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Research Note

First Report of the Ash Dieback Pathogen *Hymenoscyphus fraxineus* in Korea

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Abstract In the past two decades, European ash trees (*Fraxinus* spp.) have been severely damaged due to ash dieback disease, which is caused by the fungal species *Hymenoscyphus fraxineus* (*Chalara fraxinea* in the anamorphic stage). Recent molecular phylogenetic and population genetic studies have suggested that this fungus has been introduced from Asia to Europe. During a fungal survey in Korea, *H. fraxineus*-like apothecia were collected from fallen leaves, rachises, and petioles of Korean ash and Manchurian ash trees. The morphological and ecological traits of these materials are described with the internal transcribed spacer rDNA sequence comparison of *H. fraxineus* strains collected from Korea, China and Japan.

Keywords Ash dieback, *Chalara fraxinea*, *Hymenoscyphus fraxineus*, Korean ash, Manchurian ash

Ash dieback is a highly infectious fungal disease that is threatening various ash trees (*Fraxinus* spp.) in Europe. Since ash dieback was first observed in North-East Poland in the early 1990s, it has throughout the continent [1]. *Chalara fraxinea* T. Kowalski was described as the causal agent of ash dieback [2]. *Hymenoscyphus albidus* (Gillet) W. Phillips, a common helotialean species in Europe, was suggested to be a teleomorph of *C. fraxinea* based on their morphological similarity [3]. However, a recent molecular study [4] showed that *H. albidus* is a species complex that can be subdivided into pathogenic and non-pathogenic groups. In the pathogenic group, a new species, *H. pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T. N. Sieber & Holdenr, has been designated and suggested as a teleomorph of *C. fraxinea* [4]. Further cultural and population genetic studies have demonstrated the distinctions between *H. albidus* and *H. pseudoalbidus* [5-8]. Zhao *et al.* [9] described that the presence or absence of croziers at the base of asci can be a taxonomic key to distinguish these two species. It was recently hypothesized that the causal agent of the sudden outbreak of European ash dieback may have been introduced from East Asia [4, 9]. Zhao *et al.* [9] found that Japanese materials of *Lambertella albida* are morphologically and genetically similar to *H. pseudoalbidus*. Zheng and Zhuang [10] also reported the occurrence of *H. pseudoalbidus* in China. According to the recent amendment of fungal nomenclature, the correct name for *H. pseudoalbidus* is *H. fraxineus* (T. Kowalski) Baral, Queloz & Hosoya [11]. During a fungal biodiversity survey in Korea, *Hymenoscyphus* species on fallen leaves, rachises and petioles of Korean ash (*Fraxinus rhynchophylla* Hance, also known as *F. chinensis* subsp. *rhynchophylla* (Hance) Hemsl.) and Manchurian ash (*F. mandshurica* Rupr.) were collected. We performed a detailed morphological examination and sequence comparison with other *Hymenoscyphus* spp. including the close relatives *H. albidus* and *H. fraxineus* (= *H. pseudoalbidus*).

Apothecia collected on fallen leaves, rachises, and petioles were pressed with clean papers and dried on the laboratory bench. All collected materials have been deposited at the Korea University Herbarium (KUS). Fresh materials were

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primarily mounted with distilled water for the confirmation of the natural colors of their microstructures. Dried materials were rehydrated in 3~10% aqueous KOH. Amyloid reactions were conducted with Melzer's reagent or Lugol's solution (IKI) without KOH pretreatment. Line drawings were made with the aid of an Olympus BX50 microscope equipped with an Olympus U-DA drawing tube. Measurements were made in distilled water, Congo red, lactophenol-cotton blue, IKI, or Melzer's reagent and are reported as follows: minimum-maximum (length) × minimum-maximum (width) [mean length ± standard deviation × mean width ± standard deviation, Q (length/width ratio) = average ± standard deviation].

Genomic DNA was extracted from dried apothecia using the methodology described by Lee and Taylor [12]. The concentration and purity of extracted nucleic acids were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). PCR was performed in 50 μL reactions that consisted of 39 μL of sterile water, each primer at 0.4 μM, 1 unit of ExTaq DNA Polymerase (TaKaRa, Tokyo, Japan), a dNTP mixture consisting of all four nucleotides, each at a concentration of 2.5 mM, 10× ExTaq buffer containing 20 mM Mg^{2+}, and approximately 100 ng of template DNA. ITS1 and ITS4

Table 1. Sequences used in this study

Species	Source	Location	GenBank accession No.
Chalara fraxinea T. Kowalski	71026.1	Czech Rep.	FJ429386
	CBS 122504	Poland	FJ597975
Cudoniella clavus (Alb. & Schwein.) Dennis	AFTOL-ID 166	USA	DQ491502
Hymenoscyphus crataegi Baral & R. Galan	F156966	Spain	DQ431177
H. albidoides H. D. Zheng & W. Y. Zhuang	HMAS264141	China	KF188721
	HMAS264140	China	KF188722
	HMAS264142	China	KF188723
H. albidus (Gillet) W. Phillips	90803.16	Switzerland	GU586879
	90805.2	Switzerland	GU586880
	90807.15	Switzerland	GU586899
	90812.1	Switzerland	GU586893
	090812.3c	Switzerland	GU586895
	Ber_02	Switzerland	GU586877
	Cas_01	Switzerland	GU586882
	Lav_01	Switzerland	GU586884
	Qui_01	Switzerland	GU586887
H. brevicellulus H. D. Zheng & W. Y. Zhuang	HMAS 264018	China	JX977162
H. caudatus (P. Karst.) Dennis	HMAS82057	China	AY348576
H. fraxineus (T. Kowalski) Baral, Queloz & Hosoya	TNS-F12761	Japan	AB705218
	TNS-F40043	Japan	AB705219
	TNS-F40051	Japan	AB705220
	TNS-F12503	Japan	AB705221
	90807.1	Germany	GU586901
	Oth 01	Switzerland	GU586904
	Bel 01	Switzerland	GU586905
	90807.16	Switzerland	GU586917
	HMAS266596	China	KF188725
	HMAS266580	China	KF188726
	HMAS266581	China	KF188727
H. fructigenus (Bull.) Gray	F156965	Spain	DQ431176
	F109077	Spain	DQ431169
H. ginkgonis J. G. Han & H.D. Shin	KUS-F51352	Korea	EU096525
H. immutabilis (Fuckel) Dennis	HMAS 71809	China	AY348584
H. macroguttatus Baral, B. Declereq & Hengstm	HB7034	Germany	DQ431179
H. scutula (Pers.) W. Phillips	HMAS82093	China	AY348590
	HMAS82098	China	AY348591
H. serotinus (Pers.) W. Phillips	F115891	Spain	DQ431173
	F093261	Spain	DQ431168
Hymenoscyphus sp.	KUS-F52355	Korea	KF830851
	KUS-F52610	Korea	KF830852
	KUS-F52255	Korea	KF830850
	KUS-F52613	Korea	KF830853
Sclerotinia sclerotiorum (Lib.) de Bary	KUS-F26433	Korea	KC412065

primers [13] were used to amplify the internal transcribed spacer (ITS) region of rDNA, including the 5.8S gene. The cycling parameters for ITS amplification included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. After confirming the PCR products on an agarose/TBE electrophoretic gel (Certified Molecular Biology Agarose; Bio-Rad Laboratories, Madrid, Spain), they were purified using a LaboPass PCR Kit (COSMO Genetech, Seoul, Korea) following the manufacturer's protocols. Sequencing was performed on an automatic sequencer (ABI Prism 377 DNA Sequencer) using the BigDye Cycle Sequencing Kit (version 3.1; Applied Biosystems, Foster City, CA, USA) with the same primers used for the PCR amplifications. The obtained ITS sequences were deposited in GenBank with the accession Nos. KF830851, KF830852, KF830850, and KF830853.

In addition to the newly generated sequence data, 37 fungal ITS sequences were retrieved from GenBank to determine the phylogenetic placement of Korean *Hymenoscyphus* specimens (Table 1). The obtained sequences were aligned using ClustalW [14] and manually edited using BioEdit ver. 7.0.5.2 [15] when necessary. Ambiguously aligned positions were excluded from the subsequent analysis. Phylogenetic analyses using neighbor-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) were performed with MEGA ver. 6.06 [16]. In the NJ analysis, the distances between ITS sequences were calculated using the Kimura 2-parameter model. In the ML analysis, the general time-reversible model with a gamma-distributed substitution rate and proportion of invariant sites (GTR + I + G) was separately applied to each gene for the nucleotide substitution model. An MP heuristic search was performed with 5,000 replications, each with 500 random sequence additions, and branch swapping by tree bisection-reconnection. All nucleotide substitutions were equally weighted and unordered with gaps treated as missing data. The nodal support for the individual branches was estimated by bootstrapping (BS) using 1,000 replicates [17].

Previously, *Hymenoscyphus albidus* and *H. fraxineus* were regarded as morphologically indistinguishable until H. O. Baral (personal communication) and Zhao *et al.* [9] demonstrated differences in their ascal bases. In Korea, the apothecial discomycetes collected on the fallen leaves, rachises, and petioles of *Fraxinus* spp. were confirmed as *H. albidus* based on earlier descriptions [18, 19]. However, during the re-examination of Korean materials, we observed that all samples possessed distinct croziers at the ascal bases (Fig. 1). We report *H. fraxineus* for the first time in Korea and describe its morphological and ecological traits.

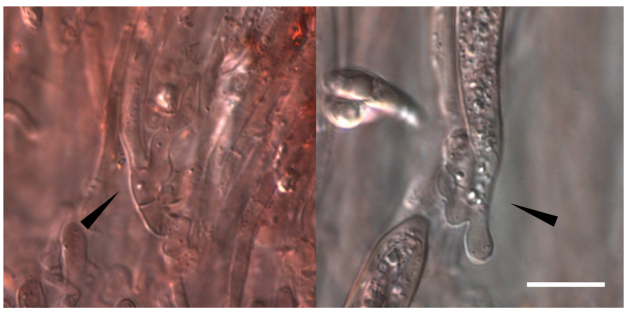

Fig. 1. Distinct croziers observed at the ascal bases of *Hymenoscyphus fraxineus* material (KUS-F52255). Mounted in Congo red solution (scale bar = 10 μm).

Hymenoscyphus fraxineus **(T. Kowalski) Baral, Queloz & Hosoya (Figs. 1~3)**

≡ *Chalara fraxinea* T. Kowalski, For. Pathol. 36: 264 (2006).
= *Hymenoscyphus pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T. N. Sieber & Holdenr., For. Pathol. 41: 140 (2011).

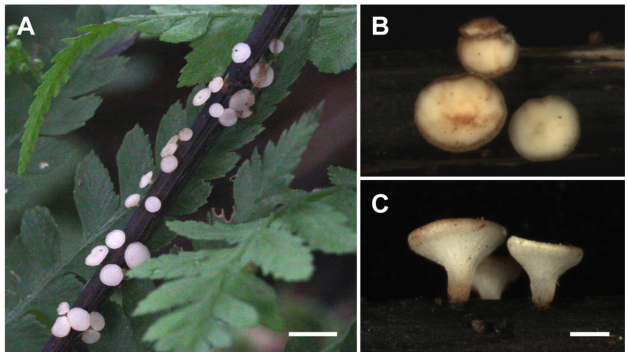

Fig. 2. *Hymenoscyphus fraxineus*. A, Fruiting bodies on fallen rachis of *F. rhynchophylla*; B, C, Upper and side views of cupulate apothecia (scale bars: A = 10 mm, B, C = 1 mm).

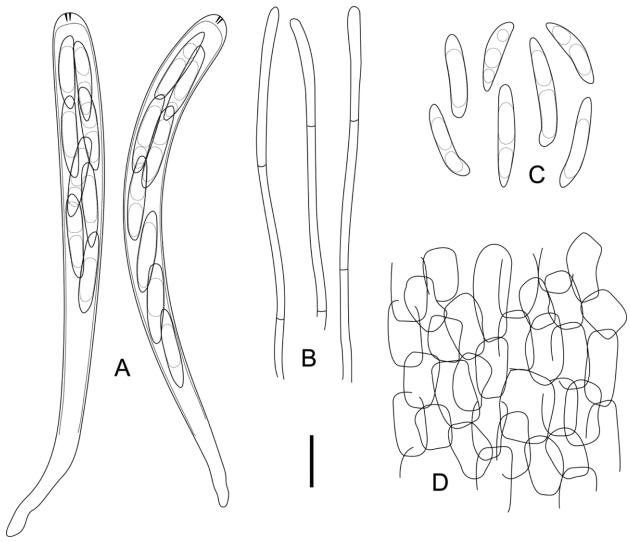

Fig. 3. *Hymenoscyphus fraxineus*. A, 8-spored asci with MLZ+ apical pores; B, Cylindric paraphyses; C, Elliptic-fusoid ascospores containing several oil drops; D, Ectal excipulum composed of prismatic to angular cells (scale bar: A~D = 10 μm).

Apothecia gregarious, superficial, seated on a distinct stipe. Receptacle shallow cupulate to discoid, ivory to white or cream when fresh, turning light brown when dry. Disc up to 2 mm in diameter, plano-concave, concolorous with the receptacle. Stipe cylindric, slightly tapering towards the base, concolorous with the receptacle, blackened near the base, up to 1 mm long. Ectal excipulum composed of prismatic to angular cells, individual cells hyaline to light yellowish, thin-walled, 8~18 × 6~10 µm, decreasing in size near the apothecial margin. Asci arising from distinct croziers, cylindric, hyaline, 8-spored, apex conical, apical pore blued in IKI without KOH pretreatment, 85~105 × 7~9 µm (95.6 ± 3.21 × 8.5 ± 0.33 µm, n = 35). Ascospores elliptic-fusoid to clavate, scutuloid, hyaline, biseriate, occupying the upper 1/2~3/4 of the entire asci length, 15~21 × 3~4 µm (19.3 ± 0.75 × 3.6 ± 0.13 µm, Q = 5.03 ± 0.13, n = 80). Paraphyses cylindric, hyaline, septate, unbranched, 2~2.5 µm wide, tips slightly swollen up to 3 µm, slightly exceeding the asci.

Specimens examined: On leaves, rachises, and petioles of Korean ash (*Fraxinus rhynchophylla*)—Korea: Danyang, Darian Valley in Mt. Sobaek National Park, 36°57'46" N, 128°25'24" E, alt. 400 m, 14 Jul 2007, KUS-F51679 and KUS-F51684; Pyeongchang, Mt. Taegi, 37°35'25" N, 128°16'47" E,

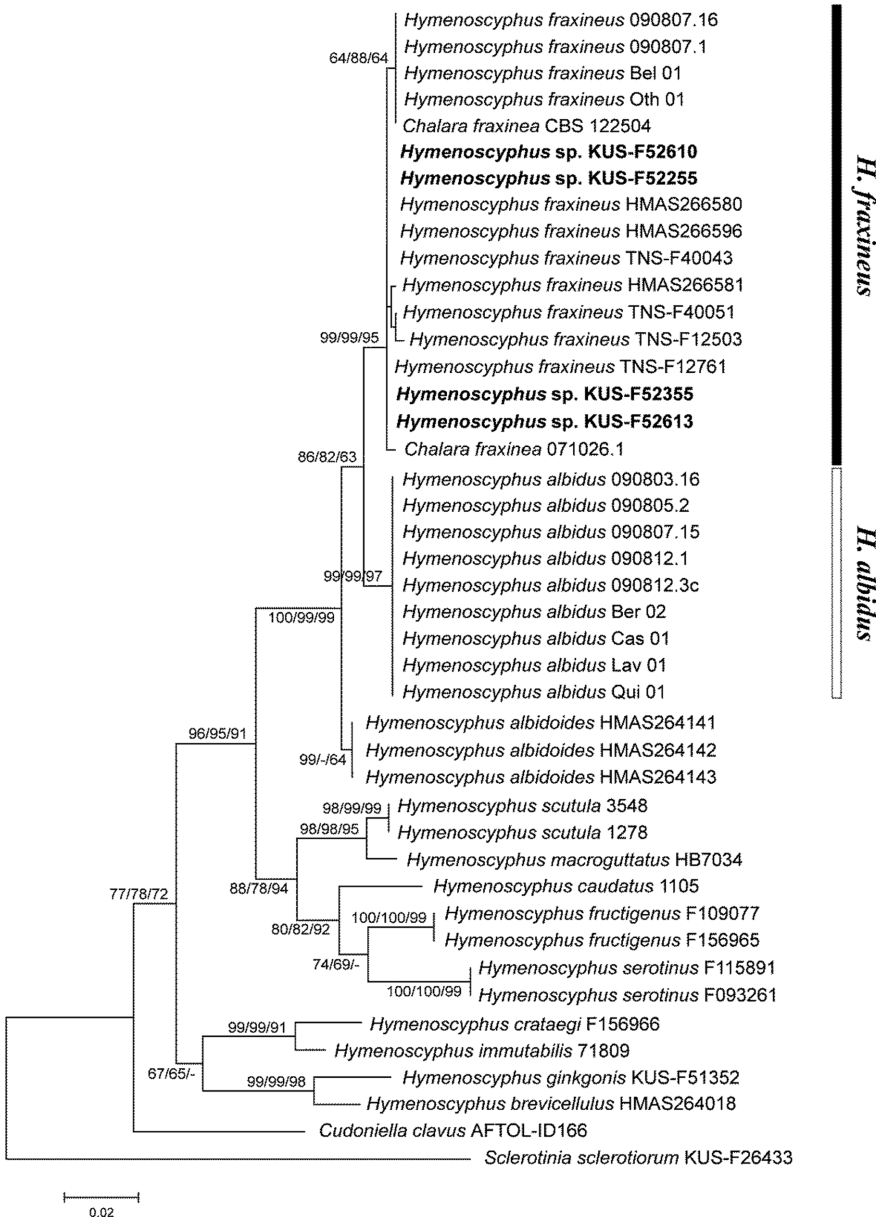

Fig. 4. Neighbor-joining (NJ) tree of *Hymenoscyphus* spp. based on the internal transcribed spacer rDNA sequences. The bootstrap values greater than 60% are indicated above the corresponding branches (NJ BP/ML BP/MP BP). The newly generated sequences in this study are shown in bold. The scale bar indicates the proportion of sites changing along each branch. BP, bootstrap proportion; ML, maximum likelihood; MP, maximum parsimony.

alt. 1050 m, 14 Aug 2008, KUS-F52255; Yangpyeong, Seolmaejae Recreation Forest, 37°33'14" N, 127°30'12" E, alt. 300 m, 15 Jul 2009, KUS-F52542; Yangpyeong, Saneum Recreation Forest, 37°36'9" N, 127°35'54" E, alt. 200 m, 15 Jul 2009, KUS-F52552; Hoengseong, Cheongtaesan Recreation Forest, 37°31'32" N, 128°17'34" E, alt. 850 m, 19 Aug 2009, KUS-F52610; Hoengseong, Supchaewon, 37°23'25" N, 128°17'34" E, alt. 1,050 m, 20 Aug 2009, KUS-F52613; on damp rotting leaves of Manchurian ash (*Fraxinus mandshurica*)—Korea: Hoengseong, Supchaewon, 37°23'24" N, 128°17'39" E, alt. 1,100 m, 23 Sep 2008, KUS-F52355.

Morphological characteristics of the Korean materials were in agreement with the previous descriptions of *H. albidus* [18, 19]. Based on the croziers at the ascal bases that were first noticed by Zhao *et al.* [9], this fungus was identified as *H. fraxineus*. Korf [20] and Hosoya *et al.* [19] regarded it as a member of *Lambertella* (Rutstroemiaceae, Helotiales) based on the existence of stromatized patches on the substrates. In a recent molecular study, however, the genus *Lambertella* was determined to be a polyphyletic group, and *Hymenoscyphus* was suggested to be the most appropriate genus for this taxon [9]. *Hymenoscyphus caudatus* (P. Karst.) Dennis is also similar to *H. fraxineus* due to its white stipitate apothecia and scutuloid ascospores as well as its foliicolous habit, but differs in the small size of its apothecia (up to 1.5 mm diameter) [18, 21]. *Hymenoscyphus ginkgonis* J. G. Han & H. D. Shin is another similar species possessing ascospores of a similar shape and size, but is distinguished by its substrate-specificity on ginkgo (*Ginkgo biloba* L.) leaves and the presence of deep violet pigments in its paraphyses [22]. *Hymenoscyphus albidoides* H. D. Zheng & W. Y. Zhuang recently described from China is distinct from this fungus owing to an outer covering composed of 1~3 parallel hyphal layers, cuboid crystals in the basal tissue of the stipe, and its occurrence on the veins of rotten leaves of *Picrasma quassioides* (D. Don) Benn. [10].

Korean materials of *H. fraxineus* were usually isolated from Korean ash (*F. rhynchophylla*), which is a new host for this fungus. However, ash dieback symptoms were not detected when we collected these materials, indicating the possibility that Korean ash has an inherited resistance to this pathogen. Korean ash is phylogenetically closely related to manna ash (*F. ornus* L.) [23], which is known to be less susceptible to ash dieback than other European ash species [24]. In China and Japan, *H. fraxineus* has been reported on Manchurian ash (*F. mandshurica*), which is widely distributed in Eastern Asia [9, 10].

The analyzed dataset included 41 *Hymenoscyphus* spp. and two outgroup taxa, *Cudoniella clavus* (Helotiaceae) and *Sclerotinia sclerotiorum* (Sclerotiniaceae) (Table 1). The final alignment contained 477 nucleotide positions, of which 333 were invariant and 94 were parsimony-informative characters. The ML analysis yielded an optimal tree with a best log-likelihood of −1,795.14. Ten equally most-parsimonious trees of 251 steps were obtained by the MP analysis, with a consistency index of 0.6972 and an retention index of 0.8403. Since the inferred NJ, ML, and MP trees were topologically congruent, the NJ tree is represented with the bootstrap values of NJ, ML, and MP analyses above the corresponding branches (Fig. 4). In the inferred trees, the *Hymenoscyphus* spp. formed a single group with moderate support values (NJ BS = 77, ML BS = 78, and MP BS = 72), reflecting the broad circumscription of the genus as well as the possibility of the heterogeneous assemblage of diverse fungal species. As shown in previous studies, *H. albidus* and *H. fraxineus* were morphologically similar but clearly separated in the phylogeny [4, 9, 10]. Korean materials (KUS-F52255, KUS-F52355, KUS-F52610, and KUS-F52613) were grouped with other *H. fraxineus* (= *Chalara fraxinea*) strains with strong nodal supports (NJ BS = 99, ML BS = 99, and MP BS = 95). *Hymenoscyphus albidoides* formed a sister-group with *H. albidus* and *H. fraxineus*.

Zhao *et al.* [9] investigated the intraspecific variation in *H. fraxineus* (= *H. pseudoalbidus*), and found that the ITS sequence of Asian strains concurred with that of European strains except for two nucleotide positions, 83 (G instead of a gap) and 124 (C instead of T) (Table 2), reflecting

Table 2. Comparison of ITS sequences from *Hymenoscyphus albidus* and *H. fraxineus* strains used in this study

Species	Origin	Position															
		77	79	83	86	87	101	117	124	155	258	355	361	379	436	443	450
H. albidus	Europe[a]	C	C	G	T(G)	T	C	T	C	T	G	C	C	C	A	T	A
H. fraxineus	Europe[b]	T	T	-	T	C	T	C	T(C)	T	G	G	T	C(T)	C	T	C
	China[c]	T	T	G	T	C	T	C	C	T	G	G	T	C	C	T(C)	C
	Japan[d]	T	T	G	T	C	T	C	C	T(G)	G	G	T	C	C	T(C)	C
	Korea[e]	T	T	G	T	C	T	C	C	T	G(R)	G	T	C	C	T	C

Numbering from *H. albidus* 90803.16 (GU586879). The second dominant base is indicated by parentheses in case there is a variation. '-' indicates gap.

[a]GU586877, GU586879, GU586880, GU586882, GU586884, GU586887, GU586893, GU586895, and GU586899.

[b]FJ429386, FJ597975, GU586901, GU586904, GU586905, and GU586917.

[c]KF188725, KF188726, KF188727.

[d]AB705218, AB705219, AB705220, and AB705221.

[e]KF830850, KF830851, KF830852, and KF830853.

their close relation. Among the Asian strains, positions 155, 379, and 443 showed some variations (Table 2). The obtained ITS sequences of Korean isolates were nearly identical, irrespective of the host. KUS-F52255 showed a degenerate base R (indicating A or G bases) instead of G at the nucleotide position 258 (Table 2), but it is likely to be an incorrect base call since all known ITS sequences of *H. fraxineus*, *H. albidus*, and even the close relative *H. albidoides* show the same nucleotide G at this position.

Based on the field observations and artificial inoculation studies showing Asian ash species tend to be immune to ash dieback disease [24, 25], Asia is considered a possible origin of the ash dieback pathogen [4, 9]. This hypothesis was verified by subsequent population genetic analyses using polymorphic microsatellite markers showing that European *H. fraxineus* has limited genetic diversity among and within populations [5, 6]. In contrast, Zhao *et al.* [9] found that Japanese *H. fraxineus* possess more genetic variation compared to European populations. The ITS sequences of Japanese *H. fraxineus* is most variable among the Asian materials, while those of Chinese and Korean materials are relatively conserved (Table 2) although more population sampling from various sites is necessary.

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