Developing a One-Step Multiplex PCR Assay for Rapid Detection of Four Stubby-Root Nematode Species, *Paratrichodorus allius*, *P. minor*, *P. porosus*, and *Trichodorus obtusus*

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Abstract

Four trichodorid species, *Paratrichodorus allius*, *P. minor*, *P. porosus*, and *Trichodorus obtusus*, were found in multiple states in the United States. Traditional diagnosis based on morphology and morphometrics is laborious and requires an experienced taxonomist. Additionally, end-point diagnosis using PCR was only available for *P. allius*. To increase diagnostic efficiency and reduce costs, a one-step multiplex PCR assay was developed to simultaneously identify these four species using one PCR reaction. Available sequences of 18S ribosomal DNA and internal transcribed spacer 1 (ITS1) region of these species were aligned and five primers were designed. The conserved forward primer located in the 18S region, in combination with the species-specific antisense primer in the ITS1 region, amplified a single distinctive PCR fragment for each species (421/425 bp for

P. allius, 190 bp for *P. minor*, 513 bp for *P. porosus*, and 353 bp for *T. obtusus*). In silico analysis with 10 other trichodorid species and experimental analysis using samples with these four species, 20 other plant-parasitic and three non-plant-parasitic nematodes demonstrated high specificity with the primers designed. The multiplex PCR amplified desirable fragments using a set of artificially mixed templates containing one, two, three, or four targeted species. The reliability of multiplex PCR results was demonstrated by using nematode populations isolated from infested fields from diverse geographic regions in eight states. The multiplex PCR-based tool developed in this study for the first time provides a simple, rapid, and cost-friendly assay for accurate diagnosis of the four major trichodorid nematodes in the United States.

The stubby-root nematodes *Paratrichodorus* Siddiqi, 1974 and *Trichodorus* Cobb, 1913 in the family Trichodoridae (Thorne, 1935) Siddiqi, 1961 are plant ectoparasitic nematodes with signature cigar-shaped bodies and curved onchiostyles. They are important because they cause direct feeding damage and can vector certain viruses including *Tobacco rattle virus*, *Pea-early browning virus*, and *Pepper ringspot virus*, which cause severe economic damage on crops, especially in potato and ornamental bulbous crops (Decraemer 1995; Harrison and Robinson 1986). In the United States, *Paratrichodorus teres* (Hooper, 1962) (syn. *Trichodorus teres*, *T. flevebsus*), *P. allius* (Jensen, 1963), *P. minor* (Colbran, 1956) (syn. *Nanidorus minor*, *N. christiei*, *P. christiei*, *P. obesus*, *T. christiei*, *T. minor*, *T. obesus*), *P. porosus* (Allen, 1957) (syn. *Atlantadorus porosus*, *T. bucrius*, *T. porosus*), *P. renifer* (syn. *N. renifer*), and *T. obtusus* (Cobb, 1913) (syn. *T. proximus*) are present (Decraemer 1995; Kumari and Subbotin 2012; Riga et al. 2007; Hajihassani et al. 2018). Four of these species are commonly found in multiple states, including *P. allius* which is distributed in seven states (Lopez-Nicora et al. 2014; Mojtahedi et al. 2000; Yan et al. 2016a, b), *P. minor* in 31 states (CABI/EPPO 2002), *P. porosus* in 10 states (CABI/EPPO 2009), and *T. obtusus* in seven states (Crow 2005). A broad range of hosts for these four species, including potato, common grain, turfgrasses, and vegetables, occur in the United States (Crow 2004,

2005; Lopez-Nicora et al. 2014; Mojtahedi and Santo 1999; Shaver et al. 2016; Sheedy et al. 2010; Ye et al. 2015; Zeng et al. 2012; Hajihassani et al. 2018). Additionally, the three *Paratrichodorus* spp. are considered to be vectors of tobamoviruses (Decraemer and Robbins 2007; Huang et al. 2018).

It has been reported that approximately 10% of field populations of *P. allius* were viruliferous in the Pacific Northwest of the United States, resulting in significant economic losses to the potato industry (Mojtahedi et al. 2000). The species *P. minor* and *T. obtusus* are known to damage the root system of many turfgrasses, such as St. Augustinegrass (Rhoades 1965), bermudagrass (Crow and Welch 2004), and zoysiagrass (Shaver et al. 2013). The species *P. porosus* damaged maize and sorghum crops and affected forage production in North Carolina, U.S. (Chévres-Román et al. 1971) and induced black-blot disease negatively impacting the Chinese yam industry (Nishizawa 1973). Given the large number of economic hosts negatively affected by stubby-root nematodes, an efficient, time- and cost-saving diagnostic tool would be highly beneficial for determination of rotational planting plans and chemical control application to mitigate crop damage and reduce nematode spread. For example, it would not be recommended to establish turfgrass, corn, or sorghum in a field infested with *P. minor*. Instead, summer legumes could be planted to maintain low population densities of this stubby-root nematode species in the soil (Crow 2004). In a potato field infested with *P. allius*, a treatment of metam sodium and 1,3-dichloropropene might be considered to control the nematode and its associated *Tobacco rattle virus* causal agent of corky ringspot disease (Ingham et al. 2000, 2007).

Differentiation of stubby-root nematodes from other plant-parasitic nematodes is easily accomplished based on morphological characters, while differentiation of stubby-root nematode species from each other is difficult as the differences in morphometrics are minor among closely related species (Decraemer and Baujard 1998). Two stubby-root nematode species, *P. allius* and *P. teres*, are morphologically similar and could only be separated by the vaginal shape of females and the number of ventromedian precloacal supplements of males (Riga and Nelison 2005). However, the vaginal shape is easily affected by fixation and handling during the

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specimen preparation, and males of some *Paratrichodorus* species are rarely found. Hence, the accuracy of morphological identification of nematode species is challenging. The partial 18S rDNA and ITS1 region of *P. allius* and *P. teres* were sequenced, and their alignment suggested that the sequence identity of these two species (GenBank accessions: AM087124.1 and AM087125.1) was only 94% (Riga et al. 2007). Subsequently, a molecular diagnosis method was introduced to identify these two morphologically closely related species, using end-point duplex PCR with species-specific primers located in the ITS1 region (Riga et al. 2007).

To date, several molecular methods have been developed to identify, differentiate, and quantify stubby-root nematode species, such as PCR followed by a restriction fragment length polymorphism analysis (PCR-RFLP) (Duarte et al. 2011; Kumari and Subbotin 2012), end-point single or duplex PCR with species-specific primers (Boutsika et al. 2004; Huang et al. 2017b; Riga et al. 2007), and quantitative real-time PCR monitoring amplicon production during each cycle (Holeva et al. 2006; Huang et al. 2017a; 2018). Among these PCR-RFLP techniques requires additional steps that are time-consuming, while quantitative real-time PCR focuses on the quantification of population density, which is more expensive. In addition, quantitative real-time PCR requires additional professional training on techniques and data normalization and presentation, which limits its application for species identification. The end-point single PCR with species-specific primers is more suitable as an inexpensive and routine diagnostic service, with the advantage of lower cost and time, and requires less technical skill. Currently, diagnoses using end-point single PCR with species-specific primers are available for *P. allius*, *P. teres*, *P. macrostylus*, *P. pachydermus*, *T. primitivus*, and *T. similis* (Boutsika et al. 2004; Huang et al. 2017b; Riga et al. 2007), but not for the other three widely distributed and economically important stubby-root nematodes, *P. minor*, *P. porosus*, and *T. obtusus*.

The end-point single PCR with only one pair of primers is simpler than multiplex PCR with multiple primer pairs in its design, implementation, and optimization. However, the development and utilization of three individual end-point single PCR assays for specific identification of these three species are laborious and come at higher costs. Additionally, multiple tests with several different end-point single PCR for nematode species identification is not recommended when limited DNA template is available. Thus, the multiplex PCR assay has the advantage of simultaneous identification of multiple gene targets using a single reaction, minimizing the amount of DNA template and PCR reagents necessary to complete a diagnosis. Despite the complexity of multiplex PCR assay development, multiplex PCR methods have been successfully developed in differentiation of various pathogens, such as bacteria (Cui et al. 2016), fungi (Cho et al. 2016), and nematodes (Filipiak et al. 2017). For nematode species differentiation, multiplex PCR protocols were developed for *Xiphinema* spp. (Wang et al. 2003), *Meloidogyne* spp. (Hu et al. 2011), and *Bursaphelenchus* spp. (Filipiak et al. 2017), with a maximum of four species identified by a single PCR reaction (Wang et al. 2003). For stubby-root nematode species identification, a duplex PCR was developed to detect *P. allius* and *P. teres* simultaneously (Riga et al. 2007). To our knowledge, there is no multiplex PCR available for other stubby-root nematode species.

Farmers may want to know what species of plant-parasitic nematodes is present in their fields in order to make better nematode management decisions through crop rotation or use of resistant crops. Information on nematode infestations would commonly come from professional soil diagnostic services. The multiplex PCR method is more preferable than several end-point single PCR assays to improve the efficiency and reduce the cost of nematode diagnosis. However, there is no report of a PCR technique for simultaneous detection and identification of *P. allius*, *P. minor*, *P. porosus*, and *T. obtusus* which are widely present and economically important trichodoridae species in the United States. The main objective of this study was to develop a convenient one-step (in a single PCR reaction) multiplex PCR-based assay with the capability of simultaneous and unambiguous identification of *P. allius*, *P. minor*, *P. porosus*, and *T. obtusus*, using single target nematodes. The molecular characterization of 18S rDNA, 28S

rDNA, 5.8S rDNA, ITS1 rDNA, and ITS2 rDNA sequences were reported previously for these four nematode species (Huang et al. 2018), which accelerated the development of multiplex PCR-based diagnostic method in this study.

Materials and Methods

Nematode materials and DNA extraction from single nematodes. In 2016–2018, stubby-root nematode infested soil samples were collected by our group from North Dakota and Minnesota, and also provided by collaborators from Florida, Idaho, Oregon, and Nebraska (Table 1). Additional nematode suspensions containing stubby-root nematodes were obtained from North Carolina and Georgia (Table 1). These nematode samples were collected from grass, alfalfa, barley, corn, soybean, onion, potato, and sugar beet fields. For the soil samples, vermiform nematodes were extracted from 200 g of soil by sieving and decanting followed by sugar centrifugal flotation methods (Jenkins 1964). Stubby-root nematodes were hand-picked from nematode suspensions under a dissecting microscope (Zeiss stemi 305 Lab microscope, Zeiss). The genomic DNA from single individuals of each stubby-root nematode species was extracted using Proteinase K method (Huang et al. 2018; Kumari and Subbotin 2012). For each soil sample or nematode suspension, at least three genomic DNA preparations of stubby-root nematode individuals were used as replicates. The DNA extracts were stored in -20°C for subsequent experiments.

Multiple sequence alignment, primer design, and in silico analysis. Multiplex PCR primers for differentiation of *P. allius*, *P. minor*, *P. porosus*, and *T. obtusus* were targeted on the ITS1 rDNA, which is the most variable region when compared with the 18S rDNA and 28S rDNA regions. The ITS1 rDNA sequences used for multiple sequence alignment and comparison were retrieved from GenBank, including eight sequences of *P. allius* (MG938575–MG938582), two sequences of *P. porosus* (MG938570, MG938571), one sequence of *P. minor* (MG938572), and two sequences of *T. obtusus* (MG938573, MG938574). The alignment was performed using ClustalX (Thompson et al. 1997), and primer selections were carried out using the PrimerSelect module in DNASTAR software package (Lasergene, Madison, WI, USA). To minimize the number of primers present in a PCR reaction, the forward primer was nonspecific while reverse primers were species-specific (Table 2; Supplementary Fig. S1). Therefore, the universal forward primer was located in the conserved region of 18S rDNA and the compatible reverse primers were selected under default settings with additional criteria including: (i) located in ITS1 rDNA region unique for each species, (ii) no formation of severe primer hairpins, self-dimers, and pair-dimers, (iii) melt temperature (T_m) differences among primers less than 5°C , and (iv) amplified fragments differ from each other by at least 50 bp in order to clearly distinguish the four species on an agarose gel.

Primer specificity was assessed by in silico analysis (Okubara et al. 2008; Schroeder et al. 2006; Yan et al. 2008), using nematode species in the genera of *Paratrichodorus* and *Trichodorus* that were available in the GenBank database (Supplementary Table S1). There were 81 isolates from 14 trichodoridae nematode species in the analysis, including 26 isolates from *P. allius*, 19 isolates from *P. porosus*, 10 isolates from *P. minor*, six isolates from *P. renifer*, three isolates from each of *T. pakistanensis* and *T. similis*, two isolates from each of *P. macrostylus*, *P. pachydermus*, *T. nanjingensis*, *T. obtusus*, *T. primitivus*, and *T. sparsus*, and one isolate of each of *P. teres* and *T. cedarus*. No PCR amplification was considered when the primer-template duplex stability (ΔG) was presented as insignificant or poor primer-template duplex stability ($\Delta G > -31$ kcal/mol). Primers were synthesized by Eurofin MWG Operon LLC (Huntsville, AL).

Multiplex polymerase chain reaction. The multiplex PCR protocol for differentiating the targeted nematode species, optimal PCR reaction, and thermocycler conditions were developed using previously published procedures (Henegariu et al. 1997). To initiate this work, a singleplex PCR for all four target species, with one primer pair consisting of the universal forward primer and one of the species-specific reverse primers, was set up using the same

protocol, including the same concentration of each PCR reagent and cycling conditions. The initial singleplex PCR was carried out at conditions including 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 μM of each primer, with annealing temperature calculated by the PrimerSelect software. Accordingly, the multiplex PCR was conducted by adding all the primers into the PCR reaction as the protocol of the single, basic PCR. PCR products were separated in 2.5% agarose gel at 100 volts for 40 min and documented using the AlphaImager Gel Documentation System (Proteinsimple Inc., Santa Clara, CA, USA). Based on the banding patterns of PCR products, the multiplex PCR protocol was further optimized by adjusting the annealing temperature, extension time, and each primer concentration, as recommended by Henegariu et al. (1997). The final optimized multiplex PCR used in this study for stubby-root nematode species identification was executed in a 25-μl volume including 1.0 μl of template, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 μM of UnivF3 (universal forward primer), 0.12 μM of PaR1 (*P. allius* specific reverse primer), 0.2 μM of PpR1 (*P. porosus* specific reverse primer), 0.16 μM of ToR2 (*T. obtusus* specific reverse primer), 1× Colorless GoTaq Flexi PCR buffer, and 1.0 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). The PCR cycling conditions were: one cycle of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min 20 s, and a final cycle of 72°C for 10 min.

Multiplex PCR validation and diagnosis of *Paratrichodorus allius*, *P. minor*, *P. porosus*, and *Trichodorus obtusus*. To determine the specificity of the multiplex PCR for the nematode species targeted, PCR was performed with a set of DNA samples from target and nontarget nematode individuals (Table 3) that were used in previous studies (Huang et al. 2017a, b; 2018). A total of 13 target nematode samples and 23 nontarget nematode samples were used, including all four target stubby-root nematode species, five cyst nematode species (*Heterodera glycines*, *H. schachtii*, *Cactodera milleri*, *Globodera ellingtonae*, and *G. pallida*), one root-knot nematode species (*Meloidogyne chitwoodi*), six root-lesion nematode species (*Pratylenchus penetrans*, *P. neglectus*, *P. scribneri*, *P. thornei*, *P. zaeae*, and *Pratylenchus* sp.), one lance nematode species (*Hoplolaimus stephanus*), one unknown stunt nematode (*Tylenchorhynchus* sp.), one spiral nematode (*Helicotylenchus microlobus*), one unidentified dagger nematode species (*Xiphinema* sp.), one ring nematode (*Mesocriconema* sp.), one pin nematode (*Paratylenchus nanus*), and three non-plant-parasitic nematodes (Table 3).

To further validate the specificity and capability of the protocol on species differentiation, multiplex PCR was evaluated with a set of artificially prepared DNA mixtures consisting of two, three, and four target nematode species at the ratio of 1:1, 1:1:1, and 1:1:1:1, respectively. Additionally, the species of stubby-root nematodes from field soils of diverse geographic regions (Table 1) were determined by the

Table 1. Stubby-root nematode samples used in this study

Sample ID	County, state	Sampling date	Crop	Multiplex PCR fragment (bp)	Species identity	Species confirmed by ^a
7103	Sargent, ND	09/2017	Potato	421/425	<i>Paratrichodorus allius</i>	Species-specific PCR
7105	Sargent, ND	09/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
7106	Sargent, ND	09/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
SB1	Clay, MN	06/2017	Sugarbeet	421/425	<i>P. allius</i>	Species-specific PCR
SB51	Clay, MN	07/2017	Sugarbeet	421/425	<i>P. allius</i>	Species-specific PCR
IDL1	Fremont, ID	10/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
IDS7	Fremont, ID	10/2017	Alfalfa	421/425	<i>P. allius</i>	Species-specific PCR
IDB1	Fremont, ID	10/2017	Barley	421/425	<i>P. allius</i>	Species-specific PCR
SH1	Umatilla, OR	12/2016	Potato	421/425	<i>P. allius</i>	Species-specific PCR
SH3	Umatilla, OR	12/2016	Potato	421/425	<i>P. allius</i>	Species-specific PCR
7524	Umatilla, OR	12/2016	Potato	421/425	<i>P. allius</i>	Species-specific PCR
MS2	NE	03/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
MS5	NE	03/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
GJ-10	NE	03/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
CSS4	NE	05/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
CSS10	NE	05/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
NEB8	NE	10/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
NEB13	NE	10/2017	Potato	421/425	<i>P. allius</i>	Species-specific PCR
34536	Cumberland, NC	05/2016	Corn	190	<i>P. minor</i>	DNA sequencing
34537	Lee, NC	05/2016	Corn	190	<i>P. minor</i>	DNA sequencing
34538	Lee, NC	05/2016	Corn	190	<i>P. minor</i>	DNA sequencing
32943	Nash, NC	05/2016	Soybean	190	<i>P. minor</i>	DNA sequencing
UGA	Tattnall, GA	02/2018	Onion	190	<i>P. minor</i>	DNA sequencing
FL48	Marion, FL	05/2016	Grass	353	<i>Trichodorus obtusus</i>	DNA sequencing
FL50	Marion, FL	05/2016	Grass	353	<i>T. obtusus</i>	DNA sequencing

^a The species of *P. allius* was confirmed by species-specific PCR developed by Huang et al. (2017b); the species of *P. minor* and *T. obtusus* were confirmed by direct sequencing of PCR products amplified by BL18/5818, as described by Huang et al. (2018). The DNA sequencing was performed by Genscript Corporation (Piscataway, NJ, USA).

Table 2. Universal and species-specific primers used in this study

Primer name	Sense	Sequence (5'-3')	Primer location	Used for
D3A	Forward	GACCCGTCTTGAAACACGGA	28S rDNA	Testing the presence of template DNA
D3B	Reverse	TCGGAAGGAACCACTACTA	28S rDNA	
BL18	Forward	CCGTCGTMCTACTACCGATT	18S rDNA	Sequencing for species identity confirmation
5818	Reverse	ACGARCCGAGTGATCCAC	5.8S rDNA	
UnivF3	Forward	GTAACAAGGTTTCCGTAGGTGAA	18S rDNA	Multiplex PCR universal primer
PaR1	Reverse	AGTCGACGTAGGTACCCAACAAAC	ITS1 rDNA	Specific for <i>Paratrichodorus allius</i>
PmR1	Reverse	GGCGATGCTTGCGGGTGTG	ITS1 rDNA	Specific for <i>P. minor</i>
PpR1	Reverse	GTACAGTTTTATCGTGCCGTTAG	ITS1 rDNA	Specific for <i>P. porosus</i>
ToR2	Reverse	ACAAGCAAGCAAGCGGTCTC	ITS1 rDNA	Specific for <i>Trichodorus obtusus</i>

multiplex PCR using three independent DNA extracts from single nematodes in each sample. The accuracy of multiplex PCR diagnosis was evaluated by sequencing ITS1 rDNA region amplified by primers BL18/5818 (Huang et al. 2018). As an alternative, for *P. allius*, the species identity was confirmed by the species-specific singleplex PCR (Huang et al. 2017b). Before being subjected to the multiplex PCR, the quality of all DNA used in the study was checked by amplification with a set of universal primers D3A/D3B (Al-Banna et al. 1997).

Results

Selection and specificity of multiplex PCR primers. According to the sequence alignment performed by ClustalX using available information from GenBank, the 18S rDNA region was more conserved than the ITS1 region. Hence, the universal forward primer UnivF3 was chosen from the 18S rDNA region in positions 747–769 for *P. allius* (MG938579 and MG938580), *P. porosus* (MG938571), and *T. obtusus* (MG938573), and positions 745–767 for *P. minor* (MG938572). The compatible species-specific reverse primers were selected from the ITS1 region in the positions 1236–1259 for *P. porosus* (MG938571), 916–934 for *P. minor* (MG938572), and 1080–1099 for *T. obtusus* (MG938573), resulting in expected PCR fragment sizes of 513, 190, and 353 bp for *P. porosus*, *P. minor*, and *T. obtusus*, respectively. Due to the sequence polymorphism,

the specific reverse primer for *P. allius* was located in the positions 1150–1173 (MG938579) or 1146–1169 (MG938580) for different isolates, resulting in expected PCR fragment sizes of 425 bp (MG938579) or 421 bp (MG938580).

As expected, in silico analysis suggested that the primer UnivF3 formed a stable hybrid ($\Delta G > -31$ kcal/mol) with all 14 stubby-root nematode species examined, including seven from *Paratrichodorus* spp. and seven from *Trichodorus* spp. Meanwhile, the species-specific reverse primers only formed a stable hybrid with all isolates of the target nematode species, revealing the specificity of each primer set for its target species amplification. Moreover, the primer specificity was further tested by experimental PCR using DNA of four target stubby-root nematode species collected from multiple states, 18 nontarget plant-parasitic nematode species, and three free-living nematode species (Table 3). All eight DNA samples of *P. allius* from five states produced one fragment with the expected size using the multiplex PCR. Single fragments with the expected sizes were produced when DNA extracts from one isolate of *P. minor*, two isolates of *P. porosus*, and two isolates of *T. obtusus* from different states were used as templates in PCR (Table 3). No PCR products were observed using DNA extracts from samples of 23 nontarget nematodes as template and the nontemplate control which used sterilized ddH₂O in place of a DNA template in the PCR reaction. Amplification of all DNA extracts of nematodes occurred when the

Table 3. Nematode species used in this study to test the specificity of multiplex PCR assay

Code ^a	Species	State	Crop	Multiplex PCR banding pattern ^b			
				Pa (421/425 bp)	Pm (190 bp)	Pp (513 bp)	To (352 bp)
1	<i>Paratrichodorus allius</i>	ND	Potato	+	-	-	-
2	<i>P. allius</i>	ND	Field pea	+	-	-	-
3	<i>P. allius</i>	MN	Sugarbeet	+	-	-	-
4	<i>P. allius</i>	MN	Sugarbeet	+	-	-	-
5	<i>P. allius</i>	ID	Potato	+	-	-	-
6	<i>P. allius</i>	ID	Potato	+	-	-	-
7	<i>P. allius</i>	WA	Potato	+	-	-	-
8	<i>P. allius</i>	OR	Potato	+	-	-	-
9	<i>P. minor</i>	NC	Soybean	-	+	-	-
10	<i>P. porosus</i>	SC	Spinach	-	-	+	-
11	<i>P. porosus</i>	NC	Soybean	-	-	+	-
12	<i>Trichodorus obtusus</i>	SC	Grass	-	-	-	+
13	<i>T. obtusus</i>	FL	Grass	-	-	-	+
14	<i>Cactodera milleri</i>	MN	Potato	-	-	-	-
15	<i>Globodera ellingtonae</i>	OR	Potato	-	-	-	-
16	<i>G. pallida</i>	ID	/ ^c	-	-	-	-
17	<i>Heterodera glycines</i>	ND	Potato	-	-	-	-
18	<i>H. schachtii</i>	OR	Sugarbeet	-	-	-	-
19	<i>Meloidogyne chitwoodi</i>	WA	Potato	-	-	-	-
20	<i>Pratylenchus penetrans</i>	MD	/	-	-	-	-
21	<i>P. neglectus</i>	ND	Wheat	-	-	-	-
22	<i>P. neglectus</i>	OR	Wheat	-	-	-	-
23	<i>P. scribneri</i>	FL	Tomato	-	-	-	-
24	<i>P. scribneri</i>	ND	Potato	-	-	-	-
25	<i>P. thornei</i>	OR	Wheat	-	-	-	-
26	<i>P. zaeae</i>	/	/	-	-	-	-
27	<i>Hoplolaimus stephanus</i>	ND	Soybean	-	-	-	-
28	<i>Tylenchorhynchus</i> sp.	ND	Soybean	-	-	-	-
29	<i>Mesocriconema curvatum</i>	ND	Potato	-	-	-	-
30	<i>Paratylenchus nanus</i>	ND	Field Pea	-	-	-	-
31	<i>Pratylenchus</i> sp.	ND	Soybean	-	-	-	-
32	<i>Helicotylenchus microlobus</i>	ND	Soybean	-	-	-	-
33	<i>Xiphinema</i> sp.	ND	Potato	-	-	-	-
34	Non-plant-parasitic nematode 1	ND	Potato	-	-	-	-
35	Non-plant-parasitic nematode 2	ND	Potato	-	-	-	-
36	Non-plant-parasitic nematode 3	ND	Potato	-	-	-	-

^a DNA Code 1-13 were used in the study of Huang et al. (2018), Code 14-30 were used in the study of Huang et al. (2017b), and Code 31-36 were used in the study of Huang et al. (2017a).

^b Pa, Pp, Pm, and To refer to specific PCR fragment for *Paratrichodorus allius*, *P. porosus*, *P. minor*, and *Trichodorus obtusus* generated by multiplex PCR; +/– stands for presence/absence of amplification.

^c / indicates that the information was not available.

universal primers D3A/D3B were used in PCR, confirming the presence of DNA in the unknown samples (data not shown).

Multiplex PCR for discrimination of *Paratrichodorus allius*, *P. minor*, *P. porosus*, and *Trichodorus obtusus* from artificially mixed DNA templates. The capability of multiplex PCR for simultaneous differentiation of *P. allius*, *P. minor*, *P. porosus*, and *T. obtusus* was investigated by using a set of artificially prepared DNA mixtures. The multiplex PCR with all five primers from the singleplex PCR protocols could generate specific fragments associated with their target DNA (Fig. 1). However, the amplification competition was observed when there were multiple targets in the PCR reaction, revealed by the unbalanced PCR products in intensity shown in the agarose gel (Fig. 1A). By increasing the annealing temperature, decreasing the concentration of reverse primers associated with abundant PCR products, and increasing the concentration of reverse primers associated with low amounts of PCR products, the optimized PCR protocol finally produced PCR fragments that could be easily distinguished from each other in the agarose gel with similar band intensities (Fig. 1B). The template from single nematode species only generated a single and relevant PCR product (Fig. 1, lanes 1-4). When the template contained more than one target, expected PCR products were amplified matching corresponding target nematodes. In detail, the banding patterns represented that the mixtures of any two targets, *P. allius* and *T. obtusus*, *P. minor* and *T. obtusus*, *P. porosus* and *T. obtusus*, *P. allius* and *P. minor*, *P. allius* and *P. porosus*, and *P. minor* and *P. porosus*, produced two fragments at the sizes of 421/425 bp and 353 bp, 190 bp and 353 bp, 513 bp and 353 bp, 421/425 bp and 190 bp, 421/425 bp and 513 bp, and 190 bp and 513 bp, respectively (Fig. 1, lanes 5-10). The mixtures of any three targets, *P. allius* and *P. minor* and *T. obtusus*, *P. allius* and *P. porosus* and *T. obtusus*, *P. minor* and *P. porosus* and *T. obtusus*, and *P. allius* and *P. minor* and *P. porosus*, produced three fragments at the sizes of 421/425 bp and 190 bp and 353 bp, 421/425 bp and 513 bp and 353 bp, 190 bp and 513 bp and 353 bp, and 421/425 bp and 190 bp and 513 bp, respectively (Fig. 1, lanes 11-14). Finally, the mixture of all four targets, *P. allius* and *P. minor* and *P. porosus* and *T. obtusus*, produced four fragments at the sizes of 421/425 bp, 190 bp, 513 bp and 353 bp, respectively (Fig. 1, lane

15). Neither cross-reaction products nor amplifications with a non-template control that used sterilized ddH₂O instead of DNA were observed in the multiplex PCR reactions.

Multiplex PCR detection of *Paratrichodorus allius*, *P. minor*, *P. porosus*, and *Trichodorus obtusus* from naturally infested soils. The ability of multiplex PCR for detecting and identifying stubby-root nematodes was evaluated using 25 stubby-root nematode populations isolated from infested fields distributed in eight states including North Dakota, Minnesota, Florida, North Carolina, Georgia, Idaho, Oregon, and Nebraska (Table 1). All DNA extracts of single stubby-root nematodes were amplified, indicating the existence of target species. Based on the banding pattern and fragment size of multiplex PCR products, uniform amplification results were presented using three different DNA extracts of single stubby-root nematodes from one soil sample. All DNA extracts generated a single, specific fragment, and species identity was assigned according to the length of PCR products shown in the agarose gel (Table 1, Fig. 2). Among them, 18 samples producing the fragment size of 421/425 bp were identified as containing *P. allius*, five samples producing the fragment size of 190 bp contained *P. minor*, and two samples producing the fragment size of 353 bp contained *T. obtusus*. No fragment size of 513 bp was produced, indicating no *P. porosus* was detected. The species identities were further supported by either sequence information of ITS1 rDNA obtained from PCR amplification with BL18/5818 primers or *P. allius*-specific PCR.

Discussion

Molecular diagnostic tools have been broadly developed for nematode species identification which can diagnose and distinguish species regardless of nematode life stage present, eliminating the need for laborious morphometric measurements. This study describes the development, validation, and application of a one-step multiplex PCR method for detection and identification of four trichodoridae nematode species, *P. allius*, *P. minor*, *P. porosus*, and *T. obtusus*, which are commonly distributed in the United States. Previous studies reported that PCR-RFLP based on 18S rRNA gene could be used to differentiate *P. allius*, *P. porosus*, *P. minor*, and 18 other stubby-root nematode species (Kumari and Subbotin 2012). However,

Fig. 1. Electrophoresis of multiplex PCR products with DNA from single or multiple target nematodes. (A) Multiplex PCR products using the singleplex PCR protocol. (B) Multiplex PCR products using the optimized PCR protocol. DNA templates that correspond to lanes 1-15 in the agarose gel were prepared as follows: lanes 1-4, DNA from a nematode individual of each of *Trichodorus obtusus*, *Paratrichodorus allius*, *P. minor*, and *P. porosus*; lanes 5-10, DNA mixtures from any two target nematodes; lanes 11-14, DNA mixtures from any three target nematodes; lane 15, DNA mixture from four target nematodes. H₂O refers to the no template control using sterilized double distilled water in the PCR reaction instead of DNA template. M refers to 100 bp DNA ladder.

compared with one-step multiplex PCR, the technique of PCR-RFLP requires posttreatment of PCR products with restriction enzyme digestion, a process that is time-consuming. Although end-point single PCR amplification with one pair of species-specific primers has been applied for identification of stubby-root nematode species to overcome the disadvantage of PCR-RFLP, it was only able to detect one target nematode species at one time (Boutsika et al. 2004; Holeva et al. 2006; Huang et al. 2017b). The multiplex PCR utilizing more than one pair of primers is becoming increasingly popular because of its ability to simultaneously detect multiple targets in a single PCR reaction. Multiplex PCR-based species identification methods were well-developed for many organisms, including nematodes (Filipiak et al. 2017; Hu et al. 2011; Riga et al. 2007; Wang et al. 2003).

To establish the multiplex PCR, with obvious segregation of PCR products amplified from each of the nematode species targets and with a minimal number of primers to reduce the possibility of primer competition and cross-amplification in the PCR reaction, the 18S-ITS1 rDNA region was selected as the target region for amplification by PCR in this study. The molecular characterization of 28S rDNA, 18S rDNA, 5.8S rDNA, and ITS rDNA in *P. allius*, *P. porosus*, *P. minor*, and *T. obtusus* revealed that 28S, 18S, and 5.8S regions were conserved among species while the ITS1 region was more variable (Huang et al. 2018). Therefore, more specifically, the 18S region was targeted by the universal forward primer while the ITS1 region was targeted by the species-specific reverse primers.

The specificity of all primer pairs and their optimal PCR conditions are keys for a successful multiplex PCR assay (Henegariu et al. 1997). In silico analysis predicted that stable primer-template can only be formed with target species of all isolates from various geographic regions, but not with other nontarget stubby-root nematode species having ITS rDNA sequences available in GenBank. Meanwhile, the evaluation was confirmed by experimental PCR with no amplification from DNA of other common plant-parasitic nematodes and free-living nematodes. The multiplex PCR with five primers forming four pairs and targeting the four different species produced DNA fragments at desirable sizes when one, two, three, or all four species were present. The results indicated that there was no presence of primers and/or DNA template interferences in

our multiplex PCR reactions. Moreover, the efficacy of primers for each target appeared to be similar, revealed by clear PCR products without much difference in band intensity shown in the agarose gel. These fragments can be easily distinguished from each other, allowing easy species identification based on fragment sizes. Previously, two trichodorid nematode species, *P. allius* and *P. teres*, could be distinguished using a single PCR reaction (Riga et al. 2007). As a comparison, the multiplex PCR developed in this study could detect more species of stubby-root nematodes through one PCR reaction, including three species of *P. minor*, *P. porosus*, and *T. obtusus*. Except for the PCR-RFLP assay (Kumari and Subbotin 2012), other molecular diagnostic tools have not been reported for these three species.

Our developed multiplex PCR assay was applied for species identification of stubby-root nematodes isolated from natural fields in diverse geographic regions. By using three individual nematodes independently, three trichodorid species, *P. allius*, *P. minor*, and *T. obtusus*, were identified in eight states, and no *P. porosus* was detected from the samples tested. In spite of the nucleotide variation in the ITS1 region among *P. allius* isolates from different regions (Huang et al. 2018), the multiplex PCR developed was able to identify all reported isolates from states of ND, MN, OR, ID, WA, and NC, according to in silico analysis. Consistently, the experimental multiplex PCR using field samples was capable of identifying all the isolates of *P. allius* from ND, MN, ID, and OR and additionally all the isolates of *P. allius* from NE, indicating that the multiplex PCR identification approach can be broadly used for samples from different regions. Either the sequences of ITS rDNA or available species-specific PCR confirmed the species identity, which demonstrated the reliability of this multiplex PCR-based approach for diagnostic purpose. DNA samples used for trichodorid species detection in other studies were from adults (Boutsika et al. 2004; Riga et al. 2007). However, the multiplex PCR in this study used DNA extracts from single nematodes in different life stages (small juveniles, large juveniles, or adults), which is applicable to all common nematode samples, even for samples without adults or with low population densities lacking of adults. On the other hand, even though a mixed population of trichodorid nematodes in a field has not been found,

Fig. 2. Multiplex PCR assay for differentiation of stubby-root nematodes by generating species-specific PCR products. The label ## stands for Population ID-Replicates. To1, To2; Pm; Pp1, Pp2; and Pa are positive controls of *Trichodorus obtusus*, *Paratrichodorus minor*, *P. porosus*, and *P. allius*, respectively; H₂O serves as no template control using sterilized ddH₂O instead of DNA template in the PCR. M refers to 100 bp ladder.

compared with end-point single PCR for each species, the one-step multiplex PCR approach has advantages of saving time and costs, due to running only one PCR reaction instead of attempting species-specific PCR for each of the four species. Overall, the results suggest that the described approach in this study allows simultaneous, rapid, and precise identification and discrimination of four species of *P. allius*, *P. porosus*, *P. minor*, and *T. obtusus*. This technique will benefit the diagnostic services and growers through associated low cost, high throughput, and relatively simple operational equipment. This study would provide useful information for further research developing multiplex PCR-based quantification of stubby-root nematode species to meet a high demand of diagnostic services.

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