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Research Article

Molecular Identification of the Invasive Species, *Nysius cymoides* (Spinola, 1837) (Hemiptera: Lygaeidae) in Türkiye

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Abstract

The false chinch bug, *Nysius cymoides* (Spinola, 1837) (Hemiptera: Lygaeidae), is an important pest with a wide host range. It was first discovered in Italy but now causing agricultural damage worldwide, including Türkiye. Specimens of *N. cymoides* were collected from surveys of the sunflower and corn fields in Malkara-Keşan province, in Edirne in July 2020. Because of the population increase of the pest and their damages to sunflowers and corn fields, the pest identification has become an important issue. We investigated molecular characterization and identification of the pest by using the mitochondrial cytochrome oxidase I (COI) barcode region. Adult and late immature stages of the specimens were examined under a stereozoom microscope and morphologically identified as well. The partial *COI* gene (659 bp) was amplified by using LCO1490/ HCO 2198 and PCR products were sequenced directly for molecular characterization. The sequence was registered GenBank database with accession number OL989232. Molecular analyzes were performed using MEGA X software. As a result, the nucleotide frequencies were A = 33.81%, T/U = 35.79%, C = 16.03%, and G = 14.37%. The distance-based species limitation method applied by using ASAP and ABGD, identified 5 species in the partition with the lowest ASAP score (1.5). The barcode gap distance of this partition with the best ASAP score was determined as 1.7%. Also, the threshold distance was determined as 1.62%. The highest nucleotide identity of the studied specimen (OL989232) was detected as 99.8% with the specimen of *N. cymoides* from France (KJ541649.1) in GenBank. The objective of this study was the first attempt to identify *N. cymoides* specimens collected in Edirne based on DNA barcode regions.

Keywords: *Nysius cymoides*, DNA barcode, COI, mtDNA, Molecular identification.

Türkiye'de İstalacı bir tür olan *Nysius cymoides* (Hemiptera: Lygaeidae)'in Moleküler Tanımlaması

Öz

Sahte çinç böceği, *Nysius cymoides* (Hemiptera: Lygaeidae), geniş bir konukçu çeşitliliğine sahip önemli bir zararlıdır. İlk olarak İtalya'da tespit edilen bu zararlı, şimdi Türkiye'de dahil olmak üzere tüm dünyada tarımsal zararlara yol açmaktadır. *N. cymoides* örnekleri, Temmuz 2020'de Edirne Keşan bölgesinde ayçiçeği tarlalarında yapılan sörvey araştırması sonucunda elde edilmiştir. Zararlının popülasyonundaki artış ve ayçiçeği ile mısır tarlarındaki zararı, bu türün doğru teşhis edilmesi zorunlu kılmıştır. Zararlının teşhisi ve moleküler karakterizasyonunun araştırılması için mitokondrial sitokrom oksidaz I (COI) barkod alanı kullanılmıştır. Ergin ve geç ergin öncesi dönem örnekleri stereozoom mikroskop altında incelenmiş ve morfolojik olarak ta tanımlanmıştır. COI genin bir parçası (659 bç) LCO1490/ HCO 2198 primerleri ile çoğaltılmıştır. Moleküler karakterizasyon için PCR ürünleri doğrudan dizilmiştir. Dizilim GenBank veri tabanına OL989232 erişim numarası ile kaydedilmiştir. Moleküler analizler MEGA X yazılımı kullanılarak yapılmıştır. Sonuçta, nükleotid frekansları A = %33.81, T/U = %35.79, C = %16.03 ve G = %14.37 olarak belirlenmiştir. ASAP ve ABGD kullanılarak uygulanan mesafeye dayalı tür sınırlama yöntemi, en düşük ASAP puanına (1.5) sahip bölmede 5 tür belirlenmiştir. En iyi ASAP puanına sahip bu bölümün barkod boşluk mesafesi %1.7 olarak belirlenmiştir. Eşik mesafesi %1.62 olarak belirlenmiştir. Karşılaştırılan diziler arasında en yüksek nükleotid benzerliği, çalışmamız sonucu elde edilen dizi (OL989232) ile *N. cymoides* France (KJ541649.1) dizisi arasında %99.8 olarak tespit edilmiştir. Bu çalışma ile Edirne'den toplanan *N. cymoides* örneklerinin ilk kez moleküler teşhisi gerçekleştirilmiştir.

Anahtar Kelimeler: *Nysius cymoides*, DNA barkod, COI, mtDNA, Moleküler tanımlama.

Introduction

Lygaeoidea is a superfamily belonging to Hemiptera order having mostly phytophagous pests. *Nysius cymoides* (Spinola, 1837) (Hemiptera: Lygaeidae) is known and widely distributed around the world (Hori, 2000; Sweet, 2000; Scaccini and Furlan, 2019). The common name is called as the false chinch bug and was described previously as *A. cymoides* (Bocchi et al., 2016; Haouas et al., 2019).

Nysius cymoides is thermophilic insect (Pericart, 1998; Aukema, 2013; Scaccini and Furlan, 2019). It is an epidemic and invasive species in Türkiye and commonly distributed in Europe, Central Asia and North Africa, the Middle East, and Arabian deserts (Pericart, 1998; Aukema, 2013; Scaccini and Furlan, 2019; Haouas et al., 2019). The false chinch bug is a polyphagous reported on quinoa, *Chenopodium quinoa*, and soy bean, *Glycine max* (Bocchi et al., 2016; Scaccini and Furlan, 2019) in Italy, on alfaalfa, *Medicago sativa* (Yasunage, 1990), cotton, *Gossypium hirsitum*, clover, *Trifolium* spp. (Wipfli et al., 1990), canola, *Brassica napus* (Sarafrazi et al., 2009), almond, *Prunus amygdalus* var. *dulcis* and apple, *Malus domestica* in Iran (Mollashahi et al., 2017). It is reported to prefer cruciferous plants as legumes and many other plant families (Haouas et al., 2019; Yazıcı, 2022). It is stated as univoltine or multivoltine at lower latitudes (Bocchi et al., 2016).

In Türkiye, it has been determined on canola in Hatay (Demirel, 2009), olive orchards in Edremit (Abacıgil et al., 2010), and cultivated fruit trees in Mardin and Siirt (Matocq and Özgen, 2010), pistachio (Bolu, 2012), vineyard (Özgen, 2012) and on tomato, cucumber, watermelon, eggplant, pepper, corn, purslane, alfalfa and weeds as well (Özgen et al., 2020).

The outbreaks occur in hot summer by increasing their population causing damages to seeds, vegetables and fruits. Several epidemic populations were reported for the false chinch bug on quinoa and canola (Bocchi et al., 2016). Like all sucking mouth insects, the damages of *N. cymoides* are caused by vascular tissues (phloem and xylem) and new growth parts of the plants by nymphs and adults (Özgen et al., 2020). The damaged plant turns to yellowish-brown in color and develops wilthing and necrosis. However, recent distributions and new host plants of this pest were determined, and the enhanced cultivated host plant list, the biology of the false chinch bug, the different biological stages, generation times, managements, biological control agents and molecular studies were not well studied.

Eventhough *Nysius* genus is well known, it is complex and hard to identify morphologically because of the similarities in appearance. In general, the adult stage is used to identify insect species based on their taxonomic characters. Molecular methods are important for the accurate and rapid identification of invasive pest species without depending on the biological stages (Kuyulu et al., 2019). Especially, the cytochrome oxidase subunit I (COI) has been used successfully in insect identification and keeping track of existing pest species and their distributions (Kuyulu and Genc, 2020; Yücel and Genc, 2022). The COI gene of mitochondrial DNA is called the barcode region to design PCR primers to identify insect species (Hebert et al., 2003). In this study, *N. cymoides* were collected from Keşan, Edirne, which had high infestation on canola plantations and then probably moved to maize and sunflower fields. The objective of the study was to identify and characterize *N. cymoides* specimens based on molecular methods which would be the initial step to develop management strategies.

Material and Methods

Collecting samples

Nysius cymoides specimens were collected from sunflower fields in Keşan, Edirne in July 2020. The intensive population was occurred in 2020 in Edirne and showed the adults and nymph stages on the plant, ground and irrigation pipes (Figure 1).

Figure 1. The nymph and adult population of *Nysius cymoides* on corn, soil and irrigation pipe (A, B, C).

The specimens were brought to Çanakkale Onsekiz Mart University, Insect Molecular Biology Laboratory. They were transferred to Eppendorf tubes with the help of a soft-tipped forceps and kept at -20ºC until used. The adults were examined externally under the Olympus SZX9 stereo zoom microscope and photographed with an attached Olympus digital camera (Figure 2).

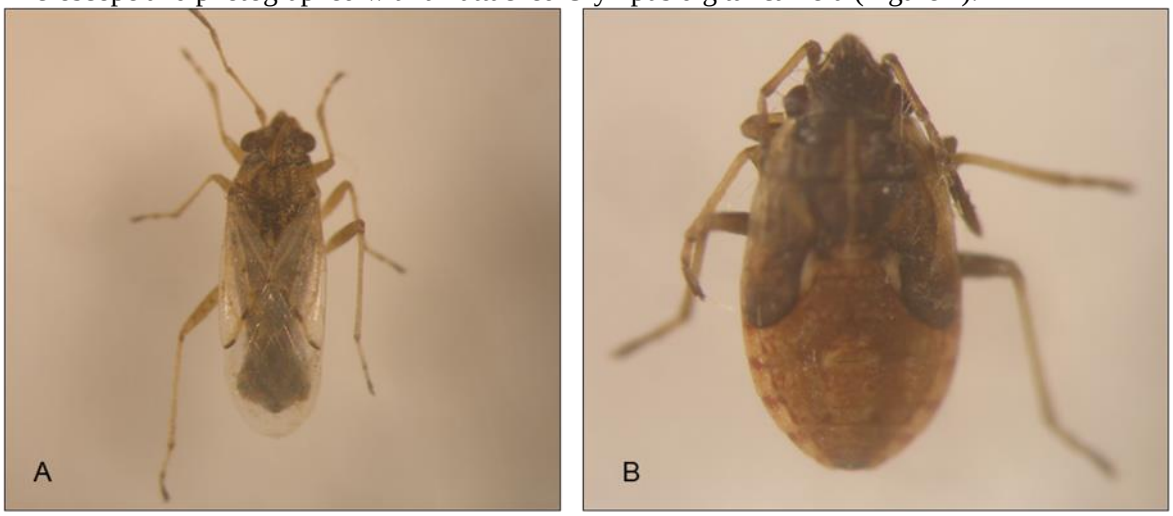

Figure 2. Images of *Nysius cymoides* adult (A) and nymph (B).

Morphological identification of the specimens was performed by Paul Skelley (in DPI, FDACS in Gainesville, Florida, USA)**.**

DNA Extraction, PCR amplification

Total genomic DNA was isolated individually (n=3) from the whole body using the PureLink Genomic DNA Mini Kit™ (Invitrogen) according to the manufacturer's instructions. The amount of DNA was quantified with a NanoDrop spectrophotometer. (Thermo Scientific™, One/OneC Microvolume).

PCR amplification was performed using the universal primers (LCO1490F, 5'GGTCAACAAATCATAAAGATATTGG 3' and HCO2198R, 5' TAAACTTCAGGCTGACCAAAAAATCA 3') of the partial fragment of cytochrome oxidase I (*COI*) gene of mtDNA (Folmer et al., 1994). PCR was performed in a 25-µL final reaction volume, including 2.5 µL of 10XPCR buffer (Ampliqon), 1 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTPs (10 mM each), 0.5 µL of each primer (10 pmol), 1 µL of template DNA (20 ng/µL), and 1 U of Taq polymerase (Ampliqon). PCR was carried out with a Bio-Rad S1000™ Thermal cycler with programme setting as follows: 4 min

at 94 °C initial denaturation, then 36 cycles of 40 s at 94 °C, 1 min at 48 °C, 45 s at 72 °C and with a final extension for 10 min at 72 °C (Kuyulu et al., 2019). PCR products were confirmed on 1% agarose gel at 120 V for 30 min then sent to a company for sequencing one-way using forward primer.
The sequence was checked manually to avoid misreading. The resulting sequence was deposited in the NCBI database using BLASTn with standard settings and submitted in GenBank (Accession Numbers: OL989232). The sequence was edited using MEGA X (Kumar et al., 2018) and aligned using Clustal W 1.6 (Thompson et al., 1994). *Greenidea artocarpi* is used as an outgroup in this study. The haplotypes of the sequenced specimen were checked not to have internal stop codons to assure mtDNA nucleotides (Mutun and Atay, 2015).

Phylogenetic analyzes

Distance between samples was based on the parameter algorithm Kimura 2-Parameter (K2P) using MEGA X (Saitou and Nei, 1987). The phylogenetic tree was performed to investigate the relationships between the species and Neighbor-joining (NJ) was run using Kimura's two-parameter algorithm model. Support for individual clades was estimated by conducting bootstrap analyses with 1000 replications.

Analysis of haplotype and genetic diversity was performed using the DNA Sequence polymorphism (DnaSP) v6 software (Rozas et al., 2017). The haplotype network created to reveal evolutionary relationships was carried out in PopART 1.7 using the Median Joining (MJ) method (Bandelt et al., 1999). Pairwise Sequence Alignment analysis was performed using the Emboss matcher (https://www.ebi.ac.uk).

It was stated that ABGD (Automatic Barcode Gap Discovery) (Puillandre et al., 2012) showed the best compatibility with the morphologically studied species (Pentinsaari et al., 2017; Zhou et al., 2019). ASAP (Assemble Species by Automatic Partitioning) (Puillandre et al., 2021) score is created by calculating the probability of panmixia (p-value) and the corresponding barcode gap width (W) and the average of these two parameters. The lower the ASAP score achieved, the better the section is. At the same time, this method also calculates the distance threshold, revealing accelerated speciation rates. ASAP and ABGD, which were used to identify unknown species and explore species limitations, were used to identify species with the enumeration system based on the Kimura (K80) distance measurement model using default parameters.

Results and Discussion

The false chinch bug increases its population quickly and all biological stages of the pest damage the host plants so the identification should be needed as quickly as possible. The specimens were morphologically identified by Paul Skelley (DPI, FDACS) and then assured further on the sequences of partial *COI* gene of mtDNA in this study.

Mitochondrial COI is an effective tool to understand the genetic structure, insect identification and evolutionary rate among pest species (Kuyulu et al., 2019). A total of 3 specimens representing Edirne population of *N. cymoides* were successfully sequenced. A 659 bp of the mitochondrial *COI* gene fragments was successfully sequenced and used for molecular analyses. The insertion, deletion stop codon and/or nonsense mutations were not observed.

The obtained sequence was confirmed not to have any insertion, deletion and/or stop codon. The AT content was determined as 68.40% and the GC content was 31.60%. The mean nucleotide frequencies were observed as A = 32.9%, T/U = 35.5%, C = 16.4%, and G = 15.2%. A strong AT bias was found at 68.4%, a usual situation for in the mitochondrial gene of the insects (Mutun and Atay, 2015). The intraspecific and interspecific mean distances were calculated as 4% and 24.8%, respectively.

A sequence logo of *Nysius cymoides* was created after aligning the sequences of *Nysius* genus (Figure 3). The overall height of the resulting logo position depends on the degree of protection in the corresponding multi-row alignment column. Highly protected column alignments produce high logo positions. The letters of each stack were listed from the most frequent to the least frequent (Figure 3). The best BLAST search for the sequence obtained in Edirne, Türkiye (OL989232) succeed with the sequence KJ541649.1, which corresponded to *N. cymoides*, a French specimen, at an accuracy of 99.8% (Table 1).

The lowest nucleotide identity was detected as 91.4% with the sequence of *Nysius fuscovittatus* (KR033483.1) (Table 1). The alignment of the nucleotide sequence indicated 11 haplotypes, which were different than each other. The BLAST search for the obtained sequence (OL989232) called H1 haplotype was obtained with the sequences (KJ541649.1 and KJ541525.1) which corresponded *N. cymoides*, French specimens. The COI gene sequence was shown here as a monophyletic group in *N. cymoides* sequences (Figure 4).

Figure 3. Multiple aligned of the partial COI gene sequence logo of *Nysius cymoides*

Table 1. Matrix of pairwise identity percentages of *Nysius cymoides* and other sequences

(%)	OL989232	KJ541649.1	KJ541587.1	KJ541524.1	KJ541525.1	KR045234.1	KR030829.1	KR033483.1	KR040116.1	KR044831.1	KR034038.1	KR565634.1	KR578324.1	KR034420.1	KU373954.1	KR032099.1	KY829715.1	JX051422.1
OL989232																		
KJ541649.1	99.8																	
KJ541587.1	99.7	99.8																
KJ541524.1	99.4	99.4	99.2															
KJ541525.1	99.7	99.7	99.5	99.5														
KR045234.1	94.6	94.7	94.6	94.3	95.0													
KR030829.1	94.6	94.8	94.7	94.3	95.0	100												
KR033483.1	91.4	91.8	91.9	90.9	91.0	92.1	92.2											
KR040116.1	98.9	99.1	99.2	98.4	98.6	94.1	94.2	91.5										
KR044831.1	99.1	99.2	99.4	98.6	98.8	94.2	94.2	91.1	99.8									
KR034038.1	98.9	99.1	99.2	98.4	98.8	94.0	94.1	91.3	99.7	99.8								
KR565634.1	99	99.2	99.4	98.5	98.8	93.9	94.1	91.2	99.8	100	99.8							
KR578324.1	99.1	99.3	99.5	98.7	98.8	94	94	91	100	100	99.8	100						
KR034420.1	95.2	95.4	95.3	94.8	95.4	98.8	98.8	92.7	94.5	94.7	94.7	94.7	94.5					
KU373954.1	95	95.3	95.1	94.6	95.2	98.9	98.9	92.7	94.4	94.5	94.5	94.5	94.3	99.5				
KR032099.1	95	95.3	95.1	94.6	95.2	98.9	98.9	92.7	94.4	94.5	94.5	94.5	94.3	99.8	99.7			
KY829715.1	97.2	97.4	97.6	96.8	97.2	95.5	95.6	91.9	97.1	97	97.2	97.1	96.8	96.1	95.9	95.9		
JX051422.1	75.1	75.4	75.5	74.7	75	75.1	75.4	74.6	75.5	75.5	75.8	75.1	73.3	76	76	76	74.9	

*Comparisons are shown using accession numbers. The names represented by the accession numbers are shown in detail in the phylogenetic tree.

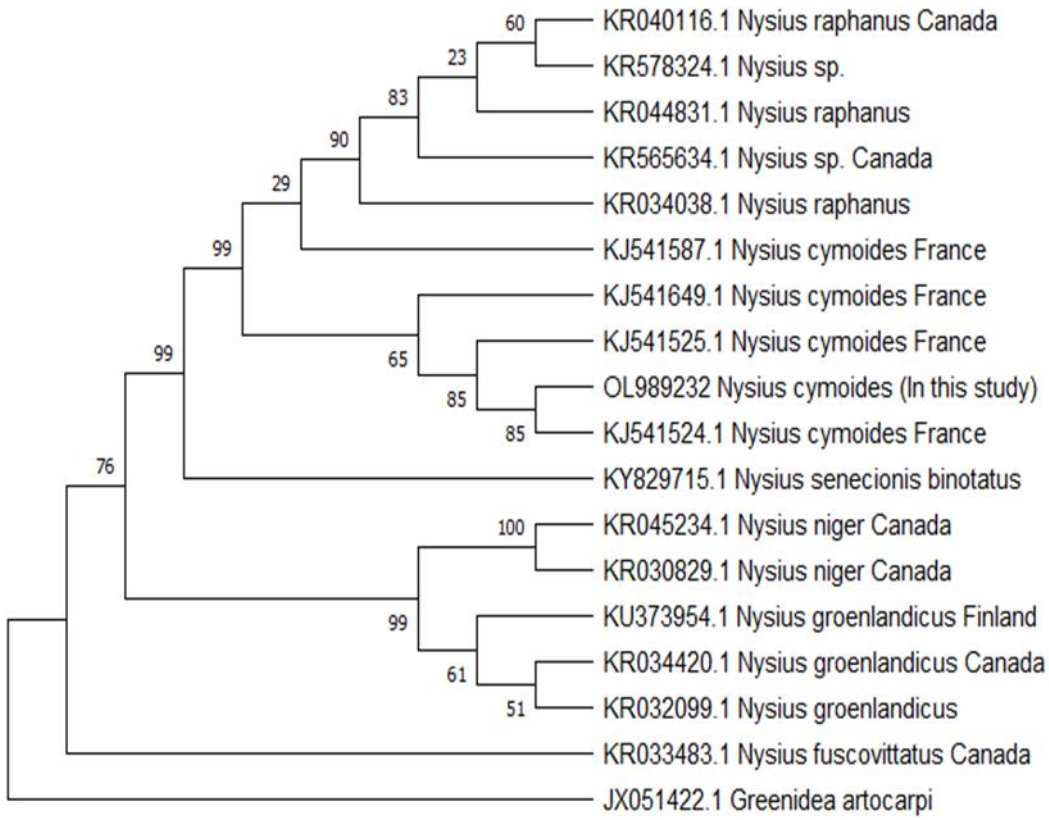

Figure 4. Phylogenetic tree based on partial sequence of COI gene of *Nysius cymoides* specimen. *Greenidea artocarpi* was used as outgroup.

Eight partitions have been defined by ASAP and ABGD. The 5 species were identified in the partition with the lowest ASAP score (1.5). The results supported that the phylogenetic tree was created previously with the 5 species and divided into groups, as the first group (n=10) included *N. cymoides, N. raphanus and Nysius sp*., the second group (n=5) was *N. niger* and *N. groenlandicus* and the other group was *Nysius* spp. (*N. fuscovittatus, N. senecionis binotatus*) and the outgroup (*Greenidea artocarpi*). They were each represented as a separate group.

The barcode gap distance of this partition with the best ASAP score was determined as 1.7%. The threshold distance was determined as 1.62%. For each new group, the p-value calculated based on the pairwise differences between subgroups (intra) and (inter) was determined as 2.22e-01 (Figure 5).

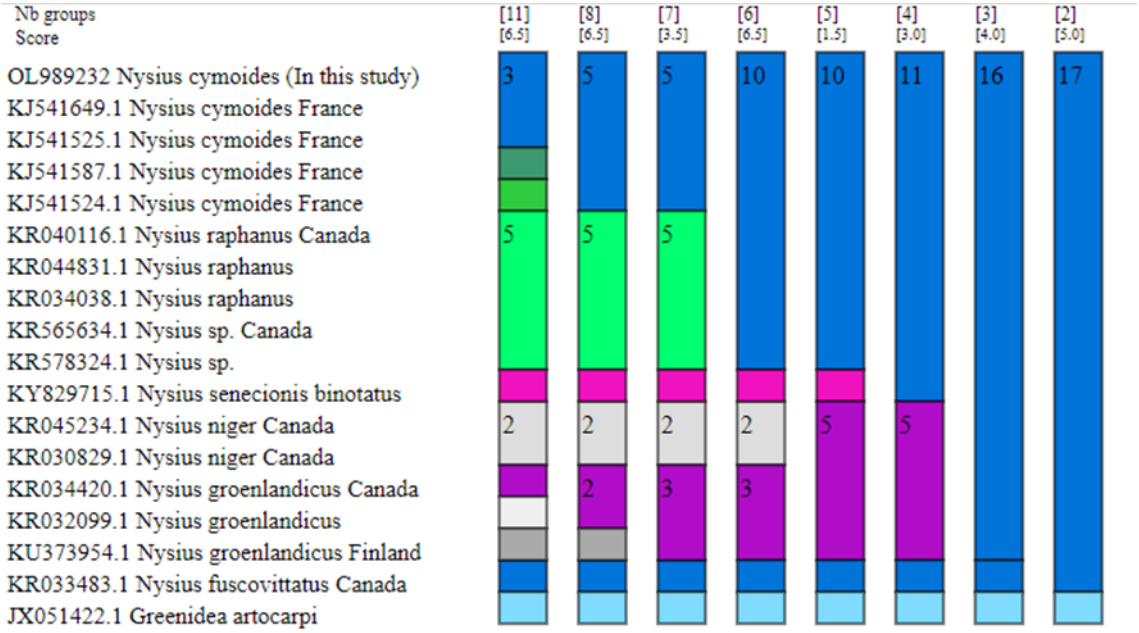

Figure 5. Boxed species chart using ASAP and ABGD.

To support the accuracy of the presented results, it is recommended that several subsequent partitions were considered, not just the partition with the best ASAP score (Puillandre et al., 2021). In the second-best ASAP score (3.00), 4 species were defined. *Nysius senecionis binotatus*, which formed a single group in the best part, was included in the first group. Other groups remained stable. In this partition, the threshold distance was determined as 2.97% (Figure 5).

As a result of the genetic diversity analysis of the *Nysius* species examined in the study, the average haplotype diversity (H_d) was determined as 0.866 and the nucleotide diversity (π) was also determined as 2.658%. Haplotype diversity varied between 0.00-1.00, while nucleotide diversity was found to vary between 0.00-0.403 (Table 2).

Table 2. Genetic diversity analysis of *Nysius* species.

Population	n	h	S	H_d	k	π (%)
Nysius cymoides	5	3	2	0.70	0.80	0.161
Nysius niger	2	1	0	0.00	0.00	0.00
Nysius raphanus	3	1	0	0.00	0.00	0.00
Nysius spp.	2	1	0	0.00	0.00	0.00
Nysius groenlandicus	3	3	3	1.00	2.00	0.403
Nysius senecionis binonatus	1	1	0	0.00	0.00	0.00
Total	16	10	30	0.866	13.238	2.658

*n: Number of sequences, h: Number of haplotypes, S: Number of segregating sites, H_d: Haplotype diversity, k: Average number of differences, π: Nucleotide diversity.

A Median-Joining tree was constructed to determine the phylogenetic network relationship between the haplotype of the studied *Nysius* species. The haplotype network was formed in 3 groups. The sample representing the Keşan region in Türkiye, is included in Group I. In this group, the H2 haplotype representing the *N. cymoides* France population is central. H2 is linked to two separate haplotypes, one of them is the H1 haplotype and shared *N. cymoides* specimens from France and Türkiye. The median joining tree was created to reveal the phylogenetic relationship also supported the accuracy of the species morphological identification (Figure 6).

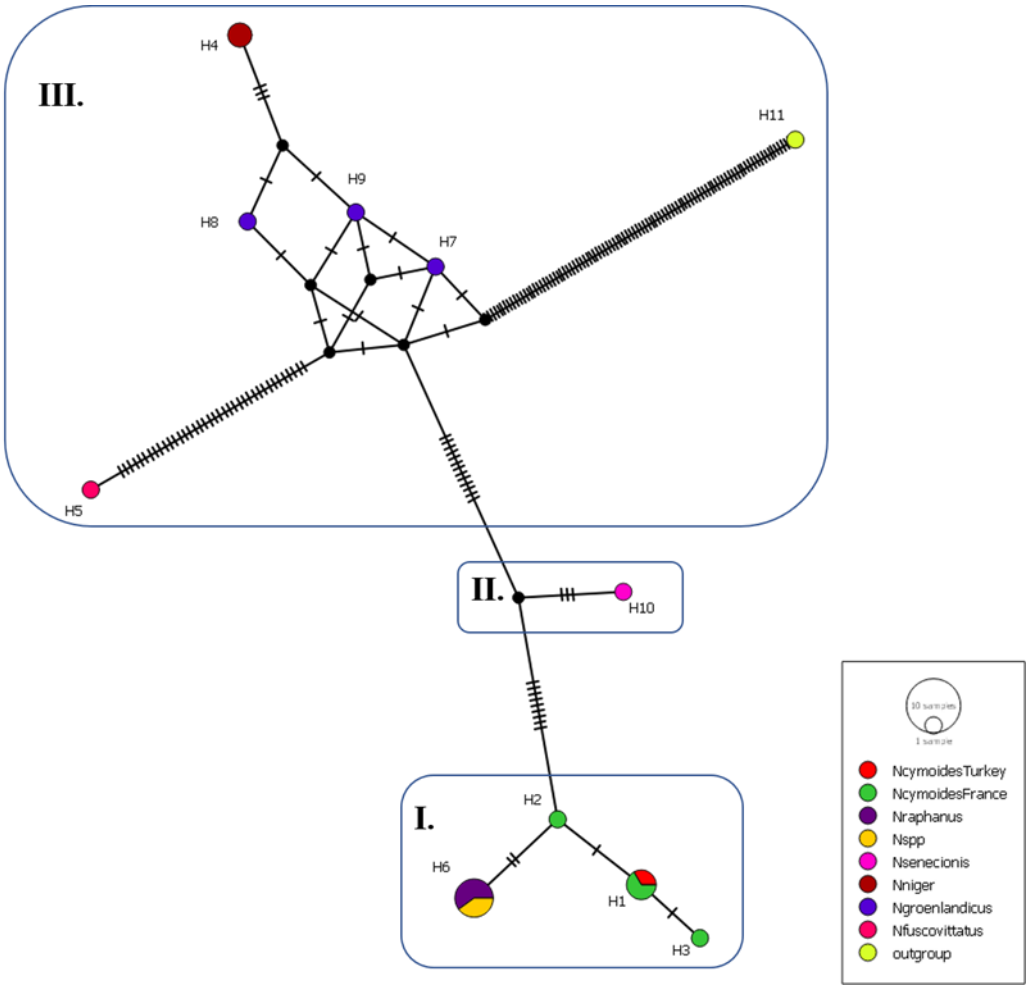

Figure 6. Median Joining Network analysis of COI haplotypes of *Nysius species*

Invasive pests were expected to exhibit genetic variation as a result of bottlenecks that tended to pass through at entry (Nei et al., 1975). This issue is important for the maternal inheritance and rapidly evolving *COI* gene of mtDNA. In this study, the DNA barcode was used to identify *N. cymoides* specimens based on both the morphological characters and the mitochondrial lineages within the species.

It was reported that *Nysius* spp are easily distributed all over the world except Antarctica continent (Ashlock, 1967). It is also listed as the main pest worldwide (Sweet, 2000), and the epidemics of *Nysius* species have also been reported in the regions where it is not considered to be a very important pest, including the Europe continent (Scaccini and Furlan, 2021).

The spread and infestation of *N. cymoides* are mainly due to their short life cycle, high mobility, and the high reproductive rate. It can adapt to the new environment resulting in as highly invasive pest and is very crucial to control *N. cymoides* during the summer season. So, the precise identification of the pest species is significant in surveillance and to applying management strategies. In order to do that molecular techniques are the best approaches providing supportive data besides morphological identification. The DNA barcode regions especially the *COI* gene has been used effectively for insect identification especially for invasive pests based on their high degree of polymporphism on *COI* gene and to understand the evolutionary rate (Hebert et al., 2003).

Especially, xerophiles and thermophiles species of *Nysius* require warmer climates to thrive (Scaccini and Furlan, 2021) and if the temperatures over 13.6 °C to 15.0 °C are considered to be thresholds for the development of egg and immature stages of *N. cymoides*. It has been reported that the development of the *Nysius* spp. is much faster when the temperature increases from 17.5 °C to 37.5 °C (Mohaghegh Neyshabouri, 2009). In addition to that they are more active on sunny days and the survival rate was higher in longer daylight period (Mollashahi et al., 2017). According to the survey study done

in 2018 in Eastern and Southeastern Anatolia, the population of *N. cymoides* was very high due to the dry and hot summer conditions (Özgen et al., 2020).

We carried out both morphological and molecular identification of *N. cymoides* specimens obtained during the surveys conducted in the sunflower fields in Edirne. The precise identification of the invasive pest with a high population is very important for the phytosanitary process and to control spreading of the pest. Further studies are needed regarding its biology, survival, host range, laboratory maintenance, and its behavior with the combination of climatic changes and agricultural factors.

Conclusion

The molecular approach is the best method based on the use of a partial *COI* gene can be effective in insect identification. This study is the first molecular report on the specimens of *N. cymoides* in Edirne, Türkiye. The phylogenetic relationship of the pest population is crucial to understand evolutionary rate, gene heterogeneity, population dynamics and gene flow. So, these results will be useful for the identification of invasive pest species and management programmes.

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Authors' Contributions

The contribution of the authors is equal.

Conflicts of Interest Statement

The authors have declared no conflict of interest.

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