Description of *Pseudopestalotiopsis kubahensis* sp. nov., a new species of microfungi from Kubah National Park, Sarawak, Malaysia

*Lateef AA[1,2] , Sepiah M [1] and Bolhassan MH[1]

[1]Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.
[2]Department of Plant Biology, Faculty of Life Science, University of Ilorin, Nigeria.

Abstract

A survey on the diversity and distribution of microfungi was carried out, during which a distinct *Pestalotiopsis*-like taxa was isolated from green leaves of *Macaranga* sp. from Kubah National Park, Sarawak. The phylogenetic analysis of DNA sequences from the internal transcribed spacer gene region (ITS1, 5.8S and ITS2) of the rDNA shows this species to form a distinct clade from the *Pestalotiopsis*, and cluster with the genus *Pseudopestalotiopsis*, a new genus which was recently carved out from the *Pestalotiopsis*. This species differs from closely related *Pseudopestalotiopsis* species such as *Ps. cocos*, *Ps. indica* and *Ps. theae* by its conidial characters such as its unknobbed apical appendages and shorter basal appendages. The new microfungal species, *Ps. kubahensis* is hereby described based on morphological and molecular data as the fifth species in the genus *Pseudopestalotiopsis*.

Introduction

The genus *Pseudopestalotiopsis* was recently carved out of *Pestalotiopsis* Steyaert, which was recently reviewed, which resulted in two other genera being separated from *Pestalotiopsis*, namely, *Neopestalotiopsis* and *Pseudopestalotiopsis* (Maharachchikumbura et al. 2014). The colour of the median cells have consistently been observed as the major delimiting character with which species cluster together in *Pestalotiopsis* into three main clades, as lightly-coloured concolourous median cells, versicolourous median cells and darkly-coloured concolourous median cells (Maharachchikumbura et al. 2014; Song, Maharachchikumbura, et al. 2014), then followed by the other conidial characters, as observed in molecular studies. It was based on this three different pigmentation patterns that the genus *Pestalotiopsis* was split into three genera as, i) the genus *Pestalotiopsis*; to accommodate species with lightly-pigmented concolourous median cells, ii) the genus *Neopestalotiopsis*; to accommodate species with versicolourous median cells, and iii) the genus *Pseudopestalotiopsis*; for species with dark concolourous median cells (Maharachchikumbura et al. 2014). In line with the recent re-classification, we describe in this paper a new species of *Pseudopestalotiopsis* isolated from *Macaranga* sp., a dominant plant species of the lowland dipterocarp forest in Sarawak, Malaysia.

Materials and methods

Sample collection and Isolation of the microfungal isolates – Green leaves of *Macaranga* sp. were collected from Kubah National park at an elevation of 410 m above sea level, $01^{o}36'081''$ N, $110^{o}11'204''$ E, placed in a labelled plastic bag and transported to the laboratory. The leaves were processed for isolation of epiphytic microfungi as described by Lateef et al. (2014). The leaf samples were plated on plain water agar and incubated at room temperature (27 ± 2 ^{o}C) for four weeks. The emerging microfungal colonies were recorded and obtained into pure culture on Potato dextrose agar (PDA). Permanent slides were prepared and pictures were taken with an Olympus DP72 camera attached to an Olympus BX50 microscope. Measurements of 25 conidia were recorded. Cultural characteristics of the isolates at room temperature were also recorded and colony pictures were taken with a hand-held Samsung ES91 camera.

DNA extraction, Polymerase Chain Reaction (PCR) amplification and DNA sequencing – The total genomic DNA was extracted using the CTAB method (Murray and Thompson 1980). The extracted DNA was confirmed for successful extraction by gel electrophoresis. The internal transcribed spacer (ITS) gene regions were amplified by PCR using the primer pairs ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) in a SensQuest lab cycler. A PCR mixture of 25 µL total volume was used. The master mix contains 5 µL of 5 X Mg free-PCR buffer, 0.15 µL of MgCl$_2$, 1.5 µL of dNTPs, 1 µL of each primers, 0.15 µL of Fementas Taq DNA polymerase and 12.85 µL of double-sterilised distilled water (ddH$_2$O) and 1.5 µL of DNA template including a control reaction with ddH$_2$O as template instead of DNA. The PCR programme was set at initial denaturation of 2 min at 94 ^{o}C, followed by 35 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, 72 ^{o}C for 1 min and final extension of 72 ^{o}C for 10 min. The PCR products were gel electrophoresed to verify successful amplification as done for genomic DNA. Successful PCR products were sent to a private sequencing company (1st -Base, Asia) for both forward and reverse primer sanger sequencing.

Phylogenetic analysis – The sequences obtained from the sequencing company were confirmed to belong to the proposed microfungal genus using BLAST-n search on the GenBank website. SeqTrace version 0.9.0 software (Stucky 2012) was used to obtain a consensus sequence from the forward and reverse sequences. The sequence obtained was submitted to GenBank to obtain an accession number. Similar reference sequences were downloaded from GenBank for phylogenetic analysis following a similarity search. Sequences were aligned using MAFFT alignment software (Katoh and Standley 2013) and adjusted using Gblock (Castresana 2000; Talavera and Castresana 2007).

In total, 18 strains belonging to 13 species (Table 1) were used for the phylogenetic analysis. A maximum parsimony analysis using PAUP (Phylogenetic Analysis Using Parsimony) software v.4.0b10 (Swofford 2002) was done. All characters were equally weighted and gaps were treated as missing data. Parsimonious trees were inferred using the heuristic search option with Tree-Bisection Reconnection (TBR) branch swapping and 1,000 random sequence additions. Maximum trees were set up to 10,000 with auto-increase option, branches of zero length were collapsed and all multiple parsimonious trees were saved. The robustness of the most parsimonious tree was evaluated by 1,000 bootstrap replications resulting from maximum parsimony analysis, each with 10 replicates of random stepwise addition of taxa (Felsenstein 1985). Tree descriptive statistics, such as tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for each Maximum Parsimonious Tree (MPT) generated. The Kishino-Hasegawa tests (Kishino and Hasegawa 1989) were performed to determine whether the trees inferred under different optimality criteria were significantly different.

In addition, Bayesian analysis using MrBayes software v.3.1.2 (Ronquist and Huelsenbeck 2003) was also done. Suitable models of nucleotide substitution were first selected using MrModeltest v.2 (Nylander 2004). The GTR model (no rate variation) was selected for the ITS sequences and then used for the Bayesian inference analysis of four simultaneous Markov Chains Monte Carlo (MCMC) chains for 2 000 000 generations and trees were sampled every 1000th generation. The trees were viewed and annotated in TreeGraph 2 (Stöver and Müller 2010).

Table 1 Strains used in the phylogenetic analysis with their GenBank accession numbers

S/N	Species	Isolate/strain	Host	GenBank accession number (ITS)
1.	*Neopestalotiopsis natalensis*	CBS 138.41	*Acacia mollissima*	KM199377
2.	*Pestalosphaeria hansenii*	ATCC 48245	*Pinus carribaea*	AF377290
3.	*Pestalotiopsis diploclisia*	CBS 115449	*Psychotria tutcheri*	KM199314
4.	*Pestalotiopsis diploclisia*	CBS 115585	*Diploclisia glaucescens*	KM199315
5.	*Pestalotiopsis disseminata*	CY152	Nests of insects	HQ607992
6.	*Pestalotiopsis kenyana*	CBS 911.96	Raw material from agar agar	KM199303
7.	*Pestalotiopsis neglecta*	CCTU 12	Rock sample	JX854541
8.	*Pestalotiopsis telopeae*	CBS 114137	*Protea neriifolia*	KM199301
9.	*Pestalotiopsis theae*	CMU ELA1	*Elaeis guineensis*	JX205216
10.	*Pestalotiopsis vismiae*	RCEF6350	*Carya cathayensis*	KM015217
11.	*Pseudopestalotiopsis cocos*	CBS 272.29	*Cocos nucifera*	KM199378 1
12.	*Pseudopestalotiopsis indica*	CBS 459.78	*Hibiscus rosa-sinensis*	KM199381 1
13.	*Pseudopestalotiopsis kubahensis* (this study)	UMAS KUB-P20	*Macaranga* sp.	KT006749
14.	*Pseudopestalotiopsis theae*	SC011	*Camellia sinensis*	JQ683726
15.	*Pseudopestalotiopsis theae*	MFLUCC 12-0055	*Camellia sinensis*	JQ683727
16.	*Pseudopestalotiopsis theae*	BPC50	Mango leaf	KM510412
17.	*Pseudopestalotiopsis theae*	USM5-5	*Musa* spp	KM111476
18	*Seiridium cardinale*	ICMP 7323	*Cupressocyparis leylandii*	AF409995

Results

Molecular phylogenetic analysis − The data matrix for the phylogenetic analysis consisted of 513 characters including gaps treated as missing data, 432 characters are constant, 35 variable characters are parsimony-uninformative and 46 characters are parsimony informative. The parsimony analysis yielded 1365 Most Parsimonious Trees (MPT), and the best tree (TL = 98, CI = 0.918, HI = 0.0816, RI = 0.9712 and RC = 0.8919) is presented (Fig. 2) showing that the strains were separated mainly into three clades corresponding to the pigmentation of the three median cells.

Pseudopestalotiopsis kubahensis A.A. Lateef, M. Sepiah & M.H. Bolhassan, sp. **nov.** Fig. 1
MycoBank − **MB 812829**
Facesoffungi number: FoF 01310

Etymology − Named after the name of the location of isolation; Kubah National Park in Sarawak Malaysia.

Sexual state − unknown.

Asexual state − *Conidiomata* pycnidial, globose to subglobose, black to dark brown, immersed or sub-immersed; exuding black to dark brown mass of conidia. *Conidiophores* indistinct, reduced to conidiogenous cells. *Conidiogenous cells* discrete, ampuliform to lageniform, 13–19 × 3–6.1 μm, hyaline, thin-walled, smooth. *Conidia* cylindrical to sub-cylindrical, straight to slightly curved, 4-septate, (26–)27-30(–33) × 5.6–7.3 μm, mean ± SD = 28.7 ± 1.8 × 6.5 ± 0.5 μm; basal cell cylindrical to obconic, hyaline, thin-walled, smooth, 3.8-6.8 μm long, mean ± SD = 5.36 ± 0.8 μm; the three median cells together (17-)18-20(-22.5) μm long, mean ± SD = 19.2 ± 1.1 μm, concolourous, dark brown with septa darker than the rest of the cells (second cell from base 5.7-8.5 μm, mean ± SD = 6.8 ± 0.7 μm; third cell 5.1–7.0 μm, mean ± SD = 5.9 ± 0.5 μm; fourth cell 5.3-7.2 μm, mean ± SD = 6.4 ± 0.4 μm); apical cell 3.9–6.2 μm long, mean ± SD = 5.1 ± 0.6 um, cylindrical to sub-cylindrical, hyaline; 2-4 (mostly 3) tubular apical appendages, arising from the apex of the apical cell each at different points, unbranched (or rarely branched), flexuous, 15.9-29.4 μm long, mean ± SD = 23.64 ± 3.44 μm; basal appendage present, single, tubular, unbranched, short, 3.1–6.0 μm long, mean ± SD = 4.4 ± 0.7 μm.

Colonies grow fast on PDA, reaching 8-8.5 cm diameter after 5 days at 25 °C, mycelium white, with aerial round tuft of mycelia, smooth, cottony, edge undulate, fruiting bodies black, scattered, immersed or sub-immersed, reverse light cream in colour (Fig. 1b).
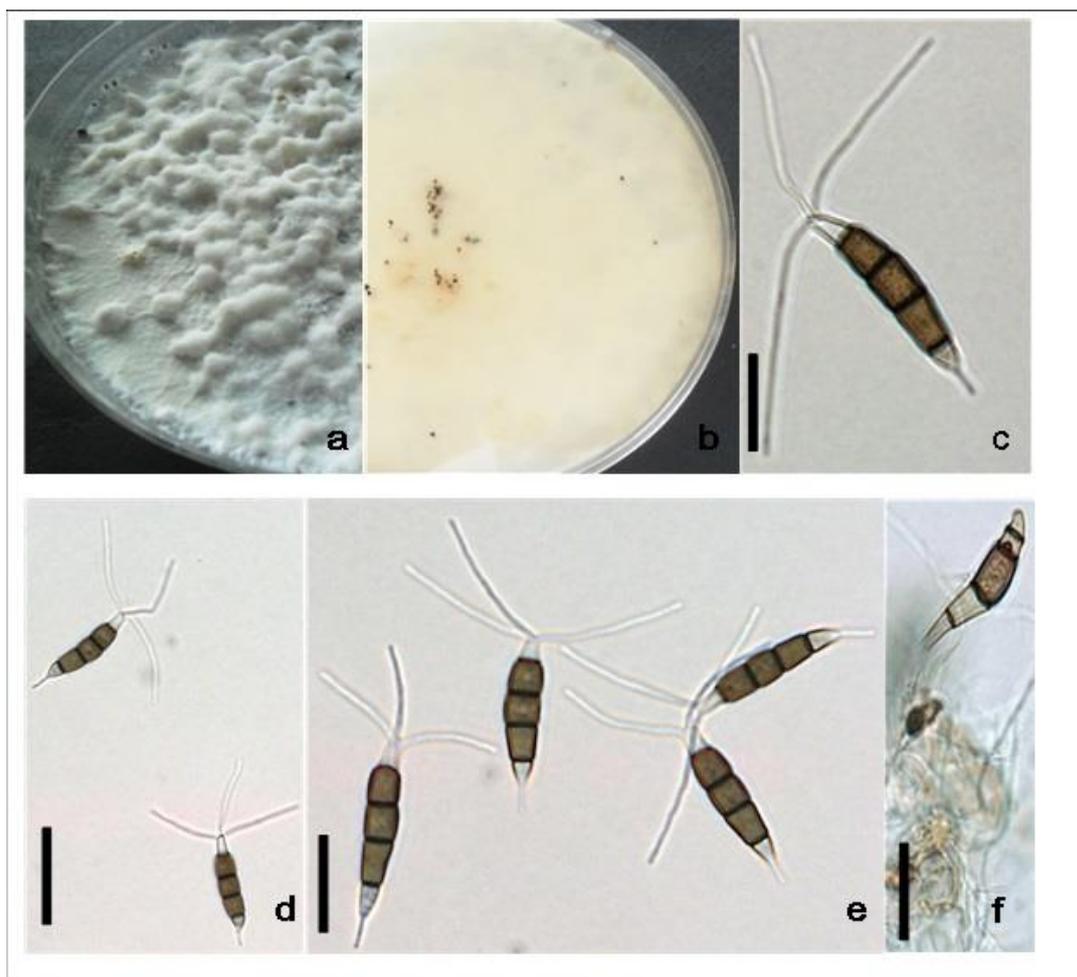
Fig. 1 – *Pseudopestalotiopsis kubahensis* (UMAS KUB-P20). a. Colony surface on PDA. b. Reverse view. c-e. Conidia. f. Conidiogenous cell. Scale bars = 20 µm c, e & f; 50 µm d

Distribution and habitat – On green leaves of *Macaranga* sp., Kubah National Park, Sarawak, Malaysia.

Type – MALAYSIA, Sarawak, Kubah National Park, from green leaves of *Macaranga* sp, September 2014, A.A. Lateef (KUB-2006 **holotype**, ex-type culture UMAS KUB-P20, deposited in the Mycology laboratory, Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak).

Notes – *Pseudopestalotiopsis kubahensis* is distinct from its closely related species, *Ps. theae* and *Ps. simitheae*, by its longer conidia (26–33 µm; mean = 28.7 µm) as compared to *Ps. theae* (epitype 22.5–28 µm; mean = 25.5µm) (Maharachchikumbura et al. 2013) and *Ps. simitheae* (22-30 µm; mean = 26 µm) (Song, Tangthirasunun, et al. 2014); unknobbed apical appendages, and a light cream colony reverse colour, with a characteristic round mycelial tuft on the colony surface (Fig. 1a & 1b) . *Ps. kubahensis* also has a bigger conidia (26–33 × 5.6–7.3 µm) compared to *Ps. cocos* (21–25 × 6–7.5 µm), but smaller than that of *Ps. indica* (32.5–36 × 6.5–9 µm), with longer apical appendages than *Ps. cocos* (12-23 µm), but shorter than that of *Ps. Indica* (30–40 µm) (Maharachchikumbura et al. 2014). Furthermore, *Ps. kubahensis* also has a shorter basal appendage compared to the other known species in the genus (Maharachchikumbura et al. 2013; Maharachchikumbura et al. 2014; Song, Tangthirasunun, et al. 2014) and a longer basal cell (3.8–6.8 µm long; mean = 5.36 µm) than *Ps. theae* (epitype 3.9–5.3 µm long; mean = 4.55 µm) (Maharachchikumbura et al. 2013), *Ps. cocos* (3.5–5 µm) (Maharachchikumbura et al. 2014) and *Ps. simitheae* (3–7 µm long; mean =5µm) (Song, Tangthirasunun, et al. 2014), but shorter than that of *Ps. indica* (5.5–7 µm) ((Maharachchikumbura et al. 2014).
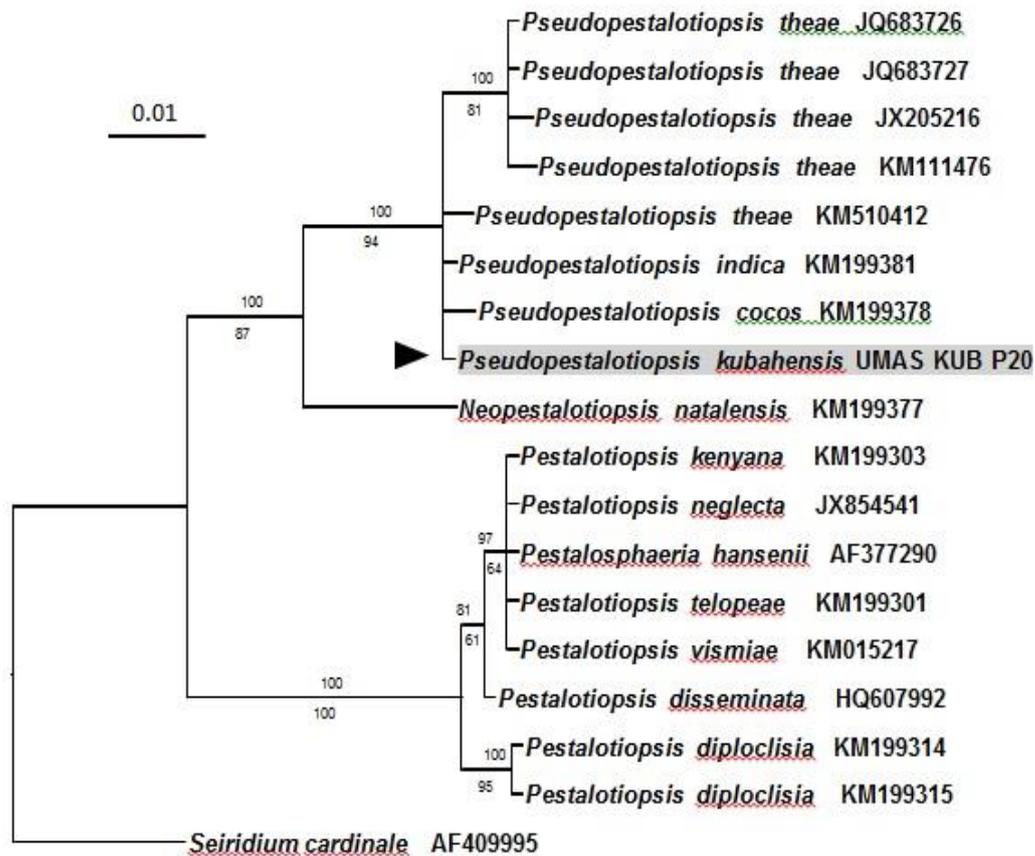
Fig. 2 – Strict consensus tree of 1365 equally parsimonious trees generated from the ITS gene sequences of 18 strains showing the relationship of the new species, *Ps. kubahensis* (highlighted) with *Pestalotiopsis* spp and *Neopestalotiopsis* spp with *Seiridium cardinale* as the outgroup taxon. Given at the nodes are the Bayesian Posterior Probabilities (BPP) (top) and Maximum Parsimony Bootstrap support values (MPB) greater than 50% (bottom). Scale bar represents expected number of changes per site.

Discussion

Pseudopestalotiopsis kubahensis, isolated from green leaves of *Macaranga* sp, is a distinct species in the genus *Pseudopestalotiopsis*; which was recently split from *Pestalotiopsis* by Maharachchikumbura et al. (2014b), based on its morphological characteristics and molecular phylogeny. This species is clearly distinct based on its morphological characteristics such as length of its conidia, basal cell, unknobbed apical appendages and shorter basal appendage, including having a round mycelia tuft on its colony surface and also based on its molecular phylogeny. However, using ITS gene region only, even though it is the universal fungal barcode, makes it difficult to separate some fungal lineages.

With the use of molecular data combined with morphological characters, *Ps. kubahensis* has been successfully identified as a novel species.

It is important for *Pestalotiopsis* species and other previously described species to be re-evaluated using molecular data from different gene regions and also pigmentation of their median cells. This will eventually result in some *Pestalotiopsis* species to be transferred to the genus *Pseudopestalotiopsis*.

Acknowledgments

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