A New Species of Rhizocarpon from Southwest China—Postprint of Sichuan Map Lichen

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Abstract

This study was conducted in southwestern China, employing a combined approach of phenotypic characteristics (morphological, anatomical, and chemical features) and genotypic characteristics (ITS sequences) to perform taxonomic research on lichen species of the genus Rhizocarpon (Rhizocarpon Ramond ex DC.), clarifying the taxonomic status of some species within this genus and preliminarily exploring the correspondence between phenotypes and genotypes. Through this study, we discovered one new species in this genus—Rhizocarpon sichuanense Y. M. Zhang, L. Hu & W. C. Wang, sp. nov. The main differences between this new species and its similar species, Rhizocarpon cinereonigrum Vain. and Rhizocarpon sinense Zahlbr., are: the thallus lobes are relatively dispersed, with a surface that is cracked or sub-squamulose; the lower thallus is black and conspicuous; the spores are larger, (27–)32–42.5$×12.5–17.5(−20)μm; andTLCdetectionrevealsthepresenceofbarbaticacid.Inphylogeneticanalysis,thisn
dispersedthalluslobes, significantlysmallerspores(23–36×$13–16 m), and contains diffractaic acid or no chemical substances in the thallus. Phylogenetic analysis results indicate: (1) this new species—Rhizocarpon sichuanense belongs to the Badioatrum group within the brown thallus subgenus; (2) classification of some groups within Rhizocarpon based solely on anatomical characteristics (spore size and septum type) is unreasonable and requires comprehensive analysis combined with chemical characteristics. This paper provides a taxonomic description of the new species and high-resolution images of morphology, anatomy, and chemistry. The phylogenetic tree constructed using ITS sequences of the new species provides fundamental data for establishing a more natural and reasonable classification system. Additionally, this paper compiles a detailed species key for the Badioatrum group with brown, 1-septate ascospores within the brown thallus subgenus worldwide, providing reference support for further research on this group.

Full Text

Preamble

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Title: A New Species of *Rhizocarpon* from Southwest China—*Rhizocarpon sichuanense*

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Abstract

This study investigates species of the genus *Rhizocarpon* Ramond ex DC. from southwest China using an integrative approach combining phenotypic characteristics (morphology, anatomy, and chemistry) with genotypic data (ITS sequences). The aims were to clarify the taxonomic status of selected species and explore the correspondence between phenotypic and genotypic traits within the genus. Our research has identified one new species—*Rhizocarpon sichuanense* Y. M. Zhang, L. Hu & W. C. Wang, sp. nov. This new species resembles *Rhizocarpon cinereonigrum* Vain. and *R. sinense* Zahlbr. in producing a brown thallus, I– medulla, 8-spored asci, and brown, 1-septate ascospores, but can be distinguished by its scattered, areolate to subsquamulose thallus with a distinct black prothallus, larger ascospores [(27–)32–42.5 × 12.5–17.5(–20) µm], and the presence of barbatic acid (TLC). Phylogenetic analysis places the new species in the same clade as *R. badioatrum* (Flörke ex Spreng.) Th. Fr., though the latter differs in having a more continuous thallus, significantly smaller spores (23–36 × 13–16 µm), and containing diffractaic acid or no secondary metabolites. Our phylogenetic results indicate: (1) *R. sichuanense* belongs to the Badioatrum group within *Rhizocarpon* subgenus *Phaeothallus*; and (2) classification of certain *Rhizocarpon* groups based solely on anatomical characteristics (spore size and septation type) is problematic, necessitating integration of chemical data. This paper provides a detailed taxonomic description of the new species along with high-resolution images of its morphology, anatomy, and chemistry. The phylogenetic tree constructed using ITS sequences of the new species provides foundational data for establishing a more natural classification system. Additionally, we present a comprehensive species key for the Badioatrum group (brown, 1-septate ascospores) of *R.* subg. *Phaeothallus* worldwide, offering valuable resources for future research on this group.

Keywords: barbatic acid, Rhizocarpaceae, lichenized ascomycetes, lichenized fungi, taxonomy

Introduction

The genus *Rhizocarpon* was established by Candolle in 1805. Its primary characteristics include: crustose thallus growth form, ranging from cracked to squamulose; thallus lobes continuous or scattered, surface color varying from yellow-green, white, gray to dark brown; apothecia black, lecideine, with narrow margins that are inconspicuous or distinctly thickened; exciple typically darkly pigmented externally, lighter internally; epihymenium brown to blue-green, K± purple-red; asci containing 1–8 spores, with the ascus apex reacting with iodine to show dark blue outer wall and only lightly colored inner wall at the apex—the so-called *Rhizocarpon*-type ascus apical apparatus; ascospores hyaline to brown or dark green, 1- to multi-septate or submuriform to muriform, with distinct haloes; hymenium deep brown (Hafellner, 1984). In 1956, Runemark studied the yellow-green species of European *Rhizocarpon*, classifying those with 1-septate spores measuring 9–18 µm into the Superficiale group; those with 1-septate spores 18–32 µm into the Alpicola group; muriform-spored species with I− medulla into the Viridiatrum group; and muriform-spored species with I+ medulla into the Geographicum group (Runemark, 1956).

In 1967, Thomson divided *Rhizocarpon* into two subgenera based on the presence or absence of rhizocarpic acid: species with yellow-green thallus containing rhizocarpic acid were placed in *Rhizocarpon* subg. *Rhizocarpon*, while those with non-yellow-green thallus (white, gray to brown) lacking rhizocarpic acid were placed in *R.* subg. *Phaeothallus* (Thomson, 1967). Poelt (1988) subsequently summarized species belonging to subgenus *Rhizocarpon* and compiled a comprehensive key. Later, Fryday (2000, 2002) and Ihlen (2004), studying non-yellow-green *Rhizocarpon* species in Europe, classified brown, 1-septate spored species within subgenus *Phaeothallus* into the Badioatrum group; hyaline, muriform-spored species into the Obscuratum group; and hyaline, 1-septate spored species into the Hochstetteri group.

With the development of molecular biology, McCune et al. (2016) and Davydov et al. (2017) employed an integrative phenotypic and genotypic (ITS) approach in phylogenetic studies of Alaskan and Siberian Altai *Rhizocarpon* species, discovering two new species (*R. quinonum* McCune, Timdal & Bendiksby and *R. smaragdulum* Davydov & Yakovch.) and two new records (*R. atroflavescens* Lynge and *R. norvegicum* Räsänen). Roca-Valiente (2013) conducted a multi-gene phylogenetic analysis of the Geographicum group within subgenus *Rhizocarpon*, revealing that phenotypic and genotypic characteristics do not fully correspond, and that classification based solely on phenotypic traits inadequately reflects natural evolutionary patterns and phylogenetic relationships. Therefore, systematic taxonomic studies of *Rhizocarpon* using integrated phenotypic and genotypic approaches are urgently needed.

Currently, approximately 230 *Rhizocarpon* species have been reported globally, predominantly distributed in plateau and mountainous regions with cold climates (Lücking et al., 2016; Davydov & Yakovchenko, 2017; Kalb & Aptroot, 2017; Paukov et al., 2017; Kondratyuk et al., 2018; Fryday, 2019; Elix & McCarthy, 2019; McCarthy et al., 2020; Spribille et al., 2020). Only 47 species have been reported from China, mainly concentrated in southwestern (Yunnan, Sichuan, Guizhou, Hubei, Tibet) and northwestern regions (Xinjiang, Qinghai, Gansu, Shaanxi, Shanxi, western Inner Mongolia) (Wei, 1991; Abbas & Wu, 1998; Aptroot & Sparrius, 2003; Sérusiaux et al., 2003; Golubkov et al., 2009; Li et al., 2013; Zhao et al., 2013; Mahire et al., 2015; Wang et al., 2015a,b,c, 2016; Gulina & Anwar, 2019; Hu et al., 2020; Bi et al., 2022). China is vast with high biodiversity and rich lichen resources, yet research infrastructure remains relatively weak. It is estimated that known lichenized fungal species represent only 8.5% of the predicted total, with 91.5% of species awaiting investigation (Wei, 2018). Chinese scholars have long reported *Rhizocarpon* species sporadically, relying primarily on classical taxonomic (phenotypic) methods. Consequently, applying integrated phenotypic and genotypic approaches to *Rhizocarpon* taxonomy in China holds significant importance.

This study employs an integrative approach combining phenotypic (morphological, anatomical, and chemical) and genotypic (ITS sequence) characteristics to taxonomically identify *Rhizocarpon* species. Our objectives are to clarify the taxonomic status of selected species and provide data supporting a more natural classification system; explore the correspondence between phenotypic and genotypic traits to furnish reference materials for future *Rhizocarpon* research; and compile a worldwide species key for the Badioatrum group to provide foundational resources for studying this group.

1. Materials and Methods

Lichen specimens examined in this study were collected from the Garze Tibetan Autonomous Prefecture and Liangshan Yi Autonomous Prefecture in Sichuan Province, and are deposited in the Herbarium of Shandong Normal University (SDNU) and the Lichen Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN-L).

External morphological features (including thallus color and thickness, apothecial morphology, color, and size) were examined and photographed using a stereomicroscope (Olympus SZX16). Anatomical characteristics were observed and documented with a light microscope (Olympus BX61), including the color, thickness, and crystal distribution of the epihymenium, hymenium, hypothecium, and exciple. Color reactions of the cortex and medulla were tested using K reagent (10% KOH solution), C reagent (saturated NaOCl solution), IKI reagent (10% Lugol's iodine solution), and P reagent (saturated *p*-phenylenediamine in 95%

ethanol). Lichen secondary metabolites were detected using thin-layer chromatography (TLC) in solvent system B (Culberson, 1972; Culberson & Kristinsson, 1970).

Genomic DNA was extracted from each specimen using the Sigma-Aldrich DNA extraction kit following the manufacturer's protocol, with extracted DNA templates stored long-term at −20°C. The fungal-specific primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) were used for PCR amplification of ITS sequences. The PCR program was as follows: initial denaturation at 95°C for 2 min; 35 cycles of 94°C for 20 s, 53°C for 60 s, and 72°C for 2 min; final extension at 72°C for 15 min; with products held at 4°C.

PCR products were purified and sequenced by Qingdao Biosense Bioscience Co., Ltd. Single-end sequences were corrected and assembled using SeqMan (Swindell & Plasterer, 1997). Reference *Rhizocarpon* sequences were downloaded from GenBank. Phylogenetic trees were constructed using MAFFT v. 7 (Katoh et al., 2009) for sequence alignment and Gblocks V0.19b (Talavera & Castresana, 2007) to remove ambiguously aligned regions. The best-fit model for Bayesian inference (BI) was selected using jModelTest 2 (Darriba et al., 2012). Maximum likelihood (ML) analysis was performed using RAxML v. 8.2.6 (Stamatakis, 2014) with 1000 bootstrap replicates to assess branch support. Both ML and BI analyses were run on the Cipres Science Gateway (http://www.phylo.org). Resulting tree files were visualized and edited using FigTree v.1.4.3. Branches with bootstrap support (BP) >75% or Bayesian posterior probability (PP) >0.95 were considered well-supported.

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2. Results

2.1 Molecular Analysis

Four new ITS sequences of *Rhizocarpon* were generated in this study. After alignment with 23 reference sequences downloaded from GenBank and removal of ambiguous regions, a matrix of 546 sites was obtained for 27 species. The ML and BI trees showed identical topologies; therefore, only the ML tree is presented, with Bayesian posterior probabilities indicated after bootstrap values at each node (Fig. 1). The new species has been registered at Fungal Names (https://nmdc.cn/fungalnames), and newly generated sequences have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank).

2.2 Species Description

Rhizocarpon sichuanense Y. M. Zhang, L. Hu & W. C. Wang, sp. nov.
FIGURE:2

MycoBank: FN 571638

Diagnosis: This species is similar to *Rhizocarpon cinereonigrum* Vain. but differs by its large brown ascospores, areolate to subsquamulose thallus, grey-brown areolae, and the presence of barbatic acid.

Etymology: The epithet 'sichuanense' refers to Sichuan Province, where this species was discovered.

Type: China, Sichuan, Garze Tibetan Autonomous Prefecture, Kangding City, Ertaizizhongqiao Co., 30°2'34.24"N, 101°49'53.27"E, 3752 m alt., on rock, 29 April 2021, Ling Hu et al., 20210551 (SDNU–holotypus).

Description: Thallus crustose, areolate to subsquamulose, generally up to 5 cm diam., sometimes larger; areoles brown to grey-brown, flat to slightly convex, matt, scattered on prothallus, round or angular, up to 0.75 mm diam., 0.1–0.5 mm thick, cracks usually present on the areolae, epruinose to faintly pruinose. Upper cortex brown, dull, smooth, without an epinecral layer, 12–20 µm high, containing crystals. Lower cortex black-brown, without crystals. Photobiont layer continuous, 65–95 µm high; photobiont chlorococcoid, algal cells 10–15 µm diam. Prothallus distinct, black, well-developed between the areolae and along the margin. Apothecia black, lecideine, 0.2–0.9 mm diam., round or angular between the areolae, sessile on the black prothallus; disc flat to concave, scabrid, epruinose; proper margin 50–100 µm thick, persistent, at or slightly above the level of the disc, concolorous with the disc, epruinose to faintly pruinose, sparingly shiny; exciple 50–100 µm thick, deep brown to black at the rim, inner part red-brown, containing crystals dissolved in K, radiating hyphae 4–6 µm wide; hymenium colorless, 80–135 µm tall; paraphyses septate, branched and anastomosing, ca. 2.5 µm thick in mid-hymenium and 3–4(–5) µm thick apically; epihymenium pale brown to deep brown, K+ pale violet-red, to 25 µm thick, without crystals; hypothecium black-brown, without crystals, K−; asci clavate, *Rhizocarpon*-type, 8-spored; ascospores soon becoming dark brown, 1-septate, ellipsoid or oblong, halonate, (27–)32–42.5 × 12.5–17.5(–20) µm, length/width ratio (1.4–)1.7–2.1(–2.2).

Chemistry: Medulla I−, K−, C−, KC−, P−; barbatic acid (TLC).

Distribution and Habitat: The new species is known from Sichuan Province, growing on large granite boulders at elevations between 2731–3752 m, in association with *Rhizocarpon geographicum*, *Dermatocarpon* sp., and *Aspicilia* sp. It is currently known only from China.

Additional Specimens Examined:
China, Sichuan, Garze Tibetan Autonomous Prefecture, Luding County, Mt. Yajiageng, on rock, 29°54'10.19"N, 101°59'59.64"E, alt. 3946 m, 27 April 2021, Ling Hu et al., 20210102 (SDNU).
China, Sichuan, Liangshan Yi Autonomous Prefecture, Huili County, on rock, 26°47'22.89"N, 102°12'18.58"E, alt. 3648 m, 23 April 2021, Ling Hu et al., 20210837 (SDNU).
China, Sichuan, Liangshan Yi Autonomous Prefecture, Puge County, on rock, 27°35 5.63 N, 102°22 47.40 E, alt. 3622 m, 13 September 2021, X.Y. Wang et al., XY21-418 (KUN 80845).

Remarks: *Rhizocarpon sichuanense* is characterized by its saxicolous, scattered grey-brown areolate to subsquamulose thallus, distinct black prothallus, large 1-septate brown ascospores, and presence of barbatic acid. *R. cinereonigrum* is similar but has smaller ascospores (25–36 µm long) and produces stictic acid in the thallus (Thomson, 1977). Phylogenetic trees indicate the new species is closely related to *R. badioatrum* (Spreng.) Th. Fr., which also has brown epihymenium K+ violet and 1-septate brown ascospores, but differs in having a dark brown, more continuous thallus with diffractaic acid or no secondary products (Timdal & Holtan-Hartwig, 1988). Among all non-yellow *Rhizocarpon* species with 1-septate brown ascospores, *R. sichuanense* has the largest ascospores (32–42.5 µm long), followed by *R. sinense* (30–40 µm), while *R. badioatrum* and *R. cinereonigrum* have smaller spores (25–36 µm), and other species are even smaller (<30 µm) (Fletcher et al., 2009). Based on these combined morphological, anatomical, chemical, and molecular data, we recognize *R. sichuanense* as a distinct new species.

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3. Discussion

The new species is primarily characterized by its saxicolous, areolate to subsquamulose thallus with scattered areoles and grey-brown surface, distinct black prothallus, large 1-septate ascospores, and barbatic acid as the secondary metabolite. *Rhizocarpon cinereonigrum* is morphologically similar but differs in having smaller ascospores (25–36 µm long) and stictic acid in the thallus (Thomson, 1977). Phylogenetic analysis shows *R. sichuanense* is closely related to *R. badioatrum* (Spreng.) Th. Fr., which shares the brown epihymenium K+ violet reaction and 1-septate brown ascospores, but differs in its continuously distributed thallus, darker brown coloration, prothallus restricted to the thallus margin, smaller spores (23–36 × 13–16 µm), and diffractaic acid or no secondary metabolites (Timdal & Holtan-Hartwig, 1988). Additionally, *R. sinense* Zahlbr. also has relatively large spores (30–40 × 10–15 µm) with 1-septation, but differs in its darker brown, shiny thallus with an epinecral layer, gyrophoric acid (C+ red), immersed apothecia, and olive-brown ascospores (Zahlbruckner, 1930).

Within the Badioatrum group of subgenus *Phaeothallus* (species with brown, 1-septate ascospores), *R. sichuanense* possesses the largest ascospores (32–42.5 µm long), followed by *R. sinense* (30–40 µm), while *R. badioatrum* and *R. cinereonigrum* have relatively smaller spores (25–36 µm), and other species are even smaller (<30 µm) (Fletcher et al., 2009). Therefore, based on the comprehensive morphological, anatomical, chemical, and molecular evidence, we conclude that *R. sichuanense* represents a valid new species.

Furthermore, our integrative phenotypic and genotypic analyses reveal that at the subgeneric level, species of *R.* subg. *Rhizocarpon* and *R.* subg. *Phaeothallus* do not form separate monophyletic clades; rather, the former is nested within the latter. This demonstrates that Thomson's (1997) classification based solely on thallus color and rhizocarpic acid production is artificial, a finding also reported by McCune et al. (2016) and Davydov et al. (2017). At the species or group level, we found that phenotypic and genotypic characteristics generally correspond within subgenus *Rhizocarpon*, where the Superficiale, Viridiatrum, and Geographicum groups (distinguished by spore size, septation type, and medulla reaction) each form distinct clades. However, within subgenus *Phaeothallus*, particularly the Badioatrum group, species do not cluster together. Instead, *R. sichuanense* groups with *R. badioatrum*, *R. copelandii* with *R. jemtlandicum*, and *R. rittokense* forms a separate lineage. This indicates that classification of the Badioatrum group cannot rely exclusively on spore color and septation, but must incorporate chemical characteristics and epihymenium K reactions. Future studies should increase taxon sampling across *Rhizocarpon*, integrating phenotypic and genotypic data to further elucidate infrageneric and species-level delimitations and establish a more natural classification system.

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4. Key to Species of the Badioatrum Group Worldwide

1. Thallus brown, composed of umbilicate areolae with epinecral layer, medulla I−, ascospores 20–24 × 10–15 μm, containing barbatic acid………………………………… *R. rittokense* (Hellb.) Th. Fr.

2. Thallus not as above…………………………………………………………………………….2

3. Thallus cephalodiate (composed of a second photosynthetic symbiont and fungal partner)…………………………………………………………………… *R. hensseniae* Brodo

4. Thallus not cephalodiate………………………………………………………………………3

5. Ascospores small, 12–22 μm long……………………………………………………………..4

6. Ascospores large, 22–42 μm long……………………………………………………………8

7. Medulla yellow above, containing rhizocarpic acid………………………………….. *R. flavomedullosum* Elix & P.M. McCarthy

8. Medulla white, not containing rhizocarpic acid…………………………………………..5

9. Medulla I−, epihymenium black-blue, K−, ascospores 12–15 × 7–7.5 μm…………… *R. alaxense* J.W. Thomson

10. Medulla I+ blue…………………………………………………………………………………….6

11. Containing bourgeanic acid, ascospores 15–24 µm long……………………………. *R. vigilans* P.M. McCarthy & Elix

12. Containing ±gyrophoric acid, ascospores small, 12–16 µm long………………………7

13. Thallus reduced, epihymenium black-brown, hymenium pale red, ascospores brown, 12.5–20 × 5–7.5 µm, containing no substance…………………………………. *R. umense* (H. Magn.) A. Nordin

14. Thallus well developed, epihymenium brown, hymenium hyaline, ascospores olive-green or brown, 12.5–16 × 6.5–7.5 µm, containing ±gyrophoric acid………. *R. simillimum* (Anzi) Lettau

15. Epihymenium olive-brown to green-black, K−, containing crystals dissolving in K……………………………………………………………………………………9

16. Epihymenium red-brown to deep brown, K+ purple-red, no crystals…………………………………………………………………………………..10

17. Thallus pale grey to dark grey or dark brown, areolae up to 1 mm diam., without pruina, dull or shiny, plane to bullate; medulla K+ yellow, K± red, containing stictic acid and ±norstictic acid…………………. *R. copelandii* (Körb.) Th. Fr.

18. Thallus dark brown, areolae up to 1.5 mm diam., with faintly grey pruinose, dull, plane to weakly convex; medulla K+ yellow, containing stictic acid only…………………………………………………………. *R. jemtlandicum* Malme

19. Medulla K+ yellow, containing stictic acid only, ascospores 28–38 × 12–18 µm……………………………………………………. *R. cinereonigrum* Vain.

20. Medulla K−, not containing stictic acid…………………………………………11

21. Thallus containing gyrophoric acid, spores olive-brown, 35–40 × 10–15 µm……………………………………………………………. *R. sinense* Zahlbr.

22. Thallus not containing gyrophoric acid……………………………………….12

23. Areoles continuous, prothallus present along the margin, ascospores smaller, 23–36 × 13–16 µm, containing diffractaic acid or no substances………………….. *R. badioatrum* (Flörke ex Spreng.) Th. Fr.

24. Areoles more or less scattered, prothallus present between the areoles, ascospores (27–)32–42.5 × 12.5–17.5(–20) µm, containing barbatic acid……………………… *R. sichuanense* Y.M. Zhang, L. Hu & W.C. Wang

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Note: Figure translations are in progress. See original paper for figures.