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RESEARCH ARTICLE

Occurrence, identification and phylogenetic analyses of cereal cyst nematodes (*Heterodera* spp.) in Turkey

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Abstract

Plant-parasitic nematodes are very common on cereal crops and cause economic losses *via* reduction in grain quality and quantity. During 2014, 83 soil samples were collected from wheat and barley fields in 21 districts of 13 provinces across five regions (Central Anatolia, Marmara, Aegean, Southeast Anatolia, and Black Sea Region) of Turkey. Cyst-forming nematodes were found in 66 samples (80%), and the internal transcribed spacer (ITS) sequencing and species-specific PCR identified the species in 64 samples as *Heterodera filipjevi*, *Heterodera latipons*, and *Heterodera avenae*. The predominant pathogenic cereal cyst nematode was *H. filipjevi*, which was found in all five regions surveyed. *H. avenae* was only detected in Southeast Anatolia whereas *H. latipons* was detected in Southeast Anatolia and Central Anatolia. ITS-rDNA phylogenetic analyses showed that *H. avenae* isolates from China clustered with *H. australis*, and Turkish isolates were closely related to European and USA isolates of this species. *H. filipjevi* from Turkey and China were clustered closely with those from the UK, Germany, Russia, and the USA. The density of many of these populations exceeded or approached the maximum threshold level for economic loss. To our knowledge, this is the first report of *H. filipjevi* in Diyarbakir, Edirne, and Kutahya provinces, and the first report of *H. avenae* in Diyarbakir Province. These results exhibit the most rigorous analysis to date on the occurrence and distribution of *Heterodera* spp. in Turkey's major wheat-producing areas, thus providing a basis for more specific resistance breeding, as well as other management practices.

Keywords: species specific PCR, cereal cyst nematode, molecular identification, ITS-rDNA, wheat pathogen

1. Introduction

The cereal cyst nematodes (CCNs, *Heterodera* spp.), first reported in 1874 in Germany, cause serious economic damage to cereal crops worldwide, especially in temperate regions (Rivoal and Nicol 2009). *Heterodera* species can reduce yields of wheat and barley, with *Heterodera avenae* Wollenweber, 1924, *Heterodera filipjevi* Madzhidov, 1981, *Heterodera latipons* Franklin, 1984, the three most

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economically important species (Smiley *et al.* 2009). The *H. avenae* complex group is comprised of eleven species: *H. arenaria*, *H. aucklandica*, *H. avenae*, *H. bifenestra*, *H. filipjevi*, *H. hordecalis*, *H. iri*, *H. latipons*, *H. mani*, *H. spinicauda*, and *H. turcomanica* (Subbotin *et al.* 1999; Subbotin *et al.* 2003). The most commonly reported species, *H. avenae*, was described as the oat cyst nematode, and is now found in more than 40 wheat-growing countries (Subbotin *et al.* 2010). In China, *H. avenae* has been shown to cause yield losses of 16–55% when population densities exceed 10 eggs g^{-1} of soil (Hou *et al.* 2012). *H. filipjevi* has been detected in 22 countries across Europe, Asia, the Middle East, and North America, whereas *H. latipons* has been detected in 27 countries throughout Europe, Asia, the Middle East, North America, and northern Africa (Subbotin *et al.* 2010). In Iran, the effect of *H. filipjevi* on wheat increased commensurately with increasing initial population (*Pi*) density: aerial shoot yields losses of 13, 21, 29, and 37%, and grain yield losses of 11, 31, 38, and 47% occurred at the *Pi* levels of 2.5, 5, 10, and 20 eggs and second-stage juveniles (J2s) g^{-1} of soil, respectively (Hajihasani and Hajihasani 2010).

Turkey is currently among the top ten wheat producers in the world, with a gross production of more than 22 million tons (http://faostat.fao.org/). *H. avenae* was first reported in Turkey by Yuksel (1973). *H. filipjevi* was subsequently also detected in Turkey in 1995 (Rumpenhorst *et al.* 1996), and found in 87% of the wheat growing areas of the Central Anatolian Plateau (CAP). Recent surveys of cereal fields in the CAP have shown that *H. filipjevi* is widely distributed in major areas of wheat and barley cultivation (Şahin *et al.* 2009), and causes average yield losses of 42% for rain-fed winter wheat grown in the CAP (Nicol *et al.* 2006). Sahin *et al.* (2009) reported that, in Turkey, the threshold density for economic impact by yield loss due to *H. filipjevi* may be in the range of 5 eggs g^{-1} of soil.

Heterodera species are traditionally identified by their cysts, as well as morphological and morphometric features of J2s, though this is difficult and requires experienced nematologists (Subbotin *et al.* 1999). The use of molecular techniques for identification is easier and more accurate, as DNA-sequence variation in the internal transcribed spacer (ITS) regions of ribosomal DNA can be used to clarify phylogenetic relationships and identify many nematode taxa (Subbotin *et al.* 2001). Similarly, the sequence characterized amplified region (SCAR) marker system can be used to rapidly detect *H. avenae* and *H. filipjevi* in mixed nematode samples with high accuracy and sensitivity (Qi *et al.* 2012; Peng *et al.* 2013). The primer set (Hlat-act), designed using Allele ID 7.73, was shown to be very specific to *H. latipons* (Toumi *et al.* 2013). SCAR-PCR amplification with species-specific primers has been used to identify several CCNs such as *H. glycines*, *H. latipons*, *H. schachtii*, *H. avenae*, and *H. filipjevi*, without requiring the subsequent restriction fragment length polymorphism (RFLP) procedure (Yan *et al.* 2013).

In this study, CCN populations from 13 provinces, over five regions in Turkey, were identified by the SCAR-PCR method, as well as by sequencing the ITS-rDNA regions. Using the ITS-rDNA sequence of the CCN populations — available in the GenBank — we performed a phylogenetic analysis comparing the Turkey CCN populations with the downloaded GenBank ITS sequences. This study aimed to: 1) survey wheat growing areas in Turkey to refine knowledge of the distribution of predominant CCN species; 2) identify CCN isolates to the species level using molecular tools; and 3) quantify cyst densities in each wheat growing area. Above all, this survey was designed to elucidate the current status of species distribution as a basis for developing CCN-management strategies.

2. Materials and methods

2.1. Nematode collection

Soil samples were taken from 83 sites in the major wheat and barley growing areas of 13 provinces (Konya, Uşak, Edirne, Kutahya, Eskisehir, Mardin, Diyarbakir, Sanliurfa, Kilis, Gaziantep, Bolu, Haymana, and Yozgat) of Turkey during August 2014. The areas surveyed covered the five major wheat-producing regions in Turkey: CAP, Marmara, Aegean, Southeast Anatolia, and the Black Sea region. One kilogram of soil was collected from each field by taking five samples in a zigzag pattern. Cysts were extracted from the soil using Cobb's sieving gravity method (Persmark *et al.* 1992). A 100-g subsample of soil was washed, and cysts were then collected with a brush. A stereomicroscope was then used to count the number of eggs from five gravid cysts. Five samples from the major wheat-producing areas in China (Beijing City and Henan, Qinghai and Shandong provinces) were used as controls.

The following equations were used to estimate the empty cyst rate and the frequency of the cyst occurrence:

Empty cysts rate (%)=Empty cysts/Total cysts×100

Frequency (%)=Samples with cysts/Total samples×100

Eggs in cysts of each sample are reported as the means and standard deviation of the mean. Data were evaluated using ANOVA to determine if number differed significantly among samples ($P \leq 0.05$).

2.2. DNA extraction

Nematode genomic DNA was extracted as described by Qi *et al.* (2012). Briefly, at least three full cysts from each subsample were transferred to a 0.2-mL microtube (one

microtube per subsample) containing 10 μL of double distilled water (ddH_2O), frozen individually in liquid nitrogen and pierced with a disinfected glass rod. 10 μL of lysis buffer (7.5 μL 10× PCR buffer and 2.5 μL of 600 g mL^{-1} proteinase K) was added to each tube. Samples were frozen at –80°C for 1.5 h and then incubated for 1.5 h at 65°C, followed by 10 min at 95°C in a PCR Thermo-cycler (Eppendorf, Germany). The samples were then centrifuged (1 000 r min^{-1}) at 4°C for 1.5 min and the supernatant was transferred into a new Eppendorf tube for storage at –20°C.

2.3. Species-specific PCR amplification

Extracted DNA (1.5 μL) was added to an Eppendorf tube containing 12.5 μL 2×*Taq* PCR StarMix buffer (Gen Star, Biotech Co., Ltd., Beijing, China), 9.0 μL ddH_2O, and 2× 1 μL of 10 μmol L^{-1} primers, to make a final volume of 25 μL. Primers TW81 and AB28 were used to amplify the ITS-rDNA region, as described by Joyce *et al.* (1994). SCAR-PCR primers HaF1 and HaR1 *vs. H. avenae* (Qi *et al.* 2012), HfF2 and HfR2 *vs. H. filipjevi* (Peng *et al.* 2013), and specific primers Hlat-actF and Hlat-actR *vs. H. latipons* (Toumi *et al.* 2013) were used to PCR amplify the specific fragments. The PCR program consisted of an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 30 s at 94°C (denaturation), and then 45 s at 56°C, 59°C, 58°C, or 50°C (the corresponding annealing temperature for ITS-rDNA/*H. avenae*/*H. filipjevi*/*H. latipons*, respectively), and 1 min at 72°C for elongation. The reaction was terminated by a final extension cycle at 72°C for 10 min. PCR products were stored at 4°C. After PCR amplification, 5 μL of each PCR product was separated on a 1.5% agarose gel.

2.4. Purification and sequencing

In accordance with the SCAR-PCR results, ITS-PCR products of the *Heterodera* populations were purified using the TIAN Gel Midi Purification Kit (Tiangen BioTech Co., Ltd., Beijing, China), as per the manufacturer's instructions. Purified products were cloned into the pGEM®-T Easy vector (Promega Biotech Co., Ltd., Beijing, China) and transformed into DH5α competent cells (Tiangen BioTech Co., Ltd., Beijing, China). Ten clones of each population were isolated using blue and white selection and confirmed using PCR, five positive clones of which were then sequenced.

2.5. Sequence alignment and phylogenetic analyses

Sequences were edited and analyzed using MEGA 5.05 (Center for Evolutionary Medicine and Informatics, Biodesign Institute, Tempe, AZ, USA; Tamura *et al.* 2011). We used the maximum likelihood evolutionary model for phylogeny reconstruction and bootstrap analysis, with 1 000 bootstrapped data sets, to determine the statistical consistency of the classification (Hall 2005). Gaps were treated as missing data. A tree clustering of the populations at different levels (based on genetic distance) was constructed from the ITS sequence alignment.

For identification purposes, sequences of 29 known, full ITS regions from different species of the *Heterodera* and *Globodera* genera were obtained from the GenBank database (Table 1), with an emphasis on representative species of the *H. avenae* group. In this study, the 27 ITS-rDNA PCR products represented different species and populations. All sequences were identified using the Blastx algorithm blasted in the NCBI nr/nt database. New sequences from this study were deposited in GenBank with the Acc. no. KR704287–KR704313, as shown in Table 2.

3. Results

3.1. Identification and distribution of *Heterodera* spp.

Cyst-forming nematodes were found in 66 of the 83 samples (80% frequency), and up to 46% of these samples contained live cysts. Using SCAR-PCR amplification, cyst populations from these 64 samples were identified as *H. filipjevi*, *H. latipons*, and *H. avenae* (Table 2). *H. latipons* was detected in two locations (3% of the positive samples) from Konya and Gaziantep provinces. Seven samples (11% of the positive samples) collected from Diyarbakir, Sanliurfa, Kilis, and Mardin provinces (all in the Southeast Anatolia region) were identified as *H. avenae*. *H. filipjevi* was found in 55 soil samples (83% of the positive samples) from across all five of the surveyed regions. The size of SCAR-PCR products was 1 010, 646, and 204 bp for *H. avenae*, *H. filipjevi*, and *H. latipons* populations, respectively. Lanes 31 and 35 were blank due to the absence of eggs in cysts (Fig. 1). These SCAR-PCR patterns coincided with the sequencing results. According to our survey, *H. filipjevi* was the predominant species in Turkey. *H. latipons* and *H. avenae* populations occurred in mixtures with *H. filipjevi*, and there were no pure, single-populations for either *H. latipons* or *H. avenae*. However, *H. filipjevi* populations were found as single populations in Haymana, Yozgat, Kutahya, Eskisehir, Uşak, Bolu, and Edirne provinces (Fig. 2).

The greatest number of eggs per cyst occurred in Konya (up to 321 eggs per cyst) and the fewest occurred in Edirne (200 eggs per cyst). Taking a conservative average of 220 eggs per cyst, the number of CCN eggs ranges between 0.4–13.9 eggs g^{-1} of soil in Turkey (Table 3). The lowest and highest density of *H. filipjevi* was 0.4 eggs g^{-1} of soil in Kutahya and 13.9 eggs g^{-1} of soil in Uşak, respectively. In addition, the *H. filipjevi* density in Uşak (13.9 eggs g^{-1} of

Table 1 Nematode species (*Heterodera* spp.) and populations downloaded from NCBI used in the phylogenetic analysis

Species	Population	Country	Accession no.	Source of data
H. arenaria	Linconshire	UK	AF274396.1	Subbotin *et al.* (2001)
H. aucklandica	Zaaren	Belgium	AY148379.1	Subbotin *et al.* (2003)
H. australis	York Peninsula	Australia	AY148395.1	Subbotin *et al.* (2003)
H. avenae	Anhui	China	HQ450289.1	Wei *et al.* (unpublished)
	Cukurova	Turkey	AY148364.1	Subbotin *et al.* (2003)
	Gaziantep	Turkey	KM199830.1	Imren *et al.* (2014)
	Hebei	China	HM560741.1	Peng *et al.* (unpublished)
	Idaho	USA	EF153843.1	Skantar *et al.* (unpublished)
	Rothamsted	UK	AY148358.1	Subbotin *et al.* (2003)
	Santa Olalla	Spain	AY148354.1	Subbotin *et al.* (2003)
	Taaken	Germany	AY148353.1	Subbotin *et al.* (2003)
	Villasavary	France	AY148374.1	Subbotin *et al.* (2003)
H. filipjevi	Akenham	UK	AY148403.1	Subbotin *et al.* (2003)
	Gimbte	Germany	AY148400.1	Subbotin *et al.* (2002)
	Jiaozuo	China	HM027889.1	Fu *et al.* (unpublished)
	K. Maras	Turkey	KM199843.1	Imren *et al.* (2014)
	Saratov	Russia	AF274399.1	Subbotin *et al.* (2001)
	Selcuklu	Turkey	AY148398.1	Subbotin *et al.* (2003)
	Xuchang	China	GU083595.1	Peng *et al.* (2010)
	unknown	USA	GU079654.1	Yan *et al.* (2010)
H. glycines	Golestan	Iran	AF498387.1	Tanha *et al.* (2003)
H. latipons	Kilis	Turkey	KM199826.1	Imren *et al.* (2014)
	Salmas	Iran	AF498382.1	Tanha *et al.* (2003)
H. mani	Hamminkeln	Germany	AY148377.1	Subbotin *et al.* (2003)
H. pratensis	Otterndorf	Germany	AY148383.1	Subbotin *et al.* (2003)
H. ripae	Ussurijskii	Russia	AF393840.1	Eroshenko *et al.* (2001)
H. schachtii	Tongeren	Belgium	EU616694.1	Ou *et al.* (unpublished)
H. ustinovi	unknown	Belgium	AY148407.1	Subbotin *et al.* (2003)
Globodera rostochiensis	British	Canada	FJ212167.1	Madani *et al.* (unpublished)

Table 2 Occurrence and SCAR-PCR identifcation results of populations of *Heterodera* spp. in Turkey

Code	Location	Province	Region	Results	Samples	Accession no.
2	Uşak Merkez	Uşak	Aegean	*H. filipjevi*	5	KR704287
5	MYO-Bay	Karaman	Central Anatolia	*H. latipons*	2	KR704288
6	MYO-Bay	Konya	Central Anatolia	*H. filipjevi*	2	KR704289
7	Hamidiye-emirdağ	Edirne	Thrace	*H. filipjevi*	3	KR704290
23	Hayrabolu Malkara	Edirne	Thrace	*H. filipjevi*	5	KR704291
39	Eskisehir-Kütahya	Eskisehir	Central Anatolia	*H. filipjevi*	4	KR704292
43	Kütahya-Eskisehir	Kutahya	Aegean	*H. filipjevi*	5	KR704293
49	Şenyurt	Mardin	South East Anatolia	*H. avenae*	3	KR704294
50	Acarköy -Mardin	Mardin	South East Anatolia	*H. filipjevi*	5	KR704295
52	Silvan	Diyarbakir	South East Anatolia	*H. avenae*	3	KR704296
54	Alabal	Diyarbakir	South East Anatolia	*H. filipjevi*	3	KR704297
57	Bozova	Sanliurfa	South East Anatolia	*H. avenae*	4	KR704298
59	Karaköprü	Sanliurfa	South East Anatolia	*H. filipjevi*	4	KR704299
63	Üçdamlar	Kilis	South East Anatolia	*H. avenae*	3	KR704300
65	Karkamış	Gaziantep	South East Anatolia	*H. latipons*	2	KR704301
66	Oğuzeli	Gaziantep	South East Anatolia	*H. filipjevi*	4	KR704302
67	Mercidabık	Kilis	South East Anatolia	*H. filipjevi*	3	KR704303
71	Çifteler-Belpınar	Eskisehir	Central Anatolia	*H. filipjevi*	3	KR704304
78	Lületaşı	Bolu	Black sea	*H. filipjevi*	7	KR704305
81	Haymana	Ankara	Central Anatolia	*H. filipjevi*	3	KR704306
82	Yozgat-Kırşehir	Yozgat	Central Anatolia	*H. filipjevi*	3	KR704307
83	Karagözler	Eskisehir	Central Anatolia	*H. filipjevi*	3	KR704308
84	Daxing	Beijing	North China	*H. avenae*	1	KR704309
85	Huangzhong	Qinghai	Northwest China	*H. avenae*	3	KR704310
86	Taian	Shandong	North China	*H. avenae*	10	KR704311
87	Xinxiang	Henan	Central China	*H. avenae*	3	KR704312
88	Yuzhou	Henan	Central China	*H. filipjevi*	3	KR704313

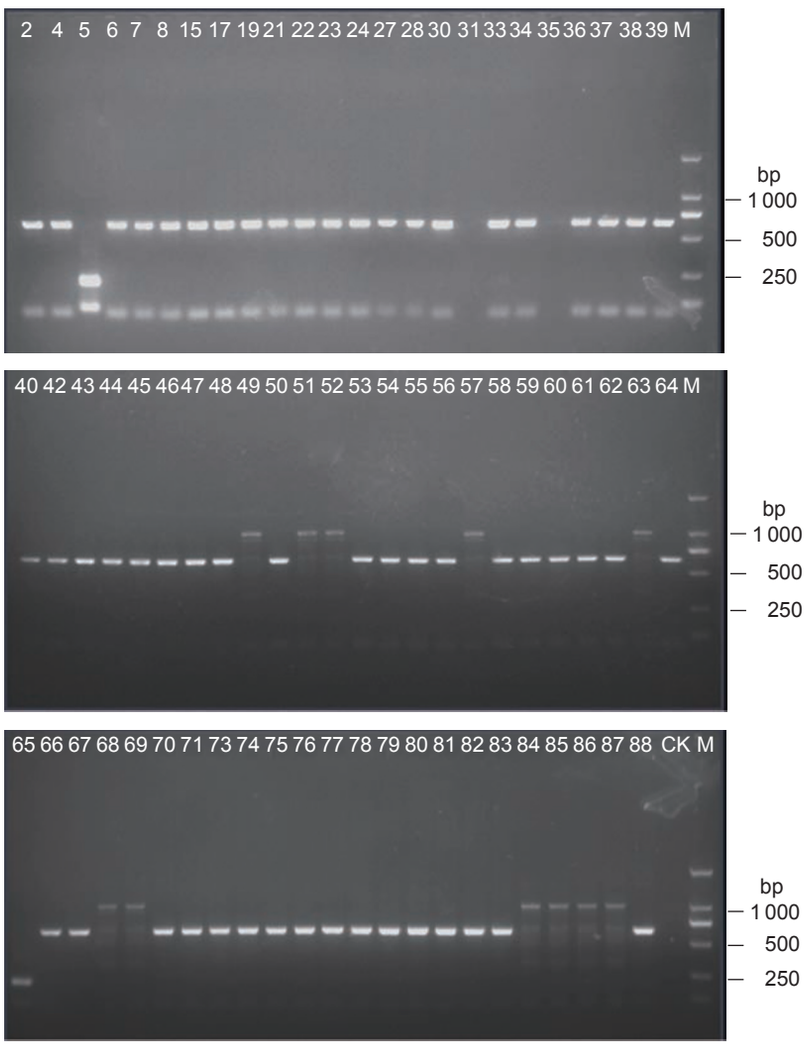

Fig. 1 PCR patterns of *Heterodera avenae* and other related isolates amplified using the sequence characterized amplified region (SCAR) primers. 2–83, cysts collected in Turkey; 84–88, cysts collected in China; CK, the negative control; M, DNA ladder DL 2000.

Fig. 2 Map of Turkey showing the occurrence and distribution of the cereal cyst nematodes belonging to *Heterodera* spp.

Table 3 Population density and frequency of occurrence of *Heterodera* spp. populations in the wheat fields of Turkey

Region	Province	Density (no. of cysts in 100 g soil)			Empty rate (%)	No. of eggs in 1 g soil
		H. filipjevi	*H. avenae*	*H. latipon*		
Aegean Region	Uşak	6.30±0.9	–	–	62.5	13.86
Black Sea Region	Bolu	3.68±0.7	–	–	58.9	8.10
Marmara Region	Edirne	2.06±0.3	–	–	40.0	4.53
Southeast Anatolia Region	Gaziantep	0.90±0.0	–	1.3±0.3	66.7	4.84
	Diyarbakir	0.75±0.3	0.80±0.6	–	43.4	3.41
	Kilis	0.97±0.0	0.70±0.0	–	66.7	3.67
	Sanliurfa	1.07±0.7	0.60±0.0	–	40.5	3.67
	Mardin	1.28±0.1	0.95±0.3	–	51.3	4.91
Central Anatolia Region	Haymana	4.50±0.0	–	–	48.1	9.00
	Yozgat	5.00±0.0	–	–	55.6	11.00
	Kutahya	0.20±0.0	–	–	70.6	0.44
	Konya	0.15±0.0	–	1.1±0.3	48.8	2.75
	Eskisehir	3.28±0.6	–	–	64.5	7.22

Data are means±standard deviation. –, not detected.

soil), Bolu (8.1 eggs g^{-1} of soil), Haymana (9.0 eggs g^{-1} of soil), and Yozgat (11.0 eggs g^{-1} of soil) exceeded the damage threshold level, i.e., in the range of 5 eggs g^{-1} of soil (Sahin *et al.* 2009). While in Edirne (4.5 eggs g^{-1} of soil), Gaziantep (4.8 eggs g^{-1} of soil), and Mardin (4.9 eggs g^{-1} of soil), the population density was nearly equivalent to the economic threshold level.

3.2. Phylogenetic analyses of *Heterodera* spp. using sequences of ITS-rDNA region amplicons

We amplified, sequenced, and aligned the ITS rDNA regions of the Turkish *Heterodera* populations, five *Heterodera* samples from China, and *Heterodera* sequences available in NCBI to analyze the phylogenetic relationships and further validate the species identification. For the 66 populations, the amplification of the ITS-rDNA region (ITS1-5.8S-ITS2) — including the flanking parts of the 18S and 28S genes — yielded a single fragment of approximately 1 000 bp. We evaluated samples from 27 geographically distant sites and clustered 55 *Heterodera* populations and one out-group (*Globodera rostochiensis*). Species with bootstrap values of over 60% are given. In this study, species within the *H. avenae* group species were clearly divided into three groups in the phylogenetic tree (Fig. 3).

Group A included the *H. latipons* isolates from Turkey (KM199826.1) and Iran (AF498382.1). They clustered together with the isolates encoded as 65 and 05 at the value of 99% distinctly different from *H. avenae* complex populations in Group B and *H. filipjevi* populations in Group C. Groups B and C were separated each other by 65 and 90%, respectively. Group B consisted of the *Heterodera* complex group, including *H. arenaria*, *H. aucklandica*, *H. mani*, *H. australis*, *H. pratensis*, *H. ustinovi*, as well as 18 *H. avenae* isolates from NCBI and this study. *H. ustinovi* formed a singleton branch, and differed from the others at a bootstrap value of 65%. The *H. avenae* isolates encoded with 49, 52, 57 and 63 collected from Turkey, and isolates from Europe and the USA, clustered together with *H. arenaria*, *H. aucklandica*, and *H. mani* as one subgroup with a bootstrap value of 99%. Four *H. avenae* isolates encoded with 84, 85, 86, and 87 collected from China, along with the *H. australis* and *H. pratensis*, were clustered in another branch as one subgroup with a bootstrap value of 87%.

All of the *H. filipjevi* populations were clustered in Group C, which included 16 isolates from this study, samples 02, 06, 07, 23, 39, 43, 50, 54, 59, 66, 67, 71, 78, 81, 82, and 83. All of these samples were collected from Turkey, except sample 88, which was obtained from Henan, China. The ITS sequences for seven *H. filipjevi* isolates from UK, Germany, China, Turkey, and USA were downloaded from GenBank. The isolates collected from Mardin and Diyarbakir were in a subgroup separated from the other *H. filipjevi* populations, with a bootstrap value of 98%. *G. rostochiensis* was positioned independently as an out group.

4. Discussion

This study represents the latest information on the occurrence and distribution of CCNs in Turkey's major wheat-producing areas, i.e., the CAP, Thrace, and Southeast Anatolia regions (Dababat *et al.* 2014). Imren *et al.* (2015) reported the distribution and diversity of CCNs in a limited area of the Mediterranean region (Adana, Osmaniye, Kahramanmaraş, Hatay, Gaziantep, and Kilis provinces) of Turkey. Of these isolates, 75% were identified as *H. avenae*, 15% as *H. latipons*, and 10% as *H. flipjevi*. Toktay *et al.* (2015) reported the occurrence and population densities of CCNs from the East Anatolian region, and identified *H. filipjevi* and *H. latipons*. *Heterodera filipjevi* was the dominant species

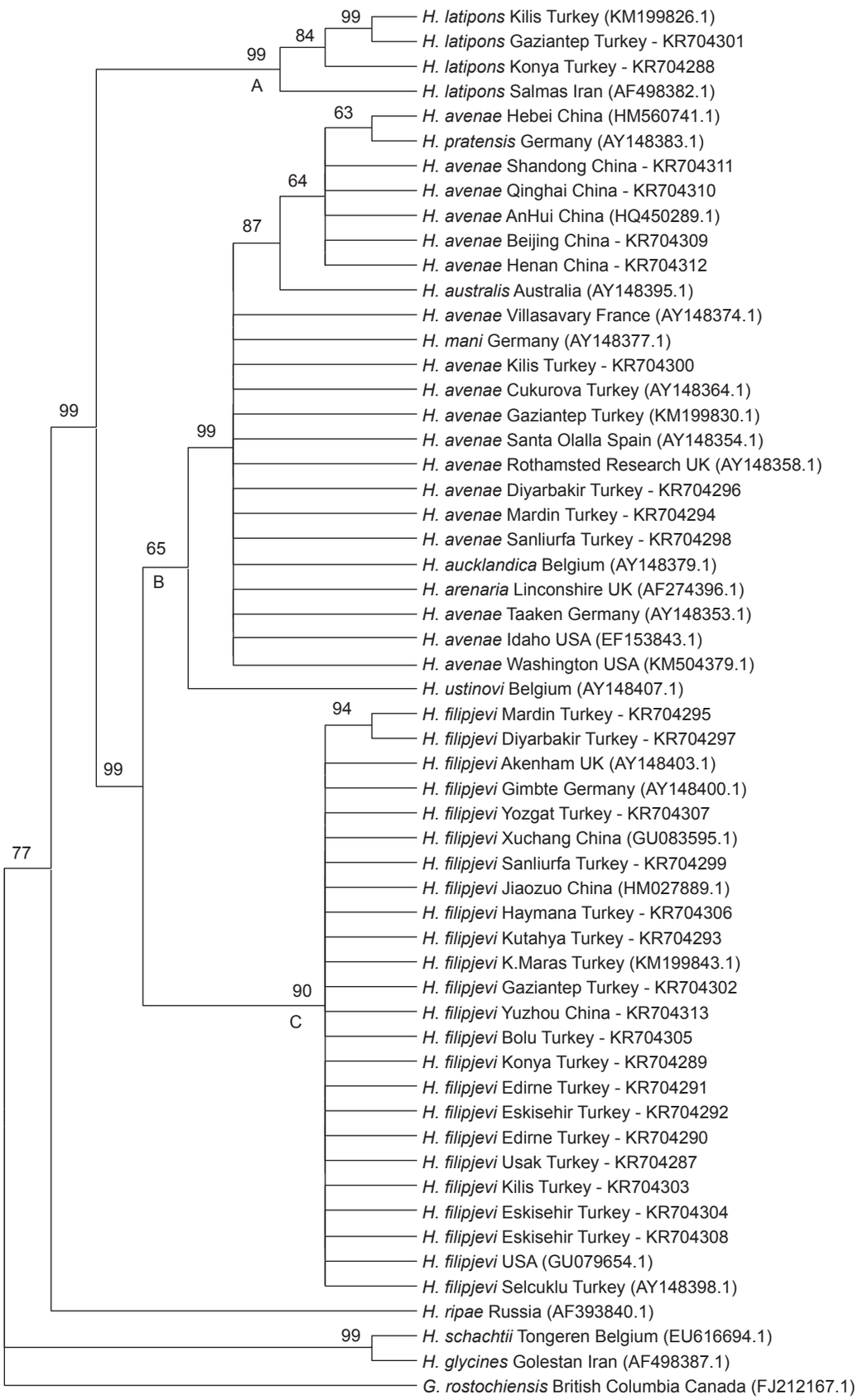

Fig. 3 Phylogeny reconstruction tree (maximum likelihood) of the internal transcribed spacer (ITS) sequences from 47 cyst-forming nematodes (*Heterodera* spp. and *Globodera rostochiensis*). Bootstrap values (more than 60%) are given.

in the wheat fields of Elazig, Malatya, Sivas, Erzurum, Erzincan, Igdir and Kars provinces, while *H. latipons* was found only in the three provinces of Erzincan, Elazig and Malatya. *H. filipjevi* is the most widely distributed of the CCNs in Turkey; in this study, we found its prevalence in five regions of Turkey andprevalent in the CAP region (Şahin 2009). However, in the cereal fields of the Southeast Anatolia region, *H. latipons* was the prevalent species (Imren *et al.* 2012). This species has been reported near Hatay, Kilis, Osmaniye, Adana, Konya, Afyon, Eskisehir, Ankra, Adıyaman, and Kahramanmaras provinces (Abidou *et al.* 2005; Imren *et al.* 2012, 2015). To our knowledge, this is the first report of *H. filipjevi* in Diyarbakir, Edirne, and Kutahya provinces, and the first report of *H. avenae* in Diyarbakir province. It is therefore particularly important to be able to identify individual *Heterodera* species in Turkey. Wang *et al.* (2012) reported that agricultural machinery operations, like combine-harvester and rotary cultivator, might be the primary means of distributing CCN. The developed sets of SCAR primers were successfully used in straightforward, fast and reliable PCR assays to identify *Heterodera* spp. (Ou *et al.* 2008). This study endeavors to provide the first systematic analysis of CCN genetic variation between Turkish isolates and CCNs from other wheat-producing nations.

Maximum likelihood and Bayesian inference, in general, outperformed neighbor joining, maximum parsimony and parsimony analyses in terms of tree reconstruction accuracy (Hall 2005; Ogden and Rosenberg 2006). Samples tested in this study were partitioned into three groups. Group A contained all sequences and isolates of *H. latipons*, which was consistent with the phylogenetic analysis results obtained by Tanha Maafi *et al.* (2003) and Imren *et al.* (2012, 2015), who showed that the *H. latipons* from Syria, Iran, and Turkey were all closely related. Baklawa *et al.* (2015) reported that the *H. avenae* complex clustered into one branch, with a bootstrap value of 100%. Subbotin *et al.* (2003) reported that European, Asian, and African isolates of *H. avenae* formed several groups based on phylogenetic analysis of the ITS-rRNA gene and PCR-ITS-RFLP. The phylogenetic trees obtained in this study are consistent with previously published results. All members of *H. avenae* species complex (*H. arenaria*, *H. aucklandica*, *H. mani*, *H. australis*, *H. pratensis*, and *H. avenae*) clustered together, and showed only minor morphological and morphometric differences, similar to the reports by Clapp *et al.* (2000) and Subbotin *et al.* (2003).

Turkish populations of *H. avenae* had a much closer relationship with the European and American populations than that of China. Subbotin *et al.* (2003) reported a cluster of *H. filipjevi* from UK, Spain, Russia, and Turkey in one branch, based on a simple matching similarity measurement estimated using RAPD markers. Tanha Maafi *et al.* (2003) proposed that the ITS sequence alignment of *H. filipjevi* isolates from Iran and Russia (Acc. no. AF274399) could be clustered together with the nucleotide identity of 100%, using the minimum evolution method, and Imren *et al.* (2012, 2015) reported that *H. filipjevi* populations from Iran and Turkey could cluster with a bootstrap value of 99%. In this study, all the *H. filipjevi* isolates clustered in Group C with a high bootstrap value of 90%. Our phylogenies generally agree with morphological groupings of cyst-forming nematode species, as well as with previous phylogenies based on their coevolution with host plants.

We analyzed the *Heterodera* spp. distribution across most of the wheat-producing regions in Turkey. The degree of crop loss caused by CCN is closely related to the number of eggs and J2s per gram of soil in infested fields before planting. A density of 5 eggs g^{-1} soil caused a loss of about 10% in yield of wheat *cv*. Bayonet under the experimental conditions (Fisher and Hancock 1991). According to Imren *et al.* (2014), *H. avenae* significantly suppressed grain yield of wheat, resulting in yield losses of 4–26% in Turkey. The relationship between nematodes and a host in the field vary with location and with stress conditions. Damage is largely determined by crop rotation and chemicals used for soil treatments (Barker and Olthof 1976). Our investigation showed that the CCN density in fields sampled in Uşak Bolu, Eskisehir, Uşak, Haymana, and Yozgat provinces all exceed the damage threshold, and the densities in Mardin, Gaziantep, and Edirne were nearly equal to the damage threshold. These results indicate the need to increase the vigilance on CCN population dynamics in Turkey to prevent important economic losses of wheat yields. Many different control methods such as rotational schemes, chemical control, cultivation of resistant varieties, and biological control are available and necessary, their combined effects should target decreasing and maintaining CCN population density below economic threshold levels.

5. Conclusion

The present study exhibits the latest information on the occurrence and distribution of CCN in Turkey's major wheat-producing areas. *H. filipjevi* is the preponderant species and distributed widely in Turkey. ITS-rDNA phylogenetic analyses showed the different isolates of *H. filipjevi* were clustered closely, *H. avenae* isolates from China clustered with *H. australis*, and Turkish populations were closely related with European and American isolates. What's more, it's the first report about *H. filipjevi* in Diyarbakir, Edirne, and Kutahya provinces, and *H. avenae* in Diyarbakir Province. The density of CCN eggs in some provinces in Turkey exceeded the damage threshold level. The farmers, agronomists, and nematologists should coop-

erate to prevent important economic losses of wheat yields.

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