



A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): Evidence from 18S and D2–D3 expansion segments of 28S ribosomal RNA genes and morphological characters

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ABSTRACT

The root lesion nematodes of the genus *Pratylenchus* Filipjev, 1936 are migratory endoparasites of plant roots, considered among the most widespread and important nematode parasites in a variety of crops. We obtained gene sequences from the D2 and D3 expansion segments of 28S rRNA partial and 18S rRNA from 31 populations belonging to 11 valid and two unidentified species of root lesion nematodes and five outgroup taxa. These datasets were analyzed using maximum parsimony and Bayesian inference. The alignments were generated using the secondary structure models for these molecules and analyzed with Bayesian inference under the standard models and the complex model, considering helices under the doublet model and loops and bulges under the general time reversible model. The phylogenetic informativeness of morphological characters is tested by reconstruction of their histories on rRNA based trees using parallel parsimony and Bayesian approaches. Phylogenetic and sequence analyses of the 28S D2–D3 dataset with 145 accessions for 28 species and 18S dataset with 68 accessions for 15 species confirmed among large numbers of geographical diverse isolates that most classical morphospecies are monophyletic. Phylogenetic analyses revealed at least six distinct major clades of examined *Pratylenchus* species and these clades are generally congruent with those defined by characters derived from lip patterns, numbers of lip annules, and spermatheca shape. Morphological results suggest the need for sophisticated character discovery and analysis for morphology based phylogenetics in nematodes.

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1. Introduction

Pratylenchus Filipjev, 1936 (root lesion nematodes) are migratory root endoparasites that are among the most widespread and destructive phytopathogenic nematodes of agricultural crops (Sasser and Freckman, 1987). They invade and migrate through root cortical parenchyma producing necrotic lesions by direct feeding while providing avenues for secondary colonization by pathogenic microorganisms. With *Pratylenchus* steadily growing to include from 45 to 89 putative morphospecies according to different authors (Siddiqi, 2000; Ryss, 2002a; Castillo and Vovlas, 2007), morphological distinctions among *Pratylenchus* species have been periodically reviewed (Sher and Allen, 1953; Loof, 1960, 1978, 1991; Corbett, 1969; Ryss, 1988, 2002a,b; Handoo and Golden,

1989; Café Filho and Huang, 1989; Frederick and Tarjan, 1989; Hernández et al., 2000). Whereas morphology including morphometrics has been the basis for diagnosis of the large number of *Pratylenchus* species, the fallibility of many individual characters has been recognized (Roman and Hirschmann, 1969; Tarte and Mai, 1976; Ryss, 1988, 2002a; Loof, 1991). Only more recently has there been interest in applying morphology for phylogenetic analysis of the genus *Pratylenchus*. Ryss (2002a,b) provided a comprehensive morphologically based cladistic 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. Morphological characters traditionally used in species diagnosis in many cases are not likely to be informative to infer *Pratylenchus* phylogeny; in addition to issues of phenotypic plasticity (e.g. heterotachy and convergence), potential flaws of particular morphological characters in their application to phylogenetic inference may include character linkage, intraspecific exceeding interspecific variability, nonhomology, and artifact in interpreting characters. Beyond traditional characters, and also

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with some application to recognizing intrageneric relationships, scanning electron microscopy (SEM) of lip patterns has demonstrated a high degree of diversity among *Pratylenchus* species (Corbett and Clark, 1983; Duncan et al., 1999; Hernández et al., 2000; Inserra et al., 2007). Details of lip patterns have been incorporated in many species descriptions and redescrptions (Luc, 1987; Zeidan and Geraert, 1991; Mizukubo, 1992a; Mizukubo et al., 1997; Duncan et al., 1999; Handoo et al., 2001; Inserra et al., 2001, 2007; Carta et al., 2002; de la Peña et al., 2006), but they have not been broadly applied to phylogenetic questions.

Al-Banna et al. (1997), who employed the D3 expansion segment of the 28S rRNA gene of *Pratylenchus*, was the first to propose a molecular phylogenetic analysis of the genus based on 10 species. Later Carta et al. (2001), De Luca et al. (2004) re-analyzed these data with additional species using the same gene fragment. However, as revealed by the analyses for other nematodes (Subbotin et al., 2005), the D3 expansion fragment does not contain sufficient phylogenetic signal to resolve relationships for nematodes at the species level. Addition of the more variable D2 expansion segment significantly improves resolution, as demonstrated by limited application for diverse *P. coffeae* isolates together with closely related species (Duncan et al., 1999; Inserra et al., 2007). Thus, the general pattern of molecular evolution of *Pratylenchus* as well as broad evaluation of molecular phylogenetic congruence with morphological characters remains unstudied.

A general goal of the present work is to develop a phylogenetic framework for *Pratylenchus*, and more specific objectives are to: (i) study relationships between species based on the partial 18S rRNA gene and the D2 and D3 expansion segments of the 28S rRNA gene using maximum likelihood and Bayesian inference under different evolution models; (ii) test monophyly of classical morphospecies and estimate species boundaries using RNA gene sequences from large numbers of geographically diverse isolates; (iii) estimate congruence of molecular and morphological evolution by mapping characters onto molecular trees using both parsimony and Bayesian approaches.

In this study we analyze several rRNA gene datasets for *Pratylenchus*, including the full dataset containing all available sequences for each gene and a reduced dataset containing only a single sequence for each species. Separate and combined analyses of the genes were performed for the reduced dataset. Since the rRNA molecule forms secondary and tertiary structures mediated by base pairings, and since ignoring these phenomena can discard crucial phylogenetic information, we reconstructed the secondary structure for the first domain of 18S and of the D2 and D3 expansion fragments of 28S for each species. The secondary structural information was applied in the alignment procedure and phylogenetic analysis by Bayesian inference under the complex model of RNA evolution, considering helices under the doublet model and loops and bulges under the general time reversible model (GTR). Knowledge of secondary structure thus allows applying a more sophisticated model, consequently generating a picture of relationships that is arguably more realistic.

Based on our estimates of root lesion nematode phylogeny we evaluate the current taxonomy of the genus and discuss the relevance of our data to previous phylogenetic and taxonomic hypotheses. The phylogenetic framework will provide a beginning point for recognizing major clades and the implications of these clades for particular morphological evolution. The value of these characters for resolution of *Pratylenchus* will be tested by reconstructing their histories using both parsimony and Bayesian approaches. Bayesian methods have offered a powerful tool for analyzing morphology, including in combination with molecular data (Glennier et al., 2004; Nylander et al., 2004), although given the currently limited scope of characters suitable for phylogenetic analysis in the genus, we test characters herein by estimating their ancestral

states on independently derived trees (Ronquist, 2004). Notwithstanding the potential for parsimony approaches to reveal uncertainty in mapping morphology (Ronquist and Liljeblad, 2001), herein we employ Bayesian ancestral state reconstruction, which has also been shown to provide a more reliable estimate of the strengths of competing hypotheses and account for ambiguity in tree structure (Pfenninger et al., 2005; Prud'homme et al., 2006; Vanderpoorten and Goffinet, 2006).

2. Materials and methods

2.1. Taxon sampling and outgroup selection

All sequences available from GenBank (as of June 10, 2007) for 18S rRNA (Holterman et al., 2006; Meldal et al., 2007; Lee et al., unpublished) and 28S rRNA (Al-Banna et al., 1997; Duncan et al., 1999; Handoo et al., 2001; Carta et al., 2001; De Luca et al., 2004; de la Peña et al., 2006; Inserra et al., 2007; de la Peña and Moens, unpublished) were included in the analysis. Based on a comprehensive molecular phylogenetic analysis of 82 species of tylenchid and aphelenchid nematodes including three *Pratylenchus* species (Subbotin et al., 2006), five representatives of Tylenchida were selected as the most appropriate outgroup taxa for rooting molecular based trees. Voucher specimens are stored at the University of California Riverside Nematode Collection (UCRNC).

2.2. DNA isolation, amplification, cloning, and sequencing

DNA was extracted from several specimens from each sample using the proteinase K protocol. Nematode specimens were each transferred to an Eppendorf tube containing 16 μ l double distilled water, 2 μ l 10 \times PCR buffer and 2 μ l proteinase K (600 μ g/ml) (Promega); specimens were crushed during 3 min with an ultrasonic homogenizer. The tubes were incubated at 65 $^{\circ}$ C (1 h) and then at 95 $^{\circ}$ C (15 min). Detailed protocols for PCR, cloning, and sequencing were as described by Tanha Maafi et al. (2003). The forward D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and reverse D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (Subbotin et al., 2006) and the forward G18SU (5'-GCTTGCTCAAAGATTAAGCC-3') and reverse R18Ty11 (5'-GGTCCAAGAATTTCACTCTC-3') primers (Chizhov et al., 2006) were used for amplification and sequencing of the D2–D3 expansion segments of the 28S rRNA and partial 18S rRNA genes, respectively. Forty-five new partial 18S rRNA and 59 D2–D3 expansion segments of 28S rRNA sequences for *Pratylenchus* and three new partial 18S rRNA gene sequences of outgroup taxa are reported here; these all have been deposited in the GenBank under the accession numbers given in Table 1. Accession numbers of previously published sequences are given in Figs. 2 and 3.

2.3. Sequence alignment and phylogenetic analysis of full datasets for 28S and partial 18S sequences

The 140 and 63 sequences of 28S D2–D3 and partial 18S rRNA, respectively, for *Pratylenchus* were aligned with corresponding genes of five sequences for outgroup taxa using ClustalX 1.64 (Chenna et al., 2003) with default parameters for gap opening and gap extension penalties. The models for nucleotide substitutions were selected for each ribosomal gene individually using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion (AIC) in conjunction with PAUP* 4b4a (Swofford, 2003). Bayesian inference analyses (BI) of each dataset were conducted separately using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). All Bayesian analyses were initiated with random starting trees and were run with four chains of 3.0×10^6 generations for the 28S dataset and 6.0×10^6 for the 18S dataset.

Table 1
Species and populations of *Pratylenchus* and outgroup taxa sequenced in the present study

Identification based on morphology and rRNA sequences	Host plant	Locality	Collection codes for DNA and nematode cultures in the UCR	GenBank accession number for D2–D3 of 28S rRNA	GenBank accession number for 18S rRNA	Source of materials
<i>Pratylenchus agilis</i>	Corn (<i>Zea mays</i> L.)	USA, Florida, Vero Beach	CA77; DD	EU130841	EU130793, EU130794	Dr. J. Pinochet, Spain
<i>Pratylenchus brachyurus</i>	Cherry (<i>Cerasus</i> sp.)	Japan, Okinawa	CA83; MM	EU130842	EU130795	Dr. T. Mizukubo, Japan
<i>Pratylenchus brachyurus</i>	Cotton (<i>Gossypium</i> sp.)	USA, California	CA106; A	–	EU130796, EU130797	Dr. P.A. Roberts, USA
<i>Pratylenchus coffeae</i>	Sweet potato (<i>Ipomoea batatas</i> L.)	Japan, Kumamoto	CA97, CA88; P	EU130847–EU130849	EU130798, EU130799	Dr. T. Mizukubo, Japan
<i>Pratylenchus coffeae</i>	Taro (<i>Colocasia esculenta</i> (L.) Schott)	Japan, Kagoshima	CA80, CA96; O	EU130843–EU130846	–	Dr. T. Mizukubo, Japan
<i>Pratylenchus coffeae</i> ^a	Tea (<i>Camellia sinensis</i> (L.) Kuntze)	Japan, Shizuoko	CA101, LL	EU130851	–	Dr. T. Mizukubo, Japan
<i>Pratylenchus crenatus</i>	Barley (<i>Hordeum vulgare</i> L.)	UK	CA81; G	EU130852, EU130852	EU130800	Rothamsted Research, UK
<i>Pratylenchus neglectus</i>	Barley (<i>Hordeum vulgare</i> L.)	USA, California, Davis	CA94; B	EU130854, EU130855	EU130801, EU130802	Dr. P. Roberts, USA
<i>Pratylenchus penetrans</i>	Carrot (<i>Daucus carota</i> L.)	Japan, Chiba	CA64; R	EU130856, EU130857	–	Dr. T. Mizukubo, Japan
<i>Pratylenchus penetrans</i>	Apple (<i>Malus domestica</i> Borkh)	France, Bretanya	CA82; CC	EU130858, EU130859	EU130803, EU130804	Dr. J. Pinochet, Spain
<i>Pratylenchus penetrans</i>	Cabbage (<i>Brassica oleracea</i> L.)	Japan, Aichi	CA85; Q	EU130860, EU130861	EU130805, EU130806	Dr. T. Mizukubo, Japan
<i>Pratylenchus penetrans</i>	Cowpea (<i>Vigna unguiculata</i> L. Walp)	USA, California, Stanislaus County	CA91	EU130862, EU130863	EU130807, EU130808	Dr P.A. Roberts, USA
<i>Pratylenchus pinguicaudatus</i>	Wheat (<i>Triticum</i> sp.)	UK	CA73; L	–	EU130809, EU130810	Rothamsted Research, UK
<i>Pratylenchus scribneri</i>	Sudan grass (<i>Sorghum vulgare</i> var <i>sudanense</i> Hitch)	USA, California, Imperial Valley	CA62; T	EU130864	–	Dr. P. Roberts, USA
<i>Pratylenchus scribneri</i>	Corn (<i>Zea mays</i> L.)	USA, Florida, Vero Beach	CA75; EE	EU130865	EU130811, EU130812	Dr. J. Pinochet, Spain
<i>Pratylenchus thornei</i>	Fescue (<i>Festuca</i> sp.)	USA, California, Stanislaus	CA93; D	EU130879, EU130880	EU130825, EU130826	Dr. P.A. Roberts, USA
<i>Pratylenchus thornei</i>	Garbanzo (<i>Cicer arietinum</i> L.)	Spain, Canete	CA79; FF	EU130873, EU130874	EU130819	Dr. J. Pinochet, Spain
<i>Pratylenchus thornei</i>	Wheat (<i>Triticum</i> sp.)	UK	CA86; M	EU130875, EU130876	EU130820, EU130821, EU130822	Rothamsted Research, UK
<i>Pratylenchus thornei</i>	Unknown	Moldova	CA74	EU130881	EU130817, EU130818	Dr. L. Poiras, Moldova
<i>Pratylenchus thornei</i>	Wheat (<i>Triticum</i> sp.)	Australia, South Australia	CA76; X	EU130869–EU130872	EU130813, EU130814	Dr. J. Curran, Australia
<i>Pratylenchus thornei</i> ^b	Apple (<i>Malus domestica</i> Borkh)	Australia, Queensland	CA71; W	EU130866–EU130868	EU130815, EU130816	Dr. J. Curran, Australia
<i>Pratylenchus thornei</i>	Wheat (<i>Triticum</i> sp.)	Australia, Queensland	CA87; U	EU130877, EU130878	EU130823, EU130824	Dr. J. Curran, Australia
<i>Pratylenchus vulnus</i>	Black walnut (<i>Juglans nigra</i> L.)	USA, California, Kearney Exp. Station	CA92; E	EU130887, EU130888	EU130830, EU130831	Dr. P. Roberts, USA
<i>Pratylenchus vulnus</i>	Strawberry (<i>Fragaria</i> sp.)	Japan, Saga	CA78; S	EU130883, EU130884	EU130828, EU130829	Dr. T. Mizukubo, Japan
<i>Pratylenchus vulnus</i>	Rose (<i>Rosa</i> sp.)	Spain, Barcelona	CA69; AA	EU130882	EU130827	Dr. J. Pinochet, Spain
<i>Pratylenchus vulnus</i>	Boysenberry (Hybrid <i>Rubus</i>)	USA, California, Vista	CA90, C	EU130885, EU130886	–	Dr. P. Roberts, USA
<i>Pratylenchus zaeae</i>	Unknown	South Africa	CA70; N	EU130893–EU130896	EU130833, EU130834	Dr. S. Loots, South Africa
<i>Pratylenchus zaeae</i>	Unknown	Japan, Okinawa	CA68; NN	EU130889–EU130892	EU130832	Dr. T. Mizukubo, Japan
<i>Pratylenchus</i> sp. 1 ^c	Coffee (<i>Coffea</i> sp.)	Guatemala	CA61; F	EU130897	–	Rothamsted Research, UK
<i>Pratylenchus</i> sp. 1 ^c	Coffee (<i>Coffea</i> sp.)	Guatemala	CA72; GG	EU130898, EU130899	EU130835, EU130836	Dr. J. Pinochet, Spain
<i>Pratylenchus</i> sp. 2 ^d	Unknown	USA, California	CA111	–	EU130837	Dr. P.A. Roberts, USA
<i>Belonolaimus longicaudatus</i>	Unknown	USA, California	CA31	–	EU130838	Dr. O. Becker, USA
<i>Basiria gracilis</i>	Unknown	USA, California	CA1	–	EU130839	Dr. J.G. Baldwin, USA
<i>Psilenchus</i> sp.	Unknown	USA, California	CA12	–	EU130840	Dr. J.G. Baldwin, USA

^a Tentatively morphologically identified as *P. loosi*.

^b Tentatively morphologically identified as *P. jordanensis*.

^c Tentatively morphologically identified as *P. coffeae*.

^d Tentatively morphologically identified as *P. brachyurus*.

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 stabilized after approximately 10^3 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. Clades with PP equal to or more than 95% are considered highly supported.

2.4. Secondary structure prediction for rRNA

Secondary structures for D2 and D3 of 28S rRNA were predicted using the energy minimization approach for each sequence and then optimized using covariation analysis as described by Subbotin et al. (2007). Mfold software program Version 3 (<http://www.bioinfo.rpi.edu/~zukerm/>) (Zuker, 1989) was used to predict fragments of molecules. Secondary structures for 18S rRNA were predicted by homology modeling using structures of this molecule for *Caenorhabditis elegans* (Ellis et al., 1986) and other organisms from the European ribosomal RNA database (<http://www.psb.ugent.be/rRNA>; Van de Peer et al., 1997; Wuyts et al., 2002). Mfold was used to predict variable regions of the 18S rRNA. Structures were visualized using RnaViz (De Rijk et al., 2003) and PseudoViewer 3 (Han et al., 2002) and drawn using Adobe Illustrator v10. Labeling of helices of 18S and 28S rRNA was in accordance with Wuyts et al. (2001). Structures of rRNA in dot-bracket format for 28S for 23 species and 18S for 15 species studied here were deposited in the database NEMrRNA (<http://www.nemamex.ucr.edu/rna>) (Subbotin et al., 2007).

2.5. Secondary structure alignment of reduced sequence datasets, model selection, and phylogenetic analyses of reduced datasets

One sequence from each valid species has been used to build the reduced dataset for each gene. Sequences in secondary structure format were aligned using the MARNA web server (Siebert and Backofen, 2005, <http://biwww2.informatik.uni-freiburg.de/Software/MARNA/index.html>) based on both primary and secondary structures. As the default setting, the base deletion was scored 2.0, base mismatch 1.0, arc removing 2.0, arc breaking 1.5, and base mismatch 1.8. Correctness of alignment and insertions of indels was verified by visually checking each secondary structure model with PseudoViewer as described by Subbotin et al. (2007). The following secondary structure alignments were generated: (i) D2–D3 of 28S rRNA with 23 *Pratylenchus* species (without *P. pratensis* and *P. pinguicaudatus*) and five outgroup taxa; (ii) partial 18S rRNA with 13 *Pratylenchus* species and five outgroups; (iii) combined D2–D3 of 28S + partial 18S rRNA for 25 *Pratylenchus* species and five outgroups. In the combined dataset for 15 species only one of these gene fragments was included. Alignments are available from the senior author by request. Alignments were analyzed by maximum parsimony (MP) using

PAUP and BI using MrBayes separately and in combination under the standard and the complex model of RNA evolution. MP robustness of the clades was assessed by bootstrap analysis yielding bootstrap percentage (BS) for each node estimated from 1000 replicates.

The complex model included the doublet model with 16 states of nucleotide doublets for the stem region and the standard model of DNA substitution with four nucleotide states for loops and bulges and a gamma distribution (G) of among-site rate heterogeneity with six rate categories, allowing a proportion of site to be invariant (Ronquist and Huelsenbeck, 2005). The models were compared by use of the Bayes factor (Table 2) (Kass and Raftery, 1995; Dohrmann et al., 2006). All Bayesian analyses were initiated with random starting trees and were run with four chains for 1.0×10^6 generations. In each run, trees were sampled every 100 generations. Two runs were performed for each analysis. Burn-in was 1000 trees, as determined by examination of the log-likelihoods of sampled trees. The topologies were used to generate a 50% majority rule consensus tree.

2.6. Morphological matrix and mapping of morphological characters

Molecular phylogenies were evaluated for implications on evolution of five morphological characters. Three discrete characters from lip patterns were considered following the approach of Baldwin and Schouest (1990) and Baldwin (1992) in defining different states along a hypothetical fusion process going from a basic tylenchid pattern of a labial disc surrounded by six lips, as well as the shape and configuration of these boundaries (Fig. 1A). Other characters included from classical literature are the number of lip annules and some aspects of offset spermathecae (whether the lumen is slit-like or round/oval distended), the latter being evaluated as independent of linkage to the presence of males (character states 2 and 3, respectively, in Ryss, 2002b). Although a large suite of other traditional diagnostic characters were carefully evaluated (Ragsdale, unpublished), these proved to be phylogenetically uninformative, often because variation was shown to be influenced by epigenetics, and intraspecific variation exceeded putative limits between species.

The total number and taxon sampling of assembled characters are presently inadequate for a phylogenetic analysis of a purely morphological matrix in determining resolved clade membership for most taxa; this was affirmed by preliminary parsimony analyses of a matrix with 19 morphological characters (Ragsdale, unpublished). A more appropriate test of the phylogenetic value of morphological characters was determined to be tracking morphological character state changes on a tree derived independently and from more complete data, namely the phylogeny based on the combined 28S D2–D3 and partial 18S sequences.

Two approaches were used to map morphological characters. In the first approach, the criterion of parsimony was used to optimize character state evolution on the molecular consensus tree using MacClade 4.06 (Maddison and Maddison, 2003). A hypothetical ancestor was used as an outgroup to polarize those char-

Table 2

Harmonic mean of the sampled likelihood values for phylogeny obtained with different modeling schemes and comparison of standard models with the complex model

Model + G + I	D2–D3 28S		18S		D2–D3 28S + 18S	
	Harmonic mean	2ln (B_{10})	Harmonic mean	2ln (B_{10})	Harmonic mean	2ln (B_{10})
SYM	–6745.13	1219.72	–4384.29	414.01	–11,254.02	2055.2
GTR	–6715.48	1160.42	–4382.57	412.29	–11,248.36	2043.88
Double + GTR	–6135.27	–	–3970.28	–	–10,226.42	–

Bayes factor were calculated as $2\ln(B_{10}) = 2$ (harmonic mean (L_n)—harmonic mean (L_0)). Where L_n , likelihood value of H_n and L_0 , likelihood value of H_0 . Bayes factor were interpreted according to the table of Kass and Raftery, 1995, when evidence for model M_1 over M_0 were considered: (i) not worth more than a bare mention if $2\ln(B_{10}) = 0-2$; (ii) positive, if $2\ln(B_{10}) = 2-6$; (iii) strong, if $2\ln(B_{10}) = 6-10$; (iv) very strong, if $2\ln(B_{10}) > 10$.

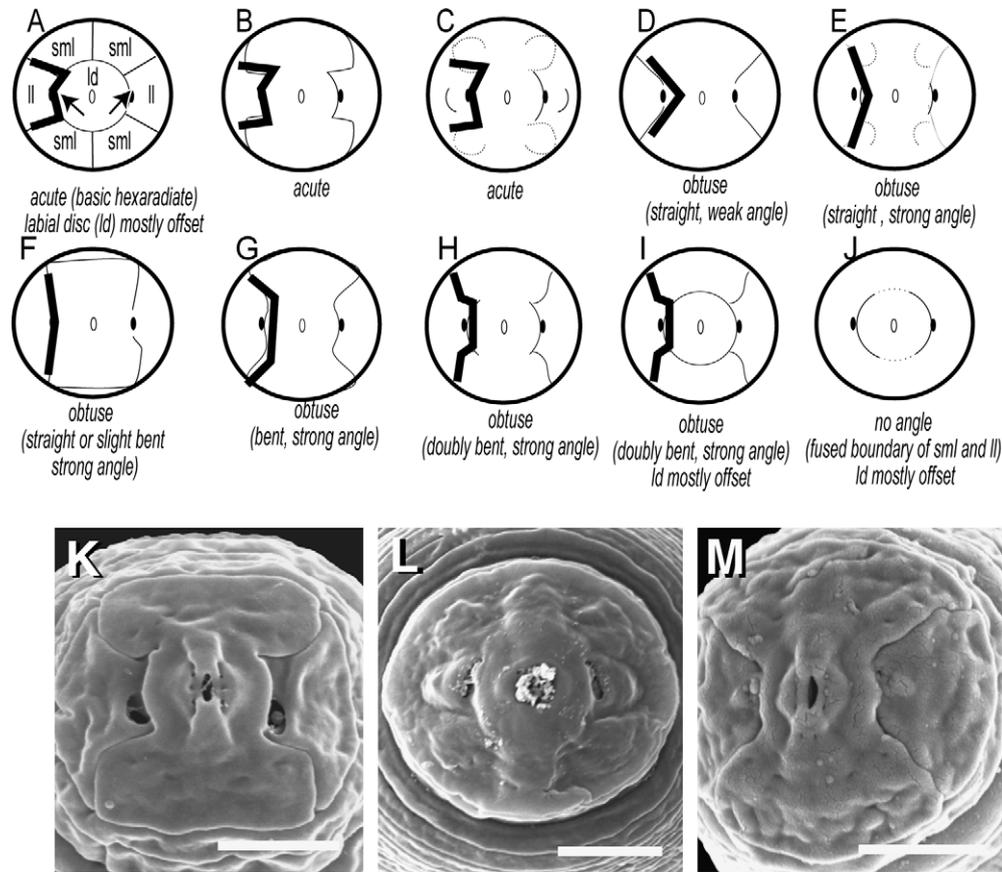


Fig. 1. Diagrammatic representation of lip pattern characters considered in the present study; heavy lines illustrate states for angle of lateral lip in boundary of submedial lips and labial disc. (A) Basic hexaradiate pattern for Tylenchida with labial disc (ld) surrounded by six lips including four submedial (sml) and two lateral (ll). Arrowheads mark position of amphid openings (after Baldwin and Schouest, 1990). (B) *Pratylenchus vulnus* (after University of California Riverside Collection [UCR], Momota, 1978; Corbett and Clark, 1983; Hernández et al., 2000), *P. penetrans* (after UCR, Corbett and Clark, 1983; Hernández et al., 2000), *P. fallax* (after Corbett and Clark, 1983; Momota, 1978), *P. pinguicaudatus* (after Corbett and Clark, 1983), *P. dunensis* (after de la Peña et al., 2006), *P. pratensis* (after Corbett and Clark, 1983). (C) *P. brachyurus* (after UCR, Corbett and Clark, 1983; Hernández et al., 2000), *P. arlingtoni* (after Handoo et al., 2001); *P. crenatus* (after Corbett and Clark, 1983, but requiring confirmation with higher resolution SEM). (D) *P. neglectus* (after UCR, Hernández et al., 2000). (E) *P. zaeae* (after UCR, Corbett and Clark, 1983), *P. goodeyi* (after Corbett and Clark, 1983; Hernández et al., 2000). (F) *P. teres vanderbergae* (after Carta et al., 2002). (G) *P. thornei* (after UCR, Corbett and Clark, 1983). (H) *P. gutierrezii* (Golden et al., 1992). (I) *P. agilis* (after UCR), *P. scribneri* (after UCR, Hernández et al., 2000; Inserra et al., 2007), *P. hexincisus* (after UCR, Inserra et al., 2007), *P. pseudocoffeae* (after Mizukubo, 1992a; Duncan et al., 1999 [group II]). (J) *P. coffeae* (after UCR, Momota, 1978; Inserra et al., 2001; Corbett and Clark, 1983; Mizukubo, 1992b; Duncan et al., 1999), *P. hippeastri* (after Inserra et al., 2007), *P. loosi* (after Corbett and Clark, 1983; Duncan et al., 1999), *P. jaehni* (after Inserra et al., 2001). (K) SEM micrograph of *P. penetrans* depicting acute pattern as illustrated in B. Scale is 1 μm . (L) SEM micrograph of *P. brachyurus* depicting acute pattern as illustrated in C. Scale is 1 μm . (M) SEM micrograph of *P. thornei* depicting obtuse pattern as illustrated in G. Scale is 1 μm .

acters for which ancestral states for the genus could be safely estimated (ancestral lip characters as shown in Fig. 1A; presence of round/oval spermatheca). Because of the apparent morphological disparity for these characters between *Pratylenchus* spp. and our closest outgroup in the molecular analysis (*Belonolaimus*; concerning the highly diverged lip pattern in this genus see Section 4.4), hypotheses of homology could not be reliably made for meaningful polarization using this outgroup. However, in our analysis the presence of a hypothetical outgroup, in contrast to using no outgroup, did not affect the most parsimonious mapping except for at the root, to which the hypothesized ancestral character state was assigned instead of an equivocal assignment. Yet as the scope of the study has assumed to illuminate phylogenetic resolution within *Pratylenchus*, we regard our morphological outgroup designation as trivial.

Ancestral character states were estimated according to their posterior probability distributions in a Bayesian approach using the program SIMMAP (Bollback, 2006). Because posterior probabilities of ancestral states are conditional on probabilities of their respective nodes, inferring character state evolution across a phylogeny in a Bayesian framework enabled considering uncertainty both in mapping given a phylogenetic hypothesis and in the phy-

logenetic hypothesis itself. In combining the results with those based strictly on parsimony, which optimizes characters on a single tree and does not permit any less parsimonious character histories, the goal was to reveal any character histories in parts of the phylogeny that might otherwise be unduly regarded as well supported in tracking relationships in *Pratylenchus*. Character histories were sampled across the entire set of 18,000 trees (i.e. excluding burn-in) drawn in the Bayesian analysis of the combined molecular dataset. A hyperprior approach was taken to sample morphology priors (Schulz and Churchill, 1999) using the default bias (for binary characters) and rate parameters, the former sampling priors from a flat ($\alpha = 1$), discrete ($k = 19$) symmetrical beta distribution, the latter sampling from a discrete ($k = 60$) G distribution with $\alpha = 3.0$ and $\beta = 2.0$. Priors were sampled 10 times per tree in the simulations. Two separate runs were conducted, one for each of the sets of 9000 sampled trees from parallel MCMC runs as implemented in MrBayes 3.1.2, to check for congruence of posterior probabilities between them. In both parsimony and Bayesian character history analyses, all outgroup taxa assumed missing data for all characters, for the reason that no confident homology statements could be made for the characters across such phylogenetic distance.

3. Results

3.1. Analysis of D2–D3 of 28S rRNA full dataset

The D2–D3 of 28S rRNA sequence alignment for 145 accessions was 838 bp. The alignment was analyzed under the best-fit GTR + G + I model to yield a majority rule consensus BI tree given in Fig. 2. Phylogenetic analysis confirmed among large numbers of geographical diverse isolates that most classical morphospecies are monophyletic. Analysis resolved six highly supported (PP \geq 95%) major clades: (I) *P. pratensis*, *P. coffeae*, *P. gutierrezii*, *P. loosi*, unidentified *Pratylenchus* sp. 1, *P. jaehni*, *P. hippeastri*, *P. scribneri* with closely related species *P. agilis*, *P. hexincisus*, and *P. pseudocoffeae*; (II) *P. teres teres* and *P. teres vanderbergae*, (III) *P. vulnus*, *P. crenatus*, *P. brachyurus*, and unidentified *Pratylenchus* sp. 3; (IV) *P. dunensis* and *P. penetrans* with closely related species *P. fallax*, *P. arlingtoni*, *P. convallariae*, and *P. pinguicaudatus*; (V) *P. thornei*, *P. mediterraneus*, and *P. neglectus* with *P. brzeskii* and (VI) *P. zae*. Relationships between these clades were poorly resolved, whereas relationships between most species within clades were mainly moderately or highly resolved with exception of relationships between species in clade I: *P. scribneri*, *P. agilis*, *P. hexincisus*, and *P. pseudocoffeae*. The position of *P. scribneri* was not resolved and the question for its possible misidentification remains open. In clade I, *P. coffeae* was represented by three lineages with (i) a basal position of the population from Ghana, (ii) a clade with identical sequences for D2–D3 PCR clones from a Japanese population, and (iii) a large clade including several populations from Asia and North and South America. Sequence diversity within *P. coffeae* ranged from 0% to 3.6%.

In clade IV relationships of *P. penetrans* with closely related species *P. fallax*, *P. arlingtoni*, *P. convallariae*, and *P. pinguicaudatus* were moderately resolved, as only the D3 fragment of 28S rRNA was used for these last four species in the analysis.

Relationships between species within clade V were highly resolved. Including *P. thornei* with 22 accessions formed two distinct clades. One of these clades included most populations and another grouped Australian populations of *P. thornei* with *P. mediterraneus* from Israel. The ITS rRNA sequences of one Australian population were distributed between both clades. Intraspecific diversity for *P. thornei* reached 6.5%.

The position of *P. zae* (clade VI) with respect to other *Pratylenchus* species was not resolved. A high level of heterogeneity was revealed within both populations of these species that originated from Japan and South Africa. Sequence diversity within clones of same populations of *P. zae* ranged from 0.1% to 5.9%.

3.2. Analysis of partial 18S rRNA full dataset

The partial 18S rRNA sequence alignment for 68 accessions was 890 bp. The majority rule consensus BI tree obtained under the best-fit SYM + G + I model contained eight major clades with PP \geq 95%, mainly corresponding and named as clades on the tree from the D2–D3 of 28S rRNA dataset. The clades were (Fig. 3): (IA) *P. coffeae*, *P. scribneri* with *P. agilis* and an unidentified *Pratylenchus* species; (IB) *P. pratensis*; (IIIA) *P. vulnus*; (IIIB) *P. crenatus*; (IIIC) *P. brachyurus* and an unidentified *Pratylenchus* sp. 3; (IV) *P. penetrans* and *P. pinguicaudatus*; (VI) *P. zae* with *P. goodeyi* and (V) *P. thornei* with *P. neglectus*. Relationships between these clades were not well resolved. Sister relationships for *P. zae* with *P. goodeyi* and *P. thornei* with *P. neglectus* were highly supported. *Pratylenchus thornei* with 19 accessions formed two distinct clades, one of them including only sequences from Australian populations. Several misidentified accessions from GenBank were corrected during our analysis (Fig. 3).

3.3. Secondary structures for D2 and D3 expansion segments of 28S rRNA gene and analyses of the D2–D3 of 28S reduced dataset

All *Pratylenchus* species and outgroup taxa displayed similar secondary structures for D2 and D3 of 28S rRNA. The secondary structures of these segments, which are common to almost all eukaryotes, are given in Appendix A for *P. thornei*. Regions of C1 and D4/e1, D5 for 28S were most variable in terms of numbers of substitution changes among pratylenchids. Most common base changes in the stem region were C \leftrightarrow U and G \leftrightarrow A.

The 28S rRNA sequence alignment for 28 taxa was 634 bp. The GTR + G + I model was selected as the best-fit among standard models for the 28S dataset according to the AIC. The majority rule consensus BI trees obtained under a GTR + G + I model, SYM + G + I model, complex model and consensus MP tree have similar topologies (data not shown) and contained three highly supported clades including several species: (I) *P. coffeae*, *P. loosi*, *P. gutierrezii*, *P. hippeastri*, an undescribed new species from Guatemala and Costa Rica, *P. jaehni*, *P. pseudocoffeae*, *P. scribneri* with *P. agilis* and *P. hexincisus* (PP = 100, 100, 100; BS = 97%); (IV) *P. dunensis* and *P. penetrans* with *P. fallax*, *P. arlingtoni*, and *P. convallariae* (PP = 100, 100, 100, BS = 100%); (V) *P. thornei*, *P. neglectus*, and *P. brzeskii* (PP = 94, 96, 96, BS = 98% without *P. thornei*); and “clades” each representing only one accession: (II) *P. teres vanderbergae*, (IIIA) *P. vulnus*, (IIIB) *P. crenatus*, (IIIC) *P. brachyurus* and (VI) *P. zae*. Position and grouping of these species were not well resolved. According to the Bayes factor, the complex model could explain the dataset significantly better than the GTR + G + I model (Table 2). Differences in topologies between BI trees generated by different modeling schemes were mainly in respect to the positions of *P. brachyurus*, *P. vulnus*, *P. crenatus*, and *P. teres vanderbergae*, which remain unresolved.

3.4. Secondary structures for partial 18S rRNA gene and analyses of the 18S reduced dataset

Using homology modeling revealed similar secondary structures for 18S rRNA for *Pratylenchus* and outgroup taxa. The secondary structures of the 18S rRNA of *P. thornei* is given in Appendix A. Regions of 16, 23/e1, 23/e4, 29, 30, 43 for 18S rRNA were most variable in terms of numbers of substitution changes.

The partial 18S rRNA sequence alignment for 18 taxa was 905 bp. The SYM + G + I model was selected as the best-fit among standard models for the 18S datasets based on the AIC. Differences in estimated marginal likelihoods between SYM + G + I and GTR + G + I models were not significant (Table 2) and the topologies generated by these models were identical. Four clades were evident (data not shown) on the BI tree: (I) *P. coffeae*, *P. scribneri*, and *P. agilis* (PP = 100%); (III + IV) *P. brachyurus*, *P. crenatus*, *P. pratensis*, *P. vulnus*, *P. penetrans*, and *P. pinguicaudatus* (PP = 92%); (VI) *P. zae* and *P. goodeyi* (PP = 94%); (V) *P. neglectus* and *P. thornei* (PP = 100%). The partitioned Double + GTR model was a significantly better fit to this dataset than the standard models (Table 2). However, the topology of the 50% consensus BI tree under the complex model was very similar to previous ones. In a strict consensus MP tree, only two clades were highly supported (I and V), whereas relationships among other species were not well resolved (data not shown).

3.5. Combined analyses of D2–D3 of 28S and partial 18S reduced dataset

The D2–D3 of 28S + partial 18S sequence secondary structure alignment for 30 accessions was 1539 bp. The majority rule consensus BI tree (data not shown) obtained under GTR + G + I and

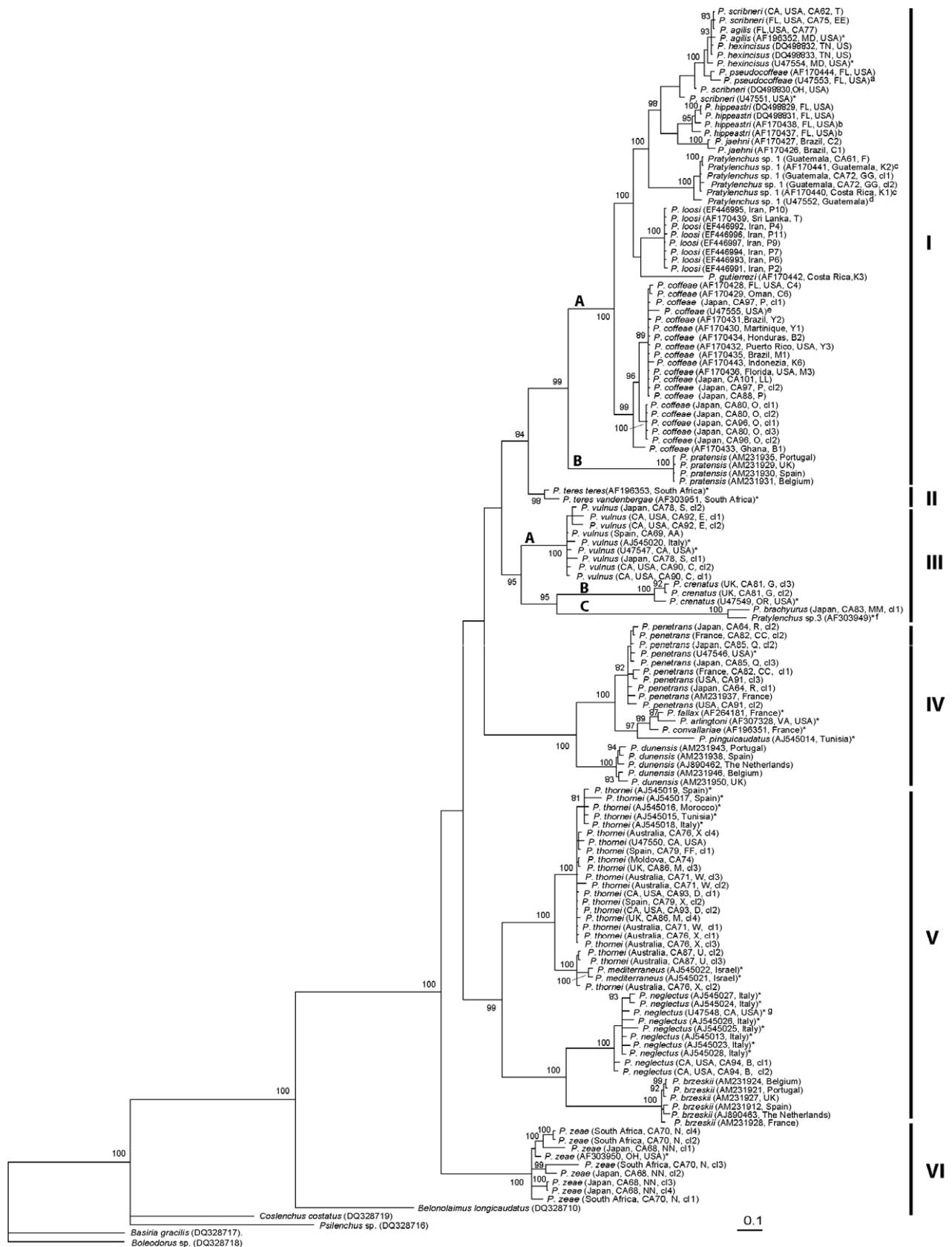


Fig. 2. Bayesian 50% majority rule consensus tree (30,001 trees sampled; burn-in = 1001 trees) from two runs as inferred by the D2–D3 of 28S alignment of the full dataset under the GTR + G + I model. Roman numerals I–VI indicate major clades. Letters A–C correspond to nodes indicating subclades individually resolved, consistent with, and similarly denoted in the 18S tree (Fig. 3). Estimated marginal likelihood for runs: harmonic mean = $-11,317.14$. Posterior probabilities (%) are given for appropriate clades. * indicates species having D3 sequences only; a—identified as *P. brachyurus* by Al-Banna et al. (1997); b—preliminarily identified as *P. loosi* by Duncan et al. (1999); c—preliminarily identified as *P. gutierrezii* by Duncan et al. (1999); d—identified as *P. coffeae* by Al-Banna et al. (1997); e—identified as *P. musicola* by Al-Banna et al. (1997), the species is considered as a synonym of *P. coffeae*; f—identified as *P. hexincisus* by Carta et al. (2001); g—identified as *P. minyus* by Al-Banna et al. (1997), this species is considered a synonym of *P. neglectus*.

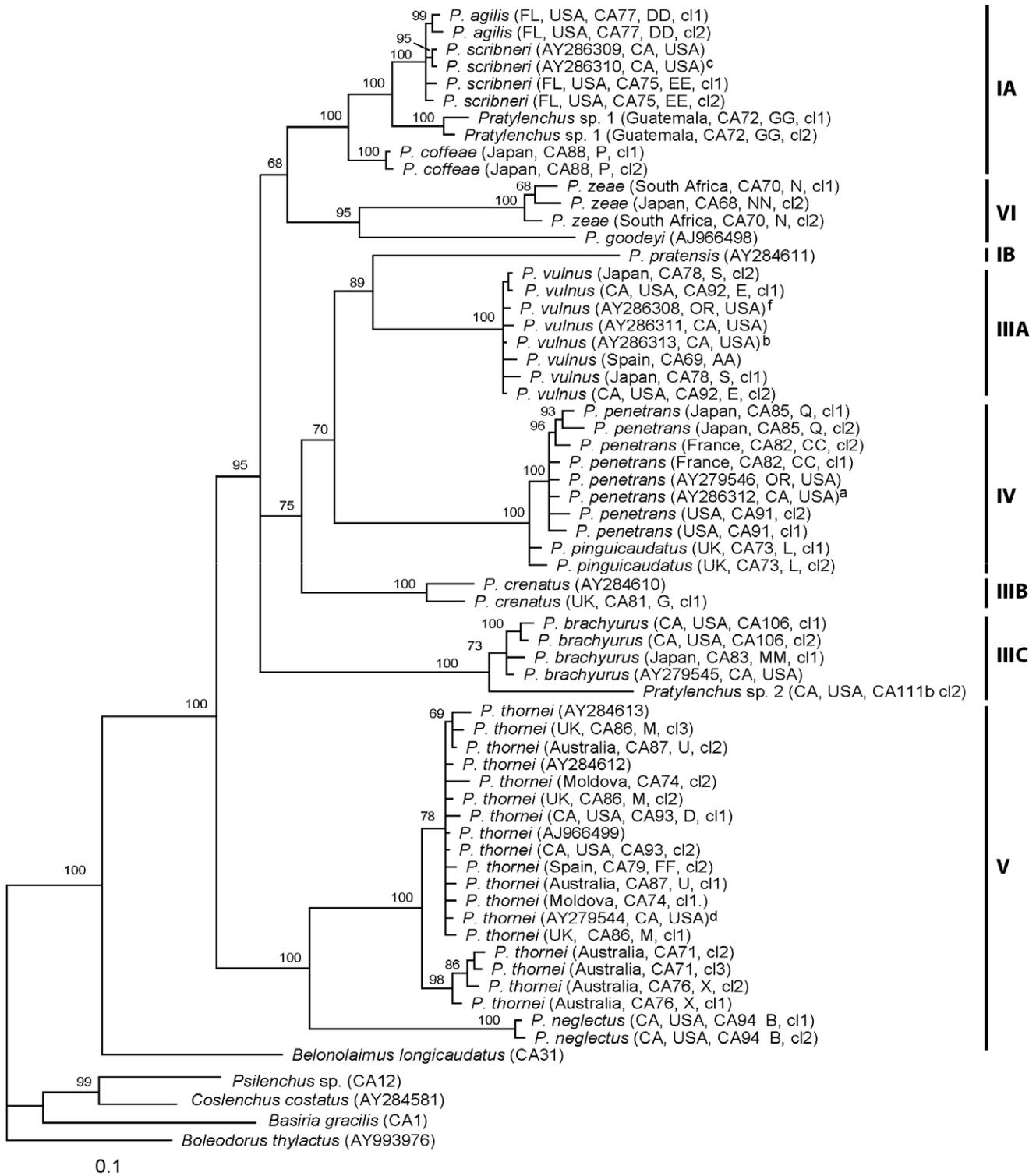


Fig. 3. Bayesian 50% majority rule consensus tree (60,001 trees sampled; burn-in = 1001 trees) from two runs as inferred by the partial 18S alignment of the full dataset under the SYM + G + I model. Roman numerals I–VI correspond to clades consistent with the 28S tree (Fig. 2). Numerals qualified by letters (A–C) indicate clades that are fragmented with respect to the 18S tree. Letters correspond to subclades individually resolved as shown in Fig. 2. Estimated marginal likelihood for runs: arithmetic mean = -6206.96 , harmonic mean = -6292.76 . Posterior probabilities (%) are given for appropriate clades. a—identified as *P. vulnus* by Lee et al. (unpublished); b—identified as *Pratylenchus* sp. by Lee et al. (unpublished); c—identified as *P. thornei* by Lee et al. (unpublished); d—identified as *P. neglectus* by Lee et al. (unpublished); f—identified as *P. penetrans* by Lee et al. (unpublished).

SYM + G + I models have similar topologies and contained three major highly supported clades (Fig. 4): (a) *P. pratensis*, *P. crenatus*, *P. vulnus*, *P. brachyurus*, *P. dunensis* and *P. penetrans* with *P. pinguicaudatus*, *P. convallariae*, *P. fallax*, and *P. arlingtoni* (PP = 96%); (b) *P. coffeae*, *P. loosi*, *P. gutierrezii*, *P. jaehni*, *P. hippeastri*, and *P. scribneri*

with closely related species *P. agilis*, *P. hexincisus*, and *P. pseudocoffeae* (PP = 100%); (c) *P. brzeskii*, *P. neglectus*, and *P. thornei* (PP = 96%). The tree also contains one moderately supported clade (d) (PP = 94%) with *P. zaeae* and *P. goodeyi* and another clade (e) with *P. teres vanderbergae*.

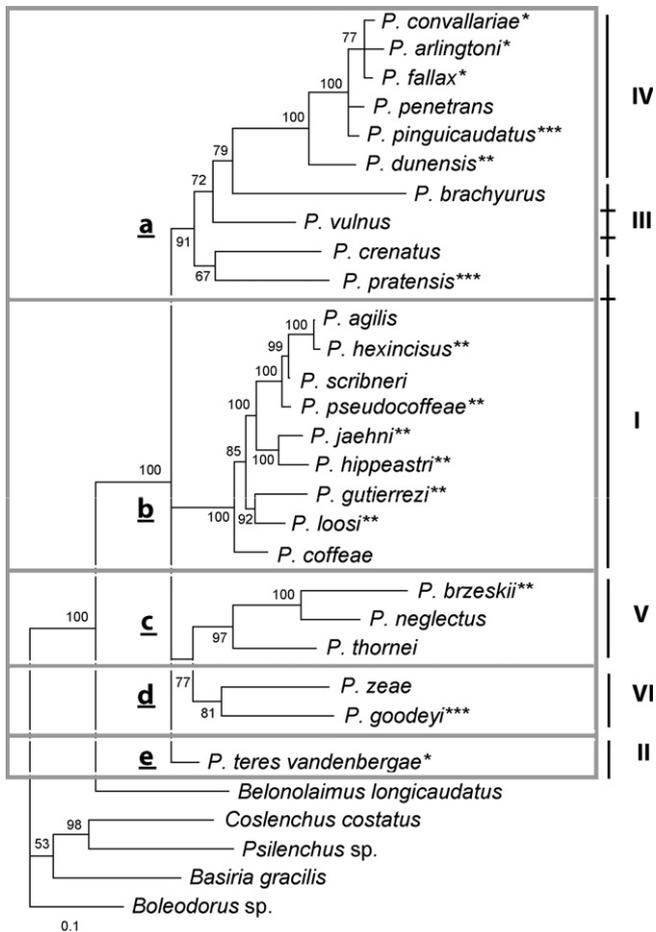


Fig. 4. Bayesian 50% majority rule consensus tree (10,001 trees sampled; burn-in = 1001 trees) from two runs as inferred the D2–D3 of 28S and partial 18S alignment of the reduced dataset under the complex model: doublet and (GTR + I + G) models. Roman numerals I–VI circumscribe taxa belonging to clades designated in the 28S tree (Fig. 2). Boxes with letters a–e designate major clades represented in Fig. 5. Estimated marginal likelihood for runs: harmonic mean = $-10,226.42$. Posterior probabilities (%) are given for appropriate clades. Clade numbering as it is presented in the BI tree and obtained from the full D2–D3 of 28S dataset is also given. * indicates species having D3 sequences only; ** indicates species having D2 and D3 sequences only; *** indicates species having 18S sequences only.

According to the Bayes factor, the partitioned Double + GTR model was a significantly better fit to the dataset than the standard models (Table 2). The 50% majority rule consensus tree obtained with the complex model of evolution had a similar topology to those under standard models (Fig. 4) with the exception that the tree revealed a sister relationship between *P. crenatus* and *P. pratensis* (PP = 53%), whereas in the BI tree under the simple model this relationship was not resolved. Furthermore, in the majority rule tree, PP values for several clades were lower: *P. zaeae* + *P. goodeyi*, 81% versus 94% or 95%; the clade *P. scribneri*, *P. agilis*, *P. hexincisus*, *P. pseudocoffeae*, *P. jaehni*, *P. gutierrezii*, *P. loosi*, and *P. hippeastri*, 85% versus 99%; the clade *P. loosi* + *P. gutierrezii*, 92% versus 100%.

The consensus MP tree revealed only two highly supported major clades: (a) *P. dunensis* and *P. penetrans* with *P. pinguicaudatus*, *P. convallariae*, *P. fallax*, and *P. arlingtoni* (BS = 99%) and clade b as it is defined in the BI tree (BS = 100%); other relationships were weakly supported except for *P. brzeskii* + *P. neglectus* (BS = 92%) (data not shown).

3.6. Analysis of mapping morphology on a combined D2–D3 of 28S and partial 18S rRNA tree

Character evolution was largely congruent between parsimony and Bayesian methods (Fig. 5), although Bayesian ancestral state

reconstruction revealed a few cases in which character histories were rivaled by alternate hypotheses thus reducing their reliability for resolving species relationships. Among Bayesian character history simulations, the two runs on separate sets of trees were highly congruent, although they showed differences of up to 0.5–2% of posterior probabilities in a few cases where competing reconstructions were also relatively highly probable, that is, where more than one state had a posterior probability of greater than 5%.

Aspects of lip patterns are largely consistent with major clades a, b, c, d, and e (Figs. 1 and 5). The labial disc is mostly offset having a clear boundary with the submedial lips throughout members of clade b, but with *P. gutierrezii* as a sole exception (Fig. 1H–J, Fig. 5A and Table 3). Species in which the lateral and submedial lips are fused (i.e. *P. coffeae*, *P. gutierrezii*, *P. hippeastri*, *P. jaehni*, and *P. loosi*, Fig. 1J) occur only in clade b, again with *P. gutierrezii* as the sole exception. Conversely, the lateral lips are defined by a distinct boundary in a group (i.e. *P. agilis*, *P. hexincisus*, *P. pseudocoffeae*, and *P. scribneri*) within the same clade b (Fig. 1H, Fig. 5B and Table 3); notably, in some isolates/preparations of either *P. scribneri* or *P. hexincisus* the labial disc is less offset (slightly fused) than in most others, arguably suggesting an intermediate morphological transition (see Section 4.4).

The boundary of the lateral lips forming an acute pattern is uniquely shared by all taxa in clade a; whereas all taxa in clades c and d share a pattern in which the comparable boundary is obtuse (Fig. 1G, D and L, Fig. 5C and Table 3). Although the position of *P. teres vanderbergae* is not clearly resolved in the consensus tree, its otherwise unique pattern also shares a strongly obtuse boundary with taxa of clades c and d (Fig. 5C; Table 3). The position of *P. zaeae* is not well resolved with respect to other taxa (PP = 79%), but the unusual lip pattern of this species includes a strongly obtuse boundary with the lateral lips; current information of its sister taxon, *P. goodeyi*, suggests a pattern similar to that of *P. zaeae* (Fig. 1, Table 3). Taxa in clade b for which this character could be discerned all have a boundary that is obtuse medially but also bends toward the dorsoventral plane (“double bend”; Fig. 1G and H; Table 3). Other taxa in clade b have lips fused to the labial disc, so character states cannot be assigned (Fig. 1J, Fig. 5C; Table 3).

The character state of two lip annules posterior to the lip pattern (Table 3) is expressed throughout clade b; sole exceptions outside this clade are sister taxa *P. neglectus* + *P. brzeskii* in clade c and *P. dunensis* and *P. brachyurus* in clade a, with the positions of the latter species unresolved (PP = 63%). With respect to number of lip annules, Bayesian simulations confirm that nodes defining the smallest clades including taxa with discordant states show ambiguous ancestral state reconstructions but that nodes circumscribing clades a, b, c, and d show strong support for one state over the other, inasmuch as the nodes are supported (Fig. 5D).

A slit-like spermatheca is unique to *P. neglectus*, *P. thornei*, and *P. zaeae* (Fig. 5E). While all in clades c and d, the sister taxa of two of them have an alternate state, equivocally suggesting a reversal from, or plesiomorphy of, a slit-like spermatheca. Notably, PP values for the node defining clade c + d and the node common to only *P. goodeyi* and *P. zaeae*, which show conflicting states, are not high (70% and 79%, respectively).

4. Discussion

4.1. Molecular phylogeny of *Pratylenchus* based on rRNA genes

With increasing taxonomic sampling of gene fragments, the present study provides a more comprehensive view of phylogeny of root lesion nematodes than previously available; it further provides a basis for recognizing morphological characters that track evolution versus those that are less directly informative. In our

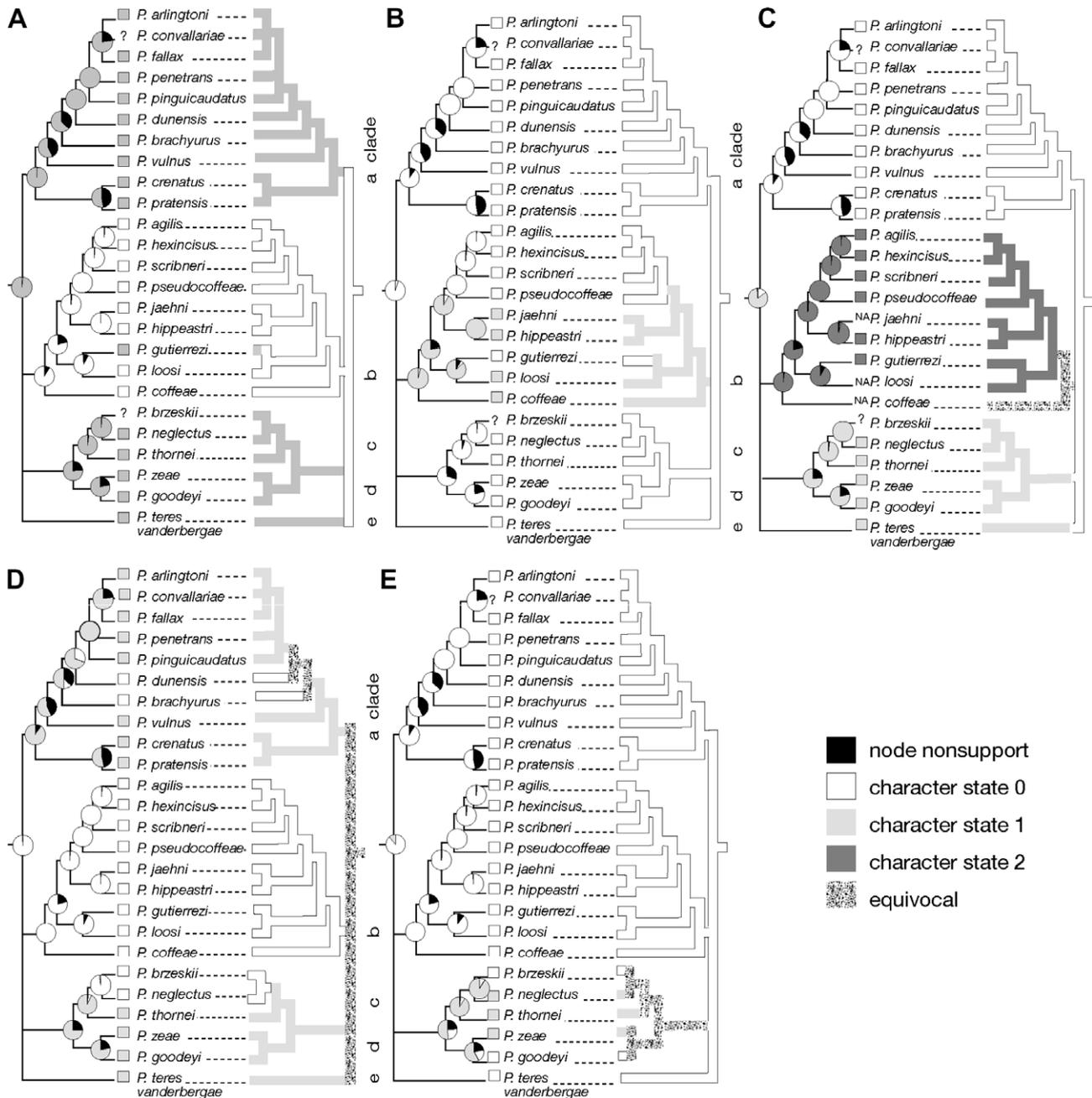


Fig. 5. Morphological character history reconstruction for five lip and spermatheca characters using Bayesian simulations (trees on left) and by parsimony (right) on the consensus tree. Charts at nodes show relative posterior probabilities of different ancestral state reconstructions and/or nonexistence of the node based on the set of MCMC trees from the combined 18S and 28S analysis. Letters a–e correspond to major clades as designated in consensus tree (Fig. 4). Character states follow Table 3: (A) boundary of labial disc with labial lips; (B) boundary of lateral lips with submerdial lips; (C) angle of lateral lips with labial disc; (D) number of lip annules; (E) spermatheca shape.

study the number of main *Pratylenchus* clades varied depending on genes used. The BI analysis of the combined 28S and 18S dataset revealed at least six clades with *Pratylenchus* species. The three major highly supported root lesion nematode groups were evident from all analyses. The first group consists of *P. coffeae* and closely related species as first defined by Duncan et al. (1999) including *P. loosi*, *P. hippeastri*, *P. gutierrezii*, *P. jaehni*, undescribed *Pratylenchus* from Central America, and *P. scribneri* within its sibling species *P. agilis* and *P. hexinciscus*. The second group unites *P. thornei*, *P. mediterraneus*, and *P. neglectus* with *P. brzeskii*. The third group includes *P. dunensis* and *P. penetrans* with closely related species *P. fallax*, *P. arlingtoni*, *P. pinguicaudatus*, and *P. convallariae*. Relationships between these groups as well as relationships between other species (*P. zae*, *P. goodeyi*, *P. teres vanderbergae*, *P. brachyurus*, *P. crenatus*,

and *P. vulnus*) are still not clearly resolved from the D2–D3 28S, 18S or combined rRNA datasets. Positions of weakly or moderately supported clades varied within trees generated from different datasets. With some exceptions our phylogenetic trees, including these groupings, are mainly congruent with those based only on the analysis of D3 of 28S rRNA by Carta et al. (2001), De Luca et al. (2004), but herein adding the D2 expansion segment of 28S significantly increased support for several main clades. In contrast, molecular trees in many aspects contradict the morphology based phylogeny proposed by Ryss (2002b), raising questions of incongruence of these features in the evolution of root lesion nematodes.

In the present work, we applied a more objective approach with reconstruction of an optimal alignment using secondary structure information for each studied D2–D3 expansion segments of 28S

Table 3
Morphological features and states considered for potential phylogenetic information

Feature ^a	Boundary labial disc with submedial lips ^b	Boundary lateral lips with submedial lips ^b	Angle lateral lip with labial disc ^b	# lip annules	Spermatheca shape
Character state coding TAXA	0 = mostly offset 1 = not offset	0 = no fused 1 = fused	0 = acute 1 = obtuse 2 = obtuse/double bend	0 = 2 annules 1 = 3–4 annules	0 = round, oval or indistinct 1 = slit-like
<i>P. agilis</i> I. ^c	0	0	2	0	0
<i>P. arlingtoni</i> C.	1	0	0	1	0
<i>P. brachyurus</i> C.	1	0	0	0	0
<i>P. brzeskii</i> ?	?	?	?	0	0
<i>P. coffeae</i> J.	0	1	N/A	0	0
<i>P. convallariae</i> ?	?	?	?	1	0
<i>P. crenatus</i> ^d C.	1	0	0	1	0
<i>P. dunensis</i> B.	1	0	0	0	0
<i>P. fallax</i> B.	1	0	0	1	0
<i>P. goodeyi</i>	1	0	1	1	0
<i>P. gutierrezii</i> H.	1	0	2	0	0
<i>P. hexincisus</i> I.	0	0	2	0	0
<i>P. hippeastri</i> I.	0	1	2	0	0
<i>P. jaehni</i> J.	0	1	2	0	0
<i>P. loosi</i> J.	0	1	N/A	0	0
<i>P. neglectus</i> D.	1	0	1	0	1
<i>P. penetrans</i> B.	1	0	0	1	0
<i>P. pratensis</i> B.	1	0	0	1	0
<i>P. pinguicaudatus</i> B.	1	0	0	1	0
<i>P. pseudocoffeae</i> I.	0	0	1	0	0
<i>P. scribneri</i> I.	0 ^e	0	1	0	0
<i>P. teres vanderbergae</i> F.	1	0	1	1	0
<i>P. thornei</i> G.	1	0	1	1	1
<i>P. vulnus</i> B.	1	0	0	1	0
<i>P. zeae</i> E.	1	0	1	1	1

^a Letter following name corresponds to SEM lip pattern shown in Fig. 4.

^b See Fig. 4 for explanation.

^c Not available to include in consensus tree (Figs. 3 and 5).

^d Requires confirmation with better resolved SEM.

^e Offset in an Ohio isolate (Inserra et al., 2007), more offset in California isolates (Hernández et al., 2000 and Bell, unpublished); see Discussion.

and partial 18S rRNA gene sequences. Secondary structure information provided a guide to align molecular regions previously considered ambiguous. Application of the MARNA program generates a reliable alignment and represents a powerful tool. Our enhanced knowledge of secondary structure allowed us to apply a more sophisticated model and consequently to generate a more realistic tree for phylogenetic estimation. However, in this work the phylogenetic trees obtained under the standard and complex models were not principally different, and their topology varied mainly in positions of weakly or moderately supported clades. The relationships among several main *Pratylenchus* clades and between some species remain unresolved and thereby suggest that the rRNA markers do not contain sufficient phylogenetic signal to resolve relationships at certain levels and thus other markers should be used to resolve those questions.

4.2. Implication of molecular data for *Pratylenchus* systematics

The reliability of *Pratylenchus* morphospecies, often diagnosed on few characters, some of them polymorphic, may sometimes be doubtful considering the potential of similar character sets converging across disparate geographies. Alternative hypotheses are that a species is consistent but may be widely distributed because it is ancient, or that it has been dispersed, for example, by modern global anthropogenic practices, often in conjunction with agriculture. Herein the monophyly of several such morphospecies can be tested by examining an independent character set, namely, sequences shown to evolve at about the level of extant species in relation to worldwide populations. In the present study, even where several geographically divergent isolates are included, most species are reassuringly monophyletic. Exceptions can generally be accounted for by misidentifications (Figs. 2 and 3), although in some cases the validity of species might be questioned and subsequently tested by sequencing topotypes, where available.

Analysis of rRNA genes revealed several groups of *Pratylenchus* species with very similar sequences of D2–D3 of 28S rRNA or partial 18S rRNA. Of particular concern is the large *P. penetrans* species complex including, in addition to *P. penetrans*, closely related species *P. fallax*, *P. convallariae*, *P. pinguicaudatus*, and *P. arlingtoni*, which overlap in many morphological features such that the validity of some of these taxa may be questioned. After extensive study of morphological and morphometrical variation of *P. penetrans* from different geographical localities, Tarte and Mai, 1976 found high levels of polymorphism within and between populations, concluding that *P. convallariae* and *P. fallax* might be polymorphic variants of *P. penetrans*. However, Handoo et al. (2001), after detailed study of *P. fallax*, *P. convallariae*, and *P. arlingtoni*, found several minor morphological and morphometrical differences distinguishing these species from each other and from *P. penetrans*. Perry et al. (1980) found infertile F₁ progeny after interspecific crosses between *P. penetrans* and *P. fallax* in sterile culture and concluded that the species are valid. Minimal sequence differences in rRNA genes might be a result of a recent speciation events in this group, and other biochemical and molecular markers should be found to discriminate the species from this complex. Ibrahim et al. (1995) found several isozymes discriminating *P. fallax* and *P. penetrans*; furthermore, Waeyenberge et al. (2000) revealed differences in the ITS-rRNA length and in the ITS-RFLPs among these species.

A second group includes *P. thornei* and *P. mediterraneus*, which are similar in D3 sequences (De Luca et al., 2004) but seem to differ in their ITS-rRNA length and ITS-RFLPs (Waeyenberge et al., 2000). *Pratylenchus thornei* populations from throughout the world are split into two highly supported clades. One of them is represented by accessions originating solely from Australia clustering together with *P. mediterraneus* from Israel. Interestingly, the ITS-rRNA sequences of the South Australian population were distributed within both clades. The Australian population of root lesion nematodes originally morphologically identified for our study as

P. jordanensis has sequences similar to *P. thornei* and clustered with it in all BI trees. Ryss (2002b) proposed synonymy of *P. jordanensis* with *P. scribneri* based on the analysis of 26 morphological characters. Conversely, Inserra et al. (2005a,b) after studying *P. jordanensis* paratypes and materials from three populations from Oman suggested, on the basis of morphological similarities, that *P. jordanensis* is a junior synonym of *P. zaeae*. As the taxonomic status of this species is under discussion, and as we cannot exclude possible misidentification of this population, herein we consider this sample as *P. thornei*.

The sequence analysis revealed very similar sequences for *P. scribneri*, *P. agilis*, *P. hexincisus*, and *P. pseudocoffeae*. *Pratylenchus scribneri* is paraphyletic in our D2–D3 of 28S rRNA tree. The taxonomic problems with description and identification of *P. scribneri* have been discussed by Ryss (1988) and Inserra et al. (2007). Evidently, the molecular characterization of topotypes are required to test species status for populations tentatively ascribed to *P. scribneri* in previous work as well as in the present study. A consideration may be that *P. scribneri* is inherently variable as suggested, for example, by Roman and Triantaphyllou (1969), who noted isolates that varied in chromosome number and inferred polyploidy. Synonymization of *P. agilis* with *P. scribneri* was proposed by Hernández et al. (2000) in part based upon scanning and light microscopic studies and isozyme analysis (Andrés et al., 2000). However, Waeyenberge et al. (2000) found differences between *P. scribneri* and *P. agilis* with respect to ITS-rRNA length and in the RFLPs between these species.

The limits defining *P. coffeae* and related species based on the D2–D3 sequence analysis, SEM of lip patterns, RAPD analysis, and principal component analysis of morphological characters is discussed in detail by Duncan et al. (1999). Mizukubo et al. (2003) distinguished four RFLP phenotypes for isolates of *P. coffeae*. Several new species were suggested and later described from this group (Inserra et al., 2001, 2007); according to molecular results herein at least one *Pratylenchus* species from Guatemala and Costa Rica is new and should be described (Figs. 2 and 3). *Pratylenchus coffeae*, as we identified it for the present study is monophyletic but divided into three rRNA groups in our 28S BI trees. Taxonomic clarification of the population from Ghana is required.

Our study revealed the presence of a possible sibling species for *P. brachyurus*. Payan and Dickson (1990) revealed three distinct phenotypic groups based on isozyme study of several populations of this species. Evidently, more detailed morphological, biochemical, and molecular analysis is required to further characterize this species.

4.3. Potential of rRNA genes for barcoding *Pratylenchus* species

The use of DNA sequences to identify nematodes has been suggested as a more efficient approach relative to traditional morphological identification (Floyd et al., 2002; Blaxter, 2004). Among 68 recognized morphospecies of *Pratylenchus* by Castillo and Vovlas (2007) nearly half are only reported once and from the type locality. Misidentification of such rare species seems likely, and particularly so where they are distinguished from commonly distributed species by only a few characters. Several cases of misidentification of *Pratylenchus* sequences deposited in GenBank were revealed by our analysis. This underscores that a DNA barcoding approach would be useful to supplement traditional morphological identification. Almost all species included in our study have a unique sequence combination or signature; most exceptions are in cases where the validity of the species identification is questionable.

Several rRNA fragments have been proposed as appropriate markers for barcoding nematodes (Floyd et al., 2002; De Ley et al., 2005). Comparison of two rRNA gene regions included in our study show that the D2–D3 expansion segment of 28S rRNA seems

to be a better target than partial 18S rRNA for barcoding with respect to recognizing a higher degree of interspecific genetic variability. Furthermore, this region, being appropriately short, facilitates amplification as well as conserving flanking sites for universal primers. Presence of divergent, paralogous copies that require cloning or inability to detect recently divergent species, however, may partly limit application of the rRNA genes. Therefore, accurate barcoding may necessitate application of not one but several genes or its fragments. For example, rRNA genes with their spacers and some nuclear genes recently generated for *Pratylenchus* through EST projects (Mitreva et al., 2004) could be considered as a more promising approach for DNA barcoding of the root lesion nematodes.

4.4. Evolution of *Pratylenchus* lip patterns

Consistent with previous application of independent components of lip patterns, as viewed in Heteroderinae with SEM (Baldwin and Schouest, 1990; Baldwin, 1992), within *Pratylenchus* these appear to also have surprising variability among species with respect to shape and fusion of lip components relative to a basic hexaradiate pattern for Tylenchida of a labial disc surrounded by two lateral and four submedial lips (Baldwin, 1992; Luc, 1987; Baldwin and Schouest, 1990; Baldwin and Powers, 1987). Treating diverse features as independent characters appears to be justified and more informative to phylogenetics versus treating the entire pattern as a single feature. It is not known if particular lip patterns are associated with functional considerations; however, we suggest that some aspects may reflect more fundamental features including boundaries between particular underlying hypodermal syncytia (Bumbarger et al., 2006) or the shape of the underlying cuticular framework that might also be associated with the shape of the adjacent amphid sensory openings (often obscured in SEM by secretions).

Whereas lip patterns diverging from a basic hexaradiate pattern include plausible characters for phylogenetics, their value needs further examination of a broader representation of taxa including outgroups. The phylogenetic significance of the suggested shared lip patterns is contingent on character polarity and thus outgroup resolution. With respect to hypothesizing polarity for the above characters, a consideration in using a representative of a hypothesized outgroup (e.g. analogous to those in molecular studies) is that morphological characters, such as lip patterns, while informative within *Pratylenchus*, apparently evolve so quickly in sister clades (e.g. see Geraert and Raski, 1987) that in this narrow context they provide little basis for recognizing their plesiomorphic states. This difficulty may be resolved with broader phylogenetic resolution of Tylenchida and wider representation of lip patterns. Another challenge to using lip patterns in phylogenetic analysis is that in preparation patterns can collapse and introduce artifacts that can lead to ambiguity or misinterpretation. In the present study we have noted lack of clear resolution for *P. crenatus* (Table 3) but patterns for some other species will also benefit as better resolved SEM images become available. Some patterns including *P. zaeae*, *P. arlingtoni*, and *P. brachyurus* (Fig. 1C and E) are inherently difficult to interpret because lips are not delimited with deep indentations as in most other taxa and submedial lips appear to be reduced to “buds”; nevertheless the relative shape and the angle of the boundary of the adjacent lateral lips can be resolved. Whereas lip patterns are demonstrably consistent within a species where they have been examined across a number of isolates, one exception may occur in clade b in the case of *P. scribneri* and *P. hexincisus*. Isolates of *P. scribneri* from California show a clearly offset labial disc (Hernández et al., 2000; A. Bell, UCRNC, unpublished), but more recently an isolate of *P. scribneri* from Ohio is shown where there is little if any offset; this morphological diversity is particularly inter-

esting considering that *P. scribneri* also is diverse in its 18S sequences (Fig. 3). Conversely, *P. hexincisus* from California shows little offset of the labial disc, whereas that from Tennessee shows strong offset. Also within this group is *P. agilis* where on the basis of similar SEM lip patterns, combined with additional data, Hernández et al. (2000) considered *P. agilis* a synonym of *P. scribneri*. An important consideration is how subject to artifact interpretation some lip pattern characters might be if specimens partially shrink during preparation. To address such a potential problem, at UCRNC our approach applied to pratylenchids is to prepare material both by glycerin infiltration (Sher and Bell, 1975) and by critical point drying (Mundo-Ocampo et al., 2003) as a point of comparison to minimize misinterpretation.

A widely used classical character in *Pratylenchus* related to lip patterns is the number of lip annules that occur immediately posterior to the lips (Allen and Jensen, 1951), and one test of the present study was to determine if numbers of lip annules are congruent with particular clades. To some extent clade b, well supported by the present study, corresponds to a clade identified by Carta et al. (2001) as including tropical species also congruent to a large degree with two lip annules, but with *P. neglectus* (as in the present study) being a notable exception (resolved herein in clade c). To this exception in the present study we add *P. brzeskii* (clade c) as well as *P. dunensis* and *P. brachyurus* (clade a). Whereas Carta et al. (2001) note that *P. hexincisus* also has two lip annules, Loof (1978) comments that it may have three, further underscoring the morphological diversity of this species as presently defined.

4.5. Potential for understanding evolution of additional *Pratylenchus* features

The molecular phylogenetic framework herein provided for *Pratylenchus* may provide a basis to begin understanding the evolution of additional morphological characters. With broader sampling, diversity in the female gonoduct of *Pratylenchus* (Bert et al., 2003) holds some promise for phylogenetic information. Whereas the overall cellular structure of the spermatheca appears to be relatively consistent, with 12 cells, the arrangement of these cells was observed to be species specific. In the present study we followed Ryss (2002b) in coding the spermatheca of *P. neglectus* and *P. thornei* in clade c. Notably, coding of a slit-like spermatheca versus alternatives is independent of whether or not the female has been inseminated or the presence of absence of males (e.g. as in parthenogenetic species). It may be significant that this slit-like spermatheca is also shared by *P. zaeae* (clade d), considering that the position of this species is not strongly resolved by the molecular based consensus tree (Fig. 4, Fig. 5E).

Additional characters that may be informed by the molecular based phylogeny, but first requiring broader taxon sampling, include chromosome number relative to plausible patterns of ploidy throughout the genus, as well as modes of reproduction including amphimixis and facultative (meiotic) and obligate (mitotic) parthenogenesis (Roman and Triantaphyllou, 1969; Triantaphyllou and Hirschmann, 1980). Data on modes of reproduction might also provide insight into understanding patterns of intraspecific variability.

The classical diagnostic character of number of lines in the lateral field, in relation to the consensus tree of the present study appears to be homoplastic with six or more lines evolving convergently in *P. arlingtoni* (clade a), *P. crenatus* (clade a), and *P. hexincisus* (clade b) (Fig. 5). One difficulty with this character is evident in SEM investigations of many nematode species where apparently transitional or artifactual lines introduced by crenation) morphologies of four major lines with less pronounced lines between are observed. Inserra et al. (2007) refers to a case of confusion in identity of *P. scribneri* and *P. hexincisus*, with some

specimens from a putative *P. hexincisus* isolate having obscure lateral fields or fields marked by only four lines; similarly, Carta et al. (2001) notes that vouchers putatively of *P. hexincisus* (with six lines) were mixed with material of *P. agilis* (with four lines).

4.6. Implications for morphology based phylogenetics in nematodes

The present study suggests that understanding morphological evolution or conversely using morphology to inform phylogeny may require a more sophisticated understanding of characters and models of their evolution than is often available for nematodes. In general, diagnostic characters, although useful for species identification, are most often inadequately understood to be coded and inform phylogeny. By example, for *Pratylenchus*, carefully reviewing and testing each of the characters previously proposed (Ryss, 2002b), eliminating ratios and other linked characters that could give a false impression of parsimony, the most promising and discrete features appeared to be number of lip annules (but combining 3 and 4 since this variation is often the result of interpretation from incomplete annules or different orientations of the head in preparation) and presence of a slit-like spermatheca (distinguished as a separate character state relative to all others). To these two we added three characters from the broadly based information that has accumulated on lip regions, including outgroups. Beyond these five characters, additional characters of particular promise are suggested but must be extended to additional taxa.

Although the present study is necessarily constrained in scope by the level of current knowledge with respect to range of morphological characters where patterns of evolution can be inferred, it does provide promise and direction for approaching morphology in phylogenetics for nematodes and other comparably small organisms. In addition to the problems of diagnostic features, phylogenetically informative morphologies in nematodes are often at or beyond the limits of light microscope resolution, and thus recognizing, for example, characters, character states, homology, polarity, and functional convergence may require more refined tools than allowed by classical microscopy, as has already been illustrated for other nematode groups (Kiontke et al., 2007) and deeper phylogenetic nodes (Baldwin et al., 1997; Zhang and Baldwin, 2000; Baldwin et al., 2004; Bumbarger et al., 2006). Furthermore, analysis of characters needs to be given due consideration. Developing computational methods can employ alternative criteria (e.g. Bayesian, maximum likelihood) to parsimony in reconstructing character histories; taking advantage of alternatives allows us to test the effect of methodology on our inferences of character evolution. Although our results show significant congruence between character histories inferred by different methods, it is apparent that reliance upon parsimony only can potentially lead to a false sense of confidence in some characters, which depend at least partially on resolution of the trees used for reconstruction, implicating the need to both interpret and analyze these characters cautiously. As demonstrated for *Pratylenchus*, molecular phylogenies as an independent character set clearly test and inform morphological evolution, and often challenge for greater sophistication in understanding morphology toward revelation of phenotypic plasticity and congruence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ympev.2008.04.028.

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