

Discovery of the first lichenized fungus in the family *Chaetothyriaceae* (Ascomycota), *Ceramothyrium ryukyuense* sp. nov.

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Abstract. *Ceramothyrium ryukyuense* (*Chaetothyriaceae*) is described as a new species from Okinawa, southern Japan. It is characterized by subglobose minute ascomata (up to 110 μm diam.) covered with a brown mycelial pellicle, small ellipsoid 1(–2)-septate ascospores (8.7–13.8 \times 3.4–4.9 μm) within a small ascus (20–30 \times 13–17 μm), and a lichenized thallus. This species resembles non-lichenized Brazilian species, *C. paiveae* and *C. philodendri*, in producing minute ascomata and 1–4 or 7 septate ascospores. However, besides its lichenized status, *C. ryukyuense* is distinguished by its smaller asci (up to 30 μm long in *C. ryukyuense* vs. 30–42 μm long in *C. paiveae*, and 50–100 μm long in *C. philodendri*), and predominantly 1-septate ascospores in *C. ryukyuense*, whereas multi-septate in *C. paiveae* and *C. philodendri*. It was collected on a living leaf of *Arecaceae* in the subtropical forest near the seashore. In a phylogenetic tree based on nuITS and nuLSU sequences, *C. ryukyuense* formed a sister clade to *Ceramothyrium exiguum* which is known as an anamorphic species. DNA sequences of *C. paiveae* and *C. philodendri*, morphologically similar species to *C. ryukyuense*, were not available in this study. Algal cells distant from the perithecium exhibited continuous branching, while those near the perithecium were strongly deformed into a spherical shape and were partially unicellular. The photobiont of *C. ryukyuense* is suggested to be a species of *Trentepohliales*, inferred from a phylogenetic analysis based on the *rbcl* sequence. *Ceramothyrium ryukyuense* is the first report of a lichenized lineage within *Chaetothyriaceae*.

Key words: *Arecaceae*, Asia, *Chaetothyriales*, foliicolous lichen, lichenization, subtropics, symbiosis, *Trentepohliales*

Introduction

Mycologists sometimes describe fungal taxa without noticing their lichenization. For example, *Dictyocatenu-lata alba* (Morris & Finley 1967) which was recognized as a non-lichenized fungus until Diederich et al. (2008) showed that it was the same species with a lichen species, *Cheiromycina ananas*. An example was also documented in foliicolous lichenized fungi, such as *Pazschkea chusqueae* (non-lichenized) and *Belonidium fuscohya-linum* (non-lichenized), which were synonymized with *Aulaxina quadrangula* (lichenized) by Santesson (1952).

It seems that the lack of recognition of a lichenized state may have resulted from overlooking the presence of undeveloped lichen thalli.

Fungi with uncertain lichenization have attracted considerable interest among researchers. A notable example is *Stictis*, which exhibits “optional lichenization”, functioning either as a lichenized or non-lichenized fungus depending on the habitat (Wedin et al. 2004). Additionally, species like *Collemopsidium pelvetiae* and *Mastodia tessellata* are referred to as “borderline lichens” as they only form an indistinct lichen thallus (Kohlmeyer et al. 2004; Pérez-Ortega et al. 2016). Moreover, there is an ongoing debate regarding whether they are lichenized or not, such as *Semigyalecta paradoxa*, (Santesson 1952; Kalb & Vězda 1994; Thor et al. 2000; Wang & Wei 2018; Miyazawa et al. 2022).

During our study of foliicolous lichens in Japan, an interesting material was collected from Okinawa Island, southern Japan. At first, this specimen could not be morphologically identified into any known genera

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of lichenized fungi. A BLAST search of the sequence obtained from the sample suggested that it might belong to the *Chaetothyriaceae* in the *Chaetothyriales*. Although lichenized lineages within the *Chaetothyriales* are known (i.e., *Lyromma*, *Microtheliopsis* and *Pyrenothrix*: Lücking 2008), the morphology of the collected material did not fit with those genera. *Lyromma* differs in having barrel-shaped perithecia with a slightly constricted base and conidiomata resembling perithecia. *Microtheliopsis* is distinguished from our fungus by the lens-shaped perithecia with a spreading base and greyish-brown ascospores. *Pyrenothrix* is distinguished by the appressed filamentous thallus with a cyanobacterial photobiont (*Scytonema*). Furthermore, as all taxa within *Chaetothyriaceae* are known to be non-lichenized fungi (Marasinghe et al. 2023), the occurrence of a lichenized fungus in the family would be an unusual matter and should be carefully investigated to confirm whether our material is truly lichenized.

The aim of this study is to determine the taxonomic position of the foliicolous material by conducting anatomical observations and molecular phylogenetic analyses, as well as examining a coexisting alga, and to discuss the lichenization of the fungus.

Materials and methods

Morphology and chemistry

Morphological observations and photography were performed using a dissecting microscope (SZX16; Olympus, Tokyo, Japan) and a differential interference contrast

microscope (BX51; Olympus) equipped with a digital camera (EOS Kiss X10i; Canon, Tokyo, Japan). Anatomical examinations were performed using hand-cut sections mounted in GAW solution (glycerin: ethanol: water = 1: 1: 1) (Asahina 1936).

The amyloidity of ascus and hamathecium was examined using Lugol's solution (I) or using I after pre-treatment with 5% KOH solution (K/I). Secondary substances were analyzed using high-performance thin layer chromatography (HPTLC) following Schumm & Elix (2015). The solvent system B' (*n*-hexane: methyl *tert*-butyl ether: formic acid, 140: 72: 18) (Culberson & Johnson 1982) was used for HPTLC. The spot colors were checked under 254 and 366 nm wavelengths 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.

DNA extraction, PCR amplification and sequencing

DNA was extracted separately from an ascoma and the mycelium with the algal cells, using a modified method based on Izumitsu et al. (2012) (see also Miyazawa et al. 2022). The voucher specimen for DNA extraction is housed in the herbarium of the National Museum of Nature and Science (TNS), Tsukuba, Japan.

For PCR amplification, 10 µL of PCR mix contained 1 µL of genomic DNA extraction, 0.25 µL of each primer (10 pmol/µL) and 5 µL EmeraldAmp® MAX PCR Master Mix (TaKaRa Bio Inc.). For fungal DNA, the partial sequences of the ITS1-5.8S-ITS2 (nuITS), the large subunit of the nuclear ribosomal RNA gene (nuLSU), and the

Table 1. Strains/vouchers of *Chaetothyriaceae* and their GenBank accession numbers. New sequences obtained in this study are in bold.

Taxon	Strain/Voucher	nuITS	nuLSU	Reference
<i>Aphanophora eugeniae</i>	CBS 124105	FJ839617	FJ839652	Crous et al. 2009
<i>Arthrospiala arthrospora</i>	COAD 658	KY173473	KX447143	Crous et al. 2016
<i>Bryce Kendrickomyces acaciae</i>	CBS 124104	FJ839606	FJ839641	Crous et al. 2009
<i>Campthophora hylomeconis</i>	CBS 113311	EU035415	EU035415	Crous et al. 2007
<i>C. schimae</i>	IFRDCC 2664	MF285231	MF285233	Yang et al. 2018
<i>Ceramothyrium aquaticum</i>	VTCCF-1210	LC360299	LC360296	Yen et al. 2018
<i>C. carniolicum</i>	CBS 175.95	KC455237	KC455251	Réblová et al. 2013
<i>C. chiangraiese</i>	MFLUCC 18-1354	MN481190	MN449441	Wijesinghe et al. 2019
<i>C. exiguum</i>	VTCCF-1209	LC360297	LC360295	Yen et al. 2018
<i>C. ficus</i>	MFLUCC 15-0228	KT588601	KT588599	Hongsanan et al. 2015
<i>C. linnacea</i>	CBS 742.94	MH862502	MH874144	Vu et al. 2019
<i>C. longivolcaniforme</i>	MFLUCC 16-1306	KP324929	KP324931	Zeng et al. 2016
<i>C. phuquocense</i>	VTCCF-1206	LC360298	LC360294	Yen et al. 2018
<i>C. podocarp</i>	CPC 19826	KC005773	KC005795	Crous et al. 2012
<i>C. ryukyense</i>	KeM1225 (ascoma)	LC844104	LC844106	This study
<i>C. ryukyense</i>	KeM1225 (mycelium of thallus)	LC844105	LC844107	This study
<i>C. thailandicum</i>	MFLUCC 10-0008	HQ895838	HQ895835	Chomnunti et al. 2012
<i>Chaetothyrium agathis</i>	MFLUCC 12-0113	KP744437	KP744480	Liu et al. 2015
<i>Exophiala eucalyptorum</i>	CBS 121638	KC455245	KC455258	Réblová et al. 2013
<i>Fumagopsis stellae</i>	CBS 145078	MK047447	MK047497	Crous et al. 2018a
<i>Hermetothecium mikaniae-micranthae</i>	VIC 47212	MN537723	MN537725	Crous et al. 2019
<i>Longihyalospora ampeli</i>	MFLU 19-0824	MN219716	MN238771	Tennakoon et al. 2019
<i>Nullicomyces eucalypti</i>	CPC 32942	MH327807	MH327843	Crous et al. 2018b
<i>Phaeosaccardinula coffeicola</i>	SQUCC 12167	MH345730	MH345729	Maharachchikumbura et al. 2018
<i>P. dendrocalami</i>	IFRDCC 2649	KF667242	KF667246	Yang et al. 2014
<i>P. ficus</i>	MFLU(CC)10-0009	HQ895840	HQ895837	Chomnunti et al. 2012
<i>Vonarxia vagans</i>	CBS 123533	FJ839636	FJ839672	Crous et al. 2009

small subunit of the mitochondrial ribosomal RNA gene (mtSSU) were amplified with the primer sets ITS1F (Gardes & Bruns 1993) and LR1 (Vilgalys & Hester 1990) for nuITS, LIC24R (Miadlikowska & Lutzoni 2000) and LR7 (Vilgalys & Hester 1990) for nuLSU, and mrSSU1 and mrSSU3R (Zoller et al. 1999) for mtSSU. For alga DNA, the partial ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcl*) was amplified with primer set a-ch-*rbcl*-203'-MPN and a-ch-*rbcl*-991-3'-MPN (Nelsen et al. 2011). The PCR conditions followed Ohmura et al. (2020) for nuITS, were modified from Frisch et al. (2014) (45 cycles to 35 cycles) for nuLSU, followed Wang et al. (2020) for mtSSU, and were modified from Nelsen et al. (2011) (45 cycles to 35 cycles) for *rbcl*. Amplifications were carried out using an Applied Biosystems Veriti® 96-Well Thermal Cycler (Thermo Fisher Scientific). The PCR products were purified using an illustra™ ExoProStar™ (GE HealthCare). For purification, 1.2 µL of PCR product was mixed with 0.2 µL illustra™ ExoProStar™

and 0.5 µL dH₂O, then incubated at 37°C for 30 minutes followed by 80°C for 15 minutes.

DNA sequencing was performed on the Applied Biosystems™ 3500xL Genetic Analyzer (Thermo Fisher Scientific) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The taxon name and GenBank accession numbers for the obtained sequences are shown in Table 1 and Table 2.

Molecular phylogenetic analysis

The newly obtained fungal nuITS and nuLSU sequences from an ascoma and a mycelium of our material were aligned with sequences of selected taxa from GenBank (Table 1) using MAFFT ver. 7 (Katoh et al. 2019) employing the default settings. The mtSSU sequences (LC844108 from an ascoma and LC844109 from the mycelium) were not used in this phylogenetic analysis due to the lack of DNA sequencing data for related fungi. Taxa for

Table 2. Vouchers/isolations of *Trentepohliales*, their origins and GenBank accession numbers. A new sequence obtained in this study is in bold.

Algal taxon	Voucher/isolation	Origin of alga	Country and region	<i>rbcl</i> -accession No.	Reference
<i>Printzina cf. bosseae</i>	UNA00068465	Bark of <i>Cryptomeria</i> tree	Portugal: Azores Islands, Sao Jorge	FJ534623	Rindi et al. 2009
<i>Trentepohlia cf. annulata</i>	UNA00068475	Metal post	French Guiana: Montagne de Kaw, Fourgassie waterfalls	FJ534610	Rindi et al. 2009
<i>Trentepohlia cf. jolithus</i>	UNA00068471	Metal pillar of bridge	French Guiana: Sinnamary	FJ534602	Rindi et al. 2009
<i>Trentepohlia cf. umbrina</i>	UNA00068476	Concrete wall	French Guiana: Sinnamary	FJ534612	Rindi et al. 2009
<i>Trentepohlia</i> sp.	DS37	<i>Porina</i> sp.	Costa Rica	KC469202	Unpublished
<i>Trentepohliaceae</i> sp.	Borgato 50 (BR)	<i>Enterographa zonata</i>	Belgium: Bohan	OL956825	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 14043 (BR)	<i>Enterographa pitardii</i>	Spain: Canary Islands, Tenerife, Chamorga	OL956887	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 15684 (BR)	<i>Mazosia carnea</i>	Guadeloupe: French Antilles, Les Saintes	OL956891	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 15686 (BR)	<i>Mazosia carnea</i>	Guadeloupe: French Antilles, Les Saintes	OL956892	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 17242 (BR)	<i>Gorgadesia mira</i>	Cape Verde: Green Cap, Sao Vincente	OL956900	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 18403 (BR)	<i>Dichosporidium nigrocinctum</i>	Martinique: Fort-de-France	OL956906	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 18500 (BR)	<i>Enterographa cf. quassicola</i>	Martinique: French Antilles, Trinite	OL956907	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 18584 (BR)	<i>Dichosporidium nigrocinctum</i>	Martinique: French Antilles, Sainte-Luce	OL956909	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Borgato 100 (BR)	<i>Enterographa crassa</i>	France: La Capelle-les-Boulogne	OL956854	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Borgato 15 (BR)	<i>Opegrapha vermicellifera</i>	Belgium: Montaigne	OL956932	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Borgato 90 (BR)	<i>Enterographa crassa</i>	France: La Capelle-les-Boulogne	OL956846	Borgato et al. 2022
<i>Trentepohliaceae</i> sp.	Ertz 17550 (BR)	<i>Opegrapha vulgata</i>	France: Carnac	OL956905	Borgato et al. 2022
<i>Trentepohliales</i> sp.	Lücking s.n. (F)/68	<i>Porina imitatrix</i>	Panama	GU549455	Nelsen et al. 2011
<i>Trentepohliales</i> sp.	Lücking s.n. (F)/36	<i>Porina</i> aff. <i>farinosa</i>	Panama	GU549444	Nelsen et al. 2011
<i>Trentepohliales</i> sp.	Lücking s.n. (F)/5B	<i>Porina dolichophora</i>	Costa Rica	GU549453	Nelsen et al. 2011
<i>Trentepohliales</i> sp.	Lücking s.n. (F)/7B	<i>Porina</i> aff. <i>dolichophora</i>	Costa Rica	GU549458	Nelsen et al. 2011
<i>Trentepohliales</i> sp.	Lücking s.n. (F)/70	<i>Porina nucula</i>	Panama	GU549457	Nelsen et al. 2011
<i>Trentepohliales</i> sp.	Lumbsch 19815a (F)/89B	<i>Dichosporidium boschianum</i>	Fiji	GU549461	Nelsen et al. 2011
<i>Trentepohliales</i> sp.	K. Miyazawa 1225	<i>Ceramothyrium ryukyuense</i>	Japan: Okinawa	LC844110	This study

alignment, including *Brycekendrickomyces acacia* as an outgroup (see Table 1), were chosen based on BLAST results and the previous phylogenetic studies (Zeng et al. 2016; Crous et al. 2019; Tennakoon et al. 2019; Wijesinghe et al. 2019). The nuITS and nuLSU datasets were aligned separately. After removing sites with gaps, missing, and ambiguous sites, the datasets were concatenated. The final alignment comprised 839 sites and was used for the molecular phylogenetic analyses.

The newly obtained *rbcl* sequence from the alga co-occurring with the fungus was aligned with sequences of selected taxa from GenBank (Table 1) using MAFFT ver. 7 (Katoh et al. 2019) under the default settings. Taxa for alignment, including *Trentepohliaceae* sp. (voucher Ertz 17242, GenBank accession number: OL956900) as an outgroup, were selected based on BLAST results and the previous phylogenetic study (Borgato et al. 2022) (see Table 2). After removing sites with gaps, missing, and ambiguous sites, the final alignment consisting of 593 sites was used for the molecular phylogenetic analyses.

The maximum likelihood (ML) phylogenetic trees were generated using the Tamura-Nei model (Tamura & Nei 1993) with gamma distribution and evolutionarily invariable ($G + I$) for the fungal datasets, and Tamura 3-parameter model (Tamura 1992) plus ($G + I$) for the algal datasets which were selected as the best fitting model based on the lowest Bayesian information criterion (BIC) score. Bootstrap values ($\geq 50\%$) from 1,000 replicates for ML and neighbor-joining (NJ) methods are shown on each branch (Figs 1 & 2). Branches with bootstrap values of $\geq 70\%$ in both analyses are indicated with bold black lines. All calculations were conducted in MEGA X (Kumar et al. 2018).

Results

Morphology and chemistry

Algal cells were well incorporated into the mycelial tissues to form a lichen thallus (Figs 3C–D, G). Contact between the hyphae and algal cells was observed, but no penetration of hyphal haustoria into the algal cells was observed (Fig. 3C). Interestingly, algal cells distant from the perithecium exhibited continuous branching, while those near the perithecium were strongly deformed into a spherical shape and were partially unicellular (Fig. 3G). The details of the morphology and chemical characteristics of our material are described in the Taxonomic Treatments section.

Molecular phylogenetic analysis

The DNA sequences obtained from an ascoma and the mycelium were identical to each other across all aligned sites (nuITS: 625 sites; nuLSU: 1115 sites; mtSSU: 606 sites). The ML tree showing the position of our target fungus within the *Chaetothyriaceae* is presented in Fig. 1. The topology of our phylogenetic tree showed no fundamental conflict with those of Zeng et al. (2016), Crous et al. (2019, Supplementary material FP1017), Tennakoon et al. (2019), and Wijesinghe et al. (2019), except for the

placement of *Ceramothyrium thailandicum*. The molecular phylogenetic position of our fungus was inferred to be a sister clade to *Ceramothyrium exiguum* with moderate support values (NJ/ML = 81/81) (Fig. 1).

The phylogenetic tree reconstructed for the algal component of our material with the selected algal samples from GenBank was fundamentally consistent with that of Borgato et al. (2022). The alga of our material was inferred to be closely related to *Trentepohliaceae* sp. (voucher Ertz 18500, GenBank accession number: OL956907) (Fig. 2), which was isolated from a lichenized fungus of *Enterographa* cf. *quassiicola*. However, this relationship was weakly supported by low bootstrap values (NJ/ML = 60/60).

Discussion

Taxonomic position of the fungus

Our molecular phylogenetic analysis suggests that the fungus in our material is a sister to *Ceramothyrium exiguum*, a species of an anamorph isolated from fallen leaves in Vietnam (Yen et al. 2018) (Fig. 1). Although the genus *Ceramothyrium* is reported to be phylogenetically diverse, potentially leading to future taxonomic revisions (Wijesinghe et al. 2019), our material shares the typical morphological features of this genus. Specifically, these include a mycelial pellicle covering the ascoma without setae (except for *C. menglunense*), and an 8-spored bitunicate ascus containing hyaline pluriseptate ascospores (Batista & Maia 1956; Wijesinghe et al. 2019). Based on these morphological features and the molecular phylogenetic analysis, our material is appropriate to classify as a member of the genus *Ceramothyrium*.

Among the 42 described species of *Ceramothyrium* (Batista & Maia 1956; Batista & Ciferri 1962; Petrak 1962; Hughes 1976; Constantinescu et al. 1989; Barr 1993; Chomnunti et al. 2012; Crous et al. 2012; Hong-sanan et al. 2015; Hyde et al. 2016; Zeng et al. 2016; Yen et al. 2018; Crous et al. 2019; Tennakoon et al. 2019; Wijesinghe et al. 2019), none exhibit the same morphology as our material. Specifically, our material is distinguished by the minute ascomata (up to 100 μm diam.) and small ellipsoid 1(–2)-septate ascospores (8.7–13.8 \times 3.4–4.9 μm) within small asci (20–30 \times 13–17 μm). In addition, a lichenized thallus is characteristic for our material (see below section). Based on these results, we concluded that our material was an undescribed species in the genus, which is hereby described below as *Ceramothyrium ryukyuense*.

Lichenization

Algal cells were incorporated within the sheet-like tissue composed of mycelium (Figs 3C, G), with contacts between hyphae and algal cells (Fig. 3C). These contacts, observed under the optical microscope, appear to correspond to either “simple wall-to-wall apposition” or “type 1” intraparietal haustoria for various lichens with trebouxoid photobionts (Honegger 1986) and with trentepohlioid photobionts (Lambright & Tucker 1980; Meier

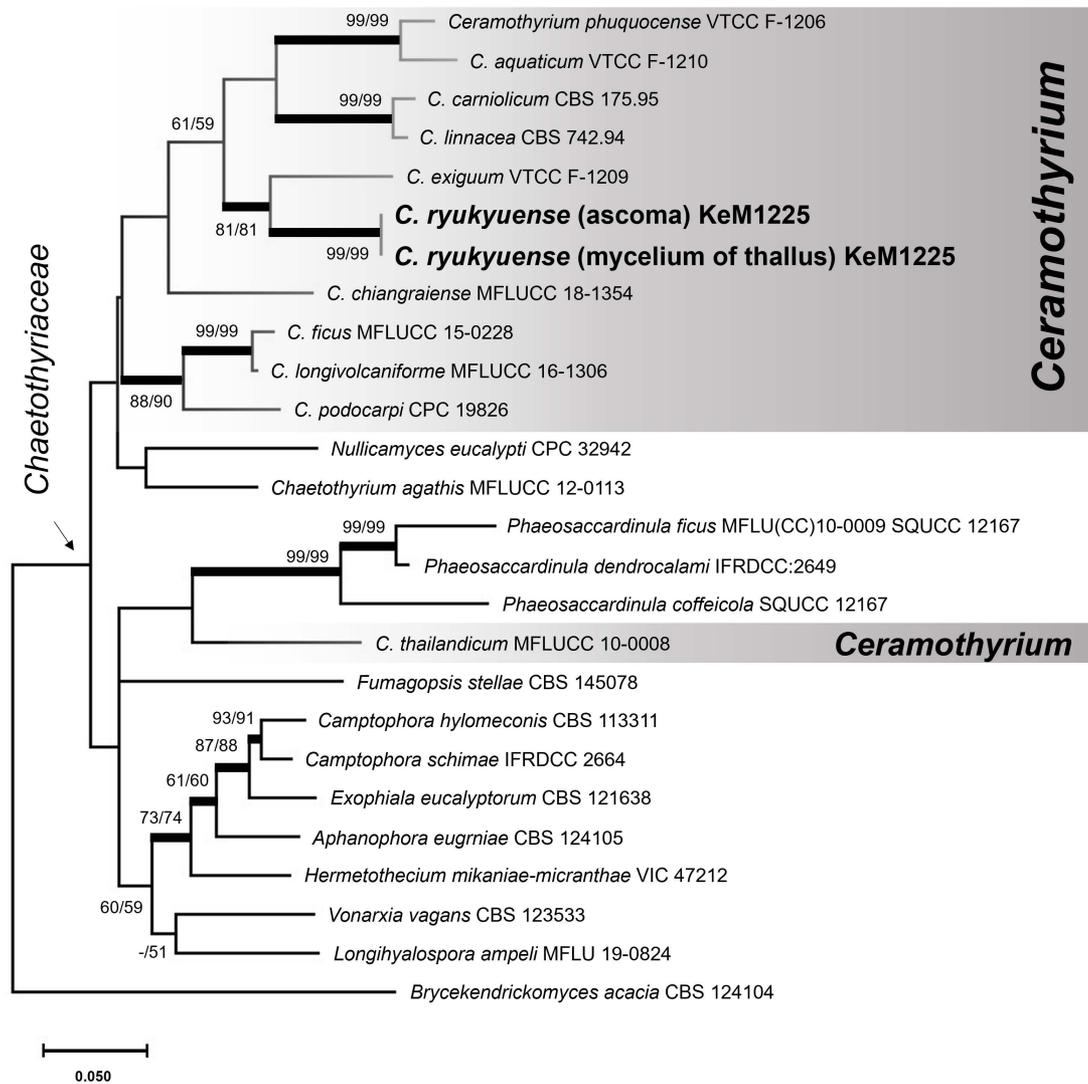


Figure 1. A ML tree showing the phylogenetic position of *Ceramothyrium ryukyuense* sp. nov. collected from Japan (in bold) in *Chaetothyriaceae*. *Bryce Kendrickomyces acacia* is used as an out group. NJ and ML support values are presented for each node. Branches highly supported (≥ 70) by both analyses are indicated with bold black lines.

& Chapman 1983; Matthews et al. 1989; Tucker et al. 1991; Sanders et al. 2023). At least, in our observations, there was no apparent penetration of fungal haustorium into the interior of algal cell. This observation implies that the fungus does not exhibit parasitic behavior against the alga. Furthermore, algal cells distant from the perithecium continuously branched, while cells near the perithecium were deformed into spherical shape, and some were unicellular (Fig. 3G). The deformation and unicellularity of filamentous trentepohlioid photobionts have been also reported in those of *Graphidaceae* and *Coenogonium* (Takeshita et al. 1999; Ohmura et al. 2016). Given that the cell contacts between hyphae and algal cells, as well as algal cell deformation and unicellularity were also observed in our sample as seen in the other trentepohlioid lichens, it can be concluded that *Ceramothyrium ryukyuense* is lichenized. Molecular phylogenetic analysis of the alga from *C. ryukyuense* indicated that it apparently belongs to *Trentepohliales* (Fig. 2). The sister sample (voucher Ertz 18500, GenBank accession number: OL956907) was originated to a lichen, *Enterographa* cf. *quassiicola* (Fig. 2, Table 2). The results are consistent

with our hypothesis that *C. ryukyuense* is a lichenized species.

There are no reports of other lichenized species within the genus *Ceramothyrium*. However, in the figures of papers on other *Ceramothyrium* species, we noticed the coexistence of multiple to numerous algal cells within the mycelial tissue: for example, *C. thailandicum* (Figs 3d, f in Zeng et al. 2016) and *C. chiangraiense* (Figs 2e, f in Wijesinghe et al. 2019). These observations, along with the fungus we describe here, suggest the presence of a potentially overlooked lichenized lineage within the *Chaetothyriaceae*. A well-developed stratified thallus cannot be confirmed in these fungi, but there is a possibility that they could represent a so-called “borderline lichen” (sensu Kohlmeyer et al. 2004; Pérez-Ortega et al. 2016). This possibility should be carefully examined through comprehensive morphological analysis focusing on the lichenized thallus with deformation and unicellularity of algal cells, similar to the observations made in our present study.

The family *Chaetothyriaceae* in the order *Chaetothyriales*, to which the genus *Ceramothyrium* belongs,

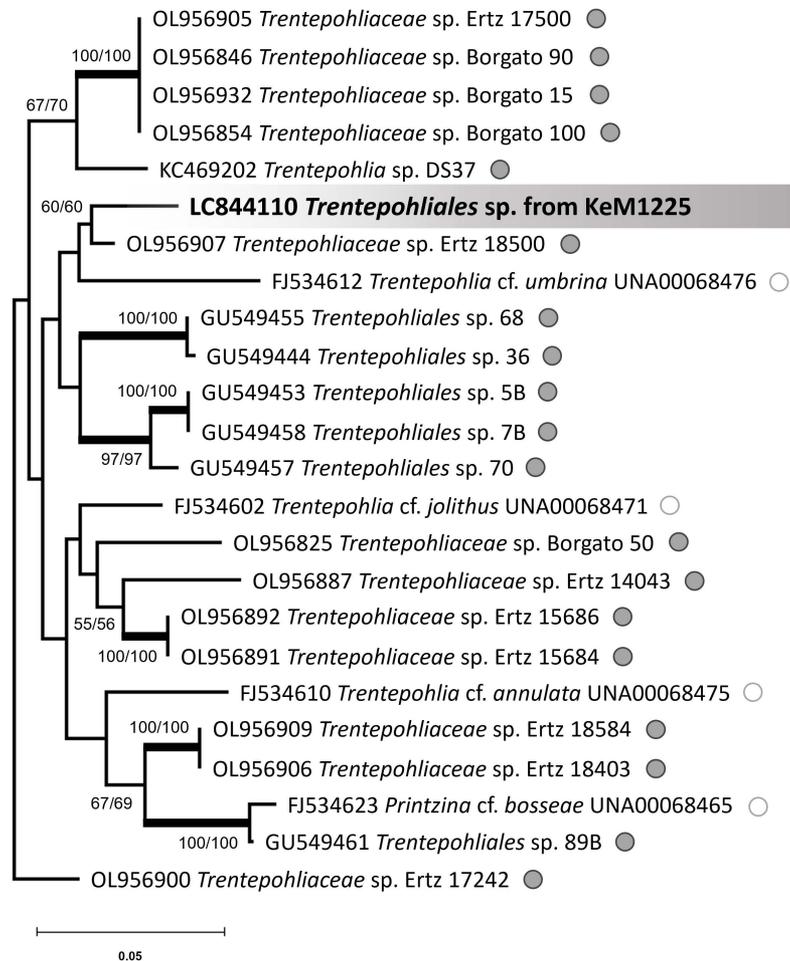


Figure 2. A ML tree showing the phylogenetic position of photobiont from *Ceramothyrium ryukyense* (in bold). *Trentepohliaceae* sp. Ertz 17242 is used as an out group. NJ and ML support values are presented for each node. Branches supported with high bootstrap NJ/ML values (≥ 70) are indicated with bold black lines. The grey circle next to label indicates that it was isolated from lichenized fungus, whereas the white circle indicates that it was isolated from bark or artifacts.

has been known as epiphytic or saprobic on leaves, branches, or stems, with no lichenized species known until this study (Marasinghe et al. 2023). Most taxa of *Chaetothyriales*, including *Chaetothyriaceae*, are recognized as non-lichenized fungi (Marasinghe et al. 2023). However, within the *Chaetothyriales*, most taxa in the families *Lyrommataceae*, *Microtheliopsisaceae*, and *Pyrenotrichaceae* are known as foliicolous lichenized fungi (Lücking 2008). These families are believed to have evolved from non-lichenized fungi that grew on living leaves (Lücking 2008; Lücking et al. 2017). This evolutionary background implies that the discovery of lichenized fungi in *Ceramothyrium* is not surprising. *Lyromma* species in *Lyrommataceae* differ in having barrel-shaped perithecia with slightly constricted bases and conidiomata resembling perithecia. *Microtheliopsis* species in *Microtheliopsisaceae* are distinguished from *Ceramothyrium ryukyense* by the lens-shaped perithecia with a spreading base and greyish-brown ascospores. *Pyrenothrix* species in *Pyrenotrichaceae* are characterized by the appressed filamentous thallus with a cyanobacterial photobiont (*Scytonema*). The DNA sequences of these three families have not yet been obtained, and the investigation of their phylogenetic relationship to *C. ryukyense* should be a focus of future research.

Ceramothyrium ryukyense shares some morphological similarities with the genus *Phylloblastia*, such as brownish hemispherical to subglobose perithecia with depressed apices, a well-developed paraplectenchymatous involucrellum, paraphysate K/I+ blue hamathecium, and transversely septate to submuriform, oblong to cylindrical ascospores (Lücking 2008). Despite these similarities, *Phylloblastia* is thought to belong to a different lichenized lineage of *Verrucariaceae* within *Verrucariales*. However, *C. ryukyense* is distinct from *Phylloblastia* due to its lack of abundant, well-developed periphyses. The DNA sequence of *Phylloblastia* has not been available to date, so it is impossible to directly compare genes at this time. Therefore, future studies are needed with more detailed examinations to better understand these relationships.

Taxonomy treatments

Ceramothyrium ryukyense K. Miyaz. & Y. Ohmura, sp. nov. (Fig. 3)

MycoBank MB 855861

Diagnosis: Similar to non-lichenized *C. paiveae* and *C. philodendri* which produce 1–4 or 7-septate ascospores, but distinguished by the smaller asci (20–30 × 13–17 μm vs. 30–42 × 13–25 μm in *C. paiveae*, and 50–100 × 20–27.5 μm

in *C. philodendri*), predominantly 1-septate ascospores (predominantly 3-septate in *C. paiveae* judging from figures in the protologue; up to 7-septate in *C. philodendri*), and the lichenized thallus.

Type: Japan, Ryukyu Islands (Okinawa Pref.): Horohoro-no-mori, Gushikami, Yaese-cho, Shimajiri-gun, Okinawa Island (26°07'N, 127°44'E), on leaf of *Arecaceae* sp., 60 m elev., 17 November 2022, K. Miyazawa 1225 (TNS – holotype: TNS-L-132773).

Description. Thallus continuous, greenish yellow, 10–15 µm thick. Ascomata perithecia, sessile, often depressed at the top, apical part papillose, pale yellow to yellowish brown, 80–110 µm diam., 60–130 µm tall; covered with thin brown paraplectenchymatous tissue, 10–15 µm thick; inner wall composed of prosoplectenchymatous tissue, 10–15 µm thick. Asci bitunicate, oval, 8-spored, I–, K/I–, 20–30 × 13–17 µm. Hamathecium aparaphysate, gel I–, K/I+ blue. Ascospores ellipsoidal, 1(–2)-septate, with slightly constriction at septa, colorless, (8.7–)10.4–12.9(–13.8) × (3.4–)3.7–4.4(–4.9) µm (n = 30), 1.8–3.5 times as long as broad. Conidiomata not observed. Photobiont *Trentepohliales*, fundamentally dichotomously branched, cells rectangular to Z-shaped

distant from perithecia, but subglobose around ascomata, (6.6–)7.0–10.7(–14.9) × (2.5–)3.2–5.2(–5.9) µm (n = 20).

Chemistry. No secondary substances were detected using HPTLC.

Etymology. The epithet '*ryukyuense*' refers to Ryukyu Islands in southern Japan where the new species was collected.

Habitat and distribution. *Ceramothyrium ryukyuense* was found on a living leaf of *Arecaceae* sp. (Fig. 3A), in a subtropical forest near the seashore. This species was so far only found at the type locality.

Notes. *Ceramothyrium ryukyuense* is characterized by a thin lichenized thallus incorporating a plate-like formation of *Trentepohliales* algae (Figs 3C, G), a brown mycelial pellicle covering the minute ascomata (up to 110 µm diam.) on the surface (Figs 3B–D), and small ellipsoid 1-septate ascospores (8.7–13.8 × 3.4–4.9 µm) within small asci (20–30 × 13–17 µm) (Fig. 3F).

Among the 42 species of *Ceramothyrium* described so far, *C. paiveae* and *C. philodendri* are also known to

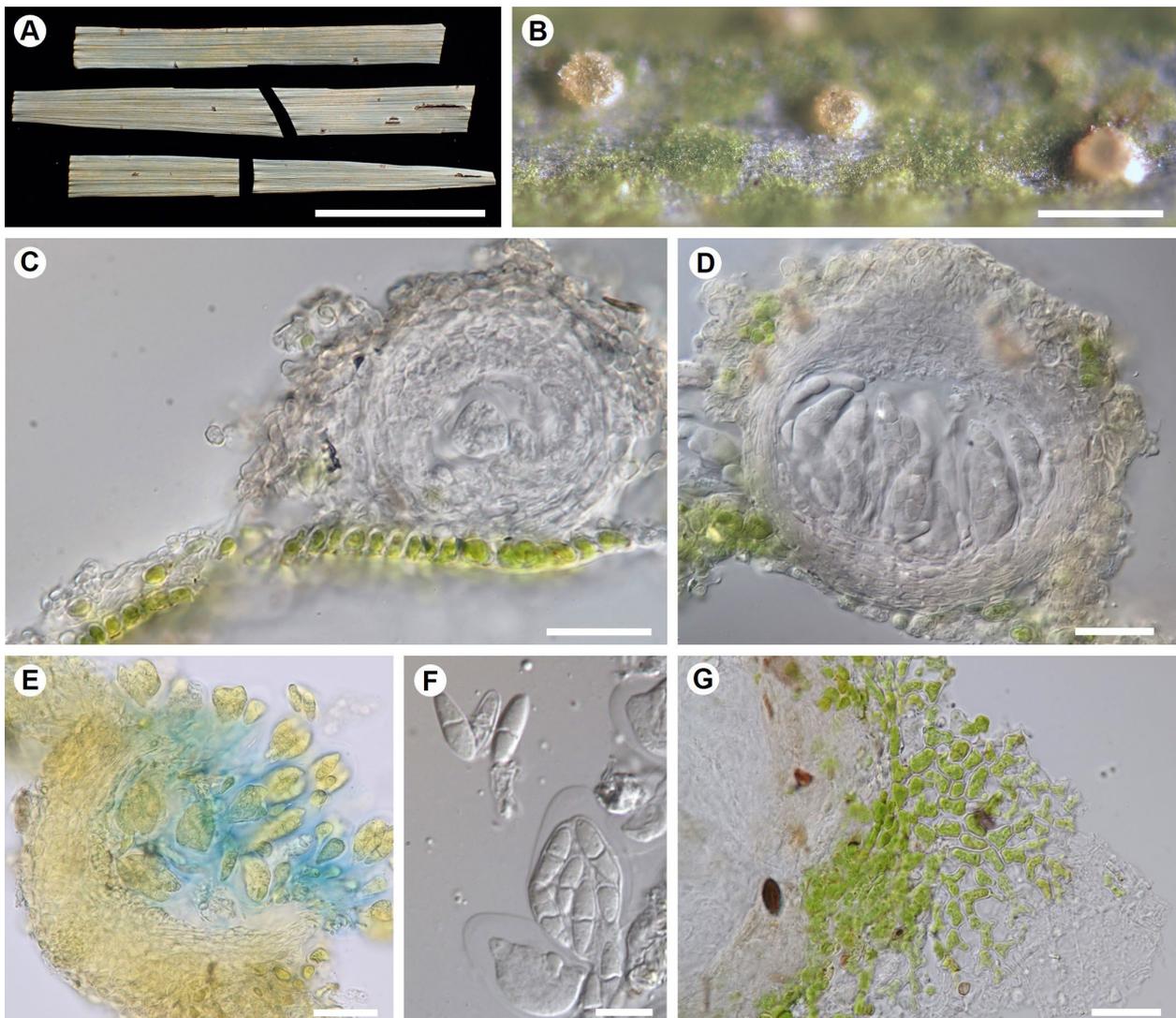


Figure 3. *Ceramothyrium ryukyuense* (holotype, TNS). A – thallus on leaf of *Arecaceae* sp.; B – ascomata on thallus; C – vertical section of immature ascoma and thallus; D – vertical section of mature ascoma; E – mashed perithecium stained with K/I; F – ascospores and asci; G – photobiont cells and mycelium near the ascoma. Scales: A = 5 cm, B = 200 µm, C–E & G = 20 µm, F = 10 µm.

produce 1-septate ascospores. *Ceramothyrium paiveae* is distinguished from *C. ryukyense* by its larger asci (30–42 µm long vs. 20–30 long in *C. ryukyense*) and predominantly 3-septate ascospores judging from figures in the protologue. In contrast, *C. philodendri* is distinguished by even larger asci (50–75 × 20–27.5 µm, up to 100 µm long) and larger ascospores (17.5–32.5 × 5–7.5 µm vs. 8.7–13.8 × 3.4–4.9 µm in *C. ryukyense*) with up to 7 septa (Batista & Maia 1956). *Ceramothyrium biseptatum* also resembles to *C. ryukyense* in possessing tiny apothecia (90–100 µm diam.) and 2-septate ascospores. However, *C. biseptatum* differs in having the larger asci (90–100 µm vs. 20–30 µm long in *C. ryukyense*) and larger ascospores (14–16 × 4.5–5.5 µm vs. up to 13.8 µm long in *C. ryukyense*). These similar species are also distinguished by their non-lichenized state, but detailed examination of their lichenized state should be carefully conducted in future research.

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