Racoleus japonicus sp. nov. (Teratosphaeriaceae, Ascomycota), a new sterile filamentous lichen collected from Japan

Kento MIYAZAWA1,*, Yoshihito OHMURA2,3

ABSTRACT: Racoleus japonicus is described as a new species. It is characterized by a blackish brown minutely filamentous thallus with lateral spines, vertical arrangement of hyphae with uneven and undulate to corrugated hyphal walls, and a Trentepohlia photobiont. In this genus, only R. trichophorus was known before the present study. Racoleus japonicus is distinguished from that species by having broader filaments (10–15 μm wide), larger hyphal cells (10–20 × 3–7 μm), and lateral sinuous spines. It was collected on shady rocks from northern to southern Japan at elevations between 290 and 1700 m. A molecular phylogeny based on nuSSU, nuLSU, and mtSSU inferred that I. tenuis is classified within Teratosphaeriaceae.

KEY WORDS: Capnodiales, Cystocoleus, lichenized fungi, mtSSU, nuLSU, nuSSU, Racodium, Trentepohlia.

INTRODUCTION

The genera Cystocoleus Thwaites, Racodium Fr. and Racoleus R. Sant. & D. Hawksw. are known as sterile filamentous lichens that are characterized by blackish brown fungal hyphae surrounding a filamentous green alga of the genus Trentepohlia. According to Hawksworth et al. (2011), each genus is characterized by the following morphology: Cystocoleus has a twisted arrangement of hyphae with a corrugated hyphal wall; Racodium has a vertical arrangement of hyphae with a straight hyphal wall; and Racoleus has a vertical arrangement of hyphae with a corrugated wall. Furthermore, by scanning electronic microscopy, a warded ornamentation on the surface of hyphal wall is observed only in Cystocoleus (vs. smooth surface in Racodium and Racoleus). Emphasizing the lateral spines as a feature of Racoleus by Hawksworth et al. (2011) will be discussed later in this paper. These genera were considered to belong to Capnodiales because of the similar appearance in morphology (Hawksworth et al., 2011). Abdollahzadeh et al. (2020) delimited Capnodiales s. str. and transferred Cystocoleus to Mycosphaerellales and Racodium to Racodiales, but those authors did not mention Racoleus.

Cystocoleus and Racodium, but not Racoleus, were analyzed with other related fungi based on nuSSU, nuLSU and mtSSU sequence data (Muggia et al., 2008) or by using nuLSU, ITS rDNA, TEF-1α, and RPB2 sequences (Abdollahzadeh et al., 2020). The molecular phylogenetic analyses of those authors suggested that the relationship between Cystocoleus and Racodium is not sister and that the lichenization occurred independently in each group within Capnodiales s. lat., a group of fungi in which other taxa are non-lichenized (i.e., saprophytes, parasites, ectophytes, or epiphytes; Abdollahzadeh et al., 2020). While the taxonomic position of Cystocoleus and Racodium has been discussed based on molecular phylogeny and morphological data, the position of Racoleus remains unclear due to a lack of DNA sequences.

While examining the Japanese sterile filamentous lichens housed in the herbarium of the National Museum of Nature and Science (TNS), Tsukuba, Japan, several specimens were recognized as an undescribed species of the genus Racoleus. The aim of this study is to describe and illustrate the new species, Racoleus japonicus, and to infer its phylogenetic position based on molecular data.

MATERIALS AND METHODS

Morphology and chemistry

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

Morphological observations and photography were performed using a dissecting microscope (SZX16; Olympus) and a differential interference contrast microscope (BX53; Olympus) with a digital camera (EOS Kiss X10i; Canon). Anatomical examinations were performed using hand-cut sections mounted in GAW (glycerin: ethanol: water = 1:1:1) solution (Ashina, 1936).

Color spot tests for K, C, KC, and Pd were performed according to Orange et al. (2001). Secondary substances were examined using high-performance thin layer
Table 1. Collections/strains and their GenBank accession numbers. New sequences obtained in this study are in bold.

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DNA extraction, PCR amplification and sequencing

DNA was extracted from fresh material collected within one year according to a modified method of Izumitsu et al. (2012) (see also Miyazawa et al., 2022) or a modified CTAB protocol (Hosaka, 2009). The voucher specimens for DNA extractions are housed in TNS.

For PCR 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® MAX PCR Master Mix (TaKaRa Bio Inc.). The partial sequences of the small subunit and the large subunit of the nuclear ribosomal RNA gene (nuSSU and nuLSU) and of the small subunit of the mitochondrial ribosomal RNA gene (mtSSU) were amplified with the primer sets nuSSU0021 (Gargas and DePriest, 1996) and nuSSU0852 (Gargas and Taylor, 1992) for nuSSU, LIC24R (Miadlikowska and Lutzoni, 2000) and LR7 or LR3 (Vilgalys and Hester, 1990) for nuLSU, and mrSSU1 and mrSSU3R (Zoller et al., 1999) for mtSSU, according to the modified PCR conditions of Frisch et al. (2014) (45 cycles to 35 cycles) using an Applied Biosystems Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific). The PCR products were purified with illumina™ ExoProStar™ (GE HealthCare). 1.2 μL of PCR products were incubated with 0.2 μL ExoProStar™ and 0.5 μL ddH2Oat 37°C for 30 minutes and then at 80°C for 15 minutes.

DNA sequencing was performed on an 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 the GenBank accession numbers for the obtained sequences are shown in Table 1.

Molecular phylogenetic analyses

The newly obtained nuSSU, nuLSU and mtSSU sequences of Racoleus japonicus from the Japanese material were aligned with sequences of selected taxa in

chromatography (HPTLC) following Schumm and Elix (2015). Solvent system 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 UV light and visible light, before and after spraying with 10% sulfuric acid on the HPTLC plate and charring at 90°C for 20 minutes.
RESULTS AND DISCUSSION

Molecular analysis

Among the aligned sites of each locus within the Japanese material of *Racoleus japonicus*, those of nuSSU (857 sites) had ten variable sites including singleton and/or parsimony-informative sites (98.9–100% identity among six specimens), those of nuLSU (1285 sites) had twelve variable sites and one gap site (99.4–99.9% identity among six specimens), and those of mtSSU (826 sites) had no variable sites or gaps (100% identity among three specimens).

Before conducting the phylogenetic analysis with combined sequences of three loci, each locus was separately analyzed. The phylogenetic tree based on nuSSU could not resolve the relationships among the families within Capnodiales *s. lat.*. However, each tree based on nuLSU or mtSSU shows supportable bootstrap values on some branches in the trees.

The concatenated phylogenetic tree based on three loci for *Racoleus japonicus* within Capnodiales *s. lat.* is

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Capnodiales *s. lat.* from GenBank (Table 1) in MAFFT ver. 7 (Katoh et al., 2019) using the default settings. Taxa included in the alignment, including *Myriangium duriae* (Myriangiales) as the outgroup (see Table 1), were selected according to the BLAST results in GenBank and previous phylogenetic studies (Muggia et al., 2008, 2016; Abdollahzadeh et al., 2020). Each single locus data set (nuSSU, nuLSU and mtSSU) was separately aligned. After removing sites with gaps, missing or ambiguous data, the initial molecular phylogenetic trees were reconstructed based on each single locus, then the data sets were concatenated. The final alignment of 1153 sites was used for the molecular phylogenetic analyses.

Neighbor-joining (NJ) and maximum likelihood (ML) analyses were performed using the Tamura-Nei model (Tamura and Nei, 1993) plus gamma distribution with invariant sites (G + I), which was selected as the best fitting model based on the lowest Bayesian information criterion score. The bootstrap values (≥50%) of 1,000 replicates for NJ and ML are shown on each branch. All calculations were conducted in MEGA X (Kumar et al., 2018).
shown in Fig. 1. The topology of our phylogenetic ML tree fundamentally shows no conflict with those of Muggia et al. (2008, 2016) and Abdollahzadeh et al. (2020), and the bootstrap values were higher than those in the separate analysis of each locus.

The monophyly of *R. japonicus* is confirmed with high support values (NJ/ML = 100/100). This new species shows a close phylogenetic relationship with unidentifed fungi of Capnodiales *s. lat.* (A557 and A951 in Fig. 1) that were collected from alpine rock lichen communities (Muggia et al. 2016) which is supported by relatively high support values (NJ/ML = 92/87, Fig. 1). These lichen-associated fungi of Capnodiales *s. lat.* were treated under Teratosphaeriaceae (Cometto et al., 2023). This clade and other fungi of Teratosphaeriaceae including Friedmanniomycetes endolithicus and Recurvomyces mirabilis form a common clade that is not well-supported in our analyses (NJ/ML = 62/68, Fig. 1). Regardless, based on the currently available sequence data and phylogenetic analyses, we consider it is appropriate to place *R. japonicus* in Teratosphaeriaceae. Furthermore, our phylogenetic analyses revealed that Racoleus is not closely related to Cystocoleus (Cystocoleaceae) and Racodium (Racodiaceae) despite having extremely similar appearance in morphology with their unusual, sterile, filamentous thallus. Although they all belong to a broadly defined Capnodiales *s. lat.*, each genus appears to represent an independent lichenization event.

Teratosphaeriaceae has been delimited based on phylogenetic analysis of nuLSU sequence data (Crous et al., 2007), and presently does not have well defined phenotypic synapomorphies. However, within this family, there are taxa with similar traits to *R. japonicus* such as the brown fungal filaments (Crous et al., 2007; Si et al., 2023) and living in harsh environments like rock surfaces (Ruibal et al., 2008, 2009).

Because DNA sequences of the type species of Racoleus, *R. trichophorus* R. Santt. & D. Hawksw., have not been obtained, further study is needed to confirm the monophyly including both *R. trichophorus* and *R. japonicus*. However, based on the morphological features described and discussed below, we consider the new species belongs to Racoleus.

**TAXONOMIC TREATMENT**

*Racoleus japonicus* K. Miyaz. & Y. Ohmura, *sp. nov.*

*MycoBank No.: MB 850089*

Japanese common name: Iwa-goke.

Similar to *Racoleus trichophorus* but differs by wider filaments (10–15 μm vs. 7–9 μm wide in *R. trichophorus*), larger hyphal cells (3–7 μm vs. 2–3 μm wide in *R. trichophorus*), and irregularly sinuous lateral spines.


**Thallus:** minutely filamentous, blackish brown, forming a dense fluffy colony reaching up to ca. 30 mm diam. Filaments suberect to decumbent or spreading on the surface of substrate, occasionally sympodially branched, 10–15 (~20) μm wide, with lateral spines; outer wall undulating and irregularly corrugated, reflecting the morphology of the fungal hyphae. HYPHAE surrounding the algal filaments in a single layer, orientated vertically along and parallel to the axis of the filament, brown, 3–7 μm wide, septate; the septa generally 10–20 μm apart, thick-walled; walls uneven and undulate to corrugated, corrugations tending to interlink with those to adjacent hyphae. SPINES arising at broadly acute to almost right angles from the vertical axis, brown, septate, stiff, irregularly sinuous, up to 150 μm in length and 2–3 μm wide, the base expanded into a foot like cell (12–15 × 4–5 μm). CONIDIOGENOUS CELLS and CONIDIA not observed. PHOTOBIONT Trentepohlia sp., in single filaments surrounded by fungal hyphae; cells rounded rectangular, 25–50 × 8–10 μm.

**Chemistry:** C=−, K=−, KC=−, Pd=−. No secondary substances were detected by HPTLC.

**Etymology:** The epithet ‘japonicus’ refers to Japan where the new species was collected.

**Habitat and distribution:** This species grows on shady rocks or rock walls (Fig. 2A) in subboreal to temperate regions of Japan (i.e., from Hokkaido to Kyushu) at elevations between 290 and 1700 m.

**Remarks:** Racoleus japonicus closely resembles *R. trichophorus* by its dense, minutely filamentous, blackish brown fluffy colonies (Fig. 2B), that are formed by sympodially branching filaments (Fig. 2C) with corrugated hyphal walls (Fig. 2C). However, *R. japonicus* differs from *R. trichophorus* in wider filaments (10–15 μm vs. 7–9 μm wide in *R. trichophorus*), larger hyphal cells (3–7 μm vs. 2–3 μm wide in *R. trichophorus*), and irregularly sinuous lateral spines (Figs. 2D, 3F; vs. straight in *R. trichophorus*). Furthermore, *R. japonicus* is distributed in temperate to subboreal regions, whereas *R. trichophorus* is found in tropical regions of Africa (Ivory Coast, Asia (southern China), and South America (Peru) (Hawksworth et al., 2011).

The lateral spines of *Racoleus* were considered as an important feature for the genus distinguishing it from *Cystocoleus* and *Racodium* (Hawksworth et al., 2011). Indeed, there is a difference that the spines are straight in *R. trichophorus* (Hawksworth et al., 2011), sinuous in *R. japonicus* (Figs. 2D, 3F), *Cystocoleus* (Sukuja and Ore,
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**Fig. 2.** *Racoleus japonicus*. **A.** Habitat of type locality. **B.** Thallus (holotype, TNS). **C.** Sympodially branched filamentous thallus with corrugated hyphal walls (*K. Miyazawa 1700*, TNS). **D.** Filamentous thallus with spines (lateral hyphae) (holotype, TNS). Scale bars: B = 0.5 mm; C = 50 μm; D = 10 μm.

1934; Fig. 3D) and *Racodium* (Fig. 3E), but the following morphological features are common in these genera. The spines are composed of a single hypha extending outside the lichenized part of the thallus. The hyphae are 1.5–4 μm wide and up to 150 μm in length with some septa [the presence of septa in *R. trichophorus* was confirmed by the photo in Hawksworth *et al.*, (2011, Fig. 1D)]. There is no large morphological difference except being straight or sinuous in the lateral spine among these taxa, and this difference serves as taxonomic character at species level rather than for genus level. The arrangement of the hyphae and the presence/absence of corrugated walls in the filamentous thallus should be an important taxonomic character to recognize each genus.

*Racoleus japonicus* may be confused with *Cystocoleus ebeneus* (Dillwyn) Thwaites and *Racodium rupestre* Pers. because of the similar blackish brown filamentous thalli in which the fungal hyphae surround *Trentepohlia* filaments. However, *Racoleus japonicus* differs in the vertical arrangement of hyphae with a

corrugated wall (Fig. 3C). On the other hand, Cystocoleus has a weakly twisted arrangement of hyphae with a corrugated wall (Fig. 3A), and Racodium has a vertical arrangement of hyphae without a corrugated wall (Fig. 3B). Racoleus japonicus might be confused with taxa that belong to Spilonema Bornet (Coccocarpiaceae) and Thermutis Fr. (Lichinaceae), which have blackish filamentous thalli. However, Spilonema and Thermutis have stiffer thalli composed of wider filaments (at least 20 μm vs. up to 20 μm wide in R. japonicus), blackish apothecia, and cyanobacteria photobionts (observed materials in TNS are cited below).

Some Japanese specimens previously identified as ‘Racodium rupestre’ housed in TNS were found to be R. japonicus by our study, while R. rupestre was confirmed to occur in Japan (at about 2480 m elevation in Mt. Kinpu). The habitat of R. rupestre in Japan seems to be alpine to subalpine areas. The collections of ‘Racodium rupestre’ reported from Mt. Ryokami by Yoshimura (1964) were confirmed as Racoleus japonicus in the present study by examining materials collected from the same locality. The taxon reported by Kato and Harada (2011) from Mt. Haruna, central Honshu in Japan, should be Racoleus japonicus according to their illustration and description.

Additional specimens examined. JAPAN. Hokkaido. Kitami Prov.: Ikutahara-Kiyosato, Engaru-cho, Monbetsu-gun (N43°51'.

Exsiccate of other species examined. (Cystocoleus niger (Huds.) Har.) JAPAN. Westösterreich, Stubai Alpen: flockenrasig an schattig-trockenen, aber luftfeuchten überhängenden Urgesteinswänden im Cystocoleo-Racodium rupestris SCHADE, 1900 m elev., NW, pH 6.2, Osthang des Lisenser Tales bei Jufenau unter dem Windegg, October 1975, G. Follmann s.n. (Follmann: Lich. Exs. 491, TNS).


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LITERATURE CITED


