



# Expansion of Ash Dieback towards the scattered *Fraxinus excelsior* range of the Italian peninsula

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**Abstract** *Hymenoscyphus fraxineus*, causal agent of Ash Dieback, has posed a threat to *Fraxinus excelsior* (common ash) in Europe since the 1990s. In south-western Europe, optimal climatic conditions for *H. fraxineus* become scattered and host density decreases, reducing disease spread rates. To date, the Ash Dieback agent has not been reported from southern and most of central Italy, where native *F. excelsior* is present as small fragmented populations. This study examines the expansion of Ash Dieback into central Italy, and it considers the consequences of further local spread with regards to the loss of *F. excelsior* genetic resource. Symptomatic *F. excelsior* were sampled from sixteen sites in northern and central Italy during 2020. Specimens were analyzed with a culturomics and a quantitative PCR approach. A bibliographic search of *F. excelsior* floristic reports was conducted for the creation of a detailed range map. The combined use of both techniques confirmed the presence of *H. fraxineus* in all the sites of central Italy where host plants were symptomatic. These new records represent the southern limit of the current known distribution of this pathogen in Italy, and together with Montenegro, in Europe. The

characterization of the *F. excelsior* scattered range suggests that further spread of Ash Dieback across southern Italy is a realistic scenario. This presents a threat not just to the southern European proveniences of *F. excelsior*, but to the species as a whole, should Ash Dieback lead to the loss of warm climate adapted genetic material, which may become increasingly valuable under climate change.

**Keywords** *Hymenoscyphus fraxineus* · Common ash · Central Italy · Disease spread

## Introduction

*Hymenoscyphus fraxineus* (T. Kowalski) Baral, Que-loz & Hosoya, the causal agent of Ash Dieback disease, represents a serious threat for the common ash tree (*Fraxinus excelsior*) in the European continent (Kowalski 2006; Enderle et al. 2019). Wind-dispersed ascospores of *Hymenoscyphus fraxineus* form in apothecia on infected leaf petioles in the leaf litter (Kowalski and Holdenrieder 2009). The pathogen enters the host through leaves, young stalks and epicormic shoots and progresses from there to reach the woody tissue (Chandelier et al. 2013). Symptoms occur during spring in in the xylem and the bark of leaves, twigs, shoots and stems (Kowalski et al. 2010), causing wilting of leaves, shoot dieback, necrotic

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lesions in the bark and wood discoloration (Timmermann et al. 2011). In young ash trees, stem symptoms typically vary from a necrotic bark lesion with a small dead twig in the center to extensive bark necrosis (Kirisits et al. 2012). In this last case, apex dieback may occur in a few weeks. On older trees, ash dieback often becomes chronic. This leads to a progressive deterioration of the plant, predisposing it to other damaging factors, and eventually results in death (Timmermann et al. 2011).

The disease was reported for the first time in north-eastern Poland in 1993 (Kowalski 2006; Gil et al. 2017) and today is present in most parts of the ash distribution range in Europe. Anthropogenic factors, i.e. mainly the trade of infected plant material, and airborne dispersal of ascospores are the main pathways for spread of the pathogen to many countries (Sansford 2013; Drenkhan et al. 2017; Gil et al. 2017; Longauerová et al. 2017). The disease spread is fast, estimated to be in the range of 40–75 km a year on a continental/national scale (Gross 2013; Laivins et al. 2016). This value is likely the combined effect of human mediated and natural dispersal, since natural aerial spread for ascospores dispersal from inoculum sources has been reported to be as high as 1.4–2.6 km (Grosdidier et al. 2018a). More recently, extraordinary colonization rates of 50 km a year, driven by wind turbulences, were observed along the disease front of Northern Italy (Giongo et al. 2017) and France (Grosdidier et al. 2018b). Such impressive and fast long-distance movement is supported by the results of Grosdidier et al. (2018a), who detected aerial ascospores of *H. fraxineus* as far as 100 km beyond the disease front in France.

Moving south and west in the European continent, the spread of *H. fraxineus* rapidly decreases. The suitable environmental conditions for disease development are represented by abundant summer precipitation, high soil moisture and low air temperature (Dal Maso and Montecchio 2014). These conditions are common in central and northern European countries, where the pathogen spread fast from the early years of its establishment in the continent (Pautasso et al. 2013). As reported by Ghelardini et al. (2017) such environmental traits become scattered and geographically isolated in the European Mediterranean

countries, limiting the potential presence of *H. fraxineus* to the main mountain ranges over a certain altitude. In addition, the diminishing host density and increasing tree cover fragmentation are limiting factors to disease establishment (Grosdidier et al. 2020).

The southern and western limits of *H. fraxineus* in Europe are currently represented by Montenegro in the Balkans (Milenković et al. 2017), Central Italy (Ghelardini et al. 2017) and Central France (Grosdidier et al. 2018a). It is believed that beyond these areas, Mediterranean climatic conditions render unlikely the spread and establishment of Ash Dieback by natural dispersal (Dal Maso and Montecchio 2014). However, in southern Italy, sites suitable for the establishment of the fungus are present, especially along the Apennines chain (Dal Maso and Montecchio 2014; Ghelardini et al. 2017). In this area *F. excelsior*, even if rare, is diffused as small fragmented populations or as singular trees, across the entire mountain chain (Caudullo et al. 2017 and citations reported in Table 3). Consequently, human mediated or natural, localized introductions of *H. fraxineus* cannot be excluded.

The Apennines seem to have held a glacial refugium for at least one *F. excelsior* haplotype, which is now occurring throughout Italy and in south western France (Heuertz et al. 2004a). It is widely accepted that refugium populations may accumulate higher diversity and/or unique haplotypes owing to their persistence and relative stability over the glacial era (Hewitt 1996; Newton et al. 1999; Tzedakis et al. 2002). These populations are probably better suited to warmer climates and could be an important source of adaptive genes to survive a changing climate characterized by increasing temperatures. For these reasons, the spread of disease in these areas could represent a threat to the conservation of genetic diversity and destroy a useful source of genes for adaptation to climate change.

The aim of this study is to describe the present spread of the Ash Dieback epidemic in central Italy, and to draw attention to its possible threat for *F. excelsior* in the stands where the pathogen is not yet present.

## Material and methods

### Plant material and genomic DNA extraction

In August–September 2020, symptomatic trees of *F. excelsior* were sampled from 16 stands in northern and central Italy, along the Apennine Mountains (Table 1). Samples from sites 2 to 13 were collected in the area of the 2015 first report from the Apennines (Ghelardini et al. 2017; Luchi et al. 2016) in order to verify the consistency of local disease spread during the last years. Samples from sites 14 to 18 were collected across the three most relevant mountain massifs of central Italy, following the host natural range: Monti Sibillini National Park (14), Gran Sasso and Monti della Laga National Park (15 and 16) and Abruzzo, Lazio and Molise National Park (17 and 18). In this last location, *F. excelsior* was present but no symptoms of the disease were detected, therefore no samples were collected. (see Results). Symptoms included blackish discoloration of leaves; necrotic leaf rachises; wilting and leaf dropping; dieback of shoots, twigs and branches; necrosis of bark tissue.

Isolations were made from symptomatic branches and rachises after surface sterilization with sodium hypochlorite and ethanol according to EPPO protocol (Chandelier et al. 2013). Fragments of necrotic tissue were placed on 2% malt extract agar (MEA, DIFCO, Detroit, Michigan, USA) amended with streptomycin (0.050 gr/l) and incubated at 5 °C in the dark. After 2–4 weeks, small, dull white to fulvous brown colonies were observed and sub-cultured on MEA and maintained at 20 °C.

DNA was extracted from a portion of branches and rachises from each sampling site and from one apothecium collected in site 16 (Table 1). About 100 mg fresh weight each (the entire apothecium for the sample of site 16) was transferred to 2-ml microfuge tubes with two tungsten beads (3 mm) (Qiagen, Venlo, Netherlands) and 0.6-ml lysis buffer (EZNA Plant DNA Kit—Omega Bio-tek, Norcross, Georgia, USA), and ground with a Mixer Mill 300 (Qiagen) (2 min; 20 Hz). DNA was extracted from all samples using the EZNA Plant DNA Kit (Omega Bio-tek), following the manufacturer's protocol.

### Isolate sequencing and taxonomical analysis

Twelve representative isolates obtained in this study (Table 2) were used for phylogenetic analysis. Each isolate was grown on 300PT cellophane discs (Celsa, Varese, Italy) on MEA in 90-mm Petri dishes and maintained in the dark at 20 °C. After 15 days, mycelium was scraped from the cellophane surface, placed into 1.5 ml microfuge tubes (Sarstedt, Verona, Italy) and stored at -20 °C until use. DNA was extracted from all samples using the EZNA Plant DNA Kit (Omega Bio-tek), as previously described. The DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Amplification of the internal transcribed spacer ITS region (including spacers ITS1 and ITS2 and the 5.8S gene of the rDNA) was carried out by using ITS1 and ITS4 primers (White et al. 1990).

PCR reaction mixtures (25 µl) contained 1 µl of genomic DNA, 2.5 µl of each 10 µM primer, 0.5 µl of 10 mM dNTPs (GeneSpin, Milan, Italy), 2.5 µl of 10X PCR Buffer (GeneSpin), 3 µl of 25 nM MgCl<sub>2</sub> and 0.5 µl (5 U/µl) of Taq polymerase (TaqPol, GeneSpin). Amplifications were carried out in a Mastercycler (Eppendorf, Hamburg, Germany) using the following PCR conditions: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 90 s, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min; and a final elongation step at 72 °C for 10 min. (Migliorini et al. 2020). Amplification products were separated by electrophoresis on gels containing 1% (w/v) of agarose LE (GeneSpin). The approximate length (bp) of the amplification products was determined using the 100-bp DNA ladder Ready to Load (Genespin). PCR amplicons were purified with a miPCR Purification Kit (Metabion International, Planegg, Germany) and sequenced in both directions at Macrogen (Seoul, South Korea). The quality of amplified nucleotide sequences was checked with the software package Geneious version 10.0.9 (Biomatters, Auckland, New Zealand). Generated sequences were submitted to NCBI GenBank (Table 2) and the new obtained cultures were stored in the Fungal Collection of the Institute for Sustainable Plant Protection-National Research Council (IPSP-CNR, Sesto Fiorentino, Italy).

**Table 1** ID numbers and geographic coordinates of *Fraxinus excelsior* sites considered in the study. Plant samples in sites 1 to 16 were included in the qPCR assay. Positive amplification with *Hymenoscyphus fraxineus* specific probe is marked with “+”; no amplification with “-”. Matrix of DNA and

isolation origin is “B” for branch, “R” for rachis and “A” for apothecium. Isolates obtained in the current study are also reported. No symptomatic trees were present at Sites 17 and 18, hence, no plant material was collected

Site ID	Specimens origin	Location	Matrix	Isolate obtained in this study	Real-time PCR
1	Druogno (Verbano)	46.12850, 8.42457	B	CNRHfraxGRUVB	+
			R		+
2	Casalino (Reggio Emilia)	44.317441, 10.360392	B		+
			R		+
3	Dogana Nuova (Modena)	44.18689, 10.608621	B	CNRHfraxDNOV	+
			R		+
4	Vidiciatico (Bologna)	44.174649, 10.871913	B		+
			R		+
5	Dogana Nuova (Modena)	44.1666, 10.619277	B		+
			R		+
6	Lago Santo (Modena)	44.15476, 10.59614	B	CNRHfraxLST	+
			R		+
7	Abetone (Pistoia)	44.14765, 10.653841	B		+
			R		+
8	Abetone (Pistoia)	44.14165, 10.66979	B	CNRHfraxABT	+
			R		+
9	Pian di Novello (Pistoia)	44.11162, 10.69804	B	CNRHfraxPDNOV2, CNRHfraxPDNOV2.2	+
			R		+
10	Pian di Novello (Pistoia)	44.111629, 10.698042	B		-
			R		+
11	Ponte Petri (Pistoia)	44.03782, 10.87703	B	CNRHfraxPOPTR	+
			R		+
12	Acquerino (Pistoia)	44.016581, 11.014262	B		+
			R		+
13	Fiumicello (Forlì-Cesena)	43.949042, 11.746285	B		+
			R		+
14	Montefortino (Fermo)	42.91971, 13.27707	B	CNRHfraxINF13 CNRHfraxINF10RCHD	-
			R		-
15	Valle Vaccaro (Teramo)	42.59445, 13.47578	B	CNRHfraxVVAC	+
			R		-
16	Lago di Provvidenza (L'Aquila)	42.50950, 13.41206	A	CNRHalbLDPRO CNRHfraxLDP	-
			B		+
			R*		-
17	Camosciara (L'Aquila)	41.780699, 13.904723			
18	Opi (L'Aquila)	41.778854, 13.860062			

\* Litter rachis

#### Database for phylogeny

Genbank sequences used in this study were selected from the available literature according to the isolate's

provenience in the *H. fraxinus* whole range and the nucleotides polymorphisms present in each country (Table 2). This method provided a proper diagnostic output and, at the same time, it allowed the

**Table 2** Isolates included in the study. Isolates and ITS gene sequences generated in this study are shown in **bold**

Fungal species	Isolate number	Location	Host	GenBank accession number ITS	Reference
<i>Hymenoscyphus fraxineus</i>	<b>CNRHfraxGRUVB</b>	Verbano, Italy	<i>Fraxinus excelsior</i>	<b>MW238530</b>	This study
	<b>CNRHfraxDNOV</b>	Modena, Italy		<b>MW238533</b>	
	<b>CNRHfraxLST</b>	Parma, Italy		<b>MW238528</b>	
	<b>CNRHfraxABT</b>	Pistoia, Italy		<b>MW238534</b>	
	<b>CNRHfraxPDNOV2</b>	Pistoia, Italy		<b>MW238532</b>	
	<b>CNRHfraxPDNOV2.2</b>	Pistoia, Italy		<b>MW238529</b>	
	<b>CNRHfraxPOPTR</b>	Pistoia, Italy		<b>MW238525</b>	
	<b>CNRHfraxINF13</b>	Fermo, Italy		<b>MW238527</b>	
	<b>CNRHfraxINF10RCHD</b>	Fermo, Italy	<i>Fraxinus excelsior</i> (Rachis)	<b>MW238531</b>	
	<b>CNRHfraxVVAC</b>	Teramo, Italy		<b>MW238523</b>	
<i>Hymenoscyphus albidus</i>	<b>CNRHalbLDPRO</b>	L'Aquila, Italy	<i>Fraxinus excelsior</i> (Apotecium)	<b>MW238522</b>	
<i>H. fraxineus</i>	<b>CNRHfraxLDP</b>	L'Aquila, Italy	<i>Fraxinus excelsior</i>	<b>MW357546</b>	
	ST/FV/10–1	Austria	<i>Fraxinus</i> cf. <i>velutina</i>	KP994907	Genbank (in Pastircáková et al. 2020)
	ST/FL/8–3		<i>Fraxinus</i> cf. <i>latifolia</i>	KP994896	(Pastircáková et al. 2020)
	CBS 128,012	Belgium	Ash	FR667687	(Chandelier et al. 2011)
<i>H. albidoides</i>	HMAS 264,140	China	<i>Picrasma quassioides</i> (rotten leaf veins)	NR154903	(Zheng and Zhuang 2014)
<i>H. fraxineus</i>	HMAS266596		<i>Fraxinus</i>	KF188725	(Han et al. 2014)
	HMAS266581		<i>mandschurica</i>	KF188727	
	HMAS 266,580		Apotecium	KF188726	(Zheng and Zhuang 2014)
	5706_1; Kore_06	Croatia		KJ511201	(Gross et al. 2014)
	5707_1; Kore_07			KJ511202	
	71,026.1	Czech Republic	<i>Fraxinus excelsior</i>	FJ429386	(Han et al. 2014)
	H 191CRBKN			MG182858	Genbank (specimen from the Brno Herbarium)
	H 190CRMK			MG182857	
	H 192CRPV			MG182859	
	PRM:921,903			HF937558	Genbank (in Pastircáková et al. 2020)
071,026.1			GU586921	(Queloz et al. 2011)	
EST1809	Estonia	<i>Fraxinus sogdiana</i>	KM655828	(Drenkhan et al. 2015)	
E9		<i>Fraxinus excelsior</i>	GU797174	(Rytkönen et al. 2011)	
SOH-7665/416-M1	Far East Russia	<i>Fraxinus mandshurica</i>	KU323588	(Drenkhan et al. 2015)	

**Table 2** continued

Fungal species	Isolate number	Location	Host	GenBank accession number ITS	Reference
	SOH-7700-1 B/425-12			KU323576	
	TAAM132852; 3144-2			KP403806	
	Knip1	Finland	<i>Fraxinus excelsior</i>	GU797146	(Rytkönen et al. 2011)
	Knip4			GU797148	
	AIN-M-8	France		HM193468	(Husson et al. 2011)
	AMA-P-1			HM193455	
	90,807.1	Germany		GU586901	(Han et al. 2014)
	C421			KC576530	(Junker et al. 2014)
	C489			KC576535	
	EL120			KC576539	
		Italy		KY593990	(Giongo et al. 2017)
	CNRHfrax51.1			KT696595	(Luchi et al. 2016)
	7027_1; Hokk_27	Japan		KJ511195	(Gross et al. 2014)
	7074_1; Hokk_74			KJ511197	
	7010_1; Hokk_10			KJ511184	
	7149_1; Hokk_149			KJ511193	
	6943_1; Suga_43			KJ511214	
	6946_1; Suga_46			KJ511216	
	TNS-F12761		<i>Fraxinus</i>	AB705218	(Han et al. 2014)
	TNS-F40051		<i>mandschurica</i>	AB705220	
	KR55L	Lithuania	<i>Fraxinus excelsior</i>	KJ413058	Genbank (Specimen used in
	VIL39P			KJ820670	Burokiene et al. 2015)
	080,517.2			FJ429378	(Ioos et al. 2009)
	KR14P			KJ780084	(Schoebel et al. 2014)
	7404_1; Hanc_04	Poland		KJ511176	(Gross et al. 2014)
	7407_1; Hanc_07			KJ511177	
	R1D7		<i>Fraxinus excelsior</i>	MT053856	(Przybylski et al. 2020)
	R1D5			MT053857	
	NK2	Serbia		KX255648	(Keča et al. 2017)
	LH56	Slovakia	<i>Fraxinus bungeana</i>	MK491649	(Pastircáková et al. 2020)
	LH59		<i>Fraxinus excelsior</i>	MK491651	
	H145			MF175245	
	H149		<i>Fraxinus</i>	MF175246	
			<i>mandshurica</i>		
	KUS-F52255_1	South	<i>Fraxinus</i>	KP068053	(Gross and Han 2015)
	KUS-F52255_2	Korea	<i>rhynchophylla</i>	KP068080	
	KUS-F52610_2			KP068075	
	KUS-F52613_1		<i>Fraxinus chinensis</i>	KP068055	
			subsp.		
			<i>rhynchophylla</i>		
	KUS-F52784_2		<i>Fraxinus</i>	KP068073	
	8302_1; Upps_02	Sweden	<i>rhynchophylla</i>	KJ511221	(Gross et al. 2014)
	8303_1; Upps_03			KJ511222	

**Table 2** continued

Fungal species	Isolate number	Location	Host	GenBank accession number ITS	Reference
<i>H. albidus</i>	090,812.3c	Switzerland	Pseudosclerotium	GU586895	(Queloz et al. 2011)
<i>H. fraxineus</i>	KUE6P		<i>Fraxinus excelsior</i>	KJ820435	Genbank (Specimen used in Burokiene et al. 2015)
	KUE11L			KJ820505	
	6006_1; Aubo_06			KJ511175	(Gross et al. 2014)
	6003_1; Aubo_03			KJ511173	
	Fk138	Ukraine	<i>Fraxinus excelsior</i>	KC464347	(Davydenko et al. 2013)

taxonomical placement of our isolates according to their ITS gene region. An accurate search and collection of numerous *H. fraxineus* ITS sequences available in Genbank was conducted (data not shown). The database used in this study was then built selecting at maximum two sequences per each identical nucleotide sequence per country within those with a robust bibliographic reference. Belgium, Serbia and Ukraine represented exceptions, with only one available sequence each.

#### Phylogenetic analysis

BLAST searches of the generated sequences were done against the NCBI GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the most closely-related sequences. Sequences were compared to those of the *Hymenoscyphus fraxineus* isolates collected across the entire Eurasian range of this fungus (Table 2). The software package Geneious (Auckland, New Zealand) was used for manual optimisation and alignment (ClustalW) of the sequences. Gaps were treated as missing data. Phylogenetic analyses of all obtained sequences were performed using MEGA X (Kumar et al. 2018).

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-774.51) was shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology

with superior log likelihood value. This analysis involved 74 nucleotide sequences. There was a total of 423 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018; Stecher et al. 2020).

#### qPCR analysis

DNA samples extracted from ash branches, rachises (including samples from Ash Dieback free area, used as negative control) and apothecia were amplified in MicroAmp Fast 96-well Reaction Plates (0.1 mL) closed with optical adhesive, and using the StepOne-Plus™ Real-Time PCR System (Applied Biosystems, Life Science, Foster City, CA, USA). Real time PCR was assayed by using the *H. fraxineus* primers and probe designed by (Ioos et al. 2009), according to the EPPO protocol (Chandelier et al. 2013). Each DNA sample was assayed in duplicate. Two wells, each containing 5 µl of sterile water, were used as the no-template control.

Results were analyzed using an SDS 1.9 sequence detection system (Applied Biosystems) after manual adjustment of the baseline and fluorescence threshold. Quantification of *Hymenoscyphus fraxineus* DNA in the samples was made by interpolation from a standard curve generated with a *H. fraxineus* DNA standard that was amplified in the same PCR run. The standard curve was generated from ten fivefold serial dilutions (ranging from 10 ng/tube to 0.128 pg/tube) of a known concentration of *H. fraxineus* DNA (strain CBS 122,191) and analyzed in duplicate. The amount of *H. fraxineus* DNA from plant samples was indicated as pg of fungal DNA/µg of total DNA extracted.

## Mapping *Fraxinus excelsior* in the southern Apennines

The databases Google Scholar, Scopus and Web of Science were searched using the searchstring 'Fraxinus excelsior AND Apennines'. This search included both natural and planted nuclei. In addition, relevant studies were selected from among the papers that had cited the articles found in the search. A profuse search in grey literature was also done. We also contacted scientists and researchers to find upcoming but still unpublished studies of interest.

The results of this search were uploaded in the Free and Open Source QGIS format map to implement the Common ash distribution map in southern Italy. The linear distance between the different cores of common ash were calculated to verify the possibility of being infected by the aerial spread of the fungus and the timeframe for infection under a constant spread rate.

## Results

### Plant material

In sites 1 to 16 we found symptoms of the disease on mature and young plants as leaves wilting, bark necrosis, dieback of shoots and branches. Young plants (dbh about 4 cm) also presented symptoms of stem dieback. *Hymenoscyphus fraxineus*-like sporocarps were present on litter petioles in all sites except Site 13 (data not shown). Common ash plants in Site 17 and Site 18 did not present any disease symptoms, therefore, no specimens were collected.

### Phylogenetic analysis

BLASTn search in GenBank of sequences from all the isolates obtained in this survey revealed a 100% homology with *Hymenoscyphus fraxineus*. The ITS sequences (strains CNRHfraxGRUVB, CNRHfraxDNOV, CNRHfraxLST, CNRHfraxABT, CNRHfraxPDNOV2, CNRHfraxPDNOV2.2, CNRHfraxPOPTR, CNRHfraxINF13, CNRHfraxINF10RCHD, CNRHfraxVVAC, CNRHfraxLDP) have been deposited in Genbank under Accession Numbers MW238530, MW238533, MW238528, MW238534, MW238532, MW238529, MW238525,

MW238527, MW238531, MW238523, MW357546 (Table 2).

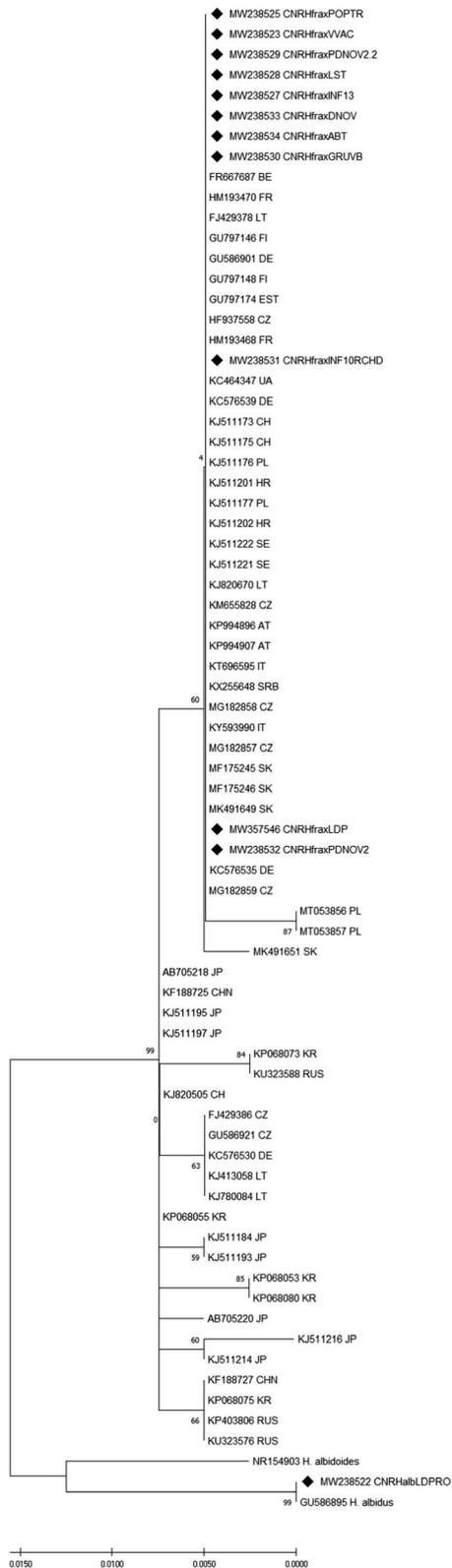
The ITS gene phylogenetic tree included 74 ingroup and no outgroup sequences, comprising 423 alignment characters, including gaps (Fig. 1). Phylogenetic analysis showed that the isolates obtained in the sample collection are *H. fraxineus*.

Fungal isolates CNRHfraxVVAC (MW238523), CNRHfraxPOPTR (MW238525), CNRHfraxINF13 (MW238527), CNRHfraxLST (MW238528), CNRHfraxPDNOV2.2 (MW238529), CNRHfraxDNOV (MW238533) and CNRHfraxABT (MW238534) differed from the rest of the sequences for a nucleotide deletion at position 395 (\*2 in Fig. 2). Isolates CNRHfraxGRUVB (MW238530), CNRHfraxINF10RCHD (MW238531), CNRHfraxLDP (MW357546) and CNRHfraxPDNOV2 (MW238532) did not present nucleotide differences with the larger group of *H. fraxineus* sequences retrieved in Genbank (\*1 in Fig. 2).

Sequences MW238530 (CNRHfraxGRUVB), MW238531 (CNRHfraxINF10RCHD), MW357546 (CNRHfraxLDP), MW238532 (CNRHfraxPDNOV2) did not present nucleotide deformities with the larger group of *H. fraxineus* sequences included in this study and they are represented by the nucleotide description of the first line (\*1). Sequences MW238523 (CNRHfraxVVAC), MW238525 (CNRHfraxPOPTR), MW238527 (CNRHfraxINF13), MW238528 (CNRHfraxLST), MW238529 (CNRHfraxPDNOV2.2), MW238533 (CNRHfraxDNOV) and MW238534 (CNRHfraxABT) differed from the rest of the sequences for a nucleotide deletion at position 395 (line marked with \*2).

### qPCR analysis

Almost all samples from symptomatic plants were positive to the *H. fraxineus*-specific qPCR assay (0,001218—28830,11 pg *H. fraxineus* DNA/μg total DNA) (Table 1). The two samples from asymptomatic plants included as negative controls did not show any amplification signals, as expected. DNA from branches in Site 10 was negative, while rachises DNA from this site revealed the presence of *H. fraxineus* in the site. The apothecia and the rachides collected in Site 16 did not produce a positive amplification signal. Indeed, ITS gene sequencing revealed that the apothecia were from *Hymenoscyphus*



◀ **Fig. 1** Phylogenetic tree, based on an ML analysis of DNA dataset of ITS sequences for *Hymenoscyphus fraxineus* species. Numbers above the branches indicate ML bootstraps (MLBS  $\geq 50\%$ ). Isolates from the current study are signed with a rhombus. The scale bar indicates the number of substitutions per site

*albidus*, (sample CNRHalbLDPRO) *Hymenoscyphus fraxineus* was, however, confirmed from Site 16, with the fungus both isolated and detected with qPCR in branch samples. No quantitative data could be provided for Site 14 because the plant samples were lost before the quantitative analysis.

### Mapping *Fraxinus excelsior* in the southern Apennines

The results of this search are reported in Table 3 and synthesized in Fig. 3.

Common ash scattered nuclei together with the closest limit on the main range have an average linear distance of 57 (min. 3- max. 110) km from one another (excluding the locations in Sardinia and Corsica islands).

The linear distance between the most southern report of the disease in 2015 (Castellaro di Galeata (FC) (Ghelardini et al. 2017), Fig. 3) and in 2020 (Lago di Provvidenza (TE), Site 16) is 201 km. The distance between the present report of *H. fraxineus* (Site 16) and the southern border of the host's natural range (Longi, Alcara Li Fusi, Tassita (ME) (Schicchi and Marino 2011; Caudullo et al. 2017); Table 3) is about 330 km (this last measure is not linear and It corresponds to the sum of linear distances between common ash scattered nuclei).

### Discussions

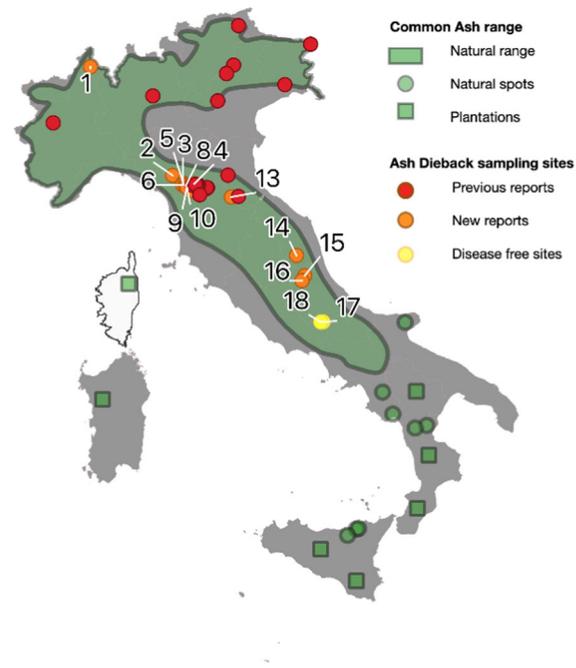
To the best of our knowledge, this is the first report of *Hymenoscyphus fraxineus* in Marche and Abruzzo regions along the central Apennines, an area characterized by mountain Mediterranean climatic conditions, with cold and snowy winters and cool summers without drought (Santini et al. 2010; Pesaresi et al. 2014). Detection of *H. fraxineus* in branches and rachises proved that the pathogen is diffused across all central Italy; it has moved from its previous



**Table 3** Common ash scattered nuclei used for the construction of the fragmented species range in Fig. 3. Names of the locations and origin of the plants were retrieved from the

corresponding references. Geographical coordinates were deduced from the data reported in the references

Location	Coordinates	Origin	References
Valle d'Orezza (Bastia, France)	42,3637; 9,4023	Synanthropic	(Caudullo et al. 2017)
Gargano (Foggia)	41,7701; 15,8381	Natural	
Campomaggiore (Potenza)	40,5447; 16,1056	Synanthropic	
M. Alburno (Salerno)	40,5325; 15,3096	Natural	(Rosati et al. 2010)
Semestene (Sassari)	40,3983; 8,7081	Synanthropic	(Caudullo et al. 2017)
Morigerati (Salerno)	40,1356; 15,5543	Natural	(De Natale 2004)
Monte Sparviere (Cosenza)	39,9255; 16,3570	Natural	(Barberis et al. 2014)
Morano Calabro (Cosenza)	39,88; 16,08	Natural	(Heuertz et al. 2004a)
Carmigliatello Silano (Cosenza)	39,3714; 16,4078	Synanthropic	(Caudullo et al. 2017)
Cinquefrondi (Reggio Calabria)	38,4073; 16,1282	Synanthropic	
Longi (Messina)	38,0312; 14,7506	Natural	(Schicchi and Marino 2011; Caudullo et al. 2017)
Alcara Li Fusi (Messina)	38,0198; 14,6981	Natural	
Tassita (Messina)	37,9048; 14,4928	Natural	
Villalba (Caltanissetta)	37,6389; 13,8549	Synanthropic	(Caudullo et al. 2017)
Chiaramonte Gulfi (Ragusa)	37,0422; 14,6980	Synanthropic	

**Fig. 3** Spatial range map of *Fraxinus excelsior* (see Table 3 for references) and location of 18 sites visited in Italy during 2020. Orange dots are sites where *Hymenoscyphus fraxineus* was detected during the 2020 surveys. Yellow dots are sites where no samples were collected because plants did not present ash dieback symptoms. Red dots are the sites of previous *H. fraxineus* reports in Italy

except Site 13 where no fungal cultures were obtained, despite the positive qPCR result for this site. In this locality, symptomatic plants were at low altitude (590 m a.s.l.), which lies outside of the optimum ash growing conditions. The samples were collected at the end of the summer, and consisted of seedlings used to establish a new forest plantation during spring 2020. Studies comparing the efficiency of qPCR and isolation methods for Ash Dieback are lacking, and possible explanations for the inconsistent results for Site 13 can only be hypothesized based on biological traits of the fungus. To date, significant effects of high temperature on *H. fraxineus* have been demonstrated in lab conditions, but not yet in the field (Hauptman et al. 2013; Grosdidier et al. 2018b). This suggests that the mycelium can persist in the host tissues exposed to heat across the southern areas of its geographic diffusion in a summer-latent form. Such low viability may negatively affect isolation success, but not the qPCR detection, which can detect very low quantities of target DNA, as low as 20 fg of *H. fraxineus* DNA (Ioos et al. 2009). This may explain our failure to isolate *H. fraxineus* from symptomatic tissue of Site 13.

So far, symptoms of *H. fraxineus* have never been reported before at latitude below 43° 57'N (Ghelardini et al. 2017). This, together with our observations of initial symptoms during sampling, seems to suggest that the disease in site 14, 15 and 16 has begun very recently, possibly in 2019. This also indicates that *H. fraxineus* has likely moved about 201 km in the past four-five years (40–50 km/year). No symptomatic adult or young trees were found at Site 17 and Site 18, therefore we should consider Site 16 as the southernmost front of the disease spread. Further diffusion is still possible, however, along the natural range and also in the scattered range areas of the host plant, as despite the discontinuous nature of the populations, the distances are within the spread ability of the disease (Giongo et al. 2017; Grosdidier et al. 2018b) or of the ascospores aerial transport (Grosdidier et al. 2018a).

If the pathogen continues to spread at the same rate as observed in the present study, it can reach the southern border of the *F. excelsior* natural range in five more years and spread over the scattered populations in the entire Italian peninsula and Sicily Island. The scenario is likely to accelerate in case of unintentional human-aided spread, by the introduction of infected propagating material in areas still free from the disease.

As reported by Marigo et al. (2000), drawing the limits of *F. excelsior* in southern Europe is difficult, especially because its distribution is scattered in small groups of trees. However, following the literature review (Table 3) this study demonstrates that the natural occurrence of common ash, whose presence in the southern Apennines was generally neglected, is quite common, at least in sites where environmental conditions are favorable. We also observed that some common ash plantations were established, but data are not available, due to their occasionally and lack of financial value, or because they were planted not as forest but as ornamental trees. The role of planted trees should not be underestimated because they can act as aerial spore “bridges” between already infected and uninfected sites.

A significant additional risk factor in the disease diffusion may come from the natural widespread presence in central and southern Italy of *Fraxinus angustifolia*, a species also susceptible to *Hymenoscyphus fraxineus*. Infections similar to those caused on common ash have been reported in Austria and

Hungary (Kirisits et al. 2010), Croatia (Barić et al. 2012), Serbia (Keča et al. 2017) and Slovakia (Kádasi-Horáková et al. 2017).

Although *F. angustifolia* is more thermophilic than *F. excelsior*, they are nevertheless sympatric along river-banks and in very humid valleys (Heuertz et al. 2006). For this reason it is reasonable to assume that where their presence intersects with the optimal environmental conditions for pathogen growth e.g. abundant summer rainfall, high soil moisture and low air temperature (Dal Maso and Montecchio 2014) *F. angustifolia* can act as a bridgehead for Ash Dieback. Identifying these sites and overseeing the possible arrival of the disease should be a priority to protect common ash biodiversity. It must be said that, unlike *F. excelsior*, the disease would not threaten the survival of this species, since its range covers also areas with a Mediterranean climate with summer aridity, where *H. fraxineus* cannot thrive.

Even if poorly studied, southeastern Europe, including south Italy, is considered a putative ice refuge for common ash (Heuertz et al. 2004b, 2006). In southeastern Europe, common ash populations are characterized by a strong genetic structure and by a marked genetic differentiation between neighboring populations (Heuertz et al. 2004b). In this area, the arrival of the pathogen by natural aerial spread, as well as the introduction of common ash from disease infected nurseries, may threaten near to extinction not the species itself, but the many *F. excelsior* small scattered marginal populations that probably conserve rare forms, harbor a wide genetic diversity and hold rare genes that could be particularly useful to improve the plasticity of this species to a changing climate. There are real risks that repeated localized tree losses and a reduction in the number of genets that effectively contribute to offspring can lead to long-term genetic depletion. This scenario is likely to be realistic in the environmental conditions typical of the central and southern Apennines, where the limiting environmental conditions for *H. fraxineus* (35°–36 °C) reported by Hauptman et al. (2013) and Grosdidier et al. (2018b) occur with a very low frequency.

In order to preserve the genetic heritage of common ash populations in this marginal portion of the range, the implementation of disease monitoring for *H. fraxineus* spread in the remaining southern stands will be crucial. According to Pautasso et al. (2013), common ash conservation strategies will need a total

mapping of *F. excelsior* distribution along the southern Apennines, subsequent plant phenotypic and genomic characterization and a disease resistance screening within all the proveniences.

Genotype x environment interaction is known to play a primary role in modulating the host response to pathogens (Bruck and Manion 1980; Manion 1986; Santini et al. 1997). Results from other pathosystems (Santini et al. 2005; Ahrens et al. 2019) provide evidence that plants have evolved different adaptive growth and defense strategies according to the environmental conditions in the area of origin and that these traits are stable over the years and sites (Ghelardini et al. 2006). This stability gives hope for setting up successful management strategies to provide greater resilience for natural ecosystems to epidemics caused by an introduced pathogen.

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**Author contributions** DM and AS conceived the study. DM, NL, EN, ALP, FP and AS collected and prepared the samples, and analysed data. DM wrote the first draft and all the others contributed to the following versions. All authors read and approved the final manuscript.

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#### Declarations

**Conflict of interest** The author declares their no conflict of interest.

**Data availability** Fungal sequences generated in this study were submitted to NCBI GenBank (sequence codes are reported in the text) and the new obtained cultures were stored in the Fungal Collection of the Institute for Sustainable Plant Protection-National Research Council (IPSP-CNR, Sesto Fiorentino, Italy).

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