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Stagonosporopsis pogostemonis: A Novel Ascomycete Fungus Causing Leaf Spot and Stem Blight on *Pogostemon cablin* (Lamiaceae) in South China

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Abstract: *Pogostemon cablin* is one of the well-known Southern Chinese medicinal plants with detoxification, anti-bacterial, anti-inflammatory, and other pharmacological functions. Identification and characterization of phytopathogens on *P. cablin* are of great significance for the prevention and control of diseases. From spring to summer of 2019 and 2020, a leaf spot disease on *Pogostemon cablin* was observed in Guangdong Province, South China. The pathogen was isolated and identified based on both morphological and DNA molecular approaches. The molecular identification was conducted using multi-gene sequence analysis of large subunit (LSU), the nuclear ribosomal internal transcribed spacer (ITS), beta-tubulin (*β-tubulin*), and RNA polymerase II (*rpb2*) genes. The causal organism was identified as *Stagonosporopsis pogostemonis*, a novel fungal species. Pathogenicity of *Stagonosporopsis pogostemonis* on *P. cablin* was fulfilled via confining the Koch's postulates, causing leaf spots and stem blight disease. This is the first report of leaf spot diseases on *P. cablin* caused by *Stagonosporopsis* species worldwide.

Keywords: Didymellaceae; phoma-like; pathogenicity; phylogeny

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1. Introduction

Pogostemon cablin is a perennial aromatic herb, generally known as "Patchouli" or "Guanghuoxiang" [1]. This is wildly used as a herbal and a raw material of aromatics which have high economic demand for its essential oil [2,3]. *Pogostemon cablin* adapts to hot and humid climatic conditions and is mostly cultivated in the Philippines and some other tropical regions, including China. This plant is wildly grown in Southern China, including Guangdong, Guangxi, Hainan, and Fujian regions, as well as Taiwan [4]. As a well-known medicinal plant in traditional Chinese medicine, *Pogostemon cablin* is recognized for its detoxification, and other pharmacological features [5]. It contains a variety of natural antibacterial compounds, and has antibacterial and anti-inflammatory effects [6]. However, *P. cablin* is often affected by various diseases during its growth and development, that affect medicinal value [7]. Bacterial wilt caused by *Ralstonia solanacearum* [8], root-knot disease caused by *Meloidogyne incognita* [9,10], some viral diseases [11], and the fungal disease by *Corynespora cassiicola* causing leaf spot [12] are reported.

The species of *Stagonosporopsis* (Didymellaceae, Pleosporales) [13,14] can cause devastating diseases on a wide range of economically important plants, those in farmlands, forests, grasslands, and other natural ecosystems [13]. Moreover, they can result in severe

yield loss of agricultural crops [15]. *Stagonosporopsis* species have been reported as severe pathogens of some crops and ornamentals in several countries, including Australia [16], China [17], France [18], India [19], Italy [20], Turkey [21], and the United States [22,23].

During 2019–2020, a new leaf disease was observed in the *Pogostemon cablin* orchards in Zhanjiang city of Guangdong Province, China. Samples were collected and the causal pathogen was isolated. The objectives of this study were to identify the fungal taxa causing leaf spots and stem blight disease of *P. cablin* in China by a combination of morphological characterization and phylogenetic analyses and confirm the pathogenicity by fulfilling Koch's postulates.

2. Results

2.1. Field Symptoms

The disease incidence was 15% under high temperature, above 25–28 °C, and high humidity. Disease symptoms initially start with foliar marginal brown discoloration, then spreading towards the inside leaf lamina. Moreover, the disease spreads to cover completely the leaf, turning it dark brown and dry (Figure 1).

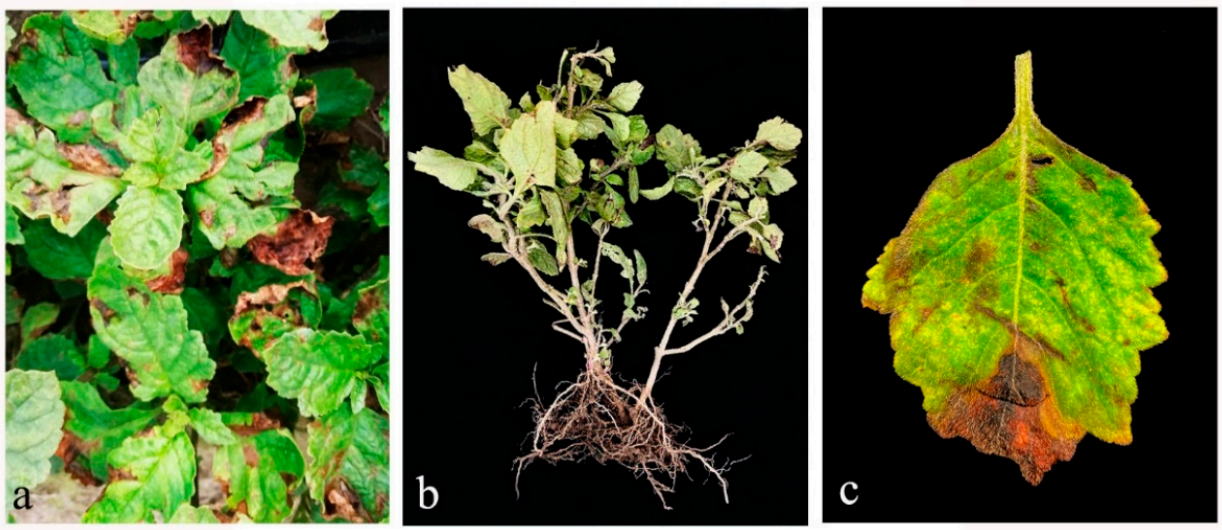

Figure 1. Field symptoms of leaf spot and stem blight caused by *Stagonosporopsis pogostemonis* (**a**) infected plants in the field with leaf spot starting from the tips of leaves. (**b**) Infected plant with wilting appearance. (**c**) Infected leaf with characteristic lesion, which starts with foliar marginal brown discoloration, then spreading towards the inside leaf lamina.

2.2. Morphological and Molecular Characterization

In total, 10 samples were collected from the field. The causal organism was isolated by tissue isolation method and pure cultures were obtained. Almost all the cultures obtained on PDA were morphologically similar, therefore two isolates were obtained for further analyses. For the molecular characterization, genomic DNA was extracted and four gene regions; LSU, ITS, *rpb2*, and *β-tubulin* were sequenced (Table 1). Phylogenetic analyses were done using maximum likelihood (ML), maximum parsimony (MP), and Bayesian analyses. Combined sequence data set of LSU, ITS, *rpb2*, and *β-tubulin* comprised of these two *Stagonosporopsis* isolates from this study and 76 reference sequences. The tree was rooted with *Allophoma piperis*. The tree topology of the ML analyses was similar to the MP and Bayesian analysis. Therefore, the best scoring RAxML tree with a final likelihood value of −10,074.586469 is presented (Figure 2). The matrix had one partition and 493 distinct alignment patterns, with 10.95% of undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.243855, C = 0.237878, G = 0.274045, and T = 0.244222; substitution rates AC = 2.129010, AG = 4.916610, AT = 1.711303, CG = 0.847171, CT = 13.823203, and GT = 1.00; gamma distribution shape parameter α = 0.096067. The dataset consisted of 2680 characters with 2256 constant characters and 364 (13.6%) parsimony-informative and

60 parsimony-uninformative characters. The maximum number of trees generated was 5000, and the most parsimonious trees had a tree length of 1148 (CI = 0.496, RI = 0.819, RC = 0.406, and HI = 0.504). Two isolates obtained in this study were clustered together in an individual clade with 100% maximum likelihood (ML), 100% maximum parsimony bootstrap values (MP), and 1.0 Bayesian posterior probabilities values (BYPP) (Figure 2). Therefore, both morphology and pairwise nucleotide differences among strains isolated in this study the type species *S. hortensis* were compared. Based on molecular and morphological evidences, isolates obtained in this study (ZHKUCC 21-0001 and ZHKUCC 21-0002) were identified as *Stagonosporopsis pogostemonis* a novel species. The species description is given below:

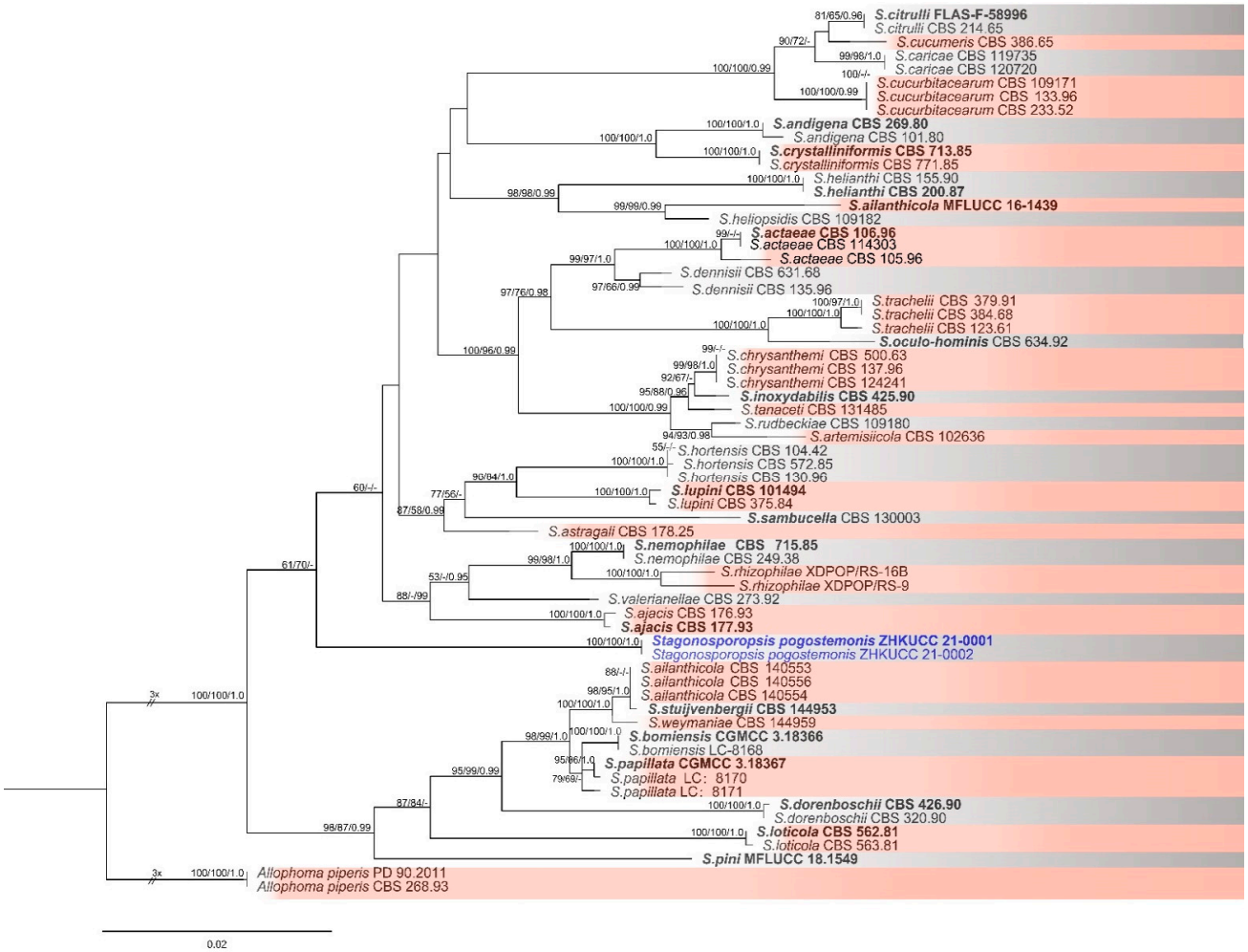

Figure 2. The best scoring RAxML tree obtained using the combined dataset of LSU, ITS, *rpb2*, and *β-tubulin* sequences. *Allophoma piperis* (CBS 268.93 and PD 90/2011) was used to root the tree. Bootstrap support values equal to or greater than 50% in ML and MP and BYPP equal or greater than 0.95 are shown as ML/MP/BYPP above the respective node. The isolates belonging to the current study are given in blue. Ex-type strains are bold. Expected number of nucleotide substitutions per site is represented by the scale bar.

Stagonosporopsis pogostemonis M. Luo, Y.H. Huang, & Manawas., sp. nov.;

Index Fungorum number: IF558590, Faces of fungi: FoF 09997, MycoBank accession number: MB840964 (Figure 3);

Etymology–In reference to the host genus name *Pogostemon*;

Holotype–ZHKUCC 21-0001;

Pathogenic on *Pogostemon cablin* leaves. Sexual morph: Not observed. Asexual morph: *Conidiomata* solitary, scattered, mostly covered with dense vegetative hyphae, subglobose or lageniform, dark brown to black, thick-walled, pycnidial. *Pycnidia* 34–161 µm × 32–98 µm

($\bar{x}$ = 58 ± 27 µm × 45 ± 16 µm, n = 50), subglobose, arranged irregularly in a disc, with conidium and a circular or longitudinal ostiole. *Pycnidial wall* pseudoparenchymatous, composed of several layers of angularis cells. *Conidiogenous cells* not observed. *Conidia* 3–5 µm × 1–3 µm ($\bar{x}$ = 4 ± 0.4 µm × 2 ± 0.3 µm, n = 50), oblong, cylindrical to ellipsoidal, with rounded both ends, smooth-walled, aseptate, with two polar guttules;

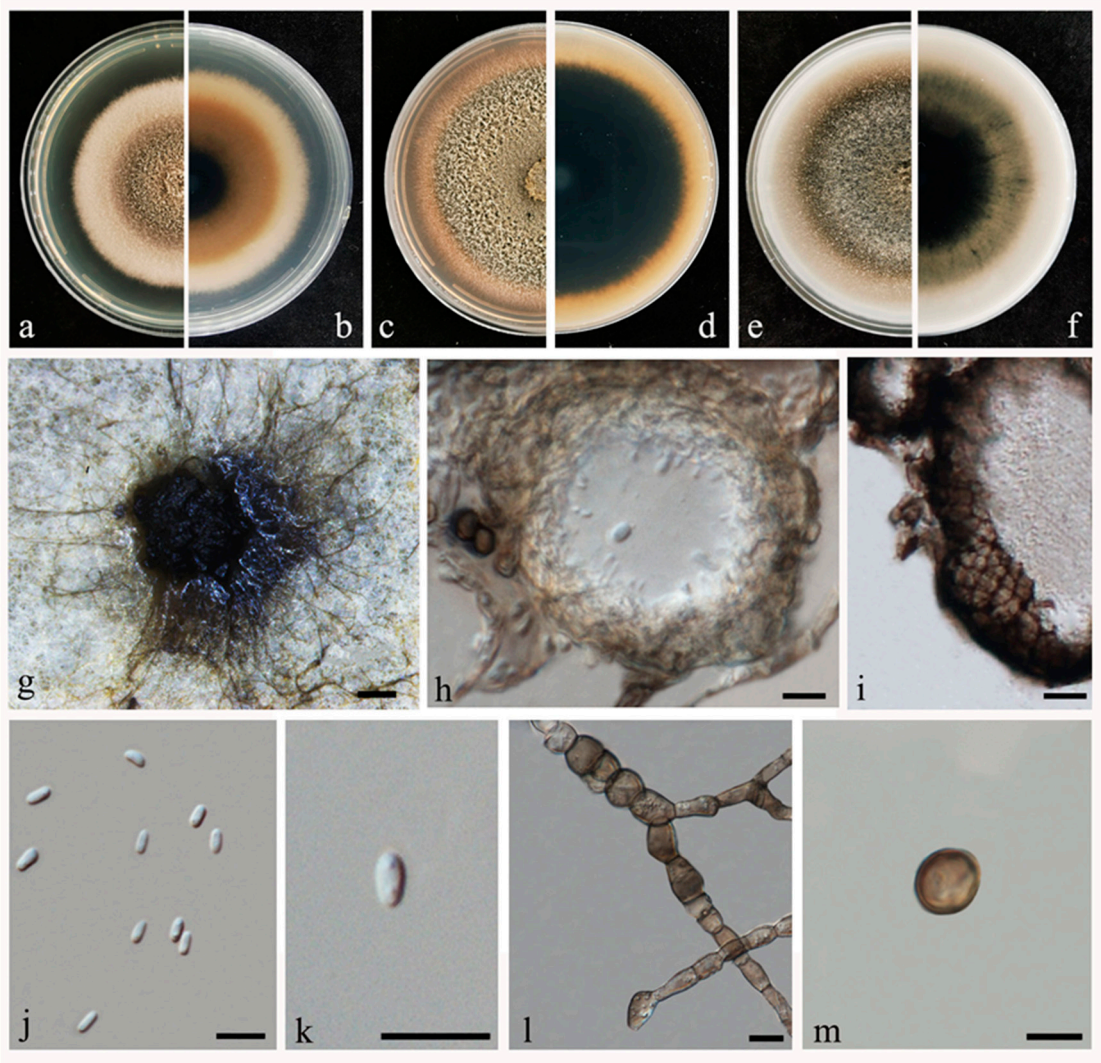

Figure 3. Morphological characteristics of *Stagonosporopsis pogostemonis* (Ex holotype ZHKUCC 21-0001). (**a**,**b**) Front and reverse view on PDA after five days at 28 °C. (**c**,**d**) Front and reverse view on MEA after seven days at 28 °C. (**e**,**f**) Front and reverse view on OA after seven days at 28 °C. (**g**) Pycnidia on PDA. (**h**) Longitudinal section of pycnidia on PDA. (**i**) Pycnidia wall. (**j**,**k**) Conidia. (**l**) Developing chlamydospores. (**m**) Chlamydospore. Scale bars: g = 200 µm; h, k, l, and m = 10 µm.

Culture characteristics: Colonies on PDA, MEA, and OA had covered the entire surface of the plate (8.5 cm in diameter) after seven days at 25 °C, growth rate of 10–16 mm/d. Colonies on PDA, margin regular, cottony, white toward the periphery, brownish-grey in the colony centre. Reverse white, becoming tawny then dark brown–black from the center. The colony characters on MEA were similar as on PDA. Colonies on OA grey and light grey. Reverse white, becoming grey then dark grey–black from the center;

Material examined: China, Guangdong Province, Zhanjiang, isolated from diseased leaves of *Pogostemon cablin*, April 2020, Y.H. Huang and Y.X. Shu, (dried culture ZHKU 21-0001, holotype and ZHKU 21-0002, paratype); ex-type culture ZHKUCC 21-0001 and ex-paratype culture ZHKUCC 21-0002; and

Habitat and host: On diseased leaves of *Pogostemon cablin*;
Known distribution: China (Zhanjiang, Guangdong province).
Notes: In the multigene phylogenetic tree constructed using LSU, ITS, *rpb2*, and *β-tubulin*, our new isolates (ZHKUCC 21-0001 and ZHKUCC 21-0002) constituted a monophyletic clade with 100% maximum likelihood, 100% maximum parsimony bootstrap, and 1.00 Bayesian posterior probability values. Thus, based on the phylogenetic species concept, we introduce this species as a new *Stagonosporopsis* species causing disease in *P. cablin.*

Table 1. Strains used for the phylogenetic analyses in this study and their GenBank accession Numbers.

Name	Strain Number [1]	Substrate (Including Host)	GenBank Accession Numbers [2]			
			rpb2	*tub2*	LSU	ITS
Stagonosporopsis Actaeae *	CBS 106.96; PD 94/1318	*Actaea spicata*	KT389672	GU237671	GU238166	GU237734
S. actaeae	CBS 114303; UPSC 2962	*Actaea spicata*	—	KT389847	KT389760	KT389544
S. actaeae	CBS 105.96; PD 74/230	*Cimicifuga simplex*	MT018018	GU237670	GU238165	GU237733
S. ailanthicola *	MFLUCC 16-1439	*Ailanthus altissima*	KY100876	KY100878	KY100874	KY100872
	CBS 140554	House dust	MT018036	MT005561	MN943664	MN973462
	CBS 140553	House dust	MT018037	MT005562	MN943665	MN973463
	CBS 140556	House dust	MT018038	MT005563	MN943666	MN973464
S. ajacis *	CBS 177.93; PD 90/115	*Delphinium* sp.	KT389673	GU237673	GU238168	GU237791
S. ajacis	CBS 176.93; PD 86/547	*Delphinium* sp.	MT018035	GU237672	GU238167	GU237790
S. andigena *	CBS 269.80; PD 75/914	*Solanum* sp.	MT018026	GU237675	GU238170	GU237817
S. andigena	CBS 101.80; IMI 386090; PD 75/909	*Solanum* sp.	—	GU237674	GU238169	GU237714
S. artemisiicola	CBS 102636; PD 73/1409	*Artemisia dracunculus*	KT389674	GU237676	GU238171	GU237728
S. astragali	CBS 178.25; MUCL 9915	*Astragalus* sp.	MT018030	GU237677	GU238172	GU237792
S. bomiensis *	CGMCC 3.18366; LC 8167	*Boraginaceae*	KY742189	KY742365	KY742277	KY742123
S. bomiensis	LC 8168	*Boraginaceae*	KY742190	KY742366	KY742278	KY742124
S. caricae	CBS 119735	*Caricae papaya*	MN983680	MN984054	MN973431	MN973042
S. caricae	CBS 120720	*Sechium edule*	MN983681	MN984055	MN973432	MN973043
S. chrysanthemi	CBS 137.96; PD 84/75	*Chrysanthemum indicum*	MT018011	GU237696	GU238191	GU237783
S. chrysanthemi	CBS 124241; PD 89/1016-4	*Chrysanthemum sinense*	MT018010	MT005550	MN943653	MN973451
S. chrysanthemi	CBS 500.63; MUCL 8090	*Chrysanthemum indicum*	MT018012	GU237695	GU238190	GU237871
S. citrulli *	FLAS-F-58996; C5-5	*Citrullus lanatus*	—	KJ855602	—	KJ855546
S. cucurbitacearum	CBS 214.65; BBA 9963	*Cucumis sativus*	MT018020	MT005553	MN943656	MN973454
S. crystalliniformis	CBS 771.85; IMI 386091; PD 85/772	*Solanum tuberosum*	—	GU237684	GU238179	GU237906
S. crystalliniformis *	CBS 713.85; ATCC 76027; PD 83/826	*Lycopersicon esculentum*	KT389675	GU237683	GU238178	GU237903
S. cucumeris	CBS 386.65	*Cucumis sativus*	MT018021	MT005554	MN943657	MN973455
S. cucurbitacearum	CBS 233.52	—	MT018024	MT005555	MN943658	MN973456
S. cucurbitacearum	CBS 133.96; PD 79/127	*Cucurbita* sp.	KT389676	GU237686	GU238181	GU237780

Table 1. *Conts.*

Name	Strain Number [1]	Substrate (Including Host)	GenBank Accession Numbers [2]			
			rpb2	*tub2*	LSU	ITS
S. cucurbitacearum	CBS 109171; PD 91/310	*Cucurbita* sp.	MN983682	GU237685	GU238180	GU237922
S. dennisii *	CBS 631.68; PD 68/147	*Solidago floribunda*	KT389677	GU237687	GU238182	GU237899
S. dennisii	CBS 135.96; PD 95/4756	*Solidago canadensis*	MT018019	GU237688	GU238183	GU237782
S. dorenboschii *	CBS 426.90; IMI 386093; PD 86/551	*Physostegia virginiana*	KT389678	GU237690	GU238185	GU237862
S. dorenboschii	CBS 320.90; PD 86/932	*Physostegia virginiana*	MT018039	GU237689	GU238184	GU237830
S. helianthi	CBS 155.90	*Helianthus annuus*	MT018025	MT005556	MN943659	MN973457
S. helianthi *	CBS 200.87	*Helianthus annuus*	KT389683	KT389848	KT389761	KT389545
S. heliopsidis	CBS 109182; PD 74/231	*Heliopsis patula*	KT389679	GU237691	GU238186	GU237747
S. hortensis	CBS 572.85; PD 79/269	*Phaseolus vulgaris*	KT389681	GU237704	GU238199	GU237893
S. hortensis	CBS 104.42	—	KT389680	GU237703	GU238198	GU237730
S. hortensis	CBS 130.96	*Phaseolus vulgaris*	MT018027	MT005557	MN943660	MN973458
S. inoxydabilis *	CBS 425.90; PD 81/520	*Chrysanthemum parthenii*	KT389682	GU237693	GU238188	GU237861
S. loticola *	CBS 562.81; PDDCC 6884	*Lotus pedunculatus*	KT389684	GU237697	GU238192	GU237890
S. loticola *	CBS 563.81; PDDCC 6799	*Lotus pedunculatus*	MT018040	MT005564	MN943667	MN973465
S. lupini	CBS 375.84; PD 80/1250	*Lupinus mutabilis*	MT018028	GU237700	GU238195	GU237844
S. lupini *	CBS 101494; PD 98/5247	*Lupinus albus*	KT389685	GU237699	GU238194	GU237724
S. nemophilae	CBS 249.38	*Nemophila insignis*	MT018032	MT005560	MN943663	MN973461
S. nemophilae *	CBS 715.85; PD 74/364	*Nemophila insignis*	MT018031	MT005559	MN943662	MN973460
S. oculo-hominis *	CBS 634.92; IMI 193307	*Corneal ulcer*	KT389686	GU237701	GU238196	GU237901
S. papillata	LC 8170	*Rumex nepalensis*	KY742192	KY742368	KY742280	KY742126
S. papillata *	CGMCC 3.18367; LC 8169	*Rumex nepalensis*	KY742191	KY742367	KY742279	KY742125
S. papillata	LC 8171	*Boraginaceae*	KY742193	KY742369	KY742281	KY742127
S. pini *	MFLUCC 18-1549	*Pinus* sp.	MK434860	MK412886	MK348019	MK347800
***S. pogostemonis* ***	**ZHKUCC 21-0001**	***Pogostemon cablin***	**MZ203135**	**MZ203132**	**MZ191532**	**MZ156571**
	ZHKUCC 21-0002	***Pogostemon cablin***	**MZ203136**	**MZ203133**	**MZ191533**	**MZ156572**
S. rhizophilae	XDPOP-RS-9	*Populus deltoides*	MN422105	MN422099	MN422103	MN422101
S. rhizophilae	XDPOP-RS-16B	*Populus deltoides*	MN422104	MN422098	MN422102	MN422100
S. rudbeckiae	CBS 109180; PD 79/175	*Rudbeckia bicolor*	MT018015	GU237702	GU238197	GU237745
S. sambucella *	CBS 130003	*Sambucus nigra*	MT018029	MT005558	MN943661	MN973459
S. stuijvenbergii *	CBS 144953; JW 132011	Garden soil	MN824475	MN824623	MN823300	MN823449
S. tanaceti	CBS 131485	*Tanacetum cinerariifolium*	MT018014	MT005551	MN943654	MN973452
S. trachelii	CBS 379.91; PD 77/675	*Campanula isophylla*	KT389687	GU237678	GU238173	GU237850
S. trachelii	CBS 384.68	*Campanula isophylla*	MT018016	GU237679	GU238174	GU237856
S. trachelii	CBS 123.61	*Campanula isophylla*	MT018017	MT005552	MN943655	MN973453

Table 1. *Conts.*

Name	Strain Number [1]	Substrate (Including Host)	GenBank Accession Numbers [2]			
			rpb2	*tub2*	LSU	ITS
S. valerianellae	CBS 273.92; PD 82/43	*Valerianella locusta*	MT018033	GU237705	GU238200	GU237819
S. weymaniae	CBS 144959; JW 201003	Garden soil	MN824479	MN824627	MN823304	MN823453
Allophoma piperis	CBS 268.93; PD 88/720	*Peperomia pereskifolia*	KT389554	GU237644	GU238129	GU237816
Al. piperis	PD 90/2011	*Peperomia* sp.	MT018045	GU237645	GU238130	GU237921

[1] ATCC: American Type Culture Collection, Virginia, USA; CBS, Centraalbureau voor Schimmelcultures (Netherlands); CGMCC, China General Microbial Culture Collection Center (China); MFLUCC, Mae Fah Luang University Culture Collection (Thailand); IMI: International Mycological Institute, JW: Johanna Westerdijk working collection housed at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; LC: LeiCai, corresponding author's personal collection deposited in laboratory, housed at CAS, China; MUCL: Mycotheque de l'Universite catholique de Louvain, Louvain-la-Neuve, Belgium; PD: Plant Protection Service, Wageningen, the Netherlands; PDDCC: Plant Diseases Division Culture Collection, Auckland, New Zealand; UPSC: Uppsala University Culture Collection, Sweden; ZHKUCC, University of Agriculture and Engineering Culture Collection(China). Sequences produced in this study are shown in bold. * ex-type or ex-epitype culture. [2] Sequences data; et al. were downloaded from NCBI following Hou et al. [24] and Wei et al. [25].

2.3. Disease Symptoms and Pathogenicity Tests

Two isolates obtained from leaf spot tissue samples were used for pathogenicity analysis. Both mycelial plug method and mycelial suspension method were employed. In the pathogenicity test, inoculated leaf tissues began to show necrosis regions after three days of the inoculation. Initial symptoms were small, oval or circular-shaped light brown spots that were gradually expanded (Figure 4). None of the control plants showed any disease symptom (Figure 4). The disease symptoms appeared on the host by using the mycelial suspension method later than that of mycelium plug inoculation. However, in the mycelial suspension the infected area was larger and there were more irregular small spots with the mycelial suspension method. Some leaves with severe disease even shriveled and dropped off after seven days under the high humidity condition. The stem first turned brown on the surface after seven days of incubation and then became dry and withered. When the stems were cut open with a longitudinal section, browning could be observed inside after one month (Figure 4) of inoculation. The pathogen was re-isolated from the diseased leaves, and the consistent fungi were re-isolated from the diseased leaves but not isolated from the control plants. The culture characteristics of the isolated strains were consistent with the inoculated strain, thus fulfilling Koch's postulates.

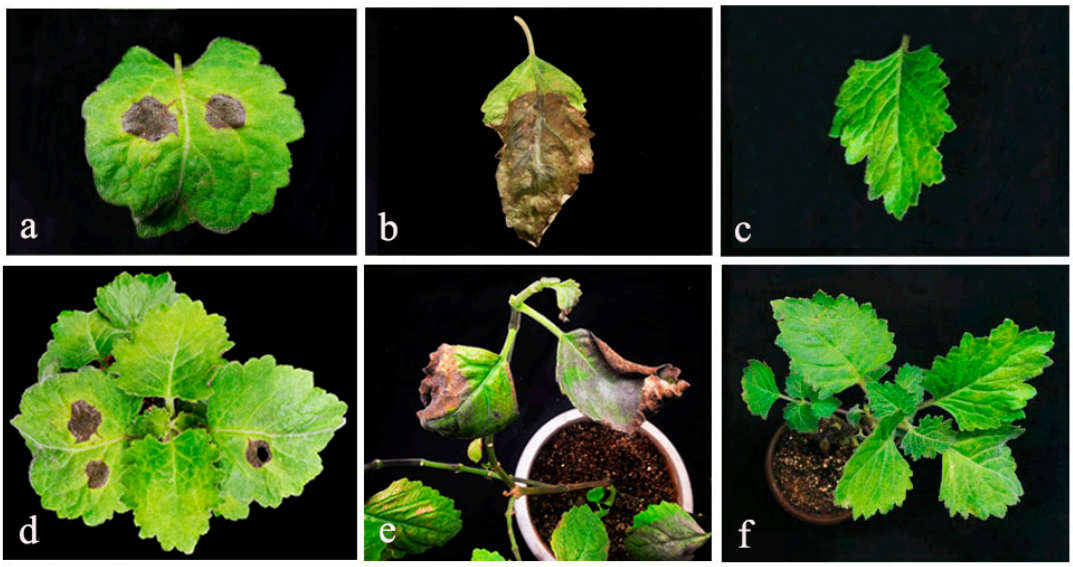

Figure 4. *Conts.*

Figure 4. Pathogenicity tests results of *Stagonosporopsis pogostemonis* inoculated into potted *Pogostemon cablin* plants. Infected leaf at seven days post inoculation with (**a**) mycelial plug and (**b**) mycelial suspension. (**c**) Control leaf. Infected plant at seven days post inoculation with (**d**) mycelial plug and (**e**) mycelial suspension. (**f**) Control plant. Vascular tissues and stem of plants inoculated with mycelial suspension (**g**,**h**) one month post inoculation and (**i**,**j**) control plants.

3. Discussion

Didymellaceae, which belongs to Pleosporales, is one of the largest families in the fungal kingdom [26]. They include plant pathogens, opportunists, endophytes, and saprobes from a wide range of host [27]. *Stagonosporopsis*, one of the genera in Didymellaceae, can cause many important plant diseases [28]. *Stagonosporopsis caricae* was reported causing leaf spots on the non-conventional fruit crop, *Vasconcellea monoica* (*Caricaceae*) in Brazil [29]. *Stagonosporopsis chrysanthemi* caused ray blight of chrysanthemum and pyrethrum and is present worldwide [30]. *Stagonosporopsis tanaceti* caused ray blight of pyrethrum (*Tanacetum cinerariifolium*), a perennial herbaceous plant cultivated for the extraction of insecticidal pyrethrins in Australia [31]. *Stagonosporopsis vannaccii* had been reported as a plant pathogenic fungus from Brazil, causing anthracnose symptoms on pods of soybean [32]. In China, *Stagonosporopsis vannaccii* caused leaf spot on *Crassocephalum crepidioides* plants in the kudzu (*Pueraria lobata*) garden in Guangxi [33]. *Stagonosporopsis cucurbitacearum* is the main cause of pumpkin gummy stem blight (GSB), one of the most devastating pumpkin crop diseases in north-east China [34].

Stagonosporopsis encompasses a wide range of hosts and occurs worldwide [35]. *Stagonosporopsis citrulli* was demonstrated as a pathogen to appear on the 14 species of Cucurbitaceae in the USA [36]. It was also detected in pyrethrum seed and seedlings [37]. The Cucurbitaceae species, such as cucumber, melon, and pumpkin, contained important pathogens, and the fruit rot diseases caused by *S. cucurbitacearum* became a major disease in many parts of the world [38]. In China, *S. cucurbitacearum* has been reported as parasitic on water spinach (*Ipomoea aquatica*) and caused spot blight disease [39]. *Stagonosporopsis oculihominis* was isolated from *Dendrobium huoshanense* as an endophytic fungus [40]. *Pogostemon cablin* leaf spot disease was frequently observed and seriously influenced by the disease in Zhanjiang city, Guangdong Province, China. The causal organism was identified as a new species; *Stagonosporopsis pogostemonis*. Diseased symptoms were characterized by brown spots from the leaf tips. In some cases, these spots can coalesce and form a big scorch-like spot covering a large portion of the leaves. Moreover, some of them form perforation, wither and drop off.

In the pathogenicity assays, *Stagonosporopsis pogostemonis* showed symptoms similar to those observed in the field. However, stem blight symptom was only observed in the mycelial suspension method while the mycelial plugs method only developed leaf spots. Therefore, it is necessary to study further to understand the primary inoculation source at the field.

Use of mycelial plug in the pathogenicity assays is always a debatable point among pathologists [41]. Inoculation of a fungus with numerous amounts of growth media might result in weak pathogen to become more aggressive [42]. Moreover, when mycelial plugs are used the inoculum cannot be quantified. In this study we observed that mycelial suspension method took longer time than mycelial plug method to develop symptoms.

Thus, it gives us the possibility to understand the latent period of this pathogen with the use of mycelial suspension method.

Similar to other relevant fields in mycology, it is necessary to identify the pathogenic taxa to the species level [43,44]. Species identification and pathogenicity confirmation are the critical steps to develop effective control measures [44]. Moreover, some species identification should go beyond species level to identify different genotypes responsible for variations in pathogenicity [44]. Therefore, future studies are necessary to collect more samples to identify beyond the species level. This is the first report about *Stagonosporopsis* species causing leaf spot and stem blight in *P. cablin* worldwide. The pathogenicity of this species was confirmed with Koch's postulates.

4. Materials and Methods

4.1. Sample Collection

Ten *Pogostemon cablin* plants with necrotic leaf spots were collected from the field in Zhanjiang City, Guangdong Province, China (E 110°3′, N 21°2′) from the spring to summer in 2020 (even though the disease incidence occurred in 2019, collections were done only in 2020). Photographs (Nikon D300s, Tokyo, Japan) were taken to record the field symptoms on-site, and samples were placed in sterile, transparent zip-lock bags before being taken back to the laboratory for further studies. Relevant information on the sampling time, latitude, and longitude of the sampling sites and plant species were recorded at the time of sample collection.

4.2. Fungal Isolation and Purification

The collected samples were washed with running tap water first. Then diseased leaves were cut into small pieces of tissue (approximately 0.5 × 0.5 cm^2) by using sterile knife at the interface between healthy and diseased leaves. The pathogen was isolated by the tissue isolation method. Tissue samples were first surface sterilized by soaking in 75% ethanol solution for 10 seconds, followed by immersing in 2.5% NaClO solution for 15 seconds, then rinsed with sterile water three times, and finally dried on sterile filter paper [24]. The surface-sterilized tissue samples were placed on potato dextrose agar (PDA) medium containing streptomycin sulphate (100 µg/mL) and incubated at 25 °C until white mycelia were observed around leaf tissue samples. Pure cultures were obtained after three times hyphal tip isolations. The cultures and the herbarium specimens were preserved in the culture collection and herbarium of Zhongkai University of Agriculture and Engineering (ZHKUCC and ZHKU).

4.3. DNA Extraction, PCR Amplification and Sequencings

Total genomic DNA (gDNA) of two strains obtained were extracted from fresh fungal mycelia grown on PDA at 25 °C for seven days. Total DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method [25]. Molecular identification of the fungal cultures was determined by using LSU [45,46], ITS [47], *β-tubulin* [48], and *rpb2* [49] genes.

A total of 25 µL PCR reaction mixture contained 1 µL of genomic DNA template, 1 µL of each forward and reverse primer (10 µM), 12.5 µL of I-5™ 2× Easy *Taq* PCR SuperMix(+dye) (TransGen Biotech, Beijing, China) and 9.5 µL of deionized distilled water (ddH_2O). The thermal cycler conditions used in PCR amplification for all gene regions are given in Table 2. The PCR amplified gene regions were sequenced by Guangzhou Tianyi Science and Technology Co. Ltd. (Guangzhou, China).

Table 2. Gene regions and respective primer pairs used in the study.

Amplified Gene.	Primer Pairs	Optimized PCR Protocols	References
rpb2	fRPB2-5F fRPB2-7cR	95 °C: 5 min, (94 °C: 30 s, 54 °C: 30 s, 72 °C: 1 min) × 32 cycles, 72 °C: 10 min	[49]
β-tubulin	Bt2a Bt2b	95 °C: 5 min, (94 °C: 30 s, 58 °C: 30 s, 72 °C: 1 min) × 32 cycles, 72 °C: 10 min	[48]
LSU	LROR LR5	95 °C: 5 min, (94 °C: 30 s, 49 °C: 30 s, 72 °C: 1 min) × 32 cycles, 72 °C: 10 min	[45,46]
ITS	ITS1 ITS4	95 °C: 5 min, (94 °C: 30 s, 53 °C: 30 s, 72 °C: 1 min) × 32 cycles, 72 °C: 10 min	[47]

4.4. Phylogenetic Analysis

The LSU, ITS, *rpb2*, and *β-tubulin* sequences were blasted in NCBI BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). According to BLAST results of LSU, ITS, *rpb2*, and *β-tubulin* sequences, the isolates obtained in this study were closely related to the species in *Stagonosporopsis*. Relevant sequence data were downloaded from NCBI following Hou et al. [28] and Wei et al. [50]. Individual sequence datasets were aligned using MAFFT version 7 at the web server (http://mafft.cbrc.jp/alignment/server, May 2021) [51] and improved manually where necessary using BioEdit [52] (http://www.mbio.ncsu.edu/BioEdit/page2.html). Then, the aligned datasets were concatenated by PhyloSuite version 7. All sequences obtained in this study were submitted to GenBank (Table 1). Phylogenetic analyses were conducted by maximum likelihood (ML) in RAxML [53], maximum parsimony (MP) in PAUP (v4.0) [54], and Bayesian analyses (BI) in MrBayes (v. 3.0b4) [55].

For the MP analysis, ambiguous regions in the alignment were excluded and gaps were treated as missing data. To evaluate tree stability, 1000 bootstrap replications were done. Zero-length branches were collapsed, and all parsimonious trees were saved. Tree parameters; tree-length (TL), consistency index (CI), retention index (RI), relative consistency index (RC), and homoplasy index (HI) were calculated. Kishino-Hasegawa tests (KHT) were conducted to evaluate differences between the trees inferred under different optimality criteria [56]. MrModeltest v. 2.3 [57] was used to identify the evolutionary models for each locus that were used in the Bayesian analysis. The maximum likelihood analyses were conducted using RAxML-HPC2 on XSEDE (8.2.8) [58] in the CIPRES Science Gateway platform [59]. The GTR + I + G evolutionary model was employed with 1000 non-parametric bootstrapping iterations. Bayesian analysis was performed in MrBayes v. 3.0b4 [55]. Six simultaneous Markov chains were run for 106 generations, sampling the trees at every 200th generation. From the 10,000 trees obtained, the first 2000 representing the burn-in phase were discarded. The remaining 8000 trees were used to calculate posterior probabilities in a majority rule consensus tree. The constructed phylogenetic tree was visualized in FigTree v1.4.2 and edited by Adobe Illustrator CS6. The final sequence alignment generated in this study was submitted to TreeBASE (https://treebase.org/treebase-web/home.html) under submission ID 28457.

4.5. Morphological Identification

The morphological characterizations of the fungal isolates were carried out based on comprehensive observation of colony characters and microscopic morphology of strain ZHKUCC 21-0001. Culture characteristics were recorded from colonies grown on PDA, malt extract agar (MEA), and oatmeal agar (OA) plates at 25 °C in 12 h dark/12 h light photoperiod for 15–30 days until sporulation [60]. Colony diameters were measured by the crisscross method on the fifth day and growth rate was calculated. The phenotypic characteristics such as colony shape, size, color, exudates, and colony margins were observed, recorded and photographed. Pycnidia were cut into 30 µm thin sections by a freezing sliding microtome (Bio-Key science and technology Co., LTD., LEICA CM1860, Weztlar, Germany) for photographing and measuring. Microscopic characters were observed and photographed using Nikon ECLIPSE 80i microscope (Nikon, Tokyo, Japan) and measure-

ments were taken using NIS-Elements BR 3.2. Measurements of spore length and width of 50 spores were taken. The mean values and standard deviation were calculated with Microsoft Excel.

4.6. Pathogenicity Test

Pathogenicity tests were conducted using *Pogostemon cablin* healthy potted seedlings. Inoculations were done by both mycelial plug method and mycelial suspension inoculation. These assays were done as non-wounded leaves and a small wounded by sterilized needle of stems. Healthy leaves of the same developmental stages were selected, and then the leaves and stems were surface sterilized with 75% alcohol. Fresh wounds were made with a sterilized needle. 6 mm-diameter mycelial plugs were put in the leaf and were inoculated on the surface of healthy young leaves. The 10% mycelial suspension (10 mg [wet weight]/100 ml [volume]) were crushed using a juice extractor (MJ-BL25B2, Guangdong Midea Household Appliance Manufacturing Co., Ltd., Guangdong, China), and wiped on the leaves and stems by sterile cotton. The leaves and stems were then covered with wet cotton and sealed with Parafilm and bagged for moisturizing. Leaves inoculated with water were used as the control. Inoculated plants were kept on the shelf in the greenhouse (25 °C) with artificial lighting (14-h period of supplementary lighting/10-h dark) each day. Disease symptoms were checked daily for 2–7 days. Once the disease was developed, the pathogen was re-isolated to confirm Koch's postulates.

Author Contributions: Z.-Y.D. and M.L. conceived the research and planned the basic research. Y.-X.S. provided the materials. Y.-X.S., Y.-H.H., J.-W.L., M.-P.Z. and M.L. conducted the experiments. Z.-Y.D., Y.-H.H., I.S.M. and M.L. prepared the manuscript. Z.-Y.D., M.L., I.S.M. and D.N.W. analyzed the data. D.N.W. and M.-M.X. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The sequence data generated in this study are deposited in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank). All accession numbers are given in Table 1. The Final alignment generated in this study available in TreeBASE under the accession number 28457.

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