Fungal Community in Symptomatic Ash Leaves in Spain

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Trapiello, E., Schoebel, C. N. and Rigling, D. 2017. Fungal Community in Symptomatic Ash Leaves in Spain. *Baltic Forestry* 23(1): 68-73.

Abstract

Mycobiota inhabiting symptomatic leaves of *Fraxinus excelsior* from two sites in Asturias, northern Spain, was analyzed to investigate the occurrence of pathogenic fungal species on European ash such as *Hymenoscyphus fraxineus*. Leaves were collected in fall 2014 and isolations were made from petioles showing discolorations. The morphological characterization of 173 isolates resulted in eight morphotypes, whereas the phylogenetic analysis resulted in seven genera, *Alternaria*, *Phomopsis* and *Phoma* being the most frequently isolated. Neither *H. fraxineus*, nor other *Hymenoscyphus* species were detected. The absence of *H. fraxineus* is consistant with field observations where no typical ash dieback symptoms were recorded. Most of the fungi isolated are known plant pathogens and some of them have occasionally produced disease symptoms on ash after artificial inoculations. Nevertheless, their natural behaviour as pathogens on *F. excelsior* remains unclear, and could be significantly influenced by different factors as environmental conditions or endophyte presence.

Keywords: *Fraxinus excelsior*, northern Spain, symptomatic leaves, pathogenic fungi, ITS-sequencing.

Introduction

Fraxinus is a genus of flowering plants belonging to the Oleaceae family. Species in this genus are usually medium to large trees and widespread across much of Europe, Asia and North America. The European ash or common ash (*Fraxinus excelsior*) is an ecologically important tree in Asturias, a region in northern Spain, where it has played an important role throughout history presenting a wide range of uses, such as wood resource, human and cattle nourishment, or as a medicinal plant. At present, *F. excelsior* in Europe is threatened by the Ascomycete fungus *Hymenoscyphus fraxineus* (synonym: *Hymenoscyphus pseudoalbidus*, basionym: *Chalara fraxinea*), the causal agent of ash dieback. *H. fraxineus* is an alien invasive fungus originated in East Asia (Zhao et al. 2012) which has been spreading across Europe since the mid-1990s, causing massive damage and mortality to susceptible ash species, mainly *F. excelsior* but also *F. angustifolia* (Gross et al. 2014). At present, this fungus has not been reported in Spain, therefore it would be interesting to study whether this pathogen has arrived before clear symptoms are visible. The aim of the present work was to study the fungal biota inhabiting common ash leaves with disease symptoms in Asturias. For this purpose, a morphological and molecular characterization of fungal cultures isolated from symptomatic ash petioles was conducted.

Material and methods

Study site description and sampling design

The study area is located in Asturias, officially the Principality of Asturias, a region in north-western Spain. Plant material was collected in autumn 2014 in two central areas, Aller (site 1, coordinates: 43.0621, 5.3450), and Llanera (site 2, coordinates: 43.2716, 5.5108). There were 5 sampling points within each area, which were at least 5 km apart from each other. At each sampling point (approx. 50 ha), 10 leaves from 10 trees were collected. Totally, a hundred ash trees were sampled. Even though trees showed a healthy common appearance, all collected leave samples showed disease symptoms such as leaf spots and petiole discoloration.

Analysis of samples, fungal isolation and morphological characterization

Isolation was conducted as follows, two leaf petioles per sample were washed with distilled water and blotted

dry. Next, they were disinfected by submerging them into 100% ethanol for 30 s, air-dried, peeled and cut off in three segments with a sterile scalpel. A small part of each segment was placed onto ash-agar medium (15 g/l of Agar, 20 g/l of Bacto Malt Extract, 50 g/l of ash leaf solution and 0.4 ml/l of Streptomycin) plates, and next incubated in the dark at room temperature during 4-6 weeks. The plates were inspected daily, and one outgrowing culture per leaf petiole was sub-cultured onto potato dextrose agar (PDA, Difco) plates. The plates were incubated at room temperature on a laboratory bench at daylight conditions for 15 days. Isolates were morphologically assessed and classified into morphological similarity groups.

DNA extraction, PCR and ITS sequencing

A representative number of isolates for each morphological group was chosen for DNA analysis. DNA extraction was performed according to Schoebel et al. 2014. The species identity was verified by ITS sequencing using ITS1 and ITS4 primers (White et al. 1990). Thermal cycling conditions were: 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55°C, 2 min at 72 °C, and a final extension of 72 °C for 10 min. PCR products were fractionated by electrophoresis and visualized under UV. For PCR purification the IllustraExoStar 1-Step kit (Fisher scientific) was used. Cycling conditions were: 1 min at 96 °C, followed by 25 cycles of 10 s at 95 °C, 5 s at 50°C and 1 min at 60 °C. Sequencing was performed in both directions, using an ABI PRISM 3130 (Applied Biosystems).

Phylogenetic analysis

Sequences were manually checked and edited to obtain a consensus sequence, and compared with reference sequences deposited in the BLAST database (http://www.ncbi..nlm.nih.gov/BLAST/). Assembled sequences were aligned and used to reconstruct a phylogenetic tree using the UPGMA method with kimura 80 as nucleotide distance measure, using CLC Genomics Workbench (version 8.0 Beta 4). All ITS sequences obtained in this work were deposited at GenBank.

Estimation of frequencies

Isolation frequencies of each genera or species were estimated. Isolation frequency was calculated as the percentage of isolates of individual genera or species in relation to the total number of isolates obtained from each site.

Results

Morphological characterization of fungal isolates

In total, 173 fungal cultures were isolated from the 200 leaf petioles analysed. Most of the cultures shared common morphological characteristics, therefore they were classified in groups of similar morphology. Totally, there were eight morphotypes (table 1), which included 163 isolates. In addition, 10 isolates were morphologically different from all other isolates so they were considered as single morphotypes. Only few isolates showed a morphology similar to *H. fraxineus.*

Genetic characterization of morphotypes

A selection of 31 representative isolates of morphological groups and single isolates, were identified by ITS sequencing. The results of the identification are presented in table 1 and the phylogenetic relationship is depicted in figure 1. Even though a few isolates showed morphological characteristics similar to *H. fraxineus*, the subsequent ITS sequencing showed that all of them did not belong to this species. As it can be seen in the phylogenetic tree (figure 1) there were two main clusters and one isolate (C9b: *Harzia acremonioides*) very dissimilar to both clusters. One cluster included 15 isolates of the morphological groups III, IV, VI, VII and a group of single isolates. The second cluster comprised 15 isolates of the morphotypes I, II, V, VIII and four single isolates.

Estimation of frequencies

From the 200 petioles analyzed (two petioles per sample), 173 (86.5%) gave fungal growth. There were seven fungal genera included in the eight morphological groups, and three genera, one family and three species belonging to single morphotypes. Data are shown in table 2.

Discussion and conclusions

This study reveals a high prevalence of plant pathogenic fungi in European ash in Asturias. Based on morphological culture characteristics of the isolates, eight morphotypes were established. ITS sequencing and phylogenetic analysis revealed seven genera among these morphotypes. These genera included *Phomopsis* spp*, Phoma* spp*, Fusarium* spp, *Epicoccum* spp,. and *Rossellinia* spp*,* in descending order in respect to isolation frequency. In addition, there were two closely related genera included in morphotype II, belonging to the Pleosporales, *Stemphylium* spp and *Alternaria* spp*,* this last being more frequently isolated (3:1). Each genera group corresponded to a different morphological group, with the exception of *Phoma* spp and *Phomopsis* spp*,* which include two morphotypes each (I and VIII, VI and VII, respectively). There were no large morphological differences observed between these pairs of groups.

Totally, 173 fungal isolates were obtained from both sampling sites, 89 isolates from site 1 and 84 from site 2. The fungal community was very similar at both sites with *Alternaria* spp, *Phomopsis* spp, *Phoma* spp and *Fusarium* spp being the most frequent followed by *Rosellinia* spp and

Table 1. ITS-based identification of fungal cultures isolated from leaves of *Fraxinus excelsior* in Asturias

Isolates						Most closely related database sequence				
Code	Site	MG	GB accession no.	SL (bp)	Identification	GB accession no.	QC (%)	Id (%)	Identification	Reference
C2b	1	VII	KT948355	552	*Phomopsis rudis*	KC343230.1	100	99	*Diaporthe rudis (Phomopsis rudis)*	Gomes et al. 2013
						FJ228188.1			*Diaporthe viticola (P. rudis)*	Bakys et al. 2009
C3b	1	*	KT948356	492	Fungal sp.	FJ228206.1	96	99	Fungal sp.	Bakys et al. 2009
						HQ414588.1				Scholtysik et al. 2013
C7b	1	VII	KT948357	545	*Phomopsis sp.*	KC843328.1	100	100	*Diaporthe cotoneastri* (*P. cotoneastri*)	Gomes et al. 2013
						KC343073.1			*Diaporthe eres* (*P. oblonga*)	
						HE774484.1			*Phomopsis sp.*	Hauptman et al. 2013
C9b	1	*	KT948358	569	*Harzia acremonioides*	HQ698593.1	97	99	*Harzia acremonioides*	nd
CAO1b	1	VI	KT948359	536	*Phomopsis sp.*	FN386273.1	99	99	*Phomopsis sp.*	nd
CAO4a	1	I	KT948360	502	*Phoma sp.*	EU852354.1	100	100	Uncultured *Phoma*	Bakys et al. 2009
CAO5	1	IV	KT948361	522	*Fusarium sp.*	KM189440.1	100	100	*Fusarium avenaceum*	nd
						GQ922561.1			*Fusarium tricinctum*	
CAO7b	1	I	KT948362	506	*Phoma sp.*	EU852354.1	100	99	Uncultured *Phoma*	Bakys et al. 2009
						AJ608976.1			*Phoma exigua*	nd
						EU343118.1			*Phoma exigua* var. *exigua*	
						JQ804843.1			*Phoma exigua* var. *foveata*	
R6b	1	II	KT948371	531	*Alternaria sp.*	KM215624.1	100	100	*Alternaria sp.*	nd
R7b	1	V	KT948372	505	*Epicoccum nigrum*	AF455395.1	100	100	*Epicoccum nigrum*	nd
R10b	1	III	KT948373	558	*Rosellinia mammiformis*	KF719200.1	100	100	*Rosellinia mammiformis*	nd
ST1b	1	I	KT948380	502	*Phoma sp.*	EU852354.1	100	100	Uncultured *Phoma*	Bakys et al. 2009
ST6a	1	*	KT948381	506	*Phoma sp.*	JX160059.1	100	99	*Phoma sp.*	nd
T4a	1	*	KT948382	514	*Phacidium mollerianum*	KR873247.1	100	100	*Phacidium mollerianum*	nd
T5b	1	II	KT948383	531	*Alternaria sp.*	KJ541477.1	100	100	*Alternaria sp.*	nd
T6b	1	*	KT948384	520	Fungal sp.	GU174285.1	100	99	Uncultured fungus	nd
T7	1	*	KT948385	520	Fungal sp.	EF040841.1	100	99	Uncultured fungus	nd
F1b	2	IV	KT948363	522	*Fusarium lateritium*	JQ693397.1	100	100	Fusarium lateritium	nd
F5a	2	VIII	KT948364	501	*Phoma* sp.	JX160059.1	100	100	*Phoma* sp.	nd
F9b	2	II	KT948365	543	*Stemphylium* sp.	AB979903.1	100	98	Stemphylium sp.	nd
L2b	2	*	KT948366	503	*Mycosphaerellaceae* sp.	EU167596.1	100	99	*Micosphaerella coacervata*	nd
						EU167590.1			*Micosphaerella linorum*	
						AB435070.1			*Micosphaerella delegatensis*	
						EF394864.1			*Septoria* sp	
L9	2	VII	KT948367	539	*Phomopsis theicola*	HE774477.1	100	100	*Diaporthe foeniculina* (*P. theicola*)	Hauptman et al. 2013

MG: morphological group, GB accession no: GeneBank accession number, SL: sequence length, QC: query cover, Id: identity, *: single isolate, nd: no data

Table 1. Continued

Isolates						Most closely related database sequence to isolates of this work				
Code	Site	MG	GB accession no.	SL (bp)	Identification	GB accession no.	QC (%)	Id (%)	Genus or species	Reference
M5a	2	IV	KT948368	524	*Fusarium sp.*	JX114790.1	100	100	*Fusarium acuminatum*	nd
						AY188923.1			*Fusarium tricinctum*	
M6b	2	VI	KT948369	545	*Phomopsis sp.*	NR_119726.1	100	100	*Diaporthe cotoneastri* (*P. cotoneastri*)	nd
						KC343073.1			*Diaporthe eres (P. oblonga*)	Gomes et al. 2013
						HE774484.1			*Phomopsis sp*	Hauptman et al. 2013
M10b	2	III	KT948370	558	*Rosellinia mammiformis*	KF719200.1	100	100	*Rosellinia mammiformis*	nd
S1b	2	II	KT948374	503	*Alternaria sp.*	KM215624.1	100	100	*Alternaria sp.*	nd
S4	2	*	KT948375	506	*Epicoccum nigrum*	KP132016.1	100	100	*Epicoccum nigrum*	nd
S10	2	*	KT948376	541	*Periconia byssoides*	KC954157.1	100	100	*Periconia byssoides*	nd
SC5b	2	V	KT948377	508	*Epicoccum nigrum*	KM036093.1	100	99	*Epicoccum nigrum*	nd
SC7b	2	*	KT948378	518	*Cladosporium sp.*	KF367491.1	100	98	*Cladosporium sp.*	nd
SC8b	2	I	KT948379	502	*Phoma sp.*	EU852354.1	100	100	Uncultured Phoma	Bakys et al. 2009

MG: morphological group, GB accession no: GeneBank accession number, SL: sequence length, QC: query cover, Id: identity, *: single isolate, nd: no data

Epicoccum spp. Three cultures could not be identified at site 1 because they had database hits as fungal sp or uncultured fungus. Interestingly, one of them, C3b isolate (table 1) is similar to fungal isolates previously recovered from ash (Bakys et al. 2009, Scholtysik et al. 2013). Several isolates from this work showed high genetic identity with isolates previously reported from ash. This is the case of *Phomopsis rudis* (C2b) or *Phoma* sp (CAO4a, CAO7b, ST1b, SC8b) (Bakys et al. 2009, Gomes et al. 2013), *Phomopsis* sp (C7b, M6b) (Gomes et al. 2013, Hauptman et al. 2013), and *Rossellinia mammiformis* (R10b, M10b) (unpublished data, sequences submitted in GenBank by Hamelin et al. 2013) (table 1). Results from our work are in line with previous studies (Przybyl 2002, Pukacki and Przybyl 2005, Bakys et al. 2009, Scholtysik et al. 2013) where *Alternaria* spp, *Cladosporium* spp, *Epicoccum* spp, *Fusarium* spp, *Phoma* spp and *Phomopsis* spp were also the most frequent fungi isolated from symptomatic ash tissues. In our study area, *Cladosporium* spp seems to be rare with only one isolate recovered from site 2 (table 2).

The isolates in the genera *Alternaria* were most frequently isolated, a result that was also obtained by Scholtysik et al. 2013. These authors suggested that high infec-

Table 2. Fungal genera and species isolated from *Fraxinus excelsior* leaves from Asturias

MG	Fungal genera/species identified	No. of isolates and frequency of isolation (%)	
		Site 1	Site 2
I, VIII, *	*Phoma spp*	15 (16.85%)	10 (11.90%)
II	*Alternaria spp-Stemphylium spp (Pleosporales)*	37 (41.57%)	32 (38.10%)
III	*Rosellinia spp*	2 (2.25%)	6 (7.14%)
IV	*Fusarium spp*	9 (10.11%)	8 (9.52%)
V, *	*Epicoccum spp*	4 (4.49%)	4 (4.76%)
VI, VII	*Phomopsis spp*	17 (19.10%)	21 (25%)
*	*Periconia byssoides*	0 (0%)	1 (1.19%)
*	*Phacidium mollerianum*	1 (1.12%)	0 (0%)
*	*Mycosphaerellaceae spp*	0 (0%)	1 (1.19%)
*	*Cladosporium spp*	0 (0%)	1 (1.19%)
*	*Harzia acremonoides*	1 (1.12%)	0 (0%)
*	Not identified	3 (3.37%)	0 (0%)
		89	84

MG: morphological group, *: single isolate

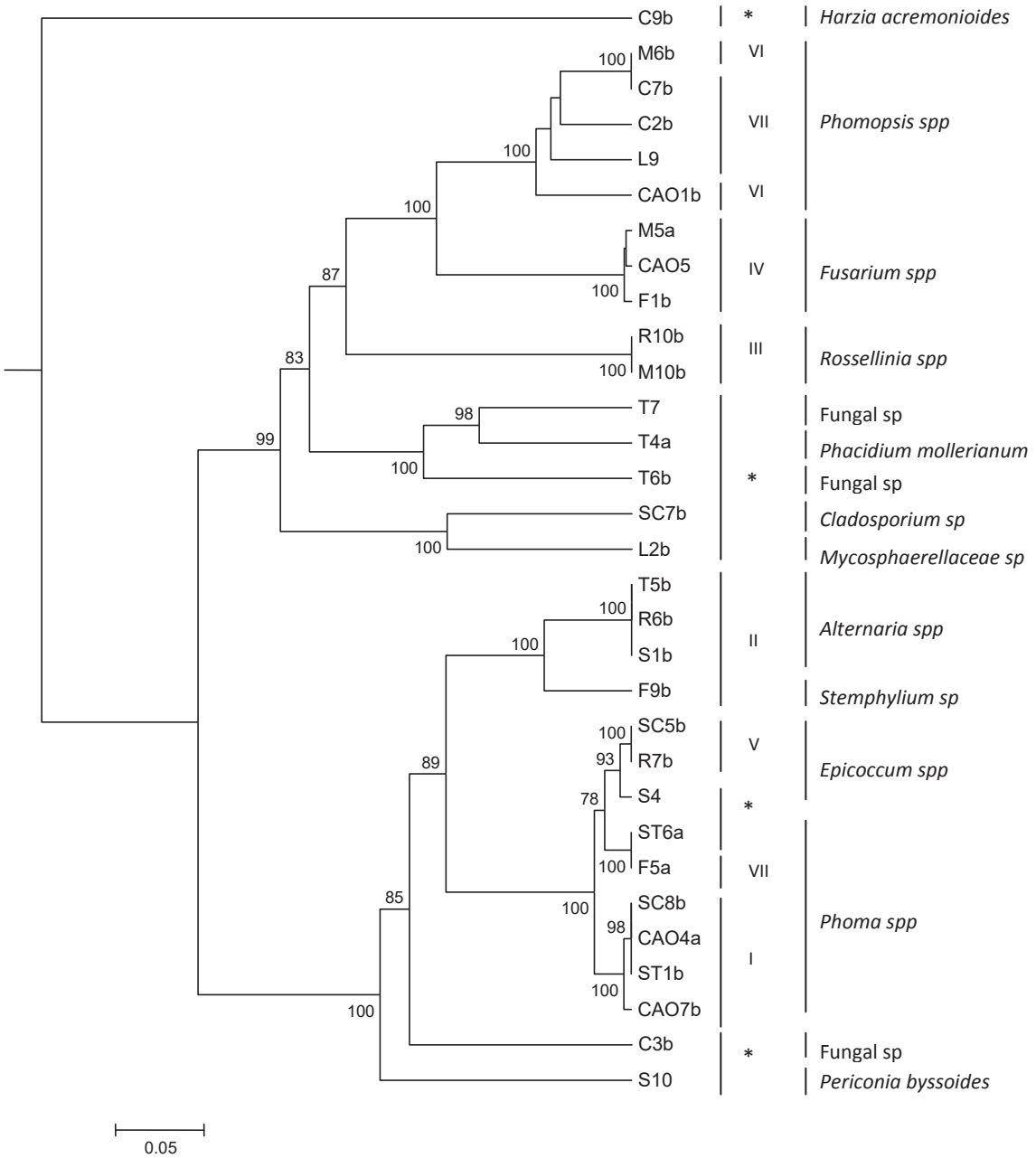

Figure 1. UPGMA tree depicting the phylogenetic relationship between the fungal isolates. Bootstrap support resulting from 1000 replicates is shown on the internodes. The morphological groups and the related fungal genera or species are shown

tion rates of *Alternaria* spp could lead to a premature leaf abscission. Other shared taxa was *Gibberella avenacea* isolated from ash shoots by Bakys et al. 2009, and also obtained in the present work (synonym: *Fusarium avenaceum*, CAO5) (table 1). Therefore, we can observe a similar fungal community isolated from common ash trees, independently of the study area. Concerning pathogenicity, *Phoma exigua* was supposed to cause brown spotting on shoots of European ash seedlings, being responsible for mass dying of seedlings at nurseries in Normandy, France (Boudier 1994). According to Bakys et al. 2009, four species (*A. alternata*, *E. nigrum*, *Phomopsis* sp., and *H. frax-*

ineus) isolated from ash were able to cause necrotic lesions on bark and cambium after artificial inoculation of ash seedlings, however the lesions were relatively small and most of the inoculated trees remained visually healthy. In that study, severe disease symptoms were only observed on seedlings inoculated with *H. fraxineus*. These results are consistent with our field observations in Asturias, where all collected leaf samples showed disease symptoms, but most of the trees remain apparently healthy, and *H. fraxineus* was not isolated. In addition, there are different observations about the susceptibility of *F. excelsior* to ash dieback, partially influenced by climatic stressors such as drought and frost (Pukacki and Przybyl 2005), and possibly the synergistic action of several fungal species (Bakys et al. 2009). Therefore, environmental factors and fungal interactions in addition to genetic resistance should be considered as possible modifiers of the pathogenicity of ash mycobiota in nature. In conclusion, even though some of fungal species isolated in this work are known plant pathogens and they were likely responsible for the disease symptoms observed on *F. excelsior* leaves, their behaviour as pathogens of ash remains still unclear and further research about their role in ash would be highly interesting.

Acknowledgments

This work was financially supported by the Cost Action FP1103 'FRAXBACK' by granting a Short-Term Scientific Mission to E. Trapiello. Special thanks to Angel Trapiello and Horacio Chinchilla for their help in sample collection.

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