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NOTE

Gyalectidium setiferum (Gomphillaceae, Ascomycota), a foliicolous lichen, new to East Asia and its molecular phylogenetic position

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ABSTRACT: *Gyalectidium setiferum* Vězda & Sérus., a foliicolous lichen characterized by having some vertically oriented whitish translucent cilia surrounding a swollen greenish diahyphal mass, is reported as new to East Asia. It was collected from two localities on the lowlands of central Honshu in Japan where it grew on leaves of *Aucuba japonica* and *Maesa japonica*. The description with illustration based on the Japanese material is given. *Gyalectidium setiferum* has been included in this genus due to its morphological features even though no fertile specimen has been found so far. The monophyly of this species and its position in this genus were newly confirmed by the molecular phylogenetic analysis based on mtSSU and nrLSU.

KEY WORDS: Conidia, conidiomata, diahyphae, distribution, hyphophore, lichenized fungi, mtSSU, nrLSU, photobiont.

INTRODUCTION

The genus *Gyalectidium* (*Gomphillaceae*, lichenized *Ascomycota*) consists of 45 accepted species that mainly grow on living leaves, very rarely on barks or rocks (Ferraro *et al*., 2001; Herrera-Campos and Lücking, 2003; Safranek and Lücking, 2005; Lücking *et al*., 2007; Lücking, 2008; Suto and Ohtani, 2018). This genus is characterized by a smooth to coarsely verrucose thallus with a cartilaginous corticiform layer; immersed to erumpent, zeorine, rounded apothecia; squamiform hyphophoral conidiomata with a scale protecting an adnate, applanate, diahyphal mass (the scale is sometimes reduced or disintegrated into a cluster of erect cilia that are difficult to distinguish from setae); and branched moniliform diahyphae forming sausage-like shaped segments (Lücking, 2008).

During the course of studies for the Japanese foliicolous lichenized mycota, *G. setiferum* Vězda & Sérus. was confirmed to occur in Japan, which is the first report from East Asia. This taxon is considered to belong to the genus *Gyalectidium* due to the presence of reduced hyphophores with diahyphal mass which is produced at the base and the diahyphal mass composed of sausage-like shaped cells (Sérusiaux, 1993; Ferraro *et al*., 2001). However, no apothecia of *G. setiferum* have been found so far (Sérusiaux, 1993; Ferraro *et al*., 2001; van den Boom, 2021), so a molecular phylogenetic analysis was needed to confirm the position of this species in this genus.

The purpose of this study was to describe the morphological and chemical features of *G. setiferum* based on the Japanese material as well as performing a molecular phylogenetic analysis to confirm the monophyly of the species and its taxonomic position in the genus.

MATERIALS AND METHODS

All voucher specimens are housed in the herbarium of National Museum of Nature and Science (TNS), Tsukuba, Japan.

Morphology and chemistry

Morphological observations and photography were performed using a dissecting microscope (M165C; Leica, Wetzlar, Germany) with a digital camera (Flexacam C3; Leica) and a differential interference contrast microscope (BX53; Olympus, Tokyo, Japan) with a digital camera (EOS Kiss X9i; Canon, Tokyo, Japan). Anatomical examinations were performed using hand-cut sections mounted in GAW (glycerin: ethanol: water = 1: 1: 1) solution (Asahina, 1936). The digital image of Fig. 1A was prepared using CombineZP image stacking software (developed by Alan Hadley).

Calcium oxalate crystals were identified under the microscope by adding 25% H_2SO_4 following Thor *et al*. (2000). Color spot tests for K, C, KC, and Pd were performed according to Orange *et al*. (2001).

Secondary substances were examined using a high performance thin layer chromatography (HPTLC) following Schumm and Elix (2015). Solvent systems B′ (*n*-hexane: methyl tert-butyl ether: formic acid, 140: 72: 18) (Culberson and Johnson, 1982) was used for HPTLC. The spot color was checked under 254 and 366 nm wavelength of UV and visible light, before and after spraying with 10% sulfuric acid on the HPTLC plate and charring at 90°C for 20 minutes.

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

Taxa	Voucher	mtSSU	nrLSU	Reference
Aderkomyces sp. (sterile)	Brazil; *A.B. Xavier-Leite 2004* (ISE)	MZ827178	MZ851543	Xavier-Leite *et al*., 2022
Asterothyrium longisporum	Costa Rica; *R. Lücking s.n.* (F)	AY341363	AY341349	Lücking *et al*., 2004
Asterothyrium sp. (as *Calenia monospora*)	Costa Rica; *R. Lücking s.n.* (F)	AY341365	AY341351	Lücking *et al*., 2004
Calenia lobulata	Costa Rica; *R. Lücking s.n.* (B)	MZ827242	MZ851715	Xavier-Leite *et al*., 2022
Calenia monospora	Cuba; *R. Lücking et al.* 41885c (B, HAJB)	MZ827264	MZ851728	Xavier-Leite *et al*., 2022
Gyalectidium catenulatum	Costa Rica; *R. Lücking 16032b*	KF833335	KF833323	unpublished
Gyalectidium aff. *denticulatum*	Brazil; *A.B. Xavier-Leite et al. 1245* (ISE)	MZ827272	MZ851626	Xavier-Leite *et al*., 2022
Gyalectidium filicinum	Brazil; *A.B. Xavier-Leite 1951* (ISE)	MZ827174	MZ851514	Xavier-Leite *et al*., 2022
	Brazil; *A.B. Xavier-Leite 1970* (ISE)	MZ827171	MZ851521	Xavier-Leite *et al*., 2022
Gyalectidium imperfectum	Brazil; *A.B. Xavier-Leite 1973* (ISE)	MZ827170	MZ851522	Xavier-Leite *et al*., 2022
	Guatemala; *R. Lücking 4353* (B)	MZ827236	MZ851731	Xavier-Leite *et al*., 2022
Gyalectidium aff. *imperfectum*	Costa Rica; *R. Lücking 55* (B)	MZ827257	MZ851712	Xavier-Leite *et al*., 2022
Gyalectidium setiferum	Japan; *K. Miyazawa 1017* (TNS)	**LC726432**	**LC726435**	**This study**
	Japan; *K. Miyazawa 1018* (TNS)	**LC726433**	**LC726436**	**This study**
	Japan; *K. Miyazawa 1023* (TNS)	**LC726434**	**LC726437**	**This study**
Gyalectidium sp.	Brazil; *A.B. Xavier-Leite 1986* (ISE)	MZ827180	MZ851529	Xavier-Leite *et al*., 2022
Gyalectidium sp.	Brazil; *M. Cáceres & R. Lücking 143* (B, URM)	MZ827253	MZ851699	Xavier-Leite *et al*., 2022
Tricharia lancicarpa	Brazil; *A.B. Xavier-Leite 1564* (ISE)	MZ827275	MZ851491	Xavier-Leite *et al*., 2022
Tricharia vainioi	Brazil; *A.B. Xavier-Leite et al. 1402b* (ISE)	MZ827228	MZ851658	Xavier-Leite *et al*., 2022
Tricharia sp. (sterile)	Brazil; *A.B. Xavier-Leite 1950* (ISE)	MZ827173	MZ851513	Xavier-Leite *et al*., 2022

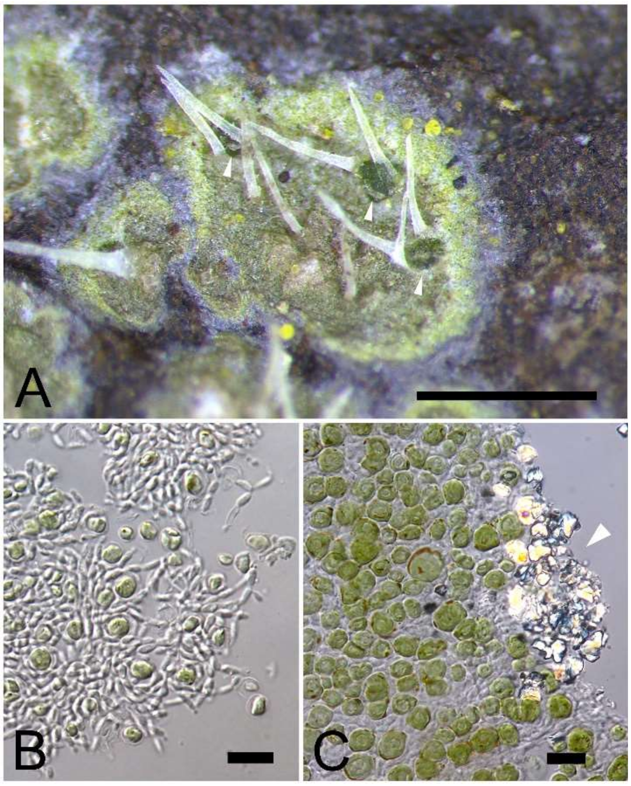

Fig. 1. ***Gyalectidium setiferum*** collected from Japan (*K. Miyazawa 1017*, TNS). **A**: Thallus with vertically oriented cilia surrounding the swollen diahyphal masses (each arrow indicates swollen diahyphal mass). **B**: Moniliform hyphae with sausage-like shaped segments in a diaphyphal mass with photobiont cells. **C**: Photobiont cells in the thallus with calcium oxalate crystals (arrow). Scale bars: A = 0.5 mm, B & C = 10 μm.

DNA extraction, PCR amplification and sequencing

DNA extraction for PCR was performed following a modified method of Izumitsu *et al.* (2012) (see also Miyazawa *et al*., 2022). The partial sequences of the small subunit of the mitochondrial ribosomal RNA gene (mtSSU) and the large subunit of the nuclear ribosomal RNA gene (nrLSU) were amplified using the primer sets mrSSU1 and mrSSU3R (Zoller *et al*., 1999) for mtSSU, LIC24R (Miadlikowska and Lutzoni, 2000) and LR7 (Vilgalys and Hester, 1990) for nrLSU, according to the following protocol. PCR was performed in a 15 μL reaction solution containing 2 μL DNA template, 7.5 μL GenRED PCR Mix Plus (Nippon Gene, Tokyo, Japan), 1.5 μL each primer (2 pmol/μL), and 2.5 μL distilled water. The PCR conditions followed the method of Wang *et al*. (2020) for mtSSU and a modified method of Frisch *et al*. (2014) (45 cycles to 35 cycles) for nrLSU, using a TaKaRa PCR Thermal Cycler Dice® Touch (TaKaRa, Tokyo, Japan). The PCR products were checked by electrophoresis on a 1.5% agarose gel stained with Midori Green Direct DNA Stain (Nippon Genetics, Tokyo, Japan) and visualized using WSE-5200 Printgraph 2 M (ATTO Corporation, Tokyo, Japan).

The PCR products were purified using an ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher, Tokyo, Japan). 13 μL of PCR products with 2 μL of 4 times diluted ExoSAP-IT™ were incubated at 37°C for 15 minutes, then 80°C for 15 minutes.

Sequences were obtained by a DNA sequencing service (Eurofins Genomics, Tokyo, Japan). The taxon name and GenBank accession numbers for the obtained sequences are shown in Table 1.

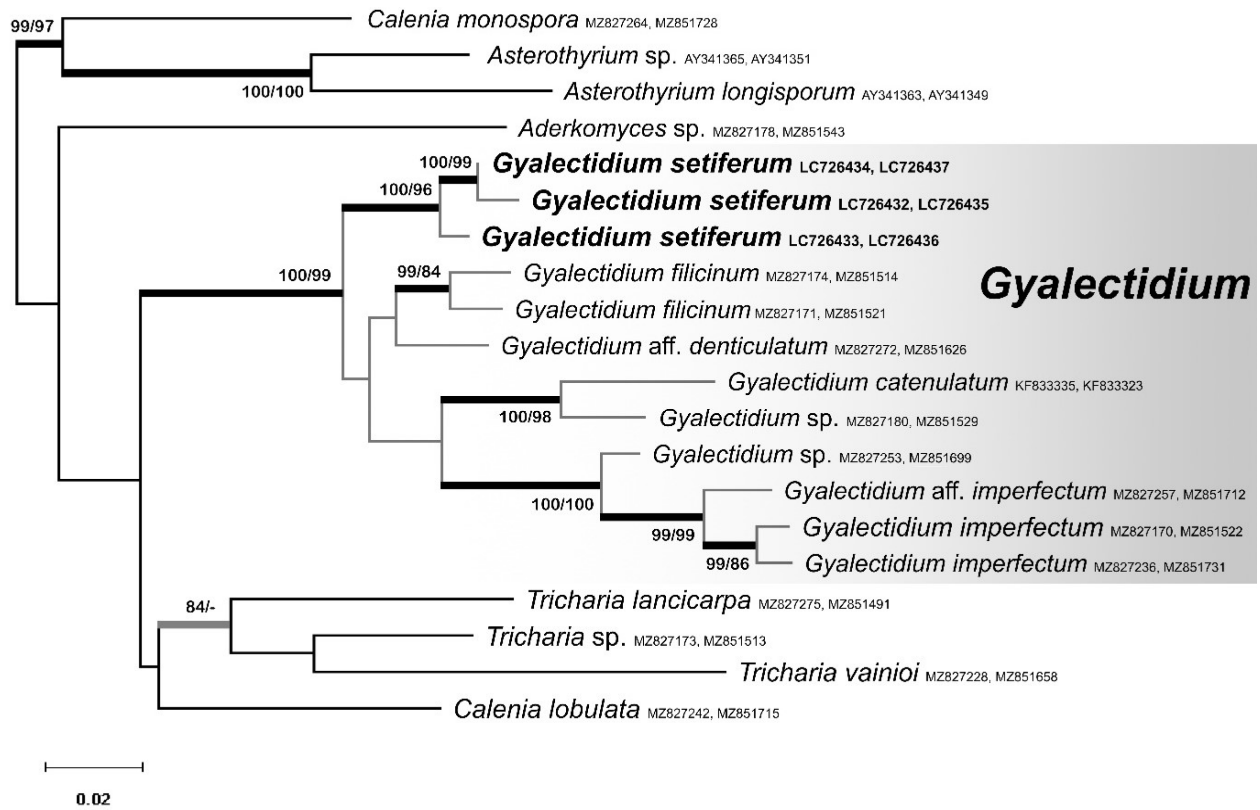

Fig. 2. Maximum likelihood tree of selected taxa in Gomphillaceae showing the phylogenetic position of ***Gyalectidium setiferum*** collected from Japan (in bold) within the *Gyalectidium* clade. *Calenia monospora-Asterothyrium* group is used as an out group. NJ (first) and ML (second) support values are presented for each node. Branches highly supported (≥70) by both analyses are indicated with bold black lines, and a branch supported by only one of the analyses with a bold grey line.

Molecular phylogenetic analyses

The newly obtained mtSSU and nrLSU sequences of *Gyalectidium setiferum* from the Japanese material were aligned with registered sequences of selected taxa in GenBank (Table 1) in MAFFT ver. 7 (Katoh *et al*., 2019) using the default settings. For the outgroup, the sequences of *Calenia monospora* (MZ827264, MZ851728), *Asterothyrium* sp. (AY341365, AY341351) and *A. longisporum* (AY341363, AY341349) from GenBank were used to be consistent with the phylogenetic tree generated by Xavier-Leite *et al*. (2022). Each data set (mtSSU and nrLSU) was separately aligned. After removing sites with gaps, missing data and ambiguous data, the data were concatenated. The final alignment of 897 sites was used for the molecular phylogenetic analyses.

The neighbor-joining method (NJ) and maximum likelihood method (ML) analyses were performed by Tamura 3-parameter model (Tamura, 1992) plus gamma distribution which was selected as the best fitting model. The bootstrap values (≥70%) with 1,000 replicates for NJ and ML were placed on each branch. A branch with high bootstrap values (≥70%) by both analyses was indicated with bold black line, and a branch supported by only one of the analyses was shown with bold grey line. All calculations were conducted in MEGA X (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

Molecular analysis

Among the aligned sites of each locus within the Japanese material, there are ten variable sites and one gap site in 739 sites of mtSSU (the percent identity among three specimens: 98.5–100%) and nine variable sites in 501 sites of nrLSU (98.2–99.2%). The topologies of phylogenetic trees constructed by both methods are consistent. The ML phylogenetic tree is shown in Fig. 2. The monophyly of *G. setiferum* and the its position in the genus *Gyalectidium* were confirmed with high support values (NJ/ML = 100/96, 100/99 respectively).

There was no conflict between the topology of our phylogenetic tree including *G. setiferum* and that of Xavier-Leite *et al*. (2022). However, the relationship between *G. setiferum* and other *Gyalectidium* species was unclear due to the low bootstrap values (Fig. 2). Further phylogenetic analysis with additional DNA information of other loci of more related species would clarify the phylogenetic position of *G. setiferum* within the genus.

TAXONOMIC TREATMENT

Gyalectidium setiferum Vězda & Sérus., in Sérusiaux, Nord. J. Bot. 13: 454 (1993). Type: GEORGIA, Colchis, Soci, valley of Chosta River, 50 m elev., on leaf of *Laurocerasus officinalis*, 1980, *Vězda s. n.* [holotype: PRA, not seen; a photo is shown in Ferraro *et al*. (2001)]. **Fig. 1**

THALLUS crustose, more or less circular, forming rounded to irregular patches, up to 4 mm diam. and 20–60 µm thick, with corticiform layer, finely but irregularly verrucose due to incrustation with calcium oxalate crystals, greenish grey; prothallus present, membranaceous, pale grey; sterile setae scattered on the thallus that resemble the cilia of hyphophoral conidiomata, tapering towards the tips, 0.2–0.4 mm long and 15–35 µm wide, white translucent. ASCOMATA not seen. CONIDIOMATA hyphophoral, laminal, sessile on thallus, their scales divided into 2–5 vertically oriented cilia surrounding the swollen diahyphal mass; cilia, tapering towards the tips, 0.3–0.5 mm long and 15–30 µm wide, often abruptly broadened at the base (up to 90 µm), white translucent; diahyphal mass green, 0.1–0.15 mm diam., composed of moniliform hyphae, terminal segments sausage-like shaped, cells 3–8 × 1.5–2.5 µm. Photobiont *Chlorella* (*s. lat.*), green, with a subparietal chloroplast bearing a conspicuous central pyrenoid, spherical, 6–11 µm diam. in thallus and spherical to ellipsoid, 3–7 × 2–5 µm diam. in diahyphal mass.

Chemistry. C–, K–, KC–, Pd– (thallus). No secondary substances were detected by HPTLC.

The morphological and chemical features of specimens collected from Japan are consistent with the protologue (Sérusiaux, 1993) and descriptions provided by Ferraro *et al*. (2001) and van den Boom (2021). Among the Japanese specimens, no clear differences in morphology were observed, so this study treats the genetic differences (see above; Fig. 2) as variations within a single species.

This species is characterized by a greenish grey thallus with some vertically oriented whitish translucent cilia surrounding the swollen greenish diahyphal mass (Fig. 1A). Similar hyphophoral structures are also known in *G. eskuchei* Sérus. and *G. rosae-emiliae* Herrera-Camp. & Lücking (Lücking, 2008) within this genus, but both differ in lacking a prominent diahyphal mass. The latter also differs in lacking sterile setae.

A subparietal chloroplast bearing a conspicuous central pyrenoid in a spherical to ellipsoid cell was observed in the photobiont cells of *G. setiferum* from Japan (Fig. 1C). From the morphology, it can be identified as a species of *Chlorella* (*s. lat.*). However, molecular phylogenetic analyses of *Chlorella* (*s. lat.*) were revealed it as being polyphyletic and consisting of at least twelve independent lineages of the *Trebouxiophyceae* and *Chlorophyceae* (Huss *et al*., 1999; Krienitz *et al*., 2004; Darienko *et al*., 2010, 2016; Pröschold *et al*., 2011; Krienitz *et al*., 2015; Darienko and Pröschold, 2019). Without DNA data, it would be difficult to identify a genus of the photobiont of *G. setiferum*. Since photobionts of other *Gyalectidum* species belong to *Jaagichlorella* Reisigl (as *Heveochlorella* J. Zhang, V.A.R. Huss, X. Sun, K. Chang & D. Pang) (Sanders *et al*., 2016) which have similar intracellular structures with a single chloroplast bearing a central pyrenoid, the photobiont of Japanese *G. setiferum* could belong to *Jaagicholorella* or a related genus within *Chlorella* (*s. lat.*).

The size of photobiont cells in the diahyphal mass are much smaller than those in the thallus (Fig. 1B & C). Such small algal cells in the diahyphal mass were also reported from the type material of *G. setiferum* (Sérusiaux, 1993).

Gyalectidium setiferum may be confused with other foliicolous species having whitish setae or hyphophores on the thallus (e.g., *Aderkomyces* spp., *Echinoplaca* spp. and other *Gyalectidium* spp.), but these species do not produce swollen greenish diahyphal masses surrounded by vertically oriented whitish translucent cilia.

This species was known only from western Europe and Caucasus as an obligately foliicolous species, especially reported on *Buxus* and *Abies* (see Ferraro *et al*., 2001; van den Boom, 2021). In Japan, *G. setiferum* was found on leaves of *Aucuba japonica* Thunb. in Ibaraki Prefecture and *Maesa japonica* (Thunb.) Moritzi & Zoll. in Chiba Prefecture at ca. 100 m elevation of central Honshu. It is here reported as new to East Asia, resulting a total of eight species of *Gyalectidium* are recorded at present in this region, i.e., *G. australe* Lücking, *G. catenulatum* (Cavalc. & A.A. Silva) L.I. Ferraro, Lücking & Sérus., *G. caucasicum* (Elenkin & Woron.) Vězda, *G. ciliatum* Lücking, G. Thor & Tat. Matsumoto, *G. filicinum* Müll. Arg., *G. setiferum* Vězda & Sérus., *G. shimanense* Y. Suto and *G. radiatum* Lücking, G. Thor & Tat. Matsumoto (Thor *et al*., 2000; Aptroot and Sparrius, 2003; Aptroot *et al*., 2003; this study).

***Specimens examined*.** **JAPAN**. Honshu. Hitachi Prov. (Ibaraki Pref.): near Fudotaki Waterfall, Shiio, Makabe-cho, Sakuragawa-city (N36°14′, E140°04′), 120 m elev., on leaf of *Aucuba japonica*, 5 May 2022, *K. Miyazawa 1017, 1018 pr. p.* (in collection *Fellhanera bouteillei*) (TNS). Kazusa Prov. (Chiba Pref.): near headstream of Minato River, Toyooka, Futtsu-city (N35°10′, E139°59′), 130 m elev., on leaf of *Maesa japonica*, 21 May 2022, *K. Miyazawa 1023* (TNS).

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