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Development of two species-specific primer sets to detect the cereal cyst nematodes *Heterodera avenae* and *Heterodera filipjevi*

Fateh Toumi · Lieven Waeyenberge · Nicole Viaene · Amer Dababat · Julie M. Nicol · Francis Ogbonnaya · Maurice Moens

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Abstract Twelve *Heterodera* species are of major economic significance in wheat and barley. Of these, *H. avenae*, *H. filipjevi* and *H. latipons* are among the most important ones, and sometimes coexist. The identification of *Heterodera* species using morphological characteristics is time consuming, requires specialized skill and can be imprecise, especially when they occur mixed in field populations. Molecular techniques can provide a more accurate way for nematode identification. This study reports the results of experiments targeting the mitochondrial cytochrome oxidase subunit 1 (COI) gene to develop species-specific primers that could be used for the identification of *H. avenae* and *H. filipjevi*. The COI gene of 9 *Heterodera* spp. and *Punctodera punctata* was partially sequenced and the resultant sequences were aligned to find unique sites suitable for the design of primers. The alignment showed variability between *H. avenae, H. filipjevi* and other *Heterodera* species. Two sets of species-specific primers were identified for the identification of both species and the conditions for their use in PCR were optimised. The specificity of the designed primers was checked by comparison with one population of *P. punctata* and populations of 14 other *Heterodera* species, nine populations of *H. avenae* and 10 populations of *H. filipjevi* originating from different countries. To test the sensitivity, the PCR was run with DNA extracted from five second-stage juveniles (J2) of *H. avenae* or five J2 of *H. filipjevi* mixed with DNA extracted from varying numbers of J2 of *H. latipons*. It was possible to detect as few as five J2 of *H. avenae* or *H. filipjevi* among 100 J2 of *H. latipons*. The two primers sets allow the detection of *H. avenae* and *H. filipjevi* where they occur in mixed populations with other *Heterodera* spp.

Keywords Cytochrome oxidase subunit 1 · Molecular identification · PCR · Sequence · Species-specific primer

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Introduction

Wheat, maize and rice occupy the most eminent position among grain crops in terms of production, acreage and source of nutrition, particularly in developing countries. It has been estimated that about 70 % of the land cultivated for food crops is devoted to cereal crops (McDonald and Nicol 2005). By 2030, the world production of cereals is expected to increase to 8 billion tons, whereas world wheat (*Triticum aestivum*) production is estimated to increase from 584 million tons (1995–1999 average) to 860 million tons (Marathée and Gomez-MacPherson 2001; Hossain and Teixeira Da Silva 2012). However, cereals are exposed to biotic and abiotic stresses. Among the biotic stresses, plant-parasitic nematodes play an important role in decreasing crop yield (Nicol and Rivoal 2008). The cereal cyst nematodes (CCN) are of global importance in cereal production systems. Significant economic losses due to these nematodes have been reported from West Asia, North Africa, Europe, Australia and the United States of America (Rivoal and Cook 1993; Nicol and Rivoal 2008; Sahin et al. 2009). Twelve out of 80 *Heterodera* species affect roots of cereals and grasses (Subbotin et al. 2010). Of these, *H. avenae*, *H. filipjevi* and *H. latipons* are considered the most economically important, and sometimes coexist (Rivoal and Cook 1993; Abidou et al. 2005a; McDonald and Nicol 2005; Yan and Smiley 2009; Toumi et al. 2012).

The traditional identification of *Heterodera* species using morphological characteristics is time-consuming and requires specialized skill, especially in the case of mixtures. Because a correct identification of the species is of major importance in nematode control strategies, the development of molecular tools allowing the identification and discrimination between species is warranted (Romero et al. 1996; Rumpenhorst et al. 1996; Rivoal et al. 2003; Subbotin et al. 2003; Waeyenberge et al. 2009). The internal transcribed spacer region (ITS) is possibly the most widely used genetic marker for nematode identification and phylogenetic studies at species level (Vrain et al. 1992; Wendt et al. 1993; Zijlstra et al. 1995). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the ITS-regions has provided a reliable instrument for a precise identification of cyst nematode species and subspecies (Subbotin et al. 2000; Rivoal et al. 2003; Madani et al. 2004; Abidou et al. 2005b; Smiley et al. 2008; Yan and Smiley 2010). This technique requires a combination of restriction enzymes to distinguish between *Heterodera* species. It has been reported that 23 restriction enzymes could separate all species belonging to the avenae-group with the exception of *H. avenae* from *H. arenaria* (Subbotin et al. 2000). Further, problems with partly digested recognition sites, lack of specificity, and insufficient resolution of small fragments on agarose gels can interfere with a clear interpretation of the results (Waeyenberge et al. 2009). The ITS-region was also used to develop species-specific primers detecting *Heterodera* species in a simple PCR-reaction, *e.g. H. glycines* (Subbotin et al. 2001) and *H. schachtii* (Amiri et al. 2002). However, the analysis of the ITS sequences reveals limited variations for discriminating *H. avenae* and closely related species (Fu et al. 2011). Moreover, polymorphism between ribosomal DNA (rDNA) repeats can occur within one species, e.g. *H. avenae* (Bekal et al. 1997; Zhao et al. 2011) and *H. filipjevi* (Subbotin et al. 2000; Subbotin et al. 2003). This polymorphism makes the design of a species-specific primer based on ITS-sequences very difficult.

The mitochondrial cytochrome oxidase subunit 1 (COI) gene was successfully used to discriminate between many species of free-living marine nematodes (Derycke et al. 2010). Similarly, the gene has been used to generate DNA-barcodes for a variety of biological species, from bacteria to mammals (Hebert et al. 2003). Recently, it was reported that the gene was useful in discriminating between plant-parasitic quarantine nematode species (M. Holterman, personal communication). Against this background, we decided to investigate the COI gene to determine its usefulness for the development of species-specific primers for *H. avenae* and *H. filipjevi.*

Materials and methods

Nematodes

Fifty-four populations belonging to 14 *Heterodera* species and one population of *Punctodera punctata* were obtained from 18 different countries and 11 hosts (Table 1). Several populations represented species of the *avenae*-group, *viz. H. avenae, H. filipjevi, H. latipons* and *H. hordecalis*. They originated from different hosts and countries, particularly from Syria and Turkey. The suppliers provided full details of the species

Table 1 Cyst nematode species and populations used in this study with their origin, provider and accession number of their ITS and COI sequences in GenBank

Code	Species	Host	Country	Source	AN ITS sequence	AN COI sequence
Did29	*Heterodera avenae*	wheat	China	D. Peng	JX024193	-
Did33	*H. avenae*	wheat	France	R. Rivoal	JX024192	-
Did11	*H. avenae*	wheat	Saudi Arabia (Hail)	A. Dawabah	JX024191	-
Fa19	*H. avenae*	wheat	Syria – Deir Al-Zor (Elzebarie)	G. Hassan	JX024198	KC172908
Fa5	*H. avenae*	wheat	Syria – Al-Hasakah (Sebat)	G. Hassan	JX024199	-
Fa1	*H. avenae*	wheat	Syria – Al-Hasakah (Tel Aswad)	F. Toumi	JX024197	KC172909
Tuni6	*H. avenae*	wheat	Tunisia	N. Kachouri	JX024190	-
Mus21	*H. avenae*	wheat	Turkey – Hatay	M. Imren	JX024196	-
Did12	*H. avenae*	wheat	Turkey – Kilis	D. Saglam	JX024194	-
Did49	*H. avenae*	wheat	USA	R. Smiley	JX024195	-
DCP1248	*H. betae*	peas	Belgium	ILVO	JX024200	-
Elsd2	*H. betae*	sugar beet	Germany	B. Niere	JX024221	-
DCP1734	*H. carotae*	carrot	France	ILVO	-	-
FaC3	*H. ciceri*	chickpea	Syria - Aleppo	S. Hajjar	JX024201	KC172919
HD11	*H. daverti*	alfalfa	the Netherlands	G. Karssen	JX024202	KC172915
Did15	*H. filipjevi*	wheat	Iran - Aligoudarz	Z. Tanha Maafi	JX024208	KC172910
E88	*H. filipjevi*	wheat	Russia	INRA	JX024209	-
Fa125	*H. filipjevi*	wheat	Syria – Al-Hasakah (Jolebsan)	F. Toumi	-	KC172911
Fa126	*H. filipjevi*	wheat	Syria – Al-Hasakah (Tel-Ailol)	F. Toumi	-	-
Did23	*H. filipjevi*	wheat	Turkey - Ankara	D. Saglam	JX024207	-
Did23b	*H. filipjevi*	wheat	Turkey - Ankara	D. Saglam	JX024205	-
Did23d	*H. filipjevi*	wheat	Turkey - Ankara	D. Saglam	JX024206	-
Did42b	*H. filipjevi*	wheat	Turkey – Eskisehir	D. Saglam	JX024204	-
Did42c	*H. filipjevi*	wheat	Turkey – Eskisehir	D. Saglam	JX024203	-
HFUSA	*H. filipjevi*	wheat	USA -Oregon	R. Smiley	-	-
HGHar	*H. glycines*	soybean	Canada	R. Riggs	JX024212	-
Did38	*H. glycines*	soybean	Iran - Mazan	Z. Tanha Maafi	JX024210	KC172914
HG10	*H. glycines*	soybean	USA	G. Karssen	JX024211	-
HGRiggs	*H. glycines*	soybean	USA	R. Riggs	JX024213	-
MP1	*H. goettingiana*	pea	Germany	J. Hallmann	JX024214	-
E69	*H. hordecalis*	wheat	Israel	INRA	JX024215	-
TuniB	*H. hordecalis*	wheat	Tunisia	N. Kachouri	JX024216	KC172912
MP5	*H. humuli*	hop	Germany	J. Hallmann	JX024217	-
HLCyp	*H. latipons*	wheat	Cyprus	M. Christoforou	JX024187	-
HL50	*H. latipons*	barley	Iran	Z. Tanha Maafi	JX024186	-
HLIran	*H. latipons*	wheat	Iran	Z. Tanha Maafi	JX024189	-
HL5	*H. latipons*	barley	Jordan	L. Al-banna	JX024188	-
HLMorc	*H. latipons*	wheat	Morocco	F. Mokrini	JQ319037	-
Fa3	*H. latipons*	wheat	Syria – Al-Hasakah	F. Toumi	JX024175	-
Fa7A3	*H. latipons*	wheat	Syria – Al-Hasakah (Aamer)	G. Hassan	JX024178	-
Fa7B1	*H. latipons*	barley	Syria – Al-Hasakah (Khanamia)	K. Assas	JX024181	-
Fa7A4	*H. latipons*	wheat	Syria – Al-Raqqa	G. Hassan	JX024179	-
Fa7A1	*H. latipons*	wheat	Syria – Deir Al-Zor	F. Toumi	JX024176	KC172913

Table 1 (continued)

Code	Species	Host	Country	Source	AN ITS sequence	AN COI sequence
Fa7A2	*H. latipons*	wheat	Syria – Deir Al-Zor	G. Hassan	JX024177	-
Fa7A5	*H. latipons*	wheat	Syria – Aleppo	F. Toumi	JX024180	-
Fa7B2	*H. latipons*	barley	Syria –Al-Hasakah (Tel Khaled)	K. Assas	JX024182	-
Mus2	*H. latipons*	wheat	Turkey – Gaziantep (Arikder)	M. Imren	JX024184	-
Mus1	*H. latipons*	wheat	Turkey – Gaziantep (Karkamis)	M. Imren	JX024180	-
Mus17	*H. latipons*	wheat	Turkey – Kilis	M. Imren	JX024185	-
DCP1041A	*H. pratensis*	grass	Belgium	ILVO	-	KC172916
HSPol	*H. schachtii*	sugar beet	Poland	S. Kornobis	JX024219	KC172918
HSC9872	*H. schachtii*	sugar beet	the Netherlands	G. Karssen	JX024220	-
HSNDL	*H. schachtii*	sugar beet	the Netherlands	HZPC	JX024218	-
HT9	*H. trifolii*	clover	the Netherlands	G. Karssen	FJ040402	-
DCP1041B	*Punctodera punctata*	grass	Belgium	ILVO	-	KC172917

identity, their host, and origin. We confirmed the species identity of all populations molecularly. For each population, DNA was extracted from five J2 of one cyst, using the method developed by Holterman et al. (2006). The ITS region was amplified by adding 1 μl DNA to the PCR reaction mixture containing 23 μl ddH_2O, 25 μl of Dream *Taq* PCR Master Mix (2×) (Fermentas Life Sciences, Germany), 1 μM of forward primer 5′-CGT AAC AAG GTA GCT GTA G-3′ and 1 μM of the reverse primer 5′-TCC TCC GCT AAA TGA TAT G-3′ (Ferris et al. 1993). The PCR products were purified following the instructions included in the Wizard SV Gel and PCR Clean-Up System kit (Promega Benelux, Leiden, The Netherlands), sequenced in both directions, edited, analysed and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/).

Development of species-specific primer sets

Selection of species-specific primers

Nine *Heterodera* spp. (*H. filipjevi, H. avenae, H. glycines, H. daverti, H. latipons, H. hordecalis, H. schachtii, H. pratensis, H. ciceri*) represented by one population each and two populations of both *H. avenae* and *H. filipjevi*, along with one population of *P. punctata*, were selected for designing two species-specific primer sets for detecting *H. avenae* and *H. filipjevi*, in separate PCRs. COI was amplified by adding 1 μl DNA of the selected species to the PCR reaction mixture containing 25 μl of Dream *Taq* PCR Master Mix (2×) (Fermentas Life Sciences, Germany), 1 μM of forward primer JB3F (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′), 1 μM of the reverse primer JB5R (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) (Derycke et al. 2005), and ddH_2O up to a final volume of 50 μl. The PCR-program settings were as follows: initial denaturation step at 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 41 °C for 30 s and 72 °C for 45 s; and an additional amplification step at 72 °C for 8 min (QBOL protocol 2011; http://www.qbol.wur.nl/UK/). Loading dye (6× Orange DNA, Fermentas) was added to the PCR products. Electrophoresis of PCR products was carried out in a 1.5 % TAE buffered agarose gel (40 min, 100 V), stained in an ethidium bromide bath (1 mg/l) for 30 min and photographed under UV light. Where a positive result was evident, the remainder of the PCR product was purified (Promega Benelux, Leiden, The Netherlands), and sequenced (Macrogen, the Netherlands) in both directions. Finally, the sequences were edited and analysed using software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall 1999). Software AlleleID 7.73 was used to investigate putative species-specific DNA fragments that could be used as primers for the identification of *H. avenae* or *H. filipjevi*. Additionally, an in silico study was done to check the potential of secondary structures, self-primer-dimer and hetero primer-dimer formation within two primers in one set and between two primers from different sets in case of the primers mix (https://www.idtdna.com/

analyzer/Applications/OligoAnalyzer/). The sequences of the potential species-specific primers were further screened by searching for their presence in sequences stored in GenBank (BlastN option, http://www.ncbi.nlm.nih.gov/). Finally, one primer set was selected for each species.

Optimization and specificity of species-specific PCRs

A gradient PCR was performed to determine the optimum annealing temperature (Ta) for each primer set. DNA of *H. avenae* population Fa1 and *H. filipjevi* population Did15 was used as template DNA. One µl DNA was added to the PCR master mix used for COI gene amplification (see primer selection above). For *H. avenae,* the designed forward primer (AVEN-COIF) was (5′-GGG TTT TCG GTT ATT TGG-3′), and the designed reverse primer (AVEN-COIR) was (5′-CGC CTA TCT AAA TCT ATA CCA-3′). For *H. filipjevi*, the designed forward primer (FILI-COIF) (5′-GTA GGA ATA GAT TTA GAT AGT C-3′), and the designed reverse primer (FILI-COIR) (5′-TGA GCA ACA ACA TAA TAA G-3′) were used. The Ta varied between 53 °C and 65 °C. The highest temperature still showing a bright, single and clean PCR-product without secondary products such as primer-dimers for both species-specific PCRs was selected as final Ta.

To check the specificity of both primers sets (AVEN-COI and FILI-COI), separate PCRS with DNA from each *Heterodera* spp. and *P. punctata* (Table 1) were carried out using optimized conditions. An additional test was performed to ensure that the primers sets AVEN-COI and FILI-COI were valid for all *H. avenae* and *H. filipjevi* populations in our collection, respectively.

Sensitivity test of species-specific PCRs

To check the sensitivity of the primers, DNA was extracted from 100 J2 obtained from one cyst of *H. latipons* (Fa7A1). The juveniles were placed in a 0.5 ml tube containing 300 µl lysis buffer (Holterman et al. 2006). The tube was incubated as for previous DNA extractions and 1 µl of crude DNA-extract was used for PCR.

The sensitivity test of the AVEN-COI primers set consisted of a species-specific PCR (see above) using 1 µl DNA earlier extracted from 5 J2 of *H. avenae* (Fa1) (see section Nematodes) mixed with 1 µl DNA extracted from 5 or 100 J2 *H. latipons*. Similarly, the sensitivity test of the FILI-COI primers set involved a species-specific PCR using 1 µl DNA previously extracted from 5 J2 of *H. filipjevi* (Fa125) mixed with 1 µl DNA extracted from 5 or 100 J2 *H. latipons*.

Also, 1 µl of four dilutions (1/5, 1/10, 1/50 and 1/100) of DNA extracted from 5 J2 of both species was used in a species-specific PCR. Equally, 1 µl of two dilutions (1/5 and 1/10) of DNA extracted from 5 J2 of both species was mixed with 1 µl DNA extracted from 100 J2 of *H. latipons* and used in a species-specific PCR.

Results

Nematode identification

Amplification of the rDNA-ITS region was successful for all samples. Sequencing of both DNA strands of the purified PCR products confirmed the morphological identification of all samples. The sequences were deposited in GenBank; the accession numbers are presented in Table 1.

Species-specific PCRs development

Species-specific primers selection

Amplification of the COI gene was done successfully for all populations, and a single band with fragment size of 470 bp was produced (Fig. 1). No PCR products were obtained in the negative control without nematode DNA template.

The *in silico* investigation revealed no secondary structures and no possible primer dimer formation in both newly designed species-specific primer sets, even in case of mixing different primers. *In silico* comparative search with the designed primers revealed no match or potential primer binding sites with any of the sequences of *Heterodera* spp. and *Punctodera* spp. available in GenBank (http://blast.ncbi.nlm.nih.gov/), and with sequences that we had obtained, with the obvious exception of the *H. avenae* and *H. filipjevi* sequences. We therefore, kept the primers sets AVEN-COI and FILI-COI for further experiments. Positions of both species-specific primer sets are shown in the alignment of the sequence of the COI gene of 9 *Heterodera* spp. and *P. punctata* (Fig. 2).

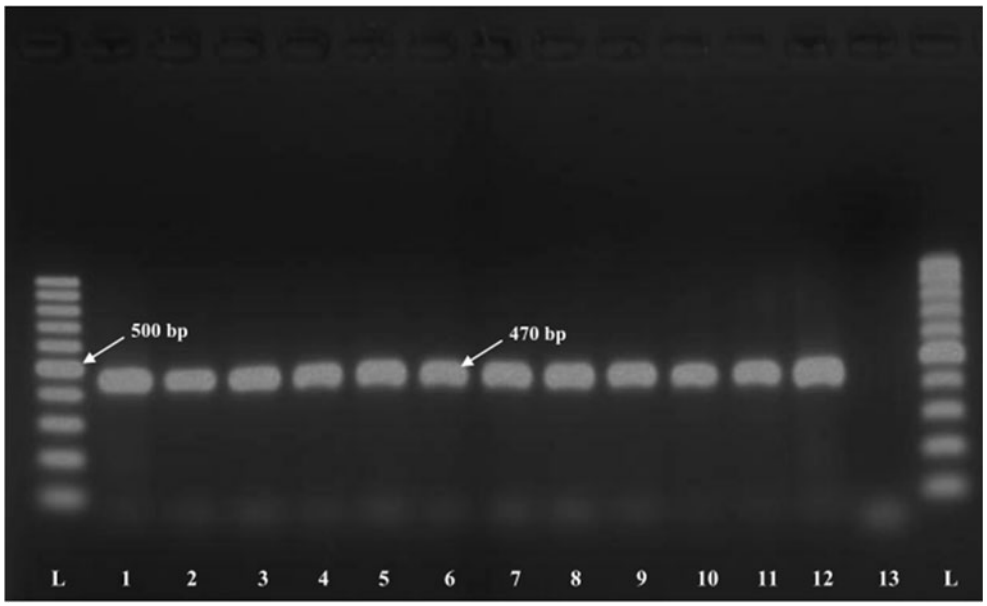

Fig. 1 Amplification results with the COI gene primers (JB3 & JB5) on a selection of *Heterodera* spp. and *Punctodera punctata*. L: 100 bp DNA ladder (Fermentas Life Sciences), 1: *Heterodera pratensis* (DCP1041A), 2: *Punctodera punctata* (DCP1041B), 3 & 4: *H. filipjevi* (Did15 & Fa125), 5: *H. glycines* (Did38), 6: *H. daverti* (HD11), 7: *H. latipons* (Fa7A1), 8 & 9: *H. avenae* (Fa1 & Fa19), 10: *H. ciceri* (Fac3), 11: *H. hordecalis* (TuniB), 12: *H. schachtii* (HSPol), 13: Negative control

Species-specific PCR(s) optimization and validation

The gradient PCR run with both primers sets and template DNA from *H. avenae* Fa1 and *H. filipjevi* Did15, respectively, showed a single and bright band for a range of annealing temperatures between 53 °C and 60 °C (Figures 3 and 4). No primer-dimers were noticed over that range. The highest annealing temperature yielding the brightest specific band without additional band(s) was retained for each species. Because the annealing temperature was optimum at 58 °C for both species, we selected this temperature for further PCRs. The optimised PCR with an initial denaturation step at 95 °C for 5 min, 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, followed by 72 °C for 8 min, repeatedly resulted in a single band of 109 bp for all *H. avenae* populations (Fig. 5), and of 245 bp for all *H. filipjevi* populations (Fig. 6), but did not generate a band for any of the other tested species.

Sensitivity test of species-specific PCRs

The sensitivity of the PCR assays using the AVEN-COI and FILI-COI primer sets was satisfactory. Both assays were able to detect the DNA extracted from 5 J2 of *H. avenae* (Fig. 7) or 5 J2 of *H. filipjevi* (Fig. 8) when mixed with the DNA obtained from 100 J2 of *H. latipons*. Both assays were also able to detect DNA in four dilutions (1/5, 1/10, 1/50 and 1/100) from DNA originating from 5 J2 of *H. avenae* or *H. filipjevi*. Moreover, detection of both species was still possible when 1 µl of the diluted (1/5 and 1/10) target DNA was mixed with 1 µl DNA extracted from 100 J2 of *H. latipons*.

Discussion

In the last decade, several surveys assessed the distribution of CCN in the main wheat and barley growing areas in Syria (Abidou et al. 2005a) and Turkey (Sahin et al. 2009; Yavuzaslanoglu et al. 2012). The result showed that mixtures of two (*H. avenae* with *H. latipons* or *H. avenae* with *H. filipjevi*) and sometimes even three species coexist in the same field. Species mixtures increase the difficulties to reach a precise identification. The use of molecular tools having the capacity to accurately identify and separate the species offers an attractive option.

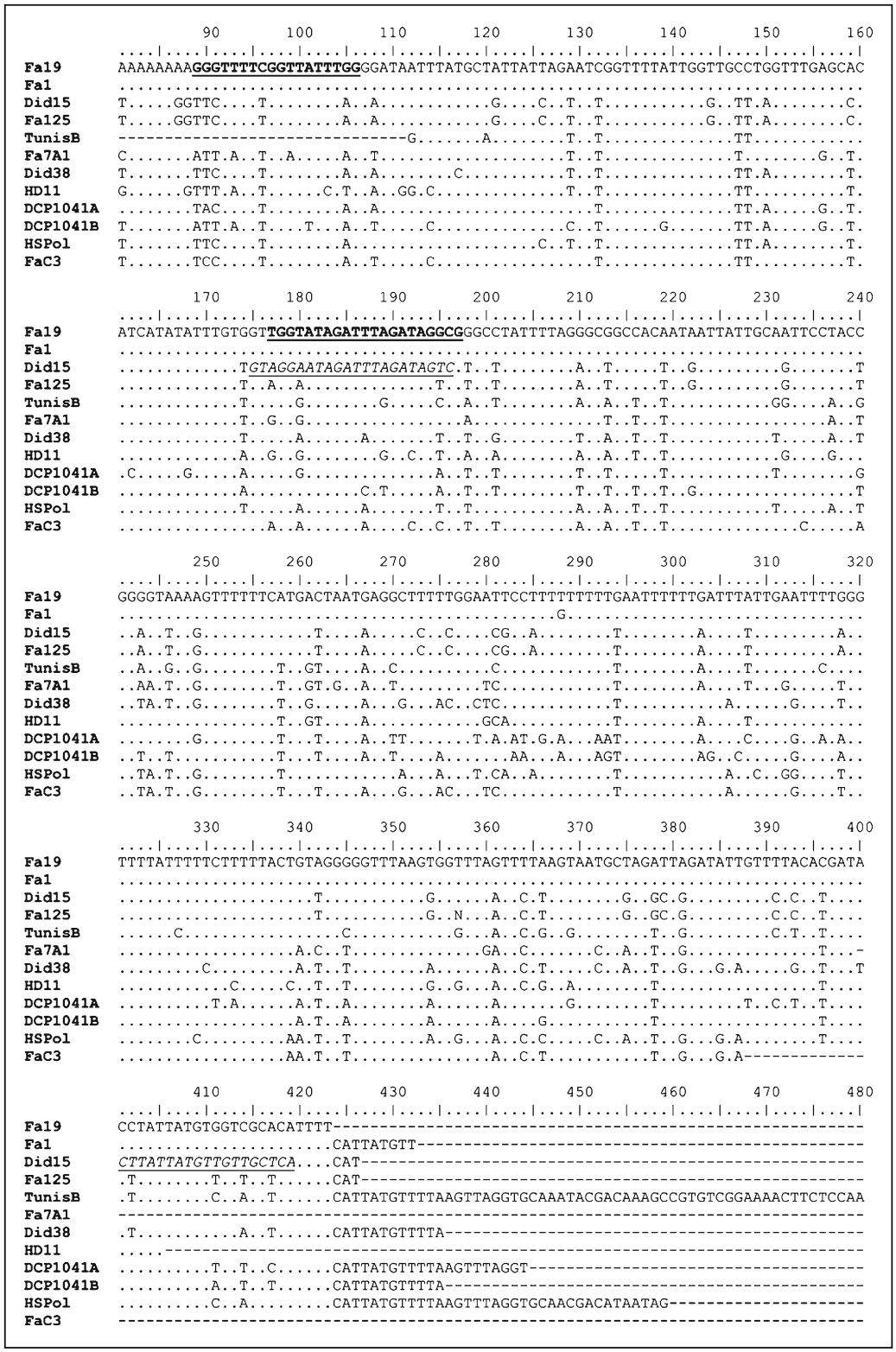

Fig. 2 Alignment of a selection of our COI-mtDNA sequences (see Table 1 for codes). Position for the species-specific primer set for *H. avenae* (AVEN-COI) is underlined and bold, and for *H. filipjevi* (FILI-COI) underlined and italic

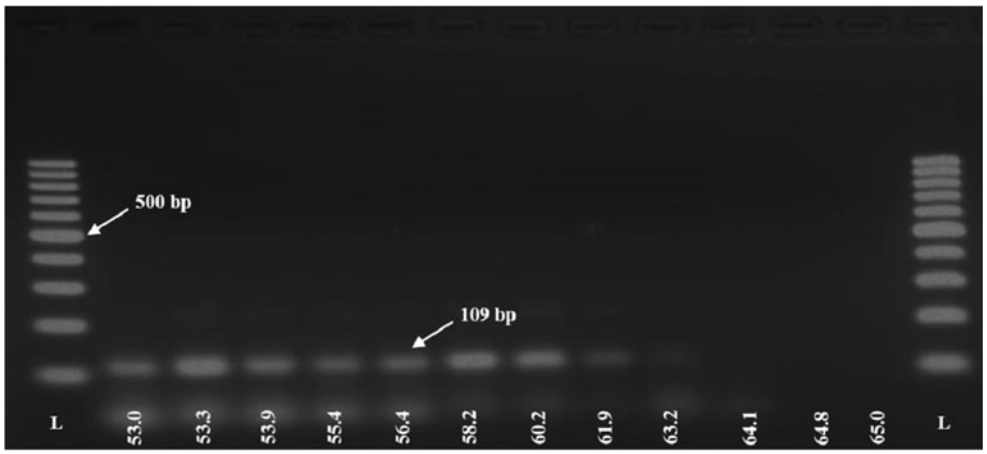

Fig. 3 Gradient PCR (temperatures indicated) with the AVEN-COI primers set using *Heterodera avenae* (Fa1). Temperature range from 53 °C to 65 °C. L: 100 bp DNA ladder (Fermentas Life Sciences)

So far, two publications reported the development and use of species-specific primers for the molecular identification of *Heterodera* spp., *i.e. H. glycines* (Subbotin et al. 2001) and *H. schachtii* (Amiri et al. 2002). Amiri et al. (2002) designed a species-specific primer (SHF6) to detect *H. schachtii* based on polymorphism in using the ITS-rDNA sequences. However, Chemeda et al. (unpublished) found the primer (SHF6) was not able to detect some *H. schachtii* populations suggesting that this polymorphism is not present in all *H. schachtii* populations. Heteroplasmy in mtDNA can cause the primer to fail recognising its binding place or to bind a non-specific target. However, during our study we did not encounter any problems as the two primers sets were species specific and were able to detect all representatives of the targeted species originating from different countries and regions. Hence, it can be suggested that the

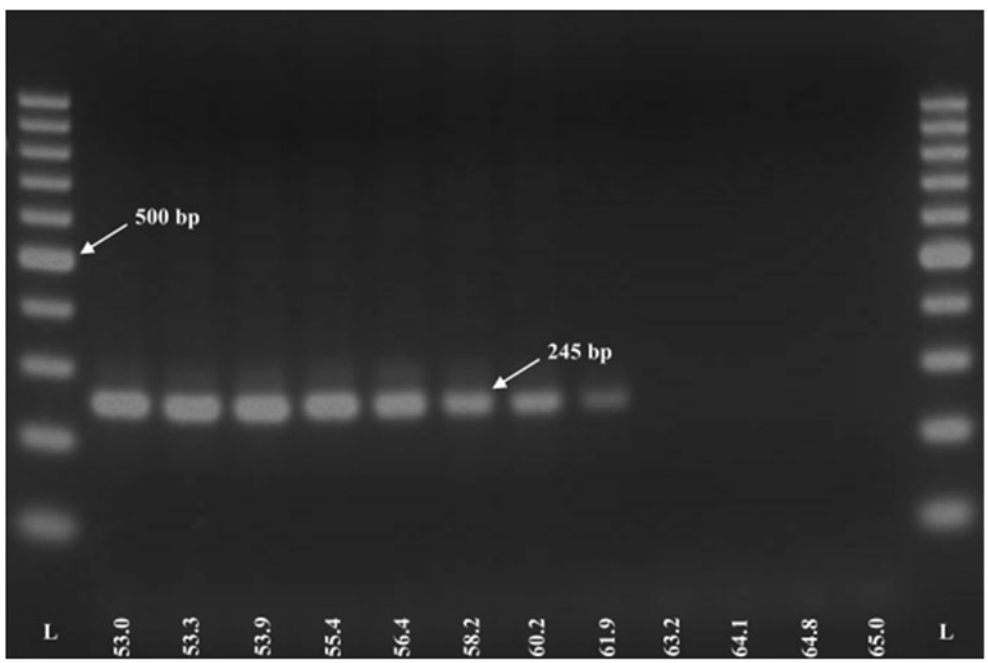

Fig. 4 Gradient PCR (temperatures indicated) with the FILI-COI primers set using *Heterodera filipjevi* (Did15). Temperature ranged from 53 °C to 65 °C. L: 100 bp DNA ladder (Fermentas Life Sciences)

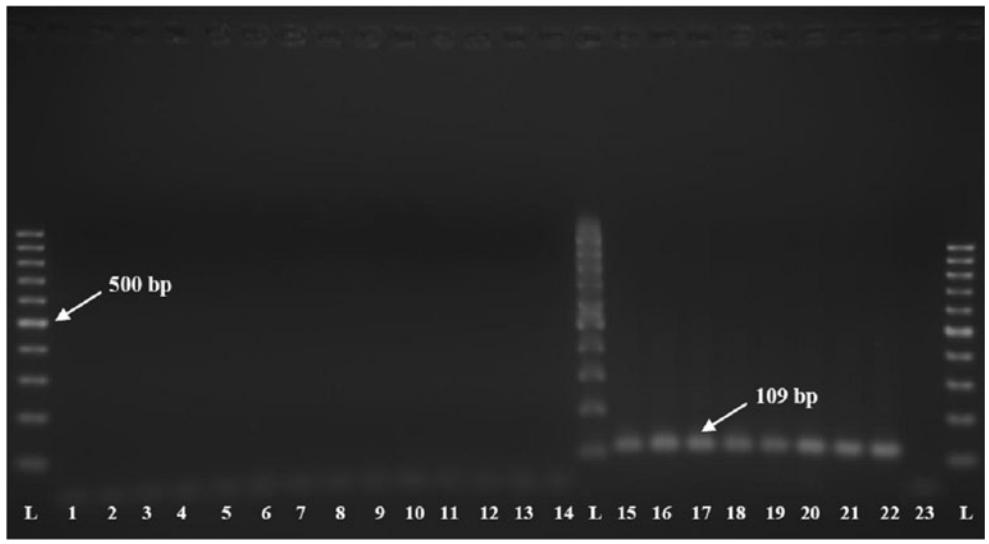

Fig. 5 Results of the optimised PCR using the AVEN-COI primers set. L: 100 bp DNA ladder (Fermentas Life Sciences). 1: *Heterodera pratensis* (DCP1041A), 2: *Punctodera punctata* (DCP1041B), 3: *H. filipjevi* (Did23b), 4: *H. hordecalis* (E69), 5: *H. glycines* (HGRiggs), 6: *H. schachtii* (HSPol), 7: *H. betae* (DCP1248), 8: *H. daverti* (HD11), 9: *H. goettingiana* (MP1), 10: *H. humuli* (MP5), 11: *H. ciceri* (FaC3), 12: *H. trifolii* (HT9), 13: *H. latipons* (Fa3), 14: *H. carotae* (DCP1734), 15–22: different populations of *H. avenae* 15: Fa1, 16: Did29, 17: Did33, 18: Did11, 19: Tuni6, 20: Did49, 21: Mus21, 22: Fa19, 23: Negative control

selected COI primer binding places are present in all the *H. avenae* and *H. filipjevi* populations.

Designing species-specific primers for *H. avenae* and *H. filipjevi* starting from ITS sequences was difficult because of the polymorphism within each species. In addition, the *in silico* comparative analysis of all our sequences separately with the already available ITS sequences in GenBank (BlastN) showed sometimes high similarity, *e.g.* 99 % similarity between *H. avenae* (Fa1) and *H. mani* (AY148377), *H. arenaria*

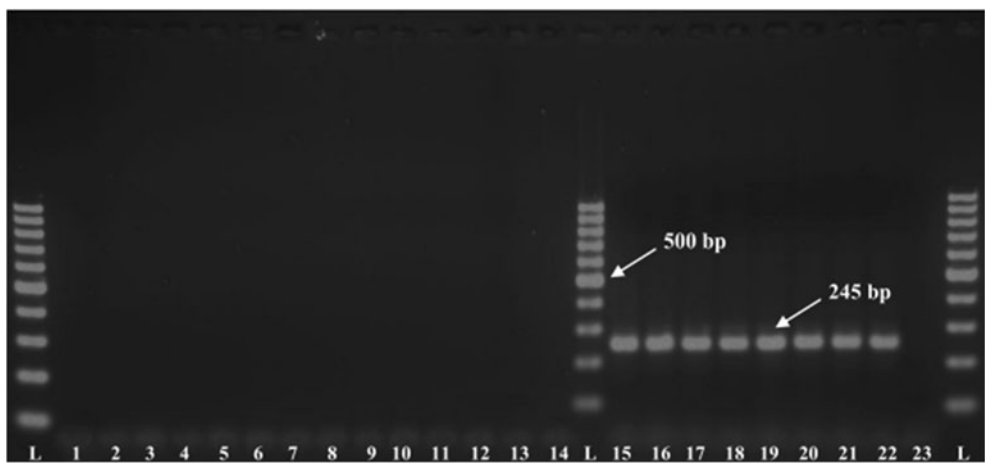

Fig. 6 Results of the optimised PCR using the FILI-COI primers set. L: 100 bp DNA ladder (Fermentas Life Sciences). 1: *Heterodera pratensis* (DCP1041A), 2: *Punctodera* punctata (DCP1041B), 3: *H. avenae* (Fa1), 4: *H. hordecalis* (E69), 5: *H. glycines* (HGRiggs), 6: *H. schachtii* (HSPol), 7: *H. betae* (DCP1248), 8: *H. latipons* (Fa3), 9: *H. goettingiana* (MP1), 10: *H. humuli* (MP5), 11: *H. ciceri* (FaC3), 12: *H. trifolii* (HT9), 13: *H. carotae* (DCP1734), 14: *H. daverti* (HD11), 15–22: different populations of *H. filipjevi* 15: Did15, 16: Did23, 17: E88, 18: Did42b, 19: Did42c, 20: Fa125, 21: Fa126, 22: HFUSA, 23: Negative control

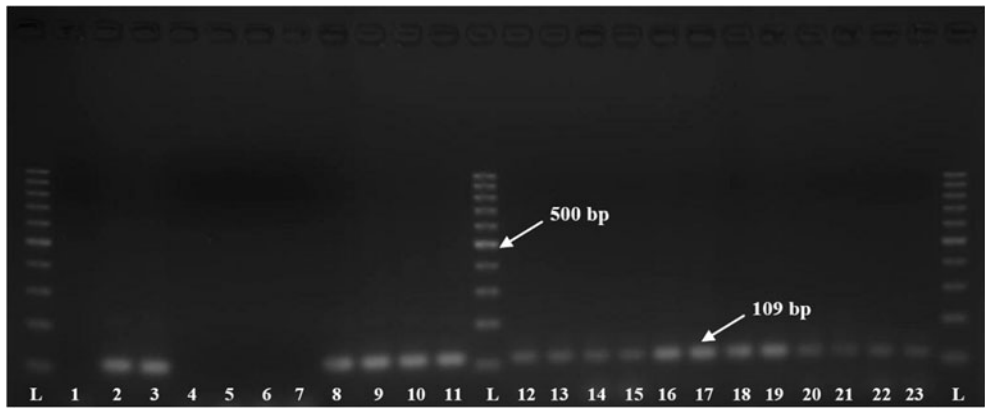

Fig. 7 Result of the sensitivity test using the AVEN-COI primers set in a PCR with 1 μl undiluted or diluted DNA of *Heterodera avenae* (HA) mixed with 1 μl DNA from *H. latipons* (HL). 1: negative control, 2–3: 5 J2 of HA, 4–5: 5 J2 of HL, 6–7: 100 J2 of HL, 8–9: 1/5 dilution of 5 J2 of HA, 10–11: 1/10 dilution of 5 J2 of HA, 12–13: 1/50 dilution of 5 J2 of HA, 14–15: 1/100 dilution of 5 J2 of HA, 16–17: 5 J2 of HA and 5 J2 of HL, 18–19: 5 J2 of HA and 100 J2 of HL, 20–21: 1/5 dilution of 5 J2 of HA and 100 J2 of HL, 22–23: 1/10 dilution of 5 J2 of HA and 100 J2 of HL, L: 100 bp DNA ladder (Fermentas Life Sciences)

(AF274396) and *H. australis* (AY148395), 97 % between *H. filipjevi* (Did15) and *H. ustinovi* (AY148406.1), and 97 % between *H. filipjevi* (E88) and *H. avenae* (HM560755.1). Also, the *in silico* comparison showed comparable similarities in actin gene sequences to occur between *Heterodera* species; actin gene sequences of *H. filipjevi, H. avenae* and *H. hordecalis* are 95 % to 96 % similar. Hence, the actin gene could not be used to design species-specific primers detecting *H. avenae* or *H. filipjevi*.

Both newly designed primers were able to detect successfully all *H. filipjevi* and *H. avenae* populations that were used in this study. No positive reaction was observed for any of the other cyst nematode species we examined. PCR optimization showed that both primers sets are useful and specific on a range of annealing temperatures (56–60 °C). Because of the very clear and unambiguous specific band obtained at 58 °C, we selected this temperature as annealing temperature. Since this temperature is suitable in PCR

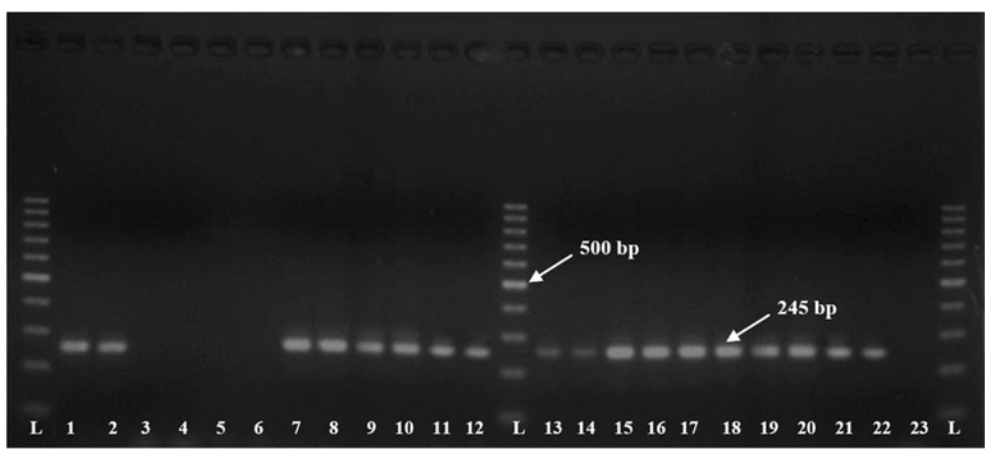

Fig. 8 Result of the sensitivity test of the FILI-COI primers set in a PCR with 1 μl undiluted or diluted DNA of *H. filipjevi* (HF) mixed with 1 μl DNA from *H. latipons* (HL). 1–2: 5 J2 of HF, 3–4: 5 J2 of HL, 5–6: 100 J2 of HL, 7–8: 1/5 dilution of 5 J2 of HF, 9–10: 1/10 dilution of 5 J2 of HF, 11–12: 1/50 dilution of 5 J2 of HF, 13–14: 1/100 dilution of 5 J2 of HF, 15–16: 5 J2 of HF and 5 J2 of HL, 17–18: 5 J2 of HF and 100 J2 of HL, 19–20: 1/5 dilution of 5 J2 of HF and 100 J2 of HL, 21–22: 1/10 dilution of 5 J2 of HF and 100 J2 of HL, 23: negative control, L: 100 bp DNA ladder (Fermentas Life Sciences)

reactions with both primers sets, we considered the detection of both species in one run.

The *in silico* study showed no reason to anticipate that the mixing of the two species-specific primer sets would have the potential for hetero primer-dimer formation when used in the same reaction. However, primer-dimers were observed when the two primers sets were mixed in one PCR using different primers concentrations (data not shown). Because primer-dimers can influence the efficiency and hence also the sensitivity of the PCR, it is not recommended to use both primers sets in a duplex PCR.

However, when used in separate PCRs, both primers sets were able to detected successfully five J2 of *H. avenae* or *H. filipjevi* either alone or in a mixture with 100 J2 of *H. latipons*. The sensitivity is even higher than five J2 since the equivalent of 1/10 of the DNA of five J2 yielded a clear band. From these results it can be concluded that 0.5 J2 of *H. avenae* or *H. filipjevi* can be detected among 100 J2 of *H. latipons*.

According to our result we can conclude that the designed species-specific PCRs are reliable, fast and sensitive identification tools. The method is particularly important for agricultural extension services where the skills to identify the species might be limited.

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