MYCOBIOLOGY
2019, VOL. 47, NO. 4, 408–414
https://doi.org/10.1080/12298093.2019.1682448

RESEARCH ARTICLE

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Identification and Characterization of *Macrophomina phaseolina* Causing Leaf Blight on White Spider Lilies (*Crinum asiaticum* and *Hymenocallis littoralis*) in Malaysia

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ABSTRACT

Crinum asiaticum and *Hymenocallis littoralis*, commonly known as spider lilies are bulbous perennial and herbaceous plants that widely planted in Malaysia as ornamental. During 2015–2016, symptom of leaf blight was noticed on the hosts from several locations in Penang. The symptom appeared as irregular brown to reddish lesions surrounded by yellow halos. As the disease progressed, the infected leaves became blighted, dried, and fell off with the presence of black microsclerotia and pycnidia on the lesions parts. The present study was conducted to investigate the causal pathogen of leaf blight on *C. asiaticum* and *H. littoralis*. Based on morphological characteristics and DNA sequences of internal transcribed spacer (ITS) region and translation elongation factor 1-alpha (TEF1-α) gene, the causal pathogen was identified as *Macrophomina phaseolina*. Phylogenetic analysis of combined dataset of ITS and TEF1-α grouped the isolates studied with other isolates of *M. phaseolina* from GenBank. The grouping of the isolates was supported by 96% bootstrap value. Pathogenicity test proved the role of the fungus in causing leaf blight on both hosts.

ARTICLE HISTORY
Received 11 March 2019
Revised 3 August 2019
Accepted 15 October 2019

KEYWORDS
Macrophomina phaseolina; leaf blight; *Crinum asiaticum*; *Hymenocallis littoralis*

1. Introduction

Crinum asiaticum and *Hymenocallis littoralis* are evergreen and bulbous species which produce narrow and strap-like leaves with white flowers. *C. asiaticum* and *H. littoralis* are locally known as bunga tembaga suasa and melong kecil, respectively [1,2] in which both are belong to family Amaryllidaceae and Order Asparagales. The spider lilies are usually planted in Malaysia as ornamental and landscape purposes. They have been claimed to have multiple ethnobotanical uses [3,4]. For instance, *C. asiaticum* capable of treating joint inflammation and sprain, induced vomiting, treat hemorrhoids, contusion, fracture, and earache [3,5] while *H. littoralis* was used to treat freckles and blemishes [4,6,7].

Like many other ornamental plants, *C. asiaticum* and *H. littoralis* were susceptible to a number of fungal diseases. *C. asiaticum* was reported to be associated with anthracnose caused by *Colletotrichum truncatum*, *C. boninense*, *C. fructicola* and *C. siamense* [8–10], ring spot caused by *Drechslera avenacea* [11], and leaf blight caused by *Drechslera* sp. [12]. Meanwhile, *H. littoralis* was reported to be infected with leaf blight caused by *Neoscytalidium dimidiatum* [13], leaf spot caused by *Phyllosticta hostae* and *Fusarium oxysporum* [14,15], brown leaf caused by *Phyllosticta hymenocallidicola* [16] and leaf tip blight caused by *Curvularia eragrostidis* [17].

Preliminary observation in Permatang Pauh and Universiti Sains Malaysia showed outdoor plantings of *C. asiaticum* and *H. littoralis* have been infected with leaf blight. The symptom was irregular brown to reddish lesions surrounded by yellow halos formed on the leaves. The presence of black microsclerotia and pycnidia was also observed on the infected leaves (Figure 1(A)). Severe infection causing the blighted leaves dried and fell off. The purpose of this study was to investigate the causal agent of leaf blight on *C. asiaticum* and *H. littorallis* based on morphological characteristics, DNA sequences, and phylogenetic analysis.

2. Materials and methods

2.1. Isolation and morphological identification

Blighted leaves of *C. asiaticum* and *H. littoralis* were collected from Permatang Pauh and Universiti Sains Malaysia, Penang. To isolate the suspected pathogen,

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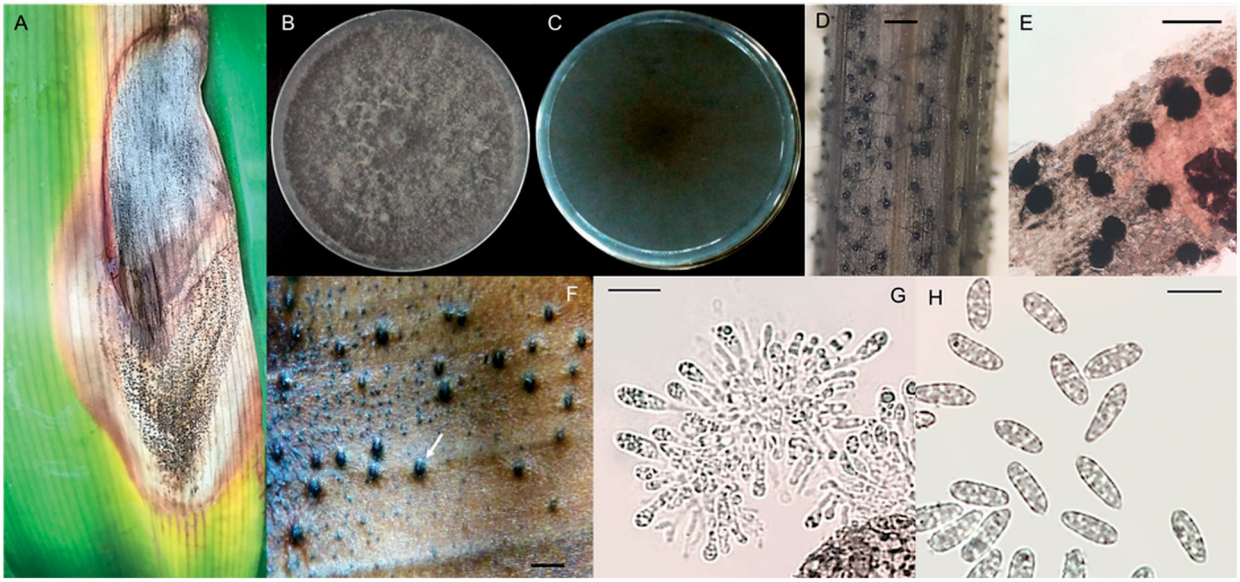

Figure 1. Disease symptom and morphological characteristics of *Macrophomina phaseolina* isolated from *Crinum asiaticum* and *Hymenocallis littoralis*. (A) Symptom of leaf blight observed in the field; (B) Colony appearance; (C) Pigmentation; (D, E) Microsclerotia produced on toothpick; (F) Pycnidia formed on the infected host tissue; (G) Conidiogenous cell; (H) Conidia extruded from the crushed pycnidium. Scale bars (D, E) = 100 µm, (F) = 200 µm, (G, H) = 20 µm.

the symptomatic samples were cut into small pieces for surface sterilization in 70% ethanol (C_2H_5OH) and 1% sodium hypochlorite (NaOCl) for 3 min each. Then, the samples were rinsed in three changes of sterile distilled water for 1 min each and left to dry on sterile filter papers before plating on potato dextrose agar (PDA) and incubated at 25 ± 2 °C for 2–3 days. Single spore isolation was performed to obtain a pure culture of the fungal colony by transferring the hyphal tip onto a new PDA plate and incubated as above. A mycelial disc of 6 mm diameter was placed onto the center of PDA plate and incubated as above for 7 days. Water agar (WA) overlaid with toothpicks [18] was used to induce the formation of microsclerotia and pycnidia and incubated as above for 7 days. The size of 50 randomly selected conidia and the diameter of 50 microsclerotia and pycnidia were measured.

2.2. Molecular identification and phylogenetic analysis

Identification of the fungal isolates was further confirmed by DNA sequences. All the isolates were grown in potato dextrose broth (PDB) and incubated as above for 7 days. The fungal mycelium was harvested and ground in liquid nitrogen to a fine powder. A total of 60 mg of the fine powdered mycelium were extracted by using Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany) following the manufacturer's instructions.

PCR amplification of internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF1-α) was performed using the primer pairs of ITS1/ITS4 [19] and EF1-728F [20]/EF2 [21], respectively. The amplification was carried out in a total volume of 50 µl PCR mixture containing 8 µl Green buffer (Promega, Madison, WI), 8 µl $MgCl_2$, 1 µl deoxynucleotide triphosphate polymerase (dNTP), 8 µl of each primer (Promega, USA), 0.3 µl Taq polymerase, and 1 µl genomic DNA. The reaction was performed in a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA) with the following condition: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The amplified PCR products were separated in 1% agarose gel, run at 80 V and 400 mA for 90 min. The size of fragment was estimated based on comparison with 100 bp DNA ladder (Generulers™; Fermentas, Waltham, MA).

The PCR products were sent to a service provider (First BASE Laboratories Sdn Bhd, Seri Kembangan, Malaysia) for DNA purification and sequencing. The DNA sequences obtained were deposited in GenBank. The generated consensus sequences were compared with other sequences in GenBank database (http://www.ncbi.nlm.nih.gov) by using BLAST to determine the identity of the fungal isolates.

Multiple sequence alignment was performed and used to construct maximum likelihood (ML) tree using Molecular Evolutionary Genetic Analysis (MEGA7) [22] based on substitution model by Kimura-2-parameter [23]. The robustness of each grouping and branch was analyzed by 1000 bootstrap replications [24]. The fungal sequences obtained in this study were compared with several isolates of *M. phaseolina* and *Macrophomina* spp.

Table 1. Isolates used for the phylogenetic analysis in this study.

Species	Isolate[x]	Host	Locality	GenBank accession No. ITS	GenBank accession No. TEF1-α	Reference
Macrophomina phaseolina	CMM3650[T]	*Jatropha curcas*	Brazil: Espirito Santo	KF234552	KF226710	[25]
M. phaseolina	PD112[T]	*Prunus dulcis*	USA	GU251105	GU251237	[26]
M. phaseolina	CMM3615[T]	*J. curcas*	Brazil: Minas Gerais	KF234547	KF226693	[25]
M. phaseolina	MUCC531[T]	*Sesbania formosa*	Western Australia: Kununurra	EF585505	EF585560	[27]
M. phaseolina	CBS205.47[T]	*Phaseolus vulgaris*	Italy	KF951622	KF951997	[28]
M. phaseolina	PPCA213	*Crinum asiaticum*	Malaysia: Penang	MK408582	MK408571	This study
M. phaseolina	PPCA30	*C. asiaticum*	Malaysia: Penang	MK408583	MK408572	This study
M. phaseolina	PPCA29	*C. asiaticum*	Malaysia: Penang	MK408584	MK408573	This study
M. phaseolina	PPHL26	*Hymenocallis littoralis*	Malaysia: Penang	MK408585	MK408574	This study
M. phaseolina	PPHL25	*H. littoralis*	Malaysia: Penang	MK408586	MK408575	This study
M. phaseolina	PPHL23	*H. littoralis*	Malaysia: Penang	MK408587	MK408576	This study
Macrophomina pseudophaseolina	CMM3653[T]	*J. curcas*	Brazil: Minas Gerais	KF369262	KF553906	[29]
M. pseudophaseolina	CMM4231[T]	*Arachis hyphogaea*	Brazil: Rio Grande do Norte	KU058951	KU058921	[29]
M. pseudophaseolina	CPC21400[T]	*A. hyphogaea*	Senegal: Louga	KF951788	KF952150	[29]
M. pseudophaseolina	CPC21502[T]	*Hibiscus sabdarifa*	Senegal: Saint Louis	KF951797	KF952159	[29]
M. pseudophaseolina	CPC21417[T]	*Arachis hypogaea*	Senegal: Louga	KF951791	KF952153	[29]
Macrophomina euphorbiicola	CMM4045[T]	*Jatropha gossypifolia*	Brazil: Paraiba	KU058928	KU058898	[29]
M. euphorbiicola	CMM4134[T]	*Ricinus communis*	Brazil: Bahia	KU058936	KU058906	[29]
M. euphorbiicola	CMM4145[T]	*Ricinus communis*	Brazil: Bahia	KU058937	KU058907	[29]
Macrophomina vaccinii	CGMCC3.19508[T]	*Vaccinium* sp.	China: Fujian	MK687455	MK687431	[30]
M. vaccinii	CGMCC3.19509[T]	*Vaccinium* sp.	China: Fujian	MK687456	MK687432	[30]
M. vaccinii	CGMCC3.19510[T]	*Vaccinium* sp.	China: Fujian	MK687457	MK687433	[30]
Botryosphaeria dothidea	CBS110302	*Vitis vinifera*	Portugal	AY259092	AY573218	[31]

[x]Reference isolate used as comparison in phylogenetic analysis.

from various hosts and *Botryosphaeria dothidea* was used as an outgroup (Table 1).

2.3. Pathogenicity test

A total of six healthy seedlings of *C. asiaticum* and *H. littoralis* were used for pathogenicity test. Mycelial plug was applied as an inoculum, preparing from 7-day-old PDA culture using a sterile cork borer (6 mm diameter). The leaves of *C. asiaticum* and *H. littoralis* were surface sterilized with 70% ethanol and wounded with a sterile toothpick or cork borer [32]. The mycelial plug was inoculated on the wounded area as a treatment while the PDA plug without mycelia was inoculated as a control. The plugs were wrapped with wet cotton to maintain moisture content. Each fungal isolate was done in triplicate and the test was repeated twice. The inoculated plants of *C. asiaticum* and *H. littoralis* were placed in a plant house of School of Biological Sciences, Universiti Sains Malaysia for 2 weeks of incubation. Disease signs and symptoms were checked daily.

3. Results

3.1. Morphological identification

A total of six fungal isolates were obtained from the infected leaves of *C. asiaticum* (PPCA29, PPCA30, and PPCA213) and *H. littoralis* (PPHL23, PPHL25, and PPHL26). All the fungal isolates produced dark olive colonies which turned darker as the culture age (Figure 1(B)) and for the reverse side of the plates, the cultures showed black and dark pigmentation with abundant of microsclerotia embedded (Figure 1(C)). The formation of microsclerotia was observed on the PDA plates and also on the toothpicks (Figure 1(D)) after 7 days of incubation. The microsclerotia were black, smooth, round to oblong, uniformly reticulate, formed from hyphal aggregates, 40.1 ± 5.8 µm in diameter (Figure 1(E)). None of the isolates produced pycnidia from PDA culture and WA overlaid with toothpicks. Pycnidia were present on the infected leaves of *C. asiaticum* and *H. littoralis* collected from the fields (Figure 1(F)). The pycnidia were dark to grayish, globose, membranous, 96.8 ± 12.9 µm in diameter with a truncate ostiole. The structure of conidiogenous cell and conidia can be seen from the crushed pycnidia. The conidiogenous cell was hyaline, short obpyriform to subcylindrical with immature conidia near the apex (Figure 1(G)). The conidia were a single cell, hyaline, aseptate, ellipsoid to obovoid and 22.3 ± 2.0 × 7.2 ± 0.5 µm in size (Figure 1(H)).

3.2. Molecular identification and phylogenetic analysis

The accession numbers for all fungal isolates in the present study were MK408582 to MK408587 for ITS and MK408571 to MK408576 for TEF1-α. Based on BLAST search, all the isolates showed 99.81% and 98.89% identities to KF234552 (ITS) and KF226710 (TEF1-α) of *Macrophomina phaseolina*, respectively.

Comparison of sequences showed that the six isolates of *M. phaseolina* recovered from *C. asiaticum* and *H. littoralis* were grouped into the same clade with reference isolates of *M. phaseolina* from GenBank including CMM3650, PD112, CMM3615, and MUCC531 based on ITS and TEF1-α. The grouping of the isolates was supported by 96% bootstrap value (Figure 2).

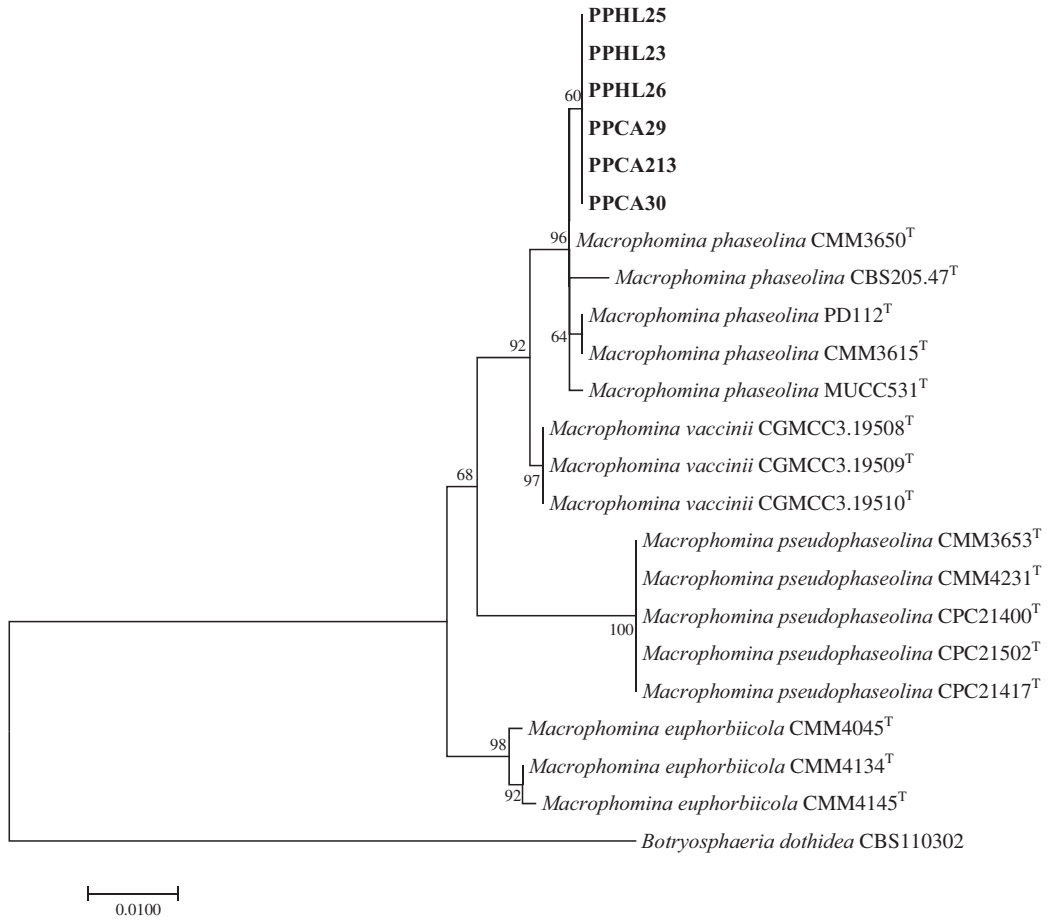

Figure 2. Maximum likelihood tree generated from a combined dataset of ITS and TEF1-α sequences of *Macrophomina phaseolina* isolated from *Crinum asiaticum* and *Hymenocallis littoralis* and other related species. The bootstrap values are located at the nodes. The scale bar indicates the number of substitutions per position. Isolates obtained in the present study are indicated in bold and reference isolates from GenBank are indicated as superscript T.

3.3. Pathogenicity test

All the inoculated leaves of *C. asiaticum* and *H. littoralis* showing the typical symptom of leaf blight as observed in the fields except for the control which remained asymptomatic. Initially, the symptom appeared as irregular brown to reddish lesion surrounded by yellow halo. Then, the lesion expanded and turned darker (Figure 3(A–C)). As the disease progressed, the production of small black microsclerotia and pycnidia was scattered on the infected area (Figure 3(D)). The infected leaf tissue was taken and examined under the dissecting microscope. Hyphae produced from germinated microsclerotium were observed and used to penetrate into the host cell (Figure 3(E)). Reisolation and reidentification of the fungal isolates were conducted and fulfilled the Koch's postulates.

4. Discussion and conclusion

The present study highlighted the causal pathogen of leaf blight on spider lilies (*C. asiaticum* and *H. littoralis*) in Malaysia was proved as *M. phaseolina*. *M. phaseolina* is a well-known plant pathogen causing several important diseases such as charcoal rot [33,34], crown rot [35], Ashy stem blight [36], wilt [37], leaf blight [38], and stem, collar, and root rot [39–41]. This seed-borne or soil-borne pathogen was responsible for causing diseases on numerous hosts namely sunflower, strawberry, soybean, watermelon, guava, and mungbean [42]. Result of pathogenicity test showed the presence of microsclerotia and pycnidia on the inoculated leaves. The germinated microsclerotia will produce infection hyphae which then penetrate through the epidermal cells and colonize intercellularly permitting the fungus to survive prolonged in the soil [42,43].

Besides spider lilies, the other plant from order Asparagales in India, *Chlorophytum borivilianum* was reported to be infected with *M. phaseolina* causing leaf spot disease [44]. The symptom caused almost similar to the present study which appeared as small brown spot on leaf, then the lesions became transparent at advanced stage. Black pycnidia developed on the infected part and at serious stage, the infected leaves dried prematurely and the plant failed to produce healthy tubers.

Morphological characteristics of *M. phaseolina* described fit with the description by Sarr et al. [28] and Crous et al. [45]. From the cultures on plates,

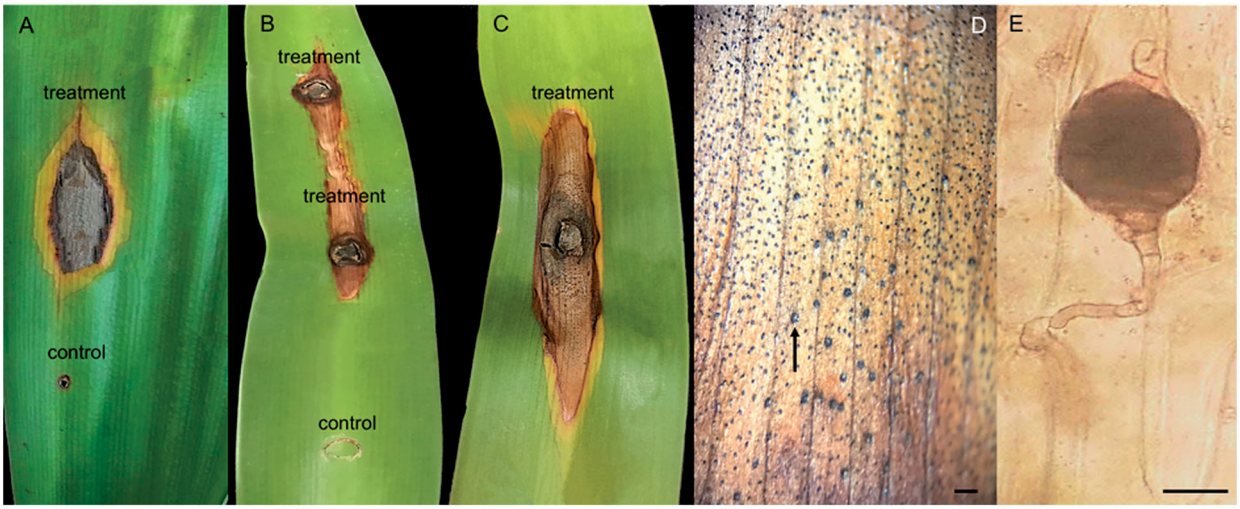

Figure 3. Pathogenicity test of *Macrophomina phaseolina* on *Crinum asiaticum* and *Hymenocallis littoralis*. (A) Blight symptom produced on the leaf of *C. asiaticum*; (B, C) Blight symptoms produced on the leaves of *H. littoralis*; (D) Microsclerotia and pycnidia (arrowed) formed on the inoculated leaf of host plant; (E) A germinated microsclerotium producing infection hyphae to penetrate into host's epidermal cell. Scale bars (D) = 200 µm, (E) = 25 µm.

only conidia and microsclerotia were observed. Pycnidia are rarely produced on the culture and their formation depends on the host and the specific nature of the fungal isolates [42,46]. Identification of *M. phaseolina* solely based on morphology is difficult and challenge as the fungus has two asexual subphases namely saprophytic phase and pathogenic phase. Saprophytic phase (*Rhizoctonia bataticola*) forms microsclerotia and mycelia, while pathogenic phase (*M. phaseolina*) presents in host tissues and forms microsclerotia, mycelia, and pycnidia [42]. Both subphases were observed from the fungal isolates in the present study and to support their morphological identification, molecular analysis was carried out.

Molecular identification of *M. phaseolina* in the present study was verified using ITS region and TEF1-α as adopted by Sousa et al. [47]. Most of the previous studies relied on ITS to recognize *M. phaseolina* [48–50] and the others used species-specific primers [51–53].

In conclusion, to our knowledge, this is the first report of *M. phaseolina* causing leaf blight on white spider lilies (*C. asiaticum* and *H. littoralis*). Findings in the present study will be beneficial in disease monitoring, quarantine, and management purposes of the host.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by Fundamental Research Grant Scheme (FRGS) [203.PBIOLOGI.6711575] and Research University Grant (RUI) [1001/PBIOLOGI/8011061].

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