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Enterocytozoon bieneusi in Italian water buffalo calves (*Bubalus bubalis*): An exploratory haplotype analysis within Bovidae family in the European context☆

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ABSTRACT

Microsporidia are an important group of emerging opportunistic parasites in human and non-human animals, with possible zoonotic potential; however, no data are currently available on their presence in commonly farmed species in Italy, particularly regarding *Enterocytozoon bieneusi* in water buffalo (*Bubalus bubalis*). This preliminary study investigated the prevalence and genetic variability of *E. bieneusi* in water buffalo calves in southern Italy. Additionally, given its spread among other members of the Bovidae family, a haplotype-level network analysis was performed using *E. bieneusi* sequences available in GenBank from two of the most commonly farmed Bovidae species in Europe: cattle (*Bos taurus*) and water buffalo (*B. bubalis*). The survey was conducted on four farms between September and December 2023 for a total of 37 buffaloes sampled. DNA extracted from collected faeces was subjected to molecular analysis amplifying ITS region. For the molecular characterization, a phylogenetic analysis was performed by Maximum Likelihood method. For the comparison between our sequences and those available from water buffaloes and cattle from Europe, a haplotype analysis was conducted to obtain a network calculation. Five samples from Italian *B. bubalis* tested positive for *E. bieneusi* with an overall prevalence value of 13.5 %. Phylogenetic analysis assigned isolates to three genotypes (YNDCEB-90; A; I) which were shared among different hosts, including humans and clustering in the phylogenetic Group 1 and Group 2. The network analysis identified Hp9 as the most frequently detected haplotype, distributed across multiple countries, including Italy. The second most common haplotype, Hp12, was exclusively found in Italy and Turkey from *B. bubalis*. The differences in haplotype patterns observed between *B. taurus* and *B. bubalis* could shed light on the species-specific interactions of *E. bieneusi*. The prevalence observed, along with the detection of zoonotic genotypes in water buffaloes, could pose a potential public health concern. The associated risk extends beyond the direct contact with infected animals or their faeces, encompassing possible contamination of the food chain and the environment, including ground and surface water sources.

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1. Introduction

The domestic buffalo (*Bubalus bubalis*), also known as the water buffalo, is a species of the Bovidae family, widely distributed across Europe and Asia. It is a key livestock species primarily farmed for meat and high-fat milk production, due to its resilience, adaptability, and superior feed efficiency compared to cattle (Minervino et al., 2020). For this reason, the water buffalo farming is important for the economy of several countries, including Italy, where Mozzarella cheese manufacturing from milk of water buffalo is third-ranked in sales volume (https://news.italianfood.net).

From a public health perspective, water buffaloes in Italian farming systems are susceptible to various infectious diseases, particularly intestinal protozoa (Cacciò et al., 2007; Rinaldi et al., 2007; Gabrielli et al., 2021). Additionally, they have been identified as potential reservoirs for the transmission of Microsporidia (Qin et al., 2022).

The phylum Microsporidia comprises a varied group of unicellular obligate intracellular parasites. They were formerly thought to be primitive early branching protozoa, but phylogenetic analyses revealed that they are associated with the lowest branch of the Fungi Kingdom, the Cryptomycota. Microsporidia target several taxa of invertebrate and vertebrate hosts, with a zoonotic potential (Han et al., 2021). Seventeen species of the roughly 200 genera and 1500 species included in the phylum are confirmed to cause infections in humans, with *Enterocytozoon bieneusi* as the most often diagnosed; it causes the 90 % of opportunistic infections in individuals with compromised immune systems, such as those with AIDS, organ transplant recipients, cancer patients, as well as, young children, and the elderly (Li et al., 2019).

The transmission occurs through the faecal-oral route. Resistant spores from animals can contaminate water, dairy products, and vegetables, thus the global increase in cattle and buffalo populations has raised concerns about the potential faecal contamination and microsporidian zoonotic transmission to humans (Qin et al., 2022; Rezaeian et al., 2023).

In immunocompromised individuals the infection may result in chronic acute diarrhea, dyspepsia, respiratory tract inflammation and non-calculus cholecystitis (Han et al., 2021; Qin et al., 2022). There is currently no fully effective treatment for *E. bieneusi* infection in humans, therefore preventing transmission between susceptible hosts appears to be the most suitable approach to reduce the risk of infection (Han et al., 2021).

More than 685 genotypes of *E. bieneusi* have been found so far in 236 different animal species (e.g. pigs, cats, dogs, horses, and particularly cattle) and, based on the internal transcribed spacer (ITS) of rRNA gene's nucleotide sequence, they have been divided into 13 phylogenetic groups (Qin et al., 2022; Jiang et al., 2023).

This study provides the first prevalence and molecular data of *E. bieneusi* in water buffalo calves (*B. bubalis*) from Italy, followed by a haplotype and network analysis involving the currently available *E. bieneusi* sequences retrieved from two of the most common farmed members of the Bovidae family: cattle (*Bos taurus*) and water buffalo (*B. bubalis*) from Europe.

To conduct molecular investigations regarding *E. bieneusi* infection in its hosts and to identify potential risk factors, might be very significant in providing data to prevent the spread of this parasite. Avoiding cross-species transmission, in fact, remains fundamental in terms of public health for the zoonoses management.

2. Materials and methods

2.1. Sampling

In the period September – December 2023, individual fresh faecal samples were collected directly from the rectal ampulla of 37 water buffalo calves as a pilot study. Calves were stabled in single paddocks of 4 farms located in Campania region (southern Italy). The number of accessible animals was limited due to both seasonal constraints and the small number of farms with suitable calf populations. For each animal 10 g of faeces were stored and faecal samples were transferred refrigerated to laboratories of Department of Clinical Sciences and Translational Medicine, Tor Vergata University, for molecular analyses.

2.2. Molecular characterization and phylogenetic analysis

Faecal samples were kept at 4 °C, and the genomic DNA extraction performed within 24 h after collection. Genomic DNA was isolated using the QIAmp DNA stool mini kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. All samples were subjected to a two-step nested PCR protocol amplifying the ITS region (~243 bp) and portions of the flanking large and small subunits of the ribosomal RNA gene, as previously described by Buckholt et al. (2002), obtaining a 390 bp product. To guarantee PCR accuracy and reliability, each PCR run included both positive and negative controls. Amplicons were purified using the mi-PCR Purification Kit (Metabion International AG) and transferred to a separate facility for sequencing (Bio-Fab Research, Rome, Italy). FinchTV software was used for manual forward and reverse sequence checks. Through the Standard Nucleotide BLAST search, the consensus sequences were matched to those in GenBank database to verify isolates identity. Sequences were then aligned by AliView (Larsson, 2014), using reference representative sequences. A Maximum Likelihood (ML) phylogenetic tree was created with IQ-TREE software (Minh et al., 2020) to improve the assessment of genetic diversity among *E. bieneusi* genotypes and to determine genetic relationships between newly identified and previously described genotypes. Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) was used as best model to create the phylogenetic tree, then represented in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). A bootstrap analysis with 1000 replicates was carried out to guarantee the accuracy of the genetic distances.

2.3. European dataset and haplotype analysis

For an appropriate comparison between our sequences and those available from water buffaloes and cattle from Europe, a dataset named DatasetEU was built, encompassing all *E. bieneusi* sequences present in GenBank from *B. taurus* and *B. bubalis* in Europe, including the new sequences obtained in this study. To acquire sequences from GenBank, a multistep strategy was employed by entering the keywords:"*Enterocytozoon bieneusi*" AND/OR"Europe" AND/OR "water buffalo" AND/OR"calves" AND/OR"cattle" AND/OR "*B. taurus*" AND/OR "*B. bubalis*" in the search field of the "Nucleotide database". Only sequences with the 99 % of identity with *E. bieneusi* were considered. In case of ambiguous sequences (unphased data) sequences were excluded from the analysis.

The haplotype analysis for DatasetEU was conducted on polymorphic sites using DnaSP v.6 software and Tajima's D test (Tajima, 1989). PoPART (Population Analysis with Reticulate Trees) genetic software (Leigh and Bryant, 2015) was used to perform the Minimum Spanning Network calculation (Bandelt et al., 1999). The analysis was performed with sequences trimmed to the shortest length with high-quality fragments and sites, considering alignment gaps.

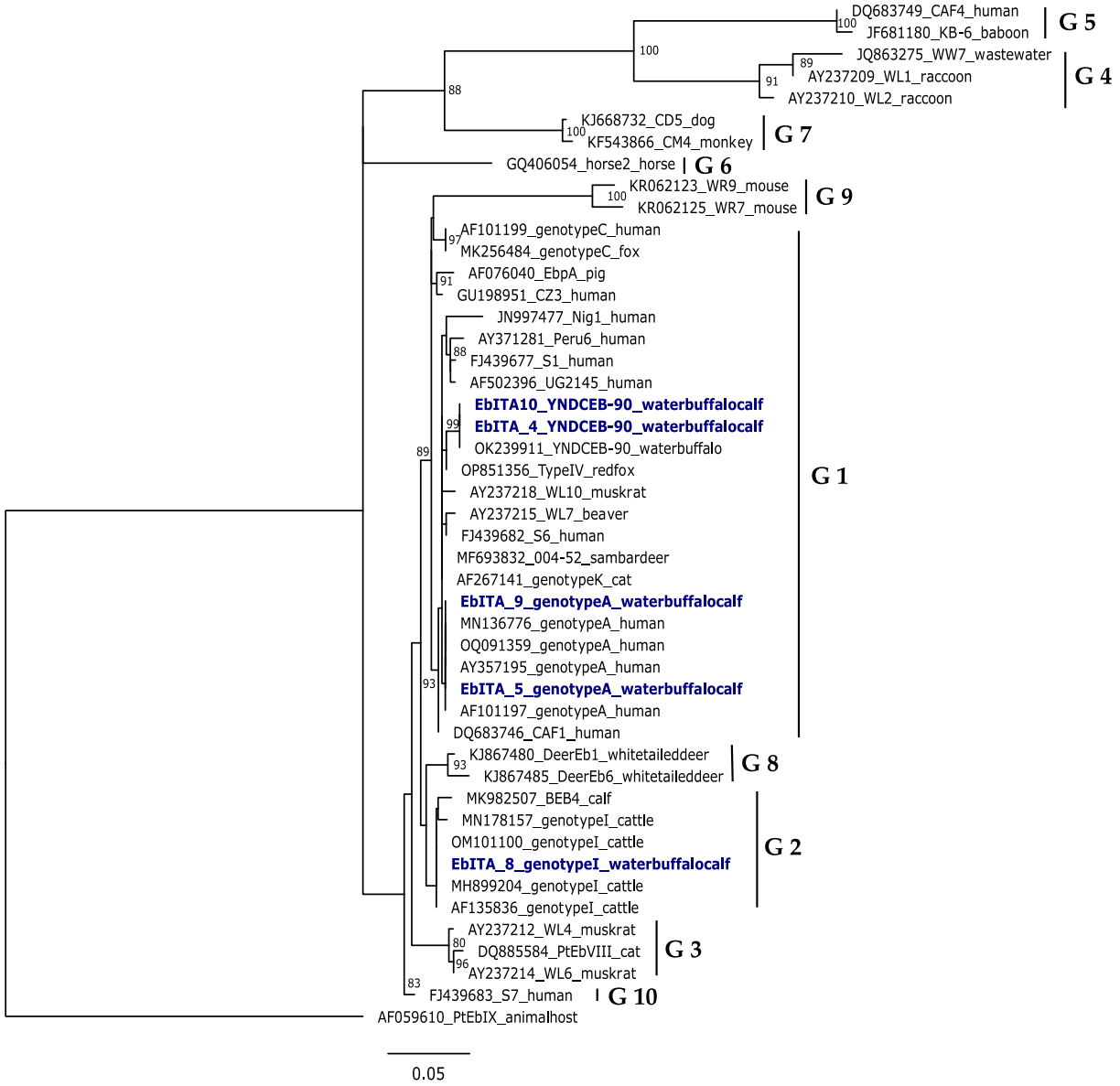

Fig. 1. Phylogenetic tree of *E. bieneusi* ITS rRNA inferred using the Maximum Likelihood (ML) method. Sequences generated in the present study are displayed in bold and blue. Genetic distances were estimated based on the HKY + G4 substitution model. Bootstrap values exceeding 80 % are indicated at the corresponding tree nodes. Accession numbers of sequences retrieved from GenBank are reported alongside their genotype designation, host species, and associated groups. The letter G denotes the phylogenetic group to which each cluster belongs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Molecular characterization and phylogenetic analysis

Following molecular analysis, five samples tested positive for *E. bieneusi* with an overall prevalence value of 13.5 % (5/37), 95 % confidence interval (CI). All positive samples produced high-quality sequences. Two isolates, EbITA_4 and EbITA_10, showed 100 % of identity and 100 % of query coverage with *E. bieneusi* isolates from Turkey and China having *B. bubalis* as host (OR885729, OR885721 and MZ229914 respectively). Other two isolates, EbITA_9 and EbITA_5, were 100 % identical and had 100 % of query coverage with human isolates from Germany, Italy and Spain (AF101197, OR676939, OQ091359). One isolate (EbITA_8) showed 100 % of identity and 100 % of query coverage with several isolates: a rabbit from China (MW790902), *B. taurus* from China (MN178157; OM101100; KU531572), *B. taurus* from Australia (MH899204) and *B. taurus* from Ethiopia (MT231513).

Phylogenetic analysis of ITS region sequences (Fig. 1) revealed the presence of three different genotypes, as follows: YNDCEB-90; A; I. Isolates EbITA_10, EbITA_4, EbITA_9 and EbITA_5 clustered together in the phylogenetic Group 1 (G1), which includes both different animal hosts and humans. In details, EbITA_10 and EbITA_4 form a separated sub-cluster together with isolate OK239911, which also originates from a water buffalo, whereas EbITA_9 and EbITA_5 form a sub-cluster with isolates exclusively from humans. Isolate EbITA_8 is the only one to cluster within phylogenetic Group 2 (G2), together with all isolates originating from *B. taurus* (adult cattle and calves) as host.

Sequences obtained were deposited in GenBank under the accession numbers PV405426-PV405430.

3.2. Haplotype analysis

Through the search and the criteria specified in Materials and Methods (Section 2.3), 38 *E. bieneusi* sequences were retrieved having as hosts the two ruminant species *B. taurus* and *B. bubalis* from Europe, allowing the construction of DatasetEU (Table 1), for a total of 43 sequences including also the five from Italian water buffalo calves obtained in the present study.

The selected region spanned 319 sites. Alignment gaps were included in the evaluation, with 36 variable sites. Regarding haplotype distribution, 14 distinct haplotypes were observed resulting in a haplotypic diversity (Hd) of 0.8184.

The most frequently detected haplotypes (Hp) are haplotype 9, shared by Austria, Czech Republic, Portugal, Germany, and Italy, followed by haplotype 12, found only in Italy and Turkey, and haplotype 11, which is common to several countries (Austria, Germany, Portugal, and Turkey) (Fig. 2). The haplotype analysis revealed that haplotype 9 includes several variations of genotype I, including two "new" genotypes, and it is found both in *B. taurus* cattle and *B. bubalis* calves. On the contrary haplotype 12 is closely related to genotype YNDCEB-90 and seems to be specific of *B. bubalis* as host. Haplotype 11 includes genotype J and is shared between *B. taurus* and *B. bubalis*. Turkey appears to be the country with the highest haplotypic variability, exhibiting numerous variants differentiated by single mutations or pairs of mutations, particularly in the cluster formed by haplotypes Hp2, Hp3, Hp4, and Hp5, all belonging to the "ERUSS" genotype macrogroup (Fig. S1).

Table 1
DatasetEU, which includes all available *E. bieneusi* sequences from European *B. taurus* and *B. bubalis*, as well as those from the present study from Italian water buffalo calves. Where absent, the prevalence value was not presented or not calculable. In the case of genotypes not associated by the authors with existing genotypes, these were indicated as n.a. Where specified, the cattle/calves entry is mentioned.

Origin	Host	Prevalence value (%)	Genotype	Acc. Number	Reference
Italy	*B. bubalis* (calves)	13.5	A; I; YNDCEB-90	PV405426-PV405430	**Present study**
Germany	*B. taurus*	10.7	I; J	AF135836-AF135837	Rinder et al., 2000
Germany	*B. taurus*	12	EbpA; J; I; M; N	AF267143 - AF267144	Dengjel et al., 2011
Portugal	*B. taurus*	6.25	BEB5	AY331009	Sulaiman et al., 2004
Portugal	*B. taurus* (calves)	100	J; PtEb XI	DQ885587- DQ885586	Lobo et al., 2006
Czech Republic	*B. taurus* (cattle/ calves)	15.42	I	100 % homology with AF135836	Juránková et al., 2013
Slovakia	*B. taurus* (calves)	0.2	n.a.	MF508610 - MF509308	Valenčáková and Danišová, 2019
Turkey	*B. taurus* (cattle/ calves)	19.3	N; ERUSS1; ERUSS2; ERUSS3	MH204102 - MH204106	Bilgin et al., 2020
Turkey	*B. bubalis*	2.7	J; YNDCEB-90	OK239909 - OK239912	Onder et al., 2022
Spain	*B. taurus*	0.6	BEB4	MZ666878	Abarca et al., 2021
Austria	*B. taurus* (calves)	7.9	J; I; BEB4; BEB8	OP455917 - OP455934	Lichtmannsperger et al., 2023

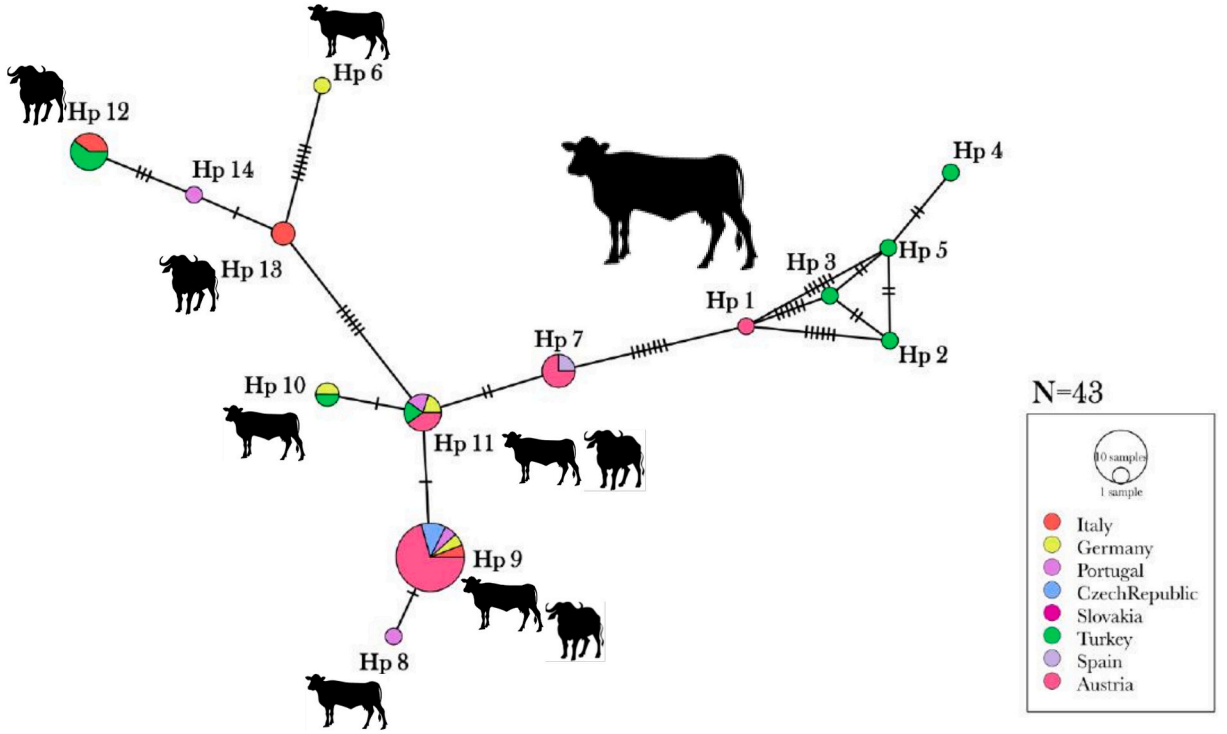

Fig. 2. Minimum spanning haplotype networks built in PoPART software for DatasetEU. (43 sequences). Haplotypes (Hp) are represented by circles proportional to relative haplotype abundance; different shades indicate different areas of origin. Notches refer to the mutational steps between haplotypes.

4. Discussion

The Bovidae family is recognized as a significant reservoir of Microsporidia (Taghipour et al., 2022). The choice to conduct an initial screening of the microsporidian *E. bieneusi* in water buffalo was driven by the economic importance of *B. bubalis* in Italy. Indeed, advanced techniques in selection, milk monitoring, production, management, nutrition, reproduction, food quality, and marketing are highly developed and refined for Italian buffalo livestock population (Neglia et al., 2023). There are around 200,000 water buffaloes in Italy, primarily raised in the central and southern areas. These herds play a significant role in the economy, particularly in the dairy sector. Notably, buffalo milk is the key ingredient in the production of "Mozzarella di bufala", a fresh cheese that holds Protected Designation of Origin (PDO) status under European Union regulations (CEE N 1107, 12 June 1996) (https://ec.europa.eu/commission).

Regarding cattle, in the Mediterranean area the livestock population consists of approximately 26,3 million individuals (De Rancourt and Mottet, 2006). In 2023, the European Union produced 6.4 million tonnes of bovine meat (carcasses of beef and veal). That same year, more than three-quarters of the total beef production came from six countries: France (20.7 %), Germany (17.3 %), Ireland (10.9 %), Italy (9.7 %), Poland (9.3 %), and Spain (9.1 %) (https://ec.europa.eu/eurostat). At the beginning of 2024, dairy cattle numbers in the European Union amounted to 19,7 million, according to a recent US Department of Agriculture (USDA) Global Agricultural Information Network (GAIN) report (https://www.thedairysite.com).

For this reason, monitoring Microsporidia, considered as emergent pathogens in the global food chain in these hosts, is essential, as these microorganisms, although not currently classified as priority foodborne parasites, have the potential to contaminate the human food supply through water, food, and environmental exposure. Consequently, natural hosts of Microsporidia capable of infecting humans may become part of the food chain (Stentiford et al., 2016). *E. bieneusi*, in particular, has also been detected in cow's milk, with genotypes having a zoonotic potential and a broad host adaptation (Lee, 2008).

To date, there have been no studies, either microscopic or molecular, investigating the presence of Microsporidia, and specifically *E. bieneusi*, in water buffaloes or in cattle in Italy.

This molecular-based study represents the first investigation into the genetic diversity of *E. bieneusi* in animals and specifically in water buffaloes in Italy. At the European level, the only other study focusing on *B. bubalis* was conducted in 2022 by Onder and colleagues in Turkey, with a prevalence of 2.7 % and the identification of the YNDCEB-90 genotype in most of the isolates ($N = 5$) and the J genotype in 3 isolates. In contrast, our study showed a higher prevalence and greater genetic variability. Regarding the prevalence value (13.5 %), it aligns with cattle global values, as reported in the meta-analysis by Taghipour et al. (2022), where the pooled prevalence of cattle microsporidiosis was estimated at 14 %, and the prevalence of *E. bieneusi* was 13.9 % based on the ITS gene. It should be noted, however, that the data presented in this study come from a limited sample size, which must be taken into account,

particularly in the light of the scarce data available in literature regarding *B. bubalis*, when interpreting the observed prevalence rates.

The YNDCEB-90 genotype was not the only one highlighted in our study: within the phylogenetic Group 1, two isolates were associated with genotype A, a potentially zoonotic genotype as it has frequently found in humans but also in dogs, non-human primates and birds (Li et al., 2019). Group 2 genotypes were previously thought to be specific to ruminants, as this assumption was based on studies conducted exclusively in cattle (Li et al., 2019). In our analysis, one isolate also clusters within Group 2, along with all samples from cattle/calves, and belongs to genotype I. Genotype I, along with the other genotypes more commonly detected in ruminants (BEB4, BEB6, and J), has later been identified in various animal species and humans. This also raises public health concerns regarding the zoonotic potential of certain genotypes within this group, including genotype I (Li et al., 2019; Taghipour et al., 2022).

The haplotype analysis showed three *E. bieneusi* haplotypes shared within the Bovidae family in Europe: haplotype 9, haplotype 12 and haplotype 11. Haplotype 9 encompasses genotype I and it is present in both *B. taurus* cattle and *B. bubalis* calves. In contrast, haplotype 12 is closely related to genotype YNDCEB-90 and appears to be specific to *B. bubalis*. Haplotype 11 is shared between *B. taurus* and *B. bubalis*. Data from Turkey (Bilgin et al., 2020; Onder et al., 2022) exhibit the highest haplotypic diversity: this may offer valuable insights into the genetic variation of this parasite within the Bovidae family. In particular, the elevated diversity could reflect Turkey's role as a historical crossroads for livestock trade (Köksal and Nacar, 2024).

Based on our findings, it may be suggested that the detection of zoonotic genotypes in water buffaloes could potentially represent a public health concern. This risk may arise not only through direct contact with infected animals or their faeces, but also through the possible contamination of the food chain and the environment, including ground and surface water sources. Indeed, water and food sources play a major role in the distribution and transfer of microsporidia infection to animals and humans as reported (Rezaeian et al., 2023). This highlights the need to integrate food safety and environmental measures, including milk pasteurization in "Mozzarella di bufala" production and water treatment near farms, particularly in areas where livestock and agriculture overlap and faecal runoff can spread waterborne pathogens.

5. Conclusions

The present pilot study provides the first insights into prevalence and spreading of genetic variants of *E. bieneusi* circulating in *B. bubalis* in Italy, comparing them with those previously identified in water buffalo and cattle across Europe. Since Mediterranean buffalo farming in Italy is largely confined to southern regions, the geographic focus of this study reflects the major distribution of the host population within the country. Given the growing global population of immunocompromised individuals, the control of Microsporidia infections in domestic animals, identifying and characterizing the genetic diversity of this pathogen in livestock may serve as a strategy to tackle the significant challenges posed by this emerging opportunistic pathogen. The findings of this study are encouraging and underscore the relevance of further expanding the investigation.

CRediT authorship contribution statement

Isabel Guadano-Procesi: Writing – original draft, Software, Methodology, Investigation, Conceptualization. **Antonio Bosco:** Writing – review & editing, Validation, Methodology, Investigation. **Lavinia Ciuca:** Methodology, Investigation. **Paola Pepe:** Investigation. **Camilla Sangiovanni:** Writing – review & editing. **David Di Cave:** Writing – review & editing. **Laura Rinaldi:** Writing – review & editing, Validation, Resources. **Federica Berrilli:** Writing – review & editing, Supervision, Resources.

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Declaration of competing interest

The authors confirm that they have no recognized financial conflicts of interest or personal connections that could have influenced the work presented in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2025.e00273.

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