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Original article (Orijinal araştırma)

Sexual dimorphism in the Anatolian endemic tiger beetle, *Cephalota circumdata* ssp. *cappadocica* Franzen, 1996 (Coleoptera: Carabidae: Cicindelinae): a study showing the effectiveness of geometric morphometrics[1]

Anadolu endemik kaplan böceği, *Cephalota circumdata* ssp. *cappadocica* Franzen, 1996 (Coleoptera: Carabidae: Cicindelinae)'de eşeysel dimorfizm: geometrik morfometrinin etkinliğini gösteren bir çalışma

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Abstract

Sexual dimorphism is an important source of intraspecies variation in tiger beetles. However, little is known about sexual dimorphism in tiger beetles. This article contributes the literature in the field of sexual dimorphism by comparing the morphology of males and females in the context of phenotypic changes in the head and pronotum of endemic tiger beetle *Cephalota circumdata* ssp. *cappadocica* Franzen, 1996 (Coleoptera: Carabidae: Cicindelinae). All the specimens examined in the study were gathered during May and August of 2016 from salty soils around Seyfe Lake located in Kırşehir Province, Turkey. Specifically, the efficacy of geometric morphometrics was assessed in the analysis of sexual dimorphism of tiger beetles. Statistically significant differences were found in the head and pronotum shape variation and regression results indicated that size has little influence on the differentiation of shape among sexes. Moreover, the jackknifed cross-validated correct classification percentages for head and pronotum were 88% and 85%, respectively when using only the shape variables. Consequently, geometric morphometrics is an effective and useful method to determine sexual dimorphism in tiger beetles.

Keywords: *Cephalota circumdata* ssp. *cappadocica*, Cicindelinae, geometric morphometrics, sexual dimorphism

Öz

Eşeysel dimorfizm, kaplan böceklerinde türler arası varyasyonun önemli bir kaynağıdır. Buna rağmen, kaplan böceklerindeki eşeysel dimorfizm hakkında az şey bilinmektedir. Bu makale, endemik kaplan böceği *Cephalota circumdata* ssp. *cappadocica* Franzen, 1996 (Coleoptera: Carabidae: Cicindelinae)'nin baş ve pronotumundaki fenotipik değişiklikler bağlamında erkek ve dişilerin morfolojisini karşılaştırarak eşeysel dimorfizm alanındaki literatüre katkıda bulunmaktadır. Araştırmada incelenen tüm örnekler, 2016 yılında Mayıs-Ağustos ayları arasında Kırşehir (Türkiye) İlinde bulunan Seyfe Gölü çevresindeki tuzlu topraklardan toplanmıştır. Spesifik olarak, kaplan böceklerinin eşeysel dimorfizminin analizinde geometrik morfometrinin etkinliği değerlendirilmiştir. Baş ve pronotum şekil varyasyonlarında istatistiksel olarak anlamlı farklılıklar bulunmuş ve regresyon sonuçları cinsiyetler arası bu şekil farklılaşmasında büyüklüğün etkisinin çok az olduğunu göstermiştir. Ayrıca sadece şekil değişkenlerinin kullanıldığında, Jack-knife çapraz geçerlenmiş doğru sınıflandırma yüzdesi sırasıyla baş için %88 ve pronotum için %85 olarak bulunmuştur. Sonuç olarak geometrik morfometri, kaplan böceklerinde eşeysel dimorfizmin belirlenmesinde etkili ve kullanışlı bir yöntemdir.

Anahtar sözcükler: *Cephalota circumdata* ssp. *cappadocica*, Cicindelinae, geometrik morphometri, eşeysel dimorfizm

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Introduction

Sexual differences are often dramatic and widespread among taxa (Wyman et al., 2013). It is possible to see many striking examples of this among animal species where dramatic changes have been identified in males and females (Teder, 2014). For insects in particular, knowledge of the amount of sexual dimorphism is a critical source of information for exploring and exploiting variation in the life history, morphology, physiology and behavior. Of these, morphology is still used extensively in insect taxonomy. Morphological sexual dimorphism, if not taken into account, could lead to a taxonomic misunderstanding because males and females may be described as separate species. Additionally, a better documenting of subtle differences between the sexes could be of significance in understanding the biology, especially diagnosis and accurate identification of insects (Punzalan & Hosken, 2010; Allen et al., 2011; Virginio et al., 2015; Baig et al., 2016; Moraes et al., 2016).

The insect order Coleoptera possess a large number and great diversity of sexually dimorphic species. Sexual differences are generally unnoticeable in most beetle species (at least visually). The male and female can be distinguished only by minor (often microscopic) morphological differences (Kawano, 2006; Maeno et al., 2012; Benitez et al., 2013; Hsiao et al., 2015). Hence, the challenge is finding appropriate groups of species on which to test models of sexual dimorphism. One group of beetles that may be ideal for these purposes is the family Carabidae (tiger beetles), which have sclerotized body parts suitable for morphometric descriptions (Pearson, 1988; Pearson & Vogler, 2001). Some previous studies also lend support to this notion. Kritsky & Simon (1995) examined the mandibles of five tiger beetle species to determine sexual dimorphism in their size and tooth arrangement. Satoh et al. (2003) measured the mandible length of nine tiger beetle species to show sexual dimorphism. Satoh & Hori (2004) reported that *Lophyridia angulata* (Fabricius, 1798) (Coleoptera: Carabidae: Cicindelinae) has sexual dimorphism in mandible length and has large interpopulation differences in mandible size. Franzen & Heinz (2005) evaluated sexual dimorphism in the enigmatic tiger beetle, *Mantica horni* Kolbe, 1896 (Coleoptera: Carabidae: Cicindelinae) based on morphometric values of elytral length. Jaskula (2005) detected sexual dimorphism in Polish population of the tiger beetle *Cicindela hybrida* ssp. *hybrida* Linneaus, 1758 (Coleoptera: Carabidae: Cicindelinae) via biometric studies measured some parameters of the mandibles, elytra and pronota. Cassola & Bouyer (2007) mentioned the strongly sexually dimorphic labrum for the African tiger beetle genus *Neochila* Basilewsky, 1953 (Coleoptera: Carabidae: Cicindelinae). Franzen (2007) investigated several populations of tiger beetles of the *Cicindela campestris* Linnaeus, 1758 (Coleoptera: Carabidae: Cicindelinae) group from southern Turkey and Lebanon with respect to 10 morphometric ratios of head, pronotum, elytra, aedeagus and antenna considering possible sex dependent variations. Ball et al. (2011) stated that the genus *Manticora* Fabricius, 1792 (Coleoptera: Carabidae: Cicindelinae) has the pronouncedly sexually dimorphic mandibles. Young (2015) obtained various body measurements including length of the mandibles, elytra and mesothoracic legs to show possible size differences between the sexes. Jaskuła et al. (2016) tested whether variation of morphometric traits between males and females in *Calomera littoralis* (Fabricius, 1787) (Coleoptera: Carabidae: Cicindelinae) that included head, pronotum, elytra and mandible measurements.

In the studies of sexual dimorphism in tiger beetles or other beetles, standard morphometrics has tended to focus on measuring linear distances such as length, width and height, until geometric morphometrics was created and has gained prominence over time. Among the phenotypic tools, this technique has recently been increasingly used in insect taxonomy and systematics to resolve complex taxonomic issues at the species level (Baracchi et al., 2011; Gómez et al., 2013; Liu et al., 2016). Besides, equally importantly, geometric morphometrics can be a valuable means to reveal, quantify and analyze subtle variations the in case of males and females presenting quite similar external morphology (Camargo et al., 2015). Exact geometric descriptions of morphological differences between the same structures in both sexes are produced via geometric morphometric analysis (Pretorius & Scholtz, 2001). Geometric

morphometrics, thus, has become an indispensable tool for sexual dimorphism studies in beetles (Benitez et al., 2013; Jun-Yan et al., 2015; Eldred et al., 2016; Vesović et al., 2019). Of these, less familiar is the use of geometric morphometrics to detect the degree of sexual dimorphism in species of tiger beetles except for recent work on the four tiger beetle species. Jones & Conner (2018) quantified shape differences in the mandibles of these species via geometric morphometric technique to look at both intraspecific sexual dimorphism as well as interspecific differences.

Within the tiger beetle genus *Cephalota* Dokhtouroff, 1883 (Coleoptera: Carabidae: Cicindelinae), the endemic tiger beetle *Cephalota circumdata* ssp. *cappadocica* Franzen, 1996 (Coleoptera: Carabidae: Cicindelinae) distributes along banks of the salt lakes in the central Anatolia (Franzen, 1996; Cassola, 1999; Gebert, 1999; Azadbakhsh & Nozari, 2015; Matalin & Chikatunov, 2016). The degree of morphological sexual dimorphism in *C. c.* ssp. *cappadocica* is slight and the differences between males and females are not quite as marked. Furthermore, there are no known studies on the description of sexual dimorphism on *C. c.* ssp. *cappadocica*. These factors, therefore, stimulated our interest in this beetle in which we specifically opted and compared head and pronotum.

The principal goal of this study is to evaluate the performance of geometric morphometrics to elucidate sexual dimorphism in the head and pronotum of tiger beetle species, using *C. c.* ssp. *cappadocica* as a model species. Results of this research could provide the groundwork for follow up studies.

Materials and Methods

Data collection and landmark digitizing

All the specimens employed in the study were gathered during May and August of 2016 with entomological hand net from salty soils around Seyfe Lake in Kırşehir Province, Turkey (Figure 1) and preserved in 70% ethanol. Morphological identification was performed by second author (Yavuz Koçak). Specimens of the two sexes were recognized by dissecting the genitalia since it was not possible to visually assess the sex of adult beetles.

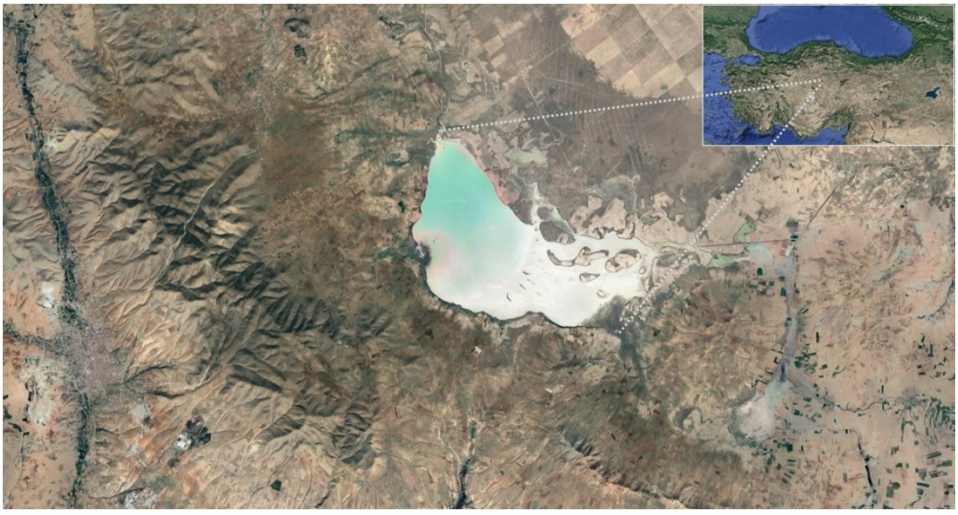

Figure 1. Seyfe Lake in Kırşehir Province, Turkey (satellite image from Anonymous, 2020).

Each specimen was photographed with a camera attached to the Leica microscope separately for head and pronotum. Twelve landmarks on the head and 10 landmarks on the pronotum were digitized once for each image using TPSdig2 (Rohlf, 2017) (Figure 2a, b) (Table 1). The landmark coordinates of 60 specimens (30 females and 30 males) were used for the head and pronotum shape analyses. MorphoJ v1.03a (Klingenberg, 2011) was used for analyses configurations of landmarks.

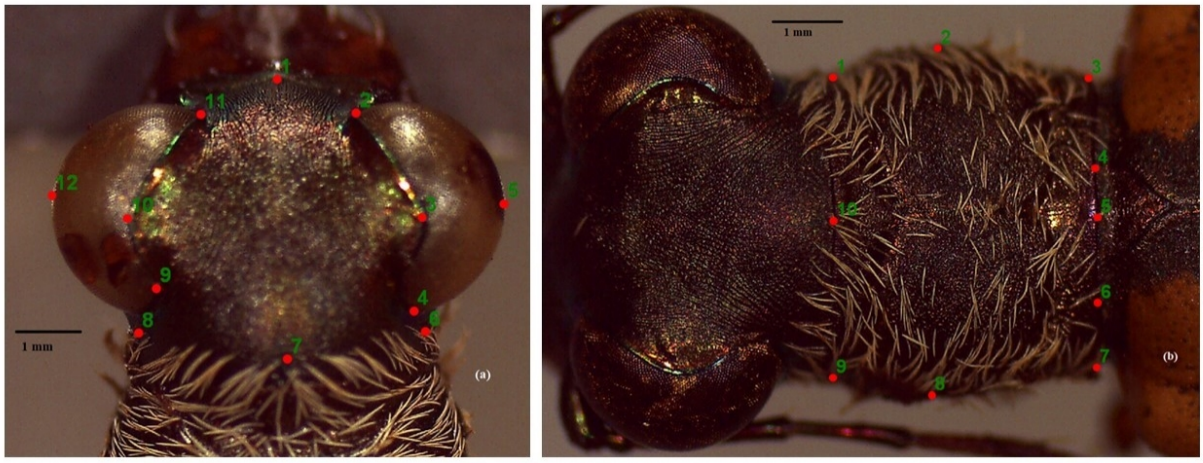

Figure 2. Dorsal views of the landmarks used to define the shape of a) head and b) pronotum of *Cephalota circumdata* ssp. *cappadocica* specimens (see description of landmarks in Table 1).

Table 1. Morphological landmarks used in this study

	Landmark	Description		Landmark	Description
Head	1	The center of the anterior margin of the clypeus	Pronotum	1	The top of the right fore pronotal angle
	2	Anteriormost of the right eye		2	Point at half the length of the right lateral pronotal margin
	3	Leftmost at maximum width of the right eye		3	The top of the right hind pronotal angle
	4	Posteriormost of the right eye		4	The right pronotal base emargination
	5	Rightmost at maximum width of the right eye		5	The center of the pronotal base
	6	The intersection of the pronotum with the right posterior of the head		6	The left pronotal base emargination
	7	Center of the posterior part of the head		7	The top of the left hind pronotal angle
	8	The intersection of the pronotum with the left posterior of the head		8	Point at half the length of the left lateral pronotal margin
	9	Posteriormost of the left eye		9	The top of the left fore pronotal angle
	10	Rightmost at maximum width of the left eye		10	The center of the anterior pronotal margin
	11	Anteriormost of the left eye			
	12	Leftmost at maximum width of the left eye			

Geometric morphometric analyses

Landmark-based morphometric methods were used as these are efficient in capturing information about the biological shape and lead to potent statistical approaches for analyze the difference of shape. In addition, these methods allow quantitatively accurate and clear viewing of shape changes (Rohlf & Marcus,

1993). Generalized procrustes analysis (GPA) superimposed the specimens into a common coordinate system and mathematically eliminated the effects of digitizing position, orientation and scale (Rohlf & Slice, 1990). The software package MorphoJ was used to perform the GPA. To compare overall head and pronotum size between sexes, the centroid size (square root of the sum of the square distances between each landmark and the centroid) (Bookstein, 1986) was computed. centroid size (CS) was used for it is a measure of size that is independent of shape in absence of allometry (Bookstein, 1991).

Centroid size and shape variables were used in following statistical analyses. First, the mean CS of sexes were compared for each structure by using independent groups t-test and visualized using a boxplot. Second, a principle component analysis using the covariance matrix of the procrustes shape coordinates was conducted to degrade dimensionality of the data to ensure the necessity of the parametric test. Covariance matrix of the shape coordinates and PC scores was generated in MorphoJ. A multivariate analysis of variance (MANOVA) was performed using the PC scores as shape variables to test whether sex have significant effects on the average shape of head and pronotum. Then multivariate regression of shape onto size was used to explore how shape varies with size by using PAST 3 (Hammer et al., 2001). Finally, discriminant function analyses (DFA), using jackknifed cross validation, was performed for head and pronotum structures, separately, using only the shape variables and then using shape and CS by using PAST 3. The independent samples t-test and MANOVA were performed using the IBM SPSS 25.

Results and Discussion

For head and pronotum, Kolmogorov-Smirnov test revealed a normal distribution of each sex group ($p > 0.05$) and Levene's test showed that variance of centroid size is equal across sex groups for head ($F = 3.25$, $p = 0.076$) but not equal for pronotum ($F = 14.4$, $p = 0.000$). The independent groups t-test showed that the CS mean of males is significantly different from that of the females, for both structures. (for head $t = 6.68$, $df = 58$, $p = 0.000$, and for pronotum $t = 2.54$, $df = 40$, $p = 0.015$). Figure 3 shows box plots of CS for head and pronotum. Further, distributions of females appear to be more variable with respect to CS than males and females are larger than males for both head and pronotum.

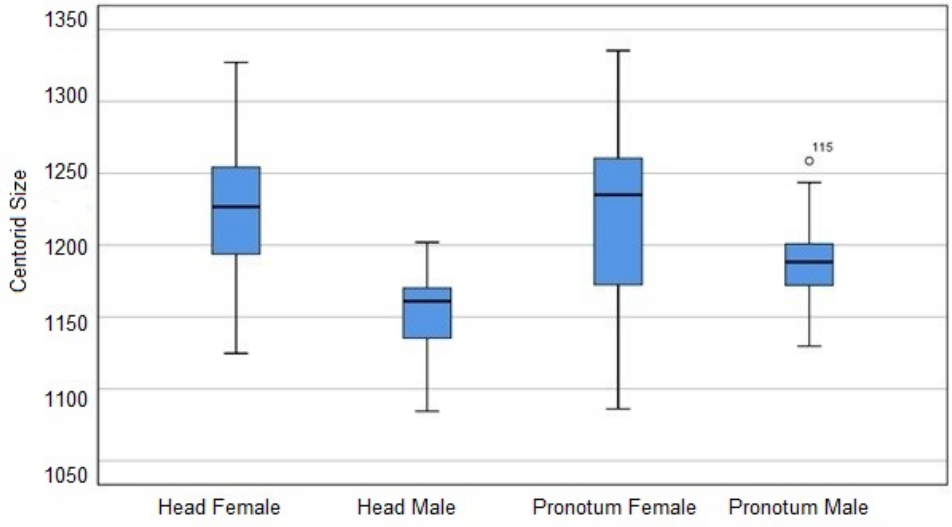

Figure 3. Boxplots of centroid size for head and pronotum for males and females of *Cephalota circumdata* ssp. *cappadocica*.

For head, first two principle components explain 60.8% of total variance (PC1 and PC2 explains 40.1% and 20.7%, respectively). Eight components were necessary to explain more than 90% of the head shape variation. For pronotum, first two principle components explain 41.2% of total variance (PC1 and PC2 explains 21.9% and 19.3%, respectively). Nine components were necessary to explain more than 90%

of the pronotum shape variation. MANOVA (PCs as shape variables) procedure detected significant difference between shape of females and males both head and pronotum (for head; Pillai's Trace = 0.613, p = 0.000, for pronotum; Pillai's Trace = 0.727, p = 0.000). Multivariate regression of shape onto size results indicated that size has only 9.23% influence on the differentiation in head shape among sexes. Similarly, size has only 5.24% influence on the differentiation in pronotum shape among sexes.

DFA for head and pronotum were first run on only the shape variables and then run on both shape variables and CS. DFA, MANOVA and multivariate regression were performed using the first eight principal components for head. The jackknifed cross-validated correct classification percentage for head is 88% (90% for females and 87% for males) when using only the shape variables (Table 2). In addition, when CS is added to DFA, the jackknife cross-validated correct classification percentage increased to 93% for females, but stayed the same for males (87%).

Table 2. Jackknifed correct classification summary of head shape

Head	Shape Variables		Shape Variables and Centroid Size	
	Classification of females (*n*)	Classification of males (*n*)	Classification of females (*n*)	Classification of males (*n*)
Female	(27/30) 90.0%	(3/30) 10.0%	(28/30) 93.3%	(2/30) 06.7%
Male	(4/30) 13.3%	(26/30) 86.7%	(4/30) 13.3%	(26/30) 86.7%
Total	(31/60) 51.7%	(29/60) 48.3%	(32/60) 53.3%	(28/60) 46.7%
	88% of cross-validated grouped cases correctly classified		90% of cross-validated grouped cases correctly classified	

DFA, MANOVA and multivariate regression were performed using the first nine principal components for pronotum. The jackknifed cross-validated correct classification percentage for pronotum is 85% (87% for females and 83% for males) when using only the shape variables (Table 3). In addition, when CS is added in the DFA, the jackknifed cross-validated correct classification percentage was same for females but increased to 90% for males. Notably, cross validation results in a higher classification for females than males only except when using both shape variables and centroid size for pronotum.

Table 3. Jackknifed correct classification summary of pronotum shape

Pronotum	Shape Variables		Shape Variables and Centroid Size	
	Classification of females (*n*)	Classification of males (*n*)	Classification of females (*n*)	Classification of males (*n*)
Female	(26/30) 86.7%	(4/30) 13.3%	(26/30) 86.7%	(4/30) 13.3%
Male	(5/30) 16.7%	(25/30) 83.3%	(3/30) 10.0%	(27/30) 90.0%
Total	(31/60) 51.7%	(29/60) 48.3%	(29/60) 48.3%	(31/60) 51.7%
	85% of cross-validated grouped cases correctly classified		88% of cross-validated grouped cases correctly classified	

Also, the results of DFA show that the landmarks with the greatest variation were numbers 1, 2, 3, 6, 7, 8, 11 and 12 indicating that females have a wider and shorter head with relatively larger eyes than males. This is also related to elongated and sharpened from both anterior and posterior parts of the head shape in male (Figure 4a). According to shape variation in landmarks 1, 2, 5, 8, 9 and 10 females pronotum was wider and shorter than males. However, the posterior parts of pronotum was slightly narrower in females (Figure 4b).

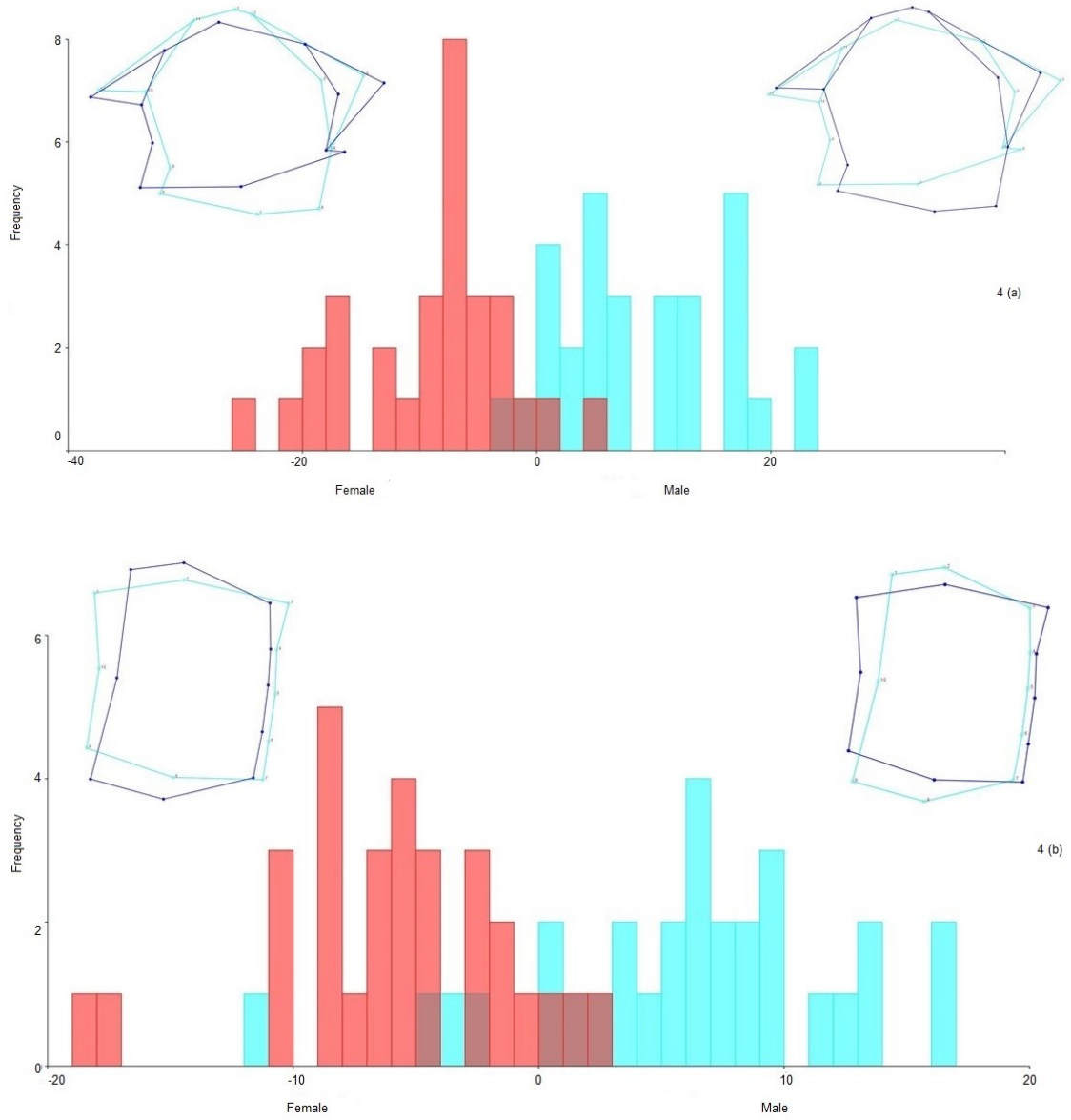

Figure 4. Cross validation scores of shape variables of a) head and b) pronotum. Wire-frame graphs were shown for female and male respectively at the top of left and right of each figure. The extreme changes of shape in positive and negative direction was shown by the violet lines and mean shape of head and pronotum was shown by blue lines. The scale for each figure is; head DA (-20 to 20), pronotum DA (-10 to 10).

Methods for the selection and use of body parts, and for the evaluation of sexes, are of central importance in sexual dimorphism. Geometric morphometrics is not only a novel tool for detecting morphological variations (Mitteroecker & Gunz, 2009; Breno et al., 2011; Kaliontzopoulou, 2011; Benítez et al., 2013; Meng et al., 2018), but also is the best clue to determine sexual dimorphism between and/or among organisms (Hood, 2000; Kaliontzopoulou et al., 2007; Moneva et al., 2012; Alencar et al., 2014; Jun-Yan et al., 2015; Solis et al., 2015; Minoli et al., 2016; Benítez & Vargas, 2017; Tamagnini et al., 2018). For insects in general, and beetles in particular, past works on sexual dimorphism have concentrated on sclerotized body parts (Pretorius & Scholtz, 2001), such as head and pronotum (Torres et al., 2010; Cruz et al., 2011; Acevedo, 2015; Ober & Connolly, 2015; Eldred et al., 2016; Sukhodolskaya & Saveliev, 2017;

Vesović et al., 2019). Since, an external morphological trait could allow for comparison of intraspecific variation in sexual versus nonsexual characters (Polihronakis, 2006). Of these, the adult head morphology of Coleoptera is interesting in its own right and provide phylogenetically informative characters (Antunes-Carvalho et al., 2016). Also, easily perceived differences in size, structure and function in the prothorax (pronotum constricted behind the head and is the dorsal plate of the prothorax) are usually viewed as taxonomic evidence (Hlavac, 1972). The present study, thus, intended to show that geometric morphometrics can confirm a clear sexual difference in both head and pronotum of *C. c.* ssp. *cappadocica*.

Of the very large number of geometric morphometrics studies on insect species (Tatsuta et al., 2018), relatively few have examined tiger beetles (Jones & Conner, 2018). As far as is known, no Anatolian tiger beetle has been analyzed via geometrics morphometrics in terms of sexually dimorphic features. For the first time, we aimed at answering the question of whether the sexes of endemic tiger beetle *C. c.* ssp. *cappadocica* can be morphologically differentiated. Actually, this can be achieved using suitable body structures. As also emphasized-above, it is concluded that forebody parts (head and pronotum) should be regarded as one of the most important external adult features for tiger beetles. Since, the head and the pronotum of a tiger beetle are obvious characters and likely to provide direct support for inferring their taxonomic knowledge, viz., make it a separate coleopteran family in suborder Adephaga. Tiger beetles are easily distinguished from other adephagan beetles by the general structures of the head and the pronotum as followings: head with eyes wider than pronotum and the hind margin of the pronotum is narrower than the base of the elytra (Pearson,1988; Pearson & Vogler, 2001; Uniyal & Bhargav, 2007; Assmann et al., 2018).

Our results support the view that males of *C. c.* ssp. *cappadocica* are less variable with respect to CS when compared with the females and females are larger than males in both head and pronotum (Figure 3). Geometric morphometric analysis of shape variation in the *C. c.* ssp. *cappadocica* revealed statistically significant differences in both the head and pronotum. Also, in this study multivariate regression of shape onto size results indicated that size has little influence on the differentiation in both head and pronotum shape among sexes.

Our attempt allowed us to characterize morphological comparisons between the sexes. Statistically significant shape differences were identified in both the head and pronotum of the *C. c.* ssp. *cappadocica* using discriminant function analysis. According to results of head, while only shape variables were included in the discriminant function, 88.3% of cross-validated grouped cases correctly classified, this rate increased by only 1.7% when the CS was added (Table 2). Similarly, according to results of pronotum, while only shape variables were included in the discriminant function, 85% of cross-validated grouped cases correctly classified, this rate increased by only 3.3% when the CS was added (Table 3). Thus, discrimination results appear to support multivariate regression of shape against size results; size has little influence on the differentiation in both head and pronotum shape among sexes.

Focusing the head shape *C. c.* ssp. *cappadocica,* females have a wider and shorter head with relatively larger eyes than males. This is also related to elongated and sharpened from both anterior and posterior parts of the head shape in male (Figure 4a). Our results also show that the female pronotum is wider and shorter than that of males. However, the posterior parts of pronotum was slightly narrower in females (Figure 4b).

In conclusion, our results highlight the importance of considering sexual dimorphism in terms of shape and centroid size of endemic tiger beetle *C. c.* ssp. *cappadocica*. Here, the evidence of statistically significant shape differences was found among sexes of the *C. c.* ssp. *cappadocica*. Therefore, geometric morphometrics may be an effective and useful tool especially when the size-independent shape differentiation is too small to be detected by the human eye. In conclusion, this study of the geometric morphometrics of Anatolian tiger beetles revealed unexpected data and enhanced the importance of such analysis in taxonomy and systematic.

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Original article (Orijinal araştırma)

Chemical composition of *Vitex agnus-castus* L. (Verbenaceae) essential oil and its larvicidal effectiveness on *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) larvae[1]

Vitex agnus-castus L. (Verbenaceae) uçucu yağının kimyasal bileşeni ve *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) larvaları üzerindeki larvasidal etkinliği

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Abstract

In this study, the chemical composition of the essential oil obtained from chaste tree (*Vitex agnus-castus* L.) (Verbenaceae) was analyzed by gas chromatography-mass spectrometry (GC-MS) and its larvicidal effectiveness at 250, 500 and 1000 µL/L, and 24, 48, 72 and 96 h against the five instars of the pine processionary moth, *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) investigated. The study was conducted at Muğla Sıtkı Koçman University (Fethiye A.S.M.K. Vocational High School) under laboratory conditions (25±2°C, 65±5% RH and 14:10 h L:D photoperiod) in 2015-2017. Each larvicidal effectiveness test was repeated three times. β-caryophyllene (16.8%), germacrene D (14.7%), 1,8-cineole (14.5%) and ζ-gurjunene (11.8%) were identified as main compounds of the essential oil. Ninety-six h after treatment, the essential oil caused between 13.3 and 96.6% mortality. The highest mortality at 250, 500 and 1000 µL/L doses were 70.0% for L_1, 80.0% for L_3 and 96.6% for L_1, L_3 and L_4 instars after 96 h, respectively. It was concluded that the *V. agnus-castus* essential oil has potential as an alternative for control of *T. pityocampa* larvae.

Keywords: Chaste tree, essential oil, pine processionary moth, toxicity

Öz

Bu çalışmada, hayıt (*Vitex agnus-castus* L.) (Verbenaceae) bitkisinden elde edilen uçucu yağın gaz kromatografisi-kütle spektrometresi (GC-MS) yöntemiyle kimyasal bileşeni analiz edilmiştir ve onun 250, 500 ve 1000 µL/L dozlarında 24., 48., 72. ve 96. saatlerde uygulanmasıyla, çam keseböceğinin, *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) beş farklı dönem larvalarına karşı larvasidal etkinliği araştırılmıştır. Bu çalışma, 2015-2017 yılları arasında Muğla Sıtkı Koçman Üniversitesi'nde (Fethiye A.S.M.K. Meslek Yüksekokulu'nda) laboratuvar şartlarında (25±2°C), %65±5 ON ve 14/10 A/K) yürütülmüştür. Her bir larvasidal etkinlilik testi üç kez tekrarlanmıştır. β-caryophyllene (%16,8), germacrene D (%14,7), 1,8-cineole (%14,5) ve ζ-gurjunene (%11,8) bu uçucu yağın ana bileşenleri olarak kaydedilmiştir. Çalışmanın 96. saatinde, uçucu yağ %13,30 ile %96,6 arasında ölümlere sebep olmuştur. 250, 500 ve 1000 µL/L dozlarındaki en yüksek ölüm oranları sırasıyla, L_1 dönemi için %70,0, L_3 dönemi için %80,0 ve L_2, L_3, L_4 dönemleri için ise %96,6 olarak kaydedilmiştir. Bütün bunlar göz önüne alındığında, *V. agnus-castus* uçucu yağının kontrollerle kıyaslandığında, *T. pityocampa* larvalarının kontrolü için potansiyel bir alternatif olduğu sonucuna varılabilir.

Anahtar sözcükler: Hayıt ağacı, uçucu yağ, çam keseböceği, toksisite

[1] This study was conducted as a Master thesis of first author.

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Introduction

Forests, which protect the natural balance of the world we live in, make a significant contribution to meeting the various needs of people, and are continually evolving with unique life forms and ecological environment. About 28% of Turkey is covered with forests and there are more than 50 tree species adapted to these areas. Among the trees, red pine (*Pinus brutia* Tenellus) (Pinaceae) is widespread in the Mediterranean, Aegean and Marmara Regions of Turkey and supports diverse wildlife. It is used economically in various ways such as firewood, timber with its high quality as wood, and has become the most essential plant in the forestry industry (Oktem, 1987). Red pine represents nearly half of the forest area in the Mediterranean Region, 40% in the Aegean Region and 10% in the Marmara Region of Turkey. In addition, this species is also found in some areas of the Western Black Sea Region. However, many factors affect the red pine population (Neyişci, 1987). Among these factors, the most important ones are illegal and uncontrolled cutting, land clearance, forest fires, unplanned and improper zoning permits, road construction and human migration. In addition to these, insects known as smokeless fire threaten the red pine trees. Among the insects, the pine processionary moth (*Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) is one of the most important insect pests of the red pine trees. *Thaumetopoea pityocampa* adults are not harmful, but the larvae feed on conifers such as *Pinus halepensis* Miller*, Pinus sylvestris* L.*, Pinus pinea* L.*, Pinus nigra* Arnold with *Cedrus libani* Richert (Pinaceae) as well as red pine (*P. brutia*) in Turkey and constantly cause economic losses. They cause between 22 and 65% growth reduction in the conifers and also decrease their diameter and height. As a result, weakened trees are invaded by other pests that cause more rapid transmission of diseases (Çanakçıoğlu, 1993; Kanat et al., 2002; Köse, 2007). When the population of *T. pityocampa* is intense, it can cause the death of the trees.

Different methods (mechanical, biological and chemical control) have been used to control *T. pityocampa* larvae in the past, but damage was not fully prevented and a permanent solution has not been found. Also, this pest remains as a significant problem in the coniferous forests of Turkey and the world. In chemical control, the excessive and random use of synthetic chemicals in agricultural and forest areas caused many adverse effects on human and environmental health (Breuer & Devkota, 1990). In particular, they negatively affect beneficial organisms that protect natural balance (Güncan & Durmuşoğlu, 2004). Given all these negativities and the continuing loss by *T. pityocampa* larvae, there is a need to develop alternative control methods against this pest that are eco-friendly and protect the natural balance.

In this regard, plant-derived compounds should be considered for the control of *T. pityocampa* larvae. Many plant species that contain phenolic compounds and essential oils with potent biological activity are used as critical natural bio-agents (Mokbel & Fumio, 2006; Batish et al., 2008). According to recent studies around the world, more than 200,000 plant species have been found to contain insecticidal compounds, but only 1% of them have considered useful. Among these compounds, pyrethrum, rotenone, nicotine and azadirachtin were reported to be the most important plant-derived compounds that have pesticidal effects as a result of studies on many insect pests (Isman, 2006). Essential oils are volatile and fragrant natural compounds extracted from various parts of plants (e.g., flowers, seeds, leaves, fruits and husks), which are usually in the form of liquid at room temperature, quickly crystallized, colorless or light yellow. Plant-derived compounds, especially essential oils, when used against pests the agricultural field, they can help to reduce insect resistance and environmental pollution, with no residual (permanent) effect on the environment. From this perspective, natural insecticides do not pose much threat to human and environmental health (Aksoy, 1982; Güncan & Durmuşoğlu, 2004; Isman, 2006).

The genus *Vitex* L. belongs to Verbenaceae family and has approximately 300 species in tropical regions of the world. Its origin is Mediterranean countries and there are two species (*Vitex agnus-castus* L. and *Vitex pseudo negundo* Haussknecht) grown in natural areas of Aegean and Mediterranean Regions in Turkey (Eryiğit et al., 2015; Tin et al., 2017). Among these species, *V. agnus-castus* was well known by ancient herbalists in the Middle Ages due to its medicinal properties, and also used as a contraceptive and

an antiaphrodisiac drug (Cambie & Brewis, 1997; De Kok, 2007). The species has a great importance because of its specific taste, aroma and medicinal use, and contains large amounts of essential oil. It has been found that a methanol extract of *V. agnus-castus* has an antibacterial effect (Karaman et al., 2008).

Essential oils, extracts and compounds obtained from various plants have been found to have insecticidal, ovicidal, larvicidal, repellent, attractant and antifeedant effects in many studies on harmful insects. Yelekçi (1981) reported that an extract of fruits of *Melia azedarach* L. (Meliaceae) had larvicidal effect on *T. pityocampa*. Regnault-Roger et al. (1993) reported that 22 different essential oils cause mortality at different rates in *Acanthoscelides obtectus* (Say, 1831) (Coleoptera: Bruchidae) and among them, *Origanum majorana* L. and *Thymus serpyllum* L. (Lamiaceae) oils were the most toxic. Shaaya et al. (1993) determined that different plant essential oils were insecticidal to some stored product pest adults. In another study, it was found that the essential oil of *Lavandula stoechas* L. (Lamiaceae) had very toxic effect on *Lasioderma serricorne* Fabricus, 1972 (Coleoptera: Anobiidae) and *Rhyzopertha dominica* (Fabricus, 1792) (Coleoptera: Bostrichidae) adults (Ebadollahi et al., 2010). Heydarzade & Moravvej (2012) determined that *Foeniculum vulgare* Miller (Apiaceae), *Teucrium polium* L. (Lamiaceae) and *Satureja hortensis* L. (Lamiaceae) oils were highly toxic to *Callosobruchus maculatus* (Coleoptera: Fabricius, 1775) (Coleoptera: Bruchidae). Kesdek et al. (2014) reported that *Achillea wilhelmsii* C. Koch (Asteraceae), *Nepeta meyeri* Benth., *S. hortensis*., *Origanum onites* L., *Origanum rotundifolium* Boiss. and *Tanacetum argyrophyllum* (C. Koch) (Lamiaceae) plant extracts had very toxic effects (between 3.33 and 100%) on four larvae instars at 0.25, 0.5 and 1 µl/dish doses. Germinara et al. (2017) determined that *Lavandula angustifolia* Miller (Lamiaceae) oil caused different rates mortality. In another study, five different commercial volatile oils (thyme, sage, poppy, garlic and rosemary) were found to be 70-100% effective against *T. pityocampa* larvae (Yiğit et al., 2019).

No long-term solution has been found against *T. pityocampa* in studies conducted so far. The main objective of the study was to investigate the chemical components of the essential oil obtained from chaste trees (*V. agnus-castus*) growing in natural areas and its larvicidal effectiveness under laboratory conditions against the five instars larvae of *T. pityocampa*, which is a very important coniferous tree pest.

Materials and Methods

Biological material

Thaumetopoea pityocampa larvae used in the study were collected from Esenköy (Dont), Fethiye, Muğla Province. Pouches (nests) on branches of red pine trees were cut with the help of gloves and pruning shears and placed into 30 x 45 x 30 cm-size cardboard boxes, under wrapped filter paper. The larvae were fed adding fresh leafy shoots cut from non-infected shoots. The larvae were removed from pouches using forceps and were placed in Petri dishes at 25±2ºC and 65±5% RH under laboratory conditions. This process was performed separately for each larval instar (between October 2016 and March 2017).

Plant Material

The chaste tree (*V. agnus-castus*) samples used in this study was collected at the flowering stage from Babadağ and Mendos Mountains, Fethiye, Muğla Province in June and September 2015, and June 2016. The aerial parts (flowers and leaves) of the plant were dried in shade before processing with a grinder. Dried plant herbarium samples have been stored in the Department of Environmental Protection Technologies, Fethiye A.S.M.K. Vocational High School, Muğla Sıtkı Koçman University.

Essential oil extraction

Extraction of *V. agnus-castus* essential oil was performed as described by Çakır et al. (2016). The aerial parts of the plant were dried in shaded environmental and milled in a grinder. Then this material (300 g) was subjected to hydro-distillation in a Clevenger-type apparatus for 4 h yielding 0.31% (v/w) of oil. The yields were determined for dry plant samples. The essential oil was stored in the refrigerator at +4ºC until used in experiments.

Gas chromatography-mass spectrometry analysis

The essential oil of *V. agnus-castus* chemical composition was determined by gas chromatography-mass spectrometry (GC-MS). A DB-1 united silica non-polar very thin column (30 m x 0.25 mm I.D., film denseness 0.25 µm) was used for the analysis. Helium was used as the carrier gas with flow rate of 1.4 mL/min. The ion source, injector and MS. transfer line heats were 200, 220 and 290ºC, respectively. Diluted samples (1/100 v/v in ethanol) of 1 µL were injected manually and in the splitless mode. The ionization energy of EI-MS measurements was attained at 70 eV. Mass. range was from m/z 50 to 650 amu. Scan time was 0.5 s with 0.1 s interscan delays. The oven temperature was maintained at 60ºC for 5 min, then raised to 240ºC with 4ºC/min increases and held at this temperature for 10 min. Identification of components of the essential oil was based on GC retention indices and computer matching with the libraries of Wiley, NIST-2008 and TRLIB, as well as by comparison of the segmentation models of the mass spectra (Küçükaydın et al., 2020).

Bioassay of the larvicidal effectiveness of the essential oil

In order to determine the larvicidal effectiveness of the *V. agnus-castus* essential oil against *T. pityocampa* larvae, the essential oil was dissolved at 1:2 in ethanol and the final concentration was prepared as 250, 500 and 1000 µL/L doses (Kesdek et al., 2013). Two layers of sterilized filter paper were placed in Petri dishes (according to 120 ml volume, 9 cm width x 1.5 cm depth). Ten larvae were placed in each Petri dish and 1 mL from the essential oil and positive control (25% diflubenzuron) solutions prepared as the stock were sprayed with a hand spray (Manual Potter Spray Tower-Burkard Scientific Limited, Uxbridge, UK) to contact the larvae. Ethanol in the essential oil was evaporated under ambient conditions for 1 min. To feed the larvae, non-contaminated fresh pine leaves were added in sufficient amounts (according to larvae instar, 5-10 g; by increasing each larval stage) and Parafilm was wrapped around the Petri dishes. Previously prepared essential oil solutions were mixed using vortex in 1 min. before application. Pure water plus ethanol was used as the negative control while the commercial chemical, Kormilin 25 WP (25% diflubenzuron) was used as a positive control. The experiments were conducted at 25±2ºC, 65±5% RH and 14:10 h L:D photoperiod, each test was repeated with three times for each larval stage and dose. Dead larvae were counted 24, 48, 72 and 96 h after treatment (Kesdek et al., 2014, 2020).

Data analysis

To determine whether there was a statistically significant difference between the results obtained, two-way analysis of variance was performed using SPSS (Statistical Package for Social Sciences 17.0). The differences between means were determined by Duncan's multiple range test. Median lethal dose (LD) LD_{50} and LD_{90} values after 24, 48, 72 and 96 h were calculated using the Finney method (Finney, 1971). To determine LD values at 95% confidence limits of each application EPA probit analysis program was used. Significant differences were tested at $P< 0.05$.

Results and Discussion

Chemical compositions of the essential oil

The essential oil of *V. agnus-castus* was analyzed by GC-MS and its chemical components are given in Table 1. In the essential oil, 32 components were determined which was represent 100% of the composition. The major components were β-caryophyllene (16.76%), germacrene D (14.71%), 1,8-cineole (14.52%), ζ-gurjunene (11.82%), α-pinene (9.88%), α-terpineol acetate (5.29%), Tau-cadinol (5.23%), terpinene-4-ol (2.76%), spathulenol (2.02%) and verticillol (1.91%).

Table 1. Chemical components (%) of the oil obtained from aerial parts of *Vitex agnus-castus*

Retention time (min)	Compound name	Essential oil (%)	Identification methods
4.430	3-methyl-2-heptanone	0.08	GC/MS/RI
4.482	α-thujene	0.23	GC/MS/RI
4.667	α-pinene	9.88	GC/MS/RI
6.572	β-pinene	1.11	GC/MS/RI
6.98	α-phellandrene	0.23	GC/MS/RI
7.435	α-terpinene	0.71	GC/MS/RI
7.734	α-camphene	0.26	GC/MS/RI
7.956	1,8-cineole	14.52	GC/MS/RI
8.711	β-ociimene	1.05	GC/MS/RI
9.050	γ-terpinene	1.35	GC/MS/RI
10.183	terpinolene	0.40	GC/MS/RI
10.717	linalool	0.20	GC/MS/RI
11.456	4-isopropyl-1-methyl-2 cyclohexene-1-ol	0.10	GC/MS/RI
13.232	ocimenol	0.36	GC/MS/RI
13.610	terpinene-4-ol	2.76	GC/MS/RI
14.148	α-terpineol	1.38	GC/MS/RI
19.511	ζ-elemene	0.79	GC/MS/RI
20.009	α-terpineol acetate	5.29	GC/MS/RI
21.982	α-gurjunene	1.79	GC/MS/RI
22.303	β-caryophyllene	16.76	GC/MS/RI
23.408	α-caryophyllene	0.15	GC/MS/RI
23.691	germacrene D	14.71	GC/MS/RI
24.855	ζ-gurjunene	11.82	GC/MS/RI
25.408	α-amorphene	0.44	GC/MS/RI
25.739	ζ-cadinene	0.41	GC/MS/RI
27.031	globulol	0.56	GC/MS/RI
27.347	spathulenol	2.02	GC/MS/RI
27.522	caryophyllene oxide	1.46	GC/MS/RI
28.125	ledol	1.27	GC/MS/RI
29.291	Tau-cadinol	5.23	GC/MS/RI
36.011	biformene	0.76	GC/MS/RI
37.777	verticillol	1.91	GC/MS/RI
	Total identified (%)	**100.00**	

[a] Calculated retention index to *n*-alkanes (C_8-C_{28}) on SGE-BPX5 capillary column. GC: co-injection with.standards; MS; previously identified based on computer coordination of the.mass spectra of peaks with Wiley 7N and TRLIB libraries and according to Adams, (2007); RI:.Identification based on comparing of repression index with Adams' data. t, trace (<0.1%).

Larvicidal effect of the essential oil

The application of three different doses (250, 500 and 1000 µL/L) of *V. agnus-castus* essential oil caused mortality at different rates in *T. pityocampa* larvae compared to the control. The mortality rates were between 13.3 and 96.6% for all larval stages 96 h after treatment. Depending on the application dose and time, the mortality increased. When the mortality of the essential oil was compared for 24, 48, 72 and 96 h

after treatment there were statistical differences between the treatments for each larval stage. The most effective dose for L_1, L_2, L_3, L_4 and L_5 instars was established to be 1000 µL/L. While the highest mortality rates were found between 70.0 and 96.6% for L_1 instar 96 h after treatment, the lowest mortality for L_2 instar (between 13.3 and 40.0%) (Table 2; $P<0.05$).

Twenty-four h after treatment, the highest larvicidal toxicity with1000 µL/L was 70.0% in L_1 instar. However, the lowest larvicidal toxicity was with 250 µL/L, 6.7% in L_2 instar. However, there was no mortality with 250 µL/L in L_5 instar. Similarly, 48 h after treatment, the highest mortality was with 1000 µL/L, 76.6% in L_1 instar. With the same treatment time, the lowest mortality was with 250 and 500 µL/L, 10.0 and 23.3% in L_2 and L_5 instars, respectively (Table 2; $P<0.05$). Seventy-two h after treatment with 250 and 500 µL/L the mortality was 13.3 to 90.0% in L_1, L_2, L_3, L_4 and L_5 instars. The highest mortality was with 1000 µL/Petri dish, 90.0% and 80.0% in L_1 and L_3 instars, respectively. However, the lowest mortality was with 250 and 500 µL/L, 13.3% and 16.6% in L_2 instar, respectively. In general, while most mortality was in L_1 instar 72 h after treatment, the lowest mortality was for L_2 instar. Ninety-six h after treatment, mortality was from 13.3 to 96.6% in L_1, L_2, L_3, L_4 and L_5 instars. However, differences between mortality were not significant 96 h after treatment period for 250, 500 and 1000 µL/L in L_1, L_3 and L_4. Overall, the most effective dose was detected to be 1000 µL/L (Table 2; $P < 0.05$). Kormilin 25 WP (25% Diflubenzuron) is one of the most widely used commercial insecticides for *T. pityocampa* larvae. In this study, 100% toxicity (except 250 µL/L dose, 83.3% for L_2) was obtained 96 h after treatment with all doses of Kormilin (250, 500 and 1000 µL/L) (Table 2; $P < 0.05$).

The LD values (LD_{50} and LD_{90}) of the study are summarized in Table 3. When LD values 96 h after treatment were compared the highest toxicity based on the LD_{50} and LD_{90} were in L_1 instar (0.53 and 1.25 µL/larva), respectively. However, the lowest toxicity based on the LD_{50} and LD_{90} 96 h after treatment were 2.57 and 22.09 µL/larva in L_5 instar, respectively. Similarly, LD_{50} 96 h after treatment were 0.62, 0.97, 1.03 and 2.57 µL/larva for L_2, L_3, L_4 and L_5 instars, respectively (Table 3). These results indicated that the larvicidal activity increased with increasing dose and exposure time. *Vitex agnus-caspus* essential oil gave potentially useful toxicity to the larvae of *T. pityocampa* (Tables 2 to 3).

Consequently, 96 h after treatment, when mortality of the five instars of *T. pityocampa* were compared, L_2 instar of *T. pityocampa* was the most resistant (due to mortalities between 13.3 and 40.0%), while L_1 instar was the most susceptible (in varying rates between 70.0 and 96.6%) (Table 2).

There were many studies relating to the chemical composition of the essential oil obtained from different parts of *V. agnus-castus*. Senatore et al. (1996) recorded 1.8 cineole (flowers 14.1%, leaves 15.6%), α-terpineol (flowers 6.4%, leaves 8.5%) and sabinene (flowers 6.3%, leaves 6.9%) as the main components in the essential oil of *V. agnus-castus*. Hamid et al. (2010) determined β-pinene (20.0%), viridiflorol (9.8%), α-pinene (9.1%), cis-ocimene (8.4%), 1.8 cineole (6.7%) and β-farnesene (5.4%) as dominant constituents in the *V. agnus-castus* essential oil in Nigeria. Eryiğit et al. (2015) found *trans*-caryophyllene (19.17%), sabinene (18.05%) and 1.8- cineole (16.13%) as the main compositions in the *V. agnus-castus* plant oil in Turkey. Similarly, in another study from Turkey, Tin et al. (2017) detected 1.8 cineole (8.24%), propenamide (6.07%), caryophyllene (5.56%), bicyclogermacrene (5.51%), sabinene (5.37%), maleimide (5.28%), *trans-β*-farnesene (4.45%) and α-pinene (3.98%) as the main components the *V. agnus-castus* essential oil. In the present study, the main compounds were β-caryophyllene (16.8%), germacrene D (14.7%) and 1.8-cineole (14.5%), respectively (Table 1). When the previous studies and our study are examined carefully, constituents of *V. agnus-castus* essential oil are similar to each other, but rates of components are different. These differences may be due to the fact that the composition of *V. agnus-castus* oil may depend on geographical conditions, such as soil and climatic conditions and environmental factors.

Table 2. Larvicidal effectiveness of *Vitex agnus-castus* essential oil against five instars of *Thaumetopoea pityocampa*

Essential Oil	Dose (µL/L)	Mortality (%)			
		Exposure Time (h)			
		24	48	72	96
		L_1 Instar Larvae			
Vitex agnus-castus	250	33.3±5.74 b	46.6±3.33 b	53.3±6.66 b	70.0±10.0 b
	500	50.0±26.4 c	63.3±8.81 c	70.0±11.5 c	76.6±14.5 b
	1000	70.0±17.4 d	76.6±12.0 d	90.0 ±5.77 d	96.6±3.33 c
Positive Control (Kormilin)	250	100±0.0 e	100±0.0 e	100±0.0 d	100±0.0 c
	500	100±0.0 e	100±0.0 e	100±0.0 d	100±0.0 c
	1000	100±0.0 e	100±0.0 e	100±0.0 d	100±0.0 c
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
		L_2 Instar Larvae			
Vitex agnus-castus	250	6.7±3.33 ab	10.0±5.77 ab	13.3±3.33 a	13.3±3.33 a
	500	10.0±10.0 ab	10.0±10.0 ab	16.6±12.0 a	26.6±21.8 a
	1000	26.6±17.6 b	36.6±27.2 b	36.6±12.0 a	40.0±30.5 a
Positive Control (Kormilin)	250	70.0±5.77 c	76.6±3.33 c	80.0±5.77 b	83.3±8.81 b
	500	96.6±3.33 d	96.6±3.33 c	96.6±3.33 b	100±0.0 b
	1000	100±0.0 d	100±0.0 c	100±0.0 b	100±0.0 b
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
		L_3 Instar Larvae			
Vitex agnus-castus	250	10.0±5.77 b	40.0±10.0 b	53.3±12.0 b	53.3±12.0 b
	500	33.3±3.33 c	50.0±10.0 bc	63.3±8.81 b	80.0±11.5 c
	1000	46.6±3.33 d	56.6±6.66 c	80.0±0.0 c	96.6±3.33 cd
Positive Control (Kormilin)	250	100±0.0 e	100±0.0 d	100±0.0 d	100±0.0 d
	500	100±0.0 e	100±0.0 d	100±0.0 d	100±0.0 d
	1000	100±0.0 e	100±0.0 d	100±0.0 d	100±0.0 d
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
		L_4 Instar Larvae			
Vitex agnus-castus	250	16.6 ±3.33 b	36.6±3.33 b	53.3±12.0 b	53.3±12.0 b
	500	26.6±3.33 c	40.0±15.2 b	56.6±8.81 b	56.6±8.81 b
	1000	30.0±0.0 c	60.0±0.0 c	76.6±3.33 c	96.6±3.33 c
Positive Control (Kormilin)	250	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	500	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	1000	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a

Table 2. Continued

Essential Oil	Dose (µL/L)	Mortality (%)			
		Exposure Time (h)			
		24	48	72	96
		L_5 Instar Larvae			
Vitex agnus-castus	250	0.0±0.0 a	13.3±3.33 b	20.0±0.0 b	26.6±3.33 b
	500	11.1±6.08 b	23.3±3.33 c	33.3±3.33 c	50.0±5.77 c
	1000	20.0±5.77 c	26.6±3.33 c	33.3±3.33 c	50.0±5.77 c
Positive Control (Kormilin)	250	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	500	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	1000	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a

[a] Values followed by the letter within columns do not differ significantly at P ≤ 0.05 according to Duncan Multiple test;
[b] Mean±SE of three replicates, each set up with 10 larvae.

Table 3. LD_{50} and LD_{90} values (µL/L) of *Vitex agnus-castus* essential oil on the five instars of *Thaumetopoea pityocampa*

	Vitex agnus-castus				
	LD_{50} (Limits)	LD_{90} (Limits)	X^2	Slope±SE (Limits)	Probability
L_1 Instar Larvae	0.53 (0.01-0.83)	1.25 (0.72-2.20)	6.41	3.42±1.46 (0.56-6.29)	0.83
L_2 Instar Larvae	0.62 (0.10-1.03)	2.64 (1.70-6.63)	16.29	2.03±0.82 (0.74-4.15	0.66
L_3 Instar Larvae	0.97 (0.15-1.39)	2.43 (1.70-15.58)	11.58	3.23±0.84 (1.59-4.88)	0.52
L_4 Instar Larvae	1.03 (0.23-1.44)	3.91 (2.79-65.90)	17.38	2.22±0.75 (0.54-3.41)	0.50
L_5 Instar Larvae	2.57 (1.61-1.11)	22.09 (6.18-7.89)	2.59	1.37±0.70 (0.01-2.73)	0.29

[a] Lethal concentration causing 50% mortality 96 h after treatment;
[b] Lethal concentration causing 90% mortality 96 h after treatment;
[c] Chi square value.

There are some studies reported by a few researchers on the toxicity of essential oils to *T. pityocampa* larvae. Çetin et al. (2006) recorded that O*riganum onites* L. (Lamiaceae) and *Citrus aurantium* L. (Rutaceae) essential oils caused mortality between 72.5 and 97.5% in L_4 and L_5 instars of pine processionary moth, *Thaumetopoea wilkinsoni* Tams, 1926 (Lepidoptera: Notodontidae) at 0.1, 0.5 and 1% doses 24 h after treatment. In the present study, mortality rates were between 11.1 and 30.0% in L_4 and L_5 instars of *T. pityocampa* with *V. agnus-castus* oil at 250, 500 and 1000 µL/L doses 24 h after treatment. In another study, 24 h after treatment the essentials oil obtained from *Satureja hortensis* L., *Origanum onites* L., *O. rotundifolium* Boss. (Lamiaceae) and *Tanacetum argyrophyllum* (C. Koch) Tvzel. (Asteraceae) caused the highest mortality in L_2, L_3 and L_4 instars (100% at 20 µL/dish) (Kesdek et al., 2013). In the present study, 24 h after treatment with 1000 µL/L of *V. agnus-castus* essential oil, the highest mortality was 70.0, 46.6 and 30.0% in L_1, L_3 and L_4 instars (Table 2). When these studies are compared, it was concluded that they support each other. Germinara et al. (2017) found that *Lavandula angustifolia* Miller (Lamicaeae) oil caused different rates mortality (from 91.7 to 100%) at 0.449 mg/adult 24 and 48 h after exposure, respectively. In another study, the mortality with 10, 15 and 20 µL/Petri dish doses of *Achillea biebersteinii* Afan (Asteraceae) oil against L_2, L_3 and L_4 instars 48 h after treatment were 73.3, 73.3 and 83.3% in L_2 instar, 43.3, 50.0 and 73.3% in L_3 instar, 36.6, 43.3 and 53.0% in L_4 instar, respectively (Kesdek et al., 2020). Usanmaz Bozhüyük et al. (2018) found that the essential oils of *Seriphidium santonicum* (L.) Soják and *Artemisia absinthium* L. (Asteraceae) caused 6.66 to 100% mortality 48 h after application of three different

doses (10, 15 and 20 µL/dish) in five instars of *T. pityocampa*. In the present study, we determined that *V. agnus-castus* essential oil caused 36.6 to 76.6% mortality in three different doses (250, 500 and 1000 µL/L) in L_1, L_3 and L_4 instars (46.6, 63.6 and 76.6% for L_1 instar; 40.0, 50.0 and 56.6% in L_3 instar; 36.6, 40.0 and 60.0% in L_4 instar) 48 h after treatment, respectively. In addition, we found that *V. agnus-castus* oil gave the lowest mortality of 10.0% and the highest mortality with 76.6% 48 h after treatment at 250, 500 and 1000 µL/L against five instars of *T. pityocampa* larvae (Table 2). Kesdek et al. (2013) determined that *Origanum acutidens*, *O. onites*, *O. rotundifolium*, *S. hortensis*, *Satureja spicigera* (C. Koch) (Lamiaceae), *Thymus sipyleus* Boiss., *T. argyrophyllum* and *Achillea gypsicola* Hub.-Mor. (Asteraceae) plant essential oils caused the mortality from 73.3 to 100% 72 h after treatment with 10 and 20 µL/Petri dish in L_2, L_3, and L_4 instars of *T. pityocampa*. In the present study, the essential oil of *V. agnus-castus* gave mortality from 13.3 to 90.0% 72 h after treatment with 250, 500 and 1000 µL/L in L_2, L_3, and L_4 instars of *T. pityocampa.* These studies support each other. Kesdek et al. (2014) found that extracts of six different plant species [*Nepeta meyeri* Benth, *S. hortensis*, *O. onites*, *O. rotundifolium* (Lamiaceae), *Achillea santolinoides* (C. Koch) Lag. and *T. argyrophyllum* (Asteraceae)] had larvicidal effect between 3.33 and 100% in L_2, L_3 and L_4 instars of *T. pityocampa* 96 h after treatment. In another study, five different commercial volatile oils (thyme, sage, poppy, garlic and rosemary) were found to be 70-100% effective against *T. pityocampa* larvae (Yiğit et al., 2019). The results of this study showed that *V. agnus-castus* oil caused mortality from 13.3 to 96.6% 96 h after treatment with three different doses (250, 500 and 1000 µL/L) in the five instars of *T. pityocampa* (Table 2).

Considering the lethal dose (LD) 96 h after treatment with *V. agnus-castus* oil in larvae of *T. pityocampa*, LD_{50} values were 0.53, 0.62, 0.97, 1.03 and 2.57 µL/larva in L_1, L_2, L_3, L_4 and L_5 instars, respectively. However, the highest toxicity was with 0.53 µL/larva in L_1 instar, the least toxicity was with 2.57 µL/larva in L_5 instar. For LD_{50} and LD_{90} 96 h after treatment, the highest toxicity was 0.53 and 1.25 µL/larva in L_1 instar, respectively. However, at the same time, the lowest toxicity for LD_{50} and LD_{90} were 2.57 and 22.09 µL/larva in L_5 instar (Table 3).

In conclusion, many studies by different researchers show that chemicals used against diseases and pests in the forest and agricultural areas are very dangerous for human and environmental health and also ecological balance. Therefore, there is a need for alternative methods to protect the environment and human health and ecological balance. For these reasons, components derived from plants are potentially useful. In this study, larvicidal toxicity of *V. agnus-castus* essential oil to pine processionary moth (*T. pityocampa*) larvae was investigated. According to the results, we determined that as the application doses and time of *V. agnus-castus* oil increased, larvicidal toxicities also increased. When the toxicities were compared according to the application dose, the highest toxicity (96.6%) was with the highest dose of the essential oil (1000 µL/L) in L_1, L_3 and L_4 instars. The lowest toxicity was in L_2 instar larvae. In the light of these data, when the larvicidal toxicity of the *V. agnus-castus* essential oil to the five larval instars of *T. pityocampa* is carefully examined, it is concluded that this essential oil could be an alternative for the control of *T. pityocampa*, which is one of the most damaging pests for coniferous trees. Finally, we consider that this study will provide a useful basis for further studies.

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Original article (Orijinal araştırma)

Insecticide residue analyses in cucumbers sampled from Çanakkale open markets[1]

Çanakkale açık pazarlarından örneklenen hıyarlarda insektisit kalıntı analizleri

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Abstract

The aim of this study was to investigate four insecticide residues in cucumbers with the aid of QuEChERS 2007.1 method. For method verification assessment, pesticide-free cucumber matrix was spiked with 0.1, 1 and 10 times of MRL for each pesticide. The QuEChERS-LC-MS/MS analytical method revealed that the detection limits of the insecticides were below the MRLs and the overall recovery of method was 97.7%. These figures were within the SANTE recovery limits (60-140%) and the values specified for the repeatability (≤20%). Cucumbers were collected from six different stands (A-F) at Çanakkale open markets for 6 weeks between 23 November and 28 December 2018. Residues of each sampling time and each stand were assessed. Acetamiprid residue of 257g and 236 µg/kg were detected in week 5 from stand B and in week 2 from stand E, respectively. These values are close to MRL (300 µg/kg). Formetanate hydrochloride residue of the week 3 from stand F (36.3 µg/kg) was more than MRL of 10 µg/kg. Pirimiphos methyl and chlorpyrifos residues were not detected in cucumbers. Theoretical maximum daily intake assessment showed that there was no chronic exposure risk for these four pesticides through cucumber consumption.

Keywords: Cucumber, insecticide residues, QuEChERS, risk assessment, toxicology

Öz

Bu çalışma hıyarlarda dört insektisit kalıntısını QuEChERS 2007.1 yöntemi ile belirlemek amacıyla yapılmıştır. Metot doğrulama değerlendirmesi için pestisit içermeyen hıyar örneği MRL değerlerinin 0.1, 1 ve 10 katı seviyelerinde her pestisit ile zenginleştirilmiştir (fortifikasyon). QuEChERS-LC-MS/MS analiz yöntemi ile insektisitlerin dedeksiyon limitleri MRL'lerin altında ve tüm metodun geri alımı %97.7 olarak bulunmuştur. Bu değerler SANTE geri alım limiti (%60-140) ve belirlenen tekrarlanabilirlik değerleri (≤20%) arasındadır. Hıyarlar 6 hafta boyunca Çanakkale açık pazarlarından altı farklı tezgâhtan (A-F) 23 Kasım-28 Aralık 2018 tarihleri arasında toplanmıştır. Her bir örnekleme zamanı ve her bir tezgâha ait örneklerde kalıntılar araştırılmıştır. Acetamiprid kalıntısı, 5. hafta B tezgahında ve 2. hafta E tezgahında sırasıyla 257 µg/kg ve 236 µg/kg olarak tespit edilmiştir. Bu değerler MRL'ne (300 µg/kg) yakındır. Üçüncü hafta F tezgahında formetanate hidroklorür kalıntısı (36.3 µg/kg),10 µg/kg MRL değerinden daha fazla bulunmuştur. Hıyarlarda pirimiphos methyl ve chlorpyrifos kalıntısı bulunmamıştır. Teorik maksimum günlük alım değerlendirmesi hıyar tüketiminde bu 4 pestisitin kronik maruziyet riski oluşturmadığını göstermiştir.

Anahtar sözcükler: Hıyar, insektisit kalıntıları, QuEChERS, risk değerlendirmesi, toksikoloji

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Introduction

Cucumber with an annual production of 1.9 Mt is the third placed vegetable produced Turkey after tomato and pepper. Of this production, 7.3 kt come from Çanakkale Province (TÜİK, 2019). About 83% of cucumber exported from Turkey is sent to EU (European Union) countries. Pests including *Bemisia tabaci* (Gennadius, 1889), *Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera: Aleyrodidae), *Aphis* spp. (Hemiptera: Aphididae) generate significant problems for cucumber production and result in serious economic losses each year. Insecticides are commonly used in cucumber production. Acetamiprid is used against the *B. tabaci* and *T. vaporariorum*, formetanate hydrochloride is applied against the *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) and pirimiphos methyl is used against *Aphis* spp. Chlorpyrifos is used against *Aphis* spp. *Agrotis ipsilon* (Hufnagel, 1766) (Lepidoptera: Noctuidae), *Gryllotalpa gryllotalpa* (Mandal, 1982) (Orthoptera: Gryllotalpidae), *Agriotes* spp. (Coleoptera: Elateridae). However, chlorpyrifos was completely banned in Turkey in May 2020 and is not included in the BKÜ (Plant Protection Products) Database (BKÜ, 2020).

Despite the significant role in crop productivity and food security, chemical pesticides exert serious risks on human health and environment. Pesticide residues in agricultural products have negative effects on human health. Therefore, it is a major concern for consumers. Residues can constitute serious risks for human health (Council Directive 90/642/EEC, 1990). It is important to estimate pesticide exposure level from vegetables. Cucumber is consumed fresh and pickled. Pesticide exposure is very important, especially in fresh consumption. Farmers generally use pesticides for pest control in cucumber fields. However, some farmers use pesticides included in permissible lists but not recommended for cucumber. If integrated pest management (IPM) systems are not practiced, pesticides cause serious residue problems. Sometimes this topic is a barrier to international trade.

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method (Anastassiades et al. 2003) is largely employed in vegetable and fruit matrix safety analyses at well-equipped laboratories (Polat & Tiryaki, 2020). However, local laboratory conditions may require further verification for the method to be used reliably (Omeroglu et al., 2012).

Leili et al. (2016) used QuEChERS method to investigate pesticides residues in greenhouse cucumbers. The recovery of ethion and imidacloprid analyses ranged from 88 to 102%. Researchers have reported 35 and 31% reduction in ethion and imidacloprid levels, respectively, one day after pesticide application, 51 and 43% reduction with washing and 93 and 64% reductions with peeling. Wu & Hu (2014) conducted a study about method validation for fosthiazate residues in cucumber and soil. The recovery rates in cucumbers and soil varied between 91.2 and 99.0%. Researchers used QuEChERS and found that fosthiazate residues were lower than the MRL (maximum residue level) of 0.2 mg/kg.

Abdel-Ghany et al. (2016) worked on eight neonicotinoid insecticide residues in cucumbers and soil by LC/MS coupled with QuEChERS. Researchers set up a field trial in Qaluybiya, Egypt and sprayed pesticides on cucumbers. Cucumbers were sampled 1 h after application and after the 1, 2, 3, 5, 7, 14 and 21 d. The acetamiprid residue was found to be 938 ng/g in zero-time sample. However, 1, 2, 3, 5, 7, 14 and 21 d after pesticide application, residues were measured as 862 ng/g (8.1% reduction), 666 ng/g (28.9% reduction), 504 ng/g (46.3% reduction), 425 ng/g (54.7% reduction), 325 ng/g (64.9% reduction), 221 ng/g (76.4% reduction) and 87.5 ng/g (90.7% reduction), respectively.

In a study conducted by Hassanzadeh et al. (2012), imidacloprid was applied to greenhouse cucumbers at the recommended rate and twice that rate. The initial deposits were measured as 1.93 and 3.65 mg/kg in single and double doses, respectively, and recovery rates after 21 d were 94.5 and 99.2%, respectively. The residual imidacloprid level was lower than the MRL of 1 mg/kg after 3 d. Islam et al. (2015)

investigated pesticide residues on cucumbers sampled from the local markets and detected mancozeb residues in one out of three samples.

According to a study conducted in the Aegean Region of Turkey, the residues in 18 (26%) cucumber samples exceeded the MRL. Chlorpyrifos, dimethomorph and methomyl residues exceeded MRL in one, eight and four cucumber samples, respectively. Four or more pesticide residues were encountered in cucumber samples (Türköz-Bakırcı et al., 2014).

Nasiri et al. (2016) investigated residues of 12 pesticides on cucumber samples. The recovery of pesticides at five spiking levels using the QuEChERS method was in the range of 80.6-112%. The method was shown to be repeatable with RSD lower than 20%. Among 43 greenhouse cucumber samples, six samples contained chlorpyrifos residues at 97.1 µg/kg (range: 66.4-148 µg/kg) which was higher than EU MRL of 50 µg/kg.

Kaya & Tuna (2019) used QuEChERS to investigate pesticide residues in cucumbers collected from three open markets in İzmir Province and detected acetamiprid, chlorpyrifos, metalaxyl-M and thiamethoxam residues respectively as 0.01, 0.004, 0.033 and 0.025 mg/kg. The EU-MRL of them were 0.3, 0.05, 0.5 and 0.5 mg/kg, respectively.

Cara et al. (2011) worked on degradation of acetamiprid in greenhouse cucumber. When greenhouse indoor temperatures were between 29 and 35ºC, a rapid decline was seen in acetamiprid residues. The researchers demonstrated the importance of PHI (pre-harvest interval) for pesticide residues. In another study, consumer responses to pesticide residues in agricultural products were investigated. The study showed that about 40% of fruit and vegetable consumers had concerns about pesticide residues (Oraman, 2011).

Many studies have been conducted on the removal of pesticide residues by various product processing methods. In a study, chlorpyrifos residues (artificially spiked) on cucumber were reduced by 53.1, 59.2 and 62.9% with 5 ,10 and 20 min tap water washing, respectively (Liang et al., 2012). Indeed, mode of action of pesticide (systemic and/or contact) is important for reduction of residues by washing treatments (Polat &Tiryaki, 2020).

The present study was conducted to investigate residues of the four most widely used insecticide in cucumbers sampled from open markets of Çanakkale Province of Turkey. There are no previous studies investigating pesticide residues in cucumbers of Çanakkale Province. The selection of insecticides was made according to the RASFF (Rapid Alert System for Food and Feed) notification (there are RASFF notifications for three pesticides in cucumber for Turkey), residue data of EFSA (European Food Safety Authority) and registration (authorization) of them in Turkish - BKÜ Database. Of these, formetanate chloride is considered to be removed in Turkey and EU, due to environmental and toxicological risk (GKGM, 2020). The QuEChERS AOAC 2007.01 method (Lehotay et al., 2005) was used in the study. Verification of QuEChERS method was performed based on SANTE (EC Directorate-General for Health and Food Safety) guidelines (SANTE, 2019).

Materials and Methods

Reagents and chemicals

Standards of acetamiprid, chlorpyrifos, formetanate hydrochloride and pirimiphos methyl pesticides were supplied from a Dr. Ehrenstorfer Laboratories GmbH (Wesel,Germany) at purity of 98, 99, 99.2 and 97.6%, respectively. Some properties of insecticides are summarized in Table 1. $MgSO_4*7H_2O$, sodium acetate (NaAC), acetonitrile (ACN), toluene and methanol were supplied from Merck Company (Darmstadt, Germany) at purity of 99.0-100.5, 99.0, 99.9, 99.0 and 99.9%, respectively. Primary-secondary amine (PSA, 40 µM, 100 g) was sourced from Agilent (Santa Clara, CA, USA).

Table 1. Some properties of insecticides (WHO, 2009; EU, 2020; IRAC, 2020; PPDB, 2020)

Parameter		Acetamiprid	Chlorpyrifos	Formetanate hydrochloride	Pirimiphos methyl
Group		Neonicotinoid	Organophosphate	Formamidine	Organophosphate
Formula		$C_{10}H_{11}ClN_4$	$C_9H_{11}Cl_3NO_3PS$	$C_{11}H_{15}N_3O_3$	$C_{11}H_{20}N_3O_3PS$
Action mode		Systemic, Nicotinic acetylcholine receptor (nAChR)	Non-systemic, acetylcholinesterase (AChE) inhibitors, nerve action	Stomach action and contact, acetylcholinesterase (AChE) inhibitors, nerve action	Contact and respiratory action, acetylcholinesterase (AChE) inhibitors, nerve action
Physicochemical parameters	Boiling	Degrades before boiling	Degrades before boiling	Degrades before boiling	Degrades before boiling
	Solubility in water (mg/L)	2950	1.05	822000	11
	logP	0.8	4.7	-0.0014	4.2
	Degradation point (ºC)	200	170	204	162
	Molecular weight (g/mol)	222.67	350.58	257.8	305.33
Toxicological parameters	Acceptable daily intake, (mg/kg/bw/day)	0.025	0.001	0.004	0.004
	Acute reference dose (mg/kg/bw/day)	0.025	0.005	0.005	0.1
	Maximum permissible intake, (mg/person/day)	1.5	0.06	0.24	0.24
	Inhalation LC_{50} (Mammals) (mg/L)	> 1.15	0.1	0.15	> 4.7
	Dermal LD_{50} (Mammals) (mg/kg)	> 2000	> 1250	> 2000	> 2000
	Acute oral LD_{50} (Mammals) (mg/kg)	146	66	14.8	1414
	WHO classification [a]	II	II	Ib	II

[a] Ib, highly hazardous; II, moderately hazardous.

Instruments

Chromatographic analyses were performed with LC-MS/MS (Waters Acquity UPLC+Acquity TQD) equipped with BEH C_{18} column (1.7 µm, 2.1 mm x 100 mm). Injection volume, flow rate and total run time were 20 µL, 0.3 mL/min and 15 min, respectively. Desolvation gas flow, cone gas flow and collision gas flow were 600, 50 and 0.19 mL/min, respectively. A gradient program of 5 mM ammonium acetate plus 95% MeOH (B) and 5 mM ammonium acetate plus 5% MeOH in water (A) were used. Quasimolecular ions were 222.1 m/z for formetanate hydrochloride, 223.1 m/z as [M+H]+ for acetamiprid, 306.15 m/z for pirimiphos methyl and 349.9 m/z for chlorpyrifos. For quantification, reactions of 222.1/165.1 m/z, 223.1/126.1 m/z, 306.15/164.11 m/z and 349.9/96.9 m/z were monitored through a multiple reaction monitoring mode for formetanate hydrochloride, acetamiprid, pirimiphos methyl and chlorpyrifos, respectively. Similar values for confirmation were 222.1/93.0 m/z, 223.1/90.0 m/z, 306.15/108.05 and 349.9/197.9 m/z for formetanate hydrochloride, acetamiprid, pirimiphos methyl and chlorpyrifos, respectively.

Standard and fortification solutions

Stock solutions (400 µg/mL) of experimental pesticides were prepared. Then, 1.0 µg/mL of working solutions and calibration solutions with the range of 2-50 pg/µL were prepared in ACN for all active ingredients. Spiking solutions corresponding to 0.1, 1 and 10 x MRL were prepared. The standards and solutions were stored at 4ºC in dark. Representative apple matrix was used for matrix-matched calibrations (MC) and quantification, as indicated in Codex Alimentarius Commission Guidelines (CAC, 2003) and SANTE Guidelines (SANTE, 2019). Spiking level of 10 times MRL was diluted to fit calibration range.

Fortification trials and analyses

Despite the widespread use of the QuEChERS method in sophisticated laboratories, there is still a need for validation/verification for local conditions of your own laboratories. Recovery assessment is the first step of method validation evaluation (SANTE, 2019). For this aim, 1 kg of blank (pesticide-free sample, no pesticide applied sample) cucumber sample was homogenized with a blender. Then, 15 g homogenized sample spiked with 100 µL ACN was mixed with acetamiprid, chlorpyrifos, formetanate hydrochloride solution and pirimiphos methyl at 0.1, 1 and 10 x MRL levels in three replicates (analytical portion) (Table 2). Resultant mixture was vortexed for 30 s and left for pesticide interaction for 15 min.

Table 2. Fortification parameters for four insecticides

Fortification	Code	Level of fortification (µg/kg)			
		Acetamiprid	Chlorpyrifos	Formetanate hydrochloride	Pirimiphos methyl
0.1 x MRL*	F1/1-3	30	5	1	1
1x MRL	F2/1-3	300	50	10	10
10 x MRL	F3/1-3	3000	500	100	100
Control	F0/1-3	-	-	-	-

* EU MRL(µg/kg).

Analyses of all spiked and market samples were performed with the QuEChERS AOAC Method 2007.01 and LC-MS/MS (Lehotay, 2005). Schematic diagram of the method is illustrated in Figure 1. Three 200 µL extracts of each analytical portion were subjected to chromatographic analysis. The recovery was calculated with the use of Equation1.

$$\text{Recovery } \% = \frac{\text{Measured concentration}}{\text{Spiked concentration}} \text{x}100 \quad (1)$$

Method precision and recovery rates were tested in accordance with SANCO European Guidelines (SANTE, 2019). Method linearity was checked for the range of 2-50 pg/mL.

Collecting cucumber samples and analyses

Cucumbers were collected from six different stands of Çanakkale open markets for 6 weeks (1 sampling per week) between 23 November and 28 December 2018. About 2 kg samples were taken in each sampling. Samples were immediately brought to laboratory for analysis. About 1 kg chopped cucumber sample was well homogenized and 15 g analytical portion was taken in three triplicates. Further analytical procedure of the QuEChERS-AOAC method are illustrated in Figure 1. In total 108 analyses (6 stands x 6 weeks x 3 analytical portions) were performed.

Methodology for assessing dietary intake of insecticides

WHO Guidelines were used to assess dietary intake of pesticides (WHO, 1997). Acceptable daily intake (ADI) (mg/kg/b.w/day) and maximum permissible intake (MPI) (mg/person/day) values of insecticides are provided in Table 1. Theoretical maximum daily intake (TMDI) values were calculated as percentage of ADI. In Turkey, annual cucumber consumption per person is 18.5 kg (i.e., 51 g of cucumber per day) (TÜİK, 2019). Mean national theoretical maximum daily intake (NTMDI) and ADI% were calculated with the use of Equations 2 and 3, respectively. According to WHO guidelines, chronic exposure levels of pesticides that have values not exceeding 100% of ADI are low (WHO,1997).

$$\text{Mean NTMDI, mg/kg} = \text{Daily cucumber consumption, mg/kgXMean residue, mg/kg} \quad (2)$$

$$\text{ADI}\% = \frac{\text{Mean NTMDI}}{\text{MPI}} \quad (3)$$

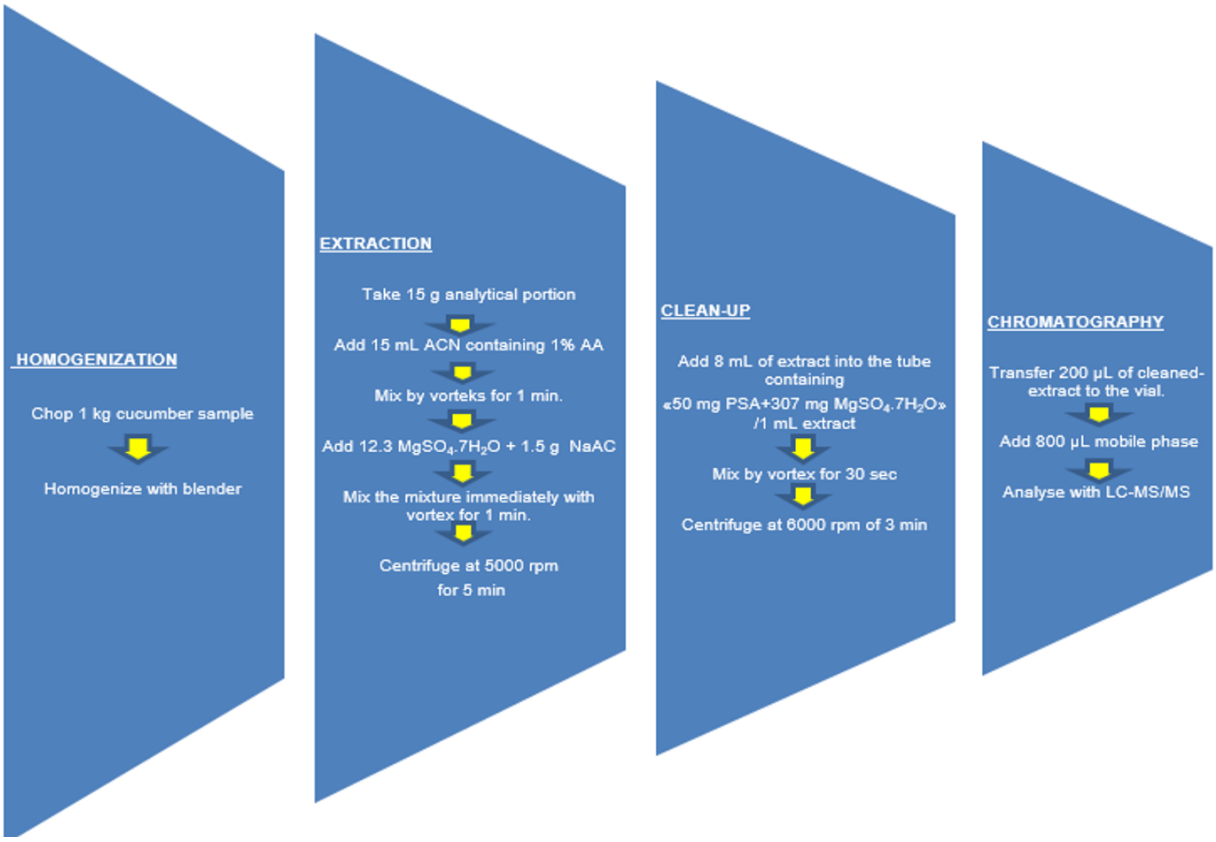

Figure 1. Schematic presentation of QuEChERS-AOAC method.

Results and Discussion

Method verification

Linearity

Calibration curves of experimental pesticides are presented in Figure 2. Resultant curves were linear within the range of 2-50 pg/µL ($R^2 \geq 0.999$). Regression equations are used as the analytical function of MC. The regression equation, as the analytical function of MC, was used for analyte quantification.

Repeatability of retention times

Retention time of pesticides (tR, min) should comply with the calibration standards with a ±0.1 min tolerance (SANTE, 2019). The repeatability of retention times for experimental pesticides was assessed through MC solutions of 2, 5, 10, 20 and 50 pg/µL. The retention time ranges were 10.18-10.19 min (with RSD of 0.05%), 2.85-2.86 min (with RSD of 0.19%) and 9.38-9.39 min (with RSD of 0.04%) for chlorpyrifos, formetanate hydrochloride and pirimiphos methyl, respectively. Acetamiprid tR was 4.91 min in all runs.

Limit of Quantification

Limit of quantification (LOQ) was identified as 2 µg/kg (less than MRL of 300 µg/kg) for acetamiprid, 10 µg/kg (less than MRL of 50 µg/kg) for chlorpyrifos, 5 µg/kg (below than MRL of 10 µg/kg) for formetanate hydrochloride and 1 µg/kg (below than MRL of 10 µg/kg) for pirimiphos methyl.

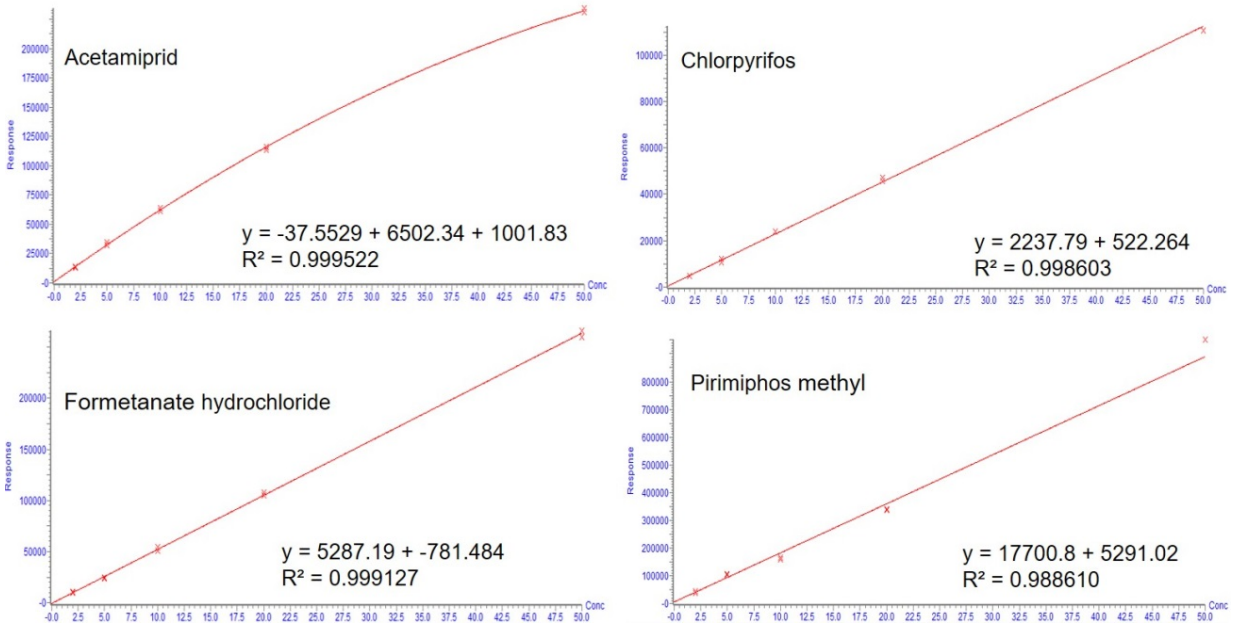

Figure 2. Calibration curves for four compounds in matrix-matched calibration.

Precision and accuracy

Method precision and trueness are generally assessed through repeatability (RSD%) and recovery (Q%) (SANTE, 2019; EURACHEM, 2014; TURKAK, 2019). Present recovery rates are provided in Table 3. Recovery rates of acetamiprid, chlorpyrifos, formetanate hydrochloride and pirimiphos methyl were 89.10% (RSD = 15.4%, n = 27), 84.1% (RSD = 15.8%, n = 18), 111% (RSD = 11.6%, n = 18) and 107% (RSD = 18.3%, n = 27), respectively. Mean recoveries varied between 84.1 and 111% (maximum RSD = 18.3%). The overall recovery rate was determined as 97.7% (RSD = 19.0%, n = 90). These figures were within the SANTE recovery limits (60% ≤ Q ≤ 140%) and the values specified for the repeatability (≤20%) for cucumber. The present findings on recovery rates also comply with the method verification parameters for pesticide residue analyses (SANTE, 2019; EURACHEM, 2014). In Hassanzadeh et al. (2012), mean recovery of imidacloprid in cucumbers was reported as 104%.

Accuracy is the closeness of the measured values to actual values (Tiryaki, 2016). Current accuracy values (as a tool for trueness) are provided in Table 3. Present findings revealed that QuEChERS yielded efficient recovery rates for experimental insecticides. Thus, it was thought that present analytical method may offer a rapid and accurate method for insecticide residue analysis in cucumbers.

Residues of cucumber samples

In this study, a total of 108 analytical portions, [36 samples (6-week x 6 stands designated as A to F) and three replicates] were analyzed. Evaluations were made for each insecticide on a weekly and stand basis. In cucumber samples, acetamiprid, chlorpyrifos, formetanate hydrochloride and pirimiphos methyl residues were detected. In addition, some traces of insecticide residues were encountered.

Table 3. QuEChERS-AOAC method verification

Active ingredient	Concentration (µg/kg)		Recovery %	RSD %
	Spiked	Measured[a]	(As a tool for trueness)	(As a tool for precision)
Acetamiprid	30	30.67	102.24	9.73
	300	271.10	90.37	9.81
	3000	2241.07	74.70	3.90
	Mean recovery, n=27		89.10	15.44
Chlorpyrifos	5	nd	-	-
	50	38.72	77.44	11.10
	500	454.20	90.84	15.62
	Mean recovery, n=18		84.14	15.81
Formetanate hydrochloride	1	nd[b]	-	-
	10	10.86	108.68	15.62
	100	113.68	113.67	6.10
	Mean recovery, n=18		111.18	11.55
Pirimiphos methyl	1	0.83	83.77	9.28
	10	11.17	111.68	3.09
	100	126.54	126.54	2.27
	Mean recovery, n=27		107.33	18.30
Whole recovery of the QuEChERS-AOAC (method accuracy): 97.71 % (n=90) RSD=19.01)				

[a] Mean of three analytical portions; [b] nd, not detected (below detection limit).

Acetamiprid

Acetamiprid (LOQ of 2 µg/kg) residues of 256.57 µg/kg and 235.93 µg/kg were detected in week 5 from stand B and week 2 from stand E, respectively. These two values were close to EU MRL of 300 µg/kg. Residues were 165 µg/kg in week 3 from stand E and 139 µg/kg in week 4 from stand D, which were well below the EU-MRL (Figure 3). The present samples all had acetamiprid residues below EU MRL of 300 µg/kg for cucumber. According to Türköz-Bakırcı et al. (2014), nine cucumber samples had acetamiprid residues below the LOQ. Kaya & Tuna (2019) found 10 µg/kg acetamiprid residues in cucumber samples.

Chlorpyrifos

LOQ and EU-MRL for chlorpyrifos were respectively identified as 10 and 50 µg/kg. Chlorpyrifos residues of all samples were below LOQ. Nasiri et al. (2016) found 97.13 µg/kg chlorpyrifos residues in six cucumber samples out of 43 greenhouse samples. In Türköz-Bakırcı et al. (2014), chlorpyrifos, residues exceeded MRL in one cucumber samples. In Kaya & Tuna (2019), chlorpyrifos residue in cucumber samples was identified as 33 µg/kg.

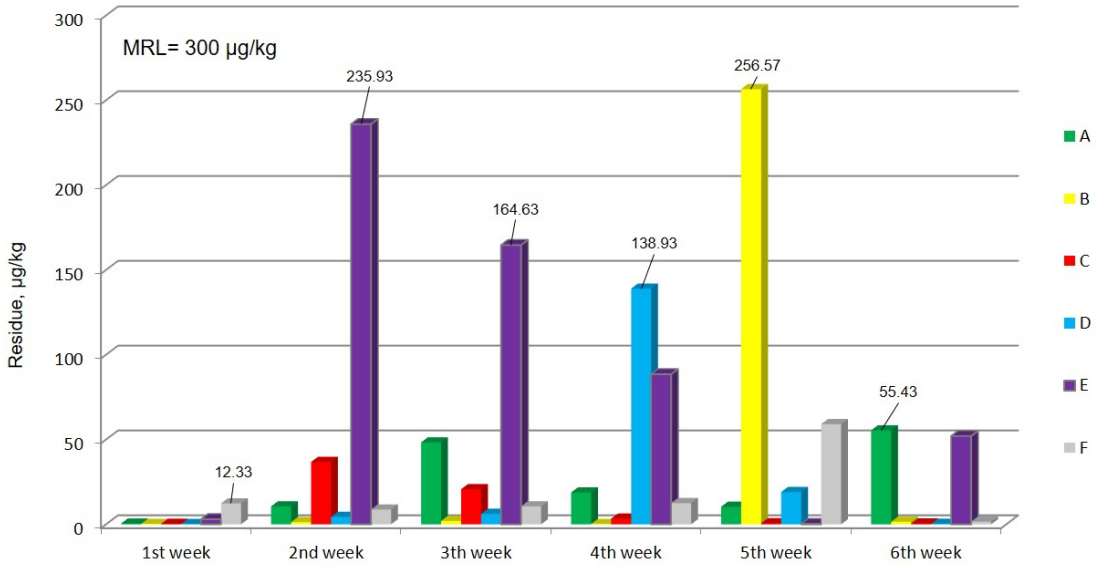

Figure 3. Acetamiprid residues in cucumbers based on week and stand.

Formetanate hydrochloride

LOQ and EU-MRL for formetanate hydrochloride were respectively identified as 5 and 10 µg/kg. In one sample (in week 3 from stand F), formetanate hydrochloride residue (36.3 µg/kg) was three times more than MRL. Residue of 11.5g, 11.4g, 10.7g and 10.1 µg/kg were found in week 3 from stand A, week 2 from stand C, week 4 from stand A and week 4 for from C, respectively. These values also slightly exceed the MRL (Figure 4). As shown in Figure 4, formetanate hydrochloride residues were not detected in week 5 and 6. This may indicate decreasing residues with increasing time after the harvest. Formetanate hydrochloride is considered to be banned insecticides in Turkey (GKGM, 2020). According to WHO classification, it is also highly hazardous (Class Ib) substance (WHO, 2009).

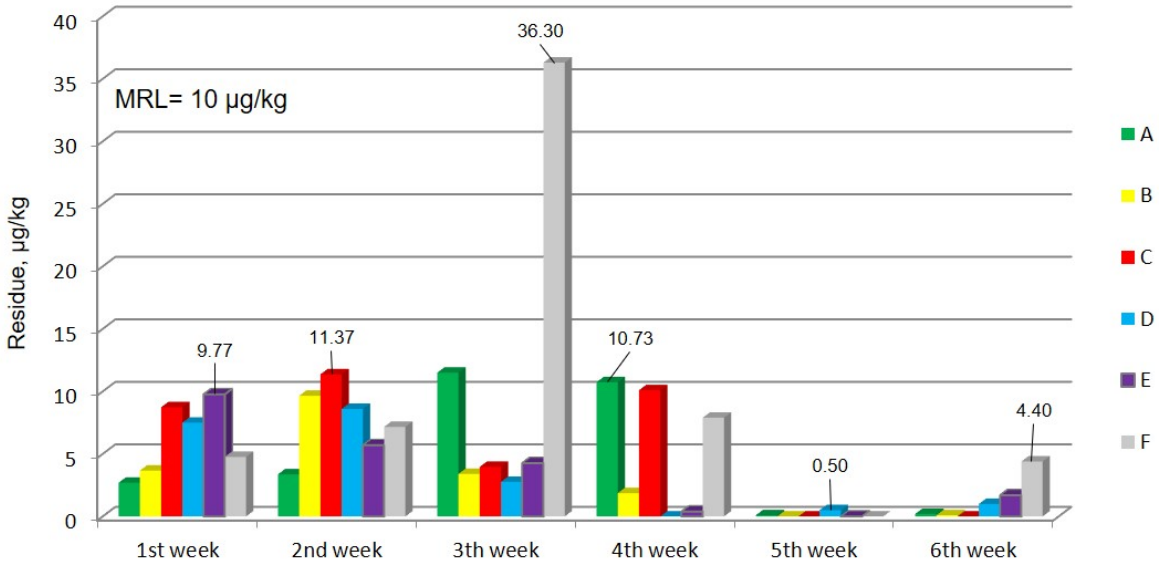

Figure 4. Formetanate hydrochloride residues in cucumbers based on week and stand.

Pirimiphos methyl

LOQ and EU-MRL for pirimiphos methyl were 1 and 10 µg/kg, respectively. Residues of pirimiphos methyl were neither exceeding MRL nor close to LOQ.

In addition to these four insecticides, 9 µg/kg of oxadixyl residues (LOQ = µg/kg) were detected in week 1 from stand F. This value is close to the MRL of 10 µg/kg.

Hassanzade et al. (2012) reported recovery rates for imidacloprid in 21 d respectively as 94.5% and 99.2% at single and double doses. Residue levels decreased below MRL of 1 mg/kg in 3 d. In a residue monitoring project conducted between 1996 and 2000 in Turkey, about 1000 vegetable and fruit samples were studied. Insecticide residue levels were below MRL in 45 greenhouse cucumber samples (Anonymous, 2002). Islam et al. (2015) investigated pesticide residues on cucumber samples from local markets and detected Mancozeb residue (about 50 ppm) in one out of three samples. Kaya & Tuna (2019) investigated pesticide residues in cucumbers and detected thiamethoxam residues as 0.025 mg/kg. The EU-MRL of the pesticide was 0.5 mg/kg.

Risk assessment for dietary intake of insecticides

Acetamiprid

Acetamiprid was the most abundant residue in present cucumber samples. Acetamiprid residue levels varied between 2.03 and 257 µg/kg. Overall mean residue of acetamiprid was 53.3 µg/kg. Risk assessments were made over 24 residues. Mean NTMDI was calculated as 2.7 µg/day (Equation 2). Average NTMDI, as a percentage of ADI, was calculated as 0.18% (Equation 3). Gölge & Kabak (2015) reported daily acetamiprid intake with tomato as 0.04 µg/kg/b.w. Chronic exposure level of this insecticide is low, since acetamiprid has a value not exceeding 100% of the ADI (WHO, 1997).

Chlorpyrifos

Risk assessment was not made for chlorpyrifos since no residue (more than LOQ) was detected in any of the cucumber samples.

Formetanate hydrochloride

Formetanate hydrochloride levels varied between 4.4 and 36.3 µg/kg. Overall mean residue of formetanate hydrochloride was 0.01115 mg/kg. Risk assessments were made over 13 residue data. Mean NTMDI was calculated as 0.00056 mg/day. Average NTMDI, as a percentage of ADI, was calculated as 0.233. Chronic exposure level of this insecticide is low, since formetanate HCl has a value less than 100% of the ADI (WHO, 1997).

Pirimiphos methyl

Since neither exceeding MRL nor close to LOQ, residues were not detected in any cucumber samples, therefore, risk assessments were not made for pirimiphos methyl.

Conclusion

Agrochemicals have a significant role in improving agricultural production and reducing labor inputs for pest control. Pesticides may prevent yield losses to some extent, but exert serious risks on human health and environment. The current work was conducted to investigate some insecticide residues in cucumbers, sampled from open markets of Çanakkale Province of Turkey. In the study, the required method validation criteria were met. The QuEChERS method was successfully used in acetamiprid, chlorpyrifos, formetanate hydrochloride and pirimiphos methyl residue analyses in cucumbers. None of the cucumbers sampled from Çanakkale open markets contained residues of acetamiprid, chlorpyrifos and

pirimiphos methyl exceeding their MRLs. It was concluded based on present findings that consumption of cucumbers in Çanakkale Province did not pose a risk to human health, except formetanate hydrochloride. In one sample, formetanate hydrochloride was 3 times greater than the MRL. It can also be concluded that the absence of formetanate hydrochloride residue in any samples of week 5 and 6 emphasized the importance of PHI. Cucumber should be sampled at different PHI from the same field. It was concluded based on present data that there was no risk for cucumber consumption in terms of four insecticides. However, it is important to work with large data in order to evaluate risk of exposure in such studies.

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Original article (Orijinal araştırma)

Additional and new records of some Oxytelinae (Coleoptera: Staphylinidae) from Turkey with notes on habitat associations

Türkiye'den bazı Oxytelinae (Coleoptera: Staphylinidae) türlerinin habitat tercihlerine ait notlar ile ek ve yeni kayıtlar

Derya ÇIFTÇİ[1*]

Abstract

In this study, the specimens collected between 2011-2015 belonging *Anotylus* Thomson, C.G., 1859, *Oxytelus* Gravenhorst, 1806 and *Platystethus* Mannerheim, 1830 (Staphylinidae: Oxytelinae) were evaluated in terms of habitats and distributions. New and additional distribution data for 16 species are reported from various provinces of Turkey. Among them, *Anotylus rugifrons* (Hochhuth, 1849), *Anotylus sexualis* (Eppelsheim, 1892) and *Oxytelus pseudopiceus* Kashcheev, 1999 are new records for Turkey. For several other species, the author provides new provincial records. Distributions of 16 species in Turkey are mapped. Photographs of habitus, male sternites VII-VIII and aedeagus of *A. sexualis*, and habitus and male sternite VII of *O. pseudopiceus* are provided. EUNIS habitat types where the species were collected were mentioned. The highest number of species (9 species) was found in E3.4 followed by G1.7 (6 species) and G3.5 (5 species). Most of species were taken from cow dung. Habitat and environment information of species listed in present study were compared and evaluated with the literature. In addition, it is thought that nutritional requirements are at the main determinant in the habitat associations of the species.

Keywords: Anatolia, *Anotylus*, EUNIS habitats, *Oxytelus*, *Platystethus*

Öz

Bu çalışmada, 2011-2015 yılları arası toplanan *Anotylus* Thomson, C.G., 1859*, Oxytelus* Gravenhorst, 1806 ve *Platystethus* Mannerheim, 1830 (Staphylinidae: Oxytelinae) cinslerine ait örnekler bulundukları habitatlar ve dağılışları açısından değerlendirilmiştir. 16 tür için yeni ve ek dağılış kayıtları Türkiye'nin çeşitli illerinden kaydedildi. Bunlar arasında, *Anotylus rugifrons* (Hochhuth, 1849), *Anotylus sexualis* (Eppelsheim, 1892) ve *Oxytelus pseudopiceus* Kashcheev, 1999 Türkiye için yeni kayıttır. Diğer birçok tür için, yazar yeni il kayıtları sağlamıştır. 16 türün Türkiye dağılışı harita ile gösterilmiştir. *Anotylus sexualis* türünün habitus, erkek VII-VIII. sternitleri ve aedeagusunun ile *O. pseudopiceus* türünün habitus ve erkek VII. sternit fotoğrafları verilmiştir. Türlerin toplandığı EUNIS habitat tiplerinden bahsedilmiştir. En fazla tür sayısı (9 tür) E3.4 habitatında bulunmuş, bunu G1.7 (6 tür) ve G3.5 (5 tür) habitatları takip etmiştir. Çoğu tür inek dışkısından alınmıştır. Bu çalışmada listelenen türlerin habitat ve ortam bilgileri literatürdeki bilgiler ile karşılaştırılmış ve değerlendirilmiştir. Ek olarak, besin tercihinin türlerin habitat tercihlerinde temel belirleyici olduğu düşünülmektedir.

Anahtar sözcükler: Anadolu, *Anotylus*, EUNIS habitatlar, *Oxytelus*, *Platystethus*

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Introduction

Species of the subfamily Oxytelinae (Coleoptera: Staphylinidae) are distributed worldwide (not all genera) and contain more 2000 described taxa (Schülke, 2012a; Schülke & Smetana, 2015). This subfamily is characterized by transverse, more or less rectangular flattened head with the antennae fixed to the sides of the head under a raised edge or tubercle. Several genera have visible abdominal tergite II at the basal of the abdomen. Another characteristic structure is a tergite IX divided by tergite X into two parts (Lott, 2009; Schülke, 2012a).

Species of Oxytelinae are observed in two different habitat types: moss, sand and gravel on banks of water bodies; and dung (e.g., herbivorous and chickens) or decaying plant (Makranczy, 2006; Schülke, 2012a). Some species of *Bledius* Leach, 1819 and *Thinobius* Kiesenwetter, 1844 feed on algae and other organic remarks and inhabit waterside mud or sand (Hammond, 1976). *Anotylus* Thomson, 1859, *Oxytelus* Gravenhorst, 1806 and some *Platystethus* Mannerheim, 1830 are found in litter, dung or decaying material in temperate regions (Hammond, 1976). In the literature, it is reported that some Oxytelinae species were found in places such as dung, organic material, and habitats such as stream edge and forest (Hammond, 1976; Makranczy, 2006; Schülke, 2012a). There is no study that gives habitats of Oxytelinae species as EUNIS habitat type.

With the studies in the last decade, several species have been added to Turkish Oxytelinae fauna and the distribution areas of some species have been expanded. These studies are not particularly concerned with determination of the subfamily species. These are generally the determination of general Turkish Staphylinidae fauna (Assing, 2009, 2010, 2011, 2013, 2014, 2016; Kesdek et al., 2009; Özgen et al., 2010; Özgen & Anlaş, 2016), examination of museums or collections specimens (Anlaş & Rose, 2009; Özgen, 2011; Schülke, 2012b), composition of dung fauna (Özgen & Anlaş, 2010; Anlaş et al., 2014; Tezcan et al., 2019) and pitfall or light trap studies (Tezcan & Anlaş, 2009; Japoshvili & Anlaş, 2011). According to the checklist of Anlaş (2009) while the number of recorded Oxytelinae taxa was 94 but this number has now increased to 112 taxa.

Nevertheless, the fauna of Oxytelinae of many Turkish provinces has been insufficiently studied. The aim of this study was to enhance knowledge on the distributions and habitats of species of three genera of Oxytelinae in Turkey.

Materials and Methods

The studied materials were deposited in Zoological Museum of Gazi University (ZMGU), Ankara, Turkey. For this study, 258 specimens of oxytelines belonging *Anotylus*, *Oxytelus* and *Platystethus* were examined. Specimens were collected in different parts of Turkey in 2011-2015.

A list of Turkish provinces with positive records for each species is provided together with sources. Reliable localities are shown as points on the distribution maps for all taxa. The points of the records without exact locality information are shown near the province, district or village. Distributions of species are mapped in Arc Map 10. Photographs were taken with a Canon EOS M camera.

If EUNIS habitat information of the localities where the specimens were collected and environment (e.g., under the stones and from cow dung) in which they were collected are known, it is written after the locality information. Habitat information of old records and records without exact locality information are unknown. EUNIS habitat type of each locality was determined using Davies et al. (2004). Specimens were collected from eleven EUNIS habitats. The codes and names of these EUNIS habitats are: E1.2E, Irano-Anatolian steppes; E3.4, moist or wet eutrophic and mesotrophic grassland; E4.4, calcareous alpine and subalpine grassland; F5.3, pseudomaquis; G1, Broadleaved deciduous woodland; G1.3, Mediterranean riparian woodland; G1.7, thermophilus deciduous woodland; G2.1, Mediterranean evergreen *Quercus*

woodland; G3.5, *Pinus nigra* woodland; G3.9, coniferous woodland dominated by Cupressaceae or Taxaceae; and G4.B, mixed Mediterranean *Pinus*-thermophilus *Quercus* woodland. For a detailed description of habitats see Davies et al. (2004).

Results

Anotylus clypeonitens (Pandellé, 1867) (Figure 1)

Material examined. Batman: Hasankeyf, Irmak Village, 37º43'32.28"N, 41º31'12.40"E, 476 m, 17.05.2015, ♂, moist or wet eutrophic and mesotrophic grassland, under stone, leg. D. Çiftçi; Eskişehir: Mihalıççık, N of Yalımkaya, 39º59'30"N, 31º15'29"E, 1210 m, 13.07.2012, ♂, thermophilus deciduous woodland, from cow dung, leg. D. Çiftçi; Sarıcakaya, Alapınar, 4 km along Alapınar-Laçin Road, 40º02'29.94"N, 30º49'49.32"E, 1035 m, 29.06.2012, ♂, mixed Mediterranean *Pinus*-thermophilus *Quercus* woodland, from cow dung, leg. D. Çiftçi; Osmaniye: Kadirli, 37º29'40.63"N, 36º05'49.04"E, 269 m, 26.04.2015, 2♂♂, Mediterranean riparian woodland, leg. D. Çiftçi.

Distribution in Turkey. Ankara, Antalya, Balıkesir, Kocaeli, Sakarya and Sinop (Horion, 1963; Smetana, 1967; Coiffait, 1978; Herman, 2001; Anlaş & Rose, 2009; Assing, 2013).

Remarks. This species is reported here from Eskişehir, Osmaniye and Batman Provinces for the first time. The specimens were collected from various EUNIS habitats; mixed Mediterranean *Pinus*-thermophilus *Quercus* woodland, thermophilus deciduous woodland, Mediterranean riparian woodland, moist or wet eutrophic and mesotrophic grassland. According to published data, *A. clypeonitens* is found on rotting plant substances, especially in compost heaps (Lott, 2009; Schülke, 2012a). In this study, the species was found in cow dung and under stones.

Figure 1. Distribution of *Anotylus clypeonitens* in Turkey.

Anotylus complanatus (Erichson, 1839) (Figure 2)

Material examined. Batman: Hasankeyf, Irmak Village, 37º43'32.28"N, 41º31'12.40"E, 476 m, 17.05.2015, ♂, moist or wet eutrophic and mesotrophic grassland, under stone, leg. D. Çiftçi; Mersin: Silifke, S of Imamlı Village, 493 m, 36º26'50.11"N, 34º00'31.39"E, 490 m, 21.04.2015, ♂, thermophilus deciduous woodland, leg. D. Çiftçi; Muğla: Marmaris, SE of Bozburun Village, 18 m, 36º40'33.73"N, 28º04'28.58"E, 16.04.2015, 2♂♂, thermophilus deciduous woodland, leg. D. Çiftçi; Marmaris, W of Taşlıca Village, 261 m, 36º37'54.76"N, 28º05'42.07"E, 16.04.2015, ♂, thermophilus deciduous woodland, leg. D. Çiftçi; Milas, SE of Kultak Village, 1 m, 37º01'58.39"N, 28°05'52.79"E, 14.04.2015, ♂, thermophilus deciduous woodland, leg. D. Çiftçi.

Distribution in Turkey. Antalya, Istanbul and Karaman (Apfelbeck, 1901; Scheerpeltz, 1958, 1962; Horion, 1963; Anlaş, 2009; Schülke, 2009).

Remarks. This species is here reported from southeastern Anatolia for the first time. The specimens collected from thermophilus deciduous woodland habitat and moist or wet eutrophic and mesotrophic

grassland habitat. According the published data (Hammond, 1976; Lott, 2009; Schülke, 2012a), the species was found in all kinds of organic materials (dung and litter). In this study, one specimen was taken from under stone in wet meadow, habitats of other specimens are unknown.

Figure 2. Distribution of *Anotylus complanatus* in Turkey.

***Anotylus fairmairei* (Pandellé, 1867) (Figure 3)**

Material examined. Erzincan: Refahiye, SE of Yurtbaşı Village, 39º53'46.87"N, 38º56'42.28"E, 1728 m, 21.06.2011, ♀, moist or wet eutrophic and mesotrophic grassland, from cow dung, leg. D. Çiftçi; Erzurum: Aşkale, N of Yeniköy Village, 39º51'53.98"N, 40º42'11.52"E, 2065 m, 25.06.2011, ♂, calcareous alpine and subalpine grassland, from cow dung, leg. D. Çiftçi.

Distribution in Turkey. Trabzon (Assing, 2009).

Remarks. The species had been reported from Trabzon Province. The specimens from Erzincan and Erzurum represent the third record from Turkey. In this study, it was collected from calcareous alpine and subalpine grassland habitat and moist or wet eutrophic and mesotrophic grassland habitat. *Anotylus fairmairei* is widespread and rare species in Central Europe, and recorded for all kinds of organic materials (dung and litter) (Lott, 2009; Schülke, 2012a). The species has only been found in several locations in east and north east part of Turkey (Figure 3). In the present study, specimens were collected from cow dung in different habitats.

Figure 3. Distribution of *Anotylus fairmairei* in Turkey.

***Anotylus intricatus* (Erichson, 1840) (Figure 4)**

Material examined. Eskişehir: Tepebaşı, exit of Hekimdağ, 39º54'48"N, 30º34'09"E, 1241 m, 23.08.2011, ♂, moist or wet eutrophic and mesotrophic grassland, from cow dung, leg. D. Çiftçi.

Distribution in Turkey. Adana, Balıkesir and Izmir (Fauvel, 1872; Scheerpeltz, 1962; Horion, 1963; Smetana, 1967; Herman, 2001).

Remarks. The specimen from Eskişehir represents a new provincial record. *Anotylus intricatus*, widespread in Central Europe, but rare everywhere and can be found in all kind of decayed remains

(Schülke, 2012a). The species is recorded from several provinces of Turkey (Figure 4). In the present study, the species collected from moist or wet eutrophic and mesotrophic grassland habitat in cow dung.

Figure 4. Distribution of *Anotylus intricatus* in Turkey.

***Anotylus inustus* (Gravenhorst, 1806) (Figure 5)**

Material examined. Ankara: Nallıhan, between Kavakköy-Osmanköy, 40º04'16.00"N, 30º58'58.00"E, 835 m, 29.04.2012, 3♀♀, thermophilous deciduous woodland, leg. D. Çiftçi; Nallıhan, between Kuzucular-Tekirler, 40º06'38.89"N, 30º54'19.48"E, 375 m, 29.04.2012, 12♂♂, 15♀♀, Irano-Anatolian steppes, leg. D. Çiftçi; Nallıhan, Osmanköy Village, 40º04'09.29"N,30º54'49.70"E, 570 m, 15.06.2012, ♂, *Pinus nigra* woodland, leg. D. Çiftçi; Nallıhan, turnout Yenice-Düzköy-Kuzucular road, 40º05'0.76"N, 30º51'36.78"E, 260 m, 28.04.2012, 54♂♂, 63♀♀, Irano-Anatolian steppes, from chicken feces, leg. D. Çiftçi; Antalya: Alanya, Çayarası, 36º'28.56"N, 32º24'08.88"E, 1110m, 14.05.2011, ♂, leg. D. Çiftçi; Aydın: Çine, SE of Alabayır Village, 37º31'37.78"N, 28º11'01.96"E, 642 m, 13.04.2015, ♂, leg. D. Çiftçi; Bilecik: Söğüt, Akçasu, 40º05'15.97"N, 30º18'30.34"E, 185 m, 29.04.2012, 2♂♂, Irano-Anatolian steppes, from cow dung, leg. D. Çiftçi; Çanakkale: Gelibolu, SW of Evreşe, 40º38'35.08"N, 26º52'03.40"E, 7 m, 02.05.2013, ♂, moist or wet eutrophic and mesotrophic grassland, leg. D. Çiftçi; Eskişehir: Sarıcakaya, 4 km along Alapınar-Laçin Road, 40º02'29.94"N, 30º49'49.32"E, 1035m, 22.09.2011, ♂, mixed Mediterranean *Pinus*-thermophilus *Quercus* woodland, leg. D. Çiftçi; Sarıcakaya, Beyköy, 40º05'17.28"N, 30º45'55.85"E, 250 m, 29.04.2012, ♀, Irano-Anatolian steppes, leg. D. Çiftçi; Sarıcakaya, between Beyköy-Kapıkaya, 40º03'41.92"N, 30º43'40.36"E, 230 m, 29.04.2012, 4♂♂, 2♀♀, Irano-Anatolian steppes, under stones, leg. D. Çiftçi; Sarıcakaya, Düzköy, 40º05'18.41"N, 30º50'20.38"E, 250 m, 29.04.2012, 2♂♂, Irano-Anatolian steppes, leg. D. Çiftçi; Tepebaşı, Yarımca Village, 39º55'15.00"N, 30º40'17.76"E, 1311 m, 29.04.2012, 2♂♂, *Pinus nigra* woodland, leg. D. Çiftçi; Kırklareli: Center, NE of Erikler Village, 41º52'58.09"N, 27º09'22.20"E, 435 m, 01.05.2013, ♂, thermophilus deciduous woodland, leg. D. Çiftçi; Center, W of Ahmetçe Village, 41º48'00.30"N, 27º10'29.46"E, 300 m, 01.05.2013, 3♂♂, ♀, pseudomaquis, leg. D. Çiftçi; Kütahya: Tavşanlı, S of Eşen Village, 39º42'47.35"N, 29º18'58.83"E, 1087 m, 16.05.2013, ♀, coniferous woodland dominated by Cupressaceae or Taxaceae, leg. D. Çiftçi; Mersin: Gülnar, SW of Ilısu Village, 36º32'30.50"N, 33º03'24.28"E, 805 m, 21.04.2015, ♂, leg. D. Çiftçi; Silifke, S of Imamlı Village, 36º26'50.11"N, 34º00'31.39"E, 490 m, 21.04.2015, ♂, leg. D. Çiftçi; Muğla: Bodrum, E of Dağbelen Village, 37º05'00.19"N, 27º21'54.57"E, 360 m, 12.04.2015, 8♂♂, 3♀♀, leg. D. Çiftçi; Fethiye, E of Korubükü Village, 36º28'03.85"N, 29º24'08.10"E, 185 m, 25.03.2015, ♀, leg. D. Çiftçi; Fethiye, Kumlu Plain, 36º18'47.47"N, 29º16'38.19" E 4 m, 25.03.2015, 54♂♂, 59♀♀, leg. D. Çiftçi; Marmaris, W of Taşlıca Village, 36º37'54.76"N, 28º05'42.07"E, 261 m, 16.04.2015, 2♂♂, ♀, leg. D. Çiftçi; Center, SW of Kuyucak Village, 37º03'31.95"N, 28º16'09.98"E, 478 m, 14.04.2015, ♂, leg. D. Çiftçi; Yatağan, NW of Çakırlar Village, 37º27'43.11"N, 28º08'44.82"E, 370 m, 13.04.2015, 2♂♂, leg. D. Çiftçi; Siirt: Center, Köprübaşı Village, near of Kezer Stream, 37º57'43.24"N, 41º51'26.23"E, 03.04.2014, 24♂♂, 10♀♀, leg. D. Çiftçi; Center, Kılıçlı Village, 38º00'56.83"N, 41º46'34.74"E, 546 m, 14.03.2015, 2♂♂, 2♀♀, leg. D. Çiftçi; Center, Siirt University Kezer Campus, 37º58'23.03"N, 41º51'05.32" E 11.03.2015, 2♂♂, leg. D. Çiftçi; Tekirdağ: Malkara, SW of Çimendere

Village, 40º46'23.82"N, 27º01'01.52"E, 185 m, 02.05.2013, ♂, Mediterranean evergreen *Quercus* woodland, leg. D. Çiftçi.

Distribution in Turkey. Adana, Artvin, Batman, Bursa, Diyarbakır, Erzincan, Erzurum, Isparta Istanbul, Izmir, Karaman, Kilis, Kocaeli, Konya, Manisa, Mardin, Mersin, Muğla and Siirt (Peyron, 1858; Fauvel, 1872; Apfelbeck, 1901; Ganglbauer, 1905; Scheerpeltz, 1958; Horion, 1963; Smetana, 1967; Herman, 2001; Anlaş, 2009; Anlaş & Rose, 2009; Kesdek et al., 2009; Tezcan & Anlaş, 2009; Özgen et al., 2010; Özgen & Anlaş, 2010; Japoshvili & Anlaş, 2011; Assing, 2013; Anlaş et al., 2014; Özgen & Anlaş, 2016; Tanyeri et al., 2017; Tezcan et al., 2019).

Remarks. This species is one of the most common and widespread species of *Anotylus* in Turkey. The species is recorded from several provinces of Turkey for the first time: Ankara, Antalya, Aydın, Bilecik, Çanakkale, Eskişehir, Kırklareli, Kütahya and Tekirdağ. *Anotylus inustus* was collected from wide variety of habitats (see material examined) in chicken feces, cow dung and under stones.

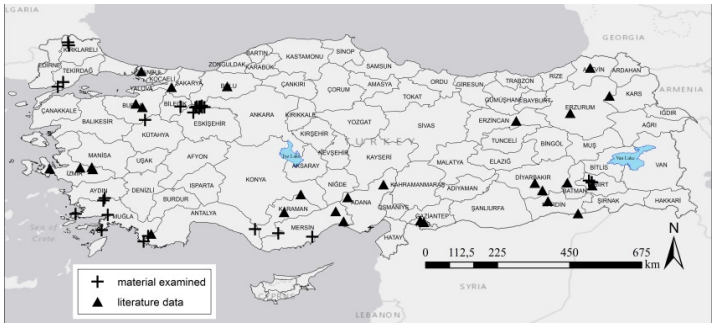

Figure 5. Distribution of *Anotylus inustus* in Turkey.

***Anotylus pumilus* (Erichson, 1839) (Figure 6)**

Material examined. Kars: Selim, E of Katranlı Village, 40º28'01.44"N, 42º43'18.10"E, 1910 m, 16.07.2011, ♂, Irano-Anatolian steppes, leg. D. Çiftçi.

Distribution in Turkey. Afyon and Ankara (Scheerpeltz, 1962; Horion, 1963; Smetana, 1967; Herman, 2001; Assing, 2013).

Remarks. The material from Kars represents the third record from Turkey fauna. This species was found in dry dung (Schülke, 2012a). Similarly, in this study, the specimen collected in Irano-Anatolian steppes in dry cow dung.

Figure 6. Distribution of *Anotylus pumilus* in Turkey.

***Anotylus rugifrons* (Hochhuth, 1849) (Figure 7)**

Material examined. Eskişehir: Tepebaşı, Uludere, 39º55'58.00"N, 30º21'17.00"E, 1240 m, 29.04.2012, 2♂♂, ♀, Irano-Anatolian steppes, under stones, leg. D. Çiftçi; Erzurum: Pasinler, N of Tepecik

Village, 39º57'33.94"N, 41º46'35.83"E, 1624 m, 14.07.2011, ♀, moist or wet eutrophic and mesotrophic grassland, leg. D. Çiftçi; Kars: Center, SE of Boğatepe Village, 40º47'34.71"N, 42º54'47.82"E, 2253 m, 17.07.2011, ♂, calcareous alpine and subalpine grassland, leg. D. Çiftçi.

Remarks. This species is widespread in Europe, but rare everywhere (Schülke, 2012a; Schülke & Smetana, 2015). It is here reported from Turkey for the first time. Similar to the published data (Schülke, 2012a), the species was found in wet meadows and river bank. Also, in this study, it was collected from under the stone in the Iranian-Anatolian steppes.

Figure 7. Distribution of *Anotylus rugifrons* in Turkey.

***Anotylus rugosus* (Fabricius, 1775) (Figure 8)**

Material examined. Eskişehir: Sarıcakaya, 5 km along Laçin-Alapınar Road, 40º01'48.00"N, 30º48'08.64"E, 580 m, 16.06.2012, ♂, *Pinus nigra* woodland, light trap, leg. D. Çiftçi.

Distribution in Turkey. Adana, Ankara, Bingöl, Erzurum, Karaman and Mersin (Peyron, 1858; Fauvel, 1871; Smetana, 1967; Özgen & Anlaş, 2010; Schülke, 2012b).

Remarks. The specimens represent new record from Eskişehir. It is one of the most common rove beetles in Central Europe and Palearctic Region (Schülke, 2012a; Schülke & Smetana, 2015). It seems to be common in Turkey, but in this study only one specimen was recorded. *Anotylus rugosus* can be found in soil litter and on all kind of decayed remains (Lott, 2009; Schülke, 2012a). In this study, the species was recorded at light trap in the *Pinus nigra* woodland, as in Lane & Mann (2006).

Figure 8. Distribution of *Anotylus rugosus* in Turkey.

***Anotylus sculpturatus* (Gravenhorst, 1806) (Figure 9)**

Material examined. Ankara: Nallıhan, between Kavakköy-Osmanköy Villages, 40º04'16.68"N, 30º58'57.00"E, 835 m, 29.04.2012, ♂, thermophilus deciduous woodland, leg. D. Çiftçi; Bilecik: Inhisar, Akkum Village, 40º05'16.59"N, 30º24'08.59"E, 325 m, 29.04.2012, ♀, orchard, under stone, leg. D. Çiftçi; Söğüt, Akçasu, 40º05'15.97"N, 30º18'30.34"E, 185 m, 29.04.2012, ♂, 2♀♀, *Pinus nigra* woodland, from cow dung, leg. D. Çiftçi; Eskişehir: Mihalıççık, 7 km along Gürleyik-Yalımkaya Road, 39º59'N, 31º18'E,

1135 m, 22.09.2011, ♀, thermophilus deciduous woodland, from cow dung, leg. D. Çiftçi; Sarıcakaya, 4 km along Alapınar-Laçin Road, 39º56'03.18"N, 31º03'20.70"E, 1035 m, 22.09.2011, 6♂♂, mixed Mediterranean *Pinus*-thermophilus *Quercus w*oodland, leg. D. Çiftçi; Sarıcakaya, 4 km along Alapınar-Laçin Road, 40º02'N, 30º49'E, 1035 m, 02.12.2011, ♂, mixed Mediterranean *Pinus*-thermophilus *Quercus w*oodland, leg. D. Çiftçi; Tebebaşı, Tandır Village, 39º55'N, 30º40'E, 1312 m, 29.04.2012, ♂, ♀, *Pinus nigra* woodland, leg. D. Çiftçi; Muğla: Fethiye, Ölüdeniz, Baba Mountain, 36º33'30.35"N, 29º10'34.23"E, 900m, 05.05.2012, ♀, light trap, leg. D. Çiftçi; Marmaris, SE of Bozburun Village, 36º40'33.73"N, 28º04'28.58"E, 18 m, 16.04.2015, 3♂♂, leg. D. Çiftçi; Marmaris, W of Taşlıca Village, 36º37'54.76"N, 28º05'42.07"E, 261 m, 16.04.2015, ♂, leg. D. Çiftçi.

Distribution in Turkey. Antalya, Bolu, Düzce, Elazığ, Istanbul, Kastamonu, Manisa, Mardin, Mersin and Siirt (Peyron, 1858; Herman, 2001; Özgen & Anlaş, 2010; Anlaş & Rose, 2009; Assing, 2013; Assing, 2014; Anlaş et al., 2014).

Remarks. The species is new record all provinces listed above for the first time. It is one of the most common species of the genus in Central Europe and Palearctic Region (Schülke, 2012a; Schülke & Smetana, 2015). It is also common in Turkey. According to published data (Lott, 2009; Schülke, 2012a), the specimens were taken from cow dung. The specimens recorded from Turkey mostly collected from forest habitats (see material examined).

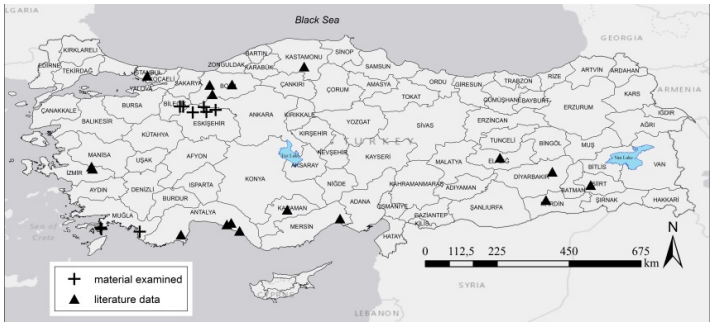

Figure 9. Distribution of *Anotylus sculpturatus* in Turkey.

***Anotylus sexualis* (Eppelsheim, 1892) (Figures 10 & 11)**

Material examined. Eskişehir: Beylikova, 3.5 km along Doğanoğlu-Bozan Road, 39º49'N, 31º10'E, 862 m, 30.04.2012, ♂, Mediterranean riparian woodland, from cow dung, leg. D. Çiftçi.

Figure 10. Distribution of *Anotylus sexualis* in Turkey.

Remarks. The original description of *Oxytelus sexualis* is based on a holotype from Tashkent, Uzbekistan (Eppelsheim, 1892). Scheerpeltz (1962, 1963) provided morphological characters of *Oxytelus* (*Anotylus*) *sexualis* in his identification key and described distribution area of this species: “Transcaspian region, Turkestan, Afghanistan, Iran”. Herman (1970) considered this species as *Anotylus sexualis*

(Eppelsheim, 1892) (Herman, 1970). This species is known from Afghanistan, Iran, Uzbekistan (Schülke & Smetana, 2015). *Anotylus sexualis* is a new record for the fauna of Turkey. The published data on habitat or nutritional associations of this species are missing. Apparently, they are similar to other species of the genus. In this study, it was collected from cow dung in moist or wet eutrophic and mesotrophic grassland habitat.

Figure 11. *Anotylus sexualis*: a) forebody (Scale: 0.5 mm), b) ♂ sternite VII, c) ♂ sternite VIII, d) aedeagus ventral view, e) aedeagus lateral view (Scales: 0.1 mm).

***Oxytelus laqueatus* (Marsham, 1802) (Figure 12)**

Material examined. Artvin: Borçka, Uğurköy, 41º28'47.29"N, 42º00'18.12"E, 22.07.2014, ♂, near broadleaved deciduous woodland, leg. D. Çiftçi.

Distribution in Turkey. Kastamonu and Rize (Assing, 2007, 2011).

Remarks. The material from Artvin represents third record from Turkey. It is a widespread species in the world (Schülke & Smetana, 2015). The species was found in all kind of decayed or rotting organic matter (Lott, 2009; Schülke, 2012a). In this study, the species is collected from broadleaved deciduous woodland.

Figure 12. Distribution of *Oxytelus laqueatus* in Turkey.

***Oxytelus piceus* (Linnaeus, 1767) (Figure 13)**

Material examined. Eskişehir: Alpu, 3 km along Alapınar-Taycılar Road, 40º00'N, 30º50'E, 1110 m, 22.08.2011, ♂, 3♀♀, thermophilus deciduous woodland, leg. D. Çiftçi; Sarıcakaya, 5 km along Laçin-Alapınar, 40º01'48.47"N, 30º48'08.64"E, 580 m, 16.06.2012, ♂, *Pinus nigra* woodland, light trap, leg. D. Çiftçi; Sarıcakaya, Alapınar, 4 km along Laçin Road, 40º02'29.94"N, 30º49'49.32"E, 1035 m, 28.06.2012, ♂, mixed Mediterranean *Pinus*-thermophilus *Quercus* woodland, from cow dung, leg. D. Çiftçi; Izmir:

Bornova, Yakaköy, 38º31'04.68"N, 27º19'53.85"E, 508 m, 11.04.2011, ♂, leg. D. Çiftçi; Kırşehir: Akçakent, W of Ömeruşağı Village, 39º43'26.61"N, 34º04'36.68"E, 877 m, 05.06.2011, 8♂♂, 5♀♀, leg. D. Çiftçi.

Distribution in Turkey. Adana, Antalya, Erzurum, Mardin and Mersin (Peyron, 1858; Smetana, 1967, Herman, 2001; Anlaş & Rose, 2009; Kesdek et.al., 2009; Özgen et al., 2010).

Remarks. It is here reported from central and western Anatolia for the first time. It is a widespread species in the Palearctic Region (Schülke & Smetana, 2015). It can be detected in all kinds of digested matter, often flying (landing net in the light) (Lott, 2009). In this study, it was generally collected from forest habitats (thermophilus deciduous woodland, *Pinus nigra* woodland, mixed Mediterranean *Pinus*-thermophilus *Quercus* woodland) in cow dung also using light trap.

Figure 13. Distribution of *Oxytelus piceus* in Turkey.

***Oxytelus pseudopiceus* Kashcheev, 1999 (Figures 14 & 15)**

Material examined. Eskişehir: Sarıcakaya, Laçin-Alapınar Road, 40º01'48.47"N, 30º48'08.64"E, 580 m, 17.06.2012, 4♂♂, ♀, *Pinus nigra* woodland, light trap, leg. D. Çiftçi; Gümüşhane: Kelkit, E of Yeniyol Village, 39º54'04.61"N, 39º24'54.82"E, 1876 m, 22.06.2011, ♂, leg. D. Çiftçi.

Figure 14. Distribution of *Oxytelus pseudopiceus* in Turkey.

Remarks. According Kashcheev (1999), *O. pseudopiceus* was described based on a male holotype and paratypes (eight males and 12 females) from “south of Zaysan”, three males and one female from “Zailiysky Alatau” (Kazakhstan). This author provided drawings of head, pronotum, last sternites of abdomen and aedeagus of the species (Kashcheev, 1999). *Oxytelus pseudopiceus* is a new record for Turkey. The specimens from Eskişehir were collected with a light trap at the border of *Pinus nigra* woodland. In this study, the photographs of the forebody and the male sternite VII of this species are published for the first time (Figure 15).

Figure 15. *Oxytelus pseudopiceus*: a) forebody (Scale: 0.5 mm), b) ♂ sternite VII (Scale: 0.1 mm).

***Oxytelus sculptus* Gravenhorst, 1806 (Figure 16)**

Material examined. Batman: Hasankeyf, Irmak Village, 37º43'32.28"N, 41º31'12.40"E, 476 m, 17.05.2015, ♂, moist or wet eutrophic and mesotrophic grassland, under stone, leg. D. Çiftçi; Eskişehir: Tepebaşı, Hekimdağ, 39º57'N, 30º30'E, 1240 m, 24.09.2011, ♂, ♀, Irano-Anatolian steppes, leg. D. Çiftçi.

Distribution in Turkey. Adana, Ankara, Diyarbakır, Karaman, Mardin, Mersin, Muğla and Sakarya (Peyron, 1858; Smetana, 1967; Herman, 2001; Anlaş, 2009; Özgen & Anlaş, 2010; Özgen, 2011, Tezcan et al. 2019).

Remarks. This species was recorded for Batman and Eskişehir Provinces for the first time. It is a cosmopolitan species (Schülke & Smetana, 2015). This species can be found in compost and dung heaps (Lott, 2009; Schülke, 2012a).

Figure 16. Distribution of *Oxytelus sculptus* in Turkey.

***Platystethus cornutus* (Gravenhorst, 1802) (Figure 17)**

Material examined. Eskişehir: Mihalıççık, Üçbaşlı, 39º47'N, 31º38'E, 945 m, 20.08.2011, ♂, moist or wet eutrophic and mesotrophic grassland, nearby pond, leg. D. Çiftçi.

Distribution in Turkey. Ankara, Aydın, Edirne, Eskişehir, Izmir, Manisa, Mardin, Mersin, Karaman and Kütahya (Peyron, 1858; Smetana, 1967; Peyron, 1858; Anlaş, 2009; Anlaş & Rose, 2009; Özgen et al., 2010).

Remarks. It is the most frequently encountered wetland *Platystethus* species. It breeds in mud and sand around ponds and rivers (Lott, 2009). In this study, it was also collected from around pond shores.

Figure 17. Distribution of *Platystethus cornutus* in Turkey.

***Platystethus nitens* (Sahlberg, 1832) (Figure 18)**

Material examined. Eskişehir: Mihallıçcık, Ahurözü, 39º47'N, 31º42'E, 1035 m, 20.09.2011, ♂, moist or wet eutrophic and mesotrophic grassland, from cow dung, leg. D. Çiftçi; Tepebaşı, Danışment Village, 39º53'N, 30º42'E, 1200 m, 28.04.2012, ♂, thermophilus deciduous woodland and near stream, from cow dung, leg. D. Çiftçi.

Distribution in Turkey. Ankara, Antalya, Eskişehir, Isparta, Kilis, Kocaeli, Mardin and Sakarya (Scheerpeltz, 1955; Horion, 1963; Smetana, 1967; Herman, 2001; Özgen & Anlaş, 2010; Anlaş & Rose, 2009; Assing, 2013, 2014; Altunsoy et al., 2017).

Remarks. *P. nitens* usually occurs in warm places with tightly compact soil, especially on lake shores, river banks, in fields and gardens. It can be found in detritus, compost, horse and cattle dung (Burakowski et al., 1979; Koch, 1989). In this study, this species was found in cow dung on wet meadow and river banks (in thermophilus deciduous woodland).

Figure 18. Distribution of *Platystethus nitens* in Turkey.

Discussion

In this study, the specimens *Anotylus*, *Oxytelus* and *Platystethus* belonging to the Oxytelinae subfamily were evaluated in Zoological Museum of Gazi University (ZMGU), Ankara, Turkey. At the end of the evaluation, 16 species were identified. Of these species, 10 belonged to *Anotylus*, four to *Oxytelus* and two to *Platystethus*. Among them, *A. rugifrons* (Hochhuth, 1849), *A. sexualis* (Eppelsheim, 1892) and *O. pseudopiceus* Kashcheev, 1999 are new records for Turkish fauna.

In this study, habitat associations and environments of the species was compiled (Table 1) from information given in published papers.

Table 1. Oxytelinae species collected at the EUNIS habitats

Species	EUNIS Habitat Code										
	E1.2E	E3.4	E4.4	F5.3	G1	G1.3	G1.7	G2.1	G3.5	G3.9	G4.B
Anotylus clypeonitens		x				x	x				x
Anotylus complanatus		x					x				
Anotylus fairmairei		x	x								
Anotylus intricatus		x									
Anotylus inustus	x	x		x			x	x	x	x	x
Anotylus pumilus	x										
Anotylus rugifrons	x	x	x								
Anotylus rugosus									x		
Anotylus sculpturatus							x		x		x
Anotylus sexualis						x					
Oxytelus laqueatus					x						
Oxytelus piceus							x		x		x
Oxytelus pseudopiceus									x		
Oxytelus sculptus	x	x									
Platystethus cornutus		x									
Platystethus nitens		x					x				

Among the detected species, six species were collected from only one locality. Other species were collected from more than one locality (Table 1). Of the species collected from a single locality, *A. intricatus* is found in decomposed material (Schülke, 2012a) and in the present study it was taken from E3.4 habitat from the edge of stream. *Anotylus pumilus* is a common species for dry dung (Schülke, 2012a). The species was collected from dry cow dung and oak leaf litter in dry habitats (Irano-Anatolian steppes and oak forest) as that in Assing (2013). The habitat of the *A. rugosus* is given as steppes in Özgen & Anlaş (2010) and Schülke (2012b) and in present study it was collected from the black pine forest. The species that common in Europe is also likely to be highly widespread in Turkey. Therefore, it is can also be found from many different habitats. Habitat or nutritional associations of *A. sexualis* are not specified in the literature. It is thought to occur decomposed material like other species of the genus. In this study, the specimen was taken from G1.3 (Mediterranean riparian woodland) habitat in cow dung. The common species *O. laqueatus* was collected from broadleaved deciduous woodland in this study and Assing (2007). The species was found in all kind of decayed or rotting organic matter (Lott, 2009; Schülke, 2012a). *Platystethus cornutus* was most frequently encountered in wetlands (Lott, 2009). The specimens collected in this study and of Anlaş (2009) (record from Manisa: Çatalköprü) were taken from the edge of the stream. In addition, it was observed that the specimens with exact locality record were collected from various habitats; agricultural land and forest (Anlaş, 2009; Anlaş & Rose, 2009; Özgen et al., 2010). The habitat information of the species mentioned above is incomplete. The habitat information given in this study and in the literature are similar for some species. In addition, new habitat information has been added for some species.

When number of the species detected in habitats is examined numerically, nine species from E3.4 habitat, six species from G1.7 habitat and five species from G3.5 habitat were identified. Fewer than five species were found from other habitats (Table 1). The specimens collected from E3.4 habitat are usually taken from cow dung. The reason for this is that the water in this habitat is considered to be used by cattle for drinking. Ten species were identified from forest habitats (habitats starting with G code). Some of the specimens collected in forest habitats were taken from cow dung. Species collected from these habitats usually feed on various kinds of decayed or rotting organic matter. Studies in the Nearctic region (González-Vainer et al., 2012; Greenberg & Thomas, 1995) showed that forest habitats have greater coprophagous Oxytelinae species diversity than pasture habitats. This was considered to be a complex habitat that provides shelter from predators of forest habitats. In Europe, due to the fact that domestic mammals in pasture are more dominant, it is stated that coprophagous Oxytelinae species richness in pasture habitat is high (González-Vainer et al., 2012; Greenberg & Thomas, 1995). Numerous small oxytelines are many times found in cow dung. These beetles consume decomposing material in dung (Hammond, 1976; Hanski & Cambefort, 1991). Also, some oxytelines are predominantly associated with litter or decaying plant material (Hammond, 1976; Lott, 2009). In this study, it was observed that there are a moderate number of species in forest habitats, possibly because there are more nutritional resources in forests.

Overall, it is considered that the nutritional requirements of the *Anotylus*, *Oxytelus* and *Platystethus* species listed above are the main determinant of their habitat associations. However, there is insufficient information about their habitats to draw strong conclusions. Further habitat and environmental information need to be collected in future studies.

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Original article (Orijinal araştırma)

The effect of illumination with different light wavelengths on the orientation of Turkestan cockroach, *Blatta lateralis* (Walker, 1868) (Blattodea: Blattidae)[1]

Farklı dalga boyuna sahip ışınların Türkistan hamamböceği, *Blatta lateralis* (Walker, 1868) (Blattodea: Blattidae)'in yönelimi üzerine etkisi

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Abstract

In this study, the effect of illumination with different light wavelengths (red, green, yellow, blue, white and a dark control) on the orientation of the Turkestan cockroach, *Blatta lateralis* (Walker,1868) (Blattodea: Blattidae) was investigated. The study was conducted under laboratory conditions in 2019 at Bursa Uludağ University. In orientation trials, adult cockroaches were exposed to three different light illuminances (25, 250 and 2500 lux). The trials were conducted in six- and two-arm choice arenas. In each trial, three replicates and 100 individuals were used. The data obtained show that the sensitivity to the illumination with different wavelengths may increase depending on light intensity. In particular, in the six-arm and two-arm trial, in the experiment where blue light of 2500 lux intensity was applied, the cockroach orientation was the lowest at 0.9% and 1.8%, respectively. In general, the highest orientation was against the dark (control) chamber in all trials. In addition, under all lux values, the orientation to red light was higher than with green, yellow, blue and white light. As a result of this study, it has been determined that blue light may have a repellent effect on cockroaches, and red light may be more attractive than other wavelengths. These studies can be useful in the development of an alternative method of control to replace chemicals used against cockroaches that would be harmless to human health and the environment.

Keywords: *Blatta lateralis*, LED light, light wavelength, Turkestan cockroach

Öz

Bu çalışmada, farklı dalga boylarına (kırmızı, yeşil, sarı, mavi, beyaz ve karanlık) sahip ışınların Türkistan hamamböceği, *Blatta lateralis* (Walker,1868) (Blattodea: Blattidae)'in yönelimi üzerindeki etkisi araştırılmıştır. Çalışma, 2019 yılında Bursa Uludağ Üniversitesi'nde laboratuvar koşullarında yürütülmüştür. Yönelim denemelerinde, ergin hamamböcekleri üç farklı ışık şiddetine (25, 250 ve 2500 lüks) maruz bırakılmıştır. Denemeler 6-kollu ve 2-kollu seçimli olarak yürütülmüştür. Tüm denemelerde 3 tekerrür ve her tekerrürde 100 birey kullanılmıştır. Elde edilen veriler, ışık şiddetine bağlı olarak farklı dalga boyuna sahip ışınlara yönelim hassasiyetinin artabileceğini göstermektedir. Özellikle, altı -kollu ve iki -kollu denemede, 2500 lüks şiddetinde mavi ışık uygulandığında, hamamböceğinin yönelimi en düşük seviyede olup, sırasıyla, %0.9 ve %1.8 oranında olmuştur. Genel olarak en yüksek yönelim, tüm denemelerde karanlık (kontrol) odacığa olmuştur. Ayrıca tüm lüks değerleri altında kırmızı ışığa yönelim, yeşil, sarı, mavi ve beyaz ışığa göre daha fazla olmuştur. Bu çalışma sonucunda, mavi ışığın hamamböcekleri üzerinde kaçırıcı bir etki gösterebileceği, kırmızı ışığın ise diğer dalga boylarına göre daha çekici olabileceği tespit edilmiştir. Yapılan bu çalışmalar, hamamböceklerine karşı kullanılan kimyasalların yerini alabilecek insan sağlığına ve çevreye zararsız olan alternatif bir mücadele metodunun geliştirilmesinde faydalı olabilir.

Anahtar sözcükler: *Blatta lateralis*, LED ışık, ışık dalga boyu, Türkistan hamamböceği

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Introduction

Cockroaches (Blattellidae) have out-survived dinosaurs, ice ages and they first appeared early in Upper Carboniferous period (Vishniakova, 1982; Vršanský, 2005, 2008). They are pests in homes, restaurants, hospitals, warehouses, offices and food processing areas. Their spread takes place mostly by movement of people and trade (Rehn, 1945). It is known that cockroaches have an allergic effect in humans and can carry various pathogenic organisms (Rosenstrich et al., 1997; Ahmad et al., 2011). They can transmit almost 150 bacterial species, 60 species of fungus, 45 species of parasitic worms and 90 species of protozoa to human either biologically or mechanically (Collins et al., 1995; Tatfeng et al., 2005; Etim et al., 2013). Cosmopolitan species such as American cockroach, *Periplaneta americana* (Linnaeus, 1758) (Blattodea: Blattidae) and German cockroach, *Blattella germanica* (Linnaeus, 1767) (Blattodea: Blattellidae) are common insects worldwide. In addition, the Turkestan cockroach, *Blatta lateralis* (Walker,1868) (Blattodea: Blattidae) are found in Afghanistan, Libya, Pakistan, southern Russia, Uzbekistan (Alesho, 1997), some urban areas of Punjub State of India (Sandhu & Sohi, 1981) and in southwestern United States (Kim & Rust, 2013). Turkestan cockroach live occasionally indoors, but it is found especially animal manure piles and around structures in gardens (Alesho, 1997; Artyukhina, 1972). Also, Turkestan cockroach and oriental cockroach, *Blatta orientalis* Linnaeus, 1758 (Blattodea: Blattidae) have been identified in Turkey (Demirsoy, 2014).

Given the insecticidal effects of synthetic chemicals such, as sulfluramid, fipronil and imidacloprid, that are often used to control cockroaches (Rust et al., 1995). However, cockroaches develop resistance to these chemicals (Ko et al., 2015). In addition, these chemicals have negative effects on human health and the environment. Therefore, new methods need to be developed in the control of cockroaches. To manage these pests, some insect repellents (plant essential oils) (Prakash et al., 1990, Paranagama & Ekanayake, 2004; Yilmaz & Tunaz, 2013), diatomaceous earth application (Özcan et al., 2018; Alkan et al., 2019) and biocontrol methods are used (Fox & Bressan-Nascimento, 2006; Hernández-Ramírez et al., 2007; Maketon et al., 2010; Tee et al., 2011; Hubner-Campos et al., 2013; Gutierrez et al., 2016; Baggio-Deibler et al., 2018). Also, insecticidal paints can provide useful control of *P. americana* populations for up to 3 months (Bueno-Marí et al., 2013).

Under normal conditions, cockroaches are active overnight, undertaking exploration, feeding and mating (Lipton & Sutherland, 1970a, b; Seelinger, 1984) avoiding illuminated areas (Kelly & Mote, 1990). One of the most well-known behavioral features of cockroaches are escape reactions in response to sudden illumination, they hide immediately in the nearest dark shelter. The cockroach visual system consists of two simple eyes, ocelli, and two large compound eyes, so such reactions to light depend on their intensity and wavelength (Kelly & Mote, 1990) and are mainly based on light input through the compound eyes (Okada & Toh, 1998). The ocelli of the cockroach can be recognized as two large white spots, located between the compound eyes at the base of the antennae (Cooter, 1975; Weber & Renner, 1976) and each ocellus contains about 10,000 photoreceptors that converge to only four larges second-order lamina cells (Toh & Sagara, 1984).

Artificial lights can be useful in the control of pests in the context of integrated control or in greenhouses (Johansen et al., 2011). It is generally known that some insect species that are active at night are attracted to an UV light source. Therefore, various UV light traps have been developed for population tracking or mass capture (Shimoda & Honda, 2013). Light sources of different colors have different effects (mortality, attractive and behavioral) on various insect species. Blue light has lethal effects on *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) (Hori et al., 2014). The UV (black) source was determined and for all light sources, the most common insect sets of traps were Diptera, Coleoptera and Lepidoptera, respectively (Ashfaq et al., 2005). Furthermore, lighting has been shown to affect the activities of agriculturally beneficial organisms, such as predators. This was found in studies of *Orius sauteri* (Poppius, 1909) (Heteroptera: Anthocoridae), an agriculturally beneficial insect (Wang et al., 2013).

Recently, especially with the production of LED bulbs, the use of lights in the control of harmful insects has increased (Shimoda & Honda, 2013). It has been found that illumination with different wavelengths may have lethal, adductive or attractive effects on insects (Pate & Curtis, 2001; Ashfaq et al., 2005; Van Langevelde et al., 2011; Shimoda & Honda, 2013; Hori et al., 2014). In Turkey, there have been no reports on the orientation of cockroaches to light. However, Uluca & Karaca (2016) conducted studies on the effect of ultrasonic pest repellents on Turkestan cockroaches. Therefore, the aim of this study is to develop a control method that could replace the chemicals in the control of cockroaches that is harmless to human health and environment. For this purpose, the effect of illumination having different wavelengths and intensity on Turkestan cockroaches was investigated.

Materials and Methods

The trials were conducted in 2019 in the laboratory of the Department of Entomology, Bursa Uludağ University, Faculty of Agriculture, Department of Plant Protection.

Insects

A commercial supply of *B. lateralis* female and male adults used in the experiment and purchased from insect breeder, Antalya Çekirge (Mira Canlı Hayvan Böcek Tur. İnş. Tarım Tic. Ltd. Şti., Antalya, Turkey), a package containing 1500 individuals (Anonymous, 2019).

Light and sticky trap

LED light sources with different wavelengths were used in this research (Figure 1a). The power of the LEDs used is 1.5 W. The wavelengths used were red (678 nm), green (620 nm), yellow (580 nm), blue (478 nm) and white (all colors). In the experiment, yellow sticky traps were placed in the base area of each chamber (except the release point) in order to accurately determine the number of adult insects (Figure 1b).

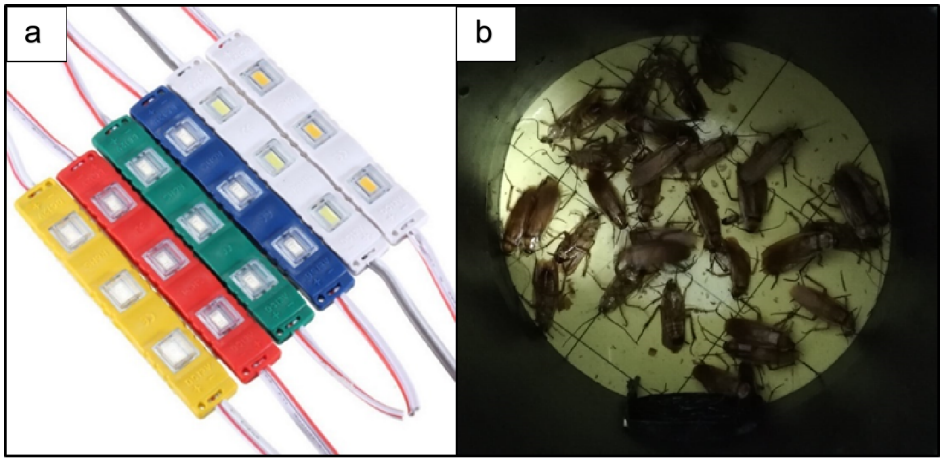

Figure 1. a) LED lights and b) sticky trap.

Plastic black flower pots (9 × 10 cm) were used as hiding places for cockroaches. The pots (chambers) illuminated by LED light sources with different wavelengths were connected by a plastic tube (5 × 10 cm) at the center in a single combination.

Six and two-arm choice test

The light wavelength preference of adult male Turkestan cockroaches was tested in a six-arm arena, similar to that previously used for *Orius* bugs and a parasitoid fly (Ogino et al., 2015; Tokushima et al., 2016). This setup enables the cockroaches to be presented with six LED light sources simultaneously.

The six equal chambers used in this study were connected to the starting point in the middle by means of insect passage (Figure 2). Adult insects were placed in the middle starting point (Figure 2). Five of the chambers were illuminated by LEDs of different wavelength. One of them had light source (dark control). The floor area of each chamber was covered with a sticky trap in order to determine the number of insects entering. All light sources in the chambers were adjusted to an intensity of 25 lux. Light intensities were measured with a light meter.

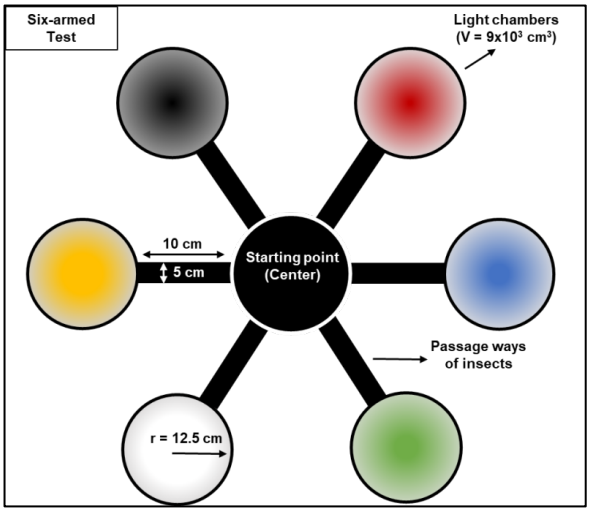

Figure 2. Schematic view of the six-arm choice arena.

In two-arm choice test, the orientation to the chambers illuminated only with 2500 lux LED light sources was observed in binary combinations with five light colors.

Experimental procedure

First, the chambers illuminated by LED light sources with different wavelengths were connected by tubes to the center in a single combination. The orientation of the cockroach adults to the chambers with three different light intensities (25, 250 and 2500 lux) were observed. In the next step, the orientation towards the chambers illuminated by LED light sources of only 2500 lux intensity was observed in binary combinations (dark-light). In this experiment, five combinations (control-red light, control-blue light, control-green light, control-white light and control-yellow light with dark as the control) were tested. One hundred adult male cockroaches were released at the same time in the center point and the orientation of the insects to light was determined after 15 min. Each application was replicated three times.

Statistical analysis

Statistical differences in the ratio of cockroaches to chambers with different wavelengths and different light intensities were analyzed by two-way analysis of variance. LSD test ($P < 0.05$) was used to determine the difference between means. Differences in the proportions of *B. lateralis* adult attraction by moving toward one of the light sources or toward the dark (control) and different lux intensity were analyzed using the Pearson chi-squared test. Insects that did not make a choice were excluded from the statistical analysis.

Results

Orientation of Turkestan cockroach to different wavelength in six-arm combinations

The orientation of cockroach adults place in the center of chambers with different wavelengths was observed at three different light intensities and the results are given in Table 1.

Table 1. Average proportion (%) of adult orientation of Turkestan cockroaches to different wavelength light sources at different light intensities (25, 250 and 2500 lux)

	Proportion of adults (%)		
	25 lux	250 lux	2500 lux
Control (dark)	20.7 d	27.8 b	34.1 a
Red	17.4 e	21.5 cd	22.9 c
Blue	9.5 ij	2.5 l	0.9 m
Green	14.1 f	9.5 ij	5.3 k
White	10.5 hi	12.0 gh	12.8 fg
Yellow	13.6 f	8.0 j	4.9 k

The orientation of Turkestan cockroach was highest to the dark control at light intensities of 250 and 2500 lux (27.8 and 34.1%, respectively) and then to red-light (21.5 and 22.9%, respectively). Also, the orientation to the control at 25 lux was 20.7%. The lowest orientation was to blue color (0.9% at 2500 lux), and then yellow and green color (4.9 and 5.3%, respectively). Generally, as light intensity decreased, the differences between orientation to different wavelengths likewise decreased (Figure 3).

Considering only light with different wavelengths, the highest Turkestan cockroach orientation was towards the dark control (82.6%). After the control, the statistically highest trend was 61.7% to chambers with red and white light (35.2%), respectively. The orientation to chambers with green and yellow light was no statistically different, a mean of 27.7%. Statistically, the lowest orientation was towards chambers with blue light with 12.9% (Table 1). Considering only the different lux light intensities, 25 lux give no statistical significance (P>0.05). However, 250 and 2500 lux were statistically significant (P < 0.01).

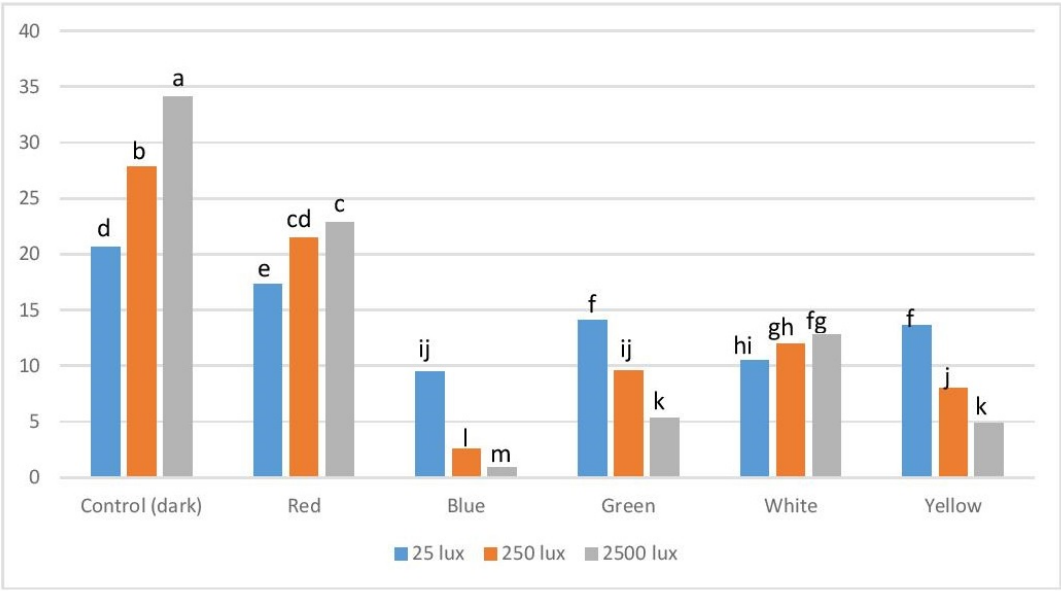

Figure 3. Orientation of Turkestan cockroach adults to the three light intensities (25, 250 and 2500 lux) for six treatments (F=184; df=20; P>0.0001).

Orientation of the Turkestan cockroach to the two-arm combinations at the highest light intensity (2500 Lux)

The orientation of Turkestan cockroaches in binary light combinations under 2500 lux light intensity is given in Table 2. In this binary combination study, the orientation towards the dark control was the highest

and varied between 44.3 and 62.3%. In the center (dim) values were 25.0 to 35.9%. In addition, in application with LED lights, the highest orientation was 30.7% for red light, the lowest orientation for blue light (1.8%), followed by yellow (8.7%), green (9.1%) and white (20.1%) light. These results were similar to the six-arm combinations and so support each other. The results of two-arm choice test between light colors show statistically significant with blue light (df=1, x^2=90.34, $P < 0.01$).

Table 2. Average proportion (%) of adult orientation of Turkestan cockroaches in binary light combinations of different wavelengths at 2500 lux (%)

Combination	Orientation	Proportion of adults (%)
Dark-Red	Control (dark)	44.3 a
	Center (dim)	25.0 c
	Red	30.7 b
Dark-Blue	Control (dark)	62.3 a
	Center (dim)	35.9 b
	Blue	1.8 c
Dark-Green	Control (dark)	58.9 a
	Center (dim)	31.9 b
	Green	9.1 c
Dark-White	Control (dark)	51.7 a
	Center (dim)	28.2 b
	White	20.1 c
Dark-Yellow	Control (dark)	58.6 a
	Center (dim)	32.8 b
	Yellow	8.6 c

Discussion

In this study, the highest orientation of the Turkestan cockroach (*B. lateralis*) was to non-illuminated chamber (i.e. dark control) and the lowest orientation was to blue light. However, the orientation towards red light was found to be second highest following dark (control) in all trial conditions. The orientation to green and yellow light was not statistically different under at three light intensities (25, 250 and 2500 lux). Unlike our studies, in an experiment with light sources having a wavelength of 350 to 700 nm, Koehler et al. (1987) suggested that green or UV light changed the behavior of German cockroaches by 30%, while yellow and red light did not show any effect. Furthermore, in our study, some of the cockroaches were found in the dim light at the release point. Similar to our results, Zhukovskaya et al. (2017) state that green light promotes mobility in *P. americana* adults and dim UV light source causes adults to remain inactive.

In the study by Okada & Toh (1998), *P. americana* escape in a shadow-dependent pause (shadow response) was observed even at less than 0.01 lux (very low light levels). Similarly, as a result of the observations made in this study, it was observed that *B. lateralis* adults were statistically more oriented towards the dark chamber. The average number of adult insects under dim light was statistically less than the number of adults in the chamber illuminated by red LED light. Overall, red light is more attractive than dim light and less attractive than darkness. In contrast to our study, Dean (2017) found that red light repels a greater number of Dublin cockroaches than yellow, blue, white, green, black and no light control (dark).

In our study, blue light was found to be the least attractive. In contrast to our study, Dean (2017) reported that blue light was the most attractive to Dublin cockroaches. Yellow and green light showed the least attraction after blue. Similar to our study, the same researcher emphasizes that green light is the second least attractive color. Contrast to our study, in different insect groups, green LEDs are attractive for adults of *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) and *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) (Park et al., 2014; Yang et al., 2015). In this study, yellow light was found to be the second least attractive after blue, but Dean (2017) emphasizes that yellow light attracts cockroaches.

Various results have been obtained in studies with other insect species on this subject. In addition, Shibuya et al. (2018) reported that blue light has lethal effects on different stages of vinegar fly, (*D. melanogaster*). Also, Hori et al. (2014) examined the lethal effects of visible light with short wavelength on insects. They found that short wavelength visible (blue) light had lethal effects on the vinegar fly eggs, larvae, pupae and adults. In the same study, they found that blue light had a lethal effect for mosquitoes and flour beetles, but that the effective wavelength at which death occurs differed between insect species. The findings in the same study also show that high toxic wavelengths of visible light are species-specific in insects and that shorter wavelengths are not always more lethal. For some organisms, such as insects, blue light appears to be more harmful than UV light. Our study confirms the results of, Hori et al. (2014). Orientation of adult cockroaches tends to vary with light wavelength. In our study, especially blue light showed the least attraction. In this regard, light sources with storage insects were studied. Similar to our study, Kim et al. (2013) found repellent effect of the blue light to *Lasioderma serricorne* (Fabricius, 1792) (Coleoptera: Anobiidae). In contrast to our study, Jeon & Lee (2016) reported that blue light is the most attractive to *Sitotroga cerealella* (Olivier, 1789) (Lepidoptera: Gelechiidae). Also, in contrast to our study, Jeon et al. (2012) reported blue light attraction in *Sitophilus oryzae* (Linnaeus, 1763) (Coleoptera: Curculionidae). Similar to our study, Wang et al. (2013), determined that red light was attractive to *O. sauteri* and the research found the blue and red light had a negative impact on the development of this predator species. Lee et al. (2015) also found the repellent effect of the blue light on *Tyrophagus putrescentiae* (Schrank, 1781) (Acari: Acaridae).

In conclusion, the orientation of cockroaches under light intensity of 250 and 2500 lux was low with blue light. In this case, it is understood that the blue light has a negative effect. However, as light intensity decreases (25 lux), the orientation to blue light increases becoming equivalent to white light. In this study it was determined that light sources can be used as an alternative to chemical methods in the control of cockroaches. In the future, it is thought that red light could be used as an attractant and blue light as a repellent for Turkestan cockroaches. In addition, it was understood that LED sources with different levels of light intensity would exhibit significantly variable activity on cockroaches.

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Original article (Orijinal araştırma)

Contribution to the knowledge of Ichneumonidae (Hymenoptera) of Bursa Uludağ National Park area including new records[1]

Bursa Uludağ Doğal Park alanı Ichneumonidae türlerine yeni kayıtlarla birlikte katkılar

Fatma Zehra ÇAYLAK[2] **Saliha ÇORUH[2*]**

Abstract

In order to record species of the family Ichneumonidae (Hymenoptera) in Bursa Uludağ National Park area, this study was conducted between 2018-2019. Specimens belonging to the subfamily Campopleginae and Cryptinae were collected from three localities on natural vegetation, flowering plants and weeds. Determinations were made for 21 species, *Acrolyta rufocincta* (Gravenhorst, 1829), *Campoletis thomsoni* (Roman, 1915), *Diadegma glabriculum* (Holmgren, 1859), *Dichrogaster heteropus* (Thomson, 1896), *Mesoleptus congener* (Förster, 1876), *Phygadeuon hercynicus* Gravenhorst, 1829, *Phygadeuon lapponicus* Thomson, 1884, *Phygadeuon nitidus* Gravenhorst, 1829 and *Thaumatogelis audax* (Olivier, 1792) are recorded for the first time from Anatolia, Turkey.

Keywords: Campopleginae, Cryptinae, Ichneumonidae, natural park area, new records, Uludağ

Öz

Bu çalışma, Bursa Uludağ Doğal Park alanı'nın Ichneumonidae (Hymenoptera) türlerini tespit etmek amacıyla 2018-2019 yılları arasında yapılmıştır. Campopleginae ve Cryptinae altfamilyalarına ait türler doğal alanlar, çiçekli bitkiler ve yabancı otların hâkim olduğu üç farklı lokaliteden toplanmıştır. Teşhisi yapılan 21 türden, *Acrolyta rufocincta* (Gravenhorst, 1829), *Campoletis thomsoni* (Roman, 1915), *Diadegma glabriculum* (Holmgren, 1859), *Dichrogaster heteropus* (Thomson, 1896), *Mesoleptus congener* (Förster, 1876), *Phygadeuon hercynicus* Gravenhorst, 1829, *Phygadeuon lapponicus* Thomson, 1884, *Phygadeuon nitidus* Gravenhorst, 1829 ve *Thaumatogelis audax* (Olivier, 1792) türleri Anadolu, Türkiye için yeni kayıt durumundadır.

Anahtar sözcükler: Campopleginae, Cryptinae, Ichneumonidae, doğal park alanı, yeni kayıtlar, Uludağ

[1] This study is part of Master Science thesis of the first author, accepted at 20.12.2019 in Atatürk University, Graduate School of Natural and Applied Sciences.

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Introduction

The family Ichneumonidae is an important group in the order Hymenoptera in terms of number of species, species diversity and potential for use on biological control.

Ichneumonidae is a rich family, with 1601 genera and 25 285 species (Yu et al., 2016). *A catalogue of the Turkish Ichneumonidae (Hymenoptera)* was the first comprehensive study in Turkey (Kolarov, 1995). With the contributions detailed below this has increased the known diversity of the family in Turkey to 1 282 species. (Çoruh, 2017, 2019a, b; Kolarov et al., 2017; Narmanlıoğlu & Çoruh, 2017; Çoruh et al., 2018, 2019; Riedel et al., 2018; Sarı & Çoruh, 2018; Özdan & Gürbüz, 2019; Vas, 2019a, b; Çaylak & Çoruh, 2020; Kıraç & Gürbüz, 2020; Kolarov et al., 2020).

The aim of this study was to collect and determine specimens from Bursa Uludağ National Park Area (BUNPA) that will contribute to the knowledge of the Ichneumonidae fauna of Turkey.

Materials and Methods

Uludağ, 36 km south of Bursa (Figure 1), is one of the Turkey's popular winter recreation areas. Uludağ has a natural park because it has a rich flora and fauna. Mount Uludağ (Great Mountain) is 2,543 m high (Table 1).

Uludağ National Park Area has extraordinary natural features, forests, flora and fauna. This flora has including specific endemic plants. Vegetation data from where the insects were collected are given in Table 3.

The study area sampled of three localities (Alaçam, 40º07'34.7"N, 29º17'31.2"E; Cevizdibi, 40º08'13.9"N, 29º17'35.1"E; and Gözede, 40º09'13.6"N, 29º17'26.1"E; Figure 2).

Adult specimens of Ichneumonidae were collected from various habitats that flowering plants, weeds and open areas of BUNPA by sweep net in the summers of 2018 and 2019. Some of the specimens were collected from blackberry gardens. Each locality had different altitude and vegetation. The most common plants were *Allium paniculatum* L., *Epilobium angustifolium* L., *Galium elongatum* C. Presl, *Hypericum perforatum* L., *Medicago polymorpha* L., *Mentha longifolia* (L.), *Polypodium vulgare* L., *Raphanus raphanistrum* L., *Trifolium repens* L. and *Vicia cracca* L.

Table 1. Data of collected species

Locality	Year	Date	Altitude (m.)
Alaçam	2018	20.VI.2018	1100
		21.VII.2018	1450
		08.VIII.2018	1700
	2019	08.VI.2019	1200
		20.VII.2019	1500
		19.VIII.2019	1600
Cevizdibi	2018	22.VI.2018	800
		15.VII.2018	830
		02.VIII.2018	850
	2019	29.VI.2019	820
		17. VII.2019	830
		24. VIII.2019	840
Gözede	2018	24.VI.2018	670
		17.VII.2018	650
		05.VIII.2018	610
	2019	28.V.2019	620
		18.VII.2019	640
		06.VIII.2019	630

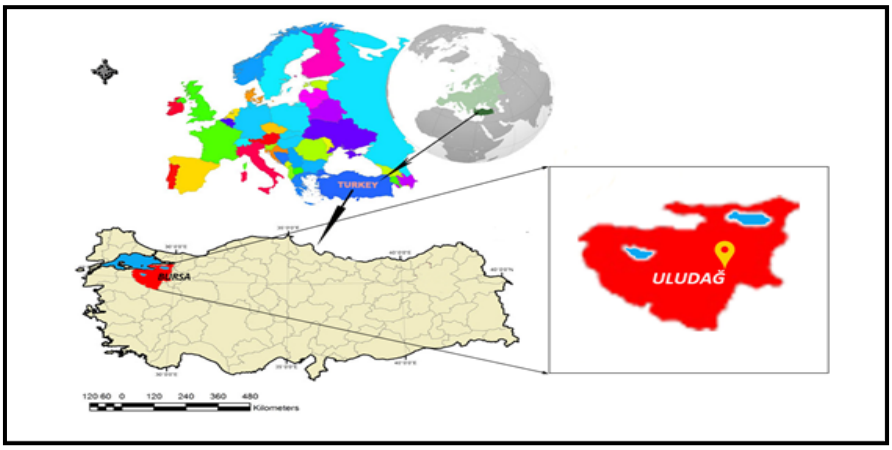

Figure 1. Map of study area.

Figure 2. Study area (Alaçam, Cevizdibi and Gözede).

Collected insect specimens were pinned, labeled and deposited in the Entomological Museum, Erzurum, Turkey (EMET). Photographs were taken of the specimens by the Leica CLS stereomicroscope connected computer at 50x magnification.

All ichneumonid specimens were collected by first author. Specimens were identified by Dr. Janko Kolarov and the second author. Plant species were pressed and identified by Dr. İrfan Çoruh according to Davis (1965-1988) and deposited in the herbarium of Atatürk University, Faculty of Agriculture, and Department of Plant Protection.

Turkey distribution data for the species was obtained from the literature. Global distribution and associated plants data mainly followed Taxapad (Yu et al., 2016).

The newly recorded species are indicated with an asterisk (*) in the text.

Results

Two hundred and sixty-three specimens belonging to 21 species in 12 genera in two subfamilies were collected in BUNPA. Nine of these are reported here for the first time for Ichneumonidae fauna of Turkey. With these additions, 1 291 species are now recorded for Turkey.

Subfamily Campopleginae Förster, 1869

Campoletis agilis (Holmgren, 1860) (Figure 3a)

Material examined. BUNPA: Cevizdibi, 830 m, 15.VII.2018, 6 ♂♂; Gözede, 17.VII.2018, 650 m, 4 ♂♂, 06.VIII.2019, 630 m, 7 ♂♂.

Associated plants. *Peucedanum oreoselinum* (L.).

Distribution in Turkey. Giresun (Kolarov et al., 2016) (Figure 6a).

Global distribution. European and Western Palearctic Region.

Remarks. This species was collected from Giresun Province. Previously, it had only been reported from the Black Sea Region (Giresun).

Campoletis crassicornis (Tschek, 1871) (Figure 3b)

Material examined. BUNPA: Cevizdibi, 850 m, 02.VIII.2018, 5 ♂♂; Gözede, 620 m, 28.V.2019, 7 ♂♂.

Associated plants. *Peucedanum oreoselinum* (L.).

Distribution in Turkey. Adana, Burdur, Erzurum and Giresun (Kolarov & Beyarslan, 1995; Çoruh et al., 2013; Çoruh et al., 2016; Çoruh et al., 2018) (Figure 6b).

Global distribution. European and Western Palearctic Region.

**Campoletis thomsoni* (Roman, 1915) (Figure 3c)

Material examined. BUNPA: Cevizdibi, 800 m, 22.VI.2018, 3 ♀♀, 830 m, 17.VII.2019, ♀; Gözede, 610 m, 05.VIII.2018, 7 ♂♂.

Global distribution. Palearctic Region.

Remarks. This is new species record for Turkey.

Figure 3. Collected species: a) *Campoletis agilis*; b) *Campoletis crassicornis*; c) *Campoletis thomsoni*; d) *Casinaria albipalpis*; e) *Diadegma glabriculum*; f) *Diadegma mediterraneum*; g) *Hyposoter didymator*; h) *Acrolyta rufocincta*.

Casinaria albipalpis (Gravenhorst, 1829) (Figure 3d)

Material examined. BUNPA: Alaçam, 1100 m, 20.VI.2018, 5 ♂♂, 1500 m, 20.VII.2019, 9 ♂♂.

Associated plants. *Picea* sp. and *Quercus robur* L.

Distribution in Turkey. Anatolia (locality name unknown) (Riedel et al., 2010).

Global distribution. Palearctic Region.

**Diadegma glabriculum* (Holmgren, 1859) (Figure 3e)

Material examined. BUNPA: Uludağ: Alaçam, 1450 m, 21.VII.2018, 5 ♀♀; Cevizdibi, 830 m, 15.VII. 2018, ♀; Gözede, 610 m, 05.VIII.2018, 4 ♀♀, 630 m, 06.VIII.2019, ♀.

Global distribution. European, Western Palearctic and Nearctic Region.

Remarks. This is new species record for Turkey.

Diadegma mediterraneum (Constantineanu, 1930) (Figure 3f)

Material examined. BUNPA: Uludağ: Alaçam, 1200 m, 08.VI.2019, 8 ♀♀; Gözede, 610 m, 05.VIII.2018, 4 ♀♀.

Distribution in Turkey. Erzincan, Erzurum and Kahramanmaraş (Kolarov & Beyarslan 1995; Çoruh et al., 2005, 2014a, 2016) (Figure 6c).

Global distribution. European and Western Palearctic Region.

Remarks. This species was collected from Kahramanmaraş (Marat) Province in 1995 and Erzurum Palandöken Mountain in 1987 as a female. It has not been recorded again.

Hyposoter didymator (Thunberg, 1822) (Figure 3g)

Material examined. BUNPA: Uludağ: Alaçam, 1500 m, 20.VII.2019, 8 ♀♀; Gözede, 650 m, 17.VII.2018, 5 ♂♂.

Associated plants. *Angelica sylvestris* L., *Heracleum sphondylium* L. and *Peucedanum oreoselinum* (L.).

Distribution in Turkey. Adana, Ankara, Aydın, Çankırı, Eskişehir, Hatay and Istanbul (Kolarov 1989, 1995; Yaşarakıncı & Kornoşor, 1990; Özdemir & Kılınçer, 1990; Sertkaya et al., 2004; Sertkaya & Bayram 2005; Kaya & Kornoşor, 2008; Şimşek et al., 2015; Shaw et al., 2016) (Figure 6d).

Global distribution. Australasian and Palearctic Region.

Remarks. The species was obtained from 89 hosts, mostly in the Noctuidae (Lepidoptera).

Subfamily Cryptinae Kirby, 1837

**Acrolyta rufocincta* (Gravenhorst, 1829) (Figure 3h)

Material examined. BUNPA: Uludağ: Alaçam, 1600 m, 19.VIII.2019, 7 ♀♀; Cevizdibi, 850 m, 02.VIII.2018, 5 ♀♀.

Associated plants. *Daucus carota* L., *Euphorbia nicaeensis* All. and *Senecio jacobaea* L.

Global distribution. European and West Palearctic Region.

Remarks. This is new species record for Turkey.

Agrothereutes fumipennis (Gravenhorst, 1829) (Figure 4a)

Material examined. BUNPA: Uludağ: Cevizdibi, 830 m, 15.VII.2018, 7 ♂♂, 830 m, 17.VII.2019, 3 ♀♀, 12 ♂♂, 840 m, 24.VIII.2019, 6 ♀♀.

Associated plants. *Peucedanum oreoselinum* (L.)

Distribution in Turkey. Erzurum, Isparta and Kastamonu (Çoruh & Özbek, 2005; Gürbüz & Kolarov, 2008; Kolarov & Yurtcan, 2008; Gürbüz et al., 2009a; Çoruh et al., 2014b) (Figure 6e).

Global distribution. Palearctic Region.

Aptesis senicula (Kriechbaumer, 1893) (Figure 4b)

Material examined. BUNPA: Uludağ: Alaçam, 1600 m, 19.VIII.2019, 9 ♀♀; Cevizdibi, 800 m, 22.VI.2018, 3 ♂♂, 840 m, 24.VIII.2019, 5 ♀♀, 4 ♂♂.

Distribution in Turkey. Adana, Mersin, Rize and Tunceli (Bayarslan & Kolarov, 1994; Kolarov et al., 2014, 2016; Çoruh et al., 2014b) (Figure 6f).

Global distribution. European and West Palearctic Region.

Bathythrix pellucidator (Gravenhorst, 1829) (Figure 4c)

Material examined. BUNPA: Uludağ: Alaçam, 1450 m, 21.VII.2018, 3 ♀♀, 1200 m, 08.VI.2019, 3 ♀♀, 5 ♂♂.

Associated plants. *Picea* spp.

Distribution in Turkey. Ordu and Rize (Çoruh et al., 2014a) (Figure 6g).

Global distribution. Palearctic Region.

**Dichrogaster heteropus* (Thomson, 1896) (Figure 4d)

Material examined. BUNPA: Uludağ: Cevizdibi, 850 m, 02.VIII.2018, 2 ♂♂, 820 m, 29.VI.2019, 2 ♂♂.

Associated plants. *Cornus* sp.

Global distribution. European and West Palearctic Region.

Remarks. This is new species record for Turkey.

Dichrogaster schimitscheki (Fahringer, 1935) (Figure 3e)

Material examined. BUNPA: Uludağ: Gözede, 610 m, 05.VIII.2018, 4 ♂♂, 640 m, 18.VII.2019, 5 ♂♂.

Distribution in Turkey. Isparta (Kolarov & Gürbüz, 2007; Gürbüz et al., 2009b) (Figure 6h).

Global distribution. European, Nearctic and West Palearctic Region.

Gelis agilis (Fabricius, 1775) (Figure 4f)

Material examined. BUNPA: Uludağ: Alaçam, 1100 m, 20.VI.2018, 2 ♂♂; Cevizdibi, 800 m, 22.VI.2018, 4 ♂♂, 820 m, 29.VI.2019, ♂.

Associated plants. *Lonicera* sp., *Mentha longifolia* (L.), *Picea excelsa* (Lam.), *Prunus* sp., *Quercus robur* L. and *Salix* sp.

Distribution in Turkey. Trabzon (Kolarov et al., 2016) (Figure 6i).

Global distribution. Palearctic Region.

Remarks. This species has 137 known hosts (Yu et al., 2016).

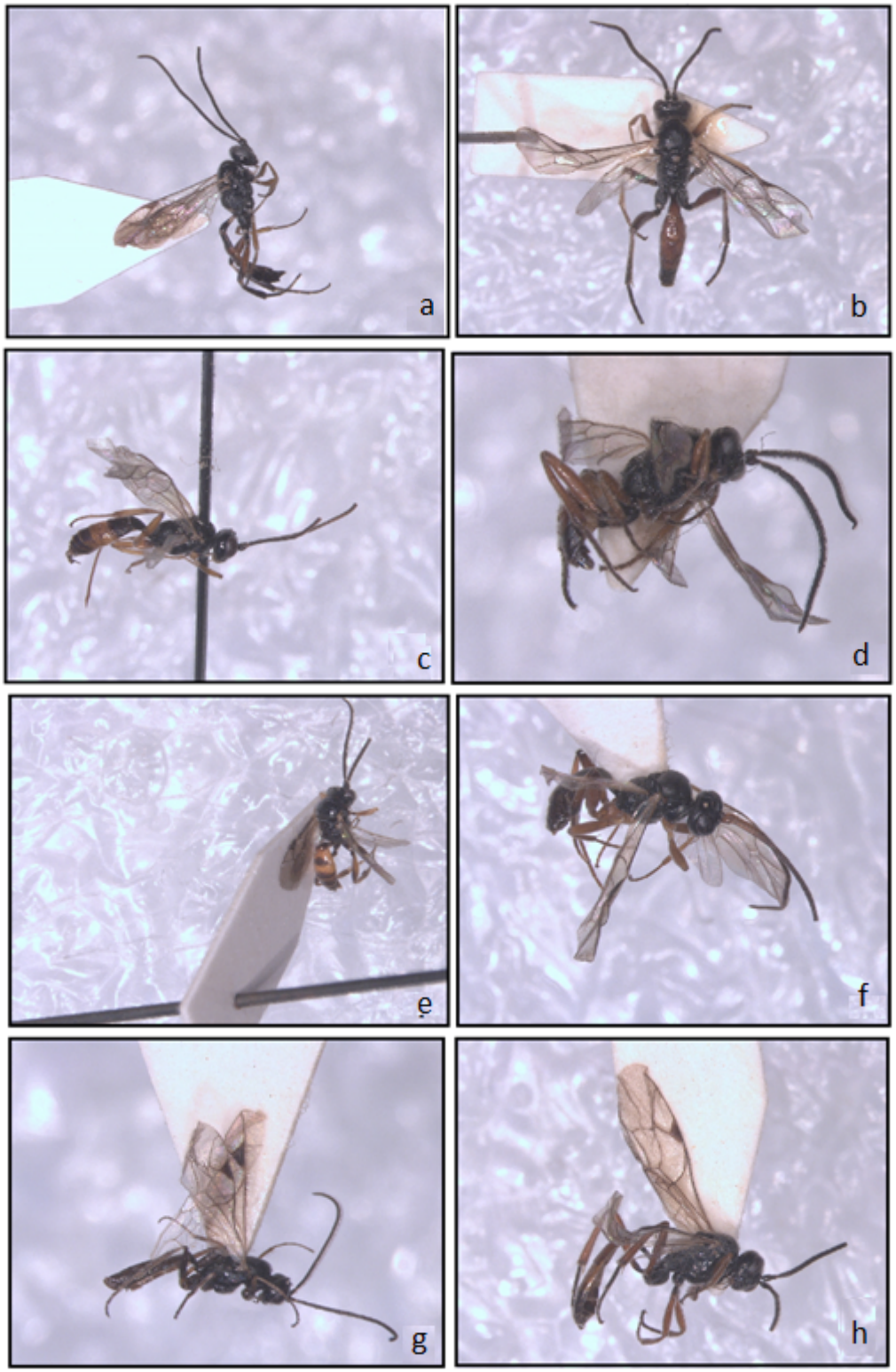

Figure 4. Collected species: a) *Agrothereutes fumipennis*; b) *Aptesis senicula*; c) *Bathythrix pellucidator*; d) *Dichrogaster heteropus*; e) *Dichrogaster schimitscheki*; f) *Gelis agilis*; g) *Gelis exareolatus*; h) *Mesoleptus congener*.

Gelis exareolatus (Förster, 1850) (Figure 4g)

Material examined. BUNPA: Uludağ: Alaçam, 1700 m, 08.VIII.2018, 4 ♂♂; Gözede, 640 m, 18.VII.2019, 14 ♂♂.

Distribution in Turkey. Ankara (Kolarov, 1987; Öncüer, 1991; Kolarov, 1995) (Figure 6j).

Global distribution. Palearctic Region.

Remarks. This species was recorded from Ankara in 1925 by Kolarov (1987).

**Mesoleptus congener* (Förster, 1876) (Figure 4h)

Material examined. BUNPA: Uludağ: Alaçam, 1500 m, 20.VII.2019, 4 ♀♀, 5 ♂♂; Gözede, 670 m, 24.VI.2018, 5 ♂♂, 630 m, 06.VIII.2019, 3 ♀♀, 4 ♂♂.

Global distribution. European and West Palearctic Region.

Remarks. This is new species record for Turkey.

Mesoleptus incessor (Haliday,1838) (Figure 5a)

Material examined. BUNPA: Alaçam, 1600 m, 19.VIII.2019, 4 ♂♂; Cevizdibi, 830 m, 17.VII.2019, 4 ♂♂; Gözede, 670 m, 24.VI.2018, ♂.

Associated plants. *Angelica sylvestris* L., *Euphorbia nicaeensis* All., *E. virgata* Waldst, *Heracleum sphondylium* L. ve *Picea excelsa* (Lam.).

Distribution in Turkey. Anatolia (locality name unknown) (Jussila et al., 2010).).

Global distribution. Palearctic Region.

Figure 5. Collected species: a) *Mesoleptus incessor*; b) *Phygadeuon hercynicus*; c) *Phygadeuon nitidus*; d) *Phygadeuon lapponicus*; e) *Thaumatogelis audax*.

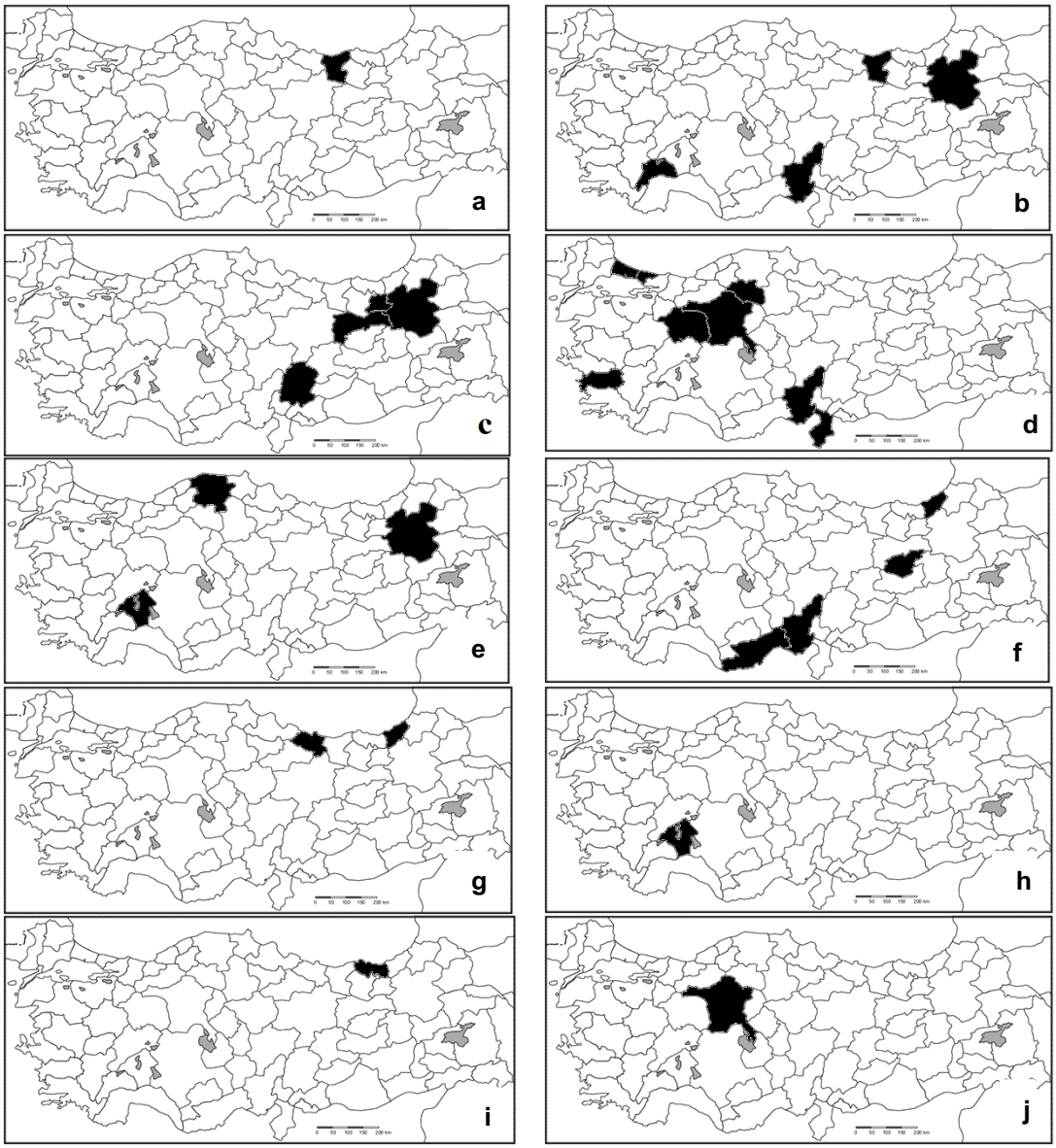

Figure 6. Distribution in Turkey of species: a) *Campoletis agilis*; b) *Campoletis crassicornis*; c) *Diadegma mediterraneum*; d) *Hyposoter didymator*; e) *Agrothereutes fumipennis*; f) *Aptesis senicula*; g) *Bathythrix pellucidator;* h) *Dichrogaster schimitscheki*; i) *Gelis agilis*; j) *Gelis exareolatus*.

**Phygadeuon hercynicus* Gravenhorst, 1829 (Figure 5b)

Material examined. BUNPA: Alaçam, 1100 m, 20.VI.2018, 2 ♂♂, 1200 m, 08.VI.2019, 3 ♂♂; Gözede, 630 m, 06.VIII.2019, 3 ♂♂.

Associated plants. *Angelica sylvestris* L.

Global distribution. European and West Palearctic Region.

Remarks. This is new species record for Turkey.

**Phygadeuon nitidus* Gravenhorst, 1829 (Figure 5c)

Material examined. BUNPA: Alaçam, 1600 m, 19.VIII.2019, 4 ♂♂; Cevizdibi, 850 m, 02.VIII.2018, 5 ♂♂; Gözede, 650 m, 17.VII.2018, 2 ♂♂, 640 m, 18.VII.2019, 5 ♀♀.

Global distribution. European and West Palearctic Region.

Remarks. This is new species record for Turkey.

**Phygadeuon lapponicus* Thomson, 1884 (Figure 5d)

Material examined. BUNPA: Alaçam, 1700 m, 08.VIII.2018, 7 ♂♂.

Associated plants. *Salix* sp.

Global distribution. Palearctic Region.

Remarks. This is new species record for Turkey.

**Thaumatogelis audax* (Olivier, 1792) (Figure 5e)

Material examined. BUNPA: Gözede, 610 m, 05.VIII.2018, 2 ♂♂.

Associated plants. *Deschampsia cespitosa* (L.)

Global distribution. European and West Palearctic Region.

Remarks. This is new species record for Turkey.

Discussion

In Turkey, there was no detailed information available on Ichneumonidae until 1995. Three hundred and eighty-three Ichneumonid species are listed in catalog for Turkey complied by Kolarov (1995). Most of studies have been conducted in Thrace, Eastern Anatolia and Mediterranean Regions of Turkey. With the new records reported here, this number is now 1 291. Previous studies have shown that 124 of the 1,288 species were obtained from economic pests in different orders (Figure 7) (Sarı, 2017).

Figure 7. Ichneumonidae species obtained from different pests.

In total, 263 specimens were collected during the summers of 2018 and 2019. Among these, *A. rufocincta*, *C. thomsoni*, *D. glabriculum*, *D. heteropus*, *M. congener* (Förster, 1876), *P. hercynicus*, *P. lapponicus*, *P. nitidus*, and *T. audax* are new record for the Turkish fauna.

At the end of the study 21 species were identified. Among these, seven species belonging to Campopleginae with 90 individuals and 14 species Cryptinae with 173 individuals were recorded. Cryptinae showed (Table 2) a higher density in terms of both number of species and number of individuals. Species were collected at three different altitudes from 610 to 1700 m with most specimens being collected between 610 and 700 m. Specimens were collected from May to August, with most being collected in July. *Agrothereutes fumipennis* was the most commonly trapped species in net and *D. heteropus* the least captured.

Table 2. Data of species: Individual numbers (IN), vertical distribution (VD), seasonal dynamics (SD), geographical regions (GR), zoogeographical regions (ZR), host records (HR), first record of Turkey (FRT) of specimens

Taxa name	IN	VD	SD	GR	ZR	H	FRT
Subfamily: Campoleginae Förster, 1869							
Genus: *Campoletis* Förster, 1869							
Campoletis agilis (Holmgren, 1860)	17	A, B	Jl, A	BSR	BP, E, WP	x	Kolarov et al., 2016
Campoletis crassicornis (Tschek, 1871)	12	A, B	M, A	BSR, EAR, MtR	BP, E, WP	x	Kolarov & Beyarslan, 1995
**Campoletis thomsoni* (Roman, 1915)	11	A, B	J, A	*	E, EP, WP		New record
Genus: *Casinaria* Holmgren,1859							
Casinaria albipalpis (Gravenhorst, 1829)	14	C, D	J, Jl	?	BP, E, WP	x	Riedel, 2018
Genus: *Diadegma* Förster,1869							
**Diadegma glabriculum* (Holmgren, 1859)	11	A, B, D	Jl, A	*	BP, E, NEAR		New record
Diadegma mediterraneum (Constantineanu, 1930)	12	A, C	J, A	EAR, MtR	BP, E		Kolarov & Beyarslan, 1995
Genus: *Hyposoter* Förster, 1869							
Hyposoter didymator (Thunberg, 1822)	13	A, D	J	CAR, MR, MtR	AUS, BP, E, WP	x	Steiner, 1936
Subfamily: Cryptinae Kirby, 1837							
Genus: *Acrolyta* Förster, 1869							
**Acrolyta rufocincta* (Gravenhorst, 1829)	12	B, E	A	*	E, WP	x	New record
Genus: *Agrothereutes* Förster, 1850							
Agrothereutes fumipennis (Gravenhorst, 1829)	28	B	Jl, A	BSR, EAR, MtR	E, EP, WP	x	Çoruh & Özbek, 2005
Genus: *Aptesis* Förster, 1850							
Aptesis senicula (Kriechbaumer, 1893)	21	B, E	J, A	BSR, EAR, MtR	E, WP		Beyarslan & Kolarov, 1994
Cins: *Bathythrix* Förster, 1869							
Bathythrix pellucidator (Gravenhorst, 1829)	11	C, D	J, Jl	BSR	E, EP, WP	x	Çoruh et al., 2014b
Genus: *Dichrogaster* Doumerc, 1855							
**Dichrogaster heteropus* (Thomson, 1896)	4	B	J, A	*	E, WP	x	New record
Dichrogaster schimitscheki (Fahringer, 1935)	9	A	Jl, A	MtR	E, NEAR, WP		Kolarov & Gürbüz, 2007
Genus: *Gelis* Thunberg, 1827							
Gelis agilis (Fabricius, 1775)	7	B, C	J	BSR	E, EP, WP	x	Fahringer, 1922
Gelis exareolatus (Förster, 1850)	18	A, E	Jl, A	CAR	E, EP, WP	x	Kolarov, 1987
Genus *Mesoleptus* Gravenhost, 1829							
**Mesoleptus congener* (Förster, 1876)	21	A, D	J, Jl, A	*	E, WP		New record
Mesoleptus incessor (Haliday,1838)	9	A, B, E	J, Jl, A	?	E, EP, WP	x	Jussila et al., 2010
Genus *Phygadeuon* Gravenhorst, 1829							
**Phygadeuon hercynicus* Gravenhorst, 1829	8	A, C	J, A	*	E, WP	x	New record
**Phygadeuon nitidus* Gravenhorst, 1829	16	A, B, E	Jl, A	*	E, WP		New record
**Phygadeuon lapponicus* Thomson, 1884	7	E	A	*	E, EP, WP	x	New record
**Thaumatogelis audax* (Olivier, 1792)	2	A	A	*	E, WP	x	New record

Vertical distribution: A, 0-750 m; B, 751-1000 m; C, 1001-1250 m; D, 1251-1500 m; and E, 1501-1750 m. Seasonal dynamics: M, May, J, June; Jl, July; and A, August. Geographical regions: AR, Aegean Region; BSR, Black Sea Region; CAR, Central Anatolia Region; EAR, Eastern Anatolia Region; MR, Marmara Region; and MtR, Mediterranean Region. Zoogeographical regions: AUS, Australasian; E, Europe; EP, Eastern Palearctic; NEAR, Nearctic Region; and WP, Western Palearctic.

Considering distribution by regions, it was found that the species were collected mostly from Mediterranean Region and at least from the Marmara Region. While *A. fumipennis*, *A. senicula*, *C. crassicornis* and *H, didymator and* were collected three different regions, *C. agilis, D. schimitscheki*, *G. agilis* and *G. exareolatus* were collected only one region in previous studies. When considering previous records, *H. didymator* has been collected from seven cities, *A. senicula* and *C. crassicornis* from four cities, and *C. agilis, D. schimitscheki, G. agilis* and *G. exareolatus* from collected only one city. In contrast, *H. didymator* has been recorded in 50 countries worldwide. With this study, Bursa has been added to list of localities each species. Although *C. thomsoni* and *P. lapponicus* occur in the Palearctic region, they were recorded for the first time in Turkey with this study. *Campoletis agilis, D. schimitscheki*, *G. agilis* and *G. exareolatus* have been collected from only one province in Turkey and Bursa became the second known locality for these species with this study. Weeds were also detected in study areas and these weeds are shown in Table 3. Based on all these data, we can say that results of study provide new information and scientific value to scientists who work and want to work in this field.

Table 3. Weeds species in study area

Name of weed	Localities		
	Alaçam	Cevizdibi	Gözede
Prunella vulgaris L.		✓	
Polypodium vulgare L.		✓	✓
Epilobium angustifolium L.	✓		
Sonchus asper (L.) Hill		✓	✓
Nepeta nuda L.		✓	
Globularia trichosantha Fisch. & C.A.Mey.	✓		
Medicago polymorpha L.			✓
Galium elongatum C. Presl	✓		
Mentha longifolia (L.) L.	✓	✓	✓
Dactylis glomerata L.			✓
Malva sylvestris L.			✓
Silene vulgaris (Moench) Garcke	✓		
Onobrychis gracilis Besser	✓		
Securigera varia (L.) Lassen		✓	✓
Matricaria chamomilla L.	✓		
Hypericum perforatum L.		✓	✓
Anthemis cretica L.		✓	✓
Trifolium repens L.	✓	✓	✓
Plantago lanceolata L.			✓
Raphanus raphanistrum L.			✓
Vicia cracca L.	✓		
Allium paniculatum L.			✓

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Original article (Orijinal araştırma)

Seasonal population dynamics of *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) in generative organs of olives in Hatay Province, Turkey[1]

Hatay İli'nde zeytinin generatif organlarında *Aceria olea* (Nalepa, 1900) (Acari: Eriophyidae)'nın mevsimsel popülasyon değişimi

Kamuran KAYA[2*]

Abstract

Olive, *Olea europaea* L., is an evergreen tree native to areas with Mediterranean climates. Its fruit and oil are key ingredients in the Mediterranean cuisine. Olive gall mite, *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) is a mite species that feeds on olive leaves, buds or flowers. This study was conducted to determine the population dynamics of *A. oleae* in generative organs of olive in Altınözü, Antakya and Samandağ Districts of Hatay between in April and October 2017 and 2018. The highest mite density was detected in Altınözü during the flowering period in 2017 and the budding period in 2018; and a second peak was seen during fruiting period for both years. A peak occurred in samples collected from Antakya for both years (in fruit stage, June 2017, and in budding stage, April 2018) but no prominent peak was noted Samandağ samples. A high rate of flower and fruit dropping was observed and this concurred with noticeable decreases in *A. oleae* population in Altınözü and Antakya Districts in both years. Three phytoseiid species, *Typhlodromus* (*Anthoseius*) *athenas* Swirski & Ragusa, 1976 (44%), *Typhlodromus* (*Anthoseius*) *rapidus* Wainstein & Arutunjan, 1968 (29%) and *Typhlodromus* (*Typhlodromus*) *athiasae* Porath & Swirski, 1965 (26%) (Acari: Phytoseiidae) were detected in these orchards. It is thought they probably fed on *A. oleae* since their primary prey, tetranychid mites were unavailable. Correlation results showed that population sizes of *A. oleae* did not decrease significantly with temperature and humidity in 2017. In 2018, the population was negatively correlated with temperature, but no significant increase occurred as humidity rose.

Keywords: Olive, olive gall mite, population dynamics, Turkey

Öz

Zeytin, *Olea europaea* L., Akdeniz iklimine sahip bölgelere özgü, yaprağını dökmeyen bir ağaçtır. Meyvesi ve yağı, Akdeniz mutfağının temel malzemeleridir. Zeytin tomurcuk akarı, *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) zeytinin yaprakları, tomurcukları veya çiçekleriyle beslenen bir akar türüdür. Bu çalışma, Hatay'ın Altınözü, Antakya ve Samandağ ilçelerinde bulunan zeytinlerin generatif organlarında, 2017-2018 yıllarının Nisan-Ekim ayları arasında *A. oleae*'nin popülasyon dalgalanmalarını belirlemek amacıyla yapılmıştır. Altınözü'nde en yüksek popülasyon yoğunluğu 2017'de çiçeklenme döneminde, 2018'de tomurcuk döneminde tespit edilmiştir. Altınözü'nde her iki yılda meyve döneminde ikinci pik görülmüştür. Antakya'dan toplanan örneklerde her iki yıl için de tek pik meydana gelmiştir (Haziran 2017'de meyve döneminde ve Nisan 2018'de tomurcuk döneminde). Ancak Samandağ örneklerinde belirgin bir tepe noktası belirlenmemiştir. Altınözü ve Antakya ilçelerinde her iki yılda da yüksek oranda çiçek ve meyve dökümü görülmüş ve bu durum *A. oleae* popülasyonunda meydana gelen gözle görülür düşüşlerle uyumlu olmuştur. Bu bahçelerde, *Typhlodromus* (*Anthoseius*) *athenas* Swirski & Ragusa, 1976 (44.44%), *Typhlodromus* (*Anthoseius*) *rapidus* Wainstein & Arutunjan, 1968 (29.17%) ve *Typhlodromus* (*Typhlodromus*) *athiasae* Porath & Swirski, 1965 (26.4%) (Acari: Phytoseiidae) olmak üzere üç phytoseiid akar türü belirlenmiştir. Bu türlerin muhtemelen birincil avları olan tetranychid akarları mevcut olmadığı için *A. oleae* ile beslendikleri düşünülmektedir. Korelasyon sonuçları, *A. oleae* popülasyon büyüklüğünün 2017'de sıcaklık ve nem ile önemli düzeyde azalmadığını göstermiştir. 2018'de popülasyon, sıcaklıkla negatif korelasyon gösterirken, nemin yükselmesi ile önemli bir artış meydana gelmemiştir.

Anahtar sözcükler: Zeytin, zeytin tomurcuk akarı, popülasyon değişimi, Türkiye

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Introduction

Olive (*Olea europaea* L., Oleaceae) is a broadleaved evergreen tree native to the Mediterranean basin and other areas with Mediterranean climates. Its fruit and oil are highly valued and of major agriculture importance, especially in Mediterranean countries. Olives are one of the oldest known cultivated plants that spread from the original Mesopotamia Region of Turkey, which is considered as its origin in the Mediterranean basin. For more than 10 years, the Turkish government has implemented a comprehensive program to support and promote olive growing and production on suitable uncultivated areas. Turkey is the fourth largest producer of olives in the world with 1.5 Gt produced per year, followed by Spain, Italy and Morocco (FAO, 2018). Hatay Province has 6.3% of the olive production areas in Turkey and is ranked sixth among the provinces in the country in terms of olive production (Anonymous, 2015).

Olive-infesting eriophyid mites are economically important agricultural pests that have been reported on olives in Egypt and Tunisia (Abou-Awad et al., 2005; Chatti et al., 2017) from North Africa; in Albania and Greece (Hatzinikolis, 1986; Shahini et al., 2009) from Europe and in Israel (Avidov & Harpaz, 1969) from the Middle East. Tzanakakis (2003) indicated that 12 of the 30-mite species reported from olives belong to the family Eriophyidae. Among these mites, Hatzinikolis (1971) reported that species like *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) damage olive flowers and young fruits, and significantly inhibit growth of olive trees in olive plantations.

Many previous studies have determined the plant feeding pests affecting olive trees and their distribution in olive groves of Turkey; however, only a few of these studies have focused on phytophagous mites, especially eriophyid mites, in such olive plantations. For instance, the first records of the presence of *A. oleae* in Turkey were by Alkan (1952) and İyriboz (1968). The other studies that have studied the presence and have reported the economic importance of eriophyoids, *A. oleae* in particular, in different provinces of Turkey, report that it is in Bursa (Kumral & Kovancı, 2004), in Mersin together with *Aculus olearius* Castagnoli, 1977 (Acari: Eriophyidae) (Çetin & Alaoğlu, 2006) and in seven provinces of the Eastern Mediterranean Region (Kaçar et al., 2010). Çetin et al. (2012) found this species mixed with populations of *A. olearius* in İzmir, Manisa and Balıkesir Provinces. They determined a 26-82% infestation rate of trees and an 8-59% injury rate of fruit by these mites. Also, recently, Ersin et al. (2020) found *A. oleae* mixed with *Tegolophus hassani* (Keifer, 1959) (Acari: Eriophyidae) populations in the same provinces.

Although a study by Kaçar et al. (2010) reported the presence of *A. oleae* in Hatay Province, no detailed information was provided regarding their population dynamics on olive trees. This study was conducted to determine the population dynamics of *A. oleae* in olive orchards in three districts that are the most important districts in terms of production areas in Hatay Province of Turkey. Concurrently, we aimed to detect phytoseiid species on olives in the study area.

Materials and Methods

The study was conducted in Altınözü (36°07'10"N, 36°15'59"E, 269 m), Antakya (36°14’34"N, 36°09'38"E, 90 m) and Samandağ Districts (36°09'27"N, 36°00'40"E, 207 m) in Hatay (Turkey), between the months of April and October in 2017 and in 2018 (Figure 1).

From each district, an orchard with trees infested with only *A. oleae* species were randomly selected, and five trees located at different points in such orchards were sampled. The sampled trees were Gemlik cultivars that were about 25 years old in Altınözü and Antakya, and 15 years old in Samandağ. These cultivars were not irrigated and had no chemical pesticides sprayed on them to control any pests and diseases during this study. According to the phenological stage of the plants, 20 cm long shoots containing buds, flowers or fruits were collected every 2 weeks from the four cardinal directions of each selected tree (20 shoots from each orchard). A pole pruner was used to collect these shoots from a height of 1-1.5 m

aboveground. Samples were collected from 11 April to 24 October 2017 and from 29 March to 10 October 2018. Shoot samples were brought to the laboratory and kept in a refrigerator at ~4°C until their inspection. From each of these shoots, five buds or flower clusters or fruits (100 samples of generative organs for each orchard) were taken randomly and examined, and mobile stages of *A. oleae* on these samples were counted directly under a stereomicroscope. Population curves were plotted using averages of the mite populations on five trees for each district.

Eriophyid specimens were collected with insect pins or parts of the plants with mite colonies were cut with a lancet under a stereomicroscope. Collected specimens were preserved in 70% ethanol until identification. Preparations and identifications were made as Keifer (1975 a, b). Identification of the mite was made by Assoc. Prof. Evsel Denizhan (Trakya University, Faculty of Science, Department of Biology, Edirne, Turkey).

Predatory mites (Phytoseiidae) were also collected during the study. Phytoseiid specimens were collected with a fine brush under stereomicroscope. Collected specimens were preserved in 70% ethanol until their preparations. Specimens were cleared in lactophenol solution and mounted in Hoyer medium. The phytoseiids were identified by Prof. Dr. Nabi Alper Kumral (Uludağ University, Faculty of Agriculture, Department of Plant Protection, Bursa, Turkey) according to Çobanoğlu (1997), Swirskii & Ragusa (1976), and Chant & Yoshida-Shaul (1987) for *Typhlodromus* (*Anthoseius*) *rapidus* Wainstein & Arutunjan, 1968, *Typhlodromus* (*Anthoseius*) *athenas* Swirski & Ragusa, 1976 and *Typhlodromus* (*Typhlodromus*) *athiasae* Porath & Swirski, 1965 (Acari: Phytoseiidae), respectively. Identified samples were kept in the collection of Kamuran Kaya at the Department of Plant Protection, University of Hatay Mustafa Kemal, Turkey. Daily and monthly average meteorological data collected by meteorological stations in each district were taken from the Central Hatay Meteorological Station. Correlations between climatic factors and the mite population was performed using SPSS software (SPSS, 2012).

Figure 1. Map showing the three districts where the study was conducted in Hatay Province

Results and Discussion

In Altınözü District, *A. oleae* (Eriophyidae) had similar population density curves in both years of study with two peaks occurring in each year (Figures 2 & 3). These peaks were observed in 9 May and 20 June 2017 (514 mites/20 flower clusters and 232 mites/20 fruits, respectively); and on 12 April and 21 June 2018 (923 mites/20 buds and 558 mites/20 fruits, respectively). The highest density of population was detected during the flowering and budding stages of 2017 and 2018, respectively. The first 4 months of

2018 had higher average temperatures and relative humidity compared to the previous year; this might be the reason for the higher population density. For example, the humidity and temperature values in February were 53.4% and 8.7°C for the year 2017, and 80% and 11.3°C for the 2018 (Figure 4). Similarly, Ersin et al. (2020) found that the highest population densities of eriophyid mites on buds were in April and on leaves and fruits in May and June during their 2016-2017 survey in Western Turkey. In contrast, Abou-Awad et al. (2005) reported the highest population densities of *A. oleae* and *T. hassani* on leaves occurred in mid-July in Egypt. Shahini et al. (2009) also reported that the highest population densities of *A. oleae* on flowers of olive trees were in May and on fruits in June, 2001 in Albania. This information supports our findings. In our present study, the first peak and the highest population were observed on flowers in May (2017) and on buds in April (2018). Second peak in both years were observed in June on fruits, but then the population started to decrease. During this period, the average relative humidity (RH) and temperature values were 62.5% and 24.9°C in 2017 and 66.6% and 24.6°C in 2018, respectively. Vacante (2015) established that *A. oleae* moves to the leaves as the fruit grows and forms denser populations there during the summer. Likewise, we reasoned that the majority of the population moved to the leaves in the presence of optimal conditions for eriophyid mites (data not shown).

The population density of *A. oleae* in Samandağ District was quite low during the whole season. This may be due to the low initial mite population density on the buds since the olive trees in Samandağ were much younger than the ones grown in other districts. Also, Samandağ is located on the coast and such harsh climatic conditions on olive trees, pruning time, and pressure of phytoseiid mite as a result of feeding, may have influenced the population densities of *A. oleae*. While there was no prominent peak for both years, the highest population occurred in 23 May 2017 (38 mites/20 flower clusters) and in 12 April 2018 (51 mites/20 buds) (Figures 2 & 3).

A single peak occurred in Antakya District in both years (Figures 2 & 3); on 20 June 2017 with 201 mites/20 fruits and on 12 April 2018 with 860 mites/20 buds. Also, the higher average temperatures and RH in the first 4 months of 2018 compared to the previous year caused higher population densities in Antakya and this peak population density was reached earlier during these months. Shahini et al. (2009) also detected that between April 2001 and September 2002 the highest *A. oleae* population on fruits for the two consecutive years was in June. Although the climatic factors affect the eriophyid mite population, the reason for different population densities may be due to the local abundance of eriophyids on leaves, buds or fruits (Ersin et al., 2020).

In 2018, the olive trees started their budding, flowering and fruiting periods 2 weeks earlier than in the previous year for all districts. Consequently, *A. oleae* populations were generally higher in 2018 and reached the first peak in Altınözü about a month earlier compared to 2017. In Antakya, the only peak that occurred in both years was in April during the budding period in 2018, which was earlier than the previous year. All of these differences regarding the time of phenological periods, time of peaks and size of mite population may be related to differences in climatic conditions, especially between January-April (Figure 4).

Considerable declines were observed in the populations of the pest starting the end of June in 2017 in the three districts where the study was conducted. In the following year (2018), these declines occurred at the end of April in Antakya and at the end of June in Altınözü. As temperature started rising, the majority of the mite populations moved to the leaves. Also, the high rate of flower and fruit dropping observed in both years resulted in significant decreases in population of the mite in both Altınözü and Antakya. Similar declines were also reported by Çetin et al. (2012). Although the population sizes were apparently declined with temperature and RH in 2017 there was no significant correlation (Table 1). In 2018, the population also was negatively correlated with the temperatures for each district, but at a significant level ($p < 0.05$ for Altınözü and Antakya; $p < 0.01$ for Samandağ). The population was not significantly correlated with humidity in any districts in 2018.

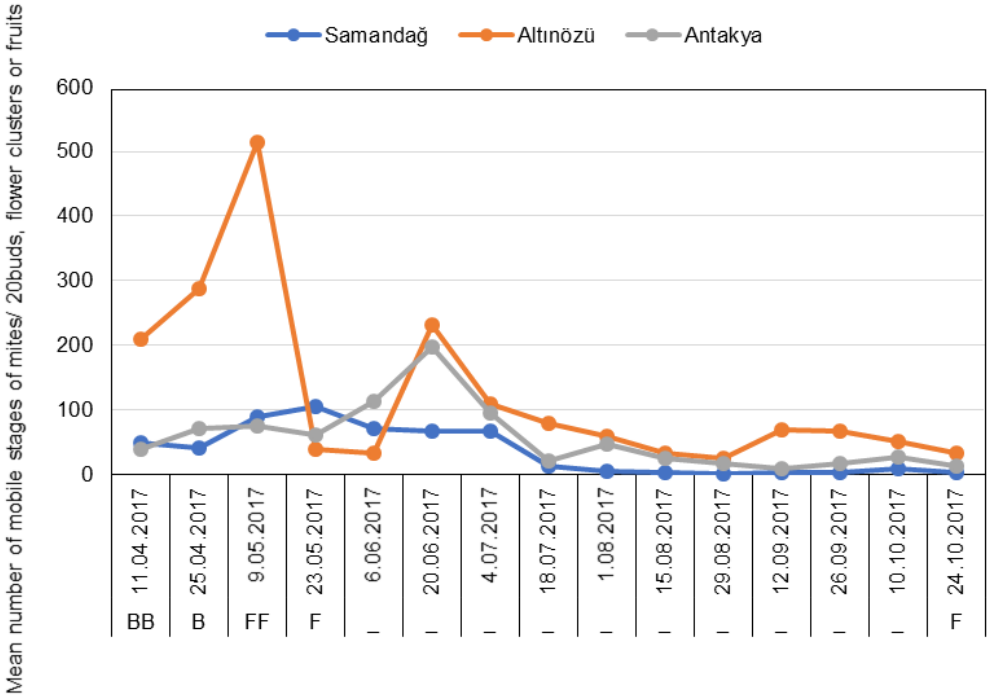

Figure 2. Population dynamics of *Aceria oleae* in three districts of Hatay Province in 2017 (BB, beginning of budding; B, budding; FF, flowering; and F, fruit).

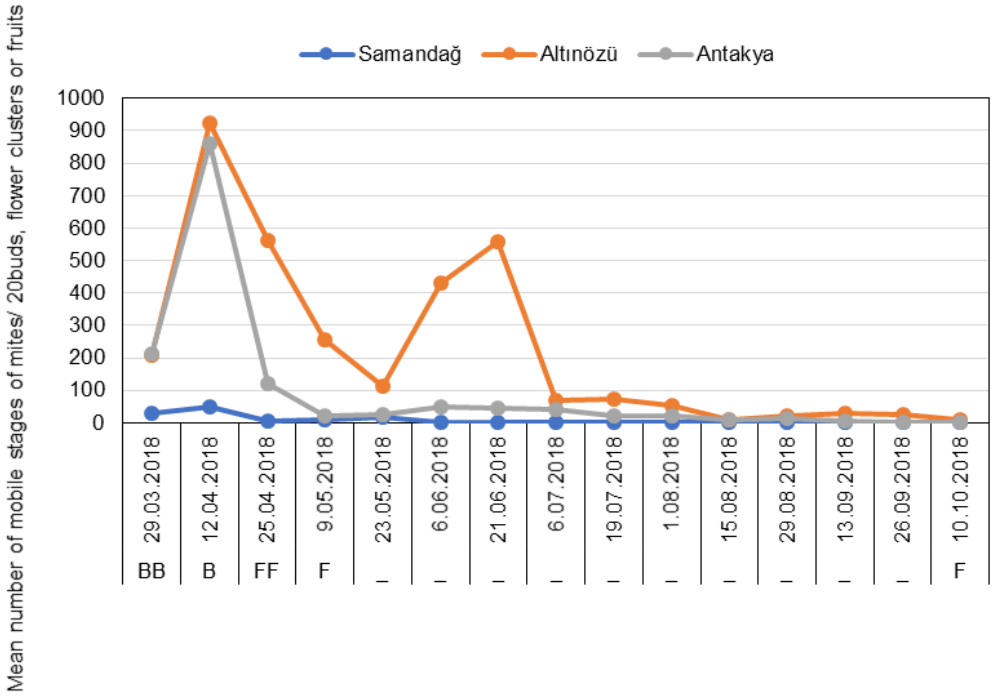

Figure 3. Population dynamics of *Aceria oleae* in three districts of Hatay Province in 2018 (BB, beginning of budding; B, budding; FF, flowering; and F, fruit).

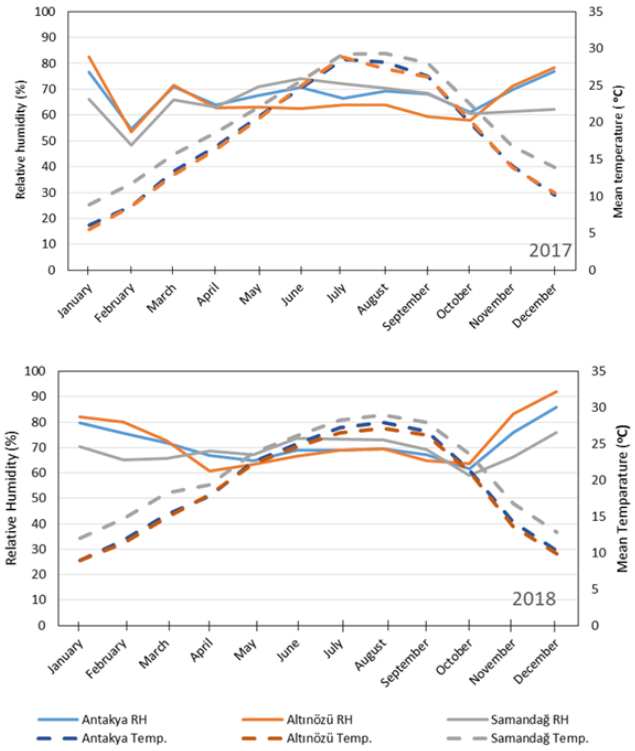

Figure 4. Meteorological data values (RH, relative humidity; and Temp., mean temperature) in three districts in 2017 and 2018.

Table 1. Correlation coefficient between temperature, relative humidity and eriophyid mite population sizes during two consecutive years

	Correlation coefficient values			
	2017		2018	
	Temperature	Humidity	Temperature	Humidity
Altınözü	-0.323	-0.353	-0.581*	0.133
Samandağ	-0.445	-0.151	-0.780**	0.186
Antakya	-0.035	-0.317	-0.612	0.420

* $p<0.05$; ** $p<0.01$.

Hatzinikolis (1971) and Shahini et al. (2009) also reported that about 90% of the mites were found in flower clusters. They suggested that the attacks on flowers and fruits resulted in drying and dropping of flowers or premature dropping of fruits. Similar results about flower and fruit dropping were reported by Hatzinikolis (1981), Lindquist et al. (1996) and Denizhan et al. (2015). Also, Çetin & Alaoğlu (2006) reported that *A. oleae* populations were at the highest during budding period, especially close to the bud-burst period; and this high population caused the dropping of buds. In the current study, the olive trees in Altınözü and Antakya were observed to frequently drop their buds during the budding period of 2018.

It was observed that the high population of mite feeding on the olive fruits caused split, deformed and/or small fruits, and also there were silvery or brown spots on and around the stem pit of the fruits. The symptoms observed on fruits collected from Altınözü in 21 June 2018 are shown in Figure 5. Similar symptoms were indicated by many other researchers (Laccone & Nuzzaci, 1977; Lindquist et al., 1996; Elhadi & Birger, 1999; Çetin & Alaoğlu, 2006).

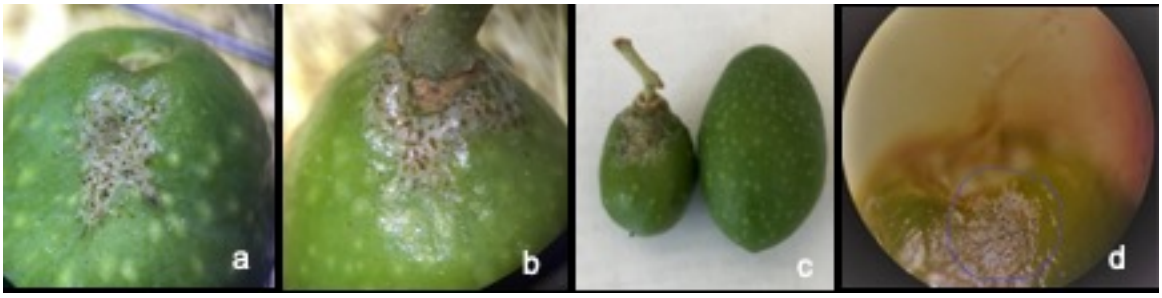

Figure 5. a-b) Symptoms caused by A*ceria oleae* feeding on olive fruit, c) *A. oleae* infested and healthy olive fruit, d) *A. oleae* colony on fruit.

Three phytoseiid mite species were obtained in the orchards where the population dynamics of *A. oleae* were followed. These species and the orchards they collected are given in Table 2.

Table 2. Phytoseiid species and their collection dates in the olive orchards in three districts of Hatay Province in 2017-2018.

	Altınözü		Antakya		Samandağ	
	2017	2018	2017	2018	2017	2018
Typhlodromus (*Anthoseius*) *athenas*	01.08 (1)* 24.08 (1) 10.10 (1)	09.05 (1) 06.07 (1) 19.07 (2) 01.08 (3) 15.08 (2)	05.04 (1) 09.05 (1) 04.07 (1) 26.09 (2) 10.10 (1) 24.10 (2)	25.04 (1) 06.07 (1) 19.07 (3) 01.08 (2) 15.08 (3)	04.07 (1)	01.08 (1)
Typhlodromus (*Anthoseius*) *rapidus*				22.06 (1) 06.07 (1) 19.07 (1) 01.08 (1) 15.08 (9)	04.07 (1)	06.06 (1) 06.07 (1) 19.07 (5)
Typhlodromus (*Typhlodromus*) *athiasae*		09.05 (1)	18.07 (9)		09.05 (2) 24.08 (3)	19.07 (2) 01.08 (1) 15.08 (1)

* The numbers in parentheses indicate the number of individuals.

All three species were observed in Samandağ and *T. athenas* was observed in all districts in both of the years. *T. rapidus* was found in Antakya in 2018 while it was not observed in Altınözü. *Typhlodromus athiasae* was detected in Antakya in 2017 and in Altınözü in 2018. The most common species were *T. athenas* in Antakya and Altınözü, and *T. athiasae* in Samandağ. According to the collected samples, incidence rates of *T. athenas*, *T. rapidus* and *T. athiasae* were 44, 29 and 26%, respectively. Previous studies have determined that *T. athiasae* is the most common species in Turkey. It was found for first time on citrus orchards in Turkey (Antalya) by McMurtry (1977). In subsequent studies, while *T. athiasae* was detected on many host plants, it was reported as one of two most abundant species in olive orchards only in Bursa (Kumral et al., 2010). Kumral (2005) found *T. athiasae* on trees of six different pome and stone fruits and specified that the species was commonly found in areas where the Tetranychidae species were found. These phytoseiid mite species we detected prefer tetranychid mites as food but as no tetranychid mites were on the olive trees we studied, we reasoned that these phytoseiids fed on *A. oleae*. Ersin et al. (2020) also reported that the phytoseiids detected in their survey probably feed on eriophyid mites since their primary prey (tetranychid mites) were not found in these olive orchards.

Also, it has been determined that some phytoseiids are capable of reducing populations of *A. oleae* in olive production (Abou-Awad et al., 2005). Momen (2009) reported that developmental periods of *T. athiasae* were similar when reared on *Eriophyes dioscoridis* Soliman & Abou Awad, 1977 and *Tetranychus urticae* Koch, 1836. Also, a higher net reproduction rate in a shorter mean generation time was observed when reared on *E. dioscoridis*. These results give important information on the superiority of predacious mite *T. athiasae* over eriophyid mite. *Typhlodromus athenas* has been frequently observed on olive trees in previous studies (Chatti et al., 2017). It has been reported that this species is well adapted to high temperatures occurring in the Mediterranean Region and it may be a useful biological control agent (Kolokytha et al., 2011). *Typhlodromus athenas* was first reported on olives from the Western Turkey by Ersin et al. (2020). *Typhlodromus rapidus* was recorded before by Çobanoğlu (1997) on hazel and Çakır et al. (2020) on walnut; however, there is no record on olive in Turkey. This study is the first to report the three phytoseiid species detected in olive growing areas of Hatay. These records are valuable because the phytoseiid mites play an important role in preventing outbreaks of various phytophagous mites, especially tetranychid and eriophyoid mites, in natural habitats (Edland & Evans, 1998).

According to Abou-Awad et al. (2005), differences in eriophyid mite populations can be caused by many factors such as variety, shady/sunny or height from the ground where sample was taken from the tree and leaf age. In addition to these factors, differences in meteorological data between districts and natural enemies are thought to have an impact on these populations. Recently, Ersin et al. (2020), determined that there were differences in the population densities of eriophyid mites in different orchards during the same period. According to their comments, the reason for their high number in some years might be their local abundance on some leaves, buds or fruits. Some phytoseiids were shown to be capable of controlling the population of *A. oleae* in olive nurseries (Abou-Awad et al., 2005). Also, it was reported that *T. athiasae* was successful in controlling tetranychids and eriophyids on some fruit species (McMurtry, 1977; Kumral & Kovancı, 2007). Thus, some authors have reported that there is a stable mite community with a positive relationship between phytophagous mites and their predominant predators (Amano & Chant, 1990; Abou-Awad et al., 2000).

In the current study, the seasonal status of the populations of *A. oleae* in generative organs of olives in three districts of Hatay Province (Turkey) was observed. The highest density of population was detected on flowers in May and fruits in June 2017; and on buds in April and fruits in June 2018. The high rate of bud, flower and young fruit dropping, deformations and smaller fruit formations occurred depending on the mite population. It was also determined that different species of phytoseiid mites were widespread in olive orchards. Further studies on this pest, i.e. resistance or susceptibility to different olive cultivars, effects of the mite on yield and product quality or olive oil obtained, and interactions with its predators, would contribute to fundamental knowledge and also to the implementation of the integrated pest management studies.

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Original article (Orijinal araştırma)

Genetic diversity of Turkish populations of *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae)[1]

Türkiye *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae) popülasyonlarının genetik çeşitliliği

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Abstract

The genetic diversity and population genetics of the citrus mealybug, *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae), were investigated based on sequencing of mitochondrial DNA. A total of 108 individuals were collected from different host plants in Turkey during 2011-2015. Partial sequences of the cytochrome c oxidase subunit 1 gene revealed five haplotypes in Turkey, with one of these (Hap 1) as the common haplotype, being present in 102 individuals. Additionally, 90 homologous nucleotide sequences retrieved from GenBank were incorporated into the analyses and compared with sequences of isolates from Turkey. Molecular diversity indices revealed overall high mitochondrial DNA diversity for these populations. Further, Tajima's D test and Fu's Fs test showed negative values, except in South Africa, indicating deviations from neutrality and suggesting recent population expansion for the populations. Pairwise comparisons of the different populations using the pairwise fixation index$_t$ was significant for some comparisons, indicating genetic differentiation among the *P. citri* populations studied. Based on these findings and those from earlier studies, it was hypothesized that demographic expansion has occurred in *P. citri* via the introduction of mealybugs by anthropogenic movements.

Keywords: Citrus mealybug, haplotype diversity, Mediterranean, mtDNA, molecular phylogenetic

Öz

Turunçgil unlubiti, *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae)'nin genetik çeşitliliği ve popülasyon genetiği, mitokondriyal DNA bölgesine göre araştırılmıştır. Türkiye'de 2011-2015 yıllarında farklı konukçu bitkilerden toplam 108 birey toplanmıştır. Sitokrom C oksidaz alt birim geninin kısmi gen dizileri Türkiye'de beş haplotip olduğunu ortaya çıkarmıştır, bunlardan biri (Hap 1) yaygın haplotip olarak 102 örnekte saptanmıştır. Ayrıca, 90 homolog nükleotit *P. citri* sekansları analizlerimize dahil edilmiş ve Türkiye izolatlarına ait diziler ile karşılaştırılmıştır. Moleküler çeşitlilik indeksleri, bu popülasyonlar için genel olarak yüksek mitokondriyal DNA çeşitliliğini ortaya çıkarmıştır. Ayrıca, Tajima'nın D testi ve Fu'nun Fs testi Güney Asya hariç popülasyonların nötral değişim endeksinde sapmaları ve son popülasyon yayılmasını ifade eden negatif değerler göstermiştir. Çalışılan *P. citri* popülasyonları arasında genetik farklılaşmayı gösteren, ikili sabitleme endeksi kullanan farklı popülasyonların ikili karşılaştırmaları bazı bölgeler için önemli bulunmuştur. Bu çalışma ve daha önceki çalışmalardan elde edilen bulgulara dayanarak, antropojenik hareketlerle *P. citri*'de demografik genişlemenin meydana geldiği varsayılmaktadır.

Anahtar sözcükler: Turunçgil unlubiti, haplotip çeşitliliği, Akdeniz, mtDNA, moleküler filogenetik

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Introduction

Citrus mealybug, *Planococcus citri* Risso, 1813 (Hemiptera: Pseudococcidae), is an important pest on cultivated and agricultural plants such as banana, citrus, cotton, grapes, and ornamentals, such as chrysanthemum (Ben-Dov, 1994; García Morales et al., 2016; Karacaoğlu & Satar, 2017a). Citrus mealybug can feed directly on the phloem tissue of the plants and cause early dropping of flowers and fruits, and the honeydew from mealybugs leads indirectly to the development of sooty mold on leaves and fruit surfaces, which negatively affects photosynthesis (Lodos, 1986; Uygun, 2001; Douglas & Kruger, 2008; Meyer et al., 2008; Nakaune et al., 2008; Satar et al., 2013). The citrus mealybug is a well-known pest in agricultural crops around the world, and over the past two decades there has been increased damage by citrus mealybug, causing serious yield losses in the East Mediterranean Region of Turkey (Karacaoğlu & Satar, 2017b).

Comprehensive control methods to suppress of the pest are chemical and biological control in citrus production areas of Turkey which spreads along the Mediterranean Region and westward to İzmir in the Aegean Region. However, these methods could lead to yield different results in the application areas. In the west part of the Mediterranean Region generally is applying biological control and getting positive result (Yayla & Satar, 2012; Karacaoğlu & Satar, 2017b; Telli & Yiğit, 2019). However, the eastern regions had some problem with biological control and even chemical control has not enough successful in some zone of this area. Especially, no positive results have been obtained after releasing of predators and parasitoids at the lower Seyhan District in the East Mediterranean in the last two decades (Karacaoğlu, 2016; Karacaoğlu & Satar, 2017b). The reasons for these differences might be the development of resistance to the insecticides, effect of the host plants, geographic or climatic differences factors. Moreover, introducing the subspecies or invasive form of *P. citri* into new geographical areas by importing or exporting fruit or ornamental plants may also explain these observed differences. This may have resulted in the *P. citri* group or complex in the region.

Reliable techniques for correct identification of taxa are essential for establishing accurate control programs (Lourenco et al., 2006). The mealybug species on citrus in all the Mediterranean Region of Turkey was identified as *P. citri* by taxonomy since 1950s (Bodenheimer, 1951; Soylu & Ürel, 1977; Kansu & Uygun, 1980; Karacaoğlu et al., 2016). However, morphological identification is needed to be done by professional expertise, even when insects are provided at the right stage and as adult females, one cannot distinguish *P. citri* individuals from those of closely related species (Cox, 1989; Malausa et al., 2011). Nowadays, the most commonly used tools to detect differences among individuals and populations are molecular techniques. Many researchers have aimed at using DNA markers to the taxa concerned are closely related species, biology, mealybug-symbiont coevolution, and epigenetic mechanisms of mealybugs (Baumann & Baumann, 2005; Khosla et al., 2006; Hardy et al., 2008; Rugman-Jones et al., 2009; Daane et al., 2011; Beltrà et al., 2012; Roda et al., 2013; Wang et al., 2016a, b; Huang et al., 2017; Poveda-Martinez et al., 2019). Mitochondrial DNA (mtDNA) has been used extensively in population studies of many insect species because it evolves rapidly and, unlike nuclear DNA, it lacks recombination and many regions are conserved (Roderick, 1996; Malausa et al., 2011).

This study aimed to reveal whether the differences arising in the struggle of mealybug control are based on a genetic differentiation and compare the Turkish population with world population. Therefore, phylogenetic relationships among different *P. citri* populations from Turkey and other countries from different continents were investigated. Mealybugs were collected from widely spread major citrus production areas and other host plants such as vegetables, shrubs and ornamental plants in Turkey, and a partial of the mitochondrial cytochrome c oxidase subunit 1 (COI) region was sequenced from each individual insect. The results obtained yield information about the genetic variation of citrus mealybug populations in the citrus production regions of Turkey and the presence of subspecies or cryptic species are discussed. Also, the geographic origin of *P. citri* were traced with population genetic analyses with specimens from Turkey and other locations in GenBank.

Materials and methods

Sampling of *Planococcus citri* populations and identification

The survey was conducted to collect *P. citri* samples between July and October from the Aegean Region in 2011-2013, and June from the Mediterranean Region of Turkey in 2011-2015 (Figure 1 & Table 1). Mealybugs were mainly sampled from citrus orchards but collections were also made from the fruits, leaves and branches of weeds, ornamental plants and vegetables infested with mealybugs. The infected plant materials were brought to the Citrus Pest Laboratory, Department of Plant Protection, Çukurova University and inspected under a Leica S8APO (Leica Microsystems Ltd., Wetzlar, Germany) binocular microscope. Mealybug samples were stored in 1.5-mL Eppendorf tubes containing 99% ethanol at -80ºC. Preparations of mealybugs were made for morphological identification according to Kozar & Kosztarab (1988), then identified by Dr. Bora Kaydan (Çukurova University, Vocational School of Imamoğlu, Adana, Turkey) and deposited in the collection of Citrus Research Laboratory at Çukurova University.

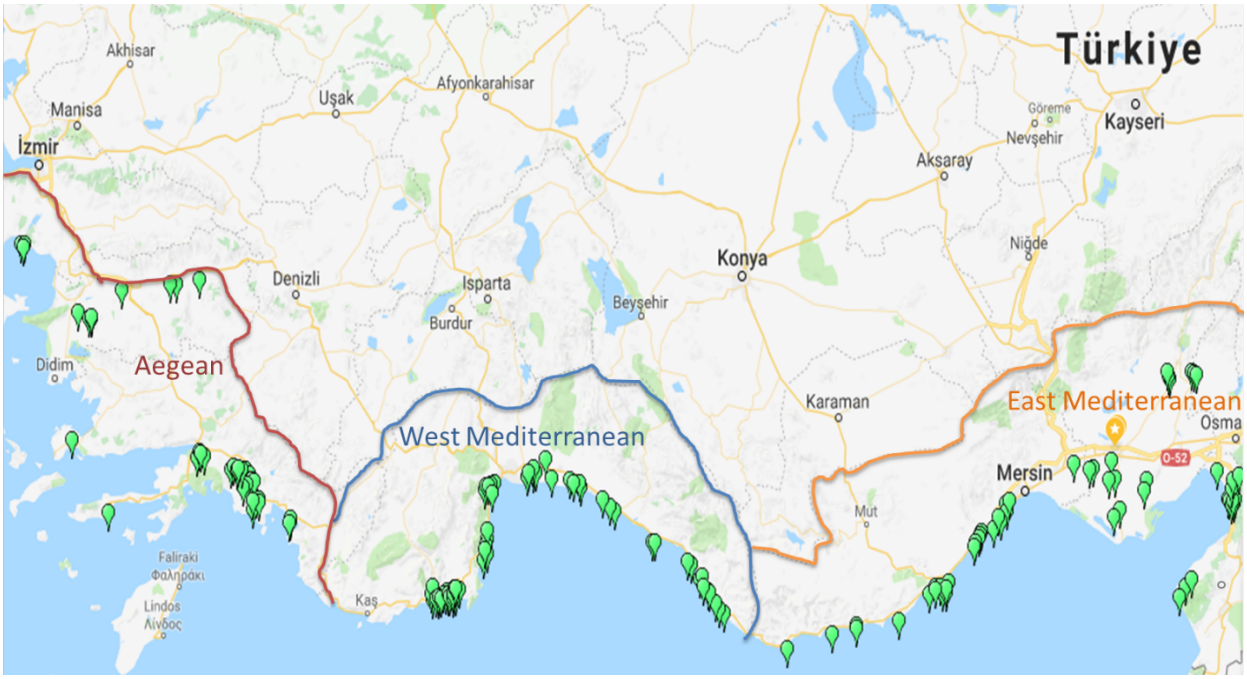

Figure 1. Sampling site for *Planococcus citri* populations in three regions of Turkey.

DNA isolation and PCR amplification

Genomic DNA of 108 specimens was extracted from an adult using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacture's protocol. The primer pairs, C1-J-2183 (5'-CAACATTTAT TTTGATTTTTTGG-3') and C1-N-2568 (5'-GCWACWACRTAATAKGTATCATG-3') were used for PCR amplification of mealybug mitochondrial DNA as described by Malausa et al. (2011). The reaction mixture was prepared with a total volume of 50 µl containing Taq buffer (10X), 2.5 mM MgCl2, 250 µM dNTPs, 1 µM primer, 0.5 U Taq, and 5 µl DNA template. The PCR conditions were: 1 min at 95ºC for predenaturation, followed by 35 cycles of 95ºC for 15 s, 52ºC for 15 s, 72ºC for 1 min, and last extension of 72ºC for 7 min. The PCR products were run on 1% agarose gel, and viewed over a UV light. The PCR products were subjected to Sanger sequencing by Molgentek®, Istanbul, Turkey.

Table 1. List of insect specimens examined in this study from three regions of Turkey, including collection date, location, and host plant

No	Date	Province	District	Host plant	Latitude (E)	Longitude (N)
East Mediterranean						
1	07.06.2011	Adana	Seyhan	*Capparis spinosa* L. (1753)	35º01'66.13"	36º09'85.19"
2	09.06.2011	Adana	Yüreğir	*Kalanchoe blossfeldiana* Poelln (1934)	35º03'41.17"	37º00'12.20"
3	12.06.2011	Adana	Kadirli	*Solanum muricatum* Aiton, (1789)	36º05'23.87"	37º02'21.00"
4	24.06.2011	Adana	Yüreğir	*Kalanchoe blossfeldiana*	35º03'41.17"	37º00'12.20"
5	24.06.2011	Adana	Yüreğir	unknown	35º03'41.17"	37º00'12.20"
6	01.07.2011	Adana	Ceyhan	*Citrus aurantium* L. (1754)	35º04'84.60"	37º01'57.66"
11	13.07.2011	Mersin	Tarsus	*Lycopersicon esculentum* L. (1753)	35º02'38.85"	36º05'83.13"
12	13.07.2011	Mersin	Tarsus	*Schefflera* spp.	35º02'38.85"	36º05'83.13"
13	13.07.2011	Mersin	Tarsus	unknown	35º02'38.85"	36º05'83.13"
14	13.07.2011	Mersin	Tarsus	*Ficus benjamina* L. (1767)	35º02'38.85"	36º05'83.13"
15	14.10.2011	Mersin	Tarsus	*Cydonia oblonga* Mill. (1768)	34º05'33.92"	36º05'50.55"
16	17.11.2011	Mersin	Anamur	*Musa paradisiaca* L. (1753)	32º05'24.20"	36º05'46.00"
17	18.11.2011	Mersin	Silifke	*Citrullus lanatus* (Thunb.) Matsum. & Nakai, (1916)	34º00'15.85"	36º02'02.41"
18	15.12.2011	Adana	Yüreğir	*Punica granatum* L. (1753)	35º03'41.17"	37º00'12.20"
19	19.04.2012	Adana	Çukurova	*Begonia* sp.	35º01'74.21"	37º02'09.51"
20	04.06.2012	Adana	Seyhan	*Malva sylvestris* L. (1753)	35º01'66.13"	36º09'85.19"
24	11.07.2012	Adana	Yüreğir	*Citrus paradisi* Macfad. (1830)	35º06'08.00"	37º00'29.53"
26	11.09.2012	Adana	Yüreğir	*Vitis* sp.	35º03'41.17"	37º00'12.00"
27	17.10.2012	Adana	Yüreğir	*Diospyros kaki* L.F., 1782	35º02'09.14"	36º05'81.02"
29	16.02.2013	Mersin	Anamur	*Musa paradisiaca*	32º05'21.62"	36º06'12.96"
45	12.06.2013	Mersin	Tarsus	*Citrus sinensis* (L.) Osbeck 1765	35º00'17.52"	36º09'94.42"
46	12.06.2013	Mersin	Silifke	*Citrus limon* (L.) Osbeck 1765	33º06'35.00"	36º38'27.83"
47	14.06.2013	Adana	Seyhan	*Ceratonia siliqua* L. (1753)	35º01'83.45"	37º00'40.87"
48	16.06.2013	Adana	Yüreğir	*Solanum muricatum* Aiton 1789	35º03'41.17"	37º00'12.20"
49	18.06.2013	Adana	Seyhan	*Citrus sinensis*	35º01'84.24"	6º05'54.41"
57	26.06.2013	Adana	Sarıçam	*Citrus paradisi*	35º06'08.00"	37º00'29.53"
58	27.06.2013	Adana	Seyhan	*Citrus paradisi*	35º01'66.13"	36º09'85.19"
59	27.06.2013	Mersin	Erdemli	*Citrus paradisi*	34º07'83.00"	36º06'24.98"
70	06.07.2013	Mersin	Aydıncık	*Citrus sinensis*	33º03'58.78"	36º01'83.97"
71	06.07.2013	Mersin	Aydıncık	*Citrus limon*	33º00'17.00"	36º02'05.33"
73	01.08.2013	Adana	Çukurova	*Morus alba* L. (1753)	35º01'80.08"	37º02'58.59"
74	23.08.2013	Hatay	Samandağ	*Citrus sinensis*	38º03'15.00"	37º05'80.22"
75	03.09.2013	Adana	Çukurova	*Rosa rugosa* Thunb. (1784)	35º01'74.66"	37º02'12.73"
87	01.10.2013	Hatay	Samandağ	*Citrus sinensis*	35º07'18.00"	36º01'29.03"
88	04.11.2013	Adana	Yüreğir	*Punica granatum*	35º03'41.17"	37º00'12.20"
89	30.07.2013	Mersin	Erdemli	*Citrus medica* var. *sarcodactylis* (Siebold ex Hoola van Nooten) Swingle	34º03'38.78"	36º06'24.98"
91	17.06.2014	Mersin	Erdemli	*Citrus limon*	34º02'96.00"	36º06'24.20"
92	17.06.2014	Mersin	Erdemli	*Citrus limon*	34º02'68.63"	36º06'03.13"

Table 1. Continued

93	18.06.2014	Adana	Kozan	*Citrus sinensis*	35º07'30.02"	37º04'29.20"
94	26.06.2014	Mersin	Anamur	*Citrus sinensis*	32º01'45.00"	36º09'06.10"
99	01.07.2014	Adana	Seyhan	*Citrus sinensis*	35º01'32.77"	36º09'64.12"
100	05.09.2014	Hatay	İskenderun	*Cupressus sempervirens* L. (1753)	35º08'81.38"	36º04'05.53"
101	05.09.2014	Hatay	İskenderun	*Citrus sinensis*	35º08'68.03"	36º03'81.20"
102	05.09.2014	Hatay	İskenderun	*Punica granatum*	35º08'68.03"	36º03'81.12"
103	07.09.2014	Hatay	Dörtyol	*Citrus paradisi*	36º01'88.13"	36º08'19.22"
104	07.09.2014	Hatay	Erzin	*Citrus reticulata* Blanco (1837)	36º02'22.22"	36º09'14.47"
105	31.05.2015	Mersin	Silifke	*Citrus limon*	33º04'55.00"	36º08'13.11"
106	31.05.2015	Mersin	Mezitli	*Citrus limon*	34º04'15.00"	36º07'54.77"
107	11.06.2015	Mersin	Mezitli	*Citrus sinensis*	34º04'54.93"	36º00'79.20"
108	12.06.2015	Mersin	Akdeniz	*Citrus sinensis*	34º07'55.08"	36º08'55.87"
West Mediterranean						
7	05.07.2011	Antalya	Finike	*Cupressus sempervirens*	30º08'00.43"	36º02'15.14"
8	06.07.2011	Antalya	Finike	*Citrus sinensis*	30º08'02.43"	36º02'15.14"
9	08.07.2011	Antalya	Finike	*Begonia* sp.	30º09'44.90"	36º01'83.82"
10	08.07.2011	Antalya	Finike	*Hibiscus rosa-sinensis* L. (1753)	30º09'44.90"	36º01'83.82"
21	12.06.2012	Antalya	Finike	*Citrus sinensis*	30º01'33.33"	36º01'94.96"
22	12.06.2012	Antalya	Finike	*Citrus sinensis*	30º01'33.33"	36º01'94.96"
25	13.07.2012	Antalya	Alanya	*Ceratonia siliqua* L. (1753)	31º05'34.14"	36º03'45.70"
30	09.03.2013	Antalya	Alanaya	*Rosa rugosa*	31º05'32.56"	36º03'55.85"
39	07.06.2013	Antalya	Finike	*Citrus sinensis*	30º12'24.36"	34º01'67.16"
40	07.06.2013	Antalya	Kumluca	*Citrus sinensis*	30º01'46.17"	36º03'48.38"
41	07.06.2013	Antalya	Kumluca	*Citrus sinensis*	30º03'15.13"	36º03'24.78"
42	07.06.2013	Antalya	Kumluca	*Citrus sinensis*	30º03'42.37"	36º02'75.53"
43	07.06.2013	Antalya	Kemer	*Ficus benjamina*	30º05'51.03"	36º05'45.38"
44	07.06.2013	Antalya	Serik	*Citrus paradisi*	35º03'03.93"	37º02'85.44"
50	19.06.2013	Antalya	Gazipaşa	*Citrus sinensis*	32º01'81.00"	36º02'65.03"
51	19.06.2013	Antalya	Gazipaşa	*Citrus sinensis*	32º07'35.00"	36º09'56.11"
52	19.06.2013	Antalya	Alanya	*Citrus sinensis*	32º01'82.08"	36º04'29.02"
53	19.06.2013	Antalya	Manavgat	*Citrus sinensis*	31º05'18.73"	36º07'58.62"
54	20.06.2013	Antalya	Serik	*Citrus limon*	30º09'24.73"	36º09'46.72"
55	20.06.2013	Antalya	Serik	*Citrus sinensis*	30º09'95.28"	37º00'15.02"
56	20.06.2013	Antalya	Aksu	*Citrus sinensis*	30º08'57.72"	36º09'31.58"
63	04.07.2013	Antalya	Finike	*Citrus sinensis*	30º09'45.00"	36º04'75.30"
64	04.07.2013	Antalya	Finike	*Citrus sinensis*	30º01'60.12"	36º01'85.00"
65	04.07.2013	Antalya	Kumluca	*Citrus sinensis*	30º02'51.42"	36º04'13.00"
66	05.07.2013	Antalya	Konyaaltı	*Citrus sinensis*	30º06'07.48"	36º08'95.53"
67	05.07.2013	Antalya	Konyaaltı	*Punica granatum*	30º05'49.48"	36º05'15.52"
68	05.07.2013	Antalya	Konyaaltı	*Citrus limon*	30º05'39.73"	36º08'84.72"

Table 1. Continued

69	05.07.2013	Antalya	Konyaaltı	*Citrus sinensis*	30º05'57.88"	36º08'81.73"
72	12.07.2013	Antalya	Gazipaşa	*Schinus molle* L. (1753)	32º01'84.86"	36º01'61.41"
90	24.05.2014	Antalya	Muratpaşa	*Cupressus sempervirens*	30º03'84.29"	36º05'43.14"
95	26.06.2014	Antalya	Gazipaşa	*Citrus sinensis*	32º06'55.00"	36º00'25.10"
96	26.06.2014	Antalya	Manavgat	*Citrus sinensis*	31º00'85.00"	36º09'55.30"
97	27.06.2014	Antalya	Kemer	*Citrus sinensis*	30º00'25.00"	36º05'63.52"
98	28.06.2014	Antalya	Aksu	*Citrus sinensis*	30º08'63.88''	36º09'46.77
Aegean						
23	26.06.2012	Muğla	Bozburun	*Pinus brutia* Ten (1815)	28º01'33.69"	36º04'44.04"
28	02.11.2012	Muğla	Bodrum	unknown	28º07'45.92"	37º09'58.78"
31	03.06.2013	İzmir	Menderes	*Citrus reticulata*	27º08'05.00"	37º03'15.23"
32	04.06.2013	Aydın	Söke	*Citrus sinensis*	27º32'22.17"	37º42'47.12"
33	06.06.2013	Muğla	Köyceğiz	*Citrus sinensis*	28º08'15.00"	37º00'33.08"
34	06.06. 2013	Muğla	Köyceğiz	*Citrus sinensis*	28º07'38.13"	36º04'31.90"
35	06.06.2013	Muğla	Ortaca	*Citrus sinensis*	28º07'96.58"	36º08'19.92"
36	06.06.2013	Muğla	Dalaman	*Citrus sinensis*	35º02'94.12"	37º00'41.18"
37	06.06.2013	Muğla	Dalaman	*Citrus sinensis*	28º06'85.00"	36º07'63.52"
38	06.06.2013	Muğla	Fethiye	*Citrus sinensis*	28º09'63.58"	36º07'41.28"
60	03.07.2013	Muğla	Köyceğiz	*Citrus reticulata*	28º06'23.88"	36º09'80.57"
61	03.07.2013	Muğla	Köyceğiz	*Citrus sinensis*	28º07'18.62"	36º08'90.98"
62	04.07.2013	Muğla	Fethiye	*Citrus sinensis*	29º01'55.00"	36º07'18.43"
76	09.09.2013	Aydın	Sultanhisar	*Citrus sinensis*	28º02'85.00''	37º08'80.37"
77	10.09.2013	İzmir	Menderes	*Citrus reticulata*	27º01'38.46"	38º05'05.05"
78	10.09.2013	Aydın	Söke	*Citrus sinensis*	27º05'40.93"	37º07'14.77"
79	10.09.2013	Aydın	Söke	*Portulaca oleracea* L. (1753)	27º05'40.93"	37º07'14.77"
80	10.09.2013	Aydın	Söke	*Citrus sinensis*	27º03'45.00"	37º07'55.17"
81	10.09.2013	Aydın	Söke	*Portulaca oleracea*	27º03'45.00"	37º07'55.17"
82	10.09.2013	Aydın	Söke	*Citrus sinensis*	27º05'35.62"	37º03'05.17"
83	11.09.2013	Muğla	Datça	*Citrus reticulata*	27º06'71.07"	36º05'43.24"
84	12.09.2013	Muğla	Ortaca	*Punica granatum*	28º07'93.47"	36º05'04.39"
85	12.09.2013	Muğla	Dalaman	*Citrus paradisi*	28º08'10.02"	36º08'04.62"
86	12.09.2013	Muğla	Fethiye	*Citrus sinensis*	29º00'50.07"	36º06'99.52"

Phylogenetic analyses

DNA sequences for each of the 108 insects collected in Turkey were obtained in both directions and final base calls were made using FinchTV (FinchTV, 2019). The alignment of sequences was done using Mega 6.0 (Tamura et al., 2013). Both direction sequences were contiged for each individual. DNA sequences from an additional 90 *P. citri* individuals from different countries around the world were obtained for the same COI gene region from NCBI GenBank. Phylogenetic analysis was carried out using the neighbor-joining method (bootstrap 1000) as implemented in MEGA 6.0 (Felsenstein, 1985; Saitou & Nei, 1987) using the Kimura two-parameter model (Kimura, 1980; Tamura et al., 2013), which was the most appropriate model for the datasets as determined in MEGA. A COI sequence from *Planococcus minor* (Maskell, 1897) (Hemiptera: Pseudococcidae) (GenBank accession KY373094) was used as the outgroup.

The haplotype networks of *Planococcus citri* mtDNA COI gene

Mitochondrial COI gene-specific haplotype networks were constructed from DNA sequences from the specimens in this study and reference genes using the median-joining method (Bandelt et al., 1999) contained within the software program PopArt (Leigh & Bryant, 2015). Specimens were color-coded according to geographic region (Table 1) to enable display of the proportion of each haplotype from each region.

Population genetic analysis

The specimens in the research and reference genes were grouped geographically as the Americas (Brazil and the USA), the Middle East (Iran and Turkey), Europe (France and Spain), North Africa (Egypt and Tunisia), South Africa (South Africa) and the Far East (China, Philippines, South Korea and Vietnam) for population genetic analysis. One hundred and ninety-eight specimens were analyzed (Table 2). Population diversity indices: haplotypes number, nucleotide diversity (π), haplotype diversity (Hd), numbers of segregating sites and the average number of pairwise nucleotide differences within the population (K), were calculated using DnaSP 4.5 software. The neutrality indices of Tajima's D and Fu's Fs in each population were also calculated using DnaSP (Librado & Rozas, 2009). The total values were calculated for Turkish specimens and Turkish specimens + reference genes, separately. Arlequin 3.1 was used to calculate pairwise genetic difference (F_{ST}) between all populations (Sharma et al., 2013).

Results

Planococcus citri specimens from the three geographic regions yielded 230 bp COI fragments from 108 samples. The obtained haplotypes sequences in this study have been submitted to GenBank with accessions MN930633 to MN930637. Searching GenBank with these sequences allowed us to obtain an additional 90 COI sequences from *P. citri* individuals from across the globe (Figure 1, Tables 1 & 2).

The Turkish populations have five haplotypes (Hap 1-5). Hap 1 (n=102) was dominant for three subregions (94%) followed by Hap 2 (3%). Besides, two haplotypes from the Aegean Region (Hap 1 and 3), three haplotypes from the East (Hap 1, 2 and 5) and West (Hap 1, 2 and 4) Mediterranean Regions were determined (Tables 1 & 2). Hap 1 on different plants such as weeds, vegetables, shrubs, and trees, Hap 2 on *Schefflera* spp. and *Ficus benjamini* were collected. However, Hap 3 and 4 on *Citrus sinensis*, Hap 5 on *Citrus limon* were detected (Table 1). When the Turkish haplotypes were compared to each other, they were found to have a few bases difference (Table 3).

In addition to the five haplotypes detected in Turkey, an additional nine haplotypes were detected worldwide, giving 14 haplotypes in total. With respect to geographic distribution, there were three haplotypes from the Americas, six from the Middle East, five from Europe, four from the Far East and three from Africa (Table 2). Among the world samples, out of Turkey specimens, Hap 1 (n=44) was again the most common (49%) followed by Hap 2 (n=19; 21%). Hap 1 was the most frequent haplotype present in China and South Korea, while Hap 2 was present in Brazil, South Korea and Philippines.

The phylogenetic relationships of the 14 haplotypes are shown in the phylogenetic tree presented in Figure 2. The tree shows that the 14 haplotypes detected from 198 specimens form two major groups (Figure 2). One of the branches contains Hap 3 and 13, the other branch was two forked and has rest of the other haplotypes except Hap 12. Hap 10, 2, 6 and 14 are grouped on the same subbranch, while Hap 4, 5, 7 and 8 were on the other branch and this branch is also including the most common Hap 1. Only one branch had bootstrap values >50. Hap 12 was grouped in the tree on the same branch with the outgroup sequence from *Planococcus minor*.

Table 2. Accessions of nucleotide sequenced of COI gene of *Planococcus citri* collected in Turkey and from different countries obtained from published databases (n, specimen number)

Country	n	Accession numbers	Number of Haplotype	Group
Brazil	6	KJ530615, KJ530616	1	America
		KJ530612, KJ530613, KJ530614	2	
		KJ530611	6	
USA	2	MJMB463, MJMB373	1	
France	6	JQ085542, GU134705	1	Europe (EU)
		JQ085543, GU134706	2	
		GU134707	10	
		GU168801	11	
Spain	4	JF714200, JF714201	1	
		JF714199	2	
		JF714198	14	
China	34	KY372821, KY373047, KY372583, KY373077, KY372860, KY372516, KY373009, KY372671, KY373012, KY372899, KY372610, KY372545, KY372866, KY372939, KY372979, KY373108, KY373051, KY372496, KY372602, KY372871, KY373081, KP692646, KP692637, KP692648, KP692640, KP692644, KP692641, KP692643, KP692647, KP692639, KY372651	1	Far East
		KY373030, KP692645	2	
		KY372472	7	
Vietnam	4	DSPKJ267, DSPKJ268	1	
		DSPKJ167, DSPKJ166	2	
India	1	KU296034	12	
Philippines	3	DSPKJ110, DSPKJ112, DSPKJ111	2	
South Korea	12	HM474285, HM474286, HM474287, HM474278, HM474284, HM474279	1	
		HM474288, GU936938, HM474280, HM474281, HM474282, HM474283	2	
Turkey	108	East Mediterrenean 1, 2, 3, 4, 5, 6, 11, 13, 15, 16, 17, 18, 19, 20, 24, 26, 27, 29, 45, 47, 48, 49, 57, 58, 59, 70, 71, 73, 74, 75, 87, 88, 89, 91, 92, 93, 94, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 West Mediterrenean 7, 8, 9, 10, 21, 22, 25, 30, 40, 41, 42, 44, 50, 51, 52, 53, 54, 55, 56, 63, 64, 65, 66, 67, 68, 69, 72, 90, 95, 96, 97, 98 Aegean 23, 28, 31, 32, 33, 34, 35, 36, 38, 60, 61, 62, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86	1	Middle East
		East Mediterrenean 12, 14 West Mediterrenean 43	2	
		Aegean 37	3	
		West Mediterrenean 39	4	
		East Mediterrenean 46	5	
Iran	1	JF905464	13	
Tunisia	2	MJMB301, MJMB303	1	North Africa
Egypt	3	JQ085540	1	
		JQ085544	8	
		JQ085545	9	
South Africa	12	GBMH1664	1	South Africa
		SIBI435, SIBI436, SIBI43, SIBI438, SIBI439	11	
		SIBI196, SIBI197, SIBI198, SIBI199, SIBI200, SIBI213	13	

Hap 1 and 2 are the two main groups according to haplotype network (Figure 3) and both haplotypes distributed all over the geographic groups except Hap 2, which was not detected in South Africa. Hap 11 and 13 were recorded as common haplotypes in South Africa and are separately localized on the haplotype network (Figure 2). The single nucleotide differences from Hap 1 generally detected in the network.

High mitochondrial DNA diversity was revealed overall for the populations examined according to molecular diversity indices (Table 4). Aegean has higher K (0.182) and π (0.00076) values and the lowest Hd (0.019) value, while West Mediterranean Region has highest Hd (0.127) value among the regions of Turkey. However, the highest Hd (0.800) values for Europe, the highest K (1.200) and π (0.00525) values were for North Africa in comparison of world populations. Further, Tajima's D test and Fu's FS test showed negative values, except in South Africa, indicating deviations from neutrality. Pairwise comparisons of the different populations using the F_{ST} was significant for all comparisons except those between the Americas and the Far East, the Americas and Europe, North Africa and the Far East, and lastly Europe and the Far East (Table 5).

Table 3. Sequences Differences between the five haplotypes of the COI gene of *Planococcus citri* from Turkey

Haplotype number	(position in alignment) BP				
	50	107	114	125	146
Haplotype 1	T	T	A	C	A
Haplotype 2	T	T	A	C	**T**
Haplotype 3	T	**C**	A	**T**	A
Haplotype 4	**C**	**T**	A	C	A
Haplotype 5	T	T	**G**	C	A

Table 4. Diversity and neutrality indices of *Planococcus citri* populations calculated from nucleotide sequence of mitochondrial COI gene

Geographic origin	n	S	K	H	Hd±S.D.	π	D	Fu's Fs
E. Med_TR	54	2	0.110	3	0.108±0.057	0.00056	-1.31	-2.42
W. Med_TR	31	2	0.129	3	0.127±0.080	0.00054	-1.51	-2.40
Aegean_TR	22	2	0.182	2	0.019±0.081	0.00076	-1.51	-0.11
Turkey	108	5	0.129	5	0.108±0.041	0.00084	-1.82*	-5.63**
America	8	2	0.821	3	0.679±0.122	0.00364	0.24	-0.15
Europe	10	4	1.156	5	0.800±0.100	0.00506	-0.72	-1.90
Far East	54	8	0.651	4	0.445±0.061	0.00284	-1.69	-0.16***
South Africa	12	2	1.076	3	0.621±0.087	0.00471	1.82	0.84
Middle East	109	5	0.145	6	0.124±0.043	0.00063	-1.78	-7.31*
North Africa	5	3	1.200	3	0.700±0.218	0.00525	-1.05	-0.19
Worldwide	198	15	0.526	14	0.397±0.042	0.00232	-4.74*	-13.40***

Statistical differences *, P<0.05; **, P<0.02; ***, P<0.01;
East Mediterranean Region: Adana (n=29), Mersin (n=20), Hatay (n=6); West Mediterranean Region: Antalya (n=31); Aegean Region: İzmir (n=2), Aydın (7), Muğla (13); Turkey: Adana, Antalya, Mersin, Hatay, Muğla, Aydın, İzmir, Artvin (1); the Americas (Brazil and USA); Europe (France and Spain); the Far East (China, Philippines, South Korea and Vietnam); South Africa; the Middle East (Iran and Turkey); North Africa (Egypt and Tunisia); Worldwide: Americas, Europe, the Far East, South Africa, the Middle East and North Africa.

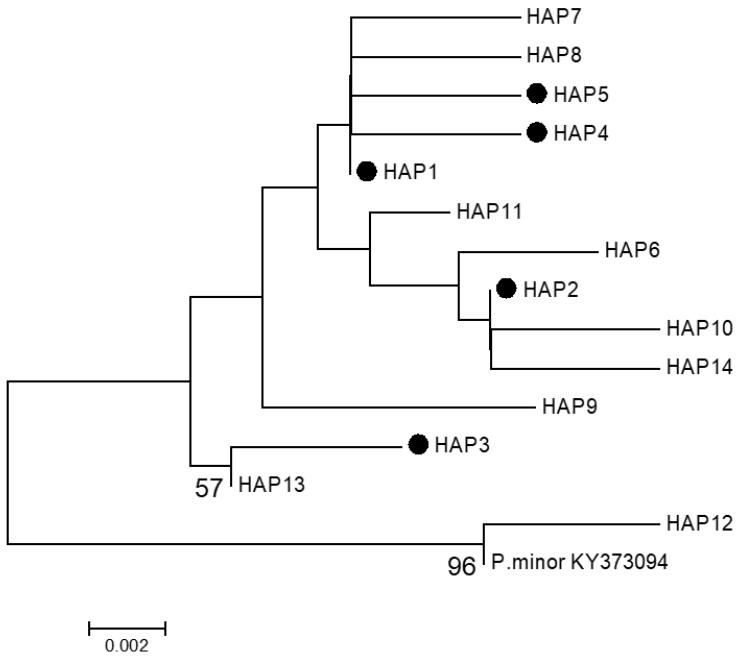

Figure 2. Dendrogram constructed by neighbor-joining method from the COI sequences of *Planococcus citri* analyzed in the present study along with those retrieved from GenBank belong to the Americas, Europe, the Far East, the Middle East, North Africa, and South Africa. Bootstrap values greater than 50% are indicated at branch nodes. Outgroup, *Planococcus minor*. Dots indicate the Turkish haplotypes.

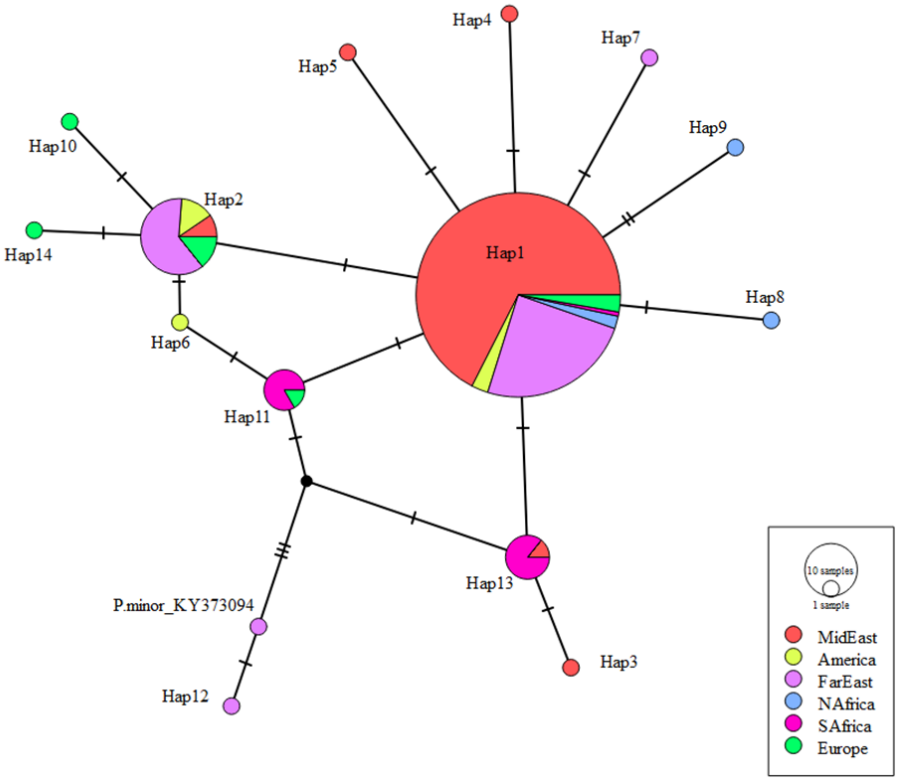

Figure 3. Haplotype network of the 14 haplotypes identified in *Planococcus citri*. Tick marks show base differences between haplotypes.

Table 5. Pairwise genetic distance (F_{st}) between different world samples of *Planococcus citri* calculated from nucleotide sequence of mitochondrial COI gene

	America	Far East	Middle East	North Africa	Europe
Far East	0.034				
Middle East	0.549*	0.142*			
North Africa	0.193*	0.134	0.358*		
Europe	0.000	0.057	0.520*	0.161*	
South Africa	0.331*	0.369*	0.631*	0.258*	0.318*

* The asterisk indicates statistical difference between the geographical regions ($p<0.05$).

Discussion

The research revealed that *P. citri* has significant genetic differentiation with a few base shifts based on the used COI molecular marker. *Planococcus citri* has two predominant haplotypes Hap 1 and 2 both Turkey and over the world and other haplotypes modified from these two haplotypes are apparent in the haplotype network. Haplotype networks are an intuitive method for visualizing relationships between individual genotypes at the population level (Leigh & Bryant, 2015). The differences between the sequences generally located on the different point of the sequences and with limited nucleotide substitutions. Detected fourteen haplotypes all around the world were close to each other according to the phylogenetic tree. Hap 1 clustered with Hap 4 and 5 from Turkish haplotypes, Hap 7 from China and Hap 8 from Egypt. All these are connected each other via an historical trade route (the Silk Road). Moreover, Hap 2 clustered with Hap 10 and 14 from Europe, Hap 6 from Brazil and Hap 11 from South of Africa this also another trade route (the Spice Road). Along this road, Brazil is out the group but it has connection with Spain and Portugal culture. America and Europe have totally same haplotypes, likely due to transportation of the pest by plant trade.

Hap 12 (GenBank accession KU296034) from India was more closely related to the outgroup haplotype from *P. minor* than it was to *P. citri*. These two-mealybug species are very difficult to distinguish from each other morphologically (Cox & Wetton, 1988) and, the identification technique to separate these two species is based on Cox Score. Cox score uses point system and if the score less than 35 meaning *P. minor*, if higher it is *P. citri*. (Cox, 1989). Nagalakshmi (2019) reported that the separation of these two species is based on variation in numbers of ventral oral collar tubular ducts. Wang et al. (2016b) detected the nucleotide sequence identity of 5′ and 3′ COI gens of between *P. citri* and *P. minor* were 97-98% and 96-98%, respectively. And the sequences have stable species-specific identification sited on 5' and 3' for both species. Moreover, *P. citri* and *P. minor* clustered on the same branch for COI region among 54 mealybug species as a monophyletic group, the genetic distance between two species was 1.96% for nearest neighbor analyses (Wang et al., 2016a).

East and West Mediterranean Regions of Turkey have similar Hd, π, and K values and the values were higher than Aegean Region. Tajima's D and Fu's Fs values of Turkey which calculated from three subregions were negative and statically significant show that the population have rare alleles and expecting a new population expansion in the region. The Middle East has more haplotypes than the other regions, however, the Far East has more segregating sites, Europe has the highest Hd value, even low sample number. Far East and Middle East also have statistically significant Fu's Fs values. If the expansion of *P. citri* had started from the Far East throughout the world, occurring a high of number of rare alleles from the region might be considered normal. Europe and the Americas specimens were genetically totally same according to F_{ST}. The Middle East and South Africa are further apart.

Planococcus citri has wide morphological variation indicating that it is a complex of different ecological, biological and geographical races (Ferris, 1950; de Lotto, 1964; Padi, 1990) or possibly contains cryptic species (Rung et al., 2009). For example, *P. citri* on roots of coffee is considered to be a different

race from mealybugs feeding on aerial parts of coffee from East Africa (de Lotto, 1964). Both races have different morphological characters such as size, shorter antenna and legs and dermal suture number. Moreover, the race living on roots feeds on fungi. However, research on chromosomal patterns and symbiotic organisms of the two races did not show them to be different from *P. citri* (de Lotto, 1964). So, the single haplotypes, for example only one individual from Hap 3, 4 and 5 obtained in Turkey, should be investigated in this respect.

While Hap 2 was found on *Ficus* sp. in our research, it was characterized generally on the ornamental plants and other trees in the different researches such as *Ficus* sp. and *Kalanchoe* sp. (Abd-Rabou et al., 2012), *Mackaya bella* Harv., 1859 (Acanthaceae) and *Clerodendrum* sp. (Malausa et al., 2011), *Bischofia javanica* Blume, 1827 (Wang et al., 2014). These findings may support that Hap 2 generally does not prefer *Citrus* sp. and might represent a race or cryptic species.

Increasing International trade and intercontinental transportation activities of ornamental plants, especially from the Far East, may be causing a breakdown of biogeographic barriers within and between species. Quarantine measures generally target prevention of entry of new species to a region. However, the movement of cryptic species or haplotypes to a new region is difficult to control, because we lack information on the population genetic history of the species. Citrus mealybug probably originated from China and spread from there throughout the world (Barlet, 1978). Given its invasive dispersal potential, it now occurs all around the world including Africa, America, Asia and Europe (García Morales et al., 2016). As the Far East including China, India is considered the center of origin of citrus (Gmitter & Hu, 1990; Nicolosi, 2007), therefore transportation of the fruit and propagating material from this region may explain the spread of the citrus mealybug. Another dispersal mechanism is long distance transport of ornamental plants. As a result of this trade, over the last two decades two important pests have established well away from their origin, *Bemisa tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) subspecies (de Moraes et al., 2018) and *Thrips hawaiiensis* (Morgan, 1913) (Thysanoptera: Thripidae) (Reynaud et al., 2008; Atakan et al., 2015). The Far East is the likely origin of these pests, paralleling to the distribution of citrus plants throughout the world (Bartlett, 1978).

The study indicates that Hap 1 and 2 are possibility different cryptic species based on few base changes, because of different host plant range. Different cryptic species might not be so significant for chemical control tactics. However, they might be important for biological control strategies, because of their effect on fitness cost of parasitoids (Forbes et al., 2012; He et al., 2019). Insecticide pressure on the pest directly affects its survival ability and might cause genetic modification of the insect. The data from lower Seyhan revealed no genetic differentiation of *P. citri* populations in that district. However, this does not mean that the district does not have variation, as the small dataset is inadequate to support that conclusion. The information is insufficient to ascertain the biotype of the citrus mealybug so further research with different genomic regions or techniques is needed to provide more specific information about the *P. citri* complex.

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Original article (Orijinal araştırma)

Control potentials of some entomopathogenic nematodes against Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu, 1951 (Hymenoptera: Cynipidae)[1]

Asya kestanesi gal arısı, *Dryocosmus kuriphilus* Yasumatsu, 1951 (Hymenoptera: Cynipidae)'ya karşı bazı entomopatojen nematodların mücadele potansiyelleri

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Abstract

The Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu, 1951 (Hymenoptera: Cynipidae), has spread rapidly worldwide and can cause 80% product loss in chestnut. In the chemical control insecticides are ineffective because the larvae of the insect are well protected inside the chestnut galls. Various parasitoids of *D. kuriphilus* have been reared in Europe. However, native European parasitoids cannot keep the *D. kuriphilus* population below the economic threshold. The purpose of this research was to determine the potential of some entomopathogenic nematodes (EPNs) as an alternative biological control agent. Although EPNs have not been studied on *D. kuriphilus* until now, it is known that EPNs can infect some above-ground pests. In this study, two strains of *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae) and one strain of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) with the four dosages were applied against *D. kuriphilus* adults in both chestnut shoots and petri dishes. The study was conducted in 2019 under laboratory conditions in Bursa (Turkey). The results of the study indicated that the EPNs can infect *D. kuriphilus* adults. In addition, the numbers of egg laying of *D. kuriphilus* adults exposed to the EPNs decreased.

Keywords: Above-ground application, biological control, *Dryocosmus kuriphilus,* entomopathogenic nematode

Öz

Asya kestane gal arısı, *Dryocosmus kuriphilus* Yasumatsu*,* 1951 (Hymenoptera: Cynipidae) dünya çapında hızla yayılmaktadır ve kestane içinde %80 ürün kaybına neden olabilmektedir. *Dryocosmus kuriphilus* larvaları kestane galleri içinde iyi korunduğu için kimyasal mücadelede, insektisitler etkisiz kalmaktadır. *Dryocosmus kuriphilus*'un çeşitli parazitoidleri Avrupa'da yetiştirilmektedir. Ancak, yerel Avrupa parazitoitleri *D. kuriphilus* popülasyonunu ekonomik zarar eşiğinin altında tutamamıştır. Bu araştırmanın amacı, *D. kuriphilus*'a karşı alternatif bir biyolojik mücadele ajanı olarak bazı entomopatojen nematodların (EPN) potansiyelini belirlemektir. Şimdiye kadar *D. kuriphilus* üzerinde EPN'ler ile ilgili çalışılmamış olmakla birlikte EPN'lerin toprak üstü zararlılarını enfekte edebileceği bilinmektedir. Bu çalışmada 4 dozda *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae)'nin iki ırkı ile *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae)'nın bir ırkı hem kestane filizlerinde hem de petri kabı deneylerinde *D. kuriphilus* erginlerine uygulanmıştır. Bu çalışma, 2019 yılında Bursa (Türkiye)'da laboratuvar koşullarında gerçekleştirilmiştir. Çalışmanın sonuçları, EPN'lerin *D. kuriphilus* erginlerini enfekte edebildiklerini kanıtlamıştır. Ayrıca, EPN'lere maruz kalan *D. kuriphilus* erginlerinin yumurtlama sayısı azalmıştır.

Anahtar sözcükler: Toprak üstü uygulama, biyolojik mücadele, *Dryocosmus kuriphilus*, entomopatojen nematod

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Introduction

Chemical pesticides used in agricultural fields are a major cause of environmental pollution. Consequently, the importance of the biological control agents as an alternative to chemical pesticides has increased (Olson, 2015). Entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae are effectively used in place of some insecticides providing environmentally friendly control (Lacey & Shapiro-Ilan, 2008). EPNs, which have a symbiotic relationship with *Photorhabdus* and *Xenorhabdus* bacteria, enter insect hemolymph through natural openings. After penetration, the EPNs, release their symbiotic bacteria and the infested insects die from septicemia within 24 to 48 h (Stock & Blair, 2008). EPNs are naturally present in soil so are especially used to control soil-dwelling insect pests (Wright et al., 2005). Also, they have the potential to suppress above-ground insect pests such as grasshoppers and cockroaches (Morton & García-del-Pino, 2013; Şahin et al., 2018). The spectrum of insects susceptible to the EPNs is quite broad. Insects from 17 orders and 135 families have been found to be vulnerable to the EPNs (El-Kady et al., 2014).

The Asian chestnut gall wasp *Dryocosmus* kuriphilus Yasumatsu, 1951 (Hymenoptera: Cynipidae), native to China, is the only member of the Cynipidae family that attacks the *Castanea* genus (Stone et al., 2002; Abe et al., 2007; Gehring et al., 2018). Originally from China, *D. kuriphilus* was the first identified in Japan in 1941 and then in Korea. It was transmitted to Nepal in 1999 and to the USA with plant material imported from China in 1974. It was first identified in Europe in northern Italy in 2002, and then in Slovenia, France, Switzerland, and Hungary (Panzavolta et al., 2012). The first record of the *D. kuriphilus* in Turkey was made by Çetin et al. (2014). Over the past 20 years, the pest has spread to 25 countries in Asia, Europe and North America. Its rapid spread across different regions has resulted in considerable ecological and economic damage making *D. kuriphilus* one of the most important chestnut pests worldwide (Avtzis et al., 2019).

Dryocosmus kuriphilus females lay eggs in axillary buds of *Castanea* spp. during the summer. The eggs generally hatch in 30-40 days and the first instar of larvae overwinter inside the buds until spring comes (Avtzis et al., 2019). Larvae, which feed inside the bud during the spring, cause flower abortion and the inhibition of female flower formation (Gehring et al., 2018). Thus, fruit yield of the chestnut, *Castanea* spp., can decrease by 80% (Battisti et al., 2014; Avtzis et al., 2019).

The first attempts to limit the damage of *D. kuriphilus* were to develop resistant chestnut cultivars that were initially effective (Avtzis et al., 2019). In chemical control, contact-effect insecticides are ineffective, because the larvae of *D. kuriphilus* are well protected inside the chestnut gall (Bosio et al., 2009). Another problem that makes insecticide use unsuccessful is the difficultly of determining the best control time because *D. kuriphilus* adult emergence date can change and the adults die within 10 days (Germinara et al., 2011; Bernardo et al., 2013). For biological control, 44 species of parasitoids of *D. kuriphilus* in six families have been reared in Europe (Matošević & Melika, 2013; Quacchia et al., 2013; Kos et al., 2015). Although the parasitoid *Torymus sinensis* Kamijo, 1982 (Hymenoptera: Torymidae) is known to be effective against the *D. kuriphilus* in some countries (Moriya et al., 2003; Yara, 2006; Quacchia et al., 2014), the parasitoid cannot keep the *D. kuriphilus* population below the economic threshold, especially in Europe and Middle East (Santi & Maini, 2011; Matošević & Melika, 2013; Quacchia et al., 2013; Askew et al., 2013; Kos et al., 2015; Francati et al., 2015).

Nevertheless, EPNs can be a successful alternative for biological control of *D. kuriphilus* because, EPNs have potential to be effective against above-ground insect pests (El-Kady et al., 2014; Şahin et al., 2018). The purpose of this study is to determine the potential of some EPN strains, two strains (TURS3 and STE5) of *Steinernema feltiae* Filipjev, 1934 (Rhabditida: Steinernematidae) and one strain (HBH) of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae), against *D. kuriphilus* as the biological control agents.

Materials and Methods

EPNs production and application dosages

Three different strains of the EPNs, *Steinernema feltiae* TURS3, *S. feltiae* STE5, *Heterorhabditis bacteriophora* HBH, were used in this study. The 2 or 3-day-old infective juveniles (IJs) of these strains obtained by *in vivo* production were used against *D. kuriphilus*. The final instar larvae of great wax moth, *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) were used for *in vivo* production of the EPNs used (McMullen & Stock, 2014).

The EPNs were applied to adult (female) *D. kuriphilus* both in Petri dishes and chestnut shoots (Figure 1). The dosages of the EPNs applied in Petri dishes were 20, 50, 100 and 200 IJs per cm^2. The numbers of the IJs per *D. kuriphilus* adult in Petri dishes were about 57, 141, 283 and 564, respectively. Plain water was applied for the control.

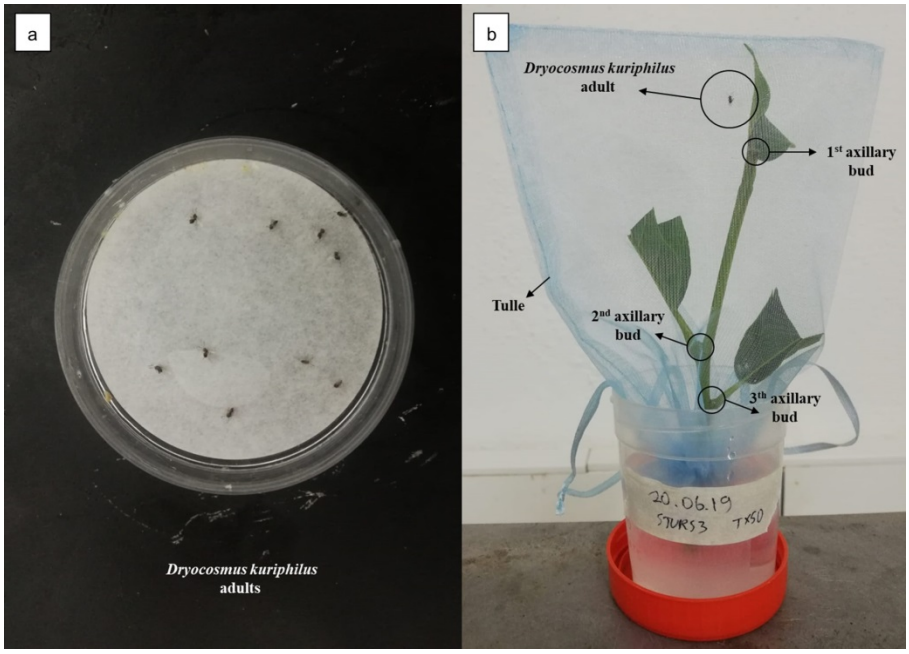

Figure 1. a) *Dryocosmus kuriphilus* adults in a plastic Petri dish; b) gall-free shoots with three axillary buds.

The solution of EPNs diluted with distilled water and were applied on the chestnut shoots by spraying (Shapiro-Ilan et al., 2006). For this, gall-free shoots containing three axillary buds were used (Figure 1). The application dosages on the shoots were 20, 50, 100 and 200 IJs/10 µl. (Susurluk, 2008). About 10 µl of the used dosages accumulated on each bud. Distilled water was applied as a control.

Dryocosmus kuriphilus adults

In spring 2019, 2-year old-shoots with galls (30-40 cm long) (a total of 50 shoots) and current shoots with buds (30-40 shoots) were collected from chestnut orchard in Bursa Province (Cumalıkızık Village, 40°10'21" N, 29°10'16" E) in northwestern Turkey, between May and June at weekly intervals. In order to obtain *D. kuriphilus* adults, we separated the galls from shoots and placed them in cardboard culture boxes. The emergence of the adults that reproduce by thelytokous parthenogenesis (Zhu et al., 2007) was checked daily and were used as soon as after hatching.

Mortality of *Dryocosmus kuriphilus* in Petri dishes

Plastic Petri dishes with a diameter of 6 cm were used for *D. kuriphilus* inoculation. Sterile moist filter paper (80% RH) in the Petri dishes were inoculated with the EPNs at the doses give above. Then 10 of *D. kuriphilus* adults were placed on the filter paper (Figure 1a). The lid of the Petri dishes was closed and it was incubated at 24°C in the dark. Water was applied for the control. The *D. kuriphilus* adults that died after 5 days were dissected to determine, whether they were infected by the EPNs. This experiment was repeated three times for each EPN strain and dosage (Figure 2).

Effects of EPN on *Dryocosmus kuriphilus* egg number in buds

As soon as *D. kuriphilus* adults emerge from the chestnut galls they begin to lay eggs in the buds and die within 7 days (Germinara et al., 2011; Bernardo et al., 2013). Even if these adults are infected by EPNs when they reach the buds, it can take up to 1 or 2 days for EPNs to kill the insects (Stock & Blair, 2008). For this reason, an egg counting method was used to determine the effectiveness of EPNs in controlling females of *D. kuriphilus.* The gall-free shoots were put in plastic bottles (100 ml) filled with water and then the EPNs were applied by spraying the shoots (Shapiro-Ilan et al., 2006). As control, distilled water was applied. Only one *D. kuriphilus* female was released on each shoot, then immediately the shoots in the bottle were covered with tulle (Figure 1b). These shoots were kept at 25°C under laboratory conditions with a 10:14 h L:D photoperiod. After 10 days, the tulle was removed and the numbers of eggs laid in buds counted. Five shoots were used in each batch. The batches were run for each EPN strain and dosage (Figure 3).

Statistical Analysis

The mortality and spawning rate of *D. kuriphilus* were examined using analysis of variance, as of the data was normally distributed. LSD test ($P<0.05$) was used to determine the difference between means in JMP® 7.0 software.

Results and Discussion

Mortality of *Dryocosmus kuriphilus* adults in Petri dishes

At 25 IJs/cm^2, TURS3 caused 20% mortality of *D. kuriphilus* adults and was statistically more effective than the control. Mortality of *D. kuriphilus* adults caused by STE5 and HBH was not significant at 13% and 6.7%, respectively. The effect of HBH was not statistically different from the control. At 50 IJs/cm^2, TURS3, STE5 and HBH caused 60, 47 and 33% mortality, respectively, differences between the mortality rates being statistically significant. At 100 IJs/cm^2, HBH and TURS3 caused 67 and 60% mortality, respectively, but these values were not statistically different. The highest mortality was 87% with STE5. At 200 IJs/cm^2, STE5 and TURS3 gave 100 and 93.3% mortality, respectively, but these values were not statistically different. However, with HBH at this dosage was 73% being significantly lower than STE5 and TURS3, and higher than control (Figure 2).

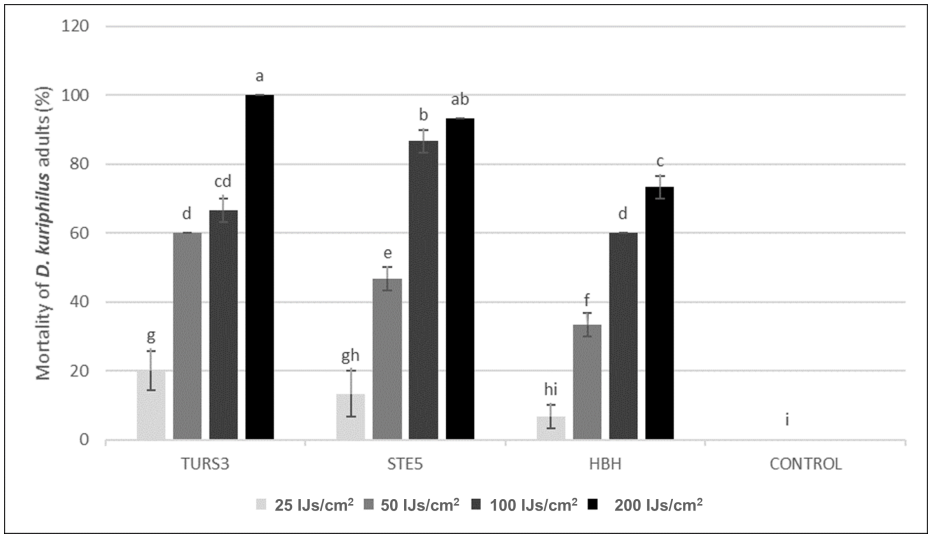

Figure 2. The mortality of *Dryocosmus kuriphilus* adults in Petri dishes. TURS3 and STE5 are strains of *Steinernema feltiae*, and HBH of *Heterorhabditis bacteriophora*. Mean ± SE followed by the same letter are not significantly different (F=95.0, df=12,26, P<0.0001).

Mean number of *Dryocosmus kuriphilus* eggs in each bud

At of 25 IJs/cm^2, the mean numbers of eggs in the buds with TURS3, STE5 and HBH applied were not significantly different from the control (34, 36 and 36, respectively). At 50 IJs/cm^2, the mean numbers of eggs in the buds with STE5 and HBH applied were not significantly different from the control (34 and 35, respectively). At 100 IJs/cm^2, the highest number of eggs determined was 37. The lowest egg number was 22 with TURS3 were applied. At 200 IJs/cm^2, the mean numbers of eggs in the buds treated with TURS3, STE5 and HBH were 15, 30 and 25, respectively, with the differences between these values being statistically significant (Figure 3). *Steinernema feltiae* TURS3 and STE5 were generally found to be more effective than *H. bacteriophora* HBH in terms of killing *D. kuriphilus.*

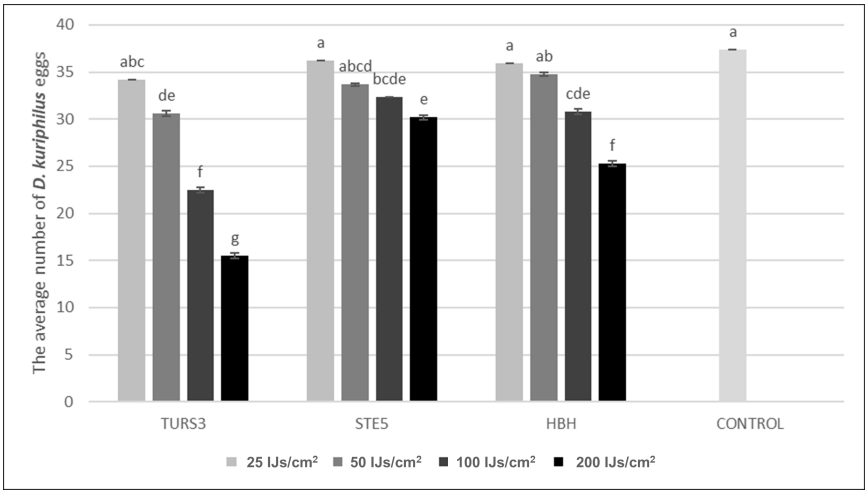

Figure 3. The average numbers of *Dryocosmus kuriphilus* eggs on each shoot [TURS3 and STE5: *Steinernema feltiae*, HBH: *Heterorhabditis bacteriophora*. Means (Means ± SE) followed by the same letter are not statistically significant (F=25.3, df=12,176, P<0.0001)].

Dryocosmus kuriphilus, an important pest of *Castanea* spp., continues to spread worldwide (Avtzis et al., 2019). The first attempts to suppress parasitism of *D. kuriphilus* initially focused on using resistant cultivars of *Castanea crenata* Siebold & Zucc. in the world. Although the use of resistant cultivars is not a sufficiently effective solution, some resistant chestnut cultivars are commercially used by some growers (Nugnes et al., 2018; Avtzis et al., 2019). However, for the control of *D. kuriphilus* recent studies have focused on biological control agents that may provide an alternative to insecticides (Avtzis et al., 2019), because of insecticides are not effective against *D. kuriphilus* larvae, since larvae of the insect are well protected inside the chestnut galls (Bosio et al., 2009).

In the application made on the chesnut shoots in this study, even if *D. kuriphilus* adults are infected by used EPNs as soon as they reach the buds, *D. kuriphilus* death occurs within 2 days. As the EPN dosages increased, the average number of eggs laid in the galls by *D. kuriphilus* adults decreased. These results show that the infected *D. kuriphilus* adults can continue to lay eggs until their death. However, the decrease in the mean numbers of eggs laid compared to the control is promising in terms of biological control. As the viability of the *D. kuriphilus* adults decreased with infection by EPNs, the amount of egg laying decreased. Thus, in the shoot experiment, numbers of laid eggs were recorded rather than the lifespan of the adults.

The use of parasitoids as biological control against *D. kuriphilus* as an alternative control method has been studied by many researchers (Speranza et al., 2008; Quacchia et al., 2013; Matošević et al., 2014; Avtzis et al., 2019). According to their results, the use of parasitoids against *D. kuriphilus* has not been effective to the needed level (Santi & Maini, 2011; Askew et al., 2013; Matošević & Melika, 2013; Quacchia et al., 2013; Kos et al., 2015; Francati et al., 2015). However, no study on the use of EPNs in the controlling of *D. kuriphilus* has been undertaken to date. Although EPNs have the potential to infect above-ground pests, they are mostly used to control pests of soil borne insect pests (Wright et al., 2005; Şahin et al., 2018) because there are important factors such as temperature, ultraviolet radiation and humidity that mostly make the above-ground application unsuccessful (Georgis et al., 2006; Lacey & Georgis, 2012). Despite all these factors, many studies are underway to enable the use of EPNs for the control of above-ground insect pests and remarkable results have been achieved (Maketon et al., 2010; Beck et al., 2013; Şahin et al., 2018; Platt et al., 2019). Similar to our study, Cutler et al. (2017) have achieved control of adults of some cockroach species [*Blaptica dubia* (Serville, 1838), *Gromphadorhina portentosa* Schaum, 1853) and *Nauphoeta cinerea* (Olivier, 1789)] using *Heterorhabditis* and *Steinernema* spp. In addition, *H. bacteriophora* were successfully used against adults of *Locusta migratoria* by Sahin et al. (2018). Schroer & Ehlers (2005), used EPNs *Steinernema carpocapsae* (Weiser, 1955) with a formulation containing 0.3% of the surfactant Rimulgan and 0.3% of the polymer, xanthan gum, on cabbage foliage to control the diamondback moth, *Plutella xylostella* (Linnaeus, 1758), larvae. With these formulations, 80% of the larvae of the insect died within 58 h. Similarly, in the present study, at a dosage of 200 IJs/cm^2, *S. feltiae* STE5 and TURS3 caused more than 80% mortality of *D. kuriphilus* adults. Also, Van Damme et al. (2016) reported that foliar application of EPNs *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* to tomato leaf for control of *Tuta absoluta* (Meyrick, 1917) larvae were effective with mortality of fourth instars being between 77 and 97%. Also, in the same study, *S. feltiae* and *S. carpocapsae* were more effective than *H. bacteriophora* against *T. absoluta*. Also, Hussein and El-Mahdi (2019) formulated three EPNs strains (*H. bacteriophora* BA1, *S. carpocapsae* BA2, *S. feltiae* OBIII) using mixed polymer based on calcium alginate to control *Thrips tabaci* Lindeman, 1889, on onion plants. Significant differences were observed in the mortality of the *T. tabaci* population. The highest mortality was caused by *S. carpocapsae* BA2 and *S. feltiae* OBIII, and the lowest mortality was with *H. bacteriophora* BA1. Consistent with these two studies, in the present study, *S. feltiae* STE5 and TURS3 were more effective against *D. kuriphilus* adults than *H. bacteriophora* HBH especially at 200 IJs/cm^2 in both Petri dish and shoot experiments. Considering studies that had been reported, in general *Steinernema* spp. appears to be more effective than *Heterorhabditis*

spp. in above-ground applications. According to the results of the present study, as similar result was found for *D. kuriphilus* and perhaps if protective formulations were used the effectiveness of the EPNs tested would be improved.

In the future, the use of EPNs against above-ground targets will become more effective. Efforts to develop the above-ground application techniques, which are currently underway, will continue to increase the effectiveness of EPNs. Control potential of used EPNs in the study against *D. kuriphilus* supports improvement of these above-ground applications.

Conclusions

This study is the first attempt to control of *D. kuriphilus* by using of EPNs. In this study, EPNs sprayed on the gall-free shoots were effective in reducing egg numbers of *D. kuriphilus* in the buds. With the EPN strains (TURS3, STE5 and HBH) used, the mean numbers of *D. kuriphilus* eggs in the buds decreased statistically as dosage increased. This study shows that the EPNs can cause a reduction in the numbers of *D. kuriphilus* eggs, which is important for reducing *D. kuriphilus* damage in chestnuts. One of the important implications of this study is that EPNs might be successfully be used for controlling of *D. kuriphilus* in the future. It is suggested that the present study will contribute to the development of the above-ground EPN application techniques.

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Original article (Orijinal araştırma)

Determination of arthropod biodiversity and some ecological parameters of Erdal Şekeroğlu (Isparta, Turkey) and Kadıini (Antalya, Turkey) cave ecosystems with evaluation of usability of insects in cave mapping[1]

Erdal Şekeroğlu (Isparta-Türkiye) ve Kadıini (Antalya-Türkiye) mağara ekosistemlerinde arthropod biyolojik çeşitliliği ile bazı ekolojik parametrelerin belirlenmesi ve böceklerin mağara haritalamasında kullanım olanaklarının araştırılması

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Abstract

The aim of the study was to determine the species composition, diversity, similarity and completeness of cave-dwelling arthropods in cave zones (entrance, twilight and dark zones) in Erdal Şekeroğlu Cave (ESC) (Atabey-Isparta Province) and Kadıini Cave (KIC) (Alanya-Antalya Province) ecosystems in Turkey. The study also aimed to investigate whether these species can be used for mapping cave zones. The samplings were conducted by using aspirator and pitfall trap methods in ESC among 2010-2020 and in KIC in 2017. Hence statistical analyses were performed with the data gathered from the field studies conducted in the same year (2017) in order to evaluate ecological data in the two cave ecosystems homogeneously. During the study, a total of 51 arthropod species, mostly hexapods, belonging to five classes were collected. Biodiversity parameters, similarity index, indicator species analyses, and species richness estimators were calculated for each cave and cave zones. In addition to reporting the distributions of hexapods in cave ecosystems, this paper discusses for the first time if such ecological data can inform cave mapping and exploration.

Keywords: Cave zones, indicator species, similarity, species richness estimators

Öz

Çalışmada, Erdal Şekeroğlu (ESC) (Isparta, Türkiye) ve Kadıini (KIC) (Antalya, Türkiye) Mağaralarının farklı (giriş, alacakaranlık ve karanlık) zonlarında yaşayan arthropod türlerinin çeşitliliğinin, benzerliğinin ve tahmini tür sayılarının belirlenmesi amaçlanmıştır. Ayrıca, çalışmada belirlenen türlerin mağara bölgelerinin haritalanmasında kullanılabilirlikleri araştırılmıştır. Örneklemeler, ESC'de 2010-2020 yılları arasında, KIC'da ise 2017 yılında, aspiratör ve çukur tuzak yöntemleri kullanılarak gerçekleştirilmiştir. İstatistiksel analizler, iki mağara ekosistemindeki ekolojik verileri homojen olarak değerlendirmek amacıyla, aynı yıl (2017) yapılan saha çalışmalarından elde edilen verilerle yapılmıştır. Çalışmada, çoğu hexapod olmak üzere beş sınıfa ait toplam 51 eklem bacaklı türü tespit edilmiştir. Her iki mağara ve mağara zonları için biyolojik çeşitlilik, benzerlik, biyolojik gösterge ve tür tahminleyici analizleri yapılmıştır. Ayrıca, böceklerin mağara haritalaması ve keşiflerinde kullanılabilirliği de dünyada ilk kez tartışılmıştır.

Anahtar sözcükler: Mağara zonları, biyolojik gösterge türleri, benzerlik, tür zenginliği tahminleyicileri

[1] Part of the study was presented as an oral abstract at the VII. Plant Protection Congress (14-17 November.2018, Muğla, Turkey).

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Introduction

Caves are formed over millions of years and contain unusual ecosystems. In general, underground areas, large enough to be entered by a person are considered a cave. Cave depths and lengths range can be from a few meters to thousands of meters (Palmer, 1991; Northup & Lavoie, 2001; Gunn, 2004) and cave ecosystems have a relatively stable temperature, humid and limited supply of nutrients (Barton & Jurado, 2007; Weliange, 2016).

Caves have been used throughout human history for many purposes including scientific studies, recreation and tourism, natural cold storage, maturation and preservation of animal products (e.g., cheese and oil), mushroom cultivation, treatment of respiratory diseases, liquefied gas, natural gas and fuel oil storage, shelter and protection for military purposes, guano collection, mineral extraction, groundwater extraction and protection of spring waters (Tolan-Smith & Bonsall, 1997).

The science of studying the structure, formation, biology and physical features of caves is called speleology. Speleology is a broad interdisciplinary field incorporating archaeology, biology, chemistry, geology, physics, meteorology, hydrology, scientific exploration and cartography in the subterranean environment to better understand the cave ecosystems (Gunn, 2004; Kowalczk, 2009; Lee et al., 2012). While speleology is the branch of science that investigates cave exploration, the structure, physical properties, history and life forms of the caves, biospeleology examines the cave species and their roles of the food chain in cave ecosystems (Latella & Stoch, 2002; Veni, 2019). Biospeleology came into being in the mid-nineteenth century (Vandell, 1964; Camacho, 1992). Remarkable progress was achieved in the biospeleology of the European and American caves in the mid 20th century (Camacho, 1992). These studies revealed that caves have taxonomically diverse fauna (Hobbs, 2012).

Biodiversity can be defined as the diversity of genes, species, and ecosystems (Feest et al., 2010, Cramer et al., 2017, Tydecks et al., 2018). Cave ecosystems often support high diversity and can contain species found in no other terrestrial and aquatic ecosystems (Howarth, 1983; Tercafs, 1988; Culver & Sket, 2000; Culver et al., 2004; Culver & White, 2005; Fernandes et al., 2016). However, the biological diversity of caves remains incompletely documented (Culver et al., 2006). This is particularly the case in Turkey, despite it being a cave rich country. One approach that can be useful in filling this knowledge gap is the use of indicator species, i.e., species that are indicators of the condition of a habitat, community or ecosystem (McGeoch & Chown, 1998; Zacharias & Roff, 2001; Carignan & Villard, 2002; Niemi & McDonald, 2004; Latella et al., 2012; Kurniawan et al., 2018). In the cave ecosystems, indicator species have been used to determine microhabitat, cave area or season and to monitor organic pollution and the effects of cave tourism (Eberhard, 1992; Moulds, 2006; Village et al., 2019).

Cave-dwelling organisms can be classified into three groups according to the degree of adaptation to the subterranean environments. Their classification is typically as follows (Barr, 1968).

Trogloxenes: these species inhabit caves temporarily for particular physiological needs that are linked to seasonal variation and are characterized by a prolonged decrease in their activity. Trogloxenes only enter caves during periods of reduced activity (hibernation, estivation or diapause). Their reproduction is aboveground, and no morphological differences are apparent between subterranean and aboveground individuals.

Troglophiles: these species can be defined as facultative subterranean dwellers in the sense that they are suitable to live in subterranean biotopes because of behavioral and physiological (principally linked to diet) predispositions. They have no typical morphological adaptations to cave ecosystems.

Troglobites: these are permanent, obligatory occupants of the subterranean environment, and cannot live elsewhere. Cave-dwelling species (troglobites) are adapted only to cave conditions. As they

are confined to a very particular biotope, have a restricted range and small populations therefore these species are very sensitive to environmental changes (Samways, 1994, 2007).

Light is one of the main factors affecting evolutionary development in cave ecosystems. As a result of the effect of the light, the cave is divided into three zones: entrance, twilight and dark zones. The distribution of arthropods in the cave zones can use to assign these zones.

Turkey with about 40 000 caves is considered a cave heaven when compared to other countries of the world (Anonymous, 2019). However, biospeleological studies have been very limited in Turkey up to date (Kunt et al., 2010) with only limited scientific studies on the life cycles of the cave arthropods, their roles in the food chain, their use in zone identification and cave mapping, biological indicator values, and biological diversity (Eberhard, 1992; Moulds, 2006; Village et al., 2019).

Based on these facts, the aims of the study were (1) to determine the biodiversity of the arthropod assemblages of the Erdal Şekeroğlu Cave (ESC) (Atabey District, Isparta Province, Turkey) and Kadıini Cave (KIC) (Alanya District, Antalya Province, Turkey), (2) to compare the arthropod assemblages inhabiting in the three cave zones in each cave, (3) to evaluate the usability of insects in cave mapping with indicator species analyses (ISA) performed to test whether the species can be used as an indicator of that of cave zones, and (4) to calculate the completeness of the inventory by using species richness estimators.

Materials and Methods

This study was conducted in ESC and KIC to determine the biodiversity of the arthropod assemblages, compare the arthropod assemblages inhabiting in the three cave zones in each cave, calculate the completeness of the inventory by using species richness estimators, and evaluate the usability of insects in cave mapping.

Studied caves

Erdal Şekeroğlu Cave

ESC is located in Atabey District, Isparta Province of Turkey (37°56’51.97" N, 30°34’38.16" E). The cave is 88 m long and 26 m deep. The main axis starting from the entrance of the cave was formed as a result of collapses and divided the cave into two layers. At the end of the cave, after a vertical climb of about 8 m, even the lower chamber can be reached. The upper floor, which extends towards the end of the cave, runs parallel to the main axis and ends about 5 m above the main axis. Immediately after the cave entrance zone, the twilight zone starts and extends for about 15 m. After the twilight zone, the dark zone continues until the end of the cave. Accordingly, the entrance zone of the ESC is 0-9 m, twilight zone 9-23 m and the dark zone 23-88 m (for more information, see www.magara.org). Sampling in ESC was performed at different times between 2010 and 2020.

Kadıini Cave

KIC is located in Alanya District, Antalya Province of Turkey (36°35’08.2" N, 32°04’39.5" E). The cave is 2027 m long and 45 m deep. The entrance zone consists of a large gallery. The twilight zone starts almost immediately after entering a sharp and narrow gallery from the entrance zone and takes about 50 m. The dark zone extends to the end of the cave. Accordingly, the entrance zone of KIC is 0-25 m, twilight zone, 25-50 m and the dark zone 50-2027 m (for more information, see www.magara.org). Sampling in KIC was conducted at different times during 2017.

Sampling methods

Samplings were conducted in the ESC at different times between 2010 and 2020 (November 2010; June 2011; 03-04 March, 07-08 July and 17-18 November 2012; 9-10 February, 15-16 June, and 23-24

November 2013; 14-15 June and 27-28 December 2014; 2-3 May, 11-12 July and 14-15 November 2015; July 2016; 22-26 February and 19-22 October 2017; 19-20 May, 18-19 August and 3-4 November 2018; 4-5 May and 6-7 July 2019; and 15-16 February 2020) and also in the KIC during 2017 (15-19 February and 12-15 October) for determination of arthropod fauna.

Homogeneous collecting procedures were applied and data from ESC between 22-26 February 2017, 19-22 October 2017, and from KIC between 15-19 February 2017 and 12-15 October 2017 were used for comparison of biodiversity and the other ecological parameters in both caves.

Samples were collected using an aspirator by eye and by pitfall traps inside both caves. In each zone within the caves, the arthropod samples were collected by aspirator from cave surfaces (such as wall and ceiling) for 5 min. Also, five pitfall traps were placed in each zone. Specimens were brought to the laboratory and then they sorted by family and labeled. Specimen identification was made with the support of specialists detailed in the Acknowledgments. The collected specimens are deposited in the special collection of the first author.

Data analysis

The arthropod assemblages of both caves were evaluated by the following diversity indices: Shannon-Wiener (H′), Simpson diversity index (S), Simpson dominance (Sd), Shannon evenness (*EH*), and Sörensen index (Bs).

Shannon-Wiener diversity index (H′) $$H' = -\sum p_i \ln(p_i)$$

where H' is the index of diversity, p_i is the importance value of a species as a proportion of all species, and ln is the natural logarithm.

Simpson's diversity index (S) $$S = 1 - \sum n_i(n_i - 1) / N(N-1)$$

where S is the index of diversity, n_i is the importance value of a species as a proportion of all species, and N is the sum of the number of individuals.

Simpson's dominance index (Sd) $$Sd = \sum n_i(n_i - 1) / N(N-1)$$

where Sd is the index of dominancy, i is number of species, n_i is the importance value of a species as a proportion of all species, and N is the sum of the number of individuals.

Shannon evenness index (EH) $$EH = H' / \ln(N))$$

where EH is Evenness index, H' is the index of Shannon-Wiener diversity, ln is the natural logarithm, and N is the sum of the number of individuals.

Sörensen index (Bs) was used to determine the compositional similarity between the arthropod assemblages of the cave zones of each cave (Southwood, 1971; Magurran, 1988; Krebs, 1999; Magurran, 2004).

Sörensen index $$Bs = 2C / A + B$$

where Bs is the similarity index, A is the number of species in A, B is the number of species in B, and C is the number of common species in A and B.

ISA are used to test the usage of the collected arthropod species to identify a cave zone. Percentage dominance of each sampled species was calculated according to Heydemann (1953) with the following formula;

$$D(\%) = 100N_i / N$$

where D is percent dominance, N_i is the number of captured individuals of a species, N is the sum of the number of individuals.

ISA gives indicator values (IV) for each species in each group and these values are tested for significance using the Monte Carlo test (Heydemann, 1953; Dufrêne & Legendre 1997) as follows:

(1) The proportional abundance of a particular species in a group was calculated relative to the abundance of that species in all groups.

Let A is sample unit x species matrix, a_{ijk} is the abundance of species j in sample unit (SU) i of group k, n_k is the number of sample units in group k, g is the total number of the groups.

Firstly, the mean abundance X_{kj} of species j in group k was calculated:

$$x_{kj} = \sum_{i=1}^{nk} a_{ijk} / n_k$$

Then the relative abundance *RAkj* of species j in group k was calculated:

$$RA_{jk} = x_{kj} / \sum_{k=1}^{g} x_{kj}$$

(2) The proportional frequency of species in each group was calculated:

Firstly, A is transformed into a matrix of presence-absence (*b*),

$$b_{ij} = a_{ij}^0$$

then relative frequency RF_{kj} of species j in group k was calculated:

$$RF_{kj} = \sum_{i=1}^{n_k} b_{ijk} / n_k$$

(3) The product of the two proportions calculated in steps 1 and 2 is then determined. The result is expressed as a percentage, yielding an indicator value IV_{kj} for each species *j* in each group *k*.

$$IV_{kj} = 100(RA_{kj} X RF_{kj})$$

(4) The highest indicator value (IV_{max}) for a given species across groups is saved as a summary of the overall indicator value for that species.

(5) The statistical significance of IV_{max} by using the Monte Carlo method is evaluated. The SUs are randomly reassigned to the groups a large number of times (default = 1000). Each time, IV_{max} is calculated. The probability of type I error is based on the proportion of times that the IV_{max} from the randomized data set equals to or exceeds the IV_{max} from the actual data set. The null hypothesis is that IV_{max} is no larger than it would have been expected by chance (i.e., the species has no indicator value).

In addition to these, to assess the completeness of the inventory, species richness estimators (Chao 1, Chao 2, Jacknife 1, Jacknife 2, Bootstrap, ACE, ICE) were used (Burnham & Overton, 1978, 1979; Heltshe

& Forrester, 1983; Chao, 1984; Smith & van Belle, 1984; Chao & Lee, 1992; Chao et al., 1993; Colwell & Coddington, 1994; Lee & Chao, 1994; Colwell, 1997; Chazdon et al., 1998). These methods provide a lower estimate of total species richness.

Following formulas of species richness estimators are given:

Chao 1 type estimators (for abundance data) (Chao, 1984; Colwell & Coddington, 1994)

$$S_{Chao\,1} = S_{obs} + F_1^2/2F_2$$

where S_{obs} is the observed number of species, F_1 is singletons (species with only one individual), and F_2 is doubletons (species with only two individuals) (Chao, 1984; Chazdon et al., 1998).

Chao 2 type estimators (for replicated incidence data) (Chao, 1987; Colwell & Coddington, 1994)

$$S_{Chao\,2} = S_{obs} + Q_1^2/2Q_2$$

where Q_1 is the frequency of uniques and Q_2 is the frequency of duplicates.

Jackknife 1 type estimators (for abundance data) (Burnham & Overton, 1978, 1979; Heltshe & Forrester, 1983)

$$S_{Jack\,1} = S_{obs} + Q_1\,(m - 1/m)$$

where m is the total number of samples.

Jackknife 2 type estimators (for incidence data) (Smith & van Belle, 1984)

$$S_{Jack\,2} = S_{obs} + \left(\frac{Q_1(2m-3)}{m} - \frac{Q_2\,(m-2)^2}{m(m-1)}\right)$$

Bootstrap type estimators (based on repetition) (Smith & van Belle, 1984)

$$S_{boot} = S_{obs} + \sum_{k=1}^{S_{obs}} (1 - p_k)^2$$

where p_k is the proportion of samples that contain species k.

ACE (abundance coverage estimator) type estimators (for abundance data) (Chao & Lee, 1992; Chao, et al., 1993)

$$S_{ace} = S_{abund} + \frac{S_{rare}}{C_{ace}} + \frac{F_1}{C_{ace}} Y_{ace}^2$$

where S_{abound} is the number of abundant species (each with more than 10 individuals) when all samples are pooled, S_{rare} is the number of rare species (each with 10 or fewer individuals) when all samples are pooled, C_{ace} is the sample abundance coverage estimator and Y^2_{ace} is the estimated coefficient of variation of the F_i for rare species

ICE (incidence coverage-based estimator**)** type estimators (for incidence data) (Lee & Chao, 1994)

$$S_{ice} = S_{freq} + \frac{S_{\inf r}}{C_{ice}} + \frac{Q_1}{C_{ice}} Y_{ice}^2$$

where S_{freq} is the number of frequent species (each found in more than 10 samples), $S_{inf\ r}$ is the number of infrequent species (each found in 10 or fewer samples), C_{ice} is the sample incidence coverage estimator and Y^2_{ice} is the estimated coefficient of variation of the Q_i for infrequent species.

The type estimators calculated from the data obtained from ESC and KIC were graphed and computer simulations made. The all type estimators results were compared with each other. Diversity indices were analyzed with EvenDiv 1.1 (Heimann, 2004) and similarity indices were analyzed using the MultiVariate Statistical Package (MVSP 3.11c) for Windows (Kovach, 1999). PC-Ord (Version 4.14) was used for Biological Indicator Analysis (McCune & Mefford, 2016) and species estimations were calculated with EstimateS v8.2 (Colwell, 2019). Statistical analyses were performed with the data gathered from the field studies conducted in the same years.

Results

Arthropoda fauna of Erdal Şekeroğlu and Kadıini Caves

A total of 25 arthropod species were caught in the ESC with 622 individuals belonging to five classes, nine orders, 15 families between 2010 and 2020 (see description of the Table 1 for details) while 26 arthropod species were sampled in KIC with 160 individuals belonging to three classes, six orders, 18 families during 15-19 February 2017 and 12-15 October 2017 (Tables 1 & 2).

It was determined that the frequency of sampling did not increase significantly in species richness in ESC. Taxa that could be identified to species in situ, such as some of the carabid, chrysomelid, coccinellid, curculionid, scarabaeid (Coleoptera), erebid (Lepidoptera), gryllid and rhaphidophorid (Orthoptera) were counted and released in the zone where captured.

According to homogeneous collecting procedures (ESC, 22-26 February 2017 and 19-22 October 2017, and KIC, 15-19 February and 12-15 October 2017), 47 arthropod species (21 species from ESC and 26 species from KIC) were determined (Table 3). Among these, 36 species (7 Arachnida, 1 Diplopoda and 28 Hexapoda) were identified to species while eight species (6 Arachnida, 1 Diplopoda and 1 Hexapoda) were identified at the genus level. Two arachnids could be identified as family level however one chilopod species could be identified as a morphospecies (Tables 4 & 5).

Most of the hexapods *Stigmatomma denticulatum* Roger, 1859 (Hymenoptera: Formicidae), *Camponotus aethiops* (Latreille, 1798) (Hymenoptera: Formicidae), *Messor semirufus* (André, 1883) (Hymenoptera: Formicidae), *Tomicus minor* (Hartig, 1834) (Coleoptera: Curculionidae), *Ips sexdentatus* (Boerner, 1776) (Coleoptera: Curculionidae), *Carabus glabratus* Paykull, 1790 (Coleoptera: Carabidae), *Carabus graecus* Dejean, 1826 (Coleoptera: Carabidae), *Anoxia asiatica* Desbrochers, 1871 (Coleoptera: Scarabaeidae), *Oxythyrea cinctella* (Schaum, 1841) (Coleoptera: Scarabaeidae), *Cetonia aurata* (L., 1758) (Coleoptera: Scarabaeidae), *Chrysomela populi* L., 1758 (Coleoptera: Chrysomelidae), *Rhynchaenus asellus* Gravenhorst, 1807 *Gymnetron asellus* Scopoli, 1763 (Coleoptera: Curculionidae), *Larinus curtus* Hochhut, 1851 (Coleoptera: Curculionidae), *Scoliopteryx libatrix* L., 1758 (Lepidoptera: Erebidae), and one callipodid, *Eurygyrus* sp. (Callipodida: Schizopetalidae) were sampled from entrance zone of ESC. One centipede, described as morphospecies, was found with two individuals from twilight zone of ESC. One carabid beetle which is a troglobite species only occurs in cave ecosystems, *Ophonus (Hesperophonus) azureus* (F., 1775) and the other species *Laemostenus (Antisphodrus) longicornis* Casale, 1988 (Coleoptera: Carabidae) a typical troglophiles to troglobite species were found only dark zone of ESC with six and 12 individuals, respectively. Three arachnids; *Carios* sp. *(*Ixodoidea: Argasidae) and one from the family Linyphiidae, and one from Dysderidae, identified as morpho species were sampled on the dark zone of ESC (Table 4).

Table 1. Number of individuals and sampling dates of the species in ESC

Class	Order	Family	Species	Individuals and sampling date codes*
Arachnida	Ixodoidea	Argasidae	*Carios* sp. ?	1 (A); 2 (B); 1 (E 2); 1 (F2); 2 (H1); 1 (J1)
	Araneae	Dysderidae	?	1 (A); 1 (B); 1 (C2); 1 (C3); 1 (D1); 1 (E1); 1 (F3); 1 (G); 1 (H1); 3 (H2); 1 (I1); 1 (I3); 2 (J2)
		Linyphiidae	?	4 (C1); 2 (D3); 2 (E 2); 11 (F2); 3 (H1); 5 (H2); 7 (I2); 3 (J2); 1 (K)
		?	?	1 (A); 3 (C1); 1 (C3); 2 (D3); 1 (E2); 2 (I2); 1 (K)
		?	?	2 (B); 1 (C1); 1 (C3); 2 (D1); 1 (D3); 1 (E1); 1 (E2); 1 (F2); 1 (F3); 1 (I3)
Chilopoda	Scolopendromorpha	?	?	1 (C3); 2 (D1); 2 (D2); 1 (D3); 1 (E2); 1 (F1); 1 (I2); 1 (I3); 1 (J1); 1 (K)
	?	?	?	1 (C2); 2 (D2); 1 (E1); 2 (F1); 1 (F3); 1 (G); 1 (H1); 1 (H2); 1 (I1); 1 (J2)
Collembola	?	?	?	12 (A); 38 (C1); 15 (C3); 17 (D3) 11 (E2); 8 (F3); 6 (I1)
Diplopoda	Callipodida	Schizopetalidae	*Eurygyrus* sp.	2 (A); 1 (C1); 1 (C2); 2 (C3); 1 (D1); 1 (D2); 2 (D3); 4 (E1); 3 (E2); 2 (F1); 1 (F3); 1 (G); 4 (H1); 5 (H2); 4 (I1); 7 (I2); 1 (I3); 3 (J1); 4 (J2); 2 (K)
Hexapoda	Coleoptera	Carabidae	*Carabus glabratus* Paykull, 1790	1 (A); 2 (C1); 1 (C2); 1 (C3); 1 (D1); 2 (D3); 3 (E1); 1 (F1); 2 (F2); 2 (F3); 1 (G); 1 (H1); 2 (I1); 1 (I2); 1 (I3); 1 (J1); 2 (J2); 1 (K)
			Carabus graecus Dejean, 1826	1 (A); 1 (B); 2 (C1); 1 (C3); 1 (D1); 3 (D2); 1 (D3); 2 (E1); 1 (E2); 3; (F1); 1 (F3); 1 (G); 1 (H2); 3 (I1); 1 (I3); 1 (J2); 1 (K)
			Laemostenus (Antisphodrus) longicornis Casale, 1988	2 (A); 1 (B); 1 (C1); 3 (C2); 2 (C3); 4 (D1); 1 (D3); 4 (E1); 3 (E2); 2 (F1); 7 (F2); 2 (F3); 2 (G); 7 (H1); 5 (H2); 2 (I1); 1 (I2); 1 (I3); 2 (J1); 3 (J2); 3 (K)
			Ophonus (Hesperophonus) azureus (F., 1775)	1 (A); 2 (B); 1 (C1); 1 (C2); 3 (C3); 3 (D1); 2 (D2); 1 (D3); 3 (E2); 2 (F1); 1 (F2); 3 (F3); 2 (G); 3 (H1); 3 (H2); 1 (I1); 2 (I2); 1 (I3); 1 (J1); 2 (J2); 2 (K)
		Chrysomelidae	*Chrysomela populi* L., 1758	1 (B); 1 (C); 1 (D2); 1 (F1); 1 (F2); 1 (G); 1 (H1); 1 (I1); 1 (I2); 1 (I3); 2 (J1); 1(K)
		Curculionidae	*Rhynchaenus asellus* Gravenhorst, 1807	1 (A); 1 (C2); 2 (D2); 3 (E1); 1 (F1); 1 (H2); 1 (I2); 2 (K)
			Ips sexdentatus (Boerner, 1776)	1 (B); 1 (C1); 1 (D1); 2 (D2); 1 (E1); 1 (F2); 1 (H1); 1 (I3); 1 (J2)
			Larinus curtus Hochhuth, 1851	1 (C1); 1 (D2); 1 (F2); 1 (H1); 1 (J1)
			Tomicus minor (Hartig, 1834)	2 (B); 2 (C2); 1 (D2); 1 (F1); 1 (G); 1 (H2); 1 (J1)
		Scarabaeidae	*Anoxia asiatica* Desbrochers, 1871	1 (B); 2 (C2); 2 (D2); 2 (F1); 1 (F2); 1 (G); 1 (H2); 1 (I1); 2 (I2); 1 (I3); 1 (J1); 1 (J2)
			Cetonia aurata (L., 1758)	1 (A); 2 (B); 3 (C2); 2 (D2); 2 (E1); 3 (F2); 1 (G); 1 (H2); 1 (I1); 1 (I2); 1 (J1); 1 (J2)
			Oxythyrea cinctella (Schaum, 1841)	1 (B); 1 (C1); 1 (C2); 1 (D1); 1 (E1); 1 (F2); 1 (H1); 2 (I2); 1 (I3); 2 (J2); 1 (K)
	Hymenoptera	Formicidae	*Camponotus aethiops* (Latreille, 1798)	2 (B); 1 (C2); 4 (D2); 1 (E1); 1 (F1); 1 (F2); 1 (H1); 1 (I2); 2 J2)
			Messor semirufus (André, 1883)	1 (B); 2 (F2); 2 (H2); 4 (I2); 1 (J2)
			Stigmatomma denticulatum Roger, 1859	1 (A); 9 (B); 4 (C2); 6 (D2); 4 (E1); 8 (F1); 4 (F2); 6 (G); 1 (H1); 4 (I1); 4 (I2); 3 (J1); 6 (J2)
	Lepidoptera	Erebidae	*Scoliopteryx libatrix* L., 1758	1 (A); 1 (C1); 1 (C2); 1 (C3); 2 (D1); 1 (D2); 2 (D3); 1 (E1); 1 (E2); 1 (F1); 1 (F3); 1 (G); 2 (H1); 1 (H2); 2 (I1); 1 (I2); 1 (I3); 1 (J1); 3 (K)

*A, during November 2010; B, during June 2011; C1, 03-04 March 2012; C2, 07-08 July 2012; C3, 17-18 November 2012; D1, 9-10 February 2013; D2, 15-16 June 2013; D3, 23-24 November 2013; E1, 14-15 June 2014; E2, 27-28 December 2014; F1, 2-3 May 2015; F2, 11-12 July 2015; F3, 14-15 November 2015; G, during July 2016; H1, 22-26 February 2017, H2, 19-22 October 2017; I1, 19-20 May 2018; I2, 18-19 August 2018; I3, 3-4 November 2018; J1, 4-5 May 2019; J2, 6-7 July 2019; and K, 15-16 February 2020.

Table 2. Number of individuals and sampling dates of the species in KIC

Class	Order	Family	Species	Individuals and sampling date codes*
Arachnida	Araneae	Agelenidae	*Tegenaria percuriosa* Brignoli, 1972	1 (L2)
			Tegenaria sp.	1 (l1); 3 (L2)
		Dysderidae	*Dysderocrates* sp.	2 (L1); 5 (L2)
			Harpactea sp.	1 (L1)
		Filistatidae	*Pritha* sp.	1 (L2)
		Linyphiidae	*Centromerus* sp.	1 (L1)
			Lepthyphantes leprosus (Ohlert, 1865)	1 (L1)
			Troglohyphantes sp.	1 (L1)
		Pholcidae	*Hoplopholcus asiaeminoris* Brignoli, 1978	2 (L1); 4 (L2)
			Hoplopholcus sp.	3 (L1); 6 (L2)
		Sparassidae	*Heteropoda variegata* (Simon, 1874)	4 (L2)
	Scorpiones	Iuridae	*Protoiurus kadleci* (Kovarik Fet, Soleglad & Yağmur, 2010)	1 (L1); 3 (L2)
Diplopoda	Callipodida	Schizopetalidae	*Eurygyrus bilselii* (Verhoeff, 1940)	7 (L1); 9 (L2)
Hexapoda	Coleoptera	Carabidae	*Calathus syriacus* Chaudoir, 1863	1 (L2)
			Harpalus distinguendus (Duftschmid, 1812)	1 (L2)
			Laemostenus longicornis Casale, 1988	8 (L1); 10 (L2)
		Coccinellidae	*Coccinella septempunctata* L., 1758	1 (L1)
		Curculionidae	*Orthotomicus erosus* (Wollaston, 1857)	1 (L2)
			Tomicus destruens (Wollaston, 1865)	1 (L1)
		Meloidae	*Zonitis flava* F., 1775	1 (L2)
		Scarabaeidae	*Oryctes nasicornis* L., 1758	1 (L1)
	Hymenoptera	Formicidae	*Cataglyphis nodus* (Brullé, 1833)	1 (L1); 4 (L2)
			Messor oertzeni Forel, 1910	1 (L1); 1 (L2)
			Tapinoma erraticum (Latreille, 1798)	1 (L1)
	Orthoptera	Gryllidae	*Ovaliptila alanya* Gorochov & Ünal, 2012	25 (L1); 37 (L2)
		Rhaphidophoridae	*Troglophilus gajaci* Us, 1974	3 (L1); 5 (L2)

*L1, 15-19 February 2017; and L2, 12-15 October 2017.

Most of the Hexapods were sampled only from entrance zone of KIC; *Tomicus destruens* (Wollaston, 1865) (Coleoptera: Curculionidae), *Orthotomicus erosus* (Wollaston, 1857) (Coleoptera: Curculionidae), *Zonitis praeusta Zonitis flava* F., 1775 (Coleoptera: Meloidae), *Tapinoma erraticum* (Latreille, 1798) (Hymenoptera: Formicidae), *Cataglyphis nodus* (Brullé, 1833) (Hymenoptera: Formicidae), *Messor oertzeni* Forel, 1910 (Hymenoptera: Formicidae), *Oryctes nasicornis* L., 1758 (Coleoptera: Scarabaeidae), *Coccinella septempunctata* L., 1758 (Coleoptera: Coccinellidae), *Harpalus distinguendus* (Duftschmid, 1812) (Coleoptera: Carabidae), and *Calathus syriacus* Chaudoir, 1863 (Coleoptera: Carabidae) with fewer individuals. *Pritha* sp. (Araneae: Filistatidae) was only one species from the order Araneae caught in the entrance zone (Table 5).

A notable result, *Dysderocrates* sp. (Araneae: Dysderidae) was captured in both entrance and dark zones with two and five individuals, respectively. Species sampled in all three zones, entrance, twilight, and dark were *Hoplopholcus asiaeminoris* Brignoli, 1978 (Araneae: Pholcidae) and *Hoplopholcus* sp. (Araneae: Pholcidae). One arachnid, *Heteropoda variegata* (Simon, 1874) (Araneae: Sparassidae), one callipodid, *Eurygyrus bilselii* (Verhoeff, 1940) (Callipodida: Schizopetalidae) and two hexapod, *Laemostenus longicornis* Casale, 1988 (Coleoptera: Carabidae), and *Ovaliptila alanya* Gorochov & Ünal, 2012 (Orthoptera: Gryllidae) were captured from twilight and dark zones of KIC. Species only found in dark zone were *Tegenaria percuriosa* Brignoli, 1972 (Araneae: Agelenidae), *Tegenaria* sp. (Araneae:

Agelenidae), *Harpactea* sp. (Araneae: Dysderidae), *Lepthyphantes* p*leprosus* (Ohlert, 1865) (Araneae: Linyphiidae), *Centromerus* sp. (Araneae: Linyphiidae), *Troglohyphantes* sp. (Araneae: Linyphiidae), *Protoiurus kadleci* (Kovarik Fet, Soleglad & Yağmur, 2010) (Scorpiones: Iuridae), and *Troglophilus gajaci* Us, 1974 (Orthoptera: Rhaphidophoridae). Except *T. gajaci*, most of these were captured with few individuals (Table 5).

During the study, homogeneous collecting procedures were applied, 159 and 60 individuals were sampled from KIC and ESC, respectively.

Biological diversity of Erdal Şekeroğlu and Kadıini Caves

Results of the biodiversity indices, Shannon-Wiener, Simpson diversity and Shannon evenness, evaluated by the arthropod assemblages of both caves are given in Table 3.

Species richness was 21 and 26 in ESC and KIC, respectively. ESC was found to be more diverse (H' 2.597 and S 0.8961) than KIC (H' 2.307 and S 0.8112) according to both Shannon-Wiener and Simpson diversity indices. Shannon evenness results showed that the population density of the species was more uniformly distributed in ESC than KIC.

Shannon-Wiener's and Simpson's diversity indices showed that the entrance zones of both caves were more diverse than the other zones (Table 3). In addition to that, the dark zone of the KIC was more diverse than the dark zone of the ESC.

Table 3. Results of biological diversity indices for caves and each zone of the caves

Caves & Cave Zones*	Sr[1]	Ni[2]	H'[3]	S[4]	Sd[5]	EH[6]
ESC	21	60	2.5940	0.8961	0.1040	0.8523
ESCE	15	26	2.3174	0.8432	0,1568	0.8557
ESCT	1	2	-	-	-	-
ESCD	5	32	1.4615	0.7422	0,2578	0.9081
KIC	26	158	2.3070	0.8112	0.1890	0.7081
KICE	14	23	2.4615	0.8960	0.1040	0.9327
KICT	6	71	1.0304	0.4797	0.5203	0.5751
KICD	15	64	2.3704	0.8857	0.1143	0.8753

* E, entrance zone; T, twilight zone; and D, dark zone (as appended to the habitat names, ESC, Erdal Şekeroğlu Cave and KIC, Kadıini Cave);
[1] species richness; [2] Sum of individuals; [3] Shannon-Wiener Diversity index; [4] Simpson Diversity index; [5] Simpson Dominance index, [6] Shannon evenness index.

Similarity of Erdal Şekeroğlu and Kadıini Caves and cave zones

The similarity dendrogram built on the base of the Sörensen index showed that there was no similarity between ESC and KIC, and also between the zones of each cave. It was revealed that there was the only similarity between the zones in KIC. The twilight and dark zones of the KIC were 48.5% similar to each other, and the entrance zone was found 13.3% similar to this group (Figure 1). These results show that the cave ecosystems have their unique species diversity and ecosystems. Also, these results show that all of the species collected from both caves have limited dispersal ability because they are adapted to caves. The cladograms for ESC indicate that all of the species have special habitat preferences, but the cladograms for KIC indicates that some species can inhabit both twilight zone and dark zone. Therefore, it can be concluded that all of the species in ESC have specific zone adaptation based on light.

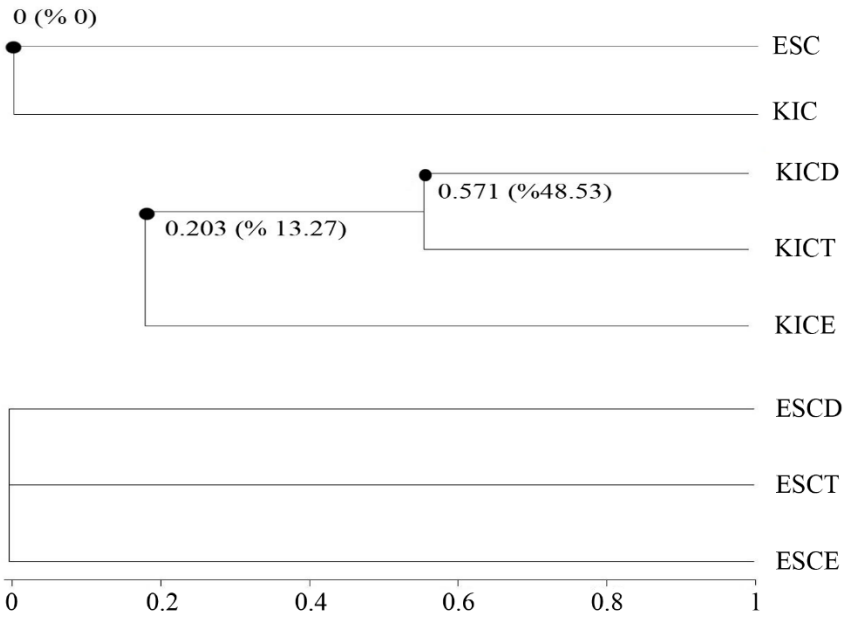

Figure 1. Similarity between arthropod assemblages inhabiting different caves and cave zones based on species composition (Sörensen index). ESC, Erdal Şekeroğlu Cave; KIC, Kadıini Cave; KICE, entrance zone of the Kadiini Cave; KICT, twilight zone of the Kadiini Cave; KICD, dark zone of the Kadiini Cave; ESCE, entrance zone of the Erdal Şekeroğlu Cave; ESCT, twilight zone of the Erdal Şekeroğlu Cave; ESCD, dark zone of the Erdal Şekeroğlu Cave (percentages given in parentheses are calculated separately from the percent similarity).

Indicator species of Erdal Şekeroğlu and Kadıini Caves

As a result of the inclusion of rare individuals in the analysis, all of these species were found statistically significant as indicators for zone description in ESC ($P < 0.001$). (Table 4). According to ISA, *O. alanya* was determined as an indicator species for the twilight zone of the KIC with 82% InV (Table 5), however, this species was also detected in the dark zone between 1700 and 1800 m ahead in the KIC (Figure 2). Photograph of the species is given Figure 3.

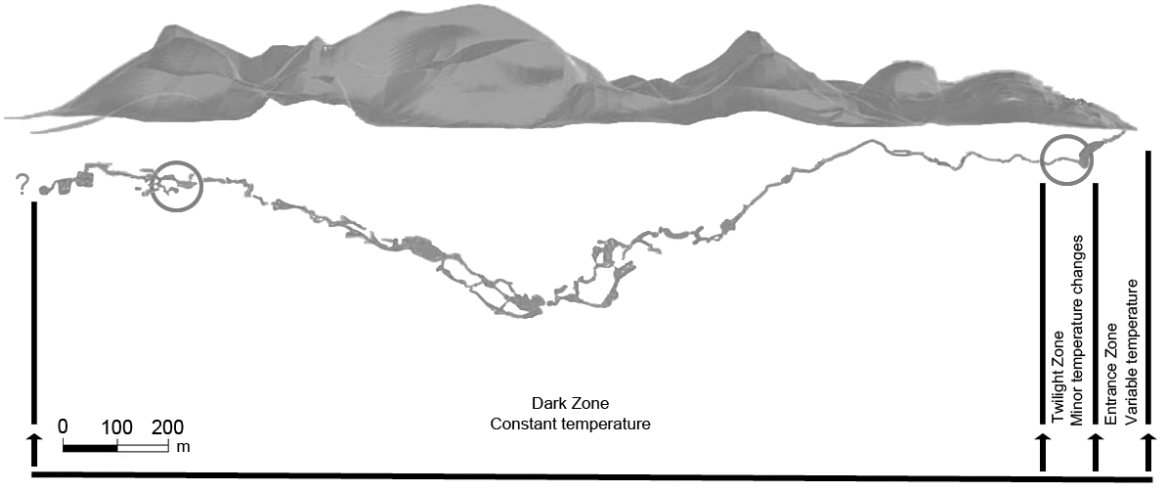

Figure 2. Map of the known part of the KIC (2027 m) showing the representation of the cave zones and the distribution of the *Ovaliptila alanya* in the cave (distribution of the species is indicated by two circles) (The base map prepared by members of Akdeniz University Caving Society-AKUMAK).

Figure 3. Photograph of *Ovaliptila alanya*, the first insect described from cave mapping (photo by the first author).

Table 4. Indicator species and their indicator values (Monte Carlo test, P < 0.05, 4999 permutations, random number seed of 699) in the zones of the ESC

Class	Order	Family	Species	Z	%InV	P*	E	T	D
Arachnida	Ixodoidea	Argasidae	*Carios* sp. ?	D	100	0.0324	-	-	2
	Araneae	Dysderidae	?	D	100	0.0324	-	-	4
		Linyphiidae	?	D	100	0.0324	-	-	8
Chilopoda	?	?	?	T	100	0.0354	-	2	-
Diplopoda	Callipodida	Schizopetalidae	*Eurygyrus* sp.	E	100	0.0382	9	-	-
Hexapoda	Coleoptera	Carabidae	*Carabus glabratus* Paykull, 1790	E	100	0.0382	1	-	-
			Carabus graecus Dejean, 1826	E	100	0.0382	1	-	-
			Laemostenus (Antisphodrus) longicornis Casale, 1988	D	100	0.0324	-	-	12
			Ophonus (Hesperophonus) azureus (F., 1775)	D	100	0.0324	-	-	6
		Chrysomelidae	*Chrysomela populi* L., 1758	E	100	0.0382	1	-	-
		Curculionidae	*Rhynchaenus asellus* Gravenhorst, 1807	E	100	0.0382	1	-	-
			Ips sexdentatus (Boerner, 1776)	E	100	0.0382	1	-	-
			Larinus curtus Hochhuth, 1851	E	100	0.0382	1	-	-
			Tomicus minor (Hartig, 1834)	E	100	0.0382	1	-	-
	Hymenoptera	Formicidae	*Camponotus aethiops* (Latreille, 1798)	E	100	0.0382	1	-	-
			Messor semirufus (André, 1883)	E	100	0.0382	2	-	-
			Stigmatomma denticulatum Roger, 1859	E	100	0.0382	1	-	-
	Coleoptera	Scarabaeidae	*Anoxia asiatica* Desbrochers des Loges, 1871	E	100	0.0382	1	-	-
			Cetonia aurata (L., 1758)	E	100	0.0382	1	-	-
			Oxythyrea cinctella (Schaum, 1841)	E	100	0.0382	1	-	-
	Lepidoptera	Erebidae	*Scoliopteryx libatrix* L., 1758	E	100	0.0382	3	-	-

*E, entrance zone; T, twilight zone; D, dark zone and Z, the zone where the species is the indicator
Maxgrp = group identifier for group with maximum observed IV
a Proportion of randomized trials with IV equal to or exceeding the observed IV.
p = (1 + number of runs ≥ observed) / (1 + number of randomized runs).

Table 5. Indicator species and their indicator values (Monte Carlo test, P < 0.05, 4999 permutations, random number seed of 5733) in the zones of the KIC

Class	Order	Family	Species	Z	%InV	P*	E	T	D
Arachnida	Araneae	Agelenidae	*Tegenaria percuriosa* Brignoli, 1972	D	100	0.0336	-	-	1
			Tegenaria sp.	D	100	0.0336	-	-	4
		Dysderidae	*Dysderocrates* sp.	E	71	0.6689	2	-	5
			Harpactea sp.	D	100	0.0336	-	-	1
		Filistatidae	*Pritha* sp.	E	100	0.0348	1	-	-
		Linyphiidae	*Centromerus* sp.	D	100	0.0336	-	-	1
			Lepthyphantes leprosus (Ohlert, 1865)	D	100	0.0336	-	-	1
			Troglohyphantes sp.	D	100	0.0336	-	-	1
		Pholcidae	*Hoplopholcus asiaeminoris* Brignoli, 1978	-	33	-	2	2	2
			Hoplopholcus sp.	-	33	-	3	3	3
		Sparassidae	*Heteropoda variegata* (Simon, 1874)	T	50	0.6743	-	2	2
	Scorpiones	Iuridae	*Protoiurus kadleci* (Kovarik Fet, Soleglad & Yağmur, 2010)	D	100	0.0336	-	-	4
Diplopoda	Callipodida	Schizopetalidae	*Eurygyrus bilselii* (Verhoeff, 1940)	T	67	0.6743	-	5	11
Hexapoda	Coleoptera	Carabidae	*Calathus syriacus* Chaudoir, 1863	E	100	0.0348	1	-	-
			Harpalus distinguendus (Duftschmid, 1812)	E	100	0.0348	1	-	-
			Laemostenus longicornis Casale, 1988	T	50	0.6743	-	9	9
		Coccinellidae	*Coccinella septempunctata* L., 1758	E	100	0.0348	1	-	-
		Curculionidae	*Orthotomicus erosus* (Wollaston, 1857)	E	100	0.0348	1	-	-
			Tomicus destruens (Wollaston, 1865)	E	100	0.0348	1	-	-
		Meloidae	*Zonitis praeusta* F., 1792	E	100	0.0348	1	-	-
		Scarabaeidae	*Oryctes nasicornis* L., 1758	E	100	0.0348	1	-	-
	Hymenoptera	Formicidae	*Cataglyphis nodus* (Brullé, 1833)	E	100	0.0348	5	-	-
			Messor oertzeni Forel, 1910	E	100	0.0348	2	-	-
			Tapinoma erraticum (Latreille, 1798)	E	100	0.0348	1	-	-
	Orthoptera	Gryllidae	*Ovaliptila alanya* Gorochov & Ünal, 2012	T	81	0.6743	-	50	12
		Rhaphidophoridae	*Troglophilus gajaci* Us, 1974	D	100	0.0336	-	-	8

* E: entrance zone, T: twilight zone, D: dark zone, Z: the zone where the species is the indicator;
Maxgrp = group identifier for group with maximum observed IV;
a Proportion of randomized trials with IV equal to or exceeding the observed IV;
p = (1+number of runs ≥ observed) / (1+number of randomized runs).

Species richness estimations of Erdal Şekeroğlu and Kadıini Caves

The results of the species estimators for both caves showed that there were still some undetected species in each cave (Table 6, Figure 4). The percentage of the determined species falls between 20% (ACE) and 81% (Bootstrap) in KIC and between 13% (ICE and Chao 2) and 84% (MMRuns) in ESC (Table 6). Although the range of estimation percentages was similar for both caves, the estimation percentages of ESC were more similar, apart from ICE and Chao 2.

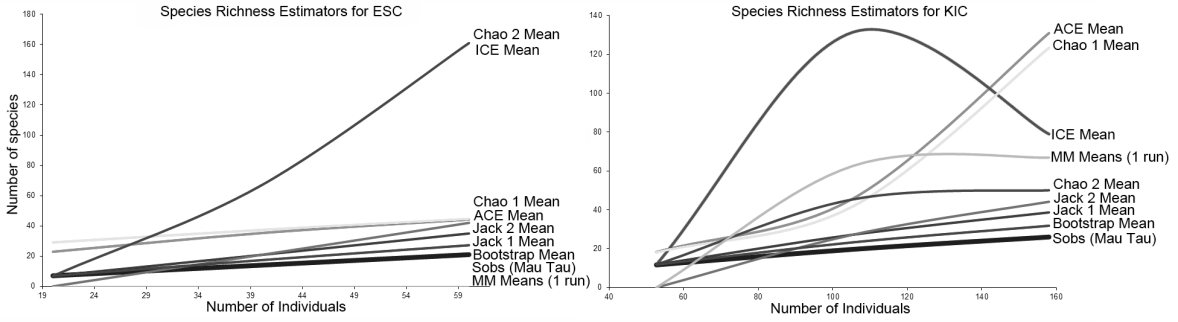

Figure 4. Species accumulation curves for ESC (left) and KIC (right).

Table 6. The number of the recorded and estimated species, and the percentage of the estimated species number recorded for each cave

	KIC	ESC
Observed species (S*obs*)	26	21
Number of samples	3	3
Number of individuals	158	60
Singletons	14	12
Doubletons	1	3
ACE	131	44
ACE %	20	48
ICE	79	161
ICE %	33	13
Chao 1	123	45
Chao 1 %	21	47
Chao 2	50	161
Chao 2 %	52	13
Jack 1	39	35
Jack 1 %	67	60
Jack 2	44	42
Jack 2 %	59	50
Bootstraps	32	27
Bootstraps %	81	78
MMRuns	42	25
MMRuns %	62	84
MMMeans	67	0
MMMeans %	39	0

Discussion

The arthropod biodiversity of KIC (Antalya, Alanya) and ESC (Isparta, Atabey) was determined. As a result of the study, 51 arthropod species were detected. These belonged to five classes: 29 Hexapoda, 17 Arachnida, two Chilopoda, two Diplopoda and one Collembola. The species richness and diversity of insects are similar in these two cave ecosystems as well as other ecosystems of the worldwide. Many scientific studies in cave ecosystems show that hexapods are more diverse than other arthropod classes. Additionally, a significant proportion of the arthropod species that are collected in the cave ecosystems are hexapods (Poulson & Culver, 1969; Schneider et al., 2010; Culver & Pipan, 2018; Niemiller & Taylor, 2019; Ledesma et al., 2020).

Biodiversity parameters can be measured differently, even in different regions in the same cave ecosystem. This is due to many ecological factors such as human activity, habitat degradation, nutrient and availability (Poulson & Culver, 1969). When the caves were evaluated for species diversity, Shannon-Wiener and Simpson diversity indices showed that the ESC was more diverse than KIC. For species diversity, results of the diversity indices revealed that the twilight zones in both caves are less diverse than the entrance zones and the dark zones. Our study thus agrees with similar studies conducted on species diversity of arthropods inhabiting different cave zones (Prous et al., 2004; Tobin et al., 2013; Kurniawan et al., 2018). However, most of these studies have revealed that the state of diversity varies between zones depending on the many biotic and abiotic factors (Tobin et al., 2013). Also, it is known that diversity increases with the increasing area because the larger area has more habitats and niches to be able to support a larger variety of species (MacArthur & Wilson, 1967). When considering the length of the caves, the results of the present study are inconsistent with this theory. There is some knowledge of human activities from the Chalcolithic Age-Early Bronze Age in the KIC (Yılmaz Usta, 2019). So, the lower species diversity in KIC may have been caused by anthropogenic activities (such as habitat destruction and modification) from nearly 5,000 BC to today in this cave. The abundance of the species living in the entrance zone and accidentally fall into the cave should also be considered.

The arthropod assemblages inhabiting in the three cave zones in each cave were compared. The similarity dendrograms built on the base of the Sörensen index showed that there was no similarity between ESC and KIC and between similar zones in both caves. However, the twilight zone and dark zone of the KIC had 48% similarity and these two zones had 13% similarity with the entrance zone of this cave. It should be taken into consideration that one of the factors that increased the similarity between the twilight zone and the dark zone may be caused by the unexpected distribution of *O. alanya*. Despite this eventuality it is clear that the arthropod assemblages of the twilight zones of both caves are more similar to the assemblages of the dark zones. The higher similarity among these arthropod assemblages is caused by the higher abiotic similarity among the twilight and dark zones. These results are found similar to those of other studies (Kurniawan et al., 2018).

When species compositions of both caves have taken into account at the species level, results showed that both caves have unique species composition. However, when the species compositions of both caves are considered at the family level, it was found that taxa in the twilight zone and the dark zone of both caves belong to the same families. These situations may be due to two reasons. Firstly, species-level differences can arise from the geographical distance between the caves. Secondly, family-level similarity can arise from the fauna of in each cave being descended from similar ancestral fauna. Considering similarities of cave zones, although KIC was longer than ESC, the similarity among the cave zones in KIC was higher than ESC. There may be two reasons for the zone similarity of KIC. Firstly, food may be carried into the dark zone by cavers and animals due to the structure of KIC. Secondly, the dark zone of the KIC could be connecting to the outside with small cracks. In other words, the zones of the ESC are better separated from each other. However, many arthropods seem to delimit that transition zone based on light penetration, salinity, supply of nutrients and other factors (Wittmann, 2004).

No study to date has examined insects as indicators in cave mapping. In present study we investigated whether there is a species that can be used as a biological indicator. ISA showed that *O. alanya* can be used as an indicator species for the twilight zone of the KIC with 81% InV. It is quite unlikely that this species would also be concurrently collected from the dark zone of the KIC. Under normal circumstances, the indicator value of the species must have been found 100% in the twilight zone (Taylan et al., 2020) This significant distribution pattern of the *O. alanya* could be due to another undiscovered entrance of the cave or small cracks connecting the dark zone (actually twilight zone if the light comes in) of the KIC with the external environment. In this context, this species appears to be potentially useful in cave mapping.

Completeness of the arthropod inventory was calculated by using species estimators. According to all of the species estimators, the species estimates for KIC ranged from 20 to 81% and the species estimates for ESC ranged from 13 to 84%. Similarly, Wynne (2014) stated that none of the accumulation curves neared an asymptote in the studied four caves. In the present study, the results show that species estimators are reasonably incomplete and all of the estimators agree in their values that there are still undetected species in each cave. The high amount of rare species and sampling limitations made species richness estimation more challenging in the both caves ESC and KIC. Due to the large number of rare species in cave ecosystems the number of species predicted by species richness estimators is large (Schneider & Culver, 2004; Chao & Chiu, 2016).

In conclusion, the present study (1) highlights the need for further studies to determine the complete fauna in both caves and (2) shows that each cave and its zones have a unique fauna, and warrant conservation on this basis. Also, the study highlights that there are limited studies of the biodiversity and ecological parameters of arthropod assemblages on the cave ecosystems (Prous et al., 2004; Tobin et al., 2013; Wynne, 2014; Kurniawan et al., 2018).

An additional conclusion is our results demonstrate how insects can be used in cave mapping, and in supporting caves protection and conservation. The study is a small, but crucial, step towards understanding biodiversity patterns in these important but poorly documented ecosystems. This is key given the role of these species have in the food chains and in light of their vulnerability to changing environmental conditions. We argue strongly that trogloxene and troglophile arthropods should be taken into consideration before any decision to open the cave for tourism. Follow up work is urgently needed in this area before known and unknown species become extinct.

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Original article (Orijinal araştırma)

Phytophagous nematodes in cereal fields in Niğde Province, Turkey

Niğde İli tahıl alanlarında bulunan fitofag nematodlar

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Abstract

This study evaluated the occurrence and incidence of phytophagous nematodes and identified the cereal cyst nematode species by morphological and molecular tools in the main cereal-growing areas in Niğde in 2018-2019. Phytophagous nematodes within twelve genera were detected in 95% of soil samples. The most common phytophagous nematodes in cereal soil were in the genera *Heterodera, Ditylenchus, Merlinius, Pratylenchus, Aphelenchus, Aphelenchoides, Tylenchus, Helicotylenchus, Trophurus, Pratylenchoides, Filenchus* and *Xiphinema* (in decreasing order of incidence). In particular, 75% of the soil samples from surveyed fields were infested with the cereal cyst nematodes (*Heterodera* spp.). Morphological characteristics of cysts and second-stage juveniles were calculated within the expected ranges for *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984, however, two populations from Çamardı was determined as *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae). Intraspecific variation was not observed within the populations of *H. filipjevi* which could be in the same genotypic group. In addition to the high incidence of these *Heterodera* spp., intensive cereal cropping systems with/without non-cereal rotations in wheat production areas of Niğde also resulted in high incidence of root lesion nematode, *Pratylenchus* species.

Keywords: Identification, *Heterodera* spp., phylogeny, phytophagous nematodes, wheat

Öz

Bu çalışma ile 2018-2019 yıllarında Niğde İli'ndeki başlıca tahıl yetiştirme alanlarında bulunan fitofag nematodların yoğunluk ve dağılımı belirlenmiş, tahıl kist nematodu türlerinin morfolojik ve moleküler yöntemlerle tanımı yapılmıştır. Fitofag nematodlar 12 cins olarak toprak örneklerinin %95'inde tespit edilmiştir. En yaygın fitofag nematodlar sırasıyla, *Heterodera*, *Ditylenchus*, *Merlinius*, *Pratylenchus*, *Aphelenchus*, *Aphelenchoides*, *Tylenchus*, *Helicotylenchus*, *Trophurus*, *Pratylenchoides*, *Filenchus* ve *Xiphinema* cinsleridir (bulunma yoğunluğuna göre sıralanmıştır). İncelenen toprak örneklerinin %75'inde tahıl kist nematodu (*Heterodera* spp.) bulunmuştur. Kistlerin ve 2. dönem larvaların ölçümleri ve morfolojik karakterlerinin değerlendirilmesiyle elde edilen sonuçlarda *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984 olarak tespit edilmiş olup sadece Çamardı'dan iki popülasyon *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae) olarak belirlenmiştir. *Heterodera filipjevi*'nin tür içi varyasyonlarına bakıldığında hepsinin aynı gurupta olabileceği değerlendirilmiştir. Buna ilave olarak tahıl kist nematodları yanında kök lezyon nematodlarının, *Pratylenchus* türlerinin de rotasyon yapılan/yapılmayan Niğde İli tarım alanlarında yüksek yoğunlukta bulunduğu sonucuna varılmıştır.

Anahtar sözcükler: Teşhis, *Heterodera* spp., filogenetik, bitki paraziti nematodlar, buğday

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Introduction

Wheat (*Triticum* spp.) is the third most important crop after rice and maize in terms of global production with an average annual production of almost 772 Mt in 2017 (FAO, 2018). As the most important food crops of many countries, bread wheat [*Triticum aestivum* L. (Poales: Poaceae)] and durum wheat [*Triticum durum* Desf. (Poales: Poaceae)] provides an important nutrition to the feeding both humans and livestock, and contribute nearly one-third of the total food grain production in the world. A complex of biotic (i.e., diseases and insects) and abiotic factors (i.e., soil and climate) affects directly wheat grain yield in the important wheat-growing areas of the world. Among the biotic factors, the plant parasitic nematodes (PPNs) are a major biotic constraint to wheat production systems worldwide, especially where the plants under stress by other biotic and abiotic factors, particularly drought. PPNs cause severe annual economic losses of up to 216 billion USD worldwide when simultaneously exposed to drought (Nyaku et al., 2017). Cereal cyst nematodes (CCNs) [*Heterodera avenae* Wollenweber, 1924 (Tylenchida: Heteroderidae) group], among the PPNs, are most commonly found in wheat cropping areas (Rivoal & Cook, 1993; Dababat & Fourie, 2018).

Heterodera avenae group occurs widely throughout the wheat-growing areas of the world (Dababat & Fourie, 2018). Cereal cyst nematodes are causing yield loss of about 3.4 million USD annually in the Pacific Northwest region of the USA (Smiley & Nicol, 2009). The main species of cyst nematode pests of cereals are *H. avenae*, *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984 and *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae) within *H. avenae* group, which contains 12 nematode species (Rivoal & Cook, 1993; Nicol, 2002). Accurate identification of the species causing the loss is critical for determining the most effective method to control cyst nematodes, and requires both morphological and molecular methods. The vulval region of cysts and second-stage juveniles (J2s) are the most common features used for identification of *Heterodera* spp. (Handoo, 2002; Subbotin et al., 2010). However, the use of these morphological characters of *Heterodera* spp. is difficult and needs experienced scientists. Currently, molecular identification techniques are used overcome the taxonomic difficulties with morphological species identification (Dababat et al., 2015; Imren et al., 2020). Determination of the genetic variation in cyst nematode populations also aids the development or improvement of resistant host plants.

Turkey, with an average annual production of about 20 Mt and a planted area of about 7.3 Mha, is one of the important wheat-growing countries of the world, but the average yield of around 2.7 t/ha is relatively low (FAO, 2018). The Central Anatolian Plateau (CAP) produces about 5 Mha wheat annually under rainfed conditions with only a limited area of irrigation. Located on the CAP, Niğde Province produces around 230 kt of wheat, barley and rye grain on over 125 kha (TurkStat, 2020). In Niğde, wheat crops are frequently exposed to drought stress due to insufficient annual rainfall (200-300 mm) and scarcity of supplementary irrigation. Several studies were conducted between 1974-2020 to detect the diversity in *H. avenae* group in the root zone of various crops in different areas of Turkey (Yüksel, 1974; Rumpenhorst et al., 1996; Abidou et al., 2005, Şahin et al., 2009; Dababat et al., 2015; Imren et al., 2018). In Turkey, cyst nematodes in the *H. avenae* group were first found in associated wheat in Erzurum in 1974 and since then *H. avenae* group nematodes have been increasingly found in the other wheat-growing areas of Turkey (Imren et al., 2012; Dababat et al., 2015). *Heterodera avenae* group populations from the CAP have mainly been identified based on their morphology and morphometrics; therefore, limited information is available on the variation in their morphometrics in relation to their genetic structure (Şahin et al., 2009).

This research was conducted to reveal the status of phytophagous nematodes including the *H. avenae* group in the cereal-growing areas of Niğde by completing an intensive survey in wheat fields in Altunhisar, Bor, Central, Çamardı and Ulukışla Districts. The objectives were to (1) determine the incidence of the important species of PPNs in Niğde Province, (2) determination and evaluate both cysts and J2s of CCN populations with morphology, morphometrics and molecular assays including sequencing of the internal transcribed spacer (ITS) of ribosomal DNA fragments, and (3) investigate the phylogenetic relationships with and between populations found.

Materials and Methods

Nematode populations

The survey was conducted in 2018 between physiological maturity and harvest stage of wheat from the cereal-growing areas of Altunhisar, Bor, Central, Çamardı and Ulukışla Districts in Niğde. In total 64 cereal fields (wheat and barley) were sampled (Figure 1 & Table 1) about 10-20 km apart with soil samples were taken arbitrarily along a zigzag transect. A minimum of 10 cores was taken per sample using 2.5 cm diameter soil corer, the soil mixed and a representative subsample of 2 kg was kept for nematode extraction. Details recorded included crop, district and geographic coordinates.

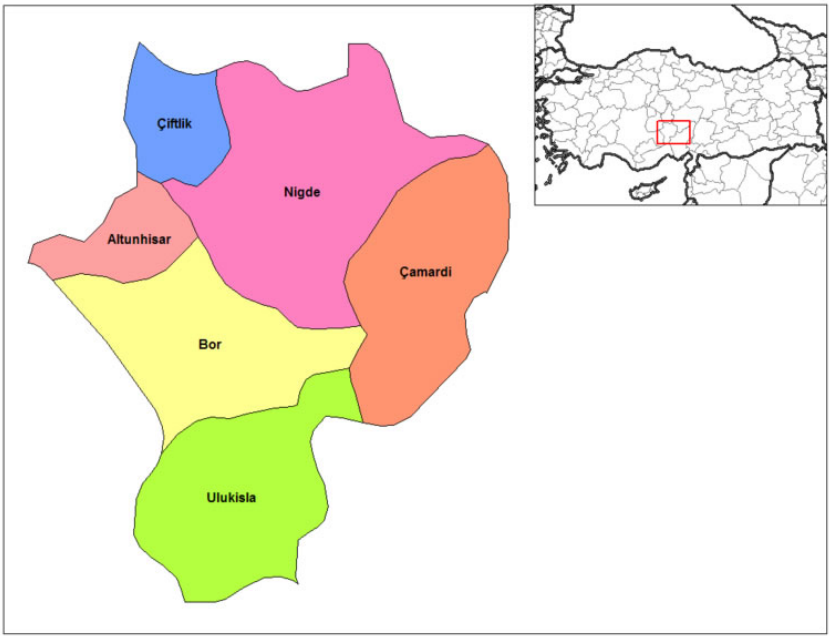

Figure 1. Districts of Niğde Province, Turkey surveyed for phytophagous nematodes (image source: www.istanbul-city-guide.com/map/turkey/nigde/map_of_nigde.jpg). The district labeled Niğde is Central District as used in the text. No fields were sampled in Çiftlik because cereal production is not a major land use in that district.

Migratory nematodes were extracted using a modified Baermann funnel from 100 ml of soil as described by Hooper (1986). After extraction, nematode suspensions were placed in measuring cylinders and allowed to settle for 8 h, the supernatant discarded, and the concentrated nematodes (plus debris) were transferred in 15-ml tubes. Nematode identification was made to genus under a light microscope at 100× magnification (Leica 5500, Wetzlar Germany). Cyst nematodes were extracted from 250 ml of soil using a modified decanting and sieving technique (Dababat et al., 2014). At least 20 full cysts were selected from each population, and stored at 4ºC for later morphological and molecular analysis. Cysts were morphologically identified to genus under a stereomicroscope at 20× magnification. The incidence of the nematodes was determined for each field as the quantity of the samples with nematodes as a proportion of the total number of samples.

Morphological identification of *Heterodera* specimens

Each *Heterodera* population was identified based on the vulval cones structure and J2 dimensions as well as their morphological features. Vulval cone slides were used to identified cyst nematode specimens according to Hooper (1986). Vulval slit length, vulval bridge width, fenestra width and length, and underbridge width and length were measured. The presence of underbridge and bullae of the perineal area were also determined (Handoo, 2002).

Table 1. Districts, locations, number of wheat and barley fields sampled (infested only) and their geographic coordinates in Niğde Province, Turkey sampled for phytophagous nematode assessment in 2018

District	Location	Infested fields	Geographic coordinates (N and E)
Altunhisar	Akmanlar	1	38°94'88.3" 34º27'97.0"
	Central	1	38°76'71.4" 34º89'07.0"
	Çömlekçi	1	38°05'67.1" 34º29'71.8"
	Ulukışla	1	38°04'88.3" 34º21'77.3"
	Yakacık	2	38°02'95.3" 34º34'36.5"; 38°00'88.4" 34º31'85.6"
Bor	Bahçeli	1	38°26'51.9" 34º66'77.0"
	Central	7	37°09'67.6" 34º55'06.3"; 38°20'11.4" 34º21'77.3"; 38°16'71.4" 34º35'88.3"; 38°36'27.1" 34º06'79.0"; 38°50'61.0" 34º54'09.3"; 38°90'23.4" 34º12'09.3"; 38°56'87.4" 34º66'56.3"
Çamardı	Central	7	38°07'14.6" 34º82'62.3"; 38°06'82.6" 34º85'08.3"; 38°07'71.7" 34º91'65.8"; 38°06'73.4" 34º93'82.6"; 38°04'86.0" 34º96'66.8"; 38°03'18.8" 34º99'12.1"; 37°88'89.6" 35º09'58.2"
	Kavlaktepe	2	37°99'43.3" 35º06'43.4"; 37°97'66.8" 35º08'45.1"
Central	Ağcaşar	1	38°31'97.0" 34º71'51.6"
	Alay	11	38°26'31.2" 34º69'18.7"; 38°26'73.1" 34º67'91.2"; 38°27'52.7" 34º65'80.2"; 38°26'66.3" 34º71'62.6"; 38°28'54.7" 34º72'90.6"; 38°28'06.6" 34º73'69.8"; 38°27'11.2" 34º74'23.6"; 38°26'95.8" 34º73'42.7"; 38°26'71.4" 34º73'72.5"; 38°28'03.8" 34º69'51.3"; 38°27'15.4" 34º33'20.2"
	Edikli	3	38°19'01.4" 34º96'76.5"; 38°22'27.4" 34º96'43.1"; 38°26'13.1" 34º91'94.5"
	Konaklı	1	38°17'29.4" 34º94'06.8"
	Orhanlı	6	38°28'36.3" 34º89'33.2"; 38°29'62.4" 34º88'79.9"; 38°29'01.1" 34º88'32.0"; 38°30'62.2" 34º87'77.3"; 38°32'46.3" 34º83'95.3"; 38°33'52.5" 34º82'86.2"
Ulukışla	Güney	3	37°10'42.8" 34º35'11.3"; 37°10'51.9" 34º09'54.3"; 37°06'89.9" 34º68'11.3"

To prepare permanent slides, 15 juveniles from the individual cysts were heated, fixed in TAF solution (7% formalin and 40% formalin) and put in glycerol (Handoo, 2002). The most important features of J2s including stylet length, length and width of body, length, width and hyaline part of tail were measured. The morphometric ratios a, b', c and c' were determined (Handoo, 2002). *Heterodera* spp. cyst and J2 were identified using previous studies and identification keys. (Franklin, 1969; Mulvey & Golden, 1983; Handoo, 2002). At least 15 J2s and 15 cysts from each population were examined and photographed using a Leica DFC295 optic camera installed on a DM5500 B light microscope (Leica, Wetzlar, Germany) and measured by Leica software v.4.1.0.

Data were analyzed with SPSS 22.0 for Windows (IBM, Armonk, NY, USA) to determine any significant differences between the 48 populations ($P \leq 0.05$).

Molecular identification of *Heterodera* specimens

Genomic DNA of each population were isolated from one cyst according to method in Holterman et al. (2006). The primers were used for sequencing, AB28 (5'-CGTAACAAGGTAGCTGTAG-3') and TW81 (5'-TCCTCCGCTAAATGATATG-3') to amplify the ITS region of nematode DNA according to protocol of Joyce et al. (1994).

A total of 48 ITS sequences were identified using a BLAST in the GenBank database. After identification of CCN species in Niğde, six population from each district of Niğde were used for phylogenetic relationship DNA sequences of were aligned with Clustal X (Kimura, 1980). Phylogenetic analyses of the CCN populations and reference population available in the GenBank database were performed with MEGA

v 7.0 software using ITS regions (Kumar et al., 2016). A neighbor-joining tree was constructed as Tamura & Nei (1993) using 1000 times bootstrap. Gaps were considered as missing data in the sequences. *Heterodera schachtii* Schmidt, 1871 (Tylenchida: Heteroderidae) was used for as an outgroup for character polarization.

Results

Incidence of motile nematodes and cysts

Only 48 samples were evaluated for PPNs because soil samples were hard and dry during harvest. Specimens of motile phytophagous nematode genera were obtained from soil from 46 (96%) of the 48 infested fields sampled (Tables 1 & 2) with an average density 840 nematodes/100 g soil. The PPNs were identified in the samples as shown in Table 2. The highest incidence was for *Ditylenchus* species. (25 fields) followed by species of *Merlinus* (21 fields) and *Pratylenchus* (13 fields). The least common genera were *Filenchus* and *Xiphinema* each only found in a single field. *Heterodera* cysts were found in all of these 48 sampled fields. The economically important PPNs found were *Heterodera* (CCNs) and *Pratylenchus* (root lesion nematode). The cyst nematodes in found in 48 of the 64 fields (Table 3). The species were determined to be *H. filipjevi* and *H. latipons,* both in the *H. avenae* group, based on the morphologic, morphometric and molecular analysis. The fields in which cyst nematodes were detected were mostly used for cereal monocultures without fallow or crop rotation. The highest cyst incidence was in Altunhisar and Çamardı Districts and the lowest in Ulukışla District (Table 3). The average cyst density (cysts/250 g of soil) was calculated for each district, with the highest density in Çamardı District being threefold that found in Bor and Central Districts (Table 3).

Table 2. Genera of phytophagous nematodes extracted from in 48 infested wheat and barley fields in locations within five districts of Niğde Province, Turkey in 2018. Data shown is the number of fields for each the 12 genera extracted and the final column the number of genera extracted at each location including all fields sampled. The nematodes were extracted as motile forms from soil, except for *Heterodera*, which was extracted as cysts

District	Location	Infested fields	*Aphelenchoides*	*Aphelenchus*	*Ditylenchus*	*Filenchus*	*Helicotylenchus*	*Merlinius*	*Heterodera*	*Pratylenchoides*	*Pratylenchus*	*Trophurus*	*Tylenchus*	*Xiphinema*	Genera
Altunhisar	Akmanlar	1	-	-	-	-	-	1	1	-	1	-	-	-	3
	Central	1	-	1	1	-	-	1	1	-	-	-	-	-	4
	Çömlekçi	1	-	1	-	-	-	1	1	-	-	-	-	-	3
	Ulukışla	1	-	-	1	-	-	1	1	-	-	-	-	-	3
	Yakacık	2	-	-	1	-	1	2	2	-	-	-	-	-	4
Bor	Bahçeli	1	-	-	1	-	-	-	1	-	-	-	-	-	2
	Central	7	2	2	2	1	-	1	7	-	3	-	3	-	8
Çamardı	Central	7	2	2	4	-	-	1	7	1	3	1	3	1	10
	Kavlaktepe	2	-	-	2	-	1	1	2	1	1	1	-	-	7
Central	Ağcaşar	1	-	-	1	-	-	1	1	-	-	-	-	-	3
	Alay	11	2	-	4	-	3	6	11	-	4	-	2	-	7
	Edikli	3	1	1	2	-	-	1	3	-	-	1	-	-	6
	Konaklı	1	-	-	-	-	-	1	1	-	-	1	-	-	3
	Orhanlı	6	1	3	4	-	1	1	6	-	-	-	-	-	6
Ulukışla	Güney	3	1	1	2	-	1	2	3	-	1	-	-	-	7
All districts		48	9	11	25	1	7	21	48	2	13	4	8	1	

Morphology and morphometrics of cyst population

The survey yielded 48 *Heterodera* populations, with 46 determined to be *H. filipjevi* and two *H. latipons*.

***Heterodera filipjevi* (Madzhidov, 1981) Stelter (Tylenchida: Heteroderidae)**

Forty-six populations of *H. filipjevi* were detected across the five districts sampled. The morphological and morphometric characters were consistent with those reported by Handoo (2002). The CCN has lemon shaped cysts and their vulval area protruding at the posterior. Fenestral area was bifenestrate and horseshoe shaped with heavy bullae and underbridge (Figure 2). Measurements (mean and range, n = 15) for specimens from four districts (Table 4) were: body length without neck 599 µm (550-632 µm), neck length 85 µm (72-108 µm), fenestra length 50 µm (47-55 µm) and width 21.2 µm (20.4-21.8 µm). J2s head were slightly offset head and its body cylindrical with conical hyaline tail tip. The juveniles have strong stylet and moderately concave basal knobs (Figure 2). The body of *H. filipjevi* varied from 550 to 632 µm long and stylet from 24.2 to 25.7 µm. The body has four lateral lines but usually the two inner lines were prominent.

Table 3. Incidence and population density of cyst nematodes (*Heterodera* spp.) in wheat and barley fields sampled in districts of Niğde Province, Turkey, in 2018. *Heterodera filipjevi* as detected in 46 fields and *Heterodera latipons* in two fields

District	Fields sampled	Infested fields	Incidence (%)	Population density (cysts/250 g soil)
Altunhisar	6	6	100	7
Bor	10	8	80	10
Çamardı	9	9	100	30
Çiftlik	5	0	0	0
Central	30	22	73	10
Ulukışla	4	3	75	4
All	64	48	81	

* The two fields with *H. latipons* were in Çamardı Central (38°07'71.7" N, 34º91'65.8" E and 38°04'86.0" N, 34º96'66.8" E).

***Heterodera latipons* (Franklin, 1969) (Tylenchida: Heteroderidae)**

Only two populations of *H. latipons* from Çamardı District were examined. The morphological and morphometric characters were consistent with those reported by Handoo (2002) (Table 4). The cyst color was light brown, lemon shaped with ridges in a zigzag pattern (Figure 3). Fenestral area was bifenestrate and strong under bridge with no bullae body 470 µm (451-532 µm) without neck, body 293 µm (250-390 µm), neck 65 µm (60-80 µm), fenestra 64 µm (47.3-66.5 µm) and width 21 µm (18-25 µm), underbridge 96 µm (85-115 µm), vulval slit 8 µm (7.4-8.5 µm), vulval bridge width 12.4 µm (9.4-13.4 µm) (Figure 3). J2s (n = 15) had cylindrical body and head with conical tail extended with short hyaline terminal compared with *H. filipjev*i bodies. J2 body had four lateral lines, length 470 µm (412-482 µm), width 19 µm (19-21 µm), length of stylet 24 µm (23-25 µm), tail 60.2 µm (56-63 µm) and hyaline terminal 25 µm (23-29 µm) (Table 4).

Molecular characterization of cyst population

The nematode ribosomal gene of ITS(ITS1-5.8S-ITS2), region of all 48 *Heterodera* populations were successfully amplified for sequencing using AB28, TW81 primers pair and PCR product of nematodes were about 1060 bp. Forty-six sequences were identified as *H. filipjevi* and two *H. latipons* using BLAST program in GenBank database.

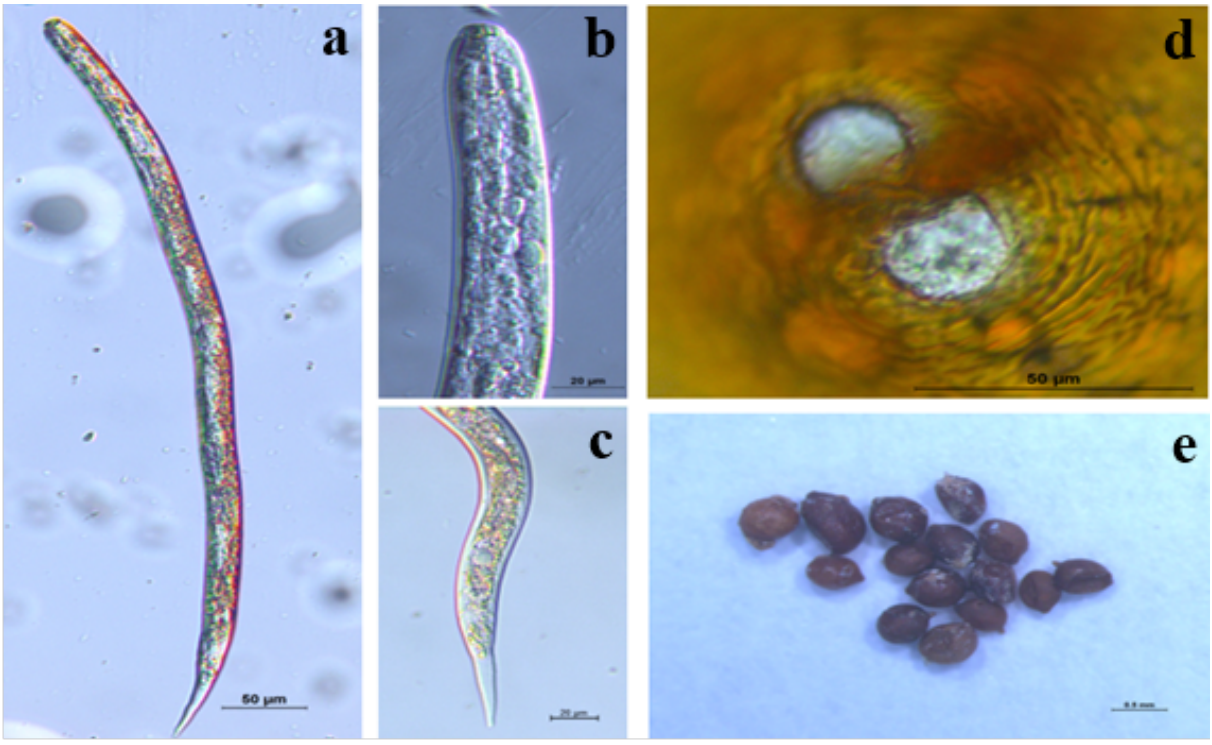

Figure 2. Light micrographs of *Heterodera filipjevi* from northern Niğde Province: a) second-stage juveniles; b) head region; c) tail region; d) fenestral region; and e) cysts of *Heterodera filipjevi*.

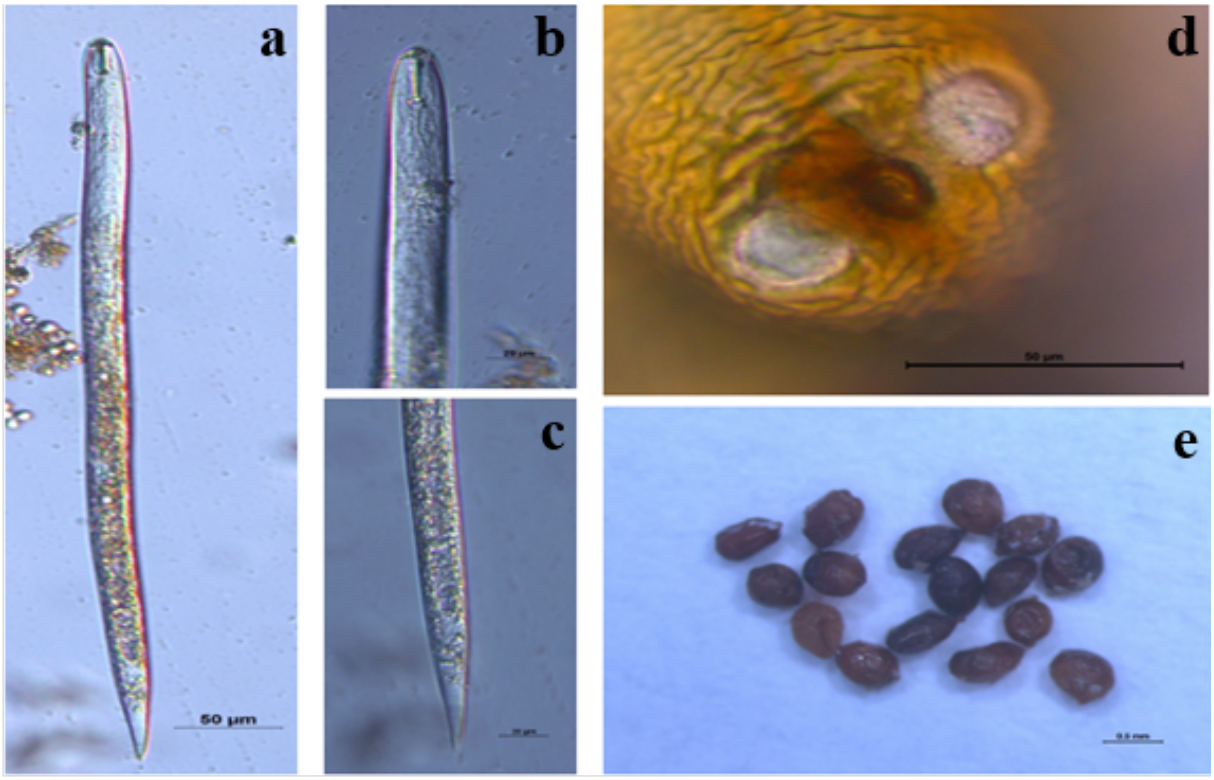

Figure 3. Light micrographs of *Heterodera filipjevi* from North part of Niğde Province: a) second-stage juveniles; b) head region; c) tail region; d) fenestral region; and e) cysts of *Heterodera latipons*.

Table 4. Some morphometric characters of the second-stage juveniles and cysts of *Heterodera filipjevi* and *H. latipons* populations (n = 10) extracted from wheat and barley fields in districts of Niğde Province, Turkey in 2018. All measurements are given in µm ± SE

Character	*H. filipjevi*				*H. latipons*
	Altunhisar	Bor	Central	Ulukışla	Çamardı
	Second-stage juveniles				
Body length	613±4.1	602±2.0	551±3.0	632±1.1	471±5.0
Stylet length	25.7±0.3	25.3±0.2	24.2±0.4	25.4±0.5	22.6±7.0
Tail length	65.1±1.1	63.0±1.9	56.1±1.1	66.1±1.1	49.4±0.18
Hyaline length	42±0.4	37±0.19	38±0.82	40±0.2	25±0.45
	Cysts				
Fenestra length	47.3±3.6	50.1±4.9	55.3±6.7	49.6±9.1	66.5±6.0
Semifenestra width	21.7	21.8	21.1	20.4	17.7±1.1
Vulval bridge width	10.2±1.3	11.9±0.7	13.8±0.2	9.4±1.7	19.8±4.6
Vulval slit length	7.8±1.4	8.2±1.7	8.5±1.1	7.4±1.1	7.0±1.6

A phylogenetic tree was constructed with six population of Niğde from ITS region sequence of CCN populations (Figure 4). Samples from the four different locations; Altunhisar, Central, Bor and Ulukışla Districts in Niğde Province were grouped as *H. filipjevi* and one population from Çamardı as *H. latipons* and the outgroup of *H. schachtii* (Figure 4). *Heterodera filipjevi* populations was compared to international genotypes in the phylogenetic tree. Results indicated that a clear separation of the cyst nematodes, *H. filipjevi* and *H. latipons*, and confirmed the link between genotyping and phenotyping traits. Intraspecific polymorphism was not observed within *H. filipjevi* populations, which were in the same group within the phylogenetic tree and representative isolates from GenBank, supported by a moderate to high bootstrap value (Figure 4).

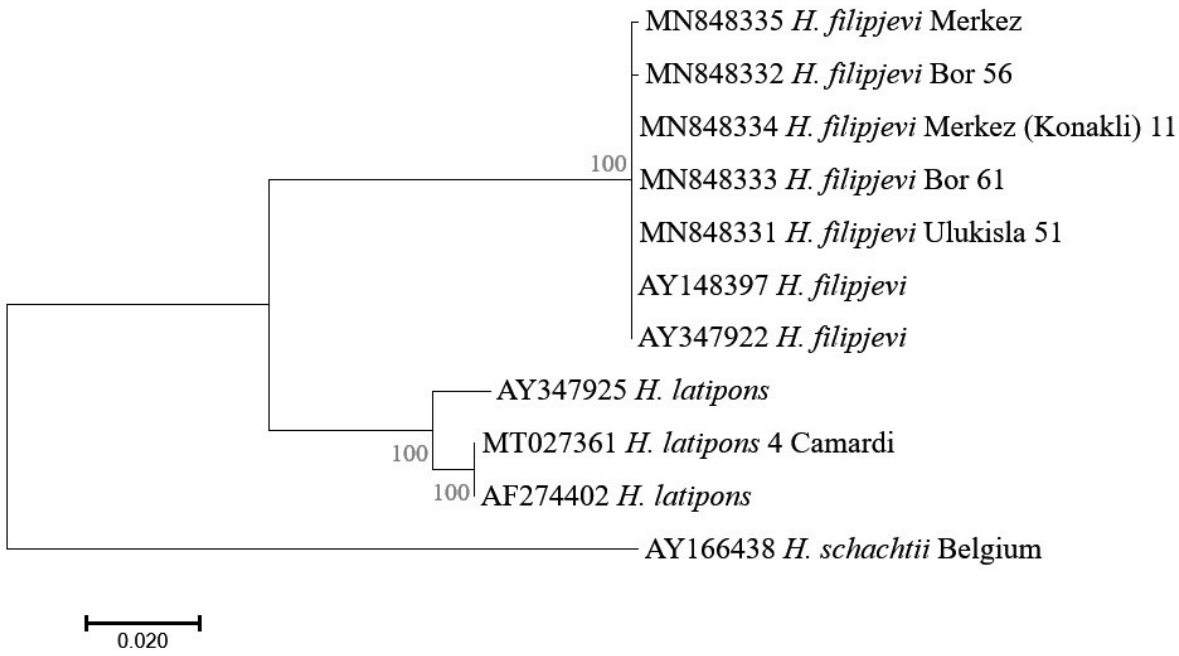

Figure 4. Phylogenetic tree of *Heterodera filipjevi* and *H. latipons* populations from Niğde Province. *Heterodera schachtii* from Belgium was used as an outgroup.

Discussion

This survey was completed in order to determine the incidence of PPNs in cereal fields in Niğde Province. Phytophagous nematodes were detected in 96% of soil samples, and 12 PPN genera were determined, namely *Heterodera, Ditylenchus, Merlinius, Pratylenchus, Aphelenchus, Aphelenchoides, Tylenchus, Helicotylenchus, Trophurus, Pratylenchoides, Filenchus* and *Xiphinema* (in decreasing order of incidence). *Amplimerlinus, Merlinus, Paratrophurus, Pratylenchoides* and the other tylenchid nematodes were the predominant genera of phytophagous nematodes including *Pratylenchoides sheri* Robbins, 1985 (Tylenchida: Hoplolaimidae), *Merlinius brevidens* (Allen, 1955) (Tylenchida: Belonolaimidae), *Amplimerlinius vicia* (Saltukoğlu, 1973) Siddiqi, 1976 (Tylenchida: Dolichodoridae), *Paratrophurus striatus* Castillo, Siddiqi & Gomez-Barcina, 1989 and *Paratrophurus acristylus* Siddiqi & Siddigi, 1983 (Tylenchida: Belonolaimidae) previously determined in wheat-growing areas of the Southeast Anatolian Region of Turkey (Imren & Elekcioğlu, 2008). Öcal & Elekçioğlu (2015) reported that the most common phytophagous nematodes were *Aphelenchus avenae* Bastian, 1865 (Tylenchida: Aphelenchoididae)*, H. latipons, M. brevidens, Pratylenchus thornei* Sher & Allen, 1953 (Tylenchida: Hoplolaimidae) and *Scutylenchus quadrifer* (Andrássy, 1954) (Tylenchina: Merliniidae) in the cereal cropping system of Adıyaman Province, Turkey. Consequently, the reported genera are potentially of economic importance for cereal production in Niğde Province.

The high incidence of *Heterodera* (*H. filipjevi* and *H. latipons*) is a key finding of this study, 76% of sampled fields having cyst nematodes. The fields where cyst nematodes were not detected were generally rotated with other crops. The highest incidence (100%) was found in Altunhisar and Çamardı Districts but the lowest was still over 70% in the three other districts sampled. Sahin et al. (2009) were found CCNs (*H. filipjevi* and *H. latipons),* and root lesion nematodes [*P. thornei* and *Pratylenchus. neglectus* (Rensch, 1924) Filipjev & Schuurmans Stekhoven, 1941]*,* were the most widely distributed species. Toktay et al. (2015) reported that 56% of wheat fields were infested with *H. filipjevi* in Elazig, Erzincan, Erzurum, Igdir, Kars, Malatya and Sivas Provinces in the East Anatolia Region of Turkey. Imren et al. (2016) reported that 83% of wheat fields were infested with *H. filipjevi*, in Bolu Province, Turkey. Therefore, cropping practice and environmental conditions in Niğde Province are particularly suited to the persistence of *H. filipjevi* which poses a risk to wheat production. The incidence of *H. latipons* was low, so it must be less competitive than *H. filipjevi* in this context.

This study indicated that there was no polymorphism in the populations of *H. filipjevi* according to the morphological parameters, which confirm differences in measurements of cysts and J2 bodies.

Heterodera filipjevi is similar to *H. avenae* with some minor differences such as less bullae and thinner underbridge in *H. avena*e (Handoo, 2002; Subbotin et al., 2010). However, *H. latipons* is quite different from *H. filipjevi* with a particularly prominent underbridge and no bullae in the fenestral area. *Heterodera filipjevi* and *H. latipons* can be easily separated using differences in underbridge structure and presence of bullae in the fenestral area of *H. latipons* (Wouts & Sturhan, 1995; Rivoal et al., 2003; Subbotin et al., 2003). *Heterodera latipons* J2s have a shorter tail, stylet and hyaline compared to *H. filipjevi* J2s. Briefly some morphometric features are easily distinguishable in these species such as cyst length, color and shape, fenestra length, J2s tail and hyaline length (Madzhidov, 1981; Valdeolivas & Romero, 1990; Wouts & Sturhan, 1995).

This study confirmed the functionality of CCN cysts and J2 body dimensions, and rDNA sequences for identifying populations of *H. latipons* and *H.* filipjevi. Specimens within the *H. filipjevi* population can be clearly grouped by morphological and molecular data. Likewise, Bekal et al. (1997) did not found any genetic differentiation between populations of *H. filipjevi* and *H. latipons*. Similarly, Imren et al. (2015) did not found any genetic differentiation between *H. filipjevi* populations in the Mediterranean Region of Turkey. However, Subbotin et al. (2010) reported intraspecific polymorphism between *H. filipjevi* populations and Toktay et al. (2015) also found intraspecific variation between populations in *H. filipjevi* from the Eastern Anatolian Region of Turkey.

This study determined the incidence of phytophagous nematodes in the five main cereal-growing districts, Altunhisar, Çamardı, Bor, Central and Ulukışla, of Niğde Province, particularly the high incidence and population densities of *H. filipjevi*. These findings indicated that detailed investigation of the pathotypes of *H. filipjevi* and *H. latipons* in comparison to other areas of Turkey would be justified. It is therefore recommended that policymakers and researchers consider diversification of wheat genotypes cultivated in Niğde, including durum wheat given its higher resistance to cyst nematodes; follow cultural practices especially crop rotation. Also, the is a need to breed wheat germplasm adapted to the areas with high levels of resistant to the cereal cyst nematodes, and to increase the awareness of advisors and growers of the potential impact of phytophagous nematodes on cereal productions in the province.

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Original article (Orijinal araştırma)

Biochemical changes in hemolymph of spinning and non-spinning silkworm larvae, *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae), reared on fresh mulberry leaves: possible reasons for non-spinning syndrome

Taze dut yapraklarında yetiştirilen koza ören ve koza öremeyen ipekböceği larvalarının *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) hemolenfinde biyokimyasal değişimler: koza örememe sendromunun olası nedenleri

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Abstract

Non-spinning syndrome in *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) is a serious issue for the sericulture industry. Determination of urea metabolism as an important parameter at the onset of spinning has shown the need for examining the role of urea metabolism in the non-spinning syndrome. The aim of this study was to investigate role of urea metabolism in the non-spinning syndrome by evaluating urease activity and L-arginine concentrations in the silkworm hemolymph and mulberry leaves. Additionally, urea concentrations were determined in hemolymph samples. Urease activities in hemolymph samples were almost twice as high in spinning larvae (SL) than in non-spinning larvae, 25 ± 5.8 vs 10.9 ± 2.4 units/l ($P < 0.05$). Urea concentrations in the SL hemolymph decreased significantly from day 5 (137 ± 13 mg/l) to day 7 (97 ± 17 mg/l) of the fifth instar ($P < 0.01$), it remained almost constant in NSL hemolymph (149 ± 19 to 167 ± 4 mg/l). Additionally, L-arginine concentrations in hemolymph samples obtained from NSL of 4.55 ± 0.48 mM were significantly higher than in SL at 2.72 ± 0.45 mM ($P < 0.01$). Changes in urease activity and L-arginine concentrations in hemolymph were similarly observed in mulberry leaves. These results suggested that changes in urea metabolism may cause or contribute to non-spinning syndrome in silkworms.

Keywords: L-arginine, non-spinning syndrome, silkworm, urea, urease

Öz

Bombyx mori (L., 1758) (Lepidoptera: Bombycidae)'de görülen koza örememe sendromu, ipekböcekçiliği sektöründe ciddi bir sorun olarak görülmektedir. Üre metabolizmasının koza örme başlangıcında önemli olması, bu metabolizmanın koza örememe sendromunda olası rolünün incelenmesi gereğini doğurmuştur. Bu çalışmanın amacı, ipekböceği hemolenfinde ve dut yapraklarında üreaz aktivitesi ve L-arginin konsantrasyonlarını saptayarak, koza örememe sendromunda üre metabolizmasının rolünü araştırmaktır. Bu parametreler ek olarak hemolenf örneklerinde üre konsantrasyonları da ölçülmüştür. Koza ören grubun hemolenfinde üreaz aktivitesi (25 ± 5,8 units/l), koza örmeyen gruba (10,9 ± 2,4 units/l) göre iki kat daha yüksek bulunmuştur ($P < 0.05$). Koza ören larvaların hemolenfinde üre konsantrasyonu 5. dönemin 5. gününden (137 ± 13 mg/l) 7. gününe önemli seviyede azalırken (97 ± 17 mg/l; $P < 0.01$), koza öremeyen larvalarda hemen hemen sabit kalmıştır (149 ± 19 mg/l'den 166 ± 4 mg/l'ye). Koza öremeyen larvaların hemolenf örneklerinde L-arginin konsantrasyonu (4.55 ± 0.48 mM), koza ören larva grubundan anlamlı ölçüde yüksek bulunmuştur (2.72 ± 0.45 mM; $P < 0.01$). Hemolenfteki üreaz aktivitesi ve L-arginin konsantrasyonlarında gözlemlenen değişiklikler benzer şekilde dut yapraklarında da saptanmıştır. Bu sonuçlar, üre metabolizmasındaki olası bir baskılanmanın ipekböceklerinde görülen koza örememe sendromuna katkıda bulunabileceği ve/veya bunun sonucu olabileceğini düşündürmektedir.

Anahtar sözcükler: L-arginin, koza örememe sendromu, ipekböceği, üre, üreaz

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Introduction

Silkworms, *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae), are most common monophagous insect in human domestication and the sericulture industry is an important economic resource in the world (Savithri et al., 2013). Sericulture has experienced serious problems with non-spinning syndrome in *B. mori* in sericulture practicing countries affecting silkworm rearing and silk production. Although cocoon and raw silk production have continued to be historically, traditionally and culturally practiced in Turkey (Sahan, 2011), recently, both cocoon and silkworm egg production have faced this problem in different regions of Turkey like other countries (Monconduit & Mauchamp 1998; Leonardi et al., 2001; Sun et al., 2012; Sahan et al., 2019). Therefore, it is important to determine the cause of and find solutions to this syndrome in silkworms.

Detailed examination of the developmental stages of silkworms for determination of the cause of non-spinning syndrome have shown that biochemical composition of silkworm hemolymph is an important factor for silkworm development and growth (Zhou et al., 2015; Shen et al., 2016; Dong et al., 2017). Several studies found that the change of urea concentration and urease activity in the hemolymph in the fifth instar is associated with the onset of spinning (Hirayama et al., 1997, 2000a). Urease hydrolysis urea to carbon dioxide and ammonia which is the main nitrogen source for silk protein synthesis in the spinning larvae (Hirayama et al., 1997; Sirko & Brodzik, 2000). Silkworms are host plant-dependent insect as it cannot synthesize urease (Hirayama et al., 1997, 2000a). In addition to protein, amino acids and other necessary substances, silkworms obtain urease from mulberry leaves (Jyothi et al., 2014). While mulberry leaves are external sources for changing urea level in hemolymph, L-arginine is the main amino acid for production of the urea internally in the insect body (Cochran, 1985; Mobley et al., 1995). In light of this information, it is possible to hypothesize that changes urea and urease concentrations may cause the non-spinning syndrome. However, there has been no studies on the relationship between these parameters and non-spinning syndrome.

In our laboratories, we observed that some silkworm larvae cannot spin their cocoons and complete their life cycle. Our prefeeding trials conducted with the leaves obtained from different regions showed that larvae could not spin their cocoons based on the source of mulberry leaves used for feeding. Given that the metabolite composition of silkworm hemolymph is highly influenced by nutrient composition of the mulberry leaves, it seems likely that mulberry leaves used to feed silkworms probably caused this problem.

The main aim of this study was to analyze biochemical parameters that are important for onset of spinning including urea concentrations in the hemolymph of spinning (those fed on mulberry leaves obtained from Örencik Village) and non-spinning (those fed on mulberry leaves obtained from Hürriyet Campus) silkworm larvae. Additionally, L-arginine concentrations and urease activities in each spinning and non-spinning larvae and mulberry leaves obtained from the same regions were measured to detect relationship of non-spinning syndrome and urea metabolism.

Materials and Methods

This study was conducted between April and December 2018 in Kozabirlik (Cocoon Cooperatives Union) silkworm breeding laboratory on the campus of Bursa Directorate of Provincial Agriculture and Forestry, which has a large mulberry planting of about 3 ha.

Materials

Monovoltin hybrid *B. mori* larvae were used for this experiment. Hybrid silkworm eggs (1 g = 2000 eggs) of (M-Chinese X N-Japan) were obtained from Kozabirlik and were incubated at 25-28ºC and 80-85% RH for 11 days.

Silkworm rearing

Newly hatched larvae were separated randomly into two groups and brushed with a feather into wooden rearing trays (70 x 100 cm). At the bottom and for covering of tray, paraffin papers were used to avoid humidity loss during rearing. Silkworm larvae were fed with fresh mulberry leaves under a 12:12 h L:D photoperiod. Mulberry leaves were obtained two different areas. The first group larvae were fed with mulberry leaves obtained from Kozabirlik Campus where larvae had previously been determined to not spin cocoons. The second group of larvae were fed with mulberry leaves collected from a silkworm producer in Örencik Village where it had previously been determined that silkworms could spin cocoons. This study examined the fifth instar of *B. mori* silkworm larvae which lasts about 8 days. Silkworm larvae were reared at optimum rearing conditions for each instar. During the first instar, silkworm larvae were reared at 28ºC and 85-90% RH. After this period, temperature and RH were decreased by 1ºC and 5%, respectively for in each instar. Silkworm larvae were fed three times each day during the first three instars, and four and five times at the fourth and fifth instars. Paraffin paper was used during the first three instars except molting so as to prevent withering leaf and to maintain humidity. The rearing trays were cleaned at the end of each instar with unconsumed leaves, excreta and dead larvae removed (Krishnaswami et al., 1973).

Collection of the hemolymphs

For the determination of urease activity on day 8 of the fifth instar (just before spinning stage) hemolymph samples of silkworm larvae were pooled into three eppendorf tubes containing for 5-7 samples per tube. To determine the urea concentration of hemolymph on days 5 and 7 of the fifth instar, hemolymph samples were collected into the eppendorf tubes as above. Hemolymph samples were collected by making an incision through one of the prologs and transferred to the eppendorf tubes containing 4 µL of thiourea (0.2 M) to prevent the sample blackening. The samples were then centrifuged (Allsheng Mini10K Mini Centrifuge, Hangzhou, Zhejiang, China) for 10 min in 10,000 rpm and upper phase was transferred to new tubes, and stored at -20ºC until analysis of urease activity and urea level.

Determination of the urease activity and urea in hemolymphs samples

Urease activities of the samples were assayed calorimetrically (Mannheim Boehringer Photometer 4010, Roche Diagnostics, Rotkreuz, Switzerland) in the laboratory of the Department of Pharmacology in Faculty of Medicine of using a commercial kit (Sigma-Aldrich Chemicals, St. Louis, MI, USA) as indicated in user guide. Urease activities of the samples were expressed as enzyme units per unit volume. Urea concentrations of the samples were determined by a spectrophotometric (Jasco FP-750 spectrophotometer, Easton, MD, USA) method provided by a central laboratory of Uludağ University Faculty of Medicine as weight of urea N per unit volume of hemolymph.

Determination of urease activity and L-arginine concentrations in mulberry leaves

For determination of urease activity in mulberry leaves, they were washed with distilled water and then dried with paper towel. About 5 mg of leaf pieces were homogenized in phosphate buffer (0.1 M, pH 7.5). After centrifugation (Allsheng Mini10K Mini Centrifuge) at 12 000 rpm for 10 min, supernatants were used for determination of urease activity with the same kit as used for hemolymph samples.

L-arginine concentrations were measured in dried and powdered samples. Specifically, prewashed mulberry leaves were dried under 80ºC for 24 h and then were powdered in a glass mortar. About 100 mg of dried samples were transferred into the glass flasks containing 100 ml of 0.1 N HCl. The flasks were placed on a shaker and L-arginine was extracted for 12 h. At the end of extraction period, 1 ml of each samples were taken into the eppendorf tubes and centrifuged (Allsheng Mini10K Mini Centrifuge) at 12 000 rpm for 10 min. Upper phases (50 µl) were derivatized and then analyzed for L-arginine with a HPLC system. For determination of L-arginine in hemolymphs samples, samples (200 µl) were first acidified with $HClO_4$ (final concentration of 0.4 N), vortexed (BioSan Vortex, Riga, Latvia), then centrifuged at 12 000 rpm for 15 min and upper phase (50 µl) were derivatized before HPLC analysis.

L-arginine in mulberry and hemolymphs samples were determined by a high-performance liquid chromatography (HPLC) after derivatization with diethyl ethoxymethylenemalonate (DEEMM) as indicated by Megias et al. (2015). Specifically, 3 ml of borated buffer (1 M, pH 9.0, containing 2 µl of DEEMM) was added into the standard and samples. After mixing with vortex, all samples were incubated at 50ºC for 50 min. Samples were centrifuged at 12 000 rpm and then injected (50 µl) into the HPLC system. HPLC system (HP 1100 series, Hewlett-Packard, Palo Alto, CA, USA) consisted of a quaternary pump (HP-G1311A, Hewlett-Packard, Palo Alto, CA, USA), a UV detector (1049A, Hewlett-Packard) and a solvent module (G1322A, Hewlett-Packard, Palo Alto). L-arginine was separated on a reversed phase C18 column (Macherey-Nagel GmbH, Duren, Germany) with mobile phase A (25 mM glacial acetic acid in water) and mobile phase B (acetonitrile). During the first 3 min of elution, the ratio of the A mobile phase was 96%, which was decreased to 69% by the end of the chromatogram (model PU-980 liquid chromatography pump; Jasco). Column was maintained at room temperature and flow rate was adjusted to 0.9 ml/min. Chromatograms were detected at 280 nm and L-arginine concentrations were calculated by comparing peak heights of the samples with L-arginine standards processed together with the samples.

Statistical Analysis

The data obtained were analyzed using the GraphPad software, version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± SEM Statistical analyses were performed using the Student's t test. $P < 0.05$ was considered significant.

Results

Urease activity in the hemolymph samples was 24.9 ± 5.8 units/l in spinning larvae (those fed with mulberry leaves from Örencik Village) and 10.9 ± 2.4 units/l ($t = 3.36$, $df = 4$, $P < 0.05$) in non-spinning larvae (those fed with mulberry leaves from Hürriyet Campus). Urease activity in mulberry leaves were 4.0 ± 0.3 units/g, and 2.1 ± 0.9 units/g ($t = 3.26$, $df = 4$, $P < 0.05$) in samples from Örencik Village and Hürriyet Campus, respectively. Thus, as shown in Figure 1, urease activities in both hemolymph samples and mulberry leaves fed for spinning larvae are almost two times higher than the non-spinning larvae and mulberry leaves used.

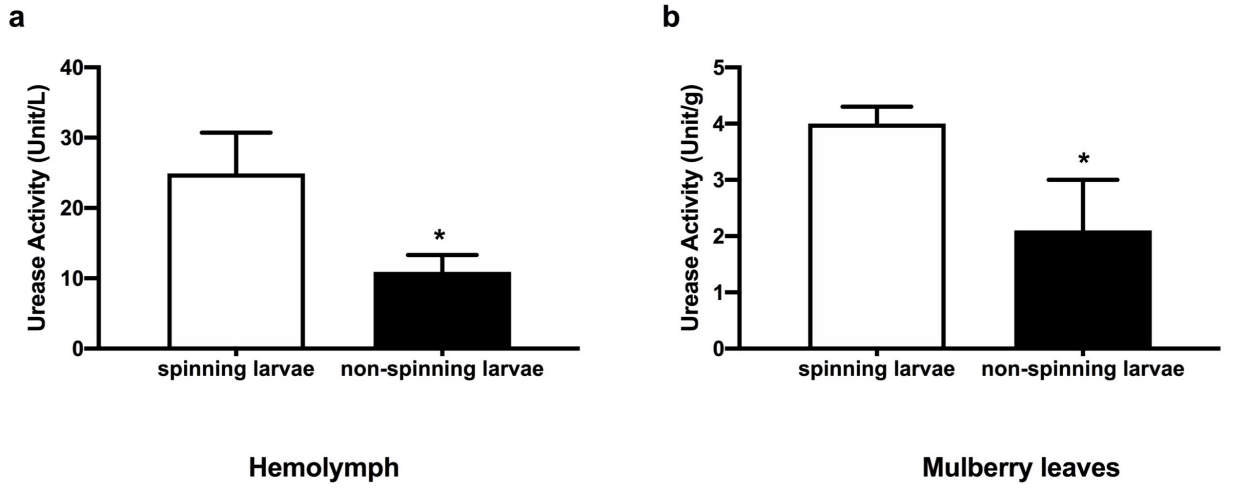

Figure 1. Urease activities in hemolymph samples of *Bombyx mori* (a) and mulberry leaves (b) for spinning larvae (fed with mulberry leaves from Örencik Village) and non-spinning larvae (fed with mulberry leaves from Hürriyet Campus). *P < 0.05, significantly different from spinning larvae group.

It was also observed that spinning larvae started to spin their cocoons on day 8 of the fifth instar (Figure 2a), however, non-spinning larvae became dauer larva and lay down without spinning their cocoon (Figure 2b). In addition, in non-spinning larvae, the fifth instar was prolonged to 18 days and they turned into darker larvae and died without spinning their cocoons (Figure 2c).

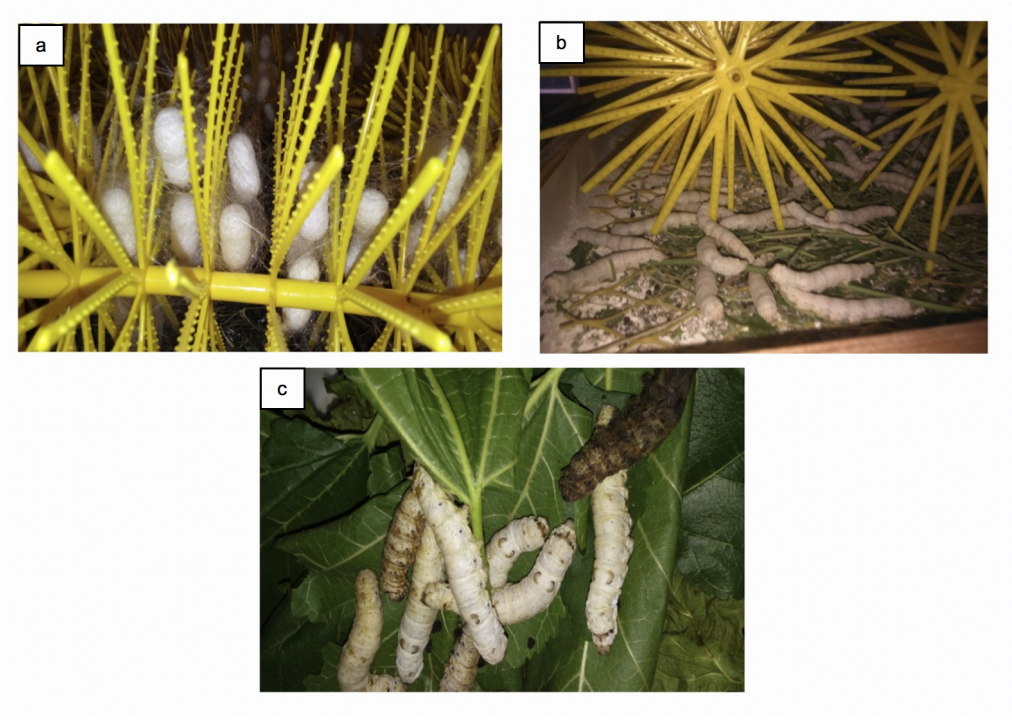

Figure 2. The picture of spinning larvae (a) on day 8 of the fifth instar and non-spinning larvae on day 9 (b) and 15 (c) of the fifth instar of *Bombyx mori.*

Changes in the urea concentrations on days 5 and 7 of the fifth instar in hemolymph samples taken from the non-spinning and spinning larvae were given in Figure 3. Urea concentrations in the spinning larvae hemolymph decreased to 97 ± 17 mg/l on day 7 of the fifth instar from its day 5 value 137 ± 13 mg/l ($t = 3.95$, $df = 12$, $P < 0.01$). Urea concentrations in non-spinning larvae hemolymph, however, did not decline as seen in spinning larvae, but showed a tendency to increase during the same period (from 149 ± 19 mg/dL to 166 ± 4 mg/l).

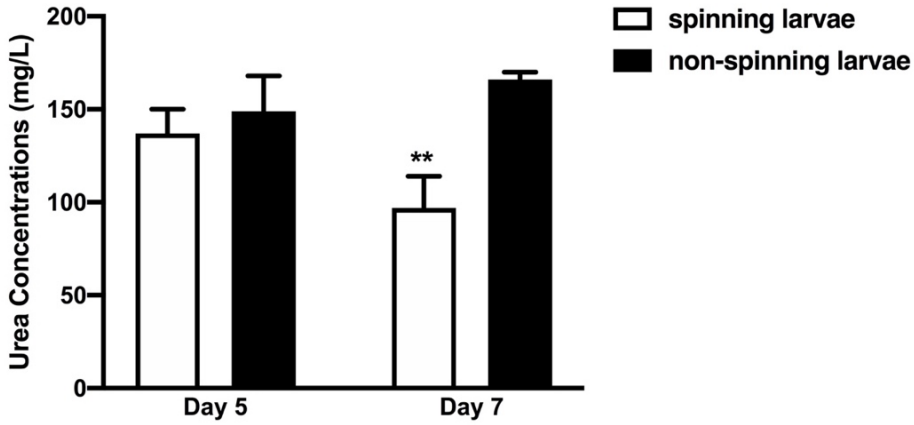

Figure 3. Urea concentrations in hemolymphs samples from spinning and non-spinning larvae of *Bombyx mori* on days 5 and 7 of the fifth instar. **P < 0.01, significantly different from spinning larvae group on day 5.

L-Arginine concentrations in mulberry leaves and hemolymph samples are presented in Figure 4. L-arginine concentrations in mulberry leaves from Hürriyet Campus fed to non-spinning larvae had significantly higher concentrations than mulberry leaves fed to spinning larvae (788 ± 59 nmol/g vs 129 ± 41 nmol/g; t = 9.17, df = 12, P < 0.01). L-Arginine concentrations in the hemolymph were almost twice as high in non-spinning than spinning larvae (4.55 ± 0.48l vs 2.72 ± 0.45 mM; t = 2.80, df = 12, P < 0.01).

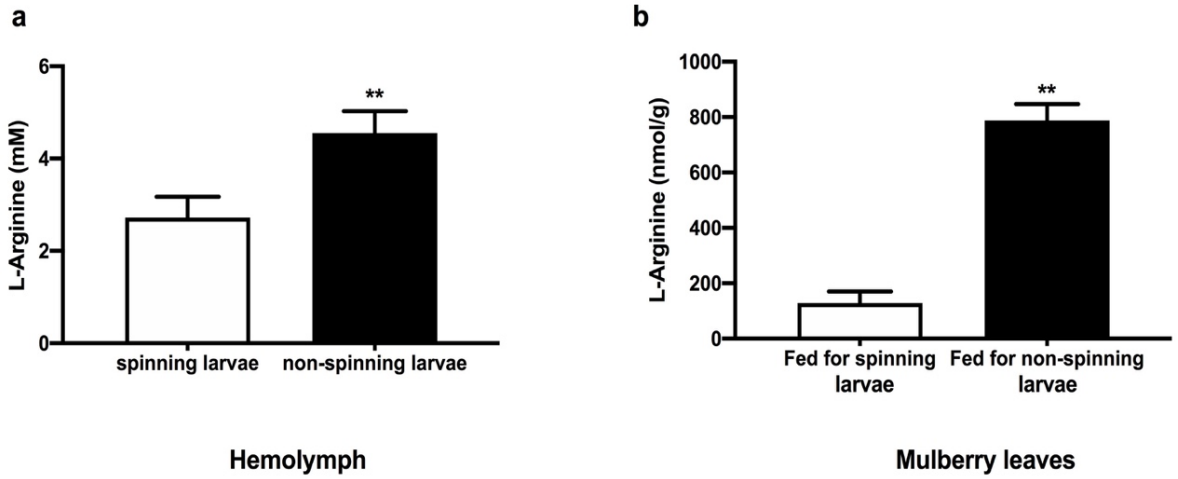

Figure 4. L-Arginine concentrations in a) hemolymphs samples at spinning and non-spinning larvae of *Bombyx mori* and b) mulberry leaves used to feed spinning (Örencik Village) and non-spinning (Hürriyet Campus) larvae. **P < 0.01, significantly different from spinning larvae group.

Discussion

Non-spinning syndrome threatens not only the production of breeding cocoons and eggs, but also affects commercial cocoon production in Turkey and other countries such as Iran, China and Brazil (Monconduit & Mauchamp, 1998; Leonardi et al., 2001; Sun et al., 2012; Sahan et al., 2019). Additionally, both native and foreign sourced silkworm lines are under great risk because of this syndrome. Exact mechanism of the non-spinning syndrome is not known, but many factors seem to be involved in both egg and commercial cocoon crops. Non-synchronous spinning movement and solidification of the silk, very high temperatures in the spinning room, improper handling of cocoons during molting and industrial pollution can cause the non-spinning syndrome. It has been demonstrated that some of these factors change biochemical composition of hemolymph in different developmental stages of silkworms (Etebari et al., 2007; Malik & Malik, 2009).

Several studies have revealed changes in amino acid, lipid and some enzyme concentrations in spinning and non-spinning larvae (Etebari et al., 2007; Zhou et al., 2015), but there has been relatively work on urea metabolism. The sharp change in urea concentrations and urease activity at the onset of spinning indicates the need for investigating the role of urea metabolism in non-spinning syndrome. Thus, our study was mainly focused on urea and L-arginine concentrations and urease activities in hemolymph samples obtained from non-spinning and spinning larvae. Given that *B. mori* uses mulberry leaves as a source of urease and L-arginine, we also determined urease activities and L-arginine concentrations in mulberry leaves fed to these larvae.

It is known that urease of mulberry leaves is involved in urea hydrolysis, and nitrogenous molecules occurred from this hydrolysis are used for production of silk proteins and to complete the insects's life cycle (Kurahashi et al., 2005). Additionally, transport of the mulberry leaf urease from the midgut into the hemolymph is selective and larval-stage specific. When hemolymph is collected just before the spinning stage, no urease activity could be determined even if the larvae were fed on mulberry leaves (Hirayama et

al., 2000b; Sugimura et al., 2001). In present study, we observed that urease activity in hemolymph of the spinning larvae is almost twice as high as in the hemolymph of non-spinning larvae. To our knowledge, this remarkable phenomenon is reported here for the first time, and this significant decline in urease activity in hemolymph of non-spinning larvae may be a critical factor in non-spinning syndrome. In addition to hemolymph samples, we also measured urease activities in mulberry leaves. As shown in Figure 1b, urease activity in mulberry leaves fed to non-spinning larvae was significantly lower than in the leaves fed to spinning larvae, suggesting that low urease activity in non-spinning larvae hemolymph may be cause by the mulberry leaves they consume.

It has been shown that hemolymph of silkworm larvae reared on mulberry leaves contains a considerable quantity of urea (Yamada et al., 1983; Sumida et al., 1990). Urea concentration in the hemolymph increases until days 4 and 5 of the fifth instar, but then decreases sharply to day 7, probably as a result of the sufficient sequestration of urease derived from the ingested mulberry leaves (Sumida et al., 1993). Consistent with these findings, we determined that urea concentrations in spinning larvae hemolymph decreased significantly between days 5 and 7 of the fifth instar. In contrast, urea concentrations in non-spinning larvae hemolymph did not decline but rather increased during the same period, supporting the conclusions being drawn about the reduced urease activity in non-spinning larvae. Consistently, it has been previously reported that high urea concentration in hemolymph of the non-spinning larvae is probably a result of the insufficient transport of leaf urease into the silkworm hemolymph (Sumida et al., 1995).

In the present study we also observed that L-arginine concentrations were significantly higher in both non-spinning larvae hemolymph and the mulberry leaves the consumed (Figure 4). L-arginine is known as a main source of the urea in silkworms. Given that the nitrogen released by urease is used for silk production, this increase in L-arginine concentrations could be considered as an advantage for silk formation (Chakrabarty & Kaliwal, 2012), if there was no decline in urease activity. We did not know if the high L-arginine present in non-spinning larvae comes from the high L-arginine in mulberry leaves or why these mulberry leaves contain higher L-arginine; this needs to be determined by additional studies. It is noteworthy that L-arginine also has the ability to inhibit urease. Due to this property of L-arginine, urease-based ion-selective field effect transistors biosensor for arginine determination have been developed (Sheliakina et al., 2014). Indeed, we determined that L-arginine can inhibit urease in a concentration dependent manner (data not shown; the lowest concentration of L-arginine tested was 10 μM and caused a 20% decline in soybean urease activity).

In summary, based on the data presented, it is suggested that biochemical alterations, such as a decrease in urease activity and increase in L-arginine concentrations in both hemolymph and mulberry leaves, and increase in urea in hemolymph may cause or contribute to non-spinning syndrome in silkworms. Although the reason for this change in the urea metabolism of silkworm larvae may originate from the leaves they consume, there are no studies in the literature on this possible reason for the change in the urease activity of mulberry leaves. Further research needs to be done to investigate these reasons.

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