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 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 saprobic, 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), 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., *Falcatula* Souza, Hong Y. Su, Z.L. Luo & K.D. Hyde, *Phycomyces* Sonder. *Pseudocoleophoma kazakstani* Souza, Minami & 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 Chommunti 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 LROR 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 30 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 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain/Voucher no.</th>
<th>GenBank accession no.</th>
<th>TL</th>
<th>CI</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dictyosporiaceae</em> (Pleosporales, Dothideomycetes)....</td>
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<td></td>
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</table>

**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.
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. Phylogenograms 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 data 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 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 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 and Bayesian posterior probabilities (BYPP) greater than 0.95 are given above each branch in that order (Fig. 1).

TAXONOMY

_Pseudocoleophoma zingiberacearum_ Tennakoon, D.J. Bhat, C.H. Kuo & K.D. Hyde, sp. nov.

**Holotype** - NCYU 19-0004

Saprobic on _Hedychium coronarium_ J. Koenig (Zingiberaceae). Sexual morph: Undetermined. Asexual morph: Conidiomata forming dark spots on host surface, 110-200 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, doliform 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 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.

Aseptate

This study

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Tanaka

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-9

et al

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Reference

Conidiomata

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zingiberacearum

7.5

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conidiomatalwall (7.5-15μm) and smaller conidia (12-14

immersed to erumpent, ostiolate conidiomata,

conidioma

from . in having immersed, non-ostiolate

with (CBS 139700) in

zingiberacearum

Dictyosporiaceae Pseudocoleophoma

BYPP) monophyletic clade sister to and

constitutes a strongly supported (100% ML, 99% MP, 1.00

BYPP). Species of

Pseudocoleophoma

are described under in having immersed to

semi-immersed conidiomata, phialidic, doliform to

semi-immersed conidiomata, phialidic, doliiform to

Descriptively

Pseudocoleophoma zingiberacearum,

Remarks: The characteristics of our species,

Pseudocoleophoma zingiberacearum, tally with those

described under Pseudocoleophoma in having immersed to

semi-immersed conidiomata, phialidic, doliform 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

Gregar ithecium which are also members of

Dictyosporiaceae (Fig. 1). In particular, Pseudocoleophoma

zingiberacearum shares a close phylogenetic relationship with

Pseudocoleophoma calamaragrostidis (CBS 139700) in

high bootstrap support (83% ML, 70% MP, 0.90 BYPP).

However, Pseudocoleophoma zingiberacearum is distinct

from P. calamaragrostidis in having immersed, non-ostiolate

conidiomata, wider conidiomatal wall (17-24 μm) and larger

conidia (12-14 × 2-3 μm), whereas P. calamaragrostidis has

immersed to erumpent, ostiolate conidiomata, thinner

conidiomatal wall (7.5-15μm) and smaller conidia (12-14×

2-3μm).

Table 2. Synopsis of hitherto recorded Pseudocoleophoma species

<table>
<thead>
<tr>
<th>Pseudocoleophoma species</th>
<th>Size (μm)</th>
<th>Septation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. calamaragrostidis (CBS 139700)</td>
<td>20-30 × 250-350</td>
<td>1-2</td>
<td>Jayasiri et al. (2019)</td>
</tr>
<tr>
<td>P. zingiberacearum (CBS 139701)</td>
<td>7-12 × 10-20</td>
<td>1-2</td>
<td>Tanaka et al. (2015)</td>
</tr>
<tr>
<td>P. typhicola (CBS 101110)</td>
<td>180-100 × 200-150</td>
<td>1-2</td>
<td>Hyde et al. (2016)</td>
</tr>
<tr>
<td>P. zingiberacearum (NCYUCC 19-0052)</td>
<td>100-150 × 220-228</td>
<td>15-24</td>
<td>12-14-2.5</td>
</tr>
</tbody>
</table>

Pseudocoleophoma zingiberacearum also differs from P. calamaragrostidis in terms of host association, as the latter has been reported from dead leaves of Calamagrostis 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. calamaragrostidis 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. calamaragrostidis 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. calamaragrostidis Kaz. Tanaka & K. Hiray., P. polygonica 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. calamaragrostidis (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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