IJIE Int. J. Indust. Entomol. 33(2) 121-131 (2016)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2016.33.2.121

Sequence and phylogenetic analysis of Intergenic spacer (IGS) region of ten microsporian isolates infecting Indian vanya silkworms (*Samia cynthia ricini* and *Antheraea assamensis*).

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Abstract

Ten microsporidian isolates from *Samia cynthia ricini*, and *Antheraea assamensis* in India along with a *Nosema* reference strain (NIK-1s_mys) from *B. mori* India were characterised morphologically and molecular based tools. The test isolates observed elongated oval in shape while reference strain was oval and ranging from 3.80 to 4.90 <mu>m in length and 2.60 to 3.05 <mu>m in width. The ribosomal DNA region 'IGS' of test isolates assessed by PCR amplification, followed by cloning and sequencing. IGS sequence and phylogenetic analysis of test microsporidian isolates showed very close relationship with three *Nosema* references species: *N. philosamia*, *N. antheraea* isolated from *Philosamia cynthia ricini* and *Antheraea perny* in China respectively and *N. disstriae* from *Malacosma disstriae* in Canada. The clustering pattern of dendogram reveals all test isolates appear distinct from *Nosema* std. (NIK-1s_mys) India used as reference strain in the study. The result suggests IGS indeed a suitable and highly applicable molecular tool for identifying and characterise the microsporidian isolates in similar population.

Int. J. Indust. Entomol. 33(2), 121-131 (2016)

Received : 13 Jun 2016
Accepted : 24 Nov 2016

Keywords:
microsporidia,
Samia cynthia ricini,
Antheraea assamensis,
Intergenic Spacer (IGS),
genetic variability

Introduction

Wild silkworms, *Samia cynthia ricinii* (Donovan; Lepidoptera: Saturniidae) and *Antheraea assamensis* (Helfer; Lepidoptera: Saturniidae) confined to the Brahmaputra valley of Assam, India and in the tribal inhabited districts of other northeast States of the country. Presently, more than 30,000 rural families in Assam, India are directly or indirectly associated with vanya silk production.

Microsporidia are highly derived relative of fungi that are obligate intracellular spore forming parasites of almost all animals. To date, formally 1300 species under 160 genera have been described, and they cause a variety of important economically and medically diseases particularly in sericulture and apiculture (Corradi and Keeling 2009). The first microsporidial species was described *Nosema bombycis*, the causative agent of pebrine, a disease of the silkworm *Bombyx mori* (Nageli 1857). In 1863, Pasteur firstly investigated to find

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a method of control pebrine (Pastuer 1870). Now a day Pebrine still takes a heavy economic toll on the silk industry (Quan 2011). In recent past, many redundant microsporidian species have been designated based on morphology, ultra structure, life cycle and host parasite relationships that affect sericulture and apiculture.

The r-DNA coding gene arranged in tandem repeats within the nuclear genome of microsporidia. It was described the repeat unit is arranged in the reverse order (LSU-ITS1-SSU-IGS-5S) in *N. bombycis* (Huang *et al*. 2004), *Nosema antheraeae* (Wang *et al*. 2006a), *Nosema spodopterae* (Tsai *et al*. 2005), *Nosema plutellae* (Ku *et al*. 2007) and an undescribed *Nosema* species (Tsai *et al*. 2009) (Fig. 2). While other closely related microsporidia such as *Nosema ceranae* (Huang *et al*. 2008) and *Vairimorpha* sp. (Liu *et al*. 2012) rDNA coding gene arranged in the universal order (5S-IGS-SSU-ITS1-LSU) (Fig. 2). ITS/IGS exhibits higher rate of divergence as compared to the widely used SSU r-RNA gene, and now been established as a universal DNA barcode marker for fungi (Schoch *et al*. 2012, Wang *et al*. 2006a). In 1995, Didier was first observed differences in microsporidial SSU rRNA gene and in the ITS/IGS for separate isolates of *Encephalitozoon cuniculi* (Didier *et al*. 1995). Since it has been demonstrated in a number of other microsporidia species including *Encephalitozoon hellem* (Rinder *et al*. 1997), *Enterocytozoon bieneusi* (Breton *et al*. 2007, Santin *et al*. 2009), *Nosema ceranae* (Huang *et al*. 2008) and *Nosema* sp. (Li *et al*. 2012). In this study, we were targeted the IGS region that is located in between rDNA coding region of SSU-rRNA and the 5S-rRNA gene cluster of the microsporidia (Fig. 2). The IGS sequence region that most rapidly evolving sequences tool and provides significant data that are considered to be phylogenetical useful for delineating relationships within species (Hillis *et al*. 1991).

The aim of our study was to collect diseased vanya silkworms, *Samia cynthia ricini* and *Antheraea assamensis* (diseased/dead) from the varied geographical forest locations in the State of Assam, India and screen them for the presence of microsporidia and to characterise them morphological and molecular basis. We obtained ten fresh isolates infecting the *S. c. ricini* and *A. assamnesis*. Microsporidia fresh isolates morphologically (shape and size) identified. For each isolate, we amplified the IGS r-DNA regions and cloned the resulting PCR products. Sequenced triplicate clones for each of the amplified product from each isolate. In addition, IGS r-DNA regions sequence data from the reference *Nosema*/*Vairimorpha* and other microsporidia were included in our analysis. Our investigation successfully approached to check the differences among microspoidian isolates infecting the vanya silkworms, *S. c. ricini* and *A. assamensis*, with special reference to vast geographical forest areas in different districts of Assam State, India. We anticipate using this information to aid us in microsporidian isolates identification for the control of pebrine disease in the wild silk moth in India and other silk producer country.

Materials and Methods

Microsporidian spores (collection, isolation and purification)

Six microsporidians were collected from individual diseased silkmoths of *Samia cynthia ricini* and four from *Antheraea assamnesis* in different geographical forests areas in Assam State, India (Fig. 1). One reference *Nosema* strain Microsporidian spores were isolated from individual infected silkmoths by maceration and suspended them in 0.85% NaCl followed by filtration through layers of cheese cloth and centrifugation at 3500 r/min for 10 min. The spore pellet was further purified based on 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, shape and size are given in Table 1.

Spore morphology (length and width)

The morphology of test spores isolates was observed under phase contrast microscope (Carl Zeiss–AXIO, Humburg, Germany). The length and width of spores were measured according to the method described by Undeen (Undeen and Vavra 1997).

DNA extraction and purification

DNA was extracted from test spore isolates using the glass bead method described by Undeen and Cockburn (1989). DNA

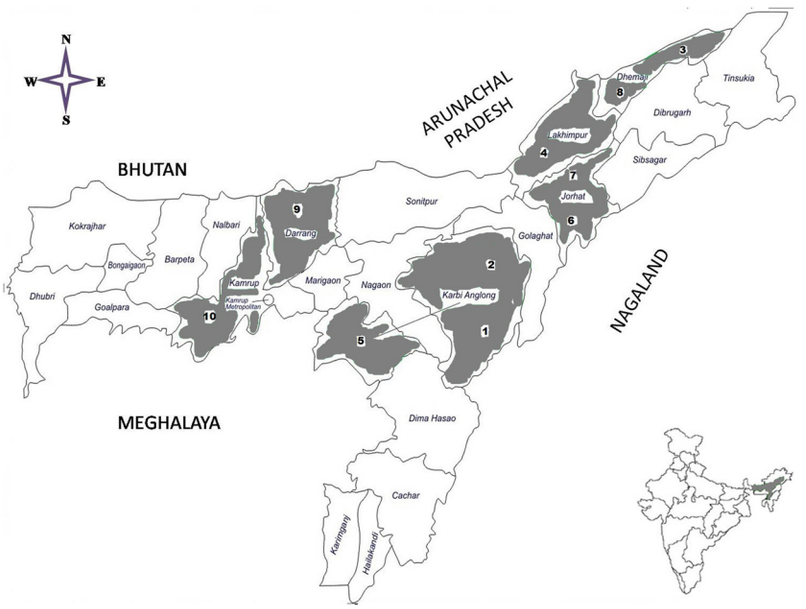

Fig. 1. Seven district of Assam, India from which test microsporidia spores were isolated.

Table 1. Details of ten microsporidian isolates and a reference strain: their place of collection, host and morphology

Sl. No.	Name of isolates	Host	Place of collection (Forest area/Village), district; Latitude; Longitude; Elevation	Spore shape	Spore size (µm)	
					Length	Width
1	MIA-1eBr	*Samia. c. ricini* Donovan	Barpathar forest area, District: Korbi Anglong, Assam, India 26˚2'76.31N / 93˚8'97.38E/90.0	EO	3.80±0.08	2.60±0.01
2	MIA-2eBr	*Samia. c. ricini*	Barpathar forest area, District: Korbi Anglong, Assam, India 26˚2'76.31N / 93˚8'97.38E/90.0	EO	3.80±0.05	2.70±0.12
3	MIA-3eDj	*Samia. c. ricini*	Dhemaji forest area, District: Dhemaji, Assam, India 27˚4'81.11N / 94˚5'57.28E/70.0	EO	3.90±0.20	2.95±0.10
4	MIA-4eLr	*Samia. c. ricini*	Lakhimpur forest area, District: Lakhimpur, Assam, India 27˚2'06.35N / 94˚1'51.37E/78.0	EO	4.05±0.08	3.05±0.01
5	MIA-5eDu	*Samia. c. ricini*	Diphu forest area, District: Korbi Anglong, Assam, India 25˚8'46.52N / 93˚4'29.87E/74.0	EO	4.00±0.07	2.70±0.01
6	MIA-6eTr	*Samia. c. ricini*	Titabar forest area, District: Jorhat, Assam, India 26˚5'88.08N / 94˚1'87.21E/68.0	EO	3.85±0.18	2.60±0.01
7	MIA-7mJr	*Antheraea assamensis* Helfer	Jorhat forest area, District: Jorhat, Assam, India. 26˚7'56.02N / 94˚2'09.45E/92.0	EO	4.90±0.23	2.90±0.01
8	MIA-8mDm	*Antheraea assamensis*	Dhemaji forest area, District: Dhemaji, Assam, India 27˚4'81.11N / 94˚5'57.28E/70.0	EO	4.40±0.12	2.66±0.08
9	MIA-9mMd	*Antheraea assamensis*	Mangaldoi forest area, District: Darrang, Assam, India 26˚4'33.00N / 92˚0'33.00E/70.0	EO	4.10±0.21	2.90±0.04
10	MIA-10mKp	*Antheraea assamensis*	Kamrup forest area, District: Kamrup, Assam, India 26˚3'16.08N / 91˚5'98.39E/68.0	EO	3.90±0.08	2.80±0.06
11	NIK-1s_mys	*Bombyx mori* Linnaeus	CSTRI, Mysore, District: Mysore, Karnataka, India. 12˚17'44.9154N/76˚38'21.7716E/770.10	O	3.80±0.08	2.60±0.01

Note: MIA, Microsporidia India Assam; NIK, Nosema India Karnataka; CSR&TI, Central Sericulture Research and Training Institute, EO- elongated oval; O-oval.

concentration and quality was determined both by spectrophotometery at 260 and 280 nm and on 0.8% agarose gel, using a known quantity of DNA (10 ng/ll) as a standard before use in subsequent PCRs. A working solution of DNA (10 ng/ll) was prepared in sterile double distilled water.

PCR amplification, cloning and sequencing of IGS region

Genomic DNA of test isolates and a reference strain, NIK-1s_mys was amplified with primer designed on *Nosema bombycis* SSU-rRNA gene sequence (D85503, and D85504) between 1115-1141bp (26 mer) used as forward primer-CGTCGTCTATCTAAGATGGTATTATC and 5SR (Huang *et al*. 2008) reverse primer –TACAGCACCCAACGTTCCCAAG (Fig. 2). PCR amplification was carried out in 10 µL, using 10 ng DNA, 5 pmol of each primer, 0.2mM of each dNTP, 2mM $MgCl_2$, and 1U of Taq Polymerase (MBI Fermentas, USA). The

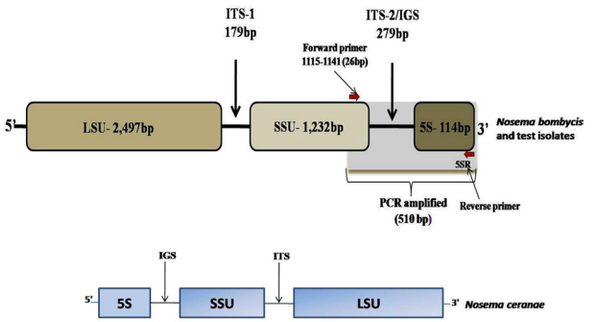

Fig. 2. Schematic diagram of *N. bombycis*, test isolates and *N. ceranae* rDNA genes arrangement. Open rectangles represent open reading frames (ORFs). Red arrows indicate the primer binding sites.

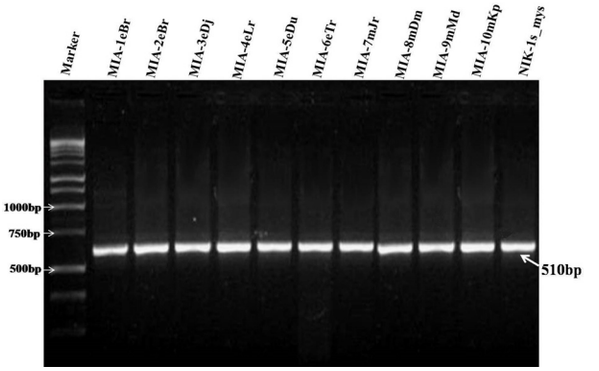

Fig. 3. PCR amplification of IGS region from genomic DNA of 10 test microsporidian isolates along with NIK-1s_mys. PCR amplicon of size 510 bp approximately as shown in 1.2% agarose gel.

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 primer generated expected size fragment of a range 510-515 bp [117bp- SSU rRNA gene, 279bp- IGS, and 114bp-5s rRNA gene (Fig. 2)] for all test isolates genomic DNA. The amplified products were visualized on 1.2% agrose gel (Fig. 3). These amplified products were purified by QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified amplified 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 transformed into *E. coli* (JM101) competent cells and plated on ampicillin agar plates. All obtained colonies on plate were expected positive. Minimum five clones were selected and plasmids were isolated from the cells using the GeneJET plasmid miniprep kit (Fermentas Life Sciences). Plasmids were digested with restriction enzyme (Bgl II) to check whether they contained the desired insert or not. Three clones from each isolates were sequenced using DNA sequencing kit (BDT version 3.1) with semi-automatic DNA sequencer (ABI Prism 310, Applied Biosystems, Perkin Elmer) using M13 universal primers at Eurofins Genomics India Pvt. Ltd., Bangalore, India. The sequence similarity of the three clones of each isolate was 99.50%. Two identical clones sequence were chosen for particular isolate representation. The sequences were cleaned of any vector contamination using a vector screen program from National Center for Biotechnology Information, (NCBI, Bethesda, Maryland, USA). Further, the obtained final sequences have deposited in NCBI GeneBank and the accession number details given in Table 3.

IGS sequence region analysis

Microsporidia IGS sequence homology was carried out using BLAST search from NCBI database. In contrast, 21 non-redundant microsporidians [*Nosema/Vairimorpha* species including an out-group *Glugoides intestinalis*] IGS region sequences were obtained from the NCBI database and were aligned with IGS sequences of test microsporidian isolates including a reference strain in CLUSTAL W program, (Higgins *et al*. 1994) (Table 2). A phylogenetic tree was generated with aligned sequences using maximum likelihood; the branch and bound option (with 500 bootstrap replicates) of the Molecular Evolutionary Genetic Analysis (MEGA) program was

Table 2 Taxon, Source, host and Gene Bank Acc. No. of microsporidians species used in the phylogenetic analysis.

Accession No.	Organism name	Host	Order	Family
KP151535	MIA-1eBr	*Samia cynthia ricini*	Lepidoptera	Saturniidae
KP151536	MIA-2eBr	*Samia cynthia ricini*	Lepidoptera	Saturniidae
KP151537	MIA-3eDj	*Samia cynthia ricini*	Lepidoptera	Saturniidae
KP151538	MIA-4eLr	*Samia cynthia ricini*	Lepidoptera	Saturniidae
KP151539	MIA-5eDu	*Samia cynthia ricini*	Lepidoptera	Saturniidae
KP151540	MIA-6eTr	*Samia cynthia ricini*	Lepidoptera	Saturniidae
KP151541	MIA-7mJr	*Antheraea assamensis*	Lepidoptera	Saturniidae
KP151542	MIA-8mDm	*Antheraea assamensis*	Lepidoptera	Saturniidae
KP151543	MIA-9mMd	*Antheraea assamensis*	Lepidoptera	Saturniidae
KP151544	MIA-10mKp	*Antheraea assamensis*	Lepidoptera	Saturniidae
KP177890	NIK-1s_mys	*Bombyx mori*	Lepidoptera	Bombycidae
FJ767862	*Nosema philosamia*	*Philosamia cynthia ricini*	Lepidoptera	Saturniidae
DQ073396	*Nosema antheraeae*	*Antheraea pernyi*	Lepidoptera	Saturniidae
HQ457431	*Nosema disstriae*	*Malacosma disstriae*	Lepidoptera	Lasiocampidae
AY960987	*Nosema plutellae*	*Plutella xylostella*	Lepidoptera	Plutellidae
JF443699	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
JF443694	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
EU350375	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
HQ457432	*Nosema fumiferanae*	*Choristoneura femiferane*	Lepidoptera	Tortricidae
JF443684	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
HQ891818	*Vairimorpha sp.*	*Bombyx mori*	Lepidoptera	Bombycidae
AF394525	*Glugoides intestinalis*	*Daphnia magna*	Cladocera	Daphniidae
EU105211	*Microsporidium sp.*	*Hemerophila atrilinata*	Lepidoptera	Geometridae
EU338534	*Uncultured Nosema*	*Eurema blanda arsakia*	Lepidoptera	Pieridae
FJ969508	*Nosema sp. PA*	*Phyllobrotica armata Baly*	Coleoptera	Chrysomelidae
FJ772435	*Nosema heliothidis*	*Helicoverpa armigera*	Lepidoptera	Noctuidae
AY747307	*Nosema spodopterae*	*Spodoptera litura*	Lepidoptera	Noctuidae
JN882299	*Nosema sp. HA*	*Hemerophila atrilinata*	Lepidoptera	Geometridae
JN792450	*Endoreticulatus sp.*	*Bombyx mori*	Lepidoptera	Bombycidae
X213699	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
AB097401	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
DQ445481	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
JX213703	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae

The test microsporidian isolates in the present study is shown in bold.

Table 3. The length of the partial SSU rRNA coding region, complete IGS non coding region and 5S rRNA coding region, total size of PCR amplified fragments, their AT content and microsporidians sequence accession no. in NCBI.

Sl. No.	Microsporidian isolates ID	SSU-rRNA (bp)	IGS (bp)	5S-rRNA (bp)	Total amplified size (bp)	A+T content (%)	Gene bank Accession No.
1	MIA-1eBr	117	282	114	513	69.8	KP151535
2	MIA-2eBr	115	282	115	512	69.3	KP151536
3	MIA-3eDj	116	282	115	513	68.7	KP151537
4	MIA-4eLr	117	282	115	514	69.5	KP151538
5	MIA-5eDu	117	282	115	514	69.5	KP151539
6	MIA-6eTr	117	280	115	512	70.7	KP151540
7	MIA-7mJr	117	280	115	512	69.9	KP151541
8	MIA-8mDm	117	283	115	515	69.1	KP151542
9	MIA-9mMd	117	282	115	514	69.7	KP151543
10	MIA-10mKp	117	282	115	514	70.2	KP151544
11	NIK-1s_mys	117	279	114	510	65.7	KP177890
12	*Nosema sp.*	116	282	115	513	70.2	FJ767862
13	*N. antheraeae*	117	279	115	511	69.7	DQ073396
14	*N. disstriae*	113	280	115	508	69.7	HQ457431
15	*N. plutellae*	116	285	115	516	69.8	AY960987
16	*N. bombycis*	116	286	115	517	68.7	JF443699
17	*N. bombycis*	116	277	115	508	69.1	JF443694
18	*N. bombycis*	116	278	115	509	66.6	EU350375
19	*N. fumiferanae*	115	280	114	509	68.8	HQ457432
20	*N. bombycis*	116	279	115	510	67.6	J F443684
21	*Vairimorpha sp.*	77	307	115	499	69.1	HQ891818
22	*Glugoides intestinalis*	114	291	105	510	60.0	AF394525
23	*Uncultured Nosema*	116	281	115	512	67.6	EU338534
24	*Nosema sp. PA*	116	281	115	512	66.2	FJ969508
25	*N. heliothidis*	116	274	115	505	66.3	FJ772435
26	*N. spodopterae*	116	276	115	507	66.5	AY747307
27	*Nosema sp. HA*	116	279	115	510	68.2	JN882299
28	*Endoreticulatus sp.*	58	283	115	456	66.2	JN792450
29	*N. bombycis*	116	283	89	488	68.0	JX213699
30	*N. bombycis*	116	281	115	512	68.4	AB097401
31	*N. bombycis*	116	283	115	514	67.5	DQ445481
32	*N. bombycis -*	116	278	90	484	66.9	JX213703

Table 4. Sequence similarity matrix of IGS region among 10 test microsporidians and a reference strain.

Sequence	MIA-1eBr (KP151535)	MIA -2eBr (KP151536)	MIA-3eDj (KP151537)	MIA-4eLr (KP151538)	MIA-5eDu (KP151539)	MIA-6eTr (KP151540)	MIA-7mJr (KP151541)	MIA-8mDm (KP151542)	MIA-9mMd (KP151543)	MIA-10mKp (KP151544)	NIK_1s_mys (KP177890)
MIA-1eBr (KP151535)	ID	0.99	0.92	0.94	0.94	0.95	0.94	0.95	0.95	0.96	0.82
MIA -2eBr (KP151536)	0.99	ID	0.92	0.93	0.94	0.94	0.93	0.94	0.95	0.95	0.82
MIA-3eDj (KP151537)	0.92	0.92	ID	0.91	0.92	0.94	0.92	0.94	0.93	0.92	0.81
MIA-4eLr (KP151538)	0.94	0.93	0.91	ID	0.93	0.94	0.92	0.93	0.95	0.93	0.81
MIA-5eDu (KP151539)	0.94	0.94	0.92	0.93	ID	0.93	0.92	0.94	0.92	0.94	0.83
MIA-6eTr (KP151540)	0.95	0.94	0.94	0.94	0.93	ID	0.94	0.94	0.94	0.93	0.82
MIA-7mJr (KP151541)	0.94	0.93	0.92	0.92	0.92	0.94	ID	0.93	0.93	0.92	0.81
MIA-8mDm (KP151542)	0.95	0.94	0.94	0.93	0.94	0.94	0.93	ID	0.94	0.93	0.83
MIA-9mMd (KP151543)	0.95	0.95	0.93	0.95	0.92	0.94	0.93	0.94	ID	0.94	0.83
MIA-10mKp (KP151544)	0.96	0.95	0.92	0.93	0.94	0.93	0.92	0.93	0.94	ID	0.81
NIK_1s_mys (KP177890)	0.82	0.82	0.81	0.81	0.83	0.82	0.81	0.83	0.83	0.81	ID

used (Version 6.0) (Tamura *et al*. 2013). The cloned and sequenced IGS region of test isolates and a *Nosema* reference strain NIK-1s_mys were checked for sequence similarity among them by the Sequence Identity Matrix programmes in BioEdit software (Hall 1999) (Table 4)

Results

Morphological characterization

The shape of the test isolates were observed oval to elongated oval, while the reference strain (NIK-1s_mys) was oval. The test spores size range from 3.80 to 4.90 <mu>m in length and 2.60 to 3.05 <mu>m in width, and the reference strain (NIK-1s_mys) is 3.80<mu>m in length and 2.60<mu>m in width. Details of each microsporidian isolates, their shape and size are given in Table 1.

Molecular characterization

PCR amplification, cloning and sequencing of IGS fragment

The primers set used in PCR were successfully amplified the targeted IGS region for all test microsporidian isolates and a reference strain (Fig. 3). The PCR amplified fragment consisted of a partial region of SSU r RNA gene followed by complete sequence of IGS and complete 5S r RNA gene, and the expected fragment size was approx 510 bp for almost all the test microsporidian isolates. Full-length sequences of the cloned gene ranging from 490 to 518 bps were obtained and the sequences were successfully submitted to the NCBI-Gen Bank. The individual accession details for the submitted IGS sequences from KP153535- KP153544 are given in Table-3

Length and sequence variation in IGS region

The downloaded similar homology IGS region sequences of 21 different microsporidians species from NCBI were found 484 to

514bp in lengths (Table 3). However, IGS region sequence lengths were 493-519bp for test microsporidian isolates. The sequence similarity of IGS region for the test microsporidian isolates were observed from 91 to 98%. Highest (98%) similarity was found between MIA-1eBr, and MIA-2eBr isolates, while MIA-3eDj has least (91%) similarity with MIA- 4eLr (Table-4). All test isolates had low similarity over a range of 80-82% with the reference strain NIK-1s_mys. The A+T content of test isolates varied from 68.7 to 70.7%, while used all references *Nosema* species ranged from 65.7 to 69.8%. However, the A+T content of IGS sequence was 60.0 % for *Glugoides intestinalis* (Table 3).

Phylogenetic analysis based on IGS region

The constructed dendrogram based on IGS sequence region separates all test isolates with each other as well as references microsporidia species with 50% above bootstrap values. The analysis of the phylogenetic tree manifested that 10 test isolates and 20 *Nosema* reference species including one *Endoreticulatus* sp. clustered in a single clade, which could be seperated into two groups. One group composed of the ten test isolates with three *Nosema* references sp. [*N. philosamia* (FJ767862.1), *N. antheraeae* (DQ073396.1) and *N. disstriae* (HQ457431)]. The other group composed of seventeen *Nosema* references species including NIK-1s_mys from India (Fig. 4). Two test isolates [MIA-9mMd and MIA-6eTr] were sub grouped with two Chinese references *Nosema* species [*N. philosamia* (FJ767862) and *N. antheraeae* (DQ073396)] (Fig. 4). Further analysis of dendrogram shows genuine similarity between two test isolates [MIA-1eBr and MIA-2eBr] those were isolated from *S. c. ricini* and same geographical location. In addition, *Vairimorpha* species separated alone in the same clade with all reference *Nosema* species as well as test isolates while, *Glugoides intestinalis* used as an out-group separated out of major clade in the constructed tree (Fig. 4).

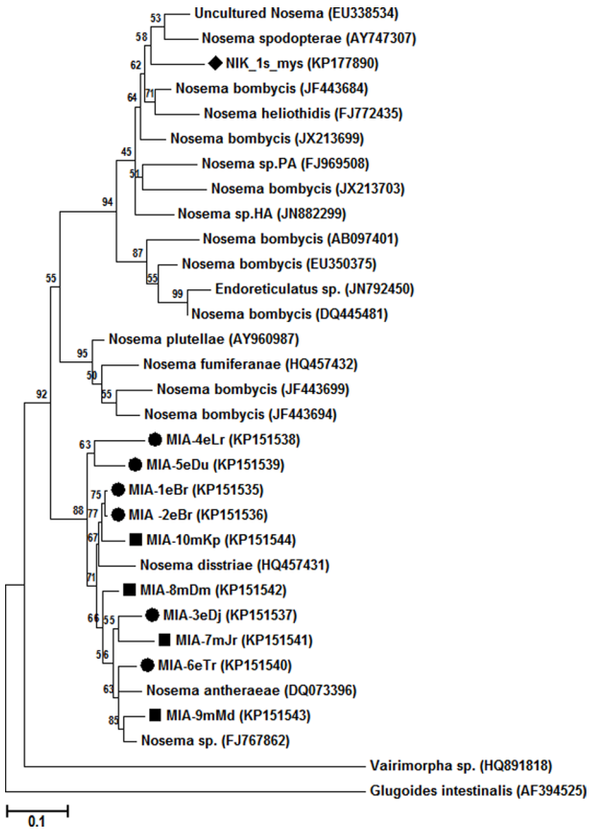

Fig. 4. Phylogenetic tree based on IGS region sequences. Thirty-two microsporidians IGS region sequences were analyszed based on maximum likelihood approach using MEGA 6 (Tamura 2013), run with 500 bootstrap replication. Number of each node indicates bootstrap value. ● spot indicate test microsporidian isolates from *S. c. ricini*; ■ spot indicate test microsporidian isolates from *A. assameensis*; ◆ spot indicates *N. bombycis* reference strain from India.

Discussion

Ten microsporidians isolated from *S. c. ricini* and *A. assamensis* were relatively characterised by using IGS region sequence and phylogenetic analysis. NIK-1s_mys a *Nosema Bombycis* standard strain from India, used in this study was previously characterized using SSU r-RNA gene analysis (Nath *et al*. 2012), that showed 99.90% of sequence homology with *N. bombycis* (GenBank accession number-D85503) (isolated from host *B. mori*), maintained at the Sericultural Experimentation Station, Tokyo, Japan. Morphological studies for the test isolates showing variation in spore width and length. However, the morphological characteristics not only be considered as a true apparent for identity of microsporidian isolates, as spore size for a given species may vary with respect to the host (Brooks *et al*. 1972). So many researchers have been described sequences and arrangement of complete r-RNA subunits genes of *Nosema* species, (Huang *at el*. 2004, Tsai *et al*. 2005, Wang *et al*. 2006b, Ku *at el*. 2007, Zhu *et al*. 2010). They suggest arrangement of a general *Nosema* species is reverse than other microsporidian species and fungi have (Fig. 2). A report suggests that reverse organization of r-RNA genes might be an important character of *Nosema* species (Tsai *et al*. 2005). Moreover, *Glugoides intestinalis* a distantly related to the genus *Nosema* has similar reverse arrangement of r-RNA gene cluster (Refardt *et al*. 2007) and *Nosema ceranae* (Huang *et al*.

2008) and *Vairimorpha* sp. (Liu *et al*. 2012) r-RNA gene arranged in the universal order. Hence the arrangement of r-RNA subunits gene is not sufficient to identify *Nosema* species, but significant to distinguish the closely related species. The PCR products by primer set utilised of amplified IGS region for all test isolates and a reference strain was proven that all have reverse arranged r-RNA genes in their genome (Fig. 1). IGS region shows differences among intraspecific genome pattern, it may be variation in the IGS sequences or variation in the length of the PCR product (Buchko *et al*. 1990). An old report suggests analysis of IGS sequence is highly applicable for phylogenetic differentiation of closely related species and a potential epidemiological tool (Appel *et al*. 1995). In 2002, molecular variations established among *Trichosporon* isolates from close geographical locations based on restriction patterns of IGS (Sugita *et al*. 2002). Three different microsporidia species showed 100% identity of SSU r-RNA gene sequences while their ITS, IGS and 5S sequences were varied (Dong *et al*. 2010). Molecular phylogenetic analysis based on IGS region might have a better tool to investigate intraspecific divergence and would be provide significance molecular evidence for the classification and evolutionary studies of microsporidia (Dong *at el*. 2010, Sagastume *et al*. 2010, Liu *et al*. 2013). Recently a report have came on differentiating of *N. ceranae* strains from different geographic origin 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 *at el*. 2012). These reports supporting that IGS tools now been established for molecular characterisitic of microsporidia isolates. Based on these reports, we target IGS tools for plylogenetic analysis of test microsporidian isolates. In this study, we analyze the complete IGS sequences along with partial SSU r-RNA and 5S r RNA sequences to derive a phylogenetic inference for six test microsporidians from *S. c. ricini* and four from *A. assamensis*. The lengths and A+T content of the IGS fragment for test isolates were found to be similar range of 21 characterised reference *Nosema* species. The IGS sequences of test microsporidian isolates favour they may be belong to the genus *Nosema*.

In order to classify the test microsporidian isolates based on dendregram that 10 test microsporidian isolates have very close genetic relationships with three *Nosema* species i.e., *Nosema philosamia,* and *Nosema antheraeae* from china and *Nosema disstriae* from Canada. Since, the test isolates were extracted from *S. c. ricini* and *A. assamnesis* the genetic similarity of test isolates with *Nosema philosamia,* and *Nosema antheraeae* support host specification of microsporidia. Sequence and phylogenetic analysis of the IGS region would be provide additional molecular evidence for the classification and evolutionary study of microsporidia. The generated tree from the IGS region of the test microsporidia isolates indicates that these isolates formed closed as well as complex phylogenetic relationship with each other due to concerted evolution. Our results can speculate about the differentiation of the isolate from the Assam state in India. There seems to be an intermixing among the each other isolates over vast geographic regions. Whether there is movement of isolates due to patterns of insect migration, infection from alternate hosts, weather patterns or geographic isolation due to mountains, rivers or roads, remains to be determined. As above said, it is quite possible that the Assam isolates represents may be a separate group (species) and that the data presented here 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, the IGS region variability among isolates would be a rich source of information and could serve to differentiate these isolates in order to give us insight into the origins and spread of this economically important disease of the vanya silkworms in India.

In conclusion, based on morphological and molecular characteristics of the ten microsporidian isolates from S. *c. ricini* and *A. assamensis* was differentiating to *Nosema* species certainly with *Nosema bombycis*. Study of phylogenetic relationship based on IGS region and r-RNA gene sequences have provided substantial evidence on the diversity of the different microsproidian isolates from *S. c. ricini* and *A. assamensis,* which is of practical substance for further studies on diagnose and prevention of Pebrine (microsporidiosis) in silkworm.

Acknowledgement

Wazid Hassan is a recipient PhD fellowship by University Grants Commission (Maulana Azad National Fellowship), Government of India.

Reference

Appel DJ, Gordon TR (1995) Intraspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. Exp Mycol 19, 120-128.

Breton J, Bart-Dellabesse E, Biligui S, Carbone A, Seiller X, Okome-Nkoumou M, Nzamba C, Kombila M, Accoceberry I, Thellier M (2007) New highly divergent rRNA sequence among biodiverse

genotypes of Enterocytozoon bieneusi strains isolated from humans in Gabon and Cameroon. J Clin Microbiol 45(8), 2580-2589.

Brooks WM, Cranford JD (1972) Microsporidiases of the hymenopterous parasites, *Campoletis sonorensis* and *Cardiochiles nigriceps*, larval parasites of *Heliothis* species. Journal of Invertebrate Pathology 20, 77-94.

Buchko J, Klassen GR (1990) Detection of length heterogeneity in the ribosomal DNA of *Pythium ultimum* by PCR amplification of the intergenic region. Curr Genet 18(3), 203-205.

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

Didier ES, Vossbrinck CR, Baker MD, Rogers LB, Bertucci DC, Shadduck JA (1995) Identification and characterization of three Enterocytozoon cuniculi strains. Parasitology 111, 411-421.

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 *Nosema ceranae* (Microsporidia) isolates from two different geographic origins. Vete Microbiol 162, 670-67.

Hall TA (1999) Bioedit: a user-friendly biological alignment editor and analysis programme for Windows 95/98/NT. Nucleic Acids Symposium 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 Research 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 Nosema ceranae isolates from different hosts and locations. Journal of Invertebrate Pathology 97, 9-13.

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

Ku CT, Wang CY, Tsai YC, Tzeng CC, Wang CH (2007) Phylogenetic analysis of two putative *Nosema* isolates from Cruciferous Lepidopteran pests in Taiwan. Journal of Invertebarate Pathology 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. International Journal for Parasitology 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 microsporidian Vairimorpha sp. BM (Microsporidia, Nosematidae) from Bombyx mori 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 *Nosema bombycis* isolates from different geographical regions in China. Journal of Invertebarate Pathology 113(1), 63-69.

Nageli C (1857) Ueber die neve Krankheit der Seidenraupe and verwandte Organismen. Botanische Zeitung 15, 760-761.

Nath BS, Gupta SK, Bajpai AK (2012) Molecular characterization and phylogenetic relationships among microsporidian isolates infecting silkworm, Bombyx mori using small subunit rRNA (SSU-rRNA) gene sequence analysis. Acta Parasitologica 57(4), 342-353.

Pastuer L (1870) Etudes sur la maladie des vers a soie. Paris, France.

Quan C (2011) Prevention and treatment technology of the silkworm Perbrine. Guangxi Sericulture 48 (2), 20-24.

Refardt D, Mouton L (2007) Reverse arrangement of rRNA subunits in the microsporidium Glugoides intestinalis. Journal of Eukaryotic Microbiology 54, 83-85.

Rinder N, Katzwinkel-Wladarsch S, Loscher T (1997) Evidence for the existence of genetically distinct strains of Enterocytozoon bieneusi. Parasitol Res 83(7), 670-672.

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) Enterocytozoon bieneusi Genoreference Nomenclature Based on the Internal Transcribed Spacer Sequence: A Consensus. Journal of Eukaryotic Microbiology 56, 34-38.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as universal DNA barcode marker for Fungi. PNAS 109(16), 6241-6246.

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. Molecular Biology and Evolution 30, 2725-2729.

Tsai SJ, Huang WF, Wang CH (2005) Complete sequence and gene organization of the Nosema spodopterae rRNA Gene. Journal of

Eukaryotic Microbiology 52, 52-54.

Tsai YC, Solter LF, Wang CY, Fan HS, Chang CC, Wang CH (2009) Morphological and molecular studies of a microsporidium (Nosema sp.) isolated from the three-spot grass yellow butterfly, Eurema blanda arsakia (Lepidoptera: Pieridae). Journal of Invertebrate Pathology 100, 85-93.

Undeen AH, Alger NE (1971) A density gradient method for fractionating microsporidian spores. Journal of Invertebrate Pathology 18, 419-420.

Undeen AH, Cockburn AF (1989) The extraction of DNA from microsporidia spores. Journal of Invertebrate Pathology 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 117-151.

Wang LL, Chen KP, Yao Q (2006a) The sequence and phylogenetic analysis of the ribosomal RNA gene and the ITS region of *Nosema antheraeae*. Sci Agric Sin 39(8), 1674-1679.

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

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*. Journal of Eukaryotic Microbiology 57(3), 294-296.