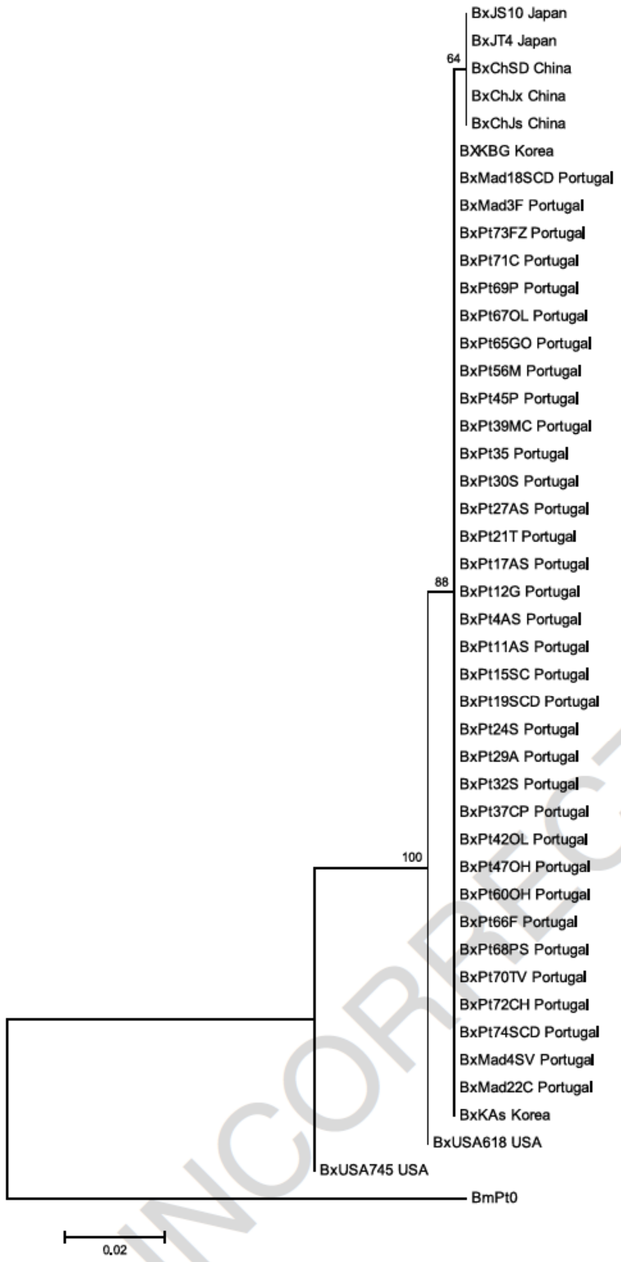

Fig. 2. *Neighbour-joining phylogenetic tree based on rRNA IGS sequences of* Bursaphelenchus xylophilus *isolates (alignment length 450 bp). Percentage bootstrap is indicated on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Tamura 3-parameter nucleotide substitution 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 to 2008. Seventeen isolates were collected from 2009-2010 and represent the new outbreaks of the disease