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NOTE

Contribution to the knowledge of the Lichen Mycota of Myanmar (II) *Heterocyphelium triseptatum* (Lecanographaceae) newly recorded from Asia and its molecular phylogenetic position

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ABSTRACT: *Heterocyphelium triseptatum* is newly recorded from Asia. The species was previously known only from South America and Africa. It was collected from the bark of a broadleaf tree at 35 m elevation in the Tanintharyi Region of southern Myanmar. An unidentified substance was newly detected for this species by thin layer chromatography (TLC). This study demonstrates the phylogenetic position of *H. triseptatum* in the Lecanographaceae using Bayesian and RAxML analyses of mtSSU and nrLSU sequence data. The close relationship with *H. leucampyx* within the *Heterocyphelium* clade was confirmed.

KEY WORDS: Distribution, Lecanographaceae, mtSSU, nrLSU, Southeast Asia, taxonomy, TLC.

INTRODUCTION

The genus *Heterocyphelium* Vain. (Lecanographaceae, lichenized Ascomycota) is characterized by its crustose thallus with mazaediate ascomata. Two species, *H. leucampyx* (Tuck.) Vain. and *H. triseptatum* Aptroot & M. Cáceres, are known in the genus (Aptroot *et al*., 2017). These species are distinguished by the number of septa in the ascospores: i.e., 2-septate in *H. leucampyx* and 3-septate in *H. triseptatum*. The former species occasionally contains a few 3-septate ascospores besides the bulk of 2-septate ascospores (Aptroot *et al*., 2017). The phylogenetic position of *H. leucampyx* was tested by Bayesian analysis based on mtSSU and *RPB2* sequences, and the genus was confirmed as belonging to the family Lecanographaceae (Van den Broeck *et al*., 2017). However, the relationship between *H. leucampyx* and *H. triseptatum* has not been examined by molecular phylogenetic analyses.

During lichen mycobiotic studies in Myanmar (Ohmura *et al*., 2020), an unidentified collection from southern Myanmar was finally identified as *H. triseptatum* based on the examination of morphology, chemistry and molecular data. This study aims at testing the phylogenetic position of *H. triseptatum* in relation to *H. leucampyx* within selected taxa of Lecanographaceae and discusses the morphology and chemistry of *H. triseptatum*.

MATERIALS AND METHODS

Morphological observations were made using a dissecting microscope (Olympus SZX16) and a differential interference contrast microscope (Olympus BX51). Anatomical examinations were made on hand-cut sections mounted in water. Ascospore measurements are given as (minimum–) range including mean ± standard deviation (–maximum) (n = number of measurements). Color spot tests for K, C, KC, and Pd were performed according to Orange *et al*. (2001). UV fluorescence of thallus and ascomata was tested under 365 nm wavelength.

Chemical lichen substances were examined using thin layer chromatography (TLC) (Culberson and Kristinsson, 1970). Solvent systems A (toluene: 1,4-dioxane: acetic acid = 180: 45: 5) (Culberson and Ammann, 1979), B′ (hexane: methyl tert-butyl ether: formic acid, 140: 72: 18) (Culberson and Johnson, 1982), and C (toluene: acetic acid = 170: 30) (Mietzsch *et al*., 1994) were used for TLC analyses. The spot color was checked under 254 nm and 365 nm wavelength of UV and visible light, before and after spraying the TLC plates with 10% sulfuric acid and charring at 110°C for 10 minutes.

DNA extraction followed a modified CTAB protocol (Hosaka, 2009). For DNA amplification, 10 µl of PCR mix contained 1 µl genomic DNA extraction, 0.25 µl of each primer (10 pmol/µl) and 5 µl EmeraldAmp PCR Master Mix (TaKaRa Bio Inc.). PCR amplification of nrLSU was performed using the primer set of LR0R (Vilgalys, unpublished) as the 5' primer and LR5 (Vilgalys and Hester, 1990) as the 3' primer. For mtSSU,

Table 1. Vouchers and their GenBank accession numbers. New sequences are in bold.

Species	Voucher	mtSSU	nrLSU	RPB2	Reference
Alyxoria mougeotii	L10058 (LD)	KJ851007	KJ851078	–	Frisch *et al*. (2014)
A. varia	Frisch 11Se1 (UPS)	KJ851006	KJ851027	KJ851147	Frisch *et al*. (2014)
Heterocyphelium leucampyx	Chaves 1758	KY360243	–	–	Van den Broeck *et al*. (2017)
	Van den Broeck 6326 (BR)	KY360242	–	KY360246	Van den Broeck *et al*. (2017)
H. triseptatum	**Ohmura 12401 (TNS)**	**MZ265289**	**MZ265286**	**–**	**This study**
Lecanographa amylacea	Thor 26176 (UPS)	KF707650	KF707639	–	Frisch *et al*. (unpublished)
L. farinosa	Ertz 14053 (BR)	KY360245	–	HQ454687	Ertz and Tehler (2011); Van den Broeck *et al*. (2017)
L. lyncea	Tehler 9123 (S)	–	HQ454560	HQ454700	Ertz and Tehler (2011)
Opegrapha brevis	L10094 (LD)	KJ851005	KJ851077	KF707659	Frisch *et al*. (2014)
O. celtidicola	Diederich 16053 (BR)	EU704066	EU704094	EU704030	Ertz *et al*. (2009)
O. lithyrga	Ertz 8784 (BR)	EU704068	EU704096	EU704032	Ertz *et al*. (2009)
O. vermicellifera	Ertz 7562 (BR)	EU704077	EU704105	EU704041	Ertz *et al*. (2009)
O. vulgata	Ertz 7564 (BR)	EU704080	EU704108	EU704044	Ertz *et al*. (2009)
Phacographa glaucomaria	Frisch 11Se33 (UPS)	KJ851022	KJ851028	KJ851136	Frisch *et al*. (2014)
P. protoparmeliae	**Frisch 15/No57 (TRH)**	**MZ265287**	**MZ265285**	**MZ272017**	**This study**
P. zwackhii	Frisch 11Se3 (UPS)	KJ851021	KJ851048	–	Frisch *et al*. (2014)
Plectocarpon lichenum	Thor 26770 (UPS)	KJ850988	–	KJ851140	Frisch *et al*. (2014)
P. nephromeum	Nordin 5813 (UPS)	KJ851004	–	KJ851139	Frisch *et al*. (2014)
P. scrobiculatae	**Frisch 15/No90 (TRH)**	**MZ265288**	–	–	**This study**
Simonyella variegata	AFTOL-ID80	AY584631	–	DQ782861	Lutzoni *et al*. (2004); James *et al*. (2006)
Zwackhia sorediifera	Thor 26210 (UPS)	KJ851024	KJ851055	KJ851142	Frisch *et al*. (2014)
Z. viridis	Ertz 7619 (BR)	EU704078	EU704106	EU704042	Ertz *et al*. (2009)

mtSSU1 as the 5' primer and mtSSU3R (Zoller *et al*., 1999) as the 3' primer were used. PCR cycling conditions were 94°C (3 min), followed by 11 cycles of 95°C (30 sec), 62°C to 52°C (30 sec) with annealing temperatures lowered by 1°C between cycles, and 72°C (1 min), followed by 30 cycles at 52°C annealing temperature and a final extension at 72°C (7 min). Sequencing was done on an ABI Prism 3130x genetic analyzer (Applied Biosystems) using the BigDye Terminator ver. 3.1 Cycle Sequencing Kit according to the manufacturer's instructions.

The sequences were aligned in MAFFT Version 7 (Katoh *et al*., 2019) using the default settings. Reference sequences for selected Lecanographaceae and Opegraphaceae (outgroup) were obtained from GenBank (Table 1). The sequences for each locus (mtSSU, nrLSU and *RPB2*) were aligned independently with MAFFT as implemented in the CIPRES Science Gateway (Miller *et al*., 2010). The E-INS-i accurate executable was used and the alignments were manually corrected for obvious aligning errors. Longer insertions and ambiguously aligned regions were removed prior to the analysis. The single-locus alignments were tested for conflicting tree topologies. Serious conflict was assumed when deviant tree topologies were supported by ≥70% bootstrap values (BS) and ≥0.95 posterior probabilities (PP). Since no conflicting tree topologies were observed, the three single-locus alignments were concatenated, resulting in a final alignment of 2899 sites (816 mtSSU, 1207 nuLSU and 876 *RPB2*).

A partitioned dataset was used for the phylogenetic analyses to enable independent parameter estimation for the three gene loci. The *RPB2* was further partitioned in codon positions to account for the higher evolutionary rate of the 3rd codon position. Maximum likelihood was performed with the RAxML-HPC black box v. 8.2.10 as implemented in the CIPRES Science Gateway (Miller *et al*., 2010) using rapid bootstrapping and full ML analysis under the GTR+GAMMA approximation allowing for a proportion of invariable sites. The analysis was stopped automatically after 100 bootstrap replicates using the bootstopping option implemented in RAxML (Pattengale *et al*., 2009).

Bayesian analysis was performed with MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) as implemented in the CIPRES Science Gateway (Miller *et al*., 2010). Models of sequence evolution for each alignment partition were estimated under the Bayesian Information Criterion (BIC; Stone, 1979) as implemented in MEGA X (Kumar *et al*., 2018). Models not supported by MrBayes were substituted with the nearest, less complex supported model. Models set for the individual partitions were: GTR+G (mtSSU, nuLSU), K2P (*RPB2* 1st), JC+G (*RPB2* 2nd) and HKY+G (*RPB2* 3rd). The analysis was run for 5,000,000 generations in 8 chains and every 200th generation was sampled. The first 30% of trees were discarded as burn-in and a 50% majority rule consensus tree was calculated.

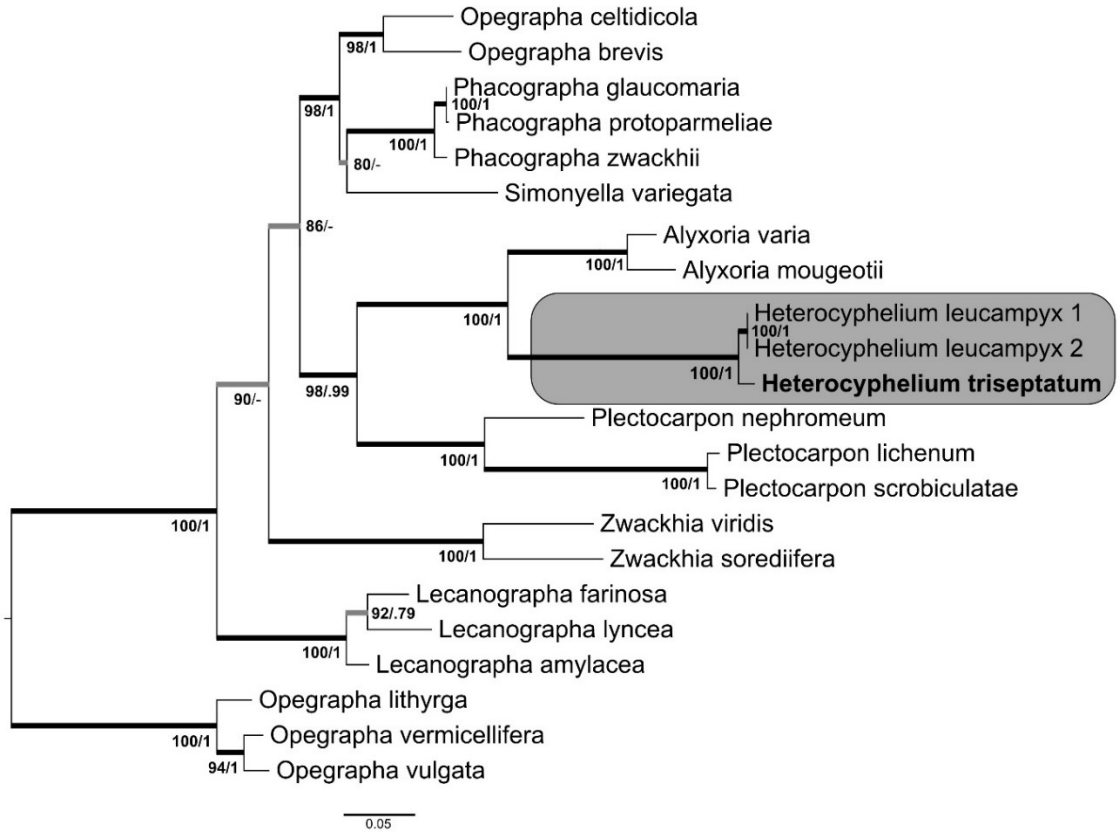

Fig. 1. Maximum likelihood tree of selected taxa in Lecanographaceae showing the phylogenetic position of ***Heterocyphelium triseptatum*** (in bold) within the *Heterocyphelium* clade. *Opegraphaceae* (***Opegrapha lithyrga***, ***O. vermicellifera*** and ***O. vulgata***) form the operational outgroup. ML (first) and Bayesian (second) support values are presented for each node. Branches supported by both analyses are indicated by strong black lines, branches supported by one of the analyses only by strong grey lines.

The voucher specimen of *H. triseptatum* used in this study is housed in the herbarium of National Museum of Nature and Science (TNS), Tsukuba, Japan, with duplicates in the herbaria of Forest Research Institute (RAF), Nay Pyi Taw, Myanmar, and National Taiwan University (TAI), Taipei, Taiwan.

RESULTS AND DISCUSSION

Molecular analysis

New mtSSU and nrLSU sequences were generated for one individual of *H. triseptatum* collected in Myanmar. Amplification of the *RPB2* failed for the Myanmar collection. The final alignment used for the Bayesian and RAxML phylogenetic analyses comprised 1040 variable sites (mtSSU 311, nrLSU 258 and *RPB2* 471), of which 874 were parsimony informative (mtSSU 267, nrLSU 177, and *RPB2* 430). The phylogenetic tree is shown in Fig. 1. The phylogenetic analyses confirm the close relationship of *H. triseptatum* and *H. leucampyx* as a sister group (1 PP and 100% BS). The mtSSU of *H. triseptatum* differed by 21 and 22 bp from the mtSSU sequences of *H. leucampyx* collected in Uganda and Costa Rica, respectively. *Heterocyphelium* is included as sister to *Alyxoria* (1 PP and 100% BS) in a well-supported clade with the lichenicolous genus *Plectocarpon* (1 PP and 100% BS). This phylogenetic relationship was first shown by Van den Broeck *et al*. (2017), who established the taxonomic position of the mazaediate genus *Heterocyphelium* in the Lecanographaceae. Sequence data are lacking for *H. triseptatum* from South America and East Africa. The relatedness of the Myanmar collection towards the specimens of other tropical regions, and particularly towards the type collection from Brazil, cannot be shown.

Heterocyphelium is the fourth mazaediate genus in Arthoniales, in addition to *Sporostigma*, *Tylophorella*, and *Tylophoron* (Van den Broeck *et al*., 2017). The mazaediate ascoma morphology is polyphyletic in the Arthoniales as shown by the taxonomic position of *Tylophoron* in the Arthoniaceae (Lumbsch *et al*., 2009). For that reason, *Tylophoron* has been excluded from the present analysis. The family-level classification of the Arthoniales is shown in Ertz and Tehler (2011) and Frisch *et al.* (2014). Molecular data are lacking for *Sporostigma* and *Tylophorella*. These two genera are classified as Arthoniales incertae sedis in the current *Outline of the Ascomycota* (Wijayawardene *et al*., 2018).

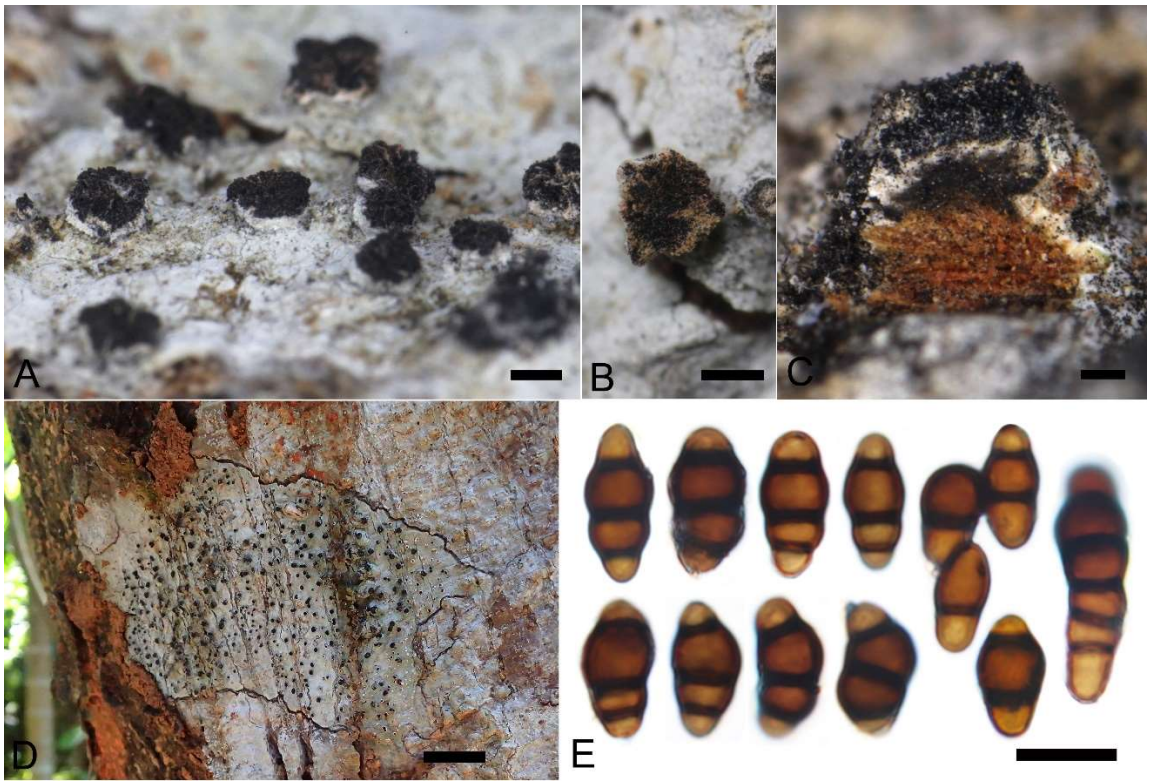

Fig. 2. ***Heterocyphelium triseptatum*** collected from Myanmar (YO12401, TNS). **A**. Mazaediate ascomata and thallus. **B**. Ascoma with orange pigment in byssoid margin. **C**. Longitudinal section of ascoma. The bark tissue is rising up under the apothecial base. The distribution of orange pigment is heterogenous in the byssoid margin. **D**. A colony growing on tree bark in the field. **E**. Ascospores. Scales: A–B = 0.5 mm, C = 0.2 mm, D = 1 cm, E= 10 µm.

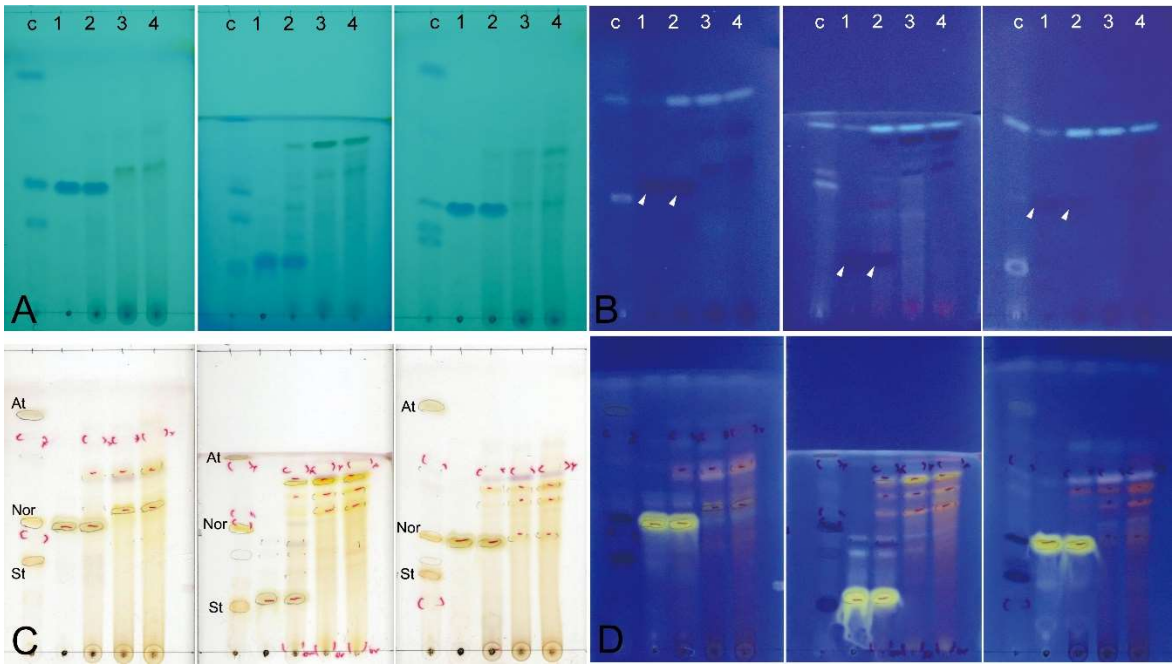

Fig. 3. TLC analyses of the chemical substance in ***Heterocyphelium triseptatum*** (YO12401, TNS) and its substrate tree bark. A set of three plates was developed in solvents A, B′, and C, respectively. **A.** $UV_{254\ nm}$ before charring with sulfuric acid spray. **B**. $UV_{365\ nm}$ before charring with sulfuric acid spray. Arrows indicate the quenched TLC spots. **C**. Visible light after charring with sulfuric acid spray. **D**. $UV_{365\ nm}$ after charring with sulfuric acid spray. c = control, 1 = ascomata only, 2 = ascomata with substrate of bark fragment, 3 = bark only (surface tissue of bark), 4 = bark only (cork part of periderm bark). Control contains stictic acid (St) for R_f class 2, norstictic acid (Nor) for R_f class 4, and atranorin (At) for R_f class 7

Recent molecular phylogenetic analyses (Ertz and Tehler, 2011; Frisch *et al*., 2014; Van den Broeck *et al*., 2017) and the present study show low support for several basal branches, resulting in conflicting tree topologies of the Lecanographaceae. All these studies use the same set of gene loci and a similar taxon sampling. An expanded taxon sampling and additional gene loci seem necessary for fully resolving the phylogeny of the Lecanographaceae.

TAXONOMIC TREATMENT

Heterocyphelium triseptatum Aptroot & M.Cáceres

Fig. 2

For a detailed description, see Aptroot *et al*. (2017). The statistical values for the ascospore size in the Myanmar collection are (11.8–)12.8–16.9(–25.6) × (5.6–)6.4–7.6(–8.4) µm (n = 58). The ascospores are mostly 3-septate, but 1-, 2-, and 5-septate ascospores were rarely observed (Fig. 2). Aptroot *et al*. (2017) reported that the ascospores of *H. triseptatum* in the collections from Brazil and Tanzania were 12–14 × 6–7 µm in size and 3-septate. These values are within the range measured for the specimen from Myanmar.

***Chemistry*:** Color spot test: K–, C–, KC–, Pd–, UV– (for thallus and ascoma with mazaedium pruina). TLC: an unidentified substance. On the TLC plates, the spot of this substance is visible under $UV_{254\ nm}$ (Fig. 3A) and quenches under $UV_{365\ nm}$ (Fig. 3B). After the treatment with sulfuric acid and charring, the spot is yellow (Fig. 3C) and shows strong yellow fluorescence under $UV_{365\ nm}$ (Fig. 3D). The spot position in solvent A is R_f class 4 (slightly below norstictic acid position); in solvent B′, R_f class 2 (slightly above stictic acid position); and in solvent C, R_f class 4 (see Fig. 3C).

***Remarks*:** *Heterocyphelium triseptatum* is distinguished from *H. leucampyx* only by the number of ascospore septa [(1–)3(–5) for *H. triseptatum* and 2(–3) for *H. leucampyx*]. The extreme values given in parentheses were only rarely observed in both species. In this study, we confirmed a 21–22 bp difference in the mtSSU sequences between *H. triseptatum* and *H. leucampyx*. The close phylogenetic relationship as sister taxa in *Heterocyphelium* is confirmed by the molecular analyses (Fig. 1).

TLC analyses were performed for ascomata of *H. triseptatum* after carefully removing all remaining host bark, for ascomata including the host bark (see Fig. 2C), and for the outer and inner parts of the host bark alone (Fig. 3). The new, unidentified chemical substance is confirmed to be located in the ascomata of the lichen and not in the host bark, with varying concentration in the tested ascomata. This substance appears to be identical to the orange pigment on the ascomata (Fig. 2B). The substance has neither been reported for *H. triseptatum* outside Myanmar nor from *H. leucampyx*. In both species no chemical substance was detected by TLC (Aptroot *et al*. 2017; Van den Broeck *et al*., 2017). Based on the current data, this study could not evaluate whether the Myanmar individuals represent a new species due to the presence of this unidentified substance. Further taxonomic research based on morphological, chemical and molecular phylogenetic data is needed to reliably comparing the collection from Myanmar material with materials from other regions. This study tentatively treats the substance in the Myanmar collection as part of the observed variation in *H. triseptatum*.

***Distribution*:** This species was previously known from Bolivia, Brazil, and Tanzania (Aptroot *et al*., 2017; Guzow-Krzemińska *et al*., 2019), growing on tree bark in savanna or under a tropical monsoon climate. The Myanmar material was also found on tree bark under a tropical monsoon climate. The distribution now extends to Asia (Myanmar).

***Specimen examined*: MYANMAR. Tanintharyi Region**: Migyanughlaung, Tanintharyi Nature Reserve, Yephyu Township, Dawei District (N14°40′18″, E98°08′16″), on bark of broadleaf tree, elev. 35 m, 23 January 2017, Y. Ohmura 12401 (TNS, RAF, TAI).

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