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Lack of Genetic Variation of *Bursaphelenchus xylophilus* in Portugal Revealed by RAPD-PCR Analyses

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Abstract: Random Amplified Polymorphic DNA (RAPD-PCR) technique was used to assess the level of genetic variability and genetic relationships among 24 Portuguese isolates of pinewood nematode, *Bursaphelenchus xylophilus*. The isolates represent the main infested areas of Portugal. Two additional isolates of *B. xylophilus* representing North America and East Asia were included, and *B. mucronatus* was used as out-group. Twenty-eight random primers generated a total of 640 DNA fragments. The Nei and Li similarity index revealed a high genetic similarity among the Portuguese isolates (above 90%). Hierarchical cluster analysis was performed to illustrate the relatedness among the isolates. No indication for separate groups among the Portuguese isolates was obtained, and the low level of genetic diversity strongly suggests that they were dispersed recently from a single introduction. The lack of apparent relationship between the genetic and the geographic matrices of the Portuguese isolates limits the use of this technique for following recent pathways of distribution. Genetic distance of the Portuguese isolates towards an isolate from China was much lower as compared to an isolate from the USA. This confirmed previous results suggesting an East Asian origin of the Portuguese *B. xylophilus*.

Key words: pinewood nematode, DNA fingerprinting, RAPD, similarity.

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) Nickle, 1970, the pinewood nematode (PWN), is the causal agent of pine wilt disease (PWD) and one of the most important pests and pathogens of conifer forests worldwide (Evans et al., 1996). Although considered a native species to North America (Rutherford et al., 1990), in the past century it has been introduced and spread into non-native areas, first Japan (Yano, 1913; Kiyohara and Tokushige, 1971), then China (Cheng et al., 1983), Taiwan (Tzean and Jan, 1985) and Korea (Yi et al., 1989), and more recently into the European continent in Portugal (Mota et al., 1999). The impact of this invasion into non-native areas is to damage endemic natural resources, mainly in pine forests, not only by the huge economic loss of wood, but also due to the social importance of pine forests in some countries such as Japan (Mamiya, 2004; Yang, 2004; Rodrigues, 2006).

The introduction and spread of this species into new areas depend on appropriate environmental conditions (mean summer temperature above 20°C), the presence of a suitable/susceptible host tree (mainly *Pinus* spp.) and the presence of a proper insect vector (usually a *Monochamus* sp.) (Mamiya, 1984; Linit, 1988; Kishi, 1995; Evans et al., 1996). Although the expression and expansion of pine wilt disease depend on a range of biological and physical factors, PWN displays a wide range of pathogenicity (Kiyohara and Bolla, 1990; Sutherland et al., 1991) and is associated with various host species across a wide geographical distribution (for detailed information see Ryss et al., 2005).

In Portugal, PWN is associated with maritime pine (*Pinus pinaster* Ait.) (Mota et al., 1999; Penas et al., 2004) and with a longhorn cerambycid beetle (*Monochamus galloprovincialis* Oliv.) as the insect vector (Sousa et al., 2001). The geographic distribution of PWN is confined to an area 20 km south of Lisbon (Peninsula de Setúbal), occupying 510,000 ha of the continental area. A buffer zone of approximately 500,000 ha free of PWN was established for safety reasons (Rodrigues, 2006). Due to the phytosanitary measures implemented by the Portuguese government following the European Union directives (77/93 updated as 2000/29/EC), the nematode has been confined to this part of the country since its detection in 1999 (Penas et al., 2004; Rodrigues, 2006); however, each year thousands of trees displaying symptoms of PWD have been cut and removed (Rodrigues, 2006).

Several molecular biological techniques have been used for the study of genetic variability among different geographical isolates of *B. xylophilus*. Initially, the genetic differentiation of some populations was achieved by the use of restriction analyses and hybridization with total genomic DNA (Bolla et al., 1988), or by applying DNA probes (Webster et al., 1990; Abad et al., 1991; Tàres et al., 1993). Other studies using the heat shock protein *Hsp70* gene (Beckenbach et al., 1992), PCR-RFLP and rDNA sequencing (Iwahori et al., 1998; Beckenbach et al., 1999) demonstrated some genetic differences among different isolates.

The RAPD-PCR technique has also been used for the study of intra-specific variation of PWN isolates from China (Zheng et al., 1998; Zhang et al., 1999), Japan (Kusano et al., 1999) and a mixture of different geographical isolates (Braasch et al., 1995; Irdani et al., 1995a, 1995b; Wang et al., 2001; Zhang et al., 2002).

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Recently, a more integrated study has been conducted using several isolates each from the native regions (Canada and USA) and non-indigenous areas (China, Japan, Korea and Portugal) (Metge and Burgermeister, 2006).

The introduction of a species into a new area can be used as a natural case study, where the species must be able to cope with a range of new environmental pressures (Sakai et al., 2001). The genetic diversity among the Portuguese isolates of *B. xylophilus* is not known since available information is restricted to only three isolates from adjacent blocks of the affected area (Metge and Burgermeister, 2006). Two groups have observed significant degrees of differentiation among different isolates from countries where PWN has become established (Zheng et al., 1998; Metge and Burgermeister, 2006).

In this study, we have applied the RAPD-PCR technique to determine genetic distances among isolates of PWN from 24 locations within the affected area in Portugal. Cluster analysis of genetic relationships was used to examine whether the Portuguese *B. xylophilus* originated from a single introduction or multiple introductions, and an attempt was made to trace the spreading of *B. xylophilus* from its point of introduction throughout the affected area in Portugal.

Materials and Methods

Nematode isolates: In 2005, during the annual survey for PWN carried out by PROLUNP (http://www.dgrf.min-agricultura.pt/prolunp), a total of 250 pine wood samples were collected from *P. pinaster* (maritime pine) trees displaying symptoms of PWD from the 28 blocks that compose the affected area in Portugal. The division of the affected area into blocks follows the experimental design established by PROLUNP for the practical purpose of survey and eradication of PWN (Fig. 1). Wood samples, 40 to 80 g each, were collected from pine trees at 1.5 m from the base of the trunk using a 1.2-cm-diam. low-speed drill and stored in small plastic bags. Nematodes were extracted using Baermann funnel technique and processed within 48 hr.

Culturing geographic isolates: From each positive sample (presence of *B. xylophilus*), the nematodes were collected and cultured on *Botrytis cinerea* Pars., grown on potato dextrose agar (PDA) and incubated at 25°C for 2 wk. After successful rearing, 24 isolates were selected, representing 24 different blocks (the four re-

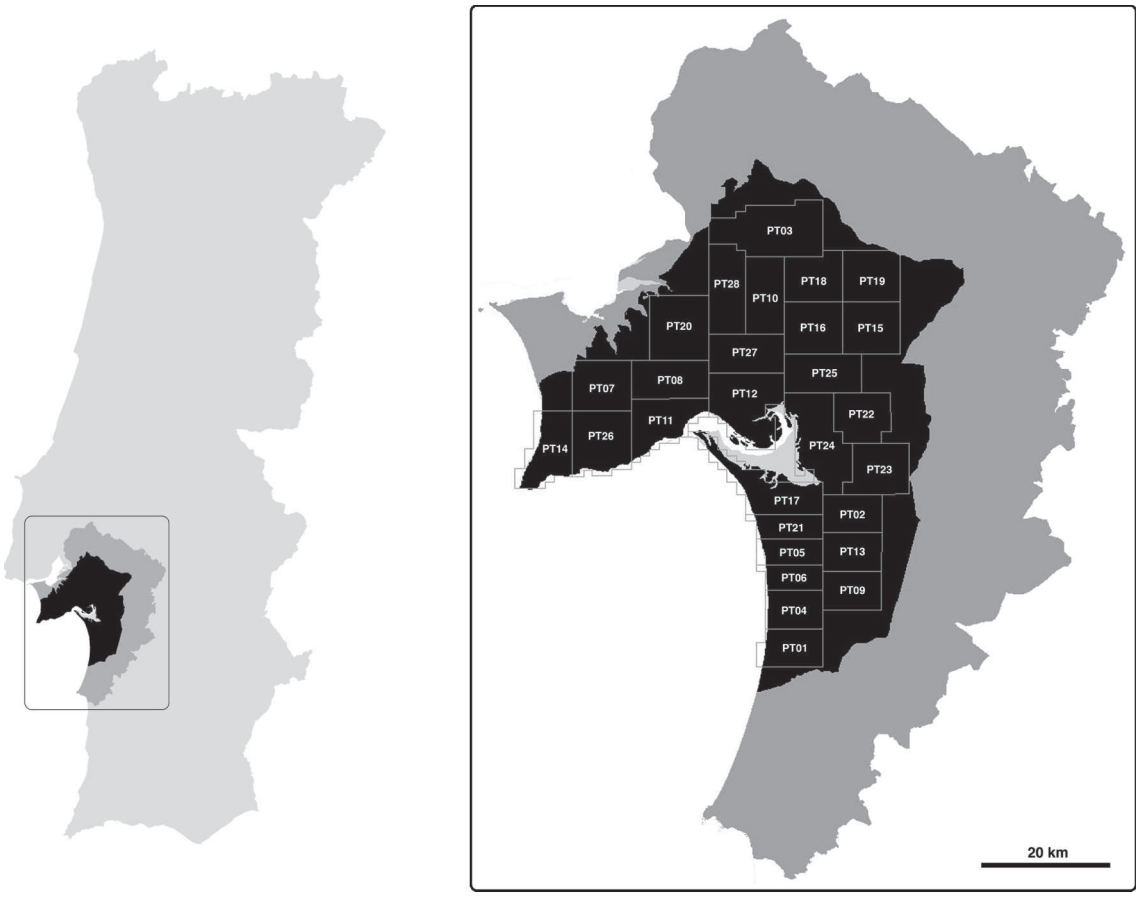

Fig. 1. Right: Portugal continental and location of the quarantine area. Left: Location of *Bursaphelenchus xylophilus* isolates obtained from different blocks within the affected area. Black: the area affected by the PWN; dark grey: the buffer area, established for safety reasons (free of PWN).

maining blocks were excluded partly due to unsuccessful rearing of some cultures and the limited number of sample slots in the electrophoresis apparatus). From each isolate, 100 to 200 nematodes (without separation according to sex or developmental stage) were collected and washed several times in distilled water, transferred to a 1.5-ml Eppendorf tube with distilled water and stored at −80°C until use. All isolates have been confirmed as *B. xylophilus* by ITS-RFLP (data not shown). The additional isolates used were: one *B. xylophilus* from Nanjing, China (BBA code: Ne12/02) isolated from *P. thunbergii* Parl. and kept in fungus culture since 2002, one *B. xylophilus* from Missouri, USA (BBA code: N5/00) from an unknown source and kept in fungus culture since 2000 and one *B. mucronatus* from Brandenburg, Germany (BBA code: DE-4w) isolated from *P. sylvestris* L. and kept in fungus culture since 1996, as an outgroup.

DNA extraction: DNA extraction was performed using the QIAmp DNA Micro Kit (Qiagen, Germany). The nematodes were placed in 1.5-ml microcentrifuge tubes and pelleted by centrifugation at 9,000*g* for 2 min, and the supernatant discarded. To the pellet, 30 µl of ATL buffer was added, and the nematodes were homogenized using Eppendorf micropestles (Eppendorf, Hamburg, Germany). The homogenate was mixed with an additional 150 µl of the ATL buffer and further processed according to the manufacturer's instructions. DNA concentrations were measured fluorimetrically using the fluorescent dye Hoechst 33258 and a DyNa Quant 200 fluorimeter (Pharmacia Biotech, Germany).

RAPD-PCR procedure: For this study, 30 oligonucleotide decamer primers (MWG, Germany) were used (Table 1). These primers were selected because they gave suitable results for the comparison of *B. xylophilus* isolates in previous studies (Braasch et al., 1995; Metge and Burgermeister, 2006; Gonçalo Silva, unpub. data). All RAPD reactions were performed as described by Schmitz et al. (1998), with slight modifications. Each PCR reaction (25 µl) contained Stoffel buffer (10 mM Tris, pH 8.3, 10 mM KCl), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of primer, 5 units of AmpliTaq DNA Polymerase Stoffel fragment (Applied Biosystems, Germany) and 4 ng of DNA template. Amplification was performed in a Perkin Elmer 9600 thermocycler (Applied Biosystems). The PCR was started by an initial denaturation step at 94°C for 2.5 min, followed by 40 cycles of 20 sec at 92°C, 15 sec at 38°C, 1 min at 72°C and a final extension at 72°C for 7 min. The rate of heating from 38°C to 72°C was regulated to 0.3°C/sec. After amplification, 10 µl aliquots of the reaction mixture were loaded onto a 2% agarose gel in TAE running buffer and electrophoresed for approximately 4 hr at 80 volts. The gel was stained in a 1 µg/ml ethidium bromide-water solution for 30 min and photographed with a UV system (Gel Jet Imager 2005, Intas, Germany). For each primer, PCR reactions were set up in individual 0.2-ml tubes. Twenty-seven *B. xylophilus* isolates were included: 25 from Portugal, including one isolate used as replicate; one from the USA; one from China; and one *B. mucronatus* isolate from Germany. In total, 840 reactions were performed, corresponding to 30 primers x 28 individual samples.

TABLE 1. Primer sequences and total number of randomly amplified DNA-PCR bands produced by each primer, applied to 27 *B. xylophilus* isolates and one *B. mucronatus* isolate.

Primer*	Sequence	*B. xylophilus* markers [n]	*B. mucronatus* markers [n]	Sum of all markers (*B. xylophilus* + *B. mucronatus*) [n]
Z01	TCT GTG CCA C	12	7	16
Z02	CCT ACG GGG A	22	3	24
Z03	CAG CAC CGC A	16	9	23
Z04	AGG CTG TGC T	7	4	9
Z05	TCC CAT GCT G	20	10	28
Z06	GTG CCG TTC A	19	9	25
Z07	CCA GGA GGA C	12	6	18
Z08	GGG TGG GTA A	21	10	31
Z10	CCG ACA AAC C	23	13	29
Z11	CTC AGT CGC A	18	7	25
Z12	TCA ACG GGA C	10	10	19
Z13	GAC TAA GCC C	11	8	17
Z14	TCG GAG GTT C	13	8	19
Z15	CAG GGC TTT C	10	7	16
Z16	TCC CCA TCA C	12	4	15
Z18	AGG GTC TGT G	18	6	23
Z19	GTG CGA GCA A	27	6	32
Z20	ACT TTG GAG G	13	5	15
B07	GGT GAC GCA G	19	5	24
Re6	CGG AAT TCG C	14	8	20
Re8	CGA TCG ATG C	18	6	23
Re9	GGA AGC TTC G	17	7	23
Re10	CCC TGC AGG C	18	10	23
Y01	GTG GCA TCT C	11	8	16
Y04	GGC TGC AAT G	19	11	27
Y06	AAG GCT CAC C	26	12	37
Y08	AGG CAG AGC A	23	12	33
Y16	GGG CCA ATG T	22	11	30
Total		471	222	640

* Primers Z09 (CAC CCC AGT C) and Z17 (CCT TCC CAC T) were excluded because they produced complex patterns of amplification products precluding reliable band scoring.

Data collection and analyses: The distinct RAPD products of each primer were run electrophoretically twice to ensure that no bands were artifacts. The RAPD fingerprint patterns obtained were converted into binary data matrices by scoring the presence of a band as 1 and its absence as 0. Bands that were not reproducible were excluded from the analyses. Faint and visually indistinguishable bands were ignored as genetic markers. The binary matrix was subjected to the MSVP ver. 3.12d software, using the Nei and Li coefficient (Nei and Li, 1979) to generate a matrix of genetic distances. The cluster analyses of genetic distances were performed with the unweighted pair-group method using arithmetic averages (UPGMA) in the module SAHN (sequential, agglomerative, hierarchical and nested clus-

tering method) of NTSYS-PC ver. 2.1 (Rholf, 2000). The dendrograms were constructed with the TREE option of NTSYS-PC. The cophenetic correlation coefficient was calculated to provide statistical support for the dendrograms obtained, and Mantel's test (Mantel, 1967) was performed to check the goodness-of-fit of the cluster analysis to the matrix on which it was based. To evaluate the robustness of dendrograms, bootstrap values (1,000 replications) were calculated using the software TREECON ver. 1.3b (Van de Peer, 1997). The relationships between the Nei and Li genetic distance matrix and the geographic distance matrix were assessed using Mantel's test. In this case, the geographic distance between two isolates (only for the Portuguese isolates) was defined as the linear distance between the sites.

Results

With the exception of primers Z9 and Z17, which amplified a large number of products causing difficulties for reliable band scoring, all 28 remaining primers were used for evaluation of amplification products and construction of the binary matrix. A total of 471 RAPD markers were scored for the isolates of *B. xylophilus*. These included 24 Portuguese isolates and a duplicate sample of isolate PT09 (termed PT09') for control of reproducibility, and one isolate each from Asia (Nanjing, China) and North America (Missouri, USA). A total of 222 RAPD markers were scored for the isolate of the out-group species, *B. mucronatus* (Brandenburg, Germany) (Table 1). The RAPD profiles were different with each of the primers. Depending on the primer, variable total numbers of amplified bands were obtained, as shown in Table 1. Figure 2 presents the RAPD profiles obtained from two of the 28 different primers used in order to illustrate the banding patterns observed. Within the Portuguese isolates, the banding patterns revealed a large number of monomorphic genetic markers in comparison to the polymorphic genetic markers; however, intraspecific polymorphism was revealed in a small proportion in some isolates (Table 2).

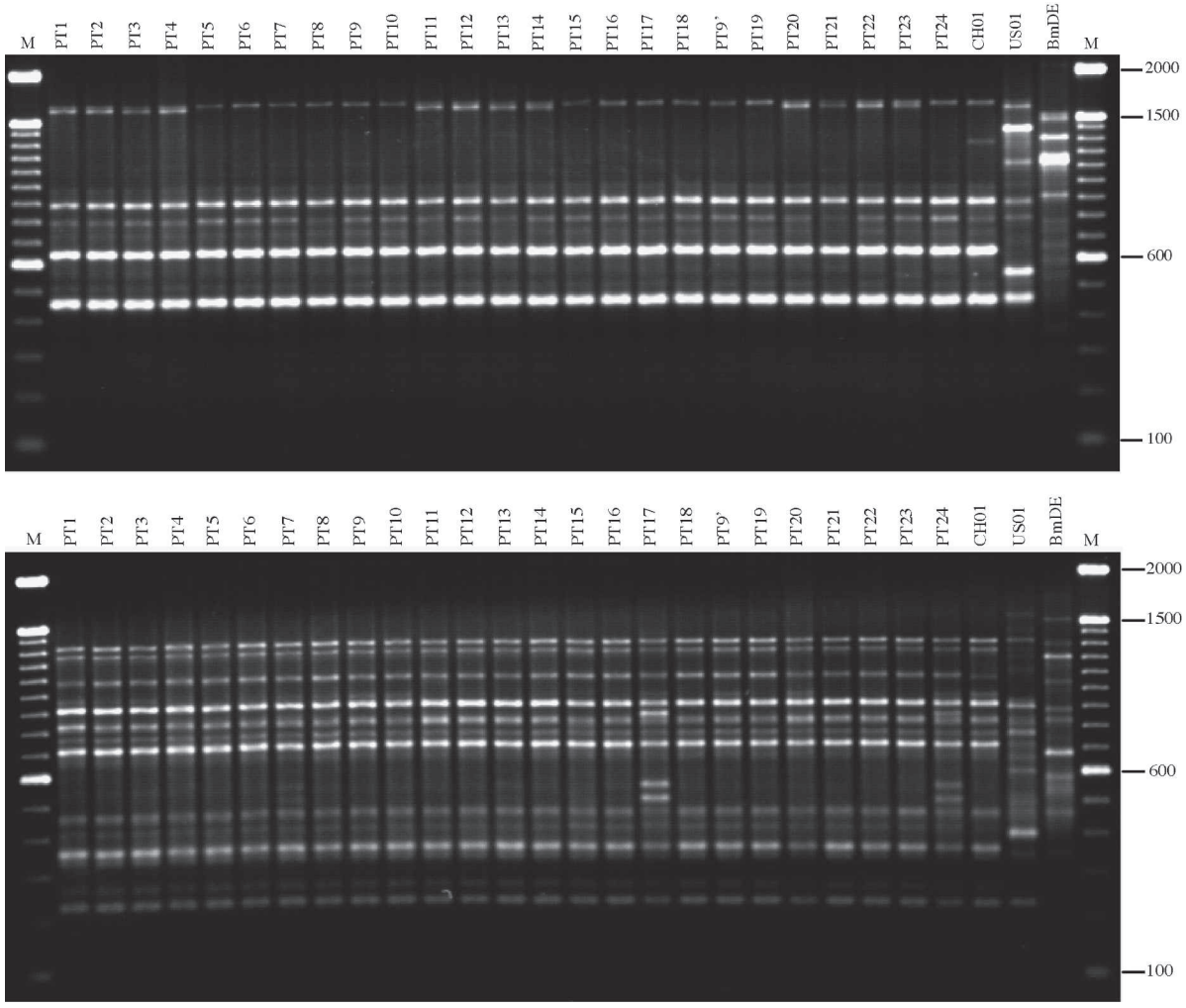

FIG. 2. RAPD profiles generated by primer Z12 (above) and Y16 (below). M: marker (100 bp ladder, Invitrogen); PT1-PT24: *B. xylophilus* isolates from Portugal; CH01: *B. xylophilus* isolate from China (BBA code: Ne12/02); US01: *B. xylophilus* isolate from USA (BBA code: N5/00); BmDE: *B. mucronatus* from Germany (BBA code: DE-4w).

TABLE 2. Number of RAPD-PCR markers among the Portuguese *B. xylophilus* isolates.

Primer	Total of bands (*a*)	Polymorphic bands (*b*)	Polymorphism % ($b/a \times 100$)
Z01	7	3	42.9
Z02	16	6	37.5
Z03	13	5	38.5
Z04	6	2	33.3
Z05	15	10	66.7
Z06	15	1	6.7
Z07	10	2	20.0
Z08	16	4	25.0
Z10	17	3	17.6
Z11	15	6	40.0
Z12	6	1	16.7
Z13	9	3	33.3
Z14	8	2	25.0
Z15	8	3	37.5
Z16	8	3	37.5
Z18	13	2	15.4
Z19	22	6	27.3
Z20	9	1	11.1
B07	10	4	40.0
Re6	10	1	10.0
Re8	16	6	37.5
Re9	11	5	45.5
Re10	13	7	53.8
Y01	10	1	10.0
Y04	14	4	28.6
Y06	18	9	50.0
Y08	19	2	10.5
Y16	16	4	25.0
Total	350	106	

The genetic similarity matrix based on the Nei and Li coefficient is presented in Table 3. The lowest similarity (approximately 50%) was reached between the American isolate and all the other *B. xylophilus* isolates. A high genetic similarity was observed between the Portuguese isolates and the isolate from China, ranging from 84% to 94%. Within the Portuguese isolates, the genetic distances reached very low values for all combinations of isolates. More than 90% of the pair-wise combinations had more than 95% genetic similarity, and the remaining pair-wise combinations were still above 90% similarity (Table 3). The pair-wise combinations between isolate PT09 and its duplicate sample (PT09') expectedly showed an extremely high genetic similarity (99%), thus illustrating the reproducibility of RAPD profiles obtained with each primer. As expected, *B. mucronatus,* used as an outgroup, showed very low similarity (around 15%) towards the *B. xylophilus* isolates.

Cluster analysis of the genetic distances was conducted using the UPGMA algorithm, based upon Nei and Li's similarity matrix. This generated a dendrogram indicating the relationships among the *B. xylophilus* isolates used in this study (Fig. 3). The cophenetic correlation coefficient between the dendrogram and the original distance matrix of the RAPD profiles was significant, with a high correlation value $r = 0.99$ (1 = best possible fit). The dendrogram obtained clearly illustrated the outgroup position of the *B. mucronatus* isolate and the large intraspecific distances between the isolate from the USA and the other isolates from China and Portugal, all of which is supported by a high bootstrap value. The position of the Chinese isolate was found to be close to the group of the Portuguese isolates, with strong support by a high bootstrap interaction node value. Within the Portuguese isolates, a remarkable degree of similarity was obtained for all 24 isolates representing the entire affected area in Portugal. Although some primers revealed a different number of polymorphic bands for some isolates, all isolates were positioned together in the same, unique cluster (Table 2; Fig. 3).

UPGMA dendrograms were also constructed (based on Pearson product-moment correlation coefficient, using the software package Gel Compare ver. 4.1) for each single primer using the profile intensity generated for the 28 isolates, and similar results were obtained, i.e., *B. mucronatus* was separated as an outgroup, the USA isolate was always clearly separated from the other *B. xylophilus* isolates and the Portuguese isolates were very close to each other and close to the Chinese isolate (data not shown).

The relationship between Nei and Li's genetic similarity matrix and the geographic distance matrix was estimated using Mantel's test. The *r* value obtained (0.212) revealed a low correlation between the genetic distances of the Portuguese isolates and their distribution among the affected area in Portugal.

DISCUSSION

Several studies have demonstrated intraspecific variability of *B. xylophilus* isolates from different geographical areas using RAPD-PCR (Braasch et al., 1995; Irdani et al., 1995a, 1995b; Zheng et al., 1998; Kusano et al., 1999; Zhang et al., 1999; Wang et al., 2001; Zhang et al., 2002; Wu et al., 2005; Metge and Burgermeister, 2006; Zhang et al., 2006). These previous investigations revealed a significant degree of genetic divergence among different isolates of *B. xylophilus.* Metge and Burgermeister (2006) examined a number of isolates (15 from North America, 12 from Asia and three from Portugal) using RAPD-PCR and ISSR-PCR. They obtained two major clusters: one including the isolates from North America (Canada and the USA) displaying a high level of genetic diversity, and a second cluster including all isolates from non-native areas (China, Korea, Japan and Portugal), with less genetic diversity. However, other estimates of similarity across isolates collected in different areas in China ranged from 46% to 95%, showing significant differences among some non-native isolates (Zheng et al., 1998; Zhang et al., 1999). This is the first study where a significant number

TABLE 3. Similarity matrix (Nei and Li coefficient) among 27 isolates of *B. xylophilus* and one isolate of *B. mucronatus* based on 640 RAPD markers.

	PT01	PT02	PT03	PT04	PT05	PT06	PT07	PT08	PT09	PT10	PT11	PT12	PT13	PT14	PT15	PT16	PT17	PT18	PT9′	PT19	PT20	PT21	PT22	PT23	PT24	CH01	US01	BmDE
PT01	1.00																											
PT02	0.996	1.00																										
PT03	0.985	0.985	1.00																									
PT04	0.983	0.983	0.979	1.00																								
PT05	0.956	0.956	0.960	0.966	1.00																							
PT06	0.965	0.965	0.973	0.975	0.956	1.00																						
PT07	0.969	0.969	0.977	0.975	0.964	0.969	1.00																					
PT08	0.973	0.969	0.969	0.979	0.964	0.965	0.973	1.00																				
PT09	0.967	0.967	0.974	0.972	0.953	0.982	0.970	0.967	1.00																			
PT10	0.979	0.979	0.978	0.992	0.973	0.978	0.982	0.986	0.976	1.00																		
PT11	0.968	0.968	0.957	0.955	0.974	0.938	0.942	0.946	0.935	0.951	1.00																	
PT12	0.990	0.990	0.990	0.984	0.958	0.975	0.971	0.967	0.972	0.980	0.962	1.00																
PT13	0.981	0.984	0.984	0.978	0.960	0.976	0.973	0.969	0.974	0.982	0.957	0.990	1.00															
PT14	0.969	0.969	0.976	0.978	0.963	0.984	0.976	0.972	0.986	0.986	0.941	0.978	0.984	1.00														
PT15	0.950	0.950	0.954	0.956	0.941	0.969	0.954	0.950	0.959	0.963	0.924	0.956	0.961	0.965	1.00													
PT16	0.958	0.958	0.969	0.963	0.944	0.969	0.957	0.953	0.978	0.967	0.930	0.967	0.969	0.976	0.954	1.00												
PT17	0.923	0.926	0.933	0.935	0.917	0.929	0.944	0.926	0.926	0.938	0.905	0.931	0.933	0.932	0.911	0.918	1.00											
PT18	0.959	0.959	0.967	0.969	0.950	0.970	0.963	0.959	0.980	0.972	0.935	0.969	0.970	0.978	0.963	0.966	0.923	1.00										
PT9′	0.965	0.965	0.972	0.970	0.951	0.980	0.969	0.965	0.998	0.974	0.933	0.970	0.972	0.988	0.957	0.976	0.925	0.978	1.00									
PT19	0.969	0.969	0.976	0.978	0.959	0.984	0.972	0.968	0.994	0.982	0.940	0.978	0.980	0.992	0.965	0.980	0.932	0.986	0.992	1.00								
PT20	0.979	0.975	0.979	0.965	0.943	0.967	0.959	0.959	0.976	0.965	0.947	0.981	0.975	0.978	0.952	0.967	0.916	0.969	0.978	0.978	1.00							
PT21	0.977	0.977	0.980	0.971	0.948	0.972	0.961	0.957	0.982	0.970	0.949	0.986	0.980	0.980	0.957	0.972	0.921	0.978	0.980	0.988	0.990	1.00						
PT22	0.977	0.977	0.984	0.974	0.952	0.976	0.965	0.961	0.982	0.974	0.949	0.986	0.984	0.988	0.961	0.976	0.925	0.974	0.984	0.988	0.990	0.992	1.00					
PT23	0.940	0.940	0.951	0.941	0.927	0.946	0.935	0.932	0.948	0.944	0.917	0.949	0.950	0.950	0.936	0.958	0.898	0.940	0.946	0.953	0.945	0.954	0.954	1.00				
PT24	0.923	0.927	0.937	0.928	0.918	0.933	0.949	0.926	0.950	0.935	0.901	0.928	0.937	0.944	0.919	0.937	0.962	0.935	0.948	0.948	0.932	0.937	0.937	0.912	1.00			
CH01	0.875	0.872	0.878	0.880	0.863	0.885	0.878	0.890	0.894	0.887	0.847	0.876	0.881	0.895	0.879	0.882	0.854	0.879	0.896	0.891	0.888	0.881	0.888	0.858	0.868	1.00		
US01	0.496	0.500	0.500	0.493	0.493	0.496	0.492	0.490	0.497	0.493	0.503	0.501	0.504	0.502	0.500	0.494	0.486	0.499	0.500	0.500	0.509	0.506	0.510	0.494	0.485	0.486	1.00	
BmDE	0.149	0.145	0.146	0.142	0.140	0.143	0.142	0.145	0.140	0.143	0.145	0.146	0.143	0.140	0.145	0.138	0.143	0.144	0.140	0.141	0.150	0.148	0.143	0.139	0.143	0.159	0.135	1.00

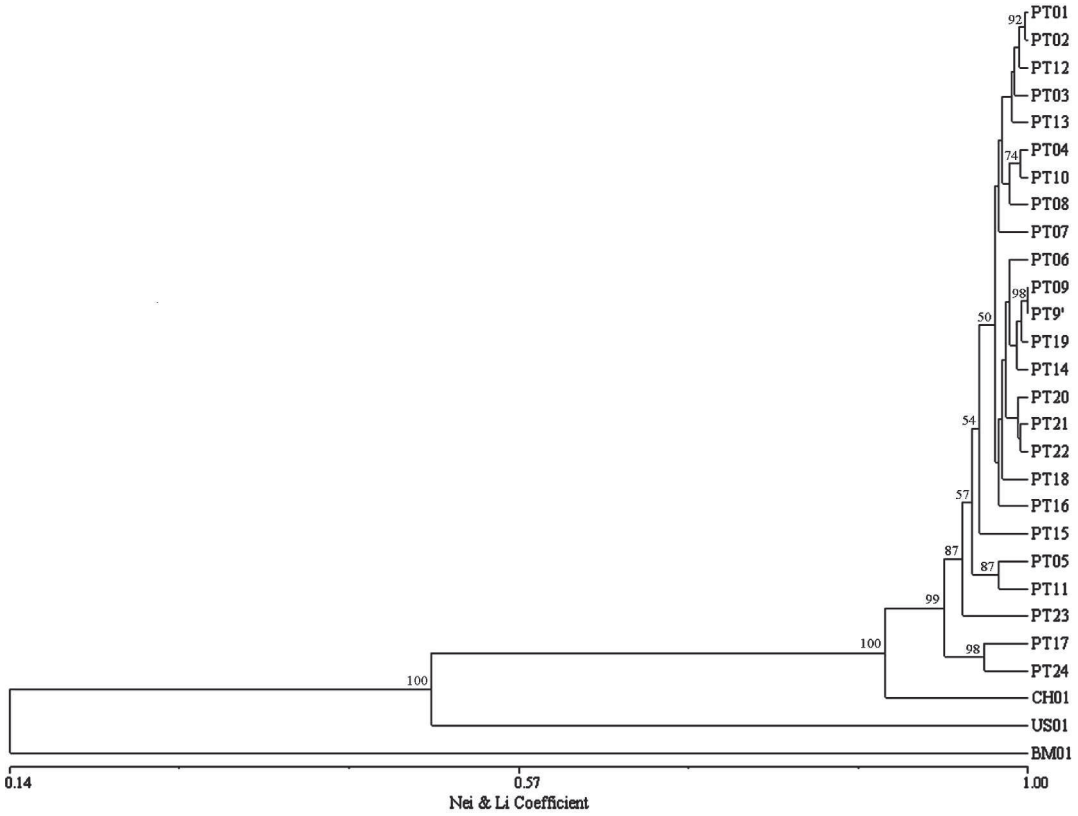

FIG. 3. UPGMA tree inferred from 640 RAPD markers for 27 *B. xylophilus* isolates and one *B. mucronatus* isolate as the out-group.

of *B. xylophilus* isolates from the affected area in Portugal was analyzed. The number of polymorphisms detected among 471 RAPD markers obtained using 28 primers was very low, reflecting a high genetic homogeneity among the 24 isolates examined. Low values of genetic distance were obtained in all pairwise comparisons, and the resulting UPGMA dendrogram suggested a low level of genetic divergence among the Portuguese isolates. Genetic distance of the Portuguese isolates from an isolate from China was much lower compared to an isolate from the USA. This confirmed previous results suggesting an East Asian origin of the Portuguese *B. xylophilus* (Metge and Burgermeister, 2006).

An objective of our investigation was to determine whether the Portuguese *B. xylophilus* originated from a single introduction or repeated introductions of the pest. Populations of an introduced invasive organism are expected to be genetically more diverse if they are derived from multiple introductions from different origins, as compared to the situation following a single introduction. Metge and Burgermeister (2006) suggested the possibility of two *B. xylophilus* introductions to Portugal from East Asia. This was based on their finding that one of their three Portuguese isolates clustered apart from the others among the isolates from East Asian countries. The three isolates were obtained from adjacent blocks of the affected area. However, the isolate that clustered separately had been maintained in culture since 1999, whereas the other two isolates were obtained in 2003 and kept in culture for only two years. Culturing of *B. xylophilus* isolates for up to 10 years on *Botrytis cinerea* malt agar may lead to small changes in RAPD profiles which are presumably caused by genetic shift (Metge et al., 2004). Culture-dependent genetic shift may therefore present an alternative explanation for the separate position of one of the three isolates studied by Metge and Burgermeister (2006). To avoid a possible genetic shift during culturing, all Portuguese isolates used in our study were collected from pine trees and reared in culture for only two weeks before DNA extraction and RAPD-PCR. No indication of separate groups of isolates was obtained in the dendrogram, and the low level of genetic diversity strongly suggests that they were dispersed recently from a single introduction.

Another intention of our study was to see whether the pathways of spreading of *B. xylophilus* from its point of introduction throughout the affected area in Portugal could be traced using RAPD-based markers. The presence of an international seaport (Setúbal) in the center of the affected area suggests a high probability for entry of PWN through this harbor. The local dispersion of PWN is always dependent on its vector beetle (*Monochamus galloprovincialis*), which can carry hundreds to thousands of nematodes (Linit, 1988). Vector flight is influenced by many factors, e.g., prevailing winds and landscape structure, including forest coverage. In analogy to findings in Japan by Takasu et al.

(2000), a nearly concentric expansion of PWN from its initial site may be tentatively assumed. This hypothesis is supported by the position of two isolates collected near the seaport (PT24 and PT17) at the root of the dendrogram. Likewise, three isolates from neighboring areas (PT23, PT11 and PT05) were placed at the bottom of the dendrogram. However, genetic differentiation was inconsistent with the geographic distances of the remaining isolates. Mantel's test showed a low correlation value (0.212) between the matrices of genetic and geographic distances. Apparently, the high degree of similarity in RAPD profiles of the Portuguese *B. xylophilus* isolates limits the use of this technique for following recent pathways of distribution. Another problem lies in the correct assessment of the pathways and mode of transportation. In our preliminary attempt of correlation, the geographical distance matrix was based on linear distances between sites of sampling. In reality, long-distance spreading may not happen only by vector flight, but in some cases be caused by the transport of infested wood or wood products to new areas inside the country. In this way, genetically identical PWN populations could be found at distant sites, and careful pathway analyses based on genetic markers could be very useful to clarify the situation and prevent further unintentional transport of the pest.

Recently, Castagnone-Sereno et al. (2006) identified 18.5% variable sequence positions in cloned repeats of the Msp I satellite DNA (146 bp) of *B. xylophilus* isolates. Their phylogenetic study based on satellite DNA variation revealed considerable diversity among Portuguese *B. xylophilus* isolates which appeared to correlate reasonably with geographic distances. Thus, satellite DNA seems to have a higher rate of genetic variation with time, compared to RAPD markers, and it is perhaps better suited to follow short-term changes in *B. xylophilus* populations following PWN introduction to Portugal. New comparative studies using satellite DNA are in progress (Castagnone-Sereno and Vieira, unpublished work) in order to provide more information about the genetic structure of the Portuguese isolates and to elucidate their pathways of spreading in the affected area in Portugal.

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