Ash Trees (Fraxinus spp.) in Urban Greenery as Possible Invasion Gates of Non-Native Phyllactinia Species

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Abstract: Two Phyllactinia species have been associated with powdery mildew on leaves of ash trees (Fraxinus) in Eurasia, Phyllactinia fraxinicola U. Braun & H.D. Shin from Southeast Asia and Phyllactinia fraxini (DC.) Fuss from Europe. Non-native ash trees are planted in urban greenery in both Europe and Southeast Asia, but so far, the two Phyllactinia species have not been reported from the same area. Our molecular analysis of European material consisting of 55 Phyllactinia specimens from 15 countries confirmed the absence of P. fraxinicola in Europe. In Europe, we confirmed P. fraxini on all three European native ash species and on the introduced Asian ash species, Fraxinus chinensis ssp. Rhynchophylla (Hance) A.E. Murray and Fraxinus mandshurica Rupr, planted in arboreta. Among the 11 collections examined from Southeast Asia, 3 were identified as P. fraxini and 8 as P. fraxinicola. The environmental niches of the two Phyllactinia species do not show significant overlap in the multidimensional space defined by bioclimatic variables. This suggests that the Asian species P. fraxinicola is not adapted to conditions prevailing in most of Europe and does not represent an invasive threat across the continent. Models of the potential distribution of Phyllactinia species do not overlap in Europe, but there are some areas to the northwest that could be susceptible to invasion by P. fraxinicola.

Keywords: bioclimatic variables; invasive fungi; powdery mildews; niche analyses; habitat modeling

1. Introduction

The genus Fraxinus L. (ash tree) comprises more than 40 tree and shrub species distributed in temperate areas of Eurasia and North America [1]. The natural distribution areas of species are currently disjunct, with species limited to large areas: (i) Europe and the Mediterranean parts of Asia and Africa, (ii) southern and southeastern Asia, and (iii) North and Central America [2]. Several species represent common forest trees or shrubs and are also used as part of urban greenery [3–5].

Ash trees were appreciated and used in Europe for their relatively low disease incidence [6]. This has changed recently with the appearance of ash dieback caused by Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz & Hosoya. This ascomycete causes shoot dieback, eventually leading to tree death and decimation of affected ash populations. The fungus may have been introduced in Europe along with imported plants of its native hosts, the Asian ash species Fraxinus mandshurica Rupr. or F. chinensis Roxb. Unlike the Asian hosts, European ash (Fraxinus excelsior L.) is highly susceptible to shoot dieback

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and the disease incidence was also enhanced by the low genetic diversity of European populations of this fungus [7,8]. The extensive research on this disease during the past 15 years has overshadowed research on other ash pathogens, such as the causative agents of powdery mildew, fungi that were among the most distinctive ash-dwelling pathogens prior to the ash dieback pandemic. Powdery mildew, often very abundant on individual trees or specific tree populations, decreases tree fitness and esthetic value [9]. Leaves of Fraxinus are frequently inhabited by host-specific agents of powdery mildew, among them Phyllactinia fraxini (DC.) Fuss, which is the dominating species in Europe. In Asia, the range of ash-specific agents of powdery mildew includes P. fraxinicola U. Braun & H.D. Shin, Erysiphe fraxinicola U. Braun & S. Takam., and E. salmonii (Syd. & P. Syd.) U. Braun & S. Takam. [10]. One of the Asian species, E. salmonii, has been recently introduced in Europe [11].

The present study aimed to define the distribution ranges and environmental preferences of ash-specific species of Phyllactinia Lév. in Eurasia. Phyllactinia fraxini has been recorded on plants of three families, mainly representatives of Oleaceae: Chionanthus L., Fontanesia Labill., Fraxinus, Ligustrum L., and Syringa L. [10,12–14]. The fungus has an extensive distribution range, occurring across Europe, Asia, North America, and north Africa in a range of Fraxinus host trees [10,15,16]. Phyllactinia fraxinicola was officially described in 2012 from Southeast Asia and is known only from this region. The Asian species was distinguished morphologically by sinuous-twisted foot cells of conidiophores. This feature was soon reported from Europe, but it turned out that these two species cannot be distinguished by this character. Only P. fraxini is so far molecularly confirmed from Europe [17]. Thus, records of both Phyllactinia species identified based only on morphology were questionable [17–19].

There are cases where unreliable morphological diagnostic characters have caused erroneous identification of an invasive alien pathogen as a native species; e.g., H. fraxineus was initially misidentified as the native species H. albidus (Roberge ex Desm.) W. Phillips [20]. Further, the implementation of molecular methods has caused the formal break-down of species complexes with overlapping or distinct distribution ranges into separate taxa [21]. Phyllactinia fraxini is an excellent example of such a case. Recent molecular data revealed that the ash-dwelling Phyllactinia is a complex of at least two species with unclear morphological differences. Limited records based on sequence identification indicated these two species did not occur in the same areas [17]. The current study sought to gather molecularly verified data from several European and Southeast Asian areas to confirm whether individual ash-dwelling Phyllactinia species are limited to one continent. Phyllactinia fraxinicola is so far reported only from areas of Southeast Asia with a tropical climate. We hypothesize that this species is limited to Southeast Asia due to its relatively narrow climatic preferences. To test this, we gathered environmental data for the verified occurrences of ash-dwelling Phyllactinia species and compared their niches based on species distribution models [22]. Species distribution models combine environmental information with species data and estimate species occurrence or abundance in an environmental and/or a geographical space. These models have been proven useful in exploring biodiversity phenomena, such as forecasting of potential distributions of invasive species in novel regions [23,24]. Many studies argue that urban greenery with non-native plants represent a risk for the introduction of new pathogens [25]. Therefore, we paid special attention to the sampling of both native and introduced ash trees from natural and urban areas in order to predict their potential invasiveness.

2. Materials and Methods

2.1. Sampling

The material used included collections of Phyllactinia on ash leaves in Europe and fungal specimens originating from Europe, Southeast Asia, and North Africa, with a special emphasis on gathering representative datasets of both native and introduced ash trees from urban and natural habitats (Table S1). The material is deposited in the herbaria NR
and SAV. Additional material was borrowed from the herbaria HMAS, KR, KUS, LBL, LJF, SOMF, and UPS. Acronyms of the herbaria follow the Index Herbariorum [26]. All studied material was identified based on internal transcribed spacer (ITS) nrDNA using the study by Scholler et al. [17] as a reference. In addition, we retrieved ITS sequences available at GenBank (www.ncbi.nlm.nih.gov/genbank, accessed on 17 September 2020). To model the distribution of *Phyllactinia* in the Mediterranean Sea area, we also included two of our collections from North Africa.

### 2.2. Molecular Analysis

DNA was isolated from chasmothecia or from powdery-mildew-infected leaf tissue using the EZNA Fungal DNA Mini Kit (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer’s instructions. To identify *Phyllactinia* species, we sequenced the ITS region. To support the phylogenetic distinction between species, we also sequenced the nuclear large subunit 28S (LSU) nrDNA region of some samples. The *Phyllactinia*-specific primer set ITS5/Ph8 was used to amplify the ITS region, and primer sets Ph7/NLP2 and ITS4/Ph7 were used for the partial LSU region [17]. Amplification of DNA was performed in a PCR reaction mix consisting of approximately 2 ng/µL of template DNA, forward and reverse primers (10 pmol/µL), 5× HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), and molecular-grade water added up to 20 µL.

For both amplicons, PCR included an initial denaturation step at 95 °C for 15 min and a terminal final extension step for 10 min at 72 °C. Amplification of nrITS consisted of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 30 s repeated 30 times. The nrLSU amplification comprised 35 cycles of denaturation at 95 °C for 60 s, annealing at 58 °C for 60 s, and elongation at 72 °C for 60 s. The same PCR conditions were used for the primer pair ITS4/Ph7, except for the annealing step, performed at 57 °C.

The PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany). Samples were sequenced by the Seqme Company (Dobříš, Czech Republic).

Sequence files were edited in Geneious version R10 [27]. Intra-individual polymorphic sites having more than one signal were marked with NC-IUPAC ambiguity codes. Both single-locus datasets (nrITS and nrLSU) were aligned by MAFFT version 7 using the strategy E-INS-i [28], manually curated, and concatenated into one multi-locus dataset.

Phylogenetic analyses of nrITS and nrLSU regions were performed to visualize differences between the two studied species. *Leveillula clavata* Nour was used as an outgroup in these analyses [19]. The final multi-locus dataset was analyzed using the Cipres Science Gateway [29] with two different methods: Bayesian inference (BI) and maximum likelihood (ML). For the ML analysis, the concatenated alignment was uploaded as a FASTA file and analyzed using RAxML-HPC2 on XSEDE (8.2.12) [30] as a partitioned dataset under the GTR + GAMMA model with 1000 bootstrap iterations. For the BI analysis, the dataset was divided into four partitions: ITS1, 5.8S, ITS2, and LSU. The best substitution model for each partition was computed jointly in Partitionfinder 1.1.1 [31]. The aligned FASTA dataset was converted to nexus format using Mesquite 3.61 [32] and further analyzed using MrBayes 3.2.6. [33] on XSEDE. Bayesian runs were computed independently twice with four MCMC chains for 10 million generations until the standard deviation of split frequencies fell below the 0.01 threshold. The convergence of runs was visually assessed using the trace function in Tracer 1.6 [34]. The tree was visualized and annotated with TreeGraph 2 [35] and graphically improved in CorelDRAW X5 (Ottawa, ON, Canada). Alignments have been deposited in TreeBase (no. 27455).

### 2.3. Environmental Space and Bioclimatic Data

The environmental space used in further habitat modeling and niche analyses was defined based on the known distribution of *Fraxinus* species that were confirmed as hosts of *Phyllactinia* in Europe and Southeast Asia. In Europe, all three native species, *F. angustifolia*
Vahl, F. excelsior, and F. ornus L., are known as the hosts. The potential distribution of ash-dwelling *Phyllactinia* in Europe is estimated as a joint distribution of the three ash species [36–38]. Asian samples were collected on *F. mandshurica*, *F. chinensis*, *F. excelsior*, *F. lanuginosa* Koidz., *F. longicuspis* Siebold & Zucc., *Fraxinus. chinensis* ssp. *rhynchophylla* (Hance) A.E. Murray, and *F. sieboldiana* Blume. We were able to find a well-defined distribution range only for *F. mandshurica* [39]. The potential distribution area of *Phyllactinia* in Southeast Asia was estimated as the known distribution of *F. mandshurica* pooled with the estimated distribution of other Asian species [2,40,41].

Within this environmental space, data of 19 bioclimatic variables were retrieved from WorldClim version 2 [42] (http://www.worldclim.org, accessed on 3 November 2020) on a grid background with an accuracy of 30 arc-second spatial resolution (Table 1). These variables are derived from monthly measurements of temperature (11 variables) and precipitation (8 variables) over a long-term period of several years. More recent data, after the year 2000, were updated by Solar resource data (© Solargis, Bratislava, Slovakia, https://solargis.com/, accessed on 3 November 2020) that are based on climatic observations from 1994 to 2019. Based on the geographic position of molecularly identified collections, we also assigned bioclimatic variable data to individual *Phyllactinia* records (Table S1). The altitudinal data for Europe were retrieved from the Digital Elevation Model over Europe (EU-DEM) (https://www.eea.europa.eu/, accessed on 5 November 2020) and for Asia from NASA Shuttle Radar Topography Mission (SRTM) version 3.0 Global 1 arc-second Data Released over Asia and Australia (https://earthdata.nasa.gov/, accessed on 5 November 2020).

### Table 1. Mean values and standard deviations (in parentheses) of bioclimatic variables at collecting sites of *Phyllactinia fraxini* and *P. fraxinicola*. Results of randomization tests comparing differences in standardized mean values of bioclimatic variables (Δ) are displayed along with corresponding probabilities (p). Results significant at α = 5% are highlighted in bold.

| Code | Bioclimatic Variables (Unit) | *P. fraxini* | *P. fraxinicola* | Δ     | p        |
|------|------------------------------|--------------|------------------|-------|---------|
| b1   | annual mean temperature (°C) | 9.4 (2.4)    | 10.8 (2.6)       | 0.55  | 0.1492  |
| b2   | mean diurnal range (mean of monthly max. minus min. temperature) (°C) | 8.6 (1.2) | 8.2 (1.2) | -0.30 | 0.4383  |
| b3   | isothermality (b2/b7) (×100) | 30 (4)       | 24 (1)           | -1.40 | 0.0008  |
| b4   | temperature seasonality (standard deviation × 100) | 740 (118) | 926 (84) | 1.44  | 0.0002  |
| b5   | maximum temperature of the warmest month (°C) | 23.1 (3.1) | 27.8 (3.4) | 1.36  | 0.0002  |
| b6   | minimum temperature of the coldest month (°C) | -6 (3.5) | -6 (2.8) | 0.01  | 0.9886  |
| b7   | temperature annual range (b5–b6) (°C) | 29.1 (4.1) | 33.8 (3.5) | 1.10  | 0.0040  |
| b8   | mean temperature of the wettest quarter (°C) | 15.3 (4.9) | 17.2 (8.2) | 0.35  | 0.3570  |
| b9   | mean temperature of the driest quarter (°C) | 3.4 (6.8) | 2.5 (6.4) | -0.13 | 0.7480  |
| b10  | mean temperature of the warmest quarter (°C) | 18.5 (2.6) | 21.9 (3) | 1.21  | 0.0008  |
| b11  | mean temperature of the coldest quarter (°C) | 0.4 (3.1) | -0.8 (2.4) | -0.37 | 0.3182  |
| b12  | annual precipitation (mm) | 708 (212) | 1624 (599) | 2.25  | <0.0001 |
| b13  | precipitation of the wettest month (mm) | 91 (51) | 304 (74) | 2.43  | <0.0001 |
| b14  | precipitation of the driest month (mm) | 35 (14) | 54 (39) | 1.02  | 0.0096  |
| b15  | precipitation seasonality (coefficient of variation) | 29.7 (16.9) | 66.6 (30.9) | 1.66  | 0.0003  |
| b16  | precipitation of the wettest quarter (mm) | 249 (117) | 751 (198) | 2.43  | <0.0001 |
| b17  | precipitation of the driest quarter (mm) | 117 (45) | 180 (127) | 1.00  | 0.0097  |
| b18  | precipitation of the warmest quarter (mm) | 228 (119) | 662 (181) | 2.30  | <0.0001 |
| b19  | precipitation of the coldest quarter (mm) | 141 (56) | 243 (231) | 1.05  | 0.0111  |

### 2.4. Modeling of Current Potential Distribution and Future Climate Scenarios

The potential distribution area of individual *Phyllactinia* species under current climatic conditions was modeled on habitat suitability maps by Maxent software version 3.4.1, with model evaluation using area under the ROC curve (AUC). To reduce multi-collinearity among the 19 bioclimatic variables, highly correlated variables (r ≥ 0.85 Pearson’s correlation coefficient) were eliminated from further models [43].
To estimate the potential distribution following climate change, future climate scenarios were modeled for both species in Europe and Southeast Asia. For this modeling, we used bioclimatic variables selected to model habitat suitability maps under current conditions and combined them with the data from the Coupled Model Intercomparison Project (CMIP5) of the World Climate Research Program [44] via the IPSL-CM5A-LR model. The climatic scenarios were modeled for the year 2050 (average of years 2041–2060). We used the greenhouse gas scenarios from the mean representative concentration pathway (RCP) level (rcp6.0) that represents an intermediate possible future climate development, depending on how much greenhouse gases are emitted in the years to come. Predictions and reconstructions were computed in the high-performance computing facility in the Geographic Resources Analysis Support System (GRASS) of the Geographic Information System (GIS) environment version 7.1, which was released under the GNU/GPL license. All calculations were done in the Computing Centre of the Slovak Academy of Sciences.

2.5. Environmental Niche Analysis

Environmental niches of studied species were delineated using 19 bioclimatic variables bounded by their minimum and maximum values in the host tree species’ distribution ranges. The environmental space was defined using the first two principal components derived from principal component analysis (PCA) on a correlation matrix of bioclimatic variables. We quantified the environmental niches following the kernel smoother approach of Broennimann et al. [45]. We divided the environmental space into a grid of 300 × 300 cells, in which each cell corresponded to a unique set of climate conditions. The species occurrences were subsequently grouped per cell and converted into densities. A Gaussian kernel smoother was applied to calculate the smoothed density of occurrences in each cell. The density estimates help to avoid unrealistic gaps in species niches due to a low sampling effort and decrease bias by calculating niche overlap [45,46]. The smoothed occurrence densities were plotted into the ordination space of PCA to visualize the position of environmental niches. The PCA was further corroborated by a series of simple randomization tests to identify bioclimatic variables with the highest potential to discriminate the niches of *Phyllactinia* species. We compared the observed differences in mean values of bioclimatic variables (∆) with 95% quantiles of their null distributions obtained by 10,000 unrestricted randomizations of the original data. Prior to analysis, the dataset was standardized to facilitate comparisons among individual variables. The same approach was used to compare differences in the altitudinal distribution of the species.

We quantified the environmental niche overlap between *P. fraxini* and *P. fraxinicola* using Schoener’s D index [47]. Schoener’s D measures the overall match between the occurrence densities of two species across all cells in the gridded environmental space and varies from 0 (no overlap) to 1 (complete overlap). The obtained measures of niche overlap were used to test the hypotheses of niche equivalency and niche similarity [48] via randomization procedures outlined by Broennimann et al. [45]. The test of niche equivalency assesses the null hypothesis that two environmental niches are identical by a random reshuffling of occurrences between species, keeping the number of occurrences as in the original datasets. This process was repeated 1000 times to create a null distribution of Schoener’s D values. We rejected the null hypothesis when the observed value of niche overlap was smaller than expected by chance, i.e., when the overlap value was smaller than 95% of the simulated values (a one-sided test). Rejection of the niche equivalency hypothesis means that the two niches are not statistically identical. When the equivalency hypothesis was rejected, we performed a less stringent test of niche similarity. The test addresses the null hypothesis that two ecological niches are no more similar than expected by chance. The chance is defined by the null distribution of Schoener’s D generated from 1000 random reallocations of the two niches within the available environmental space. A one-tailed test was calculated, while the null hypothesis was rejected when the recorded overlap was greater than 95% of the values simulated under the null hypothesis.

The analyses were performed in R [49] using the library ecospat [50].
3. Results

3.1. Molecular Identifications

Altogether, we gathered 69 samples identified by ITS nrDNA sequence analysis (Table 2). Twenty-two sequences were retrieved from GenBank. Among the 47 samples sequenced in this study, 40 are from Europe, 5 from Southeast Asia, and 2 from North Africa. The two North African collections are from North Algeria within the known distribution of *F. angustifolia*, and therefore they fall in the defined environmental space and were included in further analyses.

**Table 2.** List of *Phyllactinia* specimens on *Fraxinus* spp. used in this study. The accession numbers of the sequences obtained in this study are in bold.

| Phyllactinia | Fraxinus     | Country       | Year | Altitude (m a.s.l.) | Herbarium Voucher No. | GenBank Acc. No. | ITS LSU |
|--------------|--------------|---------------|------|---------------------|-----------------------|-----------------|--------|
| fraxini      | excelsior *  | Algeria       | 2018 | 35                  | NR 5746               | MW411022        |        |
| fraxini      | excelsior *  | Algeria       | 2018 | 15                  | NR 5750               | MW425905        |        |
| fraxini      | excelsior    | Belarus       | 2016 | 230                 | NR 5493               | MW411023        |        |
| fraxini      | excelsior *  | Belgium       | 2017 | 5                   | NR 5793               | MW425906        |        |
| fraxini      | excelsior    | Bulgaria      | 1975 | 875                 | SOMF 13846            | MW425907        |        |
| fraxini      | excelsior    | Bulgaria      | 1979 | 360                 | SOMF 16251            | MW425908        |        |
| fraxini      | ornus        | Bulgaria      | 1979 | 1400                | SOMF 16271            | MW411024        |        |
| fraxini      | ornus        | Bulgaria      | 1983 | 95                  | SOMF 17698            | MW411024        |        |
| fraxini      | ornus *      | Bulgaria      | 1982 | 550                 | SOMF 17537            | MW425910        |        |
| fraxini      | angustifolia *| Croatia       | 2017 | 130                 | NR 5638               | MW411026        |        |
| fraxini      | excelsior    | Croatia       | 2017 | 150                 | NR 5624               | MW425911        |        |
| fraxini      | excelsior    | Croatia       | 2017 | 175                 | NR 5643               | MW411027        |        |
| fraxini      | excelsior *  | Czech Rep.    | 2008 | 225                 | NR 5499               | MW411028        |        |
| fraxini      | excelsior *  | Czech Rep.    | 2007 | 475                 | NR 1004               | MW425912        | MW417101 |
| fraxini      | excelsior    | Germany       | 1995 | 100                 | UPS F172187           | MW425913        |        |
| fraxini      | excelsior *  | Germany       | 2007 | 220                 | KR M24918             | LC307198        |        |
| fraxini      | excelsior *  | Germany       | 1994 | 80                  | KR M48243             | LC307199        |        |
| fraxini      | excelsior    | Germany       | 2016 | 10                  | KR M48246             | LC307200        |        |
| fraxini      | excelsior *  | Germany       | 2016 | 15                  | KR M48247             | LC307201        |        |
| fraxini      | ornus *      | Germany       | 2008 | 245                 | GLM F91208            | LC307196        |        |
| fraxini      | ornus *      | Germany       | 2007 | 210                 | GLM F80912            | LC307197        |        |
| fraxini      | pennsylvanica *| Hungary     | 1991 | 225                 | UPS F005711           | MW425914        |        |
| fraxini      | excelsior *  | Italy         | 2005 | 435                 | KR 0018659            | MN759663        |        |
| fraxini      | excelsior *  | Iran          | 2004 | 5                   | MUMH 917              | AB080555        | AB080453 |
| fraxini      | excelsior    | Lithuania     | 1999 | 170                 | MUMH 911              | AB080549        | AB080447 |
| fraxini      | excelsior    | Lithuania     | 1999 | 200                 | MUMH 912              | AB080550        | AB080448 |
| fraxini      | excelsior *  | Lithuania     | 1999 | 85                  | MUMH 913              | AB080551        | AB080449 |
| fraxini      | excelsior    | Lithuania     | 1996 | 100                 | MUMH 914              | AB080552        | AB080450 |
| fraxini      | excelsior    | Lithuania     | 1994 | 45                  | MUMH 915              | AB080553        | AB080451 |
| fraxini      | mandshurica *| North Korea   | 1990 | 40                  | NR 3007               | MW425915        |        |
| fraxini      | excelsior    | Poland        | 2006 | 200                 | LBL M11495            | MW425916        |        |
| fraxini      | angustifolia *| Slovakia     | 2016 | 140                 | NR 5503               | MW411029        |        |
| fraxini      | chinensis ssp. rhynchophylla *| Slovakia | 2008 | 190                 | NR 4956               | MW425917        | MW417102 |
| fraxini      | excelsior *  | Slovakia      | 1982 | 135                 | SAV F20852            | MW425918        |        |
| fraxini      | excelsior    | Slovakia      | 1982 | 105                 | SAV F20853            | MW425919        |        |
| fraxini      | excelsior    | Slovakia      | 1984 | 230                 | SAV F20851            | MW425920        | MW417103 |
| fraxini      | excelsior *  | Slovakia      | 1984 | 150                 | SAV F20854            | MW425921        |        |
| fraxini      | excelsior    | Slovakia      | 2012 | 150                 | NR 2983              | MW411030        |        |
| fraxini      | excelsior *  | Slovakia      | 2005 | 325                 | NR 4380              | MW425922        |        |
| fraxini      | excelsior *  | Slovakia      | 2009 | 165                 | NR 4431              | MW425923        |        |
| fraxini      | excelsior *  | Slovakia      | 2002 | 325                 | NR 3749              | MW425924        |        |
| fraxini      | excelsior *  | Slovakia      | 2000 | 350                 | NR 3751              | MW425925        |        |
Table 2. Cont.

| Phyllactinia | Fraxinus         | Country          | Year | Altitude (m a.s.l.) | Herbarium Voucher No. | GenBank Acc. No.  |
|--------------|------------------|------------------|------|---------------------|-----------------------|-------------------|
| fraxini      | excelsior *      | Slovakia         | 2016 | 195                 | NR 5501               | MW425926          |
| fraxini      | excelsior *      | Slovakia         | 2016 | 180                 | NR 5505               | MW425927          |
| fraxini      | mandshurica *    | Slovakia         | 2016 | 190                 | NR 5500               | MW411031          |
| fraxini      | ornus *          | Slovakia         | 2016 | 140                 | NR 5504               | MW411032          |
| fraxini      | syriaca *        | Slovakia         | 2016 | 215                 | NR 5502               | MW425928          |
| fraxini      | excelsior *      | Slovakia         | 2009 | 535                 | LJF 1771              | MW417105          |
| fraxini      | chinensis ssp.   | South Korea      | 2014 | 580                 | KUS F28134            | MW425930          |
| fraxini      | mandshurica *    | South Korea      | 2013 | 40                  | KUS F27733            | MW425931          |
| fraxini      | excelsior        | Sweden           | 1961 | 125                 | UPS F558467           | MW411033          |
| fraxini      | excelsior *      | Sweden           | 1960 | 40                  | UPS F558471           | MW425932          |
| fraxini      | excelsior        | Sweden           | 1968 | 10                  | UPS F558477           | MW425933          |
| fraxini      | excelsior *      | Sweden           | 1971 | 40                  | UPS F558478           | MW417106          |
| fraxini      | excelsior        | Switzerland      | 1995 | 405                 | VPRI 21058            | MW417107          |
| fraxini      | excelsior *      | Switzerland      | 1999 | 440                 | MUMH 644              | MW417107          |
| fraxini      | excelsior *      | United Kingdom   | 2017 | 90                  | NR 5676               | MW425934          |
| fraxini      | chinensis *      | United Kingdom   | 2014 | 40                  | OE2014PM70CS          | KY660851          |
| fraxinicola  | chinensis *      | China            | 1991 | 5                   | HMAS 62162            | MW425935          |
| fraxinicola  | lanuginosa       | Japan            | 2004 | 280                 | MUMH 2902             | LC307202          |
| fraxinicola  | longicuspis *    | Japan            | 1996 | 25                  | MUMH 212              | AB080493          |
| fraxinicola  | sieboldiana      | Japan            | 1997 | 520                 | MUMH 426              | AB080585          |
| fraxinicola  | sieboldiana      | Japan            | 1998 | 1725                | MUMH 566              | AB080390          |
| fraxinicola  | chinensis ssp.   | South Korea      | 2015 | 95                  | KUS F29022            | MW425936          |
| fraxinicola  | excelsior *      | South Korea      | 1999 | 105                 | KUS F17216            | AB080541          |
| fraxinicola  | mandshurica *    | South Korea      | 1990 | 115                 | SMK 10643             | AB080433          |

* Ash trees growing in urban environments.

All European and North African samples were identified as *P. fraxini* (Figure 1). Among the five Asian collections sequenced in this study, three from the Korean peninsula represent *P. fraxini*. Two other collections obtained in this study, together with all six Asian collections retrieved from GenBank, are *P. fraxinicola*. The Iranian sample retrieved from GenBank is *P. fraxini*. In further analyses, we included 55 European and 2 North African records of *P. fraxini*, 3 Southeast Asian records of the same species, and 8 Southeast Asian records of *P. fraxinicola*.

Both species have seven distinguishing nucleotide positions in the sequenced regions, two in ITS1, four in ITS2, and one in the LSU region (Figure S1). *Phyllactinia fraxini* showed a low divergence in the ITS/LSU, while *P. fraxinicola* had 18 variable positions within our dataset, suggesting either the presence of multiple haplotypes of individual species or the existence of a so-far unresolved species complex.

We did not confirm any collection of *P. fraxinicola* on the introduced *Fraxinus* species in Europe, but only two Asian ash trees (*F. chinensis* and *F. mandshurica* from Slovakia) were sampled. The collections of *P. fraxini* on Asian ash species represent the first records of the fungus on non-native ash trees in Europe. In Asia, the three records of *P. fraxini* were all from native Asian *Fraxinus*. Among the eight collections of *P. fraxinicola*, one was collected on European ash *F. excelsior*. This study included 51 (73.9%) *Phyllactinia* collections from urban areas, 42 of them from Europe, 7 from Asia, and 2 from Africa.
Figure 1. Maximum likelihood (RAxML) phylogeny of the studied *Phyllactinia* samples inferred from nrITS and nrLSU. The maximum likelihood bootstrap support values greater than 60 and Bayesian posterior probabilities greater than or equal to 0.90 are indicated at nodes. Double slashes indicate a reduction in branch length. * Native ash trees growing in urban environments; ** introduced ash trees growing in urban environments. Specimens in bold were sequenced in this study.
3.2. Habitat Suitability Maps

Nine bioclimatic variables, sorted based on a multi-collinearity test, were used for modeling. The Maxent model gained AUC 0.869 for *P. fraxini* and 0.975 for *P. fraxinicola*, showing a considerably higher predictive power than an uninformed model with a completely random prediction (AUC = 0.5). The temperature annual range (b7) and precipitation of the wettest month (b13) were among the three most important bioclimatic variables concerning permutation importance (Table 3). The Maxent model of both species is also influenced by the mean diurnal range (b2). The model for *P. fraxini* also has a strong contribution from precipitation seasonality (b15), precipitation of the coldest quarter (b19), and precipitation of the driest month (b14). Both species show a high contribution from precipitation variables, 69.8% in *P. fraxini* and 90.9% in *P. fraxinicola*. Habitat suitability maps of the potential distribution of both species in Europe and Asia do not show any overlap. The estimated suitable habitats of *P. fraxini* are situated around the Baltic Sea and in Central Europe and Balkans. The western coastal areas of Spain, Great Britain, and Norway were not indicated as suitable for this species. Opposite to that, for *P. fraxinicola*, very highly suitable areas (0.8–1) occur only in southwest Norway and highly suitable areas (>0.7) in western Scotland (Figure 2). Potentially suitable areas for *P. fraxinicola* in Europe are situated much more to the north compared to the latitudes of current occurrences of the species in Southeast Asia. There was no significant difference in the altitudinal distribution of the species (Δ = 0.52, p = 0.1369). The occurrence of *P. fraxini* ranged between 5 and 1400 m (average 213 m) and that of *P. fraxinicola* between 5 and 1725 m (359 m).

Table 3. Bioclimatic variables used in the Maxent model and their contribution/permutation importance (in %) for *Phyllactinia fraxini* and *P. fraxinicola*. For a complete overview of bioclimatic variables, see Table 1.

| Code | Bioclimatic Variables (Unit)                                      | *P. fraxini* | *P. fraxinicola* |
|------|------------------------------------------------------------------|--------------|-----------------|
| b2   | mean diurnal range (mean of monthly max. minus min. temperature) (°C) | 1.3/1.7      | 3.6/15.7        |
| b7   | temperature annual range (b5-b6) (°C)                           | 22.1/33      | 5.5/15.7        |
| b8   | mean temperature of the wettest quarter (°C)                    | 1.9/1.6      |                 |
| b9   | mean temperature of the driest quarter (°C)                     | 4.9/2.1      |                 |
| b13  | precipitation of the wettest month (mm)                         | 12.2/30.7    | 90.9/68.6       |
| b14  | precipitation of the driest month (mm)                          | 13.2/0.6     |                 |
| b15  | precipitation seasonality (coefficient of variation)            | 28.7/14      |                 |
| b18  | precipitation of the warmest quarter (mm)                       | 0.8/5.9      |                 |
| b19  | precipitation of the coldest quarter (mm)                       | 14.9/10.4    |                 |

Figure 2. Cont.
3.3. Future Climate Scenarios

An intermediate possible future climate scenario (rcp6.0) shows an expansion of suitable habitats for *P. fraxini* in both Europe and Asia (Figure 3). In Europe, major parts of continental Europe are highly suitable, while in Asia, there is an increase in highly suitable areas in Japan, east of the Korean peninsula, and Southeast China. There is a swap of the ratio of suitable areas for *P. fraxini* and *P. fraxinicola* in Southeast Asia. The current model has shown *P. fraxinicola* as a potentially dominant species, but *P. fraxini* will have more highly suitable habitats in the area according to the future climate scenario. The prediction of the future climate scenario in Europe shows even less potential for the occurrence of *P. fraxinicola* in Europe. Areas of western Scotland and southwest Norway will become less susceptible to an invasion of the Asian species, while major parts of Central and East Europe will become intermediately suitable for it.

**Figure 2.** Habitat suitability maps modeled in Maxent and confirmed occurrences: (a) *Phyllactinia fraxini* in Europe, (b) *P. fraxini* in Southeast Asia, (c) *P. fraxinicola* in Europe, and (d) *P. fraxinicola* in Southeast Asia. The horizontal scale bar refers to the probability of distribution (1, high habitat suitability; 0, not suitable). White areas lie outside the natural distribution of *Fraxinus* host trees.

**Figure 3.** Cont.
3.4. Environmental Niche Analyses

We found only a negligible overlap in environmental niches of the two *Phyllactinia* species (D = 0.004). In contrast to *P. fraxini*, the niche of *P. fraxinicola* is shifted toward conditions with a humid climate defined by bioclimatic variables b5, b8, b10, b13, b16, and b18 (Figure 4). Annual precipitation, precipitation of the wettest quarter and the wettest month, and precipitation of the warmest quarter are the main bioclimatic characteristics discriminating niches of the studied species (Table 1). A randomization test of niche equivalency confirmed that the species' environmental niches are not identical (p = 0.002). In fact, niches of the two *Phyllactinia* species were no more similar than expected by chance alone (niche similarity test; p = 0.538).

![Figure 3](image_url)

**Figure 3.** Future climate scenarios modeled for the year 2050 and confirmed current occurrences: (a) *Phyllactinia fraxini* in Europe, (b) *P. fraxini* in Southeast Asia, (c) *P. fraxinicola* in Europe, and (d) *P. fraxinicola* in Southeast Asia. The horizontal scale bar refers to the probability of distribution (1, high habitat suitability; 0, not suitable).

![Figure 4](image_url)

**Figure 4.** Differences in *P. fraxini* (blue) and *P. fraxinicola* (red) environmental niches in principal component space based on bioclimatic variables. Shading is proportional to the density of species occurrence. A dashed line indicates the available environmental space. Vectors of the bioclimatic variables show their contribution to the principal components. Variance explained by the components is given in parentheses. The variable abbreviations are explained in Table 1.
4. Discussion

Data comments. Our study revealed relatively high target sequence variation among the eight analyzed collections of *P. fraxinicola*. To conclude whether *P. fraxinicola* is, in fact, a species complex [17], analyses of additional DNA loci and samples are needed. Nevertheless, the low habitat and niche overlap between *P. fraxini* and *P. fraxinicola* suggests that any eventual taxonomic revision would not affect our conclusions. The main result of this study is that the Southeast Asian ash-dwelling *Phyllactinia* species (or a species complex) is adapted to humid conditions that are absent in most parts of Europe. While several Asian fungal parasites are reported as invasive in Europe, for example, the ash-dwelling fungus *H. fraxineus*, causing ash dieback [7], a lack of suitable environmental conditions in Europe might be the reason why we do not have any evidence of the arrival of *P. fraxinicola* yet. However, this study also identified southwest Norway and western Scotland as potentially susceptible to this *Phyllactinia* species. Unfortunately, we do not have reliable data from these areas. There are some data about the presence or absence of *Phyllactinia* on *Fraxinus* in the border areas of the western distribution of the ash tree in metabarcoding studies. We did not use these data for our models because the studies used the ITS1 region as the barcode with only two diagnostic nucleotide positions (Supplementary Figure S1) and may involve potential errors acquired by next-generation sequencing. Based on our search of the published sequence data repositories, the metabarcoding data probably contains only *P. fraxini* amplicons. These studies agree with our habitat suitability models; they suggest the presence of the species in Norway south of Oslo and southeast Scotland (Stirling) in areas with a moderately suitable prediction for *P. fraxini* [51,52], while the study from north Spain from the area with moderate to low suitability prediction for the species does not report the presence of the fungus [53].

This study expanded the available and molecularly confirmed records of *Phyllactinia* approximately three times, but it also defined gaps in the knowledge. In Europe, we miss data from the western areas that are modeled as potentially susceptible to the invasion of *P. fraxinicola*. In Southeast Asia, the knowledge of *Phyllactinia* occurrence is very scattered and insufficient. Our Asian collections of *P. fraxini* co-occurred with *P. fraxinicola* in generally more humid areas compared to Europe, suggesting that *P. fraxinicola* has a narrower climatic amplitude.

The collection from Iran, together with the host range of the studied powdery mildew agents, suggests that at least *P. fraxini* may have continual distribution across Eurasia. However, some areas in Central Asia may represent climatic barriers for the spread of this species, and it is possible that our first reports of the species from the Korean peninsula represent information about the recent introduction of the non-native species to this part of Asia.

It is worth noting that the lack of overlap between models of suitable habitats corresponds to high niche differences, but potentially suitable areas of *P. fraxinicola* are separated by a large geographical and latitudinal gap. Asian samples of the species were collected between 33° and 43° N, and a suitable area in west Europe extends between ca. 50° and 60° N. This difference in latitude may be a reason for a climate shift by the Gulf Stream influence. Randomization tests confirmed that the precipitation-based variables have a major contribution to niche discrimination. Areas in West Europe are modeled as suitable for *P. fraxinicola* because of the high precipitation demands of the species. It should be noted here that models built from distributional data should be interpreted with caveats, especially if the distribution of the studied species is not known perfectly and the models are based on presence-only data [54]. However, the estimation of the niche overlap is fairly robust to a sample bias as the use of a kernel smoother makes the process of moving from a geographical space to a multivariate environmental space independent of both sampling effort and the choice of resolution in the environmental space [45].

In this study, we did not use any morphologically identified collections. Our observation of morphological diagnostic characters defined by current taxonomic concepts [10] confirmed the previous conclusion of Scholler et al. [17] that they do not always agree with
sequence-based identification. We observed sinuous-twisted foot cells of conidiophores (supposed to be typical for \textit{P. fraxinicola}) on two collections identified by the sequence as \textit{P. fraxini}: the collection NR 3007 from North Korea previously morphologically identified as \textit{P. fraxinicola} \cite{55} and the collection NR 5499 from the Czech Republic (Table 2). This conidiophore character not only shows partial overlap between species but also cannot be applied for autumn collections, where conidiophores are no longer present.

\textit{Phyllactinia} and causative agents of powdery mildews, in general, are biotrophic leaf parasites that cannot be cultivated without the presence of host leaf tissue \cite{56, 57}. Some studies using Illumina and the ITS region for metabarcoding of the environmental DNA of \textit{Fraxinus} leaves and twigs did not report any Erysiphales member \cite{58, 59}. Other studies showed a high representation of Erysiphales, including \textit{Phyllactinia}, on \textit{Fraxinus} leaves \cite{60}, suggesting that this method may need a specific adaptation of primers for Erysiphales. The next-generation technique may improve monitoring of the distribution and population structure of \textit{Phyllactinia} because it bypasses the need for cultivation and isolation of species and can detect plant pathogenic species when they occur asymptomatically \cite{61}.

\textbf{Urban areas and possible invasion paths.} Some authors have suggested that recent climatic changes in Europe and air pollution may impact the distribution of indigenous powdery mildews and favor the spread of alien powdery mildew agents \cite{62, 63}. Several drivers of emerging infective diseases depend not only on the introduction of non-native species but also on the genetic structure of a population of invasive fungal species and changes in ecosystems by human and climate activities \cite{64}.

This study confirmed that \textit{Phyllactinia} species do not show high specificity to \textit{Fraxinus} host tree species because \textit{P. fraxini} was confirmed on a variety of European, Asian, and North American ash species. However, disease susceptibility of trees and parasitic fungus fitness may depend on the phylogenetic closeness of the native host tree species and the congeneric tree species in the invasive range \cite{65}. More than two-thirds of our collections and also our field experience demonstrated that \textit{Phyllactinia} species can resist environmental pollution of urban areas and that planting of non-native \textit{Fraxinus} species can facilitate the spread of \textit{Phyllactinia} to regions out of its natural distribution. In this context, we identified Korean records of \textit{P. fraxini} as possible oncoming invasive events, southwest Norway and western Scotland being suggested as the most likely areas of an eventual invasion by \textit{P. fraxinicola}.

\textbf{5. Conclusions}

Based on current data, \textit{P. fraxini} is widely distributed in Eurasia and a part of North Africa. To resolve the question of whether the species was introduced in Asia recently, more data and population studies are needed. \textit{Phyllactinia fraxinicola} is only known from Asia, and based on the limited current data, it seems that it does not represent a threat for Europe except for areas with high precipitation in western Scotland and southwest Norway, where the species may find potentially suitable conditions. We did not verify the presence and identity of any ash-dwelling \textit{Phyllactinia} in these areas. The niches of both species are not similar; their appearance in a relatively small area of Korea may be based on local climatic changes caused by geomorphology. Our data do not show any species’ preference for urban or native habitats or a particular \textit{Fraxinus} species. However, transplanting the host trees over long geographical distances to areas with suitable ecological conditions may cause the introduction of non-native \textit{Phyllactinia} species.

\textbf{Supplementary Materials:} The following are available online at https://www.mdpi.com/1999-4907/12/2/183/s1: Table S1, List of studied material with collection details and bioclimatic variables; and Figure S1, Nucleotide differences in ITS and LSU nrDNA regions between \textit{Phyllactinia} fraxini and \textit{P. fraxinicola}. In orange are highlighted diagnostic positions to distinguish the species. Dashes represent gaps and empty spaces missing data.

\textbf{Author Contributions:} K.P. prepared the conceptual background of the study, organized basic data gathering, and contributed to the morphological and molecular work and to manuscript writing.
K.A., P.M., M.C. and K.B. performed morphological observations, sampling, DNA extractions, and preparation of samples for sequencing. K.A. edited and submitted the sequences in GenBank and edited the manuscript. M.C. performed phylogenetic analyses. D.S. retrieved climatic variables used in the study, prepared data for environmental space, performed Maxent analyses, and prepared maps. M.S. performed niche analyses and randomization tests and contributed to manuscript writing. S.A. prepared the conceptual framework of the study, coordinated work on data gathering and analyses, and supervised manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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