Phylogenetic Relations of *Rhizoplaca* Zopf. from Anatolia Inferred from ITS Sequence Data

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Like many lichen-forming fungi, species of the genus *Rhizoplaca* have wide geographical distributions, but studies of their genetic variability are limited. The information about the ITS rDNA sequences of three species of *Rhizoplaca* from Anatolia was generated and aligned with other species from other countries and also with the data belonging to *Lecanora* species. The examined species were collected from the volcanic rocks of Mount Erciyes which is located in the middle of Anatolia (Turkey). The sequence data aligned with eight other samples of *Rhizoplaca* and six different species of *Lecanora* were obtained from GenBank. The results support the concept maintained by Arup and Grube (2000) that *Rhizoplaca* may not be a genus separate from *Lecanora.* According to the phylogenetic tree, *Rhizoplaca melanopthalma* from Turkey with two different samples of *R. melanopthalma* from Arizona (AF159929, AF159934) and a sample from Austria formed a group under the same branch. *R. peltata* and *R. chrysoleuca* samples from Anatolia located in two other branches of the tree formed sister groups with the samples of the same species from different countries. Although *R. peltata* remained on the same branch with other samples of the same species from other countries it was placed in a different branch within the group. When the three species from Anatolia were considered alone, it was noticed that *Rhizoplaca melanopthalma* and *Rhizoplaca peltata* are phylogenetically closer to each other than *Rhizoplaca chrysoleuca*; the morphological characteristics also support this result.

Key words: Rhizoplaca, Phylogeny, ITS

Introduction

The genus *Rhizoplaca* belongs to the Lecanoraceae within the order Lecanorales and suborder Lecanorineae with *Trebouxia* as photobiont (Hawksworth, 1988). The members of this genus grow on siliceous or calcareous rocks or unattached (vagrant) on soil, in open, especially dry sites. The growth forms of the *Rhizoplaca* members are mostly umbilicate foliose but also rarely crustose or fruticose (Ryan and Nash, 1997). The colors of the thalli in the genus *Rhizoplaca* are frequently yellowish green to yellow-gray, but sometimes only gray. That thalli are usually rounded and often lumpy, 10–30 mm in diameter; the lower surface is pale to black, without rhizines. Medulla often contains placodiolic or pseudoplacodiolic acid and other depsides, depsidones and triterpenes in various combinations (Brodo *et al.*, 2001).

The thallus morphology has been used as a conventional character in lichen systematics to differentiate many different taxa. For example, separation of the crustose Buelliaceeae from the foliose to fruticose Physciaceae almost completely depends on thallus morphology. This is also the case for the Leconoraceae family. In this family, two lobate genera *Arctopeltis* Poelt (Poelt, 1983) and *Rhizoplaca* (Leuckert *et al.*, 1977) have been separated from the genus *Lecanora* mainly by their umbilicate thalli with well developed upper and lower cortices (Arup and Grube, 2000).

However, in the species, which have intermediate thallus characters, classification by using traditional generic concepts is usually difficult. Recently, ascomatal characters became very important in classification of families and genera (Hafellner, 1984). The genus *Rhizoplaca* has lecanorine apothecia, colorless, 1-celled spores and

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Lecanora-type asci as in the genus *Lecanora.* As mentioned above, the genus *Rhizoplaca* is separated from the genus *Lecanora* mainly by its thallus morphology. Another related genus, *Squamarina* has the same ascomatal characters with *Lecanora* and *Rhizoplaca*, but has *Bacidia*-type asci (Purvis *et al.*, 1992).

Rhizoplaca species were previously classified in the *Leconora* genus as section *Omphalodina* in the subgenus *Placoduim* (Poelt, 1958). But according to evaluation of many characteristics of the group it was raised to a generic level by Leuckert *et al.* (1977) using the name *Rhizoplaca* once again. In addition to its umblicate thallus structure, *Rhizoplaca* could also be distinguished by distinct upper cortex, thick lower cortex and relatively loose medulla. Although this situation seems to be the final decision about the discrimination between *Leconora* and *Rhizoplaca*, Ryan and Nash (1997) have pointed the necessity of further studies in order to set the definite boundaries.

The ITS sequence data for many different *Rhizoplaca* species have been documented and their differentiation from *Leconora* have been argued by Arup and Grube (2000). According to phylogenetic analysis they indicated that the generally accepted concept of *Rhizoplaca* as a genus separate from *Leconora* might be rejected.

Rhizoplaca genus comprise more than nine species, distributed throughout the world, only three of them are found in Turkey. Among the species distributed around the world, such as *R. chrysoleuca* (Sm.) Zopf, *R. glaucophana* (Hasse) W. Weber, *R. haydenii* (Tuck.) W. Weber, *R. marginalis* (Hasse) W. Weber, *R. melanopthalma* (DC.) Leuckert & Poelt, *R. peltata* (Ramond) Leuck. & Poelt, *R. subdiscrepans* (Nyl.) R. Sant., *R. asidophora*, *R. priestleyi* (Dodge), the species found in Turkey are *R. chrysoleuca* (Sm.) Zopf, *R. melanopthalma* (DC.) Leuckert & Poelt, *R. peltata* (Ramond) Leuckert & Poelt.

A common characteristic of the three species found in Turkey is their direct anchorage to the rocks with an umbilicus structure of their thallus. While in *R. chrysoleuca* the lower surface is rough, broken into areoles with cracks, the apothecial disks are yellowish brown and medulla P+ (paraphenylendiamine) orange (pannarin), in *R. peltata* and in *R. melanopthalma* the lower surface is smooth, apothecial disks are yellowish-brown to greenish or black, pruinose and also their medulla are bright yellow with psoromic acid P+.

In molecular studies of lichen, analysis of nuclear ribosomal DNA (rDNA) has become an important tool. The availability of non-algal primers allows the amplification of fungal target DNA without any contamination with the algal partner. The sequence data accumulated with these studies shed light to phylogenetic relationships (Lutzoni and Vilgalys, 1995), population structures (DePriest, 1993), and origin of lichens (Gargas *et al.*, 1995).

In this study we obtained ITS sequence data for three species of *Rhizoplaca* found in Turkey. The examined species were collected from the volcanic rocks of Mount Erciyes which is located in the middle of Anatolia (Turkey). The sequence data aligned with eight other samples of *Rhizoplaca* and six different species of *Lecanora* were obtained from GenBank. The results support the concept maintained by Arup and Grube (2000) that *Rhizoplaca* may not be a genus separate from *Lecanora.* Besides this, the study constitutes a contribution to the phylogenetic studies based on ITS sequence data of lichens from Turkey. In this respect this study constitutes the first data from Turkey, and from this point of view it may show off the presence of variation in populations of *Rhizoplaca* species growing in different geographical areas and ecologies.

Materials and Methods

Plant material

Three *Rhizoplaca* species were collected from around Kayseri province. The three species might be regarded as a represention of the whole population, as these were the only species grown in Turkey.

The samples were dried at room temperature and foreign matters were removed prior to grinding. The lichen samples are stored in the Herbarium of Erciyes University (Erciyes University, Department of Botany, Kayseri, Turkey). The collection localities were as follows: *Rhizoplaca melanopthalma*, Turkey, (Prov.) Kayseri (38) Erciyes Mountain, western slope of Erciyes Mountain (along the telepherics), 38°32′N, 35°30′E, 2500–2600 m (leg. & det. M. G. Halıcı); *Rhizoplaca chrysoleuca*, Turkey, (Prov.) Kayseri (38) Erciyes Mountain, western slope of Koç Mountain, 38°32′N, 35°32′E, 2200–2300 m (leg. & det. M. G. Halıcı); *Rhizoplaca peltata*, Turkey, (Prov.) Kayseri (38) Erciyes Mountain, north of Perikartın (north-

ern slope of Erciyes Mountain), 38°35′N, 35°27′E, 2300 m (leg. & det. M. G. Halıcı).

DNA extraction

Total DNA was extracted from thalli according to an improved CTAB (cetyl-trimethyl ammonium bromide) based extraction method. Briefly the extraction protocol was as follows: Lichen herbarium material (0.1 g) was ground to a fine powder in liquid nitrogen. Prewarmed extraction buffer [50 mM Tris [(tris(hydroxymethyl) amino methane]-HCl (pH 8), 50 mM EDTA, 10 ml LiCl (4 M), 1% CTAB, 2% PVPP (addition of PVPP is optional)] in the amount of 1 ml was added to the samples and ground once more in the buffer. After the samples were taken to the 1.5-ml Eppendorf tubes, 10 μl β-mercaptoethanol were added. The solution was incubated in a water bath at 65 °C for 15 min. Following these incubation period, samples were cooled to room temperature, 0.5 ml chloroform/isoamyl alcohol [24:1 (v/v)] was added and mixed well (no vortex). Then, samples were centrifuged at 17,000 × *g* (14,000 rpm) for 2 min. The supernatant was transferred to a fresh tube (~ 0.8 ml) and an equal volume of isopropanol was added; the mixture was gently mixed by inversion several times. Samples incubated for at least for 15 min on ice increased the efficiency of DNA yield. The samples were then centrifuged for 2 min at 17,000 × *g* (14,000 rpm). The supernatant was discarded. 1 ml 70% ethanol was added. The samples were then centrifuged for 1 min at 17,000 × *g* (14,000 rpm). The pellet was once more washed with 70% ethanol optionally and air-dried until all ethanol was removed. To obtain nucleic acids, the pellet was dissolved in an appropriate amount of TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA] (30–60 μl). The nucleic acids dissolved in TE buffer were treated with ribonuclease (RNase, 10 mg/ml) and stored at – 20 °C until use.

DNA was quantified via spectrophotometric measurement of UV absorption at 260 nm (Specord UV-200). DNA was also quantified by means of agarose gel electrophoresis with ethidium bromide fluorescence; a 100-bp DNA ladder was used (Fermentas) as the DNA size marker.

PCR amplification and sequencing

Internally transcribed spacer region of the rDNA gene cluster was amplified with the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al.*, 1990). ITS1F primer was designed specificly for fungal sequences at the 3′ end of the small subunit gene of the rDNA, overlapping with ITS5, whereas ITS4 was described as an universal primer corresponding to the 5′ end of the large subunit gene (White *et al.*, 1990).

Different parameters were tested for optimization of PCR reactions. PCR amplifications for sequence analysis were performed in a volume of 50 μl containing 200 ng of genomic DNA, 5 μl of 10 × reaction buffer, 5 μl of 25 mM $MgCl_2$, 5 μl of 2.5 mM dNTPs, 200 ng of each 4 mM of ITS1F primer and ITS4 primer, and 1 U *Taq* DNA polymerase (Promega, Madison, USA). Amplification was performed in a Techne Progene thermal cycler (Techne, Barloworld Scientific, Stanfordshire, U.K.). The reaction mixtures were heated in an initial step of 94 °C for 2 min and then subjected to 35 cycles of the following program: 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min 45 s. After the last cycle, the temperature was maintained at 72 °C for 8 min. The amplification products were analyzed by electrophoresis in 1.2% agarose gel containing ethidium bromide and the product sizes were determined on gels by nucleotide size marker (100 bp ladder, Fermentas, Vilnius, Litvanya). The PCR products were sequenced by the cycle sequencing method using a dye terminator cycle sequencing kit (Amersham Biosciences Corp., Piscataway, USA) according to the manufacturer's protocol and an OpenGeneÒ automated DNA sequencing system (Visible Genetics, Piscataway, USA).

Sequence analysis

The amplified fragments with the primers ITS1F and ITS4 comprising the 3′ end of small subunit gene, ITS1, the 5.8 S gene, ITS2 and the 5′ terminus of the large subunit gene were sequenced (Fig. 1). Ambiguities arising from reading errors were resolved by comparing the complementary strands with the program Clustal W 1.83. The sequences were also aligned using the same program and adjusted visually. Ambiguous aligned sites were excluded from analysis and gaps were treated as missing data. ITS sequences for eight other different samples from the *Rhizoplaca* genus and for six different species of *Lecanora*, available from GenBank (Table I) were included into multiple alignments. (Data can be obtained from the corresponding author.) A sequence matrix of 727 nucleotide positions was analyzed and 298 conservative,

Rhizoplaca melanopthalma

GCGAACCTGATCCGAGGGGTTTTTGGACAATGGGGGCTTCGGCCCCCGCATCTCTTCACCCTAAGTTTACAGG
TACCTTCGTTGCTTTGGCGGCTTTGTCTTGGGGCTCGCCCCCTAGCCGGCTGCTGGCCTCGTGCCCCGAGCCG
GTCCGTGCCCGCCTGGGGCCCATCAAATTCCGTTCATCAGTGCCGTCCGAGCAAAACCACAATAGTAAAAACT
TTCAACAACGATGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATTCGTGAATT
GCAGAATTCAGTGAATCCGTTATCGAATCTTTGAACGCACATTGCGCCCCTCGGTATTCCGGGGGGGCATGCCT
GTTCGAGCGTCATTGACACCCCTCAAGCTTAGCTTGGTATTGGGTCTTCGCCCCAACGTGGCGGGCCCGAAAG
TCAGAATGGCGAAGTTCGGCGCGACTTCGAGCGTAGTAACTCTTCTCCCGATCTGGAAGGTCGCGCTGGGCCA
TAAACGCAATCTTTCAA

Rhizoplaca chrysoleuca

TTGTTGCTTTGGGCGGGCCTTGGGGTCCGCCCCATCCCGGCCACGGGCCTCGCGCCCGGGCCAGGCCGCGCCG
TCCGGGGCCCATTCAAACTATGTTTACCAGTGCTGTCCGAGTGAATACCACAATAGTAAAAACTTTCAACAAG
GGATCTCTGGGTTCTCGCAATCGAATGAAGAAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAAATCATCGAATCTTTGAACGCACATTAGAGCCCCTTTGGGTATTCACGAGGGGCATGCCTGTTCGAGCG
TCATTGAACACTCACCCCTCAACGCTCCGCTTGCCGTGTTAGGCCGAGACCCCCGCGTAGGGCCGTACCGAAA
TCCAGTGGCGGGCCGTACCGAAATCCAGTGGCGGTCCGGCGTGACTTCGAGCGTAGTAGGGATCCTATCGCTC
GTTGGAAGCTCGGCGTCGGGGCCGGCCACGAACGCCCTCAATTTCATTTCCGA

Rhizoplaca peltata

AGTTTCCGAGTGACCTGCGAAAGATCATATGGAGAGGTCTTCGGACCTCGGGCTCGGCCCCCAGTTCTCACCC
CCTGTTCACGTATCATGTTGCTTGACGGGCCCCGGAGGCTGCCCCCTCGATGGCTGGCGACTTCGCGTCCCGG
CCTGTTCGCGCCGTCCGAGGCCTATCAACCCGACTTACCGTGCCGTCCGAGTACAACCATAATAGTATAAAAC
TTTCAACAACGGATCTCTGGGTCTGGGCATCGATGAAGAACGCAGCGAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAGTCATCGAATCTTGAACGCACATTGCGCCCCTCGGTATTCCGGGGGGCATACCTGTTCGAGCG
TCATTACACCCCTCAAGCTCGGCTTGGTACTGGGTCTTCGCCCCGGGGGCGGGCCCGAAAGTCAGTGGTGGTT
CGGCGCGACTTCGAGCGTAGTAAAACTTCTCCCGCTCTGGAAGTCGCCGCGTGACCGGCCAGACGCCCAAATC
TTGGAATGATGAGCTCGATCAGGTACGCCA

Fig. 1. The sequence information of the three different species from Turkey. The sequences comprise the 3′ end of small subunit gene, ITS1, the 5.8 S gene, ITS2, and the 5′ terminus of the large subunit gene.

325 variable, 164 parsimony informative sites were detected. All data were analyzed by MEGA 3 (Kumar *et al.*, 2004) and a bootstraped dendogram was generated.

Results

DNA was extracted with a new, improved CTAB based protocol for this study. The sequencing reactions were conducted with this DNA of enough purity, suitable for PCR amplification.

The target sequences generated by PCR amplification were sequenced in both directions. The sequencing reactions for species of *Rhizoplaca* yielded alignable sequences of 727 nucleotides long. Within these sites 298 were conservative and 325 were variable whereas 164 sites were parsimony informative. ITS sequences for eight other different samples from the *Rhizoplaca* genus and for six different species of *Leconora*, available from GenBank (Table I) were included into multiple alignments.

Table I. The aligned species, localities and their GenBank accession numbers.

Species or specimen	GenBank accession No.	Origin
Lecanora achariana A. L. Sm.	AF070019	Sweden
Lecanora garovaglii (Kölber) Zahlbr.	AF189718	Austria
Lecanora novomexicana H. Magn., U 363	AF159945	Arizona
Lecanora pruinosa Chaub.	AF070018	Italy
Lecanora reuteri Schaer	AF070026	Australia
Lecanora swartzii subsp. Nylanderi	AY541273	Australia
Protoparmalia badia (Hoffm.) Hafellner	AF070023	Australia
Protoparmaliopsis muralis (Schreb.) Rabenh.	AF159922	Australia
Rhizoplaca chrysoleuca (Sm.) Zopf	DQ321748	Turkey
Rhizoplaca chrysoleuca (Sm.) Zopf, U192	AF159924	Arizona
Rhizoplaca chrysoleuca (Sm.) Zopf, U302	AF159940	Kazakhstan
Rhizoplaca chrysoleuca (Sm.) Zopf, GenBank	AY530884	China
Rhizoplaca melanopthalma (DC.) Leuck. & Poelt	DQ321747	Turkey
Rhizoplaca melanopthalma (DC.) Leuck. & Poelt, U219	AF159929	Arizona
Rhizoplaca melanopthalma (DC.) Leuck. & Poelt, U278	AF159934	Arizona
Rhizoplaca melanopthalma (DC.) Leuck. & Poelt, U281	AF159935	Austria
Rhizoplaca peltata (Ram.) Leuck. & Poelt, MGH	DQ321749	Turkey
Rhizoplaca peltata (Ram.) Leuck. & Poelt, U198	AF159925	Arizona
Rhizoplaca peltata (Ram.) Leuck. & Poelt, U282	AF159936	British Columbia
Rhizoplaca peltata (Ram.) Leuck. & Poelt, GB	AY509803	China

Nine most parsimonous trees were generated by using *Ramalina farinacea* as outgroup. One of these trees generated by maximum parsimony is shown in Fig. 2. The trees yield similar topology indicating only slight rearrangements within the groups (a–e). Multiple alignments of samples of *Rhizoplaca* species with different *Lecanora* species (obtained from GenBank) yielded the information in the dendogram that *Rhizoplaca* species do not constitute a monophyletic branch. *R. melanopthalma* species group with *L. novomexicana* which is supported by a 84% bootstrap value. The other umblicate species from Anatolia, *R. chrysoleuca* and *R. peltata*, appeared elsewhere in the tree (Fig. 2; groups e and b, respectively), apart from the assamblage of *R. melanopthalma* and *Lecanora* species. *R. peltata* remained as a sister branch to this assamblage which is supported by a 100% bootstrap value.

Analysis with neigbour joining (Fig. 3) and minimum evolution (Fig. 4) displayed trees with similar topology with slight differences among groups (a–e) and within groups.

Discussion

DNA sequence analysis of lichens has yielded valuable information for more than a decade. The ITS region is particularly suitable for this purpose as it comprises two non-coding spacers, ITS1 and ITS2, which are less constrained than the coding regions of rDNA and therefore more likely to accumulate mutations (White *et al.*, 1990).

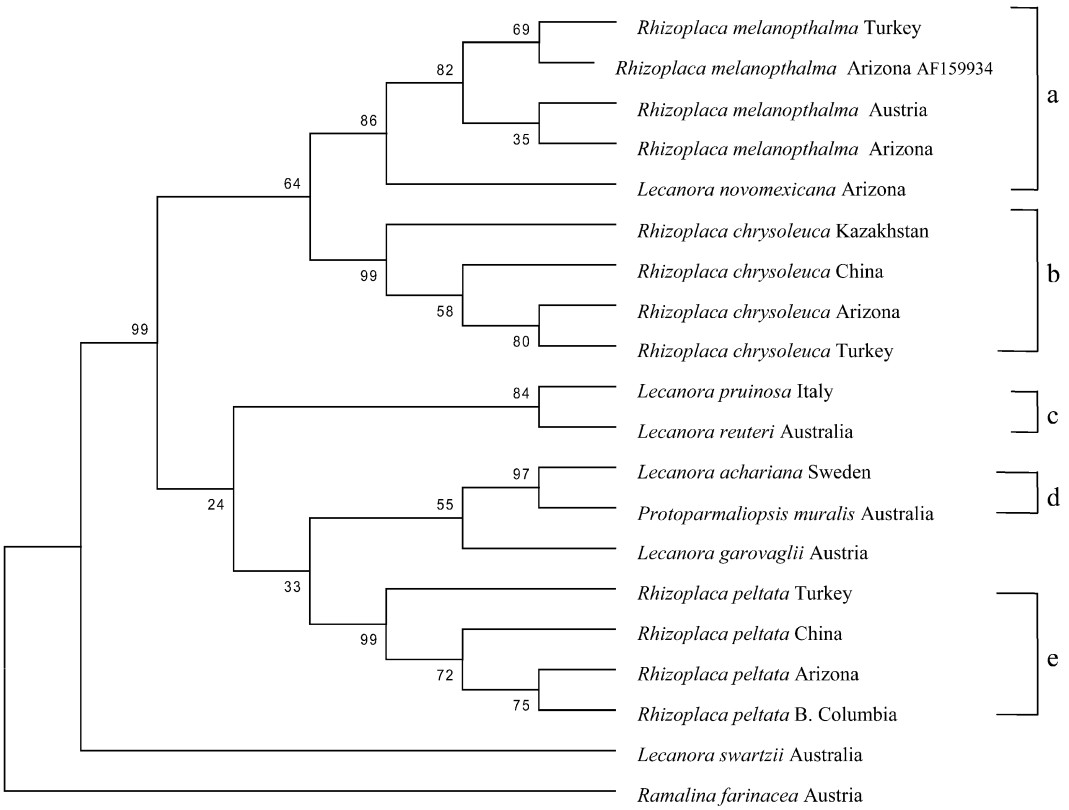

Fig. 2. Phylogenetic relations of three *Rhizoplaca* species from Anatolia and other *Rhizoplaca* and *Leconora* species. One of the nine equally parsimonious trees by maximum parsimony analysis is shown. Numbers at the nodes are bootstrap frequencies above 50%. Group placement is indicated as a–e.

Using the thallus morphology in lichen systematics to distinguish taxa at different levels works well for many species. For example, separation of the crustose Buelliaceeae from the foliose to fruticose Physciaceae almost completely depends on thallus morphology. Such examples are also found within the large family Lecanoraceae in which the genus *Rhizoplaca* is classified. In this family, two lobate genera *Arctopeltis* Poelt (Poelt, 1983) and *Rhizoplaca* (Leuckert *et al.*, 1977) have been separated from the genus *Lecanora* mainly by their umbilicate thalli with well developed upper and lower cortices (Arup and Grube, 2000).

Does the higher organization with an umblicate to foliose thallus represent a natural group? Arup and Grube (2000) tested repetitive taxa of *Rhizoplaca* that vary in thallus morphology, chemistry, disc color and ecology and concluded that the generally accepted concept of *Rhizoplaca* as a genus separate from *Lecanora* can be rejected. The data obtained from this study support this idea as the lichen materials collected from the volcanic rocks of Mount Erciyes located in the middle Anatolian province of Kayseri revealed similar sequence data information. The three species which have umblicate thalli are the only species found in Anatolia. When the phylogenetic tree was examined, *R. melanopthalma* from Turkey, two different samples of *R. melanopthalma* from Arizona (AF159934, AF159929) and a sample from Austria (AF159935) appeared on the same branch. The lobate species *L. novomexicana* (AF159945) appears in the same branch with *R. melanopthalma* as it was pointed earlier by Arup and Grube (2000) as an interesting outcome. Also *R. peltata* and *R. chrysoleuca* from Anatolia are located in two other branches of the tree that forms sister groups with the samples of the same species from different countries. On the other hand although *R. peltata* remained on the same branch with other *R. peltata* samples from other countries it took place in a different branch within the group and longer

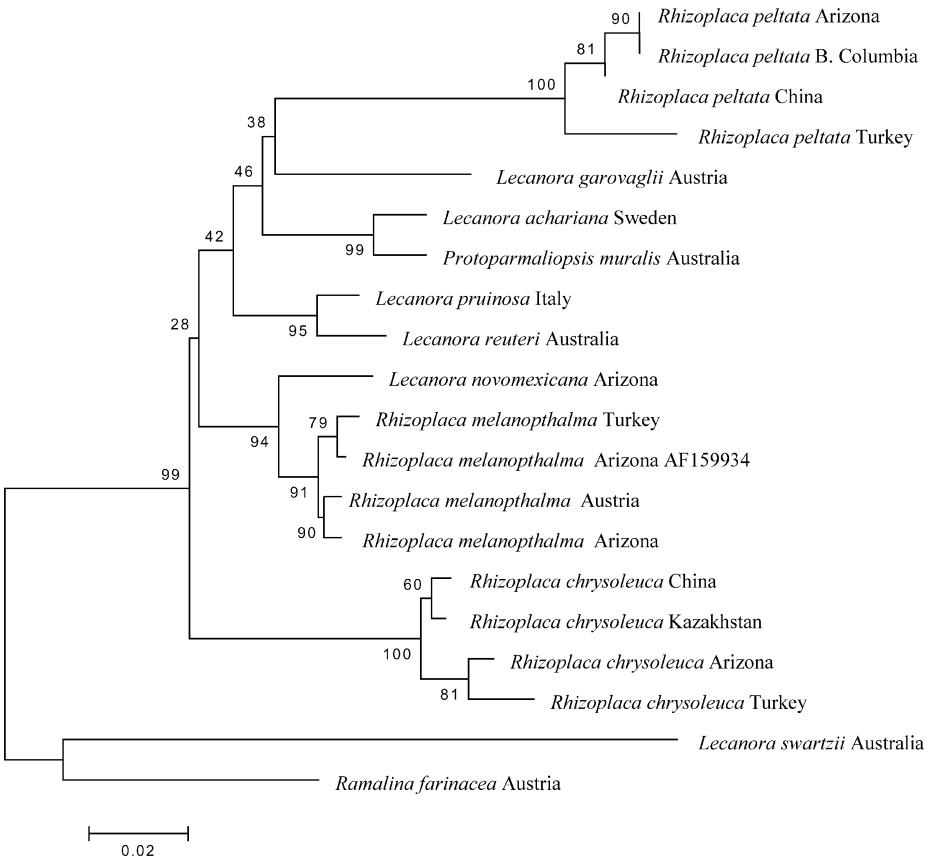

Fig. 3. Neighbor joining analysis inferred from ITS region sequences.

branch length might point the importance of geographical distribution or some other characteristics unique to that ecology.

When the three species from Anatolia were considered alone, it is noticed that *Rhizoplaca melanopthalma* and *Rhizoplaca peltata* are phylogenetically closer to each other than *Rhizoplaca chrysoleuca*; the morphological characteristics also support this result. From the examined species, *Rhizoplaca chrysoleuca* differs from *Rhizoplaca peltata* and *Rhizoplaca melanopthalma* by having pruinose, orange apothecial disks. The apothecial disks are yellowish brown and not pruinose in *R. peltata*, but yellow-brown to greenish or black and pruinose in *R. melanopthalma.* While the medulla gives P+ orange reaction (pannarin) in *R. peltata*, the medulla gives P+ bright yellow or less frequently P– (with or without psoromic acid) in *R. melanopthalma.*

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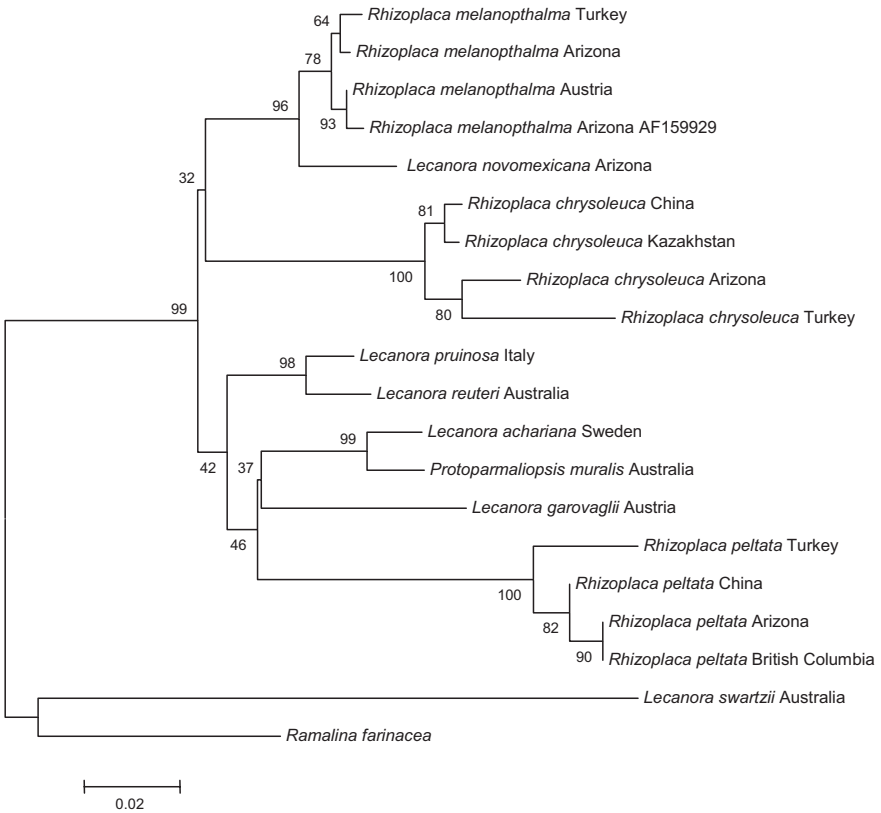

Fig. 4. Analysis by minimum evolution according to the data obtained by ITS region sequences from Anatolia and by the data obtained from GenBank.

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