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Characterisation of *Neoscytalidium dimidiatum* causing leaf blight on *Sansevieria trifasciata* in Malaysia

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Abstract

Sansevieria trifasciata showing symptoms of leaf blight were observed in several states of Malaysia. Based on morphology and DNA sequences of the Internal Transcribed Spacer (ITS) region, nine isolates of *Neoscytalidium dimidiatum* were identified. Phylogenetic analysis grouped the isolates of *N. dimidiatum* from *S. trifasciata* with the epitype of *Neoscytalidium dimidiatum* (CBS499.66) from mango. Pathogenicity test showed that all isolates of *N. dimidiatum* caused leaf blight on *S. trifasciata* and fulfilled Koch's postulates.

Keywords *Neoscytalidium dimidiatum* · Leaf blight · *Sansevieria trifasciata* · ITS

Sansevieria trifasciata, commonly known as mother-in-law's tongue, is a popular ornamental plant that can be found throughout Malaysia. Besides its attractive dark and light green variegation, *S. trifasciata* is tolerant to a wide variety of habitats. *Sansevieria trifasciata* were reported to be infected by *Colletotrichum sansevieriae* (Nakamura et al. 2006) and *Chaetomella* sp. (Li et al. 2013) associated with anthracnose and leaf spot, respectively. In 2015, another disease symptom, leaf blight was observed on *S. trifasciata* in several states of Malaysia. *Scytalidium*-like fungal isolates were recovered during preliminary fungal isolation from the samples.

During September 2015 and March 2017, diseased *S. trifasciata* with symptoms of leaf blight were collected from Penang and three other Malaysian states, namely Perak, Negeri Sembilan and Sarawak. Symptomatic samples were cut into approximately 1.5 cm^2 fragments and surface sterilised in 70% ethanol (C_2H_5OH) solution and 1% sodium hypochlorite (NaOCl) solution for 3 min each. Then, the samples were rinsed thrice in sterile distilled water for 1 min each before being plated on potato dextrose agar (PDA) and incubated at 25 ± 3 °C for 3 to 5 days. Pure cultures were obtained by transferring a single conidium from the colony grown from surface sterilised samples onto fresh PDA and incubated as described previously. A mycelial plug 6 mm in diameter taken from a 7-days-old culture was transferred onto a PDA plate. The growth rate was recorded by measuring the colony diameter daily until the mycelia fully covered the plate. The isolates were cultured on 2% water agar (WA) overlaid with sterilised horsetail twigs (*Casuarina equisetifolia*) and carnation leaves, then were incubated as described previously to induce pycnidia formation. Measurements of 50 randomly selected conidia from pycnidia and arthrospores were taken.

A total of nine *Scytalidium*-like fungal isolates were obtained from diseased *S. trifasciata* showing leaf blight symptoms in four different states (Penang, Perak, Negeri Sembilan and Sarawak). Morphologically, all the isolates were identified as *N. dimidiatum* (Crous et al. 2006; Chuang et al. 2012; Masratul Hawa et al. 2013). Colonies on PDA were greyish white, gradually became greenish dark grey, dense with arthrospores and flat aerial mycelia, with dark pigmentation (Fig. 1a and b). Mycelial growth rate was 4.3 ± 1.0 cm/day on PDA. Sizes of arthric conidia ranged from 6.2 × 3.3 μm to 10.2 × 5.5 μm, 0 to 1 septate, hyaline to brown, and circular, oval or cylindrical with round to truncate ends (Fig. 1c). Conidia exuded in milky white cirrhus from pycnidia were one-celled, aseptate, oblong and 11.4 ± 0.7 μm × 4.9 ± 0.3 μm (Fig. 1d). All nine isolates of *N. dimidiatum* were deposited in Culture Collection Unit, Department of Plant Pathology, School of Biological Sciences, Universiti Sains Malaysia. Both sterile carnation leaves and horsetail twigs were used to induce the formation of pycnidia (Fig. 1e and f). However, pycnidia on carnation leaves tended to be

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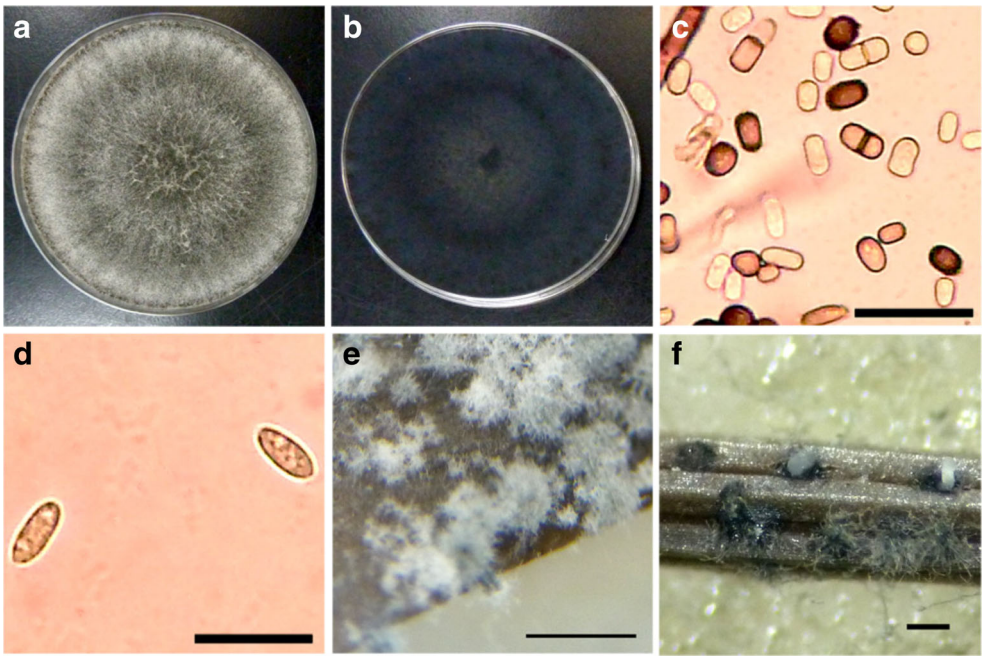

Fig. 1 Morphological characteristics of *N. dimidiatum* isolate, (**a**) colony of *N. dimidiatum* on PDA, (**b**) pigmentation of *N. dimidiatum* on PDA, (**c**) arthric conidia, (**d**) conidia from pycnidia, (**e**) pycnidia and mycelia on carnation leaf, (**f**) pycnidia with conidia exuded from cirrhus on horsetail twig. Bars: c = 20 μm, d = 20 μm, e = 1000 μm, f = 200 μm

covered by mycelia and arthric conidia, thus the horsetail twig was more suitable for examination of single-cell conidium from the conidiomata.

Invisorb Spin Plant Mini Kit (Stratec, Germany) was used to extract the DNA of the isolates. The extraction was done according to the manufacturer's instructions. Amplification of the ITS region was conducted using primers ITS1 and ITS4 (White et al. 1990).

PCRs were prepared in 50 μl containing 8 μl 5× GoTaq® Green Buffer (Promega, USA), 8 μl $MgCl_2$ (25 mM) (Promega, USA), 1 μl dNTP Mix (10 mM) (Promega, USA), 0.8 μM of each primer (ITS1 and ITS4), 0.3 μl Taq polymerase (Promega, USA) and 0.6 μl genomic DNA. PCRs were run in MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) that was programmed for an initial denaturation at 95 °C for 5 min, followed by 35 cycles (each 30 s at 95 °C, 30 s at 54 °C and 1 min at

Table 1 Isolates and GenBank accession numbers used in phylogenetic analysis

Species	Isolate	Host	Location	Reference	GenBank accession number ITS
Neofusicoccum mangiferae	CMW7024	*Mangifera indica*	Australia	Slippers et al. 2005	AY615185
Neofusicoccum vitifusiforme	STE-U5252	*Vitis vinifera*	South Africa	van Niekerk et al. 2004	AY343383
Neoscytalidium dimidiatum	10Neo	*S. trifasciata*	Penang, Malaysia	Present study	KX401435
N. dimidiatum	B3	*S. trifasciata*	Penang, Malaysia	Present study	MF580797
N. dimidiatum	D3	*S. trifasciata*	Penang, Malaysia	Present study	MF580798
N. dimidiatum	EF1	*S. trifasciata*	Sarawak, Malaysia	Present study	MF580799
N. dimidiatum	GD1	*S. trifasciata*	Negeri Sembilan, Malaysia	Present study	MF580792
N. dimidiatum	GH1	*S. trifasciata*	Negeri Sembilan, Malaysia	Present study	MF580793
N. dimidiatum	GW2	*S. trifasciata*	Perak, Malaysia	Present study	MF580794
N. dimidiatum	HX1	*S. trifasciata*	Negeri Sembilan, Malaysia	Present study	MF580795
N. dimidiatum	HY2	*S. trifasciata*	Negeri Sembilan, Malaysia	Present study	MF580796
N. dimidiatum	*CBS499.66	*M. indica*	Mali	Phillips et al. 2013	KF531820
N. novaehollandiae	CBS122071	*Crotalaria medicaginea*	Australia	Pavlic et al. 2008	EF585540
N. novaehollandiae	WAC13273	*M. indica*	Australia	Sakalidis et al. 2011	GU172397
N. orchidacearum	MFLUCC12–0533	Orchid	Thailand	Huang et al. 2016	KU179865

* Epitype (CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; WAC = Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia; CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa; MFLUCC = Mae Fah Luang University Culture Collection; STE-U = Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa)

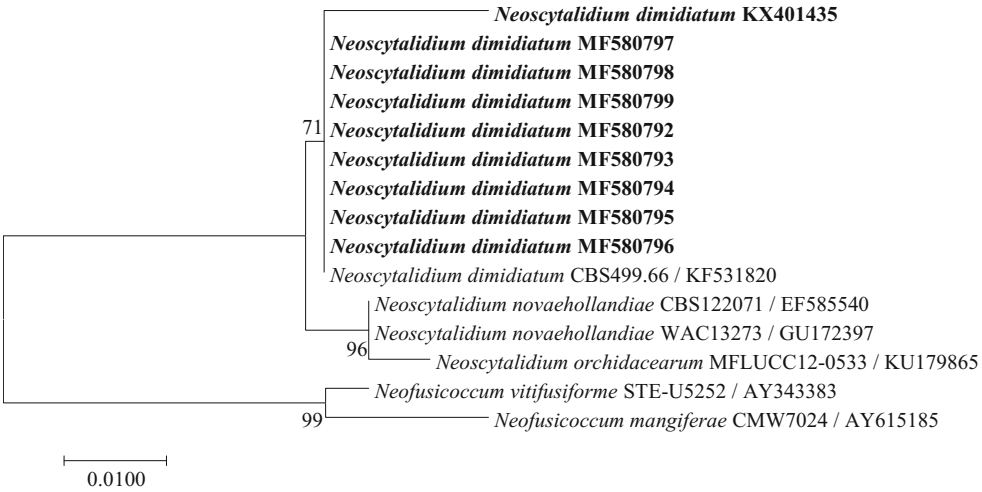

Fig. 2 Phylogenetic tree based on maximum likelihood (ML) analysis of ITS sequence data. Bootstrap value from 1000 replicates was shown at the node. *Neofusicoccum vitifusiforme* STE-U5252 and *Neofusicoccum mangiferae* CMW7024 represented outgroups. Isolates from the present study were indicated in bold

72 °C) prior to a final extension of 5 min at 72 °C. PCR products were sent to a service provider for DNA sequencing.

Sequences were aligned and analysed using MEGA7 (Kumar et al. 2016). The resulting sequences were deposited in GenBank. Based on BLAST searches, eight sequences (Accession No. MF580792-MF580799) and one sequence (KX401435) showed 100% (579/579) and 98% (572/583) identity to the epitype of *Neoscytalidium dimidiatum* (CBS499.66), respectively.

Phylogenetic relationships of the isolates were analysed using MEGA7. The ITS sequence for the type specimen of *N. dimidiatum* was incorporated for analysis using maximum likelihood (ML) method after the best model of nucleotide substitution was selected (Tamura 3-parameter). Bootstrap values were determined from 1000 bootstrap replicates. Gaps and missing data were eliminated. Isolates used in the phylogenetic study, including the type specimen and outgroups are listed in Table 1. Of the 590 characters in the multiple alignments, 522 were conserved sites, 58 were variable sites and 31 were parsimony informative sites. ML analysis based on ITS sequences indicated that all isolates of *N. dimidiatum* in the present study were grouped in the same clade with *N. dimidiatum* CBS499.66, supported by bootstrap value of 71% (Fig. 2).

Pathogenicity of all isolates obtained was assessed on potted *S. trifasciata* using conidial suspension and mycelial plug. Fungal conidial suspensions (1×10^6 conidia/ml) and mycelial plugs (6 mm in diameter) were prepared from 7-days-old cultures on PDA plates. A total of 0.1 ml of conidial suspension was injected into the leaves whereas mycelial plugs were placed with their upper surface facing downward on wounds made by a sterile needle. Sterile distilled water and PDA plugs without mycelia were used as controls. All control and inoculated plants were maintained in a plant house, School of Biological Sciences, Universiti Sains Malaysia at 25 to 31 °C for 2 weeks. Fungal isolates were then re-isolated from the symptomatic inoculated plants and re-identified. The experiment was repeated twice and three replicates were prepared for each isolate.

Inoculation of both methods on potted plants resulted in lesions with darkened centres around the wounded sites that expanded as browning to chlorosis of the tissues (Fig. 3a and b). Under humid conditions, mycelia with arthric conidia were observed on the lesions. No symptoms were observed on the controls. The pathogen was consistently re-isolated from the inoculated leaves of *S. trifasciata*, and morphological examination showed that it was *N. dimidiatum*.

Neoscytalidium dimidiatum is an opportunistic pathogen of various hosts causing different types of diseases (Padin et al. 2005). This pathogen was reported to cause stem canker (Masratul Hawa et al. 2013; Sanahuja et al. 2016) and brown rot of dragon fruit (Chuang et al. 2012), canker of English walnut (Chen et al. 2013), leaf blight of white spider lily (Nurul Nadiah et al. 2017), dieback of lesser yam (Lin et al. 2017), dieback, stem-end rot and canker of mango (Sakalidis et al. 2011; Marques et al. 2013), wood canker of grapevine (Rolshausen et al. 2013), shoot blight, canker, and gummosis

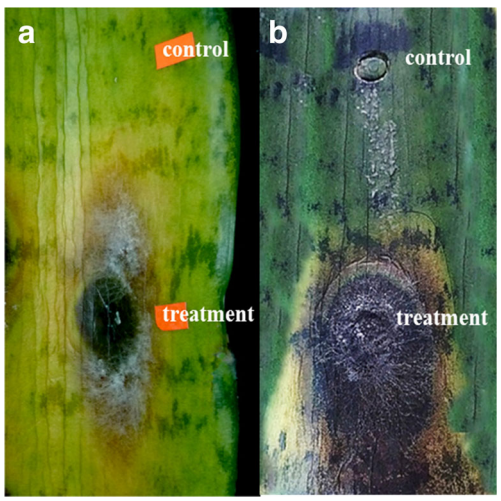

Fig. 3 Artificial inoculation of *N. dimidiatum* isolate on *S. trifasciata*, (**a**) pathogenicity test using conidial suspension, (**b**) pathogenicity test using mycelial plug on wounded leaves

of citrus (Polizzi et al. 2009), human toenail infection (da Silva et al. 2016) and rhinosinusitis (Bakhshizadeh et al. 2014). The occurrence of *N. dimidiatum* on *S. trifasciata* contributes to the knowledge of the host range of this plant pathogenic fungus. Etiological information of *N. dimidiatum* will help to develop strategies and efforts to control this important pathogen that can pose a threat to plants and humans. To our knowledge, this is the first report of *N. dimidiatum* causing leaf blight on *S. trifasciata* in Malaysia and worldwide.

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