

Full Length Research Paper

Resolving genetic relationships in manna group of lichens from genus *Aspicilia*

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As in many lichen-forming fungi, species of *Aspicilia* genus are widely distributed all over the world, but no reports exist about their phylogenetic relations based on molecular biological methods. In the current study the phylogenetic relations of some *Aspicilia* species mainly manna group of lichens were investigated. The ITS rDNA sequence information of 12 samples from six species were generated. The samples examined were collected from different provinces of Anatolia and all the sequences were aligned with the other allied groups; *Pertusaria* sp., *Thamnolia* sp., *Dibaeis* sp., *Diploschistes* sp., *Ochrolechia* sp. and *Lecanora* sp. sequence data obtained from GenBank. The phylogenetic tree obtained by minimum evolution analysis displayed two major branches. One of the branches with its six members (*Aspicilia contorta* subsp. *contorta*, *A. contorta* subsp. *hoffmanniana*, *Aspicilia hispida*, *Aspicilia fruticulosa*, *Aspicilia desertorum* *Aspicilia calcarea*) composed solely of *Aspicilia* samples from Anatolia. Three of the manna groups of lichens were placed in this branch of the tree. The other related taxa from Ostropomycetidae and Lecanoromycetidae took place in the other branch of the tree with *Aspicilia* samples from Anatolia. Results displayed that manna group of lichens, although do not represent taxonomical unit tend to form a group especially apparent by the Neighbour Joining analysis with Anatolian samples. Also the sequence information from Anatolian samples displayed that *Aspicilia* genus is phylogenetically closer to the orders and families from Ostropomycetidae subclass rather than Lecanoromycetidae which support the placement of Hymeneliaceae within Ostropomycetidae.

Key words: *Aspicilia*, phylogenetic relations, ITS, Anatolia

INTRODUCTION

The genus *Aspicilia* belongs to Hymeneliaceae within the subclass Ostropomycetidae with photobiont chlorococcoid which have one shape. Most of the species of this genus grow on calcareous and acidic rocks and most of the taxa prefer temperate and arctic sites. The members are weakly cracked to distinct areolate, scattered to whole thallus. Some of the species of this genus are placodioid with radiating marginal lobes which appear chalky white, grayish, greenish or brownish. Some possess isidia and soredia. They have characteristic ascomata apoth-

ecia aspicilioid which is mostly immersed but occasionally emergent. Their epithecium pigment is from brown to green with N and K chemical tests. They have 4 to 8 spored asci with cylindrical to clavate shape. Their ascospores are simple which have ellipsoid to globose shape and colorless and also contain thin-wall. They often contain β -orcinol depsidones such as norstictic and stictic acids; others have fatty acids or triterpenes. In genus *Aspicilia* radical changes in growth form are very common, such as some taxa may display extreme transition within the same population or even changes within the same thallus could be observed (Elenkin, 1901; Weber, 1967). For example, for *Aspicilia desertorum* which is a manna group of lichen, transformation to crustose form of lichen could arise under micro-environmental

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ntal conditions of temperature and small melt water (Kunkel, 1980), although the significance of this phenomenon in general taxonomy of *Aspicilia* is not clear.

Manna group of lichens are unique in *Aspicilia* genus with their many specific characteristics. The use of Manna lichens as food is probably very old. The practice has survived into modern times chiefly in impoverished environments, such as deserts, steppes, and tundra, means beyond the limits of cultivation; elsewhere, lichens are occasionally reported as a famine food. *Lecanora esculenta* has been identified with the 'manna' of the Hebrews, although the lichen has not been observed in Sinai, either in situ or in the form of windborne deposits. However this in itself is not decisive for explicit reports of such deposits elsewhere in western Asia do not go back beyond the 19th century (Donkin, 1981). One of the manna group lichens, generally known as *L. esculenta* (Elenkin, 1901) has been placed within *Aspicilia*, and now known as *Aspicilia esculenta*. Mereschkovsky (1911) was the first to try an account of the *A. esculenta* group and to distinguish further taxa among the vagrant. In addition to some taxa known for a long time, namely *A. esculenta* (Pall.) Flagey, *Aspicilia fruticulosa* (Eversm.) Flagey, *A. desertorum* (Kremp.) Mereschk., Hafellner et al. (2004) described two further species (*Aspicilia hispida* Mereschk. and *Aspicilia lacunose* Mereschk.) and some infraspecific taxa at the rank of forma or variety, one of which was later raised to species level (*A. desertorum* var. *aspera* Mereschk.) (Hafellner et al., 2004).

Genetic concept within Lecanoraceae has been slightly changed although the knowledge is still based on the pioneer work of Eigler (1969). Later some of the taxa were excluded by Hafellner (1984) and *Aspicilia* is now defined as a separate genus, in the family Hymeneliaceae (Hafellner, 1994; Poelt, 1974) which takes place within subclass Ostropomycetidae (Wedin et al., 2005).

The current research investigates the phylogenetic relationships of the manna group of lichens from *Aspicilia* grown in Anatolia and related groups. The purpose of the present investigation is to clarify the phylogenetic relationships of some *Aspicilia* species especially manna group of lichens grown in Anatolia and to provide a clear phylogenetic framework as a basis for detailed phylogenetic and population genetic investigations. The manna lichens; *A. fruticulosa*, *A. hispida*, *A. desertorum* and other *Aspicilia* species (*A. calcarea* and *A. contorta*) collected from various parts of Anatolia were used in the study. The phylogenetic relationships were analyzed based on ITS sequence data. The sequence data aligned with 5 *Lecanora* sp., one *Pertusaria* sp., one *Thamnotia* sp., one *Dibaeis* sp., three *Diploschistes* sp. and two *Ochrolechia* species obtained from GenBank.

MATERIALS AND METHODS

Lichen materials

12 *Aspicilia* species were collected from different localities which

were shown in Table 1. The samples were dried at room temperature and foreign matters were removed prior to grinding. The lichen samples are stored in the Herbarium of Anadolu University (Anadolu University, Department of Botany, Eskisehir, Turkey). Some of the lichen materials were provided from previously collected and stored material of Anadolu University Herbarium. The collection localities were as shown in Table 1.

DNA extraction

DNA extraction was performed according to the protocol defined by Cansaran et al. (2006). In particular: lichen herbarium material (0.1 g) 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% β -mercaptoethanol was added. The solution was incubated in 65°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 supernatant 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 μ L). The nucleic acids dissolved in TE buffer, were treated with 1 μ l of ribonuclease A (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 and a 100 bp DNA ladder was used (Promega) as the DNA size marker.

PCR amplification and sequencing

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

Different parameters were tested for optimization of PCR reactions. PCR amplifications for sequence analysis were performed in a 50 μ L volume containing 200 ng of genomic DNA, 5 μ L of 10 x reaction buffer, 2.5 mM MgCl₂, 20 μ M dNTPs, 0.2 μ M of each of the primers, and 1 U *Taq* DNA polymerase (Promega). Amplification was performed in a Techne Progene thermal cycler (Techne Cambridge Limited). The reactions were heated in an initial step of 94°C for 2 min and then subjected to 35 cycles of 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 for final extension step. The amplification products were analysed by electrophoresis in 1.2% agarose gel containing ethidium bromide and the product sizes were determined on agarose gels along with a known nucleotide size marker (100 bp ladder, Promega). The PCR products were sequenced by the cycle sequencing method using dye terminator cycle sequencing kit (Amersham Pharmacia,

Table 1. Collection area of *Aspicilia* species.

Species	Collection area
<i>Aspicilia contorta</i> subp <i>hoffmanniana</i>	Malatya, Hekimhan, Midemuşığı (Mollaibrahim) village, northern slopes, 23.07.2004, 1205 m, 38°43'20.5 N-38°04'22.4 E (leg. M. Candan) ANES 10200
<i>Aspicilia contorta</i> subp <i>hoffmanniana</i>	Elazığ, Harput, North of Serince village, calcareous rocks, 04.08.2004, 1580 m, 38°43'56 N- 39°17'18 E (leg. M. Candan) ANES 10201
<i>Aspicilia contorta</i> subp <i>hoffmanniana</i>	Adıyaman, Varlık village, South of Yoğurtul Mezra, 30.07.2004, 1160 m, 37°54'05 N-38°17'47 E (leg. M. Candan) ANES 10202
<i>Aspicilia contorta</i> subp <i>contorta</i>	Kütahya, North of Dumlupınar, 03.07.2001 (leg. et det. V. John) ANES 7391
<i>Aspicilia calcarea</i>	Adıyaman, Kahta, Southeast of Karakuş Tümü, Calcareous rocks, 28.07.2004, 830m, 37°52'34.6 N- 38°35'35 E (leg. M. Candan) ANES 10203
<i>Aspicilia calcarea</i>	Adıyaman, Besni, Southwest of Çilboğazi village, 27.07.2004, 900 m, 37°44'08 N-37°48'06.3 E (leg. M. Candan) ANES 10204
<i>Aspicilia calcarea</i>	Sivas, Kangal, Hekimhan-Sivas 46. km, 23.07.2004, 1530 m, 39°04'01.2 N-37°41'43.4 E (leg. M. Candan) ANES 10205
<i>Aspicilia calcarea</i>	Malatya, Doğanşehir, West of Kurucuova village, 30.07.2004, 1410 m, 37°59'26 N-38°03'42.4 E (leg. M. Candan) ANES 10206
<i>Aspicilia desertorum</i>	Elazığ, Harput, North of Serince village, 04.08.2004, 1580 m, 38°43'56 N- 39°17'18 E (leg. M. Candan) ANES 10207
<i>Aspicilia desertorum</i>	Elazığ, Sivrice- Hazar Mountain, 05.08.2004, 1225 m, 38°27'36 N- 39°23'10 E, leg. M. Candan)
<i>Aspicilia hispida</i>	Malatya, Darende, Southeast of Zengibar Castle, 24.07.2004, 1105 m, 38°33'59.9 N-37°30'07 E (leg. M. Candan) ANES 10209
<i>Aspicilia fruticulosa</i>	Sivas, Kangal, Hekimhan-Sivas 46. km, 23.07.2004, 1530 m, 39°04'01.2 N-37°41'43.4 E (leg. M. Candan) ANES 10210

USA) according to the manufacturer's protocol and OpenGene[®] automated DNA sequencing system (Visible Genetics, Canada).

Sequence analysis

The amplified fragments with the primers ITS1 and ITS4 comprising 3' end of small subunit gene, ITS 1, the 5.8 S gene, ITS 2 and the 5' terminus of the large subunit gene, were sequenced. Ambiguities arising from reading errors were resolved by comparing the complementary strands with the programme Clustal W 1.83. The sequences were also aligned using the same programme and adjusted visually. Ambiguous aligned sites were excluded from analysis and gaps were treated as missing data. ITS sequences for 12 other different samples from *Aspicilia* genus and for 5 different species of *Lecanora*, one *Pertusaria*, one *Thamnolia*, one *Dibaeis*, three *Diploschistes* and two *Ochrolechia* species obtained from GenBank (Table 2) were included in to multiple alignments (Figure 1). A sequence matrix of 866 nucleotide positions were analysed

and (conserved, 52; variable, 671) 539 parsimony informative sites were detected. All data were analysed by MEGA 3 (Kumar et al., 2004) and a bootstrapped dendrogram was generated.

RESULTS

A total of six species of Manna group of lichens and related species belonging to *Aspicilia* genus were included to the analysis. Nucleotide sequences generated from *Aspicilia* samples were deposited to GenBank (Table 2). The sequence data yielded from ITS region of the species were aligned with sequences of related taxa obtained from GenBank. The target sequences generated by PCR amplification were sequenced in both directions. The sequencing reactions for species of *Aspicilia* yielded alignable sequences of 866 nucleotides long. Within these

Table 2. The aligned species, localities and their GenBank accession numbers.

Species	GenBank accession No.	Origin
<i>Aspicilia calcarea</i>	DQ401563	Turkey-Malatya
<i>Aspicilia calcarea</i>	DQ401562	Turkey-Sivas
<i>Aspicilia calcarea</i>	DQ401561	Turkey-Adiyaman2
<i>Aspicilia calcarea</i>	DQ401560	Turkey-Adiyaman1
<i>Aspicilia contorta</i> subsp <i>contorta</i>	DQ401559	Turkey-Afyon
<i>Aspicilia contorta</i> subsp <i>hoffmanniana</i>	DQ401558	Turkey-Adiyaman
<i>Aspicilia contorta</i> subsp <i>hoffmanniana</i>	DQ401557	Turkey-Elazig
<i>Aspicilia contorta</i> subsp <i>hoffmanniana</i>	DQ401556	Turkey-Malatya
<i>Aspicilia desertorum</i>	DQ401568	Turkey-Elazig 2
<i>Aspicilia desertorum</i>	DQ401567	Turkey-Elazig1
<i>Aspicilia fruticulosa</i>	DQ401570	Turkey-Sivas
<i>Aspicilia hispida</i>	DQ401571	Turkey-Malatya
<i>Dibaeis baeomyces</i>	DQ782844	USA
<i>Diploschistes hensseniae</i>	AJ458291	Australia
<i>Diploschistes thunbergianus</i>	AJ458290	Australia
<i>Diploschistes scruposus</i>	AJ458287	Germany
<i>Lecanora achariana</i> A.L. Sm.	AF070019	Sweden
<i>Lecanora garovaglii</i> (Kölber) Zahlbr	AF189718	Austria
<i>Lecanora novomexicana</i> H. Magn., U 363	AF159945	Arizona
<i>Lecanora pruinoso</i> Chaub.	AF070018	Italy
<i>Lecanora reuteri</i> Schaer	AF070026	Austria
<i>Ochrolechia parella</i>	AF329174	Germany
<i>Ochrolechia juvenalis</i>	AY640957	USA
<i>Perusaria erubescens</i>	DQ219303	Antarctica
<i>Thamnolia subuliformis</i>	AY961605	New Zealand

these sites 52 were conservative and 671 were variable whereas 539 sites were parsimony informative. When transitional changes were compared transversional ones, bias towards transversional changes was observed, with the transition pair value of 80 versus transversional value of 122. When the base compositions were analysed the range values of 16.9 – 33.0; 20.4 – 33.1; 19.0 – 27.1 and 19.4 – 28.3 were observed for bases T(U), C, A, G respectively.

Nine most parsimonious trees were generated by aligning the sequence data also with allied groups. One of these trees generated by minimum evolution is shown in Figure 1. The trees yielded similar topology showing only slight rearrangements within the groups. Analysis with maximum parsimony and Neighbor joining revealed trees with similar topology with slight differences among the groups and within groups.

DISCUSSION

For the past two centuries the classification of lichenized genera, was guided primarily by morphological and chemical characters. As lichen thalli manifest a number of

easily observable morphological characters, this kind of classification was relatively easy and straight forward. Lichens with a more complicated organization such as foliose or fruticose growth usually show more thallus characters. Although those anachronistic approaches are still largely sufficient and provide well-definition of foliose and fruticose genera, they remain relatively insufficient for some large crustose genera. In spite of their heterogeneity, due to low number of synapomorphic characters, it is not always possible to separate them from the large complexes (Grube et al., 2004).

Aspicilia is one of the examples of large and crustose genus from Hymeneliaceae family, which is the member of the subclass Ostropomycetidae. The classification of Hymenelioid lichens has been an argument among lichenologists throughout the history. Körber (1855) originally circumscribed the Hymeneliaceae on the basis of 'pseudogymnocarpic' apothecial development and a double excipulum, forming a link between *Lecanora* and *Lecidea* Arch., and including *Hymenelia*, *Petractis* Fr. and *Thelotrema* Arch. In 1984 Hafellner regarded Hymeneliaceae as a 'still poorly understood family' and put a question mark especially to *Aspicilia* (Hafellner, 1984). In 1989 Hafellner totally excluded *Aspicilia* and closely related



Figure 1. Phylogenetic relations of 12 *Aspicilia* species from *Anatolia* and other *Pertusaria*, *Thamnomlia*, *Dibaeis*, *Diploschistes*, *Ochrolechia* and *Lecanora* species. One of the nine equally parsimonious trees by minimum evaluation analysis is shown. Numbers at the nodes are bootstrap frequencies above 50 %.

genera from Hymeneliaceae (Lutzoni and Brodo, 1995). Eriksson and Hawksworth (1993) in a relatively recent study formed a list by including nine genera in the Hymeneliaceae that also consist *Aspicilia*. Lutzoni and Brodo (1995) reclassified the species groups, classified under *Hymenelia* and *Ponaspis* in relation to *Eiglera* and *Aspicilia*, and declared that the status of *Aspicilia* was not changed particularly due to lack of data and is still classified within Hymeneliaceae (Lutzoni and Brodo, 1995).

Wedin et al. (2005) investigated Ascomycota with combined nLSU rDNA and mtSSU rDNA sequence datasets. *Aspicilia* and *Icmadophiliaceae* were remained within Pertusariales, but these relationships lack jackknife support and only low (86) posterior probability. Exclusion of these two groups, remaining Pertusariales as monophyletic has resulted in trees only four steps longer than the most parsimonious. Although *Icmadophiliaceae* should be treated in Pertusariales, it formed an (unsupported) sister group to the *Aspicilia*. *Hymenelia lacturis* formed together with *Tremolocia atrata* as unsupported sister group to Arctomiaceae and *Moelleropsis humida*. These results suggested the polyphyletic nature of Hymeneliaceae.

The current study comprises the first document about phylogenetic relationships of manna group of lichen from *Aspicilia*. The sequence data obtained were also aligned with related groups, with *Ochrolechia* sp., *Diploschistes* sp., *Pertusaria* sp., *Dibaeis* sp., *Thamnomlia* sp., and *Lecanora* sp. Alignment that included representatives of other genera provided assessment of the relationship of *Aspicilia* genus in a larger phylogenetic context. *Aspicilia* was separated from *Lecanora* as a genus of its own and placed to a different family. Reeb et al. (2004) according to the results of their study with nuclear ribosomal genes (SSU and LSU) and protein coding genes RPB2, renamed the subclass Ostropomycetidae. Also they showed that Pertusariales + Icmadophiliaceae is sister to Ostropales + Baeomycetales + Hymeneliaceae clade rather than Lecanoromycetidae (Reeb et al., 2004). The data obtained from this study also support the concept that *Aspicilia* should be regarded as a genus of its own.

In the current study, the revealed sequence data from 12 *Aspicilia* taxa were aligned with related taxa from Ostropomycetidae and samples from Lecanoromycetidae. The phylogenetic tree obtained by minimum evolution analysis displayed two major branches. One of the

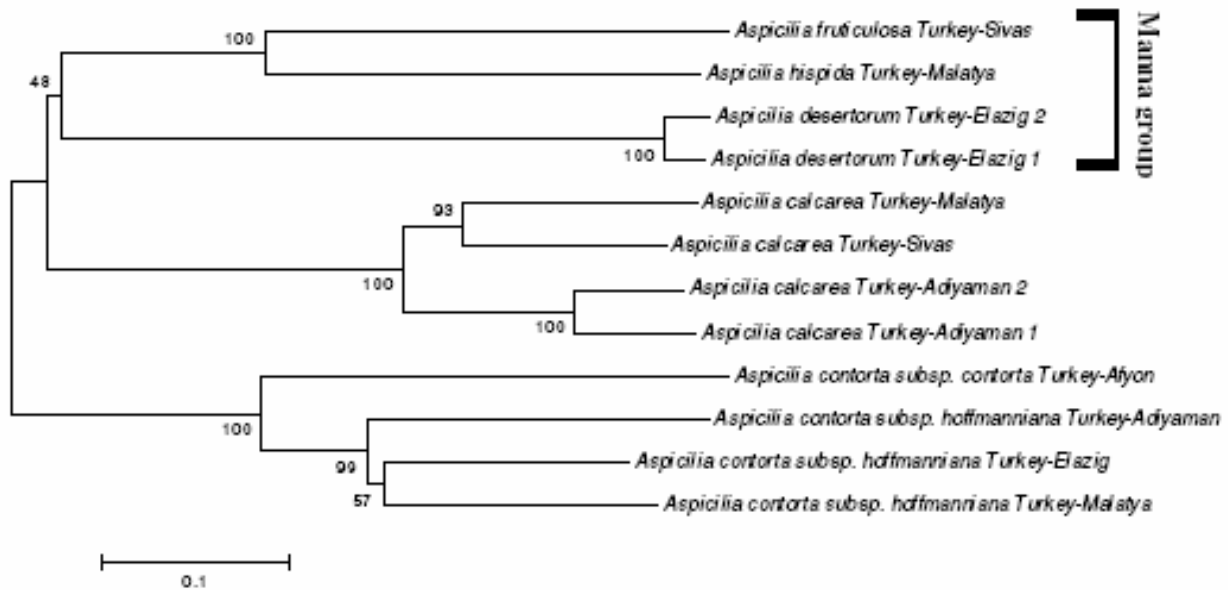


Figure 2. Neighbor joining analysis inferred from ITS region sequences.

branches with its six members (*Aspicilia contorta* subsp. *contorta*, *A. contorta* subsp. *hoffmanniana*, *A. hispida*, *A. fruticulosa*, *A. desertorum*, *A. calcarea*) composed solely of *Aspicilia* samples from Anatolia. Three of the manna groups of lichens were placed in this branch of the tree. The other related taxa from Ostropomycetidae and Lecanoromycetidae took place in the other branch of the tree with *Aspicilia* samples from Anatolia. Although manna group of lichens share few common morphological characters and do not represent a taxonomical unit results suggest close relations in phylogenetic context.

A. hispida from eastern province of Malatya and *A. fruticulosa* from middle Anatolian province of Sivas and which were all manna group of lichens composed a group on the tree that is supported with a 100% bootstrap value. Two samples of *A. desertorum* another manna group of lichen; collected from eastern city of Elazığ generated close genetic relation to each other. Although *A. desertorum* samples remained relatively close to other manna group of lichens (*A. hispida*, *A. fruticulosa*, *A. desertorum*.) they constitute another group with samples of *A. calcarea* species (60% support) which is not defined as a manna group of lichens. On the other hand these two species *A. desertorum* and *A. calcarea* use the same type of substrate; means both grow on calcareous rocks.

A. contorta, like *A. calcarea* is a species close to manna group. *A. contorta* subsp. *contorta* from Afyon province and three *A. contorta* subsp. *hoffmanniana* from eastern provinces generated another sister group with a 100% bootstrap value. On the other hand although regarded as a sister group *A. contorta* subsp. *contorta* from Afyon province remained in a different branch with a rather longer branch length. The data is consistent to the morphological data when main characters distinguishing

A. contorta subsp. *contorta* and *A. contorta* subsp. *hoffmanniana* are considered. Rico (1999) reported the differences, in thallus, areole, apothecia, ascospore size, between two subspecies (Rico, 1999). Although Eckman and Fröberg (1988) have identified a heterogeneous large group of intermediates between *A. contorta* subsp. *contorta* and *A. contorta* subsp. *hoffmanniana* and declared the hybrids between the parental forms might be the origin of these intermediates, we did not have enough data with respect to sample number and intermediates, to test this concept.

Hafellner et al. (2004) also discussed morphological variation in *Aspicilia* under steppe-like environmental conditions and thought that only the entire *Aspicilia contorta* / *calcarea* group was worth to be considered as a distinct taxon or it could well be a subgenus within *Aspicilia*.

The monophyletic nature and lower genetic diversity revealed as branch length for the related genera were prominent. The data for related genera obtained from GenBank comprised of particularly orders from Ostropomycetidae and also Lecanoramycetidae. *Pertusaria erubescens* from Pertusariaceae remained close to the *Aspicilia* species from Anatolia while the other members of Pertusariaceae formed another group relatively far apart. On the other hand samples from Thelotremaaceae and Lecanophylaceae remained closer to *Aspicilia* species compared to the species from Lecanoromycetidae. Results obtained from the samples collected from Anatolia show that *Aspicilia* genus is phylogenetically closer to the orders and families from Ostropomycetidae subclass rather than Lecanoramycetidae. These results support the study of Wedin et al. (2005) which strongly suggest the placement of Hymeneliaceae and also Pertusariales and Lecanophylaceae within Ostropomycetidae.

Samples from Anatolia analysed with Neighbor Joining analysis without aligning with the related groups, revealed species regarded as manna as one group, apart from *A. calcarea* and *A. contorta* which were defined as related species to manna group of lichens (Figure 2).

In a sense, it could be interpreted that *Aspicilia* sp. from Anatolia might represent a group of its own with characteristics unique to that ecology. *Aspicilia* species grown in Anatolia manifested greater genetic diversity revealed as longer branch length, with a polyphyletic nature (Figure 1). The higher genetic diversity observed, might be the reflection of rich flora and high potential of germplasm consisting almost 9000 different species found in Anatolia with more endemic species than whole Europe continent. But this interpretation should be confirmed with a wide spectrum study including *Aspicilia* sp. from different continents throughout the world.

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