***Cyphellostereum Ushima* Sp. Nov. (Hygrophoraceae, Agaricales) Described from Amami-Oshima Island (Kagoshima Prefecture, Ryukyu Islands), Japan, with Ultrastructural Observations of its *Rhizonema* Photobiont Filaments Penetrated Longitudinally by a Central Haustorium**

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Abstract

We collected a resupinate fungus lichenized with filamentous *Rhizonema* cyanobacteria in Amami-Oshima Island, Japan. Microscopic observations and molecular phylogenetic analyses of both the basidioma and the lichenized thallus indicated affinities within *Cyphellostereum*, the first record of this basidiomycete genus from Japan. Its unique combination of structural characters and ITS rDNA sequences suggest a new species, described here as *C. ushima*. Light and transmission electron microscopic observations revealed tubular intracellular haustoria penetrating longitudinally within the cyanobacterial cells, a feature typically seen in the related genus *Dictyonema*, but unusual in *Cyphellostereum*. The generic circumscription of *Cyphellostereum* was therefore emended to include features of *C. ushima* and other recently described species that deviate from the conventional definition of the genus. Ultrastructural details of symbiont interactions are provided for the first time in this genus. We also succeeded in cultivating the fungus from hyphae regenerated from thallus fragments, and include a brief description of those results.

TreeBASE reviewer access URL:

<http://purl.org/phylo/treebase/phylows/study/TB2:S28437?x-access-code=84d2007afb05bd743f34101ac2d97dac&format=html>

Introduction

The genus *Cyphellostereum* D.A. Reid was established in 1965 to include four species, based only on structures of the stipitate and cyphelloid (fan-shaped) fruiting body (Reid 1965); the relationship with algae was not noticed at that time. It was Oberwinkler (1970) who first recognized that *Cyphellostereum* is lichenized. He observed the mycelium of *C. pusiolum* (as *Clavaria* sp.) surrounding a cluster of green algae and trichomes of filamentous cyanobacteria in a Venezuelan collection. Since *Cyphellostereum* was rarely collected, it remained a poorly understood group. More recently, investigators have focused attention upon lichenized basidiomycetes, especially in tropical and subtropical regions (e.g., Yáñez et al. 2012; Lücking and Timdal 2016; Dal Forno et al. 2017; Dal Forno et al. 2019). This has led to the description of some additional species, and the surprising placement of the genus in Hygrophoraceae (Lawrey et al. 2009; Lodge et al. 2014). Eleven species of *Cyphellostereum* are recognized at present (Table 1). Descriptions of each taxon in *Cyphellostereum* differ greatly depending on whether the species in question was regarded as a mushroom or a lichen; the former lack data on the lichenized thallus, the latter on basidia and basidiospores. Thus, the species of *Cyphellostereum* have never been described with the morphology of both the matured basidioma and the lichenized thallus taken into account for the same taxa, thereby hindering a holistic understanding of the genus *Cyphellostereum* and its species.

Table 1

Morphological comparison of *Cyphellostereum ushima* with currently recognized 11 *Cyphellostereum* and 3 *Athelia* species associated with filamentous

Taxon	Hymenophore type	Basidia	Basidiospores	Cyanobacterial filaments	Hyphal sheath	Intracellular haustoria	Clamp connections	Type locality	Sequenced data
<i>C. ushima</i>	resupinate	clavate to short-cylindrical; 9.4–13.0 × 5.4–6.5 µm	ellipsoid to slightly elongate; 5.3–6.3 × 3.4–4.0 µm	5.5–8.6 × 3.3–7.3 µm	irregular and leaving interspaces	Present	Absent	Japan	ITS, nuLSU
<i>C. bicolor</i>	irregular, resupinate	not observed	not observed	7–9 × 5–6 µm	irregular and leaving interspaces	Absent	Absent	Mauritius	Absent
<i>C. brasiliense</i>	pileate, roundly to slightly spatulate	clavate; 18–25 × 5–6 µm	oblong ellipsoid; 7–8 × 3.5–4.5 µm	no data	no data	no data	Absent	Brazil	Absent
<i>C. galapagoense</i>	not observed	not observed	not observed	7–10 × 5–10 µm	jigsaw-puzzle-shaped, completely closed	Present	Absent	Ecuador	ITS
<i>C. georgianum</i>	resupinate to corticioid	not observed	not observed	7–12.5 × 5–10 µm	irregular and leaving interspaces	Absent	Present	USA	ITS
<i>C. imperfectum</i>	not observed	not observed	not observed	7–10 × 3–6 µm	irregular and leaving interspaces	no data	Absent	Guatemala	ITS, nuLSU, rpb2
<i>C. jamesianum</i>	resupinate	not observed	not observed	11–13 × 4–6 µm	irregular and leaving interspaces	Absent	Present	USA	ITS
<i>C. muscicola</i>	spathulate	20 × 4 µm	not observed	no data	no data	no data	Absent	Java	Absent
<i>C. phyllogenum</i>	not observed	not observed	not observed	6–12 × up to 7 µm	? ("with a regular hyphal shell")	no data	Absent	Borneo (lectotype)	ITS, nuLSU
<i>C. pusiolum</i>	dimidiate flabelliform or spatulate	cylindrical to subcylindrical; 10–26 × 3.5–5 µm	ellipsoid to pip-shaped; 5–8 × 3–4 µm	no data	no data	no data	Absent	Cuba	ITS, nuLSU, rpb2
<i>C. rivulorum</i>	shortly stipitate	clavate; 30 × 5 µm	2.5–3.5 × 2–2.5 µm	no data	no data	no data	Absent	Cuba	Absent
<i>C. unoquinoum</i>	not observed	not observed	not observed	7.5–11 × 5–7 µm	irregular and leaving interspaces	Absent	Absent	Ecuador	ITS
<i>A. andina</i>	resupinate	broadly cylindrical to clavate; 10–17–30 × 6–8 µm	broadly ellipsoidal; 6.5–7.5 × 4.5–4.8 µm (5.5–6 × 4–4.5 µm) ^a	(9–10 × 3–6 µm) ^b	no data	(Present?) ^b	Absent	Venezuela	Absent

^a Jülich (1978)^b Estimated or indicated in Oberwinkler (1970; Fig. 2)

Taxon	Hymenophore type	Basidia	Basidiospores	Cyanobacterial filaments	Hyphal sheath	Intracellular haustoria	Clamp connections	Type locality	Sequer data
<i>A. phycophila</i>	resupinate	clavate; 13–16 × 5.5–6.5 µm	pyriform, (apical part distinctly broadened) ^a ; 5–6–6.5 × 3.5–4 µm	no data	no data	no data	Absent	Venezuela	Absent
<i>A. poeltii</i>	resupinate	clavate when mature, more or less ellipsoidal when young; 15–18 × 5.5–6.5 µm	ovate, distinctly broadened near the base; 5.5–6 × 4–4.4 µm	no data	no data	no data	Present	USA	Absent
^a Jülich (1978)									
^b Estimated or indicated in Oberwinkler (1970; Fig. 2)									

Cyphellostereum and four other genera that associate with cyanobacteria (*Acantholichen* P.M. Jørg., *Cora* Fr., *Corella* Vain., and *Dictyonema* C. Agardh ex Kunth) are now known to form a well-supported monophyletic clade within Hygophoraceae, Agaricales (Dal Forno et al. 2013; Lodge et al. 2014). Recent studies have shown that all of them are lichenized with the filamentous cyanobacterial genus *Rhizonema* (Lücking et al. 2009; Dal Forno et al. 2021). Until recently, the thallus of *Cyphellostereum* was characterized by the incomplete enclosure of *Rhizonema* trichomes by mycelium and the absence of haustoria inside the trichome cells (Dal Forno et al. 2013; Lücking et al. 2013). These traits distinguished *Cyphellostereum* morphologically from its sister genus, *Dictyonema*, in which the fungus forms a cellular sheath completely covering the cyanobacterial trichomes, and intracellular haustoria are present. However, some of the very recently described species, placed within *Cyphellostereum* by molecular sequence analyses, completely enclose their photobiont trichomes with jigsaw-puzzle-like sheaths, or penetrate them with intracellular tubular haustoria as seen in *Dictyonema*. The definition of *Cyphellostereum*, therefore, needs to be revised.

Two species of cyanobacteria-associated basidiomycetes, *Dictyonema moorei* and *D. sericeum*, have been recorded for Japan (Harada et al. 2004; Ohmura and Kashiwadani 2018). In the course of studying lichenized basidiomycetes in Japan, we found a fungus similar to *Cyphellostereum* whose mycelium forms a thallus that incompletely surrounds the filamentous cyanobacteria. Molecular phylogenetic analyses of ITS rDNA placed this fungus within a lineage of *Cyphellostereum*, but it was morphologically different from typical *Cyphellostereum* species in two ways: it had intracellular haustoria inside the trichome cells, and the basidioma was whitish, resupinate, and membranaceous like *Athelia* species, neither stipitate nor cyphelloid. Based on these results, we describe the species as *Cyphellostereum ushima* sp. nov. and revise the definition of the genus *Cyphellostereum*, taking into account other very recently described members of *Cyphellostereum*.

Materials And Methods

Morphological observation

Macroscopic characters were described based on fresh materials. Microscopic characters were observed in squash preparations of fresh materials mounted in distilled water. For the observation of basidia, the hymenophore was mounted in 3% KOH solution after staining with 1% phloxine B solution. Amyloid reactions were checked using Melzer's reagent (1.5 g potassium iodide, 0.5 g iodine, 20.0 g chloral hydrate, 20.0 mL distilled water). The hymenophore and the lichenized thallus were observed under a stereo-microscope (Olympus SZ 61 and Olympus SZX 16) equipped with a digital camera (Olympus DP21). Microscopic characters of both the mycobiont and the photobiont were observed and measured with differential interference contrast (Olympus BX53, equipped with digital camera DP 73). The length/width ratio (Q) of each spore was given as the range of Q values. To describe the variation of basidiospore size and Q values, 5% of measurements from each end of the range were placed in parentheses. A voucher specimen has been deposited at the herbarium of the Osaka Museum of Natural History (OSA; Osaka, Japan).

Transmission electron microscopy (TEM)

For TEM observation, a part of lichenized thallus was embedded in 2% (w/v) low gelling temperature agarose type VII-A (Sigma-Aldrich, St. Louis, MO, USA) before fixation. The agar block was pre-fixed in 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) for 1h at room temperature and rinsed with 0.05M cacodylate buffer three times for 10 min each time. The block was post-fixed in 1% osmium tetroxide for 1h at room temperature and dehydrated in an ethanol series (10%, 30%, 50%, 70%, 90%, for 15 min per step, and 95% once and 100% twice for 20 min per step) and embedded in Agar Low Viscosity Resin (Agar Scientific, Stanstead, UK). Ultrathin sections (80 nm thick) were prepared with an RMC MT-X ultramicrotome (RMC products, Arizona, USA). Sections were stained with platinum blue (Inaga et al. 2007) and lead citrate (Venable and Coggeshall 1965). Sections were observed using a transmission electron microscope (HT7700; Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV.

Isolation of the mycobiont

The collected thallus was moistened with a drop of sterile water and fragmented by sandwiching it between two sterile glass slides that were then rubbed together. The resulting fragments were diluted with sterile water and incubated on modified Detmer's medium (MDM; Watanabe 1960) agar under the

following condition: 20 ± 1 °C, 20 µmol m⁻² s⁻¹, and 16h light / 8h dark cycle. The mycobiont isolate (NBRC 115003) was established from the mycelia recovered from the fragments and transferred to potato dextrose agar (PDA; Nissui, Tokyo, Japan), and incubated at 20 °C for 1–2 months. We also tried to isolate the mycobiont by attaching a piece of wood containing the hymenium to the lid of a plastic petri dish with mending tape so that basidiospores were discharged onto water agar; the cultures were then incubated at 20 °C in the dark for one month. The mycobiont culture obtained has been deposited in the NITE Biological Resource Center (NBRC; Kisarazu, Chiba, Japan).

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from the hymenium, the lichenized thallus, and the mycobiont isolate (NBRC 115003) according to the method of Izumitsu et al. (2012). Polymerase chain reaction (PCR) with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) was performed in 20 µL reaction volumes containing 1.5 µL of extracted template DNA, 0.4 µL of KOD-FX Neo, 10 µL of 2× PCR buffer, 4.0 µL of dNTP, 0.3 µL each of the two primers (20 µM), and 3.5 µL of distilled water. The sequence of ITS+28S rDNA (D1/D2) of the mycobiont was amplified with ITS1F (Gardes and Bruns 1993) and LR5 (Vilgalys and Hester 1990) primer pairs, and that of 16S rDNA of the photobiont was amplified with CYA-106F and CYA-781R (Nübel et al. 1997). Multiple attempts to amplify the photobiont *rbcLX* using CX and CW (Rudi et al. 1998) primer pairs failed, probably because the primers did not correspond to the genome sequence. PCR products were purified by the polyethylene glycol (PEG) precipitation method. Briefly, a 1:1 mixture of PCR product with 0.8 M NaCl and 6.5% (w/v) PEG 8000 was centrifuged at 15,000 rpm for 30 min at 4 °C, washed once with 70% ethanol, dried at 50 °C for 1 h, and stored in 20 µL TE buffer at -30 °C. Cycle sequence reactions were conducted using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) using the following primers: ITS1F and ITS4 (White et al. 1990) for the mycobiont ITS (ITS1-5.8S-ITS2), and LR0R (Rehner and Samuels 1994), LR5 for the mycobiont 28S rDNA (D1/D2 region), and CYA-106F and CYA-781R for the photobiont 16S rDNA, following the manufacturer's instructions. DNA sequences were analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All newly generated sequences were deposited in GenBank.

Phylogenetic analyses

The newly generated mycobiont ITS sequences were aligned with *Cyphellostereum* sequences from GenBank using the online software MAFFT version 7 with –auto option (Katoh et al. 2019) and manually refined with Seaview v.5.0.4 (Gouy et al. 2010). *Eonema pyriforme* was selected as outgroup based on Lawrey et al. (2009) and Dal Forno et al. (2019). The phylogenetic analyses for the mycobiont ITS were performed using maximum-likelihood (ML) and Bayesian methods. The optimum substitution models for each data set were estimated using ModelTest-NG v.0.1.6 (Darriba et al. 2020) for the ML analysis and Kakusan4 (Tanabe 2011) for the Bayesian analysis. The ML analysis was performed with RAXML-NG v.0.9.0 (Kozlov et al. 2019). The substitution model TVM+I+G4 was selected as the best-fit model for the ML analysis. Bootstrap proportions (BP) were obtained using 1,000 bootstrap replicates. The Bayesian analysis was performed with MrBayes v.3.2.7a on the CIPRES Science Gateway (Miller et al. 2010; Ronquist et al. 2012) using the substitution model K80+G containing the BIC4 parameter. Two simultaneous and independent Metropolis-coupled Markov Chain Monte Carlo (MCMC) runs were performed for 2,000,000 generations with the tree sampled for every 1,000 generations of the analyses. The convergence of the MCMC procedure was assessed from the average standard deviation of split frequencies (<0.01) and effective sample size values (>200) using MrBayes and Tracer v.1.7.1 (Rambaut et al. 2018), respectively. The first 25% of the trees were discarded as burn-in, and the remaining trees were used to calculate the 50% majority-rule trees and to determine the posterior probabilities (PP) for individual branches.

The newly obtained sequences were submitted to the DNA Data Bank of Japan (DDBJ). The refined alignment of the mycobiont ITS sequences was submitted to TreeBASE (<http://www.treebase.org>; accession number: 28437). Generated trees were visualized in FigTree v.1.4.4 (<https://github.com/rambaut/figtree/>) and edited in Inkscape v.1.0.1 (<https://inkscape.org>).

Results And Discussion

Morphological observation of *Cyphellostereum ushima*

The hymenium of *Cyphellostereum ushima* is whitish, resupinate, thin, and membranaceous, forming patches adjacent to the lichenized thallus covering the wood surface, especially on margins or underside of the substrate near the thallus (Fig. 1a, b). Basidia are short, about 10 µm in length, cylindrical to clavate, and produce four thin-walled hyaline basidiospores (Fig. 1j–l). These morphological features suggest an affinity with *Athelia* in Atheliales; the phylogenetic analyses, however, did not support such a placement, indicating its inclusion in the *Cyphellostereum* lineage in Agaricales (see below; Fig. 3). The lichenized thallus is composed of loosely intertwined, lichenized cyanobacterial filaments (*Rhizonema* trichomes) (Fig. 1c), and does not form a combined structure with hymenium as seen in *Dictyonema*. The thallus sometimes forms tufts of fibrils of densely arranged fungal-cyanobacterial filaments (Fig. 1d–f) as seen in *C. galapagoense* (Dal Forno et al. 2017). Each cyanobacterial filament is incompletely surrounded by the mycobiont hyphae (Fig. 1g–i) and different from that of *Dictyonema*, in which the trichome was completely surrounded by the jigsaw-shaped hyphae sheath. These thallus features are consistent with the conventional definition of *Cyphellostereum*. However, the mycobiont forms a central haustorium within the trichome, visible with light microscopy (Fig. 1h, i). TEM observation revealed that the surrounding mycobiont hyphae penetrated into the trichome, and longitudinally traversed trichome cells as tubular haustoria (Fig. 2f–i). Such haustoria are commonly seen in *Dictyonema*, but are unusual in *Cyphellostereum*, reported only in *C. galapagoense* so far (Dal Forno et al. 2017). Additionally, Vö (2016) reported the presence of tubular haustoria in three undescribed *Cyphellostereum* species; further sampling is needed to assess how widespread haustoria are in *Cyphellostereum*. TEM observation also showed that the haustoria were regularly constricted as they passed through the septa between trichome cells (Fig. 2a, b). The presence of the haustorium did not appear to have a significant effect on the division of trichome cells. Thylakoids were irregularly arranged in the photobiont cytoplasm, and electron-dense granules were scattered throughout (Fig. 2d). A barrel-shaped intercalary heterocyte with a thickened wall was observed in the trichome, and its cytoplasm was continuous with the adjacent normal cells through a septal pore (Fig. 2e). The heterocyte contained fewer thylakoids than the neighboring cells and the electron-dense granules were scattered. Intracellular haustoria were frequently observed in vegetative cells, but not in heterocytes. The external mycobiont hyphae occasionally penetrated trichome cells and were

constricted considerably in the cell wall of the trichome (haustorial neck) (Fig. 2f–i). The width of the haustoria neck was less than 1 μm , and although the cytoplasm of the external hyphae and intracellular haustoria was continuous, the wall of the hypha seemed to be discontinuous in the thin sections observed (Fig. 2g, i). Compared with the cell wall thickness of the external hyphae (about 150 nm), that of the intracellular haustoria was much thinner (about 50 nm).

TEM observations of *Cyphellostereum* are reported here for the first time. However, two structurally similar genera, *Cora* and *Dictyonema*, were previously observed with TEM (Roskin 1970; Oberwinkler 1980, 1984; Slocum 1980). The observation of the thallus of *C. ushima* was essentially consistent with these observations. On the other hand, the following characters could not be detected in this study: the dark staining material at the site of penetration observed in *D. irpicinum* (Slocum 1980), the multilayered scar of the haustorial mother cells, and the electron-dense ring around the haustorial neck observed in *D. sericeum* (Oberwinkler 1980). The wall of the tubular intracellular haustoria was thinner than that of the external hyphae, as observed in *D. irpicinum* (Slocum 1980), and consistent with the idea that the haustorium facilitates the transport of metabolites from photobiont to mycobiont. Unlike *Dictyonema*, which always forms tubular intracellular haustoria, there are many species of *Cyphellostereum* for which tubular intracellular haustoria have been reported as absent in light microscope observations. These need to be investigated in more detail with TEM in the future. In Ascomycota, several genera are known to lichenize *Rhizonema*, including *Coccocarpia* (Lücking et al. 2009) and *Erioderma* (Comejo et al. 2016) in Lecanoromycetes, and *Lichinodium* (Prieto et al. 2019) in Leotiomycetes. However, TEM observations of *Coccocarpia* (Arvidsson 1982) and *Lichinodium* (Prieto et al. 2019) showed that while mycobiont hyphae are in close contact with algal cells, the intracellular haustoria were not formed. Thus, at present, only basidiomycetes are known to form tubular intracellular haustoria within *Rhizonema* trichome cells.

Comparison between *C. ushima* and other known *Cyphellostereum* species

Cyphellostereum ushima is unusual in *Cyphellostereum* in that the fruiting body is resupinate and membranaceous, forming tubular haustoria, and is easily distinguished from other known *Cyphellostereum* species (Table 1). However, in recent years, some species other than *C. ushima* have been described with characteristics deviating from the conventional recognition of *Cyphellostereum*, although they are phylogenetically placed in the *Cyphellostereum* lineage, a phylogenetically well-supported monophyletic clade. For example, *C. galapagoense* was originally described as *Dictyonema* because the trichome was completely surrounded by the jigsaw-shaped hyphal sheath cells (Yáñez et al. 2012), but subsequent molecular analysis resulted in its assignment to the *Cyphellostereum* clade (Dal Forno et al. 2017). *Cyphellostereum galapagoense* is also unusual in that tubular haustoria are formed in the trichome cells (Dal Forno et al. 2017), as occurs in *C. ushima*. In addition, *C. georgianum* and *C. jamesianum* are unique in that clamp connections are observed in the vegetative hyphae (Dal Forno et al. 2019).

Isolation of *Cyphellostereum ushima*

Basidiospores discharged onto agar did not germinate at all after one month of incubation in the dark at 20°C. Some additional factors such as the presence of the photobiont may be needed for germination. On the other hand, regrowth of vegetative hyphae from the thallus fragments was observed on rare occasions (Fig. 1m), and an axenic mycobiont isolate was established by transferring the elongated mycelium to a new PDA medium (Fig. 1n). The identity of the mycobiont culture and its relationship to other taxa of *Cyphellostereum* were analyzed as detailed below.

Molecular analyses

The ML and Bayesian phylogenetic analyses were conducted using an aligned sequence dataset composed of 405 bp from the mycobiont ITS. The tree topology inferred by the Bayesian analysis was mostly identical to that of the ML tree, except for unsupported branches in the ML tree. The sequences of *C. ushima* formed a strongly supported monophyletic clade (100% ML BP/1.00 Bayesian PP) with the sequences of *C. unoquinum* (KY861495) and *C. phyllogenum* (KF443219), and they were located in a unique lineage within the clade (Fig. 3). These three species all lack clamp connections in vegetative hyphae, and incompletely cover the cyanobacterial symbiont filaments. However, *C. phyllogenum* and *C. unoquinum* differ from *C. ushima* in that they lack tubular internal haustoria. *Cyphellostereum galapagoense*, the only other species of *Cyphellostereum* known to have tubular internal haustoria (Dal Forno et al. 2017), is in a distant lineage from *C. ushima*; our phylogenetic analysis does not support genetic relatedness between the two.

The BLAST search of the sequence of 16S rDNA (GenBank accession no. LC597370) derived from the lichenized thallus of *C. ushima* indicated that the photobiont is *Rhizonema* species. Similarly, we tried to get the *rbcLX* sequence from the thallus, but *rbcLX* could not be amplified despite multiple attempts. The primers for CX and/or CW are probably not compatible with the photobiont genome. Referring to figure S9 of Dal Forno et al. (2021) on the delimitation of *Rhizonema* species based on the sequences of 16S rDNA and *rbcLX*, the 16S rDNA sequences obtained agreed with those specific to *R. interruptum* in 6 out of 7 diagnostic positions. Therefore, the photobiont of *C. ushima* is closely related to *R. interruptum*, but the *rbcLX* sequence will be needed for more precise identification.

Similarities between *C. ushima* and some lichenized *Athelia* species

In addition to the lichenized monophyletic group including such as *Cyphellostereum* and *Dictyonema* in Hygrophoraceae, three *Athelia* species (*A. andina*, *A. phycophila*, and *A. poeltii*) are also known to be lichenized with filamentous cyanobacteria (Jülich 1972, 1978) (Table 1). However, none of these lichenized *Athelia* species has been reliably reported since their original description, and all lack DNA information. The descriptions of these species do not include detailed information on the lichenized thallus, except for the sketch and observations of *A. andina* by Oberwinkler (1970, 2012), and thus they remain poorly known taxa even as lichens. Since all basidiomata of lichenized *Athelia* species are whitish, resupinate, and membranaceous, they have never been compared with *Cyphellostereum* species, whose basidiomata have been recognized as cyphelloid. The basidioma of *C. ushima* has many similarities with the above-mentioned lichenized *Athelia* species, especially with those of *A. phycophila*, but *C. ushima* is morphologically distinguished by its shorter basidia and ellipsoid to slightly elongate basidiospores (vs pyriform in *A. phycophila*). The basidioma of *Eonema pyriforme*, a non-lichenized sister lineage of *Cyphellostereum*, is whitish and resupinate like that of *Athelia*, and, in fact, used to be treated as *Athelia pyriformis* (Jülich 1972) until phylogenetic analysis

placed it in Hygrophoraceae (Lawrey et al. 2009). The fact that the basidioma of *C. ushima* is similar to those of lichenized *Athelia* species suggests that the latter may actually be part of the *Cyphellostereum* lineage. Their phylogenetic affiliations will have to be verified by examining type specimens, re-collecting in the type localities, and further molecular work.

Oberwinkler (1970) reported that both *C. pusiolum* and *A. andina* are associated not only with filamentous cyanobacteria but also with unicellular green algae to form a *Botrydina*-type thallus. Although he did not elaborate on the *Botrydina*-type thallus, his subsequent reviews of basidiolichens retain the view that the mycelia of both species form distinct thalli with filamentous cyanobacteria and unicellular green algae simultaneously (Oberwinkler 2012). On the other hand, Jülich (1972), in his official description of *A. andina*, refers to lichenization with cyanobacteria (as *Scytonema*), but does not mention green algae. Our observations of the thallus of *C. ushima* showed that the mycelium lichenized only *Rhizonema* trichomes; no interaction with other algae was observed. While dual relationships with both green algae and cyanobacteria are well-documented in certain ascolichens (particularly in Peltigerales), similar reports in basidiolichens need further study.

Taxonomy

Here we describe *Cyphellostereum ushima* with data on both the matured basidioma and the lichenized thallus. The circumscription of *Cyphellostereum* is emended as follows to encompass the features of other recently described species mentioned above.

Cyphellostereum D.A. Reid, Beih. Nova Hedwigia 18: 336 (1965), emend. H. Masumoto & Y. Degawa

Emended diagnosis: Basidiomata terrestrial, saxicolous, muscicolous, or lignicolous, whitish, sessile (resupinate) or stipitate (fan-shaped), separated from the thallus or only at the base connected to the thallus. Hyphae thin-walled, with or without clamp connections, monomitic. Cystidia absent. Basidia clavate to cylindrical, with 4 sterigmata and a simple basal septum. Basidiospores subglobose to ellipsoid, smooth, thin-walled, non-amyloid. Thallus blue-green to greenish, crustose, composed of cyanobacterial filaments, without haustoria or with tubular intracellular haustoria. Photobiont *Rhizonema*, cells cylindrical, uniseriate, with cylindrical to rounded intercalary heterocytes, occasionally forming false branches. Hyphal sheath around cyanobacterial filaments composed of irregular hyphae leaving interspaces, or composed of jigsaw-puzzle-shaped paraplectenchymatous fungal cells completely ensheathing the photobiont trichome.

Cyphellostereum ushima H. Masumoto & Y. Degawa, sp. nov. (Figs. 1 and 2)

Mycobank no.: MB XXXXX.

Holotype: JAPAN. Kagoshima, Amami-Oshima, Setouchi, Amurogama, on tree trunks of *Cryptomeria japonica*, elevation 15 m, 6 Mar 2020, H. Masumoto 364 (OSA-MY-9529, ex-type culture: NBRC 115003).

Etymology: From the island name of the type locality (Amami-Oshima) in Amami language (Ushima).

Description: Thallus epiphytic on tree trunks (*Cryptomeria japonica*), undifferentiated, membranous, developing a mat of loosely interwoven fungal-cyanobacterial fibrils, fragile, green to bluish-green. Thallus in section up to 80 µm thick; occasionally with tufts of fibrils up to 300 µm long from base, formed by densely arranged fungal-cyanobacterial filaments. Thallus composed of cyanobacterial filaments (*Rhizonema* trichomes) incompletely surrounded by hyaline hyphae (fungal sheath), 2.7–3.5 µm diam; trichome cells 5.5–8.6 µm wide × 3.3–7.3 µm high, green to bluish-green, uniseriate, occasionally false branched; trichome cells penetrated by tubular fungal hyphae (haustoria) of 2.5–3.4 µm diam; heterocytes sparse, pale yellow, 5.5–10.0 µm wide and 4.0–10 µm high, cylindrical to broadly ellipsoid; clamp connections not observed. Basidiomata formed on margins or underside of the substrate near the thallus, resupinate, effuse, very thin, arachnoid, whitish, free of cyanobacterial filament; hymenophore in section up to 40 µm thick. Hyphal system monomitic, consisting of branched, hyaline generative hyphae, smooth, thin-walled, 3–4(–5) µm wide lacking clamp connections. Cystidia absent. Basidia (8.6–)9.4–13.0(–15.4) × (4.9–)5.4–6.5(–7.1) µm, 11.19 ± 1.83 × 5.94 ± 0.58 µm on average with standard deviation (std) (n = 24), 4-spored, clavate or short-cylindrical, thin-walled, hyaline, with a simple basal septum; sterigmata 2.2–3.6 µm. Basidiospores (4.6–)5.3–6.3(–6.9) × (3.0–)3.4–4.0(–4.3) µm, 5.76 ± 0.50 × 3.69 ± 0.31 µm on average with std (n = 50), Q = (1.3–)1.4–1.6(–1.8), 1.57 ± 0.14 on average with std (n = 50), ellipsoid to slightly elongate, hyaline, smooth, thin-walled, non-amyloid. Colonies on PDA slow-growing, reaching 4.0–4.5 mm in diam after 2 months at 20 °C in the dark, three-dimensional, aggregated, pale pink to pale-brown, the mycelium hardly extending into the agar.

Distribution: Currently found only in the type locality (Amami-Oshima, Kagoshima, Japan). This is the first report of the genus *Cyphellostereum* in Japan.

Gene sequences of the holotype: LC597364 (ITS1-5.8S-ITS2), LC597367 (28S rDNA)

Gene sequences of the ex-type culture: LC597366 (ITS1-5.8S-ITS2), LC597369 (28S rDNA)

Japanese name: *Taorugoke* (*Taoru*, meaning a towel, refers to the loosely interwoven texture of the thallus; *-goke*, a suffix often used to mean a lichen).

Declarations

Ethics declarations

Ethics approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no competing interests.

Availability of data and materials

The holotype specimen of *Cyphellostereum ushima* described in this study was deposited in the herbarium of the Osaka Museum of Natural History (OSA; Osaka, Japan). The ex-type living culture was deposited in the NITE Biological Resource Center (NBRC; Kisarazu, Chiba, Japan). All sequence data generated in this study are available at GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). The alignment file can be accessed via TreeBASE (<http://www.treebase.org>).

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Authors' contributions

All authors contributed to the study conception and design. Isolation and identification of *Cyphellostereum ushima* were completed by HM. Morphological characteristics were measured and documented by HM. Molecular and phylogenetic analyses were completed by HM. Culture deposition and sequence submission were completed by HM. The first draft of the manuscript was written by HM and all authors commented on previous versions of the manuscript. This study was supervised by YD. All authors read and approved the final manuscript.

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Figures

Figure 1

Cyphellostereum ushima (OSA-MY-9529, holotype). a, b Resupinate basidiomata (white arrows) and fibrous lichenized thallus. Note that the basidiomata are formed on the edge or underside of the wood. c Higher magnification of the lichenized thallus. Note that the intricate interweaving of cyanobacterial filaments. d Fibrils condensed. e Arrangement of the fibrils. f Squashed fibrils of e. g Mycobiont hyphae (black arrowheads) incompletely surrounding the *Rhizonema* trichome. Note that the sparse heterocytes in the trichome (double arrowheads) and the double false branch of the trichome (black arrow). h, i Higher magnification of the trichome. Note the tubular intracellular haustoria (white arrowheads) inside the trichome. j Basidium mounted in 3% KOH and stained with 1% phloxine B solution. k, l Basidiospores. m Mycelium extending from the thallus fragment. n Aposymbiotic culture of mycobiont (NBRC 115003, ex-type) grown on PDA for two months at 20 °C. Scale bars: a, n = 1 mm; b = 500 µm; d = 300 µm; c = 100 µm; e, f = 50 µm; g, m = 20 µm; h–j = 10 µm; k, l = 5 µm.

Figure 2

TEM photographs of the lichenized thallus of *Cyphellostereum ushima* (OSA-MY-9529, holotype). a Longitudinal section of *Rhizonema* trichome. Note that the tubular intracellular haustoria are regularly constricted at the septa of the trichome cells. b Higher magnification of the longitudinal section in a. c Transverse section of *Rhizonema* trichome. Note the presence of intracellular haustoria in all trichome cells. d Higher magnification of the transverse section in c. e Heterocyte. Note that a distinct greyish layer all around the heterocyte and the cytoplasm of the heterocyte is continuous with the neighboring normal cell via a septal pore. f Longitudinal section of intracellular haustoria with external hyphae invading the trichome. g Higher magnification of the longitudinal section in f. Note continuity of cytoplasm of external hyphae and intracellular haustoria. h Transverse section of intracellular haustoria and external hyphae invading the trichome. i Higher magnification of the transverse section in g. Note that the wall of the external hyphae and the wall of the intracellular haustoria do not appear to be continuous in this section. al = an epiphytic algal cell on the thallus, h = mycobiont hyphae, ih = intracellular haustoria, sh = sheath of the photobiont, tc = trichome cells. Scale bars: a, c = 5 µm; f = 3 µm; b, d, e, h = 2 µm; g, i = 1 µm