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Molecular Characterization and Phylogeny of *Ditylenchus weischeri* from *Cirsium arvense* in the Prairie Provinces of Canada

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Abstract

Ditylenchus weischeri that parasitizes the weed *Cirsium arvense* (L.) Scop., 1772, (creeping thistle) was described in 2011 from Russia based on their morphology, ITS-RFLP analysis, and *Hsp*90 gene sequence of a few individuals and one field collection of the plant. More recently, we found *C. arvense* parasitized by *D. weischeri* in the Prairie Provinces of Canada. Plant host preference for *D. weischeri* was also distinct from *D. dipsaci* (Kühn) Filipjev, 1936. In the current study, a comprehensive molecular analysis of many *D. weischeri* specimens from Canada is presented. Individuals from 41*C. arvense* or yellow pea grain samples with seeds of *C. arvense* from the Prairie Provinces were sequenced for the internal transcribed spacer (ITS rDNA), large subunit (LSU) D2D3 28S rDNA, partial segment of small subunit (SSU) 18S rDNA, and the heat shock protein *Hsp*90 gene. The analysis also included *D. weischeri* individuals from *C. arvense* from Russia and garlic with *D. dipsaci* from the Provinces of Ontario and Quebec in Canada. Available sequence data of *Ditylenchus* species retrieved from GenBank were used to phylogenetically position this species within the genus *Ditylenchus*. In all studied genes, several single-nucleotide polymorphisms between the Canadian *D. weischeri* and both Russian haplotype and individuals of *D. weischeri* from *C. arvense* from Russia were found. The sequences of ITS rDNA, LSU D2D3 28S rDNA, and *Hsp*90 were used to construct separate dendrograms. For each of the three genes examined, *D. weischeri* was grouped separately from the other *Ditylenchus*. *Ditylenchus* samples from *C. arvense* was positioned to a single clade such as *D. weischeri* and distinct from *D. dipsaci*. With past reports of plant host preference and morphology, the results of this study provide further evidence for the fact that *D. weischeri* is distinct to be separated from *D. dipsaci*. Furthermore, minor differences in molecular divergence and morphology to the Russian haplotype and limited symptoms of disease on *C. arvense* in Prairie Canada suggest the Canadian and Russian populations of *D. weischeri* may be diverging.

Key words

*Cirsium arvense, Ditylenchus, Ditylenchus weischeri, Hsp*90, ITS rDNA, LSU D2D3 28S rDNA, Phylogeny, SSU 18S rDNA, Systematics.

Species of *Ditylenchus* (Filipjev, 1936) (Nematoda: Anguinidae) are mostly mycophagous or cryptophagus feeding on fungi, mosses, or lichens. However, it contains some species that are obligate plant endoparasitic nematodes of more than 500 hosts of flowering plants (Subbotin et al., 2005). The genus

Ditylenchus with more than 80 described species is distributed worldwide in different climatic regions (Brzeski 1991). Jones et al. (2013) rated *Ditylenchus* as the fifth most important nematode that is detrimental to agricultural production worldwide. The agriculturally important members of the genus are limited to a few species such as *D. dipsaci* (Kühn) (Filipjev, 1936), which is the type species of the genus, *D. destructor* Thorne, 1945, *D. angustus* (Butler) (Filipjev, 1936), *D. gigas* (Vovlas et al., 2011), and *D. africanus* (Wendt et al., 1993). Subbotin et al. (2005) provided a detailed molecular phylogenetic analysis of *Ditylenchus* species. They concluded that *Ditylenchus dipsaci* is a complex containing several species of which some are in the processes of speciation. Recently, several new species have been recognized from the *D. dipsaci* complex; *Ditylenchus gigas*, known previously as the giant race of *D. dipsaci* (Vovlas et al., 2011), *D. weischeri*, parasitizing the widely distributed weed *C. arvense* (Chizhov et al., 2010), *D. arachis* infesting peanut (*Arachis hypogaea*, 1753; Zhang et al., 2013), *D. persicus* associated with grapevines (Esmaeili et al., 2017), and *D. laurae* parasitizing the water plant *Potamogeton perfoliatus* (Skwiercz et al., 2017). Furthermore, the presence of sibling or cryptic species has been identified inside the species of *Ditylenchus*; these are nematode individuals with common morphological characters but are distinguishable molecularly (Wendt et al., 1993; Esquibet et al., 1998; Vovlas et al., 2011).

Morphologically, *D. weischeri* has a shorter tail and longer post-vulval uterine sac than *D. dipsaci*. The type host for *D. weischeri* is *C. arvense*, a weed that is native to Europe and North Asia and that has become established in the temperate regions of North and South Asia, Africa and Australia. We have observed *Ditylenchus* from yellow pea grain samples contaminated with the seeds of *C. arvense* in the Prairie Provinces of Canada (Tenuta et al., 2014). ITS-PCR-RFLP of the isolated individuals showed the nematode to be identical to *D. weischeri* described from *C. arvense* in Russia (Tenuta et al., 2014). The populations of *D. weischeri* from Canada and Russia showed minor morphological differences from *D. dipsaci* such as longer post-vulval uterine sac and tail length compared to *D. dipsaci* from garlic obtained from Ontario, Canada, which confounds identification of this nematode. Compared to the haplotype from Russia (Chizhov et al., 2010), the Canadian *D. weischeri* populations have a smaller body length (Tenuta et al., 2014).

Ditylenchus dipsaci is a quarantine nematode in many countries, whereas *D. weischeri* is not. In a growth room study, the plant host preference differed between *D. weischeri* and *D. dipsaci* with the former parasitizing *C. arvense* and the later garlic (*Allium sativum* L., 1753), yellow pea (*Pisum sativum* L., 1753) and dry edible bean (*Phaseolus vulgaris* L., 1753; Hajihassani *et al.*, 2016). As a consequence, both species require clear discrimination from each other. Molecular approaches for separating *D. weischeri* and *D. dipsaci* has been developed based on ITS-PCR RFLP (Chizhov et al., 2010) and *Hsp*90-based diagnostic PCR (Madani et al., 2015). However, both reports rely on a single gene, limited number of nematode individuals examined, and consequently upon few sequences of ITS rDNA and *Hsp*90 genes. However, intra-species variation in sequences of ITS rDNA and the presence of haplotypes can cause heterogeneity in restriction profiles of the ITS rDNA PCR-RFLP, or ambiguity in diagnostic PCR (Wendt et al., 1993; Subbotin et al., 2011; Douda et al., 2013; Madani et al., 2014). Therefore, there is a need to support differentiation of *D. weischeri* and *D. dipsaci* using more genes and many specimens.

Here in this study, molecular analysis was undertaken using 41 samples of Canadian *D. weischeri* to differentiate from *D. dipsaci* by the use of four genes, namely, the internal transcribed spacer (ITS rDNA), large subunit (LSU) D2D3 28S rDNA, *Hsp*90, and partial segment of the small subunit (SSU) 18S rDNA. The last three markers as the most commonly used for phylogenetic analysis characterize the phylogenetic position among other *Ditylenchus* species. Sequences of specimens of *Ditylenchus* from *C. arvense* from Prairie Canada were compared to sequences of specimens of *D. weischeri* of *C. arvense* from Russia, *D. dipsaci* from garlic in the provinces of Ontario and Quebec, Canada and *Ditylenchus* species deposited in GenBank.

Materials and methods

Nematode samples

In total, 45 samples of *Ditylenchus* were examined, of which 35 were from *C. arvense* plants and six from *C. arvense* in grain samples. Four samples of *Ditylenchus dipsaci* were also obtained from garlic bulbs, two from each province of Ontario and Quebec. Code and origin of the samples are given in Table 1. Most of the genomic DNA from samples was obtained from the study of Tenuta et al. (2014). The samples were coded according to their origin: (1) pea harvested grain with *C. arvense* seed (PG) from six

Table 1. List of the samples, gene sequence length (bp), and sequence accessions provided to Genbank in this study.

	ITS rDNA		D2D3 28S rRNA		SSU 18S rDNA		*Hsp*90	
Sample	Length (bp)	Accessions	Length (bp)	Accessions	Length (bp)	Accessions	Length (bp)	Accessions
PG-(33-AB)	(615)	MG386845	(562), (568), (569)	MG551892, MG551897, MG551898	(811), (812)	MG383943, MG383943	920	MG674309
PG-(44-SK)	ND		(570)	MG693783	ND		ND	
PG-(58-AB)	(619), (672), (730), (142), (705d)	MG386846, MG386861, MG386871, MG386827, MG386867	(561)	MG551891	(822), (814)	MG383953, MG383945	(905)	MG676342
PG-(76-SK)	(675)	MG386862	ND		(807)	MG383941	ND	
PG-(81-MB)	(635)	MG386853	(564)	MG551894	ND		(921)	MG674310
PG-(84-MB)	(629)	MG386850	(566)	MG551895	(813)	MG383944	ND	
CT-(81-MB)	(631)	MG386852	ND		ND		ND	
[a]CT-(84-MB)	(645)	MG386855	(558)	MG551888	(818), (821)	MG383949, MG383952	(917), (913)	MG674307, MG676342
CT-(B-MB)	(630)	MG386851	(557)	MG551887	(806)		ND	
CT-(C-MB)	(743), (745), (726)	MG386876, MG386877, MG386869	(567)	MG551896	(815)	MG383946	ND	
CT-(Road side1-MB)	(646),(647),(648)	MG386856, MG386857, MG386858	(556)	MG551886	(819), (820)	MG383950, MG383951	ND	
CT-(Road side2-MB)	(733)	MG386874	(574)	MG551900	(817)	MG383948	ND	

CT-(C11F7-SK)	(725), (732), (746), (731)	MG386868, MG386873, MG386878, MG386872	ND		(816)	MG383947	ND	
CT-(C11F11-MB)	(145)	MG386828	(560)	MG551890	ND		(918)	MG674308
CT-(C11F17-MB)	(146)	MG386829	ND		ND		ND	
CT-(C11F18-SK)	ND		ND		809		(914)	MG676343
CT-(C11F19-SK)	(729), (620), (751)	MG386870, MG386847, MG386879	(563)	MG551893	ND		(907)	MG674306
CT-(GL1-MB)	(13)	MG386825	(*580)	MG551899	ND		ND	
CT-(GL 2-MB)	(128)	MG386826	ND		ND		ND	
CT-(GL3-MB)	(601)	MG386831	ND		ND		ND	
CT-(GL4-MB)	(603), (604)	MG386832, MG386833	ND		ND		ND	
CT-(GL5-MB)	(605)	MG386834	(594)	MG551909	(803)	MG383938	ND	
CT-(GL6-MB)	(606)	MG386835	(555)	MG551885	(804)	MG383939	(901)	KJ817197
CT-(GL7-MB)	(607)	MG386836	(595)	MG551910	(805)	MG383940	(902)	MG655239
CT-(GL8-MB)	(608)	MG386837	(584)	MG551904	ND		(903)	MG662121
CT-(GL9-MB)	(609)	MG386838	(582)	MG551902	ND		ND	
CT-(GL10-MB)	(610)	MG386839	(593)	MG551908	ND		(904)	MG662122
CT-(GL11-MB)	(611)	MG386840	ND		ND		ND	
CT-(GL12-MB)	(612), (613)	MG386841, MG386842	(581)	MG551901	ND		ND	
CT-(GL13-MB)	(614)	MG386843	(583)	MG551903	ND		ND	
CT-(GL14-MB)	(621)	MG386848	ND		ND		ND	
CT-(GL15-MB)	(622)	MG386849	ND		ND		ND	

CT-(GL16-MB)	(644)	MG386854	ND		ND		ND	
CT-(GL17-MB)	(665)	MG386860	ND		ND		ND	
CT-(GL18-MB)	(676)	MG386863	ND		ND		ND	
CT-(GL19-MB)	(687)	MG386864	(590)	MG551905	ND		ND	
CT-(GL20-MB)	(688)	MG386865	(591)	MG551906	ND		ND	
CT-(GL21-MB)	(689)	MG386866	(592)	MG551907	ND		ND	
CT-(GL22-MB)	(148)	MG386830	ND		ND		ND	
CT-(GL23-MB)	(650)	MG386859	ND		ND		ND	
CT-(RU)	(127), (692), (691)	MG384317, MG384319 MG384318	(559)	MG551889	(825), (824)	MG383955, MG383954	(931)	
G-(Guelph -ON)	(659), (660), (732d), (147), (143), (144)	MG384718, MG384719, MG384731 MG384717	ND		(802)	MG434349	(916), (915)	MG711914,MG676344,
G-(C58-ON)	(669), (710d), (711d)	MG384725, MG384729, MG384730	ND		(801)	MG434348	(930)	MG711917
G-(PQ-GLG)	(694), (695), (696),(661), (662)	MG384726, MG384727, MG384728, MG384720, MG384721	ND		ND		(928)	MG711916
G-(PQ-D77)	(663), (664), (668)	MG384722, MG384723, MG384724	ND		ND		(926)	MG711915
Total	74		27		22		18	

Note: Sample origin coding is; CT= from creeping thistle in yellow pea fields, PG = from yellow pea grain with creeping thistle seeds, R=creeping thistle from roadsides, AB=Alberta, SK=Saskatchewan, and MB=Manitoba for Canadian Provinces plant material collected from, G-PQ=garlic from Quebec, G-ON=garlic from Ontario and RU = is creeping thistle root from Russia.

fields across the Prairie Provinces, PG-(58-AB) and PG-(33) from Alberta, PG-(76-SK) and PG-(44) from Saskatchewan, and PG-(81-MB) and PG-(84-MB) from Manitoba; (2) *C. arvense* from four pea grain fields, CT-(81-MB), CT-(84-MB), CT-(B-MB), CT-(C-MB), all from Manitoba; (3) *C. arvense* from roadsides of agricultural lands at two locations in Manitoba, CT-(Roadside1-2-MB); (4) *C. arvense* collected from five agricultural fields in Manitoba CT-(C11F11-MB) and CT-(C11F17-MB), in Saskatchewan CT-(C11F7-SK) CT-(C11F18-SK) and CT-(C11F19-SK); (5) *C. arvense* collected from 23 locations in agricultural ditches in Manitoba CT-(GL1 to GL23-MB); (6) garlic infested with *D. dipsaci*, two from each province of Ontario G-(Guelph-ON- and G-(C58-ON), and Quebec G-(PQ-GLG), and G-(PQ-D77); and (7) dead and dried individuals of *D. weischeri*, CT-(RU) from Russia.

Briefly, nematodes had been recovered from samples using a technique based on soaking. For this, stems, leaves and flowers of *C. arvenss* were chopped separately and soaked in distilled water in Petri dishes overnight. Similarly, samples of PG (pea harvested grain contaminated with creeping thistle seed), and pieces of stems and corm from the garlic samples G-(Guelph-ON), G-(C58-ON), G-(QP-GLG), and G-(QP-D77) were also soaked overnight in water. After soaking, the samples were checked using a stereo microscope and several individual juvenile, female, and male nematodes were picked using an eyebrow brush. Selected nematodes were stored at 4°C in 1.5-ml tubes containing either in distilled water when the nematodes needed to be used immediately or DESS solution for long-term preservation of the nematodes (Yoder et al., 2006).

Preparation of DNA templates and PCR

Genomic DNA had been recovered from juvenile or female individuals using a protocol described previously by Subbotin et al. (2005). Briefly, one nematode was cut into two or three segments under a stereo microscope in a drop of worm lysis buffer (WLB, 10 Mm Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 0.45% Tween 20) using a sterilized surgical design scalpel blade No 11 (Fisher Scientific, ON, Canada). Approximately 15 to 20 µl of the nematode suspension was then transferred to a PCR reaction tube (Eppendorf) and 2 µl proteinase K was added; the tubes were then incubated at 60°C and 95°C for 60 and 10 min, respectively. The DNA was used immediately or frozen at –20°C before use in PCR. The quality and quantity of the DNA were determined suitable for PCR by a spectrophotometer using a NanoDrop ND-1000 (Thermo Scientific, Mississauga, ON) at 260 nm.

PCR reactions were conducted to a 25 µl final reaction volume. Each reaction contained 10 µl of 2× Master Mix (Qiagen, GmbH, Hilden, Germany) including dNTPs and Taq polymerase, 0.5 µl of each primer, 2 µl of DNA extract, and 12.5 µl of dH_2O. ITS (rDNA), LSU D2D3 28S rDNA, partial segment of SSU 18S rDNA, and *Hsp*90 genes were targeted for PCR amplification. The whole ITS rDNA including ITS1, 5.8S, and ITS2 was PCR amplified using the forward primer TW-81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB-28 reverse (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Joyce et al., 1994). When the ITS rDNA could not be amplified using this primer set, the PCR was run with the forward primer rDNA1 (5′-TTGATTACGTCCC TGCCCTTT-3′) and reverse primer rDNA2 (5′-TTTCACTCGCCG TTACTAAGG-3′) developed by Vrain et al. (1992). The LSU D2D3 28S DNA gene was amplified using the forward primer D2A (5-CAAGTACCGTGAGGG AAAGTTG-3) and the reverse primer D3B (3-TCCTCGGAAG GAACCAGCTACTA-3) as used by De Ley et al. (1999). In some cases, this primer set failed; to overcome this issue an initial amplification using a nested forward primer D2AA (5′-GAAACGGATAGAGCCGACGT AT-3′) and reverse D3BB (5′-CCAAGTCAGACGA TCGATT-3′) with D2A-D3B was used. The D2AA and D3BB primers were designed on the basis of available sequences of *Ditylenchus* spp. in GenBank. A partial segment of the SSU 18S DNA gene was amplified (Blaxter et al., 1998) using the forward primer G18S4 (5-GCTTGTCTCAAAGATTAAGCC-3) and the reverse primer 18SP (5-TGATCCWMCRGCAGGTCAC-3). The pair of degenerate primer U831 (5′-AAYAA RACMAADCCNTYTGGAC-3′) and L1110 (5′-TCRCARTTVTCCATGA TRAAVAC-3′) (Skantar and Carta, 2004) was used to amplify the partial region of the *Hsp*90 gene. For the samples which were not amplified using this set of primers, a nested primer of 97F (5′-GCGCAATCCGRMYGAAGT-3′) and 93R (5′-ACGGCATTCTTGC TCTTCTT-3′) was designed and used in PCR. The PCR conditions were 3 min denaturation at 94°C, followed by 30 s at 94°C, 45 s at 54°C, and min (for D2A-D3B; D2AA-D3BB) or 2 min (for ITS rDNA) for 34 cycles. As a final step, a 10 min extension at 72°C was applied for all the PCR reactions. Each PCR run included DNA of a positive control of known DNA concentration to ensure performance of the reaction. For all PCR reaction runs, a PCR tube without DNA was used as negative controls. All PCR amplifications were performed using an Eppendorf thermal cycler (Master Cycler Pro, Hamburg, Germany).

To analyze the results of PCR amplification, the PCR products were prepared for gel electrophoresis by mixing 2 µl of the PCR product with loading buffer in water and added to 1% agarose gel with 0.5× TAE buffer to which GelRed fluorescent dye was included (1 µl, 30 ml^{-1}, Biotium, Inc, Hayward, CA). Gel electrophoresis was run for 45 min under constant current (45 to 50 mA) and amplification products viewed using a Gbox gel capture imaging system (SYNGENE, Synoptic, Ltd. Cambridge, UK) equipped with a UV transiiluminator.

Purification of the PCR product, cloning, and sequencing

PCR products were loaded on 1% agarose gel prepared in 0.5× TAE buffer, excised and purified for further analysis. Purification was performed using a Qiaquick gel extraction kit (Qiagen, GmbH, Hilden, Germany) according to manufacturer protocol. Purified PCR products were sequenced directly in both strand directions using the original primers of each amplified gene. Sequences with background noise were cloned and sequenced. Cloning was performed after ligation of 30 to 50 ng of PCR product to the pGEM-T Easy Vector System (Promega, Madison, WI). The ligation product was then transformed into DH bacteria following the supplier protocol (BioLabs, New England, MA). Plasmids of positive white colonies were isolated using the UltraClean Standard Mini Plasmid Prep Kit (MO BIO Laboratories, Carlsbad, CA); sequencing was performed with the universal vector primers M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-GTAATACGACTCACTATAGGGC-3′).

Raw sequence results were visualized using the program Chromas Lite 2.01 (Technelysium Pty Ltd, South Brisbane, Australia), edited as needed and then assembled. Additional editing of sequences was performed using Gendoc 2.7 (Nicholas et al., 1997). All sequencing was performed by the core sequencing facility of Macrogen Corp (Rochville, MD).

Multiple sequence alignment

Multiple sequence alignments were performed for the sequences generated in this study together with selected 59 published sequences of the ITS rDNA gene from *Ditylenchus* species deposited in GenBank (Table 2). For each gene, multiple sequence alignments were performed using MegAlign 7.1.0 of Lasergene (Dnastar Inc, Madison,WI) for sequences generated in this study along with those of the same gene for *D. dipsaci*, *D. weischeri, D. gigas*, *D. destructor*, and the unidentified *Ditylenchus* spp. in the GenBank. The accessions used were mainly from Helder et al. (2004), Subbotin et al. (2005, 2011), Chizhov et al. (2010), and Vovlas et al. (2011) (Table 2). After alignment, consensus parts of each alignment set from the primer ends were trimmed using Gendoc 2.7 (Nicholas et al., 1997) and analysis was performed on a 631 bp region across ITS1, 5.8S, and ITS2 genes. Comparison of the sequence for nucleotide composition and position was then performed among the studied samples along with 24 sequences retrieved from GenBank for *D. dipsaci*, *D. gigas*, and selected *D.* spp. (Table 3).

New sequences generated in this study of the SSU 18S rDNA gene were aligned with six sequences of *D. dipsaci* and one of *D. gigas* retrieved from GenBank (Table 2). The sequences were all trimmed to 1390 bp and intra- and inter-sequence variation analyses, similarity matrix, and pairwise genetic distance were estimated using MEGA5 (Tamura *et al.*, 2011).

Alignment of 29 LSU D2D3 28S rDNA sequences from *D. dipsaci*, *D. gigas*, *D. destructor*, *D. halictus* (Giblin-Davis *et al.*, 2010) and *Ditylenchus* sp from GenBank (Table 2), and new sequences from this study were done as described above. Analysis was performed on 637 bp of the trimmed sequences of the LSU D2D3 28S rDNA region. Each of the studied gene sequences were analyzed by identifying single-nucleotide polymorphisms (SNPs), base pair composition, and interspecies and intraspecies sequence variation.

The sequences obtained in this study with 14 sequences available from the *Hsp*90 gene of *Ditylenchus* species including three of *D. gigas*, *D. weischeri*, and *Ditylenchus* sp, plus five sequences of *D. dipsaci* were retrieved for GenBank (Table 2), and subjected to multiple sequence alignment and pairwise distance analysis.

Phylogenetic analyses

Phylogenetic analysis was performed on the ITS rDNA, LSU D2D3 28S rDNA, and *Hsp*90 genes. The most appropriate nucleotide substitution and parameter values were determined using jModelTest 2.1.7 (Darriba et al., 2012). The gamma distribution shape parameter and substitution rate in the Akaike information criterion were included in the analysis to determine the best fitting evolutionary model. Based on the previously published data on *Ditylenchus* (Vovlas et al., 2011, Subbotin et al., 2005, 2011), the

Table 2. Accession codes for sequences of the four genes in this study and plant host of *Ditylenchus* species retrieved from GenBank.

Species	Host plant	ITS rDNA	LSU D2D3 28S rRNA	SSU 18S rDNA	*Hsp*90
D. weischeri	*Cirsium arvense,*	AF396322	—	—	HM778133.1, HM778133.3, HM778134
D. dipsaci	*Allium sativum*	AY574298, JX123258, HQ219249	[a]JX123259, [a]JX123259, [a]FJ707362, JF327761, HQ219225	HQ219249	—
D. dipsaci	*Allium cepa*	AY574289, AY574290	HQ219226, HQ219223)	HQ219210	HM778138, HM778140
D. dipsaci	*Vicia faba*	AF396323, AY574286, AY574284, HQ219246, HQ219248, HQ219244, HQ219244	—	—	HQ219229
D. dipsaci	*Trifolium pratense*	AF396319, AY574300	—	—	—
D. dipsaci	*Medicago sativa*	AY574297, AF396320	—	—	—
D. dipsaci	*Pisum sativum*	HQ219247	FJ707364	HQ219247	—
D. dipsaci	*Fragaria* sp	AF396321, HQ219241	HQ219219	—	HQ219230, HM778139, HM778137
D. dipsaci	*Beta vulgaris*	AY574299	—	—	—
D. dipsaci	*Plantago lanceolata*	AY574301	—	—	—
D.dipsaci	*Tulipa gesneriana*	—	JF327765	—	—
D. dipsaci	*Digitalis lutea*	AY574292	—	—	—
D. dipsaci	*Avena sativa*	AY574293	—	—	—
D. dipsaci	*Veronica gentianoides*	AY574295	—	—	—
D. dipsaci	*Lysimachia vulgaris*	AY574296		—	—
D. dipsaci	Host?	[a]JN376069	—	—	—
D. dipsaci	*Zea mays*	AY574294	—	—	—
D. dipsaci	*Phlox drummondi*	AY574291	—	—	—
D. dipsaci	*Beta vulgaris*	HQ219243	—	—	—

D. dipsaci	Un known	—	—	AY593908, EU669931, AY284636	—
D.dipsaci	Narcissus sp	—	[a]FJ707361	—	—
D.dipsaci	*Cichorium* sp	—	*FJ707360	—	—
D.dipsaci	Lucerne sp	—	FJ707363,	—	—
D. gigas	*Vicia faba*	HQ219232, HQ219235, HQ219236,HQ219231, HQ219240, HQ219234, HQ219237, HQ219238, HQ219239	HQ219216,HQ219217	HQ219211	HQ219227, HQ219228
D. gigas	Unknown	[a]JN376074	—	—	—
Ditylenchus. sp	*Vicia faba*	AY574285	—	—	—
Ditylenchus. sp	*Arachis* sp	—	JX145345	—	—
Ditylenchus. sp	*Pilosella officinarum*	AY574302, AY574305, AY574304	—	—	—
Ditylenchus. sp	*Crepis praemorsa*	AY574303	—	—	—
Ditylenchus. sp	*Leontodon autumnalis*	AY574306	—	—	—
Ditylenchus. sp	*Plantago maritima*	AY574287,AY574288	—	—	HM778135, HM778136
Ditylenchus. sp	Turfgrass	—	—	—	AY603512
D. destructor	*Solanum tuberosum*	HQ235675, HQ235694, HQ235692, GQ469492, HQ235682, JN376068 JN66693	HQ235698, EU400642, EU400625, DQ328727, FJ707365	—	—
D. destructor	*Ipomoea batatas*	JX145343, AY987007	EU400638, EU400634, EU400628, EU400626, EU400622, EU400629, EU400629 (China)	—	—
D. myceliophagous	*Agaricus* sp	DQ151458, AM232236	—	—	—
D. halictus	*Halictus sexcinctus*		AY589364		

Table 3. Nucleotide position and composition on 631 bp of internal transcribed spacer (ITS1, 5.8S, ITS2 rDNA), comparison among samples from this study (Table 1) and those derived from the GenBank database (Table 2) after multiple sequence alignments. Only positions with nucleotide differences are presented. Only one sequence result for multiple individuals from the same sample shown because of lack of intra-sample differences. Sample sequences generated in this study are in bold. W = (A or T), Y = (C or T).

	ITS1												5.8S								ITS2			
Nt Position	5	23-24	44	63-5	78	138	174	186-7	189	192	227-9	275	434	436	438	485-8	494	509	510	515	534-5	591-4	613-14	617-618
Collection material (Origin)																								
***D. weischeri* PG-(33-AB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* PG-(44-SK)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	C	GA	AG-Y	CA	TA
***D. weischeri* PG-(58-AB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	C	GA	AG-Y	CA	TA
***D. weischeri* PG-(76-SK)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	C	GA	AG-Y	CA	TA
***D. weischeri* PG-(81-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	C	GA	AG-Y	CA	TA
***D. weischeri* PG-(84-MB)**	T	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	C	GA	AG-Y	CA	TA
***D. weischeri* CT-(81-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(B-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(C-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(84-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(Road side1-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(Road side2-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(C11F7-SK)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(C1-F11-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(C11F17-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(C11F18-SK)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(C11F19-SK)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(GL1-MB)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. weischeri* CT-(Ru), AF396322)**	A	AT	G	TGG	W	A	C	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	C	GA	AG-Y	CA	TA
***D. weischeri* ([a]CT-Ru)**	A	AT	G	TGG	W	A	T	TC	G	G	TGT	-	A	C	T	AAC	A	T	A	Y	GA	AG-Y	CA	TA
***D. dipsaci* G(Guelph-ON)**	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	GC	AACT	TG	CG
***D. dipsaci* G-(PQ-GLG)**	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. gigas (HQ219227)	T	AT	A	TGG	T	A	T	TC	A	G	TGT	A	A	A	A	AAA	-	T	G	C	GA	AG-Y	CG	TA
D. sp (AY574285)	T	TC	A	TGG	T	A	T	TC	A	G	TGT	A	A	A	A	AAA	-	T	G	A	GA	AG-Y	CG	TA
D. sp (AY574287)	A	TT	G	CGA	T	G	T	TC	G	G	AGA	A	G	A	A	AAT	A	T	A	C	GA	GACT	TG	CG
D. sp (AY574305)	A	TA	G	CGA	T	G	T	TC	G	G	AGA	A	G	A	A	AAT	A	T	A	C	GA	AACT	TG	CG
D. dipsaci (AY574301)	T	AT	A	TGG	T	A	T	TC	A	G	TGT	A	A	C	A	AAA	-	T	G	C	GA	AG-Y	CG	TA
D. dipsaci (AY574298)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (AF396320)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (AY574293)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (AF396321)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (AY574294)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (HQ219243)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (AF396319)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG
D. dipsaci (HQ219247)	A	TT	G	CGA	T	G	T	CT	G	C	TGT	A	A	C	A	GGT	T	C	A	C	AC	AACT	TG	CG

Note: Samples obtained in this study are in bold.

following outgroup taxa were used, *D. myceliophagous* Goodey 1958 (DQ151458 and AM232236) for ITS rDNA and *D. halictus* (AY589364) for LSU D2D3 28S rDNA. *Ditylenchus* sp. (AY603512) was used as the outgroup for *Hsp*90 analysis. For each of the studied genes, Bayesian interference analysis was then performed to confirm the tree topology using MrBayes 3.2.6 (Ronquist *et al*., 2012) to generate a 50% majority rule consensus. Tree View 1.6.6 (Page, 1996) was then used to visualize trees and the posterior probabilities are given on appropriate clades.

Results

Recovery of *Ditylenchus* from samples and PCR amplification

Amplification of the ITS rDNA resulted in a single amplicon sized 719 or 964 bp with the primer pair AB-28/TW-81 or rDNA1/rDNA2, respectively. The LSU D2D3 28S rDNA gene was amplified for 25 samples. The amplicon size obtained was 783 bp using the primer pair D2A-D3B and 660 bp when the nested primer pair D2AA-D3BB was used. No product for the LSU D2D3 28S rDNA was obtained for garlic samples with the primers sets D2A-D3B or when nested with D2AA- D3BB (Table 1).

For SSU 18S rDNA, the size of the PCR product was primarily 1514 bp and in some cases it was 1620 bp. No SSU 18S rDNA PCR product was obtained for samples CT-(C11F11-MB), CT-(C11F17-MB), CT-(C11F19-SK), PG-(44-SK), G-(Guelph-ON), G-(C58-ON), G-(PQ-GLG), and G-(PQ-D77).

The PCR amplified product of the *Hsp*90 gene was 303 bp or 212 bp when the nested primer 97F/93R was used in PCR. For samples PG-(58-AB), CT-(C11F18-SK), and CT-(C-MB), an additional PCR product of 558 or 484 bp was also amplified when the primer set U831/ L110 was used. The *Hsp*90 gene was amplified from most of the samples, but not from samples CT-(Roadside1-MB, and Roadside2-MB), CT-(C11F7-SK), CT-(C11F17-MB), CT-(81-MB, 84-MB, B-MB), and PG-(44-SK, 76-SK). No amplification was observed for the negative control without DNA.

Sequence alignment and analysis, ITS rDNA

For some nematode samples more than one sequence was assembled; as a consequence, from the 47 studied samples, a total of 74 sequences were assembled from ITS rDNA (Table 1). Forty-seven sequences were from nematodes retrieved from *C. arvense* including 25 samples coded CT-(GL-1 to GL-23-MB), seven from non-pea agricultural land CT-(C11F7-SK and C11F19-SK), 2 from CT-(C11F11-SK and C11F17-MB), six from four fields of pea grain in Manitoba CT-(81-,84-,B-,C-MB), four from CT-(Roadside1 and Roadside2-MB), and three from CT-(RU). Nine sequences were obtained from nematodes isolated from pea harvested seed contaminated with thistle seeds, including one from each collection of PG-(33-AB), PG-(76-SK), PG-(81-MB), PG-(84-MB), and five from pea grain field collections of PG-(58-AB). Next to these sequences, 18 sequences originated from *D. dipsaci* of the garlic collection including six from each G-(Guelph-ON) and G-(PQ-GLG), and 3 from each G-(C58-ON) and G-(PQ-D77). Results of the sequence comparison are presented in Table 3.

Comparison of the Canadian *D. weischeri* sequences obtained with the Russian haplotype of this species deposited in GenBank showed only one single-nucleotide transition (T/A) at position 174. Intraspecies sequence variation among all *D. weischeri* analyzed in this study, including the Russian haplotype, was limited to only two sites presenting a transversion (A/T) in position 78 bp located in ITS1 and a transition (C/T) in position 515 bp, located in ITS2.

All the *D. weischeri* sequences showed a dissimilarity of 5% and 3.8% in the whole ITS rDNA sequence of *D. dipsaci* and *D. gigas*, respectively. Including all the sequences for analysis showed an overall dissimilarity of 6.4% between *D. weischeri* and rest of the samples including *D. dipsaci*, *D. gigas*, and *D.* sp. A comparison of nucleotide composition and position among the selected samples is given in Table 3. There were 11 SNPs specific to all *D. weischeri* but not in *D. dipsaci, D. gigas,* and *D.* sp, with nucleotides and position as follows: (A/T-22), (-/A-244), (T/A-278), (T/G-460), (GA/AC-529-530), (AG/GA-531-3), (G/A-595), (-/A-601), (G/T-602), (TA/GT-613-14). Nucleotide composition (%) for each of the species was: *D. weischeri* (T=29.8%), (C=20.7%), (A=25.2%), (G=24.4%), and a GC content of 45.1%; *D. dipsaci* (T=29.9%), (C=21.3%), (A=24%), (G=24.8%), and a GC content of 46.1%; *D. destructor* (T=33.3%), (C=21.2%), (A=20.1%), (G=25.2%), and a GC content of 46.4%; *D. gigas* (T=29.8%), (C=20.7%), (A=25.3%), (G=24.2%), and a GC content of 49.9%; *Ditylenchus*. sp: (T=30.2%), (C=19.3%), (A=27.1%), (G=23.4%), and a GC content of 42.7%.

LSU D2D3 28S rDNA

A total of 27 sequences of LSU D2D3 28S rDNA were obtained (Table 1). Nineteen sequences were from

C. arvense and included 11 from samples coded CT-(GL-1 to GL-23-MB), one from each non-pea agricultural land CT-(C11F19-SK) in Saskatchewan, and CT-(C11F11-MB) in Manitoba, three fields of pea grain in Manitoba CT-(84,B,C-MB), two from CT-(Roadside1-MB and Roadside2-MB), and one from CT-(RU).

Also seven sequences were obtained from pea harvested seed contaminated with thistle seeds, including three from PG-(33-AB), and one from each PG-(81-MB), PG-(84-MB), PG-(44-SK), and PG-(58-AB). No sequences were obtained from any of *D. dipsaci* from garlic, including G-(Guelph-ON), G- (PQ-GLG), G-(C58-ON), and G-(PQ-D77). Intraspecific variation in sequences of *D. weischeri* was observed for nucleotide transversions (G/T and A/C) at position 565 bp and 561 bp, respectively, and one transition (C/T) at position 564 bp (Table 4). Sequences could not be assembled from samples from garlic (G-ON and G-PQ) due to the presence of background noise in the raw sequence data. An aalysis of the aligned sequences showed 2.6% dissimilarity between *D. dipsaci* retrieved from GenBank and *D. weischeri* from this study, which corresponded to 23 SNPs. Similarly, there were 11 SNP differences between *D. wiescheri* and *D. gigas*. The nucleotide composition of the 637 bp section of LSU D2D3 28S rDNA were: *D. weischeri* (T = 24.9%), (C = 20.3%), (A = 24%), G = (30.8%), *D. dipsaci* (T = 25.2%), (C = 20.2%), (A = 23.9%), (G = 30.8%), *D. destructor* (T = 24.9%), (C = 21.3%), (A = 22.2%), (G = 31.7%), and *D. gigas* (T = 25.7%), (C = 20.3%), (A = 23.7), (G = 30.4).

SSU 18S rDNA

In total, 22 partial sequence of the SSU 18S rDNA gene was obtained of which 11 sequences were from *C. arvense* which include one from each CT-(GL-5-MB), CT-(GL-6-MB), and CT-(GL-7-MB) from Manitoba (Table 1). Sequences were not obtained from samples PG-(44-SK) and PG-(81-MB). Sequences were obtained for CT-(84,B,C-MB), three from CT-(Roadside1-MB, and Roadside2-MB), and three from CT-(RU), two from each PG-(33-AB) and PG-(58-MB), and one from each PG-(76-SK) and PG-(84-MB). Two sequences were obtained from *D. dipsaci* from garlic, including one from each G-(Guelph-ON) and G-(C58-ON). The results from sequence comparison showed no intraspecies sequence variation among *D. weischeri* individuals. Also, Canadian *D. weischeri* SSU 18S rDNA sequences were 98% identical to those deposited in GenBank for *D. dipsaci* and *D. gigas*.

*Hsp*90

A total of 18 sequences were assembled from the examined individuals (Table 1). Ten of these sequences were from *C. arvense* and included one from each CT-(GL6-MB, GL7-MB, GL8-MB, GL-10-MB), (C11F11-MB, C11F18-SK, C11F19-SK) and two from CT-(84-MB). Also from pea grain samples with creeping thistle seeds, one sequence from each PG-(33-AB), PG-(58-AB), and PG-(81-MB) was obtained. From the garlic samples two sequences from G-(Guelph-ON) and one from each G-(C58-ON), G-(PQ-GLG) and G-(PQ-D77) was assembled. The samples with positive PCR reaction for amplification of the *Hsp*90 gene and their sequences are presented in Table 1. In GenBank, 12 sequences were available and compared with three *D. weischeri* from Russia, four *D. dipsaci,* two *D. gigas,* and three *Ditylenchus* sp. Intraspecific sequence analysis of 230 bp of *Hsp*90 obtained from *D. weischeri* in this study showed transitions (A/G and C/T) and two transversions (G/C and G/T) at positions 11, 165, 37, and 205, respectively. Sequence variation between Canadian *D. weischeri* individuals and the haplotype of this species from Russia was eight nucleotides (3.8%); for the rest of the samples the variation was 6.5%. *Hsp*90 sequences for *D. dipsaci* obtained from garlic in this study was identical to those of the same nematode species deposited in GenBank from *Allium cepa* L., 1753, and *Fragaria* L., 1753, sp., except for few point mutations. All *D. dipsaci* had 6.7% dissimilarity to *D. weischeri*. The result of pairwise sequence distance matrix (data not shown) for these samples showed that the overall mean distance was 0.095 among all analyzed samples, and 0.001 within *D. weischeri*. The shortest and the longest distance was 0.0% and 2.47%, respectively, among all the analyzed samples. The nucleotide composition for the *Ditylenchus* species was: (T = 31.3%), C = (22.3%), (A = 25.9%), (G = 20.5%) for *D. weischeri*, and (T = 31%), (C = 17.7%), (A = 26.5), (G = 24.8) for *D. dipsaci*.

Nucleotide sequence accession numbers

A total of 142 sequences determined in this study were submitted to GenBank on October 18 and 19, 2017. The GenBank accession numbers for samples sequenced in this study are given in Table 1.

Molecular phylogeny, ITS rDNA

Hierarchical clustering analysis based on ITS rDNA sequences yielded clear groupings among the

Table 4. Nucleotide position and composition, comparison sequences obtained in this study and accessions from GenBank. The LSU D2D3 28S rDNA sequence trimmed to 637 bp used for the analysis. Only one sequence result for multiple individuals from the same sample shown because of lack of intra-sample differences.

Nucleotide Position	47	76	82	89	92	100-1	106-7	160	178	234	244	249	257	279	281	304	308	320	336	341	457	483	500
Sample/isolate																							
CT-(GL8-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(RU)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(Road side-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(C11F11-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(C11F19-SK)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(B-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(C-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
CT-(84-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
PG-(33-SK)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
PG-(58-SK)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
PG-(81-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
PG-(84-MB)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
D. dipsaci ([a]FJ707364)	A	T	G	T	T	AT	TA	G	—	A	C	A	G	A	T	T	T	C	A	T	A	T	A
D. gigas (HQ219217)	A	T	A	T	T	AT	TA	C	—	A	T	A	A	A	T	T	G	T	A	T	A	T	A
D. gigas (HQ219216)	A	T	A	T	T	AT	TA	C	—	A	T	A	A	A	T	T	G	T	A	T	A	T	A
D. sp (JX145345)	T	T	G	C	A	GG	TA	A	—	A	A	A	A	T	T	A	G	T	A	T	A	T	A
D. dipsaci (HQ219223)	G	C	A	C	A	GC	CG	T	A	G	T	G	A	G	C	C	G	T	G	A	G	C	G
D. dipsaci (HQ219219)	G	C	A	C	A	GC	CG	T	A	G	T	G	A	G	C	C	G	T	G	A	G	C	G
D. dipsaci (JF327765)	G	C	A	C	A	GC	CG	T	A	G	T	G	A	G	C	C	G	T	G	A	G	C	G
D. dipsaci (JF327761)	G	C	A	C	A	GC	CG	T	A	G	T	G	A	G	C	C	G	T	G	A	G	C	G
D. dipsaci (JF327763)	G	C	A	C	A	GC	CG	T	A	G	T	G	A	G	C	C	G	T	G	A	G	C	G
D. dipsaci (JF327760)	G	C	A	C	A	GC	CG	T	A	G	T	G	A	G	C	C	G	T	G	A	G	C	G

Note: Samples obtained in this study are in bold.
[a]Sequence deosted by Douda et al. (2013).

examined samples with *D. weischeri* present in a distinct clade. The maximum parsimony base tree is presented in Figure 1. The resulting tree clustered all the 132 sequences into two main clades (I and II) with a posterior probability value of 100. Clade I comprised of *D. dipsaci*, *D. gigas*, *D. weischeri,* and an unknown *Ditylenchus* species (*D.* sp.); clade II contained only *D. destructor.* Samples in clade I were divided into two sub-clades (III and IV). Sub-clade III comprised all samples from *C. arvense* including CT-(GL-MB), CT-(81-MB, B-MB, C-MB and 84-MB), CT-(C11F7-SK, C11F17-MB, C11F18-SK, C11F19-SK), CT-(Roadside1-MB and Roadside2-MB), CT-(RU), PG-(33-AB, 44-SK, 58-AB,76-SK, 81-MB, 84-MB), and the sample of *D. weischeri* retrieved from the GenBank. *Ditylenchus gigas* and *Ditylenchus* sp. composed a separate group in sub-clade III. This sub-clade was branched by a posterior probability value of 80 from the second sub-clade (IV) that included all *D. dipsaci* samples obtained in this study, G-(Guelph-ON), G- (PQ-GLG), G-(C58-ON), and G-(PQ-D77) and those from GenBank. None of the studied samples from *C. arvense* or sequences of *D. gigas* and *Ditylenchus* sp from GenBank were clustered with sub-clade IV that contained solely *D. dipsaci*.

Molecular phylogeny, LSU D2D3 28S rDNA

Hierarchical clustering analysis of 48 partial sequences of LSU D2D3 28S rDNA of samples obtained in this study and species of *D. dipsaci*, *D. gigas,* and *D. destructor* retrieved from the GenBank is presented in Figure 2. Two distinct clades were formed that delineated *D. destructor* from clades that contained *D. dipsaci*, *D. wiescheri,* and *D. gigas* (posterior probability value of 98). All the samples of creeping thistle from this study including CT-(GL-MB), CT-(81, B, C and 84), CT-(C11F7-SK, C11F17-MB, C11F18-SK, C11F19-SK), CT-(Roadside1-MB and Roadside2-MB), CT-(RU), PG-(33-AB, 44-SK, 58-AB, 76-SK, 81-MB, 84-MB) were clustered together and were separated from *D. dipsaci* and *D. gigas* with a posterior probability value of 98. Sequences of a *Ditylenchus* sp. were positioned between the two clades, one contained *D. destructor* and the second one contained *D. dipsaci* samples. The two samples of *D. gigas* placed in a sub-clade with a posterior probability value of 100 and separated from *D. weischeri* of Canadian origin with a posterior probability value of 98. Interestingly, a sequence of D2-D3 from a sample of *Ditylenchus* deposited in GenBank under accession number FJ70364 as *D. dipsaci* was also grouped with *D. weischeri*. The sample was acquired from field pea from Prairie Canada and provided for the accession deposited by Douda et al. (2013) by the Canadian Food Inspection Agency. Clearly, the accession is for *D. weischeri* and not *D. dipsaci*.

Molecular phylogeny, *Hsp*90

The *Hsp*90-based parsimonious tree is presented in Figure 3. The sequence of the sample of *D. weischeri* from the original study of Chizhov et al. (2010), coded as CT-(RU), was grouped in a main clade together with PG-(33-AB, 81-MB, 84-MB, 58-AB), CT-(C11F11-MB, C11F18-SK, C11F19-SK), and CT-(GLG-MB). This clade was separated from two other clades that grouped *D. dipsaci* or *D. gigas*. The *Ditylenchus* sp. from garlic (G-(Guelph-ON), G-(C58-ON), and G-(PQ-D77) was clustered with *D. dipsaci* retrieved from GenBank. Since the SSU 18S rDNA gene based phylogeny is more applicable for higher taxon, i.e. family and higher, phylogenetic analysis was not performed on SSU 18S rDNA gene sequence data. Moreover, there were limited accessions of SSU 18S rDNA gene sequences in GenBank making the phylogenetic tree analysis not possible.

Discussion

The high similarity of *D. weischeri* in terms of morphology to agriculturally important species like *D. dipsaci* can lead to misidentification. Contamination of high amounts of *C. arvense* seeds with pea grain that occurs during harvesting and introduction of foreign matter at shipping ports increases the chance of detecting *Ditylenchus* nematodes, and misidentifying *D. weischeri* for *D. dipsaci*, the latter of which is a parasite of pea grain. Here in the current study, four genes were sequenced and 41 samples from three Prairie Provinces in Canada were used. The results presented here for the sequence of the four genes from many samples provide more evidence in support of differentiation of *D. weischeri* from other *Ditylenchus* including *D. dipsaci*.

In the current study, samples of *D. weischeri* from the *C. arvense* plant material used for the original descriptive study of this species in Russia (Chizhov et al., 2010) were included. Minor differences were previously observed between the Russian and Canadian *D. weischeri* from *C. arvense* i (Chizhov et al., 2010; Tenuta et al., 2014). Further, we have observed the absence of prominent typical symptoms of nematode

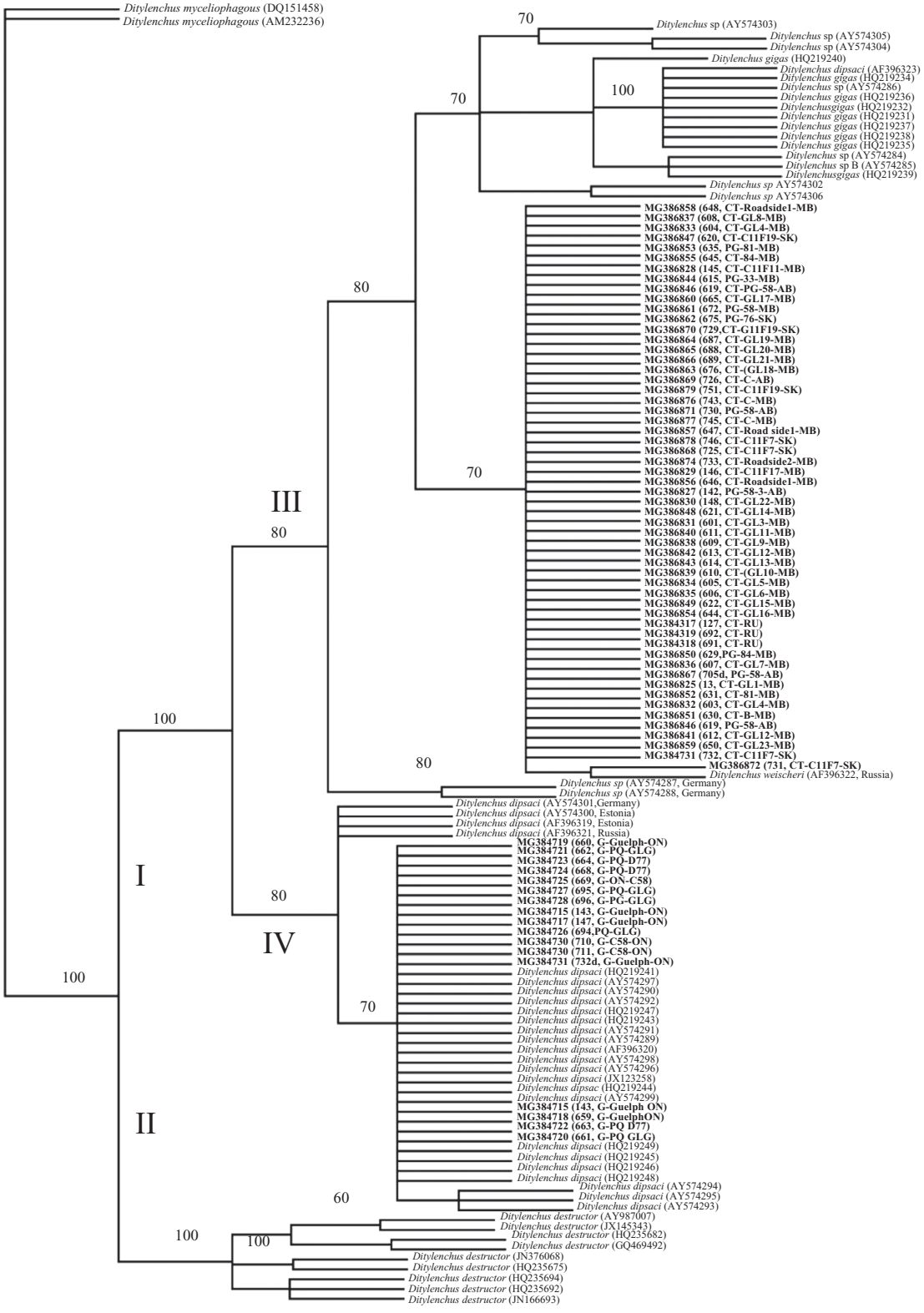

Figure 1: Phylogenetic relationships of *Ditylenchus weischeri* with related *Ditylenchus* species based on ITS rDNA sequences using MrBayes 3.2.6. jModelTest 2.1.7, and the Akaike Information Criterion were used to select TVM+G as the best model (–lnL=2956.2229; gamma shape=0.5380; G=0.2537; *R*=0.7816) and the figure created using Tree View 1.6.6. Posterior probability support values are given above the branches. Sequences generated in this study are given in bold.

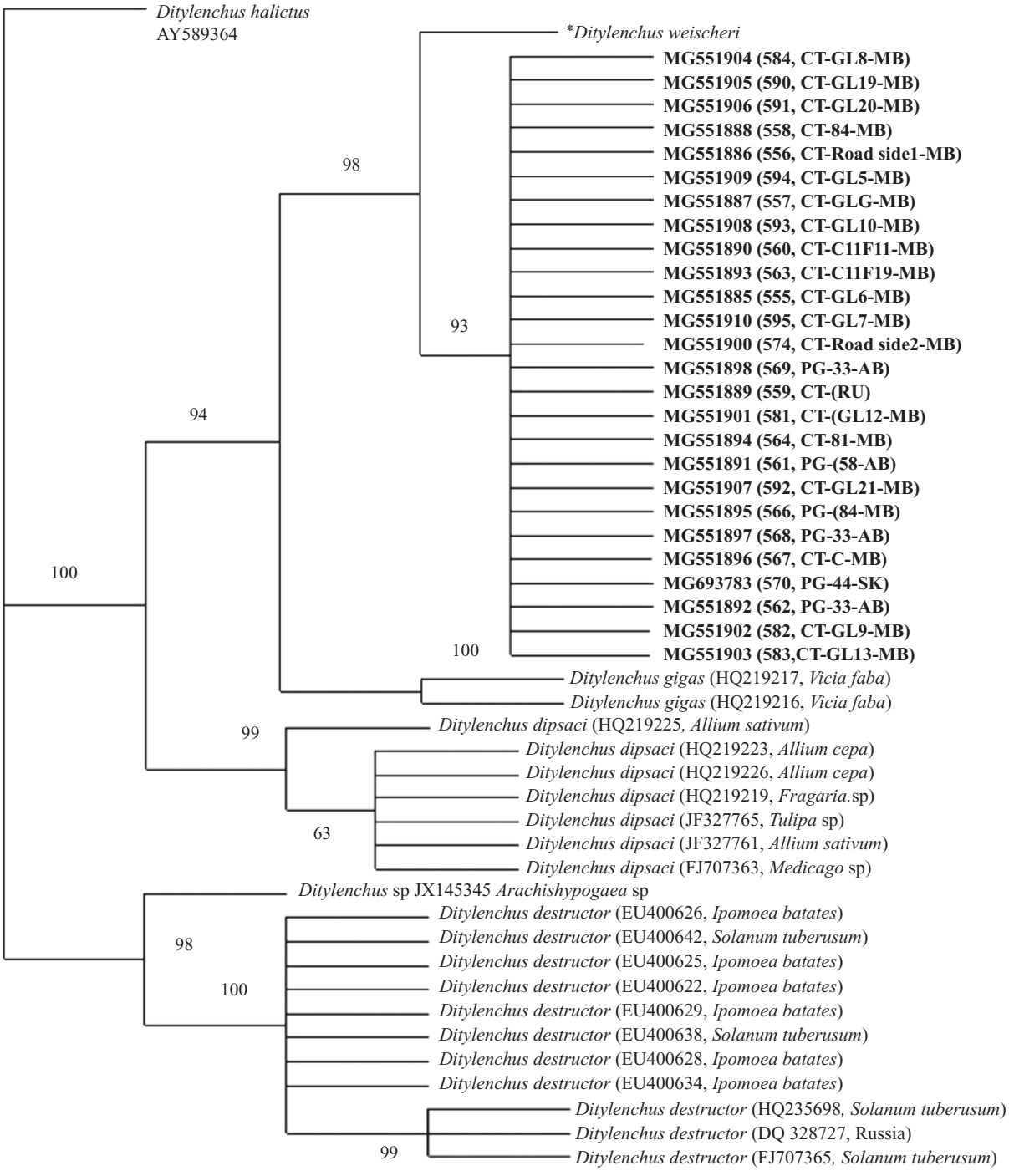

Figure 2: Phylogenetic relationships of *Ditylenchus weischeri* with related *Ditylenchus* species from the LSU D2D3 28S rDNA using MrBayes 3.2.6. jModelTest 2.1.7, and the Akaike Information Criterion were used to select GTR+I as the best model (–lnL = 2342.5725; gamma shape = 4.2350; K = 102; G = 0.3040; R = 0.7030) and the figure created using Tree View 1.6.6. Posterior probability support values are given above the branches. Sequences generated in this study are given in bold. [a]sequence deposited in GenBank by Douda *et al.* (2013).

infestation. These observations together with SNP differences and sequence divergence of the studied genes between samples from Canada and Russia in the current study indicate possible differences in pathogenicity between the Russian and Canadian populations to *C. arvense*, in terms of geographical

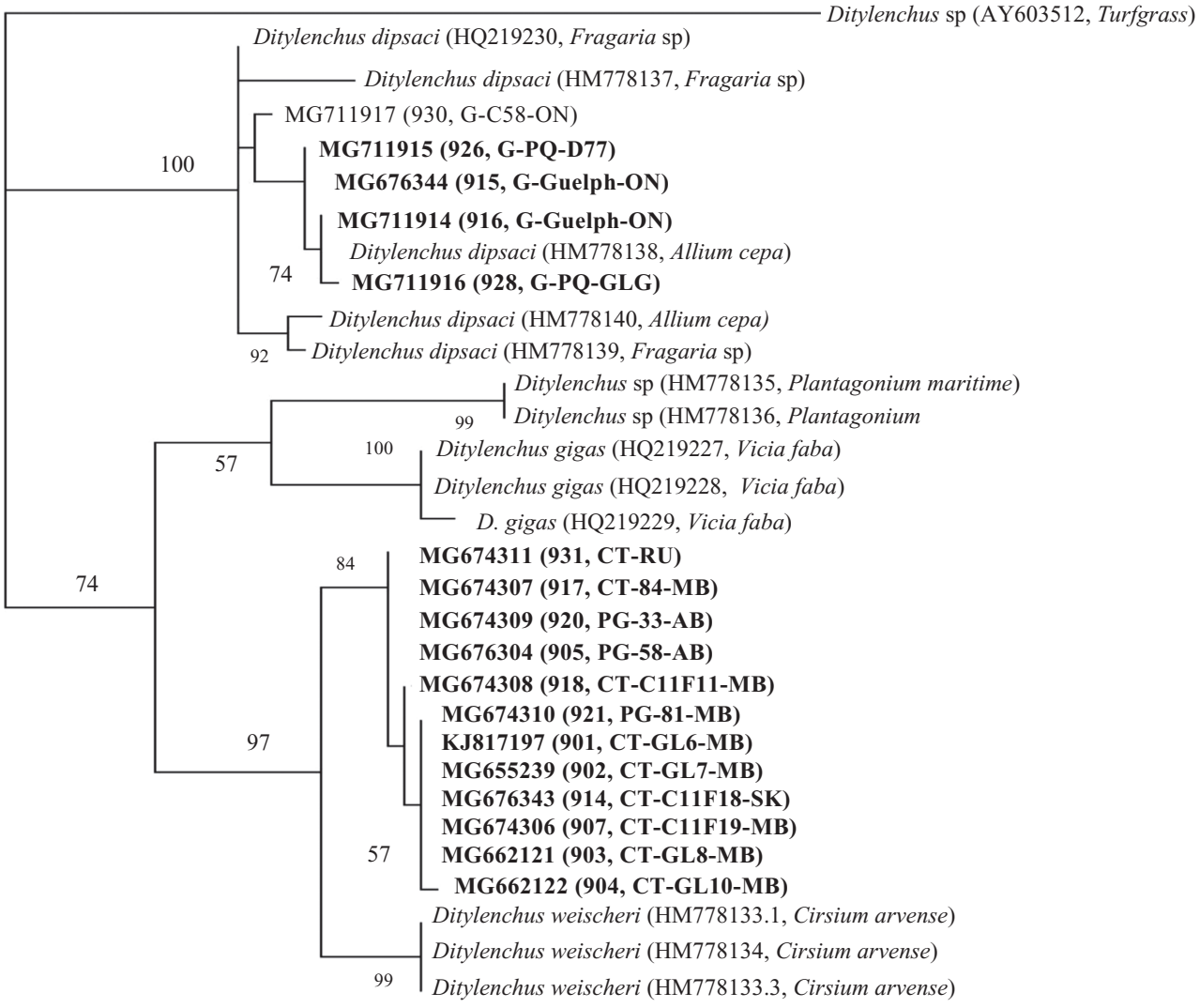

Figure 3: Phylogenetic relationships of *Ditylenchus weischeri* with related *Ditylenchus* species using MrBayes 3.2.6. jModelTest 2.1.7, and the Akaike Information Criterion were used to select HKY+G as the best models (–lnL = 917.5224; gamma shape = 0.5040; K = 63; G = 0.2154) and figure created using Tree View 1.6.6. Posterior probability support values are given above the branches. Sequences generated in this study are given in bold.

race, sibling or cryptic species or even perhaps species diversion. It is also possible that *C. arvense* populations respond differently to parasitism by *D. weischeri*.

Molecular analysis

Results of analysis of the different genes, ITS rDNA, LSU D2D3 28S rDNA, SSU 18S rDNA, and *Hsp*90 provide evidence that *D. weischeri* that is parasitizing *C. arvense* is genetically distinct from *D. dipsaci* to be distinguishable from each other. Sequence analysis of the studied genes showed the presence of heterogeneity between individuals of this species. Except for the ITSrDNA gene, there were not many sequences available for other genes in the GenBank database. Even for *D. weischeri*, there were few ITS rDNA sequences from a limited number of individuals. Among the studied genes, the least sequence divergence was observed for the SSU 18S rDNA gene. The *Hsp*90 gene showed the highest variability of sequence among species, and is the most informative marker in terms of discrimination between species, and thus likely the reason that enabled us to use it for designing species-specific primers in diagnostic PCR, (Madani et al., 2015).

Comparison of the ITS 1 and ITS 2 among the studied samples of *D. weischeri* and *D. dipsaci* showed that a higher nucleotide sequence variation existed in ITS2, thus ITS2 possibly alone would be enough informative for the differentiation of this species. This is in accordance with the published data showing suitability of ITS 2 for barcode identification and discrimination of main taxa in plants and animals (Hui et al., 2010). The GC content for *D. weischeri*, *D. dipsaci*, and *D. gigas* showed to be higher for the *D. dipsaci* group than the others. The two sequences of *D. dipsaci* each isolated from pea and garlic (Vovlas et al., 2011) were identical, but they had 22% dissimilarity to a sequence of D. dipsaci from pea (Helder et al., 2004).

While our results showed 98% similarity of Canadian *D. weischeri* to the SSU 18S rDNA sequence of *D. dipsaci*, we noticed some discrepancies in the sequences of this gene deposited in the GenBank. For example, the two sequences from two pea isolates of *D. dipsaci* each from the study of Volvas *et al.* (2011) and Helder et al. (2004) showed differences at 22%, and two sequence of D. dipsaci each isolated from pea and garlic (Vovlas et al., 2011) showed identical sequences. It was not possible to compare the Canadian samples of *D. weischeri* for the LSU D2D3 28S rDNA gene sequence due to lack of available sequences for the Russian *D. weischeri*.

Phylogeny

All dendrograms generated in this study provided almost the same pattern for grouping of *D. weischeri* compared to the other *Ditylenchus* species examined. Trees were constructed based on the ITS rDNA, LSU D2D3 28S rDNA, and *Hsp*90 genes. These are the most common molecular markers for phylogenetic analysis; in contrast, we did not use the SSU 18S rDNA gene for analysis as it is less common, had limited sequence accessions of other *Ditylenchus* in GenBank, and that SSU 18S rDNA gene is more suitable for taxon discrimination at the family or higher level (Holterman et al., 2009; Wu et al., 2015).

Congruent phylogenies using each of ITS rDNA, LSU D2D3 28S rDNA, and *Hsp*90 genes have been a robust method for integrative diagnostics, particularly for closely related species such as the cyst-forming nematodes (i.e., Skantar et al. 2012; Madani et al., 2017) and *Ditylenchus* species (Douda *et al.*, 2013). Samples of *D. weischeri* used in this study were positioned in a separate clade for each of the generated trees using those genes. The three sequences of *Hsp*90 retrieved from the Genbank for *D. weischeri* also were positioned next to all *D. weischeri* in a sub-clade on the *Hsp*90-based phylogenetic tree. Also in ITS rDNA tree a sample of *D. weischeri* collected in Saskatchewan (731-CT-C11F7) was positioned in a sub-clade with the sequence of this nematode from Russia. Although sequence similarity search using the Blastn option in NCBI, using sequences of *D. weischeri* generated in this study showed high similarity to the ITS rDNA of *D. gigas*, phylogenetic analysis positioned this species in a separated clade.

In conclusion, sequence analysis of the four genes studied provide more insight to the genome of *Ditylenchus* species and in particular *D. weischerii*, for the discrimination of this species from the closely related species of *D, dipsaci* complex, i.e. *D. dipsaci*. This was confirmed by applying phylogenetic analysis using the most commonly used genes for plant parasitic nematodes, i.e. ITS rDNA, LSU D2D3 28S rDNA, and *Hsp*90. Results from this study and of past reports of plant host preference and morphology differences provide further evidence that *D. weischeri* is distinct to be a separate species from *D. dipsaci*. Some differences in gene sequences and morphology to the Russian haplotype and less severe symptoms of disease on *C. arvense* in Prairie Canada suggests that the Canadian and Russian populations of *D. weischeri* may be diverging.

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