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Contributed Paper

Pestalotiopsis keteleeria sp. nov., Isolated from *Keteleeria pubescens* in China

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ABSTRACT

A novel *Pestalotiopsis* species, from living leaves of *Keteleeria pubescens* collected in Guizhou Province, China is described based on molecular and morphological data. This taxon is characterized by its olivaceous concolorous median cells and relatively smaller conidia. The phylogram resulting from combined sequences of the internal transcribed spacer (ITS), partial β-tubulin and partial translation elongation factor 1-alpha (*tef1*) gene regions shows that this strain forms a distinct clade separated from the other species in the genus. Based on evidence from morphology and molecular phylogeny, *Pestalotiopsis keteleeria* sp. nov. is described herein.

Keywords: coelomycetes, new species, phylogeny, taxonomy

1. INTRODUCTION

The genus *Pestalotiopsis* Steyaert was established by Steyaert by splitting of the genus *Pestalotia* De Not [1]. *Pestalotiopsis* species have a worldwide distribution, particularly in tropical and temperate ecosystems [2-3], and are pathogenic on a wide range of hosts [4-5] and an important group of endophytes which has been shown to produce a variety of bioactive secondary metabolites with potential medicinal use [6-10]. Many endophytic and pathogenic *Pestalotiopsis* species can also inhabit in dead leaves, bark and twigs as saprobes [11-12] because they are able to switch life modes [3,13].

In this paper, we introduce, describe and illustrate *Pestalotiopsis keteleeria* which is a new species found on leaves of *Keteleeria pubescens* in Guizhou Province, China. The species can be differentiated from other species by morphological and combined phylogenetic analyses based on three gene loci (ITS, β–tubulin and *tef1*).

2. MATERIALS AND METHODS

2.1 Isolation and Morphological Studies

Disease leaves of *Keteleeria pubescens* were collected from Guizhou Province, China. The samples were placed in clean plastic bags. The fungi were isolated by single spore culture technique to obtain

pure colonies of the taxon [14]. The colony was transferred to 2% potato-dextrose agar (PDA) and incubated at room temperature (25°C). The morphology of fungal colonies was recorded following the method of Hu *et al*. [11]. Fungal mycelia and spores were observed under a light microscope and photographed. Methods of examination, photography and isolation are as outlined in Maharachchikumbra *et al*. [3]. All microscopic measurements were taken with Tarosoft image framework (v. 0.9.0.7) with 30 conidial measurements.

2.2 DNA Extraction and Sequencing

Total genomic DNA was extracted from fresh mycelia using a modified protocol of Doyle and Doyle [15] and Lee and Taylor [16]. The ITS and 5.8S region of rDNA fragment were amplified using primer pairs ITS4 and ITS5 [17]; partial β-tubulin gene region was amplified with primer pairs BT2A and BT2B [18-19]; *tef1* was amplified using the primer pairs EF1-526 F and EF1-1567R [20]. PCR was performed with the 25 µL reaction system containing 19.5 µL of double distilled water, 2.5 µL of 10 × Taq buffer with $MgCl_2$, 0.5 µL of dNTP (10 mM each), 0.5 µL of each primer (10 mM), 0.25 µL Taq DNA polymerase (5 U/µL), and 1.0 µL of DNA template. The thermal cycling program followed Maharachchikumbura *et al*. [3]. Sequences generated from this study were deposited at GenBank.

2.3 Phylogenetic Analyses

DNA sequences of our own isolates together with reference sequences downloaded from GenBank were analyzed. Sequences were optimized manually to allow maximum alignment and maximum sequence similarity, as detailed in Maharachchikumbura *et al*. [3] (Table 1). The tree construction procedure was performed in PAUP* version 4.0b10 [21]. All characters were equally weighted and gaps were treated as missing data. Parsimony trees were inferred using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. Max-trees were set up to 5,000, 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 resulting from maximum parsimony analysis, each with 10 replicates of random stepwise addition of taxa [22]. Descriptive tree statistics, 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 [23] were performed to determine whether the trees inferred under different optimality criteria were significantly different.

3. RESULTS

3.1 Phylogeny

The combined data matrix of ITS, β-tubulin and *tef1* regions consisted of 50 sequences representing 33 taxa, including the new isolate and outgroup. Sequences used in this study are listed in Table 1. The alignment comprised 1,546 characters including gaps (ITS: 1-561, β-tubulin: 562-1,020 and tef1: 1,021-1,546), among which, 978 characters are constant, 162 variable characters are parsimony-uninformative and 406 are parsimony-informative. The parsimony analysis of the data matrix resulted in 15 equally parsimonious trees and the first tree (TL = 1300, CI = 0.600, RI = 0.887, RC = 0.532, HI = 0.400) is shown in Figure 1. In the phylogram, 47 *Pestalotiopsis* isolates (30 taxa) separated into three major clades corresponding to three

conidial types (conidia having concolorous median cells, versicolorous median cells and dark concolorous median cells with knobbed apical appendages) with high bootstrap support. The new species *Pestalotiopsis keteleeria* forms a distinct clade apart from the sister species of *P. theae* and *P. steyaertii*.

Table 1. Sequences used for phylogenetic analysis.

Taxon	Isolates	GenBank Accession NumberI		
		TS	β-tubulin	*tef1*
P. adusta (Ellis & Everh.) Steyaert	ICMP 6088	JX399006	JX399037	JX399070
P. adusta	MFLUCC 10-146	JX399007	JX399038	JX399071
P. anacardiacearum Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC 2397	KC247154	KC247155	KC247156
P. asiatica Maharachch. & K.D. Hyde	MFLUCC 12-0286	JX398983	JX399018	JX399049
P. camelliae Y.M. Zhang, Maharachch. & K.D. Hyde	MFLUCC 12-0277	JX399010	JX399041	JX399074
P. camelliae	MFLUCC 12-0278	JX399011	JX399042	JX399075
P. chinensis Maharachch. & K.D. Hyde	MFLUCC 12-0273	JX398995	–	–
P. chrysea Maharachch. & K.D. Hyde	MFLUCC 12-0261	JX398985	JX399020	JX399051
P. chrysea	MFLUCC 12-0262	JX398986	JX399021	JX399052
P. clavata Maharachch. & K.D. Hyde	MFLUCC 12-0268	JX398990	JX399025	JX399056
P. clavispora (G.F. Atk.) Steyaert	IFRDCC 2391	KC537808	KC537822	KC537815
P. clavispora	MFLUCC 12-0280	JX398978	JX399013	JX399044
P. clavispora	MFLUCC 12-0281	JX398979	JX399014	JX399045
P. coffeae–arabicae Y. Song, K. Geng, K.D. Hyde & Yong Wang bis	HGUP 4015	KF412647	KF412641	KF412644
P. coffeae–arabicae	HGUP 4019	KF412649	KF412643	KF412646
P. diversiseta Maharachch. & K.D. Hyde	MFLUCC 12-0287	JX399009	JX399040	JX399073
P. ellipsospora Maharachch. & K.D. Hyde	MFLUCC 12-0283	JX398980	JX399016	JX399047
P. ellipsospora	MFLUCC 12-0284	JX398981	JX399015	JX399046
P. ericacearum Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC 2439	KC537807	KC537821	KC537814
P. foedans (Sacc. & Ellis) Steyaert	CGMCC 3.9178	JX398989	JX399024	JX399055
P. foedans	CGMCC 3.9123	JX398987	JX399022	JX399053
P. foedans	CGMCC 3.9202	JX398988	JX399023	JX399054
P. furcata Maharachch. & K.D. Hyde	MFLUCC 12-0054	JQ683724	JQ683708	JQ683740
P. gaultheria Y.M. Zhang, Maharachch. & K.D. Hyde	IFRD 411-014	KC537805	KC537819	KC537812
P. inflexa Maharachch. & K.D. Hyde	MFLUCC 12-0270	JX399008	JX399039	JX399072
P. karstenii (Sacc. & P. Syd.) Steyaert	IFRDCC OP13	KC537806	KC537820	KC537813
P. keteleeria Y. Song, K.D. Hyde & Y. Wang	MFLUCC 13-0915	KJ023087	KJ023088	KJ023089
P. intermedia Maharachch. & K.D. Hyde	MFLUCC 12-0259	JX398993	JX399028	JX399059
P. linearis Maharachch. & K.D. Hyde	MFLUCC12-0271	JX398992	JX399027	JX399058
P. magna Maharachch. & K.D. Hyde	MFLUCC 12-652	KF582795	KF582793	KF582791
P. rhododendri Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC 2399	KC537804	KC537818	KC537811
P. rhodomyrtus Y. Song, K. Geng, K.D. Hyde & Yong Wang bis	HGUP 4230	KF412648	KF412642	KF412645
P. rosea Maharachch. & K.D. Hyde	MFLUCC 12-0258	JX399005	JX399036	JX399069
P. samarangensis Maharachch. & K.D. Hyde	MFLUCC 12-0233	JQ968609	JQ968610	JQ968611
P. saprophyta Maharachch. & K.D. Hyde	MFLUCC 12-0282	JX398982	JX399017	JX399048

Table 1. Continued.

Taxon	Isolates	GenBank Accession NumberI		
		TS	β-tubulin	*tef1*
P. steyaertii Mordue	IMI 192475	KF582796	KF582794	KF582792
P. theae (Sawada) Steyaert	MFLUCC 12-0055	JQ683727	JQ683711	JQ683743
P. theae	SC011	JQ683726	JQ683710	JQ683742
P. trachicarpicola Y.M. Zhang & K.D. Hyde	MFLUCC 12-0263	JX399000	JX399031	JX399064
P. trachicarpicola	MFLUCC 12-0264	JX399004	JX399035	JX399068
P. trachicarpicola	MFLUCC 12-0265	JX399003	JX399034	JX399067
P. trachicarpicola	MFLUCC 12-0266	JX399002	JX399033	JX399066
P. trachicarpicola	MFLUCC 12-0267	JX399001	JX399032	JX399065
P. trachicarpicola	IFRDCC 2403	KC537809	KC537823	KC537816
P. trachicarpicola	OP068	JQ845947	JQ845945	JQ845946
P. umberspora Maharachch. & K.D. Hyde	MFLUCC 12-0285	JX398984	JX399019	JX399050
P. unicolor Maharachch. & K.D. Hyde	MFLUCC 12-0275	JX398998	JX399029	JX399063
P. unicolor	MFLUCC 12-0276	JX398999	JX399030	–
P. verruculosa Maharachch. & K.D. Hyde	MFLUCC 12-0274	JX398996	–	JX399061
Seiridium sp.	SD096	JQ683725	JQ683709	JQ683741

Taxonomy

Pestalotiopsis keteleeria Y. Song, K.D. Hyde & Yong Wang bis, sp. nov. (Figure 2)
MycoBank: MB 807904
Etymology: *Keteleeria*, refers to the genus name of the host plant.
Type: CHINA. Guizhou Province, Guiyang, from living leaves of *Keteleeria pubescens*, October 2012, Yu Song HGUP4301 (MFLU 14-0034 **holotype**, HGUP 4301 isotype, ex-type living culture MFLUCC 13-0915 = ICMP 20194).

Associated with disease leaves of *Keteleeria pubescens*. Sexual state: unknown. Asexual state: *Conidiophores* indistinct, reduce to conidiogenous cells. *Conidiogenous cells* discrete, ampulliform, hyaline, thin-walled, smooth, 2.5-6×2-5 μm. *Conidia* fusoid to ellipsoid, straight to slightly curved, 4 septate, 18.5-24×7-9.5 μm (mean = 22×8.5 μm); basal cell conic to obconic with obtuse end, hyaline or slightly olivaceous, thin-walled and verruculose, 2.5-4.5 μm long (mean = 3 μm); with three median cells, constricted at the septa, septa and periclinal walls darker than the rest of the cell, concolorous, or sometimes two upper median cells darker than the lower one, olivaceous, verruculose, together 13-16.5 μm long (mean = 14.5 μm) second cell from base 3.5-5 μm (mean = 4.5 μm); third cell 4-7 μm (mean = 5.0 μm); fourth cell 4-5 μm (mean = 4.5 μm); apical cell 2.5-4.5 μm long (mean = 3.5 μm), hyaline, obconic to subcylindrical; 1-3 (mostly 3) tubular apical appendages, arising from the apex of the apical cell, unbranched, 7.5-21 μm long (mean = 14 μm); basal appendage mostly absent, when present single, tubular, unbranched, centric, short, 1-3.5 μm long (mean = 2 μm).

Colonies grow fast on PDA, attaining 6-7 cm diam after 5 days at 25°C, edge undulate, white, dense aerial mycelium on surface, fruiting bodies black, gregarious, compact; reverse of culture pale orange.

Distribution and habitat: On living leaves of *Keteleeria pubescens,* Guiyang, Guizhou Province, China.

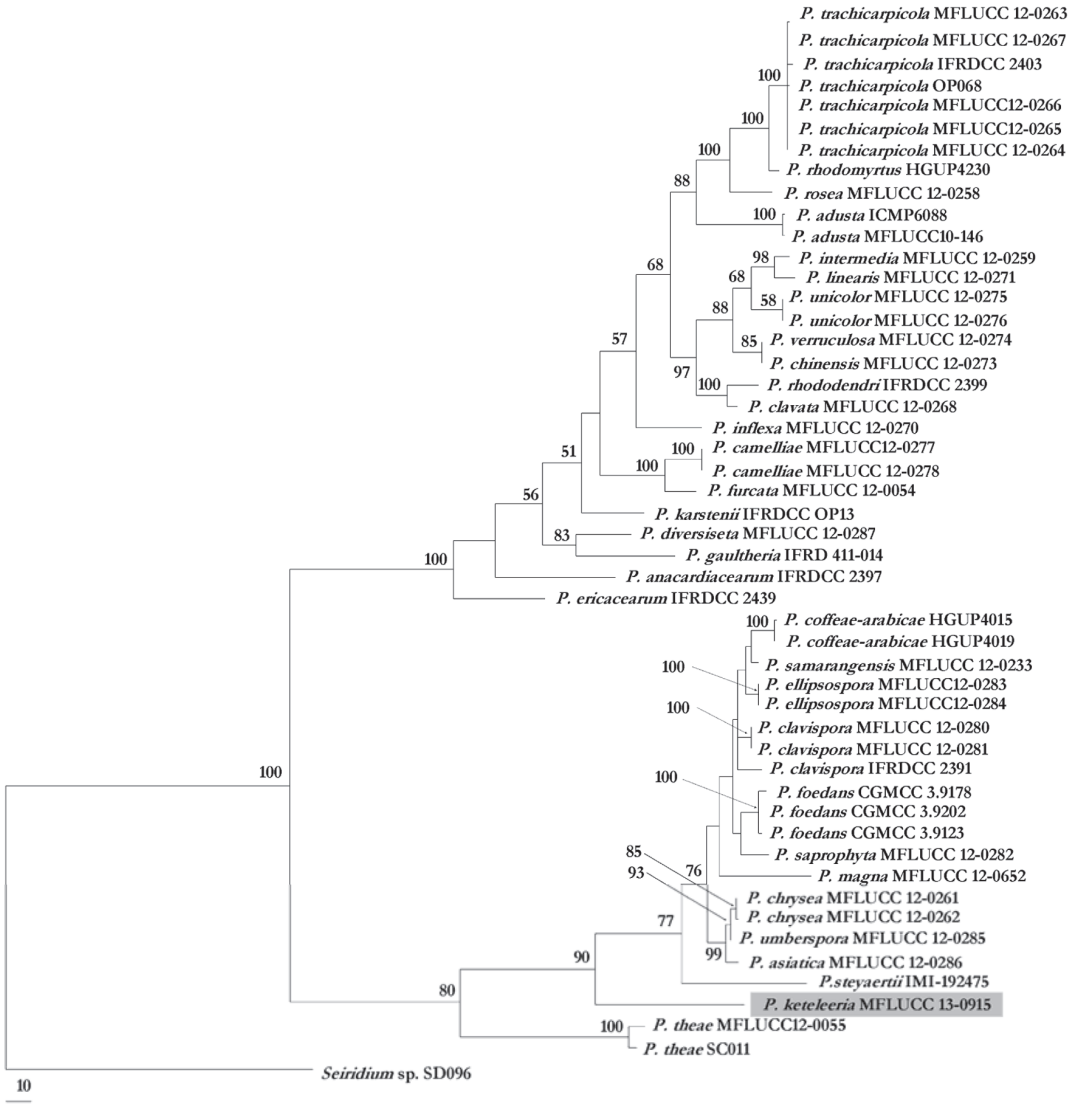

Figure 1. Maximum parsimony phylogram generated from combination of ITS, β-tubulin and *tef1* sequences. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. *Seiridium* sp. was used as the outgroup taxon. Bootstrap values higher than 50% are shown.

Notes: *Pestalotiopsis keteleeria* is a distinct species in the genus based on morphology and phylogeny. It forms a well separated clade apart from the sister species *P. theae* and *P. steyaertii*. In terms of morphology, *Pestalotiopsis keteleeria* is characterized by its spores with smaller length/width ratio, shorter apical appendages and olivaceous concolorous median cells compared with morphologically similar species. *P. steyaertii* can be distinguished from *P. keteleeria* by its larger conidia (27-34×7-9.5 μm) and versicolorous three median cells. Furthermore, *P. theae* can be differentiated from *P. keteleeria* in having knobbed apical appendages.

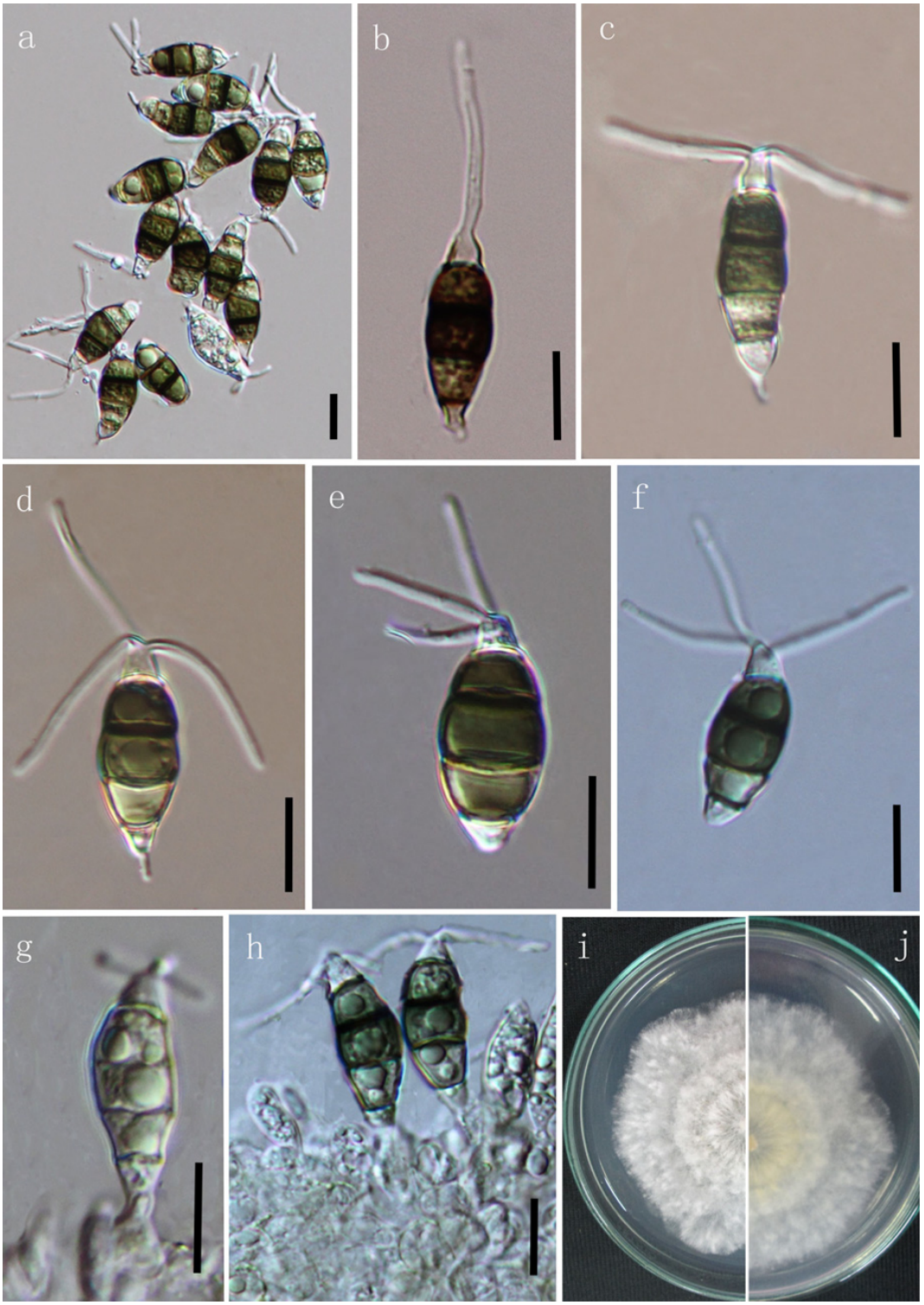

Figure 2. *Pestalotiopsis keteleeria*. a–f. Conidia. g–h. Conidiogenous cells. i–j. Colony on PDA, i. from above, j. from below. Scale bars: a–h = 10 μm.

4. DISCUSSION

In this study, one new species, *Pestalotiopsis keteleeria* from disease leaves of *Keteleeria pubescens* from Guizhou Province, China was characterized in terms of morphology and phylogeny. Including the newly-generated, ex-type sequences in the present study, currently 32 *Pestalotiopsis* species have either ex-type or ex-epitype sequences.

There are approximately 250 *Pestalotiopsis* names (Index Fungorum 2014) which were historically named according to the host association. However, *Pestalotiopsis* are not particularly host-specific and taxa may have the ability to infect a range of hosts [3-4]. Furthermore, there is no living type strain for most of these host based species. Thus, phylogenetic relationships within this large number of species are problematic [24]. Therefore it is important to clarify the relationships among *Pestalotiopsis* based on host occurrence, plus morphological and molecular data [2-3]. The significance of the application of molecular identification which can provide reliable genetic evidence to define the species boundaries in taxonomic studies is shown in our study, and the suggestion that combined multi-gene data in phylogeny would better resolve the taxonomy of *Pestalotiopsis* [3,11,12,14,25] is also confirmed here.

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REFERENCES

[1] Steyaert R.L., Contribution a l'etude monographique de Not. Et *Monochaetia* Sacc, (*Truncatella* gen. nov. et *Pestalotiopsis* gen. nov.), *Bull. Jard. Bot. Brux.*, 1949; **19**: 285-354.

[2] Maharachchikumbura S.S.N., Guo L.D., Chukeatirote E., Bahkali A.H. and Hyde K.D., *Pestalotiopsis*-morphology, phylogeny, biochemistry and diversity, *Fungal Divers.*, 2011; **50**: 167-187.

[3] Maharachchikumbura S.S.N., Guo L.D., Cai L., Chukeatirote E., Wu W.P., Sun X., Crous P.W., Bhat D.J., McKenzie E.H.C., Bahkali A.H. and Hyde K.D., A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species, *Fungal Divers.*, 2012; **56**: 95-129.

[4] Maharachchikumbura S.S.N., Guo L.D., Chukeatirote E., McKenzie E.H.C. and Hyde K.D., A destructive new disease of *Syzygium samarangense* in Thailand caused by the new species *Pestalotiopsis samarangensis*, *Trop Plant Pathol.*, 2013; **38(3)**: 227-235.

[5] Maharachchikumbura S.S.N., Zhang Y.M., Wang Y. and Hyde K.D., *Pestalotiopsis anacardiacearum* sp. nov. (Amphisphaeriaceae) has an intricate relationship with *Penicillaria jocosatrix*, the mango tip borer, *Phytotaxa*, 2013; **99(2)**: 49-57.

[6] Debbab, A., Aly, A.H. and Proksch, P., Endophytes and associated marine derived fungi—ecological and chemical perspectives. *Fungal Divers* 2012; **57**: 45-83.

[7] Ko Ko T., Stephenson S.L., Bahkali A.H. and Hyde K.D., From morphology to molecular biology: Can we use sequence data to identify fungal endophytes?, *Fungal Divers.*, 2011; **50**: 113-120.

[8] Ko Ko T.W., McKenzie E.H.C., Bahkali A.H., To-Anun C., Chukeatirote E., Promputtha I., Abd-Elsalam K.A., Soytong K., Wulandari N.F., Sanoamuang N., Jonglaekha N., Kobsueb R., Cheewangkoon R., Wikee S., Chamyuang S. and Hyde K.D., The need for re-inventory of Thai phytopathogens, *Chiang Mai J. Sci.*, 2011; **38**: 1-13.

[9] Xu J., Ebada S.S. and Proksch P., *Pestalotiopsis* a highly creative genus: Chemistry and bioactivity of secondary metabolites, *Fungal Divers*, 2010; **44:** 15-31.

[10] Heinig U., Scholz S. and Jennewein S., Getting to the bottom of taxol biosynthesis by fungi *Fungal Divers*, 2013; **60**: 161-170.

[11] Hu H.L., Jeewon R., Zhou D.Q., Zhou T.X. and Hyde K.D., Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: Evidence from rDNA and β–tubulin gene phylogenies, *Fungal Divers.*, 2007; **24**: 1-22.

[12] Maharachchikumbura S.S.N., Guo L.D., Chukeatirote E. and Hyde K.D., Improving the backbone tree for the genus *Pestalotiopsis*; addition of *P. steyaertii* and *P. magna* sp. nov., Mycol. Progress, 2013; DOI. org/ 10.1007/s11557–013–0944–0.

[13] Guba E.F., Monograph of *Monochaetia* and *Pestalotia*, Harvard University Press, Cambridge, 1961.

[14] Maharachchikumbura S.S.N., Chukeatirote E., Guo L.D., Crous P.W., McKenzie E.H.C. and Hyde K.D., *Pestalotiopsis* species associated with *Camellia sinensis* (tea), *Mycotaxon*, 2013; **123**: 47-61.

[15] Doyle J.J. and Doyle J.L., A rapid DNA isolation procedure for small quantities of fresh leaf tissue, Phytochemical, 1987; Felsenstein J., Confidence limits on phylogenies: An approach using the bootstrap, *Evolution*, 1985; **39**: 783-791.

[16] Lee S.B. and Taylor J.W., Isolation of DNA from fungal mycelia and single spores, 1990; In: Innis M.A., Gelfand D.H., Sninsky J.J. and White T.J., (eds) PCR protocols: A guide to methods and applications, Academic Press, San Diego, USA; pp. 282-287.

[17] White T.J., Bruns T., Lee S. and Taylor J.W., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, 1990; In: Innis M.A., Gelfand D.H., Sninsky J.J. and White T.J., (eds.) PCR protocols: A guide to methods and applications, Academic Press, New York; pp. 315-322.

[18] Glass N.L. and Donaldson G.C., Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes, *Appl. Environ. Microb.*, 1995; **61(4)**: 1323-1330.

[19] O'Donnell K. and Cigelnik E., Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous, *Molecular Phylogenet. Evol.*, 1997; **7(1)**: 103-116.

[20] Rehner S., Primers for elongation factor 1–α (EF1–α), 2001. http://ocid.nacas.org/research/deephyphae/EF1primer.pdf.

[21] Swofford D.L., PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0b10, Sinauer Associates, Sunderland, 2002.

[22] Felsenstein J., Confidence limits on phylogenies: An approach using the bootstrap, *Evolution*, 1985; **39**: 783-791.

[23] Kishino H. and Hasegawa M., Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, *J. Mol. Evol.*, 1989; **29**: 170-179.

[24] Zhang Y.M., Maharachchikumbura S.S.N., Tian Q. and Hyde K.D., *Pestalotiopsis* species on ornamental plants in Yunnan Province, China, *Sydowia*, 2013; **65**: 59-74.

[25] Liu A.R., Chen S.C., Wu S.Y., Xu T., Guo L.D., Jeewon R. and Wei J.G., Cultural studies coupled with DNA based sequence analyses and its implication on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy, *Molecular Phylogenet. Evol*. 2010; **57**: 528-535.