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The pine wood nematode, *Bursaphelenchus xylophilus*, in Portugal: possible introductions and spread routes of a serious biological invasion revealed by molecular methods

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Summary – The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease (PWD), is a major world-wide pathogen and pest of pine, with impacts on forest health, natural ecosystem stability and international trade. In Portugal, PWN was first diagnosed in 1999, the first occurrence also for Europe. The disease was recently detected on the island of Madeira and in northern Spain. In an attempt to search for more reliable and robust molecular markers that enable the study of intraspecific variability of *B. xylophilus* from different geographic locations, the intergenic spacer (IGS) region of the 5S rRNA gene and inter-simple sequence repeats (ISSR) analysis were used to determine the genetic relationships among 43 *B. xylophilus* isolates from Portugal, China, Japan, South Korea and USA. IGS sequence analysis showed that this region can only be used to establish interspecific relationships, since no differences were detected among Portuguese isolates from different geographic locations. Fingerprints obtained with ISSR show high genetic variability among Portuguese isolates, except for the ones obtained prior to 2008. The ISSR dendrogram suggests the spread of the disease inside continental Portugal and to Madeira. Until 2008, *B. xylophilus* populations found in continental Portugal showed low genetic diversity, pointing to a single introduction, probably from Asia, whereas recent populations from continental Portugal (2009-2010) and Madeira show high genetic diversity, suggesting multiple introductions from different origins.

Keywords – genetic diversity, IGS ISSR, intraspecific variability, ITS, molecular marker, pine wilt disease.

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934; Fuchs, 1937), is indigenous to North America and is widespread in natural coniferous forests in Canada and USA (Sutherland & Peterson, 1999). At present, *B. xylophilus* is considered one of the most important pests and pathogens in the world (Webster & Mota, 2008). The general fear of establishment of PWN, the causal agent of pine wilt disease (PWD), into countries where conifer forests assume great importance, stems from the devastating damage caused by this nematode to pine forests (Mamiya, 2004; Shin & Han, 2006). The introduction of PWN into non-native areas (outside of North America) is primarily associated with trade and the global flow of forest products (Bergdahl & Halik, 1999; Webster, 2004). Non-manufactured wood, especially in raw log form, has been identified as one of the most high-risk pathways of biological invasions, carrying forest insects and pathogens into new environments (Evans *et al*., 1996; Tkacz, 2002). Many *Bursaphelenchus* species, including the PWN, have been routinely intercepted in packaging and wood products in several countries, *e.g*., Austria (Tomiczek *et al*., 2003), China (Gu *et al*., 2006), Finland (Tomminen *et al*., 1991) and Germany (Braasch *et al*., 2001). Furthermore, recent detection of PWN in packaging wood imported from countries considered free of this pest, due to the repeated use and circulation of this type of wood material, *e.g*., Brazil, Belgium, Italy and Spain (Gu *et al*., 2006), undoubtedly underlines the importance of trade globalisation for the potential entry/establishment of this pathogen into endemic forests worldwide.

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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, by 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.*

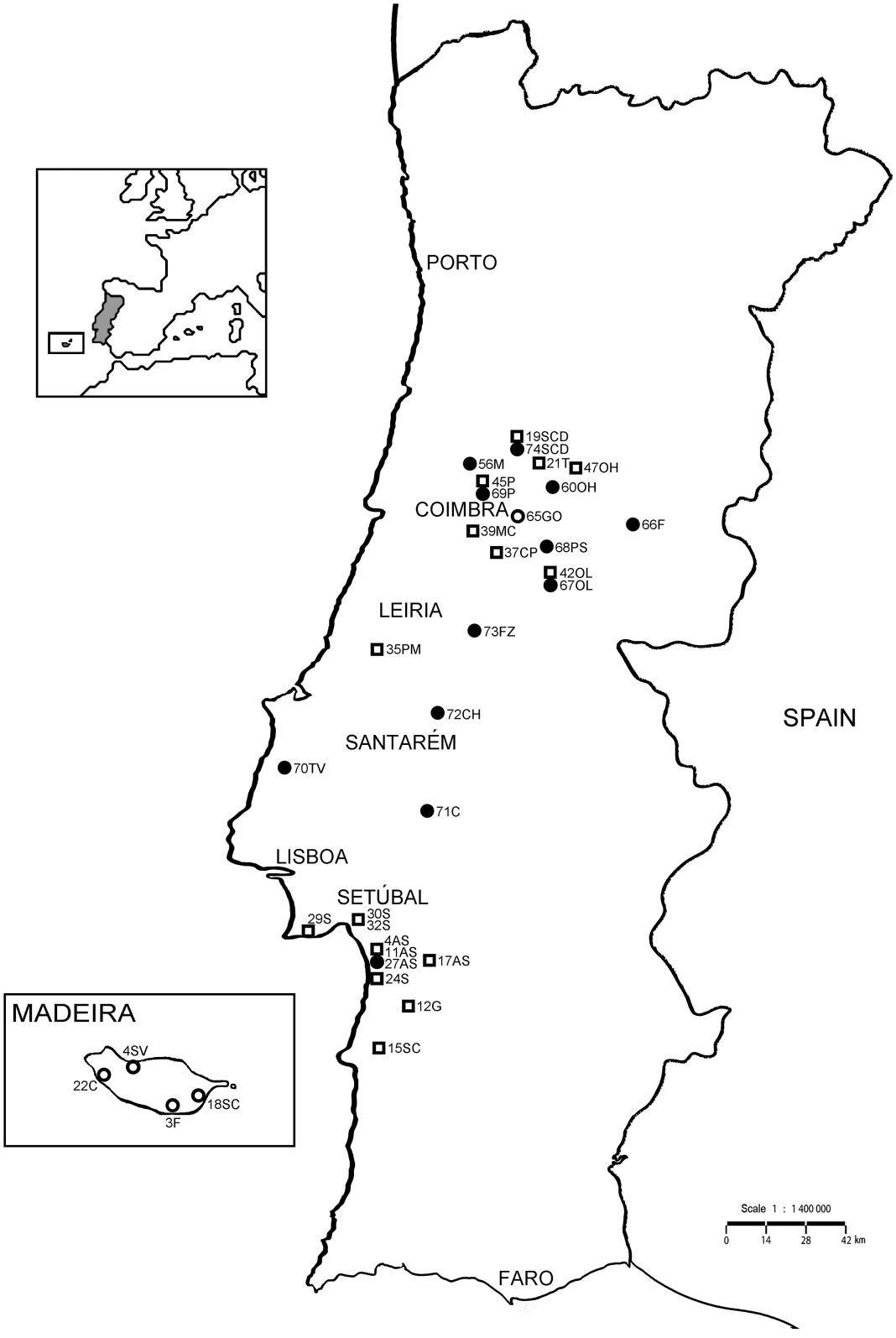
PORTO
19SCD
74SCD
56M
21T
47OH
45P
69P
60OH
COIMBRA
65GO
66F
39MC
37CP
68PS
42OL
67OL
LEIRIA
73FZ
35PM
72CH
SPAIN
SANTARÉM
70TV
71C
LISBOA
SETÚBAL
29S
30S
32S
4AS
11AS
27AS
17AS
24S
12G
15SC
MADEIRA
4SV
22C
18SC
3F
Scale 1 : 1 400 000
0 14 28 42 km
FARO

Table 1. *List of* Bursaphelenchus xylophilus *isolates used in the present study; location, year of culture, map symbol (sampling year), code, accession numbers of ITS and IGS sequences.*

Country	Geographic location	District	Year of culture	Map symbol/Code	Accession numbers	
					ITS sequences	IGS sequences
Portugal	Alcácer do Sal	Setúbal	2005	□BxPt4AS	JN684822	JN684853
	Alcácer do Sal	Setúbal	2005	□BxPt11AS	JN684827	JN684854
	Grândola	Setúbal	2006	□BxPt12G	JN684828	JN684856
	Santiago do Cacém	Setúbal	2007	□BxPt15SC	JN684829	JN684857
	Alcácer do Sal	Setúbal	2007	□BxPt17AS	JN684830	JN684855
	Santa Comba Dão	Coimbra	2008	□BxPt19SCD	JN684831	JN684861
	Tábua	Coimbra	2008	□BxPt21T	JN684832	JN684862
	Grândola	Setúbal	1999	□BxPt24S	JN684823	JN684852
	Alcácer do Sal	Setúbal	2009	●BxPt27AS	JN684826	JN684860
	Setúbal	Setúbal	1999	□BxPt29S	JN684821	JN684851
	Setúbal	Setúbal	2008	□BxPt30S	JN684825	JN684858
	Setúbal	Setúbal	2008	□BxPt32S	JN684824	JN684859
	Porto de Mós	Leiria	2008	□BxPt35PM	JN684333	JN684866
	Castanheira de Pêra	Leiria	2008	□BxPt37CP	JN684834	JN684867
	Miranda Corvo	Leiria	2008	□BxPt39MC	JN684835	JN684868
	Oleiros	Castelo Branco	2008	□BxPt42OL	JN684836	JN684869
	Penacova	Castelo Branco	2008	□BxPt45P	JN684837	JN684870
	Oliveira do Hospital	Castelo Branco	2008	□BxPt47OH	JN684838	JN684871
	Mealhada	Coimbra	2009	●BxPt56M	JN684839	JN684863
	Oliveira do Hospital	Coimbra	2009	●BxPt60OH	JN684840	JN684864
	Goís	Coimbra	2010	○BxPt65GO	JN684841	JN684865
	Fundão	Castelo Branco	2009	●BxPt66F	JN684842	JN684872
	Oleiros	Castelo Branco	2009	●BxPt67OL	JN684843	JN684873
	Pampilhosa da Serra	Coimbra	2009	●BxPt68PS	JN684844	JN684874
	Penacova	Coimbra	2009	●BxPt69P	JN684845	JN684875
	Torres Vedras	Lisboa	2009	●BxPt70TV	JN684846	JN684876
	Coruche	Santarém	2009	●BxPt71C	JN684847	JN684877
	Chamusca	Santarém	2009	●BxPt72CH	JN684848	JN684878
	Ferreira do Zêzere	Santarém	2009	●BxPt73FZ	JN684849	JN684879
	Santa Comba Dão	Viseu	2009	●BxPt74SCD	JN684850	JN684880
Portugal (Madeira Island)	São Gonçalo	Funchal	2010	○BxMad3F	JN684806	JN684882
	São Vicente	São Vicente	2010	○BxMad4SV	JN684808	JN684884
	Santa Cruz	Santa Cruz	2010	○BxMad18SC	JN684809	JN684885
	Calheta	Calheta	2010	○BxMad22C	JN684810	JN684881
China	Jiansu Province	–	–	BxChJs	JN684813	JN684886
	Jiangxi Province	–	–	BxChJx	JN684814	JN684887
	Shandong Province	–	–	BxChSD	JN684815	JN684888
Japan	Unknown	–	–	BxJS10	JN684816	JN684889
	Unknown	–	–	BxJT4	JN684817	JN684890
South Korea	Unknown	–	–	BxKAs	JN684811	JN684891
	Unknown	–	–	BxKBG	JN684812	JN684892
USA	Unknown	–	–	BxUSA618	JN684818	JN684893
	Unknown	–	–	BxUSA745	JN684819	JN684894

culture. Nematodes were centrifuged at 17 982 *g* for 5 min and excess water removed. Genomic DNA was extracted with the JETQUICK Tissue DNA Spin Kit extraction kit (Genomed, Aventura, FL, USA), according to the manufacturer's protocol. DNA was used for the molecular analysis of ITS and IGS regions and ISSR marker. Nematode DNA was kept at −20°C for further use.

Sequencing of the ITS regions

Molecular identification of the 43 isolates was made using internal transcribed spacer (ITS) polymerase chain reaction (PCR) sequencing. Amplification of the ITS region (ITS1 and ITS2) was performed with a set of universal primers used for *Bursaphelenchus* species within the *xylophilus* group (Zhuo *et al*., 2011): ITS1A 5′-CGT AAC AAG GTA GCT GTA G-3′ (Ferris *et al*., 1993) located in the 18S region and ITS1B 5′-TTT CAC TCG CCG TTA CTA AGG-3′, located in the 28S region (Vrain, 1993). The amplicon is approximately 950 bp long. PCR reactions were conducted in 50 μl PCR mixture containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer (all Fermentas, Glen Burnie, MD, USA), 0.1 μM of each primer (STABvida, Lisbon, Portugal), 0.05 units of *Taq* DNA Polymerase Recombinant (Fermentas) and 10 μl template DNA. The PCR program consists of an initial denaturation step for 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min. The last step was performed at 72°C during 2 min. The PCR product was analysed by 1% agarose gel electrophoresis, stained in ethidium bromide. The amplified fragment was sequenced by STABvida, Portugal.

Sequencing of the IGS regions

The IGS region from the 43 isolates was amplified, using a set of primers that amplifies the complete IGS fragment along with adjacent parts of the coding region (5S rRNA gene). The amplification product is approximately 500 bp. The primers were designed based on the conserved coding region of the 5S rRNA gene (Kang *et al*., 2004); forward primer for *B. xylophilus* 5′-TTA GTA CTT GGA TCG GAG ACG-3′, forward primer for *B. mucronatus* 5′-TTA GTA CTT GGA ACG GAG ACG-3′ and reverse primer for both species 5′-CAT CGT TGC TTA ACT TGG CAG A-3′. PCR amplifications were conducted in 50 μl PCR mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (all Fermentas), 0.4 μM of each primer (STABvida), 0.05 units of *Taq* DNA Polymerase Recombinant (Fermentas) and 5 μl template DNA. The PCR reaction program started by one step at 96°C for 2.5 min, followed by 35 cycles of 96°C for 1 min, 50°C for 2 min and 72°C for 3 min; the last step was at 72°C for 6 min. The PCR product was analysed by 1% agarose gel electrophoresis, stained in ethidium bromide. The amplified fragment was sequenced by STABvida, Portugal.

ISSR fingerprints

For the 43 isolates, genomic DNA was amplified using 14 different primers (Table 2), which are 12-20 bp long (Bornet & Branchard, 2001). PCR amplification was conducted in a 25 μl PCR mixture containing 1× PCR buffer, 4 mM $MgCl_2$, 0.2 mM of each dNTP (all Fermentas), 0.8 μM of primer (STABvida), 0.1 units of *Taq* DNA Polymerase recombinant (Fermentas) and 5 μl template DNA. The PCR programme consisted of one cycle at 96°C for 2.5 min, followed by 35 cycles of 94°C for 20 s, 42-55°C for 45 s and 72°C for 2 min; the last step of final extension was at 72°C for 6 min. Following PCR, the fingerprints for each primer were obtained in a 1% agarose gel stained with ethidium bromide.

Phylogenetic analysis of ITS and IGS sequence

ITS and IGS sequences were assembled and aligned using the ClustalW algorithm as implemented in BioEdit version 7.1.3.0 (Hall, 1999), under default alignment parameters. The sequences of ITS and IGS were submitted to GenBank under the accession numbers present in Table 1. Using MEGA5 version 5.05 (Tamura *et al*., 2011), phylogenetic relationships between isolates were reconstructed by the Neighbour-Joining (NJ) (Saitou & Nei, 1987) and the Maximum Likelihood methods. Bootstrap analysis was performed with 1000 replicates (Felsenstein, 1985). *Bursaphelenchus mucronatus* (BmPt0) was used as outgroup.

Analysis of ISSR fingerprints

ISSR fingerprint patterns were converted into a binary data matrix by scoring the presence of a band as 1 and its absence as 0. Only the well distinguishable bands were used as markers for matrix construction. Each fingerprint was repeated at least twice to guarantee the reproducibility of the technique. The binary matrix was

Table 2. *List of primers used for* Bursaphelenchus *sp. isolates in ISSR analysis: primer sequences and annealing temperatures, total number of amplified bands, and percentage of polymorphic and phylogenetically-informative bands. Wobbles base pair code B: C, G or T; D: A, G or T; H: A, C or T; R: A or G; V: A, C or G; Y: C or T.*

Primer	Primer sequence ($5' \rightarrow 3'$)	Annealing temperature (°C)	Total number of amplified bands	Polymorphic bands (%)	Phylogenetically informative bands (%)
11	$(GA)_9$-CCA	50	15	100	73
25	$(AC)_9$-TG	55	–	–	–
26	$(AC)_9$-GA	55	16	94	94
54	$(TC)_9$-CG	50	12	100	83
188	CGT-$(CA)_8$	55	14	100	40
190	CAG-$(GT)_9$	55	7	100	71
841	$(GA)_8$-YC	50	11	64	36
848	$(CA)_8$-RG	42	13	100	92
857	$(AC)_8$-YG	50	10	100	100
888	BDB-$(CA)_7$	55	7	100	100
890	VHV-$(GT)_7$	55	10	100	100
1423	HVH-$(TGT)_5$	50	21	100	57
1424	BDB-$(CAC)_5$	50	6	100	100
1425	BDV-$(CAG)_5$	50	12	100	75

analysed with NTSYS-PC version 2.21 (Rohlf, 2008), using the Dice or Nei and Li (1979) coefficient to generate a genetic distance similarity matrix. Cluster analysis of this matrix was made with unweighted pair group method using arithmetic averages (UPGMA) in the module SAHN (sequential, agglomerative, hierarchical and nested) clustering method. The resulting dendrogram was compared with the original similarity matrix and the cophenetic correlation coefficient (r) and Mantel's test (t) value (Mantel, 1967) were calculated to evaluate the goodness-of-fit of data.

Results

Sequencing of the ITS regions

For the 43 isolates, a PCR product of approximately 950 bp containing the entire 5.8S rRNA gene and both ITS1 and ITS 2 regions was obtained and sequenced (accession numbers in Table 1). One isolate of *B. mucronatus* (BmPt0) was used as outgroup (accession number of *B. mucronatus* ITS sequence is JN684820). The phylogenetic analysis of this region showed 100% sequence identity among geographical isolates from Portugal as well as with isolates from Japan, China, South Korea and isolate BxUSA618 (data not shown), with the exception of isolate BxMad3F, which shares 99.8% sequence similarity with all other sequences, and 98.8% of similarity with isolate BxUSA745. Isolate BxUSA745 has a separate position from all other isolates, sharing a similarity of 99% sequence with all the isolates, except BxMad3F (98.8% sequence similarity). *Bursaphelenchus mucronatus* isolate (BmPt0), shares a similarity of 88.4% with all isolates, with the exceptions of BxMad3F (sequence similarity 88.3%) and BxUSA745 (87.9% sequence similarity).

Sequencing of the IGS regions

Forty-three isolates of *B. xylophilus* were analysed using IGS sequences. *Bursaphelenchus mucronatus* (BmPt0) was used as outgroup. IGS PCR products were about 500 bp long and were sequenced and submitted to GenBank under the accession numbers presented in Table 1 (accession number of *B. mucronatus* IGS sequence is JN684895). No polymorphisms were found among Portuguese isolates, showing that this marker is not informative enough to be used in the study of genetic variability between Portuguese isolates from different geographic locations, *i.e.*, IGS sequences from Portuguese isolates do not show any intraspecific variability. Similar phylogenetic trees were obtained with both Neighbour-Joining and Maximum Likelihood methods (data not shown). One major cluster was obtained which contained all Portuguese along with the two Korean isolates (Fig. 2). These isolates are all 100% identical. Isolates from China and Japan form a separate cluster with 100% sequence identity, which groups with the first one. The two USA iso-

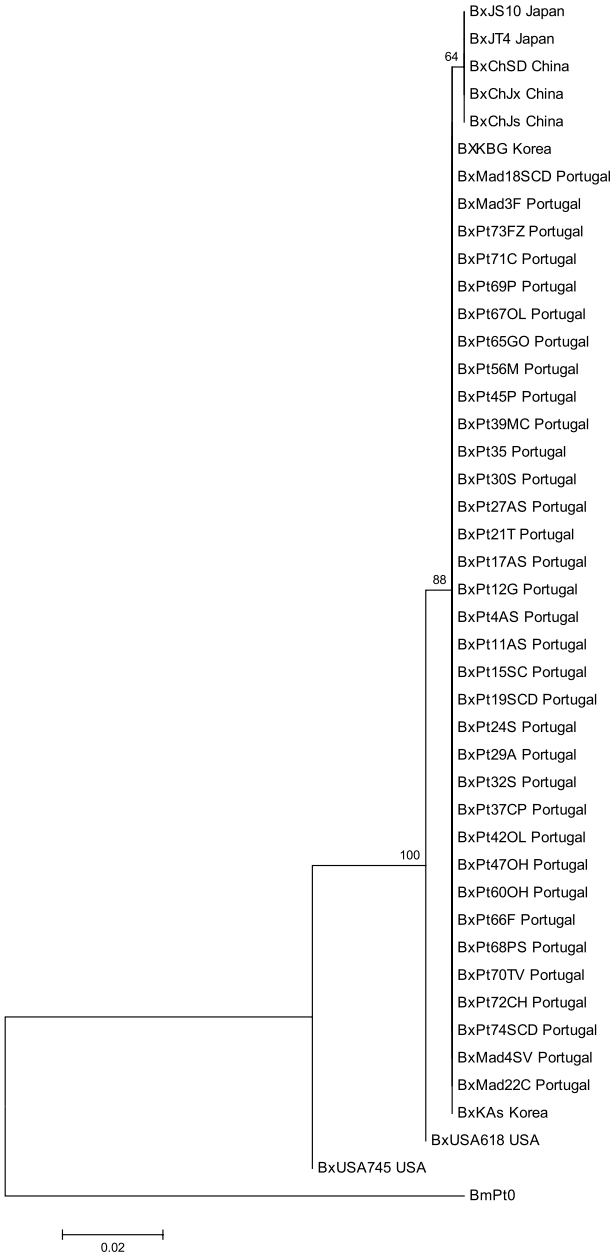

Fig. 2. *Neighbour-joining phylogenetic tree based on rRNA IGS sequences of* Bursaphelenchus xylophilus *isolates (alignment length 450 bp). Percentage bootstrap is indicated on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Tamura 3-parameter nucleotide substitution model (Tamura & Kumar, 2002) was used.*

lates have separate positions being more distantly related to all other isolates. Isolate BxUSA618 shares approximately 99%, whereas BxUSA745 only shares 97-98% sequence similarity with all other isolates. Furthermore, the sequence identity between isolates BxUSA618 and BxUSA745 is 98%, which may suggest that North American populations have a high genetic diversity.

ISSR FINGERPRINTS

Forty-three isolates of *B. xylophilus* were analysed using ISSR technique. *Bursaphelenchus mucronatus* isolate (BmPt0) was used as outgroup. All fingerprints were repeated at least three times. From the 14 primers used, 13 produced clear and reproducible banding patterns. Amplification of genomic DNA from the 44 *Bursaphelenchus* isolates with the 13 primers yielded a total of 154 ISSR markers, of which 149 are polymorphic and 117 are phylogenetically informative (Table 2). The number of bands per isolate varies between 1 and 10. Each primer generated a set of bands ranging from 500 to 2000 bp (Fig. 3). A binary matrix was assembled for the 13 primers set and cluster analysis compared Portuguese with foreign isolates. The relationships between the *B. xylophilus* isolates used in this study have a good bootstrap support (all values above 52%; Fig. 4). This dendrogram is supported by a significantly high cophenetic correlation coefficient of 0.97 and a Mantel's test (t) of 7.26. The obtained dendrogram clearly illustrates the outgroup position of *B. mucronatus*. The dendrogram shows a large cluster with 56% bootstrap support, which includes all foreign and Portuguese isolates, with the exception of isolate BxPt73FZ, and the Madeira isolates BxMad4SV and BxMad3F.

The three isolates from China group together with similarities levels between 81 and 91%. Japanese and Korean isolates are distributed across different branches of the dendrogram. Korean isolates are more similar to Japanese isolates than to Chinese isolates, considering that isolates BxJT4 and BxKAs group together with a similarity level of 91%. The two North American isolates do not form a cluster and show 63% similarity between them.

From the 34 Portuguese isolates, 17 (BxPt4AS, BxPt11AS, BxPt12G, BxPt15SC, BxPt17AS, BxPt19SCD, BxPt21T, BxPt24S, BxPt29S, BxPt30S, BxPt32S, BxPt35PM, BxPt37CP, BxPt39MC, BxPt42OL, BxPt45P and BxPt47OH) have identical fingerprints with all 13 primers. These 17 isolates that group together were collected prior to 2008, being distributed in the Setúbal, Leiria and Castelo Branco districts and representing the initial spread of the disease in continental Portugal, from 1999-2008. Seventeen isolates were collected from 2009-2010 and represent the new outbreaks of the disease

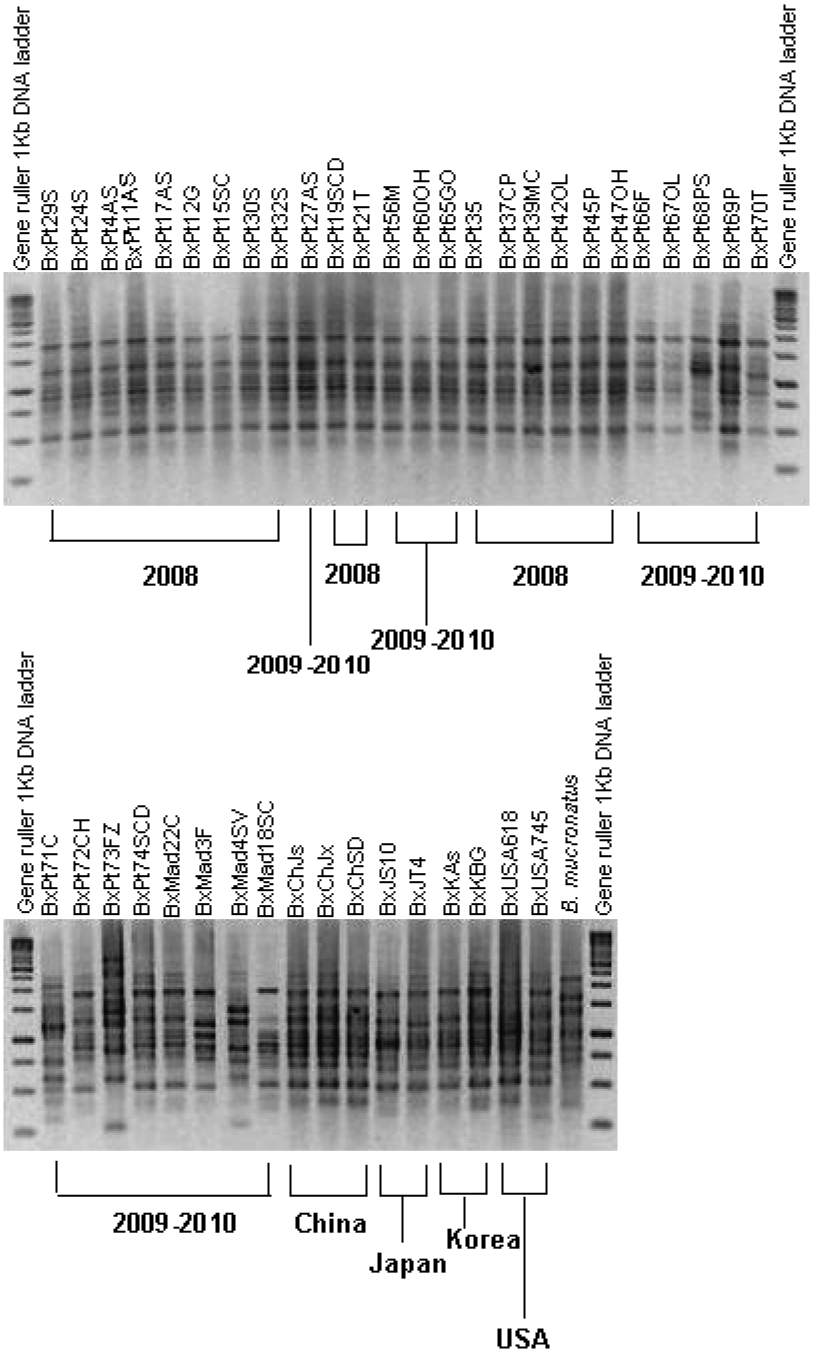

Fig. 3. *ISSR profiles from 43* Bursaphelenchus xylophilus *isolates with primer 1423.*

in continental Portugal and Madeira: BxPt27As (Setúbal district), BxPt56M/BxPt60OH/BxPt65GO/BxPt68PS/BxPt69P (Coimbra district), BxPt66F/BxPt67OL (Castelo Branco district), BxPt70TV (Lisboa district), BxPt71C/BxPt72CH/BxPt73FZ (Santarém district), BxPt74SCD (Viseu district), BxMad3F (Funchal district), BxMad4SV/BxMad9SV (São Vicente district), BxMad18SC (Santa Cruz district) and BxMad22C (Calheta district). Twelve

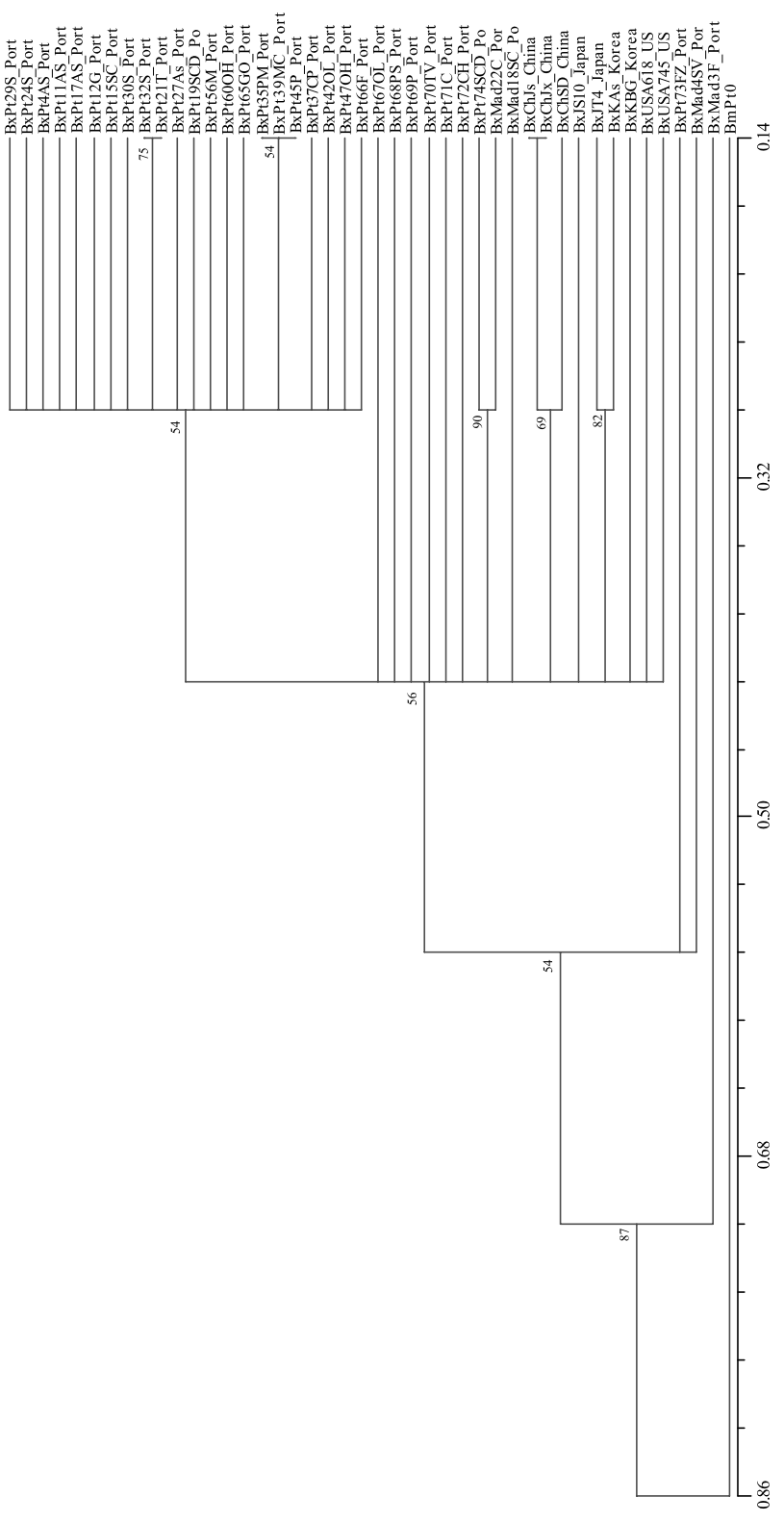

Fig. 4. *UPGMA dendrogram based on the ISSR profiles of* Bursaphelenchus xylophilus *obtained with 13 primers* ($r = 0.97$; $t = 7.26$). *Percentage bootstrap is indicated on internal branches (1000 replicates).*

of these isolates show different fingerprints with all primers from those detected up to 2008, which have different levels of variability, revealing the existence of intraspecific variability among Portuguese isolates. Isolates BxPt27AS, BxPt56M, BxPt60OH, BxPt65GO and BxPt66F show different banding patterns only for some of the primers and thus still group with isolates up to 2008 in the dendrogram (Fig. 4). Examining the distribution of these isolates in the dendrogram, Portuguese isolates collected from 1999-2008 all group in the same clade with similarity levels between 84 and 99%. However, Portuguese isolates collected in 2009-2010 are distributed across different branches of the dendrogram.

Madeira isolates BxMad18SC (Santa Cruz district) and BxMad22C (Calheta district), share many bands with Portuguese isolates collected up to 2008 and Asian isolates (Fig. 3). Isolate BxMad22C groups together with isolate BxPt74SCD, with a similarity level of 82%. Isolates BxMad3F and BxMad4SV share bands with both Asian and USA isolates, exhibiting high levels of polymorphism (Fig. 3). Furthermore, isolate BxMad3F has a clearly separate position on the dendrogram since it shows approximately the same low similarities (35-54%) with all isolates.

Discussion

The pine wood nematode is native to North America (Kanzaki & Futai, 2002) and widely distributed throughout Canada, USA and Mexico (CABI/EPPO, 1999). In these areas, the nematode co-evolved with the different North American pine species during millions of years, therefore causing no disease. Most probably, North America is the region where *B. xylophilus* populations have the highest genetic diversity (Vieira *et al.*, 2007).

In Portugal, PWN was successfully restricted to the Setúbal Peninsula for several years immediately following the first report (Mota *et al.*, 1999). This was mainly due to strong and specific measures for control of inland circulation of wood and wood products from the original affected area. However, in 2008, the nematode was found in new outbreak spots in central and northern regions of the country (Rodrigues, 2008) and, more recently, in one of the islands of the Madeira archipelago (Fonseca *et al*., 2010).

The first study using a significant number of *B. xylophilus* from Portugal was performed by Vieira *et al.* (2007), using 24 isolates from the initial affected area, the Setúbal Peninsula, the only region where the disease was detected until 2008. In this study, the authors showed that there was almost no genetic diversity among isolates and that the Portuguese isolates grouped with Chinese isolates.

To understand the origin and genetic diversity of the PWN found in the new areas in continental Portugal and Madeira, 34 isolates representing the different geographic areas were studied using IGS sequences and ISSR fingerprints. This is the first study where a significant number of *B. xylophilus* isolates from the initial affected area in the Setúbal Peninsula, as well as from the new outbreaks of the disease in continental Portugal and Madeira, have been analysed. ITS sequences confirmed all 34 Portuguese isolates as *B. xylophilus*. No intraspecific variability among isolates was found. Only isolate BxUSA745 from USA groups in a separate position from all the other isolates. Using IGS sequencing, no significant intraspecific variability was detected among isolates of *B. xylophilus*. Both USA isolates are distantly related to all other isolates, isolate BxUSA745 having a clear separate position on the phylogenetic tree. Chinese and Japanese isolates group together. All Portuguese isolates group with Korean isolates. Moreover, no clustering of the isolates according to their geographic origin was obtained.

In the dendrogram obtained from the ISSR fingerprint analysis, all Portuguese isolates collected prior to 2008 group together with similarities of 84-99%, indicating a lack of genetic diversity and suggesting a single introduction, which is in agreement with the results of Vieira *et al*. (2007). Observing the distribution of isolates collected in 2009-2010 in continental Portugal, there are five isolates (BxPt27As, BxPt56M, BxPt60OH, BxPt65GO and BxPt66F) grouping with the isolates collected up to 2008 with similarities above 86%. This suggests a spread of the disease from the initial affected area to new outbreaks, which may have occurred with great probability considering the heavy traffic of vehicles carrying wood products from the Lisbon-Setúbal area to the centre and north of Portugal, where a number of industries process pine wood for furniture. The insect vector *Monochamus galloprovincialis* is known to occur well inside the centre of the country, quite far (*ca* 200-300 km) north of the Setúbal Peninsula, for example, in the region of Penacova, near Coimbra. Furthermore, it is known to occur throughout the continental territory (Sousa *et al*., 2001, 2002; Penas *et al*., 2006). On the other hand, continental isolate BxPt73FZ has a separate position from all other isolates, suggesting a new introduction in continental Portugal, possibly from the native North American population.

The results obtained from the Madeira isolates suggest a high genetic diversity of *B. xylophilus* population on the island. Isolates BxMad4SV and BxMad3F are distinct from all other isolates and show low similarity values, which suggests new introductions in the island. The same insect vector, *M. galloprovincialis*, is known to occur on the island for more than 15 years, at least since 1996, but possibly much longer (Erber & Aguiar, 1996). This could explain the easy spread of the nematode, unnoticed for several years until its recent detection (Fonseca *et al*., 2010). The other isolate from Madeira, BxMad22C, groups with isolate BxPt74SCD with a similarity of 82%, suggesting that at least part of the Madeira population is related to the mainland population.

Previous studies on *B. xylophilus* populations have shown intraspecific variability between isolates from different geographic origins using ISSR (Metge & Burgermeister, 2006), revealing a significant degree of genetic divergence according to geographic distribution. Two major clusters were identified: one including the North American isolates (Canada and USA) displaying a high level of genetic diversity, and a second cluster including Asian and Portuguese isolates at a lower genetic diversity level. Another study, using AFLP, suggested that China was affected directly from North America or from neighbouring countries such as Japan (Cheng *et al*., 2008). Other studies with Korean isolates using microsatellites and AFLP (Jung *et al*., 2010a, b), suggest that the high genetic diversity found among some Korean populations is due to the gene flow between China, Japan and Korea. The present results support previous reports (Metge & Burgermeister, 2006; Vieira *et al*., 2007), namely IGS- and ISSR-based grouping of most Portuguese isolates with Asian isolates.

Until now, little information about *B. xylophilus* markers has been available, but the recent release of the entire genome sequence of *B. xylophilus* (Kikuchi *et al*., 2011) will be a challenge and opportunity to discover novel suitable markers to study intraspecific variability, determine pathways of disease spread and follow the evolution of introduced isolates. The increase in global trade has an important impact on natural ecosystems, making the invasion of *B. xylophilus* a serious ecological and economic problem. *Bursaphelenchus xylophilus*, in particular, damages conifers (*Pinus* spp.) making PWD one of the most serious problems of forests worldwide. The understanding of the disease and its spread will be of paramount importance to draw up new measures to avoid the dissemination of PWN to other countries. Future research on *B. xylophilus* must continue to follow the evolution and spread of this biological invader in pine forests.

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References

ABBOT, P. (2001). Individual and population variation in invertebrates revealed by inter-simple sequence repeats (ISSRs). *Journal of Insect Science* 1, 8. Available online at http://www.insectscience.org/1.8.

ABELLEIRA, A., PICOAGA, A., MANSILLA, J.P. & AGUIN, O. (2011). Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in North-western Spain. *EPPO Bulletin* 39, 344-353.

BERGDAHL, D.R. & HALIK, S. (1999). Inoculated *Pinus sylvestris* serve as long-term hosts for *Bursaphelenchus xylophilus*. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forest in relation to pine wilt and decline: proceedings of international symposium*. Tokyo, Japan, Shokada, pp. 73-78.

BLOUIN, M. (2002). Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology* 32, 527-531.

BORNET, B. & BRANCHARD, M. (2001). Nonanchored Inter Simple Sequence Repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter* 19, 209-215.

BRAASCH, H., TOMICZEK, C., METGE, K., HOYER, U., BURGERMEISTER, W., WULFERT, I. & SCHONFELD, U. (2001). Records of *Bursaphelenchus* spp. (Nematoda, Parasi-

taphelenchidae) in coniferous timber imported from the Asian part of Russia. *Forest Pathology* 31, 129-140.

CABI/EPPO (1999). *Distribution maps of plant diseases*. Wallingford, UK, CABI Publishing. Available online at http://www.CABI.org/DMPD

CHENG, H., LIN, M., LI, W. & FANG, Z. (1983). The occurrence of a pine wilting disease caused by a nematode found in Nanjing. *Forest Pest and Disease* 4, 1-5.

CHENG, X.Y., CHENG, F.X., XU, R.M. & XIE, B.Y. (2008). Genetic variation in the invasive process of *Bursaphelenchus xylophilus* (Aphelenchida: Aphelenchoididae) and its possible spread routes in China. *Heredity* 100, 356-365.

ERBER, D. & AGUIAR, A.M.F. (1996). New and remarkable species of the Coleopterous fauna of Madeira. *Boletim do Museu Municipal do Funchal* 48, 41-62.

EVANS, H.F., MCNAMARA, D.G., BRAASCH, H., CHADOUEF, J. & MAGNUSSON, C. (1996). Pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*. *EPPO Bulletin* 26, 199-249.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783-791.

FERRIS, V.R., FERRIS, J.M. & FAGHIHI, J. (1993). Variation in spacer ribosomal DNA in some cyst forming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16, 177-184.

FONSECA, L., LOPES, A., CARDOSO, J., PESTANA, M., ABREU, F., NUNES, N., MOTA, M. & ABRANTES, I. (2010). The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *30th International Symposium of European Society of Nematologists, Vienna, Austria*, p. 176. [Abstr.]

FUCHS, A.G. (1937). Neue parasitische und halbparasitische Nematoden bei Borkenkafern und einige andere Nematoden. I. Teil. *Zoologische Jahrbuecher Abteilung fuer Systematik Oekologie und Geographie der Tiere* 70, 291-380.

GU, J., BRAASCH, H., BURGERMEISTER, W., BRANDSTETTER, M. & ZHANG, J. (2006). Description of *Bursaphelenchus yongensis* sp. n. (Nematoda: Parasitaphelenchidae) isolated from *Pinus massoniana* in China. *Russian Journal of Nematology* 14, 91-99.

HALL, T.A. (1999). BioEdit: a user-friendly biological sequences alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium* 41, 95-98.

JONES, C.J., EDWARDS, K.J., CASTAGLIONE, S., WINFIELD, M.O., SALA, F., WIEL, C., BREDEMEIJER, G., VOSMAN, B., MATTHES, M., DALY, A., *ET AL.* (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding* 3, 381-390.

JUNG, J., HAN, H., RYU, S.H. & KIM, W. (2010a). Microsatellite variation in the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Bührer) Nickle in South Korea. *Genes & Genomics* 32, 147-154.

JUNG, J., HAN, H., RYU, S.H. & KIM, W. (2010b). AFLP (amplified fragment length polymorphism) analysis and genetic variation of pinewood nematode, *Bursaphelenchus xylophilus* in South Korea. *Animal Cells and Systems* 14, 31-36.

KANG, J.S., CHOI, K.S., SKIN, S.C., MOON, I.S., LEE, S.G. & LEE, S.H. (2004). Development of an efficient PCR-based diagnosis protocol for the identification of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *Nematology* 6, 279-285.

KANZAKI, N. & FUTAI, K. (2002). A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology* 4, 35-41.

KIKUCHI, T., COTTON, J.A., DALZELL, J.J., HASEGAWA, K., KANZAKI, N., MCVEIGH, P., TAKANASHI, T., TSAI, I.J., ASSEFA, S.A., COCK, P.G.A., *ET AL.* (2011). Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. *PLoS Pathogens* 7(9), e1002219. DOI:10.1371/journal.ppat.1002219

KIM, H.J., CHOI, Y.K. & MIN, B.R. (2001). Variation of the intergenic spacer (IGS) region of ribosomal DNA among *Fusarium oxysporum* formae speciales. *Journal of Microbiology* 9, 265-272.

KIYOHARA, T. & BOLLA, R.I. (1990). Pathogenic variability among populations of the pinewood nematode, *Bursaphelenchus xylophilus*. *Forest Science* 36, 1061-1076.

MAMIYA, Y. (2004). Pine wilt disease in Japan. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode,* Bursaphelenchus xylophilus*. Proceedings of an International Workshop, University of Evora, Portugal, 20-22 August, 2001*. *Nematology Monographs and Perspectives 1*. Leiden, The Netherlands, Brill, pp. 9-20.

MANTEL, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research* 27, 209-220.

METGE, K. & BURGERMEISTER, W. (2006). Intraspecific variation in provenances of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) revealed by ISSR and RAPD fingerprints. *Journal of Plant Diseases and Protection* 113, 275-282.

METGE, K., BRAASCH, H., GU, J. & BURGERMEISTER, W. (2006). Interspecific variation in ITS rDNA of *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) of different groups. *Russian Journal of Nematology* 14, 147-158.

MOTA, M., BRAASCH, H., BRAVO, M.A., PENAS, A.C., BURGERMEISTER, W., METGE, K. & SOUSA, E. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1, 727-734.

NEI, M. & LI, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America* 76, 5269-5273.

PENAS, A.C., BRAVO, M.A., NAVES, P., BONIFÁCIO, L., SOUSA, E. & MOTA, M. (2006). Species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Parasitaphelenchidae) and other

nematode genera associated with insects from *Pinus pinaster* in Portugal. *Annals of Applied Biology* 148, 121-131.

REDDY, M.P., SARLA, N. & SIDDIQ, E.A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica* 128, 9-17.

ROBERTSON, L., ARCOS, C.S., ESCUER, M., MERINO, S.R., ESPARRAGO, G., ABELLEIRA, A. & NAVAS, A. (2011). Incidence of the pinewood nematode *Bursaphelenchus xylophlius* Steiner & Buhrer, 1934 (Nickle, 1970) in Spain. *Nematology* 13, 755-757.

RODRIGUES, J. (2008). Eradication program for the pinewood nematode in Portugal. In: Mota, M. & Vieira, P. (Eds). *Pine wilt disease: a worldwide threat to forest ecosystems*. Heidelberg, Germany, Springer, pp. 5-14.

ROHLF, F.J. (2008). NTSYSpc*: numerical taxonomy system, ver. 2.20*. Setauket, NY, USA, Exeter Publishing.

SAITOU, N. & NEI, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

SHIMAZU, M. (2006). Current status on research and management of pine wilt disease in Japan. In: Han, H.R., Koh, S.H., Jung, C.S., Chung, Y.J. & Shin, S.C. (Eds). *Current status on research and management of pine wilt disease*. Seoul, Korea, Korea Forest Research Institute, pp. 1-18.

SHIN, S. & HAN, H. (2006). Current status on research and management of pine wilt disease in Korea. In: Han, H.R., Koh, S.H., Jung, C.S., Chung, Y.J. & Shin, S.C. (Eds). *Current status on research and management of pine wilt disease*. Seoul, Korea, Korea Forest Research Institute, pp. 31-44.

SOUSA, E., BRAVO, M.A., PIRES, J., NAVES, P., PENAS, A.C., BONIFÁCIO, L. & MOTA, M. (2001). *Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal. *Nematology* 3, 89-91.

SOUTHEY, J.F. (1986). *Laboratory methods for work with plant and soil nematodes*. London, UK, Ministry of Agriculture Fisheries and Food, HMSO, 202 pp.

STEINER, G. & BUHRER, E.M. (1934). *Aphelenchoides xylophilus* n. sp., a nematode associated with blue-stain and other fungi in timber. *Journal of Agricultural Research* 48, 949-951.

SUTHERLAND, J.R. & PETERSON, M. (1999). The pinewood nematode in Canada: history, distribution, hosts, potential vectors and research. In: Futai, K., Togashi, K. & Ikeda, T. (Eds). *Sustainability of pine forests in relation to pine wilt and decline: proceedings of an international symposium*. Tokyo, Japan, Shokado Shoten, pp. 247-253.

SUZUKI, K. (2002). Pine wilt disease – a threat to pine forest in Europe. *Dendrobiology* 48, 71-74.

TAMURA, K. & KUMAR, S. (2002). Evolutionary distance estimation under heterogeneous substitution pattern among lineages. *Molecular Biology and Evolution* 19, 1727-1736.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731-2739.

TKACZ, B.M. (2002). Pest risks associated with importing wood to the United States. *Canadian Journal of Plant Pathology* 24, 111-116.

TOMICZEK, C., BRAASCH, H., BURGERMEISTER, W., METGE, K., HOYER, U. & BRANDSTETTER, M. (2003). Identification of *Bursaphelenchus* spp. isolated from Chinese packaging wood imported to Austria. *Nematology* 5, 573-581.

TOMMINEN, J., HALIK, S. & BERGLAHL, D.R. (1991). Incubation temperature and time effects on life stages of *Bursaphelenchus xylophilus* in wood chips. *Journal of Nematology* 24, 477-484.

VIEIRA, P., BURGERMEISTER, W., MOTA, M., METGE, K. & SILVA, G. (2007). Lack of genetic variation of *Bursaphelenchus xylophilus* in Portugal revealed by RAPD-PCR analyses. *Journal of Nematology* 39, 118-126.

VRAIN, T.C. (1993). Restriction fragment length polymorphism separates species of the *Xiphinema americanum* group. *Journal of Nematology* 25, 361-364.

WEBSTER, J. (2004). The pine wood nematode: implications of factors past and present for pine wilt disease. In: Mota, M. & Vieira, P. (Eds). *The pinewood nematode,* Bursaphelenchus xylophilus*. Proceedings of an International Workshop, University of Evora, Portugal, 20-22 August, 2001*. *Nematology Monographs and Perspectives 1*. Leiden, The Netherlands, Brill, pp. 55-64.

WEBSTER, J. & MOTA, M. (2008). Pine wilt disease: global issues, trade and economic impact. In: Mota, M. & Vieira, P. (Eds). *Pine wilt disease: a worldwide threat to forest ecosystems*. Heidelberg, Germany, Springer, pp. 1-3.

YANO, M. (1913). Investigations on the cause of pine mortality in Nagasaki prefecture. *Sanrin-Koho* 4, 1-14.

YI, C., BYUN, B., PARK, J., YANG, S. & CHANG, K. (1989). First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle and its insect vector in Korea. *Research Reports of the Forestry Research Institute Seoul* 38, 141-149.

ZHUO, K., LUO, M., CUI, R.Q. & LIAO, J.L. (2011). A multiplex one-step PCR method for the simultaneous identification of *Bursaphelenchus xylophilus*, *B. mucronatus* and *B. doui* – three species within the *xylophilus* group. *Forest Pathology* 41, 66-69.

ZIETKIEWICZ, E., RAFALSKI, A. & LABUDA, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20, 176-183.