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Diversity and Saline Resistance of Endophytic Fungi Associated with *Pinus thunbergii* in Coastal Shelterbelts of Korea

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The Black Pine, *Pinus thunbergii*, is widely distributed along the eastern coast of Korea and its importance as a shelterbelt was highlighted after tsunamis in Indonesia and Japan. The root endophytic diversity of *P. thunbergii* was investigated in three coastal regions; Goseong, Uljin, and Busan. Fungi were isolated from the root tips, and growth rates of pure cultures were measured and compared between PDA with and without 3% NaCl to determine their saline resistance. A total of 259 isolates were divided into 136 morphotypes, of which internal transcribed spacer region sequences identified 58 species. Representatives of each major fungi phylum were present: 44 Ascomycota, 8 Zygomycota, and 6 Basidiomycota. Eighteen species exhibited saline resistance, many of which were *Penicillium* and *Trichoderma* species. Shoreline habitats harbored higher saline-tolerant endophytic diversity compared with inland sites. This investigation indicates that endophytes of *P. thunbergii* living closer to the coast may have higher resistance to salinity and potentially have specific relationships with *P. thunbergii*.

Keywords: Coastal pine forest, endophyte community, salinity tolerance, shelterbelt

Introduction

The Black Pine, *Pinus thunbergii* Parl., native to South Korea and Japan, is adapted to coastal areas and can tolerate high salt environments [33, 47]. The important role of *P. thunbergii* forests as a bioshield protecting arable land and buildings from large waves and strong winds was highlighted after recent tsunamis in Indonesia [35] and Japan [50]. For this reason, efforts have been made to understand the ecology of *P. thunbergii* to aid in recovering and maintaining coastal pine forests in Korea [7, 44].

Endophytes are broadly defined as microbes that reside entirely in plant tissues of stems, leaves, or roots [6]. Fungal endophytes are ubiquitous, with a worldwide estimate of one million species [12]. This host-endophyte relationship can take on several forms, including mutualism, commensalism, or parasitism. Most endophytes are classified in the fungal phylum Ascomycota [1, 61], but some Zygomycota [10, 14, 19, 20] and Basidiomycota [40, 41] species have been identified.

Endophytic fungi can be beneficial to plants by producing growth-promoting metabolites, enhancing nutrient uptake, increasing tolerance to harsh environmental conditions, and enhancing defenses to herbivores and pathogens [2, 3, 40, 55]. Environmental stressors, such as salinity, determine the fungal community structure and distribution [29]. Endophytes can confer habitat-adapted tolerance to host plants, while the endophyte may receive benefits such as nutrients and protection from the host plant [40, 41]. A study examining the mutualistic relationship between a coastal dunegrass (*Leymus mollis*) and its endophyte (*Fusarium culmorum*) found rapid growth and salt tolerance when together, but slowed growth when grown separately [40, 41].

Isolating and culturing endophytic fungi are essential in studying their physiological properties and applying them for biotechnological purposes [34, 51]. However, these methods are difficult for identification because many fungal species lack distinguishing morphological characteristics when cultured [8]. Recently, the use of DNA sequencing and DNA databases has enhanced our ability to accurately classify endophytic fungi [16, 28]. We take this combined culture-sequence approach to identify the diversity and

understand the ecological function of endophytic fungi associated with *P. thunbergii*.

This study represents the first work concerning the endophyte diversity in *P. thunbergii* roots. Owing to the unique microhabitat of a coastal environment, it is important to identify the fungal community of saline-tolerant fungi that have a potential of enhancing the survival and proliferation of *P. thunbergii*. To understand the geographical and ecological variation, we compared across three regions along the eastern coast of South Korea, sampling tree roots adjacent to the shoreline and inland.

Materials and Methods

Study Site and Collection

In the fall of 2012, we sampled three regions spanning the eastern coast of Korea: Goseong, Uljin, and Busan (Fig. 1). Except for Busan, where woody and herbaceous plants are syntopic with *P. thunbergii*, the sites are primarily occupied by *P. thunbergii*. In all regions, the *P. thunbergii* community is composed of mature trees estimated to be ~60 years old, located at least 200 m inland from the shoreline, and several hundred meters wide. A total of 18 root sections were taken at each site: three root sections from six trees (three trees adjacent to the shoreline and three trees inland).

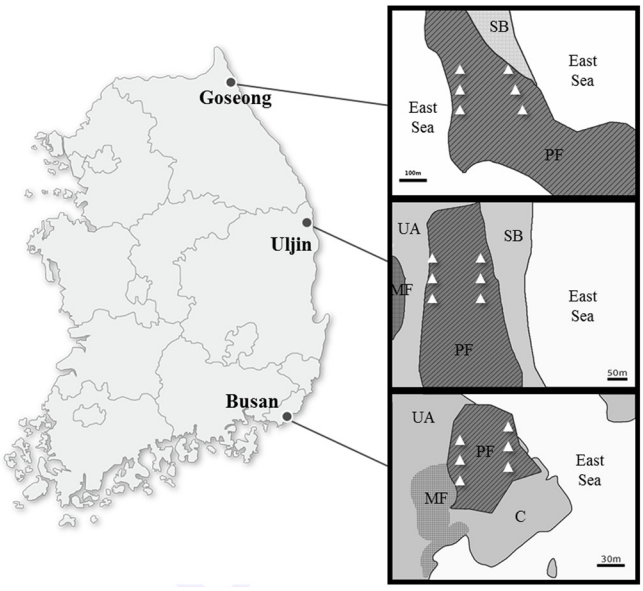

Fig. 1. The three study regions along the eastern coast of Korea in this study.

Δ indicates each collection point. At each study site, root samples were collected from six *Pinus thunbergii* individuals. Three collection points were located adjacent to the shoreline, while the other three points were ~200 m inland. SB = Sandy Beach, PF = Pine Forest, UA = Urban Area, MF = Mixed Forest, C = Cliff.

Inland samples were >200 m from the samples adjacent to the shoreline. In total, 54 roots from 18 mature trees were collected. The average length of fine root samples was approximately 30 cm. Additionally, soil from around the roots of all trees was sampled (18 total) to test soil properties. Salinity, pH, total nitrogen, and total phosphorus were analyzed for each sample at the National Instrumentation Center for Environmental Management (Seoul National University, Korea).

Endophyte Isolation and Saline Resistance Test

Root surfaces were sterilized using standard procedures [46]. These roots were cut to approximately 5 mm in length for isolation of endophytic fungi. Under sterile conditions, the root fragments were plated on potato dextrose agar (PDA) medium with 100 ppm streptomycin, incubated at 20°C in the dark for several days, and subcultured from mycelia margins to a new plate to obtain pure cultures. All pure culture isolates were grouped based on culture characters and microscopic features (Nikon 80i microscope). A representative isolate of each group was selected for DNA sequencing and evaluating salt resistance. Testing for salt resistance was performed by comparing growth rates on PDA with and without 3% NaCl, each performed in triplicate and averaged. We set 3% NaCl for the growth media to approximate the average seawater salinity of 3.5% [22]. Results are expressed in a ratio of mean growth rate with NaCl/mean growth rate without NaCl.

Molecular Approach for Identification of Endophytic Fungi

DNA was extracted using a modified CTAB extraction protocol [42]. The internal transcribed spacer region (ITS) was amplified using primer set ITS1F/ ITS4 [57]. PCR was performed on a C1000 thermocycler (Bio-Rad, USA) using the Maxime PCR PreMix-StarTaq (Intron Biotechnology Inc., Korea) in a final volume of 20 µl containing 10 pmol of each primer and 1 µl of DNA. The PCR conditions were 95°C for 10 min, followed by 35 cycles of 95°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed through a 1% agarose gel stained with loading STAR (Dyne Bio, Korea) and purified using the Expin PCR Purification Kit (GeneAll Biotechnology, Korea) according to the manufacturer's instructions. DNA sequencing was performed at the DNA Synthesis and Sequencing Facility, Macrogen (Korea), using an ABI3700 automated DNA sequencer. The sequences were deposited in GenBank (Table 1).

To identify representative endophytes, we use BLAST to compare sequences against type strains [25]. In GenBank, we searched for sequences that have the identifiers "type strain", "ex-type", "holotype", or "isotype" in the description. Since type strains available in GenBank are limited, we also included the top matching BLAST match for each sample. A phylogenetic tree was constructed to assign species names to samples. When a sequence clustered with a single species, either a type sequence or not, this identification was chosen. When the sequence was highly similar

Table 1. Classification of endophytic fungi isolated from *Pinus thunbergii* based on the GenBank database.

Fungal identity	Classification[a]	Strain[b]	GenBank Acc. No.	No. of isolates[c]						Closest GenBank match (% of Similarity)	Growth rate[d]
				Goseong		Uljin		Busan			
				S	I	S	I	S	I		
Acremonium variecolor	A/S	SFCF20120803-45	KF313108			1	1			HE608648 (99%)	1.6
Aspergillus sydowii[e]	**A/E**	**SFCF20120803-11**	**KF313094**	**1**						**AM883160 (99%)**	**1.4**
Bionectria ochroleuca[e]	**A/S**	**SFCF20120803-24**	**KF313107**	**2**						**EU071701 (99%)**	**0.3**
Chaetomium sp.	A/S	SFCF20120803-33	KF313104		1					JN168655 (99%)	0.4
Cladosporium sp.	A/D	SFCF20120803-58	KF313095	1		2				AY213640 (99%)	1.2
Eupenicillium brefeldianum	A/E	SFCF20120912-32	KF313083					4		JQ680034 (99%)	1.1
Eupenicillium javanicum	A/E	SFCF20120912-39	KF313084						2	U18358 (99%)	0.9
Eupenicillium pinetorum	A/E	SFCF20120803-43	KF313076			1				AF033411 (99%)	0.6
Fusarium oxysporum	A/S	SFCF20120912-05	KF313101			1	1	12	3	KC196121 (100%)	1.1
Fusarium sporotrichioides	A/S	SFCF20120803-57	KF313100			1				JN942834 (100%)	0.8
Gibberella sp.	A/S	SFCF20120912-15	KF313102					6		AY213654 (99%)	1
Hypocrea caerulescens	A/S	SFCF20120803-59	KF313113			1				JN715589 (99%)	0.9
Hypocrea sp.	A/S	SFCF20120803-50	KF313111			13	1			JN943372 (100%)	0.5
Ilyonectria cyclaminicola	A/S	SFCF20120912-03	KF313109					1		JF735304 (99%)	0.8
Myxotrichum stipitatum	A/D	SFCF20120912-11	KF313096					3		AF062816 (98%)	1.8
Penicillium adametzii[e]	**A/E**	**SFCF20120803-36**	**KF313079**		**1**					**NR103661 (100%)**	**0.6**
Penicillium atramentosum	A/E	SFCF20120912-01	KF313092		1		3	2	2	HQ115681 (99%)	1.2
Penicillium canescens[e]	**A/E**	**SFCF20120912-02**	**KF313093**			**11**	**7**	**6**		**AY373901 (99%)**	**1.5**
Penicillium citreonigrum[e]	**A/E**	**SFCF20120803-10**	**KF313086**	**7**			**3**			**AY373908 (98%)**	**1**
Penicillium citreonigrum[e]	**A/E**	**SFCF20120912-25**	**KF313080**					**1**		**AY157489 (99%)**	**2.3**
Penicillium daleae	A/E	SFCF20120803-30	KF313087		2					AF0334423 (99%)	0.6
Penicillium glabrum	A/E	SFCF20120803-53	KF313078			3		1		JN246001 (99%)	1.2
Penicillium janthinellum	A/E	SFCF20120912-18	KF313085					1		GU934553 (99%)	1.1
Penicillium montanense[e]	**A/E**	**SFCF20120803-19**	**KF313077**	**1**						**AF527058 (100%)**	**0.5**
Penicillium ochrochloron	A/E	SFCF20120912-47	KF313088						1	AY213675 (99%)	0.9
Penicillium raistrickii[e]	**A/E**	**SFCF20120912-26**	**KF313091**					**1**		**AY373927 (99%)**	**1.3**
Penicillium rolfsii	A/E	SFCF20120912-04	KF313082			4		3		AF033439 (98%)	1.1
Penicillium roseopurpureum	A/E	SFCF20120912-07	KF313089					3		AJ608963 (99%)	1.9
Penicillium swiecickii	A/E	SFCF20120803-12	KF313090	1						AF033490 (99%)	0.5
Penicillium sp.	A/E	SFCF20120912-37	KF313081						4	JF439500 (99%)	0.9
Pestalotiopsis sp.	A/S	SFCF20120803-66	KF313103		4		4			JX398990 (99%)	1.1
Phialocephala fortinii[e]	**A/L**	**SFCF20120803-02**	**KF313097**	**1**				**1**	**2**	**NR103577 (98%)**	**0.3**
Phialocephala sp.	A/L	SFCF20120803-08	KF313098	1			3			AB636440 (99%)	0.7
Phoma chrysanthemicola	A/D	SFCF20120803-70	KF313119			1				JN123358 (99%)	0.2
Phoma herbarum	A/D	SFCF20120803-51	KF313118			1				EU715673 (100%)	0.5
Phomopsis columnaris	A/S	SFCF20120912-20	KF313099					2		FN394688 (99%)	0.9
Simplicillium lamellicola	A/S	SFCF20120803-75	KF313106				2			AF108471 (99%)	0.1
Stemphylium sp.	A/D	SFCF20120803-44	KF313117			2				AF442784 (98%)	0.8
Thielavia terrestris	A/S	SFCF20120912-13	KF313105					1		AJ271589 (94%)	0.3
Trichoderma erinaceum	A/S	SFCF20120912-46	KF313116			1	3		2	EU280130 (99%)	0.8
Trichoderma hamatum[e]	**A/S**	**SFCF20120803-07**	**KF313114**	**2**			**3**	**1**		**AF011958 (99%)**	**1.1**
Trichoderma harzianum[e]	**A/S**	**SFCF20120803-29**	**KF313112**		**1**					**AF012003 (99%)**	**0.7**

Table 1. Continued.

Fungal identity	Classification[a]	Strain[b]	GenBank Acc. No.	No. of isolates[c]						Closest GenBank match (% of Similarity)	Growth rate[d]
				Goseong		Uljin		Busan			
				S	I	S	I	S	I		
Trichoderma koningiopsis	A/S	SFCF20120803-32	KF313115		2	3			13	JQ040369 (100%)	1.1
Trichoderma velutinum	A/S	SFCF20120803-76	KF313110				1			HM176565 (99%)	0.2
Bjerkandera adusta	B/A	SFCF20120803-28	KF313125		1			3	1	AB567717 (99%)	0.4
Irpex lacteus	B/A	SFCF20120803-21	KF313124	1						JX290577 (99%)	0.1
Merulius tremellosus	B/A	SFCF20120912-40	KF313123						1	HM051073 (99%)	0.1
Phanerochaete sordida	B/A	SFCF20120803-20	KF313122	1						HM595562 (99%)	0.1
Stereum hirsutum	B/A	SFCF20120803-27	KF313120		1					AB733150 (99%)	0.8
Trametes versicolor	B/A	SFCF20120803-46	KF313121			1				EF546242 (99%)	0.3
Gongronella butleri[e]	**Z/I**	**SFCF20120912-30**	**KF313133**					**1**		**JN206285 (97%)**	**1.1**
Mortierella alpina	Z/I	SFCF20120803-49	KF313129			2				AJ271630 (99%)	0.2
Mucor kurssanovii[e]	**Z/I**	**SFCF20120912-29**	**KF313128**					**2**		**JN206006 (99%)**	**0.8**
Mucor moelleri	Z/I	SFCF20120912-42	KF313127						2	EU484284 (99%)	0.7
Mucor zonatus[e]	**Z/I**	**SFCF20120803-83**	**KF313126**				**1**			**NR103638 (100%)**	**0.6**
Umbelopsis ramanniana	Z/I	SFCF20120803-47	KF313130	1		2		1		JQ683233 (99%)	0.6
Umbelopsis sp1	Z/I	SFCF20120803-04	KF313131	6	7					HQ157958 (99%)	0.2
Umbelopsis sp2	Z/I	SFCF20120912-33	KF313132	3	3		14	6	13	JN206387 (99%)	0.5
Total				29	24	51	47	62	46		

[a]Classification: A/D = Ascomycota/Dothideomycetes; A/E = Ascomycota/Eurotiomycetes; A/L = Ascomycota/Leotiomycetes; A/S = Ascomycota/Sordariomycetes; B/A = Basidiomycota/Agaricomycetes; Z/I = Zygomycota/Incertae sedis.

[b]Strain: SFC = Seoul National University Fungus Collection.

[c]No. of isolates: S = shoreline; I = inland.

[d]Growth rate with 3%NaCl/growth rate without 3% NaCl.

[e]Taxa in bold are those that are the most similar to type strains.

to several different species, it was only classified to the genus level.

Sequence data were assembled, aligned, and edited using MEGA 5 [48]. A maximum likelihood phylogenetic analysis was conducted with RAxML [45], using the GTRGAMMA model of evolution for tree inference and 1,000 bootstrap replicates.

Statistical Analyses

The endophyte community structure between regions and between shoreline/inland samples was compared using Mothur ver. 1.31.2 [43]. Mothur commands used for analyses are written in italics. A distance matrix describing the similarity in community membership and structure was generated using the *dist.shared* command under the theta similarity measure [60]. A non-metric multidimensional scaling (NMDS) ordination graph was plotted (*nmds*) and an AMOVA (*amova*) was performed to determine whether the groupings based on region and shoreline/inland were statistically significant.

To analyze soil variables, a nested 2-way ANOVA was performed for each variable using R ver. 3.0.1 [37] comparing the effect of region (Goseong, Uljin, Busan) and position (shoreline, inland). When significant effects were found, a TukeyHSD *post-hoc* test was used to identify the significant pairs.

Results

Isolation and Identification of Endophytic Fungi

A total of 259 endophytes were isolated from *P. thunbergii* roots (Table 1). In aggregate across all regions, fungal isolates were grouped into 136 distinct morphotypes based on morphological features of cultures. For individual regions, 43 morphotypes (53 isolates) were present in Goseong, 60 morphotypes (98 isolates) in Uljin, and 58 morphotypes (108 isolates) in Busan. With morphological characters alone, we were able to classify most morphotypes to the genus level: *Fusarium*, *Penicillium*, *Trichoderma*, and *Umbelopsis*. These genera accounted for a majority of the isolates (76%).

The phylogeny used for identification of endophytic fungi was pruned of distantly related taxa for clarity

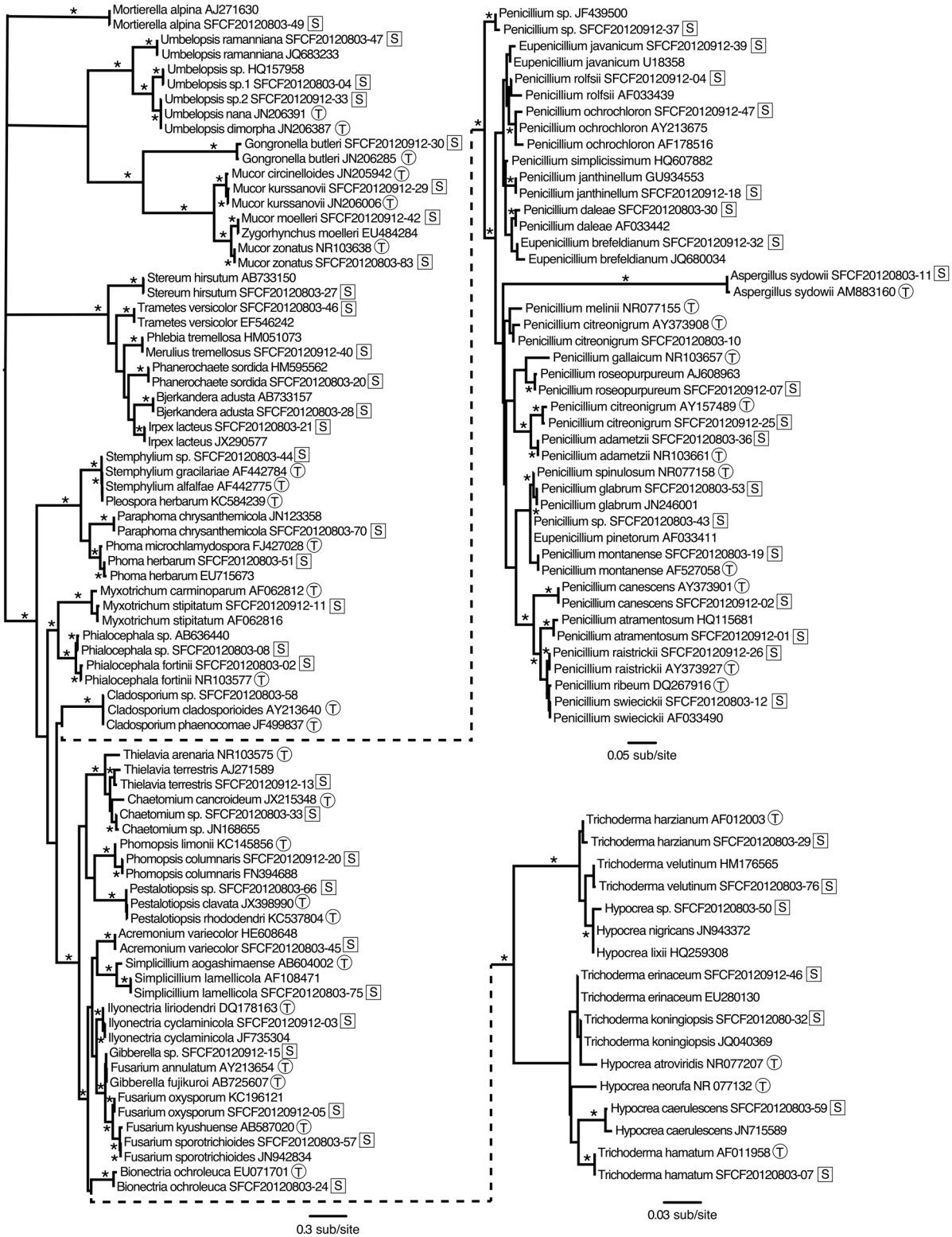

Fig. 2. Pruned phylogenetic tree (only including closely related sequences) of endophytic fungi isolated from *Pinus thunbergii* roots based on Maximum Likelihood of ITS sequences.

Taxa denoted with a "T" indicate type strains, while texa denoted with a "S" indicate endophyte samples from this study. Bootstrap values ≥90% are denoted with an "*". Note the scale change for the two subtrees.

(Fig. 2). Using ITS sequences, 58 species representing the three major fungal phyla were identified: 44 Ascomycota, 8 Zygomycota, and 6 Basidiomycota (Table 1). Sequences from 14 morphotypes were highly similar to type strains (Table 1, Fig. 2), and these identifications should be considered reliable, whereas the remaining sequences similar to non-type strains should be seen as tentative identifications. For Ascomycota, the dominant species were *Fusarium oxysporum* (17 isolates), *Hypocrea* sp. (14 isolates), *Penicillium canescens* (24 isolates), and *Trichoderma koningiopsis* (18 isolates). *Umbelopsis* sp. 2 (39 isolates) and *Bjerkandera adusta* (5 isolates) were the most frequently isolated species for Zygomycota and Basidiomycota, respectively (Table 1).

The frequency of isolation differed between species. Ascomycota species were the most common, accounting for 71.4% (185/259) of all isolates. Within Ascomycota, representation was dominated by two genera, *Penicillium* (76 isolates) and *Trichoderma* (47 isolates). The representation of Zygomycota isolates (64 isolates, 24.7%) and Basidiomycota isolates (10 isolates, 3.9%) was limited.

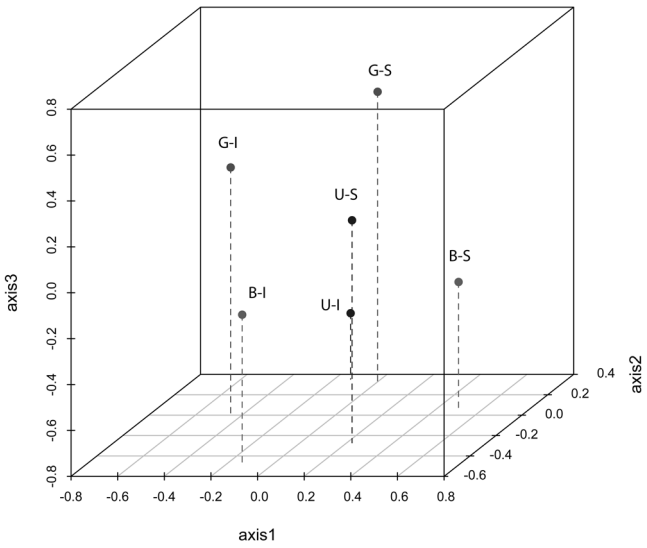

Fig. 3. Non-metric multidimensional scaling (NMDS) plot depicting the similarity of endophyte community membership and structure across all regions.
G = Goseong, U = Uljin, B = Busan, S = shoreline, I = inland.

Regional Variation

The number of species identified from each region was similar (Goseong = 23, Uljin = 27, Busan = 29). Five species were shared across the three regions (*P. atramentosum*, *Trichoderma hamatum*, *T. koningiopsis*, *Umbelopsis ramanniana*, and *Umbelopsis* sp. 2), and there were many species unique to Goseong (12), Uljin (13), and Busan (17) (Table 2). Fig. 3 is a NMDS plot comparing endophyte community membership and structure across all regions, and an AMOVA analysis comparing endophyte communities across regions was not significant ($F[2,3] = 1.47$, $p = 0.063$). Comparing between shoreline/inland samples, 44 total and 29 unique species were identified from shoreline habitats, whereas 28 total and 14 unique species were identified from inland habitats (Table 2). AMOVA analyses showed that endophyte communities between shoreline and inland were not significantly different ($F[1,4] = 1.07$, $p = 0.39$).

Data on soil characteristics at the three regions are in Table 3. The nested ANOVA for phosphorus levels were not significant (region: $F[2,12] = 0.03$, $p > 0.05$; region-position: $F[3,12] = 1.31$, $p > 0.05$). For pH, there was a significant effect for region ($F[2,12] = 7.63$, $p = <0.05$), but none for region-position ($F[3,12] = 0.39$, $p > 0.05$). *Post-hoc* tests identified significant differences between Uljin-Busan and Uljin-Goseong, where Uljin (5.94) had a higher pH than Busan (5.13) and Goseong (5.1). For salinity, there was a significant effect for region ($F[2,12] = 5.32$, $p = <0.05$), but none for region-position ($F[3,12] = 1.56$, $p > 0.05$). *Post-hoc* tests identified significant differences between Busan-Uljin and Busan-Goseong, where Busan (3.5%) had a higher level of salinity than Uljin (1.5%) and Goseong (2%). The same pattern was seen in nitrogen levels, with a significant effect for region ($F[2,12] = 6.79$, $p < 0.05$), but not for region-position ($F[3,12] = 0.9$, $p > 0.05$). *Post-hoc* tests identified

Table 2. Summary of unique species richness and saline-tolerant species from each of the regions.

Location	Total No. species	Unique (% of total)	Growth ratio >1 (% of total)	Unique, ratio >1 (% of total)
Goseong	23	12 (52.2%)	6 (26.1%)	1 (4.3%)
Uljin	27	13 (48.1%)	10 (37%)	1 (3.7%)
Busan	29	17 (58.6%)	14 (48.2%)	7 (24.1%)
Shoreline	44	29 (65.9%)	17 (38.6%)	11 (25%)
Inland	28	14 (50%)	7 (25%)	1 (3.5%)

Table 3. Summary of soil properties at the collection regions.

	Goseong		Uljin		Busan	
	Shoreline	Inland	Shoreline	Inland	Shoreline	Inland
pH	5.07 ± 0.23	5.12 ± 0.25	6.38 ± 0.08	5.64 ± 0.85	5.10 ± 0.24	5.15 ± 0.16
Salinity (%)	0.01 ± 0.003	0.03 ± 0.009	0.02 ± 0.003	0.01 ± 0.003	0.04 ± 0.006	0.03 ± 0.006
Total N (%)	0.06 ± 0.04	0.10 ± 0.02	0.07 ± 0.07	0.16 ± 0.12	0.44 ± 0.14	0.27 ± 0.06
Total P (mg/kg)	307.11 ± 20.16	286.65 ± 61.16	213.61 ± 35.62	410.47 ± 91.24	354.12 ± 114.99	259.83 ± 71.09

significant differences between Busan-Uljin and Busan-Goseong, where Busan (35.4%) had a higher level of nitrogen than Uljin (12.2%) and Goseong (8%).

Growth Rate on PDA with 3% NaCl

Growth rates of isolates on PDA media with and without NaCl were compared (Table 1). A ratio >1 indicates higher growth rate on PDA with NaCl. Species with a growth ratio >1 are subsequently referred to as "saline-tolerant." Among the 58 species, 18 species were saline-tolerant. Of these 18 species, 17 species were in Ascomycota and one in Zygomycota. Comparing across the three regions, Busan had the highest total number of saline-tolerant endophytes (14 species), followed by Uljin (10 species), and Goseong (6 species) (Table 2). Several saline-tolerant endophytes were unique to a single region (Busan = 7, Goseong = 1, Uljin = 1). Of the species with the greatest reduction in growth rate (≤0.1), three species were from Basidiomycota (*Irpex lacteus*, *Merulius tremellosus*, *Phanerochaete sordida*) and one from Ascomycota (*Simplicillium lamellicola*).

Evaluating between shoreline and inland positions, 17 endophytic fungi isolated from shoreline were saline-tolerant, with 11 being unique, compared with seven total and one unique saline-tolerant species from inland habitats (Tables 1 and 2). The three species with the highest growth ratios were unique to the shoreline of Busan (*Penicillium citreonigrum*, *Penicillium roseopurpureum*, *Myxotrichum stipitatum*) (Tables 1 and 2).

Discussion

Endophytic Diversity

A wide diversity of fungal endophytes was isolated from *P. thunbergii* roots. Representatives of all three fungal phyla were represented, with the majority of species being in Ascomycota (75.8%), followed by Zygomycota (13.8%), and Basidiomycota (10.2%). Our results mirror previous studies where most endophytes identified are in Ascomycota [1, 10, 14].

In Ascomycota, the majority of isolated endophytes were *Penicillium* and *Trichoderma* species. *Penicillium* and *Trichoderma* are usually saprophytes, but are also common as plant symbionts, playing roles in plant growth, nutrient absorption, resistance to harsh environments, defense response against pathogens, and resistance from attack of herbivores [18, 21, 23]. For example, a *Penicillium* species isolated from sand dune plants has the ability to promote growth in the host plant by producing secondary metabolites [5, 23]. Zygomycota diversity in our samples was limited to seven species, but dominated by *Umbelopsis* sp. 2. *Umbelopsis* species are common in forest soil and have potential ecological interactions with plant communities [9, 27]. Six Basidiomycota species were isolated (Table 1), all which are known to be present on dead trees as wood-rotting fungi [13] and known to infect cuts in roots [26]. Owing to the ecology of these species, we believe the presence of Basidiomycota in our samples is not an endophytic relationship, but instead an opportunistic infection of the roots.

Saline-Tolerant Endophytes

The ability to resist high salinity stress is essential for survival in coastal environments. Some endophytes can improve the survival and growth of their hosts by enhancing their tolerance to environmental stresses [40, 49]. Of the saline-tolerant endophytes identified in this study, several were previously documented. *Acremonium variecolor*, *Aspergillus sydowii*, and *Myxotrichum stipitatum* were previously identified as part of fungal communities in hypersaline environments [15, 24, 56]. In particular, *A. variecolor* is a marine-derived fungi tolerant to high salt concentrations [4].

Eighteen saline-tolerant endophytes were isolated in this study, and all but one were members of Ascomycota. Eight of these saline-tolerant Ascomycota species were in the genus *Penicillium*. *Penicillium* species are widely known to be salt-tolerant and fast growing [11, 31]. The top two saline-tolerant endophytes based on growth rate ratio were *Penicillium* species: *P. citreonigrum* (ratio = 2.3) and *P. roseopurpureum* (ratio = 1.9). *Penicillium citreonigrum* has not been identified as an endophyte, but rather the producer of

a potent toxin (citreoviridin) [38, 52, 53]. It is not a commonly isolated species, but is widely distributed [39]. *Penicillium roseopurpureum*, on the other hand, has been found as an endophyte of coffee berries, but with unknown function [54].

Comparison Between Regions and Positions

Although the overall statistical analyses on endophyte community structure, both by region and position, were not significant, there are noteworthy similarities and differences when comparing endophyte communities. All regions shared five species, of which three species (*P. atramentosum*, *T. hamatum*, *T. koningiopsis*) are saline-tolerant. Previous research found some plant species benefit from increased growth and protection from plant pathogens when associated with *T. hamatum* [17, 32] and *T. kongingiopsis* [30, 36]. The presence of shared endophytes across regions, especially saline-tolerant ones, suggests an important relationship with *P. thunbergii* roots.

When comparing between regions, Busan had the highest proportion of saline-tolerant species, including the top three species with the highest growth ratios (Table 2). Previous studies have found that differences in soil properties reflect differences in endophytic diversity [58, 59]. ANOVA analyses of soil properties detected statistical differences between the three regions. Busan soils had higher salinity and total nitrogen levels, which may be factors leading to the increased number of saline-tolerant endophytes in the region. It should also be noted that the plant community in Busan, with a combination of woody and herbaceous plants present, was more diverse than the other two regions, which may also have contributed to the differences in endophyte communities.

Comparing between positions, although there were no statistical differences in soil properties, shoreline habitats harbored higher saline-tolerant endophytic diversity. Of the 18 total saline-tolerant endophytes, 17 were isolated from shoreline soils. Moreover, the three saline-tolerant species with the highest growth ratio were unique to shoreline habitats. These observations suggest that endophytic fungi living closer to the coast may have a higher resistance to salinity and potentially have specific relationships with *P. thunbergii*.

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