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Phylogeny and taxonomy of *Phyllactinia* species (powdery mildew: *Erysiphaceae*) occurring on the ash trees (*Fraxinus* spp.)

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ABSTRACT

The genus *Fraxinus* (*Oleaceae*), known as ash trees, currently comprises 43 recognized species that are distributed in temperate and subtropical regions of the Northern Hemisphere. Two *Phyllactinia* species, *P. fraxini* and *P. fraxinicola*, have been known on *Fraxinus* spp. so far. In this study, powdery mildews belonging to *Phyllactinia* were collected on *Fraxinus* spp. from different areas of the world to make molecular and morphological analyses. These specimens are divided into four distinct molecular phylogenetic groups, which are distinguishable by their morphology and/or host preference. Two new species, viz. *P. japonica* occurring on *F. sieboldina* and *F. lanuginosa* f. *serrata*, and *P. fraxini-longicuspidis* on *F. longicuspis*, are proposed in this study. An epitype is designated for *P. fraxini*. This study indicates very high host specificity among the four *Phyllactinia* species on *Fraxinus*, suggesting that genetic isolation by host specificity played a more important role than geographic segregation in the speciation events of these *Phyllactinia* species. Evolutionary timing calculated by molecular clock analysis suggests that these powdery mildews diverged in accordance with host phylogeny after divergence of host plants.

Keywords: biogeography, epitype, new species, *Phyllactinia fraxini-longicuspidis*, *Phyllactinia japonica*

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1. Introduction

The genus *Fraxinus* (*Oleaceae*), the name for the ash tree, currently comprises 43 recognized species distributed in temperate and subtropical regions of the Northern Hemisphere (Wallander, 2008). Ash trees are usually medium to large trees, mostly deciduous, though a few subtropical species are evergreen. Because of its tough and very strong but elastic nature, the wood of ash trees is extensively used for making bows, tool handles, baseball bats, etc. In many countries, species of this genus are used as ornamental plants, as avenue trees and in parks. Representatives of two powdery mildew genera, viz. *Erysiphe* and *Phyllactinia*, have been commonly reported on *Fraxinus* species (Braun & Cook, 2012). Recent molecular and morphological analyses of *Erysiphe* species (sect. *Uncinula*) occurring on *Fraxinus* revealed that these powdery mildews are divided into three distinct species with very high host specificity (Yamaguchi et al., 2021).

Phyllactinia is another powdery mildew genus occurring on *Fraxinus* species worldwide. This genus is characterized by having a semi-endoparasitic habit with both endophytic and superficial mycelia formed in and on host leaves. The genus *Phyllactinia* is regarded as well characterized and readily discernible by its distinct morphological traits such as the acicular appendages with bulbous swelling at the base and many sea anemone-like structures, called penicillate cells, arising from the top of chasmothecia (fruiting bodies of powdery mildews). But, owing to only weekly pronounced morphological differences between collections on different hosts, representatives of *Phyllactinia* have long been recognized as a single compound species with wide host ranges, such as *P. corylea* s. lat., *P. guttata* s. lat. or *P. suffulta* (Rebent.) Sacc. (Salmon, 1900; Jaczewski, 1927; Blumer, 1967; Parmelee, 1977). However, comprehensive molecular phylogenetic analyses (Takamatsu et al., 2008) revealed that previous taxonomic approaches with wide species concepts are incorrect, i.e., *Phyllactinia* is divided into many separate species with much narrower host ranges reflecting co-evolution along with their host families and genera. These findings led to a revision of the species delimitation within *Phyllactinia* (Braun & Cook, 2012).

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Phyllactinia occurring on *Fraxinus* species has been recognized as an independent species, *P. fraxini* (DC.) Fuss, for some time, mainly based on the 3-spored asci (Homma, 1937; Braun, 1985, 1987). Molecular phylogenetic analyses revealed that *Phyllactinia* on *Fraxinus* forms a clade of its own (Takamatsu et al., 2008). This clade was, however, not homogenous, but divided into some smaller subclades, suggesting that *P. fraxini* is a species complex composed of two or more species. Braun and Cook (2012) divided *P. fraxini* s. lat. into two species, viz. *P. fraxini* s. str. and *P.* fraxinicola U. Braun & H.D. Shin, based on the morphology of foot cells of the conidiophores. *Phyllactinia fraxini* s. str. was said to be characterized by having straight foot cells and by being distributed in Europe and North America. Whereas, *P. fraxinicola* was considered an East Asian species with curved foot cells. Later, Scholler, Schmidt, Meeboon, Braun, and Takamatsu (2018) found that conidiophores of *P. fraxini* s. str. also have curved foot cells. Thus, *P. fraxini* s. str. and *P. fraxinicola* could only be distinguished by molecular characteristics and different geographic distributions, but no longer by morphological differences. In addition, the *P. fraxinicola* clade is still heterogeneous and divided into three subclades (Takamatsu et al., 2008; Scholler et al., 2018), which urgently required further phylogenetic and morphological studies to clarify the species delimitation of *Phyllactinia* species occurring on *Fraxinus*. Therefore, we conducted morphological and molecular analyses of *P. fraxini* s. lat. using a larger number of specimens, which revealed that *P. fraxini* s. lat. is divided into at least four species.

2. Materials and methods

2.1. Molecular phylogeny

Whole-cell DNA was extracted from chasmothecia using the chelex method as described in Hirata and Takamatsu (1996). The 5'-end of the 28S rDNA (including D1 and D2 domains) and internal transcribed spacer (ITS) regions were amplified and sequenced. Primer sets Ph7 (TGTTGCTTTGGYAGGCCG; Monkhung, Takamatsu, & To-anun, 2013)/NLP2 (GGTCCCAACAGCTATGCTCT; Mori, Sato, & Takamatsu, 2000) and ITS5 (GGAAGTAAAAGTCGTAACAAGG; White, Bruns, Lee, & Taylor, 1990)/Ph8 (GCCCCAAGACCAAGCC; this study) were used for amplification of the 28S rDNA and ITS region, respectively. Ph8 was newly designed in this study based on the sequences of *Phyllactinia* spp. (Takamatsu et al., 2008). The protocol used in this study was as follows: PCR mixtures (25 μL) contained 0.4 mM of each primer, 200 mM dNTPs, 1× PCR buffer (supplied by the manufacturer), 1 unit of KOD FX Neo DNA polymerase (Toyobo, Tokyo, Japan), and 1 μL of DNA extract solution. The following thermal cycling conditions were performed in a thermal cycler Dice TP-600 (Takara, Tokyo, Japan): an initial denaturing step of 94 °C for 2 min; thermal cycling for 40 cycles, where each cycle consisted of 10 s at 98 °C followed by 30 s at 65 °C for annealing, and 60 s at 68 °C for extension. The amplicons were sent to Solgent Co. Ltd. (Daejeon, South Korea) for direct sequencing using primers Ph7 and NLP2 for the Ph7/NLP2 fragment, ITS5 and Ph8 for the ITS5/Ph8 fragment.

New sequences obtained in this study were deposited in the DNA databases DDBJ and GenBank under the accession numbers LC597089–LC597107, MW327040, and MW327041. These sequences were combined with the sequences of *P. fraxini* s. lat. used in Takamatsu et al. (2008) and Scholler et al. (2018), aligned using MUSCLE (Edgar, 2004) implemented in MEGA7 (Kumar, Stecher, & Tamura, 2016), and manually refined. This alignment was deposited in TreeBASE (http://www.treebase.org/) under the accession number S27386. Phylogenetic trees were obtained from the data using the maximum parsimony (MP) and maximum likelihood (ML) methods. MP analysis was performed in PAUP* 4.0 (Swofford, 2003) with heuristic search option using the tree bisection reconnection (TBR) algorithm with 100 random sequence additions in order to find the global optimum tree. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of internal branches of the resulting trees was tested with bootstrap (BS) analysis using 1,000 replications with the step-wise addition option set as "simple" (Felsenstein, 1985). Tree scores, including tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC), were also calculated. The ML analysis was performed using raxmlGUI ver. 1.3 (Silvestro & Michalak, 2012), under a GTRGAMMA model. The BS supports and trees were obtained by running rapid bootstrap analysis of 1,000 pseudo-replicates followed by a search for the tree with the highest likelihood.

We used a molecular clock approach to estimate the timing of divergence of *Phyllactinia* spp. on *Fraxinus*. Kimura's two-parameter criterion (Kimura, 1980) was used for calculation of pair-wise genetic distances. The molecular clock (2.52×10^{-9} substitutions per site year^{-1} for ITS region including 5.8S rDNA; Takamatsu & Matsuda 2004) was used for the calculation.

2.2. Morphological examination

In order to examine the traits of the sexual morph, chasmothecia were stripped off from the leaf surfaces with a clean needle and mounted on a microscope slide in 3% NaOH using a standard light microscope (Axio Imager; Carl Zeiss, Göttingen, Germany) and differential interference contrast optical instruments and devices. To examine the asexual morphs of fresh samples, hyphae, conidiophores, and conidia of fresh collections were stripped off from the leaf surfaces with clear adhesive tape, mounted on a microscope slide with the fungal mycelium upper most, and examined in water. For dried specimens, examinations were done following the lactic acid protocol (Shin & La, 1993). If possible, thirty measurements were made for each character examined.

3. Results

3.1. Phylogenetic analyses

Nucleotide sequences spanning ITS1-5.8S rDNA-ITS2 and 28S rDNA D1/D2 domains were determined from 21 *Phyllactinia* specimens on *Fraxinus* spp. collected in Japan, China, and the Ukraine. These sequences were aligned with 25 sequences used in Takamatsu et al. (2008) and Scholler et al. (2018), and retrieved from GenBank. The alignment matrix consisted of 46 sequences and 1,300 characters, of which 33 characters (2.5%) were variable and 29 (2.2%) were informative for parsimony analysis. Outgroup sequences were not included in this data matrix, but rooting point was inferred by a preliminary analysis using *P. roboris* (Gachet) S. Blumer (AB080516 + AB080417) and *P. guttata* (Wallr.) Lév. (AB080494 + AB080384) as outgroup taxa. Previous analyses (Takamatsu et al., 2008; Takamatsu, Siahaan, Moreno-Rico, Cabrera de Álvarez, & Braun, 2016) also supported this rooting point. A total of 9,466 equally parsimonious trees with 35 steps were constructed by the MP analysis. Tree topologies were almost consistent among the trees, except for branching orders of the terminal branches and branch lengths. One of the trees is shown in Fig. 1. The ML tree topology was almost identical to the MP tree and only bootstrap supports were shown in the MP tree. The 46 sequences from powdery mildews on *Fraxinus* spp. were divided into four distinctive clades

with strong BS supports (MP ≥ 77%; ML ≥ 96%). Clade 1 consists of 28 sequences from specimens on *F. excelsior* L., *F. mandshurica* Rupr., *F. pennsylvanica* Marshall, *F. ornus* L., *Syringa vulgaris* L., *S. henryi* C.K. Schneid., and *Chionanthus virginicus* L. All specimens were collected in Europe, except for the four *F. mandshurica* specimens collected in Japan. Clade 2 is composed of seven sequences retrieved from specimens on *F. japonica* Blume ex K. Koch, *F. chinensis* Roxb., and *F. rhynchophylla* Hance collected in China and Japan, and on *F. excelsior* and *F. mandshurica* collected in Korea. Clade 3 encompasses five sequences from specimens on *F. lanuginosa* Koidz. f. *serrata* (Nakai) Murata and *F. sieboldiana* Blume, and Clade 4 consists of six sequences from specimens on *F. longicuspis* Siebold & Zucc.

All specimens of Clade 3 and Clade 4 were collected in Japan. A molecular clock analysis suggested that the divergence of the four clades occurred about 5–2 million years ago (mya) in Pliocene (Fig. 1).

3.2. Taxonomy

Phyllactinia fraxini (DC.) Fuss, Arch. Ver. Siebenb. Landesk. 14(2): 463. 1878

Figs. 2A, 3, 4.

≡ *Erysiphe fraxini* DC., Fl. franç. 2: 273. 1805.

≡ *Alphitomorpha guttata* β *fraxini* (DC.) Wallr., Verh. Ges. Naturf. Freunde Berlin 1(1): 42. 1819.

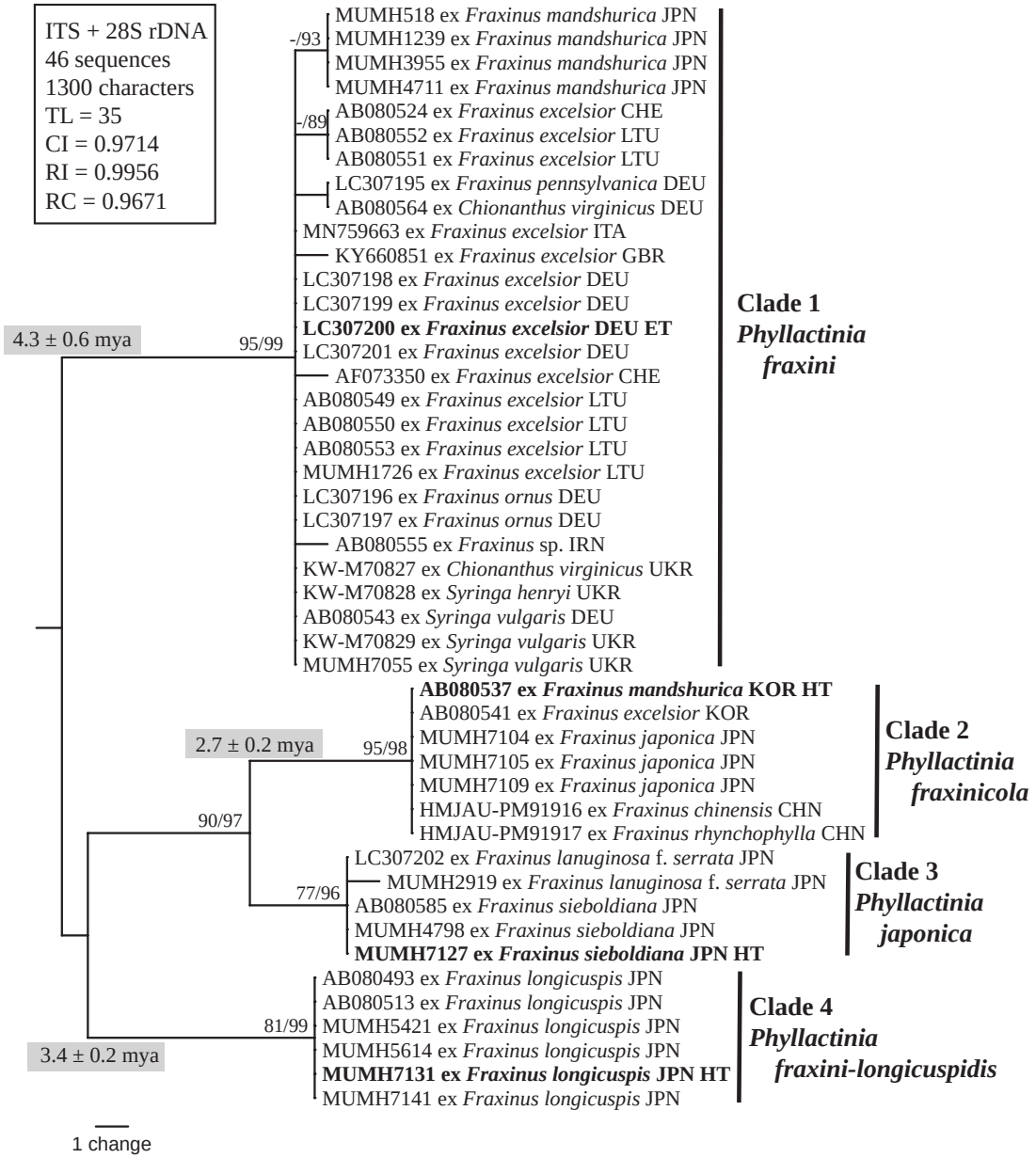

Fig. 1 – Phylogenetic analysis of the 5'-end of 28S rDNA (including domains D1 and D2) for 46 sequences from *Phyllactinia fraxini* s. lat. This is one of the equally parsimonious trees with 35 steps, which were found using a heuristic search. Horizontal branch lengths are proportional to the number of substitutions that were inferred to have occurred along a particular branch of the tree. Sequences retrieved from type specimens were shown as boldface (HT: holotype, ET: epitype). BS (≥70%) values by the maximum parsimony (MP) and maximum likelihood (ML) methods were shown on the respective branches. Rooting point was inferred by a preliminary analysis using *P. roboris* and *P. guttata* as outgroup taxa. Divergence time of the major clades calculated by molecular clock of ITS region were shown on/under the respective nodes (mya: million years ago).

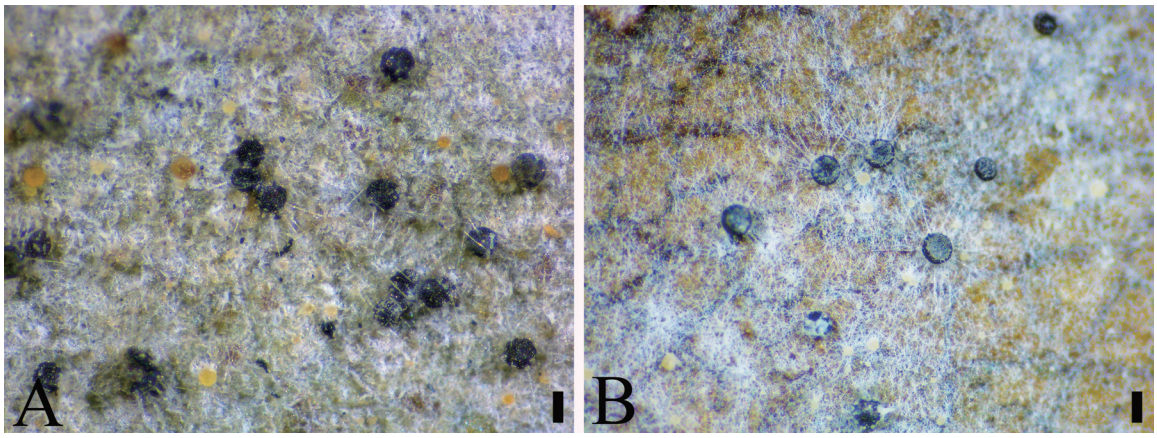

Fig. 2 – Chasmothecia produced on host leaves. A: *Phyllactinia fraxini* on *Fraxinus excelsior* (TSU-MUMH1726). B: *P. fraxinicola* on *F. japonica* (TSU-MUMH7104). *Bars*: A, B 200 μm.

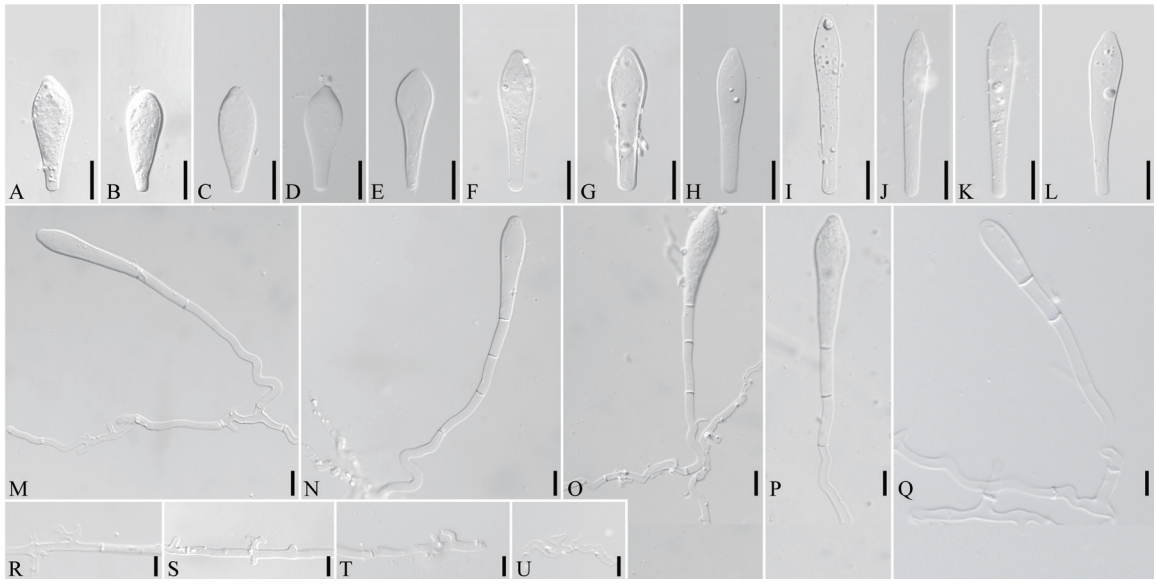

Fig. 3 – *Phyllactinia fraxini* on *Fraxinus excelsior* (KR-M-0048247). A–L: Conidia M–Q: Conidiophores. R–U: Appressoria. *Bars*: A–L 25 μm; M–U 10 μm.

≡ *Erysibe guttata* var. *fraxini* (DC.) Link, Sp. pl. 4, 6(1): 117. 1824.
≡ *Erysiphe guttata* f. *fraxini* (DC.) Fr., Syst. mycol. 3: 246. 1829.
≡ *E. lenticularis* a. *fraxini* (DC.) Rabenh., Deutschl. Krypt.-Fl. 1: 234. 1844.
≡ *Phyllactinia fraxini* (DC.) Homma, J. Fac. Agric. Hokkaido Imp. Univ. 38: 409. 1937.
= *Alphitomorpha lenticularis* Wallr., Fl. Crypt. Germ. 2: 759. 1833; type
host – *Fraxinus excelsior*.
≡ *Erysiphe lenticularis* (Wallr.) Kickx, Fl. Crypt. Env. Louv.: 139. 1835.
≡ *E. lenticularis* (Wallr.) Rabenh., Deutschl. Krypt.-Fl. 1: 234. 1844.
= *Phyllactinia suffulta* var. *blumeri* A.A. Mendonça & Marta Sequ., Agron.
Lusit. 24(2): 107. 1962; type host – *Fraxinus angustifolia*.
≡ *P. guttata* var. *blumeri* (A.A. Mendonça & M. Sequ.) U. Braun, Gleditschia 6: 172. 1978.
= *P. guttata* f. *fraxini* Fuckel, Fungi Rhen. Exs. 703. 1863.
= *P. guttata* f. *fraxini-ornus* Rabenh., Fungi Eur. Exs. 1055. 1866.
= *P. guttata* f. *fraxini-orni* Sacc., Mycoth. Ven. 624. 1876.
= *P. guttata* f. sp. *fraxini* Hammarl., Hereditas 6(1): 43. 1925.
= *P. suffulta* f. *chionanthi* Jacz., Karm. Opred. Grib., Vyp. 2. Muchn.-rosyan. griby (Leningrad): 434. 1927.
= *P. suffulta* f. *glycines* Jacz., ibid.: 436. 1927.
= *P. suffulta* f. *syringae* Jacz., ibid.: 435. 1927.
= *P. corylea* auct. p.p.
= *P. guttata* auct. p.p.
= *P. suffulta* auct. p.p.

Lectotype (designated by Braun 1987): On *Fraxinus excelsior*, FRANCE, "*Mucor erysiphe*. *Erysiphe fraxini*, Fl. franç. 731," herb. de Candolle (G00298350).

Epitype (designated here, MycoBank MBT 396028): On *F. excelsior*, GERMANY, Schleswig-Holstein, Ostholstein, Scharbeutz, spa gardens, 16 Oct 2016, leg. A. Schmidt (KR-M-0048246).

Gene sequences (ex-epitype): LC307200 (ITS+28S rDNA).

Description: Mycelium on leaves, hypophyllous; shape of hyphal appressoria variable, nipple-shaped, branched to coral-shaped, rod- to hook-shaped, solitary or in opposite pairs; conidiophores arising from external hyphae, erect, 130–220 × 5–7 μm; foot cells 47–117(–157) × 4–7 μm, sinuous to spirally twisted at the base, followed by 2–3 shorter cells, forming conidia singly; conidia clavate or ± lanceolate, apex rounded, base truncate or almost so, 39–101 ×

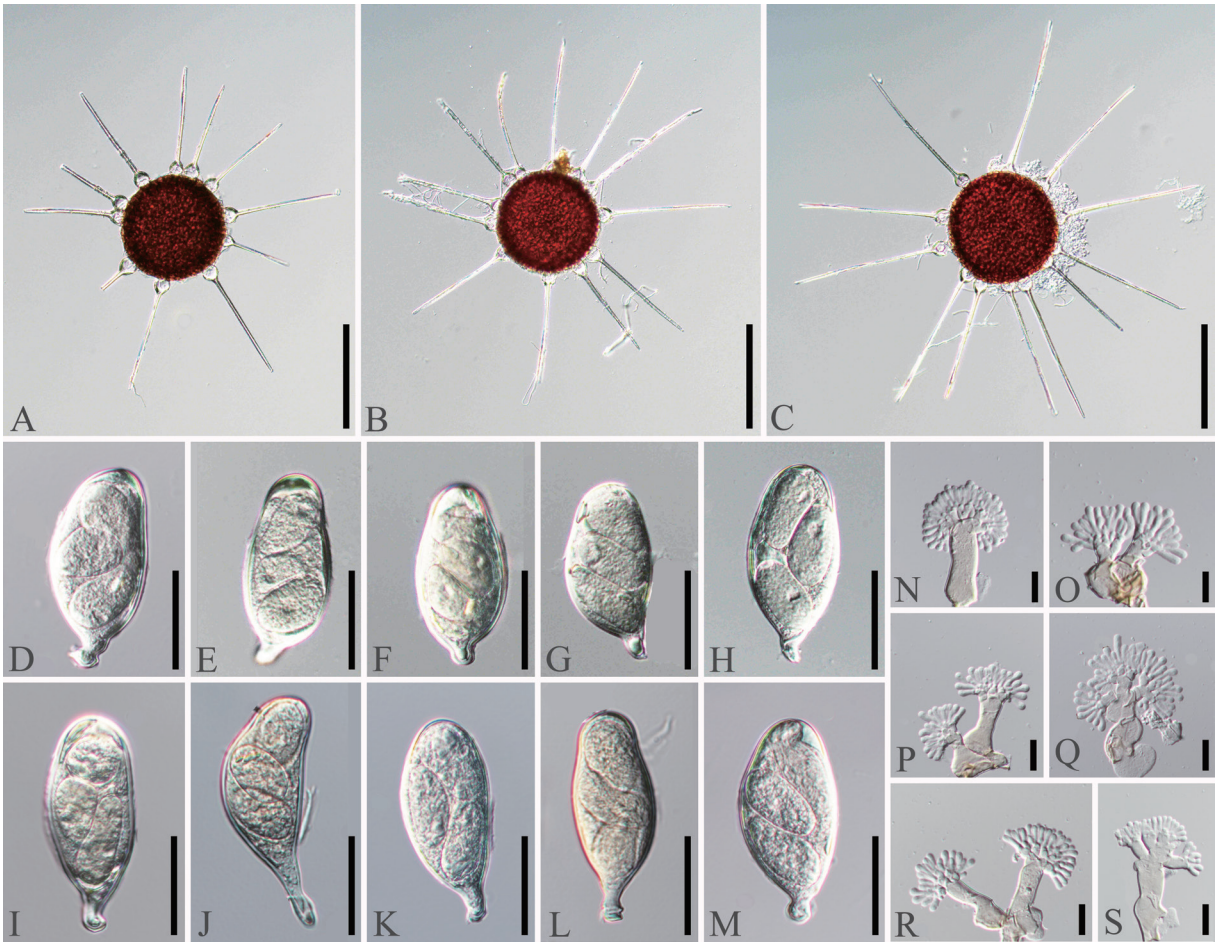

Fig. 4 – *Phyllactinia fraxini* on *Fraxinus mandshurica* (TSU-MUMH1239). A–C: Chasmothecia. D–M: Asci. N–S: Penicillate cells. *Bars*: A–C 200 μm; D–M 40 μm; N–S 15 μm.

11–22 μm. Chasmothecia hypophyllous, scattered to almost gregarious, depressed globose, 215–284 μm diam; appendages equatorial, 4–13, acicular with bulbous basal swelling, 23–50 μm diam, apex subacute to usually obtuse, (193–)215–361 × 6–10 μm, hyaline; penicillate cells in the upper half, numerous, stem 17–45 × 9–20 μm, subcylindrical, with 2–7 short branchlets at apex, filaments (14–)22–49 × 2–8(–10) μm; asci 13–25 per chamothecium, ellipsoid-ovoid, ovoid-lanceolate, 50–105 × 15–45 μm (l/w ratio = 1.7–2.8), stalked, (2–)3-spored; ascospores broad ellipsoid-ovoid, 18–40 × 10–25 μm (l/w ratio = 1.3–2.4), colorless to yellowish.

Additional specimens examined: On *Chionanthus virginicus*, UKRAINE, Kyiv, A. V. Fomin Botanical Garden, 16 May 2015, leg. V. Heluta, KW-M70827 (TSU-MUMH7052), DDBJ ID no.: LC597089 (ITS+28S rDNA); On *Fraxinus excelsior*, GERMANY, Hochschwarzwald, Oberried Weilersbach, Zastlerbad am Gasthof "Zum Schützen", 11 Oct 2008, leg. H. Jage, KR-M-0036342; Mecklenburg-Vorpommern, Neustrelitz, 15 Oct 1994, leg. M. Scholler, KR-M-0048243, DDBJ ID no.: LC307199 (ITS+28S rDNA); Schleswig-Holstein, Lübeck, Karlshof, Forstmeisterweg/Glashüttenweg, 11 Aug 2016, leg. A. Schmidt, KR-M-0048247, DDBJ ID no.: LC307201 (ITS+28S rDNA); LITHUANIA, Birstonas, 29 Sep 2002, leg. B. Grigaliunaite, TSU-MUMH1726, DDBJ ID no.: LC597094 (ITS+28S rDNA); Kaunas, 7 Sep 1999, leg. B. Grigaliunaite, TNS-F-87530 (TSU-MUMH913), DDBJ ID no.: AB080551 (ITS), AB080449 (28S rDNA); SWITZERLAND, Neuchâtel, 4 Sep 1999, leg. S. Takamatsu, TNS-F-87439 (TSU-MUMH644), DDBJ ID no.: AB080524 (ITS); On *F. mandshurica*, JAPAN, Nagano Pref., Ueda-shi, Sugadaira, 30 Sep 2000, TSU-MUMH1239, DDBJ ID no.: LC597093 (ITS+28S rDNA); 29 Sep 2005, TSU-MUMH3955, DDBJ ID no.: LC597095 (ITS+28S rDNA); Niigata Pref., Yuzawa, 23 Sep 1998, leg. S. Takamatsu, TSU-MUMH518, DDBJ ID no.: LC597092 (ITS+28S rDNA); Toyama Pref., Toyama-shi, Lake Arimine, Nishitani, 6 Oct 2007, leg. S. Takamatsu, TSU-MUMH4711, DDBJ ID no.: LC597096 (ITS+28S rDNA); On *F. ornus*, GERMANY, Sachsen, Görlitz-Rauschwalde, Hilde-Coppi-Straße, near Rosa-Luxemburg-Straße, 15 Sep 2007, leg. S. Höflich, GLM-F080911, DDBJ ID no.: LC307196 (ITS+28S rDNA); On *Syringa henryi*, UKRAINE, Kyiv, Centre, Bohdan Khmelnytsky Street, 16 Oct 2015, leg. V. Heluta, KW-M70828 (TSU-MUMH7053), DDBJ ID no.: LC597090 (ITS+28S rDNA); On *S. vulgaris*, UKRAINE, Kyiv, Centre, Bohdan Khmelnytsky Street, 16 Oct 2015, leg. V. Heluta, KW-M70829 (TSU-MUMH7054), DDBJ ID no.: LC597091 (ITS+28S rDNA); Kyiv, Pivdenna Borshchahivka, Bulgakov Street, 30 Oct 2014, leg. V. Heluta, TSU-MUMH7055, DDBJ ID no.: LC597097 (ITS+28S rDNA).

Host range and distribution (only based on collections proven by means of sequence analyses): on *Fraxinus excelsior* (Germany, Italy, Lithuania, Slovakia, Switzerland, UK, Ukraine), *F. mandshurica* (Japan), *F. ornus* (Germany), *F. pennsylvanica* (Germany), *Chionanthus virginicus* (Germany, Ukraine), *Syringa henryi* (Ukraine), *S. vulgaris* (Ukraine), *Oleaceae*.

Note: Braun (1987) designated G00298350 collected in France as lectotype. This specimen is too old to extract DNA. We thus designate KR-M-0048246 collected in 2016 from Germany as an epitype here and LC307200 as an ex-epitype sequence.

Phyllactinia fraxinicola U. Braun & H.D. Shin, in Braun & Cook, Taxonomic manual of the Erysiphales (powdery mildews): 249. 2012

Figs. 2B, 5.

= *Phyllactinia fraxini* auct. p.p.
= *P. corylea* auct. p.p.
= *P. guttata* auct. p.p.
= *P. suffulta* auct. p.p.

Holotype: On *Fraxinus* sp. (as *F. mandshurica*), SOUTH KOREA, Suwon, 26 Oct 1990, H.D. Shin, KUS-F-10643, DDBJ ID no.: AB080537 (ITS), AB080429 (28S rDNA).

Description: Mycelium on leaves, hypophyllous. Chasmothecia hypophyllous, scattered to almost gregarious, depressed globose, 220–276 μm diam; appendages equatorial, 8–19, acicular, with bulbous basal swelling, 32–51 μm diam, apex subacute to usually obtuse, 215–322(–376) × 7–11 μm, hyaline; penicillate cells in the upper half, numerous, stem 16–44 × 7.5–20 μm, subcylindrical, with 2–9 short branchlets at apex, filaments 14–53 × 2–7 μm; asci 7–19 per chamothecium, ellipsoid-ovoid, 60–85 × 26–44 μm (l/w ratio = 1.6–2.7), stalked, (1–)3(–4)-spored; ascospores broad ellipsoid-ovoid, 18–40 × 10–25 μm (l/w ratio = 1.1–2.6), colorless to yellowish.

Specimens examined: On *Fraxinus chinensis*, CHINA, Beijing, Mt. Baihua (39°83'73.37"N 115°57'46.62"E, alt. 1154 m), 19 Oct 2018, leg. S. R. Tang and L. Liu, HMJAU-PM91916, GenBank ID no.: MW327041 (ITS+28S rDNA); On *F. japonica*, JAPAN, Niigata Pref., Gosen-shi, Ojiro (37°44'12.47"N 139°13'59.11"E), 28 Oct 2017, leg. S. Takamatsu, TSU-MUMH7109, DDBJ ID no.: LC597100 (ITS+28S rDNA); Niigata Pref., Gosen-shi, Ronze (37°45'04.30"N 139°11'55.03"E), 28 Oct 2017, leg. S. Takamatsu, TSU-MUMH7105, DDBJ ID no.: LC597099 (ITS+28S rDNA); Niigata Pref., Niigata-shi, Akiba-ku, Manganji (37°48'36.39"N 139°08'13.76"E), 28 Oct 2017, leg. S. Takamatsu, TSU-MUMH7104, DDBJ ID no.: LC597098 (ITS+28S rDNA); Osaka Pref., Katano-shi, Osaka City University, Botanical Garden, 20 Nov 2005, leg. S. Takamatsu, TSU-MUMH4150; On *F. rhynchophylla*, CHINA, Beijing, Mt. Baihua (39°83'74.91"N 115°57'18.55"E, alt. 1133 m), 19 Oct 2018, leg. S. R. Tang and L. Liu, HMJAU-PM91917, GenBank ID no.: MW327040 (ITS+28S rDNA).

Host range and distribution: On *Fraxinus chinensis* (China), *F. excelsior* (?) (South Korea), *F. japonica* (Japan), *F. mandshurica* (?) (South Korea), *F. rhynchophylla* (China), *Oleaceae*.

Phyllactinia japonica M. Maeda, Meeboon & S. Takam., sp. nov.

Figs. 6, 7.

MycoBank no.: MB 838790.

= *Phyllactinia fraxinicola* auct. p.p.
= *P. fraxini* auct. p.p.
= *P. corylea* auct. p.p.
= *P. guttata* auct. p.p.
= *P. suffulta* auct. p.p.

Diagnosis: Morphologically similar to *Phyllactinia fraxini* and *P. fraxinicola*, but differs in the rDNA ITS sequences. Occurring on *Fraxinus sieboldina* and *F. lanuginosa* f. *serrata*.

Holotype: on *Fraxinus sieboldiana* (*Oleaceae*), JAPAN, Shiga Pref., Maibara-shi, Mt. Ibuki (35°23'38.18"N 136°23'23.06"E, alt. 444 m), 1 Nov 2017, leg. S. Takamatsu (TNS-F-91391). Isotype: TSU-MUMH7127.

Gene sequences (ex-holotype): LC597103 (ITS).

Etymology: *japonica*, referring to the origin (country) of this species, Japan.

Description: Mycelium hypophyllous, effuse, in irregular patches or entirely covering leaves, inconspicuous, evanescent; hyphal

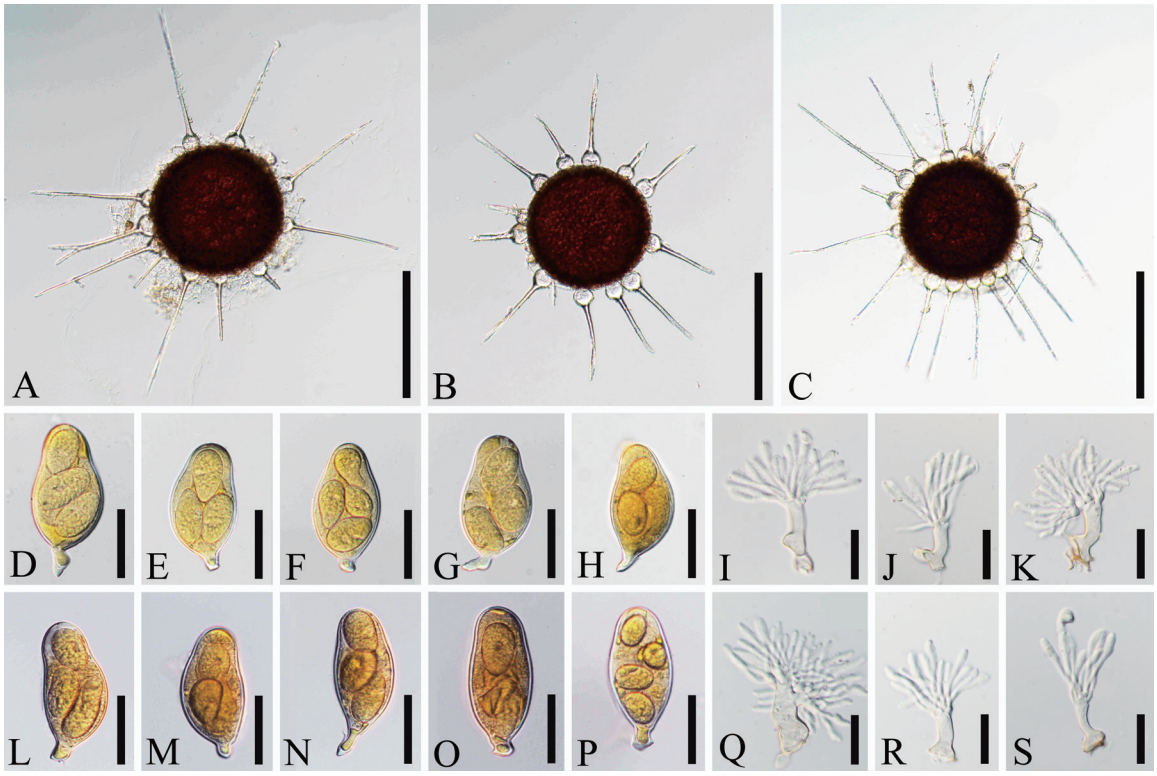

Fig. 5 – *Phyllactinia fraxinicola* on *Fraxinus japonica* (TSU-MUMH4150). A–C: Chasmothecia. D–H, L–P: Asci. I–K, Q–S: Penicillate cells. *Bars*: A–C 200 μm; D–H, L–P 40 μm; I–K, Q–S 25 μm.

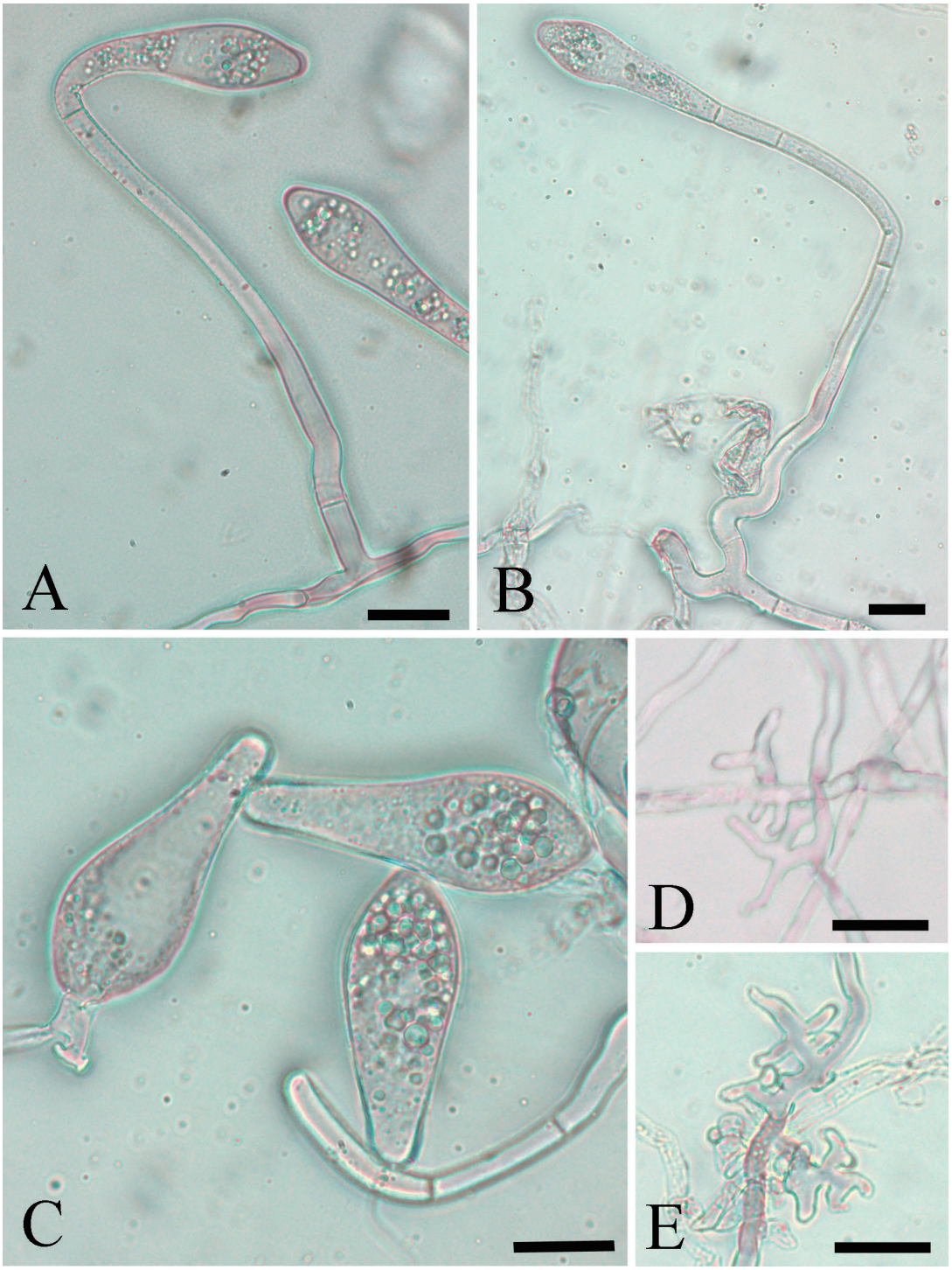

Fig. 6 – *Phyllactinia japonica* on *Fraxinus lanuginosa* f. *serrata* (TSU-MUMH7224). A, B: Conidiophores. C: Conidia. D, E: Hyphal appressoria. *Bars*: 20 μm.

cells 3–5 μm in width; hyphal appressoria rod-shaped, hooked, forked to lobed, solitary or in opposite pairs; conidiophores arising from external hyphae, on upper surface of mother cells, erect, about 95–240(–330) μm in length; foot cells long, about 60–160 (–330) × 5–17 μm, sinuous to spirally twisted at the base, followed by 1–2 shorter cells, forming conidia singly; conidia clavate to somewhat spatulate, apex rounded, not papillate, without conspicuous fibrosin bodies, 56–74 × 18–27 μm. Chasmothecia hypophyllous, scattered to almost gregarious, depressed globose, 182–241 μm diam; appendages equatorial, (2–)4–12, acicular with bulbous basal swelling, (22–)30–40 μm diam, apex subacute to usually obtuse, 220–360(–385) × 6–10 μm, hyaline; penicillate cells in the upper half, numerous, stem 23–53 × 7–19(–26) μm, subcylindrical, with 2–6 short branchlets at apex, filaments 14–33 × 2–6 μm; asci 7–17 per chamothecium, ellipsoid-ovoid, ovoid-lanceolate, 52–90 × 30–57 μm (l/w ratio = 1.6–2.4), stalked, (2–)3(–5)-spored; ascospores broad ellipsoid-ovoid, 17–43 × 14–25 μm (l/w ratio = 1.2–2.5), colorless to yellowish.

Additional specimens examined: On *Fraxinus lanuginosa* f. *serrata*, JAPAN, Hokkaido., Chitose-shi, Chitose Lake, 4 Oct 2003, leg.

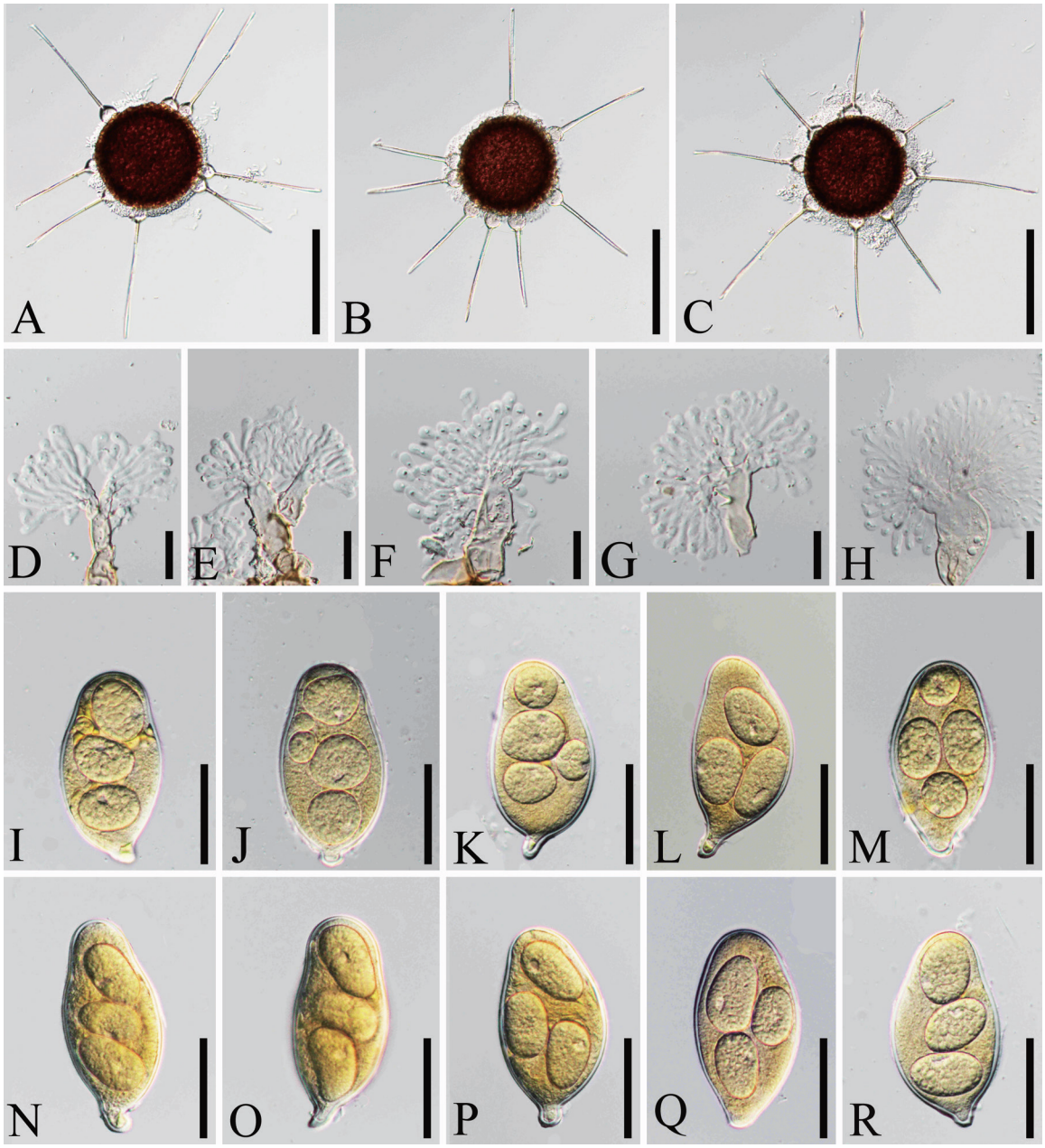

Fig. 7 – *Phyllactinia japonica* on *Fraxinus sieboldiana* (TNS-F-91391, holotype). A–C: Chasmothecia. D–H: Penicillate cells. I–R: Asci. *Bars*: A–C 200 μm; D–H 20 μm; I–R 40 μm.

S. Takamatsu and Y. Seko, TSU-MUMH2902; TSU-MUMH2919, DDBJ ID no.: LC597101 (ITS+28S rDNA); Mie Pref., Matsusaka-shi, Mt. Myojin, Okuyama-tani (34°22'01.8"N 136°5'48.5"E, alt. 1159 m), 15 Sep 2020, Leg. S. Takamatsu, TSU-MUMH7224; On *F. sieboldiana*, JAPAN, Niigata Pref., Yahiko-mura, Mt. Yahiko, 25 Oct 1997, leg. S. Takamatsu, TSU-MUMH426, DDBJ ID no.: AB080585 (ITS), AB080390 (28S); Okayama Pref., Okayama-shi, Tatsunokuchi Shrine, 4 Nov 2007, leg. S. Takamatsu, TSU-MUMH4798, DDBJ ID no.: LC597102 (ITS+28S rDNA).

Host range and distribution: On *Fraxinus lanuginosa* f. *serrata*, *F. sieboldiana* (Japan), *Oleaceae*.

Phyllactinia fraxini-longicuspidis M. Maeda, Meeboon & S. Takam., sp. nov.

Figs. 8, 9.

MycoBank no.: MB 838791.

= *Phyllactinia fraxinicola* auct. p.p.
= *P. fraxini* auct. p.p.
= *P. corylea* auct. p.p.
= *P. guttata* auct. p.p.
= *P. suffulta* auct. p.p.

Diagnosis: Morphologically similar to *Phyllactinia fraxini* and *P. fraxinicola*, but differs in constantly 2-spored asci and in the rDNA ITS sequences. Occurring on *Fraxinus longicuspis*.

Holotype: On *Fraxinus longicuspis* (*Oleaceae*), JAPAN, Shiga Pref., Maibara-shi, Mt. Ibuki (35°23'42.37"N 136°23'34.02"E, alt. 459 m), 1 Nov 2017, leg. S. Takamatsu (TNS-F91392). Isotype: TSU-MUMH7131.

Gene sequences (ex-holotype): LC597106 (ITS+28S rDNA).

Etymology: *fraxini-longicuspidis*, referring to the host of this species, *Fraxinus longicuspis*.

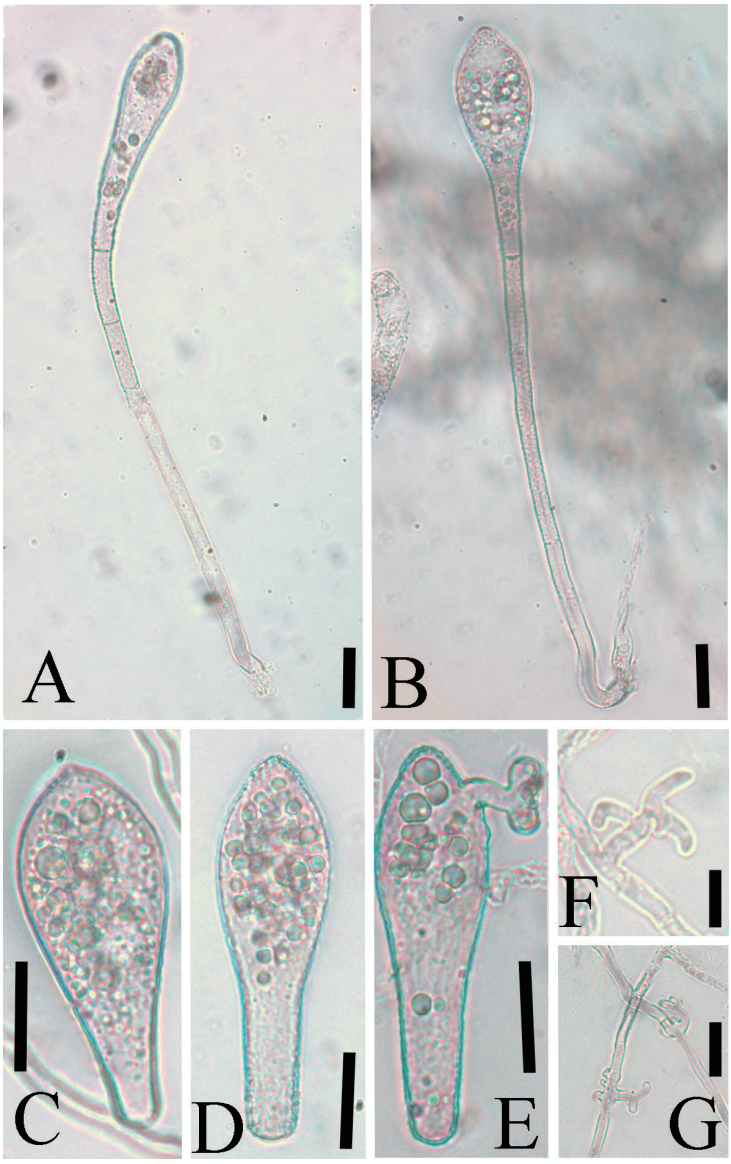

Fig. 8 – *Phyllactinia fraxini-longicuspidis* on *Fraxinus longicuspis* (TSU-MUMH7227). A, B: Conidiophores. C–E: Conidia. F, G: Appressoria. *Bars*: A–E, G 20 μm; F 10 μm.

Description: Mycelium hypophyllous, effuse, in irregular patches or entirely covering leaves, inconspicuous, evanescent; hyphal cells 3–4.5 μm in width; hyphal appressoria rod-shaped, hooked, forked to lobed, solitary or in opposite pairs; conidiophores arising from external hyphae, on upper surface of mother cells, erect, about 130–260 × 5–11 μm; foot cells very long, about (65–)80–200 (–240) × 4–6 μm, sinuous to spirally twisted at the base, followed by 1–2 shorter cells, forming conidia singly; conidia clavate to somewhat spatulate, apex rounded, not papillate, without conspicuous fibrosin bodies, 55–80 × 16–25 μm, producing subterminal germ tubes. Chasmothecia hypophyllous, scattered to almost gregarious, depressed globose, (161–)175–229 μm diam; appendages equatorial, 6–14(–17), acicular with bulbous basal swelling, 24–43 μm diam, apex subacute to usually obtuse, 164–242 × 5–9 μm, hyaline; penicillate cells in the upper half, numerous, stem 12–37 × 6–22 μm, subcylindrical, with 2–8 short branchlets at apex, filaments 9–32 × 1.5–7 μm; asci 8–20 per chamothecium, ellipsoid-ovoid, 56–74 × 26–36 μm (l/w ratio = 1.8–2.6), stalked, 2-spored; ascospores ellipsoid-ovoid, 23–41 × 13–22 μm [l/w ratio = 1.2–2.3(–2.9)], colorless to yellowish.

Additional specimens examined: On *Fraxinus longicuspis*, JAPAN, Ehime Pref., Saijo-shi, Mt. Kamegamori, 10 Nov 1998, leg. S. Takamatsu, TSU-MUMH566, DDBJ ID no.: AB080513 (ITS), AB080404 (28S rDNA); Niigata Pref., Itoigawa-shi, Mt. Myojo, 19 Oct 1996, leg. S. Takamatsu, TSU-MUMH301; Niigata Pref., Yahiko-mura, Mt. Yahiko, 18 Oct 1996, leg. S. Takamatsu, TSU-MUMH211; TSU-MUMH212, DDBJ ID no.: AB080493 (ITS), AB080383 (28S rDNA); Mie Pref., Inabe-shi, Mt. Fujiwara (35°10'27.4"N 136°28'30.0"E, alt. 137 m), 31 Oct 2011, leg. S. Takamatsu, TSU-MUMH5421, DDBJ ID no.: LC597104 (ITS+28S rDNA); Shiga Pref., Maibara-shi, Mt. Ibuki, 4 Nov 2010, leg. S. Takamatsu, TSU-MUMH5608; TSU-MUMH5614, DDBJ ID no.: LC597105 (ITS); TSU-MUMH5650; ibid. (35°23'46.09"N 136°23'38.47"E, alt. 498 m), TSU-MUMH7139; ibid. (35°23'47.99"N 136°23'37.75"E, alt. 514 m), TSU-MUMH7141, DDBJ ID no.: LC597107 (ITS+28S rDNA); ibid. (35°23'44.62"N 136°23'34.93"E, alt. 463 m), 23 Sep 2020, leg. S. Takamatsu, TSU-MUMH7227.

Host range and distribution: On *Fraxinus longicuspis* (Japan),

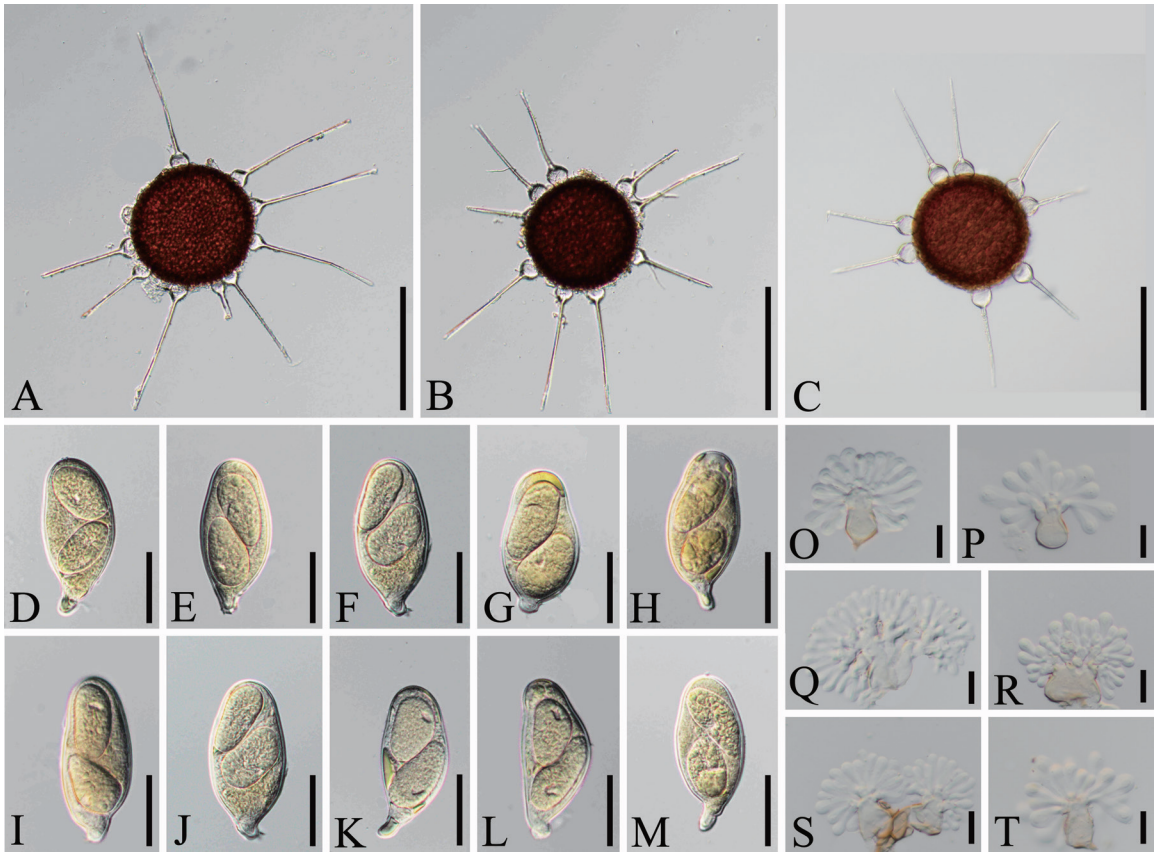

Fig. 9 – *Phyllactinia fraxini-longicuspidis* on *Fraxinus longicuspis* (TSU-MUMH5421). A–C: Chasmothecia. D–M: Asci. N–S: Penicillate cells. *Bars*: A–C 200 μm; D–M 40 μm; N–S 15 μm.

Oleaceae.

4. Discussion

4.1. Four Phyllactinia *species found on* Fraxinus

Previous phylogenetic analyses revealed that *Phyllactinia* occurring on *Fraxinus* (and other *Oleaceae* genera) formed a distinct clade (Takamatsu et al., 2008, 2016), which strongly suggested that these powdery mildew species diverged from a single ancestor. Because all 21 sequences newly retrieved in this study were similar to the sequences reported previously (Takamatsu et al., 2008, 2016; Scholler et al., 2018), we concentrated on phylogenetic relationships within the *P. fraxini* s. lat. clade. A total of 46 *P. fraxini* s. lat. sequences were divided into four distinct clades, which were consistent with the previous analyses (Takamatsu et al., 2008, 2016; Scholler et al., 2018). This suggests that there are at least four species in this group. Morphological examinations revealed that Clade 4 (*P. fraxini-longicuspidis*) is distinguishable from the other clades by having chasmothecia with consistently 2-spored asci (vs mainly 3-spored asci in the other clades). Obvious morphological differences among the other three clades are not evident, although there are distinct genetic differences among all four clades, which nevertheless reflect the involvement of different species. In addition, there are clear host preferences among the clades (see section 4.2). Therefore, it is justified to regard these clades as different species based on these genetic and host range segregations.

4.2. Host range relations

Among the four clades of *P. fraxini* s. lat., Clades 2, 3, and 4 have relatively narrow host ranges (Fig. 10). For example, the host range of Clade 2 (*P. fraxinicola*) comprises *F. japonica*, *F. chinensis*, and *F. rhynchophylla* (except for the two specimens from Korea). These *Fraxinus* species are closely related to each other and taxonomists sometimes even regard them as a single species (Wallander, 2008). Hosts of Clade 3 (*P. japonica*) encompasses *F. lanuginosa* f. *serrata* and *F. sieboldiana*, which are also closely related to each other. Clade 4 (*P. fraxini-longicuspidis*) has only a single host species (*F. longicuspis*). All the above *Fraxinus* species belong to sect. *Ornus*. Among the hosts of Clade 1 (*P. fraxini*), *F. ornus* belongs to sect. *Ornus*, while *F. excelsior* and *F. mandshurica* belong to sect. *Fraxinus*, and *F. pennsylvanica* to sect. *Melioides*. In addition, *P. fraxini* may infect *Syringa* spp. and *Chionanthus*, two other oleaceous genera. In summary, among the four species of *P. fraxini* s. lat., *P. fraxinicola*, *P. japonica*, and *P. fraxini-longicuspidis* are species with narrow host ranges confined to hosts belonging to *Fraxinus* sect. *Ornus*. On the other hand, *P. fraxini* s. str. has a wider host range infecting not only a member of sect. *Ornus*, but also members of other sections such as sects. *Fraxinus* and *Melioides*, and also members of other oleaceous genera. This wide host range of *P. fraxini* s. str. might lead to a wider geographic distribution of this species. The result that the four species of *P. fraxini* s. lat. form a distinct large clade containing species with very high host specificity strongly suggests that speciation of these species occurred closely in line with their hosts.

The fungus on two specimens from Korea labeled as *F. excelsior* and *F. mandshurica* were identified as *P. fraxinicola* based on mo-

lecular analysis. Crucially, KUS-F-10643 (GenBank ID: AB080537) is the holotype of *P. fraxinicola*, and thus *F. mandshurica* is the type host of this species. The host of KUS-F-17216 (GenBank ID: AB080541) was originally labeled as *Acer mandshuricum* (Takamatsu et al., 2008), but later re-identified as *F. excelsior* (Scholler et al., 2018). In our current analyses, all other specimens on *F. excelsior* and *F. mandshurica* belonged to Clade 1 (*P. fraxini*) and only the two Korean specimens belonged to Clade 2 (*P. fraxinicola*). This seems strange when taking the very high host specificity of *P. fraxini* s. lat. into consideration. Therefore, we borrowed the two Korean specimens to check the identity of the host plants. However, all attempts to identify the plant species failed due to a lack of necessary morphological characteristics. Further studies are necessary to investigate whether or not *F. excelsior* and *F. mandshurica* are involved in the host range of *P. fraxinicola*. In any case, it cannot be excluded that *P. fraxinicola* might have a wider host range in Asia, comparable to *P. fraxini* in Europe.

Erysiphe and *Phyllactinia* are two major powdery mildew genera occurring on *Fraxinus*. Host ranges of these two genera on *Fraxinus* spp. are largely overlapping and often found on the same host leaves simultaneously (Fig. 10). Because both *Erysiphe* spp. and *Phyllactinia* spp. on *Fraxinus* each form distinct, clearly separated clade, it is feasible that these powdery mildews diverged on *Fraxinus* species in tandem with their host phylogeny. Thus, host recognition systems by powdery mildews might be inferred by comparisons of host ranges of the two powdery mildew groups. The host ranges of the four *Phyllactinia* species on *Fraxinus*, based on the current study, were compared with host ranges of the *Erysiphe* species reported by Yamaguchi et al. (2021) (Fig. 10). The host ranges of the two genera are similar to each other, but some differences were found. For example, *P. fraxini-longicuspidis* and *E. fraxinicola* U. Braun & S. Takam. commonly have *F. longicuspis* as their only host. *Phyllactinia japonica* infects *F. lanuginosa* f. *serrata* and *F. sieboldiana*. Similarly, *E. fraxinea* Y. Yamaguchi, Meeboon & S. Takam. infects *F. lanuginosa* f. *serrata*, but *F. sieboldiana* is a host of *E. salmonii* (Syd.) U. Braun & S. Takam. *Phyllactinia fraxini* infects *F. excelsior*, *F. mandshurica*, *F. ornus*, and *F. pennsylvanica*, and *P. fraxinicola* infects *F. japonica*, *F. chinensis*, and *F. rhynchophylla*. However, all these *Fraxinus* spp. are hosts of *E. salmonii*. Of these host species, *F. excelsior* and *F. pennsylvanica* have been reported as hosts of *E. salmonii* only recently (Heluta, Takamatsu, & Siahaan, 2017). Thus, infections of these two hosts may be a result of recent host expansion by *E. salmonii*. *Phyllactinia fraxini* may infect species of other oleaceous genera, such as *Chionanthus* and *Syringa*, whereas the mentioned *Erysiphe* species exclusively infect *Fraxinus*. In summary, both *Phyllactinia* and *Erysiphe* have species of sects. *Ornus* and *Fraxinus* as their common hosts, but host ranges are somewhat different between species of these powdery mildew genera. Thus, host recognition systems may be different between the two genera. In addition, *Phyllactinia* species are distributed widely in the Northern Hemisphere, whereas *Erysiphe* species were originally distributed only in East Asia, but expanded their distribution areas to Europe recently.

4.3. Biogeography

Previously, only *P. fraxini* has been identified as *Phyllactinia* powdery mildew species parasitic on *Fraxinus*. Braun and Cook (2012) divided *P. fraxini* s. lat. into two species, viz. *P. fraxini* (European and North American species) and *P. fraxinicola* (East Asian species), based on molecular phylogeny (Takamatsu et al., 2008) and characteristics of foot cells of conidiophores. According to Braun and Cook (2012), foot cells of *P. fraxini* were said to be straight, while those of *P. fraxinicola* were considered to be sinuous or spirally twisted at the base. However, a later study (Scholler et al., 2018) revealed that *P. fraxini* also has sinuous or spirally twisted

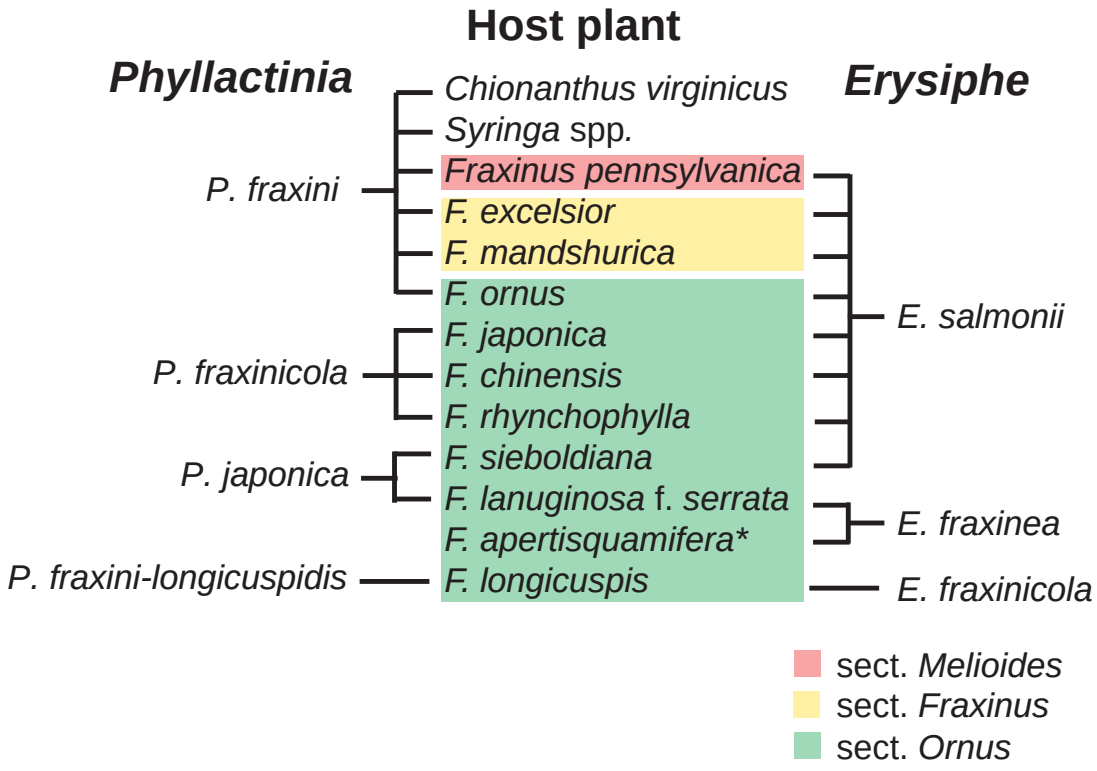

Fig. 10 – Comparison of host ranges of *Phyllactinia* and *Erysiphe* occurring on *Fraxinus*.
* *Fraxinus apertisquamifera* has not been recorded as a host of *Phyllactinia*.

foot cells, i.e., the previously assumed morphological difference between *P. fraxini* and *P. fraxinicola* proved to be untenable and misleading.

The present study revealed that all four Japanese specimens on *F. mandshurica* pertain to *P. fraxini*, which was the first evidence that this powdery mildew species is also distributed in East Asia. Thus, all recognized *Phyllactinia* species on *Fraxinus* are distributed in East Asia, whereas the host ranges of the four species are highly segregated, except for the two Korean specimens. These results suggest that genetic isolation by host specificity played a more important role than the geographic segregation in the speciation events of the four *Phyllactinia* species.

Phyllactinia fraxini has been reported in North America as well as Europe and Asia. However, as far as we know, there is no DNA sequence record of this species from North America in DNA databases. Further studies based on molecular data from North America are required.

4.4. Evolutionary timing

Calibration of the evolutionary timing using molecular clock of 28S rDNA D1/D2 domains (Takamatsu & Matsuda, 2004) showed that *Phyllactinia* diverged from *Pleochaeta* about 60 mya in early Paleogene and the divergence of *Phyllactinia* on *Fraxinus* occurred about 25 mya at the transition from Oligocene to Miocene (Takamatsu et al., 2008). The current analysis using molecular clock of ITS sequence data suggested that the divergence of the four species of *Phyllactinia* on *Fraxinus* occurred about 5–2 mya in Pliocene. On the other hand, similar molecular clock analyses using ITS sequences revealed that the three *Erysiphe* species on *Fraxinus* may have diverged about 13–7 mya in Miocene (Yamaguchi et al., 2021). These results suggest that the divergence of *Phyllactinia* species on *Fraxinus* occurred much later than the divergence of *Erysiphe* species although both fungal groups have *Fraxinus* sect. *Ornus* as main hosts. *Fraxinus* sect. *Ornus* split from other sections about 38 mya and diverged in Miocene or later in East Asia (Hinsinger et al., 2013). *Phyllactinia* species on *Fraxinus* have very high host specificities. Probably, this is not a result of co-speciation of powdery mildews and their host plants. These powdery mildews may have diverged in accordance with host phylogeny after divergence of the host plants concerned.

4.5. Key to the species of Phyllactinia on Fraxinus spp.

1a. Asci two-spored; on *Fraxinus longicuspis*, Japan ..... *P. fraxini-longicuspidis*
1b. Asci mainly three-spored 2
2a. On *Fraxinus sieboldina* and *F. lanuginosa* f. *serrata*, Japan ..... *P. japonica*
2b. On *Fraxinus japonica*, *F. chinensis* and *F. rhynchophylla*, East Asia (China, Japan, Korea) ..... *P. fraxinicola*
2c. On *Fraxinus excelsior*, *F. mandshurica*, *F. pennsylvanica*, *Syringa* spp., and *Chionanthus* spp., Europe, East Asia, North America (?) ..... *P. fraxini*

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

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