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Molecular variability and phylogenetic relationships among different species and populations of *Pratylenchus* (Nematoda: Pratylenchidae) as inferred from the analysis of the ITS rDNA

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Abstract Sequence comparisons and molecular phylogenetic analyses were used to describe the nucleotide variability of the ITS containing regions of eighteen *Pratylenchus* species and several populations. Comparative analysis of nucleotide sequences of the rDNA internal transcribed spacers (ITS1 and ITS2) among *Pratylenchus* species used in the present study demonstrates that ITS sequences can widely vary in primary sequence and length. Alignment of eighty-seven *Pratylenchus* sequences and one outgroup taxon reveals the presence of ambiguous regions that have the greatest effect on phylogeny reconstruction. Phylogenetic analyses using Bayesian Inference, Neighbour Joining-LogDet, Maximum Likelihood and Maximum Parsimony, distinguished twelve highly or moderately supported major clades within *Pratylenchus*. Our results support the taxonomic usefulness of the ITS region to identify root-lesion nematode species of the genus *Pratylenchus* but the high nucleotide variability, sometimes, can preclude its use to resolve relationships among all members of the genus. In addition, the phylogenetic groupings are not congruent with those defined by characters derived by lip patterns and numbers of lip annuli.

Keywords Bayesian inference · Maximum parsimony · Internal transcribed spacers · Root-lesion nematodes

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Introduction

Root-lesion nematodes of the genus *Pratylenchus* Filipjev, 1936 are migratory endoparasites widely distributed worldwide and regarded as severe constraints of many crops (Castillo and Vovlas 2007). They penetrate, feed and invade the cortical parenchyma, producing large necrotic areas and cavities mainly within the layer of the root cortex. The damage is often aggravated by their interactions with soilborne fungi and bacteria, resulting in complex diseases which are biological and physiological rather than physical in nature (Castillo and Vovlas 2007). Nevertheless, damage caused by *Pratylenchus* species is frequently not obvious, so it is necessary to understand their biology, ecology and

interaction with other microorganisms in order to determine their impact on crop yield (Castillo and Vovlas 2007).

Currently, the genus includes more than 70 species and 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 many incomplete descriptions published in the literature (Castillo and Vovlas 2007). Proper species identification is critical to nematode control strategies as well as to regulatory or quarantine procedures. However, as the number of new *Pratylenchus* species is constantly increasing (Inserra et al. 2007; Troccoli et al. 2008; Palomares-Rius et al. 2010; De Luca et al. 2010), the difficulties in separating species increased, driving taxonomists to search for new reliable features and analysis tools.

Since the first revision of the genus (Sher and Allen 1953), many other authors investigated several aspects concerning the taxonomy of *Pratylenchus* species, giving new insights on identification (Loof 1960, 1978; Café Filho and Huang 1989; Frederick and Tarjan 1989; Handoo and Golden 1989; Palomares-Rius et al. 2010), scanning electron microscopy (SEM) characterization (Corbett and Clark 1983; Baujard et al. 1990; Hernández et al. 2000; Inserra et al. 2007), and intraspecific variability of main morphological diagnostic characters (Taylor and Jenkins 1957; Roman and Hirschmann 1969; Tarte and Mai 1976). During the last decades, new approaches based on biochemical, molecular and phylogenetic analyses have provided powerful and useful tools to nematode systematics and practical identification of plant-parasitic nematodes. In particular the 28S rDNA gene has been largely used to discriminate among different populations and species of *Pratylenchus*. However, several authors (Al-Banna et al. 1997; Duncan et al. 1999; De Luca et al. 2004a; Subbotin et al. 2008) argued that the D2-D3 expansion segments do not contain sufficient phylogenetic signal to resolve relationships among *Pratylenchus* nematodes at species level because of the existence of cryptic or complex species, which are morphologically indistinguishable but genetically divergent, as recently reported for members of this genus (De Luca et al. 2010). More recent studies using ITS-rDNA demonstrated the usefulness of this approach for identification and phylogenetic reconstruction within the genus *Pratylenchus* (Waeyenberge et al. 2009; Palomares-Rius et al. 2010).

The main objectives of the present work were: 1) to verify species identification of geographically distant populations of *Pratylenchus* by using the partial 18S-ITS1–5.8S-ITS2-partial 28S gene; 2) to estimate the molecular variability among geographically diverse isolates; 3) to study the phylogenetic relationships among *Pratylenchus* species by using the partial 18S-ITS1–5.8S-ITS2-partial 28S gene as inferred by different tree reconstruction approaches, including Bayesian Inference (BI), Neighbour Joining LoDet (NJ-LogDet), Maximum Parsimony (MP) and Maximum Likelihood (ML).

Material and methods

Nematode populations and morphological identification

Nematodes used in this study were obtained from different crops and geographical localities (Table 1). Nematodes were preliminarily identified by morphological features. For that, eight to fifteen nematodes of each population were killed by gentle heat, fixed in a solution of 4% formaldehyde+1% propionic acid and processed to pure glycerine using Seinhorst's method (Hooper 1986). Alternatively, temporary mounts were prepared by the water-agar technique (Esser 1986). Specimens were examined using a Leica DM 2500 compound microscope with Normarski differential interference contrast at powers up to 1,000× magnification. Measurements were done using a drawing tube attached to the light microscope.

DNA extraction, PCR amplification, cloning and sequencing

Twenty individual nematodes from each of the different geographic origins were handpicked and each one placed on a glass-slide in 3 μl of the lysis buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, 15 mM $MgCl_2$, 0.1% Triton X100, 0.01% gelatine with 90 μg/ml proteinase K) and then cut into small pieces by using a sterilized syringe needle under a dissecting microscope. The suspension was recov-

Table 1 *Pratylenchus* species and populations used in this study

Identification based on ITS and D2-D3 rDNA sequences	Host and locality	Collection codes for DNA or nematode cultures	Genbank accession number for ITS	Source of materials or Reference
P. thornei	Wheat, Cerignola, Apulia, Italy	Pt-IT	FR692303	De Luca et al., 2004
P. thornei	Lentil, Sicily, Italy	Pt-SI	FR692304	De Luca et al., 2004
P. thornei	Chickpea, Cañete de las Torres, Spain	PtCA-SP	FR692299-FR692302	De Luca et al., 2004
P. thornei	Chickpea, Jerez de la Frontera, Spain	PtJE-SP	FR692305	De Luca et al., 2004
P. mediterraneus	Egyptian clover, Sa' ad, Israel	Pm-IS	FR692306-FR 692308	D. Orion
P. mediterraneus	Wheat, Foggia, Apulia, Italy	PmFO-IT	FR692313-FR692316	N. Vovlas
P. mediterraneus	Lentil, Sicily, Italy	PmSI-IT	FR692317- FR692318	A. Troccoli
P. mediterraneus	Wheat, Montalbán, Spain	Pm-SP	FR692309- FR692312	P. Castillo
P. neglectus	Wheat, Barletta, Apulia, Italy	PnBA-IT	FR692278- FR692280, FR692282, FR692290	De Luca et al., 2004
P. neglectus	Wheat, Cerignola, Apulia, Italy	PnCE-IT	FR692283- FR692289, FR692281	De Luca et al., 2004
P. neglectus	Grasses, Villarobledo, Spain	Pn-SP	FR692291- FR692298	P. Castillo
P. vulnus	Peach, Foggia, Italy	Pv-IT	FR692319- FR692323	N. Vovlas
P. gutierrezi	Anthurium, Madeira, Portugal	Pg-PT	FR692277	M. Pestana
P. bolivianus	Potato, Chile	Pb-CH	FR692328- FR692329	A. Rios
P. pseudocoffeae	Chrysanthemum, Mahallat Town, Iran	Pp-IR	FR692276, FR691856	A. Mohammad Deimi
P. goodeyi	Banana, Madeira, Portugal	Pgo-PT	FR692324	M. Pestana
Pratylenchus sp. 1	Chrysanthemum, Mahallat Town, Iran	Psp1-IR	FR692325- FR692327	A. Mohammad Deimi

ered and transferred to a cold 0.5 ml microcentrifuge tube. Each sample was overlaid with a drop of mineral oil and incubated at 60°C for 1 h and then at 95°C for 10 min to deactivate the proteinase K. The crude DNA extracted from each individual nematode was directly amplified by using the primer pairs: 18S-Int (5′-CGTAACAAGGTAGCTGTAGG-3′) and 26S-Int (5′-TCCTCCGCTAAATGATATGC-3′). PCR conditions were the same as described in De Luca et al. (2004a). The ITS amplified fragments from two or three individual nematodes for each population were purified from agarose gel and cloned into the PCR 2.1-TOPO plasmid using the TOPO TA cloning kit (Invitrogen), following the manufacturer's recommendations. In the case of *P. goodeyi*, *P. bolivianus* and *P. gutierrezi,*[1] the sequences were obtained by direct sequencing of the amplified product.

The newly obtained sequences were deposited at NCBI (National Center of Biotechnology Information) database under accession numbers listed in Table 1.

Phylogenetic analyses

The newly obtained sequences were aligned using CLUSTALW (Thompson et al. 1994) with additional

[1] *Pratylenchus gutierrezi* is considered as junior synonym of *P. panamaensis* by Siddiqi (2000), Castillo and Vovlas (2007) and Handoo et al. (2008) but as in the database the sequences are reported as *P. gutierrezi*, we decide to refer as *P. gutierrezi* in the manuscript.

sequences of *Pratylenchus* extracted from GenBank, using default parameters. Sequence alignment was manually edited using BioEdit (Hall 1999) in order to improve the default multi-alignment. Phylogenetic relationships among sequences were established using different procedures: Bayesian Inference (BI), Neighbor-Joining (NJ) with LogDet distances, Maximum Likelihood (ML) and Maximum Parsimony (MP). Bayesian Inference was carried out using the program MrBayes 3.1 (Huelsenbeck and Ronquist 2001) using the General-Time-Reversible (GTR) substitution model with the invariant site plus gamma options (eight categories). Two parallel analyses each composed of one cold and three incrementally heated chains were run for 5,000,000 generations. Trees were sampled every 50 generations and 20,000 trees were discarded as "burn-in" (sufficient to allow convergence according to the tests indicated by the program). The remaining trees were retained to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Neighbor-Joining (NJ) procedures were applied to the distance matrix obtained using the LogDet method, implemented in the PAUP 4.0b10 package (Swofford 2003). This method allows tree reconstruction even in the case of divergent bases composition, as it is frequently found in ITS regions (De Luca et al. 2004b). Maximum Likelihood (ML) and Maximum Parsomony (MP) analysis were performed using PAUP * 40b10 (Swofford 2003). Bootstrap values assessed the degree of support for each branch on the trees and they were obtained for NJ-LogDet, ML and MP trees based on 1,000 replicates. In all cases, trees were visualised using TreeView program (Page 1996). *Nacobbus aberrans* was chosen as outgroup taxon according to the results of previous published data (De Luca et al. 2004a; Duncan et al. 1999). Additional outgroups were also used in order to study the effect of the outgroup in tree reconstruction: *Radopholus similis*, *Hirschmanniella mucronata*, *Meloidogyne incognita* and *Zygotylenchus guevarai*.

Results

ITS sequence characterization

The PCR reactions of the ITS-containing region successfully amplified a single fragment in all samples analysed in the present study. The direct sequencing of the purified ITS fragments was only performed for *P. bolivianus* from Chile, *P. gutierrezi* and *P. goodeyi* from Portugal because few specimens were available. In this case Blast search at NCBI of the ITS sequences confirmed the species identity. In all the remaining *Pratylenchus* populations, PCR fragments were amplified from DNA extracted from single nematodes, cloned and sequenced. Several clones for each population were analysed and the variability in sequence and length of the ITS fragments were determined. The main sources of variability in both ITS regions from *Pratylenchus* species are the presence of differences in length and sequence. No visible length variation was observed in the sequence neither within single nematodes nor among *Pratylenchus* populations belonging to the same species. The only exception was one clone of *P. neglectus* from Italy that resulted 62 nt shorter than the other clones sequenced from the same individual nematode. The length of the ITS amplified fragments of the studied species varied from 693 to 1,170 bp (as determined by sequencing). In particular the ITS1 ranged from 260 to 467 bp, whereas the ITS2 from 162 to 299 bp, resulting in the shortest ITSs reported for plant-parasitic nematodes to date. Very few microsatellites were detected and they were only present in the longest ITS regions such as *P. pseudocoffeae* and *P. coffeae*, the remaining *Pratylenchus* species contained only stretches of (A)n, (T)n, (G)n and (C)n that are considered to represent microsatellites. Therefore, the observed length variation was mainly due to insertion/deletion events rather than to changes in the number of repeats in microsatellites.

The sequence analysis revealed high sequence variability not only between populations or isolates but also within individuals. Sequences obtained from the same individual nematode showed high degree of variability, as well as comparisons among individuals of the same population did. The nucleotide dissimilarities for each species varied up to 7% for *P. vulnus* and *P. neglectus*, up to 6% for *P. thornei*, up to 5% for *P. lentis* and *Pratylenchus* sp.1 from Iran, up to 1% for *P. bolivianus*, *P. mediterraneus*, and *P. pseudocoffeae*. At species level, such a high variability has not been noticed in other plant-parasitic nematodes. Pairwise comparisons of the ITS sequences of *Pratylenchus* species used in this study with those of *Pratylenchus* spp. from the GenBank database dis-

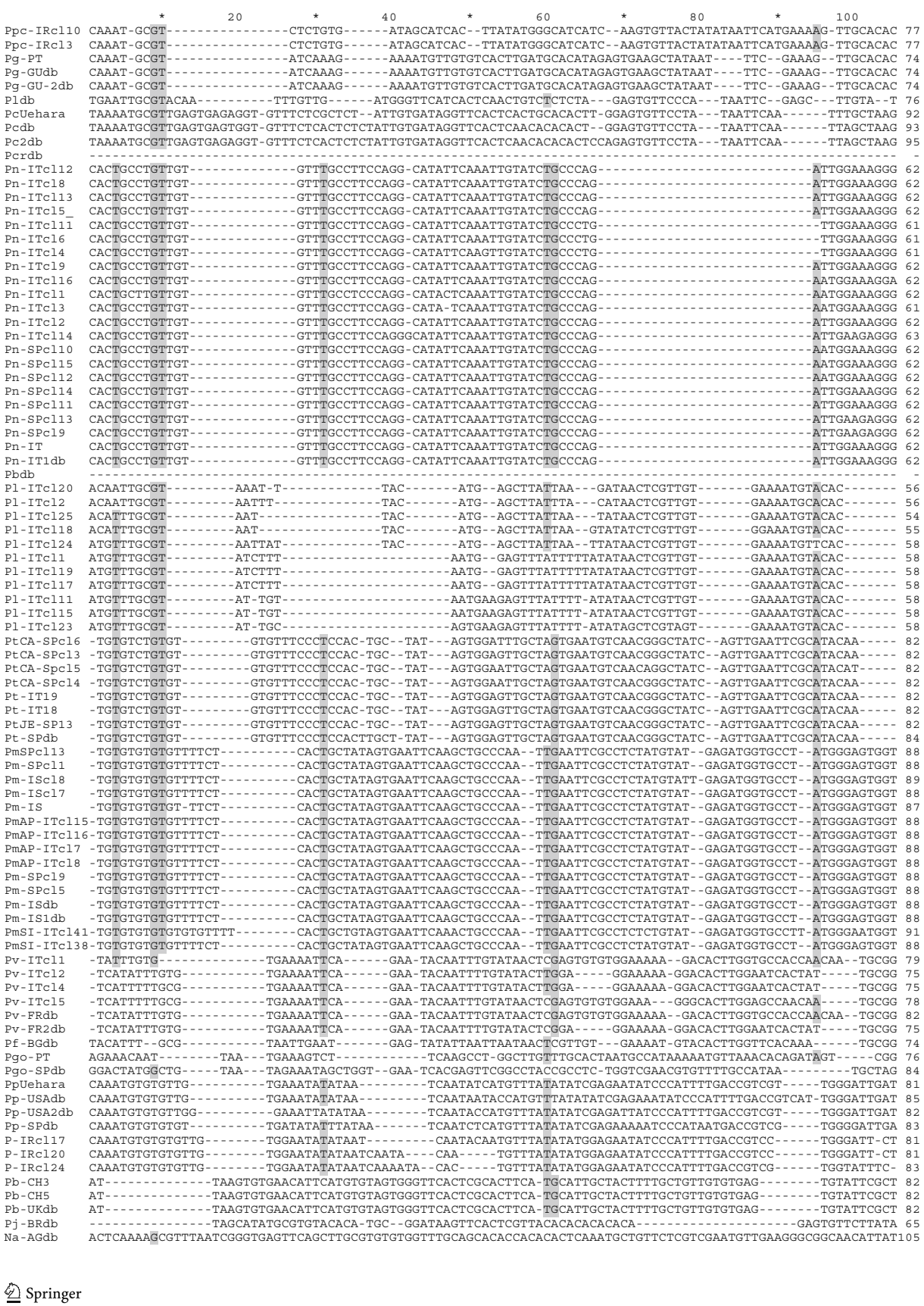
played a higher nucleotide dissimilarity (about 30%) and considerable variation in length compared to other plant-parasitic nematodes.

The ITS sequences of *Pratylenchus* species determined in our laboratory were aligned along with some ITS sequences present in the database. The ITS alignment included 88 sequences and was 1,437 bp in length. The alignment (available on request to authors) showed that the 18S (partial), 5.8S and 28S (partial) regions were less variable among taxa than the ITS1 and ITS2 regions, that were both highly variable. Several regions of the optimised ITS alignment contained multiple insertion and deletion events (indels) among taxa of varying size (1–200 bp) and the longest indels were localized in the ITS1 (Fig. 1). Furthermore, the multiple alignment constructed revealed several conserved sequence motifs characteristic for each *Pratylenchus* species.

Phylogenetic analysis

Phylogenetic relationships within and between *Pratylenchus* species were carried out on ITS sequences by means of Bayesian Inference (BI), Neighbor-Joining (NJ) with LogDet distances, Maximum Likelihood (ML) and Maximum Parsimony (MP) using *Nacobbus aberrans* and *Radopholus similis* as outgroups. Topologies of BI and ML trees were identical and congruent with that of NJ-LogDet and MP, except for positions of some weakly supported clades. Clades have been designated as monophyletic groups of species and we have tried to keep them as low in number as we could in order to make easier the comparisons between the different methods. The phylogenetic trees as inferred from BI/ML and NJ-LogDet/MP are given in Figs. 2 and 3, respectively. BI/ML trees (Fig. 2), by using different outgroups, supported both twelve clades within *Pratylenchus*, grouping the same species. Clade I included four taxa as follows: *P. pseudocoffeae*, *P. gutierrezi*, *P. loosi* and *P. coffeae*. Clade II consisted of only one taxon, *P. crenatus*. Clade III included 22 sequences from three populations of *P. neglectus* and one sequence of *P. brachyurus*. All sequences of *P. neglectus*, despite the high sequence variability, clustered together forming one highly supported subclade within clade III. Clade IV contained eight sequences from three populations of *P. thornei* and fifteen sequences from four populations of *P. mediterraneus* confirming that *P. thornei* and *P. mediterraneus* are closely related, but clearly separated, species forming each one a monophyletic group. Clade V and VI consisted each of one single taxon, *P. lentis* and *P. fallax*, respectively. Clade VII contained only one taxon *P. goodeyi* from Madeira that is different at molecular level to *P. goodeyi* sequence present in the database. Clade VIII contained all sequences of *P. vulnus* from the present study and from the database. Clade IX contained only one taxon, *P. jaehni*. Clade X consisted of *P. bolivianus* from Chile and that from England present in the database. Clade XI included sequences of an unidentified species (*Pratylenchus* sp. 1) from Iran, in addition to the closely related species *P. penetrans*. This clustering was supported by a strong PP or bootstrap value. Clade XII contained only one taxon, *P. goodeyi* from the database, located at the basal position of the tree. Most clades were supported by high PP or bootstrap values with both BI and ML giving strong support to these associations. As demonstrated by the comparison between the trees obtained using *N. aberrans* and *R. similis* as outgroups, no significant difference is observed in the tree topology and only minor differences in the support of certain branches are detected (Figs. 2a and b). Similar results were also obtained when *M. incognita* was used as outgroup. However, the use of *H. mucronata* or *Z. guevarai* produced trees with longer branches and slightly different topology at the base of the tree (clades IX to XII) and lower PP or bootstrap value (data not shown).

NJ-LogDet/MP analysis resolved the same major clades of *Pratylenchus* as obtained by using BI/ML analysis (Fig. 3) albeit with some minor changes that in many cases are not supported by high values of bootstrap, meaning that these differences may not be significant. Clade I+II included the species of clades I and II obtained by BI/ML but showing low support to the clustering of *P. crenatus* as a sister clade of *P. coffeae*/*P. loosi*. Clades III, IV and V have the same composition as obtained by BI/ML. Clades VI, VII and VIII are close together but *P. goodeyi* from Madeira (clade VII) is branching off before the other two and a clustering of *P. vulnus* and *P. fallax* (clades VI and VIII) is observed, which is slightly different to the results obtained by BI/ML. Again, clades IX, X



◀ **Fig. 1** Portion of the ITS2 multi-alignment of different DNA sequences of several species and populations of *Pratylenchus* obtained in this study or from the Genbank database. In the aligned sequences a dash indicates a gap or unknown sequence. Blocks of species-specific DNA sequences for each species are visible by eye inspection

and XI were in agreement with BI/ML topologies. Clade XII, represented by *P. goodeyi* is not in basal position anymore; however its support by bootstrap values is very weak.

In Fig. 4 are reported the phylogenetic trees containing all *Pratylenchus* sequences determined in the present study using BI and ML. These trees clearly showed that *Pratylenchus* sequences for each species and from different populations are characterized by high intraspecific variability and clustered all together. Figure 4a reported the phylogenetic relationships among *P. lentis*, *P. vulnus*, *P. bolivianus*, *P. jaehni*, *P. goodeyi*, *P. penetrans* and *Pratylenchus* sp. 1; Fig. 4b reported the phylogenetic tree describing the evolutionary relationships among *P. pseudocoffeae*, *P. gutierrezi*, *P. loosi*, *P. coffeae*, *P. crenatus* and *P. neglectus*; Fig. 4c reported the phylogenetic tree describing the evolutionary relationships among different populations of *P. thornei* and *P. mediterraneus*.

Discussion

Identification of *Pratylenchus* species is not an easy task because of the conserved morphology of members of this genus and their high intra- and interspecific variability. Intraspecific variability of the *Pratylenchus* genome and the existence of cryptic or species complexes have been demonstrated in *P. coffeae* (Duncan et al. 1999), in *P. lentis* (Troccoli et al. 2008) and, more recently, in *P. hippeastri* (De Luca et al. 2010). 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; De Luca et al. 2004a; Subbotin et al. 2008; Holterman et al. 2009; Palomares-Rius et al. 2010; De Luca et al. 2010). These studies have clarified the taxonomical status of a large number of root-lesion nematodes, although many species have yet to be characterised.

DNA sequence and phylogenetic analyses of nematode samples provide additional criteria for identifying and delimiting species within *Pratylenchus*. In particular, sequence analyses of the ITS containing region has allowed the assessment of heterogeneity among species, even for those that are closely related, as it evolved faster than the D2-D3 expansion segments of 28S rDNA and accumulated more substitution changes. The ITS loci are also particularly suited to the development of diagnostic PCR tools because they are repetitive and undergo sequence homogenisation, factors linked to the efficiency, sensitivity and specificity of amplification (Gasser et al. 2008; Waeyenberge et al. 2009).

Our study revealed high heterogeneity in the ITS sequences, within and among populations of *Pratylenchus* species studied. At the species level, such intra-individual variability (1–7%) has not been observed in other plant-parasitic nematodes. The main causes of such high variability in *Pratylenchus* are the significant length and sequence differences in both ITS1 and ITS2 which resulted in the most variable sequence and the shortest ITSs ever recorded in plant-parasitic nematodes.

The highest nucleotide variability observed among *Pratylenchus* species suggests that the ITS sequences should be useful for phylogenetic reconstruction particularly among closely related taxa. Because of the high degree of ITS sequence dissimilarity among *Pratylenchus* species, the alignment of these taxa with confidence was not always feasible.

The phylogenetic analyses with BI, ML, NJ-LogDet and MP methods yielded congruent phylogenetic trees. Notably, one highly supported root-lesion nematode subgroup was evident in both analyses, consisting of the two sister species *P. thornei* and *P. mediterraneus* (Fig. 4c) which display some similar morphological and morphometrical features (*i. e.* three lip annuli, relatively high lip region shape, truncate tail outline) but different reproductive behaviour. This clustering is largely consistent with data obtained by Palomares-Rius et al. (2010) using the ITS sequences. The same grouping was observed by De Luca et al. (2004a), Subbotin et al. (2008) and Holterman et al. (2009) using the D2-D3 and 18S ribosomal genes, also revealing that these species are closely related and share similar molecular traits. In addition the MP/NJ-LogDet tree confirmed the close relationships of *P. lentis* to *P. thornei* and *P. mediterraneus* sharing the

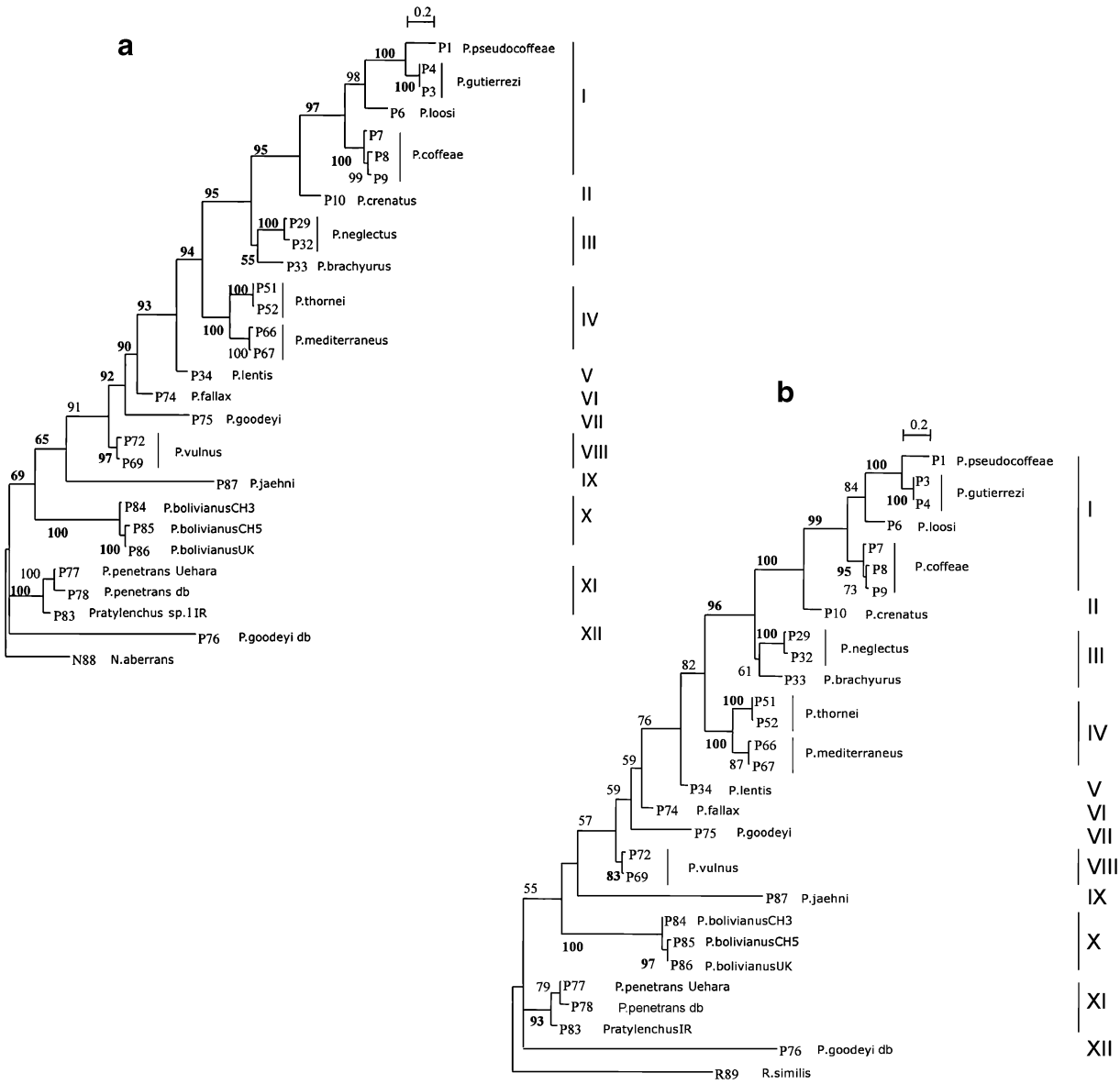


Fig. 2 Phylogenetic tree describing the evolutionary relationships among different species of *Pratylenchus* using Bayesian Inference (BI) and Maximum Likelihood (ML). Branch lengths are proportional to the distances as derived from the distance matrix obtained using the GTR method with the invariant site plus gamma options from Bayesian inference (BI). Same tree topology was also obtained with Maximum Likelihood analysis (ML). Bayesian posterior probabilities (PP) are shown on each branching point and wherever bootstrap values from ML are less than 5% different to PP, they are shown in bold. *Nacobbus aberrans* (**a**) or *Radopholus similis* (**b**) have been used as outgroups. Only bootstrap values higher than 50 are shown

same morphological features as the number of annuli (n=3). It is noteworthy that these three species also share the same geographical area and the same hosts (cereal and legumes in the Mediterranean Basin), suggesting that these species could be derived by recent speciation events with insufficient time to attain complete morphological differentiation.

Another important aspect highlighted in our results is that the choice of the outgroup can influence the resolution of the tree. Out of the five outgroups used, three of them gave similar results, *i. e*. *N. aberrans*, *R. similis* and *M. incognita*, while *H. mucronata* or *Z. guevarai* produced less resolved trees due to the presence of longer branches that

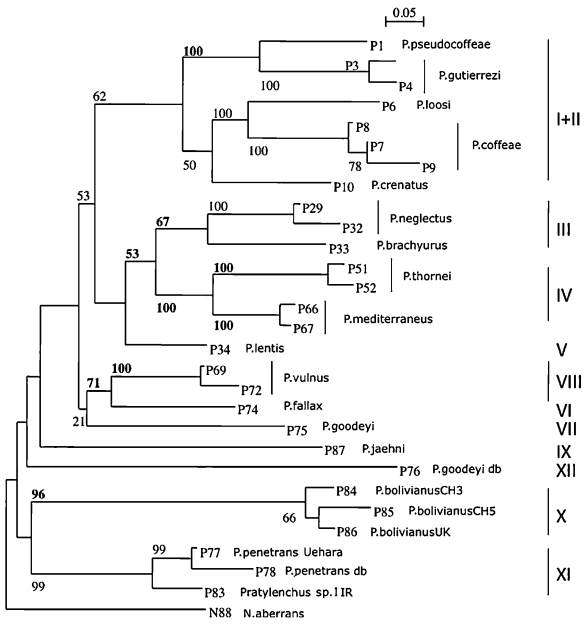


Fig. 3 Phylogenetic tree describing the evolutionary relationships among different species of *Pratylenchus* using Neighbor-Joining (NJ) on LogDet distance matrix and maximum parsimony (MP). Branch lengths are proportional to the distances as derived from the distance matrix obtained using the LogDet method. Bootstrap values for NJ-LogDet are shown on each branching point and wherever bootstrap values from MP are less than 5% different to NJ-LogDet, they are shown in bold. Only bootstrap values higher than 50 are shown

favoured long-branch attraction. As a result of this phenomenon, long branches that are dispersed in the tree, *e. g.* clades IX and XII (Fig. 2) are clustered together at the base of the tree. This has already been reported in many tree reconstructions in literature and when not detected leads to wrong tree reconstructions.

Of particular concern is the group, obtained by both analyses, of *P. coffeae*, *P. loosi*, *P. pseudocoffeae*, and *P. gutierrezi* (Fig. 4b) which resulted in closely related species, as first defined by Duncan et al. (1999) and recently by Subbotin et al. (2008) by using the D2-D3 domains. These species also overlap in several morphological features such as similar morphology, the presence of males and the same number of lip annuli (n=2).

Different sequences obtained from the different cloned fragments and populations of *P. neglectus* clustered all together (Fig. 4b) suggesting a very high level of intra-individual variability not due to the existence of a species complex. Furthermore, the presence of different ITS sequences within an individual nematode and the finding of a 62 nt shorter ITS region confirmed the presence of different ribosomal cistrons not yet completely homogenized or the presence of pseudogenes in the genome of *P. neglectus*. These results were also found when the same populations of *P. neglectus* were characterized by using the D3 expansion domains (De Luca et al. 2004a) and the ITS regions (Palomares-Rius et al. 2010). Sequences of *P. vulnus* obtained in this study formed a well supported clade together with those of *P. vulnus* deposited in the NCBI database. All sequences of *P. bolivianus* also formed a well supported clade. The clustering of the sequences of *Pratylenchus* sp. 1 from Iran with those of different *P. penetrans* populations, supported by a strong bootstrap value using BI analysis, suggested that these populations are closely related (Fig. 4a). It has been already reported that *P. penetrans* is characterized by very high genome variability suggesting that *Pratylenchus* sp. 1 may represent either a polymorphic variant of *P. penetrans* or may belong to a species complex.

Pratylenchus fallax grouped always with *P. penetrans* by using the D2-D3 region or the18S rDNA gene as molecular markers. These two species share several morphological similarities, leading Tarte and Mai (1976) to speculate that *P. fallax* could be a morphological variant of *P. penetrans*. In the present work, by using the ITS region, *P. fallax* resulted closely related to *P. lentis* confirming that *P. fallax* and *P. penetrans* are two different species, as strengthened by previous studies (Perry et al. 1980; Ibrahim et al. 1994; Waeyenberge et al. 2000; Handoo et al. 2001).

Pratylenchus goodeyi from Madeira (Portugal), isolated from a banana tree, displayed different ITS sequence compared with those of *P. goodeyi* present in the database and this difference deserves comment. Since morphological and morphometrical features of the present studied population from Madeira fits very well with previous data (Castillo and Vovlas 2007), it is conceivable that a misannotation in the database or a wrong identification of that population may be responsible for an incorrect sequence assignation. Furthermore, in both phylogenetic trees the sequences identified as *P. goodeyi* always grouped in different clades suggesting that they represent two different species and taxonomic identification of these populations is required.

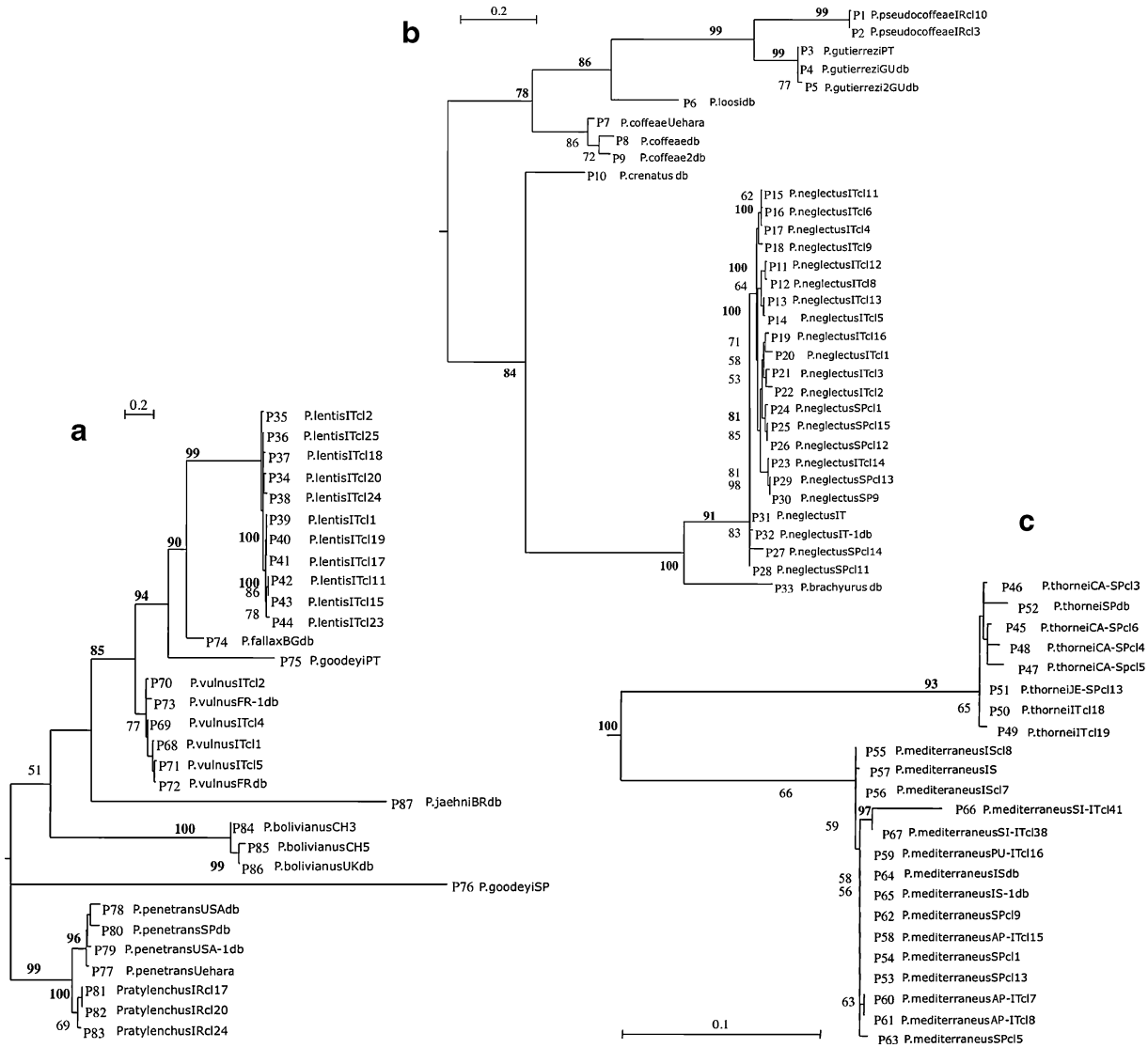


Fig. 4 Phylogenetic trees describing the evolutionary relationships among different species and populations of *Pratylenchus* using Bayesian Inference (BI) and Maximum Likelihood (ML). Branch lengths are proportional to the distances as derived from the distance matrix obtained using the GTR method with the invariant site plus gamma options from Bayesian Inference (BI). Same tree topology was also obtained with Maximum Likelihood analysis (ML). Bayesian posterior probabilities (PP) are shown on each branching point and wherever bootstrap values from ML are less than 5% different to PP, they are shown in bold. Only PP/bootstrap values higher than 50 are shown

In conclusion, the ITS sequences allowed clear separation of *Pratylenchus* species in spite of the high intraspecific variability. The alignment revealed small species-specific DNA sequences suitable for the construction of potentially useful species-specific primers or for a more promising approach for DNA barcoding of root-lesion nematodes. The phylogenetic analyses by using ITS sequences confirmed that *Pratylenchus* species are paraphyletic as previously reported (Palomares-Rius et al. 2010) and *P. penetrans* could represent a cryptic species.

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