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Characterisation of a population of *Pratylenchus hippeastri* from bromeliads and description of two related new species, *P. floridensis* n. sp. and *P. parafloridensis* n. sp., from grasses in Florida

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Summary – Morphological and molecular analyses confirmed the presence of *Pratylenchus hippeastri* in regulatory samples collected in commercial bromeliad operations from genera *Guzmania*, *Neoregelia* and *Vriesea* in central and south Florida, USA. Specimens of *P. hippeastri* from bromeliads contained males which were not detected in the type population from amaryllis and are described herein for the first time. The rDNA sequences of these males matched those of *P. hippeastri* female type material. *Pratylenchus hippeastri* and root-lesion nematodes from several hosts in Florida were characterised at the morphological and molecular level, whereas other samples from Russia and South Africa were characterised at the molecular level only. Sequence and phylogenetic analysis using the ITS rRNA gene of these root-lesion nematodes revealed the presence of eight putative new species (spH1-H8) closely related to *P. hippeastri*. Here we describe two Florida representatives of the amphimictic root-lesion nematodes from Bahia grass (N1) and maidencane (N2), previously characterised by Inserra *et al.* in 1996 and Duncan *et al*. in 1999, as two new species phylogenetically related to *P. hippeastri* and named *P. floridensis* n. sp. and *P. parafloridensis* n. sp., respectively. The small round or oval (rarely rectangular and occasionally oblong) and enlarged spermatheca and the bluntly pointed or subacute tail with smooth and occasionally indented terminus separate *P. floridensis* n. sp. from *P. parafloridensis* n. sp., which has a quadrangular spermatheca and a subhemispherical or bluntly pointed tail with generally smooth and rarely indented terminus. However, these characters may overlap in some specimens making a morphological separation problematic without the use of molecular analysis. The close phylogenetic relationships shared by the species characterised in this study indicate that they are representatives of a *P. hippeastri* species complex.

Keywords – Bahia grass, bottlebrush, *Callistemon rigidus*, *Fraxinus caroliniana*, maidencane, molecular, morphology, morphometrics, *Panicum hemitomon*, *Paspalum notatum*, phylogeny, pop ash, species complex, St Augustine grass, taxonomy.

In the early 1990s, two amphimictic populations of root-lesion nematodes were found on Bahia grass (*Paspalum notatum* Flueggé) (N1) and maidencane (*Panicum hemitomon* Schultes) (N2) in Florida, USA. The populations have regulatory significance because they are morphologically similar to *Pratylenchus coffeae* (Zimmer-

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mann, 1898) Filipjev & Schuurmans Stekhoven, 1941, which is a regulated nematode in Florida. Morphobiological studies of these two populations provided evidence that they share similarities (undivided smooth face, with two lip annuli and prominent spermatheca filled with sperm) with *P. loosi* Loof, 1960, a species morphologically closely related to *P. coffeae*. In spite of the fact that these populations have a more anterior vulva (75-80%) than *P. loosi* (79-85%), they were tentatively identified as Florida populations of *P. loosi* (Inserra *et al*., 1996). Subsequent molecular analyses of these populations showed that they were phylogenetically unrelated to both *P. coffeae* and *P. loosi* (Duncan *et al.*, 1999) and consisted of two morphologically similar species of root-lesion nematodes, designated as *Pratylenchus* N1 and N2 (N1 = *P. loosi* Zolfo Springs and N2 = *P. loosi* Lithia in Table 2). Detailed morphological characteristics were given by Inserra *et al.* (1996) and Duncan *et al.* (1999).

The recently described *P. hippeastri* Inserra, Troccoli, Gozel, Bernard, Dunn & Duncan, 2007 from amaryllis in Florida, USA, was characterised by absence of males, a flat and smooth face with two lip annuli, a rectangular empty spermatheca, a conoid tail with a bluntly pointed terminus and anterior vulva position (75-78%) (Inserra *et al.*, 2007). *Pratylenchus hippeastri* clustered in a clade with the amphimictic *Pratylenchus* N1 and N2 populations from pasture grasses in Florida when compared by D2-D3 expansion segments of the 28S rDNA sequences with those of 32 *Pratylenchus* populations studied by Inserra *et al.* (2007). The close phylogenetic relationships based on D2-D3 between *P. hippeastri* and *Pratylenchus* N1 and N2 were confirmed by Subbotin *et al.* (2008), who considered *Pratylenchus* N1 and N2 conspecific with *P. hippeastri*, in spite of the difference in their reproductive behaviour and morphological differences.

Recently, root-lesion nematodes with few males and numerous females morphologically similar to *P. hippeastri* were detected in regulatory samples collected in commercial bromeliad operations in central and south Florida. Bromeliads (Bromeliaceae) are ornamental epiphytes that produce roots that anchor the plant to branches and twigs of trees, but also take up nutrients when in contact with, or grown in, soil and other media. In spite of the presence of males, unknown from the type population of *P. hippeastri*, these root-lesion nematodes from bromeliads share major diagnostic morphological features with *P. hippeastri*. Additionally, male-less and amphimictic root-lesion nematodes with two, or occasionally three, lip annuli and morphologically similar to *P. hippeastri* and N1 and N2 occur in Florida on turf grasses, ornamentals and native trees. However, the taxonomic status of these cryptic species (morphologically similar but genetically different), as well that of N1 and N2 and those infecting bromeliads, is uncertain and requires clarification.

The main objectives of this study were to: *i*) characterise molecularly and morphologically populations resembling *P. hippeastri* from bromeliads and confirm their species identity; *ii*) provide updated molecular and morphological data on the root-lesion nematodes N1 and N2 and describe them as two new species; and *iii*) reconstruct phylogenetic relationships between *P. hippeastri* and N1 and N2 along with other closely related species from Florida, Russia and South Africa using the ITS and D2-D3 expansion segments of 28S rRNA gene sequences.

Materials and methods

Root-lesion nematode populations from bromeliads, grasses and other plants

Three bromeliad production operations located near Apopka and Miami, Florida, were surveyed during all seasons in 2007-2008. Sixty composite root and soil samples were collected from containerised bromeliads (Table 1) in all of the production operations. Ten root-lesion nematode females (originally from Goulds, south Florida) were hand picked from infested bromeliad samples and transferred to carrot disks at 23°C (Huettel, 1985). Cultured nematodes were used for molecular and morphological analyses and sex ratio determination.

Pratylenchus hippeastri from amaryllis and related male-less root-lesion nematodes from bromeliads and other hosts in Florida (spH5, spH7 and spH8), tentatively identified morphologically as representative of *P. hippeastri*, *P. zeae* Graham, 1951 and *P. jordanensis* Hashim, 1983 (= *P. zeae*) (Table 2), were selected for this study.

Table 1. *Bromeliad species and cultivars sampled in Florida**.

Genus	Cultivar
Guzmania	Confetti, Eloy Intro, Indian night, Irene, Marjan, Optima*, Orange, Ostara
Neoregelia	Passion*, Ardie*, Frank*, Inferno*, Martin*, Tricolor*
Vriesea sp.*	–

Bromeliads marked by an asterisk were infected by root lesion nematodes.

Table 2. Pratylenchus *species and populations used in this study.*

Identification based on ITS and D2-D3 rDNA sequences	Preliminary identification based on morphology	Host plant	Locality	Collection codes for DNA or nematode cultures	GenBank accession number for ITS	GenBank accession number for D2-D3 of 28S rDNA	Source of materials or reference
P. hippeastri	*P. hippeastri*	Amaryllis (*Hippeastrum* sp.)	Tampa, Hillsborough County, FL, USA	PhippTampa	FJ712932-FJ712936	GU214112, GU214113	L. Duncan
P. hippeastri	*P. hippeastri*	Bromeliads (*Neoregelia* spp.)	Goulds, Dade County, FL, USA	FloridaPh	N554883-N554887	N554879-N554882	L. Duncan
P. hippeastri	*P. hippeastri*	Amaryllis (*Hippeastrum* sp.)	Gainesville, Alachua County, FL, USA	FloridaPh	FN554888, FN554889	DQ498829, DQ498831	Inserra *et al.* (2007)
P. parafloridensis n. sp.	*P. loosi/ Pratylenchus* N2	Maidencane (*Panicum hemitomon*)	Lithia, Hillsborough County, FL, USA	Ploosi Lithia	GQ988377, GQ988378	AF170438 GU214114, GU214115	L. Duncan
P. floridensis n. sp.	*P. loosi/ Pratylenchus* N1	Bahia grass (*Paspalum notatum*)	Zolfo Springs, Hardee County, FL, USA	PloosiZolfoN1	GQ988375, GQ988376	AF170437 GU214116, GU214117	L. Duncan
Pratylenchus spH1	*Pratylenchus* sp.	Pop ash (*Fraxinus caroliniana*)	Perry, Taylor county, FL, USA	CD580	GU131132-GU131135	GU131127-GU131129	R. Inserra
Pratylenchus spH2	*P. zeae*	Unknown	Upington, South-Africa	PzUping	FJ713012-FJ713016	GU214121, GU214122	E. Van den Berg
Pratylenchus spH3	*P. subranjani*	Grassland	Russia	PsubMi8	GQ988369, GQ988370	–	A. Ryss
Pratylenchus spH4	*P. scribneri*	Corn (*Zea mays*)	Florida, USA	PscribFloridaUSA	FJ712997-FJ713001	–	J. Pinochet
Pratylenchus spH5	*P. hippeastri*	Bottlebrush (*Callistemon rigidus*)	Hastings, St John County, FL, USA	CD544	GU131136, GU131137	GU131130, GU131131	R. Inserra
Pratylenchus spH6	*Pratylenchus* sp.	St Augustine grass (*Stenotaphrum secundatum*)	Arcadia, De Soto County, FL, USA	CD547, CD548	GU131138-GU131141	GU131123-GU131126	R. Inserra
Pratylenchus spH7	*P. zeae*	Turf	Florida, USA	PzInserra	GQ988371, GQ988372	GU214123, GU214124	L. Duncan
Pratylenchus spH8	*P. jordanensis/ P. zeae*	Grassland	La Belle, Hendry County, FL, USA	PjordInserra	GQ988373, GQ988374	GU214118-GU214120	L. Duncan
P. jaehni	*P. jaehni*	Citrus (*Citrus aurantium*)	Sao Paolo, Brasil	Pjaehni	FJ712937-FJ712941	AF170426, AF170427	L. Duncan

Two additional Florida amphimictic root-lesion nematodes (spH1 and spH6), similar to N1 and N2 identified as *Pratylenchus* sp. (Table 2), were also included for molecular analysis only, along with two other samples identified as *P. subranjani* Mizukubo, Toida, Keereewan & Yoshida, 1990 and *P. zeae* (spH3, spH2) from Russia and South Africa, respectively, and a Florida population with divided face identified as *P. scribneri* Steiner *in* Sherbakoff & Stanley, 1943 (spH4) (Hernández *et al.*, 2000) (Table 2). Nematodes were extracted from soil by the sieving, decanting and centrifugal flotation method (Jenkins, 1964), and from bromeliad, bottlebrush, pop ash, St Augustine grass and mixed species of turf grass roots by incubation in jars.

Molecular analysis

DNA was extracted from individuals of both female and male root-lesion nematode specimens. DNA extraction from individual root-lesion nematode specimens, PCR, cloning and sequencing took place in three laboratories: IPP, Italy; ILVO, Belgium; and PPDC, CDFA, USA. The protocols were described in detail by De Luca *et al.* (2004), Waeyenberge *et al.* (2009) and Subbotin *et al.* (2008), respectively. The following sets of primers were used for amplification of two gene fragments in the present study: *i*) D2-D3 expansion segments of 28S rRNA using forward D2A (5′-ACAAGTACCGTGGGGAAA GTTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGC TACTA-3′) and *ii*) ITS1-5.8-ITS2-rRNA using forward

18S-Int (5′-CGTAACAAGGTAGCTGTAGG-3′) and reverse 26S-Int (5′-TCCTCCGCTAAATGATAT-3′), forward TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and reverse AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) or forward PRATTW81 (5′-GTAGGTGAACCTGCTGCTG-3′) and reverse AB28. PCR products were purified using the protocol listed by the manufacturers of Nucleospin Extract II (Macherey-Nagel, Germany) or QIAquick (Qiagen, USA) gel extraction kits and used for cloning or direct sequencing in both directions with the primers given above or M13 forward and M13 reverse primers. TOPO-TA cloning kit (Invitrogen) or pGEM-T Vector System II kit (Promega, USA) were used for cloning of PCR products. Newly obtained sequences were deposited in GenBank under accession numbers given in Table 2.

The newly obtained sequences for both ribosomal regions of *P. hippeastri* from bromeliad and of root-lesion nematodes from other hosts, including those from amaryllis, Bahia grass (N1), maidencane (N2) (Table 2), and of *P. jaehni* Inserra, Duncan, Troccoli, Dunn, dos Santos, Kaplan & Vovlas, 2001 (Duncan *et al*., 1999; Waeyenberge, unpubl.) were aligned using ClustalW (Thompson *et al*., 1997) with default parameters. *Pratylenchus jaehni* was used as an outgroup taxon (Subbotin *et al*., 2008). Phylogenetic analysis of the sequence data sets were performed with maximum parsimony (MP) using PAUP* 4b10 (Swofford, 2002) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). For MP we used heuristic search setting with ten replicates of random taxon addition, tree bisection-reconnection branch swapping to seek the most parsimonious trees. Gaps were treated as missing data. To obtain an estimation of the support for each node, a bootstrap analysis (BS) with 1000 replicates was done. BI analysis under the GTR + I + G model for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 10^3 generations. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades.

Morphometric and morphological analysis

Adult root-lesion nematodes from bromeliads were used for this study. Live specimens were immobilised by gently heating and then mounted in water agar on a slide (Esser, 1986) for measurements and photographs. Additional measurements and drawings were made from 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 a *camera lucida*. Abbreviations used are defined in Siddiqi (2000). Photographs were taken with two Leica (Wild MPS 46/52 and Leica DFC 320) cameras mounted on Nikon (Optiphot) and Leica DM 2500 compound microscopes.

The morphological information on the root-lesion nematodes from Bahia grass (N1) and maidencane (N2) provided by Inserra *et al.* (1996) and Duncan *et al.* (1999) was complemented by further microscopic observations of additional preserved specimens kept in the nematode collection (CNR-IPP, Bari) by the second author of this paper. Morphometrics of mature females of root-lesion nematode species studied by Duncan *et al*. (1999) and *P. hippeastri* from bromeliad and the original description were subjected to principal component analysis using Minitab 13 (Minitab, USA). The populations were characterised based on the lip morphology (smooth or divided) and the weakly-allometric characters/ratios V, a, and stylet length. These characters were reported to discriminate relationships among *P. coffeae*-group species that conformed closely to the phylogenetic relationships inferred by analyses of the D2-D3 region of the 28S rDNA sequence (Duncan *et al*., 1999).

Specimens for scanning electron microscope (SEM) observations were cold-fixed in glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2), post fixed 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). Nematodes were observed with a Hitachi S530 microscope at 15-20 kV accelerating voltage.

Species delimitation in studied *Pratylenchus*

Species delimitation of the studied populations was done by applying an integrated or polyphasic approach, which was based on consideration of results of morphological and morphometrical studies, phylogenetic and sequencing analysis, and analysis of host plants and geographic distribution of studied samples. This approach integrates any significant information on the organisms, and results in a consensus and transition type of classification (Subbotin & Moens, 2006). Two new species named here as *P. floridensis* n. sp. and *P. parafloridensis* n. sp. and several unidentified, putative new species defined here as *Pratylenchus* spH1-H8 were delimited in this study using

this approach. More detailed morphological and molecular analysis is still required to confirm the unique species status of *Pratylenchus* spH1-H8.

Results

Root-lesion nematode populations collected from bromeliads and other plants

Bromeliads belonging to the genera *Guzmania* Ruiz & Pav., *Neoregelia* L. B. Sm. and *Vriesea* Lindl. were found to be infected by root-lesion nematodes similar to *P. hippeastri* (Table 1). Population levels were usually <10 specimens/g fresh roots. In some cases, bromeliad roots were found infected concomitantly with a few specimens of *P. brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941, endoparasitic *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961, and *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949. Carrot cultures inoculated with female root-lesion nematodes from bromeliads produced a large number of nematodes at different life stages and only a few males (usually three males per 100 females). The population levels of other Florida root-lesion nematodes varied from 30, 160 and 100 specimens/g fresh roots for spH1, spH5 and spH6, respectively. The number of males in the amphimictic spH1 and spH6 was *ca* 40/100 females.

Molecular characterisation of *P. hippeastri* species complex

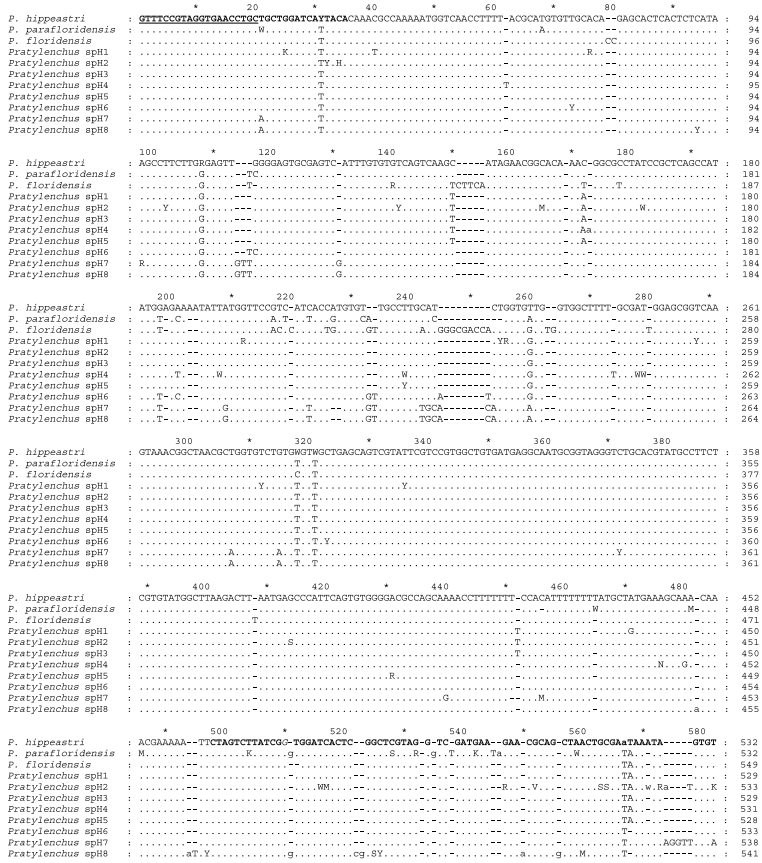
The amplification of the ITS containing region produced a single fragment of *ca* 970-1000 bp for the studied samples. The sequence alignment for *P. hippeastri* and related species with consensus sequence for each putative species is given in Figure 1. The ITS alignment included 44 sequences and was 1050 bp in length. Sequence diversity within all studied root-lesion nematodes including *P. jaehni* reached 19% (174 nucleotides); for *P. hippeastri* from bromeliad and from amaryllis it varied from 0 to 0.6% (0-6 nucleotides), whereas sequence diversity within the other root-lesion nematodes related to *P. hippeastri* reached 6.2% (57 nucleotides). Phylogenetic relationships within *Pratylenchus* species as inferred from Bayesian inference are given in Figure 2. Four main moderate or highly supported clades (PP = 90-100) were distinguished within the tree. Clade 1 grouped *P. hippeastri* populations along with root-lesion nematodes spH1-H5. Populations of *P. hippeastri* from amaryllis and bromeliads clustered together forming one highly supported (PP = 100) subclade within clade 1. The ITS sequences for *Pratylenchus* spH2 and spH3 did not form distinct subclades and relationships between them were not resolved. The root-lesion nematode N2 (= *P. parafloridensis* n. sp. in Table 2 and Figs 1-3) formed a moderately supported (PP = 90) clade 2 together with *Pratylenchus* spH6. Root-lesion nematodes spH7 and spH8 clustered together and were not well separated. The two sequences of N1 (= *P. floridensis* n. sp. in Table 2 and Figs 1-3) formed highly supported clade 4 at the basal position of the tree.

Sequence alignments of the D2-D3 of 28S rDNA included 32 sequences of 713 bp length. Sequence diversity reached 7.4% (47 nucleotides) for all root-lesion nematodes studied; 2.1% (14 nucleotides) for the species closely related to *P. hippeastri* and varied from 0-0.3% (0-2 nucleotides) within *P. hippeastri* populations. Phylogenetic relationships within *Pratylenchus* species based on D2-D3 of 28S rDNA sequences is given in Figure 3. Five main, weakly to highly supported clades (PP = 72-100), were distinguished within the tree, which corresponded to the clades on the ITS-rRNA tree. Populations of *P. hippeastri* from amaryllis and bromeliads formed a moderately supported (PP = 93) clade 1 together with *Pratylenchus* spH1 (Florida, USA), spH2 (South Africa) and spH5 (Florida, USA). Root-lesion nematodes spH7 and spH8 clustered in a moderately supported clade 3. The amphimictic N1 formed a highly supported clade 4 at the basal position of the tree. MP and BI analyses generated congruent trees with similar branch supports for the ITS and D2-D3 gene alignments, respectively.

The close phylogenetic relationships shared by the species (spH1-H8) characterised in this study indicate that they are representatives of a *P. hippeastri* species complex. These results confirmed the identity of males and females of bromeliad populations as *P. hippeastri* and provided evidence that the rDNA sequences of *P. hippeastri* males from bromeliads matched those of *P. hippeastri* female type material. Furthermore the phylogenetic findings also provided support for the description of N1 and N2 as two new species named *P. floridensis* n. sp. and *P. parafloridensis* n. sp., respectively.

Morphological characterisation of *Pratylenchus hippeastri*

Comparative measurements of females and males of *P. hippeastri* from bromeliads and those reported from amaryllis in the original description are reported in Table 3.



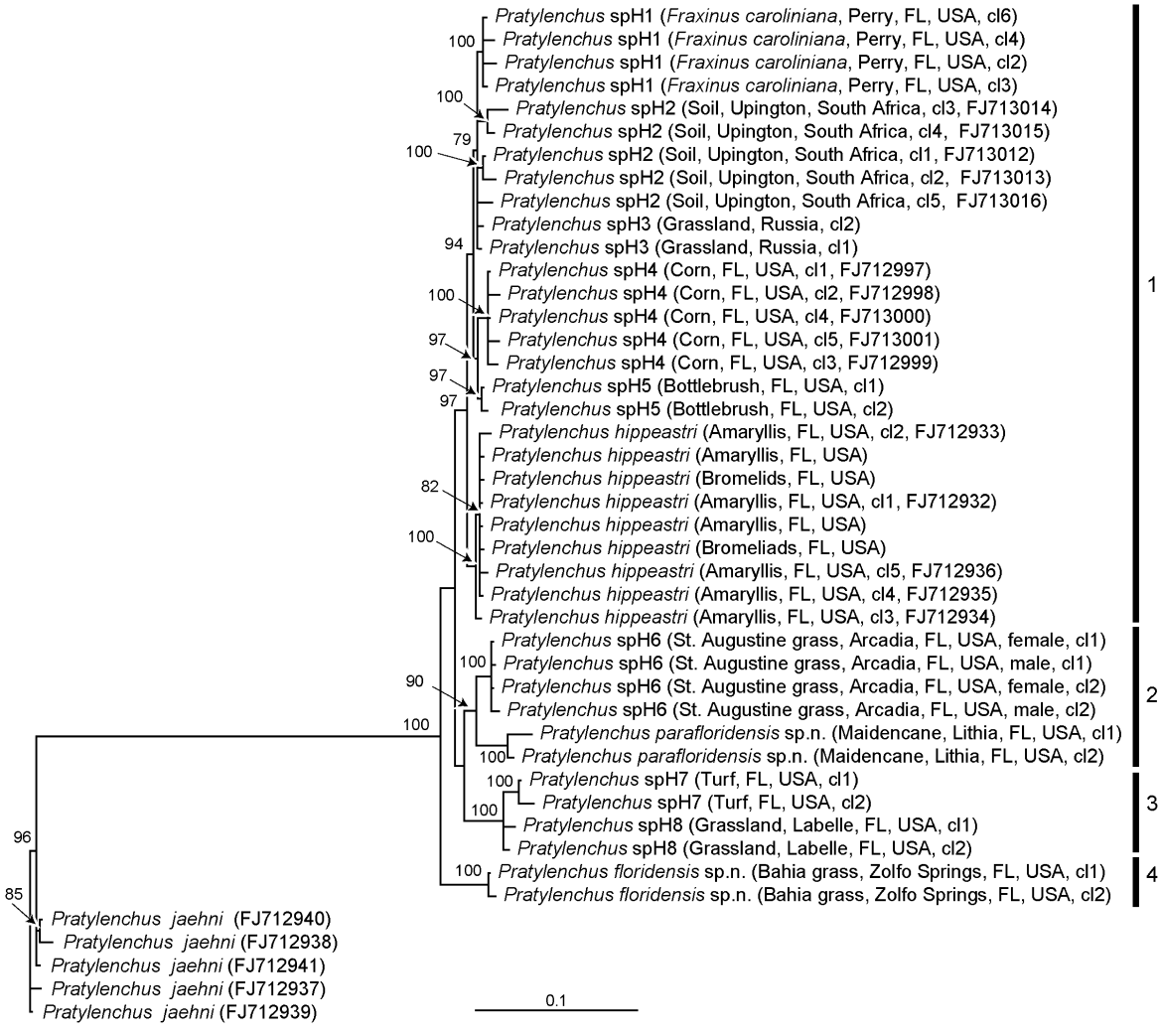


Fig. 2. *The 50% majority rule consensus tree from Bayesian analysis generated from the ITS sequence dataset for the* Pratylenchus hippeastri *species complex using the GTR + I + G model. Posterior probability more than 70% is given for appropriate clades.*

Female

Morphometric values of the females from bromeliads did not differ from those of *P. hippeastri* from amaryllis, except for the tail length in fixed specimens, which was slightly shorter than that of females from amaryllis. Small differences were observed also in the mean values of some characters of living specimens from bromeliad, such as body, tail and post-uterine sac length, which were shorter than those of specimens from amaryllis. Bromeliad females also showed smaller maximum and vulval body diameter and a shorter vulva-anus distance. However, their range values overlapped. Their lip pattern was also similar to that of *P. hippeastri* and consisted of a flat and un-

Fig. 1. *Sequence alignment of partial 18S, complete ITS1, 5.8S, ITS2 and partial 28S rRNA for the* Pratylenchus hippeastri *species complex. The 18S, 5.8S and 28S rRNA gene sequences are marked in bold, the primers sequences are underlined. Consensus sequence is given for each species only. Single letter code recommended by NC-IUB was used to specify nucleotide, if two or more bases were permitted at a particular position in a species subalignment. Lower case symbols indicate presence of one or several gaps in a particular position in sequences for a species subalignment.*

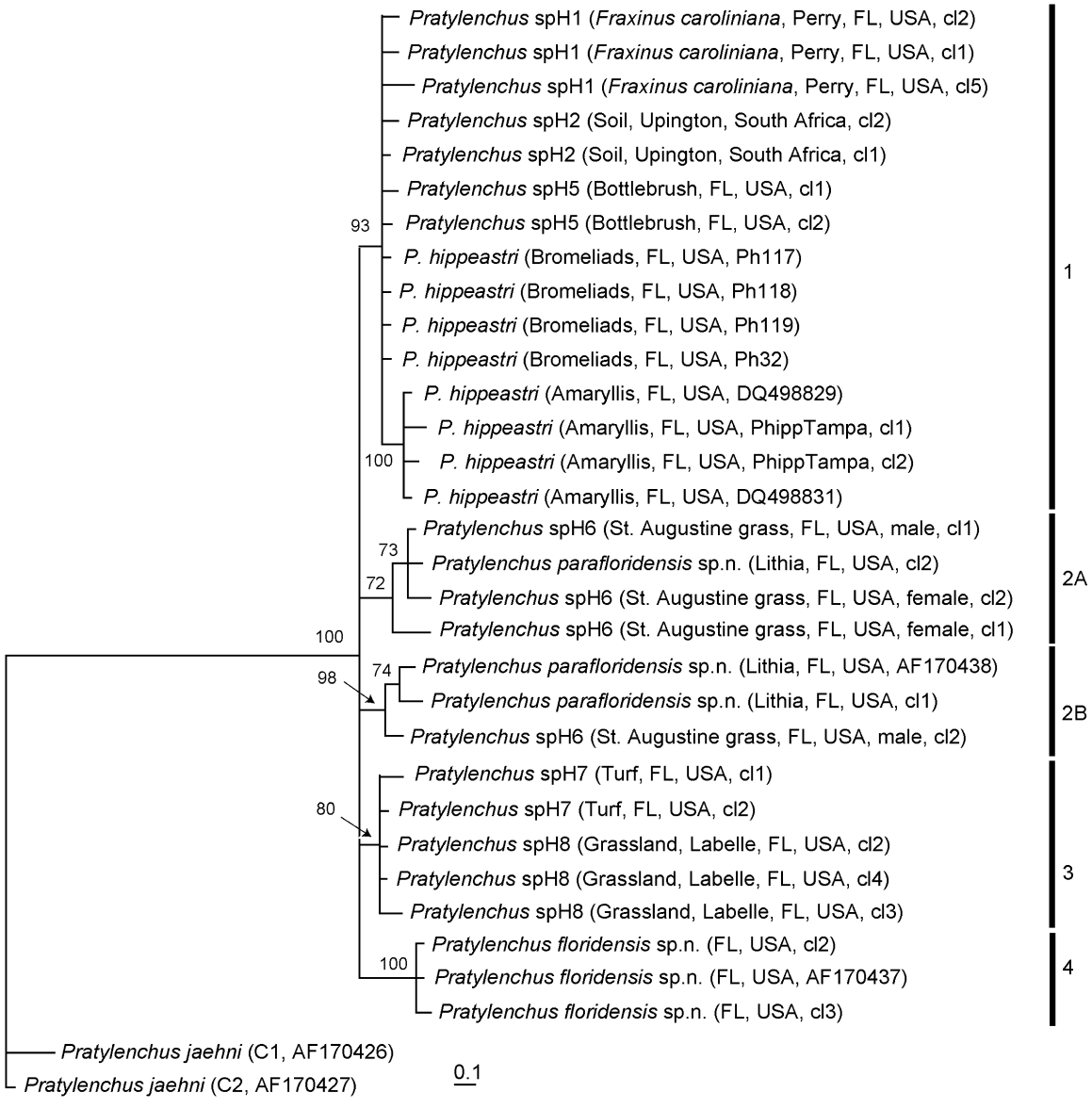


Fig. 3. *The 50% majority rule consensus tree from Bayesian analysis generated from the D2-D3 of 28S rRNA gene sequence dataset for the* Pratylenchus hippeastri *species complex using the GTR + I + G model. Posterior probability is given for appropriate clades.*

divided face with two lip annuli (in some specimens with an incomplete third annulus) and with the second annulus larger and thicker than the first. These females also had a small and empty spermatheca indicating that fertilisation by the males in the cultures may not occur. The small and empty spermatheca was also observed in females collected directly from bromeliad roots from the nurseries. These morphometric and morphological characters indicate that the lesion nematodes from bromeliads are *P. hippeastri*.

The principal components analysis for the morphometrics listed in Table 3 and those reported by Duncan *et al.* (1999) positioned bromeliad *P. hippeastri* in a group (VI) with N1 and N2 and separate from the other *Pratylenchus* species studied by Duncan *et al.* (1999) (Fig. 4). Considering the four characters used, the two principal components

Table 3. *Morphometrics of* Pratylenchus hippeastri *from Florida. All measurements are in μm and in the form: mean ± standard deviation (range).*

Character	Population from bromeliads (present study)			Population from amaryllis (Inserra *et al.*, 2007)
	Female		Male	Female
n	12 (live)	10 (fixed)	5 (fixed)	21 (fixed)
L	527 ± 48.5 (447-616)	614 ± 22.4 (585-651)	421 ± 32.3 (370-452)	590 ± 21.8 (545-627)
a	28.3 ± 1.6 (24.6-30.7)	25.2 ± 2.0 (23.7-26.5)	30.6 ± 3.3 (25.8-33.9)	25.5 ± 1.2 (23.2-27.9)
b	5.8 ± 0.4 (5.2-6.6)	6.6 ± 0.4 (5.9-7.2)	5.5 ± 0.2 (5.3-5.8)	6.5 ± 0.4 (5.7-7.1)
b′	3.7 ± 0.3 (3.3-4.2)	4.5 ± 0.4 (4.0-5.3)	3.5 ± 0.3 (3.2-3.8)	4.4 ± 0.3 (3.9-5.1)
c	17.9 ± 1.2 (15.7-19.9)	18.6 ± 2.0 (16.4-23.3)	18.4 ± 1.4 (16.1-19.3)	16.1 ± 1.0 (14.6-18.7)
c′	2.4 ± 0.2 (2.0-2.6)	2.2 ± 0.2 (1.8-2.5)	2.4 ± 0.1 (2.3-2.5)	2.6 ± 0.2 (2.2-2.9)
V or T	77.6 ± 1.3 (75.6-79.6)	77.7 ± 1.2 (75.7-79.4)	46 ± 2.8 (42.5-49.0)	77 ± 0.8 (75-78)
Stylet length	15.4 ± 0.3 (15.1-15.8)	15.8 ± 0.4 (15.3-16.7)	14.3 ± 0.3 (14.0-14.7)	15.5 ± 0.4 (15-16)
DGO from stylet base	3.4 ± 0.3 (3.0-3.8)	2.5 ± 0.3 (2.0-2.7)	2.2 ± 0.4 (1.7-2.7)	2.9 ± 0.2 (2.5-3.0)
o	22 ± 1.6 (19.2-25.2)	16 ± 1.7 (12.8-17.4)	15.4 ± 3.1 (11.6-19.0)	19 ± 1.2 (16-20)
Anterior end to:				
centre of metacorpus	58 ± 2.6 (55-64)	62 ± 1.9 (58-65)	50.6 ± 0.7 (50-51)	63 ± 1.9 (59-66)
cardia	90 ± 3.0 (85-95)	93 ± 5.3 (87-106)	79 ± 2.0 (76.7-81.3)	92 ± 3.3 (83-98)
end of pharyngeal gland lobe	139 ± 9.7 (128-153)	137 ± 8.8 (123-147)	126 ± 7.0 (117-132)	134 ± 6.6 (116-145)
secretory/excretory pore	88 ± 5.5 (77.4-95)	94 ± 2.9 (89-99)	73 ± 4.2 (66-76)	91 ± 2.5 (85-95)
Pharyngeal overlap	50 ± 8.0 (38.5-61.5)	45 ± 7.6 (33-58)	48 ± 8.9 (35-55)	43 ± 5.4 (32-51)
Max. body diam.	19 ± 1.5 (15.6-21.5)	24 ± 0.7 (23.3-25.7)	13.8 ± 0.8 (12.7-14.5)	23 ± 1.4 (21-27)
Vulval body diam.	17 ± 1.5 (14.2-20.5)	22 ± 1.6 (19.3-24)	–	21 ± 1.1 (18.0-23.0)
Anal body diam.	12 ± 1.2 (10.7-14.7)	15 ± 0.4 (14.7-16)	9.5 ± 0.3 (9.3-10)	14.4 ± 0.8 (13-16)
Vulva to anus distance	86 ± 10.9 (70.5-103)	103 ± 5.4 (92-109)	–	98 ± 6.1 (88-112)
Anterior genital tract length	133 ± 60.3 (108-170)	268 ± 60.3 (200-387)	194 ± 20.4 (165-220)	254 ± 47.2 (181-360)
PUS	23 ± 3.2 (18.6-29.4)	35 ± 3.0 (30-39.3)	–	30 ± 4.9 (21-45)
Tail length	30 ± 2.5 (27.2-35.7)	33 ± 3.0 (28-37.3)	23 ± 0.6 (22.0-23.7)	37 ± 2.2 (32.0-42.0)
Spicule length	–	–	19 ± 0.6 (18.0-19.3)	–
Gubernaculum length	–	–	5.3 ± 0.6 (4.7-6.0)	–
No. of tail annuli	24 ± 1.9 (21-26)	20 ± 2.6 (17-25)	–	22 ± 2.1 (19-26)

explained 69% of the morphological variation between the populations (n = 797 specimens from 45 populations). The correlations between the characters and the scores for principal components 1 and 2, respectively, were: V = 0.75 and −0.35; a = −0.38 and −0.75; stylet = 0.68 and −0.50; lip morphology = 0.59 and −0.54. Multiple regression of each character against the two principal component scores explained 68, 71, 72 and 64% of the variation in V, a, stylet and lip morphology, respectively.

Male

The few males present in carrot disk cultures and bromeliad roots exhibited a slight sexual dimorphism. Males had smaller lip region diameter (6.0-6.7 *vs* 7-7.8 μm), stylet knobs (2.3-3.0 across and 1.7-2.0 high *vs* 3.0-4.7 and 2.0-2.7 μm) and metacorpus (7.3-8.7 diam. × 10.0-11.3 high *vs* 10.0-11.3 × 13.3-17.3 μm) compared to those of the females. This sexual dimorphism has been reported for many amphimictic root-lesion nematodes. The male lip pattern, in spite of a slightly more collapsed appearance of the cuticle, did not differ from that of the female and showed a flat and undivided face with two lip annuli of different size with the second annulus larger and thicker than the first (Figs 5B; 6C; 7C, D). The oral disc was slightly raised and the amphidial apertures were broader than in the female. The stylet was more slender than that of the female and the knobs ellipsoidal to triangular in profile, with rounded margins.

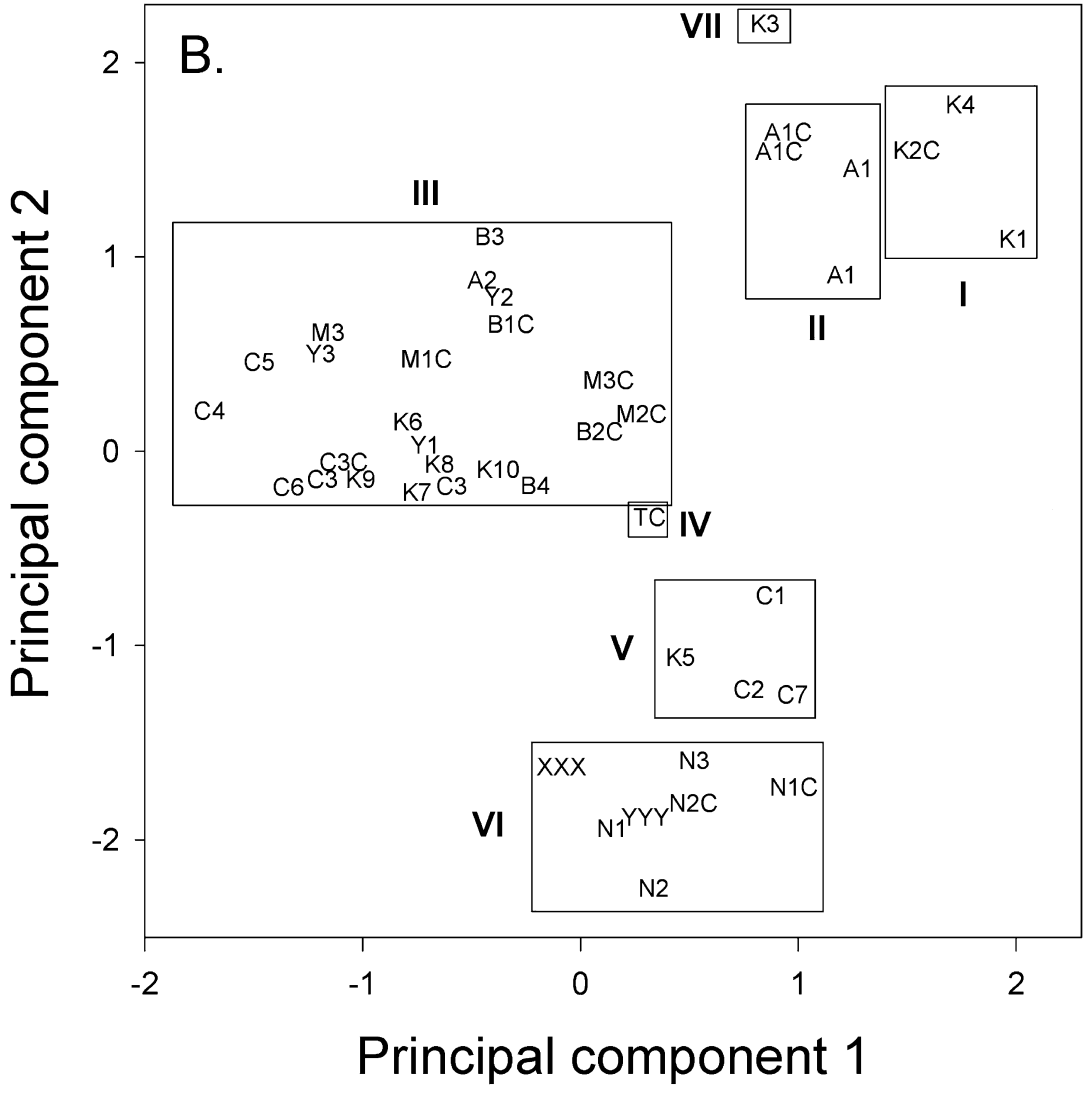


Fig. 4. *Morphometric relationships among 20* Pratylenchus *species studied by Duncan* et al. *(1999), and* P. hippeastri *from bromeliads (XXX) and amaryllis (YYY). Note the similarity of these populations to those of* P. floridensis *n. sp. (N1) and* P. parafloridensis *n. sp. (N2). Roman numbers indicate different assemblages of the root lesion nematodes studied based on one morphological (smooth* vs *divided face) and three weakly-allometric morphometric variables (V, a, length of stylet). Symbols followed by C represent populations from carrot disk culture, rather than from original host roots.*

The pharynx had a small, muscular metacorpus, rather long isthmus and a slender gland lobe, overlapping the intestine for *ca* three body diam. Spicules were curved, weakly cephalated, with two prominent expansions at the base of their proximal third. The gubernaculum was simple and slightly curved, the tail conical, enveloped by a crenate, moderately protruding bursa, extending to the tail tip. The lateral field had four smooth incisures, occupying slightly less than one-third of the body diam.

Morphological characterisation and description of root-lesion nematodes N1 and N2 as two new *Pratylenchus* species

In this study we collected enough molecular and morphological data to describe N1 and N2 as two new root-lesion nematodes, *P. floridensis* n. sp. and *P. parafloridensis* n. sp., respectively. The major objective of these descriptions was to clarify the identity of these two species

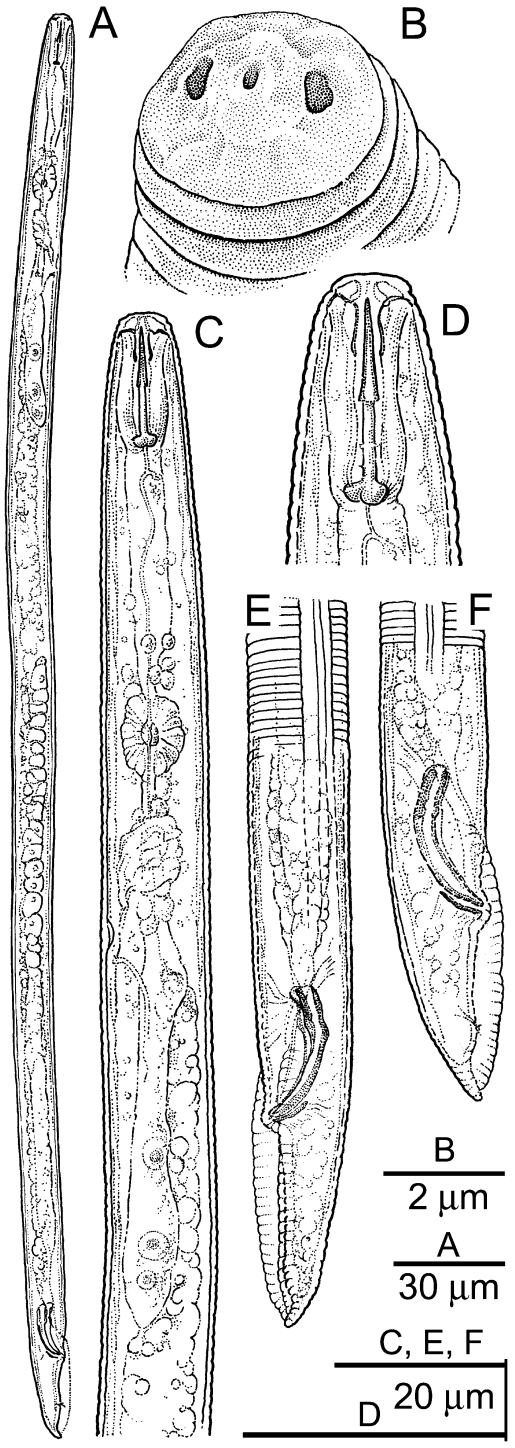


Fig. 5. Camera lucida *line drawings of male of* Pratylenchus hippeastri. *A: Entire body; B:* En face *view showing oral disc fused with median and lateral lip sectors; C: Pharyngeal region; D: Anterior end; E, F: Tail region.*

that have been reported in the literature and GenBank with acronyms. The morphological description of the other putative species in the *P. hippeastri* species complex from Florida and other countries was not attempted because they are cryptic species not separable by morphological analysis.

Pratylenchus floridensis [*] **n. sp.**

= *P. loosi apud* Inserra *et al*. (1996) *nec* Loof (1960)
= *Pratylenchus* N1 of Duncan *et al.* (1999)
(Figs 8-11)

Measurements

Measurements of this species, originally identified as *P. loosi* from Bahia grass and later as N1 root-lesion nematode, were reported in Inserra *et al.* (1996) and Duncan *et al.* (1999), respectively. Additional measurements (present study) of preserved specimens kept in the CNR-IPP, Bari's nematode collection are reported in Table 4.

Description

Female

Body of dead females almost straight. Labial region with two annuli, 2 μm high $\times$ 7 μm broad on average, offset from body by a slight constriction, second lip annulus wider and higher than first. SEM *en face* view characterised by undivided pattern, all labial sectors fused together and partially with an oval oral disc, amphidial openings rather wide, obliquely orientated, at sides of oral disc. Stylet with ellipsoidal knobs or rounded with slightly flattened anterior surface, 4 μm across, 2 μm high (mean values). Dorsal pharyngeal gland opening 2-2.5 μm posterior to stylet base (o range = 12.9-16.7%). Pharyngeal metacorpus oval, 11-13 μm high $\times$ 8.5-11 μm in diam. Isthmus slender, encircled by nerve ring in upper part, located just posterior to metacorpus. Pharyngeal gland lobe overlapping intestine ventrally, 35 $\pm$ 7.8 (27-52) μm in length. Lateral fields with four lines, not areolated. Anterior genital tract 144-240 μm or 32-47% of body length long. Spermatheca small, rounded or oval, rarely rectangular in shape, occasionally oblong and large, filled with sperm. Post-uterine sac *ca* 1.5 body diam. long, undifferentiated. Tail bluntly pointed (*sensu* Frederick & Tarjan, 1989) or subacute, with smooth terminus. In few specimens, slight indentation was observed at tail tip.

[*] Specific epithet derived from Florida, the only geographical area where this species has been detected.

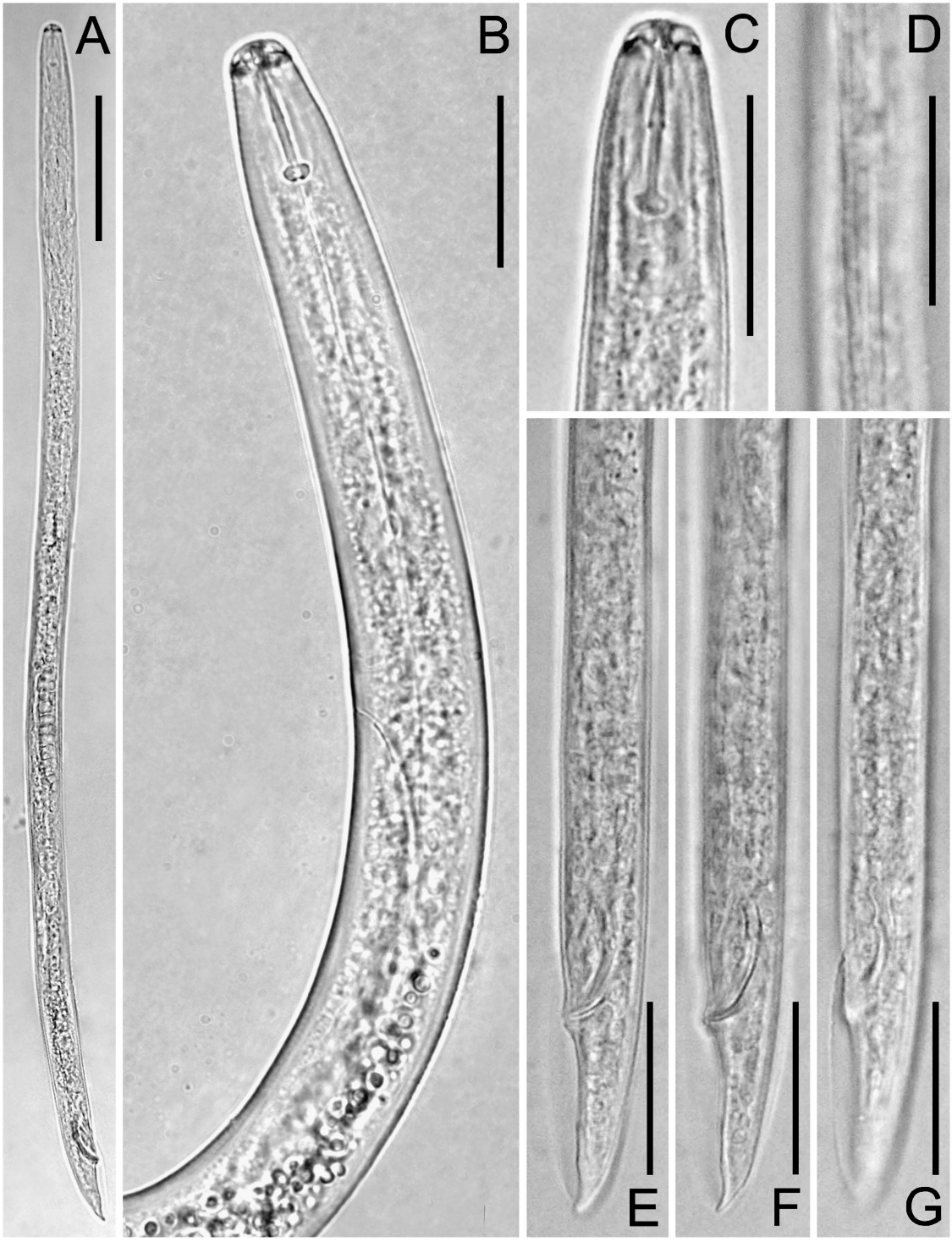


Fig. 6. *Light micrographs of male of* Pratylenchus hippeastri. *A: Entire body; B: Pharyngeal region (live specimen); C: Anterior end; D: Lateral field at mid-body; E-G: Tail region at different foci. (Scale bars: A = 50 μm; B-G = 20 μm.)*

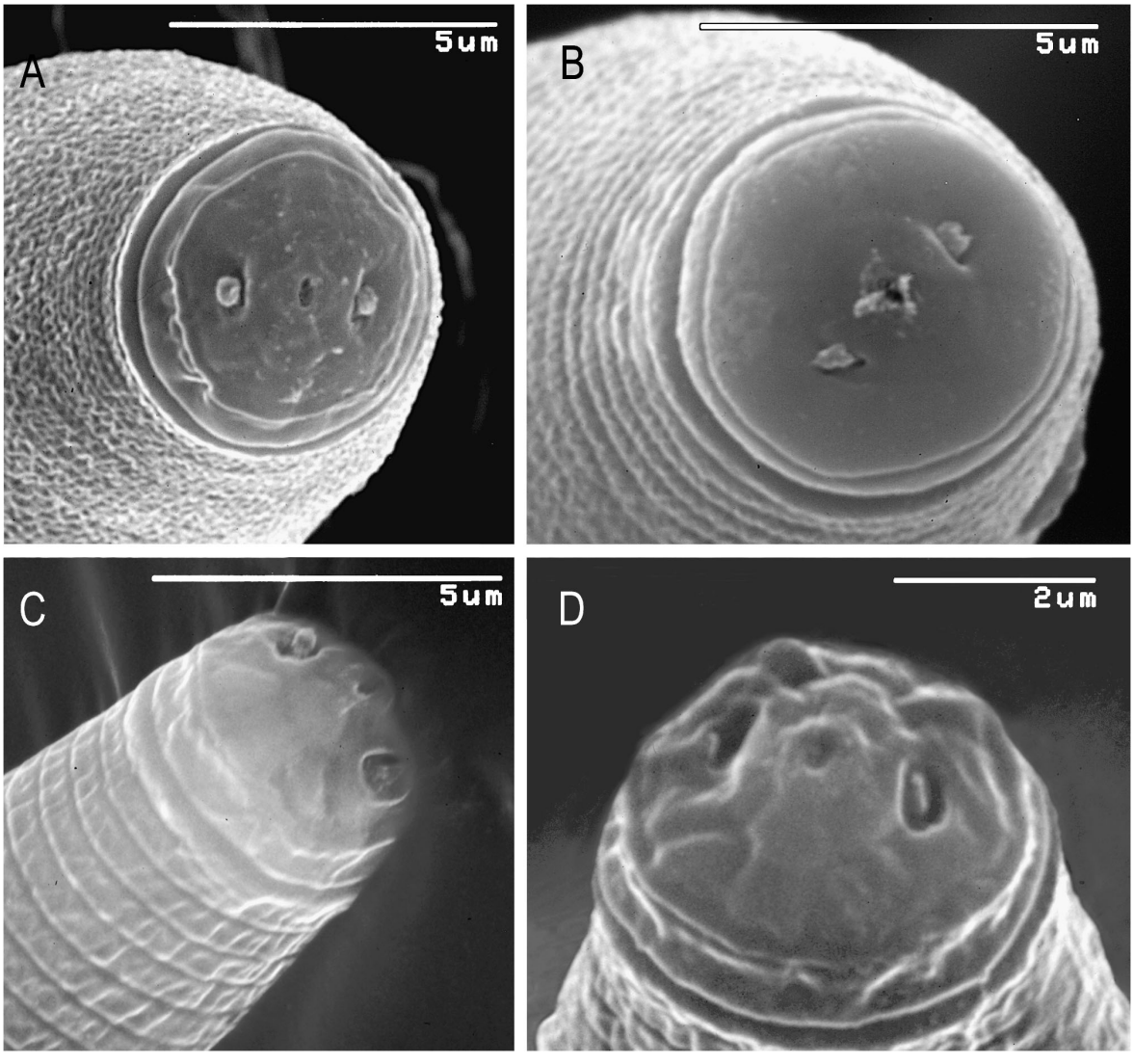


Fig. 7. *SEM morphology of* Pratylenchus hippeastri *from bromeliads in Florida: A, B: Female; C, D: Male. A, B: Undivided face pattern with all labial sectors fused together and with oral disc. Note second lip annulus thicker than first; C, D: Undivided face pattern similar to that of female, but with broader amphidial apertures.*

Male

Common in field populations. Body straight when heat-relaxed, similar to female except for sexual dimorphism and slightly smaller body size. Lip region slightly offset, 2 μm high and 5.7 μm broad, with two annuli (second annulus higher than first). Stylet more slender and shorter than in female, with minute, slightly cupped knobs, 2.7 μm across, 1.5 μm high. Lip pattern in SEM *en face* view showing a plane, undivided face, two lip annuli (second annulus larger and thicker than first) and an oral disc more rounded than in females. Pharynx with oval metacorpus (10.5 $\times$ 7.3 μm in longitudinal and cross diam., respectively) and gland lobe overlapping intestine for 37 μm. Hemizonid just anterior to secretory-excretory pore, hemizonion eight annuli posterior to it. Lateral field with four smooth lines. Testis outstretched, 238 μm long. Tail conoid, with narrowed hyaline tip, 4 μm long. Spicules arcuate, weakly cephalated, gubernaculum simple, slightly arcuate.

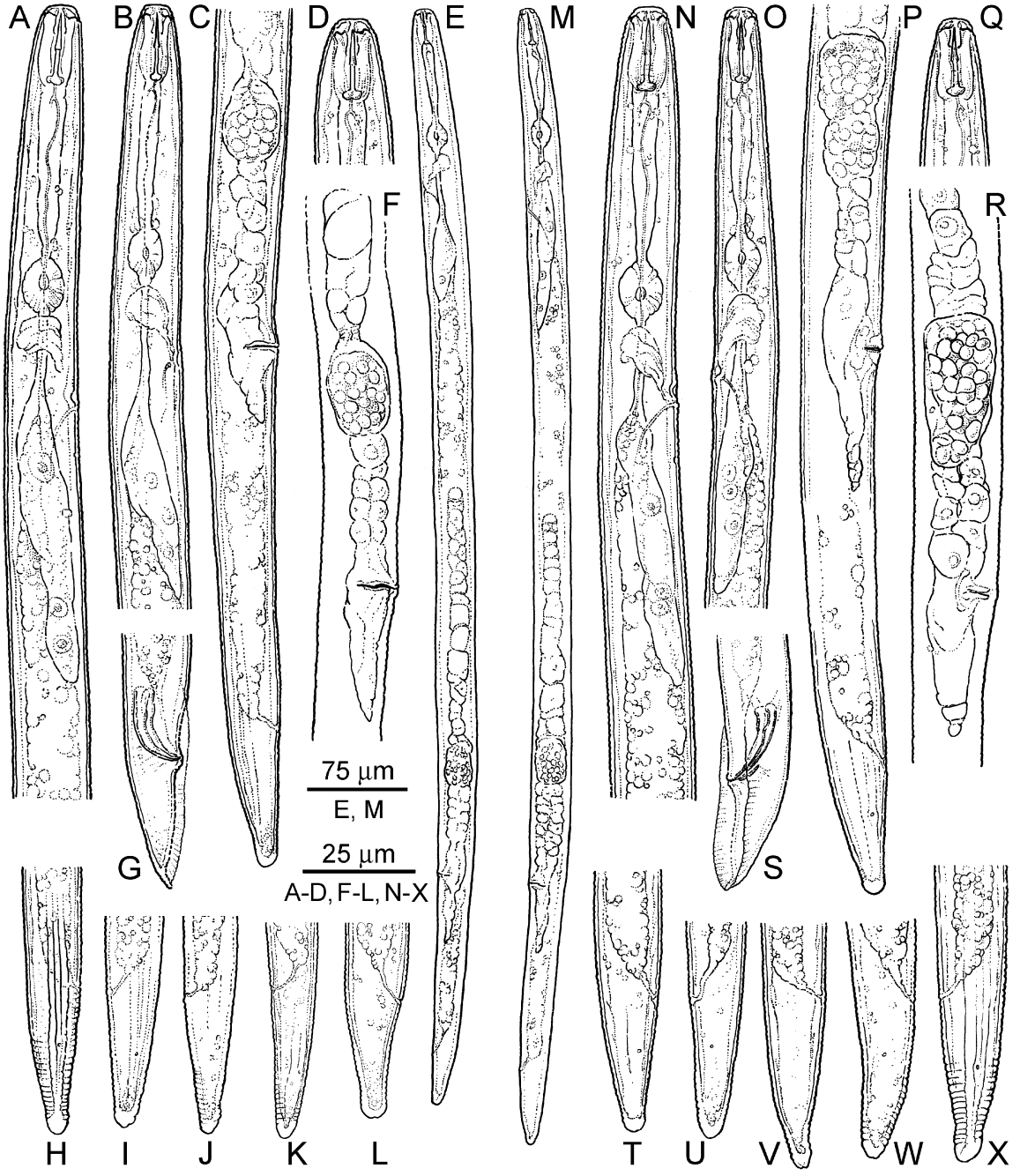


Fig. 8. Camera lucida *line drawings of* Pratylenchus floridensis *n. sp. (A-L) and* P. parafloridensis *n. sp. (M-X). A, N: Female pharyngeal region; B, O: Male pharyngeal region; C, P: Female posterior region; D, Q: Female anterior end; E, M: Female entire body; F, R: Female vulval region with spermatheca; G, S: Male tail; H-L, T-X: Female tail.*

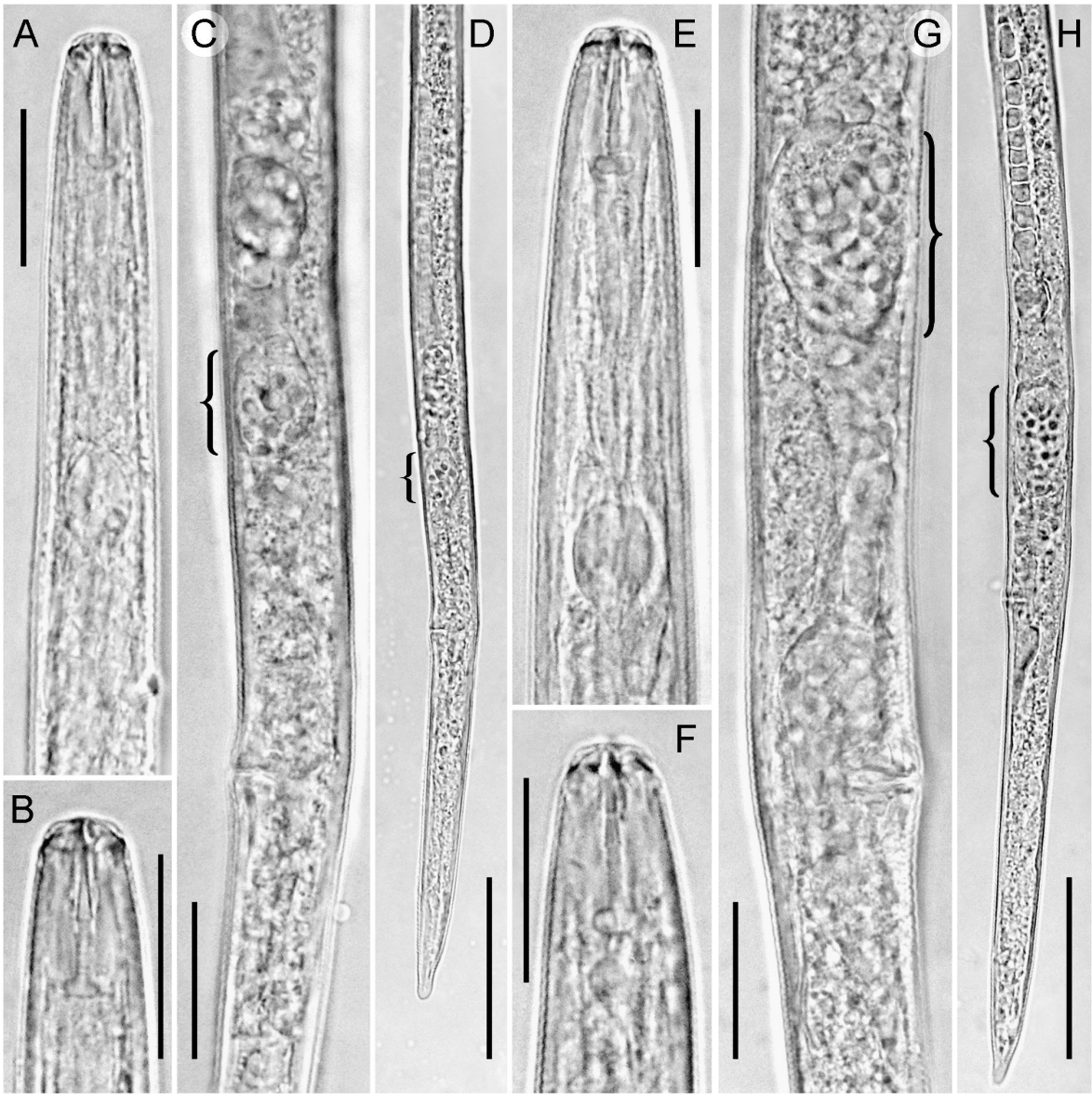


Fig. 9. *Light micrographs of* Pratylenchus floridensis *n. sp. (A-D) and* P. parafloridensis *n. sp. (E-H). A, E: Female pharyngeal region; B, F: Female anterior end; C, G: Female vulval region (brackets indicate position and the extent of spermatheca); D, H: Female posterior body portion (spermatheca in brackets). (Scale bars: A-C, E-G = 20 μm; D, H = 50 μm.)*

Type host and locality

Bahia grass (*Paspalum notatum* Flueggé) roots collected from a sod farm in Zolfo Springs, Hardee County, FL, USA (latitude 27°24′67″N; longitude 81°38′ 41″W). The soil type is sandy and the climate is subtropical.

Type material

Holotype female, 25 female and one male paratype deposited at the Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (CNR), Sede di Bari, Bari, Italy (collection numbers IPP-J-0939 to J-0944). Additional paratypes were distributed to the USDA

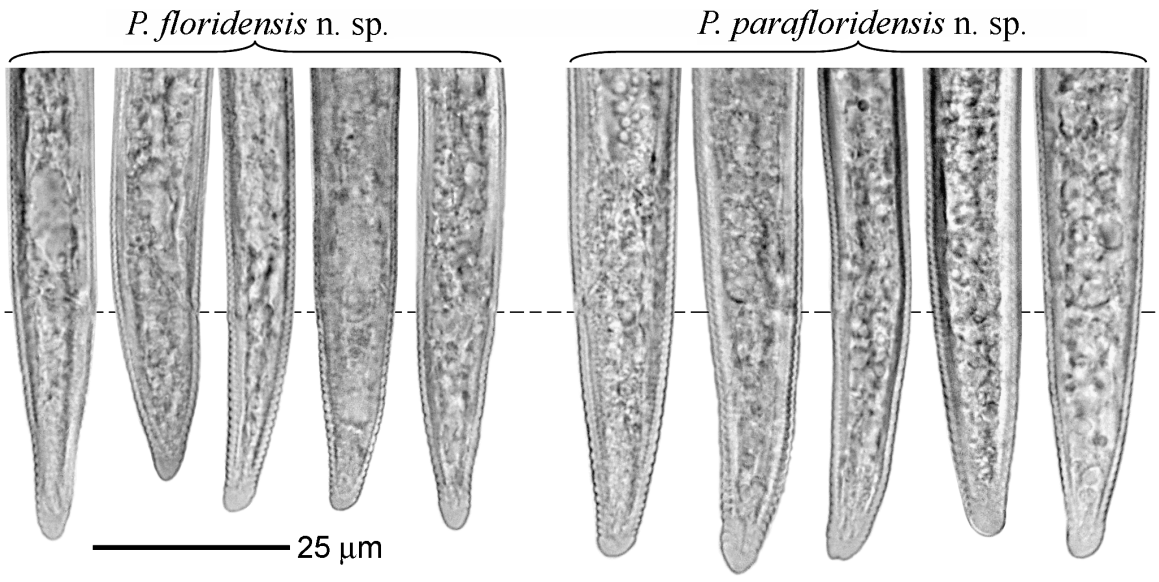


Fig. 10. *Comparative light micrographs of tail region of* Pratylenchus floridensis *n. sp. and* P. parafloridensis *n. sp. (broken line shows anus level).*

Nematode Collection, Beltsville, MD, USA (collection number IPP-J-0945), and University of California Riverside Nematode Collection, Riverside, CA, USA (collection number IPP-J-0938).

Diagnosis and relationships

The *P. floridensis* n. sp. female is characterised by the following morphological characters: slender body, undivided, plain and smooth face with all labial sectors fused together and partially with an oval oral disc, lip region with two lip annuli and with the second annulus larger and thicker than the first, ellipsoidal stylet knobs or rounded with slightly flattened anterior surface, rounded, oval or rarely rectangular spermatheca filled with sperm, tail bluntly pointed with smooth terminus (in rare specimens slightly indented). The matrix code (*sensu* Castillo & Vovlas, 2007) for this species is: A1, B2, C2, D2, E2, F4, G3, H1, I2, J1, K1.

Few morphological and morphometrical characters separate *P. floridensis* n. sp. from *P. parafloridensis* n. sp., hereinafter described. The present study revealed that *P. floridensis* n. sp. differs from *P. parafloridensis* n. sp. in having a shorter female body (average 450 *vs* 532 μm) an oval *vs* round oral disc, a small, round to oval and sometimes rectangular spermatheca *vs* quadrangular or large rectangular in *P. parafloridensis* n. sp., and a bluntly to finely pointed (rarely indented) tail tip *vs* subhemispherical or bluntly pointed tail with smooth or, less frequently, indented tail terminus. However, these characters may overlap in some specimens making the morphological separation of these two species unreliable without the corroboration of the molecular analysis and, thus, they are considered as cryptic species.

The amphimictic reproductive habits, presence of males and a large spermatheca filled with sperm morphologically separate *P. floridensis* n. sp. from other male-less *Pratylenchus* species, with undivided and smooth face, two lip annuli and having a non-functional spermatheca such as *P. acuticaudatus* Braasch & Decker, 1989, *P. angulatus* Siddiqi, 1994, *P. brachyurus*, *P. estoniensis* Ryss, 1982, *P. hippeastri* and *P. tenuis* Thorne & Malek, 1968. This new species differs from the amphimictic *Pratylenchus* with the same lip region features by the following characters: from *P. alleni* Ferris, 1961 it differs in the tail shape (bluntly pointed *vs* rounded) and a more anterior vulva position (77 *vs* 80%); from *P. araucensis* Múnera, Bert & Decraemer, 2009 by the long *vs* short pharyngeal overlap, lateral field smooth *vs* areolated in outer lateral ridges and shape of tail terminus (smooth *vs* variable); from *P. artemisiae* Zheng & Chen, 1994 by the longer stylet length (14-15.5 *vs* 11.5-14.5 μm) and more anterior vulva position (75-80 *vs* 76-81%); from *P. brzeskii* Karssen, Waeyenberge & Moens, 2000 by the shorter stylet (14-15.5 *vs* 18-19 μm); from *P. coffeae* by the tail shape (bluntly pointed *vs* rounded, truncate or indented)

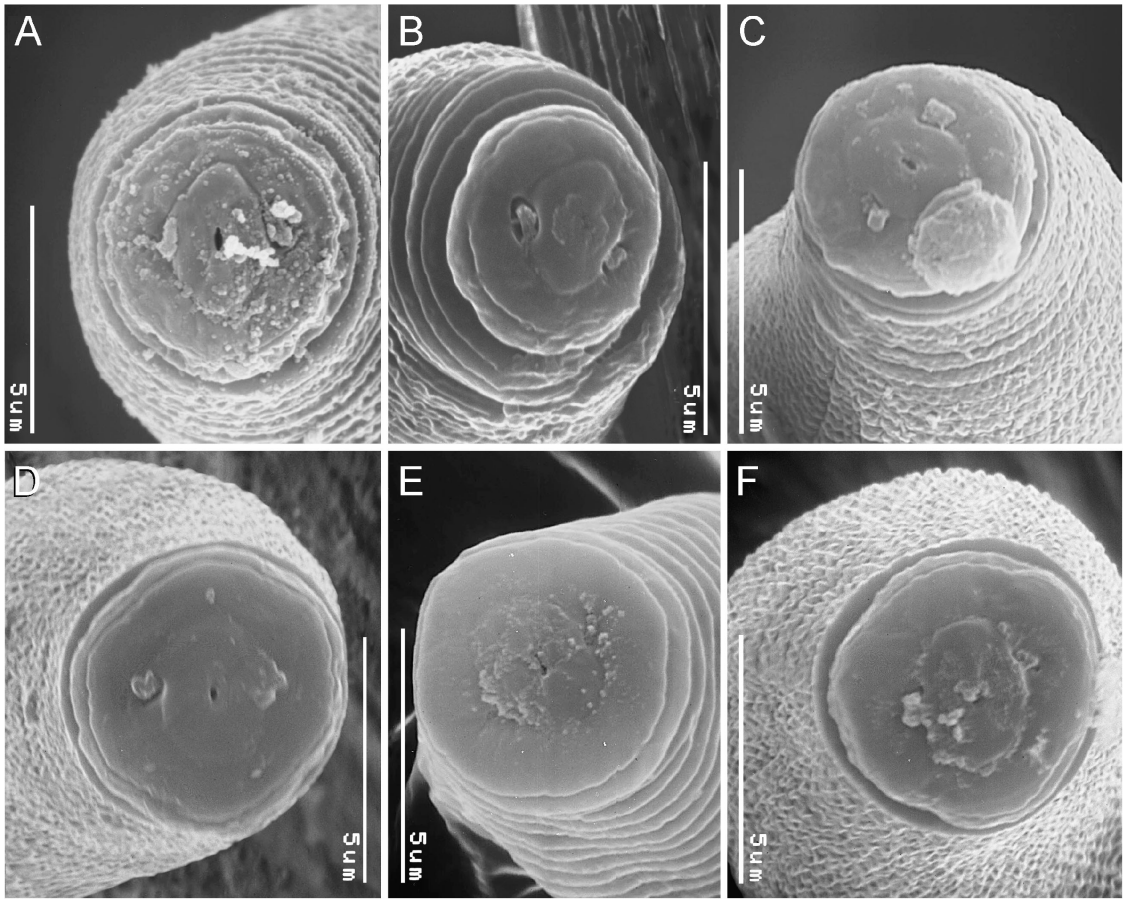


Fig. 11. *Comparison of SEM lip region morphology of* Pratylenchus floridensis *n. sp. (A-C) and* P. parafloridensis *n. sp. (D-F). A, B: Female face view showing undivided patterns with all labial sectors fused together and partially with oval oral disc. C: Male face pattern. D-F: Female undivided face patterns with all labial sectors fused together and partially with round oral disc. Note second lip annulus larger and thicker than first in both species.*

and a more anterior vulva position (77 *vs* 81%); from *P. flakkensis* Seinhorst, 1968 by the tail (bluntly pointed with smooth terminus *vs* conical with faintly annulated terminus) and stylet knob shape (ellipsoid to rounded *vs* anteriorly pointed), from *P. gutierrezi*[*] Golden, Lopez & Vilchez, 1992 by the undivided face *vs* divided; and from *P. kumamotoensis* Mizukubo, Sugimura & Uesugi, 2007 by the pharyngeal gland lobe (ventral *vs* frequently dorsal), shorter PUS (23-32 *vs* 37-45 μm) and lateral field (smooth *vs* areolated in vulval region). *Pratylenchus floridensis* n. sp. also differs from *P. jaehni*, *P. loosi*, *P. neobrachyurus* Siddiqi, 1994, *P. panamenis* Siddiqi, Lopez & Vilchez, 1991, *P. roseus* Zarina & Maqbool, 1998 and *P. silvaticus* Brzeski, 1998 by the more anterior vulva position (75-80 *vs* 77-80, 79-85, 80-84, 77-83, 81-83 and 80-83%, respectively). In addition, *P. floridensis* n. sp. has a longer tail than *P. jaehni* (25-29 *vs* 21-31 μm), a longer body than *P. neobrachyurus* (387-507 *vs* 310-410 μm), a different tail shape to *P. panamensis* (bluntly pointed with mostly smooth terminus *vs* subclavate with annulated terminus), different vulval margins, number of lateral lines and tail terminus than *P. roseus* (no vulval flaps, four lateral lines and smooth tail terminus *vs* presence of vulval flaps, six lateral lines and coarsely annulated tail terminus) and different tail shape to *P. silvaticus* (slightly clavate with irregularly striated tail terminus).

We would like to point out that lip patterns of *P. acuticaudatus*, *P. alleni*, *P. artemisiae*, *P. angulatus*, *P. brzeskii*,

[*] Considered as junior synonym of *P. panamaensis* by Siddiqi (2000), Castillo and Vovlas (2007) and Handoo *et al*. (2008).

Table 4. *Morphometrics of* Pratylenchus floridensis *n. sp. (Zolfo Springs population) and* P. parafloridensis *n. sp. (Lithia population). All measurements are in μm and in the form: mean ± s.d. (range).*

Character	*P. floridensis* n. sp.			*P. parafloridensis* n. sp.		
	Female		Male	Female		Male
	Holotype	Paratypes	Paratype	Holotype	Paratypes	Paratypes
n	–	9	1	–	10	5
L	457	450 ± 31.5 (387-507)*	457	563	532 ± 41.6 (475-603)**	448 ± 37.9 (414-494)
a	29.5	26.4 ± 1.8 (20.7-30.1)	25.4	25.6	29.0 ± 3.3 (25.2-37)	29.7 ± 3.8 (25-35.3)
b	5.9	5.6 ± 0.3 (5.3-5.9)	6.1	6.6	5.9 ± 0.3 (5.3-6.6)	5.6 ± 0.4 (5.3-6.0)
b′	3.9	3.9 ± 0.3 (3.4-4.3)	4.1	4.4	4.0 ± 0.4 (3.5-4.6)	3.7 ± 0.4 (3.4-4.0)
c	15.7	16.8 ± 0.8 (15.3-17.8)	14.6	17.9	16.8 ± 1.4 (14.9-18.5)	17.9 ± 2.0 (15-19.1)
c′	2.9	2.6 ± 0.3 (2.2-3.1)	2.9	2.4	2.9 ± 0.3 (2.4-3.3)	2.8 ± 0.4 (2.4-3.3)
V or T	77	77 ± 1.6 (75-80)*	52	78	77 ± 1.6 (75-80)**	46 ± 7.9 (35-52)
Stylet length	15	15 ± 0.6 (14.0-15.8)	22	15.8	15.4 ± 0.6 (14.5-16.0)	14 ± 0.3 (13.5-14.0)
Anterior end to centre of metacorpus	56	54 ± 4.1 (48-59)	53	57	59 ± 3.6 (52-64)	54 ± 4.3 (51-61)
end of pharyngeal gland lobe	117	118 ± 10.5 (104-140)	113	127	134 ± 6.8 (127-145)	128 ± 15 (118-145)
secretory/excretory pore	79	78 ± 6.6 (66-86)	75	83	88 ± 5.3 (81-96)	76 ± 3.6 (72-81)
Spermatheca length	16	17 ± 3.9 (13-24.5)	–	21	24 ± 8.0 (15-38)	–
Spermatheca diam.	10	12 ± 1.4 (9.5-13)	–	17	13 ± 2.2 (9.5-16)	–
PUS	28	27 ± 3.5 (23-32)	–	31	28 ± 5.2 (21-37)	–
Tail length	29	28 ± 1.4 (25-29)	31.3	31.5	32 ± 2.7 (28-35)	25 ± 5.4 (22-33)
Anal body diam.	10	11 ± 1.2 (9.0-12.0)	10.7	13	11 ± 0.7 (10.5-13)	9.0 ± 1.7 (6.7-10.7)
Spicules	–	–	19	–	–	18.5 ± 0.6 (17.8-19)
Gubernaculum	–	–	6	–	–	5.3 ± 0 (5.3)

* Measurements taken on 18 specimens.
** Measurements taken on 19 specimens.

P. estoniensis, *P. flakkensis*, *P. gibbicaudatus*, *P. kumamotoensis*, *P. neobrachyurus*, *P. panamensis*, *P. roseus*, *P. silvaticus* and *P. tenuis* are not known (Castillo & Vovlas, 2007).

Pratylenchus parafloridensis* n. sp.

= *P. loosi apud* Inserra *et al*. (1996) *nec* Loof (1960)
= *Pratylenchus* N2 of Duncan *et al*. (1999)
(Figs 8-11)

Measurements

See Table 4 and Inserra *et al.* (1996) and Table 2 and Results in Duncan *et al.* (1999).

* Specific epithet consisting of *para* = close + *floridensis* and indicating the close similarity of this species with *P. floridensis* n. sp.

Description

Female

Body of dead females almost straight or in open C. Labial region with two annuli, 2.3 μm high, 7.5 μm broad on average, offset from body by a slight constriction, second lip annulus distinctly wider and higher than first. SEM *en face* view characterised by undivided pattern, with all labial sectors fused together and with a rounded oral disc, amphidial openings obliquely orientated, at sides of oral disc. Stylet with rounded or ellipsoidal knobs, 4 μm across, 2.1 μm high (mean values). Dorsal pharyngeal gland opening 2-2.5 μm posterior to stylet base (o range = 12.5-17.2%). Pharyngeal metacorpus oval, 10-15 μm high × 7.5-13 μm diam. Isthmus slender, encircled by nerve ring in anterior half. Pharyngeal gland lobe rather long, overlapping intestine ventrally, 43 ± 9.3 (30-56) μm in length. Lateral fields with four lines,

not areolated. Anterior genital tract 171-211 μm or 31-39% of body length long. Spermatheca filled with sperm, quadrangular or large rectangular in shape, sometimes with constriction in equatorial diam., giving appearance of a bilobed structure. Post-uterine sac *ca* 1.5 body diam. long, often with rudimentary cellular elements at tip. Tail conoid, subhemispherical or bluntly pointed with smooth or slightly indented (*ca* 30% of specimens observed) terminus.

Male

Common in field populations. Body straight when heat-relaxed, similar to female except for sexual dimorphism and body size, which is slightly smaller. Lip region 2.0 $\pm$ 0.1 (2-2.1) μm high and 6.4 $\pm$ 0.3 (6-6.7) μm broad. Stylet more slender and shorter than in female, with rounded knobs, 2-2.7 μm across, 2 μm high. Pharyngeal metacorpus rounded to oval, 10.5 $\times$ 7.7 μm (longitudinal and cross diam., respectively). Pharyngeal gland lobe overlapping intestine for 43 $\pm$ 7.4 (37-51) μm. Hemizonid just anterior to excretory pore. Lateral field with four, smooth lines. Tail conical, rather short. Testis outstretched, 211 $\pm$ 34.6 (174-254) μm long. Spicules arcuate, slender, weakly cephalated, gubernaculum simple, slightly arcuate.

TYPE HOST AND LOCALITY

Maidencane (*Panicum hemitomon* Schultes) roots collected from a pasture land in Lithia, Hillsborough, FL, USA (latitude 27°79′63″N; longitude 82°21′13″W). The soil type is sandy and the climate is subtropical.

TYPE MATERIAL

Holotype female, 29 female and four male paratypes deposited at the Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (CNR), Sede di Bari, Bari, Italy (collection numbers IPP-J-0946 to J-0952). Additional paratypes were distributed to the USDA Nematode Collection, Beltsville, MD, USA (collection number IPP-J-0953), and University of California Riverside Nematode Collection, Riverside, CA, USA (collection number IPP-J-0954).

DIAGNOSIS AND RELATIONSHIPS

The *P. parafloridensis* n. sp. female is characterised by the following morphological characters: slender body, undivided, plain and smooth face with all labial sectors fused together and with a round oral disc, lip region with two lip annuli and with the second annulus larger and thicker than the first, generally rounded stylet knobs, quadrangular or large rectangular, sometimes bilobed, spermatheca filled with sperm, tail subhemispherical or bluntly pointed with smooth or, less frequently, slightly indented terminus. The matrix code (*sensu* Castillo & Vovlas, 2007) for this species is: A1, B2, C2, D2, E2, F5, G3, H1, I3, J1, K1.

The relationship of *P. parafloridensis* with other members of the genus *Pratylenchus* is similar to that described above for *P. floridensis* n. sp.

Discussion

PRATYLENCHUS HIPPEASTRI FROM BROMELIADS

This study provides evidence that *P. hippeastri* is a tropical root-lesion nematode reported so far only in Florida where it parasitises tropical ornamentals such as amaryllis and bromeliads. The application of sequence and phylogenetic analysis of the ITS-rRNA gene confirmed co-specificity of the root-lesion nematode population found parasitising bromeliads with *P. hippeastri* a previously known parasite of amaryllis only. Our observations indicate that populations of this species from bromeliads presented males in both carrot discs and bromeliad roots. So far no males have been found in other populations of this nematode. The function of the males in the bromeliad populations is unclear since they are present in very small number and are consistently in association with unmated females showing an empty and small spermatheca. There are reports of males occurring in parthenogenetic root-lesion nematodes such as *P. zeae* (Loof, 1991). The identity of these males may be questioned since contaminating male specimens belonging to different species may be associated with parthenogenetic species. However, in this case their identity was confirmed by sequencing rDNA genes from male specimens. In spite of the occurrence of a few males, our observations do not provide any evidence that *P. hippeastri* is an amphimictic species.

USEFULNESS OF THE ITS-RDNA SEQUENCES FOR SPECIES DIFFERENTIATION IN *PRATYLENCHUS*

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.

The present analysis of the ITS-rDNA dataset clearly separated *P. hippeastri* from other amphimictic and male-less root-lesion nematodes confirming that they are probably new *Pratylenchus* species belonging to the *P. hippeastri* species complex. The fact that they share morphological affinity, show minimal sequence differences in the rRNA gene and that sometimes their positions are not well resolved in the phylogenetic trees suggests that these species are derived by recent speciation events with insufficient time to attain complete morphological differentiation. The phylogenetic analysis of the ITS-rDNA does not confirm the conclusion of cospecificity of *Pratylenchu*s N1 and N2 populations with *P. hippeastri* previously made by Subbotin *et al.* (2008) based on analysis of the D2-D3 of 28S rDNA, but, instead, shows that each of these populations represents a distinct species. Successful application of the ITS for species differentiation in *Pratylenchus* has been shown by Orui (1996), Waeyenberge *et al*. (2000) and De la Peña *et al*. (2006) with PCR-RFLP. These studies also revealed heterogeneity in the ITS sequences, which resulted in additional bands on gels after restriction of PCR products. These additional bands constitute complex RFLP profiles that may complicate diagnostics of *Pratylenchus* species. Our study also revealed heterogeneity in the ITS sequences for all studied *Pratylenchus* species. However, in most cases the phylogenetic analysis of the ITS sequence dataset allowed clear separation of sample populations because, except for *Pratylenchus* spH2 and spH3, all sequences obtained from the same sample clustered together. Although *P. parafloridensis* n. sp. and *Pratylenchus* spH6 formed separate subclades on the ITS trees, relationships between these species based on the D2-D3 remain uncertain. Thus, heterogeneity of ITS rRNA did not preclude species discrimination. Combined with the PCR-RFLP method, sequence and phylogenetic analysis has become a reliable approach for differentiation of *Pratylenchus* species. More detailed analysis of the ITS sequence alignment (Fig. 1) will allow the design of species-specific primers and the discovery of appropriate restriction enzymes for diagnostics of *P. hippeastri*, *P. floridensis* n. sp., *P. parafloridensis* n. sp. and closely related species.

The *Pratylenchus hippeastri* species complex

Sequence and phylogenetic analysis revealed that a complex of cryptic species genetically similar to *P. hippeastri* occurs in Florida, USA, South Africa and Russia. In addition to *P. floridensis* n. sp. and *P. parafloridensis* n. sp., we conclude that eight other populations should be considered as putative, undescribed, species. However, additional molecular, morphological and biological studies are required to clarify the taxonomic status of these eight populations. It is noteworthy that, based on preliminary morphological studies, these populations were identified not only as *P. hippeastri*, but as several other known species. The diagnostic morphological characters for *P. hippeastri* and the newly described *P. floridensis* n. sp. and *P. parafloridensis* n. sp. overlap to a significant degree, requiring careful examination of many specimens for an accurate diagnosis. Thus, identification of the species of *P. hippeastri*-complex is likely to rely increasingly on molecular methods. Two (N1, N2) of the eight putative species from Florida were described herein as new species because of their regulatory significance. The description of the other six Florida putative *P. hippeastri*-species complex (spH1, spH4-H8) is currently not of crucial interest for agronomic or regulatory purposes; however, information provided in this paper documents their existence. The description of the putative *P. hippeastri*-species complex from South Africa and Russia (spH2, spH3) requires more detailed morphological information.

The fact that species in the *P. hippeastri*-complex were found in Florida, Russia and South Africa suggests their world-wide distribution and a broad host range among monocots. Moreover, our findings suggest the recent evolution in Florida of numerous lesion nematodes including *P. hippeastri*. These species are male-less (spH5, spH7 and spH8) or amphimictic (*P. floridensis* n. sp., *P. parafloridensis* n. sp., spH1 and spH6). All have an undivided face with two and occasionally three lip annuli with the exception of the male-less spH4 which has a divided face with two lip annuli and was identified as *P. scribneri* by Hernández *et al.* (2000). This putative *P. scribneri* in the *P. hippeastri*-complex further complicates the taxonomic status of *P. scribneri*. Many lesion nematodes from turf grasses in Florida, including spH8, have been identified as *P. zeae*. The inclusion in the *P. hippeastri*-complex of another putative *P. zeae* population from South Africa casts doubt about the real identity of *P. zeae* and provides evidence that the reports of *P. zeae* in Florida need to be revaluated.

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