The introduction and spread of this nematode has resulted in huge annual losses due to the rise in mortality and growth loss of pine forest, and to the increased costs of management procedures and disease control (Mamiya, 2004; Shimazu, 2006). In addition, the introduction of PWN has resulted in vast and irreversible changes to native forest ecosystems, including tree species conversions, wildlife habitat destruction, soil and water conservation and loss of biodiversity (Kiyohara & Bolla, 1990; Suzuki, 2002). The PWN has already been established for more then 100 years in Japan (Yano, 1913) and, in the past two decades, new reports of PWD came mainly from East Asia (Cheng *et al*., 1983; Yi *et al*., 1989).

In 1999, PWN was reported for the first time in Portugal and in Europe (Mota *et al.*, 1999), collected from dead maritime pine (*Pinus pinaster*) trees located in the Setúbal Peninsula, 30 km south east of Lisbon. During approximately 10 years, PWN was confined to the Setúbal Peninsula, occupying 510 000 ha of the continental area. Despite efforts implemented by the Portuguese authorities to control this quarantine nematode, in 2008, new areas of the disease were established in other regions of the country (Rodrigues, 2008), and, more recently, PWN was detected on Madeira, 1000 km south west of continental Portugal (Fonseca *et al.*, 2010). Recently, it has also been detected in Spain (Abelleira *et al*., 2011; Robertson *et al*., 2011). Therefore, it is of major importance to determine the origin of the new isolates and their spread routes to prevent further dissemination of the disease across Europe.

Sequence analysis of specific regions of genomic DNA has proved to be an effective approach for species identification. Ribosomal internal transcribed spacer regions ITS1 and ITS2 are positioned between the small rRNA subunit (SSU), the 5.8S rRNA gene and the large rRNA subunit (LSU). ITS is an excellent marker for quickly distinguishing among known species, due to its low level of intraspecific polymorphisms and sequence variation (Blouin, 2002). On the other hand, the intergenic spacer (IGS) region of the 5S rRNA gene is known to be highly variable, even between closely related species such as *B. xylophilus* and *B. mucronatus* (Kang *et al.*, 2004). It has also been useful to study species at the intraspecific level, such as, for example, the fungus *Fusarium oxysporum* (Kim *et al.*, 2001). Inter-simple sequence repeats (ISSR) are commonly used to study intraspecific variation and evaluate genetic diversity. This method was described for eukaryotes in general (Zietkiewicz *et al.*, 1994), but has been predominantly applied to plants (Reddy *et al.*, 2002). Studies of animal ISSR polymorphisms have been useful in analysing the pathway of introduced species (Abbot, 2001; Metge *et al*., 2006). ISSR provides informative markers because they are highly polymorphic. ISSR markers are highly reproducible due to stringent annealing temperatures, long primers, and low primer-template mismatch (semi-arbitrary primers that anchor on SSR loci) (Jones *et al*., 1997).

In this study, our main goal was to understand the widespread patterns of *B. xylophilus* isolates from Portugal and Madeira, comparing them with foreign isolates by using two distinct approaches: sequence analysis of IGS region and ISSR analysis.

Materials and methods

NEMATODE SAMPLING AND CULTURE

In the present study, 34 *B. xylophilus* isolates from Portugal (continental and the island of Madeira; Fig. 1) were used (Table 1). Madeira isolates were provided by the Nematology Laboratory, IMAR-CMA (Department of Life Sciences, University of Coimbra), and continental Portugal isolates were provided by AFN (National Forest Authority) from the national surveys made each year. Each isolate was obtained from a wood sample collected from one tree. A Portuguese isolate of the closely related species *B. mucronatus* was used as outgroup. Nine foreign isolates from China, Japan, South Korea and USA were used as reference (Table 1). Isolates were maintained in the laboratory in barley seed cultures grown with *Botrytis cinerea* at 25°C (Y. Mamiya, pers. commun.). The nematodes were extracted from cultures using the Baermann funnel technique (Southey, 1986). After this, nematodes were collected and concentrated in 1.5 ml microtubes in a minimum amount of distilled water.

DNA EXTRACTION

Genomic DNA was obtained from a 50 μl suspension containing more than 10 000 nematodes from each isolate

Fig. 1. *Map of the geographical distribution of* Bursaphelenchus xylophilus *isolates in Continental Portugal and Madeira Island, showing the sampling sites of isolates collected until 2008 and in 2009-2010. Isolates code and symbol, representing sampling year, are shown in Table 1.*

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