

1 of these isolates show different fingerprints with all
 2 primers from those detected up to 2008, which have
 3 different levels of variability, revealing the existence of
 4 intraspecific variability among Portuguese isolates. Iso-
 5 lates BxPt27AS, BxPt56M, BxPt60OH, BxPt65GO and
 6 BxPt66F show different banding patterns only for some
 7 of the primers and thus still group with isolates up to
 8 2008 in the dendrogram (Fig. 4). Examining the distri-
 9 bution of these isolates in the dendrogram, Portuguese
 10 isolates collected from 1999-2008 all group in the same
 11 clade with similarity levels between 84 and 99%. How-
 12 ever, Portuguese isolates collected in 2009-2010 are dis-
 13 tributed across different branches of the dendrogram.

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

25 Discussion

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

37 In Portugal, PWN was successfully restricted to the
 38 Setúbal Peninsula for several years immediately following
 39 the first report (Mota *et al.*, 1999). This was mainly due
 40 to strong and specific measures for control of inland
 41 circulation of wood and wood products from the original
 42 affected area. However, in 2008, the nematode was found
 43 in new outbreak spots in central and northern regions of
 44 the country (Rodrigues, 2008) and, more recently, in one
 45 of the islands of the Madeira archipelago (Fonseca *et al.*,
 46 2010).

47 The first study using a significant number of *B. xylophi-*
 48 *lus* from Portugal was performed by Vieira *et al.* (2007),
 49 using 24 isolates from the initial affected area, the Setúbal
 50 Peninsula, the only region where the disease was detected

51 until 2008. In this study, the authors showed that there
 52 was almost no genetic diversity among isolates and that
 53 the Portuguese isolates grouped with Chinese isolates.

54 To understand the origin and genetic diversity of the
 55 PWN found in the new areas in continental Portugal and
 56 Madeira, 34 isolates representing the different geographic
 57 areas were studied using IGS sequences and ISSR fin-
 58 gerprints. This is the first study where a significant num-
 59 ber of *B. xylophilus* isolates from the initial affected area
 60 in the Setúbal Peninsula, as well as from the new out-
 61 breaks of the disease in continental Portugal and Madeira,
 62 have been analysed. ITS sequences confirmed all 34 Por-
 63 tuguese isolates as *B. xylophilus*. No intraspecific variabil-
 64 ity among isolates was found. Only isolate BxUSA745
 65 from USA groups in a separate position from all the other
 66 isolates. Using IGS sequencing, no significant intraspe-
 67 cific variability was detected among isolates of *B. xylo-*
 68 *philus*. Both USA isolates are distantly related to all other
 69 isolates, isolate BxUSA745 having a clear separate posi-
 70 tion on the phylogenetic tree. Chinese and Japanese iso-
 71 lates group together. All Portuguese isolates group with
 72 Korean isolates. Moreover, no clustering of the isolates
 73 according to their geographic origin was obtained.

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 75 In the dendrogram obtained from the ISSR fingerprint
 76 analysis, all Portuguese isolates collected prior to 2008
 77 group together with similarities of 84-99%, indicating a
 78 lack of genetic diversity and suggesting a single introduc-
 79 tion, which is in agreement with the results of Vieira *et*
 80 *al.* (2007). Observing the distribution of isolates collected
 81 in 2009-2010 in continental Portugal, there are five iso-
 82 lates (BxPt27As, BxPt56M, BxPt60OH, BxPt65GO and
 83 BxPt66F) grouping with the isolates collected up to 2008
 84 with similarities above 86%. This suggests a spread of the
 85 disease from the initial affected area to new outbreaks,
 86 which may have occurred with great probability consider-
 87 ing the heavy traffic of vehicles carrying wood products
 88 from the Lisbon-Setúbal area to the centre and north of
 89 Portugal, where a number of industries process pine wood
 90 for furniture. The insect vector *Monochamus galloprovin-*
 91 *cialis* is known to occur well inside the centre of the coun-
 92 try, quite far (*ca* 200-300 km) north of the Setúbal Penin-
 93 sula, for example, in the region of Penacova, near Coim-
 94 bra. Furthermore, it is known to occur throughout the con-
 95 tinental territory (Sousa *et al.*, 2001, 2002; Penas *et al.*,
 96 2006). On the other hand, continental isolate BxPt73FZ
 97 has a separate position from all other isolates, suggesting
 98 a new introduction in continental Portugal, possibly from
 99 the native North American population.