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Short communication

Lectotypification, epitypification, and molecular phylogenetic confirmation of *Cytospora paulowniae* comb. nov., a causal pathogen of *Paulownia* tree canker in Japan

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ABSTRACT

Paulownia tree canker is a major disease of *Paulowniae tomentosa* in Japan. The pathogen was described as *Valsa paulowniae* in 1916 by Hemmi and Miyabe. However, its current taxonomic status and phylogenetic position are uncertain. In this study, we reviewed the protologue of this species and rediscovered the syntypes maintained at the Hokkaido University Museum (SAPA). From these specimens, a lectotype was selected. The molecular phylogenetic position of this species was examined with newly collected samples. Based on the result of phylogeny and morphology, an epitype of this species was designated and transferred to the genus *Cytospora*.

Keywords: Diaporthales, epitype, lectotype, The Hokkaido University Museum, *Valsa*

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Paulownia tomentosa (Thunb.) Steud. (princess tree) is a member of the Paulowniaceae native to Asia. In Japan, it has been planted mainly in the Tohoku region, northern Kanto, and southern Hokkaido. The timber has traditionally been used mainly for producing *geta* (Japanese wooden clogs) and currently for making furniture (Forestry Agency, https://www.rinya.maff.go.jp/j/tokuyou/tokusan/, Dec 2023). Domestic production of *Paulownia* lumber reached its peak in 1959, with 86,806 m^3, but by 2022 production had dropped to 230 m^3. Imported lumber from China is now more commonly used (MAFF, https://www.maff.go.jp/j/tokei/kouhyou/tokuyo_rinsan/, Dec 2023; Oka & Oka, 2020). One reason for the decline in production is the increased damage caused by canker disease since 1960 (Aono et al., 1997). Currently, efforts are being made to restore *Paulownia* cultivation in some areas of Fukushima and Gunma prefectures. However, microbial diseases of *Paulownia* trees, such as witches' broom and canker, have discouraged sustainable in *Paulownia* tree cultivation (Ito, 1974; Sasaki et al., 1981).

One of the major diseases, canker of the *Paulownia* tree, was reported by Kitajima (1915) and Hemmi (1916a) in Tohoku and Hokkaido, respectively. This disease was named "*furan-byo*" in Japanese by Kitajima (1916), and its causal pathogen was described as *Valsa paulowniae* Miyabe et Hemmi (Hemmi, 1916b; Ito, 1965; Takita, 1984). Subsequently, numerous studies on the pathogenicity and life cycle were conducted in each of the prefectures where *Paulownia* trees were grown (e.g., Aono et al., 1997; Igarashi et al., 2001; Ito et al., 1956; Mikawa, 1984; Sakuyama & Takamura, 1983; Sasaki et al., 1981; Sasaki & Matsuzaki, 1987; Takita, 1984). Under the current nomenclatural code, the teleomorphic genus *Valsa* is currently treated as a synonym of the anamorphic genus *Cytospora*, which is the oldest name among *Cytospora* and its related teleomorphic genera (Fan et al., 2020; Rossman et al., 2015). The genus *Cytospora* is currently undergoing taxonomic revision and many new species have been described worldwide (e.g., Adams et al., 2002, 2005, 2006; Fan et al., 2014, 2020; Fotouhifar et al., 2010; Gao et al., 2021; Ilyukhin et al., 2023; Jami et al., 2018; Lawrence et al., 2018; Norphanphoun et al., 2018; Pan et al., 2020; Travadon et al., 2022; Úrbez-Torres et al., 2020). On the other hand, although they are important pathogens, the taxonomic positions of *Valsa* and *Cytospora* species originally described in Japan, such as *V. paulowniae*, have not been fully clarified and need reappraisal. Comprehensive revision has been hindered because many of these old

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specimens and ex-cultures used at the time of description are lost or difficult to access from other countries. In this study, we reexamined and clarified the taxonomic position of *V. paulowniae* by reviewing the original descriptions, searching for type specimens, observing morphological characteristics, collecting new disease specimens, establishing new isolates, and conducting molecular phylogenetic analysis.

The existence of type specimens of *V. paulowniae* was searched in the fungal herbarium of the Hokkaido University Museum, (SAPA) Sapporo, Hokkaido, Japan, which is known for its large collection of specimens from Dr. Kingo Miyabe. Fourteen specimens of *V. paulowniae* without public numbers were found and assigned numbers as new fungal specimens of the Hokkaido University Museum (Table 1). Of them, ten specimens were judged to be syntypes used to prepare the original descriptions of *V. paulowniae*. From these specimens, we selected a lectotype specimen (SAPA 302) in good condition in which both sexual and asexual states could be observed.

The morphological characteristics of these specimens were examined under compound microscopes (S9-i: Leica Microsystems, Wetzlar, Germany; Stemi305Cam, and Axioscope 5: Zeiss, Göttingen, Germany). We also examined the specimens and isolates of the fungus causing *Paulownia* canker in the herbarium of the Forestry and Forest Products Research Institute (TFM-FPH), Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Ibaraki, Japan (Table 1). In addition, symptomatic samples were newly collected in Morioka City and Yahaba Town, Iwate Prefecture, in Nov 2022. From the newly collected specimens, three isolates (H524, H525, and H526) were obtained from single or multiple spores. The isolates were cultured on potato dextrose agar (PDA; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Total DNA was extracted with a DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). Nucleotide sequences of the actin (*act*), internal transcribed spacers 1 and 2 including the intervening 5.8S nrDNA gene (ITS), the translation elongation factor 1-alpha gene (*tef1*), β-tubulin gene (*tub2*), and RNA polymerase II second largest subunit (*rpb2*) were amplified using the primers ITS5/ITS4 (White et al., 1990) and EF1-728F/EF1-986R (Carbone & Kohn, 1999) or EF1-688 F/EF1-1251R (Alves et al., 2008), Bt2a/Bt2b (Glass & Donaldson, 1995), and RPB2-5F2/fRPB2-7cR (Liu et al., 1999; Sung et al., 2007), respectively (Hattori et al., 2021; Hattori & Masuya, 2023). The amplicons were sequenced in both directions using the respective PCR primers and a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) or SupreDye™ Cycle Sequencing Kit v. 3.1 (M&S Techno Systems, Osaka, Japan) on an Applied Biosystems 3730xl DNA analyzer. The obtained sequences were concatenated and aligned with sequences retrieved from GenBank that were used in previous studies (Supplementary Tables S1 & S2). For each region, the best evolutional model was estimated by Modeltest-NG (Darriba et al., 2020). Maximum likelihood (ML) analyses were implemented using RAxML-NG (Kozlov et al., 2019). After confirming the phylogenetic relationships on the backbone tree of *Cytospora* species using the ITS region (Supplementary Fig. S1), Japanese species were analyzed using multiple regions (Supplementary Table S2) with seventeen known species, which were assumed to be phylogenetically closely related to each other. The strengths of the internal branches from the resultant trees were tested by bootstrap (BS) analysis (Felsenstein, 1985) with 1000 replicates. The analysis was conducted using a matrix composed of 2300 bp with gaps (ITS: 499 bp, *act*: 281 bp, *rpb2*: 720 bp, *tef1*: 332 bp, *tub2*: 468 bp). *Diaporthe vaccinii* Shear (CBS 160.32) and *Cytospora rosicola* M. Pan & X.L. Fan (CF20197024) were used as an outgroup for the ITS tree and multilocus tree, respectively. Evolutionary models, TrNef+I+G4 for ITS, TVM+G4 for *act*, TrN+G4 for *rpb2*, TPM2uf+G4 for *tef1*, and TIM3+I+G4 for *tub2*, were applied for each locus. On the backbone tree of the genus *Cytospora*, Japanese isolates from *P. tomentosa* were located in the genus *Cytospora* as a sister branch to *Cytospora pruinosa* (Fr.) Sacc. (CBS 201.42) (Supplementary Fig. S1). Moreover, a multilocus phylogenetic analysis showed that the Japanese isolates from *P. tomentosa* formed an independent clade (BS = 100%) among the clades of the genus *Cytospora* and were a sister to *Cytospora kuanchengensis* CM Tian & N. Jiang (CFCC 52464) from China (BS = 93%) (Fig. 1). These results indicated that *V. paulowniae* should be transferred from the genus *Valsa* to *Cytospora*. Moreover, *V. paulowniae* can be distinguished from the phylogenetically closely related species *C.*

Table 1. List of isolates and specimens used in this study.

Isolate No.	Specimen No.	Regions in Japan	Collecting date	Identification in previous study	Reference
H524				-	-
H525	TFM-FPH 13121	Yahaba, Iwate Prefecture	2022 Nov 9	-	-
H526				-	-
MAFF 410491 = V-1	TFM-FPH 1196	Kushigata-mura, Ibaraki Prefecture	1952 May 28	*Valsa paulowniae*	Kobayashi (1970)
MAFF 410509 = V-79	-	Ohnuma, Fukushima Prefecture	1978 Feb 18	*V. paulowniae*	-
MAFF 410015 = LFP-V-1	-	Kushigata-mura, Ibaraki Prefecture	-	*V. paulowniae*	Kobayashi (1970)
-	SAPA 300	Mutsu, Aomori Prefecture	1903 Aug	*V. paulowniae*	Hemmi (1916b)
-	SAPA 301	Sapporo, Hokkaido	1914 Nov	*V. paulowniae*	Hemmi (1916b)
-	SAPA 302	Monbetsu, Hokkaido	1906 Jul 18	*V. paulowniae*	Hemmi (1916b)
-	SAPA 303	Monbetsu, Hokkaido	1906 Jul 18	*V. paulowniae*	Hemmi (1916b)
-	SAPA 304	Date, Hokkaido	1915 Apr 27	*V. paulowniae*	Hemmi (1916b)
-	SAPA 305	Motomura, Hokkaido	1915 Jul	*V. paulowniae*	Hemmi (1916b)
-	SAPA 306	Motomura, Hokkaido	1915 May 2	*V. paulowniae*	-
-	SAPA 307	Sobetsu, Hokkaido	1915 Aug	*V. paulowniae*	Hemmi (1916b), Kobayashi (1970)
-	SAPA 308	Sapporo, Hokkaido	1914 Oct	*V. paulowniae*	Hemmi (1916b), Kobayashi (1970)
-	SAPA 309	Maruyama, Hokkaido	1931 Jul	*V. paulowniae*	-
-	SAPA 310	Fukushima, Hokkaido	1915 Sep	*V. paulowniae*	-
-	SAPA 311	Sapporo, Hokkaido	1915 May	*V. paulowniae*	Hemmi (1916b)
-	SAPA 312	Sapporo, Hokkaido	1914 Nov	*V. paulowniae*	Hemmi (1916b)
-	SAPA 313	Oshoro, Hokkaido	1920 Oct 24	*V. paulowniae*	-

MAFF: NIAS Genbank, National Institute of Agrobiological Science, Tsukuba, Ibaraki, Japan. TFM-FPH: the herbarium of the Forestry and Forest Products Research Institute (TFM-FPH), Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Ibaraki, Japan. SAPA: the fungal herbarium of the Hokkaido University Museum, Sapporo, Hokkaido, Japan

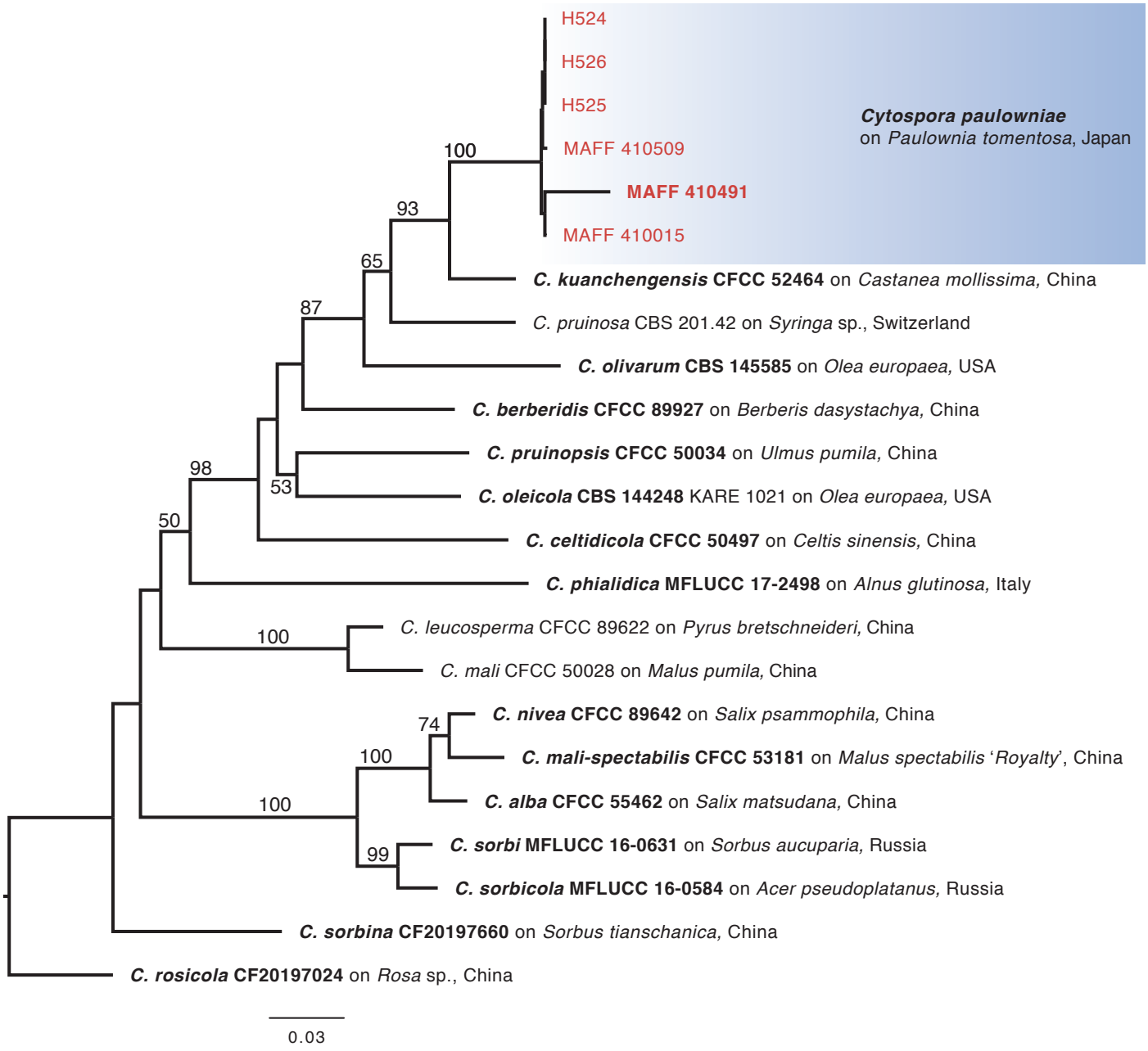

Fig. 1 – Maximum-likelihood (ML) phylogenetic tree of *Cytospora* species based on a concatenated matrix composed of ITS, *act*, *rpb2*, *tef1*, and *tub2* regions. The bootstrap values (BS) are given near branches (BS > 50). Japanese isolates examined in this study are shown in red. Ex-type strains are indicatedin bold.

kuanchengensis and *C. pruinosa* by the morphological characteristics of ascomata and pycnidia, host plants, and geographic distribution (Table 2). Although this study offers a taxonomic rearrangement of one of the oldest known important tree pathogens in Japan, many other species and herbarium specimens will eventually need reappraisal.

Taxonomy

Cytospora paulowniae (Miyabe & Hemmi) Y. Hattori, Masuya & C. Nakash. comb. nov. Fig. 2.
MycoBank no.: **MB 851088.**
Basionym: *Valsa paulowniae* Miyabe & Hemmi, in Hemmi, Bot. Mag. (Tokyo) 30: 312, 1916. MB 187945
≡ *Cytophoma paulowniae* Miyabe & Hemmi, in Kobayashi, 1970. nom. nud.

Sexual state: Ascomata immersed in the bark, scattered, erumpent when mature, grayish-black to brownish-black with 10–50 black ostioles of perithecia irregularly, 2–3.5 × 1–3 mm. Conceptacle absent. Ectostromatic disc pale brown to dark brown, usually surrounded by ostiolar necks, circular, many ostioles arranged irregularly. Ostioles numerous, dark brown to black, arranged irregularly in a disc, 1.5–2.5 mm in length. Perithecia clustered 20–50, black, compressed spheroidal, arranged irregularly, 406–570 × 325–535 µm (491 × 432 µm on average, n = 8, SAPA 302). Asci hyaline, with refractive and chitinoid ring in the nonamyloid apical apparatus, clavate to elongate-obovoid, 31–53 × 6–12 µm (44 × 9 µm on average, n = 7, TFM-FPH 1196), eight-spored. Ascospores hyaline, biseriate to multiseriate, elongate allantoid, aseptate, 9.3–21.0 × 2.5–4.7 µm (14.1 × 3.3 µm on average, n = 33, TFM-FPH 1196).

Table 2. Comparison of morphological characteristics of *Cytospora paulowniae* and related species

	Original description *V. paulowniae* (Hemmi, 1916b)	*V. paulowniae* =*Cytophoma paulowniae* (Kobayashi, 1970)	*C.paulowniae* TFM-FPH 1196 (this study)	*C. paulowniae* SAPA 302 (this study)	*C. kuanchengensis* (Ji-ang et al., 2020)	*C. pruinosa* (Adams et al., 2006)
Ascomata (mm)	2–2.5 × 1–2	1–5	2–3.5 × 1-3	2–3.5 × 1-3	-	-
perithecia (μm)	150–300	200–660	-	406–570 × 325–535 ($\bar{x}$ = 491 × 432, n = 8)	-	-
Asci (μm)	32–52 × 8–10 (commonly 44 × 8.8)	38–59 × 7. 5–10	31–53 × 6–12 ($\bar{x}$ = 44 × 9, n = 7)	27–43 × 6.9–10 ($\bar{x}$ = 34 × 9.2, n = 11)	-	-
Ascospores (μm)	10–18 × 2–4 (commonly 14-16 × 3.2)	12.5–17.5 × 2–4 ($\bar{x}$ = 14.2 × 2.6)	9.34–21.07 × 2.5–4.7 ($\bar{x}$ = 14.1 × 3.3, n = 33)	10.86–14.94 × 2.35–3.70 ($\bar{x}$ = 12.8 × 2.95, n = 21)	-	-
Conidiomata	1.5 × 0.6 (mm)	560–1400 × 840–1300 (μm)	1.0 × 0.4–0.6 (mm)	1.0 × 0.4–0.6 (mm)	(350–)455–540(–575) (μm)	(0.5–)0.6(–1.2) (mm)
conidia (μm)	2.85–8.75 × 0.88–1.75 (commonly 4.38–5.25 × 1.4)	4–6.5 × 0.5–1 ($\bar{x}$ = 5.6 × 1)	4.73–8.95 × 1.14–1.78 ($\bar{x}$ = 5.94 × 1.49, n = 84)	4.44–6.05 × 0.86–1.98 ($\bar{x}$ = 5.22 × 1.25, n = 33)	(5.5–)6–7.5(–8) × 1–2 ($\bar{x}$ = 6.9 × 1.6)	5–6 × 1.2
Host genus	*Paulownia*	*Paulownia*	*Paulownia*	*Paulownia*	*Castanea*	*Olea*
Country	Japan	Japan	Japan	Japan	China	South Africa

Asexual state: Conidiomata immersed in bark, erumpent when mature, uniloculate, flask-like shape to spherical, with rounded base, 1.0 × 0.4–0.6 mm. Conceptacle absent. Ectostromatic disc brown to dark brown, triangular to circular, with a single ostiole. Ostioles circular to ovoid, brown to black, 0.3–0.8 mm length. Conidiogenous cells enteroblastic, phialidic, subcylindrical to cylindrical, repeatedly branched, 6.8–13.2 × 1.1–3.8 μm. Conidia hyaline, unicellular, eguttulate, elongate allantoid, aseptate, 4.4–6.0 × 0.9–2.0 μm (5.22 × 1.25 μm on average, n = 33, SAPA 302).

Culture characteristics: On PDA, colonies are white to pale green, uniform, felt-like, and lacking aerial mycelia, reaching 90 mm diam at 14 d after inoculation at room temperature. In moist mature cultures, conidiomata are randomly distributed on the medium surface and exude a greenish-black spore-horn.

Hosts: *Paulownia fortune* (Seem.) Hemsl. (Ito, 1959; 1973), *P. kawakamii* T. Ito (Ito, 1973), *P. tomentasa* (Thunb.) Steud. (Hemmi, 1916b).

Syntypes: on bark or branches of *P. tomentosa* – JAPAN, Aomori, Mutsu, Aug 1903, T. Nakamura (SAPA 300); Hokkaido, Sapporo, Nov 1914, T. Hemmi (SAPA 301); Hokkaido, Sapporo, Oct 1914, T. Hemmi (SAPA 308); Hokkaido, Sapporo, May 1915, T. Hemmi (SAPA 311); Hokkaido, Sapporo, Nov 1914, T. Hemmi (SAPA 312); Hokkaido, Date-mura, Apr 1915, R. Mimma (SAPA 304); Hokkaido, Motomura, Jul 1915, K. Miyabe (SAPA 305); Hokkaido, Sobetsu, Aug 1915, K. Hashiguchi (SAPA 307).

Lectotype (designated here, MBT 10016734): JAPAN, Hokkaido, Monbetsu, on the bark of *Paulownia tomentosa* (Thunb.) Steud., 18 Jul 1906, Shakin (one of syntypes in Hemmi 1916b; SAPA 302, iso-lectotype SAPA 303).

Epitype (designated here, MBT 10016736): JAPAN, Ibaraki, Kushigata-mura, on branches of *Paulownia tomentosa* (Thunb.) Steud., 28 May 1952, T. Kobayashi (TFM-FPH 1196).

Ex-epitype culture: MAFF 410491 = V-1 in TFM-FPH.

Nucleotide sequences of ex-epitype: LC791549 (ITS), LC791558 (*act*), LC791561 (*rpb2*), LC791570 (*tef1*), and LC791576 (*tub2*).

Additional specimens and cultures examined: on bark or branches of *Paulownia tomentosa* – JAPAN, Hokkaido, Motomura, 2 May 1915, T. Matsumoto (SAPA 306); Hokkaido, Maruyama, Jul 1931, S. Ito (SAPA 309); Hokkaido, Fukushima, Sep 1915, Y. Nojjima (SAPA 310); Hokkaido, Oshoro Shiribeshi, 24 Oct 1920, K. Togashi (SAPA 313); Fukushima, Ohnuma, 18 Feb 1978 (culture MAFF 410509 = V-79 in TFM-FPH); Ibaraki, Kushigata-mura (culture MAFF 410015 = LFP-V-1 in TFM-FPH); Iwate, Yahaba, 9 Nov 2022, Y. Hattori, H. Masuya, M. Torii & T. Koiwa (TFM-FPH 13121, cultures H524, H525, H526 in TFM-FPH).

Note: SAPA302, designated as the lectotype in this study, was collected more than 100 y ago. It had dried out with age, and the asci and ascospores were smaller than in the original description because of shrinkage. Other morphological characteristics of lectotype were generally consistent with the original description by Hemmi (1916b). For further molecular phylogeny and etiological studies, a morphologically congruent specimen (TFM-FPH 1196), collected by Kobayashi (1970), was selected as epitype along with a living specimen (MAFF 410491 = V-1 in TFM-FPH).

Disclosures

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

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Fig. 2 – *Cytospora paulowniae*. A: Label of lectotype specimen (SAPA 302), designated in this study. B: Fruitbodies on the barks of *Paulownia tomentosa* (SAPA 302). C: Natural symptoms of tree canker on *P. tomentosa* (TFM-FPH 13121). D: Label of epitype specimen (TFM-FPH 1196), designated in this study. E: Conidiomata on the barks of *P. tomentosa* (TFM-FPH 1196). F–L: Ascomata forming on the bark (F, G: SAPA 302; H–L: SAPA 304); G, J: Reverse side; L: Vertical section of ascomata. M–Q: Conidiomata on the bark (SAPA 305); O: reverse side; Q: vertical section of pycnidial conidiomata. R–T: Vertical section of ascomata. U, V: Asci (U: SAPA 302; V: TFM-FPH 1196). W, X: Ascospores (W: SAPA 302; X: TFM-FPH 1196). Y: Vertical section of conidioma (SAPA 302). Z: Conidiophores. a, b: Conidia. *Bars*: H, K–M 3 mm; R, S, Y 100 μm; T–X, Z–b 20 μm.

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