DEVELOPMENT OF A SPECIES-SPECIFIC REVERSE PRIMER FOR THE MOLECULAR DIAGNOSTIC OF *PRATYLENCHUS BRACHYURUS*

A. C. Z. Machado,[1]* L. C. C. B. Ferraz,[2] and C. M. G. de Oliveira[1]

[1]Instituto Biológico, Caixa Postal 70, 13001-970, Campinas, SP, Brazil, [2]ESALQ/USP, Caixa Postal 9, 13418-900, Piracicaba, SP, Brazil. *Corresponding author: andressaczmachado@hotmail.com.

ABSTRACT

Machado, A. C. Z., L. C. C. B. Ferraz, and C. M. G. Oliveira. 2007. Development of a Species-Specific Reverse Primer for the Molecular Diagnostic of *Pratylenchus brachyurus*. Nematropica 37:249-257.

A single step PCR using a species-specific reverse primer for the diagnosis of the economically important phytonematode *Pratylenchus brachyurus* was developed. The ITS-1 region of rDNA of *P. brachyurus* was amplified by PCR from genomic DNA. Sequence analysis indicated that the ITS-1 sequences were divergent between this species and other *Pratylenchus* species (*P. coffeae*, *P. vulnus*, and *P. zeae*). These sequence differences in the ITS-1 were used to synthesize a specific reverse primer to diagnose *P. brachyurus*. The primer was assessed for its reliability by screening 29 populations of *P. brachyurus* from Brazil, producing the expected fragment size (267 bp) for all populations tested. Furthermore, when challenged with closely related *Pratylenchus* species and general nematode communities typical of soil, no PCR product was amplified. Given its specificity, sensitivity and reliability, this diagnostic primer may be of great benefit to phytosanitary services related to agronomical crops.

Key-words: ITS-1 region, root-lesion nematode, sequencing, PCR, *Pratylenchus brachyurus*.

RESUMEN

Machado, A. C. Z., L. C. C. B. Ferraz, y C. M. G. Oliveira. 2007. Desarrollo de un iniciador reverso específico para el diagnostico molecular de *Pratylenchus brachyurus*. Nematropica 37:249-257.

Se desarrolló un método basado en la reacción en cadena por la polimerasa (PCR) para detectar el nematodo de importancia económica *Pratylenchus brachyurus*. La región ITS-1 del ADNr de *P. brachyurus* fue amplificada por PCR a partir del ADN genómico. El análisis de las secuencias indicó que las mismas fueron divergentes entre esta especie y otras especies de *Pratylenchus* (*P. coffeae*, *P. vulnus* y *P. zeae*), y esta diferencia en las secuencias de la región ITS-1 fue utilizada para diseñar un iniciador reverso específico para el diagnóstico de *P. brachyurus*. Se analizaron 29 poblaciones de *P. brachyurus* de Brasil para probar la fiabilidad del iniciador, con la producción del fragmento esperado (267 pb) para todas las poblaciones. Además, se analizó la especificidad del iniciador utilizando ADN de especies cercanas a *Pratylenchus* y de comunidades de nematodos típicos del suelo, y no se produjeron los productos finales. Por tanto, dada su especificidad, sensibilidad y fiabilidad, este iniciador específico puede ser de gran utilidad para los servicios fitosanitarios en relación con cultivos de interés agronómico.

Palabras clave: diagnóstico, nematodo de las lesiones radicales, *Pratylenchus brachyurus,* región ITS-1, secuenciación, PCR.

INTRODUCTION

Due to the continuing decline in classical taxonomic expertise of many taxa including Nematoda (Behan-Pelletier, 1999; Andre *et al.*, 2001; Coomans, 2002), there is an increasing reliance on developing molecular-based diagnostic protocols to identify pests and pathogens. Several DNA-based methodologies have been developed for application in nematology (Powers, 2004; Blok, 2005). Such molecular techniques are expected to provide routine, rapid, user-friendly diagnostics.

This is particularly important with the root-lesion nematodes, *Pratylenchus* spp., as several species are distinguished by only minute morphometric or morphological differences such as number of lip annules, shape of the tail terminus, relative length of the esophageal gland overlap, length of post-uterine sac, and even lateral line number (Loof, 1978). However, recognition of these characters requires the involvement of highly trained taxonomists and intraspecific variability compounds the identification problems (Powers, 2004).

Although DNA-based techniques have been used to discriminate among species of plant-parasitic nematodes (Vrain *et al.*, 1992; Ibrahim *et al.*, 1994; Joyce *et al.*, 1994; Oliveira *et al.*, 2004), currently there are few molecular-based diagnostics available for *Pratylenchus* nematodes (Orui, 1996; Uehara *et al.*, 1998; Orui and Mizukubo, 1999; Waeyenberge *et al.*, 2000; Al-Banna *et al.*, 2004). Orui (1996), Orui and Mizukubo (1999), and Waeyenberge *et al.* (2000) identified *Pratylenchus* species using PCR-RFLP patterns in the internal transcribed spacer (ITS) regions of the rDNA, but this required the digestion of PCR products with several restriction enzymes. Indeed, for identification using the PCR-RFLP method, it is necessary to confirm that RFLP patterns consistently differ among species but are constant within a species (Orui and Mizukubo, 1999).

In a previous study, Uehara *et al.* (1998) were able to distinguish between *P. loosi* and *P. coffeae* using DNA amplification with species-specific primer sets. Recently, Al-Banna *et al.* (2004) designed five forward species-specific primers from the internal variable portion of the D3 expansion region of the 26S rDNA for *P. neglectus*, *P. penetrans*, *P. scribneri*, *P. thornei*, and *P. vulnus*. This method obviates the need for subsequent RFLP or sequence analysis of the PCR product and can be used as a rapid diagnostic tool in epidemiological and management studies. However, due to limited sequence variation, Al-Banna *et al.* (2004) were not able to design a primer that was unique to *P. brachyurus* (Godfrey, 1929) Filipjev & Sch. Stekhoven, 1941, which is an economically important migratory endoparasitic nematode in agriculture and is widely distributed throughout the world (Ferraz, 1999). *Pratylenchus brachyurus* is a well documented pest of cotton (*Gossypium hirsutum* L.), soybean [*Glycine max* (L.) Merr.], and coffee (*Coffea arabica* L. and *C. canephora* Pierre) in Brazil (Oliveira *et al.*, 1999; Silva *et al.*, 2004; Kubo *et al.*, 2004; Machado *et al.*, 2006). In the present article, we describe the development and validation of a species-specific reverse primer based on ITS-1 region rDNA for the diagnosis of *P. brachyurus*.

MATERIALS AND METHODS

Nematodes

Root and soil samples were collected from a range of crops and localities in Brazil (Tables 1 and 2). *Pratylenchus* species, including 29 *P. brachyurus* populations, *P. coffeae*, *P. zeae*, and *P. jaehni*, were identified from geographically disparate regions of Brazil, and one *P. penetrans* population was obtained from The Netherlands (Tables 1 and 2). Also, *Radopholus similis* and a distinct nematode community comprised of a range of trophic groups as defined by Yates *et al.* (1993) including bacteriophagous, predatory and other plant-parasitic forms that typically occur in Brazilian soils, were sampled (Table 2). Nematodes were extracted from roots and soil samples as described by Coolen and D'Herde (1972) and Jenkins (1964), respectively. The specimens used for molecular studies were handpicked and placed directly into 1M NaCl.

DNA Extraction

DNA from an individual female from several *Pratylenchus* species and populations of *P. brachyurus* (Tables 1 and 2) was extracted using a modified version of the method described by Stanton *et al.* (1998). In a slide containing a drop of 20 µl 0.25M NaOH, individual nematodes were cut in three parts under the stereoscope, placed into separate 0.6 ml micro-centrifuge tubes and incubated at 25°C overnight. Thereafter, samples were incubated at 99°C for 3 min and 10 µl 0.25 M HCl, 5 µl 0.5 M Tris-HCl (pH 8.0), and 5 µl 2% Triton X-100 were added to each tube. Samples were incubated at 99°C for a further 3 min, cooled and stored at -20°C.

PCR Amplification

One Ready-to-Go PCR bead (GE Healthcare Biosciences, Little Chalfont, UK) was placed into a 0.6 ml micro-centrifuge tube and 22.5 µl distilled water, 0.5 µl template DNA from a representative population of the target species (Table 1) or non target species (Table 2) and 1 µl of each 10 µM primer were added. PCR products were generated using the primers 18S (5'-TTGATTACGTCCCTGCCC-TTT-3') and RN58SR (5'-ACGAGCCGA-GTGATCCACCG-3') located respectively in the 18S and 5.8S rDNA (Boutsika, 2002). PCR conditions were as follows: 94°C for 2 min 45 s, then 40 cycles of the following: 94°C for 1 min, 57°C for 45 s, and 72°C for 2 min. The final extension phase was 72°C for 10 min. PCR products were separated on a 0.8% agarose gel and visualized by staining with ethidium bromide. PCR products of sufficiently high quality were purified for sequencing using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Biosciences, Little Chalfont, UK) according to the manufacturer's protocol.

Sequencing

Purified DNA from *P. brachyurus* populations Pb03, Pb23 and Pb24 was sequenced directly in both directions using the PCR primers and the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK), according to the instructions listed by the manufacturer. For each sequencing reaction the following reagents were added to a 0.6 ml micro-centrifuge tube: 4 µl Terminator Ready Reaction Mix, 1 µl primer (3.2 µM) and 3 µl purified DNA. The sequencing was performed on an ABI 377 DNA sequencer and the quality of the sequences produced was checked using Bioedit Software (Hall, 1999).

Design and Test of Primers

CLUSTAL X v. 1.81 (Thompson *et al.*, 1997) was used to generate a multiple sequence alignment with default settings. A species-specific reverse primer for *P. brachyurus* was designed based on an alignment of sequences generated in this study (Pb03, Pb23 and Pb24 populations) with sequences of *P. brachyurus* (PbJp), *P. coffeae* (PcJp), *P. zeae* (PzJp) and *P. vulnus* (PvIaJp, PvIbJp, and PvSJp) from Japan (Uehara *et al.*, 1999). Potential species-specific reverse primers for *P. brachyurus* were identified using PRIMER3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) and used in combination with a universal forward primer, 18S, located in the 18S rDNA. Primer combinations were initially tested against representative nematode populations that provided the original template DNA for sequencing (Pb03, Pb23 and Pb24), and also to all other *P. brachyurus* populations listed in Table 1. After initial screening, specificity was determined by challenging the selected primer combination with DNA of non-target *Pratylenchus* and other nematode species, including

Table 1. *Pratylenchus brachyurus* populations used to determine the reliability of the species-specific primer ACM7R. The populations used for sequencing of the ITS-1 are marked with an asterisk.

Population code	Host	Locality (City—State)
Pb01	Cotton (*Gossypium hirsutum*)	Rio Verde—GO
Pb02	Soybean (*Glycine max*)	Palmital—SP
Pb03*	Maize (*Zea mays*)	São Roque de Minas—MG
Pb04	Soybean (*G. max*) maintained on carrot disks	Campo Florido—MG
Pb05	Maize (*Z. mays*)	Piracicaba—SP
Pb06	Cotton (*G. hirsutum*)	Piracicaba—SP
Pb07	Cotton (*G. hirsutum*)	Adamantina—SP
Pb08	Cotton (*G. hirsutum*)	Birigui—SP
Pb09	Cotton (*G. hirsutum*)	Caiabu—SP
Pb11	Cotton (*G. hirsutum*)	Campinas—SP
Pb12	Soybean (*G. max*)	Correntina—BA
Pb13	*Brachiaria* sp.	Correntina—BA
Pb14	*Cassia occidentalis*	Luis Ed. Magalhães—BA
Pb15	Soybean (*G. max*)	Barreiras—BA
Pb16	Cotton (*G. hirsutum*)	Barreiras—BA
Pb17	Peanut (*Arachis hypogaea*)	Ribas do Rio Pardo—MS
Pb18	Soybean (*G. max*)	Ribas do Rio Pardo—MS
Pb19	Soybean (*G. max*)	Balsas—MA
Pb20	Okra (*Abelmoschus esculentus*)	Seropédica—RJ
Pb21	Cotton (*G. hirsutum*)	Serra do Ramalho—BA
Pb22	Cotton (*G. hirsutum*)	Campo Verde—MT
Pb23*	Cotton (*G. hirsutum*)	Sapezal—MT
Pb24*	Cotton (*G. hirsutum*)	Itiquira—MT
Pb25	Maize (*Z. mays*)	Dom Aquino—MT
Pb26	Soybean (*G. max*)	Dom Aquino—MT
Pb27	Cotton (*G. hirsutum*)	Leme—SP
Pb28	Cotton (*G. hirsutum*)	Guará—SP
Pb29	Soybean (*G. max*)	Ribeiro Gonçalves—PI
Pb30	*Psidium* sp.	Pelotas—RS

P. coffeae, *P. zeae*, *P. jaehni*, *P. penetrans*, *R. similis*, and a distinct nematode community (Table 2). A negative control without DNA template was also included.

RESULTS

PCR with the universal primers 18S and RN58SR successfully amplified DNA from all *P. brachyurus* populations, the four species of *Pratylenchus* (*P. coffeae*, *P. jaehni*, *P. zeae*, *P. penetrans*) and from *R. similis* yielding PCR products of approximately 500 bp in length. After extensive screening (data not shown), one species-specific reverse primer, ACM7R (5'-GCWCCATC-CAAACAAYGAG-3'), located in the ITS-1 region (Fig. 1), was found to consistently and reliably detect the target species, *P. brachyurus*, yielding products of 267 bp, when used together with the universal primer 18S (Fig. 2).

Table 2. Non-target species used for primer specificity studies.

Species	Host	Locality (City—State)
Pratylenchus coffeae (M2)	*Aglaonema* sp.	Rio de Janeiro—RJ
P. jaehni	*Citrus* sp.	Conchal—SP
P. zeae	Sugarcane (*Saccharum officinarum*)	Jaboticabal—SP
P. penetrans	*Lilium* sp.	The Netherlands
Radopholus similis	Banana (*Musa* sp.)	Piracicaba—SP
Community	Natural vegetation	Piracicaba—SP

Primer specificity was demonstrated *in silico* and *in vitro*. The *in silico* analysis was performed after alignment using Bioedit Software (Hall, 1999) of ITS-1 sequences of three non-target *Pratylenchus* species from Japan (Uehara *et al.*, 1999) and those pro-

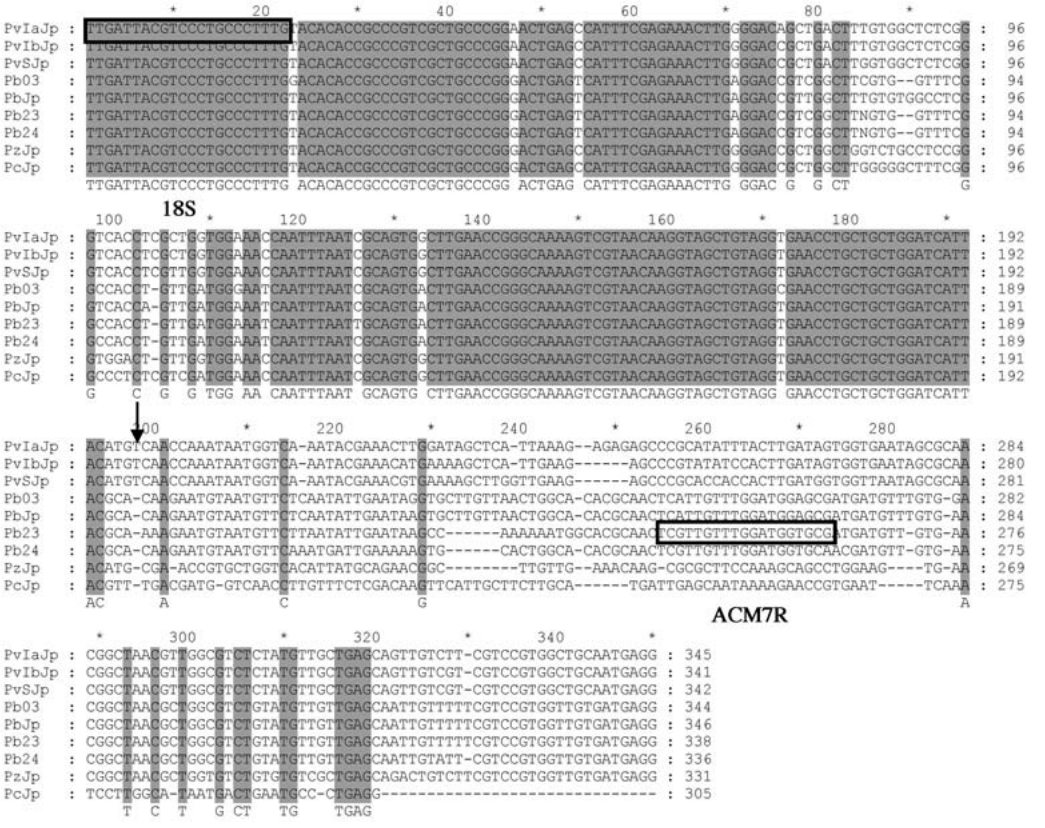

Fig. 1. Sequence alignment of partial 18S and ITS-1 regions from *Pratylenchus brachyurus* (Pb03, Pb23, Pb24, and PbJp), *P. vulnus* (PvIaJp, PvIbJp, and PvSJp), *P. coffeae* (PcJp), and *P. zeae* (PzJp). Dashes indicate gaps and grey boxes indicate the areas of homology between sequences. Arrow indicates the beginning of the ITS-1 region and boxes the location of primers 18S (nucleotides 1 to 22) and ACM7R (nucleotides 255 to 273).

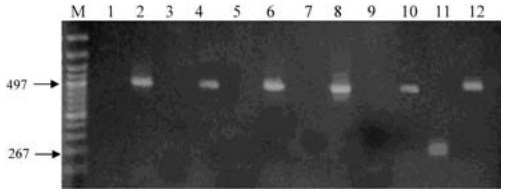

Fig. 2. Primer specificity tests. Eletrophoresis of DNA fragments amplified from single individuals of: Lanes 1) and 2) *Pratylenchus zeae*, 3) and 4) *P. coffeae*, 5) and 6) *P. jaehni*, 7) and 8) *P. penetrans*, 9) and 10) *Radopholus similis*, 11) and 12) *P. brachyurus* (Pb30), using the forward primer 18S coupled with the following reverse primers located in the ITS-1 region: 3, 5, 7, 9, and 11 (specific reverse primer ACM7R for *P. brachyurus*), and 2, 4, 6, 8, 10, and 12 (reverse primer RN58SR). M = Gene ruler 100 bp DNA Ladder Plus (Fermentas, Hanover, MD). DNA from each non-target species was checked with universal primers 18S and RN58SR to confirm successful DNA extraction (lanes 2, 4, 6, 8, 10, and 12).

vided on the present study (Pb03, Pb23 and Pb24, GenBank accession numbers EF693895-EF693897). This analysis confirmed that the species-specific primer sequence for *P. brachyurus* was not present on *P. coffeae*, *P. vulnus* and *P. zeae* and thus, PCR was unlikely to yield non-specific amplification products (Fig. 1). *In vitro* screening of the primer targeted for *P. brachyurus* with a range of non-target *Pratylenchus* species and *R. similis* demonstrated specificity by yielding the expected PCR product only for *P. brachyurus* (Fig. 2). Primer reliability was confirmed by screening 29 distinct geographic populations of *P. brachyurus*, obtaining the expected PCR product for all of them (Figs. 3 and 4). No unspecified PCR product was observed when the species-specific primer ACM7R was used with DNA extracted from the total nematode community. On the contrary, when a common DNA extraction was carried out with the nematode community and one specimen of *P. brachyurus* from the population Pb30, the specific band was clearly visible (Fig. 4).

DISCUSSION

Morphometric characters are not always reliable as primary characters for *Pratylenchus* species identification, as showed by

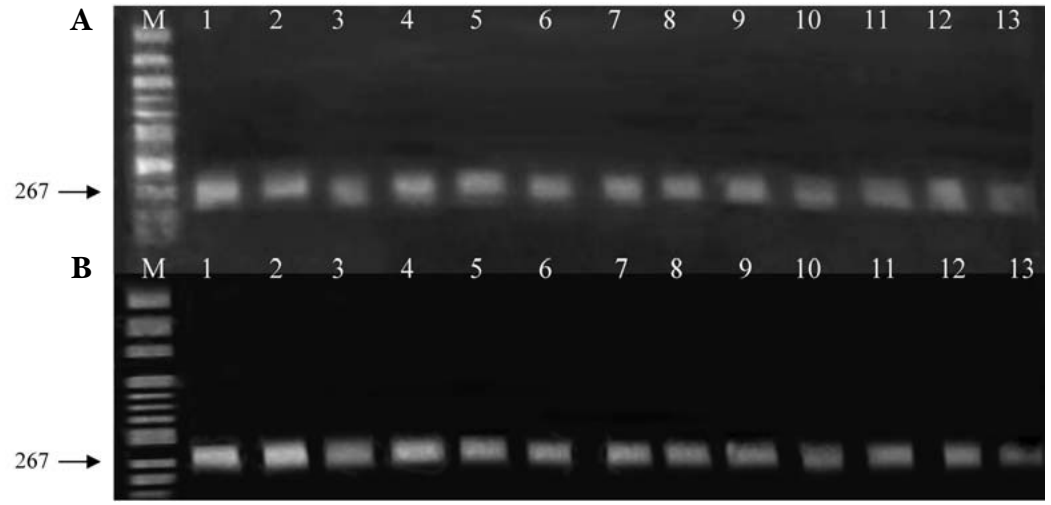

Fig. 3. Eletrophoresis of the amplified products obtained from DNA isolated from 26 populations of *P. brachyurus* (Table 1) using a combination of species-specific primer ACM7R and the universal primer 18S. A) M = Gene ruler 100 bp DNA Ladder Plus (Fermentas, Hanover, MD), lanes 1 to 13: Pb01 to Pb14. B) M = Gene ruler 100 bp DNA Ladder Plus (Fermentas, Hanover, MD), lanes 1 to 13: Pb15 to Pb27.

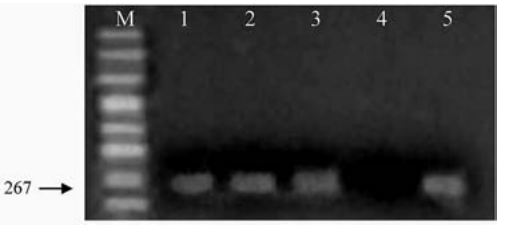

Fig. 4. Eletrophoresis of the amplified products obtained from DNA isolated from 3 populations of *P. brachyurus* (Table 1) using a combination of species-specific primer ACM7R and the universal primer 18S. M = Gene ruler 100 bp DNA Ladder Plus (Fermentas, Hanover, MD), lanes 1 to 3: Pb28 to Pb30; lane 4: community without *P. brachyurus*; lane 5: community with one individual of *P. brachyurus* (Pb30).

Doucet *et al.* (1998; 2001), Townshend (1991), and Al-Banna *et al.* (2004). Accurate identification of agronomically important nematodes is particularly relevant to ongoing work on the development of resistant cultivars and rootstocks (Al-Banna *et al.*, 2004). Variability in the ITS regions has previously been utilized for molecular diagnostics of several genera of plant-parasitic nematodes, including *Heterodera* (Subbotin *et al.*, 1999), *Longidorus* and *Paralongidorus* (Hübshen *et al.*, 2004a), *Meloidogyne* (Zijlstra *et al.*, 1995), *Pratylenchus* (Uehara *et al.*, 1998), and *Xiphinema* (Oliveira *et al.*, 2005). These PCR assays based on specific primers are straightforward, since only agarose gel eletrophoresis is required after DNA amplification. Moreover, as only one major band is obtained, specific primer sets constitute practical and rapid diagnostic tools that can be used for large number of samples.

Currently, several single-step diagnostics for *Pratylenchus* species are available, but there was no specific primer set for *P. brachyurus*. Uehara *et al.* (1998) developed specific primers for the identification of *P. coffeae* and *P. loosi*, based on sequence differences in the ITS region of the rDNA. Al-Banna *et al.* (2004) designed five forward species-specific primers from the internal variable portion of the D3 expansion region of the 26S rDNA for *P. neglectus*, *P. penetrans*, *P. scribneri*, *P. thornei*, and *P. vulnus*. However, due to limited sequence variation showed by *P. brachyurus* it was not possible to develop a specific primer for this species. Recently, Carrasco-Ballesteros *et al.* (2007) designed longer primers to amplify the sequence-characterized amplified region (SCAR) specific to *P. thornei* from DNA extracts obtained from different life stages of the nematode.

Here, we followed the criteria of Hübschen *et al.* (2004b) to demonstrate using both *in silico* and *in vitro* tests that the diagnostic primer designed for *P. brachyurus* was specific for the target species among other *Pratylenchus* and related (*R. similis*) species. Primer specificity was demonstrated by the detection of a single target nematode species within a total soil community comprised of non-target species, as well as producing no cross-reaction products derived from non-target *Pratylenchus* species. Additionally, primer reliability was confirmed by screening a number of distinct geographic populations of *P. brachyurus*. Hübschen *et al.* (2004b) argued that the majority of published nematode diagnostic primers are of doubtful utility due to the lack of strict validation assessments. In the present work, the primer developed has been addressed by the validation protocol established by Hübschen *et al.* (2004b).

Testing diagnostic primers should also, where possible, be applied to target organisms from geographically disparate locations (Oliveira *et al.*, 2005). While in this study all populations were from a single country, Brazil, the geographical distance between some populations (e.g., *P. brachyurus* Pb19 and Pb30) is >3000 km, i.e. the distance between Caracas (Venezuela), in Latin America, to New York (USA), in North America. However, it is recognized

that the evaluation of more populations from disparate areas would be desirable.

Given its specificity, sensitivity and reliability, this diagnostic primer should be of great benefit to phytosanitary services related to agronomic crops and also as a decision management tool for growers.

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