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Detection of *Chalara fraxinea* in common ash (*Fraxinus excelsior*) using real time PCR

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Summary

A real time PCR assay was developed for the detection of *Chalara fraxinea* in common ash. PCR primers and Taqman probes, based on the internal transcribed spacer region of the multi-copy gene rDNA, were tested for specificity and sensitivity. The primers amplified an 81 bp fragment for *C. fraxinea* but did not amplify DNA from other *Chalara* species or from other fungi isolated from ash, whether pathogenic or saprophytic. The limit of detection was 5 pg of genomic DNA per PCR. Moreover, naturally-infected samples were correctly diagnosed. A procedure for DNA extraction from woody tissues using an electric drill yielded DNA of an appropriate quality for real time PCR. This molecular method could be useful for routine analysis of this emergent pathogen and for epidemiological studies.

1 Introduction

A few years ago, a new fungus was reported as the cause of *Fraxinus excelsior* dieback in Poland. It was described as a new species *Chalara fraxinea* T. Kowalski sp. nov. (Kowalski 2006). Its teleomorph was identified as *Hymenoscyphus albidus*, a fungal species widespread in Europe (Kowalski and Holdenrieder 2009b). The pathogenicity of this fungus was demonstrated recently (Kowalski and Holdenrieder 2009a). Both young plants and adult trees are killed, irrespective of location and regeneration methods. The pathogen induces wilting, leaf necrosis, premature leaf-shedding, stem necrosis and wood discoloration leading to decline of the trees (Chandelier 2008; Kowalski and Holdenrieder 2008). Since its first identification, this emerging disease has increasingly been observed on common ash in other European countries, notably in Germany (Schumacher et al. 2007), Sweden (Bakys et al. 2009a,b), Denmark (Thomsen et al. 2007), Austria (Cech 2006; Halmschlager and Kirisits 2008), Norway (Talgø et al. 2008), Czech Republic (Jankovsky et al. 2008), Hungary (Szabó 2008), Lithuania (Lygis et al. 2005) and Slovenia (Ogris et al. 2009). In 2008, the fungus was also reported in France (B. Marçais, personal communication). Due to suspicion that *C. fraxinea* is an alien organism and because of its potentially economic and ecological impacts, the European Plant Protection Organization has put this pathogen on its pathogen alert list (EPPO 2007).

Currently, the detection of the pathogen rests on its isolation on malt extract agar (Kowalski 2006). However, the probability of detection is unpredictable with some types of tissues, and gives rise to false negative results due to the slow growth of the pathogen (Bakys et al. 2009a). Furthermore, the production of phialophores and conidia takes several weeks making time-consuming the identification at the species level (Kowalski 2006). A molecular method (PCR and T-RFLP) has already been proposed for the detection of *C. fraxinea* directly from plant tissues (Bakys et al. 2009a). However, this method is not adapted to high throughput analyses. In contrast, real time PCR has provided a valuable approach for rapid

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detection of plant pathogens (Atallah et al. 2007; Hayden et al. 2006; Van Gent-Pelzer et al. 2007). This method utilizes fluorescence to detect the presence of amplification products as the reaction occurs. It offers significant advantages over conventional PCR, notably in terms of sample handling. Moreover, as the reactions are conducted in a 'closed-tube' system, the risk of false positive results due to carry-over contamination is significantly reduced. For this reason, a real time PCR method for the detection of *C. fraxinea* from different plant tissues has been developed in a context of routine application.

2 Material and methods

2.1 Isolation of fungi and plant material

Field sampling was done in August–October 2008 on ash showing symptoms of crown dieback in Belgium (Wallonia, five trees in three sites) and in France (Département des Vosges, 10 trees in five sites). In both countries, branches with dieback symptoms (maximum diameter of branches = 1.5 cm) were collected and analysed within 48 h. Pieces of plant tissues (woody material without bark, bark or leaf pieces) were disinfected in 10 times diluted commercial bleach (20 s), rinsed three times in sterile water, cut into pieces of approximately 5 mm^2 in size and placed on 2% malt extract agar (MEA, Difco, Becton-Dickinson, Le Pont de Claix, France) supplemented with 100 mg/l streptomycin sulphate (Sigma-Aldrich GmbH, Steinhem, Germany). Petri dishes were incubated at room temperature in the dark for 4–5 weeks. Isolates were subcultured to 2% MEA. For long term storage, isolates were maintained on 2% MEA at 2–8°C under oil. Isolates which were collected from ash in the framework of this study (n = 16), reference *Chalara* species from the Central Bureau voor Schimmelcultures collection (The Netherlands) as well as other fungi used in this study are listed in Table 1.

Plant tissues (shoot and leaves) from healthy trees (Belgium) and from trees infected with *C. fraxinea* (France) were stored at −20°C for real time PCR assays.

2.2 DNA extraction

DNA was extracted from mycelium or plant material after grinding the tissues in liquid nitrogen. Sampled plant material was put into 1.5 ml centrifuge tubes with 250 μl lysis buffer (Tris–HCl pH8.0,100 mM; EDTA 20 mM; NaCl 1.4 M; CTAB 2%; PVP K-30 2%) and 40 μl Proteinase K (1 mg/ml). The tubes were placed for 2 h at 55°C with occasional swirling and then centrifuged for 2 min at 6000 *g*. The supernatant was transferred into a fresh tube and DNA extraction carried out with the High Pure Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. A clean up of DNA was performed by centrifugation of the DNA extract through a column containing polyvinylpyrrolidone (PVPP) suspension (10% in Tris–HCl 10 mM, pH 7.5). DNA samples were stored at −20°C. DNA concentration was estimated with a spectrophotometer at 260 nm. A grinding method for wood material was also tested. The inner part of wood displaying necrosis was sampled by drilling (diameter of drill bit = 3 mm) with a rechargeable cordless drill. The sawdust was placed into a 1.5 ml tube (~100 mg) and submitted to the DNA extraction without further grinding. The drill bit was rinsed in water, and soaked in 95% ethanol to avoid cross-contamination between samples (Jasalavich et al. 2000).

2.3 ITS sequencing

The ITS region was amplified by PCR with primers ITS1 and ITS4 (Table 2) as described by White et al. (1990) from DNA extracted from pure culture of all isolates collected from

Table 1. **List of fungal strains and their origin.**

Strain no.	Species	Original host	Year	Country	Collection (code)	C_t[1]
3838	*Alternaria alternata*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3814	*Chalara aurea*	na	1977	The Netherlands	CBS (63477)	>40
3816	*Chalara constricta*	Decaying wood	1975	Belgium	CBS (24876)	>40
3817	*Chalara fraxinea*	*Fraxinus excelsior*	2006	Poland	CBS (122507)	18.36
3819	*Chalara fraxinea*	*Fraxinus excelsior*	2006	Poland	CBS (122504)	17.22
3820	*Chalara fraxinea*	*Fraxinus excelsior*	2006	Poland	CBS (122506)	18.00
3834	*Chalara fraxinea*	*Fraxinus excelsior*	2008	France	CRAW	20.55
3835	*Chalara fraxinea*	*Fraxinus excelsior*	2008	France	CRAW	20.00
3836	*Chalara fraxinea*	*Fraxinus excelsior*	2008	France	CRAW	19.65
3815	*Chalara ovoida*	*Quercus petraea*	1987	Germany	CBS (13688)	>40
3840	*Diplodia* sp.	*Vitis vinifera*	2007	Belgium	CRAW	>40
3831	*Epicoccum nigrum*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3821	*Fusarium lateritium*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3832	*Fusarium lateritium*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3828	*Fusarium lateritium*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3839	*Penicillium* sp.	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3822	*Peniophora* sp.	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3833	*Peniophora* sp.	*Fraxinus excelsior*	2008	France	CRAW	39.29
2401	*Pestalotiopsis* sp.	*Picea abies*	2003	Belgium	CRAW	>40
3825	*Phoma exigua*	*Fraxinus excelsior*	2001	Belgium	CRAW	>40
3826	*Phoma exigua*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
2307	*Phoma exigua*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3829	*Phoma* sp.	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
3823	*Phytophthora citrophthora*	*Buxus*	2008	Belgium	CRAW	>40
3827	*Trichoderma viride*	*Fraxinus excelsior*	2008	Belgium	CRAW	>40
2188	*Verticillium dahliae*	*Fraxinus excelsior*	1999	Belgium	CRAW	>40

na, not available; CBS, Central Bureau voor Schimmelcultures, Fungal Biodiversity Centre – Utrecht (The Netherlands); CRAW, Walloon Agricultural Research Centre, Gembloux (Belgium) – our collection.
[1]C_t-values established at a threshold of 0.5 (real time PCR with probes Cf-F, Cf-R and Cf-S).

Table 2. **Sequence of primers and Taqman probe used in this study.**

Probe name	Type	Sequence
ITS-1[1]	Primer	5′-TCCGTAGGTGAACCTGCG G-3′
ITS-4[1]	Primer	5′-TCCTCCGCTTATTGATATGC-3′
Cf-F	Primer	5′-CCCTTGTGTATATTATATTGTTGCTTTAGC-3′
Cf-R	Primer	5′-GGGTCCTCTAGCAGGCACAGT-3′
Cf-S	Taqman probe	6-FAM – 5′-TCTGGGCGTCGGCCTCGG-3′ – BHQ-1

[1]White et al. 1990.

ash and listed in Table 1. Amplifications were performed in 25 μl reactions containing 2 μl DNA extract, 1 × Platinum *Taq* DNA polymerase buffer (Invitrogen, Carlsbad, California, USA), 1 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM primers and one unit of Platinum *Taq* DNA polymerase (Invitrogen). Amplification was carried out in a GeneAmp PCR System 9700. The amplification program for ITS1-ITS4 primers was as follows: initial denaturation step at 94°C for 3 min, and 40 cycles at 94°C for 30 s, 50°C for 40 s, 72°C for 1 min followed by final extension at 72°C for 10 min. The PCR products were sequenced with the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City,

California, USA) using the Genetic Analyser 3100 (Applied Biosystems) at the FYSA Unit, University of Louvain-La-Neuve (Belgium). The GenBank database (Benson et al. 2008) was used to define the identity of the sequences.

2.4 Primers design and real time PCR conditions

The ITS sequence from *C. fraxinea* (Genbank FJ228176) was compared to the ITS sequences from closely related species available in the Genbank/EMBL nucleotides databases, using the multiple sequence alignment program Clustalw2 (http://www.ebi.ac.uk/Tools/Clustalw2). Primers and probe were selected using the Primer Express software v1.0 from Applied Biosystems. The specificity of the designed primers was tested by searching for homologous sequences in the Genbank/EMBL nucleotide sequence databases using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the real time PCR assay, an aliquot of 5 μl of DNA was included in each 20 μl PCR reaction [1 × reaction buffer (qPCR Core Kit, Eurogentec, Liege, Belgium)]; 5 mM $MgCl_2$; 0.2 mM dNTPs; 0.25 μM each PCR primer (Cf-F and Cf-R; Invitrogen, Carlsbad, California, USA); 0.25 μM TaqMan probe (Cf-S; dual-labelled probe, 5′FAM/3′BHQ-1, Eurogentec) (Table 2) and 0.75 unit of HotGoldStar *Taq* DNA polymerase (qPCR Core Kit, Eurogentec). A test was also performed with the same primers and probe concentrations with a ready-to-use master mix (qPCR MasterMix, Eurogentec). Amplifications were done in an ABI Prism 7000 Sequence Detection System (Applied Biosystem) using the following conditions: 10 min at 95°C and 40 cycles of 95°C for 15 s, 59°C for 1 min. The rhodamine derivation Rox^{TM} present in the PCR buffer (passive reference dye) was used as a passive reference to normalize the signal. The threshold cycle (C_t) corresponds to the cycle at which fluorescent signal exceeds the background. C_t-values were estimated at a threshold value of 0.5 in all cases (baseline start and end cycles, 2–15). A negative water control was included with each PCR run. A DNA sample extracted from a pure culture of *C. fraxinea* (strain 3817, Table 1) was also used as a positive control to monitor the performance of the PCR. Statistical analyses (ANOVA) were performed with the Minitab^{TM} statistical software package (version 13.20, Minitab Company, Paris, France).

2.5 Real time PCR performance characteristics

The PCR efficiency was estimated by running PCR on serial dilutions of *C. fraxinea* total DNA (from 5000 pg to 0.5 pg) diluted in total DNA from healthy plant (spiking assay). The limit of detection corresponded to the smallest DNA concentration for which all the positive samples were detected, distinguishable from a zero result. In order to check for the absence of inhibitory effects in the PCR reaction, the same test was conducted in a purified reference DNA matrix (salmon sperm DNA, Promega, Madison, Wisconsin, USA), this DNA being used as blocking agent to avoid non-specific interaction of target DNA with the plastic PCR tubes.

3 Results

3.1 Fungal isolation

The fungi which were isolated from declining ash were *Alternaria alternata*, *Epicoccum nigrum*, *Fusarium lateritium*, *Peniophora* sp., *Phoma exigua*, *Phoma* sp. and *Trichoderma viride*. *Chalara fraxinea* was not isolated from trees sampled in Belgium. In contrast, the pathogen was isolated from three wood materials from two sites in France (Table 1).

3.2 Design of primers and test specificity

The size of the PCR fragment obtained with primers ITS1 and ITS4 from *C. fraxinea* (isolate 3817) was around 550 bp. The sequence was aligned with ITS sequences from other *C. fraxinea* strains and from closely related species available in the Genbank/EMBL databases. The primers Cf-F, Cf-R and the Taqman probe Cf-S were selected in polymorphic areas (Fig. 1). These probes amplified an 81 bp fragment for the six strains of *C. fraxinea* isolated from Poland (three strains) and France (three strains) (C_t-values < 25), but did not yield any signal from any other isolates used in this study (C_t-values > 39) (Table 1). A PCR with the primers ITS1-ITS4 carried out on all the isolates listed in Table 1 confirmed the quality of the DNA extracted for real time PCR (data not shown). A Genbank/EMBL search did not reveal any perfect matches to the primer and Taqman probe sequences except to *C. fraxinea* from Norway (accession number EU848544), Sweden (accession number EU852352) and Lithuania (accession number AY787704). The ITS-1 sequence of the isolate from Poland (isolate 3817, Table 1) perfectly matched the sequence of the *C. fraxinea* isolates from other countries (data not shown).

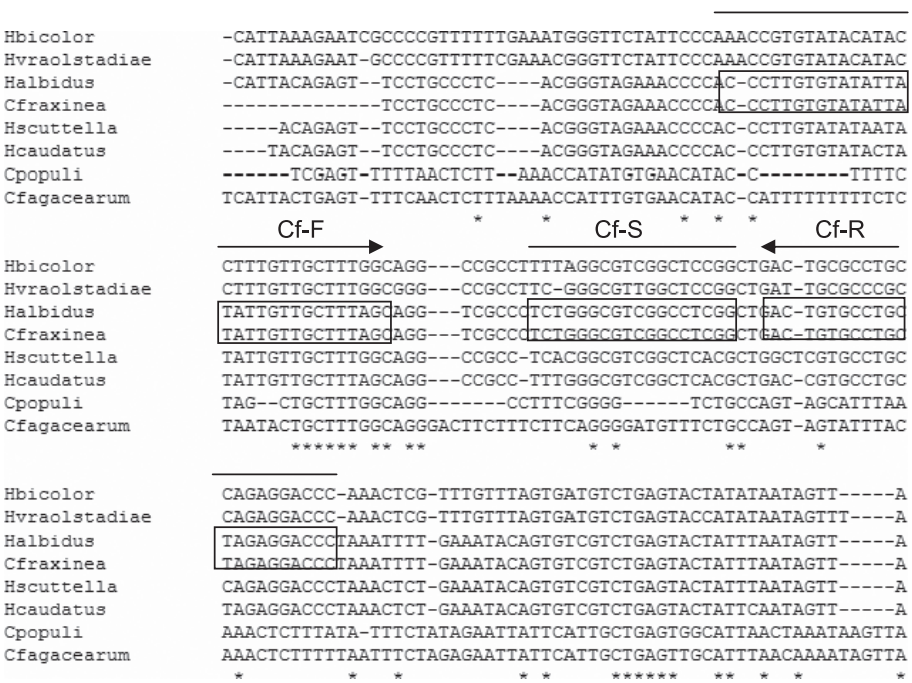

Fig. 1. ITS sequence alignment using the CLUSTALW2 program and localization of the primers Cf-F, Cf-R and the Taqman probe Cf-S (boxes). The arrows indicate the position of the forward and reverse primers. Asterisk indicates a consensus nucleotide. The Genbank numbers of the species used in the analysis are AJ292203 (*Hymenoscyphus bicolor*), AJ292200 (*H. vraolstadiae*), FJ597977 (*H. albidus*), FJ228176 (*Chalara fraxinea*), AY789432 (*H. scuttella*), AY348579 (*H. caudatus*), AY423551 (*C. populi*) and FJ347031 (*Ceratocystis fagacearum*).

3.3 Detection limit

Total DNA from *C. fraxinea* was serially diluted with plant DNA to yield concentrations ranging from 5000 to 0.5 pg/PCR. The limit of detection was 5 pg of target DNA.

Standard curve showed a linear correlation between input DNA and cycle threshold (C_t-values) with determination coefficient of 0.98 (Fig. 2a). Total DNA from *C. fraxinea* was also serially diluted with salmon sperm DNA used as reference DNA matrix. As shown in Fig. 2b, no difference was observed between both experiments ($p = 0.875$). Moreover, *C. fraxinea* was detected in leaves and wood from naturally-infected samples exhibiting symptoms of the disease, but not from healthy material. In contrast, the isolation was successful only from wood material, and not in all cases. The purification of DNA extracts (PVPP treatment) did not increase the test sensitivity. PCR performed on DNA extracted from wood material using the drill system yielded C_t-values which were similar to, or lower than those obtained with the classical extraction procedure (Table 3).

3.4 Test reproducibility

The test was conducted with two different PCR kits on three samples with different levels of infection and on two separate runs, with three replicates in each run. The ANOVA revealed significant differences between kits ($p < 0.05$), the core kit being slightly more efficient than the PCR Master Mix. However, from a practical point of view, the pathogen was detected in the three samples tested regardless of the kit used, even for the sample with a concentration of *C. fraxinea* DNA close to the detection limit (C_t-values around 35, Fig. 3).

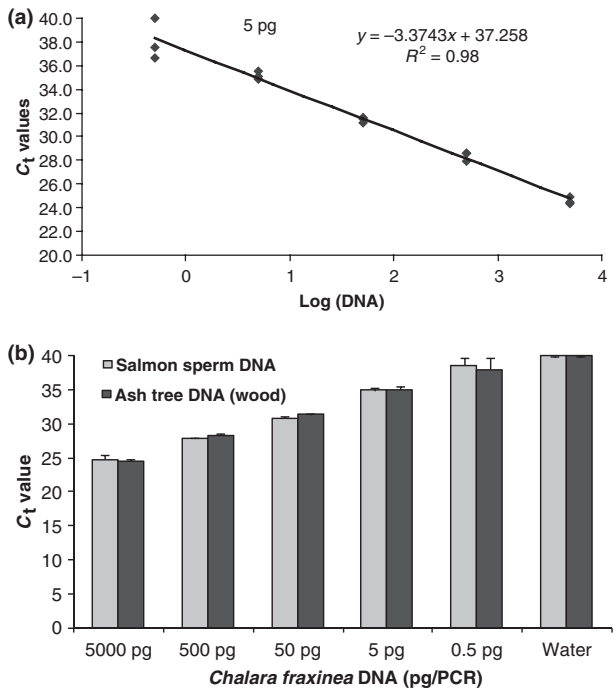

Fig. 2. Detection limit, standard curve and determination coefficient (a) and comparison between plant DNA and salmon sperm DNA used as DNA matrix for the reaction (b). Total DNA from *Chalara fraxinea* was serially diluted in plant DNA or salmon sperm DNA to yield final concentrations ranging from 0.5 pg to 5000 pg/PCR (three replicates, DNA extracted from mycelium of strain 3817). The C_t-values were determined at a threshold of 0.5.

Table 3. **Detection of *Chalara fraxinea* from necrotic tissues of dieback affected *Fraxinus excelsior* by isolation and by real time PCR on three types of DNA extracts. DNAs from healthy trees were used as the negative controls.**

No.	Origin	Country	Tissue	Isolation	Real time PCR (C_t[1])		
					No PVPP	PVPP	Drill[2]
1	Plombières	France	Leaf	–	22.26	22.74	nd
2	Plombières	France	Bark	–	35.52	35.06	nd
3	Corbenay	France	Leaf	–	28.22	29.24	nd
4	Corbenay	France	Wood	–	28.16	29.06	nd
5	Saint-Loup sur Semouze	France	Wood	+	32.72	32.91	26.39
6	Ainvelle (site 1)	France	Wood	+	24.31	24.23	nd
7	Ainvelle (site 2)	France	Wood	+	22.49	23.17	21.45
8	Ainvelle (site 2)	France	Leaf	–	22.47	22.6	nd
9	Mersuay (site 1)	France	Wood	–	22.63	24.29	28.01
10	Mersuay (site 2)	France	Wood	–	29.83	30.58	28.02
11	Floreffe (site 1)[3]	Belgium	Wood	nd	>40	nd	>40
12	Floreffe (site 2)[3]	Belgium	Leaf	nd	>40	nd	nd

+, isolation of the pathogen; –, pathogen not detected; nd, not done.
[1]C_t-values established at a threshold of 0.5.
[2]No purification of DNA with polyvinylpyrrolidone (PVPP treatment).
[3]Healthy trees.

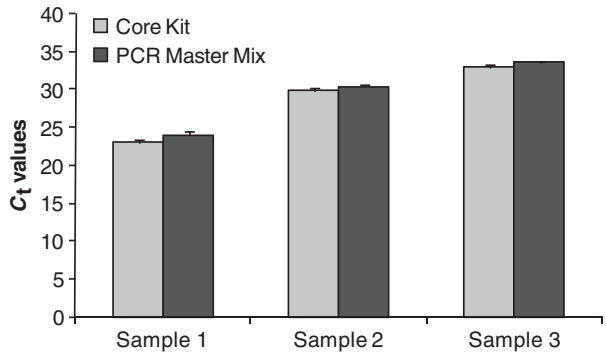

Fig. 3. Comparison between two PCR kits (PCR Core Kit and PCR Master Mix) for the detection of *Chalara fraxinea* in three naturally-infected samples with different inoculum concentrations (mean of six replicates). The C_t-values were determined at a threshold of 0.5.

4 Discussion

In this study, a real time PCR test was developed to detect *C. fraxinea* from plant tissues infected by the pathogen. Such a test is a valuable tool in diagnostic laboratories as ash dieback might be caused by other fungi and the reliable identification of the causal agent is a prerequisite to propose control measures.

In order to evaluate the test specificity, PCR was conducted on DNA extracted from fungi isolated from declining trees. Most of the fungal species which were identified had already been mentioned in other studies (Bakys et al. 2009b; Przybyl 2002 and Vasiliauskas and Stenlid 1998). None of these fungi were detected with the selected primers and probe. Moreover, as the sexual stage of *C. fraxinea* is a species belonging to the

genus *Hymenoscyphus* (Kowalski and Holdenrieder 2009b), multiple sequences alignments were carried out with several *Hymenoscyphus* species. Among the species considered, *H. caudatus*, a saprophytic species on decaying broadleaves trees, displayed a high sequence homology with *C. fraxinea.* The primers and probe were therefore selected in a region which was polymorphic between both species.

As some plant samples infected by the pathogen looked highly degraded, a purification of DNA extracts was evaluated. It did not increase the test sensitivity. Moreover, the detection limit was similar in a neutral matrix (commercial solution of salmon sperm DNA) and in DNA extracted from healthy trees. These results suggest that the method used to extract DNA does not introduce an inhibitory effect in the PCR.

By using the molecular method on infected material, results were obtained within one working day. The use of a drill for wood material further reduces the time required for sample preparation. In contrast, the fungus could be isolated after 5 weeks of incubation on MEA medium amended with antibiotic. The isolation data were inconsistent with the results of molecular analysis as only three samples out of the ten positive samples were detected. This low isolation rate could result from an inappropriate method for surface sterilization of samples. It could also be linked to the kind of plant tissue put onto the medium (leaf, bark or wood) as shown by Bakys et al. 2009a. In our case, the isolates were obtained only from wood material displaying discoloration.

Due to the recent identification of *C. fraxinea* as the main cause of ash dieback in Europe, many questions remain regarding the epidemiology of the disease. In this context, the molecular test developed could be used to estimate the ascospore density in a contamination site at different periods of the year using an airborne spore trap system as already mentioned for other pathogens (Luo et al. 2007) or to monitor the pathogen colonization in inoculated trees to evaluate the resistance to the disease, as proposed for other fungal pathogens of forest species (Hietala et al. 2003).

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