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′ → 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 good-of-fitness 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-

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