# Identification of a New Microsporidian Parasite Related to *Vittaforma corneae* in HIV-Positive and HIV-Negative Patients from Portugal

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ABSTRACT. Fecal samples from 22 HIV-positive and 3 HIV-negative patients from Portugal with symptomatic diarrhea were diagnosed positive for microsporidia by microscopy, with most parasites detected significantly bigger than *Enterocytozoon bieneusi* and *Encephalitozoon* spp. Sequence characterization of the small subunit (SSU) rRNA gene identified a microsporidian parasite with 96% homology to two published *Vittaforma corneae* sequences. Phylogenetic analysis confirmed the genetic relatedness of this new microsporidian parasite to *Vittaforma corneae* as well as *Cystosporogenes operophterae*. Results of the study demonstrate the presence of a new human-pathogenic microsporidian species, which is responsible for significant number of infections in HIV-positive and HIV-negative patients in Portugal.

Microsporidia were first recognized as a pathogen in silkworm a century ago. In 1959, the first case of human microsporidiosis was reported in Japan [5]. The importance of microsporidia has increased once it emerged as an opportunistic pathogen causing diarrheal disease in the recent AIDS epidemic in humans. In 1985, a new species of microsporidia (*Enterocytozoon bieneusi*) was discovered in a HIV-infected patient in France [2], and subsequent studies have recognized it to be the most prevalent human-pathaogenic microsporidia. Thus far, only 14 species in 7 genera (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *E. hellem*, *E. cuniculi*, *Brachiola vesicularum*, *B. (Nosema) algerae*, *Nosema ocularum*, *N. connori*, *Pleistophora africanum*, *M. ceylonensis*, and *Vittaforma corneae*) of microsporidia have been described as pathogens in humans [4]. In this study we have genetically identified a new microsporidia in HIV-positive and HIV-negative patients with diarrhea in Portugal.

## MATERIALS AND METHODS

**Parasite isolates.** Fecal samples containing microsporidia were obtained from HIV-positive (14 adults; 8 children) and HIV-negative patients (3 children) from Lisbon, Portugal (Table 1). These samples were found to have microsporidia spores that were larger than

Table 1. Sources of *Vittaforma*-like isolates from HIV-positive and HIV-negative patients in Portugal.

| Patient no. | Patient ID, code | HIV status | Parasite burden* | Age (yrs) | Diarrhea, CD4$^+$ (cell/mm$^3$)** | Mode of HIV acquisition** | SSU rRNA sequencing |
|---|---|---|---|---|---|---|---|
| 1 | F480, 5797 | HIV$^+$, Adult | +++ | 41 | Yes, 160 | Drug addict$^+$; homosexual | *Vittaforma-like* |
| 2 | F486, 5799 | HIV$^+$, Adult | ++ | 32 | Yes, — | — | *Vittaforma-like* |
| 3 | F492, 5801 | HIV$^+$, Adult | ++ | 30 | Yes, 134 | Drug addict | *Vittaforma-like* |
| 4 | F501, 5802 | HIV$^+$, Adult | + | 48 | Yes, — | — | *Vittaforma-like* |
| 5 | F518, 5806 | HIV$^+$, Adult | + | 21 | Yes, 262 | Drug addict | *Vittaforma-like* |
| 6 | F522, 5809 | HIV$^+$, Adult | + | 40 | Yes, — | — | *Vittaforma-like* |
| 7 | F548, 5811 | HIV$^+$, Adult | + | 53 | Yes, — | — | *Vittaforma-like* |
| 8 | F606, 5820 | HIV$^+$, Adult | + | 33 | Yes, 80 | — | *Vittaforma-like* |
| 9 | F622, 5823 | HIV$^+$, Adult | + | 40 | Yes, — | — | *Vittaforma-like* |
| 10 | F665, 5824 | HIV$^+$, Adult | +++ | 35 | Yes, 216 | Drug addict | *Vittaforma-like* |
| 11 | F816, 5833 | HIV$^+$, Adult | ++ | 43 | Yes, 138 | Homosexual | *Vittaforma-like* |
| 12 | F874, 5840 | HIV$^+$, Adult | + | 39 | Yes, — | Drug addict | NA |
| 13 | F970, 5842 | HIV$^+$, Adult | +++ | 27 | Yes, 216 | Drug addict | *Vittaforma-like* |
| 14 | F603, 5819 | HIV$^+$, Adult | + | — | —, — | — | *Vittaforma-like* |
| 15 | F509, 5805 | HIV$^+$, Child | + | 7 | Yes, 183 | — | *Vittaforma-like* |
| 16 | F530, 5810 | HIV$^+$, Child | ++ | 4 | Yes, 500 | Vertical | *Vittaforma-like* |
| 17 | F583, 5813 | HIV$^+$, Child | + | 6 | —, 503 | Vertical | *Vittaforma-like* |
| 18 | F587, 5814 | HIV$^+$, Child | ++ | 6 | —, 114 | Vertical | *Vittaforma-like* |
| 19 | F669, 5827 | HIV$^+$, Child | ++ | 5 | Yes, 265 | Vertical | NA |
| 20 | F671, 5828 | HIV$^+$, Child | ++ | 1.8 | Yes, 500 | Vertical | *Vittaforma-like* |
| 21 | F828, 5835 | HIV$^+$, Child | + | 1 | Yes, 373 | Vertical | *Vittaforma-like* |
| 22 | F1067, 6728 | HIV$^+$, Child | + | 11 | No, 50 | Vertical | *Vittaforma-like* |
| 23 | F469, 5796 | HIV−, Child | +++ | 3 | Yes, — | — | *Vittaforma-like* |
| 24 | F504, 5803 | HIV−, Child | +++ | 1.2 | Yes, — | — | *Vittaforma*-like |
| 25 | F521, 5808 | HIV−, Child | + | 5 | No, — | — | *Vittaforma*-like |

* +: low; ++: moderate; +++: high. ** —: unknown.
*Trachipleistophora hominis*, *T. anthropophthera*, *Microsporidium*

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Table 2. Restriction banding patterns of four common human-pathogenic microsporidia species in a SSU rRNA-base PCR-RFLP tool.

| Species | PCR fragment (bp) | *Bcg* I digestion |
|---|---|---|
| *Encephalitozoon hellem* | **632** | **632** |
| *Encephalitozoon cuniculi* | **628** | 56, 34, **204**, 34, **300** |
| *Encephalitozoon intestinalis* | **621** | **288**, 34, **299** |
| *Enterocytozoon bieneusi* | **630** | **204**, 34, **102**, 34, **256** |

*Enterocytozoon bieneusi* and *Encephalitozoon* spp. by microscopic examination of fecal smears stained by a modified trichrome blue method. DNA was extracted from fecal samples collected at two different time points from each patient after initial treatment with 1M KOH at 65°C for 15 min followed by neutralization with 25% HCl. The DNA lysate was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), and purified using QIAamp DNA Stool Kit (QIAGEN Inc, Valencia, CA) following the manufacturer's protocol.

**Small subunit (SSU) rRNA-based PCR-RFLP.** Molecular characterization was carried out initially by PCR-RFLP analysis of the SSU rRNA gene. This diagnostic protocol generates unique patterns for all the three species in the genus *Encephalitozoon* (*E. cuniculi*, *E. hellem*, *E. intestinalis*) and *Enterocytozoon bieneusi* (Table 2). For the primary PCR, a PCR product of ~640 bp was amplified using primers AL3334 [5′-TTCCGGAGAGGGAGCCTGAG-3′] and AL3336 [5′-AGGGCAT(A/C)ACGGACCTGTT-3′]. The PCR reaction consisted of 1.0 μl of DNA, 200 μM each dNTP, 1× PCR buffer (Perkin Elmer, Foster City, CA), 3.0 mM $MgCl_2$, 5.0 U of *Taq* polymerase (GIBCO BRL, Frederick, MD), and 200 nM of each primer in a total of 100 μl reaction. The reactions were carried out in a Perkin Elmer GeneAmp PCR 9700 for 35 cycles (94°C for 45 sec, 58°C for 45 sec, and 72°C for 60 sec), with an initial hot start (94°C for 5 min) and a final extension (72°C for 10 min). For the secondary PCR, a fragment of ~630 bp was amplified using 2.5 μl of the primary PCR reaction and primers AL3335 [5′-GGCAGCAGGCGCGAAACTTG-3′] and AL3337 [5′-ATTTC(A/C)AACGGCCATGCACCAC-3′]. The conditions for the secondary PCR were identical to the primary PCR except that the annealing temperature used was 62°C. For restriction fragment analysis, 20 μl of the secondary PCR products were digested in a 50 μl reaction mixture containing 20 U of *Bcg* I (New England BioLabs, Beverly, MA) and 5 μl of reaction buffer at 37°C for 1 h. The digested products were fractioned on a 1.5% agarose gel and visualized by ethidium bromide staining (Fig. 1).

The full length SSU rRNA gene (~1000 bp) was obtained using primers AL3107 [5′-CACCAGGTTGATTCTGCCTGA-3′] and AL3109 [5′-CCAACTGAAACCTTGTTACGACTT-3′]. The condition of PCR reaction was similar to diagnostic primary PCR except that the reactions were performed for 45 cycles with annealing temperature of 58°C.

**Nucleotide sequencing and phylogenetic analysis.** The diagnostic secondary and the near full-length SSU rRNA PCR products with anticipated size were purified using Microcon PCR Centrifugal Filter Devices (MILLIPORE, Bedford, MA), and sequenced in both directions using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit on an ABI 3100 automated sequencer (Perkin Elmer). Multiple alignments of the nucleotide sequences from this study and GenBank database were carried out using Wisconsin Package Version 9.0 program (Genetics Computer Group, Madison, WI) (Fig. 2). Phylogenetic analysis was performed on the SSU rRNA sequences obtained to identify the genetic relatedness of microsporidia identified in this study with other published sequences from microsporidia of human and animals (Fig. 3). In this analysis, a neighbor-joining tree was constructed using the TreeconW program (http://rrna.uia.ac.be/dcse/help/treecon.html) based on the evolutionary distances calculated by the Kimura-2-parameter model. The SSU rRNA nucleotide sequences generated from this study were deposited in GenBank database under accession numbers AY375043 and AY375044.

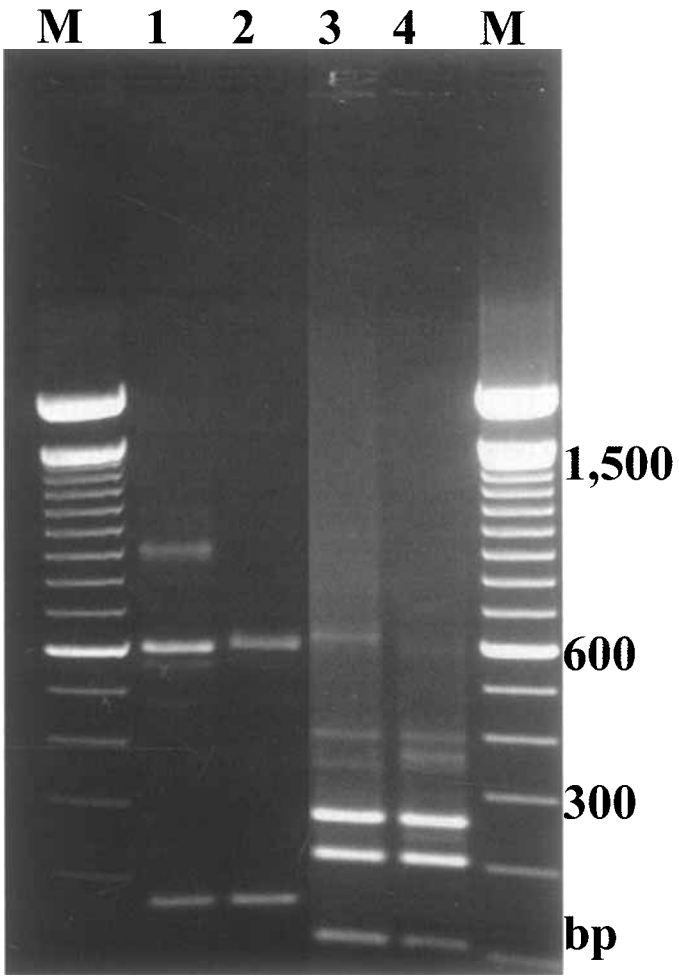


Fig. 1. Difference in the *Bcg* I RFLP patterns of SSU rRNA PCR products between *Vittaforma*-like parasite and *Enterocytozoon bieneusi* (M, 100-bp ladder; Lanes 1–2, *Vittaforma*-like; Lanes 3–4, *Enterocytozoon bieneusi*).

## RESULTS AND DISCUSSION

Fecal samples from 22 HIV-positive and 3 HIV-negative patients in Portugal with diarrhea were diagnosed positive for microsporidia by microscopy using a modified trichrome blue stain, with most parasites detected significantly bigger (1.8–3.6 μm × 1.0–1.4 μm) than *Enterocytozoon bieneusi* and *Encephalitozoon* spp. Of the 25 fecal specimens, 23 samples resulted in PCR product bands with the expected size (~620 bp), but they were not digested with *Bcg* I restriction enzyme using the SSU rRNA-based PCR-RFLP diagnostic protocol (Fig. 1). The SSU rRNA secondary PCR products were sequenced.

Multiple alignments revealed minor variations (one-point-mutation, Fig. 2) within the 72 SSU rRNA sequences obtained from these samples (each sample was amplified and sequenced at least 3 times).

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5830    TGGCAGCAGGCGCGAAACTTGCCCACTCTTTGCAGGAGGCAGTTATGAGACGTGAGAATGAATATTCAGTAAAGATGAATA
5843    ...............................................................................
L39112  C..............A.....................................AG....G...CTT.......G.G...
U11046  C..............A.....................................AG....G...CTT.......G.G...

5830    GGAGAATTGGAGGGCAAGTTTGGTGCCAGCCGCCGCGGTAATACCGACTCCAAGAGTGTGTATGAGAGATGCTGCAGTTAA
5843    ...............................................................................
L39112  ............................A..................................................
U11046  ............................A..................................................

5830    AAAGTCCGTAGTCGTAGGAAACGAAACAGGGATGTGAAGTCTCGACTGTGAGTTTTAGAGAAACCGATGGGGAACATAGTA
5843    ..........................................T....................................
L39112  ............A...A.GGGC...G..A....C..G.....ACAGTGCGA.GA.G..GG.G.................
U11046  ............A...A.GGGC...G..A....C..G.....ACAGTGCGA.GA.G..GG.G.................

5830    TACCAGGGCGAGAGATGAAATGCCAAGACCCCTGGTGGACTGAGCGAGGCGAAAGCGATGTTCTTGTAGGTATTTGGTGAT
5843    ...............................................................................
L39112  ..................................................G..............C...C......
U11046  ..................................................G..............C...C......

5830    CAAGGACGAAGGCTGGAGTATCGAAAGTGATTAGATACCGCAGTAGTTCCAGCAGTAAAAGATGCCGACATGTCTATTGGG
5843    ...............................................................................
L39112  ..........................C.....................................................CTC.....A
U11046  ..........................G.....................................................CTC.....A

5830    AACAGTGGACAGGGAGAAATCTTAGAGTTCGGGCTCTGGGGATAGTATGCTCGCAAGAGTGAAAATTAAAGAAATTGACGG
5843    ...............................................................................
L39112  C.......G.....................................................G..................
U11046  C.......G.....................................................G..................

5830    AGCTACACCACAAGGAGTGGATTGTGCGGCTTAATTTGACTCAACGCGAGGAAACTTACCAGGGCCAAGT-GTTGTGTAGA
5843    ...............................................................................-G.....
L39112  ..............................................................................AC.....
U11046  ..............................................................................A-.....

5830    AACGAGCAAGACAGAAGTGGTGCATGGCCGT
5843    ................................
L39112  .........T....G............T...
U11046  .........T....G............T...
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Fig. 2. Sequence differences in the SSU rRNA gene of *Vittaforma*-like parasites (isolate 5830 and 5843) and *Vittaforma corneae* (GenBank Accession No. L39112 and U11046).

BLASTn searches of the GenBank database with these SSU rRNA sequences identified a new microsporidian parasite with 96% homology to the two published *Vittaforma corneae* SSU rRNA sequences (GenBank Accession No. L39112 and U11046). However, 50 point-mutations were present between the published *V. corneae* (GenBank Accession No. L39112 and U11046) and the newly derived *Vittaforma*-like nucleotide sequences (present study, Fig. 2). In contrast, the two published *V. corneae* sequences displayed a 99% homology with each other with only one point mutation (GenBank Accession No U11046 and L39112).

*Vittaforma corneae* was initially found in a 45-year-old otherwise healthy man with an 18-month history of unilateral progressive central keratitis [1]. This parasite was originally assigned to the genus *Nosema*, with the name *Nosema corneum* [6]. Based on the ultra structure and the SSU rRNA nucleotide sequence data, it was transferred to a new genus and named *Vittaforma corneae* [7,8]. *Vittaforma corneae* has also been reported in a patient in Switzerland (Weber, R., Mathis, A., Zimmerli, S. & Deplazes, P. 1997. Epidemiology and clinical manifestations of HIV-associated microsporidiosis. Second workshop on microsporidiosis and crytosporidiosis in immunodeficient patient) and a water sample in the United States [3]. Results of this study indicate that other parasites related to *Vittaforma corneae* such as the one identified in Portugal patients, can also be human-pathogens.

SSU rRNA sequences have been widely used in understanding the evolutionary and taxonomic relationships of most organisms. In this study, the SSU rRNA-based phylogenetic analysis indicates that this new microsporidia (*Vittaforma*-like) is genetically related to *V. corneae* and *Cystosporogenes operophterae*, a microsporidian parasites of moths. Even though the *Vittaforma*-like parasite clustered together with *V. corneae* and *Cystosporogenes operophterae*, its taxonomic placement await results of morphological and developmental studies.

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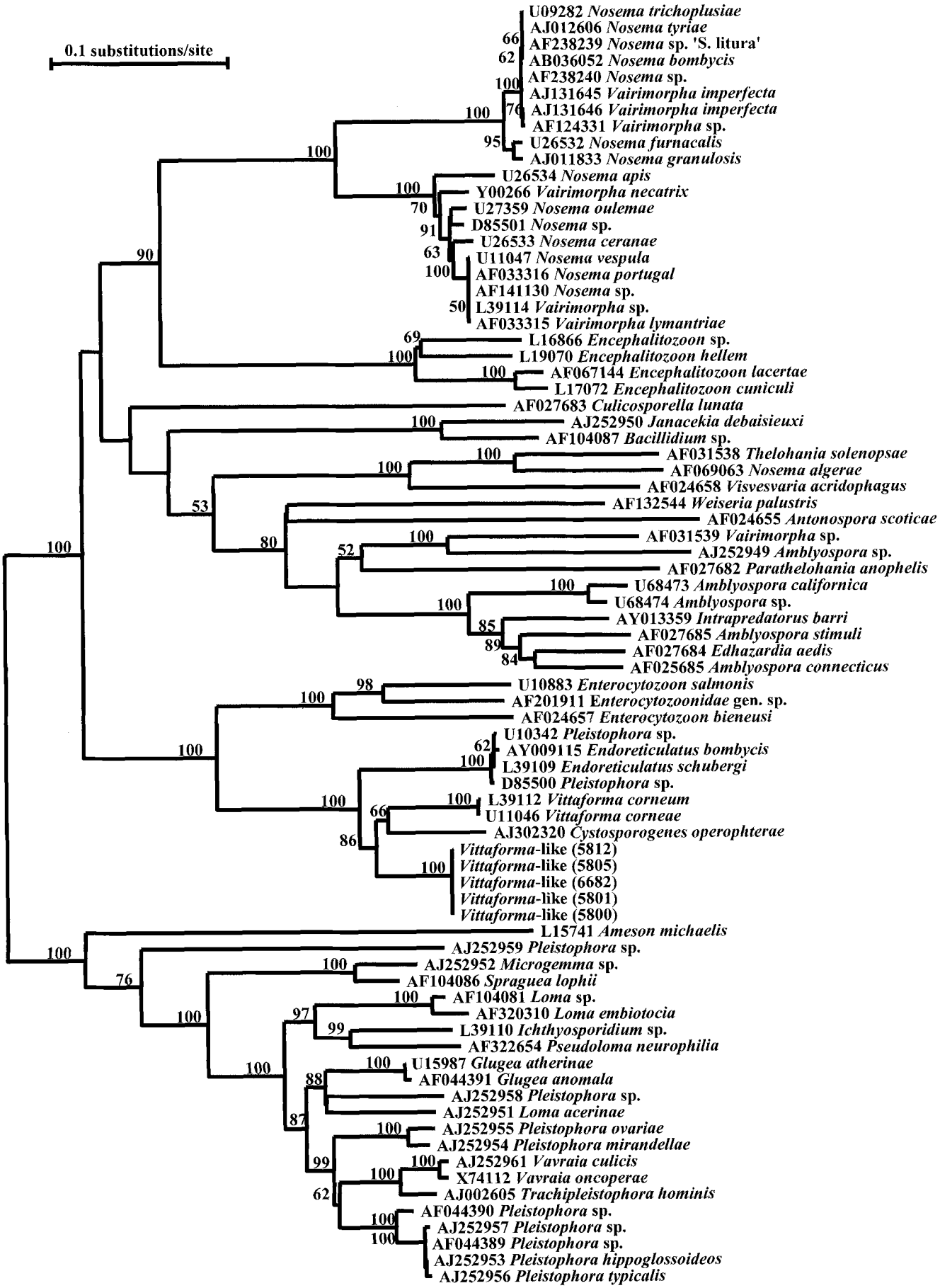
Fig. 3. Phylogenetic relationships of the *Vittaforma*-like parasite to other microsporidia inferred by a neighbor-joining analysis of the SSU rRNA gene, using genetic distance calculated by the Kimura-2-parameter model. Numbers on branches are bootstrap values using 1,000 replicates.


0.1 substitutions/site
U09282 Nosema trichoplusiae
AJ012606 Nosema tyriae
AF238239 Nosema sp. 'S. litura'
AB036052 Nosema bombycis
AF238240 Nosema sp.
AJ131645 Vairimorpha imperfecta
AJ131646 Vairimorpha imperfecta
AF124331 Vairimorpha sp.
U26532 Nosema furnacalis
AJ011833 Nosema granulosis
U26534 Nosema apis
Y00266 Vairimorpha necatrix
U27359 Nosema oulemae
D85501 Nosema sp.
U26533 Nosema ceranae
U11047 Nosema vespula
AF033316 Nosema portugal
AF141130 Nosema sp.
L39114 Vairimorpha sp.
AF033315 Vairimorpha lymantriae
L16866 Encephalitozoon sp.
L19070 Encephalitozoon hellem
AF067144 Encephalitozoon lacertae
L17072 Encephalitozoon cuniculi
AF027683 Culicosporella lunata
AJ252950 Janacekia debaisieuxi
AF104087 Bacillidium sp.
AF031538 Thelohania solenopsae
AF069063 Nosema algerae
AF024658 Visvesvaria acridophagus
AF132544 Weiseria palustris
AF024655 Antonospora scoticae
AF031539 Vairimorpha sp.
AJ252949 Amblyospora sp.
AF027682 Parathelohania anophelis
U68473 Amblyospora californica
U68474 Amblyospora sp.
AY013359 Intrapredatorus barri
AF027685 Amblyospora stimuli
AF027684 Edhazardia aedis
AF025685 Amblyospora connecticus
U10883 Enterocytozoon salmonis
AF201911 Enterocytozoonidae gen. sp.
AF024657 Enterocytozoon bieneusi
U10342 Pleistophora sp.
AY009115 Endoreticulatus bombycis
L39109 Endoreticulatus schubergi
D85500 Pleistophora sp.
L39112 Vittaforma corneum
U11046 Vittaforma corneae
AJ302320 Cystosporogenes operophterae
Vittaforma-like (5812)
Vittaforma-like (5805)
Vittaforma-like (6682)
Vittaforma-like (5801)
Vittaforma-like (5800)
L15741 Ameson michaelis
AJ252959 Pleistophora sp.
AJ252952 Microgemma sp.
AF104086 Spraguea lophii
AF104081 Loma sp.
AF320310 Loma embiotocia
L39110 Ichthyosporidium sp.
AF322654 Pseudoloma neurophilia
U15987 Glugea atherinae
AF044391 Glugea anomala
AJ252958 Pleistophora sp.
AJ252951 Loma acerinae
AJ252955 Pleistophora ovariae
AJ252954 Pleistophora mirandellae
AJ252961 Vavraia culicis
X74112 Vavraia oncoperae
AJ002605 Trachipleistophora hominis
AF044390 Pleistophora sp.
AJ252957 Pleistophora sp.
AF044389 Pleistophora sp.
AJ252953 Pleistophora hippoglossoideos
AJ252956 Pleistophora typicalis

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