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Prevalence and molecular diversity of reniform nematodes of the genus *Rotylenchulus* (Nematoda: Rotylenchulinae) in the Mediterranean Basin

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Abstract The reniform nematodes of the genus *Rotylenchulus* are semi-endoparasites of numerous herbaceous and woody plant roots and distributed in regions with Mediterranean, subtropical and tropical climates. In this study, we provide morphological and molecular characterisation of three out of 11 valid species of the genus *Rotylenchulus*: *R. macrodoratus, R. macrosoma,* and *R. reniformis* from Greece (Crete), Italy and Spain. The overall prevalence of reniform nematodes in wild and cultivated olives in Greece, Italy, and Spain was 11.5%, 19.0% and 0.6%, respectively. In Greece, *R. macrodoratus* and *R. macrosoma* were detected in cultivated olive with a prevalence of 8.2% and 6.2%, respectively, but none of them were found in wild olive. This is the first report of *R. macrosoma* in Greece. Only one reniform nematode species was detected in olive from Italy and Spain, *viz. R. macrodoratus* and *R. macrosoma*, respectively. The parasitism of *R. macrosoma* on hazelnut in northern Spain was also confirmed for the first time. This study demonstrates that *R. macrodoratus* and *R. macrosoma* have two distinct rRNA gene types in their genomes, specifically the two types of D2-D3 for *R. macrosoma* and *R. macrodoratus*, the two types of ITS for *R. macrodoratus* and the testing of the ITS variability in other *R. macrosoma* populations in different countries. *Rotylenchulus macrosoma* from Greece and Spain showed differences in nucleotide sequences in the ITS region and D2-D3 of 28S rRNA gene.

Keywords Cytochrome c oxidase subunit 1 · D2-D3 region · ITS-rRNA · Phylogeny · *R. macrodoratus* · *R. macrosoma* · *R. reniformis* · Taxonomy

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Introduction

Reniform nematodes of the genus *Rotylenchulus* Linford and Oliveira 1940 comprise 11 valid species (Van den Berg et al. 2016). These nematodes are semi-endoparasites of numerous herbaceous and woody plant roots and mainly distributed in tropical and subtropical regions. The type species of the genus, *Rotylenchulus reniformis* Linford and Oliveira 1940, is commonly named as the reniform nematode because its swollen females have a kidney-like body shape with a rounded and not elongated body terminus. For convenience, we indicate with this common term all of the species of the genus in spite of the fact their swollen females have posterior body end elongated rather than rounded as in *R. reniformis*. *Rotylenchulus reniformis* is the most important species from this genus and considered as a major pathogen of cotton and other crops in USA and several other countries (Robinson et al. 1997). The second significant pathogen is *Rotylenchulus parvus*, whereas the other species have a limited distribution and are of minor economic importance (Gaur and Perry 1991, Robinson et al. 1997).

Morphological identification of *Rotylenchulus* spp. is based on characteristics of immature females such as stylet length, position of vulva (V ratio), lip region shape and tail shape (Dasgupta et al. 1968, Germani 1978, Robinson et al. 1997). However, high intraspecific variability of some diagnostic features makes identification of this group based on morphology a difficult task. Recently, DNA-based approaches have been successfully used for the molecular diagnostics and diversity of species of *Rotylenchulus* (Agudelo et al. 2005, Leach et al. 2012, Nyaku et al. 2013a, Deng et al. 2015, Van den Berg et al. 2016). The phylogenetic analysis also confirmed the hypothesis that this genus was originated from the Afrotropical zoogeographical region (Van den Berg et al. 2016). These works reported high levels of intraspecific and intra-individual variations of rRNA with two or more distinct types of rRNA genes depending on the species studied. Reconstruction of secondary structure models for two types of the D2 of 28S rRNA for *R. reniformis* and mutation mapping showed that both models have similar conservative folding and most point mutations were compensatory, confirming that both gene rRNA types are functional (Van den Berg et al. 2016). These types of different rRNA seems also to be expressed in nematode tissues, as they were present in *R. reniformis* ESTs (Nyaku et al. 2013b). The extensive presence of several copies of rRNA genes in the majority of the species of this group with molecular data has important implications for the sequence-based phylogenetic analyses and additional markers should be studied (Van den Berg et al. 2016).

Three reniform nematode species have been reported in several herbaceous and woody plants in the Mediterranean Basin: (i) *R. macrodoratus* (the Mediterranean reniform nematode) in common ivy (*Hedera helix* L.) in France (Scotto La Massèse 1973), Italian oak (*Quercus frainetto* Ten.) and grapevine in Greece (Vovlas and Lamberti 1974, Koliopanos and Vovlas 1977, Vovlas and Vlachopoulos 1991), and grapevine, almond, plum, fig, apricot, *Phlomis fruticosa* L., *Ceratonia siliqua* L., *Nerium oleander* L., and cultivated olives in Italy (Dasgupta et al. 1968, Talamé et al. 1970, Vovlas and Lamberti 1974), apricot in Malta (Vovlas and Lamberti 1974); (ii) *R. macrosoma* in wild and cultivated olives in Spain (Castillo et al. 2003a, Van den Berg et al. 2016), carnation, *Parietaria officinalis* L., and *Eriobotrya japonica* (Thunb.) Lindl, and (iii) *R. reniformis* in several ornamental plants and papaya in Spain (Artero et al. 1977, Inserra and Vovlas 1980, Castillo and Gómez-Barcina 1993). In particular, no molecular characterization on *R. reniformis* for the reports from Spain are available. *Rotylenchulus reniformis* has also been reported on grapevine, olive and citrus in Greece (Hirschmann et al. 1966), but these reports could be a misidentification of *R. macrodoratus* as suggested by Lamberti and Vovlas (1993). Recent nematode surveys conducted from 2012 till 2016 on wild and cultivated olive orchards, as well as samples for nematode diagnostics by farmers in Crete (Greece), Italy and Spain revealed soil infestations of three reniform nematode species (*Rotylenchulus* sp.). This prompted us to undertake a detailed integrative diagnosis of these populations, as well as a molecular comparative study with previous reported data to decipher the molecular diversity in this group of nematodes in the Mediterranean basin.

The objectives of this paper were: *i*) to verify the identity of these reniform species using integrative taxonomy, which are described herein as *R. macrodoratus*, *R. macrosoma, R. reniformis*; *ii*) to determine the prevalence of these nematodes in olive and other Mediterranean crops; and iii) to increase the knowledge of rRNA intra-individual and intra-species variability within *Rotylenchulus* species.

Material and methods

Nematode population sampling, extraction and morphological identification

Nematode populations used in this study were collected from wild and cultivated olives from geographically diverse locations in Crete (Greece), Italy and Spain (Table 1). In Crete, Italy, and Spain, 182 samples (146 from cultivated olive and 36 from wild olive), 70 samples (63 from cultivated olive and 7 from wild olive) and 499 samples (376 from cultivated olive and 123 from wild olive) were collected, respectively. Soil samples were collected with a mattock and a sampler from 10 up to 40 cm depth depending on soil condition from the rhizosphere of three to five plants randomly chosen in each field for cultivated olive and from individual wild olives. Furthermore, reniform nematodes extracted from soil samples from grapevine, hazelnut, and cotton that had been brought by farmers to the I.O.S.V, Crete and IAS-CSIC, Spain laboratories for investigation, were also included in this study. Nematode specimens from the soil samples were extracted using the centrifugal-flotation method (Coolen 1979) and the wet sieving and decanting method (Cobb 1918). Specimens were fixed in hot TAF (no more than 70 °C) or 4% formaldehyde +1% propionic acid (Seinhorst 1966). Adult specimens of each sample were processed to glycerin and mounted on glass slides for species identification (De Grisse 1969). Photographs were taken using a Zeiss III compound microscope with Nomarski differential interference contrast at up to ×1000 magnification. Measurements were done using a drawing tube attached to the microscope. Prevalence of infestation and population density of reniform nematodes from samples were determined. Prevalence in olive and wild olive was calculated as the percentage of samples in which a nematode species was diagnosed with respect to total number of samples. Nematode population density in soil was assessed for each sample and calculated as the average of the soil counts.

DNA extraction, PCR and sequencing

For molecular analyses and in order to avoid mistakes in the case of mixed populations in the same sample, two nematodes preserved in DESS (Yoder et al. 2006) from each sample were temporary mounted in a drop of 1 M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure that specimens conformed in form to the unidentified populations of *Rotylenchulus*. Morphometrics and photomicrographs recorded during this initial study were not used as part of the morphological study or analyses. Following morphological confirmation, the specimens were removed from the slides and DNA extracted (Van den Berg et al. 2016). Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (2003b). For molecular analyses, nematode DNA from *Rotylenchulus* samples was extracted from single or several individuals using proteinase K as described by Castillo et al. (2003b). PCR and sequencing was completed in the laboratory of IAS-CSIC, Spain. The primer sets for amplification of the nuclear ribosomal RNA (D2-D3 of 28S rRNA, ITS region), and mitochondrial cytochrome c oxidase subunit 1(*coxI*) genes are given in Table 2.

PCR cycle conditions for the ribosomal DNA markers were: one cycle of 94 °C for 2 min, followed by 35 cycles,of 94 °C for 30 s, an annealing temperature of 55 °C for 45 s, 72 °C for 1 min, and finally one cycle of 72 °C for 10 min. The cycle for mtDNA was as described by He et al. (2005): 95 °C for 10 min, five cycles at 94 °C for 30 s, 45 °C for 40 s, and 72 °C for 1 min, and a further 35 cycles at 94 °C for 30 s, 37 °C for 30 s, and 72 °C for 1 min, followed by an extension at 72 °C for10 min. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred to above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit V.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 1.

Several primers were designed using a dataset from Van den Berg et al. (2016) and sequences obtained in this study using conventional primers described above in order to find additional haplotypes for *R. macrosoma* and *R. macrodoratus*, as well as additional ITS region sequences for *R. macrodoratus* (Table 2). Primers were designed using aligned regions from GenBank using D2A primers (De Ley et al. 1999) and degenerating

Table 1 Reniform nematode species (*Rotylenchulus* spp.) and sequences used in this study

Nematode species	Host-plant, locality	Sample code	Nematodes/ 500 ml^3 soil	GenBank accession		
				D2-D3	*ITS*	*coxI*
1. Rotylenchulus macrodoratus						
1. *R. macrodoratus*	Cultivated olive, Petrokefali, Crete, Greece	OLI028	9	KY992779	-	-
2. *R. macrodoratus*	Cultivated olive, Voutes, Crete, Greece	OLI030	6	KY992780 KY992781	KY992810	KY992841
3. *R. macrodoratus*	Cultivated olive, Stavrakia, Crete, Greece	OLI051	4	KY992782 KY992783	KY992811	KY992842
4. *R. macrodoratus*	Cultivated olive, Stavrakia, Crete, Greece	OLI053	4	KY992784	-	-
5. *R. macrodoratus*	Cultivated olive, Voutes, Crete, Greece	OLI054	8	*	-	-
6. *R. macrodoratus*	Cultivated olive, Pentamodi, Crete, Greece	OLI056	6	*	KY992812	-
7. *R. macrodoratus*	Cultivated olive, Pentamodi, Crete, Greece	OLI057	3	KY992785	KY992813	-
8. *R. macrodoratus*	Cultivated olive, Pentamodi, Crete, Greece	OLI061	3	KY992786	KY992814 KY992815	-
9. *R. macrodoratus*	Cultivated olive, Roufas, Crete, Greece	OLI006	10	*	-	-
10. *R. macrodoratus*	Cultivated olive, Petrokefali, Crete, Greece	OLI029	5	*	-	-
11. *R. macrodoratus*	Cultivated olive, Pentamodi, Crete, Greece	OLI031	5	*	-	-
12. *R. macrodoratus*	Cultivated olive, Voutes, Crete, Greece	OLI035	12	*	-	-
13. *R. macrodoratus*	Grapevine, P. Elias, Crete, Greece	GRA01	10	KY992787 KY992788	KY992816 KY992817	KY992843
14. *R. macrodoratus*	Grapevine, P. Elias, Crete, Greece	GRA02	7	KY992789	KY992818	KY992844
15. *R. macrodoratus*	Grapevine, Archanes, Crete, Greece	GRA03	5	*	-	-
16. *R. macrodoratus*	Grapevine, Sylamos, Crete, Greece	GRA04	9	KY992790	KY992819 KY992820	-
17. *R. macrodoratus*	Cultivated olive, Maruggio, Taranto, Italy	OLIVIT	39	KT003762 KY992791 KY992792	KT003795 KY992821	KT003719
18. *R. macrodoratus*	Grapevine, Bari, Italy	TOPOT	17	KT003758	KT003797 KY992822	KT003722
19. *R. macrodoratus*	Grapevine, Bari, Italy	GRAIT	29	KT003760	KT003794 KY992823	KT003721
2. Rotylenchulus macrosoma						
1. *R. macrosoma*	Cultivated olive, Petrokefali, Crete	OLI108	4	KY992793	KY992824	-
2. *R. macrosoma*	Cultivated olive, Hersonisos, Crete	OLI038	13	KY992794 KY992795	KY992825 KY992826	KY992845
3. *R. macrosoma*	Cultivated olive, Hersonisos, Crete	OLI040	3	KY992796 KY992797	KY992827	-

Table 1 (continued)

Nematode species	Host-plant, locality	Sample code	Nematodes/ 500 ml^3 soil	GenBank accession		
				D2-D3	*ITS*	*coxI*
4. *R. macrosoma*	Cultivated olive, Istro, Crete	OLI087	3	KY992798 KY992799	KY992828 KY992829	KY992846
5. *R. macrosoma*	Cultivated olive, Limnes, Crete	OLI117	3	KY992800 KY992801	KY992830	KY992847
6. *R. macrosoma*	Cultivated olive, Limnes, Crete	OLI119	8	KY992802	KY992831	KY992848
7. *R. macrosoma*	Cultivated olive, Dermatos, Crete	OLI142	6	KY992803 KY992804	KY992832	-
8. *R. macrosoma*	Cultivated olive, Peri, Crete	OLI009	5	*	-	-
9. *R. macrosoma*	Cultivated olive, Episkopi, Crete	OLI008	2	*	-	-
10. *R. macrosoma*	Wild olive, Vejer, Cádiz, Spain	BAETI	2	KT003748	KT003800	KT003724
11. *R. macrosoma*	Cultivated olive, Jerez de la Frontera, Cádiz, Spain	J0096	876	KT003747 KY992805 KY992806	KT003805 KY992833 KY992834	KT003725
12. *R. macrosoma*	Cultivated olive, Huévar del Aljarafe, Sevilla, Spain	ST079	32	KT003750	KT003802 KY992835 KY992836	KT003726
12. R. macrosoma	Hazelnut, Reus, Tarragona, Spain	REU01	8	KY992807	KY992837	KY992849
3. Rotylenchulus reniformis						
13. R. reniformis	Cotton, Los Palacios, Sevilla, Spain	CAM17	17,200	KY992808 KY992809	KY992838 KY992839 KY992840	KY992850 KY992851 KY992852

(−) Not obtained

(*) Sequenced population but not deposited in GenBank database because of their high similarity (see discussion section)

Table 2 Primer sets used in the present study

Primer code*	Sequence (5′-3′)	Amplified gene	Reference
TW81 (f) AB28 (r)	GTT TCC GTA GGT GAA CCT GC ATA TGC TTA AGT TCA GCG GGT	ITS rRNA	Tanha Maafi *et al*. (2003)
ITS_MACRODORATUS_FW2 (f)	GGTGAACCTGCTGCTGGATCATTAC	ITS rRNA	this study
ITS_MACRODORATUS_RV (r)	CRCGTCTGAGYTCAGGTCG		
ITS_MACROSOMA-A (f)	TTCTGCTGGCGTCTCTGCGTTG	ITS rRNA	this study
ITS_MACROSOMA-A (r)	ACCCTGAACCAGACGTGCCACT		
ITS_MACROSOMA-B (f)	ATTGCACCCGCTCTAGGGGCAT	ITS rRNA	this study
ITS_MACROSOMA-B (r)	CGTGCCACAGTGCAAGAACAGC		
D2A (f) D3B (r)	ACA AGT ACC GTG AGG GAA AGT TG TCG GAA GGA ACC AGC TAC TA	D2-D3 of 28S rRNA	De Ley et al. (1999)
D2A_ROT (f) D3B (r)	ACAAGTACYGTGARGGAAAGTTG TCG GAA GGA ACC AGC TAC TA	D2-D3 of rRNA	this study, De Ley *et al.* (1999)
JB3 (f) JB4.5 (r)	TTT TTT GGG CAT CCT GAG GTT TAT TAA AGA AAG AAC ATA ATGA AAA TG	*coxI* mtDNA	Bowles *et al.* (1992)

*(f) – forward primer; (r) – reverse primer

polymorphic sites for a new D2A primer were included D2A_ROT (5′-ACAAGTACYGTGARGGAAAGTTG-3′) in order to obtain the two types for *R. macrosoma* and *R. macrodoratus*. *Rotylenchulus macrodoratus* ITS region sequence was obtained by designing specific PCR in this region (ITS_MACRODORATUS_FW2 and ITS_MACRODORATUS_RV) for cloning, while for *R. macrosoma*, a specific PCR for each type were used [ITS_MACROSOMA-A (f and r) and ITS_MACROSOMA-B (f and r)] designing the primers with the program MPprimer (Shen et al. 2010). Additionally, the primer ITS_MACRODORATUS_FW2 was combined with the primer D3B in order to obtain longer D2-D3 regions in *R. macrodoratus*. PCR conditions and sequencing were identical to those used for ITS region.

Phylogenetic analyses

The D2-D3 expansion segments of 28S rRNA, ITS, and partial *coxI* sequences of different *Rotylenchulus* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen following previous published studies (Subbotin et al. 2006, Van den Berg et al. 2016). Multiple sequence alignments of the different genes were made using the Q-INS-i algorithm of MAFFT V.7.205 (Katoh and Standley 2013), which accounts for secondary RNA structure. Sequence alignments were visualised using BioEdit (Hall 1999) and edited by Gblocks ver. 0.91b (Castresana 2000) in the Castresana Laboratory server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences +1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Percentage similarity between sequences was calculated with a sequence identity matrix using BioEdit. For that, the score for each pair of sequences was compared directly and all gap or place-holding characters were treated as a gap. When the same position for both sequences had a gap it was not treated as a difference. Phylogenetic analyses of the sequence datasets were based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fit model of DNA evolution was obtained using JModelTest v.2.1.7 (Darriba et al. 2012) with the Akaike Information Criterion (AIC). The best-fit model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then given to MrBayes for phylogenetic analyses. Models were: i) general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) for the D2-D3 expansion segments of 28S rRNA, ii) an equal-frequency transversion model with invariable sites and a gamma-shaped distribution (TVMef + I + G) for the ITS region, and iii) a transversional

model with invariable sites and a gamma-shaped distribution (TVM + I + G) for the partial *coxI* gene. These BI analyses were run separately per dataset using four chains for 2×10^6 generations for the D2-D3, and 3×10^6 generations for ITS region and 2×10^6 generations for the partial *coxI*. A combined analysis of the three genes was not undertaken due to some sequences not being available for all species. The Markov Chains were sampled at intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority-rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees from all analyses were visualised using TreeView (Page 1996) and FigTree software V.1.42 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Within the samples studied in Crete (Greece), Italy and Spain on wild and cultivated olives, grapevine, hazelnut and cotton, we distinguished and characterised three known valid species of reniform nematodes: *R. macrodoratus*, *R. macrosoma*, and *R. reniformis*. As *R. macrodoratus* from Greece and Italy and *R. macrosoma* from Spain have been already reported and well characterized morphological- and morphometrically in previous studies (Talamé et al. 1970, Koliopanos and Vovlas 1977, Vovlas and Vlachopoulos 1991, Castillo et al. 2003a, Van den Berg et al. 2016), these species were morphological and morphometrically checked for identification but studied molecularly only. However, two new populations of *R. macrosoma* from cultivated olive at Crete and a population of *R. reniformis* from cotton in southern Spain were studied morphologically and morphometrically because no description or illustrations of the latter species was provided in previous records in Spain (Artero et al. 1977, Castillo and Gómez-Barcina 1993). Morphological and morphometric characterisations of *R. macrosoma* and *R. reniformis* are given below (Fig. 1, Tables 3 and 4).

The overall prevalence of reniform nematodes in olives (wild and cultivated) in Crete, Italy, and Spain was 11.5%, 19.0% and 0.6%, respectively (Table 1). In Crete, *R. macrodoratus* and *R. macrosoma* were detected in cultivated olive but none of them in wild olive, with a prevalence of 8.2% and 6.2%, respectively. However, only one reniform species was detected in olive from Italy and Spain, *viz*. *R. macrodoratus* and *R. macrosoma*, respectively. In Italy, *R. macrodoratus* was detected in cultivated olive only with the highest level of prevalence 19.0% (12 samples out of 63). Whereas in Spain, *R. macrosoma* was detected in cultivated and wild olives but showing the lowest level of prevalence 0.5% (2 samples out of 376) and 0.8% (1 sample out of 123), respectively. Nematode population densities of reniform nematodes in cultivated and wild olives were moderate to high and ranging from 3 to 876 nematodes/500 ml^3 of soil (Table 1). The lowest population density was detected for *R. macrosoma* in wild olive in Spain and *R. macrodoratus* in cultivated olive in Crete, while the highest densities were detected for *R. macrosoma* in cultivated olive in Spain (Table 1). However, even in this case with high nematode soil infestations (1.75 nematodes/ ml^3 soil), no damage symptoms were detected in aboveground plant parts.

Reniform nematodes were also found in soil samples from grapevine, hazelnut, and cotton. Cotton samples with *R. reniformis* were localized in southern Spain (Los Palacios, Sevilla, Spain) with 17,200 individuals/500 ml^3 of soil, three-four months after cotton crop in a sandy soil. No visual symptoms on the crop were detected at this density. *Rotylenchulus macrosoma* was also detected in hazelnut in the north-East Spain (Reus, Tarragona, Spain) at a density of 8 individuals/500 ml^3 of soil. Grapevine was also a good host for *R. macrodoratus* in Italy and Crete with 2 and 4 grapevine localities with nematodes densities ranging from 5 to 29 individuals/500 ml^3 of soil.

Rotylenchulus macrodoratus Dasgupta et al. 1968

The morphological and morphometrical diagnostic traits of the reniform nematode populations of this species detected in Crete (Greece) and Italy, associated with cultivated olive and grapevine were coincident with previous studies including: i) immature females with conoid-rounded lip region not set off, finely annulated, stylet long and well developed (21–24 μm long) with anchor-shaped stylet knobs; dorsal pharyngeal gland opening situated 13–18 μm posterior to base of stylet; vulva located at more than 63% of body length; tail with

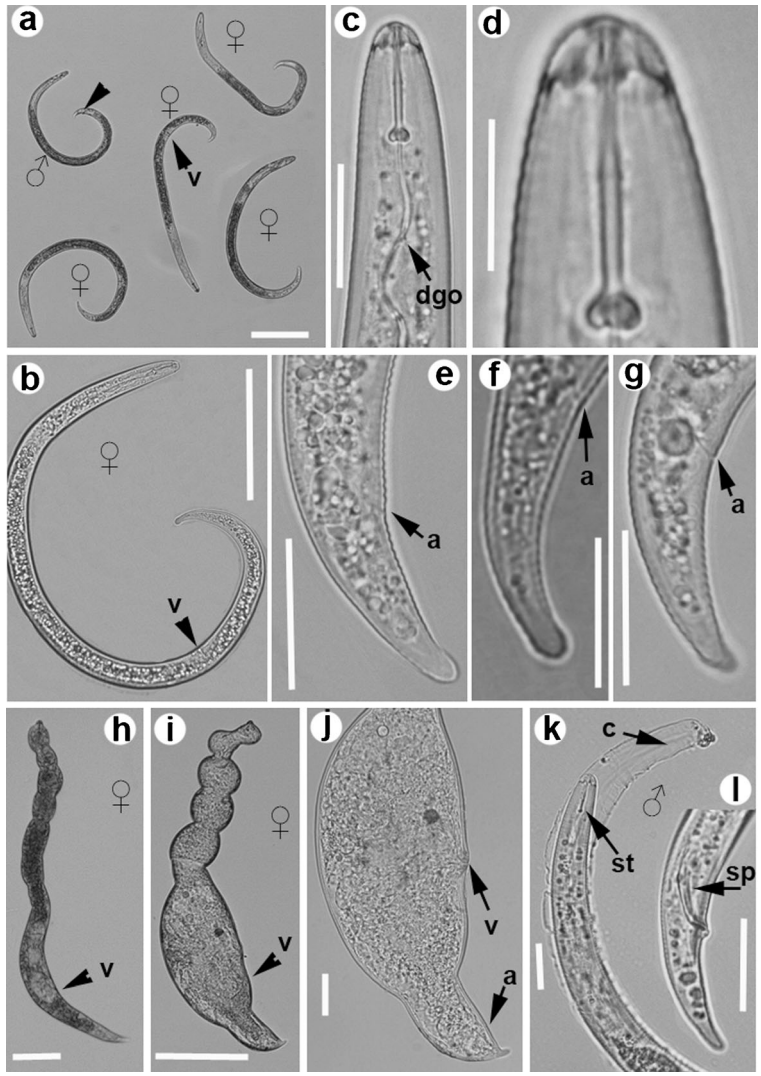

Fig. 1 Light micrographs of *Rotylenchulus reniformis* Linford and Oliveira 1940 from southern Spain. **a,b** Whole immature females and male. **c-d** Female anterior region. **e-g** Female tail region. **h-i** Whole mature females. **j** Mature female posterior region showing vulva and anus. **k** Male anterior region showing delicate stylet and old cuticle. **l** Male tail region. Abbreviations: a = anus; dgo = dorsal gland orifice; v = vulva; s = stylet; and sp. = spicule (Scale bars **a, b, h, *i*** = 100 μm; **c-d, e-g, j-l** = 20 μm)

bluntly rounded terminus, annulation around terminus not prominent; and hyaline portion 8–12 μm long; ii) males with stylet and pharynx reduced, tail broadly rounded with rounded tip.

The morphology of the new Cretan and Italian populations of *R. macrodoratus* from cultivated olive and grapevine was almost identical to that described for this species in the original and posterior descriptions, confirming that there is only slight morphological and morphometric diversity within populations of this species in the Mediterranean Basin. All the traits were within the range for this species in previous studies (Talamé et al. 1970, Koliopanos and Vovlas 1977, Vovlas and Vlachopoulos 1991, Van den Berg et al. 2016).

Rotylenchulus macrosoma Dasgupta et al. 1968 (Table 3)

Since *R. macrosoma* populations from wild and cultivated olives in Spain have been already reported and well characterized morphologically and morphometrically in previous studies (Castillo et al. 2003a, Van den Berg et al. 2016), a brief description of two new Cretan populations is provided in this study.

Immature female Lip region conoid-rounded not set off, finely annulated. Stylet long and well developed with metenchium usually slightly shorter than telenchium. Stylet knobs rounded, sloping posteriorly. Dorsal pharyngeal gland opening situated 1.4 (1.2–1.6) times of

Table 3 Morphometrics of *Rotylenchulus macrosoma* Dasgupta et al. 1968 from cultivated olive in Crete, Greece. All measurements are in μm and in the form: mean ± s.d. (range)

	Petrokefali, Crete		Limnes, Crete	
Character	Immature females	Males	Immature females	Males
n	10	5	10	10
L	467 ± 27.0 (432–506)	468 ± 28.2 (433–503)	488 ± 31.2 (428–526)	463 ± 32.7 (418–516)
a	28.9 ± 1.9 (26.1–31.6)	30.8 ± 2.1 (27.1–32.0)	29.7 ± 1.0 (28.5–31.4)	28.2 ± 1.8 (26.1–31.3)
b’	3.6 ± 0.3 (3.3–4.3)	3.4 ± 0.3 (3.1–3.6)	3.7 ± 0.2 (3.4–4.1)	3.6 ± 0.3 (3.2–4.0)
c	15.3 ± 0.9 (13.8–17.0)	14.7 ± 0.9 (13.1–15.4)	15.4 ± 1.0 (14.1–17.1)	13.8 ± 1.3 (11.6–15.4)
c’	3.3 ± 0.3 (2.8–4.0)	3.0 ± 0.2 (2.8–3.3)	3.1 ± 0.4 (2.6–3.8)	2.9 ± 0.1 (2.8–3.1)
V or T	61.4 ± 2.0 (58.0–64.0)	31.8 ± 1.3 (30.0–33.0)	62.4 ± 1.9 (60.0–65.0)	31.3 ± 5.3 (21.5–37.1)
Stylet length	18.5 ± 1.7 (16.0–21.0)	14.6 ± 0.5 (14.0–15.0)	17.4 ± 1.4 (15.5–20.0)	15.4 ± 0.5 (15.0–16.0)
DGO	23.8 ± 1.5 (21.0–26.0)	20.8 ± 1.3 (20.0–23.0)	23.6 ± 1.3 (21.0–25.0)	21.6 ± 1.7 (19.0–24.0)
o	129.8 ± 16.6 (105.0–156.3)	142.5 ± 7.2 (133.3–153.0)	136.6 ± 10.5 (120.0–156.3)	140.3 ± 5.2 (133.3–147.0)
Lip region width	6.7 ± 0.7 (6.0–7.5)	6.6 ± 0.5 (6.0–7.0)	6.7 ± 0.8 (6.0–8.0)	6.5 ± 0.6 (6.0–7.5)
Excretory pore from front	91.6 ± 4.9 (84.0–99.0)	88.8 ± 4.5 (83.0–95.0)	90.2 ± 6.5 (79.0–99.0)	88.5 ± 5.9 (78.0–95.0)
Pharynx length	129.2 ± 9.3 (114.0–140.0)	138.4 ± 5.9 (132.0–146.0)	133.1 ± 8.6 (120.0–146.0)	130.2 ± 12.7 (112.0–149.0)
Maximum body diam.	16.2 ± 0.9 (15.0–18.0)	15.2 ± 0.8 (14.0–16.0)	16.4 ± 1.1 (15.0–18.0)	16.4 ± 0.8 (15.0–18.0)
Anal body diam.	9.4 ± 1.2 (8.0–12.0)	10.6 ± 1.1 (9.0–12.0)	10.5 ± 2.1 (8.0–14.0)	11.5 ± 1.0 (10.0–13.0)
Tail length	30.6 ± 2.8 (27.0–36.0)	31.4 ± 0.9 (30.0–32.0)	31.8 ± 3.0 (28.0–37.0)	33.6 ± 2.4 (29.0–37.0)
h	10.4 ± 1.1 (9.0–12.0)	10.2 ± 1.3 (9.0–11.0)	10.6 ± 1.3 (9.0–12.0)	10.5 ± 0.8 (9.0–12.0)
Spicule length	-	21.8 ± 1.3 (20.0–23.0)	-	22.4 ± 1.4 (20.0–24.0)
Gubernaculum length	-	9.2 ± 0.8 (8.0–10.0)	-	10.2 ± 0.8 (9.0–11.0)

[a] Abbreviations are defined in Siddiqi (2000)

stylet lengths posterior to base of stylet. Excretory pore situated from opposite middle of isthmus to opposite anterior part of pharyngeal lobe. Pharyngeal glands overlapping intestine laterally and mostly ventrally. Annulation distinct over entire body, *ca* 1 μm wide. Lateral field distinct with four lines and three equal smooth bands. Tail with bluntly rounded terminus, annulation around terminus prominent. Hyaline portion 0.33 (0.30–0.35) times of tail length.

Male Similar to immature female except for genital system and a more curved posterior part of body. Stylet reduced, 0.84 (0.73–0.94) times of that of female. Tail broadly rounded with rounded tip. Gubernaculum and spicules well developed, ventrally arcuate.

Remarks The morphology of the new Cretan populations of *R. macrosoma* from cultivated olive was almost identical to that described for this species in the original description, as well as for the Spanish populations (Castillo et al. 2003a, Van den Berg et al. 2016). The detection of these populations from cultivated olive in Crete constitutes new records of this species in Greece. Minor morphometric differences of these populations from original description and those reported in Spain including body length (428–526 *vs* 520–640, 432–520 μm), stylet (15–21 *vs* 18–22, 16–20 μm), a ratio (26.1–31.6 *vs* 30–38, 27.6–32.1), c’ ratio (2.6–4.0 *vs* 3.7–5.0 2.6–4.0), V ratio (58–65 *vs* 63–68, 59–66), o ratio (105–156 *vs* 139–188, 116–156). These differences may be a result of geographical intraspecific variability.

Rotylenchulus reniformis Linford and Oliveira 1940 (Fig. 1, Table 4)

Immature female Body habitus ventrally curved to spiral-shaped upon fixation. Lip conoid, continuous with body contour, with 4–5 annuli. Labial framework moderately sclerotised, outer margin extending 1–2 annuli posteriorly into body. Lateral fields with four smooth equidistant lines, 1/4–1/5 body-width. Stylet moderately developed, 1.8 (1.7–2.2) times longer than labial region diam., its conus 50.0–54.5% of total stylet length. Basal knobs rounded, sloping posteriorly. Dorsal pharyngeal gland opening 0.9 (0.8–1.0) times stylet length posterior to stylet base. Median pharyngeal bulb moderately

Table 4 Morphometrics of *Rotylenchulus reniformis* Linford and Oliveira 1940 from southern Spain. All measurements are in μm and in the form: mean ± s.d. (range)

	Females		
Character n	Matures 5	Immatures 10	Males 10
L	443 ± 36.5 (409–487)	464 ± 30.5 (378–469)	438 ± 27.7 (398–486)
a	4.3 ± 0.7 (3.6–5.3)	24.7 ± 1.9 (21.0–27.4)	21.6 ± 0.9 (20.4–23.4)
b’	6.2 ± 0.6 (5.1–6.5)	3.4 ± 0.3 (3.1–3.8)	4.0 ± 0.2 (3.5–4.2)
c	32.1 ± 1.2 (30.9–31.4)	18.8 ± 1.5 (16.6–21.3)	16.6 ± 0.8 (15.3–17.4)
c’	0.5 ± 0.1 (0.5–0.6)	2.4 ± 0.2 (2.2–2.9)	3.0 ± 0.3 (2.7–3.5)
V or T	70.0 ± 1.6 (68.0–72.0)	70.8 ± 1.7 (68.0–73.0)	29.1 ± 4.9 (21.7–34.0)
Stylet	14.2 ± 0.8 (13.0–15.0)	16.7 ± 0.7 (16.0–18.0)	12.5 ± 0.7 (11.0–13.0)
DGO	-	14.5 ± 1.1 (13.0–16.0)	17.2 ± 0.8 (16.0–18.0)
o	-	86.9 ± 7.3 (76.5–100.0)	141.7 ± 13.9 (123.1–155.0)
Lip region width	6.5 ± 0.7 (6.0–7.5)	6.7 ± 0.7 (6.0–7.5)	6.7 ± 0.7 (6.0–7.5)
Excretory pore from front	-	76.4 ± 5.8 (70.0–90.0)	91.8 ± 7.1 (80.0–98.0)
Pharynx length	72.2 ± 7.1 (64.0–80.0)	126.7 ± 5.0 (120.0–134.0)	110.1 ± 6.0 (101.0–119.0)
Maximum body diam.	105.6 ± 19.4 (81.0–135.0)	17.7 ± 0.8 (16.0–19.0)	20.3 ± 0.9 (19.0–22.0)
Anal body diam.	26.6 ± 3.6 (23.0–32.0)	9.7 ± 0.7 (9.0–11.0)	8.8 ± 0.6 (8.0–10.0)
Tail length	13.8 ± 0.8 (13.0–15.0)	23.3 ± 1.6 (22.0–26.0)	26.4 ± 1.6 (24.0–28.0)
h	-	8.7 ± 1.4 (7.0–11.0)	7.5 ± 0.5 (7.0–8.0)
Spicules	-	–	20.8 ± 1.3 (19.0–23.0)
Gubernaculum	-	–	7.2 ± 0.6 (6.5–8.0)

Abbreviations are defined in Siddiqi (2000)

developed, oval, 9.0–13.0 μm long, with prominent valves. Nerve ring enveloping isthmus at mid-point. Excretory pore usually located near base of isthmus. Reproductive system with two genital branches equally developed, ovaries immature, reflexed. Vulva not prominent, clearly posterior to mid-body. Phasmid pore-like, eight to ten annuli posterior to anus. Tail slightly tapering to a narrow and rounded terminus, with 19–23 annuli; hyaline portion 0.37 (0.30–0.43) times of tail length.

Mature female Body ventrally arcuate, obese, kidney-shaped in well mature specimens, anterior region with irregular contour. Tail with a slender terminal portion 5–8 μm long.

Male Similar to immature female except for genital system and a more curved posterior part of body. Stylet, labial framework, and pharynx reduced, weaker than in immature females. Tail broadly rounded with rounded tip. Spicules and gubernaculum well developed, ventrally arcuate; bursa reduced, subterminal.

Remarks This species has been reported in several ornamental plants and papaya in Spain (Artero et al. 1977, Castillo and Gómez-Barcina 1993), but no molecular characterization of these populations have been provided. Morphology and morphometrics of the new Spanish population from cotton was coincident with the original description and redescription (Linford and Oliveira 1940, Siddiqi 1972), as well as the populations reported in ornamental plants in Spain (Artero et al. 1977), except for minor intraspecific differences including body length (378–469 μm *vs* 340–420, 330–450 μm), b’ ratio (3.1–3.8 *vs* 2.4–3.5, 2.7–3.8), c ratio (16.6–21.3 *vs* 14–17, 13–18), and o ratio (76.5–100 *vs* 81–106, 68–100) (Artero et al. 1977).

Phylogenetic position of Rotylenchulus species found in the Mediterranean basin.

Thirty-two sequences from the D2-D3 of 28S rRNA gene were obtained in this study. Type B - D2-D3 sequences from *R. macrodoratus* (KY992781, KY992783, KY992788, and KY992792) and *R. macrosoma* (KY992795, KY992797, KY992799, KY992801, and KY992804-KY992806) were obtained for the first time

in this study. New D2-D3 sequences from *R. macrodoratus* from Crete (KY992779-KY992791) matched well (97–99% similarity) with other accessions of *R. macrodoratus* from Italy deposited in GenBank (DQ328711, KT003758-KT003762). Intraspecific differences among type B-D2-D3 of 28S rRNA gene from *R. macrodoratus* were higher varying from 95 to 99%. Type A - D2-D3 sequences of *R. macrosoma* from Crete (KY992793, KY992794, KY992796, KY992798, KY992800, KY992802, and KY992803) showed some variability (97% similarity) with respect to the sequences from Spain populations deposited in GenBank (KT003747-KT003751). The intraspecific variability for the type B - D2-D3 of *R. macrosoma* (KY992795, KY992797, KY992799, KY992801, KY992804) ranged from 97 to 99% (from 1 to 7 nucleotides and no gaps). New sequences of *R. macrosoma* from hazelnut from northern Spain showed similarity values of 99% with other accession of *R. macrosoma* from Spain (KT003748-KT003750). Finally, *R. reniformis* from cotton from Spain (KY992808-KY992809) showed high coincidence, 99% similar to some *R. reniformis* sequences deposited in GenBank, such as, KT003743-KT003745.

The ITS region of *R. macrosoma* (KY992824-KY992832) from Crete showed coincidence with sequences deposited in GenBank, being 93–94% similar with Spanish accessions KT003800-KT003808 (type A) and KT003809-KT003810 (type B). Nucleotide diversity for both types of ITS (A and B) found among the sequences from Crete (twonucleotides and no indels (99% similar) was lower than among Spanish populations, which showed similarity values from 96 to 99% (from 4 to 30 nucleotides and 1–11 indels) for the type A and 98% for the type B (13 nucleotides and two indels). Fourteen new ITS sequences from *R. macrodoratus* were obtained in this study, eight of them correspond to type A – ITS from Crete and the other 6 sequences of type B – ITS, 3 from Crete and 3 from Italy. Type A - ITS sequences from *R. macrodoratus* (KY992810-KY992814, KY992816, KY992818, KY992819) matched well with other sequences of *R. macrodoratus* deposited in GenBank (KT003795- KT003797), showing low nucleotide variability, only three nucleotides were different (99–100% similar), but nucleotide diversity was higher for the accession KT003794 which was 98% similar (16 nucleotides and three indels) to the rest of accessions. Type B - ITS sequences (KY992815, KY992817, KY992820-KY992823) of *R. macrodoratus* were obtained for the first time in this study, nucleotide diversity in type B was higher than type A, showing similarity values of 95–96% (27–35 nucleotides and four indels). ITS sequences from *R. reniformis* from Spain (KY992838-KY992840) showed high coincidence with other accessions from this species deposited in GenBank, being 100% similar to some of them for type A (AY335191, GU003947, GU003938, KF999979, KP018550 and KP018562) and 99% similar to KP018590 (2 nucleotides and no indels) for the type B.

Finally, 12 new *cox*I sequences were obtained in this study, 5 from *R. macrosoma* (KY992845- KY992849), four from *R. macrodoratus* (KY992841- KY992844), and three from *R. reniformis* (KY992850-KY992852). *Rotylenchulus macrodoratus* from Crete (KY992841-KY992844) matched well with the topotype specimens of *R. macrodoratus* deposited in GenBank (KT003722) being 99% similar (differing by 5 nucleotides). On the contrary, *R. macrosoma* showed a high nucleotide diversity among population from Crete and Spain, being 90% similar between them (45 nucleotides and no indels), however low nucleotide diversity was found among Crete populations, differing only by 2–8 nucleotides. *CoxI* sequences for *R. reniformis* from Spain (KY992850-KY992852) was closely related (99% similarity) to *R. reniformis* from USA (KT003727-KT003731) and only differing 2–4 nucleotides.

Phylogenetic trees reconstructed by the BI method for the three rRNA genes (D2-D3 expansion regions of 28S rRNA gene, ITS rRNA and the partial *coxI* gene) are presented in Figures 2, 3 and 4, respectively. The 50% majority rule consensus BI tree of a multiple alignment including 112 D2-D3 sequences and 712 bp consisted of 4 major clades. This tree topology was similar to that obtained by Van Den Berg et al. (2016). *Rotylenchulus macrosoma* and *R. reniformis* formed two of the four major clades, one of them for the type A (PP = 1.00) and the other for the type B (PP = 0.80) showing a sister relationship. *Rotylenchulus macrodoratus* clustered alone forming two high supported subclades (PP = 1.00), one for the type A and another one for the type B. Finally the rest of *Rotylenchulus* spp. with D2-D3 sequences available in GenBank occupied a basal position in the tree forming a low supported clade (PP = 0.71). The alignment generated for the 163 ITS of *Rotylenchulus* spp. was 466 bp after discarding ambiguously aligned regions. The 50% majority rule consensus tree generated from the ITS alignment by BI analysis is presented in Fig. 4. Similarly to D2-D3 tree,

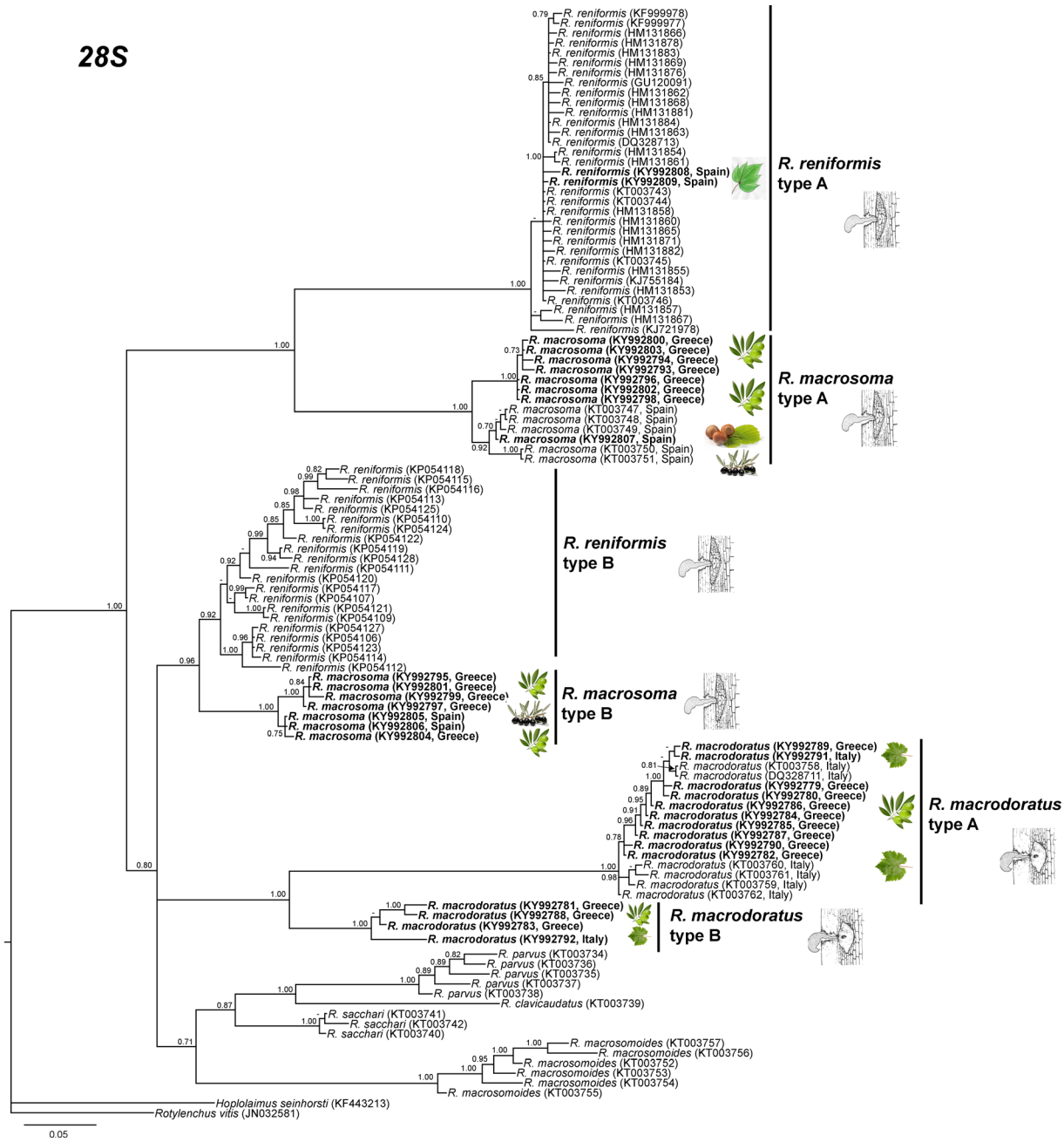

Fig. 2 The 50% majority rule consensus trees from Bayesian analysis generated from the D2-D3 of 28S rRNA gene dataset with the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold letters

R. reniformis and *R. macrosoma* clustered as a sister species for both ITS-types A and B, forming two different well-supported subclades, (PP = 1.00 and PP = 0.95, respectively). As well as in the D2-D3 tree, *R. macrodoratus* clustered alone forming two well-supported subclades (PP = 1.00) one of them correspond to the type A and the other for the type B.

Finally, the *coxI* alignment consisted of 29 sequences of 324 bp. The 50% majority rule consensus phylogenetic tree generated from the *coxI* alignment by BI

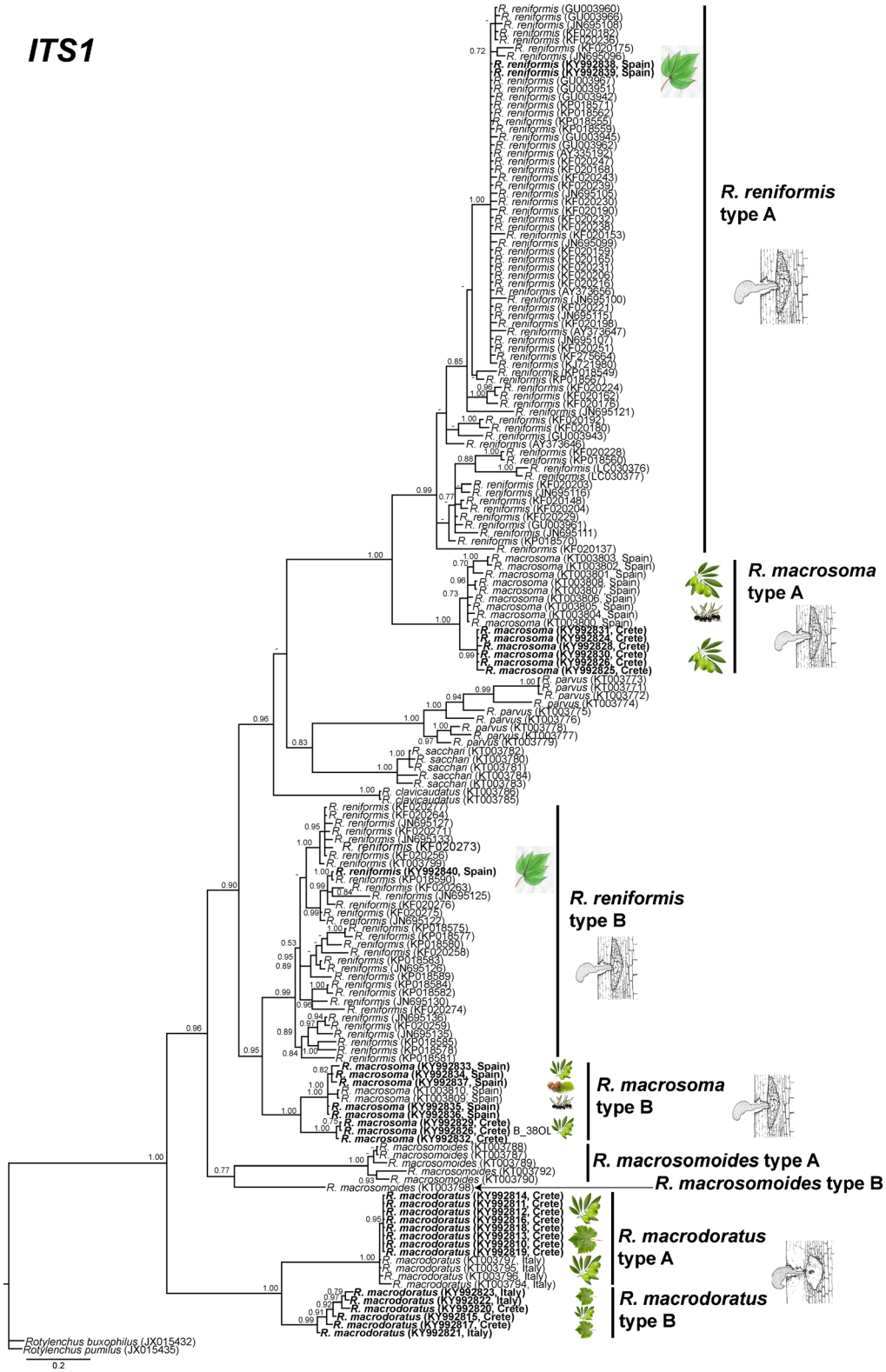

Fig. 3 The 50% majority rule consensus trees from Bayesian analysis generated from the ITS-rRNA gene dataset with TVMef + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold

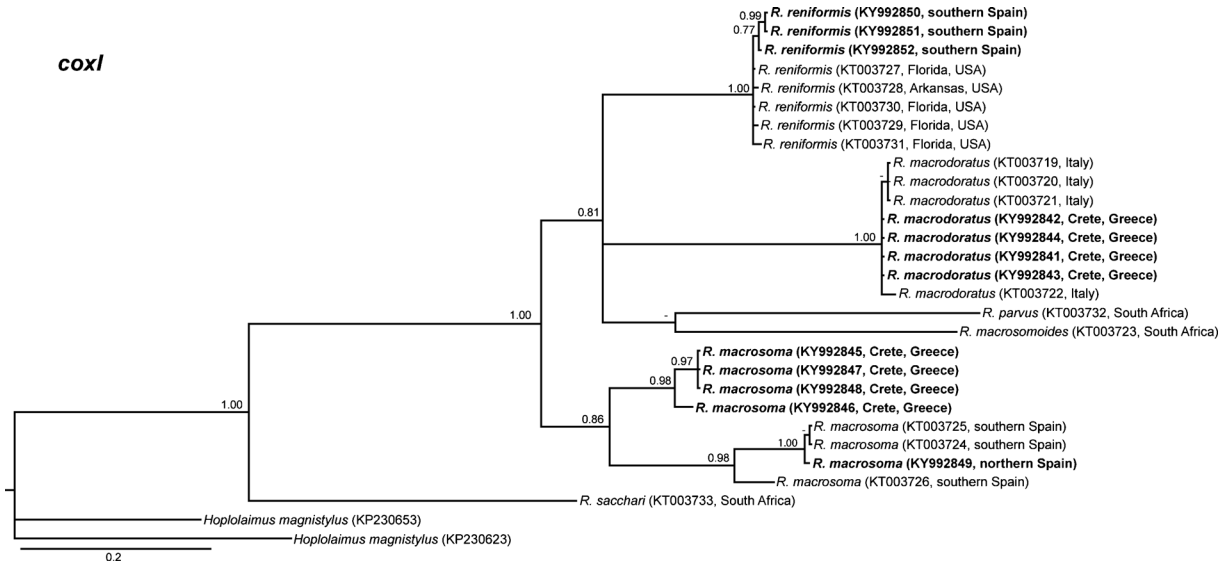

Fig. 4 The 50% majority rule consensus trees from Bayesian analysis generated from the partial *coxI* mtDNA gene dataset with TVM + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are in bold

analysis is presented in Figure 4. The *coxI* tree showed a well-supported major clade (PP = 1.00) which grouped all *Rotylenchulus* spp. with *coxI* sequences available except for *R. sacchari* (KT003733). *Rotylenchulus macrosoma* populations from Crete tend to cluster separately to the other accessions in all the molecular markers studied (D2-D3, ITS and *coxI*) in this work, forming separate subclades. The contrary was for *R. macrodoratus* populations from Crete, which do not form a separate cluster with Italian samples in all the molecular markers studied (D2-D3, ITS and *coxI*).

Discussion

In this study we provide morphological and molecular characterisation of Mediterranean species of *Rotylenchulus* (*R. reniformis*, *R. macrosoma* and *R. macrodoratus*), their prevalence in major crops and olive and their incidence in other crops as in grapevine, cotton and less important as hazelnut. Additionally, the knowledge of rRNA variants for *R. macrosoma* and *R. macrodoratus* has been expanded and the first report of *R. macrosoma* in Greece was confirmed in this study.

This study also showed new results on distribution of *Rotylenchulus* species in Mediterranean countries. Interestingly, the prevalence differs enormously among the countries studied (Spain, Italy and Greece) comparing within and among the same crops. This distribution cannot be explained by a better suitability of one of the two species affecting this crop (*R. macrodoratus* or *R. macrosoma*), but probably to environmental or human factors related to the olive crop as commercial interchange or cropping systems. Both species seems to be associated with cultivated olives, with the exception of one sample in wild olive in Spain for *R. macrosoma*, since they have been never found in wild olive. This species showed a high density of nematodes (876 nematodes/500 ml^3 soil) in one population in Southern Spain. Both species reproduce, but do not reach at high densities in the Mediterranean conditions.

This report expands the presence and the parasitism of *R. macrosoma* on hazelnut in Spain. Interestingly, *R. macrodoratus* has not been detected in Spain so far, including this wide nematode survey in olives or previous wide studies in grapevine (Gutiérrez-Gutiérrez et al. 2011). Similarly, *R. reniformis* has not been found infecting olive in natural conditions, but olive can be a potential host of this nematode as reported in several studies under controlled conditions (Badra and Khattab 1980, Al-Sayed and Abdel-Hameed 1991). However, in our case, with three sampled countries for olive nematodes, none of the field showed the presence of this nematode. This nematode is associated with sandy soils in which densities increased up to 60% of sand content in soil (Holguin et al. 2015), as it is the case

for sandy soils in Southern Spain and warmer areas as in the subtropical zones of Spain (Castillo and Gómez-Barcina 1993). Probably, it is not found where olive crop is planted because of the low levels of sand in the soil in these areas. Pot studies with graded inoculum levels of *R. reniformis* indicate that damage to a wide array of plants can occur at densities between 0.1 and 5 nematodes/ml^3 of soil (Robinson et al. 1997). Additionally, *R. reniformis* have the ability to survive in dry soil in anhydrobiotic form (Gaur and Perry 1991).

This study provided new information on the intra-individual and intra-specific variation of rRNA genes for *Rotylenchulus* spp., and revealed the two types of D2-D3 for *R. macrosoma* and *R. macrodoratus* and the two types of ITS for *R. macrodoratus*. *Rotylenchus macrosoma* also showed an important variability for ITS region with an additional ITS type C (KT003811) (Van Den Berg et al. 2016). One of the interesting aspects of the molecular study is that *R. macrosoma* populations from two different countries showed clear differences in the ITS rRNA and *coxI* sequences, which may indicate a long history of their separation. This data showed a bigger molecular variability in the ribosomal set of genes for this group of nematodes than for *coxI* in the mitochondrial genome. Interestingly, this is contrary to other studies performed with nematodes and mitochondrial markers, as mitochondrial genes have uniparental inheritance and high mutation rate (Gissi et al. 2008; Pagan et al. 2015). Additionally, *R. macrosoma* showed important molecular differences associated with its regional distribution between Spain and Greece for all markers studied, with the exception of the one sequence for type-B D2-D3 (Crete R142), while we could not see this clear geographical separation between Greece and Italy in *R. macrodoratus*. In both cases, the populations inside each country seem diverse for *R. macrosoma* for ribosomal and mitochondrial markers showing an evolution inside each of the countries. These differences among populations seem important for ribosomal markers, but not for *coxI* in the case of *R. macrodoratus*. Probably, a more interchange among Italian and Cretan populations of *R. macrodoratus* has been done in the past or a faster evolution in the ribosomal genes.

This study showed the differences in distribution, prevalence and molecular diversity of two species of *Rotylenchulus* associated with Mediterranean areas, as well as the risk of expansion of *R. reniformis* in other areas of the Mediterranean countries. More work should be done to understand the host suitability of *R. reniformis* and dispersal of this nematode from the infested area in Spain.

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Compliance with Ethical Standards

Conflict of interest All the authors certify that 1) do not have any actual or potential conflict of interest, 2) the study described is original and has not been published previously, and is not under consideration for publication elsewhere, 3) all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected. We also certify that all authors have reviewed the manuscript and approved the final version of manuscript before submission.

Human Participants and/or Animals No specific permits were required for the described fieldwork studies. Permission for sampling the crop orchards was granted by the landowner. The samples from wild plants were obtained in public areas, forests, and other natural areas studied and do not involve any species endangered or protected in Spain, Italy or Greece. The sites are not protected in any way.

Informed Consent All the authors certify that the work carried out in this research followed the principles of ethical and professional conduct have been followed. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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