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Diagnostics of stem and bulb nematodes, *Ditylenchus weischeri* and *D. dipsaci* (Nematoda: Anguinidae), using PCR with species-specific primers

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Abstract: The stem and bulb nematodes of the *Ditylenchus dipsaci* (Kühn) Filipjev species complex are obligate endoparasites of various agricultural plants, causing stunting and swelling and resulting in significant economic losses. Recently, a new closely related *Ditylenchus* species, *D. weischeri* Chizhov, Borisov and Subbotin, a parasite of the cosmopolitan herbaceous perennial weed, *Cirsium arvense* (L.) Scop., was described. Many countries impose quarantine restrictions for the presence of *D. dipsaci* in imported plant and soil materials. In the current study, we developed PCR with species-specific primers for the rapid and reliable separation of *D. weischeri* and *D. dipsaci* using gel electrophoresis and melting curve analysis. Species-specific primer sets were designed based on the nucleotide sequence of the heat shock protein (*hsp*90) gene for both nematode species. The PCR protocol was verified using samples of *D. weischeri*, *D. dipsaci* and the closely related species, *D. gigas*, which parasitizes broadbean (*Vicia faba* L.). The species-specific primer sets were able to detect *D. weischeri* and *D. dipsaci* from samples containing mixtures of *Ditylenchus* species. The PCR species-specific protocol should allow for more rapid identification of *Ditylenchus* species recovered from plant materials than previously possible.

Keywords: *Cirsium arvense*, *D. dipsaci*, *D. weischeri*, diagnostics, *Ditylenchus*, heat shock protein gene, *hsp*90, melting curve analysis

Résumé: Les nématodes de la tige et du bulbe du complexe d'espèces *Ditylenchus dipsaci* (Kühn) Filipjev sont des endoparasites obligatoires qui s'attaquent à différentes cultures et qui causent du rabougrissement et du renflement, ce qui occasionne d'importantes pertes économiques. Récemment, une nouvelle espèce de *Ditylenchus* étroitement apparentée, *D. weischeri* Chizhov, Borisov and Subbotin, un parasite de l'adventice cosmopolite vivace *Cirsium arvense* (L.) Scop., a été décrite. Plusieurs pays imposent des restrictions phytosanitaires relativement à *D. dipsaci* quant aux importations de végétaux et de matériaux constitutifs du sol. Dans cette étude, nous avons développé une PCR avec amorces spécifiques de l'espèce pour différencier rapidement et de manière fiable *D. weischeri* et *D. dipsaci*, et ce, à l'aide d'une électrophorèse sur gel et de l'analyse de la courbe de fusion. Les jeux d'amorces spécifiques de l'espèce ont été conçus, pour les deux espèces de nématodes, en fonction de la séquence de nucléotides du gène de la protéine de choc thermique (*hsp*90). Le protocole de la PCR a été vérifié avec des échantillons de *D. weischeri, D. dipsaci* et de l'espèce étroitement apparentée, *D. gigas*, qui parasite la féverole à gros grains (*Vicia faba* L.). Les jeux d'amorces spécifiques de l'espèce ont pu détecter *D. weischeri* et *D. dipsaci* dans des échantillons contenant un mélange d'espèces de *Ditylenchus*. Le protocole spécifique de l'espèce de la PCR devrait permettre d'identifier plus rapidement que cela était possible auparavant les espèces de *Ditylenchus* extraites des végétaux.

Mots clés: analyse de la courbe de fusion, *Cirsium arvense*, diagnostics, *Ditylenchus*, *D. dipsaci*, *D. weischeri*, gène de la protéine de choc thermique, *hsp*90

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Introduction

The stem and bulb nematode, *Ditylenchus dipsaci* (Kühn) Filipjev, is an obligate endoparasite of about 500 plant species and causes damage to stems of agricultural crops, such as pea (*Pisum sativum* L.), bean (*Phaseolus vulgaris* L.), strawberry (*Fragaria* × *ananassa* Duchesne) and alfalfa (*Medicago sativa* L.). As well, the nematode affects bulbs such as onion (*Allium cepa* L.), garlic (*Allium sativum* L.), tulip (*Tulipa* spp. L.) and daffodil (*Narcissus* spp. L.), causing stunting and swelling and resulting in significant economic losses. In addition, a variety of weed plants are hosts of *D. dipsaci*, making control of this nematode difficult, and it has a cosmopolitan distribution of temperate climates.

Many authors have reported that *D. dipsaci* consists of a number of biological races and populations that differ in host preferences and occur at different stages of speciation and reproductive isolation, which likely represent different species (Sturhan & Brzeski 1991). Subbotin et al. (2005) confirmed that *D. dipsaci* represented a species complex following phylogenetic analysis of ITS rRNA gene sequences of different populations and races of the nematode. Presently, the *D. dipsaci* species complex consists of two agriculturally important species: *D. dipsaci sensu lato* that parasitizes various agricultural crops and weeds, and *D. gigas* (Vovlas et al. 2011), that parasitizes broad bean (*Vicia faba* L.) and several weeds. This species complex also includes a newly described species, *D. weischeri* (Chizhov et al. 2010) that parasitizes the weed plant, *Cirsium arvense* (L.) Scop., as well as several undescribed *Ditylenchus* species that parasitize various uncultivated plants (Subbotin et al. 2005; Chizhov et al. 2010; Vovlas et al. 2011).

The nematode identified as *D. dipsaci* has been recovered from *C. arvense* and other *Cirsium* species by several authors across Europe (Goodey et al. 1965). Watson and Shorthouse (1979) found the stem nematode parasitizing *C. arvense* from a pasture near the city of Regina in Saskatchewan, Canada and described induced pathological changes in plants. *Circium arvense*, commonly called creeping thistle and Canada thistle, is a persistent herbaceous perennial weed of temperate climates distributed worldwide. Chizhov et al. (2010) first integrated morphological and molecular analyses (ITS-PCR-RFLP) for separation of this stem nematode from *D. dipsaci* and described it as a new species *D. weischeri* from *C. arvense.* The nematode was unable to parasitize onion and strawberry (Chizhov et al. 2010) and currently there is no evidence of it infecting agricultural crops.

Recently, Tenuta et al. (2015) reported the occurrence of a *Ditylenchus* sp. in field pea seed samples collected from over 150 pea growers in the provinces of Alberta, Saskatchewan and Manitoba. Over 2 years of the study, just 2% of the samples were positive, indicating a very low frequency of the nematode in the pea harvest samples. Infestation levels ranged from 4 to 1500 nematodes kg^{-1} of pea sample. Nematodes were associated with Canada thistle dry flower heads recovered from the pea samples. Morphological and PCR-ITS-RFLP analysis of adult male and female nematodes revealed they were *D. weischeri* (Tenuta et al. 2015). Differentiation of *D. weischeri* from *D. dipsaci* remains a difficult task, requiring detailed morphometric measurements, chromosome number determination, and *hsp*90 and ITS rRNA gene sequencing or PCR-ITS-RFLP analysis (Chizhov et al. 2010). The PCR-ITS-RFLP analysis is not amenable for use where more than one species of *Ditylenchus* is present in a sample. More rapid means are required for routine differentiation of the *Ditylenchus* species.

Due to the potential for severe economic loss on a large number of agricultural crops, *D. dipsaci* is listed as an A2 quarantine pest by the European and Mediterranean Plant Protection Organization (CABI 2014). It is also considered a pest of quarantine significance by the North American Plant Protection Organization, the Caribbean Plant Protection Commission and the Inter-African Phytosanitary Council (CABI 2014). As a result, many countries have quarantine restrictions on importation of infested plant and soil materials containing *D. dipsaci*. Thus, use of a reliable and rapid method for differentiation of *D. dipsaci* from *D. weischeri* is important for phytosanitary certification purposes.

The objective of the present study was to develop a PCR method with species-specific primers using the heat shock protein, *hsp*90 gene, for rapid differentiation of *D. weischeri* and *D. dipsaci* with visualization by traditional electrophoresis and melting curve analysis of diagnostic amplicons. Further, the ability of the species-specific primer sets to distinguish *D. weischeri* and *D. dipsaci* in sample mixtures of the nematode species by PCR was determined.

Materials and methods

Nematode samples

Specimens of *D. weischeri* were obtained from three yellow pea harvested seed samples contaminated with *C. arvense* from commercial fields (designated PG33, PG58 and PG81), five samples of *C. arvense* shoots (C11F11, C11F18, C11F19, CT81 and CT84) from commercial yellow pea fields in Saskatchewan or Manitoba, and three weed samples collected from fieldside ditches (GL6, GL8 and

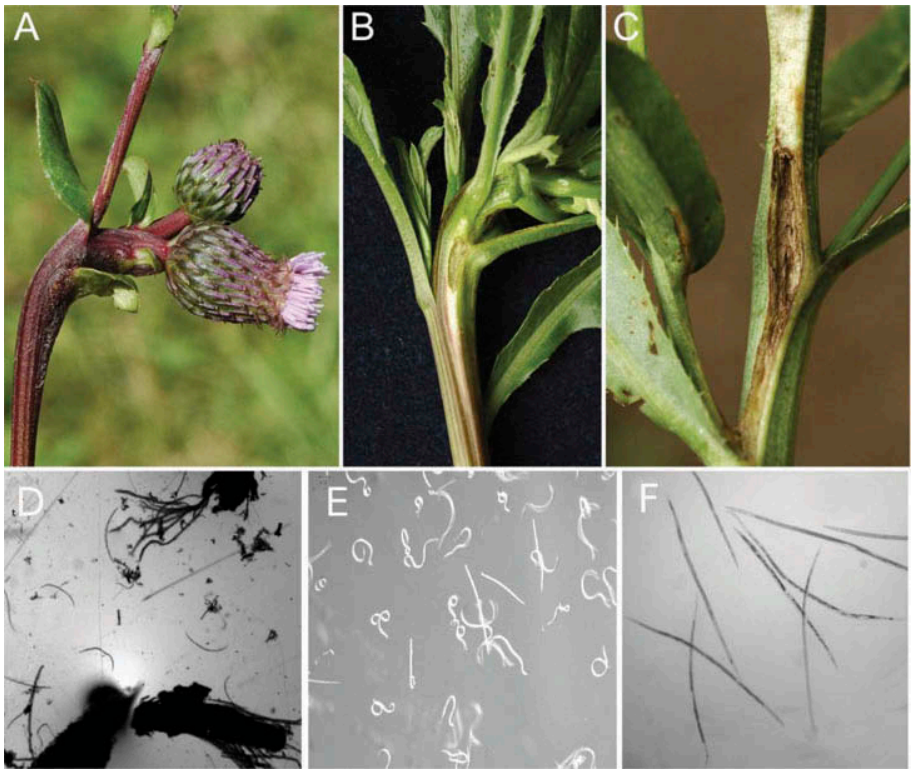

Fig. 1 (Colour online) Symptoms of infection of *Ditylenchus weischeri* on *Cirsium arvense* shoots **(A, B)** stem swelling; **(C)** cracking of stem swelling; **(D)** *D. weischeri* from *C. arvense* flowers; **(E)** live *D. weischeri*; and **(F)** heat-killed *D. weischeri* nematodes extracted from infected shoots.

GL12) at the University of Manitoba Glenlea Research Station, and native areas in the Moscow region, Russia Federation. The shoots had visible disease symptoms of leaf necrosis and shoot necrosis, twisting, stunting and thickening (Fig. 1A, B, C). The shoots were cut into small pieces and soaked in water for several hours to allow nematodes to emerge (Fig. 1D, E, F). They were then hand-picked using a needle with the aid of a stereoscope and transferred to watch glasses that contained distilled water and frozen at −15°C until further analysis. Several samples of *D. dipsaci* from garlic obtained from Minnesota, Ontario and Quebec, as well as from strawberry and *Phlox* spp. L. from Russia, and *D. gigas* from broad bean from Morocco, were included in this study.

DNA extraction, PCR, sequencing and phylogenetic analysis

Protocols of DNA extraction, PCR and sequencing used for the nematode specimens were previously described and published by Mundo-Ocampo et al. (2008). The U831 and L1110 primers (Table 1) were used for amplification of *hsp*90 gene fragment from samples PG58, PG81, C11F19, CT84, GL6, GL8, GL12 and two other collections of *D. weischeri* from *C. arvense* in MB. Sanger sequencing of the amplified fragment was conducted by the core sequencing facility of Macrogen Corp. (Rochville, MD). The sequences for the aligned *hsp*90 gene fragment were very similar for the samples of *D. weischeri*, having the same length (211-bp) and

Table 1. Primer combinations used in the present study.

Gene position or fragment	Primer code	Direction	Primer sequence 5′ – 3′	Reference
Universal *hsp*90	U831	forward	AAY AAR ACM AAG CCN TYT GGA C	Skantar and Carta (2005)
	L1110	reverse	TCR CAR TTV TCC ATG ATR AAV AC	
*D. dipsaci hsp*90	U831	forward	AAY AAR ACM AAG CCN TYT GGA C	This study
	Dipsaci_hsp90R	reverse	GWG TTA WAT AAC TTG GTC RGC	
*D. weischeri hsp*90	U831	forward	AAY AAR ACM AAG CCN TYT GGA C	This study
	Weischeri_hsp90R	reverse	AGC ACT AAA ATT AAG YGT AAA GG	

varying in only three single nucleotide positions (Madani et al., unpublished data). The sequence obtained for GL6 was submitted to the GenBank database under the accession KJ817197.

The *hsp*90 gene sequence of *D. weischeri* GL6 from MB was aligned with similar gene fragments of the *D. dipsaci* complex available in GenBank (Chizhov et al. 2010) using ClustalX 1.83 (Thompson et al. 1997) under the default parameters of the analysis program. Outgroup taxa for each dataset were chosen based on results of previously published data (Chizhov et al. 2010). The sequence dataset was analysed by Bayesian inference (BI) using the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). BI analysis under the GTR + I + G model was initiated with a random starting tree and was run with four chains for 10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The topologies were used to generate a 50% majority rule consensus tree. Sequence analysis was performed with the program PAUP* 4.0b 10 (Swofford 2003). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole sequence alignments, with adjustment for missing data.

PCR with species-specific primers

Two reverse species-specific primers, one each for *D. dipsaci* (Dipsaci_hsp90R) and *D. weischeri* (Weischeri_hsp90R), were designed using the sequence alignment of *hsp*90 gene sequences of *Ditylenchus* species including that for KJ817197 from this study. The PCR mixture was prepared as described by Tanha Maafi et al. (2003). PCR for detection of *Ditylenchus* spp. was run with either of *D. weischeri* or *D. dipsaci* specific reverse primers and the U831 universal forward primer (Table 1).

The PCR amplification profile consisted of 4 min at 94°C; 30 cycles of 1 min at 94°C, 45 s at 57°C and 45 s at 72°C, followed by a final step of 10 min at 72°C. Two µL of the PCR products were run on a 1.4% TAE (0.5×) buffered agarose gel, stained with ethidium bromide and imaged.

Melting curve analysis with species-specific primers

Each of the two primers sets specific for *D. weischeri* and *D. dipsaci* was also used in real-time PCR for melt curve analysis of amplicon products. The amplification reaction was performed using a light cycler (T100TM, Bio-Rad Laboratories Canada Ltd, Mississauga, ON). The reaction mixture consisted of 8 µL of EvaGreen dye prepared master mix (Biotium Inc., Hayward, CA), 0.25 µL of each primer, 1 µL of DNA extract and dH_2O to a final volume of 20 µL. The thermal profile consisted of three steps at each of 94°C, 55°C and 69°C, followed by melting curve profile analysis. Melting curve analysis was performed with an initial denaturing step at 94°C for 30 s at the end of the amplification cycle, then cooling of samples at 1.6°C/s (ramp 100%) to 60°C, and then increasing the temperature to 95°C at 0.5°C/step with an 18 s pause at each step. The fluorescence emission was measured at each step during the temperature ramping and the temperature at which 50% of the amplicon denatured determined as the melting point. A negative control contained only the PCR mixture without DNA template.

PCR with species-specific primers and D. weischeri *and* D. dipsaci *sample mixtures*

The ability of the species-specific primer sets to distinguish *D. weischeri* and *D. dipsaci* in sample mixtures using PCR was examined. Weischeri_hsp90 was used following DNA extractions from *D. dipsaci:D. weischeri* R was used following DNA extractions of *D. weischeri* individuals against increasing number of *D. dipsaci* from Ontario (*D. weischeri:D. dipsaci* ratio of 1:0, 1:1, 1:5 and 1:10). Similarly, Dipsaci_hsp90R was used following DNA extractions from *D. dipsaci:D. weischeri* individual nematode ratios of 1:0, 1:1, 1:5 and 1:10. All DNA extractions of mixtures and subsequent PCR were conducted using *D. weischeri* GL12 from Manitoba and from Russia, with all reactions in triplicate.

DNA was extracted from the nematode mixtures as described previously. The PCR reaction consisted of 2 µL of DNA extraction solution, 250 nM of each primer, 12 µL of premixed ready to use master mix (GoTaq® Green Master Mix, Promega, Madison WI) and distilled water to the final volume of 25 µL. The master mix could be substituted for Taq PCR Core Kit (Qiagen, Gaithersburg MD) solutions. With this substitution, the PCR cocktail consisted of 2 µL of DNA extraction solution, 250 nM of each primer, 2.5 µL of 10× PCR buffer, 5 µL of Q-Solution®, 0.5 µL of dNTP mixture and 0.1 µL of *Taq* polymerase. The final volume was adjusted to 25 µL by adding distilled water. Optimization of PCR by gradient annealing temperature was done for each primer set using a 1:1 mixture of each nematode species. After optimization, the annealing temperature was 50.5°C and 55.0°C for the primer set for *D. dipsaci* and *D. weischeri*, respectively. The amplification reaction had a preheating step at 94°C for 3 min; 33 cycles of 94°C for 30 s, 50.5 or 55.0 °C for 1 min and 72°C for

1 min; and a final extension at 72°C for 10 min. Two µL of the PCR products were run on a 1.4% TAE (0.5×) buffered 1% agarose gel containing 0.5µL of 10 000× GelRed (Biotium, Hayward CA) dye and visualised on a UV transilluminator (GBox, Syngene, Cambridge, UK).

Results and discussion

Primers U831 and L1110 successfully amplified the *hsp*90 gene fragments in all *D. weischeri* samples obtained from Canada and Russia. After excluding ambiguous poor-quality sequences and primer sequences, a fragment of 228 bp in length from sample GL6 was used for further analysis. The resulting *hsp*90 gene alignment was 247 bp in length and also included in total 13 sequences of *Ditylenchus* species from the *D. dipsaci* species complex, and *Ditylenchus* sp. and *Cephalenchus hexalineatus* were used as outgroup taxa. The sequence of the Canadian *D. weischeri* differed from that from Russia by 4.8% (11 bp). Intraspecific sequence diversity (uncorrected p-distance) for the populations of two other species of *Ditylenchus* were: *D. dipsaci* – 1.7–5.2% (4–12 bp) and *D. gigas* – 0–0.1% (0–2 bp).

A majority consensus phylogenetic tree generated by the BI analysis of the *hsp*90 sequence alignment under the GTR+G + I model was constructed and is presented in Fig. 2. The tree contained four highly supported major clades, which corresponded to four *Ditylenchus* species. The sequence of the Canadian *D. weischeri* clustered with that of *D. weischeri* from Russia. *Ditylenchus weischeri* occupied a basal position in this tree.

In this study, we developed species-specific primers for differentiation of *D. weischeri* and *D. dipsaci* based on the *hsp*90 gene sequence alignment obtained from several *Ditylenchus* species (Fig. 3). Species-specific primers were identified manually and tested with

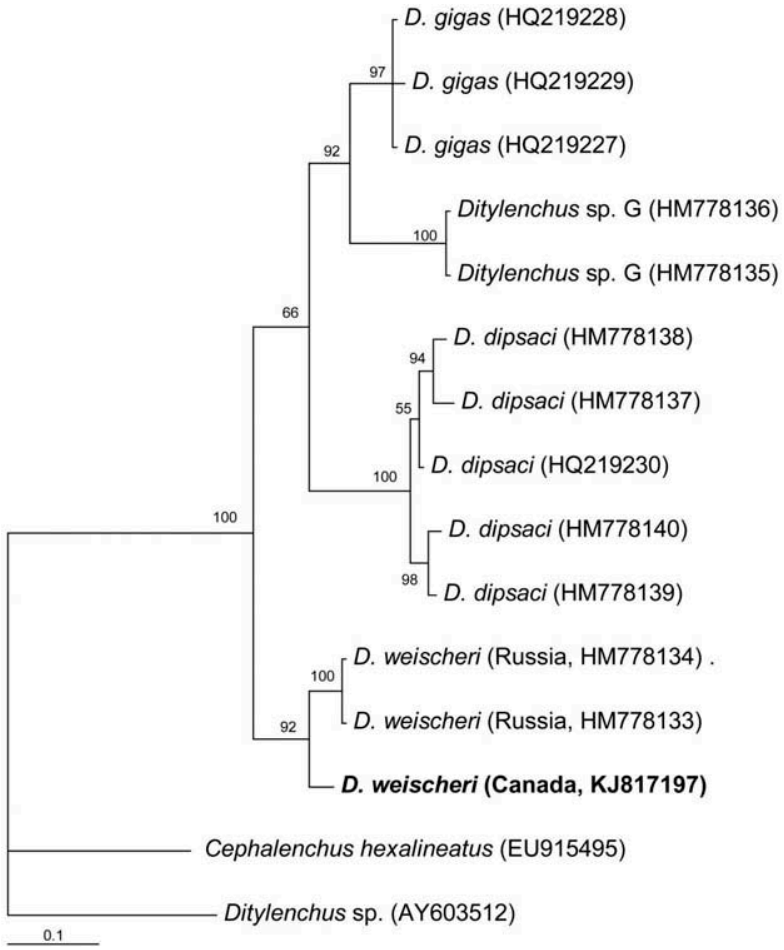

Fig. 2 Phylogenetic relationships of *D. weischeri* with other species of the genus *Ditylenchus* as inferred from the Bayesian analysis of the *hsp*90 gene sequences under the GTR+I + G model. Posterior probabilities are given on appropriate clades.

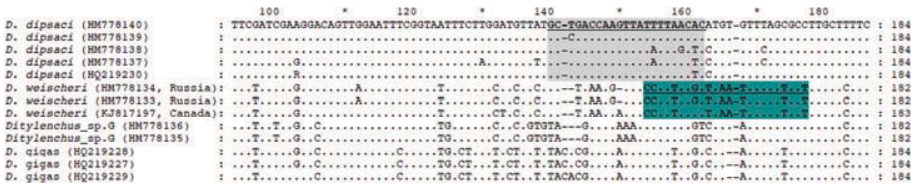

Fig. 3 (Colour online) Fragment of the *hsp*90 gene sequence alignment for *Ditylenchus* species with the annealing positions of species-specific primers Weischeri_hsp90R and Dipsaci_hsp90R for *D. weischeri* and *D. dipsaci*, respectively, shown.

D. weischeri samples from Canada and Russia, and *D. dipsaci* from Canada, the USA and Russia. The combination of the universal primer U831 with the *hsp*90 species-specific primers yielded a single PCR product around 196 bp and 182 bp in size for *D. weischeri* (Fig. 4A) and *D. dipsaci* (Fig. 4B), respectively. While we confirmed accurate identification of *D. weischeri* and *D. dipsaci* from Canadian and Russian samples and negative amplification for *D. gigas* from Morocco, further tests with more species of *Ditylenchus* and populations of *D. weischeri* and *D. dipsaci* from other geographic origins are needed to show the general applicability of our results and confirm the robustness of the method.

Melting curve analysis of samples using the two species-specific primers developed here resulted in a melting point of 82 ± 0.2°C and 81.5 ± 0.5°C for Weischeri_hsp90R and Dipsaci_hsp90R with *D. weischeri* and *D. dipsaci*, respectively (Fig. 5 A, B). The amplicon products of the real-time PCR assay were confirmed by gel electrophoresis (data not shown).

The primer set Weischeri_hsp90R specific to *D. weischeri* amplified only DNA of *D. weischeri* and detected a single nematode individual in all prepared

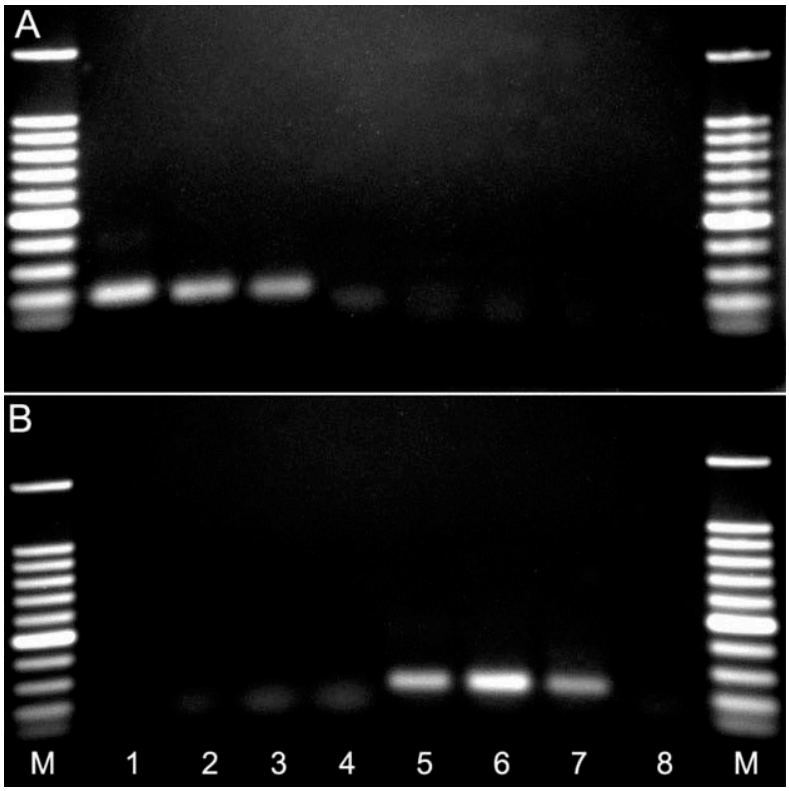

Fig. 4 Gel electrophoresis micrograph showing specific amplicons obtained from species specific PCR reactions; **(A)** *D. weischeri* species specific primers (Weischeri_hsp90R + U831); **(B)** *D. dipsaci* species specific primers (Dipsaci_hsp90R + U831). Lanes: M – 100 bp DNA ladder; 1 – *D. dipsaci* from onion, MN, USA; 2 – *D. dipsaci* from phlox, Russia; 3 – *D. dipsaci* from strawberry, Russia; 4 – *D. gigas*, Morocco; 5 – *D. weischeri*, Russia; 6, 7, *D. weischeri* from *C. arvense*, Canada; 8 – control without DNA; M – 100-bp DNA ladder.

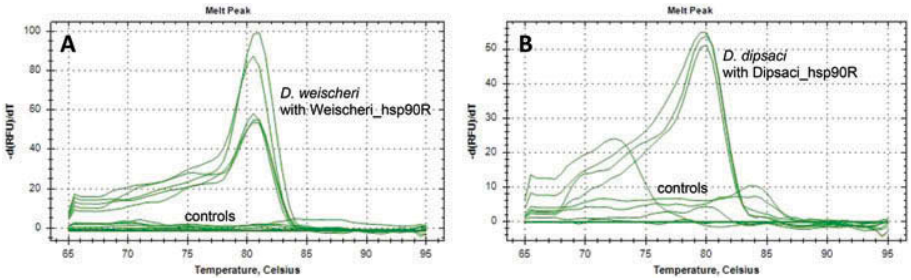

Fig. 5 (Colour online) Example melting curve analysis obtained using real-time PCR for PCR products of the DNA extracted from *Ditylenchus* samples with primer sets; (**A)** Weisheri_hsp90R + U831, specific to *D. weischeri* from four samples from Canada and one from Russia, with negative controls being other foliar nematode species obtained from *C. arvense* shoots from Manitoba; and (**B)** Dipsaci_hsp90R + U831, specific to *D. dipsaci* using garlic samples from Ontario and Quebec with negative controls being three *D. weischeri* samples from Canada.

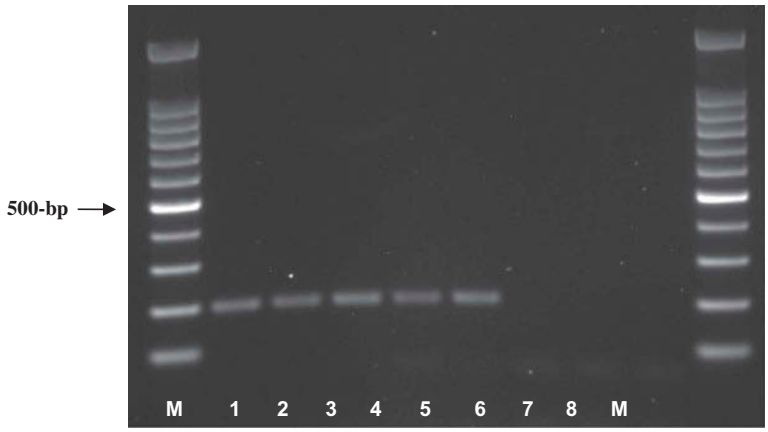

Fig. 6 Gel electrophoresis micrograph of amplification product of PCR using the *D. weischeri* species specific primer set Weischeri_hsp90R, and DNA extracted from mixture of individuals of *D. weischeri* and *D. dipsaci.* Lanes 1, 2 – *D. weischeri* from Manitoba and Russia, respectively; Lanes 3, 4, 5 – DNA from mixtures of individuals of *D. weischeri:D. dipsaci* at ratios of 1:1, 1:5 and 1:10; Lanes 6, 7 – *D. dipsaci* from Ontario and Quebec, respectively; and Lane 8 – PCR reaction without DNA; M – 100-bp DNA ladder.

mixture ratios with *D. dipsaci* (Fig. 6). Agarose gel electrophoresis of the PCR reaction product resulted in a single amplicon of approximately 200 bp in size for samples containing a single *D. weischeri* individual, regardless of the presence of *D. dipsaci* DNA and whether *D. weischeri* was from Manitoba and Russia. As previously, no amplification product was obtained with this primer when *D. dipsaci* was used as DNA template alone.

One amplicon of approximately 190 bp in size was produced using Dipsaci_hsp90R for individuals of *D. dipsaci* alone, or with increasing numbers of *D. weischeri* GL12 from Manitoba (Fig. 7) or Russia. Again, no amplification was obtained with this primer when an individual of *D. weischeri* GL12 from Manitoba or Russia was used alone as DNA template. Thus, the primer sets Weischeri_hsp90R and Dipsaci_hsp90R were able to detect an individual of *D. weischeri* and *D. dipsaci*, respectively, regardless of an increasing background of individuals of the other species.

PCR-RFLP diagnostic tools have been developed for identification of *D. dipsaci*, *D. weischeri* and *D. gigas* (Wendt et al. 1993; Subbotin et al. 2005; Chizhov et al. 2010; Vovlas et al. 2011). This method is suitable to identify species in monospecific samples but cannot be used if a sample contains more than one nematode species. The method developed in the current study for PCR with species-specific primers overcomes this limitation and allows distinguishing target species in samples containing several species. This PCR method decreases the diagnostic time and costs compared with ITS-PCR-RFLP. Previously, PCR with species-specific primers was developed for differentiation of *D. dipsaci* and *D. gigas* using species-specific SCAR or ITS-rRNA primers (Esquibet et al. 2003; Marek et al. 2005; Subbotin et al. 2005; Kerkoud et al. 2007; Zouhar et al. 2007). The results of

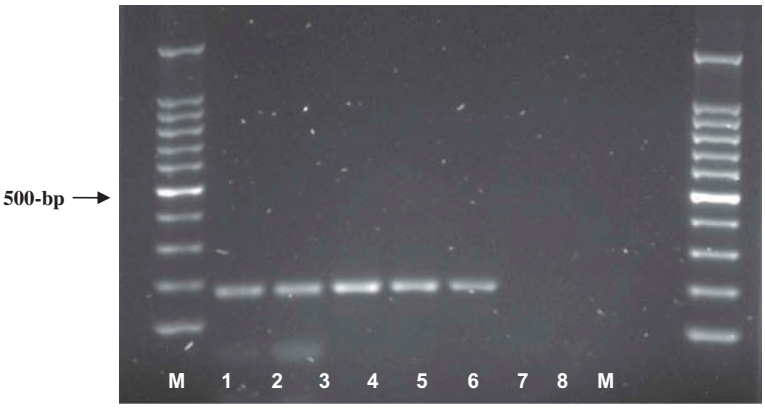

Fig. 7 Gel electrophoresis micrograph of amplification product of PCR product using the *D. dipsaci* species-specific primer set Dipsaci_hsp90R, and DNA extracted from a mixture of individuals of *D. dipsaci* and *D. weischeri*. Lanes 1, 2 – individuals of *D. dipsaci* from Ontario; Lanes 3, 4, 5 – DNA from mixtures of individuals of *D. dipsaci:D. weischer*i GL12 at ratios 1:1, 1:5 and 1:10, respectively; Lanes 6, 7 – *D. weischeri* from Russia and GL12 from Manitoba, respectively; Lane 8 – PCR reaction without DNA; M – 100-bp DNA ladder.

the current study allows for additional differentiation of *D. weischeri* by a PCR species-specific method. In this study, we also demonstrated the usefulness of melting curve analysis for the rapid specific detection of stem and bulb nematodes. This assay itself is more rapid than conventional specific nematode PCR assays described, because it excludes a post-PCR agrose gel electrophoresis step.

Douda et al. (2013) found great similarity among the stem nematodes (*D. dipsaci, D. weischeri, D. gigas*) based on the sequence of the ITS1-5.8S-ITS2 rDNA gene. The stem nematodes were distinct from other *Ditylenchus* species including *D. destructor* Thorne, a plant parasite of potato tubers and bulbs, *D. halictus* Giblin-Davis, Erteld, Kanzaki, Ye, Zeng and Center, a fungal feeder and associate of the soil-dwelling sweat bee (*Halictus sexcinctus*), and *D. myceliophagus* Goodey, a pest of commercial button mushroom (*Agaricus bisporus* (Lange) Imbach). Nevertheless, until further examination of the *hsp*90 gene with these and other *Ditylenchus* species, the PCR species-specific method developed here is intended for use on grain and fresh above-ground plant material in which the stem nematodes are usually found.

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