Species-specific polymerase chain reaction primers for simple detection of *Bursaphelenchus fraudulentus* (Nematoda: Parasitaphelenchidae)

Anna Filipiak 1,*, Agata Jakubowska 1,2 and Marek Tomalak 1

*Bursaphelenchus fraudulentus* Rühm, 1956 (Nematoda: Parasitaphelenchidae) is a tree-inhabiting species frequently found in Europe (Rühm, 1956; Schauer-Blume & Sturhan, 1989; Tomalak, 2004; Carletti et al., 2005) and Asia (Brausch et al., 2001). It is primarily found in dying or dead deciduous trees (Rühm, 1956; Schauer-Blume & Sturhan, 1989), although occasionally reported from conifers (Brausch et al., 2001). Within the genus the specific morphological characters (i.e., vulval flap, shape of spicules, position of caudal papillae and presence of four incisions in the lateral fields) place *B. fraudulentus* into a distinct *xylophilus*-group which comprises eight other, morphologically similar, species, including the quarantine pest *B. xylophilus* (Gu et al., 2008). Reliable methods of taxonomic identification of these species are therefore of particular interest to plant quarantine services.

Several molecular techniques have been developed and used for identification of *Bursaphelenchus* spp. (Wang et al., 1999; Matsunaga & Togashi, 2004; Burgermeister et al., 2005; Castagnone et al., 2005; Leal et al., 2005, 2007) with ITS-RLFP analysis the most widely used in research and quarantine practice. An alternative method, based on a simple PCR amplification with primers specific for *B. xylophilus* and *B. mucronatus* (Matsunaga & Togashi, 2004), enhances the utility of this method. The main objectives of our research were to sequence the ITS regions of the *B. fraudulentus* genome and to design specific primers for PCR amplification.

Eight isolates of *B. fraudulentus*, two of *B. mucronatus* and one of *B. xylophilus* were examined. The Austrian (Österreich), Russian (DE10w), German (Helmstedt and H26), and Hungarian (Ungarn) isolates of *B. fraudulentus* and the isolate of *B. xylophilus* (China) were obtained from the nematode collection of the Federal Research Centre for Cultivated Plants (formerly Federal Biological Research Centre for Agriculture and Forestry – BBA), Braunschweig, Germany (Dr T. Schröder). All the remaining nematode populations were isolated from infested trees in Poland. The populations of *B. fraudulentus* originated from Poznan (PL-01 and PL-05) and Kornik (PL-04) and those of *B. mucronatus* from Nowy Tomysl (NTo-01) and Slawa Slaska (SIS-01). Their taxonomic identification was originally based on morphological and morphometric characteristics and subsequently confirmed by ITS-RLFP analysis according to the protocol described by Burgermeister et al. (2005). Prior to examination, all isolates were reared on Botrytis cinerea/malt agar (4.5%) at 25°C for ca 2 weeks. Propagated nematodes were collected by the Baermann funnel method and stored in 10 μl H2O at −20°C until use.

Extraction of DNA was performed according to the method described by Iwahori et al. (1998), with minor modifications to the composition of the lysis buffer: 100 mM Tris, pH 8.5, 100 mM NaCl, 50 mM EDTA, 1% SDS, 1% β-mercaptoethanol, 100 μg ml−1 proteinase K per 100 μl buffer. Before incubation, the mixture was

1 Department of Biological Pest Control and Quarantine, Institute of Plant Protection, Wladyslawa Wegorka 20, Poznan, 60-318, Poland
2 Department of Genetics, University of Valencia, Dr. Moliner 50, 46-100, Burjassot, Spain
* Corresponding author, e-mail: A.Filipiak@ior.poznan.pl
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frozen at −80°C for 40 min. The ITS regions of *B. fraudulentus* DNA were amplified using the non-specific forward primer 5′-CGTAAACAGTACCGTGCTAG-3′ (Ferris et al., 1993) and reverse primer 5′-TTTCATGGCGGTGTCAGTG-3′ (Vrain, 1993), which give positive amplification for all *Bursaphelenchus* spp. (Leal et al., 2007).

The PCR programme and reaction components were as described by Burgermeister et al. (2005). PCR products were purified using the Qiaex II Gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and used to transform into DH5α *Escherichia coli* cells for further sequencing.

Nucleotide sequences were determined (IBB, Warsaw, Poland) and contigs assembled using SeqMan program from the Dnastar software package (DNASTAR, Madison, WI, USA). Ultimately, the sequence was composed of an alignment of two sequences of *B. fraudulentus* (PL-01) with other sequences of *B. fraudulentus* deposited in the GenBank database. In order to design species-specific primers for *B. fraudulentus*, the sequence of PL-01 was compared to sequences of other species of *Bursaphelenchus*. Multiple nucleotide sequence alignments were generated using two sequences of each of the following species: *B. fraudulentus* (GenBank accession numbers EU543693, AM179517), *B. xylophilus* (AB294736, AM179515), *B. mucronatus* (AM400246, AM179514), *B. conicaudatus* (AB067757, AM179513) and single sequences of *B. macromucronatus* (EU256381), *B. doui* (AM157743), *B. luxuriosae* (AM400245), *B. abruptus* (AM400244), *B. singaporensis* (AM180514), *B. eremus* (AM180515), *B. eggersi* (AM269911), *B. pinophilus* (AM160664) and *B. clavicauda* (AB299221). Fragments of *B. fraudulentus* that differed most from those of other *Bursaphelenchus* spp. were chosen for the primer design.

Consensus alignments were generated using ClustalW and visualised in GeneDoc. The specific primers were selected and evaluated using PrimerSelect. The PCR product was expected to be 617 bp in length.

In order to confirm that the primers fitted the species-specific conserved region in all studied isolates of *B. fraudulentus*, a comparative analysis of intraspecific sequence variation was done. The PCR products from all isolates of *B. fraudulentus* were sequenced and their sequences deposited into GenBank under accession numbers EU543693 and FJ712308-FJ712313. The DNA base sequence of the DE10w isolate was derived from the GenBank database (AM179517).

Species-specific PCR primers were designed from the ITS-1 and ITS-2 regions (forward primer: 5′-GTGATGCTGATGCTGCTAG-3′, reverse primer: 5′-CAACGTTCAATCAGCGCAA-3′). The amplification was done with a Biometra TPersonal Thermocycler (Biometra, Goettingen, Germany). The PCR amplification mixture (12.5 μl) was composed of 1 ng μl−1 template DNA, 1 μM of each primer, 200 μM dNTP, 10× PCR buffer and 1 unit Taq polymerase (Novazym, Poznan, Poland). To determine optimum annealing temperature, several PCR amplifications with different annealing temperatures ranging from 56 to 71°C were done. The optimised thermal programme for routine PCR analysis was as follows: an initial denaturation at 94°C for 5 min, then 25 reaction cycles of denaturation at 94°C for 30 s, annealing at 69°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were separated in a 1.5% agarose gel for 30 min at 65 V. The DNA fragments were visualised by staining with 1 μg ml−1 ethidium bromide and photographed under UV light.

To investigate the sensitivity of the newly designed species-specific primers for *B. fraudulentus*, an additional experiment was done with mixtures of *B. fraudulentus*, *B. xylophilus* and *B. mucronatus* DNA at various ratios, i.e., 1:1:1, 1:50:50, 1:100:100 and 1:200:200, respectively. The experiments were replicated at least six times to confirm consistency of the obtained data.

The PCR amplification with the non-specific primers successfully amplified DNA of *B. fraudulentus* (PL-01) and yielded a PCR product of 1030 bp. The DNA sequencing revealed that the amplified fragment of *B. fraudulentus* DNA contained complete regions of ITS-1, 5.8S, and ITS-2, as well as parts of the 18S and 28S regions. The obtained sequence was aligned with known sequences of other nematode species and the most conserved and heterogeneous fragments were determined. The most heterogeneous fragments showed only about 28% identity at the nucleotide level and these were the fragment of ITS-1 (83-136 nt, where number 1 stands for the first nucleotide of *B. fraudulentus* sequence submitted to NCBI under accession number EU543693) and fragment of ITS-2 (691-731 nt). Comparison of ITS regions of *B. fraudulentus*, *B. xylophilus* and *B. mucronatus* allowed us to design a primer set which, for *B. fraudulentus*, gave a product of a length different from those obtained with specific primers designed for *B. xylophilus* and *B. mucronatus* (Matsunaga & Togashi, 2004). The species-specific forward primer was located in the ITS-1 region (109-129 nt) and the reverse primer was in the ITS-2 region (707-727 nt).
The comparative analysis of intraspecific variation among sequences of all *B. fraudulentus* isolates studied revealed eight substitution sites (three A/G, three C/T and two G/T) and a gap (= insertion or deletion: C/–). In three out of nine diverging sites the PL-01, PL-05, DE10w and Helmstedt isolates clearly differed from PL-04, Österreich, Ungarn and H26 isolates. The DNA of eight isolates of *B. fraudulentus* served as templates for PCR amplification with the designed species-specific primer set. For all isolates, PCR amplification resulted in products of identical length of 617 bp (Fig. 1A). Optimum annealing temperature for the PCR amplification was experimentally determined as 69°C.

The examined isolates of *B. fraudulentus* originated from different parts of Europe. Therefore, it is assumed that the newly developed species-specific primer set can discriminate *B. fraudulentus* and serve as a universal primer set for this species. To confirm *B. fraudulentus* specificity of the designed primer set, one isolate of *B. xylophilus* and two isolates of *B. mucronatus* were used as negative control in PCR amplifications. For those populations no PCR products were generated (Fig. 1B). The PCR amplification with primer sets specific for *B. xylophilus* (XF and XR), *B. mucronatus* (MF and MR) (Matsumaga & Togashi, 2004) and those for *B. fraudulentus* designed in this study resulted in amplicons of different lengths (557, 210 and 617 bp, respectively), which can be easily distinguished in agarose gels. For each nematode species we obtained only one PCR product, both in the reaction with only a single specific primer set as well as with a mixture of all three species-specific primer sets.

The sensitivity of the designed *B. fraudulentus*-specific primer set did not decrease when a mixture of *B. fraudulentus, B. xylophilus* and *B. mucronatus* DNA samples was used. In all cases, when DNA of at least a single *B. fraudulentus* individual had been incorporated into the mixture, the obtained PCR product showed a specific band pattern for this species. Moreover, the sensitivity of these primers was good enough to detect DNA of *B. fraudulentus* in both the composition of single nematodes from all of the examined species as well as in the mixture of all three nematode species with *B. fraudulentus* to other, non-target, species ratio as high as 1:200:200 individuals. These findings are particularly relevant to examination of field-collected samples when *B. fraudulentus* is isolated together with other nematodes and rapid and reliable confirmation of its identity is needed. Based on these results, the availability of specific primers for all three species of *Bursaphelenchus* recorded in Europe from the *xylophilus* group should facilitate this simple PCR method to become a routine diagnostic tool for quarantine laboratories.

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