Morphological and molecular observations on the cereal cyst nematode *Heterodera filipjevi* from the Middle Volga River and South Ural Regions of Russia

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Summary. During 2010-2012, a survey was conducted to determine the distribution and diversity of the cereal cyst nematodes (CCN), including *Heterodera filipjevi*, within the middle Volga River and South Ural regions of the Russian Federation. A total of 270 soil samples were collected. Seven populations of CCN were found in the rhizosphere area of various cereal plants that showed symptoms of nematode disease in Saratov and Chelyabinsk regions. The highest nematode population density was found in Chelyabinsk Region, with a mean density of 100 cysts (100 g soil)$^{-1}$. The morphological and morphometric characteristics of these populations are presented showing variations in cyst body width, underbridge and vulval slit length, and in the vulva-anus distance. The morphometrics of second-stage juveniles showed minor differences between Saratov and Chelyabinsk populations compared with those of the paratypes and the population from the Republic of Bashkortostan (Bashkiria). The body lengths of the Saratov and Chelyabinsk populations were smaller than those of Bashkiria and longer than those of the paratypes. The pharynx lengths of the Saratov and Chelyabinsk populations were shorter than the paratypes. For molecular characterisation, DNA was extracted from cysts of each population. Species-specific primers for *H. avenae*, *H. filipjevi* and *H. latipons* were developed for conventional PCR amplification of the internal transcribed spacer region of ribosomal RNA (ITS1-rRNA). Primer pairs developed for *H. filipjevi* and *H. latipons* amplified PCR products only from the target species, whereas primers for *H. avenae* amplified *H. pratensis* in addition to the target species. Molecular data confirmed the identity of the seven populations as *H. filipjevi*.

Key words: *Heterodera avenae*, *Heterodera latipons*, ITS1-rRNA, molecular characterisation, morphology, morphometrics, PCR with species-specific primers.

Cereal crops are very important to the agricultural economy of the Russian Federation. During 2008-2011, Russia was included in the top five countries for production and export of wheat, oat and barley. However, various pathogens and pests, including plant-parasitic nematodes, reduce the yield of cereals in Russia from 10-12% in moist seasons to 72% under drought conditions (Popova, 1971; Tikhonova, 1972; Shiabova, 1982; Terenteva & Zhemchuzhina, 1982). Cereal cyst nematodes (CCN) are a related group of *Heterodera* species important for grain producers in Russia and throughout the world. The first description of cereal cyst nematode (*Heterodera avenae* Wollenweber, 1924) in the former USSR was made in 1936 by V.A. Mamonov (Kirjanova & Krall, 1971). Subsequently, study of the distribution, biology, economic importance, and methods of management of cereal cyst nematodes (CCN) in the former USSR peaked during 1960-1990. During this time, various former USSR scientists focused their investigations on the distribution and pathogenicity of CCN in the

Central region of the European part of the USSR (Tikhonova *et al.*, 1967; Volchkova, 1978), the middle Volga River region (Osipova, 1984, 1986), the Northwest region (Terenteva & Zhemchuzhina, 1982), the Ural region (Mamonova, 1962, 1969; Popova, 1971, 1972, 1975; Tikhonova, 1972, 1986), Siberia (Zhuk, 1969; Terenteva & Shiabova, 1973; Shiabova, 1982), Tajikistan (Madzhidov, 1981, 1985), Armenia (Poghossian, 1962) and Ukraine (Ladygina, 1962).

Several species of the *Avenae* group have been described from the former USSR: "*H. avenae*" (everywhere in the USSR), *H. filipjevi* (Tajikistan, Volga and Ural), *H. hordecalis* and *H. ustinovi* (Ukraine) (Kirjanova, 1969), *H. latipons* (the Northwest and Chernozem zones of Russia) (Subbotin *et al.*, 1999). In addition, some species were described from wild grasses, including *H. pratensis* (Gäbler *et al.*, 2000), *H. riparia* (couchgrass) (Kazachenko, 1993) and *H. arenaria* (reed) (Kirjanova & Krall, 1971).

Most species of CCN can be differentiated from each other on the basis of morphological and morphometric features (Subbotin *et al.*, 1999; Handoo, 2002; Abidou *et al.*, 2005). However, morphological identifications are usually based upon minor differences among species, so correct identifications can be difficult to achieve, particularly if a quick diagnosis is needed (Rivoal *et al.*, 2003; Subbotin *et al.*, 2003). DNA sequence variation in molecular markers such as the internal transcribed spacer (ITS) regions of ribosomal DNA can be used to identify many nematode taxa and to determine phylogenetic relationships (Powers *et al.*, 1997; Subbotin *et al.*, 2001; Holterman *et al.*, 2006). More recently, Subbotin with co-authors developed extensive molecular diagnostics of different *Heterodera* groups including CCN (Subbotin *et al.*, 2003, 2010). For mass identification of nematode populations from agricultural fields and rapid diagnosis for farmers, it is necessary to develop simple methods that are faster and more straightforward than either restriction fragment length polymorphisms (PCR-RFLP) or sequencing. Simple systems that employ species-specific primers for conventional PCR allow small laboratories with simple equipment to perform accurate species identifications.

The objectives of this study were to survey the present range of CCN in the Middle Volga River and South Ural regions, where about 60% of all cereals are grown, and to design and evaluate species-specific molecular assays for distinguishing three *Heterodera* spp. of major importance on cereal crops.

MATERIALS AND METHODS

Location and samples collecting. The Middle Volga River (Saratov and Samara Regions) and South Ural (Orenburg and Chelyabinsk regions) are the most important areas for growing cereals in Russia. Around 60% of the spring wheat is cultivated here (http://www.gks.ru/wps/wcm/connect/rosstatmain/rosstat/en/figures/agriculture/). These areas are situated between N (49.80°-56.36°) and E (42.49°-63.38°). The climate of this area is continental. The lowest temperature of winter occurs during January-February, with means between –10°C (Volga River) and –20°C (South Ural). The highest temperatures of summer are during June-July, with means between +25°C (South Ural) and +29°C (Volga River). Soil samples (200 g) were collected from the rhizosphere of cereals in both the Middle Volga River and South Ural regions at a depth between 5 and 15cm. A total of 270 samples were collected.

Cyst and juvenile isolation. Cysts of nematodes of the Heteroderidae family were collected using paper filters (Pridannikov *et al.*, 2007). Soil samples were put into plastic cups (V = 1.5 l) and stirred in water. The suspension was then filtered through sieves with mesh 1.0 and 0.16 mm. Then the content of the 0.16-mm sieve was washed onto a paper filter in a funnel. After the water had drained, cysts were manually collected under a stereomicroscope (MBS-10: Lytkarino Optical Glass Factory). The air-dried cysts were then stored at 4°C until used for morphological and molecular identification.

Some cysts were crushed. Eggs were removed, kept in water in Petri dishes (45 mm diam.) and exposed in a thermostat at 24°C for 48 h to let juveniles hatch. Hatched second-stage juveniles (J2) were collected, heat killed and fixed in 4% TAF for following morphological identification.

Morphological identification. Morphological identification of nematode species was carried out on the basis of structure of vulval cones of cysts. Cysts were fixed in 4% TAF. The posterior portion of the cysts was carefully separated from the contents (eggs, J2, fungi, *etc.*) under a microscope with forceps and needle. Vulval cones were cut, the specimens were trimmed to the size of cones, and these were transferred into a drop of pure glycerol.

Permanent slides of the cysts were prepared using 'gelatin jelly' (Luna, 1968). To obtain this mixture, 6 ml of water were added to 30 g of gelatin and then heated until the gelatin was fully dissolved. Afterwards, 6 ml of glycerin and 0.05 g of phenol were added. A drop of this solution was transferred onto a glass slide using the needle and heated until

Fig. 1. Area of investigation of CCN complex in the middle Volga River and South Ural area.

melted. Three to five vulval cones were put into melted gelatin jelly under a microscope, oriented in the correct position, and covered with a cover glass. After the gelatin jelly had hardened, the slide was sealed with fast-dried synthetic lacquer Histofluid mounting medium (Marienfeld Superior, Germany).

Juveniles after TAF fixation were transferred to distilled water and processed to glycerin (Sumenkova, 1978). The specimens were mounted in dehydrated glycerin on permanent slides, examined and measured.

Photomicrographs of vulval cones and J2 were taken with an Axio Imager A1 Carl Zeiss (Germany) light microscope equipped with Nomarski differential contrast (DIC) optics.

Primer design. The design of species-specific primers was based on published nucleotide sequences of the internal transcribed spacer (ITS1) of ribosomal RNA at the GenBank. These included *H. avenae* (JX024199), *H. filipjevi* (GU565575), *H. pratensis* (AY148391), *H. latipons* (JX024189), *H. arenaria* (AF274396) and *H. hordecalis* (JX024216). A multiple sequence alignment was generated with AlignX of the Vector NTI Suite 8.0 package using the ClustalW algorithm (http://www.clustal.org/). For design of the primers, the programs Oligo6 and mfold 3.2 were used. An *in silico* study was done to check the potential of secondary structures, self-primer-dimer and hetero primer-dimer formation within and between primers of each set. The highest annealing temperature showing a single, clear PCR-fragment was chosen as optimal. A blastN search of the nucleic acid database was used to check for potential cross-reaction with non-target species (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). Additionally, to check the specificity of primers, non-target DNAs of *Heterodera* spp. from our collection were included in PCR analysis.

DNA extraction. For each extraction, a single cyst was soaked overnight in 50 µl of double-distilled water at 4°C. After that, double-distilled water was replaced by 50 µl of Worm Lysis Buffer (Williams *et al.*, 1992) (50 mM KCl, 10 mM Tris-HCl (pH 8.2), 0.45% Tween 20, 60 µg ml^{-1} of proteinase K, 2.5 mM $MgCl_2$, 0.05% gelatin). Tubes were placed at –70°C for 15 min followed by thawing, during which time the cyst was homogenised by a pestle. Then tubes were incubated at 65°C for 1 h, followed by 95°C for 15 min. After centrifugation (40 s, 2,400 *g*), 40 µl of supernatant was transferred into a new tube. DNA yields were assessed by BioPhotometer *Plus* (Eppendorf, USA). The samples were stored at –20°C.

DNA amplification. PCR was conducted in 20-µl reaction mixtures containing: 2 µl of 10× PCR buffer (Fermentas, USA), 2 µl of 2 mM of each dNTP, 0.5 µl of each primer (4 pmol $µl^{-1}$), 0.1 µl of

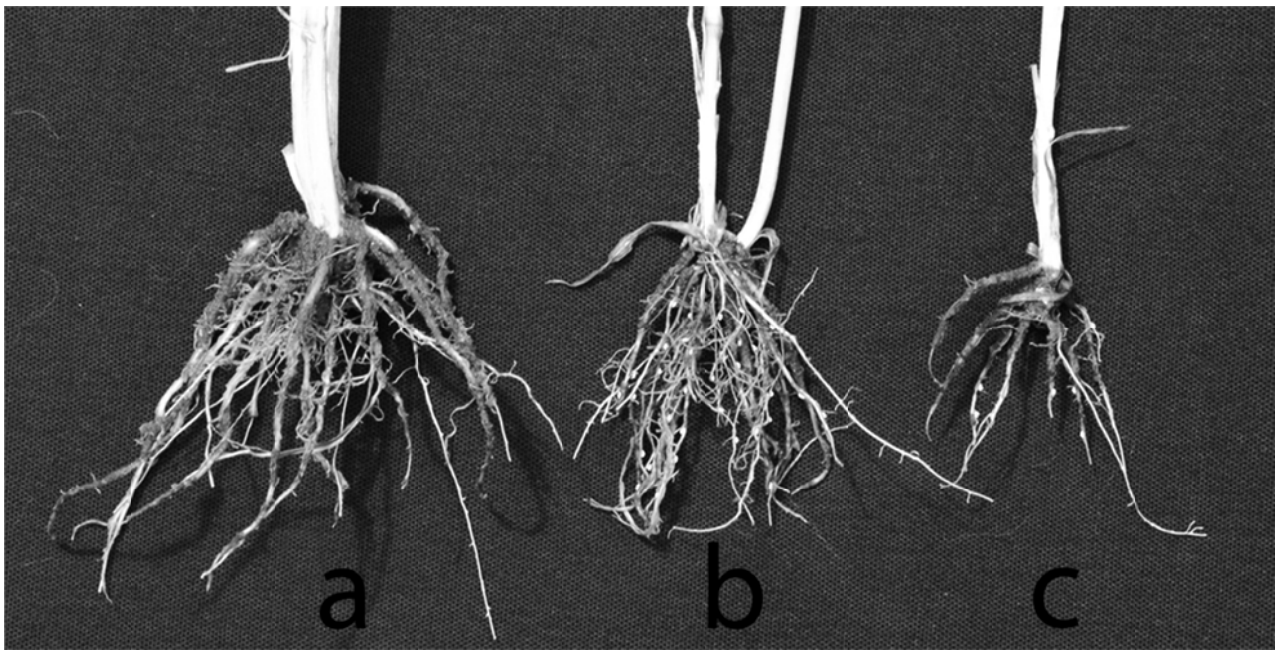

Fig. 2. Symptoms of spring wheat plant disease caused by *Heterodera filipjevi* on roots (a – non-infected control; b and c – infected roots) (photos by Dr V.N. Chizhov).

Dream-*Taq* DNA Polymerase (Fermentas, USA) (5 U μl^{-1}) and 50 ng of template DNA. The DNA amplification was carried out in a MyCycler thermal cycler (Bio-Rad, USA) programmed for 4 min at 94°C, 30 cycles of 40 s at 94°C, 40 s at 94°C, 40 s at 59°C or 62°C (depending on primer), 40 s at 72°C and then 10 min at 72°C. Results of amplification reaction were analysed by electrophoresis using a 1.5% ethidium bromide-stained agarose gel and were photographed under UV light using a Vilber Lourmat Gel Imaging System (France).

RESULTS

During 2010-2012, 73 fields of cereals from the Middle Volga River and South Ural regions were sampled. Nine populations of various cyst nematode species were found in the rhizosphere of various cereal plants showing symptoms of nematode diseases (Figs 1 & 2; Table 1). The general visualised symptoms of nematode diseases on cereals were a stunted appearance, a reduction in tillering and a uniformly chlorotic of leaves, similar to that caused by a drought condition. The 'bushy-knotted' root symptoms were not visualised in this study (Dababat *et al.*, 2011).

Two populations of cyst nematodes (on grasses from Saratov Region, Tulaykovo village and on Sudangrass from Samara Region, Bezenchuk village) were excluded from this study after morphological identification because their long vulval slit and ambifenestrate vulval cone indicated that they do not belong to the *Avenae* group. The mean of all population density was around 1-10 cysts (100 g rhizosphere soil)$^{-1}$. The highest nematode population density, around 100 cysts (100 g rhizosphere soil)$^{-1}$, was found in the territory of the Chelyabinsk Agriculture Institute (Chelyabinsk NIISH) under spring wheat fields without crop rotation. Morphology (Fig. 3) and morphometrics (Table 2) of the other seven populations were similar, and all species were identified as *Heterodera filipjevi* Madzhidov, 1985.

The most interesting morphometric differences between the Saratov and Chelyabinsk populations *vs* paratypes are: cysts from Saratov had the smaller mean underbridge length (50.4 *vs* 82.4 µm), vulva-anus distance (49.2 *vs* 63.4 µm), and larger vulval bridge width (11.3 *vs* 7.7 µm). Cysts from Chelyabinsk Region had the same parameters: smaller underbridge length 60.0 µm, vulva-anus distance 58.1 µm, and larger vulval bridge width of 9.6 µm.

Morphometrics of J2 (Table 3) showed minor differences between the Saratov and Chelyabinsk populations compared to those of the paratypes (Madzhidov, 1981) and the population from Bashkiria (Subbotin *et al.*, 1996). The body lengths of the Saratov and Chelyabinsk populations were smaller than those of Bashkiria and longer than the paratypes (512.4 and 532.8 µm *vs* 552 and 506 µm), and as a consequence, the distances to the excretory

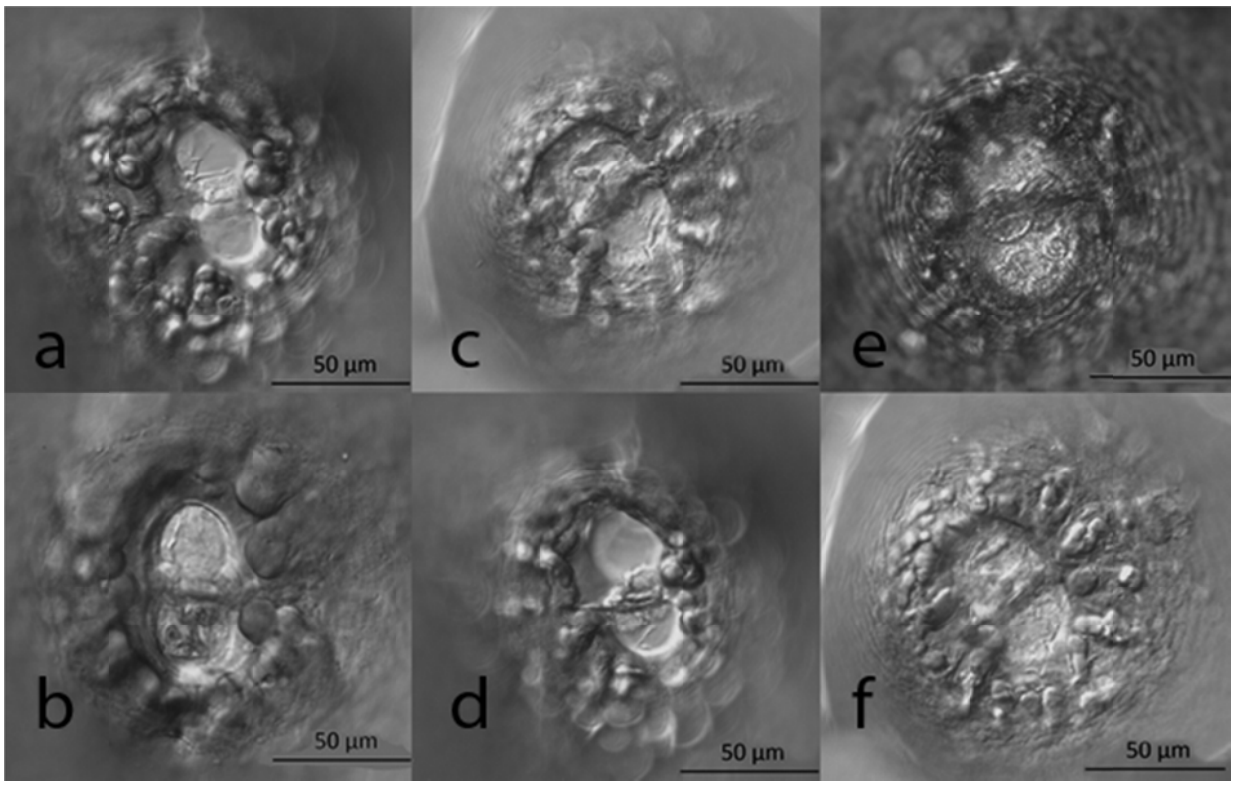

Fig. 3. Morphology of vulval cones of some populations of cereal cyst nematodes from the Middle Volga River (a, b & d) and South Ural regions (c, e & f).

pores had the same differences (105.5 and 109.0 µm *vs* 114 and 95 µm). The pharynx lengths of the Saratov and Chelyabinsk populations were shorter than that of the paratypes (117.4 and 116.4 µm *vs* 132 µm). Index b was greater on all the Volga and Ural populations than that of the paratypes (4.5, 4.6, 4.5 *vs* 3.8). Unfortunately, no males of *H. filipjevi* were found in this study.

The PCR assay was employed to identify the seven populations of CCN collected in this study. DNA of ten *Heterodera* and two *Globodera* species (*H. avenae*, *H. pratensis*, *H. latipons*, *H. cruciferae*, *H. goettingiana*, *H. humuli*, *H. ripae*, *H. schachtii*, *H. glycines*, *H. trifolii*, *Globodera rostochiensis*, *G. pallida*) from the State Collection of Phytopathogenic Microorganisms (the Russian Research Institute of Phytopathology) were used as non-target species.

The species-specific primers (Table 4) in the ITS1 region and the 5.8S ribosomal RNA gene were designed for distinguishing *H. avenae*, *H. pratensis*, *H. filipjevi* and *H. latipons*. The primer pair HavITS200/HavITS540 designed for *H. avenae* and *H. pratensis* produced a PCR amplicon of 330 bp with DNA from *H. avenae* and *H. pratensis* isolates, but did not amplify DNA from the seven other populations of CCN studied, as well as DNA from other twelve non-target *Heterodera* or *Globodera* spp. (Fig. 4A).

Amplification with the primer pair HlatITS-For/HlatITS-Rev designed for *H. latipons* yielded a single fragment of 288 bp only with DNA of *H. latipons*, but did not produce any amplicons with DNAs of non-target *Heterodera* or *Globodera* spp. and seven populations of CCN (Fig. 4B).

PCR with the primer pair HfilITS200/HfilITS540 designed for *H. filipjevi* was positive for DNA of seven populations of CCN isolated from the Middle Volga River and South Ural regions, as it resulted in amplification of the species-specific fragment of expected size (340 bp) (Fig. 4C). Moreover, no amplification was observed in other species of cyst nematodes including *H. avenae*, *H. pratensis*, *H. latipons*, *H. cruciferae*, *H. goettingiana*, *H. humuli*, *H. ripae*, *G. rostochiensis*, *G. pallida*, *H. schachtii*, *H. glycines* and *H. trifolii* (Fig. 4C).

The quality of nematode DNA template used in the study was checked by amplification with the common primer pair D3A-D3B (Al-Banna *et al.*, 1997) (Fig. 4D). No PCR products were obtained in the negative control without nematode DNA template (Fig. 4).

Table 1. DNA of nematode species used in present study

Species	Location	Host	Depository	Collector
Heterodera filipjevi	Russia, Saratov region, Saratov City	*Avena sativa* L.	SCPM*	Pridannikov M.V.
	Russia, Saratov region, Saratov City	*Triticum aestivum* L.	–	Pridannikov M.V.
	Russia, Chelyabinsk region, Timiryazevsky village	*Triticum aestivum* L.	–	Pridannikov M.V.
	Russia, Chelyabinsk region, Timiryazevsky village	*Triticum aestivum* L.	–	Pridannikov M.V.
	Russia, Chelyabinsk region, Timiryazevsky village	*Triticum aestivum* L.	–	Pridannikov M.V.
	Russia, Chelyabinsk region, Timiryazevsky village	*Avena sativa* L.	–	Pridannikov M.V.
	Russia, Chelyabinsk region, Slava village	*Avena sativa* L.	–	Pridannikov M.V.
Heterodera sp.	Russia, Samara region, Bezenchuk village	*Sorghum bicolor* L.	–	Pridannikov M.V.
Heterodera sp.	Russia, Saratov region, Tulaykovo village	Grasses, unknown plants	–	Pridannikov M.V.
Heterodera avenae	Turkey	*Triticum aestivum* L.	–	Toumi F.
H. pratensis	Russia, Yaroslavl region, Myshkin City	*Poa annua* L.	–	Chizhov V.N.
H. latipons	Turkey	*Hordeum vulgare* L.	–	Toumi F.
H. cruciferae	Russia, Moscow region, Puschino City	*Brassica oleracea* L.	–	Chizhov V.N.
H. goettingiana	Russia, Saratov region, Tulaykovo village	*Medicago sativa* L.	–	Pridannikov M.V.
H. humuli	Russia, Kaluga region, Yagodnoe village	*Humulus lupulus* L.	–	Pridannikov M.V.
H. ripae	Russia, Bashkiria republic, Gadelgareevo village	*Urtica doica* L.	–	Pridannikov M.V.
H. schachtii	Ukraine, Kiev City	*Beta vulgaris* L.	–	Galagan T.
H. glycines	Russia, Far East	*Glycine max* (L.) Merr.,	–	Pridannikov M.V.
H. trifolii	Russia, Moscow region, Zacharovo village	*Trifolium pratense* L.	–	Pridannikov M.V.
Globodera rostochiensis	Russia, Moscow region, Kraskovo village	*Solanum tuberosum* L.	–	Pridannikov M.V.
G. pallida	Italy	*Solanum tuberosum* L.	–	Melillo M.

* SCPM – the State Collection of Phytopathogenic Microorganisms (the Russian Research Institute of Phytopathology).

Table 2. Morphometric measurements of various populations of cyst nematodes from the middle Volga River and South Ural regions of Russia (values are mean ± s.d. (range))

Population / Parameters	Saratov reg.	Chelyabinsk reg.	the Republic of Bashkortostan (Subbotin *et al.*, 1996)	Paratype *H. filipjevi* (Madzhidov, 1981)
Cysts (n)	20	22	30	25
Length (ext. neck)	624.5±99.2 (455-825)	686.4±74.0 (555-835)	928±20.0 (712-1192)	690 (490-830)
Width	445.5±88.4 (270-605)	522.7±68.6 (405-675)	685±16.8 (384-792)	490 (340-620)
L/W	1.4±0.2 (1.0-1.8)	1.3±0.1 (1.1-1.5)	1.4±0.02 (1.1-1.9)	–
Vulval Cones (n)	8	12	18	25
Fenestra length	52.9±1.7 (50.0-55.0)	52.1±6.3 (42.5-62.5)	53.3±0.9 (47.5-60.0)	51.5 (41.3-64.4)
Fenestra width	29.6±4.2 (25.0-37.5)	29.4±4.2 (20.0-35.0)	30.4±0.8 (27.5-37.5)	27.5 (21.0-32.9)
Bridge width	11.3±1.9 (7.5-12.5)	9.6±1.3 (7.5-12.5)	8.1±0.4 (5.0-10.0)	7.7 (6.3-9.4)
Underbridge length	50.4±5.9 (42.5-60.0)	60.0±11.8 (47.5-80.0)	92±3.5 (83-99)	82.4 (72.5-101.5)
Vulval length	8.5±1.1 (7.5-10.0)	10.5±1.3 (7.5-12.5)	10.9±0.4 (7.5-15.0)	7.3 (6.3-8.4)
Vulva-Anus distance	49.2±4.5 (42.5-55.0)	58.1±15.9 (35.0-85.0)	–	63.4 (53.2-96.5)

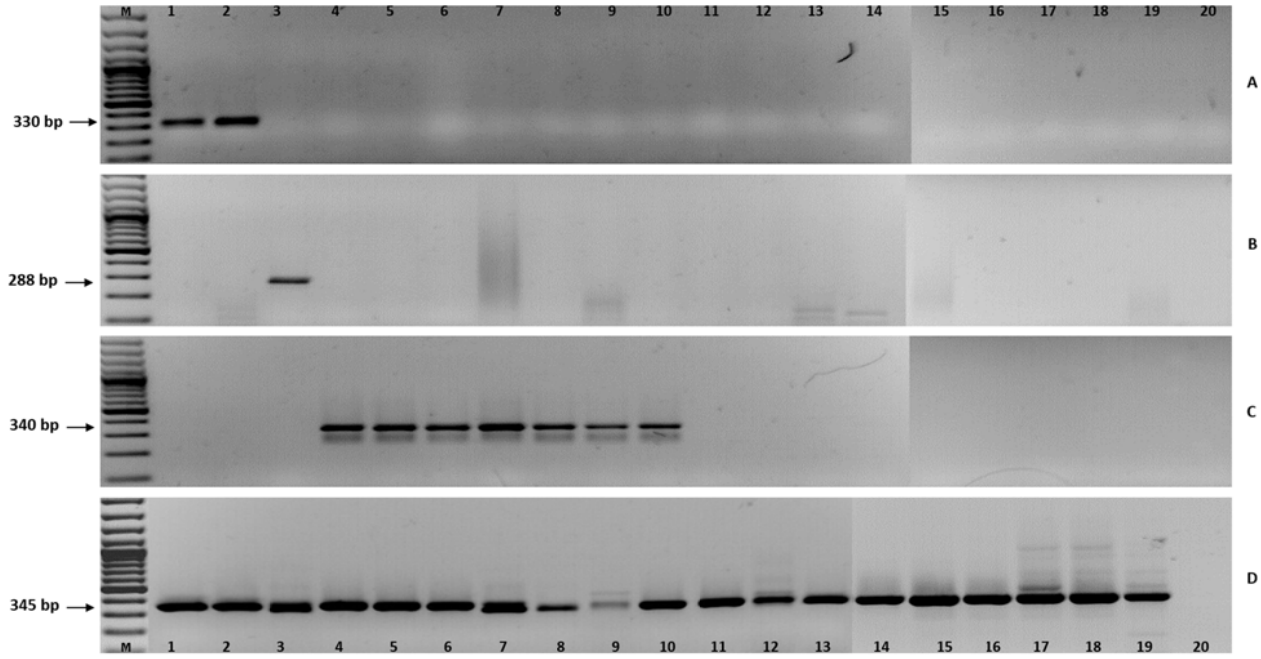

Fig. 4. PCR amplification of DNA from different *Heterodera* or *Globodera* species with the HavITS200/HavITS540 primers (A), the HlatITS-For/HlatITS-Rev primers (B), the HfilITS200/HfilITS540 primers (**C**), and the D3A/D3B primers (D). Lines: M – 100 bp DNA Ladder (Fermentas); 1 – *H. avenae*; 2 – *H. pratensis*; 3 – *H. latipons*; 4-10 – DNA extracted from seven single cysts of populations of CCN isolated from the middle Volga River and South Ural regions; 11 – *H. cruciferae*; 12 – *H. goettingiana*; 13 – *H. humuli*; 14 – *H. ripae*; 15 – *G. rostochiensis*; 16 – *G. pallida*; 17 – *H. schachtii*; 18 – *H. glycines*; 19 – *H. trifolii*; 20 – NTC (non-template control).

DISCUSSION

Nematodes interfere with the metabolic balance of the plant and inhibit hydrostatic water pressure that result in wilting (Dababat *et al.*, 2011). Various nematologists have compared nematode density in soil with crop yield losses. In 1963 in the Siberian region, 100 ha of wheat died and massive levels of CCN infection were discovered in 300 ha. In order to avoid economic losses, wheat and oat collective farms have frequently tilled CCN-infected crops into the soil and planted a non-host. In 1964, around 124 ha of wheat were ploughed again because soil contained a very high level of infestation resulting in yield losses of 168.5 t (Zhuk, 1969). A level of infection of around 20-30 juveniles per plant can cause losses of around 0.6-0.8 t ha^{-1}. In 1968 the yield of wheat infected by *H. avenae* (50-100 J2 $plant^{-1}$) was 0.4 t ha^{-1}, in contrast to the usual 1.8 t ha^{-1} in clean fields. The areas infested by CCN under cereal production in 1968 were around 3,000 ha in the Novosibirsk Region (Sveshnikova & Terenteva, 1965) and 2,200 ha in the Tyumen Region under wheat, oats and barley.

A level of infestation of around 200-500 cysts of "*H. avenae*" per 1 kg of topsoil (0-10 cm) can cause level of wheat yield losses around 60%, at 10-20 cm depth of around 30%, and at 20-30 cm of around 5-7%. Approximately 1,000 cysts (1 kg topsoil)$^{-1}$ causes death of wheat plants. Levels of potential damage are measured based on population density before cereal planting. In Bashkiria, a yield loss around 5% for wheat was established at 300-400 J2 100 cm^{-3} soil, in Western Siberia at 200 J2, and at 1000 J2, 30%. In the Northwest region, injury appears periodically, after nematodes accumulate in soil in years with favourable weather. Given the area infested by nematodes (about 1 million ha), losses of cereals can be considerable. To minimise losses, the use of cereals in crop rotations should not exceed 20%; it is necessary to use fallow land, deep under-winter ploughing, and weakly susceptible or resistant varieties of barley (Popova, 1975).

Cereal cyst nematodes induced yield losses of around 0.8-1.0 t ha^{-1} (up to 70%) for wheat and 0.3-0.4 t ha^{-1} (up to 25-30%) for barley (Mamonova, 1969). Losses of wheat in the Novosibirsk Region were 0.4-0.8 t ha^{-1} (Zhuk, 1969). The yield of wheat infected by "*H. avenae*" in the Tyumen Region was usually half that of uninfected wheat. Rye and maize were more resistant (Tikhonova, 1986).

L.V. Tikhonova and colleagues in 1967 inspected some fields of winter rye, spring wheat, oat, barley, maize and panicum in the Ural and Volga zones to detect population density of "*H. avenae*" (Tikhonova *et al.*, 1967). They used a 5-number scale: 1 – 1-35 viable cysts (kg soil)$^{-1}$; 2 – 35-70; 3 – 70-200; 4 – 200-500; 5 – 500-5000. Ninety-eight farms (52,000 ha) were inspected and cysts of "*H. avenae*" were discovered in 20,800 ha (under wheat – 44%, oats – 41%, rye – 35%, barley – 24% and maize – 28%). A high CCN density (480 cysts (kg soil)$^{-1}$) under wheat was discovered in the Perm Region (73% infected fields). A major infection of oat was discovered in the Samara Region (1,075 cyst (kg soil)$^{-1}$) and Sverdlovsk Region (675 cysts (kg soil)$^{-1}$). Range of cyst infection (by 5-number scale) in Tatarstan, Udmurtia and Orenburg region was from 1 to 3 (Popova, 1971).

Around 67% of the fields from 312 farms in Bashkiria were infected by CCN. In 1967 in the south of Bashkiria, 10,500 ha were inspected and 81.1% of the fields were infested with "*H. avenae*", and it caused 5.4-23% yield losses (Tikhonova, 1986). The wide distribution and high level of soil infestation by cysts of *Heterodera* spp. were discovered in agricultural zones where 60-70% of the entire area was under cereals (Popova, 1975).

Many former USSR nematologists have examined the effects of CCN different crop; few have paid attention to the morphology of nematodes. The study of cereal cyst nematodes in Russia was most intensive during 1960-1990. Many publications from this period concentrated on the agricultural side of CCN: the distribution of "*H. avenae*" from the European part of the former USSR to the Far East (Popova, 1971; Shiabova, 1982; Terenteva & Shiabova, 1973; Osipova, 1984); the biology of "*H. avenae*" (Popova, 1975; Osipova, 1986); the yield losses caused by CCN and the development of chemical and biological control of this species (Popova, 1972; Terenteva & Zhemchuzhina, 1982). The morphological description of *H. filipjevi* by Madzhidov (1981, 1985) coincided with the decay of Russian study of CCN. Because symptoms and agricultural impact of *H. avenae* and *H. filipjevi* are similar, nobody checked if previous studies with "*H. avenae*" in USSR utilised correctly identified nematodes or not. The first evidence of incorrect identification of "*H. avenae*" was presented by Subbotin *et al.* (1996). Six populations of CCN previously described as "*H. avenae*" from Leningrad Region (Terenteva & Shiabova, 1973), Saratov Region (Osipova, 1984) and the Republic of Bashkortostan (Tikhonova *et al.*, 1967) were subsequently identified by morphological and molecular methods as *H. filipjevi* or *H. pratensis* (Subbotin *et al.*, 1996, 2003).

Table 3. Morphometric measurements of second stage juveniles of cereal cyst nematodes from the middle Volga River and South Ural regions of Russia (values are mean ± s.d. (range))

Population Parameters	Saratov reg.	Chelyabinsk reg.	the Republic of Bashkortostan (Subbotin et al., 1996)	Paratype H. filipjevi (Madzhidov, 1981)
Juveniles (n)	31	47	20	45
Body length	512.4±4.7 (475.6-548.3)	532.8±4.2 (501.5-552.2)	552±3.7(514-573)	506 (431-581)
a	24.6±0.35 (21.7-27.4)	24.1±0.3 (22.4-26.9)	25.2±0.3 (23.9-27.2)	23.6 (21-25)
b	4.4±0.07 (3.9-5.2)	4.6±0.1 (4.1-5.3)	4.5±0.1 (4.1-5.0)	3.8 (3.3-4.4)
L/MB (b')	6.9±0.1 (6.4-7.9)	7.1±0.1 (6.6-7.9)	7.1+0.1 (6.6-8.0)	–
c	8.6±0.1 (8.2-9.3)	9.3±0.1 (8.7-10.0)	9.2±0.1 (8.6-10.2)	9.1 (6.8-10.7)
Tail length/BWA (c')	3.9±0.1 (3.7-4.4)	3.7±0.2 (3.3-4.1)	3.9±0.1 (3.5-4.4)	–
Stylet length	24.6±0.1 (23.7-25.6)	25±0.2 (23.2-25.9)	25.4±0.2(23.5-26.5)	26.5 (21.7-30.8)
DGO	6.1±0.2 (5.4-7.1)	6.4±0.3 (4.5-7.1)	4.3±0.1 (3.9-5.1)	3.7 (3.5-4.2)
Anterior end to excretory pore	105.5±1.1 (96.4-112.9)	109.0±1.5 (102.3-118.4)	114±2.0 (105-125)	95 (77-109)
Anterior end to valve of median bulb	74.6±0.9 (66.2-79.6)	75.1±0.9 (69.7-82.1)	78±0.9 (71-86)	69.6 (57-84)
Pharynx length	117.4±1.3 (104.2-127.4)	116.4±2.2 (104.5-129.9)	122.1±1.4 (115-133)	132 (115-158)
Mid-body width	20.8±0.2 (19.3-22.1)	22±0.2 (20.7-23.2)	21.8±0.2 (20.4-23.5)	22.9 (21-24.5)
Body width at anus	15.2±0.1 (14.5-15.7)	15.5±0.1 (14.3-16.4)	15.4±0.1 (14.3-16.3)	–
Tail length	59.8±0.9 (54.3-65.5)	57.3±0.8 (53.3-62.9)	60.2±0.9 (55.1-67.3)	57 (49-63)
Hyaline part of tail length	37.2±0.7 (33.4-41.1)	34.8±0.6 (32.1-40.2)	38.9±0.6 (35.7-44.9)	35 (31-39)

Table 4. List of primers developed for identification of cereal cyst nematodes of the genus *Heterodera* by single-step PCR

Nematode species	Primers code and sequences	Annealing temp., °C	Amplicon size, bp
H. filipjevi	HfilITS200 5'-ACTCGTTGCTGAGCAAAGTGATAATA-3' HfilITS540 5'-CTCATTAAGTCTTAAGCCACGTGCTAT-3'	60	340
H. avenae, *H. pratensis*	HavITS200 5'-TTGCTGAGCAAAGTGAAAAGCC-3' HavITS540 5'-TTGAGTCTTAAGCCACGTGCAGA-3'	62	330
H. latipons	HlatITS-For 5'-GGCTGCTGTGAGGCAAATG-3' HlatITS-Rev 5'-GCCACGTGCTATCAGCAA-3'	59	288

In our study, seven populations of CCN were discovered in the Middle Volga River and South Ural regions. These populations were recognised as *Heterodera filipjevi* by morphological and morphometric analyses. Previous studies of CCN in the former USSR indicated that "*H. avenae*" is widely distributed in agricultural regions. However, *H. filipjevi* is closely related to "*H. avenae*", and only minor morphological and morphometric differences can differentiate them from each other (Handoo, 2002).

Some populations of CCN originally described from 1960-1990 as "*H. avenae*" from two locations (Saratov and Chelyabinsk Agriculture Institutes)

were recollected from the same places during our study. Morphological identification showed that those populations are actually *H. filipjevi* (Pridannikov, 2011, 2012).

Recent studies that included molecular (Subbotin *et al.*, 2010) and morphological and morphometric (Pridannikov, 2011, 2012) methods have shown that populations from the Middle Volga River and South Ural regions previously identified in Russia as "*H. avenae*" are actually *H. filipjevi*. No one showed the presence of *H. avenae* in agricultural areas in Russia until now. The species-specific primers developed in this research confirm that *H. filipjevi* is the single CCN species in the agricultural fields studied. Some other *Heterodera* species from the *Avenae* group were found in wild areas but are not yet identified (unpublished data). At the present time, there are not enough laboratories to conduct time-consuming morphological identifications of CCN species, but the molecular assays presented here for identification of *H. avenae*, *H. filipjevi* or *H. latipons* may be developed further into commercial PCR kits to facilitate mass identification in test laboratories.

Molecular techniques provide efficient tools for a reliable and rapid identification of cyst nematodes. In our study, species-specific primers for *H. avenae*, *H. filipjevi* and *H. latipons* were developed based on the ITS1 region of ribosomal RNA as one of the most characterised nucleotide sequences for CCN. A single-step PCR-based approach with species-specific primers has been used for identification of closely related species of the *Avenae* group (Peng *et al.*, 2013; Toumi *et al.*, 2013a, b; Yan *et al.*, 2013).

SCAR-PCR assay based on RAPD-markers was developed for detection and identification of *H. filipjevi* from infected wheat roots and soil (Peng *et al.*, 2013). The well-characterised internal transcribed spacer region and mitochondrial cytochrome oxidase subunit 1 (COI) gene were utilised for design of primers distinguishing *H. filipjevi* and *H. avenae* (Yan *et al.*, 2013; Toumi *et al.*, 2013a). To detect *H. latipons* populations Toumi *et al.* (2013b) designed species-specific primers based on the sequence of actin gene. In our study, newly designed primers HavITS200/HavITS540, HlatITS-For/ HlatITS-Re v, and HfilITS200/ HfilITS540 were able to detect successfully *H. avenae/H. pratensis*, *H. latipons* and *H. filipjevi*, respectively. No positive reaction was observed for any of the other cyst nematode species we examined. Newly designed primers among others can be utilised for even more accurate molecular diagnosis of closely related nematodes of the *Avenae* group.

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M.V. Pridannikov, T.P. Suprunova, D.V. Shumilina, L.A. Limantseva, A.M. Skantar, Z.A. Handoo and D.J. Chitwood. Морфологическое и молекулярное изучение злаковой цистообразующей нематоды *Heterodera filipjevi* на Средней Волге и Южном Урале России.

Резюме. В период 2010-2012 гг. на Средней Волге и Южном Урале (Российская Федерация) было проведено обследование территорий с целью определить распространение и разнообразие злаковых цистообразующих нематод (ЗЦН), в том числе *Heterodera filipjevi*. В общей сложности было отобрано 270 проб почвы. В Саратовской и Челябинской областях были найдены семь популяций ЗЦН. Самая высокая плотность популяции нематод была установлена в Челябинской области, со средним значением 100 цист на 100 г почвы. Полученные морфологические и морфометрические данные нематод из этих популяций показывают изменчивость по ширине цист, длине нижнего моста и вульварной щели, по расстоянию от ануса до вульвы, а также по размеру личинок второго возраста. Для молекулярной характеристики ДНК экстрагировали из цист каждой популяции. Видоспецифические праймеры для *H. avenae*, *H. filipjevi* и *H. latipons* были разработаны для обычной ПЦР-амплификации внутреннего транскрибируемого спейсера рибосомной РНК (ITS1-rRNA). Пары праймеров, разработанных для *H. filipjevi* и *H. latipons*, позволяли получить ПЦР-продукты только для целевых видов, тогда как праймеры для *H. avenae* амплифицировали ДНК нематод вида *H. pratensis*, в дополнение к целевому виду. Молекулярные данные подтвердили принадлежность нематод из 7 изученных популяций к виду *H. filipjevi*.