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Nosema sp. isolated from Cabbage White Butterfly (*Pieris rapae*) Collected in Korea

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A microsporidium, from cabbage white butterflies, *Pieris rapae*, collected in Korea, was purified and characterized according to its gene structure, spore morphology and pathogenicity. From the observation of the isolate by SEM and TEM, the endospores, exospores and nuclei, about 12 polar filament coils of the polar tube and posterior vacuoles were all identified. The nucleotide sequence was determined for a portion of genomic DNA which spans the V4 variable region of the small subunit rRNA gene. Comparison with the GenBank database for 15 other microsporidia species suggests that this isolate is most closely related to *Nosema* species. The pathogenicity against cabbage white butterflies was quantified by inoculating variable doses of spores to the second instar larvae. Peroral inoculation at a dosage of 10^8 spores/ml resulted in the death of all larvae prior to adult eclosion, but at lower spore dosages of 10^4-10^5 spores/ml, many adults successfully emerged. The median lethal dose (LD_{50}) was determined to be 4.6×10^6 spores/ml and the isolate also transmitted transovarially to the progeny eggs at a frequency of 92%.

Key words: *Nosema* sp., *microsporidia,* ribosomal RNA gene, phylogeny, *Pieris rapae*

Microsporidia are obligate intracellular parasites and have long been considered primitive eukaryotes, both on the basis of morphological features and on the basis of molecular, mainly ribosomal RNA-based, phylogenies (Peer *et al.*, 2000). The rRNA genes have been shown to be evolutionarily very highly conserved, but the intergenic spacer regions around them are highly variable, even between closely related species (Fedoroff, 1979; Long and Dawid, 1980). The V4 variable region of the small subunit rRNA (ssrRNA) gene, comprising two conserved regions flanking a variable region, have shown marked differences among species (Malone *et al.*, 1994).

Micorsporidia were first recognized in 1857 when *Nosema bombycis* was described as a silkworm parasite (Nageli, 1857). Microsporidia have subsequently been reported as infecting major animal groups, from other protists to invertebrates and all classes of vertebrates; more than 140 genera and almost 1,200 species have been recognized (Canning and Lom, 1986; Sprague, 1992; Malone *et al.*, 1994; Sprague and Becnel, 1999). Also a number of microsporidia of medical importance, which cause opportunistic infections in AIDS patients, have recently been identified (Weber *et al.*, 1994).

Among almost 1,200 microsporidial species, at least 200 belong to the genus *Nosema* (Sprague, 1982) and most *Nosema* species are parasitic in invertebrates (Sprague, 1982). A majority of these such as *N. bombycis* (Canning *et al.*, 1999), *N. mesnili* (Nageli, 1857; Paillot, 1924; Cheung and Wang, 1995), *N. tyriae* (Canning *et al.*, 1999), *N. algerae* (Müller *et al.*, 2000), *N. apis* (Malone, 1994) and *N. trichoplusiae* (Malone, 1994) have pathogenicity to various insects. Their development takes place from direct contact with the host cell cytoplasm and nuclei are paired throughout the entire life cycle (Vavra and Larrson, 1999). Although microsporidia are spread parasites, only a few cabbage white butterfly infections with *Nosema* species have been reported.

In this paper, we report a strain of *Nosema* species which was isolated from a cabbage white butterfly (*Pieris rapae*) from Korea. The experiments were based on the use of a partial nucleotide sequence for the V4 region of ssrRNA gene, along with conventional microscope and insect pathogenicity experiments, to identify microspo-

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ridia species from the cabbage white butterfly, *Pieris rapae* (L.), which is a widespread pest of brassicas.

Materials and Methods

Microsporidia isolates

Spores of an unidentified microsporidium were obtained from *P. rapae* larvae collected from an insectarium (Na-Bi-Na-Ra) in Go-Yang, Korea. Infected larvae were macerated in phosphate-buffered saline and the resultant spore suspension was filtered through cotton wool. Spores in the filtrate were purified by the method of Sato and Watanabe (1980) using a gradient of neutralized Percoll (Sigma). After centrifugation at 73,000 g for 30 min, a band of purified spores was collected from the gradient. This band was rinsed twice and the final suspension of spores was stored in distilled water at 4°C until required. Spores were quantified using a hemocytometer.

Light and electron microscopy

Spore samples were prepared for electron microscopy by fixing for 7 days in a 2.5% (v/v) glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) counting 0.1% $CaCl_2$. Samples were postfixed in 1% aqueous OsO_4 (w/v) for 120 min, dehydrated through ascending ethanol series, and embedded in Epon-Araldite. Ultrathin sections were cut with a diamond knife mounted on a ultramicrotome (Leica Ultracut UCT), stained in methanolic uranyl acetate and then lead citrate (Takaki, 1990), and examined with an electron microscope (Zeiss LEO 912AB) operated at 100 kV.

Partial ssrRNA sequencing

DNA was prepared from spores using a Qiamp Tissue Kit (Qiagen, Germany). Spores were incubated in digestion buffer with 400 μg of proteinase K (Qiagen, Germany) at 55°C for 3 days. 500 μg glass beads (425-600 μm; Sigma) were added and the mixture was vortexed for 1 min three times daily (Müller *et al.*, 1999).

PCR primers were selected to amplify partial nucleotide sequences, spanning the V4 region of the ssrRNA gene (Malone *et al.*, 1994). Primers had the following sequences: 5'-GTTGATTCTGCCTGACGTA-3' (bases 7 to 25, *Vairimorpha necatrix* numbering as recorded in the GenBank database accession no. M24612) and 5'-ACCCGACGT-GCGCGTTATGT-3' (bases 988 to 1007). The amplified DNA fragments were cloned in pGEM-T easy Vector (Promega). DNA sequencing was performed using ABI 377 Genetic Analyzer (PE Applied Biosystems, USA).

Phylogenetic analysis using PAUP and PHYLIP

Sequences of the following microsporidian species were used in our analysis: *Ameson michaelis*, L15741 (GenBank database accession number); *Encephalitozoon cuniculi*, Z19563; *Encephalitozoon hellem*, L19070; *Ichthyosporidium* sp., L39110; *Nosema apis*, X73894; *Nosema bombi*, AY008373; *Nosema bombycis*, L39111; *Nosema trichoplusiae*, U09282; *Pleistophora* sp., U10342; *Vairimorpha lymantriae*, AF033315; *Vairimorpha necatrix*, Y00266; *Vairimorpha* sp, L39114; *Vavraia oncoperae*, X74112; *Vittaforma corneae*, U11046. A protoctistan unrelated to the Phylum *Microspora, Trypanosoma brucei,* M12676, was included in the sequence comparisons as the taxonomic outgroup for phylogenetic tree construction.

PAUP (Phylogenetic Analysis using Parsimony) ver 3.0 (Swofford, 1990) was used to infer possible phylogenetic relationships among the microsporidia. The reliability of the trees was tested by 1,000 iterations of boot strapping (Felsenstein, 1985). As an alternative to the parsimony analysis, we used the neighbor-joining (NJ) method and the maximum likelihood (ML) method incorporated in PHYLIP (Phylogeny Inference Package) ver 3.5c (Felsenstein, 1993). To obtain a phylogenetic tree, the data set was first iterated 100 times using the subprogram SEQBOOT. Next, in the case of the NJ method, the iterated data set was run using the subprogram DNADIST to obtain the distance matrix with the option of Kimuras 2-parameter method (Kimura, 1980), which attempted to correct observed dissimilarities for multiple substitution in sequences evolving with a transition bias. Individual trees from each distance matrix were obtained using the subprogram NEIGHBOR. In the case of the ML method, individual trees were directly obtained using the subprogram DNAML. In both cases, the *T. brucei* sequence was included in the analysis of tree roots. Finally, a consensus tree representing reliability at each branch in the tree was obtained using the subprogram CONSENSE.

Pathogenicity

Second instar larvae were allowed to feed on a cabbage containing 10^3-10^8 spores/cm^3 of the microsporidia. Moths obtained from the exposed larvae were provisionally regarded as infected. Infected females (IF) were paired with infected males (IM) for the studies of venereal transmission. After mating and oviposition, all moths were macerated and the wet mounts were examined for microsporidian infection a under light microscope.

Results and Discussion

We isolated a microsporidium from a cabbage white butterfly, *Pieris rapae* which was collected from an insectarium in Korea. For identification, the ultrastructure of the isolate was observed by electronmicroscopy. Until recently, the classification system of Sprague, proposed in 1977 and updated in 1982, was the most widely used (Sprague, 1977; Sprague, 1982). Species classification is based on ultrastructural features, including the size and morphology of the spores, the number of coils of the polar tube, the

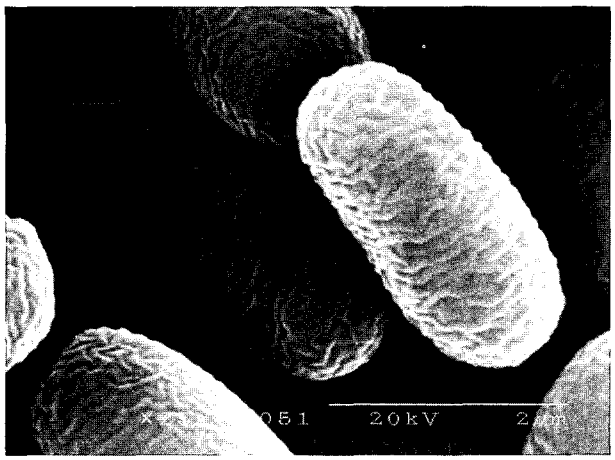


Fig. 1. Scanning electron micrographs of *Nosema* sp. C01 isolated from *Pieris rapae* collected in Korea.

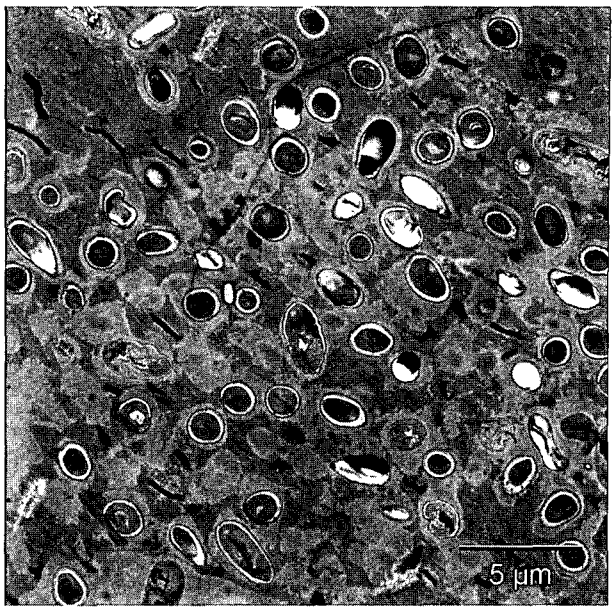
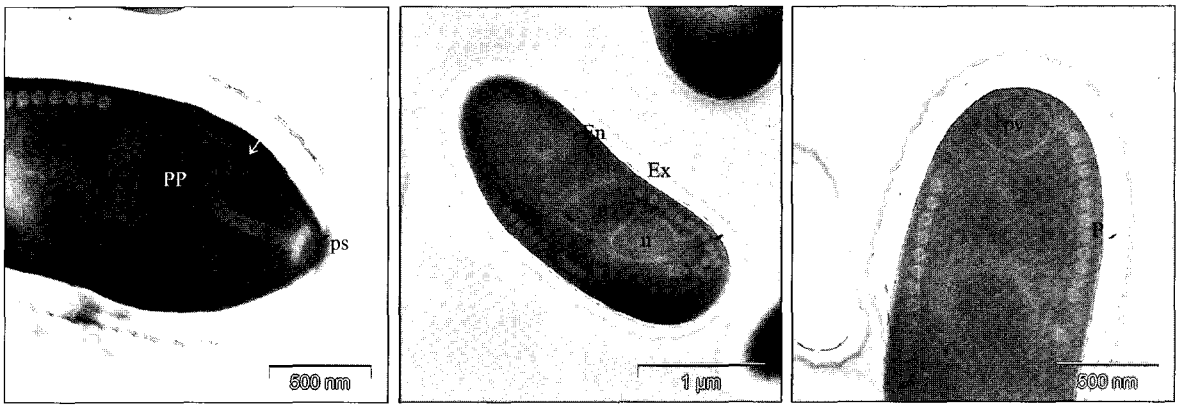


Fig. 3. Cross section of the fat body of *Pieris rapae* larvae infected *Nosema* sp. C01.

developmental life cycle, and the host-parasite relationship. When observed with the scanning electron microscope, the shape of the microsporidia spores was spherical and the surface sculpturing was crumpled (Fig. 1). The spores measured about 3.8 to 4.7 by 1.9 to 2.6 μm. Spores had an endospore and an exospore with a wavy outline. The anchoring disc of the polar tube lay within the polar sac, which formed a deep dome at the anterior extremity and extended laterally over the compact membranes of the polaroplast. The coiled region of the polar tube was arranged as 12 to 13 isofilar turns in various spores, always in a single rank close to the spore wall. The diplokaryon nuclei were slightly separated. At the posterior end of the spore was a membrane-bound vacuole with amorphous contents (Fig. 2). The cytoplasm occupying the rest of the spore was packed with ribosomes. Sites of infection were the gut epithelium and the fat body (Fig. 3). The microsporidium does not produce the pansporoblast in the developmental life cycle. These features are most closely aligned with the genus *Nosema*.

As well as ultrastructural morphology, the microsporidial taxonomy has been expanded by the use of DNA-sequence data, mainly sequences of ribosomal RNA (Malone *et al.*, 1994). Partial nucleotide sequence for the V4 region of ssrRNA gene of the isolated microsporidium from *P. rapae*, was lodged in the GenBank database under accession no. AF485270. The V4 region, comprising two conserved regions flanking a variable region, was chosen because comparison of microsporidia ssrRNA gene sequences has shown marked differences among species in this part of the gene (Malone *et al.*, 1994). Microsporidia consist of nearly 150 genera, but only 4 genera (*Nosema, Vairimorpha, Thelohania, Pleistophora*) as well as unclas-



A B C

Fig. 2. Transmission electron micrographs of *Nosema* sp. C01 isolated from *Pieris rapae* collected in Korea. A, Longitudinal section of spore showing insertion of polar tube into polar sac(ps) with lateral extensions (arrow) over the polarplast(pp); B-C, The diplokaryon(n), endospore(En), exospores(Ex), polar tube(P) and posterior vacuole(pv) are visible.

Table 1. Microsporidia described or reported from cabbage white butterfly

Microsporidian species	Spores sizes	Host insects	References
Nosema(Perezia) mesnili	3-4 × 1.5-2 μm	*P. brassicae*	Pillot (1918)
		P. rapae	Tanada (1953)
		P. canidia	Cheung & Wang (1995)
Microsporidium(Thelohania) mesnili	2.5-3.5 × 1.5-2 μm (from illustration)	*P. brassicae*	Paillot (1924)
Pleistophora schubergi aporiae	2 × 1.5 μm	*P. brassicae*	Veber (1956)
		P. rapae	Issi (1969)
Thelohania sp.	5.6-6.8 × 3.1-3.8 μm 3.8 × 1.8 μm(Free spores)	*P. rapae*	Laigo and Paschke (1966)
Vairimorpha sp	3.1 × 1.9 μm(Octospores) 8.0 × 2.1 μm(Macrospores)	*P. rapae*	Malone & Meclvor (1996)

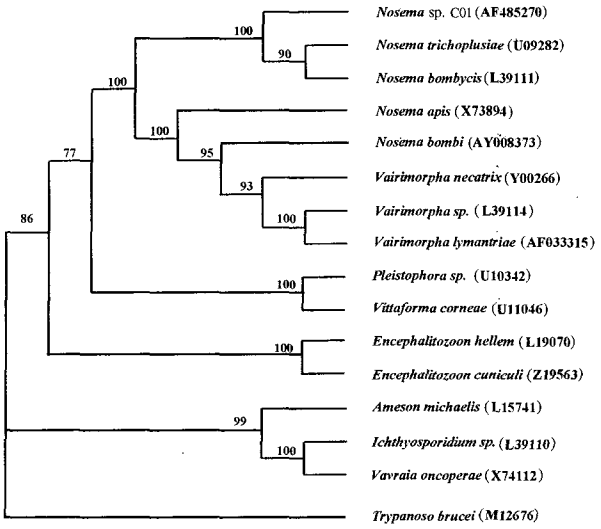


Fig. 4. PAUP analysis of nucleotide sequences for the V4 regions of partial small subunit ribosomal RNA gene using ssrRNA genes of *Trypanosoma brucei* as an outgroup. The tree shown is an unordered tree obtained with the option of "retain groups with frequency >50%" by majority-rule consensus of 100 equally parsimonious trees from the heuristic search. The numbers shown on the branches represent bootstrap values for 1,000 replicates. Parentheses are Geneback database accession numbers.

ified microsporidia have been described as pathogens in *Pieris* spp. (Table 1).

A phylogenetic tree was constructed on the basis of the ssrRNA sequence of 15 other microsporidia species deposited in GenBank, including the isolated microsporidium. This tree based on sequence distance data transformed by the mean distance values method is shown in Fig. 4. The tree places the isolated microsporidium from *P. rapae* closest to *Nosema trichoplusiae*, a microsporidium pathogen from the Asian honey bee, *Apis cerana* and *Nosema bombycis*, a microsporidium pathogen from the silkworm, *Bombycis mori*. *N. apis* appear on another nearby branch to this microsporidium, along with the lepidopteran-infecting *Vairimorpha* species, *Vairimorpha necatrix*, *Vairimorpha* sp. and *Vairimorpha lymantriae*. *Pleistophora* sp. and *Vittaforma corneae* are closely linked and in the same major grouping as the *Nosema* and *Vairimorpha* species. The two *Encephalitozoon* species are placed in a somewhat separate group. *Ameson michaelis*, *Ichthyosporidium* sp. and *Vavraia oncoperae* appear to be quite separate from the other microsporidia. Also the results of phylogenetic analyses using the neighbor-joining (NJ) method and the maximum likelihood (ML) method incorporated in PHYLIP showed an overall similar branch pattern as shown in the PAUP (data not shown). In our nucleotide sequencing result, the V4 region sequence data suggest that this isolated microsporidium is most closely related to *Nosema* species.

Mean distance values among the sequences of the V4 regions of the ssrRNA genes of the microsporidia species varied more widely (0.5 to 47.3%). This wide diversity among the microsporidia may be the result of their long evolution as obligate parasites of a wide range of host ani-

Table 2. Effect of *Nosema* sp. C01 infections on larval mortality and moth infectivity of the cabbage white butterfly, *Pieris rapae*

Inoculum (spores/ml)	No. larvae infected	No. dead with infection			No. moths emerging (%)	% Moths infected
		Larvae	Pupae	%		
1×10^3	19	0	0	0	19(95)	0
1×10^4	18	0	1	6	17(94)	47
1×10^5	20	0	0	0	20(100)	85
1×10^6	20	5	3	40	12(60)	100
1×10^7	20	9	4	65	7(35)	100
1×10^8	19	19	0	100	0(0)	-

Table 3. Assay on transovarial transmission *Nosema* sp. C01 from infected parents to the progeny

Assayed	Paired moths*			
	IF× IM	IF× HM	HF× IM	HF× HM
No. tested	7	24	20	37
No. infected eggs	7	22	0	0
Transmission rate(%)	100	92	0	0

*IF : infected female, IM : infected male, HF : healthy female, HM : healthy male

mals (Vossbrinck *et al.*, 1987).

In the quantitative bioassay, peroral inoculation of the microsporidia to 2nd instar larvae at a dosage of 10^8 spores/ml resulted in the death of all larvae prior to adult eclosion (Table 2). At a lower spore dosage of 10^7 spores/ml, a few adults successfully emerged. At 10^4 spores/ml, many individuals survived to adulthood and only a few of these adults were infected. The median lethal dose (LD_{50}) was determined to be 4.6×10^6 spores/ml and the median infectious dose (ID_{50}) was determined to be 1.0×10^4 spores/ml. The isolated microsporidium was virulent and caused chronic disease at high concentrations, whereas it was highly virulent and produced acute disease at low concentration.

The examination of eggs showed that the microsporidia was transmitted transovarially by all infected females. Maternal-mediated transovarial transmission of the microsporidia to the progeny eggs took place at a frequency of 100% (Table 3). No transmission of the microsporidia to the progeny occurred in the HF×IM crosses, an indication that there was no venereal pathway for either microsporidium. Although Kellen and Lingegren (1971) reported the venereal transmission of a microsporidium in the Indian meal moth, *Plodoa interpinctella*, that of the isolated microsporidium in the cabbage white butterfly was negative in the present study using surface-sterilized eggs, suggesting that the microsporidium might not be transmitted via sperm from male adults into the progeny eggs.

From these results, both morphological features and ssrRNA-sequence data, we propose that this species be placed in the genus *Nosema* and named *Nosema* sp. C01.

References

Canning, E.U. and J. Lom. 1986. The Microsporidia of Vertebrates, Academic Press, New York, pp. 1-16.

Canning, E.U., A. Curry, S.A. Cheney, N.J. Lafranchi-Tristem, Y. Kawakami, Y. Hatakeyama, H. Iwano, and R. Ishihara. 1999. *Nosema tyriae* n.sp. and *Nosema* sp., Microsporidian Parasites of Cinnabar Moth *Tyria jacobaeae*. *J. Invertebr. Pathol.* 74, 29-38.

Cheung, W.W. K. and J.B. Wang. 1995. Electron microscopic studies on *Nosema mesnili* Paillot (Microsporidia: Nosematidae) infecting the malpighian tubules of *Pieris canidia* larva. *Protoplasma* 186, 142-148.

Fedoroff, N.V. 1979. On spacers. *Cell* 16, 697-710.

Felsenstein, J. 1985. Confidence limits on phylogenics : an approach using the bootstrap. *Evolution* 29, 783-791.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) ver. 3.5c. Department of Genetics, University of Washington, Seattle (on disk).

Issi, I.V. 1969. On possible ways of speciation among Microsporidia. *Prog. Protozool. Proc. Int. Congr. Protozool.* 3, 337-378.

Kellen, W.R., and J.E. Lindegren. 1971. Mode of transmission of *Nosema plodiae* Kellen and Lindegren, a pathogen of *Plodia interpunctella* (Hubner). *J. Stored Prod. Res.* 7, 31-34.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substition through comparative studies of nucleotide sequence. *J. Mole. Evol.* 116, 111-120.

Laigo, F.M. and J.D. Paschke. 1966. A microsporidian, *Thelohania* sp., in *Pieris rapae*. *J. Invertebr. Pathol.* 8, 269-270.

Long, E.O. and I.B. Dawid. 1980. Repeated genes in eukaryotes. *Annu. Rev. Biochem.* 49, 727-764.

Malone, L.A. A.H. Broadwell, E.T. Lindridge, C.A. McIvor, and J.A. Ninham. 1994. Ribosomal RNA genes of two microsporidia, *Nosema apis* and *Vavraia oncoperae*, are very variable. *J. Invertebr. Pathol.* 64, 151-152.

Malone, L.A. and C.A. McIvor. 1996. Use of nucleotide sequence data to identify a microsporidian pathogen of *Pieris rapae* (Lepidoptera, Pieridae). *J. Invertebr. Pathol.* 68, 231-238.

Müller, A, K. Stellermann, P. Hartmann, M. Schrappe, G. Fatkenheuer, B. Salzberger, V. Diehl, and C. Franzen. 1999. A powerful DNA extraction method and PCR for detection of microsporidia in clinical stool specimens. *Clin Diagn Lab Immunol.* 6, 243-246.

Müller, A., T. Trammer, G. Chioralia, H.M. Seitz, V. Diehl, and C. Franzen. 2000. Ribosomal RNA of Nosema algerae and phylogenetic relationship to other microsporidia. *Parasitol Res* 86, 18-23.

Nageli, K.W. 1857. Uber die neue Krankheit der Seidenraupe und verwandte Organismen. *Bot. Z.* 15, 760-761.

Paillot, A. 1918. Deux microsporidies nouvelles parasites des chenilles de *Pieris brassicae*. *C. R. Soc. Biol.* 81, 66-68.

Paillot, A. 1924. Sur *Thelohania mesnili*, microsporidie nouvelle parasite de *Pieris brassicae* L. *C. R. Soc. Biol.* 90, 501-503.

Peer, Y.V.D., A.B. Ali, and A. Meyer. 2000. Microsporidia: accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. *Gene* 246, 1-8.

Sato, R. and H. Watanabe. 1980. Purification of mature microsporidian spores by iso-density equilibrium centrifugation. *J. Seric. Sci. Japan*, 49, 512-516. (in Japanese with English summary).

Sprague, V. and J. Vavra. 1977. Systematics of the microsporidia, in: L.A. Bulla, and T.C. cheug(Eds.), Comparative pathobiology, vol. 2. Plenum Press, New York, N.Y. pp. 1-510.

Sprague, V. 1982. Microspora, in: S.P. Parker(Eds.), Synopsis and classification of living organism, vol. 1. McGraw Hill Book Co., New York, N.Y. pp.589-594.

Sprague, V., J.J. Becnel, and E.I. Hazard. 1992. Taxonomy of phylum Microspora. *Crit. Rev. Microbiol.* 18, 285-395.

Sprague, V. and J.J. Becnel. 1999. Appendix: Checklist of available generic names for microsporidia with type species and type hosts, in: Wittner M., L.M. Weiss (Eds.), The Microbiology and Microsporidiosis, American Society for Microbiology, washington, D.C. pp. 517-530.

Swofford, D.L., 1990. PAUP: Phylogenetic analysis using parsimony, ver 3.0, Illinois National History Survey, Chamlaign (on disk).

Takaki, I., K. Yamada, T. Sato, T. Hanaichi, T. Iwamoto, and L. Tin, 1990. Penetration and stainability of modified Satos staining solution. *J. Electron Microsc.* 39, 67-68.

Tanada, Y. 1953. A microsporidian parasite of the imported cabbage worm in Hawaii. *Proc. Hawaii. Entomol. Soc.* 15, 167-175.

Vavra, J. and J.I.R. Larrson. 1999. Structure of the microsporidia, in: Wittner M., L.M. Weiss (Eds.), The Microbiology and Microsporidiosis, American Society for Microbiology, washington, D.C. pp 7-84.

Veber, J. 1956. *Plistophora aporiae* n. sp., ein parasit des Baumweisslinges *Aporia crataegi. Cesk. Parasitol.* 3, 181-185.

Vossbrinck, C.R., J.V. Maddox, S. Friedman, B.A. Debrunner-Vossbrink, and C.R. Woese. 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature* 326, 411-414.

Weber, R., R.T. Bryan, D.A. Schwartz, and R.L, Owen. 1994 Human microsporidial infections. *Clin. Microbiol. Rev.* 7, 426-461.