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Research Article

An Easy, Rapid, and Cost-Effective Method for DNA Extraction from Various Lichen Taxa and Specimens Suitable for Analysis of Fungal and Algal Strains

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Abstract Lichen studies, including biodiversity, phylogenetic relationships, and conservation concerns require definitive species identification, however many lichens can be challenging to identify at the species level. Molecular techniques have shown efficacy in discriminating among lichen taxa, however, obtaining genomic DNA from herbarium and fresh lichen thalli by conventional methods has been difficult, because lichens contain high proteins, polysaccharides, and other complex compounds in their cell walls. Here we report a rapid, easy, and inexpensive protocol for extracting PCR-quality DNA from various lichen species. This method involves the following two steps: first, cell breakage using a beadbeater; and second, extraction, isolation, and precipitation of genomic DNA. The procedure requires approximately 10 mg of lichen thalli and can be completed within 20 min. The obtained DNAs were of sufficient quality and quantity to amplify the internal transcribed spacer region from the fungal and algal lichen components, as well as to sequence the amplified products. In addition, 26 different lichen taxa were tested, resulting in successful PCR products. The results of this study validated the experimental protocols, and clearly demonstrated the efficacy and value of our KCl extraction method applied in the fungal and algal samples.

Keywords Lichens, Fungi, Algae, Genomic DNA, rRNA, Sequencing

Over 19% (approximately 14,000 species) of all known fungi are lichenized [1], and it is estimated 8% of terrestrial ecosystems are covered by lichens [2]. Lichens are mutualistic organism; a composite of a fungus and photosynthetic partner, which can be an alga, cyanobacterium, or both. The organisms exhibit a broad geographic and ecological distribution, from tropical to polar regions worldwide [3]. Lichens have shown utility in the medical community. Metabolites produced by some species exhibit broad-spectrum antibiotic, anti-tumor, and antioxidant activities [4]. Molecular biology approaches are now well-developed, and applied to address a broad range of scientific questions. Morphology-based lichen identification for biodiversity, conservation, phylogenetic, and population level studies, among others is required in conjunction with studies using molecular marker-based information, including internal transcribed spacer (ITS), large subunit RNA (LSU), small subunit RNA (SSU), and other regions that evolve at desired evolutionary rates [5-8]. Therefore, PCR and DNA sequencing are routinely employed to examine sequence information among lichen taxa [9].

However, obtaining genomic DNA from lichens has been challenging due to high protein and polysaccharide levels, and tough cell walls [10]. Furthermore, because lichens typically grow very slowly, inadequate fresh material cannot always be field collected for some species. A number of different DNA isolation methods have been developed for from herbarium and fresh lichen material [6, 11-14]. In general, highly sophisticated methods for lichen cell wall disruption, and expensive commercial kits have been applied to obtain genomic DNA. However, for identification purposes, PCR-quality genomic DNA should be suitable for amplification, which ranges from 500 bp to 1,500 bp.

For these purposes, a rapid, easy, and inexpensive method to extract PCR-quality DNA from various lichen taxa

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and specimens (fresh and preserved) is necessary. In a previous study, we developed a DNA extraction method for fungi, including *Magnaporthe oryzae*, *Fusarium* spp., and *Phytophthora* spp., which we successfully applied to five lichen species [15]. Although the method is relatively easy and applicable, only a small number of lichens were examined. In the present study, we improved the method, which we applied to 12 herbarium species, represented by 113 samples, and 26 field collected fresh lichen species. The objective of this study was to develop a rapid, reliable, and inexpensive protocol for DNA extraction suitable for PCR and DNA sequencing in a range of lichen taxa. A critical step in this procedure was breakage and cell lysis due to the rigidity of lichen cell walls.

MATERIALS AND METHODS

Specimens: fresh and preserved lichen collections. All the lichen thalli applied in this study were obtained from the Korean Lichen Center (KoLRI) at Sunchon National University (Sunchon 540-742, Korea). Material from 12 different species, i.e., 113 samples representing the species was obtained from herbarium specimens (Supplementary Table 1). In addition, twenty-six different species was obtained from fresh material (Supplementary Table 2).

Reagents and solution. KCl extraction buffer: 100 mM Tris · HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M KCl; 1× TE buffer: 10 mM Tris · HCI (pH 8.0), 1 mM EDTA (pH 8.0); Chloroform; Isopropanol (chilled); Ethanol 70%; 3 M NaCl.

DNA isolation. Schematic diagram was presented in Fig. 1.

1) Place 10~100 mg of lichen thallus in a XXTuff Reinforced Microvial (Biospec, Bartlesville, OK, USA). Add three or four 2.5-mm sterile glass beads (Daihan Scientific, Seoul, Korea) to the sample; put the microvial in liquid nitrogen; disrupt the sample with a Mini-Beadbeater-24

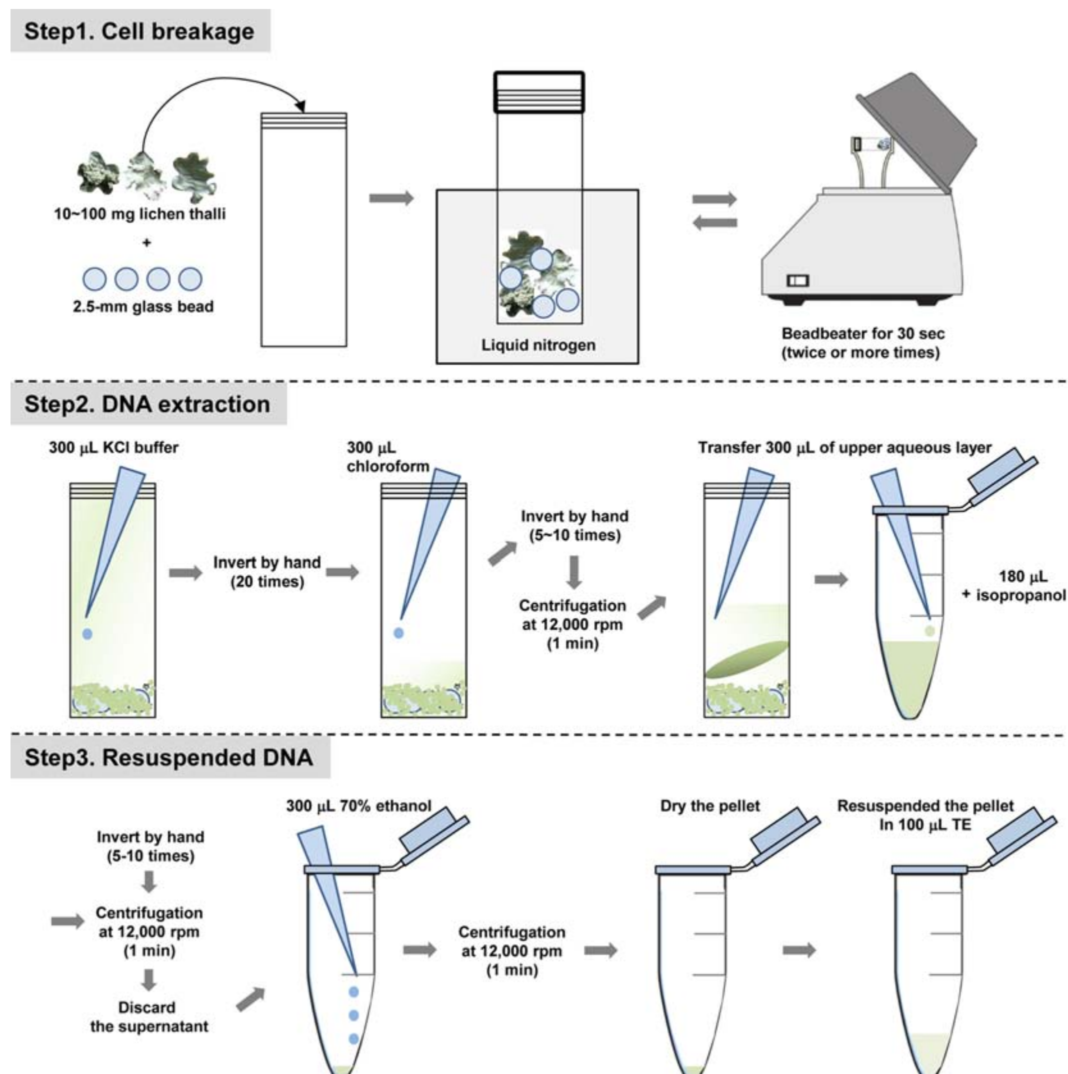

Fig. 1. Schematic diagram of the DNA extraction method.

(Biospec Products) for 30 sec; repeat the procedure, i.e., put the microvial in liquid nitrogen to the sample; and repeat sample disruption until the sample forms a fine powder.

2) Add 300 μL of KCl extraction buffer to the sample, and invert strongly by hand approximately 20 times; add 300 μL chloroform, and invert gently approximately 20 times.

3) Centrifuge sample for 1 min at 12,000 rpm at room temperature (RT). Transfer upper aqueous layer to a new 1.5-mL microcentrifuge tube, and add 180 μL (60% total volume) of chilled isopropanol. Mix by very gentle inversion.

4) Centrifuge for 1 min at 12,000 rpm at RT. Discard the supernatant. Wash the resulting pellet with 300 μL of chilled 70% ethanol. Dry the pellet at 50~65°C dry oven or heat block for 5 min. Resuspend the pellet in 100 μL of TE buffer (1×) at 50~65°C dry oven or heat block for 5 min. Purified DNA may be stored at 4°C or −20°C.

Optional step to remove high-concentration polysaccharides: Add 1 M NaCl (final concentration) to 100 μL of resuspended DNA. Mix by gentle inversion. Add 60% total volume of chilled isopropanol. Mix by very gentle inversion. Proceed with step 4.

Determination of DNA yield and quality. The quality and quantity of extracted DNAs were measured using an Epoch Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, USA).

PCR amplification and DNA sequencing. We tested the efficacy of various rRNA regions, which were amplified from the extracted DNAs and primer pairs listed in Supplementary Table 3 using the i-StarMAXII PCR master mix system (iNtRON Biotechnology, Seongnam, Korea), or the AccuPower PCR PreMixs (Bioneer, Seoul, Korea). A Takara PCR thermal cycler MP (Takara, Tokyo, Japan), or a 96-well GenAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) was used in a regular 30 cycle PCR reaction. Amplified PCR products were purified using MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology) prior to sequencing, and forward and reverse strands were sequenced using the same PCR amplification primers. The generated sequences of fungi and algae from lichen were submitted to GenBank (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Twelve lichen species were selected as representatives to compare DNA yield and quality between the KCl method we developed for lichens, and a general commercial DNA extraction kit (DNeasy Plant Mini Kit; Qiagen, Valencia, CA, USA). Genomic DNA yield and quality is provided in Table 1. Our method resulted in relatively high DNA concentrations, ranging from 54~566 ng/mg (average, 192.3 ng/mg) in lichen samples, whereas comparatively lower DNA concentrations were detected from the commercial kit, ranging from 11 to 500 ng/mg (average, 102.6 ng/mg) (Table 1). Therefore, our method out performs standard commercial DNA extraction kits, resulting in higher DNA yield from small amounts of lichen tissue. The highest DNA concentration was obtained from *Punctelia subflava* using our method, which was coincidentally one of the two lowest generated using the commercial kit (11 ng/mg and 12 ng/mg, respectively) (Table 1). Moreover, the A260/280 ratio ranged from 1.5 to 2.1 using both methods, indicating low protein contamination. However, the average A260/280 ratio for our manual method showed slightly higher quality (1.88) compared to the commercial kit (1.78) (Table 1).

DNA suitability for PCR amplification was verified by examining several primer sets. The PCR products generated using LSU and SSU are shown in Fig. 2A and 2B, respectively.

Table 1. Yield and quality of DNA from twelve representative lichen species

KoRLI No.	Lichen species	KCl method		Commercial kit		GenBank accession No.	
		DNA conc. (ng/mg)	$A_{260/280}$	DNA conc. (ng/mg)	$A_{260/280}$	Fungi	Algae
007239	*Flavoparmelia caperata*	76	1.5	16	1.5	-[a]	-
007742	*Heterodermia diademata*	94	2.0	35	1.8	KM207200	KM207205
000978	*Heterodermia hypoleuca*	60	1.9	63	1.7	KM207201	KM207206
009659	*Lobaria discolor*	58	1.8	83	1.7	-	-
008278	*Lobaria retigera*	314	2.1	213	1.8	-	-
001899	*Myelochroa entotheiochroa*	70	1.8	57	1.8	-	-
011648	*Myelochroa irrugans*	92	1.9	100	1.5	KM207202	KM207207
011592	*Parmotrema tinctorum*	54	1.8	70	1.9	-	-
016180	*Peltigera polydactylon*	354	2.0	500	1.8	-	-
010394	*Peltigera praetextata*	364	2.0	71	1.9	-	-
007637	*Punctelia subflava*	566	2.0	12	1.8	KM207203	KM207208
007349	*Umbilicaria esculenta*	206	1.8	11	2.1	KM207204	KM207209
	Average	192.3	1.88	102.6	1.77		

[a]Not performed sequencing analysis after amplification of internal transcribed spacer regions.

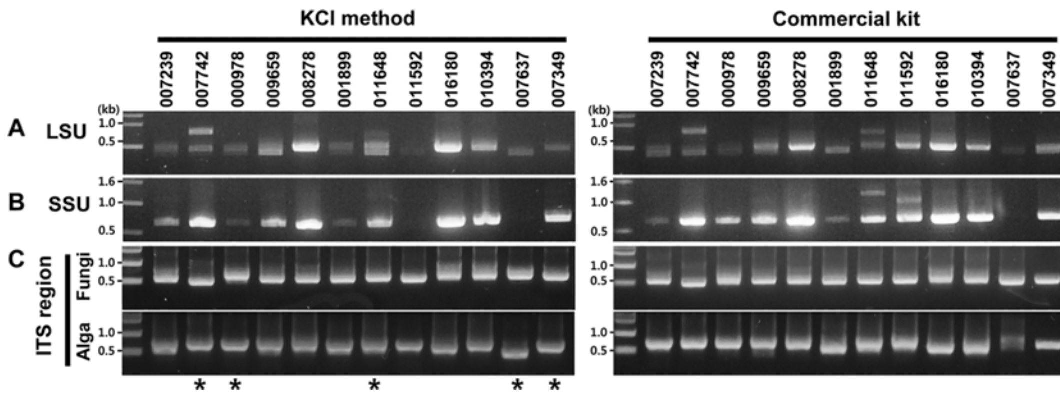

Fig. 2. Electropherograms of PCR products amplified using 12 representative DNA samples species extracted using our KCl method, and the commercial kit. Comparison of DNA samples extracted with our KCl method (on left) and commercial kit (on right). PCR amplifications were as follows: A, large subunit RNA (LSU) region; B, small subunit RNA (SSU) region; and C, internal transcribed spacer (ITS) specific fungal and algal primer region; upper and lower gel sections show fungi and algae, respectively. Asterisks: Five amplified PCR products generated by our KCl method were selected for sequencing fungal and algal ITS regions. These sequences were registered in GenBank. Representative lichen species: 007293, *Flavoparmelia carperata*; 007742, *Heterodermia diadermata*; 000978, *Heterodermia hypoleuca*; 009659, *Lobaria discolor*; 008278, *Lobaria retigera*; 001899, *Peltigera praetextata*; 011648, *Myelochroa entotheiochroa*; 011592, *Myelochroa irrugans*; 016180, *Parmotrema tinctorum*; 010394, *Peltigera polydactylon*; 007637, *Punctelia subflava*; 007349, *Umbilicaria esculenta*.

DNA was extracted from lichen thalli, which is composed of a fungus and its photobiont alga. Piercey-Normore and DePriest [16] developed specific primer sets to selectively amplify fugal and algal ITS regions, respectively. We adopted specific primer sets to compare our KCl method, and the commercial kit. Identical PCR products were obtained from both methods in fungal (Fig. 2C, upper) and algal ITS regions (Fig. 2C, lower).

Furthermore, five amplified PCR products generated by the KCl method were selected for sequencing fungal and algal ITS regions (asterisks, Fig. 2C lower left). Successful sequencing results were obtained in the fungus and alga through direct sequencing without cloning. These sequences were registered in GenBank (Supplementary Table 1).

During DNA isolation, gelatinous DNA pellets were obtained from many species, which indicates high polysaccharide concentrations. All 12 DNAs from *Umbilicaria esculenta* (Supplementary Table 1) yielded gelatinous pellets, which were hard to dissolve in TE. Previous studies reported high-salts, such as NaCl, effectively remove plant polysaccharides [17, 18]. Therefore, polysaccharides were removed by applying 1 M NaCl (final concentration) to DNA dissolved in 100 mL TE; and subsequent DNA precipitation by addition of 0.6 volumes of isopropanol. Results showed

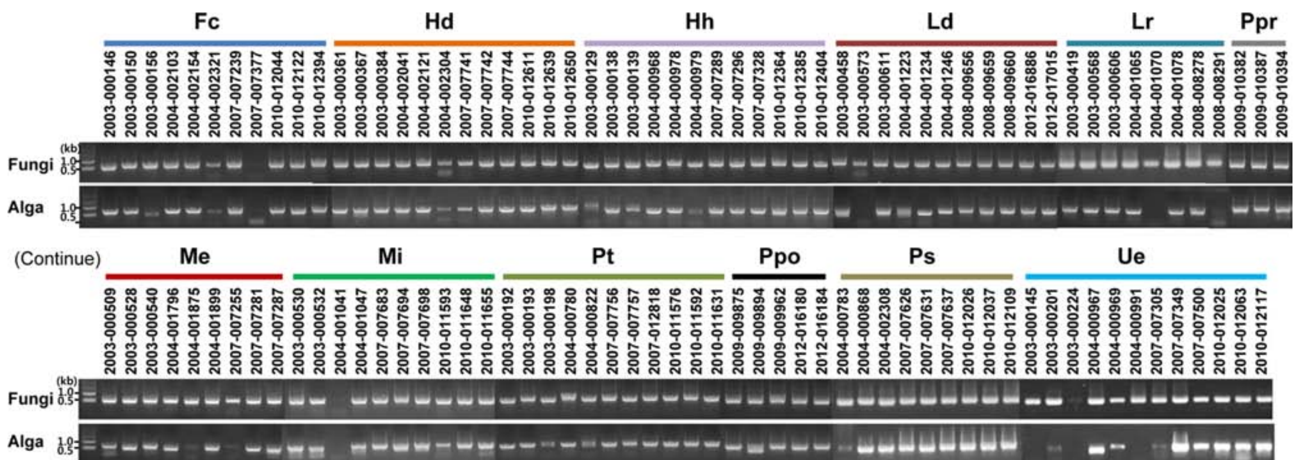

Fig. 3. PCR assays conducted using the fungal and algal specific internal transcribed spacer (ITS) primers with 113 different lichen samples from 12 species collected during 2002~2010; upper and lower gel sections show fungi and algae, respectively. Fc, *Flavoparmelia caperata*; Hd, *Heterodermia diademata*; Hh, *Heterodermia hypoleuca*; Ld, *Lobaria discolor*; Lr, *Lobaria retigera*; Ppr, *Peltigera praetextata*; Me, *Myelochroa entotheiochroa*; Mi, *Myelochroa irrugans*; Pt, *Parmotrema tinctorum*; Ppo, *Peltigera polydactylon*; Ps, *Punctelia subflava*; Ue, *Umbilicaria esculents.*

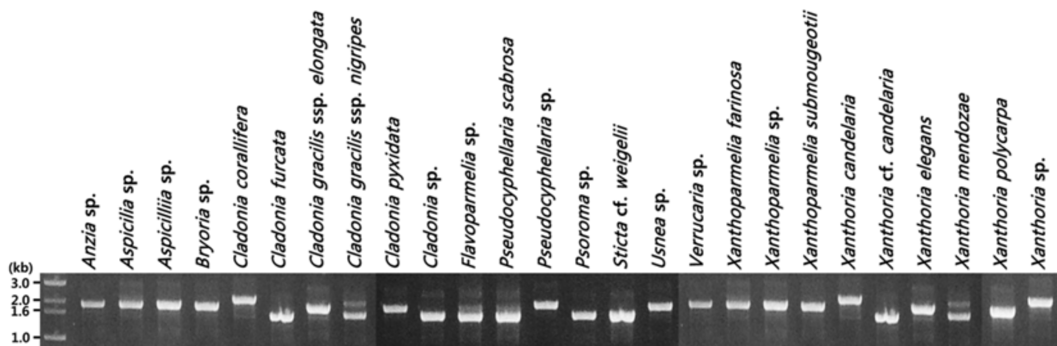

Fig. 4. PCR product electropherograms of the full-length internal transcribed spacer regions and 26S rRNA (> 1.0 kb) amplified from representative 26 DNA samples derived from taxa at different taxonomic levels (i.e., genus to subspecies) extracted with our KCl method.

most polysaccharides were effectively removed; purified DNA was easily dissolved in TE, leaving the amplified target region for PCR. This result showed NaCl effectively remove lichen polysaccharides.

We also successfully applied this method to over 113 additional isolates, which included 12 different taxa (genera, species, and subspecific taxa) collected from 2002 to 2010 (Supplementary Table 1). The specific fungal and algal ITS region was successfully amplified (Fig. 3). In addition, our KCl method was applied to examine 50 samples from 26 different lichen taxa (Supplementary Table 2), amplifying a larger PCR product, which included the ITS region plus 26S rRNA (> 1.0 kb); successful PCR products were obtained (Fig. 4). Collectively, these results indicated that the KCl method was applicable to herbarium and field collected lichen specimens (Supplementary Table 1), but also varied lichen taxonomic levels (Supplementary Table 2, Fig. 4).

A simple and cost effective protocol for extracting lichen genomic DNA was proposed in this study. The KCl DNA extraction method described here permits extraction from a wide range of lichen species (Supplementary Tables 1 and 2), and amplification of primary molecular clock-related genes [9], including ITS, LSU, and SSU (Fig. 2).

The KCl based DNA extraction method was originally designed as an easy and rapid method to PCR amplification in plants [19] and has since been widely employed in fungal DNA extraction, including successful use in *Magnaporthe* and *Fusarium* [15]. Unlike former protocols, we included a chloroform extraction step to increase DNA quality, which was effective in most lichen taxa (data not shown). Moreover, the addition of 1 M NaCl for high polysaccharide concentrations in lichens resulted in notable polysaccharide removal, and also increased DNA quality (data not shown).

Compared to existing methods [11-13], our KCl extraction approach exhibits several advantages; a very low learning curve, and cost effectiveness, which include materials and time. First, the KCl method does not involve complicated processes, expensive reagents, and low temperature centrifugation. Therefore, the method is easy to learn, and applicable for a general laboratory. Second, the time required for DNA extraction is short; for example, it is possible to process over 100 samples in 1~2 hrs. This is primarily due to the absence of an incubation time with lysis buffer, and a short centrifugation time. Furthermore, it is possible to pause (or prolong) the isolation if necessary after the addition of isopropanol, for example overnight or longer. Third, the method requires a small amount of lichen thalli (< 10 mg). DNA yields (54~566 ng/mg) were higher than those previously obtained by sodium dodecyl sulfate (7-12 ng/mg), or cetyltrimethylammonium bromide (CTAB; 15~25 ng/mg), or defined CTAB method for lichen (> 25 ng/mg) [11]. The average DNA quality was also slightly higher than that observed from the commercial kit (Supplementary Table 3). Finally, our KCl method can reduce contamination potential because it excludes contact with contaminants such as mortar, pestle, and other equipment.

In summary, our KCl DNA extraction method demonstrated high efficacy in PCR amplification and sequence analysis from herbarium and fresh lichen materials across a wide range of lichen species, including the fungal and algal components. Therefore, the methodology provides a simple, cost effective, and readily available protocol suitable for use with various lichen materials to examine fungal and algal population dynamics, phylogenetic relationships, biodiversity, and conservation concerns.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including three tables can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-42-311-s001.pdf.

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http://www.mycobiology.or.kr/src/sm/mb-42-311-s001.pdf.

Supplementary Table 1. List of fungal taxa and herbarium collections from 2003 to 2012 used in this study

Fungal taxa	Year	KoLRI No.[a]	GPS	Altitude (m)	Substratum
Flavoparmelia caperata	2003	000146	-[b]	-	-
	2003	000150	-	400	Bark
	2003	000156	-	510	Rock
	2004	002103	38°04'05.6" N, 128°26'58.8" E	480	Bark
	2004	002154	38°03'31.6" N, 128°26'41.6" E	865	Bark
	2004	002321	38°11'16.4" N, 128°21'42.7" E	450	Bark
	2007	007239	37°02'23.9" N, 128°42'55.3" E	1,224	Rock
	2007	007377	36°58'00.9" N, 128°30'31.1" E	1,385	Rock
	2010	012044	35°36'33.5" N, 127°39'65.8" E	917	Bark
	2010	012122	35°43'73.6" N, 127°46'07.8" E	1,205	Rock
	2010	012394	37°54'94.3" N, 127°58'85.7" E	704	Bark
Heterodermia diademata	2003	000361	-	680	Rock
	2003	000367	-	690	Rock
	2003	000384	-	690	Rock
	2004	002041	36°52'01.3" N, 128°06'34.3" E	?-	Rock
	2004	002121	38°03'46.7" N, 128°26'44.6" E	680	Bark
	2004	002304	38°11'16.4" N, 128°21'42.7" E	450	Rock
	2007	007741	34°45'39.1" N, 128°02'55.7" E	251	Rock
	2007	007742	34°45'39.1" N, 128°02'55.7" E	251	Rock
	2007	007744	34°45'39.1" N, 128°02'55.7" E	251	Rock
	2010	012611	37°40'51.7" N, 126°21'47.2" E	287	Rock
	2010	012639	37°14'86.5" N, 126°28'94.1" E	5	Rock
	2010	012650	37°14'86.5" N, 126°28'94.1" E	5	Rock
Heterodermia hypoleuca	2003	000129	-	1,050	Rock
	2003	000138	-	1,000	Rock
	2003	000139	36°57'14.5" N, 128°29'18.8" E	-	Rock
	2004	000968	35°48'28.5" N, 128°07'00.6" E	965 (916)	Rock
	2004	000978	35°48'54.6" N, 128°06'55.1" E	1,145 (1,097)	Bark
	2004	000979	35°48'54.6" N, 128°06'55.1" E	1,275 (1,236)	Rock
	2007	007289	36°52'52.5" N, 128°25'47.0" E	1,106	Bark
	2007	007296	36°52'38.4" N, 128°25'39.6" E	1,163	Bark
	2007	007328	36°50'45.5" N, 128°27'08.6" E	1,008	Bark
	2010	012364	37°54'79.7" N, 127°59'09.9" E	685	Bark
	2010	012385	37°54'93.1" N, 127°59'00.8" E	714	Bark
	2010	012404	37°54'93.0" N, 127°58'62.1" E	672	Rock
Lobaria discolor	2003	000458	37°06'11.4" N, 128°55'55.8" E	1,254	Bark
	2003	000573	37°06'0.4" N, 128°57'04.3" E	1,420	Bark
	2003	000611	-	1,345	Rock
	2004	001223	37°47'17.6" N, 128°33'10.6" E	1,280 (1,265)	Bark
	2004	001234	37°47'24.4" N, 128°32'57.9" E	1,410 (1,387)	Bark
	2004	001246	37°47'40.0" N, 128°32'42.7" E	1,545 (1,535)	Bark
	2008	009656	-	800	Bark

Supplementary Table 1. Continued

Fungal taxa	Year	KoLRI No.[a]	GPS	Altitude (m)	Substratum
	2008	009659	-	1,000	Bark
	2008	009660	-	1,000	Bark
	2012	016886	33°22'48.44" N, 126°35'26.70" E	1,025	Bark
	2012	017015	33°22' N, 126°34' E	1,210~1,250	Bark
Lobaria retigera	2003	000419	35°29'44.3" N, 126°53'27.6" E	612	Rock
	2003	000568	36°57'17.6" N, 128°28'47.1" E	1,394	Rock
	2003	000606	36°56'53.7" N, 128°28'11.0" E	1,325	Rock
	2004	001065	35°18'22.6" N, 127°34'49.6" E	1,565 (1,464)	Rock
	2004	001070	35°18'41.6" N, 127°35'59.3" E	1,560 (1,484)	Rock
	2004	001078	35°19'24.0" N, 127°36'43.5" E	1,655	Bark, moss
	2008	008278	37°24'05.0" N, 128°32'39.5" E	937	Rock, moss
	2008	008291	37°24'05.0" N, 128°32'39.5" E	937	Bark
Peltigera praetextata	2009	010382	37°51'69.2" N, 128°31'52.2" E	706	Soil
	2009	010387	37°51'69.2" N, 128°31'52.2" E	706	Soil
	2009	010394	37°51'69.2" N, 128°31'52.2" E	706	Rock, moss
Myelochroa entotheiochroa	2003	000509	36°57'26.7" N, 128°26'36.2" E	594	-
	2003	000528	-	-	Wood
	2003	000540	36°56'45.5" N, 128°30'06.6" E	1,006	Rock
	2004	001796	37°06'10.4" N, 128°57'16.1" E	1,070	Rock, moss
	2004	001875	-	1,405	Bark
	2004	001899	-	1,450	Bark
	2007	007255	36°53'23.4" N, 128°25'58.0" E	872	Rock
	2007	007281	36°52'52.9" N, 128°25'47.3" E	1,110	Bark
	2007	007287	36°52'52.9" N, 128°25'47.4" E	1,115	Bark
Myelochroa irrugans	2003	000530	-	-	-
	2003	000532	-	-	Bark
	2004	001041	35°17'35.2" N, 127°32'49.9" E	1,450 (1,397)	Bark
	2004	001047	35°17'45.5" N, 127°33'02.9" E	1,440 (1,381)	Rock
	2007	007683	35°22'51.0" N, 127°50'56.1" E	587	Bark
	2007	007694	35°22'53.4" N, 127°51'14.9" E	760	Bark
	2007	007698	35°22'53.0" N, 127°51'16.1" E	775	Bark
	2010	011593	34°08'64.6" N, 126°33'00.3" E	289	Rock
	2010	011648	34°08'50.4" N, 126°32'90.6" E	368	Rock
	2010	011655	34°08'44.3" N, 126°32'85.0" E	407	Rock, moss
Parmotrema tinctorum	2003	000192	-	245	-
	2003	000193	-	45	Bark
	2003	000198	-	45	-
	2004	000780	34°59'27.9" N, 127°20'01.8" E	210	Bark
	2004	000822	35°29'40.4" N, 126°35'01.5" E	37	Bark
	2007	007756	34°45'38.6" N, 128°02'54.0" E	262	Rock
	2007	007757	34°45'38.3" N, 128°02'53.6" E	280	Rock
	2007	012818	34°45'38.6" N, 128°02'54.4" E	289	Bark
	2010	011576	34°08'34.9" N, 126°33'25.5" E	251	Rock
	2010	011592	34°08'64.6" N, 126°33'00.3" E	289	Rock
	2010	011631	34°08'54.0" N, 126°32'94.5" E	351	Bark
Peltigera polydactylon	2009	009875	33°22'77.5" N, 126°33'74.9" E	1,200	Moss
	2009	009894	33°22'47.3" N, 126°33'43.0" E	1,270	Moss
	2009	009962	33°21'53.4" N, 126°30'56.8" E	1,700	Moss
	2012	016180	33°27'07.06" N, 126°32'02.03" E	1,709	Bark
	2012	016184	33°27'07.06" N, 126°32'02.03" E	1,709	Bark
Punctelia subflava	2004	000783	34°59'27.9" N, 127°20'01.8" E	210	Bark
	2004	000868	36°47'25.2" N, 128°55'25.4" E	885	Rock
	2004	002308	38°11'16.4" N, 128°21'42.7" E	450	Rock
	2007	007626	36°44'54.0" N, 128°15'49.1" E	606	Rock
	2007	007631	36°44'54.2" N, 128°15'50.5" E	603	Bark
	2007	007637	36°45'00.3" N, 128°15'53.0" E	643	Rock
	2010	012026	35°36'33.5" N, 127°39'65.8" E	917	Rock
	2010	012037	35°36'33.5" N, 127°39'65.8" E	917	Bark
	2010	012109	35°43'73.6" N, 127°46'07.8" E	1,205	Rock

Supplementary Table 1. Continued

Fungal taxa	Year	KoLRI No.[a]	GPS	Altitude (m)	Substratum
Umbilicaria esculenta	2003	000145	-	1,050	Rock
	2003	000201	-	1,218	Rock
	2003	000224	-	705	Rock
	2004	000967	35°48'28.5" N, 128°07'00.6" E	965 (916)	Rock
	2004	000969	35°48'28.5" N, 128°07'00.6" E	965 (916)	Rock
	2004	000991	35°49'11.3" N, 128°07'18.2" E	1,440 (1,396)	Rock
	2007	007305	36°52'20.4" N, 128°25'53.0" E	1,207	Rock
	2007	007349	36°57'51.3" N, 128°30'32.6" E	1,232	Rock
	2007	007500	36°55'39.3" N, 128°27'47.0" E	1,238	Rock
	2010	012025	35°36'335" N, 127°39'658" E	917	Rock
	2010	012063	35°36'614" N, 127°39'624" E	903	Rock
	2010	012117	35°43'736" N, 127°46'078" E	1,205	Rock

[a]Lichen collection number of Korean Lichen Research Institute (KoLRI).
[b]Not recorded.

Supplementary Table 2. List of fungal taxa and fresh lichen samples from 2013 used in this study

Fungal taxa	KoLRI No.	GPS	Altitude (m)	Substratum
Anzia sp.	017589	51°16'59.3" S, 072°50'27.7" W	46	*Nothofagus* sp. (trunk)
Anzia sp.	017597	51°16'59.3" S, 072°50'27.7" W	46	*Nothofagus* sp. (trunk)
Anzia sp.	017598	51°16'59.3" S, 072°50'27.7" W	46	*Nothofagus* sp. (trunk)
Anzia sp.	017599	51°16'59.3" S, 072°50'27.7" W	46	*Nothofagus* sp. (trunk)
Anzia sp.	017601	51°16'59.3" S, 072°50'27.7" W	46	*Nothofagus* sp. (trunk)
Anzia sp.	017602	51°16'59.3" S, 072°50'27.7" W	46	*Nothofagus* sp. (trunk)
Anzia sp.	017788	53°24'20.1" S, 071°15'46.0" W	285	*Nothofagus* sp. (trunk)
Anzia sp.	017789	53°24'20.1" S, 071°15'46.0" W	285	*Nothofagus* sp. (trunk)
Aspicilia sp.	017473	51°34'36.8" S, 072°35'59.0" W	133	Rock
Aspicilliia	017725	51°54'01.1" S, 072°27'13.0" W	1	Rock
Bryoria sp.	017438	51°34'36.8" S, 072°35'59.0" W	133	Rock
Bryoria sp.	017461	51°34'36.8" S, 072°35'59.0" W	133	Rock, moss
Bryoria sp.	017462	51°34'36.8" S, 072°35'59.0" W	133	Rock, moss
Bryoria sp.	017492	51°34'36.8" S, 072°35'59.0" W	133	*Nothofagus* sp. (bark)
Bryoria sp.	017544	51°21'46.8" S, 072°48'07.3" W	64	Twig
Bryoria sp.	017552	51°21'46.8" S, 072°48'07.3" W	64	Rock
Bryoria sp.	017636	51°22'36.4" S, 072°45'18.8" W	38	Rock
Bryoria sp.	017639	51°22'36.4" S, 072°45'18.8" W	38	Rock
Cladonia corallifera	017431	51°34'36.8" S, 072°35'59.0" W	133	Moss
Cladonia furcata	017580	51°16'59.3" S, 072°50'27.7" W	46	Soil
Cladonia gracilis ssp. *elongata*	017532	51°21'46.8" S, 072°48'07.3" W	64	Soil
Cladonia gracilis ssp. *nigripes*	017505	51°34'36.8" S, 072°35'59.0" W	133	Moss
Cladonia pyxidata	017467	51°34'36.8" S, 072°35'59.0" W	133	Rock, moss
Cladonia sp.	017513	51°33'30.1" S, 072°40'09.6" W	120	Soil
Flavoparmelia sp.	017653	51°22'36.4" S, 072°45'18.8" W	38	Rock
Pseudocyphellaria scabrosa	017701	51°58'54.0" S, 072°22'20.0" W	140	*Nothofagus* sp. (trunk)
Pseudocyphellaria sp.	017702	51°58'54.0" S, 072°22'20.0" W	140	*Nothofagus* sp. (trunk)
Pseudocyphellaria sp.	017745	52°03'06.1" S, 071°27'37.8" W	193	Soil
Psoroma sp.	017545	51°21'46.8" S, 072°48'07.3" W	64	Moss
Sticta cf. *weigelii*	017585	51°16'59.3" S, 072°50'27.7" W	46	Rock
Unknown fruticose	017456	51°34'36.8" S, 072°35'59.0" W	133	Rock, soil
Usnea sp.	017555	51°21'46.8" S, 072°48'07.3" W	64	Dead wood (trunk)
Usnea sp.	017694	52°01'30.8" S, 072°22'38.8" W	195	*Nothofagus* sp. (trunk)
Usnea sp.	017703	51°58'54.0" S, 072°22'20.0" W	140	*Nothofagus* sp. (trunk)
Usnea sp.	017450	51°34'36.8" S, 072°35'59.0" W	133	*Nothofagus* sp. (bark)
Usnea sp.	017499	51°34'36.8" S, 072°35'59.0" W	133	*Nothofagus* sp. (bark)
Usnea sp.	017728	51°54'01.1" S, 072°27'13.0" W	1	Rock
Verrucaria sp.	017717	51°54'01.1" S, 072°27'13.0" W	1	Rock
Xanthoparmelia farinosa	017515	51°33'30.1" S, 072°40'09.6" W	120	Rock

Supplementary Table 2. Continued

Fungal taxa	KoLRI No.	GPS	Altitude (m)	Substratum
Xanthoparmelia sp.	017485	51°34'36.8" S, 072°35'59.0" W	133	Rock
Xanthoparmelia sp.	017454	51°34'36.8" S, 072°35'59.0" W	133	Rock
Xanthoparmelia submougeotii	017517	51°33'30.1" S, 072°40'09.6" W	120	Rock
Xanthoria candelaria	017650	51°22'36.4" S, 072°45'18.8" W	38	Rock
Xanthoria cf. *candelaria*	017520	51°33'30.1" S, 072°40'09.6" W	120	Dead tree
Xanthoria elegans	017651	51°22'36.4" S, 072°45'18.8" W	38	Rock
Xanthoria cf. *elegans*	017718	51°54'01.1" S, 072°27'13.0" W	1	Rock
Xanthoria mendozae	017459	51°34'36.8" S, 072°35'59.0" W	133	Rock
Xanthoria polycarpa	017727	51°54'01.1" S, 072°27'13.0" W	1	Rock
Xanthoria cf. *polycarpa*	017714	51°54'01.1" S, 072°27'13.0" W	1	Rock
Xanthoria sp.	017608	51°22'36.4" S, 072°45'18.8" W	38	*Nothofagus* sp. (trunk)

Supplementary Table 3. Primers used in this study

Target region	Primer name	Sequence (5'-3')	References
SSU	SR1R	TACCTGGTTGATQCTGCCAGT	-
	SR7	GTTCAACTACGAGCTTTTTAA	
LSU	LS1	GTACCCGCTGAACTTAAGC	-
	LS5	TCCTGAGGGAAACTTCG	
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* (1990) [9]
	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990) [9]
ITS region for initial amplification	nu-SSU-1583-59	CAACGAGGAATTCCTAGT	DePriest (1993) [8]
	ITS4-39	TCCTC CGCTTATTGATATGC	White *et al.* (1990) [9]
Fungal specific	nr-SSU-1780-59 Fungal	CTGCGGAAGGATCATTAATGAG	Piercey-Normore and DePriest (2001) [16]
	nr-LSU-0012-39 Fungal	AGTTCAGCGGGTATCCCT	Piercey-Normore and DePriest (2001) [16]
Algal specific	nr-SSU-1780-59 Algal	CTGCGGAAGGATCATTGATTC	Piercey-Normore and DePriest (2001) [16]
	nr-LSU-0012-39 Algal	AGTTCAGCGGGTGGTCTTG	Piercey-Normore and DePriest (2001) [16]
ITS-LSU	ITS1F	CTTGGTCATTTAGAGGAAGTAA	-
	LR5	ATCCTGAGGGAAACTTC	

SSU, small subunit RNA; LSU, large subunit RNA; ITS, internal transcribed spacer.