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Review

Potential impacts of host specificity on zoonotic or interspecies transmission of *Enterocytozoon bieneusi*

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ABSTRACT

Microsporidia are composed of a highly diverse group of single-celled, obligate intracellular fungi that colonize an extremely wide range of other eukaryotes, among which *Enterocytozoon bieneusi* is the most common species responsible for human microsporidiasis. Genotyping of *E. bieneusi* based on sequence analysis of the ribosomal internal transcribed spacer (ITS) has recognized ~500 genotypes in humans and a great variety of other mammals and birds. Those genotypes vary in genetic or hereditary characteristics and form 11 genetic groups in phylogenetic analysis of the ITS nucleotide sequences. Some of genotypes in Group 1 (e.g., D, EbpC, and type IV) and Group 2 (e.g., BEB4, BEB6, I, and J) have broad host and geographic ranges, constituting a major risk for zoonotic or cross-species transmission. By contrast, host specificity seems common in Group 3 to Group 11 whose members appear well adapted to specific hosts and thus would have minimal or unknown effects on public health. Multilocus sequence typing using the ITS, three microsatellites MS1, MS3, and MS7, and one minisatellite MS4, and population genetic analysis of Group 1 isolates reveal the occurrence of clonality, potential host adaptation, and population differentiation of *E. bieneusi* in various hosts. Nonetheless, it is still highly desirable to explore novel genetic markers with enough polymorphisms, to type complex or unstructured *E. bieneusi* populations of various host species and geographic origins, notably those belonging to Group 2 to Group 11. Additional population genetic and comparative genomic data are needed to elucidate the actual extent of host specificity in *E. bieneusi* and its potential impacts on zoonotic or interspecies transmission of microsporidiasis.

1. Introduction

Microsporidia comprise a broad and diverse range of protozoan-like fungi (nearly 1500 species) that colonize a variety of host taxa including protists, bryozoans, helminths, aquatic and terrestrial arthropods, reptiles, mammals, and birds; infections of microsporidia have been historically documented to negatively affect silkworm, honeybee, fur animal, and aquatic industries and lead to considerable economic losses (Stentiford et al., 2016; Vavra and Lukes, 2013). Among 17 human- pathogenic microsporidian species, *Enterocytozoon bieneusi* is the most prevalent one, responsible for opportunistic infections in AIDS patients and other immunodeficient individuals (organ transplant recipients, children, the elderly, etc.), and resulting in gastralgia, malabsorption, and diarrhea (Matos et al., 2012; Weiss and Becnel, 2014). Nevertheless, tolerant parasitism by *E. bieneusi* seems widespread since its colonization in immunocompetent hosts commonly yields no observable or limited health impacts (Thellier and Breton, 2008). In addition to humans, the organism can infect a broad array of other mammals and birds, raising a concern for zoonotic transmission (Li et al., 2019; Santin and Fayer, 2011). Humans and nonhuman animals are also at high risks for exposure to waterborne, foodborne, and environmentally transmitted *E. bieneusi* infections (Hu et al., 2014; Mathis et al., 2005; Stentiford et al., 2016). *E. bieneusi* features a small spore size around 1 μm and shape variation; it is difficult to unravel the biological and phenotypic properties of *E. bieneusi* due to lack of in vitro culture method (Li et al., 2019; Santin and Fayer, 2011; Thellier and Breton, 2008). *E. bieneusi* has a small genome (about 6 megabases); severe reduction of genes involved in self-energy production signifies the strong host dependence of this organism (Akiyoshi et al., 2009; Keeling and Corradi, 2011; Keeling et al., 2010).

Among the diagnostic assays developed for *E. bieneusi*, PCR is indeed a reliable and sensitive one (Ghosh et al., 2014; Kaushik et al., 2018). Coupled with sequencing of PCR amplicons and subsequent polymorphism analysis, it can act as a genotyping tool to help us track down

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the infection sources and transmission routes (Li et al., 2019; Santin and Fayer, 2011; Thellier and Breton, 2008). The ribosomal internal transcribed spacer (ITS) is highly polymorphic and informative, which represents a proper genotyping marker for elucidation of the extreme genetic diversity and phenotypic heterogeneity within *E. bieneusi* (Santin and Fayer, 2011). A number of studies have used the ITS and/or several mini- and microsatellite loci to examine the genetic diversity, phylogenetic pattern, population structure, genetic segregation of populations by hosts and regions, host adaptation, zoonotic potential, and public health implications of *E. bieneusi* (Dashti et al., 2019; Feng et al., 2011; Greigert et al., 2018; Guo et al., 2014; Henriques-Gil et al., 2010; Karim et al., 2014b; Kicia et al., 2016; Li et al., 2013; Li et al., 2012b; Li et al., 2016b; Li and Xiao, 2019; Santin et al., 2018; Wan et al., 2016; Wang et al., 2019; Widmer and Akiyoshi, 2010; Widmer et al., 2013; Yue et al., 2017). The aim of the present review is to integrate advances in *E. bieneusi* ITS genotyping and multilocus sequence typing (MLST), for the purpose of understanding the inter- and intragroup host specificity of this pathogen and its implications for zoonotic or interspecies transmission.

2. ITS genotyping and phylogenetic groups

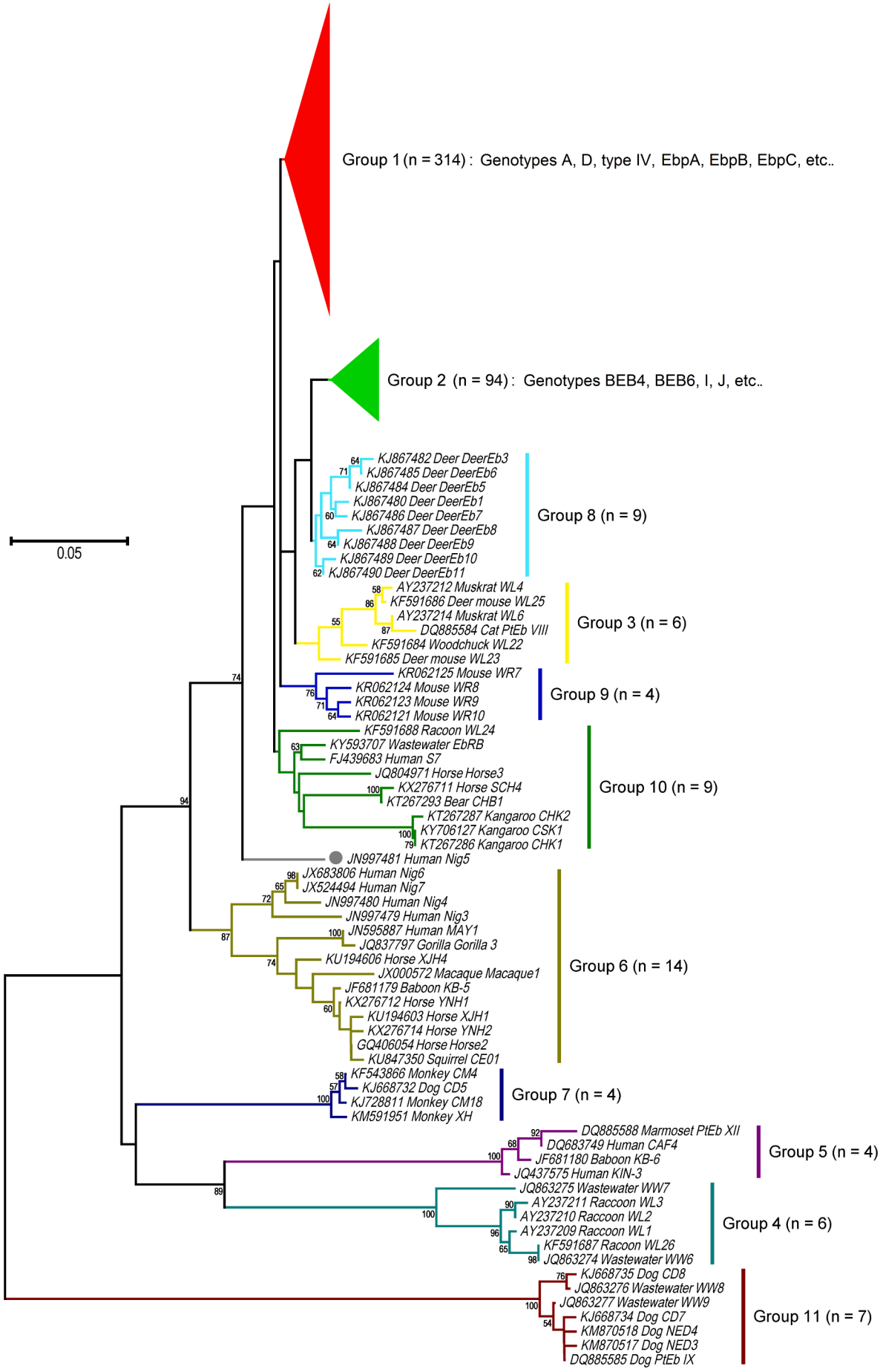
Nowadays, the ITS-based genotyping is becoming the standard method to accurately compare *E. bieneusi* isolates from a global perspective, since this maker shows a high degree of variability and it is the only fragment covered by most existing primer sets, especially the three most frequently used ones shown in Table 1: MSP1/MSP2B and MSP3/MSP4B, EBITS3/EBITS4 and EBITS1/EBITS2.4, and AL4037/AL4039 and AL4038/ALA040 (Li et al., 2019; Santin and Fayer, 2011). Nevertheless, awareness should be raised in regards to naming a new genotype. A conference paper came up with the current naming convention we use to avoid that some genotypes with identical ITS sequences receive multiple names; it proposed that only the polymorphisms at the ITS region should be taken into account in designating new genotypes, and the first published names are the preferential ones which should be used in the following studies (Santin and Fayer, 2009). A recent review has summarized and compared all the nucleotide sequences deposited in GenBank that include the entire *E. bieneusi* ITS region (Li et al., 2019). Through polymorphism analysis of the ITS, almost 500 *E. bieneusi* genotypes were validated according to the established naming standard (Santin and Fayer, 2009) and an updated list of genotypes with overlapping names (Li et al., 2019). To illuminate the genetic diversity and relationships among *E. bieneusi* isolates from various sources, an extensive phylogenetic analysis of all valid ITS genotypes was conducted and published by Li, Feng, and Santin, in June 2019 (Li et al., 2019), identifying 11 major genetic groups (Group 1 to Group 11) which show varied levels of host specificity (Fig. 1). The clustering patterns correlate largely with those as described (da Silva Fiuza et al., 2016; Guo et al., 2014; Karim et al., 2014a; Li et al., 2015).

Some of genotypes in Group 1 (e.g., D, EbpC, and type IV) and Group 2 (e.g., BEB4, BEB6, I, and J) can affect a diverse range of hosts (humans, livestock, companion animals, wild mammals, and birds) on a global scale, accounting for a high probability of zoonotic and cross-species transmission risks, whereas some others, such as genotypes B, C, and EbpB in Group 1 and genotypes BEB3, CEbA, and CEbF in Group 2, seem to have limited public health impacts as they have been confined to specific hosts since they were reported ten or more years ago (Li et al., 2019). By comparison, host specificity appears more common in Group 3 to Group 11 because an overwhelming majority of their group members, such as genotypes WL6 and PtEb VIII in Group 3, genotypes WL1 and WL3 in Group 4, genotypes CAF4 and KIN-3 in Group 5, and genotypes MAY1 and Nig3 in Group 6, have thus far been reported only in the hosts from which they were originally isolated (Li et al., 2019). Strikingly, intra-host difference in host specificity was observed in individuals with different pathological backgrounds living in the Netherlands, with genotype B found exclusively in HIV-positive patients and genotype C exclusively in kidney transplant recipients (ten Hove et al., 2009). Furthermore, the predominance of genotype BEB4 in cattle and

Table 1
Common genetic markers and nested PCR primer sets used in molecular typing of *Enterocytozoon bieneusi*.

Target[a]	Primer sequence (5′ to 3′)	Size (bp)	Usage[b]; polymorphism[c]	Reference
ITS	**MSP1**: TGAATG(G/T)GTCCCTGT **MSP2B**: GTTCATTCGCACTACT **MSP3**: GGAATTCACACCGCCCGTC(A/G)(C/T)TAT **MSP4B**: CCAAGCTTATGCTTAAGTCCAGGG	~508	Genotyping; SNPs and INDELs	Katzwinkel-Wladarsch et al. (1996); Rinder et al. (1997)
	EBITS3: GGTCATAGGGATGAAGAG **EBITS4**: TTCGAGTTCTTTCGCGCTC **EBITS1**: GCTCTGAATATCTATGGCT **EBITS2.4**: ATCGCCGACGGATCCAAGTG	~390	Genotyping; SNPs and INDELs	Buckholt et al. (2002)
	AL4037: GATGGTCATAGGGATGAAGAGCTT **AL4039**: AATACAGGATCACTTGGATCCGT **AL4038**: AGGGATGAAGAGCTTCGGCTCTG **ALA040**: AATATCCCTAATACAGGATCACT	~392	Genotyping; SNPs and INDELs	Sulaiman et al. (2003)
MS1	**F1**: CAAGTTGCAAGTTCAGTGTTTGAA **R1**: GATGAATATGCATCCATTGATGTT **F2**: TTGTAAATCGACCAAATGTGCTAT **R2**: GGACATAAACCACTAATTAATGTAAC	~676	MLST; SNPs and trinucleotide TGC, TAA and TAC repeats	Feng et al. (2011); Li et al. (2012b); Wan et al. (2016)
MS3	**F1**: CAAGCACTGTGGTTACTGTT **R1**: AAGTTAGGGCATTTAATAAAATTA **F2**: GTTCAAGTAATTGATACCAGTCT **R2**: CTCATTGAATCTAAATGTGTATAA	~537	MLST; SNPs and dinucleotide TA repeats	Feng et al. (2011); Li et al. (2012b); Wan et al. (2016)
MS4	**F1**: GCATATCGTCTCATAGGAACA **R1**: GTTCATGGTTATTAATTCCAGAA **F2**: CGAAGTGTACTACATGTCTCT **R2**: GGACTTTAATAAGTTACCTATAGT	~885	MLST; SNPs, INDELs, and tetranucleotide GGTA repeats	Feng et al. (2011); Li et al. (2012b); Wan et al. (2016)
MS7	**F1**: GTTGATCGTCCAGATGGAATT **R1**: GACTATCAGTATTACTGATTATAT **F2**: CAATAGTAAAGGAAGATGGTCA **R2**: CGTCGCTTTGTTTCATAATCTT	~471	MLST; SNPs and trinucleotide TAA repeats	Feng et al. (2011); Li et al. (2012b); Wan et al. (2016)

[a] ITS, the ribosomal internal transcribed spacer; MS1/3/7, microsatellite loci 1/3/7; MS4, minisatellite locus 4.
[b] MLST, multilocus sequence typing.
[c] SNPs, single nucleotide polymorphisms; INDELs, insertions and deletions.



(caption on next page)

Fig. 1. Phylogenetic diagram showing the inferred genetic relationships among all currently valid *Enterocytozoon bieneusi* ITS genotypes. The neighbor-joining tree was constructed based on genetic distances measured using the Kimura two-parameter model implemented in software Mega 7 (http://www.megasoftware.net/). Two outlier genotypes, CSK2 (GenBank accession no. KY706128) and SW3 (GenBank accession no. KF591679), were not considered in the analysis as they are highly divergent from the others. Another outlier genotype Nig5 was indicated by grey circle. The tree was rooted with a dog-derived genotype PtEb IX with GenBank accession no. of DQ885585. Bootstrap values above 50% from 1000 pseudoreplicates were shown at the nodes.

genotype BEB6 in other ruminant species (e.g., sheep, goats, and deer) has been remarked (Li et al., 2019). However, the current findings showing phenotypic differences among genotype groups and genotypes, need to be validated by analysis of more epidemiological data generated from various hosts sampled in the same locations. Recently, there has been a rapid increase in the number of newly identified genotypes in Group 1 and Group 2, whose host-specific traits remain uncertain.

3. MLST and subpopulations in ITS Group 1

The ITS currently meets the criteria for genotyping (Li et al., 2019; Santin and Fayer, 2011), however, it represents merely a very small portion (~243 bp in size) of *E. bieneusi* genome (Akiyoshi et al., 2009; Keeling et al., 2010). It is often unable to present subtle changes needed for subgrouping and accordingly might not adequately represent the evolutionary history of this fungus, highlighting the necessity and importance of characterizing other genetic markers and using concatenated loci for high-resolution typing (Li and Xiao, 2019). Feng et al. searched three large scaffolds and 1743 contigs from the Whole Genome Shotgun Project of an *E. bieneusi* isolate (GenBank accession no. ABGB00000000), for mini- and microsatellites that harbor short ($\leq$6 bp) or long ($>$ 6 bp) tandem repeat units (Feng et al., 2011). Seven targets were available from the searches, four (MS1, MS3, MS4, and MS7; Table 1) of which were tested to be eligible for intraspecific typing of *E. bieneusi* (Feng et al., 2011). Combined use of five loci for typing manifests significantly higher resolution than when only the ITS was used (Li and Xiao, 2019). Several MLST studies investigated genetic differentiation among *E. bieneusi* isolates (belonging majorly to Group 1) of different host origins (humans, nonhuman primates (NHPs), pigs, and farmed fur-bearing animals) at loci ITS, MS1, MS3, MS4, and MS7, recognizing quite a number of multilocus genotypes (MLGs) that varied in genotype frequency (Karim et al., 2014b; Li et al., 2013; Li et al., 2012b; Li et al., 2016b; Wan et al., 2016). Most of the MLGs identified differ from each other by hosts and regions, while their genetic relationship needs to be further elucidated (Li and Xiao, 2019). Yet, most other studies frequently failed to amplify all loci and the MLGs determined are limited in number (Deng et al., 2017; Wang et al., 2016; Zhong et al., 2017), which may be attributable to the hypermutation in *E. bieneusi* genome that hindered some isolates from effectively amplifying, especially those pertaining to Group 2 to Group 11 (Li and Xiao, 2019). It is remarkable that, using the MLST tool, frequent mixed *E. bieneusi* infections were identified in children residing in Uganda (Widmer et al., 2013).

MLST, phylogenetic and substructural analyses, and population genetic analysis have allowed subtle differentiation among isolates within Group 1 and evaluation of the extent of genetic variation among MLGs, leading to formation of several subpopulations named SP1 to SP7 (Fig. 2) (Li and Xiao, 2019). The overall *E. bieneusi* population that undergoes clonal propagation from HIV-infected adults in Peru was separated into subdivisions SP1 and SP2 as described previously (Li et al., 2012b). Reproductive clonality appears to be present also in human *E. bieneusi* populations in India and Nigeria and the MLGs generated fall into the existing SP1 and SP2 with no clear genetic segregation by regions found; several MLGs derived from captive baboons in Kenya were also grouped in SP1 (Li et al., 2013; Li et al., 2011). Two additional studies compared human-derived SP1 and SP2 with those identified in domestic pigs (SP3, SP4, and SP5) and farmed fur-bearing animals (SP6) in China, demonstrating widespread occurrence of clonality in *E. bieneusi* and apparent population differentiation

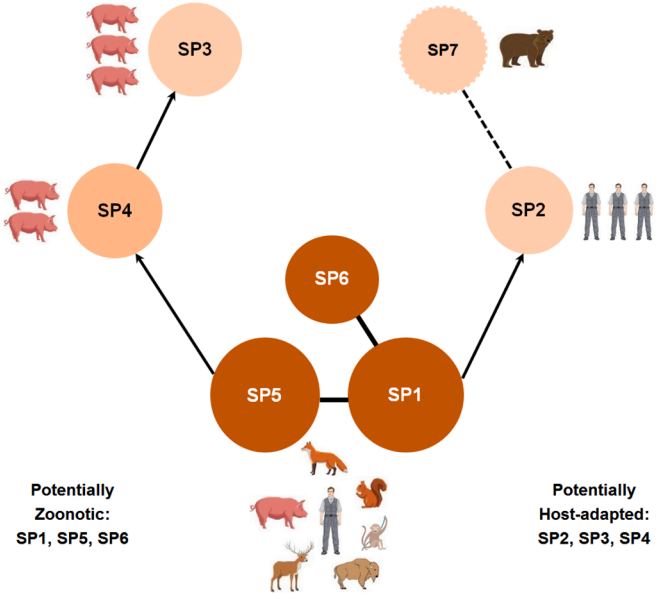


Fig. 2. Diagram showing *Enterocytozoon bieneusi* subpopulations and their potential phenotypic difference. SP1 and SP2 are subpopulations derived from humans and/or nonhuman primates in Peru, Nigeria, India, and Kenya as previously described (Li et al., 2013; Li et al., 2012b). SP3 to SP6 originate from pigs and fur animals in China in the former studies (Li et al., 2016b; Wan et al., 2016). Host-specific traits and zoonotic potential of subpopulations were discussed in a recent review (Li and Xiao, 2019). The clustering of multilocus genotypes isolated recently from cat, deer, takin, and squirrel in China in potentially zoonotic SP1/SP5 has also been noted (Li and Xiao, 2019). SP7 originating from captive bears in China is highlighted with a dashed border as its host specificity and public health implication remain to be elucidated (Deng et al., 2017; Li et al., 2016a).

of this pathogen by host species (Li et al., 2016b; Wan et al., 2016). However, it is as yet unknown if geography played a synergistic role in genetic divergence of *E. bieneusi* populations between humans and nonhuman animals. SP7 originates from captive bears in China (Deng et al., 2017; Li et al., 2016a), which is explicitly divergent from SP1 to SP6 (Li and Xiao, 2019).

4. Host specificity at the ITS and MLST levels

Analysis of host range variation of ITS genotypes among genetic groups has revealed intra- and intergroup host specificity in *E. bieneusi*, with Group 1 currently considered as possessing zoonotic significance as its host specificity is not strong, Group 2 possessing increasing zoonotic concern as its host specificity is not so strong, and Group 3 to Group 11 possessing limited or unknown zoonotic importance as their host specificity is strong (Li et al., 2019). MLST and population genetic data have provided some new insights into the difference in host specificity among subpopulations in potentially zoonotic Group 1 (Li and Xiao, 2019). The closely related SP1 and SP5 that contain mainly generalist genotypes D, type IV, and EbpC, are genetically separate from SP2, SP3, and SP4 made up of majorly Group 1 genotypes A, EbpA, and EbpB with narrow host and geographic ranges (Wan et al., 2016). The strong or complete linkage disequilibria (LD) and a very low number of genetic recombination events inferred in SP2 to SP4 are indicative of possible occurrence of host adaptation in the three

subpopulations (Wan et al., 2016). In contrast, the weakened or incomplete LD and high genetic diversity in SP1 and SP5 might be results of a broad spectrum of hosts and the capability to adapt to new host environments (Wan et al., 2016). It could be speculated that the isolates in SP1 and SP5 with clonal structure might be accountable for zoonotic or cross-species transmission and accordingly should be considered a public health threat. On the other hand, the isolates in SP2, SP3, and SP4 with epidemic structure or host-adapted traits might be dominantly restricted to specific hosts (such as SP2 to humans and SP3 and SP4 to pigs), as deduced from the rapid expansion of advantageous MLGs in some of these subpopulations (Heitman, 2010; Li et al., 2013; Li et al., 2012b; Wan et al., 2016). It is not entirely clear if host adaptation occurred in SP6 due to the small sample size, however, measurement and comparison of levels of LD and genetic diversity between SP6 and SP1/SP2 implied a certain level of cross-species capability in the subpopulation (Li et al., 2016b). Several *E. bieneusi* isolates genotyped at the ITS as SC01 and SC02 in Group 1 form a new subpopulation named SP7 (Li and Xiao, 2019). SP7 might be specific to bears since it is genetically linked to human-adapted SP2 but highly segregated from SP1 and SP5 with zoonotic or cross-species potential. In addition, the placement of a human *E. bieneusi* MLG in SP4, and the grouping of MLGs identified recently from cat, deer, takin, and squirrel in China in potentially zoonotic SP1/SP5, upheld zoonotic nature of some *E. bieneusi* isolates (Li and Xiao, 2019).

5. Public health concerns

Current epidemiologic and population genetic data indicate the possible existence of 11 ITS phylogenetic groups (Fig. 1) and seven subpopulations (Fig. 2) within ITS Group 1, which exhibit phenotypic difference in host specificity and zoonotic potential. Among numerous Group 1 members, genotypes D, EbpC, and type IV represent the most frequent contributors to *E. bieneusi* infections in almost all surveyed hosts including humans and thus may be the greatest threats to public health; a low level of host specificity in those genotypes constitutes a major risk for zoonotic or interspecies transmission and this is supported by MLST and population genetic evidences (Li et al., 2019; Li and Xiao, 2019). Group 2 consists of genotypes that infect mainly ruminants, while some (BEB4, BEB6, I, and J) of them recently have widely expanded their host ranges; nonrigid host specificity in those popular genotypes is of an increasing public health concern (Li et al., 2019). However, most genotypes in Group 3 to Group 11 and the outlier genotypes are usually adapted for a life in specific hosts and thus may cause no or minimal harm to public health or safety (Li et al., 2019).

While identification of *E. bieneusi* strains with identical ITS or multilocus sequences among humans, nonhuman mammals, and birds, could constitute some evidence for zoonotic or interspecies transmission of *E. bieneusi*, the relevant clinical or epidemiological risk factors and the potential transmission pathways have rarely been assessed in lots of previous works (Heyworth, 2017; Li and Xiao, 2019). Nonetheless, several studies highlighted probable risk factors for human infection with *E. bieneusi*. For instance, acquisition of *E. bieneusi* infections in human subjects has been linked to close contacts with cattle feces and individuals with diarrhea in Zimbabwe and household pigs in China (Gumbo et al., 1999; Wang et al., 2013); sharing a unique genotype Peru16 in both Peruvian children and guinea pigs might be associated with their presence in the same household (Cama et al., 2007); co-occurrence of zoonotic genotypes D and H in farmed pigs and the individuals living near the farms where pig fecal specimens were sampled, implies large likelihood of interspecies transmission occurring in Thailand (Prasertbun et al., 2017); acquiring a potentially human-adapted genotype A in newly admitted children in a Thai orphanage could be ascribed to their exposures to other individuals who already carry this genotype (Leelayoova et al., 2005; Pagornrat et al., 2009).

Although most of the mammalian and avian hosts investigated can harbor human-pathogenic ITS genotypes, pigs and NHPs are generally regarded as the most important reservoirs for human *E. bieneusi* affections, due to their higher carriage rate of genotypes D, EbpC, and type IV of public health significance compared to other hosts (Li et al., 2019). Some corroborative evidence has come from successful experimental infection of immunosuppressed rhesus monkeys and piglets with *E. bieneusi* strains of human origins (Kondova et al., 1998; Tzipori et al., 1997). It is striking to note the varying patterns of genotype distribution of *E. bieneusi* in NHPs, with laboratory NHPs bred indoors (having daily and close contact with their keepers) uniquely affected by zoonotic Group 1 genotypes, and wild and zoo NHPs (commonly separated from the impacts of human activities) by both zoonotic and host-adapted genotypes; isolation from human activities may have facilitated circulation of host-adapted genotypes in the latter (Li et al., 2019).

Frequent detection of the generalist genotypes D, EbpC, and type IV in various water types raises the possibility of occurring human or nonhuman animal *E. bieneusi* infections via waterborne dispersal of spores (Hu et al., 2014; Huang et al., 2017; Li et al., 2012a). The coexistence in rhesus monkeys and lake water of human-pathogenic genotypes Peru11, WL15, EbpC, and type IV in a public park, China was described; direct or close contacts with infected animals or contaminated water would increase the risk for cross-species or waterborne transmission of microsporidiosis (Ye et al., 2012). That a renal transplant recipient in France was infected with an unusual and highly divergent genotype MAY1 (genetically related to potentially horse-adapted genotype Horse2) may result from close contact with water contaminated with horse stool (Pomares et al., 2012). Detection of *E. bieneusi* in milk and various types of fresh retail produce poses a threat to foodborne infection (Jedrzejewski et al., 2007; Lee, 2008). Circumstantial evidence for foodborne transmission of *E. bieneusi* has come from an outbreak study performed in Sweden; immunocompetent individuals were confirmed to be affected by *E. bieneusi* genotype C (Decraene et al., 2012). Yet despite these considerable advances, how *E. bieneusi* is transmitted needs further elaboration in the future.

6. Conclusion and perspectives

Analysis of genetic polymorphisms of the ITS and several mini- and microsatellites has improved our appreciation of genetic diversity within *E. bieneusi* and extended our understanding of the epidemiology (infection sources and transmission routes) and population genetic aspects of this ubiquitous pathogen. Although *E. bieneusi* has the capability of adapting and developing in different hosts, varied levels of genetic diversity and host specificity by ITS and MLST groups was uncovered, which carries implications for zoonotic or interspecies transmission of *E. bieneusi*. Nevertheless, the potential factors facilitating adaptation of different *E. bieneusi* genotypes to specific hosts or host changes remain uncertain. Development of more effective polymorphic markers and application of whole genome sequencing to *E. bieneusi* typing are necessary since the current MLST tool can not be applied to all *E. bieneusi* isolates. More MLST or comparative genomic data are required to analyze population structure of *E. bieneusi* in the context of host and geographical factors in order to unveil actual impacts of host specificity on zoonotic or interspecies transmission of *E. bieneusi*.

Declaration of Competing Interest

None.

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