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Identification and infectivity of different microsporidian isolates collected from sericulture (*Bombyx mori* L) areas

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Abstract

Pebrine is the most destructive disease of silkworm, *Bombyx mori* L., as it is transmitted transovarially to the next generation and causes crop loss. Pebrine disease is caused by microsporidian strains whose pathogenicity, rate of spread, and mode of transmission differ. This has recently become a serious concern for seed producers in India. The threat has been increasing, and disease incidence has increased in various locations in Karnataka and Tamil Nadu. A disease survey was conducted at various locations in these two states to record disease incidence rates. Ten microsporidian isolates were collected and inoculated for bioassay studies into healthy larvae to determine their infectivity, multiplication, and transovarial transmission. Rearing was conducted for two successive generations, and the mortality rate in the second generation varied for all the microsporidian isolates, with pupal survival ranging from 21-65%. All ten isolates of microsporidia were found to exhibit transovarial transmission. The fecundity significantly decreased and the hatching percentage decreased from 5% to 22% in the second generation of infected batches. The presence of microsporidia was confirmed through PCR amplification of the SSU rRNA gene of Microsporidia. Five microsporidian isolates were selected for gene sequence analysis on the basis of their collection at different locations and identified as being homologous to *Nosema plutellae*, *Nosema fumiferenae* and *Nosema* species. This study helps in understanding the infectivity levels of different microsporidia prevalent in the sericulture field.

Keywords: Parasite, microsporidia, pebrine disease, *nosema*, infectivity, transovarial

Introduction

Pebrine disease caused by the intracellular parasite *Nosema bombycis* is a highly destructive disease that affects the silkworm *Bombyx mori* L. This disease is transmitted to the next generation and leads to significant losses in silk cocoon production. The disease has become more complex in recent years because of the identification of various strains and species infecting silkworms. These strains vary in their pathogenicity, spread rate, transmission method, and multiplication behavior. This poses a significant challenge for silkworm seed (egg) producers in India, as identifying and eliminating infected lots requires substantial effort due to the high multiplication rate of the disease, which can severely impact the silk industry. The rapid spread of this disease can cause extensive damage to silkworm populations and result in complete crop failure in severe cases. The complexity of the disease has increased due to the continuous emergence of different types of microsporidian infections in silkworms (Sharma *et al.*, 2003) ^[13]. Some of these infections are caused by genera such as *Vairimorpha*, *Pleistophora*, and *Thelohania*, each with unique infection patterns (Samson, 2000) ^[12]. *Nosema bombycis* was initially identified as the primary cause of microsporidiosis in silkworms by the end of the 19th century, and multiple microsporidia from different genera are now responsible for the disease. These microsporidia vary in spore shape, size, and pathogenicity, with some showing low multiplication rates in silkworms (Bhat & Nataraju, 2006) ^[4]. While some microsporidia coexist harmlessly in the gut, others, such as NIK-4m can be highly pathogenic, causing acute disease through cyst formation on the gut surface (Ananthlashmi *et al.*, 1994) ^[2].

The occurrence of microsporidians has been reported in sericulture areas, and infected lots are often discarded without determining their impact on crop loss. Understanding the infectivity and transovarial transmission of microsporidia is crucial for clarifying the extent of crop damage. Microsporidian spores exhibit a diverse range of shapes and sizes when viewed under a microscope, necessitating more advanced identification techniques beyond morphology-based methods. Vossbrinck *et al.* (1987) [15] demonstrated the utility of PCR analysis of SSU-rRNA gene sequences for the molecular identification of microsporidians. Various methods for the amplification of SSU-rRNA gene fragments have subsequently been utilized for diagnosing and identifying insect microsporidia (Kawakami *et al.*, 2001) [8]. Limited information is available on the characteristics of spores in silkworms, the pathology of pebrine disease, and fluctuations in its incidence. Therefore, this study aims to investigate the fundamental aspects of microsporidia in sericulture, including their identification and infectivity in subsequent generations.

Materials and methods

Sample collection and multiplication

A disease survey was carried out in silkworm seed areas in various locations in Karnataka and Tamil Nadu states to determine the prevalence of pebrine disease. Microsporidia collected from the fields were cultured by inoculating them into healthy larvae. The spores of the microsporidia were extracted from infected larvae, pupae, and moths by homogenizing them in a 0.6% potassium carbonate solution and then centrifuging the suspension at $3,000 \times g$ for 3 minutes. The resulting pellets were washed with water 3 to 5 times until a clear suspension was achieved, and the spores were quantified at a concentration of 1×10^6 spores/ml for bioassay studies.

Infectivity of microsporidian isolates

The infectivity of the microsporidian isolates was tested by inoculating them into third instar multivoltine silkworm (Sarupat) larvae on the first day. Three replications with 200 larvae each were used for the bioassay studies, and a control group of larvae fed mulberry leaves with distilled water was also maintained separately until spinning. Dead larvae were examined daily for the presence of microsporidian spores. Cocooning activities were carried out for 10 microsporidia, and five batches of eggs were prepared from the infected moths of each microsporidia and incubated at 25 °C until hatching for the second generation. Two hundred larvae were randomly selected after the second molt, and rearing was performed following standard procedures. Mother moths were tested for the presence of spores, and positive batches of eggs were incubated at 25 °C for embryonic development, with eggs tested for vertical transmission. Data on larval mortality, pupal mortality, fecundity, hatching percentage, and transovarial transmission rate were recorded.

PCR amplification and sequencing

Genomic DNA was extracted from the microsporidian isolates, and the *Nosema*-specific small subunit (SSU) rRNA gene was amplified from all ten microsporidian isolates via the forward primer 5'-CACCAGGTTGATTCTGCC-3' and reverse primer 5'-TTATGATCCTGCTAATGGTTC-3' designed by Baker *et*

al. (1995) [3]. The PCR master mix was prepared with 1 µl (10 pg/µl) of each of the forward and reverse primers along with 5 µl of the emerald master mix (TAKARA Bio, USA). The volume was made up to 10 µl with water, including 2 µl of template. The reaction parameters were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 35 s. The PCR samples were run on a 1.2% agarose gel with ethidium bromide at 100 V for 40 mins. The amplified products were sequenced via an ABI PRISM automated sequencer.

Phylogenetic analysis

A phylogenetic tree was constructed with nucleotide sequences through molecular evolutionary genetic analysis via MEGA 11 (Tamura *et al.*, 2021) [9]. A neighbor-joining tree was constructed by the Kimura 2-parameter with 1000 bootstrap replications and a default random seed. Five microsporidia were selected from the ten isolates from the same locations and sequenced. The SSU-rRNA genes of the five microsporidian isolates were subjected to homology searches via BLAST (Alschul *et al.*, 1990) [1]. Similar gene sequences were downloaded from NCBI, and multiple sequence alignment was performed with the Clustal W program.

Results

The infectivity of microsporidians

Ten microsporidian isolates were found in the sericulture field, with spore intensities ranging from 3 to 25 per microscopic view (Fig. 1). The isolates were cultured and optimized for spore concentration for inoculation. Different strains showed varying infectivity to silkworms, with larval mortality starting 8 days post inoculation. Dead larvae were confirmed through microscopic examination, which revealed oval/elongated spores. Mortality rates are high during spinning, with survival rates ranging from 57-83%. Cocoons were harvested on the 6th day after spinning, and pupal survival rates varied among strains ranging from 41-78% in the first generation. Dead pupae were confirmed by the presence of microsporidia spores through microscopic examination. Fecundity significantly decreased in all the isolates, ranging from 206 to 350 eggs in the second generation. The hatching percentage of the infected eggs decreased from 5-22% in the second generation. Transovarial transmission of the microsporidians was confirmed by examination of eggs on the 7th day of embryonic development, which revealed transovarial transmission in all the isolates. Pupation rates varied among the ten isolates, ranging from 21-65%, with NIT-de1 being the most virulent. Mortality was observed in the chawki stage, with mortality rates dependent on spore dose in the embryonic stage (Table 1).

Identification of microsporidia

The SSU rRNA gene of all ten microsporidia was amplified, producing products ranging in size from 1150 to 1175 bp, with an expected product size of 1230 bp in the *Nosema* genus (Fig. 2). The amplified products were purified and validated through sequencing, and the gene sequences were compared among the isolates and submitted to GenBank.

Molecular phylogeny

The SSU rRNA gene sequences of the five microsporidia isolates with the highest similarity according to BLAST

searches were selected for phylogenetic analysis. Gene sequences from 10 microsporidian species were retrieved from NCBI GenBank to construct a phylogenetic tree. The isolates clustered into different clades with a bootstrap value of 1000. NIT-de2 grouped with *N. fumiferenae* with a

similarity percentage exceeding 90%, whereas NIK-hd2 clustered separately with *N. plutellae*. NIK-ra1 and NIK-ku1 formed a distinct group closely related to *N. plutellae*, and NIK-kk2 was more closely related to *Vairimorpha* and other *Nosema* species (Fig. 3).

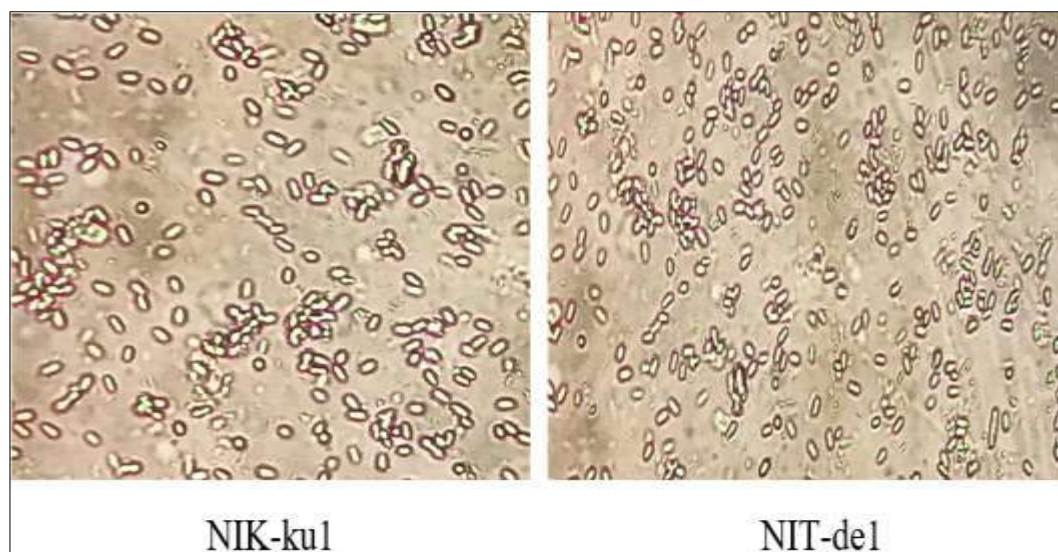


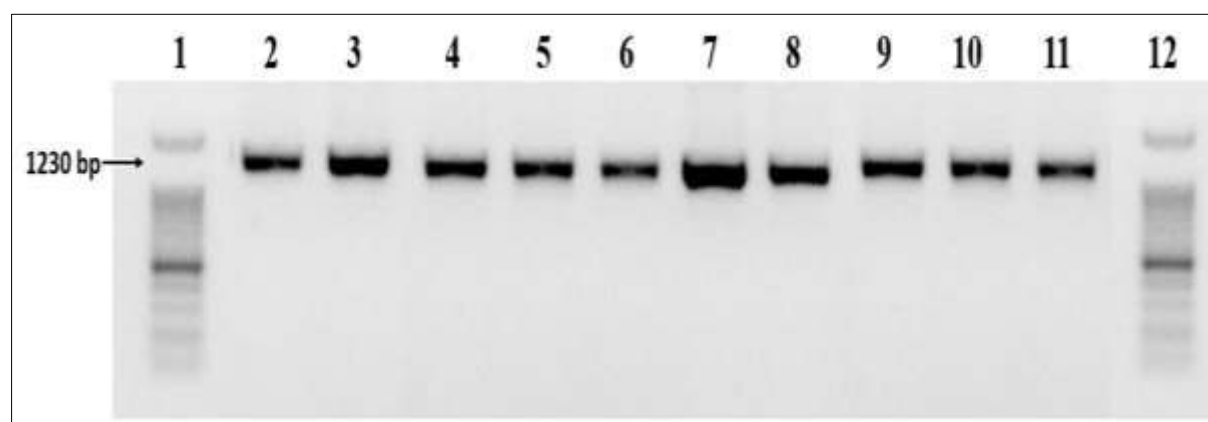
Fig 1: Microscopic view of microsporidia collected from the sericulture field and visualized at 600x magnification

Table 1: Infectivity of microsporidian isolates tested in the multivoltine silkworm Sarupat

Micro sporidia isolates	Larval Survival (%) (Mean± SD)	Pupal survival (%) (Mean± SD)	Fecundity (2 nd Gen.) (Mean± SD)	Hatching % (2 nd Gen.) (Mean± SD)	Pupation rate % (2 nd Gen.) (Mean± SD)
NIK-ku1	64±3.606	41±4.20	232±21.07	87±1.528	25±3.055
NIK-ku2	68±4.583	64±4.041	272±53.61	89±3.618	36±3.512
NIK-ra1	70±3.215	62±2.000	267±18.68	85±2.351	34±4.000
NIK-ra2	66±5.508	50±4.041	299±41.99	84±1.670	25±4.000
NIK-hd1	73±5.132	65±5.000	342±11.31	80±1.929	37±4.583
NIK-hd2	57±3.606	50±2.517	289±51.31	79±1.593	24±3.215
NIK-kk1	71±6.807	61±4.163	292±25.16	82±2.351	46±2.000
NIK-kk2	83±3.055	78±2.517	350±11.32	90±1.929	65±3.512
NIT-de1	74±4.041	55±6.110	320±17.32	87±2.517	32±5.033
NIT-de2	62±2.517	53±5.508	206±9.02	73±4.509	21±2.646
Control	94±1.000	91±0.577	422±12.01	95±1.103	92±1.00
CD (5%)	6.489	5.949	32.4	3.18	3.95

The values are average of 3 replications. NIK- *Nosema* India Karnataka, T- Tamil Nadu, ku- Kunigal, ra-

Ramnagara, kk- Kanakpura, hd- Huliya Durga, de- Denkanikottai, Gen- Generation, SD- Standard deviation.



Lanes: 1 & 12- DNA ladder, 2- NIK-ku1, 3- NIK-ku2, 4- NIK-ra1, 5- NIK-ra2, 6- NIK-hd1, 7- NIK-hd2, 8- NIK-kk1, 9- NIK-kk2, 10- NIT-de1, 11- NIT-de2

Fig 2: PCR-amplified product of the SSU gene visualized in 1.2% agarose gel

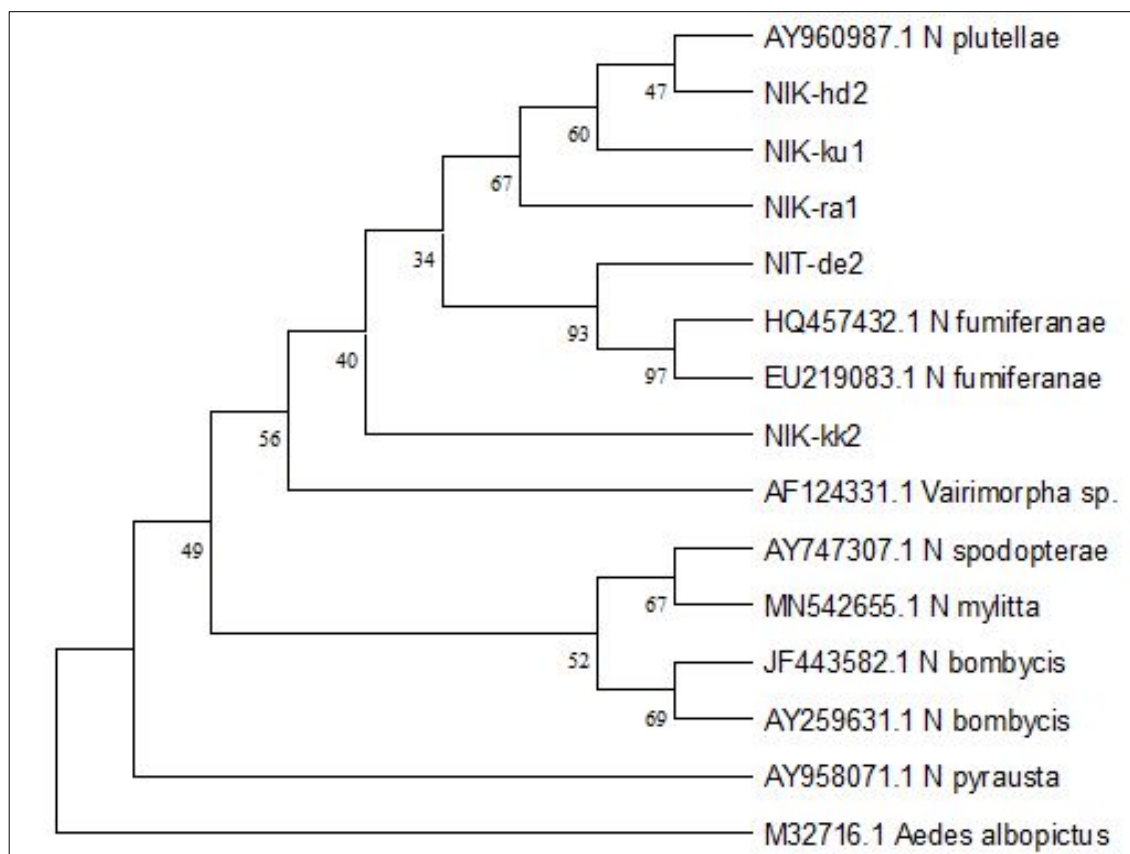


Fig 3: shows a phylogenetic tree based on nucleotide sequences of field isolates compared with different microsporidian sequences of the SSU-rRNA gene retrieved from a database. The numbers near the nodes indicate branch length. *Aedes albopictus* was included as an outgroup.

Discussion

All ten isolates were found to be infective to silkworms, and various strains were prevalent in the field. Ananthalakshmi *et al.* (1994) ^[2] isolated three different strains of microsporidians from Karnataka, whereas Samson *et al.* (1999) ^[11] isolated two microsporidians from lepidopterans that were infective to silkworms. Advanced moth testing techniques have been used for an appropriate diagnosis of pebrine disease, along with improved inspection methods for different developmental stages of silkworms. Many advancements have been made in conventional diagnostic techniques, and the development of molecular techniques has provided new tools for taxonomic and phylogenetic studies. Nageswararao *et al.* (2004) ^[10] studied the pathogenicity, mode of transmission, tissue specificity of infection, and small subunit rRNA (SSU-rRNA) gene sequences of three new microsporidian isolates from the silkworm *B. mori*. They identified NIK-2r as the type species *N. bombycis*, whereas NIK-3h and NIK-4m were identified as *Vairimorpha* sp. Surendranath *et al.* (2012) ^[14] identified five new microsporidian isolates from silkworms and confirmed them as *Nosema* species through SSU-rRNA sequence-based phylogenetic analysis. Bhuvaneshwari & Surendranath (2015) ^[5] extracted seven microsporidian isolates from infected lepidopteran pests in the sericulture zones of Karnataka, India, and conducted PCR-based analysis to determine the phylogenetic relationships among the key isolates. The phylogenetic analysis, genetic distance, and NCBI BLAST analysis indicated that the identified microsporidians are closely related to the typical *Nosema* subgroup. The identified microsporidian isolates belong to the *Nosema* genus, with NIK-hd2 showing homology with

Nosema plutellae, which infects the Diamondback moth (Gautam *et al.*, 2018) ^[7] and silkworms. NIT-de2 shows homology with *Nosema fumiferanae*, which infects larvae of the spruce budworm *Choristoneura fumiferana* (Frankenhuyzen *et al.*, 2007) ^[6]. The nucleotide differences in the SSU-rRNA gene clearly distinguished all five microsporidians from the standard type species.

Conclusion

All ten microsporidian isolates were found to be infective to *Bombyx mori* and transmitted via transovarial transmission. This study revealed that microsporidians identified from the field as *Nosema* species can cross-infect silkworms and potentially cause economic losses in the sericulture industry.

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