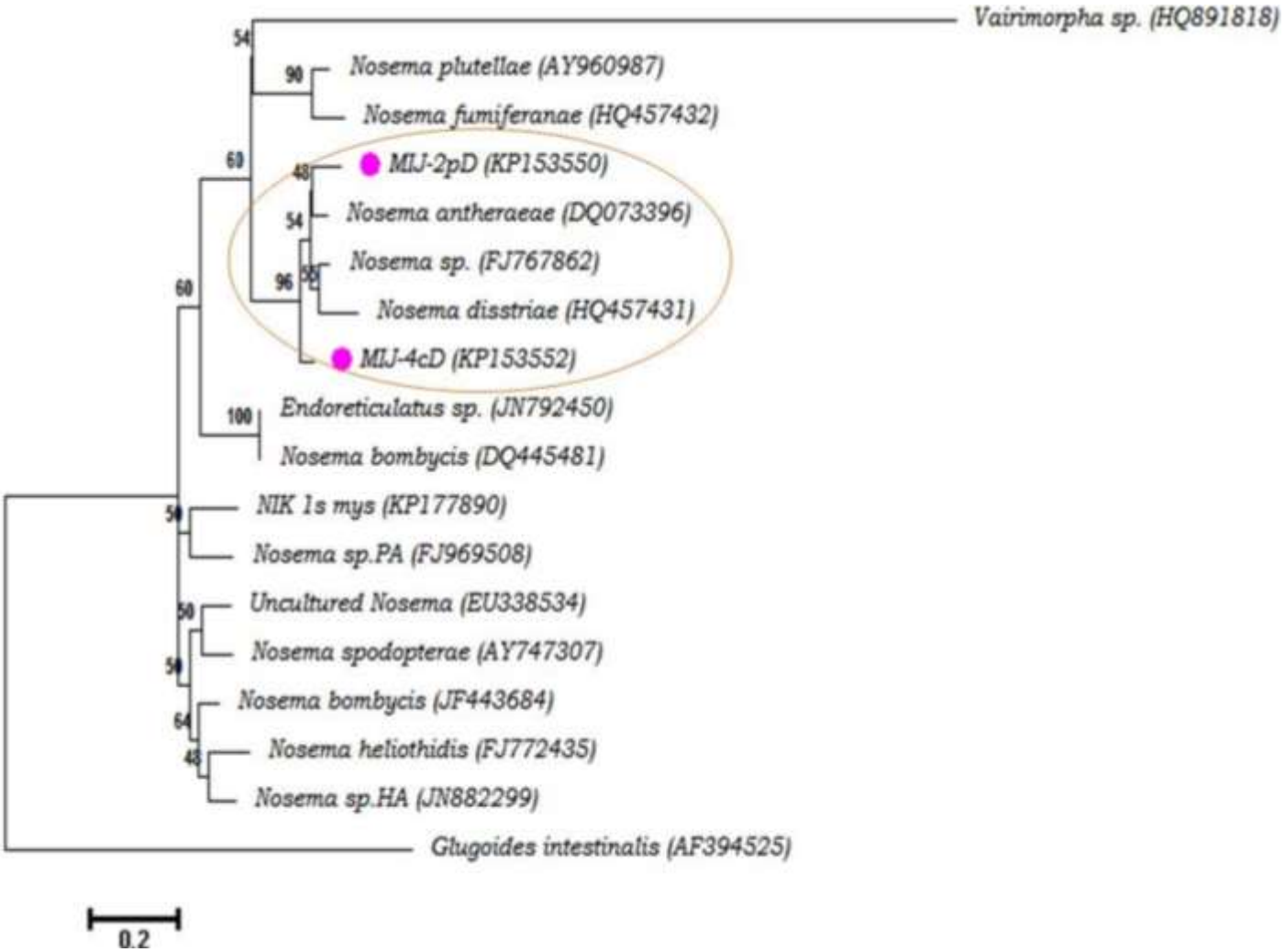


**Figure 1.** Phylogenetic tree based on IGS sequences. Eighteen different microsporidians IGS sequences were analyszed based on maximum likelihood approach using MEGA 6 (Tamura, 2013) run with 500 bootstrap replication. Number of each node indicates bootstrap value.

based on restriction patterns of IGS (Sugita et al., 2002). Various studies have indicated that, molecular phylogenetic analysis based on IGS region might be a better tool to investigate intra-specific divergence and would provide significant molecular evidence for classification and evolutionary studies of microsporidia (Dong et el., 2010; Sagastume et al., 2010; Liu et al., 2013). A recent report indicated differentiating of *N. ceranae* strains from different geographic origins in Europe based on sequence analysis of highly variable regions of IGS sequence and a part of r-RNA that corresponded to IGS region and their virulence (Dussaubat et al., 2012).

Based on these reports we targeted the IGS as well as SSU-rRNA sequences for phylogenetic analysis of key microsporidia isolates and also made a comparative study of the said gene sequences with 16 different *Nosema*/*Vairimorpha* strains to derive a phylogenetic inference for both microsporidian strains isolated from *A. myllita*. The IGS and SSU-rRNA gene sequences of the isolates indicated that they belong to the genus *Nosema*. Comparative sequence similarity analysis of IGS and SSU-rRNA sequence for the test isolates and reference strains revealed that SSU-RNA gene sequence is highly conserved. Sequence similarity analysis revealed that, the IGS sequence (280 bp) showed 70% similarity between test and reference microsporidian isolates while SSU-rRNA (1240 bp) showed 99% similarity. The dendrograms of both test isolates based on IGS and SSU-rRNA gene sequences revealed close genetic relationship with three *Nosema* species, that is, *Nosema philosamia*, and *Nosema antheraeae* from China and

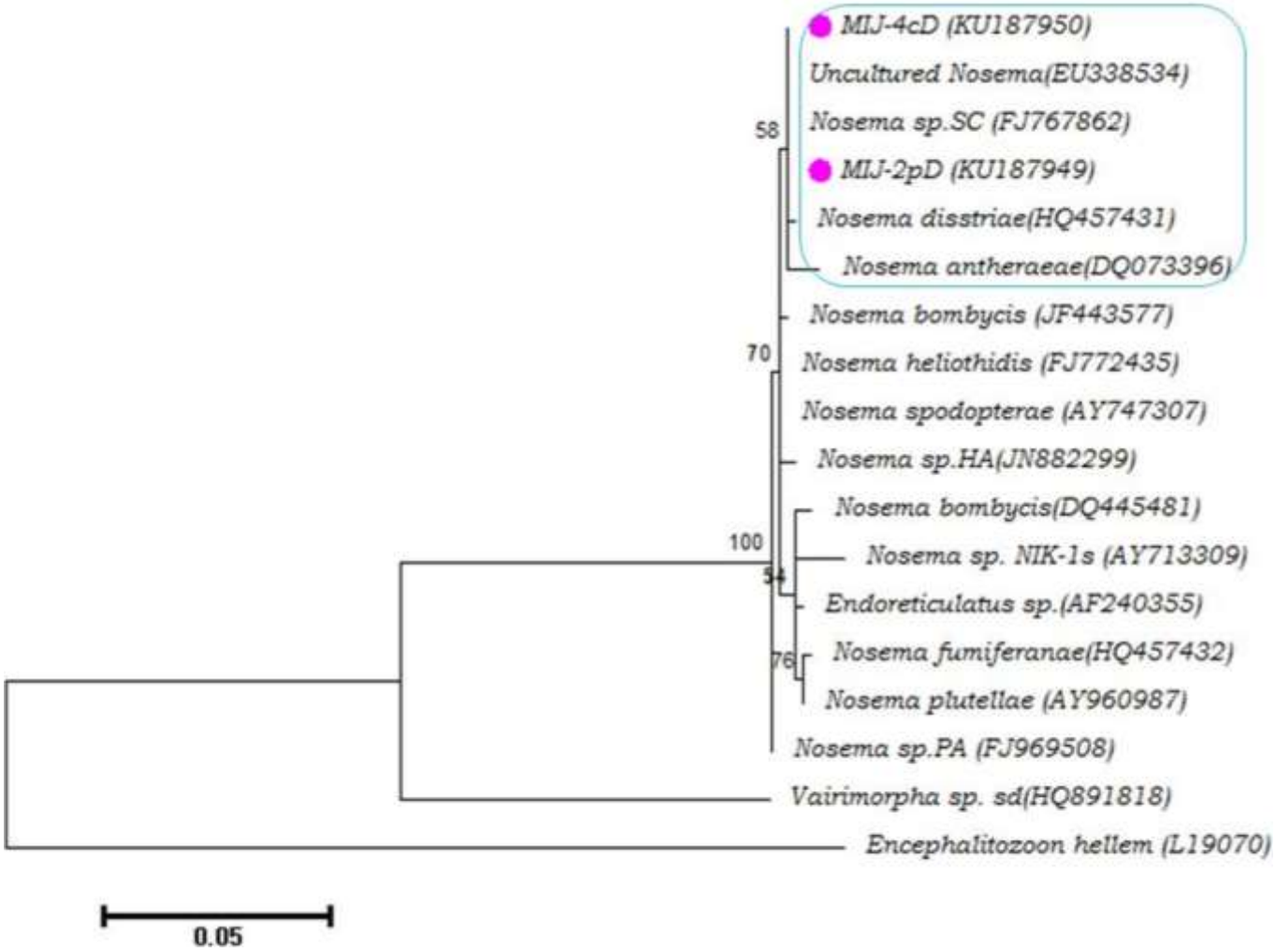


**Figure 2.** Phylogenetic tree based on SSU-rRNA gene sequences. Eighteen different microsporidians IGS sequences were analyszed based on maximum likelihood approach using MEGA 6 (Tamura, 2013) run with 500 bootstrap replication. Number of each node indicates bootstrap value.

*Nosema disstriae* from Canada. Since, the test isolates were extracted from *Antheraea mylitta* the genetic similarity of test isolates with *N. philosamia*, and *N. antheraeae* support host specification of the microsporidian strains. The trees generated from the IGS and SSU-rRNA gene sequences of the test microsporidia isolates revealed a closed as well as complex phylogenetic relationship of the test isolates with each other due to evolutionary process. Thus, the sequence based phylogenetic analysis of IGS and SSU-rRNA genes provided additional molecular evidence for the classification and evolutionary study of microsporidian isolates on species as well as genus level.

In recent times, the ITS/IGS genes of ribosome were utilized for identification of parasite species and strains as well as a tool for molecular diagnosis. The SSU rRNA gene sequence variation used for establishing phylogenetic relationship between different species in the same genus can also be used for the classification of microsporidia at the species level. Consequently, sequence and phylogenetic analysis based on the combination of SSU rRNA gene and IGS of ribosome might provide additional molecular evidence for the classification and study of evolution of microsporidia. Thus, the gives strong evidence of genetic recombination in *N. antheraeae* or *N. philosamia* and a molecular method for defining species in closely related *Nosema* species. Further, it also revealed, the SSU-rRNA and IGS sequence variability among the microsporidian isolates that would be a rich source of information and could serve to differentiate these isolates in order to give us insight into their origins as well as the spread of this

economically important disease of the tasar silkworm in India.

## Conflicts of Interests

The authors have not declared any conflict of interests.

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### REFERENCES

Aminnejad M, Babai-Ahri A, Javan-Nikkhah M, Saeid HM (2009). Molecular and Pathogenic variation within Iranian *Pyrenophoragraminea* Population; More polymorphism in IGS Region. Am-Eur.J. Agric. Environ. Sci. 6(6):697-704.

Baker MD, Vossbrinck CR, Didier ES, Maddox JV, Shadduck JA (1994). Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. J. Eukaryot Microbiol. 42:564- 570.

Brooks WM, Cranford JD (1972). Microsporidiases of the hymenopterous parasites, *Campoletissonorensis* and *Cardiochilesnigriceps*, larval parasites of *Heliothis* species. J. Invert. Pathol. 20:77-94.

Corradi N, Keeling PJ (2009). Microsporidia: a journey through radical taxonomical revisions. Fungal Biol. Rev. 23(1-2):1-8.

Dong SN, Shen ZY, Xu L, Zhu F (2010). Sequence and Phylogenetic analysis of SSU r RNA gene of five microsporidia. Curr. Microbiol. 60:30-37.

Dussaubat C, Sagastume S, Moracho TG, Botias C, Palencia PG, Hernandez RM, Conte YL, Higes M (2012). Comparative study of *Nosemaceranae* (Microsporidia) isolates from two different geographic origins. Vet. Microbiol. 162:670-67.

Erland SB, Henrion F, Martin LA, Alexender IJ (1994). Identification of the ectomycorrhizalbasidiomycetes*Tylospora fibrillose*Donk by RFLP analysis of the PCR-amplified ITS and IGS regions of ribosomal DNA. New Phytol. 126:525-532.

Hall TA (1999). Bioedit: a user-friendly biological alignment editor and analysis programme for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

Hillis DM, Dixon MT (1991). Ribosomal DNA: Molecular evolution and phylogenetic inference. Quart. Rev. Biol. 66(4):411-453.

Huang WF, Bocquet M, Lee KC, Sung IH, Jiang JH, Chen YW, Wang CH (2008). The comparison of rDNA spacer regions of *Nosemaceranae* isolates from different hosts and locations. J. Invert. Pathol. 97: 9-13.

Hung HW, Lo CF, Tseng CC, Peng SE, Chou CM, Kou GH (1998). The small subunit ribosomal RNA gene sequence of *Pleistophora anguillarum* and the use of PCR primers for diagnostic detection of the parasite. J. Eukaryot. Microbiol. 45:556-560.

Keeling PJ, Fast NM (2002). Microsporidia: Biology and evolution of highly reduced intracellular parasites. Annu. Rev. Microbiol. 56:93-116.

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. Invert. Pathol. 95:71-76.

Li JL, Chen WF, Wu J, Peng WJ, An JD, Schmid-Hempel P, Schmid-Hempel R (2012). Diversity of *Nosema* associated with bumblebees (Bombus spp.) from China. Inter. J. Parasitol. 42: 49–61.

Liu H, Pan G, Li T, Huang W, Luo B, Zhou Z (2012). Ultrastructure, chromosomal karyotype, and molecular phylogeny of a new isolate of microsporidianVairimorpha sp. BM (Microsporidia, Nosematidae) from *Bombyxmori* in China. Parasitol. Res. 110:205-210.

Liu H, Pan G, Luo B, Li T, Yang Q, Vossbrinck CR, Debrunner-Vossbrinck BA, Zhou Z (2013). Intraspecific polymorphism of r DNA among five *Nosemabombycis* isolates from different geographical regions in China. J. Invert. Pathol. 113(1):63-69.

Molin FI, Jong S, Huffman JL (1993). PCR amplification of the 3' external transcribed and intergenic spacers of the ribosomal DNA repeat unit three species of Saccharomyces. FEMS Mircobiol. Lett. 108:259-264.

Sagastume S, Aguila DC, Hernandez MR, Higes M, Gil HN (2010). Polymorphism and recombination for rDNA in the putatively asexual microsporidian *Nosema ceranae* a pathogen of honeybees. Environ. Microbiol. 13:84-95.

Santin M, Fayer R (2009). EnterocytozoonbieneusiGenoreference Nomenclature Based on the Internal Transcribed Spacer Sequence: A Consensus. J. Eukaryo. Microbiol. 56:34-38.

Singh GP, Kumar KPK, Sinha AK, Prasad BC (2011). Manual on Management of Diseases in Tasar Silkworm. CTRTI, Ranchi, 96 pp.

Sugita T, Nakajima M, Ikeda R, Matsushima T, Shinoda T (2002). Sequence analysis of Ribosomal DNA Intergenic Spacer I regions of Trichosporon Species. J. Clin. Microbiol. 40(5):826-830.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular Evolution Genetics Analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.

Undeen AH, Alger NE (1971). A density gradient method for fractionating microsporidian spores. J. Invert. Pathol. 18:419-420.

Undeen AH, Cockburn AF (1989). The extraction of DNA from microsporidia spores. J. Invert. Pathol. 54:132--133.

Undeen AH, Vavra J (1997). Research methods for entomopathogenic protozoa. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press San Diego USA. pp. 117-151.

Wang LL, Chen KP, Zhang Z, Yao Q, Gao GT, Zhao Y (2006). Phylogenetic analysis of *Nosemaantheraeae* (Microsporidia) isolated from Chinese oak silkworm, *Antheraea pernyi*. J. Eukaryot Microbiol. 53(4):310- 313.

Weiss LM (2001). Microsporidia 2001: Cincinnati. J. Eukaryot Microbiol. S: 47S–49S.

Wittner M, Weiss LM (1999). The Microsporidia and Microsporidiosis. ASM Press, Washington, D.C. 553 p.

Zhu F, Shen Z, Xu X, Tao H, Dong SN, Tang X, Xu L (2010). Phylogenetic Analysis of Complete rRNA Gene Sequence of *Nosema philosamiae* Isolated from the Lepidopteran *Philosamia cynthia ricini*. J. Eukaryot Microbiol. 57(3):294-296.