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Investigation of Cardinium endosymbiont in the micro-fauna of granaries and surroundings

Tahıl depoları ve çevresinin mikro faunasında Cardinium endosymbiontunun incelenmesi

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ABSTRACT

Cardinium is an endosymbiotic bacterium known as a reproductive manipulator in terrestrial ecosystem arthropods. Although *Cardinium* host species have been identified in recent studies, its prevalence is relatively low, perhaps due to the investigation of fewer taxonomic groups. This study was aimed to investigate *Cardinium* bacteria in micro-fauna samples in granaries of Turkey, which has suitable conditions for the distribution of arthropods. For this purpose, *Stratiolaelaps scimitus* (Womersley, 1956) (Acari: Laelapidae), *Entomobrya* sp. (Collembola: Entomobryidae), and *Balaustium* sp. (Acari: Actinotrichida) samples were investigated using the *Cardinium Clo* primer set and compared with the endosymbiont of *Bemisia tabaci* (Hemiptera: Aleyrodidae). It was determined that the *Cardinium* sequences from *S. scimitus* and *Entomobrya* sp., obtained from granaries and in close contact with each other, were quite similar and could be considered as a monophyletic group. This data can be considered as an indicator of horizontal transfer of *Cardinium* between these two taxa. *However, Balaustium* sp. and *B. tabaci* endosymbiont *Cardinium* are phylogenetically distant from them. It is thought that this study, which presents the first data on *Cardinium* endosymbiont in the granary microfauna, will contribute to studies on endosymbiotic bacteria, which are promising reproductive manipulators in the fight against harmful species, especially in agricultural production under pressure due to global warming, decreasing production, and increasing food demand. However, considering the diversity, distribution, and ecological niches of the studied species, more studies are needed to determine the prevalence of *Cardinium*.

INTRODUCTION

The population densities and distribution rates of arthropods, the most successful creatures in terrestrial habitats, are remarkable. Symbiotic, especially endosymbiotic, bacteria have an important role in this success. These bacteria can provide the essential nutrients needed by the host, take part in its defense or increase its resistance to environmental factors (Hedges et al. 2008, Kashkouli et al. 2021, Nakabachi et al. 2006, Oliver et al. 2003, Penz et al. 2012, Werren et al. 2008, Zug and Hammerstein 2012). In addition, these symbionts can be transported vertically and/or horizontally between their hosts, as well as contribute to biodiversity through gene transfer (Aikawa et al. 2014, Aikawa et al. 2022, Kashkouli et al. 2021, Telschow et al. 2005, Zug and Hammerstein 2012,). Of these bacteria, *Cardinium* (Cytophaga - Flavobacterium - Bacteroides) endosymbiont manipulates the reproduction of its host and uses it to its advantage. Mating between males carrying this maternally inherited bacterium and uninfected females results in cytoplasmic incompatibility. After this situation, the offspring cannot develop and die. On the other hand, if the female carries the same symbiont, the offspring will survive. This feature of *Cardinium* can be used as a promising method to control arthropods that cause damage to humans, animals, and plants or are vectors of various diseases (Doremus et al. 2020, Gotoh et al. 2007, Nakamura et al. 2009, Penz et al. 2012, Werren et al. 2008, Zhao et al. 2018).

Cardinium affects and shapes the population structure, ecology, and evolution of the arthropods (Doremus et al. 2020, Telschow et al. 2005). However, the prevalence of *Cardinium* in arthropods is relatively low and is limited to less taxonomic groups (Zchori-Fein and Perlman 2004). However, although the number of studies has increased recently, the number of taxa examined/investigated is also low. As far as is known, *Cardinium* has been detected in micro-fauna members (Chaisiri et al. 2015, Chang et al. 2010, Gotoh et al. 2007). However, endosymbiont composition in arthropods may vary in populations of the same species in different geographies. This is explained by the host's nutritional regimen or by variables in environmental conditions (Gomard et al. 2021). In fact, arthropods are in constant motion for anthropogenic and/or natural reasons such as trade, migration, and climate changes (Inci et al. 2016). However, it is reported that the distribution area of especially harmful arthropods is expanding (advancing north) due to global warming (Bouchard et al. 2019). Insects associated with agricultural products (especially cereals) due to the increasing food demand are the current study (Ipekdal and Kaya 2020). One of the most suitable geographies for the examination of these dynamic processes is Anatolia. As a matter of fact, Anatolia is a geography with a climate and topography suitable for biological diversity and a high rate of species diversity and endemism. In addition, since it is a bridge between Asia, Africa and Europe, it is a route for both trade and wildlife, which accelerates diversity and diversification (Inci et al. 2016, Ipekdal and Kaya 2020, Özdikmen 2016). Biodiversity in the region, which includes Anatolia, is also seen in the studies carried out, and new records are reported (Hosseini et al. 2016, Noei et al. 2017, Noei et al. 2019, Yahyapour et al. 2018). Therefore, this variety can also be used as a resource for studies on bacterial symbionts such as *Cardinium*. Because endosymbiotic bacteria are important for biotechnological and/or integrated control methods for the protection of agricultural commodities, which have increased in importance due to global warming, drought and various geopolitical risks, which are among the most important problems of the last period (Bouchard et al. 2019, FAO 2021, Gomard et al. 2021, Ipekdal and Kaya 2020). Therefore, in the present study, it was aimed to investigate the micro-fauna components and endosymbiotic *Cardinium* bacteria in the granaries and their surroundings in Kırşehir, the center of Anatolia.

MATERIALS AND METHODS

Micro fauna sampling of granaries

In the study, samples obtained from granaries in Kırşehir Province (Turkey) and its surroundings were examined. Sampling was made from five different indoor granaries and the open area around them in the same location in November-December-2021 (coordinates: 38o10'48"N-34o18'70"E). Samples were taken from the granaries in two ways, directly from the wheat heaps and from the relatively humid parts. Samples found in the stony areas were taken from the open area. The samples were examined using a dissecting microscope in the laboratory. Micro-flora members grouped according to their morphological appearances were washed with 70% alcohol for 30 seconds, rinsed with sterile distilled water, and stored at -20 oC until they were taken into alcohol and worked (Ipekdal and Kaya

Table 1. List of primers used in this study for *Cardinium* endosymbiont and insects

Primer	Sequence (5'-3')	Target genus and gene region	PCR product (bp)	Annealing (°C)	Reference
*LCO*1490-F	GGTCAACAAATCATAAAGATATTGG	COI	710	52	Folmer et al. (1994)
*HCO*2198-R	TAAACTTCAGGGTGACCAAAAAATCA				
Clo-F	GCGGTGTAAAATGAGCGTG	16S rRNA	466	54	Weeks et al. (2003)
Clo-R	ACCTMTTCTTAACTCAAGCCT				

2020). Whiteflies from tomatoes were used as a comparison and positive control. Micro-flora samples and whitefly detection were performed by molecular methods.

DNA extraction and PCR screening

Total DNA was extracted from the samples using the CTAB method (Doyle and Doyle 1990). The mitochondrial cytochrome c oxidase I subunit (COI) primer pair LCO1490-F and HCO2198-R were used to identify the species (Table 1). *Clo*-F/R primer pair was used for screening and diagnosis of *Cardinium* bacteria (Table 1). PCR reactions were carried out in a 20 µl reaction medium (Ipekdal and Kaya 2020). PCR products including negative and positive controls were electrophoresed on 1% agarose gel. Total DNAs belonging to whiteflies were used as positive controls for *Cardinium* screenings. PCR products were electrophoresed and gels were screened on a UV Transilluminator (ThermoScientific). Samples that gave electrophoretic bands in the same position as the positive control were considered positive for the presence of *Cardinium*.

Sequence analysis

PCR products obtained from at least one individual from each sample group studied were sequenced. Reverse and forward sequencing of PCR products obtained with COI and Clo primers was performed in Macrogen Inc., The Netherlands. Dendrograms were created from the obtained sequence data to show the taxonomic data generated in the study. For this, consensus sequences were obtained using the Clustal W 2.0 algorithm (Thompson et al. 1994) in BioEdit (Hall 1999). Consensus sequences were identified in NCBI databases using BLAST analyses (Altschul et al. 1990). In addition, *Cardinium* consensus sequences were compared using dendrograms created by downloading additional sequences (GenBank accession numbers in Figure 1) from NCBI databases.

Dendrograms for *Cardinium* arrays were created using the Maximum Likelihood method. Model testing was performed to find the best substitution model for each sequence set (taking into account nucleotide-sequence divergences), and as a result, the Kimura 2 + G model (Kimura 1980) (1000 replicates) was used for *Cardinium*. All phylogenetic and molecular evolutionary analyzes were performed using MEGA version X (Kumar et al. 2018).

RESULTS

Examples of micro-fauna studied

In the study, micro-fauna samples obtained from five different granaries where wheat is stored in Kırşehir (Turkey) city center and their surroundings were examined. Samples collected directly from heaps and relatively humid parts were used for sampling from inside the granaries. All samples were examined with a dissecting microscope. No sample findings were found in the samples taken from the direct piles. However, micro-fauna samples were detected in the humid parts of the granaries and the surrounding stony areas. Identification of micro-fauna members and whiteflies used as positive controls for *Cardinium* was based on matching consensus sequences from sequence data in GenBank databases. Based on BLAST similarities, those obtained from the granaries were *Stratiolaelaps scimitus* (*Hypoaspis miles*) (Acari: Laelapidae) (100%, MN781133) and *Entomobrya* sp. (Collembola: Entomobryidae) (96%, KY468324), while *Balaustium* sp. (98%, KY922407) (Acari: Actinotrichida). However, the whitefly was defined as *Bemisia tabaci* (Hemiptera: Aleyrodidae) (100%, LN829678) (Table 2).

Table 2. The examined granary micro-fauna samples, *Cardinium* screening results, and presence rate (pr: *Cardinium* positive individual/number of individuals screened) (n: number of individuals screened)

Collected area	Insect species (*n*)	Ecological niche	*Cardinium*		
			pr	GenBank	
				Similarity rate	Accession Number
In granaries	*S. scimitus* (15)	Predator	1.0	100	MN781133
In granaries	*Entomobrya* sp. (9)	Regulatory, Predator	1.0	96	KY468324
Out granaries	*Balaustium* sp. (12)	Predator, Pest agricultural	1.0	98	KY922407
In the field	*B. tabaci* (10)	Pest agricultural	1.0	100	LN829678

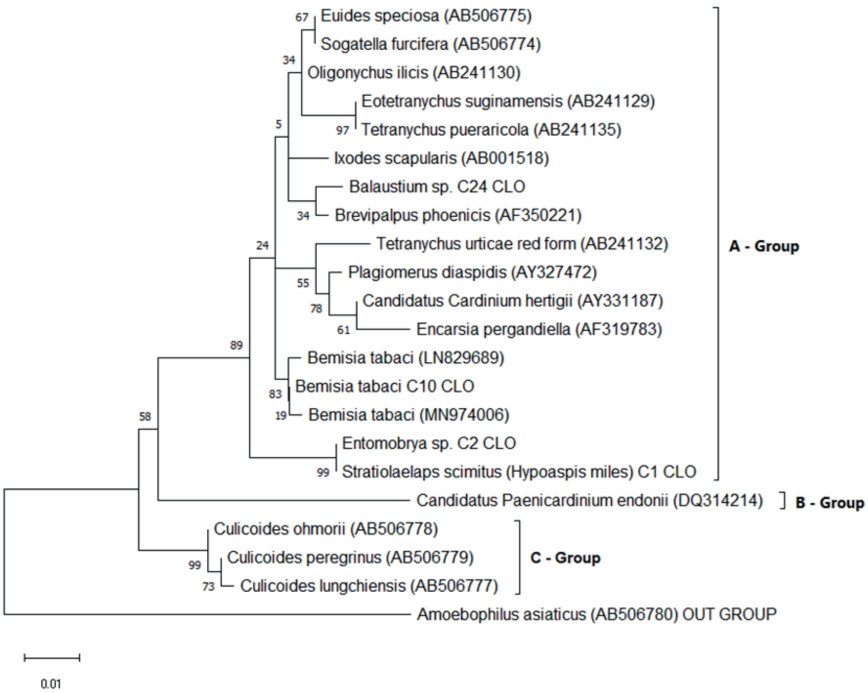

Figure 1. Maximum likelihood (ML) trees of the novel isolates based on *Clo*. Phylogenetic analysis was performed using the Kimura 2 + G parameter model and 1000 bootstraps, with four strains (*Stratiolaelaps scimitus* C1, *Entomobrya* sp. C2, *Balaustium* sp. C24, and *Bemisia tabaci* C10) from this study and additional sequences downloaded from NCBI databases (GenBank accession numbers in parentheses). The percentage of trees in which related taxa are clustered together is shown next to the branches. The display bracket and branch line display the *Cardinium* supergroup. *Amoebophilus asiaticus* was used as outgroup.

Cardinium in mites and its phylogeny

Cardinium bacteria were screened using Clo F/R primer pair in *S. scimitus, Entomobrya* sp., and *Balaustium* sp. and *B. tabaci* detected in and around the granary. The resulting PCR products were sequenced for diagnostic and confirmation purposes. Consensus sequences obtained from sequence data showed 100% similarity to *Cardinium* endosymbiont in the NCBI database (Table 2). It was determined that *Cardinium* infection had a widespread incidence in all individuals who were members of the micro-flora examined (Table 2).

Dendrograms were created using the Kimura 2-parameter model with the Maximum Likelihood method using *Cardinium* consensus sequences and DNA sequences downloaded from GenBank databases. Accordingly, the sequence data in the 16S rRNA region of 468 bp obtained from *S. scimitus, Entomobrya* sp. in the warehouses showed the presence of *Cardinium* with the same topology. In other words, they can be considered as a monophyletic group (Figure 1). On the other hand, *Balaustium* sp. outside the granaries has a different phylogeny (Figure 1). In addition, *B. tabaci*, which is used as a positive control, shows a more distant topology.

DISCUSSION

This study reports endosymbiotic *Cardinium* infection in three different mite species, *S scimitus, Entomobrya* sp., and *Balaustium* sp., which were detected in and around the granaries in Kırşehir Province (Turkey). Although *Cardinium* has been previously reported in arthropods (Chaisiri et al. 2015, Doremus et al. 2020), further studies are needed to elucidate its prevalence in populations and its effects on genotype and phenotype.

In the study, micro-fauna samples obtained from granaries in the same location and their surroundings were examined. It has been observed that *S. scimitus* and *Entomobrya* sp. species show fire spread in the parts of the warehouses where the humidity ratio is high for explainable reasons, and *Balaustium* sp. species are found around the granaries. These findings are not surprising. *S. scimitus* thrives especially in hot and humid environments. The most important feature of this mite is that it is a predatory species and is used as a biological control agent. Naturally found in the northern hemisphere, *S. scimitus* is a polyphagous mite and preys on thrips nymphs, nematodes, sciarid fly larvae, and various species of mites and many soil invertebrates (Navarro-Campos et al. 2016, Rondeau et al. 2018, Walter et al.

2003, Wright and Chambers 1994). *Entomobrya* sp., on the other hand, is a thin arc tail for which there is limited data. Although it has a cosmopolitan distribution, it is generally distributed in the northern hemisphere. It lives in a wide variety of biotopes such as soil, shrubs, bark, and canopy, and feeds on microorganisms and plant organic matter (Baquero and Jordana 2008, Hosseini et al. 2016, Kahrarian 2019). It is also stated that *Collembola* members are even soil conditioners and/or predatory species (Castaño-Meneses et al. 2004). The red mite *Balaustium* sp. is widespread and some species adapt well to man-made structures (Hiruta et al. 2018). Red mites live inside and outside crevices on bricks, stone structures, and tree bark (Halliday 2001). On the other hand, some members of *Balaustium* sp. are predators and feed on other insects. Some are known as grain pests (Halliday 2001). Notable for its diversity, *Balaustium* sp. was previously detected in Turkey (Noei et al. 2017, Noei et al. 2019). On the other hand, there is no data from Turkey on *S. scimitus* and *Entomobrya* sp. species, and this was reported for the first time in this study. However, further studies are needed on the distribution and prevalence of these micro-fauna members.

Cardinium (Cytophaga - Flavobacterium - Bacteroides) from endosymbiotic bacteria, which is a reproductive manipulator in *S. scimitus* and *Entomobrya* sp. and *Balaustium* sp., was screened and examined by this study. In addition, *B. tabaci* individuals, previously reported to have *Cardinium* (Zhao et al. 2018), were used as a comparison and positive control. As a result of the studies, the presence and common incidence of *Cardinium* were determined in all three micro-fauna members. The presence of *Cardinium* in the micro-fauna is a well-known phenomenon (Chaisiri et al. 2015, Chang et al. 2010, Gotoh et al. 2007, Nakamura et al. 2009). In addition, it is more common in *Cardinium* mites than other endosymbionts and is reported to be more important. Although the results obtained are in parallel with the previous studies, *Cardinium* was detected for the first time in the species studied here, for the first time to the best of our knowledge. However, the consensus sequences of *Cardinium*, the symbiont of *S. scimitus* and *Entomobrya* sp., are quite similar. This may be coincidental. However, it raises the question of whether *Cardinium* could have been transferred by horizontal transfer between these species, which are in close contact with each other (?). So, they can be considered a monophyletic group from a phylogenetic point of view (Figure 1). Endosymbionts can be transferred between their hosts horizontally, through feeding, predator-prey relationship, or injury, as well as maternal (vertical) transmission (Gomard et al. 2021). On the other hand, it is known that the same endosymbiotic bacteria strain can be found in different hosts. However, phylogenetic incompatibilities are also expected between hosts and endosymbiotic bacterial strains due to horizontal transfer (Tolley et al. 2019). Another view is that these bacteria can occur in distant host species (Gehrer and Vorburger 2012, Russell and Moran 2006). Another thing worth mentioning is the *Cardinium* variety. *Cardinium* has been evaluated in three upper groups in previous studies (Kageyama et al. 2009). According to the tree drawing based on this in this study, *S. scimitus* and *Entomobrya* sp. and *Balaustium* sp. endosymbiont *Cardinium* (including *B. tabaci*) clustered together with those in group A (Figure 1). However, *Cardinium* in *Balaustium* sp. is distinguished phylogenetically (Figure 1). The obtained data can be explained by the fact that the same symbionts can be found naturally in the same source host populations (Kageyama et al. 2009). However, although the first known determinations of the presence of *Cardinium* in micro-flora members in granaries and related areas are presented here, more studies are needed on the prevalence and phenotype effects of this endosymbiosis. As a matter of fact, as Tolley et al. (2019) stated, it is not clear whether this is the result of horizontal transfer of endosymbionts between distant taxa, and it would be appropriate to consider all these as hypotheses to be tested in future studies.

As a result, this study reports for the first time the presence of micro-flora members in and around granaries and the endosymbiotic *Cardinium* bacteria in them. Considering the diversity, distribution, and ecological niches of the studied species, it is thought that the obtained data will contribute to the studies of endosymbiotic bacteria, which are promising especially in the fight against harmful species.

ÖZET

Cardinium karasal ekosistem eklembacaklılarındaki üreme manipülatörü olarak bilinen endosimbiyotik bir bakteridir. Her ne kadar son dönemde çalışmalarla *Cardinium* konakçısı türler tespit edilse de, belki de daha az taksonomik grubun incelenmesinden dolayı prevalansı görece düşüktür. Bu çalışmada eklembacaklıların yayılışı için uygun koşullara sahip Türkiye'nin tahıl ambarlarındaki mikro-fauna örneklerinden *Cardinium* bakterisinin incelenmesi hedeflenmiştir. Bu amaçla tahıl ambarlarından ve çevresinden temin edilen *Stratiolaelaps scimitus* (Womersley, 1956) (Acari: Laelapidae), *Entomobrya* sp. (Collembola: Entomobryidae) ve *Balaustium* sp. (Acari: Actinotrichida) örneklerindeki *Cardinium Clo* primer seti kullanılarak incelenmiş ve *Bemisia tabaci* (Hemiptera: Aleyrodidae) endosimbiyontu ile mukayese edilmiştir. Sekans verilerine göre ambarlardan elde edilen ve yakın temasta bulunan *S. scimitus* ve *Entomobrya* sp.'teki *Cardinium* benzer homolojiye sahiptir. Bu veri bu iki takson arasında *Cardinium*'un yatay transferinin bir göstergesi olarak düşünülebilir. Ancak ne yazık ki elde edilen veri seti bunu kesin olarak kanıtlayamaz. Ayrıca *Balaustium* sp. ve *B. tabaci* endosimbiyontu *Cardinium* ise filogenetik olarak bunlardan uzaktır. Tahıl ambarı mikrofaunasında *Cardinium* endosimbiyontuna ilişkin ilk verilerin sunulduğu bu çalışmanın; özellikle küresel ısınma, azalan üretim ve artan gıda talebi nedeniyle baskı altındaki tarımsal üretimde zararlı türlerle mücadelede umut vaat eden üreme manipülatörü endosimbiyotik bakterilere yönelik çalışmalara katkı sunacağı düşünülmektedir. Ancak incelenen türlerin çeşitliliği, yayılışı ve habitatları içerisindeki ekolojik nişleri dikkate alındığında *Cardinium* prevalansının belirlenmesi için daha fazla çalışmaya ihtiyaç vardır.

Anahtar kelimeler: *Balaustium, Cardinium*, endosimbiyotik bakteri, *Entomobrya, Stratiolaelaps scimitus*

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