Environmental and Experimental Biology (2018) 16: 307–314
DOI: 10.22364/eeb.16.22
Original Paper

Genetic differentiation of *Phoma* sp. isolates using retrotransposon-based iPBS assays

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Abstract

Phoma blight is a disease affecting Norway spruce, Scots pine and other conifer seedlings in many forest tree nurseries throughout the world. Members of the *Phoma* genus, the causatives of this disease, are difficult to distinguish morphologically and genetically. In this study the use of a retrotransposon-based polymerase chain reaction approach using iPBS amplification for intra-species genetic discrimination between *Phoma* samples is described. Eight retrotransposon-based iPBS primers were used to genotype DNA from pure cultures of several *Phoma* species. The utilised markers were able to discriminate between *Phoma* species, but not all of them were able to differentiate all *Phoma* sp. isolates investigated. Belarusian samples were found to be distinct from the Latvian *Phoma* isolates. The Belorussian isolates were very similar to each other. A combination of three iPBS markers (2001, 2076 and 2242) enabled partial differentiation of the investigated Belarusian *Phoma* isolates.

Key words: genetic discrimination, inter primer binding site (iPBS) markers, *Phoma* sp.
Abbreviations: iPBS, inter primer binding site.

Introduction

Phoma blight is an infectious disease associated with several species of *Phoma* that affect several species of firs and pines and cause significant damage in tree nurseries in the USA (Srago et al. 1989). *Phoma eupyrena* is also associated with upper stem canker in Douglas fir (Hamm et al. 1989). *Phoma* blight causes considerable economic damage in Belarusian tree nurseries, affecting 5 to 15% of *Pinus sylvestris* and *Picea abies* seedlings (Siaredzich 2017). *Phoma* spp. was found to be the most common pathogen of *P. sylvestris, Larix sibirica, P. abies, Pinus sibirica* and *Abies sibirica* in Russian forest nurseries of the Novosibirsk region (Larionova et al. 2017). In contrast, in Latvian coniferous forest tree nurseries *Phoma* sp. is not considered a threat or is successfully managed. This is inferred from the absence of *Phoma* sp. related disease outbreaks in conifer tree nurseries in Latvia (Brūna, unpublished results). A study about root-associated fungi in healthy-looking *P. sylvestris* and *P. abies* seedlings in Swedish forest nurseries showed members of *Phoma* genus as commonly present on roots of healthy-looking samples not excluding a possibility of latent infection that could activate after outplanting (Stenström et al. 2014). However, representatives of *Phoma herbarum, Phoma glomerata* and *Phoma adonidicola* as well as an unidentified *Phoma* sp. were isolated by members of the Latvian State Forest Research Institute "Silava" phytopathology and mycology department from *Betula pendula* samples collected in local tree nurseries (Brūna, unpublished results), and members of the *Phoma* genus have also been isolated from grey alder and Norway spruce samples taken from Latvian forest ecosystems, (Arhipova et al. 2011a; Arhipova et al. 2011b). Interestingly, *P. herbarum* has been described as potentially beneficial for plant growth (Muhammad et al. 2009), including in Scots pine (Sanz-Ros et al. 2015), and has also been used as a biological control agent against *Taraxacum officinale* (Neumann Brebaum 1998). *P. glomerata* has been described as having mycoparasitic properties (Sullivan, White 2000), as an endophyte (Deng et al. 2011), as a pathogen causing boxwood tip blight (Horst 2001), cankers of peach trees (Thomidis et al. 2011) and, according to the American Phytopathological Society, phoma canker in elm (https://www.apsnet.org/publications/commonnames/Pages/Elm.aspx). *Phoma macrostoma* var. incolorata has been reported to inhibit the growth of the ash pathogen *Hymenoscyphus fraxineus* (Haňáčková et al. 2017). These reports show that *Phoma* species can play vastly different roles in different conditions and host species.

Phoma species are difficult to identify due to the within-species variation of morphological features when cultivated *in vitro* (Aveskamp et al. 2008). The available information about the genetics of *Phoma* is increasing. The genome of a *Phoma* member called *Phoma sp 1* has been sequenced by the Forest Institute of the National Academy of Sciences of Belarus (Baranov et al. 2015). The genotype has not been definitely assigned to a species, and whole genome shotgun sequences of the *P. herbarum* strain JCM 15942 have been made available by Manabe et al. from RIKEN Center for Life Science Technologies, Japan (NCBI SRA database accession numbers DRX033246 & DRX029297). Another sequencing project involving *Phoma tracheiphila*, a citrus pathogen, is under way in U.S. Department of Energy Joint Genome Institute (NCBI SRA database accession numbers SRX1728765, SRX1728766 and SRX1728771). Presently the identification of *Phoma* species as well as discrimination between isolates and species is still difficult and time consuming. This is because the DNA regions used for species differentiation show low sequence polymorphism, and therefore several DNA regions have to be analysed. One of the most detailed reports of the genetic discrimination of taxa of the *Phoma* genus used sequencing of three different loci: the ITS1-5.8S-ITS2 region (ITS) of the nuclear ribosomal DNA operon, part of the actin gene, and part of the β-tubulin gene (Aveskamp et al. 2009). Additional use of the RNA polymerase II second largest subunit (*rpb*2) was employed by Chen et al. (2015b) to increase resolution. Translation elongation factor 1 subunit (*tef*1) has also been used for phylogenetic studies of *Phoma* (Irinyi et al. 2007). A short yet comprehensive review regarding identity determination of *Phoma* by multiple approaches, including additional DNA markers, is provided by Rai et al. (2014). Use of large numbers of samples both for pathogen screening in nurseries and for population genetics studies is time-consuming and expensive.

The iPBS method (Kalendar et al. 2010) might serve as a tool for differentiation between *Phoma* sp. isolates. This method relies on the non-uniform distribution of retrotransposon elements in the genomes of different isolates and species and allows for greater discriminatory power. This procedure is cost-effective, less time-consuming and allows differentiation between isolates of the same or different species. In addition to providing information on genetic diversity, retrotransposons can be used for identification of a certain pathogen if sufficient genetic information is available (Fernandez et al. 1998), differentiation between isolates (Pasquali et al. 2007) and have also been shown to influence pathogenicity of plant pathogens (Mouyna et al. 1996) and plant resistance against them (McDowell, Meyers 2013). The aim of the study was to utilise iPBS markers to investigate the genetic diversity of *Phoma* sp. isolates collected in several Belarusian forest nurseries, and to compare the Belarusian samples with Phoma samples isolated from Latvian forests. Sequencing of the intergenic transcribed spacer region of ribosomal RNA genes was also performed for the Belarusian samples to obtain additional data for phylogenetic comparison to publicly available *Phoma* sp. sequences.

Materials and methods

Material

DNA from twelve pure cultures of *Phoma* sp., each obtained from a different forest tree nursery in Belarus and five Latvian *Phoma* isolates, obtained from trees of several species growing in Latvian forests was extracted for genetic analyses of these isolates. Sequences of 12 Latvian *Phoma* DNA samples (Z9B – Z300) previously obtained by N. Bruņeviča (unpublished data) were used in the analysis (Table 1). DNA isolation was carried out using the Genomic DNA purification kit (ThermoFisher Scientific) according to the manufacturer's protocol. According to morphological characteristics, the Belarusian samples were inferred to be *P. glomerata* or *P. macrostoma*, but the species could not be determined conclusively.

Sequencing analysis

DNA sequences of intergenic transcribed spacer region of ribosomal RNA genes were obtained from PCR amplicons

Table 3. *Phoma* isolates analysed in the present study

Isolate	Taxon	Origin
N04	*Phoma* sp.	Belarus
N04.1	*Phoma* sp.	Belarus
N06	*Phoma* sp.	Belarus
N07	*Phoma* sp.	Belarus
N10	*Phoma* sp.	Belarus
N12	*Phoma* sp.	Belarus
N13	*Phoma* sp.	Belarus
N14	*Phoma* sp.	Belarus
N16	*Phoma* sp.	Belarus
N17	*Phoma* sp.	Belarus
N19	*Phoma* sp.	Belarus
N20	*Phoma* sp.	Belarus
LV07	*Phoma glomerata*	Latvia
LV07v	*Phoma* sp.	Latvia
LV08k	*Phoma herbarum*	Latvia
LV09v	*Phoma herbarum*	Latvia
LV249	*Phoma herbarum*	Latvia
Z9B	*Phoma herbarum*	Latvia
Z18	*Phoma adonidicola*	Latvia
Z47	*Phoma herbarum*	Latvia
Z78	*Phoma* sp.	Latvia
Z94	*Phoma* sp.	Latvia
Z130	*Phoma* sp.	Latvia
Z158	*Phoma herbarum*	Latvia
Z163	*Phoma herbarum*	Latvia
Z178	*Phoma* sp.	Latvia
Z215	*Phoma glomerata*	Latvia
Z268	*Phoma herbarum*	Latvia
Z300	*Phoma glomerata*	Latvia

obtained with primers ITS1-F and ITS4-B (Gardes, Bruns 1993) from the Belarusian samples. Sanger sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) according to the manufacturer's protocol.

Retrotransposon-based PCR assays (iPBS)

Primers 2001, 2009, 2010, 2076, 2081, 2083, 2097, 2220, 2239, 2242, 2380 and 2384 (Kalendar et al. 2010) were used in PCR reactions of the following composition (total reaction volume 20 µL): 5x HOT FIREPol® Blend Master Mix Ready to Load with 10 mM $MgCl_2$ (Solis BioDyne) 4 µL, final primer concentration 2 µM, 10 ng of DNA. Thermal cycling was performed as follows: 95 °C 15 min initial denaturation followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 40 s and elongation at 72 °C for 3 min. The cycling program ended with final extension at 72 °C for 10 min. PCR products were analysed on 2% agarose gel, 1 × TAE buffer, and visualised by ethidium bromide staining. iPBS analysis was performed for the seven Belarusian samples.

Data analysis

The iPBS amplification results were encoded as binary data. Genetic distances were calculated using GenAlex 6.5 (Peakall, Smouse 2012) and phylogenetic trees created using the MEGA software (Kumar et al. 2018) by use of the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm (Nei, Kumar 2000). Dendrograms were also created from trimmed sequences of intergenic transcribed spacer (ITS) region of ribosomal RNA genes using the MEGA software. The graphical comparison of sequences of ITS regions of Belarusian isolates were prepared with the AlignX module of the Vector NTI software (Thermo Fisher Scientific).

Results

The ITS sequences obtained from pure cultures of Belarusian *Phoma* samples were highly similar and matched closely (99% nucleotide sequence similarity) to sequences from unidentified *Phoma* species, *P. glomerata, Phoma pomorum, P. macrostoma* and other species in the NCBI database. The closest similarity was determined to be to *Phoma* sp. isolate 701 AI-2013, NCBI GenBank sequence accession number KC662226, from Minnesota, USA (Impullitti, Malvick 2013). There was no exact match to a database accession sequence. The only difference between the obtained sequences was that samples 19 and 20 harboured a SNP mutation not present in the other samples (nt 460 T → A) (Appendix 1). The observed genetic polymorphism was low and exact species identification or discrimination between isolates was not possible. Thus sequencing analysis suggested that the sequences belonged to a single taxon.

The utilised iPBS method identified a higher level of polymorphism. Eight informative iPBS primers were utilised for genotyping of the Latvian and Belarussian *Phoma* isolates (2001, 2009, 2010, 2076, 2081, 2083, 2097 and 2220).

The Belarusian *Phoma* isolates showed obvious differences from Latvian *P. glomerata* and *P. herbarum* isolates and probably represent a different *Phoma* species (Fig. 1). The Belarusian *Phoma* isolates were very similar to each other, with only a low level of genetic diversity detected. However, three different genotypes with primer 2079 and three with primer 2001 (ignoring fainter bands) were identified within the Belarusian *Phoma* isolates (Fig. 2). Two genotypes within the Belarusian samples were identified with primer 2242 (faint bands in the bottom

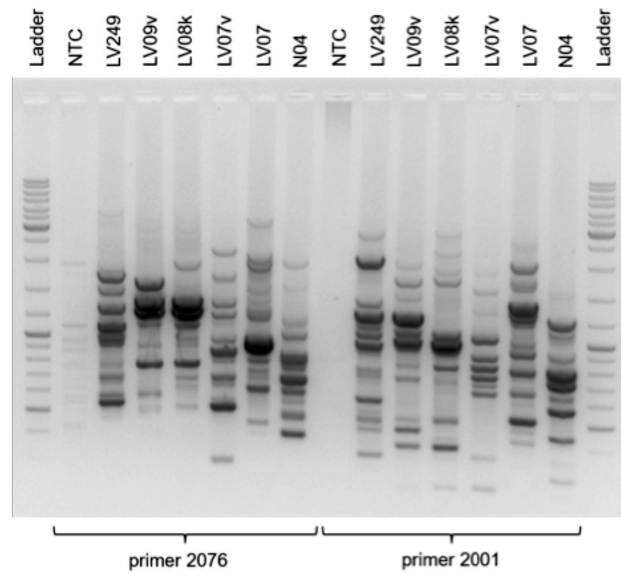

Fig. 1. Comparison of Belarusian *Phoma* sp. isolate (N04) to Latvian *P. glomerata* and *P. herbarum* isolates, electrophoresis results of iPBS assays with primers 2001 and 2076.

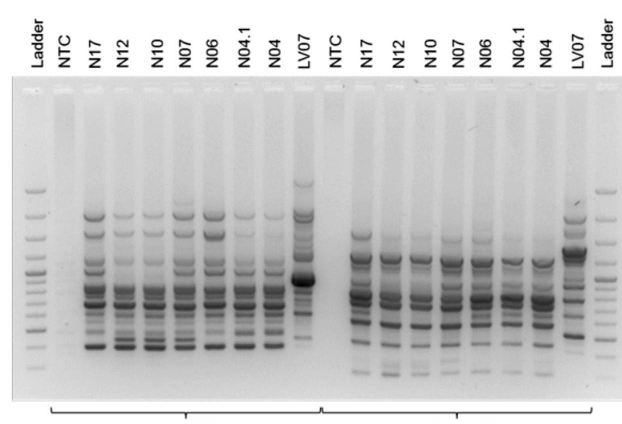

Fig. 2. Comparison of Belarusian *Phoma* sp. isolates among themselves and to a Latvian *P. glomerata* sample (LV07), electrophoresis results of iPBS assays with primers 2001 and 2076.

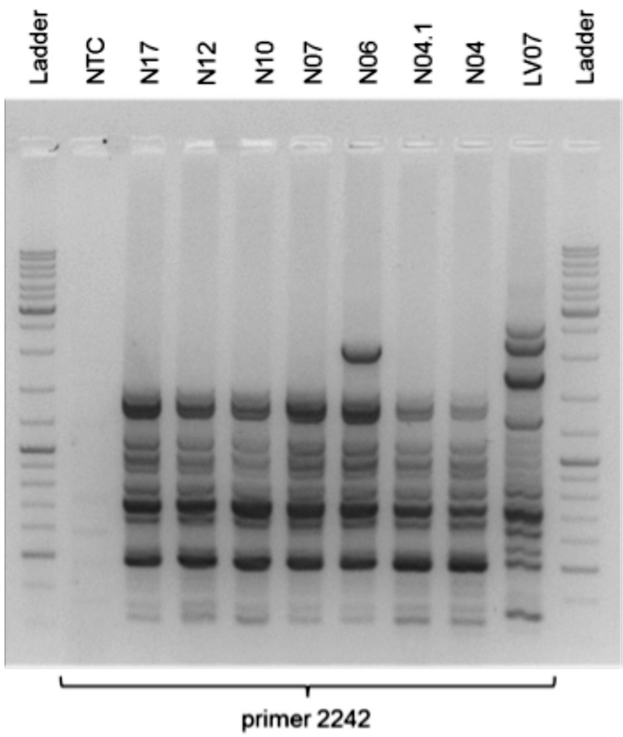

Fig. 3. Comparison of Belarusian *Phoma* sp. isolates among themselves and to a Latvian *P. glomerata* sample (LV07), electrophoresis results of iPBS assays with primer 2242.

of the gel were not considered) (Fig. 3). Pairwise genetic distances between the Latvian and Belarusian samples were calculated based on the presence or absence of 50 amplified fragments from two iPBS assays (primers 2001 and 2076), and a UPGMA phylogenetic tree was constructed (Fig. 4). The use of an additional iPBS assay (2242) in conjunction with the previous assays (2001 and 2076), allowed one of the previously undifferentiated Belarusian samples to be uniquely genotyped (isolate N6; Fig. 5). However, it was not possible to differentiate three isolates (N4, N4.1, N17).

Comparison of the ITS sequences of the Belarusian samples, the Belarusian sample with full genome information (Phoma sp1), Latvian samples (representing *P. glomerata, Phoma adonidicola* and *P. herbarum*), sequences published by Aveskamp et al. (2009) (*P. glomerata*) and other sequences from the NCBI database (*P. macrostoma*), revealed that the Belarusian samples most likely represent *P. macrostoma* or *P. glomerata* (Fig. 6, Appendix 2), which is in agreement with the morphological characteristics of these samples.

Discussion

Low genetic polymorphism in the ITS sequence analysis was expected, as previous studies on the *Phoma* genus indicated the necessity of additional DNA analyses, besides the ITS region analysis for better discrimination between species of the *Phoma* genus (Aveskamp et al. 2009; Chen et al. 2015b). Analysis of multiple conserved DNA regions can be time consuming and expensive. In contrast, genotyping using iPBS markers does not provide direct information about the sequences of produced amplicons without further investigation, but employs a simple PCR reaction followed by electrophoresis, which can be achieved quickly and at reduced cost. The nature of this method, employing the non-uniform distribution of retrotransposon elements in the genomes of different isolates and species, allows for greater discriminatory power. The number and affiliation of long terminal repeat transposable elements varies between fungal species (Muszewska et al. 2011) and isolates of the same species (Özer et al. 2016; Özer et al. 2017). The disadvantages of this method are similar to those of randomly amplified polymorphic DNA (RAPD) analysis, including the necessity for strict standardisation (Kumari, Thakur 2014) and problems associated with non-template specific PCR amplification products (Lamboy 1994), which were also observed for some of the markers utilised in this study. However, while issues of reproducibility and fragment size homoplasy need to be considered, genotyping with iPBS markers is more sensitive and accurate compared to RAPD markers (Poczai et al. 2013). The utilised iPBS primers were

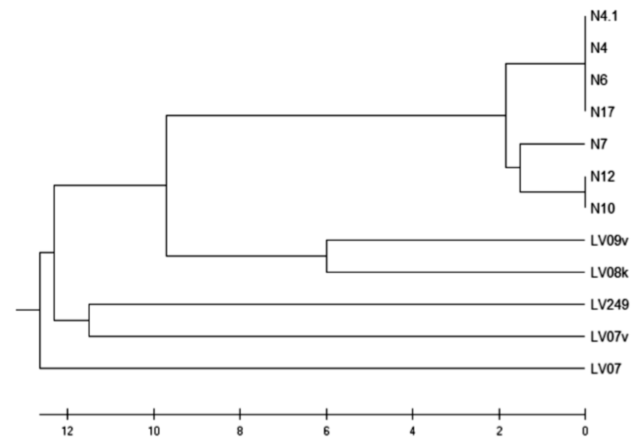

Fig.4. UPGMA dendrogram based on pairwise genetic distances between Latvian and Belarusian *Phoma* samples based on iPBS assays with primers 2001 and 2076.

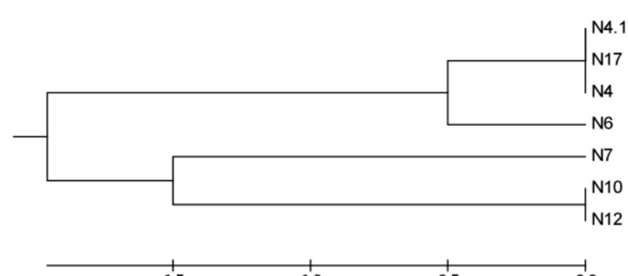

Fig.5. UPGMA dendrogram based on pairwise genetic distances between Belarusian *Phoma* samples based on iPBS assays with primers 2001, 2076 and 2242.

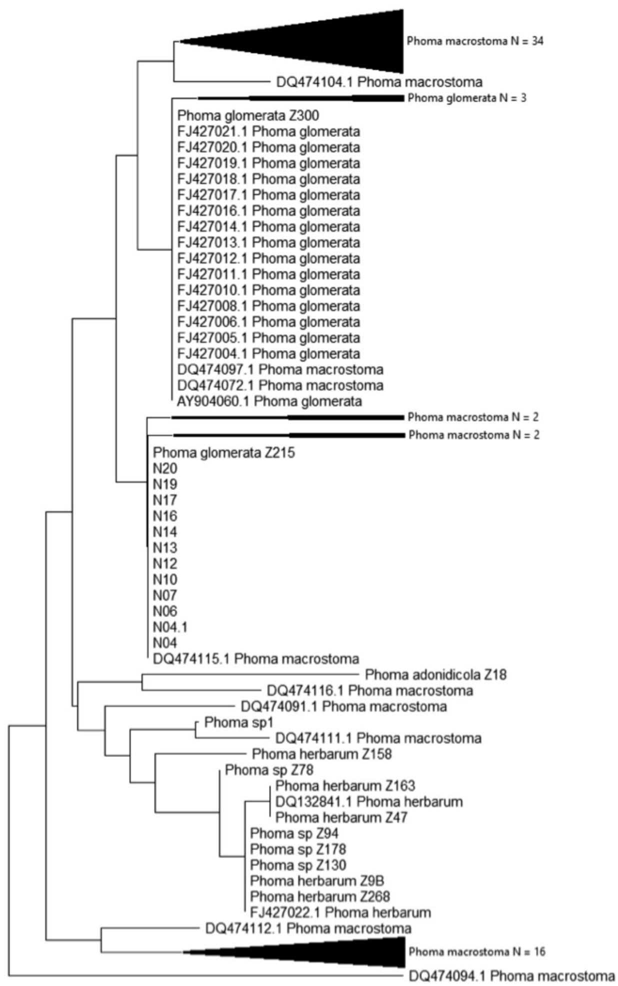

Fig.6. Phylogenetic relationship of analysed *Phoma* isolates and NCBI database accessions based on ITS sequencing.

able to partly differentiate between the analysed *Phoma* species (sample LV07 was morphologically determined to represent *P. glomerata* and samples LV08k, LV09v and LV249 represented *P. herbarum*). However, some of the Belarussian isolates were not able to be distinguished. The use of iPBS has been previously reported to be more informative than ITS sequence comparison for other fungal pathogens (Pourmahdi, Taheri 2015).

The Belarusian isolates had a high degree of similarity between each other, and in some cases, isolates were not distinguished from each other with the utilised marker set. Each isolate was obtained from a different forest nursery. Therefore there are two possible explanations for the apparent genetic uniformity of these isolates. The first possibility is that the markers utilised were not sufficiently informative to distinguish these isolates, and therefore, the use of additional markers may identify additional genetic polymorphism. However, the Latvian *Phoma* isolates, which were obtained from natural forest environments were readily distinguished with the utilised markers, indicating that these markers are sufficiently informative. The second possibility is that there was some transfer of these Phoma isolates between forest nurseries. However, as far as could be determined, the potting soil utilised in all the nurseries was not obtained from one source, and no other possible transfer vectors were identified. One common factor between nurseries was the origin of the seeds, which were obtained from common seed orchards. Further investigation of the genetic diversity of the Belarusian isolates and possible transfer routes is required to resolve this question.

Retrotransposon-based PCR assays can be successfully used for differentiation between different *Phoma* isolates that are morphologically and genetically (based ITS sequence comparison) identical. Higher genetic diversity between *Phoma* isolates from Belarus were detected by iPBS (four genotypes) than by sequencing analysis of intergenic transcribed spacer region of ribosomal RNA genes (two genotypes). The sequence based phylogenetic comparison identified clusters matching different taxonomic groups (Fig. 6 and Appendix 2) and the Belarusian isolates were grouped together indicating their distinctiveness from other isolates (and correct morphological characterisation). iPBS assays identified large genetic differences compared to the Latvian sample LV07, which was morphologically characterised as *P. glomerata*. This suggests that the Belarusian samples that were morphologically determined to be either *P. glomerata* or *P. macrostoma* are in fact *P. macrostoma*. Unfortunately, a Latvian *P. macrostoma* isolate was not available in order to compare it to the Belarusian isolates. The use of iPBS markers represents an efficient method to investigate the intra-specific diversity of fungal isolates, and can be used to characterise disease outbreaks in forest nurseries as well as the genetic diversity of natural fungal populations.

Acknowledgements

M. Siaredzich would like to thank her scientific supervisor Vasily A. Yarmalovich for his suggestions, help and support.

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Received 10 December 2018; received in revised form 18 December 2018; accepted 20 December 2018

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                                                                                     Section 1
        (1) 1         10          20          30          40          50          60          70               89
    N14 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N13 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N06 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N12 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N04 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N07 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N17 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N19 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N20 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N10 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
  N04.1 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
    N16 (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
Consensus (1) GTGACTGCGGAGGACATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGT
                                                                                     Section 2
       (90) 90          100         110         120         130         140         150         160            178
   N14 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N13 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N06 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N12 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N04 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N07 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N17 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N19 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N20 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N10 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
 N04.1 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
   N16 (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC
Consensus (90) CCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTC

                                                                                     Section 3
      (179) 179         190         200         210         220         230         240         250            267
  N14 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N13 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N06 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N12 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N04 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N07 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N17 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N19 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N20 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N10 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
N04.1 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
  N16 (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
Consensus (179) TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
                                                                                     Section 4
      (268) 268         280         290         300         310         320         330         340            356
  N14 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N13 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N06 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N12 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N04 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N07 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N17 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N19 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N20 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N10 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
N04.1 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
  N16 (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC
Consensus (268) ATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCC

                                                                                     Section 5
      (357) 357         370         380         390         400         410         420         430            445
  N14 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N13 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N06 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N12 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N04 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N07 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N17 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N19 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N20 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N10 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
N04.1 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
  N16 (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
Consensus (357) TCTGCGCGTAGACTCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACG
                                                                                     Section 6
      (446) 446           460         470         480         490         500         513
  N14 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N13 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N06 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N12 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N04 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N07 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N17 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N19 (446) ACGTCCAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N20 (446) ACGTCCAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N10 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
N04.1 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
  N16 (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
Consensus (446) ACGTCCAAAAGTACTTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT
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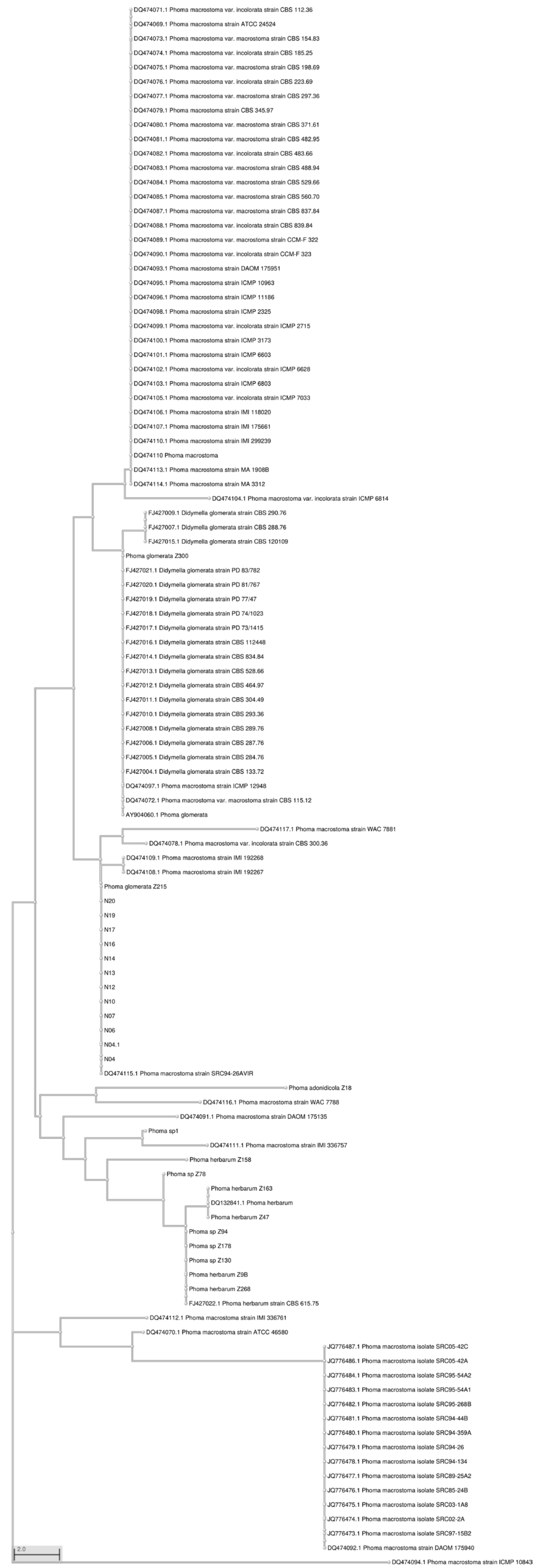
Appendix 1. Alignment of ITS sequences obtained with primers ITS1-F and ITS4-B from the Belarusian samples.



Appendix 2. Expanded view of the phylogenetic tree in Fig. 6.