Article

Neopestalotiopsis Species Associated with Flower Diseases of Macadamia integrifolia in Australia

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Abstract: Macadamia (Macadamia integrifolia) is native to eastern Australia and produces an edible nut that is extensively cultivated in commercial orchards in several countries. Little is known about the diversity of fungi associated with diseases of macadamia inflorescences. A survey of fungi associated with the dry flower disease of macadamia detected several isolates of Neopestalotiopsis (Pestalotiopsidaceae, Sordariomycetes). Five new species of Neopestalotiopsis were identified based on molecular phylogenetic analyses of concatenated gene sequences of the internal transcribed spacer (ITS), β-tubulin (TUB), and the translation elongation factor 1-alpha (TEF1α). The new species are named Neopestalotiopsis drenthii, N. maddoxii, N. olumideae, N. vheenae, and N. zakeelii, and are described by molecular, morphological, and cultural characteristics. The ecology of the isolates and their pathogenic, saprophytic, or commensal ability were not determined.

Keywords: amphipsaeriales; conidial morphology; flower blights; pestalotioid fungi; taxonomy; tree nut

1. Introduction

Macadamia (Macadamia integrifolia) is a tree nut crop that is cultivated for its high-value kernel in tropical and subtropical regions in Australia, Asia, Africa, South America, and the U.S.A. Four species, M. integrifolia, M. tetraphylla, M. ternifolia, and M. jansenii, are native to Australia [1]. Macadamia integrifolia and M. tetraphylla produce edible kernels, whereas M. ternifolia and M. jansenii produce small and inedible nuts that contain high levels of cyanogenic glycosides [2]. Several new diseases caused by fungal and oomyceteous pathogens have been reported on macadamia with the expansion of its production area [3–5].

Diseases of flowers and fruit result in significant yield losses and poor-quality kernels [6–8]. A mature macadamia tree may produce over 10,000 racemes (inflorescences) at peak anthesis, with each raceme typically having 100–300 flowers (Figure 1a) [9,10]. Flower diseases can result in poor pollination efficiency, with less than 1% of the flowers producing fruit, as well as a reduction in the potential of the flower to bear fruit. Macadamia racemes may be affected by fungal pathogens at different developmental stages. Macadamia inflorescences have four growth stages: small green buds on the rachis (stage 1); florets that turn from light green to white and are partially up to fully open, with stamens that pull away from the stigmas (stage 2); fully opened flowers with sepals that turn brown at peak anthesis (stage 3); sepals that drop and flowers with swollen fertilized embryos (stage 4) [11]. Most of the diseases that affect macadamia inflorescences are flower blights [12]. A diversity of fungi has been associated with flower blights of macadamia in Australia [13], including species of Botrytis [14], Cladosporium [15], Neopestalotiopsis, and Pestalotiopsis [8].
regions, causing leaf spot, dry flower, fruit rot, fruit scab, and trunk diseases on a range of crops [5,8,20–26]. Flower and leaf diseases on macadamia caused by Pestalotiopsis spp. have been reported in Australia [5,8], Brazil [26], and China [25]. Many new pestalotioid species have been introduced in recent years [27–33]. Many unidentified isolates of Pestalotiopsis spp. have been reported in Australia [5,8], Brazil [26], and China [25]. Many new pestalotioid genera (Pestalotiopsis, Neopestalotiopsis, and Pseudopestalotiopsis) were obtained from macadamia racemes with dry flower symptoms. However, there is little information about their identity and the diversity of fungi that cause dry flower disease on macadamia in Australia. The aim of this study was to identify the species of Neopestalotiopsis associated with the dry flowers of macadamia in Australia.

2. Materials and Methods

2.1. Sample Collection and Isolation

The isolates included in this study were collected from macadamia racemes with symptoms of dry flower disease. Samples were obtained from commercial macadamia orchards in Queensland (QLD) and New South Wales (NSW), Australia in 2019 and 2020. The samples were surface sterilized and incubated, as described by Akinsanmi et al. [8]. Monoconidial cultures of 13 isolates were established, as described by Akinsanmi et al. [34], and cryopreserved in a sterile 15% glycerol solution at −80 °C. Living cultures of the isolates were deposited in the Queensland Plant Pathology Herbarium (BRIP), Brisbane, Australia.

Figure 1. Macadamia inflorescences (racemes). (a) Pendant racemes in tree canopy, and (b) dry flower disease.

The incidence of macadamia dry flower disease caused by Pestalotiopsis macadamiae and Neopestalotiopsis macadamiae is on the increase in Australian macadamia plantations [8]. Dry flower disease is characterized by the necrotic blight of flowers (Figure 1b). Akinsanmi et al. [16] suggested that multiple species of Pestalotiopsis and Neopestalotiopsis were responsible for dry flower epidemics in Australia.

Pestalotiopsis (Pestalotiopsidaceae, Sordariomycetes) was reclassified in 2014 and two new genera, Neopestalotiopsis and Pseudopestalotiopsis, were introduced [17]. Molecular phylogenies based on the combined sequences of three gene regions, including the internal transcribed spacer (ITS) region of rDNA, β-tubulin (TUB), and the translation elongation factor 1-alpha (TEF1α), have been used to delimit species within the pestalotioid genera (Pestalotiopsis, Neopestalotiopsis, and Pseudopestalotiopsis) [17]. Morphological identification of pestalotioid species is unreliable as species often have overlapping conidial measurements [18,19].

Several species of Neopestalotiopsis are phytopathogens in tropical and subtropical regions, causing leaf spot, dry flower, fruit rot, fruit scab, and trunk diseases on a range of crops [5,8,20–26]. Flower and leaf diseases on macadamia caused by Neopestalotiopsis spp. have been reported in Australia [5,8], Brazil [26], and China [25]. Many new pestalotioid species have been introduced in recent years [27–33]. Many unidentified isolates of Neopestalotiopsis were obtained from macadamia racemes with dry flower symptoms. However, there is little information about their identity and the diversity of fungi that cause dry flower disease on macadamia in Australia. The aim of this study was to identify the species of Neopestalotiopsis associated with the dry flowers of macadamia in Australia.
2.2. Cultural and Morphological Studies

Colony characteristics of cultures on 1⁄2-potato dextrose agar (PDA; Difco Laboratories, Franklin Lakes, NJ, USA.) medium were recorded after 7 d incubation at 25 °C. Fungal morphology was recorded from colonies grown in the dark for 14 d at 25 °C on PDA as well as on autoclaved pine needles on water agar. Fungal structures were examined in lactic acid on slide mounts under a Leica DM5500B compound microscope (Wetzlar, Germany) with Nomarski differential interference contrast illumination, and images were taken with a Leica DFC 500 camera. Measurements of at least 30 conidia and other fungal structures were taken at 1000× magnification. Novel species were registered in MycoBank [35].

2.3. DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from approx. 40 mg mycelium from colonies grown on PDA for 14 d. The mycelium was homogenized using TissueLyser (Qiagen, Chadstone, Australia) for 2 min at 30 Hz, and DNA was extracted using the BioSprint 96 DNA Plant Kit on a robotic platform (Qiagen, Chadstone, Australia). DNA concentration was determined with a BioDrop Duo spectrophotometer (BioDrop, Cambridge, England) and adjusted to 10 ng μL⁻¹. The DNA of each isolate served as the template for the PCR amplifications using the reactions and thermal cyclic conditions described by Prasannath et al. [5]. Briefly, each reaction was performed in a 25 μL reaction volume, with 1 μL each of 10 μM forward and reverse primers, PCR reaction mix, and 2 μL of DNA template. PCR amplification was performed in SuperCycler Thermal Cycler (Kyratec, Wembley, Australia) at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Three loci, ITS, TUB, and TEF1α, were amplified and sequenced using the primer pairs ITS4/ITS5 [36], BT2A/BT2B [37], and EF1-526F/EF1-1567R [38], respectively. The quality of PCR amplicons was checked on 1% agarose gel electrophoresis stained with GelRed (Biotium, Melbourne, Australia) under UV light by Molecular Imager GelDoc (Bio-Rad Laboratories Inc., Gladesville, Australia). The targeted PCR products were purified and sequenced in both directions at Macrogen Inc. (Seoul, South Korea).

2.4. Phylogenetic Analyses

The DNA sequences were assembled in Geneious Prime v. 2021.0.3 (Biomatters Ltd., San Diego, CA, USA.), manually trimmed and aligned to produce consensus sequences for each locus. The consensus sequences generated in this study were deposited in GenBank (Table 1). The sequences were compared against the NCBI GenBank nucleotide database using BLASTn to check the closest phylogenetic matches. The sequences of the ex-type isolates of the Neopestalotiopsis species were retrieved from GenBank (Table 1) and aligned with the sequences generated from our isolates using MAFFT v. 7.3.8.8 [39] in Geneious. Ambiguously aligned positions in each multiple alignment were excluded using Gblocks v. 0.91b [40]. The concatenated three-locus sequence dataset (ITS + TEF1α + TUB) of Neopestalotiopsis consisted of 63 taxa, with the outgroup taxon Pestalotiopsis diversiseta MFLUCC 12-0287 (Table 1). The combined sequence data matrix was manually improved with BioEdit v. 7.2.5 [41] and gaps were treated as missing data. Phylogenetic trees were generated from Maximum Likelihood (ML), Bayesian Inference (BI), and Maximum Parsimony (MP) analyses.
| Species                  | Strain 1               | Host/Substrate          | Location          | GenBank Accession Numbers 2 | Reference |
|--------------------------|------------------------|-------------------------|-------------------|----------------------------|-----------|
| **Neopestalotiopsis**    |                        |                         |                   |                            |           |
| acrostichi               | MFLUCC 17–1754         | Acrostichum aureum      | Thailand          | MK764272 MK764338 MK764316 | [30]      |
| alpapicalis              | MFLUCC 17–2544         | Rhizophora mucronata     | Thailand          | MK357772 MK463545 MK463547 | [28]      |
| aotearoa                 | CBS 367.54             | Canvas                  | New Zealand       | KM199369 KM199454 KM199526 | [17]      |
| asiatica                 | MFLUCC 12–0286         | Prunus dulcis           | China             | JX398983 JX399018 JX399049 | [17]      |
| australis                | CBS 114159             | Telopea sp.             | Australia         | KM199348 KM199432 KM199537 | [17]      |
| brachiata                | MFLUCC 17–555          | Rhizophora apiculata    | Thailand          | MK764274 MK764340 MK764318 | [30]      |
| brasiliensis             | COAD 2166              | Psidium guajava         | Brazil            | MG686469 MG692400 MG692402 | [42]      |
| cavernicola              | KUMCC 20–0269          | Cave                    | China             | MW545802 MW557596 MW550735 | [29]      |
| chiangmaiensis           | MFLUCC 18–0113         | Pandanus sp.            | Thailand          | N/A MH141275 MH1388404     | [43]      |
| chrysea                  | MFLUCC 12–0261         | Dead leaves             | China             | JX398985 JX399020 JX399051 | [19]      |
| clavispora               | MFLUCC 12–0281         | Magnolia sp.            | China             | JX398979 JX399014 JX399045 | [19]      |
| cocoes                   | MFLUCC 15–0152         | Cocos nucifera          | Thailand          | KX789687 N/A KX789689      | [44]      |
| cubana                   | CBS 600.96             | Leaf Litter             | Cuba              | KM199347 KM199438 KM199521 | [17]      |
| dendorbi                 | MFLUCC 14–0106         | Dendrobiun cariniferum  | Thailand          | MK993571 MK975835 MK975829 | [45]      |
| drenthii                 | BRIP 72263a            | Macadamia integrifolia  | Australia         | MZ203786 MZ312679 MZ344171 | This study|
|                         | BRIP 72264a            | Macadamia integrifolia  | Australia         | MZ203787 MZ312680 MZ344172 | This study|
| egypiaca                 | CBS H–22294            | Mangifera indica        | Egypt             | KP943747 KP943746 KP943748 | [46]      |
| ellipsoidospora          | MFLUCC 12–0283         | Dead plant material     | China             | JX398980 JX399016 JX399047 | [19]      |
| eucalypticola            | CBS 264.37             | Eucalyptus globulus     | N/A               | KM199376 KM199431 KM199551 | [17]      |
| foedans                  | CGMCC 3.9123           | Mangrove plant          | China             | JX398987 JX399022 JX399053 | [19]      |
| formicidarum             | CBS 362.72             | Dead Formicidae (ant)   | Cuba              | KM199358 KM199455 KM199517 | [17]      |
| hydnorae                | EHJ6a                  | Cattleya jongheana      | Brazil            | MK454709 MK465120 MK465122 | [27]      |
| honoluliana              | CBS 114495             | Telopea sp.             | USA               | KM199364 KM199457 KM199548 | [17]      |
| hydorea                  | MFLUCC 20–0132         | Artocarpus heterophyllus| Thailand          | MW266069 MW251119 MW251129 | [32]      |
| iranensis                | CBS 137768             | Fragaria ananassa       | Iran              | KM074048 KM074057 KM074051 | [20]      |
| iranensis                | CBS 257.31             | Cocos nucifera          | Java              | KM199357 KM199437 KM199543 | [17]      |
| macadamiae              | BRIP 6373c             | Macadamia sp.           | Australia         | KX186604 KX186654 KX186627 | [8]       |
| maddoxii                 | BRIP 72260a            | Macadamia integrifolia  | Australia         | MZ203780 MZ312673 MZ344165 | This study|
|                         | BRIP 72262a            | Macadamia integrifolia  | Australia         | MZ203781 MZ312674 MZ344166 | This study|
|                         | BRIP 72266a            | Macadamia integrifolia  | Australia         | MZ203782 MZ312675 MZ344167 | This study|

Table 1. Neopestalotiopsis species and isolates used in the phylogenetic analyses, with GenBank accession numbers.
Table 1. Cont.

| Species          | Strain 1 | Host/Substrate          | Location      | GenBank Accession Numbers         | Reference |
|------------------|----------|-------------------------|---------------|-----------------------------------|-----------|
| N. magna         | MFLUCC 12–652 T | Macadamia integrifolia | Australia     | MZ303783 MZ312676 MZ344168       | This study |
| N. mesopotamica  | CBS 336.86 T | Pteridium sp.          | France        | MZ303784 MZ312677 MZ344169       | This study |
| N. musae         | MFLUCC 15–0776 T | Musa sp.              | Iraq          | MZ303785 MZ312678 MZ344170       | This study |
| N. natalensis    | CBS 138.41 T | Acacia mollissima      | Thailand      | MZ303790 MZ312683 MZ344175       | This study |
| N. nebuloides    | BRIP 66617 T | Sporobolus elongatus   | Australia     | MZ303791 MZ312684 MZ344176       | This study |
| N. olumideae     | BRIP 72273a T | Macadamia integrifolia | Australia     | MZ303794 MZ312685 MZ344177       | This study |
| N. pandanicola   | KUMCC 17–0175 | Pandanus sp.           | China         | MZ303792 MZ312686 MZ344178       | This study |
| N. pernambucana  | URM7148 | Vismia guianensis      | Brazil        | MZ303793 MZ312687 MZ344179       | This study |
| N. petila        | MFLUCC 17–1737 T | Rhizophora mironutana | Thailand      | MZ303795 MZ312688 MZ344180       | This study |
| N. phangnaengsis | MFLUCC 18–0119 T | Pandanus sp.       | Thailand      | MZ303796 MZ312689 MZ344181       | This study |
| N. piceana       | CBS 394.48 T | Picea sp.              | UK            | MZ303797 MZ312690 MZ344182       | This study |
| N. proteaurum    | CBS 114178 T | Leuocospernum cuneiforme | Zimbabwe    | MZ303798 MZ312691 MZ344183       | This study |
| N. rhizophorae   | MFLUCC 17–1551 T | Macadamia integrifolia | China         | MZ303799 MZ312692 MZ344184       | This study |
| N. rosae         | CBS 101057 T | Rosa sp.               | New Zealand   | MZ303800 MZ312693 MZ344185       | This study |
| N. roscica       | CFCC 51992 T | Rosa chinensis         | China         | MZ303801 MZ312694 MZ344186       | This study |
| N. samarangensis | CBS 115451 | Unidentified tree      | China         | MZ303802 MZ312695 MZ344187       | This study |
| N. saprophytica  | MFLUCC 12–028 T | Magnolia sp.        | China         | MZ303803 MZ312696 MZ344188       | This study |
| N. sicluanensis  | CFCC 54338 T | Castanea mollissima   | China         | MZ303804 MZ312697 MZ344189       | This study |
| N. sonneratae    | MFLUCC 17–1744 T | Sonneronata alba    | Thailand      | MZ303805 MZ312698 MZ344190       | This study |
| N. steyaertii    | IMI 192475 T | Eucalyptus viminalis  | Australia     | MZ303806 MZ312699 MZ344191       | This study |
| N. surinamensis  | CBS 450.74 T | Elaeis guineensis     | Suriname      | MZ303807 MZ312700 MZ344192       | This study |
| N. thailandica   | MFLUCC 17–1730 T | Rhizophora mironutana | Thailand      | MZ303808 MZ312701 MZ344193       | This study |
| N. umbrinospora  | MFLUCC 12–0285 T | Unidentified plant    | China         | MZ303809 MZ312702 MZ344194       | This study |
| N. vheenae       | BRIP 72293a T | Macadamia integrifolia | Australia     | MZ303810 MZ312703 MZ344195       | This study |
| N. vitis         | MFLUCC 15–1265 T | Vitis vinifera      | China         | MZ303811 MZ312704 MZ344196       | This study |
| N. zakeelii      | BRIP 72271a T | Macadamia integrifolia | Australia     | MZ303812 MZ312705 MZ344197       | This study |
|                 | BRIP 72282a T | Macadamia integrifolia | Australia     | MZ303813 MZ312706 MZ344198       | This study |
Table 1. Cont.

| Species                        | Strain 1            | Host/Substrate          | Location       | GenBank Accession Numbers 2 | Reference |
|--------------------------------|---------------------|-------------------------|----------------|-----------------------------|-----------|
| *N. zimbabwana*                | CBS 111495 T        | *Leucospermum cunciforme* | Zimbabwe       | JX556231 KM199456 KM199545  | [17]      |
| *Pestalotiopsis diversiseta*   | MFLUCC 12–0287 T    | Dead plant material     | China          | JX399009 JX399040 JX399073  | [19]      |

1 BRIP: Queensland Plant Pathology Herbarium, Australia; CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CFCC: China Forestry Culture Collection Center, Research Institute of Forest Ecology, Environment and Protection, Beijing, China; CGMCC: China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; COAD: Culture collection Coleç~ão Octávio Almeida Drummond of the Universidade Federal de Viçosa, Viçosa, Brazil; HGUP: Plant Pathology Herbarium of Guizhou University, Guizhou, China; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; KUMCC: Culture Collection of Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; URM: The Father Camille Torrend Herbarium, Pernambuco, Brazil. 2 ITS: internal transcribed spacer; TUB: β-tubulin; TEF1α: translation elongation factor 1-α. 3 as *Neopestalotiopsis clavispora*. Ex-type strains are labeled with T. N/A: Not available.
ML analysis was implemented using RAxML v. 8.2.11 [50] in Geneious. The search option was set to rapid bootstrapping, and the analysis was run using the GTR-GAMMAI evolution model with 1000 bootstrap iterations. BI analysis was conducted with MrBayes v. 3.2.1 [51] in Geneious to calculate posterior probabilities by the Markov Chain Monte Carlo (MCMC) method. The GTR-GAMMAI nucleotide substitution model was applied in BI analysis. Four MCMC chains were run simultaneously, starting from random trees for 1,000,000 generations. The temperature of the heated chain was set to 0.15 and trees were sampled every 200 generations until the average standard deviation of split frequencies reached 0.01 (stop value). Burn-in was set at 25%, after which the likelihood values were stationary. MP analysis was performed with PAUP v. 4.0b10 [52]. Trees were inferred using a heuristic search strategy, with 100 random stepwise addition and tree-reconnection (TBR) branch swapping. Max-trees were set to 5000 and bootstrap support values were evaluated for tree branches with 1000 replications [53]. Phylograms were visualized in FigTree v. 1.4.4 [54] and annotated in Adobe Illustrator 2021.

The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept and a pairwise homoplasy index (PHI) test were used to determine species boundaries [55]. The PHI test was performed using SplitsTree4 v. 4.17.1 [56] to determine the recombination level within phylogenetically closely related species. The concatenated three-locus dataset (ITS + TEF1α + TUB) was used for the analyses. PHI test results (Fw) >0.05 indicated no significant recombination within the dataset. The relationships between closely related taxa were visualized in split graphs with both the Log-Det transformation and splits decomposition options.

3. Results

3.1. Phylogenetic Analyses

The concatenated sequence data matrix comprised 1367 base pairs (bp) (476 for ITS, 464 for TEF1α, and 427 for TUB), of which 935 bp were constant, 238 bp were parsimony-uninformative, and 174 bp were parsimony-informative. ML analysis yielded a best scoring tree, with a final ML optimization value of $-6493.745$ and the following model parameters: alpha—0.597, $\Pi$(A)—0.231, $\Pi$(C)—0.276, $\Pi$(G)—0.218, and $\Pi$(T)—0.274. Similar tree topologies were obtained by ML, BI, and MP methods, and the best scoring ML tree is shown in Figure 2. ML bootstrap values, BI posterior probabilities, and MP bootstrap values (MLBS/BIPP/MPBS) are given at nodes of the phylogram (Figure 2). The phylogenetic tree inferred from the concatenated alignment resolved the 13 Neopestalotiopsis isolates from symptomatic macadamia inflorescences (dry flower disease) into five well-supported monophyletic clades that represent novel species of Neopestalotiopsis (Figure 2).

3.2. Taxonomy

Neopestalotiopsis drenthii Prasannath, Akinsanmi & R.G. Shivas, sp. nov. (Figure 3). MycoBank: MB840916.

Etymology: Named after Andre Drenth, in recognition of his many contributions to the study of tropical and subtropical plant diseases.

Type: AUSTRALIA, Queensland, Mackay, from flower blight of M. integrifolia, 3 October 2019, O.A. Akinsanmi (Holotype BRIP 72264a, includes ex-type culture). GenBank: MZ303787 (ITS); MZ312680 (TUB); MZ344172 (TEF1α).
Figure 2. Maximum Likelihood tree topology of Neopestalotiopsis based on a combined multi-locus alignment (ITS + TEF1α + TUB). Pestalotiopsis diversiseta (MFLUCC 12-0287) was used as an outgroup taxon. Maximum Likelihood bootstrap support values (>50%), Bayesian Inference posterior probabilities (>90%), and Maximum Parsimony bootstrap proportions (>50%) are displayed at the nodes, respectively. Strains of the newly described species are depicted in red.
3.2. Taxonomy

Neopestalotiopsis drenthii Prasannath, Akinsanmi & R.G. Shivas, sp. nov. (Figure 4).

Description: Conidiomata pycnidial on PDA, globose, 200–400 μm diam., solitary or aggregated in clusters, exudes black conidial masses. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform, hyaline, smooth, 5–20 × 2–5 μm. Conidia fusiform to ellipsoidal, straight or curved, 24–30 × 7–9 μm, 4-septate; basal cell conical, 4–6.5 μm, hyaline, smooth, thin-walled; with a single appendage filiform, unbranched, centric, 4–7 μm long; three median cells doliiform, 16–19 μm, smooth, versicolored, septa darker than the rest of the cell (second cell from base pale brown, 3.5–6.5 μm long; third cell medium to dark brown, 3.5–6.5 μm long; fourth cell medium to dark brown, 4–6 μm long); apical cell conical to subcylindrical, 3–5 μm long, hyaline, smooth, thin-walled; with 2–3 apical tubular appendages unbranched, filiform, 15–22 μm long. Sexual morph not seen.

Culture characteristics: Colonies on PDA after 7 d at 25 °C reach 80 mm diam., producing white aerial mycelia with copious pycnidia after two weeks; reverse cream.

Habitat and distribution: Racemes of M. integrifolia (Proteaceae); Australia.

Other material examined: AUSTRALIA, Queensland, Mackay, from flower blight of M. integrifolia, 3 October 2019, O.A. Akinsanmi (living culture, BRIP 72263a).

Notes: Neopestalotiopsis drenthii is closely related to N. surinamensis. A pairwise nucleotide comparison between N. drenthii ex-type strain (BRIP 72264a) and N. surinamensis ex-type strain (CBS 450.74) showed 2 bp differences (Identities 534/536, 2 gaps) in ITS, 0 bp differences (Identities 428/428, no gaps) in TUB, and 7 bp differences (Identities 474/481, no gaps) in TEF1α sequences in GenBank. Neopestalotiopsis drenthii and N. surinamensis have similar sized conidia, but tubular apical appendages of N. drenthii are shorter than the 18–27 μm of N. surinamensis [17].

Neopestalotiopsis maddoxii Prasannath, Akinsanmi & R.G. Shivas, sp. nov. (Figure 4).

MycoBank: MB840917.

Etymology: Named after Craig Maddox, in recognition of his research contributions to macadamia crop protection in Australia.

Type: AUSTRALIA, Queensland, Alloway, from flower blight of M. integrifolia, 22 Sep. 2019, Tim O’Dale (Holotype BRIP 72266a, includes ex-type culture). GenBank: MZ303782 (ITS); MZ312675 (TUB); MZ344167 (TEF1α).

Description: Conidiomata pycnidial on PDA, globose, 200–500 μm diam., solitary or aggregated in clusters, exudes dark slimy conidial droplets. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform, hyaline, smooth, 5–15 × 2–5 μm. Conidia fusiform to clavate, straight or curved, 25–30 × 7–11 μm, 4-septate; basal cell conical, 3–5.5 μm, hyaline, smooth, thin-walled; with a single appendage filiform, unbranched, centric, 4–7 μm long; three median cells doliiform, 18–23 μm, smooth, versicolored, septa darker than the rest of the cell (second cell from base pale brown, 4.5–7.5 μm long; third cell medium to dark brown, 4.5–7.5 μm long; fourth cell medium to dark brown, 5–7 μm

Figure 3. Neopestalotiopsis drenthii (BRIP 72264a). (a) Two-week-old colony on PDA, (b) conidiomata on pine needle agar, and (c) conidia. Scale bars: a = 1 cm; b = 1 mm; c = 10 μm.
long); apical cell subcylindrical, 3–5 µm long, hyaline, smooth, thin-walled; with 3 apical tubular appendages unbranched, filiform, 15–27 µm long. Sexual morph not seen.

Figure 4. Neopestalotiopsis maddoxii (BRIP 72266a). (a) Two-week-old colony on PDA, (b) conidiomata on pine needle agar, and (c) conidia. Scale bars: a = 1 cm; b = 1 mm; c = 10 µm.

Culture characteristics: Colonies on PDA after 7 d at 25 °C reach 70 mm diam., with cream aerial mycelium, forming abundant pycnidia near the center after two weeks; reverse cream to buff.

Habitat and distribution: Racemes of *M. integrifolia* (Proteaceae); Australia.

Other material examined: AUSTRALIA, Queensland, Nambour, from flower blight of *M. integrifolia*, 22 Aug. 2019, K. Prasannath (living culture, BRIP 72260a); Bundaberg, from flower blight of *Macadamia sp.*, 25 August 2019, O.A. Akinsanmi (living culture, BRIP 72262a); Gympie, from flower blight of *M. integrifolia*, 12 September 2019, M. Boote (living culture, BRIP 72272a); Maleny, from flower blight of *M. integrifolia*, 20 September 2019, O.A. Akinsanmi (living cultures, BRIP 72275a and BRIP 72284a).

Notes: Neopestalotiopsis maddoxii was sister to a clade containing *N. javaensis*, *N. mesopotamica*, and *N. rosae*. Neopestalotiopsis maddoxii differed from *N. javaensis* in ITS (Identities 534/539, 3 gaps); TUB (Identities 429/431, no gaps); TEF1α (Identities 480/486, 4 gaps). Neopestalotiopsis maddoxii differed from *N. mesopotamica* in ITS (Identities 534/539, 3 gaps); TUB (Identities 410/414, no gaps); TEF1α (Identities 466/472, no gaps). Neopestalotiopsis maddoxii differed from *N. rosae* in ITS (Identities 534/539, 3 gaps); TUB (Identities 430/432, no gaps); TEF1α (Identities 478/481, no gaps). Neopestalotiopsis maddoxii is morphologically indistinguishable from *N. javaensis*, *N. mesopotamica*, and *N. rosae* [17].

Neopestalotiopsis olumideae Prasannath, Akinsanmi & R.G. Shivas, sp. nov. (Figure 5). MycoBank: MB840918.

Etymology: Named after Olumide Jeff-Ego, in recognition of her research contributions to macadamia diseases in Australia.

Type: AUSTRALIA, Queensland, Maleny, from flower blight of *M. integrifolia*, 20 Sep. 2019, O.A. Akinsanmi (Holotype BRIP 72273a, includes ex-type culture). GenBank: MZ303790 (ITS); MZ312683 (TUB); MZ344175 (TEF1α).

Description: Conidiomata pycnidial on PDA, globose, 200–400 µm diam., mostly solitary. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform to cylindrical, hyaline, smooth, 5–15 × 2–5 µm. Conidia fusiform to ellipsoidal, straight or curved, 27–31 × 7–9 µm, 4-septate; basal cell conical, 4.5–7 µm, hyaline, smooth, thin-walled; with a single appendage filiform, unbranched, centric, 3–5 µm long; three median cells doliform, 18–20 µm, smooth, versicolored, septa darker than the rest of the cell (second cell from base pale brown, 4–7 µm long; third cell medium to dark brown, 4–7 µm long; fourth cell medium to dark brown, 4.5–6.6 µm long); apical cell conical to subcylinder-
drical, 3.5–5.5 μm long, hyaline, smooth, thin-walled; with 2–3 apical tubular appendages unbranched, filiform, 8–17 μm long. Sexual morph not seen.

**Figure 5.** Neopestalotiopsis olumideae (BRIP 72273a). (a) Two-week-old colony on PDA, (b) conidiogenous cells, and (c) conidia. Scale bars: a = 1 cm; b–c = 10 μm.

**Culture characteristics:** Colonies on PDA after 7 d at 25 °C reach 60 mm diam., with whitish cottony aerial mycelium; reverse cream to buff.

**Habitat and distribution:** Racemes of *M. integrifolia* (Proteaceae); Australia.

**Other material examined:** AUSTRALIA, Queensland, Maleny, from flower blight of *M. integrifolia*, 20 September 2019, O.A. Akinsanmi (living culture, BRIP 72283a).

**Notes:** Neopestalotiopsis olumideae was phylogenetically close to *N. protearum*, *N. acrostichi* and *N. pernambucana*. Neopestalotiopsis olumideae differed from *N. protearum* in ITS (Identities 517/521, no gaps); TUB (Identities 436/438, no gaps); TEF1α (Identities 469/475, 3 gaps). Neopestalotiopsis olumideae differed from *N. acrostichi* in ITS (Identities 506/506, no gaps); TUB (Identities 435/436, no gaps); TEF1α (Identities 464/471, 1 gap). Neopestalotiopsis olumideae is morphologically indistinguishable from *N. protearum*, *N. acrostichi*, and *N. pernambucana* [17,30].

**Neopestalotiopsis vheenae** Prasannath, Akinsanmi & R.G. Shivas, sp. nov. (Figure 6).

**Figure 6.** Neopestalotiopsis vheenae (BRIP 72293a). (a) Two-week-old colony on PDA, (b) conidiomata on pine needle agar, and (c) conidia. Scale bars: a = 1 cm; b = 1 mm; c = 10 μm.
MycoBank: MB840919.

**Etymology:** Named after Vheena Mohankumar, in recognition of her research studies into fungal diseases of macadamia crops in Australia.

**Type:** AUSTRALIA, New South Wales, Rosebank, from flower blight of *M. integrifolia*, 16 October 2019, *P. Fraser* (Holotype BRIP 72293a, includes ex-type culture). GenBank: MZ303792 (ITS); MZ312685 (TUB); MZ344177 (TEF1α).

**Description:** Conidiomata pycnidial on PDA, globose, 200–500 µm diam., solitary or aggregated in clusters, exudes black slimy conidial droplets. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform to cylindrical, hyaline, smooth, 5–10 × 3–5 µm. Conidia fusiform to clavate, straight or curved, 22–26 × 8–11 µm, 4-septate; basal cell conical, 3–5 µm, hyaline, smooth, thin-walled; with a single appendage filiform, unbranched, centric, 4–6.5 µm long; three median cells doliiform, 17–20 µm, smooth, versicolored, septa darker than the rest of the cell (second cell from base pale brown, 4–7 µm long; third cell medium to dark brown, 4–7 µm long; fourth cell medium to dark brown, 4.5–6.6 µm long); apical cell conical to subcylindrical, 3–4.5 µm long, hyaline, smooth, thin-walled; mostly with 3 apical tubular appendages unbranched, filiform, 15–25 µm long. Sexual morph not seen.

**Culture characteristics:** Colonies on PDA after 7 d at 25 °C reach 80 mm diam., with white dense aerial mycelium, pycnidia abundant; reverse buff.

**Habitat and distribution:** Racemes of *M. integrifolia* (Proteaceae); Australia.

**Notes:** *Neopestalotiopsis vheenae* ex-type strain (BRIP 72293a) had identical ITS, TUB, and TEF1α sequences to isolate BRIP 70210, which was previously identified as *N. clavispora* [5]. *Neopestalotiopsis vheenae* causes yellow halo spot of macadamia in Australia [5]. In this phylogenetic analysis, *N. vheenae* was sister to *N. sichuanensis*. BLASTn searches in GenBank showed that *N. vheenae* ex-type (BRIP 72293a) differed from *N. sichuanensis* ex-type (CFCC 5338) by 1 bp (Identities 510/511, no gaps) in ITS, 1 bp (Identities 422/423, no gaps) in TUB, and 10 bp (Identities 482/492, 6 gaps) in TEF1α sequences. *Neopestalotiopsis vheenae* is morphologically indistinguishable from *N. sichuanensis* [33].

**Neopestalotiopsis zakeelii** Prasannath, Akinsanmi & R.G. Shivash, sp. nov. (Figure 7).

![Figure 7. Neopestalotiopsis zakeelii (BRIP 72282a). (a) Two-week-old colony on PDA, (b) conidiomata on pine needle agar, and (c) conidia. Scale bars: a = 1 cm; b = 1 mm; c = 10 µm.](image)

MycoBank: MB840920.

**Etymology:** Named after Mohamed Cassim Mohamed Zakeel, in recognition of his research into the diagnosis of new and emerging diseases on macadamia in Australia.

**Type:** AUSTRALIA, Queensland, Landsborough, from flower blight of *M. integrifolia*, 20 September 2019, A. Woodford (Holotype BRIP 72282a, includes ex-type culture). GenBank: MZ303789 (ITS); MZ312682 (TUB); MZ344174 (TEF1α).
Description: Conidiomata pycnidial on PDA, globose, 100–250 µm diam., mostly solitary. Conidiophores reduced to conidiogenous cells. Conidiogenous cells ampulliform, hyaline, smooth, 5–20 × 2–5 µm. Conidia fusiform to ellipsoidal, straight or curved, 27–33 × 7–9 µm, 4-septate; basal cell conical, 4.5–7 µm, hyaline, smooth, thin-walled; with a single appendage filiform, unbranched, centric, 7–10 µm long; three median cells doliiform, 17–22 µm, smooth, versicolored, septa darker than the rest of the cell (second cell from base pale brown, 4.5–7.5 µm long; third cell medium to dark brown, 4.5–7.5 µm long; fourth cell medium to dark brown, 5–7 µm long); apical cell conical to subcylindrical, 3–5.5 µm long, hyaline, smooth, thin-walled; with 2–3 apical tubular appendages unbranched, filiform, 25–37 µm long. Sexual morph not seen.

Culture characteristics: Colonies on PDA after 7 d at 25 °C reach 55 mm diam., with white sparse aerial mycelium; reverse cream.

Habitat and distribution: Racemes of M. integrifolia (Proteaceae); Australia.

Other material examined: AUSTRALIA, Queensland, Nambour, from flower blight of M. integrifolia, 22 August 2019, K. Prasannath (living culture, BRIP 72271a).

Notes: Neopestalotiopsis zakeelii is most closely related to N. vitis and N. australis. Neopestalotiopsis zakeelii (ex-type: BRIP 72282a) differed from N. vitis (ex-type: MFLUCC 15-1265) by 4 bp (Identities 416/420, no gaps) in ITS; 0 bp (Identities 319/319, no gaps) in TUB; 4 bp (Identities 369/373, no gaps) in TEF1α. Neopestalotiopsis zakeelii differed from N. australis (ex-type: CBS 114159) by 4 bp (Identities 526/530, no gaps) in ITS; 3 bp (Identities 430/433, no gaps) in TUB; 6 bp (Identities 478/484, no gaps) in TEF1α. Neopestalotiopsis australis, N. vitis, and N. zakeelii are morphologically indistinguishable [17,22].

There was no evidence of significant genetic recombination (Fw > 0.05) between the novel species of Neopestalotiopsis and closely related species (Figure 8). The results confirmed that these taxa were significantly different from the existing species of Neopestalotiopsis.
Figure 8. Split graphs showing the result of PHI test of (a) Neopestalotiopsis drenthii (Fw = 0.735), (b) N. maddoxii (Fw = 0.118), (c) N. olumideae (Fw = 0.581), (d) N. vheenae (Fw = 0.876), and (e) N. zakeelii (Fw = 0.412) with their most closely related species using Log-Det transformation and splits decomposition options. The new taxon in each graph is shown in red font.

4. Discussion

Five novel species, Neopestalotiopsis drenthii, N. maddoxii, N. olumideae, N. vheenae, and N. zakeelii, were discovered in isolates obtained from macadamia inflorescences with dry flower disease and subsequently described. There was no evidence of significant genetic recombination events between these species and their closest relatives.

Pestalotioid fungi (Pestalotiopsidaceae, Sordariomycetes) are species-rich asexual taxa, which are common pathogens on many crops [30,32,57]. Multi-locus phylogenetic analyses segregated Neopestalotiopsis and Pseudopestalotiopsis from Pestalotiopsis [17]. These three genera are morphologically similar in having 5-celled conidia with tubular apical appendages [17]. Norphanphoun et al. [30] found that concatenated gene sequences of ITS, TUB, and TEF1α resolved Pestalotiopsis and Pseudopestalotiopsis, while additional genes may be required to provide a better delimitation of Neopestalotiopsis spp.
There were 49 species names recognized in Neopestalotiopsis [58] prior to the five species described in this study, Neopestalotiopsis drenthii, N. maddoxii, N. olumideae, N. vheenae, and N. zakeelii were described and illustrated. These fungi were isolated from inflorescences of macadamia with dry flower disease in Australia. The role that N. drenthii, N. maddoxii, N. olumideae, N. vheenae, and N. zakeelii play in dry flower disease of macadamia has yet to be determined. Hence, the pathogenicity of these novel species on macadamia racemes should be examined. Living cultures of the fungi are available for further study.

5. Conclusions

Five fungal species, Neopestalotiopsis drenthii, N. maddoxii, N. olumideae, N. vheenae, and N. zakeelii, were described and illustrated. These fungi were isolated from inflorescences of macadamia with dry flower disease in Australia. The role that Neopestalotiopsis drenthii, N. maddoxii, N. olumideae, N. vheenae, and N. zakeelii play in dry flower disease of macadamia has yet to be determined. Hence, the pathogenicity of these novel species on macadamia racemes should be examined. Living cultures of the fungi are available for further study.

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