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A multiple gene genealogy reveals phylogenetic placement of *Rhopalostroma lekae*

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Abstract

Rhopalostroma lekae was collected on bark of wood in Chiang Rai Province, Northern Thailand and isolates from the sexual state produced a nodulosporium-like asexual state in culture. A combined multigene sequence analysis was used to infer the phylogenetic position of *R. lekae* and its affinities with other xylariaceous genera. *Rhopalostroma* is confirmed to have particularly close affinities with the genera *Phylacia* and *Thamnomyces.* Secondary metabolite profiling of *R. lekae* showed the species to produce binaphthalene tetrol (BNT) as a major metabolite and several minor undetermined metabolites. The phylogenetic placement of *R. lekae* was resolved using a polythetic approach. Herbarium material and living cultures representing an authentic specimen of *R. lekae* are deposited in publically accessible collections that can be used in future studies.

Key words: asexual morph, nodulisporium-like, taxonomy, Xylariaceae

Introduction

Rhopalostroma was introduced by Hawksworth (1977) to accommodate *Rhopalostroma africanum* (Wakef.) D. Hawksw., *R. angolense* (Welw. & Curr.) D. Hawksw., *R. indicum* D. Hawksw. & Muthappa, *R. luzonense* (Lloyd) D. Hawksw. and *R. sphaerocephalum* (Petch) D. Hawksw. with *R. indicum* as the generic type. *Rhopalostroma* is characterized by stipitate, melanized stromata with often abruptly expanded convex heads, which become brown, purplish or black at maturity. Perithecia are immersed in dark brown to black fleshly stromata, which lack concentrical zonations. The perithecial layer is arranged peripherally in a single layer (monostichous) below the convex surface of the head with non-papillate ostioles. *Rhopalostroma gracile* D. Hawksw. & Whalley, *R. kanyae* Whalley & Thienhirun and *R. lekae* Whalley, Thienhirun, M.A. Whalley & Sihanonth from Thailand and *R. hawksworthii* Vaidya and *R. sphaerocephalum* (Petch) D. Hawks. var *indica*, from India have since been added to the genus (Hawksworth 1979, Hawksworth & Whalley 1985, Vaidya *et al.* 1991, Whalley and Thienhirun 1996, Whalley *et al.* 1998, Patil *et al.* 2012). *Rhopalostroma lekae* has also been reported from India (Patil *et al.* 2012). *Rhopalostroma* appears to be restricted to subtropical Africa and South Asia, with the discovery of *R. africanum* in India, this leaves *R. angolense* as the only species restricted to Africa (Patil *et al.* 2012).

Rhopalostroma was placed in Xylariaceae due to strong affinities with *Thamnomyces* Ehrenb. and *Phylacia Lév.* (Hawksworth 1977). *Rhopalostroma* is particularly similar to *Thamnomyces dendroidea* Cooke & Massee from micromorphological characters. This similarity and the nodulisporium-like asexual state support the inclusion of *Rhopalostroma* in the family, even though asci lack any apical apparatus. The asci in *Rhopalostroma* are repeatedly reported as evanescent in the majority of species including *R. africanum, R. indicum, R. kanyae*, *R. lekae*, *R. luzonense* and *R. sphaerocephalum* (Hawksworth 1977, Whalley & Thienhirun 1996). In *Thamnomyces*, asci lack any distinctive apical thickening or amyloid apical apparatus and the apices of the stromata are characteristic of *Rhopalostroma. Rhopalostroma* differs from *Thamnomyces* as the latter has smaller dendriod stromata and ascospores with a longitudinal germ slit. *Phylacia* differs as perithecia form a gleba-like mass and always have clavate, rather than cylindrical asci (Hawksworth 1977).

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With the exception of *R. angolense, Rhopalostroma* species lack molecular data. *Rhopalostroma angolense* was collected in western Africa and ITS sequence data was generated from pure culture (Stadler *et al.* 2010a). *Rhopalostroma* species produce BNT (binaphthalene tetrol) as a major metabolite besides certain other undetermined compounds (Stadler *et al.* 2004). Secondary metabolite profiles of *Phylacia* (Bitzer *et al.* 2008) and *Thamnomyces* (Stadler *et al.* 2010b) are similar to those obtained from *R. angolense* (Stadler *et al.* 2010a).

We re-collected *Rhopalostroma lekae* from Northern Thailand. In this manuscript we provide a detailed morphological description of *R. lekae* and its asexual state in culture. We also sequenced the taxon and provide multigene molecular data to show its affinities with *R. angolense* and other members of Xylariaceae. Herbarium material is deposited in Mae Fah Luang University, Thailand (MFLU) as 13-0440 and New Zealand Fungal Herbarium (PDD) as 104362 and cultures at Mae Fah Luang University Culture Collection, Thailand and the International Collection of Microorganisms from Plants, Landcare Research, New Zealand. Chemotaxonomic data including the chemical profiles derived from the stromatal extracts are also provided for the species classification. The current paper deals with the morphological, molecular and chemotaxonomic data of *R. lekae*.

Materials and methods

Sample collection and specimen examination

Specimens of *R. lekae* were collected in Chiang Rai Province, Northern Thailand in September and December 2012 and macroscopic and microscopic characters were recorded. A Motic SMZ-168 dissecting microscope was used to observe the structure of stromata and perithecia. A Nikon ECLIPSE 80i compound microscope was used to observe asci and ascospore characters, the reaction of ascal apical rings were tested using Melzer's reagent. Microphotography was done using a Canon 450D digital camera fitted to the microscope. Measurements of stromata (n=10), ascomata (n=10), asci (n=20) and ascospores (n=40) were made from materials mounted in water and the mean values were used in the descriptions. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures were processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems Inc). Formation of stromatal pigments was observed by placing a small piece of stroma (from both head and stipe) in a few drops of 10% KOH (Ju & Rogers 1996). The color designations were determined following Rayner (1970).

Description of cultures and asexual state

Pure cultures were obtained from single spores following the method detailed by Chomnunti *et al.* (2014). The cultures were grown in Malt and Yeast Extract Agar media (Malt extract 6 g/L, yeast extract 0.6 g/L, dextrose 4 g/l) and incubated at room temperature 28°C for 2–4 days. After 2–4 days, hyphal tips were cut and transferred to fresh Difco Oatmeal Agar (OA) media. The cultures were incubated at 25 °C for one month. After 2–3 weeks, cultures on OA were checked for asexual structures. Conidiogenous structures (conidiophores, conidiogenous cells and conidia) were observed and measured by phase contrast microscopy under 400–1000 × optical magnification. Asexual states were classified based on Stadler *et al* (2013).

DNA isolation, PCR and sequencing

DNA was extracted from isolates grown on Malt and Yeast Extract Agar media overlaid with sterilized cellophane for 5 days at 25 °C (Murali *et al.* 2006) and total genomic DNA was extracted from 0.05 to 0.10 g of mycelium scraped from the edge of the growing culture (Wu *et al.* 2001). DNA isolation was carried out according to Udayanga *et al.* (2012) with certain modifications. Precipitated DNA was recovered by centrifugation of 12,000 rpm for 10 min and three washings with 70 % ethanol, air dried, dissolved in 50 µl of sterilized distilled water and stored at -20 °C until use for amplification reactions.

Three loci were sequenced including ITS (White *et al.* 1990), LSU (Vilgalys & Hester 1990) and RPB2 (Liu *et al.* 1999). The primers and PCR protocols are summarized in Table 1. The DNA fragments were amplified using an automated thermal cycler (DongShen EDC-810- Eastwin, LifeSciences). The total volume of 50 µl reaction mixture [10×PCR buffer, 0.2 mM dNTP, 0.4 µM of each primer, 1.5 mM $MgCl_2$, Taq Polymerase and 10 ng template DNA (1:10 dilutted)], was used for PCR with adjustments of components' volumes and concentration when needed. The PCR products were visualized on 1 % agarose gels stained with Goldview (Guangzhou Geneshun Biotech, China) with D2000 DNA ladder (Realtimes Biotech, Beijing, China). All the PCR products were purified according to the company protocols and DNA sequencing was performed using the same primers in an Applied Biosystem 3730 DNA analyzer at SinoGenoMax Company, Beijing, China.

TABLE 1. Genes/loci used in the study with respective PCR primers and protocols.

Locus/gene	PCR primers (F/R)	PCR protocol	**Length of PCR product**
ITS	*ITS1/ITS4*	[a]94^{0}C: 1 min, 54–55^{0}C: 30 sec, 72^{0}C: 1.30 min (34 cycles)[b]	>600 bp
LSU	*LROR/LR5*	[a]94^{0}C: 1 min, 53^{0}C: 50 sec, 72^{0}C: 1.30 min (35 cycles)[b]	1000–1200 bp
RPB2	*fRPB2-5f/fRPB2-7cR*	[a]95^{0}C: 45 sec, 57^{0}C: 50 sec, 72^{0}C: 1.30 sec (35 cycles)[b]	>1500 bp

[a]Initiation step of 95 °C: 5 min.

[b]Final elongation step of 72 °C: 10 min and final hold at 4 °C applied to all PCR thermal cycles.

Sequence alignment and phylogenetic analysis

To reveal the phylogenetic position of *Rhopalostroma lekae,* 95 sequences from representative Xylariaceae species from what we consider to be reliable studies (Sanchez-Ballesteros *et al.* 2000, Triebel *et al.* 2005, Bitzer *et al.* 2008, Peršoh *et al.* 2009, Tang *et al.* 2009, Hsieh *et al.* 2010, Stadler *et al.* 2010a, Jaklitsch and Voglmayr 2012, Jaklitsch *et al.* 2014) were downloaded from GenBank and included in the analysis, with *Diatrype disciformis* (Hoffm.) Fr. as outgroup. The respective sequences from this study are deposited in GenBank (see Table 2). The sequences from specimens and cultures used in this study were obtained from and specimens/cultures were deposited in public herbaria, abbreviated as proposed in the Index herbariorum (http:// sciweb.nybg.org/science2/IndexHerbariorum.asp) or World Federation for Culture Collections (http://www.wfcc.info/collections/) and others as follows, Assembling the Fungal Tree of Life (AFTOL), Taxa collected and identified by Alvin M. C. Tang (AT), Herbarium of Jacques Fournier (JF), Mae Fah Luang University Culture Collection, Thailand (MFLUCC), Herbarium of Yu Ming Ju (YMJ). The phylogenetic analysis was performed using the combined ITS-LSU-RPB2 matrix.

Sequence data were aligned either with MUSCLE v.3.6 (Edgar 2004) or Bioedit 7.1.3.0 (Hall 1999) and further implemented with Clustal X v1.83 (Thompson *et al.* 1997) and manually aligned where necessary. All characters were assessed to be unordered and equally weighed. Gaps were treated as missing data. Phylogenetic analyses were performed using RAxML v7.0.3 (Stamatakis *et al.* 2010) as implemented in RAxML GUI 0.95 (Silvestro & Michalak 2012). The search strategy was set to rapid bootstrapping and the analysis carried out using the GTR model of nucleotide substitution. The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004). The bootstrap analysis for each ML tree was performed with 1000 fast bootstrap replicates with the same parameter settings using the GTR substitution model selected by MrModel Test. Model parameters were calculated separately for three different gene regions included in the combined analyses. The resulting trees were viewed using the Tree View application (Page 1996).

TABLE 2. Strains and NCBI GenBank accession numbers of representative taxa of Xylariaceae used in the phylogenetic analyses.

Name	Source	Genbank Accession numbers		
		ITS	LSU	RPB2
Amphirosellinia fushanensis	HAST Isolate 91111209[a]	GU339496	–	GQ848339
Amphirosellinia nigrospora	HAST Isolate 91092308[a]	GU322457	–	GQ848340
Annulohypoxylon atroroseum	MUCL 13113	KM186291	KM186292	–
Annulohypoxylon moriforme var. microdiscum	CBS123834	DQ631935	DQ840061	DQ631960
Annulohypoxylon nitens	MFLUCC 12-0823	KJ934991	KJ934992	KJ934994
Annulohypoxylon stygium	MFLUCC 13-0826	KJ940870	KJ940869	KJ940868
Anthostomella brabeji	CBS:110128	EU552098	EU552098	–
Anthostomella proteae	CBS:110127	EU552101	EU552101	–
Biscogniauxia capnodes	CM AT-015	DQ631933	DQ840055	–
Biscogniauxia marginata	MFLUCC 12-0740	KJ958407	KJ958408	KJ958409
Collodiscula japonica	CBS:124266[a]	JF440974	JF440974	–
Creosphaeria sassafras	CM AT-018	DQ631934	DQ840056	DQ631964
Daldinia concentrica	CBS 113277	AY616683	–	–
Hypoxylon fragiforme	MUCL 51264	KM186294	KM186295	KM186296
Hypoxylon monticulosum	MFLUCC 12-0818	KM052716	KM052717	KM052719
Kretzschmaria deusta	CBS 826.72	AJ390435	–	–
Kretzschmaria deusta	JF 05154	–	DQ840077	
Nemania aenea	JF 02118	–	DQ840070	DQ631951

.....continued on the next page

TABLE 2. (Continued)

Name	Source	Genbank Accession numbers		
		ITS	LSU	RPB2
Nemania aenea	CBS 680.86	AJ390427	–	–
Nemania chestersii	ATCC 38988	AJ390430	–	–
Nemania chestersii	JF 04024	–	DQ840072	DQ631949
Nemania diffusa	FR AT-113	DQ658238	DQ840073	DQ631947
Nemania diffusa	GZ AT-F006	FJ438909	DQ840076	DQ631957
Nemania maritima	JF04055	DQ631941	DQ840074	DQ631946
Nemania maritima	HAST Isolate 89120401[a]	GU292822	–	GQ844775
Nemania plumbea	JF TH-04-01	DQ641634	DQ840071	DQ631952
Nemania serpens	FR AT-114	DQ631942	DQ840075	DQ631948
Phylacia poculiformis	MUCL 51706	FN428830	–	–
Poronia pileiformis	HAST Isolate 88113001[b]	GU324760	–	GQ853037
Rhopalostroma angolense	CBS 126414	FN821965	KM186298	KM186297
Rhopalostroma lekae	**MFLUCC 13-0123**	**KJ472428**	**KJ472427**	**KJ472429**
Rosellinia corticium	GZ-AT-F004	DQ631940	DQ840078	–
Rostrohypoxylon terebratum	CBS 119137[a]	DQ631943	DQ840069	DQ631954
Stilbohypoxylon elaeicola	HAST isolate 173	EF026148	–	GQ844826
Stilbohypoxylon quisquilirum	CM AT-016	DQ631937	DQ840079	–
Thamnomyces camerunensis	MUCL 51396	FN428828	–	–
Xylaria acuminatilongissima	HAST Isolate 95060506[a]	EU178738	–	GQ853028
Xylaria brunneovinosa	HAST voucher 720 [c]	EU179862	–	GQ853023
Xylaria escharoidea	HAST Isolate 95060505[a]	EU179864	–	GQ853026
Xylaria hypoxylon	CBS122620[b]	AM993141	KM186301	KM186302
Xylaria grammica	HAST XT09009	DQ631944	DQ840081	DQ631956
Diatrype disciformis	AFTOL 927	AJ302437	DQ470964	DQ470915

[a]Ex-type strain

[b]Ex-epitype strain

[c]Hototype

HPLC profiling

Stromatal secondary metabolites were extracted using the protocol described by Kuhnert *et al.* (2014). Preparation of samples for HPLC profiling and analysis of results were carried out as described by Stadler *et al.* (2014).

Results

Molecular phylogeny

The combined ITS, LSU and RPB2 dataset utilized 45 taxa, with *Diatrype disciformis* as outgroup. The dataset consists of 2592 characters after alignment, of which 1884 sites were included in the ML analyses. The best scoring RAxML tree is shown in Fig. 1. Xylariaceous taxa clustered in to two major clades A and B, which are supported by the 95% and 85% bootstrap support respectively for hypoxyloid (nodulisporium-like asexual morph) and xylarioid (geniculosporium-like asexual morph) Xylariaceae. *Creosphaeria sassafras* with 95% bootstrap support forms a separate clade, appearing as a distinct linage to other members. The current study strongly supports the distinction of the two lineages of hypoxyloid and xylarioid Xylariaceae.

Clade A is a highly supported and comprising *Annulohypoxylon, Daldinia, Hypoxylon, Phylacia, Rhopalostroma, Rostrohypoxylon* and *Thamnomyces,* which are hypoxyloid Xylariaceae (95% bootstrap support). Clade B (xylarioid genera) comprised *Euepixylon, Kretzschmaria, Nemania*, *Rosellinia, Stilbohypoxylon* and *Xylaria,* which clustered together with high bootstrap support (85%). The basal group of the family Xylariaceae is represented by *Biscogniauxia* species, which cluster separately as a sister group to the other taxa of xylarioid Xylariaceae with high bootstrap support (85%).

Within the hypoxyloid Xylariaceae clade (A), two well-supported subclades (Clades I and II) were formed. Clade I with 90% bootstrap support, consists of *Daldinia* with the related genera *Phylacia, Rhopalostroma,* and *Thamnomyces. Rhopalostroma angolense* and *R. lekae* cluster together with 100% bootstrap support and clearly belong to the same genus. *Phylacia*, *Rhopalostroma* and *Thamnomyces* form a separate clade in which, *Phylacia poculiformis* separates from others with 76% bootstrap support. The monophyletic origin of *Thamnomyces* and *Rhopalostroma* is supported by 85% bootstrap support.

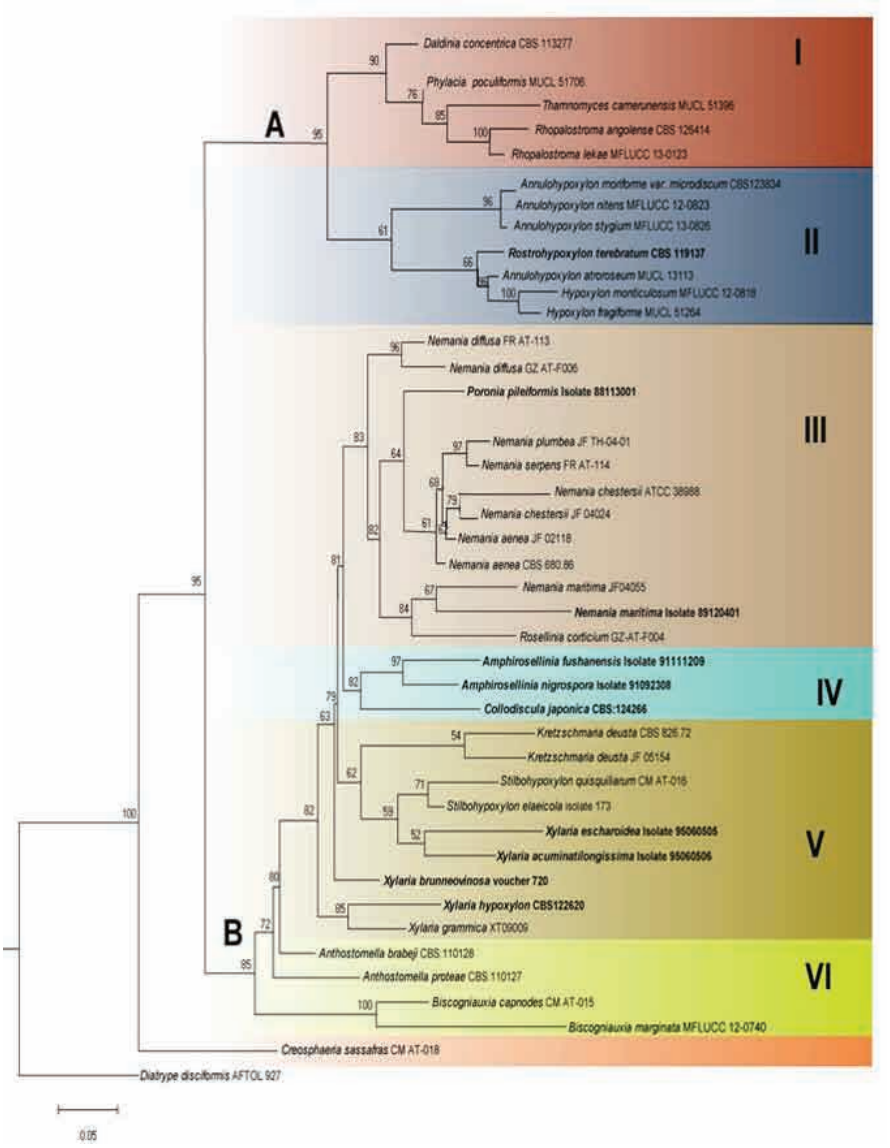

FIGURE 1. The phylogram inferred from likelihood analysis of family Xylariaceae using ITS-LSU-RPB2 sequences. Strain/culture numbers are given following the taxon names; Type specimens and ex/epi-type strains are highlighted in **bold**. The bootstrap support values from likelihood analysis >50% from 1000 RAxML replicates are shown above the branches. The tree is rooted with *Diatrype disciformis* (out group).

Clade II (61% bootstrap support) include *Annulohypoxylon, Hypoxylon* and *Rostrohypoxylon. Rostrohypoxylon terebratum* clusters within the hypoxyloid clade with *Annulohypoxylon atroroseum* with 66% bootstrap support.

Clade B is the xylarioid clade and has strong bootstrap support (85%). However the multigene phylogeny inferred from the combined LSU-ITS-RPB2 gene datasets appeared to be intermingled and resolution of internal branches were poor, probably due to the high length variations in the ITS dataset. Within clade B two distinct subclades of *Nemania* (clade IV) and *Xylaria* (clade VI) are well-supported as sister groups. The phylogenetic relationships of other genera such as *Kretzschmaria, Rosellinia, Stilbohypoxylon* to *Xylaria* and *Nemania* are still unresolved as well as interspecies relationships among *Xylaria*. Clade V comprises of *Amphirosellinia* and *Collodiscula* with 82% bootstrap support. The phylogenetic position and the relationships of *Biscogniauxia* with other genera are rather ambiguous. In combined gene phylogentic analysis, *Anthostomella* and *Biscogniauxia* formed a basal clade to all other xylarioid genera.

Taxonomy

Rhopalostroma lekae A.J.S. Whalley, S. Thienh., M.A. Whalley & P. Sihan., Botanical Journal of Scotland 50(2): 188 (1998) (Figs. 2–3) MycoBank no.: MB 483748 FoF 000017.

Habitat and distribution:—Saprobic on dead bark (Whalley *et al.* 1998), bark of *Memecylon umbellatum* Burm. (Patil *et al.* 2012), Thailand (Northern Thailand) and India (Patil *et al.* 2012).

Type species:—(as listed in protologue), Thailand, Nakon Ratchasima Province, Khao Yai National park, Orchid Falls Trail, in dead wood, 30 November 1996, T. Flegel, (holotype, RFD, no accession number) (Whalley *et al.* 1998)-stated herbarium contacted and did not have type material (see discussion).

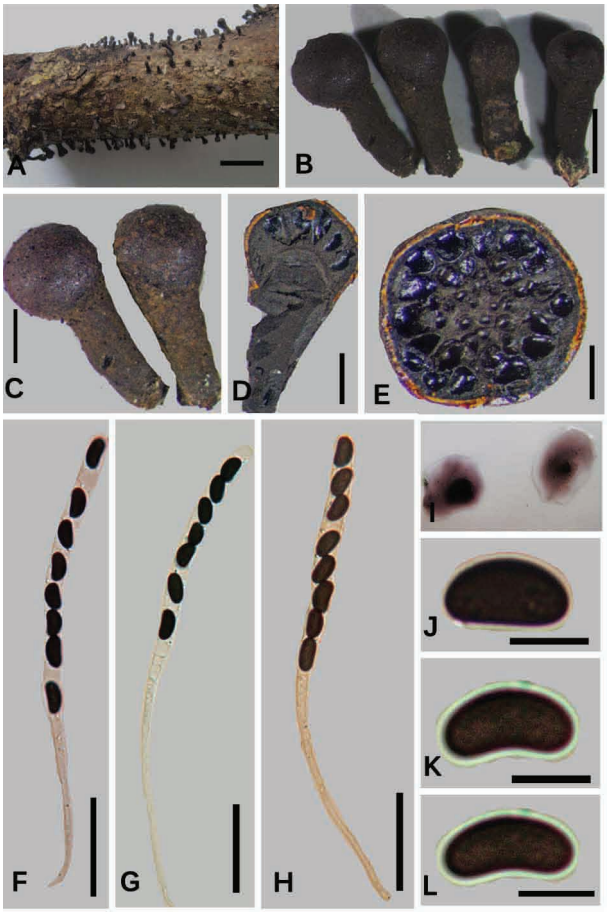

FIGURE 2. *Rhopalostroma lekae* (MFLU 13-0440). A: Habit on bark. B: Separated mature stromata. C: Stromata with dark purplish pigments in head part. D: Longitudinal section of stroma showing perithecial alignment in the periphery of stroma. E: Cross section of the stroma (head) showing perithecia. F and G: Mature ascus in water. H: Ascus in Melzer's reagent, Note the lack of an apical apparatus. I: KOH extractable pigments of stroma (left-head, right–stipe). J–l: Mature ascospores in water. *Bars:* a–b 5 mm; c–d 1 mm; e 2 mm; f–l 10 µm.

Etymology:—named in honor of the distinguished Thai Mycologist, Leka Manoch.

Sexual state:—*Stromata* (4–)5–8(–9) mm, 6.5 mm on average high, erumpent through bark, widely spreading, simple, mostly solitary or rarely clustered, but not fused and rarely branched, dark brown to black, dull yellow granules beneath the surface, carbonaceous, flesh of stipe black, stipe part (2–)3–5(–6) mm, 4.5 mm on average high, (0.3–)0.5–1.5(–1.8) mm, 0.9 mm on average diam, head expanded, globose to subglobose, (0.8–)1–1.5(–1.7) mm, 1 mm on average high, (1.8–)2–4(–4.3) mm, 2.8 mm on average diam, flesh of head black to dark purple. KOH extractable stromatal pigments present, Dark Purple (36), Chestnut (40), Vinaceous Grey (116) or Purplish Grey (128). *Perithecia* (0.3–)0.7–0.9(–1.3) mm, 0.8 mm on average high, (0.2–)0.3–0.4(–0.5) mm, 0.34 mm on average diam, immersed, arranged in a layer below the convex layer of the head, encased in carbonaceous tissue, hemispherical to ellipsoidal, and lacking a distinct neck. Ostioles appear as minute shiny black dots, umbilicate. *Paraphyses* not observed. *Asci* (120–)140–160(–175) µm, 155 µm on average, spore bearing part (70.2–)72.5–93(–97.8) × (5.3–)6–7.2(–7.9) µm, 85.5 × 6.8 µm on average, stipe (69.3–)72.5–83.5(–88.2) µm, 75.5 µm on average, 8-spored, cylindrical, long-stipitate,

without an apical ring. *Ascospores* (5.5–)7.5–10.4(–11.5) × (2.5–)3.5–5(–6.5) µm, 9.3 × 4.2 µm on average, uniseriate or overlapping uniseriate, dark brown, ellipsoidal to kidney bean-shaped, with broadly rounded ends, epispore smooth, perispore indehiscent in 10% KOH, with an indistinct straight germ slit along the entire spore length on the convex side (Fig. 2A– L). *Asexual state: Conidiophores* (85–)90–120(–130) × (1.5–)2–2.5(–3.5) µm, 102.5 × 2.1 µm on average, simple to complex, hyaline, dichotomously branched, with nodulisporium-like branching pattern. *Conidiogenous cells* (15–)20–25(–35) × (0.8–)1–1.5(–1.7) µm, 22.5 × 1.3 µm on average, developing terminally, cylindrical, hyaline, apically aggregated scars. *Conidia* (4.5–)5.4–7.7(–8.5) × (2.3–)2.8–3.5(–4.0) µm, 6.5 × 3.1 µm on average, hyaline, single, ellipsoidal, with one pointed end and one blunt end (Fig. 3E– K).

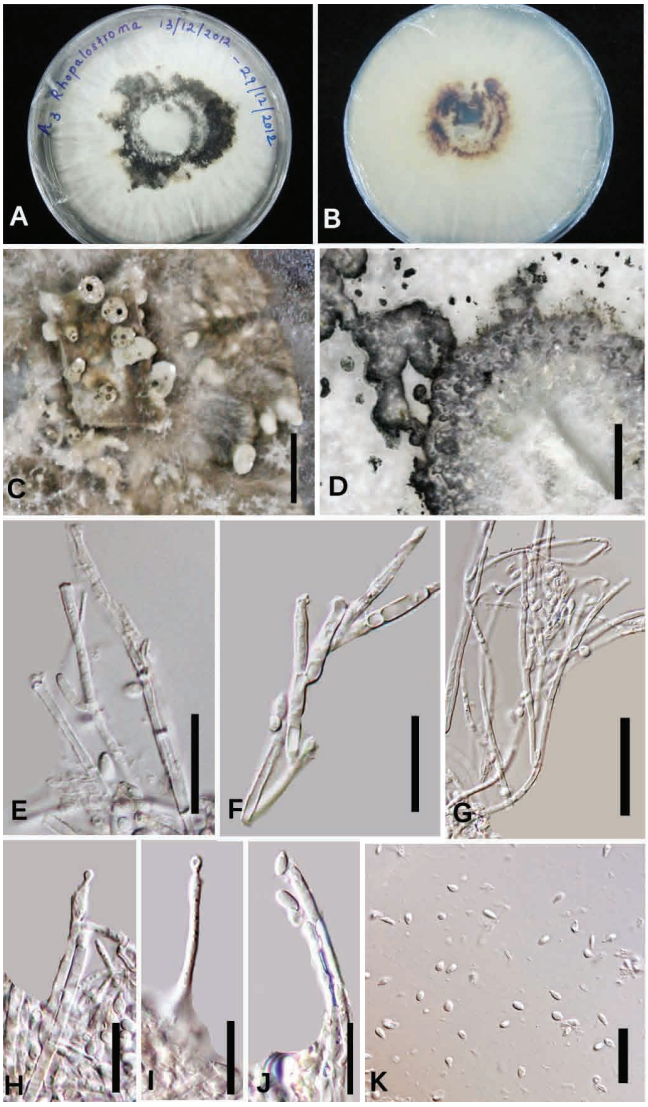

FIGURE 3. *Rhopalostroma lekae* in OA after 2 weeks (MFLUCC 13-0123). A: Averse showing melanized pigments and central mycelia. B: Reverse side of culture. C: Development of stromatal primordia in the culture. D: Melanized mycelia at the centre. E–G: Conidiophores from simple to more complex structure. H–J: Development of conidia and conidiogenesis cells. K: Conidia. *Bars:* c–d 1mm; e–k 50 µm.

Cultural characteristics:—Colonies on OA at 25^{0}C reaching 6 cm in 7 days, at first whitish developing melanized pigments around the center after 7–10 days, azonate with diffuse margins, reverse at first, whitish and turning light brown at the center. Distinct stromatal primordia observed after 7–10 days, olivaceous brown, producing aromatic odour (Fig.3A–D).

Material examined:—Thailand, Chiang Rai Province, Chiang Rai Horticultural Institute, 72 Moo 1, Den Ha-Dong Ma Da Road, Tambon Rop Wiang, Amphoe Mueang, Chiang Rai, on bark, 2 September 2012, A. Daranagama, D.J. Bhat, K.D. Hyde, MFLU 13-0440, PDD 104362, living culture, MFLUCC 13-0123, ICMP 20205, on bark, 12 December 2012, A. Daranagama, K.D. Hyde, MFLU 14-0077, living culture, MFLUCC 14-0245.

Secondary metabolites

Stromatal methanol extracts contain binaphthalene tetrol (BNT) as the major secondary metabolite and few other undetermined minor compounds (Fig. 4).

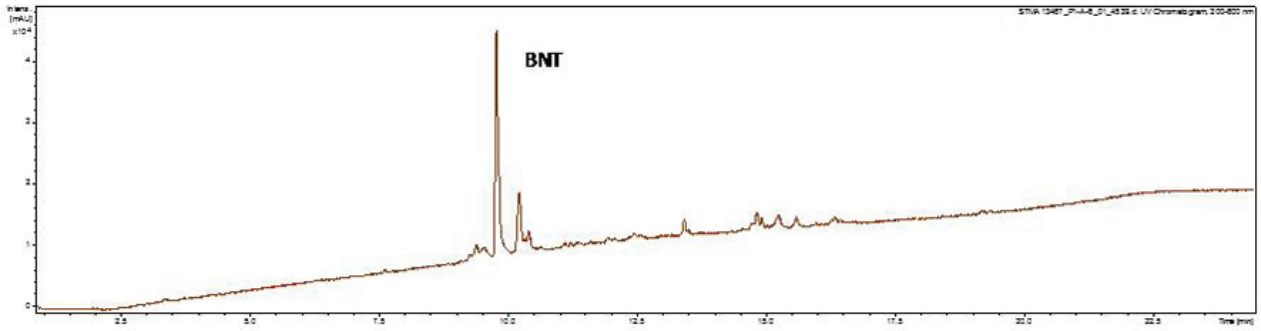

FIGURE 4. Stromatal HPLC-UV profiles of major metabolites in *Rhopalostroma lekae*.

Discussion

Recent collections of *Rhopalostroma* species support the fact that it is exclusively distributed in Asia and Africa (Whalley *et al.* 1998, Stadler *et al.* 2010a, Patil *et al.* 2012). The inconspicuous, minute and fragile nature of the stromata might be a possible reason for the lack of collections reported. The collection from northern Thailand is identical to the sexual morph in the protologue of *R. lekae* (Whalley *et al.* 1998). Dull sulfur yellow-orange granules were observed beneath the surface of the head part of the stroma by Whalley *et al.* (1998) and also in this study. This character has only been reported for *Rhopalostroma* in *R. lekae*. We contacted the Forest Herbarium, Royal Forest Department (RFD) Thailand, the herbarium where the type material was listed as lodged but they did not have the type material. We also looked for it in other Thai herbaria without success. We could not locate the type material of *Rhopalostroma lekae*, which may be lost, but we are confident that we collected *R. lekae* as the characters of our collection were identical with that of the protologue (Whalley *et al.* 1998). Our fresh collection of *R. lekae* is deposited as herbarium material in MFLU and PDD and is available for future study, while living cultures have been placed in MFLUCC and IMCP. This collection can therefore be treated as an authentic specimen of *R. lekae.*

Our data also provides new information on the asexual morph of *R. lekae*. Furthermore, this study contains additional information about *R. lekae*, including culture characteristics and KOH extractable pigments.

All *Rhopalostroma* species are reported to contain colored pigments in the presence of KOH (Stadler *et al.* 2010a). According to their stromatal pigments colours they can be categorized in to two main groups. *Rhopalostroma gracile, R. indicum* and *R. lekae* have similar purplish tones of stromatal pigment colors in KOH. The other group, including *R. africanum, R. angolense, R. dennisii* and *R. kanyae* have isabelline-olivaceous stromatal pigment colors in KOH. The differences of these colors reactions are due to their secondary metabolites, the purplish colors usually results from BNT while greenish brown (olivaceous) group is due to perylene quinone 2 (Stadler *et al.* 2010b). Thus, the KOH extractable pigments of *Rhopalostroma* species have the potential as an important feature in identification.

Rhopalostroma lekae contains BNT as major metabolite beside some minor undetermined compounds. BNT is a common metabolite in Xylariaceae and is frequently observed among the genus *Rhopalostroma* (Stadler *et al.* 2010a).

The asexual state of *Rhopalostroma* species are nodulisporium-like or virgariella-like (Hawksworth & Whalley 1985, Vaidya *et al.* 1991, Stadler *et al.* 2010a). In this study, we observed that *R. lekae* produced a nodulisporium-like asexual state ranging from simple to complex structures in OA. *Rhopalostroma angolense* and *R. kanyae* also have nodulisporium-like asexual morphs (Hawksworth 1977, Whalley & Thienhirun 1996, Stadler *et al.* 2010a,). In previous studies, the asexual state was not observed in the cultures, but were described from the conidiophores formed on the young stromata. Stadler *et al.* (2010a) also confirmed *R. angolense* had a nodulisporium-like asexual state in culture.

The placement of this genus was unclear for several years, since it does not possesses several characteristics of typical Xylariaceae (Rogers 1979). Stadler *et al.* (2010a) tested the phylogenetic relationship of *Rhopalostroma* with several other genera of Xylariaceae using the nrITS gene, thus inferring the affinities with related genera. The current study confirmed that *R. lekae* is related to *R. angolense* and *Thamnomyces* is the closest relative, while *Daldinia* and *Phylacia* appear to be other related genera. *Rhopalostroma*, *Phylacia* and *Thamnomyces* clustered in a single

subclade within the major clade including *Daldinia* confirm the relative affinities. Thus, the molecular phylogenetic analysis is congruent with the morphological relationships, with previous studies supporting strong affinities with *Thamnomyces*.

The separation of hypoxyloid and xylarioid clades generated in this study is generally concordant with chemotaxonomy and their specific asexual morphs (Sánchez-Ballesteros *et al.* 2000, Smith *et al.* 2003, Triebel *et al.* 2005). Clade A (hypoxyloid clade) represents the genera that yield stromatal pigments in potassium hydroxide and produce a nodulisporium-like asexual morph, Clade B (xylarioid clade) represents the genera that do not yield stromatal pigments in potassium hydroxide and produce a geniculosporium-like asexual morph except for *Anthostomella*,whose species may either have nodulisporium-like or libertella-like asexual morphs. However, the xylarioid clade has differences in statistical support and resolution of internal nodes. *Creosphaeria* appears to be phylogenetically distinct from the two clades mentioned and is characterized by having a libertella-like asexual morphs. The phylogenetic position of *Biscogniauxia* appeared to be variable in different gene analyses. In the ITS and RPB2 gene phylogeny (not shown here) it appears as a basal group to all other genera. However, in the LSU gene analysis, *Biscogniauxia* appears as a sister group to the xylarioid clade, which is not well-supported. Due to the lack of LSU and RPB2 data for certain taxa it is rather difficult to have a better comparison of taxon separation at this level. In this study, *Anthostomella* and *Biscogniauxia* formed basal lineages making it difficult to interpret their phylogenetic affinities. Both genera have been classified in the Hypoxyloideae as they are characterized by nodulisporium-like asexual states (Ju & Rogers 1996). *Biscogniauxia* is however, different from other members of Hypoxyloideae in having bipartite stromata and lack of KOH-extractable stromatal pigments (Ju *et al.* 1998, Stadler *et al.* 2013) while *Anthostomella* has highly reduced stromata. Ascospores of both these genera bear a hyaline cellular appendage in some species.

The ITS gene region alone proves to be inappropriate for resolving genera in phylogenetic analysis of Xylariaceae and is very likely to create confusions (Stadler *et al.* 2013, Tang *et al.* 2009, Triebel *et al.* 2005). Even with the inclusion of more taxa, due to high length variations in ITS regions, they still depict out a poor reflection (Jaklitsch *et al.* 2014) thus the molecular taxonomy, based solely on the ITS region are presently considered to be inappropriate and additional markers are therefore needed for a better resolution of xylariaceous taxa and a different gene is necessary to establish a genetic bar-coding of Xylariaceae (Pažoutová *et al.* 2013, Stadler *et al.* 2013). This study presents a multigene approach to clarify the taxonomic position of *Rhopalostroma lekae* within the family Xylariaceae. Multi-gene approaches have proven to be highly useful in resolving evolutionary relationships in many groups of the Ascomycota (Lumbsch *et al.* 2005, Miller & Huhndorf 2005, Liu *et al.*2012, Hyde *et al.* 2013). In contrast to single gene phylogenies multigene analyses are effective in increasing phylogenetic support and resolution in Xylariaceae (Tang *et al.* 2009). However, RPB2 data are limited to only certain members of Xylariaceae, even though, phylogenies inferred from RPB2 gene appear to be superior and high resolution of taxon separation could be observed. When RPB2 gene sequences were combined with other sequence datasets, ITS and LSU genes, phylogenetic separation was increased with better statistical support for all clades with a promising output. We strongly recommend that new species of hypoxyloid or xylarioid Xylariaceae should not be described (e.g. Vasilyeva *et al.* 2007, Vasilyeva *et al.* 2012), unless they deposit respective herbarium materials and, more importantly, ex-type strains in publicly accessible collections in order to facilitate future phylogenetic studies once the best genes have been selected. It is necessary to recollect and epitypify all crucial taxa of Xyalariaceae and their associates in order to establish a multi-gene genealogy hence resolve the evolutionary relationships of this diverse family (Stadler *et al.* 2013).

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