Phylogenetic Analysis of Lichen-Forming Fungi *Rhizoplaca* Zopf from China Based on ITS Data and Morphology

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A molecular phylogenetic analysis of *Rhizoplaca melanophthalma*, *Rhizoplaca chrysoleuca*, *Rhizoplaca peltata* and *Rhizoplaca haydenii* is presented based on the nuclear ribosomal internal transcribed spacer (ITS) regions and morphology. *Rhizoplaca* species were collected at 3400–3900 m in Tianshan Mountains, Xinjiang province, China. *Rhizoplaca haydenii* is reported for the first time in China. Maximum parsimony (MP) analysis of ITS sequences obtained from Tianshan Mountains samples and GenBank reveals that the evolution relationship of *Rhizoplaca melanophthalma* and *Rhizoplaca chrysoleuca* is closer to each other than to *Rhizoplaca peltata*, and *Rhizoplaca haydenii* showed closer relatedness to *Rhizoplaca melanophthalma*. When the four species groups from Tianshan Mountains were analyzed alone through the neighbour-joining (NJ) and minimum evolution method, we obtained the same result. The morphology analysis of *Rhizoplaca* Zopf which reveals the pruinose discs and apothecial discs of species did not show convincing evidences to prove phylogenetic relationship among *Rhizoplaca* species. In our study, the result further proved that *Rhizoplaca* should be rejected as a genus separate from *Lecanora*.

Key words: Rhizoplaca Zopf, Phylogeny, ITS

Introduction

The lichen has a widely geographical distribution in the world. It is a symbiotic association which is constituted by fungi and algae or cyanobacteria. The biological character of lichen is reflection of the essentiality of fungi within the symbiotic association. Therefore, lichen has been named lichen-forming fungi (Hawksworth and Hill, 1984) or lichenized fungi (Wei, 1982).

Rhizoplaca Zopf belongs to the division lichen-forming Ascomycetes and family Lecanoraceae. It comprises more than nine species distributed throughout the world, only six of them are found in China. There are *R. chrysoleuca* (Sm.) Zopf, *R. fumida* X. Q. Gao, *R. huashanensis* Wei, *R. melanophthalma* (Ram. in Lam. & DC.) Leuckert et Poelt, *R. subdiscrepans* (Nyl.) and *R. peltata* (Ram.) Leuckert et Poelt including two variations v. *peltata* and v. *regalis* (H. Magn.) Wei (Wei, 1991). According to the study of Wei and Wei (2005), *R. fumida* may be treated as one of the chemotypes within *R. chrysoleuca*.

Rhizoplaca Zopf was separated from the genus *Squamaria* DC. based on its single central rhizoid (Zopf, 1905). Afterward, Poelt (1958) advanced that *Rhizoplaca* Zopf is a genus separate from *Lecanora*. Whereas, Ryan and Nash (1997) doubted the relationship between *Rhizoplaca* Zopf and *Lecanora* and suggested to do some further investigation. Arup and Grube (2000) adapted that *Rhizoplaca* Zopf is not a genus separate from *Lecanora* and may not be a monophyletic genus. Cansaran *et al*. (2006) also supported this result. At present, researchers focus on the phylogenetic relationship among *Rhizoplaca* Zopf and other correlated genera (Arup and Grube, 2000), however, studies on phylogenetic relationships among these species are very limited.

Originally, lichenologists used thallus structure and secondary metabolism to study the phylogenetic relationship of the lichen (Sundin and Tehler, 1998; Crespo *et al*., 1999). However, there is not distinct difference of the characters within genera or between species and no further support for genera that are characterized mainly by their thallus morphology. In addition, chemical similarity or difference is not a reliable evidence of systematic relationships because chemistry is varied in many

species and sporadically shared by different groups (Leuckert *et al*., 1977; Wei, 1984; Ryan and Nash, 1997; Arup and Grube, 2000; Wei and Wei, 2005; Zhou *et al*., 2006). With the improvement of molecular technology, more and more lichenologists began to deeply reveal the phylogenetic relationship of the lichen and identify the cryptic genus within morphologically homogeneous groups using modern molecular techniques. The internal transcribed spacer regions of nuclear ribosomal DNA have been proved to be very useful in analyzing the genetic relationship among species. Internal transcribed spacer (ITS) sequences information has been one of the primary criteria in investigating the relationship at the specific level.

The aim of our study was: 1) to investigate the phylogenetic relationship among *Rhizoplaca* species by using ITS sequence analysis; 2) through analyzing morphological characters, to research whether pruinose discs and apothecial discs can be used to test the phylogenetic relationship among *Rhizoplaca* species.

Materials and Methods

Growth conditions and sample collection

The lichens in our study were collected from an ice-free cirque (43° 05′ N, 86° 49′ E, with an altitude of 3400–3900 m) near the No. 1 glacier in the source area of Urumqi river in Tianshan Mountains, Xinjiang province, China. The annual average temperature is lower than 5 °C in daytime and −4 °C at night. The temperature also undergoes a big fluctuation from nearly 4 to −10 °C during the favourable growth season from June to September. All samples were carefully cleaned with distilled water to remove possible epiphytic contaminants and then air-dried at room temperature. Finally, all dried samples were conserved at −20 °C.

DNA isolation, PCR and sequencing

Total DNA was obtained from dried lichen using the modified CTAB (cetyl-trimethyl ammonium bromide) method (Murtagh *et al*., 1999). The extraction procedure was as follows: Lichen herbarium materials were ground under liquid nitrogen and 0.1 g suspended in 600 μl extraction buffer (50 mM Tris [tris(hydroxymethyl) amino methane]-HCl (pH 8.0), 50 mM EDTA; Biodee, Beijing, China) and 100 μl of 10% SDS (Biodee). The solution was incubated in water at 65 °C for 3–5 h. 80 μl high concentration CTAB/NaCl (Biodee) and 100 μl NaCl (5 M) were added to the solution. Then the solution was incubated at 65 °C for 5 min to further eliminate protein. One volume of equilibrated phenol/chloroform/isoamylalcohol [25:24:1 (v/v/v)] (Biodee) was added and mixed thoroughly by inversion. Then, samples were centrifuged at 12,000 × *g* for 5 min (MIKRO 22R, Hettich, Tuttlingen, Germany). If precipitated protein was still observed at the aqueous/organic interface, extra washes were performed as necessary. 0.54 Volume of isopropanol was added and incubated at 4 °C for 2 h to precipitate DNA. Following centrifugation (14,000 × *g*, 5 min), the pellet was washed twice with 80% ethanol, dried in air and re-suspended in 50 μl of TE buffer [10 mM Tris-HCI, 1 mM EDTA (pH 8.0)]. Total DNA was examined for quantity and quality on an ethidium-bromide-stained 1% agarose gel (Yito Enterprise Comp. Ltd., Shanghai, China) and stored at −20 °C.

DNA reaction mixture was performed in 25 μl using 10–50 ng genomic DNA as template, 2.5 μl dNTP (Takara, Ootsu-shi, Shiga-ken, Japan), 2.5 μl 10 × PCR buffer (Takara), 1.5 μl 10 mM primers and 0.3 U Dynazyme Taq polymerase (Takara). ITS4 (TCCTCCGCTTATTGATATGC) (White *et al*., 1990) and ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) were used to amplify the ITS sequence. Polymerase chain reaction (PCR) amplification was executed with the following program: initial denaturation at 95 °C for 4 min, and subsequently a 35 cycles reaction with annealing at 50 °C for 1 min, extension at 72 °C for 1 min, denaturation at 94 °C for 1 min and final extension at 72 °C for 5 min. The PCR products were visualized on 1% agarose gel as a band of approx. 500 or 800 bp. Then the products were cleaned using the purification kit (Takara) following the manufacturer's instructions. Sequencing was accomplished using an automated sequencer ABI3730 (ABI, Foster City, USA).

Sequence alignment and phylogenetic analysis

Our sequences were aligned using Clustal X1.83 (Thompson *et al*., 1994). All parameters were default values of the software. Maximum parsimony (MP) analysis was determined using PAUP*4b4a (Swofford, 1999) with the following settings: the matrix was subjected to 10 replicates of random sequence additions using heuristic searches, tree bisection and reconnection (TBR) branch swap-

ping. Gaps were treated as "missing". One tree was held at each step during stepwise addition. Confidence limits for branches of the trees were performed by bootstrap analysis with 1,000 replications.

Neighbour-joining (NJ) and minimum evolution method analysis were carried out using MEGA3.1 (Kumar *et al*., 2004) with the following settings: two-parameter method was used to calculate the genetic distance matrix and construct the neighbour-joining tree and minimum evolution analysis. Gaps and missing data were completely deleted. Confidence limits for branches of the trees were performed by bootstrap analysis with 1,000 replications. *Parmelia sulcata* and *Protoparmelia badia* were used as outgroups.

Results

In our study, we obtained ITS sequence data for twenty-five samples of *Rhizoplaca* genus from Tianshan Mountains. There are ten *R. melanophthalma* (EF095278, EF095279, EF095280, EF095282, EF095283, EF095285, EF095286, EF095287, EF095290, EF095297), eight *R. peltata* (EF095275, EF095281, EF095284, EF095289, EF095291, EF095295, EF095296, EF101891), six *R. chrysoleuca* (EF095274, EF095276, EF095277, EF095293, EF095294, EF095298) and one *R. haydenii* (EF095292). All sequences have been deposited in GenBank. The species, localities and the GenBank accession numbers of twenty ITS data of *Rhizoplaca* Zopf obtained from GenBank are shown in Table I.

Rooted with *Parmelia sulcata* (AF410840) and *Protoparmelia badia* (AF070023) as outgroups our analysis is justified through the results of molecular investigation in the relationship among species of the *Rhizoplaca* genus (Arup and Grube, 2000; Zhou *et al*., 2006).

One hundred maximum parsimony trees with 761 parsimony-informative characters [consistency index (CI) = 0.5608; retention index (RI) = 0.6319] were found by a heuristic search, one of the trees is shown in Fig. 1. The trees are similar to each other in topologic, with only slight re-arrangements in the group containing *R. melanophthalma*.

When the phylogenetic tree is examined, *R. peltata* group from Tianshan Mountains and three different samples of *R. peltata* (AY509802, AY509803, AF159936) from GenBank appear on the same branch. *Lecanora dispersoareolate* (AF070016) and *R. peltata* (AY509802) form a sister branch with 100% support within the *R. peltata* group. Also *R. melanophthalma* and *R. chrysoleuca* locate in two other branches of the tree that form a sister group with the same species from different countries by 71% bootstrap. *R. haydenii* (Tuck.) Follm (AF159937) appears within

Table I. Species, GenBank accession numbers and localities of twenty ITS data of *Rhizoplaca* Zopf obtained from GenBank.

Species	GenBank accession No.	Origin
Rhizoplaca chrysoleuca	AY303147	China
Rhizoplaca chrysoleuca	AY509800	China
Rhizoplaca chrysoleuca	AY304153	China
Rhizoplaca chrysoleuca	AY509798	China
Rhizoplaca chrysoleuca	AY509792	China
Rhizoplaca chrysoleuca	AF159942	Idaho, USA
Rhizoplaca chrysoleuca	AF159940	Kazakhstan
Rhizoplaca chrysoleuca	AF159924	Arizona, USA
Rhizoplaca peltata	AY509803	China
Rhizoplaca peltata	AF159936	British Columbia, Canada
Rhizoplaca peltata	AY509802	China
Rhizoplaca melanophthalma	AY509791	China
Rhizoplaca melanophthalma	AF159929	Arizona, USA
Rhizoplaca melanophthalma	AF159935	Austria
Rhizoplaca haydenii	AF159937	Austria
Rhizoplaca idahoensis Rosentreter ined.	AF159943	Idaho, USA
Rhizoplaca cylindrica Ryan ined.	AF159941	Idaho, USA
Lecanora dispersoareolate	AF070016	Turkey
Parmelia sulcata	AF410840	Germany
Protoparmelia badia	AF070023	Austria

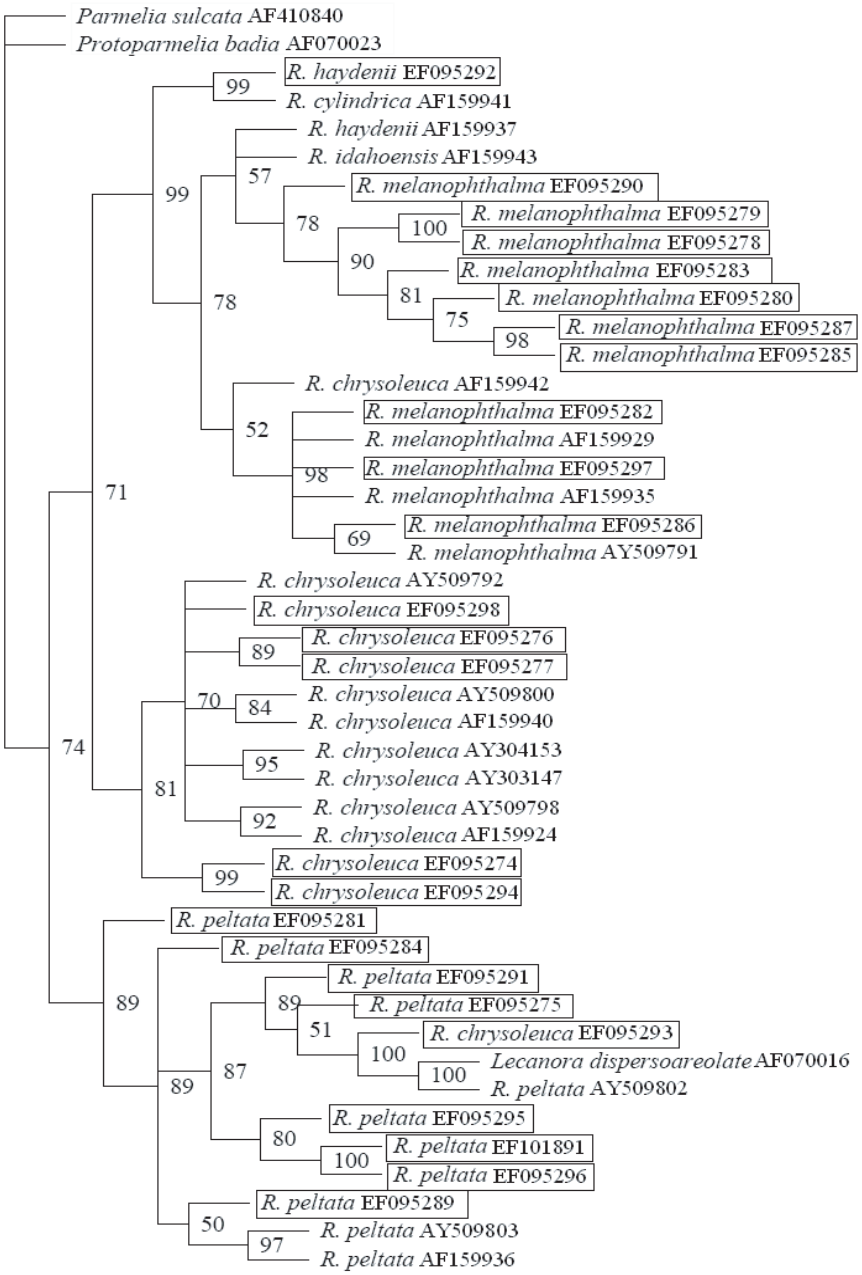

Fig. 1. Maximum parsimony analysis inferred from ITS region sequences of *Rhizoplaca* species from Tianshan Mountains and GenBank. Bootstrap percentages greater than 50%. Species group from Tianshan Mountains is located in the frames.

the *R. melanophthalma* group, and *R. haydenii* (EF095292) together with the *R. melanophthalma* group form a sister branch with 99% support.

Similar results are also obtained from neighbour-joining and minimum evolution analyses that included a species group (that are found in the

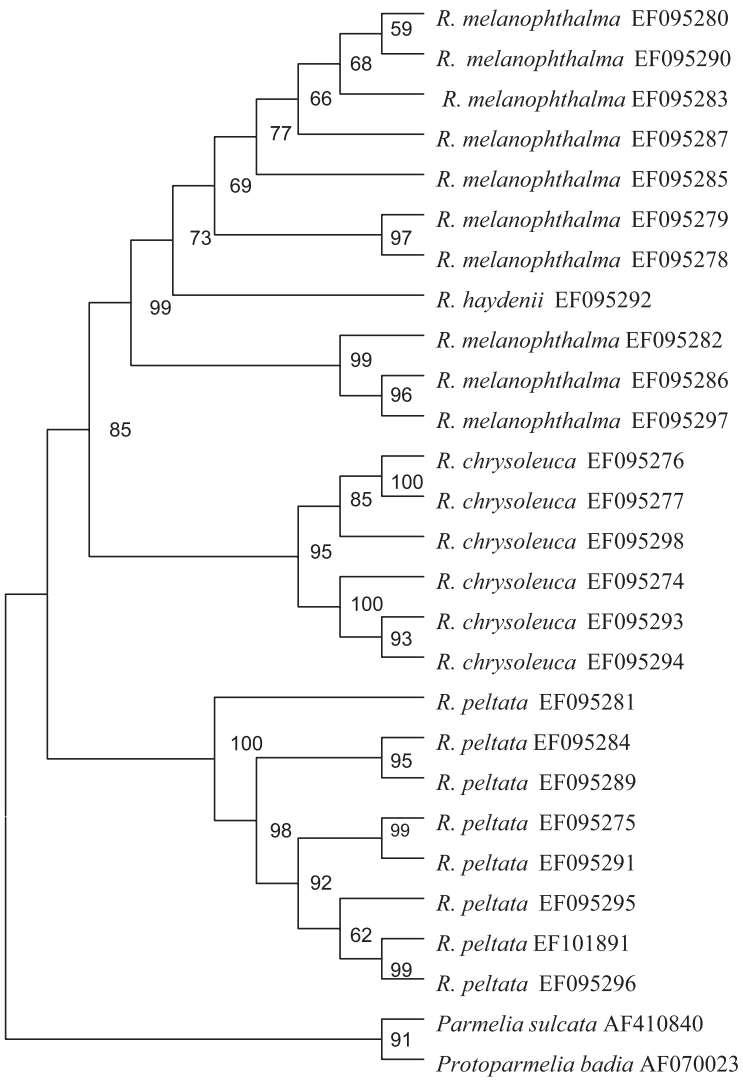

Fig. 2. Neighbour joining analysis inferred from ITS region sequences of *Rhizoplaca* species from Tianshan Mountains.

first analysis) from Tianshan Mountains in China. *R. peltata*, *R. melanophthalma* and *R. chrysoleuca* groups appear on three different branches. *R. melanophthalma* and *R. chrysoleuca* form a sister branch (with bootstrap value of 85% in NJ and 86% in the minimum evolution analysis). *R. haydenii* (EF095292) emerges as a sister group to the *R. melanophthalma* group within *R. melanophthalma.* Phylogenetic trees of neighbour-joining and minimum evolution analyses are corresponding to each other, with only slight re-arrangements in the group containing *R. melanophthalma*, and are shown in Fig. 2 and Fig. 3.

Discussion

Phylogenetic analysis

In phylogenetic trees, *R. melanophthalma* emerges as a sister group to *R. chrysoleuca* (with bootstrap values of 71% in the MP, 85% in the NJ and 86% in the minimum evolution analysis), whereas *R. peltata* appears on a different branch of the trees (with bootstrap values of 89% in the MP, 100% in the NJ and 100% in the minimum evolution analysis). The same branching pattern was also found in other papers, such as Zhou *et al.* (2006) and Arup and Grube (2000). It indicates

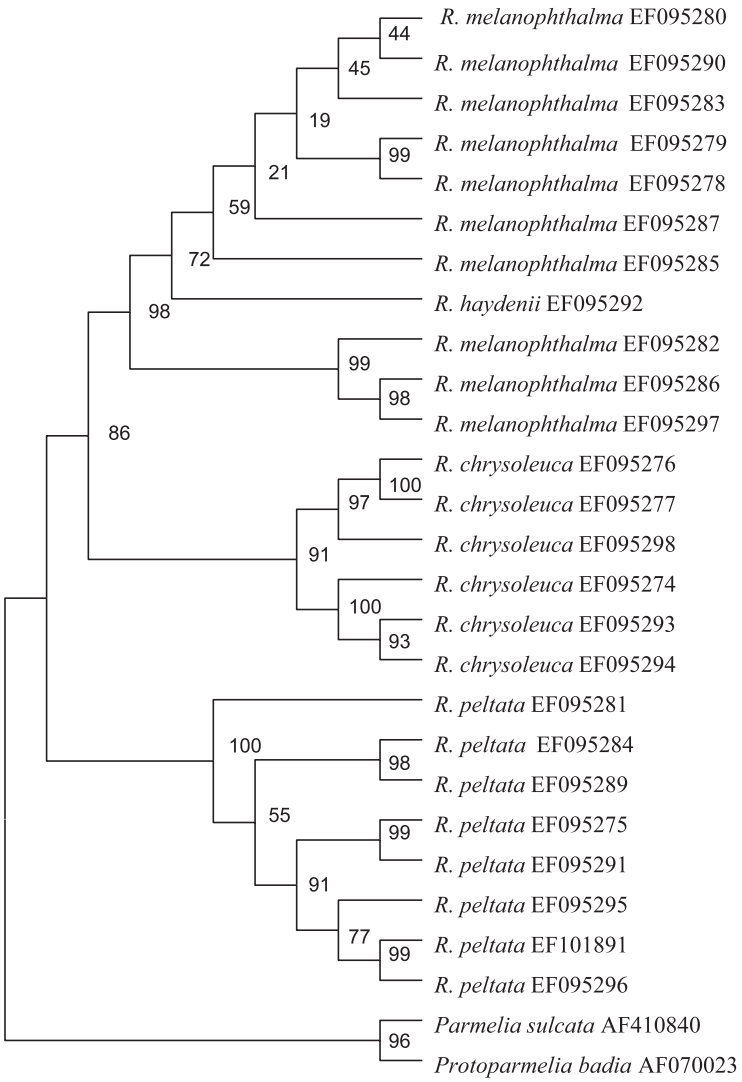

Fig. 3. Analysis by minimum evolution according to the data obtained by ITS region sequences from Tianshan Mountains.

that *R. melanophthalma* and *R. chrysoleuca* are phylogenetically closer than *R. peltata*, while Cansaran *et al*. (2006) maintained that *R. melanophthalma* and *R. peltata* were phylogenetically closer to each other than *R. chrysoleuca*. In their neighbour-joining tree, the topology of *R. peltata* and *R. melanophthalma* was supported only by 28% bootstrap values. And the topology structures of maximum parsimony and minimum evolution analyses weren't consistent with the neighbour-joining tree. However, they were the same as ours.

In addition, we also studied the phylogenetic relationship of *R. haydenii* and the other three species of the *Rhizoplaca* genus. *R. haydenii* is reported for the first time in China. The thallus morphology has no distinct difference from that shown by Arup and Grube (2000). Thalli of *R. haydenii* is an almost globose structure formed by the folding of broader and flatter lobes. In our phylogenetic analysis, *R. haydenii* and *R. melanophthalma* form a sister branch within the *R. melanophthalma* group, which is supported by the bootstrap value 99% in MP, 73% in NJ and 72% in minimum evolution. Therefore, we think that *R. haydenii* is closer related to *R. melanophthalma* than others. The same conclusion can be found in

Zhou *et al*.'s neighbour-joining tree (with bootstrap value of 99%) and Arup and Grube's maximum parsimony and maximum-likelihood analyses (with bootstrap value of 100% and 99%).

In maximum parsimony analysis, *Lecanora dispersoareolate* (AF070016) appears within the *R. peltata* group, a position strongly supported by a bootstrap value of 100%. This result further proves that *Rhizoplaca* should be rejected as a genus separate from *Lecanora* (Arup and Grube, 2000).

Morphology analysis

Cansaran *et al*. (2006) maintained that *R. melanophthalma* and *R. peltata* were phylogenetically closer to each other than *R. chrysoleuca* was also based on morphological evidence. They found that *R. chrysoleuca* differed from *R. peltata* and *R. melanophthalma* by having pruinoses, orange apothecial discs, however, the apothecial discs were yellowish brown and not pruinose in *R. peltata*, yellow-brown to greenish or black and pruinose in *R. melanophthalma.* They observed only three samples, one *R. melanophthalma*, one *R. peltata* and one *R. chrysoleuca*. The conclusion is not enough universal. In our research, ten *R. melanophthalma*, eight *R. peltata* and six *R. chrysoleuca* samples were examined. We noticed that *R. chrysoleuca* samples have green (EF095277) and brown (EF095276) apothecial discs except for orange (EF095298). The apothecial discs are all brown in *R. peltata*, and all greenish or black in *R. melanophthalma.* We didn't find pruinose in any samples during our study. Therefore, we think there are no convincing evidences to support the phylogenetic relationship among *Rhizoplaca* species by apothecial discs and pruinose of species.

Based on our research, we induce three conclusions as follows: 1) *Rhizoplaca melanophthalma* and *Rhizoplaca chrysoleuca* are phylogenetically closer to each other than *Rhizoplaca peltata*; 2) the phylogenetic relationship of *Rhizoplaca haydenii* and *Rhizoplaca melanophthalma* is closer than of *Rhizoplaca peltata* and *Rhizoplaca chrysoleuca*; 3) apothecial discs and pruinose of species can not be selected to prove a phylogenetic relationship among *Rhizoplaca* species. Further work is in process, phylogenetic relationship of more species of *Rhizoplaca* Zopf will be researched.

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