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