Species-Specific PCR Assays for Differentiating *Heterodera filipjevi* and *H. avenae*

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Abstract

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Heterodera avenae and *H. filipjevi* are economically important cyst nematodes that restrict production of cereal crops in the Pacific Northwest United States and elsewhere in the world. Identification of these two species is critical for recommending and implementing effective management practices. Primers were designed from the internal transcribed spacer (ITS) regions of *H. avenae* and *H. filipjevi* ribosomal DNA. The primers were highly specific when examined on target isolates but did not amplify DNA from nontarget *Heterodera*, *Globodera*, *Meloidogyne*, *Pratylenchu*s, and other nematode species tested. Polymerase chain reaction (PCR) and amplification conditions were estab-

lished, and *H. avenae* and *H. filipjevi* were clearly distinguished by PCR fragments of 242 and 170 bp, respectively. Robust PCR amplification was achieved with DNA extracted from a single egg or second-stage juvenile (J2) using a laboratory-made worm lysis buffer, and DNA from 0.5 egg or J2 using a commercial kit. The PCR assays were successfully employed for differentiation of *H. filipjevi* and *H. avenae* populations collected from eight locations in three Pacific Northwest states. This is the first report of a species-specific ITS PCR assay to detect and identify *H. filipjevi*. The assays for both species will enhance diagnosis of cereal cyst nematode species in infested fields.

The cereal cyst nematodes (CCN) are a widespread and important group of sedentary plant-parasitic nematodes that restrict production of cereal crops throughout the world (22). The cyst nematode genus *Heterodera* includes more than 60 species (42). Twelve species in the *Heterodera avenae* complex (10) infect roots of cereals and grasses. Three of them–*H. avenae* Wollenweber, 1924; *H. filipjevi* (Madzhidov, 1981) Stone, 1985; and *H. latipons* Franklin, 1969–are considered to be the most economically important cyst nematodes that affect cultivated cereals worldwide (15,22,25).

In the United States, four species of CCN (*H. avenae*, *H. filipjevi*, *H. mani*, and *H. ustinovi* [= *H. iri*]) have been reported but only two of them (*H. avenae* and *H. filipjevi*) are parasitic to wheat and known to be present in Pacific Northwest (PNW) fields (44). *H. ustinovi* is not associated with wheat and *H. mani* does not reproduce readily on wheat (33), and these two species have not been reported in PNW fields (R. Ingham, Oregon State University, Corvallis, *personal communication*). *H. avenae* was first detected in Oregon in 1974 (13) and is now known to occur in at least seven western states, including three PNW states: Idaho, Oregon, and Washington (7–9,25,26,30). High populations of *H. avenae* reduced yields of susceptible wheat cultivars as much as 50% (25,26,30). It was estimated that this nematode reduces the profit from wheat production by at least $3.4 million annually in the PNW (25). The first detection of *H. filipjevi* in the United States was in Oregon during 2008 (31,43). The extent to which *H. filipjevi* occurs in the western United States remains

unclear because there have been no systematic nematode surveys for this species.

H. filipjevi is closely related to *H. avenae* in the *H. avenae* group complex and morphological differences among cysts and second-stage juveniles (J2s) are very minor (10,33). Distinction between *H. avenae* and *H. filipjevi* using microscopic procedures is time consuming, requires skill and training, and is frequently inconclusive because individual specimens may vary greatly within a population (44). Due to this complexity and the potential economic liability, commercial laboratories in the PNW routinely identify CCN only to the genus level. They report "*Heterodera* spp." but do not differentiate *H. avenae* from *H. filipjevi*. Precise identification of species is important for recommending and implementing effective management practices (e.g., selection of resistant or tolerant cultivars requires knowledge of the identity of individual species because such cultivars are not necessarily resistant or tolerant to both species) (16).

Molecular techniques offer more rapid and accurate alternatives for distinguishing species of cyst nematodes than morphological identification (4,5,23,32,36). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) repeat unit has facilitated quick and precise identification of plant-parasitic nematode species, including cyst nematode species and subspecies (1,5,14,23,31,34,35,39,41). A PCR-RFLP protocol was previously reported to discriminate the species of *Heterodera* populations collected from PNW infested fields based on cleavage by up to six restriction endonucleases (44). Because PCR-RFLP requires the additional restriction cleavage step with combinations of several enzymes (23,33,44), a single-step PCR using species-specific primers is preferable.

Single-step PCR amplification with species-specific primers has been used for identification of several cyst nematodes such as *H. latipons*, *H. glycines*, *H. schachtii*, *H. avenae*, and *H. filipjevi* without need for a subsequent RFLP step (4,19,20,32,37,38). A commercial diagnostic laboratory, South Australia Research and Development Institute, uses a proprietary species-specific real-time PCR procedure to identify and quantify *H. avenae* along with two root-lesion nematode species and six fungal pathogens of wheat in

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DNA extracted from soil (11,18,21,38). Toumi et al. (38) targeted the mitochondrial cytochrome oxidase subunit 1 (COI) gene and developed two species-specific primer sets to detect *H. avenae* and *H. filipjevi*. Peng et al. (20) detected and identified *H. filipjevi* using species-specific sequence-characterized amplified region (SCAR) primers designed from randomly amplified polymorphic DNA markers. However, there are no published reports of a species-specific PCR assay using the ITS region of rDNA to detect and identify *H. filipjevi* and *H. avenae* and distinguish them from other closely related *Heterodera* spp. An increasing number of ITS sequences are now available from GenBank, which allows specificity of ITS PCR primers to be assessed by sequence analyses performed in silico using a computer program and many known DNA sequences from public databases (6,17,24,45–47).

The objectives of this study were to (i) develop species-specific ITS PCR assays for detection and differentiation of *H. filipjevi* and *H. avenae* that would also distinguish them from other nontarget *Heterodera* spp. and other nematode species commonly present in PNW wheat fields, (ii) compare the predictions of primer specificity from in silico analysis with PCR test results and expand specificity evaluation to additional nematode species and isolates that were not available in the format of culture or DNA, and (iii) determine whether the assays were useful for detecting populations of these nematodes in PNW field soils.

Materials and Methods

Cyst extraction from soil and DNA extraction from nematodes. Cyst-nematode-infested soil was placed on the top of a set of sieves consisting of 710-, 425-, and 250-μm-pore sieves. Brown cysts were extracted from the soil using the sieving-decanting method (12). Cysts collected on the bottom sieve (250 μm) were picked up with a dissecting needle under a stereomicroscope, transferred to an Eppendorf tube containing sterilized water, and stored at 4°C for nematode identification. The cysts were poked open with the needle and intact eggs and juveniles were used for DNA extraction. Approximately 20 eggs or J2s were transferred in 20 μl of sterilized nanopure water using a glass pipette, placed on a concave glass slide, and cut into pieces under a microscope. DNA was extracted from these nematodes using the method described by Yan and Smiley (44) and Waeyenberge et al. (40). The nematode suspension containing the majority of nematode pieces (10 μl) was transferred to a 0.5-ml sterile tube with 8 μl of worm lysis buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM MgCl₂, 10 mM dithiothreitol, 4.5% Tween 20, and 0.1% gelatin). The contents were immediately frozen and kept at –20°C for at least 20 min. The nematode suspension was thawed and 2 μl of proteinase K at 600 μg/ml was added into each sample. Three samples were included for each *H. filipjevi* or *H. avenae* population. Proteinase K was carried out at 65°C for 1 h; then, the enzyme was inactivated at 95°C for 10 min. The nematode lysis mix was centrifuged at 16,000 × *g* for 3 min to remove nematode debris. The supernatant was transferred to a clean 0.5-ml tube and frozen at –20°C until used for the following PCR amplification. DNA was quantified using the NanoDrop ND-1000 Spectrophotometer.

Design of species-specific PCR primers. Ten primers for *H. filipjevi* and eight primers for *H. avenae* were designed using an alignment of *Heterodera* ITS sequences of rDNA obtained from GenBank. The alignment consisted of sequences from 25 accessions: 5 *H. filipjevi* (GU079654, AY148398, AY148399, AY148403, and AY148401), 8 *H. avenae* (EF153843, AY148353,

AY148355, AY148362, AY148364, AY148365, AY148369, and AY148374), 1 *H. arenaria* (AF274396), 2 *H. mani* (AY148375 and AY148378), 3 *H. aucklandica* (AF274398, AY148379, and AY148380), 2 *H. pratensis* (AY148391 and AY148388), 2 *H. australis* (AY148393 and AY148396), and 2 *H. ustinovi* (AY148406 and AY148407). The primers were analyzed for annealing temperature, GC content, hairpin, self-dimer, and heterodimer using OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc.) and synthesized by Invitrogen. The performance of these primers was evaluated with two isolates each of *H. filipjevi* and *H. avenae* based on PCR band intensity and specificity. The sequences of the best-performing primers (HfITS-F1 and HfITS-R1 for *H. filipjevi* and HaITS-F6 and HaITS-R4 for *H. avenae*) and their expected sizes of the PCR products are shown in Table 1. These two primer pairs were used for all experiments.

Evaluation of primer specificity. Four isolates of *H. filipjevi* from the United States, Turkey, and Iran and five isolates of *H. avenae* from the United States and Syria were used to test the specificity of the primers (Table 2). Eight other *Heterodera* spp. that were used as nontarget species included *H. latipons*, *H. ciceri*, *H. glycines*, *H. goettingiana*, *H. hordecalis*, *H. lespedezae*, *H. schachtii*, and *H. zeae* (Table 2). The specificity was also examined with eight isolates of *Pratylenchus* spp. (*Pratylenchus neglectus*, *P. penetrans*, *P. thornei*, and *P. zeae*), five isolates of *Globodera* spp. (*Globodera ellingtonae*, *G. pallida*, *G. rostochiensis*, *G. tabacum solanacearum*, and *G. tabacum tabacum*), six isolates of *Meloidogyne* spp. (*Meloidogyne arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, and *M. naasi*), one isolate of *Nacobbus aberrans*, and three isolates of other plant-parasitic nematode species (*Merlinius brevidens*, *Tylenchorhynchus* sp., and *Paratylenchus* sp.) that were commonly present in PNW wheat fields.

In silico analysis. The ITS sequences of some reference *Heterodera* spp. were available on the National Center for Biotechnology Information databases (http://www.ncbi.nlm.nih.gov/). The experimental PCR responses from the reference species were compared with those predicted by in silico analysis using the computer program PrimerSelect 5.00 (DNASTAR, Inc.). Seven reference *Heterodera* spp. were compared in this study, including *H. avenae* from the United States (JX024195 and EF153843), *H. filipjevi* from the United States (GU079654) and Turkey (JX024203 and JX024204), *H. ciceri* from Syria (JX024201), *H. hordecalis* from Italy (AY347924), *H. latipons* from Syria (JX024178), *H. schachtii* from France (EF611103), and *H. zeae* from Greece (GU145616) (Table 3).

To further predict the specificity of the primers to ITS sequences of additional nematode species and isolates or DNA that could not be obtained upon request, in silico analysis was also conducted using ITS sequences of 50 *Heterodera* accessions from GenBank (Table 3) and the PrimerSelect 5.00 (DNASTAR, Inc.). The ITS sequences included nine isolates of *H. avenae*; six isolates of *H. filipjevi*; four isolates each of *H. pratensis* and *H. schachtii*; three isolates each of *H. aucklandica*, *H. bifenestra*, *H. hordecalis*, *H. latipons*, *H. mani*, and *H. ustinovi*; two isolates each of *H. australis*, *H. ciceri*, and *H. glycines*; and one isolate each of *H. arenaria*, *H. iri*, and *H. turcomanica* from 20 countries (Table 3). The specificity of the primers to the sequences of *Heterodera* spp. was determined by primer-template duplex stability values (ΔG) as described by Schroeder et al. (24), Okubara et al. (17), and Yan et al. (45–47).

PCR assay. PCR reactions (25 μl) consisted of the DNA template (8.7 ng/μl), 1× PCR buffer (10 mM Tris-HCl, 1.5 mM

Table 1. Sequences of species-specific polymerase chain reaction (PCR) primers designed in this study and the expected size of PCR amplicons to differentiate *Heterodera filipjevi* and *H. avenae*

Species	Primer name[a]	Sequence (5′–3′)	Amplicon size (bp)
H. filipjevi	F: HfITS-F1	CCCGTCTGCTGTTGAGA	170
	R: HfITS-R1	ACCTCAGGCTTTTATTATCAC	…
H. avenae	F: HaITS-F6	ATGCCCCCGTCTGCTGA	242
	R: HaITS-R4	GAGCGTGCTCGTCCAAC	…

[a] F = forward primer and R = reverse primer.

MgCl$_2$, and 50 mM KCl, pH 8.3), 0.2 mM dNTPs, 1× Cresol Red in 20% glycerol, 0.5 μM each forward and reverse primer, and 0.75 U of *Taq* polymerase (Roche). PCR amplification was carried out in a MyCycler Thermal Cycler (Bio-Rad) using the following program: one initial cycle of denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 s, 58°C (HfITS-F1/HfITS-R1) or 64°C (HaITS-F6/HaITS-R4) for 30 s, and 72°C for 30 s; and a final extension of 72°C for 7 min. PCR products were partitioned on 2% agarose gel using 0.5× Tris-borate-EDTA buffer (45 mM Tris-borate and 2 mM EDTA, pH 8.0), and stained with ethidium bromide. Amplicon sizes were extrapolated using a 100-bp DNA ladder (Roche). Band pattern was visualized and photographed under UV light using a Polaroid digital camera, and analyzed by the Polaroid PhotoMAX Pro program (Polaroid).

To confirm the quality of DNA templates from the nontarget nematode species (Table 2) provided by L. Waeyenberge and F. Toumi (Belgium), R. Rivoal (France), and A. Skantar, the D3 region of the 28S rDNA was amplified using primers D3A (5′-GACCCGTCTTGAAACACGGA-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (2,3). PCR was conducted as described above for the species-specific PCR, using PCR amplification conditions as follows: one initial cycle of denaturation for 2 min at 94°C; followed by 40 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C; with a final extension of 7 min at 72°C.

Detection sensitivity of PCR amplification. Different populations of *H. filipjevi* or *H. avenae* eggs (n = 0, 1, 2, 3, 4, 5, 10, 15, and 29) were transferred in 20 μl of sterilized nanopure water using a glass pipette. One J2 was hand-picked using a dental pick and put into 20 μl of sterilized water. Nematode DNA was extracted using the worm lysis buffer described above to determine the minimum number of nematode individuals detectable in the PCR assays. Twofold serial dilutions of a single nematode DNA (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) were also used to test the sensitivity, with or without bovine serum albumin (BSA; Roche) at 0.8 μg/μl, in PCR reactions. For comparison with extraction using a commercial kit, *H. filipjevi* and *H. avenae* eggs (n = 0, 1, and 2) and J2s (n = 0 and 1) were added separately to PowerBead tubes provided with the PowerSoil DNA Isolation Kit (MoBio), and DNA was extracted according to the manufacturer's recommendations. A low number of *H. filipjevi* and *H. avenae* (0.5 egg or 0.5 J2) was also attempted; one egg or one J2 was cut in the middle into two pieces with a dissecting needle under a microscope and both pieces (0.5 of a body) were used separately for DNA extraction using the kit. The DNA extractions were replicated four times using the worm lysis buffer and three times using the commercial kit for each population. PCR was performed in duplicate for each independent DNA extraction. The sensitivity for PCR detection was determined as the minimum number of eggs or

Table 2. Isolates of *Heterodera* spp. and other nematode species used to test the species-specific polymerase chain reaction primers for differentiating *Heterodera filipjevi* and *H. avenae*

Species	Isolate code	Origin	Host	Source[a]
H. filipjevi	Hf1	Oregon, United States	Wheat	G. Yan
H. filipjevi	Hf2	Eskisehir, Turkey	Wheat	L. Waeyenberge
H. filipjevi	Hf3	Merkez, Turkey	Wheat	L. Waeyenberge
H. filipjevi	Hf4	Lorestan, Iran	Wheat	L. Waeyenberge
H. avenae	Ha1	Oregon, United States	Wheat	R. Smiley
H. avenae	Ha2	Oregon, United States	Wheat	R. Smiley
H. avenae	Ha3	Idaho, United States	Wheat	R. Smiley
H. avenae	Ha4	Washington, United States	Wheat	R. Smiley
H. avenae	Ha5	Syria	Wheat	F. Toumi
H. latipons	Hla1	Syria	Wheat	F. Toumi
H. latipons	Hla2	Iran	Wheat	L. Waeyenberge
H. ciceri	FaC3	Syria	Chickpea	F. Toumi
H. glycines	27B1	Maryland, United States	Soybean	A. Skantar
H. goettingiana	Vacirca cyst 1	Italy	Wheat	A. Skantar
H. hordecalis	Giurffo cyst 6	Italy	Wheat	A. Skantar
H. lespedezae	6A2	Egypt	Sorghum	A. Skantar
H. schachtii	Hs	Laon, France	Sugar beet	R. Rivoal
H. zeae	48C1	Greece	Corn	A. Skantar
Globodera ellingtonae	45E11	Oregon, United States	Potato	A. Skantar
G. pallida	22D3	Idaho, United States	Potato	A. Skantar
G. rostochiensis	27A12	NY, United States	Potato	A. Skantar
G. tabacum solanacearum	27C3	Virginia, United States	Tobacco	A. Skantar
G. tabacum tabacum	27E3	Connecticut, United States	Tobacco	A. Skantar
Meloidogyne arenaria	12H8	Maryland, United States	Tomato	A. Skantar
M. arenaria	34H6	DE, United States	Traveler's tree	A. Skantar
M. chitwoodi	13C8	Oregon, United States	Potato	A. Skantar
M. hapla	6L6	Canada	Rose	A. Skantar
M. incognita	12J4	Maryland, United States	Tomato	A. Skantar
M. naasi	12C1	Oregon, United States	Wheat	A. Skantar
Merlinius brevidens	Mb	Oregon, United States	Wheat	S. Easley
Nacobbus aberrans	38D1	Peru	Potato	A. Skantar
Paratylenchus sp.	Pa	Oregon, United States	Wheat	G. Yan
Pratylenchus neglectus	Pn1	Washington, United States	Wheat	R. Smiley
P. neglectus	Pn2	Oregon, United States	Wheat	R. Smiley
P. neglectus	Pn3	Idaho, United States	Wheat	R. Smiley
P. neglectus	Pn4	Montana, United States	Wheat	A. Dyer
P. penetrans	31H12	Maryland, United States	Corn	A. Skantar
P. thornei	Pt1	Oregon, United States	Wheat	R. Smiley
P. thornei	Pt2	Oregon, United States	Wheat	R. Smiley
P. zeae	22G8	Singapore	Spider lily	A. Skantar
Tylenchorhynchus sp.	Ty	Oregon, United States	Wheat	S. Easley

[a] Isolates were obtained from R. Smiley, G. Yan, and S. Easley, Oregon State University, Columbia Basin Agricultural Research Center, Pendleton; L. Waeyenberge and F. Toumi, Institute for Agricultural and Fisheries Research, Plant Sciences Unit–Crop Protection, Merelbeke, Belgium; R. Rivoal, INRA, Rennes, France; A. Dyer, Montana State University, Bozeman; or the frozen nematode collection of A. Skantar, United States Department of Agriculture–Agricultural Research Service, Nematology Laboratory, Beltsville, MD.

J2s of *H. filipjevi* or *H. avenae* producing an amplicon on agarose gels.

Identification of *H. filipjevi* and *H. avenae* from field soil. Thirty soil samples were collected from various wheat fields or fields with recent wheat rotation in the cropping systems. These fields were thought to be infested with *H. filipjevi* or *H. avenae* based upon the symptoms of patches of stunted or uneven wheat growth during our searches for field research sites to screen wheat cultivars for resistance and tolerance. These locations included farms near Cashup, Colfax, Palouse, and Steptoe in Washington; Cove, Imbler, and Island City in Oregon; and St. Anthony in Idaho (Table 4). Samples consisted of a pool of either 50 soil cores (2.5

Table 3. *Heterodera* spp. and GenBank accession number of internal transcribed spacer (ITS) sequences used for analysis of primer-template duplex stability (ΔG) for predicting the specificity of primers (HfITS-F1/ HfITS-R1) for *Heterodera filipjevi* and primers (HaITS-F6/HaITS-R4) for *H. avenae*

Species	GenBank number	Isolate, clone[b]	Origin	ΔG (kcal/mol)[a]			
				HfITS-F1	HfITS-R1	HaITS-F6	HaITS-R4
H. avenae	AY148353	Clone 1	Germany	ins	ins	−36.9	−34.1
H. avenae	AY148355	Clone 4	Spain	ins	ins	−36.9	−34.1
H. avenae	AY148362	Clone 1	India	ins	ins	−36.9	−34.1
H. avenae	AY148364	Clone 1	Turkey	ins	ins	−36.9	−34.1
H. avenae	AY148365	Clone 1	Israel	ins	ins	−36.9	−34.1
H. avenae	AY148369	Clone 1	Morocco	ins	ins	−36.9	−34.1
H. avenae	AY148374	Clone 5	France	ins	ins	−36.9	−34.1
H. avenae	GU595424	GS23	China	ins	ins	−36.9	−31.2
H. avenae	JN831608	XJ1	China	ins	ins	−36.9	−31.2
H. avenae[c]	JX024195	Did49	United States	ins	ins	−36.9	−34.1
H. avenae[c]	EF153843	N/A	Idaho, United States	ins	ins	−36.9	−34.1
H. filipjevi	AY148399	Clone 1	Spain	−32.6	−36.2	ins	ins
H. filipjevi	AY148400	N/A	Germany	−32.6	−36.2	ins	ins
H. filipjevi	AY148401	N/A	Russia	−32.6	−36.2	ins	ins
H. filipjevi	AY148402	N/A	Tajikistan	−32.6	−36.2	ins	ins
H. filipjevi	AY148403	N/A	United Kingdom	−32.6	−36.2	ins	ins
H. filipjevi	HM208382	HN32	China	−32.6	−36.2	ins	ins
H. filipjevi[c]	GU079654	Clone 1	Oregon, United States	−32.6	−36.2	ins	ins
H. filipjevi[c]	JX024203	Did42c	Turkey	−32.6	−36.2	ins	ins
H. filipjevi[c]	JX024204	Did42b	Turkey	−32.6	−36.2	ins	ins
H. arenaria	AF274396	N/A	United Kingdom	ins	ins	−36.9	−34.1
H. aucklandica	AF274398	N/A	New Zealand	ins	ins	−36.9	−34.1
H. aucklandica	AY148379	N/A	Belgium	ins	ins	−36.9	−34.1
H. aucklandica	AY148380	Clone 1	United Kingdom	ins	ins	−36.9	−34.1
H. australis	AY148393	Clone 1	Australia	ins	ins	−36.9	−31.2
H. australis	AY148396	Clone 2	Australia	ins	ins	−36.9	−31.2
H. bifenestra	AF274384	N/A	Sweden	ins	ins	ins	ins
H. bifenestra	AF274385	N/A	Belgium	ins	ins	ins	ins
H. bifenestra	AY569020	N/A	Sweden	ins	ins	ins	ins
H. ciceri	AF274393	N/A	Belgium	ins	ins	−33.4	ins
H. ciceri	AY045758	N/A	IN, United States	ins	ins	−33.4	ins
H. ciceri[c]	JX024201	FaC3	Syria	ins	ins	−33.4	ins
H. glycines	AY590280	HG-G-MI	Michigan, United States	ins	ins	−33.4	ins
H. glycines	EF611124	Hg1-Ark1 clone 1	Arkansas, United States	ins	ins	−33.4	ins
H. hordecalis	AF274401	N/A	United Kingdom	−32.6	ins	ins	ins
H. hordecalis	AF498381	N/A	Iran	−32.6	ins	ins	ins
H. hordecalis	AY692356	N/A	Indiana, United States	−19.5	ins	ins	ins
H. hordecalis[c]	AY347924	N/A	Italy	−32.6	ins	ins	ins
H. iri	AF274400	N/A	United Kingdom	−32.6	ins	ins	ins
H. latipons	AF498382	N/A	Iran	−25.9	ins	−13.0	ins
H. latipons	AY347925	N/A	Russia	−25.9	ins	−13.0	ins
H. latipons	HM560790	JOD	Jordan	−25.9	ins	−13.0	ins
H. latipons[c]	JX024178	Fa7A3	Syria	−25.9	ins	−13.0	ins
H. mani	AY148378	Clone 1	Germany	ins	ins	−36.9	−34.1
H. mani	AY148375	Clone 3	Germany	ins	ins	−36.9	−34.1
H. mani	AY692357	N/A	United Kingdom	ins	ins	−36.9	−34.1
H. pratensis	AY148351	N/A	Russia	ins	ins	−36.9	ins
H. pratensis	AY148385	Clone 1	Germany	ins	ins	−36.9	ins
H. pratensis	AY148388	Clone 1	Netherlands	ins	ins	−36.9	ins
H. pratensis	AY148391	Clone 3	Germany	ins	ins	−36.9	−31.2
H. schachtii	EF611110	BeM4-4 clone 3	Belgium	ins	ins	−33.4	ins
H. schachtii	EF611115	Ge2 clone 1	Germany	ins	ins	−33.4	ins
H. schachtii	EF611119	Aus12 clone 6	Australia	ins	ins	−33.4	−18.0
H. schachtii	EF611121	N11 clone 1	Netherlands	ins	ins	−33.4	−18.0
H. schachtii[c]	EF611103	Fr1 clone 3	France	ins	ins	−33.4	ins
H. turcomanica	AF498386	N/A	Iran	ins	ins	ins	ins
H. ustinovi	AY148405	Clone 1	Slovakia	−32.6	ins	ins	ins
H. ustinovi	AY148406	Clone 1	Germany	−32.6	ins	ins	ins
H. ustinovi	AY148407	Clone 1	Belgium	−32.6	ins	ins	ins
H. zeae[c]	GU145616	Clone 2304	Greece	ins	ins	ins	−16.2

[a] Values less than −31 kcal/mol indicate stable primer-template formation; ins = insignificant primer-template duplex stability value predicted to have no amplification.

[b] Name of isolates or clones obtained from GenBank; N/A = not available from GenBank.

[c] Reference isolates tested by the species-specific PCR assays.

cm in diameter by 30 cm deep) or 10 shovel slices (2.5 cm thick, 10 cm wide, and 10 cm deep). Soil was placed into large, unsealed plastic bags which were then placed in an insulated cooler at sampling. The soil samples were transported to our laboratory and stored at 4°C prior to extraction of cysts.

Subsamples (1 kg each) were sent to Western Laboratories for extraction and microscopic enumeration of nematodes. Western Laboratories used a modified Oostenbrink elutriator extraction method, as described by Smiley et al. (28), and reported numbers of cyst nematodes at the genus level. The population density of CCN was expressed as the numbers of *Heterodera* spp. eggs and juveniles in 1 kg of soil.

Cysts were extracted from the remaining soil samples for species identification in our laboratory. Nematode DNA was extracted in triplicate from approximately 20 eggs or J2s from at least five cysts isolated from each soil sample using the abovementioned worm lysis buffer. Species-specific PCR was carried out in duplicate for each extract. The species identity was also confirmed by comparing the PCR-RFLP restriction pattern with those of known species and by examining key morphological features of the cysts, including color, underbridge in the vulval cone, semifenestrae shape, and development of bullae under a compound microscope, as described by Yan and Smiley (44). A no-DNA template was used as a negative control and DNA from the *H. filipjevi* isolate Hf1 or the *H. avenae* isolate Ha1 (Table 2) were used as positive controls.

Results

Primer specificity. The primer pair HfITS-F1/HfITS-R1 (Table 1) designed for *H. filipjevi* produced a unique PCR amplicon of 170 bp with DNA from four *H. filipjevi* isolates, including Hf1 from the United States and Hf2 to Hf4 from Turkey and Iran, but did not amplify DNA from *H. avenae* isolates Ha1 to Ha5 (Table 2). The amplicon size was deduced from the sequence of *H. filip-jevi* from Oregon (GU079654). The primer pair HaITS-F6/HaITS-R4 designed for *H. avenae* amplified a specific band of 242 bp with DNA from the five *H. avenae* isolates (Ha1 to Ha4 from the United States and Ha5 from Syria) but did not produce any amplification with DNA from the four *H. filipjevi* isolates. The amplicon size was deduced from the sequence of *H. avenae* from Idaho (EF153843). The sizes of the amplified fragments on agarose gel were in agreement with those inferred from the nucleotide sequences.

Moreover, no specific PCR product was observed when the amplification was carried out with DNA from eight nontarget *Heterodera* spp. (*H. latipons*, *H. ciceri*, *H. glycines*, *H. goettingiana*, *H. hordecalis*, *H. lespedezae*, *H. schachtii*, and *H. zeae*) using either the *H. filipjevi* primers (Fig. 1A) or the *H. avenae* primers (Fig. 1B). Likewise, no specific PCR amplification was produced with DNA from eight isolates of *Pratylenchus* spp., five isolates of *Globodera* spp., six isolates of *Meloidogyne* spp., one isolate of *N. aberrans*, and three other plant-parasitic nematode species (*Tylenchorhynchus* sp., *M. brevidens*, and *Paratylenchus* sp.) (Table 2). The quality of nematode DNA templates used as controls was demonstrated in Figure 1C using the universal primers D3A and D3B.

In silico analysis. The primers HfITS-F1 and HfITS-R1 are expected to form specific duplexes with the ITS sequences of three reference *H. filipjevi* isolates from the United States and Turkey based on duplex stability (ΔG) values (Table 3). Accordingly, specific amplicons of the expected size were observed in the PCR tests. These primers are also predicted to form strong specific duplexes with the ITS sequences of six other *H. filipjevi* isolates from Spain, Germany, Russia, Tajikistan, the United Kingdom, and China. Specific amplification was reflected in ΔG values of –31 kcal/mol or less for the forward and reverse primers. The forward primer HfITS-F1 was predicted to form a hybrid with the ITS sequences of *H. hordecalis* (ΔG = –32.6 or –19.5), *H. iri* (ΔG =

Table 4. Soil samples collected from different locations in three Pacific Northwest states and population densities of cereal cyst nematodes (CCN) and species identity determined by the species-specific polymerase chain reaction (PCR) assays

| Soil name | Location | Collection date | CCN density[b] | PCR assay[a] | | Species[c] |
				Heterodera avenae	*H. filipjevi*	
S1	Cashup, WA	August 2010	10,560	+	–	*H. avenae*
S2	Cashup, WA	February 2012	2,880	+	–	*H. avenae*
S3	Cashup, WA	June 2010	9,600	+	–	*H. avenae*
S4	Colfax, WA	May 2010	70	+	–	*H. avenae*
S5	Colfax, WA	June 2010	800	+	–	*H. avenae*
S6	Palouse, WA	May 2010	4,740	+	–	*H. avenae*
S7	Palouse, WA	May 2010	5,270	+	–	*H. avenae*
S8	Steptoe, WA	February 2011	3,600	+	–	*H. avenae*
S9	Steptoe, WA	February 2011	1,920	+	–	*H. avenae*
S10	Steptoe, WA	June 2010	2,160	+	–	*H. avenae*
S11	Cove, OR	November 2010	3,600	+	–	*H. avenae*
S12	Cove, OR	November 2010	320	+	–	*H. avenae*
S13	Cove, OR	May 2010	1,860	+	–	*H. avenae*
S14	Cove, OR	February 2011	160	+	–	*H. avenae*
S15	Cove, OR	February 2011	720	+	–	*H. avenae*
S16	Imbler, OR	May 2010	150	+	–	*H. avenae*
S17	Imbler, OR	November 2010	40	–	+	*H. filipjevi*
S18	Imbler, OR	November 2010	60	–	+	*H. filipjevi*
S19	Imbler, OR	November 2010	120	–	+	*H. filipjevi*
S20	Imbler, OR	November 2010	160	–	+	*H. filipjevi*
S21	Imbler, OR	November 2010	80	–	+	*H. filipjevi*
S22	Imbler, OR	May 2008	1,110	–	+	*H. filipjevi*
S23	Imbler, OR	May 2008	2,446	–	+	*H. filipjevi*
S24	Island City, OR	October 2008	2,160	+	–	*H. avenae*
S25	Island City, OR	October 2008	3,000	+	–	*H. avenae*
S26	St. Anthony, ID	September 2010	14,880	+	–	*H. avenae*
S27	St. Anthony, ID	September 2010	9,000	+	–	*H. avenae*
S28	St. Anthony, ID	September 2010	15,480	+	–	*H. avenae*
S29	St. Anthony, ID	September 2011	6,600	+	–	*H. avenae*
S30	St. Anthony, ID	September 2011	8,184	+	–	*H. avenae*

[a] Species identity determined by the species-specific PCR assay: + = presence of the specific PCR amplicon and – = absence of the specific PCR amplicon.
[b] CCN density: the number of eggs and juveniles per kilogram of soil, as reported by Western Laboratories.
[c] Species confirmed by the PCR restriction fragment length polymorphism procedure and cyst morphology described by Yan and Smiley (44).

–32.6), *H. latipons* (ΔG = –25.9), and *H. ustinovi* (ΔG = –32.6) but the reverse primer HfITS-R1 did not form a hybrid with any of these ITS sequences. Amplification from a single primer (unidirectional PCR) failed to generate enough products to be detected by PCR (17), which was supported by our experimental PCR results using the reference isolates of *H. hordecalis* from Italy and *H. latipons* from Syria. The primers were not expected to form hybrids with the ITS sequences of five reference isolates of *H. avenae* from the United States, *H. ciceri* from Syria, *H. schachtii* from France, and *H. zeae* from Greece, which is consistent with the results obtained from our PCR tests. Therefore, the *H. filipjevi* specific primers are not predicted to result in ITS amplification of any of the 51 nontarget but economically important or closely related *Heterodera* isolates from more than 20 countries evaluated in this study (Table 3).

The primers HaITS-F6 and HaITS-R4 were expected to form specific duplexes with the ITS sequences of two reference *H. avenae* isolates from the United States because of the ΔG values below the stability cut-off (–31 kcal/mol; Table 3). As expected, specific amplicons were observed in the PCR tests with these two reference isolates. The primers were also predicted to form specific hybrids with the ITS sequences of nine other *H. avenae* isolates from Germany, Spain, India, Turkey, Israel, Morocco, France, and China. However, both primers were predicted to form stable primer-template duplexes with the ITS sequences from one isolate of *H. arenaria* (the United Kingdom), three isolates of *H. aucklandica* (New Zealand, Belgium, and the United Kingdom), two isolates of *H. australis* (Australia), three isolates of *H. mani* (Germany and the United Kingdom), and one isolate of *H. pratensis* (Germany). Fortunately, the *H. avenae* primers were not expected to generate specific amplification with the ITS sequences from all nine *H. filipjevi* isolates and the ITS sequences from 30 nontarget isolates belonging to 10 *Heterodera* spp. Amplifications from a single primer HaITS-F6 with the ITS sequences from *H. ciceri*, *H. glycines*, *H. latipons*, *H. pratensis* (three of four isolates), and *H. schachtii* (two of four isolates) or from a single primer HaITS-R4 with *H. zeae* failed to generate detectable PCR products, which was supported by our experimental PCR results using the reference isolates of *H. ciceri*, *H. latipons*, *H. schachtii*, and *H. zeae*. Amplifications from both primers with two isolates of *H. schachtii* (EF611119 and EF611121) also failed to produce enough stable PCR products to be detected in the PCR system because the ΔG value of –18.0 from HaITS-R4 was relatively high and, thus, predictive of poor primer-template binding and not expected to generate logarithmic amplification at the recommended annealing temperature (17).

Detection sensitivity of the species-specific PCR assays. Specific amplicons were obtained at all populations of eggs and J2s tested for both species. For *H. filipjevi*, the PCR assay detected one egg in three of the four replicated DNA extractions and one J2 in all four replicates using the worm lysis buffer. The assay detected 1:8 dilution of DNA from a single egg or J2 without BSA. It could detect even as low as 1:128 dilution of a single *H. filipjevi* nematode DNA by adding BSA to PCR. For *H. avenae*, the PCR test detected one egg or one J2 in all four DNA extractions. The assay detected 1:16 dilution of DNA from a single egg or J2 without BSA. It could detect as low as 1:128 dilution by adding BSA. Specific amplicons were also observed at a low number of *H. filipjevi* and *H. avenae* tested using the PowerSoil DNA Isolation Kit (Fig. 2). The PCR test detected 0.5 egg or 0.5 J2 of *H. filipjevi* in all three DNA extracts (Fig. 2A). Similarly, the PCR assay detected 0.5 egg of *H. avenae* in two of three DNA extracts and 0.5 J2 in all three replicated extracts (Fig. 2B). Specific amplicons were detected in all replicated DNA extractions for other levels of inoculation. No amplification was observed from the control samples without nematodes.

Distinguishing *H. filipjevi* and *H. avenae* from PNW wheat fields. The developed method was applied for detecting and discriminating *H. filipjevi* and *H. avenae* in 30 soil samples collected from wheat fields in Idaho, Oregon, and Washington. These samples harbored a range of nematode population densities, from 40 to 15,480 *Heterodera* spp. eggs and juveniles per kilogram of soil, based on enumerations by Western Laboratories (Table 4). Seven soil samples (S17 to S23) from different fields near Imbler, OR produced specific bands with the same size as the *H. filipjevi*-positive control (170 bp) in each of the three replicated DNA extractions. These soil samples did not produce any amplification with the *H. avenae* primers. Therefore, the nematode species in these soils was identified as *H. filipjevi*. In all, 23 other soil samples (S1 to S16 and S24 to S30) from various fields near Cashup, Colfax, Palouse, and Steptoe, WA; Cove, Imbler, and Island City, OR; and St. Anthony, ID produced PCR bands with the same size as the *H. avenae*-positive control (242 bp) in all replicates, and these soil samples did not show amplification using the *H. filipjevi*-specific primers, indicating that the nematode species in these soils was *H. avenae*. The species identity in each of these soil samples was also confirmed by the PCR-RFLP restriction pattern and cyst morphology (Table 4). The banding patterns for nematode DNA from seven soil samples (S1, S8, S11, S19-S21, and S24) amplified with the specific primers are shown in Figure 3.

Discussion

Two CCN, *H. filipjevi* and *H. avenae*, were specifically detected among a range of nematode-infested soils from three PNW states
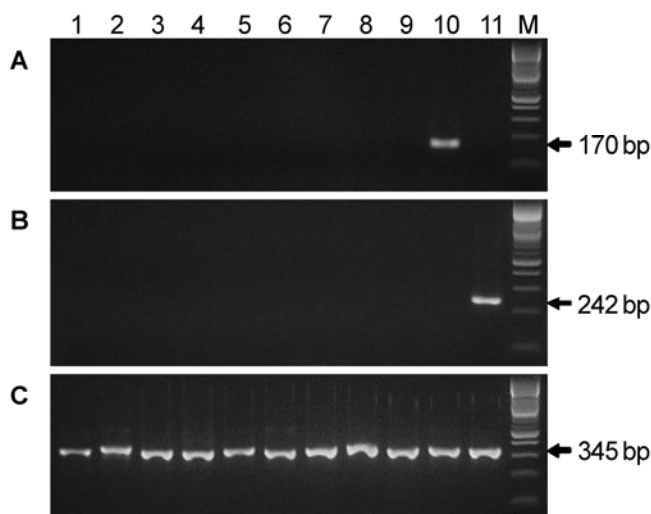

Fig. 1. Evaluation of primer specificity with control nematode species. **A,** Using specific primers HfITS-F1 and HfITS-R1 for *Heterodera filipjevi*. **B,** Using primers HaITS-F6 and HaITS-R4 for *H. avenae*. **C,** Using common primers D3A and D3B. Lane 1, *H. ciceri*; lane 2, *H. glycines*; lane 3, *H. goettingiana*; lane 4, *H. hordecalis*; lane 5, *H. lespedezae*; lane 6, *H. zeae*; lane 7, *Globodera pallida*; lane 8, *G. rostochiensis*; lane 9, *Meloidogyne chitwoodi*; lane 10, *H. filipjevi*; lane 11, *H. avenae*; lane M, 100-bp DNA molecular weight ladder.
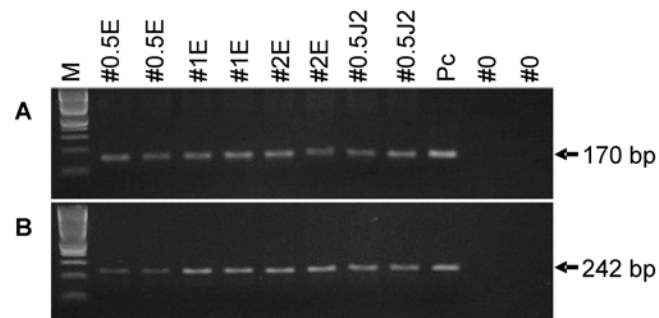

Fig. 2. Detection sensitivity of the species-specific polymerase chain reaction assays. **A,** *Heterodera filipjevi*: 0.5 egg (#0.5E), 1 egg (#1E), 2 eggs (#2E), 0.5 second-stage juvenile (#0.5J2), positive control of *H. filipjevi* (Pc), and negative control (#0). **B,** *H. avenae*: 0.5 egg (#0.5E), 1 egg (#1E), 2 eggs (#2E), 0.5 second-stage juvenile (#0.5J2), positive control of *H. avenae* (Pc), and negative control (#0). M: 100-bp DNA ladder.

(Idaho, Oregon, and Washington) and in isolates from three other countries (Iran, Syria, and Turkey) using the assays developed in this study. The assays were sensitive because they were able to detect a single egg or J2 using a laboratory-made lysis buffer, or 0.5 egg or J2 using a commercial kit. The assay for *H. filipjevi* was proven to be highly specific when tested with template DNA from 41 isolates of *Heterodera* spp. and non-*Heterodera* species, including plant-parasitic nematode species commonly present in PNW wheat fields. The primer pair for *H. filipjevi* was also predicted to be highly specific by in silico analysis using the ITS sequences from 60 isolates of closely related or economically important *Heterodera* spp. from more than 20 countries. To our knowledge, this is the first report of a species-specific ITS PCR method for detection and identification of *H. filipjevi*.

The predictions obtained from in silico analysis are consistent with the results obtained in our PCR tests with the 10 reference isolates of *H. filipjevi*, *H. avenae*, *H. latipons*, *H. hordecalis*, *H. schachtii*, *H. ciceri*, and *H. zeae*. In silico analysis was then applied to 50 other isolates of *Heterodera* spp. that could not be obtained in culture or DNA upon request to predict the specificity of the primers. The primer pair for *H. filipjevi* was more specific than that of *H. avenae*, based on predicted amplification of the latter to DNA of five *Heterodera* spp. (*H. arenaria*, *H. aucklandica*, *H. australis*, *H. mani*, and *H. pratensis*). In silico analysis has been previously used to predict the specificity of PCR primers for diagnostics of Asiatic citrus canker disease (*Xanthomonas citri* pv. *citri*), root-lesion nematodes (*P. thornei* and *P. neglectus*), Rhizoctonia root rot (*Rhizoctonia solani* and *R. oryzae*), and Pythium root rot (*Pythium* spp.) (6,17,24,45–47). Delcourt et al. (6) reported that sequence analyses conducted in silico from public databases for diagnostics of *X. citri* pv. *citri* was very informative and useful, and should be performed to efficiently evaluate primer specificity because an increasing number of sequences are now available from GenBank, which is fully supported by our findings for diagnostics of the two cyst nematode species *H. filipjevi* and *H. avenae*.

The species-specific primers were designed from the variable ITS-rDNA sequences of *Heterodera* spp. The ITS region was thought to be variable and, therefore, useful for nematode identification at species level (5,23,48). Yan and Smiley (44) reported that six restriction endonucleases (*Taq*I, *Hin*fI, *Pst*I, *Hae*III, *Rsa*I, and *Alu*I) and their combinations used for PCR-RFLP based on the ITS region allowed for distinguishing *H. avenae* and *H. filipjevi*, and for separating these two species from *H. arenaria*, *H. hordecalis*, *H. latipons*, *H. mani*, *H. pratensis*, and *H. schachtii*. The PCR assay specific for *H. filipjevi* is preferred over the PCR-RFLP analysis, because this PCR assay is expected to successfully differentiate *H. filipjevi* in one-step PCR, without additional steps of RFLP analysis and combination of multiple enzyme digestions, from all the above species evaluated by the PCR-RFLP and from all other *Heterodera* spp. evaluated in this study.

The PCR assay for *H. avenae* successfully differentiated *H. avenae* from *H. filipjevi* and *H. latipons* (two other economically important CCN), *H. glycines* and *H. schachtii* (two very widespread and economically important cyst nematodes), *H. hordecalis* (a closely related species in the *H. avenae* group), four other *Heterodera* spp., and all non-*Heterodera* species in our tests. However, in silico analysis predicted cross reactivity between the *H. avenae* primers and the sequences of *H. arenaria*, *H. aucklandica*, *H. australis*, *H. mani*, and one isolate of *H. pratensis* due to the lack of polymorphisms in the ITS sequences between these species. *H. arenaria*, *H. aucklandica*, *H. australis*, and *H. pratensis* that occur in other countries have not been reported in the United States (33,44). To our knowledge, *H. mani* was reported in the United States but has not been found in the PNW. *H. mani* also does not reproduce readily on wheat, oat, and barley (33). Therefore, cross reactions of the primers with the five species should not interfere with the diagnosis of *H. avenae* in the PNW fields in which all of these five species are currently absent. Although Toumi et al. (38) developed species-specific primer sets to detect *H. avenae* and *H. filipjevi* on the basis of the COI gene and Peng et al. (20) developed species-specific SCAR-PCR assays to detect *H. filipjevi*, these five species were not included in their tests, except that one isolate of *H. pratensis* from Belgium was examined by Toumi et al. (38). Four isolates of *H. pratensis* from Germany, the Netherlands, and Russia evaluated in our study were not tested by their assays.

A duplex PCR was attempted for differentiating these nematodes but, as a result, it was not suitable for identifying mixtures of these two species. The primer pairs for both species were mixed in the same PCR reactions. When tested with pure DNA from only one species, specific amplifications occurred and the species could be accurately identified. However, when tested with mixed DNA from equal amounts of DNA from these two species, *H. avenae* produced strong amplification but *H. filipjevi* had very weak amplification, indicating that the two primer pairs were not suitable in a duplex PCR for identifying mixed populations of these two species. This was probably due to the big difference in annealing temperatures, 58°C for the *H. filipjevi* primers and 64°C for the *H. avenae* primers.

Both primer pairs used in separate PCR reactions detected a single egg or J2 using a laboratory-made lysis buffer and detected 0.5 egg or 0.5 J2 using a commercial kit. They could detect even as low as 1:128 dilution of DNA from a single egg or J2 for both species by adding BSA to PCR. This detection sensitivity was higher than those reported for cyst nematodes. Subbotin et al. (32) described a PCR method with species-specific primers for rapid identification of the soybean cyst nematode *H. glycines* and was able to detect a single cyst or J2. Ou et al. (19) designed species-specific SCAR primers to amplify DNA from a single J2 or female of *H. glycines*. Peng et al. (20) detected 1:8 dilution of DNA from a single J2 of *H. filipjevi* using the SCAR-PCR assays. Toumi et al. (38) could detect 0.5 J2 of *H. avenae* or *H. filipjevi* among 100 J2s of *H. latipons* using species-specific primers designed from the COI gene. BSA, as a PCR enhancer, greatly improved the detection of *H. filipjevi* and *H. avenae*, which was supported by our reports for detection and quantification of the root-lesion nematode *P. neglectus* from soil (46,47).

The end-point PCR primers were tested to determine whether they were directly applicable in a SYBR Green I-based real-time PCR format. The *H. filipjevi* primers produced specific amplification from DNA templates of all isolates of *H. filipjevi* and did not produce specific amplification from all isolates of *H. avenae* and nontarget nematode species tested in this study. The mean cycle threshold (Ct) values for 1 J2, 1 egg, and 26 eggs were 33.70, 33.45, and 27.48, respectively. The primers for *H. avenae* generated specific amplification from DNA templates of all isolates of *H. avenae*, and did not generate specific amplification from all isolates of *H. filipjevi*. The mean Ct value for 26 *H. avenae* eggs was 22.47. However, these primers consistently produced secondary peaks in melting curve analyses, leading to overestimation of
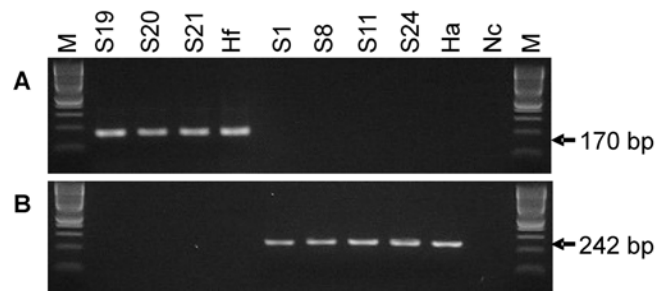

Fig. 3. Specific polymerase chain reaction (PCR) amplification using DNA from cysts isolated from field soils in the Pacific Northw4est. **A,** PCR bands (170 bp) amplified with primers HfITS-F1 and HfITS-R1 for *Heterodera filipjevi*. **B,** PCR bands (242 bp) amplified with primers HaITS-F6 and HaITS-R4 for *H. avenae*. DNA templates from the soil samples S1, S8, S11, S19, S20, S21, and S24 (Table 4) in Washington, Oregon, and Idaho were used. Hf: the *H. filipjevi* isolate (Hf1, Table 2) used as a positive control; Ha: the *H. avenae* isolate (Ha1, Table 2) used as a positive control; and Nc: negative control without DNA template. M: 100-bp DNA molecular weight ladder.

the target nematodes. We tested several other primer pairs designed from the same ITS regions but they were also not suitable for quantification. This might be due to the greater sensitivity of the real-time PCR method compared to the end-point PCR with agarose gel electrophoresis. The same scenario also occurred when we applied the end-point PCR primers to the real-time PCR for quantifying *P. neglectus* and *P. thornei* (45–47). Therefore, SYBR Green real-time PCR using a different DNA region than ITS-rDNA or TaqMan real-time PCR would have to be developed for detection and quantification of *H. avenae* and *H. filipjevi* from infested soil.

The species-specific PCR assays identified all the tested *H. filipjevi* and *H. avenae* populations that originated from three states in the PNW. To our knowledge, this is the first report of specific ITS PCR primers for distinguishing *H. avenae* and *H. filipjevi*. Mixed populations of these two species were not detected in the field samples used in this study. One possible reason is that *H. avenae* is more widely distributed than *H. filipjevi* in this region and the occurrence of mixed species is not common. Another reason could be that both species may occur in a single field but our samples might have skipped the sites that contained mixed populations. Our previous research with grid soil sampling demonstrated that CCN were unevenly distributed in a field (44).

Species identification is important for recommending and implementing effective management practices. Currently, practices for managing cereal cysts nematodes are limited (29). There are no registered chemical or biological controls that are effective for reducing populations of these cyst nematodes and increasing yields of wheat in the PNW. Rotations of wheat with multiple years of broadleaf crops or bare fallow reduce population densities and improve wheat yields compared with annual wheat (26) but crop rotations do not allow sufficiently intensive wheat production and, therefore, are generally not profitable for PNW wheat producers. The use of wheat cultivars that are both resistant and tolerant is the best approach to control damage from these nematodes (25). Lately, Smiley et al. (27) identified one commercial wheat cultivar and four breeding lines with resistance to *H. avenae* in naturally infested fields in the PNW but the resistance reactions to *H. filipjevi* were unknown. However, overseas research indicated that individual wheat cultivars could differ in their ability to resist these two nematodes; a cultivar exhibiting resistance to *H. avenae* was not necessarily resistant to *H. filipjevi*, and vice versa (16). Therefore, accurate identification of these two species in each field or region is important for selecting the best-performing cultivars to maintain high crop production.

The developed PCR assays will enable research and commercial laboratories to rapidly and efficiently diagnose the threat from individual species of CCN and facilitate systematic nematode surveys to investigate the geographic distribution of *H. filipjevi* in the western United States. This diagnostic capability is expected to become increasingly important as grain growers become more aware of damage from these root parasites, and as wheat cultivars with effective resistance genes are developed and planted in highly infested fields in the PNW.

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