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Original article (Orijinal araştırma)

Identification and genetic diversity of the Mediterranean cereal cyst nematode, *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae) in cereal production areas of Northern Cyprus

Kuzey Kıbrıs arpa ve buğday üretim alanlarında Akdeniz tahıl kist nematodu, *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae)'un tanımlanması ve genetik çeşitliliği

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Abstract

The Mediterranean cereal cyst nematode, *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae) is a destructive plant-parasitic nematode on cereal crops in particularly wheat and barley. It has a global distribution with a severe negative impact on yield quantity. In this study, a survey was conducted to identify plant-parasitic nematodes in cereal-growing areas in Cyprus. Forty-five samples including roots and soil from the root zone of plants were collected from cereal fields located in Gazimağusa, Girne, Güzelyurt and Lefkoşa Provinces before crop harvesting from late-May and early-June in 2017. Cyst-forming nematodes were determined by Fenwick's flotation and decanting techniques from 37 soil samples (82%). The internal transcribed spacer (ITS) regions of the ribosomal DNA of isolates were amplified and sequenced and subjected to a BLASTn search of the NCBI database for species identification, and the analyses showed that all samples were identified as *H. latipons*. Phylogenetic analyses based on ITS sequences revealed that *H. latipons* isolates from Northern Cyprus were closely related to isolates obtained from Morocco, Russia, Syria and Turkey. Data of this study demonstrated for the first time the presence of *H. latipons* in the cereal fields of Gazimağusa, Girne, Güzelyurt and Lefkoşa Provinces, where the nematode most likely causes serious economic problems in the cereal production. These results were the most up-to-dated analyses on the occurrence of *H. latipons* in cereal fields of Northern Cyprus and provided basic data for breeding programs to improve the resistant levels in the local cultivars.

Keywords: *Heterodera latipons*, heterogeneity, ITS, phylogeny

Öz

Akdeniz tahıl kist nematodu, *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae) özellikle buğday ve arpada olmak üzere tahıllarda önemli zararlara neden olan bir bitki paraziti nematod olup, dünyada tahıl yetiştirilen alanların büyük çoğunluğunda tespit edilmiştir. Bu çalışmada, Kıbrıs'ın tahıl alanlarındaki patojen nematodları belirlemek için bir sürvey yapılmıştır. Gazimağusa, Girne, Güzelyurt ve Lefkoşa tahıl üretim alanlarından toplam 45 adet toprak ve kök örneği 2017 yılının mayıs ayı sonu-haziran ayı başı arasında tahıl hasadı öncesinde alınmıştır. Toprak ve kök örneklerinden Fenwick yöntemii kullanılarak kistler toplanılmış, 37 adet örnekte (%82) kiste rastlanılmış olup, tüm örnekler ribozomal DNA'nın ITS bölgesi kullanılarak, tahıl kist nematodu *H. latipons* moleküler düzeyde tanımlanarak belirlenmiştir. Çalışmada Kuzey Kıbrıs arpa ve buğday alanlarından elde edilen *H. latipons* örneklerine ait ITS bölgesi sekans dizilerinin Fas, Rusya, Suriye ve Türkiye'den elde edilen izolatlara oldukça benzerlik gösterdiği tespit edilmiştir. Kuzey Kıbrıs'ın Gazimağusa, Girne, Güzelyurt ve Lefkoşa illeri tahıl alanlarında *H. latipons*'un ilk olarak tespit edildiği bu çalışma, nematodun yaygınlığını ortaya koyan ve zararlının mücadelesinde başta dayanıklı çeşit kullanımı olmak üzere diğer mücadele yöntemlerinin uygulanmasında temel oluşturacak verileri içermektedir.

Anahtar sözcükler: *Heterodera latipons*, heterojenite, ITS, filogenetik

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Introduction

Plant-parasitic nematodes are considered economically important biotic stress factors that cause severe damage to the global cereal production system. These nematodes are responsible for an annual global loss of $125 billion (Chitwood, 2003). The foliar symptoms associated with plant-parasitic nematodes infestation are similar to those caused by other soil borne pathogens, therefore, losses caused by plant-parasitic nematodes can be overlooked. Cyst-forming nematodes (*Heterodera* spp.) form a highly specialized group infesting cereal crops and causing economic damage to their production (Greco et al., 2002). *Heterodera* spp. are obligate sedentary endoparasites, and their life cycles depend on the invasion of root tissues of susceptible hosts. The feeding structures stimulated by the nematodes are called syncytia and provide a constant source of food for them to become reproductive females (Kyndt et al., 2013). Mediterranean cereal cyst nematode is a species that is occurs in Cyprus, Israel, Italy, Lebanon, Libya, Syria, Tunisia, and Turkey (Franklin, 1969; Saxena et al., 1988).Yellowing of cereal stands, ranging from mild to severe, have been observed in the early stage of *Heterodera latipons* Franklin, 1969 (Tylenchida: Heteroderidae) infestation, while the infested fields show patchy plant growth due to poor tillering and shorter spikes in the affected area in the later stages (Dababat & Fourie, 2018). The nematode population generally expands from the initial location increasing the number of affected plants, which results in enlargement of patches. These symptoms may be combined with other biotic or abiotic stresses that increase disease severity. Before the flowering stage, white and lemon-shaped females can be easily seen on the infested roots (Greco et al., 2002). Infested plants also tend to wilt during the warmer parts of the day (McDonald & Nicol, 2005).

More than 60 species have been described in the genus *Heterodera*, which has been extensively studied, especially those associated with cereals and grasses crops. Although many *Heterodera* species infest cereals, the most prevalent species is *Heterodera avenae* Wollenweber, 1924 which is considered a complex (*H. avenae* group) containing *H. avenae*; *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984; *Heterodera arenaria* Cooper, 1955, *Heterodera pratensis* Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000, *Heterodera aucklandica* Wouts & Sturhan, 1995, *Heterodera mani* Mathews, 1971, *Heterodera ustinovi* Kirjanova, 1969 and *Heterodera australis* Subbotin, Sturhan, Rumpenhorst & Moens, 2002 species (Wouts & Sturhan, 1995; Gabler et al., 2000; Sturhan & Krall, 2002; Subbotin et al., 2002). *Heterodera latipons* is considered to form a distinct species complex within the *H. avenae* group, which discriminated by molecular analyses (Figure 1) (Subbotin et al., 2003).

Heterodera latipons, known as the Mediterranean cereal cyst nematode, has been identified in Asia and Europe, this species, however, is predominantly distributed in the countries in the Mediterranean basin (Abidou et al., 2005; Smiley & Nicol, 2009). *Heterodera latipons* has been noted to attack several species such as; oat in Israel (Cohn & Ausher, 1973), barley in Libya (Franklin, 1969), wheat in China (Peng et al., 2007), barley and wheat in Turkey (Rumpenhorst et al., 1996; Imren et al., 2012) and Morocco (Mokrini et al., 2017). The Mediterranean cereal cyst nematode as well as the lesion nematode, *Pratylenchus thornei* Sher & Allen, 1953 (Tylenchida: Pratylenchidae*)* have been determined in Cyprus (Philis, 1988b, 1995) and are assumed to be the most damaging plant-parasitic nematode species on barley and wheat. Also, Sikora (1988) reported that the Mediterranean cereal cyst nematode could be one of the most important constraints on cereal production in the temperate semiarid regions, such as Cyprus. Significant yield loss in cereal production by this nematode has been also reported by Philis (1988a) in Cyprus. However, there was limited information on the distribution and genetic structure of the Mediterranean cereal cyst nematode in the cereal fields in the northern part of the island.

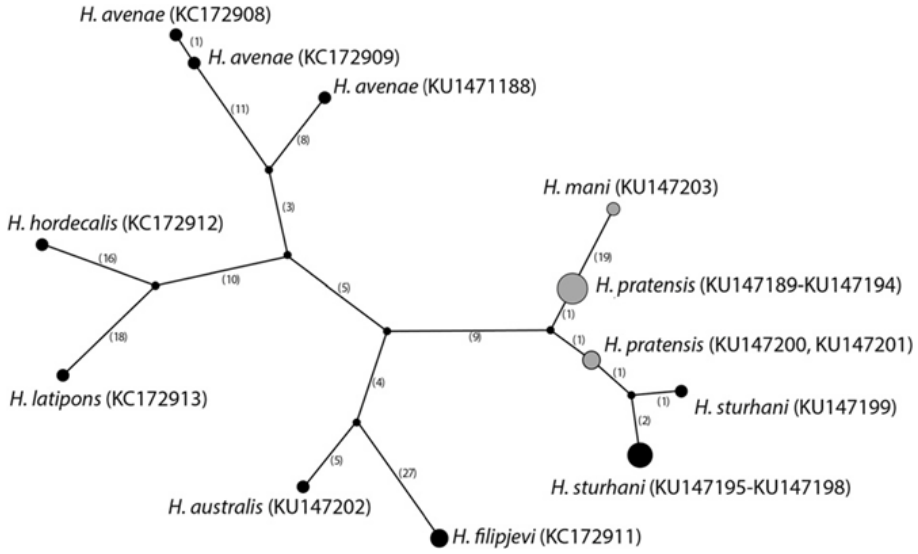

Figure 1. The phylogenetic relationship among COI haplotypes of *Heterodera avenae* group species. Small black circles represent missing haplotypes (Subbotin et al., 2003) (A number of changes are indicated in brackets).

Characterization of the cyst nematode populations molecularly at intra- or interspecies level can be vital information for the selection of appropriate and efficient management strategies (Ganguly & Rao, 2003). The increasing number of species in *H. avenae* complex has resulted in difficulties in morphological and morphometric identification and requires specialized taxonomists (Subbotin et al., 2003). Due to the minor morphological and morphometric differences within this genera complex; sufficient criteria to discriminate the species from each other would not be applicable (Subbotin et al., 1999). Therefore, molecular diagnostic techniques have provided clues to solve taxonomic problems associated with the conventional species identification (Szalanski et al., 1997; Al-Banna et al., 2004; Subbotin et al., 2010). The internal transcribed spacer (ITS) region of ribosomal DNA exhibits considerably high variations among nematode populations and is commonly used to identify species and reveal phylogenetic relationship among nematode populations at species level (Subbotin et al., 2003; Madani et al., 2004; Smiley et al., 2008). Philis (1988a) examined the morphological and morphometric characteristics of *H. latipons* obtained from Cyprus. No previous information on the genetic structure about the Mediterranean cereal cyst nematode in Cyprus is available. To fill the informative gap which is necessary to understand population structures of the Mediterranean cereal cyst nematode, surveys were conducted in the main cereal-growing areas of Cyprus. The main objectives of this study were to (a) identify nematode species using molecular tools based on their ITS sequences and (b) determine phylogenetic relationships among the nematode populations obtained in this study and representative isolates from the other countries.

Materials and Methods

Nematode populations

A comprehensive survey was carried out in 2017 for the identification, distribution, and estimation of genetic variation of *H. latipons*, from Northern Cyprus. Forty-five samples including soil and roots of plant were collected from wheat and barley fields located in Gazimağusa, Girne, Güzelyurt and Lefkoşa Provinces before the harvesting time, between the end of May and the beginning of June 2017 (Table 1). Cysts were extracted from soil and roots by flotation and decanting techniques (Fenwick, 1940). Extracted cysts were firstly classified to genus level under a V20 stereomicroscope (Zeiss, Jena, Germany). At least 20 full cysts were selected and handpicked with a needle from each sample and stored at 4°C to be used in the molecular analysis.

Table 1. List of samples with their geographical locations and *Heterodera* species identified from this study

No	Province	Location	Crop	Cyst infestation	*Heterodera* species	Accession Nos.
1	Gazimağusa	Çayönü-I	Barley	Absent	-	
2		Çayönü-II	Barley	Present	*H. latipons*	MK431040
3		Merkez	Barley	Present	*H. latipons*	MN621871
4		Türkmenköy	Barley	Absent	-	
5		Yıldırımköy	Barley	Absent	-	
6		Gelincik Iskele-I	Barley	Present	*H. latipons*	MK431035
7		Gelincik Iskele-II	Barley	Absent	-	
8		Atlılar	Barley	Absent	-	
9		Derince-I (Iskele)	Barley	Present	*H. latipons*	MK431036
10		Derince-II	Barley	Present	*H. latipons*	MK431037
11		Derince-III	Wheat	Present	*H. latipons*	MK431038
12		Birşen Iskele	Barley	Present	*H. latipons*	MN621872
13		Çetereisi	Barley	Present	*H. latipons*	MN621877
14		Gelincik	Barley	Present	*H. latipons*	MN621878
15		Tarfo	Barley	Present	*H. latipons*	MN621880
16		Yeşilköy-I	Barley	Present	*H. latipons*	MK431027
17		Yeşilköy-II	Wheat	Present	*H. latipons*	MN621870
18		Yeşilköy-III	Barley	Absent	-	
19		İncirlik	Barley	Present	*H. latipons*	MK431032
20		Kumyalı	Barley	Present	*H. latipons*	MN621875
21		Çayırova	Barley	Present	*H. latipons*	MN621876
22		Kurtuluş	Barley	Present	*H. latipons*	MN621879
23		Beyarmudu-I	Barley	Present	*H. latipons*	MN621881
24		Beyarmudu-II	Barley	Present	*H. latipons*	MN621882
25		Çanakkale Mah.	Barley	Present	-	
26		Tatlısu	Barley	Present	*H. latipons*	MK431039
27	Güzelyurt	Güneşköy	Barley	Present	*H. latipons*	MK431029
28		Meteoroloji Station	Barley	Absent	-	
29		Taşpınar-I	Barley	Present	*H. latipons*	MK431030
30		Taşpınar-II	Wheat	Present	*H. latipons*	MK431024
31		University	Barley	Present	*H. latipons*	MK431025
32		Tepebaşı-I	Barley	Present	*H. latipons*	MN621868
33		Tepebaşı-II	Barley	Present	*H. latipons*	MN621869
34		Gazievren	Barley	Present	*H. latipons*	MN621873
35		Zümrütköy	Barley	Present	*H. latipons*	MN621874
36		Aydınköy	Barley	Present	*H. latipons*	MN621883
37		Bostancı-I	Barley	Present	*H. latipons*	MN621884
38		Bostancı-II	Barley	Present	*H. latipons*	MN621885
39		Doğancı	Barley	Present	*H. latipons*	MN621886
40		Ergazi İskele	Barley	Present	*H. latipons*	MN621887
41	Lefkoşa	Yılmazköy	Barley	Present	*H. latipons*	MK431026
42		Clup Mexico	Barley	Present	*H. latipons*	MK431031
43		Mehmetçik Iskele	Wheat	Present	*H. latipons*	MK431034
44	Girne	Kormacit	Barley	Present	*H. latipons*	MK431028
45		Çamlıköy	Wheat	Present	*H. latipons*	MK431033

Molecular identification

The genomic DNA was extracted from a single mature cyst following the method described by Subbotin et al. (2001). The cyst was crushed, and the juveniles (J2s) were moved into 10 µl of double-distilled water. The J2s were homogenized via a micro-homogenizer, then the entire suspension was put into a 1.5 ml Eppendorf tube. A 10 µl of 1xPCR reaction mix [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20] and 2 µl of proteinase K (600 µg/ml; Qiagen GmbH, Hilden, Germany) were added to the lysate. The tube was exposed to incubation at 60°C for 30 min, and a further 5 min at 95°C to dispose of proteinase K. The tube was centrifuged at 16,000 rpm. The supernatant was carefully removed without disturbing the pellet, transferred into another Eppendorf tube, and stored at -20°C until further use.

PCR amplification to produce the ITS fragments of the isolates, including the 5.8S ribosomal gene as well as parts of the 18S and 28S genes, was performed with AB28 (5'-ATA TGC TTA AGT TCA GCG GGT-3') and TW81 (5'-GTT TCC GTA GGT GAA CCT GC-3') primers using a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) (Subbotin et al., 2001). PCR reactions were conducted in a 50 µl reaction mixture containing 5 µl 10× PCR reaction buffer, 0.4 µM of each primer, 2 µl template DNA, 200 µM of each dNTPs, and 1.25-unit *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA). The thermal cycler program for amplifying the ITS region was: 3 min for an initial denaturation step at 94°C, followed by 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 55°C, and 2 min extension at 72°C and a 10 min final extension at 72°C. Negative control (no DNA template) was used to ensure that there was no contamination in the reaction mix. The amplification products were evaluated on 1.5% agarose gel (100 V; 60 min) using a G: BOX F3 gel doc system (Syngene, Cambridge, UK) after ethidium bromide staining.

The PCR products were cut and eluted from the gel and purified using a QIAquick PCR purification kit (cat no 28106; Qiagen GmbH, Hilden, Germany). The purified products were subjected to bidirectional sequencing by a commercial company (Macrogen Inc., Seoul, Korea). The sequences were aligned with Clustal W (Thompson et al., 1994), which was a multiple sequence alignment method and then identified using the BLASTn algorithm on the NCBI website (NCBI, 2019). The sequences derived from this study were deposited into the GenBank database with the accession numbers shown in Table 1.

Phylogenetic analysis

The ITS sequences of the isolates were used to reveal intraspecific genomic variability of *H. latipons* populations and to determine phylogenetic relationships among themselves and the sequences of representative *H. latipons* isolates from different countries available in the GenBank database. A total of 43 nucleotide sequences was involved in the analysis. The evolutionary history was inferred using the neighbor-joining method, based on evolutionary distances computed using the Tamura-Nei method (Tamura & Nei, 1993). Gaps were treated as missing data. One sequence belonging to *Heterodera schachtii* Schmidt, 1871 isolate (accession AY166438) obtained from Belgium was used as an outgroup to root the trees and to characterize polarization. Bootstrap support was calculated for all analyses using 1000 replicates.

Results and Discussion

Heterodera latipons is one of the damaging biotic stresses reported to cause yield loss in wheat production systems around the world (Dababat & Fourie, 2018). In this study, the Mediterranean cereal cyst nematode was determined in 37 fields out of 45 cereal fields (82%) surveyed in cereal-growing areas of Northern Cyprus (Table 1). In Cyprus, the Mediterranean cereal cyst nematode was first identified by Philis (1988a) and is considered as the most destructive plant-parasitic nematode species on cereals. *Pratylenchus thornei*, root lesion nematode, was determined to often co-occurred with *H. latipons* in the same area, which can also cause substantial yield losses of barley and wheat in Cyprus (Philis, 1988a, 1995).

Figure 2. Phylogenetic tree (neighbor-joining) constructed based on the ITS sequence alignment from the 37 populations of *Heterodera latipons* including their accession numbers and strain numbers. Bootstrap values are given for the appropriate clades.

This study demonstrated that surveyed cereal-growing areas were predominantly infested with the Mediterranean cereal cyst nematode, which complies with reports that found a relatively high density of the Mediterranean cereal cyst nematode in the samples obtained from cereal fields by Imren et al. (2018) and Mokrini et al. (2017) in Turkey and Morocco, respectively.

The phylogenetic relationship among the cyst nematode populations was compared to international genotypes (Figure 2). The phylogenetic tree generated from 1000 bootstrapped sequence alignments were subjected to global rearrangement with random replications. *H. latipons* populations showed intraspecific polymorphism and were clustered into two distinct groups. The first group comprised *H. latipons* populations from Gazimagusa, Girne, Güzelyurt and Lefkosa Provinces and the second group consisted

of only isolates from Gazimagusa. Generally, the phylogenetic analyses showed that *H. latipons* isolates from Northern Cyprus were closely related to the Moroccan, Syrian and Turkish isolates. Also, Northern Cyprus cyst samples were grouped with Iran and Russia populations of *H. latipons.*

The sequence analysis of the ITS regions was frequently used for discrimination of nematode species, as well as the species of *Heterodera* genus (Subbotin et al., 2003; Baklawa et al., 2015; Imren et al., 2015; Mokrini et al., 2017). This was the first study where the intraspecific polymorphism based on the ITS sequences among *H. latipons* populations of Northern Cyprus was used. The results agree with many other studies revealing the existence of intraspecific polymorphism among *H. latipons* populations obtained from different countries (Subbotin et al*.,* 1999; Rivoal et al., 2003; Madani et al., 2004; Imren et al., 2015; Mokrini et al., 2017). Based on the results of this study, variation in nucleotide sequences observed among the nematode populations and were found to be close to Syrian and Turkish populations of *H. latipons* than Iranian and Russian populations. İmren et al. (2015) showed intraspecific differentiation between the Turkish *H. latipons* populations and the Moroccan and Syrian populations. Madani et al. (2004) and Rivoal et al. (2003) also showed intraspecific variations among populations of *H. latipons* using the PCR-RFLP method. Also, Mokrini et al. (2017) reported that *H. latipons* populations collected from different cereal-growing areas of Morocco were grouped into the same group with high similarity. In the present study, the phylogenetic tree clustered the populations of 37 distant sites at species level based on the ITS sequences as shown in Figure 2.

High prevalence of *H. latipons* was revealed in barley and wheat-growing areas of Northern Cyprus, confirming the observations that cyst nematodes species are the most important pathogens of barley, wheat and other cereals (Abidou et al., 2005; Sahin et al., 2010; Imren et al., 2012, 2015; Toktay et al., 2015). The density of these populations mostly exceeded or approached the threshold level for economic loss. CCN populations were reduced to levels below the economic damage threshold of 5 eggs/g soil under cereal monocultures (Gair et al., 1969; Dababat & Fourie, 2018). Further evaluations are necessary to determine the virulence of *H. latipons* populations to barley and wheat cultivars widely grown in Northern Cyprus and to identify the pathotypes of the *H. latipons* populations from Gazimağusa, Girne, Güzelyurt and Lefkoşa cereal-growing areas, as well as to include suitable resistance sources to cereal breeding programs. Appropriate and applicable management strategies, such as the use of resistant cultivars, fallowing and rotation with non-host crops, might be the most effective cultural methods to keep the nematode population densities below damaging levels in Cyprus. Also, several sources of resistance to cyst nematode have been identified in domestic cereals and have been recommended for inclusion in breeding programs (Dababat et al., 2015). The effectiveness of the use of resistant cultivars might not be guaranteed due to the formation of new pathotypes that show variation in the virulence, however, the cultivation of these cultivars, which have valuable traits in their genome provide resistance to populations of *H. latipons*, should be recommended.

Based on previous information, the presence of *H. latipons* in Northern Cyprus was first confirmed using molecular tools. These results demonstrated the strongest analysis to date on the existence and distribution of *H. latipons* in the main barley and wheat-producing areas of Northern Cypruss, serving a basis for more specific resistance breeding, as well as other management practices. In conclusion, an attempt was made to understand the diversity of cyst nematode species of Northern Cyprus. Additional studies are needed to explain the cyst nematode species in cereal cropping areas in Northern Cyprus.

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