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Molecular Analysis of the Alpha-Tubulin Gene from the Microsporidium, *Endoreticulatus* sp. Zhenjiang, Isolated from *Bombyx mori*

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Abstract

We isolated a microsporidium from the silkworm, *Bombyx mori*, and classified it as *Endoreticulatus* sp. Zhenjiang based on morphological characteristics and phylogenetic analyses of ribosomal sequences. This microsporidium causes silkworm pebrine, although its original host and mode of transmission are unknown. To better understand its distribution and transmission mode, it is essential to have species specific molecular markers. Towards this goal, we characterized the alpha tubulin gene from *Endoreticulatus* sp. Zhenjiang in this study. The full-length alpha-tubulin cDNA from *Endoreticulatus* sp. Zhenjiang was cloned and sequenced (GenBank ID: KJ784483) using the rapid amplification of cDNA ends (RACE) protocol. The alpha-tubulin cDNA is 1382 bp long with an open reading frame spanning 1320 bp and consisting of a short 20-bp 5'-untranslated region (5'-UTR) and a 42-bp 3'-UTR with a stop codon and a poly (A) tail. This alpha-tubulin cDNA encodes a deduced polypeptide with 439 amino acids, including a complete tubulin domain and a tubulin C domain. This protein has an estimated isoelectric point of 5.1 and a predicted molecular weight of 48.6 kDa.

Keywords

Microsporidium, RACE, Tubulin Gene, Silkworm, Pebrine

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1. Introduction

Microsporidia are unicellular eukaryotes and obligate intracellular parasites with the ability to infect a broad spectrum of hosts from protists to mammals, including humans and have worldwide distribution [1] [2]. All microsporidia identified to date belong to 160 genera and 1300 described species [3] [4]. *Nosema bombycis* is the first described microsporidium, which prevailed in Europe, America, and Asia during the mid-19th century. It is recognized as the pathogen of silkworm pebrine and still causes heavy economic losses in silk-producing countries such as China [5]. Besides *Nosema*, several microsporidian genera, such as *Pleistophora*, *Thelohania*, *Vairimorpha*, and *Endoreticulatus* have been found to infect silkworm. The genus *Endoreticulatus* clearly differs from *N. bombycis* in spore morphology and size, ultrastructure, and life cycle [6]. We have identified an *Endoreticulatus* microsporidium, which was isolated from silkworm in Zhenjiang City, Jiangsu Province, China. The fresh spores were oval, 2.9 ± 0.2 µm in length and 1.2 ± 0.2 µm in width. The organization of the rRNA gene was 5'-LSU-ITS-SSU-IGS-5S-3', which is reverse compared to the organization of most microsporidian rRNA regions. This is the first *Endoreticulatus* microsporidium with a reversed rRNA gene arrangement [7].

It is known that tubulin genes could offer useful markers to study phylogenesis [8] [9], because the tubulin proteins are structural proteins with relatively conserved amino acid sequence between species. The tubulin gene family consists of six distinct, but highly conserved subfamilies that possess alpha-, beta-, gamma-, delta-, epsilon-, and zeta-tubulins, each with specific conserved sequences and are widely distributed among eukaryotes [9] [10]. Heterodimers of alpha- and beta-tubulin proteins are the major components of microtubules, which are the central components of eukaryotic cilia, flagella, mitotic spindles and the cytoskeleton. Among the tubulin subfamilies, microsporidia have only alpha-, beta-, and gamma-tubulin genes. To understand the alpha-tubulin genes of microsporidia, we cloned and analyzed the complete cDNA sequence of the alpha-tubulin gene from *Endoreticulatus* sp. Zhenjiang. Molecular analysis revealed that the alpha-tubulin gene of *Endoreticulatus* sp. Zhenjiang had a shorter 5'-untranslated region (5'-UTR). In phylogenetic analysis, *Endoreticulatus* sp. Zhenjiang and *Endoreticulatus* sp. CHW-2004 Taiwan formed a separate clade supported by high bootstrap values. Based on the cDNA sequence, we designed specific primers to differentiate *Endoreticulatus* sp. Zhenjiang from other microsporidia.

2. Materials and Methods

2.1. Materials and Reagents

The microsporidia, *Endoreticulatus* sp. Zhenjiang, *Nosema bombycis*, *Nosema philosamiae*, *Nosema* sp. HA, *Nosema* sp. PA, *Nosema antheraeae* and silkworm p50 strain of *Bombyx mori* were maintained in the Sericulture Research Institute at the Chinese Academy of Agricultural Sciences.

SMART™ RACE cDNA Amplification Kit and Advantage 2 PCR Kit were purchased from Clontech Laboratories, Inc., Japan. RNAiso Plus, DNase I, rTaq enzyme, ExTaq enzyme, pMD18-T vectors, T4 DNA ligase and DNA size markers were purchased from Takara (Japan). All PCR primers were synthesized and DNA sequencing were performed by Shanghai Sangong Biological Engineering Technology & Services Co., Ltd. at shanghai in China. All other chemicals used were analytical grade.

2.2. Total RNA Isolation and DNA Extraction

First, the fourth instar *B. mori* larvae were allowed to feed on mulberry leaves artificially infected with *Endoreticulatus* sp. Zhenjiang spores (10^7 spores/larvae). Then, microsporidial spores of *Endoreticulatus* sp. Zhenjiang were isolated from infected silkworm for morphological analyses and maintained in the Sericulture Research Institute at the Chinese Academy of Agricultural Sciences.

Total RNA was isolated from the midguts of silkworm infected with microsporidia using RNAiso Plus. The total RNA was to deal with DNase I, insuring the absence of contaminating DNA. Quality of the total RNA was determined by 260/280 absorbance ratio as well as by running an aliquot on an agarose gel. The RNA were then stored at −80˚C until further use. Genomic DNA was extracted from the microsporidia as described by Dong *et al*. [11].

2.3. Cloning the Alpha-Tubulin cDNA from *Endoreticulatus* sp. Zhenjiang

The full-length alpha-tubulin cDNA sequence of *Endoreticulatus* sp. Zhenjiang was obtained using the SMART™

RACE cDNA Amplification Kit (Clontech). Gene specific primers for the 5'-RACE and 3'-RACE were designed based on the conserved regions in the known microsporidia alpha-tubulin genes in the National Center for Biotechnology Information (NCBI) database.

To amplify the 5'-end of the alpha-tubulin cDNA from *Endoreticulatus* sp. Zhenjiang, the following primers were used: Universal Primer Mix, UPM:
5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and GSP1:
5'-GAGAATATGCCACAAGGGGAAAGTG-3'. The 3'-end of the *Endoreticulatus* sp. Zhenjiang alpha-tubulin cDNA was amplified using the primers GSP2: 5'-CGAGATAAGAAGGATTGCTGAGAACTGC-3'- and UPM: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'. The amplified products were sequenced and subsequently, the full-length cDNA was obtained by overlapping the two sequences.

2.4. Analysis of Sequence Data

Alpha-tubulin gene sequences from *Endoreticulatus* sp. Zhenjiang were analyzed by BLASTn in the microsporidian genome database (Microsporidia DB: http://microsporidiadb.org/micro/) [12]. The nucleotide sequences obtained in the present study and those for microsporidian species compared, including those for 15 alpha-tubulin genes were aligned with the Clustal X 1.83 software [13]. Phylogenetic trees were constructed with nucleotide sequences of the protein-coding regions using MEGA5 [14] [15]. The evolutionary history was inferred from the maximum likelihood (ML) and neighbor joining (NJ) methods [9]. One thousand bootstrap replications were performed to test the robustness of the estimated phylogenetic trees [7].

2.5. Species Specific Amplification

Based on the sequence alignment, species specific primers were designed from the *Endoreticulatus* sp. Zhenjiang alpha-tubulin gene sequence region that varied from the other microsporidia. These primer sequences are: Spe-Forward—GGAGCCAGGAGTGATCAACGAGG and Spe-Reverse—AAGAACAGTCGGCCTCTTCTCG. PCR amplifications were performed using the following parameters: denaturation at 95˚C for 5 min; followed by 35 cycles of 95˚C for 30 s, 55˚C for 30 s, 72˚C for 1 min and 30 s, and then a final extension at 72˚C for 10 min [16]. The following genomic DNA were used as templates in the PCR amplifications: *Endoreticulatus* sp. Zhenjiang, *N. bombycis*, *N. philosamiae*, *Nosema* sp. HA, *Nosema* sp. PA, *N. antheraeae*. Ten samples of each microsporidan species were tested to insure the reliability of the experimental results. PCR products were analyzed on 1.0% agarose gels.

3. Results

3.1. cDNA Cloning and Sequence Analysis

The full length alpha-tubulin gene sequence was obtained using the RACE protocol, and deposited in GenBank (KJ784483). The sequence is 1382 bp long with a 20-bp 5'-UTR, and a 42-bp 3'-UTR. The 3'-UTR has a stop codon and a poly (A) tail, but there is no predicted polyadenylation signal sequence (AATAAA, **Figure 1(A)**). The open reading frame (ORF) of the *Endoreticulatus* sp. Zhenjiang alpha-tubulin cDNA was 1320 bp with a 52.1% GC content. The putative polypeptide has 439 amino acids with a calculated molecular weight of about 48.6 kDa, an isoelectric point of 5.1 and without signal peptide. A complete tubulin domain (46 - 242) and a complete tubulin C domain (244 - 389) (**Figure 1(B)**) were detected using the SMART analysis software (http://smart.embl-heidelberg.de/). BLAST homology analysis and multiple sequence alignments showed that *Endoreticulatus* sp. Zhenjiang alpha-tubulin gene sequence shares 99.4% nucleotide similarity and 99.5% amino acid sequence similarity with *Endoreticulatus* sp. CHW-2004 Taiwan (**Table 2**).

3.2. Phylogenetic Trees Constructed from Alpha-Tubulin Gene Sequence

The ML and NJ phylogenies inferred from alpha-tubulin sequence alignments are based on the nucleotide alignment of 15 microsporidian sequences (**Table 1**). The ML and NJ methods revealed that in all phylogenetic trees, *Endoreticulatus* sp. Zhenjiang was within the same clade as *Endoreticulatus* sp. CHW-2004 Taiwan with high bootstrap support (100%), while *N. bombycis*, *N. philosamiae*, *N. spodopterae*, *N. plutellae*, and *Nosema* sp. PX1 formed a separate clade. It is evident that *Endoreticulatus* sp. Zhenjiang was distantly related to *Nosema* species (**Figure 2**). This was further supported by genetic distances, *i.e.* the pairwise distance of alpha-tubulin

Table 1. GenBank accession numbers of alpha-tubulin gene from *Endoreticulatus* sp. Zhenjiang and other species used in this study.

Species Name	Alpha Tubulin Accession Numbers
Endoreticulatus sp. Zhenjiang	KJ784483
Endoreticulatus sp. CHW-2004 Taiwan	AY960112
Encephalitozoon cuniculi	NM_001041670
Encephalitozoon hellem	U66908
Encephalitozoon roromaleae	FJ026010
Edhazardia aedis	EU486986
Nosema bombycis	DQ091252
Nosema spodopterae	DQ091251
Nosema plutellae	DQ083402
Nosema philosamiae	GU947652
Nosema sp. PX1	DQ083401
Nosema ceranae BRL01	XM_002995342
Microsporidia sp. AMVB	EU625356
Glugea plecoglossi	AY138804
Trachipleistophora hominis	AY138781

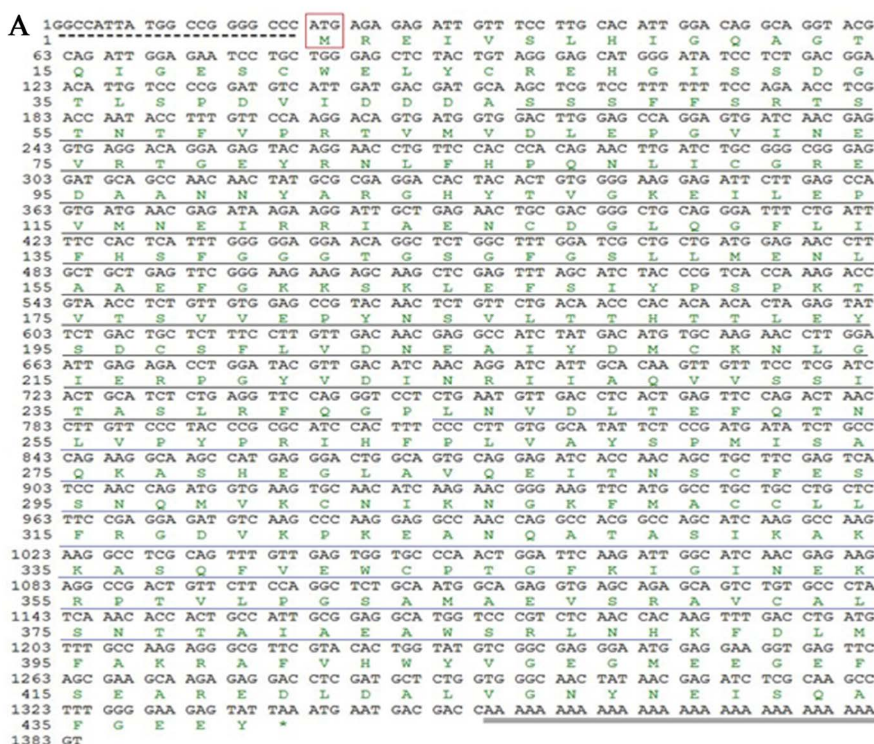

Figure 1. Nucleotide sequence, deduced amino acid sequence, and conserved domains of *Endoreticulatus* sp. Zhenjiang alpha-tubulin. (A) Nucleotide and deduced amino acid sequence of alpha-tubulin cDNA; (B) Schematic domain structure of alpha-tubulin. The start codon is boxed in red. 5'-terminal untranslated region is marked with a dotted underline. Stop codon is denoted by an asterisk (*). The polyadenylation tail is marked with double underlines. The tubulin domain is underlined in black, and tubulin_C domain is underlined in blue.

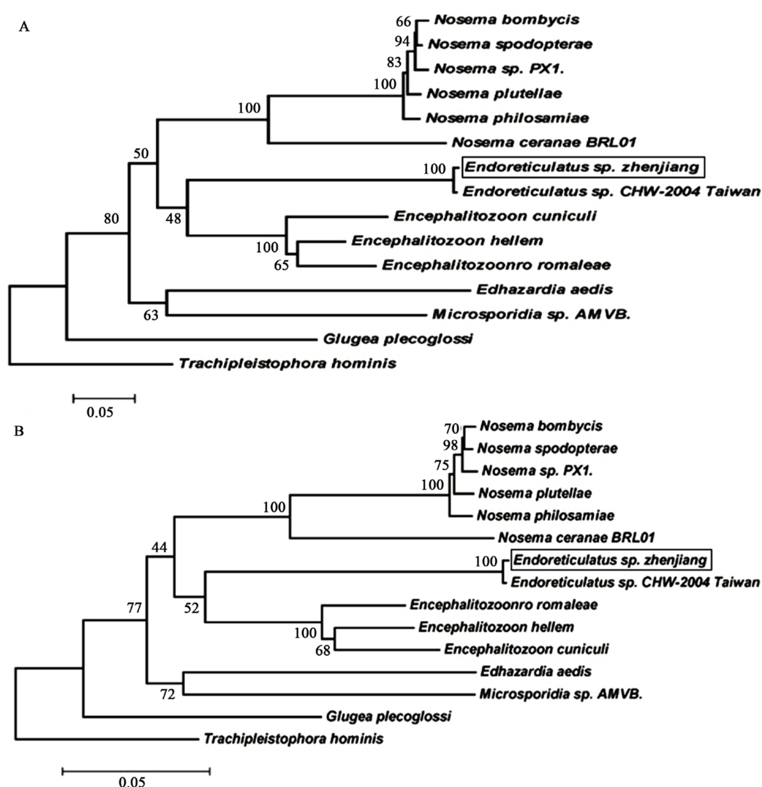

Figure 2. Nucleotide sequence, deduced amino acid sequence, and conserved domains of *Endoreticulatus* sp. Zhenjiang alpha-tubulin. (A) Nucleotide and deduced amino acid sequence of alpha-tubulin cDNA; (B) Schematic domain structure of alpha-tubulin. The start codon is boxed in red. 5'-terminal untranslated region is marked with a dotted underline. Stop codon is denoted by an asterisk (*). The polyadenylation tail is marked with double underlines. The tubulin domain is underlined in black, and tubulin_C domain is underlined in blue.

nucleotide sequences between *Endoreticulatus* sp. Zhenjiang, *N. bombycis*, *N. philosamiae*, *N. spodopterae*, *N. plutellae*, and *Nosema* sp. PX1 varied from 0.482 to 0.529 (**Table 2**). These results are consistent with our previous report [7].

3.3. Species Specific Diagnosis by PCR

Molecular diagnosis by PCR is often used for species identification and offers superior sensitivity and specificity when compared to microscopy. PCR amplification of a specific alpha-tubulin gene sequence was performed using gene specific primers (Spe-F/Spe-R) to distinguish *Endoreticulatus* sp. Zhenjiang from *N. bombycis*, *N. philosamiae*, *N. spodopterae*, *N. plutellae*, and *Nosema* sp. PX1 by PCR. The results showed that the expected fragment was amplified only from the genomic DNA of *Endoreticulatus* sp. Zhenjiang (**Figure 3**).

4. Discussion

Microsporidia are fungi-related obligate intracellular parasites with a highly reduced and compact genome, which affect microsporidian transcription and gene expression regulation [17]-[23]. For example, overlapping transcription, a highly unusual phenomenon among eukaryotes, has been observed in some microsporidian species. In the microsporidian *Antonospora locustae*, a locust parasite, and the mammalian parasite, *Encephalitozoon cuniculi*, high frequencies of multi-gene transcripts with short 5'-UTRs have been observed in the spore stage [24]-[26]. In this study, we report the full-length alpha-tubulin cDNA sequences of *Endoreticulatus* sp. Zhenjiang using the RACE protocol. Molecular analysis indicated that the 5'-UTR of the *Endoreticulatus* sp. Zhenjiang alpha tubulin was only 20 bp which was much shorter than in *Saccharomyces cerevisiae*. In addition,

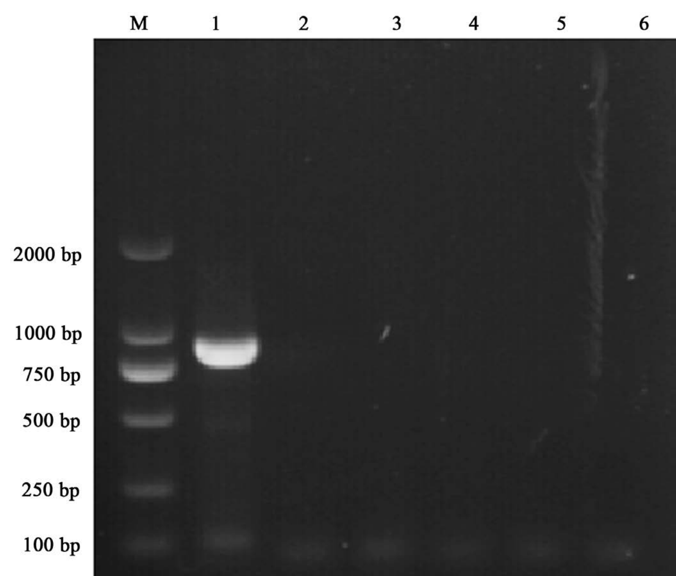

Figure 3. Species specific diagnosis by PCR. Lanes 1-6: *Endoreticulatus* sp. Zhenjiang, *Nosema bombycis*, *Nosema philosamiae*, *Nosema* sp. HA, Nosema sp. PA, and *Nosema antheraeae*.

Table 2. Comparison of microsporidian alpha-tubulin nucleotide sequences: percentage of identity (top diagonal) and pairwise distance (bottom diagonal).

Species Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Endoreticulatus* sp. Zhenjiang	-	**64.7**	**64.8**	**65.3**	**65.3**	**64.8**	**62.8**	72.0	71.3	70.9	65.7	**99.4**	66.4	63.2	66.1
2. *Nosema philosamiae*	**0.496**	-	96.5	96.5	97.0	96.4	77.8	67.7	70.5	72.2	65.3	65.1	69.7	63.2	65.3
3. *Nosema bombycis*	**0.493**	0.036	-	98.7	97.0	98.0	77.4	68.0	70.4	72.0	65.3	65.0	69.3	63.3	64.9
4. *Nosema spodopterae*	**0.482**	0.036	0.013	-	97.5	98.4	77.4	68.1	70.5	72.2	65.3	65.5	69.5	63.3	64.5
5. *Nosema plutellae*	**0.486**	0.031	0.031	0.026	-	97.0	78.0	67.3	70.3	72.0	65.0	65.5	70.1	62.6	64.5
6. *Nosema* sp. PX1	**0.494**	0.037	0.020	0.016	0.031	-	77.6	68.0	70.2	72.1	65.2	65.0	69.9	62.7	63.8
7 *Nosema ceranae* BRL01	**0.529**	0.264	0.270	0.270	0.261	0.268	-	68.3	70.8	72.3	64.8	63.3	66.7	63.3	63.2
8. *Encephalitozoon cuniculi*	0.352	0.430	0.425	0.423	0.439	0.425	0.421	-	88.1	85.5	67.4	71.9	64.1	71.3	69.3
9. *Encephalitozoon hellem*	0.364	0.379	0.382	0.378	0.383	0.385	0.376	0.132	-	90.4	67.4	71.2	64.8	69.4	69.0
10. *Encephalitozoon romaleae*	0.373	0.351	0.354	0.351	0.354	0.353	0.350	0.165	0.105	-	67.5	70.7	64.7	67.5	67.7
11. *Edhazardia aedis*	0.462	0.471	0.472	0.472	0.478	0.474	0.482	0.431	0.430	0.429	-	66.0	63.8	68.6	65.1
12. *Endoreticulatus* sp. CHW-2004 Taiwan	**0.006**	0.489	0.489	0.479	0.480	0.490	0.517	0.355	0.367	0.377	0.456	-	62.8	66.6	65.7
13. Microsporidia sp. AMVB	0.448	0.389	0.397	0.395	0.384	0.387	0.442	0.493	0.478	0.479	0.503	0.529	-	65.8	72.7
14. *Glugea plecoglossi*	0.520	0.512	0.510	0.510	0.526	0.524	0.514	0.364	0.396	0.430	0.408	0.444	0.466	-	69.4
15. *Trachipleistophora hominis*	0.455	0.472	0.482	0.491	0.490	0.505	0.512	0.397	0.403	0.426	0.472	0.462	0.343	0.394	-

the 3'-UTR was also short with 42 bp that includes 29 dA residues without a polyadenylation signal. All these features are likely due to the high compaction of microsporidia genome, which also has a gene transcription pattern that is different from other microsporidia [27].

The 5'-UTR and 3'-UTR of eukaryotic mRNA play important roles in the post-transcriptional gene expression regulation. Particularly, the 3'-UTR guides the mRNA 3' end processing with the end processing signal.

The 3'-UTR not only controls stability, degradation rate of mRNA and gives assistance to identify specific codons, but also controls translation time, sites, initiation, and efficiency [28]. The spatial and temporal regulation of eukaryotic translation initiation is largely achieved through the 5'-UTR [29]. Highly expressed genes such as housekeeping genes tend to have shorter 5'-UTRs with simple structures, but genes that need strict regulation such as growth factors and proteins associated with cell proliferation, differentiation, and apoptosis, have much longer 5'-UTRs with complex structures [30] [31].

Traditional, taxonomical species classification of microsporidia is mainly based on ultrastructural and ecological features. For example, the differential diagnosis of *Nosema* species was based on subtle differences in overlapping characteristics such as spore size, number of nuclei per cell, type of cell division, microsporidium host relationships, and primary site of infection. However, many microsporidia are considered to have a wide host range, complicated life cycles, and parasite host relationships. Therefore, the majority of the previously published classifications differ significantly and the taxonomy of microsporidia, particularly the species identification still remains controversial. Molecular markers using the tubulin protein and rRNA sequence data in combination with phylogenetic analysis can be effective tools to redefine the phylogenetic relationship between microsporidia [7] [27]. The alpha- and beta-tubulin proteins are the most abundant proteins in eukaryotic cells and their evolution may have paralleled the nucleus. Data on recent phylogenies based on alpha- and beta-tubulin protein-coding genes have enabled to carry out more robust phylogenetic analyses [32]-[36]. In this study, phylogenetic trees were constructed from *Endoreticulatus* sp. Zhenjiang alpha-tubulin sequence and 14 other microsporidian sequences. We found that *Endoreticulatus* sp. Zhenjiang was within the same clade as *Endoreticulatus* sp. CHW-2004 Taiwan, but it was distantly related to other microsporidia, which was consistent with our previous results based on SSU rRNA subunit phylogenetic tree [7]. This also explains the credibility of the tubulin gene for phylogenetic analysis.

5. Conclusion

A number of methods have been explored to effectively detect microsporidia and range from the earliest visual identification to microscopic examination and serological and molecular detection techniques [37]-[39]. Currently, molecular diagnostic methods have made great progress due to higher specificity and sensitivity in the detection of microsporidia [40] [41]. Although replacing microscopy with more sensitive and specific nucleic acid based methods is hampered by the higher cost of cloning and sequencing, multiplexing the detection of more than one parasite in a single test has been found to be very effective and would decrease the cost of the test without the need for multiple PCR equipment, which is expensive. In this study, we designed gene specific primers to amplify the *Endoreticulatus* sp. Zhenjiang alpha-tubulin gene from its genomic DNA. These primers and the conditions used, specifically were amplified only from the genomic DNA of *Endoreticulatus* sp. Zhenjiang but not from *N. bombycis*, *N. philosamiae*, *Nosema* sp. HA, *Nosema* sp. PA or *N. antheraeae*. Therefore, this study reported that the specific identification of *Endoreticulatus* sp. Zhenjiang among the other microsporidia can be a more reliable detection method to improve the epidemiology of the disease.

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