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Master thesis:

Development of a species-specific PCR to detect the Cereal Cyst Nematode *Heterodera latipons*

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Wheat and barley are the most important crops within the cereals (Nicol *et al*., 2002). In general, cereal crops are exposed to biotic and abiotic stresses. Among the biotic stress, plant-parasitic nematodes have an important role in decreasing the yield (Brown *et al*., 1985; Nicol *et al*., 2002). The cereal cyst nematodes (CCN) are widespread and one of the most important group of nematodes in the world (Rivoal and Cook, 1993). The genus *Heterodera* includes 62 species (Wouts & Baldwin, 1998). Twelve species affect roots of cereals and grasses (Yan & Smiley, 2009). Three of them (*H. avenae*, *H. filipjevi* and *H. latipons*) are among the economically most important cyst nematodes (Rivoal & Cook. 1993; McDonald & Nicol. 2005; Yan & Smiley, 2009). The identification of *Heterodera* species using morphological and morphometrical characteristics is time consuming and requires great skill and training by the observer. However, there are many other characteristics allowing to discriminate between different species of nematodes e.g. biotechnological tools (Romero *et al*., 1996; Rumpenhorst *et al*., 1996; Rivoal *et al*., 2003; Subbotin *et al*., 2003). As a consequence, the development and use of new tools to identify nematodes using molecular technologies increases exponentially (Rivoal *et al*., 2003). The analysis of coding and non-coding regions of ribosomal DNA (rDNA) became a favorite way for nematode identification (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 has provided a reliable tool for quick and precise identification of cyst nematode species and

subspecies (Bekal *et al*., 1997; Subbotin *et al*., 1999; Subbotin *et al*., 2000; Rivoal *et al*., 2003; Madani *et al*., 2004; Abidou *et al*., 2005; Smiley *et al*., 2008). This was a start of many studies leading to an explosion of RFLP-patterns and sequences. Comparisons of sequences of the ITS-rDNA of unknown nematodes with those published or deposited in GenBank (Ferris *et al*., 1994; Orui, 1997; Szalanski *et al*., 1997; Subbotin *et al*., 1999, 2000, 2001; Sabo *et al*., 2001; Tanha Maafi *et al*., 2003) facilitated the quick identification of most species of cyst nematodes. In a later stadium, the sequences were used to develop species-specific primers (Amiri *et al*., 2001). By a simple PCR-reaction, it was then possible to detect the nematode species for which the primers were designed.

Although the ITS-regions are very useful for species identification, several species cannot be separated from each other by ITS-RFLP, e.g. *H. avenae* (type A) and *H. arenaria*, *H. ciceri* and *H. trifolii* and also *H. carotae* and *H. cruciferae* due to the same restriction patterns obtained in each pair of these species (Subbotin *et al*., 2000). Sequences of these and other *Heterodera* species, like *H. trifolii*, *H. schachtii* and *H. betae*, were compared and found to be almost identical. Moreover, polymorphism occurs between rDNA repeats within one species resulting in different RFLP-patterns which can overlap with the RFLP-pattern for another species (Wouts *et al*., 2001). I therefore concluded that in some cases it is impossible to design reliable RFLP-patterns or species-specific primers using the ITS-region. Therefore I decided to investigate other DNA-regions to determine their usefulness for the development of additional identification methods. The objectives of this study were (i) to screen different DNA-regions (PCR) not belonging to rDNA and mtDNA for assessing their usefulness to develop species-specific primers for *H. latipons*, (ii) to optimize the species-specific PCR.

Materials & Methods

Nematode samples. A survey was conducted in Syria and Turkey in July 2008-2009 after the harvesting time of cereals. Cysts were extracted from soil by Seinhorst method (Seinhorst, 1964). Extracted cysts, which were retained on the 250 µm sieve, were hand-picked with a needle under a dissecting stereomicroscope. More *Heterodera* cysts (different species) were obtained from colleagues in different countries (Table 1). All cysts were stored at 4°C for molecular and morphological identification.

Morphological identification. For each population, vulval cones of several mature cysts were mounted in glycerin jelly. Underbridge structure, shape of semifenestra in the fenestral area and development of bullae were observed under a microscope and depending on all these structures the identifications was done (Shurtleff & Averre. 2000; Handoo. 2002).

DNA extraction. A selection of *Heterodera* populations comprising 9 different species and 24 isolates was selected for DNA extraction (Table 1). For each population, one cyst was transferred into 45 µl of double distilled water (ddH2O) in an Eppendorf tube and crashed using a microhomogeniser (Vibro Mixer). After centrifugation of the squashed cyst content, 40 µl of the mix were transferred to a PCR tube (0.2 ml). 50 µl of worm lysis buffer (WLB) and 10 µl of Proteinase K (20mg/ml) were added and the tubes were frozen at -80 ºC for at least 10 min and then incubated at 65 ºC for 1 hour and 95 ºC for 10 minutes consecutively in a thermocycler. After incubation, the tubes were centrifuged for 1 min at 14 000 rpm and kept at -20 ºC until use (Maafi *et al*., 2002; Waeyenberge *et al*., 2009).

Genome Amplification (Gphi**).** Due to the small amount of the extracted DNA (cysts with only a few juveniles), genome amplification was done. One µl of the DNA extract was used for genome amplification, followed by a purification step using the alcohol precipitation method as

described in the manufacturer's instructions (Illustra™ GenomiPhi V2 DNA Amplification Kit, GE Healthcare, Chalfont St Giles, UK; Skantar & Carta, 2005). The DNA concentration was measured using a UV spectrophotometer (Nanodrop ND1000, Isogen Life Sciences, Sint-Pieters-Leeuw, Belgium) and 1 ng DNA was used for PCR. The remainder of the crude and amplified DNA extract was stored at − 20◦C for future use.

PCR amplification. For molecular identification, the ITS-rDNA region was amplified. One nanogram of DNA was added to the PCR reaction mixture containing 23 µl ddH_2O, 25µl 2X DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany) and 0.5 µl of forward primer (5-CGTAACAAGGTAGCTGTAG-3) and reverse primer (5-TCCTCCGCTAAATGATATG-3) (Ferris *et al*., 1993). The DNA thermal cycler program consisted of 5 min at 95 °C; 40 cycles of 94 °C for 30 s, 45 °C for 45 s and 72 °C for 45 s; followed by a final elongation step of 8 min at 72 °C. To be able to design species-specific primers, other DNA-regions were amplified as well. Amplification for those regions was done with suitable primers in the same PCR reaction mixture and program with appropriate and adaptation of the annealing temperature (Table2). After PCR amplification, 5 µl of each PCR product was mixed with 1 µl of 6× loading buffer (Fermentas Life Sciences, Germany) and loaded on a 1.5% standard TAE buffered agarose gel. After electrophoresis (100 V for 40 min) the gel was stained with ethidium bromide (0.1 µg/ml) for 15 min, visualized and photographed under UV-light. The remaining PCR product was stored at –20 °C.

Sequencing. The remainder (two times 45 µl) of the PCR product was loaded on a 1% agarose gel for electrophoresis (100 V, 40 min). The purification process was done as described in the manufacturer's instructions (Wizard® SV Gel and PCR Clean-Up System Kit, Promega). DNA from each sample was sequenced (Macrogen, Seoul, South Korea) in both directions to obtain overlapping sequences of both DNA strand. The sequences were edited and analysed

using software packages Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall, 1999). Finally all sequences were blasted in GenBank (Sequin v. 9.00, http://www.ncbi.nlm.nih.gov/) to reveal its origin (*Heterodera* species and DNA-region).

Species-specific primer design. An alignment of all the obtained sequences of the actin gene together with sequences present in Genbank was made using Clustal X 1.64 (Thompson *et al*., 1997). This alignment was then used to determine putative species-specific DNA fragments that could be used as species-specific primers for the identification of *H. latipons*. Two species-specific primers, one forward and one reverse (sp-spec ActF & sp-specActR) were designed. Next to these, another reverse primer for the actin gene was developed as well (Act R ILVO). During the selection of the fragments, certain parameters (internal complementarity, inter-complementarity, melting temperature and length) were taken into account by using the software program DNA calculator (www.sigma-genosys.com/calc/DNAcalc.asp, Sigma-Aldrich, St Louis, MO, USA). The selected potential species-specific primers were further screened by looking for their presence in sequences stored in GenBank (BLAST option, http://www.ncbi.nlm.nih.gov/).

Table 1. *Heterodera* populations and species used in study other DNA regions

Species	Isolate code	Location	Source
H. avenae	Fa1	Syria	G. Hassan
H. latipons	Fa3	Syria	F. Toumi
H. latipons	4	Syria	F. Toumi
H. latipons	9	Syria	F. Toumi
H. latipons	15C	Syria	G. Hassan
H. latipons	8K	Syria	K. Assas
H. latipons	7A-1	Syria	F. Toumi
H. latipons	7A-2	Syria	F. Toumi
H. latipons	7A-3	Syria	F. Toumi
H. latipons	7A-4	Syria	F. Toumi
H. latipons	7A-5	Syria	F. Toumi
H. latipons	7B-1	Syria	F. Toumi
H. latipons	7B-2	Syria	F. Toumi

H. ciceri	FaC3	Syria	S. Hajjar
H. filipjevi	23.2	Turkey	D. Saglam
H. filipjevi	23.4	Turkey	D. Saglam
H. filipjevi	42.2	Turkey	D. Saglam
H. filipjevi	42.3	Turkey	D. Saglam
H. hordecalis	E69	Israel	R. Rivoal
H. glycines	H. gly Riggs	Poland	R. D. Riggs
H. schachtii	H. sch Poland	Poland	S. Kornobis
H. schachtii	H.sch NDL	Netherlands	HZPC
H. betae	DCP1248	Belgium	ILVO
H. mani	A36	UK	R. Rivoal

Table 2. List of DNA regions and primers used in this study.

DNA-region	primer code	sequence	Fragment length (bp)	Ta (°C)	Sources
Actin	Hs-actF Hs-actR	5-ACTTCATGATCGAGTTGTAGGTGGACTCG-3 5-GACCTCACTGACTACCGATGAAGATTC-3	376	55	Tytgat *et al*., 2004
Pectate Lyase	PectLyaseF PectLyase R	5-CCATCACAGTACAAGC-3 5-GGTTGGTCTGAATTTCGGAT-3	681	45-48-50	Boer *et al*., 2002
Annexins	AnnexF AnnexR	5-ATGCTCCAAAACGGCCTTACCATT-3 5-TCACTGCTCCGTGTTGCCCTT-3	1023	50-52-55	Patel *et al*., 2009
Chorismate mutase	ChorMutF ChorMutR	5-GCCATGGGACAATGCGAGAAACATTGCAC-3 5-GGCCAACAATTTCTTTGC-3	796-1167	50	Vanholme *et al*., 2009
Aldolase	AldoF AldoR	5-ATGGCAGAGGTCGGAAAC-3 5-GCTTTGTAGGTGTAGGC-3	1250-1350	45-48-50	Kovaleva *et al*., 2005
Tubulin	TubulinF TubulinR	5-CTTTACGACATTTGTTTCCGCAC-3 5-GCGGGTCACAKGCGGCCATCATG-3	251-382	50	Sabo and Ferris, 2004
Hsp90	Hsp90F Hsp90R	5-GAYACVGGVATYGGNATGACYAA-3 5-TCRCARTTVTCCATGATRAAVAC-3	900-1500	50-55-60-65	Skantar & Carta, 2004

Optimaization and specificity of species-specific PCR. To determine the optimum annealing temperature (Ta), a gradient PCR was performed in one DNA sample of *H. latipons*

(7A-1) with three combinations of primers (A, B and C in Table 3). The temperature ranged from 60 to 70 °C (60-60.3-60.8-61.2-62.9-64.3-66.0-67.5-68.5-69.3-69.8-70 °C). A certain annealing temperature was selected and two additional primer combinations (D and E in Table 3) were used in a duplex PCR to provide for an internal PCR-control. I selected the best primer-combination (combination E) and further optimized the duplex PCR with different primers and dNTP concentrations (Table 4). Finally, the specificity of the duplex-PCR was tested for all samples used in this study.

Table 3. Overview of the primer combinations tested for the development of a *H. latipons* species-specific duplex PCR.

Primer combination	**Species-specific primer**	**Actin primer specific (s)**	**Additional primers for duplex PCR**
A	sp-spec ActR	ActF	-
B	sp-spec ActF	ActR ILVO	-
C	-	ActF & ActR ILVO	-
D	sp-spec ActR	ActF	ActR ILVO
E	sp-spec ActR	ActF	D2A and D3B

Table 4. Optimaization tests for the duplex PCR

Test	ActF	sp-spec ActR	D2A(*)	D3B(*)
E1	1µM	1µM	1µM	1µM
E2	0.5µM	0.5µM	1µM	1µM
E3	1µM	1µM	2µM	2µM
E4	0.5µM	0.5µM	2µM	2µM
E1 +	E1+ 200µM dNTPs extra			
E2 +	E2+ 200µM dNTPs extra			

E3 +	E3+ 200µM dNTPs extra

(*) De Ley *et al*., (1999).

Results

Identification. The survey revealed three species of CCN in infested fields (*H. latipons, H. avenae* and *H. filipjevi*). *Heterodera latipons* was the most dominant species in Syria and *H. filipjevi* in Turkey.

Amplification of the rDNA-ITS regions was successful for all selected samples (fig1). PCR yielded an expected fragment of 1100 bp (Fig1). Sequencing these fragments confirmed the identity of the previously morphologically identified samples (Table 1). Unfortunately, double sequencing-signals in *H. latipons* sometimes made the analysis difficult but not impossible. No PCR products were obtained in the negative control without nematode DNA template.

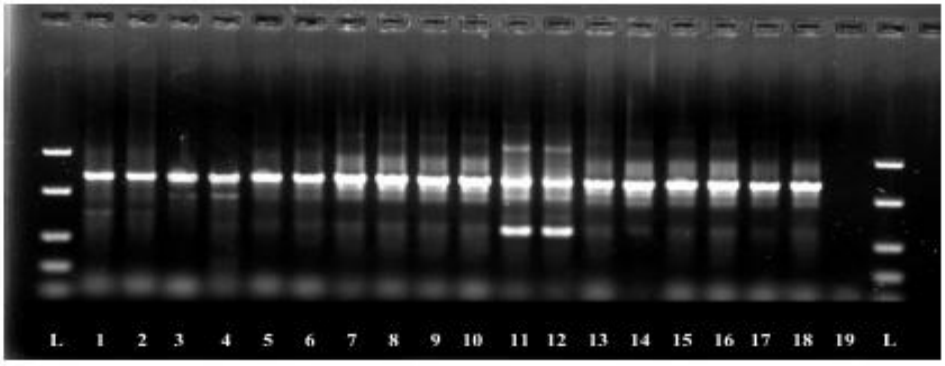

Fig. 1. PCR results for all *Heterodera* samples of the ITS regions (Ferris *et al*., 1993). 1:Fa1, 2: Fa3, 3: 4, 5: 9, 6: 15C, 7: 8K, 8: 7A-1, 9: 7A-2, 10: 7A-3, 11: 23.2, 12: 23.4, 13: 7A-4, 14: 7A-5, 15: 7B-1, 16: 7B-2, 17: 42.2, 18: 42.3, 19: negative control. (For code interpretation, see Table 1) L: Fast Ruler DNA Ladder, Low range (Fermentas Life Sciences).

Amplification of other DNA-regions. PCR products were also obtained from Actin, Hsp90 and Tublin genes. No gene-specific bands or no bands (PCR products) were obtained from the remaining genes (Pectate Lyase, Annexin, Chorismate mutase and Aldolase). PCR with the actin

gene primers revealed the expected 376 bp fragment (Tytgat *et al*., 2004; Kovaleva *et al.*, 2005) (Fig. 2). After blasting, the sequences confirmed the actin gene was amplified for all used samples. Based on the alignment we were able to construct at least two species-specific primers and additionally a universal actin primer.

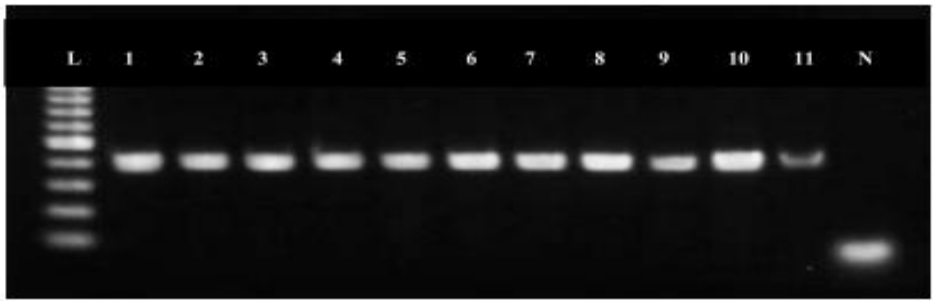

Fig. 2. PCR results of actin gene from all samples. 1:Fa1, 2: Fa3, 3: FaC3, 4: 42.2, 5: A36, 6: E69, 7: DCP1248, 8: H. sch Poland, 9: H.sch NDL, 10: H. gly Riggs, 11: 23.2, N: negative control. (For code interpretation, see Table 1) L: 100 bp DNA ladder (Fermentas Life Sciences).

In some cases PCRs with Tubulin (Sabo and Ferris, 2004) and Hsp90 (Skantar & Carta. 2004) gene primers revealed additional bands next to the expected fragment of 251-382 and 900-1500 bp, respectively. After blasting, the sequences of the expected fragments confirmed Tubulin and Hsp90 genes were amplified. After blasting sequences of few of the additional bands, it became clear that Tubulin and Hsp90 genes from various origins were amplified as well (data not shown).

Species-specific PCR. A gradient PCR was done to set the optimal annealing temperature (Fig. 4). The expected PCR bands were bright over a wide range of temperatures. I decided to keep the annealing temperature at 66 °C.

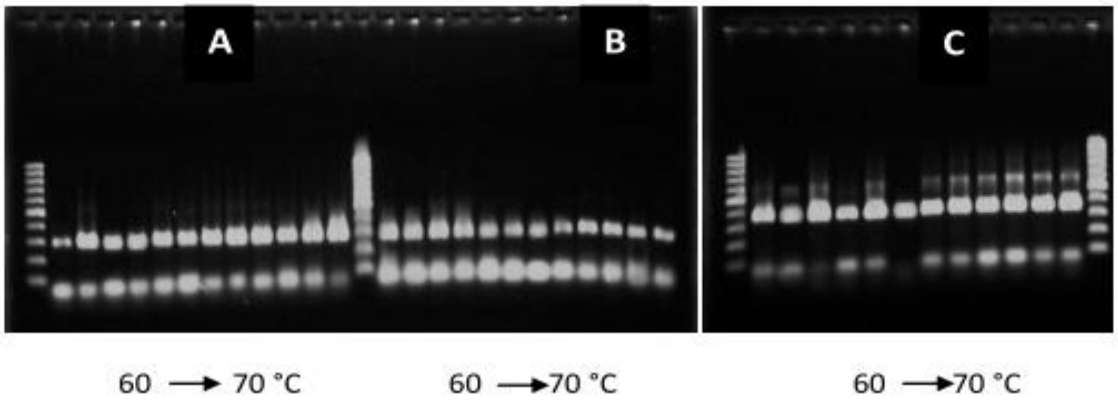

Fig. 4. Results of the Gradient PCR using *Heterodera latipons* Fa3. Temperature ranging from 60 to 70°C. **A**: ActF and sp-spec ActR; **B**: sp-spec ActF and ActR (ILVO); **C**: ActF and ActR (ILVO); 100 bp DNA ladder (Fermentas Life Sciences).

Different primer combinations in the duplex-PCR (Table 4) gave various results (Fig. 5). PCR with combinations A and B showed a clear species-specific band for *H. latipons*. PCR with primer combination C produced an actin-specific, very bright band. Combination D produced an actin-specific band (400 bp) quite close to the species-specific band (300 bp). For this reason, primer combination E, consisting of ActF and sp-spec ActR with control primers D2A and D3B was retained for further investigations. In this case both PCR-products were easier to separate from each other after electrophoresis due to the larger difference in the length of the PCR-products.

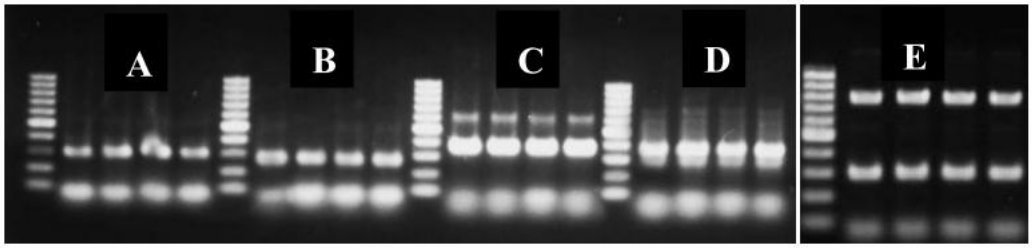

Fig. 5. Results of the optimization of the combinations between the primers using sample Fa3 (*H. latipons*); **A**: ActF & sp-spec ActR; **B**: sp-spec ActF & ActR (ILVO); **C**: ActF & ActR (ILVO); **D**: ActF & sp-spec ActR & Act R (ILVO); **E**: ActF, sp-spec ActR and D2A-D3B; 100 bp DNA ladder (Fermentas Life Sciences).

Further optimization tests for the duplex PCR revealed that a concentration (volume) of two times more than that of the internal control primers, compared to the species-specific primer set, resulted in a more or less similar brightness of both PCR products (Fig. 6). Adding an extra 200µM dNTPs to each previous reaction caused the complete disappearance of the internal control and weaker species-specific PCR products (Fig. 6. E1+, E2+ and E3+ in Table 4).

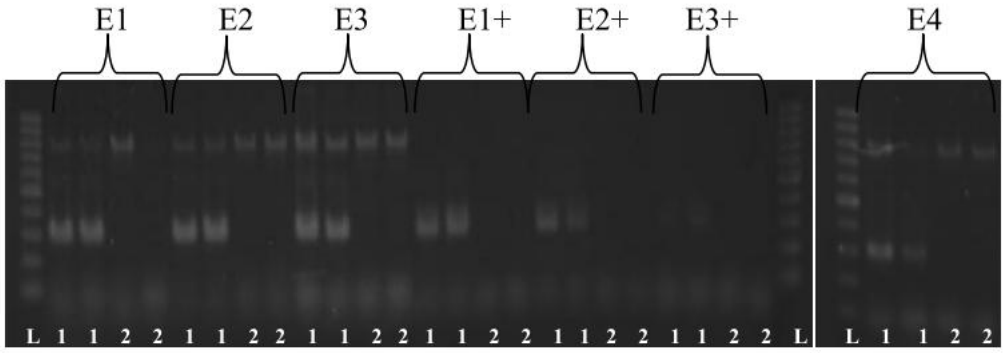

Fig. 6. Results of the duplex PCR optimization primer concentrations tests (Table 4), with a species-specific primers and internal control primers using DNA from *H. latipons* (1) as a positive control and *H. glycines* (2) as a negative control. L: 100 bp DNA ladder (Fermentas Life Sciences).

The optimized duplex-PCR using primers sp-spec ActR, ActF, D2A and D3B with the PCR program set to: 5 min at 95°C; 35 cycles of 94°C for 30 s, 66°C for 45 s and 72°C for 45 s; followed by a final elongation step of 8 min at 72°C, produced for all samples used in this study (Fig. 7) a clear universal band of 800 bp and a species-specific band for all *H. latipons* populations of 300bp.

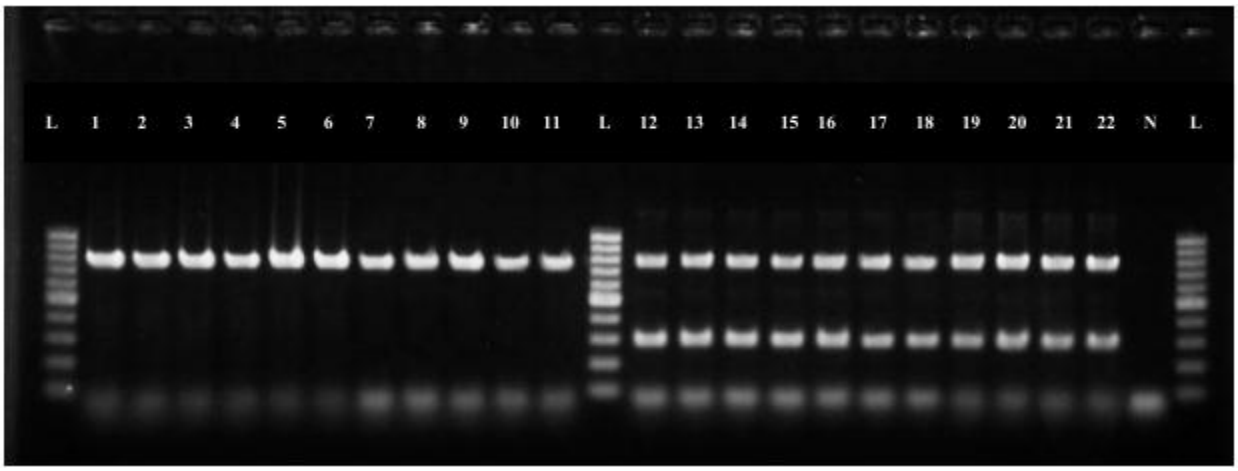

Fig. 7. Results of the *Heterodera latipons* species-specific duplex PCR (E3) using DNA from all samples. 1:Fa1, 2: 42.2, 3: 23.2, 4: FaC3, 5: A36, 6: E69, 7: DCP1248, 8: H. sch Poland, 9: H.sch NDL, 10: H. gly Riggs, 11: 23.4, 12: 7A-1, 13: 7A-2, 14: 7A-3, 15: 7A-4, 16: 7A-5, 16: 7B-1, 17: 7B-2, 18: 4, 19: 9, 20: 15C, 21: 8K, 22: Fa3, N: negative control. (For code interpretation, see Table 1) L: 100 bp DNA ladder (Fermentas Life Sciences).

Discussion

According to the survey, *H. latipons* is the most dominant species belonging to the avenae-group present in Syria. This result confirmed the results of two previously conducted surveys in the same regions. The results are not so surprising because barley is the major crop in these regions and barley as a good host for *H. latipons*. However, the survey also revealed in certain cases mixes of two or three CCN species in the same field. Therefore species identification is

unreliable when done by not skilled researchers. Molecular techniques can help but the ones used untill now have limitations. PCR-RFLP needs sometimes combinations of expensive restriction enzymes. Due to polymorphisms and because of restrictions of the technique itself, it remains sometimes difficult to distinguish between *Heterodera* species. Sequencing the ITS region is sometimes not useful since they cannot always distinguish between some species. Moreover, the technique is time-consuming and laborious. Therefore, a species-specific primer for *H. latipons* must be constructed to reliable identify the species in a simple PCR and in a short time. Also this primer will facilitate the reliable identification in all regions in Syria. This will help further research like studying the population pathotype in each region, assisting in chosing the correct control strategies e.g. resistance lines etc.

During the amplification reactions to amplify other DNA-regions, degenerated primers were used, and the primers were sensitive to any change in the annealing temperature. Therefore, obtaining nice PCR-products was difficult. Sometimes it was even impossible to create a very weak product, because any change in PCR-settings will benefit for one primer but may not be for another primer. In many cases, unspecific products or no product at all was obtained. However, we obtained sequences of all the *Heterodera* species used for the actin gene only. Therefore we decided to develop species-specific primers for this region. The alignment of the actin sequences showed very high homologies between the *Heterodera* species 83% to 97% (highest was between *H. latipons* and *H. avenae*). Nevertheless, several positions were variable enough to be able to design species-specific primers for *H. latipons*. In future, I will look to the alignment again checking the possibility to find other species-specific primers for other species especially the species present in Syria (*H. avenae, H. filipjevi* and *H. ciceri*), and to design primers to detect the Avenae group.

Variability within the binding place of the reverse primer causing sometimes weak PCR-products, could be noticed. Therefore, I decided also to design another reverse actin-primer situated in a more stable region and which combines very well with the forward species-specific primer (sp-spec ActF) to obtain always a nice PCR-product.

I can say that our species-specific PCR is not only reliable but also very robust. Gradient PCR revealed that the primers are useful over a wide range of temperatures. This makes it easy to use the PCR on different locations without further optimization, and makes it more flexible for checking the possibility to use in a duplex or multiplex PCR with other species-specific or universal primers.I decided to use the D2D3 region as an internal control for the duplex PCR. The internal control was used to prevent false negatives caused by failure of the PCR for any reason.

The test for different primer combinations in the duplex-PCR, revealed that the combination C gave bands brighter than in A and B . For combination D we should run the electrophoresis more than 70 min to be able to visualize a distance between the two bands. However, in combination E 40 min was enough to have clear and nice difference. This combination was finally retained. The concentration optimization test for the duplex PCR showed E3 was the best combination. Although E2 has the same combination ratio, in E3 both bands were bright and clear. Adding extra dNTPs caused complete inhibition of the PCR. This could be due to the mismatch between the master mix buffer and the dNTPs buffer.

I could construct a reliable and easy to use duplex PCR for the detection of *H. latipons*. The appearance of an additional weak band in few cases (*H. hordecalis* & *H. glycines*) did not disturb the interpretation of the results. However, I still need to do more tests:

- The optimized duplex PCR was capable of detecting solely *H. latipons* amongst all *Heterodera* species used in the study, but more tests still need to be done on other *Heterodera* species and *H. latipons* populations originating from all over the world to further test the specificity.
- The capability for the species-specific primer to detect one *H. latipons* individual amongst other specimens of other *Heterodera* species should be determined.
- Other species-specific primers for other species especially the species present in Syria (*H. avenae*, *H. filipjevi* and *H. ciceri*) can be designed.

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