



Genetic diversity of ITS sequences of *Bursaphelenchus xylophilus*

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ABSTRACT. The sequence variation of internal transcribed spacer (ITS) regions of ribosomal DNA has been routinely used for species identification and species-level phylogeny of the pinewood nematode, *Bursaphelenchus xylophilus*. In this study, the intraspecific ITS genetic diversity of *B. xylophilus* was evaluated. Three pinewood nematode isolates from the United States, Japan, and Portugal were used for polymerase chain reaction (PCR) ITS region amplification and sequencing. Multiple peaks were observed in sequencing chromatograms from ITS regions of American and Japanese isolates, suggesting the presence of more than one ribosomal sequence for each isolate. PCR products were further cloned and 10 clones of each isolate were subsequently sequenced. Additionally, the ITS regions of individual nematodes from each isolate were amplified, cloned and sequenced. Among the 3 *B. xylophilus* isolates analyzed, an intraspecific and intra-isolate molecular variability was found. The intra-isolate ITS molecular diversity in the American isolate was higher than that in the Japanese and Portuguese isolates. However, the level of sequence variation observed within isolates was about the same as that described among ITS repeats within individuals.

Key words: ITS diversity; Pine wilt disease; Pinewood nematode; Ribosomal DNA

INTRODUCTION

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease, is considered a native of North America. However, native American conifers are tolerant or resistant to *B. xylophilus*, and consequently this nematode has caused little damage (Mamiya, 1983; Jones et al., 2008). At the beginning of the 20th century, the PWN was introduced in Japan and became responsible for massive mortality of native pine trees (*Pinus densiflora*, *P. thunbergii* and *P. luchuensis*). The PWN then spread to China, Korea, and Taiwan (European and Mediterranean Plant Protection Organization, 2009). In Europe, the PWN was first detected in continental Portugal in the maritime pine, *P. pinaster* (Mota et al., 1999). It has been found more recently in Madeira Island (Fonseca et al., 2012) and Spain (Abelleira et al., 2011; Robertson et al., 2011) and represents an increasing threat to European forests.

Traditionally, the identification of PWN has been based on diagnostic morphological characters after extraction from wood samples. However, this methodology involves several time-consuming steps and requires a high level of taxonomical expertise. Additionally, PWN morphological identification can sometimes be difficult or impossible when only juvenile stages are detected or owing to the variation in the female tail of some isolates (Fonseca et al., 2008). Therefore, several molecular methods have been developed that frequently use species-specific primers by targeting several genomic regions such as satellite DNA (Castagnone et al., 2005; Cardoso et al., 2012), the heat shock protein 70 gene (Leal et al., 2005, 2007), the topoisomerase I gene (Huang et al., 2010), and internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) (Cao et al., 2005; Takeuchi et al., 2005) or by using nonspecific primers for polymerase chain reaction (PCR) amplification of the ITS regions and subsequent analysis with restriction fragment length polymorphism (RFLP) (Iwahori et al., 2000; Burgermeister et al., 2009). The ITS regions are located between the repeating array of nuclear 18S, 5.8S, and 28S ribosomal RNA genes and have 100-200 copies/genome. These regions are rapidly evolving and thus have been routinely used in species-level phylogeny in a wide range of organisms, including nematodes (Blouin, 2002). In *B. xylophilus*, the intraspecific variation of the ITS regions has been used to study the genetic structure of isolates from various regions of the world through sequencing (Zheng et al., 2003; Mota et al., 2006; Zhang et al., 2008; Nose et al., 2009) and RFLP analysis (Iwahori et al., 1998). Intraspecific diversity between virulent and avirulent *B. xylophilus* isolates has been detected using RFLP analysis with the enzyme *Hha*I (Aikawa et al., 2003, 2006).

ITS genetic diversity has been reported within species and individuals from a diverse range of eukaryotes, including insects (Fairley et al., 2005; Li and Wilkerson, 2007), marine sponges (Worheide et al., 2004; Vierna et al., 2010), fungi (Horton, 2002; Smith et al., 2007), and nematodes such as *Meloidogyne* (Hugall et al., 1999), *Brugia* (Areekit et al., 2009), *Ascaris* (Leles et al., 2010), and *Pratylenchus* (De Luca et al., 2011). However, to our knowledge, no data on ITS intra-isolate and intra-individual diversity in *B. xylophilus* or other *Bursaphelenchus* species have been published. In the present research, the complete nucleotide sequence of ITS rDNA regions was used to study the genetic diversity of *B. xylophilus* isolates from the United States, Japan, and Portugal.

MATERIAL AND METHODS

Nematode isolates

B. xylophilus isolates from the United States (BxUSA745), Japan (BxJ10), and Por-

tugal (BxPt12G) were maintained in cultures of *Botrytis cinerea* grown on malt extract agar medium at 25°C. Mixed developmental nematode stages were collected from the cultures, concentrated via centrifugation, and homogenized using a plastic homogenizer for DNA extraction. Three adult nematodes of each isolate were handpicked under a stereomicroscope and used individually for DNA extraction.

DNA extraction and amplification of ITS regions

Nematode DNA was extracted using a DNeasy® Blood and Tissue Mini kit (Qiagen, Germany) following manufacturer instructions. The ITS rDNA regions containing partial 18S and 28S and complete ITS1, 5.8S, and ITS2 sequences were amplified with PCR using 50 ng extracted DNA and 1 U Dream Taq DNA polymerase (Fermentas, USA) in 1X Dream Taq buffer, 0.2 mM of each deoxyribonucleotide triphosphates, and 1 µM primers 18SF 5'-CGTAACAAGGTAGCTGTAG-3' (Ferris et al., 1993) and 28SR 5'-TTTCACTCGCCGTTACTAAGG-3' (Vrain, 1993). All reactions were carried out in a Thermal Cycler (Bio-Rad, Spain) with an initial denaturation step of 95°C for 2.5 min followed by 40 reaction cycles of 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting amplification products were purified using a QIAquick® PCR Purification Kit (Qiagen) according to manufacturer instructions, directly sequenced, and further cloned.

PCR product cloning

Purified ITS-amplified products were ligated into pGEM®-T Easy Vector (Promega, USA) using 50 ng vector in a 10-µL reaction with 3 U T4 DNA Ligase (Promega) and 46 ng purified PCR product in 1X Rapid Ligation Buffer (Promega). Ligation reactions were incubated for 1 h at room temperature. Then, 2 µL ligation product was used to transform *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) following manufacturer instructions.

Sequencing and sequence analysis

Plasmid DNA was extracted from *E. coli* cells using a QIAprep® Spin Miniprep Kit (Qiagen), and 10 selected positive clones for each *B. xylophilus* isolate and for each individual nematode were fully sequenced in both strands in an Automatic Sequencer 3730xl under Big-Dye™ terminator cycling conditions at MacroGen Company (Korea). Sequence analysis, alignments, and determination of consensus sequences were carried out using BioEdit (Hall, 1999). Homologous sequences in the databases were searched using the Basic Local Alignment Search Tool (Altschul et al., 1997), and the beginning and end of the ITS regions were delineated based on GenBank data. Nucleotide diversity, which is the mean number of nucleotide substitutions per site ± standard deviation, and haplotype diversity were estimated in DnaSP 5.10 (Librado and Rozas, 2009).

RESULTS

The ITS rDNA regions containing partial 18S and 28S and complete ITS1, 5.8S, and ITS2 sequences were successfully amplified for all *B. xylophilus* DNA samples, and 63 ITS regions were

sequenced. Three of the regions were sequenced directly from the PCR product [GenBank accession Nos. JQ288085 (BxUSA745), JQ288086 (BxJ10), JF826227 (BxPt12G)] and 60 were cloned PCR products. The 3 ITS PCR products obtained from the mixed developmental nematode stages of BxUSA745, BxJ10, and BxPt12G were first directly sequenced. Multiple peaks in the sequencing chromatograms of BxUSA745 and BxJ10 were observed, suggesting the presence of more than one ITS ribosomal sequence for each isolate (Figure 1). From the further cloning of these PCR products, 10 clones of each of the 3 isolates were analyzed and, additionally, 10 clones for each individual specimen from each of the 3 isolates were also analyzed, within a total of 60 clones.

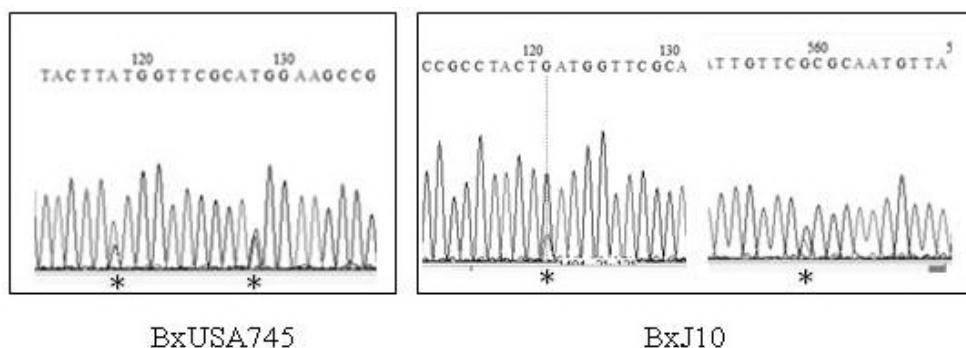


Figure 1. Sequencing chromatograms of the American (BxUSA745) and Japanese (BxJ10) *Bursaphelenchus xylophilus* isolate ITS regions, showing multiple peaks (asterisks).

Among the 10 mixed developmental nematode stage ITS clones of BxUSA745, sequences of 880/881 bp were identified, with 21 variable sites and 9 haplotypes. For an individual nematode of BxUSA745, sequences of 880/881/882 bp were identified, with 15 variable sites corresponding to 9 haplotypes. From the 10 mixed developmental nematode stage ITS clones of BxJ10, sequences of 884/885 bp were identified, with 10 variable sites corresponding to 8 haplotypes. For an individual nematode of BxJ10, sequences of 885 bp were identified, with 7 variable sites and 7 haplotypes. Mixed developmental nematode stage ITS clones of BxPt12G displayed 885-bp sequences with 9 variable sites corresponding to 8 haplotypes. For an individual nematode of BxPt12G, sequences of 885 bp were identified, with 10 variable sites corresponding to 7 haplotypes (Table 1).

Overall, the ITS molecular diversity found in BxUSA745 was higher than that in BxJ10 and BxPt12G. The intra-individual ITS molecular diversities of BxUSA745 and BxJ10 were slightly lower than the intra-isolate ITS molecular diversities. Conversely, the intra-individual ITS molecular diversity of BxPt12G was slightly higher than the intra-isolate ITS molecular diversity.

The differences among ITS sequence types were due to point nucleotide substitutions and nucleotide deletions or varying numbers of repeats that resulted in different lengths of the ITS variants. The consensus sequence resulting from the alignment of all sequences was determined (Figure 2). Within the 60 clones analyzed, 45 haplotypes were found, from which 1 major haplotype is shared by 7 clones from BxJ10 (3 from mixed developmental nematode stages and 4 from individual nematodes) and 5 clones from BxPt12G (2 from mixed developmental nematode stages and 3 from individual nematodes). This haplotype corresponds to the one determined through the direct sequencing of BxJ10 and BxPt12G PCR products. No common haplotypes were found between BxUSA745 and the other 2 isolates.

Table 1. Genetic diversity estimated for cloned ITS regions of American (BxUSA745), Japanese (BxJ10) and Portuguese (BxPt12G) *Bursaphelenchus xylophilus* isolates.

Isolate	No. of clones	Cloned fragment (bp)	No. of variable sites	Haplotypes	Pi ± standard deviation
BxUSA745					
Mixed stages	10	880/881	21	9	0.00541 ± 0.00061
Individual	10	880/881/883	15	9	0.00369 ± 0.00056
Mixed stages + individual	20	880/881/883	33	18	0.00457 ± 0.00047
BxJ10					
Mixed stages	10	884/885	10	8	0.00204 ± 0.00049
Individual	10	885	7	7	0.00158 ± 0.00038
Mixed stages + individual	20	884/885	17	14	0.00181 ± 0.00034
BxPt12G					
Mixed stages	10	885	9	8	0.00221 ± 0.00047
Individual	10	885	10	7	0.00264 ± 0.00063
Mixed stages + individual	20	885	19	14	0.00246 ± 0.00043

1 GTGAACCTTC GGCTGGATCA TTACCGATCC TATGACACAT TTATTCGTGC TCGTCACGAT GATGCGATTG 70
71 GTGACTTCGG TTGCCGCGCA TGATGGCGGT TCGATTCCGG TCGTTCCGCC TACTKRRTGGT TCGCAYGGAA 140
141 GCCGAGAGGC GACCGTGCAA CGGTGAAGTC TGGGTTTCTA CGTGCTGTTG TTGAGTTGGC GTTTTACCGT 210
211 GCCGACAGAT GAGACCAGCC AGCTGCTTGC CGATTGCTTC TGGCGAGCGT AGGATTGAAA AGCCCGAGAG 280
281 GCTGCCCTGA CAAAACATTC ATTTTACATT TATTTTGTG GAAAAGAGCT TTAAGTTACT CCGGTGGATC 350
351 ACTTGCTCG CGGGTCGATG AAGAACGCAG TGAATTGCGA TAATAAGTAC GAATTACAGA TATTATGAGT 420
421 ACCATGTTTT TGAATGCATA TTGCGCTCTT GGGCTTTGCT CTTGAGCATA TTCGATTCAG GGTGTGTTTT 490
491 TAAACTCGAG CAGAAACGCC GACTTGTTTTT TTTCAAGTTT CTGCACGTTG TGACAGTCGT CTCGCATTGT 560
561 TCRCGAATG TTAGGCCACCA TCTGTTTTAC GCGGTTTGT CCGCGACCAA TATCTTCTAC GCACGTTTTG 630
631 TYCGTGCGGG GCGAGAGGGC TTCGTGCTCG ATTGTGCTGC GCGGCTAAAC CGTTTGGTGA TGTTGTTTCA 700
701 ACGGCGCGGC CGTCAGGGAC GTTCGGATGA GAATGTTGG AGTCCTGGCT GCGGTTTGT GAGCTTCGTC 770
771 GTGAAGCCTT GCGGGCAGTG TTGTCGGAAT TGGTTGAAAC CACCTGAGTT GGGTATGACT ACCTGCTGAA 840
841 CTTAAGCATA TCAGTAAGCA GAGGAAAAGA AACAAACATG GATTC 885

Figure 2. Consensus sequence obtained from the determined ITS sequences of the 60 *Bursaphelenchus xylophilus* clones. Polymorphic sites are marked with bold and indels are underlined. *Hha*I recognition sites are boxed [position 563 A/G (R), recognition site only when G]. 18S rDNA, 5.8S rDNA and 28S rDNA surrounding ITS sequences are shaded in gray.

DISCUSSION

The complete sequence of the ITS regions from 3 isolates of *B. xylophilus* revealed intra-isolate and intra-individual genomic variability. The higher ITS genomic variability was detected in the BxUSA745 isolate, which agrees with the high diversity found among American isolates and is probably due to the ancient origin of these isolates. Interestingly, 1 of

the 21 variable sites in the BxUSA745 mixed developmental nematode stage ITS sequences changed a recognition site for the endonuclease *HhaI*, altering the predicted restriction pattern from that described for virulent *B. xylophilus* isolates to that of avirulent isolates (Aikawa et al., 2003). This site, nucleotide position 563 in the consensus sequence (see Figure 2), was not detected as a mutation site in the intra-individual analysis. In addition, although this site was not found as a variable site in the sequenced clones of BxJ10, it corresponds to one of the multiple peaks detected with direct PCR sequencing (see Figure 1), revealing that this isolate also contains both variants. These data indicate that the use of the ITS *HhaI* restriction pattern to differentiate virulent from avirulent *B. xylophilus* isolates should be reconsidered.

The major haplotype determined among the 60 clones analyzed corresponds to that determined using ITS PCR product direct sequencing of BxJ10 and BxPt12G as well as to that determined in previous studies for other Portuguese, Chinese and some Japanese isolates, which revealed no inter-isolate molecular variability in this genomic region (Mota et al., 2006; Fonseca et al., 2012). These results may reflect a unique introduction of *B. xylophilus* in Portugal from East Asia but not from America, which is in accordance with the conclusions of other authors using different molecular markers and techniques: inter-simple sequence repeats and RAPD-PCR (Metge and Burgermeister, 2006; Vieira et al., 2007) and sequencing of mitochondrial DNA gene regions (Moreira et al., 2011). Furthermore, the data reported here revealed that the molecular diversity found in the ITS regions of *B. xylophilus* isolates is mainly a result of intra-individual molecular diversity; the level of sequence variation observed among isolates is similar to that described among ITS repeats within individuals. This result is in accordance with that described for *Meloidogyne* species (Hugall et al., 1999) and those for other nematodes - typically $\leq 1\%$ (Blouin, 2002). Considering that the ribosomal spacers are noncoding regions and occur in multiple copies per genome, differences in ITS sequences can be expected to occur in individual nematodes.

In conclusion, the findings of this study demonstrate that molecular diversity exists in the ITS region of *B. xylophilus*, revealed by intra-isolate and intra-individual ITS diversity. This diversity should be taken into account when this region is used as a molecular marker for the characterization of *B. xylophilus* isolates.

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