

Original Article

TWO LENSES, ONE PATHOGEN: COMPLEMENTING MICROSCOPY WITH MOLECULAR INVESTIGATION FOR ACCURATE MICROSPORIDIA DIAGNOSIS

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Submission Date:

9 March 2026

Acceptance Date:

9 June 2026

Publication Date:

31 August 2026

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DOI:

[https://doi.org/10.63719/imrj.2026.01\(SE\).01](https://doi.org/10.63719/imrj.2026.01(SE).01)

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ABSTRACT

Microsporidium is an intestinal protozoan parasite that infects a wide range of animal species and has emerged as an important pathogen in immunocompromised individuals. The most common species infecting humans include *Enterocytozoon bienersi*, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, and *Encephalitozoon hellem*. Microscopic examination remains the primary diagnostic method as it is rapid and reliable; however, it is limited to genus-level identification. This study aimed to apply molecular techniques to complement microscopy in the identification of *Microsporidium* species. A total of 121 stool samples were re-analysed using polymerase chain reaction (PCR) targeting species-specific small subunit rRNA (SSU rRNA) genes, which include 109 samples previously identified as positive for *Microsporidium* spp. by Gram-chromotrope staining and 12 samples that were negative by the same method. The results showed that eight samples (6.6%) were PCR-positive for *Microsporidium* spp., all identified as *E. bienersi*. Of these, 3 samples were previously positive by microscopy (3/109; 2.75%), while 5 were from microscopy-negative samples (5/12; 41.67%). The amplified products were sequenced for confirmation. All samples were negative for *E. intestinalis*, *E. cuniculi*, *E. hellem*, and *Vittaforma corneae*. Phylogenetic analyses using three different methods showed that the detected strains were closely related to isolates from immunocompromised patients in Malaysia and diarrheal patients in China. The discrepancy between PCR and microscopy results may be due to several factors and requires further investigation. A larger sample size and combined diagnostic methods are needed to evaluate the role of molecular techniques in *Microsporidium* detection. These findings highlight the importance of using both molecular and microscopic methods.

KEYWORDS: *Microsporidium* spp, phylogenetic tree analysis, SSU rRNA, immunocompromised, *E. bieneusi*

INTRODUCTION

Microsporidium, a known opportunistic pathogen, primarily causes chronic diarrhoea in individuals with severely weakened immune systems, particularly Human Immunodeficiency Virus (HIV) patients with CD4+ T-cell counts below 100 cells/mm³ (1). Other immunocompromised populations, such as organ transplant recipients, bone marrow transplant patients, and those undergoing chemotherapy, are also susceptible to *Microsporidium* infections. Additionally, *Microsporidium* can affect immunocompetent individuals, including the elderly, young children, and travellers, especially those visiting tropical regions (2). Intestinal microsporidia are a significant enteric protozoan infection with public health implications due to their zoonotic potential and similarities in genotypes between humans and animals, raising concerns about waterborne, foodborne, and zoonotic transmission (3,4). Of the over 200 genera, 17 species have been implicated in causing microsporidiosis in humans (5). The infection may localise to various organs, including the gastrointestinal tract, eyes, kidneys, liver, muscles, brain, and respiratory system (1,6). *Microsporidium* can infect a range of hosts, including vertebrates, invertebrates, and aquatic environments (3,7). The global prevalence of microsporidiosis varies by population, with rates of 11.2% in HIV-positive individuals, 8.4% in immunocompetent individuals, 7.3% in cancer patients, 10.2% in organ transplant recipients, and 12.2% in individuals with gastrointestinal disorders (8). Human microsporidial infections in Malaysia remain underreported, particularly at the molecular level. Previous prevalence studies relied on light microscopy and reported prevalence rates ranging from 13.0%–32% (9-11). Only a few local studies have successfully applied molecular methods, such as polymerase chain reaction (PCR), to identify *Enterocytozoon bieneusi* (*E. bieneusi*), *Enterocytozoon intestinalis* (*E. intestinalis*), and *Enterocytozoon hellem* (*E. hellem*) from stool samples (10,12). Among these, Nasarudin et al. was the only study to incorporate the loop-mediated isothermal amplification method alongside conventional PCR for *E. bieneusi* detection. Lono et al. (9) reported an 8.5% prevalence among HIV patients, while Hassan et al. reported a higher prevalence of 29.2% among immunocompromised individuals, including HIV patients, cancer patients, and organ transplant recipients (13). Detecting species-specific causes of parasitic diarrhoea is challenging, labour-intensive, and often overlooked by surveillance systems. Many enteric protozoa and bacteria present with similar symptoms, including diarrhoea, abdominal pain, nausea, vomiting, flatulence, anorexia, and fever, making differential diagnosis difficult (14).

Light microscopy remains the most used method in clinical settings for the morphological identification of *Microsporidium* spores in biological samples. Common staining techniques include the Modified Ziehl-Neelsen, "Ryan Blue" modified trichrome stain, and acid-fast trichrome stain (7,15). Accurate species identification is crucial for effective treatment, as susceptibility to medications varies by species. For example, albendazole, an anthelmintic drug effective against *Encephalitozoon* species, is ineffective for treating infections caused by *E. bieneusi* (16). The morphological similarities across *Microsporidium* species make molecular methods, which utilise genetic data for classification, an essential tool for species-level identification (17). These methods are preferred due to the detailed information they provide, which is unattainable through microscopy alone. Molecular analysis is particularly advantageous as it allows for the precise identification of *Microsporidium* species infecting the patient. Additionally, species confirmation through molecular methods can help trace the source of infection. This approach minimises uncertainties and errors associated with microscopy, ensuring

more reliable results. This study highlights the significance of molecular-based techniques in analysing and identifying *Microsporidium*.

MATERIALS AND METHODS

Sample collection

Samples were obtained from archived stool specimens received by the Parasitology Unit, Institute for Medical Research, from hospitals in Malaysia. These samples were originally submitted for stool Full Examination and Microscopic Examination (FEME) due to suspected intestinal parasitic infections. A total of 121 samples collected between March 2022 and March 2023 were selected for PCR analysis. Permission to use the archived diagnostic samples, stored at -20 °C, was obtained from the Head of Unit, who serves as the custodian of the biological specimens. Samples were selected based on previously confirmed positive samples stained using Gram-chromotrope staining and microscopically positive for *Microsporidium* spores. In addition, 12 confirmed *Microsporidium*-negative samples were included as negative controls. All selected samples were re-labelled (M001–M121) for blinded PCR analysis. For genus-level PCR identification, the original sample IDs were maintained. For species-specific PCR, samples were re-labelled according to the detected species. For example, samples confirmed as *E. bieneusi* were assigned IDs EB001–EB121, whereas those identified as *E. intestinalis* were labelled EI001–EI121. These identifiers corresponded to the specimen codes M001–M121, respectively.

Deoxyribonucleic acid (DNA) extraction and Polymerase Chain Reaction (PCR)

DNA was extracted using the innuPREP Stool DNA Kit (Innuscreen GmbH, Germany) according to the manufacturer's instructions. Safe-lock tubes containing ceramic microbeads were used during the pre-lysis stage to facilitate sample disruption. A final volume of 100 µL genomic DNA was obtained for each sample. The concentration and purity of the PCR products were assessed using a NanoDrop spectrophotometer. The eluted DNA was aliquoted into labelled tubes and stored at -20 °C until PCR amplification.

A generic *Microsporidium* spp. primers capable of amplifying both *Encephalitozoon* and *Enterocytozoon* genera were adopted from published primers targeting the conserved region of the small subunit ribosomal RNA (SSU rRNA) gene. Species-specific primers were used to detect *E. bieneusi*, *E. intestinalis*, *Enterocytozoon cuniculi*, *E. hellem*, and *Vittaforma corneae* (Table 1). Although all samples were stool specimens, *V. corneae*, which typically causes ocular infections, was included as a PCR control to assess potential primer cross-reactivity. Negative controls were confirmed to be microsporidia-negative by FEME examination. Meanwhile, positive controls for all five *Microsporidium* were synthetically generated using reference sequences obtained from the GenBank database (accession numbers: L07123.1, L17072, L19070, and U09929) and synthesised by Macrogen (Singapore). PCR was performed in a 50 µL reaction mixture containing 25 µL MyTaq DNA Polymerase (Bioline Reagents Ltd, United Kingdom), 2.5 µL each of forward and reverse primers (0.50 µM), 5 µL DNA template, and 15 µL molecular-grade water. For species-specific primers (*E. bieneusi*, *E. intestinalis*, *E. cuniculi*, and *E. hellem*), PCR conditions consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s, and a final extension at 72 °C for 90 s. For the generic primer, the cycling conditions included 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 30 s, and a final extension at 72 °C for 90 s. PCR products were analysed using capillary electrophoresis with the QIAxcel Advanced System (QIAGEN, Germany). The remaining

amplicons were stored at -20°C before Sanger sequencing analysis service (Apical Scientific Sdn Bhd, Malaysia).

Sequence and data analysis

Data will be analysed descriptively. Only PCR-positive samples were subjected to Sanger sequencing by Apical Scientific Sdn Bhd using the sequencing primers PMP(V1), PMP2, EBIEF1, and EBIER1. Obtained sequences were analysed using BioEdit software. Genetic distances between *Microsporidium* isolates were compared with reference isolates from various countries, and phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis X (MEGA X) software based on Maximum-Likelihood, Bayesian Inference, and Maximum-Parsimony methods.

Table 1. Genus and species-specific PCR primers for the detection of *Microsporidium* spp. that are important to humans

Target region	Genus/species	Primer designation	Reference	Sequence (5'–3')	Tm (°C)	Size of PCR product (bp)
SSU rRNA	<i>E. cuniculi</i>	ECUNF ECUNR	Visvesvara et al. (18)	ATGAGAAGTGATGTGT GTGCGTGCCATGCACTC ACAGGCATC	55	549
	<i>E. hellem</i>	EHELF EHELRL		TGAGAAGTAAGATGTTT AGCAGTAAAAAGACTC TCACACTCA	55	547
	<i>E. bienewsi</i>	EBIEF1 EBIER1	da Silva et al. (19)	GAAACTTGTCCTCTCT TACGCCATGCACCACTC CTGCCATT	55	607
	<i>E. intestinalis</i>	SINTF SINTR	da Silva et al. (19)	TTTCGAGTGTAAGGAGT CGACCGTCCTCGTTCTC CTGCCCG	55	520
	<i>Microsporidium</i> spp.	PMP1(V1) PMP2	Müller et al. (20)	CACCAGGTTGATTCTGC CTGACCCTCTCCGGAAC CAAACCCTG	62	<i>E. bienewsi</i> : 250 <i>E. cuniculi</i> : 268 <i>E. intestinalis</i> : 270 <i>E. hellem</i> : 279
	<i>V. corneae</i>	NCORF1 NCORR1	Gosh et al. (21)	TGAGACGTGAGATGAG TATTCCCTGCCCACTGT CTCCAAT	55	375

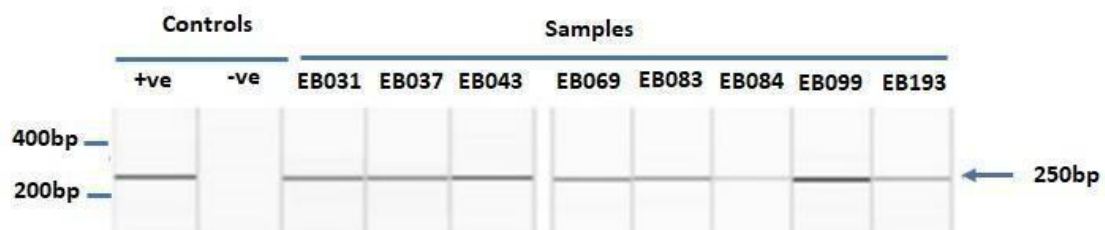
RESULTS

PCR analysis

A total of 121 samples were analysed using species-specific PCR to detect *Microsporidium* species. Brief demographic and clinical data showed that 96 samples (79.3%) came from male patients, while 25 (20.7%) were from female patients. HIV patients accounted for 67.8% (82 samples), while 4 (3.3%) were from COVID-19 patients. One sample (0.8%) was collected from a leukaemia patient. Among these samples, 109 were microscopically positive for *Microsporidium* spp. and reported as 'Microsporidium spp. seen (MSS)', while 12 samples were negative for *Microsporidium* spp. and reported as 'No Microsporidium spp. seen (NMSS)'. Out of the 121 stool samples, 8 (6.6%) tested positive for the *Microsporidium* genus, with a DNA polymorphism size of 250 base pairs. Meanwhile, species-specific identification reveals that all eight samples were positive with *E. bieneusi*, which produced a polymorphism size of around 607 base pairs (bp) (Figure 1). Although we also performed PCR using species-specific primers for *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *V. corneae*, none of the samples was positive for any of these species.

Interestingly, among the *E. bieneusi*-positive samples, 3 (2.75%) are from the MSS category, while 5 (41.67%) are from the NMSS category, giving an overall prevalence of 2.47%, and 4.13% were EB-positive from the MSS and NMSS categories, respectively. In addition, five patients were among the HIV patients with diarrhoea; one patient had COVID-19 without diarrhoea, and the other two samples were from patients with diarrhoea but had no underlying diseases (Table 2; Figure 2).

(A) Genus Identification



(B) Species-specific Identification

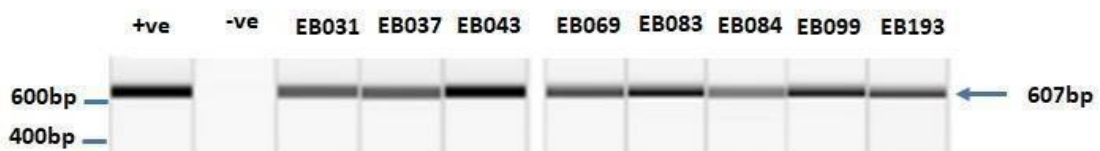


Figure 1. Capillary electrophoresis of the PCR product for: (A) Genus identification and (B) Species-specific identification.

Table 2. Clinical history of the *E. bieneusi*-positive samples detected by PCR.

Sample ID	Age	Sex	Underlying disease	Symptoms	Result by microscopy	Species identified by PCR
EB031	36	M	HIV	Chronic diarrhoea	NMSS	<i>E. bieneusi</i>
EB037	44	M	COVID-19	NA	MSS	<i>E. bieneusi</i>
EB043	35	M	HIV, Hepatitis B	Watery diarrhoea, vomiting	NMSS	<i>E. bieneusi</i>
EB069	43	M	HIV, latent syphilis, pneumonia	Weight loss, loss of appetite, coughing	MSS	<i>E. bieneusi</i>
EB083	45	M	N/A	Watery diarrhoea	NMSS	<i>E. bieneusi</i>
EB084	52	M	N/A	Watery diarrhoea	NMSS	<i>E. bieneusi</i>
EB099	24	M	HIV	Watery diarrhoea, fever	MSS	<i>E. bieneusi</i>
EB108	25	M	HIV	Watery diarrhoea, fever, loss of appetite, weight loss, odynophagia	NMSS	<i>E. bieneusi</i>

Sequencing analysis

PCR products used for genus identification showed 98%–99% sequence identity with *E. bieneusi* sequences available in the GenBank database. Further Basic Local Alignment Search Tool-nucleotide (BLASTn) analysis of PCR products for species identification demonstrated 99%–100% homology with published *E. bieneusi* sequences. Eight isolates (EB031, EB037, EB043, EB099, EB083, EB084, EB108, and EB069) showed 99%–100% similarity with an iso *E. bieneusi* late reported in an Acquired Immunodeficiency Syndrome (AIDS) patient in Mexico (Accession No. AY298728). Five isolates (EB031, EB037, EB043, EB083, and EB099) also showed 99%–100% similarity with isolates reported in diarrheal outpatients in China. Among all isolates, EB031 showed 100% identity with several *E. bieneusi* sequences reported in immunocompromised patients in China and Mexico, as well as in animal hosts including sheep, cynomolgus monkeys, and grey kangaroos.

Most isolates (except EB084) showed 99%–100% similarity with *E. bieneusi* sequences detected in sheep faeces. Similarly, most isolates (except EB084 and EB037) also showed 99%–100% similarity with sequences reported from grey kangaroos. Isolate EB385 showed 99.47% identity with *E. bieneusi* from pig faeces in China, while EB069 showed 99.64% similarity with sequences detected in bamboo rat faeces from Guangdong, China. Meanwhile, EB084 showed 99.82% similarity with sequences identified in wastewater samples in China. The M isolates (M031–M108) also demonstrated 94%–99% identity with *E. bieneusi*. The reference sequences with the highest similarity for each PCR-positive product are listed in Supplementary Table 1.

Further sequence alignment of the M isolates with microsporidia sequences from non-human hosts showed that five reference sequences belonged to the Enterocytozoonidae family. In contrast, one sequence (GenBank: KT762153) belonged to a different clade (Supplementary Table 2). The isolates M031, M037, M043, M069, M083, M084, M099, and M108 showed 96.32%–98.88% identity with *E. bieneusi* sequences obtained from cattle, which was higher than the similarities observed with *Microsporidium* from other animal hosts. In comparison, sequences from another species,

Enterocytozoon hepatopenaei, showed 87%–88% identity, while sequences from different genera (*Enterospora*, *Globulisporea*, and *Obruspora*) showed 82%–89% identity. Overall, these results demonstrate that PCR, sequencing, and database comparison can effectively differentiate between human and non-human microsporidia species.

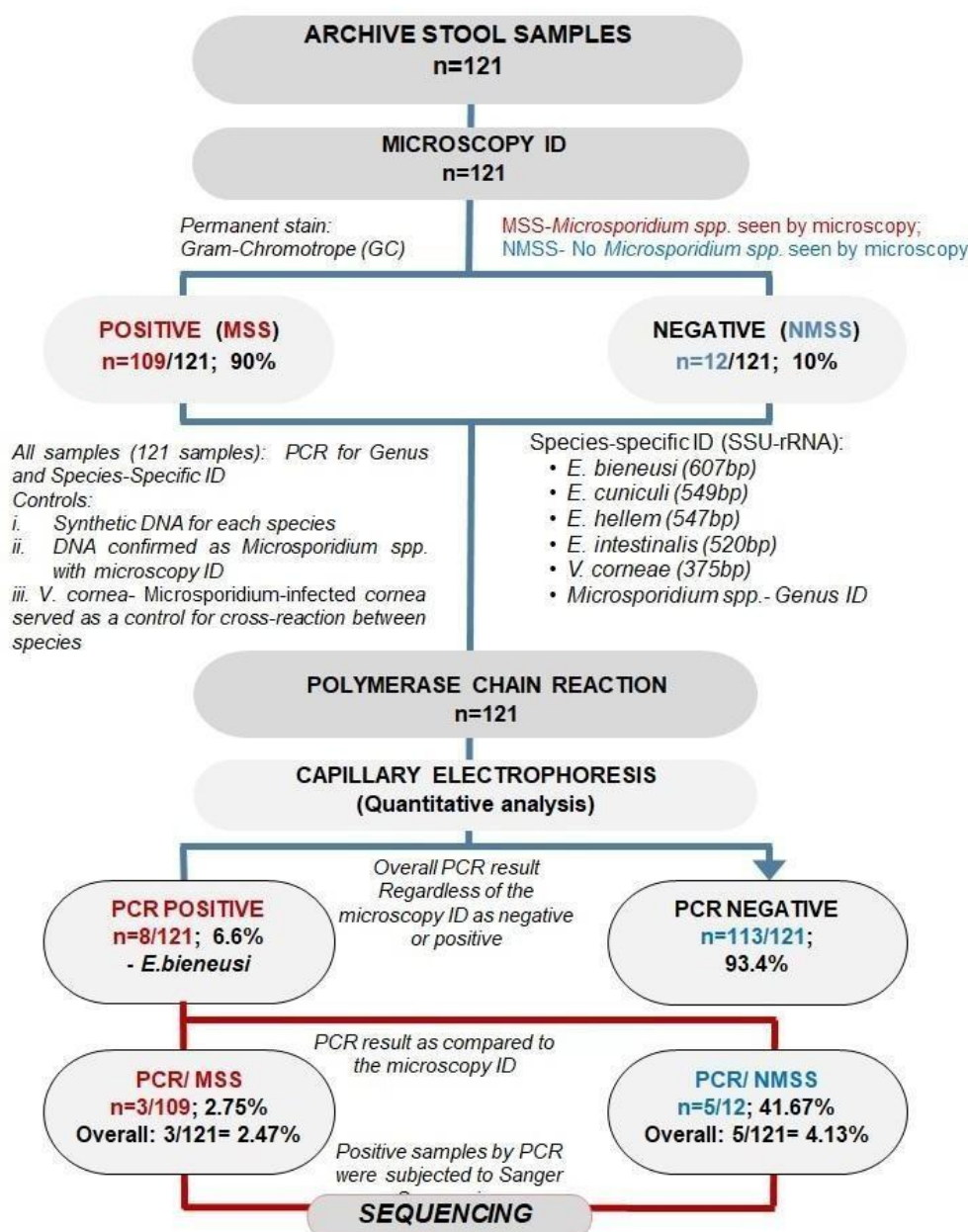


Figure 2. Flowchart depicting the process and outcomes of *Microsporidium* detection using PCR. Stool samples were initially screened by Gram-chromotrope (GC) staining, followed by PCR targeting the SSU-rRNA gene for species-specific identification. The results indicate a discrepancy between microscopy and PCR findings, highlighting potential differences in sensitivity and specificity between the methods.

Phylogenetic analysis

The results of all three phylogenetic trees show that in the upper clade, all eight *E. bieneusi* isolates in this study (EB031, EB037, EB043, EB069, EB083, EB084, EB099, and EB108) were grouped with *E. bieneusi* sequences obtained from immunocompromised patients in Malaysia and Mexico and in a patient with diarrheal symptoms in China. The clade also includes the *E. bieneusi* sequences obtained from animals in China (sheep, cynomolgus monkey, pig) and Australia (grey kangaroo), and one sequence isolated from sewage water in China. A high bootstrap support value (96%) demonstrates a strong confidence in this grouping. The middle clade, which has a bootstrap value of 90% consists of 12 isolates, including the eight remaining isolates from this study (M031, M037, M043, M069, M083, M084, M099 and M108), isolates from a renal transplant patient in India and a patient with gastrointestinal disorder from Iran, and two other isolates obtained from animals in United Arab Emirates (falcon) and The Netherlands (pigeon).

In the lower clade are four isolates obtained from Malaysian pigs, which were genetically homogeneous and formed their monophyletic clade. The outgroup sequence KY706128 branches off from all the other clades, which suggests a level of genetic divergence between these two groups since they were the same *E. bieneusi* species but represent different genotypes. Meanwhile, two outgroup sequences, KY706128 (*E. bieneusi* genotype CSK2) and AF069064 (*E. hellem*), were separated from the other *Enterocytozoon* sequences and were used to root the tree. These two outgroup sequences help visualise the direction of the evolution. Three different methods were used to generate a phylogenetic tree, which are the Maximum Likelihood, Bayesian Inference, and Maximum Parsimony methods (Figure 3).

The average pairwise genetic distances of the *E. bieneusi* isolates from this study, compared with various *E. bieneusi* isolates from different countries, were generated using the MEGA X software based on the Maximum-Likelihood Phylogenetic tree (Supplementary Table 3 and Figure 3). From the pairwise distance table, we can see that the pairwise genetic distance between the *E. bieneusi* isolates of this study and *E. bieneusi* isolates from previous immunocompromised studies in Malaysia is genetically identical; the same goes for the isolates from Mexico, Australia, and China. The isolates generated from PCR for genus identification were genetically like the *E. bieneusi* isolates from the United Arab Emirates (UAE), India, Iran, and the Netherlands. Meanwhile, the Malaysian pig isolates (Accession number MG551922-MG551925) have the highest pairwise distance from most isolates, ranging from 1.59 to 1.74, suggesting a significant genetic divergence from the isolates in this study, which is why they form their clade in the phylogenetic tree, even though they were from the same *E. bieneusi* species. The two pig isolates (MG551922, MG551924) were from the EbpA genotype, and the other two (MG551923, MG551925) were from the EbpC genotype. A higher pairwise distance was also observed with the outgroup isolates, which indicates a significant genetic divergence. The outgroup sequence KY706128 shows 1.40 to 1.57 to all sequences, while AF069094 shows the highest distance, ranging from 2.01 to 2.18 with all sequences except KY706128.

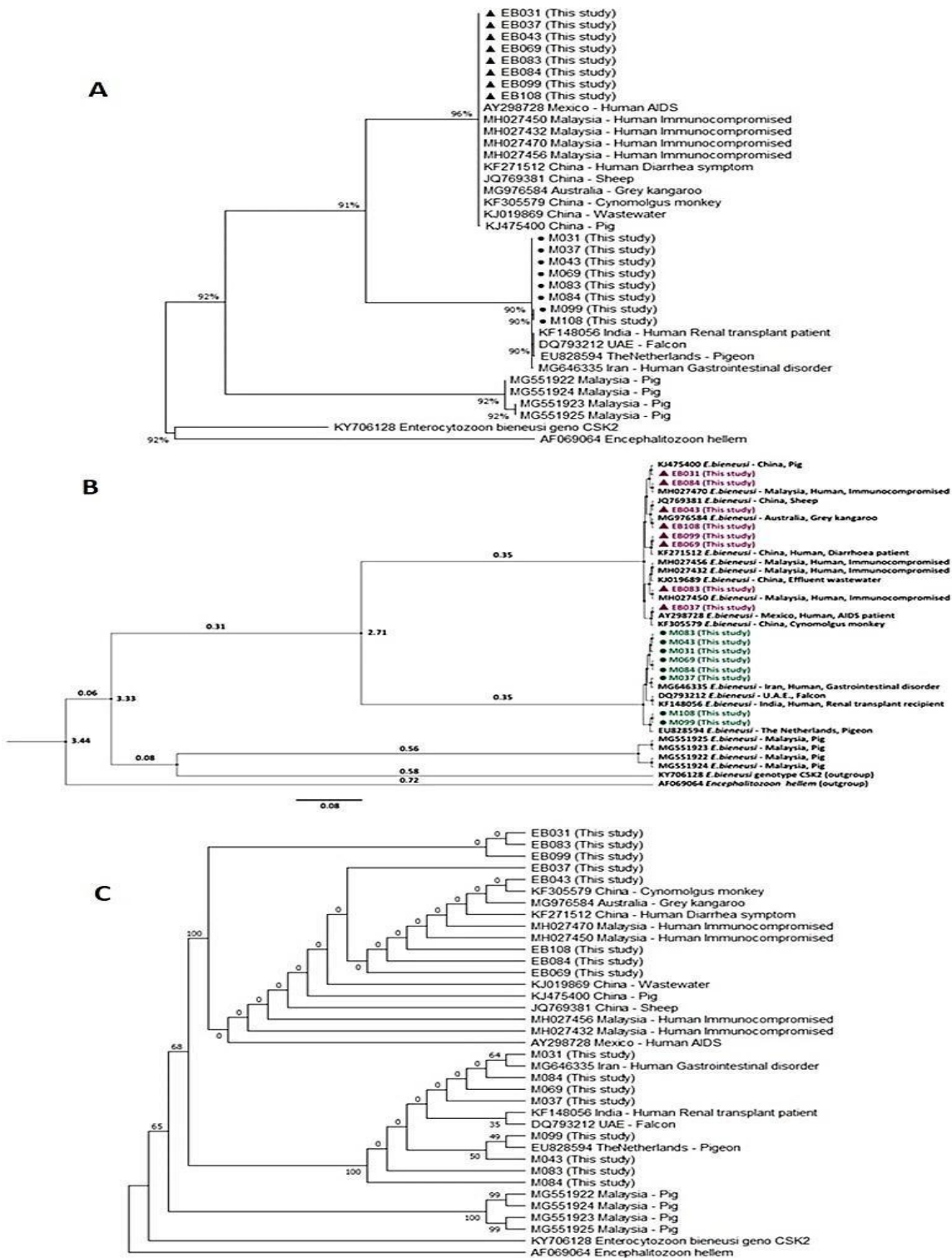


Figure 3. Phylogenetic analysis for *E. bienersi* using different methods.

(A) Maximum-Likelihood method, using the Hasegawa-Kishino-Yano (HKY) algorithm model (22). The HKY model has the lowest Bayesian Inference Criterion (BIC) number (23). The sequences marked with a black triangle (▲) represent isolates obtained from PCR for species identification, and sequences marked with black dots (●) were isolates obtained from PCR for genus identification. The percentage

of trees in which the associated taxa clustered is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site(24). The evolutionary tree was generated by Mega X (25) ; (B) Bayesian Inference method. The sequences marked with a black triangle (▲) represent isolates obtained from PCR for species identification, and sequences marked with black dots (●) were isolates obtained from PCR for genus identification. The branch length corresponds to the level of divergence of the nucleotide sequences. The evolutionary tree was generated by Bayesian Evolutionary Analysis Sampling Trees (BEAST) and FigTree v1.4.4 (26) employing a Markov chain Monte Carlo (MCMC) approach to estimate the posterior probability distribution of phylogenetic trees and (C) the Maximum Parsimony method. The sequences marked with a black triangle (▲) represent isolates obtained from PCR for species identification, and sequences marked with black dots (●) were isolates obtained from PCR for genus identification. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

DISCUSSION

This study demonstrated that PCR-based detection of *Microsporidium* spp. showed a lower positivity rate than microscopy, with only 6.6% of samples testing positive by molecular methods, despite 93.4% being identified microscopically. Similar findings were reported in Iran, Germany, and Malaysia (13,20,27). Although other studies have indicated that PCR is equally accurate as microscopy, as reported by Muller et al. (13), their findings showed variations in the detection limits of both methods. Stool samples spiked with different concentrations of cultured *Microsporidium* revealed that the reliable detection threshold varied between 10^4 – 10^6 spores per gram of stool for microscopy. In contrast, PCR demonstrated a higher sensitivity, detecting as few as 10^2 spores per gram of stool. Interestingly, our study also discovered that PCR could identify *Microsporidium* in microscopy-negative samples (NMSS). The NMSS category included five of the eight samples with positive PCR results for *E. bieneusi* (Figure 2). Similar findings were published by Müller et al. (13), who used PCR to identify *Microsporidium* in ten samples, of which 2 were initially microscopy-negative and 8 were also microscopy-positive.

There are several reasons why the results of PCR and microscopy disagree, especially when *Microsporidium* was detected using microscopy (MSS), but not with PCR. Possible causes of (a) false-positive microscopy results (microscopy-positive, PCR-negative) include: (i) Artefacts from staining: Ineffective staining methods may result in incorrect identification or omitted detection. Artefacts discovered in faecal specimens can strongly resemble parasites. Several materials are necessary to help with the identification, among these is a calibrated ocular micrometre to ascertain the structure's size range. Publications that offer high-quality pictures of parasites and artefacts, along with tables listing the sizes and traits of the many parasitic forms present in specimens, are additional essential resources (28). (ii) Misidentification: Microscopy requires highly skilled workers and is labour-intensive. Diagnostic accuracy may be lowered if genuine *Microsporidium* spores are not distinguished from non-viable spores or similar-looking entities (such as yeast or bacteria with a diplococcal shape). Spores and these artefacts are more likely to be confused by novice microscopists. Higher sensitivity of microscopy for non-viable spores or artefacts in the case of false-positive microscopy (29). On the other hand, if *Microsporidium* was detected among negative samples (NMSS) using PCR, this could cause a (b) false-negative microscopy (microscopy negative, PCR positive). Possible reasons might be due to misidentification as stated above in (ii), and the other possible explanation might be because of (iii) Low parasite load: Due to the lesser sensitivity of microscopy, a low parasite density may not be detectable. This could be the weakness of this approach, which can also be found in other parasite infections (29).

In our current study, we were only able to detect *Microsporidium* DNA in three of the 109 samples from the MSS categories. This might be due to: (iv) Poor parasite load and poor DNA yield: if the parasite density is low, it may not be detectable by PCR, which could be the cause of this (c) false-negative PCR result (PCR-negative, microscopy-positive). Several studies found that the PCR threshold limit for *Microsporidium* was 102 spores/g of stool samples (13). The low spore count in our samples might be below the detection limit, even though spores were not measured during the microscope inspection in this investigation. A low *Microsporidium* concentration could result in insufficient template DNA or poor DNA extraction. Furthermore, the sensitivity of the PCR results may potentially be compromised by the (v) DNA extraction technique. The limited DNA yield may be due to the hard double coating of the spores, which is why specific steps are needed to separate the DNA of the microsporidia. A commercial DNA extraction kit should be used in conjunction with additional sample pre-treatment techniques, such as mechanical disruption, incubation with glycine-SDS buffer, the addition of glass beads to the sample tubes, repeated freeze-thaw cycles using liquid nitrogen, and the use of specialised bead-beater equipment during the pre-lysis phase to enhance DNA extraction efficiency. (vi) Type of samples. Fresh or preserved stool samples need to be taken into consideration for DNA extraction. For example, in fresh samples, the trophozoite stage will not encyst, but it will disintegrate; some stages of the parasite can survive preservation (4,30). A low concentration of *Microsporidium* may lead to inadequate template DNA or subpar DNA extraction; also, damaged DNA from improper handling or storage may provide a false-negative PCR. The poor DNA yield may be due to the hard double coating of the spores, which calls for specific steps to separate the DNA of the microsporidia. (vii) DNA degradation and PCR inhibitors. Complex polysaccharides, haemoglobin breakdown products, and bile salts are among the compounds that have been shown to obstruct PCR in stool specimens (31,32). However, as we did not check for inhibitors during pre-amplification PCR, we cannot completely rule out the possibility that our samples contain them. PCR results may be mistakenly negative due to inhibitors. PCR sensitivity can be increased by neutralising these inhibitors. (viii) Target genes. The PCR does have some limitations because this study only targeted four of the seventeen species that are currently known to infect humans, in addition to other internal factors that affect PCR performance. Therefore, it is crucial to carefully design primers for other *Microsporidium* as well as choose different target genes, as shown in several intestinal parasites, such as internal transcribed spacer, large-subunit (LSU) and small-subunit (SSU) (33,34). (ix) Enhanced detection limit and sensitivity of various PCR techniques such as Nested PCR, quantitative PCR, Real-time PCR, Digital PCR (dPCR) and Chip PCR (31,35). Lastly, (x) Misinterpretation of structure in microscopy: small organisms, overlapping monococcus or diplococcus bacteria, and insufficient staining can all make it difficult to identify *Microsporidium* (36). Given that this study concentrates only on four of the 17 species that are currently known to infect people and other internal factors that affect PCR performance, the PCR does have certain limitations. As a result, it is important to carefully select various target genes as well as the PCR method.

Meanwhile, among the NMSS categories, out of 12 samples, we managed to identify *Microsporidium* in five samples. Is this a true infection and misdiagnosed with microscopy, or is it an alarm of (d) false-positive PCR (PCR positive, microscopy negative)? In the case of false-positive PCR, this could be due to the detection of non-viable cells or dead cells, which also happens in other organisms, such as bacterial pathogens (37). The challenge in the detection of *Microsporidium* using the PCR method may have its drawbacks, for example, in differentiating the DNA from dead and viable cells. This has been discussed by Zeng et al., who stated that PCR depends on the efficiency of amplification and DNA integrity if the target nucleic acid sequences are present in the samples. Therefore, in this scenario, PCR might pick up some cross-contaminating foreign DNA rather than the targeted DNA (38).

From our point of view of *Microsporidium* in Malaysia, most studies involving human microsporidiosis infections were caused by *E. bieneusi* (13,39). Interestingly, our study shared similarities with the study done by Ashikin et al. (40); all the patients whose samples were positive for *E. bieneusi* were adults (age range 24–56). Meanwhile, studies by Lono et al. managed to identify *E. intestinalis* in both the HIV population in Kuala Lumpur and in an indigenous population in Pahang (9). As older studies in Malaysia were restricted to only microscopy methods, species identification was impossible (11). Besides the inability to identify species, the microscopy method requires a trained microscopist to screen for microsporidian spores. The ability of the transmission electron microscope to visualise the ultrastructural characteristics of different microsporidia species makes it the 'gold standard' method in species identification of microsporidia. However, it is less sensitive and requires specialised equipment, which makes this method unsuitable for routine diagnostic tests. This instrument was also not readily available in most diagnostic laboratories due to its high cost and the requirement for trained personnel to handle it.

Our study successfully identified *E. bieneusi* in all positive *Microsporidium* samples by PCR (eight samples). All the PCR-positive samples in our study were obtained from male patients; five of them were HIV patients, and 1 was a COVID-19 patient, which correlates with a local preceding survey, where the overall prevalence of microsporidiosis was higher in male immunocompromised patients. Two of our positive isolates were individuals with no other medical illness, but they suffered from chronic, watery diarrhoea. Notably, in our study, 77 out of 121 patients (63.6%) presented with diarrhoea, and 6 out of the 77 patients found positive for *E. bieneusi* all suffered from chronic watery diarrhoea. Two other samples, which had no diarrhoeal symptoms, were obtained from an HIV patient and a COVID-19 patient. However, little was known about the patient's lifestyle, which may have led to microsporidiosis infections.

Species identification of *Microsporidium* is crucial as different species respond differently to certain drugs. Albendazole has the most consistent tubulin-inhibiting activity against *Microsporidium* species, such as species from the *Encephalitozoon* genus (40). However, the drug is not as effective in *E. bieneusi* infections. Fumagillin and nitazoxanide have shown better efficacy in treating microsporidiosis caused by *E. bieneusi* (41,42). It is interesting to note that we also suspected a chronic *E. bieneusi* infection in the same patient. In our investigation, the same patient provided samples designated M099 and M108; the initial sample (M099) was received in December 2022, and the follow-up sample (M108) was received in January 2023. The patient's follow-up sample's ongoing *E. bieneusi* presence may suggest that the treatment could not eliminate the *E. bieneusi* infection. However, more research and clarification are required.

Phylogenetic analysis revealed that the *E. bieneusi* isolates from this study clustered with known *E. bieneusi* genotypes D and NCF2. However, our PCR method, which targets the SSU rRNA region, was unable to determine the exact genotypes of these isolates. This limitation arises because the SSU rRNA gene is highly conserved and lacks sufficient variation to differentiate closely related species. To further explore the genetic diversity and evolutionary relationships, we constructed a phylogenetic tree incorporating our isolates along with *E. bieneusi* isolates from human and animal hosts. The Maximum-Likelihood method was employed to assess genetic diversity, while Bayesian Inference and Maximum Parsimony approaches were also used to ensure the robustness and reliability of our results. Each method offers unique advantages, and their combined use helps identify potential biases, validate branch support values, and enhance the overall accuracy of phylogenetic inferences. In all three phylogenetic trees (Figure 3), the eight *E. bieneusi* sequences obtained in this study grouped with isolates from Malaysia, China, Australia, and Mexico. The absence of divergence among these *E. bieneusi* isolates (as determined by species-specific PCR) suggests a high degree of genetic similarity among the samples. This finding indicates that these isolates likely originate from identical strains with

potential for widespread transmission across species.

These findings underscore the importance of integrating microscopy with molecular methods to enhance the accuracy of *Microsporidium* detection, particularly in immunocompromised patients, where early diagnosis is crucial. While the exact reasons behind the discrepancies between PCR and microscopy results remain unclear, these limitations highlight the need for further investigation. Nevertheless, this study serves as an essential starting point for a more in-depth and comprehensive analysis. One limitation of this study is the relatively small sample size, which may impact the generalisability of the results. Future research should incorporate additional genetic markers, such as SSU rRNA, mitochondrial genes, microsatellite regions, and internal transcribed spacer markers, to improve PCR sensitivity and specificity. To address discrepancies, a combination of approaches—including repeated staining with different dyes, alternative DNA extraction methods, and quantitative PCR, should be considered. Furthermore, next-generation sequencing could provide a more comprehensive understanding of species diversity, while environmental factors influencing *Microsporidium* prevalence warrant further exploration. Expanding the study to include a broader range of clinical samples beyond stool is also recommended, as *Microsporidium* can disseminate to other organs in the human body. While molecular techniques hold great promise for improving detection, Rijsman et al. cautioned that shifting from microscopy to PCR-based diagnostics has clinical implications that must be carefully considered (43). Despite its superior sensitivity, PCR presents challenges that clinicians must be aware of when interpreting results.

CONCLUSION

In conclusion, while microscopy remains useful for detecting *Microsporidium*, molecular methods enable species-specific identification. A combined approach may yield the most accurate results, especially for clinical and epidemiological purposes. This study aimed to identify five human-infecting *Microsporidium* species; however, limitations of our PCR method may have resulted in the omission of others. Future research should focus on developing broader-range primers and improving DNA extraction techniques, including pre-treatment steps and specialised equipment. Given *Microsporidium*'s ability to infect multiple organs, expanding sample types beyond stool is crucial. Additionally, incorporating genetic markers such as LSU rRNA and ITS regions can enhance species differentiation and deepen understanding of their diversity and epidemiology.

Supplementary Materials: Supplementary Table 1 [Identity similarity percentage of sequences obtained from the positive PCR products to the reference sequences available in GenBank], Supplementary Table 2 [Homology of sequences of non-human microsporidia when aligned to sequences obtained from M-isolates], and Supplementary Table 3 [Average pairwise genetic distances table of *E. bienersi* isolates from this study compared to *E. bienersi* isolates from various countries].

Author Contributions: N.A.M.Y (Conceptualisation; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Visualisation; Writing—original draft), D.I.H.A and R.B (Formal analysis; Investigation; Visualisation; Writing—original draft), N.F.A and N.A (Data curation; Validation), M.Z (Conceptualisation; Project administration; Supervision; Writing—review & editing), N.O.S and A.Y (Conceptualisation; Methodology; Writing review & editing), S. H (Writing—review & editing)

Funding: The Ministry of Health Malaysia Research Grant (NMRR ID-25-01003-X3Z) supported the study.

Institutional Review Board Statement: The study was submitted to the Medical Research & Ethics Committee (MREC), Ministry of Health Malaysia, for ethical review. MREC determined that formal

ethical approval was not required and granted an ethics waiver, as the publication involved secondary analysis of data from a previously completed student thesis (NMRR ID-25-01003-X3Z; MREC reference: 25-01003-X3Z).

Informed Consent Statement: Not applicable

Data Availability: All data have been presented in the manuscript.

Acknowledgements: We thank the Director-General of Health Malaysia for the permission to publish this paper. We also thank the Director of the Institute for Medical Research, Kuala Lumpur, Ministry of Health Malaysia, for support in the research and publishing of this paper (NMRR ID-25-01003-X3Z). The funders had no role in the study design, data collection, analysis, or preparation of the manuscript.

Conflicts of Interest: Authors have no conflict of interest to declare

CITATION

Md Yusuf, N. A., Aziz, D. I. H., Bohari, R., Zainal, M., Abdullah, N., Amir, N. F., Yaacob, A., Salim, N. O., & Hisam, S. (2026). Two lenses, one pathogen: Complementing microscopy with molecular investigation for accurate microsporidia diagnosis. *International Medical Research Journal*, (Special Issue), 1–20. [https://doi.org/10.63719/imrj.2026.01\(SE\).01](https://doi.org/10.63719/imrj.2026.01(SE).01)

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SUPPLEMENTARY FILES

Supplementary Table 1. Identity similarity percentage of sequences obtained from the positive PCR products to the reference sequences available in GenBank.

ID No.	Primer Design	Per. Ident. (%)	Accession Number	Description
EB 031	EBIEF1	100	MH027450	<i>E. bienewi</i> isolate 18SSU rRNA voucher UMB27 small subunit ribosomal RNA gene, partial sequence
EB 037	EBIEF1	99.64	MG976584	<i>E. bienewi</i> isolate GV6110 small subunit ribosomal RNA (SSU) gene, partial sequence
EB 043	EBIEF1	99.82	JQ769381	<i>E. bienewi</i> isolate Zhengzhou small subunit ribosomal RNA gene, partial sequence
EB 099	EBIEF1	99.64	MH027649	<i>E. bienewi</i> isolate 18SSU rRNA voucher UMB08 small subunit ribosomal RNA gene, partial sequence
EB 069	PMP1 (V1)	99.03	MG655310	<i>E. bienewi</i> isolate ZRC/JUMS7 18S ribosomal RNA gene, partial sequence
EB 083	EBIEF1	99.64	MH027649	<i>E. bienewi</i> isolate 18SSU rRNA voucher UMB08 small subunit ribosomal RNA gene, partial sequence
EB 084	EBIEF1	99.82	MH027456	<i>E. bienewi</i> isolate 18SSU rRNA voucher UMD14 small subunit ribosomal RNA gene, partial sequence
EB 108	EBIEF1	99.64	MG976584	<i>E. bienewi</i> isolate GV6110 small subunit ribosomal RNA (SSU) gene, partial sequence
M031	PMP1 (V1)	99.06	OQ023044	<i>E. bienewi</i> small subunit ribosomal RNA gene, partial sequence; isolate N1 small subunit ribosomal RNA gene, partial sequence.
M037	PMP1 (V1)	99.05	MG646335	<i>E. bienewi</i> isolate ZRC/JUMS6 18S ribosomal RNA gene, partial sequence



M043	PMP1 (V1)	99.51	MG646335	<i>E. bieneusi</i> isolate ZRC/JUMS6 18S ribosomal RNA gene, partial sequence
M069	PMP1 (V1)	99.03	MG655310	<i>E. bieneusi</i> isolate ZRC/JUMS7 18S ribosomal RNA gene, partial sequence
M099	PMP1 (V1)	99.51	EU828594	Uncultured <i>Enterocytozoonidae</i> isolate 001 small subunit ribosomal RNA gene, partial sequence
M083	PMP1 (V1)	99.51	MG646335	<i>E. bieneusi</i> isolate ZRC/JUMS6 18S ribosomal RNA gene, partial sequence
M084	PMP1 (V1)	99.41	MT279646	<i>E. bieneusi</i> isolate N1 small subunit ribosomal RNA gene, partial sequence
M108	PMP1 (V1)	98.96	MT473999	<i>E. bieneusi</i> isolate SCAU8890 small subunit ribosomal RNA gene, partial sequence

Note. Per. Ident.: Percentage of identity.

Supplementary Table 2. Homology of sequences of non-human microsporidia when aligned to sequences obtained from M-isolates.

Species	Host	Accession number	PCR product and identity percentage (%)							
			M031	M037	M043	M069	M083	M084	M099	M108
<i>Enterocytozoon bieuveusi</i>	Cattle (<i>Bos taurus</i>)	MT279646	98.88	98.31	97.77	96.32	97.77	97.77	97.21	97.19
<i>Enterocytozoon hepatopenaei</i>	Shrimp (<i>Penaeus vannamei</i>)	MH124059	88.07	88.07	88.07	87.57	88.07	87.57	88.07	87.57
<i>Enterospora canceri</i>	Edible crab (<i>Cancer pagarus</i>)	HE584634	88.94	89.15	88.35	87.92	88.35	87.92	88.35	87.86
<i>Enterospora nucleophila</i>	Gilt-head bream fish (<i>Sparus aurata</i>)	KF135645	85.45	85.44	85	84.33	85.44	85.02	85.44	84.95
<i>Obruspora papernae</i>	Blotchfin dragonet (<i>Callionymus filamentosus</i>)	HG005137	84.95	85.10	85.02	83.94	85.02	84.54	84.95	84.47
<i>Globulispora mitoportans</i>	Water flea (<i>Daphnia pulex</i>)	KT762153	88	88	88	82.11	88	87.50	88	84.95

Supplementary Table 3. Average pairwise genetic distances table of *E. bieneusi* isolates from this study compared to *E. bieneusi* isolates from various countries

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
(1) ▲ EB isolates (this study)																	
(2) Mexico - Human AIDS	0.00																
(3) Malaysia - Human Immunocompromised	0.00	0.00															
(4) China - Human Diarrhoea symptom	0.00	0.00	0.00														
(5) China - Sheep	0.00	0.00	0.00	0.00													
(6) China - Cynomolgus monkey	0.00	0.00	0.00	0.00	0.00												
(7) Australia - Grey kangaroo	0.00	0.00	0.00	0.00	0.00	0.00											
(8) China - Wastewater	0.00	0.00	0.00	0.00	0.00	0.00	0.00										
(9) China - Pig	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00									
(10) ● M isolates (this study)	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.83								
(11) UAE - Falcon	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.00							
(12) India - Human Renal transplant patient	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.00	0.00						
(13) Iran - Human Gastrointestinal disorder	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.00	0.01	0.01					
(14) The Netherlands - Pigeon	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.84	0.01	0.00	0.00	0.01				
(15) Malaysia - Pig	1.59	1.59	1.59	1.59	1.59	1.59	1.59	1.59	1.59	1.74	1.74	1.74	1.74	1.75			
(16) <i>E. bieneusi</i> genotype CSK2	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.41	1.56	1.57	1.57	1.57	1.57	1.48		
(17) <i>E. hellem</i>	2.01	2.01	2.01	2.01	2.01	2.01	2.01	2.01	2.02	2.17	2.18	2.18	2.18	2.18	2.09	1.52	

Note. *E. bieneusi* genotype CSK2 and *E. hellem* are used as the outgroup.