Fungal Clusters and Their Uniqueness in Geographically Segregated Wetlands: A Step Forward to Marsh Conservation for a Wealth of Future Fungal Resources

Jong Myong Park, Ji Won Hong, Woong Lee, Byoung-Hee Lee and Young-Hyun You

Water Quality Research Institute, Waterworks Headquarters Incheon Metropolitan City, Incheon, Republic of Korea; Department of Hydrogen and Renewable Energy, Kyungpook National University, Daegu, Republic of Korea; Research Institute for Dok-do and Ulleung-do Island, Kyungpook National University, Daegu, Republic of Korea; Biological and Genetic Resources Assessment Division, National Institute of Biological Resources, Incheon, Republic of Korea; Microorganism Resources Division, National Institute of Biological Resources, Incheon, Republic of Korea

ABSTRACT

Here, we investigated fungal microbiota in the understory root layer of representative well-conserved geographically segregated natural wetlands in the Korean Peninsula. We obtained 574,143 quality fungal sequences in total from soil samples in three wetlands, which were classified into 563 operational taxonomic units (OTU), 5 phyla, 84 genera. Soil texture, total nitrogen, organic carbon, pH, and electrical conductivity of soil were variable between geographical sites. We found significant differences in fungal phyla distribution and ratio, as well as genera variation and richness between the wetlands. Diversity was greater in the Jangdo islands wetland than in the other sites (Chao richness/Shannon/Simpson’s for wetland of the Jangdo islands: 283/6.45/0.97 > wetland of the Mt. Gariwang primeval forest: 169/1.17/0.22 > wetland of the Hanbando geology: 145/4.85/0.91), and this variance corresponded to the confirmed number of fungal genera or OTUs (wetlands of Jangdo islands: 42/283 > of Mt. Gariwang primeval forest: 32/169 > of the Hanbando geology: 25/145). To assess the uniqueness of the understory root layer fungus taxa, we analyzed fungal genera distribution. We found that the percentage of fungal genera common to two or three wetland sites was relatively low at 32.3%, while fungal genera unique to each wetland site was 67.7% of the total number of identified fungal species. The Jangdo island wetland had higher fungal diversity than did the other sites and showed the highest level of uniqueness among fungal genera (Is. Jangdo wetland: 34.5% > wetland of Mt. Gariwang primeval forest: 28.6% > wetland of the Hanbando geology: 16.7%).

ARTICLE HISTORY
Received 24 October 2019
Revised 20 June 2020
Accepted 1 July 2020

KEYWORDS
Natural wetland; fungal clusters; primeval forest; islands; wetland conservation

CONTACT Young-Hyun You roc2404@korea.kr

1. Introduction

Wetlands are now known to be global controllers of their surrounding environments since they have roles in purification, remediation, material cycling, buffering [1]. Marshes undergo high rates of biological and microbial succession, which aids in the maintenance of biodiversity [2]. Therefore, conservation of natural marshes is essential. Furthermore, natural wetlands remain a source of microbial richness, which has become especially crucial as competition for bio-resources among countries increases. In particular, diverse aquatic plants have colonized areas surrounding well-developed natural wetlands, which were formed long before the agricultural period of the Korea peninsula [3].

Microbial species distributed in the understory root layer soil of conserved wetlands have a great impact on vegetation, acting as a natural degrader or assimilator of macro-molecules [2]. A considerable number of microorganisms are assumed to contribute to the physiology, development, or restoration of plant vegetation [4–6]. However, fungal species in natural wetlands are relatively uncharacterized compared to bacterial species, and have great potential as valuable biological resources [7,8]. One reason for this lack of studies relates to the historical industrialization movement in Korea, which has placed less value on conservation. Fortunately, in recent years, this viewpoint is being reevaluated, with greater effort being put into understanding Korean natural resources.

Because wetlands are a large depository of biological resources, their conservation is essential. A greater knowledge of the microbiome of natural wetlands will contribute to these conservation efforts. In particular, demonstrating the uniqueness of fungal species within these sites will provide

CONTACT Young-Hyun You roc2404@korea.kr

© 2020 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group on behalf of the Korean Society of Mycology. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
Further rationale for their protection and potential for biological contribution. However, there is a need for the development of strategies to reveal fungal species uniqueness. A previous study compared several strategies for securing microbial resources, and found that geographically isolated natural environments offer a great amount of microbial diversity in the natural environment [9]. To demonstrate the uniqueness of fungal composition in natural wetlands of the Korean peninsula, one strategy is to compare clusters of fungal communities in wetlands where mutual ecological exchange does not occur. If highly differentiated fungi are observed in natural wetlands where ecological exchanges have been cut off, a stronger case could be made for the preservation of various wetlands of the Korean peninsula as sites harboring a great richness of biological resources.

This logic is also related to the fact that each country has been competing to secure useful bio-resources since the Nagoya Protocol came into effect. Given the importance of securing national microbial resources, the management of genetic resources or culture collection is politically being strengthened worldwide [10,11]. In the past, when a useful functionality was identified for microorganisms that had been secured, the entity that secured the right to patent related to the functionality could claim the economic right to the functionality. The Nagoya Protocol, however, calls for strengthening the rights of the "management nation" for biological genetic resources, and since it went into effect in 2017, nation's resource acquisition and management have been further strengthened. In addition, competition between countries to secure national microbial diversity and various culture collections has become fierce. Because nation's right to secure microbial resources has been strengthened, it has become very important to quickly secure various microbial culture collections. The concepts of "valuable national biodiversity" and "microbiological assets" have already been introduced [12]. Further, nowadays, microbial culture collections enable sustainable development goals in terms of bio-economy [11,13].

In particular, under these circumstances, scientific studies on securing microbial resources that show high diversity and applicability have been vigorously conducted [9].

Although the logic that unique microorganisms can be discovered in special natural environments has been confirmed through numerous prior studies, this common logic will someday face limitations because there is a limitation of geographical diversity within the limited lands of nations. Thus, if microbial cluster differentiation is observed in special environments undergoing geographical isolation [14], even in special environments belonging to the same or similar categories, the concept of geographical segregation itself will increase the potential to securing unique microbial species or diversity, and another strategy will be to ensure diverse and unique microbial resources. Geographic isolation, however, includes not only physical segregation, but may also involve temporal concepts, and, thus, there is the need to determine whether the persistence of isolation can increase the differentiation of symbiotic microorganisms. This is because continuous differentiation and evolution of microbial species can occur within an isolated local ecosystem. Efforts must be made to confirm these hypotheses. Through this, it should be possible to determine what kinds of natural factors can be considered as strengthening factors for securing unique microbial resources and to identify what kinds of and how many unique resources are present under the current situation where competition for microbial resources is intensifying among countries.

To ensure competitive value of natural microbial resources, the uniqueness of the total microbiome and its species composition must be demonstrated [7]. The specificity of microbial taxon composition does not directly predict the existence of uniqueness of microbial resources. However, clearly distinct, rare species or genera found in separate environments can be interpreted as clear, minimal evidence of the presence of unique microbial species in those environments [7]. By demonstrating the presence of microbial structures or unique species compositions, we can focus on securing such unique microbial species. However, this theory needs to be demonstrated experimentally.

Here, we conducted a culture-independent analysis using UNITE databases to reveal total fungal taxa of the understory root layer of wetlands under geographical segregation [15]. Our results will provide insight into the value of wetland conservation and provide clues for securing unique fungal culture collections in light of rising international bioresource competition.

2. Materials and methods

2.1 Description of sampling sites

Information on sampling area, including administrative district, GPS location, average altitude (meter), and national indications for conservation, is provided in Table 1.

The Hanbando geology wetland belongs to a climatologically inland region, and is geologically located at the intersection of two rivers on the plain [16]. Geological conditions here may vary, due to narrowing of the river during floods or sand
deposits concentrated on the river’s bend, which may create large river wetlands [16]. This wetland was previously found to harbor a greater amount of diversity for higher organisms and was designated as a wetland conservation area (2.81 km²) in 2012 [16]. The locations of wetlands on the Hanbando geology are shown in Figure 1.

The Jangdo wetlands, located in the Jangdo islands of Shinan-gun, Jeollanam-do, are a representative national wetland conservative area with an oceanic climate [17]. The Is. Jangdo consists of two islands, Daejangdo and Sojangdo [17]. The wetlands are located in Daejangdo and are 90,414 m² in area. This wetland is known to harbor diverse higher organisms with 7 species of animals, 44 species of birds, 8 species of amphibians and reptiles, 126 species of land insects, 294 species of wetland plants, and 26 plant communities. As such, it was registered as the Ramsa Treaty Wetland in 2005 to enable preservation of its natural ecosystem [17]. The Is. Jangdo wetland is a small island in the ocean, and is, thus, directly affected by oceanic climate, as shown (Figure 1). This wetland developed from peat, or dark brown layers composed of plants that have not decomposed after death [17]. These peat layers of the Jandgo island wetland are regarded as well-preserved compared to those of other islands [17], with excellent water conservation and purity [17] due to their location on the ocean side of the islands. Few human entry regions are present on this site because of harsh weather conditions [17].

The Mt. Gariwang’s primeval forest wetlands have a forest climate. They are located in the Taebaek Mountains (1561 m above sea level), the largest, highest mountain range separating the east Korean Peninsula from the west [16]. The wetlands are located at the southeast foot of Mt. Gariwang in the deepest region of the Hoedong Valley [16]. The top part of the mountain contains many small, high-rise native and herbaceous plants, and the lower region of the forest where the wetlands are located has developed pine forests and maple clusters [16].

Table 1. Geographic information of three representative wetlands in the Korea peninsula.

| Sampling area | Administrative district including sampling area | GPS location (northern latitude, eastern longitude) | Average altitude (m) | National indications for conservation |
|---------------|-------------------------------------------------|---------------------------------------------------|----------------------|-------------------------------------|
| Site 1 Mt. Gariwang’s primeval forest | Seoheung-ri, Seohe-won, Inje-gun, Gangwon-do, Korea | 37°25′58″N, 128°33′48″E | 620 | Ecological landscape protected Area of Korean nation |
| Site 2 Is. Jangdo | Bi-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do, Korea | 34°40′41.1″N, 25°22′15.4″E | 169 | Wetland conservation area of Korean nation |
| Site 3 Hanbando geology | Ongjeong-ri, Hanbando-myeon, Yeongwol-gun, Gangwon-do, Korea | 37°13′5″N, 128°20′56″E | 280 | Wetland conservation area of Korean nation |

Figure 1. Location of each wetland in the Korean Peninsula. Site 1: Mt. Gariwang’s primeval forest wetlands (37°25′58″N, 128°33′48″E, 620 m); Site 2, Is. Jangdo wetlands (34°40′41.1″N, 25°22′15.4″E, height above sea level 169 m); and Site 3, Hanbando geology wetland (37°13′5″N, 128°20′56″E, 280 m).
2.2. Sampling of understory root layer

At all sites, we sampled on an interrupted grid. Four clods of soil (1000 m$^3$) were collected at a depth of 0–10 cm from each of four fixed circles (10 m in diameter) centered on a diagonal line in the two plots. Since the highest microbial density and activity are observed near soil surface, this measurement provided the most accurate survey of microbial communities inhabiting the soil. Soil samples were shaken vigorously to separate plant roots and soil not tightly adhered to roots. Three replicates from each circle were mixed into one sample, sieved through a 2-mm screen, and packaged in sterile plastic in an ice-cooled box.

2.3. Soil analysis

Soil samples from each site were subdivided in two representative subsamples: the first one was air-dried, sieved via a 2-mm sieve, and then chemically and physically analyzed, while the second one was stored at −80°C and later processed for the pyrosequencing analysis reported below. Air-dried and sieved soil subsamples were analyzed for the following physical and chemical properties: soil texture classification, reaction (pH), organic carbon (C$_{org}$), total nitrogen (TN) and electrical conductivity (EC$_e$). Texture was determined by the pipette method, without carbonate and organic matter removal, after complete removal of soluble salts using distilled water [18]. pH was measured using 1:2.5 (w/v) soil to water mixtures, and C$_{org}$ was using the Walkley and Black method [19]. TN was analyzed using the Kjeldahl method [20]. EC1:5 was measured using 1:5 (w/v) soil to water mixtures at 25°C. EC1:5 was converted to electrical conductivity of the saturation paste extract (EC$_e$) using the correlation model proposed by Khorsandi and Yazdi [21].

2.4. DNA extraction and PCR amplification

All samples were transported to the laboratory in NIBR for DNA extraction. At the laboratory, combined soil amounting to 1 kg was gently homogenized. We separately extracted DNA from the samples using 0.5 g of the mixed soil from each bag to extract DNA for fungi. All soil DNA was extracted using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer’s protocol. Fungal internal transcribed spacer (ITS) region 1 was amplified using an ITS1F (5′-CTTGTTGACCTAGGAAGATAA-3′) and ITS2 (5′-CCTGCTTCCTCAGATGC-3′) primer pair. The resulting ITS 1 amplicons were sequenced at Macrogen (Macrogen, Inc., Seoul, Korea), using paired-end (2 × 300 nt) Illumina MiSeq sequencing system (Illumina, San Diego, CA). Purified amplified products were pyrosequenced using 454 GS-FLX Titanium system (Roche, Rotkreuz, Switzerland) at Macrogen.

2.5. Sequence processing and statistical analysis

Paired-end sequences were assembled using PANDAseq software [22]. After assembly, all sequenced data were processed using the Mothur pipeline [23]. For fungal community analysis, the flanking gene fragments were removed from the ITS1 region using ITSx version 1.0.9 [24]. The putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within Mothur [25–27], using the de novo mode. Taxonomic classification was performed using Mothur’s version of the Naïve Bayesian classifier, using the UNITE database for fungi [15]. QIME implementation of UCLUST [25] was used to assign the OTUs. OTUs were defined with a limit threshold of 97% sequence similarity for fungi. All singleton OTUs were removed from all datasets prior to analysis. All samples were standardized by random subsampling using the “subsampling” command (http://www.Mothur.org/wiki/ Sub.sample) in Mothur. Richness, diversity indices, and rarefaction values [28] were estimated using Mothur [29,30].

3. Results and discussion

3.1. Soil texture classification, pH, $C_{org}$, TN, and EC$_e$

Soil analysis revealed that although the ratios of soil constituents (Table 2) – sand, silt, and clay – varied according to wetland, all three wetlands belonged to the silt class. Clay is a fine soil particle of less than 0.002 mm, with a relatively low water permeability and strong retention of moisture and nutrients, and, therefore makes the soil texture viscous. However, not all wetlands have a high proportion of clay, and according to a soil texture classification, a soil with a clay proportion of over 50% is generally considered to be highly viscous. Silt is a soil with a particle size of 0.002–0.02 mm, and if silt is well deposited, the capillary action of water is expanded and the water permeability becomes weak. One characteristic of fields commonly used for farming is silt texture. Herein, all three wetlands were classified into the silt class as they contained more than 50% of silt among the total volume. For sand, the particle size is 0.02–2 mm and the water permeability is very high; therefore, the retention capacity of moisture and nutrients is relatively lower than that of silt or clay. Thus, the surviving plant community and soil texture classification can play an important role in shaping wetland function and services.
itself are heavily affected by the surrounding environment. In this analysis, the order of silt proportions was wetland of the Is. Jangdo (76.7%), wetland of the Hanbando geology (64.1%), and wetland of the Mt. Gariwang primeval forest (59.0%). Wetlands in the Hanbando geology were formed by long-term deposition of soil beside the river stream, wherein the flow rate is slow, and it is assumed that the constituent of wetland soils is related to their natural formation history. For the wetland of Is. Jangdo, a small islandic terrain of 1.109 km², it is suggested that the ratio of clay has been reduced due to the strong marine condition. Is. Jangdo (pH 4.8) and Mt. Gariwang primeval forest (pH 4.8) wetlands were acidic compared with an ordinary field soil for cultivation (pH 5.5–6.0), and these acidities were soil-logically similar to those of peat moss (pH 4.0–4.5%) [17], which is a dead fibrous material that forms when mosses and other living materials decompose in peat bogs, with higher water retention capacity, higher permeability, and higher nitrogen retention. In particular, the wetland of Is. Jangdo showed a relatively higher Corg (20.48 g/kg⁻¹), TN (1.124%), and electrical conductivity (1.2 Ds/m) than did the wetland of Mt. Gariwang primeval forest. Thus, the wetland of Is. Jangdo was suggested to have a high organic/inorganic nitrogen content due to the deposition of vegetation, overcoming the relatively low content of clay (6.1%). On the other hand, the alkalinity level of wetlands of the Hanbando geology was close to neutral (pH 7.8) compared with the weak acidity of general arable soil, which is estimated to be affected by fresh river water, which is close to neutral. In the wetland of the Hanbando geology, the ECe value (0.32), TN (0.442%), and Corg (12.47) were relatively low. It is necessary to note that fungi exist or flourish in natural terrains is related with organic content, acidity, and electrical conductivity, for interpretation of the fungal clusters of each wetland.

### 3.2. Pyrosequencing results and statistical analyses

We obtained 574,143 quality fungal sequences in total from total soil samples, which were classified into 563 operational taxonomic units (OTU)s at a 97% similarity level. Depending on sampling sites, 169 OTUs were confirmed in the Mt. Gariwang’s primeval forest wetlands, which belong to a mountainous climate zone [16], 283 OTUs were confirmed in the Is. Jangdo wetlands, which belong to a maritime climate zone [17], and 145 OTUs were confirmed in the Hanbando geology wetlands [17] (Table 3). Rarefaction curves for OTUs from the three wetlands were deduced (Figure 2), and five fungal taxonomical groups were confirmed in all sampling sites at the phylum level. The number of fungal phyla we identified varied between wetlands (5 in the Is. Jangdo, 3 in the Mt. Gariwang’s primeval forest, and 3 in the Hanbando geology wetland). Fungal phyla from all geological locations included Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, or Zygomycota (Table 4). A total of 84 fungal genera were identified across three sampling sites (42 in the Is. Jangdo wetland, 32 in the

| Sampling area                       | Mt. Gariwang’s primeval forest | Is. Jangdo | Hanbando geology |
|-------------------------------------|--------------------------------|------------|-----------------|
| Number of total reads               | 277,927                        | 209,609    | 229,098         |
| Total bases                         | 107,912,837                    | 78,596,700 | 87,067,254      |
| Number of valid sequences           | 236,624                        | 157,649    | 179,870         |
| GC (%)                              | 43                             | 47         | 50              |
| OTUs                                | 169                            | 283        | 145             |
| Phylum (identified)                 | 3                              | 5          | 3               |
| Genus (identified)                  | 32                             | 42         | 25              |

1. Total Reads : The total number of sequence reads.  
2. Total Bases : The total number of bases in identified reads.  
3. GC (%) : The GC percentage in sequence reads.  
4. OTUs : Operational Taxonomic Unit is an operational definition of a species or group of species often used when only DNA sequence data are available.
Mt. Gariwang’s primeval forest wetland, and 25 in the Hanbando geology wetland) (Table 3).

### 3.3. Distribution and variation of fungal phyla

Commonly confirmed fungal phyla across all wetlands included Basidiomycota, Ascomycota, and Zygomycota (Table 4). In contrast, the fungal phyla Glomeromycota and Chytridiomycota were confirmed only in the wetland of the Is. Jangdo and not at other sites (Table 4). Phyla dominance of each wetland clearly differed by region (Figure 3). In the Mt. Gariwang’s primeval forest wetland, which belongs to a mountainous climate zone, the dominant phylum was Basidiomycota (94.1%) and the least observed phylum was Ascomycota (2.8%). For the Hanbando geology wetland, the dominant phylum was Basidiomycota (42.1%), and the least prevalent was Ascomycota (37.4%), while in the Is. Jangdo wetland, which belongs to a maritime climate zone, Ascomycota dominated (32.9%), while Zygomycota was least observed (14.2%) (Figure 3).
The prevalence of the uniquely confirmed fungal phyla Glomeromycota or Chytridiomycota was low compared to other groups (Figure 3). The dominant fungal phylum was Basidiomycota in the wetland of the Mt. Gariwang primeval forest (94.1%), which contains a large amount of saprophytic tree plants (Figure 3 and Table 4). In contrary, this phylum was not prevalent (42.2%) in the Hanbando geology wetland, a region characterized by mountains and a meandering river in the center of the depositional plain [17]. Low dominance of Basidiomycota in the wetland of the Is. Jangdo (6.75%) is thought to be caused by characteristics of the flora: herbaceous plants have flourished and out-competed woody plants due to a strong coastal sea-wind. Glomeromycota is a newly diverged fungal phylum group derived from arbuscular mycorrhizae and is uniquely found in the wetland of the Is. Jangdo. In particular, the Archaeospora species belongings to this phylum is known as a root symbiont [31–33], and, therefore, cannot exist without vegetation [34]. Most reported species belonging to Glomeromycota are not saprophytic or parasitic, but are symbiotic fungi found in the understory vegetation [34]. In this study, we confirmed the existence of Glomeromycota in the Is. Jangdo wetland.

The phylum Chytridiomycota is known to contain evolutionary primeval fungi with close ancestry to the kingdom Animalia [35]. Characteristics of this phylum include a motility apparatus (zoospore) [34], and the ability to adapt to freshwater environments [35]. In addition, most species are saprophytic [36] or parasitic [37]. We confirmed the existence of Chytridiomycota in the Is. Jangdo wetland, but did not identify this phylum in the wetland of the Mt. Gariwang's primeval forest or the Hanbando geology wetland. The Is. Jangdo wetland is located far from inhabitant residential areas and is completely isolated as a national conservation area restricting the entry of visitors (Table 1). Furthermore, we confirmed the existence of Glomeromycota and Chytridiomycota here, which were not identified in other wetlands, and the existence of relatively diverse fungal phyla, most likely due to designation of the Is. Jangdo as maritime wetlands under conservation. Chytridiomycota, in particular, is adapted for survival in harsh conditions, such as extreme pH [37]. Emergence of such zoosporic true fungi also seems to reflects the maritime wetland status of the Is. Jangdo.

3.4. Variation of fungal diversity

Shannon, Chao richness, and Simpson’s indices were introduced for diversity analyses [29,30]. Wetlands of the Is. Jangdo had higher Shannon and Simpson’s indices (Shannon: 6.45; Simpson’s: 0.97) than did the Hanbando geology wetland (4.85, 0.91) or the Mt. Gariwang’s primeval forest wetland (1.17, 0.22) (Figure 4). Overall, the wetlands of the Is. Jangdo exhibited a greater evenness in fungal genera distribution and dominance of specific fungal genera, according to Simpson’s index (Figure 4). It is noteworthy that evenness and dominance of specific fungal genera are often not identical in geo-ecological comparative studies, since the dominance of specific genera tends to lower the evenness value, and the dominance of specific fungal genera can limit flourishing or existence of other species.

Additionally, we identified higher genera richness values in the wetlands of Jandgo islands (283) than in the Mt. Gariwang’s primeval forest (169) or the Hanbando geology wetland (145), according to Chao’s richness (Figure 4). Wetlands of the Mt. Gariwang’s primeval forest scored the lowest genera richness value, possibly due to their characteristic-developed vegetation, including well-developed, large, leaf-woody plants. This hypothesis is also supported by the results of the genera profile.

3.5. Distribution and variation of fungal genera

We found that fungal microbiota communities were clearly different in each geological region (Figure 5), and we deduced genera constituents and dominance (Table 5). Clear differences between each wetland were noted. Tricholoma was dominant (88.94%) in the Mt. Gariwang’s primeval forest (169) or the Hanbando geology wetland (145), according to Chao’s richness (Figure 4). Wetlands of the Mt. Gariwang’s primeval forest scored the lowest genera richness value, possibly due to their characteristic-developed vegetation, including well-developed, large, leaf-woody plants. This hypothesis is also supported by the results of the genera profile.
Tricholoma, in the saprophytic understory of wooded areas, causing a reduced genera richness of fungal clusters from each wetland. Most species belonging to Tricholoma have been reported to be saprophytes or broad-leaf trees, and are classified as ectomycorrhizal fungi of woody plants [38]. The natural history and characteristic vegetation (especially that of broad-leaf trees) of the Mt. Gariwang’s primeval forest correspond with these results. Flourishing of broad-leaf woody plants limits growth of other herbaceous plants through impeding direct sunlight. Such competition influences the soil vegetation in the primeval forest, one of our sampling wetlands.

For wetlands in the Is. Jangdo, the constituent genera ranked in the following order: Mortierella (4.19%) > Entoloma (2.65%) > Trichoderma (2.18%) > Tolypocladium (1.26%) > Chaunopycnis (1.18%) > Paecilomyces (1.12%) (Table 5). More diverse fungal genera scored a dominance percentage above 1.0%, and this phenomenon was not observed in other wetlands. Evenness of fungal genera was greater at this site than at other sites, and we observed identically high rates of evenness and dominance (Figure 4). In the case of the Hanbando geology wetland, dominance of fungal species scored above 1.0%, in the following order: Geminibasidium (32.65%) > Penicillium (8.55%) > Trichoderma (4.05%) > Mortierella (3.98%) > Leohumicola (1.93%) > Fusarium (1.12%) (Table 5). Although several fungal genera showed dominance above 1.0%, the prevalence of Geminibasidium was
significantly higher than did that of other genera. This led to a lower evenness of fungal genera at this site, particularly for *Geminibasidium*, which is known for their resistant to heat and drying, compared with that of the wetlands of the Is. Jangdo [39].

3.6. Uniqueness of wetland understory root layer fungal genera

To assess the possibility of fungal genera uniqueness, we performed a comparison of fungal genera between sites. When total fungal microflora from representative wetlands was assessed, we confirmed a total of 84 fungal genera. Among these, a considerable number of fungal genera (67 genera, 79.8%) were identified at only one wetland site, and were not commonly identified at the other sites (29 genera for the Is. Jangdo wetland, 14 genera for the Hanbando geology wetland, and 24 genera for the Mt. Gariwang’s primeval forest wetland) (Figure 6).

The uniqueness of fungal genera distribution was maximized in the wetland of the Is. Jangdo, is a site of higher ecological conservation. We also observed a higher number of OTUs, distribution of fungal taxon (genera or phyla), and richness in the wetland of the Is. Jangdo (Figure 4 and Table 3).

These results suggest that uniqueness of fungal genera in nature terrains may be related to their taxonomical uniqueness. For example, one study examined the relationship between beneficial microbial resources and symbiotic interactions [40] and assessed microbial diversity of rhizosphere soil associated with host plants through large-scale microbiological screening [38–43]. Although this tactic can reveal theoretical interactions between microorganisms and their host plants, conclusions about competitiveness are more difficult. The taxonomical locations of secured strains often become nonspecific in such studies. Many of these studies do not assess taxonomical uniqueness, making the results less meaningful and competitiveness uninterpretable.
Table 5. Fungal genera distributions in each wetland.

| Genus          | Wetland of the Gariwangsan primeval forest | Wetland of the Jangdo islands | Wetland of the Hanbando geology |
|----------------|-------------------------------------------|-----------------------------|-------------------------------|
| Mortierella    | 0.60                                      | 1.12                        | 0.00                          |
| Paecilomyces   | 0.02                                      | 0.04                        | 8.55                          |
| Penicillium    | 0.30                                      | 2.18                        | 4.05                          |
| Trichoderma    | 0.03                                      | 1.45                        | –                             |
| Phialocephala  | 0.03                                      | 0.06                        | –                             |
| Hyphoea        | 0.02                                      | 1.18                        | –                             |
| Chaunopycnis   | 0.01                                      | 2.65                        | –                             |
| Entoloma       | 0.00                                      | 2.65                        | –                             |
| Cryptosporiopsis| –                                         | 0.07                        | 0.26                          |
| Lecanicillium  | –                                         | 0.07                        | –                             |
| Ilyonectria    | –                                         | 0.01                        | 0.21                          |
| Fusarium       | –                                         | 0.10                        | 1.12                          |
| Neonectria     | –                                         | 0.67                        | 1.07                          |
| Amanita        | 0.14                                      | –                           | 0.29                          |
| Exophiala      | 0.01                                      | –                           | 0.81                          |
| Umbelopsis     | 0.74                                      | –                           | 0.43                          |
| Geminibasidium | 0.17                                      | –                           | 32.65                         |
| Archaeorhizomyces| –                                       | 0.29                        | –                             |
| Ramularia      | 0.01                                      | –                           | –                             |
| Devriesia      | 0.04                                      | –                           | –                             |
| Cenococcum     | 0.96                                      | –                           | –                             |
| Minutisphaera  | –                                         | 0.70                        | –                             |
| Gymnoscypellopsora| 0.06                                    | –                           | –                             |
| Odiumdendron   | 0.35                                      | –                           | –                             |
| Pseudoeurotium | –                                         | 0.55                        | –                             |
| Didymosphaeria | –                                         | 0.28                        | –                             |
| Paraphoma      | –                                         | 0.13                        | –                             |
| Pyrenochaeta   | –                                         | 0.13                        | –                             |
| Subplemomomas  | –                                         | 0.04                        | –                             |
| Lophohystoma   | 0.03                                      | –                           | –                             |
| Epicoccum      | –                                         | 0.15                        | –                             |
| Cyphellophora  | –                                         | –                           | 0.23                          |
| Capronia       | 0.01                                      | –                           | –                             |
| Cladophialophora| 0.04                                     | –                           | –                             |
| Cylindrocarpon | –                                         | 0.19                        | –                             |
| Aspergillus    | 0.00                                      | –                           | –                             |
| Chalara        | –                                         | –                           | 0.88                          |
| Staphylostrictum| –                                         | –                           | 0.36                          |
| Theleacteria   | –                                         | 0.08                        | –                             |
| Carestella     | –                                         | –                           | 0.16                          |
| Acremonium     | –                                         | –                           | 0.58                          |
| Neobulgaria    | 0.02                                      | –                           | –                             |
| Lachnum        | –                                         | –                           | 0.11                          |
| Scytalidium    | 0.01                                      | –                           | –                             |
| Leohumicola    | –                                         | –                           | 1.93                          |
| Meliniomyces   | 0.05                                      | –                           | –                             |
| Leotia         | 0.01                                      | –                           | –                             |
| Lophodermium   | –                                         | –                           | 0.19                          |
| Tuber          | –                                         | 0.04                        | –                             |
| Saccharomyces  | 0.00                                      | –                           | –                             |
| Chaetosphaeria | –                                         | 0.37                        | –                             |
| Thozetella     | –                                         | 0.10                        | –                             |
| Pochonia       | –                                         | 0.07                        | –                             |
| Beavseria      | –                                         | 0.63                        | –                             |
| Gibberella     | –                                         | 0.31                        | –                             |
| Maraniannaea   | –                                         | 0.38                        | –                             |
| Nectria        | –                                         | 0.15                        | –                             |
| Haptochladium  | –                                         | 0.11                        | –                             |
| Tolypocladium  | –                                         | 1.26                        | –                             |
| Arthrinium     | –                                         | 0.21                        | –                             |
| Colletotrichum | –                                         | 0.46                        | –                             |
| Gaeumannomyces | –                                         | 0.05                        | –                             |
| Verticillium   | 0.06                                      | 0.39                        | –                             |
| Sphaerides     | –                                         | 0.02                        | –                             |
| Chaetomiun     | 0.00                                      | –                           | –                             |
| Podospora      | –                                         | 0.54                        | –                             |
| Diatrypella    | –                                         | –                           | 0.28                          |
| Clavaria       | –                                         | 0.52                        | –                             |
| Cortinarius    | 1.55                                      | –                           | –                             |
| Citopilus      | 0.35                                      | –                           | –                             |
| Laccaria       | 0.09                                      | –                           | –                             |
| Tephrocybe     | 0.05                                      | –                           | –                             |
| Hydrospora     | –                                         | 0.07                        | –                             |
| Mycenae        | –                                         | 0.39                        | –                             |
| Coprinellus    | 0.01                                      | –                           | –                             |
| Tricholoma     | 88.94                                     | –                           | –                             |

(continued)
Therefore, the uniqueness of the total microbiome in addition to species composition must be assessed. The specificity of microbial taxon composition cannot be directly interpreted from the existence of unique microbial resources. However, clearly distinct species or genus compositions found uncommonly in geographically segregated special natural terrains can be interpreted as clear, minimal evidence of the presence of unique microbial species in such an environment. If characteristic microbial structures or unique species compositions can be shown, unique strains can also be expected. For example, *Tolypocladium* in this study was uniquely present in the wetland of the Is. Jangdo, with a high dominance ratio (Table 5). *Tolypocladium* has been reported as a plant-associated fungal species due to its entomopathogenic qualities [40–42]. Insect vectors transferring fungal diseases to natural or agricultural plants are becoming an emerging crisis owing to an increase in insect populations due to climate warming [44–46]. An increase in insect vectors can also cause secondary problems, such as a reduction in food supply [45]. However, *Tolypocladium* is not known to exhibit this interaction with plants, and its entomopathogenic traits are targeted to insect vectors, which can be detrimental to human health [47–49]. If the ecological role that the relationship between *Tolypocladium* and vegetation plays can be revealed, we could secure fungal resources with functions in novel taxonomical locations. Confirmation of the unique distribution of fungal genera is a kind of useful tactic to secure competitive microbiological resources. In this regard, fungal clusters information from geographically segregated special natural terrains can be useful tools.

A considerable number of studies, such as profiling of soil bacteria communities, has been vigorously conducted for the purpose of revealing interactions between native plant species clusters in the general soil rhizosphere. However, studies examining fungal microbiota composition in wetland soil or interactions with native plants are relatively few. In fact, bacterial or fungal species in freshwater or maritime wetlands in Korean Peninsula have not been extensively studied compared to those in agricultural lands. To initiate a large-scale study aimed at securing natural fungal resources or national culture collection, the understory root layer fungal biota present in the wetland environment must first be investigated to evaluate unique taxa. We could then focus on these unique taxa present in the total fungal flora, through a culture-independent analysis. Through this study, geographical segregation may be one strategy to achieve uniqueness in securing fungal resources or diversity to provide insights into effective national culture collection, based on taxonomical uniqueness. Further, confirmation of the emergence of unique fungal taxon can help in securing such unique fungal resources with beneficial activities.

In conclusion, in this study, total understory root layer microbiome of ecologically isolated wetlands was investigated. The interactions of diverse fungal strains with wetland vegetations have not been previously studied extensively in the Korean Peninsula. A total microbiome analysis of the understory root layer using culture-independent methods could provide important information and lead to the

Table 5. Continued  

| Taxa            | Is. Jangdo | Hanbando geology | 
|-----------------|------------|------------------| 
| Byssocorticium  | 0.04       | –                | 
| Suillus         | –          | –                | 
| Ganoderma       | –          | 0.05             | 0.23          | 
| Sebacina        | –          | –                | 0.20          | 
| Tremella        | 0.03       | –                | –             | 
| Archaeospora    | –          | 0.74             | –             | 
| Abidia          | –          | –                | 0.20          | 
| Mucor           | –          | 0.03             | –             | 

Taxa shared with other wetlands and unique fungal genera are indicated. The term ‘0.00’ means a confirmation ratio not exceeding two decimal points; ‘-’ means non-confirmed genera. Red font indicates ratio of genera above 1.0%. Common genera are indicated in blue highlight.
acquisition of unique fungal resources. Further, through this study, geographical segregation can be a strategy to achieve uniqueness in securing fungal resources or diversity to provide insights into effective national culture collection, based on taxonomical uniqueness. Our results will promote wetland conservation due to the economic benefits associated with competitive biological resources that are found in these regions.

**Disclosure statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Funding**

This work was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea [NIBR201701106, NIBR201902112, and NIBR202002104].

**Data availability statement**

All the raw sequences obtained from this study have been deposited at the NCBI Sequence Read Archive (SRA metadata) under the project PRJNA616032 with sample accession numbers of SAMN14479947 (Mt. Garirwang’s primeval forest wetland), SAMN14479948 (Is. Jangdo wetland), and SAMN14479949 (Hanbando geology wetland).

**References**

[1] Whitaker V, Matvienko B. The denitrification potential and hydrological conditions in the wetlands of the lobo reservoir. Verh Int Ver Theor Angew Limnol. 1998;26(3):1377–1380.

[2] Denny P. Biodiversity and wetlands. Wetl Ecol Manag. 1994;3:55–61.

[3] Kuczynska-Kippen N. Habitat choice in rotifer communities of three shallow lakes: impact of macrophyte substratum and season. Hydrobiologia. 2007;593(1):27–37.

[4] Almeida CMR, Oliveira T, Reis I, et al. Bacterial community dynamic associated with autochthonous bioaugmentation for enhanced Cu phytoremediation of salt-marsh sediments. Mar Environ Res. 2017;132:68–78.

[5] Arnold G, Van DV. The biology of freshwater wetlands. 2nd ed. Oxford: Oxford University Press; 2002.

[6] Ashraf S, Afzal M, Naveed M, et al. Endophytic bacteria enhance remediation of tannery effluent in constructed wetlands vegetated with Leptochloa fusca. Int J Phytoremedi. 2018;20(2):121–128.

[7] You YH, Park JM, Park JH, et al. Endohyphal distribution and comparative analysis of diversity in wetlands showing contrasting geomorphic conditions. Symbiosis. 2016;69(1):21–36.

[8] You YH, Park JM, Park JH, et al. Diversity of endophytic fungi associated with the roots of four aquatic plants inhabiting two wetlands in Korea. Mycobiology. 2015;43(3):231–238.

[9] Park JM, Hong JW, Son JS, et al. Strategy for securing unique microbial resources—focusing on Dokdo islands-derived microbial resources. Israel J Ecol Evol. 2018;64(1–4):1–15.

[10] MOE (Ministry of Environment. Republic of Korea). 2014. The fifth national report to the convention on biological diversity. April 2014 access to: https://www.cbd.int/nrs/.

[11] MOE (Ministry of Environment) Republic of Korea Report: Conservation and Sustainable Use of Bio-logical Resources. Access to: Korea Environmental Policy Bulletin http://hdl.handle.net/20.500.11822/9049.

[12] Ryan MJ, McCluskey K, Verkleij G, et al. Fungal biological resources to support international development: challenges and opportunities. World J Microbiol Biotechnol. 2019;35(9):139.

[13] McCluskey K. A review of living collections with special emphasis on sustainability and its impact on research across multiple disciplines. Biopreserv Biobank. 2017;15(1):20–30.

[14] Kong WS, David W. The plant geography of Korea with an emphasis on the alpine zones. New York: Springer; 1993.

[15] Abarenkov K, Nilsson RH, Larsson KH, et al. The UNITE database for molecular identification of fungi-recent updates and future perspectives. New Phytol. 2010;186(2):281–285.

[16] The Geography of Kangwondo. NGII (National Geographic Information Institute); 2015.

[17] The Geography of Jeollanam-do. NGII (National Geographic Information Institute); 2015.

[18] U.S. Salinity Lab Staff. Diagnosis and improvement of saline and alkali soils. In: Richards LA, editor. Agriculture Handbook 60. Washington, DC: USDA; 1954. p. 122–124.

[19] FAO, United Nation. Standard operating procedure for soil organic carbon, Walkley-Black method (Titration and colorimetric method). Global Soil Laboratory; 2019.

[20] Bremner JM, Mulvaney CS. Chapter 31, Nitrogen total. In: Page AL, editor. Methods of soil analysis: part 2 chemical and microbiological properties, 9.2.2. 2nd ed. Madison, WI: American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America; 1983.

[21] Khorsandi F, Yazdi FA. Estimation of saturated paste extract’s electrical conductivity from 1: 5 soil/water suspension and gypsum. Commun Soil Sci Plant Anal. 2011;42(3):315–321.

[22] Masella AP, Bartram AK, Truszkowski JM, et al. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics. 2012;13(1):31.

[23] Schloss PD, Westcott SL, Ryabin T, et al. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75(23):7537–7541.

[24] Bengtsson Palme J, Ryberg M, Hartmann M, et al. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods Ecol Evol. 2013; 4(10):914–919.
Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–336.

Edgar RC, Haas BJ, Clemente JC, et al. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27(16):2194–2200.

Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460–2461.

Heck KL, van Belle G, Simberloff D. Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology. 1975;56(6):1459–1461.

Lambstead PJ, Platt HM, Shaw KM. The detection of differences among assemblages of marine benthic species based on an assessment of dominance and diversity. J Nat Hist. 1983;17(6):859–874.

Chao A, Shen T. Program SPADE (Species Prediction and Diversity Estimation) program and user’s guide available at http://chao.stat.nthu.edu.tw; 2003.

Bennett AE, Bever JD. Mycorrhizal species differentially alter plant growth and response to herbivory. Ecology. 2007;88(1):210–218.

Giridhar Babu A, Sudhakara Reddy M. Diversity of arbuscular mycorrhizal fungi associated with plants growing in fly ash pond and their potential role in ecological restoration. Curr Microbiol. 2011;63(3):273–280.

Wilde P, Manal A, Stodden M, et al. Biodiversity of arbuscular mycorrhizal fungi in roots and soils of two salt marshes. Environ Microbiol. 2009;11(6):1548–1561.

Hibbett DS, Binder M, Bischoff JF, et al. A higher-level phylogenetic classification of the fungi. Mycol Res. 2007;111(Pt 5):509–547.

Alexopoulos CJ, Mims CW, Blackwell M. Introductory mycology. 4th ed. New York: John Wiley & Sons, Inc.; 1996.

Sparrow FK. Aquatic phycomycetes. 2nd ed. Ann Arbor: The University of Michigan Press; 1960.

Gleason FH, Scholz B, Jepcott TG, et al. Key ecological roles for zoosporic true fungi in aquatic habitats. Microbiol Spectr. 2017;5(2). DOI:10.1128/microbiolspec.FUNK-0038-2016.

Marcel B, John W, Denys O. The mushrooms and toadstools of Britain and north-western Europe. London: Hodder & Stoughton Ltd.; 1987.

Nguyen HD, Nickerson NL, Seifert KA. Basidioascus and Geminibasidium: a new lineage of heat-resistant and xerotolerant basidiomycetes. Mycologia. 2013;105(5):1231–1250.

Tak Hi, Ahmad F, Babalola OO. Advances in the application of plant growth-promoting rhizobacteria in phytoremediation of heavy metals. Rev Environ Contam Toxicol. 2013;223:33–52.

Li HB, Singh RK, Singh P, et al. Genetic diversity of nitrogen-fixing and plant growth promoting Pseudomonas species isolated from sugarcane rhizosphere. Front Microbiol. 2017;8:1268.

Rohini S, Aswani R, Kannan M, et al. Culturable endophytic bacteria of ginger rhizome and their remarkable multi-trait plant growth-promoting features. Curr Microbiol. 2018;75(4):505–511.

Wang Z, Li T, Wen X, et al. Fungal communities in rhizosphere soil under conservation tillage shift in response to plant growth. Front Microbiol. 2017;8:1301.

Dennis DJ. Observations of fungus gnat damage to glasshouse cucumber. NZJ Exp Agric. 1978;6(1):83–84.

Kalb DW, Millar RL. Dispersal of Verticillium alboatrum by the fungus gnat (Bradysia impatiens). Plant Dis. 1986;70(8):752–753.

Ludwig WW, Oetting RD. Evaluation of medium treatments for management of Frankliniella occidentalis (Thripidae: Thysanoptera) and Bradysia coprophila (Diptera: Sciaridae). Pest Manag Sci. 2001;57(12):1114–1118.

Barson G, Renn N, Bywater AF. Laboratory evaluation of six species of entomopathogenic fungi for the control of the house fly (Musca domestica L.), a pest of intensive animal units. J Invertebr Pathol. 1994;64(2):107–113.

Bandani AR. Effect of entomopathogenic fungus Tolypocladium species metabolite efrapeptin on Galleria mellonella agglutinin. Commun Agric Appl Biol Sci. 2004;69(3):165–169.

Krasnoff SB, Gupta S. Identification of the antibiotic phomalactone from the entomopathogenic fungus Hirsutella thompsonii var. symnematos. J Chem Ecol. 1994;20(2):293–302.