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# Morphological and molecular identification of *Neopestalotiopsis clavispora* causing flower blight on *Anthurium andraeanum* in Thailand

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## Abstract

Flower blight on anthurium (*Anthurium andraeanum*) was observed during August 2018 on an anthurium cultivation farm in the Songkhla Province of southern Thailand. The fungal isolate was identified as *Neopestalotiopsis clavispora* based on the morphology and DNA sequence of the internal transcribed spacer (ITS), translation elongation factor 1-α (*tef1-α*), and *β*-tubulin (*tub*) genes. The phylogenetic tree, based on the combined sequences of ITS, *tef1-α*, and *tub*, confirmed this pathogen as *N. clavispora*. Pathogenicity of the species was confirmed according to Koch's postulate: *N. clavispora* could infect anthurium. To the best of our knowledge, this is the first report of *N. clavispora* as a pathogen of anthurium.

## Keywords

Flamingo flower; Morphology; Molecular properties; *Neopestalotiopsis clavispora*; Pathogenicity test

## 1. Introduction

Anthuriums (Anthurium andraeanum), or flamingo flowers, are economical flowering plants commercially cultivated worldwide. *Anthurium*, the largest genus in the Araceae family, consists of approximately 1 000 species of flowering plants (Mantovani and Pereira, 2005). Their popularity as an economical flowering plant is due to their variable leaf shapes and colors, spathe, and spadix. In Thailand, anthuriums were first introduced in the Krabi Province in Southern Thailand and then distributed nationwide (Department of Agricultural Extension, Ministry of Agriculture and Cooperative, 2019). Recent estimates place anthurium cultivation in Thailand at approximately 30.4 hectares; however, the main cultivation still occurs in the southern regions. Southern Thailand, however, consists of tropical and subtropical climates and is therefore prone to the occurrence and spreading of disease. Several potential diseases have had a negative impact on both the quality and quantity of anthurium production.

According to the previous literature, anthuriums faced with bacterial and fungal infections that have caused devastating crop losses worldwide. For instance, bacterial blight, caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (Lipp et al., 1992; Aysan and Sahin, 2003), and bacterial wilt, caused by *Ralstonia solanacearum* (Tan et al., 2006), greatly limited commercial production. In fungal pathogens, black nose or spadix rot in anthuriums has been reported to be caused by *Colletotrichum gloeosporioides* (Alvarez, 2018) and *Lasiodiplodia theobromae* (Daengsuwan et al., 2020). In August 2018, we observed the occurrence of flower blight in anthuriums on a private cultivation farm in southern Thailand's Songkhla Province. The disease was found in 20% of the surveyed anthuriums plants on the cultivation farm. As no previous report of a pathogen causing flower blight on anthuriums exists, our research aimed

to identify the responsible pathogen through the examination of its morphological characteristics and molecular properties, as well as by testing its pathogenicity to fulfill Koch's postulates.

## 2. Materials and methods

### 2.1. Sample collection and isolation

The sample collection was conducted on a private anthurium cultivation farm in southern Thailand's Khu Ha Tai sub district, Rattaphum district, Songkhla Province (7°11′02.1″N, 100°17′12.7″E). Anthuriums 'Angel' with flower blight were collected in plastic bags and brought to the laboratory where the isolation was performed. A total of ten infected samples were collected. Isolation of the pathogen was conducted by the tissue transplantation method (Pornsuriya et al., 2018; Daengsuwan et al., 2020). The infected leaves were cut into small pieces (0.3 cm × 0.3 cm), surface sterilized with 70% ethanol, and followed by soaking in 1% sodium hypochloride (NaClO). After removing the excess NaClO with sterile distilled water (DW), pieces of the leaf samples were dried on sterile Whatman© paper in a laminar flow hood. Small pieces of the infected leaf tissues were placed in 1.5% water agar and incubated at (28 ± 2) °C with 12 h: 12 h light and dark cycle for three days. Hyphal tips (approximately 1 mm) were cut and transferred to potato dextrose agar (PDA) and incubated at (28 ± 2) °C with 12 h: 12 h light and dark cycle for further study.

### 2.2. Pathogenicity test

To test the pathogenicity of the fungal isolate, the agar plug method was utilized following the method described by Solarte et al. (2018) and Daengsuwan et al. (2020) with some modifications. Anthuriums 'Angel' at the four months stage was prepared for inoculation. The fungal isolates were cultured on PDA for five days. Anthurium spathe and spadix were wounded by sterile fine needles. Mycelial plugs from a five-day-old culture were cut from the culture plates and placed on the wounded or non-wounded anthurium spathe and spadix. The inoculated spathe and spadix were then covered with a plastic bag to maintain the humidity for 24 h within (28 ± 2) °C and a 12 h: 12 h light and dark cycle. Ten anthuriums 'Angel' were inoculated per isolate (ten replicates), and the inoculation was repeated three times. Ten anthuriums plants inoculated with PDA alone served as the control group. The development of external symptoms was observed after the first week.

## 2.3. Morphological identification

The fungal isolate that can cause flower blight on anthuriums was subjected to morphological study using both a stereomicroscope (Leica S8AP0, Leica, Germany) and a compound microscope (Leica DM750, Leica, Germany) with 30 replicates ($n = 30$). Colony characteristics and conidia were measured and compared for species identification. Pathogens were identified based on the taxonomic study of Maharachchikumbura et al. (2012, 2014).

## 2.4. Molecular identification

For DNA extraction, fungal isolates that can cause flower blight on anthurium (strain AAN2) were grown on PDA for three days. Young mycelia were subjected to DNA extraction using the mini-preparation method as previously described by Saitoh et al. (2006). DNA presence was determined via agarose gel electrophoresis. The internal transcribed spacer (ITS), translation elongation factor 1-$\alpha$ (*tef1*-$\alpha$), and $\beta$-tubulin (*tub*) genes were amplified using ITS1/ITS4 (White et al., 1990), EF1–728F/EF2 (O'Donnell et al., 1998; Carbone and Kohn, 1999), and T1/Bt2b (Glass and Donalson, 1995; O'Donnell and Cigelnik, 1997) primer pairs, respectively, in a BIO-RAD T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR was performed in a 50 µL reaction volume containing 2 × DreamTaq Green PCR Master Mix (Thermo Scientific, Massachusetts, US), 10 pmol of each primer, and 50 ng of the DNA template. The thermal cycling program used initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, 30 s of annealing at 50 °C, 1 min of extension at 72 °C, and a final extension at 72 °C for 10 min. The PCR product was stained with Novel Juice (Gene DireX, Shanghai, China) and observed via gel electrophoresis.

The PCR products were sequenced using the Macrogen Sequencing Service (Macrogen, Seoul, Korea). A BLASTn search was used to compare the sequences found in our study with those of the NCBI (National Center for Biotechnology Information) database. All gene sequences were aligned using MEGA X (Kumar et al., 2018), and the phylogenetics of the combined ITS, *tef1*-α, and *tub* gene sequences were constructed through maximum-parsimony with 1 000 bootstrap replications.

# 3. Results

## 3.1. Symptom recognition and pathogenicity test

The primary symptom of anthurium flower blight was small circular brown spots, 0.1 to 0.2 cm, growing to 2 to 5 cm in size; with lesions distributed throughout the spathe and spadix of the anthuriums (Fig. 1, a–d). A total of five fungal isolates were isolated from ten infected anthurium samples, in which primary identification was based on the colony's characteristics and morphology. Of the five isolates, two strains were identified as *Colletotrichum* spp. (AAC1, AAC2), and three strains of *Neopestalotiopsis* spp. (AAN1, AAN2, and AAN3). All isolated strains have been tested on pathogenicity, and only the pathogenicity strain was subjected to further study. Pathogenicity tests revealed that only the AAN2 strain developed external symptoms similar to those of natural infection after one week (Fig. 1, e, f). Ten wounded anthurium plants developed flower blight after inoculated by AAN2 strain. No symptoms were observed in the non-wounded anthuriums, control plants and the other strains. The strain AAN2 was re-isolated, and its morphology again matched that of the *Neopestalotiopsis* sp., confirming Koch's postulates (data not shown). Therefore, the fungal strain AAN2 was selected for identification based on its morphology and molecular properties.

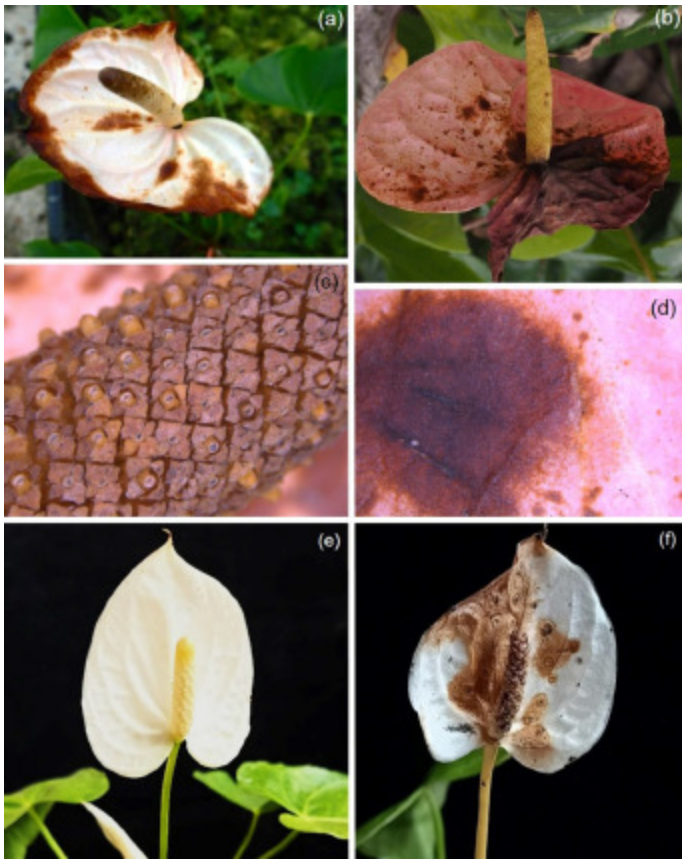


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Fig. 1. Flower blight and pathogenicity test

**Flower blight of anthuriums (a, b), magnified views of the disease on the spadix (c) and the spathe (d); the control anthurium (e) and symptom on inoculated anthurium 'Angel' (f).**

## 3.2. Morphological observation of the fungal isolate

The fungal strain AAN2 colony was white, cottony, and flocculent, contained undulate edges with dense aerial mycelium on its surface, and averaged 12.16 mm • $d^{-1}$ growth at (28 ± 2) °C ( Fig. 2, a, b). Black conidiomata formed superficially, scattered over the PDA at ten days after incubation at ambient temperature (28 ± 2) °C, 99–108 mm in diameter, and contained slimy black conidial mass (Fig. 2, c). Conidia were fusoid to ellipsoidal, slightly curved, and comprised five cells, 17.82 to 25.06 (20.93 ± 1.82) µm × 4.83 to 7.47 (6.44 ± 0.64) µm. The middle three cells were versicolored, whereas the three lower median cells were pale brown, and the two upper two cells were dark brown (Fig. 2, d–g). The apical cells showed two to three appendages [14.65 to 32.68 (24.42 ± 4.73) µm long], whereas only a single appendage was found on the basal cell [2.69 to 6.90 (4.59 ± 1.05) µm long] (Fig. 2, d–g). Based on the morphological characteristics (Table 1), the fungal black conidial strain was identified as *Neopestalotiopsis* sp. (Maharachchikumbura et al., 2012, 2014). The strain was deposited in the Culture Collection of the Pest Management Department, Faculty of Natural Resources, Prince of Songkla University, Hatyai, Thailand; with the accession code PSU-AAN2.

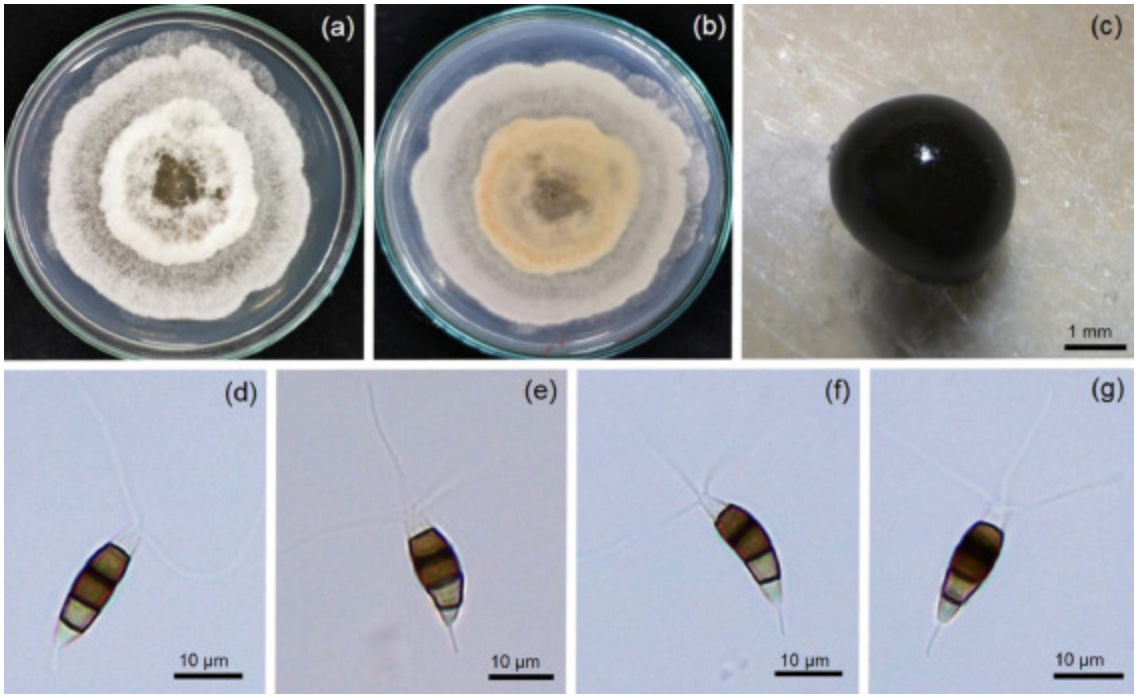


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Fig. 2. Morphological characteristics of the *Neopestalotiopsis* sp. colony on PDA

**Top view (a); bottom view (b); slimy conidial mass on PDA (c); conidia with two to three apical appendages; and a single basal appendage (d–g).**

Table 1. Sequences of *Neopestalotiopsis* spp. and *Pseudopestalotiopsis coccos* used for phylogenetic analysis

| **Taxon** | **Isolate** | **GenBank accession number** | | |
|---|---|---|---|---|
| | | **ITS** | ***TUB*** | ***TEF1-α*** |
| *N. acrostichi* | MFLUCC 17–1754 | MK764272 | MK764338 | MK764316 |
| *N. asiatica* | MFLUCC12–0286 | JX398983 | JX399018 | JX399049 |
| *N. australis* | CBS 114,159 | KM199348 | KM199432 | KM199537 |
| *N. brachiata* | MFLUCC 17–1555 | MK764274 | MK764340 | MK764318 |

| Taxon | Isolate | GenBank accession number | | |
|---|---|---|---|---|
| | | ITS | *TUB* | *TEF1-α* |
| *N. chrysea* | MFLUCC12–0261 | JX398985 | JX399020 | JX399051 |
| *N. clavispora* | MFLUCC12–0281 | JX398979 | JX399014 | JX399045 |
| *N. cubana* | CBS 600.96 | KM199347 | KM199438 | KM199521 |
| *N. egyptiaca* | PEST1 | KP943747 | KP943746 | KP943748 |
| *N. eucalypticola* | CBS 264.37 | KM199376 | KM199431 | KM199551 |
| *N. formicarum* | CBS 362.72 | KM199358 | KM199455 | KM199517 |
| *N. javaensis* | CBS 257.31 | KM199357 | KM199437 | KM199543 |
| *N. macadamiae* | BRIP 63737c | KX186604 | KX186654 | KX186627 |
| *N. mesopotamica* | CBS 336.86 | KM199362 | KM199441 | KM199555 |
| *N. musae* | MFLUCC 15–0776 | KX789683 | KX789686 | KX789685 |
| *N. petila* | MFLUCC 17–1737 | MK764275 | MK764341 | MK764319 |
| *N. piceana* | CBS 394.48 | KM199368 | KM199453 | KM199527 |
| *N. rhizophorae* | MFLUCC 17–1551 | MK764277 | MK764344 | MK764321 |
| *N. rosae* | CBS 101,057 | KM199359 | KM199429 | KM199523 |
| *N. saprophytica* | CBS 115,452 | KM199345 | KM199433 | KM199538 |
| *N. surinamensis* | CBS 450.74 | KM199351 | KM199465 | KM199518 |
| *N. umberspora* | MFLUCC12–0285 | JX398984 | JX399019 | JX399050 |
| *Pseudopestalotiopsis cocos* | CBS 272.29 | NR_145,246 | KM199467 | KM199553 |

## 3.3. Molecular identification and phylogenetic analysis of PSU-AAN2

To identify the strain PSU-AAN2 through its molecular properties, the ITS, *tef1-α*, and *tub* gene regions were sequenced. The BLASTn search of these gene sequences revealed that PSU-AAN2 belonged to the *Neopestalotiopsis* species. The BLASTn search showed strain PSU-AAN2

matched with *Neopestalotiopsis clavispora* with 97.27%, 98.68% and 100.00% identity for ITS, *tub* and *tef1-α* gene sequences, respectively. We constructed a phylogenetic tree with 21 sequences of known *Neopestalotiopsis* spp. and an outgroup of *Pseudopestalotiopsis cocos* ( Table 1), based on concatenated datasets (ITS, *tef1-α* and *tub*). The alignment contained 602, 450, and 241 bases for ITS, *tef1-α*, and *tub*, respectively. The PSU-AAN2 grouped with the type strain *N. clavispora* (Fig. 3). The sequences were deposited in GenBank and accession numbers were assigned as LC537159, LC537160 and LC537161 for ITS, *tub* and *tef1-α*, respectively. A combination of the morphological characteristics (Table 2) and phylogenetic properties (Fig. 3 ) confirmed that the PSU-AAN2 strain belonged to *N. clavispora*.

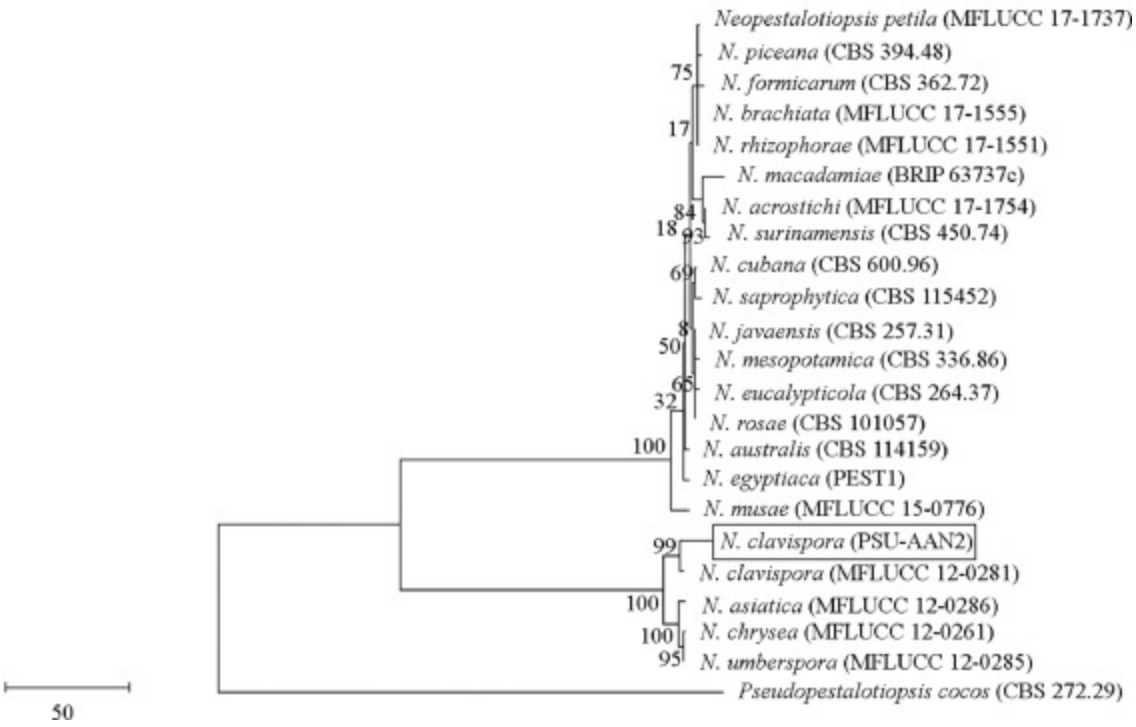


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Fig. 3. Multilocus phylogenetic tree based on maximum-parsimony through the alignment of combined sequences (ITS+*tef1-α* +*tub*) of the *Neopestalotiopsis* species

Table 2. Morphological characteristics of strain PSU-AAN2 causing flower blight on anthurium and the description of *Neopestalotiopsis clavispora*

| **Morphological characteristic** | ***Neopestalotiopsis clavispora*** | **PSU-AAN2** |
|---|---|---|
| Colony on PDA | Whitish Aerial mycelium on surface, undulate edge | White, cottony with undulate edge |
| Conidiomata diameter/mm | 150–250 | 99–108 |
| Conidia length/μm | 18–26 | 17.82–25.06 |
| Conidia wide/μm | 6.5–8.5 | 4.83–7.47 |
| Number of cell | 5 | 5 |
| Pigmentation of median cells | Versicolor | Versicolor |
| Number of apical appendage | 2–3 | 2–3 |
| Apical appendage length/μm | 19–30 | 14.65–32.68 |
| Number of basal appendage | 1 | 1 |
| Basal appendage length/μm | 3.0–5.5 | 2.69–6.90 |
| References | Maharachchikumbura et al. (2012) | This study |

## 4. Discussion

In this study, we isolated the fungal pathogens from infected anthuriums. Among the five isolations found, only the PSU-AAN2 strain caused flower blight, as observed in natural infection, whereas other strains did not. A total of five strains were recovered from a tissue transplantation method as described in the results, including two strains of *Colletotrichum* spp. (AAC1, AAC2), and three strains of *Neopestalotiopsis* spp. (AAN1, AAN2, AAN3). However, pathogenicity tests revealed that two strains of *Colletotrichum* spp. (AAC1, ACC2) and two strains of *Neopestalotiopsis* spp. (AAN1, AAN3) were not able to cause flower blight on

anthuriums, and this may be due to the capacity of the endophyte, as has been observed in several *Colletotrichum* spp. and *Neopestalotiopsis* spp. (Ma et al., 2018; Kumar et al., 2019). The *Pestalotiopsis* and *Neopestalotiopsis* species have been known to infect plant hosts through a wound or through a natural opening (Keith et al., 2006; Daengsuwan et al., 2020; Pornsuriya et al., 2020). In the present study, flower blight in anthuriums occurred via the wounding method in agreement with the aforementioned research, suggesting that wounds may help conidia penetrate plant tissue, thus creating symptoms of infection.

Mararachchikumbura et al. (2014) divided the fungi in *Pestalotiopsis* into three genera, *Neopestalotiopsis, Pestalotiopsis*, and *Pseudopestalotiopsis* through morphological examination and the molecular study of multiple sequences. They determined that the dominant characteristics of *Neopestalotiopsis* are indistinct in the reduction of conidiophores and they have versicolored median cells, which is in agreement with the present study. However, morphological investigations were not able to unequivocally distinguish *Neopestalotiopsis* from *Pestalotiopsis* or *Pseudopestalotiopsis* (Tejesvi et al., 2009). To categorize *Neopestalotiopsis* into the species level requires multiple nucleotide sequences of ITS, *tef1-α*, and *tub* ( Mararachchikumbura et al., 2014).

Initially, the phylogenetic significance of *Pestalotiopsis*, and its allied genera, was constructed through the use of a single-gene sequence, and data such as ITS or *tub* genes ( Jeewon et al., 2003; Hu et al., 2007). However, the use of a single-gene sequence does not distinguish *Neopestalotiopsis* from *Pestalotiopsis* or *Pseudopestalotiopsis*. Mararachchikumbura et al. (2012, 2014) further suggested that the combination of ITS, *tef1-α*, and *tub* sequences was suitable for species identification of *Neopestalotiopsis*. In the present study, we constructed a phylogenetic tree from the combined sequences of ITS, *tef1-α*, and *tub*, which enabled us to identify the flower blight pathogen *N. clavispora*, which corresponded with the findings of Mararachchikumbura et al. (2014).

To the best of our knowledge, our study represents the first attempt to identify and characterize *Neopestalotiopsis* fungi causing flower blight in anthuriums through both morphology and molecular properties. Since its establishment ( Maharachchikumbura et al., 2014), increased reports of various plant diseases caused by *N. clavispora* have been made; including root and stem rot in strawberry plants ( Obregón et al., 2018), leaf spots on macadamia (Santos et al., 2019), *Phedimus aizoon* var. *latifolius* (Yang et al., 2017), canker and twig dieback in blueberry bushes (Borrero et al., 2018),

and ring spots on *Kadsura coccinea* (Xie et al., 2018). Therefore, research in the global control of this disease pathogen, in all plant species, must be conducted.

## Acknowledgments

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