horticulturae

MDPI

Article

First Molecular Phylogenetic Identification and Report of *Pseudocercospora cannabina* Causing Leaf Spot Disease on *Cannabis sativa* in Thailand

Dulanjalee Lakmali Harishchandra [1,2], Sukanya Haituk [1,2], Patchareeya Withee [1], Nisachon Tamakaew [1], Nittaya Nokum [1], Chaorai Kanchanomai [3,4], Tonapha Pusadee [3], Chiharu Nakashima [5,*] and Ratchadawan Cheewangkoon [1,*]

1 Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand; dulanjalee.h@cmu.ac.th (D.L.H.); sukanya.h@cmu.ac.th (S.H.); pwithee92@gmail.com (P.W.); nisachon2655@gmail.com (N.T.); nittaya_n@cmu.ac.th (N.N.)
2 Office of Research Administration, Chiang Mai University, Chiang Mai 50200, Thailand
3 Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand; chaorai.k@hotmail.com (C.K.); tonapha.p@cmu.ac.th (T.P.)
4 Managing Director Office, Chaofah Vineyard, Chiang Rai 57210, Thailand
5 Traditional and Industrial Hemp Research Project Center, Graduate School of Bioresources, Mie University, 1577 Kurima-machiya, Tsu 514-8507, Mie, Japan
* Correspondence: chiharu@bio.mie-u.ac.jp (C.N.); ratchadawan.c@cmu.ac.th (R.C.)

Abstract: *Cannabis sativa* is gaining attention as an agronomically important crop in many countries around the world. The identification and control of leaf diseases in cannabis are very important for cannabis cultivators as leaves are the most economically important part of the cannabis plants. In 2022, several cannabis plants in cultivations showing olive leaf spot symptoms emerged from Chiang Rai province, Thailand. Preliminary studies indicated that the causal organism is *Pseudocercospora* sp. Species of *Pseudocercospora* are important plant pathogens that are now identified through morphological studies combined with DNA sequence data of Internal Transcribed Spacer (ITS), Actin (*act*), Translation Elongation Factor (*tef*), and RNA Polymerase II second largest subunit (*rpb2*) gene regions. We aimed to investigate and understand the emergence of olive leaf spot disease in cannabis plants in Chiang Rai province, Thailand, with a specific focus on the combined morpho-molecular identification of the pathogen. In our study, *Pseudocercospora cannabina*, the causal organism of olive leaf spot disease, was identified as the leaf spot-causing pathogen with both morphological and phylogenetic analyses. Our study is the first to provide molecular data for *Ps. cannabina* as the typenor *Ps. cannabina* isolates from previous studies have made molecular data available for this species. A pathogenicity test, re-isolation, and identification steps were performed to fulfill Koch's postulates. This comprehensive approach enhances our understanding of the olive leaf spot disease and its causative agent in cannabis.

Keywords: pathogenicity; cannabis diseases; molecular phylogeny; new disease report; new DNA sequence data; biodiversity; olive leaf spot

Citation: Harishchandra, D.L.; Haituk, S.; Withee, P.; Tamakaew, N.; Nokum, N.; Kanchanomai, C.; Pusadee, T.; Nakashima, C.; Cheewangkoon, R. First Molecular Phylogenetic Identification and Report of *Pseudocercospora cannabina* Causing Leaf Spot Disease on *Cannabis sativa* in Thailand. *Horticulturae* **2023**, *9*, 1261. https://doi.org/10.3390/horticulturae9121261

Academic Editors: Manuel Anguita Maeso, Zhi Li and Min Gao

Received: 1 November 2023
Revised: 19 November 2023
Accepted: 22 November 2023
Published: 23 November 2023

1. Introduction

Cannabis sativa L., also known as hemp or marijuana, has emerged as an agronomically important crop due to its assorted portfolio of uses [1]. It is also relatively easy to grow cannabis, as it has low demand for fertilizers, biocides, weed control, or crop rotation [2]. As a crop with both food and non-food products and a high yield, many counties have legalized the cultivation of cannabis for its potential contribution in improving agro-industrial fields such as agriculture, textiles, bio-composite, paper making, automotive, construction, bio-fuel, functional food, oil, cosmetics, personal care, and the pharmaceutical industry [3,4]. The legal cultivation of cannabis is carried out in over 40 countries, while it still remains

illegal to cultivate or use cannabis products in many countries all over the world due to its narcotic potential [5]. The main cannabis cultivators in the world are the United States, China, and Canada, where it is carried out under strict government regulations. Cannabis farming has a good turnover value for the harvest. For example, in the USA alone, the value of hemp production in the open totaled USD 824 million in 2021 [6].

Historically, cannabis was introduced to Thailand and other countries in Southeast Asia from India [7], and has been used for medicinal purposes, as a food source, and as a source of fiber. The drug-containing genotype of cannabis is more well-known, as its active ingredients are used for medical and recreational purposes, for which it is only legally grown in a few countries of the world, including Thailand. On the other hand, industrial hemp (with THC levels below 0.3%) is used as a food source rich in proteins, omega-3 fatty acids, magnesium, and vitamins in the form of hemp seeds, oil, milk, cheese, protein powder [8], and as a source of strong and stiff and easily recyclable natural fiber [9].

Due to the legalization of cannabis cultivation in some countries and its potential impact on public health, ecological balance, agricultural productivity, and economic benefits, pathogens affecting the cultivation of cannabis and hemp and disease prevention measures have gained the attention of cannabis cultivators and researchers [10]. Both insect pests and fungal, bacterial, and viral pathogens can cause diseases in cannabis [11]. Common diseases causing major economic losses during cannabis cultivation include leaf spots, damping-off, root and crown rot, powdery mildew, bud rots, post-harvest molds, and dudding. Among them, diseases caused by fungal pathogens have risen as the most devastating [12,13].

To avoid the economic losses caused during cultivation and storage in post-harvest stages, it is important to correctly identify diseases and their causal organisms to establish prevention strategies [14]. Additionally, it is important that the end product be free of diseases because the recreational use of cannabis is highly regulated by the governments in all countries where it is grown legally, and for the beneficial and economical manufacturing of high-quality downstream products. This is crucial not only for meeting regulatory standards but also for safeguarding public health.

Leaf diseases caused by fungi in cannabis include yellow leaf spot disease caused by *Septoria cannabis* and *S. neocannabina*, brown leaf spot caused by *Phoma* and *Ascochyta* species, white leaf spot caused by *Diaporthe ganjae* (=*Phomopsis ganjae*) [15], olive leaf spot caused by *Pseudocercospora cannabina* and *Cercospora cannabis*, Stemphylium Leaf and Stem Spot caused by *Stemphylium botryosum* and *S. herbarum*, black mildew caused by *Schiffnerula cannabis*, Black Dot caused by *Epicoccum nigrum*, and Pepper spot caused by *Leptosphaerulina trifolii* [16]. Among them, leaf spot disease caused by *Pseudocercospora cannabina* remains a high-priority disease [17]. Due to its ability to overwinter on infected planting materials and soil, this disease is very commonly observed in field-grown hemp. *Ps. Cannabina* infections can cause the complete defoliation of cannabis and hemp plants [18,19].

Pseudocercospora Speg. (Mycosphaerellaceae, Mycosphaerellales) contains many plant-pathogenic species causing leaf spots, fruit spots, and blights in a wide range of hosts dispersed in a vast geographical distribution area [20–22]. Their diversity is relatively higher in tropical and temperate areas, causing diseases in important agricultural crops, such as Sigatoka leaf disease in bananas, sooty spot disease in kiwi, leaf and fruit spot in citrus [23,24], and angular leaf spot disease in beans [25], leading to devastating economical losses. Quarantine regulations also consider *Pseudocercospora* ssp. As an important phytopathogenic fungi as it has the potential to cause devastating disease to many crops [20,26].

Pseudocercospora species are identified using various characteristics, such as their associated host plants, morphology, pathogenicity, and phylogenetic relationships generated by a multi-locus phylogeny [20,22,23,27,28]. Many phytopathogenic species of *Pseudocercospora* are suggested as host-specific from the results of multi-locus phylogeny and inoculation tests on specific plant hosts [20,22,23]. Therefore, DNA barcoding data of the causal agents of the disease play an important role in the correct identification of species of the genus *Pseudocercospora* when an unknown disease is observed in the field.

During a survey of cannabis fields in Chiang Rai province of Northern Thailand, *Cannabis sativa* leaves showing leaf spot symptoms were collected and brought to the laboratory. After preliminary morphological studies under microscopical observations, the causal organism observed on the leaf spots was identified to be a *Pseudocercospora* sp.

Even though the phylogenetic analyses revealed that this species could be a novel one, we concluded that this is *Pseudocercospora cannabina*, considering the host specificity of this genus, the unavailability of sequence data for this species until our study, and morphological similarities present between our isolates and the previously described morphological characteristics of the type *Ps. cannabina.*

Even though *Ps. cannabina* is reported and identified as the pathogen associated with olive leaf spot disease in cannabis all around the world, the identification is performed only with the observation of field symptoms and morphological characteristics of the isolated pathogen. Although these steps are important for identification, they are not highly accurate. Most countries have introduced molecular-based diagnosis techniques for rapid diagnosis or epidemiological studies to grasp the origin of the pathogen. Our study aims to solve this issue by providing DNA sequence data of multiple gene regions, contributing to a more accurate identification and comprehensive understanding of *Ps. cannabina* as a pathogen of cannabis. This study reveals the phylogenetic position within the genus *Pseudocercospora*, indicating several species' barcodes, such as *tef* and *rpb2* gene regions. Also, we describe the symptoms in detail and the morphology based on the current taxonomical criteria. This information will be helpful for field diagnoses in the cropping fields and for quarantine purposes.

We conducted etiological studies, including a pathogenicity test, detailed morphological observations, and multi-locus phylogenetic analyses, to reveal the fundamental information needed for accurate identification and disease control. Illustrations and detailed descriptions are provided.

2. Materials and Methods

2.1. Sample Collection and Isolation

Samples of leaf spot in *Cannabis sativa* were collected from a plantation area located in Chiang Rai Province, northern Thailand, on 5th February, 2022. Twenty symptomatic leaves were randomly collected from this plantation. Leaf samples were kept in sterile zip-lock plastic bags and carried to the laboratory within 24 h of collection. Single spore isolation was carried out as described by To-Anun et al. (2011) [29]. The fungal specimens with desired structures (i.e., conidiomata, conidiophore, conidiogenous cells, and conidia) were mounted on lactic acid and photographs were taken using the Axiovision Zeiss Scope-A1 microscope fitted with a Canon EOS 6D digital camera. The morphological measurements were carried out using the Tarosoft (R) Image Frame Work program. The specimens were deposited in the Sustainable Development of Biological Resources Laboratory (SDBR) at Chiang Mai University, Chiang Mai, Thailand.

2.2. DNA Extraction and PCR Amplification and Sequencing

The genomic DNA from the fungal mycelia was extracted using the DNA Extraction Mini Kit (FAVORGEN, Ping Tung, Taiwan) following the manufacturer's protocol. The internal transcribed spacer (ITS1, 5.8S, ITS2), the partial actin (*act*), translation elongation factor 1-alpha (*tef1*), and RNA polymerase second largest subunit (*rpb2*) genes were amplified by the polymerase chain reaction (PCR) using ITS4/ITS5 primers [30], ACT-512F/ACT-783R primers [31], EF1-668/EF1-1251 primers [31,32], and RPB2-5F2/RPB2-7cR primers [33], respectively. PCR reactions were performed in a 25 µL reaction mixture containing 1.0 µL DNA template, 1.0 µL each forward and reverse primer, 12.5 µL 2X Quick Taq® HS DyeMix (TOYOBO, Japan), and 9.5 µL deionized water. The amplification program for all four genes was performed in separate PCR reactions and consisted of an initial denaturation step at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, an annealing step at 52 °C for 45 s (ITS), 55 °C for 1 min (*act*) and 56 °C for 1 min

(*rbp2*), and an extension step at 72 °C for 10 min on a GeneMax thermal cycler (Hangzhou Bioer Technology Co., Ltd. (BIOER), Zhejiang, China). PCR products were checked on 1% agarose gel electrophoresis. The purified PCR products were sent to the 1st Base Company (Kembangan, Malaysia). The obtained nucleotide sequences were deposited in GenBank (accession numbers available in Table 1).

Table 1. GenBank accession numbers of the *Pseudocercospora* species isolates used for the phylogenetic analysis. Culture collection numbers with "*" designate the type. The isolates obtained in this study are in bold.

Species	Culture Collection Number	ITS	*act*	*tef*	*rpb2*
Pseudocercospora abeliae	MUCC1674 *	LC599330	LC599407	LC599448	LC599587
Ps. aeschynomenicola	COAD 1972 *	KT290146	KT313501	KT290200	NA
Ps. aleuritis	MAFF237174 *	LC599331	LC599408	LC599449	LC599588
Ps. angolensis	CBS 149.53 *	JQ324975	JQ325011	JQ324988	NA
Ps. basiramifera	CBS 111072 *	GU269661	GU320368	DQ211677	NA
Ps. cannabina	**SDBR-CMU372 ***	**OR101673**	**OR344083**	**NA**	**OR344085**
Ps. cannabina	**SDBR-CMU373**	**OR101674**	**OR344084**	**NA**	**OR344086**
Ps. casuarinae	CBS 128218 *	HQ599603	LC599413	LC599454	NA
Ps. ceratoniae	CBS 147386 = CPC19998 *	LC599335	LC599414	LC599455	LC599592
Ps. cercidicola	MAFF237791 *	GU269671	GU320377	GU384388	KX462618
Ps. cercidis-chinensis	CBS 132109 = CPC14481 *	GU269670	GU320376	GU384387	LC599593
Ps. chamaecristae	CPC 25228 = COAD 1973 *	KT290147	KT313502	KT290201	NA
Ps. chiangmaiensis	CBS 123244 *	EU882113	KF903544	KF903177	NA
Ps. chionanthi-retusi	NCHUP L605 *	KX462585	KX462552	KX462671	KX462620
Ps. cordiana	CBS 114685 *	AF362054	GU320387	GU384398	NA
Ps. delonicicola	MUCC2869 *	LC599341	LC599421	LC599463	LC599601
Ps. diplusodonii	CPC 25179 = COAD 1476 *	KT290135	KT313490	KT290189	NA
Ps. eriobotryae	MUCC1007 *	KX462589	KX462557	KX462676	KX462628
Ps. eriobotryicola	NCHUP L1601 *	KX462590	KX462558	KX462677	KX462629
Ps. ershadii	CBS 136114 = CCTU1206 *	KM452867	KM452844	KM452889	MN786459
Ps. eumusae	CBS 114824 *	EU514238	LFZN0100 0053	LFZN0100 0037	NA
Ps. euphorbiacearum	COAD 1537 *	KT290145	KT313500	KT290199	NA
Ps. exilis	COAD 1501*	KT290139	KT313494	KT290193	NA
Ps. fijiensis	CBS 120258 = CIRAD 86 *	EU514248	NW006921533	NW006921532	NW006921535
Ps. fukuokaensis	MAFF237768 *	GU269714	GU320418	GU384430	KX462632
Ps. glochidionis	MAFF237000 *	LC599348	LC599428	LC599470	LC599608
Ps. haiweiensis	CBS 131584 *	GU269803	GU320506	GU384514	KX462634
Ps. imazekii	MUCC1668 *	KX462596	KX462564	KX462683	KX462638
Ps. kobayashiana	MAFF236999 *	LC511998	LC512004	LC515780	LC515791
Ps. liquidambaricola	MUCC1664 *	LC599352	LC599432	LC599474	LC599611
Ps. maetaengensis	MFLUCC 14-0011 *	GU188048	NA	NA	NA
Ps. mangifericola	BRIP 52776b *	GU188048	NA	NA	NA
Ps. marginalis	CBS 131582 *	GU269794	GU320495	GU384504	NA
Ps. musae	CBS 116634 *	GU269747	GU320449	GU384459	NA
Ps. nandinae	MAFF239633 *	KX462600	KX462568	KX462687	KX462645
Ps. neriicola	CBS 138010 *	KJ869165	KJ869231	KJ869240	KX462647
Ps. norchiensis	CBS 120738 *	EF394859	GU320455	GU384464	KX462648
Ps. pini-densiflorae	MUCC534 *	LC599354	LC599434	LC599478	LC599615
Ps. piperis	COAD 1111	JX875062	NA	JX896123	NA
Ps. plumeriifolii	COAD 1498 *	KT290138	KT313493	KT290192	NA
Ps. pothomorphes	COAD 1450 *	KT290131	KT313486	KT290185	NA
Ps. proiphydis	BRIP58545 *	KM055430	NA	KM055437	NA
Ps. pruni-grayanae	MUCC1715 *	LC599356	NA	LC599481	LC599618
Ps. pseudomusae	CBS 147147 = CPC37270*	MW063423	MW070772	MW071091	MW070919
Ps. pseudomyrticola	CBS 145554 *	MK876405	MK876461	MK876499	MK876490
Ps. punicae	MAFF236998	KX462606	KX462573	KX462692	KX462655

Table 1. *Cont.*

Species	Culture Collection Number	ITS	*act*	*tef*	*rpb2*
Ps. pyracanthigena	CBS 131589 *	GU269766	GU320469	GU384478	NA
Ps. ravenalicola	CBS 122468 *	GU269810	GU320513	GU384521	NA
Ps. rhamnellae	CBS 131590 *	GU269795	GU320496	GU384505	NA
Ps. rhapisicola	MAFF305042 *	LC599357	LC599436	LC599483	LC599620
Ps. rigidae	COAD 1472 *	KT290134	KT313489	KT290188	NA
Ps. sawadae	MAFF239714	LC599359	LC599438	LC599485	LC599622
Ps. schizolobii	CBS 120029 *	KF251322	KF253628	KF253269	NA
Ps. sennae-multijugae	COAD 1519 *	KT290142	KT313497	KT290196	NA
Ps. serpocaulonicola	COAD 1866 *	KT037525	KT037607	KT037485	NA
Ps. solani-pseudocapsicicola	COAD 1974 *	KT290148	KT313503	KT290202	NA
Ps. struthanthi	COAD 1512 *	KT290141	KT313496	KT290195	NA
Ps. tabernaemontanae	CPC 19198 *	LC599363	LC599442	NA	LC599625
Ps. tinea	NCHUP L1603 *	KX462608	KX462577	KX462696	KX462660
Ps. trinidadensis	CPC 26082 = COAD1756 *	KT290157	NA	KT290210	NA
Ps. tumulosa	CBS 121158 *	DQ530217	NA	NA	NA
Ps. violamaculans	MUCC1660 *	KX462610	KX462579	KX462698	KX462662
Ps. vitis	CPC 11595	GU269829	GU320533	GU384541	KX462663
Ps. vitis	CBS 128211 *	LC599366	LC599445	LC599492	LC599627
Trochophora simplex	CBS 124744	GU269872	GU320568	GU384580	KX462666

2.3. Phylogenetic Analyses

The DNA sequences generated were assembled and aligned with 63 sequences of *Pseudocercospora* species retrieved from previous studies by Nakashima et al. (2016) [23], Videira et al. (2017) [28], and Chen et al. (2022) [22] using MEGA X software package [34] (Table 1). This matrix was aligned by using MAFFT (Multiple Alignment using Fast Fourier Transform) online version [35] and edited manually. Maximum-likelihood (ML) analysis was used in this study to estimate the phylogenetic relationship of the samples. ModelTest-NG [36] was used to estimate the best substitution model for each gene for ML analysis, and ML analysis was performed using RAxML-NG [37]. Branch strengths were tested by a bootstrap analysis of 100 replications [38]. ML analysis was performed with the evolutional models set as TN93ef + I + G4 for the ITS and *act* regions, and TN93+G4 for *tef1* and *rpb2* regions. *Trochophora simplex* (CBS 124744) was selected as an outgroup in all analyses and trees were viewed by using FigTree v. 1.4.2 (Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK).

2.4. Pathogenicity Test

To confirm Koch's postulates, pathogenicity tests were conducted using the newly isolated strain from *Cannabis sativa*, following the methodology outlined by Sautua et al. (2020) [39]. For each isolate, spore suspensions (10^6 conidia/mL) were sprayed onto *C. sativa* leaves. Spore suspensions were prepared by combining sterile water with 1 mL of Tween 20 to aid in the dispersion of conidia onto 35-day-old colonies. As a control, leaves were sprayed with sterile distilled water and Tween 20. Following inoculation, the plants were placed in wet plastic boxes under sterile conditions and closely monitored on a daily basis for symptom detection. Once symptoms appeared on the inoculated leaves, the causal organism was reisolated. The reisolated strains were then compared to the original isolates to confirm the fulfillment of Koch's postulates.

3. Results

Field observations of the cannabis plants showed olive leaf spot symptoms with circular to elliptical spots with a grey center and dark brown to black margins (Figure 1A–E). Severely affected plants showed signs of defoliation. The morphological characteristics and

measurements were similar to the type specimen of *Ps. cannabina* described in Wakefield, 1917 [40].

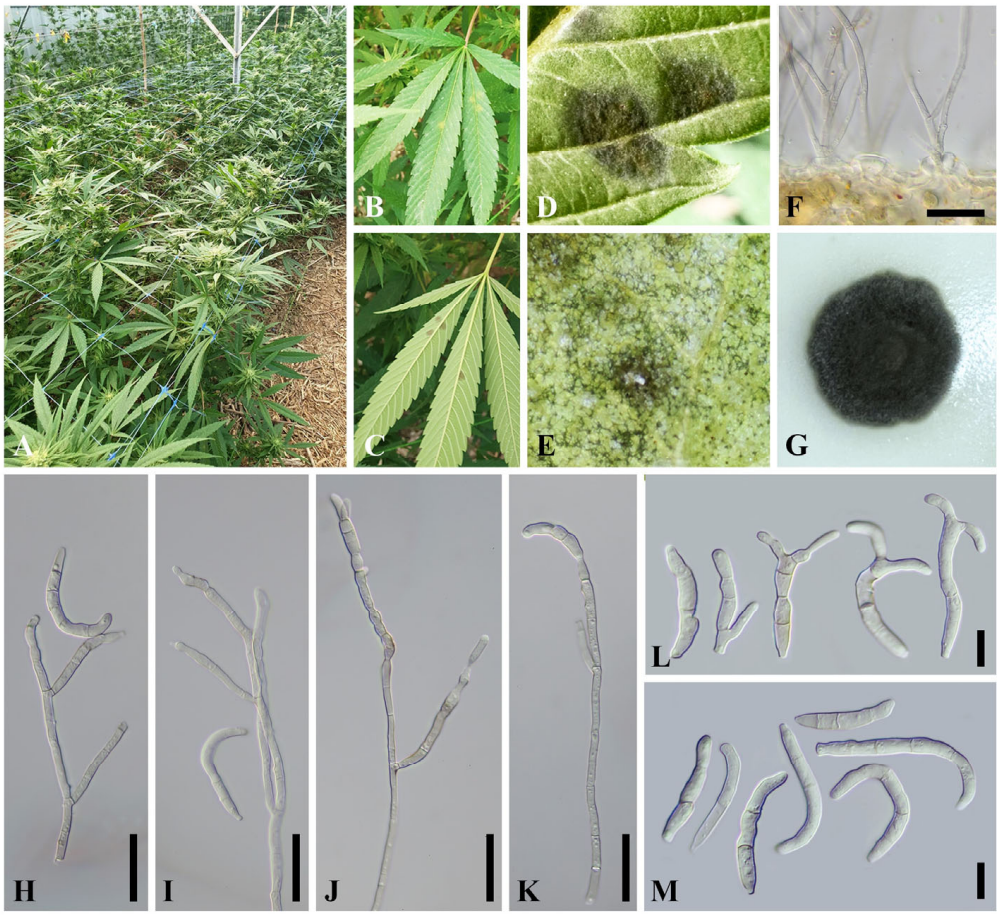

Figure 1. Natural symptoms of olive leaf spot on *Cannabis sativa* L. (**A**–**C**). Conidial masses on the lower leaf surface (**D**,**E**); cross section (**F**); colony on PDA after 15 days at 25–30 °C (**G**); conidiophores and conidiogenous cells (**H**–**K**); conidia (**L**,**M**); scale bars: F, H–K = 50 µm; L–M = 20 µm.

3.1. Taxonomy

Taxonomic Description

Pseudocercospora cannabina (Wakef.) Deighton, Mycological Papers 140:141 (1976) [MB#321527] (Figure 1).

Description in vivo—Leaf spots amphigenous, on the upper surface, at first inconspicuous to slightly discolored, vein limited, later pale yellowish, irregular to subcircular, 2–6 mm diam., without distinct margin, finally covering the whole leaf, on the lower surface, indistinct, pale yellowish, covered with sooty conidial masses. *Caespituli* hypophyllous, effuse, sooty, vein-limited, greyish to pale brown patches, velutinous. *Mycelium* internal, hyphae septate, branched, brown to dark brown. *Stromata* rudimentary or poorly developed, forming substomatal hyphal aggregations. *Conidiophores* emerged from stromata or branched from superficial hyphae, 1–5(–8) in a loose fascicle, olivaceous brown or brown, darker than conidia or concolored, 9–15-septate, 0–3 times branched near the middle, straight to slightly sinuous, not geniculate, short or well developed, 75–115 × 4–6 µm; *conidiogenous cells* integrated, apical, proliferating percurrently or sympodially, conspicuously constricted at the middle part caused by percurrent proliferation, conically truncated at the apex, conidial loci inconspicuous. *Conidia* holoblastic, solitary, filiform to obclavato-cylindric, straight to curved, smooth, pale olivaceous to very pale brown, guttulate, 2–5-septate, sometimes constricted at the septa, uniform or irregular in width, broadly rounded at the

apex, obconically truncated to truncated at the base, 35–60 × 3.5–5 µm; hilum unthickened, and not darkened.

Culture characteristics: Colonies on PDA reaching 2 cm in diameter after 25 days at room temperature; moderate aerial mycelium, circular, growth effuse with elevated colony center, grey to dark in outer region, entire margin, aerial mycelium dense.

Specimens examined: Thailand, Chiang Rai, Muang, on leaves of *Cannabis sativa* L., 5 February 2022, N. Tamakaew, CRC188, living culture SDBR-CMU372; *ibid*., Chiang Rai, Muang, on leaves of *Cannabis sativa* L., 5 February 2022, N. Tamakaew, CRC189, living culture SDBR-CMU373.

3.2. Pathogenicity Test

Within the 24 h incubation period, the penetration of germ tubes or hyphae of *Ps. cannabina* into leaf tissue could be observed. Under the compound microscope, hyphae invaded through the stomata and were growing inside of the leaf tissue (Figure 2). In contrast, no symptoms were observed on the control leaves sprayed with sterile distilled water. The leaves developed leaf spot symptoms 5 days post-inoculation. The reisolated pathogen from the inoculated leaves showed a similar morphology to the isolate used for the pathogenicity test, and the DNA sequence data for the ITS gene region obtained from the reisolated samples were also identical to the isolate (SDBR-CMU373) used for the pathogenicity test.

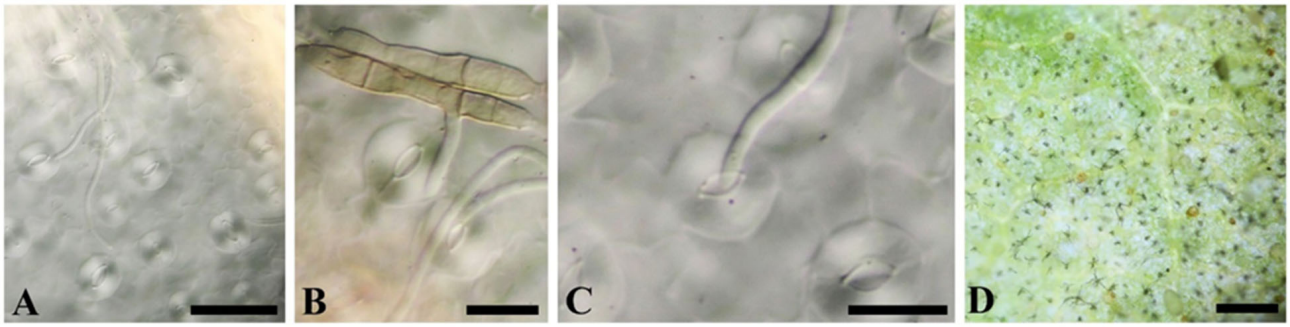

Figure 2. Pathogenicity test. (**A**–**C**) Fungal hyphae penetrated through *Cannabis sativa* stomata on the lower leaf surface after 24 h; (**D**) the reproduction of the symptoms with emerged conidiophores and conidia on the lower leaf surface 5 DPI. Scale bars: (**A**,**B**) = 20 µL; (**C**) = 50 µL; (**D**) = 1 cm.

3.3. Phylogeny

The two isolates of *Pseudocercospora* species on *C. sativa* had identical sequences on the loci analyzed in this study. Therefore, the sequences obtained from one isolate (SDBR-CMU372 = RC238) were included in these analyses. The sequencing results of all regions were combined and aligned in a data matrix of 65 OTU, consisting of *Pseudocercospora* and *Trochophora* species (Table 1.). The final alignment contained a total of 1826 characters consisting of four regional sequences, ITS: 495 sites, *act*: 233 sites, *tef1*: 422 sites, and *rpb2*: 676 sites, including alignment gaps. The ML tree is shown in Figure 3. The sequences of the isolate analyzed in this study formed a clade with hitherto known species: *Ps. diplusodonii* on *Diplusodon* sp., *Ps. ershadii* on *Diospyros lotus*, *Ps. kobayashiana* on *Diospyros kaki*, and *Ps. liquidambaricola* on *Liquidambar formosana*. The clade was weakly supported by bootstrap analyses, but it was distinguishable from closely related taxa and recognized as an independent species (Figure 3).

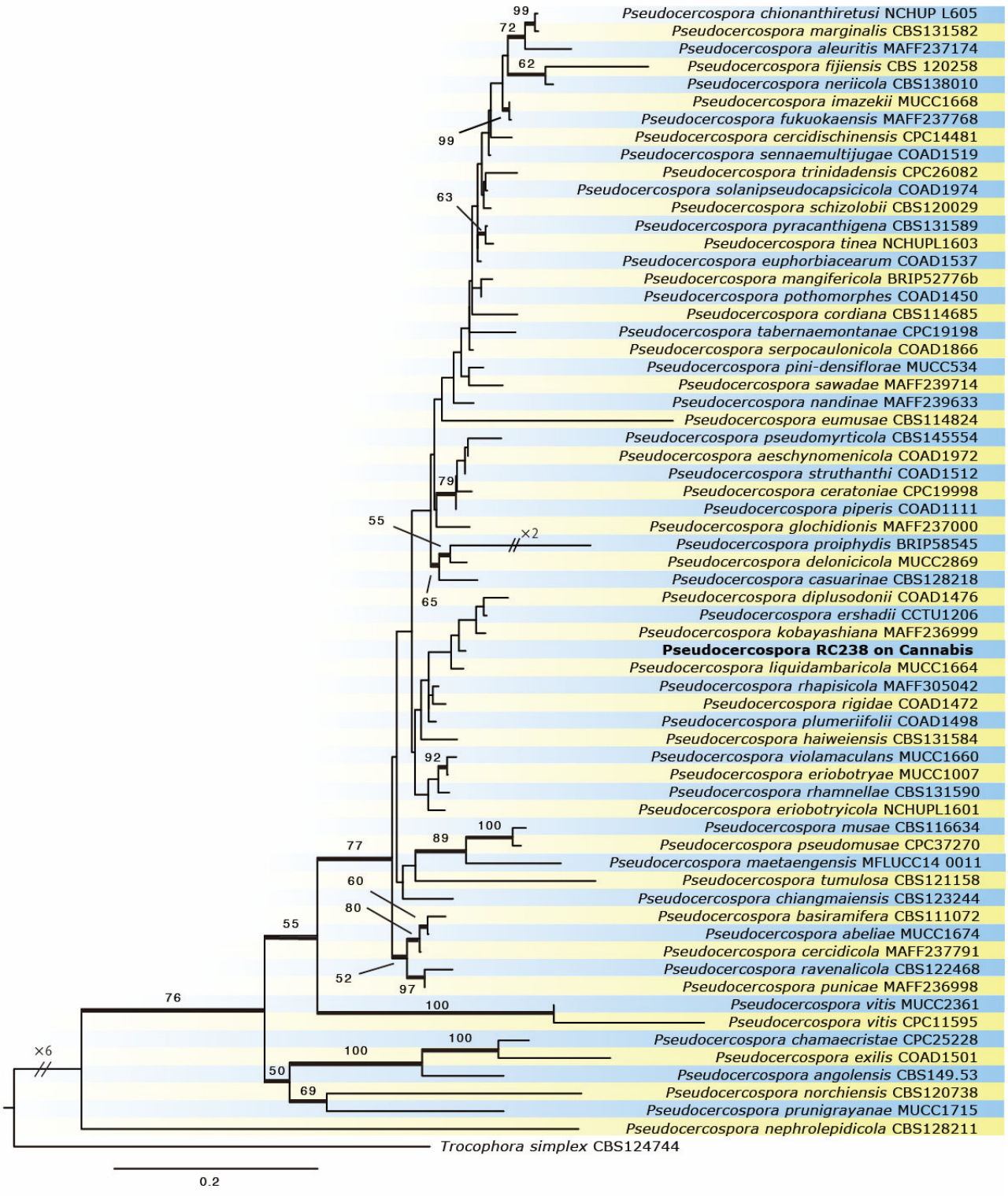

Figure 3. Phylogram of *Pseudocercospora* resulting from a maximum likelihood analysis, based on a combined matrix of ITS, *act*, *rpb2,* and *tef1*. Numbers above the branches indicate ML bootstraps (ML BS $\geq$ 50%).

4. Discussion

The causal organism of olive leaf spot, *Pseudocercospora cannabina,* is an important pathogen in cannabis or hemp. It has been previously reported in China, India, Korea, Poland, Australia, and the USA [41]. Even though it is reported as a high-priority pathogen

causing olive leaf spot disease in *Cannabis sativa*, in almost all studies mentioned, the identification was only carried out using morphological characteristics, which is not highly accurate. For the plant genus *Cannabis*, an alternative species of Cercosporoid fungi, *Cercospora cannabis* Hara & Fukui, is known [42,43]. According to Chupp (1954) [42], *C. cannabina* (=*Ps. cannabina*) is distinguishable by having colored conidia and branched conidiophores. Morphologically, the causal organism of leaf spots on *C. sativa* in Thailand is identical to the original description of *C. cannabina* by Wakefield (1917) [40]. From these results, our isolates were identified as *Ps. cannabina*. Additionally, sequence data originating from the type material of *Ps. cannabina* are not available and they were collected from Uganda, quite far from Thailand, where the disease was observed in our study. As mentioned above, *Ps. cannabina* is widely distributed around the world. Therefore, further studies are required for a more detailed understanding of the distribution and intraspecies diversity of this species.

Novel species introduced in this genus are nowadays verified by data involving host associations, morphological characters, and phylogenetic relationships using multi-locus data using DNA sequences [20]. Even though these species are important as phytopathogens, many species in *Pseudocercospora* lack sequence data from the type material. This causes difficulties in the identification and rapid diagnosis of plant disease since accurately distinguishing through just morphological observations can be challenging and sequence data can play a very important role in the accurate identification of *Pseudocercospora* species. Hence, adding sequence data to resolve this data gap is very important. Chen et al. 2022 [22] designated ITS as the barcode for *Pseudocercospora*, and species delineation can be undertaken further with additional gene regions *act*, *rpb2*, and *tef1*.

In the pathogenicity test, similar symptoms observed in the field were reproduced and the causal organism re-isolated. From these results, the pathogen causing leaf spots on *C. sativa* was confirmed to be *Ps. cannabina* from morphological comparisons and sequence data fulfilling Koch's postulates.

In Thailand, many species from *Pseudocercospora* have been reported but none from Cannabis [44]. Our study is the first to report olive leaf spot disease in Cannabis from Thailand. Even though leaf spot diseases are commonly reported in Cannabis around the world, in-depth studies have yet to be conducted and published. Moreover, this study is the first to use both morphological and molecular data to identify and confirm the pathogenicity of *Ps. cannabina* causing leaf spot disease on *Cannabis sativa*. The data obtained in this study will contribute to the rapid diagnosis and control of the disease.

Author Contributions: Conceptualization, R.C. and C.N.; methodology, D.L.H., S.H., P.W. and C.K.; validation, D.L.H., C.N., S.H., N.T. and N.N.; formal analysis, D.L.H. and C.N.; investigation, D.L.H., C.N., S.H., P.W., N.T., N.N., C.K., T.P. and R.C.; writing—original draft preparation, D.L.H.; writing—review and editing, C.N., S.H., P.W. and R.C.; supervision, T.P. and R.C.; project administration, R.C.; funding acquisition, C.N. and R.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. The APC was funded by Mie University, Mie, Japan.

Data Availability Statement: All sequence data are available in NCBI GenBank following the accession numbers in the manuscript.

Acknowledgments: This study was partially supported by the Post-Doctoral Fellowship 2022 for Reinventing Chiang Mai University.

Conflicts of Interest: Author Chaorai Kanchanomai was employed by the company Chaofah Vineyard, Chiang Rai 57210, Thailand. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Salentijn, E.M.J.; Zhang, Q.; Amaducci, S.; Yang, M.; Trindade, L.M. New developments in fiber hemp (*Cannabis sativa* L.) breeding. *Ind. Crops Prod.* **2015**, *68*, 32–41. [CrossRef]
2. Struik, P.C.; Amaducci, S.; Bullard, M.J.; Stutterheim, N.C. Agronomy of fibre hemp (*Cannabis sativa* L.) in Europe. *Ind. Crops Prod.* **2000**, *11*, 107–118. [CrossRef]
3. Tsaliki, E.; Kalivas, A.; Jankauskiene, Z.; Irakli, M.; Cook, C.; Grigoriadis, I.; Panoras, I.; Vasilakoglou, I.; Dhima, K. Fibre and Seed Productivity of Industrial Hemp (*Cannabis sativa* L.) Varieties under Mediterranean Conditions. *Agronomy* **2021**, *11*, 171. [CrossRef]
4. Rupasinghe, V.H.P.; Davis, A.; Kumar, S.K.; Murray, B.; Zheljazkov, V.Z. Industrial hemp (*Cannabis sativa* subsp. sativa) as an emerging source for value-added functional food ingredients and nutraceuticals. *Molecules* **2020**, *25*, 4078. [CrossRef]
5. Gwinn, K.D.; Hansen, Z.; Kelly, H.; Ownley, B.H. Diseases of *Cannabis sativa* Caused by Diverse *Fusarium* Species. *Front Agronomy* **2022**, *3*, 796062. [CrossRef]
6. Honig, L. National Hemp Report Agricultural Statistics Board Briefing, USDA. 2022. Available online: www.nass.usda.gov (accessed on 25 May 2023).
7. Martin, M.A. Ethnobotanical Aspects of Cannabis in Southeast Asia. In *Cannabis and Culture*; Rubin, V., Ed.; Mouton Publishers: Berlin, Germany, 1975; pp. 63–76, ISBN 9027976694.
8. Cerino, P.; Buonerba, C.; Cannazza, G.; D'Auria, J.; Ottoni, E.; Fulgione, A.; Di Stasio, A.; Pierri, B.; Gallo, A. A Review of Hemp as Food and Nutritional Supplement. *Cannabis Cannabinoid Res.* **2021**, *6*, 19–27. [CrossRef] [PubMed]
9. Dhakal, H.N.; Zhang, Z. The use of hemp fibers as reinforcements in composites. In *Biofiber Reinforcements in Composite Materials*; Woodhead Publishing: Sawston, UK, 2015; pp. 86–103, ISBN 9781782421221. [CrossRef]
10. Punja, Z.K. Emerging diseases of *Cannabis sativa* and sustainable management. *Pest Manag. Sci.* **2021**, *9*, 3857–3870. [CrossRef]
11. Finley, S.J.; Javan, C.T.; Green, R.L. Bridging Disciplines: Applications of Forensic Science and Industrial Hemp. *Front. Microbiol.* **2022**, *13*, 760374. [CrossRef] [PubMed]
12. Punja, Z.K.; Collyer, D.; Scott, C.; Lung, S.; Holmes, J.; Sutton, D. Pathogens and Molds Affecting Production and Quality of *Cannabis sativa* L. *Front. Plant Sci.* **2019**, *10*, 1120. [CrossRef] [PubMed]
13. Jerushalmi, S.; Maymon, M.; Dombrovsky, A. Fungal Pathogens Affecting the Production and Quality of Medical Cannabis in Israel. *Plants* **2020**, *9*, 882. [CrossRef]
14. Manawasinghe, I.S.; Phillips, A.J.L.; Xu, J.; Balasuriya, A.; Hyde, K.D.; Stępień, Ł.; Harischandra, D.L.; Karunarathna, A.; Yan, J.; Weerasinghe, J.; et al. Defining a species in fungal plant pathology: Beyond the species level. *Fungal Divers.* **2021**, *109*, 267–282. [CrossRef]
15. McPartland, J.M.; Hillig, K.W. White Leaf Spot. *J. Ind. Hemp* **2006**, *11*, 43–50. [CrossRef]
16. Bakro, F.; Wielgusz, K.; Bunalski, M.; Jedryczka, M. An overview of pathogen and insect threats to fibre and oilseed hemp (*Cannabis sativa* L.) and methods for their biocontrol. In Proceedings of the Meeting 17th IOBC Working Group Meeting on Integrated Control in Oilseeds (ICOC), International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palearctic Regional Section (IOBC-WPRS), Zagreb, Croatia, 18–20 September 2018; Volume 136, pp. 9–20.
17. Santo, D. Australian Industrial Hemp Industry Pesticide Review and Evaluation Program. AgriFutures Australia. Publication No. 22-060. 2022. Available online: https://agrifutures.com.au/wp-content/uploads/2022/06/22-060.pdf (accessed on 25 May 2023).
18. Rodriguez, G.; Kibler, A.; Campbell, P.; Punja, Z.K. Fungal diseases of *Cannabis sativa* in British Columbia, Canada. In Proceedings of the American Phytopathological Society Annual Meeting, Pasadena, CA, USA, 1–5 August 2015. Poster 529-P.
19. Ward-Gauthier, N.; Beale, J.; Amsden, B.; Dixon, E. Greenhouse hemp in Kentucky exhibits many common diseases. In Proceedings of the American Phytopathological Society Annual Meeting, Pasadena, CA, USA, 1–5 August 2015. Poster 502-P.
20. Crous, P.W.; Braun, U.; Hunter, G.C.; Wingfield, M.J.; Verkley, G.J.M.; Shin, H.-D.; Nakashima, C.; Groenewald, J.Z. Phylogenetic lineages in *Pseudocercospora*. *Stud. Mycol.* **2013**, *75*, 37–114. [CrossRef] [PubMed]
21. Wanasinghe, D.N.; Phukhamsakda, C.; Hyde, K.D.; Jeewon, R.; Lee, H.B.; Jones, E.B.G.; Tibpromma, S.; Tennakoon, D.S.; Dissanayake, A.J.; Jayasiri, S.C.; et al. Fungal diversity notes 709–839: Taxonomic and phylogenetic contributions to fungal taxa with an emphasis on fungi on Rosaceae. *Fungal Divers.* **2018**, *89*, 1–236. [CrossRef]
22. Chen, Q.; Bakhshi, M.; Balci, Y.; Broders, K.; Cheewangkoon, R.; Chen, S.; Fan, X.; Gramaje, D.; Halleen, F.; Jung, M.H.; et al. Genera of phytopathogenic fungi: GOPHY 4. *Stud. Mycol.* **2022**, *101*, 417–564. [CrossRef] [PubMed]
23. Nakashima, C.; Motohashi, K.; Chen, C.Y.; Groenewald, J.Z.; Crous, P.W. Species diversity of *Pseudocercospora* from Far East Asia. *Mycol. Prog.* **2016**, *15*, 1093–1117. [CrossRef]
24. Aslake, M.; Sintayehu, A.; Alem, T.; Abera, M. Distribution and Status of Citrus Leaf and Fruit Spot (*Pseudocercospora angolensis*) Disease in North Western Ethiopia. *Open J. Ecol.* **2022**, *12*, 733–741. [CrossRef]
25. Silva, K.J.D.E.; De Souza, E.A.; Sartorato, A.; De Souza, F. Pathogenic Variability of Isolates of Pseudocercospora griseola, the Cause of Common Bean Angular Leaf Spot, and its Implications for Resistance Breeding. *J. Phytopathol.* **2008**, *156*, 602–606. [CrossRef]
26. Evans, H.C. The genus Mycosphaerella and its anamorphs *Cercoseptoria*, *Dothistroma* and *Lecanosticta* on pines. *Mycol. Pap.* **1984**, *153*, 1–102.
27. Braun, U.; Nakashima, C.; Crous, P.W. Cercosporoid fungi (*Mycosphaerellaceae*) 1. Species on other fungi, Pteridophyta and Gymnospermae. *IMA Fungus* **2013**, *4*, 265–345. [CrossRef]

28. Videira, S.I.R.; Groenewald, J.Z.; Nakashima, C.; Braun, U.; Barreto, R.W.; de Wit, P.J.G.M.; Crous, P.W. Mycosphaerellaceae—Chaos or clarity? *Stud. Mycol.* **2017**, *87*, 257–421. [CrossRef] [PubMed]
29. To-Anun, C.; Hidayat, I.; Meeboon, J. Genus *Cercospora* in Thailand: Taxonomy and Phylogeny (with a dichotomous key to species). *Plant Pathol. Quar.* **2011**, *1*, 11–87. [CrossRef]
30. White, T.J.; Burns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.
31. Carbone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **1999**, *91*, 553–556. [CrossRef]
32. O'Donnell, K.; Kistler, H.C.; Cigelnik, E.; Ploetz, R.C. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2044–2049. [CrossRef] [PubMed]
33. Liu, Y.J.; Whelen, S.; Hall, B.D. Phylogenetic Relationships Among Ascomycetes: Evidence from an RNA Polymerse II Subunit. *Mol. Biol. Evol.* **1998**, *16*, 1799–1808. [CrossRef] [PubMed]
34. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
35. Katoh, K.; Rozewicki, J.; Yamada, K.D. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* **2019**, *20*, 1160–1166. [CrossRef]
36. Darriba, D.; Posada, D.; Kozlov, A.M.; Stamatakis, A.; Morel, B.; Flouri, T. ModelTest-NG: A New and Scalable Tool for the Selection of DNA and Protein Evolutionary Models. *Mol. Biol. Evol.* **2020**, *37*, 291–294. [CrossRef]
37. Kozlov, A.M.; Darriba, D.; Stamatakis, A. RAxML-NG: A fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* **2019**, *35*, 4453–4455. [CrossRef]
38. Felsenstein, J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef] [PubMed]
39. Sautua, F.J.; Doyle, V.P.; Price, P.P.; Porfiri, A.; Fernandez, P.; Scandiani, M.M.; Carmona, M.A. Fungicide resistance in *Cercospora* species causing *Cercospora* leaf blight and purple seed stain of soybean in Argentina. *Plant Pathol.* **2020**, *69*, 1678–1694. [CrossRef]
40. Wakefield, E.M. Fungi Exotici: XXIII. *Bull. Misc. Inf. R. Bot. Gard. Kew.* **1917**, *1917*, 308–314. [CrossRef]
41. Farr, D.F.; Rossman, A.Y.; Castlebury, L.A. United States National Fungus Collections Fungus-Host Dataset. Ag Data Commons. 2021. Available online: https://data.nal.usda.gov/dataset/united-states-national-fungus-collections-fungus-host-dataset (accessed on 13 November 2022).
42. Chupp, C. A monograph of the Fungus genus *Cercospora*. by the Author Plant Pathology Department, Cornell University, Ithaca, N. Y. *AIBS Bull.* **1954**, *4*, 11. [CrossRef]
43. Crous, P.W.; Braun, U. *Mycosphaerella and its Anamorphs 1: Names Published in Cercospora and Passalora*; CBS Biodiversity Series 1; CBS Fungal Biodiversity Centre: Utrecht, The Netherlands, 2003; ISBN 90-70351-49-8.
44. Chen, Y.J.; Jayawardena, R.S.; Bhunjun, C.S.; Harishchandra, D.L.; Hyde, K.D. *Pseudocercospora dypsidis* sp. Nov. (Mycosphaerellaceae) on *Dypsis lutescens* leaves in Thailand. *Phytotaxa* **2020**, *474*, 218–234. [CrossRef]