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ORIGINAL ARTICLE

Characterization of cereal cyst nematodes in wheat using morphometrics, SCAR markers, RFLP, and rDNA-ITS sequence analyses

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Abstract

In this study, morphological and molecular characterizations of twenty-four *Heterodera* populations (cereal cyst nematodes, CCNs) collected from wheat production fields in Turkey were carried out. Light microscopy, species-specific markers, RFLP, and ITS sequencing were used to identify the nematode populations. The obtained CCN populations were identified as *Heterodera avenae*, *H. filipjevi*, and *H. latipons* according to the morphometric analysis, which was confirmed by the molecular techniques. The ITS region sequencing analysis confirmed the species identification, and phylogenetic analysis of this region grouped the populations with representative *Heterodera* populations from different origin countries deposited in GenBank. The simulation of four restriction enzymes, *Alu*I, *Pst*I, *BsuR*I (*Hae*III), and *Rsa*I, employed the ITS sequences of isolates to discriminate the Turkish *Heterodera* populations. ITS-RFLP patterns produced by endonuclease enzymes provided variations among *Heterodera* species. There was no intraspecific variation in populations of each *Heterodera* species in the ITS-RFLP analyses. The species-specific primers, AvenF-COI/AvenR-COI, HfF/HfR, and H-LatF/H-LatR, yielded 109 bp, 646 bp, and 204 bp products for *H. avenae*, *H. filipjevi*, and *H. latipons* populations, respectively. This is the first research to provide conclusive diagnostic tests for cyst nematode populations isolated from Turkey. These assays provide a sensitive, practical, and quick method for detecting *Heterodera* species and, therefore, have the potential to be utilized in the early identification of populations and monitoring of infestations without morphometric studies.

Keywords Diagnostics · *Heterodera avenae* group · Molecular · Morphometrics

Introduction

Small cereal grains such as rice, wheat, and corn have been an important part of human nutrition for thousands of years and have played a vital role in the formation of human civilization (Erenstein et al. 2022). More than 50% of the global daily calorie intake comes directly from grain consumption (Awika 2011). Wheat (*Triticum aestivum* L.) is an essential food crop in many countries and accounts for almost a third of the total food grain production in the world. In Turkey, wheat farming is practiced intensively and occupies an average of 8.5 million hectares (FAOSTAT 2021). Total wheat grain production reached 20 million tons in 2021, and this is 5% lower than that of 2016 production and 15% below the previous 5 years average (2012–2016). Unfortunately, this production is still inadequate and does not meet the needs of the growing population (Hoover et al. 2010). Cereal cultivation in Turkey is often subject to various restrictions, including insufficient fertilization and irrigation, soil-borne diseases, and several plant-parasitic nematodes (Shroyer et al. 1990; Dababat and Fourie 2018; Seid et al. 2021).

There are 12 cereal cyst nematode (CCN) species in the *Heterodera avenae* group, among the most important plant-parasitic nematodes hindering cereal yield worldwide (Dababat and Fourie 2018; Dababat et al. 2020; Mehalaine et al. 2020). *Heterodera avenae* Wollenweber, *H. filipjevi*

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(Madzhidov) Stelter, and *H. latipons* (Franklin) are the most damaging plant-parasitic nematodes in winter wheat, barley, oat, and rye (Subbotin et al. 2010; İmren et al. 2021). *Heterodera avenae* is widely distributed in temperate wheat-producing regions throughout the world (Imren et al. 2015; Dababat and Fourie 2018). *Heterodera filipjevi* is found in eastern and northern Europe, central and west Asia, the Middle East, the Indian subcontinent, and North America (Talatschian et al. 1976; Stoyanov 1982; Rivoal et al. 2003; Smiley et al. 2005; Toktay et al. 2015; Dababat and Fourie 2018; Özarslandan et al. 2020; Imren et al. 2021). *Heterodera latipons* occurs mainly in the Mediterranean region but also in Asia and Europe (Sewell 1973; Abidou et al. 2005; Sabova et al. 1988; Smiley and Nicol 2009; Imren et al. 2018).

Due to the similar morphology and small details that often distinguish species in the genus *Heterodera* (Turner and Subbotin 2013), the polymerase chain reaction-internal transcribed spacer-restriction fragment length polymorphism (PCR-ITS-RFLP) has been developed to differentiate between different species (Subbotin et al. 2003; Maafi et al. 2003). For example, the enzyme responsible for restriction *Pst*I strongly distinguished *H. filipjevi* from the other species of the *H. avenae* group (Subbotin et al. 1999). By comparing the patterns obtained from DNA fingerprinting with random amplified polymorphic DNA, species-specific fragments may be discovered and used to generate species-specific primers. The sequence characterized amplified region PCR analysis has been successfully utilized to differentiate the species *Globodera rostochiensis* and *G. pallida* (Fullaondo et al. 1999) and *H. glycines* (Fullaondo et al. 1999; Ou et al. 2008). *Heterodera avenae* and other cyst-forming nematodes have also been distinguished by using species-specific markers (Qi et al. 2012).

Definitive molecular diagnosis often requires sequencing the ITS-rRNA locus with universal or specific developed primers. At present, the *Heterodera* molecular diagnostic outline has been generated for 40 species, but another 40 species have not been molecularly characterized yet (Turner and Subbotin 2013). Additionally, phylogenetic trees can be constructed using molecular data to represent the historical relationship between groups of organisms or taxa, thereby reconstructing the genealogical ties between organisms, and estimating the time of divergence between them, i.e., when they last shared a common ancestor. According to Jacob et al. (2008), these trees can be based primarily on morphological differences, but with the advances made by molecular studies, it is now possible to use DNA and protein sequences to reconstruct phylogenetic relationships.

Therefore, the objectives of this study were to (i) collect data on the occurrence and distribution of CCN species in wheat fields in Hatay, Adana, Osmaniye, Kahramanmaraş, Gaziantep, Kilis, Mardin, and Bolu provinces in Turkey, (ii) conduct a morphological and morphometrical analysis of cysts and second-stage juveniles (J2) of Turkish CCN populations, and (iii) get a better knowledge of the genetic variance among members of the *H. avenae* group; the molecular features of Turkish populations will be compared to those of populations originated from different countries.

Material and methods

Sampling and nematode extraction

A total of 24 samples were collected from different wheat-growing fields located in Hatay, Adana, Osmaniye, Kahramanmaraş, Gaziantep, Kilis, Mardin, and Bolu provinces of Turkey, in 2019. Soil samples were collected at the maturity stage/harvesting time at a depth of 0–30 cm. The samples were run to extract nematodes from 100 cm^3 of each soil sample as described by Hooper (1986) with decanting and sieving. Extractions were poured onto filter paper disks to drain excess water, and then the dry extracted cysts were inspected under a Discovery V20 stereomicroscope at 20 × magnification (Carl Zeiss AG, Oberkochen, Germany) for the presence of cysts. At least 20 full cysts from each sample were collected and transferred to a refrigerator and stored at 4 °C until morphological and molecular analysis.

Morphological and morphometrical examinations

Morphological examination and diagnosis of cysts were performed to the species level with the aid of the Discovery V20 stereomicroscope at 20 × magnification. Vulval cone specimens were prepared through fixation of ten cysts per population in a formalin-glycerol fixative, then mounted on glycerol (Mulvey and Golden 1983). The prepared slides were examined under a Primo Star light microscope (Carl Zeiss AG, Oberkochen, Germany), according to Hooper (1986). Ten J2 derived from a single cyst were gently heated, fixed in triethanolamine/formalin, and embedded in glycerol to prepare permanent slides (Hooper, 1986).

The *Heterodera* populations were identified as per the previously reported morphological descriptions and diagnostic criteria of cysts and second stage juveniles (J2) (Franklin 1969; Mulvey and Golden 1983; Handoo and Ibrahim 2002). The length of the vulval slit, width of the vulval bridge, width of the fenestra, length of the fenestra, and the length of the underbridge were measured (Mulvey and Golden 1983). Additionally, the lack or presence of underbridge and bullae were performed on the cyst's perineal pattern (Handoo and Ibrahim 2002).

The body length, stylet length, distance from the anterior region to the esophagus junction, body width, distance from the anterior region to the base of the esophageal bulb, tail

length and width, and length of the hyaline portion of the tail were all measured as the most critical characters for J2 identification. The morphometrical features of J2 were also determined, including the a, b′, c, and c′ ratios. A total of ten cysts and ten J2 per population were included in the morphological measurements. Using an Axio Lab. A1 microscope (Carl Zeiss AG, Oberkochen, Germany), one cyst and 10 J2 were viewed and photographed for each population using an Axiocam ERc5s digital camera, and measurements were approximated using ZEN lite software (Carl Zeiss AG, Oberkochen, Germany).

To evaluate any meaningful difference among the populations ($P \leq 0.05$), ANOVA was used to analyze data, software SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). The standard test of means was used to detect if there were statistically significant differences in variance across populations ($P \leq 0.05$).

Molecular identification

DNA extraction and PCR For each population, one cyst was crushed, and juveniles were split into minute pieces using a sterile scalpel blade. They were hand-selected and placed in a clean PCR tube containing a 10 µl drop of 1 × PCR reaction buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, and 0.01%Tween 20. Proteinase K solution (> 600 mAU/ml, Qiagen GmbH, Hilden, Germany) was added to the mix. To inactivate the proteinase K, the tube was heated to 60 °C for 15 min and then to 95 °C for 5 min. The lysate was stored at − 20 °C for ITS sequencing and RFLP.

The universal primer sets, AB28 primer (5′-CGTAAC AAGGTAGCTGTAG-3′) and TW81 primer (5′-TCCTCC GCTAAATGATATG-3′), were used to amplify the ITS sections, including the 5.8S ribosomal gene, as well as small parts of the 18S and 28S rDNA. A PCR reaction containing 0.5 µl of nematode lysate, 2 µl of 10 × ammonium buffer (Tris–HCl pH 8.5, $(NH_4)_2S0_4$, 15 mM $MgCl_2$, 1% Tween 20), 0.5 mM primers, 0.2 mM dNTPs, and 1 unit of Ampliqon TEMPase Hot Start DNA polymerase (Berntsen, Rdovre, Denmark) was carried out in a total volume of 20 µl. Amplification reactions were carried out in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following settings: one cycle of 12 min at 94 °C, 35 cycles of 20 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C, and a final extension step of 10 min at 72 °C. Then, on a 1.6% agarose gel, the amplification products were separated. As a size reference, a 100 bp DNA Ladder (Solis BioDyne, Tartu, Estonia) was employed. A G:Box F3 gel documentation system was used to visualize and document the DNA banding profiles (Syngene, Cambridge, UK). The confirmed amplicons were sent to the Macrogen Inc. Sequencing Service (Seoul, Korea) for bidirectional sequencing.

Phylogenetic analysis The sequencing and phylogenetic analysis of the ITS sequences were run for molecular diagnosis of the cyst populations from Hatay, Adana, Osmaniye, Kahramanmaraş, Gaziantep, Kilis, Mardin, and Bolu provinces. DNA sequences were produced with primers TW81 and AB28 to identity of the cyst populations. The obtained sequences were aligned using ClustalX 2.1, a multiple sequence alignment technique with default parameters, to produce the best formation for phylogenetic connections (Thompson et al. 2000). Phylogenies based on the alignment sequences of populations from the current study and those of reference isolates from GenBank were reconstructed using Maximum-Likelihood (ML) analysis with MEGA7 (Kumar et al. 2018). To create the ML phylogeny based on the General Time Reversible model (Nei and Kumar 2000), Nearest-Neigbour-Interchange (NNI) was used as the heuristic method for tree inference with bootstrap re-sampling analysis for 1,000 replicates to estimate the confidence of tree topologies (Felsenstein 1985).

PCR–RFLP analysis To generate virtual PCR–RFLP gel electrophoresis images, SnapGene Gel Simulator is used (software from GSL Biotech; available at snapgene.com) with the sequences obtained from this study. This program processes DNA sequences with *Alu*I, *Pst*I, *Bsu*RI (*Hae*III), or *Rsa*I restriction enzymes (Subbotin et al. 2003; Maafi et al. 2003) and stimulates gel electrophoresis and produces virtual gel images. For all populations, approximately 1050 bp fragments were amplified using the primer combination of TW81 and AB28 (Joyce et al., 1994).

Species-specific primer amplification To identify *H. avenae* group populations, the species-specific primer pairs were used to amplify the species-specific products. Eight *H. avenae* individuals were used to amplify the species-specific 109 bp fragment. Each primer set contains 50 µl of PCR reaction mixes, which include 2 µl of DNA template, 21 µl ddH2O, 25 µl 2 Dream*Taq* PCR Master Mix (Thermo Fischer Scientific, Waltham, MA, USA), and 1 µM of each primer. PCR amplification using AVEN-COIF and AVEN-COIR primers was conducted with an initial denaturation step at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, followed by 72 °C for 8 min (Toumi et al. 2013). The amplification with HfF and HfR was performed in the T100 thermal cycler with the following cycling profile: 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 1 min at 72, followed by 72 °C for 8 min (Peng et al. 2013a, b). PCR amplification for H-LatF-COI and H-LatR-COI primers was done as follows: 95 °C for 5 min, then 30 cycles of 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C, with a final extension at 72 °C for 8 min (Toumi et al. 2013). The PCR products were separated on a 1.4% standard agarose gel. The consensus sequences

Table 1 *Heterodera* populations obtained in this study

Code	Province	Location	Latitude	Longitude	Accession number
Heterodera avenae					
TRHa01	Hatay	Reyhanlı	36.310383	36.527206	OL828538
TRHa02	Hatay	Kırıkhan	36.454751	36.311648	OL828539
TRHa03	Adana	Sarıçam	37.027634	35.380143	OL828540
TRHa04	Adana	Mustafabeyli	37.074361	36.049875	OL828541
TRHa05	Osmaniye	Cevdetiye	37.132272	36.189151	OL828542
TRHa06	Gaziantep	Karkamış-I	36.843499	37.983801	OL828543
TRHa07	Gaziantep	Karkamış-II	36.859871	37.978710	OL828544
TRHa08	Gaziantep	Karkamış-III	36.855904	38.008358	OL828545
Heterodera filipjevi					
TRHf01	Kahramanmaraş	Türkoğlu	37.380694	36.871222	OL828546
TRHf02	Kahramanmaraş	Afşin	38.263078	36.930661	OL828547
TRHf03	Kahramanmaraş	Elbistan	38.218263	37.271324	OL828548
TRHf04	Bolu	Gerede-I	40.762410	32.232179	OL828549
TRHf05	Bolu	Gerede-II	40.742370	32.251914	OL828550
TRHf06	Bolu	Merkez-I	40.752099	31.756046	OL828551
TRHf07	Bolu	Merkez-II	40.740176	31.708913	OL828552
TRHf08	Bolu	Yeniçağa	40.780802	32.070322	OL828553
Heterodera latipons					
TRHl01	Kilis	Musabeyli	36.886681	36.935871	OL828554
TRHl02	Kilis	Elbeyli	36.673279	37.454031	OL828555
TRHl03	Kilis	Çıldıroba	36.661350	37.400812	OL828556
TRHl04	Mardin	Kızıltepe-III	37.222543	40.583806	OL828557
TRHl05	Mardin	Nusaybin-I	37.105157	41.186467	OL828558
TRHl06	Mardin	Nusaybin-II	37.095852	41.252327	OL828559
TRHl07	Mardin	Kızıltepe-I	37.178778	40.635891	OL828560
TRHl08	Mardin	Kızıltepe-II	37.200594	40.632295	OL828561

were generated and finally used as BLAST queries against the NCBI nucleotide database. The cyst population species from Hatay, Adana, Osmaniye, Kahramanmaraş, Gaziantep, Kilis, Mardin, and Bolu provinces were compared with closely related cyst samples in GenBank. The phylogenetic tree of the cyst nematode populations from Hatay, Adana, Osmaniye, Kahramanmaraş, Gaziantep, Kilis, Mardin, and Bolu provinces is shown in Fig. 3. The phylogenetic similarity of *Heterodera* populations was also compared to international populations. Sequence alignments were used to build the phylogenetic tree, which was then reorganized globally using random replications (Table 1).

Results

Morphological and morphometrical studies

The Turkish cyst populations and J2s obtained from a single cyst for each population were identified morphologically

Table 2 The morphological characters of *Heterodera* species (all measurements are in µm, $n = 10$)

	Heterodera avenae	*Heterodera filipjevi*	*Heterodera latipons*
Body length (J2)	590.4 ± 1.8 (580.0–612.0)	501.3 ± 4.0 (468.6–540.0)	465.1 ± 10.2 (410.5–590.8)
Stylet length (J2)	26.80 ± 1.54 (23.0–28.2)	23.88 ± 0.89 (21.9–28.6)	22.23 ± 0.12 (20.9–23.2)
Tail length (J2)	76.28 ± 1.24 (70.0–86.1)	58.25 ± 2.90 (44.8–68.6)	51.28 ± 1.56 (40.8–59.3)
Hyaline length (J2)	46.8 ± 1.24 (40.0–52.34)	32.9 ± 2.08 (21.5–42.50)	24.0 ± 1.85 (18.9–26.75)
Fenestral length (cyst)	68.34 ± 1.38 (62.34–72.36)	58.28 ± 2.18 (40.43–65.73)	54.69 ± 1.48 (49.44–62.64)
Semi fenestral width (cyst)	23.18 ± 1.19 (18.60–28.50)	21.16 ± 1.34 (17.45–24.56)	19.54 ± 1.28 (16.80–25.60)
Vulval bridge width (cyst)	13.21 ± 1.04 (10.42–15.80)	12.96 ± 1.21 (8.90–14.22)	10.28 ± 1.04 (7.90–13.40)
Vulval slit length (cyst)	24.64 ± 1.25 (19.42–26.92)	21.88 ± 1.21 (18.45–23.45)	20.04 ± 1.18 (18.45–24.96)
Underbridge length (cyst)	Absent	80.75 ± 13.21 (67.45–110.24)	95.45 ± 15.27 (85.65–121.10)

using international standard descriptions and measurements. Among the Turkish populations studied, variations in cyst vulval cone, J2 forms, and dimensions were observed (Table 2). CCN populations observed in wheat fields in the provinces of Hatay, Adana, Osmaniye, Kahramanmaraş, Gaziantep, Kilis, Mardin, and Bolu were classified as *H. avenae*, *H. filipjevi*, and *H. latipons* based on these morphological traits.

The cysts of *H. avenae* had heavy prominent bullae that surrounded the vulval cone and no underbridge. *H. filipjevi* had less prominent bullae and a clear but thin underbridge when compared to *H. avenae*. *Heterodera latipons* differed from the others two Heterodera species by having a strong underbridge and a lack of distinct bullae in the vulval cone. In the current study, the cyst of *H. avenae* had heavy prominent bullae without underbridge, while *H. filipjevi* also had less prominent bullae with a slight underbridge. However, *H. latipons* having a strong underbridge without bullae in the vulval cone differed from *H. avenae* and *H. filipjevi* populations.

The *H. avenae* and *H. filipjevi* populations were bi-fenestrate, with relative semifenestral lengths ranging from 41.2 to 53.9 µl (Fig. 1), comparable to the *H. latipons* populations (Table 2). *Heterodera avenae* and *H. filipjevi* populations had slight changes in cyst vulval cone and J2 features and measures. There was a significant variation when *H. latipons* compared with *H. avenae* and *H. filipjevi*. In general, no underbridge was found in the vulval cone structures of any of the examined populations of *H. avenae,* while *H. filipjevi* cysts had a well-developed underbridge in the cyst vulval cones. However, the underbridge of *H. latipons* was strong with a few to absent bullae. *Heterodera avenae* populations had vulval bridge lengths ranging from 24.5 to 39.2 µm. In addition, the vulval cone had a small vulval slit, no underbridge, and thick bullae. With 480–576 µm and 20.5–23.5 µm, respectively, J2 body length and diameter were comparable to *H. avenae* populations (Fig. 2, Table 2). Tail length, tail diameter at the anus, and hyaline tail region length were all near to *H. avenae* population measures, ranging from 58.8 to 70.6 µm, 15.7 to 17.6 µm, and 39.2 to 46.1 µm, respectively. *Heterodera filipjevi* was also bi-fenestrate, but with slightly longer semifenestral length and breadth, ranging from 53.9 to 63.7 µm and 23.5 to 29.4 µm, respectively (Fig. 2, Table 2). The vulval slit was shorter, ranging in length from 6.9 to 10.8 µm, and an underbridge with medium development, ranging in length from 69.4 to 89.0 µm, as well as heavy bullae (Fig. 1).

The J2 body lengths of *H. filipjevi* populations were shorter, ranging from 490 to 552 µm with a diameter of 20.6 to 23.5 µm (Fig. 2, Table 2). The anal body diameter varied from 13.7 to 17.6 µm, while the tail length was substantially shorter, ranging from 49.0 to 63.7 µm with the rounded termination. The vulval bridge lengths of *H. latipons* populations ranged from 24.5 to 39.2 µm. The vulval cone has a robust underbridge as well as few bullae 480–576 µm and 20.5–23.5 µm, respectively, J2 body length and diameter were in the same range as *H. latipons* populations (Table 2). Tail length, tail diameter at the anus, and hyaline tail region length were all near to *H. latipons* population measures, ranging from 58.8 to 70.6 µm, 15.7 to 17.6 µm, and 39.2 to 46.1 µm, respectively.

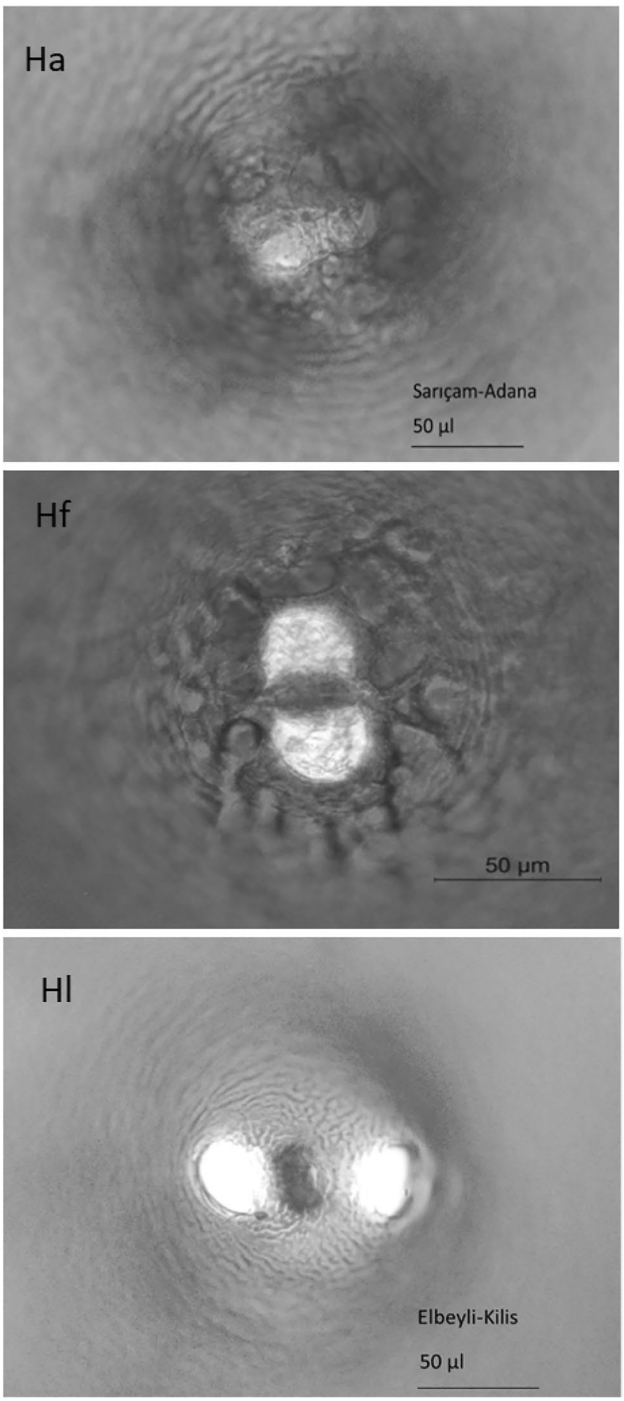

Fig. 1 Fenestral region of cyst of *Heterodera avenae* (Ha), *H. filipjevi* (Hf), and *H. latipons* (Hl) from Turkey

Molecular identification

All samples produced an expected DNA band of approximately 1050 bp. No PCR product was generated in the no-template controls. The majority of cyst populations were 99–100% identical to that cyst samples in the GenBank database. Twenty-four cyst samples were identified based on their ITS sequences as *H. avenae*, *H. filipjevi*, and *H. latipons*. All accession numbers for ITS nucleotide sequences were deposited in GenBank (Table 1).

Phylogenetic analysis Minor intraspecific variability was found in *Heterodera* species populations, which could be grouped into three major groups based on the *Heterodera* species level in the

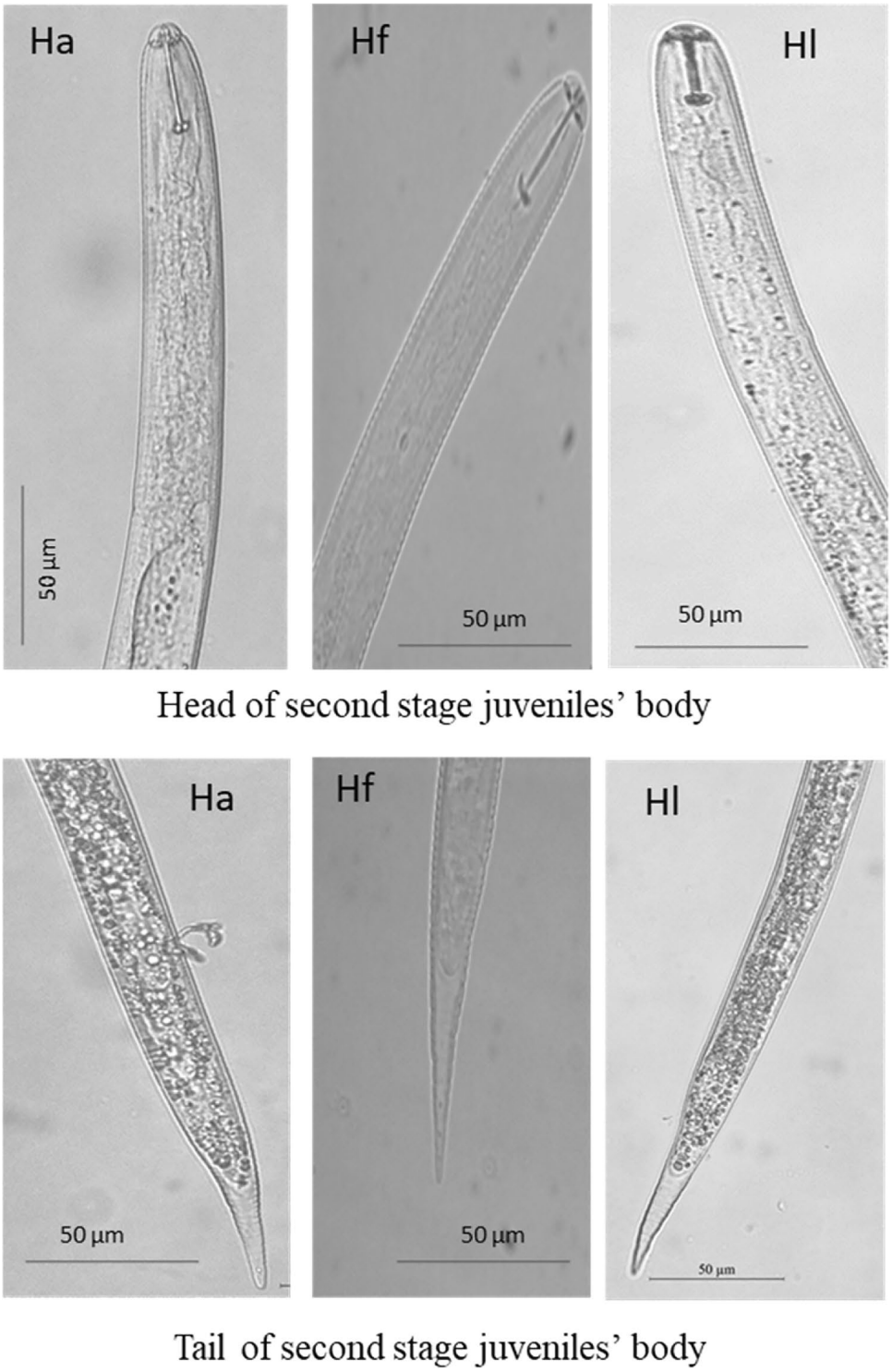

Fig. 2 Head and tail region of second-stage juveniles of *Heterodera avenae* (Ha), *H. filipjevi* (Hf), and *H. latipons* (Hl) from Turkey

phylogenetic tree and representative GenBank populations, with a reasonable bootstrap value (Fig. 3). For CCN species-level phylogenetic study, one consensus sequence was obtained from bidirectional sequences and utilized for extensive phylogenetic analysis: *H. avenae*, *H. filipjevi*, and *H. latipons*. Based on genetic variations in the ITS sequences, a phylogenetic tree grouping the CCN species was created. The first cluster of *H. avenae* included samples from the four provinces of Hatay, Adana, Gaziantep, and Osmaniye. The isolates of CCN obtained from two provinces, Kahramanmaraş and Bolu were grouped as a second *H. filipjevi* cluster. The third cluster of *H. latipons* was found in the Kilis and Mardin populations. Each *Heterodera* species exhibited a slight intraspecific polymorphism, clustering the populations into three groups in the phylogenetic tree.

PCR–RFLP analysis Differentiation of the investigated populations was possible as supported by polymorphic PCR–RFLP patterns. All the investigated species produced RFLPs after digestion with six endonuclease restriction enzymes: *Alu*I, *Pst*I, *BsuR*I, and *Rsa*I (Fig. 4). For each species population, the restriction enzymes produced identical RFLP patterns. Although no single enzyme was able to distinguish all examined species, a combination of the patterns generated by multiple separate enzymes was able to distinguish most of the species and populations studied.

Four enzymes, *Alu*I, *Pst*I, BsuRI, and *Rsa*I, allowed differentiation among the CCN species: *H. avenae*, *H. filipjevi*, and *H. latipons* (Fig. 4). The endonuclease enzyme of *Alu*I distinguished *H. avenae* (566 and 484 bp) from *H. filipjevi* (571 and 483 bp), and *H. latipons* (343, 170, 25, and 18 bp). The restriction enzyme *Pst*I clearly differentiated *H. filipjevi* (713, 211, and 130 bp) from the other two *Heterodera* species. Moreover, the endonuclease enzyme *Rsa*I separated *H. latipons* (1020 and 21 bp) from *H. avenae* (708, 320, and 21 bp) and *H. filipjevi* (707, 326, and 21 bp). Also, *BsuR*I distinguished *H. latipons* (532, 408, 77, and 24 bp) from *H. avenae* (420, 353, 176, 52, and 24 bp) and *H. filipjevi* (424, 378, 176, 52, and 24 bp).

Species-specific primer amplification The species-specific primer pairs, AVEN-COIF and AVEN-COIR, yielded specific products for Hatay, Adana, Osmaniye, and Gaziantep populations (Fig. 5). Other cyst nematode species, *H. filipjevi* and *H. latipons*, yielded no amplification with these primers. A band of DNA was created for the Bolu and Kahramanmaraş populations using the species-specific primer pairs (HfF1 and HfR1). Eight *H. filipjevi* individuals were used to amplify the species-specific 646 bp fragment, while 204 bp fragment was amplified from eight *H. latipons* individuals.

Discussion

The cereal cyst nematodes, *H. avenae*, *H. filipjevi*, and *H. latipons*, are global pests attacking cereal crops leading to a significant loss in gain quality and quantity. These nematodes are closely related and exhibit few morphological and ITS sequence variations (Bekal et al. 1997; Tamura and Nei 1993; Subbotin et al. 1999, 2003; Umarao and Vangapandu 2008; Yan and Smiley 2008).

This result is convenient with the previous study based on morphological and morphometric characteristics. It is known that the underbridge and bullae in the vulval cone are the key morphological distinctions among *H. avenae*, *H. filipjevi*, and *H. latipons*, which are consistent with previous results from other studies (Subbotin et al. 1999, 2003; Yan and Smiley 2008). The second stage juveniles of *H. avenae* have longer tail, stylet and hyaline when compared to both

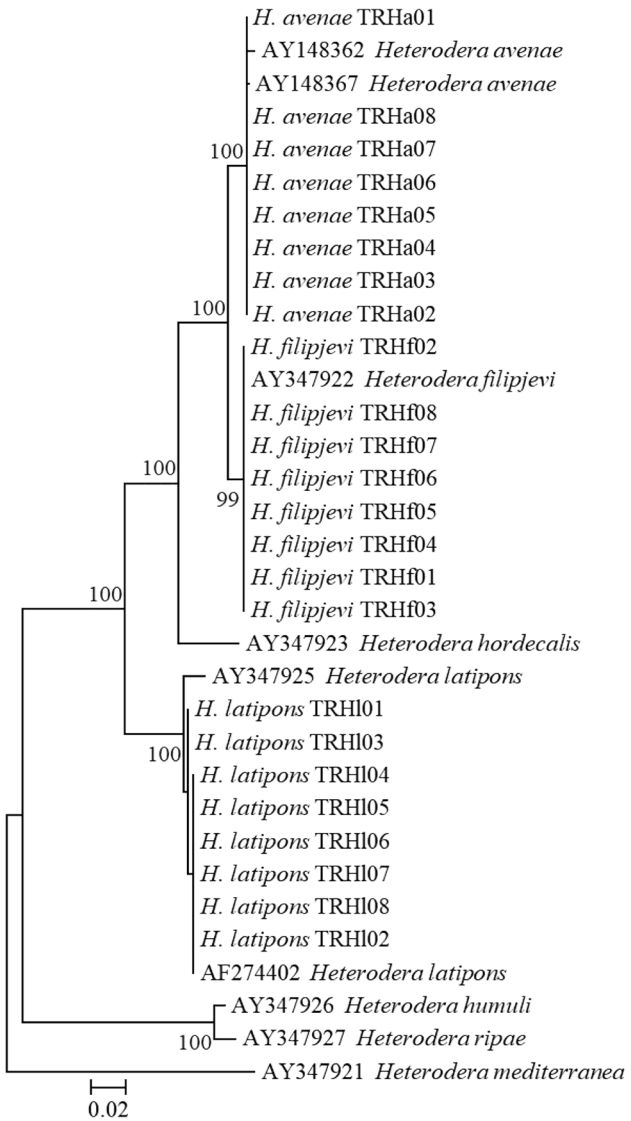

Fig. 3 Phylogenetic trees of ITS regions of *Heterodera* species obtained in this study and the closest species. Sequences were analyzed using maximum likelihood method. Numbers at nodes indicate bootstrap values. The detailed information about isolates obtained in this study is shown in Table 1

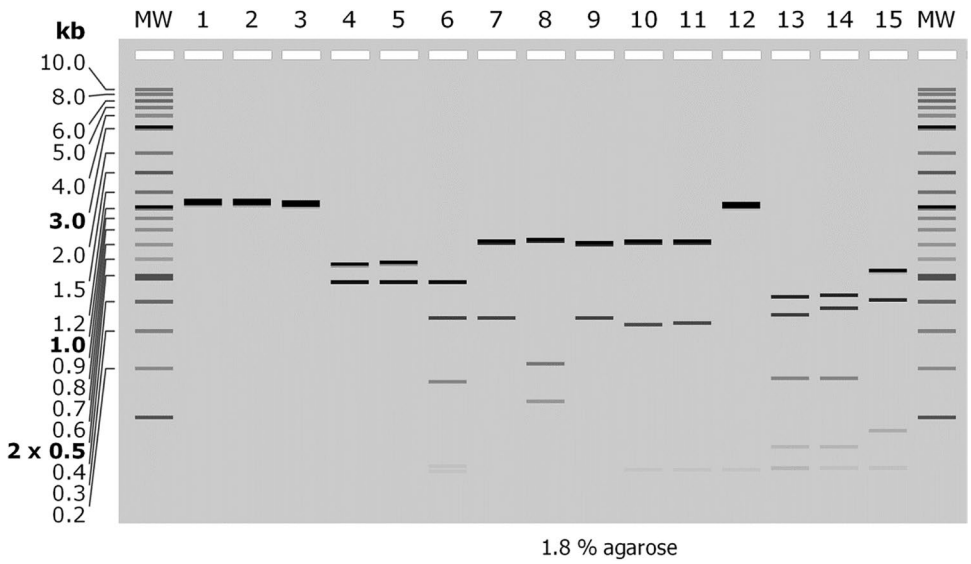

Fig. 4 Restriction fragments of amplified ITS regions of cyst forming nematodes. 1–3, PCR-ITS products; 4–6, *Alu*I, 7–9: *Pst*I; 10–12, *Rsa*I; 13–15, *BsuR*I profiles from *H avenae*, *H. filipjevi*, and *H. latipons*, respectively. MW, 1 kb Plus DNA ladder (New England Biolabs)

H. filipjevi and *H. latipons*. Besides, *H. filipjevi* has a longer tail compared to *H. latipons* which is separated from those of two *H. avenae* and *H. filipjevi* populations which have small morphological characters (Madzhidov 1981; Valdeolivas and Romero 1990; Wouts et al. 1995).

This result is consistent with that of Subbotin et al. (2003), who reported that the ITS sequence alignment of *H. filipjevi* populations from Iran and Russia clustered together with a nucleotide similarity of 100% using the minimum evolution method. Imren et al. (2012) found that *H. filipjevi* populations from Iran and Turkey were phylogenetically aggregated into a single group. The Hatay, Adana, Gaziantep, and Osmaniye populations were identified as belonging to the first cluster of *H. avenae*. The Kahramanmaraş and Bolu populations were classified into the second cluster of *H. filipjevi*, whereas Kilis and Mardin populations consisted of the third cluster of *H. latipons*. Subbotin et al. (2001) reported that different cyst nematode species were thought to be phylogenetically examined based on ITS sequences, and this region was thought to be useful in identifying species (Subbotin et al. 2001).

According to Subbotin et al. (2003), restrictions by *Alu*I and *Rsa*I separated European (type A) from Asian (type B) populations. According to Maafi et al. (2003), *Alu*I exhibited variability in the ITS region of multiple *H. avenae* populations. Turkish *H. filipjevi* was easily distinguished from other *H. avenae* and *H. latipons* using the restriction enzyme *Pst*I. According to Subbotin et al. (1999), the restriction enzyme *Pst*I clearly distinguished *H. filipjevi* from other *Heterodera* members. Also, *BsuR*I distinguished Turkish *H. latipons* from *H. avenae* and *H. filipjevi*. Maafi et al. (2003) reported that the restriction enzyme *BsuR*I distinctly separated *H. latipons* from other members of the *Heterodera* species. Our results demonstrated that the restriction profiles generated by PCR–RFLP might be used to differentiate between the Turkish populations of *H. avenae*, *H. filipjevi*, and *H. latipons*. A comparative investigation of numerous populations of *Heterodera* species should be conducted, and rDNA-RFLP can be used to distinguish species and populations.

Although PCR–RFLP can assist in species identification, however, the ones utilized to date still have some limitations. PCR–RFLP occasionally requires the use of costly restriction enzyme combinations. Due to polymorphisms and the technique's limitations, it remains difficult to differentiate

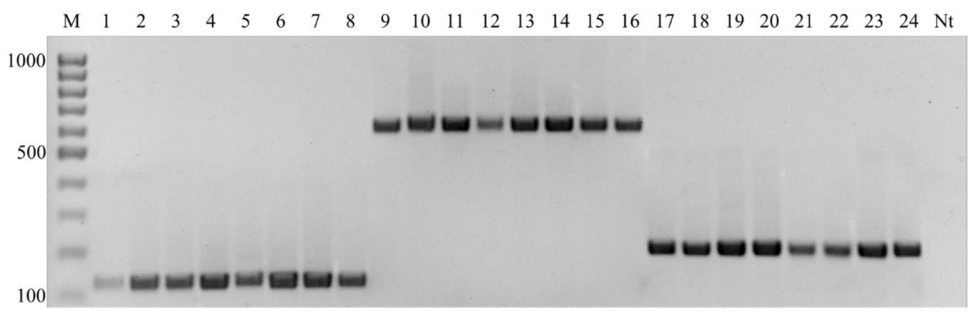

Fig. 5 The band profiles with cyst nematodes. 1–8, *Heterodera avenae*; 9–16, *Heterodera filipjevi*; 17–24, *Heterodera latipons*; Nt, nontemplate DNA. The order of the populations (from 1 to 24) in the agarose gel is as in Table 1. M, 100 bp DNA Ladder (Solis BioDyne, Tartu, Estonia), up to 1000 bp

Heterodera species; also, these procedures are time-consuming and arduous. As a result, we tested a diagnostic test based on markers to complement current detection methods. In this study, the primer pairs designed for species-specific fragments were successfully used to detect *H. avenae, H. filipjevi* and *H. latipons* populations. The primer set AvenF-COI/AvenR-COI clearly differentiated *H. avenae* from *H. filipjevi* and *H. latipons*. Toumi et al. (2013) stated that the species-specific primers AvenF-COI and AvenR-COI clearly differentiated *H. avenae* from other members of the *Heterodera*. While in our study, the primers HfF and HfR, clearly differentiated *H. filipjevi* from other members of the *Heterodera*. Peng et al. (2013a, b) reported that HfF and HfR, clearly separated *H. filipjevi* population from the other *Heterodera* species. Also, H-LatF and H-LatR primers distinguished Turkish *H. latipons* from *H. avenae* and *H. filipjevi*. Toumi et al. (2013) reported that the H-LatF and H-LatR primers distinctly separated *H. latipons* from other members of the *Heterodera* species. The findings indicated that restriction profiles formed from species-specific markers derived from RAPD fragments might be used to differentiate between Turkish populations of *H. avenae, H. filipjevi,* and *H. latipons*. A comparative investigation of many populations of *Heterodera* species should be conducted, and species-specific markers can be used to distinguish *Heterodera* species and populations.

In this study, we reported comprehensively morphological, morphometric, and molecular data set for *H. avenae*, *H. filipjevi*, and *H. latipons*. These assays, including species-specific markers, ITS sequencing, and ITS-RFLP, provide a sensitive and practical method for detecting *H. avenae*, *H. filipjevi*, and *H. latipons*, and they may be used for early identification and monitoring of *Heterodera* infestations in the field.

Author contribution The experiments were designed by A. Dababat, Ş. Yıldız, and M. İmren, and were carried out and written by D. Dağlı, N. Duman, E. Yüksel, G. Özer, and M. İmren; and M. İmren is responsible for supervision and review.

Data availability The data that support the findings of the current study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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