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Development of a species-specific PCR to detect the cereal cyst nematode, *Heterodera latipons*

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Summary – Several *Heterodera* species can reduce the yield of wheat and barley, among which *H. avenae*, *H. filipjevi* and *H. latipons* are economically the most important. Their identification, based on morphological characteristics, is not straightforward but can be made easier using molecular techniques. In this study, we developed species-specific primers for the detection of *H. latipons*. The actin gene of eight *Heterodera* species was partially sequenced and, after purifying and sequencing the PCR products, all sequences were aligned to find unique sites. The alignment showed moderate to very high similarities between the species. However, a small fragment of the actin gene was suitable for the construction of a potentially useful species-specific primer for *H. latipons*. The optimised PCR was subsequently tested with several populations of 14 *Heterodera* species and a single population of *Punctodera punctata*. *Heterodera latipons* was represented by 16 populations originating from six different countries. The primer set (Hlat-act), designed using AlleleID 7.73, was shown to be very specific. To test its sensitivity further, the PCR was conducted on DNA extracted from five second-stage juveniles (J2) of *H. latipons* mixed with five or 100 J2 belonging to *H. avenae*. The PCR was able to detect up to 1:10 dilution of the DNA obtained from five J2. The results showed that a specific and sensitive *H. latipons* species-specific PCR was constructed.

Keywords – actin gene, diagnostics, molecular detection, sequences, species-specific primer.

Wheat, maize and rice are the most important crops within the cereals (Nicol & Rivoal, 2008). Plant-parasitic nematodes can have a great impact on their yield (Brown, 1985; Nicol, 2002), especially the cereal cyst nematodes (*Heterodera* spp.), which are spread worldwide (Rivoal & Cook, 1993). The genus *Heterodera* includes 80 species (Subbotin *et al.*, 2010). Twelve species affect roots of cereals and grasses (Yan & Smiley, 2009), among which *H. avenae*, *H. filipjevi* and *H. latipons* are considered the most economically important (Rivoal & Cook, 1993; McDonald & Nicol, 2005; Yan & Smiley, 2009).

The identification of *Heterodera* species using morphological and morphometric characteristics is time consuming and requires specialised skills. Fortunately, biotechnological techniques have become available as tools to identify nematodes (Romero *et al.*, 1996; Rumpenhorst *et al.*, 1996; Rivoal *et al.*, 2003; Subbotin *et al.*, 2003; Waeyenberge *et al.*, 2009). The analysis of coding and non-coding regions of ribosomal DNA (rDNA) has become a favourite method for nematode identification since the 1990s (Vrain *et al.*, 1992; Wendt *et al.*, 1993; Zijlstra *et al.*, 1995). The internal transcribed spacer region (ITS) is variable and therefore useful for nematode identification and phylogenetic studies at species level. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based on ITS-regions of the rDNA repeat units of *Heterodera* spp. has frequently been used for the identification of cyst nematodes (Bekal *et al.*, 1997; Subbotin *et al.*, 1999, 2000; Rivoal *et al.*, 2003; Madani *et al.*, 2004; Abidou *et al.*, 2005a; Smiley *et al.*, 2008; Yan & Smiley, 2009). This technique requires the combination of the patterns of DNA fragments obtained after digestion with a series of restriction enzymes. For example, five restriction enzymes (*Alu*I, *Rsa*I, *Bsu*RI, *Bsh*1236I and *Hin*6I) are able

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to distinguish between *H. latipons* and other *Heterodera* spp. However, several species cannot be separated from each other (*e.g.*, *H. avenae* (type A) and *H. arenaria*) because of a lack of differences between the restriction patterns obtained (Subbotin *et al.*, 2000). Also, PCR-RLFP has some limitations: some enzymes are rare and thus expensive, sometimes digestion of PCR products is only partial, or small differences between digestion products make (visual) analysis difficult.

ITS sequences were studied to develop species-specific primers used in simple PCR reactions, *e.g.*, for detection of *H. glycines* (Subbotin *et al.*, 2001) and *H. schachtii* (Amiri *et al.*, 2002). However, polymorphism between rDNA repeats within a species like *H. latipons* makes designing a species-specific primer very difficult (Rivoal *et al.*, 2003). In view of this, we investigated an alternative non-multi-copy DNA region, *i.e.*, the actin gene. Although this gene has been investigated before (Matthews *et al.*, 2004; Tytgat *et al.*, 2004; Kovaleva *et al.*, 2005; Mundo-Ocampo *et al.*, 2008), it has never been used for diagnostic purposes. In the present study, we report on the development of a species-specific primer for *H. latipons* based on actin sequences.

Materials and methods

NEMATODES

A collection of 54 populations comprising 14 *Heterodera* species along with a single population of *Punctodera punctata* originating from 18 different countries and 11 hosts was used (Table 1). For several species, especially for those from the *H. avenae* group (*H. latipons*, *H. avenae* and *H. filipjevi*), more than one population was available. Samples from Syria and Turkey were collected from different regions and different hosts. Most of the suppliers provided us with information regarding the identity of the species, their host and origin. The species identity was confirmed or determined by amplification of the ITS-rDNA (see below) and by comparing the obtained sequences with those available in GenBank (BlastN option, http://www.ncbi.nlm.nih.gov/).

DNA EXTRACTION

Five second-stage juveniles (J2) from one cyst of the available populations were transferred to a 0.5 ml tube containing 25 μl of double distilled water (ddH_2O) and 25 μl lysis buffer (Holterman *et al.*, 2006) was added (final concentration 200 mM NaCl, 200 mM Tris-HCl (pH 8), 1% β-mercaptoethanol and 800 μg ml^{-1} Proteinase K). Samples were incubated for 2 h at 65°C followed by 5 min at 99°C in a thermomixer with a rotation speed of 300 rpm. For sensitivity tests, additional DNA was extracted from 100 J2 of *H. avenae* placed in a 0.5-ml tube containing 150 μl ddH_2O and 150 μl lysis buffer. As the DNA extraction method was originally designed to be used for 1-5 J2 only (Holterman *et al.*, 2006), we decided to increase the volume of lysis buffer to avoid possible fluctuations in DNA yield caused by the increased concentration of nematode proteins combined with a deficiency of, especially, Proteinase K. The remainder were stored at −20°C for future use.

MOLECULAR IDENTIFICATION OF COLLECTION

From each DNA extract, amplification of the rDNA-ITS region was performed by adding 1 μl DNA to a PCR reaction mixture containing 23 μl ddH_2O, 25 μl of Dream *Taq* PCR Master Mix (2×) (Fermentas Life Sciences), 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 program was as follows: initial denaturation step

Table 1. *Heterodera* populations and species used in this study with their origin, provider and accession number (AN) in GenBank.

Code	Species	Host	Country	Source	AN ITS sequence	AN actin sequence
Did29	*H. 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	–
Fa5	*H. avenae*	Wheat	Syria, Al-Hasakah (Sebat)	G. Hassan	JX024199	–
Fa1	*H. avenae*	Wheat	Syria, Al-Hasakah (Tel Aswad)	F. Toumi	JX024197	JX024223
Tuni6	*H. avenae*	Wheat	Tunisia	N. Kachouri	JX024190	–

Table 1. (Continued.)

Code	Species	Host	Country	Source	AN ITS sequence	AN actin sequence
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	JX024224
Elsd2	*H. betae*	Sugar beet	Germany	B. Niere	JX024221	–
DCP1734	*H. carotae*	Carrot	France	ILVO	–	–
FaC3	*H. ciceri*	Chickpea	Syria, Aleppo	S. Hajjar	JX024201	JX024225
HD11	*H. daverti*	Alfalfa	The Netherlands	G. Karssen	JX024202	–
Did15	*H. filipjevi*	Wheat	Iran, Aligoudarz	Z. Tanha Maafi	JX024208	–
E88	*H. filipjevi*	Wheat	Russia	INRA	JX024209	–
Fa125	*H. filipjevi*	Wheat	Syria, Al-Hasakah (Jolebsan)	F. Toumi	–	–
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	JX024226
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	–
HG10	*H. glycines*	Soybean	USA	G. Karssen	JX024211	–
HGRiggs	*H. glycines*	Soybean	USA	R. Riggs	JX024213	JX024227
MP1	*H. goettingiana*	Pea	Germany	J. Hallmann	JX024214	–
E69	*H. hordecalis*	Wheat	Israel	INRA	JX024215	JX024228
TuniB	*H. hordecalis*	Wheat	Tunisia	N. Kachouri	JX024216	–
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	JX024222
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	–
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	–	–
HSPol	*H. schachtii*	Sugar beet	Poland	S. Kornobis	JX024219	JX024229
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	–	–

at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 49°C for 45 s and 72°C for 60 s, and an additional amplification step at 72°C for 8 min. After electrophoresis of 5 μl PCR product in a 1.5% TAE buffered agarose gel (1 h, 100 V), the gel was stained in an ethidium bromide bath (1 mg l^{-1}) for 30 min and photographed under UV light. In case of a positive result, the remainder of the PCR product was purified after electrophoresis in a 1% TAE buffered agarose gel (1 h, 100 V) following the instructions included in the Wizard SV Gel and PCR Clean-Up System kit (Promega Benelux). Subsequently, the concentrations of the purified PCR products were measured using a UV spectrophotometer (Nanodrop ND-1000, Isogen Life Sciences). The purified PCR products were sequenced (Macrogen) in both directions to obtain overlapping sequences of the forward and reverse DNA strand. Finally, the sequences were visualised, edited and analysed using software packages Chromas 2.00 (Technelysium) and BioEdit 7.0.4.1 (Hall, 1999), and compared with sequences in GenBank (BlastN option, http://www.ncbi.nlm.nih.gov/) to reveal the identity of the *Heterodera* sample.

Design and test of species-specific primers

Eight *Heterodera* spp. were selected for the design of species-specific primers for *H. latipons*, *i.e.*, four species belonging to the *H. avenae* group (*H. latipons*, *H. avenae*, *H. hordecalis* and *H. filipjevi*) and four from other groups (*H. ciceri*, *H. betae*, *H. schachtii* and *H. glycines*) (accession numbers for actin sequences in Table 1). Part of the actin region from the eight species was amplified from crude DNA (see above) using Hs-actF (5′-ACT TCA TGA TCG AGT TGT AGG TGG ACT CG-3′) and Hs-actR (5′-ACC TCA CTG ACT ACC GAT GAA GAT TC-3′) (Tytgat *et al.*, 2004) and sequenced. To determine putative species-specific DNA fragments that could be used as primers for the identification of *H. latipons*, all of these sequences were aligned and compared visually using Clustal X 1.64 (Thompson *et al.*, 1997). For the selection of the fragments, software AlleleID 7.73 was used. The potential species-specific primers were also screened for their presence in sequences stored in GenBank (BlastN option, http://www.ncbi.nlm.nih.gov/). Finally, two primers were selected for the species-specific PCR.

Species-specific PCR

To determine the optimum annealing temperature (T_a), a gradient PCR was performed by using the DNA sample of one population of *H. latipons* (Fa3). The PCR mix and program were the same as for the amplification of the ITS region, with exception of the two newly designed primers: 0.5 μM of *H. latipons*-specific actin primer forward (Hlat-actF) (5′-ATG CCA TCA TTA TTC CTT-3′), and 0.5 μM of *H. latipons*-specific actin primer reverse (Hlat-actR) (5′-ACA GAG AGT CAA ATT GTG-3′), while the T_a ranged from 43 to 60°C.

To check the specificity of the species-specific primers for *H. latipons* (Hlat-act), the PCR was optimised with an initial denaturation at 95°C for 5 min, 50 cycles at 94°C for 30 s, 50°C for 45 s and 72°C for 45 s, followed by 72°C for 8 min, and applied with the DNA extract from all nematodes populations available, including 16 populations of *H. latipons* (Table 1).

Finally, to estimate the sensitivity of the PCR, 1 μl DNA extracted from five J2 of *H. latipons* (Fa3) was mixed with 1 μl DNA extracted from five and 100 J2 of *H. avenae* (Fa19). Also, 1 μl DNA of four dilutions (1:2, 1:5, 1:10 and 1:50) of DNA extracted from five J2 were included in the sensitivity test. Furthermore, two dilutions (1:2 and 1:5) of five J2 were mixed with 1 μl DNA extracted from 100 J2 of *H. avenae*.

Results

Amplification of the rDNA-ITS region was successful for all samples and produced a single band with a fragment size of 1100 bp. No PCR products were obtained in the negative control without nematode DNA template. Sequencing of both DNA strands of the PCR products confirmed the identification of all samples.

The amplification of the actin gene yielded a fragment of 420 bp (Fig. 1). The sequences were subjected to BlastN. This confirmed that the actin gene was amplified for all eight *Heterodera* species. The alignment and comparison of the obtained sequences with those deposited in GenBank showed moderate (83%) to very high (94%) similarities between the species, with the highest similarity between *H. latipons* and *H. filipjevi*. Nevertheless, the software AlleleID 7.73 was able to identify species-specific primers for *H. latipons* (see above). The forward primer was located in intron 5 of the Actin 1 gene, the reverse primer in intron 6 (Fig. 2). A BlastN-search with the newly designed primers revealed no match with any of

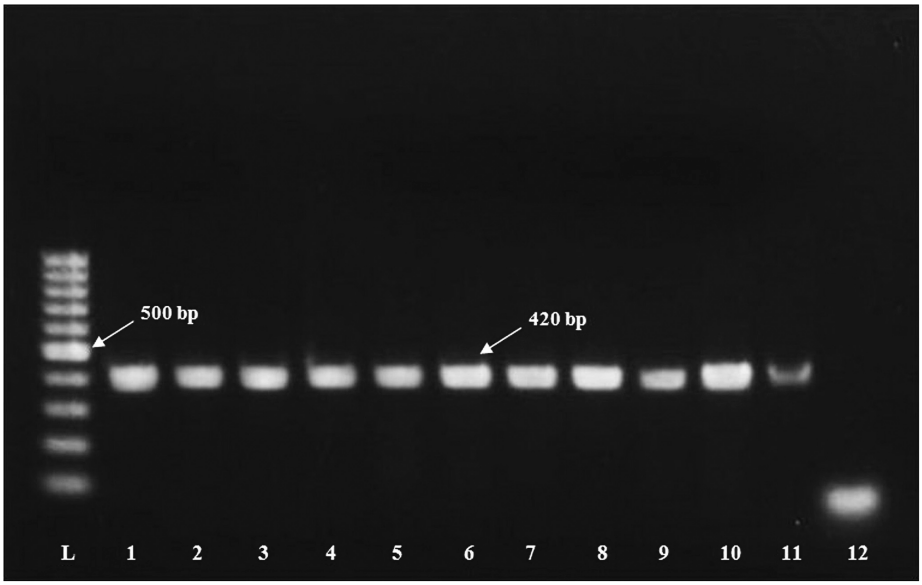

Fig. 1. PCR amplification of DNA from eight different *Heterodera* species with the universal actin gene primers (Hs-actF and Hs-actR) revealing a 420-bp fragment. Lanes: L = 100 bp DNA ladder (Fermentas Life Sciences); 1, 2 = *H. latipons* (Fa3); 3, 4 = *H. avenae* (Fa1); 5 = *H. hordecalis* (E69); 6, 7 = *H. filipjevi* (Did23b); 8 = *H. glycines* (HGRiggs); 9 = *H. schachtii* (HSPol); 10 = *H. betae* (DCP1248); 11 = *H. ciceri* (FaC3); 12 = Negative control. (See Table 1 for codes.)

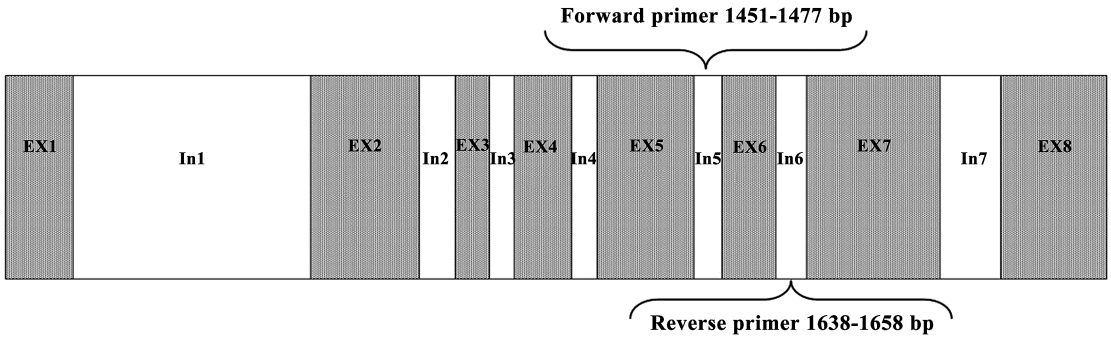

Fig. 2. A graphical map of the Actin 1 gene showing the positions of the designed species-specific primers. The shaded and white boxes represent the exons (Ex) and introns (In), respectively. The sizes of the boxes correspond with the lengths of the exons and introns.

the nematode sequences available in GenBank. The same was true when looking for potential primer binding sites within the *Heterodera* sequences that we had obtained, with the obvious exception of the *H. latipons* sequences. We therefore, retained this primers set for further experiments.

The gradient PCR allowed us to set the optimal annealing temperature for the retained primers between 47 and 55°C (Fig. 3). In this range we obtained clear bright bands; at temperatures out of this range we obtained weaker bands. Because higher temperatures normally increase the specificity of amplification yielding a high amount of PCR product, we fixed the annealing temperature at 50°C. The optimised PCR repeatedly resulted in a single band of 204 bp for all *H. latipons* populations, without appearance of any additional band (Fig. 4; eight out of 16 populations shown). No bands appeared for the other *Heterodera* species, nor for *P. punctata* (Fig. 4, selected populations of 13 *Heterodera* species shown).

The sensitivity of the assay was acceptable since it was possible to detect five J2 of *H. latipons* when mixed with

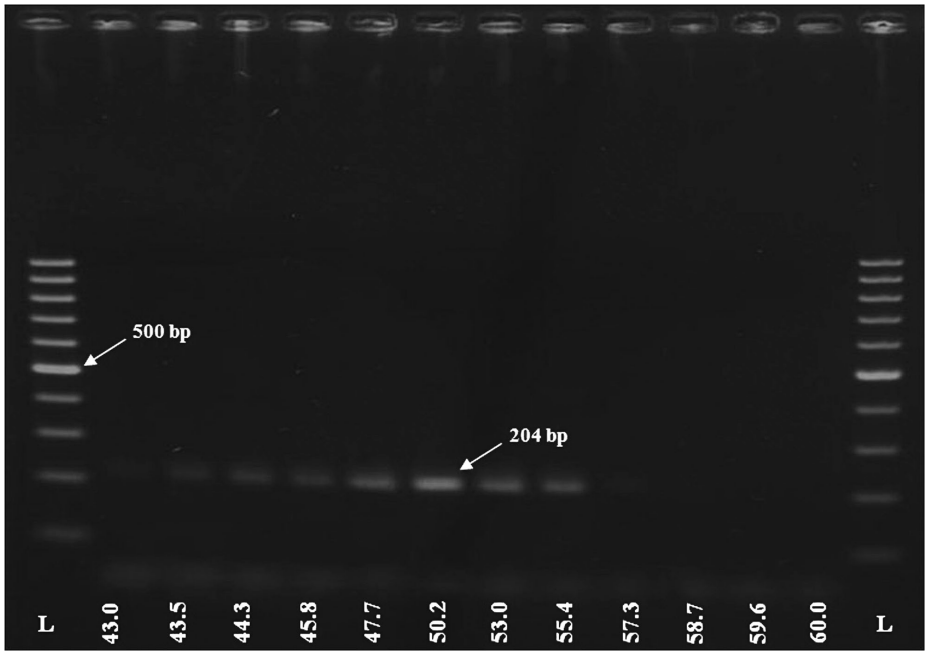

Fig. 3. Gradient PCR with the *Heterodera latipons*-specific PCR (Hlat-act primers) using DNA of *H. latipons* (Fa3). Temperature range from 43 to 60°C. L = 100 bp DNA ladder (Fermentas Life Sciences).

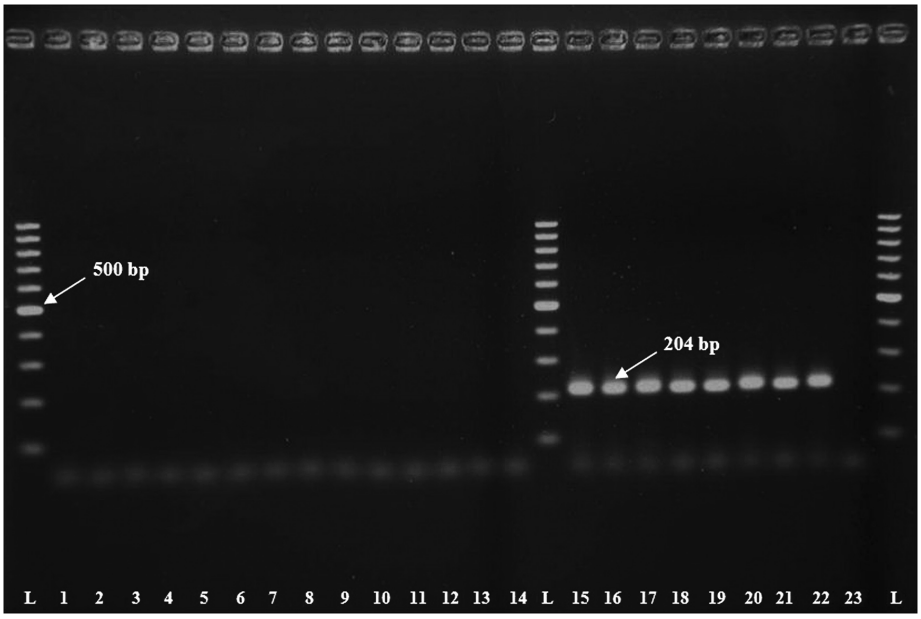

Fig. 4. Selected results of the *Heterodera latipons*-specific PCR (Hlat-act primers) using DNA from all samples (see Table 1 for codes). Lanes: L = 100 bp DNA ladder (Fermentas Life Sciences); 1 = *Heterodera pratensis* (DCP1041A); 2 = *Punctodera punctata* (DCP1041B); 3 = *H. avenae* (Fa1); 4 = *H. hordecalis* (E69); 5 = *H. glycines* (HGRiggs); 6 = *H. schachtii* (HSPol); 7 = *H. betae* (DCP1248); 8 = *H. filipjevi* (Fa125); 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. latipons* (15 = HL5; 16 = HLCyp; 17 = Fa7A1; 18 = Fa3; 19 = HLMorc; 20 = Mus1; 21 = HLIran; 22 = HL50); 23 = Negative control.

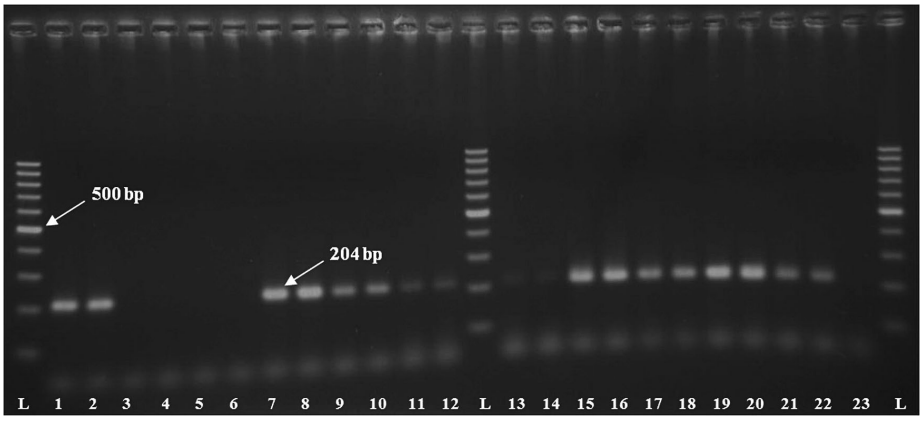

Fig. 5. Result of the sensitivity test of the Hlat-act primers in a PCR with 1 μl undiluted or diluted DNA of *Heterodera latipons* (Hl) mixed with 1 μl DNA from *H. avenae* (Ha). Lanes: 1, 2 = 1:2 dilution of 5 second-stage juveniles (J2) of Hl; 3, 4 = 5 J2 of Ha; 5, 6 = 100 J2 of Ha; 7, 8 = 5 J2 of Hl; 9, 10 = 1:5 dilution of 5 J2 of Hl; 11, 12 = 1:10 dilution of 5 J2 of Hl; 13, 14 = 1:50 dilution of 5 J2 of Hl; 15, 16 = 5 J2 of Hl and 100 J2 of Ha; 17, 18 = 1:2 dilution of 5 J2 of Hl and 100 J2 of Ha; 19, 20 = 5 J2 of Hl and 5 J2 of Ha; 21, 22 = 1:5 dilution of 5 J2 of Hl and 100 J2 of Ha; 23 = negative control; L = 100-bp DNA ladder (Fermentas Life Sciences).

100 J2 of *H. avenae*. The detection was still possible with a 1:10 dilution of DNA of *H. latipons* while the 1:50 dilution yielded a weaker band (Fig. 5).

Discussion

Three surveys conducted in Syria revealed *H. latipons* to be the most dominant species of the *H. avenae* group (Abidou *et al.*, 2005b; Hassan, 2008; Toumi *et al.*, 2012). However, the results also revealed that mixtures of two (*H. avenae* with *H. latipons* or *H. avenae* with *H. filipjevi*) or even three species can occur in a same field. Planning of successful plant protection measures against these pests requires the precise identification of the species. Unfortunately, specific skill on morphological identification is frequently one of the major problems. Nevertheless, an accurate and fast identification of *H. latipons* in cereal growing areas is very important. Molecular identification techniques can be a way to solve this problem.

Only two publications report on the development and use of species-specific primers for the molecular identification of *Heterodera* species, *i.e.*, *H. glycines* (Subbotin *et al.*, 2001) and *H. schachtii* (Amiri *et al.*, 2002). The latter primer (SHF6), based on sequence polymorphism, was able to detect all 35 populations of *H. schachtii* studied, without a positive result for other cyst nematode species, including two isolates of *H. betae*. However, they stated that, due to the high similarity of the ITS-sequences within the *schachtii* group, only this *H. schachtii* sequence polymorphism was suited for species-specific primer design. Later, Chemeda *et al.* (2012) found that the SHF6 primer did not detect some populations of *H. schachtii*, although the universal primers included in the PCR produced a band of about 1100 bp for all *Heterodera* samples tested, indicating a successful PCR. A similar polymorphism was observed by Rivoal *et al.* (2003) who detected a great genetic variability between and within *H. latipons* isolates with *Taq*I endonuclease. This result could be explained by the heterogeneity of the ITS from uncompleted concerted evolution of multi-copy gene families such as rDNA, as described by Hillis & Dixon (1991). Moreover, Ferris *et al.* (1999) suggested the existence of sibling species in *H. latipons* when they compared the sequence of ITS rDNA in two morphologically similar but geographically separated isolates from Israel (Gilat) and Russia (Rostov). Thus, designing a species-specific primer against *H. latipons* based on ITS sequences could turn out to be problematic because of this polymorphism. Hence, we explored a non-multi-copy DNA region. The present study clearly demonstrates that the actin gene can be used to distinguish *H. latipons* from other *Heterodera* species.

Our species-specific PCR detected all *H. latipons* populations used in this study, originating from different countries and regions. No positive reaction was observed with any of the 14 other cyst nematode species examined. Gradient PCR revealed that the primers are useful and specific over a wide range of the annealing temperatures between 47 and 55°C. This simplifies the use of the PCR

assay at different labs without further optimisation. We selected 50°C as the annealing temperature because of the very clear and bright specific band.

We detected five J2 of *H. latipons* either alone or in a mixture with DNA extracted from five or 100 J2 of *H. avenae*, confirming the efficiency of the primer. The sensitivity of the PCR was high: up to 1:10 dilution of DNA obtained from five J2 of *H. latipons* showed a clear DNA band (204 bp), indicating a detection limit of at least 0.5 J2. However, the detection limit is above 0.1 J2, as the 1/50 dilution of five J2 did not provide a visible band.

The method we developed is the first species-specific PCR detecting a species of the cereal cyst nematode complex. The technique is particularly important for agricultural extension services where the skills to identify the species are often limited. It also allows fast identification of *H. latipons* so that several nematode samples can be processed in one day including DNA extraction. We intend to develop species-specific primer sets for the other two major cereal cyst nematode species. These sets should, along with the species-specific primer set for *H. latipons*, complete the molecular tool for the identification of the three main cereal cyst nematode species.

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