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Screening stored wheat beetles for reproductive parasitic endosymbionts in central Turkey

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ABSTRACT

Stored-product pest insects cause significant loss in stored wheat worldwide. In Turkey, an important wheat producer and historic centre of wheat domestication, almost 60 stored-product pest insects have been reported so far, most of them being coleopteran species. Using reproductive parasitic endosymbionts (RPEs) is a promising recent approach among control methods alternative to insecticides. For planning and studying pest management with these bacteria, first of all, their presence in the natural pest populations should be investigated. The present study focused on screening the RPEs in Central Anatolian stored wheat pests. We collected pests in granaries in Kırşehir province and identified 10 coleopteran species both morphologically and genetically; namely, *Ahasverus advena*, *Cryptolestes ferrugineus*, *C. pusillus*, *Carpophilus obsoletus*, *Oryzaephilus surinamensis*, *Rhyzopertha dominica*, *Sitophilus granarius*, *S. oryzae*, *S. zeamais*, and *Tribolium castaneum*. In these pests, we screened the most commonly studied RPEs worldwide, *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*, by using specific genetic primers. As a result, we detected RPE presence in almost all sampling localities visited. The RPEs that we found were *Rickettsia*, *Spiroplasma*, and *Wolbachia*. We found no infection caused by *Arsenophonus*, *Cardinium*, *Fritschea*, nor *Hamiltonella*. *Rickettsia* presence was only in *S. granarius* populations, whereas *Spiroplasma* and *Wolbachia* presence were not species specific. 22% of all sampled beetle individuals were *Wolbachia* positive. The highest detection rate per granary was that of *Spiroplasma* (80%). *Wolbachia* and *Spiroplasma* were the most frequently detected RPEs per insect species. We also found several cases of coinfections. This study is the first attempt to screen stored-product pests for seven RPEs together.

1. Introduction

Wheat loss is one of the primary issues in agriculture (Baloch, 1999). Losses during storage usually occur equally or even more than those in the field and the main causes are pest insects that reduce the quality of stored grains and leave their bodily fragments in the food processed for human consumption (Bousquet 1990; Harein and Meronuck, 1991). Stored-product pest insects cause 10–25% loss in stored-products globally (Emekçi and Ferizli, 2000; Abass et al., 2014). Coleoptera is the dominant group among these pests in terms of both species diversity and abundance (Aydın and Soran, 1987).

Significant problems in stored-product pest management, such as insecticide resistance, make research on alternative control methods attractive (Fields and White, 2002; Pimentel et al., 2009; Opit et al., 2012). The use of *Wolbachia*-like reproductive parasitic endosymbionts (RPEs) is one of the most intriguing methods of recent years (Bourtzis, 2008; Werren et al., 2008; Brelsfoard and Dobson, 2009; Saridaki and Bourtzis, 2010) as they (e.g. *Cardinium*, *Fritschea*, *Hamiltonella, Rickettsia*, *Spiroplasma*, and *Wolbachia*) can potentially change reproductive behaviour, population sex structure, or fecundity of their insect hosts (Gherna et al., 1991; Stouthamer et al., 1999; Bandi et al., 2001; Everett et al., 2005; White et al., 2009; Brumin et al., 2012; Haselkorn and Jaenike, 2015). RPEs can be used in pest management programs through approaches relying on their effects on host insects (such as sterile insect technique or manipulation of insecticide resistance) (Bourtzis, 2008; Kontsedalov et al., 2008; Vignesh et al., 2018; Liu and Guo, 2019). In order to answer the question whether RPEs can be used to control insect pests, a thorough screening for their

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RPE flora is necessary. Hilgenboecker et al. (2008) estimated that 66% of insect species is infected with *Wolbachia*. Although rates of infection with other endosymbionts seem to be lower [e.g. *Rickettsia*, *Cardinium*, and *Spiroplasma* infection rates in arthropods: 24%, 13%, and 7%, respectively; Duron et al., 2008; Weinert, 2015], this could change after studies on RPEs accumulate.

Wolbachia-like RPEs have been detected in various species of stored-product insects (Dong et al., 2007; Mikac, 2007; Kageyama et al., 2010; Kondo et al., 2011). However, reports on the RPEs of stored-product insects from Turkey are extremely rare. Since Turkey is the lieu of wheat domestication (Luo et al., 2007; Smith and Branting, 2014), high genetic diversity can be expected in stored-product insects in Turkey as evidenced by McCulloch et al. (2020) for the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera). Anatolia was probably the very first region where populations of the stored wheat pests reached high numbers, and interacted not only with each other but also with their mutual bacterial flora, which could result in striking evolutionary patterns in the RPE flora of stored-product insects in the region. Furthermore, Turkey is one of the 10 greatest wheat producers in the world (~20 million tones/year) (USDA, 2020) and wheat constitutes 65% of its total annual agricultural production (Aydın and Soran, 1987). Stored-product insects cause 10–20% loss in Turkey (Alkan, 1946; İyriboz and İleri, 1942 cited in Aydın and Soran, 1987). We searched the publications from Turkey and found 63 stored-product pest insect species being reported, 48 of which were coleopterans (Table S1; Supporting Information).

In the present study, we explored RPE presence in coleopteran stored-product pest species that we collected from different granaries in Kırşehir, Central Turkey. To characterize RPE strains, we first determined RPE-positive insects by using specific PCR primers and then we explored sequences of positive PCR samples. This study is the first attempt to screen stored wheat pests for six RPEs (*Cardinium*, *Fritschea*, *Hamiltonella, Rickettsia*, *Spiroplasma*, and *Wolbachia*) together.

2. Materials and methods

2.1. Insect samples

All the insect samples examined in this study were collected from 10 different private granaries in Kırşehir, Turkey (Fig. 1 and Table 1) in September–October 2017. Insects were either sampled directly on the spot or emerged from collected grain samples (1 kg per locality) after rearing in the laboratory (25 ± 1 °C; 70–75% RH; dark). After taxonomic identification under a dissection microscope, 3 to 10 individuals per species per sampling locality were selected for DNA extraction, depending on the number of available individuals. We sterilized the samples with 70% ethanol for 30 s and then rinsed with distilled water.

2.2. DNA extraction

Insect DNA (including bacterial DNA) was extracted from the abdomen by using CTAB method (Doyle and Doyle, 1990). In order to confirm doubtful morphological identification of the insect species, we sequenced a 710 bp region of the mitochondrial cytochrome *c* oxidase subunit I (COI) that we amplified by using LCO1490-F 5′-GGTCAACAA ATCATAAAGATATTGG-3′ and HCO2198-R 5′-TAAACTTCAGGGTGACCA AAAAATCA-3' (Folmer et al., 1994).

2.3. Diagnostic PCR

In order to detect RPE presence, PCR amplifications were performed by using 20 μl mixtures each containing 10 mM of deoxynucleoside triphosphate, 1 μM of each primer, 0.1 U of Taq DNA polymerase, 1 x PCR buffer and 1 μl of DNA. We selected the most commonly studied RPEs to screen in this study, and they were as follows: *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*. Specific primers used and their characteristics are given in Table 2.

2.4. Sequencing

5 μl of PCR products of bacterial gene regions were electrophoresed in a 1% agarose gel with negative and corresponding positive controls. Electrophoresed gels were screened on a UV Transilluminator (ThermoScientific) and photographed. Samples that gave electrophoretic bands at the same position as the positive control were accepted positive in terms of presence of the corresponding bacteria, and one DNA sample for each band from at least one individual of a species from a granary was sequenced. Reverse and forward sequencing of the RPE and insect COI PCR products were performed by Macrogen Inc., Netherlands (www.macrogen.com).

2.5. Sequence analyses

In order to display taxonomic inferences made in the present study we constructed dendrograms for each bacteria that we detected. We obtained high-quality consensus sequences using the Clustal W 2.0 algorithm (Thompson et al., 1994) in BioEdit (Hall, 1999). Consensus sequences were compared through dendrograms constructed by using additional sequences downloaded from the databases of the NCBI by using BLAST (Altschul et al., 1990) and taxonomic identifications for both bacteria and insects were thus confirmed. GenBank accession numbers of the downloaded sequences were as follows: MF156623 (*Rickettsia*), FJ657241 (*Spiroplasma*), and AF035160, EU642841, JN109167, JN109168, JN384089, KF598750 (*Wolbachia*).

The dendrograms for *Spiroplasma*, *Rickettsia* and *Wolbachia* sequences were constructed by using the Maximum Likelihood method. We conducted model test to find the best substitution model for each sequence set, and as a result we used Jukes-Cantor model (Jukes and Cantor, 1969) for *Rickettsia*, *Spiroplasma*, and Kimura 2-parameter model (Kimura, 1980) for *Wolbachia*. All phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (Kumar et al., 2018).

3. Results

3.1. Stored wheat pest coleopters found in Kırşehir

We found at least one coleopteran species in the stored wheat in each sampling locality. We totally found 108 individuals belonged to 10 species (Table 3). The species were as follows: *Ahasverus advena* (Waltl) (Silvanidae) (foreign grain beetle), *Carpophilus obsoletus* Erichson (Nitidulidae) (corn sap beetle), *Cryptolestes ferrugineus* (Stephens) (Laemophloeidae) (rusty grain beetle), *C. pusillus* (Schönherr) (flat grain beetle), *Oryzaephilus surinamensis* (L.) (Silvanidae) (sawtoothed grain beetle), *R. dominica*, *Sitophilus granarius* (L.) (Dryophthoridae), *S. oryzae* (L.), *S. zeamais* Motschulsky (grain weevils), *Tribolium castaneum* (Herbst) (Tenebrionidae) (red flour beetle) (Fig. 2a–j). The most frequent species and their frequencies (number of granaries where the insect species detected: number of all granaries) were *S. granarius* (0.8), *O. surinamenesis* (0.7), and *R. dominica* (0.3) (Fig. 2k).

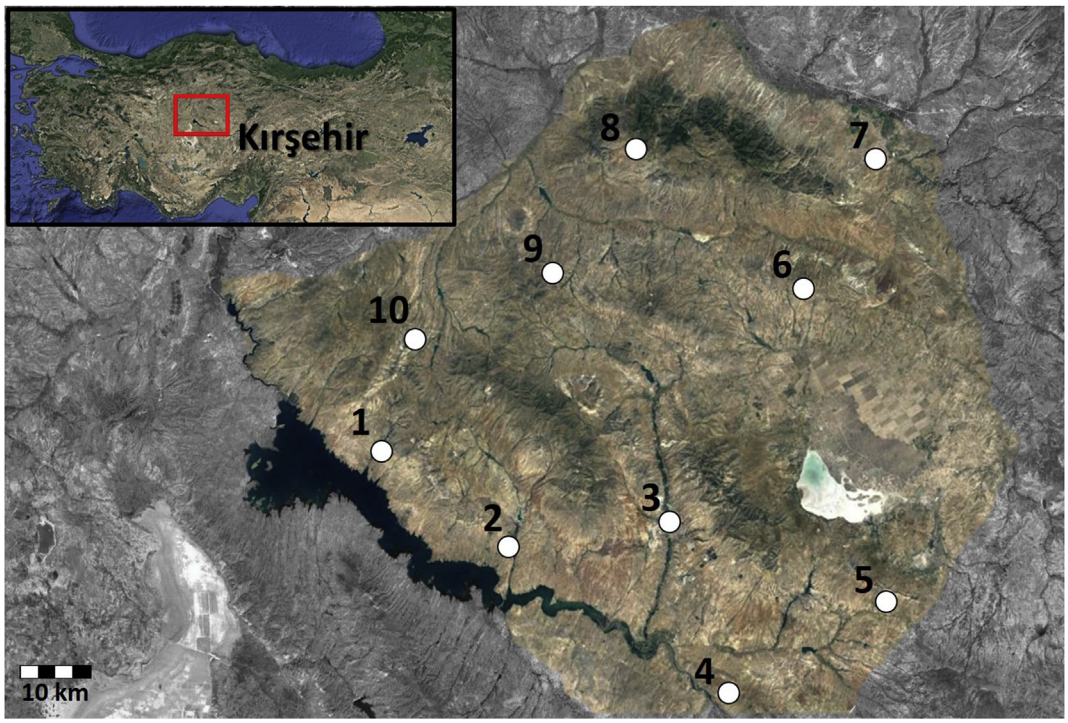

Fig. 1. Stored wheat pest beetle sampling localities in Kırşehir, Turkey.

Table 1
Sampling localities, coordinates, identified insects, and corresponding reproductive parasitic endosymbiont screening results (RPE presence ratio = number of RPE-positive individuals: number of all individuals; A: *Arsenophonus*, C: *Cardinium*, F: *Fritschea*, H: *Hamiltonella*, R: *Rickettsia*, S: *Spiroplasma*, W: *Wolbachia*, Co.: Coinfection) ("-": absent, "+": present, "‡": double infection, "‡*": triple-infection).

Locality (No)	Coordinates	Insect Species (RPE presence ratio)	Reproductive Parasitic Endosymbiont							
			A	C	F	H	R	S	W	Co.
			(RPE presence ratio)							
Akçakent (8)	39.628226° 34.095834°	*O. surinamensis* (1:3)	–	–	–	–	–	–	+ (1:3)	
		R. dominica (3:3)	–	–	–	–	–	+ (3:3)	+ (1:3)	‡ (1:3)
		S. granarius (1:4)	–	–	–	–	+ (1:4)	–	+ (1:4)	‡ (1:4)
		T. castaneum (0:8)	–	–	–	–	–	–	–	
Akpınar (9)	39.449103° 33.967108°	*A. advena* (1:2)	–	–	–	–	–	–	+ (1:2)	
		O. surinamensis (0:2)	–	–	–	–	–	–	–	
		S. granarius (1:3)	–	–	–	–	+ (1:3)	–	+ (1:3)	‡ (1:3)
Çiçekdağı (7)	39.626070° 34.452643°	*S. oryzae* (0:4)	–	–	–	–	–	–	–	
Göllü (6)	39.450548° 34.299846°	*O. surinamensis* (0:4)	–	–	–	–	–	–	–	
		S. granarius (1:3)	–	–	–	–	+ (1:3)	–	–	
Kaman (10)	39.363611° 33.753889°	*O. surinamensis* (15:15)	–	–	–	–	–	+ (1:15)	+ (15:15)	‡ (1:15)
		R. dominica (0:1)	–	–	–	–	–	–	–	
		S. granarius (1:1)	–	–	–	–	+ (1:1)	–	+ (1:1)	‡ (1:1)
Kurugöl (5)	39.045250° 34.445900°	*S. granarius* (1:3)	–	–	–	–	+ (1:3)	–	–	
Merkez (3)	39.101389° 34.183889°	*C. ferrugineus* (5:6)	–	–	–	–	–	+ (5:6)	–	
		O. surinamensis (1:1)	–	–	–	–	–	–	+ (1:1)	
		S. granarius (1:2)	–	–	–	–	+ (1:2)	–	+ (1:2)	‡ (1:2)
		S. zeamais (0:4)	–	–	–	–	–	–	–	
Ulupınar (4)	38.849167° 34.196389°	*A. advena* (0:1)	–	–	–	–	–	–	–	
		C. ferrugineus (0:2)	–	–	–	–	–	–	–	
		C. obsoletus (0:4)	–	–	–	–	–	–	–	
		C. pusillus (0:20)	–	–	–	–	–	–	–	
		R. dominica (2:2)	–	–	–	–	–	+ (2:2)	–	
		S. granarius (1:3)	–	–	–	–	+ (1:3)	+ (1:3)	–	‡ (1:3)
Savcılı (1)	39.238967° 33.682200°	*O. surinamensis* (0:4)	–	–	–	–	–	–	–	
Sıdıklı (2)	39.082117° 33.903683°	*O. surinamensis* (0:2)	–	–	–	–	–	–	–	
		S. granarius (1:1)	–	–	–	–	+ (1:1)	+ (1:1)	+ (1:1)	‡* (1:1)

3.2. Reproductive parasitic endosymbionts found in Kırşehir

We detected at least one of *Rickettsia*, *Spiroplasma* and *Wolbachia* in all sampling sites except two (Çiçekdağı and Savcılı). We could not detect *Arsenophonus*, *Cardinium*, *Fritschea*, nor *Hamiltonella* (Table 1). *Rickettsia* presence was only in *S. granarius* populations, whereas *Spiroplasma* and *Wolbachia* were not species specific. The most frequently detected RPE per granary, insect

Table 2
Specific primers used for screening the endosymbionts *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*, and their characteristics.

Primer	Sequence (5′-3′)	Target genus and gene region	PCR product (bp)	Annealing (°C)	Reference
Ars-F	GGGTTGTAAAGTACTTTCAGTCGT	*Arsenophonus*	800	52	Duron et al. (2008)
Ars-R2	GTAGCCCTRCTCGTAAGGGCC	16S rRNA			
Clo-F	GCGGTGTAAAATGAGCGTG	*Cardinium*	466	54	Weeks et al. (2003)
Clo-R	ACCTMTTCTTAACTCAAGCCT	16S rRNA			
U23—F	GATGCCTTGGCATTGATAGGCGATGAAGGA	*Fritschea*	600	55	Everett et al. (1999a); Thao et al. (2003)
23SIG-R	TGGCTCATCATGCAAAAGGCA	16S rRNA			
Ham-F	TGAGTAAAGTCTGGAATCTGG	*Hamiltonella*	730	54	Zchori-Fein and Brown (2002)
Ham-R	AGTTCAAGACCGCAACCTC	16S rRNA			
Rb—F	GCTCAGAACGAACGCTATC	*Rickettsia*	900	58	Gottlieb et al. (2006)
Rb-R	GAAGGAAAGCATCTCTGC	23S rRNA			
63-F	GCCTAATACATGCAAGTCGAAC	*Spiroplasma*	450	55	Fukatsu and Nikoh (2000); Mateos et al. (2006)
TK55-R	TAGCCGTGGCTTTCTGGTAA	16S rRNA			
Wspec-F	YATACCTATTCGAAGGGATAG	*Wolbachia*	430	53	Werren and Windsor (2000)
Wspec-R	AGCTTCGAGTGAAACCAATTC	16S rRNA			

Table 3
Infection rates (number of RPE-positive individuals of the species: total number of individuals of the species) of reproductive parasitic endosymbionts in coleopteran stored wheat pests collected from Kırşehir, Turkey.

Host (n)	Infection rates			
	Rickettsia	*Spiroplasma*	*Wolbachia*	Coinfections
A. advena (3)	0	0	0.33	0
C. ferrugineus (8)	0	0.63	0	0
C. obsoletus (4)	0	0	0	0
C. pusillus (20)	0	0	0	0
O. surinamensis (31)	0	0.48	0.10	0.03 (S + W)
R. dominica (6)	0	0.83	0.17	0.17 (S + W)
S. granarius (20)	0.70	0.10	0.25	0.20 (R + W) 0.05 (R + S) 0.05 (R + S + W)
S. oryzae (4)	0	0	0	0
S. zeamais (4)	0	0	0	0
T. castaneum (8)	0	0	0	0

species, and insect individuals were *Rickettsia* (0.8), *Spiroplasma* (0.5), *Wolbachia* (0.5); *Spiroplasma* (0.4), *Wolbachia* (0.4), *Rickettsia* (0.1); and *Wolbachia* (0.22), *Spiroplasma* (0.12), *Rickettsia* (0.07), respectively (Figure 2l). We also found several cases of coinfections (seven double infections: five in *S. granarius*, one in *R. dominica*, and one in *O. surinamensis*; and one triple infection in *S. granarius*) (Tables 1 and 3). Related infection rates (number of RPE-positive individuals of the species: total number of individuals of the species) for each host beetle species were given in Table 3. The highest infection rate was that of *Spiroplasma* in *R. dominica* (0.83), followed by *Rickettisia* in *S. granarius* (0.70), *Spiroplasma* in *C. ferrugineus* and *O. surinamensis* (0.63 and 0.48, respectively), *Wolbachia* in *A. advena* and in *S. granarius* (0.33 and 0.25, respectively) (Tables 1 and 3).

The *Rickettsia* tree was reconstructed by using a *Rickettsia* sequence from the green lacewing, *Chrysoperla pallida* (Neuroptera). We inferred two main *Rickettsia* clades only in *S. granarius* (*R*-1 and *R*-2, Fig. 3b). *R*-1 clade included samples from northern and north western granaries in Kırşehir. On the other hand, resolution in *R*-2 clade was low as revealed by the polytomy which did not allow any geographical inference. Therefore, *R*-2 was an arbitrary clade depicted for the simplicity and it cannot be accepted as a phylogenetically supported clade.

Spiroplasma tree was reconstructed by using a *Spiroplasma* sequence from the mushroom-feeding fruit fly, *Drosophila tenebrosa* (Diptera). We found two main *Spiroplasma* clades: *S*-1 including *R. dominica* and *S. granarius*, and *S*-2 including *C. ferrugineus* and *O. surinamensis* (Fig. 3a). Their positioning in the tree had no geographical significance and S-2 was not a well-supported clade.

Finally, for *Wolbachia* tree, we used *Wolbachia* sequences from two aphid species (the potato aphid, *Macrosiphum euphorbiae* and the poplar woolly aphid, *Phloemyzus passerinii* (Hemiptera) for Type A *Wolbachia* lineage, and the parasitoid wasp *Trichogramma ostrinae* (Hymenoptera) and *P. passerinii* for Type B *Wolbachia* lineage. We also used *Wolbachia* sequences from the yellow fever mosquito *Aedes aegypti* (Diptera) and rice water weevil *Lissorhoptrus oryzophilus* (Coleoptera). We found two *Wolbachia* clades (*W*-1 and *W*-2) all of which were grouped with Type B lineage. Their positioning in the tree had no geographical significance. *Wolbachia* from *O. surinamensis* and *S. granarius* generally tended to form separate clades (Fig. 3c).

We found co-infections in *O. surinamensis*, *R. dominica* and *S. granarius* (Table 1). One *O. surinamensis* individual from Kaman was co-infected with *Spiroplasma* and *Wolbachia*. One *R. dominica* individual from Akçakent was co-infected with *Spiroplasma* and *Wolbachia*. One *S. granarius* individual from Ulupınar was co-infected with *Rickettsia* and *Spiroplasma*, four from Akçakent, Akpınar, Kaman, and Merkez with *Rickettsia* and *Wolbachia*, and one from Sıdıklı with *Rickettsia*, *Spiroplasma* and *Wolbachia*.

4. Discussion

The present study showed the occurrence of 10 coleopteran pests in granaries in Kırşehir, Turkey. Among the 108 individuals sampled, 46 from almost all study sites were infected with at least one of *Rickettsia*, *Spiroplasma*, and *Wolbachia*. We did not find any *Arsenophonus*, *Cardinium*, *Fritschea*, nor *Hamiltonella*. Although their presence was shown in Coleoptera previously (Kolasa et al., 2018; Vignesh et al., 2018), more studies are needed to infer the prevalence of these endosymbionts among beetles.

Prevalence of *Rickettsia*, *Spiroplasma*, and *Wolbachia* infections varied significantly among different host species and different

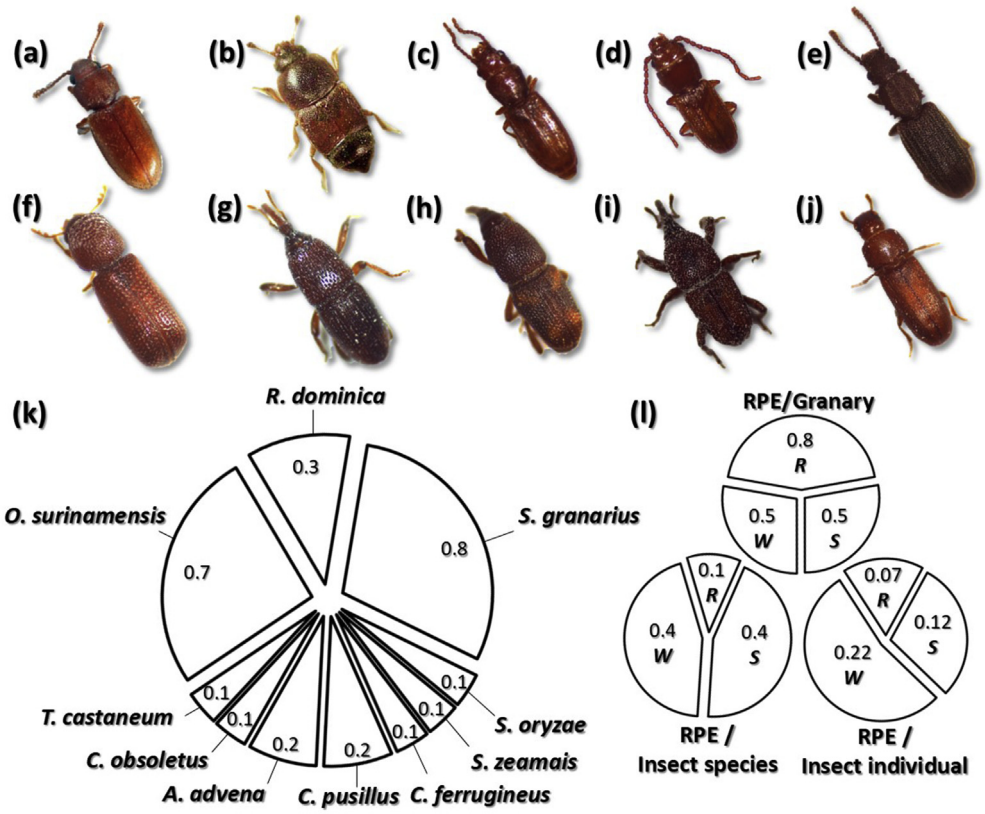

Fig. 2. (**a**) *Ahasverus advena*, (**b**) *Carpophilus obsoletus*, (**c**) *Cryptolestes ferrugineus*, (**d**) *C. pusillus*, (**e**) *Oryzaephilus surinamensis*, (**f**) *Rhyzopertha dominica*, (**g**) *Sitophilus granarius*, (**h**) *S. oryzae*, (**i**) *S. zeamais*, and (**j**) *Tribolium castaneum*, (**k**) frequencies of coleopteran stored wheat pests in Kırşehir, Turkey, (**l**) and corresponding reproductive parasitic endosymbiont detection rate per granary, insect species and insect individual (*R*: *Rickettsia*, *S*: *Spiroplasma* and *W*: *Wolbachia*).

populations of the same host. To the best of our knowledge, the following are the worldwide first reports: *Wolbachia* presence in *A. advena*, *Spiroplasma* presence in *R. dominica*, *Rickettsia* and *Spiroplasma* presence in *S. granarius*. We also reported occurrence of double infections in *O. surinamensis* (*Spiroplasma* and *Wolbachia*), *S. granarius* (*Rickettsia* and *Wolbachia*/*Rickettsia* and *Spiroplasma*), *R. dominica* (*Spiroplasma* and *Wolbachia*); and triple infection in *S. granarius* (*Rickettsia*, *Spiroplasma*, and *Wolbachia*) for the first time.

Wolbachia infection among coleopteran species was found to be 27% (Kolasa et al., 2018) and estimated to be around 38% (Kajtoch and Kotaskova, 2018). In our sampling, *Wolbachia* infection was more common than *Rickettsia* and *Spiroplasma* infections. Research on *Wolbachia* shows that many host species have *Wolbachia* infection only in some part of their ranges or in only some of their lineages (Clark et al., 2001; Roehrdanz et al., 2006). In parallel to this, we detected *Wolbachia* infections in some individuals and species, but not in other individuals of the same species nor in other species of the same community. This was the same for *Spiroplasma* but not for *Rickettsia*. Interestingly, we detected *Rickettsia* exclusively in *S. granarius*, at least one individual of all populations of which were positive for this endosymbiont. This cannot be explained by contamination as the negative controls were clean. *Rickettsia* and *Spiroplasma* infections in Coleoptera are quite widespread (Clark et al., 2001; Küchler et al., 2009; Martin et al., 2013; Kolasa et al., 2018), and the latter can even be more than *Wolbachia* infection in some cases (Martin et al., 2013). We also found that local infection rates of *Spiroplasma* were higher than those of *Rickettsia* and *Wolbachia* (Table 3). Low body mass and small sample size were two limitations in our study. Since we focused on screening of as many populations as possible, our results could be a biased estimation of real infection rates. Additionally, we cannot rule out accidental presence/detection of endosymbiont sequences in some of the insect samples which could take these bacteria into their guts after feeding on the carrion of another insect species which are infected. Therefore, not all RPE-positive individuals are necessarily infected.

4.1. Host insects and their endosymbionts: possible mechanisms

Horizontal transfer of RPEs from one host species to the other is a phenomenon known to occur between several taxa (Vavre et al., 1999; Bailly-Bechet et al., 2017) including stored-product pests (e.g. Carvalho et al., 2014). Apart from events like interspecific hybridization (Rousset and Solignac, 1995), relying on the similar resources such as larval host plant or sharing the same parasitoids or mites can result in horizontal transmission of RPEs (Heath et al., 1999; Vavre et al., 1999; Jaenike et al., 2007; Stahlhut et al., 2010; Brown and Llyod, 2015). Although it needs to be confirmed with a larger sampling, one explanation for some of our results could be horizontal transfer (see below) as a result of feeding in the same confined environment and as the parasitoids and mites in our sampling were abundant (Suppl. Mat. – Fig. S1). On the other hand, we also found the same endosymbiont clades in different insect species in distant granaries. Horizontal transfer may or may not get involved in these cases, yet, there must be other mechanisms included. These mechanisms could be related to ecology of the insect species (e.g. *R. dominica* is a good flyer [Ridley et al., 2016] and may transmit endosymbionts from one granary to the other) or to anthropogenic factors (e.g. commercial exchange of stored

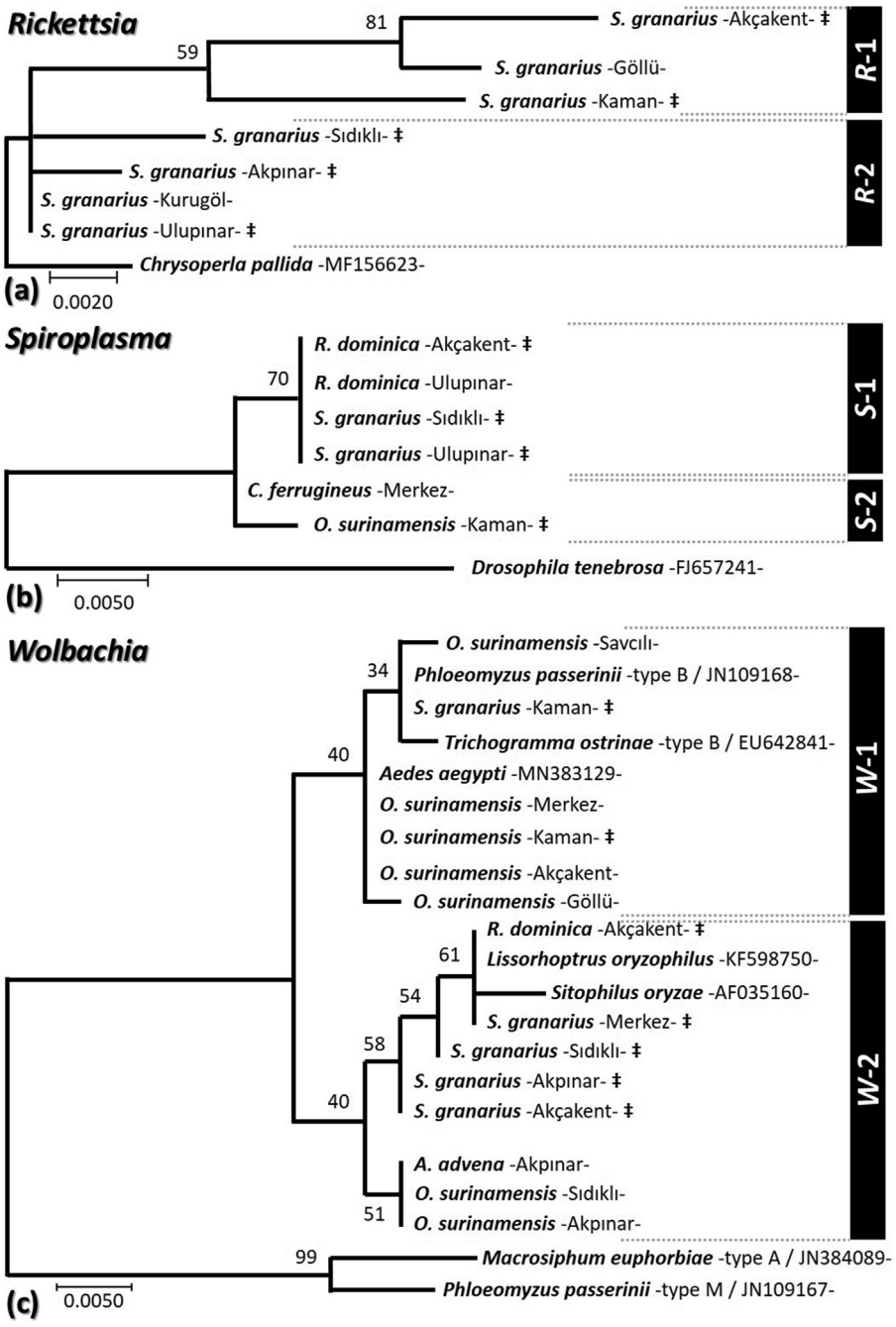

Fig. 3. Maximum likelihood trees for (**a**) *Rickettsia*, (**b**) *Spiroplasma*, (**c**) and *Wolbachia* ("‡": coinfection) sequences.

products among granaries). Although both cases would mix endosymbionts among granaries, a mechanism to explain the presence of same endosymbiont clades in different species would still be needed. If not horizontal transfer, low resolution of the gene sequences (or OTUs) used could be an explanation. Therefore, a larger portion of the bacterial genomes should be sampled for a reliable inference.

4.1.1. *A. advena*

It is a cosmopolitan species which occurs in all regions of Turkey, but, to our surprise, it had never been reported from Asian Turkey until 2019 when it was found in Aegean region (Zengin and Karaca, 2019) (Suppl. Mat. – Table S1). We report *A. advena* from Central Turkey for the first time. It mainly feeds on fungi in humid storage conditions; but wheat germ and dead and crushed grain beetles are also in its diet (Woodroffe, 1962; Engelbrecht and Buske, 1982). We found *A. advena* in the same granary (Akpınar) as *O. surinamensis* and both were infected with the same *Wolbachia* clade (*W*-2) (Fig. 3c). This may suggest a horizontal transfer of *Wolbachia* from *O. surinamensis* to *A. advena* but not the vice versa as the previous one almost exclusively feeds on the grain. On the other hand, a wider sampling is necessary to be able to discard other possibilities such as accidental presence of endosymbiont sequences in the studied individuals (see above).

4.1.2. *C. ferrugineus and C. pusillus*

C. ferrugineus has been reported from almost all regions in Turkey (Suppl. Mat. – Table S1). Ünal and Koçak (2019) showed the

occurrence of *Spiroplasma*, *Rickettsia*, and *Wolbachia* in several *C. ferrugineus* populations from several regions in Turkey. They could not find *Spiroplasma* in Central Anatolia, whereas it was the only RPE that we found in *C. ferrugineus* in the study region. We found the same *Spiroplasma* clade (*S*-2) in *C. ferrugineus* as in *O. surinamensis* (Fig. 3b). *C. ferrugineus* is a secondary pest that usually follows primary stored grain pests (Tuff and Telford, 1964). Furthermore, it lays eggs in or on the grain or on debris. This lifestyle can facilitate the horizontal transfer of RPEs from *C. ferrugineus* larvae to the other beetles that feed on the same grain. On the other hand, *C. ferrugineus* is known as a facultative predaceous and scavenger species eating dead or living eggs, larvae, and pupae of other co-existing species (Suresh et al., 2001; Mason, 2003). Thus, transfer of RPEs from other beetles to *C. ferrugineus* is also possible. Nevertheless, a wider sampling is necessary as the drawbacks mentioned above applies here, too.

To the best of our knowledge, this is the first report of *C. pusillus* from Central Anatolia (Suppl. Mat. – Table S1). It was negative for all RPEs studied. We also found *A. advena*, *C. obsoletus*, and *C. ferrugineus* in the same granary, and they were all negative in terms of screened RPEs. Yet, from the same granary *R. dominica* and *S. granarius* were positive for *Spiroplasma* and *Rickettsia* + *Spiroplasma*, respectively.

4.1.3. O. surinamensis

Its presence has been reported from all regions across Turkey (Suppl. Mat. – Table S1). Koçak and Ertürk (2019) reported the occurrence of *Rickettsia*, *Spiroplasma*, and *Wolbachia* in several populations of *O. surinamensis* in Turkey. We detected *Spiroplasma* and *Wolbachia* in *O. surinamensis*. As it is not able to feed on undamaged grains, co-existence of other grain beetles can favour its survival. This may also facilitate horizontal transfer of RPEs which could explain the occurrence of the same *Wolbachia* clade (*W*-1) in *O. surinamensis* and *S. granarius* in the granary in Kaman (Fig. 3b). This should be tested through a larger sampling.

4.1.4. R. dominica

It has been reported from almost all regions in Turkey (Suppl. Mat. – Table S1). We found it in three localities (Akçakent, Kaman, and Ulupınar) (Table 1). To the best of our knowledge, there is no study detecting *Spiroplasma* in *R. dominica* so far. We found *Spiroplasma* in *R. dominica* in two localities, in one of which (Ulupınar) we found the same clade (*S*-1) also in *S. granarius* (Fig. 3b). *Wolbachia* have been recently found in *R. dominica* by McCulloch et al. (2020) from Aegean and Mediterranean Turkey. In the present study, we report the occurrence of *Wolbachia* in *R. dominica* in Central Anatolia for the first time. It was the same *Wolbachia* (*W*-2) clade that we found in *S. granarius* samples from the same granary (Akçakent). As *R. dominica* lays eggs in the grain and feeds directly on the grain, occurrence of the same *Spiroplasma* and *Wolbachia* clades in *R. dominica* and *S. granarius* in the same granaries should be further studied through a wider sampling in order to explain whether it is a result of horizontal transfer of the RPEs or another mechanism.

4.1.5. S. granarius, S. oryzae, and S. zeamais

They occur in almost all regions of Turkey with *S. zeamais* being significantly less reported (Suppl. Mat. – Table S1). We found *S. granarius* in almost all granaries that we visited in Kırşehir. In Turkey, Tunçbilek et al. (2015) detected *Arsenophonus* and *Wolbachia* in *S. granarius* in which we found *Rickettsia*, *Spiroplasma*, and *Wolbachia*. Yaman and Koçak (2019) found *Rickettsia*, *Spiroplasma*, and *Wolbachia* in several populations of *S. oryzae* in Turkey. We could detect no RPE neither in *S. oryzae* nor in *S. zeamais*. On the other hand, presence of *Spiroplasma* in a *S. zeamais* population has been shown previously (Zhang et al., 2017).

4.1.6. T. castaneum

Although *Wolbachia* is quite widespread in the natural *T. confusum* populations, it is not the case for *T. castaneum* (and other species in the same genus) (Wade and Stevens, 1985). To confirm this, Goodacre et al. (2015) could not find *Wolbachia* in *T. castaneum*, but they found *Rickettsia* and *Spiroplasma*. We could detect none of the six RPEs in *T. castaneum*. Enlarging the sample size, and sampling from different sexes can potentially change this result.

Kageyama et al. (2010) suggests that the infection status of RPEs (*Wolbachia* in their case) may be a possible indicator for discriminating and/or tracing the distribution trajectories of the pest insect strains. On the other hand, phylogenetic incongruences between hosts and RPE strains due to horizontal transfer events are also evident (Tolley et al., 2019), and such events lead to the occurrence of the RPE in distantly related host species (Russell and Moran, 2006; Gehrer and Vorburger, 2012). In the present study, *Wolbachia* sequences from *S. granarius* and *O. surinamensis* from different locations were mostly grouped separately according to the host species (Fig. 3c). Yet, we are far from understanding whether this is a case of host specific infection as Kageyama et al. (2010) suggested or simply a result of host insects being originated from the same source population and thus harbour the same reproductive symbionts. We also found cases where *Wolbachia* in *R. dominica* clustered within the *Wolbachia* clade of *S. granarius* (Fig. 3c). Similarly, we cannot be sure whether these cases are caused by horizontal transfer between distant host taxa as suggested by Tolley et al. (2019) or by other factors some of which mentioned above. All these remain as hypotheses to test through future studies based on larger sampling sizes.

4.2. Coinfections: double and triple infections

Infection with multiple RPE genera or multiple strains of the same genus is a widespread phenomenon (Malloch et al., 2000; Jaenike et al., 2010; Martin et al., 2013). Benefits of hosting multiple symbiont types at the same time may include improved resistance to natural enemies (Oliver et al., 2006; Guay et al., 2009). Facultative RPEs of aphids, including *Hamiltonella*, *Rickettsia*, and *Spiroplasma* are known to protect their hosts against important natural enemies (Oliver et al., 2003; Łukasik et al., 2013). Coinfections with several different strains or species of symbionts in the same host are commonly found in diverse insect groups (Goto et al., 2006 and references therein). There is also evidence for antagonistic relationship between RPEs. For example, Goto et al. (2006) reported a negative interaction between *Spiroplasma* and *Wolbachia*. Although some endosymbionts (such as *Rickettsia* and *Wolbachia*) tended to be found more frequently together as coinfections in the present study, it is not possible for us to determine whether these observations reflect any positive or negative associations between endosymbionts due to our low sampling size.

5. Conclusions

Ample archaeological and molecular evidence point to southeastern Turkey and northern Syria as the cradle of agriculture (Lev-Yadun et al., 2000; Salamini et al., 2002; Abbo et al., 2006). Wheat was most probably cultivated in Turkey during the first millennium BCE, as documented by remains from several archaeological sites in Anatolia (Luo et al., 2007; Smith and Branting, 2014), among which Kaman-Kalehöyük excavations in Kırşehir proved significance of the study region in terms of early agriculture (Nesbitt 1995; Fairbairn et al., 2002). Wheat cultivation must bring

the need for storage which could eventually provide an environment for complex stored-product pest-RPE interactions. Being on the crossroad of different continents must also have a significant impact on the RPE diversity of Turkey, which has been already shown for other taxa (Altınlı et al., 2018). We believe that these interactions will be better resolved with wider screening reports, and studies from Turkey may be particularly important as we are largely unaware of the diversity of stored-product pests and their RPEs in this country. Although our results are far from precision, we put the horizontal transfer and other mechanisms mentioned above as hypotheses to test in future studies in Central Anatolia.

Author statement

Kahraman İpekdal: Conceptualization, Field Work, Methodology, Software, Writing. **Tayfun Kaya**: Conceptualization, Field Work, Methodology, Bench Work, Reviewing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jspr.2020.101732.

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