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Identification of some *Lecidea*, *Porpidia* and *Lecidella* species (lichen-forming ascomycetes) distributed in Turkey by sequence analysis of rDNA ITS region

Türkiye'de yayılış gösteren bazı *Lecidea*, *Porpidia* ve *Lecidella* türlerinin (liken oluşturan ascomycetes) rDNA ITS bölgesinin dizi analizi yöntemi ile tanımlanması

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ABSTRACT

Objective: The taxonomy of *Lecidea* is extremely complex because of the enormous morphological variation within and between species. The aim of this study was to analyse the rDNA (ITS) regions of *Lecidea* species and related genus called *Lecidella* and *Porpidia* which are widely spreaded in Anatolia, Turkey.

Methods: The ITS rDNA sequence information of 17 samples from 11 species which were collected from different provinces of Anatolia were generated. Some of the specimens from *Lecidea*, *Lecidella*, Lecanora and *Porpidia* genus were also taken from the GenBank (www.ncbi.nlm.nih.gov). The phylogenetic analysis was performed by the help of four different methods (NJ, ME, MP, UPGMA) and these different methods manifested similar results.

Results: Minimum-Evolution (ME) dendrogram revealed that species of *Lecidea*, *Lecidella*, *Porpidia* and *Ganoderma* sp. genus were distributed into four main branches. *Ganoderma applanatum* (GU256764) which was considered as outgroup formed one of the branches, while the other species were collected on the other branchs. Generally the species which belong to the same genus, combined in one branch towards to the origin. In accordance with the results derived from

ÖZET

Amaç: *Lecidea* cinsine ait türlerin taksonomisi tür içi ve türler arasındaki büyük morfolojik farklılıkların olması nedeniyle oldukça karmaşa göstermektedir. Bu çalışma kapsamında, *Lecidea* türleri ve onunla ilişkili cinsler olarak bilinen ve Anadolu'da oldukça yaygın olan *Lecidella* ve *Porpidia* cins türlerine ait örneklere ait rDNA (ITS) bölgelerinin analiz edilmesi amaçlanmıştır.

Yöntemler: Anadolu'nun farklı bölgelerinden toplanan 11 türe ait 17 liken örneğine ait rDNA ITS dizi analizi verileri incelenmiştir. Diğer ülkelerde dağılım gösteren *Lecidea*, *Lecidella*, Lecanora ve *Porpidia* cinsine ait bazı türlerin sekans analizi bilgileri GenBank'dan (www.ncbi.nlm.nih.gov) alınmıştır. Filogenetik analizler dört farklı metotla (NJ, ME, MP, UPGMA) analiz edilmiştir ve analiz sonuçlarında oluşturulan dört faklı filogenetik ağaçta da benzer sonuçlar elde edilmiştir.

Bulgular: Minimum-Evolution (ME) analizine göre oluşturan filogenetik agaca göre *Lecidea*, *Lecidella*, *Porpidia* ve Ganoderma cinslerine ait türlerin dört ana dala ayrılmış olduğu tespit edilmiştir. Minimum-Evolution (ME) analizde *Ganoderma applanatum* (GU256764) türü dış-grup olarak kullanılmıştır ve çalışılan tüm türlerden ayrı bir dal oluşturmuştur. Çalışılan *Lecidea* ve onunla ilişkili cinslerin tür ayrımı karşılaştırıldığında genellikle aynı

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molecular phylogenetic analysis, genus *Lecidea* is found closer *Porpidia* rather than *Lecidella* morphologically. Numerable 4100 nucleotides were obtained from DNA sequences of related region of studied samples. It was indicated that 177 nucleotides of those regions were stable (C), 747 nucleotides were variable (V). It was confirmed that there were transitions in 56 nucleotide pairs, tranversion in 54 nucleotide pairs of compared samples.

Conclusion: In this study, the results of phylogenetic analysis of the genus *Lecidea* and other similar groups were firstly evaluated and the results will not be only a guide but also will provide a resource for next researchers.

Key Words: *Lecidea*, *Lecidella*, *Porpidia*, ITS

cinsine ait türler ana dala karşı bir dal oluşturmuşlardır. Moleküler filogenetik analizler sonucunda elde edilen sonuçlara göre *Lecidea* cinsi *Lecidella* cinsinden ziyade *Porpidia* cinsine daha yakın bulunmuştur. Çalışılan örneklerden ilgili bölgelerinin DNA dizisinden 4100 nükleotit elde edilmiştir. Bu nükleotidlerin 177 tanesi korunmuş (C), 747 nükleotit farklılaşmış (V) olarak belirlenmiştir. 56 nükleotit çifti transitions, 54 nükleotit çiftide tranversion olarak tespit edilmiştir.

Sonuç: Bu çalışma kapsamında *Lecidea* ve onunla ilişkili cinslerin filogenetik analizi literatürde ilk defa değerlendirilmiştir ve elde edilen sonuçların ileride yapılacak fungus türlerinin moleküler filogenetik yöntemlerle tanımlanması çalışmalarına kaynak sağlayacağı düşünülmektedir.

Anahtar Kelimeler: *Lecidea*, *Lecidella*, *Porpidia*, ITS

INTRODUCTION

The lichen genus forming *Lecidea* Ach. (*Lecidea*ceae, *Ascomycota*) is one of the most heterogeneous of Zahlbruckner's artificial system and a large number of genera have been separated from it, especially during the last 30 years (1). The taxonomy of *Lecidea* is extremely complex because of the enormous morphological variation within and between species. Some problems are associated with the discrepancies of morphological data. Most systematic studies on *Lecidea* have been concerned with saxicolous species groups, but a number of recent studies deal with the non-saxicolous species groups as well (1 - 3).

During the 150 years following Erik Acharius initial description of *Lecidea* in 1803, this genus became the "garbage bin" for crustose lichen taxa with generally green algal photobionts, photobiont-free apothecial margins and hyaline and single-celled ascospores. At its height, the genus had grown to include approximately 1600 species (2, 3). Only in the second half of the last century efforts were started, most significantly by Hertel (1, 4) to revise the genus based on morphological, chemical, ecological, and biogeographical data. As currently circumscribed *Lecidea* (*Lecidea*ceae) includes approximately 100 species whose within genus revision is currently under way (1). The species of this genus grow on rock with thalli that are thick to thin, continuous or composed of dispersed areoles. The members are often endolithic which appear white to ashy gray, orange or becoming orange because of iron compounds in the rock substrate. The genus *Lecidea* belongs to *Lecidea*ceae includes photobiont green (*Trebouxia*). They have characteristic apothecia lecideine which is disks black or very dark brown, lightly or heavily coated with white pruina in some taxa. Their paraphyses are branched and net-like, not conspicuously expanded at the tips. Their epihymenium pigment is brown, olive or green. They have eight per spores per asci with a distinctive tube

or cylinder in the tip. Their ascospores are one-celled which have colorless and large. Most of the species of this genus grow on siliceous or rarely calcareous rocks. A large percentage of crustose lichens on rock with large black apothecia (more than 0.75 mm in diameter) belong to this important genus. Other crustose lichens that are superficially similar with a hand lens include species of *Lecidea*, *Rhizocarpon*, *Sarcogyne* and *Buellia* (5).

To light on the clarification of the taxonomic status of *Lecidea* and related genera such as *Porpidia* and *Lecidella*, the sequence diversity in the internal transcribed spacer (ITS) region of nuclear ribosomal DNA was studied. *Porpidia* belongs to the largest group of the Lecanoromycetes. *Porpidia*, as well as the family *Porpidia*ceae, include exclusively crustose taxa that form colourful thalli on siliceous to slightly calcareous rock surfaces worldwide in association with their unicellular green algal photobionts of the genus Trebouxia (Chlorophyta) (6). Members of *Porpidia* are inhabitants of exposed to shaded, but always humid localities in temperate to arctic zones. The lichen genus *Porpidia* provides excellent opportunities for evolutionary, reproductive, and ecological studies of crustose epilithic lichen symbioses (3).

In its unclear taxonomic history *Porpidia* is closely entwined with the crustose genus *Lecidea*. One of the largest genera split from *Lecidea* is the genus *Porpidia*. Several revisions of the genus *Porpidia* based on traditionally employed morphological, chemical, ecological, and biogeographical characters exist (7-12). They are the most studied of the recent segregates of *Lecidea* (2, 8-11) but in spite of this, although it is usually easy to identify a lichen as a *Porpidia*, it is often very difficult, or impossible, to assign it to a particular species. Species concepts are still unclear within the genus with many of the characters used in separating taxa (e.g. chemistry, width of excipular hyphae) being revealed only after detailed microscopic/biochemical investigation. According to Buschbom and Mueller (3), it is probable that many of these problems are unresolvable by traditional taxonomic methods and that molecular techniques will be required to elucidate critical species boundaries.

The other related genus with *Lecidea*; the genus *Lecidella* was established by Körber (13). Zahlbruckner (14) placed it in *Lecidea* sect. Eu *Lecidea*. Hertel (4), restricted *Lecidella* to the *Lecidella elaeochroma* group which had been precisely characterized by Fries (15) and classified it as a subgenus of *Lecidea*. Hertel and Leuckert (16) revealed the special status of the taxon *Lecidella* by chemosystematic investigations and established it as a separate genus. In this respect the presence of derivatives of norlichexanthone in the taxon was recognized as a crucial character. The *Lecidella thalli* are gray but some with imperceptible thalli. Their apothecia are lecideine type which are pitch black, often shiny and with black margins level with the disk or prominent. The species of *Lecidella* grow on rocks (especially those containing calcium), bark, wood or soil (5). As to the infrageneric classification of the genus *Lecidella* for a long time it was based mainly on thallus colour and spot tests, which are both of limited value, and on morphological characters. As a result, many species were doubtful and their delimitations indistinct. By use of additional chemical and morphological characters it has been possible to overcome these obstacles (17 - 20). Important contributions to the chemistry of the genus were made by Huneck and Santesson (21) and Elix and Crook (22).

In general, it is systematically difficult to determine crustose lichens and less informative for resolving phylogenetic problems of lichen genera. Development of molecular techniques has been asisted to determine genetic similarities in lichen species. Recent molecular studies have been mainly used to reveal phylogenetic relationships between the specimens. Some scientists have been widely used for reconstructing phylogenetic relationships from the overall genome similarity (23, 24). The spacer regions like the internal transcribed spacers (ITS) and external transcribed spacers (ETS) are widely utilized in phylogenetics studies. The fact, that these regions are present in many copies in the genome is an advantage for laboratory practice and might be useful tools for phylogenetic analysis. The internal transcribed spacer (ITS) is intercalated in the 18S-5.8S-28S region separating the elements of the rDNA locus (25). One of the most preferred techniques to determine genetic relationship of lichens at lower taxonomic levels involves sequence diversity study of the ITS region of nuclear ribosomal DNA (26). The ITS region plays a role in ribosomal maturation and processing of small and large-subunit rDNAs (27). The evolutionary origin of the ITS is considered to be an intron-like structure flanked by highly conserved region from which universal primers can be obtained (28, 29). The small size of the ITS region makes this region easy to amplify, even from herbarium material that is dry. The other advantage in sequencing the ITS region is that it is non-coding and so includes a relatively high level of variability (27). Since its first application by Porter and Collins (30) in it has become widely used for phylogeny reconstruction.

It clearly indicates that the species concept in *Lecidea* needs revision and that molecular data are helpful in interpreting subtle morphological differences that have been previously regarded as intraspecific variability. It was investigated that ITS regions of *Lecidea*, *Lecidella*, and *Porpidia* lichen species for variability using PCR and automated sequencing. The identity of lichens from some *Lecidea*, *Lecidella* and *Porpidia* species has been determined by ITS rDNA sequence comparisons in order to estimate the diversity, to detect patterns of specificity. Specific PCR primers have been used to determine the ITS rDNA sequences from DNA extractions of dried lichens. There are 17 specimens from *Lecidea*, *Lecidella*, Lecidoma and *Porpidia* in Turkey as herbarium sample and in very small amounts. Other *Lecidea*, *Lecidella* and *Porpidia* lichen species recorded for Turkey were not available because most of the species were collected by foreign researchers in 1800s. The indicated locations of mentioned samples are not consistent with current settlement regions. Direct comparisons and phylogenetic analyses allowed the assignment of some *Lecidea*, *Lecidella* and *Porpidia* ITS rDNA phylogeny.

In this study, molecular techniques were applied on 17 samples of genus *Lecidea*, *Lecidella*, *Lecidoma* and *Porpidia* lichen which were collected from Anatolia, Turkey. 26 lichen samples related to *Lecidea*, *Lecidella*, *Lecanora* and *Porpidia* specimens were obtained from GenBank. Also *Ganoderma applanatum* (GU256764) was used to test reliability of analysis as a out-group. This is the first study with molecular markers and ITS sequence analysis on *Lecidea* and their related genuses which are *Lecidella* and *Porpidia* in Anatolia, Turkey and focused on revealing the genetic distances and also defining genotypes of the specimens of the species used in the study.

MATERIAL AND METHODS

Lichen materials

A total of 17 samples were used in the present study including *Lecidea* (4), *Lecidella* (3), *Porpidia* (3) and *Lecidoma* (1) species. Lichen samples were collected from different parts of Anatolia, Turkey and Table 1 was shown in localities. 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). Some of the lichen materials were provided from previously collected and stored material of Erciyes University Herbarium.

Table 1. Collection area of *Lecidea*, *Lecidella*, *Porpidia* and *Lecidoma* species

Accession number	Name of sample	Location
HQ605926	*Lecidea fuscoatra*	Ankara, Beynam Forest, N 36°53'920", E 32°55'005", 1450 m, 06.01.2009
HQ605929	*Lecidea fuscoatra*	Bilecik, North of Çaltı village, N 40°01'36", E 30°14'17", 573 m, 23.07.2007
HQ605930	*Lecidea fuscoatra*	Konya, Gevne village, N 36°53'127", E 32°19'124", 2100 m, 06.07.2007
HQ605928	*Lecidea fuscoatra* var. *grisella*	Trabzon, Of, Uzungöl province, N 40037'091", E 40036'885", 1240 m, 30.10.2008
HQ605931	*Lecidea fuscoatra* var. *grisella*	Trabzon, Maçka, surround of the Sumela Monastery, 1230 m, N 40°41'16", E 39°39'37", 29.10.2008
KF570277	*Lecidea syncarpa*	Trabzon, Maçka, surround of the Sumela Monastery, 1230 m, N 40°41'16", E 39°39'37", 29.10.2008
KF570280	*Lecidea plana*	Trabzon, Maçka, surround of the Sumela Monastery, 1230 m, N 40°41'16", E 39°39'37", 29.10.2008
HQ605936	*Lecidella elaeochroma*	Trabzon, Of, Uzungöl, N 40037'091", E 40036'885", 1240 m, 31.10.2008
HQ605938	*Lecidella elaeochroma*	Kayseri, Bakırdağ, Çataloluk village, N38°11'326", E 35°50'605", 1400 m, 15.06.2007
HQ605932	*Lecidella patavina*	Sivas, Gürün, Gökpınar Village, N 38°39'111", E 37°18'374", 1562 m, 12.06. 2007
HQ605934	*Lecidella patavina*	Niğde, Çamardı, Aladağlar National Park, Emli village, N 37°45'886", E 35°06'454", 1840 m, 19.06.2007
HQ605935	*Lecidella stigmatea*	Rize, Çamlıhemşin, around of Zilkale, N 40058'389", E 40057'505", 691 m, 30.10.2008
HQ605937	*Lecidella stigmatea*	Kayseri, Yahyalı Aladağlar National Park, Tekekalesi Dirsek village, N 37 °050'279", E 35°011'946", 3320m, 19.06.2007
HQ605941	*Porpidia crustulata*	Trabzon, Of, Uzungöl-Soğanlı road,N 40036'117", E 40016'682", 2210 m, 31.10.2008
HQ605940	*Porpidia macrocarpa*	Rize, Çamlıhemşin, Ayder plateau and Kavran plateau, N 40055'601", E 41007'603", 1700 m, 01.11.2008
HQ605939	*Porpidia musiva*	Trabzon, Of, Uzungöl-Soğanlı road, N 40036'117", E 40016'682", 2210 m, 29.10.2008
KF570278	*Lecidoma demissum*	Trabzon, Maçka, surround of the Sumela Monastery, 1230 m, N 40°41'16", E 39°39'37", 29.10.2008

DNA extraction, ITS amplification and sequencing

Total DNA was extracted from thallus or apothecia bu using DNA isolation protocol of herbarium material on lichen species (31). This protocol gives a high quality DNA, free of polysaccharides and other metabolites which might interfere with restriction endonucleases. In particular: lichen material (0.1g) was ground to a fine powder in liquid nitrogen. Prewarmed extraction buffer [50 mM Tris-HCL (pH 8), 50 mM EDTA, 0.8 M LiCl, 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, 0.2% ß-mercaptoethanol was added. The solution was incubated in 65 ^{o}C water bath for 15 min. Following these incubation periods, 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, and the supernatant was transferred to a fresh tube (~0.8 mL). Equal volume of isopropanol was added to the supernant and mixed gently by inversion several times. Incubation of the samples for at least 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). Supernatant was discarded and 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. The obtained nucleic acids as a pellet were 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 1 µL of ribonuclease A (10 mg/mL) and stored at -20 ^{o}C until use. Concentration and purity of extracted DNA were measured at OD 260 and by measuring 260 nm/280 nm absorbance ratio by nanodrop (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, USA), respectively. The integrity of the extracted DNA was also evaluated by electrophoresis.

ITS region (ITS1F-5.8-ITS4) was amplified by PCR using the primers ITS 1F was designed specificly for fungal sequences (5'-CTTGGTCATTTAGAGGAAGTAA-3') (32) for the 3' end of 18S rDNA and ITS4 was described as a universal primer (5'-TCCTCCGCTTATTGATATGC-3'), (29) for the 5' end of 28S rDNA. PCR amplifications for sequence analysis were performed in a 50 µL volume containing 30 ng of genomic DNA, 5 µL of 10 x reaction buffer, 2.5 mM MgCl2, 0.4 µL dNTPs (10 µM), 0.2 µM of each of the primers, and 1 U Taq polymerase (Fermentas, Canada). The thermal cycling for PCR comprised incubation at 94 ^{o}C for 3 min, and 35 cycles, each with 94 ^{o}C for 30 sn, 52-54 ^{o}C for 1 min, and extension of 1 min 30 s at 72 ^{o}C for 8 min. Two separate PCR reactions were carried to amplify rDNA (ITS) regions of DNA. In order to prevent non-specific bands appeared in some reactions the annealing temperature was increased to 54 ^{o}C in some cases. The amplified PCR products were purified using Beckman Coulter Genomel Lab DTCS Quick Start Kit according to manufacturer's instruction. PCR products and DNA markers (100 bp, Fermentas, Canada) were analyzed by electrophoresis in 1.2% agarose gel (AppliChem, Canada), containing 0,5 µL/mL ethidium bromide, for 2 h at 100 V.

After PCR amplification of the entire ITS region (ITS1-5.8S-ITS2), all species analyzed displayed a single band of PCR products of about 600 bp. The amplified fragments with the primers ITS1F and ITS4

comprising 3' end of small subunit gene, ITS 1F, the 5.8 S gene, ITS 4 and the 5' terminus of the large subunit gene, were sequenced. Sequence reactions were purified using the Beckman Coulter Agencourt Clean SEQ kit. The PCR products were sequenced by the cycle sequencing method using dye terminator cycle sequencing kit (Amersham Pharmacia, USA) according to the manufacturer's protocol. The purified and dried PCR-products were sent to Ankara University Biotechnology Institute for sequencing using the PCR primers. DNA sequence analysis was carried with Beckman coulter CEQ 8000 Genetic Analysis System in Biotechnology Institute.

Data Analysis

Chromatograms were manually checked using Chromas 2.01 (Chromas version 2.01; www.technelysium.com.au.chromas.html). The alignment of sequences (including out-group taxon) was done using CLUSTAL X2 (33). Two separate sequences which were obtained from one sample with forward (ITS1F) and reverse (ITS 4) primers derived from the ITS region were matched by the help of Clustal X2 programme. For comparison, lecideoid species from other parts of the world was sequenced and included in the data matrix together with one sequence downloaded from GenBank database (www.ncbi.nim.nih.gov) as shown in Table 2. Alignment of the sequences was performed visually, as gaps were few and easily interpreted. Insersion/deletion gaps were treated as missing data.

All data were analysed by Molecular Evolutionary Genetics Analysis (MEGA) 4 and a bootstraped dendogram was generated (34). To test for potential conflict, parsimony bootstrap analyses were performed on each individual dataset, and 75% bootstrap consensus trees were examined for conflict. Maximum Composite Likelihood analyses were performed using the program MEGA 4 (34). Bootstrapping was performed based on 1000 replicates with random sequence additions. Homoplasy levels were assessed by calculating consistency index (CI), retention index (RI), and rescaled consistency (RC) index from each parsimony search.

Table 2. Localities and their GenBank accession numbers

Species	GenBank No	Origin
Lecanora rupicola	AY541259	Austria
Lecanora rupicola	AY541265	Austria
Lecanora bicincta	AY541242	Austria
Lecanora bicincta	AY541243	Austria
Lecanora albella	AY541241	Austria
Lecanora farinacea	AY541261	Austria
Lecanora farinacea	AY541262	Austria
Lecanora carpinea	AY541246	Austria
Lecanora carpinea	AY541247	Austria
Lecidella elaeochroma	AY541275	Austria
Lecidella elaeochroma	EU266082	Korea
Lecidella stigmatea	JN873901	Austria
Lecidella stigmatea	JN873902	Austria
Lecidella patavina	JN873893	Austria
Lecidella patavina	JN873894	Austria
Lecidella carpathica	DQ534471	Korea
Lecidella carpathica	AY541274	Austria
Lecidea atrobrunnea	HQ650657	Germany
Lecidea atrobrunnea	GU074455	Austria
Lecidea atrobrunnea	GU074457	Austria
Lecidea fuscoatra	EU263922	Austria
Lecidea fuscoatra	HQ650662	Germany
Lecidea plana	EU259903	Austria
Lecidea plana	EU259904	Austria
Porpidia macrocarpa	EU263923	Austria
Porpidia speirea	HQ650631	USA
Ganoderma applanatum	GU256764	USA

RESULTS

The lichen genus *Lecidea* was struck together with the substrate and it was so hard to collect genus during field works. Therefore only 0.001-0.08 gr lichen samples were obtained from substrate. According to DNA extraction results, the concentrations of DNAs were approximately in the range of 34.09 – 933.5 ng/µL and 260 nm / 280 nm ratios. Purity of DNA was between 0.59-1.72.

In this study, rDNA (ITS) regions of four species from genus *Lecidea*, three species from genus *Lecidella*, three species from genus *Porpidia* and one species *Lecidoma* were amplified by using ITS1F forward and ITS4 reverse primers with the help of PCR and sequenced by genetic analyzer to reveal genetic similarities and variations among the specimens. A sequence matrix of 4100 nucleotide positions were analysed and had 747 variable positions, of which 336 were parsimony-informative sites were detected (34). The number of base substitutions per site from averaging over all sequence pairs between groups was shown Table 3. All results were based on the pairwise analysis of 44 sequences (27 samples obtained from GenBank+17 samples obtained from Turkey). Analyses were conducted using the Minimum Evolution (ME) method in MEGA 4. According to the analysis genus *Porpidia* was the closest to genus *Lecidea* with 0.043 distance index. Genus *Lecidoma* was the second with 0.053 distance index. *Ganoderma applanatum* was very distant to all studied samples with 0.179-0.241 genetic distance index (Table 3). When transitional changes were compared transversional ones, bias towards transversional changes were observed, with the transition pair value of 56 versus transversional value of 54 (Table 4). Dendograms were obtained according to different phylogenetic methods such as Neighbour-Joining (NJ), Minimum Evolution (ME), Maksimum Parsimony, UPGMA using the software MEGA 4. Bootstrapping was performed based on 1.000 replicates with random sequence additions. To test for potential conflict, parsimony bootstrap analyses were performed on each individual dataset, and 75% bootstrap consensus trees were examined for conflict. Homoplasy levels were assessed by calculating consistency index (CI), retention index (RI), and rescaled consistency (RC) index from each parsimony search. The trees yielded similar topology showing only slight rearrangements within the groups. Since the topologies of the MP; ML, NJ and UPGMA analyses did not show any strongly supported conflicts, only Minimum-Evolution (ME) dendogram was shown in Figure 1.

Table 3. Estimates of evolutionary divergence over sequence pairs between groups

A	B	C	D	E	OG
1. A					
2. B	0.024				
3. C	0.130	0.095			
4. D	0.053	0.023	0.100		
5. E	0.043	0.061	0.076	0.076	
6. OG	0.241	0.187	0.204	0.198	0.179

A: *Lecida* **C:** *Lecidoma* **E:** *Porpidia*
B: *Lecidella* **D:** *Lecanora* **OG:** Out Group

Table 4. Numbers and base pairs of compared samples

Related region	ii	si	sv	R=si/sv	TT	TC	TA	TG	CC	CA	CG	AA
ITS region	382	56	54	1.03	84	37	11	11	110	14	19	79
	AG	GG	TOTAL									
	19	109	491.79									

ii = Identical Pairs, si = Transitionsal Pairs, sv = Transversional Pairs, R = si/sv

It was observed that the species were divided into two branches and both of them showed binary branches according to dendogram obtained from Minimum Evolution (ME) analysis. *G. applanatum* (GU256764) species constructed one of the branches while the other species clustered on the other branch (Figure 1). A result of the Minimum Evolution (ME) analysis without out-group (*G. applanatum*) four major clades were formed. Generally the species which were belong to the same genus, combined in one branch towards to the origin. According to dendogram, species from genus *Lecidea* were close to species from *Porpidia* genus phylogenetically, while the species from *Lecidella* genus located on a separate branch from the species in *Lecidea* and *Porpidia* genus (Figure 1). *Lecidea fuscoatra*

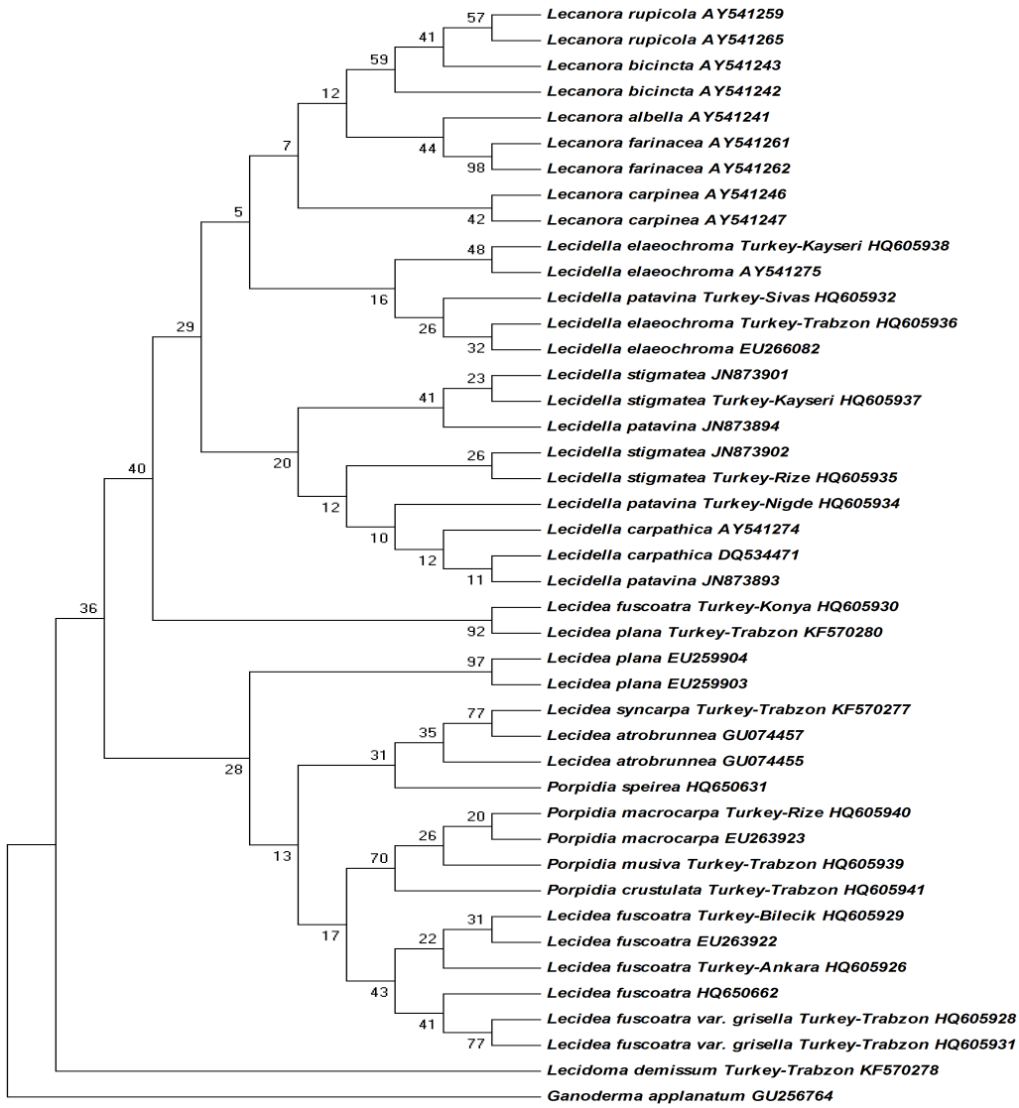

Figure 1. Minimum Evolution analysis inferred from ITS region sequences which is shown phylogenetic relations of 17 samples from *Lecidea, Lecidella, Lecidoma* and *Porpidia* genus. Numbers at the nodes are bootstrap frequencies above 40%.

which was collected from two different provinces of Konya was located on a separate branch from Ankara, Bilecik and Trabzon *L. fuscoatra* samples. Thus, it showed genetic difference from the other samples (Figure 1). *Porpidia musiva* species showed phylogenetic proximity to *Porpidia* species while *P. macrocarpa* showed phylogenetic proximity to *P. crustulata* species. Two of *Lecidella patavina* and *Lecidella stigmatea* samples which were collected from different localities were located on a separate branches. These two species showed proximite branches (Figure 1). Trabzon and Korea (EU266082) *Lecidella elaeochroma* samples showed boot strap value (32%) and they formed a group. Also Bakırdağ-Kayseri and Austria (AY541275) samples of the same species showed boot strap value (48%) and formed another group (Figure 1).

The results of this phylogenetic analysis indicates that four distinct lineages of *Lecidea* sp. and related genus occur in Anatolia, Turkey. Four dendograms were obtained according to different phylogenetic methods. The trees yielded similar topology showing only slight rearrangements within the groups. We demonstrated only Minimum-Evolution dendogram because analysis with Maximum Parsimony (MP) and Minimum-Evolution revealed trees with similar topology with slight differences among the groups and within groups.

DISCUSSION

The diversity of lichens, especially crustose species, is still poorly known (35). To overcome difficulties with the morphology based species delimitations in these groups, we evaluated molecular data (nuclear ITS and rDNA sequences) to test species boundaries within the genus *Lecidea* (35). Molecular data are largely absent for Turkey lichens, with only a few exceptions (36, 37). To overcome difficulties of assessing species diversity in Turkey lecideoid lichens, we employed molecular data (nuclear ITS ribosomal DNA sequences) to test species boundaries. Based on our phylogenetic estimate we re evaluated morphological characters to identify characters that can be used to identify these distinct lineages. Here we have focused on samples belonging to the genus *Lecidea*, *Lecidella* and *Porpidia*.

Ruprecht et al. (35), sampling was done along a north-south transect at five different areas in the Ross Sea region. Phylogenetic analyses also include specimens from other regions in Antarctica and non-Antarctic areas. According to their results, maximum parsimony, maximum likelihood and Bayesian analyses agreed in placing the samples from continental Antarctica into four major groups. Based on this phylogenetic estimate, their restudied the micromorphology and secondary chemistry of these four clades to evaluate the use of these characters as phylogenetic discriminators. These clades are identified as the following species *Lecidea* cancriformis, *L. andersonii* as well as the new species *L. polypycnidophora* Ruprecht & Turk sp. nov. and another previously unnamed clade of uncertain status, referred to as *Lecidea* spp. (35). Ruprecht et al. demonstrate that the diversity of *Lecidea* spp. in continental Antarctica is higher than previously thought (35). Geographical data evaluation also shows a decreasing diversity of *Lecidea* species the more continental and drier the habitats are. It clearly indicates that the species concept in *Lecidea* needs revision and that molecular data are helpful in interpreting subtle morphological differences that have been previously regarded as intraspecific variability (35).

This is especially true for crustose lichens that are often reduced to minute patches surrounding ascomata under the harsh climatic conditions typical of this ecosystem. The number of available collections is limited, which restricts the ability to assess variability within species. Because of the

poor understanding of morphological and chemical variation, their taxonomy is currently in urgent need of revision. Lecideoid lichens often act as pioneers on rock and pebbles (38). Saxicolous lecideoid lichens in Turkey include species of the genera *Carbonea, Lecanora*, *Lecidea*, and *Lecidella*. Despite the ecological importance of these lichens in polar habitats, the taxonomy is only poorly known and the circumscription of taxa differs between authors.

According to Buschbom and Mueller (3) the lichen-forming genus *Porpidia* (*Porpidia*ceae, Ascomycota) provided excellent opportunities for evolutionary, reproductive, and ecological studies of crustose epilithic lichen symbioses. Separate and combined analyses of nuclear ribosomal RNA large subunit and nuclear ß-tubulin gene fragments were performed using maximum parsimony, maximum likelihood, and Bayesian approaches in their study. Branch support was estimated using non-parametic bootstrapping and posterior probabilities, while monophyly of a priori defined groups was tested using posterior probabilities. The results revealed a highly supported ''*Porpidia* sensu lato,'' however, *Porpidia* itself was not monophyletic. Several smaller genera of the *Porpidia*ceae and probably the large genus *Lecidea* (*Lecidea*ceae) were nested within the group (3). The present study was also indicated that the species of the genus *Porpidia* are nested within *Lecidea* as shown in previous study.

A large percentage of crustose lichens on rock with large black apothecia (more than 0.75 mm in diameter) belong to *Porpidia* genus. Other crustose lichens that are superficially similar with a hand lens include species of *Lecidea*, *Rhizocarpon, Sarcogyne* and *Buellia*. *P. crustulata* together with the very similar *P. macrocarpa*, make up the bulk of the nonpruinose species of *Porpidia* (5). In our study, *P. crustulata* and *P. macrocarpa* composed a close branch on the three that is supported with a 96% bootstrap value. In *P. macrocarpa*, the apothecia tend to be a larger (up to 3.5 mm in diameter-por crus 0.13-1.5 mm in diameter); the spores are larger (13-23 x 7-10 µm other 10-17 x 5-9 µm) the hymenium is higher (80-120 µm other 60-90 µm), and cells of the exciple are smaller (mostly 3-6 µm in diameter other 5-8 µm). The gelatinous halo around the spores helps distinguish species with 2-celled spores from similar species of *Buellia* or *Catillaria*. *Porpidia* has 1-celled spores and a different ascus type. *Lecidea* species have much smaller, 1-celled spores, and their paraphyses are mostly unbranched (5). According to our study result of phlogenetic analyses and morphological characters of species in Anatolia, Turkey were similar results.

The *Porpidia* genus is one of the most studied of the segregates of *Lecidea* (2, 3, 7, 9-11). The reasons for this are largely because of the difficulty in recognizing species-level characters within the genus. Most species of *Porpidia* have a grey thallus and black sessile apothecia and, as macroscopic characters (e.g. thickness of thallus, size of apothecia) appear to be extremely variable within a single species, specimens are usually impossible to identify beyond genus in the field (5). It was aim of the study molecular studies of the *Lecidea* genus and related genus have greatly enhanced our understanding of the infrageneric relationships, thus permitting a re-assessment of morphological characters that should result in a clearer understanding of species concepts within the genus.

The analyses of Buschbom and Mueller indicated that the genus *Porpidia* could be divided into three infra-generic groups, with a high probability that the *Lecidea*ceae s. str. (i.e. *Lecidea* and *Cecidonia*) was nested within them (3). This suggested that either the *Porpidia*ceae should be included within the *Lecidea*ceae (and *Porpidia* within *Lecidea*), or that *Porpidia* should be divided into at least three, and possibly four, separate genera. According to our results, species of *Porpidia* genus in Anatolia, Turkey

remained relatively close to *Lecidea* group samples. However, Buschbom and Mueller (3) studied only a limited number of the relevant taxa, and because of the lack of consistent supporting morphological/ chemical differences, they preferred to await the results of further analyses of all the available character systems (molecular, morphological, and chemical) before making any taxonomic innovations. *P. crustulata* is a common species in upland and montane habitats. It is an early colonizer of bare rock surfaces and is frequent around areas of prolonged snow-lie. It is distinguished from *P. macrocarpa* in the field by its smaller apothecia and microscopically by its smaller ascospores, lower thecium and thicker excipular hyphae (5).

Lecidella may be hard to distinguish from other black disk lichens such as *Lecidea*, *Porpidia* or *Buellia*. They are, however, fairly easy to recognize under the microscope by the combination of their easily separating paraphyses, greenish tissues, and broad spores (5). In the current study *Porpidia* was the closest to genus *Lecidea*. *Lecidella carpathica* differs mainly in its dark yellowish brown hypothecium and darker exciple; it contains atranorin and diploicin (an orcinol depsidone). *Lecidella* carpathica which were obtain from GenBank located on a separate branch from the species in *Lecidea*, *Lecidella* and *Porpidia* genus. Because of these reasons could be explained synerjistic effect of the seconder metabolite. *Lecidella patavina* (syn. *L. spitsbergensis*) has the same chemistry as *L. stigmata*, but its hymenium is filled with oil drops, its exciple contains crytals, and the thallus tends to be thicker. *Lecidella elaeochroma* located on a separate branch from the other *Lecidella* species because of this result *Lecidella elaeochroma* which differs only in its C positive orange thallus (arthothelin).

Dissimilarity index and dendrogram data which are conducted as a result of this research reveals the genomic similarities and differences of studied samples. By determining molecular phylogeny of the samples showing the variation in different habitats and populations, certain taxonomic values have revealed. The distant taxa relations have been defined by ITS region amplification and sequencing. Wide range of information is provided on Anatolia, Turkey of *Lecidea* and *Lecidella*, *Porpidia* samples. A molecular study involving the *Lecidea*, *Lecidella* and *Porpidia* species that are present in Anatolia, Turkey have not been carried out so far. Furthermore these genus are quite less molecular studies in the world literature. Thus, current study is the first report on *Lecidella*, *Porpidia* genus in the world.

Lichens are major sources of biodiversity in terms of wide variety of species in Anatolia. If the study extends by using more species and localities, ITS variations among the species involving *Lecidea*, *Lecidella* and *Porpidia* will help to enlighten evolutional differentiation of the lichens. Those type of studies will make contribution to reveal and protect the rich gene potential of Turkey. It is truly important that those type of studies will be very beneficial to find solutions to the taxonomic problems of certain samples and to reveal their position in lichen systematic on molecular dimension.

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