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ShortCommunication

Septoria cannabicola, a new species on *Cannabis sativa* from Japan

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ABSTRACT

Septoria leaf spot on hemp has re-emerged with increasing hemp cultivation worldwide. In Japan, ***Septoria cannabis***, initially recorded as the causal pathogen in Japan, was studied with morphology based on the current criteria and detailed molecular phylogenetic analyses using seven gene loci. The robust phylogenetic data and morphology of examined specimens unveiled the existence of a new species of the genus *Septoria* causing leaf spot disease on ***Cannabis sativa***.

Keywords: hemp, leaf spot, pathogen, re-emergent disease, taxonomy

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Cannabis sativa L. is one of the fibre crops cultivated legally with a long cultivation history in Japan for commodities and religious rites. It is known to get infected by fungal pathogens. The earliest records of hemp diseases in Japan include *Septoria cannabis* Sacc. and *Peronospora cannabina* Otth. (Shirai, 1911). In recent years, with the increasing cultivation worldwide, reports of fungal diseases of *C. sativa*, including leaf spot by *Septoria cannabis* (Rahnama et al., 2021), sooty spot by *Pseudocercospora cannabina* (Harishchandra et al., 2023), powdery mildew by *Golovinomyces cichoracearum* sensu lato (s. lat.) (Scott & Punja, 2021), and wilting by *Fusarium* species (Gwinn et al., 2022), have been increasing. In 2023, leaf spots were observed in a commercial hemp field in the Kii Peninsula, Mie Prefecture, Japan, where the hemp was cultivated for religious purposes in Shinto shrines. The onset of leaf spots was characterised by the appearance of lesions as indistinct yellow spots on the lower leaves. Subsequently, these spots transformed into brown irregular lesions, leading to the early defoliation of older leaves. This study aims to identify the causal agent of the leaf spot disease and provide taxonomical updates of previous records of pathogens of *C. sativa* in Japan.

To identify the causal agent, symptomatic leaves were collected from commercial hemp fields located in Mie Prefecture, Japan. The fungus on the symptomatic leaves was isolated from conidial masses by a single conidium isolation method (Nakashima et al., 2016). An isolate was deposited as MUCC3619 in the Mie University Culture Collection (MUCC), Laboratory of Phytopathology, Graduate School of Bioresources, Mie University, Mie Prefecture, Japan. A dried diseased plant specimen was kept in the herbarium, Laboratory of Phytopathology, Graduate School of Bioresources, Mie University (TSU), as TSU-MUMH11996. Inoculation tests to confirm pathogenicity were conducted at Mie University, where *C. sativa* has not been cultivated before. Nine plants grown for 12 wk after sowing were used. Among them, three plants wounded with fine needles and three intact plants were inoculated with the isolated fungus. Additionally, three plants were sprayed with sterilised water as control. From each experimental section, five leaves from the bottom to the middle of a plant were selected and subjected to inoculation. The isolate MUCC3619 was incubated for 2 wk on a Malt Agar (MA; Crous et al., 2019) plate, ground with sterilised distilled water, and prepared as a mycelial fragment as inoculum. Finally, 200 mL of the inoculum was sprayed onto the leaf surface of the wounded plants and the intact plants. The plants were kept under moist conditions for 24 h and covered with plastic bags.

Extraction of genomic DNA from the mycelia of a growing culture was carried out according to the manufacturer's instructions using the DNeasy Ultra Clean Microbial Kit (Qiagen, Hilden, Germany). Subsequently, polymerase chain reactions (PCR) were performed to amplify seven genomic loci within the DNA regions, which encompassed the rDNA internal transcribed spacer region (ITS), the nuclear large subunit rDNA (LSU), and the genes for beta-tubulin (BTUB), the second largest subunit of RNA polymerase II (RPB2), translation elongation factor 1-alpha (TEF-1a), calmodulin (CAL), and actin (ACT) (Quaedvlieg et al., 2013). Analysed sequences were assembled and compared with sequences retrieved from previous studies, as listed in Table 1 (Quaedvlieg et al., 2013; Rahnama et al., 2021). The matrix was concatenated and aligned using the software Concatenator (Vences et al., 2022) and phylogenetic trees generated through Maximum-likelihood (ML) and Bayesian Inference (BI) analyses were used in this study to estimate the phylogenetic relationships. The best substitution model for each region in the analysis was assessed using ModelTest-NG (Darriba et al., 2020) and applied accordingly. ML analyses were

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conducted with RAxML-NG (Kozlov et al., 2019), with 1000 bootstrap replicates to evaluate the robustness of the branches. BI analysis was performed using MrBayes (Ronquist et al., 2012) with Metropolis-Coupled Markov chain Monte Carlo (MCMCMC) searches run for 10 million generations, with the first 25% of the trees being discarded as a burn-in phase, based on an average standard deviation of split frequencies below 0.001. The posterior probability was determined using the remaining trees. *Septoria* cf. *stachydicola* CBS 128668 was used as an outgroup in this study, and the generated trees were visualised using FigTree v 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

For the pathogenicity test, 14 d after inoculation, all inoculated leaves without wounds exhibited symptoms similar to those observed in the original field. No symptoms were observed in control leaves and inoculated leaves with wounds. Both ML and BI trees demonstrated congruent tree topology, placing isolate MUCC3619 in a different clade than *Septoria cannabis* isolated from the United States (Fig. 1). Morphological characteristics of specimens examined in this study, including specimens collected from 1896 to 1947 in the northern part of Japan, showed that the conidioma size of the Japanese fungus (88–125 μm diam) is larger than that of *S. cannabis* (90 μm diam; McPartland, 1995) and *S. neocannabina* McPartl. (66 μm diam; McPartland, 1995) on *C. sativa*. Apart from conidioma size, observable differences include conidial size as described by McPartland (1995), where conidia of *S. cannabis* are longer and wider (30–55 × 2.0–2.5 μm) compared to the Japanese fungus, and those of *S. neocannabina* are shorter with about the same width 20–30 × 1.0–2.0 μm. From these results, the Japanese *Septoria* species on *C. cannabis* should be treated as a novel species.

According to Rahnama et al. (2021), ex-type cultures and nucleotide sequences of other *Septoria* spp. infecting *C. sativa,* i.e. *S. cannabis* Sacc., *S. cannabina* Westend., and *S. neocannabina*

Table 1. List of isolates and reference sequences used in this study.

Fungal Species[b]	Locality	Host	Strain no.	GenBank accession no.[a]						
				TEF-1a	BTUB	RPB2	LSU	ITS	ACT	CAL
Septoria calendulae	Italy	*Calendula arvensis*	CBS 349.58	KF253304	KF252829	KF252358	KF251861	KF251357	KF253661	KF254009
Septoria cannabicola	**Japan**	***Cannabis sativa***	**MUCC3619[T]**	**OR759783**	**OR759784**	**OR759785**	**OR755918**	**OR755916**	**OR759782**	**OR759786**
Septoria cannabis	USA	*Cannabis sativa*	17JS002	MW556605	MW556608	MW556602	MW556614	MW556611	MW526952	MW526955
Septoria cannabis	USA	*Cannabis sativa*	18CL004	MW556603	MW556606	MW556600	MW556612	MW556609	MW526950	MW526953
Septoria cannabis	USA	*Cannabis sativa*	18MF001	MW556604	MW556607	MW556601	MW556613	MW556610	MW526951	MW526954
Septoria chelidonii	South Korea	*Chelidonium majus*	CBS 128607	KF253319	KF252844	KF252373	KF251876	KF251372	KF253676	KF254024
Septoria siegesbeckiae	South Korea	*Siegesbeckia glabrescens*	CBS 128659	KF253494	KF253014	KF252540	KF252051	KF251546	KF253849	KF254198
Septoria siegesbeckiae	South Korea	*Siegesbeckia pubescens*	CBS 128661	KF253495	KF253015	KF252541	KF252052	KF251547	KF253850	KF254199
Septoria stachydicola	South Korea	*Stachys riederi* var. *japonica*	CBS 128668	KF253512	KF253033	KF252558	KF252070	KF251565	KF253866	KF254217
Septoria violae-palustris	Austria	*Viola sp.*	CBS 109108	KF253440	KF252961	KF252489	KF251997	KF251492	KF253796	KF254145
Septoria violae-palustris	Austria	*Viola sp.*	CBS 109109	KF253441	KF252962	KF252490	KF251998	KF251493	KF253797	KF254146

[T] : Ex-type culture,
MUCC: Mie University Culture Collection (MUCC), Laboratory of Phytopathology, Graduate School of Bioresources, Mie University, Mie Prefecture, Japan.
[a] ITS: rDNA internal transcribed spacer region, LSU: nuclear large subunit rDNA, BTUB: beta-tubulin, RPB2: the second largest subunit of RNA polymerase II, TEF-1a: translation elongation factor 1-alpha, CAL: calmodulin, ACT: actin.
[b] Representative species were cited from Quaedvlieg et al. (2013) and Rahnama et al. (2021).

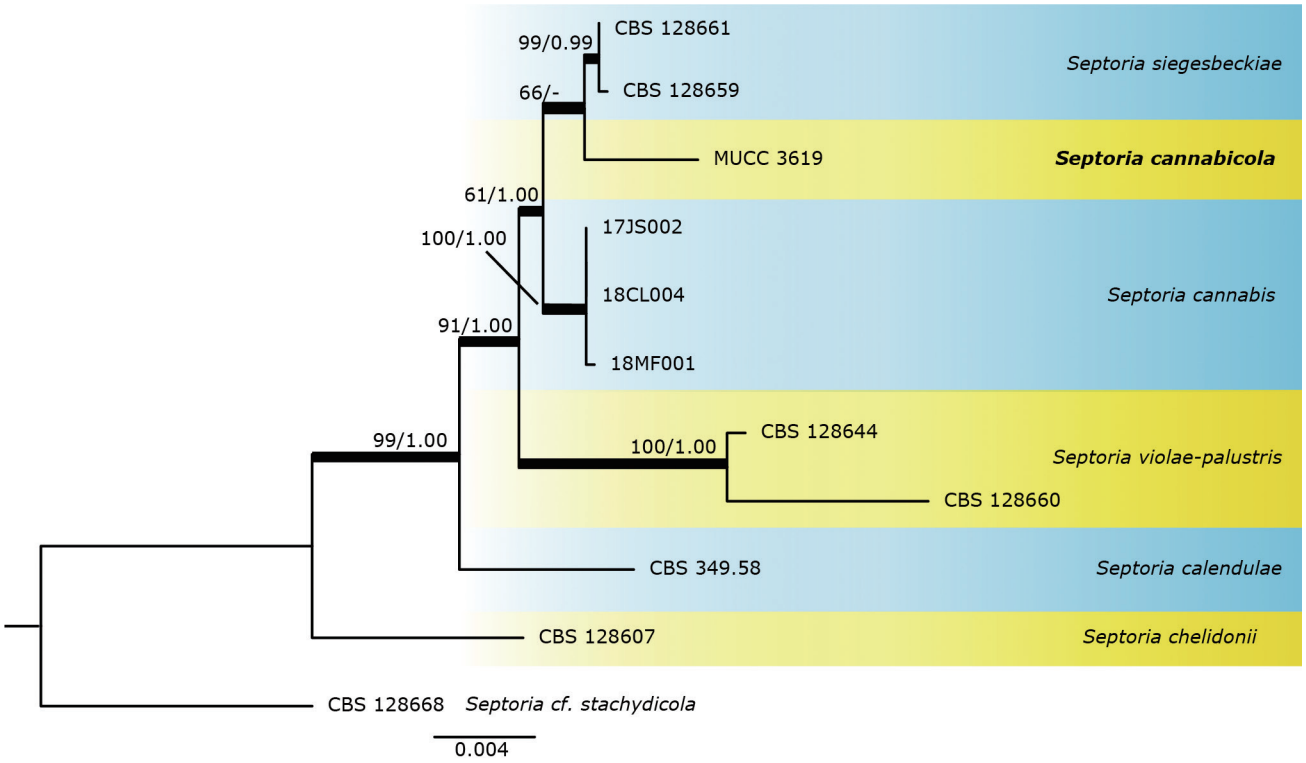

Figure 1 – Maximum-likelihood (ML) phylogenetic tree of *Septoria* spp. constructed by using a concatenated matrix composed of 7 loci. The bootstrap value of ML and posterior probability of Bayesian inference are indicated near branch as BS/PP.

McPartl., are unavailable. In addition, Rahnama et al. (2021) noted that the resurgence of *Septoria cannabis* in the US is likely to coincide with the reintroduction of large-scale planting of industrial hemp. However, it was also mentioned that the actual distribution of the disease is unknown, and whether it is reintroduced or always present at a low level is unknown.

In Japan, the leaf spot disease of *C. sativa* caused by *Septoria* species was first reported by Shirai (1911) as "Shira-hoshi-byo" in Japanese. The fungal pathogen was documented and described as *S. cannabis*. Subsequently, several researchers, including Ideta (1926) and Hara (1930), reported the same disease on *C. sativa*. However, their descriptions of the causal pathogen were relatively simple. Watanabe and Takesawa (1936) provided a detailed description of the morphological characteristics of the causal pathogen, along with the aetiology of *Septoria* leaf spots on *C. sativa,* but concluded that the disease was caused by *S. cannabis*. As the previous description of the causal pathogen relied only on morphological characteristics, this study provides additional details on molecular data and morphology, consequently establishing that the causal agent of leaf spot in *C. sativa* in Japan is a different fungus.

Taxonomy

Septoria cannabicola Ujat & C. Nakash. sp. nov., Fig. 2.
MycoBank no.: MB 850821.

Etymology: derived from the host plant genus, *Cannabis*.

Leaf spots on the lower position leaves, causing early defoliation, amphigenous, angular, yellow with indistinct border at early

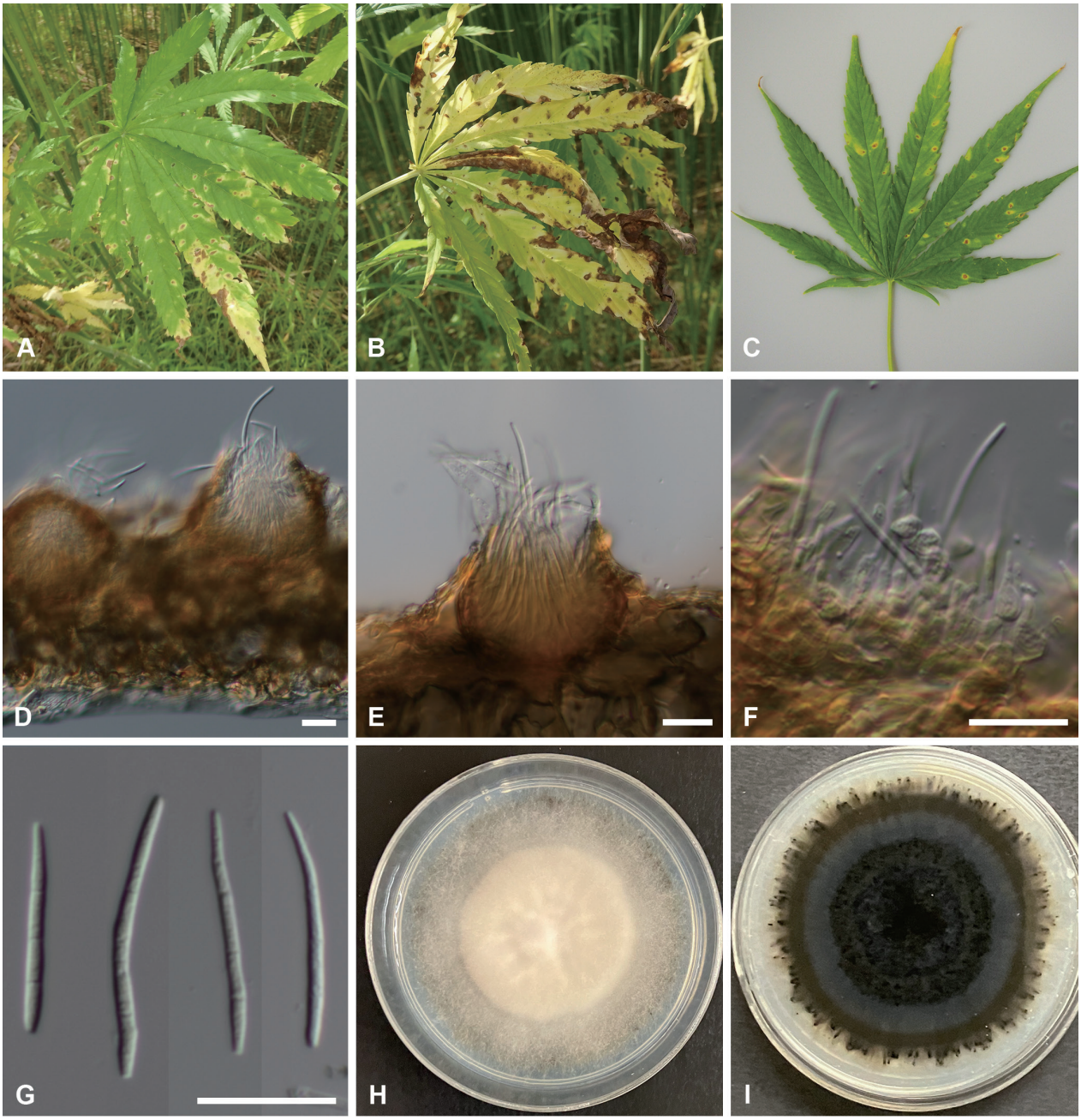

Figure 2 – *Septoria cannabicola* (TSU-MUMH 11996). A, B: Natural symptoms of leaf spot disease on *Cannabis sativa* L. caused by *Septoria cannabicola*. C: Symptoms on artificially inoculated leaves of *C. sativa*. D, E: Conidiomata. F: Conidiogenous cells. G: Conidia. H, I: Colony characteristics of *Septoria cannabicola* in culture on MA. *Bars*: D–G 20 μm.

stage, later becoming brown, circular to irregular, surrounded by yellow halo, 2–8 mm. Mycelium hyaline or pale brown, 2–2.5 μm in width. Conidiomata pycnidial, amphigenous, mainly hypogenous, pale brown to brown, epidermal or subepidermal, submerged or erumpent through epidermis, globose to subglobose, 88–125 μm diam, with an ostiole erumpent through epidermis, with opening 20–25 μm diam; conidiomatal wall 1–2 cell layers wide, composed of *textura angularis*, 2–2.5 μm. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform, hyaline, lining inner cavity of basal half of conidiomata, percurrently proliferating, 2.5–5 × 2–3 μm. Conidia holoblastic, solitary, hyaline, cylindrical to obclavate, straight to slightly curved, pointed at the tip, obconical truncated at the base, not thickened, 30–40 × 1–2.5 μm, 0–4-septate.

Colony on MA greyish white to cream buff, floccose, with loose aerial mycelia at the edge; pycnidial conidiomata rarely formed on the surface under diffuse natural light at room temperature; reverse black to olivaceous black, with concentric patterns.

Holotype: JAPAN, Mie, Minami Ise, Kirihara, on *Cannabis sativa* L., 07 Jul 2023, collected by C. Nakashima (TSU-MUMH 11996).

Ex-type culture: MUCC3619.

DNA sequences of ex-type culture: OR755916 (ITS), OR755918 (LSU), OR759782 (ACT), OR759783 (TEF), OR759784 (BTUB), OR759785 (RPB2), OR759786 (CAL).

Additional specimens examined: on *C. sativa*, JAPAN, Shida Path, 07 Jul 1905, K. Sawada (4836) (IUM*-FY914); Iwate, Morioka, 12 Sep 1911, G. Yamada (4824) (IUM-FY915); Iwate, Morioka, 05 Oct 1905, G. Yamada (4823) (IUM-FY916); Iwate, Morioka, 17 Oct 1904, G. Yamada (4822) (IUM-FY917); Hokkaido, Otaru, 17 Jul 1896, G. Yamada (4817) (IUM-FY918); Hokkaido, Otaru, 01 Aug 1898, G. Yamada (4818) (IUM-FY919); Hokkaido, Maruyama, 13 Oct 1896, G. Yamada (4819) (IUM-FY920); Hokkaido, Maruyama, 10 Jul 1896, G. Yamada (4820) (IUM-FY921); Hokkaido, Sapporo, 03 Oct 1896, G. Yamada (4821) (IUM-FY922); Iwate, Morioka, 06 Jun 1947, K. Sawada (IUM-FY946).

*IUM: Iwate University Museum, Morioka, Iwate, Japan.

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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