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Description of *Pratylenchus hispaniensis* n. sp. from Spain and considerations on the phylogenetic relationship among selected genera in the family Pratylenchidae

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Summary – A new amphimictic species, *Pratylenchus hispaniensis* n. sp., parasitising the roots of gum cistus in Andújar (Jaén), southern Spain, is described. The new species is characterised by the presence of numerous males and by the female having a lip region with three annuli, a divided face, a robust stylet (14.5-17.0 μm) with rounded knobs, lateral fields with four lines, V = 80-84, a round spermatheca full of sperm, well developed post-vulval uterine sac and an obliquely truncate tail with irregularly annulated terminus. Morphologically this species is related to *P. bhatti*, *P. kralli*, *P. mediterraneus*, *P. pseudofallax* and *P. thornei*. A phenetic study of the 25 most useful diagnostic morphological and allometric characters for *Pratylenchus* species was done using multivariate factor and linear discriminant analyses. In the factor analysis the first seven factors accounted for 71.1% of the total variance of the characters selected. These factors were related to female tail, pharyngeal overlap, reproductive behaviour, stylet length, L/post-vulval uterine sac ratio, body length and number of lip annuli. Discriminant analysis differentiated *Pratylenchus* spp. from the three valid species of *Zygotylenchus*. The results of the phylogenetic analysis based on sequences of the D2-D3 expansion regions of 28S, partial 18S and ITS rRNA genes confirmed the close relationship of *P. hispaniensis* n. sp. with *P. mediterraneus* and inferred molecular affinity with *P. brzeskii*, *P. neglectus* and *P. thornei*, in spite of variation in the position of *P. hispaniensis* n. sp. in the clades. Additional phylogenetic analyses based on the same sets of sequences for *P. hispaniensis* n. sp., *Zygotylenchus guevarai* and other Pratylenchidae indicated that *Pratylenchus* includes several paraphyletic lineages; however, likelihood tests did not reject monophyly of the genus. The inclusion of *Pratylenchus*, *Zygotylenchus*, *Hirschmanniella*, *Nacobbus* and *Apratylenchus* in Pratylenchidae was supported.

Keywords – *Cistus ladanifer*, D2-D3 region, gum cistus, molecular, morphology, morphometrics, root-lesion nematodes, SEM, Shimodaira-Hasegawa test, taxonomy.

The genus *Pratylenchus* Filipjev, 1936 contains more than 70 species of root-lesion nematodes. The morphological identification and delimitation of these species remains problematic due to their high morphological plasticity, the small number of diagnostic features available at species level, the intraspecific variability of some of these characters and the many incomplete descriptions published in the literature (Castillo & Vovlas, 2007). As a result, the actual number of valid species has varied according to author (see Siddiqi, 1986, 2000; Ryss, 1988, 2002a, b; Frederick & Tarjan, 1989; Handoo & Golden, 1989; Loof, 1991; Duncan *et al*., 1999; Castillo &

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Vovlas, 2007). Species differentiation is also difficult in the genus *Zygotylenchus* Siddiqi, 1963, a well defined genus commonly occurring in the Mediterranean region and other geographical areas (Siddiqi, 2000; Urek *et al*., 2003; Lisková *et al*., 2007). It contains only three valid species, *Z. guevarai* (Tobar Jiménez, 1963) Braun & Loof, 1966, *Z. natalensis* Van den Berg, 2003, and *Z. taomasinae* (de Guiran, 1963) Braun & Loof, 1966, plus another, poorly defined, species, *Z. biterminalis* Razjivin & Milan, 1978, which has been considered *incertae sedis* (Siddiqi, 2000). Morphologically, *Zygotylenchus* is an easily recognised taxon, although its validity has not previously been corroborated by molecular data.

Configuration of the lip pattern has been used successfully in the separation of *Pratylenchus* species. Lip patterns of *en face* with SEM were used to reveal phylogenetic relationships in several plant-parasitic nematode genera (Baldwin, 1992), as well as in the *Pratylenchus* (Corbett & Clark, 1983). They were coincident with major clades of selected species, including *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941, *P. coffeae* (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941, *P. goodeyi* Sher & Allen, 1953, *P. loosi* Loof, 1960, *P. panamaensis* Siddiqi, Dabur & Bajaj, 1991 (= *P. gutierrezi* Golden, López & Vilchez, 1992), *P. pratensis* (de Man, 1880) Filipjev, 1936, and *P. pseudocoffeae* Mizukubo, 1992, obtained from Bayesian analysis of D2-D3 expansion segments of 28S rRNA and partial 18S rRNA (Duncan *et al.*, 1999; Handoo *et al*., 2008; Subbotin *et al*., 2008).

Only recently has there been an interest in applying morphology for cladistic and phenetic analysis of the genus *Pratylenchus*. Duncan *et al*. (1999) showed that principal component analysis (PCA) of one morphological (smooth face *vs* divided face) and three allometric characters (length of stylet, ratios V and a) revealed congruence with phylogenetic relationships inferred from analysis of 28S rDNA sequences based on 32 *Pratylenchus* isolates with two lip annuli and amphimictic reproduction (*i.e*., presence of males). Ryss (2002a, b) provided a comprehensive morphological analysis of 49 species and 26 characters of *Pratylenchus* and, simultaneously, Carta *et al.* (2002) published a morphologically-based phylogenetic tree for 11 *Pratylenchus* species. However, these statistical approaches have not been applied to compare and differentiate species groups in *Pratylenchus* or to validate differences with *Zygotylenchus*. Therefore, one of the main objectives of the present study was to provide insights into the genus and species delimitation within and between *Pratylenchus* and *Zygotylenchus* taxa based on a multivariate analyses statistical approach using the most useful diagnostic morphological and allometric characters for *Pratylenchus* species.

Sequence analyses of nuclear ribosomal RNA genes have been used for molecular characterisation and reconstruction of phylogenetic relationships of *Pratylenchus* spp. (Al-Banna *et al*., 1997; Duncan *et al.*, 1999; Carta *et al*., 2001; De Luca *et al*., 2004; Subbotin *et al.*, 2008). These studies have clarified the taxonomical status of a large number of root-lesion nematodes, although many species have yet to be so characterised.

There has been consensus among taxonomists in considering the two genera mentioned above along with *Achlysiella* Hunt, Bridge & Machon, 1989, *Apratylenchoides* Sher, 1973, *Apratylenchus* Trinh, Waeyenberge, Nguyen, Baldwin, Karssen & Moens, 2009, *Hirschmanniella* Luc & Goodey, 1964, *Hoplotylus* s'Jacob, 1960, *Nacobbus* Thorne & Allen, 1944, *Pratylenchoides* Winslow, 1958, *Radopholus* Thorne, 1949 and *Zygradus* Siddiqi, 1991 as members of the family Pratylenchidae Thorne, 1949 (Luc, 1987; Siddiqi, 2000; Trinh *et al*., 2009). Recent molecular and phylogenetic studies concerning the relationships within these genera using sequences of the D2-D3 expansion regions of 28S rRNA (Subbotin *et al*., 2006; Trinh *et al.*, 2009) and 18S rRNA (Holterman, 2007; Bert *et al.*, 2008) have indicated that *Radopholus* and *Pratylenchoides* stood apart from other genera and did not belong to this family. These findings may cast doubt on the inclusion of *Achlysiella*, *Hoplotylus* and *Zygradus* in the Pratylenchidae since they are morphologically related to *Radopholus* and *Pratylenchoides*.

During nematode surveys conducted in cultivated and natural environments in southern Spain, an amphimictic root-lesion nematode was detected in roots of gum cistus, a flowering ornamental shrub used for the extraction of essential oils. Preliminary morphological examinations indicated that this amphimictic species did not fit any description of known *Pratylenchus* species and appeared to be morphologically related to *P. mediterraneus* Corbett, 1983, *P. neglectus* (Rensch, 1924) Filipjev & Schuurmans Stekhoven, 1941 and *P. thornei* Sher & Allen, 1953. In order to verify the taxonomic status of this species, we conducted a morpho-biological and molecular study of this unknown *Pratylenchus*, which is described herein as *P. hispaniensis* n. sp. These morpho-biological and molecular analyses were expanded to other valid members of the family Pratylenchidae and included two Californian populations of *P. neglectus* and *P. thornei*, and an Italian

and three Spanish populations of *Z. guevarai*. The rRNA sequences of *Pratylenchus*, *Zygotylenchus*, *Hirschmanniella*, *Nacobbus* and *Apratylenchus* species, along with members of the genus *Meloidogyne* and subfamily Telotylenchinae obtained from this study and GenBank, were used to supplement the morpho-biological description of *P. hispaniensis* n. sp. with molecular data. The specific objectives of this paper were: *i*) to conduct a phenetic study of the most useful diagnostic morphological and allometric characters for *Pratylenchus* species using multivariate factor and linear discriminant analyses; *ii*) to determine the molecular and phylogenetic affinities of *P. hispaniensis* n. sp. with closely related species using the rRNA gene sequences (ITS, D2-D3 of 28S and 18S); and *iii*) to establish the phylogenetic relationships among the genera *Pratylenchus*, *Zygotylenchus*, *Hirschmanniella*, *Nacobbus* and *Apratylenchus*, and to compare these with other genera outside the Pratylenchidae.

Materials and methods

Nematode populations

Specimens of *P. hispaniensis* n. sp. were obtained from a sandy soil in the rhizosphere and roots of the flowering ornamental shrub gum cistus (*Cistus ladanifer* L.) from Andújar (Jaén province), southern Spain (38°07′43.90″N latitude, 3°58′40.03″W longitude) at an altitude of 532 m a.s.l. Nematode populations of *Z. guevarai* were obtained from several localities in Italy and Spain and included three populations from the rhizosphere of grapevine (*Vitis vinifera* L.) from Bollullos par del Condado (Huelva province), southern Spain; one population from grapevine from Laguardia (Alava province), northern Spain; a population from the rhizosphere and roots of wheat (*Triticum aestivum* L.) from Fuente Palmera (Córdoba province), southern Spain; and one population from grapevine from Barletta (Bari province), southern Italy. *Pratylenchus neglectus* and *P. thornei* were obtained from soil samples collected in California, USA. The nematodes were extracted from rhizosphere soil samples by magnesium sulphate centrifugal flotation (Coolen, 1979).

Light and scanning electron microscopy

Specimens for light microscopy (LM) were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid, and processed to pure glycerin using Seinhorst's (1966) method. Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at up to ×1000 magnification. Measurements were done using a *camera lucida* attached to a light microscope. Morphometric data were processed using Statistix 9.0 (NH Analytical Software, Roseville, MN, USA).

For scanning electron microscopy (SEM) studies, fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold and observed with a Jeol JSM-5800 microscope (Abolafía *et al*., 2002).

Multivariate analyses

The analyses were based upon the following characters: number of lip annuli, *en face* view (undivided *vs* divided), lateral field lines and structures at the vulval region, body length (L), stylet length, stylet knob shape, excretory pore to anterior end (EP), pharyngeal overlap, shape of spermatheca, functionality of spermatheca, vulva position (V), female posterior genital tract length (PUS), female tail length, number of female tail annuli, female tail shape, female tail tip shape, presence or absence of males, and the ratios a, b, c, c′, L/PUS ratio, L/stylet length and L/EP. Since many descriptions of *Pratylenchus* spp. are incomplete and these characters are lacking (mainly the *en face* view), the study was based on only 38 species for which these characters were available (Castillo & Vovlas, 2007). We performed a multivariate factor analysis on this group of *Pratylenchus* spp. in order to determine the association among species within the genus. In addition, a multivariate canonical discriminant analysis was done to determine those morphological characters that could be used to differentiate between the *Pratylenchus* species studied and the three valid species of *Zygotylenchus* (Siddiqi, 2000).

Factor analysis was performed with the FACTOR procedure of SAS (Statistical Analysis System, version 9.1; SAS Institute, Cary, NC, USA). This analysis produced a set of variables (factors) that were linear combinations of the original variables. The new variables (factors) were independent of each other and ranked according to the amount of variation accounted for. After the initial factor extraction by the principal component method, an orthogonal varimax raw rotation was used to estimate the factor loadings. Only factors with an eigenvalue > 1 were extracted. Additionally, a discriminant function analysis was used to determine which morphometric characters discriminated between the *Pratylenchus* and *Zygotylenchus* genera using the SAS STEPDISC and DISCRIM procedures.

DNA EXTRACTION, PCR, CLONING AND SEQUENCING

Nematode DNA from *P. hispaniensis* n. sp. and *Z. guevarai* populations was extracted from single individuals and *P. neglectus* and *P. thornei* from several individuals using proteinase K as described by Castillo *et al.* (2003). Detailed protocols for PCR and cloning were as described by Castillo *et al.* (2003) and Tanha Maafi *et al.* (2003). Three rRNA gene fragments were amplified and used for sequence and phylogenetic analysis. The following primers were used for amplification D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) for amplification of D2-D3 regions of 28S (Subbotin *et al.*, 2006); G18SU (5′-GCTTGTCTCAAAGATTAAGCC-3′) and R18Tyl1 (5′-GGTCCAAGAATTTCACCTCTC-3′) for partial amplification of 18S rRNA (Chizhov *et al.*, 2006); and TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) for amplification of the partial 18S-ITS1-5.8S-ITS2 partial 28S gene (Tanha Maafi *et al.*, 2003).

PCR products were purified after amplification with Geneclean turbo (Q-BIOgene, Illkirch, France) or QI-Aquick (Qiagen, Chatsworth, CA, USA) gel extraction kits, quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for cloning or direct sequencing in both directions with the primers referred above. Two clones from each sample were sequenced. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3100 genetic analyser; Applied Biosystems, Foster City, CA, USA) at the University of Córdoba, Spain, or the University of California, Riverside, CA, USA, sequencing facilities. The newly obtained sequences were submitted to the GenBank database under accession numbers FJ717816-FJ717825 as indicated on the phylogenetic trees.

PHYLOGENETIC ANALYSES

The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters with corresponding published gene sequences (De Ley *et al.*, 2002, 2005, 2007; Tigano *et al.*, 2005; Holterman *et al.*, 2006; Subbotin *et al.*, 2006, 2008; Bert *et al.*, 2008; Troccoli *et al.*, 2008; Waeyenberge *et al.*, unpubl.). Outgroup taxa for each dataset were chosen according to the results of previous published data (Holterman *et al.*, 2006; Subbotin *et al.*, 2006, 2008; Bert *et al.*, 2008). Sequence alignments were manually edited using GenDoc 2.5.0 (Nicholas *et al.*, 1997). Two alignments were generated from each D2-D3 and 18S dataset sequences: *i*) automatic alignment; and *ii*) culled alignment, from which ambiguously aligned regions were excluded. Sequence analyses of alignments were performed with PAUP* 4.0b10 (Swofford, 2003). Phylogenetic analysis of the sequence data sets were performed with maximum parsimony (MP) and maximum likelihood (ML) using PAUP* and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution was obtained using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion in conjunction with PAUP*. BI analysis under 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 1000 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView program (Page, 1996). We used the Shimodaira-Hasegawa (SH) test as implemented in PAUP* for testing alternative topologies.

Results

MULTIVARIATE ANALYSES

In the factor analysis, the first seven factors (eigenvalue > 1) accounted for 71.1% of the total variance of morphometric characters of the 38 *Pratylenchus* species included in the analysis (Table 1). Table 1 includes the eigenvalues for the six factors extracted, that were a combination of all characters in the analysis, and the corresponding values in the eigenvectors for each character that were used to interpret the significance of the factors. Factor 1 was dominated by high positive weights (eigenvector > 0.66) for female tail length, number of female tail annuli, pharyngeal overlap and c′ ratio, and high negative weights for c ratio (Table 1; Fig. 1A, B). This factor is therefore related to the female tail and pharyngeal overlap. Factor 2 is dominated by high positive weights (eigenvector $= 0.92$) for functionality of spermatheca and high negative weights (eigenvector < 0.73) for presence/absence of males and shape of spermatheca (Table 1;

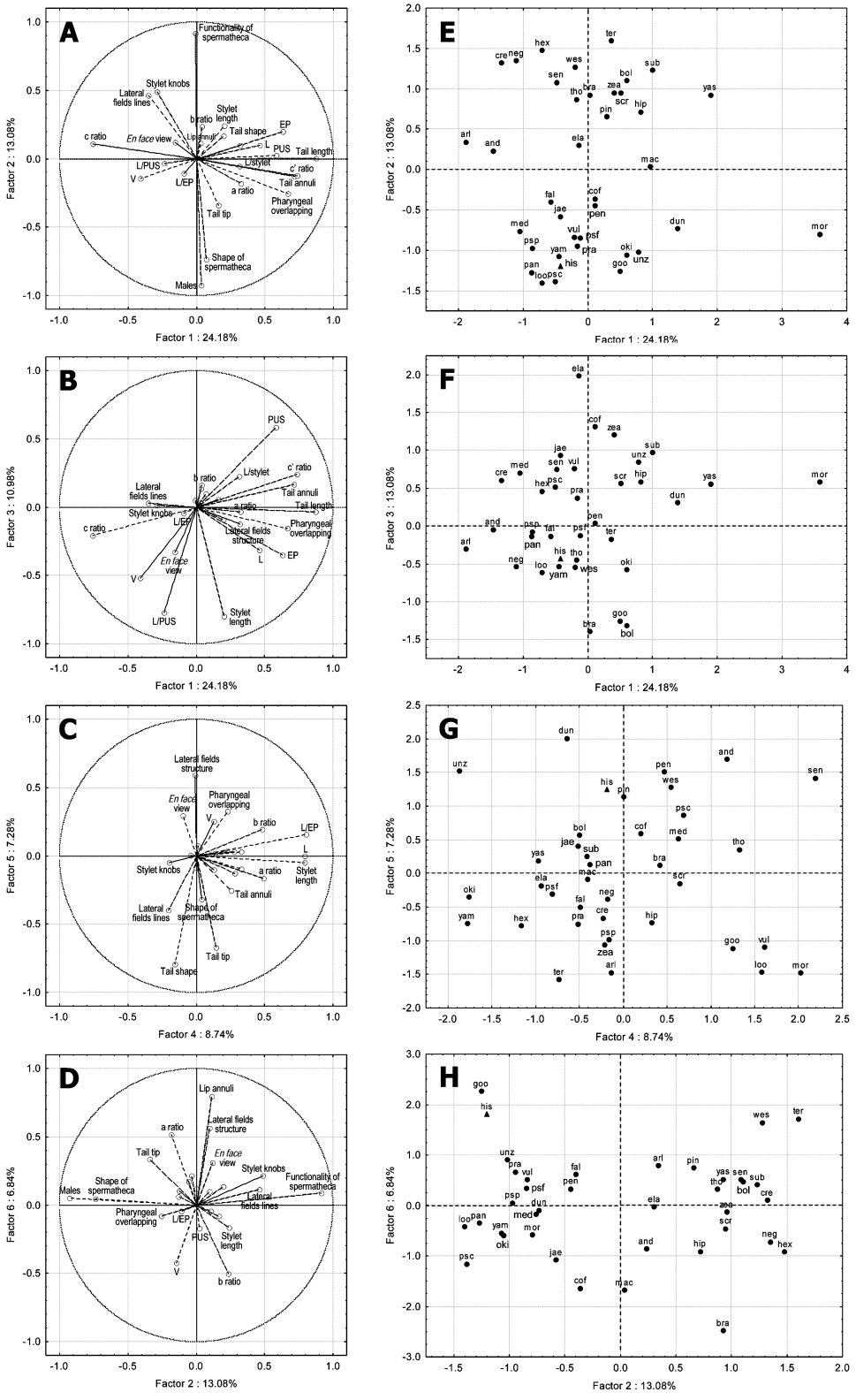
Table 1. *Eigenvector and eigenvalues of factors derived from nematode morphometric characters for 38* Pratylenchus *species.*

Character	Factor					
	F1	F2	F3	F4	F5	F6
Lip annuli	0.032	0.114	0.034	0.226	0.034	*0.796*
En face view	−0.153	0.120	−0.330	−0.100	0.295	0.310
Presence/absence of males	0.035	*−0.925*	0.135	−0.038	0.002	0.048
Shape of spermatheca	0.074	*−0.735*	0.097	0.039	−0.320	0.046
Functionality of spermatheca	−0.001	*0.916*	0.051	−0.003	−0.088	0.088
Female tail shape	0.199	0.168	−0.092	−0.156	*−0.793*	−0.079
Female tail length	*0.874*	0.001	−0.033	0.328	−0.099	0.118
Number of female tail annuli	*0.717*	−0.130	0.164	0.255	−0.254	0.061
Female tail tip shape	0.160	−0.340	0.054	0.146	*−0.674*	0.334
Lateral field lines at vulval region	−0.352	0.462	0.028	−0.203	−0.399	0.145
Lateral field structure at vulval region	0.319	0.095	−0.124	−0.008	0.591	0.560
L	0.460	0.098	−0.311	*0.792*	−0.002	0.096
a	0.325	−0.181	−0.034	0.496	−0.165	0.513
b	0.041	0.234	0.160	0.485	0.193	−0.505
c	*−0.758*	0.107	−0.207	0.331	0.029	−0.042
c′	*0.738*	−0.125	0.241	0.137	0.020	0.105
V	−0.411	−0.147	−0.521	0.128	0.253	−0.426
Stylet length	0.204	0.241	*−0.800*	0.015	0.073	−0.169
Stylet knob shape	−0.287	0.488	0.012	−0.199	−0.051	0.214
Excretory pore to anterior end (EP)	0.632	0.196	−0.351	0.285	−0.128	0.133
Pharyngeal overlap	*0.665*	−0.257	−0.157	0.230	0.322	−0.079
Female posterior genital tract length (PUS)	0.584	0.022	0.584	0.275	0.027	−0.171
L/stylet length	0.315	−0.054	0.226	*0.796*	−0.052	0.170
L/EP	−0.094	−0.108	−0.047	*0.803*	0.156	−0.043
L/PUS	−0.235	−0.036	*−0.775*	0.128	−0.105	0.212
Eigenvalues	6.046	3.270	2.744	2.185	1.819	1.711
% of total variance	24.18	13.08	10.98	8.74	7.28	6.84
Cumulative % of total variance	24.18	37.26	48.24	56.98	64.26	71.10

Factors are based on 38 *Pratylenchus* species listed in Figure 1. Values of morphometric and morphological characters dominating factors 1 to 6 (eigenvector > 0.65) are italicised. Morphological and diagnostic characters according to Castillo and Vovlas (2007).

Fig. 1A, D), relating this factor with reproductive behaviour (parthenogenesis *vs* amphimixis). Factor 3 is dominated by high negative weights (eigenvector < 0.77) for stylet length and L/PUS ratio (Table 1; Fig. 1B). Factor 4 is dominated by high positive weights (eigenvector $>$ 0.79) for body length (L) and L/stylet length and L/EP ratios (Table 1; Fig. 1C), thereby relating this factor to the size of the nematode species. Factor 5 is dominated by high negative weights (eigenvector < 0.67) for the female tail length and tail shape (Table 1; Fig. 1C). Factor 6 identifies the uniqueness of the lip annuli (Table 1; Fig. 1D). Of the remaining morphological characters, PUS and lateral field lines and their form at the vulval region were not highly associated with any of the factors extracted but, to a lesser extent, showed positive weights to factors 1 (eigenvector $= 0.58$), 3 (eigenvector $= 0.58$), 5 (eigenvector $= 0.59$) and 6 (eigenvector $= 0.56$), respectively (Table 1). Results of factor analyses were represented graphically in Cartesian plots in which *Pratylenchus* species were projected on the plane of the x and y axes, respectively, as pairwise combinations of factors 1 to 6 (Fig. 1E-H).

According to their relative position along the x axis (factor 1) on Figure 1E and F, the female tail length, number of female tail annuli and c ratio increased and c′ ratio decreased from left to right. Similarly, according to their position along the y axis on Figure 1E (factor 2) parthenogenetic *Pratylenchus* spp. are grouped at the top, whereas amphimictic species with small to rounded spermatheca and those with oval to rectangular spermatheca


A
Functionality of spermatheca
Stylet knobs
Lateral fields lines
Stylet length
b ratio
Lip annuli
Tail shape
EP
L
PUS
Tail length
c ratio
En face view
L/PUS
V
L/EP
L/stylet
c' ratio
a ratio
Tail annuli
Pharyngeal overlapping
Tail tip
Shape of spermatheca
Males
Factor 1 : 24.18%
Factor 2 : 13.08%
B
PUS
L/stylet
c' ratio
b ratio
Lateral fields lines
Tail annuli
a ratio
Tail length
Stylet knobs
L/EP
Lateral fields structure
Pharyngeal overlapping
c ratio
En face view
V
L
EP
L/PUS
Stylet length
Factor 1 : 24.18%
Factor 3 : 10.98%
C
Lateral fields structure
En face view
Pharyngeal overlapping
V
b ratio
L/EP
L
Stylet knobs
a ratio
Stylet length
Tail annuli
Lateral fields lines
Shape of spermatheca
Tail tip
Tail shape
Factor 4 : 8.74%
Factor 5 : 7.28%
D
Lip annuli
a ratio
Lateral fields structure
Tail tip
En face view
Stylet knobs
Shape of spermatheca
Males
Functionality of spermatheca
Lateral fields lines
Pharyngeal overlapping
L/EP
PUS
Stylet length
V
b ratio
Factor 2 : 13.08%
Factor 6 : 6.84%
E
ter
hex
cre
neg
wes
sub
sen
bol
zea
tho
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psp
yam
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oki
his
goo
unz
pan
loo
psc
Factor 1 : 24.18%
Factor 2 : 13.08%
F
ela
cof
zea
jae
sub
unz
sen
vul
med
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yas
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hex
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dun
pen
and
psp
fal
psf
ter
arl
pan
his
tho
neg
loo
yam
wes
oki
goo
bra
bol
Factor 1 : 24.18%
Factor 3 : 13.08%
G
dun
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pen
sen
his
pin
wes
psc
bol
cof
med
jae
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yas
sub
pan
bra
mac
scr
ela
psf
oki
neg
fal
yam
hex
cre
hip
psp
goo
vul
zea
loo
arl
ter
mor
Factor 4 : 8.74%
Factor 5 : 7.28%
H
goo
his
wes
ter
unz
arl
pin
pra
vul
fal
yas
sen
sub
pen
psf
bol
cre
psp
dun
ela
zea
med
loo
pan
yam
mor
scr
oki
neg
and
hip
hex
psc
jae
cof
mac
bra
Factor 2 : 13.08%
Factor 6 : 6.84%

Fig. 1. *Factor analysis of nine morphometric characters used to characterise 38* Pratylenchus *species. Left panel: projection of morphometric and morphological characters on the plane of factors 1 and 2 (A), 1 and 3 (B), 4 and 5 (C) and 2 and 6 (D). Right panel: projection of* Pratylenchus *species on the plane of factor 1 and 2 (E), 1 and 3 (F), 4 and 5 (G) and 2 and 6 (H). Abbreviations: and* = andinus*; arl* = arlingtoni*; bol* = bolivianus*; bra* = brachyurus*; cof* = coffeae*; cre* = crenatus*; dun* = dunensis*; ela* = elamini*; fal* = fallax*; goo* = goodeyi*; hex* = hexincisus*; hip* = hippeastri*; his* = hispaniensis *n. sp.; jae* = jaehni*; loo* = loosi*; mac* = macrostylus*; med* = mediterraneus*; mor* = morettoi*; neg* = neglectus*; oki* = okinawensis*; pan* = panamaensis*; pen* = penetrans*; pin* = pinguicaudatus*; pra* = pratensis*; psc* = pseudocoffeae*; psf* = pseudofallax*; psp* = pseudopratensis*; scr* = scribneri*; sen* = sensillatus*; sub* = subranjani*; ter* = teres*; tho* = thornei*; unz* = unzenensis*; vul* = vulnus*; wes* = wescolagricus*; yam* = yamagutii*; yas* = yassini*; zea* = zeae.

are grouped at the middle and bottom, respectively. According to their position along the *y* axis on Figure 1F (factor 3), nematode stylet length and L/PUS ratio decreased from bottom to top. When projected on the plane of factor 1 and 2 on Figure 1E, amphimictic species with longer tail, higher number of lip annuli and lower c ratio are located at the left-bottom quadrant on Figure 1E, *i.e*., *P. hispaniensis* n. sp., *P. mediterraneus* or *P. pseudofallax* Café-Filho & Huang, 1989, while parthenogenetic species with shorter tail, lower lip annuli number and higher c ratio are located at the right-top quadrant, *i.e*., *P. zeae*, *P. scribneri* or *P. hippeastri*. In Figure 1F, amphimictic species with longer stylet and higher L/PUS ratio are located at the left-bottom quadrant, *i.e*., *P. hispaniensis*, *P. neglectus* and *P. thornei*. By contrast, parthenogenetic species with a shorter stylet and lower L/PUS ratio are located at the right-top quadrant in Figure 1F, *i.e*., *P. dunensis*, *P. coffee* and *P. zeae*.

When *Pratylenchus* species are projected on the plane of factors 4 and 5 (Fig. 1G), the size of the *Pratylenchus* spp. increases (*i.e*., body length (L), and L/stylet length and L/EP ratios) from left to right along the *x* axis. On the other hand, along the *y* axis (factor 5), species with cylindrical and conoid tail shape and pointed and smooth tail tip shape are grouped at the bottom and top, respectively. Finally, according to their position along the *y* axis in Figure 1H (factor 6), *Pratylenchus* species with two (*i.e*., *P. brachyurus*), three (*i.e*., *P. pseudofallax*) and four lip annuli (*i.e*., *P. goodeyi*) are grouped at the bottom, middle and top, respectively.

Discriminant analysis proved effective in differentiating the 37 (excluding *P. hispaniensis* n. sp.) published *Pratylenchus* species from the three valid species of *Zygotylenchus* (Wilks' Lambda = 0.006, $P < 0.0001$). The morphometric characters with the greatest discrimination power were the female posterior genital tract length (PUS) (Wilks' Lambda = 0.040, $P < 0.0001$), the body length/PUS ratio (Wilks' Lambda = 0.222, $P < 0.0001$), number of female tail annuli (Wilks' Lambda = 0.007, $P = 0.0314$) and b ratio (Wilks' Lambda = 0.007, $P = 0.0451$). To a lesser extent, the stylet length, *en face* pattern and excretory pore to anterior end distance also contributed to the discriminant function. The fit between the species considered as belonging to each of the two genera and those predicted by the discriminant function was 100%. The discriminant model was validated using *P. hispaniensis* n. sp. as an undescribed species. The model classified the new species as member of the genus *Pratylenchus*.

DESCRIPTION

Pratylenchus hispaniensis* **n. sp.**

(Figs 2-4)

MEASUREMENTS

See Table 2.

DESCRIPTION

Female

Body slender, vermiform, tapering towards both ends, ventrally arcuate to almost straight in relaxed condition. Body annuli *ca* 1-1.5 μm wide at mid-body. Lateral field with four lines starting at mid-stylet level, field occupying *ca* one-third of body diam. at mid-body, 7.0 $\pm$ 0.8 (6.0-8.0) μm wide; starting at annuli 5-7 at stylet level. Inner and outer bands regularly areolated. Lip region flat, continuous with body contour, consisting of three annuli, 2.5-3.0 μm high and 7.0-8.0 μm wide on average, first annulus slightly narrower and lower than second and cephalic framework strongly developed. *En face* view characterised by a divided face with rectangular subdorsal and subventral lips fused with oral disc in a dumb-bell pattern which is separated from lateral lip sectors by two,

* The specific epithet refers to the geographic origin and is derived from the Latin *hispaniensis* = Spanish, from Spain.

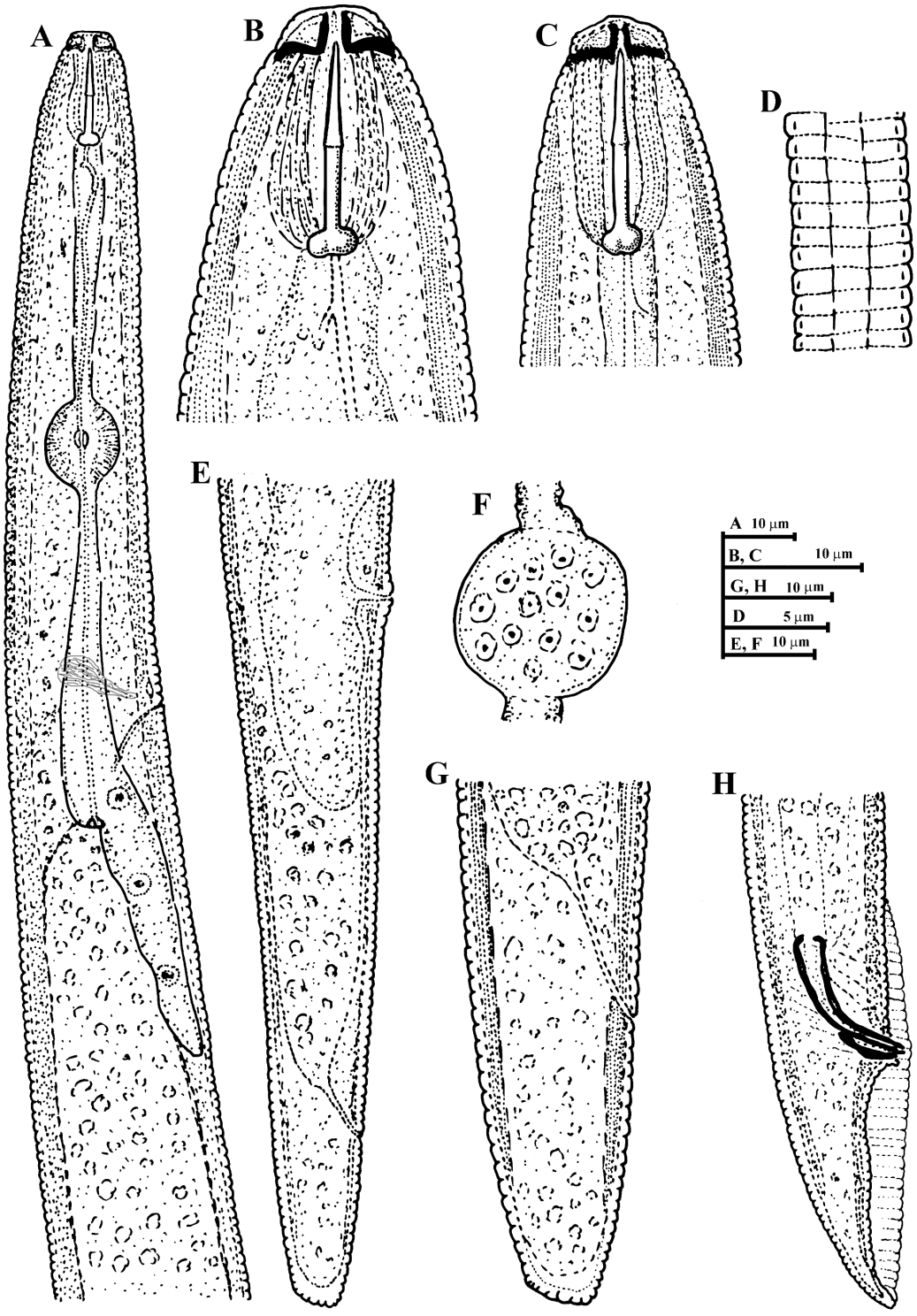

Fig. 2. Pratylenchus hispaniensis *n. sp. A: Female neck region; B, C: Female and male anterior body region, respectively; D: Detail of lateral field at mid-body; E: Female posterior region; F: Detail of spermatheca; G: Female tail region; H: Male tail region and copulatory apparatus.*

Fig. 3. *Light micrographs of* Pratylenchus hispaniensis *n. sp. A: Entire female and male; B: Female neck region; C, D: Female and male anterior body regions, respectively; E: Female anterior body region; F, G: Female posterior regions; H: Detail of lateral field at mid-body; I-K: Female tails; L: Male tail with bursa and copulatory apparatus. (Scale bars: A = 50 μm; B-G, I-L = 15 μm; H = 5 μm.)*

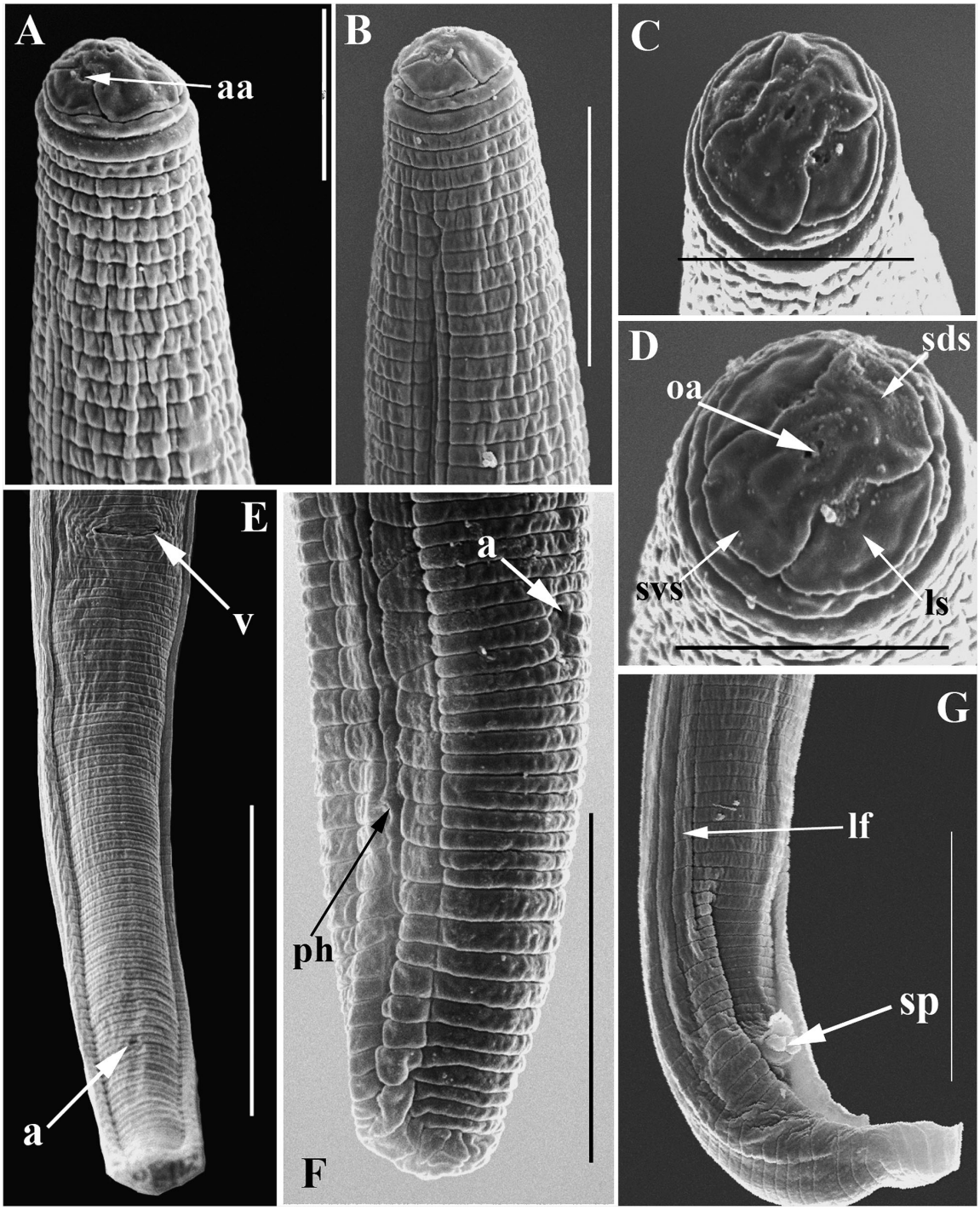

Fig. 4. *Scanning electron micrographs of* Pratylenchus hispaniensis *n. sp. Female (A-F). A, B: Anterior body region; C, D: Lip region,* en face *view; E: Ventral view of vulva-terminus body portion; F: Tail. Male (G). G: Tail. Abbreviations: a = anus; aa = amphidial aperture; lf = lateral field; ls = lateral lip sector; oa = oral aperture; ph = phasmid; sds = subdorsal lip sector; sp = spicule; svs = subventral lip sector; v = vulva. (Scale bars: A, C, D = 5 μm; B, F = 10 μm; E, G = 20 μm.)*

Table 2. *Morphometrics of* Pratylenchus hispaniensis *n. sp. from the rhizosphere and roots of the flowering ornamental shrub gum cistus (*Cistus ladanifer *L.) from Andújar (Jaén), southern Spain. All measurements are in* μm *and in the format: mean* $\pm$ *standard deviation (range).*

Character	Female		Male
	Holotype	Paratypes	Paratypes
n	–	24	21
L	544	538 ± 38 (462-622)	509 ± 46 (411-569)
a	27.2	28.3 ± 1.9 (24.7-31.5)	29.6 ± 2.6 (24.2-35.7)
b	5.9	5.7 ± 0.6 (4.6-6.4)	5.5 ± 0.2 (5.3-5.7)
b′	4.3	4.0 ± 0.3 (3.4-4.5)	3.8 ± 0.3 (3.3-4.4)
c	23.6	20.4 ± 1.5 (17.6-23.6)	20.4 ± 2.3 (17.2-24.7))
c′	1.8	2.0 ± 0.2 (1.6-2.3)	2.0 ± 0.3 (1.4-2.5)
V or T	83	81.9 ± 1.2 (80.0-84.0)	36.9 ± 6.7 (30.0-47.0)
Stylet length	15	15.3 ± 0.7 (14.5-17.0)	15.1 ± 0.5 (14.5-16.0)
M	50	50.8 ± 1.0 (50-52)	50.5 ± 1.4 (50-52)
O	13.3	17.7 ± 2.9 (12.9-21.2)	14.4 ± 2.3 (12.5-16.7)
Anterior end to nerve ring	72	71 ± 4.8 (63-81)	72 ± 3.0 (67-76)
Anterior end to excretory pore	86	87 ± 6.6 (76-98)	87 ± 4.6 (78-91)
MB	42	43 ± 1.8 (40-45)	42 ± 1.4 (40-44)
Pharynx length	125	133 ± 6.6 (120-148)	133 ± 4.9 (123-143)
Tail length	23	27 ± 2.4 (23-33)	25 ± 3.6 (18-32)
Spicule	–	–	17.1 ± 1.3 (15.0-19.0)
Gubernaculum	–	–	5.6 ± 0.7 (5.0-7.0)

Abbreviations as defined in Siddiqi (2000).

almost straight, incisures forming an obtuse angle *sensu* Subbotin *et al.* (2008). Lip pattern configuration in accordance with group 2 according to classification scheme of Corbett and Clark (1983). Stylet short, robust with rounded knobs (3.0-4.0) μm wide on average. Distance of dorsal pharyngeal gland orifice to stylet base = 2.8 ± 0.6 (2.0-3.5) μm, dorsal pharyngeal gland orifice ampulla distinct. Procorpus largely cylindrical, 37.6 ± 2.7 (33-42) μm long, narrowing posteriorly towards junction with median bulb. Median bulb muscular, rounded-oval, occupying half of corresponding body diam., 13.0 ± 1.0 (12-15) × 9.8 ± 1.0 (9-12) μm, cuticularised valve plates prominent. Nerve ring encircling posterior part of isthmus, 70.6 ± 4.8 (63-81) μm from anterior end. Isthmus straight, narrow, 21.9 ± 3.2 (17-27) μm long. Hemizonid two body annuli long, located just anterior to secretory-excretory pore. Secretory-excretory pore slightly anterior to pharyngo-intestinal junction. Pharyngeal glands in tandem, elongate, overlapping intestine ventrally, 43 ± 6.1 (34-54) μm long; pharyngeal gland nuclei in tandem, posterior one (subventral gland) somewhat larger. Intestine lacking fasciculi. Reproductive system monodelphic, prodelphic, 170 ± 31.5 (125-216) μm long, ovary outstretched with single row of oocytes, vulva at more than 80% of total body length from anterior end, vulval lips slightly protruding, no lateral flaps, no epiptygma. Spermatheca rounded, 13-24 μm wide, thick walled, containing globular sperm 1.5-2 μm diam. Post-vulval uterine sac well developed, 20.0 ± 2.9 (15-26) μm long, 1.2 times anal body diam. Distance from vulva to anus = 69 ± 5.2 (63-80) μm. Tail short, cylindrical, with 22-25 annuli, slightly narrowing in posterior third. Tail tip obliquely truncate and irregularly annulated. Anus round to oval shaped in ventral view. Small, rounded, phasmid located between inner lateral field lines at mid-tail. Hyaline portion of tail terminus distinct.

Male

Common, almost as abundant as female. Morphology similar to that of female, including *en face* morphology, except for sexual dimorphism. Lip region characters as in female, but more truncate in outline. Lateral fields marked by four lines, inner and outer bands regularly areolated. Reproductive system characterised by single testis anteriorly outstretched. Spicules and gubernaculum ventrally curved. Tail pointed. Bursa 43.6 ± 4.6 (37-52) μm long, arising slightly anterior to head of retracted spicule and enveloping tail. Ventral surface of bursa coarsely crenate; phasmid on bursa located almost midway between cloacal opening and tail tip.

TYPE HOST AND LOCALITY

Pratylenchus hispaniensis n. sp. was found in a sandy soil and roots of gum cistus (*Cistus ladanifer* L.) from Andújar (Jaén), southern Spain.

TYPE MATERIAL

Holotype female, 16 female and 15 male paratypes deposited in the Nematode Collection of the Institute of Sustainable Agriculture, CSIC, Córdoba, Spain (collection numbers PR-419-13, PR-419-01 to PR-419-12, respectively). Eight female and six male paratypes deposited in the Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.), Sezione di Bari, Bari, Italy (collection numbers PR-419-22 to PR-419-24); USDA Nematode Collection, Beltsville, MD, USA (collection number PR-419-16), and Nematode collection of the Department of Nematology, Landbouwhogeschool, Wageningen, The Netherlands (collection number PR-419-15). Specific D2-D3, ITS, and 18S sequences are deposited in GenBank with accession numbers FJ717822, FJ717816 and FJ717825, respectively.

DIAGNOSIS AND RELATIONSHIPS

Pratylenchus hispaniensis n. sp. females are characterised by a combination of the following morphological features: lip region with three annuli; a divided face with rectangular subdorsal and subventral lips fused with the oral disc and visibly separated from lateral lips by two almost straight incisures forming an obtuse angle, stylet robust (14.5-17.0 μm), with rounded knobs (3.0-4.0) μm wide; lateral fields with four lines, regularly areolated; V = 80-84; spermatheca rounded; post-vulval uterine sac well developed, 1.2 anal body diam. long; tail cylindrical with 22-25 annuli; tail cylindrical with distinct annulations, tail tip obliquely truncate and irregularly annulated. Males are common and morphologically similar to females except for sexual dimorphism.

As with *P. hispaniensis* n. sp. the following species all have three lip annuli, a stylet 13-16 μm long, a rounded functional spermatheca and numerous males: *P. bhatti* Siddiqi, Dabur & Bajaj, 1991, *P. kralli* Ryss, 1982, *P. mediterraneus*, and *P. pseudofallax*. It is not known, however, whether *P. bhatti* and *P. kralli* share a divided face with *P. hispaniensis* n. sp., *P. mediterraneus* and *P. pseudofallax.*

Morphologically *P. hispaniensis* n. sp. can be distinguished from the most similar species by a number of particular characteristics resulting from its specific matrix code (A2, B2, C2, D2, E3, F2, G1, H2, I3, J1, K2 *sensu* Castillo & Vovlas, 2007). From *P. bhatti* it differs by vulva position (V = 80-84 *vs* 69-76), female tail shape (cylindrical, tail tip irregularly annulated *vs* subcylindrical to conoid, tail tip smooth), pharyngeal overlap (34-54 *vs* 30-35 μm) and lateral field structure at vulval region (completely areolated bands *vs* smooth bands). From *P. kralli* it differs by vulva position (V = 80-84 *vs* 74-80), longer post-vulval uterine sac (15-26 *vs* 14 μm); female tail shape (cylindrical, tail tip irregularly annulated *vs* conoid, tail tip smooth). From *P. mediterraneus* it differs by vulva position (V = 80-84 *vs* 77-80), female tail shape (cylindrical, tail tip irregularly annulated *vs* subcylindrical, broadly rounded to truncate with smooth terminus) and presence of areolations on the lateral field bands at vulval level *vs* smooth. From *P. pseudofallax* it differs by female tail shape (cylindrical with irregularly annulated terminus *vs* conoid-rounded or bluntly pointed with smooth or indented terminus).

PHYLOGENETIC POSITION OF *P. HISPANIENSIS* N. SP. WITHIN THE GENUS

The primer pairs D2A and D3B, G18SU and R18Tyl1, and TW81 and AB28 amplified a PCR product *ca* 850 bp, 900 bp and 1100 bp in length, respectively. Three sequences for each gene fragment: D2-D3 of 28S, 18S and ITS rRNA were obtained from three single individuals. There was no variation within the corresponding gene. *Pratylenchus hispaniensis* n. sp. differed in the D2-D3 sequences from the most closely related species, *P. brzeskii* Karssen, Wayenberge & Moens, 2000, by 65 nucleotides or 10.8%, from *P. neglectus* by 104 nucleotides or 17.1%, and from *P. thornei* by 118 nucleotides or 15.7%.

The D2-D3 automatic alignment consisted of 43 sequences and had 751 bp in length, whereas the D2-D3 culled alignment had 691 bp. The majority consensus phylogenetic tree generated from the D2-D3 automatic alignment by BI analysis under the GTR + I + G model is presented in Figure 5. In respect of the position of *P. hispaniensis* n. sp. and related species, the topologies obtained by automatic alignment and culled alignment by ML and BI analyses were congruent. *Pratylenchus hispaniensis* n. sp. was a sister taxon to *P. brzeskii* and, together with *P. neglectus* and *P. thornei*, formed a highly supported clade.

The 18S automatic alignment included 47 sequences and was 854 bp in length, whereas the 18S culled alignment was 803 bp. The majority consensus phylogenetic tree generated from the 18S automatic alignment by BI analysis under the GTR + I + G model is presented in Figure 6. The tree topologies obtained from ML and BI analyses were congruent. In the tree, *P. hispaniensis* n. sp. occupied a basal position within a highly supported clade together with *P. neglectus* and *P. thornei.*

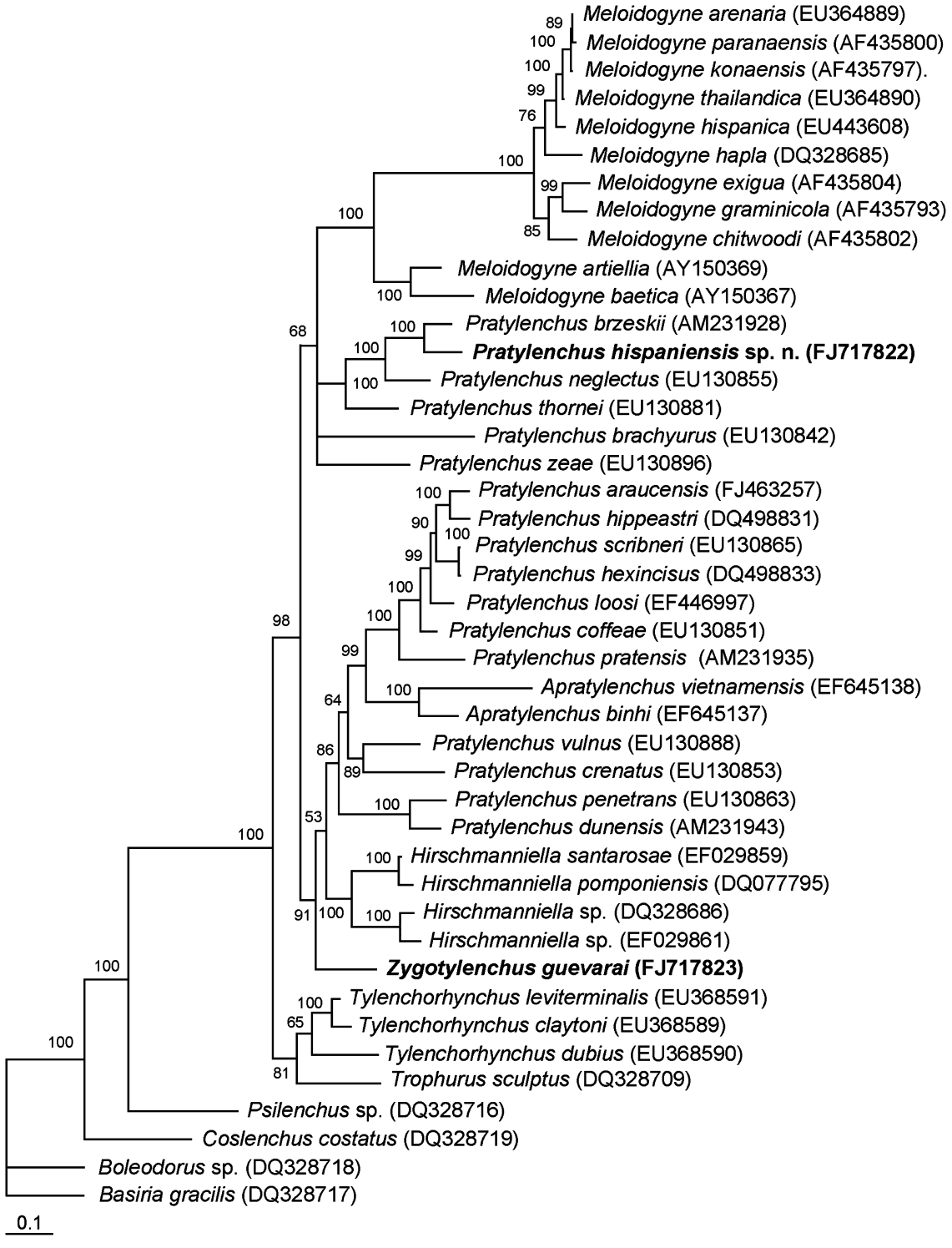

Fig. 5. *The 50% majority rule consensus trees from Bayesian analysis generated from the D2-D3 of 28S rRNA gene dataset with the c GTR + I + G model (Arithmetic mean: −11805.01; Harmonic mean: −11843.01). PP values are given in appropriate clades. Newly sequenced samples are indicated in boldface.*

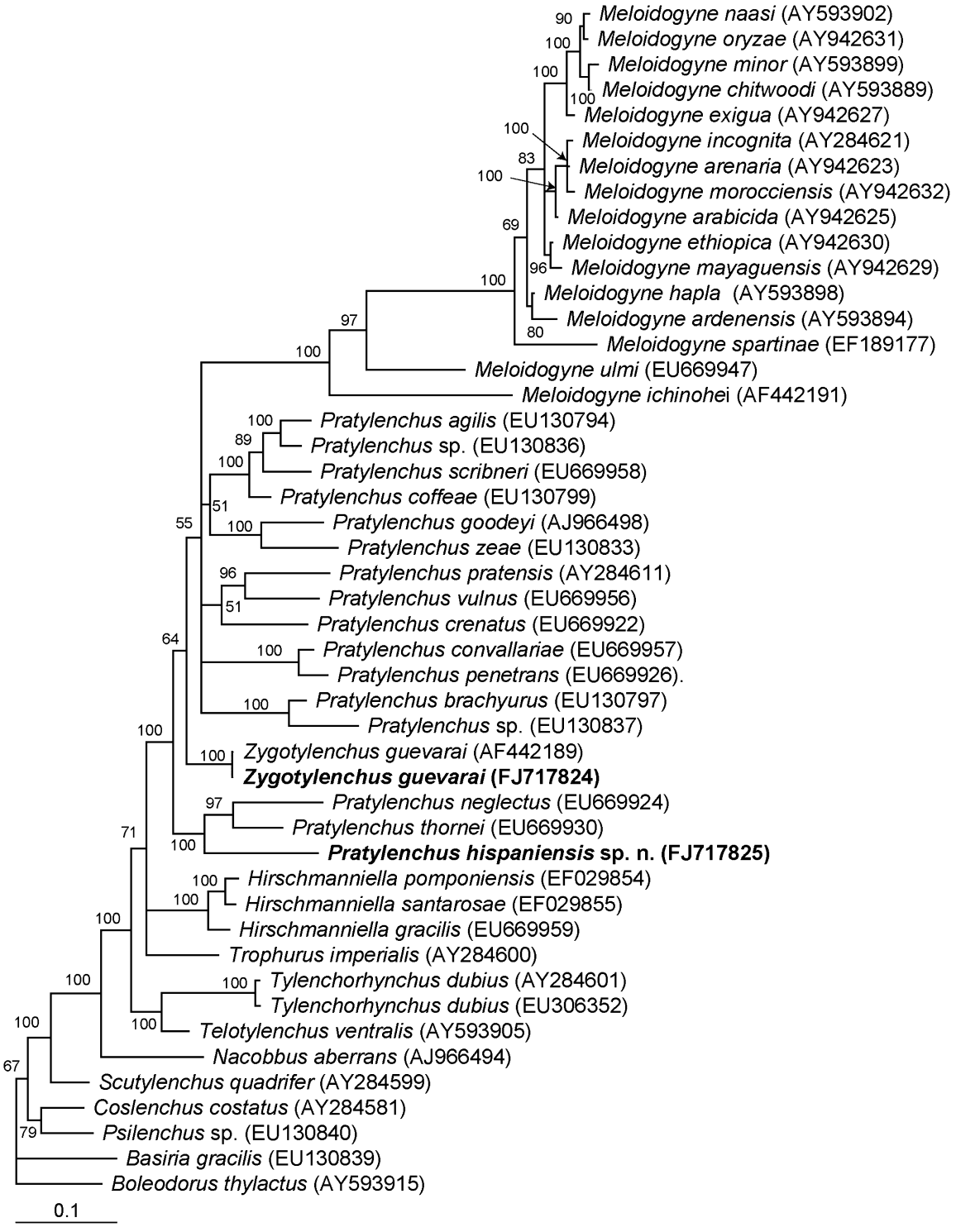

Fig. 6. *The 50% majority rule consensus trees from Bayesian analysis generated from the 18S rRNA gene dataset with the c GTR + I + G model (Arithmetic mean: −7881.23, Harmonic mean: −7929.99). PP values are given in appropriate clades. Newly sequenced samples are indicated in boldface.*

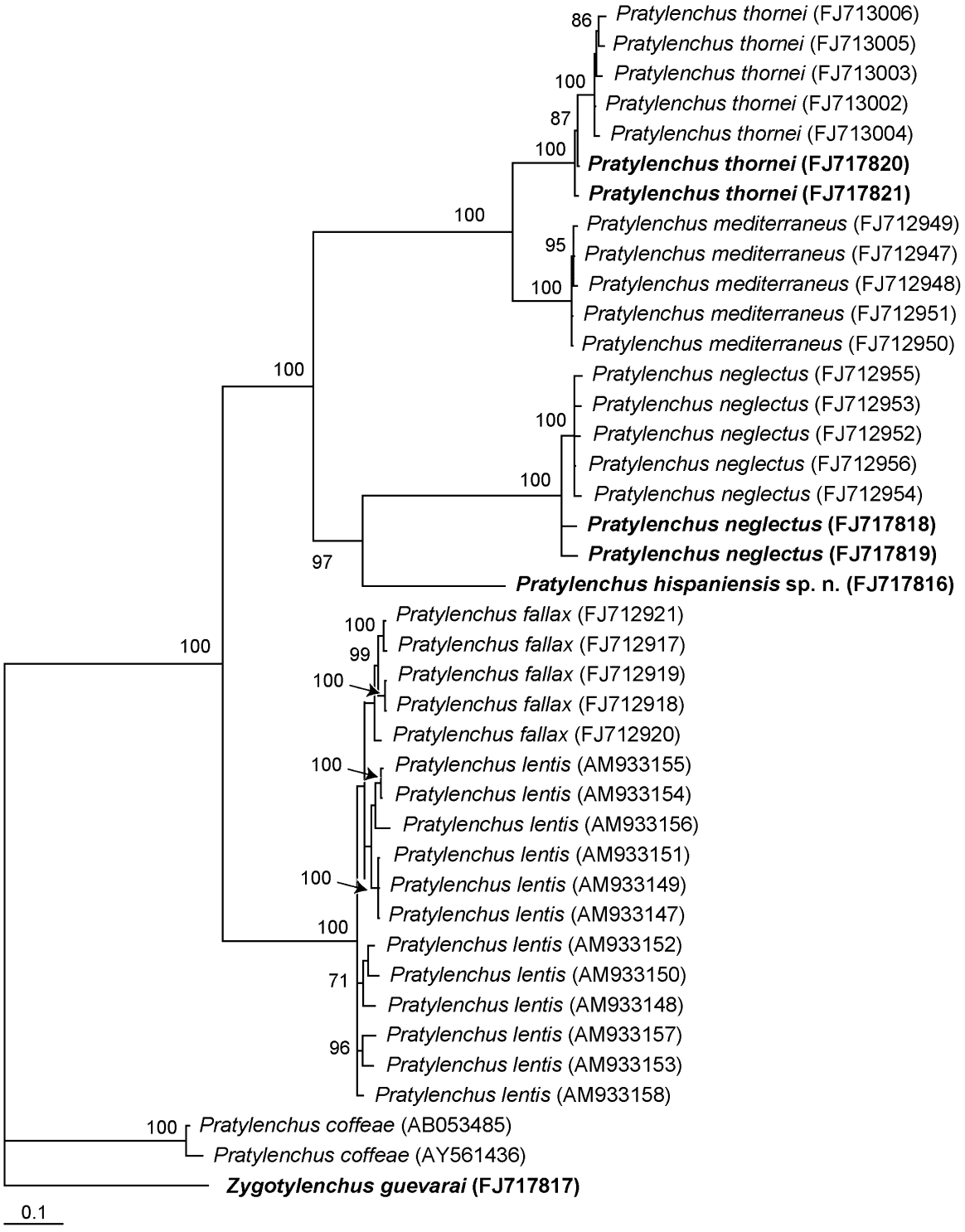

Fig. 7. *The 50% majority rule consensus trees from Bayesian analysis generated from the ITS-rRNA gene dataset with the c GTR + I + G model (Arithmetic mean: −4454.77, Harmonic mean: −4468.40). PP values (more than 70%) are given in appropriate clades. Newly sequenced samples are indicated in boldface.*

The ITS alignment contained 39 sequences of seven *Pratylenchus* species and was 836 bp in length. The majority consensus phylogenetic tree generated from this alignment by BI analysis under the GTR + I + G model is presented in Figure 7. In this tree, *P. hispaniensis* n. sp. formed a highly supported clade with *P. neglectus.*

Remarkably, *P. hispaniensis* n. sp., *P. brzeskii*, *P. mediterraneus*, *P. neglectus* and *P. thornei* share similar molecular traits and a similar, divided, face (the *en face* form of *P. brzeskii* is not known).

LM AND SEM STUDIES OF *ZYGOTYLENCHUS GUEVARAI*

Morphological studies based on LM and SEM observations (Figs 8, 9) of one Italian and four Spanish populations of *Z. guevarai* showed typical traits for this species as described in the literature (de Guiran, 1963; Siddiqi, 1963, 1975; Tobar Jiménez, 1963), except for minor differences in some measurements and ratios that conform with intraspecific variability. SEM observations of *Z. guevarai* showed that the lateral fields had four lines, the lateral bands being wider than the inner band, with some irregular areolations at the mid-body and tail regions (Fig. 8). The labial region was flattened anteriorly and had a moderately developed labial framework and four or five annuli. The *en face* view was characterised by a rounded oral disc fused with the partially incised submedian and subventral lip sectors (Fig. 8) and visibly separated from the lateral lips by two incisures forming an obtuse angle. Our results mostly agree with those reported by La Rosa (2008) for a Sicilian population of *Z. guevarai* and by Pourjam *et al*. (2000) for an Iranian population that showed the second lip annulus marked by longitudinal incisures unlike the configuration of this annulus in the European populations. The spermatheca of the Italian and Spanish populations had 12 cells, the two cells connecting to the uterus being clearly larger, this observation agreeing with data reported by Bert *et al*. (2003).

PHYLOGENETIC RELATIONSHIPS BETWEEN THE GENERA OF PRATYLENCHIDAE AND TESTING ALTERNATIVE TOPOLOGIES

In the D2-D3 and 18S trees, all *Pratylenchus* species were paraphyletic and distributed within seven clades (Figs 5, 6). Relationships within these clades were not well resolved. The SH test did not reject the monophyletic hypothesis of *Pratylenchus* when the culled D2-D3 and both 18S datasets were used (Table 3). The genus *Apratylenchus* nested within *Pratylenchus* and formed a highly supported clade (PP = 99) with the *pratensis-coffeae* group. The validity of this genus was rejected by the SH test in automatic D2-D3 alignment, but accepted in culled D2-D3 alignment. Species of *Hirschmanniella* and *Meloidogyne* were monophyletic and formed two highly supported (PP = 100) clades. The position of *Z. guevarai* within Pratylenchidae was not well resolved in all trees. Only in one of four datasets were the sister relationships between *Pratylenchus* and *Zygotylenchus* rejected by the SH test. In the 18S trees generated from automatic and culled alignments *N. aberrans* clustered outside other Pratylenchidae (Fig. 6), although the position of this species within Pratylenchidae was supported by the SH test with 18S culled alignment (Table 3).

Discussion

CONGRUENCE AND INCONGRUENCE BETWEEN MOLECULAR AND MORPHOLOGICAL GROUPING OF *PRATYLENCHUS* SPECIES

The results of our studies indicated that the molecular affinity among *P. hispaniensis* n. sp., *P. brzeskii*, *P. mediterraneus*, *P. neglectus* and *P. thornei* is partially reflected by the similar morphological and morphometrical features used in this study such as the number of lip annuli or *en face* view pattern when species are projected on the plane of factors (Fig. 1E-F). This finding is mostly in agreement with the conclusion from other studies indicating that the clades on molecular phylogenetic trees are generally congruent with those defined by characters derived from lip patterns of *Pratylenchus* (Duncan *et al*., 1999; Handoo *et al*., 2008; Subbotin *et al*., 2008). However, the number of lip annuli and reproductive behaviour were not reflected by the results of these phylogenetic analyses. In fact, *P. brzeskii* and *P. neglectus* have two, rather than three, lip annuli as in *P. hispaniensis* n. sp., *P. mediterraneus* and *P. thornei.* Furthermore *P. neglectus* and *P. thornei* are parthenogenetic species in contrast to *P. hispaniensis* n. sp., *P. mediterraneus* and *P. brzeskii* which are all amphimictic. In any case, the results from multivariate analysis separates this group of 38 *Pratylenchus* species according to the 25 characters analysed and the incongruence observed with molecular analyses based on rRNA genes may be due mostly to the lack of molecular information for many *Pratylenchus* species included in the study. Our results agree with Subbotin *et al*. (2008) who reported that morphological characters tradi-

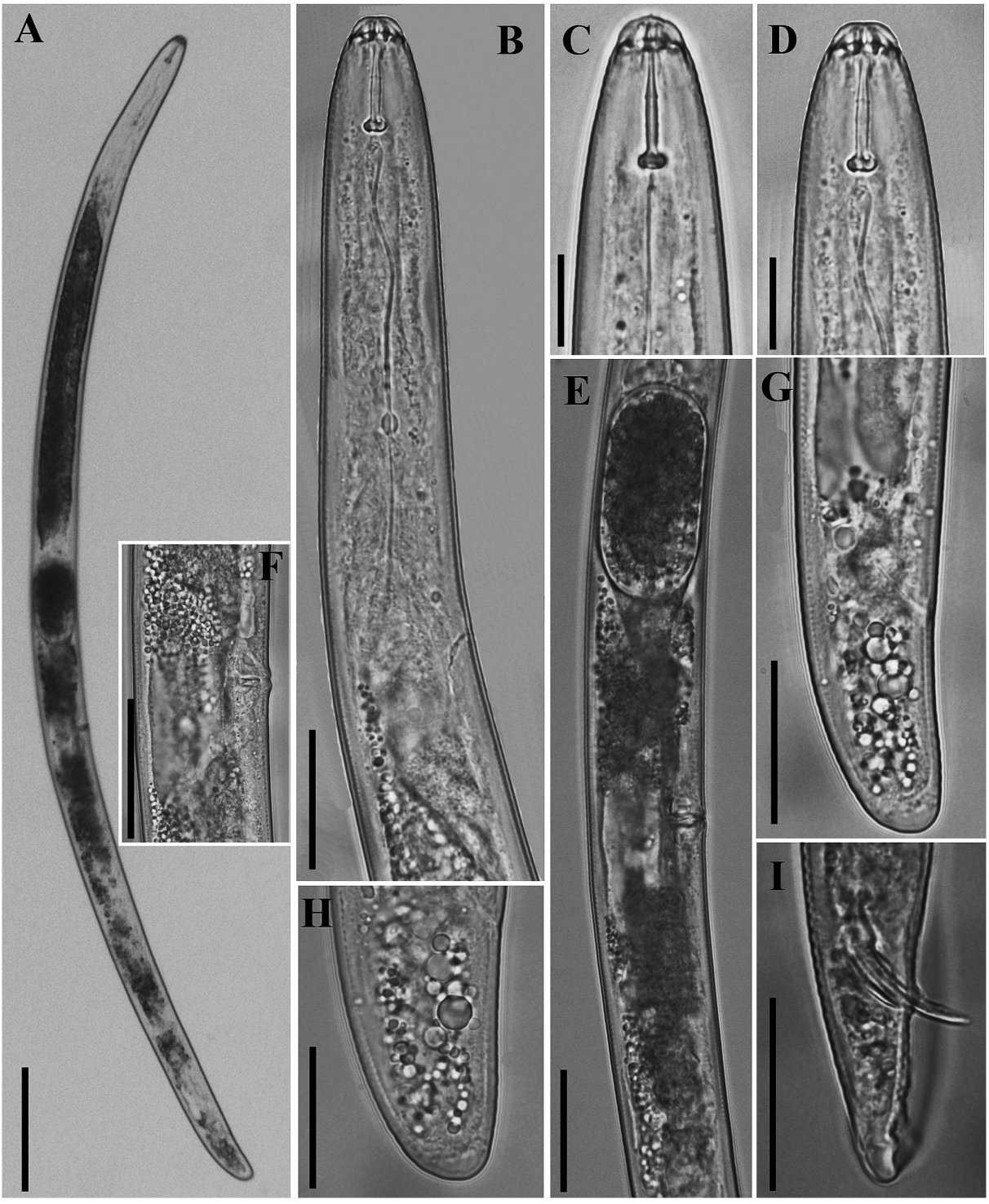

Fig. 8. *Light micrographs of* Zygotylenchus guevarai *(Tobar Jiménez, 1963) Braun & Loof, 1966. A: Entire female; B: Female neck region; C, D: Female anterior body region; E, F: Female vulval region; G, H: Female tails; I: Male tail with protruding copulatory apparatus. (Scale bars: A = 50 μm; B, E-I = 20 μm; C, D = 10 μm.)*

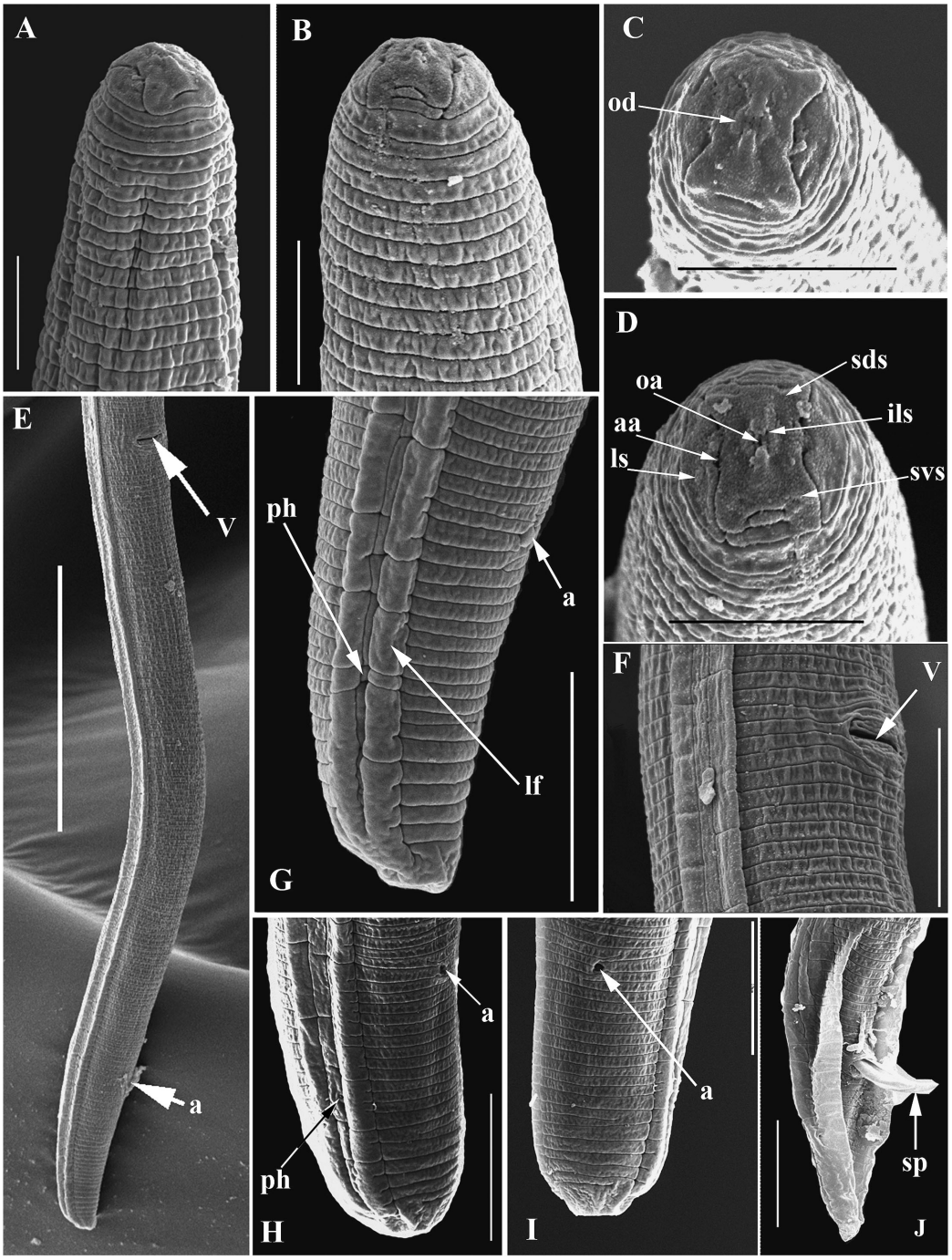

Fig. 9. *Scanning electron micrographs of* Zygotylenchus guevarai *(Tobar Jiménez, 1963) Braun & Loof, 1966. Female (A-F). A, B: Anterior body region; C, D: Lip region, face view; E: Posterior body portion showing vulva and anus position; F: Vulval region; G-I: Tail (I = ventral view); J: Male tail with protruding spicules. Abbreviations: a = anus; aa = amphidial aperture; ils = inner labial sensilla; lf = lateral field; ls = lateral lip sector; oa = oral aperture; od = oral disc; ph = phasmid; sds = subdorsal lip sectors; sp = spicule; svs = subventral lip sectors; V = vulva. (Scale bars: A-D = 5 μm; E = 50 μm; F-J = 10 μm.)*

Table 3. *Evaluation of tree topology and alternative hypotheses with the Shimodaira-Hasegawa test.*

Gene:	D2-D3 of 28S rRNA						18S rRNA					
Alignment:	Automatic			Culled			Automatic			Culled		
Hypothesis:	ln L	Δ ln L	*P*	ln L	Δ ln L	*P*	ln L	Δ ln L	*P*	ln L	Δ ln L	*P*
ML tree	11768.356	Best	–	9805.553	Best		7838.702	Best		6569.168	Best	
MP tree	11783.716	15.360	0.385	9826.487	20.933	0.455	7840.505	1.803	0.940	6575.380	6.211	0.757
Monophyly of *Pratylenchus* without nested *Apratylenchus*, or validity of *Apratylenchus*: (*Pratylenchus*), (*Apratylenchus*)	11805.266	36.909	0.020*	9837.680	32.126	0.161	–	–	–	–	–	–
Monophyly of *Pratylenchus* with nested *Apratylenchus*: (*Pratylenchus* + *Apratylenchus*)	11788.277	19.921	0.242	9829.760	24.207	0.353	–	–	–	–	–	–
Monophyly of *Pratylenchus* (*Pratylenchus*)	–	–	–	–	–	–	7841.189	2.487	0.907	6580.718	11.549	0.594
Validity of Pratylenchinae: (*Pratylenchus* + *Zygotylenchus*)	11805.266	36.909	0.020*	9837.680	32.126	0.161	7844.559	5.856	0.749	6582.274	13.105	0.532
Validity of Pratylenchinae: (*Pratylenchus*, with nested *Apratylenchus* + *Zygotylenchus*)	11785.383	17.027	0.324	9886.333	80.780	0.000*	–	–	–			
Validity of Pratylenchidae: (*Pratylenchus* with nested *Apratylenchus* + *Zygotylenchus* + *Hirschmanniella*)	11785.962	17.605	0.308	9828.043	22.489	0.406	–	–	–			
Validity of Pratylenchidae: (*Pratylenchus* + *Nacobbus* + *Zygotylenchus* + *Hirschmanniella*)	–	–	–	–	–	–	7867.208	28.506	0.030*	6599.675	30.507	0.086

* Trees significantly worse that the best tree at $P < 0.05$.

tionally used in species diagnosis are, in many cases, not likely to be informative for inferring *Pratylenchus* phylogeny. Because of phenotypic plasticity, potential flaws in applying particular morphological characters for phylogenetic inference may include character linkage, intraspecific variability exceeding interspecific variability, non-homology or be an artefact from interpreting characters.

Origin and relationships between genera of *Pratylenchidae*

Ryss (1988) pointed out that the question concerning the origin of the Pratylenchidae is complicated because of the lack of a more primitive group closely related to pratylenchids within the Hoplolaimoidea Filipjev, 1934. In other words, this question is connected with the problem of the origin of the Hoplolaimoidea itself. He considered that representatives of Dolichodoridae, especially Telotylenchinae, are most closely related to Pratylenchidae as they share general features of the lip region structure. Indeed, molecular phylogenetic analysis (Subbotin *et al*., 2006; Bert *et al.*, 2008) revealed close relationships of the subfamilies Telotylenchinae (*Tylenchorhynchus*, *Trophurus*) and Macrotrophurinae (*Macrotrophurus*) with the Pratylenchidae. The results of our present analyses, based on wider nematode taxon sampling, support this conclusion.

The Pratylenchidae is recognised as a eurymorphic family because its genera differ from each other by a rather large number of characters (Luc, 1987). Ryss (1988) noticed that the subfamily Nacobbinae stands apart from other Pratylenchidae by its morphological and ecological peculiarities. Nacobbinae differs from other Pratylenchidae by the presence of a saccate female body shape, dorsally overlapping pharyngeal glands and the habit of sedentary parasitism on plant roots. Ryss (1988) concluded that the structure of the lip region in *Nacobbus* was more similar to that of Telotylenchinae than any other Pratylenchidae. This important morphological difference

may infer that *Nacobbus* represents the earliest divergent lineage within Pratylenchidae. The molecular phylogeny reconstructed by Bert *et al.* (2008), and the present study using 18S rRNA sequence data, clearly support this hypothesis.

Molecular phylogeny revealed close relationships between the genera *Pratylenchus*, *Zygotylenchus* and newly erected genus *Apratylenchus.* However, the relationships between these genera were not well resolved from the rRNA datasets. Moreover, unequal rates of rRNA evolution in *Pratylenchus* lineages may have created the effect of long branch attraction, artificially resulting in the paraphyly of the genus with nested position of some other genera (*Apratylenchus* or *Zygotylenchus*). We believe that wider taxon sampling and partition analysis could resolve this issue in future studies. However, the positions of the genera within Pratylenchidae evaluated with maximum likelihood tests showed that they do not conflict with the relationships resulted from the present morphological classifications.

In all analyses, the genus *Meloidogyne* showed close relationships with Pratylenchidae, especially with *Pratylenchus.* This result is congruent with a hypothesis suggested by Ryss (1988) that, among Hoplolaimoidea, Pratylenchinae is most closely related to *Meloidogyne* in details of the lip region and pharyngeal structure. He believed these morphological similarities to be indicative of common ancestry between Meloidogynidae and representatives of Pratylenchinae.

Monophyly of the genus *Pratylenchus*

Pratylenchus is certainly the best known among the genera of the Pratylenchidae as it contains several economically important species with a worldwide distribution and which have been intensively studied by many research teams. Although *Pratylenchus* has been periodically reviewed (Ryss, 1988, 2000a; Castillo & Vovlas, 2007), morphological analysis has never raised doubts about its monophyly. Al-Banna *et al.* (1997) was the first to conclude, based on parsimony analysis of D3 expansion region of 28S rRNA gene sequences for ten *Pratylenchus* species together with *Nacobbus*, *Radopholus* and *Hirschmanniella*, that *Pratylenchus* was a paraphyletic assemblage. The paraphyly of *Pratylenchus* can also be observed on some other phylogenetic trees derived from more comprehensive analysis (see Carta *et al*., 2001; De Luca *et al*., 2004) using same gene fragment. However, the question concerning *Pratylenchus* paraphyly was not discussed by these authors, perhaps because of a lack of statistical support for such a conclusion. After making a phylogenetic analysis of the 18S gene sequences for Pratylenchidae, Holterman *et al.* (2009) concluded that *Pratylenchus* was not monophyletic and, as such, the nomenclature of this important group of plant parasites required reconsideration. Indeed, the paraphyletic patterns for *Pratylenchus* are also inferred from our present analysis of D2-D3 of 28S and 18S rRNA datasets. However, the SH test does not reject the traditionally recognised monophyly of this genus from any culled alignments from which ambiguously aligned regions were removed. We believe that the paraphylic patterns may be artificially derived from the rRNA genes datasets, which included highly divergent sequences undergoing rapid and unequal evolution within this taxon. Thus, at present we do not see an immediate reason to revise the genus *Pratylenchus*, robust from rRNA or other datasets being a necessary prerequisite for such an action.

In conclusion, since the number of sequences and taxa used for testing hypotheses of phylogenetic relationships within the genus *Pratylenchus* have steadily grown over the last decade, polyphasic taxonomy, which assembles and assimilates all available data information, may be a better strategy for species delimitation and for clarifying phylogenetic relationships in this complex group of genera.

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