THREE SPECIES OF ENTOMOPATHOGENIC NEMATODES OF THE FAMILY STEINERNEMATIDAE (NEMATODA: RHABDITIDA) NEW TO CONTINENTAL PORTUGAL

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Summary. A survey was conducted to determine the species of entomopathogenic nematodes occurring in continental Portugal. Nematodes were recovered from soil samples collected from Alentejo (South) and central Portugal from 2006 to 2009. Nematode isolates were identified based on morphology and sequence analysis. Phylogenetic analysis was based on sequences of partial 28S (D2D3 domain), internal transcribed spacer (ITS) and cytochrome C oxidase subunit I gene (*COI*). Nematode isolates 59F, 15G, 20F and 2B were characterized in detail. In the collected samples, nematodes from the genera *Steinernema* and *Heterorhabditis* were identified, namely *Heterorhabditis bacteriophora* (1.9%), *Steinernema feltiae* (11.5%), *S. kraussei* (0.32%), *S. intermedium* (0.32%) and *Steinernema* sp. (0.63%) *glaseri*-group. Isolate 2B shows morphological characteristics identical to *S. intermedium*, which is a member of the *affine/intermedium*-group and is characterized by the presence of strongly curved and robust spicules with a distinct rostrum in the male, and dorsal tail depression in third-stage infective junveniles (IJ). However, the phylogenies based on the three molecular markers revealed that isolate 2B is more closely related to *S. affine* than to *S. intermedium*. Based on morphological observations, isolate 20F was identified as *S. kraussei*, which is a member of the *kraussei/feltiae*-group, characterized by IJs with a straight body of medium length (mean = 700-950 µm), lateral field mostly with eight ridges, rather broad, flatly rounded and continuous cephalic region, excretory pore at mid-pharynx level; males with mucronate tail, yellowish spicules ca. 50 µm long and wide manubria; females with short conoid tail with pointed non-mucronate tip. Based on morphology and sequence analysis, isolates 59F and 15G were considered conspecific and identified as a species belonging to the *glaseri*-group. More detailed studies are necessary to decide whether these isolates represent a new species.

Key words: *Steinernema intermedium, S. kraussei, Steinernema* sp. *glaseri*-group, cytochrome oxidase c, ribosomal sequences, phylogeny.

Entomopathogenic nematodes (EPNs) of the families Steinernematidae Chitwood and Chitwood, 1937 and Heterorhabditidae Poinar, 1976 are lethal parasites of insects, being widely distributed in soils worldwide (Hominick *et al.*, 1996). These nematodes have been known since the XVIIth century and include more than 30 families (Kaya and Stock, 1997). Hunt (2007) reported 55 valid species of the genus *Steinernema* Travassos, 1927 and eleven species of the genus *Heterorhabditis* (Hunt, 2007). However, after 2007 many more entomopathogenic nematode species have been described, mainly from Africa and Asia (Khatri-Chhetri *et al.*, 2011).

Nematodes of the families Steinernematidae and Heterorhabditidae are potential candidates to be used as bio-control agents due to their capacity for controlling insect pests worldwide (Kaya and Stock, 1997; Bedding, 1998; Shapiro-Ilan *et al.*, 2002). Classified as parasitoids and pathogens, they have many attributes that give them the ability to be used as bio-pesticides: they can be found under diverse ecological conditions including cultivated fields, forests, grasslands, deserts and ocean beaches (Hominick *et al.,* 1996). The third-stage infective juveniles (IJ) of these families are non-feeding, free living stages in the soil where they can survive for extended periods. They are safe to non-target organisms and to the environment, can be mass produced, formulated and easily applied as bio-pesticides (Georgis and Kaya, 1998), being compatible with some insecticides (Chen *et al.*, 2003), and have a broad host range of insect pests in a variety of habitats (Kaya and Gaugler, 1993; Gaugler, 2002).

Insects play an important role in agricultural production and have acquired resistance to insecticides during recent decades (Ahmad *et al.*, 2003) following the increase usage of pesticides. Entomopathogenic nematodes represent an alternative to insecticides, the identification of new species in different countries and ecosystems being of major importance in pursuing this objective. These nematodes have a complex life cycle inside the insect host. When infective juveniles locate a potential host in the soil, they move towards it and penetrate the insect body, via natural openings or areas of thin cuticle. Once inside the body cavity, they release their symbiotic bacteria (belonging to the genera *Xenorhabdus* and *Photorhabdus* for steinernematids and heterorhabditids, respectively), which multiply rapidly, causing insect death within 48 hours by septicemia (Kaya and Koppenhofer, 1999). IJs feed on liquefying

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host tissues, and then mature into adults. During their adult stages they complete their life cycle, producing eggs. The eggs release juveniles and when food resources drecrease, IJs emerge from the insect cadaver, searching for a new host.

In Portugal, until 2006, the only region where surveys and studies of EPN had been conducted was the Azores archipelago (Simões *et al.*, 1994; Rosa and Simões, 2004). Therefore, an extensive survey was initiated in 2006 and concluded in 2009 to obtain more information on the distribution of EPN in continental Portugal. Soil samples were randomly collected from selected ecosystems, especially in areas considered suitable habitats for the presence of steinernematids and heterorhabditids, such as conifer forests, sandy soils, cultivated fields, natural grasslands, roadsides with tree verges, cultivated fields, and typical habitats from each location in Portugal. The main goal of the survey was to evaluate the biodiversity in continental Portugal of entomopathogenic nematodes, which hopefully could then be used as bio-control agents. Because the number of species of EPNs is growing fast, it has become difficult to identify them just by morphological characters and, therefore, it is necessary to use both morphological and molecular approaches, using different markers according to the purpose.

Previously, molecular and phylogenetic studies showed that the genus *Steinernema* contains some species which are difficult to discriminate using DNA sequence analysis (Nguyen *et al.*, 2007). Therefore, in the present study, the four new EPN isolates found in samples collected in Alentejo and Algarve, as well as in central and northern continental Portugal, were firstly identified morphologically. Then, phylogenetic analysis based on three molecular markers, ITS (Internal Transcribed Spacer), the partial sequence of the D2D3 from 28S and cytochrome C oxidase subunit I gene (*COI*), were also used to confirm nematode identification.

MATERIAL AND METHODS

Sampling

Three hundred and twenty soil samples were randomly collected from Alentejo and central continental Portugal, from different types of natural and cultivated habitats, such as forest, cultivated fields, grasslands, woodlands, vineyards, and irrigated land. We tried to obtain samples from all the different habitats common to the different regions, but also typical habitats from each region. From each sampling site, three or four subsamples were taken from an area of *c.* 200 m^2, totalling approximately 2 dm^3 of soil. Soil samples were collected from a depth of 0-20 cm and placed in a plastic bag identified by GPS location, vegetation and date. To extract EPN from the soil, the *G. mellonella* L. (Lepidoptera: Pyralidae) trapping method (Bedding and Akhurst, 1975) was used. Therefore, ten last instar larvae of *G. mellonella* were placed inside perforated metal tea bags, partly filled with soil, which were then embedded in the soil sample in each plastic bag. Soil samples were stored at 25 ºC, and every four days dead *G. mellonella* were removed and replaced by new ones. This procedure was carried out during 12 days. The dead *G. mellonella* larvae were transferred to White traps (White, 1929) and IJs collected during the following days and stored at 10 ºC in distilled water. To identify the nematodes, morphological, morphometrical and molecular characterizations were made.

Morphological and morphometrical characterization

For scanning electron microscopy (SEM), adults and *dauer* juveniles were fixed in 4% formalin buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 4-6 ºC. They were then post-fixed with 2% osmium tetroxide solution for 12 hours at 25 ºC, dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted on SEM stubs, and coated with gold (Nguyen and Smart, 1995). The mounts were examined in a JEOL scanning electron microscope.

For light microscopy (LM), the nematodes were heat-killed on glass slides in a drop of water, and mounted in aqueous media under a cover glass. Another batch of nematodes was fixed in hot TAF (Southey, 1970), transferred to glycerin by the slow evaporation method and mounted permanently in anhydrous glycerin mounts (Seinhorst, 1959). Measurements and examination of the nematode morphology were performed with the aid of an AMPLIVAL light microscope (Carl Zeiss, Jena) and a Leitz DIAPLAN provided with Nomarski optics. For photomicrographs, a Zeiss photomicroscope with DIC optics was used.

Table I. Acession numbers in GenBank of isolate 2B *Steinernema intermedium*, isolate 20F, *S. kraussei* and isolates 59F and 15G *Steinernema* sp., from continental Portugal.

Species	ITS	28S domain D2D3	*COI*
S. intermedium	JN808124	JN808125	JN808126
S. kraussei	JN683825	JN683826	JN683829
Steinernema sp. (isolate 59F)	JN683827	-	JN683830
Steinernema sp. (isolate 15G)	JN683828	-	JN6838301

Molecular characterization

For phylogenetic analysis, three molecular markers were used: partial sequence of 28S, D2D3 domain, internal transcribed spacer (ITS) regions of rDNA, and mitochondrial gene encoding cytochrome C oxidase subunit I (*COI*).

Nematode DNA was extracted from several thousand IJs using JETQUICK Tissue DNA Spin Kit (GENOMED) according to supplier information.

Ribosomal and mitochondrial regions were amplified by PCR from the previously extracted genomic DNA. The nuclear large subunit rDNA gene region (28S), corresponding to positions 3745-4700 in *Caenorhabditis elegans* Maupas, and containing the D2 and D3 divergent domains, was amplified using forward primer 391 (D2F) 5´-AGC GGA GGA AAA GAA ACT AA-3´ and reverse primer 501 (D3R) 5´-TCG GAA GGA ACC AGC TAC TA-3´ (Nadler *et al.*, 2006). For amplification of the ITS region we used primers TW81 (5´-GTT TCC GTA GGT GAA CCT GC-3´) (forward primer) and AB28 (5´-ATA TGC TTA AGT TCA GCG GGT-3´) (reverse primer) (Joyce *et al.*, 1994).

Approximately 45% of the mitochondrial *COI* gene, corresponding to positions 7893-8596 in *C. elegans*, was amplified using forward primer 507 (COXIF) 5´-AGT TCT AAT CAT AAR GAT ATY GG-3´ and reverse primer 588 (COXIR) 5´-TAA ACT TCA GGG TGA CCA AAA AAT CA-3´ (Nadler *et al.*, 2006). PCR cycling conditions varied according to the region amplified. For the 28S (D2D3) rDNA region, the parameters included denaturation at 94 ºC for 3 min, followed by 33 cycles of 94 ºC for 30 sec, 52 ºC for 30 sec and 72 ºC for 1 min, followed by a final extension at 72 ºC for 7 min. For the ITS rDNA region, PCR conditions included initial denaturation at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 1 min, 55 ºC for 1 min 30 sec and 72 ºC for 2 min, followed by a final extension at 72 ºC for 5 min. For the *COI* mtDNA region, the PCR protocol included denaturation at 94 ºC for 3 min, followed by 37 cycles of 94 ºC for 30 sec, 50 ºC for 30 sec and 72 ºC for 45 sec, followed by a final extension at 72 ºC for 7 min. The products were analysed on 1% agarose gels with TBE buffer. The PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) following the manufacturer's instructions. PCR products were sequenced in both directions by a contract sequencer (STABVIDA Inc).

Sequence alignment and phylogenetic analysis of ITS, D2D3 and *COI* sequences

The sequences obtained (Table I) were compared with those from GenBank using the BLASTN program (Altschul *et al.*, 1990) and were aligned with BioEdit ver. 7.0.5 (Hall, 1999) with other *Steinernema* species sequences (Table II) under default alignment parameters. Sequences were deposited under the accession numbers

Table II. Details of *Steinernema* spp. used in this study. Site of sequencing: NCBI, BLAST (Spaces shown as '-' means the absence of information).

Species	Accession number to ITS rDNA, 28S domain D2D3, cytochrome oxidase c subunit I (*COI*)
S. abbasi Elawad, Ahmad, and Reid, 1997	AY248749, GU569060, HQ406728
S. affine (Bovien, 1937)	AF331912, AF331899, GU569061
S. arenarium (Artyukhovsky, 1967)	AY230160, AF331892, AY943979
S. beddingi Qiu, Hu, Zhou, Pang and Nguyen, 2005	AY603397, AY603396, -
S. boemarei, Lee, Sicard, Skeie and Stock, 2009	FJ152414, GU569046, GU569065
S. bicornutum Tallosi, Peters, and Ehlers, 1995	AY230163, GU569045, GU569064
S. carpocapsae (Weiser, 1955)	GQ421615, EU598241, AY943981
S. ceratophorum (Jian, Reid, and Hunt, 1997)	AY230165, AF331888, AY943982
S. cubanum Mracek, Hernandez, and Boemare, 1994	AY230166, AF331889, AY943983
S. diaprepsi Nguyen and Duncan, 2002	GU173997, GU177830, GU569067
S. feltiae (Filipjev, 1934)	EU598240, EU598250, AY943985
S. glaseri (Steiner, 1929)	AF122015, AF331908, AY943986
S. hermaphroditum Stock, Griffin and Chaenari, 2004	- , AY598358, AY943987
S. intermedium (Poinar, 1985)	AY230172, AF331909, AY943988
S. loci Phan, Nguyen and Moens, 2001	GQ497740, -, -
S. kraussei (Steiner, 1923)	EU914856, GU569053, GU569070
S. longicaudum Shen and Wang, 1992	AY230177, GU569054, AY943993
S. monticolum Stock, Choo and Kaya, 1997	AF331914, AF331895, AY943994
S. neocurtillae Nguyen and Smart, 1992	AF122018, FJ263674 , -
S. oregonense Liu and Berry, 1996	AY230180, GU569055, GU569072
S. pakistanense Shahina, Anis, Reid, Rowe and Maqbool, 2001	AY230181, - , -
S. riobrave Cabanillas, Poinar and Raulston, 1994	GU174001, GU177834, AY943998
S. robustispiculum Phan, Subbotin, Waeyenberge & Moens, 2005	AY355444, - ,-
S. silvaticum Sturhan, Spiridonov and Mráček, 2005	- , DQ399663, -
S. websteri Cutler and Stock, 2003	- , JF503100, GU569074
S. weiseri Mráček, Sturhan and Reid, 2003	- , GU569059, GU569075

Table III. Abiotic/biotic analysis of soil samples positive for *S. intermedium*, *S. kraussei* and *Steinernema* sp., from continental Portugal.

Factor considered	*S. intermedium* isolate 2B	*S. kraussei* isolate 20F	*Steinernema* sp. isolates 59F	*Steinernema* sp. isolate 15G
County	Mora	Castro d´Aire	Figueira da Foz	Figueiró dos Vinhos
Vegetation	Mixed forest	Moors and heathland	Mixed forest	Broad-leaved forest
GPS coordinates	38º 97´N 8º 13´W	40º 91´N 7º 85´W	40º 14´N 8º 83´W	39º 97´N 8º 33´W
Elevation (m)	136	745	47	76
Average annual temperature (ºC)	16.7	15.2	15.2	18.1
Total anual rainfall (mm)	346.1	1152.8	1152.8	987.3
P_2O_5	16.00	30.00	72.00	128.00
NO_3	35.50	214.00	21.50	248.00
Organic matter (%)	3.20	6.10	6.3	6.10
pH (H_2O)	5.34	4.17	4.90	6.23
% Nitrogen (Kjeldahl)	0.11	0.26	0.25	0.27

reported in Table II. Using Mega 5 (Tamura *et al.*, 2007), phylogenetic relationships among isolates were reconstructed by the Maximum Parsimony method (Nei and Kumar, 2000). Clades from trees of MP were supported by bootstrap analysis (Felsenstein, 1985) with 1000 replicates. The distances were calculated according to Close-Neighbor-Interchange (CNI) on Random Trees model (Nei and Kumar, 2000) for both nucleotide and amino acid sequence alignments. Sequences of *Bursaphelenchus xylophilus* Steiner *et* Buher were used as out-group in the different phylogenetic trees.

RESULTS

Nematodes extracted from the collected samples belonged to the genera *Steinernema* and *Heterorhabditis*. They were *H. bacteriophora* Poinar (1.9%), *S. feltiae* (Filipjev) Wouts, Mráček, Gerdin *et* Bedding (11.5%), *S. kraussei* (Steiner) Travassos (0.32%), *S. intermedium* (Poinar) Mamiya (0.32%) and *Steinernema* sp. (0.63%) *glaseri*-group. Isolates 15G and 59F belong to *Steinernema* sp., isolate 20F was identified as *S. kraussei* and isolate 2B as *S. intermedium*. The soil samples from which these nematodes were obtained were analysed for pH, organic matter and soil texture (Table III) at the Soil Analysis Laboratory, ICAAM, University of Evora. Isolate 2B was recovered from a forest habitat composed of stone pine (*Pinus pinea* L.) and cork oak (*Quercus suber* L.) stands. Isolate 20F was recovered from a region with heather (moors and heathland, NUTs "Nomenclatura para unidades territoriais para fins estatísticos" classification, accepted by European Union). Isolates 15G and 59F were recovered from olive groves and eucalyptus stands, respectively [NUT classification of mixed forest for isolate 59F, and broad-leaved forest for isolate 15G (Table III)].

Morphological and morphometric analysis

Morphology and morphometric analysis (Table IV) showed that isolate 2B possessed features characteristic of *S. intermedium* (IJ – tail dorsal constriction, male – robust and curved spicules with a prominent rostrum and mucronless tail) (Fig. 1C, D). Isolates 15G and 59F were identified as belonging to the *S. glaseri* (Steiner) Wouts, Mráček, Gerdin *et* Bedding group, in which the IJs were over 1000 µm long, with eight equally spaced and developed ridges in the lateral field and with a pos-

Table IV. Comparison of some morphologically important characters of the Portuguese entomopathogenic nematodes (n = 10).

Species (isolates)	IJ BL	IJ c´	IJ %D	IJ %Hy
S. intermedium (2B)	727-808	4.0-4.4	50-54	48-52
S. kraussei (20F)	909-970	4.0-4.5	43-46	27-30
S. glaseri group (59F)	1030-1232	3.6-3.8	55-60	45-50
S. glaseri group (15G)	1010-1111	3.5-3.8	54-60	45-50

IJ-infective juvenile, BL- body length, c´-tail length/tail width, IJ %D- length to excretory pore/pharynx length x100, IJ % Hy - hyaline layer length/tail length x100.

terior position of the excretory pore and a low value of ratio c´; males had slightly curved spicules with oblongate manubrium and mucronless tail (Fig. 1A, B, E). Isolate 20F possessed moderately curved lamina of spicule, presence of rostrum and oblongate manubrium in the males, IJs with short hyaline layer (<40%) and thus resembling *S. kraussei* (Fig. 1F).

Phylogenetic analysis

For isolates 15G and 59F, sequences from the 28S rDNA D2D3 domain were not obtained, so phylogenetic analysis of this region included just isolates 20F and 2B. The identification at species level for *Steinernema* sp. *glaseri*-group requires additional research, such as more detailed morphological and morphometric data, because not all nucleotide differences corresponded to either to *S. glaseri* or *S. arenarium* (Artyukhovsky) Wouts, Mráček, Gerdin *et* Bedding. The total evidence dataset in phylogenetic trees ranks our isolate 2B in the *affine/intermedium*-group and isolate 20F in the *kraussei/feltiae*-group because, in the nucleotide analysis of *Steinernema* species, it is difficult to determine homologous character states for many morphological characters (Nadler *et al.*, 2006). However, morphology and morphometry support identification of these isolates as *S. intermedium* and *S. kraussei.*

ITS rDNA region. Maximum parsimony (MP) analysis of ITS rDNA datasets were constructed based on 930 positions, involving 21 nucleotide sequences (Fig. 2). MP analysis groups our isolates within the *Steinernema* species and is supported by strong bootstrap values

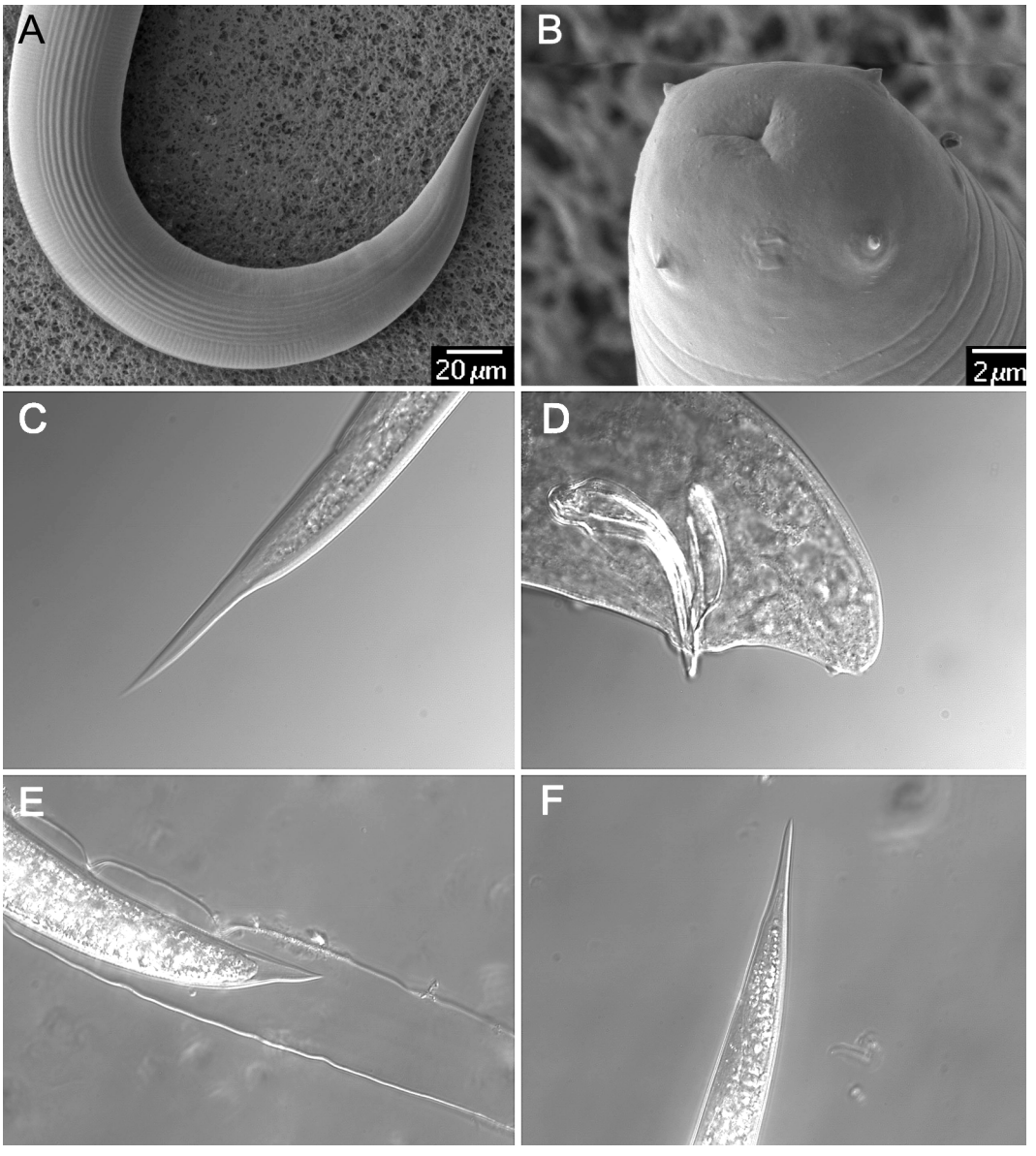

Fig. 1. Steinernematid species from continental Portugal. A,B,E *Steinernema* sp. "*glaseri* group" infective juvenile: A, lateral field with eight ridges; B, head with four cephalic papillae; E, tail with hyaline layer in sheath of the second stage juvenile. C,D, *S. intermedium*: C, IJ tail with a dorsal constriction; D, male tail with spicules. F, *S. kraussei*, IJ tail with short hyaline layer.

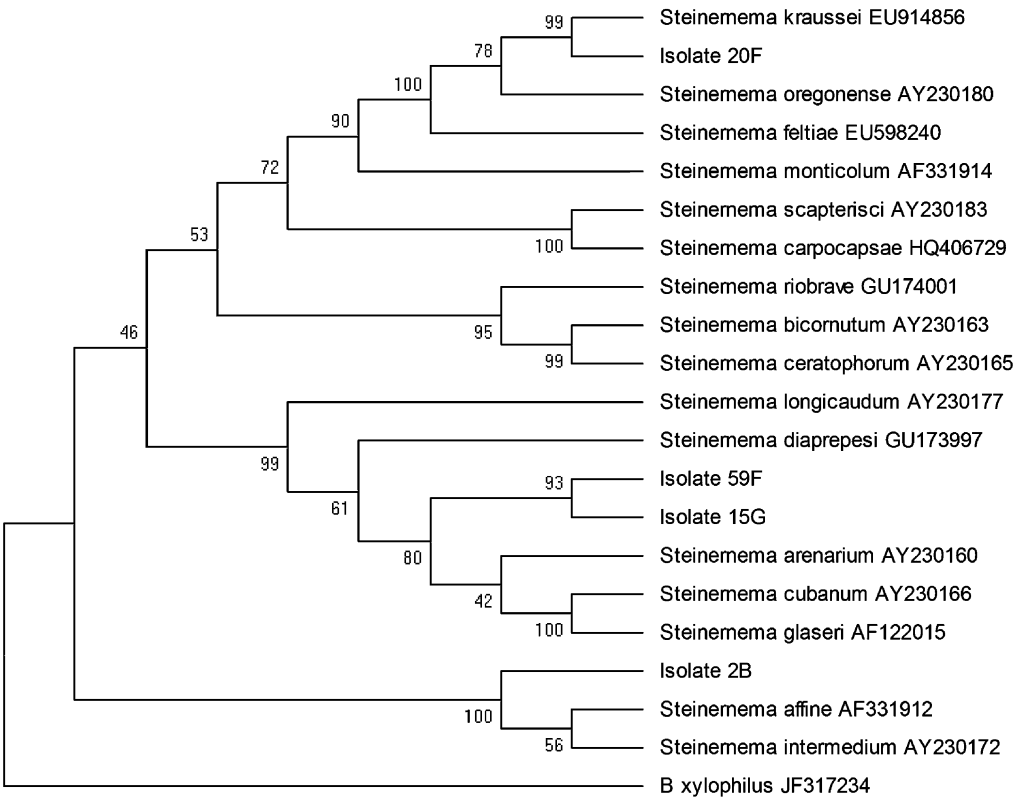

Fig. 2. Phylogenetic relationships based on Maximum Parsimony between 16 *Steinernema* species and isolates 2B, 20F, 59F and 15G, with bootstrap analysis of ITS regions.

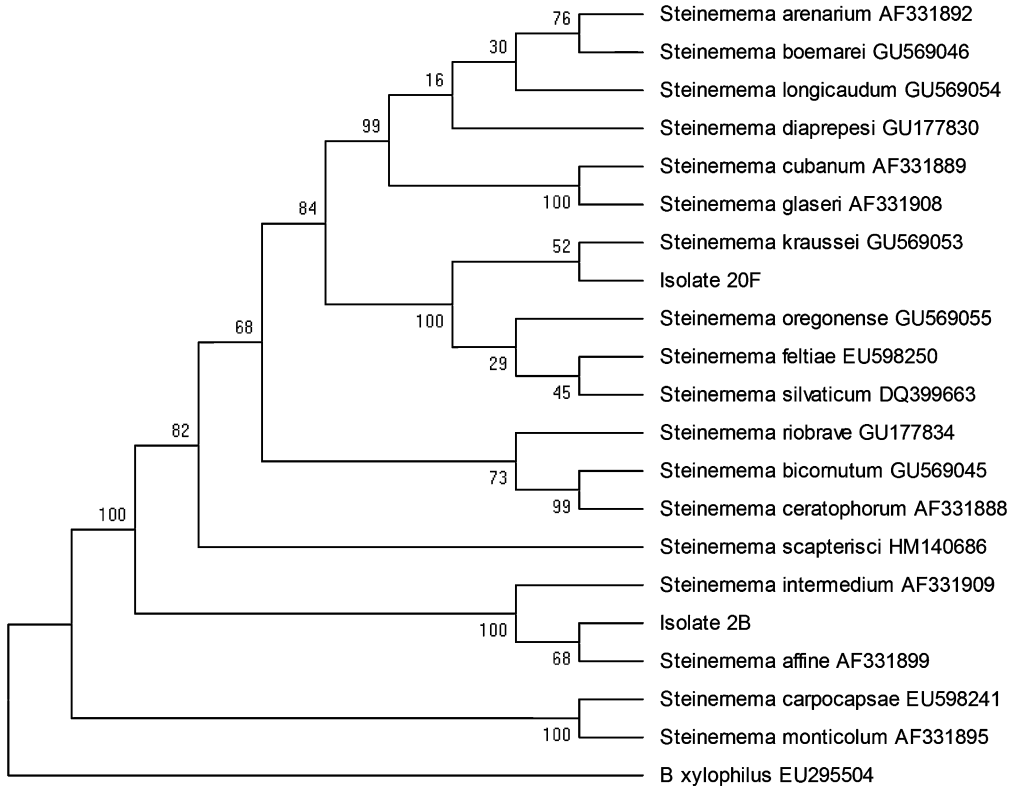

Fig. 3. Phylogenetic relationships based on Maximum Parsimony between 18 *Steinernema* species and isolates 2B and 20F, with bootstrap analysis of D2D3 regions.

above 71%. Isolate 2B clusters with *S. affine* and *S. intermedium*, sharing 97.5% of identity with *S. affine* and 40.5% of identity with *S. intermdium* (Fig. 2). By maximum parsimony analysis of ITS rDNA, isolate 20F branches with species from the *kraussei/feltiae*-group (*S. feltiae, S. oregonense* Liu *et* Berry and *S. kraussei*) with 99% bootstrap support. Isolate 20F groups with *S. kraussei* (Fig. 2) with an identity of 92.2%. MP analysis of ITS rDNA groups isolates 59F and 15G together, sharing an identity of 85.5% (Fig. 2). These isolates group with species from the *glaseri*-group (*S. cubanum* Mráček, Hernández *et* Boemare, S. *diaprepsi* Nguyen *et* Duncan, *S. glaseri* and *S. longicaudum* Shen *et* Wang, etc.) suggesting that these two isolates could represent a new species.

28S rDNA region, domain D2 and D3. For isolates 15G and 59F, sequences from the 28S domain D2D3 rDNA were not obtained. For this reason, the phylogenetic analysis of this region does not include these isolates. MP analysis of the 28S domain D2D3 rDNA dataset was constructed based upon 959 positions involving 21 nucleotide sequences (Fig. 3). Isolate 2B also groups with the clade comprising *S. intermedium* and *S. affine*, as its ITS MP analysis, supported by strong bootstrap values (100%) (Fig. 3), shares an identity of 91.8% with *S. affine* and of 90% with *S. intermedium*. 28S rDNA sequence of isolate 20F groups with *S. kraussei*, sharing with it an identity of 80.7% (Fig. 3).

Cytochrome c oxidase subunit I (COI). MP analysis of cytochrome c oxidase subunit 1 (*COI*) of the mtDNA dataset was constructed based upon 889 positions involving 21 nucleotide sequences. *COI* sequence shows the same results already found with ITS and D2D3, identifying isolate 2B as *S. intermedium*, isolate 20F as *S. kraussei* and isolates 15G and 59F as *Steinernema* sp. belonging to the *glaseri*-group (Fig. 4).

DISCUSSION

Accurate identification of entomopathogenic nematodes has important implications in systematics and population genetics and is of major importance for selection of species for future use in biological control. The combination of molecular and morphological methods is necessary to solve a variety of issues in EPN taxonomy. A recent study of phylogenetic relationships among *Steinernema* species combined morphological and molecular methods and showed that most morphological features are not phylogenetically informative (Spiridonov *et al*., 2004). Still, it is possible to obtain some areas of agreement between morphological and molecular results. For example, taxa with morphological differences, such as *S. affine* and *S. intermedium*, are closely related in molecular data (Nguyen *et al*., 2007). At present, *S. intermedium sensu lato* includes a number

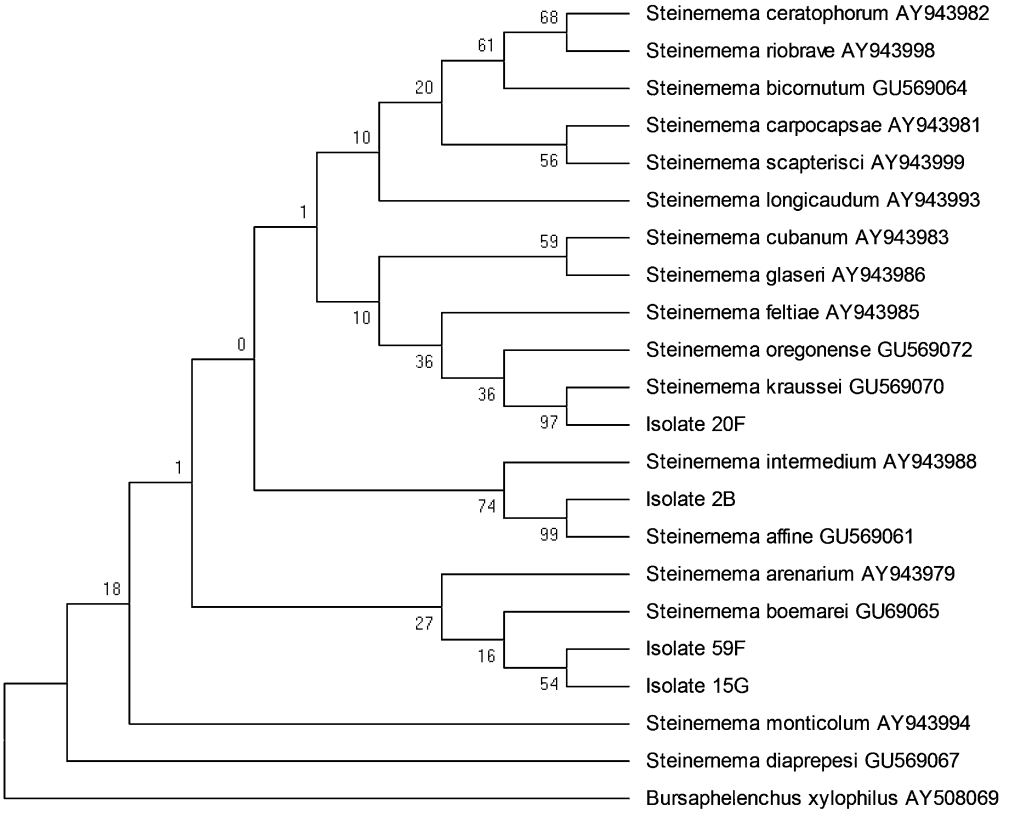

Fig. 4. Phylogenetic relationships based on Maximum Parsimony between 17 Steinernema species and isolates 2B, 20F, 59F and 15G with bootstrap analysis of *COI* regions.

of isolates from North America and throughout Eurasia. These isolates vary significantly in morphology, even sharing characters with *S. affine* (Yoshida *et al.*, pers. com.). The isolates of the *S. affine/S. intermedium* complex need a serious morphological and DNA revision. Stock (2002) points to the difficulties in using just molecular identification for EPN.

Several studies on molecular markers show that the 28S and ITS regions from ribosomal DNA and mitochondrial cytochrome C oxidase subunit I gene (*COI*) can be considered the best DNA regions to study phylogenetic relationships among EPN (Liu *et al.*, 1999; Stock *et al.*, 2001; Nguyen *et al.*, 2007). The D2D3 segment is a region that seems to evolve slower than ITS and *COI,* so it is an interesting tool for species delimitation (Nadler, 2002). Morphological characters in phylogenetic analyses of EPN remain robust, but their use requires taxonomic expertise. For a more accurate identification, phylogenetic trees should incorporate molecular and morphological data, because with molecular data alone it is sometimes impossible to make inferences from homologous characters (Liu and Berry 1996; Qui *et al.*, 2004; Nguyen *et al.*, 2007; Lee *et al.*, 2009).

During the survey undertaken after 2006 in continental Portugal, *H. bacteriophora* and *S. feltiae* were recovered (Valadas *et al.*, 2007; Valadas *et al.*, 2009). The present work describes three additional species that were identified as *S. kraussei, S. intermedium* and *Steinernema* sp. *glaseri*-group. *Steinernema intermedium* is a common species in Spain (Garcia del Pino, 2005), a neighbouring country with climatic conditions similar to Portugal. *Steinernema intermedium* appears to have a global distribution, having been originally described from South Carolina, USA (Nguyen *et al.*, 2007). In Europe this species is known from Germany (Sturhan, 1999), Czech Republic (Mráček *et al.*, 1999), Spain (Garcia del Pino, 2005) and Switzerland (Steiner, 1996). *Steinernema kraussei* is widely distributed in Europe, having been reported from Belgium, Czech Republic, Germany, Sweden, Netherlands, UK and Italy (Tarasco *et al.*, 2009). This species is common in European woodlands and forest soils (Sturhan *et al.*, 2005). Also, it has been found in Canada (Mráček and Webster, 1993) and USA (Stock *et al.*, 2000); it is prevalent in woodland habitats (Mráček *et al.*, 2005) and is rarely found in open habitats.

The current study on EPN identification was based on three regions from ribosomal and mitochondrial DNA combined with morphological and morphometric identification. Thus, the comparison of ITS and 28S rDNA and cytochrome C oxidase subunit I (*COI*) mtDNA provides information to assess the relatedness among *Steinernema* spp. In the present study the use of DNA sequences provided limited information concerning the closest species of our isolates, using two regions of ribosomal DNA (ITS and 28S, D2D3 domain) and one region of mtDNA (cytochrome C oxidase subunit I) for isolate 2B. Based on morphological and morphometric data, isolate 2B was identified as *S. intermedium* due to the presence of bluntly tipped spicules and lack of a spine in the tail of infective juveniles (Fig. 1), which is characteristic of the IJ of *S. affine*. In Portugal, *S. intermedium* occurred in *Pinus pinea* forests and in a soil with low pH (5.34). *Steinernema affine* and *S. intermedium* probably derived from the same ancestor, the differences between the two being due to their adaptations for survival in different geographic conditions, and so there is a clear disadvantage of using just a molecular and not a morphological approach to identify the species. Using just molecular data, isolate 2B groups with *S. affine* in ITS and *COI* markers with bootstraps of 68% and 99%, respectively. These groups then group with *S. intermedium*, with bootstraps between 74-100%. Without morphological characterization, it would not be possible to identify isolate 2B as *S. intermedium*. Isolate 20F was morphologically identified as *S. kraussei*. This species can be distinguished by spicule shape, usually with a finely oblongate manubrium, moderately developed rostrum and short hyaline layer. Molecularly, isolate 20F groups with *S. kraussei*, for all the three markers with strong bootstrap values between 52 and 99%. Isolates 15G and 59F, represent the same species because, in phylogenetic analysis using ITS and *COI* markers, they always group together. They were identified as *Steinernema* sp. belonging to the *glaseri*-group, with which they share morphological characteristics. The only information that we obtained from the morphology and sequence analysis of these two isolates was that they share high degrees of identity, and group with species of the *glaseri*-group. The clarification of whether these isolates are conspecific either with *S. glaseri*, found in the Azores (Rosa *et al.*, 2000), or *S. arenarium*, found in Spain (Garcia del Pino, 2005), or are even a new species, requires more detailed study. Moreover, a comparison with *S. boemarei* Lee, Sicard, Skeie *et* Stock, belonging to the *glaseri*-group and described from southern France (Lee *et al.*, 2009), is needed.

This study has identified three more species new to continental Portugal, namely *S. kraussei, S. intermedium* and a species which we have so far identified as *Steinernema* sp. *glaseri*-group. Previously, only *S. feltiae* (Valadas *et al.,* 2007) and *H. bacteriophora* (Valadas *et al.,* 2009) had been reported from continental Portugal. The habitats where *S. kraussei* was found was moors and heathlands and *S. intermedium* was detected in mixed forest, both considered acid soils (5.34 for *S. intermedium* and 4.17 for *S. kraussei*). Also, it seems that these two previous species are not so abundant in continental Portugal as *S. feltiae* and *H. bacteriophora,* that were found in 36 of the soil positive samples, whereas *S. intermedium* and *S. kraussei,* as well as *Steinernema* spp. were just detected in four soil samples, respectively, out of the 320 samples collected in Alentejo and central continental Portugal.

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