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Research Article

Identification of *Heterodera latipons* Using PCR with Sequence Characterised Amplified Region (SCAR) Primers

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Abstract. The Mediterranaen cereal cyst nematode, *Heterodera latipons*, causing serious yield losses in protected cultivation cereal cropping areas in Turkey. An accurate and reliable identification is primarily needed to establish effective, sustainable and environmentally safe control measures to any cyst nematode species. In this study, twenty-five populations of *Heterodera latipons* collected from cereal areas in East-Mediterranean and South-East Anatolian regions were identified using species-specific primers (SCAR). The SCAR primer pair, H-latac F and H-latact R, defined species-specific primers were used to identify *Heterodera* populations. In this study stated that H-latac F and H-latact R primers for identifying of *H. latipons* can be efficient tools to identify the Turkish Mediterranean cereal cyst nematode populations. Moreover, the results indicated that *H. latipons* was the prominent cereal cyst nematode species in the East Mediterranean and South East Anatolian regions of Turkey. This clarified approach may supply a quick basis for identification of *H. latipons* populations for their future management and facilitate checking its distribution in the agricultural areas.

Heterodera latipons'un SCAR (Sequence Characterised Amplified Region) Primer Kullanılarak Tanımlanması

Anahtar kelimeler:
Heterodera latipons, teşhis, SCAR primer

Özet. Akdeniz Tahıl kist nematodu *Heterodera latipons,* mücadelesinin yapılmadığı buğday üretim alanlarında önemli derece ürün kayıplarına neden olabilebilmektedir. Nematodlar ile mücadelede sürüdürülebilir ve etkili bir mücadele için nematodun türünün bilinmesi oldukça önemlidir. Bu çalışmada, Doğu Akdeniz ve Güney Doğu Anadolu bölgeleri tahıl alanlarından elde edilen 25 adet *Heterodera latipons* popülasyonu, türe spesifik primer çifti (SCAR) H-latacF ve H-latactR kullanılarak tanımlanmıştır. Çalışma sonucunda, *Heterodera latipons* popülasyonlarının tanımlanmasında H-latacF ve H-latactR primerleri etmeni başarılı bir şekilde tanımlamış olup, nematodun Doğu Akdeniz ve Güney Doğu Anadolu bölgelerindeki en önemli Tahıl kisti nematod türü olduğu ortaya konulmuştur. Ayrıca, çalışmada kullanılan türe özgü primerler *Heterodera latipons* popülasyonlarının kolay ve hızlı tanımlanmasında kullanılabileceği sonucuna varılmıştır.

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INTRODUCTION

Cereal cyst nematodes (CCN) are a group of root-feeding plant-parasitic nematodes that infect many kinds of cereals used as food crops and are known to be a major constraint to wheat production as they cause significant crop losses globally (Nicol, 2002; Dababat *et al.,* 2014). The cereal cyst nematodes (CCN), *Heterodera* spp., are amongst the most economically significant phytonematodes infecting cereal in Europe, America and Asia (Cook and Noel, 2003). Twelve *Heterodera* spp. infecting cereal crops worldwide, of these species, *H. avenae* (Wollenweber), *H. latipons* (Franklin) and *H. filipjevi* (Madzhidov) Stone (Rivoal and Cook, 1993, McDonald and Nicol, 2005) have been found in Turkey and reported to cause significant yield losses (Imren and Elekcioğlu, 2015; Dababat *et al.,* 2015). The Mediterranean cereal cyst nematode, *Heterodera latipons*, is common in the Mediterranean countries including; Turkey, Cyprus, Italy, Spain, Israel, Libya and Jordan (Franklin, 1969; Cohn and Ausher, 1973; Mor *et al.,* 1992; Yousef and Jacob, 1994; Philis, 1995; Imren *et al.,* 2012; 2015).

The accurate identification and quantification of nematode species is an essential step in nematode control strategies. However, correct and rapid identification of nematode species is very significant both for breeding programs and for studying their genetic and biological variability or to prevent form global spread pathogens (Blok and Powers, 2009). *Heterodera* spp. are extremely diverse, genetically, morphologically and biologically, and therefore, its identification is complex, problematic and time consuming using classical techniques. Moreover, its identification can be mistaken in some cases when the perineal patterns have been used as the only criterion (McDonald and Nicol, 2005). Recently, novel molecular diagnostic techniques provide clues to solve taxonomic problems associated with conventional species identification (Al-Banna *et al.,* 2004; Szalanski *et al.,* 1997). Ribosomal DNA analyses are commonly accepted as alternative methods including polymorphism of restriction enzyme recognition sites (PCR-RFLP), length variation and comparative sequence analysis of rDNA array such as ITS1 and ITS2 (Cherry *et al.,* 1997; Power *et al.,* 1997; Thiery and Mugniery, 1996). Also, the species-specific primers (SCAR) have many advantages such as highly dependable, powerful, repeatable method for studying the genetic structure of populations (Thoumi *et al.,* 2015).

The aims of this research were to identify *H. latipons* populations obtained from wheat and barley growing areas in Adana, Kilis, Hatay, Gaziantep, Kilis, and Mardin provinces in Turkey using the species-specific primers (SCAR) from the last studies for *H. latipons* species identification in this region. These primers would be used to amplify the SCAR, enabling the straightforward, fast and reliable identification of *H. latipons*.

MATERIAL AND METHOD

Nematode Sampling and Extraction

Twenty-five *H. latipons* populations were obtained from wheat and barley fields of different localities; sixteen populations from the southeast area (Gaziantep, Kilis, and Mardin provinces) and nine population eastern mediternaen region (Adana and Hatay provinces) in Turkey (Table 1).

Table 1. Primers used in this study.
Çizelge 1. Çalışmada kullanılan primerler.

Primer name	Sequence (5'- 3')	Using	Reference
Hlat-actF	ATGCCATCATTATTCCTT	SCAR primer of *H. latipons*	Toumi *et al.,* 2013
Hlat-actR	ACAGAGAGTCAAATTGTG		

Nematode samples were collected from plants showing symptoms of chlorotic, yellowing leaves, and poor growth. From each field a total of ten subsamples including soil and roots were taken by Auger at a depth of 15-20 cm and then the subsamples were mixed together to form a representative sample of 2 kg. The modified sieving-decanting method (Fenwick, 1940) was used to extract cysts from soil and identified under a stereo-binocular microscope (Zeiss, Jena, Germany, V20) at 12× magnification. After the extraction, cysts were stored at 4 °C. Nematode specimens were obtained from wheat and barley fields in 2016.

Molecular Identification of Nematode

DNA extraction

Mature females (cyst) of *H. latipons* were selected for DNA extraction. A single cyst was placed into 45 µl of double distilled water (ddH_2O) in an Eppendorf tube which was then crashed by using a micro homogeniser

(Vibro Mixer). Then, the mix (40 µl) were placed into a 0.2 ml PCR tube. The lysis buffer (WLB) (10 µl) and Proteinase K (20 mg/ml) were added to the mixture and incubated at 80 ºC for at least 10 min. Incubation was performed at 65 ºC for 1 h and 95 ºC for 10 min in a thermocycler. Finally, the product was centrifuged at 14000 rpm for 1 min and stored at -20 ºC as per (Maafi *et al.,* 2003; Waeyenberge *et al.,* 2009).

PCR amplification using SCAR primer

The species-specific forward primer (Hlat-act F) and the reverse primer (Hlat-act R) (Table 2) (Toumi *et al.,* 2013) were used in the identification of *H. latipons.* The species-specific forward primers Hlac-actiF and reverse Hlac-actiF were designed based on the variable region in the alignment of the 28S rRNA expansion domains.

Table 2. List of identified *Heterodera latipons* sampled from wheat and barley fields in Agro-ecological regions of Turkey.
Çizelge 2. Türkiye'nin Agro-ekolojik bölgelerinde örnekleme yapılan buğday ve arpa alanlarından tanımlanmış Heterodera latipons listesi.

No	Province	District	Location	Species
1	Adana	Sarıçam	Giriş Gediği	*H. latipons*
2	Adana	Sarıçam	Kepeztepe	*H. latipons*
3	Adana	Sarıçam	Dutluca	*H. latipons*
4	Adana	Sarıçam	Tülüler	*H. latipons*
5	Hatay	Kırıkhan	Kurtlusoğuksu	*H. latipons*
6	Hatay	Kırıkhan	Merkez -I	*H. latipons*
7	Hatay	Kırıkhan	Merkez -II	*H. latipons*
8	Hatay	Reyhanlı	Karakaya	*H. latipons*
9	Hatay	Reyhanlı	Müşrüfe	*H. latipons*
10	Gaziantep	Karkamış	Türkyurdu	*H. latipons*
11	Gaziantep	Karkamış	Akçaköy-I	*H. latipons*
12	Gaziantep	Karkamış	Akçaköy-II	*H. latipons*
13	Gaziantep	Karkamış	Arıkdere–I	*H. latipons*
14	Gaziantep	Karkamış	Arıkdere–II	*H. latipons*
15	Kilis	Musabeyli	Haydarlar	*H. latipons*
16	Kilis	Musabeyli	Deliçay	*H. latipons*
17	Kilis	Musabeyli	Besenli	*H. latipons*
18	Kilis	Merkez	Acar	*H. latipons*
19	Kilis	Merkez	Karaöner	*H. latipons*
20	Kilis	Merkez	Yığmatepe	*H. latipons*
21	Kilis	Elbeyli	Doğanlı	*H. latipons*
22	Mardin	Nusaybin	Merkez	*H. latipons*
23	Mardin	Nusaybin	Cizre Yolu	*H. latipons*
24	Mardin	Nusaybin	Yolindi	*H. latipons*
25	Mardin	Kızıltepe	Güneyli	*H. latipons*

PCR reactions of 50 µl included the DNA template (2 µl), 21 µl ddH_2O, 25 µl 2× DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany), and 1 µM of each of the primers, Hlac-actiF and Hlac-actiR. PCR amplification was performed in a thermocycler (Bio-Rad) as follows: 95 °C for 5 mins followed by 30 cycles of 30 s at 94 °C, 45 s at 50 °C and 45 s, with a final extension at 72 °C for 10 min (Toumi *et al.,* 2013). Products of PCR were separated in 1% standard agarose gels mixed with safe stain. Molecular size was estimated by a 100 bp DNA ladder (Fermentase). The pattern of the band was imaged under UV light (Figure 1).

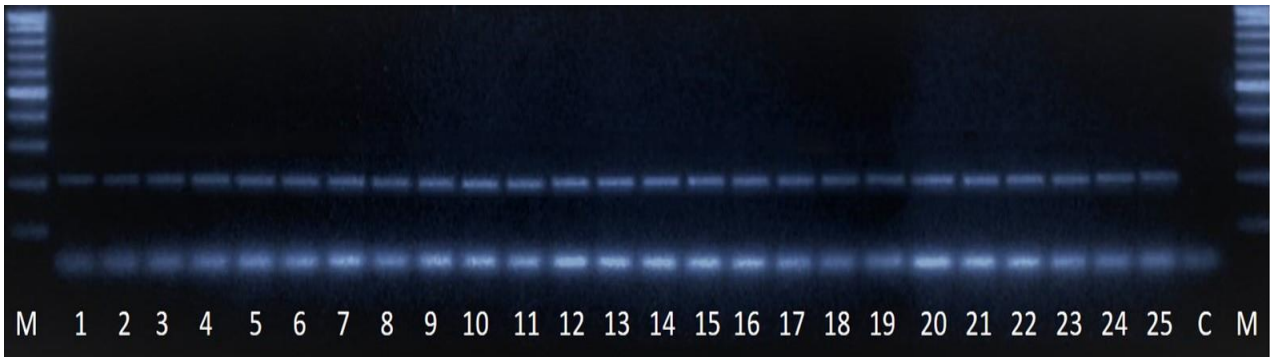

Figure 1. Agorase gel image of PCR product of *Heterodera latipons* using SCAR primers. M: 100 bp DNA ladder, C: Negative control.

*Şekil 1. SCAR primerleri kullanarak Heterodera latipons'*un *PCR ürününün agoraz jel görüntüsü.*

RESULTS AND DISCUSSION

The cereal cyst nematode species are morphologically very similar to each other and identification them to the species level is difficult (Hando, 2002). Moreover, more than one cereal cyst nematode species are sometimes can be found together on the root of the same plant. Hence, fast and precise identification of cyst nematode is needed for choosing the appropriate management strategy especially by the breeding programs (Smiley *et al.,* 2004). Description of cyst nematode populations from around the world is essential to understand the morphological, biochemical and molecular variability within a species and to recognize stable characters for their diagnosis (Rumpenhorst, 1996; Subbotin *et al.,* 2010). Among the molecular identification techniques, the species-specific primers (SCAR) is an correct and rapid identification methods of cereal cyst nematode species. So far, only two publications stated the development and use of species-specific primers for molecular identification of *Heterodera* species, *H. latipons* (Toumi *et al.,* 2013). The species-specific primers were developed by Toumi *et al.* (2013) generate bands at 204 bp. The result of this study confirmed the specificity of the primer sets.

Heterodera latipons populations were identified using the species-specific (SCAR) primers (Toumi *et al.,* 2013) in this study. Hlac-actiF and Hlac-actiR primers amplified a unique PCR product from its respective aim and did not produce an applicant from other *Heterodera* species. The *H. latipons* species-specific primers PCR (Hlac-actiF and Hlac-actiR) amplified a band of 204 bp for 25 samples (Figure 1). Moreover, these primers produced no band of DNA extracted from the other two cereal cyst nematode species; *H. avenae* and *H. filipjevi* (Figure 1). Twenty-five cyst nematode populations were molecularly identified as *H. latipons*. Our results showed agreement with earlier studies carried out by Toumi *et al.* (2013) who reported that Hlac-actiF and Hlac-actiR primers amplified a PCR product which is unique product which was not produced by other *Heterodera* species. Additionally, actin gene amplification and nematode (cyts) amplicated product were very much similar to each other. This assay revealed that the amplification of DNA with conserved actin gene primers from all studied samples of *H. latipons* confirms the reliability of PCR test in the present study.

Amplification of the actin gene regions with the current primers was successful for the studied *H. latipons* populations in the present study. This assay indicated that preserved actin gene primers amplified DNA from all individuals of the *H. latipons* populations, therefore confirming the overall reliability of the PCR for a sample tested. However, due to rDNA repeat polymorphism within the species cause difficulty to make species-specific primers (Rivoal *et al.*, 2003) and for this reason, we researched an alternative non-multi-copy DNA region such as the actin gene. Although this gene has been researched before (Matthews *et al.*, 2004; Tytgat *et al.*, 2004; Kovaleva *et al.*, 2005; Mundo-Ocampo *et al.*, 2008), it has never been used for identification objectives. In this work, we report on the development of a species-specific primer for *H. latipons* based on actin sequences.

CONCLUSION

The CCN species, *H. avenae* and *H. latipons* which cause serious yield losses in protected cereal cropping areas in Eastern Mediterranean and South East Anatolian regions of Turkey, were identified using species-specific primers in this study. Primers optimized successfully resulted in amplification of DNA obtained from all the nematode sources including, juveniles and females. The primer sets were used in our studies to identify of cereal cyst nematode.

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