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Host Range of *Hymenoscyphus fraxineus* in Slovak Arboreta

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Abstract: The health of 34 different *Fraxinus* taxa in association with the pathogenic fungus *Hymenoscyphus fraxineus* was assessed in four Slovak arboreta. Averaged across all arboreta, nearly one-quarter (24.9%) of all evaluated trees showed ash dieback symptoms. The damage was most serious on the common ash *F. excelsior*, a native species. The percentage of dead trees did not exceed 2% for all evaluated trees. Generally, ash trees of all ages were affected, though the intensity of the damage varied among the sites. The identity of *H. fraxineus* was confirmed by conventional PCR targeting the internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA, as well as the 18S gene/ITS-2 region of the rDNA operon. In Slovakia, the pathogen has expanded its host range from native species not only to their ornamental cultivars, but also to introduced North American (*F. cinerea*, *F. latifolia*, *F. pennsylvanica*, *F. quadrangulata*) and Asian (*F. bungeana*, *F. chinensis* ssp. *rhynchophylla*, *F. mandshurica*) ash species. *H. fraxineus* was also observed on the previous year's leaf petioles of the native European species *F. ornus*, considered a weakly susceptible host. In Slovak arboreta, *H. fraxineus* was found on 23 *Fraxinus* taxa; 21 of them represent first records for the country. *F. bungeana* is recorded as a new host species of *H. fraxineus*.

Keywords: ash dieback; crown defoliation; *Fraxinus* spp.; *Hymenoscyphus fraxineus*

1. Introduction

Fraxinus L. (Oleaceae) is widely distributed over North and Central America, Asia, Europe, and Northwest Africa, from tropical to temperate climates [1]. Although *Fraxinus* often grows in forests, at their edges, and along tree-lined roads, it is also planted in urban greenery and parks as an ornamental tree. Since the early 1990s, ash dieback is a serious disease that has been killing ash across Europe [2–5]. The disease is caused by the invasive fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (syn. *Hymenoscyphus pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T.N. Sieber & Holdenr.), the anamorph of which is *Chalara fraxinea* T. Kowalski. Currently, it is one of seven known species of the genus *Hymenoscyphus* Gray (Helotiales) occurring on ash trees. The non-pathogenic species *Hymenoscyphus albidus* (Gillet) W. Phillips has been known from Europe since 1851 [6]. Gross and Han [7] and Gross et al. [8] described three Asian *Hymenoscyphus* species, *H. koreanus* A. Gross & J.G. Han, *H. linearis* Hosoya, A. Gross & Baral, and *H. occultus* A. Gross & J.G. Han. Although pathogenicity tests revealed that *H. linearis* is avirulent [8], there is currently no information on the pathogenicity of *H. koreanus* and *H. occultus*. Recently, Kowalski and Bilanski [9] have identified another species, *H. pusillus* T. Kowalski & P. Bilański, from *F. pennsylvanica* Marshall in Poland, but the pathogenicity of this fungus has not yet been confirmed. *H. caudatus* (P. Karst.) Dennis, occurring on various plant species, has also been recorded on ash in China [10] and the British Isles [11].

H. fraxineus is widespread in both East Asia (Northeastern China, Japan, Korea, and the Russian Far East) and Europe [12]. In Europe, this pathogen mainly attacks common ash (*F. excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl), but it is also recorded on North American (*F. americana* L., *F. nigra* Marshall, *F. pennsylvanica*) and Asian (*F. mandshurica* Rupr., *F. sogdiana* Bunge) ash species in Estonia [13,14]. *H. fraxineus* has been recorded from *F. ornus* L. only in Austria [15]. In Danish arboretum, *H. fraxineus* apothecia were equally well-formed on many introduced host *Fraxinus* species as it was on common ash [16]. Most recently, the fungus has been found on three new oleaceous host plant species (*Chionanthus virginicus* L., *Phillyrea angustifolia* L., *Phillyrea latifolia* L.) at Westonbirt Arboretum in the United Kingdom [17]. These were the first findings on hosts other than *Fraxinus*. In Slovakia, two native European ash species (*F. excelsior*, *F. angustifolia*) are attacked by *H. fraxineus* [18–21]. Previous papers have only recorded ash dieback on these two native *Fraxinus* species in forest stands, seed orchards, and tree alleys in Slovakia. The health of non-native ash trees and ornamental cultivars of native species, however, has not yet been investigated in Slovakia.

The aims of this study were (i) to evaluate the health of introduced ash species and ornamental cultivars of native *Fraxinus* species in terms of the occurrence of *H. fraxineus* in Slovak arboreta and (ii) to determine the host range of *H. fraxineus* in the arboreta.

2. Materials and Methods

2.1. Study Sites

Study material was collected in three arboreta situated in central Slovakia and one arboretum in Southwestern Slovakia (Figure 1, Table 1).

Figure 1. Map of Slovakia showing the four sampled arboreta.

Table 1. Characteristics of study sites in Slovakia.

Arboretum	Total Area [ha]	Coordinates	Altitude [m a.s.l.]	MAT [°C]	MTGS [°C]	APS [mm]	MPSGS [mm]
Borová hora	47.80	48°35′ N 19°08′ E	291–377	9.7	16.8	763.3	432.1
Kysihýbel	7.78	48°27′ N 18°56′ E	530–563	9.1	15.8	853.3	465.8
Liptovský Hrádok	7.24	49°02′ N 19°43′ E	641–646	8.0	14.6	729.5	460.2
Mlyňany	67.00	48°19′ N 18°22′ E	163–217	10.7	17.5	555.5	337.0

MAT—mean annual temperature; MTGS—mean temperature in the growing season; APS—annual precipitation sum; MPSGS—mean precipitation sum in the growing season. The growing season is from April to September. The climatic data were computed for the ten-year period 2009–2018 (data source: Slovak Hydrometeorological Institute, Bratislava).

The Borová Hora Arboretum (Zvolen District) is spread over the southwestern slopes of Zvolenská vrchovina upland, which is one of the geomorphological units of the Zvolenská kotlina basin; it is characteristically upland. From a climatic point of view, this area is warm and slightly humid (Figure 2), with a cold winter [22].

The Kysihýbel Arboretum (Banská Štiavnica District) is located near the town of Banská Štiavnica. The area is protected by the Štiavnické vrchy Mountains Protected Landscape Area. This forestry arboretum is a unique experimental forestry area specialized in rapidly growing exotic wood species [23]. It is in a warm temperate zone with a humid climate (Figure 2), without extreme temperature changes.

The Liptovský Hrádok Arboretum (Liptovský Mikuláš District) is situated in an urban area in the town of Liptovský Hrádok. This area has warm, humid summers and cold winters. The Liptovský Hrádok Arboretum is the highest elevation arboretum in Central Europe and is registered as a protected area under the Nature and Landscape Protection Act of Parliament [24].

The Mlyňany Arboretum (Zlaté Moravce District) is a large park situated in one of the warmest and driest areas of Slovakia in the southern foothills of the Carpathian Mountains at the northern edge of the hottest part of Slovakia. The area is characterized by prolonged dry periods during the height of summer; during some years, there is sometimes no summer rain at all. The Mlyňany Arboretum manages the largest collection of non-native trees and shrubs in Slovakia, which also happens to be one of the richest collections in Central Europe [25].

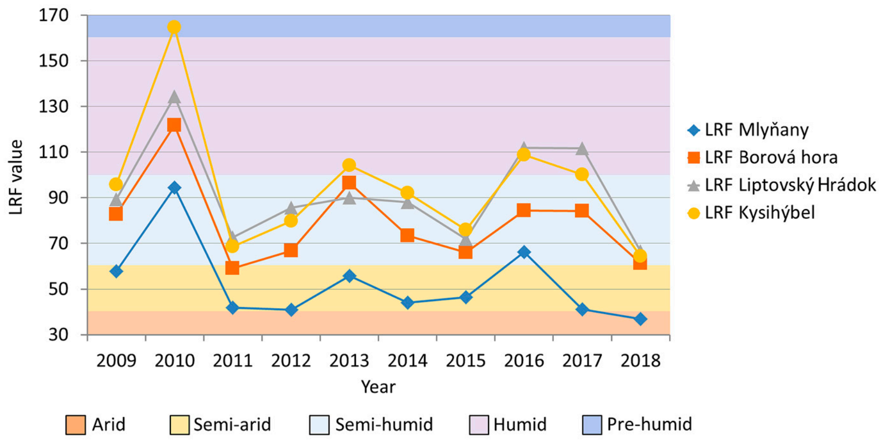

Figure 2. Lang's rain factor (LRF) for the years 2009 to 2018. The LRF was calculated as the ratio of the mean annual precipitation (in mm) and the mean annual air temperature (in °C) [26].

2.2. *Ash Dieback Damage Assessment in Study Sites*

Ash trees in the arboreta were assessed for ash dieback symptoms (necrotic leaf spots, brownish to orange colored bark necrosis and cankers on stems and branches, shoot dieback, apothecia on the previous year's leaf petioles lying in the litter under the tree) during the growing season (July–August) in Mlyňany in 2015 and in Borová hora, Kysihýbel, and Liptovský Hrádok in 2018. The extreme droughts caused by precipitation deficits in Mlyňany in 2015 were also common during the four years before the assessment. Two years prior to the assessments in Borová hora, Kysihýbel, and Liptovský Hrádok in 2018, the annual precipitation was higher and the average annual temperature was lower than in the year of the assessment. The dieback damage assessments were made before leaf fall and covered a total of 470 trees (20–118 years old) of 34 taxa. Ash dieback crown damage was assessed visually by the same person. The extent of tree crown defoliation was reported as the percentage of defoliation [27].

2.3. *Collection of Field Samples*

Apothecia of *H. fraxineus* on rachises and petioles of *Fraxinus* spp. in the litter of the previous year's leaf fall under symptomatic trees were collected from August to September in 2015 and 2016 in Mlyňany and during the first half of August 2018 in Borová hora, Kysihýbel, and Liptovský Hrádok. The samples were placed in plastic tubes and brought to the laboratory for molecular analysis to confirm species identity. In arboreta, individual ash species are spatially separated from each other. Particular ash species grow either solitarily or in monoculture groups surrounded by non-ash tree species, thus minimizing the possibility that the leaf litter under a tree would contain leaves of another ash species.

Voucher specimens were deposited in the Plant Pathology Herbarium (NR) of the Institute of Forest Ecology SAS in Nitra, Slovakia under the accession numbers NR5544–NR5551 and NR5553–NR5556 (Mlyňany Arboretum), NR5696–NR5713 (Borová hora Arboretum), NR5714–NR5719 (Kysihýbel Arboretum), and NR5729–NR5736 (Liptovský Hrádok Arboretum).

2.4. DNA Extraction, PCR Amplification, and Sequencing

DNA was extracted from the samples by grinding apothecia in liquid nitrogen with a disposable polypropylene pellet pestle directly in 1.5 mL microtubes. DNA extractions were performed using the EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit (Bio Basic Inc., Markham, ON, Canada) according to the protocol of the manufacturer. DNA was resuspended in 50 µL of elution buffer and stored at −20 °C.

The internal transcribed spacer (ITS) region of ribosomal DNA (ITS1-5.8S-ITS2) was amplified with the universal primer pair ITS1F/ ITS4 [28,29]. For identification of *H. fraxineus*, species–specific primers targeting the 18S gene and the ITS-2 region of rDNA operon [30] were used in conventional PCR.

All PCRs were performed in a total volume of 20 µL. The PCR mixtures consisted of 4 µL 5× HOT FIREpol® BlendMaster Mix (Solis BioDyne, Tartu, Estonia), 0.4 µL of each primer (final concentration 0.2 µM), deionized water of molecular grade (Pro injection, B. Braun), and ~10 ng of template DNA. Every reaction was performed with a negative control containing the master mix without template DNA. The PCRs were carried out on a T100™ Thermal Cycler (Biorad, Applied Biosystems, Waltham, MA, USA). For the ITS region, the PCR cycling conditions included an initial denaturation for 14 min at 95 °C followed by 35 cycles for 15 s at 95 °C, annealing for 30 s at 50 °C, elongation for 80 s at 72 °C, and a final elongation step for 10 min at 72 °C. PCR conditions for the specific primer 18S gene/ITS2 region included an initial denaturation step for 15 min at 95 °C, followed by 35 cycles for 20 s at 95 °C, annealing for 30 s at 62 °C, elongation for 1 min at 72 °C, and a final elongation step for 8 min at 72 °C. All PCR products were visualized by electrophoresis on 0.8% (w/v) TBE agarose gels stained with SimplySafe stain (EURx, Gdansk, Poland).

The target PCR fragments were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by the Sanger method using an ABI 3130xl sequencer (Applied Biosystems) at SEQme Ltd. (Dobříš, Czech Republic). The obtained DNA sequences were compared by BLAST (Basic Local Alignment Search Tool, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi) against ITS sequences in the NCBI GenBank Sequence Database.

2.5. Phylogenetic Analysis

The ITS sequences obtained were trimmed and edited using Molecular Evolutionary Genetics Analysis software Version 7.0 (MEGA7) [31]. Final datasets were aligned with the BioEdit Sequence Alignment Editor Version 7.2.5 [32]. To demonstrate differences in sequences of the studied material of *H. fraxineus* from Slovakia, we performed a phylogenetic analysis using corresponding GenBank sequence deposits of several known species of the *Hymenoscyphus* genus on *Fraxinus* as references, i.e., *H. albidus*, *H. fraxineus*, *H. occultus*, *H. pusillus*, *H. koreanus*, *H. linearis*, and *H. caudatus*. *Hymenoscyphus menthae* (W. Phillips) Baral was used as the outgroup. All samples used in our analysis are represented by ITS sequence. The ITS dataset was analyzed using the Maximum Likelihood (ML) method based on the Tamura–Nei model [33]. The phylogenetic tree was reconstructed using MEGA7.

2.6. Statistical Analysis

The data on crown defoliation of different *Fraxinus* taxa inspected in four Slovak arboreta were tested with one-way analysis of variance (ANOVA). Taxa with low sample size ($n < 2$) were excluded from the test. Significance of the differences in defoliation means among arboreta was evaluated by Duncan test. The statistical analysis was performed using STATISTICA software Version 10 (StatSoft Inc., Tulsa, OK, USA, 2011).

3. Results

3.1. Defoliation Level of Ash Trees in Slovak Arboreta

In Borová hora Arboretum, 28 trees of 12 *Fraxinus* taxa were evaluated (Table 2). The percentage of defoliation varied among taxa. Out of six *F. angustifolia* trees, three showed less than 10% defoliation, one was slightly defoliated (up to 25%), and two were moderately defoliated (30% and 40%). A single specimen of *F. angustifolia* ssp. *pannonica* Soó & T. Simon was heavily defoliated (85%). Variation in the level of defoliation was observed also among cultivars of *F. excelsior*. The 'Aurea Pendula', 'Diversifolia' (one out of three trees), 'Heterophylla Pendula', and 'Nana' cultivars showed less than 20% defoliation, and two 'Diversifolia', 'Pendula' and 'Pruhoniciana' were heavily defoliated (60%–95%). *F. ornus* trees showed no defoliation. Three trees of *F. mandshurica*, the only Asian species growing in the arboretum, were moderately defoliated (40%). Three trees of the North-American species, *F. pennsylvanica*, showed defoliation (one had up to 10% defoliation, two showed moderate defoliation (30% and 50%)); two trees of 'Aucubifolia' cultivar had a defoliation level of 20%. No ash tree had the crown completely defoliated. Average percent defoliation of ash trees in Borová hora was 31.8%. All *Fraxinus* taxa, except *F. ornus*, showed ash dieback symptoms.

In Kysihýbel Arboretum, 54 trees of 9 taxa were inspected (Table 2). Out of 25 *F. excelsior* trees, two showed slight defoliation (up to 20%), 12 had moderate defoliation (30%–60%), nine were heavily defoliated (70%–95%), and two were completely defoliated. It should be noted that several of the dying *F. excelsior* trees had also been heavily attacked by the ash bark beetle, *Hylesinus* sp. (Curculionidae, Coleoptera). All trees of *F. ornus* showed up to 10% defoliation. Of the assessed Asian species, *F. bungeana* A. DC. and *F. chinensis* ssp. *rhynchophylla* (Hance) A.E. Murray showed up to 5% defoliation. Out of four *F. mandshurica* trees, three had up to 10% defoliation and one showed 20% defoliation. The North American species *F. americana*, *F. cinerea* Bosc, and *F. latifolia* Benth. were slightly defoliated (15%–30%). All trees of *F. pennsylvanica* had up to 30% defoliation. Average percent defoliation of ash trees in Kysihýbel was 35.7%.

In Liptovský Hrádok Arboretum, 20 trees of 7 taxa were assessed (Table 2). Out of 12 *F. excelsior* trees, three showed up to 10% defoliation, two showed slight defoliation (up to 25%), and seven were moderately defoliated (30%–40%). The single specimen of *F. excelsior* 'Globosa' was heavily defoliated (80%). Of the assessed Asian ash species represented by one tree each, *F. chinensis* ssp. *rhynchophylla* showed <10% defoliation, *F. mandshurica* was slightly defoliated (20%), and *F. bungeana* had up to 30% defoliation. North American *F. pennsylvanica* and *F. pennsylvanica* v. *lanceolata* (Borkh.) Sarg. showed moderate to heavy defoliation. Average percent defoliation of ash trees reached 34.5%. All *Fraxinus* taxa, except *F. chinensis* ssp. *rhynchophylla*, showed ash dieback symptoms.

In Mlyňany Arboretum, a total of 368 trees of 23 taxa were assessed for ash dieback symptoms (Table 2). Out of 12 *F. excelsior* trees, three showed less than 10% defoliation, one was slightly defoliated (up to 20%), two were moderately defoliated (30% and 50%), one had heavy defoliation (80%), and five were dead. Of the *F. excelsior* cultivars, one 'Angustifolia', five 'Diversifolia' and a single specimen of 'Pendula' had 50%–75% defoliation, and one tree of 'Angustifolia' cultivar was dead. Out of 18 *F. angustifolia* ssp. *syriaca* (Boiss.) Yalt., 14 showed up to 10% defoliation and four had 15%–20% defoliation. All 5 trees of *F. angustifolia* ssp. *oxycarpa* (M. Bieb. ex Willd.) Franco & Rocha Afonso showed up to 75% defoliation. Out of 17 *F. ornus* trees, 13 showed slight defoliation (up to 20%), three had 30% defoliation, and one had heavy defoliation (70%). Up to 50 trees of two *F. pennsylvanica* varieties (v. *lanceolata* and v. *subintegerrima*) showed no or less than 10% defoliation, two trees were slightly defoliated (up to 20%), and one was moderately defoliated (50%). One tree of v. *subintegerrima* had up to 90% defoliation. Both *F. americana* trees showed moderate defoliation (30% and 50%). Out of four *F. quadrangulata* Michx., three had no defoliation and one was up to 20% defoliated. Six trees of the North American species *F. velutina* Torr. showed 20%–30% defoliation. Of the assessed Asian species, all 15 trees of *F. bungeana*, 126 of *F. chinensis* Roxb., 13 of *F. longicuspis* Siebold & Zucc., and one *F. pubinervis* Blume showed no or less than 10% defoliation. Out of 70 *F. chinensis* ssp. *rhynchophylla* trees, 65 had no defoliation and five showed up to 20% defoliation. Out of seven *F. mandshurica*, one showed up to 5% defoliation, three were slightly defoliated (up to 20%), and three had heavy defoliation (70%–90%). *F. sogdiana* and *F. paxiana* Lingelsh., represented by one tree each, showed 20% and 50% defoliation, respectively. A single specimen of *F. xanthoxyloides* v. *dimorpha* (Coss. & Durieu) Wenz. showed no defoliation. In summary, 296 trees (80.4%) of evaluated trees showed no or less than <10% defoliation, 37 trees were classified as slightly defoliated, 16 as moderately defoliated, 13 as severely defoliated, and 6 trees were dead. Average percent defoliation of ash trees in Mlyňany Arboretum was 8.9%. Ten *Fraxinus* taxa showed ash dieback symptoms. Although *F. americana*, *F. angustifolia* ssp. *syriaca*, *F. longicuspis*, *F. paxiana*, *F. pennsylvanica* v. *lanceolata*, *F. pennsylvanica* v. *subintegerrima*, *F. sogdiana*, and *F. velutina* showed slight to moderate defoliation, no other symptoms of ash dieback or production of apothecia were observed.

A total of 470 trees of 34 taxa were evaluated. In all arboreta, 69.6% of evaluated trees showed no, or less than 10%, defoliation. The mean value of defoliation reached 14.5%. Dead trees accounted for 1.7% of all assessed trees. Figure 3 shows the estimated crown defoliation of ash trees in Mlyňany in 2015 and in Borová hora, Liptovský Hrádok, and Kysihýbel in 2018.

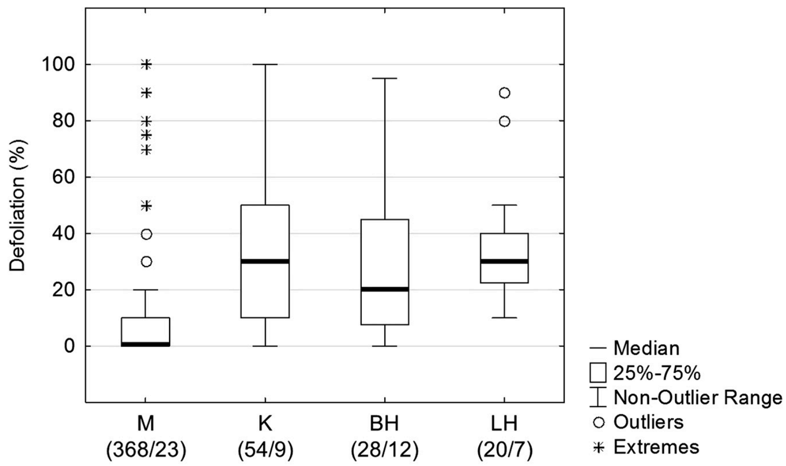

Figure 3. Crown defoliation (in percentages) of ash trees in Slovak arboreta (BH—Borová hora, K—Kysihýbel, LH—Liptovský Hrádok, M—Mlyňany). The number of trees examined/number of taxa is shown in parenthesis under the location name.

Defoliation percentage or range of defoliation percentage (if more than 2 trees of the same taxon on the same site showed different levels of defoliation) of individual *Fraxinus* taxa in each arboretum is given in Table 2. Severe to extreme defoliation was common in *F. excelsior* and most of its cultivars and rarely in *F. mandshurica*, *F. pennsylvanica*, and two subspecies of *F. angustifolia* (Figure 4). *F. excelsior* was the only species that was completely defoliated in Mlyňany and Kysihýbel. Percentage of defoliation varied significantly among *Fraxinus* taxa ($F_{(24, 436)} = 36.707$, $p < 0.001$) when analyzed after omitting the nine taxa represented by one tree each (*F. angustifolia* 'Pendula', *F. angustifolia* ssp. *pannonica*, *F. excelsior* 'Globosa', *F. excelsior* 'Heterophylla Pendula', *F. excelsior* 'Nana', *F. paxiana*, *F. pubinervis*, *F. sogdiana*, *F. xanthoxyloides* v. *dimorpha*).

Table 2. Summary of *Fraxinus* taxa examined for the presence of *Hymenoscyphus fraxineus* in four Slovak arboreta, including the extent of tree defoliation, results of species–specific PCR, and GenBank accession numbers of representative sequences.

Host	Arboretum [a]	No. of Trees	Age of Trees [b] [yrs.]	Tree Defoliation [c] [%]	Year of Apothecia Collection	Testing Results of SSPP [d]	GenBank Acc. No. ITS
F. americana	K	2	118	15	n.f.		
F. americana	M	2	36	30; 50	2015	–	
F. angustifolia	BH	6	37	5–40	2018	+	MK522151
F. angustifolia 'Pendula'	M	1	36	5	n.f.		
F. angustifolia ssp. *oxycarpa*	M	5	36	75	2015	+	MF175248
F. angustifolia ssp. *pannonica*	BH	1	43	85	2018	+	MK491641
F. angustifolia ssp. *syriaca*	M	18	53	10–20	n.f.		
F. angustifolia v. *australis*	M	7	33	0–40	2015	+	MF175249
F. bungeana	K	4	29	5	n.f.		
F. bungeana	LH	1	35	30	2018	+	MK491649
F. bungeana	M	15	45	0	n.f.		
F. chinensis	M	126	30–50	0–10	n.f.		
F. chinensis ssp. *rhynchophylla*	K	6	29	5	2018	+	MK491647
F. chinensis ssp. *rhynchophylla*	LH	1	20	10	n.f.		
F. chinensis ssp. *rhynchophylla*	M	70	30–45	0–20	n.f.		
F. cinerea	K	2	113	30	2018	+	MK491646
F. excelsior	K	25	92–118	20–100	2018	+	MK491642
F. excelsior	LH	12	45–80	10–40	2018	+	MK491651
F. excelsior	M	12	51	5–100	2015	+	MF175245
F. excelsior 'Angustifolia'	M	2	53	70; 100	2015	+	MF175250
F. excelsior 'Aurea Pendula'	BH	2	35; 40	10; 20	2018	+	MK491632
F. excelsior 'Diversifolia'	BH	3	44	5–80	2018	+	MK491635
F. excelsior 'Diversifolia'	M	5	36	50	2015	+	MF175252
F. excelsior 'Globosa'	LH	1	36	80	2018	+	MK491652
F. excelsior 'Heterophylla Pendula'	BH	1	43	5	2018	+	MK491639
F. excelsior 'Nana'	BH	1	39	10	2018	+	MK491638
F. excelsior 'Pendula'	BH	1	39	95	2018	+	MK491637
F. excelsior 'Pendula'	M	1	60	75	2015	+	MF175251
F. excelsior 'Pruhoniciana'	BH	2	50	70; 90	2018	+	MK491634
F. latifolia *	K	3	112	20	2018	+	MK491645

Table 2. *Cont.*

Host	Arboretum [a]	No. of Trees	Age of Trees [b] [yrs.]	Tree Defoliation [c] [%]	Year of Apothecia Collection	Testing Results of SSPP [d]	GenBank Acc. No. ITS
F. longicuspis	M	13	35	0–10	n.f.		
F. mandshurica	BH	3	53	40	2018	+	MK491640
F. mandshurica	K	4	29	10–20	2018	+	MK491643
F. mandshurica	LH	1	33	20	2018	+	MK491648
F. mandshurica	M	7	30–50	5–90	2015	+	MF175246
F. ornus	BH	3	55	0	n.f.		
F. ornus	K	3	114	10	n.f.		
F. ornus	M	17	48–57	20–70	2015	+	MF175247
F. paxiana	M	1	53	50	n.f.		
F. pennsylvanica	BH	3	55	10–50	2018	+	MK491636
F. pennsylvanica	K	5	113	30	2018	+	MK491644
F. pennsylvanica	LH	2	45	50; 90	2018	+	MK491650
F. pennsylvanica 'Aucubifolia'	BH	2	45	20	2018	+	MK491633
F. pennsylvanica v. *lanceolata*	LH	2	32	40	2018	+	MK491653
F. pennsylvanica v. *lanceolata*	M	10	30–45	0-20	2016	−	
F. pennsylvanica v. *subintegerrima*	M	43	36	0–90	2016	−	
F. pubinervis	M	1	46	5	n.f.		
F. quadrangulata	M	4	34	0–20	2016	+	MF175253
F. sogdiana **	M	1	34	20	n.f.		
F. velutina	M	6	40	20–30	n.f.		
F. xanthoxyloides v. *dimorpha*	M	1	33	0	n.f.		

Notes: Nomenclature accepted according to the world's largest botanical database, Tropicos [34]. * The host species grows in the Kysihýbel Arboretum under the synonymous name *F. oregona* Nutt. ** The host species grows in the Mlyňany Arboretum under the synonymous name *F. potamophila* Herder. [a] Abbreviations: BH, Borová hora; K, Kysihýbel; LH, Liptovský Hrádok; M, Mlyňany. [b] The age of each tree was obtained from its registration card. If trees of the same taxon at different ages were evaluated on the site, their age range is given. [c] If more than two trees of the same taxon on the same site showed different levels of percentage defoliation, the range of percentage defoliation is given. [d] SSPP, species–specific primed PCR; '+' if DNA fragment corresponded to the expected size of PCR product. n.f. (not found) if no apothecia were present on fallen ash leaf rachises and petioles in the litter from the previous year.

Only three species of ash, *F. excelsior*, *F. mandshurica*, and *F. pennsylvanica*, were present in all Slovak arboreta. Percentage of defoliation of *F. excelsior* (including taxa of lower rank) varied significantly among arboreta ($F_{(3, 64)} = 3.35$, $p = 0.024$; Figure 5). The crown damage of *F. excelsior* trees in Liptovský Hrádok was significantly lower than in Mlyňany and Kysihýbel. There was also a significant difference in the percentage of defoliation of *F. pennsylvanica* among arboreta ($F_{(3, 63)} = 20.93$, $p < 0.001$; Figure 5). The highest defoliation was observed in Liptovský Hrádok, the lowest in Mlyňany. The average defoliation showed a similar level in Borová hora and Kysihýbel, but it was significantly higher than in Mlyňany and lower than in Liptovský Hrádok. Although *F. mandshurica* grew in all arboreta, we did not include it in the analysis due to the low sample size in Liptovský Hrádok.

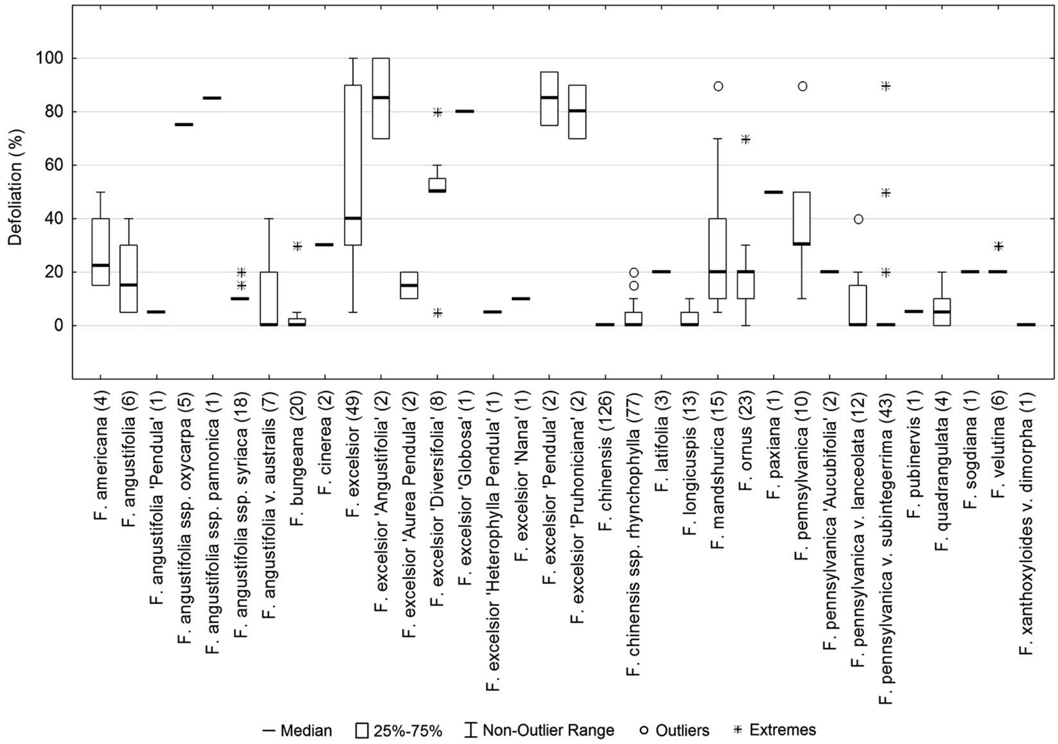

Figure 4. Crown defoliation (in percentages) of *Fraxinus* taxa in four Slovak arboreta. The number of assessed trees is enclosed in parenthesis after the taxon name.

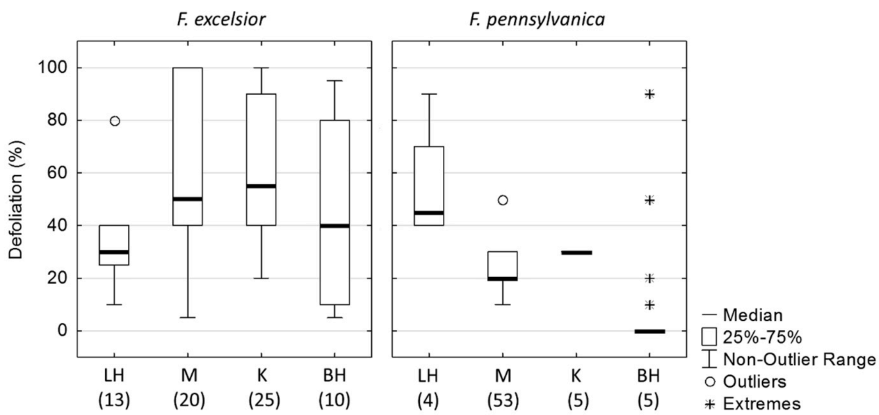

Figure 5. Comparison of crown defoliation (in percentages) of *F. excelsior* and *F. pennsylvanica* (both at species level) among Slovak arboreta (BH—Borová hora, K—Kysihýbel, LH—Liptovský Hrádok, M—Mlyňany). The number of trees examined is indicated in parenthesis under the location name.

3.2. Sequencing Analysis

DNA was extracted from 64 apothecia samples. The amplicons of ITS region using the ITS1F/ITS4 primers ranged from 700 to 875 bp. The obtained ITS1-5.8S-ITS2 rDNA sequences exhibited between 99.07% (GenBank accession no. KY593990) to 100% (GenBank accession no. MN833901) similarity to previously deposited sequences of *H. fraxineus* in GenBank. The obtained ITS sequences (a single sequence per *Fraxinus* taxon and per site) were deposited in the NCBI GenBank; accession numbers are given in Table 2.

In 57 of the 64 samples, the presence of *H. fraxineus* was detected by species–specific PCR, which produced an amplicon of size 456 bp and showed positive results for all samples except three samples from *F. pennsylvanica* v. *subintegerrima,* three from *F. pennsylvanica* v. *lanceolata*, and one from *F. americana* that were collected in Mlyňany (Table 2).

3.3. Phylogenetic Analysis

The ITS dataset contained 65 sequences representing eight *Hymenoscyphus* species. Among them, 32 sequences were newly obtained and 33 were retrieved from GenBank. In the phylogenetic analysis, *Hymenoscyphus menthae* from *Solidago canadensis* L. was chosen as the outgroup.

Phylogenetic analysis of 32 samples of *H. fraxineus* from different *Fraxinus* species collected in Slovak arboreta showed high sequence similarity with all reference sequences of *H. fraxineus* from GenBank. They all grouped together in one strongly supported (81% bootstrap support) clade with *H. fraxineus* from different *Fraxinus* species collected in other European localities and Asia (Figure 6).

The obtained ITS sequences of Slovak samples were identical irrespective of the host and concurred with those of European samples but differed from Asian samples at some positions. All Slovak ITS sequences differed from the three ITS sequences (KF188725, KJ511194, KP068051) from Japan, Korea, and China at two nucleotide positions, 433 (a gap instead of G) and 474 (T instead of C), and from one ITS sequence (KU323580) from Russia at three positions, 424 (a gap instead of G), 465 (T instead of C), and 679 (C instead of T).

3.4. Host Range of H. fraxineus in Slovak Arboreta

In four Slovak arboreta, the identity of the fungus *H. fraxineus* was molecularly confirmed in 23 out of 34 evaluated *Fraxinus* taxa. Ash dieback was recorded in 11 out of 12 taxa in Borová hora, in 6 out of 9 taxa in Kysihýbel, in 6 out of 7 taxa in Liptovský Hrádok, and in 9 out of 23 taxa in Mlyňany. The numbers of ash trees (at species level), of both symptomatic and asymptomatic individuals, in each of the examined arboreta are given in Table 3.

In total, 64.3% of the trees of native ash species showed symptoms of ash dieback in the arboreta. The fungus was confirmed on the common ash *F. excelsior* as well as its eight cultivars in all evaluated arboreta; on *F. angustifolia* and its two subspecies and one variety in Borová hora and Mlyňany; and on *F. ornus* in Mlyňany.

Analysis of samples from Asian *Fraxinus* species confirmed the presence of *H. fraxineus* on *F. mandshurica* in all arboreta, on *F. chinensis* ssp. *rhynchophylla* in Kysihýbel, and on *F. bungeana* in Liptovský Hrádok. Four Asian species, *F. longicuspis*, *F. paxiana*, *F. pubinervis*, and *F. sogdiana*, were not attacked by the pathogen. Less than 6% of the trees of Asian species showed ash dieback symptoms in all examined arboreta.

The causal agent of ash dieback was recorded on four North American ash species: *F. cinerea* and *F. latifolia* in Kysihýbel; on *F. pennsylvanica* and its one cultivar and one variety in Borová hora, Kysihýbel, and Liptovský Hrádok; and on *F. quadrangulata* in Mlyňany. Although *F. velutina* showed slight defoliation, no other symptoms of ash dieback or production of apothecia were observed. *F. americana* was moderately defoliated in Mlyňany but without clear symptoms and with a negative result for a single sample from this host tested using *H. fraxineus*-specific PCR primers. In Slovak arboreta, 25% of the trees of North American species were attacked by *H. fraxineus*.

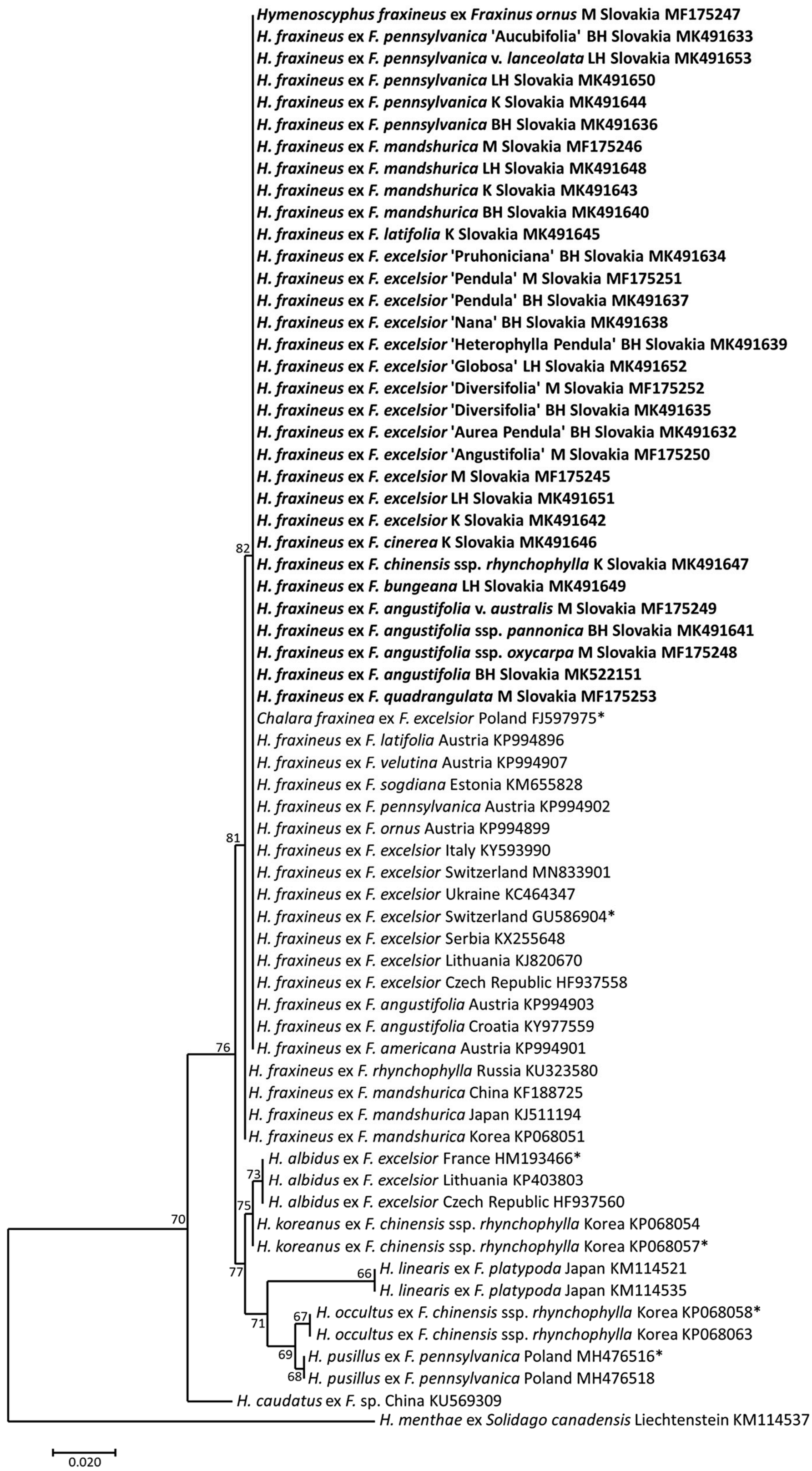

Figure 6. Maximum likelihood tree based on ITS rDNA sequences of *Hymenoscyphus* spp. The bootstrap values >60% are indicated at the nodes. The scale bar indicates the number of substitutions per site. Material collected in Slovak arboreta (BH—Borová hora, K—Kysihýbel, LH—Liptovský Hrádok, M—Mlyňany) and sequenced in this work are shown in bold type. Sequences from type materials are marked with an asterisk (*).

Table 3. *Fraxinus* species with and without ash dieback symptoms in study sites.

Natural Distribution	*Fraxinus* Species	Number of Asymptomatic/Symptomatic Trees					% of Symptomatic Trees in all Arboreta
		BH	**K**	**LH**	**M**	**All Arboreta**	
Asia	*bungeana*		4/0	0/1	15/0	19/1	5
	chinensis *		3/3	1/0	196/0	200/3	1.5
	longicuspis				13/0	13/0	0
	mandshurica	0/3	3/1	0/1	1/6	4/11	73.3
	paxiana				1/0	1/0	0
	pubinervis				1/0	1/0	0
	sogdiana				1/0	1/0	0
Europe to Asia	*angustifolia* *	0/7			24/7	24/14	36.8
	excelsior *	0/10	0/25	0/13	2/18	2/66	97.1
	ornus	3/0	3/0		14/3	20/3	13
North America	*americana*		2/0		2/0	4/0	0
	cinerea		0/2			0/2	100
	latifolia		1/2			1/2	66.7
	pennsylvanica *	0/5	0/5	0/4	53/0	53/14	20.9
	quadrangulata				3/1	3/1	25
	velutina				6/0	6/0	0
North Africa	*xanthoxyloides* *				1/0	1/0	0
	Totals	**3/25**	**16/38**	**1/19**	**333/35**	**353/117**	**24.9**

Abbreviations: BH—Borová hora, K—Kysihýbel, LH—Liptovský Hrádok, M—Mlyňany. * including taxa of lower rank.

The North African ash tree species *F. xanthoxyloides* v. *dimorpha*, an endangered species listed in the IUCN Red List of Threatened Species 2017, had no symptoms of ash dieback in Mlyňany.

The highest number of assessed trees was in Mlyňany Arboretum, and the number of trees of introduced ash taxa greatly exceeded the native species in this locality. In Mlyňany, 9.5% of evaluated trees showed ash dieback symptoms in 2015. Most of the asymptomatic trees in Mlyňany belong to introduced species. The percentage of symptomatic ash trees in Kysihýbel, Borová hora, and Liptovský Hrádok ranged from 70% to 95% in 2018. The native species predominated in these three arboreta with lower total numbers of ash trees.

Ash dieback symptoms were recorded in ash trees of all ages (Table 2). Together in all arboreta, 24.9% of all evaluated trees showed ash dieback symptoms (Table 3). The greatest damage caused by *H. fraxineus* was recorded on *F. excelsior* and five of its eight cultivars. *F. mandshurica*, *F. angustifolia* ssp. *oxycarpa*, *F. angustifolia* ssp. *pannonica* (represented by a single tree), and *F. pennsylvanica* rarely showed serious dieback. The following 21 taxa represent new hosts of *H. fraxineus* in Slovakia: *F. angustifolia* ssp. *oxycarpa*, *F. angustifolia* ssp. *pannonica*, *F. angustifolia* v. *australis*, *F. bungeana*, *F. chinensis* ssp. *rhynchophylla*, *F. cinerea*, 8 cultivars of *F. excelsior* ('Angustifolia', 'Aurea Pendula', 'Diversifolia', 'Globosa', 'Heterophylla Pendula', 'Nana', 'Pendula', 'Pruhoniciana'), *F. latifolia*, *F. mandshurica*, *F. ornus*, *F. pennsylvanica*, *F. pennsylvanica* 'Aucubifolia', *F. pennsylvanica* v. *lanceolata*, and *F. quadrangulata*. *F. bungeana* is reported here as a new host species for *H. fraxineus*; it had not previously been reported as a host for this fungus.

4. Discussion

Three *Hymenoscyphus* species, *H. albidus*, *H. fraxineus*, and *H. pusillus*, are known to occur on *Fraxinus* trees in Europe. The non-pathogenic *H. albidus* has not been recorded in Slovakia to date. One herbarium specimen from Slovakia (PRM857180, Nízke Tatry Mts., 2005), originally identified as *H. albidus*, was revised as *H. fraxineus* by Koukol et al. [35] on the basis of ITS rDNA sequences. None of the samples examined in the present study were identified as *H. albidus*. *Hymenoscyphus pusillus*, recently discovered and described from *F. pennsylvanica* in Poland [9], has not yet been recorded in Slovakia. The pathogenic *H. fraxineus*, the cause of massive mortality of ash trees in forest stands throughout Europe, mainly attacks the native ash species *F. excelsior* and *F. angustifolia* [2,36,37]. In Slovakia, the invasive fungus *H. fraxineus* causes much damage to both *F. excelsior* and *F. angustifolia* in forest stands and seed orchards [19–21]. The third native European species, *F. ornus*, seemed to be tolerant to ash dieback until recently. However, Kirisits and Schwanda [15] recently isolated *H. fraxineus* from leaf rachises of *F. ornus* seedlings in Austria. We also recorded *F. ornus* as a host of the pathogen in the Mlyňany Arboretum.

The present study of introduced ash species in Slovak arboreta confirmed that the pathogen has a much larger host range in Slovakia. *H. fraxineus* is considered a weak pathogen of the Asian species *F. mandshurica* in Japan [38,39], China [40], the Russian Far East [10,41], and South Korea [42]. However, *F. mandshurica* trees were moderately, and occasionally badly, affected with symptoms of dieback of shoots and twigs in the evaluated Slovak arboreta. Drenkhan and Hanso [13] also recorded *F. mandshurica* trees affected with ash dieback in Estonian parks. Cleary et al. [43] confirmed the presence of *H. fraxineus* in asymptomatic leaves of *F. mandshurica* from Eastern Russia.

In South Korea [42] and in the Russian Far East [10], apothecia of *H. fraxineus* have been recorded on fallen leaves of *F. chinensis* ssp. *rhynchophylla*. Nielsen et al. [16] did not detect *H. fraxineus* DNA in the leaves of *F. chinensis* ssp. *rhynchophylla* after intensive spore exposure, and no bark lesions were developed after direct stem inoculation. As a result, they suggested that *F. chinensis* ssp. *rhynchophylla* may be less susceptible to leaf infection by *H. fraxineus* than *F. mandshurica*. When exposed to natural infection in a Danish arboretum, some of the additional Asian *Fraxinus* species examined were also asymptomatic or showed only a few dead shoots. In the present study, *F. chinensis* ssp. *rhynchophylla* trees in Kysihýbel Arboretum showed slight symptoms, including minor shoot dieback and bark necrosis.

In 2015, Drenkhan et al. [14] recorded *F. sogdiana*, a Central Asian ash species, as a new host of *H. fraxineus* in Estonia, indicating that some additional Asian ash species planted in Europe can serve as hosts for this invasive pathogen. *Fraxinus sogdiana* growing in Mlyňany Arboretum under the name *F. potamophila* had no symptoms of ash dieback. However, *F. bungeana*, native to Northeast China, showed symptoms of the disease in Liptovský Hrádok Arboretum in 2018. These observations are the first report of *H. fraxineus* on *F. bungeana*; our finding of *H. fraxineus* apothecia on petioles of fallen leaves of *F. bungeana* in Slovakia represents a new host among Asian ash species.

Although *H. fraxineus* has not yet been recorded in North America, some North American *Fraxinus* species (*F. albicans* Buckley, *F. americana*, *F. latifolia*, *F. nigra*, *F. pennsylvanica*, *F. profunda* (Bush) Bush, *F. quadrangulata*, *F. velutina*) seem to be susceptible to shoot infection [13,16,44,45]. *Fraxinus pennsylvanica* naturally infected by the ash dieback pathogen was recorded in Estonia and Austria [13,46]. The results from Slovak arboreta confirmed *F. cinerea*, *F. latifolia*, *F. pennsylvanica*, *F. pennsylvanica* 'Aucubifolia', *F. pennsylvanica* v. *lanceolata*, and *F. quadrangulata* as hosts of *H. fraxineus*. On the other hand, *F. americana* and *F. velutina* planted in Slovak arboreta were not infected by the pathogen. *Hymenoscyphus fraxineus* has already been reported on these two host species from European parks and arboreta [13,16]. Although several *F. pennsylvanica* v. *lanceolata* and v. *subintegerrima* trees in Mlyňany Arboretum showed severe defoliation, the presence of *H. fraxineus* was not confirmed by our molecular work. *Hymenoscyphus*-like apothecia collected under these trees will be subjected to further analysis.

Four ash species, *F. longicuspis*, *F. paxiana*, *F. pubinervis*, and *F. xanthoxyloides* v. *dimorpha*, not yet recorded as hosts of *H. fraxineus*, showed no symptoms of dieback in Slovak arboreta. Nielsen et al. [16] assumed that failure of *H. fraxineus* to infect two Asian ash species, *F. sieboldiana* Blume and *F. paxiana*, may reflect cases of non-host resistance. We recommend, however, that monitoring and analysis of all asymptomatic exotic ash trees be continued; their resistance to ash dieback should not be assumed.

The phylogram (Figure 6) shows that ITS sequences of all Slovak samples of *H. fraxineus* are grouped in a separate clade together with 16 sequences from other European samples retrieved from the GenBank database. The Slovak ITS sequences concur with sequences of European samples and form a subgroup within a *H. fraxineus* clade with a strong nodal support (ML = 82). The phylogram also demonstrates an intraspecific variation in *H. fraxineus*. The four GenBank sequences of Asian strains are clearly separated from the Slovak and all European samples due to differences at two nucleotide positions for strains from *F. mandshurica* (KF188725, KJ511194, KP068051) and at the other three positions for a strain from *F. chinensis* ssp. *rhynchophylla* (KU323580). These observations are in accordance with several recent studies and confirm that the ITS region of *H. fraxineus* from Asian populations (from Japan, China, and Korea) as well as samples from the Russian Far East are genetically divergent from European population of *H. fraxineus* [10,39,40,42].

The artificial inoculations as well as field observations of recent studies have demonstrated that Asian ash species tended to be immune to ash dieback disease compared to European ash species [10,13,43,47]. Field observations of ash dieback symptoms in this study also suggest that *H. fraxineus* is more pathogenic to European ash species (e.g., *F. excelsior* and *F. angustifolia*) than to Asian ash species (*F. mandshurica* and *F. chinensis*). It is important to note that no pathogenicity tests were carried out in this study and the above findings are solely based on the field observations of symptoms.

There are clear differences in the progression of ash dieback in trees of different age, irrespective of stand disease history, but there are also differences among stands in different regions, depending on their disease histories [48]. Trees are subject to dieback at all ages [3]; our results confirm this. Although semi-mature trees (20–60 years old) of introduced ash species growing in arboreta situated in temperate and humid areas displayed ash dieback symptoms, they were not infected (or only rarely infected) in the warmest and driest arboretum (Mlyňany). It has been shown that a high number of the youngest and intermediate-aged future crop trees in forest stands in Norway were dead, while development of the disease in large, dominant trees was slower [48]. The higher mortality of young trees and nursery saplings causes immense problems for establishing and tending young stands [49]. Due to the different age structure of individual *Fraxinus* taxa in Slovak arboreta, it was not possible to evaluate the influence of tree age on susceptibility to infection. However, the influence of tree age is considered a secondary factor [50]. We should note that the number of dead trees can be affected by regular tree maintenance (removal of dead and/or dying trees) in the arboreta. For example, during our investigations, no ash tree had its crown completely defoliated in Liptovský Hrádok Arboretum—however, seven dead trees (5 *F. excelsior*, 1 *F. excelsior* 'Pendula', 1 *F. pennsylvanica* 'Aucubifolia') were felled 1–2 years before our assessment [51]. In addition, from 2012 to 2019, 31 dead ash trees (26 *F. excelsior* cultivars, 4 *F. ornus*, 1 *F. angustifolia*) were removed in Borová hora Arboretum. In Mlyňany Arboretum, 9 dead trees of *F. excelsior* and 1 *F. mandshurica* were felled from 2019–2020. The cause of the death of ash trees that were felled before our survey was not determined. From the time of our survey to 2019, no ash trees were felled due to *H. fraxineus* attack in Kysihýbel Arboretum [52].

Climate and site conditions have a fundamental influence on *H. fraxineus* sporulation, spore release and dispersal, as well as on infection rate throughout the growing season [53–57]. The dynamics of apothecia production varied depending on weather conditions during the years of our survey. Longer periods with low rainfall adversely affected the fructification of the pathogen. Thus, apothecia were not found under some trees with ash dieback symptoms in Mlyňany in 2015. A lower infection rate (9.5%) was recorded in Mlyňany Arboretum, while the proportion of symptomatic ash trees in Kysihýbel, Borová hora, and Liptovský Hrádok ranged from 70% to 95%. We assume that the higher percentage of infected trees in the three arboreta from central Slovakia was influenced by more favorable microclimatic conditions at the localities. Borová hora, Kysihýbel, and Liptovský Hrádok experience lower average temperatures and higher amounts of precipitation compared to Mlyňany Arboretum, which is situated in one of the warmest and driest areas of Slovakia. Likewise, Ogris [58], Kessler et al. [49], Hauptman et al. [59], and Gross et al. [60] recorded higher severity of ash dieback on sites with high relative air humidity and lower temperatures. The differences in the infection rates of ashes among Slovak arboreta can be caused not only by environmental factors (e.g., tree competition, shading, microclimate, etc.) or crown and canopy management, but also by ash species composition and tree size. The lesser amount of damage found in Mlyňany might be due to the fact that the investigation in Mlyňany took place in 2015, whereas the other three arboreta were surveyed in 2018. The disease progression in these arboreta will be further monitored.

Insect pests and other plant pathogenic fungi represent a noticeable threat to stressed and weakened ash trees and may contribute to the progress of ash dieback disease in ash stands. Numerous fungal taxa on ash stems and twigs in the initial and advanced stages of dieback were recorded by Bakys et al. [3], Kowalski et al. [61], and Pastirčáková et al. [62]. The development and severity of ash dieback may also be correlated with soil moisture in stands and with *Armillaria* root infections [48,63]. In Slovak seed orchards, Longauerová et al. [20] observed frequent occurrence of *Armillaria cepistipes* Velen. and *Armillaria gallica* Marxm. & Romagn. on trees affected by ash dieback. However, we did not find any *Armillaria* fruiting bodies on assessed ash trees in Slovak arboreta. Post-dieback invasion by secondary pests also accelerates the death of dieback-weakened trees. In Kysihýbel Arboretum, we observed that common ash trees with symptoms of ash dieback had been heavily attacked by the ash bark beetle, *Hylesinus* sp., which affected the degree of crown defoliation. In older ash stands in Slovakia, Kunca et al. [64] and Zúbrik et al. [65] noted an increased number of the secondary invaders *Hylesinus fraxini* Panzer and *Hylesinus crenatus* Fabricius on trees already weakened by *H. fraxineus*.

Recent research studies have focused on finding resistance in host trees. The evidence for genetic control of resistance was provided by studies of clonal seed orchards for the native species *F. angustifolia* and *F. excelsior* [66,67]. In addition, species–specific variation in disease susceptibility has been previously documented among European, North American, and Asian *Fraxinus* species [16]. Recently, Stocks et al. [68] identified ash genes that may confer resistance to ash dieback. Given the importance of ash in forest management and urban greenery, it is crucial to continue developing resistant trees for the purpose of safeguarding European ash populations for future generations.

5. Conclusions

The invasive fungus *Hymenoscyphus fraxineus* decimates not only native European *Fraxinus* species, but introduced ash species as well. A survey of four Slovak arboreta revealed that the host range of *H. fraxineus* in Slovakia also includes Asian and North American ash species. Ash dieback was recorded from *F. bungeana* for the very first time. Since many introduced ash species have been attacked in arboreta in Northern and Central Europe, we assume that a similar situation can be found in arboreta and botanical gardens in other parts of Europe.

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