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Molecular identification of root-lesion nematodes, *Pratylenchus* spp. in agricultural crops from Costa Rica[1]

Identificación molecular de nematodos lesionadores de raíz, *Pratylenchus* spp., en cultivos agrícolas de Costa Rica

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Abstract

Introduction. The root-lesion nematodes, *Pratylenchus* spp., have a wide host range and reduce the yield of different crops. Information on the diversity of *Pratylenchus* species is scarce in Costa Rica. **Objective.** To identify the *Pratylenchus* species associated with 12 crops based on the D3 region of the 28S rDNA gene. **Materials and methods.** During 2013 to 2015, root samples were collected in Alajuela, Cartago, Guanacaste, Heredia, and San José in crops of rice (*Oryza sativa*), black pepper (*Piper nigrum*), sugarcane (*Saccharum officinarum*), aster (*Aster* sp.), coffee (*Coffea arabica*), banana (*Musa paradisiaca*), lily (*Lilium* sp.), gypsophila (*Gypsophila* sp.), onion (Allium cepa), potato (*Solanum tuberosum*), strawberry (*Fragaria* x *ananassa*), and leather-leaf fern (*Rumohra adiantiformis*). The D3 region of the 28S rDNA gene from each population was amplified and sequenced. A GenBank Blast Search was performed for each sequence. The phylogenetic relationships were established by Bayesian Inference. **Results.** Blast Search indicated the presence of *P. pseudocoffeae* in aster, *P. brachyurus* in black pepper, *P. crenatus* in onion and potato, *P. hippeastri* and *P. gutierrezi* in sugarcane and coffee, respectively. *Pratylenchus bolivianus* in leather-leaf fern and potato, *P. penetrans* in onion, strawberry, gypsophila, and lily, *P. zeae* in rice and sugarcane, while P. speijeri in banana. The phylogenetic analysis corroborated the *Pratylenchus* species identity with exceptions of sequences from 1) banana, grouped to *P. coffeae* complex group, 2) sugar cane, grouped to *P. hippeastri* complex group 3) onion and potato were related with *P. crenatus*, in an independent group, and 4) leather-leaf fern and potato were grouped with *P. bolivianus* with low resolution. **Conclusions.** Nine genetic groups of *Pratylenchus* were found, some of those should be verified with other molecular markers to get a conclusive identification.

Keywords: 28S gene, ribosomal DNA, PCR, DNA sequence.

Resumen

Introducción. Los nematodos lesionadores, *Pratylenchus* spp., poseen un amplio rango de hospederos y reducen los rendimientos de diferentes cultivos. La información sobre la diversidad de especies de *Pratylenchus* en Costa Rica es escasa. **Objetivo.** Identificar las especies de *Pratylenchus* asociadas a 12 cultivos con base en la región D3 del gen 28SrDNA. **Materiales y métodos.** Durante el 2013 al 2015, se recolectaron muestras de raíces en Alajuela, Cartago, Guanacaste, Heredia y San José en cultivos de arroz (*Oryza sativa*), pimienta negra (*Piper nigrum*), caña de azúcar (*Saccharum officinarum*), aster (*Aster* sp.), café (*Coffea arabica*), banano (*Musa paradisiaca*), lirio (*Lilium* sp.), gysophila (*Gypsophila* sp.), cebolla (*Allium cepa*), papa (*Solanum tuberosum*), fresa (*Fragaria* x *ananassa*) y helecho de hoja de cuero (*Rumohra adiantiformis*). Se amplificó y secuenció la región D3 del gen 28S del ADNr de cada población. Se realizó una búsqueda en el Gen Bank con un "Blast Search" para cada secuencia. Las relaciones filogenéticas se establecieron mediante inferencia bayesiana. **Resultados.** "Blast Search" indicó la presencia de *P. pseudocoffeae* en aster, *P. brachyurus* en pimienta negra, *P. crenatus* en cebolla y papa, *P. hippeastri* y *P. gutierrezi* en caña de azúcar y café, respectivamente. *P. bolivianus* en helecho hoja de cuero y papa, *P. penetrans* en cebolla, fresa, gypsophila y lirio, *P. zeae* en arroz y caña de azúcar, mientras que *P. speijeri* en banano. El análisis filogenético corroboró la identidad de especies de Pratylenchus con excepciones de secuencias de: 1) banano, agrupada al grupo complejo *P. coffeae*, 2) caña de azúcar al grupo complejo *P. hippeastri* 3) cebolla y papa relacionados a *P. crenatus*, en un grupo independiente y 4) helecho de hoja de cuero y papa se agruparon con *P. bolivianus* con baja resolución. **Conclusiones.** Se encontraron nueve grupos genéticos de *Pratylenchus*, algunos de los cuales deben verificarse con otros marcadores moleculares para lograr una identificación concluyente.

Palabras clave: gen 28S, ADN ribosomal, PCR, secuencia de ADN.

Introduction

Plant-parasitic nematodes (PPN) cause crop losses estimated at 12.3 % worldwide, equivalent to 173 US billion dollars (Kumar et al., 2020). One of the major groups of PPN is root-lesion nematode (*Pratylenchus* spp.), which ranks third as having a great economic impact on crops worldwide (Jones et al., 2013). The first description of the genus was made in 1880 by De Man, who originally called the nematode *Tylenchulus pratensis* (Castillo & Vovlas, 2007). The current name, *Pratylenchus*, was given later in 1936 by Filipjev, with the description of the characteristics that make this genus distinguishable from others (Filipjev, 1936).

Pratylenchus is a migratory endoparasite globally distributed that has been reported in more than 400 hosts (Castillo & Vovlas, 2007; Handoo et al., 2008). It is known as "root-lession nematode" because of the damage caused when enters the roots, where it generates hollow channels when moving and feeding inside the root system (Piedrahita et al., 2012). The number of legitimate *Pratylenchus* species is still in debate, but it is estimated with around 103 species (Nguyen et al., 2019).

Species identification is valuable for adequate integrated nematode management (Al-Banna et al., 2004; Mokrini et al., 2019), e.g., to enforce quarantine measures to prevent the entry of species that could have detrimental effects on crops or to avoid the rejection of agricultural exports (Blok, 2005). Additionally, species identification is essential to implement effective nematode management strategies in response to the ban of several nematicides from the market. Among the management alternatives to chemical control, are the use of resistant varieties and crop rotation which requires the identification at the species level (Moura Cintra Goulart, 2008; Starr et al., 2002).

Many studies involve the use of a taxonomic polyphasic approach (morphological, morphometric, biochemical, and molecular methods) in the characterization of *Pratylenchus* species (Castillo & Vovlas, 2007).

However, the identification of the genus *Pratylenchus* is complex due to the number of validated species and the variation within the same species (Nguyen et al., 2019). The morphological identification of *Pratylenchus* is challenging for diagnostics because of the presence of cryptic species (Bogale et al., 2021) that make some of the species identifiable only by DNA analysis (Singh et al., 2020). Molecular tools for species identification can help to overcome these problems and have become essential for accurate classification (De Luca et al., 2012; Janssen, Karssen, Couvreur, et al., 2017; Janssen, Karssen, Orlando, et al., 2017; Palomares-Rius et al., 2014). Besides, more reliable results are obtained in less time with molecular techniques than other methods (Donn et al., 2008). Usually, molecular tools reduce the time for species identification, increase precision, and require less experience (Starr et al., 2002).

Molecular studies of *Pratylenchus* have been done extensively by the analysis of ITS rDNA, 18S rDNA, D2-D3 of the 28S rDNA and mitochondrial genes (Castillo & Vovlas, 2007; De Luca et al., 2012; Janssen, Karssen, Couvreur et al., 2017; Orui & Mizukubo, 1999). The region D2-D3 of the 28S rDNA of *Pratylenchus* has been defined as a better molecular marker to identify species within the genus compared to 18S rDNA (Subbotin et al., 2008). Inside the genome region, the D3 subunit fragment of the 28S-rDNA gene has been widely used for the molecular identification of *Pratylenchus* species (Al-Banna et al., 1997; 2004; De Luca et al., 2004; Hodda et al., 2014).

In Costa Rica, the *Pratylenchus* species characterized initially, based on morphological methods were *P. zeae*, *P. brachyurus*, *P. coffeae*, and *P. penetrans* (González-Fernández, 1979; Guzmán-Hernández et al., 2011; López et al., 1987; López & Salazar, 1990). Later on, *P. coffeae* and *P. brachyurus* were analyzed and identified with molecular techniques (Humphreys-Pereira et al., 2017), and *P. bolivianus*, *P. gutierrezi*, *P. pseudocoffeae*, and *P. zeae* with both morphological and molecular methods (Zamora-Araya et al., 2016). Current information about the *Pratylenchus* species in Costa Rica is scarce. In this research, we conducted a *Pratylenchus* survey in different agricultural crops in Costa Rica by the selection of molecular tools. Pitfalls of the molecular-exclusive approach were decreased by the selection of at least one accession per *Pratylenchus* species from a per-review publication containing information related to the morphological identification of the species. The objective of this study was to identify the *Pratylenchus* species associated with 12 crops based on the D3 segment of the 28S rDNA gene.

Materials and methods

Nematode populations and hosts

Roots samples from 12 crops were collected, during 2013 to 2015, from five provinces of Costa Rica (Table 1). Three root subsamples (10 plants each) were collected with a shovel from each location site in a systematic pattern in zigzag, from 0 to 30 cm depth. Crops and localities were selected based on samples detected as positives for *Pratylenchus* spp. when the producers provide them to the Nematology Laboratory of the Universidad de Costa Rica. The number of sequences analyzed per location varied from 2 to 13 (Table 1). The distribution of the *Pratylenchus* populations used in this study is shown in Figure 1.

Sample preparation

Nematode extractions were done in the Laboratory of Nematology from the Crop Protection Research Center (CIPROC) from the Agronomy School at the Universidad de Costa Rica, San Pedro, San José. Plant roots were washed, cut into 1-3 cm-long pieces and 10 g were selected randomly for processing by flotation-centrifugation method (Caveness & Jensen, 1955) with modifications from the Nematology Laboratory from the Universidad de Costa Rica. The roots were washed and blended for 20 s, the solution was poured into 100 over 500 sieves and

Table 1. *Pratylenchus* spp. populations collected in five provinces of Costa Rica. Laboratory of Nematology, Crop Protection Research Center, Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Cuadro 1. Poblaciones de *Pratylenchus* spp. colectadas en cinco provincias de Costa Rica. Laboratorio de Nematología, Centro de Investigación en Protección de Cultivos, Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Population code	Crop	Locality	Number of sequences
PA	Rice (*Oryza sativa*)	Quebradón, Upala, Alajuela	10
PB	Banana (*Musa paradisiaca*)	Sarapiquí, Heredia	10
PC1	Coffee (*Coffea arabica*)	Navarro, Orosi, Cartago	11
PC2	Coffee	San Marcos, Tarrazú, San José	2
PE1	Onion (*Allium cepa*)	San Juan de Chicuá, Cartago	12
PE2	Onion	Pacayas, Cartago	3
PF	Strawberry (*Fragaria x ananassa*)	Llano Grande, Cartago	6
PG	*Gypsophila* spp.	Llano Grande, Cartago	5
PH	Leather-leaf fern (*Rumohra adiantiformis*)	Birrisito, Cartago	10
PL	Lily (*Lilium* sp.)	Heredia	7
PM	Black pepper (*Piper nigrum*)	Muelle, San Carlos	3
PÑ1	Sugarcane (*Saccharum officinarum*)	Grecia, Alajuela	11
PÑ2	Sugarcane	Cañas, Guanacaste	7
PP	Potato (*Solanum tuberosum*)	San Juan de Chicuá, Cartago	12
PP-E	Potato-Onion	Pacayas, Cartago	7
PS	Aster (*Aster* sp.)	Puriscal, San José	13

was collected in a 50 mL conical tube (Eppendorf). It was centrifuge at 2800 rpm for 3 min. Supernatant was discarded and a sugar solution with a specific gravity of 1,18 was added into the tube. The tube with the sugar solution was agitated and centrifugated at 2800 rpm for 3 min. This was poured into a 500-mesh sieve and washed with abundant water. The remnant was verted into plastic 50 mm Petri dishes (Thermo Fisher Scientific) for the posterior identification. Nematodes were first identified at the genus level using a stereomicroscope Nikon model SMZ754T. The genus was differentiated by the flat cephalic structure, short and wide stylet, ventral overlap with the intestine, in females the vulva is located at 70-85 % of the body length, conical tail rounded in the terminus. Males has terminal bursa, *Pratylenchus* individuals at the adult stage were picked using a metal surgical needle and placed in a Petri dish with distilled water for DNA extraction.

DNA extraction and PCR amplification

The DNA extraction and PCR amplification were done in the Biotechnology Laboratory of the Agronomic Research Center (CIA) from the Agronomy School at the Universidad de Costa Rica, San Pedro, San José. Five nematodes, per sample, were placed in a coverslip with a drop of Worm Lysis Buffer (WLB, 50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM $MgCl_2$, 0.45 % Tween 20) (Madani et al., 2005) + 100 μg mL^{-1} proteinase K (20 mg mL^{-1}); Thermo Fisher Scientific). Each nematode was cut with a scalpel into three pieces and the five nematodes per sample were transferred into PCR tubes containing 5 μL of Worm Lysis Buffer + proteinase K. Samples were incubated at 80 °C for 30 min, 65 ºC for 1 h, followed by a proteinase K inactivation step at 95 ºC for 15 min. Primers D3A (5´-GACCCGTCTTGAAACACGGA-3´) and D3B (5´-TCGGAAGGAACCAGCTACTA-3´) (Al-

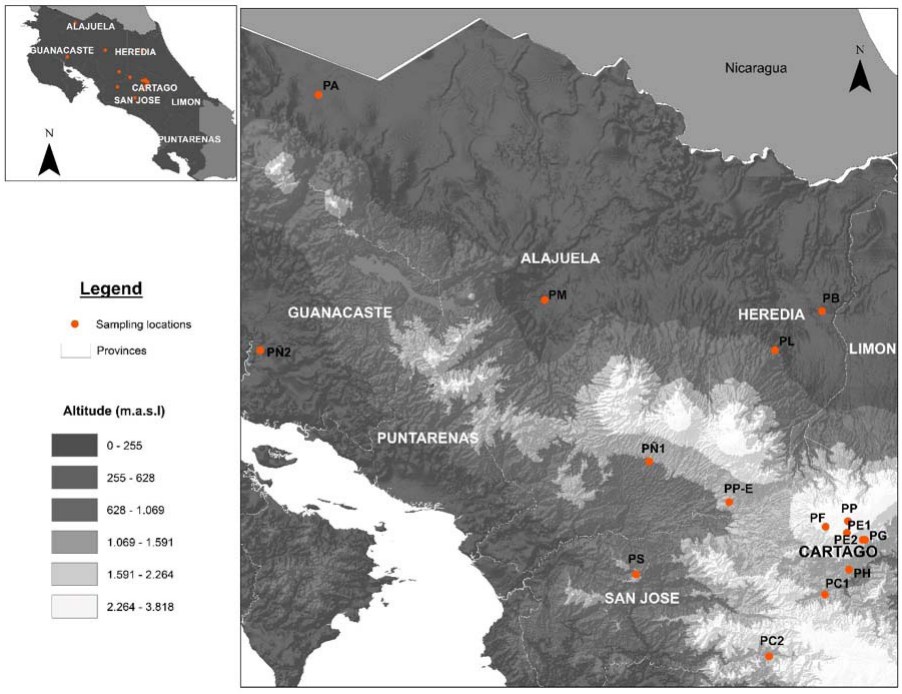

Figure 1. Geographic localization of the *Pratylenchus* populations collected in five provinces of Costa Rica. Laboratory of Nematology, Crop Protection Research Center, Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

PA: rice (*Oryza sativa*), PB: banana (*Musa paradisiaca*); PC1 and PC2: coffee; PE1 and PE2: onion; PF: strawberry (*Fragaria x ananassa*), PG: gypsophila, PL: lily, PÑ1 and PÑ2: sugarcane (*Saccharum officinarum*); PM: black pepper (*Piper nigrum*); PS: aster (*Aster* sp.); PH: leather-leaf fern (*Rumohra adiantiformis*); PP: potato (*Solanum tuberosum*).

Figura 1. Localización geográfica de las poblaciones de *Pratylenchus* colectadas en cinco provincias de Costa Rica. Laboratorio de Nematología, Centro de Investigación en Protección de Cultivos, Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

PA: arroz, PB: banano; PC1 y PC2: café; PE1 y PE2: cebolla; PF: fresa, PG: gyphsophila, PL: lily, PÑ1 y PÑ2: caña de azúcar; PM: pimienta negra; PD: áster; PH: helecho de hoja de cuero; PP: papa.

Banna et al., 1997) were used to amplify the D3 region of the 28S rDNA. PCR was carried out in 25 μL as follows: 2.5 μL of Dream Taq Buffer 10X, 1μL of dNTP´s (2 mM), 2 μL of each primer (10 μM), 1 μL of BSA (20 mg mL^{-1}), 1.5 mM of $MgCl_2$, 0.5 μL of Dream Taq (5 U μL^{-1}, Thermo Fisher Scientific), and 5 μL of the DNA extract.

The amplification conditions consisted of an initial denaturation at 92 ºC for 5 min, 40 cycles at 94 ºC for 30 s, 55 ºC for 45 s, 72 ºC for 1 min, and a final extension at 72 ºC for 7 min. The PCR products were visualized by electrophoresis in 1.6 % agarose gel (1.6 g of agarose in 100 mL of TRIS-Borate-EDTA 0.5X buffer). PCR products were stained with GelRedTM (Biotium). PCR products were purified with a NucleoSpin® gel and PCR Clean-up Kit (Macherey-Nagel) and sequenced bidirectionally by Macrogen (Seoul, South Korea). The resulting sequences were edited using BioEdit (Hall, 1999) and compared with other sequences of *Pratylenchus* from the GenBank by BLAST Search (National Center for Biotechnology Information, NCBI, USA).

Phylogenetic analysis

The phylogenetic analysis of the D3 sequences of *Pratylenchus* spp. generated in this study and sequences recovered from the GenBank was estimated with the Bayesian Inference (BI) analysis with the program MrBayes 3.2 (Ronquist & Huelsenbeck, 2003). The MUSCLE algorithm (Edgar, 2004) implemented in Mega 6 (Tamura et al., 2013) was used for the sequence alignment. The best DNA model of evolution (K80 + G) was selected on JmodelTest (Darriba et al., 2012; Guindon & Gascuel, 2003) with the BIC criterion. The phylogenetic tree was visualized on FigTree 1.4.3 (Rambaut, 2012). At least one sequence from previous peer-review publications were included per *Pratylenchus* species used for the phylogenetic analysis (Table 2).

Table 2. GenBank accessions used for the phylogenetic analysis. Laboratory of Nematology, Crop Protection Research Center, Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Cuadro 2. Accesiones del GeneBank usadas para el análisis filogenético, Laboratorio de Nematología, Centro de Investigación en Protección de Cultivos, Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Species	Accession	Source	Species	Accession	Source
Zygotylenchus guevarai	JQ917439	Unpublished	*Pratylenchus hippeastri*	DQ498829	Inserra et al. (2007)
Pratylenchus bolivianus	KP780255	Unpublished		DQ498831	Inserra et al. (2007)
	KP780256	Unpublished		GU214113	De Luca et al. (2010)
	KT971354	Zamora Araya et al. (2016)		KJ001720	Unpublished
Pratylenchus brachyurus	EU130842	Subbotin et al. (2008)	*Pratylenchus loosi*	AF170439	Duncan et al. (1999)
	HQ662580	Unpublished		EF446992	Hajieghrari et al. (2007)
	KF712473	Unpublished	*Pratylenchus neglectus*	AJ545024	De Luca et al. (2004)
	MH018683	Unpublished		AJ545027	De Luca et al. (2004)
Pratylenchus brzeskii	AM231921	de la Pena et al. (2006)	*Pratylenchus parafloridensis*	AF170438	De Luca et al. (2010)
	AM231924	de la Pena et al. (2006)		GU214115	De Luca et al. (2010)
Pratylenchus coffeae	AF170429	Duncan et al. (1999)	*Pratylenchus penetrans*	EU130863	Subbotin et al. (2008)
	AF170431	Duncan et al. (1999)		EU1308659	Subbotin et al. (2008)
	AF170434	Duncan et al. (1999)		JX046996	Wang et al. (2012)
	KX792100	Humphreys-Pereira et al. (2017)		JX047000	Wang et al. (2012)
	KX792101	Humphreys-Pereira et al. (2017)		KX792098	Humphreys-Pereira et al. (2017)
Pratylenchus crenatus	EU130852	Subbotin et al. (2008)	*Pratylenchus pratensis*	AM231929	de la Pena et al. (2006)
	EU130853	Subbotin et al. (2008)		AM231935	de la Pena et al. (2006)
Pratylenchus dunensis	AM231938	de la Pena et al. (2006)	*Pratylenchus speijeri*	KF974715	Unpublished
	AM231943	de la Pena et al. (2006)		KY424295	Unpublished
Pratylenchus floridensis	AF170437	Duncan et al. (1999)	*Pratylenchus zeae*	EU130889	Subbotin et al. (2008)
	GU214117	De Luca et al. (2010)		EU130893	Subbotin et al. (2008)
Pratylenchus gutierrezi	AF170442.1	Duncan et al. (1999)		EU130894	Subbotin et al. (2008)
	KT971355	Zamora Araya et al. (2016)		KT971361	Zamora Araya et al. (2016)
	KX792096	Humphreys-Pereira et al. (2017)		KX792099	Humphreys-Pereira et al. (2017)
	KX792097	Humphreys-Pereira et al. (2017)			

Results

Molecular identification and phylogenetic analyses

The resulted D3 segment amplification products from all *Pratylenchus* DNA samples were ca.300 bp (Figure 2). The similarity percentage between sequences obtained in this study and sequences retrieved from the GenBank are shown in Table 3. The phylogenetic relationships based on the D3 region included an alignment of 74 *Pratylenchus* sequences and one sequence from *Zygotylenchus guevarai* sequence as an outgroup (Figure 3). Most of the D3 *Pratylenchus* sequences generated in this study were positioned on highly supported clades (Bayesian Posterior Probability, BPP>90). The tree topology showed in a basal position, a large monophyletic group formed with *P. zeae* accessions and *Pratylenchus* sequences obtained from rice (PA) and sugar cane (PÑ2) (BPP=100). *Pratylenchus* sequences from onion (PE1), potato (PP02), and from a field with both potato and onion (PP-E) were positioned in a clade with *P. crenatus*, with a high support value (BPP=100), but *P. crenatus* formed an independent group (BPP=98%).

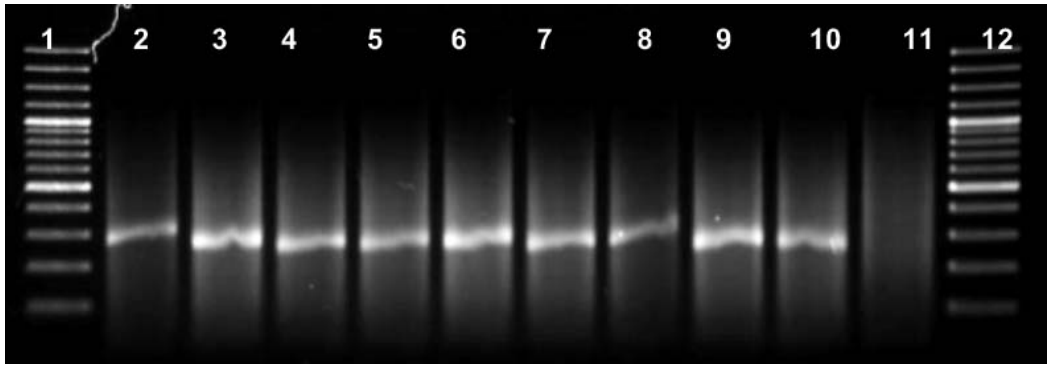

Figure 2. *Pratylenchus* spp. rDNA amplification of a segment of the D3 region of the 28S gene. Well 1 and 12: molecular marker 100 bp. Well 2 to 10: amplification using the primers D3A and D3B. Well 11: negative control. Laboratory of Nematology, Crop Protection Research Center, Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Figura 2. Amplificación de un segmento de la region D3 del gen 28S del ADNr de *Pratylenchus* spp. Pozo 1 y 12: marcador molecular 100 pb. Pozo 2 a 10: amplificación del con los primers D3A y D3B. Pozo 11: control negative. Laborario de Nematología, Centro de Investigación en Protección de Cultivos, Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

The *Pratylenchus* sequence from black pepper formed a clade with sequences of *P. brachyurus* retrieved from the GenBank (BPP=100). Two sister groups both with high support values were formed with sequences of *P. penetrans* from the GenBank and sequences from onion (PE2), lily (PL), strawberry (PF), and *Gypsophila* (PG) for group one (BPP=100), and two sequences of *P. dunensis* for group two (BPP=93). The *Pratylenchus* sequences from coffee PC1 and PC2 were grouped with *P. gutierrezi* (BPP=100), whereas the *Pratylenchus* sequences from aster (PS01 and PS02) were grouped with sequences from *P. pseudocoffeae* (BPP=100). The *Pratylenchus* DNA samples from leather-leaf fern, one from potato, banana, and one from sugarcane did not show a good resolution.

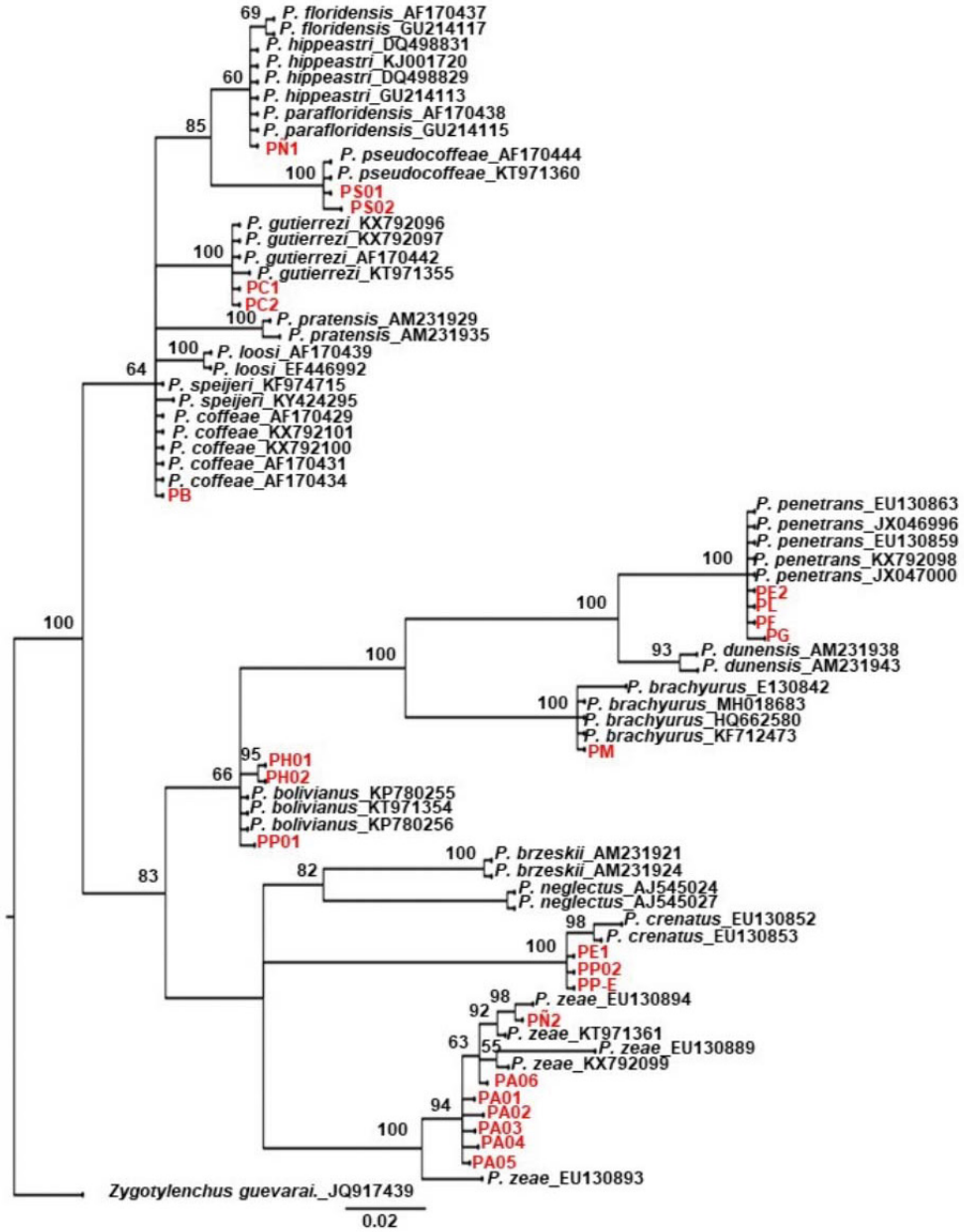

Figure 3. Phylogenetic relationships within the genus *Pratylenchus* as inferred from the Bayesian analysis of the D3 region of the 28S rRNA gene using the GTR+ I+ G model. Laboratory of Nematology, Crop Protection Research Center, Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Figura 3. Relaciones filogenéticas dentro del género *Pratylenchus* de acuerdo con el análisis Bayesiano de la región D3 del gen 28SrRNA con el modelo GTR+ I+ G Laboratorio de Nematología, Centro de Investigación en Protección de Cultivos, Escuela de Agronomía, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Table 3. Similarity of the *Pratylenchus* sequences generated in this study, with the GenBank accessions based on the D3 region of 28S rDNA gene and number of haplotypes per population. Laboratory of Nematology, Crop Protection Research Center, Agronomy School, Universidad de Costa Rica. San Pedro, San José, Costa Rica. 2013-2015.

Cuadro 3. Similitud de las secuencias de *Pratylenchus* generadas en este estudio, con las accesiones del GeneBank, basados en la región D3 del gen 28S ADNr y número de haplotipos por población Laboratorio de Nematología, Centro de Investigación en Protección de Cultivos, Escuela de Agronomía, Universidad de Costa Rica. San Pedro. San José, Costa Rica. 2013-2015.

Crop	Code	*Pratylenchus* species	Haplotypes		Identity (%)	Matched accession from the GenBank
Aster	PS	*P. pseudocoffeae*	2	H1 H2	100 99.60	KT971360, KT175533, KT175531 KT971360, KT175533, KT175531
Banana	PB	*P. speijeri*	1	H1	99.7 - 100	KJ698686, KY424295, KF974715
Black pepper	PM	*P. brachyurus*	1	H1	100	MH018563, KF712473, HQ662580
Coffee	PC1 PC2	*P. gutierrezi*	1 1	H1 H1	100	KX792097, KX792096, AF170442
Gypsophila	PG	*P. penetrans*	1	H1	99.60	EU13086
Leather- leaf fern	PH	*P. bolivianus*	2	H1	99.60	LT985478, KU198960
Lily	PL	*P. penetrans*	1	H1	100	MT528233, MN251259, JQ003984
Onion	PE1	*P. crenatus*	1	H1	99.6 - 100	KY468856, KM580544, KY560461
	PE2	*P. penetrans*	1	H1	100	EU130863, JX47000, JX046996
Potato	PP-1 PP-2	*P. bolivianus* *P. crenatus*	1 1	H1 H1	99.60 100	MG871467, KT971354, KU198960 KY468856, KY468848
Potato/Onion	PP-E	*P. crenatus*	1	H1	100	KY468856, KY468848, KY468844
Rice	PA	*P. zeae*	6	H1	98 - 99	KX792099, AF303950, KY424266
				H2	98.30	KY424266, KX792099, KU198950
				H3	98.30	KX792099, KY424266, KU198950
				H4	99	KX792099, KU198950, EU130894
				H5	98.6 - 99.3	KX792099, KU198950, EU130894
				H6	99 - 99.6	KX792099, KU198950, EU130894
Sugar Cane	PÑ1 PÑ2	*P. hippeastri* *P. zeae*	1 1	H1 H1	100 99.60	LT965044, MH324473 KX792099, KU198950
Strawberry	PF	*P. penetrans*	1	H1	100	MT528233, MT528232, MT528231

Discussion

The molecular analysis of *Pratylenchus* sequences based on the D3 region of the 28S rDNA gene allowed the identification of nine different genetic groups. Morphological and morphometric methods of nematodes identification should complement the molecular methods for accurate identification of these organisms, but in genera like *Pratylenchus*, some species can only be distinguished based on molecular identification (De Luca et al., 2012; Palomares-Rius et al., 2014; Troccoli et al., 2021; Wang et al., 2015). In this research it was corroborated that it was possible to identified different *Pratylenchus* phylogenetic groups using the molecular approach. However,

some of the genetic groups identified need to be validated using a polyphasic approach. The phylogenetic tree recovered in this research for the D3 region presented a similar topology to that published by Subbotin et al. (2008) for the D2-D3 28S rDNA gene region. The same authors mentioned that despite some exceptions the topology recovered from the larger fragment D2-D3 region of the 28S rDNA is similar to the D3 region topology.

The root-lesion nematode, *P. zeae* was found in rice from Upala, Alajuela, and sugarcane from Cañas, Guanacaste. Previously, an identification of this nematode with morphological techniques in four regions of Costa Rica: Atlantic, North Pacific (Guanacaste), Central Pacific, and Southeast was made (López et al., 1987). The results from this research could have implications for nematode management because both crops are common in Guanacaste. Therefore, the presence of this crops permits the reproduction and survival of the nematode species and might reduce the positive impact of the sugar cane/rice rotation system (Nzogela et al., 2020; Santos et al., 2012). *P. zeae* has been associated with significant yield reduction in sugarcane and rice crops (Namu et al., 2018; Nzogela et al., 2020; Singh et al., 2020). The genetic heterogeneity shown within the *P. zeae* populations from Costa Rica is consistent with previous reports (Carta et al., 2001; Mwamula et al., 2020; Subbotin et al., 2008), and supports that *P. zeae* could be a species complex. In other taxa, cryptic species have been proposed within *P. parazeae*, *P. coffeae*, and *P. hippeastri* (De Luca et al., 2010; 2012; Inserra et al., 2007; Palomares-Rius et al., 2014; Wang et al., 2015).

The D3 segment did not provide a good resolution for the identification of the *Pratylenchus* species associated with sugarcane in Grecia, Alajuela. Even though Blast Search identified the sample as *P. hippeastri* with 100 % similarity, the phylogenetic analysis placed this population within a clade formed by *P. hippeastri*, *P. floridensis*, and *P. parafloridensis*. The last two species are considered cryptic species within the *P. hippeastri* group. The use of a larger fragment as the D2-D3 region for the characterization of *Pratylenchus* species, due to the D2 segment variability was recomend by Subbotin et al. (2008). The D3 segment allowed the identification of the root-lesion nematode. *P. hippeastri* was confirmed in the central region of Costa Rica in strawberry (Brenes-Campos et al., 2022).

P. pseudocoffeae was associated with aster plants in Puriscal. Previously, this nematode was found in chrysanthemum from Heredia (Zamora-Araya et al., 2016). Worldwide, *P. pseudocoffeae* has been documented in ornamental plants as chrysanthemum, artemisia, aster, and grasses (Inserra et al., 1998; Uesugi et al., 2012). Pathogenicity assays should be performed to evaluate the host susceptibility of *P. pseudocoffeae* in aster and determine the level of damage.

The *Pratylenchus* sample extracted from banana was grouped in a large clade (with a poor resolution, BPP=64) with species considered members of the *P. coffeae* species complex, such as *P. speijeri* and *P. coffeae*. These two species are considered cryptic species (De Luca et al., 2012; Palomares-Rius et al., 2014), and their separation as different species is unreliable using morphological and morphometric methods (De Luca et al., 2012). *Pratylenchus speijeri* was described and isolated from *Musa* in Ghana (De Luca et al., 2012) and weeds (*Rottboellia cochinchinensis*, *Panicum maximum*, *Acalypha ciliata*, *Sida acuta*, *Brachiaria deflex*, and *Feuria aestuans*) on previously planted fields with banana in the same country (Brentu et al., 2013). In Costa Rica, there is little information related to the *Pratylenchus* species affecting the banana crop. The presence of *P. coffeae* and other *Pratylenchus* spp. have been reported but no molecular characterization was provided (Fernández Solano & Quesada Solís, 2013).

In the present study, *P. gutierrezi* was found in two locations affecting coffee, Orosí, Cartago, and Tarrazú, San José. The root-lesion nematode *P. gutierrezi* was initially reported on coffee in San Antonio, Cinco Esquinas, Alajuela (Morgan Golden et al., 1992; Zamora Araya et al., 2016), then found on yampee (*Dioscorea trifida*) in the south region of Costa Rica (Humphreys-Pereira et al., 2017). As the nematode was identified in two new locations, caution must be taken with the movement of infected seedling material from one locality to another, to avoid further nematode dissemination.

The root-lesion nematode, *P. penetrans* was the species most frequently found in this study, associated with four (onion, strawberry, gypsophila, and lily) out of 12 crops, collected from the provinces of Cartago and Heredia. *Pratylenchus* penetrans has a wide host range (Bélair et al., 2007; Castillo & Vovlas, 2007), and previous reports

from other countries showed the synergistic relationship between this species and pathogenic fungi and bacteria (Figueiredo et al., 2021; LaMondia, 2003). Therefore, studies to determine the damage caused by *Pratylenchus* species to different crops in Costa Rica are essential, as well as the identification of other hosts to establish accurate management strategies against *Pratylenchu*s plant-parasitic nematode.

Despite the low resolution of the phylogenetic analysis with the *Pratylenchus* samples obtained from leather-leaf fern (Birrisito, Cartago) and potato (San Juan de Chicua, Cartago), the blast search for both samples resulted in the highest similarity values with *P. bolivianus* (100 % identity). Two root-lesion nematodes species had been identified on leather-leaf fern in Costa Rica, *P. penetrans* in Coris, Cartago (López & Salazar, 1990), and *P. bolivianus* in San Isidro, Heredia (Zamora-Araya et al., 2016). Further studies on nematode surveys in these crops and molecular species identification should be performed to determine this species distribution in Cartago and in other parts of the country.

Some sequences of *Pratylenchus* spp. collected from potato and onion were grouped in a clade with sequences of *P. crenatus*. A query in the GenBank showed the highest similarity to sequences of *P. crenatus* (100 % identity). This nematode has been reported on potatoes in several countries such as Belgium (Pelsmaeker & Coomans, 1987), United States (Florini et al., 1987; Wheeler et al., 1994), Canada (Kimpinski & Smith, 1988; Olthof et al., 1982), and in onions from New Zealand (Knight et al., 1997). Crop rotation between potato and onion is common in the northern area of Cartago, which might be favoring an increase in the nematode population density. The characterization of this nematode population using more regions of the genome and morphology, pathogenicity assays, and the screening for tolerant varieties should be a priority since both crops are essential for the economy of northern Cartago.

The nematode *P. brachyurus* was found associated with black pepper from Muelle, San Carlos, Alajuela. No previous reports were found of this nematode on black pepper in Costa Rica. This species was identified using morphological methods in pineapple from Pital, San Carlos (López & Salazar, 1990). Later, Humphreys-Pereira et al. (2017) using the D3 region identified *P. brachyurus* associated with yellow yam (Los Chiles, Alajuela), yampee (Los Chiles, Alajuela and Sabalito, Coto Brus), and pineapple (Upala, Alajuela). These results could indicate that this species could have a wide distribution in Alajuela province. *P. brachyurus* is an important specie in yield reduction in different crops in South Africa as maize and cassava (Coyne et al., 2018). Therefore, further pathogenicity analysis in the country should be done.

The results from this research increase knowledge on the status of *Pratylenchus* species in the country. It is important to extend sampling to other provinces of the country and in different plant hosts, as well as to explore other ecological conditions. This information is valuable for the establishment of alternative control strategies.

Conclusions

A total of nine *Pratylenchus* species were identified in 12 crops using the D3 region of the 28S gene. These results reflect the great diversity of *Pratylenchus* in the country and establish a starting point for future research on the management of these nematodes.

In the Chorotega region of Costa Rica, the species *P. zeae* was found associated with sugarcane and rice.

In the Atlantic region, a species from the *P. coffeae* complex group and highly similar to *P. speijeri* was identified on banana, whereas in the Northern region of the country, *P. brachyurus* was found for the first time in black pepper.

In the Central region, *P. pseudocoffeae* was found associated for the first time with aster, *P. gutierrezi* in coffee, a species highly similar with *P. crenatus* in onion and potato, *P. penetrans* in onion, strawberry, gypsophila, and lily,

P. bolivianus with leather-leaf fern and potato, and a species from the *P. hippeastri* complex group in sugarcane. The species *P. zeae*, *P. penetrans*, and *P. gutierrezi* appear to have a wide geographic distribution in the country.

The morphological, morphometric, and the amplification of more regions of the genome could help to have more robust results and it is suggested for further investigations.

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