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Comparison of the sequences of the D3 expansion of the 26S ribosomal genes reveals different degrees of heterogeneity in different populations and species of *Pratylenchus* from the Mediterranean region

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Abstract

Plant parasitic nematodes belonging to the genus *Pratylenchus,* also known as root lesion nematodes, cause serious economic damage to different crop plants. In order to explore genetic structures in different isolates, we investigated several *P. thornei, P. neglectus* and *P. penetrans* populations of different geographic origins. The analysis at the species level was also extended to *P. penetrans*, *P. pinguicaudatus*, *P. vulnus* and *P. mediterraneus*. Sequence analysis of a specific portion of DNA was carried out. In particular, the sequences of the D3 region of the 26S gene were obtained and compared with similar sequences available in databases. The results support the hypothesis that *P. penetrans* may represent a species complex, while in *P. neglectus* the intra-species heterogeneity observed is due to intra-individual variability. Furthermore, the specific conservation of some nucleotides in different *P. thornei* populations indicates their fixation in the rDNA repeats in this species. The presence of these nucleotides, the molecular signature of *P. thornei*, may assist in determining the nature of nematode infections.

Introduction

Root lesion nematodes (*Pratylenchus* spp.) are common migratory endoparasites of plants worldwide. They invade the root cortical parenchyma, producing large necrotic areas and causing severe yield losses. The identification of species of *Pratylenchus* is made difficult by their overlapping morphometric characters and morphological diversity (Corbett and Clark, 1983). Furthermore, the taxonomy of the genus *Pratylenchus* is an open question (Luc, 1987; Ouri and Mizikubo, 1999). Also, the phylogenetic position based on molecular characters indicates that the genus *Pratylenchus* is paraphyletic, suggesting that lineage sorting is incomplete (Al-Banna et al., 1997; Duncan et al., 1999). The existence of cryptic nematode species that are morphologically indistinguishable yet genetically divergent suggesting the existence of species complexes has recently been reported in other nematode genera (Courtright et al., 2000). Therefore, further molecular studies are needed to revise the species that currently are included in the genus *Pratylenchus*.

The utilization of molecular data to detect differences between species at the DNA level has focused on the differences between the ribosomal genes (Hillis and Dixon, 1991; Petersen et al., 1997; Powers et al., 1997; Uehara et al., 1998). The use of rDNA has many advantages. It occurs in multicopy arrays, making its use easy even on single individuals. In addition, known polymerase

chain reaction primer sequences can select suitable gene portions, characterized by a slow or high evolutionary rate. In most animals, the rDNA genes show a pattern of concerted evolution. Thus, changes accumulating during the evolution of a repeat unit are either eliminated or incorporated into all of the repeats, so that a single predominating sequence is present within a species (De Giorgi et al., 2002). However, substantial differences among individuals have also been reported in the sequences of the multigene ribosomal families (Seperack et al., 1988; Martins et al., 2002).

In eukaryotes, the coordinate expression of different genes allows the formation of the complex ribosome machinery (Nygard and Nilsson, 1987; Vahidi et al., 1991). However, the ribosomal coding genes 18S, 5.8 and 26S or 28S, with their flanking regions, are the genes most used for phylogenetic and diagnostic studies.

The ribosomal coding genes 18S, 5.8 and 26S or 28S, separated by internal transcribed spacers (ITSs), are organized in tandem arrays, bordered by intergenic regions (IGS). The coding regions are usually conserved even between distantly related species, while ITS spacers show considerable variability between different animal genomes. The level of ITS sequence variation observed among individuals of the same species is about the same as that observed among ITS repeats within individuals, typically $\leq 1\%$ (Heise et al., 1999; Nadler et al., 2000). In *Pratylenchus* spp., ITS regions are one of the most variable nuclear loci so far identified. (Uehara et al., 1998; Waeyenberge et al., 2002). Ribosomal transcribed spacers ITS1 and ITS2 are the markers most commonly used to differentiate between different nematode species (Zijlstra et al., 1995; Petersen et al., 1997; Powers et al., 1997). On the other hand, the small ribosomal subunit (18S gene) is the sequence most widely used for molecular phylogenetic analysis, especially for evaluating deep level relationships among organisms, including nematodes (Fitch et al., 1995; Floyd et al., 2002). A key study using this gene from different taxa sampled across the entire phylum Nematoda has provided the most comprehensive molecular phylogeny of nematodes thus far (Blaxter et al., 1998). The large ribosomal subunit in nematodes is generally referred to as 26S (Ellis et al., 1986; De Giorgi et al., 2002). Most of the variations in length in these genes are accounted for by D expansion segments (Clark et al., 1984), which are believed to be redundant structures tolerated during evolution because they do not interfere with ribosome functions (Hancock and Dover, 1988). In particular, the D3 region is used to investigate sequence diversity among samples of different nematode species of different geographic origins (Al-Banna et al., 1997; Duncan et al., 1999; Nadler et al., 2000; Carta et al., 2001; Handoo et al., 2001).

Although the low level of similarity detected in *Pratylenchus* spp. of different geographically isolated samples suggested that the *Pratylenchus* genome is highly variable (Duncan et al., 1999; Waeyenberge et al., 2002), the extent of variability within and between individuals in the different species has not received much attention. Such information will be of increasing relevance, considering the expected rapid growth of sequence data from studies aimed at population migration studies and at pathotype identification (Carta et al., 2002; Waeyenberge et al., 2002).

This study was undertaken to understand the variability in different species of *Pratylenchus*. To this aim different populations of the same species were investigated. The populations used came from different countries of the Mediterranean basin specifically affecting Mediterranean crops. The sequencing of the D3 regions of individual nematodes characterized the populations at the molecular level. Sequence comparison was also made with other *Pratylenchus* sequences collected from other locations, whenever the information was available in the databases.

Materials and methods

Nematodes

The following nematodes were used for the study: six populations of *P. thornei* ; three *P. neglectus*; four and one each of *P. mediterraneus P. pinguicaudatus* and *P. vulnus* (Table 1). These nematodes were identified using classical morphological methods and reared and maintained on sterile carrot disks in a growth chamber (Di Vito et al., 2002). The nematodes were extracted from the carrot disks (Young, 1954), and experiments were carried out using single individuals. Between five and eight different individuals were analyzed from each of the different geographic origins (Table 1).

Table 1. Pratylenchus species and isolates used in this study

Species	Location	Number of individuals	Code
P. thornei	Cerignola, Italy	6	P.thoIT
P. thornei	Rommani, Morocco	7	P.thoMO
P. thornei	Beja, Tunisia	6	P.thoTU
P. thornei	Cañete, Spain	8	P.thoCA-SP
P. thornei	Jerez, Spain	7	P.thoJE-SP
P. vulnus	Castellaneta, Italy	5	P.vulIT
P. pinguicaudatus	Beja, Tunisia	6	P.pinTU
P. neglectus	Monopoli, Italy	pool	P.negPIT
P. neglectus	Barletta, Italy	7	P.neg40BIT; P.neg37BIT
P. neglectus	Cerignola, Italy	8	P.neg24TIT; P.neg45TIT; P.neg46TIT; P.neg47TIT;
P. penetrans	Gravina, Italy	5	P.penIT
P. mediterraneus	Israel	7(♀), 5(♂)	P.med1IS(♀); P.med 7IS (♂)

The different *Pratylenchus* species with the origin of the different populations and the number of individuals analyzed are indicated. A code has been created for each individual sequence, according to the following protocol: an upper case character indicating the name of the genus is followed by the three letters of the species and then by letters indicating the geographic origin. When the code contains digits, it indicates separate clones of the same individuals. Codes are the same as those used in the phylogenetic reconstruction.

DNA extraction, PCR amplification, sequencing and characterization of D3 sequences

The sequences were usually obtained by direct sequencing of the amplified product but, in some cases, they were obtained after cloning the PCR product. In the case of *P. thornei*, samples were collected from the localities mentioned more than once, at different times of year. From *P. mediterraneus*, an obligatorily amphimictic species, both male and female specimens were analyzed. Since the original populations were cultured on carrot disks before use, the populations will have suffered a certain narrowing of genetic diversity. Therefore, the term 'isolate' will be preferentially used hereafter in preference to the term 'population'.

A single nematode was hand-picked and 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 cut into small pieces. The suspension was recovered from the glass slide, which was washed with a further 7 µl of lysis buffer. The suspension and washings were transferred to a 0.5 ml microcentrifuge tube at 4 °C. Each 10 µl sample was overlaid with a drop of mineral oil and incubated at 60 °C for 1 h and at 95 °C for 10 min. Amplification of this crude DNA preparation was performed by diluting the sample to 100 µl such that it contained 0.2 mM of each dNTP, 20 pmols of each primer and 2.5 units of *Taq* DNA polymerase (Roche). The PCR conditions were: an initial denaturation at 94 °C for 6 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and a final step at 72 °C for 6 min.

The primers used were short oligonucleotides that specifically anneal to the 5′ end of the 26S ribosomal gene, in the D3 expansion region, corresponding to the fragment spanning nucleotide 4351 and nucleotide 4703 in *Caenorhabditis elegans* gene (Ellis et al., 1986). The forward primer was 5′-GACCCGTCTTGAAACACGGA-3′ and the reverse primer: 5′-TCGGAAGGAACCAGCTACTA-3′. The amplified fragments were isolated from gels and then sequenced. In the case of *P. neglectus*, different sequences were obtained from different individuals. Therefore, PCR products were cloned into the pCR 2.1-TOPO plasmid and TOP10 competent cells transformed using the TOPO TA cloning Kit (Invitrogen), following the manufacturer's recommendations.

The sequences obtained within single individuals were identical and confirmed by comparison with the same fragment amplified and sequenced on genomic DNA extracted as already described using pooled individuals (De Giorgi et al., 1997).

In general the sequence did not differ between individuals of the same isolate. However when ambiguity was detected in specific nucleotides, the

availability of the same sequence obtained from several different individuals allowed the construction of the consensus sequence. In the case of *P. neglectus*, the sequences obtained from more than one individuals were different between them and from the sequence obtained from pooled individuals. The sequences derived from all the samples were compared. Sequences from closely related nematodes, already available in the literature, were also included for comparison.

Nucleotide sequence data reported in this paper are available in the EMBL database under the following accession numbers: *P. neglectus*: AJ545013–AJ545028; *P. pinguicaudatus*: AJ545014; *P. thornei*: AJ545015–AJ 545019; *P. vulnus*: AJ545020; *P. mediterraneus*: AJ545021–AJ545022; *P. penetrans*: AJ575268.

Computer analysis

The GCG package of the University of Wisconsin genetic Group was used for sequence analysis (GCG, 1994). Sequences were aligned using the PILEUP program and the alignment was optimized manually using the LINEUP program. A graphic presentation highlighting the sequence conservation was obtained using the PRETTYPLOT program. Evolutionary analysis was carried out using the programs implemented in the PAUP* 4.0b10 package (Swofford, 1998). Genetic distances were obtained using the GTR method (Saccone et al., 1990). Phylogenetic trees were reconstructed by means of both Minimum Evolution (ME), and Neighbour Joining (NJ) approaches. In addition to these distance methods, Maximum Parsimony (MP) analysis was also performed using the PAUP* 4.0b10 package (Swofford, 1998). In all cases, bootstrap values were based on 1000 replicates. *Meloidogyne javanica* was used as an outgroup.

Results

Attention was focused on the D3 expansion region of the 26S rDNA. The similarities of the different sequences were analyzed. A direct comparison was made possible by the analysis of the multi-alignment in which the sequences of other Pratylenchidae present in the database were also included. The multi-alignment, not presented here, is available on request.

The comparison of the aligned sequences demonstrates that sequence identity is detected only in a few instances and that the degree of micro-heterogeneity is different in the different species.

The genetic distances between sequences were measured and the phylogenetic relationships inferred. The phylogenetic analyses with maximum parsimony, minimum evolution and neighbour joining methods yielded a very similar topologies for the phylogenetic relationships of *Pratylenchus* spp. Therefore, only one phylogenetic tree is presented (Figure 1). Using *M. javanica* as an outgroup, the clustering of *P. penetrans* sequences with those of *P. pinguicaudatus* was supported by a strong bootstrap value, suggesting that *P. pinguicaudatus* is more closely related to *P. penetrans* than to the other species. However, it is interesting to note that the sequence of our *P. penetrans* isolate was different from the sequence available in the database for this species. Furthermore the sequences of *P. arlingtoni*, *P. convallariae* and *P. fallax* are included in this cluster and are closely related to each other. The clustering of these species with *P. penetrans* and with *P. pinguicaudatus,* is also supported by high bootstrap value.

It can be seen from Figure 1 that a cluster supported by a very strong bootstrap values is formed by *P. mediterraneus* isolates. The sequences from *P. mediterraneus*, males and females, did not show any differences, thus indicating that in this species the D3 rDNA is completely homogeneous. From Figure 1 it is also clear that all *P. thornei* clustered in a monophyletic group, closely related to *P. mediterraneus*. On the other hand very high sequence variability was detected within *P. neglectus*, where differences were found not only between isolates but also within individuals. Indeed, the amplification products obtained were clearly heterogeneous, so that cloning of individual molecules was required (Table 1).

The situation seems to be different in the case of *P. thornei*, because when DNA was extracted from pooled individuals of *P. thornei* grown on a single Petri dish, the sequence of DNA obtained was very similar to sequences in the databases. Furthermore, when the sequences of different isolates were compared, divergence of about 1% was observed, the level of diversity which is usually detected in the ITS repeats within individuals and much

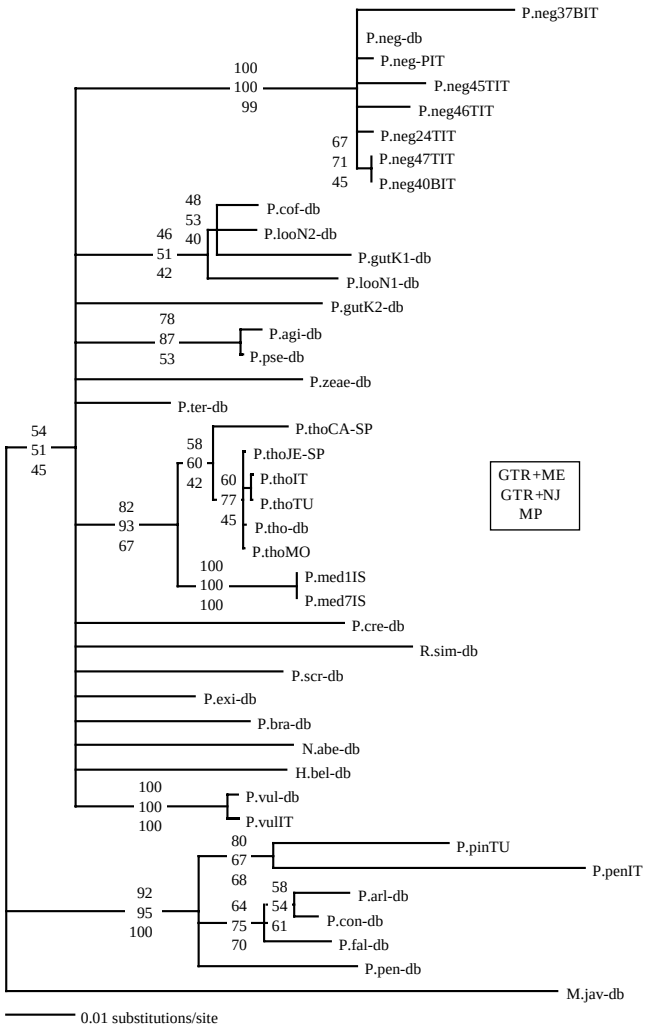

Figure 1. Phylogenetic tree describing the evolutionary relationships of *Pratylenchus* species and isolates. Branch lengths proportional to the distances are derived from the distance matrix GTR, used as described in Materials and methods. Bootstrap values shown on the nodes refer to the different methods of phylogenetic reconstruction used. The codes of the sequences obtained in this study are reported in Table 1. The sequences already available in the databases are as follows: P.pen-db, *P. penetrans*, Accession number U47546; P.vul-db, *P. vulnus,* U47547; H.bel-db, *Hirschmanniella belli,* U47556; N.abe-db, *Nacobbus aberrans*, U47557; P.tho-db, *P. thornei,* U47550; P.bra-db, *P. brachyurus,* U47553; P.exi-db, *P. hexincisus,* U47554; P.scr-db, *P. scribneri,* U47551; R.sim-db, *Radopholus similis,* U47558; P.neg-db, *P. neglectus,* U47548; P.cre-db, *P. crenatus,* U47549; M.jav-db, *M. javanica,* U47559; P.arl-db, *P. arlingtoni*, AF307328; P.zeae-db, *P. zeae*, AF303950; P.fal.-db, *P. fallax*, AF264181; P.pse-db, *P. pseudocoffeae*, AF170444; P.cof-db, *P. coffeae*, AF170443; P.gutK1-db, *P. gutierrezi* K1, AF 170440; P.gutK2-db, *P. gutierrezi* K2, AF170441; P.looN1-db, *P. loosi* N1, AF170437; P.looN2-db, *P. loosi* N2, AF170438; P.ter-db, *P. teres* vandenbergae, AF303951; P.agi-db, *P. agilis*, AF196352; P. con-db, *P. convallariae*, AF196351; *C. elegans*, X03680.

shorter branches were obtained in the tree (Figure 1), indicating very short genetic distances. A nucleotide variation site was found in the position corresponding to nucleotide 4451 in the *C. elegans* rRNA gene. In this position, the sequence from the database has a T, while in the *P. thornei* isolates [representatives of the same species from Beja (Tunisia), Cañete (Spain), Rommani (Morocco) and Cerignola (Italy)] repeatedly sequenced on both DNA strands consistently revealed the presence of a C. More interestingly, in sequences from a population from Jerez (Spain), both nu-

cleotides (C and T) were present in two sequences cloned from the same individual, but the sequences were otherwise identical.

A portion of the multi-alignment in which boxes of identical sequences are highlighted is shown in Figure 2. It can be seen that there are several positions in the sequences in which the persistence of the same nucleotide seems a peculiarity of *P. thornei*. Such conserved positions, which correspond to the *C. elegans* nucleotides n. 4370, 4385, 4404, 4442, 4444, 4463, 4467, 4469 and 4510, are a distinctive feature of *P. thornei* not shared with most other species but shared occasionally with *P. mediterraneus*, emphasizing a similarity at the molecular level between these two nematode species.

Discussion

Intra-specific variability of the *Pratylenchus* genome, has been demonstrated in *P. coffeae* by others (Duncan et al., 1999; Waeyenberge et al., 2002) and evidence has been produced that several species closely related to *P. coffeae* are species complexes (Duncan et al., 1999). The concept of species complex (syngameon), indicate a large number of species linked by frequent and occasional hybridization in nature (Grant, 1957). The recent availability of sequence data has allowed in other systems the identification of cryptic species which are morphological identical, yet genetically distinct (van Oppen et al., 2001). Although more data are required to promote the concept of species complexes, nevertheless the sequence divergence can be used for studying species heterogeneity. Sequence analyses of several isolates allowed us to assess the level of sequence heterogeneity in several *Pratylenchus* species. In the phylogenetic tree of Figure 1, sequences of other species within the Pratylenchidae, extracted from the database, were included in order to understand the evolutionary relationships in this family. However, from the data presented, it can be postulated that the origin of these different genera and species was consistent with a bush-like radiation phenomenon as no clear phylogenetic relationships was revealed between them, in spite of the variability shown among the different species.

The fact that the two sequences of *P. penetrans* display nucleotide differences deserves comment. It is always possible that a trivial error such as a misannotation in the database or a wrong identification of nematodes is responsible for an incorrect sequence. However, assuming that such an

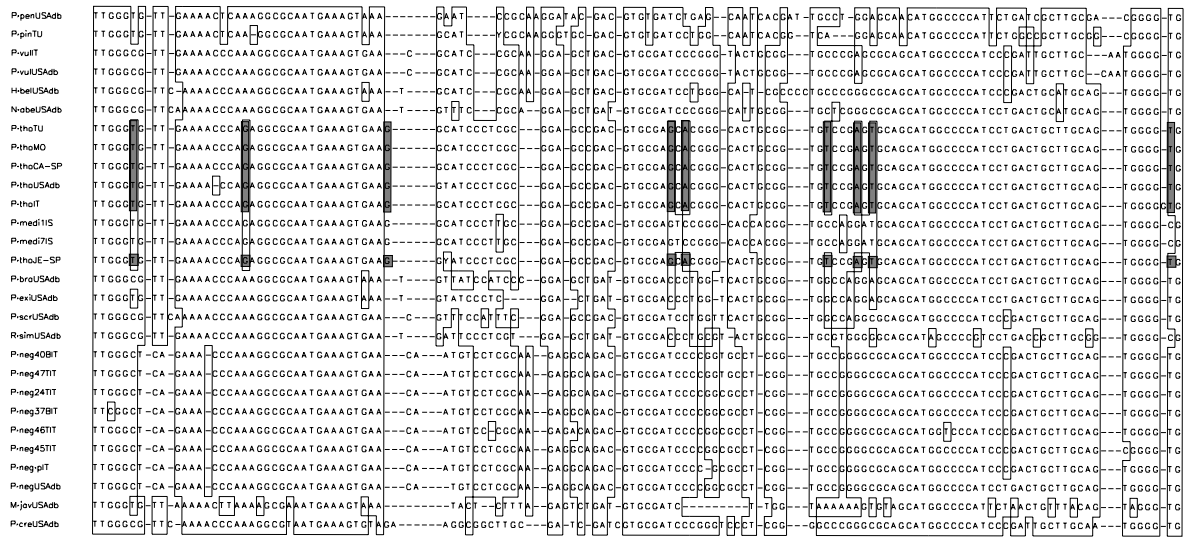

Figure 2. Prettyplot on a portion of the multi-alignment of different populations and species of phytoparasitic nematodes. The multi-alignment represent the nucleotide sequences corresponding to the nucleotide positions n 4365 to 4511 in the *C. elegans* gene. The boxes represent identical nucleotides in the multi-alignment, while the conserved positions found exclusively in *Pratylenchus thornei* are shaded.

error is not occurring, and considering that our sequences are derived from many separate experiments, the differences observed may reflect the dynamics of homogenization in the rDNA repeat families. It has been reported that, within the same genome, different classes of tandem repeats can be detected, resulting in sequence similarity within the same array, but extensive variation in different loci (Martins et al., 2002). Therefore, it is conceivable that we have amplified the same locus while the sequences present in the database represent a different class of the repeat. However, it is also possible that *P. penetrans* is characterized by very high genome variability suggesting that it may represent a species complex.

The sequences of *P. pinguicaudatus* were obtained from different individuals of the same population and they share the same D3 sequence, thus suggesting the absence of not only intra-individual, but also inter-individual variability. Although the possibility of misidentification does exist, the characteristic tail tip morphology, almost cylindrical to broadly round, definitively proves the correct identification. However, the absence of different geographical isolates and of additional sequences in the database, does not allow any conclusion on the homogeneity of these sequences.

The clustering of *P. mediterraneus* with the *P. thornei* sequences, with differences detected in some crucial nucleotide positions (see below), suggests a very close phylogenetic relationship. However, the fact that all *P. thornei* isolates clustered in a monophyletic group supported by high bootstrap values, and the long branch leading to *P. mediterraneus*, strongly indicate that *P. mediterraneus* should be considered different species.

The different sequences obtained from the different cloned sequences of *P. neglectus* did not cluster according to geographical origin, suggesting a very high level of intra-individual sequence variation. The relatively long branches observed in the tree reported in Figure 1, also reflect this high level of intra-individual variability. Therefore, the data suggest that the variability observed in this species is not due to the existence of a species complexes, but instead to intra-individual variability. This observation is interesting as high intra-individual variability in the sequences of the ITS regions in specimens of the root-knot nematode *Meloidogyne* have been reported (Hugall et al., 1999). In this case, the presence of many different ITS sequences within a single individual was revealed by cloning the DNA from a single egg-mass culture, thus amplifying any possible ribosomal target irrespective of abundance and functional character. Interestingly, it has recently been demonstrated that the extreme diversity of nuclear ribosomal DNA in the hard coral *Acropora* is largely due to the sequencing of pseudogenes (Marquez et al., 2003).

Sequence analyses presented in this paper indicate a very low level of sequence diversity in *P. thornei* isolates, strongly suggesting extensive genetic homogenization. These findings provide evidence to support the proposal that this species does not represent a syngameon or species complex, although analyses of independent data set (i.e. for other genes) are required to provide additional evidence. Finally, the results obtained allowed us to reveal the conservation of nucleotides between different isolates of *P. thornei*. The presence of these conserved nucleotides can be considered as the molecular signature of *P. thornei* and used as an additional tool for the identification of this species.

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