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Hymenoscyphus fraxineus is a leaf pathogen of local *Fraxinus* species in the Russian Far East

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Dieback of European ash was first observed in Europe in the early 1990s. The disease is caused by the invasive ascomycete *Hymenoscyphus fraxineus,* proposed to originate from Far East Asia, where it has been considered a harmless saprotroph. This study investigates the occurrence of *H. fraxineus* in tissues of local ash species in the Russian Far East, and assesses its population-specific genetic variation by ITS sequencing. Shoot dieback symptoms, characteristic of *H. fraxineus* infection on European ash, were common, but not abundant, on *Fraxinus mandshurica* and *Fraxinus rhynchophylla* trees in Far East Russia. High levels of pathogen DNA were associated with necrotic leaf tissues of these ash species, indicating that the local *H. fraxineus* population is pathogenic to their leaves. However, the low levels of *H. fraxineus* DNA detected in shoots with symptoms, the failure to isolate this fungus from such tissues, and the presence of other fungi with pathogenic potential in shoots with symptoms indicate that local *H. fraxineus* strains may not be responsible (or their role is negligible) for the observed ash shoot dieback symptoms in the region. Conspicuous differences in ITS rDNA sequences detected between *H. fraxineus* isolates from Russian Far East and European populations suggest that the current ash dieback epidemic in Europe might not directly originate from the Russian Far East. Revision of the herbarium material shows that the earliest specimen of *H. fraxineus* was collected in 1962 from the Russian Far East and the oldest *H. fraxineus* specimen of China was collected in 2004.

Keywords: Chalara fraxinea, *Fraxinus mandshurica*, *Fraxinus rhynchophylla*, herbaria, *Hymenoscyphus pseudoalbidus*, invasion

Introduction

Dieback of European ash (*Fraxinus excelsior*) was first observed in Poland in the early 1990s (Kowalski & Holdenrieder, 2009). The causal agent of ash dieback is *Hymenoscyphus fraxineus* (syn. *H. pseudoalbidus*, anamorph *Chalara fraxinea*), a discomycete from the order Helotiales. Owing to efficient spread by windborne ascospores, this pathogen is now present almost throughout the entire natural distribution range of European ash (McKinney *et al.*, 2014), and threatens the future of this tree species and associated biodiversity (Pautasso *et al.*, 2013).

Hymenoscyphus fraxineus has been proposed to be native to East Asia (Zhao *et al.*, 2013). In Asia, the first record of *H. fraxineus* is from Japan (under the name *Lambertella albida*) on petioles of decaying leaves of Mandshurian ash (*F. mandshurica*) and dates back to 1990 (Hosoya *et al.*, 1993). In the Russian Far East (Primorskiy Kray), the fungus was first documented in 2005 on rachises of Mandshurian ash (Baral & Bemmann, 2014). Two years later, the fungus was reported in South Korea on rachises and petioles of fallen leaves of Korean ash (*F. rhynchophylla*) and Mandshurian ash (Han *et al.*, 2014). In 2012, *H. fraxineus* was recorded in China for the first time, on rotten rachises, petioles and leaflet veins of Mandshurian ash (Zheng & Zhuang, 2014).

In contrast to European ash, Mandshurian and Korean ashes have been reported to exhibit no dieback symptoms in their native range, and, therefore, *H. fraxineus* was considered to be a harmless saprotroph on decomposing leaf tissues (Hosoya *et al.*, 1993; Zhao *et al.*, 2013; Han *et al.*, 2014; Zheng & Zhuang, 2014; Gross & Han, 2015). However, in European arboreta, typical dieback symptoms were observed on Mandshurian ash, although the degree of damage was considerably smaller compared to that observed on European ash (Drenkhan & Hanso, 2010). Following artificial inoculation of stem wounds of young Mandshurian ash seedlings, both European and Japanese strains of *H. fraxineus* caused necrotic lesions on shoots (Gross & Holdenrieder, 2015). In another artificial inoculation experiment in Europe,

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involving stem wounding, Japanese *H. fraxineus* strains caused significantly longer lesions on *F. excelsior* and *F. pennsylvanica* seedlings compared to European strains of the fungus (Gross & Sieber, 2016). Taken together, the inoculation studies suggest that the tested Japanese strains of *H. fraxineus* have pathogenic potential in shoots of European ash, *F. pennsylvanica* and also Mandshurian ash.

Several studies have been carried out to determine the genetic diversity, population structure, and pathways and patterns of spread of *H. fraxineus* in Europe. The general lack of population structure (Bengtsson *et al.*, 2012; Gross *et al.*, 2012, 2014; Burokiene *et al.*, 2015) and considerably lower allelic richness in European *H. fraxineus* populations compared to the Japanese populations (Zhao *et al.*, 2013; Gross *et al.*, 2014) suggest that the European population might have been established by a small number of fungal individuals. Moreover, based on comparative gene sequence analyses of elongation factor, calmodulin and actin, and internal transcribed spacer (ITS) regions of rDNA, Japanese, Chinese and Korean *H. fraxineus* populations show more genetic variation than the European population (Zhao *et al.*, 2013; Gross *et al.*, 2014; Han *et al.*, 2014). These studied Asian populations of *H. fraxineus* are genetically markedly different from the European population, and, thus, comparative genetic analyses of additional Asian populations are needed to localize the source population of the pathogen.

There is a long history of import of seeds and plants of Mandshurian ash from Russian Far East to the Baltic States (Drenkhan *et al.*, 2014). Therefore, the Russian *H. fraxineus* populations represent a potential source for introduction of the pathogen to Europe. The main aims of this study were to investigate (i) whether the local ash species in the Russian Far East show symptoms that are characteristic of ash dieback in Europe, (ii) the occurrence of *H. fraxineus* in such tissues, and (iii) the level of genetic variation in the ITS rDNA gene cluster among *H. fraxineus* strains from the region. To achieve these goals, fruit bodies of *Hymenoscyphus* spp. as well as leaves, shoots and seeds were collected from Mandshurian ash, Korean ash and unidentified *Fraxinus* species in Primorskiy Kray and Khabarovskiy Kray regions and subjected to molecular analyses.

In addition, herbarium specimens of *Hymenoscyphus* spp. collected earlier from Far East Asia (Russian Far East and northeastern China) were investigated to determine the historical presence of *H. fraxineus* in Far East Asia.

Materials and methods

Collection of field samples and herbarium specimens

A field expedition to the Russian Far East (Primorskiy Kray and Khabarovskiy Kray regions) was organized between August and September 2014 to sample *H. fraxineus*-like ascomata and leaves, shoots and seeds of local ash species (Tables 1 & 2). Identification of *Fraxinus* species was performed according to Krüssmann (1965), Wallander (2012), and the Oleaceae information site (www.oleaceae.info).

Nine herbarium specimens of *Hymenoscyphus* fruit-bodies, collected in the Russian Far East during 1962–2012 and northeastern China during 2003–2004, and originally identified as *Hymenoscyphus caudatus*, *H. albidus* or *H. epiphyllus* (Table 1), were subjected to molecular identification.

Fungal isolation and DNA extraction

Fungi were isolated from necrotic shoot lesions of *Fraxinus* spp. (nine different shoot samples) growing in the Russian Far East by following the procedures described in Drenkhan & Hanso (2010). Briefly, shoot surfaces were sterilized with 96% ethanol before bark removal. Tissue pieces were cut from the lower edge of discoloured xylem associated with up to 1-year-old bark lesions, placed on malt extract agar (MEA) in Petri plates and incubated for 2 weeks at room temperature in the dark. Pure cultures were obtained by subculturing the emerging fungal colonies onto fresh MEA medium.

For molecular analysis, ash tissues (36 leaf, 32 shoot and 2 seed samples; Tables 1, 2 & S1) and isolated fungal cultures were homogenized in an MM400 homogenizer (Retsch GmbH) with the aid of steel beads, and subjected to DNA extraction with E.Z.N.A Fungal DNA Mini kit (Omega Bio-Tek Inc.) or DNeasy Plant Mini kit (QIAGEN), according to the manufacturers' instructions. Ascocarps of *H. fraxineus* (24 samples; Table 1) were either homogenized with an MM400 homogenizer and subjected to DNA extraction using the E.Z.N.A Fungal DNA Mini kit, or were ground manually in Eppendorf tubes using a pestle and sterile quartz sand, and subjected to DNA extraction following 'protocol 8' (Isolation of DNA from Mouse Tails) of the Easy-DNA kit (Invitrogen). Shoot bark was removed before DNA extraction.

ITS rDNA PCR for sequence analysis

For DNA extracted from pure fungal cultures and fruit-body samples, the PCR was carried out using primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes & Bruns, 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990) and HotStarTaq Plus DNA polymerase (QIAGEN) in a 50 μL reaction volume. PCR mixture and cycling conditions were adjusted following instructions of the polymerase manufacturer.

For detection of *H. fraxineus* from DNA extracted from plant tissues and ascocarps, species-specific primed PCR (SSPP) was performed using the primer pair HFrax-F (5′-CTTTAG CAGGTCGCCCTCT-3′) and HFrax-R (5′-TGCTGGCAAGA CACCGCAA-3′) (Drenkhan *et al.*, 2016). For SSPP detection of *H. albidus* the primer pair HAlbid-F (5′-GACCGTGCCTGCTA GAGGAT-3′) and HAlbid-R (5′-GGTTTCTGGCAAGACACC TC-3′) (Drenkhan *et al.*, 2016) was used. For both assays, the PCR mixture and cycling conditions were as described by Drenkhan *et al.* (2016).

PCR products were visualized on 1% agarose gel (SeaKem LE agarose; Lonza) under UV light, and DNA fragments corresponding to the expected PCR product size were purified with MinElute PCR purification kit (QIAGEN) and then sequenced in both directions on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The DNA samples from fruit-bodies and plant tissues were sequenced using the primer pairs ITS-1F and ITS4, and HFrax-F and HFrax-R, respectively. The sequences obtained were edited using BioEdit v. 7.2.5 (Hall, 1999) and

Table 1 Herbarium and fresh specimens of *Hymenoscyphus* fruit-bodies on *Fraxinus* spp. leaf, shoot and seed samples examined in the study

No.	Herbarium and/or collection number[a]	Host species	Analysed tissue	Sampling locality[b] or geographical coordinates	Sampling date and preliminary identification in herbaria	GenBank accession no.	Identification by SSPP[c]		Similarity to ITS sequence of *H. fraxineus* (%)[d]	
							H. fraxineus	*H. albidus*	KF188724 (Chinese)	FJ597975 (European)
Herbarium samples										
1	D-544	*Fraxinus* sp.	Petiole	FE Rus, Ussuriyskiy, Suputinskiy	16/08/1962; *H. caudatus*	KR733601	+	–	99.7	99.4
2	D-547	*Fraxinus* sp.	Ascocarp	FE Rus, Primorye, Ussuriyskiy	17/08/1962; *H. caudatus*	—	–	–	—	—
3	TAAM60020	*Fraxinus* sp.	Petiole	FE Rus, Primorye, Suputinskiy Reserv.	17/08/1962; *H. caudatus*	—	–	–	—	—
4	D-2613	*Fraxinus* sp.	Ascocarp	NE China, Heilongjiang province, Hulin	02/09/2003; *H. albidus*	KU569309[e]	–	–	—	—
5	D-2798	*Fraxinus* sp.	Petiole	FE Rus, Bol'sehihtsir	22/07/2004; *H. albidus*	KR733600	+	–	100.0	99.5
6	D-2695	*Fraxinus* sp.	Ascocarp	NE China, Heilongjiang province	06/08/2004; *H. albidus*	KR733602	+	–	100.0	99.4
7	D-2697	*Fraxinus* sp.	Petiole	NE China	06/08/2004; *H. albidus*	—	+	–	—	—
8	D-3393	*Fraxinus* sp.	Ascocarp	FE Rus, Udege	03/08/2012; *H. epiphyllus*	—	+	–	—	—
9	D-3416	*Fraxinus* sp.	Petiole	FE Rus, Udege	05/08/2012; *H. epiphyllus*	—	+	–	—	—
Samples collected in Far East Russia during the present study										
10	SOH-7664/416-R7	*Fraxinus rhynchophylla*	Ascocarp	43.0507°N, 131.8620°E	26/08/2014	KU323580	nt	nt	99.7	99.3
11	SOH-7665/416-M1	*Fraxinus mandshurica*	Ascocarp	43.0507°N, 131.8620°E	26/08/2014	KU323588	nt	nt	99.6	99.2
12	SOH-7663/416-R6	*F. rhynchophylla*	Ascocarp	43.0507°N, 131.8620°E	26/08/2014	KU323577	nt	nt	99.7	99.3
15	SOH-7664-2 B	*F. rhynchophylla*	Ascocarp	43.0507°N, 131.8620°E	26/08/2014	KU323587	nt	nt	100.0	99.5
16	TAAM132851; 3142-3	*F. mandshurica*	Ascocarp	43.2240°N, 131.9919°E	29/08/2014	KR733591	+	–	99.7	99.3
17	SOH-7712/428-5	*F. mandshurica*	Ascocarp	43.2240°N, 131.9919°E	29/08/2014	KU323586	nt	nt	100.0	99.5
18	TAAM132850; 3146-2	*F. mandshurica*	Ascocarp	43.6442°N, 132.3439°E	28/08/2014	KR733592	+	–	100.0	99.5
19	SOH-7698/425-8	*F. mandshurica*	Ascocarp	43.6442°N, 132.3439°E	28/08/2014	KU323579	nt	nt	100.0	99.5
20	SOH-7705/425	*F. mandshurica*	Ascocarp	43.6442°N, 132.3439°E	28/08/2014	KU323585	nt	nt	100.0	99.5
21	SOH-7696/425/4	*F. mandshurica*	Ascocarp	43.6442°N, 132.3439°E	28/08/2014	KU323584	nt	nt	100.0	99.5
22	SOH-7697 B/425	*F. mandshurica*	Ascocarp	43.6442°N, 132.3439°E	28/08/2014	KU323583	nt	nt	100.0	99.5
23	SOH-7700-1 B/425-12	*F. mandshurica*	Ascocarp	43.6442°N, 132.3439°E	28/08/2014	KU323576	nt	nt	99.7	99.3
24	4088	*F. mandshurica*	Ascocarp	43.0198°N, 131.8650°E	26/08/2014	—	+	–	—	—
25	3741	*F. rhynchophylla*	Leaf	43.0287°N, 131.8044°E	26/08/2014	—	+	–	—	—

(continued)

Table 1 (continued)

No.	Herbarium and/or collection number[a]	Host species	Analysed tissue	Sampling locality[b] or geographical coordinates	Sampling date and preliminary identification in herbaria	GenBank accession no.	Identification by SSPP[c]		Similarity to ITS sequence of *H. fraxineus* (%)[d]	
							H. fraxineus	*H. albidus*	KF188724 (Chinese)	FJ597975 (European)
26	3739	*F. rhynchophylla*	Leaf	43.6442°N, 132.3439°E	28/08/2014	—	–	–	—	—
27	3740	*F. rhynchophylla*	Leaf (petiole)	43.6442°N, 132.3439°E	28/08/2014	—	+	–	—	—
28	3733-1	*F. mandshurica*	Seed	43.6442°N, 132.3439°E	29/08/2014	—	+	–	—	—
29	3733-2	*F. mandshurica*	Seed	43.6442°N, 132.3439°E	29/08/2014	—	+	–	—	—
30	3738	*F. mandshurica*	Shoot	43.0287°N, 131.8044°E	26/08/2014	—	–	–	—	—
31	SOH-7673-2/418-3	*F. mandshurica*	Shoot	43.0287°N, 131.8044°E	26/08/2014	KU323589	nt	nt	99.8	99.5
32	3734	*F. mandshurica*	Shoot	43.6442°N, 132.3439°E	28/08/2014	KR733596	+	–	100.0	99.4
33	3735	*F. mandshurica*	Shoot	43.6442°N, 132.3439°E	28/08/2014	KR733597	+	–	100.0	99.4
34	TAAM132852; 3144-2	*F. mandshurica*	Ascocarp	48.2830°N, 134.8332°E	01/09/2014	KP403806	+	–	99.7	99.3
35	SOH-7794/441	*F. mandshurica*	Ascocarp	48.2824°N, 135.3209°E	03/09/2014	KU323582	nt	nt	100.0	99.5
36	SOH-7746/435	*F. mandshurica*	Ascocarp	48.2830°N, 134.8332°E	01/09/2014	KU323578	nt	nt	99.7	99.3
37	3145-3	*F. mandshurica*	Ascocarp	48.2879°N, 134.8034°E	01/09/2014	KP403807	+	–	99.5	99.1
38	3145-2	*F. mandshurica*	Ascocarp	48.2879°N, 134.8034°E	01/09/2014	—	+	–	—	—
39	SOH-7760 B/437-4	*F. mandshurica*	Ascocarp	48.2879°N, 134.8034°E	01/09/2014	KU323581	nt	nt	99.7	99.3
40	SOH-7723/429-3	*F. mandshurica*	Ascocarp	48.3075°N, 134.8220°E	30/08/2014	KU323575	nt	nt	99.7	99.3
41	3736	*Fraxinus* sp.	Leaf	48.3051°N, 134.8116°E	03/09/2014	—	+	–	—	—
42	3730	*F. mandshurica*	Leaf	48.3051°N, 134.8116°E	04/09/2014	—	–	–	—	—
43	3731	*F. mandshurica*	Leaf	48.3051°N, 134.8116°E	04/09/2014	KR733594	+	–	99.7	99.4
44	3732	*F. mandshurica*	Leaf	48.3051°N, 134.8116°E	04/09/2014	KR733595	+	–	100.0	99.4
45	3729	*F. mandshurica*	Leaf	48.3051°N, 134.8116°E	04/09/2014	KU323574	+	–	99.7	99.4
46	3111	*F. mandshurica*	Leaf (vein)	48.3051°N, 134.8116°E	04/09/2014	KR733593	+	–	100.0	99.4
47	3743	*F. mandshurica*	Shoot	48.2830°N, 134.8332°E	01/09/2014	—	+	–	—	—
48	3744	*F. mandshurica*	Shoot	48.2830°N, 134.8332°E	01/09/2014	—	+	–	—	—

nt, not tested.

[a]TAAM, Mycological herbarium of the Estonian University of Life Sciences; D, Herbarium of the Institute of Biology and Soil Sciences of the Russian Far East.

[b]FE Rus, Far East Russia.

[c]SSPP, species-specific primed PCR.

[d]The underlined sequences are of full length, i.e. they include the entire ITS1 and ITS2 sequences.

[e]Identified by ITS sequencing as *Hymenoscyphus caudatus*.

Table 2 qPCR detection of *Hymenoscyphus fraxineus* DNA in *Fraxinus* spp. tissues collected in the Russian Far East in 2014

Sample ID	Sample presented in Figure	Host species	Sampling coordinates	Tissue analysed	C_t value		Amount of DNA (ng)
					Cfrax- P[a]	Hf_Rus-P[b]	
SOH-7672-1	3a	*Fraxinus rhynchophylla*	43.0199°N, 131.8650°E	Shoot with bark lesion	n.d.	37.31	0.01
SOH-7672-2	3a	*F. rhynchophylla*	43.0199°N, 131.8650°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7809-1	3b	*Fraxinus* sp.	48.3686°N, 135.0693°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7809-2	3b	*Fraxinus* sp.	48.3686°N, 135.0693°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7810-1	3c	*Fraxinus* sp.	48.3686°N, 135.0693°E	Shoot with bark lesion	n.d.	33.00	0.22
SOH-7810-2	3c	*Fraxinus* sp.	48.3686°N, 135.0693°E	Shoot with bark lesion	n.d.	34.74	0.06
SOH-7670-1	n.a.	*F. rhynchophylla*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7671-1	n.a.	*F. rhynchophylla*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7671-2	n.a.	*F. rhynchophylla*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	32.32	0.36
SOH-7673-1	n.a.	*F. mandshurica*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7673-2/418-3	n.a.	*F. mandshurica*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	33.73	0.01
SOH-7674-1	n.a.	*F. mandshurica*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7674-2	n.a.	*F. mandshurica*	43.0198°N, 131.8650°E	Shoot with bark lesion	n.d.	33.68	0.14
SOH-7778-1	n.a.	*F. mandshurica*	48.2844°N, 134.8720°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7778-2	n.a.	*F. mandshurica*	48.2844°N, 134.8720°E	Shoot with bark lesion	n.d.	34.75	0.06
SOH-7808-1	n.a.	*Fraxinus* sp.	48.3686°N, 135.0693°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-7808-2	n.a.	*Fraxinus* sp.	48.3686°N, 135.0693°E	Shoot with bark lesion	n.d.	n.d.	n.d.
SOH-1.422	4a	*F. mandshurica*	44.1982°N, 132.8144°E	Leaf necrotic tissue	n.d.	31.30	0.74
SOH-1.G422	4a	*F. mandshurica*	44.1982°N, 132.8144°E	Leaf green tissue	41.84	n.d.	n.d.
SOH-2.422	4b	*F. mandshurica*	44.1982°N, 132.8144°E	Leaf necrotic tissue	n.d.	35.94	0.03
SOH-2.G422	4b	*F. mandshurica*	44.1982°N, 132.8144°E	Leaf green tissue	41.27	n.d.	n.d.
SOH-3.422	4c	*F. mandshurica*	44.1982°N, 132.8144°E	Leaf necrotic tissue	42.08	28.33	6.11
SOH-3.G422	4c	*F. mandshurica*	44.1982°N, 132.8144°E	Leaf green tissue	n.d.	36.56	0.02
SOH-04-01-2g	4d	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf green tissue	41.86	34.15	0.10
SOH-04-01-3g	4d	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf green tissue	n.d.	n.d.	n.d.
SOH-04-01-2s	4d	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	40.13	25.93	33.57
SOH-04-01-3s	4d	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	n.d.	33.94	0.11
SOH-04-02-2g	4e	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf green tissue	n.d.	37.58	0.01
SOH-04-02-4g	4e	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf green tissue	n.d.	34.45	0.08
SOH-04-02-2s	4e	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	37.91	22.87	294.91
SOH-04-02-4s	4e	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	31.31	21.46	800.44
SOH-04-03-3g	4f	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf green tissue	n.d.	32.58	0.30
SOH-04-03-3s	4f	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	32.18	21.74	658.06
SOH-04-03-p1	4f	*F. mandshurica*	48.3051°N, 134.8116°E	Petiole necrotic tissue	32.81	19.75	2699.45
SOH-04-04-2g	4g	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf green tissue	n.d.	33.60	0.15
SOH-04-04-2s	4g	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	36.19	25.12	59.69
SOH-04-04-p1	4g	*F. mandshurica*	48.3051°N, 134.8116°E	Petiole necrotic tissue	34.31	20.58	1501.04
SOH-04-05-1s	4h	*F. mandshurica*	48.3051°N, 134.8116°E	Leaf necrotic tissue	41.55	30.26	1.56
SOH-04-05-p1	4h	*F. mandshurica*	48.3051°N, 134.8116°E	Petiole necrotic tissue	36.17	21.02	1098.46

n.a., not applicable; n.d., not detected.

[a]qPCR probe designed for detection of *H. fraxineus* by Ioos *et al.* (2009).

[b]qPCR probe designed for detection of *H. fraxineus* in the present study.

queried against ITS sequences in the NCBI GenBank sequence database and the Unite database (Kõljalg *et al.*, 2013).

Quantification of *H. fraxineus* DNA *in planta*

qPCR quantification of *H. fraxineus* DNA from plant tissue samples was performed using Takyon Low ROX Probe 2× MasterMix dTTP blue (Eurogentec). First, the primer and probe set (Cfrax-F 5′-ATTATATTGTTGCTTTAGCAGGTC-3′, Cfrax-R 5′-TCCTCTAGCAGGCACAGTC-3′, Cfrax-P 5′-FAM-CTCT GGGCGTCGGCCTCG-BHQ1-3′) was tested, which had been designed and validated for *H. fraxineus* specificity in real-time PCR by Ioos *et al.* (2009). Due to mismatch problems with the target sequence in the probe region, the Cfrax probe was substituted with a new probe, HfRus-P 5′-FAM-CTCTGGGGCGTCGG CCTCG-BHQ1-3′, designed in the present study, and containing an extra G at probe position 8 in comparison to the Cfrax-P probe. Standard curve samples containing known concentrations of *H. fraxineus* DNA were prepared from DNA extracted from a fruit-body collected in the Russian Far East (Table 1, collection no. 4088). To ensure that cycle threshold (C_t) values from the experimental samples fell within the standard curves and to eliminate effects of PCR inhibitory compounds potentially present in undiluted samples, a 10-fold dilution series (undiluted, 10- and 100-fold diluted) was prepared for all experimental samples. For each sample, all three DNA

concentrations were used as templates in the real-time PCR. Three microlitres of the DNA solution was used as a template for each 25 μL PCR reaction and each single-plex reaction was repeated twice. PCR cycling conditions were: initial denaturing step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 65 °C for 25 s. Fluorescence emissions were detected with ViiA 7 Real-Time PCR system (Applied Biosystems).

A standard curve was constructed, based on the relationship of C_t values and log-transformed known concentrations of DNA from pure cultures of *H. fraxineus*. This was used to determine the concentration of *H. fraxineus* DNA present in ash tissue samples. C_t values from 10-fold diluted templates were used for calculation of DNA concentration, because many undiluted DNA templates from ash tissues contained PCR inhibitory compounds leading to high C_t values outside the range of the standard curve. Tissue-specific differences in the amounts of *H. fraxineus* DNA were subjected to analysis of variance with an HSD post hoc test using SPSS v. 22.0 (IBM Inc.) and were considered statistically significant at $P \leq 0.05$.

Results

Occurrence and identification of *Hymenoscyphus*-like fruit-bodies in ash forests of the Russian Far East

In the Russian Far East, the summer of 2014 was one of the driest and warmest over the entire period of meteorological recordings in the region (www.meteoinfo.ru). In all surveyed stands of Mandshurian and Korean ash in Primorskiy Kray and Khabarovskiy Kray regions, a large amount of ash leaf petioles with dark pseudosclerotial plates were observed in leaf litter. *Hymenoscyphus* fruit bodies (Fig. 1) were commonly observed in moist ash stands, while the number of fruit-bodies was comparably low on dry sites. All attempts to isolate *H. fraxineus* from the ascomata and petioles with symptoms failed. All ITS sequences obtained from the collected *Hymenoscyphus*-like apothecia formed on petioles of Mandshurian and Korean ash corresponded to *H. fraxineus* (Table 1). Eight of these *H. fraxineus*-corresponding sequences were identical to each other, while the remaining sequences showed 99.3–99.7% sequence similarity to the most common sequence variant (Table 1). The differences among sequences were found in the internal transcribed spacer regions: ITS1 had up to one point mutation among samples and ITS2 up to two point mutations among the samples (Fig. S1). No obvious differences were observed between sequences of *H. fraxineus* obtained from Primorskiy Kray and Khabarovskiy Kray (distance between the sampling locations is about 650 km) or between sequences obtained from ascomata associated with Mandshurian and Korean ash.

Figure 1 Ascocarps of *Hymenoscyphus fraxineus* on leaf petioles of *Fraxinus* sp. in Russkiy Island, Primorskiy Kray, Far East Russia. [Colour figure can be viewed at wileyonlinelibrary.com].

Shoot symptoms and associated fungi

Many of the observed Mandshurian and Korean ash trees exhibited shoot dieback in the upper crown both in forest stands and in urban plantings of Vladivostok, these symptoms being most pronounced in Khabarovsk (Fig. 2). Shoot dieback was also observed on young Mandshurian and Korean ash trees in natural forests of Primorskiy Kray and Khabarovskiy Kray regions (Fig. 2). However, no samples were taken from the crowns of large trees with symptoms to examine whether the symptoms were related to *H. fraxineus* or other causes such as winter freezing or summer drought.

Isolations from the discoloured shoot xylem tissues associated with necrotic bark lesions (Fig. 3) on young Mandshurian ash and Korean ash trees yielded a range of different fungi, but not *H. fraxineus*. Among the isolated fungi, *Botryosphaeria dothidea*, *Eutypa astroidea*, *Melanconium oblongum*, *Phomopsis* sp. and *Paraconiothyrium brasiliense* were identified. Most abundant taxa were *Eutypa astroidea*, with three isolations (7%) out of 41 pure cultures, and *B. dothidea* and *Phomopsis* sp., with two isolations of each (Table S1). In two of these samples (3743 and 3744; Tables 1 & S1) the presence of *H. fraxineus* was detected by species-specific PCR.

Five out of six shoot samples from Mandshurian ash or *Fraxinus* sp. screened by species-specific PCR and/or examined by ITS sequence analysis showed presence of *H. fraxineus* (Table 1). However, in those cases when *H. fraxineus* was detected in shoots with symptoms, the amount of its DNA was very low (Table 2). Two of the *H. fraxineus* sequences obtained from shoots (Table 1) showed 100% similarity to the most common sequence variant obtained from ascomata.

Leaf symptoms and their association with *H. fraxineus*

In the surveyed regions, most of the broadleaved trees observed (e.g. *Juglans mandshurica*, *Betula* spp., *Ulmus* spp., *Acer* spp., *Populus* spp.) showed green foliage with occasional leaf spots, whereas many ash trees exhibited signs of leaf senescence. In natural forests surveyed in Primorskiy Kray and Khabarovskiy Kray, necrotic lesions on leaflet and leaf veins, and wilting of leaves were

Figure 2 Shoot dieback in the upper crown of a large Mandshurian ash tree near Bykova, Khabarovskiy Kray, Far East Russia (a) and in a naturally regenerated sapling of Korean ash (b) growing on a former agricultural land on Russkiy Island in Primorskiy Kray, Far East Russia. [Colour figure can be viewed at wileyonlinelibrary.com].

commonly observed on saplings of Mandshurian ash (Fig. 4). Seven out of nine leaf tissue samples from Mandshurian and Korean ash screened by species-specific PCR and/or examined by ITS sequence analysis showed presence of *H. fraxineus* (Table 1). Two of the obtained sequences showed 100% similarity to ITS sequences of DNA extracted from ascomata.

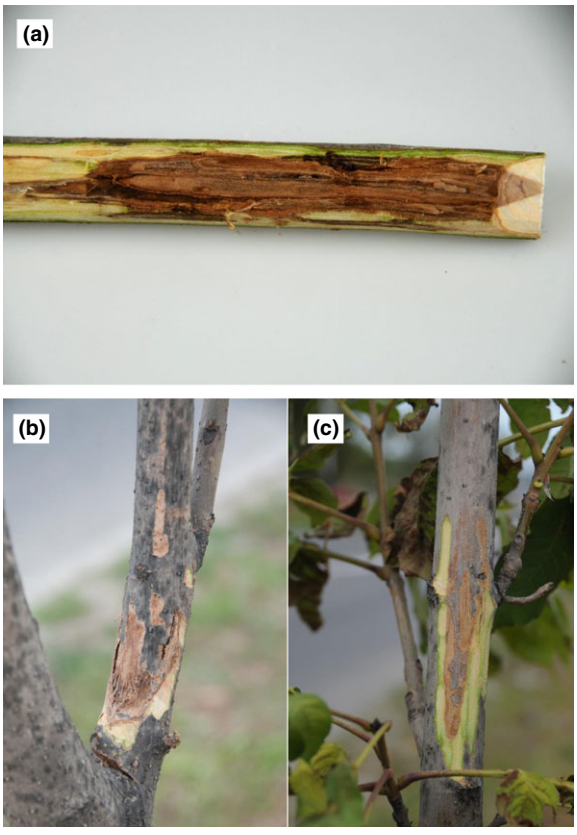

Figure 3 Up to 1-year-old necrotic lesions on shoots of *Fraxinus* spp. In the Russian Far East examined by qPCR for the presence and amount of *Hymenoscyphus fraxineus* DNA (see Table 2). (a) *F. rhynchophylla*, Primorskiy Kray, Russkiy Island, collection ID 7672; (b) *Fraxinus* sp., Khabarovsk, collection ID 7809; and (c) *Fraxinus* sp., Khabarovsk, collection ID 7810. [Colour figure can be viewed at wileyonlinelibrary.com].

In most samples originating from necrotic lesions on leaf blades and petioles, high levels of *H. fraxineus* DNA were detected by qPCR with the HfRus probe. In contrast, pathogen DNA levels in green (symptomless) regions of these leaves were negligible or not detected at all (Table 2). The difference between mean amounts of *H. fraxineus* DNA detected in symptomless (mean value 0.11 ng) and necrotic leaflet tissues (mean value 19.84 ng) was statistically significant ($P \leq 0.05$). Petiole tissues with symptoms showed a significantly higher mean amount of *H. fraxineus* DNA (mean value 1766 ng) than could be detected in leaflet tissues of any condition. The qPCR assay using the Cfrax probe gave very poor detection of *H. fraxineus* DNA, with C_t values approximately 10 cycles higher than those obtained using the HfRus probe (Table 2).

H. fraxineus in seeds

The collected seeds of local Mandshurian ash from the Russian Far East, visually assessed as healthy, were positive for *H. fraxineus* DNA in the species-specific priming test (Table 1).

Identification of herbarium specimens

Several specimens originally identified as *H. albidus*, *H. caudatus* or *H. epiphyllus* were positive in the *H. fraxineus* assay. None of the herbarium samples examined was positive in the *H. albidus* assay. The sequences obtained from the herbarium samples that were positive in the *H. fraxineus* assay showed 99.7–100% similarity to the sequences obtained from field material collected during the present study. One herbarium specimen collected in 2003 from Hulin, northeast Heilongjiang province, China, originally identified as *H. albidus* (herbarium specimen no. D-2613; Table 1), showed the closest sequence match to that of *Hymenoscyphus caudatus* (GenBank accession no. KU569309; Table 1).

According to the revised identification of *Hymenoscyphus*-associated herbarium specimens deposited at the Mycological Herbarium of the Estonian University of Life Sciences (TAAM) and Herbarium of the Institute of Biology and Soil Sciences of the Russian Far East, the earliest specimen of *H. fraxineus* was collected in 1962

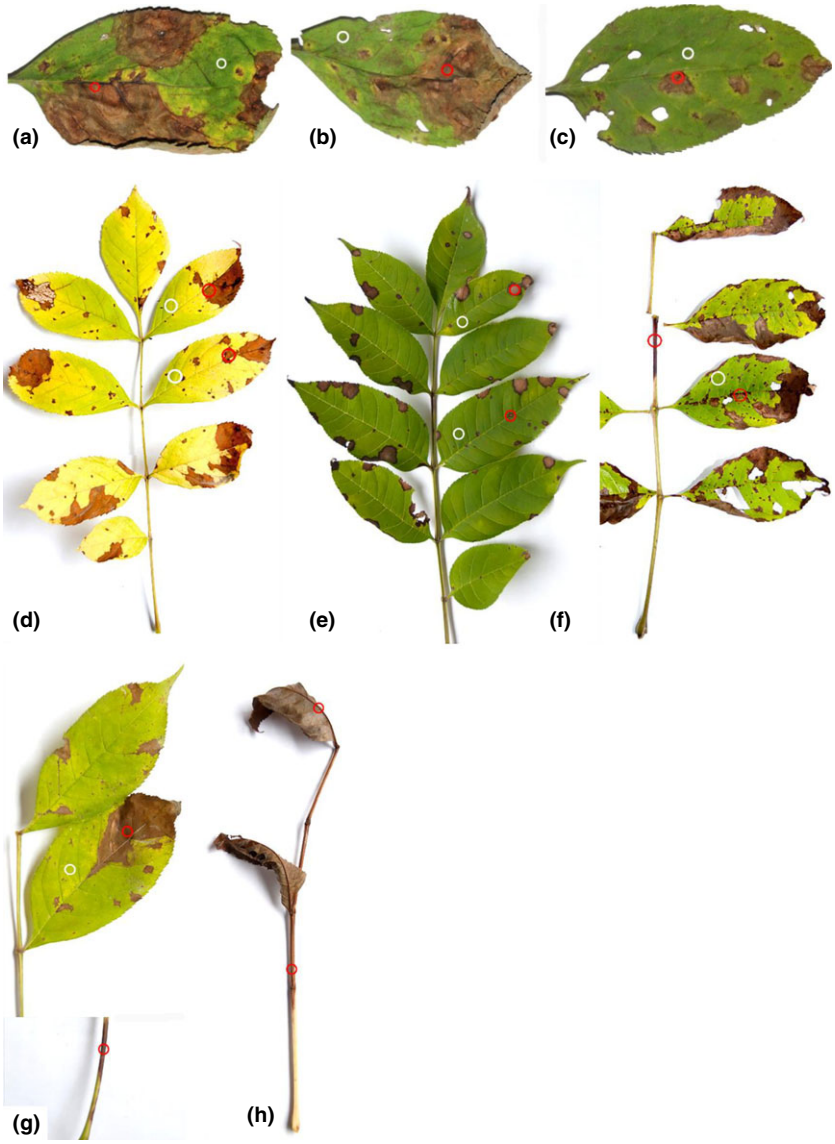

Figure 4 Mandshurian ash leaves with symptoms, sampled in the Russian Far East (Primorskiy Kray and Khabarovskiy Kray regions) and examined by qPCR for the presence and amount of *Hymenoscyphus fraxineus* DNA. Open circles indicate sampling points for the qPCR analyses; red circles show sampled necrotic areas on leaves and petioles and white circles show sampled symptomless leaflet areas. Collection ID SOH-1.422 (a), SOH-2.422 (b), SOH-3.422 (c), SOH-04-01 (d), SOH-04-02 (e), SOH-04-03 (f), SOH-04-04 (g), SOH-04-05 (h) (see Table 2). [Colour figure can be viewed at wileyonlinelibrary.com].

from the Russian Far East (Primorskiy Kray, Ussuriyskiy region, Suputinsk, herbarium specimen no. D-544, GenBank accession no. KR733601; Table 1). This apparently represents the earliest documented *H. fraxineus* in the world. Results also showed that, to date, the oldest *H. fraxineus* specimen of Chinese origin (an ascocarp, herbarium specimen no. D-2695, and ash leaf petiole, D-2697), was collected in 2004 (Table 1).

Comparison between *H. fraxineus* ITS rDNA sequences from Asia and Europe

The most common ITS1-5.8S-ITS2 sequence variant obtained from material collected during the present study in the Russian Far East showed from 99.8% (GenBank accession no. KF188729) to 100% (KF188724) similarity to previously deposited sequences of *H. fraxineus* originating from China, 99.4% (KP068076) to 100% (KP068053) similarity to sequences of Korean *H. fraxineus* strains, 99.4% (KJ511216) to 100% (KJ780100) similarity to sequences of Japanese *H. fraxineus* strains, and 99.5% similarity to the holotype strain of *C. fraxinea* originating from Poland (FJ597975). The latter differs from the most common sequence variant of the present Russian samples by a deletion at position 83 and a point mutation at position 124 within the ITS1 region (Fig. S1). All sequences of *H. fraxineus* originating from the Russian Far East were conserved at these two sites. With the exception of one previously deposited *H. fraxineus* sequence of Japanese origin (KJ511183), which also shows the deletion present in the *C. fraxinea* holotype strain at position 83, these two discriminating positions (83 and 124) proved to be conserved not only across all *H. fraxineus* sequences now obtained from the Russian Far East samples but also all previous *H. fraxineus* GenBank depositions from China, Korea and

Japan. Among the 397 European *H. fraxineus* sequence accessions available at the NCBI database (retrieved 30 November 2015), a sequence of one Swiss isolate (KJ820424) showed the two nucleotides specific to Asian samples at positions 83 and 124, while two other Swiss samples (KJ820435, KJ820505), two Lithuanian samples (KJ413058, KJ780084), one Czech sample (GU586921) and one German sample (KC576530) showed the point mutation at position 124 that is present in all *H. fraxineus* sequences from Asia. The remaining 392 *H. fraxineus* sequences from Europe were identical to the holotype strain of *C. fraxinea* at positions 83 and 124 (alignments not shown).

Discussion

Mandshurian ash and Korean ash in the Russian Far East commonly showed symptoms characteristic to *H. fraxineus* infection on European ash, i.e. necrotic lesions on leaf vein tissues and dieback of young shoots that showed necrotic bark lesions with discoloured xylem in these regions (Figs 2–4). The high levels of pathogen DNA recorded from necrotic leaf tissues of these Asian ash species indicate that the local *H. fraxineus* population is pathogenic to leaves of Mandshurian and Korean ashes. The low levels of *H. fraxineus* DNA detected in discoloured xylem associated with necrotic bark indicates that the shoot dieback symptoms observed in local ash species in the Russian Far East may be caused by other fungi.

The significantly higher levels of *H. fraxineus* DNA detected in necrotic leaf tissues compared to the negligible pathogen DNA levels in symptomless leaf tissues of Mandshurian ash suggest that the lesions were caused by *H. fraxineus*. The levels of *H. fraxineus* DNA detected in necrotic leaf tissues of the Asian ash species investigated in the present study are similar to those recorded in European ash leaf tissues with symptoms in Europe (Steinböck, 2014). Therefore, the present findings suggest that Asian *H. fraxineus* strains are pathogenic to leaves of Asian ash species in a similar manner to the European population of *H. fraxineus* on European ash. In a recent study, Cleary *et al.* (2016) used high-throughput sequencing and showed the presence of *H. fraxineus* in symptomless leaf tissues of Mandshurian ash in the Russian Far East. They concluded that this fungus is a non-pathogenic endophyte in its native range. The sampling time of the study of Cleary *et al.* (2016) is described as 'summer 2012', without providing the sampling dates. However, there may be no inconsistency between the present study and that of Cleary *et al.* (2016); it is possible that *H. fraxineus* is foremost a leaf endophyte both in its native range and in Europe, but can be pathogenic in both regions if the inoculum density reaches a certain critical threshold that enables this fungus to challenge the host defence responses.

Levels of *H. fraxineus* DNA detected in discoloured xylem associated with necrotic bark lesions in shoots of Mandshurian and Korean ash were very low in comparison to *H. fraxineus* DNA levels detected in such tissues of European ash that were collected in spring (Matsiakh *et al.*, 2016), summer, autumn and winter (I. Matsiakh, H. Solheim, A. M. Hietala, N. E. Nagy & V. Kramarets, Norwegian Institute of Bioeconomy Research, personal communication). In addition, in the present study, *H. fraxineus* could not be isolated from the edges of discoloured xylem regions associated with necrotic bark lesions, even though fungal isolation from European ash of this tissue is considered to result in high recovery of *H. fraxineus* mycelia (Gross *et al.*, 2014). Instead, several other fungi capable of causing shoot cankers were recovered: *B. dothidea*, *E. astroidea*, *M. oblongum*, *Phomopsis* sp. and *P. brasiliense*. *Botryosphaeria dothidea* is a pathogenic ascomycete parasitizing a wide variety of tree and shrub species around the world (Crous *et al.*, 2006).

The low presence of *H. fraxineus* in ash shoot lesions in the Russian Far East is in contrast with the prevalent isolation of the fungus from shoot lesions of Mandshurian ash growing in Europe (Drenkhan & Hanso, 2010). These results also differ from research demonstrating that both European and Asian strains of *H. fraxineus* readily cause necrotic lesions on artificially inoculated seedling stems of Mandshurian ash (*F. mandshurica* var. *japonica*) and European ash (Gross & Holdenrieder, 2015; Gross & Sieber, 2016). However, field observations from the present study have shown that Mandshurian ash exhibits far lower susceptibility to shoot dieback caused by natural *H. fraxineus* infections than European ash in Europe (Drenkhan & Hanso, 2010). Furthermore, shoot inoculation experiments carried out with *H. fraxineus* typically involve stem wounding; unless wounding is required for natural shoot infection by this fungus, the results from such treatments may represent an artefact as the structural and induced host defence responses are circumvented when the inoculum is placed on a stem wound. Thus, data from the present investigation suggest that *H. fraxineus* may not be a virulent shoot pathogen on Asian ash species in their native range. Although a relatively small amount of material was examined in detail in the present study, the apparent inconsistency in the association of *H. fraxineus* with shoot lesions of Mandshurian ash in Europe and the Russian Far East may indicate differences in the virulence or infection pressure between European and Asian fungal populations. Further studies are warranted to investigate fungal communities associated with shoot lesions of local ash species in the Russian Far East and the aetiology of these lesions.

Asian *H. fraxineus* populations from Japan, China and Korea analysed so far appear to be genetically divergent from the European population of *H. fraxineus* (Zhao *et al.*, 2013; Gross *et al.*, 2014; Han *et al.*, 2014). The GenBank ITS rDNA sequences of *H. fraxineus* available from East Asia, including the Russian Far East, are conserved at two nucleotide positions within the ITS1 region that allows discrimination between Asian and European populations. While the number of Asian *H. fraxineus*

strains and apothecia subjected to ITS sequencing, present study included, is still small compared to strains characterized from Europe, this consistent difference suggests that the European population of *H. fraxineus* may not originate directly from the East Asian populations studied so far. In line with this, Drenkhan *et al.* (2014) found no spatial or temporal correlation between the historical introduction of Mandshurian ash to Estonia from the Russian Far East and spread of the ash dieback epidemic in the northern Baltic. Should the pathogen invasion, after all, originate from East Asia, the *H. fraxineus* strains that founded the European population must represent rare ITS rDNA sequence variants in East Asia, because all *H. fraxineus* strains from the region analysed so far show the standard East Asian sequence variant.

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References

Baral HO, Bemmann M, 2014. *Hymenoscyphus fraxineus* vs. *Hymenoscyphus albidus* – a comparative light microscopic study on the causal agent of European ash dieback and related foliicolous, stroma-forming species. *Mycology* **5**, 228–90.

Bengtsson SBK, Vasaitis R, Kirisits T, Solheim H, Stenlid J, 2012. Population structure of *Hymenoscyphus pseudoalbidus* and its genetic relationship to *Hymenoscyphus albidus*. *Fungal Ecology* **5**, 147–53.

Burokiene D, Prospero S, Jung E *et al.*, 2015. Genetic population structure of the invasive ash dieback pathogen *Hymenoscyphus fraxineus* in its expanding range. *Biological Invasions* **17**, 2743–56.

Cleary MR, Nguyen D, Marciulyniene D, Berlin A, Vasaitis R, Stenlid J, 2016. Friend or foe? Biological and ecological traits of the European ash dieback pathogen *Hymenoscyphus fraxineus* in its native environment. *Scientific Reports* **6**, 21895.

Crous PW, Slippers B, Wingfield MJ *et al.*, 2006. Phylogenetic lineages in the Botryosphaeriaceae. *Studies in Mycology* **55**, 235–53.

Drenkhan R, Hanso M, 2010. New host species for *Chalara fraxinea*. *New Disease Reports* **22**, 16.

Drenkhan R, Sander H, Hanso M, 2014. Introduction of Mandshurian ash (*Fraxinus mandshurica* Rupr.) to Estonia: is it related to the current epidemic on European ash (*F. excelsior* L.)? *European Journal of Forest Research* **133**, 769–81.

Drenkhan R, Riit T, Adamson K, Hanso M, 2016. The earliest samples of *Hymenoscyphus albidus* vs. *H. fraxineus* in Estonian mycological herbaria. *Mycological Progress*. doi: 10.1007/s11557-016-1209-5.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for Basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113–8.

Gross A, Han JG, 2015. *Hymenoscyphus fraxineus* and two new *Hymenoscyphus* species identified in Korea. *Mycological Progress* **14**, 1–20.

Gross A, Holdenrieder O, 2015. Pathogenicity of *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus* towards *Fraxinus mandshurica* var. *japonica*. *Forest Pathology* **45**, 172–4.

Gross A, Sieber TN, 2016. Virulence of *Hymenoscyphus albidus* and native and introduced *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *Fraxinus pennsylvanica*. *Plant Pathology* **65**, 655–63.

Gross A, Grünig CR, Queloz V, Holdenrieder O, 2012. A molecular toolkit for population genetic investigations of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Forest Pathology* **42**, 252–64.

Gross A, Hosoya T, Queloz V, 2014. Population structure of the invasive forest pathogen *Hymenoscyphus pseudoalbidus*. *Molecular Ecology* **23**, 2943–60.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–8.

Han JG, Shrestha B, Hosoya T, Lee KH, Sung GH, Shin HD, 2014. First report of the ash dieback pathogen *Hymenoscyphus fraxineus* in Korea. *Mycobiology* **42**, 391–6.

Hosoya T, Otani Y, Furuya K, 1993. Materials for the fungus flora of Japan (46). *Transactions of the Mycological Society of Japan* **34**, 429–32.

Ioos R, Kowalski T, Husson C, Holdenrieder O, 2009. Rapid *in planta* detection of *Chalara fraxinea* by a real-time PCR assay using a dual-labelled probe. *European Journal of Plant Pathology* **125**, 329–35.

Kõljalg U, Nilsson RH, Abarenkov K *et al.*, 2013. Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology* **22**, 5271–7.

Kowalski T, Holdenrieder O, 2009. The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology* **39**, 304–8.

Krüssmann G, 1965. *Die Laubgehölze. Eine Dendrologie für die Praxis*. Berlin, Germany: Paul Parey.

Matsiakh I, Solheim H, Nagy NE, Hietala AM, Kramarets V, 2016. Tissue-specific DNA levels and hyphal growth patterns of *Hymenoscyphus fraxineus* in stems of naturally infected *Fraxinus excelsior* saplings. *Forest Pathology* **46**, 206–14.

McKinney LV, Nielsen LR, Collinge DB, Thomsen IM, Hansen JK, Kjær ED, 2014. The ash dieback crisis: genetic solution in resistance can prove a long-term solution. *Plant Pathology* **63**, 485–99.

Pautasso M, Aas G, Queloz V, Holdenrieder O, 2013. European ash (*Fraxinus excelsior*) dieback – a conservation biology challenge. *Biological Conservation* **158**, 37–49.

Steinböck S, 2014. *Ash Dieback Caused by* Hymenoscyphus pseudoalbidus *in Norway: Phenology and Etiology of Leaf Symptoms and Ascospore Dispersal Distances*. Vienna, Austria: University of Natural Resources and Life Sciences, Masters thesis.

Wallander E, 2012. Systematic and floral evolution in *Fraxinus* (Oleaceae). *Belgische Dendrologie Belge* **2012**, 39–58.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Sequencing Guide to Methods and Applications*. San Diego, CA, USA: Academic Press, 315–22.

Zhao YJ, Hosoya T, Baral HO, Hosaka K, Kakishima M, 2013. *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. *Mycotaxon* **122**, 25–41.

Zheng HD, Zhuang WY, 2014. *Hymenoscyphus albidoides* sp. nov. and *H. pseudoalbidus* from China. *Mycological Progress* **13**, 625–38.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. CLUSTAL v. 2.1 multiple sequence alignment of the ITS1-5.8S-ITS2 region of representative *Hymenoscyphus fraxineus* sequences from Europe (holotype strain of *Chalara fraxinea* from Poland, FJ597975),

China (KF188724), South Korea (KP068051), Japan (KJ511220) and Russian Far East (Rus, the present study, Table 1), and two reference species (*Hymenoscyphus albidus* and *H. koreanus*). Sequence in green shows the 18S, 5.8S and 28S rDNA genes. The line symbol (–) indicates a gap in the alignment, a dot (.) denotes identical nucleotides between the sequences at the given positions. Sequences in grey show the primers used for sequencing of the fungus from host material. The underlined sequences show the primers and probe used for real-time PCR quantification of the fungus in host tissues: the primer sequences were described by Ioos *et al.* (2009), while the probe was adjusted in the present study.

Table S1. Isolations from the discoloured shoot xylem tissues associated with necrotic bark lesions on *Fraxinus* spp. in the Russian Far East. A total of 41 pure cultures were obtained from nine different shoot samples.