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New Localities of *Ropalospora phaeoplaca* in Japan and Far East of Russia with ITS nrDNA Sequences

Yoshihito Ohmura

Department of Botany, National Museum of Nature and Science,
4–1–1 Amakubo, Tsukuba, Ibaraki 305–0005, Japan
E-mail: ohmura-y@kahaku.go.jp

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Abstract Six localities from Honshu in Japan and one from Primorye Territory in Far East of Russia are newly added for the distribution of *Ropalospora phaeoplaca*. Although this species is known from main land of China, Taiwan, Korea and Far East of Russia, only the type locality was known in Japan before the present report. The ITS nrDNA region was sequenced from the fresh materials using in this study, and the maximum likelihood phylogenetic tree is also presented. The tree shows that the samples of *R. phaeoplaca* formed a monophyletic clade that is a sister to the clade of *R. viridis*, and they are sister to *R. lugubris* clade. Although *R. chlorantha* is considered to be closely related to *R. phaeoplaca*, the ITS nrDNA sequence could not be obtained in this study.

Key words: Ascomycota, distribution, eastern Asia, lichenized fungi, phylogeny, Ropalosporaceae.

Introduction

Ropalospora phaeoplaca (Zahlbr.) S.Ekman belongs to the family Ropalosporaceae (Ascomycota). It was originally described as a species of *Bacidia* (Zahlbruckner, 1927), and transferred into *Ropalospora* by Ekman (1996). This species is characterized by the following features: thallus corticolous, crustose, grayish-white to grayish green, smooth to verrucose areolate, esorediate; prothallus black or absent; apothecia lecideine, sessile, black, up to 1 mm; disk black, epruinose; exciple brown externally, inside pale brown to hyaline, with K-soluble crystals; epihymenium brown; hymenium hyaline, inspersed; hypothecium hyaline; asci clavate, 8–16 spored, *Fuscidea*-type; paraphysis simple, slightly branched at the top; ascospores hyaline, acicular with one attenuate end, straight or curved, 4–8 septate, 30–47 × 2–3 μm; pycnidia not seen (Zahlbruckner, 1927; Ekman, 1996; Hu *et al.*, 2013), and chemistry is K–, C–, KC–, P– (thallus and medulla) and presence of unidentified substance detected by thin layer chromatography (TLC) (Rf class 5–6, $UV_{254\,nm}$ + visible, with solvent C) and microcrystal test (MCT) (crystal in GE) (Hu *et al.*, 2013).

Although this species is known from main land of China, Taiwan, Korea and Far East of Russia, only the type locality was known in Japan before the present report (Zahlbruckner, 1927; Ekman, 1996; Aptroot and Sparrius, 2003; Hu *et al.*, 2013; Skirina, 2015, 2017). This paper contributes to the distribution of *R. phaeoplaca* especially for Japan and also one additional locality for Russia, and the ITS nrDNA region was also sequenced from the fresh materials.

Materials and Methods

This study is based on the herbarium specimens housed in the herbarium of National

Museum of Nature and Science (TNS), Tsukuba, Japan.

Morphological observations were made using a dissecting microscope (SZX16, Olympus) and a differential interference contrast microscope (BX53, Olympus). Sections of apothecia were prepared by hand cutting with a razor blade and mounted in water.

Chemical substances were detected by means of TLC with solvent B′ (hexane : methyl tert.-butyl ether : formic acid, 140 : 72 : 18) (Culberson and Kristinsson, 1970; Culberson and Johnson, 1982). MCT was performed using GE (acetic acid : glycerol = 1 : 3) and oT (*o*-toluidine : glycerol : ethanol = 1 : 2 : 2) (Asahina, 1936–1940). The amyloidity of ascus was examined using 1% Lugol's iodine solution.

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 the ITS nrDNA region (including ITS1, 5.8S nrDNA and ITS2) was performed using the primer set of ITS1F (Gardes and Bruns, 1993) as the 5′ primer and LR1 (Vilgalys and Hester, 1990) as the 3′ primer. PCR cycling conditions were 94℃ (3 min), followed by 11 cycles of 95℃ (30 s), 62℃ to 52℃ (30 s) with annealing temperatures lowered by 1℃ between cycles, and 72℃ (1 min), followed by 30 cycles at 52℃ annealing temperature and a final extension at 72℃ (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 ver. 7 (Katoh *et al.*, 2019) using the default settings. A sequence of *Stereocaulon apocalypticum* Nyl. was used as an out group because BLAST search (Altschul *et al.*, 1997) showed high score of homology. After removing sites with gaps, missing data and ambiguous data, the resulting alignment of 410 sites was used for the molecular phylogenetic analyses.

The maximum likelihood (ML) (Felsenstein, 1981) and neighbor-joining (NJ) (Saitou and Nei, 1987) analyses with the best nucleotide substitution model were performed. Tamura 3-parameter (T92) (Tamura, 1992) plus gamma distribution (+G) was selected for the model. The bootstrap values (Felsenstein, 1985) were calculated from 1,000 replicates for ML and NJ. All calculations were conducteed in MEGA 7.0.18 (Kumar *et al.*, 2016).

The sample data for molecular analyses and

Table 1. DDBJ/EMBL/Genbank accession numbers of samples used for molecular analyses in this study

Taxon	Voucher (Herbarium Code)*	Accession no. for ITS rDNA sequence	Reference
Ropalospora lugubris	Timdal 11170 (O)	MG926020	Kistenich *et al.* (2018)
	O-L-173455 (O)	MK812610	Marthinsen *et al.* (2019)
	BGL-98792 (BG)	MK812617	Marthinsen *et al.* (2019)
	O-L-168439 (O)	MK812670	Marthinsen *et al.* (2019)
	HK51464 (TNS)	LC516199	This study
R. phaeoplaca	HK50726 (TNS)	LC516205	This study
	YO6297 (TNS)	LC516201	This study
	YO8913 (TNS)	LC516202	This study
	YO10900 (TNS)	LC516203	This study
	YO11100 (TNS)	LC516204	This study
R. viridis	JL24387 (TNS)	LC516200	This study
	M. Kukwa & A. Łubek 17266 (KTC, UGDA)	MN387183	Singh *et al.* (2019)

*The detail collection data for voucher specimens of HK (= H. Kashiwadani), JL (= J. Lendemer) and YO (= Y. Ohmura) are shown in the paragraph of specimens examined except *Stereocaulon apocalypticum* which was collected in Japan, Honshu, Prov. Kai (Yamanashi Pref.): Sensui Pass, Minami-Alps-city (N35°44′44″, E138°14′03″), on rock, elevation 2260 m, 3 September 2012, Y. Ohmura 9268 (TNS) (GenBank accession no. for ITS rDNA sequence: LC516206).

their DDBJ/EMBL/GenBank accession numbers for the obtained ITS nrDNA sequences are shown in Table 1.

Results and Discussion

Ropalospora phaeoplaca (Zahlbr.) S.Ekman, Op. Bot. 127: 128, 1996.
≡ *Bacidia phaeoplaca* Zahlbr., Bot. Mag., Tokyo

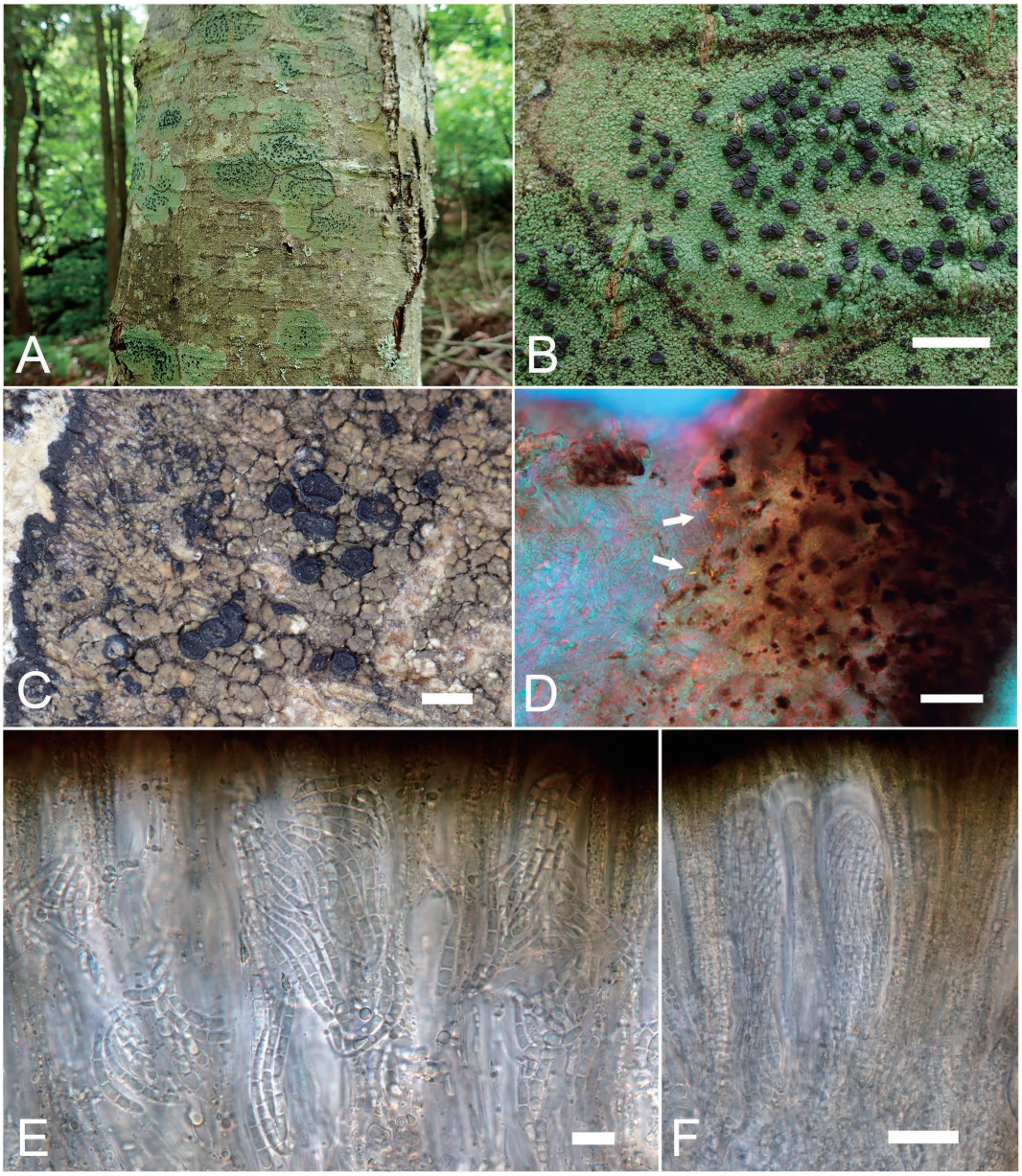

Fig. 1. *Ropalospora phaeoplaca*. A. Habitat (Y. Ohmura 11100, TNS). B. Thallus with apothecia (fresh material) (Y. Ohmura 11100, TNS). C. Thallus with apothecia (old specimens) (isotype in TNS). D. K-soluble crystals in proper exciple observed using a differential interference contrast microscope (around arrows) (isotype in TNS). E. Ascospores (H. Kashiwadani 51829, TNS). F. Asci with spores (H. Kashiwadani 51829, TNS). Scales: B = 5 mm, C = 1 mm, D = 20 µm, E = 10 µm, F = 20 µm.

41: 333, 1927.

Type: JAPAN. Honshu. Prov. Uzen (Yamagata Pref.): Takayu, 24 June 1904, U. Faurie 5882 (Holotype in W; Isotype in TNS!, KYO).

(Fig. 1)

The detail description and illustration of *R. phaeoplaca* are provided in Hu *et al.* (2013). This species is considered to be closely related to *R. chlorantha* (Tuck.) S.Ekman but differs in having the proper exciple inspersed with minute K-soluble crystals and in usually eight (occasionally ≤16) spores per ascus (Ekman, 1996).

The materials from Japan and Russia used in this study coincide with the protologue and the features mentioned above as well as with morphology and chemistry of the isotype (TNS). The size of these ascospores was 24–37 × 2.8–3.5 μm (n = 13) with 3–7 septa. Although it was shorter length and slightly wider width than the former reports (30–47 × 2–3 μm) (Zahlbruckner, 1927; Hu *et al.*, 2013), the observed shorter ascospores could be immature because they were only 3 septa (usually 4–7 septa). So that, this study treats the shorter size as variation. Amount of the K-soluble crystals in the proper exciple vary very much from few to abundant in the materials of this study. For example, those in isotype are less amount (Fig. 1D) and abundant crystals are like the illustration shown in Hu *et al.* (2013).

An unidentified substance was detected by TLC as reported in Hu *et al.* (2013), but it was not crystalized by MCT using GE and oT in this study. The TLC sport was visible with $UV_{254\,nm}$ and yellow color after 110°C heating for 10 min with 10% H_2SO_4 spray. The Rf class is 7 in solvent B′ (slightly higher than atranorin and perlatolic acid) and Rf class 6 in solvent C (same height with perlatolic acid) (Fig. 2). It would be difficult to distinguish from perlatolic acid because only slight difference was seen in solvent B' and the same position in solvent C. But the unidentified substance was detected from all samples of *R. phaeoplaca* used in this study, which would have an important taxonomic value for the species.

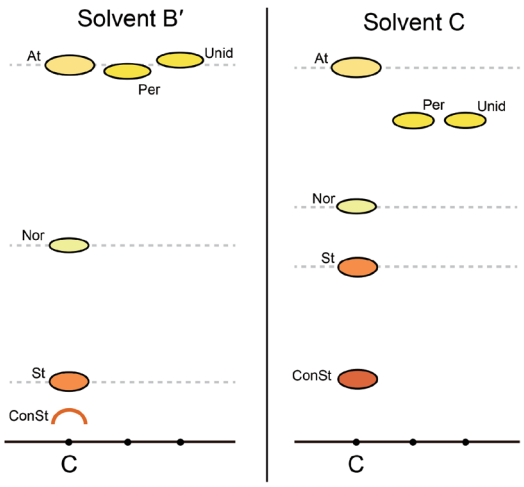

Fig. 2. Diagnostic TLC spots of lichen substances detected in *Ropalospora phaeoplaca* in Solvents B' and C. Control (C) using *Stereocaulon japonicum* which contains atranorin (At) for Rf class 7, norstictic acid (Nor) for Rf class 4, stictic acid (St) for Rf class 2, and constictic acid (ConSt) for Rf class 1. Per = perlatolic acid as a reference substance which is common in *R. chlorantha*. Unid = unidentified substance detected from *R. phaeoplaca*.

A total of five sequences of ITS nrDNA for Japanese and Russian specimens of *R. phaeoplaca*, one for *R. lugubris* (Sommerf.) Poelt collected in Japan, and one for *R. viridis* (Tønsberg) Tønsberg collected in U.S.A. (Lendemer: Lich. East. N. Amer. Exs. 420) were obtained in this study. The genus *Ropalospora* includes seven accepted species: i.e., *R. chirisanensis* S.Y. Kondr. *et al.*, *R. chlorantha*, *R. hibernica* (P. James & Poelt) Tønsberg, *R. lugubris*, *R. phaeoplaca*, *R. rossii* Øvstedal, and *R. viridis* (see Hu *et al.*, 2013; Kondratyuk *et al.*, 2016). Among them, the ITS nrDNA sequences of *R. phaeoplaca* and *R. viridis* were first registered into GenBank in this study. In order to confirm the independency of *R. phaeoplaca*, molecular phylogenetic analyses based on these ITS nrDNA sequences are performed. As the result, samples of *R. phaeoplaca* formed a monophyletic clade with high support value (ML/NJ = 100/100) (Fig. 3). The clade of *R. phaeoplaca* forms a sister clade (99/98) with *R. viridis* that is sorediate

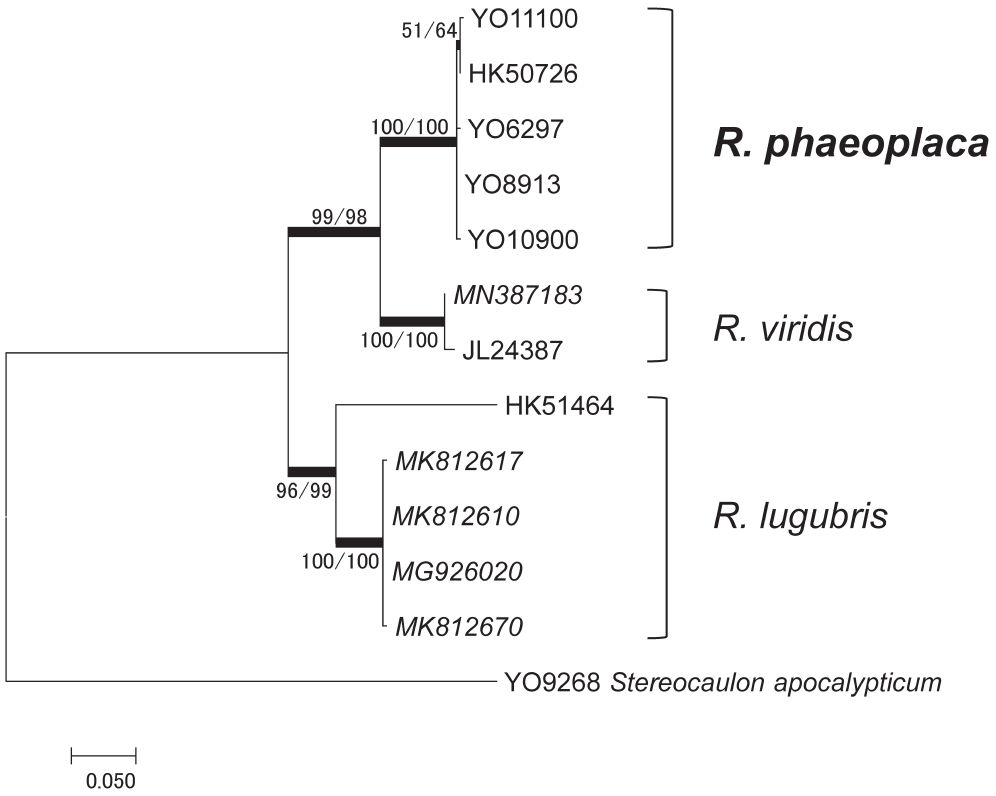

Fig. 3. Molecular phylogenetic tree of *Ropalospora phaeoplaca* and the related taxa based on ITS nrDNA sequences. The tree was constructed by ML method, and the reliability of each branch was tested by ML and NJ methods. The bootstrap values for ML/NJ analyses are shown on the branches only when both are ≥ 50% simultaneously. Bold branches indicate they are ≥ 50% simultaneously. The OTU names indicated the vouchers of H. Kashiwadani (HK), Y. Ohmura (YO) and J. Lendemer (JL), and the GenBank accession number (italic) (see Table 1). *Stereocaulon apocalypticum* (YO9268) was used as an outgroup.

and usually sterile. The samples of *R. lugubris*, a saxicolous species, forms a monophyletic clade combining Japanese and European samples (96/99). The clade of *R. lugubris* is a sister to *R. phaeoplaca* – *R. viridis* clade. Although *R. chlorantha* is considered to be closely related to *R. phaeoplaca* (Ekman, 1996), the ITS nrDNA sequence could not be obtained for the phylogenetic analysis in this study because no fresh material was available.

In Japan, the specimens were collected on barks of broad-leaf deciduous trees such as *Alnus hirsuta*, *Fagus crenata*, *Magnolia obovata*, and *Quercus crispula* at elevation between 400 to 1160 m (Fig. 1A). In Russia, it was collected on bark of broad-leaf deciduous tree at elevation c. 400 m.

Specimens examined (*R. phaeoplaca*). **JAPAN**. Honshu. Prov. Ugo (Akita Pref.): Akataki, Higashinaruse-mura, Ogachi-gun, on bark of *Alnus hirsuta*, 480 m elev., 20 November 2012, H. Kashiwadani 50726 (TNS); Genryu Area (Sangaizawa-Minamisawa Deai) of Kasugegawa in Shirakami Mts, Fujisato-machi, Yamamoto-gun, on bark of decayed wood of *Fagus crenata*, c. 400 m elev., 23 October 1999, M. Inoue 27649 (TNS). Prov. Shimotsuke (Tochigi Pref.): Nikko Botanical Garden, Nikko-city (N36°45′01″, E139°35′17″), on trunk of *Magnolia obovata*, 630 m elev., 11 September 2012, Y. Ohmura 8913 & A. Frisch (TNS). Prov. Kozuke (Gunma Pref.): Akagisawa, Tone-machi, Numata-city (N36°35′48″, E139°11′22″), on bark of *Alnus* sp., 930 m elev., 31 May 2016, Y. Ohm-

ura 11100 (TNS); the same locality, on bark of *Alnus* sp., 930 m elev., 10 June 2016, Y. Ohmura 11112 (TNS). Prov. Kai (Yamanashi Pref.): Aokigahara, Saiko Fuji-Kawaguchi-cho, Minamitsuru-gun (N32°29′35″, E138°38′60″), on bark of *Quercus crispula*, c. 950 m elev., 15 April 2017, H. Kashiwadani 51829 (TNS); Nishizawa Valley, Mitomi-Kawaura, Yamanashi-city (N35°52′06″, E138°44′53″), on bark of *Alnus hirusta*, 1160 m elev., 8 February 2009, Y. Ohmura *et al.* 6297 (TNS). **RUSSIA**. Primorsky Kray: c. 4 km N of Mt. Livadiyskaya (N43°06′19″, E132°41′31″), on bark of broadleaf deciduous tree, c. 400 m elev., 23 September 2013. Y. Ohmura 10900 (TNS).

Other specimens examined (*R. lugubris*). **JAPAN**. Hokkaido. Prov. Tokachi: around summit area of Mt. Haku-un, Kamishihoro-cho, Kato-gun (N43°15′23″, E143°07′09″), on rocks, 1120–1140 m elev., 6 July 2014, H. Kashiwadani 51464 (TNS).

Exiccata examined (*R. viridis*). **U.S.A.** Pennsylvania. Potter County: Susquehannock State Forest, E side of Ridge Rd. 0.75N of jct w/Hunts Run Rd. (N41°32′36″, W78°06′16″), 1749 ft. elev., *Larix* stand in maple (*Acer*) forest with sandstone boulders, on *Acer*, 2 September 2010, J. C. Lendemer 24387 (J. C. Lendemer: Lichens of Eastern North America Exsiccati 420) (TNS).

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