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Characterization of *Fomes fomentarius s.s. and F. inzengae* in Belgian Beech Forests

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Abstract: Real-time PCRs were developed to characterize *Fomes fomentarius* lineages occurring in the beech forests of the Ardennes (southern Belgium). A collection of *F. fomentarius sensu lato* isolates was built up in 2020 and 2021 from basidiocarps developing on beech trunks, stumps, or logs. Two of the 148 isolates tested (monokaryotic and dikaryotic) belonged to the species *Fomes inzengae*, while the other isolates corresponded to the species *Fomes fomentarius sensu stricto*. As far as we know, this is the first record of *Fomes inzengae* in Belgium. This fungus was also found in a mature tree not showing any signs of decay. This demonstrates its endophytic nature. Growth tests at different temperatures, as well as wood degradation tests in accordance with standard NBN EN113 showed different behavior between both species. Passive sticky traps were installed during two growing seasons at the two sites where *F. inzengae* had been reported. Spores were detected at all collection times between April and July, using the specific molecular test developed. Moreover, in 2023, the DNA concentrations measured were 100 times higher than in 2022. The implications of this new species in Belgium is discussed in the context of climate change.

Keywords: beech; cryptic species; endophyte; wood decay

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1. Introduction

With a potential distribution range of over 200 million hectares of woodlands, European beech (*Fagus sylvatica* L.) is one of the most prevalent forest tree species in Europe [1,2]. In Belgium, it is the second most prevalent deciduous tree species after oak, with which it is often mixed. More than two-thirds of pure beech stands are concentrated in the bioclimatic area of the Ardennes [3]. The European beech is sensitive to drought [4] and summer heat waves [5]. Over the past few years, due to climate change, European beech forests have shown a loss of vitality notably in Belgium [6,7] but also in other parts of Europe [8–10]. Under such conditions, beech stands may become increasingly susceptible to infection by secondary pathogens [11] and especially by wood decaying fungi with an endophytic phase which are common in beech sapwood [12].

Fomes fomentarius (L.) Kickx Fr (*Polyporales*, *Agaromycetes*, *Basidiomycota*) is a wood decaying fungus which causes white rot on a large number of wood species, mainly on broad-leaved trees. When present on tree stumps and dead trees, *F. fomentarius* behaves as a saprophytic agent with an important ecological role. Indeed, by decomposing wood, it participates in nutrient recycling. By eliminating dead wood, it also frees up space in the forest [13]. However, under stress conditions, *F. fomentarius* can behave as a pathogen by infecting standing trees [14], leading to a reduction in timber quality and trunk breakage in the event of windstorms. *F. fomentarius* is an endophytic species [15]. The transition from a

latent to an active state in wood is not well understood yet. As indicated by Boddy and Rayner [16], it might be triggered by particular environmental conditions that interfere with the normal functioning of sapwood, notably a decrease in moisture content associated with an increase in aeration.

Fomes fomentarius is present in diverse continents. Several phylogenetic studies using isolates from different geographical areas have shown that *F. fomentarius* is a species complex with several (cryptic) lineages [17–21]. Currently, four lineages have been identified: *Fomes* sp. from North America, *F. fomentarius sensu stricto (s.s.)* from Northern Europe, *Fomes* sp. from Asia and *F. inzengae* [21]. The lineage *F. inzengae* (Ces. & De Not.) Cooke is more prevalent in the Mediterranean region [22,23] and has been considered as a new cryptic species [21]. It differs from *F. fomentarius s.s.* in its hosts range, but also in growth characteristics and volatile compounds [21]. *F. fomentarius s.s.* and *F. inzengae* also show micro-morphological differences, but these are not easily identifiable in the field [24].

There were several objectives in this study. Firstly, the development of rapid molecular tools based on qPCR for the detection of lineages other than *F. fomentarius s.s* and the application of these tests on wood and fungal isolates collected in Belgian beech forests. Secondly, the use of the qPCR method in detecting *F. inzengae* on spores collected on spore traps to study the release period of this fungal species in Belgium. Thirdly, a comparison of the behavior of the lineages identified in terms of growth temperature and beech wood degradation capacity.

2. Materials and Methods

2.1. Collection of Fungal Isolates

A total of 15 beech stands of different ages were selected in southern Belgium in the bioclimatic zone of the Ardennes [25] where European beech (*Fagus sylvatica*) is one of the main hardwood species (Figure 1).

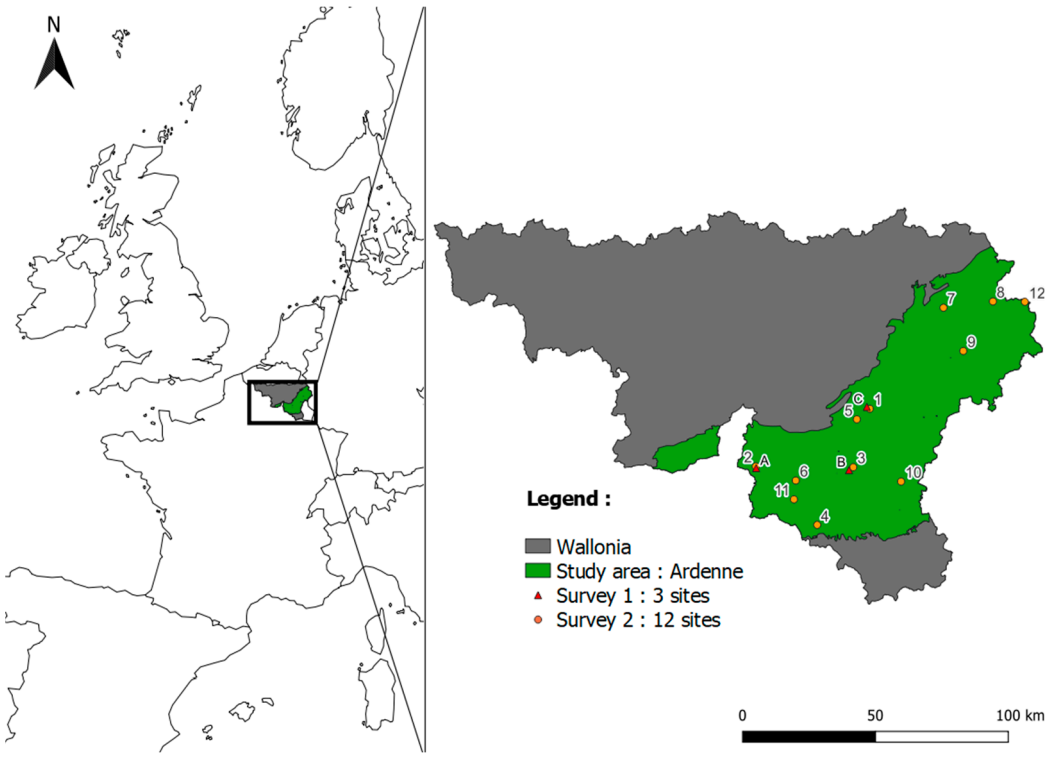

Figure 1. Location of the 12 beech stands where *F. fomentarius* isolates were collected from basidiocarps (numbers from 1 to 12) and of the three additional stands from which wood samples were collected to evaluate the presence of *F. fomentarius* as an endophyte (letters A, B and C). The bioclimatic area of the Ardennes in Belgium is in green.

Monokaryotic (n = 46) and dikaryotic (n = 102) isolates of *F. fomentarius s.l.* were collected from basidiocarps on living trees as well as on broken trees or stumps (Table 1). Monokaryotic isolates were obtained by collecting spores released in spring (end of April to mid-May) in sterile 1.5 mL tubes. Spores were suspended in sterile distilled water, and spread onto 2% Malt Extract Agar (MEA: 15 g/L agar and 20 g/L malt extract) containing streptomycin sulphate (80 mg/L). Plates were incubated at 25 °C in the dark. Single colonies were transferred onto 2% MEA. Regarding dikaryotic strains, pieces of basidiocarp context tissue (~2 cm × 2 cm) were sterilized for 1 min in a NaClO solution (0.6% active chlorine), cut into small pieces (~2 mm × 3 mm), plated onto 2% MEA supplemented with streptomycin sulphate and incubated at 25 °C in the dark. Pure cultures were transferred onto 2% MEA. All the isolates were subcultured on MEA supplemented with beech wood sawdust (10 g/L) every three months and preserved at 4 °C on MEA under paraffin oil.

Table 1. Collection of *F. fomentarius s.l.* isolates from Belgian beech stands. Numbers in brackets represent locations within the surveillance network. Each isolate was collected from a basidiocarp on a different tree.

Type of Material	Location (Stand Nr)	Number of Isolates	Collection Period
Monokaryotic	Louette-Saint-Pierre (2)	6 [a]	April 2020
Monokaryotic	Séviscourt (3)	6	April 2020
Monokaryotic	Sainte-Cécile (4)	2	April 2020
Monokaryotic	Nassogne (5)	10 [b]	April 2020
Monokaryotic	Carlsbourg (6)	2	April 2020
Monokaryotic	Spa (7)	9	April 2020
Monokaryotic	Elsenborn (8)	1	April 2020
Monokaryotic	Mogimont (11)	1	April 2020
Monokaryotic	Bullange (12)	9	April 2020
Dikaryotic	Séviscourt (3)	10	March 2021
Dikaryotic	Nassogne (5)	31	March 2021
Dikaryotic	Spa (7)	29 [c]	March 2021
Dikaryotic	Vielsalm (9)	2	March 2021
Dikaryotic	Vaux-Sur-Sure (10)	30 [d]	March 2021

[a,c] = *F. inzengae* & [b,d] = *F. fomentarius s.s.* isolates used for qPCR validations, growth rates at different temperatures and test of wood degradation.

2.2. Wood Material

Three mature beeches (circumference at breast height >100 cm) and nine saplings (circumference at breast height ~50 cm) were sampled at three additional forest sites in the same bioclimatic area (Figure 1), representing a total of 36 trees. The trees did not show any sporocarp on the trunk. Selected at random in the stand, they were felled during the winter, outside the period of *F. fomentarius* sporulation. Five-cm-thick wood disks were collected at two heights in two replicates (two disks at approximately 50 cm from the collar base, and two others at the base of the crown). The samples collected did not reveal any wood discoloration or decaying process. Within 24 h after the felling, the 144 wood disks were surface-disinfected with 70 percent alcohol and wrapped in polyethylene film with a few 1–2 mm holes as described by Baum et al. [15]. The disks were then placed at 20 °C for 24 weeks. At the end of the incubation period, the wood disks were cleared of the fungi growing on the surface, planed and then surface-disinfected with a blowtorch. Using a disinfected drill flamed between two samples, sawdust was gathered at 5 points at a depth of 2 cm in the wood. The 144 samples of sawdust were stored at −20 °C until processing.

One more tree was cut to provide green beech wood specimens of 50 × 25 × 15 mm to test the wood durability.

2.3. Spore Trapping Experiments

In 2022 and 2023, passive spore traps (Figure 2). were used to determine the sporulation period of *F. inzengae.* The collection unit (52 × 74 mm for easy transport in a Petri

dish) consisted of a tape (Tesa, Double-Sided Tape Universal, Brussels, Belgium) with one side glued to a rigid plastic sheet and the other side ready to be exposed to the air after removing the protective film. It was pinned onto a piece of expanded polystyrene fixed on a wooden support at 1 m from the ground. Spore traps (3 per site) were installed in the two sites known to be infected by *F. inzengae* (site 2, Louette-Saint-Pierre and site 7, Spa, Figure 1). In Spa, several basidiocarps of *F. inzengae* were present on a living tree and the spore traps were placed around this tree (in 3 directions, at ~0.5 m from the trunk) for the two years of trapping. In Louette-Saint-Pierre, in 2022, spore traps were placed around a log bearing basidiocarps and laying on the ground after trunk breaking. Unfortunately, the infected log was removed by forest managers during the winter of 2022–2023. Therefore, in 2023, the spore traps were placed around the remaining base of the broken tree. Spore trapping experiments were carried out from April to July of each year. The tapes were replaced every two weeks. At the end of each collection period, the tapes were collected in separate sterile Petri dishes and stored at −20 °C until further processing.

Figure 2. Passive spore traps used for the collection of spores of *Fomes inzengae* in beech stands.

2.4. DNA Extraction

Mycelium plugs were placed on cellophane laid on an MEA 2% medium. After ~10 days at 25 °C, ~100 mg of mycelium was collected in a 2 mL tube and ground with 5 mm stainless steel beads in a Retch mixer mill MM200 (Verder Scientific Benelux, Aartselaar, Belgium) (30 Hz, 1 min). Context tissues from basidiocarps were ground in liquid nitrogen. DNA extraction was performed from ground mycelium or basidiocarp and from wood sawdust using the NucleoSpin Plant II kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentration and purity were estimated by measuring absorbance at 260 and 280 nm using a Biospectrometer (Eppendorf, Hamburg, Germany).

In order to extract DNA from spore traps, 8 mL TE 4× [Tris 40 mM pH 8.0, EDTA 4 mM] heated to 65 °C were placed in the Petri dish containing the collection unit as recommended by Garbelotto et al. [26]. The spores were recovered by scrapping the sticky paper with a spatula. The spore suspension was collected in a 50 mL Falcon tube. The operation was repeated with 8 mL TE 4×. After centrifugation (4000× *g*, 1 h), 14 mL of supernatant was removed and the pellet was resuspended in the remaining 2 mL of TE 4×. The suspension was divided into two 1.5 mL tubes and centrifuged for 5 min at 15,500× *g*. The pellets were resuspended in 200 µL of TEX lysis buffer [100 mM Tris pH 8.0, 20 mM EDTA, 1.4 mM NaCl, 2% CTAB, 2% PVPP-K30] pre-warmed at 65 °C. The contents of the two tubes (400 µL) were transferred into one tube. DNA extraction was performed using the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) after a lysis step for 90 min at 65 °C with 10 µL proteinase K from the extraction kit.

2.5. Real-Time PCR and Conventional PCR

Polymorphic DNA regions for *F. fomentarius* detection were identified after alignment of ITS sequences of the 4 lineages described by Peintner et al. [21] (accession numbers JX290073, MH114657, EU273503, GQ184602, KM360129, MG719676, JX183719, HM584810, KC505546, GU203514, JQ901966 and JF927720) as well as ITS sequences from closely related fungal species in the Polyporales order (*Fomes fasciatus*, *Trametes versicolor*, *Ganoderma applanatum* and *Fomitopsis pinicola*). Using Primer3Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, accession date: 28 January 2021), primers and dual-labelled probes were then selected in these polymorphic regions to detect all lineages (test 1) or the lineages *Fomes* sp. from Asia and the species *F. inzengae* (test 2) (Table 2). The in silico specificity of the primers and probes selected was verified against the universal GenBank database.

Table 2. List of primers and probes used in the study.

Target	Primer Name	Sequence	Reference
F. fomentarius s.l. (test 1)	FomesF5	5′ ggatgttggaggcttttgct 3′	This study
	FomesP2	6-FAM-5′ atcggctgtcggtgtgat 3′-BHQ1	
	FomesR3	5′ agctgtctctgacgagaccat 3′	
F. inzengae & *Fomes* sp. (Asia) (test 2)	FinzF3	5′ cgaatctttgaacgcacctt 3′	This study
	FinzP	6-FAM-5′ gccctcgtttgagtcagc 3′-BHQ1	
	FinzR3	5′ gcaaggaaccaagctaatgc 3′	
F. fomentarius s.l.	FfomF	5′ gggttgtagctggccttc 3′	[27]
	FfomR	5′ ccagcaaaagcctccaatc 3′	
ITS of fungi	ITS1F	5′cttggtcatttagaggaagtaa 3′	[28]
	ITS5	5′ ggaagtaaaagtcgtaacaagg 3′	[29]
	ITS4	5′ tcctccgcttattgatatgc 3′	

Real-time PCRs were performed in a total volume of 20 µL on an ABI7000 thermocycler (Life Technologies, Merelbeke, Belgium) using the FastGen Probe 2XqPCR Universal (Nippon Genetics Europe, Düren, Germany). The reaction mixture contained 1× reaction buffer, primers and probe at 0.25 µM and the fluorophore Rox (0.5 µM). The thermal regime consisted of a denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 15 s and hybridization at 64 °C (test 1) or 56 °C (test 2) for 1 min. The fluorescence threshold line was adjusted manually at 0.3 in each PCR run. The limit of detection of the two qPCR methods was determined from serial dilutions (from 1 ng to 1 fg) of fungal DNA (two isolates of *F. fomentarius s.s.* and two isolates of *F. inzengae*) considering 3 replicates for each DNA concentration level. To assess the specificity of the molecular tests developed, the qPCRs were carried out on DNA from pure cultures of *F. fomentarius s.s.* and *F. inzengae* as well as from DNA of other fungal species extracted from pure cultures or basidiocarps (Table 3)

Nested conventional PCRs using the first set of primers ITS1F/ITS4 [28,29] (Table 2) and the second set of primers FfomF and FfomR developed by Parfitt et al. [27] for the detection of *F. fomentarius s.l.* (Table 2) were conducted on sawdust collected from beech wood. Conventional PCRs with the primers ITS5 and ITS4 [29] (Table 2) were carried out on DNA from fungal pure cultures. The PCR mixture contained 1× GoTaq Flexi buffer (Promega Madison, Madison, MI, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of the forward and reverse primers, 1 U Taq DNA polymerase and 3 µL genomic DNA. For the PCR with primers FfomF/FfomR, the amplification products from the first PCR were diluted 10 times prior to the second PCR. Amplifications were performed on a T100 thermocycler (Bio-Rad, Nazareth, Belgium) in a total volume of 50 µL. The thermocycler profile consisted in a denaturation for 5 min at 95 °C, 40× (30 s at 94 °C, 30 s at the annealing temperature, 1 min at 72 °C) and a final extension at 72 °C for 10 min. The annealing temperatures were 55 °C for ITS1F/ITS4, 52 °C for ITS5/ITS4 and 60 °C for FfomF/FfomR, respectively. The PCR products were sequenced in both directions at Eurofins Genomics (France). The sequences were assembled with BioEdit v 7.1.3.0 [30] The sequences generated with primers ITS5 and ITS4 from *F. inzengae* pure cultures

(isolates 5704 and 5711) were deposited in GenBank (GenBank accessions numbers OR473259 and OR473260).

Table 3. List of fungal isolates /fungal DNA used to evaluate the specificity of the qPCR methods.

Fungal Species	Material for DNA Extraction	Collection Code	Country of Origin	Collection Period
Armillaria gallica	Mycelium	3342	France *	1993
Bjerkandera adusta	Mycelium	2523	Belgium	2003
Daedaleopsis confragosa	Basidiocarp		Belgium	2010
Fomes fomentarius s.s. (monokaryotic)	Mycelium	5705	Belgium	2010
Fomes fomentarius s.s. (dikaryotic)	Mycelium	5706	Belgium	2021
F. inzengae (dikaryotic)	Mycelium	5704	Belgium	2021
F. inzengae (monokaryotic)	Mycelium	5711	Belgium	2021
Fomitopsis pinicola	Basidiocarp		Belgium	2019
Fusarium solani	Mycelium	5733	Belgium	2022
Ganoderma adspersum	Basidiocarp		Belgium	2010
Ganoderma applanatum	Basidiocarp		Belgium	2010
Phytophthroa x cambivora	Mycelium	P3398	Belgium	2005
Picipes badius	Basidiocarp		Belgium	2010
Trametes versicolor	Basidiocarp		Belgium	2018
Trametes hirsuta	Basidiocarp		Belgium	2018
Trametes versicolor	Mycelium	3561	Belgium	2018

* Collection INRAE, UMR Biogeco (France).

2.6. Growth Rates at Different Temperatures

For each species (*F. fomentarius s.s.* and *F. inzengae*), one monokaryotic and one dikaryotic isolate were used (respectively *F. fomentarius s.s.* isolate 5705 from site 5 and 5706 from site 10, and *F. inzengae* isolate 5711 from site 2 and 5704 from site 7). The four isolates were first grown on 2% MEA with sawdust (10 g/L) to maintain their decaying capacities. Mycelium plugs (6 mm) were taken at the margin of one-week old culture, transferred onto MEA 2% and placed at 25 °C. After three days, this period being required to observe growth for all isolates, the Petri dishes were placed at eight different temperatures (10, 15, 20, 25, 30, 32, 35 and 37 °C). There were five replicates per treatment (isolate/temperature). Mean growth rate was measured every day using two predefined directions on the Petri dish.

2.7. Wood Degradation

The entire wood degradation test is based on NBN EN-113-2 [31] using mass losses as an expression of the virulence of the fungus. The same strains as those used for the growth test at different temperatures were selected. *Trametes versicolor* (L.) Lloyd (EN-standard wood decay fungus—CTB 863 A strain) was used as a control. All isolates were maintained on 2% MEA with sawdust. Beech wood specimens (50 × 25 × 15 mm) were made from a freshly cut beech tree. They were compared to beech specimens stored during 10 years at 2–4 °C. Twelve specimens of both types were used for each fungal isolate. They were oven dried at 103 ± 3 °C for 24 h, weighed (M1) and stored in a conditioning chamber (25 ± 2 °C and 65 ± 5% relative humidity (RH) for 4 weeks. During the conditioning period of the wood specimens, mycelium plugs of the different fungi were placed on 50 mL of MEA 2% in Kolle flasks (approximate container capacity of 605 mL). When the mycelium had completely covered the surface of the medium, wood specimens sterilized twice at 24 h of interval for 20 min at 121 °C were placed in the Kolle flask on a thin glass support to avoid direct contact with the medium. All the specimens were incubated at 25 ± 2 °C and 65 ± 5% RH for 16 weeks. At the end of the incubation time, the wood specimens were

weighted (Mw), cleaned of the mycelium that had proliferated on the surface and dried at 103 °C for 24 h. They were then weighed (M2). The mass loss (ML) and moisture content (MC) were calculated using the following equations:

$$ML\ (\%) = ((M1 - M2)/M1) \times 100 \quad (1)$$

$$MC\ (\%) = ((Mw - M2)/M2) \times 100 \quad (2)$$

2.8. Statistical Analysis

All the analyses were carried out with R software (version 4.2.1) [32] with the packages dplyr [33], DescTools [34], car [35], multcomp [36], ARTool [37] and ggplot2 [38]. When descriptive statistics are presented, they are always expressed as means ± standard deviations. The significance level $\alpha = 0.05$ was used for all the tests. Statistical assumptions were verified. The difference in colony growth of *F. fomentarius* and *F. inzengae* at different temperatures were analyzed using a one-way ANOVA and a Tukey's test. Concerning the beech wood decaying test, non-parametric tests (Aligned ranks transformation ANOVA in the initial model and Kruskal–Wallis one-way analysis of variance supported by pairwise comparisons for the second one) were used to highlight differences between the two variables (type of wood and biological activity of the different isolates).

3. Results

3.1. Design of Primers and Dual-Labelled Probes for the Detection of F. fomentarius s.l. and F. inzengae/Fomes sp. from Asia and Survey in Belgian Beech Forests

Primers and dual-labelled probes were designed from sequences of *F. fomentarius s.l* (test 1) and *F. inzengae*/*Fomes* sp. from Asia (test 2) available in public DNA databases. The two qPCRs were applied on DNA from the 148 *Fomes fomentarius s.l.* isolates available (46 monokaryotic isolates, and 102 dikaryotic isolates) collected in 12 Belgian beech forests. All the samples were positive with test 1. Two results were positive with test 2, one corresponding to a monokaryotic isolate originating from a trunk on the ground in Louette-Saint-Pierre (site 2, isolate 5711), and one dikaryotic isolate originating from a standing tree in Spa (site 7, isolate 5704). By sequencing the amplification products generated with primers ITS5 and ITS4 from these two isolates, the sequences corresponded to *F. inzengae*.

Molecular tests (test 1 and test 2) were carried out on the five *F. fomentarius* basidiocarps present on the trunk of the tree from which dikaryotic strain 5704 had been isolated in Spa. Only three of the 5 basidiocarps were positive with test 2, demonstrating *F. fomentarius s.s.* and *F. inzengae* were present on the same tree.

3.2. Performance of the qPCR Methods Developed for the Detection of F. fomentarius s.l. and the Lineages F. inzengae/Fomes sp. from Asia

Pure cultures of *F. fomentarius s.s.* and *F. inzengae* obtained from the survey carried out in Belgian beech forests were used to evaluate the performance of the two qPCR methods. Standard curves from 10-fold dilution series were generated using DNA from isolates 5705 and 5706 (*F. fomentarius s.s.*) and from isolates 5704 and 5711 (*F. inzengae*). As shown in Table 4, the tests were linear over 6 logs. The limit of detection corresponding to the smallest concentration of DNA detected in all replicates was 10 fg per PCR for the two tests. There was no clear difference in Cq values between *F. fomentarius s.s.* and *F. inzengae* with the test targeting all lineages of *F. fomentarius* (test 1). The test targeting the lineages *Fomes* sp. from Asia and *F. inzengae* (test 2) did not detect the European isolates of *F. fomentarius* (lineage *F. fomentarius s.s.*). The two tests were specific (no amplification from DNA of other fungal species listed in Table 3)).

Table 4. Performances of the 2 qPCR methods developed. LOD = Limit of detection (in fg/PCR).

Test	Target	Slope	Constant	R^2	Log	LOD
1	*Fomes fomentarius s.l.*	−3.368	37.372	0.999	6	10
2	*Fomes* sp. Asia & *F. inzengae*	−3.506	40.098	0.999	6	10

3.3. Identification of Fomes inzengae as an Endophyte in Belgian Beech Forests

Wood disks (n = 144) collected from 36 standing beech trees that did not display any wood discoloration, sporocarps, nor decay in the wood at the time of harvest were analyzed with the qPCR methods developed after an incubation of 24 weeks at 20–22 °C. *F. fomentarius s.l.* was detected in 22 samples out of the 144 analyzed. These samples belonged to 16 of the 36 beech trees. There were two positive trees in Beauraing, six in La Roche, and eight in Saint-Hubert. Among the positive trees, 12 corresponded to saplings. Positive results were obtained from wood disks collected at the base of the tree, at the base of the crown or at both positions. The qPCR targeting the lineages *Fomes* sp. Asia and *F. inzengae* (test 2) revealed one positive result. The corresponding sample was also positive with test 1 and originated from a mature tree in Saint-Hubert. The Cq value generated with the test targeting *F. fomentarius s.l.* (test 1) for this sample was very low (Cq = 22.04) compared to the Cq values generated for the other samples (Cq values ranging from 33.03 to 39.12). This difference in Cq values corresponds to a DNA concentration of approximately 3–4 orders of magnitude. As the amplification product generated with primers FinzF3/FinzR3 on this DNA sample was too short for Sanger Sequencing, and was not in a polymorphic region, a nested conventional PCR with the primers ITS1F/ITS4 and FfomF/FfomR was carried out to find out if the identified lineage corresponded to *F. inzengae* or to *Fomes* sp. Asia. The sequence of the amplification product corresponded to *F. inzengae*.

3.4. Periods of Spore Release

Spore traps were installed in 2022 and 2023 in the two beech stands where *F. inzengae* had been isolated. As shown in Figure 3, differences were observed in 2022 between the two sites with a peak in May in Louette-Saint-Pierre, and a peak in June in Spa. Spores were detected during each collection period. However, the release of spores was very low in April and at the beginning of May 2022 for both sites. In 2023, the source of inoculum in Louette-Saint-Pierre was unfortunately removed and there was no detection. In Spa, the peak of spore release was observed in June as in 2022. However, the mean amount of DNA detected in 2023 was around 100 times higher than that detected in 2022. Very large variations in the amount of DNA detected on the various spore traps were observed for all the collection periods.

3.5. Growth at Different Temperatures for F. inzengae and F. fomentarius s.s.

All the isolates were incubated for 3 days at 25 °C to allow growth to resume on the culture medium before incubation at the various test temperatures. After four days at 32 °C (corresponding to 7 days on the medium), the mycelium of *F. inzengae* isolate 5711 had reached the edge of the Petri dish. The growth measurements were therefore interrupted after three days for all the treatments. As shown on Figure 4, significant differences (p-value < 0.001) were observed between the two fungal species after 3 days in culture, with the *F. inzengae* isolates growing faster than *F. fomentarius s.s.* isolates at 35 and 37 °C while the *F. fomentarius s.s.* isolates grew faster than isolates of the other species at 10 °C. The situation was less contrasted between 15 and 32 °C. Although there was no significant difference between species, some differences existed between isolates from the same species. The *F. fomentarius s.s.* dikaryotic isolate (5706) generally grew faster than the monokaryotic isolate (5705), with significant differences at 30, 32 and 35 °C (p-value < 0.001). The opposite trend was observed for *F. inzengae*, with the monokaryotic isolate (5711) growing faster from 15 to 30 °C (p-value < 0.001) but showing similar growth values at 32, 35, and 37 °C.

From 15 to 30 °C, the *F. inzengae* monokaryotic isolate 5711 displayed a higher growth rate than the three other isolates. Optimum growth temperature for all strains was 32 °C.

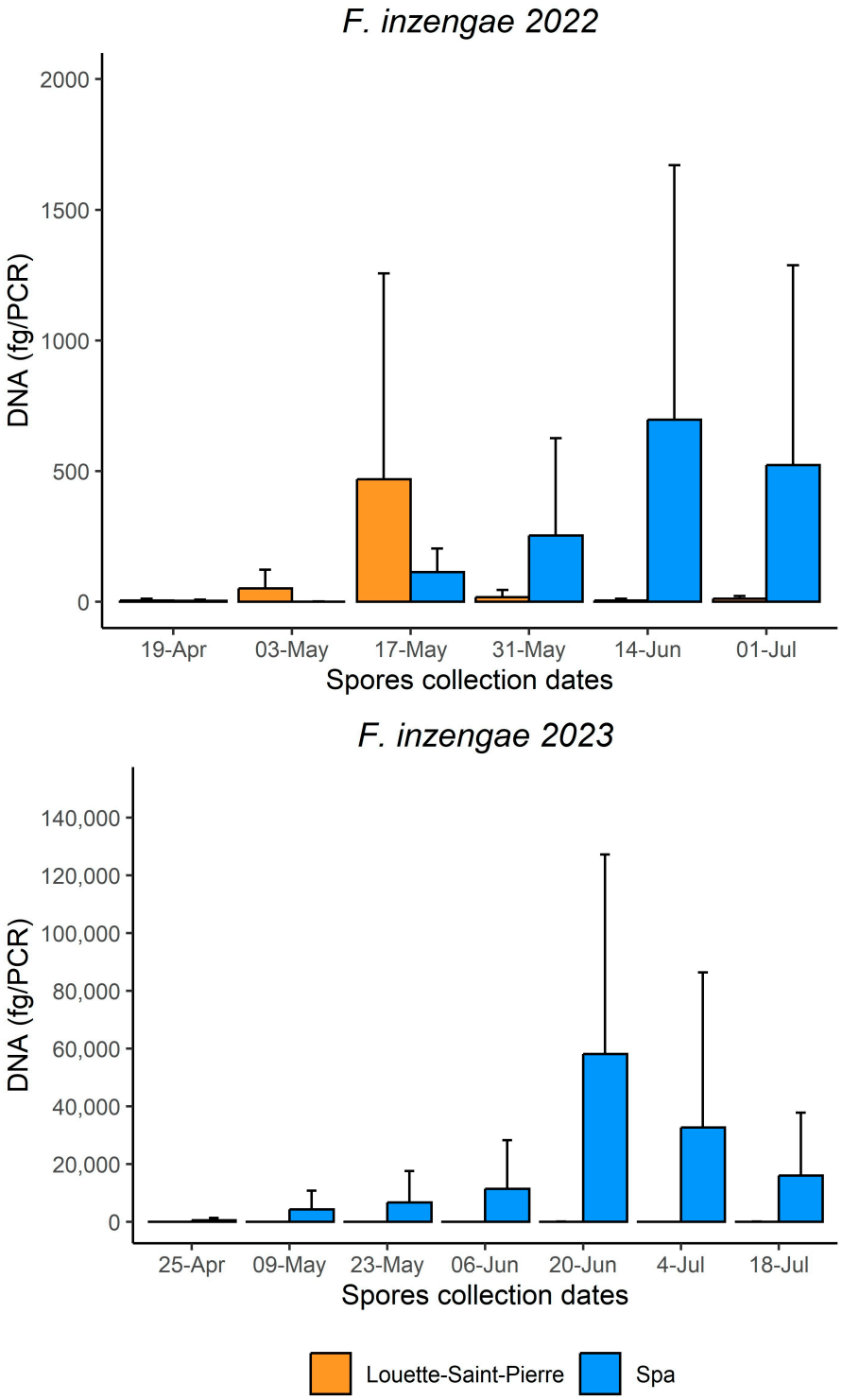

Figure 3. Spore trapping for the detection of *F. inzengae* in two beech stands in southern Belgium. The test was conducted in two beech forests (Louette-Saint-Pierre and Spa). Spores were collected at different periods, from April to July, in 2022 and 2023. Collection period = 2 weeks. Each date represents the date the traps were recovered from the sites.

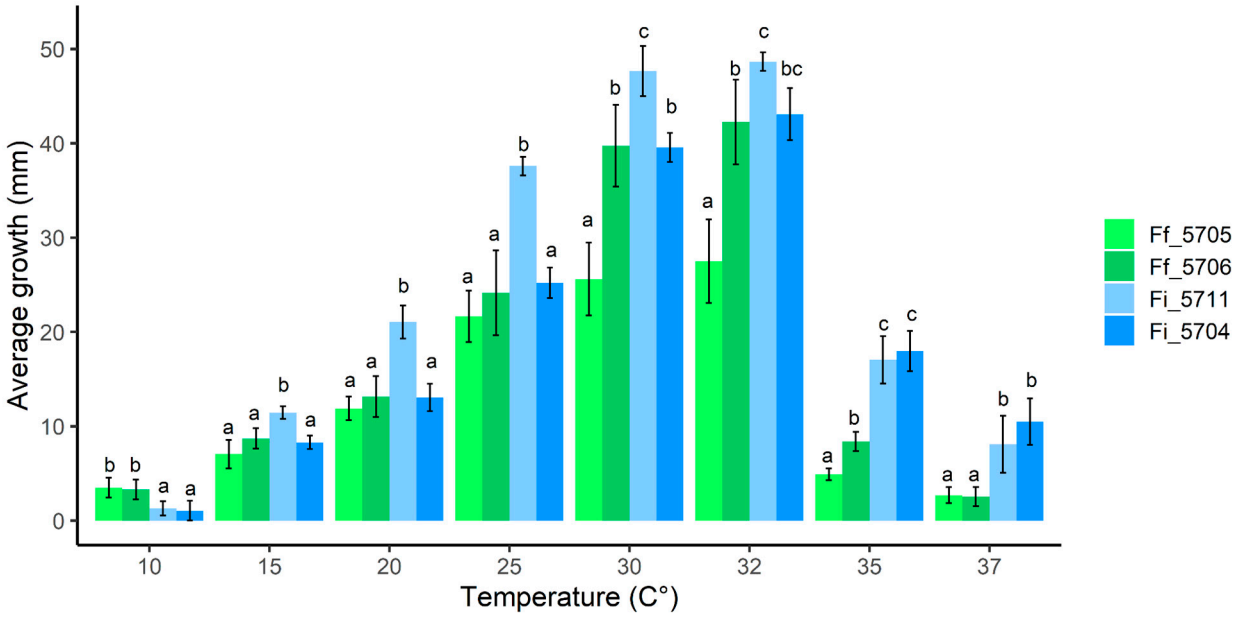

Figure 4. Average growth (in mm) after 3 days at different temperatures. The green bars represent the strains of *F. fomentarius s.s.*, while the blue bars represent the strains of *F. inzengae*. Modalities with the same letters are not significantly different.

3.6. Wood Degradation

After 16 weeks of incubation, the mycelium of the two *F. fomentarius* lineages had covered the wood specimen in all the Kolle flasks, without contamination of other species (Figure 5). Three wood samples had moisture contents that were considered abnormal according to the NBN EN-113 protocol (<25% or >100% of MC). These were one sample of isolate 5704 (118.73% of MC) and two samples of isolate 5711 (21.85% and 22.2% of MC). They were removed from the data set for the statistical analysis. The mean mass loss for the reference isolate *T. versicolor* was 29.35 ± 2.81% when the norm requires more than 20%. The average density of the wood specimens tested was 703 ± 31.95 kg/m^3.

(a) (b)

Figure 5. (**a**) Experiment in Kolle flasks to evaluate the wood decaying capacity of two isolates of *F. fomentarius s.s.* and two isolates of *F. inzengae*; (**b**) wood specimen covered with mycelium after 16 weeks of incubation.

As there was no significant difference between green and old wood material as well as no interaction between the variables "type of wood" and "fungal isolates" (p-value > 0.05), the replicates from the two types of material were combined for the analysis. Significant differences in mass loss were observed between the different fungal isolates

(*p*-value < 0.001) (Figure 6). The monokaryotic isolate of *F. fomentarius* induced the lowest mass loss (9.39 ± 4.74%, *p*-value < 0.001) while the dikaryotic isolate of *F. inzengae* induced the highest mass loss (38.81 ± 10.72%, *p*-value < 0.01). The other isolates did not differ significantly regarding mass loss (*F. inzengae* monokaryotic isolate: 29.18 ± 7.88%; *T. versicolor*, 29.35 ± 2.81% and *F. fomentarius* dikaryotic isolate: 31.09 ± 3.53%). An important variability was observed between replicates of the two isolates of *F. inzengae* (monokaryotic and dikaryotic). Such a variability was not observed for the *F. fomentarius s.s.* isolates nor for the reference isolate *T. versicolor.* There was no correlation between the density of the wood samples used and the mass loss measured (r = −0.06), indicating that the nature of the wood is not involved.

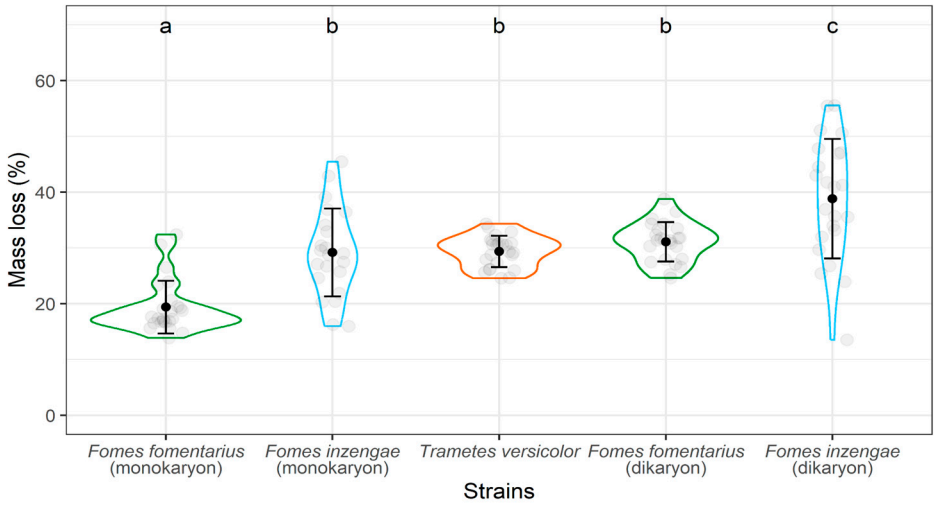

Figure 6. Violin plot showing the average mass loss (%) of beech wood specimen (24 replicates per treatment) after 16 weeks of incubation with different wood-decay fungi at 25 °C. The grey dots represent the values observed; the black dots and their error bars represent the mean and standard deviation for each strain. The green, blue and red lines are the density curves for *F. fomentarius s.s.*, *F. inzengae* and the reference strain *T. versicolor*, respectively. Modalities with the same letters are not significantly different.

4. Discussion

4.1. Rapid Detection Tools for Two Species of the F. fomentarius s.l. Complex

Cryptic fungal species are common among forest pathogens [21,39,40]. These species are difficult to distinguish based on macroscopic morphological characteristics. They may therefore go unnoticed during monitoring based on visual observations in the forest. In the case of invasive pathogens, their similarity to native species can delay their detection or allow them to be introduced into a new geographical area [41,42]. As an example, the exotic species *Heterobasidion irregulare* originating from North America and detected in Italy [43] is very similar to the native species *Heterobasidion annosum*. However, the two species have different ecological characteristics [44] which led EPPO to place *H. irregulare* on its alert list in 2013, and then on its A2 list in 2015.

The *F. fomentarius* species complex comprises two lineages, known as genotype A and genotype B and, for each of these lineages, two sublineages (A1 and A2, and B1 and B2) [45]. As part of a phylogenetic study of a large number of ITS sequences from isolates from various geographical origins, two of these sublineages were renamed: *F. fomentarius s.s.* corresponds to sublineage A2 while *F. inzengae* corresponds to sublineage B2 [21]. Although they have different micro-morphological characteristics, distinguishing lineages A and B on a visual basis is difficult [24]. Polymorphisms in the ITS sequence make it possible to identify them, but sequencing the ITS zone requires the isolation of a pure strain if specific PCR primers are not available, which is time-consuming and not always possible, particularly when the fungus is in a latent form in the wood. Direct observation of spores

under the microscope is also inappropriate method to detect airborne basidiospores using spore traps as spores of different fungi are present in a mixture making detection based on a non-specific method unreliable. In this study, two qPCR tests were developed, one to detect all the lineages of the *F. fomentarius* complex (genotypes A and B), and the other to detect the lineage *Fomes* sp. from Asia and *F. inzengae* (genotype B only). The benefit of these two tests is that they are very rapid and can be applied to wood and basidiocarps, as well as to environmental DNA samples, notably from spore traps. They are therefore suitable for surveillance in forests. Both tests are highly sensitive (detection limit at 10 fg/PCR), which means they can be used for early detection.

4.2. Fomes inzengae Is Present in Beech Wood in Belgian Forests

Using the molecular tools developed, monitoring has been conducted in Belgium in the Ardennes area where beech is broadly distributed, particularly in the form of pure forest stands. Although not very common, *F. inzengae* has been identified from basidiocarps collected from living beech trees, but also from broken beeches (trunk). To our knowledge, this is the first report of this species in Belgium. This fungus has been reported in Europe, notably in England, Italy, Latvia, Slovakia and Slovenia [46], Spain [47] and the Czech Republic [24]. It is capable of infecting various hardwood species, but rarely infects beech trees, unlike *F. fomentarius s.s.* which is prevalent on beeches [21]. Our work confirms this result, since only two of the 148 isolates tested (monokaryotic and dikaryotic) belong to the species *F. inzengae*, the others (N = 146) corresponding to the species *F. fomentarius s.s.*. However, the two isolates of *F. inzengae* were found in two different sites out of the twelve selected and in contrasting geographical areas (Figure 1). Interestingly, basidiocarps of both species were sometimes present on the same tree, demonstrating their co-existence on the same substrate.

Like *F. fomentarius s.s.* [15], *F. inzengae* behaves like an endophyte. However, unlike *F. fomentarius s.s.*, it was not identified in undecayed wood from young trees in our study. This result may be due to the low number of trees tested (36 trees) limiting the probability of identifying this fungus given its infrequent occurrence on beech. Its identification in the wood of a mature tree suggests that it might had been present in the wood for several years.

4.3. Aerial Inoculum of F. inzengae Varies Greatly from Year to Year

Passive spore traps consisting of double-sided adhesive tape (gravity sampling) have already been described, notably for pollen sampling [48]. These spore traps are inexpensive. They can therefore be deployed in large numbers on forest sites, which is not possible with Burkard samplers. They are also very simple to install in the forest, meaning that they can be manipulated by people with no specific experience of handling spore traps, like foresters. Moreover, the film protecting the adhesive being removed in the forest at the time of trapping, the risk of contamination is limited. They are therefore easier to use than horizontal slides or membranes coated with a sticky compound. They are also very resistant to humid conditions in contrast to filter papers which can show physical degradation when dampened [26]. Finally, they can be maintained on a site for several days (15 days in our experiment), which is not possible with rotorods, which generally operate for one or two days [49]. Essentially relying on gravity sampling, they are less efficient than impaction spore traps and not suitable for accurate quantitative detection. However, the objective of the experiment was to characterize the period of spore release of *F. inzengae* in forest sites where the species had already been detected. Moreover, the spore traps were placed near the inoculum source (basidiocarps), to insure an inoculum level sufficient to be detected by a very simple trapping device. This hypothesis was confirmed as *F. inzengae* DNA was detected at different periods of the growing season, in variable concentrations depending on the collection dates, the site location and the position of the spore trap. DNA concentrations were extremely variable among sampling points, suggesting that several replicates have to be used to detect an airborne inoculum. Unlike 2022, no detection occurred in 2023 in Louette-Saint-Pierre. The removal of the main source

of inoculum during the winter of 2022–2023 probably explains this result and suggests that most of the spores collected on the spore traps were from the infected material located near the spore trap, and not from spores originating from other sources of inoculum.

4.4. Fomes inzengae and F. fomentarius s.s. Display Different Ecological Characteristics

The tests on growth at different temperatures, and on wood degradation, were conducted on only two *F. inzengae* isolates because these isolates were the only ones identified on beech in Belgium as part of the surveillance programme.

In terms of growth characteristics, our results are consistent with those of Peintner et al. [21]. *F. fomentarius s.s.* grows much better at low temperatures (10 °C in our case), whereas *F. inzengae* displays a higher growth rate at temperatures ranging from 30 to 35 °C.

In terms of wood degradation, the NBN EN-113 was followed as it is the standard usually used to evaluate the degradation capacity of wood decay fungi [50]. The results obtained with the reference strain of *T. versicolor* (20% minimum of mass loss) indicate that the test was carried out correctly and that the data are reliable.

Both *F. inzengae* isolates showed contrasting behavior, and it was not possible to demonstrate any significant difference in mass loss between the two species (*F. fomentarius s.s.* and *F. inzengae*). Our results are consistent with those of Cristini et al. [47] even though a different experimental protocol was followed, particularly with regard to the size of the wood samples and the incubation period. However, a difference between species appeared in terms of response repeatability. Indeed, both *F. inzengae* isolates showed large variations in mass loss depending on the replicate considered, whereas this phenomenon was not observed in *F. fomentarius s.s.* and *T. versicolor*. The maximum values of wood degradation were also the highest for the two *F. inzengae* isolates (45.5 and 55.5% respectively compared to 32.4 and 38.8% for *F. fomentarius s.s.*), suggesting that *F. inzengae* might degrade beech wood more actively. This hypothesis is reinforced by the results of the experiment aiming to evaluate the presence of *F. inzengae* as an endophyte where wood disks infected with *F. inzengae* were found to be highly concentrated in DNA from this fungal species while wood disks infected with *F. fomentarius s.s.* displayed a very low concentration of *F. fomentarius s.s.* DNA. As both species are detected with the same efficiency with the qPCR test 1, this result suggests that the *F. inzengae* isolate naturally present in wood disks was more active in colonizing beech wood than that of *F. fomentarius s.s.*.

5. Conclusions

The native species *F. fomentarius s.s.* and the Mediterranean species *F. inzengae* were found in the same environment (pure stands of European beech) indicating that there is potentially direct competition between both species. Currently, the Mediterranean species is not frequent in the beech forests of the Ardennes, and the number of isolates per site remains limited, suggesting that it behaves as a non-invasive colonizer. However, this situation could change in the future in the context of climate change as *F. inzengae* is more adapted to higher temperatures. Moreover, these higher temperatures might at the same time increase vulnerability of beech and favor a switching from endophytic to pathogenic behavior. This could lead to the replacement of the native species by *F. inzengae*. Future studies should clarify the potential increase of *F. inzengae* occurrence in European beeches as well as in other forest species and its impact on wood degradation in standing trees.

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