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ORIGINAL ARTICLE

Molecular detection and identification of *Wolbachia* endosymbiont in fleas (Insecta: Siphonaptera)

Zuhal Onder[1] · Arif Ciloglu[1] · Onder Duzlu[1] · Alparslan Yildirim[1] · Mubeccel Okur[1] · Gamze Yetismis[1] · Abdullah Inci[1]

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Abstract

The aim of this study was to determine the presence and prevalence of *Wolbachia* bacteria in natural population of fleas (Insecta: Siphonaptera) in Turkey, and to exhibit the molecular characterization and the phylogenetic reconstruction at the positive isolates with other species in GenBank, based on 16S rDNA sequences. One hundred twenty-four flea samples belonging to the species *Ctenocephalides canis*, *C. felis*, and *Pulex irritans* were collected from animal shelters in Kayseri between January and August 2017. All flea species were individually screened for the presence of *Wolbachia* spp. by polymerase chain reaction (PCR) targeting the 16S ribosomal RNA gene. According to PCR analyses, *Wolbachia* spp. were found prevalent in *C. canis* and *P. irritans* fleas, while it was not detected in the *C. felis* species. Totally, 20 isolates were purified from agarose gel and sequenced with the same primers for molecular characterization and phylogenetic analyses. The sequence analyses revealed 17 polymorphic sites and 2 genetically different *Wolbachia* isolates, representing two different haplotypes in two flea species. The distribution patterns, molecular characterization, and phylogenetic status of *Wolbachia* spp. of fleas in Turkey are presented for the first time with this study. Understanding of the role of *Wolbachia* in vector biology may provide information for developing *Wolbachia*-based biological control tools.

Introduction

Wolbachia is a gram-negative, obligate intracellular bacteria, and maternally inherited endosymbiont belonging to the family Anaplasmataceae within the order Rickettsiales (Doudoumis et al. 2012; Karimian et al. 2018). These endosymbiotic bacteria infect approximately 40–66% of arthropods as well as filarial nematodes all over the world (Hilgenboecker et al. 2008; Zug and Hammerstein 2012; Weinert et al. 2015; Sazama et al. 2017). Intracellular bacteria have been reported as a reason for different reproductive alterations and developmental changes in their hosts, including cytoplasmic incompatibility of host gametes, parthenogenesis, male-killing, and feminization of genetic males (Werren et al. 2008; Doudoumis et al. 2012; Karimian et al. 2018). Additionally, recent studies have exhibited that the presence of *Wolbachia* bacteria in arthropod hosts influences the immune system of the significant arthropod vectors (Xi et al. 2008; Kambris et al. 2009; Moreira et al. 2009). Arthropod-borne viruses (arboviruses) are transmitted between vertebrate hosts and blood-feeding arthropods such as mosquitoes, sand flies, biting midges, mites, lice, flea, and ticks (Kamtchum-Tatuene et al. 2017). Insects, including mosquitoes, have developed a highly effective innate immune system to defend themselves against harmful microbes during their life cycle. These bacteria in mosquitoes boost the basal immune response and enhance the mosquito's resistance to pathogens, including dengue, Zika virus, and malaria parasites (Pan et al. 2018). Similarly, if *Wolbachia* infections increased resistance to pathogens of fleas, it may be desired to incorporate this bacterium as a biocontrol agent against flea-transmitted diseases. To understand better the biological, ecological, and evolutionary processes of *Wolbachia* in arthropods will help in using it as a potential agent in a biological control of pest and disease vectors (Beard et al. 1998; Doudoumis et al. 2012).

Fleas (Insecta: Siphonaptera) are the most significant ectoparasites of domestic animals all over the world and can cause important pathology in their hosts (Azarm et al. 2016). They are obligate parasites (ecto- or endoparasitic) of birds and mammals (Whiting et al. 2008; Azarm et al. 2016). The Order Siphonaptera plays a role as parasites in the host by

✉ Zuhal Onder
zuhalbiskin@erciyes.edu.tr

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

causing allergic dermatitis. It is estimated that fleas cause approximately 50% of all cat and dog dermatological cases presented in veterinary clinics (Kramer and Mencke 2001), and pet owners spend more than 1 billion dollars annually on flea control products (Rust 2005; Otranto and Wall 2008; Blagburn and Dryden 2009). Fleas have medical and economic importance as a vector of several zoonotic diseases such as bartonellosis (*Bartonella* spp.), murine typhus (*Rickettsia typhi*), tularemia (*Francisella tularensis*), flea-borne spotted fever (*Rickettsia felis*), and plague (*Yersinia pestis*). These ectoparasites can transmit several disease agents to animals and humans (Eisen and Gage 2012).

In recent studies, the multilocus genotyping (MLST), which includes the sequences of the surface protein gene (*wsp*), 16S rDNA, *groEL*, *dna*A, *ftsZ*, *gatB*, *coxA*, *hcpA*, *gltA*, and *fbpA* genes of *Wolbachia* spp., has been frequently used to confirm the identification and phylogeny of strains of the *Wolbachia* spp. (Casiraghi et al. 2005; Doudoumis et al. 2012). Up to now, 17 supergroups of *Wolbachia* have been determined based on a MLST, named A-Q (except G) (Karimian et al. 2018). *Wolbachia* strains from supergroups A and B have been found in a wide variety of insect species (Karimian et al. 2018). Fourteen subgroups (Mel, AlbA, Mors, Riv, Uni, Haw, Pap, Aus, Whi, Lop, Eum, Nov, Niv, and Sub) were found within Super Group A, while 10 subgroups (Con, Dei, Pip, CauB, Prn, Dro, Unif, Crag, Perp, and Leva) were found within Super Group B (Vivero et al. 2017; Karimian et al. 2018). The 16S rDNA gene region was used to determine the macro-taxonomy (supergroup designation) and micro-taxonomy (subgroup designation) of the genus *Wolbachia* in various hosts (O'Neill et al. 1992; Gorham et al. 2003).

Molecular data on the *Wolbachia* bacterial endosymbiont in fleas around the world is very limited (Gorham et al. 2003; Dittmar and Whiting 2004; Tay 2013; Oteo et al. 2014). In addition, there is no study on the molecular characterization and genotyping of *Wolbachia* bacteria in fleas in Turkey. In this study, we aimed to detect the presence and prevalence of *Wolbachia* endosymbiont by PCR analyses of the 16S rDNA gene region. *Wolbachia* isolates in flea populations were molecularly characterized for the first time in Turkey, based on the 16S rDNA gene region.

Material and methods

Collection of fleas and morphological identification

The flea specimens were collected manually from animal shelters in two districts, Kocasinan and Melikgazi, in Kayseri Province (Table 1) in Central Anatolia of Turkey between January and August 2017. The captured fleas were placed into vials with 70% ethanol solution and transferred to the laboratory. The flea specimens were morphologically identified under a stereomicroscope (BX41 Olympus) using related identification keys (Furman and Catts 1982; Wall and Shearer 2001).

Genomic DNA isolation

The adult flea specimens were pulverized in a 1.5-mL tube with liquid nitrogen, using plastic pestles. The total genomic DNA (gDNA) was isolated from individually crushed fleas using a commercial kit (GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, USA) following the manufacturer's instructions, with a final elution volume of 50 μL. The extracted gDNAs were stored at − 20 °C until the molecular analyses.

PCR amplification

The concentration of extracted gDNA was measured using a Qubit 3.0 Fluorometer (Life Technologies). The detection of the presence of *Wolbachia* DNA from each flea was carried out using universal eubacterial 16S rRNA specific primers: 16SWol forward (5′-GCTTAACACATGCAAG-3′) and 16SWol reverse (5′-CCATTGTAGCACGTGT-3′) primers (O'Neill et al. 1992). These primers amplified an ~900-base-pair (bp) fragment of the 16S rRNA gene. In order to confirm the presence of *Wolbachia*, we used the second specific primers (81F:5′-TGGTCCAATAAGTGATGAAGAAAC-3′ and 691R:5′-AAAAATTAAACGCTACTCCA-3′) for the *Wolbachia* surface protein gene (*wsp*) that amplified a 590- to 632-bp fragment (Zhou et al. 1998).

All PCR assays were performed with a 25-μL reaction mixture containing 12.5 μL of commercial Master Mix (Maxima Hot Start Green PCR Master Mix; Thermo Fisher Scientific, Waltham, MA, USA), 1.25 μL of each primer (10 μ mol/L concentration), 8 μL ddH_2O, and 2 μL of gDNA template (10–30 ng/μL) by C1000 Thermal Cycler (Bio-Rad, USA).

Wolbachia DNA from naturally infected *Culex pipiens* was used as a positive control, whereas DNase-RNase-free water was used as negative control in each PCR assay. PCR amplification protocols for both *wsp* and the 16S rDNA gene regions have an initial denaturation step of 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min (modified from O'Neill et al. 1992 and Zhou et al. 1998). All samples were held at 4 °C. The PCR products (10 μL) were electrophoresed on ethidium bromide-stained 1.5% agarose (Sigma-Aldrich) 1×TAE gel, run at 135 V for 30 min, and visualized using the CLP Gel Documentation System (UVP INC Upland, CA).

Table 1 The number of collected fleas from different locations and *Wolbachia* infections by PCR analyses

District	Selected village for sampling	Coordinates latitude and longitude	Number of collected fleas			Number of PCR-positive samples		
			C. canis	*C. felis*	*P. irritans*	*C. canis*	*C. felis*	*P. irritans*
Kocasinan	Emmiler	38° 90′ 88.13″ N, 35° 40′ 47.43″ E	10	5	12	8	–	10
	Elagoz	38° 80′ 58.32″ N, 35° 51′ 20.88″ E	5	3	8	3	–	4
	Molu	38° 80′ 37.82″ N, 35° 36′ 58.51″ E	15	1	2	12	–	2
Melikgazi	Buyukburunguz	38° 75′ 18.15″ N, 35° 77′ 34.39″ E	15	6	10	10	–	8
	Agirnas	38° 80′ 85.55″ N, 35° 75′ 00.94″ E	7	2	8	6	–	6
	Sarimsakli	38° 88′ 66.31″ N, 35° 71′ 21.05″ E	8	1	6	7	–	4
	Total		60	18	46	46	–	34

Sequence alignment, phylogenetic, and genetic distance analyses

All PCR-positive products were purified from agarose gel using the High Pure PCR product purification kit (Roche, Mannheim, Germany) following the manufacturer's instructions prior to sequencing. The purified DNA fragment was sequenced in both directions (Macrogen, Korea) using 16SWol forward and reverse primers. The nucleotide chromatograms were edited with Geneious R11 software (http://www.geneious.com, Kearse et al. 2012). DNA polymorphism, the number of haplotypes, and nucleotide diversity within each population were determined using DnaSP 5.10.01 (Librado and Rozas 2009). Pairwise sequence divergences within and among the haplogroups were calculated using the Kimura two-parameter (K2P) distance model (Kimura 1980) in MEGA version 7 (Tamura et al. 2013). Phylogenetic analyses (Fig. 4) were conducted using Bayesian (BA) inference in Mr Bayes 3.2.6 (Huelsenbeck and Ronquist 2001) and through the Geneious R11 (Kearse et al. 2012) software in order to compare our sequences with the other *Wolbachia* sequences obtained from fleas. The General Time Reversible Model, including invariable sites and variation among sites (GTR + I + G), was utilized according to the results of Akaike's Information Criterion in jModelTest version 0.1.1 (Posada 2008). Two Markov Chain Monte Carlo (MCMC) simulations in the BA were run simultaneously for 10 million generations, with sampling every 200 generations for posterior probability calculations. Twenty-five percent of the parameter estimates were discarded as burn-in periods. *Ehrlichia* sp. (MF069159) was used as an outgroup. The nucleotide sequence data obtained in this study has been deposited in the GenBank database under the accession numbers MH521186–MH521191.

Results

Totally, 124 flea samples, which included human flea *Pulex irritans* ($n = 46$, 31 female, 15 male), dog flea *Ctenocephalides canis* ($n = 60$, 37 female, 23 male), and cat flea *C. felis* ($n = 18$, 8 female, 10 male) were collected from the study areas (Fig. 1). *Ctenocephalides canis* (60 specimens; 48.38%) was determined as the most prevalent species in the samples. These fleas were also identified with molecular analyses of mitochondrial cytochrome c oxidase I subunit (mt-COI) gene region (Folmer et al. 1994) (GenBank accession numbers as follows: KY865409–13 for *C. canis*, KY865418–19 for *C. felis*, and KY865414–17 for *P. irritans*, unpublished). The 16S rDNA gene of the *Wolbachia* spp. was successfully amplified with PCR in 80 out of 124 flea specimens (Fig. 2). The number of fleas collected from different locations, and *Wolbachia* infections are shown in Table 1. *Wolbachia* infections were detected in only the *C. canis* and *P. irritans* specimens in this study. *Wolbachia* infection rates were determined higher in *C. canis* (75.0%). *Wolbachia* infection prevalence was determined as 60.41% (29/48) in male, and 67.0% (51/76) in female fleas (Fig. 3). Not all positives from the 16S rDNA screening were amplified with the *wsp* gene region (Fig. 2). To our knowledge, this is the first record of *Wolbachia* in fleas in Turkey. Twenty out of 80 positive samples were sequenced for the 16S rRNA gene region. All sequenced samples exhibited 100.0% identity to each other. Three nucleotide sequences from *P. irritans* and *C. canis* samples were submitted to the GenBank. According to the DNA polymorphism and haplotype analyses of the obtained sequences, 17 polymorphic sites and 2 different haplotypes (named ERU-Wol-16S-1 and ERU-Wol-16S-2) were determined in the study. The haplotype ERU-Wol-16S-2 was constructed from 11 *Wolbachia* isolates for *C. canis* while the ERU-Wol-16S-1 haplotype consisted of nine *Wolbachia* isolates from *P. irritans*. The mean haplotype and nucleotide diversities among the obtained 16S rDNA sequences were determined to be 0.600 and 0.01162. The haplotype ERU-Wol-16S-1 clustered into a monophyletic group with some published *Wolbachia* isolates from various flea samples in different geographical regions (Zhou et al. 1998; Gorham et al. 2003; Dittmar and Whiting 2004; da Silva et al. 2017). Pairwise genetic distance between our isolates and several sequences of *Wolbachia* available in the GenBank displayed variation ranging from 0.9 to 2.1%. The haplotype ERU-Wol-

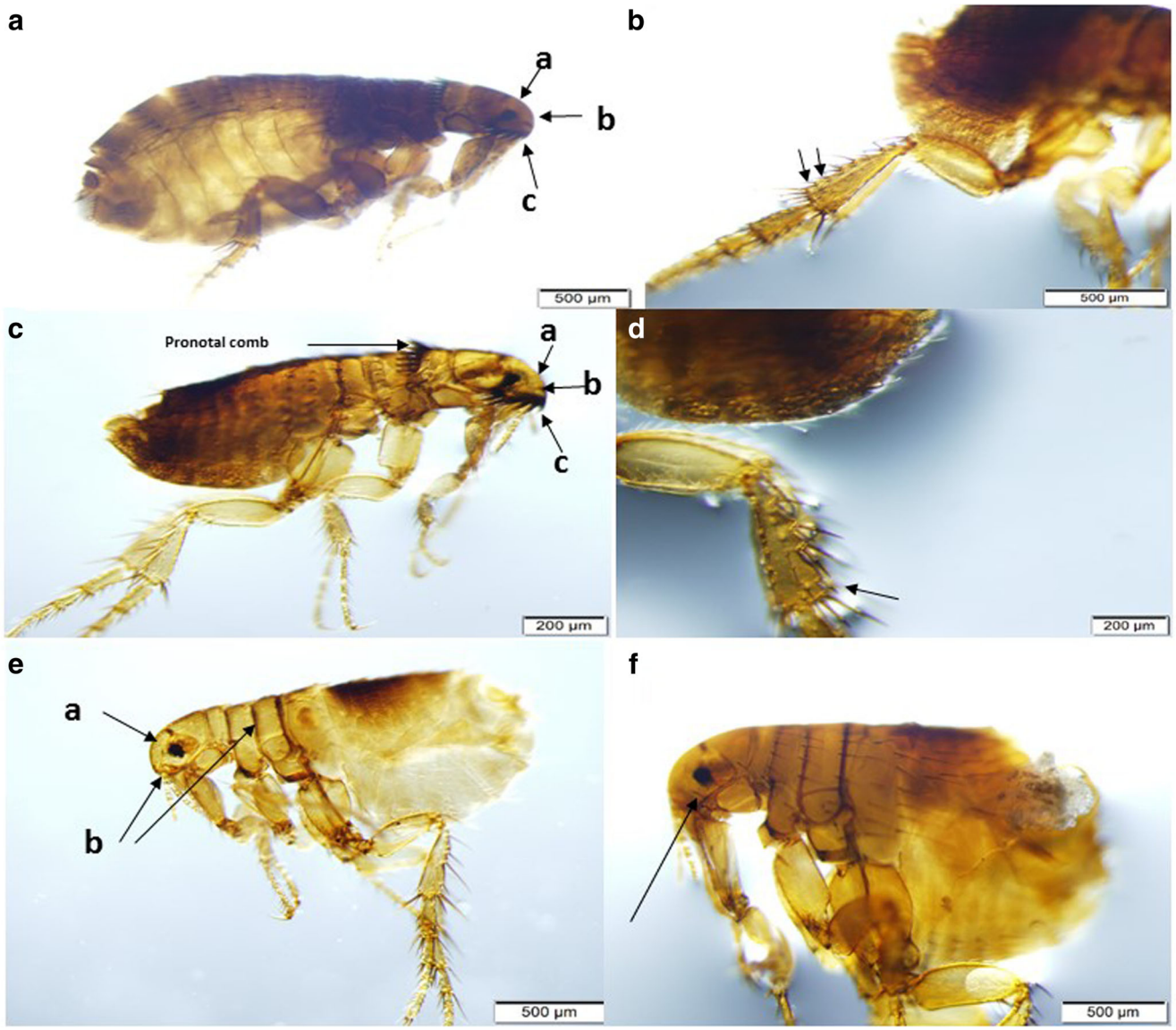

Fig. 1 Characteristic features of *Ctenocephalides canis*, *C. felis*, and *P. irritans* collected from animal shelters. **a** *C. canis* with a bluntly rounded frons or head (a) and a short stout dorsal incrassation (b). The first spine (c) of the genal ctenidia was close to half the length of the second spine. **b** The dorso-posterior margin of the hind tibia bore two notches with stout setae (black arrows) between the post median and apical setae in *C. canis*. **c** The species *C. felis* is characterized by an acutely angled frons (a) and the head appears "pointier" than *C. canis*. The dorsal incrassation (b) in this species is long and narrow and the first spine (c) of the genal ctenidia exceeds at least half of the second in length. **d** One short stout bristle in the interval between the post median and apical setae on the dorso-posterior margin of the hind tibia in *C. felis*. **e** *P. irritans* has a broadly rounded frons (a) and lacks both genal and pronotal combs (b). **f** The ocular setae are below (black arrow) the eye in *P. irritans*

16S-1 showed 1.3 ± 0.3 interspecific distance in the *Wolbachia* haplotypes, which are found in different species of fleas. The phylogenetic tree of the 16S rDNA sequences (Fig. 4) explored two major clades. The first clade (Clade I) includes species presented in Fig. 4. The second clade (Clade II) contains only the *Wolbachia* strain presented in *C. canis*. In Clade I, ERU-Wol-16S-1 clustered with isolates from several flea species collected from the USA, Nearctic/Neotropical regions, China, Brazil, Spain, and Kazakhstan (Fig. 4, Clade I). ERU-Wol-16S-2 clustered separately in Clade II. As seen in Fig. 4, our *Wolbachia* isolates obtained from *C. canis* have clustered phylogenetically separate from other *Wolbachia* isolates in *C. canis* reported from other countries (Brazil, Spain, and USA).

Discussion

In the present study, the dog flea *C. canis* was the main flea species in the studied areas (48.38% of the samples). We

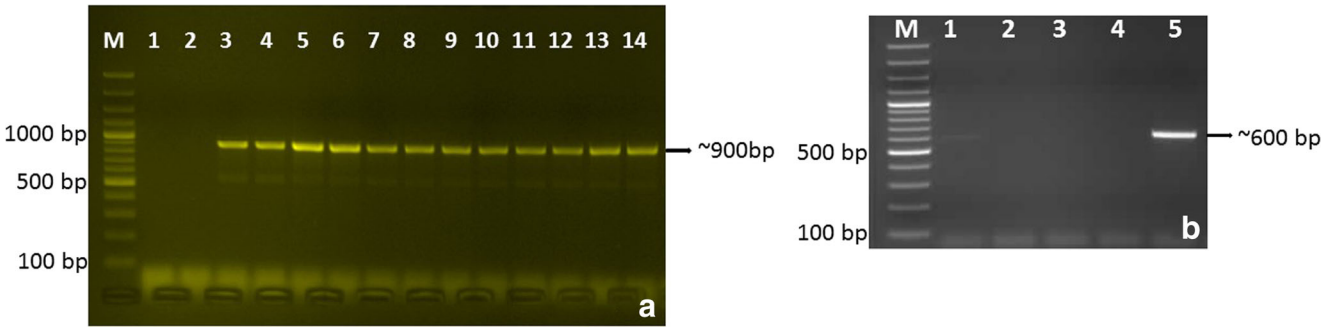

Fig. 2 **a** PCR amplification of *Wolbachia* 16S rDNA gene in flea specimens collected from animal shelters. Lane M, 100-bp marker. Lanes 1–2, negative samples. Lanes 3–13, positive samples. Lane 14, positive control. **b** Results of PCR amplification of the *wsp* gene of *Wolbachia* in flea specimens that were positive in 16S rDNA screening. Lane M, 100-bp marker. Lanes 1–4, negative samples. Lane 5, positive control

screened the fleas for the presence of *Wolbachia* by PCR with 16S rDNA gene region primers and with *wsp* gene region primers for confirmation of 16S rDNA positive samples. All samples were found positive for the 16S rDNA gene, but not for the *wsp* gene region. This result exhibited similar findings with the results of Gorham et al. (2003). Gorham et al. (2003) had similarly determined positive samples with only 16S rDNA screening in some flea samples. These results showed that 16S rDNA primers might be more sensitive than the *wsp* primers in detecting *Wolbachia* infection. This incompatibility between the *wsp* and 16S rDNA gene regions may be also due to natural properties of the two genes. The *wsp* gene is a membrane protein of the bacterial outer envelope and it is built up quickly, and thus the PCR primers are specific to strains of the *Wolbachia* spp. (Zhou et al. 1998). However, the 16S rDNA gene has more of a slow-change gene in some conservative regions. Thus, the used primers in this study may match virtually 100.0% with the templates and can amplify all strains of *Wolbachia* spp. Some samples may contain different strains of *Wolbachia* endobacteria that do not amplify with primers of the *wsp* gene region (Gorham et al. 2003). However, Pourali et al. (2009) showed that the detection of *Wolbachia* by *wsp* primers declines with time of storage (95% ethanol and freezing at − 20 °C), whereas the detection levels with the 16S rDNA primers remained high (Werren and Windsor 2000). According to Pourali et al. (2009), this result has been revealed to have some degradation of the DNA within the samples, which affects the amplification of the *wsp* product. In our

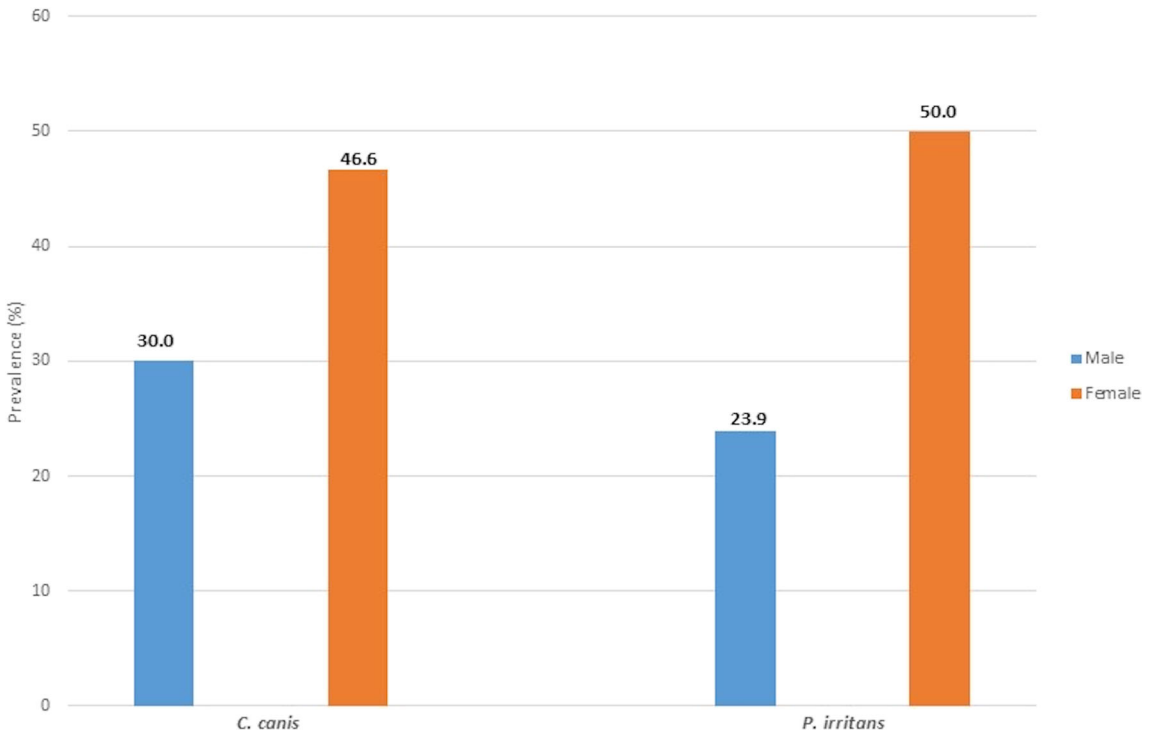

Fig. 3 Prevalence of *Wolbachia* infection in male and female fleas for *C. canis* and *P. irritans* species. Out of the 80 positive samples, 30.0% (18/60) and 23.9% (11/46) of males and 46.6% (28/60) and 50.0% (23/46) of females in *C. canis* and *P. irritans* specimens were positive

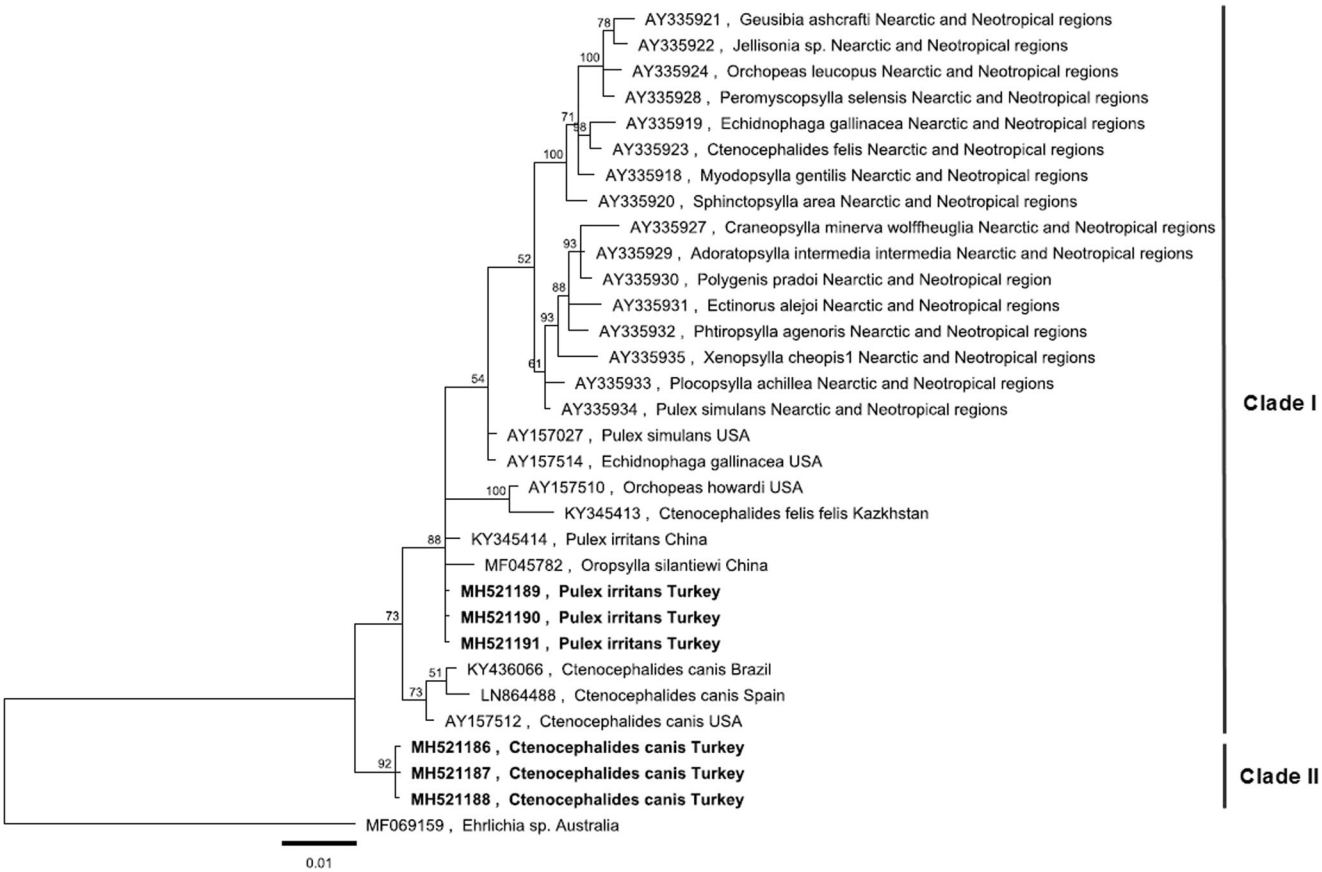

Fig. 4 BA inference tree is based on 16S rDNA sequences of *Wolbachia* in fleas, with *Ehrlichia* sp. (MF069159) as the outgroup. Numbers above branches indicate posterior probabilities. Sequences were grouped according to host, country, and accession numbers. Isolates with bold characters indicate the haplotypes detected in this study. Scale bar represents 0.01 substitutions per nucleotide position

study, fleas were stored within 70% ethanol at + 4 °C for morphological identification. Then, genomic DNA from the fleas were extracted and stored at − 20 °C until PCR analysis. The genomic DNA could have been degraded due to the storage medium, as compatible with the findings of Pourali et al. (2009), and thus, we may not identify *Wolbachia* DNA with the *wsp* analysis in this study.

Wolbachia endobacteria were firstly detected in fleas in 2000 (Jeyaprakash and Hoy 2000). Our study clearly showed that *Wolbachia* infections are present in several flea populations. Our results were also confirmed by sequencing the positive isolates and phylogenetic analysis based on the 16S gene dataset. The prevalence of *Wolbachia* infection may be different in various populations of fleas. In the Siphonapteran host, from 7.0% (*C. canis*) to 100.0% (*Tunga penetrans*) of examined specimens were found to be infected with *Wolbachia* (Fischer et al. 2002; Gorham et al. 2003). Dittmar and Whiting (2004) investigated 79 flea specimens collected from the Nearctic and Neotropical regions, and the results of this study showed that 25.31% (20/79) of the samples were found positive for *Wolbachia* spp. Our study is the first report for a *Wolbachia* presence in various flea species in Turkey. However, the *Wolbachia* infection was seen *C. canis* and *P. irritans* species. Interestingly, no infection was detected in neither female nor male *C. felis* specimens. The infection rate in *C. canis* and *P. irritans* of *Wolbachia* endosymbiont was determined as 75.0 and 25.0%. The prevalence of *Wolbachia* in *C. canis* was higher than *P. irritans*. Gorham et al. (2003) reported a low infection rate (7.0%) of *Wolbachia* in *C. canis* fleas. This result is not in agreement with our study. The prevalence of *Wolbachia* in *P. irritans* (25.0%) in this study was quite lower than the rate of *Wolbachia* (83.0%) infection in *P. irritans* specimens collected from dogs in two Ecuadorian areas (Pastaza and Chimborazo provinces) in 2012 (Oteo et al. 2014). Additionally, similar findings were reported that *Wolbachia* was detected in 94% of the *P. simulans* collected from dogs and cats from animal shelters and animal control facilities in four different counties: Bulloch, Liberty, and Chatham (Georgia), and Erie (New York) (Gorham et al. 2003). Although *C. felis* fleas are known to carry *Wolbachia* (Jeyaprakash and Hoy 2000; Rolain et al. 2003; Oteo et al. 2014), none of the *C. felis* specimens in this study showed evidence of carriage of *Wolbachia* bacteria. Furthermore, our analysis is in high agreement with Oteo et al. (2014), who showed the absence of *Wolbachia* endosymbiont in *C. felis* collected from animals in Ecuador. In previous studies, the

infection rates of *Wolbachia* in cat fleas have been reported to vary from 20.0 to 63.0% (Rolain et al. 2003; Gorham et al. 2003; Tay 2013). Moreover, the results may also point out that the host species has high importance in the infection prevalence of *Wolbachia* endobacteria in fleas (Gorham et al. 2003).

In this study, female flea numbers ($n = 76$) were detected more abundantly than male numbers ($n = 48$), which is in agreement with previous findings (Gracia et al. 2008; Tavassoli et al. 2010). In temporary parasites, males mostly have a shorter life period and are more active. Thus, males leave earlier from their hosts (Marshall 1981). The females need blood to produce their eggs, so females leave their hosts later (Dryden 1993). In our study, we detected that *Wolbachia* positivity rate in female fleas was much closer to the infection rate in male fleas.

The 16S rDNA product obtained in this study was sequenced and the result of sequences is identical with the *Wolbachia* strain obtained from fleas in different geographical regions. Our 16S sequence analyses revealed two haplotypes (ERU-Wol-16S-1 and ERU-Wol-16S-2) for *Wolbachia* from the *C. canis* and *P. irritans* fleas. Based on the phylogenetic analyses of 16S rDNA sequences, *Wolbachia* strains have divided into two clades in the phylogenetic trees. According to Dittmar and Whiting (2004), based on phylogenetic analysis of *Wolbachia* 16S rDNA sequences, Neotropical and Nearctic *Wolbachia* strains of fleas are a monophyletic group and have multiple haplotype, like our findings. Our results could be explained with strain differences between *Wolbachia* strains in collected fleas in animal shelters.

In conclusion, our study provides the first molecular data about *Wolbachia* infection in fleas in Turkey. Based on 16S rDNA analyses, the presence of endosymbiotic *Wolbachia* has been found in *C. canis* and *P. irritans*. Hence, more detailed epidemiologic studies are needed to detect the relationship between *Wolbachia* endosymbiont and flea hosts in the future. The detection of this endobacteria in various arthropod species may be used as a potential agent in application of *Wolbachia*-based biological control approaches for arthropod pests and disease vectors in further research. These findings have valuable outputs to understand better the occurrence and distribution of *Wolbachia* endosymbiont in fleas in Turkey.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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