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Molecular Identification and Diversity of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* Species from Four Host Plants in Sarawak, Borneo Island (Malaysia)

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Abstract: Until recently, *Pestalotiopsis* species have been identified based on host relationship and conidial dimensions. *Pestalotiopsis* species occur as endophytes, saprobes and also pathogens of many plant hosts. This study used molecular phylogenetic relationships based on ITS sequence data to identify *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species in addition to their conidial pigmentation from four host plants namely *Macaranga triloba*, *Macaranga* sp., *Shorea macrophylla* and *Syzygium* sp. Based on the molecular phylogenetic analysis of 18 *Pestalotiopsis*-like isolates from the four different host plants, the isolates clustered separately into three clades corresponding to their conidial morphology and conidial median cell pigmentation as *Neopestalotiopsis*, *Pestalotiopsis*, and *Pseudopestalotiopsis* irrespective of their host plant association. Among the four host plants studied, *S. macrophylla* hosts the highest diversity of *Pestalotiopsis*-like species while *Syzygium* sp. had the lowest diversity. This is the first report on the molecular phylogenetics and diversity of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species from Sarawak, Malaysia in line with recent re-classification in the genus. New records of *Pestalotiopsis*-like species were also recorded on new host plants.

Keywords: ITS sequence; Microfungi; Molecular identification; Pestalotiopsis; Phylogenetics.

1. Introduction

Pestalotiopsis species are known to produce Taxol, an anti-cancer agent [1,2]. Correct identification and naming of species in the genus *Pestalotiopsis* is quite difficult and complex due to the overlapping of conidial and cultural morphologies in many species in the genus. The genus *Pestalotiopsis* was initially separated from the genus *Pestalotia* De Not in 1949 by Steyaert to accommodate 5-celled *Pestalotiopsis*-like species [3]. Many *Pestalotiopsis* species were described as distinct species mainly based on host plant associations [3]. However, several studies have showed that *Pestalotiopsis* cannot be differentiated based on host plant association only, but can be correctly identified using conidial morphology combined with DNA sequence data [3, 4, 5, 6, 7].

Consequently, a recent re-classification of *Pestalotiopsis* was done resulting in two other genera being carved out of *Pestalotiopsis* namely *Neopestalotiopsis* and *Pseudopestalotiopsis* [3]. The genus *Pestalotiopsis* accommodated species with lightly-pigmented concolourous median cells while the genus *Neopestalotiopsis* accommodated species with versicolourous median cells, and the genus *Pseudopestalotiopsis* for species with darkly-coloured concolourous median cells.

In Malaysia, Sarawak is located on the Borneo Island and has been recognised as a hotspot for high biological diversity indicating its rich natural resources. There has not been any study on the molecular identification, diversity and distribution of *Pestalotiopsis*-like microfungi in this region and also to reflect the current classification and evolutionary relationships. This present study aimed at revealing the identity, diversity and host plant distribution of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species in selected National Parks in Sarawak.

The result of this study will be a contribution to the taxonomy and ecology of these *Pestalotiopsis*-like species in Sarawak, Borneo Island and Malaysia, as well as being a useful step for successful prospecting of the

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useful metabolites produced by *Pestalotiopsis*-like microfungi.

2. Materials and Methods

Sample collection sites and Sampling design

The sample collection sites were two National Parks (NPs); Kubah and Gunung Gading NPs, located in the 7th division of Sarawak, East Malaysia. Sarawak lies in the tropical region on the Borneo Island, with a high humidity and rainfall throughout the year. Leaf samples were collected from four host plants namely *Macaranga triloba*, *Macaranga* sp., *Shorea macrophylla* and *Syzygium* sp. on four collection trips to both sampling sites; two trips to Kubah National Park in March, 2014 and September, 2014, likewise, two trips to Gunung Gading National Park in November, 2014 and February, 2015. The latitude and longitude readings at each collection points were recorded with an hand-held Global Positioning System (GPS) equipment. Leaf-litters and green leaves were collected from each selected plant. Leaf-litters were picked directly from under the plant. The collected samples were put in plastic bags, labelled appropriately and transported to the laboratory for processing.

Isolation and Identification of the *Pestalotiopsis*-like Microfungi

The collected leaf samples were processed for isolation of endophytic and saprobic *Pestalotiopsis*-like microfungi as done by [8]. *Pestalotiopsis*-like fungi were isolated from leaves of the different plant species. Isolates belonging to different morpho-species were selected based on their morphological characters such as colony growth rate, colony growth pattern and colony reverse colouration. Measurements of 25 conidia of each isolate were recorded including the pigmentation of the median cells.

All the pure cultures were deposited in the Mycology laboratory, Department of Plant Science and Environmental Ecology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak. Identification of the different genera and species were done based on conidial pigmentation and molecular sequence analysis. The frequency of isolation of each microfungal taxa was determined according to [9,10] as follows.

Genomic DNA Extraction, PCR (Polymerase Chain Reaction) amplification and Sequencing.

Each morpho-species isolate was grown on potato dextrose agar (PDA) and incubated at room temperature (25 ± 2 ˚C) for three to ten days before harvesting for DNA extraction. The total genomic DNA was extracted using the CTAB method [11] and the internal transcribed spacer (ITS) gene regions of the total genomic DNA were amplified through polymerase chain reaction (PCR) using the primer pairs ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) [12] in a SensQuest lab cycler. The PCR mixture used was made up of 5 µL of 5 x Mg free-PCR buffer, 0.15 µL of MgCl, 1.5 µL of dNTPs, 1 µL of each primers, 0.15 µL of Fermentas Taq DNA polymerase, 12.85 µL of double-sterilised distilled water (ddH_2O) and 1.5 µL of the DNA template with a total volume of 25 µL. The PCR control reaction contained ddH_2O as template instead of DNA. The PCR programme used was 2 min at 94 ˚C, followed by 35 cycles at 94 ˚ C for 1 min, 55 ˚C for 1 min, then 72 ˚C for 1 min and final extension at 72˚C for 10 min.

The PCR products were sent to a private sequencing company (1st -Base, Asia) for both forward and reverse primer Sanger sequencing using an ABI PRISM sequencer.

Phylogenetic Analysis

The sequences obtained from the sequencing company were confirmed to belong to the intended microfungal group using BLAST-n on the GenBank website (http://blast.ncbi.nlm.nih.gov). SeqTrace version 0.9.0 [13] was then used to obtain consensus sequences from the forward and reverse sequences. Similar reference sequences based on BLAST-n results were downloaded from GenBank for phylogenetic analysis following a similarity search and then processed in AliView version 1.17-beta software [14] before they were aligned using MAFFT version 7 [15] and then adjusted using Gblocks version 0.91b [16, 17].

$$\text{Frequency of occurrence} = \frac{\text{the total number of leaf segments from which a fungal taxa was present}}{\text{the total number of leaf segments observed}} \times 100$$

Table 1 Strains used in this study for the phylogenetic analysis with their GenBank accession numbers.

S/N	Species	Strain	Host (Country)	GenBank accession number (ITS)
1	*Neopestalotiopsis clavispora*	CBS 447.73	Decaying wood (Sri Lanka)	KM199374
2	***Neopestalotiopsis eucalypticola**	CBS 264.37	*Eucalyptus globulus* (-)	KM199376
3	***Neopestalotiopsis formicarum**	CBS 115.83	Plant debris (Cuba)	KM199344
4	***Neopestalotiopsis mesopotamica**	CBS 336.86	*Pinus brutia* (Iraq)	KM199362
5	*Neopestalotiopsis mesopotamica*	CBS 299.74	*Eucalyptus* sp. (Turkey)	KM199361
6	*Neopestalotiopsis* sp.	UMAS 2_23	*Macaranga* sp. (Malaysia)	KT337383
7	*Neopestalotiopsis* sp.	UMAS 8_1	*Macaranga* sp. (Malaysia)	KT337378
8	*Neopestalotiopsis* sp.	UMAS P12	*Macaranga triloba* (Malaysia)	KT337385
9	***Neopestalotiopsis surinamensis**	CBS 450.74	Soil under *Elaeis guineensis* (Suriname)	KM199351
10	*Pestalotiopsis disseminata*	UMAS P13	*Shorea macrophylla* (Malaysia)	KT337380
11	*Pestalotiopsis microspora*	UMAS P15	*Shorea macrophylla* (Malaysia)	KT337388
12	*Pestalotiopsis parva*	UMAS P5	*M. triloba* (Malaysia)	KT337386
13	*Pestalotiopsis biciliata*	CBS 124463	*Platanus* × *hispanica* (Slovakia)	KM199308
14	***Pestalotiopsis biciliata**	CBS 236.38	*Paeonia* sp. (Italy)	KM199309
15	*Pestalotiopsis biciliata*	UMAS P16	*Shorea macrophylla* (Malaysia)	KT337374
16	*Pestalotiopsis diploclisia*	UMAS MP1	*Syzygium* sp. (Malaysia)	KT337389
17	*Pestalotiopsis disseminata*	PSH2000I-043	*Podocarpus macrophyllus* (China)	AY687869
18	*Pestalotiopsis grevilleae*	CBS 114127	*Grevillea* sp. (Australia)	KM199300
19	*Pestalotiopsis kenyana*	CBS 911.96	Raw material from agar-agar (-)	KM199303
20	***Pestalotiopsis kenyana**	CBS 442.67	*Coffea* sp. (Kenya)	KM199302

21	*Pestalotiopsis neglecta*	HKUCC 996	- (China)	AF409975
22	*Pestalotiopsis neglecta*	UMAS 7_2	*Shorea macrophylla* (Malaysia)	KT337391
23	*Pestalotiopsis neglecta*	UMAS P10	*Shorea macrophylla* (Malaysia)	KT337377
24	**Pestalotiopsis oryzae*	CBS 353.69	*Oryza sativa* (Denmark)	KM199299
25	*Pestalotiopsis parva*	CBS 278.35	*Leucothoe fontanesiana* (-)	KM199313
26	*Pestalotiopsis parva*	UMAS P6	*Macaranga triloba* (Malaysia)	KT337384
27	*Pestalotiopsis* sp.	UMAS P14	*Shorea macrophylla* (Malaysia)	KT337375
28	*Pestalotiopsis* sp.	UMAS 1705	*Shorea macrophylla* (Malaysia)	KT337373
29	*Pestalotiopsis* sp.	UMAS P11	*Shorea macrophylla* (Malaysia)	KT337381
30	*Pestalotiopsis* sp.	UMAS P4	*Macaranga triloba* (Malaysia)	KT337387
31	*Pestalotiopsis telopeae*	CBS 113606	*Telopea* sp. (Australia)	KM199295
32	**Pestalotiopsis telopeae*	CBS 114161	*Telopea* sp. (Australia)	KM199296
33	*Pestalotiopsis theae*	P145	*Pharus latifolius* (USA)	EF423551
34	*Pestalotiopsis vismiae*	R11	*Glycine max* cultivar *Monarca* (Brazil)	KM980000
35	*Pestalotiopsis vismiae*	UMAS P9	*Shorea macrophylla* (Malaysia)	KT337390
36	*Pseudopestalotiopsis cocos*	CBS 272.29	*Cocos nucifera* (Indonesia)	KM199378
37	*Pseudopestalotiopsis indica*	CBS 459.78	*Hibiscus rosa-sinensis* (India)	KM199381
38	*Pseudopestalotiopsis kubahensis*	UMAS KUB-P20	*Macaranga* sp. (Malaysia)	KT006749
39	*Pseudopestalotiopsis theae*	BPC50	Mango leaf (Malaysia)	KM510412
40	*Pseudopestalotiopsis theae*	UMAS 2005	*Macaranga* sp. (Malaysia)	KT337379
41	*Seiridium cardinale* (Outgroup)	ICMP 7323	-	AF409995

*means sequence is from the type material.

The aligned sequences were then used for maximum parsimony analysis using PAUP (Phylogenetic Analysis Using Parsimony) software v.4.0b10 [18]. For the parsimony analysis, 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. Max trees were set up to 10,000, 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, each with 10 replicates of random stepwise addition of taxa [19]. Tree descriptive statistics, such as tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were also calculated for each Maximum Parsimonious Tree (MPT) generated. The Kishino-Hasegawa tests [20] were performed to determine whether the trees inferred under different optimality criteria were significantly different from each other.
The phylogenetic trees generated were viewed and edited in TreeGraph2 version 2.10.1-641 beta [21].

3. Results and Discussion

Molecular identification and phylogenetics

The sequences of the ITS region of the isolates from this study ranged from 546 - 617 bp. From the phylogenetic analysis using PAUP (Phylogenetic Analysis Using Parsimony) software version 4.0b10, the aligned sequence matrix consists of 41 taxa (including the outgroup taxon *Seiridium cardinale*), 577 characters including gaps treated as missing data, 465 characters are constant, 44 variable characters are parsimony-uninformative and 68 characters are parsimony informative. The parsimony analysis yielded 10,000 Most Parsimonious Trees (MPT) with the strict consensus tree having tree length (TL) = 132, consistency index (CI) = 0.9167, homoplasy index (HI) = 0.0833, retention index (RI) = 0.9818 and rescaled consistency index (RC) = 0.9000. The bootstrap majority-rule consensus tree is shown in Fig. 1, showing the clustering and identification of the isolates separated mainly into three clades corresponding to the morphology and pigmentation of their conidia.

In total, 18 isolates were identified using the ITS region sequence data. The result of the phylogenetic analysis showed that all the isolates from this study, highlighted in Fig. 1 were clustered in different clades across the phylogenetic tree with three main clades recognised; clade A, B and C. All the clades with high bootstrap support values. From the phylogenetic tree, clade A, with a bootstrap support value of 96 % had two of our isolates (*Ps. theae* & *Ps. kubahensis*) clustering in this clade. The isolates from this study that clustered in this clade were those with concolourous darkly pigmented conidia (Fig. 2A). Concolourously dark pigmented *Pestalotiopsis*-like species have been recently named as a new genus by Maharachchikumbura, Hyde, Groenewald, Xu, & Crous (2014) as *Pseudopestalotiopsis.*

Clade B, with a bootstrap value of 100 % had three of our isolates in this clade, representing isolates with versicolourous pigmented conidia. In this clade, the two upper median cells of their conidia are more darkly pigmented than the third median cell (Fig. 2B) and this clade represents the *Neopestalotiopsis* which was also recently separated from the genus *Pestalotiopsis* [3].

In clade C, majority of the isolates in this study clustered here with a bootstrap value of 100 %, representing the concolourously lightly pigmented isolates (Fig. 2C). This clade remains the monophyletic group of *Pestalotiopsis* species [3].

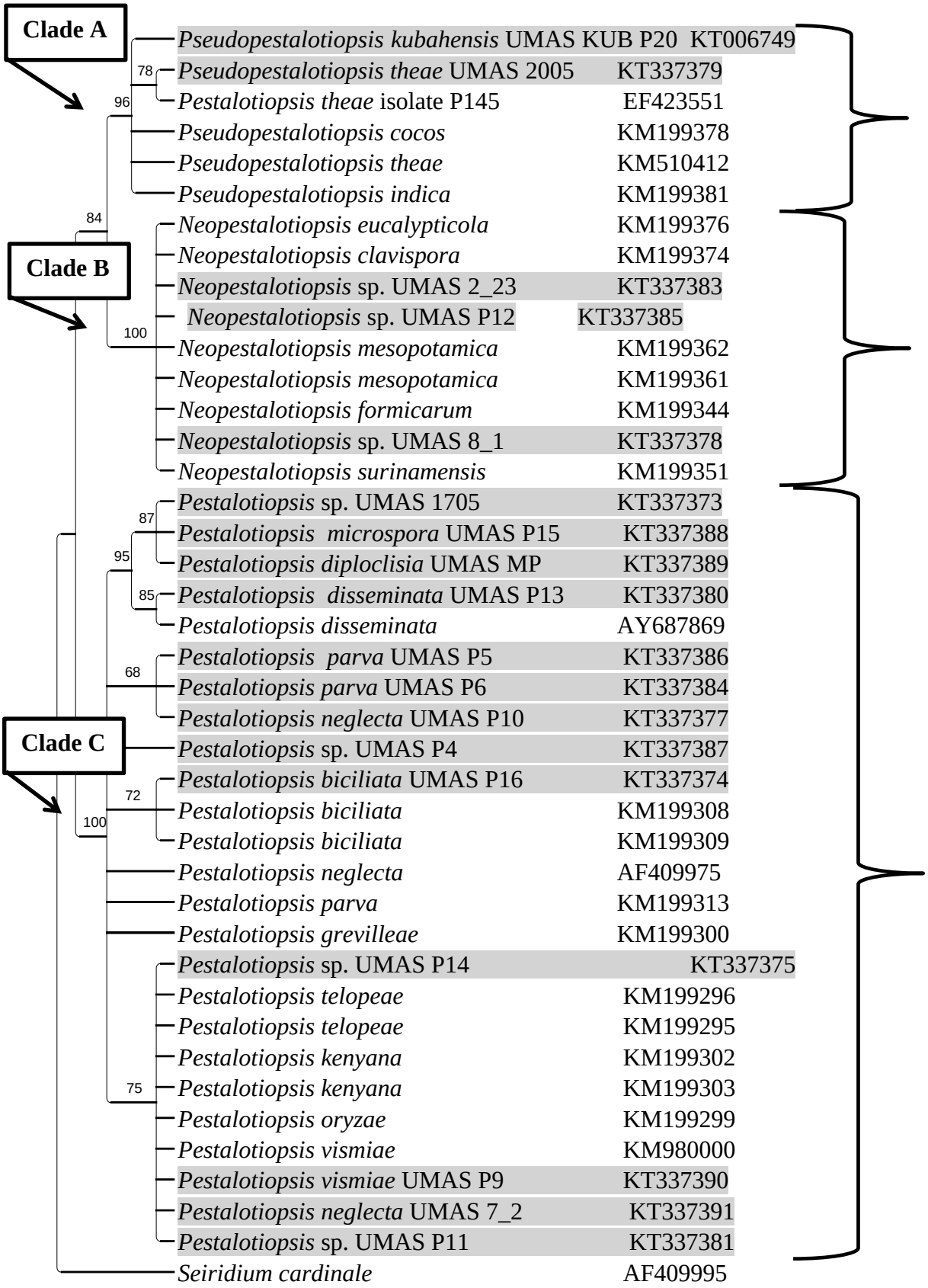

Fig. 1 Bootstrap Majority-rule consensus tree of 10,000 equally parsimonious trees generated from the ITS (ITS1, 5.8S and ITS2) gene sequences of 41 strains showing the relationship of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species. Given at the nodes are the Maximum Parsimony Bootstrap support values (MPB) greater than 50%.

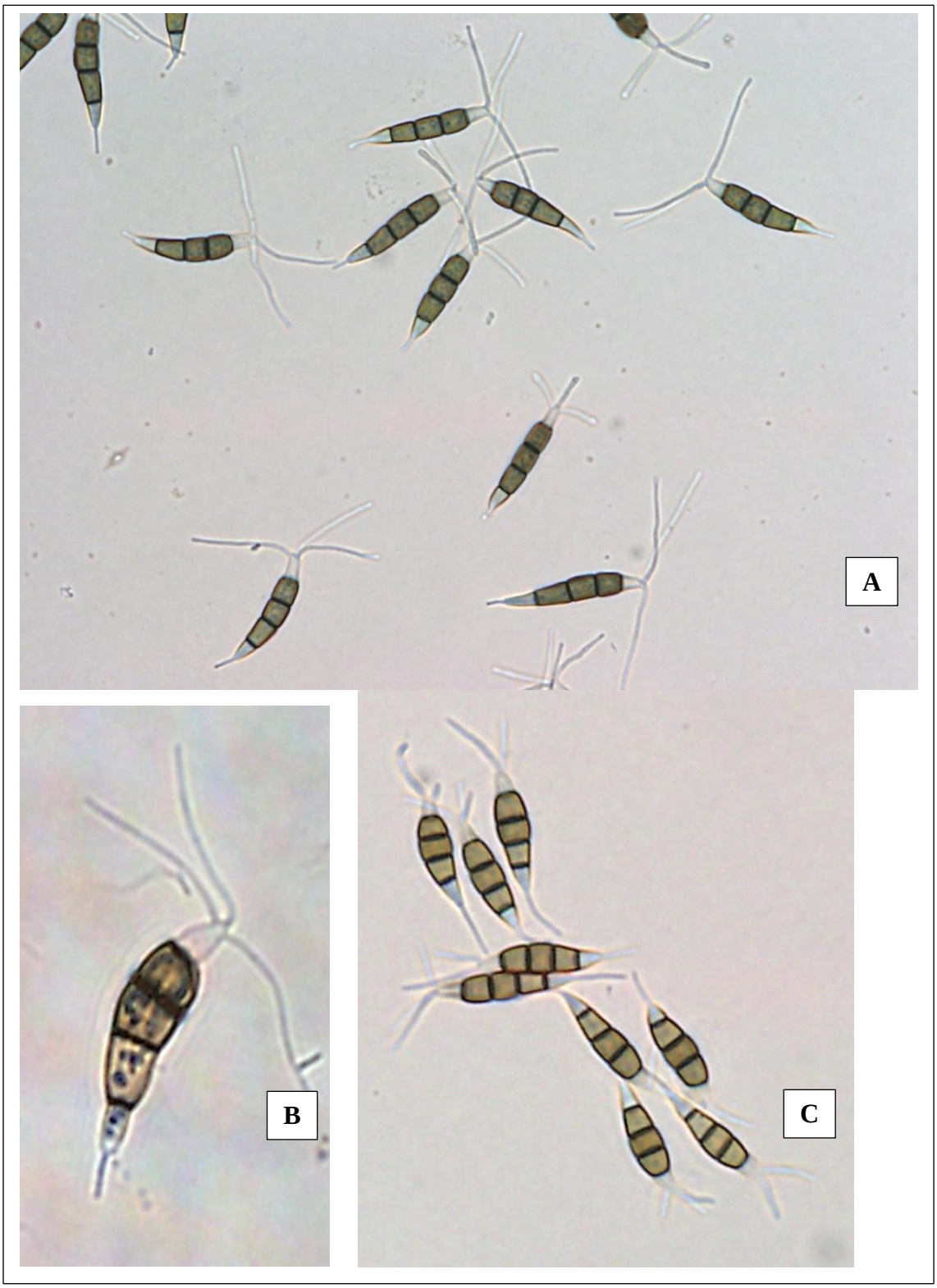

Fig. 2. Conidial morphology of A. *Pseudopestalotiopsis* sp B. *Neopestalotiopsis* sp. C. *Pestalotiopsis* sp. Scale bars A-C=50 µm.

However, some *Pestalotiopsis* spp. in clade B and clade C were not able to be identified specifically due to unresolved distinctiveness and overlap on the phylogenetic tree and this can be attributed to the use of only ITS sequence data for the species identification.

Furthermore, a quick look at the cultural and conidial morphology of all isolates from this study will primarily indicate that they belong to the genus *Pestalotiopsis*, but a close look at their conidial morphology and use of molecular phylogenetic analysis will reveal that the isolates were different and belonged to three different genera, *Pseudopestalotiopsis*, *Neopestalotiopsis* and *Pestalotiopsis*. The isolates from this study were classified into these three genera in line with the recent taxonomic re-classification of the genus *Pestalotiopsis* [3].

Naming of species in the genus *Pestalotiopsis* have earlier been confused as a result of overlapping conidial characteristics and description of species based on morphology and host plant associations, without molecular sequence data. Also, the cultural morphology and growth rates of the isolates in this study were not informative for differentiating the species which have also been observed in many other studies on species of *Pestalotiopsis* [4, 5]. Conidial morphology combined with molecular analysis is recommended as the main distinguishing characters for the genus *Pestalotiopsis*. Based on this, [3] splitted the genus *Pestalotiopsis* and two new genera were carved out of the genus, namely *Pseudopestalotiopsis* and *Neopestalotiopsis*. Before the re-classification of *Pestalotiopsis*, Jeewon *et al.* [5] ; Jeewon *et al.* [6[; Maharachchikumbura *et al.* [22[, and Song *et al.*, [23[have also reported a similar clustering of *Pestalotiopsis* spp. based on their median cell pigmentation. With the recent re-classification of *Pestalotiopsis*, it becomes more straight-forward to identify and name *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species, thereby avoiding the overlap and confusion in their nomenclature with a resultant improvement in their use for biotechnological use such as for metabolites production, as this will make their identification and communication between scientist unconfused.

Diversity and Distribution of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species on the host plants

In terms of the diversity and distribution of the *Pestalotiopsis*-like species on the four host plants, *S. macrophylla* in Kubah NP was host to nine taxa of the *Pestalotiopsis*-like fungi, all belonging to the genus *Pestalotiopsis* (Table 2). Four species each were isolated from *M. triloba* and *Macaranga* sp. while only one *Pestalotiopsis* taxa was isolated from *Syzygium* sp.

The isolation of *Pestalotiopsis neglecta, Pestalotiopsis biciliata, Pestalotiopsis microspora, Pestalotiopsis disseminata, Pestalotiopsis vismiae* including three other *Pestalotiopsis* sp. from *S. macrophylla* constitutes new records on this host plant for these species as they have not been earlier reported on this plant as well as from Sarawak. Likewise, *Pestalotiopsis parva, Pestalotiopsis* sp., *Neopestalotiopsis* sp. recorded on *M. triloba*; *Pseudopestalotiopsis kubahensis, Neopestalotiopsis* sp., *Pseudopestalotiopsis theae on Macaranga* sp.; and *Pestalotiopsis diploclisia on Syzygium* sp. are new records on their respective host plants.

Among the four host plants, *S. macrophylla* hosts the highest diversity of *Pestalotiopsis*-like species while *Syzygium* sp. had the lowest diversity of the microfungal group. [24, 25] have earlier reported the frequent occurrences of *Pestalotiopsis* spp. on decaying leaves of *Shorea obtusa* in Thailand.

Table 2 Host plant affinities and isolation frequencies of *Pestalotiopsis*-like isolates obtained from this study.

S/N	Species name (Isolate)	Host Plant species	Frequency of occurrence
1	*Pestalotiopsis* sp. (UMAS P4)	*Macaranga triloba*	0.2
2	*Pestalotiopsis parva* (UMAS P5)	*Macaranga triloba*	0.2
3	*Pestalotiopsis parva* (UMAS P6)	*Macaranga triloba*	0.12
4	*Pestalotiopsis vismiae* (UMAS P9)	*Shorea macrophylla*	0.36
5	*Pestalotiopsis neglecta* (UMAS P10)	*Shorea macrophylla*	0.08
6	*Pestalotiopsis* sp. (UMAS P11)	*Shorea macrophylla*	0.06
7	*Neopestalotiopsis* sp. (UMAS P12)	*Macaranga triloba*	0.2
8	*Pestalotiopsis disseminata* (UMAS P13)	*Shorea macrophylla*	0.08
9	*Pestalotiopsis* sp. (UMAS P14)	*Shorea macrophylla*	0.08
10	*Pestalotiopsis microspora* (UMAS P15)	*Shorea macrophylla*	0.14
11	*Pestalotiopsis biciliata* (UMAS P16)	*Shorea macrophylla*	0.08
12	*Pseudopestalotiopsis kubahensis* (UMAS KUB P20)	*Macaranga* sp.	0.28
13	*Pestalotiopsis diploclisia* (UMAS MP)	*Syzygium* sp.	0.48
14	*Pestalotiopsis* sp. (UMAS 1705)	*Shorea macrophylla*	0.2
15	*Neopestalotiopsis* sp. (UMAS 8_1)	*Macaranga* sp.	0.72
16	*Neopestalotiopsis* sp. (UMAS 2_23)	*Macaranga* sp.	0.18
17	*Pseudopestalotiopsis theae* (UMAS P2005)	*Macaranga* sp.	0.04
18	*Pestalotiopsis neglecta* (UMAS 7_2)	*Shorea macrophylla*	0.36
	Total=18 morpho-species	**4 Host plants**	

4. Conclusion

With a total of 18 *Pestalotiopsis*-like microfungi from four host plants from Kubah and Gunung Gading National Parks in Sarawak, this study revealed the high diversity of these microfungi in Sarawak and also represents the first report to reveal the distribution and diversity as well as molecular phylogenetics of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* species in Malaysia, with new records of microfungi on new host plants.

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