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Detection of *Chalara fraxinea* from tissue of *Fraxinus excelsior* using species-specific ITS primers

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Summary

Chalara fraxinea (teleomorph: *Hymenoscyphus albidus*) is known as a serious pathogen of *Fraxinus excelsior*, causing massive dieback of trees in Europe. The fungus is able to cause latent infections, and has been previously detected as an endophyte in asymptomatic tissues. *Chalara fraxinea* is a slow grower in culture, and is thus likely to be overgrown by faster growing fungi whenever pure culture isolations are being attempted. This study reports specific ITS primers allowing fast and reliable detection of the pathogen directly from infected tissues of *F. excelsior*.

1 Introduction

Starting in mid-1990s until present, massive dieback of *Fraxinus excelsior* has been observed in mid-eastern Europe, which later spread westwards and is currently threatening the host tree species on a continental scale (BAKYS et al. 2009a; and references therein). Regular isolation from declining *F. excelsior*, and subsequent artificial inoculation tests provided evidence that the ascomycete anamorph *Chalara fraxinea* T. Kowalski (KOWALSKI 2006) is the causal agent of the disease (BAKYS et al. 2009a,b; KOWALSKI and HOLDENRIEDER 2009a). More recent study demonstrated that teleomorph of *C. fraxinea* is the widespread discomycete *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips (KOWALSKI and HOLDENRIEDER 2009b).

The crowns of declining *F. excelsior* trees exhibit a wide range of symptoms: (i) wilting and premature shedding of leaves; (ii) necroses of leaves, buds, leaf stalks and bark; (iii) top and shoot dieback; (iv) cankers on shoots, branches and stems (BAKYS et al. 2009a; and references therein). This indicates that the fungus is capable to colonize a wide spectrum of different plant substrates and tissues. Moreover, *C. fraxinea* has often been detected in symptomless tissue (petioles) of *F. excelsior*, revealing latent infections (BAKYS et al. 2009b). Yet, in some studies only sporadic and occasional isolation of *C. fraxinea* was reported. As a slow grower in culture, the fungus could have been overgrown by other species during isolation (KIRISITS and HALMSCHLAGER 2008; BAKYS et al. 2009a).

To overcome this, one needs to elaborate a rapid and reliable method for detection of the pathogen directly from plant material. Such detection would be of a high value to phytosanitary authorities, nurseries, practical forestry and horticulture, and research, e.g. when searching for possible latent life stages of the fungus or alternative hosts. Detection of plant pathogens directly from infected tissues by PCR using species-specific primers is now commonly used for diagnostic purposes. In forest pathology, the technique has been successfully employed to identify ascomycetous pathogens in various tree organs, e.g. foliage, bark and wood (e.g. SMITH and STANOSZ 2008). The aim of this study was to develop species-specific ITS primers of *C. fraxinea*, thus providing PCR-based tool for detection of the pathogen directly from infected tissues of *F. excelsior*.

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2 Materials and methods

Based on the ITS sequence of *C. fraxinea* obtained by BAKYS et al. (2009b), one pair of molecular primers was developed: forward 5'-AGC TGG GGA AAC CTG ACT G-3', situated in an intron in the 18s gene, and reverse 5'-ACA CCG CAA GGA CCC TAT C-3' located in the ITS region (Fig. 1). In general, this study followed methods described by STENSTRÖM and IHRMARK (2005).

First, the specificity has been assessed for each primer separately. Two separate sets of PCR amplifications were carried out, first using the designed forward primer in combination with the universal reverse primer ITS4, and then using the reverse primer in combination with the universal forward primer ITS1F. The PCR amplifications were run with DNA of *C. fraxinea*, *H. caudatus* and *H. fructigenus*. Furthermore, combined specificity of both designed primers for *C. fraxinea* was assessed by PCR using DNA of 55 fungal taxa (FJ228162–FJ228217) isolated from visually healthy *F. excelsior* shoots, and from shoots exhibiting different dieback symptoms (BAKYS et al. 2009a).

For validation tests, DNA was extracted from mycelia of C. fraxinea and from symptomatic bark samples of shoots. Mycelia (1-2 g) grown in liquid malt culture (10 g maltextract/l) were freeze dried for 48 h and homogenized with fast prep Precellys™ control device (Bertin Technologies, Montigny-le-Bretonneux, France). Microcentrifuge tubes, 2 ml, with 2.5 mm glass beads were used. CTAB 3% cetyl trimetyl ammonium bromide (containing 0.15 м Tris-HCl, 2.6 м NaCl and 2 mм EDTA; pH 8.0) were added to remove membrane lipids. Chloroform (500 μ l) was used to clean the DNA further, and 2-propanol was used to precipitate the DNA into a pellet. The pellet was dissolved in 100 μ l milliQ water. Previous to DNA extractions, 20 bark samples from 20 shoots were surface sterilised using 70% ethanol and deionised water. Outer bark was peeled off from the necrotic areas. Then, the inner bark was cut out in pieces of about 5 mm² in size, freeze dried in 48 h and homogenized using screw nut (M3) and screw (M4×10) in fast prep Precellys[™] control device. Afterwards, the same protocol as for mycelia DNA extraction was followed. To evaluate PCR efficiency, isolations of C. fraxinea had been attempted from the same samples of necrotic bark as those subjected to DNA extraction. Isolation followed the protocol by KOWALSKI (2006).

Validation tests were done with the designed primers against 20 extracts from symptomatic bark, simultaneously checking DNA of 78 *C. fraxinea* isolates from Sweden (34), Finland (3), Lithuania (12), Denmark (22), Germany (1), Poland (4), Austria (1) and Czechia (1). PCR reactions contained 10× PCR buffer Y, 0.2 mM dNTPs, 0.75 mM MgCl₂, 0.3 U ThermoRed Taq polymerase, 0.02 μ M of each primer, and 0.25 ng/ μ l DNA if nothing else is mentioned. PCR PC-960G gradient thermal cycler, TECTHUM lab was used. The reaction started with 5 min of 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min. The reaction was finished by an extension step at 72°C for 8 min. The PCR-products were separated by gel electrophoresis on 1% agarose gel (agarose D1,

		←Intron	18s gene	ITS $1 \rightarrow$
C. fraxinea:	5'-CAGCTGGGGGAAACCTGACTGCTA	AGCGCGTTCCGTAGGT	GAACCTGCGGAAGGA	TCATTACAG-3'
H. scutula:	5'	TTCCGTAGGT	GAACCTGCGGAAGGA	TCATTACAG-3'
	ITS 2			
C. fraxinea:	5'-CTTCTCGCGATAGGGTCCTTGCG	GTGTCTTGCCAGC-3'		
H. scutula:	5'-CTTCTCGCGATAGGGTCCTCGTC	GTGACTTGCCAGC-3'		
H. caudatus:	5'-CACCCCGCGATAGAACCCTTAG	GGTATCTTGCCAGC-3'		
H. fructigenus:	5'-CTCCTCGCGATAGATGTTTTAG	GGTGTCTTGCCAGC-3'		

Fig. 1. Forward and reverse sequences of primers specific for *Chalara fraxinea* (underlined) aligned with the respective sequences of *Hymenoscyphus* spp. Nucleotide differences among species are shown in bold.

Conda, Madrid, Spain) in SB buffer (50 mm natriumtetraborat) (4 V cm⁻¹) for 1 h. As size standard GeneRuler mix (Fermentas, Burlington, Ontario, Canada) was used. The gel was stained with ethidium bromide and visualized under UV light. Due to possible problems of PCR inhibition from tree bark compounds (e.g. LANGRELL 2002), possible effects from *F. excelsior* bark extracts were evaluated. PCR reactions with 0.5 and 0.05 ng/ μ l DNA from asymptomatic bark combined with pure culture of *C. fraxinea* DNA were run with the developed primers in three different concentrations of 0.25, 0.025 and 0.0025 ng/ μ l. Then, all respective fungal DNA concentrations were run without *F. excelsior* DNA, and 0.5 ng/ μ l *F. excelsior* DNA was tested without fungal DNA.

3 Results and discussion

Analysis of C. fraxinea ITS sequences produced in our laboratory has revealed significant differences in size, depending on whether the forward primer ITS1 or the primer ITS1F (BAKYS et al. 2009a,b) was used. Although the primer ITS1F is normally aligned only 16 bp backwards in 18s small subunit rRNA gene than ITS1, for C. fraxinea it produced a sequence several hundred bp larger than using ITS1. For example, the sequences AY787704 (LYGIS et al. 2005) and FJ228176 (BAKYS et al. 2009a), yielded using ITS1, were 463 and 487 bp in size, but the sequence EU852352 (BAKYS et al. 2009b) yielded by ITS1F contained 833 bp. When another three C. fraxinea isolates were sequenced using ITS1F, those also yielded long sequences of 746 bp (GenBank access numbers: GQ181019, GQ181020, GQ181021). Moreover, all four ITS1F-generated sequences shared 100% identity, showing no intraspecific polymorphism within the amplified region. When they were aligned (DNAStar, Madison, WI, USA) with the corresponding (ITS1F-generated) sequence of the closest relative Hymenoscyphus scutula (AY789432), the comparison revealed absence of the intron in the latter species. This indicated that: (i) 18s small subunit rRNA gene of *C. fraxinea* contains intron of several hundred bp, (ii) there is no variation neither in that intron, nor in the rest of ITS, and (iii) the intron is absent in related species. Thus, a sequence from that particular intron was chosen for tests as the forward primer specific to C. fraxinea. The reverse primer was designed based on the sites within the ITS region that differed most between C. fraxinea and three of its closest relatives: H. scutula (AY789432), H. caudatus (AY348576) and H. fructigenus (DQ431176) (Fig. 1).

The substitution of specific-specific primers with universal ITS primers 1F or 4 resulted in PCR products from other tested isolates, while the developed species-specific primers only gave product from DNA samples of *C. fraxinea*. The substitution of primers with universal ITS primers therefore showed that none of our primers can be substituted without loss of specificity. The length of their amplified DNA fragment was 456 bp.

Specificity tests using a pair of designed primers yielded a strong species-specific band when run against DNA samples of all 78 *C. fraxinea* isolates tested. DNA concentrations as low as 0.0025 μ l always gave clearly visible band. No band was observed in tests with two *Hymenoscyphus* spp. Yet, DNA from 34 of the 55 species isolated from declining *F. excelsior* by BAKYS et al. (2009a) gave a weak variable band of a bigger fragment size than *C. fraxinea* (Fig. 2). Those bands cannot be explained by comparison of ITS and 18s gene intron sequences, but could involve some other region of the genome. It could be therefore recommended to use a positive control from a pure culture isolate of *C. fraxinea* when using developed primers for PCR amplification.

In validation tests, DNA extracted from the twenty samples of diseased bark responded positively to the primers, with a band size identical with pure isolate DNA sample of *C. fraxinea* (Fig. 2). Attempted isolation of the pathogen from adjacent bark tissues yielded isolates of *C. fraxinea* in 14 out of 20 cases. Therefore, our primers showed by 30%



Fig. 2. Amplification products generated by the developed primers from symptomatic bark of Fraxinus excelsior (bands 1-5), mycelium of Chalara fraxinea (6, 10 and 13), Cladosporium tennissimum (7), Apiospora montagnei (8), Botryotinia fuckeliana (9), Phoma herbarum (11), Fusarium lateritium (12) and negative control (14).



Fig. 3. Amplification products generated by the developed primers from DNA mixtures of *Chalara* fraxinea (isolate 1 in bands 1–9, and isolate 2 in bands 10–18) and *Fraxinus excelsior* (extracts from asymptomatic bark). Concentration of *C. fraxinea* DNA in wells 1–3 and 10–12 is 0.25 ng/ μ l, in wells 4–6 and 13–5 is 0.025 ng/ μ l, and in wells 7–9 and 16–18, 0.0025 ng/ μ l. Respective concentrations of *F. excelsior* bark DNA in each of these groups are 0.5 ng/ μ l, 0.05 ng/ μ l and 0. Well 19 is a negative control and well 20 contains 0.5 ng/ μ l DNA only from *F. excelsior* bark extract.

higher sensitivity in detecting the pathogen than pure culture isolations. Bark extracts had no inhabiting effect on PCR reactions. Adding up to 1 ng/ μ l DNA from asymptomatic bark to DNA from *C. fraxinea* always resulted in similar bands as mycelial DNA samples (Fig. 3). The use of species-specific primers designed in this study has thereby proved to be a reliable method in detection infections of *C. fraxinea* directly from tissue of *F. excelsior*. Moreover, as the method is able to detect latent infections of the pathogen, this provides valuable tool for future studies on the epidemiology of *C. fraxinea*.

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