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Six First Reports of Pin Nematodes from Portugal, with an Update of the Systematics, Genetic Diversity, and Phylogeny of the Genus *Paratylenchus* (Nematoda: Tylenchulidae)

Teresa Rosmaninho [1], Manuel Mota [1], Maria L. Inácio [2], Jonathan D. Eisenback [3] and Carlos Gutiérrez-Gutiérrez [1,*]

[1] NemaLab/MED, Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7002-554 Evora, Portugal; tcr@uevora.pt (T.R.); mmota@uevora.pt (M.M.)
[2] Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Quinta do Marquês, 2780-159 Oeiras, Portugal; lurdes.inacio@iniav.pt
[3] School of Plant and Environmental Science, Virginia Tech, Blacksburg, VA 24060, USA; jon@vt.edu
* Correspondence: carlosg@uevora.pt

Abstract: Pin nematodes (*Paratylenchus* spp.) currently comprise 132 species of polyphagous plant ectoparasites with at least seven species that are plant pathogenic emphasizing the need for correct identification to establish an appropriate management strategy. Sequences of highly conserved regions of ribosomal and mitochondrial RNA genes are a powerful species-level diagnostic tool within Tylenchulidae. A nematological survey was conducted from 2019 to 2021 in commercial vineyards distributed in four major wine-producing regions in the Central and South Portugal. Nine populations of *Paratylenchus* spp. were isolated from the rhizosphere of grapevines that were characterized from morphological data and molecular phylogenetic analysis using two rRNA genes (D2–D3 expansion segments of the 28S, and partial 18S) and a region partial of the COI mtRNA gene. Contrasting morphological hypotheses with molecular data provided rapid detection of six species, specifically *P. goodeyi*, *P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis*, and *P. veruculatus*. Bayesian inference (BI) phylogenetic trees for these molecular markers established phylogenetic relationships underscore the importance of using genomic and molecular phylogenetic data for accurate pin nematode identification at the species level. To our knowledge, this is the first report of *Paratylenchus* spp. in Portugal, extending the geographical distribution of these species in the Mediterranean Basin, and the first record of *P. goodeyi*, *P. pedrami*, *P. variabilis*, *P. veruculatus* and *P. tenicaudatus* parasitizing grapevine.

Keywords: Bayesian inference; D2–D3 expansion segments of large ribosomal subunit 28S; cytochrome c oxidase subunit 1; mitochondrial RNA; partial small ribosomal subunit; morphology; ribosomal RNA; *Vitis* spp.

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1. Introduction

Rhabditida Chitwood, 1933 is one of the most diverse and biologically versatile orders within the phylum Nematoda [1,2]. In fact, they are one of the largest in terms of numbers of species [1,2] including mycophagous species, parasites of invertebrate and a wide diversity of plant parasites. Members of the genus *Paratylenchus* Micoletzky, 1922 *sensu lato*, commonly known as pin nematodes, belong to the family Tylenchulidae Skarbilovich, 1947 (superfamily Criconematoidea Taylor, 1936 (Geraert, 1966); infraorder Tylenchomorpha De Ley & Blaxter, 2002; suborder Tylenchina Chitwood, 1950; order Rhabditida) [2–4]. According to morphological features and new molecular phylogenetic data [5–8], currently members belonging to *Paratylenchus sensu lato* are divided on two subgroups without taxonomic validity: (i) *Paratylenchus* species with a straight and short stylet, apparently

arrow-like form, usually less than 40 µm, composed of a conus about half of the total stylet length (*Paratylenchus sensu stricto*), and (ii) *Paratylenchus* species with a flexible and long stylet, often ventrally twisted, normally between 40 to 120 µm, composed of a conus occupying more than 70% of the total stylet (*Gracilacus* spp.). The systematic position of these two groups has been discussed several times [7,9–16].

The pin nematodes of genus *Paratylenchus sensu lato* are a large group of small and vermiform, metazoan parasites of annual, perennial and biennial herbaceous plants, woody shrubs and trees [4,17,18]. They are commonly found in natural and cultivated environments, especially layers of soil around roots of woody plants and bushes [17]. Their life-cycle is relatively short, with an average of 30–40 days at 25–28 °C, but this varies considerably among species [17,19,20]. During this period all vermiform juvenile stages and adults penetrate the root by moving their stylet through epidermal and root hair cell walls to feed ecto-parasitically which causes diverse degrees of negative effects on the roots of their hosts, including root injury and poor plant development, consequently suppressing yield and even plant longevity [4–6,17,19,20]. In addition, some species appear to be able to penetrate the lateral roots from the mechanical action of their stylet allowing them to enter into the intercellular spaces deep within the cortex and feeding endo-parasitically [17,21,22], which causes the symptoms to become more severe. Within *Paratylenchus*, at least seven species with short stylet (less than 40 µm) are plant pathogenic [17,20]. Some examples on fruits and woody shrubs are *P. hamatus* Thorne & Allen, 1950 [23–33], *P. neoamblycephalus* Geraert, 1965 [22,34] and *P. nanus* Cobb, 1923 [35–37], which emphasizes the need for the correct identification of species.

Pin nematodes are an adaptable cosmopolitan group of plant-parasitic nematodes commonly found in most of soil types of temperate regions worldwide [4,17]. They show a remarkable adaptability to changing environmental conditions such as hot and cold temperatures, and drought [5,6]. Like other nematodes, their success in unfavourable conditions is associated with their parthenogenetic reproduction where males are absent or rare, and the occurrence of a dauer juvenile stage (usually pre-adult or J4) [5,6,37]. Several previous studies have reported the association of this nematode group with perennial woody plants in the Mediterranean Region [5,6,38–40]. Thus, the occurrence and geographical distribution of pin nematodes in the Iberian Peninsula was reviewed by Peña-Santiago et al. [41] who reported a total of 22 pin nematode species that included *Paratylenchus aonli* Misra & Edward, 1971 (Hernández & Jordana, 1992), *P. arculatus* Luc & De Guiran, 1962, *P. baldaccii* Raski, 1975, *P. ciccaronei* Raski, 1975, *P. curvitatus* Van der Linde, 1938, *P. enatus* (Raski, 1976) Siddiqi, 1986, *P. goodeyi* Oostenbrink, 1953, *P. macrodorus* Brzeski, 1963, *P. microdorus* Andrássy, 1959, *P. minusculus* Tarjan, 1960, *P. mirus*, (Raski, 1962) Siddiqi & Goodey, 1964, *P. nanus* Cobb, 1923, *P. neoamblycephalus* Geraert, 1965, *P. peraticus* (Raski, 1962) Siddiqi & Goodey, 1964, *P. projectus* Jenkins, 1956, *P. sheri*, (Raski, 1973) Siddiqi, 1986), *P. similis* Khan, Prasad & Mathur, 1967, *P. steineri* Golden, 1961, *P. straeleni* (de Coninck, 1931) Oostenbrink, 1960, *P. tenuicaudatus* Wu, 1961, *P. teres* (Raski, 1976) Siddiqi, 1986, *P. vandenbrandei* De Grisse, 1962, and *P. veruculatus* Wu, 1962. Later on, Munawar et al. [42] reported the presence of several Spanish populations of *P. tateae* Wu & Townsend, 1973 on Northern and Southeast of the Iberian Peninsula, Spain. Recently, Clavero-Camacho et al. [5,6] revealed a remarkable biodiversity of pin nematode species associated with cultivated and uncultivated environments in Spain. In fact, Clavero-Camacho et al. [5,6] described five new species (*P. caravaquenus* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021, *P. indalus* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021, *P. pedrami* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021, *P. parastraeleni* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Palomares-Rius and Castillo Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021, and *P. zurgenerus* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021), and thirteen additional new reports for Spain; however, most of all these previous studies in

the Iberian Peninsula were with Spanish populations of pin nematodes [5,6,38,40,41], with exception of Lima [43] and Macara [44]. To our knowledge, no previous studies of detailed descriptions included measurements and/or molecular characterizations of Portuguese populations of pin nematodes.

According to the morphological features and morphometric measurements of females and males, and that of juveniles [mainly fourth-stage juveniles (J4)], each *Paratylenchus* species was defined from a compendium of diagnostic characters used for identification at the level of species with the key published by Ghaderi et al. [45]. However, the relatively small body size of these nematodes, together with their high inter- and intra-specific variability and marked plasticity in morphology, makes species identification based on these traits difficult and sometimes unreliable and challenging to study and identify. Sequencing RNA-based markers is a powerful tool for identifying and discriminating pin nematode species within the family Tylenchulidae [5–7,42]. Over the last years several studies [5–7,20,42,46–60] have shown the usefulness of the combination of two or more molecular markers based on ribosomal RNA (rRNA) (particularly D2–D3 domains of 28S gene and the internal transcribed spacer 1 (ITS1) region) for an accurate and fast diagnosis of *Paratylenchus* species. D2–D3 domains of 28S rRNA and ITS1 have proved more effective in species identification compared to partial 18S, as both these molecular markers display more species variability with respect to partial 18S. In fact, the 18S rRNA gene has been used for species diagnosis, and phylogeny in a more restricted number of species inside *Paratylenchus* [7,42]. Additionally, studies have revealed that the mitochondrial marker gene, particularly the partial cytochrome c oxidase subunit I or COI, is useful for the delineation of closely related species within Tylenchulidae [5–7,49,61]. However, an integrative taxonomic approach based on morphological and molecular phylogenetic analysis, allowing the linking of genomic and phenotypic data, is the best strategy to solve practical issues involving species delimitation in pin nematodes [5–7]. To our knowledge, approximately 60%, or more than half of the 132 valid species of this genus, have no molecular markers in the GenBank database, and there is a need for new molecular information.

Members of the genus *Paratylenchus* have not been studied in detail in Portugal since the last to study them were Lima [43] and Macara [44]. They recorded *Paratylenchus* spp. in cultivated and natural environments, however none have been formally identified to species. Thus, updated information on the present biodiversity including molecular data, occurrence and distribution of pin nematodes in Portuguese vineyards is lacking. For these reasons, an accurate diagnosis of pin nematode species in Portugal is essential. This study reports on the biodiversity of *Paratylenchus* spp. in grapevine soil samples from four major grapevine-growing areas in the Central and South Portugal. A robust taxonomical approach based on contrasting morphology with molecular analysis revealed that these *Paratylenchus* populations belong to morphospecies groups 3 and 10 defined by Ghaderi et al. [45]. Thus, the aims of the present work was (1) to characterize and illustrate several populations of pin nematode species from four major grapevine-growing areas of Central and South Portugal using an integrative approach based on contrasting morphological data with molecular analysis, (2) to update the diversity of pin nematodes recorded from Portugal and (3) to establish phylogenetic relationships of several isolates of pin nematode species found in this survey with available sequences of other pin nematode species deposited in Genbank. The results of this study will add information distinguishing pin nematode species and reveal new knowledge on the genetic diversity and the geographic distribution of pin nematode species.

2. Materials and Methods

2.1. Nematode Population Sampling

During three consecutive years (2019–2021) in Central and South Portugal, soil samples were collected from around the roots of grapevine (*Vitis vinifera* L.) in 44 commercial vineyards which are located in four of the main grapevine-growing regions (Alentejo, Tejo, Setubal and Lisbon). During this survey several populations of *Paratylenchus* spp. were

isolated from infested soil samples (Table 1). They were extracted from soil by a modified Cobb sieving and flotation, followed with a final extraction in an Oostenbrink dish [62] and a rapid centrifugal flotation [63]. Additional soil was collected afterwards from the same sample to guarantee enough specimens for morpho-anatomical analysis and/or multi-locus sequencing.

Table 1. Taxa sampled for *Paratylenchus* species and sequences used in this study.

Species	Sample Code	Locality	Host	Genbank Accessions		
				18S	28S	COI
P. goodeyi	T1-3	Monte da Ribeira, São Manços	grapevine	OM345189-OM345190	OM348556-OM348560; OM348553	OM348572-OM348573
	09-02-20	Carvalhal, Bombarral	grapevine	-	OM348543	-
P. hamatus	45-007-20	Roliça, Bombarral	grapevine	OM345185	OM348545-OM348547	OM348567-OM348568
P. pedrami	AL-V4	Santa Catarina de Sítimos, Alcácer do Sal	grapevine	OM345191-OM345192	OM348562-OM348566-	OM348574-OM348577
	CF-1	Aldeia Galega da Merceana, Alenquer	grapevine	-	OM348550-OM348551	OM348570
P. tenuicaudatus	198-33-19	Carvalhal, Bombarral	grapevine	OM345184	OM348544	-
P. variabilis	197-32-19	São Domingos de Carmões, Torres Vedras	grapevine	OM345186	OM348548-OM348549	OM348569
P. veruculatus	T1-3	Monte da Ribeira, São Manços	grapevine	OM345187-OM345188	OM348552; OM348554-OM348555; OM348561	OM348571

(-) Not obtained or not performed.

2.2. Morphological and Morphometrical Study

After extracting nematodes from soil, fresh nematodes were examined under a stereo microscope (Olympus SZX112) and immediately picked into an embryo glass dish and stored in sterile, distilled water at 4 °C until further processing. For light microscopy (LM) studies, live nematodes were placed in a drop of distilled water, gently heat killed, and fixed for 48–72 h at room temperature (25 °C) in a fixative solution composed of 4% formaldehyde, 1% glycerol and 85% distilled water. After nematodes were fixed, they were processed into pure glycerine using a modification of the Seinhorst method [64] and transferred to a small drop of glycerol on a glass slide ready for mounting of permanent slides. Light micrographs of nematode specimens mounted permanently on slides were acquired using a light microscope (Olympus BX50, Hamburg, Germany) with differential interference contrast (DIC) up to 1000× magnification. Phenotypic image analysis and measurements were done using an Olympus DP70 camera Cell® software (Olympus Corp., Tokyo, Japan). All measurements were expressed in micrometers (µm). All abbreviations used are defined by Siddiqi [4]. According to metric (e.g., de Man body ratios, lip region and width, stylet length, lip maximum body width, vulva position, pharyngeal length, and tail length and diameter) and non-metric (e.g., lip region shape, number of lateral lines, presence or absence of males, shape of spermatheca, presence of sperm, presence or absence of advulval flaps, tail shape, vulva size and shape, and tail terminus shape) morphological data of adult specimens and juveniles (mainly J4) (when available), all pin nematode species found in this survey were well defined phenotypically by a compendium of diagnostic characters given by Ghaderi et al. [45]. LM studies were carried out at the Nematology Lab, University of Évora, Portugal. For scanning electron microscopy (SEM) studies, fixed specimens were dehydrated in a graded ethanol series, immersed in HMDS (hexamethyldisilazane 98%), mounted on SEM stubs, sputter-coated with a thin layer of

gold [65], and observed with a Hitachi S3700N (Tokyo, Japan) SEM coupled to a Bruker (Karlsruhe, Germany) XFlash 5010 SDD Detector system [66]. The SEM coupled with energy-dispersive X-ray spectrometry (SEM-EDS) experiments were conducted on high vacuum mode with acceleration voltage of 5–10 kV. SEM studies were carried out at the Hercules Lab, University of Évora, Portugal.

2.3. DNA Extraction

Genomic DNA was prepared according to Gutiérrez-Gutiérrez et al. [67]. DNA was extracted from single individual live adult nematodes (females and males when available) and even juvenile stages (when available), which were previously examined by LM on temporary glass slide mounts for taking photomicrographs and measurements to record their phenotypic data that matched their associated genotype. For DNA extraction, individual nematodes were placed in a 1.5 µL drop of sterile water on the microscope glass slide, and each specimen was chopped into three small pieces with a surface-sterilized, pointed needle. Subsequently, all individual nematode bits were transferred to a PCR tube with 20 µL of solution containing 12 µL ddH_2O, 6 µL 10× PCR buffer, and 2 µL of proteinase K (20 mg/mL) (Nalgene®). Tubes were centrifuged at 11,000 rpm for 1 min and later frozen at −80 °C (45 min). Samples were mixed for 10 s, centrifuged at 11,000 rpm for 30 s, and incubated at 57 °C (2 h) and 95 °C (15 min).

2.4. PCR Amplification, DNA Purification and Sequencing

Genomic DNA from a single individual specimen was used to amplify two rDNA fragments: D2–D3 of 28S, and partial 18S rRNA gene and the partial fragment of COI mtRNA gene. The PCR was performed in a final volume of 50 µL containing: 1 µL of DNA template, 25 µL NZYTaq 2× Green Master Mix (2.5 mM $MgCl_2$, 200 mM dNTPs, 0.2 U/µL DNA Polymerase) (NZYTech, Lisbon, Portugal), 1.25 µL of each primer (10 mM), and 22.75 µL of ddH_2O. Each rDNA and mtDNA fragment was amplified using several primer pairs (Table S1). PCR assays were conducted as described by Clavero-Camacho et al. [5,6] and Singh et al. [7]. PCR cycle conditions included one cycle of 95 °C for 3 min; followed by 30 cycles of 94 °C for 30 s; an annealing temperature of 51 °C (391F/D3B), and 50 °C (988F/1912R, 1813F/2646R, J3/J4.5, COIF/COIF) for 30 s, 72 °C for 15–45 s; and one cycle of 72 °C for 7 min. The PCR products were purified [67] and were used as template for direct sequencing on a DNA multicapillary sequencer (ABI 3730xl DNA Sequencer; Applied Biosystems, Foster City, CA, USA), using a BigDye Terminator V3.1 Cycle Sequencing Kit at the STABVIDA facilities (Caparica, Portugal). Additional D2A primer (Table S1) was used for guaranteeing a robust and complete sequence. All new sequences of the pin nematode species found were deposited in the GenBank under the accession numbers indicated on the Table 1.

2.5. Phylogenetic Analyses

The newly obtained D2–D3 expansion segments of 28S rRNA, partial 18S rRNA and partial COI mtRNA sequences from all known pin nematode species found in this survey (Table 1), together with the available sequences of other pin nematode species obtained from the National Center for Biotechnology Information (NCBI) were used for phylogenetic analyses. Outgroup taxa for each gene studied was chosen according to previously published data by Clavero-Camacho et al. [5,6], Singh et al. [7] and Subbotin et al. [49]. The sequences were aligned using an online version of MAFFT v. 7 [68] with default parameters. Sequence alignments were visualized with ClustalX2 [69] and edited by Gblocks v. 0.91b [70] with less stringent selection Gblocks parameters (www.phylogeny.fr, accessed on 2 December 2021). Homogeneities of base frequencies and optional substitution models for 28S rRNA, 18S rRNA, and COI datasets were tested with Kakusan4 [71]. The homogeneity test indicated that the base composition of each dataset was significantly homogeneous. Bayesian inference (BI) trees of all molecular data were constructed with MrBayes v. 3.2.1 [72]. For BI analysis, the substitution model was tested by the Bayesian Information Criterion and

two models were selected for our three molecular regions studied. GTR model with a gamma-shaped distribution was selected for D2–D3 regions of 28S rRNA and COI, while the K80 model with a gamma-shaped distribution was selected for the partial 18S rRNA. Convergence of the MCMC chain and appropriate burn-in were assessed with Tracer 1.7.1 [73]. BI analysis was run for 5,000,000 generations, sampling every 100th tree and discarding 'burn-in'for the first 25% of the sampled tree. The resulting trees were visualized and edited using FigTree v1.4.3 [74].

3. Results

From the 44 samples analysed, *Paratylenchus* nematodes were detected in 7 (15.9%), showing a moderate incidence in Central and South Portugal. A total of six species were identified from eight selected isolates of *Paratylenchus* spp. from seven soil samples in six localities in Portugal (Table 1). The population densities of *Paratylenchus* spp. ranged between 10 to 250 nematodes/500 cm^3 of soil. All specimens of pin nematodes (including males, females and juveniles when available) were examined to get a fast first preliminary taxonomic check. Subsequently, adult specimens found within each population studied were morphometrical and morphologically examined in detail checking their complexity. Also, these phenotypic data were contrasted with molecular data. Molecular data were useful to resolve the taxonomic identity of the species complex of pin nematode species found in this study. Juveniles were used only for molecular analaysis since few specimens were available. For each population studied, we provided a set of metrical and non-metrical morphological data of adult specimens (females and males when available) as well as mitochondrial and ribosomal molecular markers for their identification (Table 1). Morphological analysis was followed by barcoding sequences and molecular phylogenetic data allowed the discernment a total of six known pin nematode species, some of them sharing samples, namely *P. goodeyi*, *P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis* Raski, 1975, and *P. veruculatus*. All these pin nematode species must to be considered as first reports for Portugal and measurements from adults (females and males when available), as well as molecular phylogenetic analysis using rDNA and mtDNA molecular markers were provided for their phylogenetic studies and unequivocal diagnosis at the species level.

3.1. Systematics

3.1.1. Morphological Features and Morphometric Measurements

Paratylenchus goodeyi Oostenbrink, 1953
(Figure 1; Table 2)
Morphological features: Figure 1.
Morphometric measurements: Table 2.

Description of females. Body slender, C- or J-shaped when heat relaxed; cuticle annulated; lateral field with four lines; a conoid-rounded lip region, lip region not set off from body contour, without labial sclerotization; pharynx paratylenchid; stylet long 49.6–60.6 µm, with flexible appearance, often ventrally twisted; rounded stylet knobs; dorsal pharyngeal gland opening 4.8–5.6 µm behind stylet knobs; median pharyngeal bulb large elongate, 2.2–3.0 times as long as wide; basal bulb pyriform; excretory pore situated 84.0–115.0 µm from anterior end; body slightly narrower posterior at the vulva; an anterior uterine branch occupying 29.6–34.8% of the body length; a single ovary outstretched; a short and rudimentary post uterine branch present; vulval flaps present; tail slender, usually 40.0–60.7 µm long and 4.2–5.5 times as long as anal diameter, conoid with variable terminus from subacute to finely rounded (Figure 1).

Males. Not found.

Brief description of juveniles. Only three 4th stage juvenile (J4) specimens were detected. Juveniles had a few differences in morphology compared to the females, being characterized by a robust body, cephalic region anteriorly truncate with labial framework noticeably sclerotized, a short rigid and straight stylet 15–17 µm long; many dark granules

into the body; anus difficult to distinguish; and a posterior body similar to female with a rounded tail terminus (Figure 1).

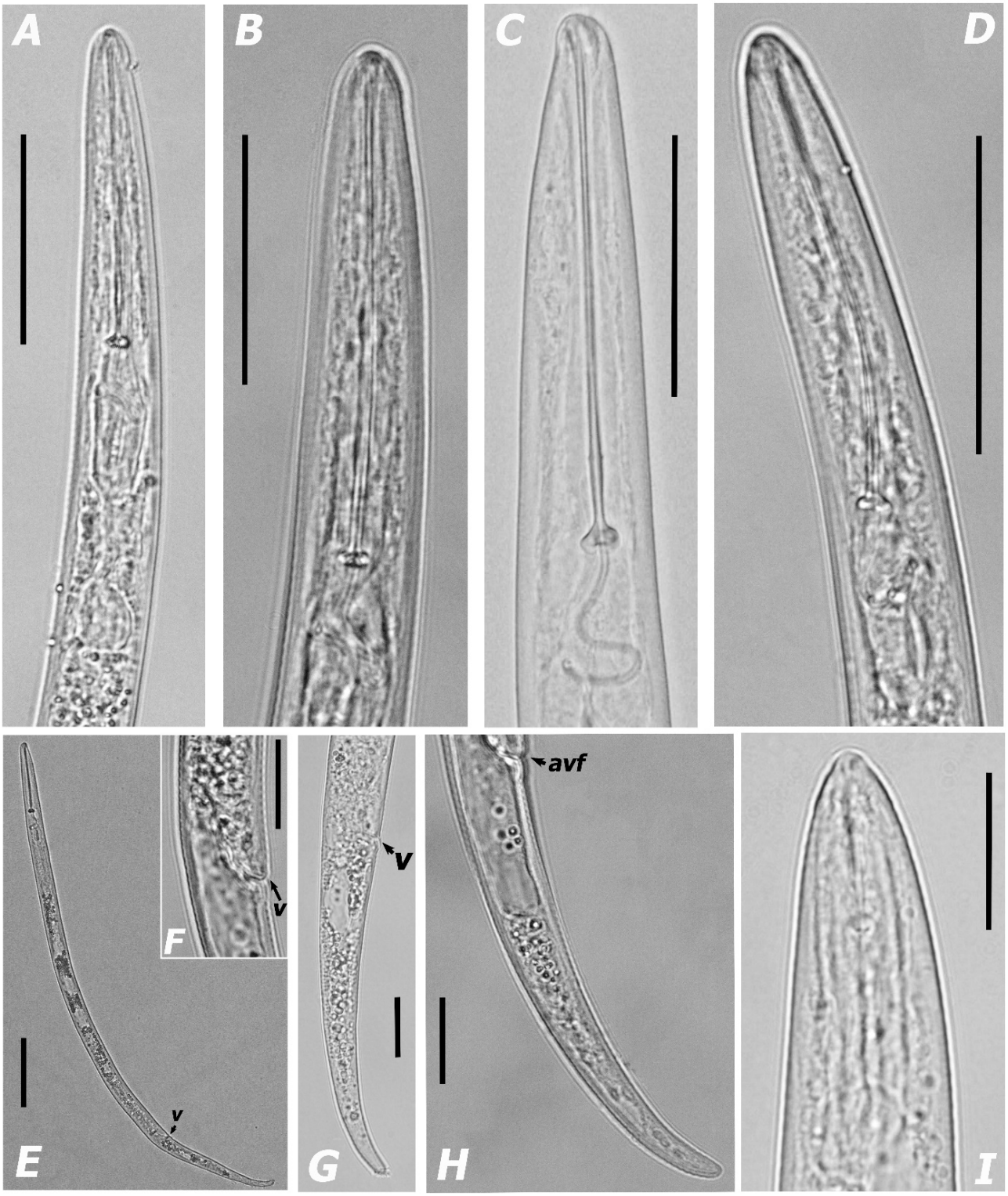

Figure 1. Light micrographs of *Paratylenchus goodeyi* Oostenbrink, 1953 (**A**–**I**). (**A**–**D**): Female pharyngeal region; (**E**): Entire female with vulva (arrowhead); (**F**): Detail of vulva (arrowhead); (**G**): Female posterior region showing vulva (arrowhead); (**H**): Female posterior region showing advulval flap (arrowhead); (**I**): Fourth-stage juvenile anterior region. Scale bars ((**A**) = 38 µm, (**B**–**D**) = 29 µm, (**E**) = 58 µm, (**F**–**H**) = 15 µm, (**I**) = 16 µm). (Abbreviations: avf = advulval flap; V = vulva).

Table 2. Morphometrics of *Paratylenchus goodeyi* Oostenbrink, 1953 and *P. tenuicaudatus* Wu, 1961 from the rhizosphere of grapevines (*Vitis vinifera* L.) in vineyards in Portugal. All measurements in µm and in the format: mean ± s.d. (range) *.

Measurements and Ratios	***P. goodeyi***		***P. tenuicaudatus***	
Sample Code	**T1-3**	**09-02-20**	**198-33-19**	
Locality	Monte da Ribeira, São Manços	Carvalhal, Bombarral	Carvalhal, Bombarral	
n	5 females	1 female	5 females	1 male
L	449.2 ± 33.5 (399.5–481.5)	414.5	296.8 ± 20.7 (276.7–331.3)	354.4
a	31.1 ± 1.8 (28.4–32.7)	28.1	24.4 ± 1.1 (22.8–25.5)	26.4
b	3.6 ± 0.2 (3.5–3.9)	3.4	4.1 ± 0.3 (3.7–4.4)	4.6
c	8.7 ± 1.3 (7.3–10.0)	9.1	11.1 ± 1.9 (8.4–13.3)	13.1
c′	5.0 ± 0.5 (4.5–5.5)	4.2	3.0 ± 0.3 (2.6–3.5)	2.7
V or T	79.8 ± 1.2 (78.2–81.0)	80.8	81.2 ± 1.2 (79.2–81.9)	63.6
G_1(%)	31.9 ± 2.2 (29.6–34.8)	41.7	22.8 ± 2.8 (20.9–26.0)	-
Stylet length	57.9 ± 2.2 (54.8–60.6)	49.6	30.2 ± 1.1 (29.0–31.6)	-
Cone length	47.7 ± 1.7 (46.5–48.9)	39.7	25.5 ± 0.3 (25.3–25.7)	-
(Stylet length/body length) × 100	13.0 ± 0.6 (12.3–14.0)	12.0	10.2 ± 0.5 (9.5–10.8)	-
m	84.6 ± 0.3 (84.4–84.9)	80.1	81.6 ± 2.1 (80.1–83.0)	-
DGO	5.2 ± 0.5 (4.8–5.6)	5.5	5.1 ± 0.6 (4.7–5.6)	-
O	9.2 ± 0.6 (8.8–9.6)	11.0	16.5 ± 2.3 (14.9–18.1)	-
Lip width	2.9 ± 0.5 (2.4–3.5)	2.7	3.3 ± 0.7 (2.7–4.2)	2.9
Median bulb length	22.8 ± 1.6 (20.7–25.0)	35.5	14.3 ± 1.6 (12.0–16.2)	16.5
Median bulb width	8.5 ± 0.5 (7.8–9.3)	9.2	5.9 ± 0.9 (5.0–7.3)	4.7
Anterior end to center median bulb	76.0 ± 4.2 (70.5–81.0)	69.7	41.1 ± 2.1 (37.6–43.1)	40.9
MB	61.8 ± 1.7 (59.9–63.6)	57.3	56.2 ± 4.1 (49.5–59.5)	49.1
Excretory pore to anterior end	93.9 ± 12.2 (84.0–115.0)	86.1	63.3 ± 3.7 (58.0–68.4)	75.5
Pharynx length	123.0 ± 4.7 (115.0–127.3)	121.6	73.2 ± 2.7 (70.1–76.0)	83.3
Maximum body diam.	14.4 ± 0.5 (13.8–14.9)	14.7	12.2 ± 1.3 (11.5–14.5)	13.4
Tail length	52.4 ± 8.5 (40.0–60.7)	45.5	27.2 ± 4.3 (22.4–33.0)	27.1
Anal body diam.	10.5 ± 1.0 (9.0–11.4)	10.8	9.2 ± 1.1 (7.7–10.6)	10.2
Spicules	-	-	-	19
Gubernaculum	-	-	-	6.5

* Abbreviations are defined in Siddiqi (2000). (-) Not obtained or not performed.

Remarks. According to Ghaderi et al. [45], our two populations of this species belong to group 10 (stylet length more than 40 µm, four lateral lines, and advulval flaps present). This pin nematode species was found in two soil samples of grapevine (*Vitis vinifera* L.) from commercial vineyards with unknown rootstock Monte da Ribeira locality, São Manços (Évora district) and Carvalhal locality, Bombarral (Leiria district) in Southern and Central Portugal, respectively (Table 1). These populations presented low nematode densities in both soil samples (35 to 45 individuals/500 cm^3 of soil). The morphological and morphometrical traits closely agree with the original description of this species [75]. It matches well with other populations on the Iberian populations described by Castillo et al. [76] and Clavero-Camacho et al. [5,6], with the exception of some minor differences (Table 2). In spite of this species has been reported by a large list of authors [7,10,75–84], only some of them included morphometric data [5–7,10,75,76,79,80]. Apparently, our populations of *P. goodeyi* are almost indistinguishable from several *Paratylenchus* species belonging to group 10 after Ghaderi et al. [45] including *P. ivorensis* Luc & de Guiran, 1962, *P. pandatus* (Raski, 1976) Siddiqi, 1989 and *P. straeleni* [5–7,45], except for some subtle differences in morphological traits and measurements and DNA molecular markers (Tables 1 and 2). This species was originally described parasitizing grass roots in Netherlands [75,81] and was later found mainly associated to natural environments and fruits in Europe [5–7,10,77–81] and Asia [82–84]. To our knowledge this is the first record of this species parasitizing

grapevine. Furthermore, these findings represent the first detection of this species in Portugal and also confirm that this species is widely distributed in Iberian Peninsula.

Paratylenchus hamatus Thorne & Allen, 1950.

(Figure 2; Table 3)

Morphological features: Figure 2.

Morphometric measurements: Table 3.

Description of females. Body slender, slightly ventrally curved, usually J-shaped when heat relaxed; cuticle thin and finely annulated; lateral field with four incisures; lip region not set off from body contour, with the presence of small submedian lobes; framework weakly sclerotized; pharynx paratylenchid; stylet moderately short, usually 28.8–31.9 µm long, showing rigidity and straightness; rounded stylet knobs; dorsal pharyngeal gland opening 5.9–7.3 µm behind stylet knobs; median pharyngeal bulb large elongate, 2.5–3.2 times as long as wide; basal bulb pyriform; excretory pore situated slightly anterior to or at level of anterior part of pharyngeal basal bulb, usually 52.0–70.4 µm from anterior end; body slightly narrower posterior to the vulva; an anterior uterine branch occupying 34.9–36.4% of the body length; a single ovary outstretched, well developed; a small and rudimentary post uterine branch; advulval flaps present, but not prominent; tail slender, 27.7–35.9 µm long, curved ventrally, gradually tapering to form a subacute to finely rounded terminus (Figure 2).

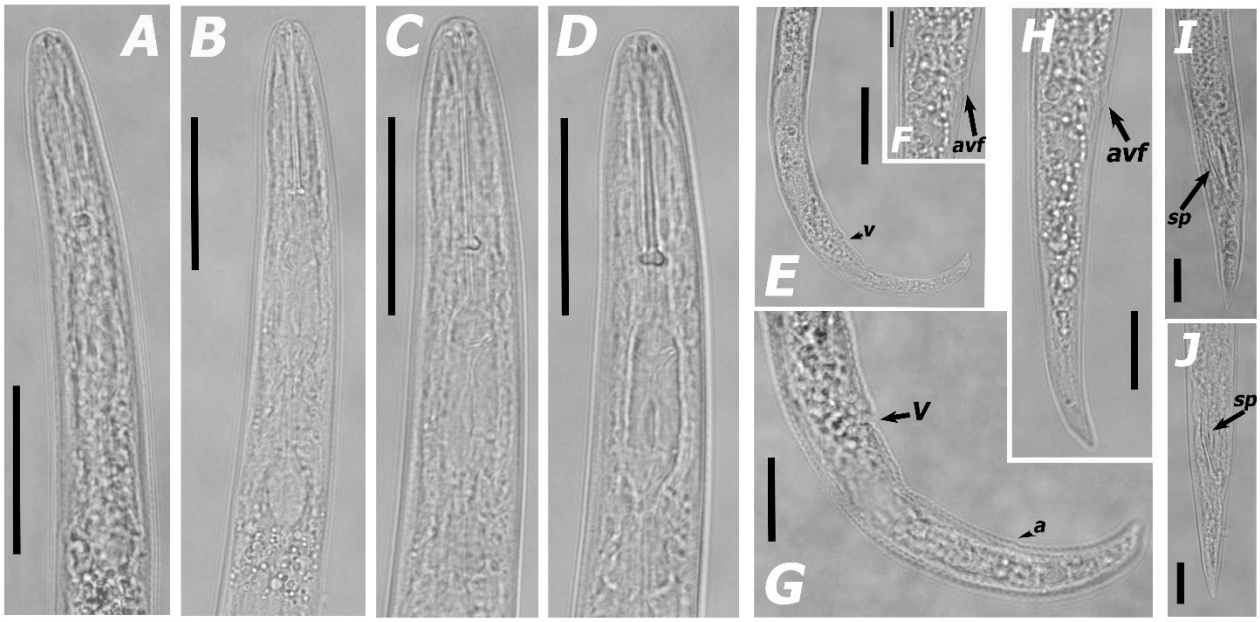

Figure 2. Light micrographs of *Paratylenchus hamatus* Thorne & Allen, 1950 (**A**–**J**). (**A**–**D**): Female pharyngeal region; (**E**): Female posterior region showing vulva (arrowhead); (**F**): Detail of advulval flap (arrowhead); (**G**): Female posterior region showing vulva and anus (arrowhead); (**H**): Female posterior region showing advulval flap (arrowhead); (**I**,**J**): Male posterior region showing spicules (arrowhead). Scale bars ((**A**–**D**) = 21 µm, (**E**) = 28 µm, (**F**) = 7 µm, (**G**,**H**) = 14 µm, (**I**,**J**) = 9 µm). (Abbreviations: a = anus; avf = advulval flap; sp = spicule; V = vulva).

Description of males. Males are as abundant as females. Appearance of body similar to female, except for reproductive organs, and the development of stylet and tail shape. Stylet 16.0–20.7 µm long, smaller than in the female, with small stylet knobs. Excretory pore located 68.2 µm from anterior end. Testis outstretched, with small spermatozoa; spicule slender, roughly 21.8 µm, slightly curved towards end; gubernaculum slightly curved; bursa absent; almost straight to ventrally slightly curved tapering slightly and gradually to a narrow, rounded tip (Figure 2).

Juveniles. Not found.

Table 3. Morphometrics of *Paratylenchus hamatus* Thorne & Allen, 1950, *P. variabilis* Raski, 1975 and *P. veruculatus* Wu, 1962 from the rhizosphere of grapevines (*Vitis vinifera* L.) in a vineyard in Portugal. All measurements in µm and in the format: mean ± s.d. (range) *.

Measurements and Ratios	*P. hamatus*		*P. variabilis*	*P. veruculatus*
Sample Code	**045-007-20**		**197-32-19**	**T1-3**
Locality	Roliça, Bombarral		São Domingos de Carmões, Torres Vedras	Monte da Ribeira, São Manços
n	4 females	5 males	13 females	2 females
L	320.1 ± 16.4 (298.3–337.8)	442.9 ± 19.3 (321.9–362.7)	296.1 ± 23.2 (247.7–336.2)	306.8 ± 22.0 (291.3–322.4)
a	22.8 ± 1.9 (20.4–25.0)	27.1 ± 2.1 (24.7–29.6)	22.8 ± 1.4 (20.5–25.0)	21.9 ± 3.0 (19.8–24.0)
b	4.1 ± 0.1 (4.0–4.1)	4.6 ± 0.3 (4.1–4.8)	4.2 ± 0.4 (3.8–4.9)	3.8 ± 0.3 (3.6–4.0)
c	10.6 ± 0.9 (9.4–11.6)	13.3 ± 1.4 (11.7–14.8)	12.9 ± 1.6 (9.1–15.0)	11.7 ± 0.6 (11.3–12.1)
c′	3.2 ± 0.4 (2.8–3.6)	3.1 ± 0.4 (2.5–3.5)	2.6 ± 0.4 (2.0–3.4)	2.7 ± 0.4 (2.4–2.9)
V or T	81.8 ± 1.7 (80.6–84.1)	68.2 ± 1.5 (66.4–69.7)	83.9 ± 1.0 (82.4–85.4)	83.8 ± 1.1 (83.0–84.6)
G_1(%)	35.7 ± 0.7 (34.9–36.4)	-	30.1 ± 6.4 (18.0–38.4)	19.8 ± 0.3 (19.5–20.0)
Stylet length	30.6 ± 1.3 (28.8–31.9)	19.0 ± 1.9 (16.0–20.7)	17.6 ± 0.8 (16.3–19.0)	14.0 ± 1.3 (13.1–14.9)
Cone length	19.5 ± 1.9 (18.1–20.9)	14.3 ± 0.5 (14.0–14.7)	13.0 ± 1.0 (12.0–14.0)	8.3 ± 1.1 (7.5–9.0)
(Stylet length/body length) × 100	9.6 ± 0.4 (9.0–10.0)	5.6 ± 0.7 (4.4–6.1)	6.0 ± 0.7 (5.1–7.7)	5.2 ± 0.6 (4.8–5.6)
m	65.7 ± 4.0 (62.8–68.5)	73.6 ± 3.8 (70.9–76.3)	72.1 ± 8.1 (63.2–80.5)	68.8 ± 8.8 (62.5–75.0)
DGO	6.6 ± 1.0 (5.9–7.3)	-	5.4 ± 0.2 (5.2–5.6)	-
O	22.3 ± 4.2 (19.4–25.3)	-	29.9 ± 2.2 (27.8–32.4)	-
Lip width	3.4 ± 0.4 (3.0–3.9)	3.1 ± 0.2 (2.9–3.4)	3.5 ± 0.5 (2.7–4.3)	3.8 ± 0.4 (3.5–4.1)
Median bulb length	19.8 ± 2.7 (16.9–22.4)	17.4 ± 3.1 (15.2–22.0)	17.1 ± 1.5 (15.2–20.5)	20.3 ± 1.4 (19.3–21.3)
Median bulb width	6.8 ± 0.6 (5.8–7.2)	4.6 ± 0.6 (4.0–5.2)	7.4 ± 1.0 (5.7–9.9)	7.4 ± 0.1 (7.4–7.3)
Anterior end to center median bulb	41.5 ± 2.8 (37.5–44.0)	39.9 ± 0.2 (38.8–40.0)	36.4 ± 2.2 (32.1–40.5)	41.9 ± 0.3 (51.7–42.2)
MB	53.8 ± 3.3 (50.5–56.8)	51.5 ± 1.6 (50.3–52.6)	51.9 ± 1.8 (49.1–54.7)	51.6 ± 0.3 (51.4–51.9)
Excretory pore to anterior end	69.1 ± 3.5 (66.7–74.1)	68.2 ± 2.0 (65.3–70.0)	64.4 ± 3.1 (57.4–68.4)	73.1 ± 0.3 (72.9–73.3)
Pharynx length	77.1 ± 2.7 (74.3–80.9)	75.1 ± 2.9 (71.9–79.0)	70.2 ± 4.2 (62.1–75.0)	81.2 ± 0.1 (81.1–81.3)
Maximum body diam.	14.1 ± 0.5 (13.5–14.7)	12.8 ± 1.6 (10.9–14.6)	13.0 ± 1.0 (11.9–15.4)	14.1 ± 0.9 (13.4–14.7)
Tail length	30.4 ± 3.7 (27.7–35.9)	26.0 ± 2.9 (21.8–28.2)	23.3 ± 3.3 (16.5–31.7)	26.3 ± 3.1 (24.2–28.5)
Anal body diam.	9.6 ± 1.0 (8.1–10.4)	8.5 ± 0.7 (8.1–9.7)	9.1 ± 0.9 (6.9–1.3)	9.8 ± 0.1 (9.8–9.9)
Spicules	-	21.8 ± 1.1 (20.1–22.6)	-	-
Gubernaculum	-	11.7 ± 2.0 (9.0–13.9)	-	-

* Abbreviations are defined in Siddiqi (2000). (-) Not obtained or not performed.

Remarks. According to measurements and morphological data of female and male adults, our populations of *Paratylenchus* species belong to group 3 as defined by Ghaderi et al. [45] and characterized by stylet length less than 40 µm, four lateral lines and advulval flaps present. This pin nematode species was found in one soil samples of grapevine (*Vitis vinifera* L.) in a commercial vineyard with unknown rootstock in Roliça locality, Bombarral(Leiria district, Central Portugal) (Table 1). This Portuguese population presented low nematode densities in the soil (25 individuals/500 cm^3 of soil). The morphological and morphometrical traits closely agree with the original description (paratype specimens) of the species *P. hamatus* [32] and other subsequent records worldwide [6,45,60], no crucial metrical differences at intraspecific level (Table 3). Apparently, our population of this species is almost indistinguishable from several species (*P. baldaccii*, *P. tenuicaudatus* and others) belonging to the *P. hamatus* "species complex" [6,60], except for some subtle morpho-anatomical differences and DNA molecular markers (Tables 1 and 3). This species was originally described parasitizing fig (*Ficus carica* L.) roots in Planada, CA, USA [32] and was later found in the rhizosphere from a large list of fruits, vegetables and ornamental plants worldwide [6,45,60]. These findings represent the first record of this pin nematode species for Portugal.

Paratylenchus pedrami Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021.

(Figure 3; Table 4)

Figure 3. Light and scanning electron microscopy micrographs of *Paratylenchus pedrami* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021 (**A**–**O**). (**A**–**C**): Female pharyngeal region showing excretory pore (arrowhead); (**D**): Male pharyngeal region showing excretory pore (arrowhead); (**E**): Female anterior region; (**F**): Anterior uterine branch showing vulva and ovary (arrowhead); (**G**): Entire male specimen; (**H**–**I**): Male posterior region showing spicules (arrowhead). (**J**,**K**): Female posterior region showing vulva (arrowhead); (**L**,**N**): Detail of advulval flap (arrowhead); (**M**): Female posterior region showing vulva and lateral field (arrowhead); (**O**): Entire fourth-stage juvenile showing genital primordium. Scale bars ((**A**,**B**) = 44 µm, (**C**) = 22 µm, (**D**) = 11 µm; (**E**) = 2.5 µm, (**F**) = 12 µm, (**G**) = 8 µm, (**H**,**I**) = 4 µm, (**J**–**L**) = 8 µm). (Abbreviations: avf = advulval flap; ep = excretory pore; gp = genital primordium; lf= lateral field; ov = ovary; sp = spicule; V = vulva).

Table 4. Morphometrics of *Paratylenchus pedrami* Clavero-Camacho, Cantalapiedra-Navarrete, Archidona-Yuste, Castillo and Palomares-Rius, 2021 from the rhizosphere of grapevine (*Vitis vinifera* L.) in vineyards in Portugal. All measurements in µm and in the format: mean ± s.d. (range) *.

Character/Sample Code	AL-V4		CF-1
Locality	Santa Catarina de Sítimos, Alcácer do Sal		Aldeia Galega da Merceana
n	20 females	3 males	12 females
L	298.5 ± 22.8 (239.3–337.4)	255.2 ± 32.2 (227.3–290.5)	294.3 ± 16.9 (270.0–330.6)
a	24.4 2.7 (19.7–28.9)	23.2 ± 4.3 (20.7–28.1)	24.4 ± 1.9 (20.4–27.1)
b	4.0 ± 0.3 (3.6–4.7)	3.8 ± 0.1 (3.8–3.9)	4.2 ± 0.5 (3.2–5.2)
c	13.0 ± 2.4 (9.7–17.7)	24.1 ± 4.5 (19.4–28.3)	14.3 ± 1.5 (11.2–16.4)
c′	2.9 ± 0.5 (2.0–3.8)	1.4 ± 0.3 (1.1–1.6)	2.4 ± 0.3 (2.0–3.0)
V or T	81.2 ± 1.4 (79.2–85.4)	28.7 ± 1.9 (27.4–30.1)	80.5 ± 1.3 (78.9–82.9)
G_1	29.3 ± 9.7 (17.8–53.3)	-	25.3 ± 3.2 (19.9–28.0)
Stylet length	29.8 ± 1.2 (27.5–32.0)	-	29.2 ± 1.3 (26.2–30.5)
Cone length	20.1 ± 1.2 (18.3–22.4)	-	20.6 ± 0.5 (20.0–21.0)
(Stylet length/body length) × 100	10.0 ± 0.7 (9.1–11.9)	-	10.0 ± 0.3 (9.7–10.7)
m	67.6 ± 2.6 (61.4–72.6)	-	69.7 ± 1.6 (67.8–71.5)
DGO	5.1 ± 0.6 (4.0–6.1)	-	4.3 ± 0.4 (3.8–4.5)
O	17.1 ± 1.9 (13.7–19.9)	-	14.4 ± 1.3 (12.9–15.2)
Lip width	3.0 ± 0.2 (2.5–3.4)	1.8 ± 0.1 (1.6–1.9)	3.3 ± 0.4 (2.3–3.8)
Median bulb length	18.7 ± 1.6 (16.4–21.7)	-	16.5 ± 1.1 (14.5–18.1)
Median bulb width	7.1 ± 0.8 (6.2–8.3)	-	5.9 ± 0.6 (4.9–7.2)
Anterior end to center median bulb	44.2 ± 1.5 (41.5–46.6)	-	38.7 ± 3.7 (32.0–42.6)
MB	58.7 ± 2.9 (53.6–65.6)	-	55.9 ± 4.7 (44.5–61.3)
Excretory pore to anterior end	65.2 ± 3.7 (57.7–70.5)	55.1 ± 3.0 (528–58.5)	60.0 ± 3.2 (52.0–63.1)
Pharynx length	74.3 ± 5.5 (64.0–83.2)	65.2 ± 2.5 (60.4–64.0)	69.5 ± 6.5 (59.6–83.2)
Maximum body diam.	12.4 ± 1.4 (9.5–14.4)	11.1 ± 0.8 (10.3–12.0)	12.0 ± 1.0 (10.8–14.3)
Tail length	23.7 ± 4.4 (17.0–30.3)	10.7 ± 1.7 (8.8–11.7)	20.5 ± 2.2 (17.0–26.0)
Anal body diam	8.1 ± 1.0 (5.7–9.8)	7.6 ± 0.4 (7.2–8.0)	8.5 ± 1.2 (6.9–10.4)
Spicules	-	16.2 ± 0.5 (15.7–16.7)	-
Gubernaculum	-	3.8 ± 0.1 (3.8–3.9)	-

* Abbreviations are defined in Siddiqi (2000). (-) Not obtained or not performed.

Morphological features: Figure 3.

Morphometric measurements: Table 4.

Description of females. Body slender, ventrally curved, J- or C-shaped when heat relaxed; cuticle thin and finely annulated; lateral field equidistant with four distinct lines; lip region rounded, with anterior end flattened, continuous with the remainder of the body, presence of small submedian lobes; framework weakly sclerotized; pharynx typical paratylenchid; stylet moderately short, usually 26.2–32.0 µm long, apparently rigid and straight; rounded stylet knobs; dorsal pharyngeal gland opening 3.8–6.1 µm behind stylet knobs; median pharyngeal bulb large elongate, 2.2–3.4 times as long as wide; basal bulb pyriform; excretory pore situated 52.0–70.4 µm from anterior end; body slightly narrower in the rear portion of the vulva; advulval lips prominent; lateral vulval flaps slightly rounded; a single ovary outstretched, well developed; the aperture of the vulva occupying half of the corresponding body width; anterior uterine branch occupying 19.0–23.0% of the body length; a small and rudimentary post uterine branch; tail slender, 17.0–30.3 µm long, 2.0–3.8 times as long as wide, curved ventrally, gradually tapering to form a rounded or subacute terminus (Figure 3).

Description of males. Males are less abundant than females. Appearance of body similar to female, except for reproductive organs, the level of development of the pharynx structures and tail shape. They are characterized by lacking a stylet and a rudimentary pharynx with the procorpus, metacorpus and basal bulb inconspicuous and lacking of functional ability. Excretory pore located 55.1 µm from anterior end. Testis outstretched, with small spermatozoa; spicule slender, slightly curved towards end; gubernaculum curved; bursa absent; tail short and rounded (Figure 3).

Brief description of juveniles. Only 3 juvenile specimens in the fourth developmental stages (J4) were detected. J4 showed many dark granules along the body cavity and a marked position of the developing vulva. They were similar in morphology to the adult females; however, they were characterized with a less developed stylet (19.5–25.5 µm long) and pharynx than in the females. The pharynx was usually visible in fresh specimens; undeveloped genital primordium; anus difficult to distinguish; and a posterior body similar to the female, but a slightly more rounded tail terminus (Figure 3).

Remarks. According to the measurements and morphological data of female and male adults and that of J4, both populations of this *Paratylenchus* species were defined from the compendium of key diagnostic characters to be within the group 3 (stylet length less than 40 µm, four lateral lines and advulval flaps present) as defined by Ghaderi et al. [45]. This pin nematode species was found in two soil samples of grapevine in commercial vineyards with unknown rootstock in Santa Catarina de Sítimos locality, Alcácer do Sal, Setubal district, and Aldeia Galega da Merceana locality, Alenquer, Lisbon district (Southern Portugal) (Table 1). The population presented moderate to high numbers of individuals in soil (200 and 250 individuals/500 cm^3 of soil). The morphological and morphometrical traits closely agree with the original description (paratype specimens) of the species [6] and another subsequent record of this species [5,6], except for minor intraspecific variations (Table 4). Apparently, our two populations of this species are almost indistinguishable from other species belonging to the group 3 as defined by Ghaderi et al. [45], such as *P. arculatus*, *P. baldaccii* and *P. salubris* Raski, 1975, except by minor differences in morpho-anatomical data, morphometric measures and DNA molecular markers (Tables 1 and 4). This species was originally described parasitizing almond roots in Southern Spain [6] and later was reported in soil samples around plant roots in natural environments [5]. These findings represent the first and third records for this species for Portugal and Europe, respectively. To our knowledge these results represent the first records of this species parasitizing grapevine. We confirm a wider geographical distribution on the Iberian Peninsula and an apparently wide range of host species.

Paratylenchus tenuicaudatus Wu, 1961

(Figure 4; Table 2)

Morphological features: Figure 4.

Morphometric measurements: Table 2.

Description of females. Body slender, curved ventrally, J- or C-shaped when heat relaxed; cuticle annulated; lateral field with four equally spaced incisures; lip region slightly rounded, continuous with the rest of the body, with indistinct submedian lobes when observed from lateral view; pharynx typical paratylenchid; moderately short stylet 29.0–31.6 µm long, apparently rigid and straight; rounded stylet knobs; dorsal pharyngeal gland opening 4.7–5.6 µm behind stylet knobs; median pharyngeal bulb large elongate, 2.0–2.8 times as long as wide; basal bulb oval to oblong; excretory pore was situated 58.0–68.4 µm from anterior end; body narrower in the posterior portion of the vulva (more accentuated in older specimens); advulval flaps present; vulval flaps prominent; a single ovary outstretched; anterior uterine branch occupying 20.9–26.0% of the body length; a vestigial and rudimentary post uterine branch; tail slender, 22.4–33.7 µm long, 2.6–3.5 times as long as wide, narrows gradually to finely rounded terminus, showing hook-like curved end in older specimens (Figure 4).

Description of males. Males are relatively less abundant than females. Appearance of body similar to female, except for reproductive organs, the developmental level of pharynx structures and tail shape. Stylet lacking and pharynx inconspicuous. Excretory pore located 75.5 µm from anterior end. Testis outstretched, with small spermatozoa; spicule slender 19.0 µm, slightly curved towards end; gubernaculum curved; bursa absent; tail long 27.1 µm, showing a pointed terminus (Figure 4).

Juveniles. Not found.

Remarks. According to Ghaderi et al. [45], this species belongs to group 3 (stylet length less than 40 µm, four lateral lines and advulval flaps present). This Portuguese population was found in one soil sample of grapevine *(Vitis vinifera* L.) in a commercial

vineyard with unknown rootstock in Carvalhal locality, Bombarral (Leiria district, Central Portugal) (Table 1). This population presented low nematode densities in soil (10 individuals/500 cm^3 of soil). The morphological and morphometrical traits closely agree with the original description of this species from Canada [85] and others populations [6,59,60], except for some insignificant intraspecific differences (Table 2). Apparently, our population of *P. tenuicaudatus* is almost indistinguishable from several *Paratylenchus* species belonging to *P. hamatus*-species complex [5–7,45], except by some subtle differences in measurements and DNA molecular markers (Tables 1 and 2). Wu [85] described this species in the rhizosphere of soil natural environments. Later on, it has also been reported in several localities of USA [60,86–88], Iran [59] and Spain [6,88]. These findings represent the first detection of this species in Portugal and the seventh after the original description from Canada [85]. To our knowledge these results represent the first records of this species parasitizing grapevine. We confirm a wider geographical distribution of this species in the Iberian Peninsula.

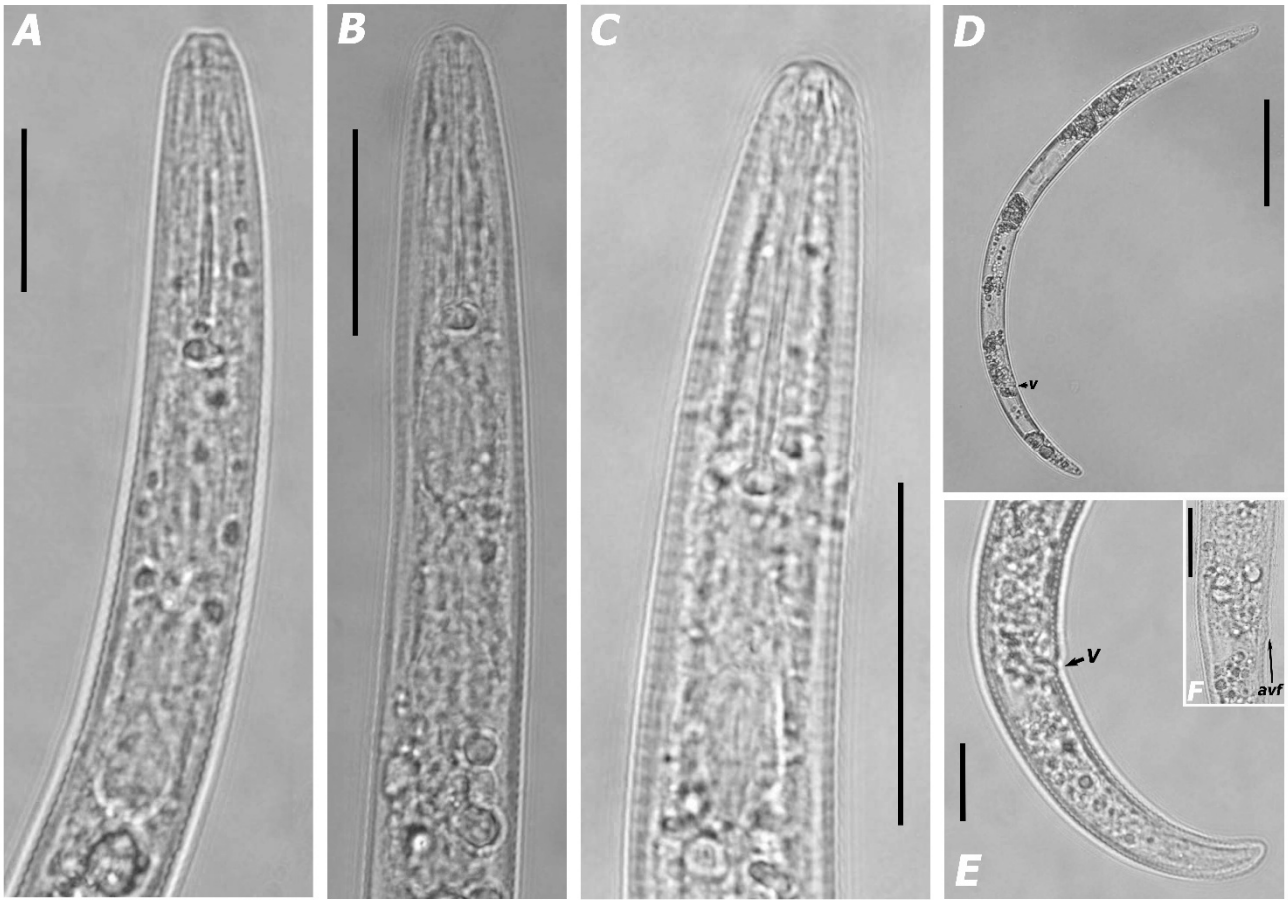

Figure 4. Light micrographs of *Paratylenchus tenuicaudatus* Wu, 1961 (**A**–**F**). (**A**–**C**): Female pharyngeal region; (**D**): Entire female specimen; (**E**): Female posterior region showing vulva (arrowhead); (**F**): Detail of advulval flap (arrowhead). Scale bars ((**A**) = 15 µm, (**B**,**C**) = 21 µm, (**D**) = 60 µm, (**E**–**F**) = 12 µm). (Abbreviations: avf = advulval flap; V = vulva).

Paratylenchus variabilis Raski, 1975
(Figure 5; Table 3)
Morphological features: Figure 5.
Morphometric measurements: Table 3.

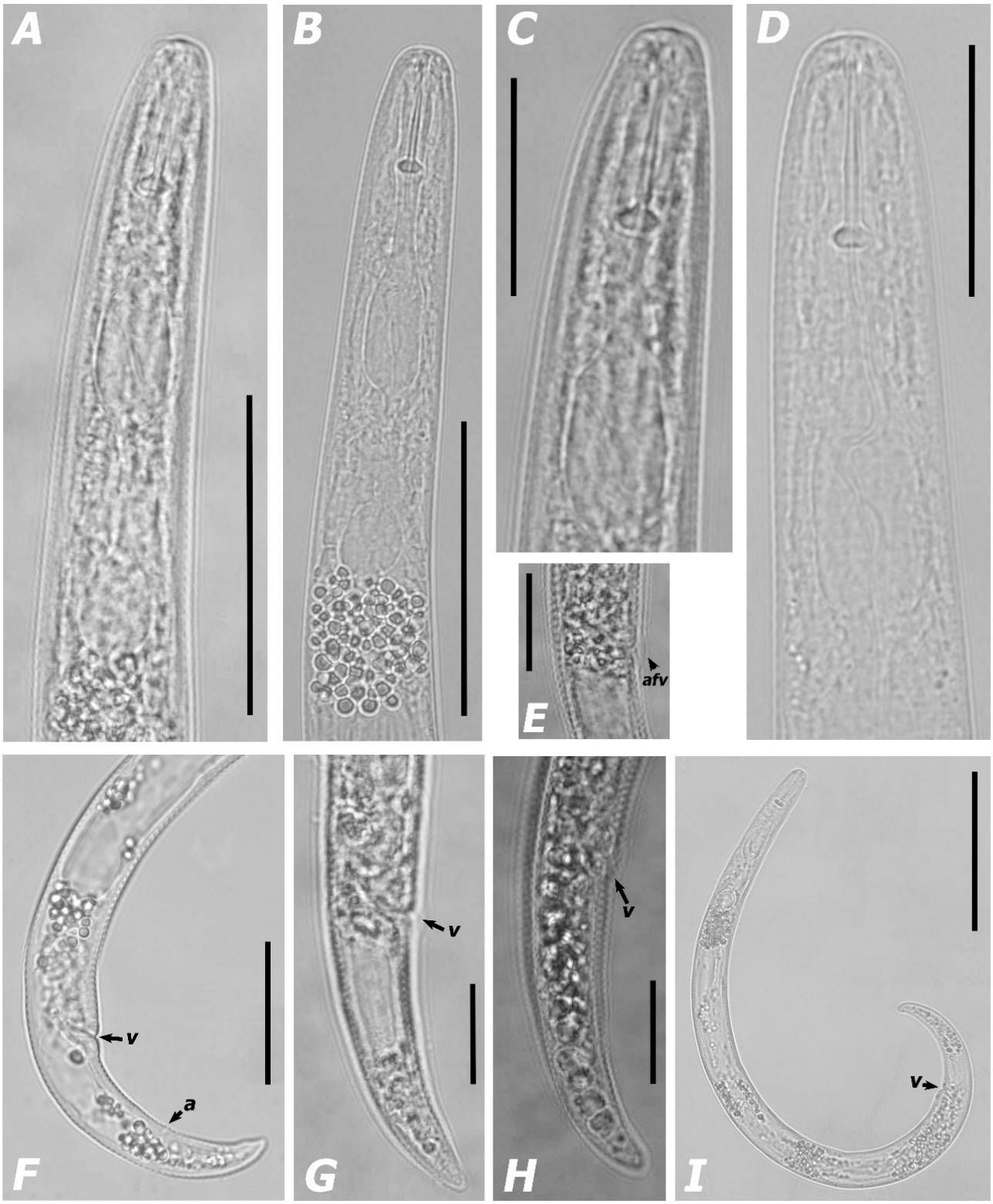

Figure 5. Light micrographs of *Paratylenchus variabilis* Raski, 1975 (**A**–**I**). (**A**–**D**): Female pharyngeal region; (**E**): Detail of advulval flap (arrowhead); (**F**): Female posterior region showing vulva and anus (arrowhead); (**G**,**H**): Female posterior region showing vulva (arrowhead); (**I**): Entire female specimen showing vulva (arrowhead). Scale bars ((**A**,**B**) = 36 µm, (**C**,**D**) = 18 µm, (**E**) = 13 µm; (**F**) = 26 µm, (**G**,**H**) = 13 µm, I = 70 µm). (Abbreviations: a = anus; avf = advulval flap; V = vulva).

Description of females. Body slender, ventrally curved, C-shaped when heat relaxed; cuticle thin and finely annulated; lateral field with four lines; rounded lip region with indistinct, submedian lobes, continuous with the rest of the body; framework weakly sclerotized; pharynx typical paratylenchid; short stylet 16.3–17.6 µm long, apparently rigid and straight; rounded stylet knobs; dorsal pharyngeal gland opening 5.2–5.6 µm behind stylet knobs; median pharyngeal bulb large elongate, 1.8–3.2 times as long as wide; basal bulb pyriform; excretory pore situated 57.4–68.4 µm from anterior end; body slightly narrower in the posterior portion of the vulva, abruptly in older specimens; advulval flaps present; vulval flaps prominent, apparently rounded; a single ovary outstretched; anterior uterine branch occupying 18.0–38.4% of the body length; a short and rudimentary post uterine branch present; tail slender, 16.5–31.7 µm long, 2.0–3.4 times as long as wide, narrows gradually to a bluntly rounded terminus (Figure 5).

Males. Not found.

Juveniles. Not found.

Remarks. According to Ghaderi et al. [45], our population of *Paratylenchus* species belongs to group 3 (stylet length less than 40 µm, four lateral lines and advulval flaps present). This Portuguese population was found in one soil sample of grapevine in commercial vineyards with unknown rootstock in São Domingos de Carmões locality, Torres Vedras (Lisbon district, Southern Portugal) (Table 1). This population presented low nematode densities in soil (50 individuals/500 cm^3 of soil). The morphological and morphometrical traits closely agree with the original description of the species from US [89] and subsequent records [5,45], except for minor intraspecific differences (Table 3). This species is morphologically close to *P. microdorus* and another *Paratylenchus* species belonging to *P. microdorus*-species complex. Apparently, our pin nematode population is almost indistinguishable from several species belong to *P. microdorus* species complex (e.i. *P. microdorus*, *P. recisus* Siddiqi, 1996, and *P. zurgenerus*) [5,60,89], except by some indiscernible morpho-anatomical differences and DNA molecular markers (Tables 1 and 3). This species was originally described parasitizing sourberry (*Rhus trilobata* Nutt.) roots in US [89] and later was reported parasitizing roots of herbaceous plants and shrubs in natural environments (i.e., scrub oak (*Quercus* spp.), sagebrush (*Artemisia* spp.) and burrobrush (*Hymenoclea monogyra* T. & G. ex Gray) in the U.S.A. [89], turfgrass roots in Iran and almond (*Prunus amygdalus* Batsch) roots in Spain [5,60]. These findings represent the first and second records of this species for Portugal and Europe, respectively. To our knowledge, these findings represent the first record of this species parasitizing grapevine. We confirm a wide global distribution and an apparently ability to parasitize a diverse and wide range of host species.

Paratylenchus veruculatus Wu, 1962

(Figure 6; Table 3)

Morphological features: Figure 6.

Morphometric measurements: Table 3.

Description of females. Body robust, slightly ventrally curved, C-shaped when heat relaxed; body cuticle distinctly annulated; lateral field equidistant showing four distinct lines; lip region broadly rounded, continuous with the rest of the body; submedian lobes not protruding; framework weakly sclerotized; pharynx typical paratylenchid type; short stylet, usually 13.1–14.9 µm long, apparently rigid and straight; well-developed rounded stylet knobs; median pharyngeal bulb large elongate, 2.6–2.9 times as long as wide; basal bulb pyriform; excretory pore was situated 72.9–73.3 µm from anterior end; diameter of the body behind vulva reduced gradually; advulval flaps present; vulval flaps prominent; a single ovary outstretched; anterior uterine branch occupying 18.0–38.4% of the body length; a small and vestigial post uterine branch present; tail 24.2–28.5 µm, 2.4–2.9 times anal diameter, narrows gradually to conoid with often broadly rounded terminus (Figure 6).

Males. Not found.

Juveniles. Not found.

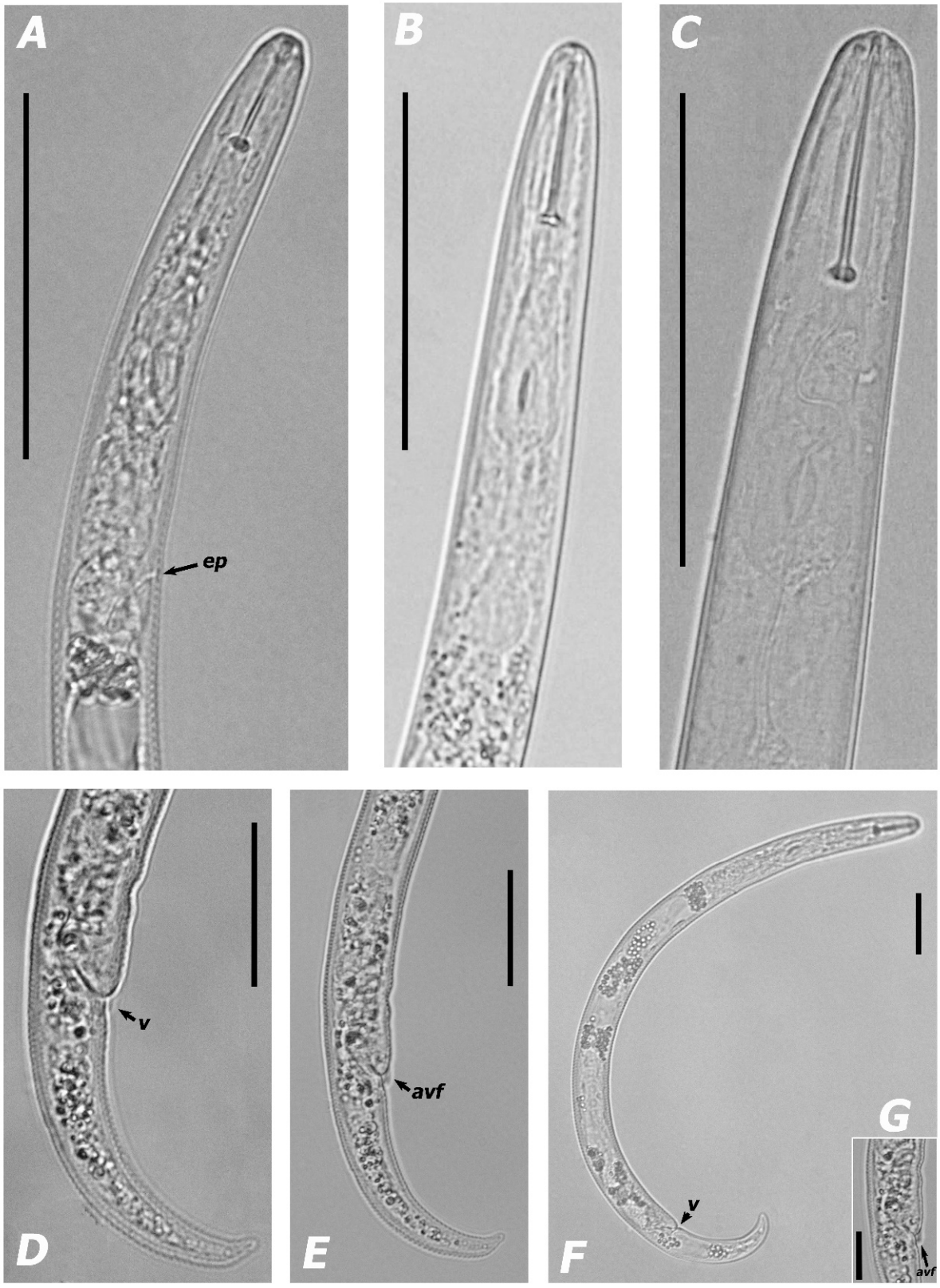

Figure 6. Light micrographs of *Paratylenchus veruculatus* Wu, 1962 (**A**–**G**). (**A**–**C**): Female pharyngeal region; (**D**): Female posterior region showing vulva (arrowhead); (**E**): Female posterior region showing advulval flap (arrowhead); (**F**): Entire female specimen showing vulva (arrowhead); (**G**): Detail of advulval flap (arrowhead). Scale bars (A–C = 42 µm, D–F = 28 µm, G = 14 µm;). (Abbreviations: avf = advulval flap; ep = excretory pore; V = vulva).

Remarks. According to Ghaderi et al. [45], this population of *Paratylenchus* species is characterized by stylet length less than 40 µm, four lateral lines and advulval flaps present. This Portuguese population was found in one soil sample of grapevine (*Vitis vinifera* L.) in a commercial vineyard with unknown rootstock in Monte da Ribeira locality, São Manços (Évora district, Southern Portugal) (Table 1). Low nematode densities were present in the soil (20 individuals/500 cm^3 of soil). The morphological and morphometrical traits closely agree with the original description of this species [90] and other subsequent records [5–7,10,45,79,82,89], except for some negligible intraspecific variations (Table 3). On the basis of morphological and morphometric data our population of this species is to close other *Paratylenchus* spp. (e.i. *P. microdorus*, *P. recisus* and *P. variabilis*) [5–7,45]. Apparently, our population is morphologically almost indistinguishable from these three species, except by some indiscernible morphological differences and DNA molecular markers (Tables 1 and 3). This species was originally described parasitizing heather (*Calluna vulgaris* (L.) Hull) roots in Scotland [90] and later was reported in soil around roots of uncultivated plants in natural environments from Belgium, Russia, Poland and Spain [5–7,10,45,79,82,89], sugarcane (*Saccharum officinarum* L.) from Iran [45], and fruit orchards (e.g., almond, cherry (*Prunus* spp.) and peach (*Prunus persica* (L.) Hatsch) [5,6]. This species has not been reported in Portugal and these findings represent the first record for this country. To our knowledge, these results represent the first record of this species parasitizing grapevine, and confirm also that this species is widely distributed in the Iberian Peninsula.

3.1.2. Molecular Results and Phylogenetic Relationships of the Six Known *Paratylenchus* spp. and Other Members of Genus *Paratylenchus*

For the six known pin nematode species obtained, the three genes (the D2–D3 expansion segments of 28S rRNA, and the complete 18S rRNA) and partial COI mtRNA gene) had an approximate size of 1000, 1600, and 400 bp, respectively. Ribosomal and mitochondrial sequences of six known *Paratylenchus* spp. (*P. goodeyi*, *P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis* and *P. veruculatus*) (OM348543–OM348577; OM345184–OM345192) matched well with sequences from the same species previously deposited in GenBank, increasing knowledge of the genotypic diversity in *Paratylenchus* (Table 1). For these *Paratylenchus* spp., there were multiple failed attempts to sequence a partial COI mtRNA and the complete18S rRNA genes before our study was concluded (Table 1). The D2–D3 expansion segments of 28S rRNA gene sequences from *P. pedrami* (OM348562–OM348566; OM348550–OM348551) matched closely (98–99% similarity) to several sequences of other populations of this same species deposited in GenBank (MZ265118, Spain; MW798283–MW798285, Spain); and the variations among these D2–D3 sequences ranged from 4 to 13 nucleotides. Intra-specific variation of D2–D3 detected among the two Portuguese populations of *P. pedrami* (two from grapevines) (Table 1) was from 10 to 18 nucleotides (98–99% similarity and 0 to 2 indels). For *P. pedrami*, four COI mtRNA gene sequences from Alcácer do Sal (AL-V4, grapevine) (OM348574–OM348577) and one sequence from Aldeia Galega da Merceana (CF-01, grapevine) (OM348570) were sequenced and showed a sequence similarity of 95%, with some minor intra-specific variations (2–19 nucleotides). Likewise our COI sequences (OM348574–OM348577, OM348570) had 94–96% similarity to other deposited in GenBank for *P. pedrami* (MW797009, Spain); and the variations among these COI sequences ranged from 11 to 15 nucleotides. Two homogeneous sequences of the complete 18S rRNA gene (99% of similarity) for *P. pedrami* (OM345191–OM345192) were 96% of similar to nine sequences deposited in GenBank belonging to *P. shenzhenensis* Wang, Xie, Li, Xu, Yu & Wang, 2013 (KF668494–KF668497, KF668499, KF668502–KF668504, China) and *Paratylenchus* sp. JH-2014 (KJ636431). The variations among the 18S sequences of these species were from 68 to74 nucleotides and 10 to 12 indels, being a new molecular marker for this species. The D2–D3 sequences from *P. veruculatus* (OM348552; OM348554–OM348555; OM348561) matched closely (99–100% similarity) to sequences of Spanish and Belgian populations of this species in GenBank (MW798310, Spain; MW798313–MW798314, Spain; MZ265134–MZ265135, Spain; MW798311–MW798312, Spain; MW413687, Belgium); and the variations

among them ranged from 0 to 12 nucleotides and 0 to 2 indels. For *P. veruculatus*, the COI mtRNA gene sequence (OM348571) showed a high and variable sequence homology with other sequences of this same species in Genbank; the homology ranged from greater than or equal to 98% (MW421717–MW421722, Belgium; MW797027–MW797029, Spain), to 94% (MW79702424–MW79702426, Spain). The variations among these COI sequences ranged from 1 to 18 nucleotides. Two identical sequences of the complete 18S rRNA gene (100% of similarity) of *P. veruculatus* (OM343587–OM343588) showed a similar homology (more than 99%) with several sequences deposited in GenBank belonging to *P. veruculatus* (MW413747–MW413748, Spain), *P. nanus* (MW413707–MW413708, and MW413712, Belgium; MN783664–MN783667, Belgium) and *P. goodeyi* (MW413699, Belgium). The variations among these 18S sequences of these species were from 0 to 5 nucleotides. D2–D3 sequences of *P. variabilis* (OM348548–OM348549) had a sequence similarity greater than 99% to several sequences of Spanish populations (MZ265127–MZ265129); and a variation among these sequences of this species from 1 nucleotide and 1 indel. For *P. variabilis*, COI sequence (OM348569) showed a sequence similarity of 97% to other sequence of this same species deposited in Genbank (MZ262265, Spain). The variation among these COI sequences was 8 nucleotides. The complete 18S of rRNA gene sequence of *P. variabilis* (OM345186) was 96%, similar to *Paratylenchus* sp. N2508 (MF094926, US). The variations among these 18S sequences of these species were from 0 to 5 nucleotides. This complete 18S rRNA gene sequence is a new molecular marker for this *Paratylenchus* species. D2–D3 sequences of *P. tenuicaudatus* (OM348544) matched well with a high sequence homology (more than 99% similarity) to sequences of several populations of this same species in GenBank (MW798306–MW798309, Spain; KF242223–KF242224, US; KU291239, Iran); and a variation among these sequences of this species from 0 to 4 nucleotides. Our complete 18S rRNA gene sequence of *P. tenuicaudatus* (OM345184) was 98% similar to several sequences deposited in GenBank belonging to *P. projectus* (MF094890, US; MF094897, US; KJ636433;-KJ636434). The variations among these 18S sequences of these species were from 18 to 24 nucleotides and 4 indels. D2–D3 sequences of *P. hamatus* (OM348545–OM348547) matched well with a high sequence homology (more than 99% similarity) to sequences of several populations of this same species in GenBank (KF242213–KF242218, US; KF242204, US; MW413565–MW413566, US; MW413558, US; MW798295–MW798299, Spain); and a variation among these sequences of this species from 0 to 3 nucleotides and 0 to 2 indels. For *P. hamatus*, the partial portion of COI gene sequenced agrees with results obtained from the D2–D3 fragments; in fact the COI sequences (OM348567–OM348568) showed a sequence homology higher than to 99% to several isolates of the same species (MW411822–MW411824, US; MW797016–MW797017; Spain) and *Paratylenchus* sp. NM TSH-2020 (MN711355–MN711357). The variation among these COI sequences was 0 to 1 nucleotide. Two homogeneous sequences of the complete 18S rRNA gene of *P. hamatus* (OM345185) were 98% similar to *P. projectus* (MF094890, US; MF094897, US; KJ636433–KJ636434). The variations among these 18S sequences of these species were from 18 to 24 nucleotides and 1 indel. This complete 18S rRNA gene sequence is a new molecular marker for *P. hamatus*. The D2–D3 sequences from *P. goodeyi* (OM348556–OM348560; OM348553; OM348543) had a sequence similarity greater than 98% to several sequences of this same species in GenBank (MW413631–MW413633, Belgium; MW798293–MW798294, Spain; MZ265102, Spain; MZ265104–MZ265105, Spain; MZ265083–MZ265084, MZ265086, Spain; MZ265088–MZ265090, Spain; MZ265093, Spain; MZ265096, Spain; MZ265099–MZ265100, Spain); and the variations among them ranged from 2 to 15 nucleotides and 0 to 3 indels. Intra-specific variation of D2–D3 detected among the Portuguese populations of *P. goodeyi* (Table 1) was from 8 to 13 nucleotides and 1 to 3 indels (98–99% similarity). For *P. goodeyi*, our COI gene sequences (OM348572–OM348573) had 94–95% similarity to other deposited in GenBank (MW421647–MW421649, Belgium; MW797014–MW797015, Spain; MZ262227–MZ262229, Spain; MZ262233–MZ262238, Spain; MZ262231, Spain). The variations among these COI sequences ranged from 15 to 17 nucleotides. The partial 18S rRNA gene sequences of *P. goodeyi* (OM343589–OM343590) showed a similar homology (97%) with a large number of sequences deposited in Gen-

Bank belonging to *Paratylenchus* spp., such as *Paratylenchus* sp. N2508 (MF094926, US), *Paratylenchus* sp. (KJ636435), and *P. projectus* (MF094890, US; MF094897, US; KJ636433). The variations among these 18S sequences of these species were from 38 to 47 nucleotides.

Using Bayesian inference (BI), we compared the phylogenetic position of six known *Paratylenchus* spp. (*P. goodeyi*, *P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis*, and *P. veruculatus*) by using the D2–D3 fragments of 28S rRNA and, the complete 18S rRNA gene sequences, and the partial COI mtRNA gene sequences (Figures 7–9). The BI tree (50% majority rule consensus tree) of a multiple-edited alignment included 108 18S rRNA sequences of *Paratylenchus* spp. (Figure 7) and two outgroup species [*Hemicycliophora aquatica* (Micoletzky, 1913) Loos, 1948 (MF094911), and *Hemicriconemoides kanayaensis* Nakasono & Ichinoe, 1961 (MG029558)] (Figure 7). A total of nine new sequences were obtained for this ribosomal molecular marker. The BI tree inferred from the analysis of the 18S sequence alignment contained highly or moderately supported major clades (PP = 1.0 and P = 0.99) (Figure 7). The general topology of this BI tree, including its clades, is slightly coincident with recent phylogenetic studies on paratylenchids [7,42]. Likewise, the BI tree (50% majority rule consensus tree) of the D2–D3 segments of 28S rRNA gene (Figure 8) was based on a multiple-edited alignment included 182 28S sequences of *Paratylenchus* spp. and three outgroup species (*Basiria gracilis* (Thorne, 1949) Siddiqi, 1963, MW716278; *Filenchus* sp., JQ005014; *Aglenchus geraerti* Mizukubo, 1989, MK639370) (Figure 8). A total of twenty-four new sequences were obtained for this ribosomal molecular marker and included in this analysis. BI tree inferred from the analysis of D2–D3 sequence alignment contained highly or moderately supported major clades (PP = 1.0, PP = 0.94 and PP = 0.92). The general topology of this BI tree, including its clades, is mainly coincident with recent phylogenetic studies on paratylenchids [5–7]. Similarly, the BI tree (50% majority rule consensus tree) of a multiple-edited alignment included 169 COI mtRNA sequences of *Paratylenchus* spp. and two outgroup species (*Mesocriconema* sp., KY574819; *Mesocriconema discus* (Thorne & Malek, 1968) Loof & De Grisse, 1989, KY574622) (Figure 9). A total of eleven new sequences that include all the Portugueses populations of *Paratylenchus* spp. except for *P. tenuicaudatus* were obtained for this mitochondrial molecular marker and included in this phylogenetic analysis. BI tree inferred from the analysis of COI sequence alignment contained highly or moderately supported major clades (PP = 1.0, and PP = 0.98). The general topology of this BI tree, including its clades, was similar to that of the D2–D3 segments of the 28S gene and slightly coincident with recent phylogenetic studies on paratylenchids [5–7].

The generated phylogenetic tree inferred from 18S rRNA gene sequences showed incongruences between the pattern of branching in the phylogenetic tree and the current taxonomy within *Paratylenchus* genus (Figure 7). So, this ribosomal molecular marker failed to delimit well established species; for example *P. goodeyi* (OM345189– OM345190, Portugal; MW413698–MW413699, Belgium), *P. nanus* (MW413707–MW413708, MW413711, Belgium) and *P. veruculatus* (OM345187– OM345188, Portugal; MW413747–MW413748, Belgium) were grouped together as belonging to the same species. Furthermore, the generated D2–D3 phylogenetic tree showed a congruent position for all known species (*P. goodeyi, P. hamatus*, *P. pedrami*, *P. tenuicaudatus*, *P. variabilis* and *P. veruculatus*) found in this study (Figure 8). D2–D3 phylogenetic tree clustered, with strong support, our Portuguese sequences of *P. pedrami* (OM348562–OM348566, Portugal; OM348550–OM348551, Portugal) from this study with other Spanish isolates of this same species (MZ265118, Spain; MW798283–MW798285, Spain). Thus, all these *P. pedrami* populations form a cluster clearly separated from a morphologically related species as *P. baldaccii* (MZ265079, MW798290–MW798291, Spain). *Paratylenchus hamatus* sequences (OM348545–OM348547, Portugal) were grouped in a well-supported clade also containing this same species (MW798297–MW798298; Spain) which are clearly separated of *P. tenuicaudatus* (OM348544, Portugal; MW798308–MW798309, Spain; KU291239, Iran) and other members of the *P. hamatus* "species complex" (KF242220–KF242222, MW413670). D2–D3 tree clustered, with strong support, our Portuguese sequences of *P. veruculatus* (OM348552, OM348554–OM348555, OM348561, Portugal) from this study with other Spanish and Belgian isolates of this same

species (MW413687, Belgium; MW798310–MW798315, Spain). Thus, all these *P. veruculatus* populations constitute a cluster clearly separated from Iberian populations of *P. variabilis* (OM348548–OM348549, Portugal; MZ265127–MZ265129, Spain) and other *Paratylenchus* spp. as *P. microdorus* (MW413654, Belgium) and *P. recisus* (MZ265119–MZ265120, Spain). *Paratylenchus goodeyi* (OM348543, OM348553, OM348556–OM348560, Portugal) clustered in a well-supported clade also contain Spanish and Belgian isolates of this same species (MW798293–MW798294, Spain; MW413631, Belgium) which are separated of close *Paratylenchus* species as *P. pandatus* (MZ265116–MZ265117, Spain) and *P. straeleni* (KF242234, USA). Likewise, phylogenetic analysis showed that the COI tree is congruent with 28S rRNA phylogenies (Figure 9). Both D2–D3 and COI trees showed topologies partially coincident with other recent studies with, in some cases, similar or different clade support [5–7].

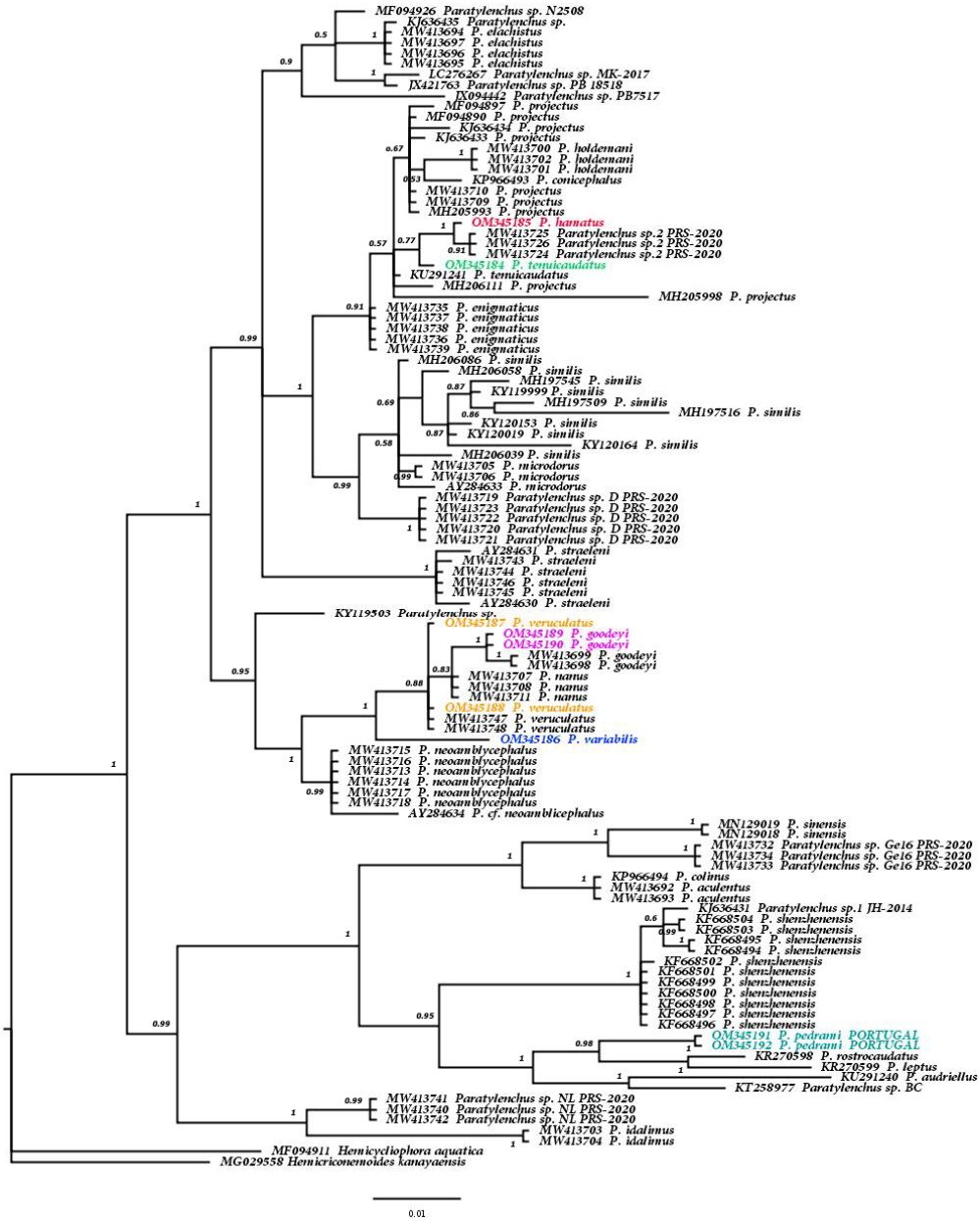

Figure 7. Phylogenetic relationships within the genus *Paratylenchus*. Bayesian 50% majority rule consensus trees as inferred from 18S rRNA sequences alignments under the K80 model with a gamma-shaped distribution. Posterior probabilities of more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are coloured in red, green, yellow, pink, navy blue, and sky blue. Scale bar = expected changes per site.

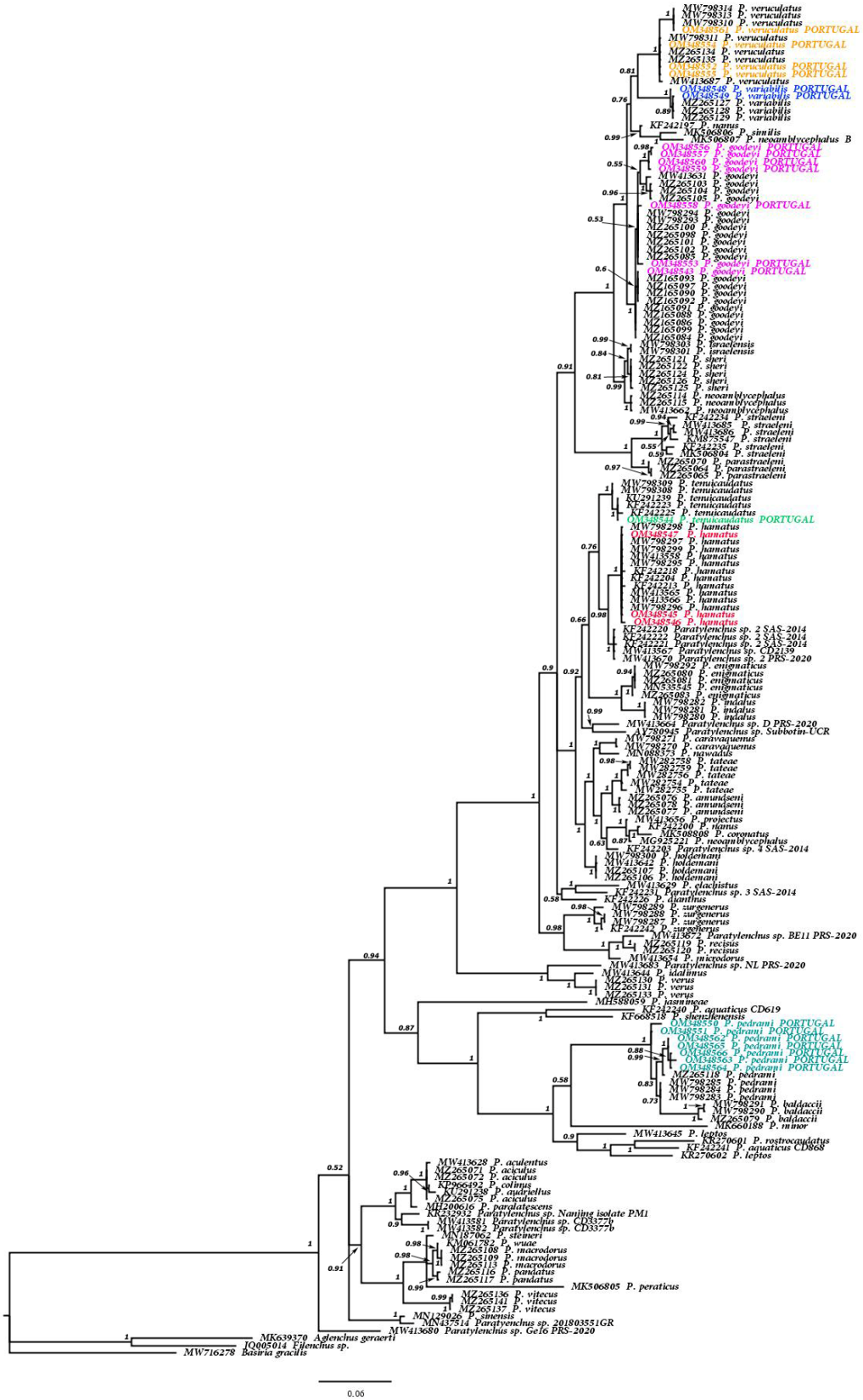

Figure 8. Phylogenetic relationships within the genus *Paratylenchus*. Bayesian 50% majority rule consensus trees as inferred from 28S rRNA sequences alignments under the GTR model with a gamma-shaped distribution. Posterior probabilities of more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are coloured in red, green, yellow, pink, navy blue, and sky blue. Scale bar = expected changes per site.

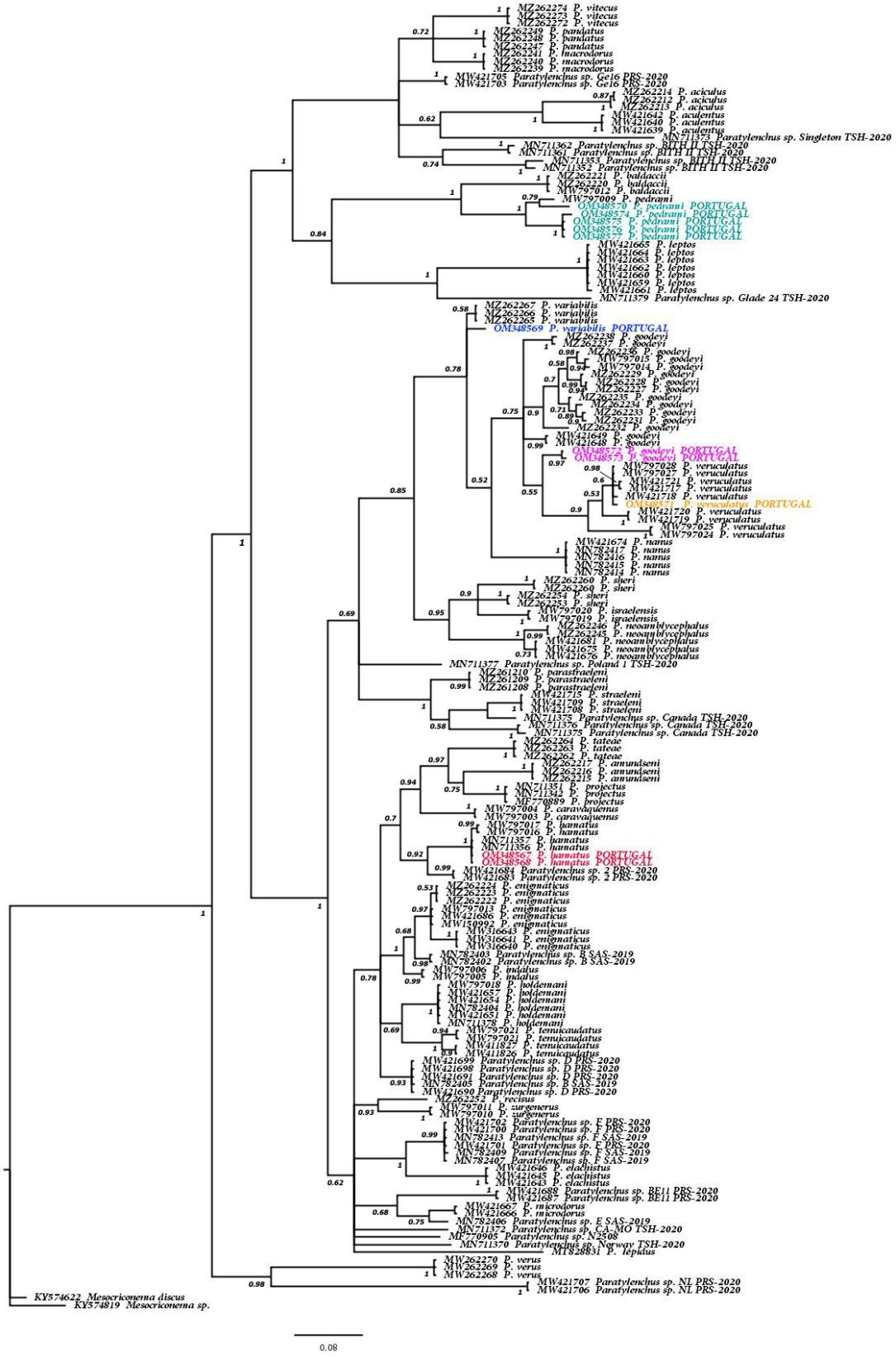

Figure 9. Phylogenetic relationships within the genus *Paratylenchus*. Bayesian 50% majority rule consensus trees as inferred from COI sequences alignments under the GTR model with a gamma-shaped distribution. Posterior probabilities of more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are coloured in red, yellow, pink, navy blue, and sky blue. Scale bar = expected changes per site.

4. Discussion

This research is the first study of pin nematodes of the genus *Paratylenchus* carried out in Portugal improving the knowledge of their biodiversity, and expanding the molecular information useful for their unequivocal identification. The main goal of our study was to identify and describe, morphologically and molecularly, *Paratylenchus* spp. parasitizing the roots of grapevines in vineyards of the Southern and Central Portugal. This was conducted in a nematological survey that included 44 sampling sites. Seven soil samples, each infested with only one pin nematode population, except one of them with two populations, were selected for this detailed study. Our results confirmed the usefulness of developing a robust taxonomic approach based on the contrasting of morphological data with genotyping rRNA markers to correctly discriminate among *Paratylenchus* species. We identified several populations by integrating morphological observations, morphometrics, and molecular data based on rRNA sequences to elucidate their phylogenetic relationships within *Paratylenchus* spp. Molecular markers were generated for the known pin nematode species, and the molecular genetic diversity of these six species (*P. goodeyi*, *P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis*, and *P. veruculatus*) was evaluated. New molecular markers were generated for some of these pin nematode species.

This integrative taxonomic study of eight Portuguese populations of *Paratylenchus* spp. confirmed that the identification of pin nematode species from phenotypic features is not easy because of their small body size together with high inter- and intra-specific trait variations and plasticity in morphological traits. As for previous biogeographic studies [5,6], our study has revealed six first report of *Paratylenchus* spp. for Portugal (*P. goodeyi*, *P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis*, and *P. veruculatus*). Our findings confirm previous results [5,6], that some *Paratylenchus* species are spreading in more than one continent, and thus more widely spread than supposed.

Forty-four sequences belonging to two nuclear rRNA and one mtRNA markers were generated in this study: 24 D2–D3, 9 18S, and 11 partial COI sequences. The results corroborate previous studies [5,6,8,42,46–48] in finding the utility of this integrative approach and molecular data for species discrimination in *Paratylenchus*. Our findings are in accordance with the results of recent studies [5,6], since they showed that the D2–D3 of the 28S rRNA and the COI region within mtRNA were the most decisive, precise, and reliable molecular markers for discriminating among species and for the diagnosis at species level within *Paratylenchus* genus. Therefore, our findings confirm that the D2–D3 region of the 28S rRNA gene should be considered the most effective molecular marker for the species-level identification of paratylenchids. In spite of the excellent reputation of the partial COI mtRNA gene has for allowing discrimination among *Paratylenchus* species [61], our findings agree with other previous studies [5–7] confirming that this molecular marker sometimes shows high intraspecific variation. The 18S rRNA gene is highly conserved intra-species (so that similarities are close to 99–100%); in fact our complete sequence (approximately 1600 bp) did not provide good resolution at the lower taxonomic levels failing to delimit well established *Paratylenchus* species. However, this 18S ribosomal molecular marker was useful for discrimination and delimitation of some specific species within the *Paratylenchus* genus. For example, in *P. pedrami*, the complete 18S rRNA sequence (OM345191–OM345191) was compared to other sequences belonging to known *Paratylenchus* species (KF668497–KF668497, KF668499, KF668502, KF668504, KJ636331) and was 96% similar with fewer than 74 nucleotides differences, whereas sequences of the COI region (OM348574–OM348577, OM348570) also had a low homology with maximum similarity values of 96% to *P. pedrami* (MW797009) and the fragments D2–D3 of 28S rRNA (OM348562–OM348566; OM348550–OM348551) showed a high homology with maximum similarity values of 99% to *P. pedrami* (MZ265118, MW798283–MW798285).

Phylogenetic trees reconstructed by the BI approach using new *Paratylenchus* sequences and others from Genbank inferred similar patterns. Our 28S phylogenetic trees indicated that *P. pedrami* (OM348562–OM348566, OM348550–OM348551, Portugal; MW738282–MW738285, Spain; MZ265118, Spain) was clearly grouped in a sub-clade with *P. bal-*

daccii (MW738290–MW738291, Spain; MZ265079, Spain), and *P. minor* (MK660188, China), which are three species sharing a similar morphology characterized mainly by a short to medium-sized style length (around 30 µm). However, as is common in phylogenetic studies, this three species were also inside in an upper sub-clade with other morphologically related species showing smaller stylet length (*P. leptus* Raski 1975, KR270602, MW413645; *P. rostrocaudatus* Huang & Raski, 1987, KR270601; *P. aquaticus* Merny, 1966, KF242240–KF242241; *P. shenzhenensis* Wang, Xie, Li, Xu, Yu, & Wang, 2013, KF668518). Likewise, *P. variabilis* (OM348548–OM348549, Portugal; MZ265127–MZ265129, Spain), was grouped in a well-supported sub-clade with a large number of species (*P. nanus*, *P. veruculatus*, *P. goodeyi*, *P. similis*, *P. variabilis*) which are six species sharing quite phenotypic features among them. For example, *P. similis* (MK506806), *P. nanus* (KF242197) and *P. veruculatus* (OM348561, OM348552–OM348553, Portugal; MW798310–MW798311, MW798313–MW798314, Spain; MZ265134–MZ265135, Spain; MW413687, Belgium) sharing a relatively short-sized style length (less than 25 µm), however *P. goodeyi* show a longer stylet (OM358543, OM358556–OM358560, Portugal; MZ265084–MZ265085, MZ265091–MZ265095, MZ265097–MZ265105, Spain; MW413631, Belgium). Similarly, *P. tenuicaudatus* (OM348544, Portugal; MW798306–MW798309, Spain; KU291239) was clearly grouped in a sub-clade to several species characterized by a short to medium-sized style length (around 30 µm) (*P. hamatus*: OM348545–OM348547, MW798285–MW798289, KF242215; *P. enigmaticus* Munawar, Yevtushenko, Palomares-Rius and Castillo, 2021: MW798292, MN535545; MZ265080- MZ265084; *P. indalus*: MW798273–MW798282). Thus, the phylogenetic tree topologies, particularly based on our 28S rRNA dataset, agrees with previous phylogenetic studies [5–7,42]. Regarding stylet in *Paratylenchus* spp. it is worth to note that there is a high morphological diversity and its feeding role denotes a decisive factor in the fitness within this genus. Likewise, the 28S tree topology denotes possibly a monophyletic origin of *Paratylenchus* spp., in which a long stylet (more than 40 µm) is a plesiomorphic character. In addition, the pattern of branching in the 28S phylogenetic tree denotes two synapomorphies, so that intermediate stylet length (around 30 µm) and short stylet length (less than 25 µm), in the major evolutionary events of the *Paratylenchus* genus. Munawar et al. [42] and Clavero-Camacho et al. [5,6] supported a significant association between the phylogenetic tree topologies and the division defined by Ghaderi et al. [45] regarding stylet length (less than 40 vs. more than 40 µm), however they did not keep a intermedium-size stylet length in mind. Furthermore, this study confirms the results of previous studies [5–7,42,45] that it is common to find a same soil sample showing the co-existence of *Paratylenchus* species. Niche partitioning is the ecological process that reduces interspecific competition in which natural selection drives competing species into different patterns of resource use or niche in time and/or space [91]. Thus, the ecological theory could help to better understand of this phylogenetic flexibility characterized by multiple evolutionary synapomorphies. For example, *P. goodeyi* and *P. veruculatus* are two species close phylogenetically but they have clear phenotypic differences regarding the stylet length that are commonly found together in the same sample soil. In fact, *P. goodeyi* has a long stylet that allows this species to feed in root cell layers deep in both taproots and fibrous roots, primary or secondary roots of herbaceous and woody plants and does not need to move frequently to new feeding sites, while *P. veruculatus* has a short and rigid stylet that allows this nematode to feed in root cells of outside layers in thin lateral roots of herbaceous and woody plants and need to move drilling new feeding sites..

5. Conclusions

Our work contributes with relevant understanding of the occurrence, distribution and biodiversity of *Paratylenchus* species infesting grapevine soils in Portugal. This study describes six first reports of *Paratylenchus* spp. (*P. goodeyi, P. hamatus*, *P. pedrami*, *P. tenicaudatus*, *P. variabilis,* and *P. veruculatus*) for the country by utilizing morphological features, morphometric measures and molecular data, and establishes these species' phylogenetic relationships within the genus *Paratylenchus*. Our study also highlights the value of using

rRNA molecular markers for the identification of *Paratylenchus* spp., when other conventional methods alone based on morphology are inconclusive in this taxonomically confusing genus. Likewise, we generated molecular markers for precise and unequivocal diagnosis of different known *Paratylenchus* species, evaluated their molecular genetic diversity and established molecular phylogenetic relationship that shows new insights into the systematics and the evolution of *Paratylenchus* genus. In addition, our results confirm that the diversity of paratylenchid nematodes in commercial vineyards is underrepresented, since the species reported in this study are all new records for Portugal. However, this is the first systematic study for *Paratylenchus* detection in Portugal using a taxonomic integrative approach and increasing species diversity is expected to be found in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8040343/s1, Table S1: List of the primers used in this study.

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