The Journal of Microbiology, December 2004, p.292-298 Vol. 42, No. 4

Introduction of Saxicolous Lichens Distributed in Coastal Rocks of U-do Islet in Jeju, Korea

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(Received September 13, 2004 / Accepted December 3, 2004)

This study reports, for the first time, the ivestigation of the distribution of Korean saxicolous lichens in the coastal rocks of U-do islet, which is known as an unpolluted zone in Jeju. More than thirty lichens were obtained and investigated from the coastal rocks frequently contacted by seawater. A molecular analysis using PCR amplification of the rRNA ITS regions revealed the coastal rock lichens could be placed into 8 families and 14 genera, Ramalinaceae (*Bacidia, Ramalina*), Physciaceae (*Buellia, Dirinaria, Phaeophyscia, Physcia, Pyxine*), Lecanoraceae (*Candelaria, Lecanora*), Parmeliaceae (*Xanthoparmelia*), Graphidaceae (*Graphis*), Pertusariaceae (*Pertusaria*), Rhizocarpaceae (*Rhizocarpon*), and Teloschistaceae (*Caloplaca*), showing a diversity of lichens, with foliose (flat leaf-like), crustose (crust-like), and fruticose (miniature shrub-like) life forms might be distributed in the coastal rocks. These findings suggested the possibility that the lichens identified in the present work might be resistant to a salty environment.

Key words: coastal rock lichens, ITS, phylogeny, U-do, Jeju

Lichens are unique organisms that are composed an alga (either green or blue-green) and a fungus (either a basidiomycete or ascomycete). These two organisms live together in a state of symbiosis, the algae photosynthesizing and providing food for the lichen, with the fungus being the structure or matrix to which the algae sticks. Thus, they need each other as shown in Fig. 1, which was drawn by our microbiology research group. Lichens are commonly separated into three groups: foliose, crustose and fruiticose. They are found on several different strata, including trees (corticolous species), rocks (saxicolous species), and soil (terricolous species) (Armstrong and Platt, 1993; Nash, 1996; Brodo *et al.*, 2001). Due to the complexity of identification and lack of knowledge of lichens, they are often avoided as the subject for scientific study by both biologists and naturalists.

Compared with other organisms, lichens are rarely studied due to the extreme difficulties encountered in the research process. These difficulties include the lack of sufficient, up-to-date literature, taxonomic keys, and the lack of reference collections. Because of the limited literature on lichens, especially crustose lichens, there has become an increasing need for this type of research project.

Rapid advances in molecular biology have resulted in the development of culture-independent approaches for describing bacterial communities without bias, i.e., the selectivity of the total community due to cultivation. DNA

Fig. 1. A representative painting of the remarkable marriage between algae and fungi. The symbionts needed each other in order to live a life in diverse environments such as trees, soils and rocks, etc.

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fingerprinting allows for the rapid assessment of the genetic structure of complex communities in diverse environments and the extent of changes caused by environmental disturbances. DNA fingerprinting analyzes part of the genetic information, mostly on the ribosomal operon, contained in the nucleic acids directly extracted from environmental samples. The target genes are amplified by PCR, with the amplified fragments subsequently differentiated by their size or sequence variability. More recently, attempts have been made to use these approaches to characterize fungal communities, as culture-dependent methods have similar limitations to those for bacteria (Gardes and Bruns, 1993; Fischer and Triplett, 1999; Borneman and Hartin, 2000; Peters *et al.*, 2000; Bhattacharya *et al.*, 2002; Martin *et al.*, 2003). For analysis of lichen-forming fungi the molecular technique can be a useful means, considering the possibility of two or more fungi living together with one algae. There are two internal transcribed spacers (ITS), which show the sequence polymorphism of the fungal nuclear ribosomal DNA (rDNA) region and the 5.8S rRNA gene (ITS1-5.8S-ITS2). Information on the length heterogeneity of the ITS1-5.8S-ITS2 region was examined by searching the GenBank database to assess the extent of variability within the main fungal taxonomic groups.

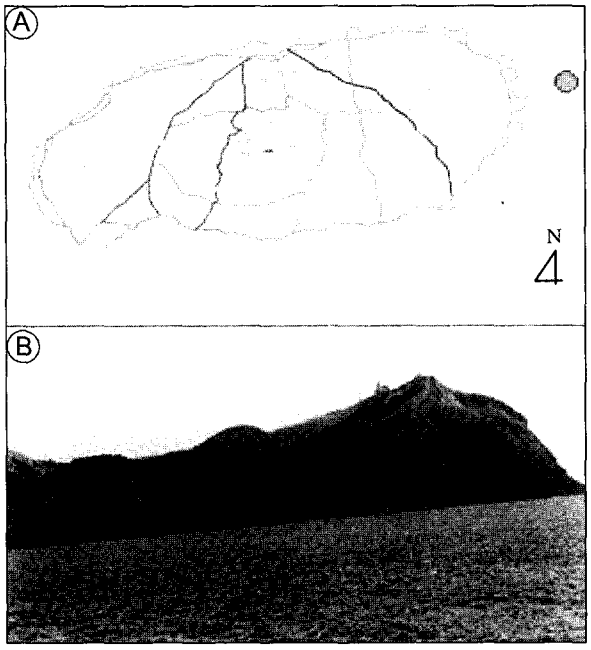

Fig. 2. The map for lichen collection in Jeju, Korea. The red circle in panel **A** indicates the sampling site, U-do located in 33°30'23"-27'1" N, 126°56'19"-29" E. Panel **B** shows the view of U-do. Lichens distributed in coastal rocks were observed, photocopied, and collected for lichen study in the laboratory on April 6, 2003.

Korean lichens have been studied for long time by a few biologists or lichenologists (Kim and Lee, 1975; Cho and Lee, 1980; Kim, 1981; Park, 1983; Park, 1990; Moon *et al.*, 1991; Hur *et al.*, 2004), but very limited knowledge or information on saxicolous lichens, especially distributed in coastal rocks, is available. Therefore, this study aimed at creating an inventory of the more common saxicolous

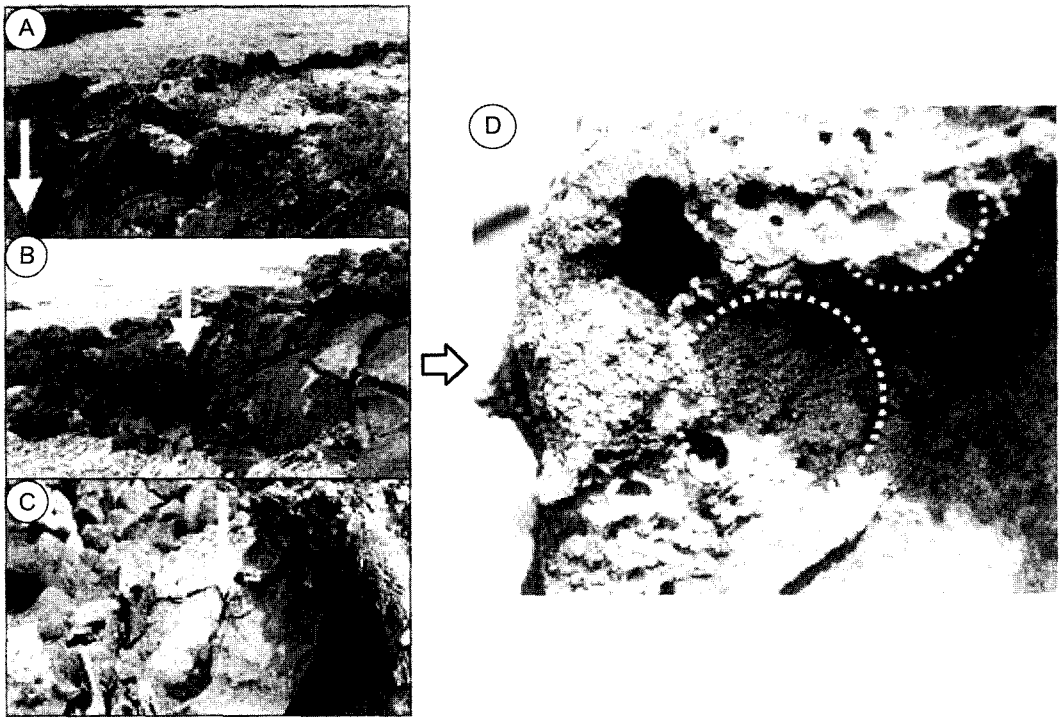

Fig. 3. A view of the coastal rocks of U-do, where saxicolous lichens were observed and collected. Panels **A, B** and **C** are the photo images taken at the different angles, with the arrows indicating the lichens distributed in the coastal rocks frequently contacted by seawater. Panel **D** is the close-up photo image of lichen, showing different the types.

lichens in coastal rocks of U-do, which is located in 33°30'N, 126°56'E (Fig. 2). This study reports for the first time on the saxicolous lichens distributed in the coastal rocks of U-do frequently contacted by seawater using both genetic and morphological analyses.

Materials and Methods

Collection and storage of lichen samples

Coastal rocks along the U-do islet, such as those shown in Fig. 3, were inspected, and specimens taken that could be removed using a screwdriver, mallet or utensils. All specimens were labelled with the collection date and site, and taken to the laboratory for identification of the level of species (or genus) using a molecular approach based on the intergenic spaces (ITS) of the 5.8S-18S rRNA sequence region along with lichen morphology such as thallus color, thallus classification (crustose, foliose, fruticose or squamulose), apothecia, either irregular or round and cuplike, with or without exciple, and spore characteristics, including color and size. The lichen samples used in this study were chosen due to their different geographic origins. All samples were collected and stored at room temperature until used.

DNA extraction

DNA was directly extracted from little sample of lichen as follows: Lichen sample was ground and powdered in the bowl. During this process, liquid nitrogen was periodically added to prevent DNA breakage. The powdered sample was transferred to an Eppendorf tube containing the lysis buffer from MOBIO (USA) DNA extraction kit. The phenol-extraction method was also used for some lichens, as follows: 100~500 mg of lichen-containing rock sample were distributed into 4 separate microcentrifuge tubes using a spatula. Seventy five ml of 500.mM EDTA (pH 9.4) were added and gently mixed by tapping. The tubes containing lichen samples were frozen in liquid nitrogen and thawed quickly by placing in a water bath that was heated for 80 sec in a microwave. This step was repeated four times, and the sample resuspended in 225 ml miniprep solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0) and 100 ml of a lysozyme solution (4 ml of miniprep solution I, and a pinch of lysozyme). 50 ml of 10% SDS was added, followed quickly by 800 ml of phenol-chloroform, in an extraction hood. The mixture was vortexed for 1 min to form an emulsion and then centrifuged at 14,000 × g for 3 min. The top phase (aqueous, pink) was transferred to a new tube, and 800 ml of phenol-chloroform added. It was vortexed and spun for 3 min, and the top layer then transferred to a new tube and the lichen DNA precipitated with miniprep solution II (2 ml glycogen. Thirty ml 3 M sodium acetate and 1 ml 100% ethanol). The mixture was centrifuged at 14,000 × g and 4°C for approximately 15 min, and the supernatant was decanted off. Excess supernatant was pulled off with a pipette following pulse centrifugation for a few seconds, and then dried in the speed vacuum for 5 min. The pellet was resuspended in 50 ml sterile miliQ water, and visualized by gel electrophoresis. The DNA solution was adjusted to 1 ml, 1 gram of caesium chloride added and the suspension ultracentrifuged overnight. The DNA band was extracted from the microcentrifuge tubes and dialyzed for 40 min by placing a 0.025 μm filter on the surface of a petri dish containing sterile water, with the introduction of an aliquot of the DNA sample onto the filter. The DNA was recovered from the membrane filter, and used for PCR amplification. The recovered DNA was stored at -70°C until used.

Amplification of ITS of lichen rDNA

The 5.8S-18S rRNA sequences were obtained following PCR amplification with universal primers designed in this study (Table 1). The intergenic space (ITS) of the lichens from the coastal rocks was amplified using the primer sets described in Table 1, with three different concentrations of lichen DNA, from 10 ng to 50 ng. All the procedures for PCR amplification were performed according to the method described previously (Kahng *et al.*, 2001). All reactions were carried out in 25 μl volumes, containing

Table 1. Oligonucleotide sequences for PCR primers used in this study

Primers	Sequences	References or Sources
NS5	5'-AAC TTA AAG GAA TTG ACG GAA G-3'	White *et al.* (1990)
NS6	5'-GCA TCA CAG ACC TGT TAT TGC CTC-3'	White *et al.* (1990)
KL9F	5'-AMC YTG CGG AAG GAT CAT TAC-3'	This study
KL9R	5'-TTA TTG ATA TGC TTA AGT TCA G-3'	This study
KL6F	5'-GAG AGA GGG GCT TCG YGC TCC CG-3'	This study
KL6R	5'-CGA WCT TTC RRR GCG GAT GA-3'	This study
KL7F	5'-CYA MCC GCC CCC RMC TCT TCY AC-3'	This study
KLUR	5'-GCA ATG TGC GTT CAA AGA YTC-3'	This study

12.5 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate, 2.5 μl of 10x PCR buffer(100 mM TRIS-HCl, 15 mM $MgCl_2$ and 500 mM KCl, pH 8.3), and 0.5U of *Taq* DNA polymerase (Roche Diagnostics, Germany), increased to 25 μl with sterile water. PCR was performed in a PCR machine (Perkin Elmer, USA) with the following thermo-cycling program: 5 min denaturation at 95°C, followed by 33 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C, 1 min extension at 72°C, and a final extension step of 10 min at 72°C. Ten microliters of PCR products were visualized by electrophoresis in 2% (w/v) agarose gels, with ethidium bromide (0.5 μg/ml) staining. To avoid contamination, all solutions were sterile prepared by autoclaving twice and treated with hard UV for 90 min in 1 mL aliquots.

Construction of 5.8S-18S rRNA ITS clonal libraries and screening of ITS clones

To construct an ITS rDNA clonal library from the lichen samples, the PCR products were ligated into the pGEM-T vector and transformed into *E. coli* JM109. At least five clones were selected from each of the three plates containing transformants originated from the different DNA concentrations. Plasmid DNA from selected clones was purified and stored at -20°C for DNA sequencing. The insert DNA was analyzed by the restriction enzymes, *Apa*I and *Sac*I.

Sequencing and phylogenetic analysis of ITS from saxicolous lichens

Nucleotide sequencing was carried out using an ABI 377A automated sequencer according to the method previously described (Kahng *et al.*, 2001). The sequence results obtained from the different lichen clones were comparatively analyzed based on database homology searches using the Blast algorithm, and a phylogenetic tree generated with the ITS sequences from D/B. For DNA sequence analysis, the Lasergene software programs, Megalign and GCG pileup were used. All the 5.8S rRNA sequences included in ITS were deposited in the GenBank. Identification of lichens at the genus level was drawn based on the combined data obtained from the morphological and genetic analyses.

Results and Discussion

Lichens have been very broadly and intensively studied in America and Europe owing to appearance of molecular tool, as they can be used for diverse purposes including biological indicators for environmental pollution. Lichen can even live in poor environments, which are very dry and exposed to sunlight, owing to the remarkable marriage between fungi and algae (Fig. 1). In addition, many bioactive compounds for pharmaceutical and medical use have been found in these mysterious microorganisms,

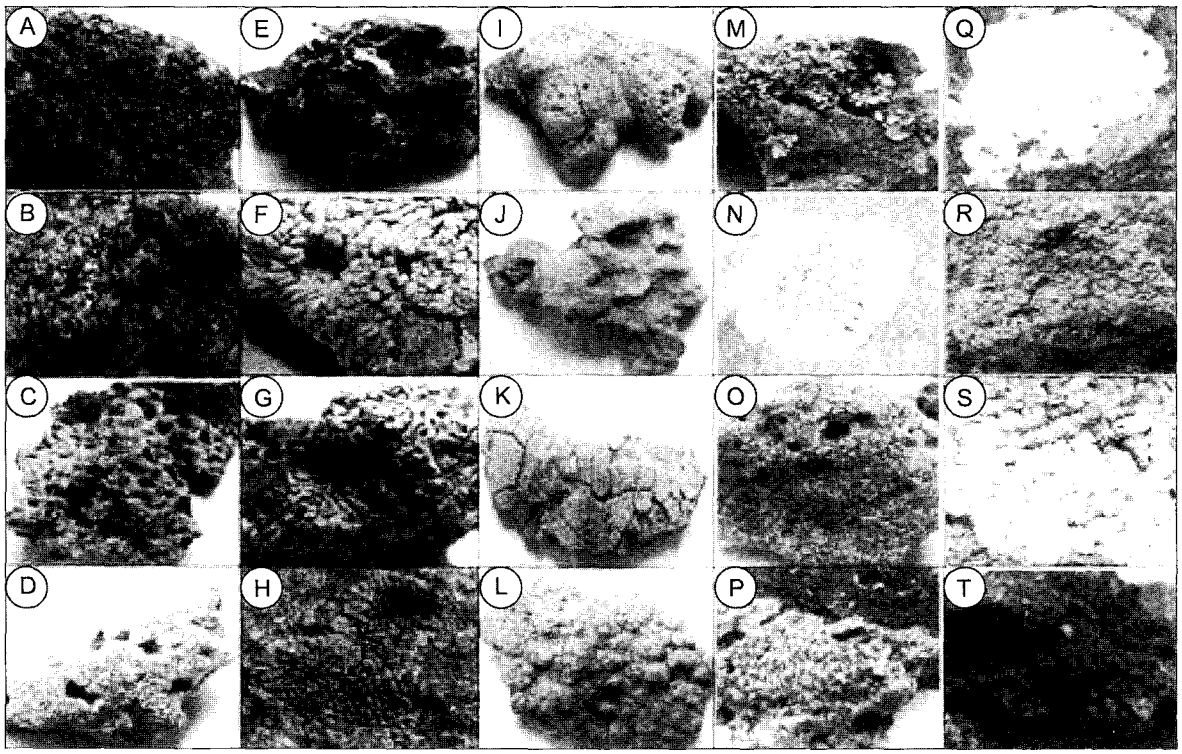

Fig. 4. Representative saxicolous lichens distributed in the coastal rocks of U-do, In the eastern part of Jeju. Panels **A, B** and **C,** *Candelaria* sp.; **D,** *Graphis* sp.; **E,** *Ramalina* sp.; **F,** *Physcia* sp.; **G,** *Phaeophyscia* sp.; **H,** *Caloplaca* sp.; **I, J, K** and **L,** *Lecanora* sp.; **M,** *Pyxine* sp.; **N,** *Dirinaria* sp.; **O,** *Rhizocarpon* sp.; **P,** *Porpidia* sp. **Q,** *Bacidia* sp.; **R,** *Pertusaria* sp.; **S,** *Xanthoparmelia* sp.; **T,** *Verrucaria* sp.

which attract the attention of both microbiologists and lichenologists. U-do, which is located in 33°30'N, 126°56' E, is known as an unpolluted zone on a very small island in Korea (Fig. 2). This environment pushed us to study then lichens distributed at U-do. Our initial study focused on rock lichens, because diverse saxicolous lichens have been observed, as shown in Fig. 3, and were assumed to experience frequent contact with seawater. More than thirty lichens frequently contacted by seawater were removed from coastal rocks at the U-do Islet, Jeju, Republic of Korea. A morphological analysis suggested that at least twenty lichen-forming fungi, foliose, crustose and fruiticose types were distributed in the coastal rocks (Fig. 4). Most lichens were crustose, but foliose types, such as *Xanthoparmelia*, *Physcia* and *Phaeophyscia,* were also found with the fruticose lichen, *Ramalina*. Identification for the lichens, based on rRNA ITS as well as morphological characteristics, revealed 8 families, Ramalinaceae, Physciaceae, Lecanoraceae, Parmeliaceae, Pertusariaceae, Rhizocarpaceae, Grapidaceae and Teloschistaceae, including fifteen genera, distributed in the coastal rocks of U-do (Table 2). Fourteen of the genera were: *Bacidia, Ramalina, Buellia, Dirinaria, Phaeophyscia, Physcia, Pyxine, Candelaria, Lecanora, Xanthoparmelia, Graphis, Pertusaria, Rhizocarpon,* and *Caloplaca* (Figs. 4 and 5). The genera *Bacidia* and *Ramalina* found in this study belonged to Ramalinaceae. Two types of *Ramalina* sharing extensive homology with *R. americana* and *R. siliquosa* were found, and at least three types of *Bacidia* were distributed at U-do. The genera *Buellia, Dirinaria, Phaeophyscia, Physcia,* and *Pyxine* belong to the family Physciaceae. *Buellia* was distributed in the upper region of the investigated coastal rocks and stones on the farm fences, which might experience rare contact with seawater. The genera *Dirinaria, Phaeophyscia, Physcia,* and *Pyxine* were observed in similar places. The genera *Candelaria* and *Lecanora* belong to the family Lecanoraceae, *Xanthoparmelia* to Parmeliaceae, *Pertusaria* to Pertusariaceae, *Rhizocarpon* to Rhizocarpaceae, *Graphis* to Graphidaceae, and *Caloplaca* to Teloschistaceae.

Previous studies on rock lichens have suggested that coastal rocks are typically characterized by a maritime community of yellow and gray lichens such as *Xanthoria parietina* and *Caloplaca marina* (Ulrik and Lutzoni,

Table 2. Identification of saxicolous lichens from coastal rocks or stones of farm fences very close to the sea based on morphology and ITS sequence studies

Taxon	Strata	References (location)
Graphidaceae		
Graphis sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Teloschistaceae		
Caloplaca sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Lecanoraceae		
Candelaria sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Lecanora sp.	Stones of farm fence near sea	Rarely contacting with seawater
Parmaliaceae		
Xanthoparmelia sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Pertusariaceae		
Pertusaria sp.	Coastal rocks (Basalt)	Rarely contacting with seawater
Physciaceae		
Buellia sp.	Stones of farm fences near sea	Rarely contacting with seawater
Dirinaria sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Phaeophyscia sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Physcia sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Pyxine sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Ramalinaceae		
Bacidia sp.	Coastal rocks (Basalt)	Rarely contacting with seawater
Ramalina sp.	Coastal rocks (Basalt)	Frequently contacting with seawater
Rhizocarpaceae		
Rhizocarpon sp.	Coastal rocks (Basalt)	Rarely contacting with seawater

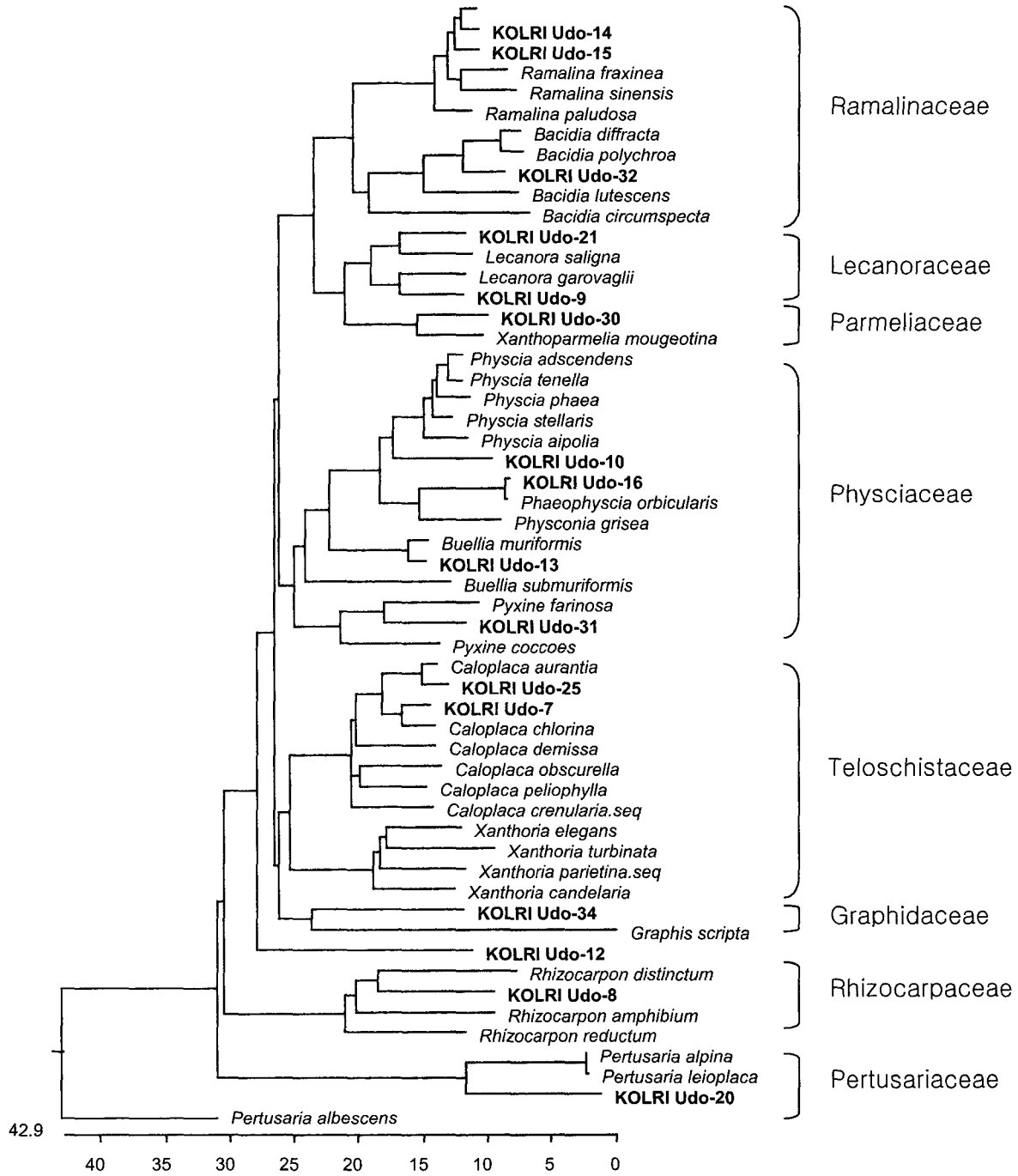

Fig. 5. The tree showing the phylogenetic relationship between the lichens found in U-do Islet and their identities. Approx 400~450 bp 5.8S rRNA sequences, containing the ITS region, of the lichens living on the coastal rocks in the U-do islet, were analyzed and used to construct the dendrogram. The rDNA ITS sequences from genera *Graphis* and *Ochrolechia* were not used to make the tree due to lack of the rDNA ITS sequences in the analysed data.

2003). The lichen, *Caloplaca,* from the coastal rocks of U-do was found to share extensive similarities in its rDNA ITS sequence with those of *Xanthoria parietina* and *Caloplaca marina*, suggesting some of the rock lichens might be different from those known or have evolved for a long time in a different environment, which warrants further extensive study. Thus, most of the lichens identified by molecular technique based on rDNA ITS sequence variation were temporarily designated at the genus level. Phylogenetic analysis based on the 400 ~ 450 bp of 5.8S rDNA containing ITS region showed that Ramalinacea had relatively close relationships with Lecanoraceae and Parmaliaceae, and Teloschistaceae with Graphidaceae. Notably, six strains among the genus *Lecanora* were

found, each only displaying slight sequence variations. The listed lichens among a family showed 80 ~ 98% similarity to one another, which warrants further intensive analysis. Some lichens such as *Porpidia* and *Pertusaria* were not used for drawing the phylogenetic tree due to the lack of rDNA ITS sequences in the analysed data. The phylogenetic tree, based on the ITS sequence shown in Fig. 5, revealed that the family Ramalinaceae had a closer relationship with Lecanoraceae and Parmeliaceae than other lichen families found in this study.

Unfortunately many crustose lichens collected through this study remain to be identified due to the lack of time and a limited knowledge of lichens. In addition to the identified lichens described in Table 2, more were found, but not identified. The coastal rock lichens found and identified in this study might be resistant to a salty environment. Therefore, the physiological characterization of such lichens through isolation of algae and/or fungi might be necessary to gain an understanding of their favourable habitat. Our future studies will extend to other coastal sites for determining the lichens distributed in the coastal rocks of Korea, as well as for comparative analysis with these results.

Acknowledgment

This work was supported by grants from the Ministry of Science and Technology (M1-0219-14-0002).

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