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 in 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