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Rhytisma huangshanense sp. nov. described from morphological and molecular data

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Abstract — A fungus collected from the Huangshan Mountains in Anhui Province, China is described as a new species, *Rhytisma huangshanense*. The new species, occurring on leaves of *Rhododendron simsii*, is similar to foliicolous species of *Coccomyces* but differs in having thicker epiphyllous ascomata that open by a more or less longitudinal split. Analyses based on partial small subunit or large subunit nuclear ribosomal DNA sequences confirm placement of *R. huangshanense* in the genus.

Key words — LSU rDNA, *Rhytismatales*, SSU rDNA, taxonomy

Introduction

Species of *Rhytisma* Fr. are parasites causing tar-spot symptoms on leaves of broadleaf trees, and most of them are highly host-specific (Hou & Piepenbring 2005). In most species, stromata develop on living leaves to produce spermatial morphs, with the meiotic stage appearing the following season on fallen and overwintered leaves. Most records of *Rhytisma* species are based on observations of stromata on living leaves (Cannon & Minter 1986, Hou 2004, Hou & Piepenbring 2005).

In China, 11 species of *Rhytisma* have been reported (Hou & Piepenbring 2005). Among them, six have been described in detail with ascomatal morphology and the remaining species will be restudied when mature ascomata are available in future. In the present paper we describe a new species of *Rhytisma* on *Rhododendron simsii* Planch. from the Huangshan Mountains in Anhui Province, China.

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Table 1. SSU rDNA sequences of the species used in the study and their GenBank accession numbers

Species	Voucher specimen	GenBank accession No.
Blumeria graminis f. sp. bromi		AB033476
Bulgaria inquinans		DQ471008
Byssoascus striatosporus		AB015776
Chloroscypha chloromela		AF203461
Coccomyces dentatus		AY544701
Coccomyces strobi		DQ471027
Colpoma quercinum		AF203452
Cudonia circinans		AF107343
Cyclaneusma minus		AF203458
Elytroderma deformans		AF203455
Hymenoscyphus fructigenus		HFU67430
Lirula macrospora		AF203453
Lophodermium pinastri		AF106014
Meloderma desmazieresii		AF203454
Microglossum viride		MVU46031
Oidiodendron tenuissimum		AB015787
Phacidium coniferarum		AF203467
Rhytisma acerinum		AF356695
Rhytisma huangshanense	Hou et al. 564	FJ495193
Rhytisma salicinum		RSU53370
Spathularia flavida		AF107344
Spathularia velutipes		AF281072
Tryblidiopsis pinastri		DQ471035

Table 2. LSU rDNA sequences of the species used in the study and their GenBank accession numbers

Species	Voucher specimen	GenBank accession No.
Coccomyces dentatus		AY544657
Coccomyces strobi		DQ470975
Colpoma quercinum		EU833991
Cudonia circinans		AY533013
Cudonia lutea		AF433140
Cudonia sichuanensis		AF433137
Cyclaneusma minus		FJ176868
Lophodermium pinastri		AY004334
Meria laricis		DQ470954
Potebniamyces pyri		DQ470949
Rhytisma acerinum		AF356696
Rhytisma acerinum		EU833992
Rhytisma acerinum	Hou et al. 203	FJ495190
Rhytisma salicinum	Hou et al. 70	FJ495191
Rhytisma huangshanense	Hou et al. 564	FJ495192
Spathularia flavida		AY541496
Spathularia velutipes		AF279411
Tryblidiopsis pinastri		DQ470983

Materials and methods

Morphological study

Sections of ascomata of different thicknesses were made by hand using a razor blade. Microscopic preparations were made in water, Melzer's reagent, 5% (w/v) KOH, or 0.1% (w/v) cotton blue in lactic acid. For observation of ascomatal outlines in vertical section, sections were mounted in lactic acid or cotton blue with pretreatment in water. Gelatinous sheaths surrounding the ascospores and paraphyses were observed in water or cotton blue. Measurements were made from 20 ascospores and asci for each specimen using material mounted in 5% KOH.

Molecular methods

Total genomic DNA was extracted from herbarium material using the PeqLab E.Z.N.A.® Fungal DNA kit following the manufacturer's protocol after the samples were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube together with one 3 mm in diam. tungsten carbide ball. The SSU/LSU rDNA regions were amplified using the primers pairs NS1/NS4 and LR0R/R5 (White et al. 1990, Vilgalys and Hester 1990). PCR was performed in 25 µl reactions including, DNA template 1.0 µl, primer1/2 1.0 µl, 2 × MasterMix 12.5 µl, and H_2O, under the following parameters: 94 °C for 40 seconds, 50 °C for SSU/45 °C for LSU for 55 seconds, 72 °C for 1 minute, for a total of 30 cycles followed by a final extension step at 72 °C for 7 minutes. The PCR products were sent to Invitrogen Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing and editing. The other SSU/LSU rDNA sequence data included in this study were downloaded from GenBank (Tables 1–2).

Phylogenetic analyses

DNA sequences were aligned with Clustal X (Thompson et al. 1997). Further manual alignment was done in Se-Al v.2.03a (Rambaut 2000). The insertion sequences and sections of the sequences longer than the sequence of the new species were excluded from the analyses. Two data sets of the SSU/LSU rDNA sequence data were prepared and were analyzed separately using maximum parsimony methods performed in PAUP* 4.0b10 (Swofford 1998). Maximum parsimony analyses were conducted using heuristic searches with 1000 replicates of random-addition sequence, tree bisection reconnection (TBR) branch swapping and no maxtree limit. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions. A bootstrap analysis was performed with 1000 replicates, each with 10 random taxon addition sequences. MAXTREES was set to 1000, and TBR branch swapping was employed.

Results

Morphological analyses indicate that the specimen collected from the Huangshan Mountains in Anhui Province, China represents a new species of *Rhytisma* (Figs. 1–8).

The length of the SSU alignment was 921bp, with 66 phylogentically informative positions. The maximum parsimony analyses of sequences resulted in one most parsimonious tree (Fig. 9) with a length (TL) of 239 steps, consistency index (CI) of 0.6975, retention index (RI) of 0.7012, homoplasy index (HI) of 0.3025, and rescaled consistency index (RC) of 0.6971. The length of the LSU alignment was 849 bp, with 122 phylogentically informative positions. The maximum parsimony analyses of sequences resulted in one most parsimonious tree (Fig. 10) with a length TL of 380 steps, CI of 0.6579, RI of 0.6524, HI of 0.4483, and rescaled RC of 0.4292.

The results showed that *Rhytisma huangshanense* formed a well supported sister clade to *R. salicinum* (Pers.) Fr. in analyses of sequences of SSU rDNA and a well supported monophyletic clade to *Rhytisma* species in analyses of sequences of LSU rDNA.

Taxonomy

Rhytisma huangshanense C.L. Hou & M.M. Wang, **sp. nov.** Figures 1–8

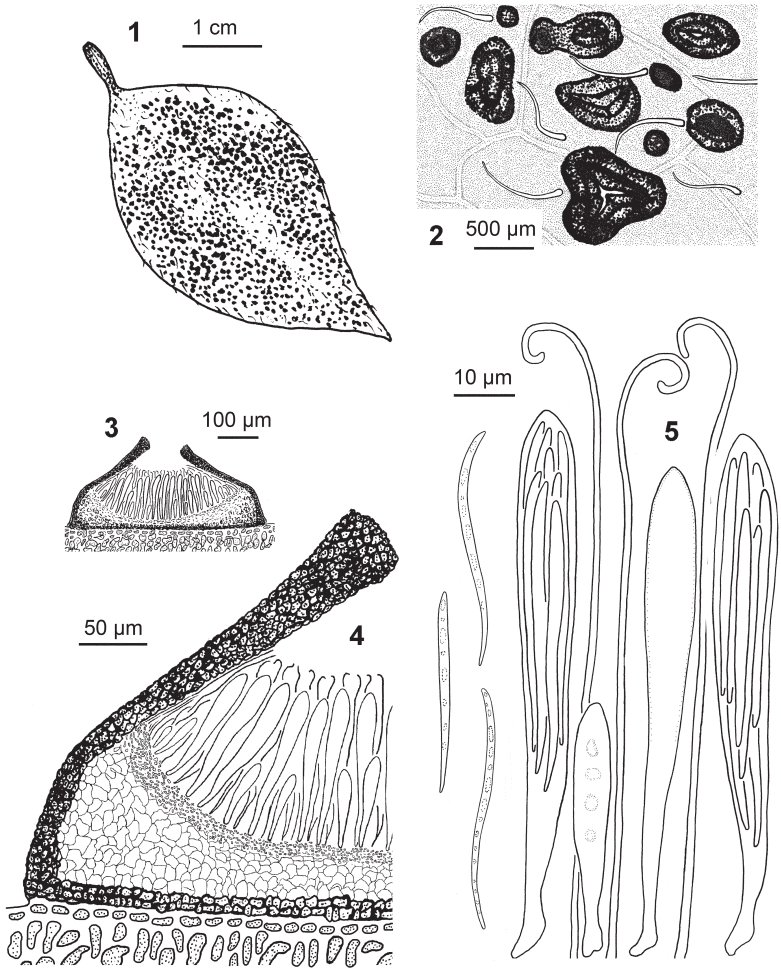
MycoBank 512114

Ascomata 250–1000 × 250–650 µm, epiphylla, subcuticularia, nigra, elliptica, orbicularia vel irregularia; paraphyses filiformes, circinatae; asci 70–110 × 7–10 µm, cylindrici vel clavati; ascosporae 35–60 × 2–2.5 µm, cylindricae, filiformes.

Etymology: *huangshanense*, referring to the Huangshan Mountains where the specimen was collected.

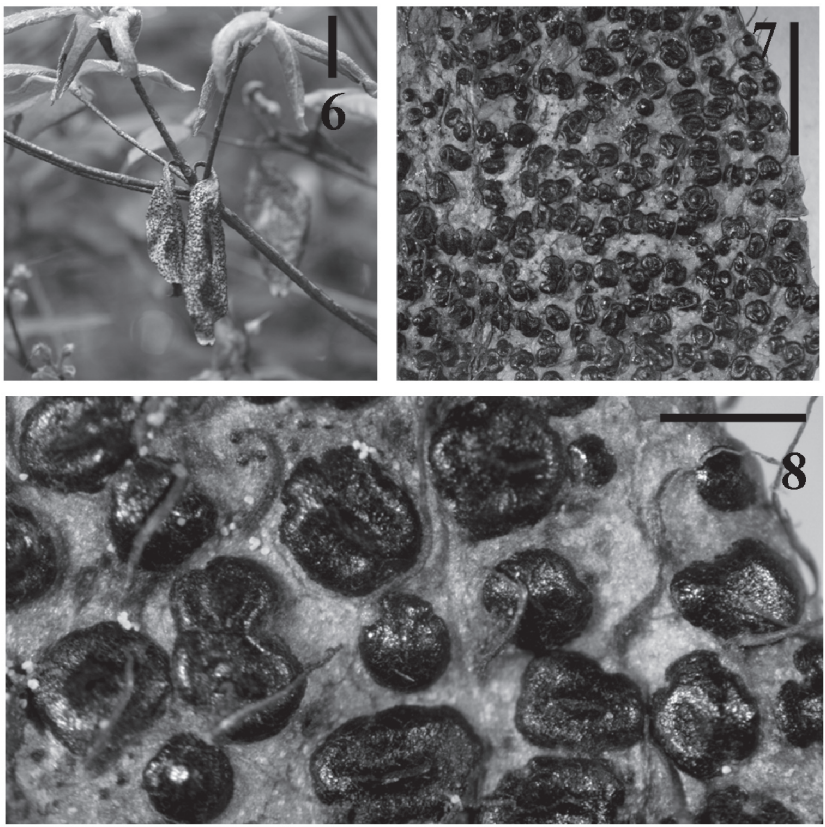
Holotype: on leaves of *Rhododendron simsii* (*Ericaceae*), CHINA. ANHUI, the Huangshan Mountains, Tianhai, alt. ca. 1700 m, 10 June 2006, C. L. Hou, M. M. Wang & L. Zhang 564 (AAUF).

Ascomata on dead leaves, epiphyllous, elliptical, orbicular or slightly irregular, black, shiny, single, occasionally coalescing, spreading over the whole surface of the leaf, 250–1000 × 250–650 µm, strongly raising the surface of the substrate, often with central papilla when immature, opening with a longitudinal split, or with slightly irregular splits when ascomata are coalesced. Lips cells absent. In median vertical section, ascomata subcuticular, covering stroma to 30–45 µm thick near the centre of the ascoma, sometimes slightly thinner towards the edges, extending to the basal stroma, consisting of an outer layer of host cuticle, and an inner layer of black, thick-walled angular cells. Basal stroma well-developed, 40–65 µm thick, composed of an outer layer of two rows of black, thick-walled, angular cells and an inner layer of 3–4 rows of hyaline, thin-walled angular cells. Subhymenium consisting of textura intricata, 10–15



Figs 1–5. *Rhytisma huangshanense* on leaves of *Rhododendron simsii*. 1. A Leaf bearing ascomata. 2. Ascomata observed under the dissecting microscope. 3. Ascoma in vertical section. 4. Structure of an ascoma in vertical section. 5. Paraphyses, a young ascus, an ascus after the liberation of the ascospores, two mature asci with ascospores, and discharged ascospores.

µm thick. Paraphyses 120–150 × 1.5–2 µm, filiform, unbranched, circinate at the apex. Asci ripening sequentially, 70–110 × 7–10 µm, ± cylindrical-clavate, short-stalked, slightly acute at the apex, 8-spored, thin-walled, without circumapical thickening, J-. Ascospores 35–60 × 2–2.5 µm, filiform, slightly sigmoid when released, slightly tapering toward the tips and more towards the bases, hyaline, aseptate, without gelatinous sheaths.



Figs 6–8. Photographs of *Rhytisma huangshanense*. 6. Two Leaves bearing ascomata attached to a twig of *Rhododendron simsii*. Scale bar = 1 cm. 7. Ascomata on a leaf. Scale bar = 5 mm. 8. Ascomata on a leaf. Scale bar = 1 mm.

Conidiomata and Zone lines not seen.

Distribution: Only from the type locality.

Habitat: Collected on dead leaves which are still attached to twigs.

Discussion

Ascomatal shapes of *Rhytisma huangshanense* are somewhat similar to some foliicolous species of *Coccomyces* De Not. and some species of *Rhytisma*. SSU rDNA sequence analyses showed that our new species was closely related to *R. salicinum* with 99% bootstrap support but distantly related to a sequence reported to be of the type species of *Rhytisma*, *R. acerinum* (Pers.) Fr.

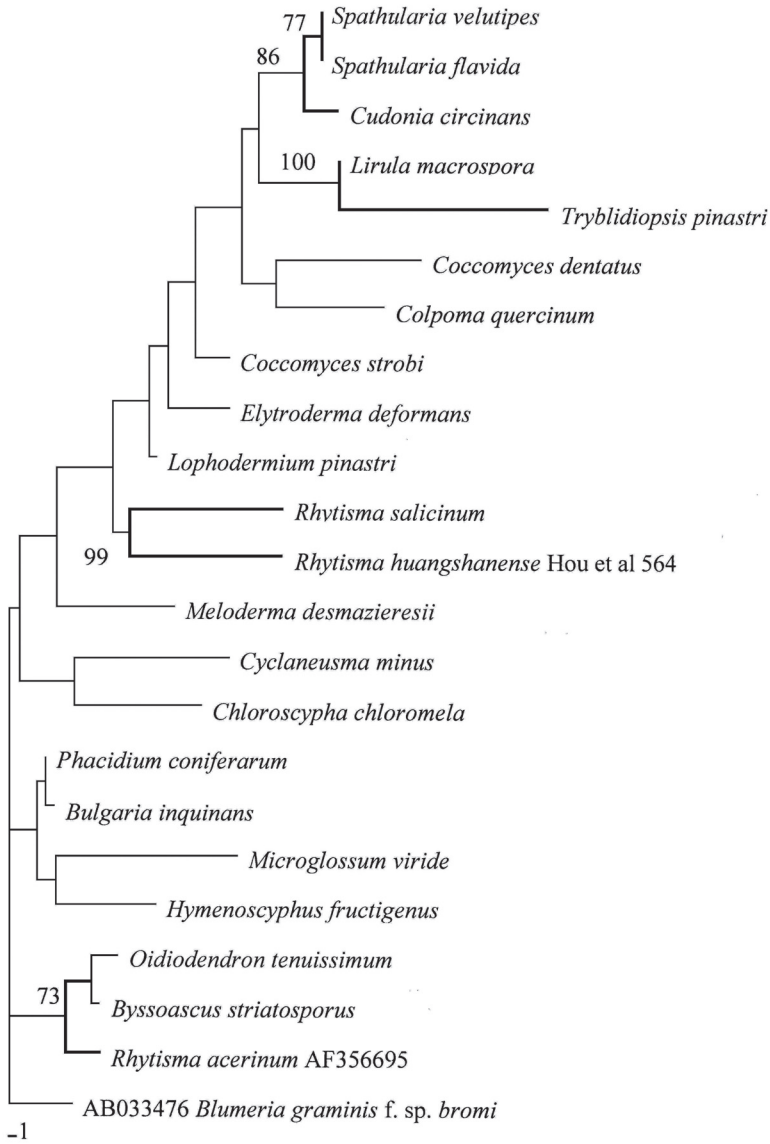


Fig. 9. Phylogenetic hypothesis derived from maximum parsimony analysis of partial nuclear small subunit ribosomal RNA gene sequences of *Rhytisma huangshanense* and other related species, using *Blumeria graminis f.* sp. *bromi* as an outgroup. Bootstrap values of more than 70 % from 1000 replications are shown on the respective branches (bold).

Fig. 10. Phylogenetic hypothesis derived from maximum parsimony analysis of partial nuclear large subunit ribosomal RNA gene sequences of *Rhytisma huangshanense* and other related species, using *Cyclaneusma minus* as an outgroup. Bootstrap values of more than 70 % from 1000 replications are shown on the respective branches (bold).

(GenBank AF356695), which formed a group with members of *Myxotrichaceae* (*Ascomycota* incertae sedis). Therefore a sequence of SSU rDNA from a trustworthy collection of *R. acerinum* should be obtained for comparison with AF356695. LSU rDNA sequence analysis showed that *R. huangshanense* formed

a monophyletic clade with *R. acerinum* and *R. salicinum* with 88% bootstrap support. *R. huangshanense* was distantly related to *Coccomyces* species and other rhytismatalean species. The LSU sequence of *R. acerinum* in GenBank (AF356696) also is likely from a misidentified source. The other LSU sequences from *R. acerinum*, EU833992 and Hou et al. 203, are congruent with the expected phylogeny. Sequences of EU833992 and Hou et al. 203 are identical while the sequence similarity for AF356696 and EU833992 is only 88%.

Species of *Rhytisma* are usually parasitic on living leaves, though one species, *Rhytisma yuexiense* C.-L. Hou & M. Piepenbr., however, has been observed only on fallen leaves (Hou & Piepenbring 2005). Ascomata of *Rhytisma* species open by a more or less longitudinal split. *Coccomyces* species mostly inhabit fallen leaves and their ascomata open by more or less radiate splits. Due to lack of molecular data, the type species of *Coccomyces*, *C. tumidus* (Fr.) De Not. was not included in the phylogenetic analyses. However, the type species *Rhytisma*, *R. acerinum* is distinct from *C. tumidus* in ecology and ascomal development.

Five species of *Rhytisma* on *Rhododendron* spp. are known worldwide (Farr et al. 1996, Hou & Piepenbring 2005). *R. huangshanense* with unilocular stromata is similar to *Rhytisma anhuiense* C.-L. Hou & M. Piepenbr., *R. rhododendri-oldhamii* Sawada (invalidly published), *R. rhododendri* Fr. and *R. shiraiana* Hemmi & Kurata (Hemmi & Kurata 1931, Hou & Piepenbring 2005, Saccardo 1889, Sawada 1943). *R. anhuiense* has hypophyllous ascomata opening by irregular splits and clavate asci with clavate to slightly cylindrical ascospores, and is distinct from *R. huangshanense*. Owing to the much larger, rugose stromata (4–6 × 3–4 mm), *R. rhododendri* is undoubtedly a different species from *R. huangshanense*, which has small stromata (250–1000 × 250–650 μm), spread throughout the leaves rather than big tar spots. *R. rhododendri-oldhamii* has much wider asci (73–140 × 13–16 μm) and ascospores (40–55× 3.5–5 μm) than those of *R. huangshanense*, with paraphyses straight rather than hooked or twisted at the tips. Paraphyses of *R. shiraiana* are similar to the present species. However, *R. shiraiana* has larger stromata (up to 3 mm diam.), much wider and longer asci (99–147 × 11–14 μm), and much wider ascospores (21–45 × 2.8–3.8 μm) than those of *R. huangshanense*.

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