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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Research on the morphological and molecular diagnosis of *Hyalopterus pruni* (Geoffroy)

Hyalopterus pruni (Geoffroy)'nin morfolojik ve moleküler teşhisi üzerine araştırma

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Abstract

Aphids are one of the most important groups of insects that cause damage to agricultural crops, ornamental plants, as well as herbaceous and woody plants in their natural habitats. Aphids that feed on plant sap can cause significant crop losses worldwide, ranging from 70% to 80%, due to stunted growth, deformation, wilting, and other detrimental effects on plants. Despite the chemical, biological, and integrated pest management methods applied against these damages, aphids have rapidly expanded their distribution areas and their damages have been increasing in recent times. *Hyalopterus* Koch (Hemiptera: Aphididae), a genus of aphids, are known worldwide as pests that infest *Prunus* trees, which are stone fruit trees. They cause damage by feeding on the trees and also by transmitting plant viruses. Subsequently, improper and indiscriminate use of chemical control methods negatively impacts both human and environmental health. Accurate identification of aphids, especially in terms of invasive species, is crucial for early detection of their damages in the initial stages. The mitochondrial cytochrome c oxidase subunit I (COI) gene is an effective gene region used in the identification of many economically important plant pests worldwide. In this study, a total of 50 individuals of *Hyalopterus pruni* (Geoffroy) were collected from three localities Şarköy (Ulaman, Bulgurlu, Gölcük, Cumhuriyet, Mürefte, Hoşköy, Gaziköy, Tepeköy, Palamut), Süleymanpaşa (Yüzüncüyıl, Altınova, Banarlı, Barboros, Bıyıkali, Çınarlı, Değirmenaltı, Ferhadanlı, Hürriyet, Karacakılavuz, Karaevli, Naip, Namık Kemal and Marmaraereğlisi (Bahçelievler, Cedit Ali Paşa, Dereağzı, Mustafa Kemal Paşa, Sultanköy, Türkmenli, Yakuplu and Yeniçiftlik) in Tekirdağ province. The species H11, H41, and H61, which were selected to represent three counties, were sequenced, and the molecular sequence results revealed that *H. pruni*, as morphologically described, showed 99% consistency at the molecular level.

Keywords: Aphid, *Hyalopterus pruni*, COI, Molecular diagnosis, Tekirdağ

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Öz

Yaprakbitleri tarım ürünleri, süs bitkileri, otsu ve odunsu bitkilere zarar veren en önemli böcek gruplarından biridir. Bitki özsuyu ile beslenen yaprakbitleri, bitkilerde bodulaşma, deformasyon, solma ve diğer zararlı etkileri nedeniyle dünya çapında %70 ila %80 arasında değişen önemli ürün kayıplarına neden olmaktadırlar. Bu zararlarına karşı uygulanan kimyasal, biyolojik ve entegre zararlı mücadele yöntemlerine rağmen yaprakbitleri son zamanlarda yayılma alanlarını hızla genişletmekte ve zararlarını arttırmaktadırlar. *Hyalopterus* Koch (Hemiptera: Aphididae) cinsine ait türler sert çekirdekli meyve ağaçları olan Prunus ağaçlarını istila eden zararlılar olarak dünya çapında bilinmektedir. Ağaçlarda beslenerek bitki virus vektörlüğü yaparak zarar verirler. Yaprakbitlerini doğru teşhisi, özellikle istilacı türler açısından, zararlarının başlangıç aşamalarında erken tespit için hayati öneme sahiptir. Yaprakbitleri taksonomistleri tür düzeyinde teşhisleri, konukçubitkilere dayalı tanı anahtarları kullanarak morfolojik özelliklere göre yapmaktadırlar. Bununla birlikte, yaprakbitleri gibi yüksek fenotipik çeşitliliğe sahip gruplarda, bu tanı anahtarları bazen yetersiz kalabilir. Mitokondriyal sitokrom c oksidaz alt ünitesi I (COI) geni, dünya genelinde birçok ekonomik öneme sahip bitki zararlılarının teşhisinde etkin kullanılan bir gen bölgesidir. Bu çalışmada, *Hyalopterus pruni* (Geoffroy) türünden toplam 50 birey, Tekirdağ ilindeki Şarköy (Ulaman, Bulgurlu, Gölcük, Cumhuriyet, Mürefte, Hoşköy, Gaziköy, Tepeköy, Palamut), Süleymanpaşa (Yüzüncüyıl, Altınova, Banarlı, Barboros, Bıyıkali, Çınarlı, Değirmenaltı, Ferhadanlı, Hürriyet, Karacakılavuz, Karaevli, Naip, Namık Kemal) ve Marmaraereğlisi (Bahçelievler, Cedit Ali Paşa, Dereağzı, Mustafa Kemal Paşa, Sultanköy, Türkmenli, Yakuplu ve Yeniçiftlik) bölgelerinden toplanmıştır. Üç ilçeyi temsil etmek üzere seçilen H11, H41 ve H61 türleri dizi olarak alınmış ve moleküler dizi sonuçları, morfolojik olarak tanımlanan *H. pruni'*nin moleküler düzeyde %99 uyumluluk gösterdiği tespit edilmiştir.

Anahtar Kelimeler: Yaprakbiti, *Hyalopterus pruni,* COI, Moleküler tanı, Tekirdağ

1. Introduction

Aphids are one of the most important insect groups that cause damage to agricultural products, ornamental plants, and naturally growing herbaceous and woody plants in the areas they are found. Aphids, which feed on plant sap, cause 70% to 80% crop loss worldwide due to consequences such as stunting, deformation, and wilting in plants. They are found worldwide, although they are more frequently observed in temperate zones (Blackman and Eastop, 2023). The aphids, which are significant pests of many plants, belong to the superfamily Aphidoidea (Hemiptera) (Budak et al., 2022). Species belonging to this superfamily generally live in colonies on their hosts (Zeybek and Tozlu, 2022). Certain aphid species pose a significant threat as invasive pests, jeopardizing agricultural ecosystems on a global scale (Capinera, 2002). They are not only plant feeders but also play a crucial role as virus vectors, responsible for transmitting nearly 30% of all known plant virus species (Brault et al., 2010).

The prominent biological characteristics of aphids, including polyphenism, host alternation, and the ability to reproduce both sexually and asexually, have rendered them a compelling model for evolutionary and ecological research. (Dixon, 1998; Coeur d'Acier et al., 2007). Furthermore, precise taxonomy is crucial for detecting biological invasions and effectively managing pest populations (Lozier et al., 2008).

Diagnoses of aphids are made using a single specimen and based on morphological characters, utilizing keys. Phenotypic variation due to host and environmental influences can make the diagnosis of aphids difficult. Furthermore, other factors that can make the diagnosis of aphid species difficult include the occurrence of different morphologies on different hosts under various climatic conditions, complex life cycles, polymorphism, cyclic parthenogenesis, and host switching during summer and spring months. Considering these factors, a potentially more dependable method for diagnosing taxonomically complex groups could be to collectively assess morphological, molecular, and host plant information (Hebert et al., 2003).

The mitochondrial cytochrome c oxidase sub unit I (COI) gene has been widely adopted as an effective method for the identification of many economically important plant pests worldwide. Mitochondrial gene regions include cytochrome oxidase I and II (COI, COII), cytochrome b (CytB), F-ATPase subunits 6 and 8 (F-ATP), NADH-1 dehydrogenase (NADH1), and 12S and 16S ribosomal RNA (12S/16S). (Hoy, 2003); (Freeland, 2005). COI has two important advantages among these gene regions. Firstly, universal primers for this gene region are highly robust. Secondly, it has more phylogenetic markers compared to other mitochondrial genes. Furthermore, it is observed that the amino acid sequence changes are relatively slow (Hebert et al., 2003). It has been reported that the COI region is useful for the accurate identification of aphid species, especially in cases where morphological characters are insufficient (Cocuzza et al., 2015).

Accurate and timely identification of *Hyalopterus* members is essential for proper and timely control, as well as reducing the economic losses they cause. In all molecular and morphometric studies conducted on this genus in recent years, it has been widely accepted that members of this genus use different *Prunus* species as their primary host (Poulios et al., 2007, Lozier et al., 2008; Rakauskas et al., 2013). Especially with their primary hosts being economically important *Prunus* spp. species, aphids cause direct damage and result in economic losses. The differentiation of members of this genus is challenging because their identification is solely based on corniculus in the morphological key (Lozier et al., 2008; Rakauskas et al., 2013). In additionally, *Hyalopterus pruni* has been detected in approximately 32 provinces in Turkey and is a seriously spreading aphid species (Kök and Özdemir, 2021).

Hyalopterus pruni apterae rather elongate-bodied, pale green with darker green mottling, covered with white wax meal; BL 1.5-2.6 mm. On undersides of leaves of *Prunus domestica*, and sometimes on other *Prunus* spp., especially *P. armeniaca*, but not *P. dulcis*. Infested leaves do not curl. Alatae have a green abdomen with white wax patches on each segment. Migration occurs to *Phragmites*, or sometimes to *Arundo donax*; for its appearance on *Phragmites* see. Widely distributed in Europe and Asia and introduced to North America, but records from Africa and Australia are based on secondary host populations and are possibly another species (Blackman and Eastop, 2023).

2. Materials and Methods

2.1. Collection and preparation of aphids

The survey study was conducted in Şarköy, Marmaraereğlisi and Süleymanpaşa province of Tekirdağ in 2021. The aphids samples were collected from plum (*Prunus domestica*) trees.

During sampling, attempts were made to collect as many winged and wingless adult individuals as possible. A code number has been assigned to each sample. Furthermore, information such as the color prior to preservation in alcohol, date, host plant, and collection location were recorded in the field notebook. The generated code numbers were placed as labels, written with a pencil, inside the cryo tubes containing the aphid samples.

The collecting and preservation methods employed were largely based on Hille Ris Lambers (1950) approach. According to this method, aphids were initially subjected to the cleaning process to reveal diagnostic characteristics and to remove body colors and waxy substances present on the body in some species. Sufficient 4 minutes below the boiling point in a water bath. The ethyl alcohol in the tubes was emptied, and 10% KOH was added to the tubes. The specimens, especially the dark-colored ones, were boiled in KOH for 3-7 minutes until their colors became suitable. After determining sufficient lightening of the specimen colors, ethyl alcohol was added to the glass tubes containing KOH, and then they were left to stand for a while. Subsequently, the KOH-ethyl alcohol mixture was removed from the tubes manually or with the help of a pasteur pipette. Ethyl alcohol was added again to the tubes to ensure thorough cleaning of the specimens. The ethyl alcohol in the tubes was emptied, and a previously prepared 1:1 mixture of Chloral hydrate-Phenol was added to the tubes. The samples were kept in this mixture at temperatures below the boiling point in a water bath, varying depending on the species, for 5-10 minutes. After the cleaning process, the aphid samples were thus made ready for preparation. The preparation process of the cleaned aphids was completed. The Berlese medium was used as the environment for preparing permanent mounts. To prepare this medium, a specific amount of gum arabic, glycerin, chloral hydrate, and distilled water were thoroughly mixed at room temperature and then filtered through several layers of glass wool to obtain a clean mixture. After preparing the Berlese medium, a small amount was dropped onto each slide, and the cleaned aphids were placed on each slide in both ventral and dorsal positions, as nymphs and winged-wingless adults. The aphids placed on the slide were positioned with their antennae, wings, and legs open, ensuring that diagnostic characteristics were visible.

2.2. Morphological characterization of aphids

The diagnoses of *Hyalopterus pruni* samples obtained from their host plants in Tekirdağ province during field studies conducted in 2021 were morphologically carried out according to Cottier (1953), Börner (1952), Bodenheimer and Swirski (1957), Hille Ris Lambers (1945, 1947a, 1947b, 1949, 1969, 1973), Börner and Heinze (1957), Tuatay and Remaudiere (1964), Shaposhnikov (1964), Stroyan (1957, 1961, 1963, 1969, 1977, 1984), Bissel (1978) and Blackman and Eastop (1984, 1994, 2000, 2014, 2020). The species identification of aphids was carried out using a LEICA DM LB2 light microscope. The definite identification of aphids was performed by Associate Professor Dr. Işıl ÖZDEMİR.

2.3. DNA extraction, amplification and sequence

A total of 50 individuals of *H. pruni* were collected from three localities Şarköy (Ulaman, Bulgurlu, Gölcük, Cumhuriyet, Mürefte, Hoşköy, Gaziköy, Tepeköy, Palamut), Süleymanpaşa (Yüzüncüyıl, Altınova, Banarlı, Barboros, Bıyıkali, Çınarlı, Değirmenaltı, Ferhadanlı, Hürriyet, Karacakılavuz, Karaevli, Naip, Namık Kemal and Marmaraereğlisi (Bahçelievler, Cedit Ali Paşa, Dereağzı, Mustafa Kemal Paşa, Sultanköy, Türkmenli, Yakuplu and Yeniçiftlik) in Tekirdag province. DNA extraction was done following method; Put the aphid in a 1.5 ml Eppendorf tube. If the aphid was stored in ethanol, place the aphid on a tissue for a few minutes to let the ethanol evaporate. Wash pestle in a large volume of distilled water and dry after every use. Add 300 µl TNES buffer by letting it run down the pestle to wash all of the squised aphid into the tube. Mix and incubate tube at 55 °C for 1 – 3 h (or at 37 °C over night). Add 85 µl of 5M NaCl and shake hard for 15 s (proteins become precipitated). Microfuge at full speed for 5 – 10 min. Microfuge at full speed for at least 5 min. Air-dry the pellet cover tubes with a tissue to avoid contamination. Re-suspend the DNA in the required amount of 1x TE buffer.

A 700 bp segment of the mitochondrial COI gene was PCR amplified using the primers LepF (5′-

ATTCAACCAATCATAAAGATATTGG-3′) and LepR (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) (Hebert et al. 2004). The PCR assay was carried out following program initial denaturation at 94°C for1 min, followed by 6 cycles of 1 min denaturation at 94°C, 1 min and 30 s annealing at 45°C, and 1 min and 15 s extension at 72°C, then followed by 36 cycles of 1 min at 94°C, 1 min and 30 s at 51°C, and 1 min and 15 s at 72°C, with a final step of 5 min extension at 72°C, and cooling to 4°C before the PCR products were removed from the thermocyler. PCR products were checked by electrophoresis on 1.5% agarose gel in TBE buffer (*Table 1*) (Xu et al., 2011).

Table 1. Chemicals used for polymerase chain reaction (PCR).

for 10 µl reactions	
H2O	4.0 µl
10 × Taq buffer	1.0 µl
MgCl2 25 mM	0.8 µl
dNTPs	1.6 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Taq polymerase	0.1 µl
DNA	1.0 µl

Additionally, three samples representing each district were selected and sent to the Nabiltem Center Laboratory at Tekirdag Namik Kemal University for sequencing.

2.4 Data analysis

The sequence results were aligned using MEGA 4 (Tamura et al., 2007) and identified by comparison with the nucleotide-nucleotide basic alignment search tool (BLAST) (GenBank DNA sequence database, National Center for Biotechnology Information).

3. Research Results and Discussion

Identify aphids using mitochondrial genes, PCR was done using LepF and LepR primers. The PCR products showed a 700 bp band segment as mentioned amplifying DNA fragment of the mitochondrial genome in all samples. To sequence the mitochondrial gene, PCR products were sent to NABILTEM Center Laboratory. After sequencing, results were aligned, and the gene-related sequence was blasted in NCBI GenBank. Based on the BLAST results, the samples were identified. >99% identity and 100% coverage to: *Hyalopterus pruni* (KR582302). H11, H41, and H61 (species representing thee different locations of Tekirdag) are matching. The gene is confirmed as "COI". The size that we could successfully recover is ~340 bp.

The morphological characteristics of aphids play a significant role in their ability to adapt to different host plants. Furthermore, environmental factors such as day length and temperature have complex effects on the morphological characteristics used to identify population differences. The morphology of aphids and their relationship with host plants are crucial in their classification. Their ability to closely associate with host plants relies on their distinct life cycles (Lee et al., 2015). The mitochondrial COI gene region is important in revealing the genetic variation at the species and interspecies levels in aphid groups, due to its rapid and highly accurate results (Foottit et al., 2009; Valenzuela, 2009).

In recent years, DNA barcoding studies have become one of the frequently used methods by many aphidologists to address taxonomic issues in various insect groups, including aphids, aiming to resolve existing taxonomic problems. (Hebert et al., 2003). Lozier et. al. (2008) before updating the diagnostic key used for distinguishing members of the genus Hyalopterus, it has been reported that often *H. amygdali* or *H. pruni* were indicated when it came to the genus discrimination in the diagnostic key, and these two species were frequently confused with each other during diagnosis.

In their study conducted in Greece, Poulios et al. (2007) determined that *H. amygdali* A utilizes *P. dulcis* and *P. armaniaca* as hosts, *H. amygdali* B utilizes *P. persica* as a host, and *H. pruni* utilizes *P. domestica* as a host. Indeed, in our study, samples were collected from *P. domestica*, and both classical and morphological analyses confirmed that they were *H. pruni.* Lozier et al. (2008) evaluated *Hyalopterus* populations feeding on *Prunus* host

plants in Spain, Italy, Greece, Tunisia, Israel, the United States, and Georgia using molecular methods in their study. They have determined that *H. persikonus* feeds on *P. persica*, *H. amygdali* feeds on *P. dulcis*, and finally *H. pruni* feeds on *P. domestica*. In his study conducted in Afyonkarahisar, Kütahya, Niğde, and Uşak provinces, Şenol (2017) sampled from different localities on *P. domestica*, *P. persica*, *P. dulcis*, *P. armaniaca*, and *Phragmites* hosts. It has been determined that the morphological variations of individuals belonging to the genus *Hyalopterus*, depending on the host, locality, and species, are consistent with previous studies.

4. Conclusions

Species definitions derived from traditional diagnostic methods could potentially be deceptive, particularly within categories like aphids. As a result, it is recommended to collectively analyze morphological and molecular data before attempting species identification in the investigated aphid groups.

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