Journal of Plant Diseases and Protection
https://doi.org/10.1007/s41348-020-00306-0

ORIGINAL ARTICLE

Plant-parasitic nematodes on cereals in northern Kazakhstan

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Received: 2 May 2019 / Accepted: 12 February 2020

Abstract
Plant-parasitic nematodes (PPNs) are considered serious damaging on the global cereals production systems. The current study was conducted to evaluate the incidence of PPNs in the main cereal-growing areas in northern Kazakhstan. PPNs were detected in about 90% of 78 soil samples and thirteen genera were identified, including *Pratylenchus*, *Heterodera*, *Geocenamus*, *Ditylenchus*, *Helicotylenchus*, *Rotylenchus*, *Pratylenchoides*, and *Tylenchorhynchus*. Out of the 78 samples, 32 samples were found infested by *Heterodera filipjevi* based on the morphological and molecular analysis. To our knowledge, this is the first report on this cereal cyst nematode species in northern Kazakhstan. During the morphological and molecular assays, intraspecific polymorphism was observed within *H. filipjevi* populations and the populations divided into at least two groups. The highest frequency of infestation of *H. filipjevi* (76%) was recorded from Kokshetau Province when compared to other provinces: Astana (50%), Petropavl (37%), and Kostanay (16%). The highest number of cysts (30.4) was found among Astana samples while the lowest number of cysts (18.2) was recorded from Kostanay samples. Cyst nematodes can maintain their population above the economic threshold as stimulated by the cereal monoculture system (mainly wheat) which is similar to the cereal production systems of northern Kazakhstan.

Keywords *Heterodera* spp. · ITS region · Plant-parasitic nematodes · Taxonomy · Wheat

Introduction

Bread wheat (*Triticum aestivum* L.) is the most important crop of many countries, contributing nearly one third of the total global food grain production (FAOSTAT 2019). Kazakhstan is one of the largest wheat-producing countries with an average annual grain production of 14 million tons over a total area of 12 million ha in 2013–2017. Wheat-growing area exceeds 80% of the total cultivated area in Kazakhstan (Abugalieva and Pena 2010; Abugaliyeva and Morgounov 2016). However, the country average wheat yield is still far below that of the international wheat yield average (Abugaliyeva and Morgounov 2016). Plant-parasitic nematodes are considered one of the most relevant biotic constraints limiting cereal production worldwide (Dababat et al. 2015; Dababat and Hendrika 2018). PPNs have been overlooked in many countries around the world due to lack of expertise and funding. Globally, the crop losses value caused by nematodes is estimated at $157 billion per annum (Abad et al. 2008). Among the PPNs, the cereal cyst nematodes (CCNs) on wheat are the most widely studied genera and have been reported from many countries (Cook and Noel 2002; Handoo 2002; Nicol et al. 2003; Subbotin et al. 2010; Dababat et al. 2014, 2015; Dababat and Hendrika 2018).

Identification of cereal cyst nematodes is a complex process and traditionally done based on comparative morphology and diagnostic keys (Luc et al. 1988; Handoo 2002). Techniques relied on variations of protein or genomic DNA

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Published online: 03 March 2020

of nematodes facilitate the species identification and provide phylogenetic information (Bekal et al. 1997; Subbotin et al. 2003). To date, 12 species and intraspecific CCN pathotypes infecting cereals and grasses have been identified in *H. filipjevi*, *H. latipons*, and *H. avenae* populations being the most damaging on cereals (Rumpenhorst et al. 1996; McDonald and Nicol 2005; Imren et al. 2012, 2015; Haddadi and Mokabli 2015; Mokrini et al. 2017). CCN has highly heterogeneous in their virulence toward certain wheat (*Triticum* spp.), barley (*Hordeum vulgare* L.), and oat (*Avena sativa* L.) genotypes (Cook and Noel 2002; Dababat et al. 2015; Dababat and Hendrika 2018). To achieve effective control of CCNs reduction in the population below the economic threshold is needed, which requires fundamental studies on nematode population dynamics and virulence assays with representative local cultivars under natural field conditions. Cultural practices based on rotational combinations with non-host plants, use of resistant cultivars, and clean fallow can effectively control these nematodes.

Several studies were carried out between 1960 and 1985 to evaluate the diversity of PPNs in soil of various crops in different territories of Kazakhstan (Balbaeva and Chinasilov 1981). CCNs were reported from different territories mainly based on their morphology (Kirjanova and Sagitov 1975; Kirjanova et al. 1976; Subbotin et al. 2003, 2010). No information about the variation of their morphometrics and genetics exists. This research was conducted to explore the occurrence of PPNs in the major wheat- and barley-growing areas in northern Kazakhstan including Astana, Kokshetau, Petropavl, and Kostanay provinces. The main objectives of this study were to: (a) prove the incidence of major genera of PPNs, (b) identify and compare both cysts and second-stage juveniles (J2s) of CCN populations using morphological, morphometrical, and molecular approaches, and (c) determine the phylogenetic relationships among these CCN populations.

Materials and method

Sampling and extraction of PPN

The survey was conducted in 2018 just at the maturity stage and harvesting time of cereals in growing areas from Astana, Kokshetau, Petropavl, and Kostanay provinces in northern Kazakhstan (Fig. 1). Almost all the surveyed wheat and barley fields displayed stunted patches of poor plant growth, chlorotic lower leaves, and few or no productive tillers. A total of 78 cereal fields in the four provinces were sampled. Soil samples were collected in a zigzag pattern with a

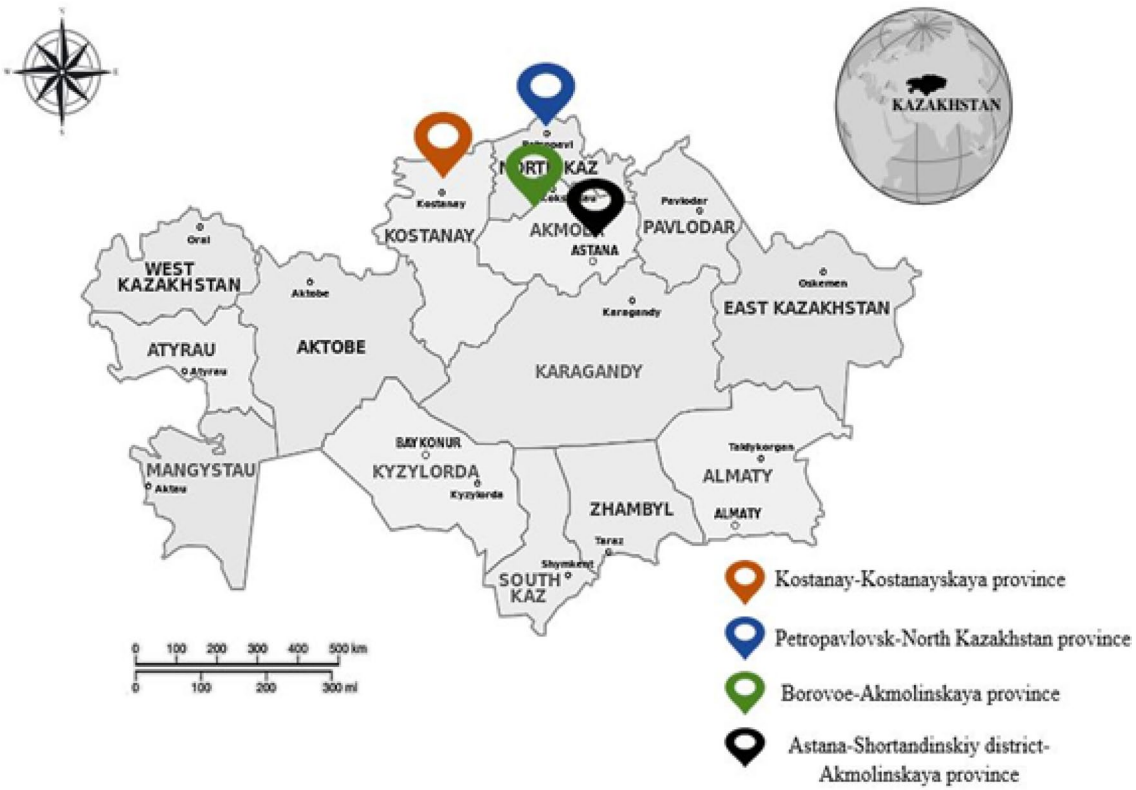

Fig. 1 Surveyed locations to obtain plant-parasitic nematodes in northern Kazakhstan

distance of 10–20 km from each sampled location. A minimum of 20 cores were taken per sample, the soil was mixed, and then, a representative sample of 2 kg was retained. Each sample was labeled with sample number, location, coordination, and the cultivated crop.

Migratory nematodes were extracted from 100 cm^3 of soil by using a modified Baermann funnel method conducted with Petri dishes (Hooper 1986). Extracted nematodes were transferred into water in graduated cylinders and left to settle for 8 h, and then, the supernatant was discarded. The nematode suspension was poured into a 15-ml tube. Nematodes were counted under a light microscope at 100 × magnification.

Cyst nematodes were extracted from 250 cm^3 of soil by using the modified sieving–decanting method (Fenwick 1940; Dababat et al. 2014). At least 20 full cysts were selected and handpicked from each sample and stored at +4 °C to be used in the molecular and morphological analysis. Initially, cysts were classified at genus level under a V20 stereo-binocular microscope (Zeiss, Jena, Germany), as round and ovoid cysts (*Globodera* and *Punctodera*) or lemon-shaped cysts (*Heterodera* and *Cactodera*) (Golden 1986; Subbotin et al. 2010).

Nematode abundance was calculated for each field with the formula: (number of samples with nematode/total number of samples) * 100.

Morphological identification of cysts

The characteristics of the cysts' vulval cones, J2 measurements, and morphometric features were examined in the identification. Vulval cone slides were prepared by fixing ten cysts from each population in formalin–glycerol fixative mounted on glycerol and detected with a light microscope, as per Golden (1986). The length of vulval slit, the width of vulval bridge, length and width of the fenestra as well as the underbridge were measured. The presence or absence of underbridge and bullae analysis on cyst's perineal pattern was examined according to Handoo (2002).

Ten juveniles obtained from the same cyst were gently heated, fixed in triethanolamine formalin solution, embedded in glycerol, and then prepared on permanent slides. Body and stylet length, distance from anterior region to junction with the pharynx, body width, and distance from anterior region to the base of the esophageal bulb, tail length, tail width, and length of the hyaline portion of the tail were measured as described by Handoo (2002). Ten J2s and ten cysts from each population were observed, photographed, and measured using a Leica DFC295 digital camera installed on a Leica DM5000 B optical microscope and Leica Application Suite (LAS) software v.4.1.0.

Morphometric data of both J2s and vulval cones of the cyst nematodes population were subjected to analysis of variance according to the general linear model procedure using the SAS statistical package (SAS Institute Inc. Cary, North Carolina, USA). Arithmetic means were compared using Tukey's HSD (Honest Significant Difference) test at $P \leq 0.05$ to test the differences or similarities between measurements of the different morphological characters used in identification.

Molecular identification of cysts

Genomic DNA of each population was extracted from a single cyst using the protocol described by Waeyenberge et al. (2000) with some modifications. PCRs were performed in a total volume of 50 µL containing 1 µL of genomic DNA, 25 µL of 2 × Dream *Taq* PCR Master Mix (Thermo Scientific, USA), 22 µL of ultra-pure sterile water, and 1 µM each of forward primer AB28 (5′-CGT AAC AAG GTA GCT GTA G-3′) and reverse primer TW81 (5′-TCC TCC GCT AAA TGA TAT G-3′) (Subbotin et al. 2001).

PCR steps consisted of an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The program was finalized at 72 °C for 10 min. Negative control (no DNA) was used to ensure that there was no contamination in amplifications. Amplification quality was evaluated using UV illumination after ethidium bromide staining on 1.5% agarose gel (100 V-40 min).

Sequencing of the internal transcribed spacer (ITS) of the rDNA region was used to identify nematode species and reveal intraspecific genomic variability of the *Heterodera* populations to compare them with the sequences available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and to determine phylogenetic relationships among themselves. A commercial company (REFGEN Biotechnology, Ankara, Turkey) sequenced PCR products in both directions to obtain overlapping sequences of both DNA strands. A total of 32 DNA sequences were screened using BLAST searches for their statistical similarities (positive matrix scores) to ITS-rRNA gene sequences of identified nematodes in the GenBank. Sequence traces were quality checked using the Trace Editor of MEGA 7 (Kumar et al. 2016). The alignment of these DNA sequences was conducted with ClustalW using the default parameters for gap opening and gap extension penalties (Kimura 1980). All aligned characters were applied in the phylogenetic analysis. The evolutionary history was inferred using the neighbor-joining method, based on evolutionary distances computed using the Tamura–Nei method (Tamura and Nei 1993). Gaps were treated as missing data. One *Heterodera schachtii* isolate (Accession No. AY166438) was used as an outgroup to root the trees and for characterizing polarization. Bootstrap support was calculated for all analyses using 1000 replicates.

Result

Incidence of PPN

The abundance of migratory PPN populations per 100 g of soil associated with cereal crops at 78 sites from Astana, Kokshetau, Petropavl, and Kostanay provinces in northern Kazakhstan was calculated (Table 1). Plant-parasitic nematodes were present in 90% of the samples, with an average abundance of 960 individuals/100 g soil. Twelve genera of migratory PPNs were identified in which *Geocenamus* spp. were the most prevalent (65%), followed by *Trophurus* spp. (62.5%) and *Pratylenchus* spp. (52.5%). The lowest frequency was recorded for *Pratylenchoides* spp. (20%). Economically important PPNs found in soil samples belong to the *Pratylenchus* genus. The populations of *Pratylenchus* species tended to be generally higher than the other PPNs with exceptions of *Geocenamus* and *Trophurus* species (Table 1).

Heterodera species were found in 44.8% of the cereal fields. Based on the morphological characteristics and molecular analysis, *H. filipjevi* was the only CCN species identified in the surveyed area (Table 2). The highest proportion of fields infected with *H. filipjevi* (76%) was recorded from Kokshetau while the lowest proportion of infested fields (16%) was recorded from Kostanay Province. The highest numbers of cysts were 30.4/250 g and 26/250 g, found in the samples from Astana and Kokshetau provinces, respectively. The lowest number of cysts with 23.2/250 g and 18.2/250 g was recorded in Petropavl and Kostanay, respectively. The overall average cysts number was 24.45 throughout the infested fields.

Table 1 The proportion of fields infested by migratory nematode genera in 78 fields surveyed in northern Kazakhstan

No.	Genus of Nematode	Proportion of infested fields (%)[a]	Number of nematodes[b]
1	*Geocenamus*	65	960 ± 120 (220–1100)
2	*Trophurus*	62.5	240 ± 20 (200–360)
3	*Pratylenchus*	52.5	240 ± 40 (400–980)
4	*Ditylenchus*	45.5	340 ± 60 (200–880)
5	*Helicotylenchus*	40	420 ± 50 (280–780)
6	*Tylenchus*	38.6	320 ± 40 (200–480)
7	*Amplimerlinus*	30	640 ± 160 (200–980)
8	*Merlinus*	27.5	550 ± 60 (100–860)
9	*Paratylenchus*	27.5	280 ± 20 (100–200)
10	*Tylenchorhynchus*	24	120 ± 160 (140–360)
11	*Rotylenchus*	22.4	240 ± 30 (160–900)
12	*Pratylenchoides*	20	310 ± 40 (120–460)

[a](Number of samples with nematode/total number of samples) * 100

[b]Average number of nematodes/100 g soil in relation to infested fields ± standard deviation (min and max)

Table 2 The proportion of fields infested by *Heterodera filipjevi* in 78 fields surveyed in northern Kazakhstan

Number	Provinces	Number of fields surveyed	Infestation (%)	Av. Number of cysts/250 g[a]
1	Astana	12	50	30.4
2	Kokshetau	21	76	26
3	Petropavl	27	37	23.2
4	Kostanay	18	16	18.2
Total		78	44.75	24.45

[a]Based on the total samples in relation to infested fields

Morphological identification of cyst samples

The morphological and morphometric characters of the J2s and cysts were measured for each population. Based on the J2s and cysts morphological features, the cereal cyst nematode, *H. filipjevi*, was identified from the samples of all surveyed provinces. Cysts had prominent bullae and a clear underbridge, which was very narrow in the centre and located close to the vulval bridge (Fig. 2).

The fenestral length and the semifenestral width of *H. filipjevi* were 48.34–58.34 µm and 20.4–24.4 µm, respectively, and the vulval cone was bifenestrated with an underbridge (Table 3). Second-stage juveniles of *H. filipjevi* were cylindrical, with a slightly offset head and a tapering round tail tip. The stylet was strong with shallow anteriorly concave basal knobs. The juvenile body length of *H. filipjevi* varied from 518.9 to 556.3 µm, and stylet length was 23.2–24.9 µm with moderately concave stylet knobs. *H. filipjevi* cysts had slightly underbridge with bullae and greater fenestral length, larger vulval bridge width (10.56–15.65 µm), and shorter vulval slit length (18.2–22.6 µm) (Table 3).

According to the taxonomic characters, *H. filipjevi* populations were divided into at least two groups. The first group consisted of Kokshetau populations, whereas the second group consisted of Astana populations. Kostanay populations were mainly closed to Kokshetau populations, while Petropavl populations mostly were similar to Astana populations. Generally, the values of the body length, stylet length, tail length, hyaline tail tip length for J2, and fenestral length, semifenestral width, vulval bridge width, and vulval slit length for cysts were higher in the first group especially in Kokshetau populations when compared to the second group particularly to Astana populations (Table 3). For example, the vulval slit of the second group cysts was shorter (18.2 µm) when compared to those of the first group (22.6 µm). Moreover, the first group had wider underbridge with more sclerotization than second group, while J2 stylet

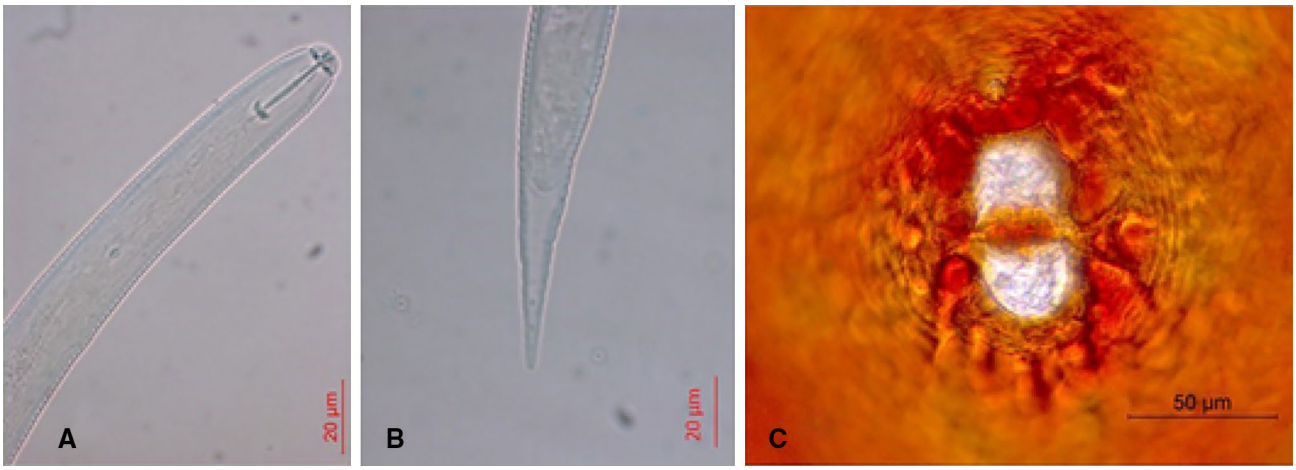

Fig. 2 Light micrographs of *Heterodera filipjevi* from northern Kazakhstan regions. **a** Stylet, **b** anus and hyaline tail tip, **c** female fenestra, narrow vulval slit, and heavy bullae

Table 3 Morphological and morphometrical characteristics of J2s and cysts of *Heterodera filipjevi* populations ($n=10$); dimension measurements are given in µm

Identification characters	Astana population	Kokshetau population	Petropavl population	Kostanay population
Body length	$521.3 \pm 4.84^{b*}$ (482.8–542.5)	548.3 ± 3.74^{a} (471.8–588.6)	518.9 ± 6.62^{b} (467.8–548.5)	556.3 ± 7.52^{a} (481.5–589.8)
Stylet length	23.2 ± 0.45^{b} (22.2–25.6)	24.9 ± 0.32^{a} (22.5–27.6)	23.5 ± 0.49^{b} (21.6–24.8)	24.2 ± 0.38^{ab} (21.6–25.9)
Tail length	50.45 ± 1.86^{c} (43.4–62.6)	52.66 ± 3.35^{b} (41.6–64.6)	54.68 ± 3.94^{a} (45.6–66.8)	51.24 ± 4.52^{b} (42.5–67.6)
Hyaline tail tip length	25.6 ± 1.32^{b} (21.4–34.5)	26.6 ± 2.57^{a} (22.4–32.3)	25.8 ± 1.45^{b} (21.9–37.6)	25.4 ± 1.88^{b} (20.4–36.4)
Fenestral length	51.32 ± 2.32^{b} (42.1–63.4)	58.34 ± 3.44^{a} (43.8–68.4)	54.54 ± 2.32^{ab} (45.2–63.2)	48.34 ± 1.64^{b} (40.2–59.8)
Semifenestral width	20.4 ± 2.43^{c} (15.2–26.6)	24.4 ± 2^{a} (16.7–24.6)	23.2 ± 1.04^{ab} (16.8–27.5)	22.4 ± 2.09^{b} (15.2–28.6)
Vulval bridge width	10.56 ± 1.32^{b} (6.8-17.2)	14.44 ± 2.43^{a} (9.2–18.6)	12.09 ± 2.33^{ab} (7.88-16.54)	15.65 ± 2.33^{a} (9.2–17.5)
Vulval slit length	19.5 ± 1.02^{b} (12.6–25.8)	22.6 ± 1.58^{a} (10.3–26.2)	20.2 ± 2.34^{ab} (13.4–25.6)	18.2 ± 3.32^{b} (13.3–25.5)

*The same letters denote no significant differences at $P \leq 0.05$ in a row, by Tukey's HSD test

knobs were more anteriorly concave in the first group compared to the second one.

Molecular identification of cyst samples

The ITS-rDNA regions of all 32 CCN populations were successfully amplified using the primers described above. For all populations, amplification of the ITS1, 5.8S, and ITS2, including the flanking parts of the 18S and 28S genes, yielded a single fragment of approximately 1060 bp. No PCR products were obtained from the negative control that lacked a DNA template. Moreover, the BLAST analysis of the obtained sequences revealed that all 32 populations were *H. filipjevi*. The corresponding sequences of these 32 nematode isolates matched the homologous populations listed in the GenBank database with high sequence similarity. The resultant sequences of isolates from Kazakhstan were deposited in the GenBank with Accession Nos. as shown in Fig. 3.

Phylogenetic analysis of cyst samples

A phylogenetic tree clustering the populations at different levels is based on genetic distance which was constructed from the ITS sequence alignment (Fig. 3). The

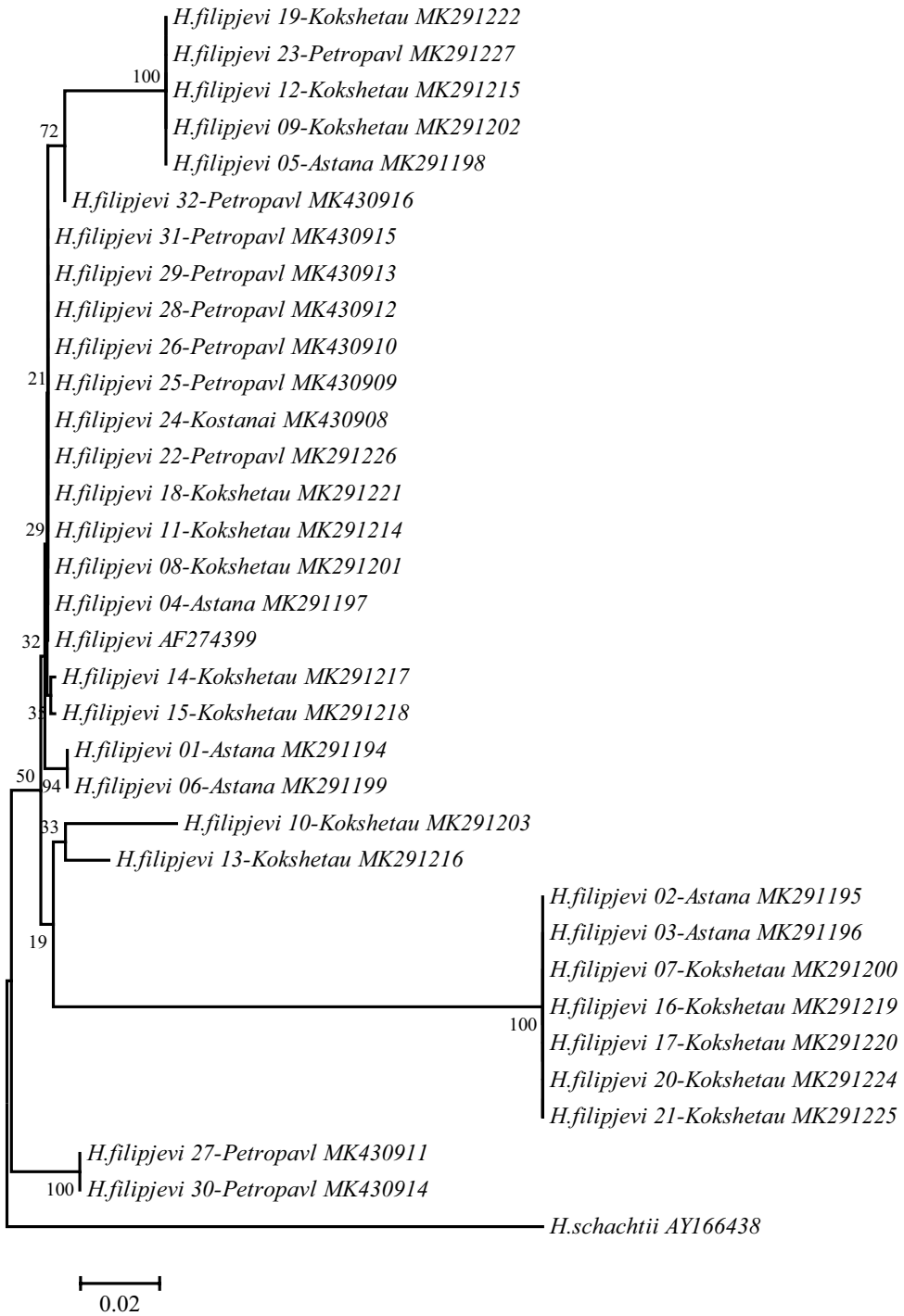

Fig. 3 Phylogenetic tree of *Heterodera filipjevi* populations from northern Kazakhstan regions

phylogenetic tree was generated from 1000 bootstrapped sequence alignments, which were subjected to global rearrangement with random replications. Samples from the four provinces were grouped into two main clusters of *H. filipjevi* and one outgroup of *H. schachtii*. Results indicated that the ribosomal DNA sequences of two *H. filipjevi* isolates from Petropavl differed from the others. Isolates within the *H. filipjevi* populations clearly were divided into two different groups within the phylogenetic tree, indicating that intraspecific polymorphism existed among the *H. filipjevi* populations (Fig. 3).

Discussion

The results of this survey showed high incidence rate of *H. filipjevi* in the main cereal-growing areas in northern Kazakhstan with 44.75% of cereal fields being infested. The number of cysts was > 30.4 cysts/250 g of soil, which likely reduces wheat yield as this exceeds the economic threshold level, 5 eggs g^{-1} of soil (Sahin et al. 2009), particularly when combined with other abiotic or biotic stress factors such as other nematodes, fungal pathogens, and diminishing supply of water or plant nutrition late in the growing season. The seven samples from Astana and Kokshetau had exceptionally high populations density of *H. filipjevi* that were at least 15 times higher than the population density that could cause significant yield loss in wheat. Barley and wheat are continuously cultivated on the same land as monoculture in the surveyed areas. Rivoal and Cook (1993) reported growing cereals as a monoculture resulted in gradually increasing populations of CCN that influences the amount of yield loss in infested fields. One explanation for the high density of cyst populations (average cyst = 24.45/250 g in the infested soil samples) is due to the suitable environmental conditions which enable cyst nematode to complete its life cycle easily. For instance, temperature and moisture effects varied with the nematode activity (hatching, penetration, development, and reproduction). For example, hatching occurred in tap water at 10–25 °C with an optimum temperature at 10 °C. Also, J2 penetrated wheat roots at 10, 15, and 20 °C, while the maximum penetration occurred at 15 °C (Sahin et al. 2010; Dababat et al. 2015).

This study indicated that there was polymorphism in the populations of *H. filipjevi* according to the morphological parameters, which confirm differences in measurements of cysts and J2 bodies. *H. filipjevi* is closely related to *H. avenae* and only minor morphological differences, which can differentiate them from each other (Hando 2002; Subbotin et al. 2010). Most of morphological and morphometrical features of *H. filipjevi* resemble those of *H. avenae* such as the big bullae, the short fenestra length and vulval bridge width in cysts, and the long tail and hyaline part of the tail in juveniles (Rivoal et al. 2003; Subbotin et al. 2003, 2010). In this study, the discrimination of specimens based on the development of bullae and the presence of an underbridge was successfully performed even at low magnification.

This is the first report on *H. filipjevi* from northern Kazakhstan using molecular phylogeny. The sequence of the ITS region is used effectively to support and confirm the morphological traits for accurate identification of *H. filipjevi* (Handoo 2002; Subbotin et al. 2010). The present study indicated the polymorphism in the populations of *H. filipjevi* which were divided into distinct groups. Subbotin et al. (2010) revealed intraspecific polymorphism among *H. filipjevi* populations. Toktay et al. (2015) also showed intraspecific differentiation among the populations of *H. filipjevi* from the eastern Anatolian region of Turkey. Imren et al. (2015), however, did not detect any genetic variation among *H. filipjevi* populations in the Mediterranean region of Turkey.

The reduction in yield loss caused by cereal cyst nematodes requires effective management of this nematode to keep its damage below the threshold levels, 5 eggs g^{-1} of soil (Sahin et al. 2009). Therefore, observations of population dynamics and yield losses on representative local cultivars under natural field conditions were required to determine an effective management strategy (Nicol et al. 2003; Dababat et al. 2014). The limited number of resistance sources for breeding purposes has been found in domestic cereals and their wild relatives, acting against *H. avenae* (Dababat et al. 2015). *Cre*1 was the only gene reported to confer resistance against *H. filipjevi* in wheat derivative *Thinopyrum* (wheatgrass) and wheat landrace Sardari (Akar et al. 2009; Li et al. 2012). New resistance sources, designed as *Cre* genes, conferring resistance to *H. filipjevi* have been identified in wheat. Breeding programs should target pyramiding those different sources of resistance into high-yielding varieties, to provide valid resistance against CCN. In fact, there has been no variety providing strong and sustainable resistance against *H. filipjevi* in wheat, barley, and oat, yet.

In 2016, the soil-borne pathogens program at CIMMYT-Turkey screened 34 local wheat cultivars obtained from Kazakhstan and some cultivars showed good levels of resistance to the Turkish *H. filipjevi* populations (Dababat et al. unpublished data). However, additional investigations are necessary to identify the pathotypes of the *H. filipjevi* populations in northern Kazakhstan, as they might be different from the *H. filipjevi* populations in Turkey. Further investigations are needed to evaluate the widely grown cultivars and to determine novel resistance sources to be used in cereal breeding programs in Kazakhstan.

This study highlighted the occurrence of the main genera of PPNs with an emphasis on the cereal's cyst nematode species *H. filipjevi* in northern Kazakhstan. Further detailed surveys in southern Kazakhstan and comprehensive pathotype studies on *H. filipjevi* populations from different regions are still needed. In conclusion, the recommendations of this survey to the policy makers and the researchers are to: diversify wheat cultivated types to include durum wheat which is more resistant than spring wheat to cyst nematodes (Akar et al. 2009; Li et al. 2012; Dababat et al. 2015); follow cultural practices especially crop rotation; breed for germplasm with higher levels of resistance to *H. filipjevi*; and train scientist to conduct research on the these nematodes in Kazakhstan. To maintain the population densities of the nematodes

species of below damaging levels, appropriate management measures such as rotational schemes and the use of resistant varieties should be implemented.

Acknowledgements The authors would like to thank the International Maize and Wheat Improvement Centre (CIMMYT) for funding this study.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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