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A survey of entomopathogenic nematode species in continental Portugal

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Abstract

Entomopathogenic nematodes (EPN) are lethal parasites of insects, used as biocontrol agents. The objectives of this work were to survey the presence of EPN in continental Portugal and to characterize the different species. Of the 791 soil samples collected throughout continental Portugal, 53 were positive for EPN. *Steinernema feltiae* and *Heterorhabditis bacteriophora* were the two most abundant species. Analysis of EPN geographical distribution revealed an association between nematode species and vegetation type. *Heterorhabditis bacteriophora* was mostly found in the Alentejo region while *S. feltiae* was present in land occupied by agriculture with natural vegetation, broadleaved forest, mixed forest and transitional woodland-shrub, agro-forestry areas, complex cultivated patterns and non-irrigated arable land. Although no clear association was found between species and soil type, *S. feltiae* was typically recovered from cambisols and *H. bacteriophora* was more abundant in lithosols. Sequencing of the internal transcribed spacer (ITS) region indicated that *S. feltiae* was the most abundant species, followed by *H. bacteriophora*. *Steinernema intermedium* and *S. kraussei* were each isolated from one site and *Steinernema* sp. from two sites. Phylogenetic analyses of ITS, D2D3 expansion region of the 28S rRNA gene, as well as mitochondrial cytochrome *c* oxidase subunit I (COXI) and cytochrome *b* (*cytb*) genes, was performed to evaluate the genetic diversity of *S. feltiae* and *H. bacteriophora*. No significant genetic diversity was found among *H. bacteriophora* isolates. However, COXI seems to be the best marker to study genetic diversity of *S. feltiae*. This survey contributes to the understanding of EPN distribution in Europe.

Introduction

Entomopathogenic nematodes (EPN) are a group of nematode families which are insect parasites and possess many attributes that enable their commercial use as biocontrol agents (Kaya & Stock, 1997). They are promising candidates for biocontrol of insects due to their ability to search for hosts, safety to non-target organisms and the environment, high reproductive potential, capacity to be mass produced and ability to be used with other agricultural chemicals (Koppenhöfer, 2000). Only the infective third-stage juveniles (IJ) of these nematodes are found free living in soil and non-feeding stages, under diverse ecological conditions and in all kinds of habitats (Hominick *et al.*, 1996), being able to survive in soil for extended periods, until they find a suitable host. EPN are distributed worldwide, and include more than 16 species of *Heterorhabditis* and at

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least 60 species of *Steinernema* (Nguyen & Hunt, 2007). They are obligate parasites of insects that form complex, highly virulent symbiotic relationships with enterobacteria, *Xenorhabdus*–Steinernematidae and *Photorhabdus*–Heterorhabditidae (Boemare, 2002). As parasites of insects, they have a wide range of hosts (Kaya & Gaugler, 1993) being used in crop protection in Australia, Europe, USA and Asia (Georgis *et al.*, 2006).

The study of their genetic diversity is extremely important because new species and/or isolates may be useful as biological control agents against agriculturally important pests (Stock, 2009). The detection and identification of indigenous EPN isolates is of major importance, due to differences in strain virulence against natural and local insect pests, environmental conditions that may affect their survival and reproductive potential (Stock, 2009). Several surveys on EPN have already been conducted in Mediterranean countries, namely Egypt (Shamseldean & Abd- Elgawad, 1994), Greece (Menti *et al.*, 1997), Italy (Triggiani & Tarasco, 2000), Israel (Glazer *et al.*, 1991), Palestine (Iraki *et al.*, 2003), Spain (García del Pino, 2005; Campos-Herrera *et al.*, 2007) and Turkey (Hazir *et al.*, 2003). Recently, surveys have been conducted in several European countries, such as Austria (Hozzank *et al.*, 2003), Belgium (Midituri *et al.*, 1997), Bulgaria (Shishiniova *et al.*, 2000), Czechoslovakia (Mráček *et al.*, 1999), Denmark (Nielsen & Philipsen, 2003), France (Emelianoff *et al.*, 2008), Germany (Sturhan & Ruess, 1999), Poland (Bednarek, 1998), Russia (Ivanova *et al.*, 2000), Slovakia (Sturhan & Liskova, 1999), Switzerland (Steiner, 1996) and United Kingdom (Gwynn & Richardson, 1996). Entomopathogenic nematodes show significant variation in behaviour, host range, infectivity, reproduction and tolerance to adverse environmental conditions and therefore it is of major interest to fully characterize natural populations (Stock, 2009).

Studies on the genera *Steinernema* and *Heterorhabditis* have been conducted using molecular methods, such as random amplification of polymorphic DNA (RAPD) (Liu & Berry, 1996) and restriction fragment length polymorphism (RFLP) (Reid *et al.*, 1997). After several tested methods, sequencing of different regions of the genome has become the most suitable approach, not only for assessing phylogenetic relationships, but also for species delimitation (Stock, 2009). Among nuclear genes, ribosomal genes have been used extensively at different taxonomic levels. Ribosomal genes include the 18S rRNA gene, the internal transcribed spacers (ITS1 and ITS2), the 5.8S and the 28S rRNA genes, which contain variable and conserved regions (Stock, 2009). The 5.8S rRNA gene is a highly conserved region, contrary to the ITS1 and ITS2 regions, which evolve at a higher rate than the 18S and 28S rRNA genes, making them ideal for phylogenetic studies at species and population levels (Nguyen *et al*, 2001; Spiridonov *et al.*, 2004; Stock, 2009). Sequences of the D2D3 expansion region of the 28S rRNA gene were used by some authors to characterize EPN populations (Stock *et al.*, 2001) and may yield more information than the ITS region. On the other hand, mitochondrial cytochrome *c* oxidase subunit I (COXI) and cytochrome *b* (*cytb*) genes evolve more slowly, being better suited for deeper lineage phylogenies (Stock, 2009). Mitochondrial DNA sequences may be more useful in genetic diversity studies (Edgington *et al.*, 2010).

The genus *Steinernema* is divided into five phylogenetic groups based on the D2D3 expansion region of the 28S rRNA gene and infective juveniles' length (Nguyen & Hunt, 2007): '*bicornutum-group*', '*carpocapsae-group*', '*feltiae-group*', '*glaseri-group*' and '*intermedium-group*'.

Earlier reports of EPN in Portugal were conducted in the Azores archipelago (Simões *et al.*, 1994; Rosa & Simões, 2004), where several surveys have been undertaken as part of a wide programme to find endemic biological agents to control insect pests of pastures. These previous studies described the presence of *Steinernema carpocapsae, S. glaseri* and *Heterorhabditis bacteriophora* in the Azores (Simões *et al.*, 1994; Rosa & Simões, 2004). Until 2006, there were no studies on entomopathogenic nematodes from continental Portugal. The first published report was on the identification of three isolates (I1, I8 and H9) of *S. feltiae* based on ITS sequence (Valadas *et al.*, 2007). Later, three other isolates (I3, R7 and X7), were identified as *H. bacteriophora* using both ITS sequence and morphological characterization (Valadas *et al.*, 2009). More recently, three other species, *S. intermedium* (isolate 2B), *S. kraussei* (isolate 20F) and *Steinernema* sp. (isolates 59F and 15G) have been described (Valadas *et al.*, 2011), using morphological and molecular data.

It is known that EPN distribution depends on temperature and precipitation and is closely related to vegetation type and presence of insect hosts. Soil type and texture are also very important parameters which influence EPN distribution (Campos-Herrera *et al.*, 2011; El Borai *et al.*, 2012). Continental Portugal has a wide diversity of crops, such as fruit trees, cereals and vegetables, and natural habitats, such as conifer forests and grasslands. These habitats are subject to insect pests which every year cause significant losses in agricultural production.

The major objectives of this research were to determine the distribution of EPN in continental Portugal and to characterize the different species using several molecular markers.

Materials and methods

Survey zones and sampling procedures

Between 2006 and 2009, 791 soil samples were haphazardly collected across continental Portugal, divided into five NUTS (Nomenclature of Territorial Units for Statistics): Norte, Centro, Lisboa e Vale do Tejo (herein designated as Lisboa), Alentejo and Algarve (http://www.igeo.pt/atlas/Cap3/Cap3f_1.html) (see Table S1, available online).

According to Köppen's climate classification (Köppen & Geiger, 1928), continental Portugal is divided in two regions: one temperate with rainy winters and dry, hot summers (Csa) and another temperate with rainy winters and dry, cool summers (Csb). Vegetation is affected by climate and, thus, continental Portugal has three kinds of influence: Atlantic, Continental and Mediterranean, Atlantic being predominant. Soil samples were collected from both cultivated and non-cultivated areas, covering the two climatic regions of continental Portugal,

including different vegetation types, such as irrigated land, forests, grasslands and cultivated fields, among others. Three to four subsamples were collected at 0–20 cm and used to create a single sample representative of 200 m^2. Soil samples were properly dated and identified with GPS (Global Positioning System) (Garmin, Olathe, Kansas, USA) location.

Soil and vegetation types were mapped with ArcGIS software version 10.0 (ESRI; http://www.esri.com/) using Food and Agriculture Organization soil classification (FAO, 2006) and Coordination of information on the environment (CORINE) land cover classification (Caetano *et al.*, 2009). Temperature and precipitation data were obtained from 'PORDATA, Base de Dados Portugal Contemporâneo' (http://www.pordata.pt).

Nematode recovery, propagation and identification

EPN were recovered from soil samples using the baiting technique, described by Bedding & Akhurst (1975). Before processing, samples were homogenized and then baited with ten last instar larvae of *Galleria mellonella* placed inside a perforated metal tea bag, partly filled with soil which was embedded in the soil sample. Soil samples were stored in the dark at 25°C and dead *G. mellonella* were removed and replaced every 4 days for a total of 12 days of baiting. Collected *G. mellonella* were transferred to White traps (White, 1927) and IJ recovered for the 5–12 following days. IJ were stored in distilled water at 10°C. To establish new cultures, emerging nematodes were pooled for each sample and used to infect new *G. mellonella* larvae. Only IJ collected during the week after the first emergence from the insect cadavers were used to establish new cultures. The colour of *G. mellonella* cadavers, which ranges from cream to brown (*Steinernema* spp.) or red (*Heterorhabditis* spp.) within 24–48 h after nematode penetration, was used for preliminary determination of EPN genera. Further identification of the genera of all 53 isolates was carried out by the method of Nguyen & Hunt (2007) and by sequencing the rRNA ITS region (Nguyen *et al.*, 2001).

Sequencing of ribosomal regions and mitochondrial genes

For each isolate, genomic DNA was extracted from a suspension of 50 µl with more than 10,000 nematodes. Total DNA was extracted with the JETQUICK Tissue DNA Spin Kit extraction kit (GENOMED, Löhne, Germany), according to the manufacturer's protocol. DNA was used for sequence analysis of ribosomal ITS regions and D2D3 region of the 28S rRNA gene, and mitochondrial genes *cytb* and COXI. Nematode DNA was kept at −20°C for further use.

Polymerase chain reactions (PCR) containing 1 × PCR buffer (Fermentas, Vilnius, Lithuania), 1.5 mM $MgCl_2$ (Fermentas), 200 µM of each deoxyribonucleoside triphosphate (dNTP) (Fermentas), 0.4 µM of each primer (with the exception of *cytb* primers, which were used at a concentration of 1 µM each) (Stabvida, Caparica, Portugal), 2.5 units of *Taq* DNA polymerase (recombinant) (Fermentas) and 5 µl template DNA (10–20 ng) were used (total volume 50 µl). Primers and PCR amplification conditions for each region and gene are presented in table 1.

All PCR products were analysed by electrophoresis in 1% agarose gels with Tris-borate buffer, stained in ethidium bromide, purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Uppsala, Sweden) and sequenced at Stabvida, Portugal.

Phylogenetic analysis

Multiple sequence alignments of ITS, D2D3 region of the 28S rRNA gene, COXI and *cytb* sequences were assembled using the ClustalW algorithm as implemented in BioEdit version 7.1.3.0 (Hall, 1999), under default alignment parameters. The best-fitting evolutionary model of nucleotide substitutions was determined, using MEGA5 version 5.05 (Tamura *et al.*, 2011) and phylogenetic relationships among isolates were reconstructed by neighbour-joining (Saitou & Nei, 1987) and maximum likelihood (ML) methods. Gaps or indels were treated using the complete deletion option. Bootstrap analysis was performed with 100 replicates (Felsenstein, 1985). Sequences were compared with those from reference organisms/strains available in the GenBank database.

Data analysis

Statistical analysis was performed using PASW Statistics 18 release 18.0.0 software (SPSS Inc., Chicago, Illinois, USA). Relationships between species and NUTS, vegetation or soil types were determined using the chi-square test of association. Results are presented as the test statistic (χ^2), degrees of freedom (df) and probability of equal or greater deviation (P).

Table 1. Primers used for sequencing reactions of ribosomal regions and mitochondrial genes.

Target	Primer	Sequence (5′–3′)	Product (bp)	Reference
ITS rRNA	TW81-F	5′-GTTTCCGTAGGTGAACC TGC-3′	1000	Joyce *et al.*, 1994
	AB28-R	5′-ATATGCTTAAGTTCAGCGGGT-3′		
D2D3 expansion	#391-F	5′-AGCGGAGGAAAAGAAACTAA-3′	1100	Nadler *et al.*, 2006
	#501-R	5′-TCGGAAGGAACCAGCTACTA-3′		
COXI	#507-F	5′-AGTTCTAATCATAARGATATYGG-3′	1000	Nadler *et al.*, 2006
	#588-R	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′		
cytb	CytBHetF-F	5′-TTTTGTAAATTCTCTTGTT-3′	1200	The present study
	CytBHetR-R	5′-AAATAGAAAACAAATAACTCAAA-3′		

Correspondence analysis (CA) was used as an explorative method to study associations and to reveal interdependencies between each two of the above-mentioned variables. Visualization using CA is based on representing chi-square distances among species and NUTS, vegetation or soil types.

Results

Data analysis

EPN were recovered from 53 of the 791 sampled sites (6.7%) (fig. 1). Nine soil samples (17%) were positive for the occurrence of heterorhabditids and 44 (83%) for steinernematids, identified as described. Nematodes isolated from the positive samples were further identified to the species level based on ITS sequences.

Although there is no association between species and NUTS ($P > 0.1$) (table 2), the CA biplot revealed that *H. bacteriophora* isolates are more abundant in the Alentejo region. The nine positive samples for *H. bacteriophora* were recovered from sites below the altitude of 400 m. *Steinernema feltiae* was detected from 40 sites (75.5%) in locations where the altitude varies between 13 and 878 m. No association was found between species and climate ($P > 0.05$).

Habitat and soil types

Positive samples were most commonly found in mixed forest, land occupied by agriculture with natural vegetation, broadleaved forest and transitional woodland-shrub (table 3). *Heterorhabditis bacteriophora* was found in a wide range of habitats including: mixed forest, transitional woodland-shrub, vineyards, broadleaved forest, land occupied by agriculture with natural vegetation and permanently irrigated land (table 3). *Steinernema feltiae* was present in all kinds of habitats containing EPN, being more abundant in land occupied by agriculture with natural vegetation, broadleaved forest, mixed forest, transitional woodland-shrub, agro-forestry areas, complex cultivated patterns and non-irrigated arable land (table 3). *Steinernema intermedium* was found in a mixed forest and *S. kraussei* in moors and heathland habitat (table 3). The *Steinernema* sp. isolates were found in a broadleaved forest and in a mixed forest (table 3). There is an association between species and vegetation type ($\chi^2 = 83.438$, df $= 60$, $P > 0.05$) (fig. 2). Furthermore, the CA biplot showed that the most abundant species, *S. feltiae*, is present mainly in land occupied by agriculture with natural vegetation, broadleaved forest, fruit trees and berry plantations, olive groves and non-irrigated arable land (fig. 2). *Steinernema feltiae* was found in almost all soil types, namely cambisols, podzols, luvisols, lithosols and regosols, whereas *H. bacteriophora* was only found in lithosols, podzols, cambisols and luvisols (table 4). The *Steinernema* sp. isolates were found in a lithosol and a cambisol. *Steinernema kraussei* and *S. intermedium* isolates were found in a cambisol and a podzol, respectively (table 4).

No association was found between species and soil type ($P > 0.1$). However, the CA biplot indicates that the two most abundant species are not randomly distributed by the different soil types: *S. feltiae* was mostly recovered from luvisols, but also from cambisols and regosols; *H. bacteriophora* is more abundant in lithosols. *Heterorhabditis bacteriophora* was found in soil samples with pH values between 4.37 and 7.92, whereas *S. feltiae* was present in soils with pH values between 4.02 and 8.11. *Steinernema intermedium* and *S. kraussei* were found in soils with pH 4.17–5.34. *Steinernema* sp. isolates were recovered in soils with pH values of 4.90 and 6.23. No association was found between species and the soil's physical and chemical characteristics ($P > 0.05$).

Phylogenetic analysis of ITS, D2D3, COXI and cytb

Nematodes isolated from the positive samples were identified based on ITS sequences as *S. feltiae* (40 isolates), *S. intermedium* (one isolate), *S. kraussei* (one isolate), *Steinernema* sp. (two isolates) and *H. bacteriophora* (nine isolates) (fig. 3). Because *S. feltiae* and *H. bacteriophora* were the two most abundant EPN species, D2D3 region of the 28S rRNA gene, COXI and *cytb* genes were used to further evaluate the genetic diversity of the populations. Repeated attempts to amplify and sequence some genes for some isolates were unsuccessful, thus justifying the discrepant number of isolates in the different phylogenies.

ITS phylogenies of S. feltiae *and* H. bacteriophora

For 37 *S. feltiae* and eight *H. bacteriophora* Portuguese isolates, a PCR product of approximately 1000 bp, containing the partial sequence of ITS1 and ITS2, and the whole 5.8S rRNA gene, was obtained and sequenced. *Heterorhabditis bacteriophora* was included as outgroup (fig. 3).

Three sequence types were obtained with six *S. feltiae* isolates being used for phylogenetic analyses, namely isolate 11A, which also represents 24A; isolates 32A and 86E, representing 93E, I1 and Z8; and isolates I2, 22A and H9 representing the remaining isolates.

Comparing the six Portuguese with foreign *S. feltiae* isolates, few polymorphisms were found. All Portuguese and foreign *S. feltiae* isolates group in the same cluster, together with the other species from the *'feltiae-group'* (*S. oregonense* and *S. kraussei*) sharing sequence identities between 94 and 100% (fig. 3). *Steinernema feltiae* Portuguese isolates showed some genetic diversity, which is represented by isolates 11A, 22A, H9 and I2, together with *S. feltiae* from Spain, which share sequence identities between 99.6 and 99.8%. Portuguese isolates 86E and 32A, together with the remaining *S. feltiae* foreign isolates, share sequence identities between 94 and 99.8%. Other species belonging to the *'feltiae-group'*, *S. kraussei* and *S. oregonense*, as well as *S. kraussei* isolate 20F, share sequence identities between 56 and 95% with foreign and Portuguese isolates. Isolates 15G and 59F share the highest identity with species of the *'glaseri-group'* (75–85%), suggesting that *Steinernema* sp. belong to this group (fig. 3). Grouping with the major cluster are species belonging to *'carpocapsae-group'* and *'bicornutum-group'*.

The most distant group includes the two species from the *'intermedium-group'*, *S. intermedium* and *S. affine*, and the Portuguese isolate 2B of the species *S. intermedium*. Species from the *'intermedium-group'* share identities

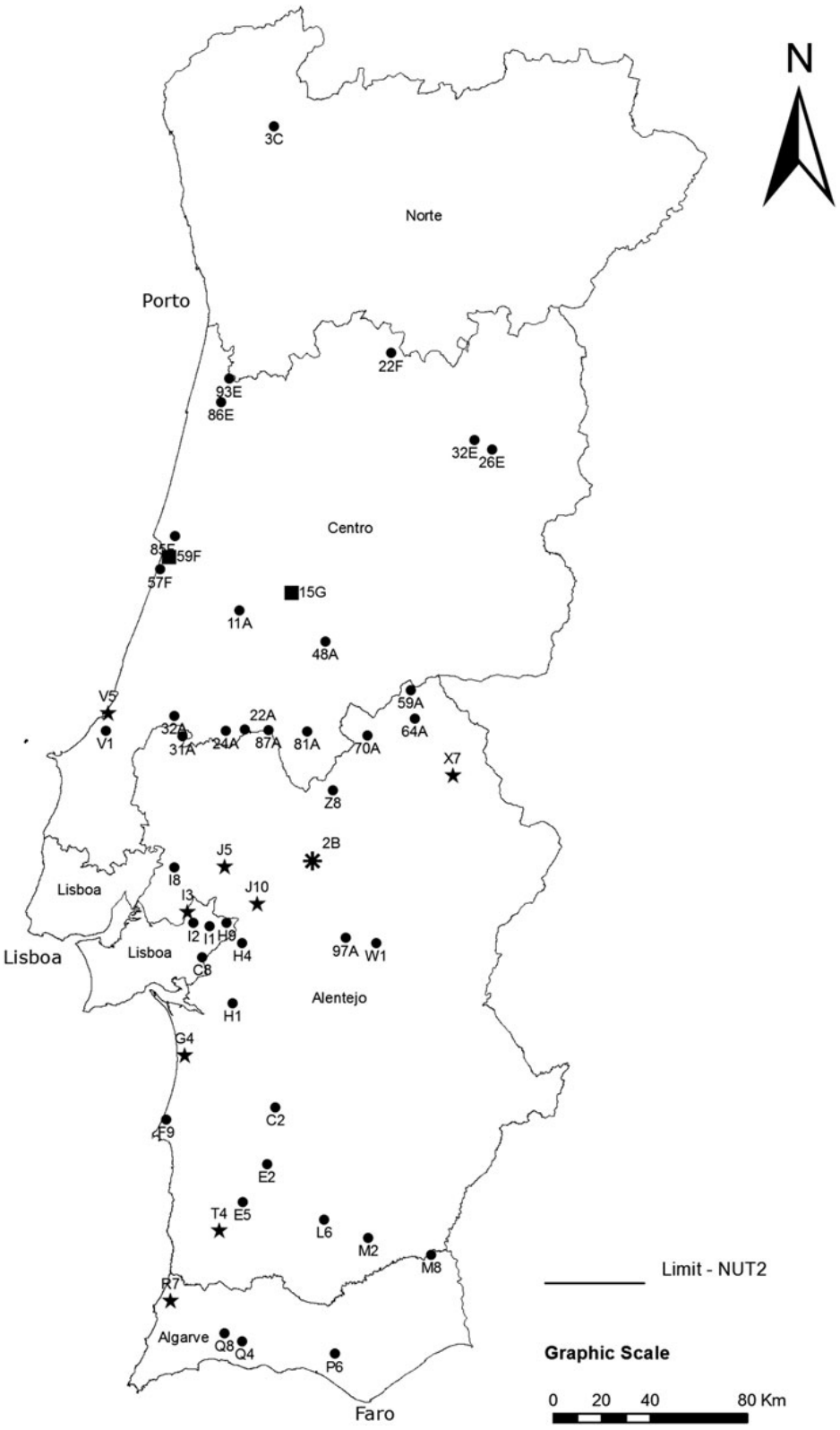

Fig. 1. Entomopathogenic nematode distribution across the five NUTS (Nomenclature of Territorial Units for Statistics) of continental Portugal. Each point is marked with the name of the isolate and the corresponding species. Map based on the CAOP (Carta Administrativa e Oficial de Portugal; http://www.igeo.pt). *Steinernema* sp., ■; *S. feltiae*, ●; *S. kraussei*, ▲; *S. intermedium*, ✱; *Heterorhabditis bacteriophora*, ★.

Table 2. The occurrence of entomopathogenic nematodes in continental Portugal using NUTS (Nomenclature of Territorial Units for Statistics) (CAOP; http://www.igeo.pt), relative to total area covered and the proportion of EPN species recovered.

NUTS	Area (km^2)	Area (%)	Number of samples	Number of EPN	Positive samples (%)
Norte	21 285.876	23.89	268	1	1.9[1]
Centro	28 199.404	31.65	207	19	28[1]; 1.9[3]; 3.8[4]; 1.9[5]
Lisboa e Vale do Tejo	3001.938	3.37	84	5	7.5[1];1.9[5]
Alentejo	31 604.906	35.48	181	23	30.2[1]; 1.9[2]; 11.3[5]
Algarve	4996.795	5.61	51	5	7.5[1]; 1.9[5]

[1] *Steinernema feltiae;*
[2] *S. intermedium;*
[3] *S. kraussei;*
[4] *Steinernema* sp.;
[5] *Heterorhabditis bacteriophora.*

between 93 and 99% and have 57–86% sequence identity with species from the *'feltiae-group'* (fig. 3).

ITS sequences indicate that all Portuguese *Heterorhabditis* isolates belong to the species *H. bacteriophora*. The eight Portuguese isolates of *H. bacteriophora* are 100% identical in sequence and all group together with foreign *H. bacteriophora* isolates (with bootstrap support of 98%), sharing identities of 98–100% (data not shown). *Heterorhabditis zealandica* also groups inside this cluster, sharing 96–97% identity with *H. bacteriophora* isolates. The most distant species from *H. bacteriophora* are *H. indica, H. floridensis, H. amazonensis* and *H. baujardi*, sharing 70–72% identity with *H. bacteriophora* isolates (data not shown). *Heterorhabditis marelatus* and *H. megidis* are more similar to *H. bacteriophora* (sequence identity between 74 and 79%) than the previous species.

S. feltiae *and* H. bacteriophora *D2D3 phylogenies*

The D2D3 expansion region of the 28S rRNA gene sequences confirms the previous identification of EPN isolates as *S. feltiae* and *H. bacteriophora*.

For 27 *S. feltiae* and seven *H. bacteriophora* Portuguese isolates, a PCR product of approximately 1100 bp, containing the partial sequence of the D2D3 region of the 28S rRNA gene, was obtained and sequenced. From the 27, only five representative isolates were used in the phylogenetic analysis, because the others share 100% sequence identity with these isolates (isolate 3C represents isolates 11A, 22A, 22F, 24A, 57F, 59A, 70A, 85F, 87A, C8, E2, F9, H1, H9, I2, I8, L6 and P6; and isolate 32A stands for isolates 86E, 93E, V1 and I1) (fig. 4). *Globodera pallida* was included as outgroup, considering that the D2D3 *H. bacteriophora* sequence was too close to species from the '*carpocapsae-group*' to serve this purpose.

Comparing the five Portuguese isolates of *S. feltiae* with foreign *S. feltiae*, only a few polymorphisms were found. All Portuguese and foreign *S. feltiae* isolates group in the same cluster sharing 99–100% sequence identity with 99% bootstrap support, showing no genetic diversity between isolates (fig. 4). *Steinernema feltiae* isolates group with species from the *'feltiae-group'* (*S. monticolum* and *S. kraussei*) sharing sequence identities of 92–98% (bootstrap support 77%). Regarding the D2D3 phylogeny, no polymorphisms were found among *H. bacteriophora* Portuguese isolates. One major cluster was obtained, which contains all Portuguese isolates and the reference strains of *H. bacteriophora* (bootstrap support 98%). All *H. bacteriophora* isolates are 100% identical in sequence (data not shown). The closest species to *H. bacteriophora* is *H. megidis*, sharing a sequence identity between 91 and 93%; the most distant is *H. indica*, with a sequence identity of 90%.

S. feltiae *COXI gene phylogeny*

From the 23 Portuguese *S. feltiae* isolates, only 16 representative isolates were used for phylogenetic analysis (isolate 3C represents 87A; isolate 11A represents 22F and 70A; isolate H9 represents F9; isolate 24A represents P6; isolate I2 represents H1 and I8). *Heterorhabditis bacteriophora* was included as outgroup. A PCR product of approximately 1000 bp, containing the partial sequence of the COXI gene was obtained and sequenced for the Portuguese isolates.

There is a large cluster that includes all *S. feltiae* Portuguese isolates, together with the reference species of *S. feltiae*, sharing sequence identity values between 94 and 100% (bootstrap support of 99%) (fig. 5). Inside this major cluster there is some diversity between Portuguese isolates (sequence identity values of 97–100%), in some cases supported by bootstrap values that vary between 36 and 95% (fig. 5). For example, isolates 59A and 85F form a group sharing a sequence identity of 99.5%, grouping also with isolate H9, with whom they share a sequence identity of 99%. All *S. feltiae* Portuguese isolates share high sequence identity values (98–100%). This major cluster groups with *S. kraussei* and *S. oregonense*, both species of the '*feltiae-group*' sharing identities of 85–87% (fig. 5). The entire previous clade clusters together with species belonging to the *'intermedium-group'*, the *'bicornutum-group'*, the '*glaseri-group'* and the *'carpocapsae-group*' (fig. 5). The *Steinernema* sp. isolates 15G and 59F, group together sharing similarities of 92%. These isolates seem to represent a putative new species inside the '*glaseri-group*', with whom they share 87–90% sequence identity (fig. 5).

Table 3. The occurrence of entomopathogenic nematodes in the study, relative to the three classes of vegetation based on the 'Land Cover Nomenclature' (Caetano *et al.*, 2009).

Vegetation type			Number of samples	Number of EPN	Positive samples (%)
(type I)	(type II)	(type III)			
Artificial surfaces	Urban fabric	Discontinuous urban fabric (112)	47	2	3.8[1]
Artificial surfaces	Industrial, commercial and transport units	Industrial or commercial units (121)	6	0	0
Artificial surfaces	Industrial, commercial and transport units	Road and rail networks and associated land (122)	1	0	0
Artificial surfaces	Mine, dump and construction sites	Construction sites (133)	1	0	0
Agricultural areas	Arable lands	Non-irrigated arable land (211)	47	3	5.7[1]
Agricultural areas	Arable lands	Permanently irrigated land (212)	39	3	3.8[1]; 1.9[5]
Agricultural areas	Arable lands	Rice fields (213)	10	0	0
Agricultural areas	Permanent crops	Vineyards (221)	15	2	3.8[5]
Agricultural areas	Permanent crops	Fruit trees and berry plantation (222)	13	2	3.8[1]
Agricultural areas	Permanent crops	Olive groves (223)	20	2	3.8[1]
Agricultural areas	Pastures	Pastures (231)	2	0	0
Agricultural areas	Pastures	Artificial grasslands (232)	1	0	0
Agricultural areas	Heterogeneous agricultural areas	Annual crops associated with permanent crops (241)	71	0	0
Agricultural areas	Heterogeneous agricultural areas	Complex cultivation patterns (242)	89	3	5.7[1]
Agricultural areas	Heterogeneous agricultural areas	Land occupied by agriculture with natural vegetation (243)	43	7	11.3[1]; 1.8[5]
Agricultural areas	Heterogeneous agricultural areas	Agro-forestry areas (244)	21	3	5.7[1]
Forest and semi-natural areas	Forests	Broadleaved forest (311)	77	6	7.5[1]; 1.9[4]; 1.9[5]
Forest and semi-natural areas	Forests	Coniferous forest (312)	57	1	1.9[1]
Forest and semi-natural areas	Forests	Mixed forest (313)	58	8	7.5[1]; 1.9[2]; 1.9[4]; 3.8[5]
Forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Natural grasslands (321)	30	2	3.8[1]
Forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Moors and heathland (322)	16	1	1.9[3]
Forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Sclerophyllous vegetation (323)	14	0	0
Forest and semi-natural areas	Scrub and/or herbaceous vegetation associations	Transitional woodland-shrub (324)	94	6	7.5[1]; 3.8[5]
Forest and semi-natural areas	Open spaces with little or no vegetation	Beaches, dunes, sands (331)	3	0	0
Forest and semi-natural areas	Open spaces with little or no vegetation	Burnt areas (334)	2	0	0
Wetlands	Maritime wetlands	Salt marshes (421)	1	0	0
Water bodies	Inland waters	Water bodies (511)	3	1	1.9[1]
Water bodies	Marine waters	Estuaries (522)	10	1	1.9[1]

[1] *Steinernema feltiae;*
[2] *S. intermedium;*
[3] *S. kraussei;*
[4] *Steinernema* sp.;
[5] *Heterorhabditis bacteriophora.*

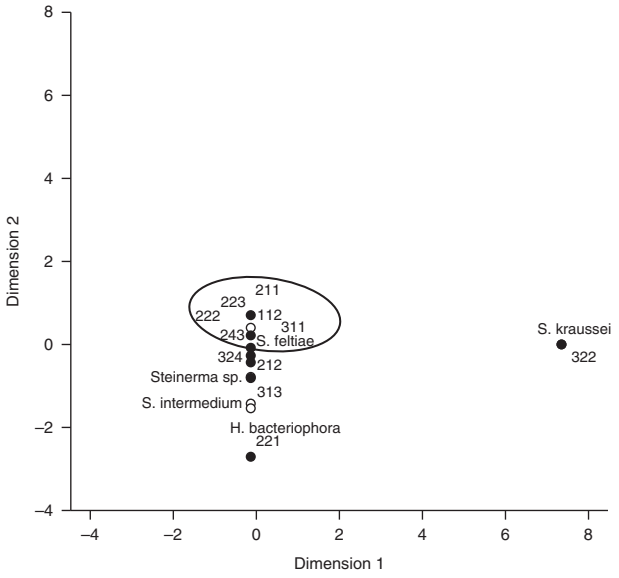

Fig. 2. CA biplot of the relationship between species and (a) NUTS (Nomenclature of Territorial Units for Statistics), (b) vegetation type, (c) soil type. Note: some dots are overlaid. Species, ○; vegetation type, ●.

H. bacteriophora cytb *gene phylogeny*

For nine *H. bacteriophora* Portuguese isolates, a PCR product of approximately 1200 bp, containing a partial sequence of the *cytb* gene was obtained and sequenced. *Steinernema carpocapsae* was included as outgroup. No polymorphisms were found among Portuguese isolates (100% identical). One major cluster (bootstrap value of 68%) was obtained, which contains all Portuguese isolates sharing 100% sequence identity (fig. 6). Portuguese isolates share 99.7% sequence identity with the reference strain of *H. bacteriophora* and 81.6% with *S. carpocapsae* (fig. 6).

Discussion

Even though national surveys already undertaken provide valuable data on EPN distribution (García del Pino & Palomo, 1996; Kary *et al.*, 2009; Edgington *et al.*, 2010; Khatri-Chhetri *et al*, 2010; Ma *et al.*, 2010), species habitat preferences are still poorly understood. The present study aimed at understanding the natural occurrence of EPN in continental Portugal, representing the most systematic and extensive survey made for the first time in the country to evaluate indigenous species of EPN.

The survey covered all NUTs, the different climatic regions and a wide variety of vegetation and soil types. Positive soil samples were analysed with the additional information collected, namely temperature and precipitation values, altitude and soil type. Although EPN were recovered at a low rate (6.7% of sampling sites) in our study, five different species were isolated from the entire country: *S. feltiae* (75%), *H. bacteriophora* (17%), *S. intermedium* (1.9%), *S. kraussei* (1.9%) and *Steinernema* sp. (3.8%). *Steinernema feltiae* and *H. bacteriophora* were the two most abundant species found in the country. One reason for the low recovery rate obtained in the present study, could be the fact that only one insect, *G. mellonella,* was used as trap insect, and it may not be the appropriate host for all EPN species (Kary *et al.*, 2009). Also, the fact that just one temperature value (25°C) was used for soil baiting samples may represent a limitation. The use of just one baiting temperature may not cover all the requirements for other EPN species (Mráček *et al.*, 2005). Furthermore, the choice of sampling sites may contribute to differences in EPN recovery percentage (Mráček *et al.*, 2005). However, this low recovery percentage is not unusual, and it has already been reported from other surveys (Rosa *et al.*, 2000; Hazir *et al.*, 2003; Kary *et al.*, 2009). Furthermore, the low *H. bacteriophora* recovery rate compared to *Steinernema* spp. may be due to the fact that this species, as mentioned by Emelianoff *et al.* (2008), is preferentially located in beaches, which were habitats not sampled in the present study. In addition, *H. bacteriophora* is highly mobile, responding to chemical signals from the host, and being adapted to infect less mobile insects that are found in lower soil layers (Ishibashi, 2002). Since our samples were collected from the upper soil layer, this could explain the low recovery and genetic diversity of *H. bacteriophora* found in continental Portugal.

Steinernema feltiae, S. intermedium and *H. bacteriophora* are considered to be the most common EPN species in Europe, having been detected previously in southern France and in northern Spain (Hominick, 2002). The recovery rates of both *Steinernema* spp. and *H. bacteriophora* are similar to those of other studies in the Mediterranean area: 1.3% *Heterorhabditis* and 22% *Steinernema* in Catalonia (García del Pino, 2005), and 5.3% *Heterorhabditis* and 8.5% *Steinernema* in Italy (Tarasco & Triggiani, 1997).

In other studies, the percentage recovery of EPN species has varied from 0.7 to 70.1% (Mráček & Becvár, 2000; Bruck, 2004). The 6.7% recovery detected in continental Portugal is within the interval of the expected values (3.3–23%) obtained in Spain, which has similar climatic conditions and types of soil and vegetation to continental Portugal (De Doucet & Gabarra, 1994; García del Pino, 1994, 2005; García del Pino & Palomo, 1996;

Table 4. The occurrence of entomopathogenic nematodes, relative to soil types (FAO, 2006).

Soil type	Number of samples	Number of EPN	Positive samples (%)
Podzols	99	12	17[1]; 1.9[2]; 3.8[5]
Cambisols	359	24	36[1]; 1.9[3]; 1.9[4]; 3.8[5]
Luvisols	111	11	17[1];3.8[5]
Lithosols	155	6	3.8[1]; 1.9[4]; 5.7[5]
Regosols	12	1	1.9[1]
Solonchaks	2	0	0
Fluvisols	15	0	0
No classification	38	0	0

[1] *Steinernema feltiae;*
[2] *S. intermedium;*
[3] *S. kraussei;*
[4] *Steinernema* sp.;
[5] *Heterorhabditis bacteriophora.*

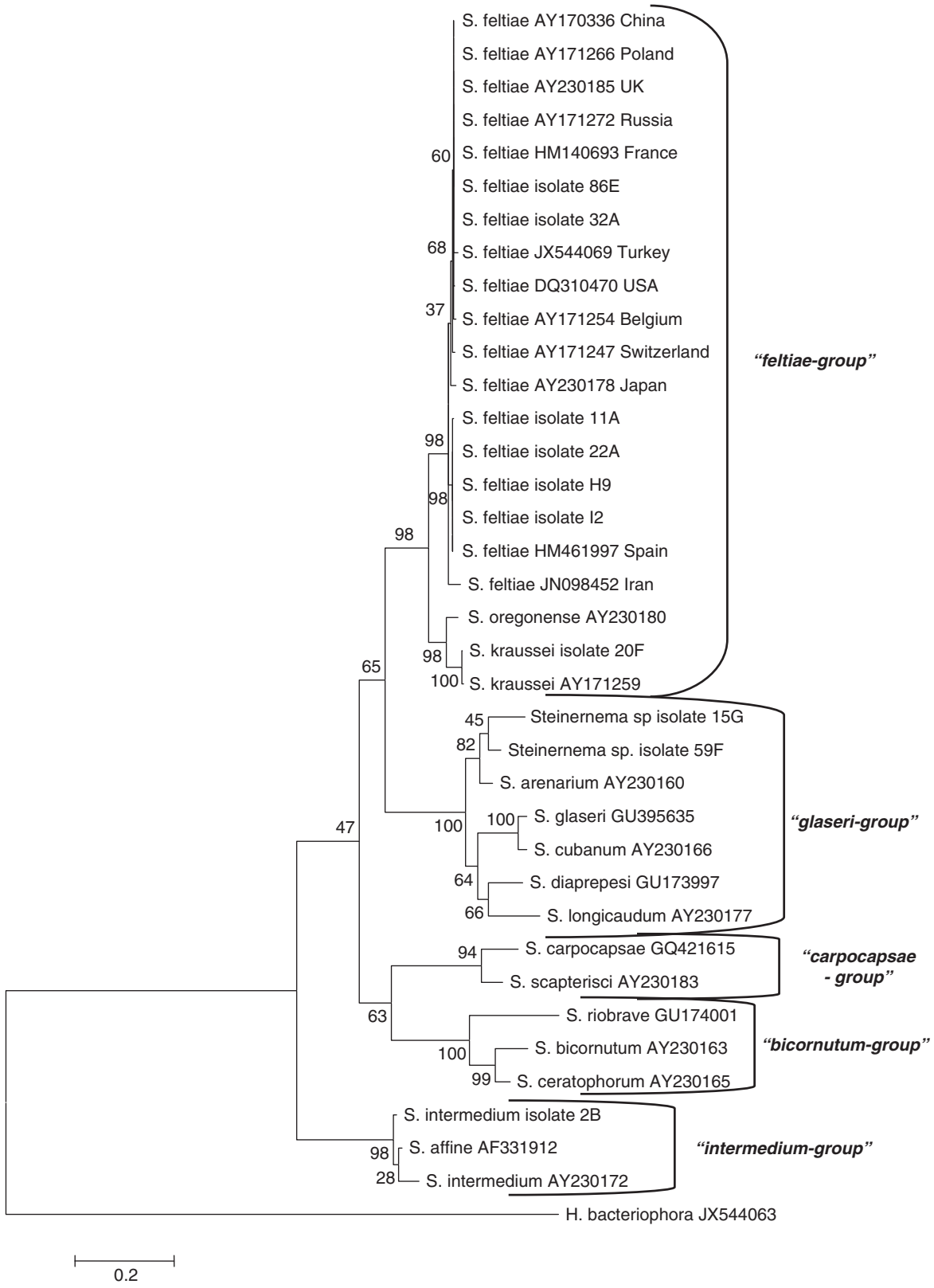

Fig. 3. Maximum likelihood ITS phylogenetic trees using Tamura 3-parameter model (Tamura, 1992), based on nucleotide sequences of *Steinernema* spp. and reference strains (alignment length 768 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 0.2 substitutions per site.

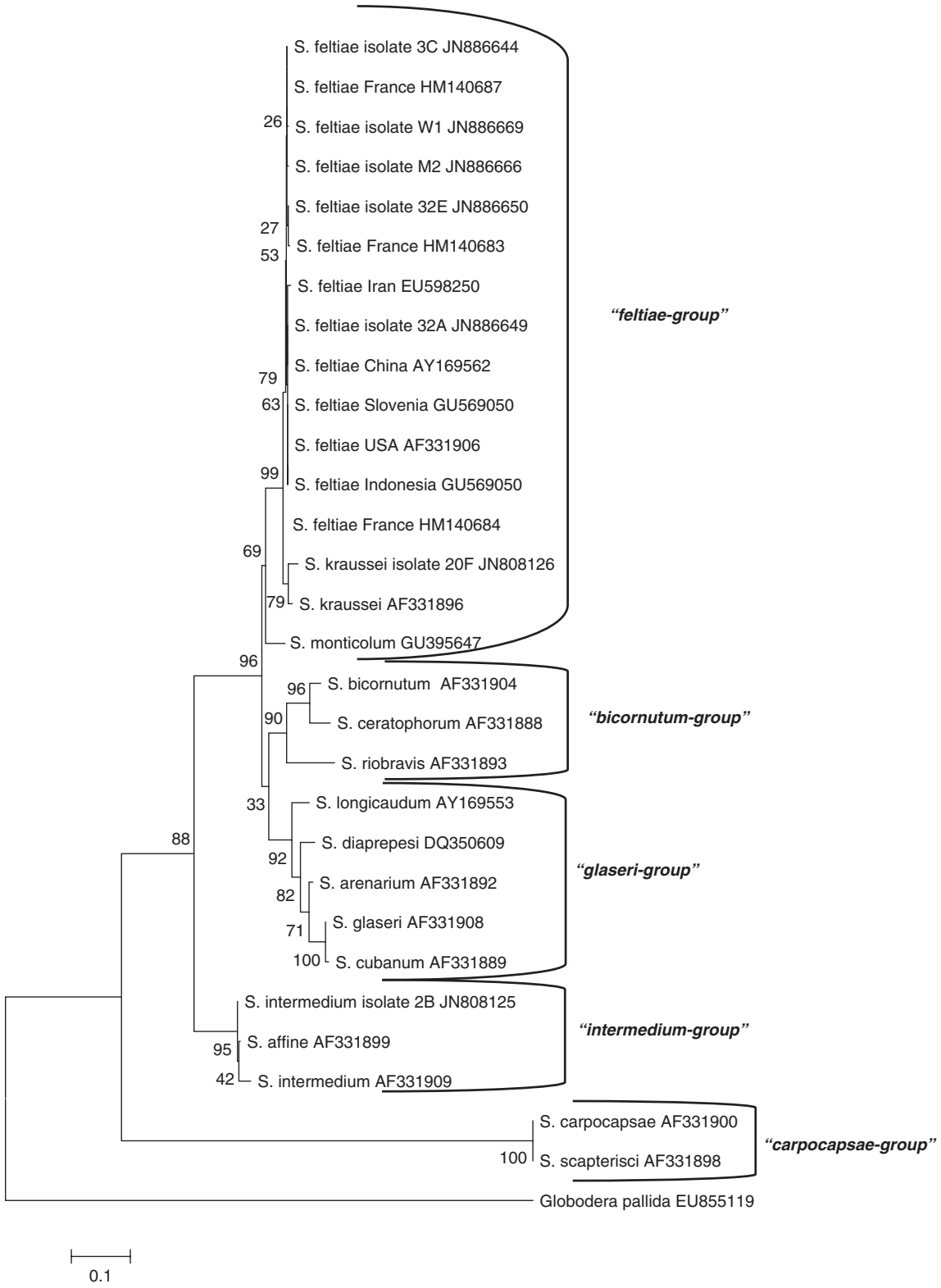

Fig. 4. Maximum likelihood D2D3 phylogenetic tree using Kimura 2-parameter model (Kimura, 1980), based on nucleotide sequences of *Steinernema* spp. and reference strains (alignment length 469 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 0.1 substitutions per site.

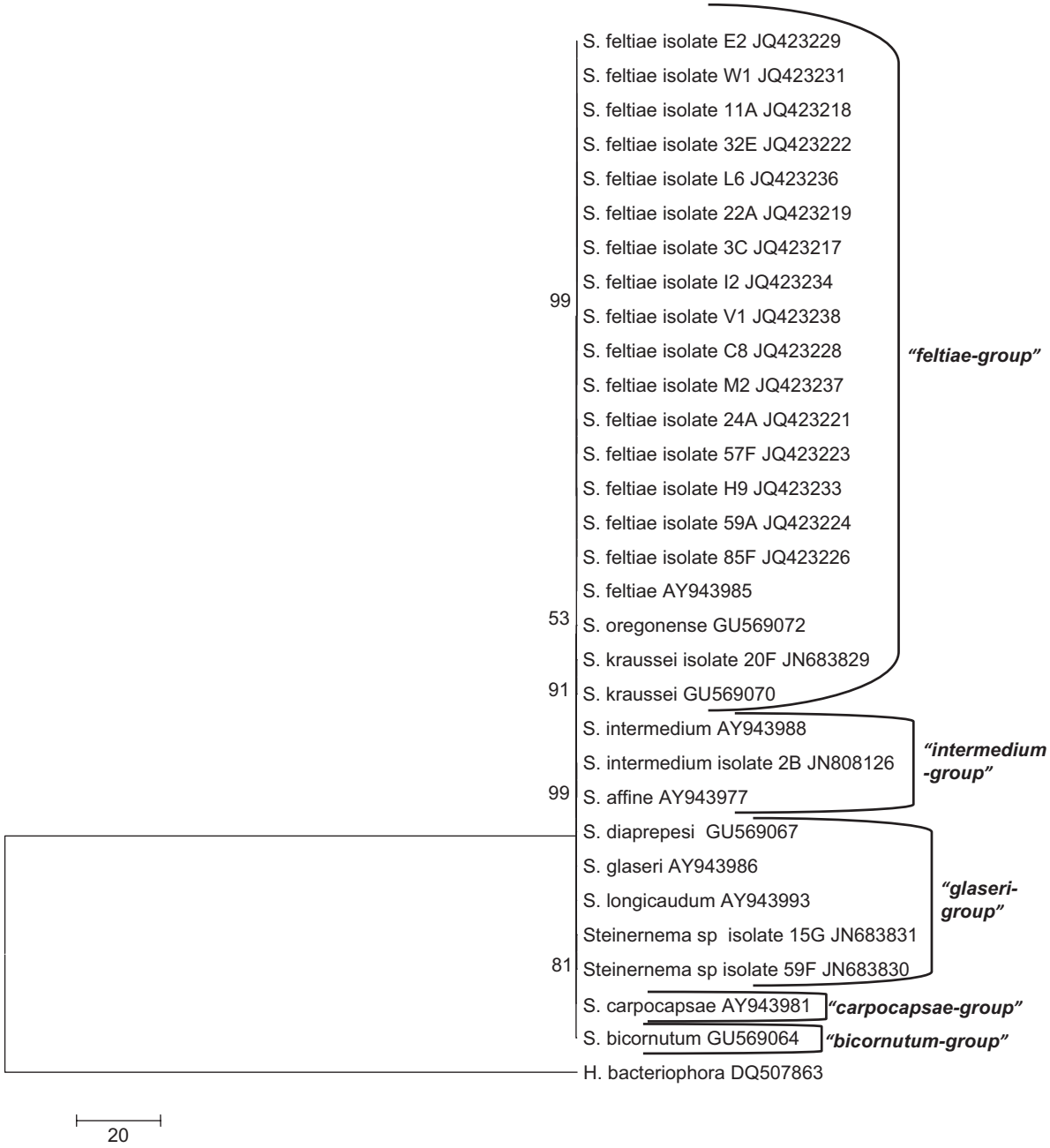

Fig. 5. Maximum likelihood COXI phylogenetic tree using the HKY model (Hasegawa *et al.*, 1985), based on nucleotide sequences of *Steinernema* spp. isolates and reference strains (alignment length 566 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 20 substitutions per site.

Campos-Herrera *et al.*, 2007). Other Mediterranean countries also have similar recovery rates: Greece, 5% (Menti *et al.*, 1997); Egypt, 10% (Shamseldean & Abd-Elgawad, 1994); Mediterranean Turkey, 5.8% (Kepenekci, 2002); and Italy, 5–15.5% (Ehlers *et al.*, 1991; Tarasco & Triggiani, 1997; Triggiani & Tarasco, 2000). On the other hand, EPN seem to occur more frequently in northern European countries: Sweden, 25% (Burman *et al.*, 1986); Czechoslovakia, 37% (Mráček, 1980); Finland, 37% (Vänninen *et al.*, 1989); Germany, 1.2% (Ehlers *et al.*, 1991); Britain, 48.6% (Hominick & Briscoe, 1990); Scotland, 2.2% (Boag *et al.*, 1992); and Ireland, 10.5–14% (Downes & Griffin, 1991; Griffin *et al.*, 1991). The EPN abundance, distribution and habitat preference are related to host–parasite relationships, environmental conditions and soil characteristics (Nielsen & Philipsen, 2003; Püza & Mráček, 2005; Campos-Herrera *et al.*, 2007).

Our results show that natural habitats present a higher percentage of positive samples, compared to agricultural ones, probably due to chemical control of insect pests in

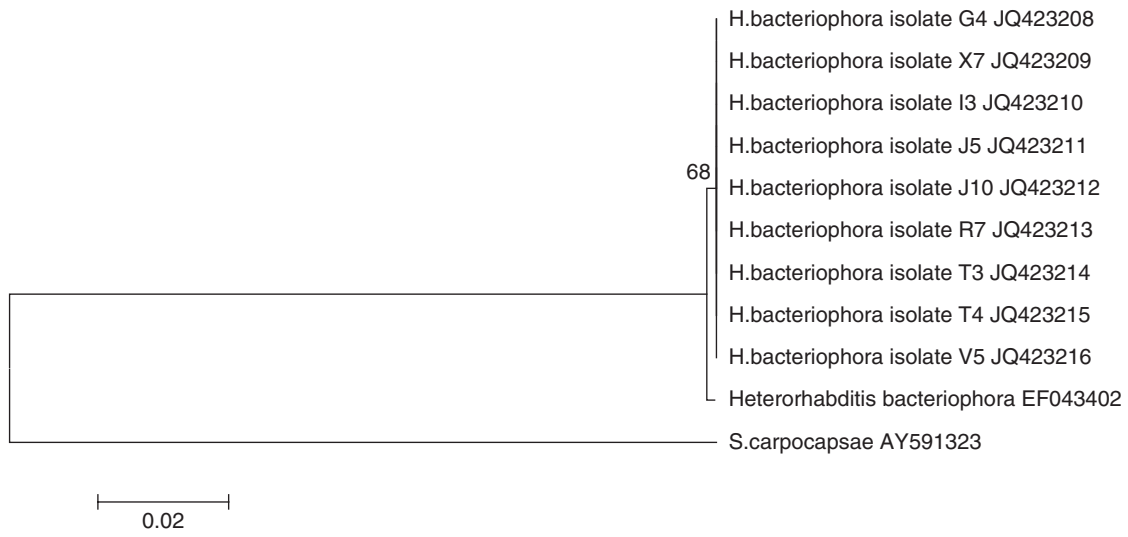

Fig. 6. Maximum likelihood *cytb* phylogenetic tree using Tamura-Nei model (Tamura & Nei, 1993), based on nucleotide sequences of *H. bacteriophora* isolates and reference strains (alignment length 749 bp). Percentage bootstrap is indicated on internal branches (100 replicates); scale bar indicates 0.02 substitutions per site.

agricultural regions, which partially reduces the abundance of natural biocontrol agents. Stock *et al.* (2008) also claimed a higher abundance of EPN in natural habitats such as forests. EPN were recovered from soils with high sand content, which favour their mobility and survival, such as cambisols, podzols, luvisols followed by lithosols and regosols. Observing the soils' physical and chemical characteristics, no clear relationship was found. Abiotic factors, such as altitude, temperature or rainfall, do not influence the distribution of EPN species. No correlation was observed between genetic diversity of *S. feltiae* isolates and any of the biotic or abiotic parameters that were analysed for these isolates.

Steinernema kraussei and *S. intermedium* were each found at only one site, in moors and heathland and mixed forests, respectively. The reason for the low recovery of these two species is not known. *Steinernema kraussei* and *S. intermedium* are common in Europe, with *S. intermedium* showing a preference for tree habitats (Nguyen & Hunt, 2007). *Steinernema* sp. was recovered from only two sites, in mixed forests and broadleaved forest habitats.

The species nature of the genus *Steinernema* is a result of its longer evolution history (Adams *et al.*, 2007) and its reproductive patterns (amphimictic and hermaphroditic), making this genus more capable of occupying a wide range of habitats than *Heterorhabditis* (Edgington *et al.*, 2010). The geographical and habitat preferences of EPN species in continental Portugal may also reflect the chances of dispersal events as well as feeding patterns. However, EPN diversity determined in this study is similar to the diversity reported in previous studies in northern Spain and southern France, which constitute the closest area already sampled and also most similar in terms of climate and soil (García del Pino & Palomo, 1996; Campos-Herrera *et al.*, 2007; Emelianoff *et al.*, 2008). *Steinernema feltiae* and *H. bacteriophora*, the two most abundant EPN species in continental Portugal, are also considered to be the two most common species in Europe, having also been found in southern France and northern Spain (Hominick, 2002). However, conclusions regarding diversity and biogeography of EPN must be reached with some caution, since the results, in part, reflect searching effort and sampling technique rather than actual numbers and/or habitat preferences of EPN.

The major objectives of this study were to determine the indigenous species present in continental Portugal and evaluate the genetic diversity of isolates. For *H. bacteriophora* and *S. feltiae*, the two most abundant species found in continental Portugal, genetic diversity was assessed based on different molecular markers: ITS, D2D3 expansion region of the 28S rRNA gene and two mitochondrial genes, COXI and *cytb*. There were no indications of a molecular and geographical intraspecific variation of either *S. feltiae* or *H. bacteriophora*.

According to our results, *H. bacteriophora* isolates show no differences concerning the *cytb* gene, ITS and D2D3 expansion region of the 28S rRNA gene. Furthermore, no genetic diversity was observed among *S. feltiae* isolates using the D2D3 expansion region of the 28S rRNA gene. However, the ITS region and COXI gene revealed some genetic diversity among *S. feltiae* isolates. The diversity found among *S. feltiae* isolates using ITS and COXI sequences has no correlation with the physical and chemical parameters that characterize soil samples, nor with NUTS, soil or vegetation type.

In conclusion, this survey shows that five EPN species are known to be present in Portuguese soils. Furthermore, the occurrence of *S. feltiae* and *H. bacteriophora* as the two most abundant species in continental Portugal, suggests the potential role of these nematodes in natural regulation of insect populations. Further research on host ranges and characterization of these nematodes in view of possible use in biological control should be undertaken to minimize the use of chemical pesticides.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0022149X13000217

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