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RESEARCH

Molecular detection and genotyping of *Dientamoeba fragilis* and *Blastocystis* sp. in housefly *Musca domestica* (Diptera: Muscidae): first report for *Dientamoeba fragilis*

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Abstract

Dientamoeba fragilis and *Blastocystis* sp. are single-celled protozoan parasites of humans and animals. Although they are found in the intestines of healthy hosts, the pathogenicity of them is still unclear. To date, there is no report on *D. fragilis* and only two studies (without subtyping) on the occurrence of *Blastocystis* sp. in *Musca domestica*. In this study, fly samples were collected from livestock farms and their surroundings in the Kirsehir province (Central Anatolia Region) of Türkiye from May to August 2023. A total of 150 microscopically identified *M. domestica* samples were analyzed for the detection of *D. fragilis* and *Blastocystis* sp. molecularly. The overall prevalence of *Blastocystis* sp. and *D. fragilis* in *M. domestica* was determined to be 3.3% (5/150) and 8.0% (12/150), respectively. The SSU rRNA gene sequences of the isolates indicated genotype 1 of *D. fragilis*. Eleven isolates were identical and represented a single isolate (KAU-Dfrag1). BLAST analysis of KAU-Dfrag1 indicated identity with the isolates reported from humans, cattle, sheep, and budgerigars. The other isolate (KAU-Dfrag2) was polymorphic at two nucleotides from KAU-Dfrag1 and three nucleotides from known genotypes from GenBank and represented a variant of genotype 1. The *Blastocystis* sp. isolates were found to be identical and represent a single genotype (KAU-Blast1). BLAST analysis revealed that the KAU-Blast1 genotype belonged to the potentially zoonotic subtype 5 (ST5) and exhibited the highest genetic identity (ranging from 99.4 to 99.6%) with pigs, cattle, and sheep from different countries. Our study provides the first data on the molecular prevalence, epidemiology, and genotypic characterization of *D. fragilis* and *Blastocystis* sp. in *M. domestica*.

Keywords *Blastocystis* sp. · *Dientamoeba fragilis* · Genotypic characterization · Molecular prevalence · *Musca domestica*

Introduction

In many countries, parasitic gastrointestinal infections caused by helminths and protozoa pose a significant health concern (Sepahvand et al. 2022). These infections may reveal some clinical symptoms such as diarrhea, abdominal pain, nausea, and irritable bowel syndrome, or they may be asymptomatic. *Blastocystis* sp. Alexieff, 1911 and *Dientamoeba fragilis* Jepps and Dobell, 1918 are unicellular protozoan parasites of humans and many animals (Boer et al. 2020). The reported prevalence of *D. fragilis* ranges from 0.2 to 82%, while *Blastocystis* sp. prevalence ranges from 0.5 to 100% (Sarzhanov et al. 2021; Jirků et al. 2022). Despite their presence in the intestines of healthy hosts, the pathogenicity of *D. fragilis* and *Blastocystis* sp. remains unclear (Boer et al. 2020; Leonardi and Tan 2023). Transmission probably occurs via the fecal–oral route (Munasinghe et al. 2013; Jinatham et al. 2022). Some studies suggest that *D. fragilis* may be transmitted through helminth eggs such as *Ascaris lumbricoides* Linnaeus, 1758 and *Enterobius vermicularis* Linnaeus, 1758 (Röser et al. 2013). However, Guadano-Procesi et al. (2024) noted that the main transmission route of *D. fragilis* is a fecal–oral route.

Molecular analysis of the small subunit ribosomal RNA (SSU rRNA) has revealed large genetic variability within the *Blastocystis* species complex and classified into subtypes (STs) (Stensvold et al. 2007; Stensvold and Clark

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2020; Maloney et al. 2022). To date, researchers have discovered at least 36 STs of *Blastocystis* sp. (Maloney et al. 2022; Stensvold et al. 2023; Yu et al. 2023). These subtypes may be related to symptoms, pathogenicity, and zoonotic potential (Skotarczak 2018; Hublin et al. 2021). In contrast, Asghari et al. 2020) reported no significant difference between frequency of the *Blastocystis* subtypes in symptomatic and asymptomatic cancer patients. Of the subtypes, ST1-10, ST12, ST14, and ST41 have been identified in humans (Hernández-Castro et al. 2023), and ST1–ST4 also account for almost 90% of subtyped human isolates (Deng et al. 2021; Popruk et al. 2021). Nine subtypes, ST1–ST8 and ST12, have been found in both humans and animal hosts, indicating potential transmission between them in settings such as zoos and slaughterhouses (Maloney et al. 2021). Shams et al. (2024) analyzed fecal samples from cattle, sheep, and their breeders and found that ST1-3 subtypes were common. Additionally, ST5 has been previously reported in humans and various animals, suggesting its adaptation to pigs (Udonsom et al. 2018). Similarly, ST5 has been detected in pigs and farm workers in Australia (Wei et al. 2023; Wang et al. 2018). In dogs and cats, closely related animal species to humans, a higher genetic diversity was reported (ST1-8, ST10, ST23, ST24 in dogs and ST1-4, ST10, ST14 in cats) (Mahdavi et al. 2022; Shams et al. 2022).

D. fragilis exhibits limited genetic diversity. Two genotypes, named 1 and 2, have been described using different gene regions (SSU rRNA, actin, EF1α) of *D. fragilis*, and genotype 1 is more common than genotype 2 (Stensvold et al. 2013). Genotype 1 has been reported in a range of hosts including humans, gorillas, pigs, dogs, cats, cattle, and budgerigars (Stark et al. 2008; Cacciò et al. 2012; Chan et al. 2016; Yildiz and Aynur 2022; Yetismis et al. 2022).

Musca domestica Linnaeus, 1758 are the most common synanthropic flies worldwide and often found on decaying matter, garbage, and feces. *M. domestica* is closely related with humans and animals and complete its entire lifecycle with these habitats (Khamesipour et al. 2018). These flies serve as both mechanical vectors and reservoirs for more than a hundred pathogens, including bacteria, parasites, and viruses. The transmission of these pathogens occurs through the flies' mouthparts and other parts of their bodies (Issa 2019; Park et al. 2019). As far as we know, there have been two studies conducted on the occurrence of *Blastocystis* sp. on *M. domestica*, excluding ST differentiation (Toriano and Malimban 2019; El-Salem et al. 2021), but no studies have been conducted on *D. fragilis.*

The aim of the study was to investigate the prevalence and genotypic characterization of *Blastocystis* sp. and *D. fragilis* in *M. domestica* within the Central Anatolia Region of Türkiye.

Materials and methods

Sample collection and morphological identification

Sampling was carried out from May to August 2023 at 12 locations (Table 1) in Kirsehir province in the Central Anatolia Region of Türkiye (Fig. 1). Kirsehir province has cold and snowy winters and hot and dry summers. Agriculture and animal husbandry are the primary economic activities in this region. Adult flies were collected with sweeping nets. After capture, the flies were anesthetized with diethyl ether in a disposable plastic bag, fixed in absolute ethanol, and stored at − 20 °C until morphological identification and DNA extraction. Morpho-taxonomic identification was achieved according to the morphological identification keys (Dodge 1953a, b; Hennig 1955; Pont 1991; Nihei and Carvalho 2007). Microscopic examination was carried out under an Olympus BX51 light microscope (Olympus, Tokyo, Japan). Among the samples morphologically identified as *M. domestica*, a total of 150 samples, 11 to 14 samples for each farm, were selected for molecular analysis. No human or animal ethics approval was required for the completion of this study.

DNA extraction and PCR amplification

Musca domestica specimens were ground in a microcentrifuge tube individually under liquid nitrogen using a mortar and pestle. Genomic DNA was isolated with using commercial kit (PureLink Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The final nucleic acid volumes were adjusted to 50 µl and stored at − 20 °C.

Table 1 Sampling farms with GPS coordinates

		Animal species in farms	Latitude (N)	Longitude (E)
Kirsehir	Farm 1	Cattle	39° 24′ 18″	33° 59′ 39″
	Farm 2	Cattle	39° 20′ 48″	34° 10′ 04″
	Farm 3	Cattle, dog	39° 04′ 57″	34° 15′ 54″
	Farm 4	Cattle, dog	39° 13′ 42″	34° 15′ 53″
	Farm 5	Cattle, dog, chicken	39° 21′ 13″	33° 56′ 25″
	Farm 6	Cattle, dog	39° 22′ 13″	33° 48′ 26″
	Farm 7	Cattle	39° 03′ 24″	34° 25′ 11″
	Farm 8	Cattle	39° 09′ 14″	34° 07′ 36″
	Farm 9	Cattle	39° 04′ 04″	34° 15′ 19″
	Farm 10	Cattle, dog	39° 02′ 10″	34° 26′ 33″
	Farm 11	Cattle, dog	39° 20′ 12″	34° 15′ 50″
	Farm 12	Cattle	39° 27′15″	34° 18′ 32″

Fig. 1 Sampling location (https://www.wikipedia.org)

The SSU rRNA gene region of *D. fragilis* was amplified from all the individual gDNA samples, using nested PCR assay with the primer pairs DF1 (5′-CTCATAATC TACTTGGA ACCAATT-3′) and DF4 (5′-CCCCGATTA TTCTCTTTGATATT-3′) (Vandenberg et al. 2006), and DF322For (5′-GAGAAGGCGCCTGAGAGATA-3′) and DF687Rev (5′-TTCATACTGCGCTAAATCATT-3′) (Cacciò et al. 2012). The thermal cycling conditions for the first step are as follows: 95 °C for 4 min, followed by 40 cycles, each consisting of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The second step was the same, except the annealing temperature was 54 °C. Molecular detection of *Blastocystis* sp. was achieved by PCR amplification of the SSU rRNA gene with the primers RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) and BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) (Scicluna et al. 2006). The thermal cycling conditions are as follows: 95 °C for 4 min, followed by 34 cycles, each consisting of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

All the PCR analysis contained positive and negative controls (distilled water), and PCR products were electrophoresed on a 1.5% agarose gel and visualized using a gel documentation system. Amplicons from positive samples were purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA) according to the manufacturer's instructions and sequenced in both directions with amplification primers (BMLabosis, Ankara, Türkiye).

Sequence and phylogenetic analysis

The raw sequence data from both reads were checked for the presence of any double or ambiguous peaks. The forward and reverse sequences were assembled into a final consensus sequence using the de novo assembly plugin in Geneious 2020.0.3 software (www.geneious.com). Final sequences were compared with reference sequences in the GenBank database by BLAST analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For genotyping, the consensus sequences of each isolate were aligned with the reference and other related sequences using ClustalW algorithm in MEGA X (Kumar et al. 2018).

The phylogenetic analyses were performed by the maximum likelihood (ML) method implemented in PhyML (v2.4.4) (Guindon et al. 2003), neighbor-joining (NJ) method with bootstrap values (10,000 replicates) in MEGA X, and a Bayesian inference (BI) tree in BEAST v1.10.4 (Suchard et al. 2018). Markov chain Monte Carlo simulations were run simultaneously for 10 million generations, with sampling every 100 generations for the posterior probability calculations in BI. Before constructing a majority consensus tree, 25% of the initial trees in each run were discarded as burn-in with TreeAnnotator in BEAST v1.10.4. BI trees were viewed and edited by FigTree v1.3.1 (Rambaut 2009). The best DNA-substitution model for ML and BI analyses was determined as follow to Akaike information criterion (AIC) and BIC (Bayesian information criterion) algorithm by using jModelTest v.0.1.1 (Posada 2008).

Results

The overall prevalence of *Blastocystis* sp. and *D. fragilis* in *M. domestica* was identified by PCR analysis to be 3.3% (5/150) and 8.0% (12/150), respectively. No mixed infection was detected. While *Blastocystis* sp. positive samples were determined in one farm (farm 6), *D. fragilis* was detected in samples from two nearby farms in same locations (farm 4). Sequence analysis of the SSU rRNA gene region of 12 isolates indicated genotype 1 of *D. fragilis* and revealed that 11 of them were completely identical, representing a single isolate (KAU-Dfrag1, GenBank accession: OR916315). The

other isolate (KAU-Dfrag2, GenBank accession: OR916316) was identified as the variant of the KAU-Dfrag1. The two isolates differed by two bases at the 219th (T/C) and 222nd (A/G) positions. BLAST analysis revealed that the KAU-Dfrag1 genotype showed 100% similarity to previously identified genotypes from humans in Italy (OQ345680), Iran (AB692771), Türkiye (MZ405082), Czechia (OP375684), cattle in Türkiye (ON242172), and budgerigars in Türkiye (MW130447-48).

Through analyzing the SSU rRNA gene region of five *Blastocystis* sp. isolates revealed that wholly identical and represents a unique genotype (KAU-Blast1, GenBank accession: OR916317). By BLAST analysis, our identified genotype belongs to subtype 5 and exhibited the highest identity (ranging from 99.4 to 99.6%) with pigs in the Philippines (KY610170 and MF737388), Romania (MK801418), Austria (MK801415), and Germany (MK801367); sheep in China (ON062964) and Iran (MN316540); *Lemur catta* in Spain (OK285248); and cattle in Malaysia (MK240462).

Phylogenetic trees were constructed using ML, NJ, and BI analyses of partial SSU rRNA gene sequences. Regarding *D. fragilis*, the NJ tree with the identified genotype and the known genotypes from GenBank were shown in Fig. 2. The group of genotype 1 and genotype 2 were clearly separated as a distinct branch, which is supported by 95% and 88% bootstrap values for ML and NJ, and 0.9 Bayesian posterior probability. The KAU-Dfrag1 and KAU-Dfrag2 isolates were assigned to the genotype 1 group, which includes those found in humans, cattle, and budgerigars from various regions. The related trichomonad species *Histomonas meleagridis*, which were included in the analysis, were clearly separated from *D. fragilis* genotypes. For *Blastocystis* sp., ML, NJ, and BI analyses were produced concordant tree that was constructed with 44 genotypes originating from different hosts and countries. These genotypes were successfully categorized into 17 subtype groups. The novel genotype KAU-Blast1 was clustered with ST5 sequences identified in pigs in Japan (AB070998) and Philippines (KY610170), cattle in Malaysia (MK240462) and Japan (AB107966), sheep in China (ON062969), and *Bufo japonicus japonicus* in Japan (AY266469).

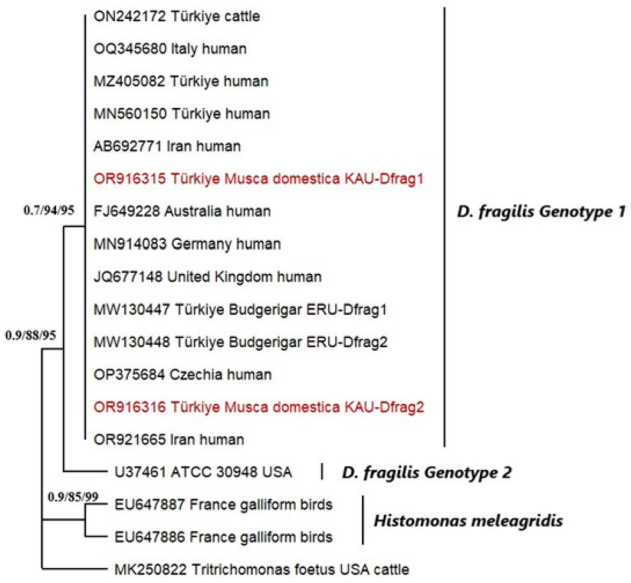

Fig. 2 Phylogenetic relationships of *Dientamoeba fragilis* isolates from *Musca domestica* in this study (in red) and known genotypes previously reported from different countries and hosts. The tree was constructed using neighbor-joining analyses of SSU rRNA sequences. The isolates are displayed with GenBank accession numbers, country, and host. Nodal supports presented above the line indicate Bayesian posterior probability, NJ bootstrap using Mega X, and ML bootstrap using PhyML (1000 replicates), respectively. The *Tritrichomonas foetus* genotype is used as an outgroup

Discussion

Houseflies are the most widespread flies in the world and transmit more than a hundred pathogens, including many helminth eggs such as *Enterobius vermicularis*, *Strongyloides* sp., *Trichuris* sp., *Taenia* sp., and *Hymenolepis* sp.; protozoan cysts; and trophozoites such as *Giardia* sp., *Cryptosporidium* sp., and *Entamoeba* sp., as well as some bacteria such as *Escherichia coli*, *Shigella*, and *Salmonella* species, which can cause diseases in humans and animals (Issa 2019). The transmission of these pathogens to the host is mainly mechanical (Graczyk et al. 2005). In the present study, the molecular prevalence and characterization of *Blastocystis* sp. and *D. fragilis* in *M. domestica* were investigated using molecular methods. Human infections with *D. fragilis* and *Blastocystis* sp. have been reported in many countries. The prevalence of these parasites varies widely, ranging from 0.2 to 82% for *D. fragilis* (Cacciò 2018) and 0.5 to 100% for *Blastocystis* sp. (Popruk et al. 2021), depending on the diagnostic methods used. Recently, there have been a growing number of studies investigating the presence of these pathogens in various sources, including farm and pet animals, food, environmental materials, and insects. To the best of our knowledge, there have been two previous reports of *Blastocystis* sp. indicating the presence of *M. domestica* without molecular genotyping and no reports on *D. fragilis* (Toriano & Malimban 2019; El-Salem et al. 2021). In Türkiye, the most common *Blastocystis* subtype was ST3 (47.9%), followed by ST1 (17.5%), ST2 (14.7%), ST4 (4%), and ST5-ST7 (15.9%) in humans, livestock, and pets, and a limited number of environmental samples (Malatyalı et al. 2023). In addition, few studies have been conducted on *D. fragilis* in humans (Girginkardeşler et al. 2003; Kurt et al. 2008; Sarzhanov et al. 2021; Sivcan et al. 2018; Yıldız

et al. 2021), cattle (Yildiz and Aynur 2022), and budgerigars (Yetismis et al. 2022).

We found the relatively low prevalence of (3.3%) *D. fragilis* in *M. domestica* and established for the first time that *M. domestica* may play a role in the transmission dynamics of dientamoebiasis. The SSU rRNA is a gene commonly used for the characterization of *D. fragilis* (Tolba et al. 2022; Jirků et al. 2022; Guadano-Procesi et al. 2024; Shams et al. 2024). Amplification and RFLP analysis of the SSU rRNA gene revealed two distinct genetic variants, genotypes 1 and 2. To date, studies on the molecular characterization of *D. fragilis* have shown that genotype 1 is dominant. Analysis of the SSU rRNA partial gene region in *D. fragilis* isolates from *M. domestica* revealed the existence of the common genotype 1. The sequences of the eleven isolates of *D. fragilis* in *M. domestica* (KAU-Dfrag1) were found to be identical to the sequences, clustered in genotype 1, which is also identified from human and animal hosts. Additionally, a single-nucleotide polymorphism was identified at the 72nd base (T/C) in KAU-Dfrag1 sequence, while three nucleotide polymorphisms were found at the 72nd base (T/C), 219th base (T/C), and 222nd base (A/G) in KAU-Dfrag2 sequence. Phylogenetic analyses of the identified genotypes revealed the same or close genetic structure of *D. fragilis* in *M. domestica* as the common genotype in humans and animals, suggesting zoonotic potential.

Blastocystis sp. exhibits extensive genetic diversity and is categorized into different subtypes. These subtypes may be associated with distinct symptoms, pathogenesis, risk factors, and zoonotic potentials. In our study, we identified five isolates of *Blastocystis* sp., representing a novel genotype (KAU-Blast1), were identical to each other. The detected genotype was phylogenetically determined to be in ST5, which is commonly found in animal hosts and rarely human genotypes (Fig. 3). Pintong et al. (2018) reported a high molecular prevalence of ST5 in pig farms (78.1%; 25/32) and suggested that ST5 is dominant in pigs. Their work and Yan et al. (2007) identified ST5 in humans and suggested the zoonotic potential this genotype. Sharifi et al. (2020) reported that ST5 was the most dominant subtype in cattle.

Despite various studies examining the gut microbiota of houseflies, there is a lack of information regarding *D. fragilis* or *Blastocystis* sp. (Gupta et al. 2012; Zhao et al. 2017; Park et al. 2019; Monyama et al. 2023). Junqueira et al. (2017) investigated the microbiomes of *Chrysomya megacephala* ($n = 63$) and *M. domestica* ($n = 53$) using whole-genome shotgun (WGS) sequencing and describing the complexity of microbiological diversity. However, their research did not include any data on *D. fragilis* or *Blastocystis* sp. It is worth noting that the legs, wings, and head of flies exhibit the highest microbial diversity and are known to play a significant role in mechanical vectoring. Therefore, we propose that the isolates we detected in our study may

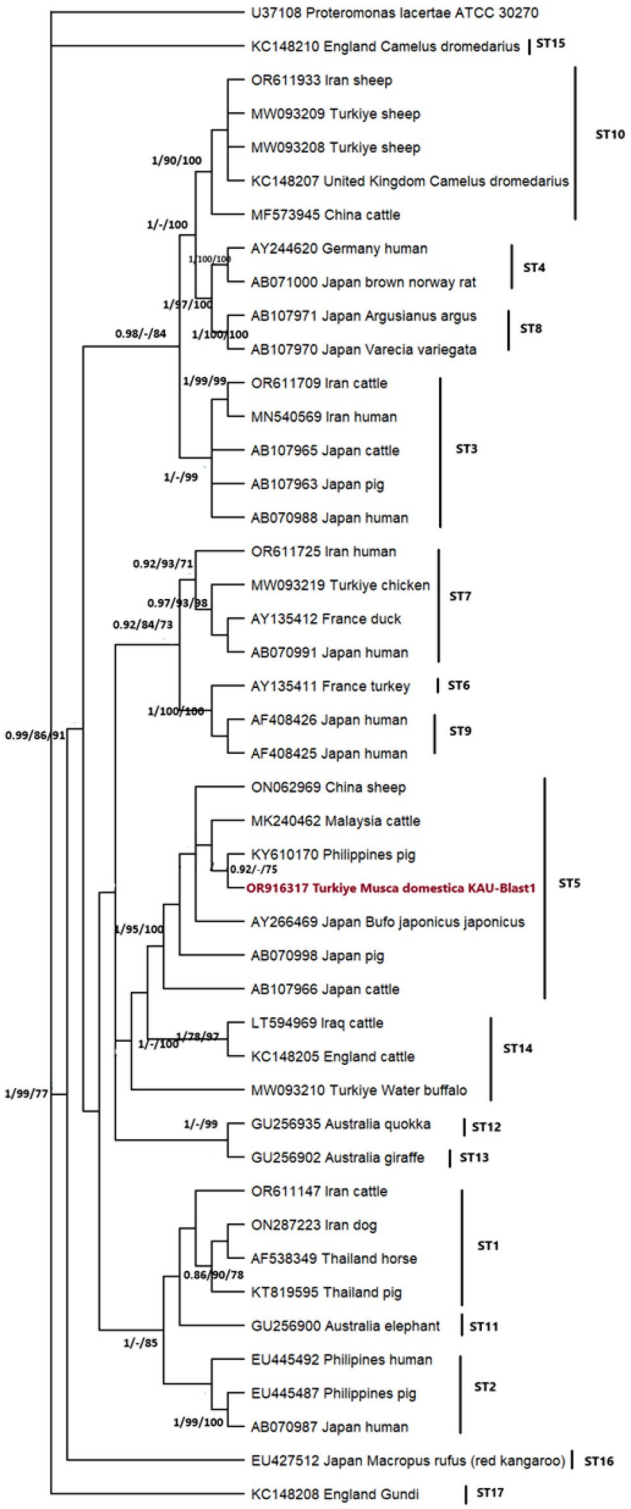

Fig. 3 Phylogenetic relationships of *Blastocystis* sp. isolate from *Musca domestica* in this study (in red) and known genotypes previously reported from different countries and hosts. The tree was constructed using neighbor-joining analyses of SSU rRNA sequences. The isolates are displayed with GenBank accession numbers, country, and host. Nodal supports presented above the line indicate Bayesian posterior probability, NJ bootstrap using Mega X, and ML bootstrap using PhyML (1000 replicates), respectively. The *Proteromonas lacertae* ATCC30270 genotype is used as an outgroup

be surface contamination on flies, but this should be detail investigated in further studies.

Our findings make a significant contribution to our understanding of *Blastocystis* sp. and *D. fragilis*, specifically in terms of their molecular epidemiology and transmission dynamics. For the first time, the vector potential of *M. domestica* for *D. fragilis* and *Blastocystis* sp. was revealed by the determination of subtypes. Further studies are needed to better understand the vector competence of *M. domestica* for *D. fragilis* and *Blastocystis* sp. using large-scale sampling and different muscid fly species.

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Author contribution N.E., A.Y., and O.D. conceptualized the study (methodology); N.E. carried out the experiments; N.E. and O.D. analyzed the results; N.E., A.Y., and O.D. wrote and reviewed the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no conflict of interest.

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