Small subunit rDNA phylogeny of *Bacillidium* sp. (Microspora, Mrazekiidae) infecting oligochaets

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SUMMARY

Small subunit (SSU) rDNA has been sequenced from a microsporidium, identified as a member of the genus *Bacillidium* obtained from an oligochaete. The length of the amplified PCR product was 1386 bp which is currently the longest microsporidium SSU sequence known. Phylogenetic analysis using 28 microsporidia SSU sequences, using 3 different tree-building methods indicated that *Bacillidium* sp. may be one of the earliest branches on the microsporidia tree. However, bootstrapping failed to give a high score (more than 50%) for the position of *Bacillidium* sp. The branch leading to *Bacillidium* sp. was long, indicating that this species is not closely related to any of the other microsporidia so far studied by means of rDNA.

Key words: *Bacillidium*, Microspora, molecular phylogeny, taxonomy.

INTRODUCTION

Microsporidia are known from at least 8 different metazoan phyla (Canning, 1990; Sprague, Becnel & Hazard, 1992). Issi (1986) suggested that gut-infecting microsporida in annelids could be among the first entering metazoan hosts from infections originating in parasitic gregarines. Marine polychaetes are frequently infected by gregarines; however, microsporidia are not common in this group whereas the annelid group containing microsporidia are the freshwater oligochaetes but they are not known to carry gregarine infections to any large extent. Microsporidia in the family Mrazekiidae are typically parasites in limnic oligochaetes (Larsson & Götz, 1996). Species from the genera *Jirovecia* Weisser, 1977 and *Bacillidium* Janda 1928 have an unusual size and shape, being large and slender (length up to and over 25 μm) with a special arrangement of the extrusion apparatus. This structure, the manubrium, has been shown to be homologous to the polar tube in other microsporidia (Götz, 1981), an important morphological feature used to identify these genera.

Sequence data from macromolecules have been useful in the study of microsporidia phylogeny. In the majority of these studies, ribosomal RNA (rRNA) or their genes (rDNA) have been used. Several of these studies have shown disagreements between phylogenies based on molecular data and studies using morphology (Baker *et al.* 1994, 1995, 1997). The number of available microsporidia rRNA sequences is increasing but data from many taxa are still lacking. The present study presents the small subunit (SSU) rDNA sequence from a *Bacillidium* sp. from an oligochaete together with some morphological observations. Phylogenetic analysis using 28 microsporidia SSU rRNA sequences is presented and discussed in relation to morphological knowledge of the different groups.

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MATERIALS AND METHODS

Microsporidia

Oligochaetes were collected in the river Oselva about 25 km outside Bergen, Norway. Specimens were examined by binocular and light microscope. From an infected individual, a small piece of the worm was removed and transferred to double distilled water (ddH_2O) containing penicillin and streptomycin (15 U/ml) for extraction of nucleic acids (see below). This tissue piece was homogenized and washed several times in ddH_2O by repeated centrifugation in an Eppendorf Microfuge at 4000 ***g*** for 2 min and finally dissolved in 100 μl of ddH_2O and stored at 4 °C until used. The rest of the worm was transferred to a modified Karnowsky fixative (see below).

Nucleic acid preparation

The above-described spore suspension was centrifuged at 6000 ***g*** for 5 min and dissolved in a digestion buffer (100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0·5% SDS, pH 8·0) containing 0·5 mg/ml proteinase K and incubated overnight at 37 °C. Nucleic acid was then extracted twice with an

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equal volume of phenol/chloroform followed by an equal volume of chloroform, and then ethanol precipitated. The precipitated DNA was dissolved in 50 μl of ddH_2O. The DNA concentration was estimated by running a 5 μl aliquot on a 1 % agarose gel.

Polymerase Chain Reaction (PCR)

A set of primers (V1f = 5′-CACCAGGTTGATTCTGCC-3′ and 1492r = 5′-GGTTACCTTGTTACGACTT-3′) was selected to allow amplification of the SSU rDNA gene. PCR was carried out in 100 μl reactions using 10 pmol of each primer, 20 nmol of each dNTP, 10 μl 10 × *Taq* polymerase buffer (Pharmacia, Uppsala, Sweden), 2·5 units *Taq* DNA polymerase and approximately 25 ng of the genomic DNA. The reactions were run on a Techne thermocycler for 35 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. After completion of the 35 cycles, 10 min extension at 72 °C was applied. PCR products were visualized by running 20 μl aliquots on a 1 % agarose gel and were then purified on a 1 % low-melting agarose gel and the band of desired size (approximately 1350 bp) was then excised from the gel.

Sequencing

Purified PCR product was manually sequenced in both directions using the Thermo sequenase kit from US Biochemicals (Cleveland, OH). Approximately 20 ng of PCR product was used and the sequence reactions were done in accordance with the manufacturer's recommendations. The sequence reactions were run on a Techne thermocycler. Purified PCR product was also ligated into a TA-vector (Invitrogen, San Diego, CA) and used in accordance with the recommendation of the manufacturer (Invitrogen, TA Cloning Kit, San Diego, CA). Transformed colonies were screened by PCR to identify bacteria with insert of correct size. Plasmid purification was done using miniprep, and 1 clone was sequenced as described above with the exception that 200 ng of template was used in each sequence reaction.

Preparation for electronmicroscopy

The oligochaete was fixed in a modified Karnovsky fixative, washed in a phosphate-buffered sucrose solution, and stained with a phosphate-buffered 1 % OsO_4 solution for 1·5 h. After washing, the pieces were dehydrated using a graded series of acetone and embedded in Epon 812. Semi-thin (1 μm) and ultra-thin sections (about 30 nm thick) were cut on a Reichert Ultracut E microtome. The semi-thin sections were stained in toluidine blue and examined in a light microscope. The ultra-thin sections were stained for 1 h in 2 % aqueous uranyl acetate solution and then stained with lead citrate for 5 min. The sections were examined in a Jeol 100S electron microscope operating at 80 kV.

Phylogenetic analysis

The SSU sequence was aligned by eye to an alignment of microsporidia SSU rRNA sequences obtained from the rRNA WWW server at the Department of Biochemistry, Molecular Biology, University of Antwerp (UIA), Belgium (http://rrna.uia.ac.be/), (Van de Peer *et al*. 1994)). This alignment is based on the secondary structure model by Van De Peer *et al*. (1996). This alignment was used in the phylogenetic analysis using PAUP (Swoffor, 1993) and the PHYLIP-package (Felsenstein, 1993). The maximum likelihood analysis was performed with fastDNAml (Felsenstein, 1981; Olsen *et al*. 1994) on a CRAY Origin 2000 supercomputer.

In addition to the *Bacillidium* sp. sequence obtained in the present study (AF104087) the following species have been used: *Amblyospora californica* (U68473), *Amblyospora* sp. (U68474), *Ameson michaelis* (L15741), *Encephalitozoon cuniculi* (L39107), *E. hellem* (L39108), *Endoreticulatus schubergi* (L39109), *Enterocytozoon bieneusi* (L07123), *Giardia ardeae* (Z17210/S53313), *G. lamblia* (M54878 M19500), *Glugea anomala* (AF104084), *G. atherinae* (U15987), *Hexamita inflata* (L07836), *Ichthyosporidium* sp. (139110), *Loma* sp. (AF104081), *Nosema bombycis* (D85504), *Nucleospora salmonis* (U78176), *Pleistophora anguillarum* (U47052), *P. mirrandellae* (AF104085), *P. typicalis* (AF104080), *Pleistophora* sp. 2 (AF104083), *Pleistophora* sp. 3 (AF104082), *Pleistophora* sp. A (U10342), *Pleistophora* sp. B (D85500), *Septata intestinalis* (L39113), *Spraguea lophii* (AF104086), *Spraguea lophii* (AF033197), *Trachipleistophora hominis* (AJ002605), *Vairimorpha necatrix* (Y00266), *Vavraia oncoperae* (X74112), *Vittaforme corneae* (L39112).

RESULTS

Morphological and ultrastructural observations

One specimen of *Lumbriculus* sp. was infected with this species of microsporidium. The infection was in the coelom and the host-cell was similar to white blood cells (coelomocytes). They appeared multinucleated with a hairy surface with a few intermingling fibroblasts (referred to as xenoma) (Fig. 1C). Infected cells occurred throughout the coelom. Spores and pre-sporogonic developmental stages occurred in the xenoma simultaneously (Fig. 1C). All developmental stages were diplokaryotic. The spores were 24–27·2 × 3·2 μm (measured fresh), narrowing against the posterior part, and possessed a gelatinous mass posteriorly when observed fresh

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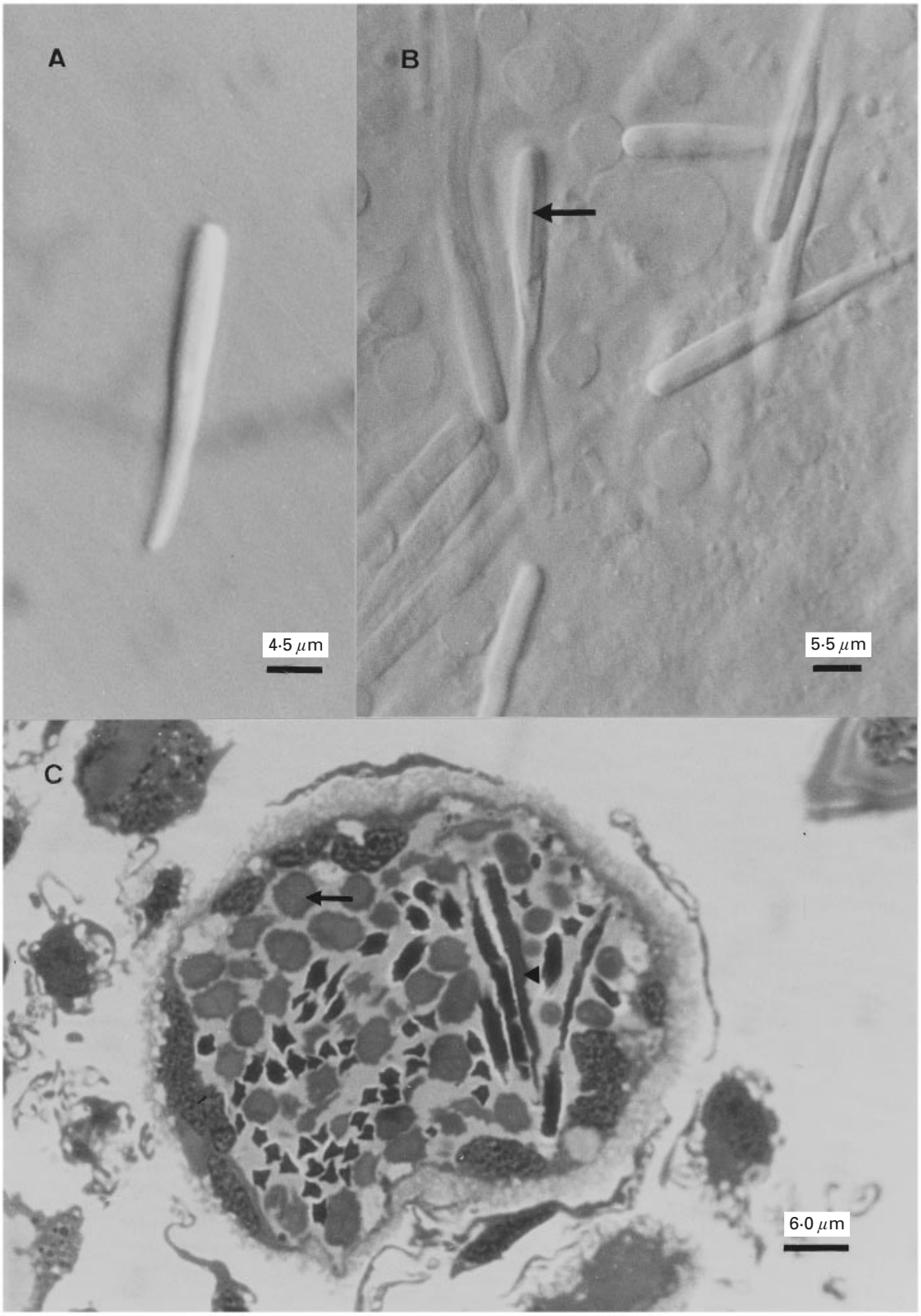

Fig. 1. Light micrographs of *Bacillidium* sp. (A) Interference micrograph of Karnovsky fixed spore of *Bacillidium* sp. The spore is slightly curved and widest at the anterior end. (B) Interference micrograph of fresh *Bacillidium* sp. spores. The manubrium is visible in some spores and indicated by the arrow. (C) Epon section through an infected coelomyocyte showing different developmental stages of the microsporidium. Arrow shows a diplokaryotic cell and the arrow head a longitudinal section through a spore.

(Fig. 1A, B). The spores occurred frequently as pairs in the xenoma indicating a disporoblastic development. The anterior part of the extrusion apparatus was visible by light microscopy (Fig. 1B) and confirmed by TEM. At the posterior part, 2 coils of the polar tube were observed in some spores. The

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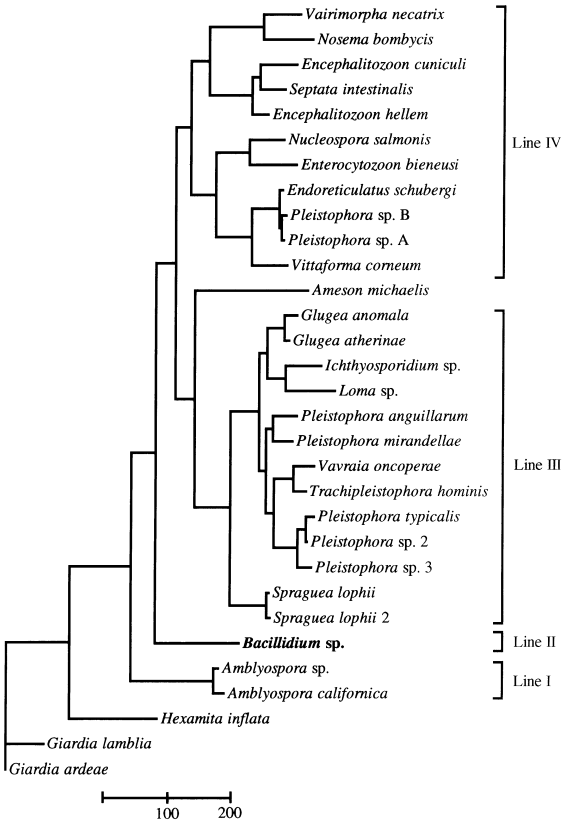

Fig. 2. Tree obtained by maximum likelihood analysis. The branch lengths are proportional to unambiguous changes. The tree is rooted with *Giardia ardea* and hence, no changes on that branch. The bar indicates unambiguous changes along the branches.

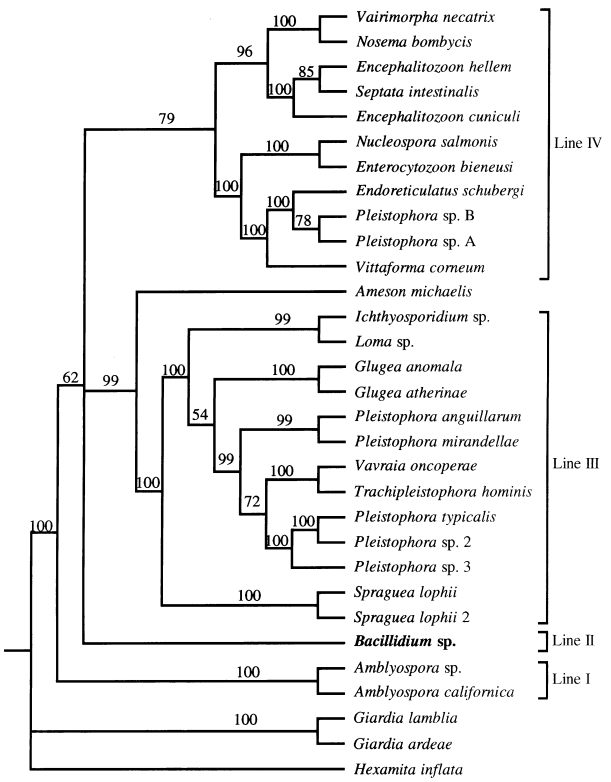

Fig. 3. Consensus tree obtained with 500 bootstrap replicates using Kimura-2 parameter with neighbour joining. The number on the branches indicates the percentage out of 500 replicates that topology was supported. Branches with less than 50% support are collapsed. *Hexamita inflata*, *Giardia ardea* and *Giardia lamblia* are used as an outgroup.

simplest developmental stages observed were diplokaryotic cells with the nuclei filling up most of the space with a thickened plasma membrane. The cytoplasm contained ribosomes and endoplasmic reticulum. These are probably sporonts. Multinucleated plasmodia or sporophorous vesicles were not observed.

rDNA analysis

The small subunit rDNA fragment amplified from *Bacillidium* sp. was 1386 bp and is the longest sequence thus far known from microsporidia. The primer 1492r anneals about 25 bp downstream the 3′ end of the SSU rRNA gene which makes the coding region to be about 1410 bp. This sequence showed a relatively low degree of similarity with other microsporidia SSU rDNA sequences in the GeneBank. In the alignment, 28 microsporidia and 3 diplomonadid sequences were used and the alignment consisted of 1925 characters. Three different tree building methods have been applied and the overall tree topology was similar. Four major lineages can be recognized and occur in the majority of the trees. Line I consists of the 2 *Amblyospora* species, Line II the *Bacillidium* sp., Line III 14 microsporidia and line IV 11 microsporidia species (see Figs. 2–4). Maximum parsimony using PAUP generated 2 equally parsimonious trees (tree length = 4967, consistency index = 0·521) and the difference between these two trees is due to the position of *Bacillidium* sp. Maximum likelihood and Kimura 2-parameter with neighbour joining both suggest that *Bacillidium* sp. split off after the *Amblyospora* spp. as the second microsporidia lineage (similar to one of the parsimony trees). The other parsimony tree suggests that *Bacillidium* sp. comprised a group together with *Encephalitozoon* spp. *S. intestinalis*, *V. necatrix* and *N. bombycis*. This group was a sister group to the fish line (Line III) and *A. michaelis*. The branch leading to *Bacillidium* sp. is defined by 204 unambiguous changes (calculated using ML) and is 1 of the 3 longest microsporidia branches. The *A. michaelis* branch (235 characters) and the branch leading to *Amblyospora* spp. (215 characters) were the longest. Testing the tree topology by means of bootstrapping (parsimony and Kimura 2-parameter) failed to give strong support (more than 50%) for the position of *Bacillidium* sp. The support for *Bacillidium* sp. being the second microsporidia branch was only 36·4% using Kimura 2-parameter. The analysis suggests that *Amblyospora* spp. is the first branch on the microsporidia tree, supported in 61·8 and 70% of the bootstrap samples by Kimura 2-parameter and parsimony, respectively. The group consisting of *A.*

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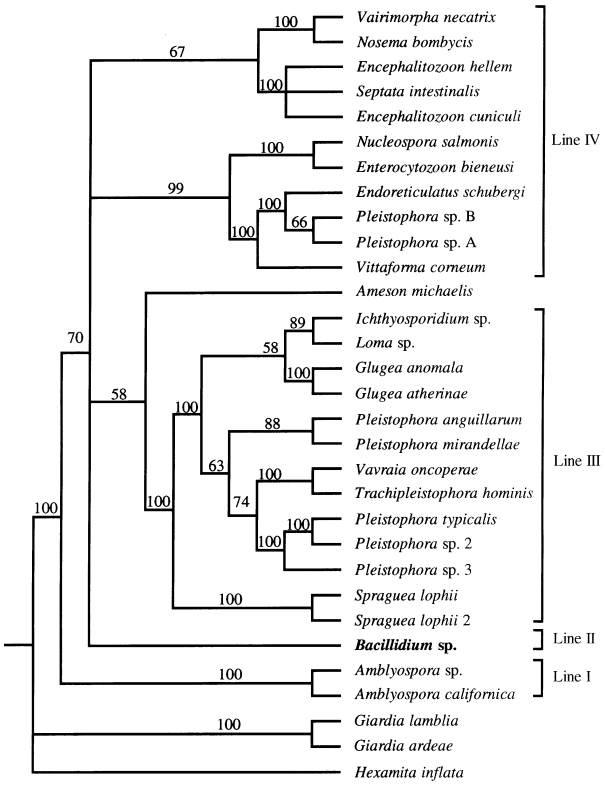

Fig. 4. Consensus tree obtained after 500 bootstrap replicates using maximum parsimony in PAUP. The number on the branches shows the percentage out of 500 replicates that topology was supported. Branches with less than 50 % support are collapsed. The analysis was run with *Hexamita inflata*, *Giardia ardea* and *Giardia lamblia* as an outgroup.

michaelis and the fish microsporidia (Line III) was supported in 99·8 % of the bootstrap replicates using Kimura 2-parameter and 58 % using parsimony (Figs. 3, 4 respectively). Line IV was supported in 78·6 % of the bootstrap samples using Kimura 2-parameter but failed to give a support over 50 % using parsimony. This is probably related to the uncertain position of the *Bacillidium* sp. sequence.

DISCUSSION

Of the 5 genera currently recognized in the family Mrazekiidae, the genera *Jirovecia* and *Bacillidium* have large and slender spores (e.g. Larsson, 1989, 1990, 1994; Larsson & Götz, 1996). *Jirovecia* spores have a posterior tail-like projection whereas *Bacillidium* spores are rounded (Larsson, 1994; Larsson & Götz, 1996). Even if the samples for TEM in the present study were of a poor quality, the present species most likely belongs to the genus *Bacillidium*. The key features were diplokaryon in all observed developmental stages, a long slender spore without projection, no sporophorous vesicles, disporoblastic development and a manubroid polar tube. All these characters are features of the genus *Bacillidium*. Furthermore, the host, an aquatic oligochaete, is the common host group for this microsporidium genus. The samples obtained for TEM were not of a quality that permits comparison with other *Bacillidium* spp.

This is the first report of SSU rDNA sequence analysis of a microsporidium from an annelid host. Previous studies have mainly been dealing with microsporidia infecting insects, humans, and fish (Baker *et al*. 1994, 1995, 1997; Nilsen, Endresen & Hordvik, 1998). However, we still lack genetic information about microsporidia from many of the host groups.

Bootstrap re-sampling of the present data set failed to give strong support for the phylogenetic position of *Bacillidium* sp. Nevertheless, in 3 out of the 4 trees we obtained in the analysis, using different tree building methods, the position of *Bacillidium* sp. was similar and hence, probably the most likely tree topology. Long branch lengths usually makes phylogenetic analysis less reliable and the different tree building methods show a variable degree of efficiency to resolve such phylogenies. Simulation studies have shown that ML is the most reliable method to handle these data sets (Nei, 1996).

The basal split off and long branch leading to *Bacillidium* sp. suggest that this group has been isolated for a long time. This could explain the unique morphology this group of microsporidia possesses. They have probably evolved together with their host group for a long period. Three of the branches in the present microsporidium phylogeny are very long (the *Bacillidium* branch, the *A. michaelis* branch and the branch leading to *Amplyospora* spp.). The *Amblyospora* spp. have been considered to be the most advanced of the microsporidia, having a life-cycle consisting of 3 different spore-types and the need for an intermediate host (e.g. Becnel, 1994). Baker *et al*. (1997) showed the basal split off for the *Amblyospora* spp. and discussed this in relation to other insect-infecting microsporidia and their developmental cycles and suggested that the evolution has been towards a simpler life-cycle with fewer stages. If we assume that *Bacillidium* sp. has split off from the microsporidium tree just after the *Amblyospora* spp. this may not be correct. The life-cycle as we know it in *Bacillidium* (and other similar microsporidia) is simple compared to the *Amblyospora* spp., with no intermediate host and all the developmental stages occurring in the same cell type and division by binary fission. The long branch leading to *Amblyospora* spp. suggests that this lineage has existed for a long time, but not necessarily in the form we know today. The complex life-cycle and development of the *Amblyospora* spp. can be an adaptation to the host and environment and an advanced state of an old microsporidium lineage in which the ancestor had a simpler life-cycle.

Sprague, Becnel & Hazard (1992) placed *Bacillidium* and 4 other genera (*Hrabeya*, *Jirovecia*,

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Mrazekia and *Rectispora*) in the order Dissociodihaplophasida (class Dihaplophasea), in the superfamily Nosematoidea. This superfamily contains 5 families and 3 of these are included in the present study; *Bacillidium* (Mrazekiidae), *N. bombycis* (Nosematidae) and *Ichthyosporidium* sp. (Ichthyosporidiidae). There is no evidence from the phylogenetic analysis to suggest that any pair from these 3 families comprises a monophylectic taxon or that they are in any way related. Hence, the classification by Sprague *et al*. (1992) should be revised. Weiser (1977) reported that the order Nosematidida contained 2 families, Nosematida and Mrazekiidae. However, there is no molecular support for this classification. Larsson (1986) put *Mrazekia* in a taxon together with *Hirsutosporis* and *Nosema* and with *Ichthyosporidium* as a sister group. Even if *Bacillidium* was not considered in the phylogeny proposed by Larsson (1986), *Mrazekia* and *Bacillidium* are currently in the same family (Mrazekiidae). The present molecular data do not support this classification, as this will not make a monophyletic taxon.

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