Ankara Üniv Vet Fak Derg, **60**, 189-194, 2013

Detection and molecular characterization of the *Wolbachia* Endobacteria in the *Culex pipiens* (Diptera: Culicidae) specimens collected from Kayseri province of Turkey

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Summary: This study was performed to investigate *Wolbachia* endobacteria in *Culex pipiens* specimens collected from Kayseri province of Turkey. For this aim, totally 10 genomic DNA pools each including 6-15 *Cx. pipiens* specimens which were collected and identified within the scope of a project (No: 107O533) supported by TUBITAK, were examined by using the amplification of surface protein gene (*wsp*) region of the *Wolbachia*. The sequences from this gene were highly variable and could be used to resolve the phylogenetic relationships of different *Wolbachia* strains. After the genomic DNA extraction from the pools, PCR analyses were carried out with *Wolbachia* specific primer pair which was amplified a 590-632 bp region of the wsp gene. Out of 6 of the 10 examined genomic DNA pools were found to be positive (60.0%) by PCR analyses. The minimum infection rate of *Wolbachia* spp. in the totally analyzed 118 *Cx. pipens* specimens was determined as 5.08. One of the amplicon from the positive isolates was gel purified and sequenced in terms of wsp gene region of *Wolbachia* by using the same primers. Pair wise analyses of the obtained DNA sequences and multiple alignments with some other *Wolbachia* strains available in the GenBank were done and phylogenies were investigated. The obtained isolate (WolKys1) was deposited in GenBank International Nucleotide Sequence Database with the accession number JX474753. The phylogenetic analyses revealed that the obtained WolKys1 isolate belongs to *Wolbachia* Super Group B and wPIP group. According to the phylogenetic comparisons the WolKys1 showed 100.0% identity with some other *Wolbachia* isolates under the Group B. In conclusion, this study reports the first molecular detection and characterization of *Wolbachia* endobacteria in *Cx. pipiens* populations in Turkey.

Key words: *Cx. pipiens,* molecular characterization, Turkey, *Wolbachia.*

Kayseri yöresinden toplanmış *Culex pipiens* örneklerinde *Wolbachia* Endobakterisinin belirlenmesi ve moleküler karakterizasyonu

Özet: Bu çalışma, Kayseri yöresinden toplanmış *Culex pipiens* örneklerinde *Wolbachia* endobakterisini araştırmak amacıyla yapılmıştır. Bu amaçla TÜBİTAK tarafından desteklenen 107O533 kod no'lu araştırma projesi kapsamında, Kayseri yöresinden toplanmış, *Cx. pipiens* olarak identifiye edilmiş ve her birinde 6-15 adet *C. pipiens* türü içeren 10 adet genomik DNA havuzu materyal olarak belirlenmiştir. Genomik DNA havuzları *Wolbachia* yüzey protein (wsp) gen bölgesinin amplifikasyonu yönünden incelenmiştir. Bu gen bölgesinin sekansı yüksek değişkenlik göstermekte olup farklı *Wolbachia* suşları arasındaki filogenetik ilişkilerin analizinde kullanılabilmektedir. Havuzlardan genomik DNA ekstraksiyonunu takiben wsp gen bölgesinin 590-632 bp'lik kısmını amplifiye eden *Wolbachia* spesifik primerler ile PCR analizleri yapılmıştır. İncelenen 10 havuzun 6'sı (%60,0) PCR analizleriyle pozitif bulunmuştur. İncelenen toplam 118 *Cx. pipiens* türünde *Wolbachia* spp. ile minimum enfeksiyon oranı 5.08 olarak belirlenmiştir. Pozitif izolatlardan birine ait amplikon jel pürifiye edilmiş ve söz konusu gen bölgesi yönünden aynı primerler ile sekanslanmıştır. Elde edilen DNA dizisinin GenBank'ta mevcut diğer bazı *Wolbachia* suşları ile pairwise analizleri ve multiple alignmentları yapılarak filogenisi araştırılmıştır. Elde edilen izolat (WolKys1) JX474753 aksesyon numarası ile GenBank International Nucleotide Sequence Database'e kaydedilmiştir. Filogenetik analiz sonucu WolKys1 izolatının *Wolbachia* B süper grubu ve wPIP grubu içinde yer aldığı belirlenmiştir. Filogenetik kıyaslamalara göre WolKys1 izolatının B grubu altındaki diğer bazı *Wolbachia* izolatları ile %100 identiklik gösterdiği saptanmıştır. Sonuç olarak bu çalışma ile Türkiye'de ilk kez *Cx. pipiens* populasyonlarında *Wolbachia* endobakterisinin moleküler olarak belirlenmesi ve karakterizasyonu yapılmıştır.

Anahtar sözcükler: *Cx. pipiens*, moleküler karakterize, Türkiye, *Wolbachia*

Introduction

The intracellular bacteria *Wolbachia* are maternally inherited endosymbionts that a genus of the class Alphaproteobacteria and belonging to the order Rickettsiales. These gram-negative bacteria are found invertebrates including insects, arachnids, crustaceans and filarial nematodes (33). Infection prevalence is very high in insect orders; estimates suggest that 65% of insect species are infected with *Wolbachia*. These bacteria cause a number of reproductive alterations in

their hosts, including cytoplasmic incompatibility (CI) in a wide range of insects (5, 8, 24), parthenogenesis induction (PI) in a parasitoid wasps and thrips (2, 31, 32), feminization of genetic males in isopods and moths, and killing of males in beetles, butterflies and a fruit fly (14, 15, 20, 21). Intracellular bacteria were first reported as Rickettsia-like microorganisms, within the ovaries and testes of the mosquito *Culex pipiens* by Hertig and Wolbach in 1924s. These bacteria were named as *Wolbachia pipientis* in 1936 (18). Based on the 16S rDNA gene and the protein-coding gene (groEL) sequence analysis, it has been confirmed into the family Anaplasmataceae which also includes of the genera *Ehrlichia, Anaplasma, Cowdria*, and *Neorickettsia* (24, 33). The gene phylogenies of the genus *Wolbachia* have shown the presence of eight major clades (A-H), have been named 'supergroups'. Supergroups A and B found in arthropods (36), supergroups C and D found in filarial nematodes (4), the E supergroup contain *Wolbachia* spp. from wingless insects, the springtails (Collembola) (35), members of supergroup F are known to infect arthropods (termites and scorpions) (3), and recent studies suggest that they also infect the filarial parasite *Mansonella ozzardi*, a causative agent of human filariasis (9, 22), members in supergroup G infect spiders and members in supergroup H are found termites and also more recently *Dipetalonema gracile* included under the Group H (28, 29).

The purpose of this study is to assess the presence of *Wolbachia* endobacteria in *Cx. pipiens* specimens using molecular tools and to estimate infection rates among *Cx. pipiens* populations collected from Kayseri province. Furthermore molecular characterization of a *Wolbachia* endobacteria isolate from *Cx. pipiens* specimens based on wsp gene sequnces is also documented.

Materials and Method

Sampling area and Cx. pipiens specimens: The material of this study was obtained within the scope of a former research project supported by TUBITAK (No: 107O533) which investigates the prevalent mosquito species in Kayseri province and vector competence of the collected species for the nematode *Dirofilaria immitis* by molecular tools (42). Totally 10 genomic DNA pools each including 6-15 *Cx. pipiens* specimens were selected for the study.

DNA isolation: The pools were ground to a fine powder using liquid nitrogen in a pre-cooled mortar and pestle. DNA was extracted by using AxyPrep Multisource Genomic DNA Miniprep Kit (AP-MN-MS-GDNA-250, Axygen Biosciences, USA) following the manufacturer's instructions. The final DNA pellet was dissolved in 50 µl elution buffer and the extracted genomic DNA's were stored at -20ºC until PCR analysis.

DNA amplification: DNA concentrations of the extracted mosquito pools were measured by using Nano Drop Spectrophotometer (Bioneer ExiprepTM 16, Alameda, CA, USA) before PCR analyses in order to adjust optimum genomic DNA amounts used in the PCR analyses. Obtained genomic DNA's from pools were examined by using the wsp 81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC) and wsp 691R (5'-AAA AAT TAA ACG CTA CTC CA) primers in order to amplification of a DNA fragment ranging from 590 to 632 bp region of *Wolbachia* surface protein gene (wsp) (43). PCR was conducted with a total volume of 25 µl consisting of 50 ng of total genomic DNA, 2.5µl of 10X PCR buffer, 4mM of MgCl2, 0.4µM of each primer, 200 mM of each dNTP, 1U Taq DNA polymerase and deionized water. PCR amplifications were done under the following thermal profile: initial denaturation at 94 °C for 5 min, followed by 35 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min. The amplification products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized in CLP Gel Documentation System (UVP INC Uplant, CA).

DNA sequencing and analysis: One of the amplicon from the positive isolates was gel purified by a commercial kit (High Pure PCR product purification kit, Roche). The purified amplicon was sequenced in ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) in both directions by using 81F-691R primers to obtain wsp gene sequences. The alignment of sequences was carried out using Clustal W method and phylogenetic analyzes of isolates were performed using the neighbor-joining (NJ) method with Geneious 5.5.5 software (13). The Kimura 2 Parameter model was utilized to estimate the evolutionary distances. Bootstrap re-sampling (1,000 cycles) was performed for each method to assess tree topology. Unique nucleotide sequence generated in the study was deposited in the GenBank International Nucleotide Sequence Database with the accession number JX474753 after checking carefully against the original chromatogram from the sequencing gel.

Calculation of the infection rates: Minimum infection rates (MIRs) with *Wolbachia* in the examined *Cx. pipiens* specimens were calculated by the standard formula: (number of positive mosquito pools)/(total number of mosquitoes tested) X 100 (39).

Results

Presence of Wolbachia and minimum infection rates (MIRs) in Cx. pipiens specimens: Of the 10 *Cx. pipiens* genomic DNA pools screened, 6 (60.0%) were found to be positive for *Wolbachia* with PCR analyses.

Amplification of the DNA fragments ranging from 590 to 632 bp with the primers 81F and 691R on the agarose gel are shown in Figure 1.

The minimum infection rate (MIRs) of *Wolbachia* spp. in the totally analyzed 118 *Cx. pipens* specimens was determined as 5.08.

Sequence and phylogenetic analysis of Wolbachia surface protein gene: After the pairwise alignment of the two sequences obtained by using the two primers, the final sequence of the WolKys1 was generated. The nucleotide and amino acid sequences of the WolKys1 are presented in Figure 2.

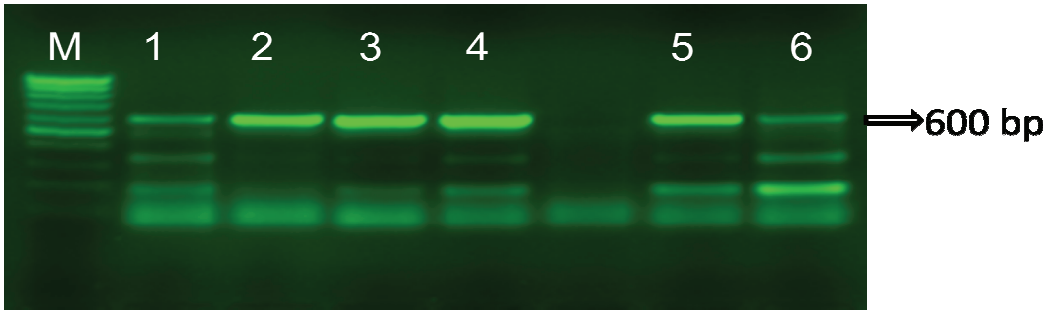

Figure 1. PCR results from *Cx. pipiens* genomic DNA pools with wsp general primers. M: 100 bp DNA ladder; 1-6: *Wolbachia* positives.
Şekil 1. *Cx. pipiens* genomic DNA havuzlarında wsp genel primerleri ile PCR sonuçları. M: 100 bp DNA ladder, 1-6: *Wolbachia* pozitifler.

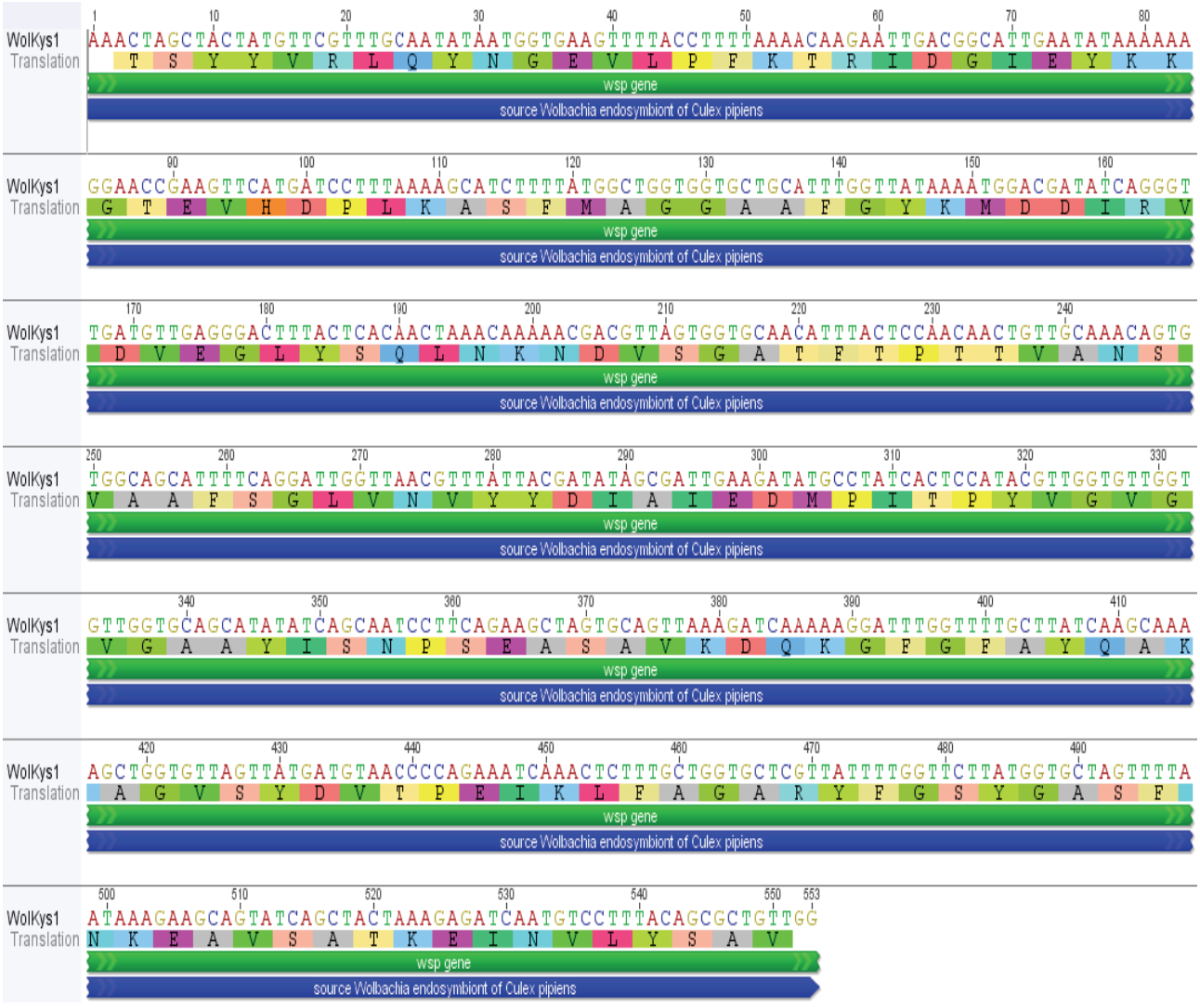

Figure 2. Nucleotide and amino acid sequences of WolKys1 isolate detected from *Cx. pipiens* specimens.
Şekil 2. *Cx. pipiens* örneklerinde saptanan WolKys1 izolatının nükleotid ve amino asit dizilimleri.

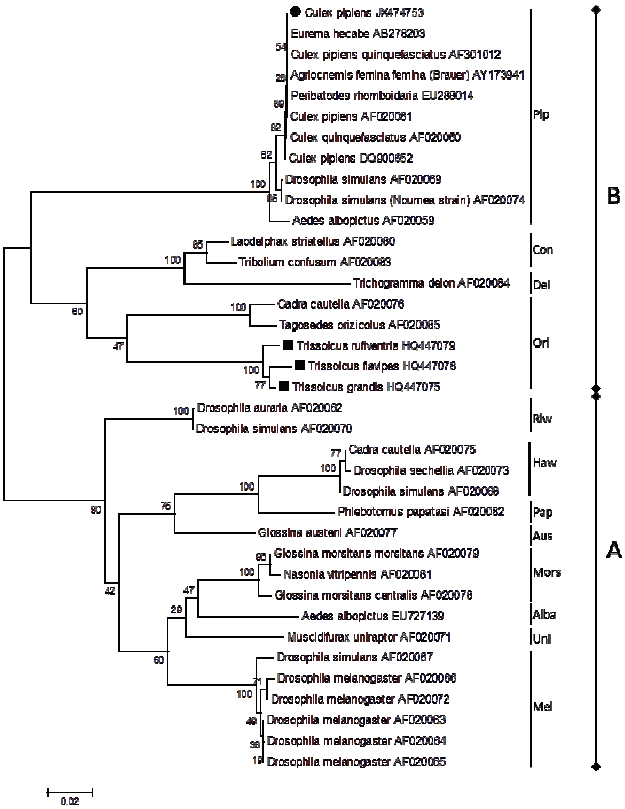

Figure 3. Phylogenetic relationship among WolKys1 and some other *Wolbachia* isolates (Neighbour Joining-Kimura 2 Parameter model) from different groups available in GenBank. • : WolKys1 isolate from Kayseri province. ■: *Wolbachia* isolates from *Trissolcus* sp. in Turkey. Scale bar indicates number of nucleotide substitutions per site.
Şekil 3. WolKys1 izolatı ile GenBankta mevcut farklı gruplardan diğer bazı Wolbachia izolatları arasındaki filogenetik ilişki (Neighbour Joining - Kimura 2 Parameter model). • : Kayseri yöresinden WolKys1 izolatı. ■ : Türkiye'de *Trissolcus* türlerinde *Wolbachia* izolatı. Ölçek çizgisi bölgeye göre nükleotid değişim sayısını göstermektedir.

A phylogenetic tree was constructed after the multiple alignment of the WolKys1 with some other *Wolbachia* strains in different taxa in the GenBank by using Neighbor Joining method (Kimura 2 parameter model, Bootstrap re-sampling 1000 cycles) (Fig 3). The phylogenetic analyses revealed that the WolKys1 isolate obtained from *Cx. pipens* specimens belongs to *Wolbachia* Super Group B and wPIP group.

According to the phylogenetic comparisons the WolKys1 showed 100.0% identity with the isolates obtained from *Cx. pipiens* (DQ900652, AF301012, AF020060-61), *Peribadotes rhomboidaria* (EU288014), *Agriocnemis femina femina* (Brauer) (AY173941) and *Eurema hecabe* (AB278203) under the Pip group. Mean genetic diversity in Pip group was determined as 0.5±0.1% whereas 22.8±2.0%, 19.5±2.0% and 26.0±2.5% mean difference were found between Pip and Ori, Con, Dei groups, respectively. The mean phylogenetic difference between Pip group and other isolates under the Super group A was calculated as 25.0±2.0%.

Discussion and Conclusion

Wolbachia is the most common intracellular endobacterium that present in more than 65% of insect species and found in all major insect orders including Coleoptera, Diptera, Hemiptera/Homoptera, Hymenoptera, Lepidoptera, and Orthoptera (11, 19, 27, 38). Some medically important mosquito specimens are reported to be naturally and/or artificially infected with *Wolbachia*, such as the common house mosquito *Cx. pipiens* (1, 26, 41), the Asian tiger mosquito *Aedes albopictus* (24) and the yellow fever mosquito *Ae. aegypti* (23, 30, 40). The number of infected insect species with *Wolbachia* has been increasing rapidly in recent years. Recent progress in molecular techniques particularly has allowed to systematic surveys of *Wolbachia* distribution and diversity more reliable. The 16S rRNA, *ftsZ* and *wsp* gene region studies have provided a number of useful molecular tools for such genotyping *Wolbachia* strains from different hosts (27, 37). Among these gene regions outer membrane protein of *Wolbachia* (wsp) was determined to be highly variable and sequences from this gene region provides much more informative features for determining the evolutionary relationships among *Wolbachia* strains (16, 43). It was also reported that phylogenetic diversity in the wsp gene is almost 10 times greater than the divergence described in 16S rRNA gen region (24, 43). Wsp gen region was also chosen in this study for investigating and genotyping of *Wolbachia* in *Cx. pipiens* specimens due to its advantages and usefulness in the phylogenetic analyses. In this study 60.0% of the examined *Cx. pipiens* pools were found to be positive for *Wolbachia* with PCR analyses of wsp gene region and the minimum infection rate (MIRs) of *Wolbachia* spp. in the totally analyzed *Cx. pipens* specimens was determined as 5.08. Behbahani (6) also determined *Culex pipiens quinquefasciatus* specimens collected from Shoushtar in south west of Iran were infected with *Wolbachia,* while no infection was found in *Cx. tritaeniorynchus* and *Cx. theileri* specimens in the study (6). In South India, Sunish et al (34) also reported high *Wolbachia* prevalence in totally 750 adult *Cx. pipiens quinquefasciatus* by PCR. Duron et al (12) reported *Wolbachia* infection in 178 field-caught *Cx. pipiens* specimens from four locations (Ganges, Saint Bauzille de Putois, Maurin and Viols le Fort) in France. Ravikumar et al (28) determined *Wolbachia* infection as 20% and 50% in *Aedes* and *Culex* populations by PCR using *Wolbachia* specific wsp gene primers in India. Rasgon and Scott (27) tested 14 North American mosquito species in five genera (*Aedes, Anopheles, Culiseta, Culex* and *Ochlerotatus*) for *Wolbachia* infection, but the infections were reported only in *Cx. pipiens* species complex. The presence and prevalence of *Wolbachia* in *Cx. pipiens* populations in our study are in agreement with the related studies (6, 12, 27, 34).

In arthropods several gene regions such as 16S rDNA and ftsZ, have been used for molecular characterization and phylogenetic analysis of *Wolbachia* strains (4, 7, 10, 22, 24). However, molecular genotyping by using these gen regions has only been able to resolve a limited number of broad *Wolbachia* strain groupings, determined as A and B and two groups within the A group based on ftsZ sequences (36, 43). However, applying wsp gene sequence analysis in the phylogenetic relationships among *Wolbachia* strains revealed some distinct clades within both the groups A and B (43). A total of 8 and 4 genetically different potential groups (Fig. 3) were determined in A and B super groups, respectively (43). In this study the *Wolbachia* isolate obtained from *Cx. pipiens* specimens was characterized in *Wolbachia* Supergroup B and wPip group. This result is consistent with some other studies which also reported *Wolbachia* strains from *Cx. pipiens* in different regions belong to wPip group of B super group (1, 12, 25, 43) and also the obtained WolKys1 isolate was found to be 100.0% identical with some other isolates under the wPip group. It was also reported that there was high genetic divergence among the groups within each super group and the diversity was much greater in super group B than super group A (43). Similarly, mean genetic diversity was found higher (19.5%-26.0%) when comparing the Pip group with the Ori, Con, Dei groups under Super group B where as high identity rate (99.5%) was determined among isolates under the Pip group. The mean phylogenetic difference between Pip group and other isolates under the Super group A was also found higher (25.0%). In addition a high genetic diversity (23.1%) was also determined among the WolKys1 isolate and *Wolbachia* isolates reported from *Trissolcus rufiventris, T. flavipes* and *T. grandis* in Turkey which are known to be specific enemies of stink bugs (17).

In conclusion, this study describes the first molecular detection and characterization of *Wolbachia* endobacteria in *Cx. pipiens* specimens captured in Kayseri province of Turkey based on wsp gene analyses. The knowledge about the *Wolbachia* infections in several kinds of arthropods found in Turkey is still inadequate. Therefore further studies should be conducted to determine the distribution and genotyping of *Wolbachia* endobacteria found in arthropods.

Acknowledgements

The authors are grateful to TUBITAK for supporting the study material with project number 107 O 533. This article was presented at 1st National Symposium on Vectors and Vector-Borne with International Participation, 9-10 September, 2012, Avanos, Cappadocia, Nevsehir, Turkey.

Conflict of interest statement: None of the authors of this paper has a financial or personel relationsship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Geliş tarihi: 14.02.2013 / Kabul tarihi: 27.03.2013

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