# OCCURRENCE AND IDENTIFICATION OF CEREAL CYST NEMATODE, *HETERODERA FILIPJEVI* (NEMATA: HETERODERIDAE), IN BOLU PROVINCE OF TURKEY

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## ABSTRACT

İmren, M., H. Toktay, H. Kutuk, and A. A. Dababat. 2016. Occurrence and identification of cereal cyst nematode, *Heterodera filipjevi* (Nemata: Heteroderidae) in Bolu Province of Turkey. Nematropica 46:154-161.

Cereal cyst nematodes, *Heterodera filipjevi*, parasitize the roots of wheat and cause significant yield losses around the world. Cereal cyst nematodes are one of the most important and endemic problems in wheat-growing areas especially where rainfall is limited and monoculture crop production practices exist. In Turkey, cereal cyst nematodes are considered among the most damaging pests in wheat. Identifying cyst nematode species and determining their prevalence and distribution in Turkey are important for predicting potential spread in the future. This survey aimed to identify cyst nematode species and to assess their prevalence in cereal growing areas of Bolu province in Turkey. Cereal cyst nematode cysts were found in 83% of the surveyed fields. Prevalence of cysts was greater in cereal-growing areas in the provincial center and in the Gerede district. Morphological features of second-stage juveniles and cysts were examined, and DNA was extracted from second-stage juveniles and the species-specific Sequence Characterized Amplified Region (SCAR) primers were used for molecular identification. Based on both morphological and molecular methods, all samples were identified as *H. filipjevi*. This study was the first comprehensive investigation of *H. filipjevi* prevalence in cereal fields in Bolu. This information is of value to alert personnel of wheat breeding programs to develop local resistant varieties to target *H. filipjevi*.

*Key words:* cereal, cyst nematodes, molecular identification, morphological characters.

## RESUMEN

İmren, M., H. Toktay, H. Kutuk, y A. A. Dababat. 2016. Presencia e identificación del nematodo quístico de los cereales, *Heterodera filipjevi* (Nemata: Heteroderidae) en la provincia de Bolu, Turquía. Nematropica 46:154-161.

El nematodos quístico de los cereales, *Heterodera filipjevi*, parasita las raíces del trigo y causa pérdidas importantes de cosecha a nivel mundial. Los nematodos quísticos de los cereales son uno de los problemas más importantes y endémicos de las áreas productoras de trigo, especialmente cuando el agua de lluvia es limitada y se realiza una producción en monocultivo. En Turquía, los nematodos quísticos de los cereales se incluyen entre las plagas más dañinas para el trigo. La identificación de las especies de nematodos quísticos así como la determinación de su prevalencia y distribución en Turquía son importantes para predecir su potencial expansión en el futuro. Esta prospección pretende identificar las especies de nematodos quísticos de los cereales y determinar su prevalencia en las áreas productoras de cereales en la provincia de Bolu, Turquía. Se encontraron nematodos quísticos de los cereales en un 83 por ciento de los campos prospectados. La prevalencia de quistes fue mayor en las áreas cerealistas del centro de la provincia y en el distrito de Gerede. Se examinaron las características morfológicas de los juveniles de segundo estadío y de los quistes, se extrajo DNA de los juveniles de segundo estadío y se utilizaron cebadores (SCAR) para la identificación molecular. De acuerdo con ambos métodos morfológicos y moleculares, todas las muestras

fueron identificadas como *H. filipjevi*. Este estudio constituye la primera investigación completa sobre la prevalencia de *H. filipjevi* en campos de cereales en Bolu. Esta información es de valor para alertar al personal de mejora de trigo sobre la necesidad desarrollar variedades resistentes locales frente a *H. filipjevi*.

*Palabras clave:* cereal, nematodos quísticos, identificación molecular, caracteres morfológicos.

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is cultivated worldwide and is the second most important food crop in the developing world after rice (FAOSTAT, 2015). Wheat is vulnerable to numerous plant pathogens that adversely affect its production (Nicol, 2002). Cereal cyst nematodes (CCN), including *Heterodera filipjevi*, are highly specialized soil-borne parasites that infect many cereals and cause significant crop losses globally (Nicol, 2002; Dababat *et al*., 2014).

The cyst nematode genus, *Heterodera*, has 70 species, including a complex of 12 species known as the *Heterodera avenae* group (McDonald and Nicol, 2005). Species in this group include: *H. avenae*, *H. filipjevi*, and *H. latipons*, which cause the most important economic losses in small grain crops (Rivoal and Cook, 1993; McDonald and Nicol, 2005; Dababat *et al*., 2014; 2015). *Heterodera avenae* is widely distributed in temperate wheat-producing regions throughout the world (Nicol, 2002; Smiley and Nicol, 2009), while *H. filipjevi* is found in eastern and northern Europe, Central and West Asia, the Middle East, the Indian subcontinent, and North America (Rivoal *et al.*, 2003; Smiley *et al*., 2005). *Heterodera latipons* occurs primarily in the Mediterranean region, eastern and northern Europe, the Middle and Near East, North and South Africa, Asia, and North America (Nicol, 2002; Abidou *et al*., 2005; Smiley *et al*., 2005).

Turkey is currently the tenth largest wheat producer in the world with a gross production of 22 million tons produced in over 78 million ha in 2015 (FAOSTAT, 2015). Previous studies indicated that the most economically important cyst nematode species, *H. avenae*, *H. filipjevi*, and *H. latipons* are present in Turkey and restrict wheat production (Dababat *et al*., 2015). *Heterodera avenae* was the first cyst nematode species reported in wheat fields in the eastern Anatolian region of Turkey (Yuksel, 1973). Later on, it was reported as widely distributed in the Eastern Mediterranean and South Anatolian regions as well as in the Thrace and Aegean regions (Abidou *et al*., 2005; Dababat *et al.*, 2015; Imren *et al*., 2015). Both *H. avenae* and *H. latipons* were found as mixed populations in the same wheat and barley fields in Eastern Mediterranean and South Anatolian regions (Imren *et al*., 2012; 2015). *Heterodera filipjevi* has economic importance on winter wheat in central and eastern Anatolian region where it causes significant yield loss (Rumpenhorst, 1996; Abidou *et al*., 2005; Sahin, 2010; Toktay *et al.*, 2015). The occurrence and distribution of cyst nematodes in wheat fields in the Black Sea region including Bolu Province have not been documented. The objectives of this study were to survey for occurrence and distribution of the CCN in Bolu Province and to identify the CCN species which were found using both morphological and molecular diagnostic tools.

## MATERIALS AND METHODS

### *Soil sampling and extraction of cysts*

The survey was conducted in cereals fields just after harvesting the crops in Bolu Province between August and September 2014-2015 growing season. A total of two kg of soil consisting of 5 to 7 subsamples was collected from each field and stored at 4°C until nematode extraction. A total of 145 cereal fields located in five districts (Central Area, Gerede, Dörtdivan, Yenicag, and Mudurnu) of Bolu Province were surveyed in June and July just prior to harvesting in 2014-2015 (Fig. 1).

After mixing the soil, a 250-$cm^3$ subsample was used for nematode extraction by employing a modified sieving-decanting method (Fenwick, 1940). Cysts collected on the 250-µm sieves were counted and handpicked with a dissecting needle using a stereo-binocular microscope (Zeiss, Jena, Germany, V20) at 12× magnification. Cysts were sterilized with 1% NaOCl for 10 min and rinsed 3 times with sterilized tap water. Sterilized cysts were stored in a refrigerator at 4°C before molecular and morphological identification. The frequency of occurrence (prevalence = number of fields with cyst /total number of fields surveyed) and incidence of the nematode (number of samples with cyst/total number of samples) were calculated for each field.

### *Morphological and molecular identification*

Second-stage juveniles ($J_2$) and cysts were used to measure morphological features and to calculate morphometric ratio (De Grisse, 1969; Handoo, 2002). Identification was performed under a microscope (Leica DM5000 B, Germany) based on underbridge structure, shape of semifenestra in the

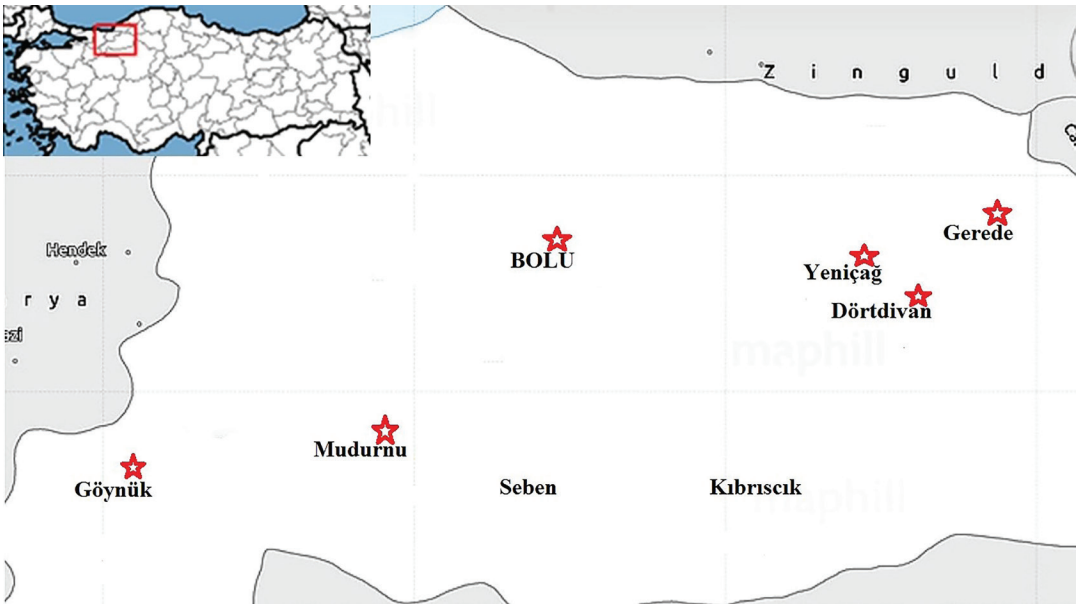


Fig. 1. A map of Bolu Province showing the four surveyed regions

Table 1. Sequence Characterized Amplified Region (SCAR) primers used to identify *Heterodera filipjevi* from nematode survey samples in the Bolu Province of Turkey.

| Primer name | Sequence (5'- 3') | Reference |
|---|---|---|
| HfF2 (Forward) | CAGGACGAAACTCATTCAACCAA | Peng *et al.* (2013) |
| HfR2 (Reverse) | AGGGCGAACAGGAGAAGATTAGA | |

fenestral area and development of bullae of cyst (Handoo, 2002). The length of the second-stage juvenile body, stylet, tail, hyaline tail, stylet knob shape, lateral lines, and tail terminus shape were measured (Subbotin *et al*., 1996). The morphometric features such as the a, b, b', c and c' ratios of the $J_2$, the length of cysts excluding the neck (L'), the width of cysts (B), and the L'/B ratio were also determined (Handoo, 2002; Subbotin *et al*., 1996; 2003).

DNA was extracted from one cyst from fields where species were collected. A single cyst was transferred to an eppendorf tube (0.5 ml) containing 80 µl $ddH_2O$. The cyst was crushed manually, and the content was further disturbed using a micro-vibromixer for 30 sec. Using a micropipette containing 40 µl $ddH_2O$, one to five $J_2$ were transferred to a PCR tube (0.2 ml). The lysis buffer [50 mM KCl, 10 mM Tris pH 8.0, 1.5 mM MgCl2, 1 mM DTT, 0.45% Tween 20 (Sigma, UK)], and 10 µl proteinase K (600 µg/ml) were added to each tube. Tubes were centrifuged at 13,500 rpm for 2 min at 25°C, then placed at -80°C for 10 min, incubated at 65°C for 1 h, followed by 95°C for 10 min. The samples were kept at - 20°C until use (Waeyenberge *et al.*, 2000). Sequence Characterized Amplified Region (SCAR) PCR primers for *H. filipjevi* (HfF2 and HfR2) were used to PCR-amplify the specific fragments (Table 1). The PCR program consisted of an initial denaturation step at 95°C for 4 min followed by 35 cycles of 30 sec at 94°C (denaturation), 45 sec at 58°C and 1 min at 72°C for elongation. The reaction was terminated by a final extension cycle at 72°C for 10 min. The PCR product was stored at 4°C for later use. After PCR amplification, 5 µl of each PCR product was mixed with 1 µl of 6× loading buffer (Fermentas Life Science, St. Leon-Rot, Germany) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis (100 V for 40 min), the gel was stained with ethidium bromide (0.1 µg/mL) for 15 min and visualized and photographed under UV light. The remaining PCR product was stored at –20°C (Subbotin *et al*., 2003).

Data for cysts in different zones of Bolu Province and eggs in cysts are presented as the mean and standard error of the mean for each sample. Data were analysed according to standard analysis of variance procedures using SPSS 10.0 program for Windows (SPSS Inc., Chicago, IL, USA). Differences among samples were tested using one-way analysis of variance (ANOVA) followed by the

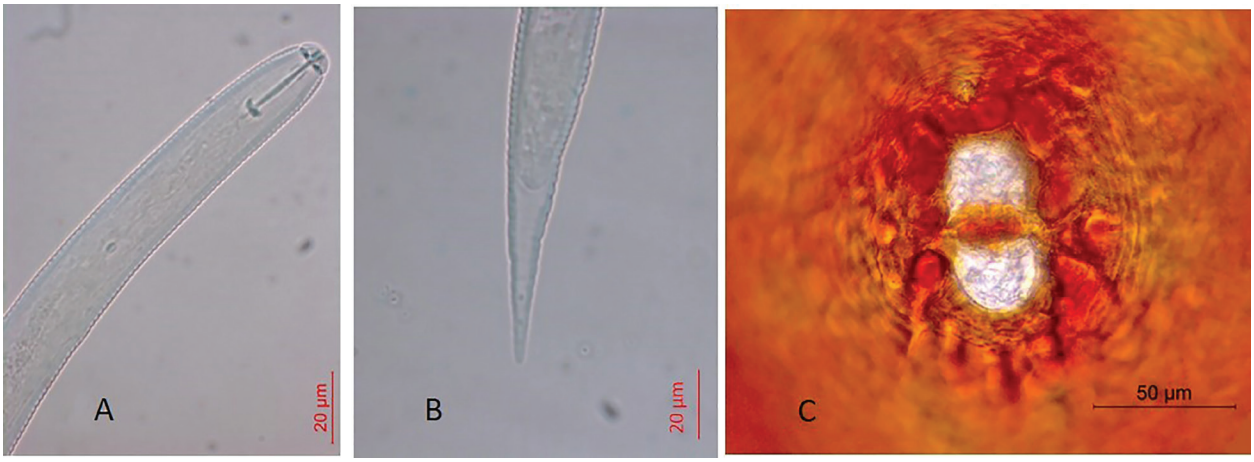


Fig. 2. Second-stage juveniles and vulval patterns of *Heterodera filipjevi*. A) head region showing the head, stylet, and basal knobs, B) tail region, and C) vulval cone

Duncan Test for mean comparison if the F-value was significant ($P < 0.05$).

## RESULTS

### *Prevalence and density of cyst population*

Both empty and cysts containing eggs were counted in all samples, and the field infestation was determined based on the presence of egg-containing cysts. Cysts were extracted from 120 samples (83%) and of those, 39 samples were empty. The remaining 81 samples contained eggs. Nematode population densities were low in most fields, and 48 of the infested fields (33%) had less than 1 egg per gram soil. An additional 21 fields (14%) had between 1 and 6 eggs per gram soil. Only 12 fields (8%) had more than 6 eggs per gram soil.

Nematode population densities were higher in Gerede and the provincial center than in the Dörtdivan, Mudurnu, and Yenicag Districts (Table 2). Among 120 samples, egg-containing cysts were found in 36 samples, and all were identified as *H. filipjevi* by morphological and molecular methods (Table 3). This species was widespread in the Gerede District, and the incidence was lower in Mudurnu (Table 2).

### *Morphological and molecular identification*

Cysts were found in 36 samples and identified as *H. filipjevi* by morphological (vulval cone pattern identification) and molecular identification methods (PCR amplification of sequence characterized amplified region (SCAR) primers). Second-stage juvenile body length ranged from 509-630 µm, and the tail tapered to a rounded tip. The head was offset and usually had three annules. The distance from the head to the valves of the median bulb ranged from 68.25-79.60 µm (Fig. 2A). The length of the tail was 58.7-65 µm; the hyaline part of the tail was 31.2-39.2 µm long (Table 3), with hyaline tail of more than 50% of the total tail length (Fig. 2B). The ratio of hyaline tail to the true tail was 1.49-2.08. The lateral fields contained four lines, of which the inner two were more distinct, and the outer bands were heavily areolated. The stylet was robust, measuring 21-25.9 µm in length with anchor-shaped basal knobs.

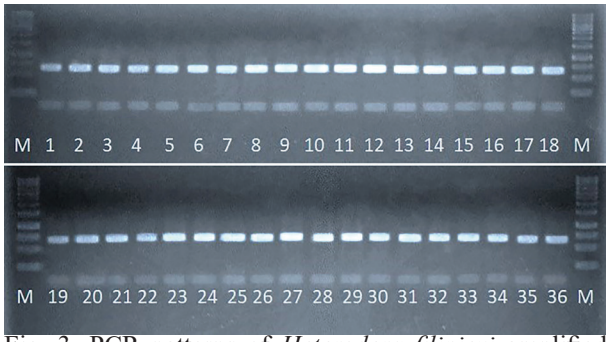


Fig. 3. PCR patterns of *Heterodera filipjevi* amplified using SCAR primers. M is DNA marker DL 1000.

The cysts were light brown and lemon-shaped, with a zigzag pattern on the cyst wall and a bifenestrate vulval cone with horseshoe-shaped semifenestra with few bullae (Fig. 2C). The cysts were also characterized by body length (575-801 µm), body width (476-610 µm), fenestra length (42.3-57.8 µm) and width (17.8-29 µm), slight underbridge (72.24-80.16 µm), vulval slit (6.5-18.6 µm), vulval bridge length (19-40) and vulval bridge width (10-13 µm) (Table 3).

Species specific SCAR primers HfF2/HfR2 produced a 646 bp product for *H. filipjevi* (Fig. 3). One cyst nematode from Bolu Province gave equivocal results with SCAR primers. The SCAR

Table 2. *Heterodera filipjevi* found in soil samples collected in the Bolu Province of Turkey.

| | | | Cysts in 250 g soil | | | | Coordinates | |
|---|---|---|---|---|---|---|---|---|
| No. | District | Host | Egg-containing | Empty | Total | Juveniles/g soil | Latitude | Longitude |
| 1 | Gerede | Wheat | 5 | 3 | 8 | 7 | 40 47' 16 N | 32 12' 13 E |
| 2 | Gerede | Wheat | 3 | 1 | 4 | 5 | 40 46' 41 N | 32 12' 18 E |
| 3 | Gerede | Wheat | 10 | 5 | 15 | 13 | 40 46' 18 N | 32 12' 12 E |
| 4 | Gerede | Wheat | 12 | 4 | 16 | 18 | 40 45' 08 N | 32 12' 25 E |
| 5 | Gerede | Wheat | 37 | 3 | 38 | 52 | 40 44' 06 N | 32 10' 35 E |
| 6 | Gerede | Wheat | 10 | 3 | 13 | 15 | 40 42' 15 N | 32 08' 47 E |
| 7 | Gerede | Wheat | 5 | 2 | 7 | 8 | 40 44' 12 N | 32 11' 04 E |
| 8 | Gerede | Wheat | 10 | 3 | 13 | 15 | 40 43' 06 N | 32 11' 51 E |
| 9 | Gerede | Barley | 8 | 1 | 9 | 12 | 40 44' 31 N | 32 11' 04 E |
| 10 | Gerede | Wheat | 16 | 1 | 17 | 24 | 40 46' 14 N | 32 09' 37 E |
| 11 | Gerede | Wheat | 16 | 3 | 19 | 24 | 40 44' 58 N | 32 08' 08 E |
| 12 | Dörtdivan | Barley | 32 | 3 | 35 | 48 | 40 44' 43 N | 32 07' 43 E |
| 13 | Dörtdivan | Wheat | 24 | 2 | 26 | 36 | 40 44' 24 N | 32 06' 45 E |
| 14 | Dörtdivan | Oat | 8 | 1 | 9 | 12 | 40 44' 31 N | 32 11' 04 E |
| 15 | Dörtdivan | Wheat | 6 | 7 | 13 | 9 | 40 41' 34 N | 32 03' 34 E |
| 16 | Dörtdivan | Wheat | 24 | 10 | 34 | 32 | 40 43' 45 N | 32 03' 34 E |
| 17 | Yeniçağ | Barley | 8 | 3 | 11 | 12 | 40 45' 47 N | 32 14' 18 E |
| 18 | Yeniçağ | Barley | 6 | 8 | 14 | 8 | 40 46' 20 N | 32 59' 11 E |
| 19 | Yeniçağ | Wheat | 4 | 2 | 6 | 6 | 40 47' 44 N | 32 00' 14 E |
| 20 | Yeniçağ | Wheat | 7 | 6 | 13 | 10 | 40 45' 05 N | 31 44' 56 E |
| 21 | Provincial center | Barley | 6 | 2 | 8 | 9 | 40 45' 32 N | 31 45' 07 E |
| 22 | Provincial center | Wheat | 34 | 4 | 38 | 50 | 40 45' 01 N | 31 44' 48 E |
| 23 | Provincial center | Wheat | 12 | 5 | 17 | 16 | 40 45' 17 N | 31 44' 26 E |
| 24 | Provincial center | Wheat | 9 | 4 | 13 | 13 | 40 44' 55 N | 31 44' 36 E |
| 25 | Provincial center | Wheat | 16 | 5 | 21 | 24 | 40 44' 50 N | 31 44' 09 E |
| 26 | Provincial center | Barley | 6 | 3 | 9 | 9 | 40 45' 06 N | 31 43' 47 E |
| 27 | Provincial center | Barley | 5 | 4 | 9 | 8 | 40 46' 02 N | 31 43' 10 E |
| 28 | Provincial center | Wheat | 7 | 3 | 10 | 10 | 40 45' 56 N | 31 42' 49 E |
| 29 | Provincial center | Wheat | 6 | 5 | 11 | 9 | 40 45' 26 N | 31 40' 51 E |
| 30 | Provincial center | Wheat | 12 | 4 | 16 | 16 | 40 44' 57 N | 31 40' 20 E |
| 31 | Provincial center | Wheat | 6 | 8 | 14 | 9 | 40 43' 15 N | 31 41' 24 E |
| 32 | Provincial center | Barley | 14 | 6 | 24 | 20 | 40 43' 18 N | 31 40' 28 E |
| 33 | Mudurnu | Wheat | 3 | 5 | 8 | 4 | 40 49' 20 N | 31 11' 54 E |
| 34 | Mudurnu | Wheat | 2 | 4 | 6 | 3 | 40 29' 03 N | 31 10' 55 E |
| 35 | Mudurnu | Wheat | 2 | 6 | 8 | 6 | 40 28' 48 N | 31 10' 17 E |
| 36 | Mudurnu | Wheat | 4 | 1 | 5 | 6 | 40 27' 13 N | 31 09' 38 E |

primers identified *H. filipjevi* with the highest frequency (64.5%) in all localities.

## DISCUSSION

*Heterodera filipjevi* is one of the major causes of yield loss in winter wheat in Central Anatolian Plateau in Turkey (Nicol and Rivoal, 2008). In this study, the cyst nematode, *H. filipjevi*, was identified in 120 cereal production fields, and it was commonly found in wheat-growing areas of Bolu Province (Fig. 1). This cyst nematode species was detected for the first time by Rumpenhorst *et al.* (1996) in Turkey. Our surveys indicated that cereal-growing areas surveyed were commonly infested by *H. filipjevi*. This cyst nematode species was found in many of the samples at relatively high densities. Similar results were reported by Abidou *et al.* (2005) and Sahin (2010) who detected *H. filipjevi* at high densities in Central Anatolian Plateau. Intensive surveys for *H.*

Table 3. Morphopmetrics of cysts, con-top and $J_2$ of *Heterodera filipjevi* from Bolu Province, Turkey [Measurements in micrometers (µm)].

| Trait | Mean ± Std Error | Min-Max Values |
|---|---|---|
| Cyst (20) | | |
| Length | 707. 85 ± 67.75 | 575 - 801 |
| Width | 512. 05 ± 59.45 | 476 - 610 |
| Length / Width | 1.38 ± 0.1 | 1.20 - 1.31 |
| Vulva Slit length | 13.32 ± 4.14 | 6.5 - 18. 6 |
| Fenestral length | 50.54 ± 4.98 | 42.3 - 57.8 |
| Fenestral width | 24.85 ± 2.99 | 17.8 – 29 |
| Vulval bridge width | 11.35 ± 0.90 | 10 - 13 |
| Vulval bridge length | 33.06 ± 6. 17 | 19 - 40 |
| Under bridge (Present) | | |
| Under bridge length | 76.28 ± 3.8 | 72.24 - 80.16 |
| Second-Stage Juvenile (20) | | |
| Body length | 557. 05 ± 37.11 | 509 - 630 |
| Labial region height | 5.28 ± 0.61 | 4.4 - 6.6 |
| Labial region diameter | 10.46 ± 0.73 | 9.4 - 11.4 |
| Stylet length | 22. 51 ± 1. 02 | 21 - 22.9 |
| Stylet knobs width | 5. 79 ± 0.59 | 5.2 - 9.8 |
| DGO | 4.02 ± 0,3 | 3.6 - 4.8 |
| Distance anterior end to | 72. 14 ± 1.24 | 68. 25 - 79. 60 |
| median bulb (Mb) | 100. 40 ± 0,75 | 97 - 107 |
| Excretory pore | 22. 52 ± 1.4 | 19 - 23.8 |
| Body diameter at mid-body | 17.03 ± 2.28 | 13.6 - 19.8 |
| Body diameter at anus | 62.5 ± 2.01 | 58.7 - 65 |
| Tail length | 35. 43 ± 2. 60 | 31.2 - 39. 2 |
| Hyaline region (H) | | |
| Lateral Lines (4) | | |
| a | 24.09 ± 2.33 | 20. 84 - 28. 64 |
| b | 4. 72 ± 0.6 | 3.2 - 6.6 |
| c | 8.9 ± 0.6 | 7.99 - 9.69 |
| c' | 3.73 ± 0.54 | 2.96 - 4.78 |

*avenae* and economic thresholds of host crops under field conditions have been performed throughout the world (Nicol *et al*., 2002). Therefore, studies on the population dynamics under winter wheat conditions are necessary to estimate the economic thresholds of cereals to *H. filipjevi* in Turkey as well as in Bolu Province.

The morphological characters of several *Heterodera* species are very similar to each other, and there has been considerable disagreement on taxonomic classification and identification of *Heterodera* species due to morphological variations within species. The vulval cone pattern technique and morphology of $J_2$ have been suggested as the most definitive way to characterize *Heterodera* spp. (Handoo, 2002). However, the vulval cone patterns are only useful when cysts are present in the crop, and $J_2$ morphology likewise is only useful when $J_2$ are present in a sample. In addition, sometimes more than one species of *Heterodera* could be found in the same plant root or soil. Therefore, the rapid and accurate identification methods for cyst nematodes are needed for management and breeding studies (Subbotin *et al.*, 1999). Molecular information can be used as a complement to morphological data in the identification process (Subbotin *et al*., 2003).

This is the first report of morphological and molecular identification of CCN in Bolu Province of Turkey. Our morphological results are in agreement with previous studies of the cysts of *H. filipjevi* (Handoo, 2002; Holgado *et al*., 2004; and Smiley *et al.*, 2008). Moreover, $J_2$ stylet length and morphology of knobs, tail length, and hyaline tail terminus length are similar to previous studies (Handoo, 2002; Subbotin *et al*., 2003; Holgado *et al.*, 2004; Smiley *et al*., 2008), and the offset head with three annules, and the distance from the head to the valves of the median bulb (Holgado *et al.*, 2004) agree with previous reports.

*Heterodera filipjevi* was successfully identified using species-specific SCAR primers. It is believed that these primers are very well-suited for identification of the nematodes in the sampled area in Turkey. These primers successfully amplified their expected fragment from single individual stages of CCN from only 1/30 of the DNA extract. Similar results were found by Cui *et al.* (2016) who identified some cyst nematode populations from Turkey and China. Moreover, Peng *et al.* (2013) has also suggested that SCAR primers HfF2/HfR2 could be used to identify cyst nematode population of *H. filipjevi*.

Cyst nematodes species, *H. avenae*, *H. filipjevi*, and *H. latipons* are the most important pathogens of wheat and other cereals in Turkey (Abidou *et al*., 2005; Sahin *et al*., 2010; Imren *et al*., 2012; 2015; Toktay *et al*., 2015). This study revealed the regional incidence of *H. filipjevi* in the wheat-growing areas of Bolu Province in Turkey. Further investigations are necessary to identify suitable resistance sources to be used in cereal breeding programmes for this region.

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