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Original article

Annual variation of the *Orosanga japonica* Melichar 1898 (Hemiptera: Ricaniidae) populations in the eastern Black Sea region of Turkey and possible molecular separation with based on 28S rDNA sequences from other Ricaniidae groups

Türkiye'nin Doğu Karadeniz Bölgesi'ndeki *Orosanga japonica* Melichar 1898 (Hemiptera: Ricaniidae) popülasyonlarının yıllık değişimi ve diğer Ricaniidae gruplarından 28S rDNA sekanslarına dayanarak olası moleküler ayrımları

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A R T I C L E   I N F O

A B S T R A C T

Orosanga japonica (Melichar, 1898) is one of the invasive species that are distributed in the eastern Black Sea coastal zone. The aim of the study was to determine the annual variation of the populations and solve the taxonomic problem in Turkey of the species with different tools. For this purpose, the specimens were collected from 14 different locations along the eastern Black Sea coastal zone of Artvin, Rize, Trabzon for investigating annual variation and from 15 different locations for achieving molecular separation. Population counts were performed from May to September during two years (2014-2015). Populations were found generally in nymphal stage in May and adult densities were very low in 2014. On the contrary, adult individuals could not be counted in May 2015. Adult population peak was seen in July and August in 2014 and August and September in 2015. Based on 28S region DNA sequences, 6 haplotypes were found. Two main branches were determined in the dendrogram generated by using 28S rDNA sequence. Molecular data showed that our samples belonged to the main branch together with some Ricaniidae and Flatidae GenBank samples but in one different line. *Ricania simulans* GenBank sample situated second branch. The samples collected in the eastern Black Sea region of Turkey were found to be *O. japonica* according to the detailed morphological examinations and molecular results showed that can be separated from other Ricaniidae samples.

INTRODUCTION

The species of Ricaniidae Amyot et Serville 1843 is generally distributed in tropical regions and represented with

some species in the Palearctic region. The family includes approximately 432 species in over 40 genera occurring predominantly in tropics and subtropics of the eastern part of the world (Bourgoin 2017, Gnezdilov 2009). Five species of *Orosanga* genus are known in Japan [*O. dido* Fennah 1971, *O. triton* Fennah 1971, *O. laverna* Fennah 1971, *O. xantho* Fennah 1971, *O. japonica* (Melichar, 1898)]. Although *O. japonica* distribution range area is very extending, other four *Orosanga* species distribution range limited only Japan (Fennah 1971, Hayashi and Fujinuma 2016). *O. japonica* firstly described as a *Ricania japonica* (Melichar 1898) and was identified within the genus *Ricania* (Melichar 1898, Nast 1987, Gnezdilov and Sugonyaev 2009, Gjonov 2011). According to the Hayashi and Fujinima (2016), Bourgoin (2017) and Mozaffarian (2018), the specimens from Crimea, Georgia, Bulgaria, Turkey, and Iran were identified in *Orosanga* genus.

O. japonica was firstly detected in the early 20th century in Georgia, Russia (Krasnodar) and Ukraine (Crimea) that originated from the Far East (Bourgoin 2017, EPPO 2016, Gnezdilov and Sugonyaev 2009, Nast 1987). It was found the very extended range in Georgia at the end of the 20th century and lastly was occur in Bulgaria, Turkey, and Iran (Demir 2009, Demir 2018, Gjonov 2011, Gnezdilov and Sugonyaev 2009, Mozaffarian 2018). This species has been introduced along the eastern Black Sea coastal region of Turkey and shows a spreading pattern from eastern to the middle Black Sea coastal area (0-400 m, our preliminary observations; Cebir 2016, Oztemir 2014). Recently, it was also spreading western Black Sea and Marmara region (Demir 2018). The species has damaged a large number of agricultural crops and natural forest products in the eastern Black Sea coastline since 2006. *O. japonica* is a polyphagous sap-feeding insect species and found generally on shrubs and trees as both nymphal stages and adult periods. It has been recorded on some important crops especially tea and kiwifruit, bean, grapevine, maize in many areas in the eastern Black Sea region (Cebir 2016, Demir 2009, Oztemir 2014). *O. japonica* has an high adaptation capacity in the eastern Black Sea area habitats and their distribution patterns vary according to climatic factors and host plant species in a small habitat patch (Cebir 2016, Oztemir 2014). Mozaffarian (2018) reported that this species may be considered invasive species in north Iran. Persistence of the invasive species is supported by high carrying capacity (K) which means the good colonists in animal species have higher rates of population growth and higher K (Lawton et al. 1986). *O. japonica* seems to have these features in the eastern Black Sea coastline area.

O. japonica (syn. *R. japonica*) identified as a *R. simulans* by some authors (Walker 1851) and conducted some studies about biological control techniques (Ak et al. 2013, Ak et al. 2015, Gokturk and Aksu 2014, Gokturk and Mıhlı 2015, Guclu et al. 2010). But some internet sources implying that records of *R. simulans* in Turkey are misidentifications of *O. japonica*. Also, EPPO (European and Mediterranean Plant Protection Organization) implied that it is not entirely clear whether one or two new invasives (*R. simulans* and *O. japonica*) species have been introduced into Turkey (EPPO 2016). The superfamily Fulgoroidea is a very large and complex family (Bourgoin et al. 1997). Species descriptions in this family are based on morphological and molecular characters. Morphological identification of species can be controversial in many samples. Therefore, identification of morphological species should be verified by molecular methods (Wang et al. 2016). Nuclear or mitochondrial markers are generally used in molecular phylogenetic analyzes for planthoppers (Bourgoin et al. 1997, Song and Liang 2013, Urban and Cryan 2007, Yeh et al. 1998, Yeh et al. 2005). Molecular species identification limits in some situations related to the lack of comparison material. Therefore, the selected markers should be suitable for study purpose. The 28S rDNA gene region have used for identifying the Fulgoroidea superfamily genus because of the conserved region (Kwon et al. 2017, Song and Liang 2013, Wang et al. 2016). This study firstly aimed to reveal the annual variation of population and secondly aimed to show whether it is molecularly separated from other Ricaniidae groups with 28S rDNA sequences.

MATERIALS AND METHODS

Study areas

This study involves three provinces in the eastern Black Sea area for annual variation (2014-2015) and one more province for molecular studies in Turkey. Study areas are given in Figure 1. Fourteen locality samples were used for annual variation and molecular studies (Artvin: Sarp, Güreşen Kemalpaşa and Hopa; Rize: Fındıklı, Ardeşen, Pazar, Çayeli, Rize city center (Merkez), Derepazarı, and İyidere; Trabzon: Sürmene, Yomra and Akçaabat), and extra two locality samples were used only for molecular studies (Arsin, Giresun). Çayeli samples were not giving accurate sequence data, therefore excluded from the molecular evaluation.
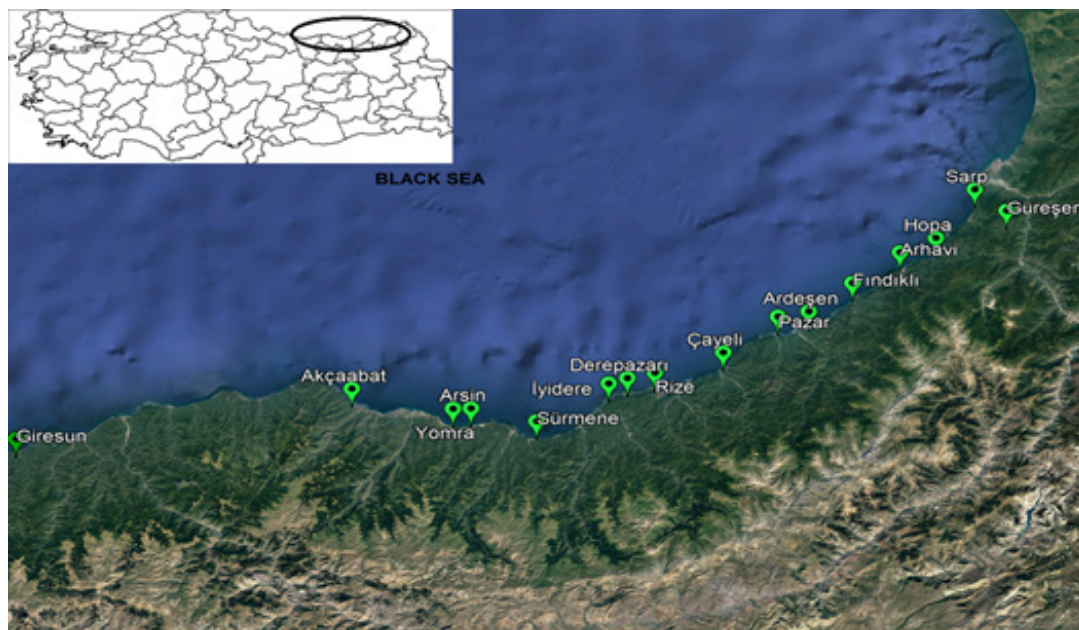
Figure 1. Sampling sites of the specimens, *Orosanga japonica* from Black Sea region of Turkey

Determination of annual variation of the Orosanga populations

Nymph and adult stage samples were collected from 14 localities in the eastern Black Sea region in Turkey and were taken to the laboratory. Mouth aspirator was used for collecting as nymphs and adults on only blackberry shrubs for standardization. The method was standardized as 10 mins/person. Collections were carried out 10 square meter area for every collection points by one collector. The study was conducted during 2014-2015 from May to September. Samples were collected at subsequent days of 15th-17th days of the same month for every sampling point.

All specimens were separated gently for avoid to damage identification characters for morphological identification. Identification of the specimens was performed under stereozoom microscope (Leica S4 E) according to the Melichar (1898), Fennah (1971), Demir (2009), Mozaffarian (2018). The genus of *Orosanga*; forewings with dense longitudinal veins, veins Sc and R arising from the short common stalk on basal cell (Fennah 1971). Firstly, we used these characters to describe that the samples are in the genus *Orosanga*. The specimens were identified as *O. japonica* by very pale yellowish hyaline coverts and two white hyaline transverse bands, the outer of which is not interrupted, but only on both sides, slightly constricted in the middle, and sending a tooth inward towards the center of the corium on the inner margin (Melichar 1898). After morphological identification of the samples, a total of 45 specimens were randomly selected from 15 different locations for DNA extraction.

DNA extraction

Collected samples were preserved in 95% ethanol and kept at +4 ºC until the molecular studies started. Genomic DNA was extracted from the whole bodies of 45 samples (3 samples of each area) according to the isolation protocol from the manufacturer of GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification of ribosomal gene

28S nuclear DNA marker for targeted each specimen for surveying separation performances of the specimens. 28S_F (5'-AACAGCCGTTGCACAAGA-3') and 28S_R (5'-GGACACCTGCGTTATCATTT-3') were used to amplify the 28S rDNA region (Song and Liang 2013).

PCR was carried out 50 µl final volumes in 0.2 ml microfuge tubes on a BioRad T100 Thermocycler (BioRad Inc, Hercules, CA, USA). PCR amplification was performed in 1X GoTaq DNA polymerase reaction buffer (Promega®, USA), 3 µl of $MgCl_2$ (25 mM), 1 µl of each primers (40 pmoles), 1.5 µl of dNTPs (5 mM), 5 µl of gDNA (10-50 ng), 0.6 µl of GoTaq DNA polymerase (Promega®, USA), and enough volume of ddH_2O up to 50 µl. Reaction cycles included 50 seconds for initial denaturation at 94 ºC, and 35 cycles with following temperature profiles: denaturation for 50 s at 94 ºC, annealing for 50 s at 55 ºC, and extension for 90 s at 72 ºC and 72 ºC for the final extension at 10 min. Amplicons were detected by 1.2% agarose gel electrophoresis with 100 bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing and data analyses

We obtained nuclear 28S sequence only from 29 samples (Table 1). PCR amplicons were sequenced by Macrogen Inc. (Amsterdam, Holland). DNA sequence alignment was performed with BioEdit (Hall 1999) and ClustalW software. Nucleotide composition, haplotype variation (Nei 1987), haplotype separation in population, and nucleotide variation (Nei and Tajima 1981) of the samples were determined by DNASP v5 (Librado and Rozas 2009). Neighbour joining analysis in Mega 7 were used for phylogenetic analysis.

month in 2015. Population densities gradually increased from June to September. Population peak was seen in August and September.

Determination of the molecular separation with 28S rDNA

It was used 852 bp region for 28S rDNAs evaluation. It was used *Privesa* sp., *Scolypapa* sp., *Pochazia confusa*, *Ricania shantungensis*, *Ricania marginalis*, *Aprivesa* sp., *Pseudoflatoides* sp., *R. simulans*, GenBank samples for comparing (Table 2). Our samples situated the same line within six haplotypes and separated different degrees of other compared GenBank samples. 28S rDNAs sequence results

Table 1. Sample names and haplotype number from collected to the field for 28S ITS region assay

Haplotype Number	GenBank accession number	Frequency	Location Name	Date of collection	Host Plant
Hap_1:	KY007505.1	22	Pazar x2, Merkez x2, Yomra x2, Hopa x2, Sarp, Güreşen, Fındıklı, Giresun, Ardeşen x2, Sürmene x2, Arsin x2, Derepazarı x2, Akçaabat, İyidere	August 2015	Blackberry
Hap_2:	KY007506.1	2	Arhavi x2	August 2015	Blackberry
Hap_3:	KY007507.1	2	Sarp, İyidere	August 2015	Blackberry
Hap_4:	KY007504.1	1	Güreşen	August 2015	Blackberry
Hap_5:	KY007510.1	1	Giresun	August 2015	Blackberry
Hap_6:	KY007508.1	1	Akçaabat	August 2015	Blackberry

RESULTS

Determination of annual variations of the Orosanga populations

The results of the biennial population count of (2014 and 2015) nymphal and adult stage are given in Figure 2 and Figure 3. Populations were generally in nymphal stages in May and the number of adult insects in this month was very low. The highest number of nymphs was collected in Çayeli (27), Rize (21), and Güreşen Kemalpaşa (21) collection points respectively in 2014. The lowest number of nymphs was collected Akçaabat (8), Yomra (8) and Sürmene (9) respectively in 2014. Adult density gradually increased from June to August and the nymphal stage was seen lastly in early August 2014 (e.g. Çayeli 3 specimens, Rize 2 specimens, Güreşen Kemalpaşa 0 specimen). Population peak was seen in July and August for this year. In September, adult densities were found high, but at the end of this month, both nymphs and adults were not found except the eggs in all study areas. In contrast to 2014, individuals were seen at the end of May and any adult stage individuals couldn't be found for this

and GenBank samples have formed two main branches. One branch included just only *R. simulans* and other branches included other compared GenBank samples together with our samples (Figure 4). Six haplotypes defined all studied samples (Table 3). In these haplotypes, hap 2 (hap 2 indicates haplotype 2) has T in position 742, hap 3 has A in position 265, hap 4 has A in position 700, hap 5 has C in position 195, and hap 6 has T in position 15 when compared to h1 (Table 3). The results showed that all haplotypes were different from each other only one base in totally 852 bp of 28S regions. Haplotype number, haplotype variation (*h*), and nucleotide variation (π) of the specimens have been shown in Table 2. Datasets were uploaded to the GenBank with the following accession numbers: KY007504.1 Guresen, KY007505.1 Iyidere, KY007506.1 Arhavi, KY007507.1 Sarp, KY007508.1 Akcaabat and KY007510.1 Giresun.
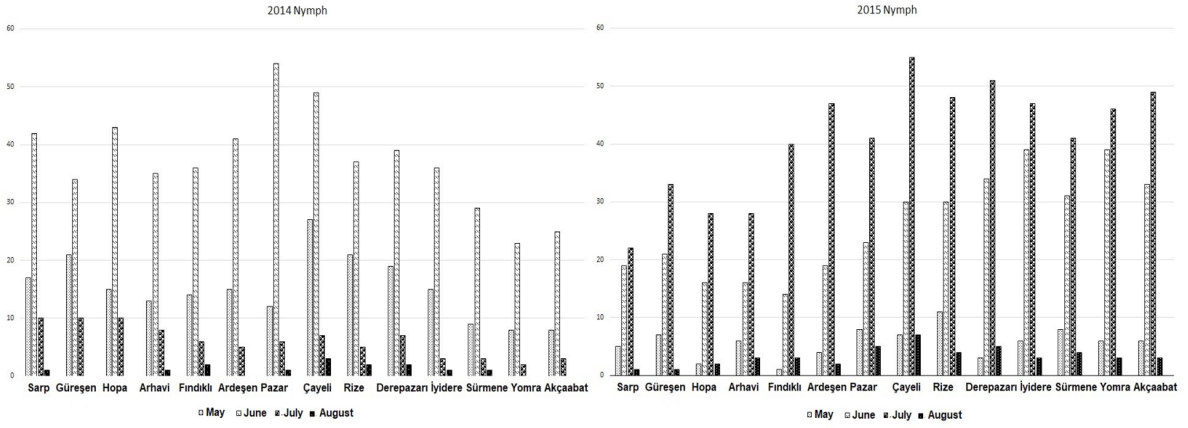
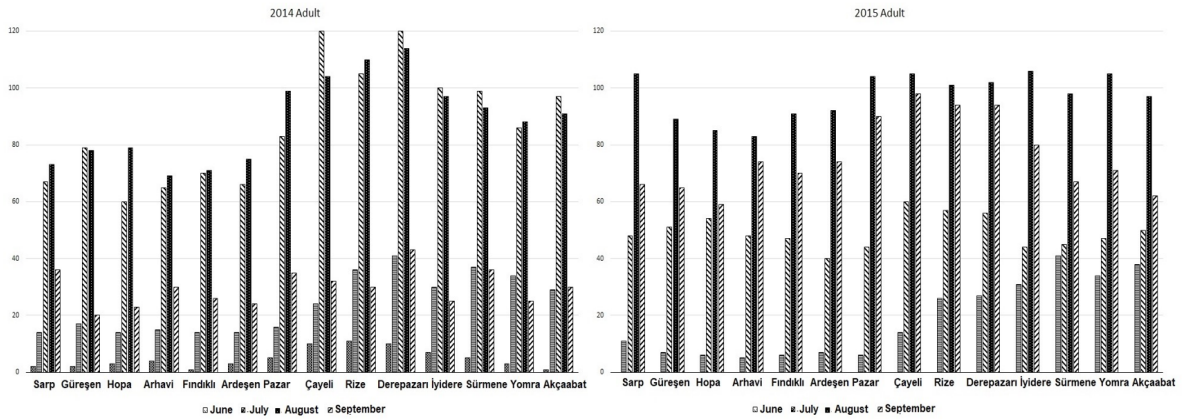**Figure 2.** Biennial population (nyphal stage) count of *Orosanga japonica*

Figure 3. Biennial population (adult stage) count of *Orosanga japonica*

Table 2. Comparison samples and GenBank accession numbers

Species	Taxonomy	GenBank Access number	References
Aprivesa sp.	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	DQ532645.1	Urban and Cryan 2007
Privesa sp.	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	DQ532643.1	Urban and Cryan 2007
Scolypopa sp.	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	DQ532644.1	Urban and Cryan 2007
Pseudoflatoides sp.	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	DQ532615.1	Urban and Cryan 2007
Pochazia confusa	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	JX556811.1	Song and Liang 2013
Ricania marginalis	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	JX556814.1	Song and Liang 2013
Ricania simulans	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	JX556815.1	Song and Liang 2013
Ricania shantungensis	Fulgoromorpha; Fulgoroidea; Ricaniidae; Aprivesa	KU377155.1	Won et al. 2017

Table 3. 28S nucleotide variations and positions, localities, sample number, haplotype number, haplotype variation (*h*), and nucleotide variation (π) of *Orosanga japonica*

Haplotypes	Variable Nucleotide Positions 1277 19604 55502	Artvin				Rize						Trabzon				Giresun	Total
		Sarp	Guresen K.pasa	Hopa	Arhavi	Findikli	Ardesen	Pazar	Merkez	Derepazari	Iyidere	Surmene	Arsin	Yomra	Akcaabat	Giresun	15
Hap 1	GATCA	1	1	2		1	2	2	2	2	1	2	2	2	1	1	**22**
Hap 2	....T				2												**2**
Hap 3	..A..	1									1						**2**
Hap 4	...A.		1														**1**
Hap 5	.C...															1	**1**
Hap 6	T....														1		**1**
Sample size		2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	**29**
h		10	10	0	0	0	0	0	0	0	10	0	0	0	10	10	**0.43915**
		0.00117	0.00117	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00117	0.00	0.00	0.00	0.00117	0.00117	**0.00057**

Figure 4. Neighbour joining tree according to 28S rDNAs sequence data (haplotypes used for generating dendrogram)

Note: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.23194307 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei 1984) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar 2002). The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 836 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

DISCUSSION

Molecular characteristics of the Orosanga japonica

Alignment, genome assembly, and all calculations of sequences were analyzed using with MEGA 7. According to molecular-based studies, 28S rDNA sequences were given six haplotypes from *O. japonica* populations in the Black Sea region of Turkey, all haplotypes were different from each other only one base in different positions. Our *Orosanga* samples were similar with different degrees to other compared groups but create a separate branch from other samples. Two main branches were obtained in the dendrogram generated by using 28S rDNA sequence data. *Apprivesa* sp., *Pseudoflatoides* sp., *R. marginalis*, *Scolypapa* sp., *P. confuse*, *R. shantungensis*, *Privesa* sp. were in the first branch. Our *Orosanga* samples were situated the main branch with this group and placed a separate line from these samples. *R. simulans* GenBank sample situated the second branch. Ak et al. (2015) indicated that the species distributed in eastern Black Sea area is *R. simulans*. But detailed morphological examinations were showed this species is *O. japonica* and 28S rDNA region results showed the difference from *R. simulans* and other Ricaniidae, Flatidae groups. According to the EPPO Global Database, *O. japonica* was collected for the first time in Rize, Turkey in 2007 (EPPO Reporting Service No: 05-2016/Num. article: 2016/100). Also, they implied the confusing situation of the *Ricania* and *Orosanga* species in Turkey. Demir (2009) also collected *Orosanga* specimens from the eastern Black Sea region of Turkey and identified whole samples as *O. japonica*. Furthermore, the distribution of *R. simulans* is limited with China, Taiwan, India, and Japan, the distribution of *O. japonica* include Ukraine Georgia, Bulgaria, Iran, and Turkey together with abovementioned areas, though (Bourgoin 2017, Demir 2018, Hayashi and Fujinuma 2016, Mozaffarian 2018).

Annual variation of the population

O. japonica has received more attention locally related to the polyphagous sap-feeding behavior and population persistence of the study area in the eastern Black Sea coastal zone since 2009. Our results showed that the most abundant area varies monthly and yearly, but generally Rize city and its towns slightly have more *Orosanga* specimens than the other study areas. The results in 2014 and 2015 showed the increasing trend for the abundance for the Trabzon city and towns. Ak et al. (2015) noted that the most abundant area for the *Orosanga* specimens is Kemalpaşa. Our results indicated that the hatchling time of the eggs varied yearly and changed from the first week to the fourth week of May and our findings correlated with the results of Ak et al. (2015). Ak et al (2015) stated that hatchling time of eggs was the last week of May

in 2009 and 2011, but they found that in 2010 the hatchling time was the second week of May. Our results indicated that *O. japonica* prefers to stay and feed fresh shoot on blueberry, grape, tea, kiwi, bean, cucumber, etc. and gives one generation of a year. Avidzba and Bobokhidze (1982) reported the same situation for Abkhazia and also noted that it has a wide range of host in that area. Dzhashi et al. (1982) reported that the species has one generation for a year in southern Georgia, but the hatchling time of the eggs starts about one month before Turkey. Tayutivutikul and Kusigemati (1992) indicated that *O. japonica* has a two generations in Taiwan condition. They also noted that nymphs were appeared in from May to July and from August to October, and adults appeared from June to November in 1988. This can be explained by the climatic and vegetational differences between the regions.

Agricultural fields are not large in the study area and vegetable production is limited to families need themselves in the eastern Black Sea region of Turkey. Bean, maize production is very popular for the areas related to eating habits. But nymphs and adults of *O. japonica* are showed serious pressure on agricultural productions related to the polyphagous sap-feeding behavior. Tea and kiwifruit, which are the most cultivated plants in the region, both plants host for possible feeding and egg laying area for the *O. japonica*. Tea and kiwifruit farming is very important related to the primary source of income for the inhabitants. Therefore, population increase trends and lack of control facilities is very important for the area and primarily agricultural production. According to the results of this study, it is understood that effective control methods should be developed because the timing of tea, kiwi and other important crop harvests coincides with the most intense period of *O. japonica*.

In conclusion, this is the first annual variation and population genetic study of *O. japonica* which confirms the high population density and possible molecular separation of the species with 28S rDNA region. Our results also showed the existence of one lineage of *O. japonica* and provided clues about distribution patterns of the species. Our results suggested highly homogenous and genetically differentiated groups from Sarp to Giresun along the Black Sea coastal zone.

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ÖZET

Orosanga japonica (Melichar, 1898), Doğu Karadeniz kıyı bölgesinde dağılan istilacı türlerden biridir. Bu çalışmanın

amacı, popülasyonların yıllık değişkenliğini belirlemek ve türün Türkiye'deki taksonomik problemini farklı araçlarla çözmektir. Bu amaçla örnekler, yıllık değişimleri araştırmak amacıyla Doğu Karadeniz Bölgesi kıyı hattı boyunca Artvin, Rize, Trabzon illerinden 14 farklı noktadan ve moleküler ayrımı sağlamak için 15 farklı noktadan toplanmıştır. Popülasyon sayımları iki yıl boyunca mayıs-eylül ayları arasında (2014-2015) yapılmıştır. 2014 yılında popülasyonlar genellikle mayısta nimf aşamasında ve ergin yoğunlukları çok düşük olarak bulunmuştur. Bu durumun aksine 2015 yılı mayıs ayında erginlere rastlanmamıştır. En yoğun ergin popülasyonu 2014 yılında temmuz ve ağustos, 2015'te ise ağustos ve eylül aylarında görülmüştür. 28S bölgesi DNA dizilerine dayanarak, 6 haplotip bulunmuştur. 28S rDNA kullanılarak oluşturulan dendrogramda iki ana dal belirlenmiştir. Moleküler veriler, örneklerimizin bazı Ricaniidae ve Flatidae GenBank örnekleriyle birlikte ana kolda, fakat farklı bir dalda olduğunu göstermiştir. *Ricania simulans* GenBank örneği ikinci dalda yer almıştır. Türkiye'nin Doğu Karadeniz Bölgesi'nden toplanan örneklerin ayrıntılı morfolojik incelemelere göre *O. japonica* olduğu belirlenmiş ve moleküler sonuçlar diğer Ricaniidae örneklerinden ayrılabileceğini göstermiştir.

Anahtar kelimeler: Hemiptera, *Orosanga japonica*, Ricaniidae, yıllık dalgalanma, 28S rDNA

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