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Root-lesion nematodes of the genus *Pratylenchus* (Nematoda: Pratylenchidae) from Costa Rica with molecular identification of *P. gutierrezi* and *P. panamaensis* topotypes

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Abstract Root-lesion nematodes of the genus *Pratylenchus* are among the most damaging nematodes for crop and ornamental plants worldwide. There are about 75 described species in this genus with a wide host range, but a difficult diagnosis because of morphological similarities and overlapping of morphometric characters among species. Four species of *Pratylenchus* were detected parasitizing cultivated and ornamental plants in Costa Rica: *Pratylenchus bolivianus, P. gutierrezi*, *P. pseudocoffeae*, and *P. zeae*; while *P. panamaensis* was detected in coffee in the type locality at Panama. The specimens were identified using morphological and molecular methods. Morphometrics and morphology using light and scanning electron microscopy are given for all of them. The presence of *P. bolivianus* and *P. pseudocoffeae* are reported for the first time in Costa Rica. The molecular and morphological variability associated with former populations of *P. gutierrezi* showed that we are in front of complex of species. This has been resolved with the study of topotype populations of *P. gutierrezi* and *P. panamaensis* combined with rDNA sequences (D2-D3 expansion segments of 28S and ITS). Our results, including SEM *en face* view data on topotypes of *P. gutierrezi* and *P. panamaensis* demonstrated that both species are almost undistinguishable morphologically, but clearly separated molecularly with D2-D3 expansion segments of 28S (89 % similar, 84 nucleotides and 19 indels) and ITS rDNA (only 36 % coverage with a 88 % similarity value). Consequently, *P. gutierrezi* and *P. panamaensis* need to be maintained as different valid taxa. Molecular data on D2-D3 expansion segments of 28S and ITS rDNA of topotypes of *P. panamaensis* were also coincident (99 % similarity) with root-lesion nematodes parasitizing coffee in Guatemala and Costa Rica, and banana in Madeira, and previously identified as *P. gutierrezi* (K1-AF170440, K2-AF170441, FR692277, respectively), which need to be considered conspecific with *P. panamaensis.* These results showed us the necessity of integrative taxonomy in the identification and characterization of *Pratylenchus* spp.

Keywords Bayesian inference · Chrysanthemum · Coffee · Fern · Panama · Phylogeny · rDNA · Rice · Root-lesion · Taxonomy

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Introduction

Root-lesion nematodes of the genus *Pratylenchus* are recognized as one of the major nematological constrains in crops of primary economic importance, mainly for their crop losses, wide host range and the worldwide distribution for some species (Castillo and Vovlas 2007). All members of the genus are endoparasitic migratory cortical feeders, causing severe root damage to their hosts, including ornamentals (Castillo and Vovlas 2007; Deimi et al. 2009). The genus *Pratylenchus* Filipjev 1936 comprises 75 nominal species distributed in almost every cool, temperate and tropical environment that increasing its phytopathological importance (Castillo and Vovlas 2007; Troccoli et al. 2008; Munera et al. 2009; De Luca et al. 2010; Palomares-Rius et al. 2010; De Luca et al. 2012; Palomares-Rius et al. 2014b; Wang et al. 2015).

The morphological diagnosis of some root-lesion nematode species into the genus *Pratylenchus* has not clarified their species status since morphological convergence or morphometric ranges overlapping for some characters among related species (Castillo and Vovlas 2007). This is the case of *Pratylenchus pseudocoffeae* Mizukubo 1992 which shares several morphologic-morphometric characters with other amphimictic *Pratylenchus* spp. such as *Pratylenchus coffeae* (Zimmermann 1898) Filipjev and Schuurmans Stekhoven 1941, *Pratylenchus gutierrezi* Golden et al. 1992, and *Pratylenchus penetrans* (Cobb 1917) Filipjev and Schuurmans Stekhoven 1941. This fact has prompted nematologists to discover alternate methods and features for more accurate identification, including scanning electron microscopy (SEM) and the use of polytomous key integrated with molecular analyses (Corbett and Clark 1983; Castillo and Vovlas 2007; Palomares-Rius et al. 2014b). Sequence analyses of nuclear ribosomal RNA genes have been used for molecular characterisation and reconstruction of phylogenetic relationships of *Pratylenchus* spp. (Al-Banna et al. 1997; Duncan et al. 1999; Carta et al. 2001; Al-Banna et al. 2004; De Luca et al. 2004; Subbotin et al. 2008; De Luca et al. 2011; Palomares-Rius et al. 2010, 2014b). These studies have resolved the taxonomical status of a large number of root-lesion nematodes, although many species have yet to be characterised molecularly. The integration of molecular tools with morphological studies of this genus could provide new insights into the diversity and unequivocal identification of the target species.

During the rainy seasons from 2013 to 2015 severe rhizosphere soil infestations and feeder root infections from several cultivated and ornamental plants by *Pratylenchus* spp. were detected in Alajuela and Heredia provinces of Costa Rica. Preliminary morphological observations indicated that the root-lesion nematode species detected appeared to be morphologically related to *P. gutierrezi* Golden et al. 1992, *Pratylenchus panamaensis* Siddiqi et al. 1992 (the former species previously considered synonym of the latter), *P. pseudocoffeae* Mizukubo 1992, *Pratylenchus zeae* Graham 1951, and another unidentified species, a fact which prompted us to undertake a detailed morphological and molecular comparative study with previous reported data.

Pratylenchus gutierrezi described from coffee (*Coffea arabica* L.) at San Antonio, Alajuela Province, Costa Rica (Golden et al. 1992), and *P. pseudocoffeae* described from mum (*Chrysanthemum morifolium* Ramat) and artemisia (*Artemisia feddei* Lev. and Van.) in Japan, have amphimictic individuals with two lip annuli and medial (subdorsal and subventral fused together) lip sectors divided from lateral lip sectors (Inserra et al. 1998). Inserra et al. (1998) determined the morphological variability of *P. gutierrezi* populations originating from coffee in Costa Rica and Guatemala differing in body length. *Pratylenchus gutierrezi* was compared and differentiated from *Pratylenchus flakkensis* Seinhorst 1968, *Pratylenchus gibbicaudatus* Minagawa 1982 and *Pratylenchus neglectus* (Rensch 1924) Filipjev and Schuurmans Stekhoven 1941 in its original description (Golden et al. 1992). However, *P. gutierrezi* was not compared with the almost contemporaneous species *P. panamaensis*. For this reason, Siddiqi (2000), after comparison of both species, did not find significant differences between them and the former was considered a junior synonym of the latter. Castillo and Vovlas (2007) and Handoo et al. (2008) maintained this action and therefore *P. gutierrezi* was considered as synonym of *P. panamaensis*. However, DNA sequences or the arrangement of lip pattern of topotype specimens of *P. panamaensis* were not available to validate the synonym of both species at the time, being both species morphologically almost identical (most probably cryptic). The unresolved morphological characterisation of *Pratylenchus* populations molecularly homogeneous parasitizing coffee in Costa Rica and Guatemala (Inserra et al. 1998; Duncan et al. 1999; Subbotin et al. 2008) emphasised the need to obtain

morphological and molecular data of topotype specimens of *P. panamaensis* from Panama to be used in diagnostic works for comparison with other populations of this species from coffee growing areas of Central America. Taxonomic controversial was maintained, since the morphology and D2-D3 sequence of a *P. gutierrezi* topotype population (K3, AF170442) revealed the likelihood that it is not conspecific with two other undescribed species of root-lesion nematodes (isolates K1 and K2, erroneously identified as *P. gutierrezi* in GenBank, AF170440, AF170441, respectively) with divided faces from coffee from Costa Rica and Guatemala, respectively) (Duncan et al. 1999). In order to reach this objective a study was conducted in the type locality of Boquete, Chiriquí Province, Panama to determine integrative morphological, morphometric and molecular characterization, allowing synonymizing or separating both species conclusively.

Therefore, the objectives of the present study were: (i) to provide an accurate identification of the root-lesion nematode species infecting cultivated and ornamental plants in Costa Rica, including the characterisation of a population of *P. gutierrezi* from San Antonio, Alajuela province (type locality) and *P. panamaensis* from Boquete, Chiriquí Province, Panama (type locality), both infecting coffee; (ii) to characterise molecularly the root-lesion nematode populations using the D2-D3 expansion segments of 28S rDNA and ITS rDNA; and (iii) to explore the phylogenetic relationships of these root-lesion nematode populations within *Pratylenchidae* spp.

Material and methods

Nematode population sampling

Nematode surveys were conducted from 2013 to 2015 during the rainy seasons in cultivated (coffee and rice) and ornamental plants (mum and fern) in Costa Rica and Panama (Table 1). Each soil and root sample was a composite of 20–25 soil cores arbitrarily chosen from the same field to a depth of 25–30 cm with an Oakfield tube of 2.5-cm diameter. Samples were placed in labelled plastic bags, sealed and brought back to the nematology laboratory where they were stored at 4 °C until processed for nematode extraction. Specimens of *P. gutierrezi* from San Antonio, Alajuela province, Costa Rica (type locality) were characterized morphological and molecularly to be used in diagnostic works for comparison with those of other populations of this species or with topotypes of *P. panamaensis* from Boquete, Chiriquí Province, Panama. Nematodes were extracted from 500 cm^3 of soil by centrifugal flotation (Coolen 1979) method. In some cases, additional soil samples were collected afterwards from the same locality for completing the necessary specimens for morphological and/or molecular identification. Root samples were homogenate with a blender and tissue suspension was then sieved through a 250-μm pore sieve over a 5-μm pore sieve. Nematodes and root debris retained on the 5-μm pore sieve were separated by centrifuging at 1100 × *g* for 5 min in a magnesium sulphate solution of 1,16 specific gravity (Coolen 1979).

Nematode morphological identification

Specimens for light microscopy were killed by gentle heat, fixed in a solution of 4 % formaldehyde + 1 % propionic acid and processed to pure glycerine using Seinhorst's method (1966). Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to 1,000× magnification. Measurements and drawings were made at the *camera lucida* on glycerine infiltrated specimens. All measurements were expressed in micrometers (μm). All other abbreviations used are as defined in Siddiqi (2000).

For SEM studies, fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold and observed with a Carl Zeiss Merlin Field Emission Scanning Electron Microscope (FE-SEM) (Abolafia et al. 2002).

Nematode molecular identification

For molecular analyses, two live nematodes from each sample were temporary mounted in a drop of 1 M NaCl containing glass beads and after taking measurements and photomicrographs of diagnostic characters the slides were dismantled and DNA extracted. Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (2003). The D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (Nunn 1992). The ITS region was amplified using forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′)

Table 1 Root-lesion nematode populations sampled for *Pratylenchus* and sequences used in this study from Costa Rica and Panama

Species	Locality	Host	GenBank accessions	
			D2-D3	ITS
Pratylenchus bolivianus	San Isidro, Heredia Province (Costa Rica)	fern (*Rumohra adiantiformis*)	KT971354	KT971362
Pratylenchus gutierrezi	San Antonio, Alajuela Province, Costa Rica (type locality)	coffee (*Coffea arabica*)	KT971355	KT971363
Pratylenchus gutierrezi	Cinco Esquinas, Alajuela Province (Costa Rica)	coffee (*Coffea arabica*)	KT971356 KT971357	KT971364
Pratylenchus panamaensis	Boquete, Chiriquí Province, Panama (type locality)	coffee (*Coffea arabica*)	KT971358 KT971359	KT971365 KT971366
Pratylenchus pseudocoffeae	San Isidro, Heredia Province (Costa Rica)	*Chrysanthemum* sp.	KT971360	KT971367
Pratylenchus zeae	San Carlos, Alajuela Province, Costa Rica	rice (*Oryza sativa*)	KT971361	–

(−) Not obtained

and reverse primer AB28 (5′-ATATGCTTAA-GTTCAGCGGGT-3′) as described in Curran et al. (1994).

PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on Table 1 and in the phylogenetic trees.

Phylogenetic analyses

D2-D3 and ITS rDNA sequences of different *Pratylenchus* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa used in D2-D3 expansion segments of 28S rDNA were the same as used in Subbotin et al. (2008) and Palomares-Rius et al. (2014b). *Nacobbus aberrans* was used as outgroup for ITS phylogeny, which need to be restricted to the available sequences of *Pratylenchus* with homology to the studied species in this research. Multiple alignments of the different genes were made using the Q-INS-i algorithm of MAFFT v. 7.205 (Katoh and Standley 2013) which accounts for secondary RNA structure. Sequence alignments were manually edited using BioEdit (Hall 1999). Percentage similarity between sequences was calculated using the sequence identity matrix using BioEdit. For that, the score for each pair of sequences was compared directly and all indel or place-holding characters were treated as an indel. When position of both sequences have an indel they do not contribute (they are not an identity, they simply do not exist). Phylogenetic analyses of the sequence data sets were performed based on Bayesian inference (BI) using MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). The best fitted model of DNA evolution was obtained using JMODELTEST v. 2.1.7 (Darriba et al. 2012) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analysis under a transversional of invariable sites and a gamma-shaped distribution (TVM + I+ G) model for D2-D3 expansion segments of 28S rRNA and a general time reversible of invariable sites and a gamma-shaped distribution (GTR + I+ G) model for ITS region, were initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 2×10^6 and 1×10^6 generations, respectively. The MCMC were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50 % majority rule consensus tree.

Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996).

Results

Field symptoms and nematode infections

Cultivated coffee and rice, and ornamental plants (mum and fern) in commercial fields at several localities of Costa Rica and Boquete in Panama infected by root-lesion nematodes showed some decline with stunting, and necrotic lesions on the root system. Population density in naturally infested soil of the sampled plants ranged from 100 to 200 nematodes/100 mL of soil, and from 250 to 600 nematodes/g of infected fresh root. Moreover nematode populations of *P. panamaensis* from coffee in Panama ranged from 2 to 8 nematodes/100 mL of soil, and from 0.08 to 0.12 nematodes/g of infected fresh root.

Morphological and morphometrical study

The morphological and morphometrical data as well as molecular delineation of the root-lesion nematodes detected in cultivated (coffee and rice) and ornamental plants (mum and fern) in Costa Rica and coffee at Boquete, Panama, were previously compared with original descriptions and rDNA sequence analysis within preceding studies on root-lesion nematodes (Castillo and Vovlas 2007; Mizukubo et al. 2007; Subbotin et al. 2008; Deimi et al. 2009; Palomares-Rius et al. 2010, 2014b). The integrative approaches identified these species as *P. bolivianus*, *P. gutierrezi*, *P. panamaensis P. pseudocoffeae*, and *P. zeae* (Tables 1, 2, 3, 4 and 5). For these species, a brief description and a morphometric comparison with previous records are provided below. Similarly, in Table 3 is reported the matrix code for the species detected in ornamental and cultivated plants in Costa Rica and coffee in Boquete, Panama, according to the polytomous tabular key proposed by Castillo and Vovlas (2007).

***Pratylenchus bolivianus* Corbett 1983**

(Figs. 1, 2, Table 2)

The Costa Rican population of this amphimictic species from the rhizosphere and roots of fern was characterised by a body almost straight, lip region not offset from body, bearing three annuli (Figs. 1, 2). SEM *en face* view pattern showing an oval oral aperture centrally located in labial disc and surrounded by six (three on each side) inner labial sensilla, with no division between sub-median and lateral segments, presumably due to fusion of the first lip annulus to the oral disc (Fig. 2a, e). This lip pattern configuration is in agreement with Group 2 according to classification scheme of Corbett and Clark (1983). Amphidial apertures oval-shaped are obliquely orientated (Fig. 2a, e). Stylet is rather long, robust; stylet knobs with forward pointing anteriorly margins. Lateral fields with four equidistant lines, outer bands partially areolated under SEM but difficult to detect under LM, occupying almost one third of body diam. at mid-body (Fig. 2b, f–i, n–q). Pharyngeal overlapping length is 38.1 ± 1.3 (36.5–39.5) μm. Excretory pore located anterior to level of cardia. Spermatheca rounded to spherical, with sperm; post-vulval uterine sac 31 ± 2.8 (25.0–38.0) μm long or usually one vulval body diam. Tail conical with 19–25 annuli, and coarse annulation around terminus (Fig. 2b, k, q). Phasmids pore-like, centred in lateral fields and located near mid-tail (Fig. 2b).

Males less abundant than female (ratio = 1: 2) and morphologically similar except for sexual dimorphism. Testis outstretched anteriorly. Tail conoid, enveloped by crenate-edged bursa, tip narrow and pointed. Spicules are ventrally arcuate with a gubernaculum small and curve. Phasmids open on bursa, generally anterior to mid-tail.

The morphology and morphometric of this population agree closely with the original description of the species (including SEM studies) by Corbett (1983) and other descriptions (Valenzuela and Raski 1985), except for slightly longer body length (560–744 *vs* 530–720 μm), a slightly higher c ratio (15–25 *vs* 16–22), which may be because of geographical intraspecific variability (Corbett 1983; Valenzuela and Raski 1985; Castillo and Vovlas 2007).

This species has been reported in the type locality of Toralapa, Cochabamba Valley, Bolivia (Corbett 1983), Orange Bay, Hardy Peninsula, Chile (Valenzuela and Raski 1985), The Netherlands (Amsing 1996), in USA (Lehman 2002, reported as *P. australis*), in UK (Cotten et al. 1991; Waeyenberge et al. 2000), Chile (De Luca et al. 2011) and China (Wang & Peng unpublished, Wang et al. unpublished). Up to our knowledge this is the first report from Costa Rica. According to the polytomous key by Castillo and Vovlas (2007), the Costa Rican lesion nematode

Table 2 Morphometrics of *Pratylenchus bolivianus* Corbett 1983 from fern in Costa Rica. All measurements in μm and in the format: mean ± s.d. (range)[a]

Character	*Pratylenchus bolivianus*	
	fern (*Rumohra adiantiformis*)	
	San Isidro, Heredia Province	
	Females	Males
n	20	10
L	650.4 ± 54.6 (560–744)	511.6 ± 76.7 (427–639)
a	24.3 ± 3.5 (16.5–31.8)	25.7 ± 3.1 (21.8–31.1)
b'	4.8 ± 0.9 (3.6–6.7)	3.5 ± 0.3 (3.1–3.9)
c	20.4 ± 2.7 (15.0–25.4)	19.0 ± 1.7 (16.1–21.5)
c'	2.1 ± 0.4 (1.6–3.1)	2.0 ± 0.2 (1.6–2.2)
V	82.1 ± 1.4 (79–84)	–
G_1	18.1 ± 2.6 (14.4–22.8)	–
Stylet length	17.8 ± 0.7 (17.0–19.0)	16.1 ± 0.9 (14.5–17.0)
m	48.4 ± 2.6 (44.1–52.4)	51.0 ± 4.4 (46.3–60.8)
O	17.0 ± 2.2 (13.0–21.4)	16.4 ± 3.8 (10.4–21.3)
Anterior end to excretory pore	96.3 ± 9.6 (69.0–108.0)	80.7 ± 9.6 (67.0–94.3)
MB	28.8 ± 2.1 (24.3–31.1)	29.6 ± 3.3 (25.0–34.4)
Pharynx length	141.1 ± 25.7 (105.0–198.0)	148.4 ± 8.6 (140–168)
Tail length	32.4 ± 4.5 (25–44)	27.4 ± 1.9 (24.2–29.7)
Spicules	–	16.9 ± 0.7 (16.0–18.0)
Gubernaculum	–	4.0 ± 1.1 (3.0–5.5)

[a] Abbreviations are defined in Siddiqi (2000)

parasitizing fern belongs to species with three lip annuli and *en face* view Group 2 according to classification scheme of Corbett and Clark (1983), and has the specific alphanumeric codes indicated in Table 3.

***Pratylenchus gutierrezi* Golden et al. 1992**
(Figs. 3, 4, Table 4)

The Costa Rican population of this amphimictic species from the rhizosphere and roots of coffee was characterised by a rounded lip region relatively low setoff from body contour by a small constriction, anteriorly flattened, and bearing two annuli (Fig. 3). SEM *en face* view pattern showing an oval oral aperture centrally located in labial disc and surrounded by six (three on each side) inner labial sensilla (Fig. 4a, f, j). Subdorsal and subventral sectors of first lip annulus similar in shape and clearly separated from lateral sectors and also separated from oral plate by a slight depression. This lip pattern configuration is in agreement with Group 2 according to classification scheme of Corbett and Clark (1983). Amphidial apertures are small, round to oval-shaped, located between labial plate and lateral sectors (Fig. 4a, f, j). Stylet is robust, with prominent rounded knobs. Lateral fields consisting of three bands delimitated by four lines, outer bands appearing irregularly areolated along body. Hemizonid prominent, 1–2 annuli anterior to excretory pore. Pharyngeal overlapping length rather long, 36.0 ± 9.7 (30.0–47.0) μm. Spermatheca round to spherical and filled with sperm, post-vulval uterine sac 26.7 ± 4.0 (22.5–36.0) μm long or *ca* two anal body width diam. Tail subcylindrical (Figs. 3g–j, 4o, p). Tail terminus round to bluntly rounded, coarsely annulated (Fig. 4b, o, p). Phasmids pore-like, centred in lateral fields and located slightly anterior mid-tail.

Males common but less abundant than female (ratio = 1: 2) and morphologically similar except for sexual dimorphism and smaller body size. Testis single and outstretched anteriorly. Tail conoid,

Table 3 Polytomous diagnostics of *Pratylenchus* spp. from ornamental and cultivated plants in Costa Rica

Species	Morphological characters[a]										
	$A_{1–3}$ Lip annuli	$B_{1–2}$ Presence of males	$C_{1–5}$ Stylet length (in μm)	$D_{1–4}$ Shape of spermatheca	$E_{1–4}$ Vulva position (V%)	$F_{1–6}$ PUS (in μm)	$G_{1–3}$ Female tail shape	$H_{1–4}$ Female tail tip	$I_{1–4}$ Pharyn-geal overlap (in μm)	$J_{1–3}$ Lateral field	$K_{1–2}$ Lateral field structures
coffeae (original description, coffee, Indonesia)	A1	B2	C2	D3	D3	F6	G2	H1	I2	J123	K1
bolivianus (fern, Dulce Nombre, San Isidro, Costa Rica)	A2	B2	C34	D2	E3	F34	G3	H2	I2	J1	K2
gutierrezi (topotypes, San Antonio, Alajuela, Costa Rica)	A1	B2	C3	D23	E23	F2	G2	H2	I3	J1	K1
gutierrezi (Cinco Esquinas, Costa Rica)	A1	B2	C3	D23	E23	F45	G2	H2	I23	J1	K2
panamaensis (coffee, topotypes, Panama)	A1	B2	C3	D3	E3	F2	G2	H2	I3	J1	K1
pseudocoffeae (chrysanthemum, San Isidro de Heredia, Costa Rica)	A1	B2	C23	D3	E3	F5	G2	H1	I4	J1	K2
zeae (rice, Altamira, Costa Rica)	A2	B1	C2	D2	E1	F5	G3	H3	I3	J1	K1

[a] Morphological characters according to Castillo and Vovlas (2007). Group A: 1 = two; 2 = three; 3 = four. Group B: 1 = absent; 2 = present. Group C: 1 = <13; 2 = 13–15.9; 3 = 16–17.9; 4 = 18–20; 5 = >20. Group D: 1 = absent or reduced; 2 = rounded to spherical; 3 = oval; 4 = rectangular. Group E: 1 = <75; 2 = 75–79.9; 3 = 80–85; 4 = >85. Group F: 1 = <16; 2 = 16–19.9; 3 = 20–24.9; 4 = 25–29.9; 5 = 30–35; 6 = >35. Group G: 1 = cylindrical; 2 = subcylindrical; 3 = conoid. Group H: 1 = smooth; 2 = striated; 3 = pointed; 4 = with ventral projection. Group I: 1 = <30; 2 = 30–39.9; 3 = 40–50; 4 = >50. Group J: 1 = four; 2 = five; 3 = six to eight. Group K: 1 = smooth bands; 2 = partially or completely areolated bands

Table 4 Morphometrics of *Pratylenchus* species from coffee plants of Costa Rica and Panamá. All measurements in μm and in the format: mean ± s.d. (range)[a]

Character	*P. gutierrezi*		*P. gutierrezi* (paratypes)		*P. panamaensis* (topotypes)	
	coffee (*Coffea arabica*)		coffee (*Coffea arabica*)		coffee (*Coffea arabica*)	
	Cinco Esquinas, Alajuela, Costa Rica		San Antonio, Alajuela, Costa Rica		Boquete, Chiriquí Province, Panama	
	Females	Males	Females	Males	Females	Males
n	20	10	30	20	10	10
L	573.3 ± 59.0 (452–692)	463.8 ± 48.1 (367–515)	500.1 ± 27.7 (430–552)	425.2 ± 20.8 (378–459)	417.7 ± 18.3 (389–446)	352.8 ± 32.1 (312–409)
a	19.7 ± 2.5 (16.0–26.7)	24.7 ± 3.4 (20.0–29.4)	19.8 ± 2.1 (15.0–24.9)	24.5 ± 3.1 (17.6–30.7)	24.2 ± 1.9 (21.9–27.5)	25.2 ± 3.2 (19.6–30.5)
b	–	–	–	–	5.7 ± 0.5 (4.9–6.5)	4.8 ± 0.8 (3.9–6.4)
b’	4.8 ± 1.1 (3.0–7.4)	3.4 ± 0.3 (3.1–4.1)	3.9 ± 0.3 (3.5–4.5)	3.8 ± 0.2 (3.4–4.1)	3.7 ± 0.2 (3.4–4.0)	3.2 ± 0.4 (2.6–3.9)
c	21.9 ± 2.2 (16.0–26.0)	19.1 ± 2.7 (13.6–22.3)	19.9 ± 2.6 (16.6–24.6)	21.5 ± 1.2 (19.5–24.2)	16.7 ± 1.0 (14.8–18.5)	17.7 ± 2.8 (14.7–23.7)
c’	1.6 ± 0.4 (1.8–2.3)	2.0 ± 0.2 (1.8–2.5)	–	–	2.3 ± 0.2 (2.0–2.6)	2.0 ± 0.2 (1.4–2.2)
V	80.8 ± 1.5 (78–84)	–	80.0 ± 2.3 (74.1–83.8)	–	79.1 ± 2.2 (76.1–84.1)	–
G_1	18.9 ± 4.2 (14.8–26.5)	–	–	–	34.2 ± 5.4 (24.6–40.0)	–
Stylet length	17.0 ± 0.7 (15.5–18.0)	15.3 ± 0.5 (14.5–16.0)	16.8 ± 0.5 (15.9–17.6)	15.3 ± 0.6 (13.8–16.2)	15.3 ± 0.2 (15.0–16.0)	14.0 ± 0.8 (12.0–15.0)
m	46.9 ± 3.1 (39.3–50.1)	46.3 ± 3.7 (41.0–51.7)	–	–	48.2 ± 2.1 (45.2–51.6)	48.7 ± 3.3 (41.1–52.4)
O	19.4 ± 4.0 (14.4–25.8)	19.4 ± 4.0 (11.4–25.3)	–	–	14.7 ± 2.2 (12.7–18.9)	17.5 ± 2.2 (14.9–21.1)
Anterior end to excretory pore	94.9 ± 18.8 (63–160)	76.8 ± 10.7 (57–89)	83.1 ± 4.1 (74.0–94.0)	78.9 ± 2.7 (76.5–84.0)	78.5 ± 4.2 (72.0–86.0)	69.6 ± 8.1 (56.0–85.0)
MB	35.5 ± 3.0 (30.8–40.0)	26.4 ± 2.6 (20.7–29.0)	–	–	35.5 ± 4.0 (30.3–40.9)	33.0 ± 5.0 (21.5–39.1)
Pharynx length	124.6 ± 25.2 (74–160)	130.2 ± 9.3 (112–146)	128.9 ± 6.2 (119–143)	113.3 ± 8.8 (104–132)	114.3 ± 2.9 (110–120)	118.8 ± 24.3 (104–186)
Tail length	26.4 ± 3.2 (19.0–31.0)	23.5 ± 2.0 (20.0–27.0)	25.4 ± 2.6 (21.1–29.2)	20.0 ± 1.3 (17.5–21.6)	25.0 ± 1.8 (22.0–29.0)	20.2 ± 1.9 (16.5–22.0)
Spicules	–	17.8 ± 1.8 (15.5–21.0)	–	16.8 ± 1.4 (15.5–21.0)	–	15.6 ± 1.7 (13.5–19.0)
Gubernaculum	–	3.6 ± 0.5 (3.0–4.5)	–	3.7 ± 0.4 (3.5–4.5)	–	3.3 ± 0.3 (3.0–4.0)

[a] Abbreviations are defined in Siddiqi (2000)

Table 5 Morphometrics of *Pratylenchus* species from cultivated and ornamental plants in Costa Rica. All measurements in μm and in the format: mean ± s.d. (range)[a]

Character	*P. pseudocoffeae* *Chrysanthemum* sp. San Isidro, Heredia Province Females	*P. zeae* rice (*Oryza sativa*) San Carlos, Alajuela Province Females
n	20	20
L	615.1 ± 66.2 (494–710)	504.0 ± 37.9 (434–556)
a	24.4 ± 2.3 (21.3–29.0)	25.8 ± 1.8 (23.1–29.8)
b’	4.3 ± 0.5 (3.7–6.0)	4.1 ± 0.5 (3.2–4.7)
c	21.7 ± 4.0 (17.4–32.6)	17.4 ± 4.0 (12.5–23.9)
c’	1.9 ± 0.3 (1.2–2.4)	2.4 ± 0.4 (1.5–3.1)
V	81.0 ± 1.7 (79–86)	71.1 ± 1.8 (67.3–74.4)
G_1	23.3 ± 9.8 (15.0–46.7)	16.1 ± 1.7 (12.5–18.1)
Stylet length	16.6 ± 0.7 (15.0–17.0)	15.0 ± 0.6 (14.0–16.0)
m	46.4 ± 2.1 (42.5–48.5)	46.8 ± 4.2 (41.7–56.1)
O	14.1 ± 1.6 (11.9–16.0)	18.8 ± 2.5 (15.4–22.5)
Anterior end to excretory pore	94.3 ± 9.8 (73–105)	81.4 ± 6.7 (65.0–95.3)
MB	36.4 ± 6.3 (25.3–45.6)	33.2 ± 3.0 (28.9–38.2)
Pharynx length	144.1 ± 20.2 (103–173)	123.1 ± 9.1 (108–139)
Tail length	28.9 ± 4.3 (20.0–34.0)	27.3 ± 5.8 (21.0–33.0)

[a] Abbreviations are defined in Siddiqi (2000)

enveloped by crenate-edged bursa, tip narrow and pointed. Spicules and gubernaculum ventrally curved. Phasmids open on bursa, generally anterior to mid-tail (Fig. 4g, h, k, l). The morphology and morphometric of this population from Cinco Esquinas, Alajuela, Costa Rica agrees closely with the original description of this population by Golden et al. (1992) from San Antonio, Alajuela Province, Costa Rica. In addition, the topotype specimens of *P. gutierrezi* studied here were morphologically identical to the original population and also molecularly congruent with data by Duncan et al. (1999).

According to the polytomous key by Castillo and Vovlas (2007), the Costa Rican lesion nematode parasitizing coffee belongs to species with two lip annuli and *en face* view Group 2 according to classification scheme of Corbett and Clark (1983), and has the specific alphanumeric codes indicated in Table 3.

***Pratylenchus panamaensis* Siddiqi et al. 1992**

(Figs. 5, 6, Table 4)

The population of this amphimictic species from the rhizosphere and roots of coffee from the type locality in Boquete, Panama was characterised by a rounded lip region relatively low setoff from body contour by a small constriction, anteriorly flattened, and bearing two annuli (Fig. 5). Body straight to slightly curve ventrally. Lip region low, flat and having two annuli (Fig. 5d). Lateral fields with four equidistant incisures (Fig. 5e), not areolated, inner incisures usually fusing on tail. SEM *en face* view pattern showing an oval oral aperture centrally located in labial disc and surrounded by six (three on each side) inner labial sensilla (Fig. 6c, d). Subdorsal and subventral sectors of first lip annulus similar in shape and clearly separated from lateral sectors and also separated from oral plate by a slight depression (Fig. 6d). This lip pattern configuration is in agreement with Group 2 according to classification scheme of Corbett and Clark (1983). Amphidial apertures are small, round to oval-shaped, located between labial plate and lateral sectors (Fig. 6c, d). Stylet with 7.0–8.0 μm long conus and strong rounded to anteriorly flattened basal knobs. Opening of dorsal pharyngeal gland 2.0–3.0 μm from stylet base. Excretory pore usually opposite pharyngeal-intestinal junction. Hemizonid just anterior to excretory pore, 1–2 annuli long. Spermatheca longitudinally oval, filled with sperms (Fig. 5g). Post-vulval uterine sac 20.5 ± 2.9 (17.0–25.0) μm long. Vulva a transverse slit, located

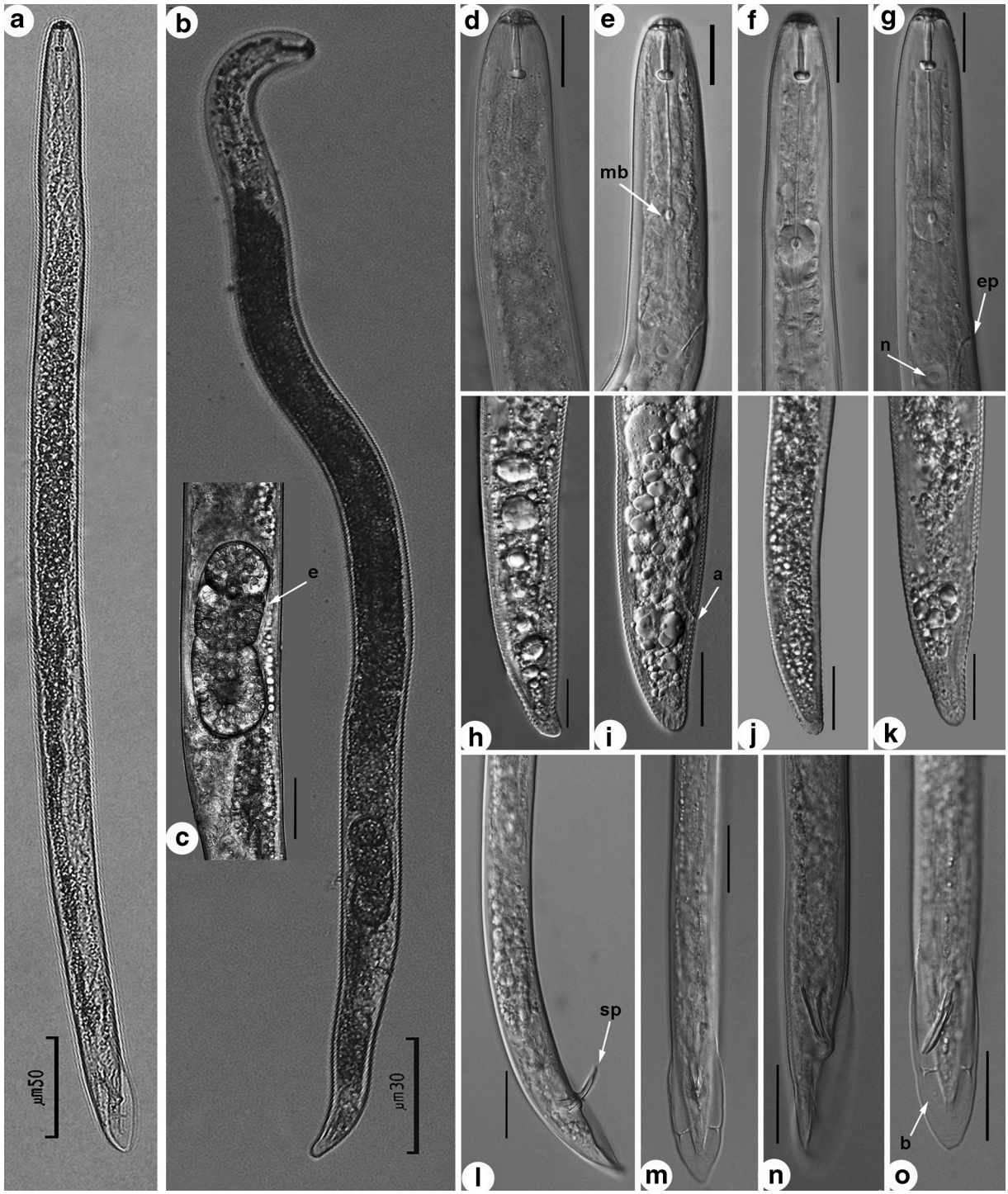


Fig. 1 Light micrographs of *Pratylenchus bolivianus* Corbett 1983 from San Isidro, Heredia, Costa Rica. **a, b** Whole male and female. **c** Vulval region. **d**–**g** Female anterior regions. **h**–**k** Female tail regions. **l**–**o** Male tail regions. Abbreviations: **a** = anus; **b** = bursa; **e** = egg; ep = excretory pore; mb = median bulb; n = nucleus of pharyngeal gland; sp. = spicule. (*Scale bars*: **a** = 50 μm; **b** = 30 μm; **c-o** = 20 μm)

on a prominent ventral protuberance of body. Tail subcylindrical to subclavate with a broadly rounded, distinctly crenate terminus. Phasmids located at or just posterior to middle of tail (Fig. 6e, h).

Male as abundant as female. Stylet and labial sclerotization weaker than that of female. Spicules cephalated, gubernaculum trough-shaped (Fig. 5h). Tail tip narrow and pointed. Bursa arises a little anterior to head of spicule and envelopes tail. Phasmids distinct, extending into bursa, at or just anterior to middle of tail.

The morphology and morphometric of this population agree closely with the original description of this population by Siddiqi et al. (1992) from Boquete, Panama. In addition, morphology and morphometrics fit well with the population of *P. gutierrezi* from Guatemala reported by Inserra et al. (1998) and topotypes studied in the present research, except for minor differences in a shorter body and tail length, and a slightly lower c ratio, which may be related with intraspecific variability of the species or because Inserra et al. (1998) used live specimens for measurements and this may results in a enlargement in linear measurements that generally has been estimated to be 5–7 % in other species as *P. zeae* Graham 1951 (Saha and Khan 1989).

According to the polytomous key by Castillo and Vovlas (2007), the Panama root-lesion nematode parasitizing coffee belongs to species with two lip annuli and *en face* view Group 2 according to classification scheme of Corbett and Clark (1983), and has the specific alphanumeric codes indicated in Table 3.

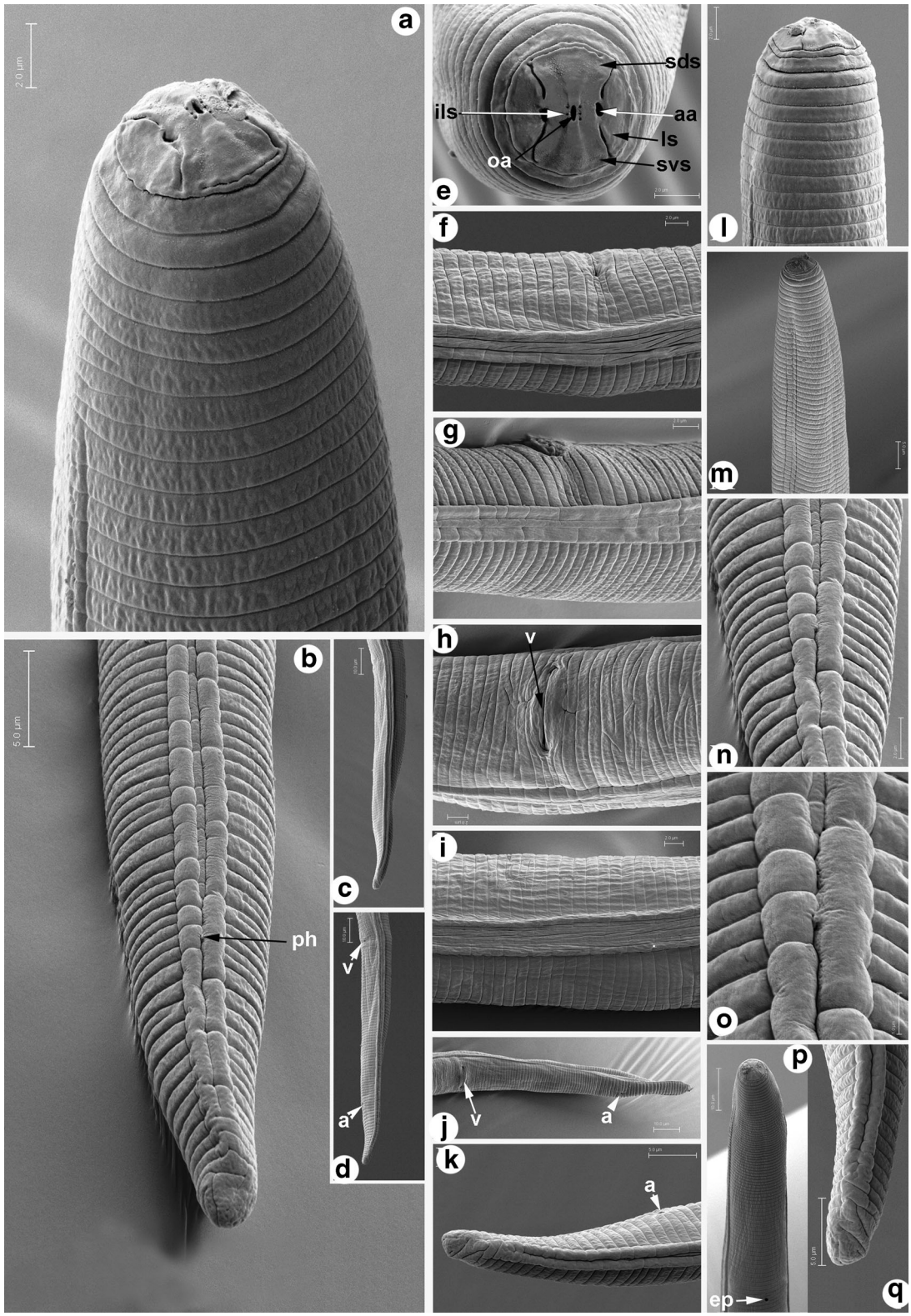
***Pratylenchus pseudocoffeae* Mizukubo 1992**

(Figs. 7, 8, Table 5)

The Costa Rican population of this amphimictic species from the rhizosphere and roots of mum was characterised by a hemispherical lip region relatively low, flattened anteriorly, and bearing two annuli (Fig. 7). SEM *en face* view pattern showing an oval oral aperture centrally located in labial disc and surrounded by six (three on each side) inner labial sensilla (Fig. 8a, i, j). Subdorsal and subventral sectors of first lip annulus similar in shape and clearly separated from lateral sectors and also separated from oral plate by a slight depression. This lip pattern configuration in agreement with Group 2 according to classification scheme of Corbett and Clark (1983). Amphidial apertures oval-shaped, obliquely orientated, located between labial plate and lateral sectors (Fig. 8a, i, j). Stylet knobs mostly broadly rounded, hardly flattened anteriorly. Lateral fields consisting of three bands delimitated by four lines, outer bands appearing irregularly areolated in mid-body and tail regions. Pharyngeal overlapping rather long, 54.0±3.3 (51.0–57.5) μm. Excretory pore located slightly anterior to level of cardia. Spermatheca oblong, post-vulval uterine sac 26.0±4.4 (20.0–35.0) μm long, usually less than twice vulval body diam. Tail almost subcylindrical, tapering towards terminus (Fig. 8b, f, l, m). Tail terminus usually subhemispherical or broadly rounded, tip smooth (Fig. 8b, f, l). Phasmids pore-like, centred in lateral fields and located near mid-tail.

Males less abundant than female (ratio = 1: 2) and morphologically similar except for sexual dimorphism. Testis outstretched, with spermatogonia in double or triple rows. Tail conoid, tip narrow and pointed. Bursa arises a little anterior to head of spicule and envelopes tail. Spicules arch-shaped, gubernaculum simple. The morphology and morphometric of this population agree closely with the original description of the species by Mizukubo (1992) and populations from Iran and Florida (Deimi et al. 2009; Inserra et al. 1998), except for a slightly longer body length (410–620 *vs* 494–710 μm). These differences could be attributable to geographical intraspecific variability. *En face* view SEM observations of lip patterns also agree well with those of previous descriptions, with lateral sectors of the first lip annulus being divided from oral disc and medial sectors. These morphological data indicate that this root-lesion nematode from Costa Rica is morphologically and molecularly conspecific with *P. pseudocoffeae* from Japan, Florida and Iran (Mizukubo 1992; Inserra et al. 1998, and Deimi et al. 2009). According to the polytomous key by Castillo and Vovlas (2007), the Costa Rican root-lesion nematode parasitizing chrysanthemum belongs to species with two lip annuli and *en face* view Group 2 according to classification scheme of Corbett and Clark (1983), and has the specific alphanumeric codes indicated in Table 3.

This species was firstly described from mum (*Chrysanthemum* × *morifolium* Ramat) and artemisia (*Artemisia feddei* Lev. & Van.) in Japan (Mizukubo 1992). Thereafter Inserra et al. (1998) described a new population of *P. pseudocoffeae* from Florida (although no molecular data were available) and another morphologically undefined population from Belgium, identified as *P. pseudocoffeae*, was used for molecular studies by Waeyenberge et al. (2000). Finally, Deimi et al. (2009)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◀ **Fig. 2** SEM morphology of *Pratylenchus bolivianus* Corbett 1983 from San Isidro, Heredia, Costa Rica (**a**–**q**). **a, e, l, m, p** Anterior female region showing starting of lateral fields, *en face* view showing submedian segments fused to the oral disc, and excretory pore. **f**–**i** Vulval region showing lateral fields. **j** Posterior female region showing anus and vulva. **k, n, o, q** Female tail region. Abbreviations: a = anus; aa = amphidial aperture; ep = excretory pore; ils = inner labial sensilla; ls = lateral sectors; oa = oral aperture; ph = phasmid; sds = subdorsal segment; svs = subventral segment; v = vulva. (Scale bars: **a, e**–**i, l, n** = 2 μm; **b, k, m, q** = 5 μm; **c, d, j, p** = 10 μm; **o** = 1 μm)

carried out an integrative taxonomic study (including morphological and molecular analyses) on populations infecting mum in glasshouses and open fields in Iran. This species is close to *P. penetrans* due to a general resemblance in the morphology of body, and particularly of tail region and spermatheca (Castillo and Vovlas 2007). Our morphological and molecular analyses confirmed the identity of this species, which is a new occurrence and a first record of the species in Costa Rica, and becomes the fifth world record after the original description.

***Pratylenchus zeae* Graham 1951**

(Figs. 9, 10, Table 5)

The Costa Rican population of the corn root-lesion nematode from the rhizosphere of rice was characterised by the absence of males, lip region not setoff from body contour and bearing three annuli (Fig. 9). SEM *en face* view pattern showing an oval oral aperture centrally located in labial disc and surrounded by six (three on each side) inner labial sensilla, with no division between sub-

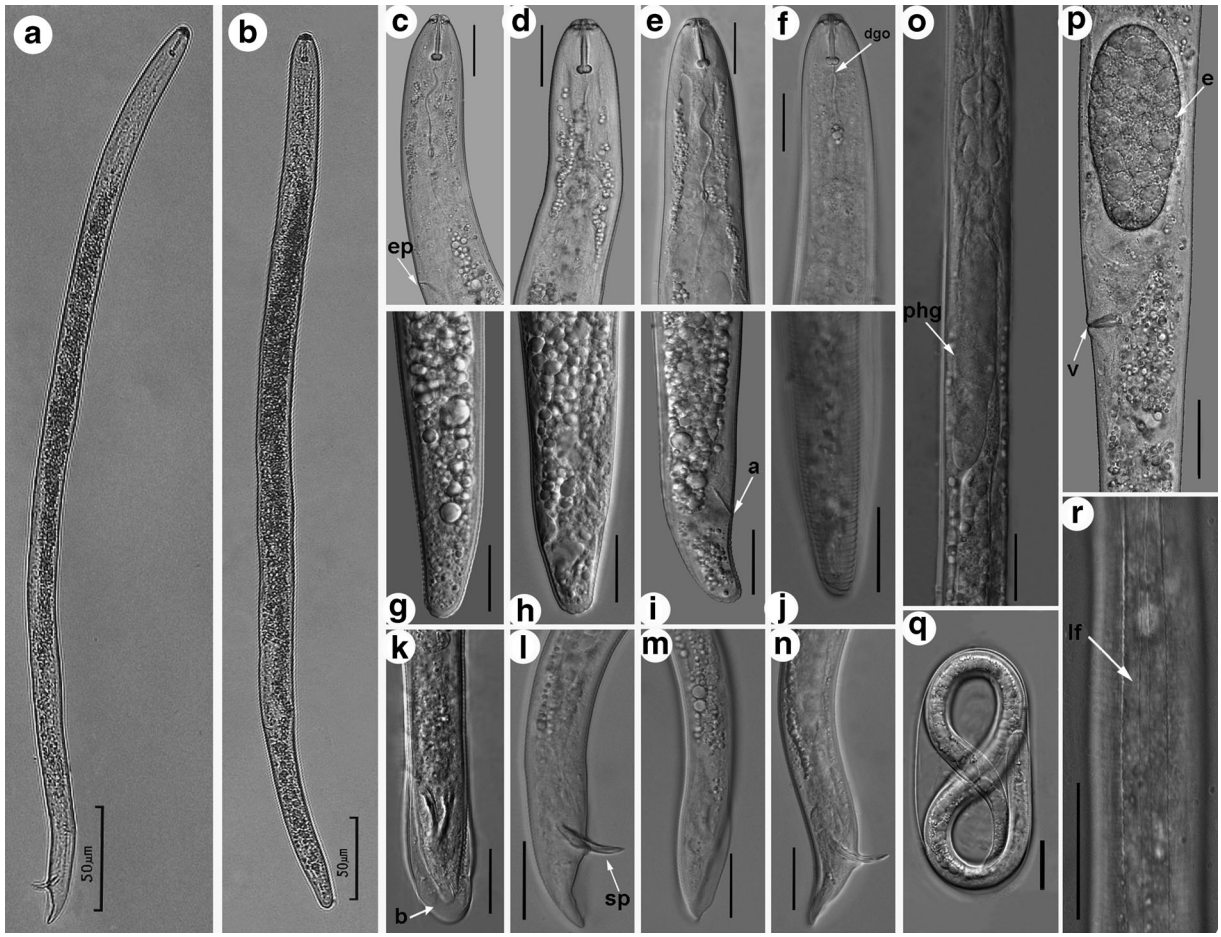


Fig. 3 Light micrographs of *Pratylenchus gutierrezi* Golden et al. 1992 from Cinco Esquinas, Alajuela, Costa Rica. **a, b** Whole male and female. **c**–**f** Female anterior regions. **g**–**j** Female tail regions. **k**–**n** Male tail regions. **o** Pharyngeal region. **p** Vulval region. **q** Embryonated egg. **r** Detail of lateral field at midbody. Abbreviations: a = anus; b = bursa; dgo = dorsal gland opening; e = egg; ep = excretory pore; lt = lateral field; phg = pharyngeal gland; sp. = spicule; V = vulva. (Scale bars: **a, b** = 50 μm; **c**-**o** = 20 μm; **p** = 10 μm; **r** = 20 μm; **q** = 10 μm)

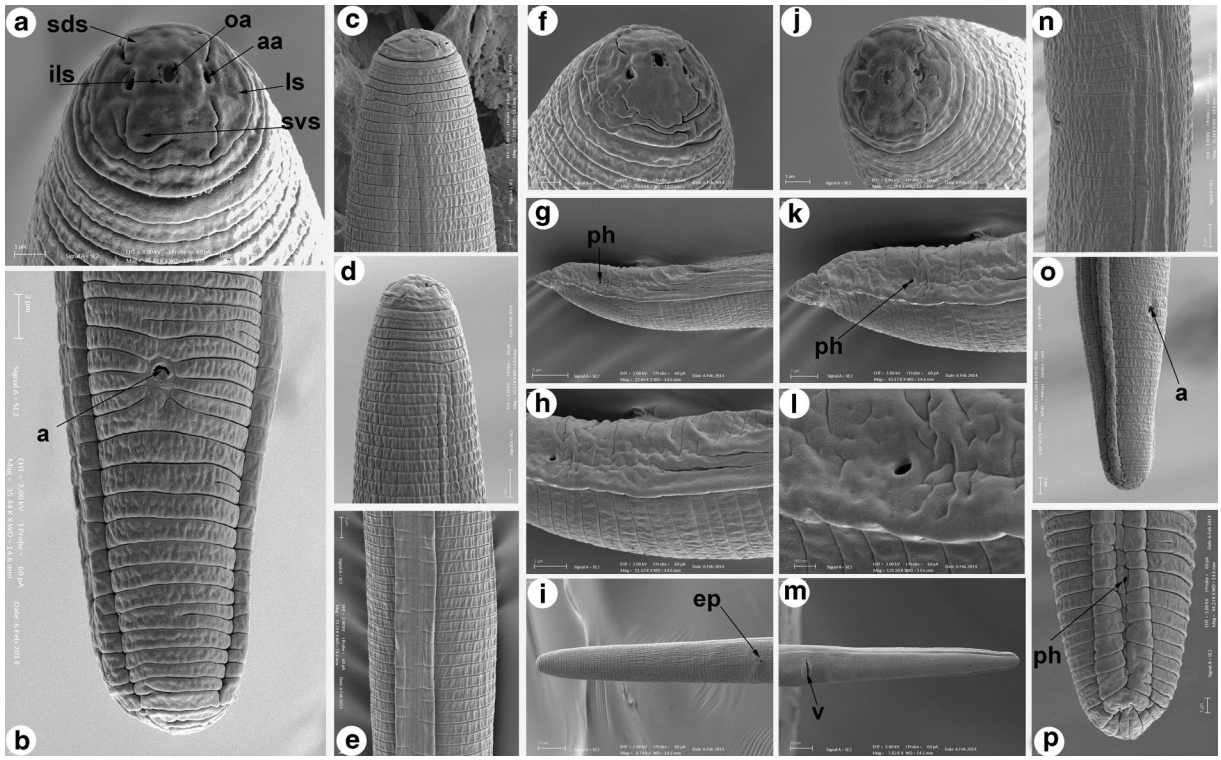


Fig. 4 SEM morphology of *Pratylenchus gutierrezi* Golden et al. 1992 from Cinco Esquinas, Alajuela, Costa Rica (**a**–**p**). **a, f, j** *En face* view showing submedian segments fused to the oral disc. **c, d** Anterior female region showing starting of lateral fields. **e, n** Lateral fields at mid-body. **i** Anterior female region showing excretory pore. **n** Posterior female region showing vulva. **b, o-p** Female tail region. **g, k** Male tail region. **h, l** Detail of male tail region showing phasmid. Abbreviations: a = anus; aa = amphidial aperture; ep = excretory pore; ils = inner labial sensilla; ls = lateral sectors; oa = oral aperture; ph = phasmid; sds = subdorsal segment; svs = subventral segment; v = vulva. (*Scale bars*: **a, f, j, p** = 1 μm; **b**–**e, h, k, n, o** = 2 μm; **g** = 5 μm; **i, m** = 10 μm; **l** = 0.5 μm)

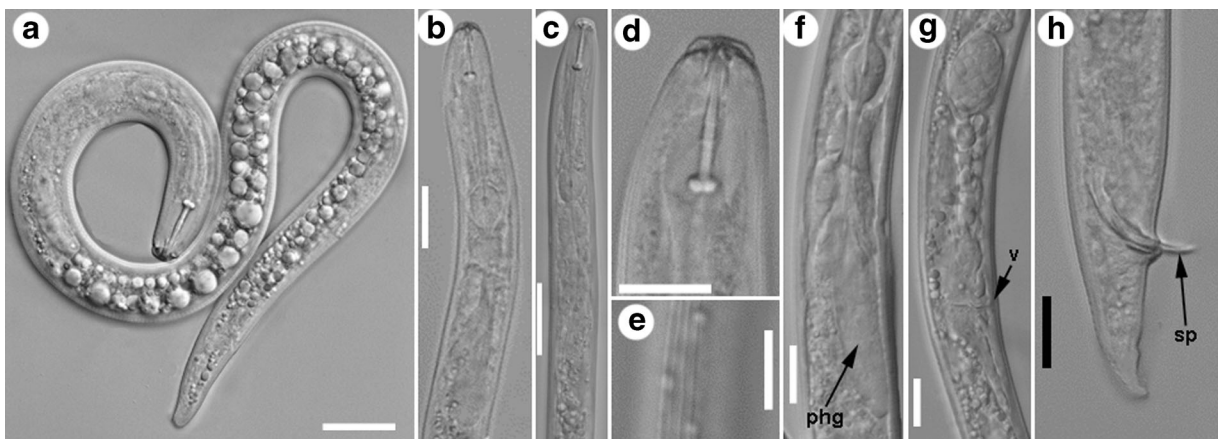


Fig. 5 Light micrographs of *Pratylenchus panamaensis* Siddiqi et al. 1992 from Boquete, Chiriquí Province, Panama (type locality). **a** Whole female. **b**–**c** Female anterior regions. **d** Female lip region. **e** Detail of lateral field at midbody. **f** Pharyngeal region. **g** Vulval region showing spermatheca. **h** Male tail region. Abbreviations: phg = pharyngeal gland; sp. = spicule; spm = spermatheca; V = vulva. (*Scale bars*: **a**–**c** = 20 μm; **d**–**h** = 10 μm)

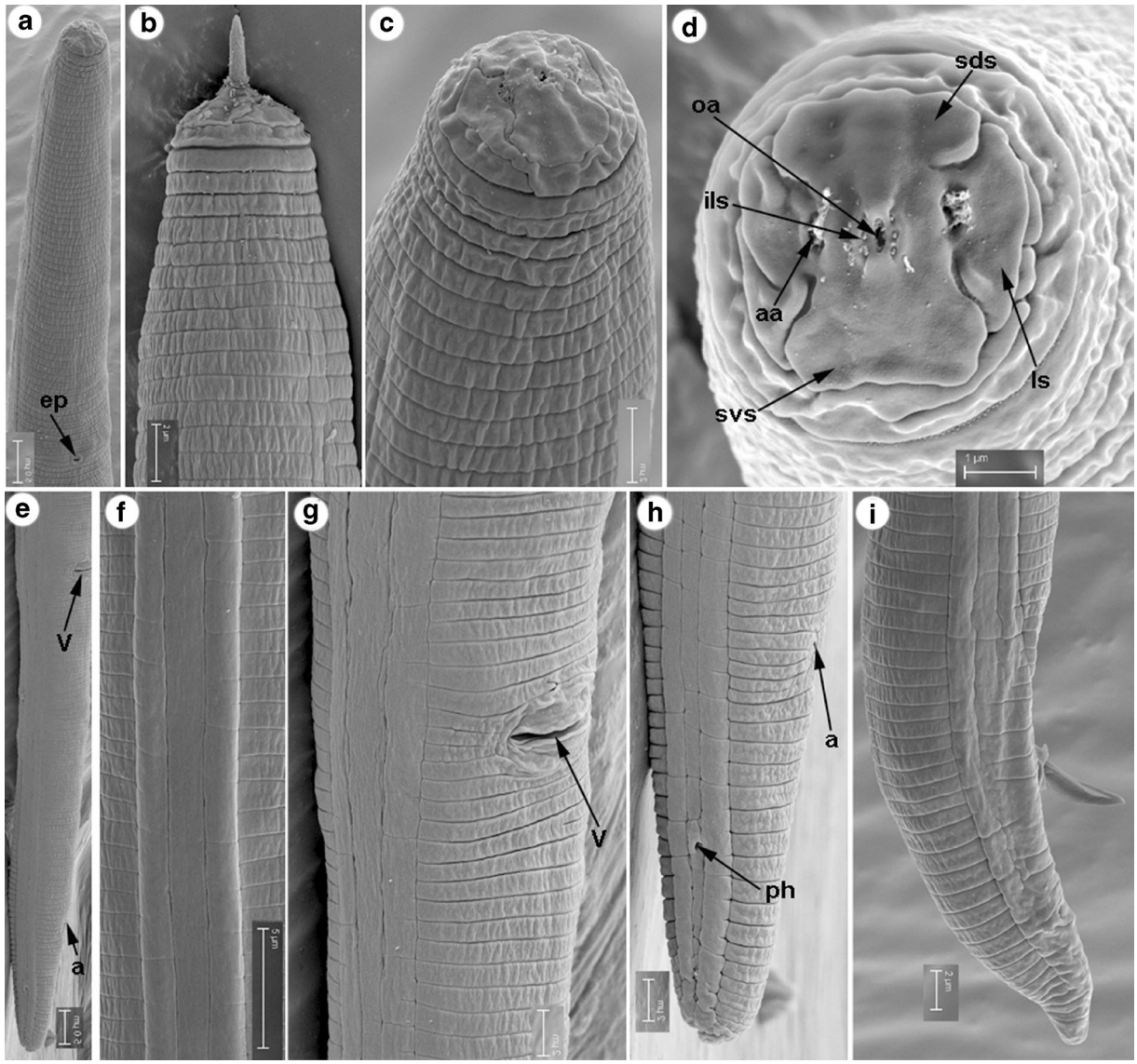


Fig. 6 SEM morphology of *Pratylenchus panamaensis* Siddiqi et al. 1992 from Boquete, Chiriquí Province, Panama (type locality) (**a**–**i**). **a** Anterior female region showing starting of lateral field and excretory pore. **b** Lip region. **c, d** *En face* view showing submedian segments fused to the oral disc. **e** Posterior female region showing vulva and anus. **f, g** Lateral fields at mid-body. **h** Female tail region. **i** Male tail region. Abbreviations: a = anus; aa = amphidial aperture; ep = excretory pore; ils = inner labial sensilla; ls = lateral sectors; oa = oral aperture; ph = phasmid; sds = subdorsal segment; svs = subventral segment; v = vulva. (*Scale bars*: **a, c, e, f** = 5 µm; **b, g**–**i** = 2 µm; **d** = 1 µm)

median and lateral segments, presumably due to fusion of the first lip annulus to the oral disc (Fig. 10d, h). This lip pattern configuration is in agreement with Group 1 according to classification scheme of Corbett and Clark (1983). Amphidial apertures oval-shaped, obliquely orientated (Fig. 10d, h). Stylet with broad, anteriorly flattened basal knobs. Lateral fields with four lines extending along tail beyond phasmids; inner band showing a slight irregularity in mid-body region but no corresponding fifth line (Fig. 10e, i). Pharyngeal overlapping rather long, 48.7 ± 1.3 (47.0–50.0) µm. Excretory pore located anterior to level of cardia. Spermatheca round, small, without sperm; post-vulval uterine sac short, 24.7 ± 1.9 (22.0–27.5) µm long, usually less than one and half vulval body diam. Tail shape narrowly rounded to subacute and bluntly pointed terminus (Fig. 10c, f, k). Phasmids pore-like, centred in lateral fields and located near mid-tail. The morphology and morphometric of this population agree closely with

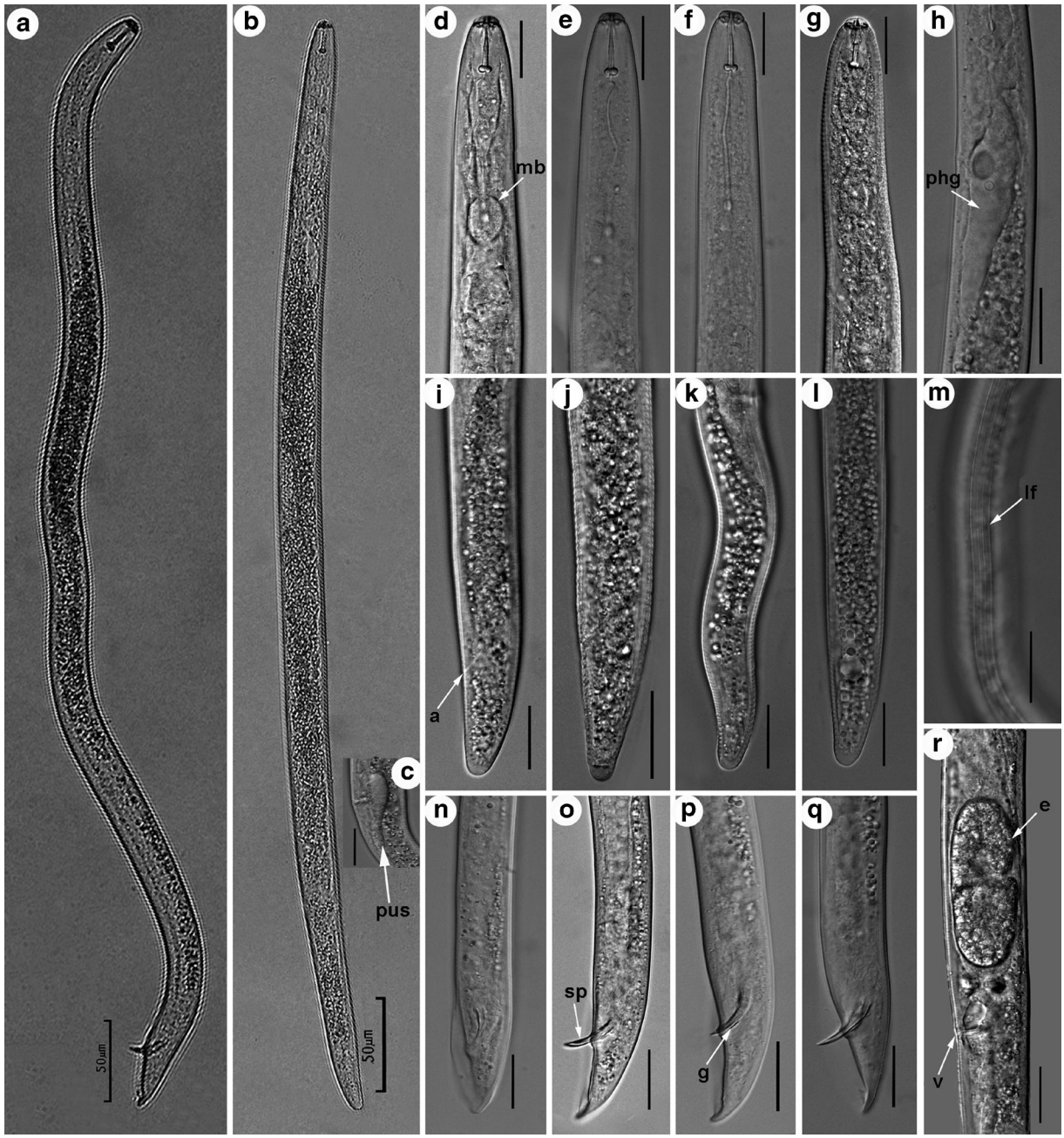


Fig. 7 Light micrographs of *Pratylenchus pseudocoffeae* Mizukubo 1992 from San Isidro, Heredia, Costa Rica. **a, b** Whole male and female. **c** Vulval region. **d**–**g** Female anterior regions. **h** Pharyngeal region. **i**–**l** Female tail regions. **m** Detail of lateral field at midbody. **n**–**q** Male tail regions. **r** Vulval region. Abbreviations: a = anus; e = egg; g = gubernaculum; lt = lateral field; mb = median bulb; phg = pharyngeal gland; pus = postvulval uterine sac; sp. = spicule; V = vulva. (Scale bars: **a, b** = 50 μm; **c** = 10 μm; **d**-**r** = 20 μm)

the original description of the species by Graham (1951) and populations from Jordan (Hashim 1984), Estonia (Ryss 1988), and Guadalupe (Van Den Berg and Quénéhervé 2000).

According to the polytomous key by Castillo and Vovlas (2007), the Costa Rican lesion nematode parasitizing rice belongs to species with three lip annuli and *en face* view Group 1 according to classification scheme

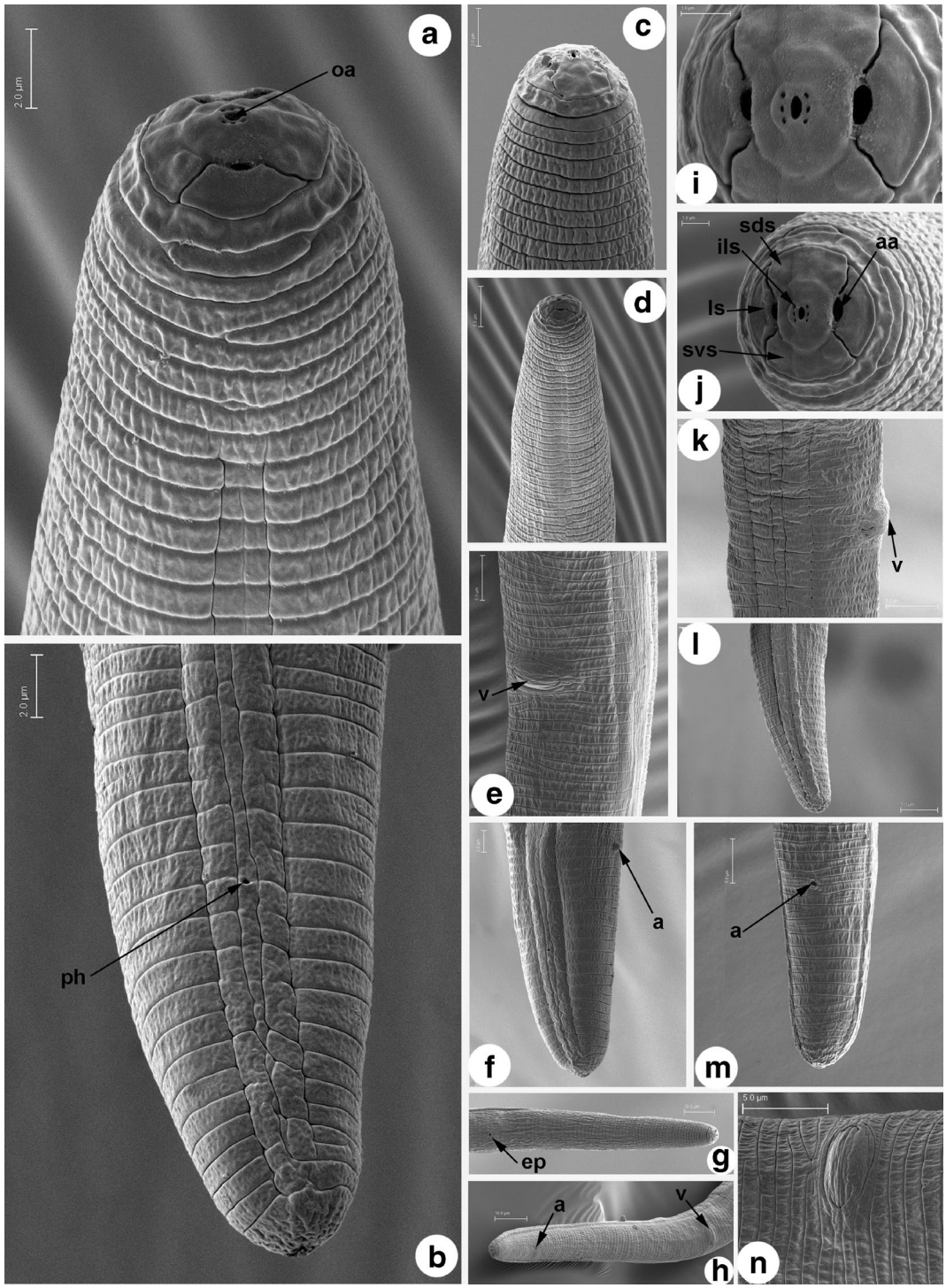


Fig. 8 SEM morphology of *Pratylenchus pseudocoffeae* Mizukubo 1992 from San Isidro, Heredia, Costa Rica (**a**–**n**). **a, c, d, i, g, j** Anterior female region showing starting of lateral fields, *en face* view showing submedian segments fused to the oral disc, and excretory pore. **h** Posterior female region showing anus and vulva. **e, k, n** Vulval region. **b, f, l, m** Female tail region. Abbreviations: a = anus; aa = amphidial aperture; ep = excretory pore; ils = inner labial sensilla; ls = lateral sectors; oa = oral aperture; ph = phasmid; sds = subdorsal segment; svs = subventral segment; v = vulva. (*Scale bars*: **a**–**c, f** = 2 μm; **d**, **e**, **k**–**n** = 5 μm; **g, h** = 10 μm; **i, j** = 1 μm)

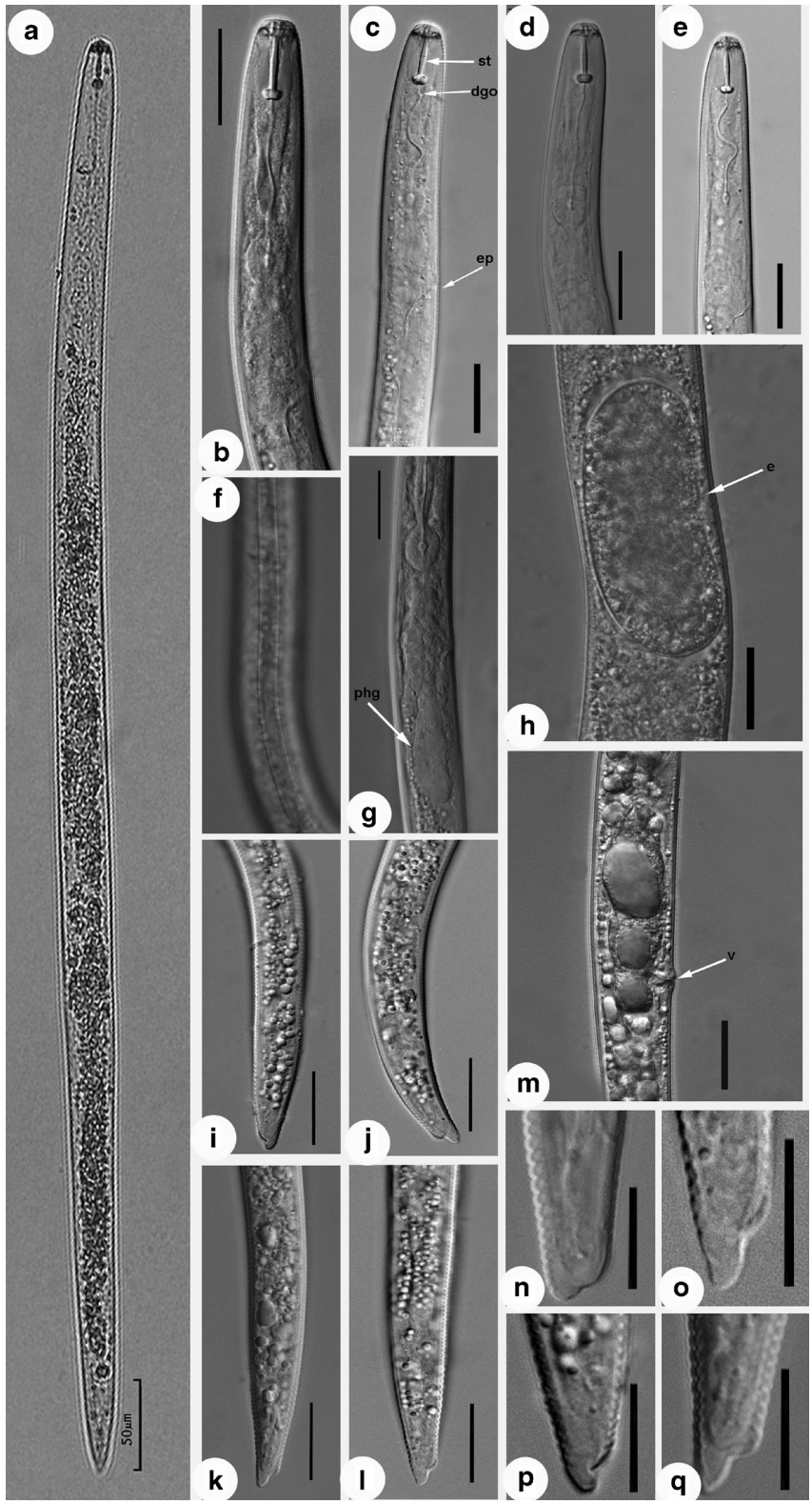


Fig. 9 Light micrographs of *Pratylenchus zeae* Graham 1951 from Aguas Zarcas, San Carlos, Costa Rica. **a** Whole female. **b–e** Female anterior regions. **f** Detail of lateral field at midbody. **g** Pharyngeal region. **h, m** Vulval regions. **i–l** Female tail regions. **n–q** Female tail tip regions. Abbreviations: dgo = dorsal gland orifice; e = egg; ep = excretory pore; phg = pharyngeal gland; st = stylet; V = vulva. (*Scale bars*: **a** = 50 μm; **b–l** = 20 μm; **m–q** = 10 μm)

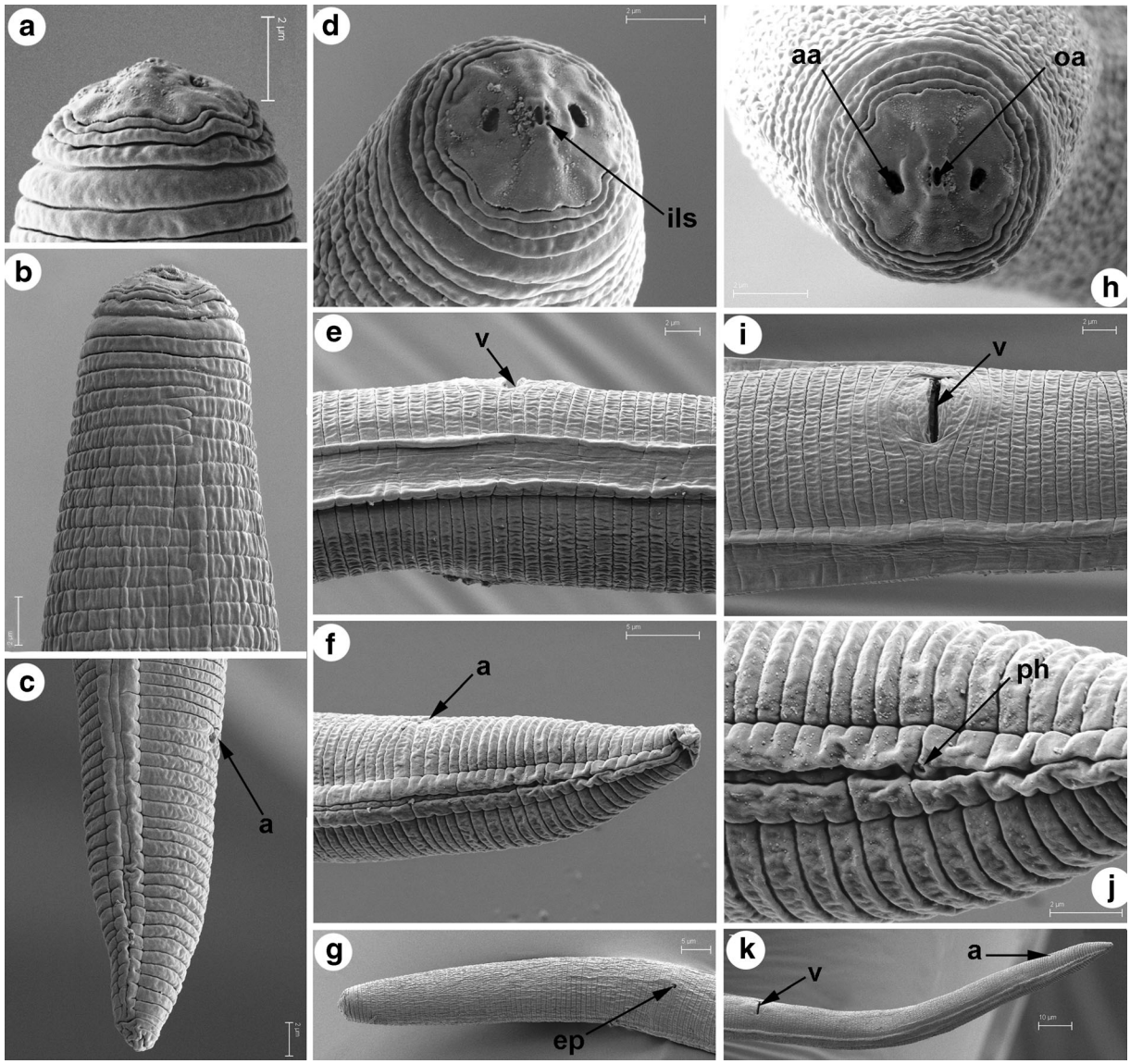


Fig. 10 SEM morphology of *Pratylenchus zeae* Graham 1951 from Aguas Zarcas, San Carlos, Costa Rica (**a**–**k**). **a, b, d, g, h** Anterior female region showing *en face* view with a plain undivided pattern with no division between sub-median and lateral segments, and excretory pore. **e, i** Vulval region showing lateral fields. **k** Posterior female region showing anus and vulva. **c, f, j** Female tail region. Abbreviations: a = anus; aa = amphidial aperture; ep = excretory pore; ils = inner labial sensilla; oa = oral aperture; ph = phasmid; v = vulva. (*Scale bars*: **a**–**e, h, i, j** = 2 μm; **f, g** = 5 μm; **k** = 10 μm)

of Corbett and Clark (1983), and has the specific alphanumeric codes indicated in Table 3.

Remarks on the identity of *Pratylenchus gutierrezi* and *Pratylenchus panamaensis*

The morphology of *P. gutierrezi* is quite similar to that of *P. coffeae* except that SEM revealed distinct medial and lateral lip sectors in the *en face* view of the first lip annulus of the former species, in contrast to the smooth face (fused lip sectors) reported for *P. coffeae* from citrus (Corbett and Clark 1983). Morphologically *P. gutierrezi* was also almost identical to *P. panamaensis* from which was considered a junior synonym based on the principle of priority of the International Code of Zoological Nomenclature (1985, art. 23) as proposed by Siddiqi (2000), Castillo and Vovlas (2007) and Handoo et al. (2008). However, up to date

no data on SEM *en face* view pattern or molecular markers had been obtained for *P. panamaensis* parasitizing coffee in Boquete, Panama.

Few morphological and morphometrical characters can separate *P. gutierrezi* from *P. panamaensis* (Siddiqi et al. 1992; Golden et al. 1992). The present study revealed that *P. gutierrezi* differ morphometrically from *P. panamaensis* in having a slightly longer female body length (430–692 *vs* 389–590 μm), a lightly shorter stylet and female tail length (15–18, 19–31 *vs* 15–17, 22–29 μm; respectively), slightly lower a and c' ratio (16–27, 1.8–2.3 *vs* 21–30, 1.6–2.6; respectively), and conversely higher c ratio (16–26 *vs* 15–23). However, these characters overlap making the morphological separation of these two species unreliable by visual perception without the corroboration of the molecular analysis and being able to be considered as cryptic species. Finally, our present results, including SEM *en face* view data on topotypes of *P. panamaensis*, demonstrated that both species are almost undistinguishable, but clearly separated molecularly with the two ribosomal genes D2-D3 expansion segments of 28S (89 % similar, 84 nucleotides and 19 indels) and ITS rDNA (88 to 89 % similarity, from 83 to 86 nucleotides and from 17 to 24 indels) (see below).

Phylogenetic relationships of *Pratylenchus spp.* from Costa Rica

The amplification of D2-D3 expansion segments of 28S rRNA and ITS rRNA yielded a single fragment of approximately 800 bp and 950 bp, respectively, based on direct fragment sequencing. Sequences from other species of *Pratylenchus* obtained from NCBI were used for further phylogenetic studies. D2-D3 expansion segments of 28S rRNA of *Pratylenchus* spp. from Costa Rica (KT971354-KT971357, KT971360-KT971361) and the topotype population of *P. panamaensis* from Panama (KT971358-KT971359) matched well with the *Pratylenchus* spp. deposited in GenBank. *Pratylenchus gutierrezi* (KT971355-KT971357) showed a coincidence with topotype specimens of *P. gutierrezi* (AF170442, K3 isolate) deposited in GenBank, being 99 % similar (differing in 12–13 nucleotides and three indels). However, these accessions showed a low coincidence, 89 % similarity (differing in 81 nucleotides and 17 indels) with the two other isolates named as *P. gutierrezi* (K1, AF170440 and K2, AF170441, from Costa Rica and Guatemala, respectively). The new studied populations in this research from San Antonio (type locality) and Cinco Esquinas showed a higher similarity between themselves, *viz*. 99 % (differing in five nucleotides and 0 indels). Similarly, D2-D3 expansion segments of 28S rRNA of *P. panamaensis* (KT971358-KT971359) from Boquete showed a high coincidence with accessions named as *P. gutierrezi* [isolates K1 (AF170440 K2 (AF170441), CA61 (EU130897) and CA72 (EU130899-EU130898)] deposited in GenBank, being 100–99 % similar (differing in 0–3 nucleotides and 0–1 indels). D2-D3 sequence for *P. bolivianus* (KT971354) from San Isidro, Heredia province, Costa Rica parasitizing fern was closely related (99 % similarity) to *P. bolivianus* from China (KP780255-KP780256) by 5–6 bp and two indels, and *Pratylenchus* sp. DP-2010 (HM469436) by five nucleotides and two indels; thus, this latter species should be considered as conspecific of *P. bolivianus*. *Pratylenchus pseudocoffeae* (KT971360) matched well (99 % similar, differing in five nucleotides and two indels) with the sequence of *P. pseudocoffeae* deposited in GenBank (AF170444). Finally, *P. zeae* (KT971361) showed coincidence with sequences deposited in GenBank, being 99 % similar (differing in eight nucleotides and three indels) and 98 % similar (differing in three nucleotides and three indels) with accessions AB933457 and AB933458, respectively. However, intraspecific variation was detected amongst sequences of *P. zeae* deposited in GenBank, this variation ranged from 4 to 55 nucleotides and from 0 to 7 indels (99–92 % similar).

ITS region of *P. gutierrezi* (KT971363-KT971364) and *P. panamaensis* (KT971365-KT971366) were obtained for the first time in this study. The new ITS sequences obtained from *P. gutierrezi* in the type locality and Cinco Esquinas (Costa Rica) showed scarce homology with *Pratylenchus* spp. deposited in GenBank, particularly no homology was detected with *P. panamaensis* from Panama, including 86 % similarity (74–75 nucleotides, 22–23 indels) with unidentified species of *Pratylenchus* sp. H5, H3 and H2 from Florida, Russia and South Africa (GU131137, GU988369, FJ713012, respectively) (De Luca et al. 2010), but no information of ITS rDNA from isolate K3 of *P. gutierrezi* was available. Conversely, the new ITS sequences obtained from topotypes of *P. panamaensis* showed high similarity 99–96 % (3–25 nucleotides and 2–16 indels) with sequences deposited in GenBank, including *P. gutierrezi* from banana in Madeira, Portugal

(FR692277), and different clones of *P. gutierrezi* from coffee in Guatemala (FJ712927-FJ712931). *Pratylenchus bolivianus* ITS (KT971362) was close to *P. bolivianus* sequences deposited in GenBank from UK, China, Chile, and New Zealand (FJ712893-FJ71896, HM469446-HM469447, FR692328-FR692328, and KP780257-KP780258) with 90–89 % similarity. And *P. pseudocoffeae* (KT971367) matched well with sequences deposited in GenBank, being 99 % similar (differing in seven nucleotides and five indels) to accessions FR692276 and FR691856, both from chrysanthemum in China and Iran, respectively (De Luca et al. 2011).

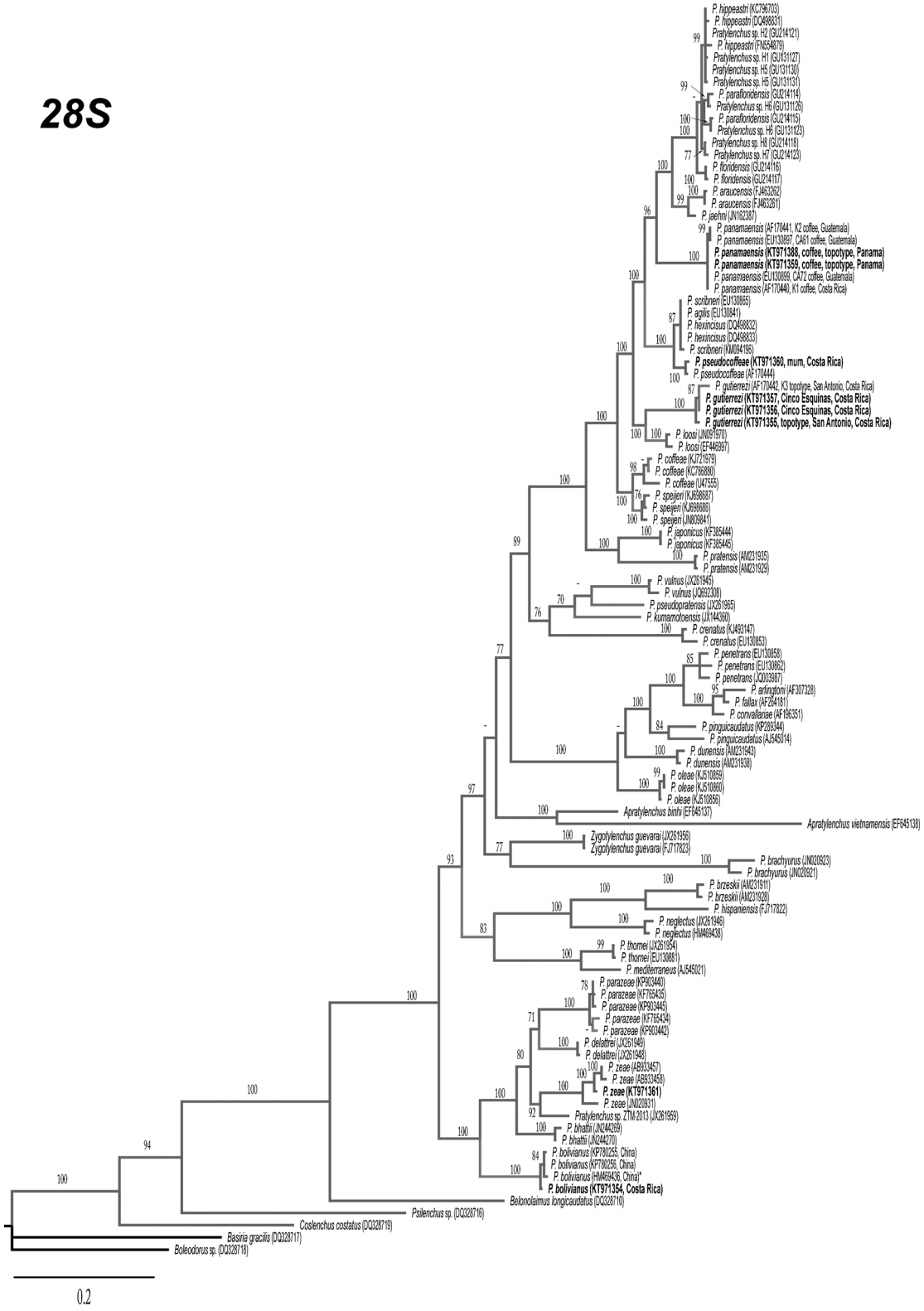
Phylogenetic relationships among *Pratylenchus* spp. inferred from analyses of D2-D3 expansion segments of 28S rRNA and ITS rRNA gene sequences using BI are given in Figs. 11 and 12, respectively. The 50 % majority rule consensus BI tree of a multiple alignment including 103 D2-D3 sequences and 695 bp consisted of 3 highly supported major clades in the genus *Pratylenchus*, *Zygotylenchus* and *Apratylenchus* (Fig. 11). This tree topology was similar to that obtained by Palomares-Rius et al. (2014b). *Pratylenchus gutierrezi, P. panamaensis* and *P. pseudocoffeae* were placed in a highly supported group, but all of them well separated in three subclades (Fig. 11). *Pratylenchus gutierrezi* formed a well-supported subclade with *P. loosi* Loof 1960. *Pratylenchus panamaensis* clustered with *P. hippeastri*, *P. floridensis* and *P. parafloridensis* populations as well as with *Pratylenchus* sp. H1-H8 (Fig. 11). *Pratylenchus pseudocoffeae* formed a highly supported clade with *P. pseudocoffeae* (AF170444) from aster, Florida, *Pratylenchus scribneri* Sherbakoff and Stanley 1943, *P. agilis* Thorne and Malek 1968, and *Pratylenchus hexincisus* Taylor and Jenkins 1957. *Pratylenchus bolivianus* and *P. zeae* were placed at a basal well defined position in the tree related to *Pratylenchus parazeae* Wang et al. 2015, *Pratylenchus delattrei* Luc, 1958, other accessions of *P. zeae* and *Pratylenchus bhattii* Siddiqi et al. 1992.

Because of the great molecular diversity within ITS sequences of *Pratylenchus* spp. deposited in GenBank, for phylogenetic analysis of this gene only homologous sequences to *Pratylenchus* spp. to our detected species in Costa Rica and Panama were selected (Fig. 12). The alignment generated for the 50 ITS of *Pratylenchus* samples was 758 bp after discarding ambiguously aligned regions from the alignment. The 50 % majority rule consensus tree generated from de ITS alignment by BI analysis under de model GTR + I + G is presented in Fig. 12. *Pratylenchus gutierrezi*, *P. panamaensis* and *P. pseudocoffeae* were grouped in three separated well supported subclades placed inside a wide range of other *Pratylenchus* species (Fig. 12). *Pratylenchus pseudocoffeae* formed a well-supported subclade together with *P. scribneri*, *P. agilis* and with other accessions for *P. pseudocoffeae* (Fig. 12). While, *P. panamaensis* was nested with the clade described before and *P. alleni* Ferris 1961. The accessions from this species formed a well-supported clade with the population sampled in this study, and those from Portugal (FR692277) (De Luca et al. 2011) and Guatemala (FJ712927-FJ712930) (Fig. 12). The positions of these species are mainly congruent with those obtained from the D2-D3 analysis. ITS sequence of *P. bolivianus* and others from *P. zeae* from GenBank were not included in the phylogenetic analysis because of the low homology with sequences included in the dataset.

Discussion

The primary objective of this study was to identify and characterize molecularly root-lesion nematodes parasitizing cultivated and ornamental plants in Costa Rica as well as an integrative characterization of topotypes of *P. gutierrezi* and *P. panamaensis* parasitizing coffee in Costa Rica and Panama, respectively. Our results showed the difficulties with the *Pratylenchus* spp. identification and the importance of integrating morphological and molecular markers using specimens from the topotype populations. In our case, we increased the number of root-lesion nematode species detected in Costa Rica, where only *P. coffeae* (Avelino et al. 2009) and *P. gutierrezi* (Golden et al. 1992; Inserra et al. 1998) had been reported. We reported for the first time the presence of *P. bolivianus* and *P. pseudocoffeae* in Costa Rica. *Pratylenchus zeae* was detected in rice for the first time in Costa Rica, but this nematode had already been reported in sugar cane in the same area of San Carlos, Alajuela Province by López and Salazar (1990).

Our results demonstrate that the application of rRNA molecular markers integrated with morphological studies can help in the diagnosis and characterization of root-lesion nematode species. We used the topotype populations for clarifying the synonymy between *P. gutierrezi* and *P. panamaensis*. Our results demonstrated that both nematode species are a good example


28S
P. hippeastri (KC796703)
P. hippeastri (DQ988831)
Pratylenchus sp. H2 (GU214121)
P. hippeastri (FN554979)
Pratylenchus sp. H1 (GU131127)
Pratylenchus sp. H5 (GU131130)
Pratylenchus sp. H5 (GU131131)
P. paraflordensis (GU214114)
Pratylenchus sp. H6 (GU131126)
P. paraflordensis (GU214115)
Pratylenchus sp. H5 (GU131123)
Pratylenchus sp. H8 (GU214118)
Pratylenchus sp. H7 (GU214123)
P. flordensis (GU214116)
P. flordensis (GU214117)
P. araucensis (FJ463282)
P. araucensis (FJ463281)
P. jaehni (JN162387)
P. panamaensis (AF170441, K2 coffee, Guatemala)
P. panamaensis (EU130897, CA61 coffee, Guatemala)
P. panamaensis (KT971388, coffee, topotype, Panama)
P. panamaensis (KT971359, coffee, topotype, Panama)
P. panamaensis (EU130899, CA72 coffee, Guatemala)
P. panamaensis (AF170440, K1 coffee, Costa Rica)
P. scribneri (EU130865)
P. agilis (EU130841)
P. flexincisus (DQ988832)
P. flexincisus (DQ988833)
P. scribneri (AM084195)
P. pseudocoffeae (KT971360, mum, Costa Rica)
P. pseudocoffeae (AF170444)
P. gutierrezi (AF170442, K3 topotype, San Antonio, Costa Rica)
P. gutierrezi (KT971357, Cinco Esquinas, Costa Rica)
P. gutierrezi (KT971356, Cinco Esquinas, Costa Rica)
P. gutierrezi (KT971355, topotype, San Antonio, Costa Rica)
P. loosi (JN091970)
P. loosi (EF446997)
P. coffeae (KJ721979)
P. coffeae (KC766880)
P. coffeae (U47555)
P. speijeri (KJ698687)
P. speijeri (KJ698688)
P. speijeri (JN803941)
P. japonicus (KF385444)
P. japonicus (KF385445)
P. pratensis (AM231935)
P. pratensis (AM231929)
P. vulnus (JX261945)
P. vulnus (JQ922308)
P. pseudopratensis (JX261965)
P. kumamotoensis (JX144360)
P. crenatus (KJ493147)
P. crenatus (EU130853)
P. penetrans (EU130858)
P. penetrans (EU130892)
P. penetrans (JQ003987)
P. arlingtoni (AF307328)
P. fallax (AF284181)
P. convallariae (AF196351)
P. pinguicaudatus (KP289344)
P. pinguicaudatus (AJ545014)
P. dunensis (AM231943)
P. dunensis (AM231938)
P. oleae (KJ510859)
P. oleae (KJ510860)
P. oleae (KJ510856)
Apratylenchus binhi (EF645137)
Apratylenchus vietnamensis (EF645138)
Zygotylenchus guevarai (JX261956)
Zygotylenchus guevarai (FJ717823)
P. brachyurus (JN020923)
P. brachyurus (JN020921)
P. brzeskii (AM231911)
P. brzeskii (AM231928)
P. hispaniensis (FJ717822)
P. neglectus (JX261946)
P. neglectus (HM469438)
P. thornei (JX261954)
P. thornei (EU130881)
P. mediterraneus (AJ545021)
P. parazeae (KP903440)
P. parazeae (KF765435)
P. parazeae (KP903445)
P. parazeae (KF765434)
P. parazeae (KP903442)
P. delattrei (JX261949)
P. delattrei (JX261948)
P. zeae (AB933457)
P. zeae (AB933458)
P. zeae (KT971361)
P. zeae (JN020931)
Pratylenchus sp. ZTM-2013 (JX261959)
P. bhattii (JN244269)
P. bhattii (JN244270)
P. bolivianus (KP780255, China)
P. bolivianus (KP780256, China)
P. bolivianus (HM469436, China*
P. bolivianus (KT971354, Costa Rica)
Belonolaimus longicaudatus (DQ328710)
Psilenchus sp. (DQ328716)
Coslenchus costatus (DQ328719)
Basiria gracilis (DQ328717)
Boleodorus sp. (DQ328718)
0.2

◀ **Fig. 11** Phylogenetic relationships of root-lesion nematodes from Costa Rica within *Pratylenchus* spp. Bayesian 50 % majority rule consensus trees as inferred from D2- D3 expansion segments of 28S rRNA sequences alignments under the TVM + I + G model. Posterior probabilities more than 70 % are given for appropriate clades. Newly obtained sequences in this study are in bold font. *Scale bar* = expected changes per site

of cryptic speciation (Palomares-Rius et al. 2014a), since morphology and morphometrics are almost indistinguishable, including SEM studies, but rRNA markers (D2-D3 and ITS) confirmed two clearly separate taxa. As previously suggested, molecular phylogeny studies on D2-D3 expansion segments of 28S rRNA revealed close relationships between the genera *Pratylenchus*, *Zygotylenchus* and the newly erected genus *Apratylenchus* (Palomares-Rius et al. 2010). Major clades are highly correlated with the phylogenetic study done by Subbotin et al. (2008) and Palomares-Rius et al. (2014b). Although we agree with De Luca et al. (2010) suggesting that the ITS-containing region allows better discrimination among the closely related species studied because it has evolved faster than the D2-D3 expansion segments of 28S rDNA and has accumulated more substitution changes. Our results on D2-D3 regions were also congruent with ITS and clearly corroborate

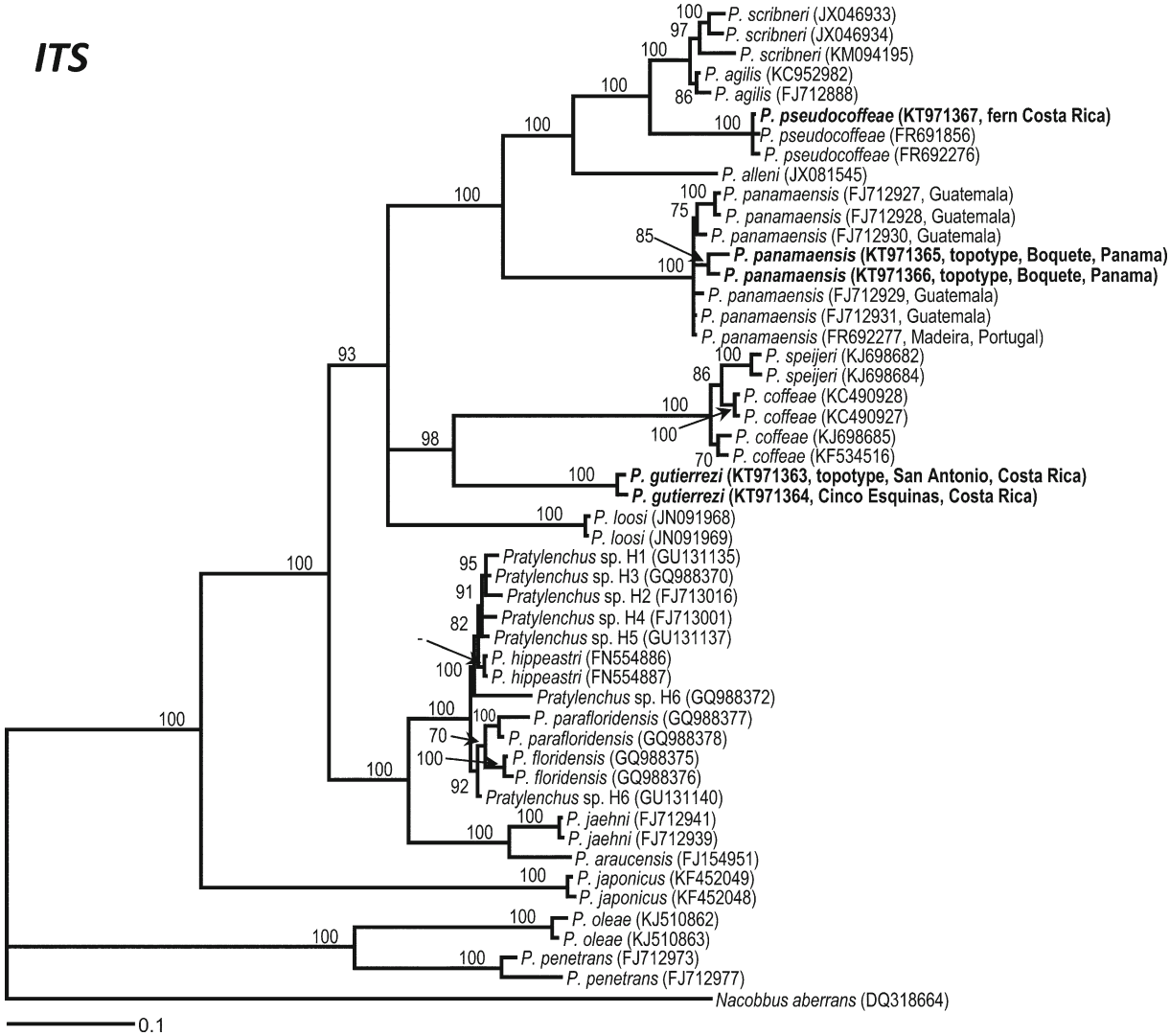


Fig. 12 Phylogenetic relationships of root-lesion nematodes from Costa Rica within *Pratylenchus* spp. Bayesian 50 % majority rule consensus trees as inferred from ITS sequence alignment under the GTR + I + G model. Posterior probabilities more than 70 % are given for appropriate clades. Newly obtained sequences in this study are in bold font. *Scale bar* = expected changes per site

the separation of *P. gutierrezi* and *P. panamaensis*. Furthermore, these data suggest that sequenced *Pratylenchus* isolates parasitizing coffee in Guatemala (K1, AF170440; K2, AF170441; CA61, EU130897; and CA72, EU130899-EU130898) and banana in Madeira, Portugal (FR692277), previously considered as isolates of *P. gutierrezi*, need to be considered as conspecific with *P. panamaensis*. Thus, *P. gutierrezi* and *P. panamaensis* need to be maintained as different valid taxa, and future research on reproductive fitness of these species in different host-plant can shed light on additional aspects on the biology of these species.

Although *P. coffeae* is the most widely reported root-lesion nematode species worldwide on coffee (Campos and Villain 2005), some research reported that different populations of *P. coffeae* can have different host ranges and suggested the existence of biotypes of *P. coffeae* based on differences in reproduction on coffee and citrus between two *P. coffeae* populations (Silva and Inomoto 2002). The present results suggest that other species with similar morphology may be infesting coffee soil growing areas, such as *P. gutierrezi* and *P. panamaensis*; thus, an accurate identification of the root-lesion nematodes attacking coffee in growing areas of Central and South America need to be a prerequisite for designing effective management strategies.

In summary, the present study establishes the importance of using polyphasic identification highlighting the time consuming aspect and difficulty of a correct identification at species level within the genus *Pratylenchus* spp. This study also provides molecular markers for a precise and unequivocal diagnosis of root-lesion nematodes parasitizing cultivated and ornamental plants in Costa Rica. And particularly, it clarifies that two clearly separate species of *Pratylenchus* are damaging coffee in Central America (*P. gutierrezi* and *P. panamaensis*), which need to be considered for future phytopathological, biological and ecological studies.

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