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New species and records of the lichen genus *Graphis* (*Graphidaceae*, Ascomycota) from Thailand

Arunpak PITAKPONG, Ekaphan KRAICHAK, Khwanruan Butsathorn PAPONG, Nooduan MUANGSAN, Pongthep SUWANWAREE, H. Thorsten LUMBSCH and Robert LÜCKING

Abstract: One new species and six new records of the crustose lichenized genus *Graphis* are reported from Thailand. *Graphis koratensis* Pitakpong, Kraichak & Lücking sp. nov. is characterized by lirelline ascocarps with whitish grey or grey-green pruina along the slit, transversely septate ascospores, and the presence of norstictic acid. Phylogenetic analyses with two loci (mtSSU and nuLSU) show the distinct position of this new species within the genus. Six new records for Thailand are reported, including *G. cincta* (Pers.) Aptroot, *G. jejuensis* K. H. Moon *et al.*, *G. nigrocarpa* Adaw. & Makhija, *G. renschiana* (Müll. Arg.) Stizenb., *G. seminuda* Müll. Arg., and *G. subserpentina* Nyl.

Key words: lichenized fungi, phylogeny, taxonomy, tropical species

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Introduction

With more than 400 species, the lichen genus *Graphis* is one of the largest genera of crustose lichens (Lücking *et al.* 2009, 2014; Rivas Plata *et al.* 2011). The genus traditionally included taxa with lirellate ascomata and hyaline, transversely septate ascospores, but has been recircumscribed based on excipular structure and thallus morphology, as well as ascospore amyloidity (Staiger 2002). According to this concept, *Graphis* is also now defined as having ascomata with convergent labia, a partly to fully carbonized excipulum, a usually corticated, white-grey thallus, and amyloid ascospores that react I+ violet-blue. *Graphis* species are commonly found in montane and dry tropical forests in semi-exposed situations. Since the comprehensive work by Staiger (2002), the number of species described within the genus has grown steadily due to the publication of a worldwide key (Lücking *et al.* 2009) and concerted efforts in taxonomic revisions and collection of the currently expanded family *Graphidaceae* (Sohrabi *et al.* 2014). As the genus appears to include two polyphyletic clades, the phylogenetic relationships within the genus remain an area of active research (Berger *et al.* 2011; Rivas Plata *et al.* 2011, 2013).

The study of the genus *Graphis* in Thailand has been somewhat sporadic, even though the country contains many suitable habitats for the genus (Rundel & Boonpragob 2009). Since the earliest report by Vainio in the Koh Chang Flora (Vainio 1909), 59 named species of the genus have been listed from Thailand, mostly as part of larger area-based studies of the family *Graphidaceae*, or the tribe *Graphidoideae* (Vainio 1909, 1921; Nakashi *et al.* 2001; Aptroot *et al.* 2007; Papong *et al.* 2007; Poengsungnoen *et al.* 2010; Mongkolsuk *et al.* 2011). In many cases, the genus represents the largest portion of the lichen diversity. For example, in a

A. Pitakpong, N. Muangsan and P. Suwanwaree: School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand.
E. Kraichak (corresponding author): Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand. Email: fsciepk@ku.ac.th
K. B. Papong: Department of Biology, Faculty of Science, Mahasarakham University, Maha Sarakham 44150, Thailand.
H. T. Lumbsch and R. Lücking: Science and Education, The Field Museum, 1400 Lake Shore Drive, Chicago, IL 60605, USA.

study of graphidoid lichens in Phu Luang Wildlife Sanctuary, *Graphis* constituted the largest genus, with over a third of the species richness (35 out of 91 species) in the family (Poengsungnoen *et al.* 2010). Similarly, in a study of Khao Yai National Park, 34 *Graphis* species were reported, making it the most speciose genus in the study (Lichen Research Unit, Ramkhamhaeng University 2004). Despite this level of species diversity, a country-wide study on the genus has yet to be completed.

In the present paper, we aim to enhance the current understanding of the genus *Graphis* in the light of recent literature, collections and new molecular data. We describe one new species, along with six new records of *Graphis* from dry dipterocarp forests in northern and eastern floristic provinces of Thailand. We use morphological, anatomical, chemical, and molecular data to circumscribe the new species, *Graphis koratensis* Pitakpong, Kraichak & Lücking sp. nov.

Materials and Methods

Specimen collection and identification

A total of 1704 specimens belonging to *Graphidaceae* were collected during 2013 and 2014 from dry evergreen and dry dipterocarp forests in ten national parks and two other natural areas in Thailand. The national parks included 1) Khun Khan, 2) Doi Inthanon, 3) Phu Hing Rong Kla, 4) Phu Pha Terb, 5) Phu Toei, 6) Thong Pha Phum, 7) Phu Chong Na Yoi, 8) Pang Sida, 9) Keang Krachan, and 10) Hat Chao Mai. The two other natural areas included Sakaerat Environmental Research Station and Tadton waterfall in Mukdahan Province. The new species is described from specimens mostly collected at Sakaerat Environmental Research Station (SERS) in Nakhon Ratchasima (14°26' to 14°32'N, 101°50' to 101°57'E). SERS is located in mountainous terrain at 280–762 m above sea level, 300 km from Bangkok and 60 km from downtown Nakhon Ratchasima. All voucher specimens are deposited in the herbarium of Suranaree University of Technology (SUT), with some duplicates at the Field Museum (F).

The morphological and anatomical characteristics of the thallus and reproductive structures were studied using a low magnification stereomicroscope (Olympus-SZX12, Tokyo, Japan) at magnifications of ×7 to ×90. Sections of thalli and ascomata were examined with a light compound microscope (Olympus-BH2, Tokyo, Japan) at magnifications of ×40 to ×1000. All measurements were made on material mounted in water; Lugol's iodine and 10% KOH solutions were used for the colour reactions of asci. Secondary products were identified by thin-layer chromatography in solvent C (170 ml toluene, 30 ml acetic acid), according to standardized methods (Elix & Ernst-Russell 1993; Orange *et al.* 2001). The specimens were also examined under UV light (254 nm) for UV-reactive secondary metabolites.

Molecular data

DNA extraction, amplification, and sequencing of the mitochondrial small subunit (mtSSU), and nuclear large subunit (nuLSU) were performed using apothecia from four specimens of the new species. DNA was extracted using the Red-Extract Sigma Kit (Sigma Aldrich, USA). Dilutions of 9:1 of the genomic extracts were used in PCR reactions. Primers for PCR amplifications included a) mrSSU1 and mrSSU3R (Zoller *et al.* 1999) for the mtSSU, and b) AL2R (Mangold *et al.* 2008) and LR3 (Vilgalys & Hester 1990) for the nuLSU. PCR reactions contained 2·5 µl Sigma RED-Extract-N-Amp™ PCR, 0·5 µl of each primer (10 µM), 2 µl genomic DNA extract and 4 µl distilled water for a total of 10 µl. Thermal cycling conditions were as follows: a) for mtSSU: initial denaturation for 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 1 min at 50°C, 1 min 30 s at 72°C, and a final elongation for 10 min at 72°C; and b) for nuLSU: initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, 1 min at 72°C, and a final elongation for 10 min at 72°C. Amplification products were visualized on 1% agarose gels stained with ethidium bromide. The product was purified by the cutting of the target bands and digesting of the gel materials at 70°C for 10 min or until it turned liquid. Then it was cooled to 45°C-heat block for 5 min, treated with 1 µl of gelase (Epicentre Biotechnologies, Madison, WI, USA) and incubated at 45°C for at least 3 h or left overnight. The 10-µl cycle sequencing reactions followed standard protocols from a previous study (Rivas Plata *et al.* 2013). The products were then sequenced, using Applied Biosystems 3730 DNA Analyzer (Foster City, California, USA) automatic sequencer. Sequence fragments obtained were assembled with Geneious 8.0.3 (Drummond *et al.* 2014), manually inspected and adjusted. From this process, two mtSSU and three nuLSU sequences were obtained from the new species and submitted to GenBank (Table 1).

Phylogenetic analysis

In order to determine the genetic identity of the new species, the newly obtained sequences were aligned with 42 other samples of lichens from the genus *Graphis* and two outgroup taxa of the genus *Diorgyma* available from GenBank (Table 1). For each locus, the alignment was performed in the software Geneious 8.0.3 (Drummond *et al.* 2014) with the built-in MUSCLE algorithm with eight iterations. The resulting alignment was further adjusted manually to remove ambiguity. The 956-bp concatenated sequences were used to reconstruct phylogenetic relationships among the samples with maximum likelihood (ML) and Bayesian approaches.

TABLE 1. *Voucher information and GenBank accessions for the sequences used in the phylogenetic analysis. The accessions in bold are sequences generated in this study. The missing sequences are indicated with a dash (–).*

		GenBank Acc. No.	
Species	Voucher information	mtSSU	nuLSU
Diorygma antillarum	*Lücking* 33019 (F)	JX046454	JX046467
D. minisporum	*Lumbsch* 19543v (F)	HQ639598	HQ639626
Graphis albissima	*Rivas Plata* 1004D (F)	HQ639604	–
G. anfractuosa	*Hernandez* 1340 (F)	HQ639618	–
G. angustata	*Lücking* 28102 (F)	HQ639612	HQ639632
G. caesiella 1	*Berger* 17247 (Hb. Berger)	DQ431975	AY640028
G. caesiella 2	*Lumbsch* 20540i (F)	JX421065	–
G. caesiella 3	*Lumbsch* 20530a (F)	JX421066	–
G. cinerea	*Kalb* 26950 (Hb. Kalb)	DQ431988	DQ431947
G. cf. *gracilescens*	*Lücking* 33942B (Hb. Kalb)	DQ431976	DQ431936
G. chrysocarpa	*Lücking* No. 00-35 (Hb. Kalb)	DQ431987	–
G. dichotoma	*Rivas Plata* 2088 (F)	–	HQ639633
G. furcata	*Rivas Plata* 1172Q (F)	HQ639607	–
G. handelii	*Green* GR4BH	KC592281	–
G. illinata	*Lumbsch* 19639 (F)	HQ639614	HQ639634
G. implicata 1	*Lücking* 28527 (F)	–	HQ639653
G. implicata 2	*Rivas Plata* 0103A (F)	HQ639602	–
G. implicata 3	*Lücking* 28104 (F)	–	HQ639655
G. implicata 4	*Lücking* 28039 (F)	–	HQ639654
G. implicata 5	*Lücking* 16103a (F)	KJ440975	KJ440928
G. implicata 6	*Lücking* 16103b (Hb. Kalb)	DQ431978	DQ431939
G. leptoclada	*Lumbsch* 20535b (F)	JX421068	JX421509
G. librata 1	*Lücking* 28007 (F)	–	HQ639637
G. librata 2	*Lücking* 28001 (F)	KJ440976	KJ440929
G. librata 3	*Lücking* 28001b (F)	HQ639621	HQ639636
G. pavoniana	*Lücking* 16100c (Hb. Kalb)	DQ431986	DQ431946
G. proserpens	*Rivas Plata* 2065 (F)	HQ639619	–
G. pseudocinerea 1	*Lücking* 26537 (F)	HQ639620	HQ639639
G. pseudocinerea 2	*Lücking* 26531 (F)	–	HQ639638
G. pseudocinerea 3	*Lücking* 26532a (F)	–	HQ639640
G. pseudoserpens 1	*Lücking* 28048 (F)	–	HQ639642
G. pseudoserpens 2	*Lücking* 28003 (F)	–	HQ639641
G. rhizocola 1	*Lücking* 28512 (F)	–	HQ639644
G. rhizocola 2	*Lücking* 28548 (F)	–	HQ639645
G. rhizocola 3	*Lücking* 28502 (F)	–	HQ639643
G. rimulosa	*Rivas Plata* 1021H (F)	JX421069	–
G. scripta 1	*Tønsberg* 42518 (BG)	KJ440969	KJ440922
G. scripta 2	*Nelsen* MN499 (F)	KJ461720	KJ440935
G. scripta 3	*Neuwirth* 11834 (ABL)	KJ440977	KJ440932
G. sp.A	*Pitakpong* A04 (SUT)	**KP862882**	**KP862884**
G. sp.A	*Pitakpong* 311 (SUT)	**KP862883**	**KP862887**
G. sp.A	*Pitakpong* D205 (SUT)	–	**KP862885**
G. sp.A	*Pitakpong* E108 (SUT)	–	**KP862886**
G. streblocarpa	*Rivas Plata* 1015E (F)	–	HQ639646
G. tenella	*Rivas Plata* 1007G (F)	–	HQ639647
G. tsunodae	*Lücking* 26096 (F)	–	JX421511
G. vestitoides	*Rivas Plata* 2078 (F)	–	HQ639648
G. xanthospora	*Lücking* 26535 (F)	–	HQ639649

The ML analysis was performed with RaXML BlackBox 8.1.1 (Stamatakis 2006), with two partitions for each of the two loci and 1000 pseudoreplicates. For the Bayesian approach, the dataset was also partitioned into two for each of the two loci and then analyzed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). A run with 10 000 000

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generations, starting with a random tree and employing four simultaneous chains, was executed. Heating of chains was set to 0·2. Posterior probabilities were approximated by sampling trees, using a variant of the Markov chain Monte Carlo (MCMC) method. Every 1000th tree was sampled to avoid sample autocorrelation. The first 4000 trees were discarded as burn-in. For the remaining trees, a majority-rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Only clades with bootstrap support equal or above 70% under ML and posterior probabilities equal or above 0·95 in a Bayesian framework were considered as supported. Both ML and Bayesian analyses were performed on the CIPRES supercomputer cluster (Miller *et al.* 2010). The topology from the ML analysis was illustrated, using the R-package ape (Paradis *et al.* 2004). The alignment and tree were submitted to TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S17170).

Results and Discussion

After reviewing the literature, we found that 11 of the 59 reported names of *Graphis* from Thailand represented synonyms. Five of these were transferred to other genera, four with already reported *Graphis* species, and two with *Graphis* species new to Thailand (see Taxonomic Treatment below). From our recent collection of 1704 specimens, a total of 536 were identified as *Graphis* spp. Thirty-seven species of *Graphis* were found, including one new species, *Graphis koratensis* Pitakpong, Kraichak & Lücking sp. nov., and six new records from Thailand (see Taxonomic Treatment below). *Graphis koratensis* was collected from two dry dipterocarp forests in north-eastern Thailand. While morphologically similar to *G. caesiella*, the new species differs in a number of apothecial characters. It is also phylogenetically distinct from *G. caesiella*, with strong support from both the ML and Bayesian analyses (98 and 0·91, respectively; Fig. 1). Similar to a previous study (Berger *et al.* 2011; Rivas Plata *et al.* 2011), we recovered two main clades, the *Graphis* and "*Allographa*" clades, within the genus in its current circumscription. While the monophyly of the genus *Graphis* is still somewhat uncertain (Rivas Plata *et al.* 2013), the analyses placed *G. koratensis* in the *Graphis* s. str. clade along with the type species, *G. scripta*.

Taxonomic Treatment

Graphis koratensis Pitakpong, Kraichak & Lücking sp. nov.

MycoBank No.: MB811731

Lirelline ascocarps with whitish grey or grey-green pruina along the slit with a completely carbonized expiculum.

Type: Thailand, Nakhon Ratchasima Province, Sakaerat Environmental Research Station, dry dipterocarp forest, located at a height of 380 m a.s.l., tree trunks, 2014, *Pitakpong* D205 (SUT—holotype; F—isotype).

(Fig. 2)

Thallus corticolous, 3–6 cm diam., 150–250 µm thick in cross-section, continuous; *surface* smooth to uneven, pale whitish grey or grey-green, cortex distinct 10–15 µm; *algal layer* continuous 50–75 µm, medulla 100–150 µm thick, with clusters of calcium oxalate crystals.

Ascomata lirelliform, emergent to prominent, straight to curved, sparsely branched, with an apically thin thalline margin, 2–7 mm long, 0·3–0·5 mm wide, 0·10–0·15 mm high; *labia* thick, entire, appearing greyish black but with pruina along the slit. *Proper exciple* completely carbonized, 100–140 µm wide; *hymenium* clear, 100–150 µm high; *asci* 20–25 µm long, 110–140 µm wide. *Ascospores* 8 per ascus, oblong to narrowly fusiform, transversely 11–19 septate, 70–115 µm long, 9–15 µm wide, colourless.

Chemistry. Norstictic acid (thallus in section with K+ yellow efflux forming red, needle-shaped crystals, C−, P+ orange, UV−).

Etymology. The epithet was derived from the Thai word *Korat*, referring to the traditional name of the type locality (Nakhon Ratchasima).

Notes. This new species is similar to *Graphis caesiella* Vain. in having white-pruinose labia and elongate and irregularly branched lirellae, plus an identical chemistry, but differs in the completely carbonized excipulum and the larger ascospores (20−40 × 6−8 µm in *G. caesiella*). The only other species in the genus

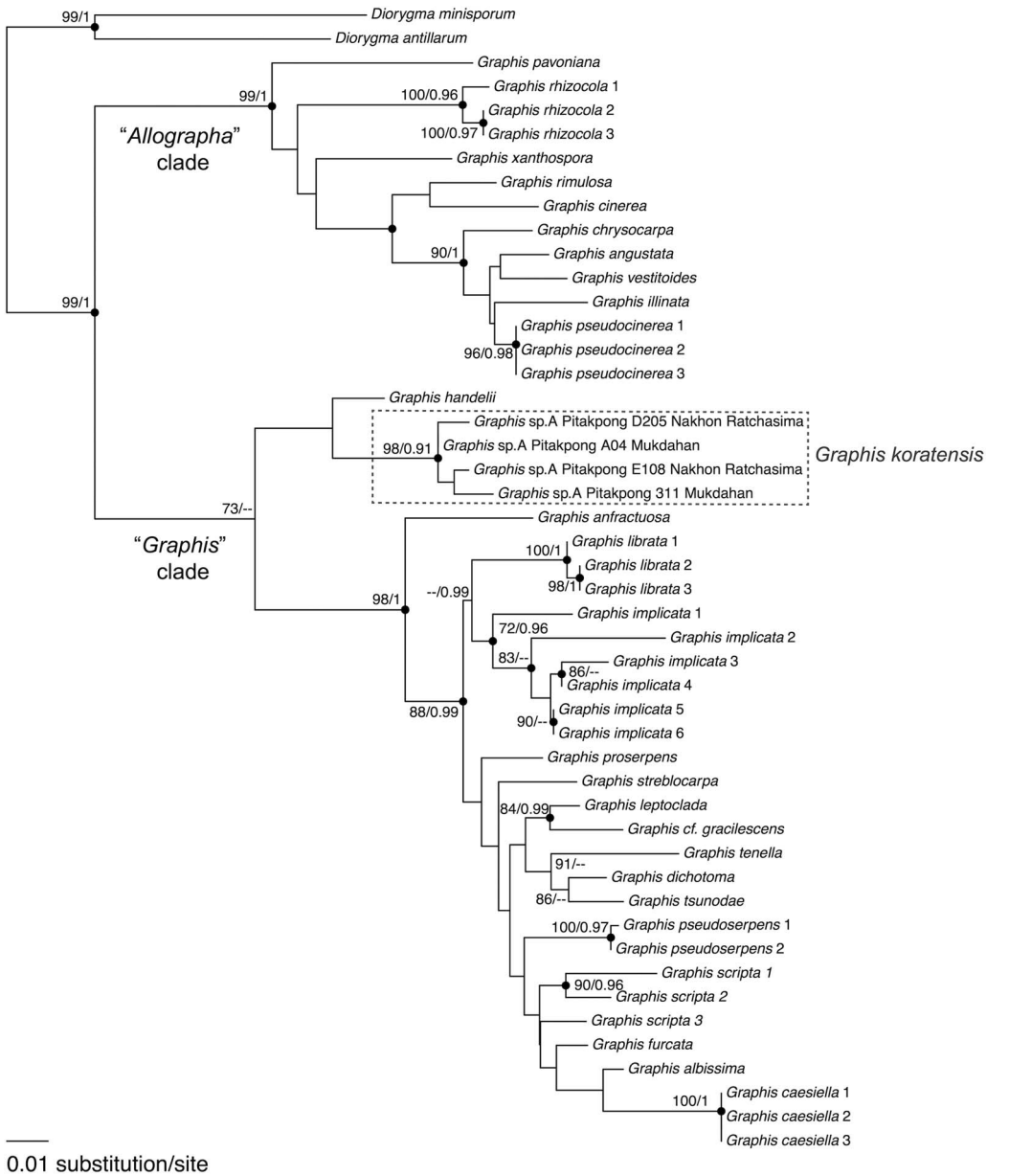

FIG. 1. Phenogram illustrating phylogenetic relationships among lichens in the genus *Graphis*. The topology follows the bipartitioned tree from a maximum likelihood (ML) analysis. The numbers at each node represent the bootstrap support value from the ML analysis, followed by the posterior probability (PP) from the Bayesian analysis (ML/PP). Only the ML support > 70 and PP > 0·95 are considered strong supports and reported here. Circles indicate nodes with strong support from both analyses.

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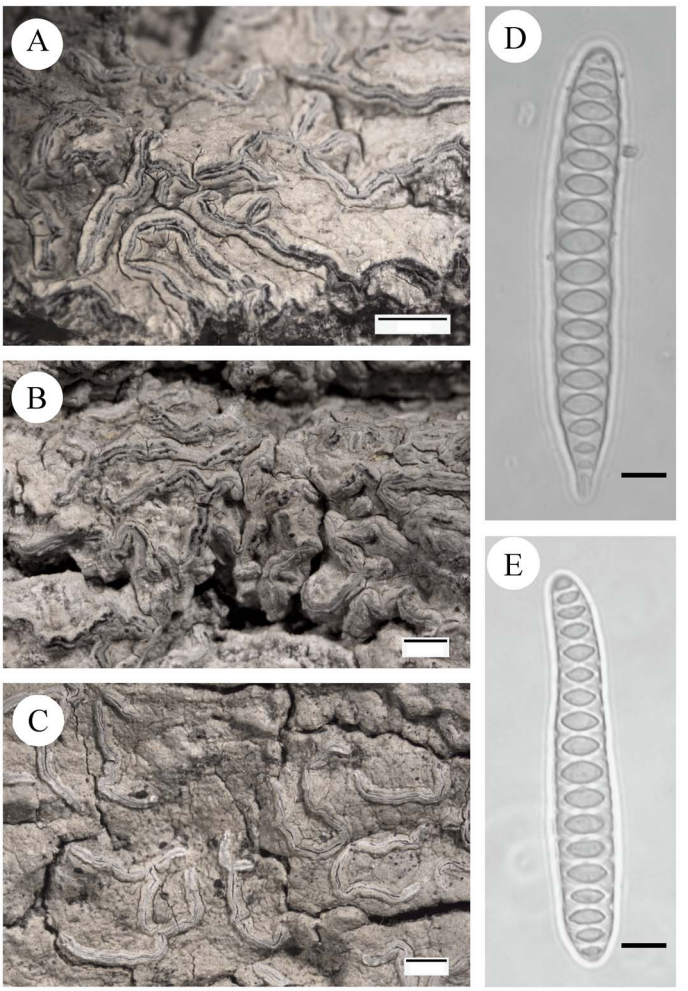

FIG. 2. *Graphis koratensis* Pitakpong, Kraichak, Lücking sp. nov. A & B, thallus and ascomata showing lirellae from *Pitakpong* D205; C, from *Pitakpong* A04; D & E, ascospores from *Pitakpong* D205. Scales: A–C = 1 mm; D & E = 10 µm.

with a completely carbonized excipulum, large, transversely septate ascospores and norstictic acid is *G. marginata* Raddi, which has strongly prominent lirellae with sharply delimited, black labia, and belongs in the "*Allographa*" clade (Berger *et al.* 2011).

In reference to the worldwide key (Lücking *et al.* 2009), *G. koratensis* belongs to Group 4 (labia entire, excipulum laterally carbonized, hymenium clear, ascospores transversely septate, p. 391–397). This species can be placed in couplet 6 along with *Graphis erythrocardia* Müll. Arg., for having a similar chemistry (norstictic acid), but differing in having larger ascospores.

Selected specimens examined. **Thailand:** *Nakhon Ratchasima*: dry dipterocarp forest in Sakaerat Environmental Research Station, 2014, *Pitakpong* E108, D202 (SUT). *Mukdahan*: dry dipterocarp forest in Phu Pha Terb, 16°26'7"N, 104°48'21"E, 2014, *Pitakpong* A04, 311 (SUT).

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New records of *Graphis* from Thailand

Graphis cincta (Pers.) Aptroot

Habitat. Dry dipterocarp forest, Tadton waterfall, Mukdahan Province, SUT-211.

Graphis jejuensis K. H. Moon *et al.*

Habitat. Dry dipterocarp forest, Tadton waterfall, Mukdahan Province, SUT-315.

Graphis nigrocarpa Adaw. & Makhija

Habitat. Dry dipterocarp forest, Phu Pha Terb National Park, Mukdahan Province, SUT-005.

Graphis renschiana (Müll. Arg.) Stizenb.

Habitat. Mixed deciduous forest, Khun Khan National Park, Chiang Mai Province, SUT-501.

Graphis seminuda Müll.Arg.

Habitat. Dry dipterocarp forest, Tadton waterfall, Mukdahan Province, SUT-317.

Graphis subserpentina Nyl.

Habitat. Dry dipterocarp forest, Phu Chong Na Yoi National Park, Ubon Ratchathani Province, SUT-008.

Synonyms and nomenclatural notes

The following names were reported from Thailand, but have since been synonymized with other names and/or transferred to other genera. The names in bold are currently accepted names.

Graphis albidolivens Vain., *Ann. Acad. Sci. Fenn. Ser. A*, **15**(6)**:** 217 (1921), reported in Aptroot *et al.* (2007) = **Diorygma hieroglyphicum (Pers.) Kalb *et al.*** in *Symb. Bot. Ups.* **34**(1)**:** 151 (2004).

Graphis aphanes Mont. & Bosch, *Plant. Junghuhn.* **4:** 474 (1855), reported by Nakashi *et al.* (2001) = **Hemithecium aphanes (Mont. & Bosch) M. Nakan. & Kashiw.,** *Bull. Ntl. Sci. Mus. Tokyo* **29:** 88 (2003).

Graphis ceylanica Zahbr., *Cat. Lich. Univ.* **2:** 297 (1923), reported in Papong *et al.* (2007) = **Graphis dendrogramma Nyl.** in Lücking *et al.*, *Lichenologist* **41:** 363–452 (2009).

Graphis chondroplaca (Redinger) Lücking *et al.*, *Fieldiana, Bot.* **38:** 64 (2008), reported by Poengsungnoen *et al.* (2010) = **Graphis handelii Zahlbr.** in Lücking *et al.*, *Lichenologist* **41:** 363–452 (2009).

Graphis concolor Nyl., *Mem. Soc. Science. Nat. Cherbourg* 5 (1857), reported in Vainio (1909) = **Graphina boschiana var. concolor (Nyl.) D.D. Awasthi & Kr.P. Singh**, *Current Science* **42:** 656 (1973); possibly a species of *Carbacanthographis* or *Diorygma*.

Graphis persimilis Vain., *Bot. Tidsskr.* **29:** 125 (1909), reported in Vainio (1909) = **Phaeographis hypoglauca (Kremp.) Zahlbr.**, *Cat. Lich. Univ.* **2:** 374 (1923).

Graphis pyrrhocheila Vain., *Hedwigia* **46:** 179 (1907) *nom. illeg.*, reported in Alava (1988) = **Graphis pyrrhocheiloides Zahlbr.** in Lücking *et al.*, *Lichenologist* **41:** 363–452 (2009).

Graphis siamensis Vain., *Ann. Bot. Soc. Zool.-Bot. Fenn. Vanamo* **1**(3)**:** 52 (1921), reported in Vainio (1921); we were unable to locate the type material, but from the original description, this species does not belong to the genus *Graphis*, because of its ascoma shape and hymenium chemistry.

Graphis simplex Vain., *Hedwigia* **46:** 77 (1907), reported in Vainio (1907) = **Graphis analoga Nyl.** in Lücking *et al.*, *Lichenologist* **41:** 363–452 (2009).

Graphis subcinerea Staiger, *Bibl. Lichenol.* **85:** 258 (2002) *nom. inval.*, reported in Aptroot *et al.* (2007) = **Graphis phaeospora Vain.** in Lücking *et al.*, *Lichenologist* **41:** 363–452 (2009).

Graphis tenuis Vain., *Ann. Bot. Soc. Zool.-Bot. Fenn. Vanamo* **1**(3)**:** 52 (1921), reported in Vainio (1921) = **Graphis hossei Vain.** in Lücking *et al.*, *Lichenologist* **41:** 363–452 (2009).

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