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Molecular Characterization and Phylogenetic relationships of 7 microsporidian isolates from different Lepidopteran pests cross infecting silkworm, *Bombyx mori*, based on Intergenic spacer sequence analysis

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Abstract
The present study is based on molecular characterization of 7 microsporidian isolates extracted from infected Lepidopteran pests, collected from potential zones of sericulture in Karnataka, India. The seven spore isolates viz., NIK-55Dp, NIK-57Sl, NIK-64So, NIK-60Pd, NIK-61Pp, NIK-63Ec and VIK-52Pr, were studied along with the *Nosema* type species-NIK-1s_mys. These isolates were tested for their pathogenicity, mode of transmission and tissue specificity in laboratory reared CSR2 race of *Bombyx mori*. PCR based analysis of Intergenic spacer sequence (IGS) was carried out in order to establish spore identity and to derive phylogenetic relationships among the key isolates. A phylogenetic tree was generated for the test isolates using the maximum parsimony method from MEGA-6. Study shows that six spore isolates were clustering along with the genus of *Nosema* except isolate-VIK-52Pr, which grouped with *Vairimorpha* species. VIK-52 Pr was also found to be highly pathogenic to the lab host as compared to rest of the test isolates including NIK-1s_mys.

Keywords: Microsporidia, Lepidopteran pests, *B.mori* cross infectivity, Intergenic spacer sequence, Phylogenetic analysis.

1. Introduction

Microsporidia are diverse group of spore-forming, unicellular, obligatory intracellular parasites belonging to the phylum of Microspora, infecting more than 1200 species from 143 genera [1, 2]. They have a broad host range from protists and invertebrates to vertebrates including insects, fishes, and mammals. These are eukaryotes with distinct nucleus and nuclear envelope, but are considered to be unique as they lack vital organelles like the centrioles and mitochondria. Further the presence of prokaryote-sized ribosomes i.e., 70S (23S-LSU and 16S-SSU) presents them as extremely ancient eukaryotes [3]. However with recent molecular studies based on their tubulin genes (α- and β-) and *Hsp*70, it is suggested that they are specialized organisms sharing a common origin with the fungi [4, 5]. The first ever existence of a microsporidian- ***Nosema bombycis Naegeli*** [6], came to light with the outbreak of the deadly disease popularly called as "**Pebrine**" in mulberry silkworms *Bombyx mori*. This disease devastated the whole of silk industry in France and other countries during the 19th century. For decades since then, until the dawn of molecular science, *N. bombycis* was considered as the only causative agent for this disease in commercial silkworms. These fungal pathogens not only pose serious threats to sericulture but also to apiculture and fisheries.

Microsporidia in general have been found to infect a wide range of insects under natural field conditions. Most of the susceptible insect hosts of microsporidia belong to the orders of Lepidoptera and Diptera [7]. The perpetual incidence of microsporidiasis in silkworms may be due to various sources of secondary contamination by alternative insect hosts in and around the mulberry fields [8, 9]. Presumably, butterfly faeces and scales containing the microsporidian spores might stick to mulberry leaves and transmit the microsporidian infection to silkworm larvae [10]. Microsporidia have been reported from different agricultural and mulberry pests such as *Spodoptera litura, Spodoptera exigua, Helicoverpa armigera, Plutella xylostella, Pieris rapae, Spilosoma oblique* and *Phyllobrotica armata Baly* [11, 12]. Reports suggest that *B. mori* is not only infected by the *Nosema* sp. but atleast 10 other genera *viz*., *Vairimorpha, Pleistophora, Endoreticulatus, Cystosporogenes, Orthosomella, Thelohania, Octosporea* and *Gurleya* [13-14].

The r RNA genes have been widely exploited in both eukaryotes as well as prokaryotes for their taxonomic implications with respect to distantly related organisms. The ribosomal DNA in microsporidia is comprised of the highly conserved primary rRNA genes (LSU, SSU and 5S) punctuated by hypervariable spacer regions that includes the ITS (Internal transcribed spacer) and IGS (Intergenic spacer). Recently the non-coding spacer regions (ITS and IGS) are becoming valuable tools to study and differentiate relationships among closely related taxa in Fungi and other organisms. The ITS region can project the variations within genera and can potentially differentiate them at intra species level. The taxonomic value of ITS is due to their significant heterogeneity in both length as well as nucleotide sequences and exhibits higher rate of divergence as compared to the widely used 16S rRNA, and has now been considered as a Universal DNA barcode marker for Fungi [15, 16]. Here in this study we targeted the IGS also referred to as ITS-2 region that is located in between the SSU-rRNA and the 5S-rRNA gene cluster of the microsporidia. The IGS region is also one of the most rapidly evolving sequences and provides large data that are considered to be phylogenetically useful for delineating relationships within species [17].

The aim of our study was to collect different Lepidopteran pests (diseased/dead) across the mulberry fields and screen them for the presence of any microsporidia and also to establish the possibilities of their cross-infectivity and pathogenicity in our lab host- *B. mori*. We also did a phylogenetic analysis of all the key isolates with reference to similar sequences of microsporidian isolates from the NCBI database based on their IGS sequences using Maximum parsimony method of MEGA-6 software [18].

2. Materials and Methods

2.1 Microsporidian spores- Isolation and Purification

A survey was done during the month of June 2011, inorder to collect dead/diseased Lepidopteran insects from the surroundings of mulberry garden sites in Karnataka, India. The dead insects were initially macerated and resuspended in 0.85% saline and were tested for the presence of microsporidia under a phase contrast microscope. After confirmed detection of the spores, the respective insect tissue homogenate was processed for spore isolation by filtration through layers of cheese cloth followed by brief centrifugation at 3000 rpm for 10 min. The spore pellet was further purified based on density gradient ultracentrifugation using Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described [19]. The purified spore pellets were finally resuspended in 0.85% saline, quantified by hemocytometer and preserved at 4°C for further use. The length and width of the spores were measured using an ocular micrometer as described earlier [20].

2.2 Pathogenicity of microsporidian isolates

The pathogenicity and cross infectivity of purified spore isolates from Lepidopteran source were tested in the laboratory bred CSR2 race of silkworm *B. mori*. Healthy silkworms immediately after the 3rd moult were chosen for this experiment and divided into batches of 50 larvae each. The cross infectivity and virulence of each test isolate was checked by inducing infection in the healthy worms using three different spore dosage *viz*., 1x 10^7(dose A), 1x 10^6(dose B) and 1x10^5 (dose C)/ml in triplicates. A piece of mulberry leaf measuring about 5 cm^2 was smeared with the respective spore dose and inoculated *per os* to each batch of 50 healthy worms on the 1st day of fourth instar. The infected larvae were subsequently reared on fresh mulberry leaves after 24 h of induced infection. A control batch of healthy larvae was maintained separately under the same laboratory conditions. Mortality rates were recorded in all the doses after every 24 h up to spinning stage. Tissue extract from dead larvae were regularly tested for the pebrine infection as explained above using a phase contrast microscope. The mortality rates observed at each dose were obtained from the mean of three replicates.

2.3 DNA extraction and purification

Genomic DNA was extracted from spores using the glass bead method as reported earlier [21]. The concentration and purity of DNA was determined using the standard established protocol.

2.4 PCR amplification of IGS

A working stock of 10 ng/µl of DNA was used in PCR. The genomic DNA of each of the 7 microsporidian isolates along with standard NIK-1s_mys, was used for amplification of the IGS region using the primer set *viz*.,. ISSUF- **5’-CGTCGTCTATCTAAGATGGTATTATC-3’** and 5SR- **5’-TACAGCACCCAACGTTCCCAAG-3’** as previously described [22]. The primer set was designed based on the core region of SSU gene and the conserved region of 5S rRNA gene, respectively. The PCR products were checked on 1.2% agarose gel. The fragment band was excised from the gel followed by extraction using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, prior to cloning.

2.5 Cloning and sequencing of IGS fragment

The IGS sequence fragments of test isolates were cloned using the Clone Jet PCR Cloning Kit (Fermentas Life Sciences) as per Kit instructions. The *E.coli* strain-JM 107 was used for competent cell preparation and bacterial transformation was performed using TransformAid™ Bacterial Transformation Kit (Fermentas). Ampicillin was used for clone selection at a working concentration of 50µg/ml during the regular plating and reculturing steps. Plasmid extraction from the suitable clones was done using GeneJET plasmid miniprep kit (Fermentas Life Sciences). The clones from each of the key 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. The sequences were analyzed through bioinformatics tools and deposited in NCBI- GenBank.

2.6 Phylogenetic analysis using IGS sequences

The IGS sequences of all study isolates were analyzed by using BLAST search [23] from NCBI. Around 15 non-redundant microsporidian IGS sequences were retrieved from NCBI Gen Bank and aligned with our target sequences during phylogenetic analysis. Multiple sequence alignments were prepared using Clustal *W* with default setting and all sequences were compared to each other (pair wise alignments) to find the homology in between them. The tree was constructed by maximum parsimony method with branch and bound option using MEGA-6 software. Phylogenies were assessed by a 1000 bootstrap replication. The branch length information was obtained based on genetic distances derived from Kimura-2-parameter model.

3. Results

3.1 Isolation and purification of microsporidian isolates

Screening of various dead/diseased Lepidopteran pests collected from regions surrounding important sericulture zones of Karnataka State in India, lead to the detection of seven

microsporidian isolates. The 7 test isolates were designated based on their host names *viz*., NIK-55Dp (*Diaphania pulverulentalis*), NIK-57Sl (*Spodoptera litura*), NIK-64So (*Spilosoma obliqua*), NIK-60Pd (*Papilio demoleus*), NIK-61Pp (*Papilio polytes*), NIK-63Ec (*Euploe core*) and VIK-52Pr (*Pieris rapae*). The external morphology of our test isolates were found to be oval to ovo-cylindrical in shape. The spore dimensions of the study isolates were ranging between 3 to 6 µm in length and from 2 to 3 µm in width as compared to NIK-1s_mys (type species) with a length of 3.80 µm and a width of 2.60 µm. The microsporidia isolated from *Pieris rapae* i.e., VIK-52 Pr, exhibited the largest size with a spore length and width of 5.45 µm and 2.9 µm respectively. The details on microsporidian isolates, their parent host and external morphology are presented in **Table 1**.

Table 1: Details on external morphological features and hosts of seven Lepidopteran derived microsporidian isolates along with type species-NIK-1s_mys isolated from *B.mori*. Note. **NIK**: Nosema India Karnataka; **VIK**: Vairimorpha India Karnataka.

S. No.	Microsporidian isolates	Lepidopteran Pest/Host name	Spore shape	Spore Length (in µm)	Spore Width (in µm)
1.	**NIK-1s_mys** (*Nosema* type sp.)	*Bombyx mori*	Oval	3.80±0.08	2.60±0.01
2.	**NIK-55Dp** (*Nosema sp.)*	*Diaphania pulverulentalis*	Ovo-cylindrical	3.90±0.07	2.71±0.06
3.	**NIK-57Sl** (*Nosema* sp.)	*Spodoptera litura*	Ovo-cylindrical	4.10±0.08	1.98±0.02
4.	**NIK-64So** (Nosema *sp.*)	*Spilosoma obliqua*	Oval	3.39±0.03	2.21±0.03
5.	**NIK-60Pd** (Nosema *sp.*)	*Papilio demoleus*	Oval	3.85±0.09	2.50±0.04
6.	**NIK-61Pp** (*Nosema sp.*)	*Papilio polytes*	Oval	3.65±0.09	2.42±0.07
7.	**NIK-63Ec** (*Nosema sp.*)	*Euploe core*	Oval	4.64±0.10	2.64±0.05
8.	**VIK-52Pr** (V*airimorpha sp.*)	*Pieris rapae*	Ovo-cylindrical	5.45±0.16	2.90±0.10

3.2 Pathogenicity testing in *B.mori*

The pathogenicity as well as cross infectivity of all the 7 test isolates identified from Lepidopterans were checked in *B.mori*. All the test isolates were found to be virulent to the lab host which is shown in **Table 2**.

Table 2: Comparative cross infectivity/pathogenicity of seven microsporidian isolates from Lepidopteran pests along with NIK-1s_mys in lab host *B.mori*

S. No	Microsporidian isolates	Inoculation dose (0.35ml)	Median lethal time (in days)
1.	NIK-1s_mys (Standard strain)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	8.14
2.	NIK-55Dp (*Nosema sp.)*	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	9.80
3.	NIK-57Sl (*Nosema* sp.)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	8.50
4.	NIK-64So (Nosema *sp.*)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	7.05
5.	NIK-60Pd (Nosema *sp.*)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	10.22
6.	NIK-61Pp (*Nosema sp.*)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	10.44
7.	NIK-63Ec (*Nosema sp.*)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	10.85
8.	VIK-52Pr (V*airimorpha sp.*)	A 1 x 10^7 B 1 x 10^6 C 1 x 10^5	6.30

The inoculation of each of the microsporidian isolate to the 4th instar larvae at three different inoculation concentrations resulted in a median lethal time ranging from 6 to 11 days. The test isolate VIK-52Pr was found to be the most virulent resulting in 50% mortality rate by 6.3 days post infection followed by NIK-64So (7.05 days), NIK-1s_mys (8.14 days), NIK-57Sl (8.50 days), NIK-55Dp (9.80 days), NIK-60Pd (10.22 days), NIK-61Pp (10.44 days) and NIK-63Ec (10.85 days). Thus the overall pathogenicity of the microsporidians based on their inoculation doses was in the order of VIK-52Pr > NIK-64So > NIK-1s_mys > NIK-57Sl > NIK-55Dp > NIK-6Pd > NIK-61Pp > NIK-63Ec.

3.3 PCR amplification, cloning and sequencing of IGS fragment

The primer set used in our study, successfully amplified the targeted IGS sequence. The PCR amplicon consisted of a partial region of SSU r RNA gene followed by complete sequence of IGS and 5Sr RNA gene, approximately ranging to an expected fragment size of 510 bp for all the microsporidians (**Fig 1**). Appreciable full length sequences of the cloned gene ranging between 508-518 bps were obtained, which were successfully submitted to the NCBI-Gen Bank. The respective accession details for the submitted IGS sequences from KP177883- KP177890 are indicated in **Table 3**.

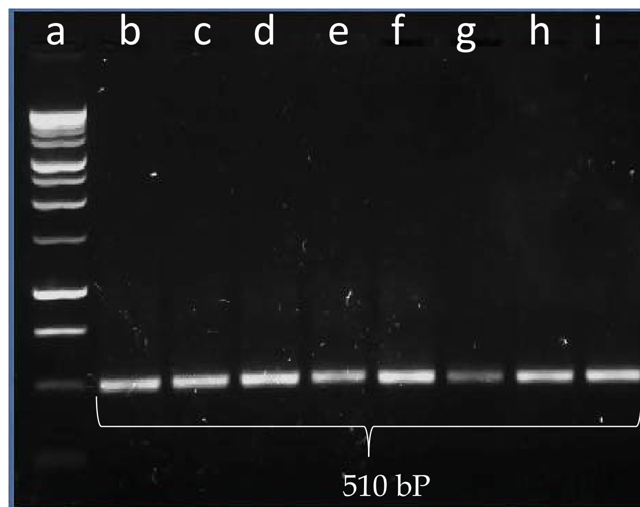

Fig 1: (a-i) PCR amplification of IGS from genomic DNA of 7 Microsporidian isolates from Lepidopteran pests along with NIK-1s_mys. PCR amplicon of size 510 bp approximately as shown in1.2% agarose gel. Lanes: a-DNA marker; b-NIK-1s_mys; c-NIK-55Dp; d-NIK-57Sl; e-NIK-64So; f-NIK-60Pd; g-NIK-61Pp; h-NIK-63Ec; i-VIK-52Pr.

3.4 Phylogenetic analysis based on IGS

A phylogenetic tree was constructed for our 7 test isolates and the type species NIK-1s_mys in comparison to homologous IGS sequences of 15 non-redundant microsporidian species/isolates downloaded from the NCBI GenBank as shown in **Table 3**.

Table 3: List of microsporidia with homologous IGS sequences from NCBI used for Phylogenetic analysis.

Organism	Isolation source	*Insect Family Lepidoptera*	Gen Bank Acc. No.
NIK-55Dp	*Diaphania pulverulentalis*	*Pyralidae*	KP177883
NIK-57Sl	*Spodoptera litura*	*Noctuidae*	KP177884
NIK-64So	*Spilosoma obliqua*	*Arctiidae*	KP177885
NIK-60Pd	*Papilio demoleus*	*Papilionidae*	KP177886
NIK-61Pp	*Papilio polytes*	*Papilionidae*	KP177887
NIK-63Ec	*Euploe core*	*Nymphalidae*	KP177888
VIK-52Pr	*Pieris rapae*	*Pieridae*	KP177889
NIK-1s_mys	*Bombyx mori*	*Bombicidae*	KP177890
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	AY259631
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	JF4436651
Nosema sp.	*Samia cynthia recini*	*Saturnidae*	FJ7678621
Nosema_ antheraeae	*Antheraea pernyi*	*Saturnidae*	DQ073396
Nosema_disstriae	*Malacosoma disstria*	*Lasiocampidae*	HQ4574311
Nosema_plutellae	*Plutella xylostella*	*Plutellidae*	AY9609871
Nosema_heliothidis	*Helicoverpa armigera*	*Noctuidae*	FJ7724351
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	JF4436831
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	JF4436991
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	JF4436941
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	JF4436961
Nosema_bombycis	*Bombyx mori*	*Bombicidae*	JF4436751
Nosema_bombycis	*Clone 5*	__	EU3503761
Vairimorpha sp.	*Bombyx mori*	*Bombicidae*	HQ8918181
Microsporidium	__	__	U908851

Note: The seven test isolates and the NIK–1s_mys (type species) are indicated in bold. "__" represents "Not documented".

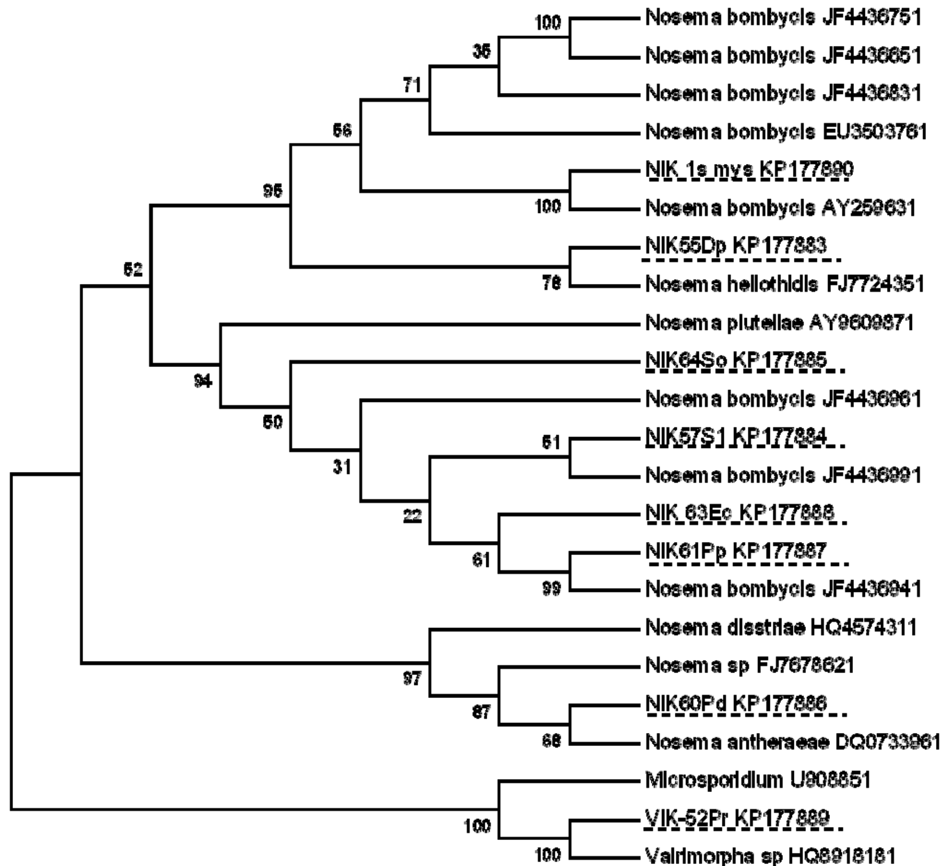

Fig 2: Maximum Parsimony Tree of 7 Lepidopteran microsporidia and NIK-1s_mys along with other microsporidia from Database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated next to the branches.

The maximum parsimony tree (MEGA-6.06) as shown in **Fig 2**, categorized almost all test isolates and NIK-1s_mys to the *Nosema* clades except isolate VIK-52Pr that was found to be exactly matching with *Vairimorpha* species (HQ891818). A comparative analysis of IGS sequence homologies have been evaluated among the 7 test isolates and NIK-1s_mys, as shown in **Table 4.**

Table 4: Comparative evaluation of IGS sequence homology between the 7 test isolates from Lepidopteran pests and type species-NIK-1s_mys used in the present study.

	NIK-55Dp	NIK-57Sl	NIK-64So	NIK-60Pd	NIK-61Pp	NIK-63Ec	VIK-52Pr	NIK-1s_mys
NIK-55Dp	-------	89.76	87.5	87.67	90.35	89.49	62.65	90.59
NIK-57Sl	89.76	-------	91.73	88.78	94.29	93.31	63.58	87.99
NIK-64So	87.5	91.73	-------	89.63	94.09	93.16	64.45	84.71
NIK-60Pd	87.67	88.78	89.63	-------	89.37	89.04	62.82	86.08
NIK-61Pp	90.35	94.29	94.09	89.37	-------	96.46	62.60	87.80
NIK-63Ec	89.49	93.31	93.17	89.04	96.46	-------	62.36	88.82
VIK-52Pr	62.65	63.58	64.45	62.82	62.60	62.36	-------	64.31
NIK-1s_mys	90.59	87.99	84.71	86.08	87.80	88.82	64.31	--------

4. Discussion

Many microsporidia are considered to have a very broad host range. The possible cross-infection of microsporidia often begins from wild insects from different orders that are seen in and around the mulberry fields thereby gaining entry into the commercially important *B. mori*. There have been previous reports on microsporidia isolated from some wild insects *viz*., *Plutella xylostella* [24], *Pieris rapae* [25], *Mimastra cyanura* Hope [26], *Spodoptera litura* [27], *Laphygma exigua* [28] and *Delias pasithoe* [29] and *Phyllobrotica armata* Baly [30]. Microsporidia from such sources can easily cross-infect silkworm, *B. mori*, causing considerable problems in industries of sericulture. Hence our target was to screen different insect pests that are commonly seen in and around mulberry fields for microsporidial infection and study their cross-infectivity in lab host - *B. mori*.

The characterization of seven microsporidian test isolates from different Lepidopteran pests based on their IGS sequence analysis was successfully carried out. The standard strain, NIK-1s_mys used in the present study has been previously characterized using SSU r RNA gene analysis [31], that shared 99.92% 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 reflected variation in spore width and length. However, the morphological characteristics alone cannot be considered as a true index to classify microsporidia, as spore size for a given species may vary with respect to the host [32]. Studies on pathogenicity provide valuable information on host-pathogen interactions as well as relationships. The seven microsporidian isolates from Lepidopteran pests of mulberry fields exhibited successful cross infection in our lab host, *B. mori*. As shown in **Table 2**, all isolates were virulent to the lab host *B.mori* and varied in their degree of pathogenicity with respect to the type species. The mortality values were dose dependent (not included in Table) in all cases and varied substantially between the test isolates. Isolate VIK-52Pr from *Pieris rapae* was found to be the most virulent microsporidian followed by NIK-64So from *Spilosoma obliqua*, in comparison to NIK-1s_mys and rest of the pest microsporidian isolates. These observations were supported by their median lethal time that ranged from 6.3 to 10.85 days thereby reflecting the differences in their virulence.

Taxonomic classifications based on highly specific PCR based methods have increasingly favored the proper identification of microsporidia. The ribosomal DNA genes have been widely exploited since many years, for deriving a Phylogenetic inference. However the more variable regions of r DNA such as the ITS and IGS are the most rapidly evolving regions making them highly polymorphic and hence are now being considered as significant tools for deriving evolutionary and taxonomic implications in fungi as well as other organisms [33]. IGS has been found to highlight differences at the intraspecific levels either as variation in the restriction patterns or as variation in the length of the PCR product [34]. A previous report states that IGS sequence analysis is not only superior with respect to phylogenetic differentiation of closely related species but also a very potential epidemiological tool [35]. Existence of high degree of molecular variations among isolates from close geographical locations based on restriction patterns of IGS has also been shown [36]. IGS based phylogenetic classification has been better tools to study intraspecific divergence and will provide more molecular evidence for the classification and evolutionary studies of microsporidia [37, 38, 39]. The highly variable regions of r DNA that corresponded to whole IGS sequence and a part of SSU was considered for differentiating *N. ceranae* strains from different geographic origin based on their virulence [40]. Hence based on these reports we chose to analyze the IGS sequences along with partial SSUr RNA and 5S r RNA sequences to derive a phylogenetic inference for our pest derived microsporidians. The lengths of the IGS fragment of our test isolates have been found to range in between 270bp to 290 bp. The G+C content was about 29-32%. It was observed that VIK-52Pr showed the least sequence homology of about 64% contrary to NIK-55Dp that shared the maximum sequence homology of about 91% respectively with the *Nosema* type species-NIK-1s_mys. The remaining test isolates shared about 84-88% of sequence similarity with the type species. The maximum parsimony tree was developed using MEGA (6.06). The consistency index was 0.687285 with a retention index of 0.824155, and the composite index was 0.577044 for all sites and parsimony-informative sites (in parentheses). The tree has placed 6 of our pest isolates and NIK-1s_mys with *Nosema bombycis* and most of the *Nosema* species from other Lepidopterans. Our *Nosema* standard strain NIK-1s_mys was showing 99.8% sequence similarity to *Nosema bombycis* (AY259631). The IGS sequence of *Nosema* sp. NIK-60Pd, isolated from *Papilio demoleus* was showing 96% homology with that of the *Nosema sp*. isolated from Chinese Oak silkworm, *Antheraea pernyi* (DQ073396, Wang *et al*., 2006). Similarly isolate NIK-63EC from *Euploe core* was sharing sequence similarity of 94.21% to *Nosema plutellae* (AY960987). The *Nosema* isolate NIK-61Pp from *Papilio polytes* was showing maximum sequence homology of 99.8%

with *N. bombycis* (JF443694). The microsporidian isolate VIK-52Pr from butterfly-*Pieris* rapae was the only *Vairimorpha* strain, among the 7 test isolates that was evidently clustering along with the genus of *Vairimorpha* sharing a sequence homology of almost 99.24%with the reference *Vairimorpha* species (HQ891818) and was showing 100% match to the *Vairimorpha* group. Thus IGS sequences can prove to be very significant genomic tools to understand the molecular basis of these persistent pathogens of sericulture.

5. Conclusion

Pebrine has been seen as a perpetual incidence in sericulture. Although several preventive measures are being strictly practiced during rearing procedure, however the infection finds its way through the feed which is contaminated by various insects visiting the field regularly. The present study is a preliminary effort to understand and identify the microsporidia from other insect hosts that affects the economically important Silkworm *B.mori*. The screening of various Lepidopteran insects of mulberry garden resulted in the isolation of seven microsporidian isolates belonging to *Nosema* and *Vairimorpha* genus. Our study adds to the fact that most of the microsporidia infecting Lepidopteran hosts predominantly belong to the genus of *Nosema*, which later enters the silkworm through cross infection.

As detection of pebrine even in single batch leads to the total rejection of the entire rearing lot, hence extensive studies must be undertaken to understand the molecular basis of microsporidia, that would aid in development of preventive and diagnostic strategies so as to avert the unwanted bulk elimination of silk crop that causes heavy economic loss to the silk rearers.

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