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Diversity and Distribution Patterns of Endolichenic Fungi in Jeju Island, South Korea

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Abstract: Lichens are symbiotic organisms containing diverse microorganisms. Endolichenic fungi (ELF) are one of the inhabitants living in lichen thalli, and have potential ecological and industrial applications due to their various secondary metabolites. As the function of endophytic fungi on the plant ecology and ecosystem sustainability, ELF may have an influence on the lichen diversity and the ecosystem, functioning similarly to the influence of endophytic fungi on plant ecology and ecosystem sustainability, which suggests the importance of understanding the diversity and community pattern of ELF. In this study, we investigated the diversity and the factors influencing the community structure of ELF in Jeju Island, South Korea by analyzing 619 fungal isolates from 79 lichen samples in Jeju Island. A total of 112 ELF species was identified and the most common species belonged to Xylariales in Sordariomycetes. The richness and community structure of ELF were significantly influenced by the host taxonomy, together with the photobiont types and environmental factors. Our results suggest that various lichen species in more diverse environments need to be analyzed to expand our knowledge of the diversity and ecology of ELF.

Keywords: algae; cyanobacteria; *Daldinia*; host specificity; lichen; oreum; photobiont; sordariomycetes; xylariales

1. Introduction

Lichens are symbiotic organisms in which the mycobiont (lichen-forming fungi) and the photobiont (green algae and/or cyanobacteria) live together in a mutualistic relationship: the mycobiont protects the photobiont against external environmental stress by forming a thallus, and the photobiont provides photosynthetic carbon as a reward [1,2]. Lichens play ecologically important roles in ecosystems as food and habitats for animals, as well as participants in nutrient cycling and soil formation [3,4]. In addition, they produce numerous secondary metabolites that are industrially or pharmaceutically effective compounds such as antibiotics, anti-tumor agents, and antioxidants [5]. Recent studies have shown that diverse microorganisms exist within lichen thalli, and they can influence the physiology of host lichen in a similar manner to the influence of endophytes on the host plant [6–8]. Endolichenic fungi (ELF) are non-mycobiont fungal species living in the lichen thallus [9]. They are distinct from the lichenicolous fungi in terms of symptomless characteristics. As for the relationship between ELF and lichens, it is unclear whether they have any kind of intimate association [8]. Given that the endophytic fungi influence the plant physiology and increase a tolerance against environmental stress (e.g., high temperature, drought, or pathogens) [10–12], it is suggested that ELF promote the biological function

of lichens in the ecosystem [8]. Therefore, the biodiversity and distribution pattern of ELF is important not only for understanding the ecology and physiology of lichens, but also for the maintenance of ecosystem sustainability in the era of global climate change.

The ELF diversity has been studied in various biomes from tropical areas to polar regions using culture-dependent [13–20] or independent approaches [19,21–25]. ELF are phylogenetically diverse, covering all lineages of Ascomycota and a minority of Basidiomycota and Mucoromycota [9,14]. The evolution of ELF is not well understood, but it is thought to be polyphyletic, and ELF have been suggested as the origin of endophytism, which would explain the similar phylogenetic range [9]. The ELF diversity is different from the co-existing endophytic diversity, which indicates that ELF are a distinctive ecological group differing from endophytic fungi [14,26]. However, the ELF diversity has been studied mainly in Europe [13,27,28], North America [9,14], and Southern Asia [17,29,30], while the ELF in Eastern Asia are largely unexplored [31]. Therefore, it is expected that exploring the ELF diversity in Eastern Asia can expand the knowledge of fungal diversity associated with lichens.

Several abiotic and biotic factors drive the pattern of fungal diversity and community structures [32–34]. It is well known that endophytic fungal communities are influenced by the climate [15,35], geographic position of the region [36], and host taxonomy [37,38]. The factors influencing the ELF community structures have rarely been investigated, but a few studies have suggested that geographic characteristics and the host taxonomy are important factors shaping the ELF community structure [9,14,15,27,28]. The ELF diversity was high in tropical or subtropical regions, probably due to favorable climatic conditions [9,15]. The lichen host is one of the factors that determine the ELF communities [14,15,27,28]. However, other studies showed no effect of the host taxonomy [39], which suggests that the pattern of ELF diversity and factors governing the ELF community may vary depending on the geographical region and spatial scales.

In this study, we investigated the ELF diversity and community structure in Jeju Island (South Korea). Jeju Island is composed of warm lowlands and subalpine (Mt. Halla, 1947 m above sea level) biomes with deciduous-evergreen broadleaved and coniferous forests [40], as well as many reported lichens [41–44]. The oreums, a special structure of parasitic volcanoes, are distributed from the lowland to the mountain area [45,46] and are covered by forests with a high diversity of animals [47,48], plants [49,50], and microorganisms [51,52]. Although study of lichen flora in oruems is limited, several studies have revealed novel records of lichen species from oreums [53,54]. However, the diversity of ELF is largely unexplored in South Korea as well as in oreums. We collected different species of lichens from several oreums in Jeju Island, and investigated the diversity of ELF by culture isolation. Moreover, we analyzed the pattern of the diversity and community structure of ELF to understand the effect of various factors (host taxonomy, photobiont type, and ecoregions) on the relationship between ELF and lichens.

2. Materials and Methods

2.1. Lichen Sample Collection and ELF Isolation

Lichen specimens were collected from sampling sites at 29 oreums covering the whole island from the lowland near the coast to the high mountain area in Mt. Halla, from April to September 2017 (Figure 1; Table S1 (Supplementary Materials)). We focused on the foliose lichens, except for *Stereocaulon* (fruticose), because the wide area of the thallus is helpful to acquire sufficiently large thalli and to avoid contamination. Healthy and fresh thalli of lichens on rock, soil, and trees were collected (Table A1) and transferred to the laboratory in paper bags.

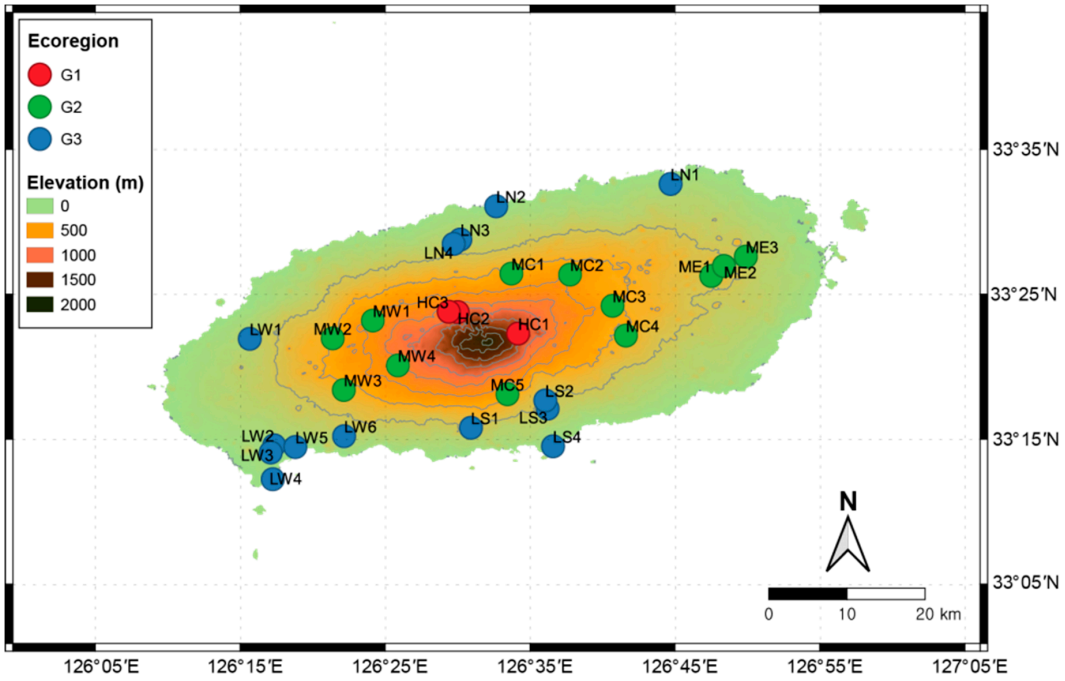

Figure 1. Map of the sampling sites on Jeju Island. Ecoregions classified using elevation and bioclimatic variables are presented in different colors.

The lichen thalli were subjected to sterilization. Before sterilization, the litter and debris attached on the thalli were removed using a needle of a syringe, and the thalli were washed in running tap water. The surface of the thalli was sterilized by a modified method used in the previous study [14]: 95% ethanol for 30 s, 0.5% NaOCl for 2 min, 70% ethanol for 30 s, and rinsed three times with sterile distilled water. The thalli were dried on sterilized paper and cut into 1 cm^2 pieces using sterilized scissors. For each specimen, a total of 20 fragments were placed on four plates of 90 mm Petri dishes containing a potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA), and incubated at 25 °C for up to 12 weeks. The plates were checked every day, and fungal isolates were transferred to PDA medium for pure culture. Additionally, ELF strains previously isolated from Jeju Island using the same procedure and deposited in the Korean Lichen Research Institute (KoRLI) were included in the analysis to increase the volume of the dataset. To recover the strains from KoRLI, they were cultured on PDA medium at 25 °C.

2.2. Molecular Identification of Fungal Strains

Genomic DNA of all isolated fungal strains was extracted using the method of Park et al. [55]. For molecular identification, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified using ITS1F and ITS4 [56]. PCR amplification was performed using an AccuPower PCR PreMix kit (Bioneer, Daejeon, South Korea) with 1 µL of DNA, 1 µL of each primer, and 17 µL of sterilized distilled water in the following condition: 5 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C, and a final extension for 5 min at 72 °C. PCR products were checked on 1% agarose gel electrophoresis, and sequencing was performed at GenoTech (Daejeon, South Korea).

Sequences were checked on FinchTV 1.4.0 (Geospiza Inc., WA, USA). Preliminary molecular operational taxonomic units (mOTUs) were constructed by 99% sequence similarity using Vsearch v. 2.14.1 [57] and its taxonomic candidates were assigned using BLAST against GenBank. Final taxonomy was assigned based on phylogenetic analysis with reference sequences. Sequences were aligned using MAFFT v.7 [58] and checked manually on MEGA v.5 [59]. The phylogenetic tree was constructed using a maximum likelihood (ML) analysis using RAxML v. 8.0.2 [60] with the GTRGAMMA model and 1000 bootstrap replicates. Finally, preliminary mOTUs with the same species name were combined to

single mOTUs. From there, mOTUs were referred as species names, but species identification was solely based on ITS sequences, without considering the morphological and cultural characteristics of the isolated strains. All generated sequences were deposited in GenBank under the accession numbers MN341225-MN341843.

2.3. Analysis of Diversity and Community Structure

Statistical analyses were conducted on R v.3.5.1 [61]. The taxonomy of the host lichen (genus), photobiont types, and ecoregions of the sampling sites were analyzed as the factors influencing the ELF diversity and community. For the lichen taxonomy, only the lichen genera represented by more than two samples were used for further analysis. The ecoregions were categorized using a cluster package [62] with partitioning around medoids (PAM) based on the elevation and climate data. Elevation data were obtained from a NASA shuttle radar topographic mission (SRTM) dataset [63] as a digital elevation model (DEM). For climate data, 19 bioclimatic variables were acquired from WorldClim database v.2 (30 arc second) [64]. Among the bioclimatic variables, BIO01 (Annual mean temperature), BIO03 (Isothermality), BIO07 (Temperature annual range), BIO12 (Annual precipitation), BIO14 (Precipitation of the driest month), and BIO15 (Precipitation seasonality) were chosen by a stepwise backward variable selection after removing high variance inflation factor (VIF > 10) using the usdm package [65]. The clustering of sampling sites according to their bioclimatic variables resulted in the delineation of three ecoregions based on the silhouette coefficient [66] and Elbow method [67]. A higher value of silhouette coefficient indicates the better quality of clustering [66]. The Elbow method suggests the optimal number of clusters when the additional number of clusters does not decrease the within-cluster variation (total within sum of square, WSS) much [67]. We computed the silhouette coefficient and WSS from the various number clusters (1–10 clusters), and choose the best number of clusters with the highest silhouette coefficient and determined by the Elbow method. The sampling sites grouped into the ecoregions were represented on the map created by QGIS v.3.8 [68] with DEM data.

The richness levels of ELF from lichens belonging to different genera, with different photobiont types, and from different ecoregions were compared using ANOVA with a Tukey's multiple comparison test as a post-hoc test. Community structures were analyzed and visualized using non-metric multidimensional scaling (NMDS) based on binary Jaccard distance with the phyloseq package [69]. The samples with more than two ELF species were used for the community analysis. The influence of the lichen host taxonomy, photobiont types, and ecoregion effect on the community structures were tested using Adonis with the vegan package [70] and pairwise Adonis with the pairwiseAdonis package [71]. Barplots and scatter plots were drawn using the ggplot2 package [72]. Indicator species analysis was performed using the vegan package to detect the ELF species showing group-specific distribution. Only the ELF species with more than five occurrences in the total dataset were accepted as an indicator species. Network analysis was conducted to detect the host preference of the ELF species that have significantly higher interactions with a specific host lichen in comparison to that of the null networks (n = 100) using an econullnetr [73]. Among the significant results from the network analysis, we excluded the results of ELF species isolated from the low number of lichens (<5 specimens). A bipartite network plot was drawn using a bipartite package [74].

3. Results

3.1. Lichen Diversity

The 79 lichen specimens collected from 29 oreums in Jeju Island (Table A1) were identified morphologically as belonging to three orders (Caliciales, Lecanorales, and Peltigerales), eight families, 15 genera, and 44 species. The number of specimens was largest in Parmeliaceae (30 specimens), followed by Stereocaulaceae (12) and Physciaceae (10). At the genus level, *Parmotrema* was most frequently collected (18 specimens), followed by *Stereocaulon* (12) and *Myelochroa* (10). At the species

level, *Stereocaulon japonicum* was the most common species (12 specimens), followed by *Parmotrema tinctorum* (6) and *Myelochroa entotheiochroa* (5). Most species (38/44 species) were collected only once or twice. Three photobiont types were characteristic for the collected lichens: green algae, cyanobacteria, and both of green algae and cyanobacteria (Table S2 (Supplementary Materials)). All specimens belonging to Caliciales and Lecanorales (except for *Stereocaulon japonicum*) as well as *Lobaria discolor* and *Lobaria japonica* (Peltigerales) had a green algal photobiont. All other specimens belonging to Peltigerales had a cyanobacterial photobiont, except for *Peltigera leucophlebia*. *Stereocaulon japonicum* and *Peltigera leucophlebia* had a photobiont consisting both of green algae and cyanobacteria.

3.2. Ecoregion Clustering

The sampling sites were clustered to three ecoregions using PAM based on environmental factors (elevation and six bioclimatic data) (Figure 2a). Ecoregion G1 is characterized by high elevation and precipitation (BIO12, BIO14, and BIO15) (Table 1). Ecoregion G2 generally had a moderate level of environmental values, except for high isothermality (BIO03) and annual range of temperature (BIO07). Ecoregion G3 had a low elevation with high mean temperature (BIO10). The distribution of lichen specimens varied among the ecoregions (Figure 2b). The genera belonging to Peltigerales (e.g., *Collema, Leptogium,* and *Peltigera*) were abundant in the G1 ecoregion, while most genera in Lecanorales (e.g., *Cladonia*, *Myelochroa*, *Parmotrema*, and *Stereocaulon*) were abundant in the G2 and G3 ecoregions. In the G2 ecoregion, *Cladonia* and *Stereocaulon* were abundant, while *Parmotrema* and *Physcia* were abundant in the G3 ecoregion.

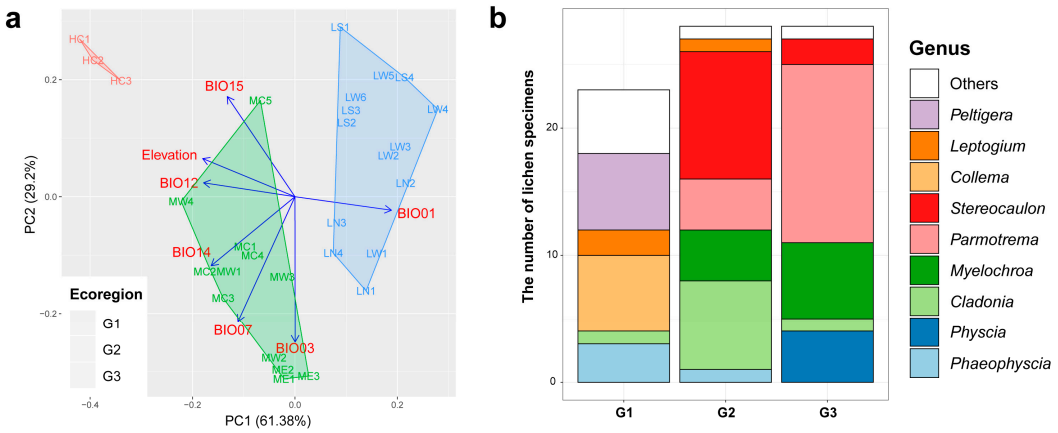

Figure 2. Classification of the ecoregions and distribution of lichen specimens in the ecoregions. (**a**) Principal component analysis (PCA) plot for sampling sites classified by the PAM (partitioning around medoids) algorithm based on elevation and bioclimatic variables (BIO01: annual mean temperature; BIO03: isothermality; BIO07: temperature annual range; BIO12: annual precipitation; BIO14: precipitation of the driest month; BIO15: precipitation seasonality); (**b**) distribution of lichen specimens among genera in the ecoregions.

Table 1. The elevation and bioclimatic variables for the ecoregions (mean ± SD). Ecoregions were classified by the PAM algorithm based on the elevation and bioclimatic variables of the sampling sites.

Variable	Ecoregion		
	G1 (n = 3)	G2 (n = 12)	G3 (n = 14)
Elevation (m, above sea level)	1351.0 ± 196.89	547.0 ± 185.54	223.6 ± 111.34
Annual mean temperature (BIO01) (°C)	9.5 ± 0.38	13.0 ± 0.91	15.1 ± 0.48
Isothermality (BIO03) (%)	24.3 ± 0.08	26.4 ± 0.82	25.1 ± 0.43
Temperature annual range (BIO07) (°C)	29.7 ± 0.44	30.5 ± 0.59	28.5 ± 0.96
Annual precipitation (BIO12) (mm)	2033.3 ± 47.51	1901.6 ± 37.15	1788.4 ± 48.9
Precipitation of the driest month (BIO14) (mm)	57.3 ± 0.58	54.6 ± 1.51	49.8 ± 2.08
Precipitation seasonality (BIO15) (%)	58.3 ± 0.21	56.4 ± 0.66	56.1 ± 0.77

3.3. Patterns of ELF Diversity

ML phylogenetic analysis was used to identify 619 ELF isolates from the lichen specimens (Table S2 (Supplementary Materials)) based on ITS sequences (Figures S1–S2 (Supplementary Materials)). ELF species belonged to three phyla, seven classes, 16 orders, 30 families, 46 genera, and 112 species. Most species belonged to Ascomycota (99 species, 88.4%), except for 11 species in Basidiomycota and two species in Mucoromycota. At the class level, Sordariomycetes had the highest number of species (80 species, 71.4%), followed by Dothideomycetes (15 species, 13.4%) (Figure 3a). Xylariales had the highest number of species (47 species, 42.0%), followed by Sordariales (18 species, 16.1%). At the genus level, the number of species was highest in *Nigrospora* (17 species) and *Chaetomium* (nine species). At the species level, *Daldinia childiae* was most frequently detected (47 specimens, 59.5%), followed by *Sordaria* cf. *fimicola* (26 specimens, 34.2%), *Nigrospora sphaerica* (25 specimens, 31.6%), and *Nemania diffusa* (23 specimens, 29.1%) (Figure 3b).

The ELF richness was compared between the host genera, photobiont types, and ecoregions (Table 2). The richness was significantly different between the host genera ($p = 0.017$), photobiont types ($p = 0.024$) and ecoregions ($p = 0.006$). The only pairwise comparison showed significant difference: ELF richness in *Stereocaulon* was significantly lower than that of *Parmotrema* ($p = 0.023$). For the photobiont type, the lichens with both green algae and cyanobacteria had significantly lower richness compared to that with green algae only ($p = 0.019$). For the ecoregions, G3 contained significantly higher ELF richness compared to G2 ($p = 0.006$).

Table 2. Distribution of ELF richness (mean ± SE) among lichen genera, lichens with different photobiont types, and ecoregions in Jeju island, South Korea. Different letters in a Group indicate significant differences ($p < 0.05$, Tukey's multiple comparison test, ANOVA). The genera with less than three samples were excluded from the analyses.

Variables	Name	N [(1)]	Richness	Group
Genus	*Cladonia*	9	4.44 ± 0.56	ab
	Collema	6	4.17 ± 0.70	ab
	Leptogium	3	4.33 ± 1.45	ab
	Myelochroa	10	6.20 ± 0.73	ab
	Parmotrema	18	6.22 ± 0.53	a
	Peltigera	6	5.67 ± 0.92	ab
	Phaeophyscia	4	4.00 ± 0.71	ab
	Physcia	4	4.25 ± 0.75	ab
	Stereocaulon	12	3.67 ± 0.41	b
Photobiont	Green algae	45	5.49 ± 0.32	a
	Green algae/Cyanobacteria	13	3.69 ± 0.38	b
	Cyanobacteria	14	4.86 ± 0.57	ab
Ecoregion	G1	18	4.67 ± 0.46	ab
	G2	27	4.30 ± 0.35	a
	G3	27	6.04 ± 0.43	b

[(1)] N: the number of samples.

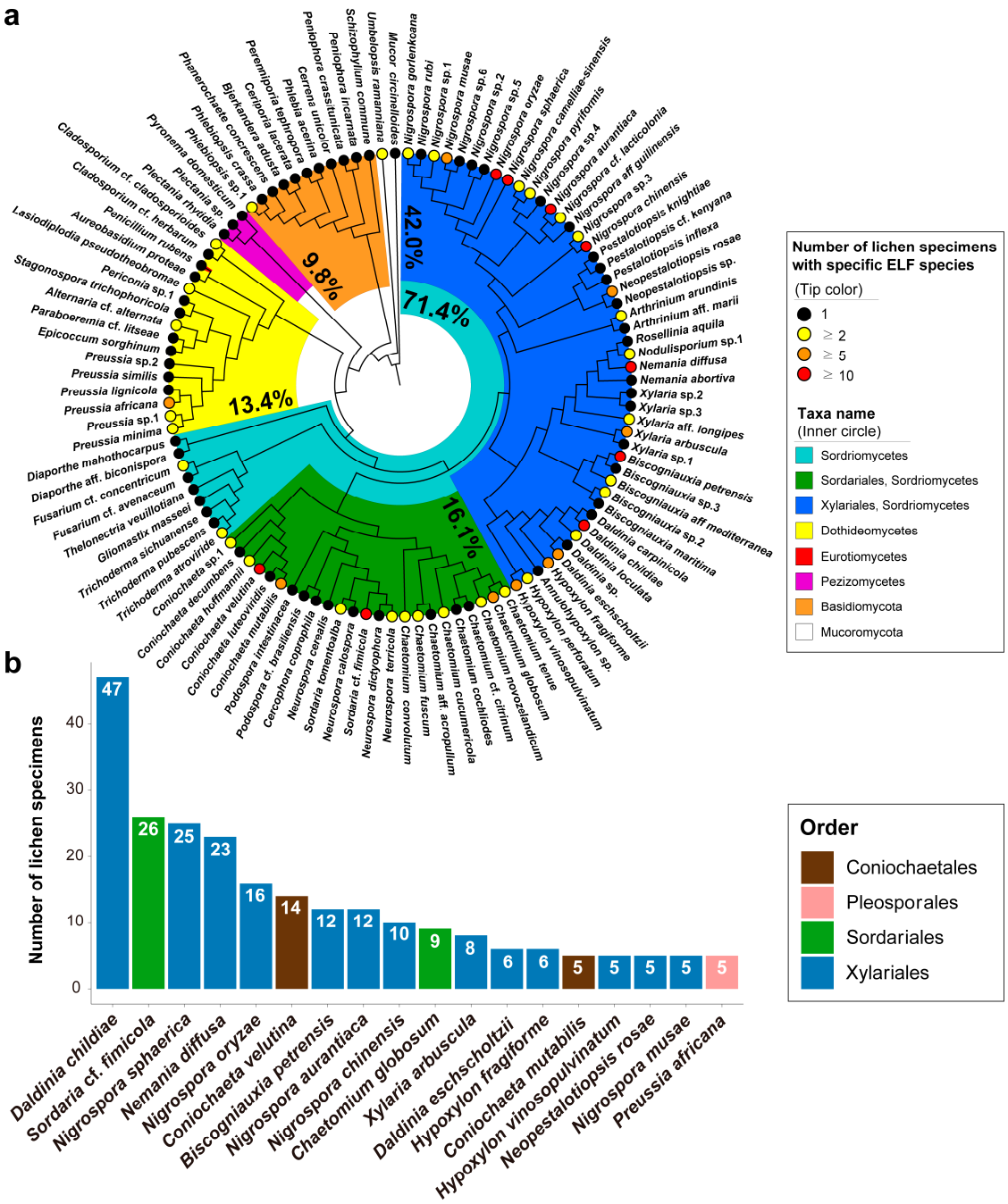

Figure 3. Diversity of endolichenic fungi (ELF) species isolated from Jeju Island. (**a**) Phylogenetic tree of ELF species based on ML analysis. The relative abundance (%) of ELF (class or order levels) is represented in the inner circle with different colors for each taxon. The color of the tip indicates the number of lichen specimens with the ELF species. Detailed phylogenetic trees are shown in Figures S1 and S2 (Supplementary Materials); (**b**) the number of lichen specimens isolated with major ELF species (≥5 specimens).

3.4. Structure of ELF Communities

Ordination analysis based on binary Jaccard dissimilarities revealed separation of communities according to the lichen taxonomy, photobiont, and ecoregions (Figure 4). Adonis analysis showed a significant effect of the host genera (R^2 = 0.179, p = 0.001), photobiont types (R^2 = 0.066, p = 0.001), and ecoregions (R^2 = 0.073, p = 0.001). For lichen genera, several pairwise comparisons, including

Collema, *Leptogium*, *Parmotrema*, and *Peltigera*, showed significant differences (Table 3). All pairs in the photobiont types showed significant differences and ecoregion G1 was significantly different from G2 and G3. Variation partitioning analysis showed that the effects of the photobiont type and ecoregion were confounded to that of the host genera, explaining 5.83% of the total variation (Figure S3 (Supplementary Materials)). Only the effect of host genera had unique explanatory power, even after removing the other effects.

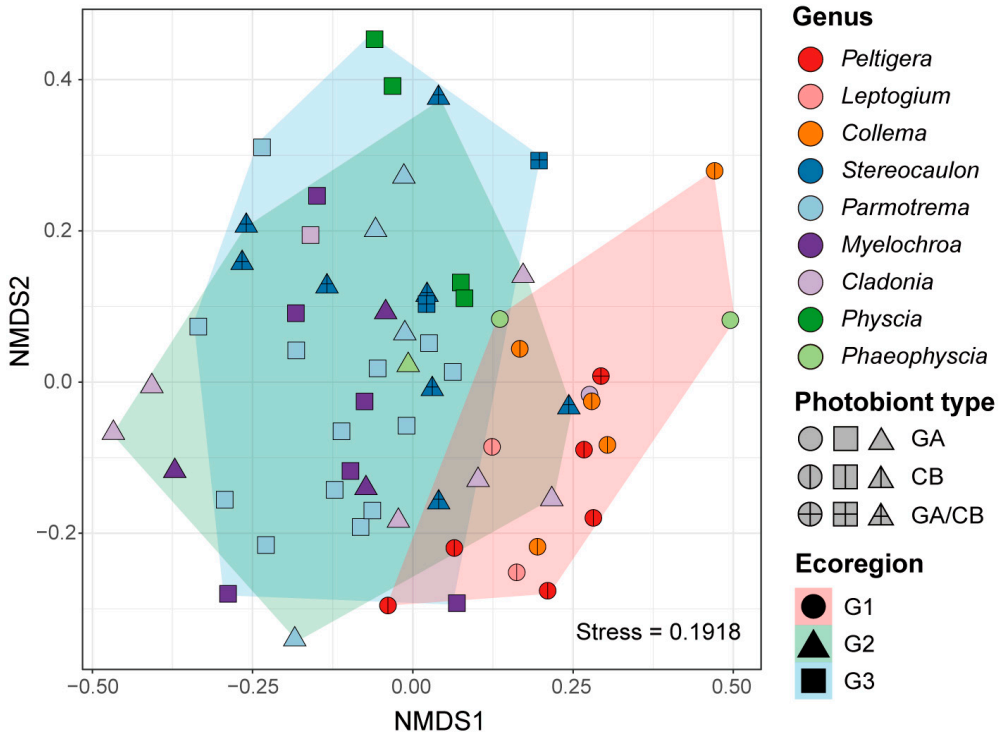

Figure 4. Non-metric multidimensional scaling (NMDS) plot for ELF communities based on binary Jaccard dissimilarities.

The indicator species analysis detected ELF species associated with definite lichen genera, photobiont types, and ecoregions (Table 4). *Sordaria* cf. *fimicola* was chosen as an indicator species for both Caliciales (*Phaeophyscia* and *Physcia*) and Lecanorales (*Myelochroa*, *Parmotrema*, and *Stereocaulon*). For the photobiont type, four indicator species were detected and the type of green algae and cyanobacteria had the highest number of indicator species (*Hypoxylon fragiforme*, *Sordaria* cf. *fimicola*, and *Nigrospora chinensis*). Seven indicator species were detected for the ecoregions. Network analysis showed similar results with indicator species analysis, in that five ELF species occurred with significantly higher frequency in specific lichen host compared to null networks simulated by the same number of interactions (Figure 5). *Biscogniauxia petrensis* and *Nigrospora aurantiaca* favored *Parmotrema*, *Hypoxylon fragiforme* and *Sordaria* cf. *fimicola* preferred *Stereocaulon*, and *Daldinia eschscholtzii* favored *Leptogium*.

Table 3. Pairwise Adonis comparisons between compositions of ELF from different lichen genera, lichens with different photobiont types, and ecoregions based on the binary Jaccard dissimilarities. *p* values were adjusted using the Bonferroni method. Significant differences are presented in bold.

Variables	Pairs	SS [(1)]	F Value	R^2	P_{adj} Value
Genus	*Cladonia* vs. *Collema*	0.464	1.123	0.093	0.367
	Cladonia vs. *Leptogium*	0.440	1.041	0.115	0.367
	Cladonia vs. *Phaeophyscia*	0.350	0.805	0.082	0.804
	Cladonia vs. *Peltigera*	0.396	0.956	0.074	0.588
	Cladonia vs. *Myelochroa*	0.359	0.859	0.054	0.726
	Cladonia vs. *Parmotrema*	0.537	1.371	0.056	0.176
	Cladonia vs. *Physcia*	0.480	1.108	0.100	0.363
	Cladonia vs. *Stereocaulon*	0.573	1.439	0.083	0.170
	Collema vs. *Leptogium*	0.435	1.231	0.198	0.363
	Collema vs. *Phaeophyscia*	0.387	1.011	0.144	0.504
	Collema vs. *Peltigera*	0.413	1.106	0.109	0.363
	Collema* vs. *Myelochroa	**0.744**	**1.918**	**0.138**	**0.029**
	Collema* vs. *Parmotrema	**0.920**	**2.487**	**0.111**	**0.018**
	Collema vs. *Physcia*	0.622	1.604	0.186	0.111
	Collema vs. *Stereocaulon*	0.690	1.888	0.127	0.062
	Leptogium vs. *Phaeophyscia*	0.439	1.157	0.278	0.367
	Leptogium vs. *Peltigera*	0.401	1.095	0.154	0.401
	Leptogium vs. *Myelochroa*	0.492	1.266	0.123	0.265
	Leptogium* vs. *Parmotrema	**0.658**	**1.795**	**0.095**	**0.036**
	Leptogium vs. *Physcia*	0.550	1.414	0.261	0.265
	Leptogium vs. *Stereocaulon*	0.618	1.722	0.147	0.111
	Phaeophyscia vs. *Peltigera*	0.432	1.106	0.136	0.367
	Phaeophyscia vs. *Myelochroa*	0.476	1.181	0.106	0.363
	Phaeophyscia vs. *Parmotrema*	0.484	1.287	0.067	0.340
	Phaeophyscia vs. *Physcia*	0.517	1.237	0.198	0.363
	Phaeophyscia vs. *Stereocaulon*	0.310	0.827	0.070	0.725
	Peltigera* vs. *Myelochroa	**0.680**	**1.738**	**0.118**	**0.018**
	Peltigera* vs. *Parmotrema	**0.888**	**2.382**	**0.102**	**0.018**
	Peltigera* vs. *Physcia	**0.657**	**1.669**	**0.173**	**0.031**
	Peltigera* vs. *Stereocaulon	**0.818**	**2.210**	**0.136**	**0.018**
	Myelochroa vs. *Parmotrema*	0.417	1.097	0.044	0.367
	Myelochroa vs. *Physcia*	0.570	1.410	0.114	0.134
	Myelochroa vs. *Stereocaulon*	0.643	1.686	0.090	0.050
	Parmotrema* vs. *Physcia	**0.689**	**1.823**	**0.088**	**0.031**
	Parmotrema* vs. *Stereocaulon	**0.747**	**2.024**	**0.075**	**0.036**
	Physcia vs. *Stereocaulon*	0.508	1.341	0.101	0.265
Photobiont [(2)]	**GA vs. CB**	**1.032**	**2.577**	**0.048**	**0.003**
	GA vs. GA/CB	**0.667**	**1.668**	**0.032**	**0.010**
	CB vs. GA/CB	**0.863**	**2.296**	**0.099**	**0.005**
Ecoregion	**G1 vs. G3**	**1.326**	**3.397**	**0.078**	**0.003**
	G1 vs. G2	**1.052**	**2.704**	**0.070**	**0.003**
	G3 vs. G2	0.521	1.305	0.028	0.108

[(1)] SS: sums of squares of variations. [(2)] Photobiont types: Cyanobacteria (CB) and green algae (GA).

Table 4. ELF species associated with definite lichen genera, photobiont types, and ecoregions. ELF occurring in more than five lichen specimens were chosen as indicator species.

Variables	Group	Indicator ELF	No.[1]	Stat	P
Genus	*Leptogium*	*Daldinia eschscholtzii*	6	0.851	0.001
	Parmotrema	*Biscogniauxia petrensis*	12	0.648	0.043
	Myel, *Parm*, *Phae*, *Phys*, *Stere* [2]	*Sordaria* cf. *fimicola*	26	0.698	0.033
Photobiont [3]	GA/CB	*Hypoxylon fragiforme*	6	0.464	0.044
	GA, GA/CB	*Sordaria* cf. *fimicola*	26	0.665	0.017
	GA/CB, CB	*Nigrospora chinensis*	10	0.521	0.036
Ecoregion	G1	*Nigrospora chinensis*	10	0.585	0.001
	G2	*Hypoxylon fragiforme*	6	0.441	0.012
	G3	*Xylaria arbuscula*	8	0.555	0.002
	G3	*Nigrospora aurantiaca*	12	0.524	0.013
	G2, G3	*Sordaria* cf. *fimicola*	26	0.655	0.011
	G2, G3	*Biscogniauxia petrensis*	12	0.50	0.042

[1] No.: the number of lichen specimens where the ELF occurred. [2] Abbreviations of the genera names: Myel (*Myelochroa*), Parm (*Parmotrema*), Phae (*Phaeophyscia*), Phys (*Physcia*), and Stere (*Stereocaulon*). [3] Photobiont types: Cyanobacteria (CB) and green algae (GA).

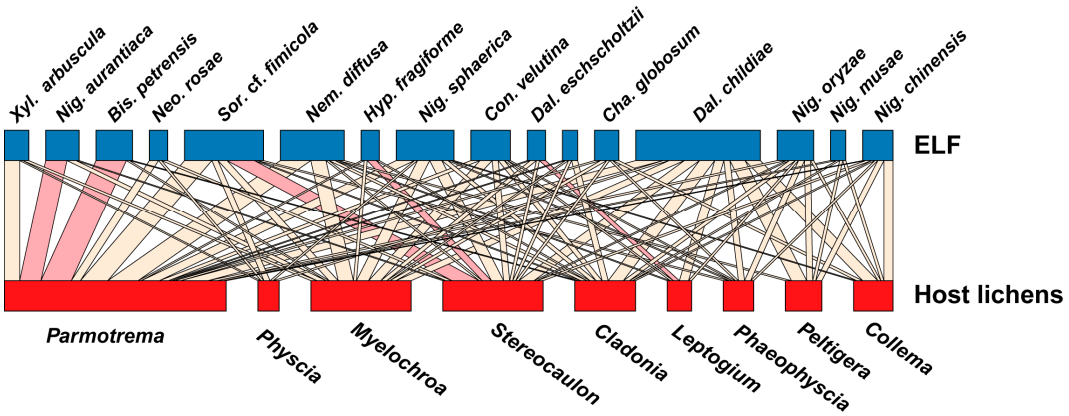

Figure 5. Bipartite network plot for major ELF species (≥5 occurrences). The line width indicates the number of occurrences. The ELF species showing a host preference are represented by the pink line.

4. Discussion

On Jeju Island, diverse ELF biota comprising 112 species was isolated from 79 lichen specimens. These ELF biota can be considered as one of the most numerous found at the regional level. Most studies identified approximately 20–60 species at the regional scale [13,16,17,22,28,30,39,75,76]. At the continental scale, Arnold et al. [9] isolated approximately 200 species, which was the highest number of ELF species found in a single study. Most ELF species isolated in our study belonged to Ascomycota, which coincides with the previous studies showing the dominance of Ascomycetes among ELF species [9,14,22,30]. Among the Ascomycota, Sordariomycetes had the highest number of ELF species (71.8%), followed by Dothideomycetes (13.6%). Some previous studies also showed a dominance of Sordariomycetes in ELF species (in China, France, Japan, North America, Norway, and Sri Lanka) [9,22,28,31,39,75]. By contrast, other studies showed a dominance of Dothideomycetes, Leotiomycetes or Pezizomycetes (in Antarctica, Germany, Italy, and USA) [13,14,27,77]. However, there is no specific pattern of ELF dominance depending on the country, continent, or climate zone. The species of *Daldinia*, *Sordaria*, and *Nigrospora* were the most frequently detected ELF species in Jeju Island (Figure 3), which were frequently isolated from lichens in the previous studies [17,28,30,39,75]. These genera are well-known wood-decaying fungi (*Daldinia*, *Hypoxylon*, and *Xylaria)* and soil-borne saprotroph (*Chaetomium*, *Nigrospora*, and *Trichoderma*). However, lichens collected in this study did

not show any symptom of decomposition or disease, which suggests that many ELF species have a saprotrophic lifestyle but it is inactive within lichen thallus. Similar phenomena have been found from endophytic fungi in plants; Many endophytic fungi in leaves turn into saprotrophic fungi when leaves fall on the ground [78]. Considering a taxonomic similarity between ELF and endophytic fungi [79,80], ELF may be a latent saprotroph, like some endophytic fungi, waiting for a suitable environment where they start to decompose. In addition, some ELF are known as coprophilous fungi (*Sordaria* and *Preussia*) living in the feces [81,82]. Given that feces are a nitrogen-rich environment, coprophilous fungi may prefer lichens containing cyanobacteria because they can fix nitrogens from the atmosphere [83]. Abundance of melanin-producing fungi (e.g., *Daldinia, Sordaria*, and *Xylaria*) was another important feature of ELF communities isolated in this study [84–86]. In a harsh environment, melanin-producing fungi are abundant because melanin has a protective role against abiotic stress such as UV radiation, extreme pH or temperature, and drought [87]. Therefore, the melanin-producing ability of ELF can be the adaptive feature for survival of lichens in a harsh environment. In addition, the dominance of *Daldinia childiae* in lichens may be associated with its adaptation to a hostile environment. Genome sequence of *Daldinia eschscholtzii* showed that carbon assimilation in nutrient-limited conditions (acid trehalases) and the heat stress response gene (ATP-dependent molecular chaperone) are core gene families in this species [88].

The patterns of ELF richness and community structure were significantly influenced by the host taxonomy, photobiont types, and ecoregions, while their effects were mixed together (Table 2; Figure 4 and Figure S3 (Supplementary Materials)). The effects of the photobiont types and ecoregions were confounded to the host effect and the unique effect on community structure was found only for the lichen taxonomy (Figure S3 (Supplementary Materials)). Generally, the photobiont type is specific for a specific lichen taxon, which explains why the effect of the photobiont types was confounded to the effect of the lichen taxonomy. For the effect of the ecoregions, the distribution of lichens varied among the ecoregions (Figure 2b), which led to a mixed effect of the host taxonomy and ecoregions. In the G1 ecoregion (high elevation and precipitation), Peltigerales species were abundant; *Cladonia* and *Stereocaulon* were abundant in G2 (moderate elevation and climate with wide temperature range), and *Parmotrema* and *Physcia* were abundant in G3 (lowlands with high average temperature) [41].

The ELF richness was comparable among the lichen genera, except for *Stereocaulon* and *Parmotrema* (Table 2); *Myelochroa* and *Parmotrema* had high ELF richness, while *Stereocaulon* had significantly low ELF richness. The high richness of ELF in *Myechora* and *Parmotrema* may be associated with the elevation in which they were frequently collected (lowlands) (Table A1), because for endophytic fungi, their richness increased in the low elevation region [35,89]. By contrast, the lowest richness of ELF in *Stereocaulon* may be due to its morphological characteristics. Among the lichens we collected, *Sterocaulon* was the only genus with fruticose thallus [90]. Because fruticose lichens are generally narrow and thin compared to foliose lichens, their thallus harbors lower number of ELF species than foliose lichens. The ELF community structures were significantly influenced by the taxonomy of host lichens (Figure 4; Table 3). Previous studies also showed a difference of ELF communities depending on the lichen taxonomy [14]. Although it was significant, only a small part of the variations was explained by the host genus (6%). In addition, frequently detected ELF have numerous hosts showing host generality, which suggests that the host generality in the overall ELF communities is stronger than the host specificity. Chagnon et al. [26] also found that ELF showed higher host generality compared to endophytic fungi living in the plants. The low level of host specificity may be due to the harsh environment of the lichen thallus. Generally, lichens are poikilohydric organisms that frequently experience alternating dry and wet conditions [91] and damages by the reactive oxygen species (ROS) [92], which can be a harsh condition for ELF living inside of the thallus. In a harsh environment, the fungal community showed low host specificity. For example, the host specificity of fungal symbionts in plants (e.g., endophytes, ectomycorrhizal, or ericoid fungi) was decreased in alpine and arctic environments [93–96].

A previous study showed that the location of ELF in the lichen thallus is at the photobiont layer [9] and ELF can acquire nutrients from the photobiont [8], which suggests that the photobiont type can be important to the ELF composition in lichens. In line with this assumption, the photobiont type significantly influenced the ELF richness and communities' composition. The lichens with two kinds of photobionts showed significantly low ELF richness (Table 2). In addition, ELF community structures were also significantly different between lichens with different photobiont type (Figure 4; Table 3). Peltigerales and *Stereocaulon* are different compared to other lichens in terms of the photobiont type. Most Peltigerales species have cyanobacteria (e.g., *Nostoc*) as the primary photobiont, whereas *Stereocaulon* has green algae as the primary photobiont with cyanobacteria as the secondary photobiont [90]. Cyanobacteria can fix atmospheric nitrogen; thus, the total nitrogen concentration in the thallus was higher in these lichens [83], which can influence the ELF community and be attractive to coprophilous fungi such as *Sordaria* cf. *fimicola* (Table 4; Figure 5).

The ecoregions classified by the elevation and bioclimatic variables were one of the factors influencing the ELF richness and community structures (Table 2; Figure 4). The richness in the G3 ecoregion (high mean temperatures in lowlands) was significantly higher than that in the other ecoregions, which agrees with a previous observation that the ELF richness was increased following the length of the growing season that is associated with the annual temperature [15]. The ecoregion effect on the community structures was significant but totally confounded to the host effects; none of the explanatory power was unique for the ecoregions. Generally, endophytic fungal communities also showed variations according to the variations in such environmental factors as elevation, annual precipitation, and temperature [35,97–99]. The lack of unique environmental patterns in this study may have arisen because the regional scale of environmental variations may be insufficient to reveal substantial variations in ELF diversity. In addition, elevation did not generally influence the presence or absence of ELF species but affected their abundance [100]; meanwhile, our study focused on the presence and absence of ELF species.

Although diverse ELF biota were isolated in our study, this study has some fundamental limitations that raise caution in interpreting the study results. First, we used a single method for isolation, which limited the number of isolated ELF species. Recent studies have shown that ELF diversity can vary depending on the isolation protocol [18,75]. Various media constituting the different nutrient compositions and different sterilization methods can expand the ELF diversity. Second, this study only covered cultivatable ELF. Given that many culture-independent studies have revealed a deeper fungal diversity [32,101], the culture-dependent approach may not have detected the whole ELF diversity within the lichen. A recent metabarcoding study for ELF diversity in Arctic and Antarctic regions showed higher ELF diversity [21,22], which suggests that the ELF diversity in Jeju Island may also exceed the diversity revealed in our study. Finally, the lichen taxonomy as a factor influencing the ELF communities was analyzed at the genus level due to the insufficient number of specimens. The low resolution of the lichen taxonomy may have ignored the ecological patterns at a fine level. Further studies with specific hypothesis and experimental designs are needed. Despite these study limitations, the culture-dependent approach can reveal remarkable diversity and consistent patterns of ELF communities. In addition, obtaining ELF isolates is important considering the usefulness of ELF as a potential source of natural products. Thus, understanding the ecological pattern of ELF isolates is meaningful to obtain various ELF species that can have potential experimental or industrial applications.

5. Conclusions

A high diversity of ELF species was identified from the lichens in Jeju Island. The most common ELF species belonged to Sordariomycetes, which is similar to the ELF diversity reported in other countries. In addition, many species were newly identified as ELF species in South Korea. The ELF richness and community composition were significantly influenced by the combination of host characteristics and environmental factors. Our results suggest that in order to reveal a higher diversity

of ELF species, we need to collect a higher variety of lichen species from different environments and to combined the culture-based methodology with a culture-independent approach such as a metabarcoding method.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/9/3769/s1. Figure S1: Phylogenetic tree based on ML analysis for (A) Xylariales and (B) other Sordariomycetes; Figure S2: Phylogenetic tree based on ML analysis for (A) other Ascomycota (non-Sordariomycetes) and (B) Basidiomycota and Mucoromycta; Figure S3: Variation partitioning plot for ELF communities based on binary Jaccard dissimilarities. The adjusted explanatory powers are presented for each factor; Table S1: Information about sampling locations; Table S2: Information of the ELF species isolated from this study.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Information about lichen specimens.

Location	Ecoregion	Lichen Name	Order	Family	Photobiont [(1)]	Substrate
HC1	G1	*Anaptychia isidiza*	Caliciales	Physciaceae	GA	Tree
		Heterodermia isidiophora	Caliciales	Physciaceae	GA	Tree
		Phaeophyscia erythrocardia	Caliciales	Physciaceae	GA	Tree
		Phaeophyscia exornatula	Caliciales	Physciaceae	GA	Tree
		Phaeophyscia imbricata	Caliciales	Physciaceae	GA	Tree
		Cladonia symphycarpia	Lecanorales	Cladoniaceae	GA	Tree
		Collema japonicum	Peltigerales	Collemataceae	CB	Tree
		Collema japonicum	Peltigerales	Collemataceae	CB	Tree
		Collema japonicum	Peltigerales	Collemataceae	CB	Tree
		Collema japonicum	Peltigerales	Collemataceae	CB	Tree
		Collema subflaccidum	Peltigerales	Collemataceae	CB	Tree
		Leptogium saturninum	Peltigerales	Collemataceae	CB	Tree
		Lobaria discolor	Peltigerales	Lobariaceae	GA	Tree
		Lobaria japonica	Peltigerales	Lobariaceae	GA	Tree
HC2	G1	*Peltigera degenii*	Peltigerales	Peltigeraceae	CB	Rock
		Peltigera didactyla	Peltigerales	Peltigeraceae	CB	Soil
		Peltigera horizontalis	Peltigerales	Peltigeraceae	CB	Rock
		Peltigera leucophlebia	Peltigerales	Peltigeraceae	GA/CB	Rock
		Peltigera neopolydactyla	Peltigerales	Peltigeraceae	CB	Rock
		Peltigera polydactylon	Peltigerales	Peltigeraceae	CB	Rock
HC3	G1	*Parmelia adaugescens*	Lecanorales	Parmeliaceae	GA	Tree
		Collema subflaccidum	Peltigerales	Collemataceae	CB	Tree
		Leptogium pedicellatum	Peltigerales	Collemataceae	CB	Tree
MC1	G2	*Cladonia* sp. 1	Lecanorales	Cladoniaceae	GA	Unknown
		Parmotrema cristiferum	Lecanorales	Parmeliaceae	GA	Unknown
		Punctelia subrudecta	Lecanorales	Parmeliaceae	GA	Unknown
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
MC2	G2	*Cladonia pyxidata*	Lecanorales	Cladoniaceae	GA	Unknown
		Cladonia rei	Lecanorales	Cladoniaceae	GA	Unknown
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
MC3	G2	*Cladonia scabriuscula*	Lecanorales	Cladoniaceae	GA	Tree
		Parmotrema cetratum	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema perlatum	Lecanorales	Parmeliaceae	GA	Tree
MC4	G2	*Stereocaulon japonicum*	Lecanorales	Stereocaulaceae	GA/CB	Rock

Table A1. *Cont.*

Location	Ecoregion	Lichen Name	Order	Family	Photobiont [(1)]	Substrate
MC5	G2	*Myelochroa entotheiochroa*	Lecanorales	Parmeliaceae	GA	Unknown
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
ME1	G2	*Stereocaulon japonicum*	Lecanorales	Stereocaulaceae	GA/CB	Rock
ME2	G2	*Stereocaulon japonicum*	Lecanorales	Stereocaulaceae	GA/CB	Rock
ME3	G2	*Stereocaulon japonicum*	Lecanorales	Stereocaulaceae	GA/CB	Rock
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
MW1	G2	*Cladonia symphycarpia*	Lecanorales	Cladoniaceae	GA	Tree
		Myelochroa indica	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema sp. 1	Lecanorales	Parmeliaceae	GA	Tree
MW2	G2	*Cladonia mongolica*	Lecanorales	Cladoniaceae	GA	Unknown
		Myelochroa indica	Lecanorales	Parmeliaceae	GA	Unknown
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
MW3	G2	*Phaeophyscia* sp. 1	Caliciales	Physciaceae	GA	Unknown
		Myelochroa entotheiochroa	Lecanorales	Parmeliaceae	GA	Unknown
MW4	G2	*Cladonia* sp. 2	Lecanorales	Cladoniaceae	GA	Tree
		Leptogium pedicellatum	Peltigerales	Collemataceae	CB	Tree
LN1	G3	*Myelochroa aurulenta*	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema praesorediosum	Lecanorales	Parmeliaceae	GA	Tree
LN2	G3	*Physcia orientalis*	Caliciales	Physciaceae	GA	Tree
		Physcia orientalis	Caliciales	Physciaceae	GA	Tree
		Parmotrema austrosinense	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema austrosinense	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema dilatatum	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema tinctorum	Lecanorales	Parmeliaceae	GA	Tree
LN3	G3	*Parmotrema reticulatum*	Lecanorales	Parmeliaceae	GA	Tree
		Parmotrema tinctorum	Lecanorales	Parmeliaceae	GA	Tree
LN4	G3	*Parmotrema defectum*	Lecanorales	Parmeliaceae	GA	Unknown
		Parmotrema praesorediosum	Lecanorales	Parmeliaceae	GA	Unknown
LS1	G3	*Parmotrema reticulatum*	Lecanorales	Parmeliaceae	GA	Unknown
LS2	G3	*Stereocaulon japonicum*	Lecanorales	Stereocaulaceae	GA/CB	Rock
LS3	G3	*Cladonia kurokawae*	Lecanorales	Cladoniaceae	GA	Unknown
LS4	G3	*Myelochroa entotheiochroa*	Lecanorales	Parmeliaceae	GA	Unknown
		Myelochroa indica	Lecanorales	Parmeliaceae	GA	Unknown
		Stereocaulon japonicum	Lecanorales	Stereocaulaceae	GA/CB	Rock
LW1	G3	*Dirinaria applanata*	Caliciales	Caliciaceae	GA	Tree
		Physcia orientalis	Caliciales	Physciaceae	GA	Tree
		Physcia orientalis	Caliciales	Physciaceae	GA	Tree
LW2	G3	*Parmotrema tinctorum*	Lecanorales	Parmeliaceae	GA	Unknown
LW3	G3	*Myelochroa aurulenta*	Lecanorales	Parmeliaceae	GA	Unknown
LW4	G3	*Parmotrema tinctorum*	Lecanorales	Parmeliaceae	GA	Unknown
LW5	G3	*Parmotrema tinctorum*	Lecanorales	Parmeliaceae	GA	Unknown
		Parmotrema tinctorum	Lecanorales	Parmeliaceae	GA	Unknown
LW6	G3	*Myelochroa entotheiochroa*	Lecanorales	Parmeliaceae	GA	Unknown
		Myelochroa entotheiochroa	Lecanorales	Parmeliaceae	GA	Unknown

[(1).] Photobiont type: Cyanobacteria (CB), green algae (GA).

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