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Leaf litter saprobic *Dictyosporiaceae* (*Pleosporales*, *Dothideomycetes*): *Pseudocoleophoma zingiberacearum* sp. nov. from *Hedychium coronarium*

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ABSTRACT

A new species, *Pseudocoleophoma zingiberacearum*, is described from dead leaves of *Hedychium coronarium* (*Zingiberaceae*) collected from Dahu forest, Alishan Mountain (656 m), Chiayi in Taiwan. Maximum likelihood, maximum parsimony and Bayesian analyses were performed to confirm the phylogenetic affinities of the species. *Pseudocoleophoma zingiberacearum* is distinguished from other *Pseudocoleophoma* species based on distinct size differences in ascomata, asci, ascospores and DNA sequence data. Morphology coupled with combined gene analyses of LSU, ITS and *tef1-α* DNA sequence data, showed that the fungus belongs to the family *Dictyosporiaceae*, *Dothideomycetes*. This is the first species of *Pseudocoleophoma* recorded from the plant family *Zingiberaceae*. The new species is compared with other *Pseudocoleophoma* species and a comprehensive description and photo-micrographs are provided.

KEYWORDS: New species, leaf litter, taxonomy, phylogeny, *Zingiberaceae*

INTRODUCTION

We are delighted to submit this paper in honour of late Dr. J. Muthumary, formerly Professor, Centre of Advanced Study in Botany, University of Madras, Chennai-600025, India, who has contributed a great deal to the study of Coelomycetous fungi and in whose name this special volume of Kavaka is being brought out.

Fungi within the class *Dothideomycetes* have a global distribution and can be found in diverse habitats, ranging from terrestrial to freshwater or even in marine systems (Hyde *et al*., 2013; Crous *et al*., 2014; Ariyawansa *et al*., 2015; Tanaka *et al*., 2015; Dayarathne *et al*., 2018; Luo *et al*. 2018). It is the largest class in *Ascomycota* and characterized by bitunicate, usually fissitunicate asci (Kirk *et al*., 2008; Hyde *et al*., 2013; Tennakoon *et al*., 2018; Phookamsak *et al*., 2019). *Dothideomycetes* species life style can be saprobes, plant pathogens, endophytes, epiphytes, fungicolous, lichenized, or lichenicolous fungi (Hyde *et al*., 2013; Diederich *et al*., 2018; Tibpromma *et al*., 2018; Yoshino *et al*., 2019). According to the recent classification of Wijayawardene *et al*. (2018), Class *Dothideomycetes* contains 33 recognized orders confirmed by molecular phylogenetic studies in combination with morphological data.

The order *Pleosporales* has been of great research interest in recent years and has undergone considerable revision based on both morphology and phylogenetic studies (Hyde *et al*., 2013; Tanaka *et al*., 2015; Thambugala *et al*., 2017; Wanasinghe *et al*., 2018; Phookamsak *et al*., 2019). It is the largest order of *Dothideomycetes* (Kirk *et al*., 2008; Zhang *et al*., 2012; Hyde *et al*., 2013) and comprises more than 70 accepted families (Wijayawardene *et al*., 2018). One of the species-rich families of *Pleosporales* is *Dictyosporiaceae*, introduced by Boonmee *et al*. (2016) to accommodate *Dictyosporium* Corda. as the type genus based on morphology and multi-gene phylogenetic analysis. *Dictyosporiaceae* species are often saprobes on decaying wood in both terrestrial and freshwater habitats (Boonmee *et al*., 2016; Wang *et al*., 2016; Tibpromma *et al*., 2018; Hyde *et al*., 2019; Phookamsak *et al*., 2019). The diagnostic characteristics of sexual morphs of *Dictyosporiaceae* are immersed to erumpent or superficial, globose to subglobose, dark brown to black ascomata, bitunicate asci with septate, hyaline, sheathed ascospores; meanwhile, asexual morphs include cheirosporous hyphomycetes (Boonmee *et al*., 2016). According to the recent outline treatment of Wijayawardene *et al*. (2018), 12 genera are accepted in *Dictyosporiaceae*, viz. *Aquadictyospora* Luo, K.D. Hyde & H.Y. Su, *Cheirosporium* L. Cai & K.D. Hyde, *Dendryphiella* Buba´k & Ranoj., *Dictyocheirospora* Souza, Boonmee & K.D. Hyde, *Dictyopalmispora* Pinruan, Boonmee & K.D. Hyde, *Dictyosporium* Corda., *Digitodesmium* P.M. Kirk, *Gregarithecium* Kaz. Tanaka & K. Hiray., *Jalapriya* Souza, Hong Y. Su, Z.L. Luo & K.D. Hyde, *Pseudocoleophoma* Kaz. Tanaka & K. Hiray., *Pseudodictyosporium* Matsush. and *Vikalpa* Souza, Boonmee, Bhat & K.D. Hyde.

In an ongoing study of leaf litter inhabiting fungi in Taiwan, interesting fungal species was collected from Dahu forest, Alishanmountain in Chiayi. Morphological and multi-gene phylogenetic analyses were performed to establish its taxonomic placement.

MATERIAL AND METHODS

Sample collection, morphological studies and isolation: Dead and decaying leaf litter samples of *Hedychium coronarium* J. Koenig were collected from Dahu forest area in Chiayi, Taiwan and brought to the laboratory in Zip lock plastic bags. Specimens were examined with a LEICA EZ4 stereomicroscope. Micro-morphological characters were determined with AXIOSKOP 2 PLUS compound microscope and images were captured with a Canon AXIOCAM 506

COLOR digital camera. Observations and photomicrographs were made from materials mounted in water. Sections of ascomata were made free-hand. Many specimens were used to observe the asci and ascospore characters and slides were preserved in Lactoglycerol, sealed by applying nail-polish around the margins of cover slip. All measurements were made with ZEN2 (blue edition) and images used for figures were processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, USA).

Single ascospore isolation was carried out following the method described in Chomnunti *et al*. (2014). Germinated ascospore was transferred to potato dextrose agar (PDA) and incubated at 25°C in normal light. Subsequent sub-culturing was done carefully to ensure no contaminants are used in generating DNA sequence data. Culture characteristics were observed after three weeks. Colonies were photographed and characters noted. Type specimen was deposited in the National Chiayi University Herbarium (NCYU) and living cultures were deposited in National Chiayi University Culture Collection (NCYUCC) and Mae Fah Luang University Culture Collection (MFLUCC). Faces of Fungi and Index Fungorum numbers were provided as in Jayasiri *et al*. (2015) and Index Fungorum (2019). The new species is established following the recommendations in Jeewon and Hyde (2016).

DNA extraction and PCR amplification: Fungal mycelium was scraped off and transferred to 1.5 mL micro-centrifuge tube using a sterilized lancet for genomic DNA extraction. Mycelium was ground to a fine powder with liquid nitrogen and DNA was extracted using the DNA extraction kit (E.Z.N.A Fungal DNA Mini Kit, D3390-02, Omega Bio-Tek) following the manufacturer's protocol. The DNA product was kept at 4°C for DNA amplification and maintained at -20°C for long term storage. DNA was amplified by Polymerase Chain Reaction (PCR) for three genes, the large subunit (28S, LSU), internal transcribed spacers (ITS1-5.8S-ITS2) and translation elongation factor 1-alpha gene (*tef1- α*). The LSU gene was amplified by using the primers LR0R and LR5 (Vilgalys and Hester, 1990; Rehner and Samuels, 1994); nuclear ITS was amplified by using the primers ITS5 and ITS4 (White *et al*., 1990) and *tef1- α* gene was amplified using the primers EF1-983F and EF1-2218R (Rehner, 2001). The amplification reactions were performed in 25µL of total reaction that contained 9.5 µL of sterilized water, 12.5 µL of 2×Power Taq PCR MasterMix (Tri-I Biotech, Taipei, Taiwan), 1 µL of each forward and reverse primers and 1 µL of DNA template. The PCR thermal cycle program of ITS, LSU and *tef1-α* gene was processed as initially 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 50 seconds, elongation at 72°C for 1 minute and a final extension at 72°C for 10 minutes, and finally kept at 4°C. The PCR products were analyzed by 1.5% agarose gels containing the Safeview DNA stain (GeneMark, Taipei, Taiwan) to confirm the expected molecular weight of a single amplification product. PCR products were purified and sequenced with primers mentioned above by Tri-I Biotech, Taipei, Taiwan. Nucleotide sequences were deposited in GenBank (**Table 1**).

Phylogenetic analysis: Phylogenetic analyses were performed from a combined ITS, LSU and *tef1-α* sequence data. Sequence results generated were subjected to BLAST (NCBI) searches to obtain the closest matches in GenBank. Sequences generated from this study were analyzed with related taxa in the family *Dictyosporiaceae* which were obtained from GenBank and from recently published data (Jayasiri *et al*., 2019; Phookamsak *et al*., 2019) **(Table 1).** The combined dataset consisted of 45 sequences including our newly generated sequences. The multiple alignments were made with MAFFT v. 7 at the web server (http://mafft.cbrc.jp/alignment/server), using default settings (Katoh and Standley, 2013).The alignment was refined manually with BioEdit v. 7.0.5.2 (Hall, 1999) where necessary.

The phylogenetic analyses were obtained from Randomized Accelerated Maximum Likelihood (RAxML), maximum parsimony analysis (MP) and Bayesian analyses. Maximum likelihood trees were generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis *et al*., 2008, Stamatakis, 2014) in the CIPRES Science Gateway platform (Miller *et al*., 2010) using GTR+I+G model of evolution. Maximum parsimony analysis (MP) was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2002), with parameters as described in Tennakoon *et al*. (2019). Descriptive tree statistics for parsimony (Tree Length [TL], Consistency Index [CI], Retention Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI] were calculated.

Table 1. GenBank and culture collection accession numbers of species included in the present phylogenetic study. The newly generated sequences are shown in bold.

Species	Strain/Voucher no.	GenBank accession no.		
		ITS	LSU	*tef1-α*
Aquadictyospora lignicola	MFLUCC 17-1318	MF948621	MF948629	MF953164
Aquaticheirospora lignicola	HKUCC 10304	AY864770	AY736378	–
Cheirosporium triseriale	HMAS 180703	EU413953	EU413954	–
Dendryphiella eucalyptorum	CBS 137987	KJ869139	KJ869196	–
D. fasciculata	MFLUCC 17-1074	MF399213	MF399214	–
D.paravinosa	CBS 141286	KX228257	KX228309	–
Dictyocheirospora bannica	MFLUCC 16-0874	MH381765	MH381774	–
D. garethjonesii	DLUCC 0848	MF948623	MF948631	MF953166
D. heptaspora	CBS 396.59	DQ018090	–	–
D. metroxylonis	MFLUCC 15-0282	MH742324	MH742316	MH764301
D. nabanheensis	KUMCC 16-0152	MH388340	MH376712	MH388375
D. pandanicola	MFLUCC 16-0365	MH388341	MH376713	MH388376
D. rotunda	MFLUCC 14-0293	KU179099	KU179100	–
D. subramanianii	BCC 3503	DQ018094	–	–
D. vinaya	MFLUCC 14-0294	KU179102	KU179103	–
D. xishuangbannaensis	KUMCC 17-0181	MH388342	MH376714	MH388377
Dictyosporium alatum	ATCC 34953	NR 077171	DQ018101	–
D. digitatum	KH 401	LC014545	AB807515	AB808491
D. elegans	NBRC 32502	DQ018087	DQ018100	–
D.nigroapice	BCC 3555	DQ018085	–	–
D. olivaceosporum	KH 375	LC014542	AB807514	AB808490
D.sexualis	MFLUCC 10-0127	KU179105	KU179106	–
D.tetrasporum	KT 2865	LC014551	AB807519	AB808495
D. thailandicum	MFLUCC 13-0773	KP716706	KP716707	–
Digitodesmium bambusicola	CBS 110279	DQ018091	DQ018103	–
Gregarithecium curvisporum	KT 922	AB809644	AB807547	–
Jalapriya inflata	NTOU 3855	JQ267362	JQ267363	–
J. pulchra	MFLUCC 15-0348	KU179108	KU179109	–
J. toruloides	CBS 209.65	DQ018093	DQ018104	–
Periconia igniaria	CBS 379.86	–	AB807566	AB808542
P. igniaria	CBS 845.96	–	GU301841	AB808543
Pseudocoleophoma bauhiniae	MFLUCC 17-2280	MK347735	MK347952	MK360075
P. bauhiniae	MFLUCC 17-2586	MK347736	MK347953	MK360076
P. calamagrostidis	KT 3284	LC014592	LC014609	LC014614
P.polygonicola	KT 731	AB809634	AB807546	AB808522
P. typhicola	MFLUCC 16-0123	KX576655	KX576656	–
P. zingiberacearum	**NCYUCC 19-0052**	**MN615939**	**MN616753**	**MN629281**
P. zingiberacearum	**NCYUCC 19-0053**	**MN615940**	**MN616754**	**MN629282**
P. zingiberacearum	**NCYUCC 19-0054**	**MN615941**	**MN616755**	**MN629283**
Pseudodictyosporium elegans	CBS 688.93	DQ018099	DQ018106	–
P. indica	CBS 471.95	DQ018097	–	–
P. thailandica	MFLUCC 16-0029	KX259520	KX259522	KX259526
P. wauense	DLUCC 0801	MF948622	MF948630	MF953165
P. wauense	NBRC 30078	DQ018098	DQ018105	–
Vikalpa australiensis	HKUCC 8797	DQ018092	–	–

Using MrModeltest 2.2, model of nucleotide substitution was performed (Nylander, 2004). Bayesian analysis (BI) (Huelsenbeck and Ronquist, 2001) was conducted with MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) to evaluate posterior probabilities (PP) (Rannala and Yang 1996; Zhaxybayeva and Gogarten, 2002) by Markov Chain Monte Carlo sampling (BMCMC). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. Phylograms were visualized with FigTree v1.4.0 (Rambaut, 2012) and annotated in Microsoft Power Point (2010). New strain sequences generated in this study are deposited in GenBank. The final alignment and trees were deposited in TreeBASE, submission ID:25289.

RESULTS

Phylogenetic analysis: The combined dada set of ITS, LSU and *tef1- α* sequences comprised 2940 characters, of which 2187 characters are constant, 555 characters are parsimony-informative, while 198 variable characters are parsimony-uninformative in the maximum parsimony (MP) analysis (TL = 1994, CI = 0.539, RI = 0.728, RC = 0.392, HI = 0.461). The RAxML analysis of the combined dataset yielded a best scoring tree (**Fig. 1**) with a final ML optimization likelihood value of -13834.72153. The matrix had 938 distinct alignment patterns, with 45.03% of undetermined characters or gaps. Estimated base frequencies; A = 0.236760, C = 0.253494, G = 0.268951, T = 0.240796; substitution rates AC = 1.528834, AG = 2.993918, AT = 2.32776, CG = 0.739798, CT = 8.030230, GT = 1.000; proportion of invariable sites I = 0.536378; gamma distribution shape parameter α = 0.662804. The Bayesian analysis was resulted 10000 trees after 1000000 generations. All analyses (ML, MP and BYPP) gave similar results and in agreement with previous studies based on multi-gene analyses (Jayasiri *et al.,* 2019; Phookamsak *et al.,* 2019). Phylogenetic analyses of the combined data matrix showed considerably high bootstrap support and well-resolved clades (**Fig. 1**). Bootstrap support values for maximum likelihood, maximum parsimony higher than 60 % and Bayesian posterior probabilities (BYPP) greater than 0.95 are given above each branch in that order (**Fig. 1**).

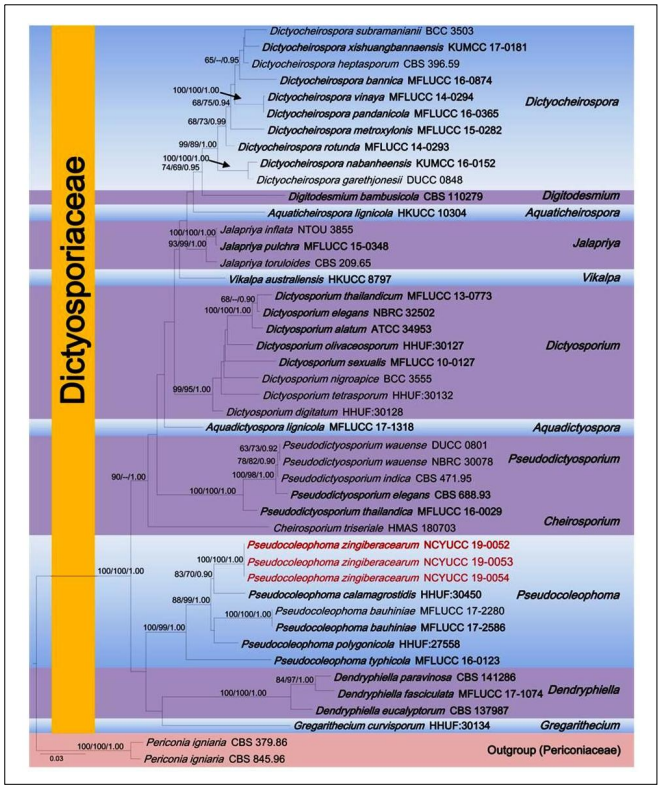

Fig. 1 RAxML tree based on a combined dataset of ITS, LSU and *tef1- α* partial sequences of 63 taxa of the family ***Dictyosporiaceae***. Bootstrap support values for maximum likelihood (ML), maximum parsimony (MP) values higher than 60% and Bayesian posterior probabilities (BYPP) greater than 0.90 are given above each branch, respectively. The new isolates are in red. Ex-type strains are in bold. The tree is rooted by *Periconia igniaria* (CBS 379.86, CBS 845.96).

TAXONOMY

Pseudocoleophoma zingiberacearum Tennakoon, D.J. Bhat, C.H. Kuo & K.D. Hyde, sp. nov. **Fig. 2**

IF 556893; FoF 06719

***Etymology*:** The species name reflects the host family *Zingiberaceae* from which the holotype was collected.

Holotype - NCYU 19-0004

Saprobic on *Hedychium coronarium* J. Koenig (*Zingiberaceae*). **Sexual morph**: Undetermined. **Asexual morph**: Conidiomata forming dark spots on host surface, 110-150 µm high × 200-220 µm diam. (= 131.7 × 208.6 µm, n = 10), pycnidial, solitary, immersed in substrate, visible as black dots covered by epidermal tissues, multi-loculate, depressed globose, glabrous, non-ostiolate. Conidiomata wall 17-24 µm wide (= 21.2 µm, n = 10), thin-walled, of equal thickness, composed of 3-4 layers of brown pseudoparenchymatous cells organized in textura angularis. Conidiophores reduced to conidiogenous cells. Conidiogenous cells 1.5-2.5 × 1-1.5 µm (= 1.8 × 1.1 µm, n = 30), phialidic, doliiform to lageniform, hyaline, aseptate, smooth-walled. Conidia 12-14 × 2-3 µm (= 13.2 × 2.4 µm, n = 30), solitary, hyaline, aseptate, oblong to ellipsoidal, with rounded to obtuse ends, smooth-walled, with guttules.

Culture characteristics: Colonies on PDA, 30 mm diam. after 3 weeks, medium dense, irregular, flat, slightly raised, with smooth surface and crenate edge, fluffy to velvety with smooth aspects from above; yellowish at the margin, white to yellowish in the centre, from below; light yellowish at the margin, light brown to yellowish in the centre, without any pigments in media.

Material examined: Taiwan, Chiayi, Fanlu Township area, Dahu forest, dead leaves of *Hedychium coronarium* (*Zingiberaceae*), 20 July 2019 (23°26.535'N 120°35.330'E), D.S. Tennakoon, GSP035-A (NCYU 19-0004, **holotype**), ex-type living culture, NCYUCC 19-0052; *ibid*. 20 July 2019 (23°27.402'N 120°36.588'E), GSP035-B (NCYU 19-0005, **paratype**), NCYUCC 19-0053, GSP035-C (NCYU 19-0006, NCYUCC 19-0054).

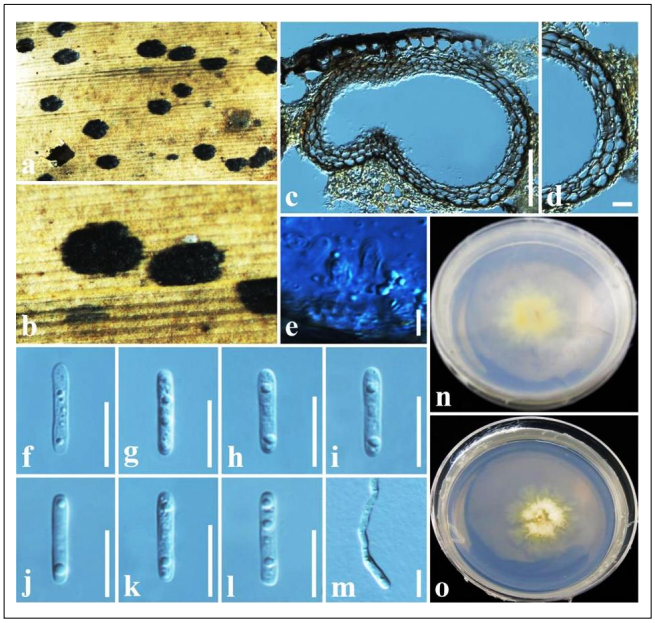

Fig. 2 ***Pseudocoleophoma zingiberacearum*** (NCYU 19-0004, **holotype**) **a**. Appearance of conidiomata on host; **b**. Close-up of conidiomata; **c**. Section of conidioma; **d**. Conidioma wall; **e**. Conidiogenous cells; **f-l**. Conidia; **m**. Germinated conidium; **n**. Colony from below; **o**. Colony from above. **Scale bars**: c = 50 µm, d = 10 µm, e = 5 µm, fm = 10 µm.

Remarks: The characteristics of our species, *Pseudocoleophoma zingiberacearum,* tally with those described under *Pseudocoleophoma* in having immesed to semi-immersed conidiomata, phialidic, doliiform to lageniform conidiogenous cells and hyaline, oblong to ellipsoidal, smooth walled conidia (Tanaka *et al*., 2015, Hyde *et al*., 2016, Jayasiri *et al*., 2019). Multi-gene phylogeny generated herein, indicates that *Pseudocoleophoma* constitutes a strongly supported (100% ML, 99% MP, 1.00 BYPP) monophyletic clade sister to *Dendryphiella* and *Gregarithecium* which are also members of *Dictyosporiaceae* (**Fig. 1**). In particular, *Pseudocoleophoma zingiberacearum* shares a close phylogenetic relationship with *Pseudocoleophoma calamagrostidis* (CBS 139700) in high bootstrap support (83% ML,70% MP, 0.90 BYPP). However, *Pseudocoleophoma zingiberacearum* is distinct from *P. calamagrostidis* in having immersed, non-ostiolate conidiomata, wider conidiomatal wall (17-24 µm) and larger conidia (12-14 × 2-3 µm), whereas *P. calamagrostidis* has immersed to erumpent, ostiolate conidiomata, thinner conidiomatal wall (7.5-15 µm) and smaller conidia (12-14 × 2-3 µm).

Table 2. Synopsis of hitherto recorded *Pseudocoleophoma* species

Pseudocoleophoma species	Size (µm)			Septation	Reference
	Conidiomata	Conidiomata wall	Conidia		
P. bauhinia (MFLUCC 17–2586)	90-115 × 130-150	20-25	7.5-11 × 2–3	Aseptate	Jayasiri *et al.* (2019)
P. calamagrostidis (CBS 139700)	220-300 × 250-500	7.5-15	6-10 × 2-2.5	Aseptate	Tanaka *et al.* (2015)
P. polygonicola (CBS 139701)	170-250 diam.	12-15	11.5-18 × 3–4.5	Aseptate	Tanaka *et al.* (2015)
P. typhicola (MFLUCC 16-123)	140-150 × 60-100	40-45	9-11 × 2-3	1-2	Hyde *et al.* (2016)
***P. zingiberacearum* (NCYUCC 19-0052)**	**110-150 × 200-220**	**17-24**	**12-14 × 2-3**	**Aseptate**	**This study**

Pseudocoleophoma zingiberacearum also differs from *P. calamagrostidis* in terms of host association, as the latter has been reported from dead leaves of *Calamagrostisma tsumurae* Maxim. (*Poaceae*) (Tanaka *et al*., 2015). This is the first report of *Pseudocoleophoma* species from *Hedychium coronarium* and even from the family *Zingiberaceae*. The main morphological differences of *Pseudocoleophoma* species are presented in **Table 2**. Besides, a comparison of the 570 nucleotides across the ITS (+5.8S) gene region of *Pseudocoleophoma zingiberacearum* and closely similar *P. calamagrostidis* reveals 18 base pair differences (3.15%) and therefore provides further evidence to introduce *P. zingiberacearum* as a new species as in the guidelines of Jeewon and Hyde (2016).

DISCUSSION

The genus *Pseudocoleophoma* Kaz. was introduced by Tanaka *et al*. (2015) based on asexual dissimilarities with *Coleophoma* species and typified by *P. calamagrostidis* Kaz. Tanaka & K. Hiray. However, *Coleophoma* species can be distinguished from *Pseudocoleophoma* in having pycnidia possessing paraphyses that are not found in *Pseudocoleophoma*, and being a member of the *Dothideales*, rather than the *Pleosporales* (Duan *et al*., 2007; De Gruyter *et al*., 2009; Tanaka *et al*., 2015). *Pseudocoleophoma* is still a small genus and comprises only four species, *viz. P. bauhiniae* Jayasiri, E.B.G. Jones & K.D. Hyde, *P. calamagrostidis* Kaz. Tanaka & K. Hiray., *P. polygonicola* Kaz. Tanaka & K. Hiray. and *P. typhicola* Kamolhan, Banmai, Boonmee, E.B.G. Jones & K.D. Hyde (Index Fungorum, 2019). In this study, we provide taxonomic details for a new species, *Pseudocoleophoma zingiberacearum,* collected from dead leaves of *Hedychium coronarium* (*Zingiberaceae*) and thus expand the genus size up to five species.

According to the phylogenetic investigations, *Pseudocoleophoma zingiberacearum* clusters in a highly supported clade (100% ML, 99% MP, 1.00 BYPP) and nested closely to *P. calamagrostidis* (CBS 139700) (83% ML, 70% MP, 0.90 BYPP). Species of *Pseudocoleophoma* have so far been recorded only from few countries (i.e. Japan, Thailand and UK) and this is the first record from Taiwan. The host specificity of *Pseudocoleophoma* species is yet to be studied, despite having been collected from few host families (*Fabaceae, Poaceae, Typhaceae*). Interestingly, *Pseudocoleophoma zingiberacearum* is the first species in the genus recorded from *Zingiberaceae*. Further collections are needed for the expansion of the genus.

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