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Characterisation of amphimictic and parthenogenetic populations of *Pratylenchus bolivianus* Corbett, 1983 (Nematoda: Pratylenchidae) and their phylogenetic relationships with closely related species

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Summary – Amphimictic populations of root-lesion nematodes with numerous males and females having three lip annuli, a functional spermatheca and non-areolated lateral field occur on sword fern (*Nephrolepis exaltata*) in Florida. Identified for decades as *Pratylenchus penetrans*, they appeared to be a morphologically separated species on the basis of a longer stylet (17.8-18.3 μm) than *P. penetrans* (15-17 μm) and different lip pattern in *en face* view (rectangular *vs* dumb-bell in *P. penetrans*). Morphologically similar amphimictic root-lesion nematodes have also been detected on flax lily in Costa Rica. Subsequent morphological observations indicated that these amphimictic root-lesion nematodes from fern and flax lily are closely related to the parthenogenetic species *P. bolivianus*, which has areolated lateral fields. In spite of the reproductive and morphological dissimilarities between these populations, their separation into different species was not supported by the results of molecular analyses of their DNA sequences. The populations used in these analyses included those that are amphimictic from Florida and Costa Rica and others that are parthenogenetic from the type locality in Bolivia, and geographically distant localities in Chile, China, Colombia and Europe. Phylogenetic analyses of the ITS and D2-D3 expansion

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segments of the 28S rRNA gene indicated that they belong to the same species, *P. bolivianus*, which consists of two morphotypes, *P. bolivianus* (am) amphimictic and *P. bolivianus* (pm) parthenogenetic, herein described and illustrated. Contradictory results were obtained by the analyses using a portion of the *hsp90* gene. The phylogenetic study, which included sequences of other root-lesion nematodes, a topotype and geographical distant populations of *P. zeae*, revealed that *P. bolivianus* and *P. zeae* formed highly supported clades in the majority consensus trees. PCR with species-specific primers for rapid diagnostics of *P. bolivianus* and *P. zeae* were developed and tested.

Keywords – 28S rRNA gene, Bolivia, Chile, China, Colombia, Costa Rica, *coxI* mtDNA, D2-D3, Europe, *hsp90* gene, ITS rRNA, molecular, morphology, morphometrics, morphotypes, *Nephrolepis exaltata*, *Phormium* sp., phylogeny, *Physalis peruviana*, plant-parasitic nematode, *Pratylenchus zeae*, root-lesion nematode, SEM, taxonomy, USA.

Cut foliage and fern production are important components of the ornamental industry of Florida. Many ferns are used in the state as decorative greens or foliage ornamentals in gardens and parks. Sword fern, *Nephrolepis exaltata* (L.) Schott. Stiff., is a common fern propagated from stolons of older plants kept in green beds in many gardens or in nurseries for the production of hanging baskets. Decline symptoms consisting of stunting, greying foliage and chlorosis have been reported in Florida sword fern operations and have been attributed to root-lesion nematodes (*Pratylenchus* sp.) (Henley *et al.*, 2014). *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941 has been considered the most common causal agent involved in the decline of fern species such as leatherleaf fern, *Rumohra adiantiformis* (Forst.) Ching, in Florida fern operations (Rhoades, 1968; Stokes & Laughlin, 1970; Hamlen, 1978; Kaplan & Osborne, 1986; O'Bannon *et al.*, 1988). The identification of this root-lesion nematode on fern has been based mainly on morphological analyses without any corroboration by molecular analyses.

In 2013, an infestation of a root-lesion nematode was detected in a sword fern operation in central Florida. The infestation was localised to beds of 3- to 4-year-old declining sword fern stock plants. The morphology of the lesion nematode extracted from the roots, although fitting that of *P. penetrans* on the basis of the presence of abundant males in addition to females with three distinct lip annuli, lateral field not areolated and a conoid tapered tail with a sub-hemispherical terminus, showed some differences such as a longer stylet (17.8-18.3 μm) and an annulated tail terminus – the stylet is 15-17 μm long and the tail terminus smooth in *P. penetrans* (Corbett, 1973; Inserra *et al.*, 1979). Such discrepancies cast doubt about the reliability of this identification and prompted more accurate examinations of this root-lesion nematode from sword fern. During these studies, a morphologically similar amphimictic population was obtained from flax lily (*Phormium* sp.) plants intercepted in California in a plant shipment from Costa Rica. This population was also included in our analyses. Preliminary comparative morphological examination of these two root-lesion nematode populations with other species with three lip annuli described in the literature (Castillo & Vovlas, 2007) indicated that they were closely related to the parthenogenetic *P. bolivianus* Corbett, 1983, a species with areolated lateral fields. However, the reproductive and morphological dissimilarities between the two populations and *P. bolivianus* were not considered sufficient characters for the designation of the two populations as a new species distinct from *P. bolivianus* without validation by molecular analyses.

Pratylenchus bolivianus was originally described from specimens collected from soil around the roots of oats and potato at an altitude of *ca* 3000 m a.s.l. in the Bolivian Andes (Corbett, 1983), *P. australis* Valenzuela & Raski, 1985, a species described from tundra soil on Hoste Island, Chile, being considered as a junior synonym (Frederick & Tarjan, 1989; Cotten *et al.*, 1991; Castillo & Vovlas, 2007). *Pratylenchus bolivianus*, a parthenogenetic Andean root-lesion nematode, was found in the UK for the first time in 1989 in glasshouse-grown ornamental *Alstroemeria* spp. originating from South America. Other records were from The Netherlands, where the nematode was found to parasitise tomato and carnation, causing damage equivalent to that induced by *P. penetrans* (Cotten *et al.*, 1991). Initial damaging population densities for *Alstroemeria* cv. Jubilee were estimated to be 24 *P. bolivianus* (100 cm^3 soil)$^{-1}$ (Amsing, 1996). This species was detected and identified as *P. australis* in Florida, in 1996, by Robert Esser on heather, *Erica persoluta* L., imported from California (Lehman, 2002). *Pratylenchus bolivianus* was also reported and molecularly characterised from Chile (De Luca *et al.*, 2011), the UK (Waeyenberge *et al.*, 2000, unpubl.), China (Wang *et al.*, unpubl.) and recently in Costa Rica on leatherleaf fern *R. adiantiformis* (Zamora Araya *et al.*, 2016). Populations of this root-lesion nematode in declining Cape gooseberry,

Physalis peruviana L., have been reported in Colombia by Múnera Uribe (2015). These Colombian populations were preliminarily identified as *P. bolivianus* by one of the authors (T. Janssen, unpubl.) who obtained a parthenogenetic population from this host. This Colombian population of *P. bolivianus* and others obtained from the type locality in Bolivia and other localities in Costa Rica and Europe were used in this study to determine the correct identity of these populations from flax lily and sword fern in Costa Rica and Florida, respectively.

In Florida, sword ferns, and especially leatherleaf ferns, are grown in pasture lands under shade cloth or under the canopy of oak trees. These sites are infested with many species of root-lesion nematode parasites of grasses such as *P. brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941, *P. hippeastri* Inserra, Troccoli, Gozel, Bernard, Dunn & Duncan, 2007 and also *P. zeae* Graham, 1951, a species with three lip annuli as in the populations from fern. The presence of these nematodes in the land where fern nurseries are established may complicate the identification of the species parasitising fern and their differentiation from those that parasitise grasses.

In order to clarify the identity of the amphimictic *Pratylenchus* from fern and their relationship with *P. bolivianus* and *P. zeae*, a study was conducted with the following objectives: *i*) to characterise morphologically and molecularly the amphimictic root-lesion nematode populations from sword fern and flax lily; *ii*) to compare the morphology and DNA sequences of these populations with parthenogenetic populations of *P. bolivianus* from the type locality and other geographical areas to confirm or disprove their co-specificity; *iii*) to characterise morphologically and molecularly *P. zeae* populations from the type locality and other distant geographic areas; and *iv*) to reconstruct phylogenetic relationships among these root-lesion nematodes and other related species using D2-D3 expansion segments of 28S rRNA gene, ITS of rRNA gene sequences, a portion of the *hsp90* gene and *coxI* mtDNA.

Materials and methods

Nematode populations

The population considered to be *P. penetrans* was collected from sword fern in a central Florida fern operation. Other root-lesion nematode species and populations, including *P. bolivianus* and *P. zeae*, were obtained from distant geographical areas and hosts (Table 1). Topotype populations of *P. bolivianus* and *P. zeae* were obtained from Toralapa, at high elevation in Bolivia and the Pee Dee Experiment Station, Florence, SC, USA, respectively. Soil and root samples from the sword fern operation were collected with a sampling scoop from the fern beds on elevated benches. Samples from other localities were collected with sampling tubes from the upper 10-40 cm soil surrounding the rhizosphere of different hosts. Nematodes from sword fern were mainly extracted from roots by incubation in jars (Young, 1954). The other nematode populations were extracted from soil by a rapid centrifugal-flotation method (Jenkins, 1964). Root-lesion nematodes from sword fern in Florida, flax lily in Costa Rica, Cape gooseberry in Colombia and corn in South Carolina were used for morphological examination by light (LM) and scanning electron (SEM) microscopy and molecular analyses. A topotype *P. zeae* population from South Carolina was used for both morphological and molecular analyses. The remaining *P. zeae* populations from other geographical areas were used only for molecular analyses. Other *Pratylenchus* species, listed in Table 1, were sequenced and used in the molecular study. Some of these species, such as *P. neglectus* (Rensch, 1924) Filipjev & Schuurmans Stekhoven, 1941 and *P. thornei* Sher & Allen, 1953, were collected in Bolivia in the type locality of *P. bolivianus*. Two unidentified *Pratylenchus* species included in this study were from Kansas (USA) and Thailand. The other remaining species used in this study had been sequenced and characterised by Subbotin *et al.* (2008). The morphometric characters reported for a *P. bolivianus* populations from Costa Rica (Zamora Araya *et al.*, 2016) were included in this study in order to provide a complete representation of the variability of the morphological characters of this species. This population, however, was not included in Table 1 since its sequences were not used for phylogenetic analyses.

Females and males of Colombian, Costa Rican and Floridian populations of *P. bolivianus* mounted on glass slides are deposited in the nematode collection at the CNR, Istituto per la Protezione Sostenibile delle Piante (IPSP; slides: IPSP-L1101 to L1128), Bari office, Italy, and in nematode collections at CDFA, Sacramento, CA, USA.

Specimens for SEM study

Live specimens of root-lesion nematodes from fern, flax lily and Cape gooseberry, and also of *P. zeae* topotypes, were immobilised by gently heating and then mounted in water agar on a slide for measurement and

Table 1. Species and populations of the *Pratylenchus* spp. used in the present study.

Species	Location	Host	Sample code	GenBank accession number				Reference or source
				ITS rRNA	D2-D3 28S rRNA	*coxI* mtDNA	*hsp*90	
P. bolivianus (am)	Costa Rica, Alajuela (intercepted by CDFA, USA)	*Phormium* sp.	CD1032	KU198985	KU198952	KU198947	KU198968	S.A. Subbotin
P. bolivianus (pm)	Colombia	*Physalis* sp.	664; CD1855	KU198991	KU198958	–	KU198967	G.E. Múnera Uribe, T. Janssen
P. bolivianus (am)	USA, Florida, Winter Garden	*Nephrolepis exaltata*	CD1367	–	KU198953	–	KU198965, KU198966	L. Violett, R.N. Inserra
P. bolivianus (pm)	Belgium, East-Flanders	*Alstroemeria* sp.	BE; CD1857	KU198987-KU198989	KU198954	–	KU198970	L. Waeyenberge
P. bolivianus (pm)	UK, West Sussex	*Alstroemeria* sp.	UK	FJ712892-FJ712896, KU198990	KU198955, KU198959	–	–	L. Waeyenberge
P. bolivianus (pm)	Bolivia, Toralapa (topotype)	*Solanum tuberosum*	NAC; CD1858	KU198986	KU198956, KU198957, KU198960	–	KU198969	L. Waeyenberge, J. Franco
P. crenatus	UK	*Hordeum vulgare*	CA81	–	EU130853	KU198946	KU198971	P. Roberts, Subbotin *et al.* (2008)
P. coffeae	Japan, Shizuoko	*Camellia sinensis*	CA101	–	–	KU198943	–	P. Roberts, Subbotin *et al.* (2008)
P. coffeae	Japan, Kumamoto	*Ipomoea batata*	CA97	–	–	KU198942	–	P. Roberts, Subbotin *et al.* (2008)
P. neglectus	Bolivia	Unknown	CD1735	–	KU198962	KU198940	–	J. Franco
P. neglectus	USA, California, Davis	*Hordeum vulgare*	CA94	–	EU130855	KU198941	–	P. Roberts, Subbotin *et al.* (2008)
P. penetrans	USA, California	*Vigna unguiculata*	CA91	–	EU130863	–	KU198974	P. Roberts, Subbotin *et al.* (2008)
P. thornei	Bolivia	Unknown	CD1736	–	KU198961	KU198938	–	J. Franco
P. thornei	Moldova	Unknown	CA74	–	–	KU198939	–	L. Poiras, Subbotin *et al.* (2008)

Table 1. (Continued.)

Species	Location	Host	Sample code	GenBank accession number				Reference or source
				ITS rRNA	D2-D3 28S rRNA	*coxI* mtDNA	*hsp*90	
P. zeae	USA, South Carolina, Florence, Pee Dee Experiment Station (topotype)	*Zea mays*	CD648, CD649	KU198975-KU198977, KU198979, KU198980	–	KU198935, KU198937	KU198973	P. Agudelo
P. zeae	USA, Maryland, Wye river eastern shore	*Zea mays*	–	–	KU198950	–	–	L. Waeyenberge, L. Carta
P. zeae	South Africa	*Saccharum officinarum*	–	–	KU198951	–	–	L. Waeyenberge, S. Berry
P. zeae	USA, Florida, Milton	*Miscanthus* sp.	CD531	KU198981, KU198982	–	KU198933	KU198972	R.N. Inserra
P. zeae	South Africa	Unknown	CA70	–	EU130893-EU130896	KU198936	–	S. Loots, Subbotin *et al.* (2008)
P. zeae	Suriname	Grasses	CD1856	KU198984	–	KU198934	–	T. Janssen, G. Karssen
P. zeae	Japan, Okinawa	Unknown	CA183; CA68	KU198978, KU198983	EU130889-EU130892	–	–	T. Mizukubo, Subbotin *et al.* (2008)
Pratylenchus sp.	USA, Kansas, Manhattan, Washington Marlatt Park	Grasses	CD871	–	KU198948	KU198945	KU198964	C. Blomquist, J. Stack
Pratylenchus sp.	Thailand (intercepted by CDFA, USA)	Unknown	CD1302	–	KU198949	KU198944	KU198963	S.A. Subbotin

photographs (Esser, 1986). Additional measurements and drawings were made using specimens killed and fixed in hot aqueous 2% formaldehyde + 1% propionic acid, dehydrated in ethanol vapour and mounted in dehydrated glycerin (Hooper, 1970). Measurements of specimens were made with an ocular micrometer and drawings with the aid of a *camera lucida*. Photographs were taken using cameras (Wild MPS 46/52 and Leica DFC 320) mounted on Nikon (Optiphot) and Leica DM 2500 compound microscopes.

Specimens for scanning electron microscope (SEM) observations were cold-fixed in glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2), post-fixed for 1 h in 2% osmium tetroxide, dehydrated in a graded series of ethanol, critical point-dried with CO_2 and sputter-coated with gold palladium (Eisenback, 1985; Chitambar, 1992). Nematodes were observed with a Hitachi S530 microscope at 15-20 kV accelerating voltage.

DNA EXTRACTION, PCR AND SEQUENCING

The DNA was extracted from several nematode individuals using proteinase K protocol as described by Subbotin *et al.* (2008). PCR and sequencing were completed in three laboratories: *i*) PPDC-CDFA, USA; *ii*) Ghent University, Belgium, and *iii*) ILVO, Belgium, and prepared as described by Tanha Maafi *et al.* (2003) or Waeyenberge *et al.* (2009). The following primer sets were used for PCR: the forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and the reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers (Subbotin *et al.*, 2006) for amplification of the D2-D3 expansion segments of 28S rRNA gene; the forward TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and the reverse AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) primers (Tanha Maafi *et al.*, 2003), or the forward Vrain2F (5′-CTT TGT ACA CAC CGC CCG TCG CT-3′) and the reverse Vrain2R (5′-TTT CAC TCG CCG TTA CTA AGG GAA TC-3′) for amplification of the ITS of rRNA gene; the forward JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and the reverse JB4 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) primers (Derycke *et al.*, 2010) for amplification of the partial *coxI* gene of mtDNA; the forward U831 (5′-AY AAR ACM AAG CCN TYT GGA C-3′) and the reverse L1110 (5′-TCR CAR TTV TCC ATG ATR AAV AC-3′) primers (Skantar & Carta, 2004) for amplification of the partial *hsp90* gene. The forward Hsp90F1-P_boliv (5′-TCC CGA TGA CAT TTC CAA TGA G-3′) and the reverse Hsp90R1-P_boliv (5′-CGG ACG TAG AGC TTG ATC GC-3′) primers were used for amplification of the partial *hsp90* gene of some *P. bolivianus*, if such samples failed with the U831 and L1110 primer set. The PCR products were purified using QIAquick (Qiagen) Gel or PCR extraction kits and submitted for direct sequencing or cloned using pGEM-T Vector System II kit (Promega). One or several clones were sequenced. The newly obtained sequences were submitted to the GenBank database under accession numbers KU198933-KU19899.

PCR WITH SPECIES-SPECIFIC PRIMERS

Species-specific primers for *P. bolivianus* and *P. zeae* were designed using the ITS rRNA gene sequence alignment. The specific primers for *P. bolivianus* – P-boliv_R1 (5′-ATA GCG CAC TGG CGC AGC ATA-3′) and *P. zeae* – P-zeae_R1 (5′-TAC GCA TAC RGT TCT GCT CAT-3′) were used in combination with the universal forward primer TW81. The PCR mixture was prepared as described by Tanha Maafi *et al.* (2003). The PCR amplification profile consisted of 4 min at 94°C; 30 cycles of 1 min at 94°C, 45 s at 57°C and 45 s at 72°C, followed by a final step of 10 min at 72°C. Of the PCR products 2 μl were run on a 1.4% TAE buffered agarose gel, stained and photographed. Several *Pratylenchus* samples were used to test the specificity of PCR with the newly designed species-specific primers.

PHYLOGENETIC ANALYSIS

The newly obtained D2-D3 of 28S rRNA, ITS of rRNA, *coxI* and *hsp90* gene sequences were aligned with corresponding published gene sequences (Duncan *et al.*, 1999; Subbotin *et al.*, 2008; De Luca *et al.*, 2010, 2011; Palomares-Rius *et al.*, 2010; Majd Taheri *et al.*, 2013; Wang *et al.*, 2015; Pili *et al.*, 2016, and others) using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters (gap opening 15.0 and gap extension 6.66) for protein coding genes or modified parameters (5.0 and 3.0) for rRNA genes. The alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the GTR + I + G model. BI analysis for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. After discarding burn-in samples, other trees were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Sequence analyses of alignments were also performed with PAUP* 4.0b 10 (Swofford, 2003).

Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment with adjustment for missing data.

Results

Nematode populations

A large number of specimens were extracted from sword fern roots. Their densities recorded during a period of 17 days of root incubation were 4-5 specimens (1 g of fresh roots)$^{-1}$. Unidentified stunt nematodes (*Tylenchorhynchus* sp.) were also associated with the lesion nematodes. Fewer amphimictic and parthenogenetic lesion nematodes were obtained from Costa Rica and Colombia. Seventy *P. zeae* topotype specimens (500 cm^3 soil)$^{-1}$ were recovered from soil samples.

Light and scanning electron microscopy studies

The morphological and morphometric features of the amphimictic population from sword fern were more similar to those of *P. bolivianus* than *P. penetrans*. However, the Florida population from sword fern and *P. bolivianus* differed in their reproductive habits (amphimictic with males present for the Florida population and parthenogenetic with males absent for *P. bolivianus* topotype from Bolivia), configuration of the lip pattern, body size, tail shape, and spermatheca size. Despite these morphological dissimilarities, the population from fern, the topotype and other populations of *P. bolivianus* were genetically identical according to the results of the molecular analyses reported in the following sections. This genetic similarity was observed also for the amphimictic population from Costa Rica. These molecular findings indicated that the lesion nematode parasitising sword fern in Florida, rather than being *P. penetrans*, is, in fact, representative of *P. bolivianus*. However, this Florida population and the other two from Costa Rica, including the amphimictic one found on leatherleaf fern (Zamora Araya *et al.*, 2016), are a morphological variant of this species, as represented in the original description by the parthenogenetic form. The morphology of the two populations of this amphimictic morphotype is herein described and indicated as *P. bolivianus* (am) to complement a recent description of another population from Costa Rica by Zamora Araya *et al.* (2016) and to distinguish it from the original parthenogenetic form, *P. bolivianus* (pm). A comparative polytomous tabular key, according to Castillo & Vovlas (2007), with alphanumeric matrix codes of the species used in the present study is also provided in Table 2.

Amphimictic *P. bolivianus* (am) morphotype: Florida population

(Figs 1, 2, 3A, C, F, H; 4A-F)

Measurements

See Table 3.

Description

Female

Body slender, almost straight in posture, small-sized. Lip region almost continuous with body contour, with three distinct annuli, first two often slightly narrower than basal annulus. First lip annulus usually thinner and narrower than second annulus in lateral view with light microscope. This lip configuration clearly shown under SEM examination. Partial fusion of first and second lip annuli observed in some specimens of Costa Rican population studied by Zamora Araya *et al.* (2016) resulting in a lip region configuration with two rather than three lip annuli. In *en face* SEM view, lip region appearing with dorsal and ventral submedian lip sectors fused together and also with oral disc in a rectangular-shaped configuration and separated on both sides from lateral sectors by an almost straight incisure forming an obtuse angle at oral disc level. This lip pattern arrangement fits that of Group 2 proposed by Corbett & Clark (1983) for *Pratylenchus* species. Prominent longitudinal ridge extending dorsoventrally from margins of subdorsal sectors to margins of subventral ones. Ridge consisting of two wedge-like cuticular structures with their apices directed toward stoma. Configuration of longitudinal ridge was not described in Costa Rican population by Zamora Araya *et al.* (2016), but was well shown in their lip region SEM micrographs of this population. Amphidial apertures oval, located between internal margins of lateral sectors and margins of fused oral disc with submedian sectors at level of stoma. Stylet robust, conus forming 50% of entire stylet length. Stylet shaft tubular and slender, basal knobs prominent, rounded, slightly anteriorly flattened. Pharyngeal procorpus cylindrical, slightly narrowing anteriorly to median bulb. Metacorpus round, with conspicuous central valve. Isthmus relatively short, enlarging in

Table 2. Polytomous key of amphimictic (am) and parthenogenetic (pm) morphotypes of *Pratylenchus bolivianus* and *P. zeae* populations used in the present study.

Species and source	Morphological character[a)]										
	Lip annuli	Presence of males	Stylet length (μm)	Shape of spermatheca	Vulva position (%)	PUS (μm)	Female tail shape	Female tail tip	Pharyngeal overlap (μm)	Lateral field	Lateral field structures
(am) *bolivianus* (fern, Winter Garden, FL, USA)	A2	B2	C3, 4	D2	E3	F4	G2, 3	H1, 2	I4	J1	K1[b)]
(am) *bolivianus* (flax lily, Alajuela, Costa Rica)	A2	B2	C3	D2	E3	F4	G2	H1	I2	J1	K1
(am) *bolivianus* (fern, San Isidoro, Heredia, Costa Rica)[c)]	A2	B2	C3, 4	D2	E3	F3, 4	G3	H2	I2	J1	K1
(pm) *bolivianus* (Cape gooseberry, Colombia)	A2	B1	C4	D1	E3	F4	G2	H1	I4	J1	K2
(pm) *bolivianus* (original description, Toralapa, Bolivia)	A2	B1	C4	D1	E3	F4	G3	H1	I2	J1	K2
zeae (corn, Florence, SC, USA)	A2	B1	C2	D2	E1	F4	G3	H3	I3	J1	K1

[a)] Morphological characters according to Castillo & 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.

[b)] A matrix code value of K1 was assigned to all of the populations of *P. bolivianus* (am), including that reported by Zamora Araya *et al.* (2016) with a K2 value, because the lateral field in the studied populations of this morphotype show the outer bands of the lateral field not areolated anterior from the vulva.

[c)] From Zamora Araya *et al.* (2016).

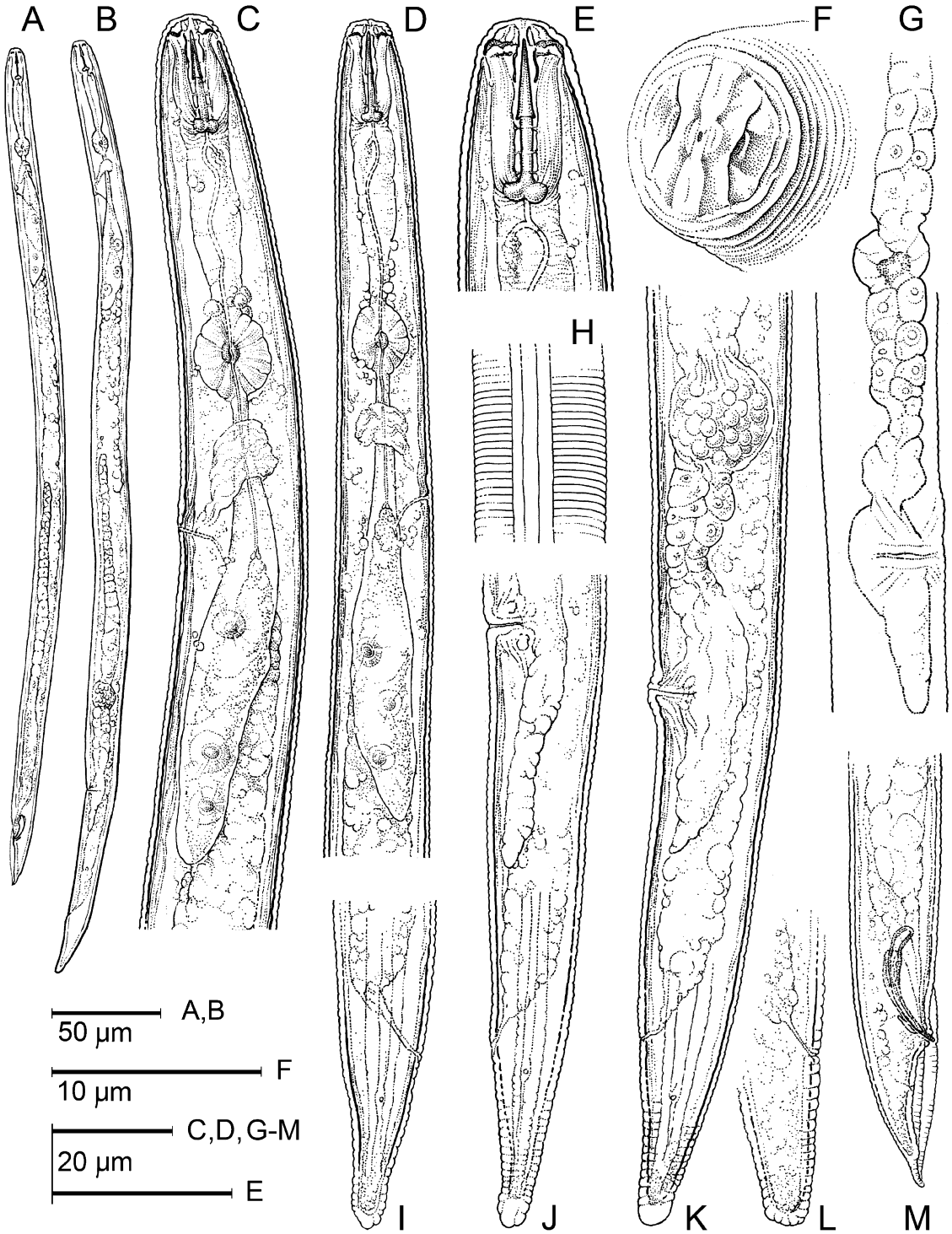
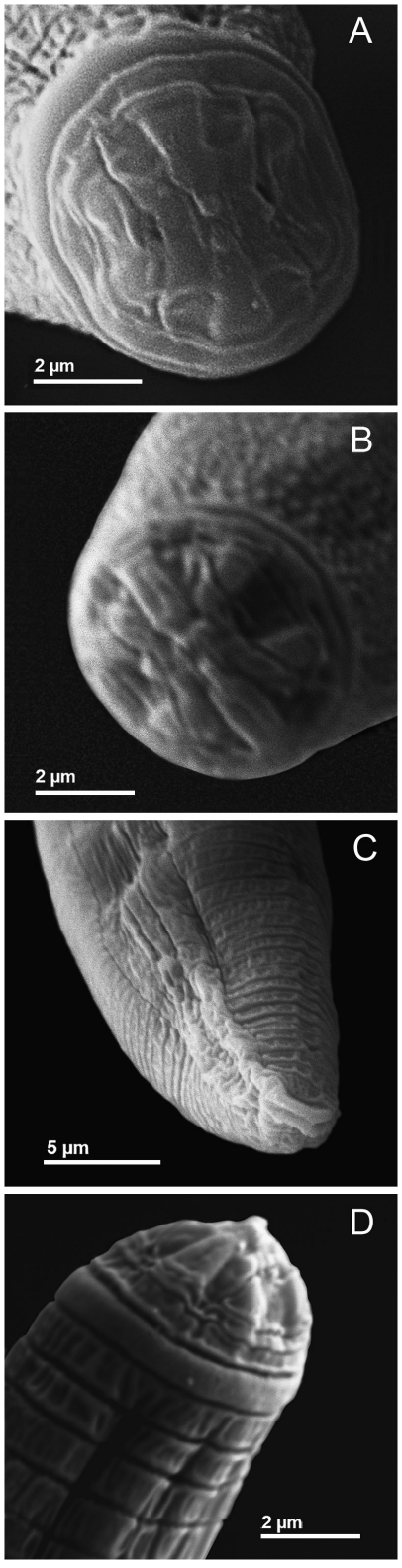


Fig. 1. *Camera lucida* line drawings of *Pratylenchus bolivianus* (am) morphotype from Florida. A, B: Female and male entire body; C: Female pharyngeal region; D: Male pharyngeal region; E: Female anterior region; F: *En face* view of female lip region, as seen at SEM; G: Posterior genital tract showing empty spermatheca; H: Female lateral field; I, J, L: Female tail; K: Female posterior region showing a large spermatheca full of sperm; M: Male tail.



a narrow, almost cylindrical gland lobe, overlapping intestine. Secretory-excretory pore located just posterior to hemizonid (2-3 annuli wide), at level of posterior end of isthmus. Body annulation clear, prominent, lateral field with four, smooth incisures at mid-body, inner two incisures merging posteriorly to phasmid. Outline of non-areolated outer bands becoming indented towards tail end between phasmid and tail tip. Indentations well shown in SEM micrographs of tail of Costa Rican population studied by Zamora Araya *et al.* (2016). Lack of areolations in outer bands at vulval level and toward mid-body confirmed in this amphimictic population. Ovary monoprodelphic, with oocytes arranged in a single row. Spermatheca large, round, full of sperm, in a few specimens appearing small and empty. Vulva posteriorly located, often framed by prominent lips. Post-uterine sac *ca* 1.5 vulval body diam. long, usually undifferentiated. Phasmids located just anterior to mid-tail. Tail markedly tapering towards end, conical to sub-cylindrical, sometimes clavate (14%). Tail terminus rather variable in shape, mostly sub-hemispherical, with striated (or irregularly annulated) margin, more rarely smooth. A truncate tail with striated (26%) or smooth terminus (16%) also encountered.

Male

Similar to female except in reproductive system, posterior end of body and in a slightly smaller body length (476 *vs* 533 μm). Anterior part of body more slender than in female. Lip region slightly higher than in female. Stylet less robust, with narrower knobs in cross section diam. with respect to female. Pharyngeal bulb small, ovate, isthmus slender, elongate, ending in a cylindroid, narrow glandular lobe. Lateral field with four plain lines. Testis outstretched and short, 40% of body length. Spicules paired, weakly cephalated and ventrally arcuate. Gubernaculum slightly curved. Tail conical, relatively short, enveloped by a slightly protruding crenate bursa.

Fig. 2. SEM morphology of *Pratylenchus bolivianus* (am) morphotype from Florida. A: Divided face pattern of female showing submedian sectors fused together and also with the oral disc in a rectangular-shaped configuration and crossed longitudinally by a cuticular ridge and separated on both sides from the lateral sectors by an almost straight incisure forming an obtuse angle at level of the oral disc; B: Schematic view of divided face pattern of female; C: Female tail; D: Female face lateral view showing the third lip annulus higher than the second and first.

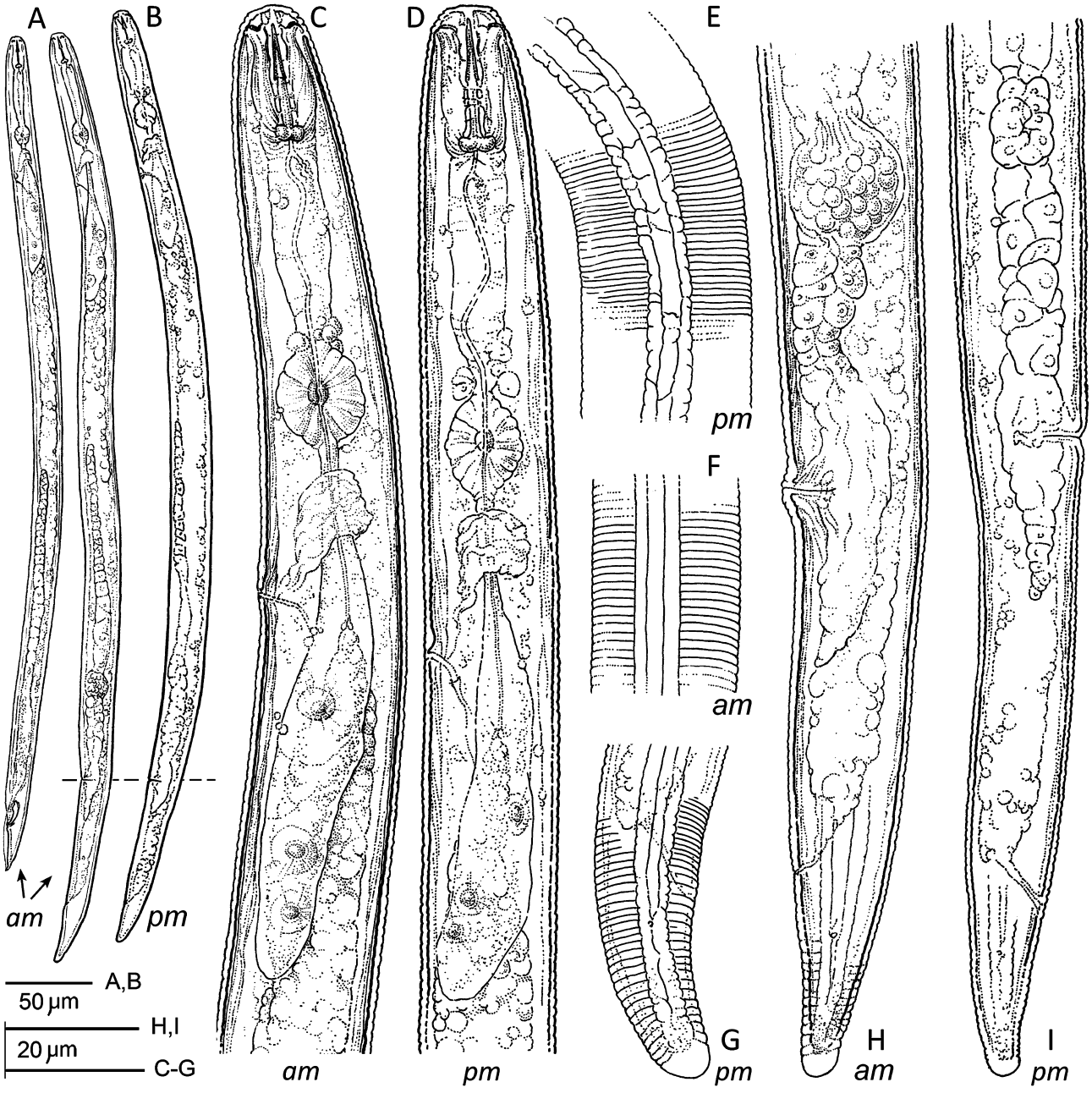


Fig. 3. *Camera lucida* comparative drawings showing differential characters between *Pratylenchus bolivianus* (am) morphotype from Florida (A, C, F, H) and *P. bolivianus* (pm) morphotype from Colombia (B, D, E, G, I). A, B: Entire body; C, D: Female pharyngeal region; E, F: Lateral field; G: Female tail; H, I: Female posterior region. Note the differences in the pattern of lateral field (areolated *vs* non-areolated in the am and pm morphotype, respectively) and in shape and size of spermatheca between the two morphotypes. Abbreviations: am = amphimictic morphotype; pm = parthenogenetic morphotype.

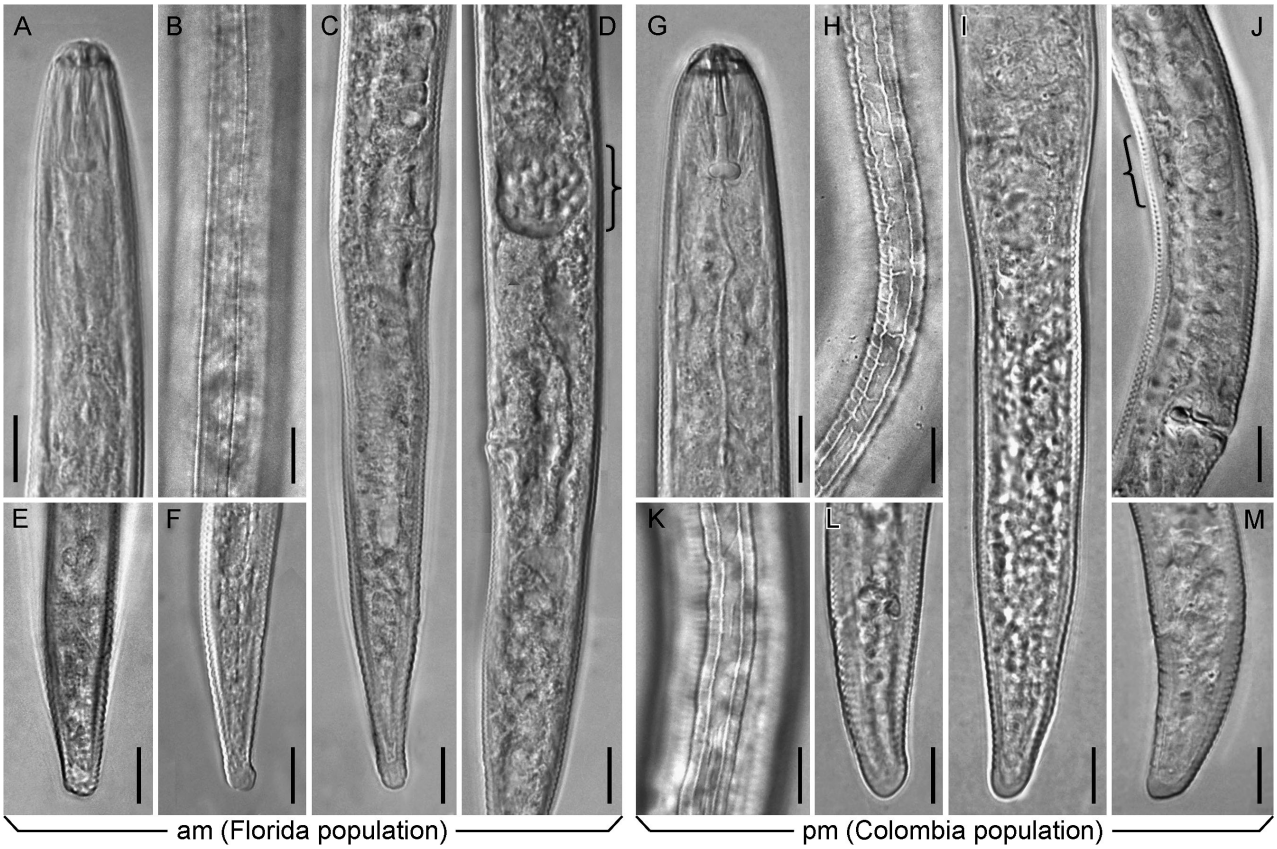


Fig. 4. Light micrographs showing differential characters between *Pratylenchus bolivianus* (am) morphotype from Florida (A-F) and *P. bolivianus* (pm) morphotype from Colombia (G-M). A, G: Female anterior region; B, H: Lateral field; C, I: Female posterior region; D, J: Female vulval region; E, F, L, M: Female tail. Note the large and full (D) *vs* small and empty (J) spermatheca (in curly bracket) and the female tail annulated *vs* smooth in the am and pm morphotypes, respectively. (Scale bars = 10 μm.)

Costa Rica population
(Fig. 5)

Measurements

See Table 3.

Remarks

The characteristics of both sexes of this amphimictic population from Costa Rica were in agreement with those of the Florida population and that described by Zamora Araya *et al.* (2016) in Costa Rica. However, females of this population have larger body dimensions than those of the Florida population (L = 634 (588-660) *vs* 533 (445-586) μm), larger spermatheca and shorter pharyngeal overlap, as also reported by Zamora Araya *et al.* (2016) for the leatherleaf fern population (38.1 (36.5-39.5) *vs* 64.5 (54-76.2) μm). Male spicules of this population were slightly longer (22 *vs* 18-20 μm).

Diagnosis

The populations of *P. bolivianus* (am) from Costa Rica and Florida are characterised mainly by presence of males, a large and functional spermatheca and by the lateral field with non-areolated outer bands for almost the entire body length with the exception of the tail where they are areolated. They differ from the parthenogenetic morphotype *P. bolivianus* (pm) by having a spermatheca large and full of sperm *vs* small and empty, a smooth lateral field *vs* areolated and with oblique striae in the middle band and tail tapering, often clavate with smooth to coarsely annulated terminus *vs* subhemispherical to truncate tail with a smooth terminus. The prominent longitudinal ridge that extends dorsoventrally from the

Fig. 5. Photomicrographs of specimens of *Pratylenchus bolivianus* (am) morphotype from Costa Rica. A: Entire female body; B-D: Female anterior region; E-G: Vulval and tail regions; H: Male anterior region; I: Male posterior region; J: Female lateral field; K: Female tail region. (Scale bars: A = 45 μm; B-D, H = 10 μm; E-G = 10 μm; I-K = 10 μm.)

margins of the subdorsal lip sectors to the margins of the subventral ones is narrower in the amphimictic population than in the parthenogenetic one. Some variability in these characters occurs. The matrix codes for these amphimictic populations of *P. bolivianus*, including that reported by Zamora Araya *et al.* (2016), are indicated in Table 2.

Table 3. Morphometrics of populations of the amphimictic morphotype of *Pratylenchus bolivianus* (am) from Winter Garden, FL, USA and from Costa Rica (CD 1032) compared with those of the parthenogenetic morphotype of *P. bolivianus* (pm) from Colombia (present study or reported in the literature) and Bolivia. All measurements are in μm and in the form: mean ± s.d. (range)[a)].

Character	Florida (am)			Costa Rica (am)		Colombia (pm)	Costa Rica (am) (Zamora Araya *et al.*, 2016)		Bolivia (pm) (Corbett, 1983)
	Female		Male	Female	Male	Female	Female	Male	Female
n[b)]	10 (live)	16 (fixed)	10 (fixed)	12 (fixed)	2 (fixed)	17 (fixed)	20 (fixed)	10 (fixed)	15 (fixed)
L	616 ± 45.3 (519-692)	533 ± 33.9 (445-586)	476 ± 43.4 (418-535)	634 ± 19.8 (589-660)	585, 525	540 ± 40.8 (455-590)	650 ± 54.6 (560-744)	512 ± 76.7 (427-639)	588 (531-629)
a	26.5 ± 2.4 (23.9-31.6)	27 ± 2.5 (21.5-31.7)	28.9 ± 2.9 (25.6-35.3)	26.0 ± 2.5 (20.9-30.8)	28.7, 25.7	25.1 ± 1.8 (21.6-27.9)	24.3 ± 3.5 (16.5-31.8)	25.7 ± 3.1 (21.8-31.1)	27 (26-29)
b	6.1 ± 0.5 (5.6-7.1)	5.6 ± 0.3 (5.0-6.3)	5.4 ± 0.3 (5.4-5.6)	6.1 ± 0.6 (5.3-7.7)	6.1, 5.5	5.3 ± 0.5 (4.4-6.1)	–	–	5.2 (3.9-5.9)
b′	3.7 ± 0.3 (3.4-4.3)	3.6 ± 0.2 (3.1-3.9)	3.4 ± 0.3 (2.9-3.9)	4.5 ± 0.5 (3.9-5.8)	4.3, 4.4	3.9 ± 0.3 (3.3-4.7)	4.8 ± 0.9 (3.6-6.7)	3.5 ± 0.3 (3.1-3.9)	4.1 (3.4-4.9)
c	19.8 ± 1.6 (17.8-23.1)	17.7 ± 1.6 (14.6-21.5)	19.5 ± 3.2 (16.6-27.6)	20.5 ± 2.8 (17.4-25.4)	14.9, 22.6	21.2 ± 1.9 (18.2-25.3)	20.4 ± 2.7 (15.0-25.4)	19.0 ± 1.7 (16.1-21.5)	19 (16-21)
c′	2.1 ± 0.3 (1.7-2.6)	2.3 ± 0.3 (1.6-3.1)	2.1 ± 0.2 (1.7-2.6)	2.1 ± 0.2 (1.6-2.6)	2.7, 1.7	1.9 ± 0.2 (1.5-2.2)	2.1 ± 0.4 (1.6-3.1)	2.0 ± 0.2 (1.6-2.2)	–
V	82.2 ± 1.4 (79.4-84.9)	81.9 ± 0.7 (80.5-83.6)	–	80.3 ± 1.9 (76-83)	–	81.8 ± 1.1 (80.1-83.8)	82.1 ± 1.4 (79-84)	–	81 (80-82)
G	32.8 ± 3.4 29.9-40.0)	32.2 ± 4.8 (24.4-40.9)	–	37.8 ± 7.7 (24-54)	–	34.0 ± 5.1 (28.3-49.3) (16)	18.1 ± 2.6 (14.4-22.8)	–	–
T	–	–	41.8 ± 5.6 (29.5-49.5)	–	39.2, 46.9	–	–	–	–
Stylet length	18.5 ± 0.5 (17.7-19.5) (11)	18 ± 0.2 (17.8-18.3)	17.4 ± 0.5 (17.0-18.3)	17.7 ± 1.0 (16.0-20.0)	18.4, 18.4	19.6 ± 0.6 (18.0-20.3) (14)	17.8 ± 0.7 (17.0-19.0)	16.1 ± 0.9 (14.5-17.0)	19 (17-20)
Stylet cone	9.6 ± 0.2 (9.4-10) (6)	9.4 ± 0.4 (8.9-9.9)	9.1 ± 0.4 (8.5-9.8) (7)	8.7 ± 0.5 (8.0-10.0)	9.2, 9.4	9.9 ± 0.6 (9.9-10.4) (14)	–	–	–
Stylet base	9.0 ± 0.2 (8.8-9.3) (6)	8.6 ± 0.4 (8.0-9.4)	8.0 ± 0.2 (7.8-8.5) (7)	9.1 ± 0.8 (8.0-11.0)	9.2, 9.0	10.3 ± 0.3 (9.9.10.7) (14)	–	–	–
Stylet knob width	4.8 ± 0.2 (4.4-5) (11)	4.5 ± 0.2 (4.2-5.0)	4.0 ± 0.2 (3.5-4.4)	4.7 ± 0.2 (4.0-5.0)	4.8, 4.8	5.5 ± 0.3 (5.3-6.0)	–	–	–
Stylet knob height	2.6 ± 0.3 (2.4-3.0) (11)	2.5 ± 0.2 (2.1-2.9)	2.1 ± 0.1 (2.0-2.4)	2.0 ± 0.5 (1.0-3.0)	1.6, 2.4	3.0 ± 0.6 (2.7-5.0)	–	–	–

Table 3. (Continued.)

Character	Florida (am)			Costa Rica (am)		Colombia (pm)	Costa Rica (am) (Zamora Araya *et al.*, 2016)		Bolivia (pm) (Corbett, 1983)
	Female		Male	Female	Male	Female	Female	Male	Female
DGO from stylet base	3.9 ± 0.4 (3.6-4.9) (11)	2.6 ± 0.4 (2.1-3.4)	2.8 ± 1.0 (2.0-4.4)	2.8 ± 0.6 (2.0-4.0)	2.4, 2.0	3.2 ± 0.5 (2.7-4.0) (16)	–	–	2.7-4
Anterior end to:									
centre of metacorpus	62 ± 4.5 (53-68)	57 ± 3.4 (52-63)	56 ± 4.0 (51-64)	63 ± 2.9 (58-68)	60, 59.2,	57 ± 3.4 (52-63)	–	–	–
cardia	100 ± 9.0 (80-113)	94 ± 5.6 (83-103)	94 ± 7.0 (84-104)	103 ± 8.7 (84-115)	95, 96	94 ± 5.6 (83-103)	–	–	–
end of pharyngeal gland lobe	170 ± 13.1 (139-184)	148 ± 6.5 (135-163)	142 ± 13.9 (126-161)	141 ± 13.9 (112-159)	137, 119	148 ± 6.5 (135-163)	141 ± 25.7 (105-198)	148 ± 8.6 (140-168)	–
secretory/ excretory pore	94 ± 8.4 (76-105)	84 ± 5 (74-93)	84 ± 6.4 (74-93)	103 ± 8.4 (83-115)	93, 91	84 ± 5 (74-93)	96 ± 9.6 (69-108)	81 ± 9.6 (67-94)	89-105
vulva	507 ± 36.2 (423-549)	436 ± 29 (366-490)	–	515 ± 23.6 (468-563)	–	436 ± 29 (366-490)	–	–	–
Pharyngeal overlap	64.5 ± 7.5 (54.4-76.2)	53.8 ± 6.2 (39.6-67.3)	48.3 ± 9.5 (34.4-66.3)	36.6 ± 12.1 (19.0-56.0)	–	53.8 ± 6.2 (39.6-67.3)	38.1 ± 1.3 (36.5-39.5)	–	18-49
Max. body diam.	23.3 ± 2.6 (20.7-28.7)	19.9 ± 1.7 (16.8-24.2)	16.7 ± 1.7 (14.8-19.8)	24.4 ± 2.4 (21.0-30.0)	20.4, 20.4	19.9 ± 1.7 (16.8-24.2)	–	–	–
Vulval body diam.	21.3 ± 2.2 (18.8-26.7)	17.8 ± 1.6 (15.8-20.7)	–	21.7 ± 1.6 (20.0-24.0)	–	17.8 ± 1.6 (15.8-20.7)	–	–	–
Anal body diam.	14.3 ± 1.0 (12.9-15.8)	12.5 ± 1.4 (9.9-14.8)	12 ± 1.2 (10.0-13.8)	14.7 ± 1.0 (13.0-16.0)	14.4, 13.6	12.5 ± 1.4 (9.9-14.8)	–	–	–
Lateral field width	6.7 ± 0.4 (5.9-6.9)	5.5 ± 0.5 (5.0-6.4) (9)	5.5 ± 0.6 (4.9-6.4) (7)	7.0 ± 1.2 (5.6-8.4) (5)	6.8, 6.4	7.7 ± 1.4 (5.3-9.3) (5)	–	–	–
Ovary length	141 ± 19.7 (120-179)	112 ± 22.5 (69-160)	–	–	–	–	–	–	–
Anterior genital tract length	202 ± 15.4 (186-238)	171 ± 25.3 (125-230)	202 ± 34.2 (129-246)	240 ± 50.9 (159-347)	229, 246	182 ± 34.4 (138-283) (16)	–	–	–
Spermatheca height	15.6 ± 3.1 (10.8-21.2) (11)	15.3 ± 4.3 (8.9-26.2)	–	26.6 ± 4.4 (20-35)	–	12 ± 2.1 (9.0-14.5) (12)	–	–	–

Table 3. (Continued.)

Character	Florida (am)			Costa Rica (am)		Colombia (pm)	Costa Rica (am) (Zamora Araya *et al.*, 2016)		Bolivia (pm) (Corbett, 1983)
	Female		Male	Female	Male	Female	Female	Male	Female
Spermatheca width	14.2 ± 1.5 (11.8-16.8) (11)	12.8 ± 2.6 (7.5-20.7)	–	18.1 ± 2.6 (15.0-24.0)	–	10.5 ± 1.9 (7.0-14.5) (12)	–	–	–
Sperm largest diam.	3.6 ± 0.7 (3.0-4.4) (4)	–	–	3.7 ± 0.3 (3.2-4.4)	–	–	–	–	–
Sperm smallest diam.	2.2 ± 0.3 (2.2-5.0) (4)	–	–	3.1 ± 0.3 (2.8-3.6)	–	–	–	–	–
Spermatheca vulva distance	45.9 ± 7.0 (30.6-56.4)	44.3 ± 8.1 (29.0-59.4)	–	–	–	38.0 ± 9.6 (22.5-47.5) (12)	–	–	–
Tail length	31.1 ± 3.0 (25.9-34.6)	30.2 ± 2.8 (24.7-34.6)	26.1 ± 1.6 (23.7-28.7)	31.3 ± 4.5 (23.0-37.0)	39.2, 23.2	25.5 ± 2.0 (22.5-28.5)	32.4 ± 4.5 (25.0-44.0)	27.4 ± 1.9 (24.2-29.7)	–
Number of tail annuli	23 ± 1.6 (22-27)	27 ± 2.9 (23-34)	27 ± 1.3 (26-29) (4)	19 ± 1.7 (16-22)	–	19 ± 2.5 (16-25)	19-25	–	–
Vulva to anus distance	78 ± 11 (66-104) (11)	66 ± 6.7 (55-78)	–	96 ± 13.4 (74-122)	–	74 ± 7.3 (62-91)	–	–	–
Vulva to tail terminus	109 ± 13.9 (96-142)	97 ± 7.4 (79-108)	–	127 ± 12.9 (108-159)	–	100 ± 3 (97-103)	–	–	–
PUS	30.3 ± 4.8 (25.2-39.8)	25.6 ± 5.8 (15.8-38.6)	–	27.7 ± 3.3 (22.0-33.0)	–	28.0 ± 5.2 (20.0-37.5)	31 ± 2.8 (25.0-38.0)	–	22-31
Spicule length	–	–	19.3 ± 0.7 (18.0-20.2)	–	22.0, 22.4	–	–	16.9 ± 0.7 (16.0-18.0)	–
Gubernaculum length	–	–	5.0 ± 0.6 (4.1-6.0)	–	6.4, 6.4	–	–	4.0 ± 1.1 (3.0-5.5)	–

[a] Abbreviations are defined in Siddiqi (2000).

[b] A number in parentheses after the range refers to the number of specimens measured when this is less than the headline number.

Parthenogenetic *P. bolivianus* (pm) morphotype

The characteristics of this morphotype were provided by Corbett (1983) in the original description of *P. bolivianus*. Another similar population was described from Chile by Valenzuela & Raski (1985) under the name of *P. australis*.

Colombia population
(Figs 3B, D, E, G, I; 4G-M)

Measurements

See Table 3.

An additional population of *P. bolivianus* (pm) was found in Colombia (Múnera Uribe, 2015). This population was initially identified molecularly by comparing its DNA sequences with those of topotype *P. bolivianus*. Subsequently, this population was also analysed morphologically.

Remarks

The morphological characters and measurements of this population were in agreement with those of the original description. However, female body dimensions were slightly smaller than those of *P. bolivianus* (pm) (540 (455-590) *vs* 588 (531-629) μm). Unlike the amphimictic populations, this population showed the characteristic areolated outer bands of the lateral field, and lacked males and a functional spermatheca. Developing oocytes were observed in the tricolumella in several individuals with an empty spermatheca, suggesting that this population can reproduce asexually by meiotic or mitotic parthenogenesis. These characters separate this population from those from Costa Rica and Florida. The morphological differences between *P. bolivianus* (pm) from Colombia and *P. bolivianus* (am) from Florida are illustrated in Figures 1, 3, and 4. The matrix code of this Colombian population is reported in Table 2.

Diagnosis and relationship of *P. bolivianus* (am)

Our findings complicate the morphological diagnosis and relationship of *P. bolivianus*. This species is both an amphimictic and parthenogenetic species characterised by a divided face with three lip annuli and lip patterns consisting of the submedian sectors fused together and with the oral disc in a rectangular configuration and separated on both sides from the lateral sectors by an almost straight incisure forming an obtuse angle at oral disc level. A distinctive feature of *P. bolivianus* (am) is the presence of a prominent longitudinal ridge extending from the margins of the subdorsal sectors to the margins of the subventral ones. The basal lip annulus is slightly higher than the first two annuli. This morphotype has females with a large, oval or round functional spermatheca and a tail terminus rather variable in shape, mostly subhemispherical or truncate, with a striated (or irregularly annulated) margin, more rarely smooth. The diagnosis of *P. bolivianus* (pm) was well elucidated by Corbett (1983). The morphological differences between the 'am' morphotype and 'pm' morphotype are well reflected by their matrix codes, which differ in the values of B, D, I and K (Table 2). Among the amphimictic *Pratylenchus* species with three lip annuli and a divided face, nine are morphologically similar to *P. bolivianus* (am). They include: *P. fallax* Seinhorst, 1968, *P. hispaniensis* Palomares-Rius, Castillo, Liébanas, Vovlas, Landa, Navas-Cortés & Subbotin, 2010, *P. mediterraneus* Corbett, 1983, *P. penetrans*, *P. pratensis* (de Man, 1880) Filipjev, 1936, *P. pseudofallax* Café-Filho & Huang, 1989, *P. pseudopratensis* Seinhorst, 1968, *P. unzenensis* Mizukubo, 1992, and *P. vulnus* Allen & Jensen, 1951. *Pratylenchus bolivianus* (am) differs from *P. fallax*, *P. mediterraneus*, *P. penetrans*, *P. pratensis*, *P. pseudofallax* and *P. unzenensis* by the rectangular configuration of the fused submedian lip sectors with the oral disc *vs* dumbbell or pandurate configuration in these species. *Pratylenchus bolivianus* (am) has a longer stylet of 17.8-18.3 μm whereas high values in stylet length range do not exceed 17 μm in all of the other named species. It also differs from *P. pseudopratensis* by the rectangular configuration of the fused submedian lip sectors with the oral disc, which are separated from the lateral sectors, whereas in *P. pseudopratensis* the oral disc is distinct from the fused submedian and lateral lip sectors and shows a stoma surrounded by a doughnut-like cuticular ring. The lip pattern configuration of *P. bolivianus* (am) is very similar to that of *P. hispaniensis*. However, *P. hispaniensis* lacks the cuticular ridge that longitudinally crosses the fused submedian sectors with the oral disc and also has a shorter stylet than *P. bolivianus* (am) (15.3 (14.5-17) *vs* 18 (17.8-18.3) μm). Finally, *P. bolivianus* (am) differs from *P. vulnus* by the lip pattern (rectangular *vs* dumbbell-shaped in *P. vulnus*), and also by the subhemispherical or truncate tail terminus with a striated margin *vs* bluntly or finely pointed with a smooth margin. A group of species, in-

cluding *P. bhatti* Siddiqi, Dabur & Bajaj, 1991, *P. convallariae* Seinhorst, 1959, *P. ekrami* Bajaj & Bhatti, 1984, *P. kasari* Ryss, 1982, *P. kralli* Ryss, 1982, *P. lentis* Troccoli, De Luca, Handoo & DiVito, 2008, *P. manaliensis* Khan & Sharma, 1992, *P. pratensisobrinus* Bernard, 1984, *P. subpenetrans* Taylor & Jenkins, 1957, *P. sudanensis* Loof & Yassin, 1971, *P. typicus* Rashid, 1974, and *P. ventroprojectus* Bernard, 1984, share with *P. bolivianus* (am) similar reproductive habits and a presence of three lip annuli. However, the configuration of their lip pattern is not known. *Pratylenchus bolivianus* (am) differs from *P. bhatti*, *P. convallariae*, *P. ekrami*, *P. kasari*, *P. kralli*, *P. lentis*, *P. manaliensis*, *P. pratensisobrinus*, *P. subpenetrans*, *P. sudanensis*, *P. typicus* and *P. ventroprojectus* by the longer stylet of 18 (17.8-18.3) *vs* 13.5 (13.0-14.0), 16-17, 12 (11-13), 16.0-17.5, 14-15, 16.3 (15.5-17.0), 14-16, 16 (15-17), 16.0 (15.0-16.5), 14-16, 15-17, 15 (14-16) μm, respectively. Some of these species, such as *P. convallariae*, *P. kasari*, *P. lentis*, *P. pratensisobrinus* and *P. typicus*, have the high range values of stylet length overlapping the low range values of *P. bolivianus* (am). This amphimictic morphotype differs from them by the subhemispherical or truncate tail terminus with striated margins *vs* truncate and annulated in *P. convallariae* and *P. lentis*, finely pointed in *P. kasari*, subhemispherical and coarsely annulated in *P. pratensisobrinus*, and bluntly pointed in *P. typicus*.

The results of the phylogenetic analysis of these species in the following sections reflect only in part the morphological similarities among *P. bolivianus* (am) and the *Pratylenchus* species mentioned above. A species morphologically and biologically different from *P. bolivianus* (am), but genetically related, is *P. zeae*, which occurs commonly in Florida where fern operations are established. The morphological details of a topotype population of *P. zeae* were studied concomitantly during this work.

Morphological characters of a topotype population of *P. zeae*

Pratylenchus zeae Graham, 1951
(Fig. 6)

Pratylenchus zeae was described by Graham (1951) from soil and roots of corn (*Zea mays* L.) and since then has been reported from many tropical and subtropical countries in Africa, Asia, the Americas and Australia from many different hosts, including agronomic and industrial crops (Castillo & Vovlas, 2007). The morphological description by LM and SEM of this species from populations geographically distant from the type locality is well documented (Baujard *et al.*, 1990; Castillo & Vovlas, 2007). However, there are no reports of SEM observations of *P. zeae* type populations. The results of our examination of a topotype population are included in this section.

Measurements

See Table 4.

Description

Body slender, almost straight in posture, small-sized. Lip region almost continuous with body contour, with three annuli not distinct. In *en face* view with SEM, lip region appearing plain, smooth, with all labial sectors fused together and with dorsal and ventral portions of oral disc. This arrangement of lip pattern fits that of Group 1 proposed by Corbett & Clark (1983) for *Pratylenchus* species. In our specimens, lateral margins of oral disc were distinct, delimiting inner side of oval amphidial apertures, which were distinct in labial plate and occluded by debris. Stoma located in middle of oral disc, surrounded by a doughnut-like cuticular ring. Labial sensilla indistinct. Three lip annuli and an incomplete fourth annulus posterior to first annulus (labial plate) distinct and well separated from first body annulus. Stylet conus forming *ca* 50% of entire stylet length. Stylet shaft tubular, basal knobs rounded, slightly anteriorly flattened. Pharyngeal procorpus cylindrical, slightly narrowing anteriorly to median bulb. Metacorpus round, with conspicuous central valve. Isthmus relatively short, enlarging in a narrow, almost cylindrical gland lobe, overlapping intestine for *ca* twice body diam. at cardia level. Secretory-excretory pore located just posterior to hemizonid (two annuli wide), at level of posterior end of isthmus. Body annulation clear, prominent, lateral field with four, smooth incisures at mid-body. Outline of outer bands smooth, except on tail end posterior to phasmids where indented. Ovary monoprodelphic, rather short, with oocytes arranged in a single row. Spermatheca round, small and empty. Vulva anteriorly located at *ca* 72% of body length. Post-uterine sac of *ca* same length as body diam., usually undifferentiated. Phasmids located at level of, or just anterior to, mid-tail, 16 μm from tail tip. Tail conical, ending in a finely pointed terminus.

Male

Not found.

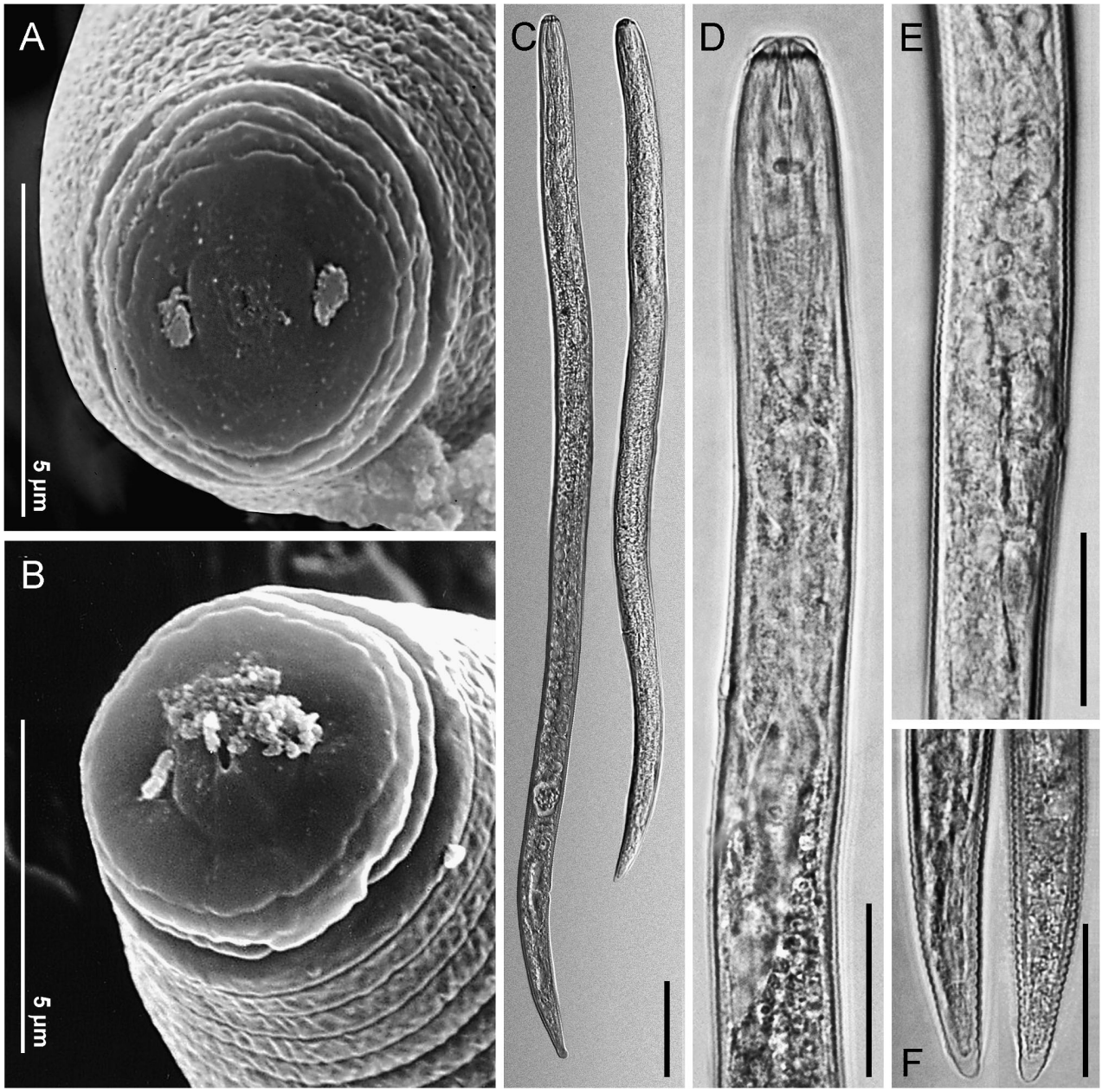


Fig. 6. SEM morphology and light micrographs of *Pratylenchus zeae*. A: Topotype female from Florence, South Carolina, showing undivided face pattern with all labial sectors fused together and with oral disc. An incomplete fourth annulus is visible under the first lip annulus; B: Female from St Augustine, Florida; C: Entire female body of *P. bolivianus* (am) from Florida (left) and *P. zeae* topotype (right). Note the more anterior vulva position in *P. zeae*; D: Topotype female pharyngeal region; E: Topotype female genital tract; F: Topotype female tail (Scale bars: C = 50 μm; D-F = 20 μm.)

Remarks

The morphometric values of our topotype specimens compare very well with those of the original description by Graham (1951) and revised values by Sher & Allen (1953). The configuration of the lip pattern of our topotype specimens matches that reported by Baujard *et al.* (1990) for a *P. zeae* population from millet in Mali.

Table 4. Morphometrics of a population of *Pratylenchus zeae* from the type locality, Florence, SC, USA, compared with those of the original description and subsequent revision. All measurements are in μm and in the form: mean ± s.d. (range)[a].

Character	Topotype (SC) USA	After Graham (1951)	After Sher & Allen (1953)
	Female		
n[b]	11 (fixed)		
L	413 ± 37 (350-470)	396-660	360-580
a	25.7 ± 2.5 (20.3-29.3)	20-25	25-30
b	5.9 ± 0.6 (5.1-7.3)	–	5.4-8.0
b′	4.0 ± 0.4 (3.5-5.0)	–	–
c	14 ± 1.8 (12.1-18.5)	–	17-21
c′	2.8 ± 0.3 (2.5-3.4)	–	–
V	72.5 ± 2.6 (68.9-77.3)	–	68-76
G	26.7 ± 5.0 (17.0-33.8)		
Stylet length	15.5 ± 0.5 (15.0-16.0)	16-18	15-17
Stylet cone	8.3 ± 0.4 (7.6-8.9)	–	–
Stylet base	7.3 ± 0.5 (6.5-8.0)	–	–
Stylet knob width	4.2 ± 0.2 (3.9-4.5)	3.9-5.1	–
Stylet knob height	2.0 ± 0.2 (1.8-2.4)	1.9-3.4	–
DGO from stylet base	1.9 ± 0.1 (1.8-2.1)	–	–
Anterior end to:			
centre of metacorpus	44 ± 3.4 (40-51)	–	–
cardia	69 ± 4.6 (63-78)	–	–
end of pharyngeal gland lobe	101 ± 7.5 (93-120)	–	–
hemizonid	69 ± 5.4 (62-75) (8)	–	–
secretory/excretory pore	71 ± 4.5 (66-80)	–	–
vulva	299 ± 30.2 (249-337)	–	–
Pharyngeal overlap	32.2 ± 4 (25.7-41.6)	–	–
Max body diam.	16.0 ± 1.6 (13.9-18.8)	–	–
Vulval body diam.	14.8 ± 1.2 (13.4-17.8)	–	–
Anal body diam.	10.3 ± 0.7 (9.0-11.4)	–	–
Anterior genital tract length	109 ± 22.5 (70-152)	133-144	–
Spermatheca height	7.7 ± 1.4 (5.9-9.9) (10)	–	–
Spermatheca width	7.9 ± 1.0 (6.8-9.9) (10)	–	–
Spermatheca vulva distance	28.9 ± 3.6 (25.7-37.6) (10)	–	–
Tail length	29.5 ± 3.1 (23.5-33.6)	–	–
Number of tail annuli	35 ± 3.8 (30-39)	18-22	–
Vulva to anus distance	82 ± 12.2 (63-101)	–	–
PUS	24.8 ± 2.3 (20.3-27.7)	–	–
Lateral field width	4.5 ± 0.2 (4.2-4.9)	–	–

[a] Abbreviations are defined in Siddiqi (2000).
[b] A number in parentheses after the range refers to the number of specimens measured when this is less than the headline number.

However, the population from Mali lacks the incomplete fourth lip annulus located below the labial plate that is observed in the topotype population. The morphological and morphometric characters of *P. zeae* differ from those of *P. bolivianus* (am and pm forms) in many features, including the configuration of the face (undivided and smooth *vs* divided), shorter stylet length (15.5 (15.0-16.0) *vs* 18.0 (17.8-18.3) μm), smaller V value (72.5 (68.9-77.3) *vs* 81.9 (80.5-83.6)%) and shape of tail terminus (finely pointed *vs* subhemispherical or truncate). The major character that they have in common is the presence of three lip annuli.

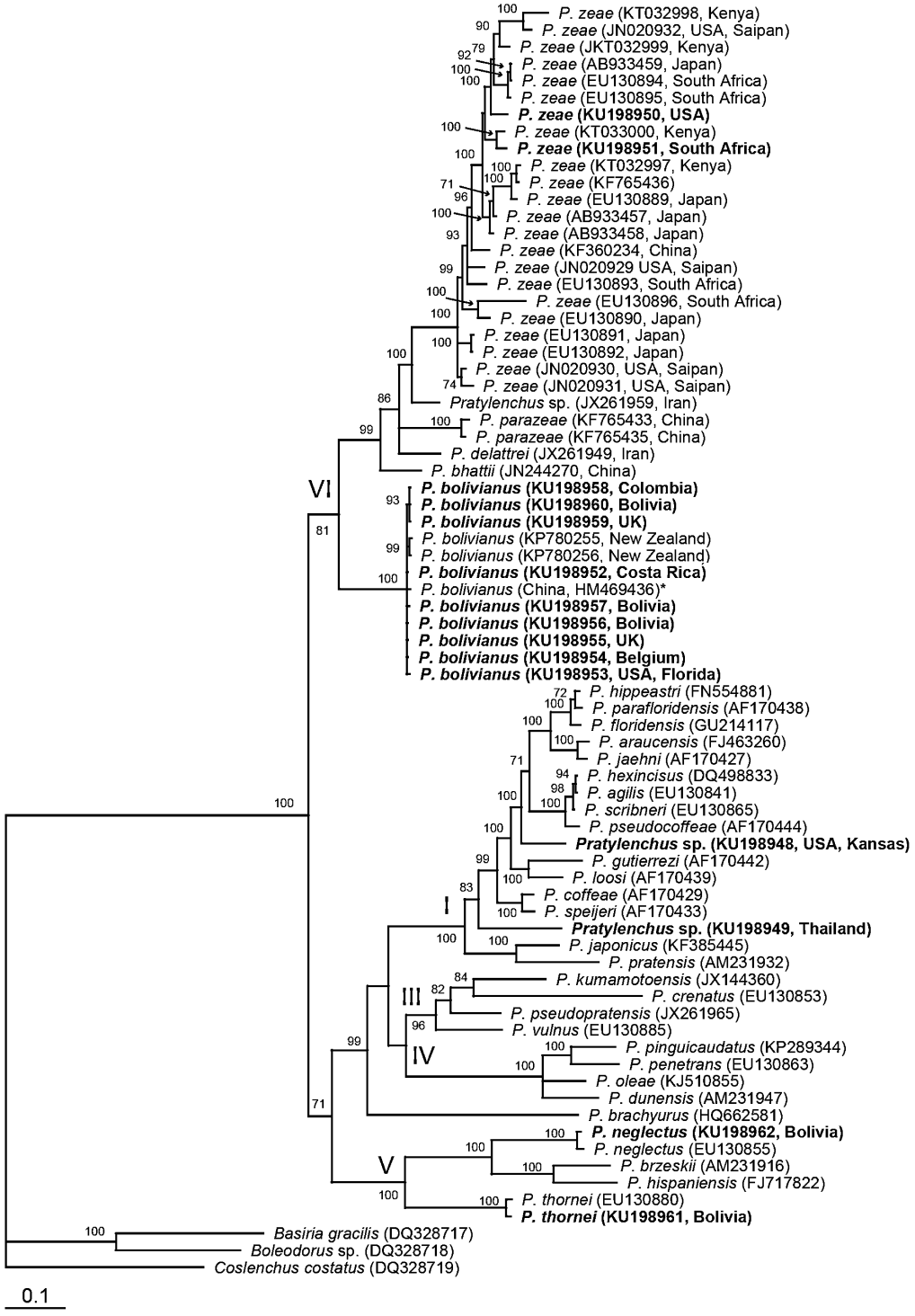


Fig. 7. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the D2-D3 of the 28S rRNA gene sequences using GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold. Clade numberings are given as in Subbotin *et al.* (2008). *Identified as *Pratylenchus* sp. in GenBank.

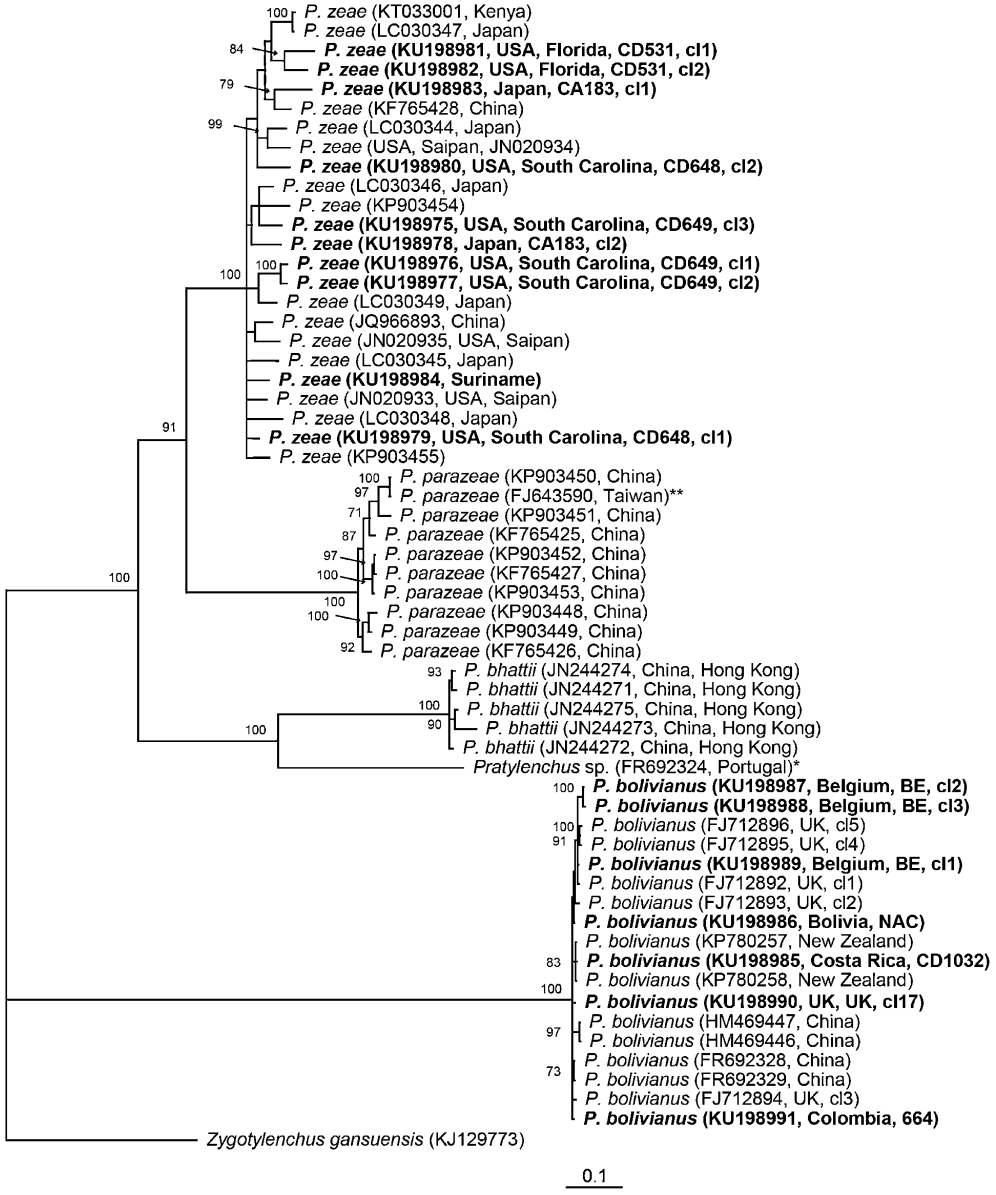


Fig. 8. Phylogenetic relationships within the genus *Pratylenchus* of the Clade VI as inferred from Bayesian analysis of the ITS of rRNA gene sequences using GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold. *Identified as *P. goodeyi* in GenBank; **Identified as *P. zeae* in GenBank.

Molecular characterisation and phylogenetic position of *P. bolivianus* and *P. zeae*

D2-D3 OF 28S RRNA

The alignment included 72 sequences of 36 *Pratylenchus* species and three sequences of outgroup taxa and was 826 bp in length. Nine new sequences of *P. bolivianus* (two amphimictic and four parthenogenetic populations) and two new sequences of *P. zeae* were included in this study. Our phylogenetic analysis suggests that *P. bolivianus* was the earliest branching taxon of Clade VI, which also included *P. bhattii*, *P. delattrei* Luc, 1958, *P. parazeae* Wang, Zhuo, Ye & Liao, 2015, *P. zeae* and unidentified *Pratylenchus* sp. *Pratylenchus bolivianus*

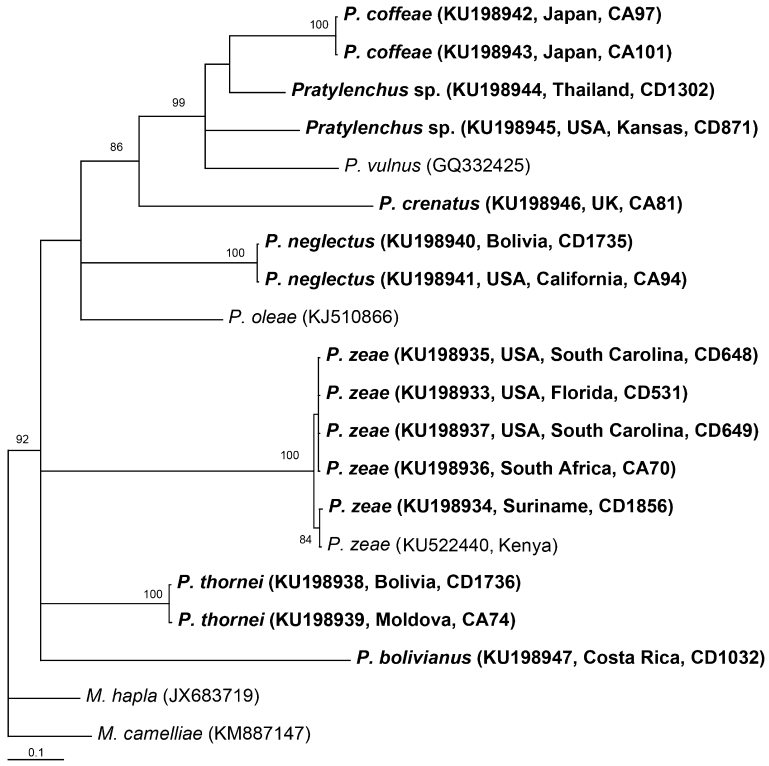


Fig. 9. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the *coxI* mtDNA gene sequences using GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

formed a highly supported monophyletic subclade. A sequence of *P. bolivianus* (KT971354) from Costa Rica published by Zamora Araya *et al.* (2016), but not included in the present analysis, was similar to all other sequences of this species. Intraspecific variations for *P. bolivianus* was 0-0.6% (0-4 bp), but for *P. zeae* was 0-9.0% (0-63 bp). Phylogenetic relationships within *Pratylenchus* species are given in Figure 7. *Pratylenchus neglectus* and *P. thornei* sequences from Bolivian populations matched very well with the GenBank sequences of corresponding species. While these populations were found to match nicely with GenBank sequences, no matching sequences were found for two *Pratylenchus* species originating from Kansas, USA, or Thailand. According to our phylogenetic analysis both species are placed within Clade I (Fig. 7).

ITS OF RRNA

The alignment included 59 sequences of five *Pratylenchus* species of Clade VI and *Zygotylenchus gansuensis* Wang, Zhuo & Liao, 2014 as an outgroup taxon. The alignment was 1049 bp in length. Seven new sequences of *P. bolivianus* and ten new sequences of *P. zeae* were included in the study. All *P. bolivianus* sequences formed a highly supported clade. Intraspecific variation for *P. bolivianus* was 0-1.8% (0-16 bp) but for *P. zeae* was 1.3-9.7% (8-57 bp). Phylogenetic relationships are given in Figure 8. The *P. zeae* populations studied clustered together with the topotype population from South Carolina, forming a highly supported clade.

COXI OF MTDNA

The alignment included 18 sequences of ten *Pratylenchus* species and two *Meloidogyne* sequences as outgroup taxa and was 396 bp in length. Five new sequences of *P. zeae*, one new sequence of *P. bolivianus* (am) from Costa Rica and nine new sequences of other *Pratylenchus* samples were included in the analysis. Intraspecific variation for *P. zeae* was 0-1.1% (0-4 bp). Relationships between *P. bolivianus* (am) and *P. zeae*, as well as between other *Pratylenchus* species, were not well resolved. Phylo-

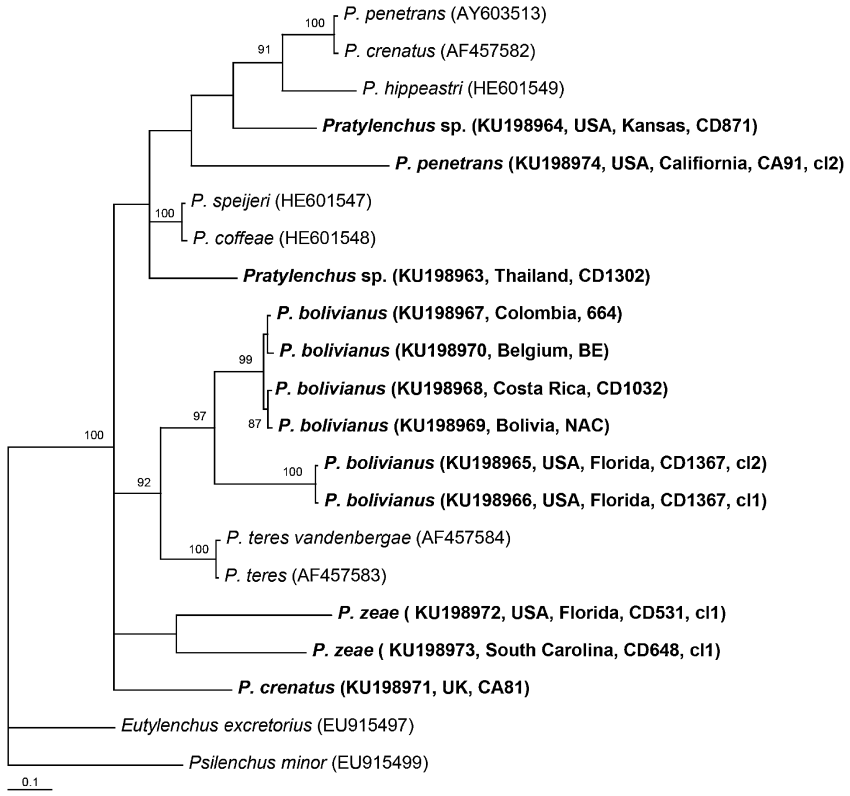


Fig. 10. Phylogenetic relationships within the genus *Pratylenchus* as inferred from Bayesian analysis of the *hsp90* gene sequences using GTR + I + G model. Posterior probabilities of over 70% are given for appropriate clades. Newly obtained sequences are indicated in bold.

genetic relationships within studied *Pratylenchus* species are given in Figure 9.

Hsp90

The alignment included 19 sequences of ten *Pratylenchus* species and two sequences of outgroup taxa and was 380 bp in length. Six new sequences from five *P. bolivianus* (am and pm) samples and two new sequences from two *P. zeae* samples were included in this study. Two types: A and B of *hsp90* gene sequences were obtained from *P. bolivianus* (am and pm) samples. The fragments of *hsp90* gene sequences for samples from Belgium and Bolivia were obtained using Hsp90F1-P_boliv and Hsp90R1-P_boliv primers, that were designed specifically for *P. bolivianus hsp90* type A, which included *P. bolivianus* (pm) from Bolivia, Belgium and Colombia and also *P. bolivianus* (am) from Costa Rica. No fragments were amplified with these primers from *P. bolivianus* (am) sample from Florida. The *hsp90* type B was only obtained from the Florida sample with the U831 and L1110 primer set. The *hsp90* type sequences differed by 14.8-17.5% (38-48 bp). Two sequences of *P. zeae* differed by 25.0% (68 bp). Phylogenetic relationships within studied *Pratylenchus* species are given in Figure 10.

Molecular diagnostics of *P. bolivianus* and *P. zeae*

Species-specific primers were designed for the detection of *P. bolivianus* and *P. zeae* based on differences in the ITS of rRNA gene sequences. Results of PCR with the species-specific primers are given in Figure 11. The combination of the universal primer TW81 with the species-specific primer P-boliv_R1 yielded a single PCR product of *ca* 295 bp for all *P. bolivianus* (am and pm forms) samples and no amplicons were found for other *Pratylenchus* samples (Fig. 11A). The combination of the universal primer TW81 with the species-specific primer P-zeae_R1 yielded a single PCR product of *ca* 560 bp for

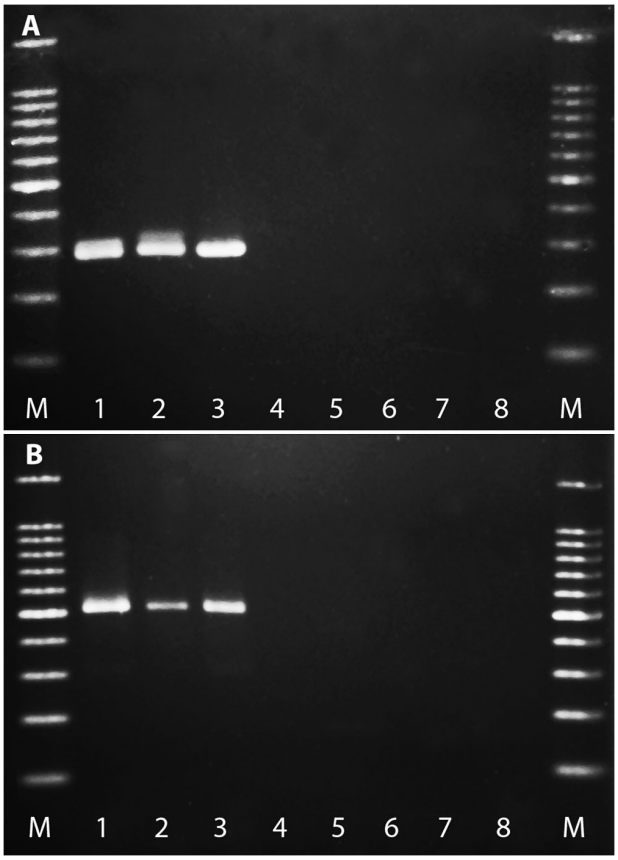


Fig. 11. PCR with species-specific primers for diagnostics of *Pratylenchus bolivianus* (A) and *P. zeae* (B). A: TW81 and P-boliv_R1 primers set. Lanes: M = 100 bp DNA ladder (Promega); 1-3: *P. bolivianus* (Florida, CD1367, Colombia, CD1855; Costa Rica, CD1032); 4: *P. penetrans* (Japan, CA85); 5: *P. crenatus* (UK, CA81); 6: *P. vulnus* (USA, CA90); 7: *P. neglectus* (USA, CA94); 8: control without DNA; B: TW81 and P-zeae_R1 primers set. Lanes: M = 100 bp DNA ladder (Promega); 1-3: *P. zeae* (CD649; CD531; CD1856); 4: *P. coffeae* (Japan, CA97); 5: *P. neglectus* (USA, CA94); 6: *P. vulnus* (USA, CA90); 7: *P. crenatus* (UK, CA81); 8: control without DNA.

all *P. zeae* samples and no amplicons were found for other *Pratylenchus* samples (Fig. 11B).

Discussion

The probable presence of amphimictic and parthenogenetic populations in a few species of *Pratylenchus* has been discussed in earlier reports (Luc, 1987). However, our study has provided evidence for the first time of the presence of morphotypes in a species of *Pratylenchus*. The two morphotypes identified in *P. bolivianus* differ not only morphologically, but also biologically. The presence of morphotypes in this genus further complicates the separation of *Pratylenchus* species by morphological analyses. Indeed, without the validation by molecular analyses the two morphotypes of *P. bolivianus* could have been ascribed to two distinct species supported by morphological and biological differences.

The observation that the occurrence of males can vary drastically within the same species of *Pratylenchus* is highly problematic as the presence/absence of males and related morphological characters (*i.e.*, spermatheca full of sperm or empty) are widely used as diagnostic and taxonomic features within the genus. Many *Pratylenchus* species have been classified on the basis of the reproductive habits of their populations, *i.e.*, sexual *vs* asexual (Castillo & Vovlas, 2007). However, some asexual species such as *P. thornei* (Sher & Allen, 1953; Loof, 1960; Castillo & Vovlas, 2007), *P. neglectus* (Rensch, 1924; Sher & Allen, 1953) and *P. hippeastri* (De Luca *et al.*, 2010) have been reported to have males occasionally present in a population. These males, however, apparently do not have any reproductive significance since the females associated with them always have an empty spermatheca. Interestingly, both *P. thornei* and *P. neglectus* exhibit lateral field variation similar to the variation observed between different *P. bolivianus* morphotypes.

From our observations it seems that climatic conditions may influence the presence or absence of males in *P. bolivianus*. The populations of *P. bolivianus* (am), which reproduces by amphimixis, are found in subtropical or tropical geographical areas, whereas the parthenogenetic populations of *P. bolivianus* (pm) are present in temperate geographical area or at high elevations in tropical areas. However, our assumption cannot be confirmed by other studies because of the small number of lesion nematode species with present or absent males in different populations. Indeed, asexual *Pratylenchus* species (*P. brachyurus*, *P. neglectus* and *P. zeae*) have previously been associated with polyploid karyotypes (Román & Triantaphyllou, 1969), indicating that genome duplications or hybridisation events might also influence the reproduction mode of *Pratylenchus* species.

The agricultural importance of *P. bolivianus* as a parasite of ornamentals and fruit crops was confirmed by this study. Both amphimictic and parthenogenetic morphotypes are able to damage fern in Florida and Cape gooseberry in Colombia, respectively. Presence of males has been observed in some Cape gooseberry fields infested by *P. bolivianus* in Colombia by one of the

authors (G.E. Múnera Uribe). However, the role and identity of these males remain undetermined.

Analysis of the D2-D3 of 28S rRNA and ITS of rRNA gene sequences confirmed that all studied samples belong to the same species: *P. bolivianus* (am and pm forms). The *hsp90* analysis results are contradictory and showed inconsistency in variations between *Pratylenchus* species. However, it has been shown that *hsp90* constitutes paralogous gene families that arose by gene duplication events (Gupta, 1995; Chen *et al.*, 2005). It seems that the primers, specifically designed for amplification of only the gene for the cytoplasmic form of *hsp90* (Skantar & Carta, 2004), can amplify other paralogous genes in *Pratylenchus* and thus compromise the approach of using this gene fragment as a reliable marker for species delimiting and phylogenetic reconstruction of the genus.

The molecular analyses of the *P. zeae* population sequences included in this study showed a close relationship with *P. bhattii*, *P. delattrei* and *P. parazeae*. *Pratylenchus bolivianus* is the sister taxon of this clade according to the phylogenetic analysis of D2-D3 expansion region of 28S rRNA gene. Congruency between the sequences of the topotype population from South Carolina and those of other population from distant geographical areas was observed in the ITS rRNA phylogenetic tree (Fig. 8). These populations clustered together in a well supported clade well separated from that of *P. parazeae*, thereby confirming the validity of this species.

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