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Identification and distribution of some medico-veterinary important pathogens in muscid flies in two geographical regions of Türkiye

Nuri Ercan[1] | Alparslan Yildirim[2] | Onder Duzlu[2] | Fahriye Ercan[3] | Gamze Yetismis[2] | Gokmen Zafer Pekmezci[4] | Abdullah Inci[2]

[1]Faculty of Agriculture, Kirsehir Ahi Evran University, Kirsehir, Turkey

[2]Department of Parasitology, Faculty of Veterinary Medicine, Erciyes University, Kayseri, Turkey

[3]Department of Plant Protection, Faculty of Agriculture, Kirsehir Ahi Evran University, Kirsehir, Turkey

[4]Preclinical Sciences, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun, Turkey

Correspondence
Nuri Ercan, Faculty of Agriculture, Kirsehir Ahi Evran University, Sahir Kurutluoglu St., 40100 Kirsehir, Turkey.
Email: nuri.ercan@ahievran.edu.tr

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Abstract

Some dipteran flies play an important role in the transmission of pathogens such as viruses, bacteria, fungi, protozoan and metazoan parasites in humans and other animals. Despite this importance, knowledge of the prevalence and molecular characteristics of some pathogens in flies is limited, and no data are available for Türkiye. In this study, we investigated the possible vector role of muscid fly species for the transmission of *Enterocytozoon bieneusi* Desportes (Chytridiopsida: Enterocytozoonidae), *Encephalitozoon* spp., *Coxiella burnetii* Derrick (Legionellales: Coxiellaceae) and *Thelazia* spp. using polymerase chain reaction (PCR) and sequence analysis. The flies were trapped in different animal-related places and surroundings from two different geographical regions of Türkiye including Central Anatolia and Middle Black Sea. According to the morphological keys, 850 (85%), 141 (14.1%) and 6 (0.6%) of the total of 1000 fly specimens identified as *Musca domestica* Linnaeus (Diptera: Muscidae), *Stomoxys calcitrans* Linnaeus (Diptera: Muscidae) and *Musca autumnalis* De Geer (Diptera: Muscidae), respectively. The other species including *Haematobia irritans* Linnaeus (Diptera: Muscidae), *Muscina stabulans* Fallén (Diptera: Muscidae) and *Hydrotaea ignava* Harris (Diptera: Muscidae) were each represented by a single specimen. Screening of the pathogens identified *E. bieneusi* only in *M. domestica* with a prevalence of 2.4%. Sequence analyses identified three known genotypes, Type IV, BEB6 and BEB8, and one novel genotype named AEUEb of *E. bieneusi* in *M. domestica*. *Coxiella burnetii* was detected in *M. domestica* and *S. calcitrans* with prevalences of 2.9% and 2.8%, respectively. The one specimen of *H. ignava* was also positive for *C. burnetii*. *Encephalitozoon* spp. and *Thelazia* spp. were not found in the examined specimens. Our results contribute to the current knowledge on the vector potential of muscid flies and their possible role in the transmission dynamics of certain pathogens, especially in regions where diseases are prevalent and affect public and animal health.

KEYWORDS

Coxiella burnetii, microsporidia, molecular prevalence, synanthropic flies, *Thelazia* spp.

INTRODUCTION

Muscidae is a large and cosmopolitan family of Diptera with more than 5000 species distributed in all biogeographic regions (Kutty et al., 2008). Muscid flies have a wide range of life strategies, both in the immature and adult stages, and have exhibited various feeding habits including saprophagy, coprophagy, herbivory, predation and blood feeding (Kutty et al., 2014; Skidmore, 1985). Some muscid flies also provide beneficial ecological contributions such as decomposition, pollination and they serve as a food resource (Savage, 2002). In contrast, some muscid flies have medical and veterinary importance because they transmit pathogens such as viruses, bacteria, fungi and parasites to humans and other animals (Förster et al., 2009).

Microsporidian species are obligate intracellular pathogens that infect a wide range of hosts, including mammals, insects and birds, and are distributed worldwide (Marrie, 1990; Santin & Fayer, 2009). In particular, most of the diversity of microsporidia is found in crustaceans and insects (Pan et al., 2018). To date, 93 of the 200 described genera of microsporidia have an insect as the type host (Becnel & Andreadis, 2014). *Enterocytozoon bieneusi* Desportes (Chytridiopsida: Enterocytozoonidae) and *Encephalitozoon* spp. are commonly identified microsporidian species (Stentiford et al., 2013). There are many reports of *E. bieneusi* in humans and farm animals, but limited reports in insects. To our knowledge, only one published study has indicated the presence of *E. bieneusi* in muscid flies, and no studies have detected *Encephalitozoon* spp.

Q fever is a zoonosis caused by *Coxiella burnetii* Derrick (Legionellales: Coxiellaceae) and distributed worldwide (Guatteo et al., 2011). Similar to microsporidians, *C. burnetii* infects a wide range of animal species, as well as humans. Ruminants are the main source of *C. burnetii*, but it is also detected in rodents, birds and arthropods (Van den Brom et al., 2015). *Coxiella burnetii* is shed via infected animal faeces, milk and birth products (Guatteo et al., 2011). Humans become infected commonly by aerosol or dust contaminated with *C. burnetii* (Tissot-Dupont et al., 2004). Studies on arthropod vectors for Q fever transmission routes have focused on ticks (Körner et al., 2021; Tokarevich et al., 2019). Considering the feeding environment of flies, it can be suggested that flies may be suitable mechanical vectors for *C. burnetii*. However, there have been no studies of *C. burnetii* in field-collected *Musca domestica* Linnaeus (Diptera: Muscidae) samples and a single study with *Stomoxys calcitrans* Linnaeus (Diptera: Muscidae) (Nelder et al., 2008). In a single experimental study, it was reported that *C. burnetii* can survive in the faeces of *M. domestica* for up to 80 days and in the dead body for up to 90 days (Hucko, 1984).

Thelazia spp. (Rhabditida: Thelaziidae) are parasitic nematodes that infect the eyes of various animals and rarely humans (do Vale et al., 2019). Human thelaziosis mainly originates *Thelazia callipeda* Railliet and Henry (Rhabditida: Thelaziidae) while bovine thelaziosis is caused by *Thelazia rhodesi* Desmarest (Rhabditida: Thelaziidae), *Thelazia gulosa* Railliet and Henry (Rhabditida: Thelaziidae) and *Thelazia skrjabini* Erschow (Rhabditida: Thelaziidae). The main vector for *T. callipeda* is *Phortica variegata* Fallen (Diptera: Drosophilidae) and *Phortica okadai* Máca (Diptera: Drosophilidae) in Europe and Asia, respectively (Otranto, Lia, Cantacessi, et al., 2005). Shi et al. (1988) reported the possible role of *M. domestica* for transmission dynamics of *T. callipeda*. However, in contrast to this report, Otranto, Lia, Testini, et al. (2005) found no evidence to support the transmission role of *M. domestica* for *T. callipeda* in both experimental and natural conditions. The reports on the vector potential of muscid flies for thelaziosis have mainly focused on *Musca autumnalis* De Geer (Diptera: Muscidae) (Giangaspero et al., 2000). The other *Musca* spp. species rarely play a role in the transmission of these nematodes (Skrjabin et al., 1967). Otranto et al. (2003) reported the detection of *T. gulosa* in *M. autumnalis*, *Musca larvipara* Portschinsky (Diptera: Muscidae), *Musca osiris* Weidemann (Diptera: Muscidae) and *M. domestica* and *T. rhodesi* in *M. autumnalis* and *M. larvipara* by PCR analysis.

The goal of the present study was to investigate the prevalence and genotyping of *E. bieneusi*, *Encephalitozoon* spp., *C. burnetii* and *Thalezia* spp. in the fly species, including *M. domestica*, *M. autumnalis*, *S. calcitrans*, *Hydrotaea ignava* Harris (Diptera: Muscidae), *Haematobia irritans* Linnaeus (Diptera: Muscidae) and *Muscina stabulans* Fallén (Diptera: Muscidae), collected from two different geographical regions of Türkiye.

MATERIALS AND METHODS

Study sites, collections and identifications

A total of 1000 muscid fly specimens were collected with sweep nets and light traps in 26 locations in two provinces (Kayseri and Kirsehir) of Central Anatolia and 14 locations in two provinces (Samsun and Sinop) of the Middle Black Sea region of Türkiye from May to August 2020–2021. The flies were caught on cattle and sheep farms and their surroundings, as well as in poultry houses. After trapping, flies were anesthetized with diethyl ether in a disposable plastic bag, collected with sterile forceps and fixed in absolute ethanol. For each location GPS coordinates, altitude, collection site and the date were recorded (Table 3). Specimens were stored at −20°C until morphological identification and DNA extraction. Muscid fly taxa were determined according to morphological identification keys (Dodge, 1953a; Dodge, 1953b; Gregor et al., 2002; Hennig, 1955–1964; Nihei & de Carvalho, 2007; Pont, 1991). After morpho-taxonomic identification, two or three legs of each specimen were removed with sterile lancets and transferred into microcentrifuge tubes for DNA barcoding, and the remaining whole bodies of each individual were used for pathogen screening. After receiving the pathogen screening results, DNA extraction and PCR analyses of the leg samples were conducted to include the pathogen-positive specimens in the DNA barcoding analysis.

DNA extraction and PCR analyses

DNA extraction was carried out from leg samples and whole bodies using a commercial kit (PureLink Genomic DNA Mini Kit, Invitrogen,

Carlsbad, CA) according to the manufacturer's instructions. Tissue samples were ground in microcentrifuge tubes under liquid nitrogen using a mortar and pestle. For DNA quantification, Qubit dsDNA BR Assay Kit was used (Thermo Fisher Scientific, USA) to check and optimize the amount of DNA used in the PCR. Extracted DNA samples were stored at −20°C.

Target pathogens in samples were screened by PCR amplification of different gene regions with specific primers using the described protocols in references in Table 1. The presence of *E. bieneusi* and *Encephalitozoon* spp. was investigated by a nested PCR amplification. For mtDNA-based barcoding of the pathogen-positive specimens, PCR amplification was performed to amplify 709 bp of the cytochrome c oxidase subunit I (COI) gene using the universal primers described by Folmer et al. (1994). Approximately 20 ng of gDNA per sample was added to the PCR mix. PCR products were electrophoresed on 1.5% agarose gel and visualized using a gel documentation system.

Sequence and phylogenetic analyses

Amplicons from pathogen-positive samples and the specimens used in COI barcoding were purified using GeneJet Gel Extraction Kit (Thermo Scientific, USA) according to manufacturer's instructions and sequenced in both directions with amplification primers by Macrogen Inc. (Macrogen, Amsterdam, The Netherlands).

The raw sequence data from both reads were checked, and primer sequences were trimmed. The resulting forward and reverse sequences were assembled to create a final consensus sequence using the De Novo Assemble 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 species identifications and genotype characterizations. All sequences were aligned using ClustalW algorithm in MEGA X (Kumar et al., 2018).

To reveal the phylogenetic relationships between the detected genotypes of *E. bieneusi*, a maximum likelihood (ML) tree was constructed using MEGA X software based on evolutionary distances calculated with Kimura 2-parameter model. The reliability of these trees was assessed via bootstrap analysis with 1000 replicates.

The genotypes of *E. bieneusi* isolates were identified by the alignment of the obtained internal transcribed spacer (ITS) sequences with the sequences of published known genotypes in GenBank database. Novel genotypes of *E. bieneusi* were named following established nomenclature system based on the ITS region (Santin & Fayer, 2009). The partial sequences of the IS1111 transposon gene of *C. burnetii* and the ITS gene region of *E. bieneusi* obtained in this study were deposited in GenBank (Table 4).

RESULTS

Morphological identification of muscid flies

A total of 1000 muscid flies belonging to six species were identified morphologically. *Musca domestica* (85%) was the most dominant species followed by *S. calcitrans* Linnaeus (Diptera: Muscidae) (14.1%). The locality and sex distribution of the identified species are given in Table 2.

Detection and prevalence of the pathogens

Molecular screening of the muscid fly samples revealed that 30 (3.0%) and 21 (2.1%) out of 1000 specimens were positive for *C. burnetii* and *E. bieneusi*, respectively. The sequence analyses confirmed the *C. burnetii* in the examined specimens, and the identified isolates were found genetically close to the isolates identified in sheep (MN917207), goat (MG385669), cattle (KP719175, MH598511) and ticks (MZ964137). *Encephalitozoon* spp. and *Thelazia* spp. were not detected in the examined samples. No mixed infections were determined.

All the identified *E. bieneusi* isolates were detected in *M. domestica* samples from Kayseri, Kirsehir and Samsun provinces, while no positivity was detected in the samples from Sinop (Table 3). *Coxiella burnetii* was determined in 25 out of 850 *M. domestica* and 4 out of 141 *S. calcitrans* specimens resulting in a prevalence of 2.9% and 2.8%, respectively (Table 4). The one *H. ignava* Harris (Diptera: Muscidae) specimen caught from Sinop province was also positive for *C. burnetii* (Table 3).

TABLE 1 Primers used in this study.

Pathogens	Target gene	Primers	Annealing T_m (°C)	References
Enterocytozoon bieneusi	Internal transcribed spacer	EBITS3/EBITS4	47	Buckholt et al. (2002), Katzwinkel Wladarsch et al. (1996)
		EBITS1/ EBITS2.4	46	
Encephalitozoon spp.	Internal transcribed spacer	MSP-I/MSP-2A	40	
		MSP-3/MSP-4A	57	
Coxiella burnetii	IS1111 transposon	Trans 1/Trans 2	60	Hoover et al. (1992)
Thelazia spp.	Internal transcribed spacer	ThA/ThC	58	Otranto et al. (2001)

TABLE 2 Collection locality, sex and species distribution of morphologically identified specimens in Muscidae from Türkiye.

Regions	Provinces	Number of specimens (♂/♀)						Total
		Musca domestica	Stomoxys calcitrans	Musca autumnalis	Haematobia irritans	Hydrotaea ignava	Muscina stabulans	
Central Anatolia	Kayseri	117/132	28/23	-	-	-	-	300
	Kirsehir	103/151	17/22	0/6	-	-	0/1	300
Middle Black Sea	Samsun	76/101	7/15	-	0/1	-	-	200
	Sinop	71/99	13/16	-	-	0/1	-	200
Total		367/483	65/76	0/6	0/1	0/1	0/1	1000

TABLE 3 Positivity of *Enterocytozoon bieneusi* and *Coxiella burnetii* in Muscidae from Türkiye.

Species	Latitude (N)	Longitude (E)	Altitude (m)	Pathogen positivity	Locality
Musca domestica	38°22′25″	35°24′24″	1266	*C. burnetii*	Kayseri
M. domestica	38°42′59″	36°25′54″	1547	*C. burnetii*	Kayseri
M. domestica	38°45′17″	36°27′55″	1600	*E. bieneusi*	Kayseri
M. domestica	39°04′41″	34°16′42″	1164	*C. burnetii*	Kirsehir
M. domestica	39°26′24″	33°57′51″	1185	*E. bieneusi*	Kirsehir
M. domestica	41°15′00″	36°46′50″	672	*E. bieneusi*	Samsun
M. domestica	41°20′52″	36°12′10″	206	*C. burnetii*	Samsun
M. domestica	41°20′51″	36°11′01″	206	*E. bieneusi*	Samsun
M. domestica	41°45′27″	35°06′49″	216	*C. burnetii*	Sinop
Stomoxys calcitrans	39°13′12″	34°16′20″	1181	*C. burnetii*	Kirsehir
S. calcitrans	41°11′32″	36°14′42″	301	*C. burnetii*	Samsun
Hydrotaea ignava	41°27′29″	35°02′50″	454	*C. burnetii*	Sinop

Genotyping and phylogenetic analysis of *E. bieneusi*

Following the sequence analysis of 21 *E. bieneusi* isolates from *M. domestica*, four ITS genotypes were identified, including three known genotypes (BEB6, BEB8 and Type IV), and a novel genotype named as AEUEb. From the phylogenetic analysis (Figure 1), 11 isolates of BEB6 and 6 isolates of BEB8 belonged to ruminant-specific group 2, and 1 isolate of Type IV was clustered into zoonotic group 1. The novel AEUEb genotype constituted a separate clade in the phylogenetic tree, which is close to group 12 of *E. bieneusi* and showed the highest identity of 90.5%–91.5% to the genotypes reported from several rodents in this group. The novel AEUEb genotype showed an overall genetic distance of 14.7% from the other genotypes identified in *M. domestica*. The multiple alignment of the ITS sequences of AEUEb and corresponding isolates showing the nucleotide substitutions is given in Figure 2.

DISCUSSION

In Diptera, synanthropic flies are closely associated with animal dung, human excrement, garbage and decaying organic matter and are responsible for the transmission of a wide variety of pathogens such as viruses, bacteria, fungi, protozoan and metazoan parasites in humans and other animals (Förster et al., 2007). The route of transmission of these pathogens to the host is predominantly mechanical (Graczyk et al., 2005). The aim of this study was to obtain data on the role and risk factors of muscid flies collected from two different biogeographical regions of Türkiye regarding the transmission dynamics of zoonotic microsporidian species (*E. bieneusi* and *Encephalitozoon* spp.), Q fever agent *C. burnetii*, and ocular infection agent *Thelazia* spp. To date, little is known about the presence of zoonotic microsporidia, particularly *E. bieneusi* and *Encephalitozoon* spp. in muscid flies. As far as we know, worldwide there is only one published study that indicates the presence of *E. bieneusi* in muscid flies. Yu et al. (2019) reported that the prevalence of *E. bieneusi* was 12.0% in synantrophic flies collected in and around a dairy farm in China without species definition of the flies. Our study provides unique data for the first time that *E. bieneusi* is found in *M. domestica* with an overall prevalence of 2.4%. Compared to the findings of Yu et al. (2019), our results indicated somewhat lower prevalence of *E. bieneusi* in *M. domestica*. It has been well known that many factors, such as the number of animals in farms, different farm management systems, climatic and environmental conditions and different methods of fly control, influence the number of flies and the prevalence of parasites, including *E. bieneusi*. We identified three known and one novel *E. bieneusi*

TABLE 4 Prevalence and distribution of genotypes and GenBank accession numbers of *Enterocytozoon bieneusi* and *Coxiella burnetii* in muscid flies collected from several regions in Türkiye.

			E. bieneusi			*C. burnetii*		
Location	No. of samples	No. of positive	Prevalence (%)	İdentified genotype	GenBank Acc. No.	No. of positive	Prevalence (%)	GenBank Acc. No.
Kayseri	300	6	2	AEUEb ($n = 3$) BEB6 ($n = 3$)	OM569679 -	8	2.6	OM654092 OM654093 OM654094
Kirsehir	300	8	2.6	BEB6	OM569678	9	3	OM654095 OM654096 OM654097 OM654104
Samsun	200	7	3.5	Type IV ($n = 1$) BEB8 ($n = 6$)	OM569677 OM569676	7	3.5	OM654098 OM654099
Sinop	200	-	-	-	-	6	3	OM654100 OM654101 OM654102 OM654103
Total	1000	21	2.1			30	3.0	

genotypes in *M. domestica*. The two known genotypes, BEB6 and BEB8, in the Group II of *E. bieneusi*, which are generally associated with the ruminant hosts, were the most common genotypes in *M. domestica*. Li and Xiao (2019) emphasized that Group II genotypes (BEB4, BEB6, I and J) have overcome the host barrier in recent years and infected new hosts, including humans. BEB6 genotype has already been reported in Türkiye but the BEB8 genotype was detected for the first time in the present study. The other known genotype, Type IV of Group I, which includes the zoonotic genotypes of *E. bieneusi*, was also identified in a specimen of *M. domestica*. This genotype has been reported from humans in France (AF242478), Bangladesh (MK982499) and Nigeria (JX683801); dog, cat, horse and fox in China (KJ668722, KX964628, MK789441 and KT750160, respectively); snake in India (KJ651436); stone marten in Poland (MN218604) and chicken in Brazil (KT943975). The Type IV genotype also was reported in cats (Pekmezci et al., 2019) from the same research area in Türkiye. The novel AEUEb genotype was identified in three specimens of *M. domestica* and it showed high genetic difference (14.7%) from the other genotypes identified in *M. domestica* in the research area. The AEUEb genotype exhibited the highest identities of 90.5%–91.5% to the genotypes reported in rodent hosts in China (Deng et al., 2018; Jiang et al., 2024). However, it might be difficult to conclude that this novel genotype is associated with rodent hosts due to the genetic difference of over 8% with the reported genotypes in rodents. Therefore, further studies, including humans and several other animal hosts in the research area, are needed to clarify the host specificity and range of the AEUEb genotype.

We identified the molecular prevalence of *C. burnetii* (3%) in muscid flies. *Coxiella burnetii* was detected in *M. domestica*, *S. calcitrans* and *H. ignava*. There have been limited studies indicating the presence of *C. burnetii* in fly species. Nelder et al. (2008) reported *C. burnetii* in *S. calcitrans* (1.8%), *Lucilia coeruleiviridis* Macquart (Diptera: Calliphoridae) (8.3%) and *Lucilia sericata* Meigen (Diptera: Calliphoridae) (16.6%), which were collected from different animal related areas in the United States. It can be argued that these flies could contact the faecal, urinary and other secretions of the infected host(s), which lead to contamination of the outer surface of the fly. An experimental study showed that *C. burnetii* did not reproduce in the body of *M. domestica* that acquired *C. burnetii* from infected milk, and contamination was limited to the outer surface of fly. It is well known that several ticks have symbiotic relationships with *C. burnetii* and tick-bites are considered one of the transmission routes of this bacteria to animal hosts (González et al., 2020; Knap et al., 2019). *Coxiella burnetii* also was reported from other ectoparasites, such as mites (*Dermanyssus gallinae* DeGeer (Mesostigmata: Dermanyssidae), fleas and *Pediculus* sp). Although a lower prevalence of *C. burnetii* was detected in muscid flies in our study, it can be suggested that muscid flies may also play a role in the transmission dynamics. Therefore, further detailed surveys based on both experimental approaches and field studies with larger samplings from different geographical areas are needed to explore the role and risk factors of muscid flies for the transmission of *C. burnetii*.

In the present study, *Encephalitozoon* spp. and *Thelazia* spp. were not detected in the examined fly samples. Even so, *Encephalitozoon* spp. were reported from different hosts such as humans (Cetinkaya et al., 2016), dogs, cats and pet budgeriars in Türkiye (Duzlu et al., 2019; Pekmezci et al., 2021). Otranto, Lia, Testini, et al. (2005) demonstrated in their experimental study that *M. domestica* has no vector potential for *T. callipeda* by molecular and dissected sample microscopy. *Musca domestica* ($n = 850$) was the predominant species in the sampling areas, while *M. autumnalis*, the main vector of thelaziosis, was represented by only six specimens. This could be one of the reasons why *Thelazia* spp. was not detected in the current study.

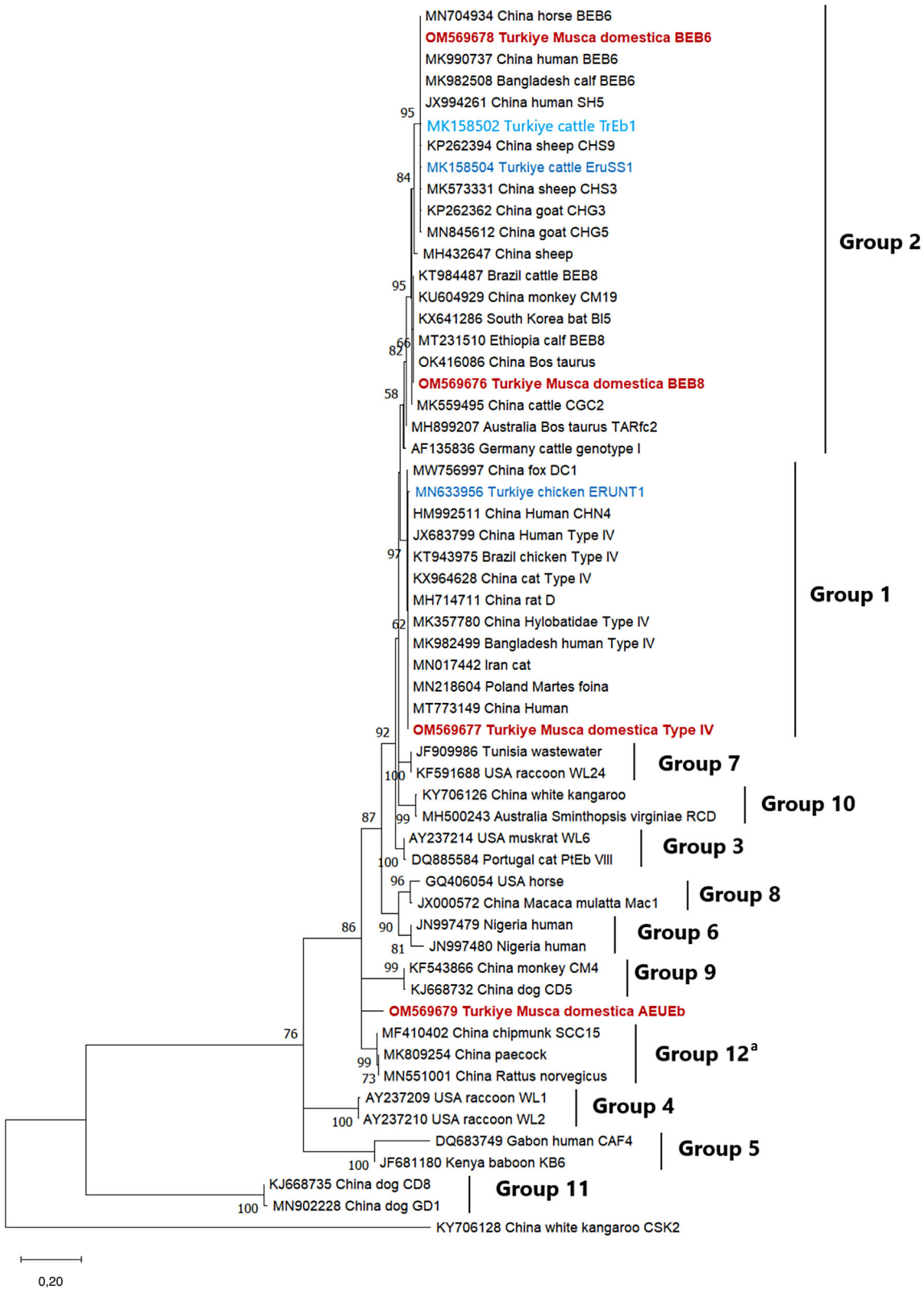

FIGURE 1 Phylogenetic relationships among the genotypes of *Enterocytozoon bieneusi* identified in *Musca domestica* from Türkiye (in red) and known genotypes previously reported from different countries and hosts. The tree was constructed using maximum likelihood analyses of internal transcribed spacer (ITS) sequences. The isolates are displayed with GenBank accession numbers, country, host and genotype name. Numbers at the branches indicate bootstrap values (1000 replicates). The *E. bieneusi* genotype CSK2 from the white kangaroo (KY706128) is used as an outgroup. [a]Group 12 proposed by Wang et al. (2020).

FIGURE 2 Sequence variation in the internal transcribed spacer region of *Enterocytozoon bieneusi* AEUEb genotype (in the first row, accession number: OM569679) and some genetically close isolates (based on percent identity) available in the GenBank database. The novel AEUEb genotype identified in *Musca domestica* from Türkiye.

In conclusion, we provide unique data on the vector potential of fly species belonging to Muscidae for *E. bieneusi* and *C. burnetii*, which contribute to current knowledge on the genetic diversity, epidemiology and transmission dynamics of both pathogens.

AUTHOR CONTRIBUTIONS

Nuri Ercan: Conceptualization; methodology; resources; investigation; visualization; data curation; project administration; writing – original draft; writing – review and editing. **Alparslan Yildirim:** Conceptualization; methodology; resources; supervision; data curation; writing – review and editing. **Onder Duzlu:** Investigation; resources; writing – review and editing; data curation. **Fahriye Ercan:** Resources; data curation; investigation; writing – review and editing. **Gamze Yetismis:** Data curation; resources; investigation; writing – review and editing. **Gokmen Zafer Pekmezci:** Data curation; resources; investigation; writing – review and editing. **Abdullah Inci:** Methodology; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

ORCID

Nuri Ercan https://orcid.org/0000-0001-6039-3510

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