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Assessment of Soil Fungal Communities Using Pyrosequencing

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Pyrosequencing, a non-electrophoretic method of DNA sequencing, was used to investigate the extensive fungal community in soils of three islands in the Yellow Sea of Korea, between Korea and China. Pyrosequencing was carried out on amplicons derived from the 5′ region of 18S rDNA. A total of 10,166 reads were obtained, with an average length of 103 bp. The maximum number of fungal phylotypes in soil predicted at 99% similarity was 3,334. The maximum numbers of phylotypes predicted at 97% and 95% similarities were 736 and 286, respectively. Through phylogenetic assignment using BLASTN, a total of 372 tentative taxa were identified. The majority of true fungal sequences recovered in this study belonged to the Ascomycota (182 tentative taxa in 2,708 reads) and Basidiomycota (172 tentative taxa in 6,837 reads). The predominant species of Ascomycota detected have been described as lichen-forming fungi, litter/wood decomposers, plant parasites, endophytes, and saprotrophs: *Peltigera neopolydactyla* (Lecanoromycetes), *Paecilomyces* sp. (Sordariomycetes), *Phacopsis huuskonenii* (Lecanoromycetes), and *Raffaelea hennebertii* (mitosporic Ascomycota). The majority of sequences in the Basidiomycota matched ectomycorrhizal and wood rotting fungi, including species of the Agaricales and Aphyllophorales, respectively. A high number of sequences in the Thelephorales, Boletales, Stereales, Hymenochaetales, and Ceratobasidiomycetes were also detected. By applying high-throughput pyrosequencing, we observed a high diversity of soil fungi and found evidence that pyrosequencing is a reliable technique for investigating fungal communities in soils.

***Keywords*:** fungal diversity, soil fungi, pyrosequencing, 18S rDNA

Soil is a complex and dynamic habitat in which microorganisms are involved in many key process required for ecosystem functioning. Highly diverse groups of fungi are represented in soil and play important physiological and ecological roles in this ecosystem (Miller, 1995). Knowledge of the structure and diversity of the fungal community in soil leads to a better understanding of their specific ecological roles in fungus-plant, fungus-pest, and fungus-microbe interactions. The ability to accurately detect and identify the organisms is fundamental to all aspects of fungal environmental studies. Therefore, assessing fungal diversity is likely to be incomplete without reliable detection and identification techniques. Fungal diversity has been investigated using traditional and molecular methods. The former often rely on identification of fruiting bodies, isolation and culturing of organisms, and laboratory identification by morphological and biochemical tests. Several disadvantages of traditional methods have been noted, such as the fact that they are time consuming, non-quantitative, and can lead to misidentification (Atkins and Clark, 2004). These limitations drive the development of better molecular methods.

Sequence-based studies of DNA offer alternative methods for defining fungal diversity and provide a potential link between ecological processes and the organisms involved (Fryar, 2002). By comparing community banding patterns, ARDRA, ARISA, DGGE/TGGE, and T-RFLP can be used to investigate changes, seasonal and spatial variations in soil fungal populations (van Elsas *et al.*, 2000; Anderson *et al.*, 2003; Edel-Hermann *et al.*, 2004). However, these methods can underestimate the level of diversity because of the presence of cross-kingdom PCR amplifications, heterogeneous bands, and incomplete band resolution (Kowalchuk *et al.*, 1997; Marshall *et al.*, 2003; Öpik *et al.*, 2003; Jeewon and Hyde, 2007). Also, these methods are limited in their ability to enumerate species richness in complex communities and do not provide any information about the taxonomic affiliation of the phylotypes (O'Brien *et al.*, 2005). These problems make it necessary to undertake cloning and sequencing. The main drawback to creating a gene library for any environment is the time and cost required to analyze the sequences. This limits the number of replicates and sample libraries and may lead to underestimation of rare but important species.

Pyrosequencing is a non-electrophoretic method for DNA sequencing that has emerged as a popular platform for DNA analysis (Ronaghi *et al.*, 1998; Ronaghi, 2001). This technology can determine 25 million bases in one 4-h run with accuracy of 99% (Margulies *et al.*, 2005). In addition, it has the potential

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advantages of rapidity, being relatively inexpensive, not requiring a cloning step, and high flexibility. Pyrosequencing has numerous applications, e.g., microbial typing (Jonasson *et al.*, 2002; Ronaghi and Elahi, 2002), analysis of single nucleotide polymorphisms (SNPs) (Gruber *et al.*, 2002; Isola *et al.*, 2005), forensics (Andréasson *et al.*, 2006), and mutation detection (White *et al.*, 2004; Ogino *et al.*, 2005). However, there are few reports of its application to environmental samples, apart from metagenomic (Edwards *et al.*, 2006) and bacterial communities' studies (Sogin *et al.*, 2006; Roesch *et al.*, 2007; Acosta-Martínez *et al.*, 2008; Kim *et al.*, 2008). In the present study, we used pyrosequencing to examine the fungal diversity in the soils of three islands in western Korea.

Materials and Methods

Soil sampling, PCR amplification, and pyrosequencing

Soil samples were collected from 5 to 10 cm below the forest soils of three islands, Soyunpyungdo (SY), Oeyeondo (OY), and Gageodo (GG), in the Yellow Sea of Korea, between Korea and China. Soil samples were kept in icebox for transfer to the laboratory where they were sieved through a 3.35 mm screen. DNA was extracted from 0.3 g of soil samples obtained from each island using a direct bead-beating extraction method with UltraClean Soil DNA Isolation kit (Mo Bio Labs, USA) according to the manufacturer's instruction. The 5′ region of the 18S rRNA gene was amplified using a newly designed primer set, TFungi 18F (5′-TYY GGR AGG, GGT, GTA, TTT-3′) and TFungi18R (5′-CAG AWA TTT GAA TGA ASC AT-3′). 100 ng of template DNA was used for a 50 µl PCR amplification reaction. After an initial denaturation step of 4 min at 94°C, amplification reactions were performed with 25 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 50°C), and extension (30 sec, 72°C), and a final extension step of 5 min at 72°C. The presence of PCR products was determined by analyzing 3 µl of product on 2% agarose gel. Negative control (containing no template) reactions were also performed to check for experimental contamination. All amplicon products from different soil samples were mixed in equal volumes. An approximate size of 120-bp PCR products was cleaned using the Qiaquick PCR Purification kit (Mo Bio). Pyrosequencing was performed on a Genome Sequencer 20 system (Roche, Switzerland) at the MACROGEN DNA Synthesis and Sequencing Facility (Seoul, Korea).

Sequence clustering, phylogenetic assignment, and species richness estimation

Quality-trimmed sequencing reads were derived from the GS20 sequencing output files. To unidirectionally sort all obtained sequences, sequences containing the reverse primer were complemented and inverted. To exclude the low quality sequences, sequences containing less than seventy bases with a high quality score (>20) and sequences containing ambiguous bases were removed at the analysis using JAVA scripts. The primer sites were trimmed from total sequences. We then used the sequence assembler, ContigExpress, in the VectorNTI package (Invitrogen, USA) to calculate the number of phylotypes with 1%, 3%, and 5% dissimilarity. To facilitate taxonomic/phylogenetic assignment of each sequence, we built a local database containing 18S rRNA gene sequences of published fungal species that were downloaded from GenBank and SILVA database. A bioinformatics pipeline was constructed for the automated taxonomic assignment of sequences using MySQL and JAVA scripts under a Linux operating system. The processed sequences were subjected to a similarity-based search using the BLAST program (Altschul *et al.*, 1990) against the fungal 18S rDNA database. The five sequences with the highest BLAST alignment scores were selected and used to align against the query sequences using a pairwise alignment algorithm (Myers and Miller, 1988). The sequence similarity values were calculated and the sequence with the highest similarity was selected for the hierarchical taxonomic assignment using the information held in the 18S database.

We also used the DOTUR program to estimate species richness (Schloss and Handelsman, 2005). Trimmed and sorted sequences were aligned using MAFFT program (Katoh and Toh, 2008). To calculate distances from aligned sequences, we used the DNADIST program with Jukes and Cantor distance parameters in PHYLIP program (Retief, 2000). The obtained distance matrix from DNADIST served as input data for DOTUR 1.53 for generating rarefaction curves and calculating the ACE and Chao1 species richness estimator. Chao1 and ACE (abundance-based coverage estimators) was calculated to estimate the richness of fungal community based on sequence dissimilarity (Sogin *et al.*, 2006; Waldrop *et al.*, 2006; Huber *et al.*, 2007). Shannon-Wiener diversity index was used to estimate the evenness of fungal community (Brodie *et al.*, 2003). Sequences are available in the GenBank Short Read Archive, accession number SRA008387.

Results

Pyrosequencing and sequence analysis

A total of 10,166 reads were obtained from pyrosequencing, with an average length of 103 bp. The quality score of sequences was provided by 454 Life Sciences, which is equivalent to the Phred score of the Sanger sequencing method (Ewing and Green, 1998; Ewing *et al.*, 1998). The average mean Phred score was 26.34 (27.84 was the maximum). To minimize effects of analytical errors, we excluded 468 reads of sequences that were short (<70 bp), had e-values greater than 1.0E-10, or had no sequences matching fungi. The remaining 9,698 reads served as a query to identify the closest matches in our local database. The maximum number of fungal phylotypes from soil predicted at 1% dissimilarity was 3,334. The maximum numbers of phylotypes predicted at 3% and 5% dissimilarities were 736 and 286, respectively (Table 1).

Richness and diversity estimates

The genetic distance matrix of sequences which was trimmed and filtered with quality from 454 total reads was generated by bioinformatics programs. When the genetic distances were assigned at 0.01, 0.03, and 0.05, the values of Chao1 were 3248.1, 1652.4, and 1007.6, respectively. The values of ACE ranged from 3753.4, 1735.2, and 986.4. These values indicated that very diverse fungi were in these forest soils. Shannon-Wiener diversity index were 5.59, 5.19, and 4.82, respectively. In spite of a relatively large genetic distance (0.05), Shannon-Wiener diversity index showed high value, 4.82. This result indicated that the fungal community of forest soils from three islands had the high evenness. Also, rarefaction curve had not reached an asymptote in genetic distance 0.01 and 0.03. Only, the curves appear to reach an asymptote and are close to saturation at genetic distance of more than 0.05 (Fig. 1).

Community analysis

Phylogenetic assignment of phylotype was performed according to best sequence matches based on BLASTN (Table 1). The

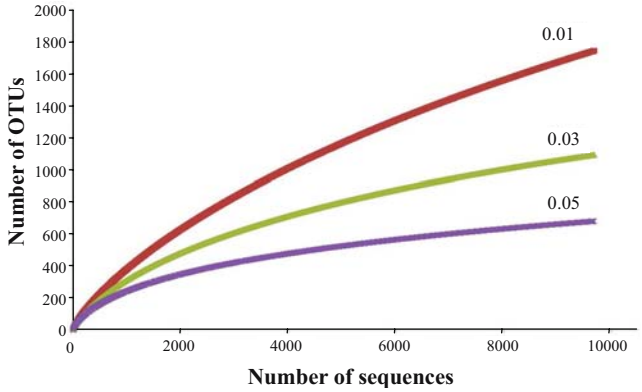

Fig. 1. Rarefaction curve for the total fungal community at distance levels of 0.01, 0.03, and 0.05.

majority of fungal sequences recovered in this study were in the Ascomycota (2,708 reads) and Basidiomycota (6,837 reads). By counting the cut-offs used in sequence differences between phylotypes (1, 3, and 5%), estimates of total fungal community richness and of the proportion in the taxonomic assignment could be confirmed (Table 1).

A total of 372 known fungal taxa were identified from pyrosequencing. The 2,708 reads of Ascomycota identified 182 tentative taxa. The most abundant taxon contained members of the Pezizomycotina. Over 100 sequence reads were detected in following classes: Lecanoromycetes, Sordariomycetes, Eurotiomycetes, Leotiomycetes, and Chaetothyriomycetes. Higher numbers of sequences were also found of mitosporic Ascomycota and of incertae sedia. A few sequences matched members of Saccharomycotina and Taphirinomycotina (Fig. 2). The most abundant genera and species were *Peltigera neopolydactyla* (Lecanoromycetes), *Paecilomyces* sp. (Sordariomycetes), *Phacopsis huuskonenii* (Lecanoromycetes), and *Raffaelea hennebertii* (mitosporic Ascomycota) (Table 2 and Fig. 2).

For Basidiomycota, sequencing data identified 172 tentative taxa. A few sequences matched members of Pucciniomycotina and Ustilaginomycotina. Many sequences were assigned to members of Agaricomycotina. The most abundant groups of the Tremellomycetes were yeast taxa, *Cystofilobasidium infirmominiatum* and *Cryptococcus terreus*. The Agaricomycetes sequences constituted 83.5% of the Basidiomycota. Most sequences in Agaricomycetes matched ectomycorrhizal and wood rotting fungi. A high number of sequences in the Thelephorales, Boletales, Stereales, Hymenochaetales, and Ceratobasidiomycetes were also detected (Table 2 and Fig. 2). The dominant ectomycorrhizal fungi were *Cortinarius*, *Hygrophorus*, *Russula*, and *Tricholoma* in Agaricales, and members of Boletales and Thelephorales. Four decay fungi, *Punctularia strigosozonata*, *Hymenochaete corrugata*, *Gloeoporus taxicola*, and *Steccherinum fimbriatum*, were in the top 20 dominant Basidiomycota (Table 2).

Traditional Zygomycota, traditional Chytridiomycota, and Glomeromycota were detected in pyrosequencing, but were rare; traditional Zygomycota (7 taxa, 84 reads), traditional Chytridiomycota (8 taxa, 66 reads), and Glomeromycota (3 taxa, 3 reads). The sequences in the traditional Zygomycota were matched to four orders, Dimargaritales (which belongs to Kickxellomycotina now, see Hibbett *et al.*, 2007), Entomophthorales (Entomophthoromycotina), Mortierellales (Mucoromycotina), and Zoopagales (Zoopagomycotina), with Mortierellales (67 reads) the most abundant. Sequences belonging to the traditional Chytridiomycota also corresponded to four orders, Chytridiales, Monoblepharidales, Neocallimastigales (Neocallimastigomycota), and Spizellomycetales, with Spizellomycetales (51 reads) the most abundant.

Discussion

The introduction of high-throughput pyrosequencing has dramatically increased the resolution at which microbial communities can be analyzed. Although the length restriction of pyrosequencing is a major drawback, pyrosequencing challenges the accurate assignment of microbial groups and estimation of their richness. Despite short amplicon sequences using GS20, this study demonstrates the potential of extensive sequencing for investigating fungal diversity in soils. The taxonomic resolution of 18S rDNA might not always be sufficient to identify fungi to species level, but has been successfully used to provide information on fungal diversity and dynamics of closely related species (Smit *et al.*, 1999). O'Brien *et al*. (2005) recovered 412 and 153 unique phylotypes from forest soil using ITS and SSU cloning, respectively. According to the literature on bacterial diversity (Roesch *et al.*, 2007; Acosta-Martínez *et al.*, 2008), 0% dissimilarity in sequences gives a dramatic overestimation of the species present in a sample, while 3% and 5% dissimilarity provide accurate estimation of the majority of species and genera, respectively. Our results suggest that the number of phylotypes predicted at 1% dissimilarity in pyrosequencing appears to be an overestimate and the number of phylotypes at 3% dissimilarity may provide a more accurate estimation of the soil fungi.

However, short sequence lengths and insufficient resolution of the 18S rDNA region may result in underestimation of the richness of fungal communities and poor phylogenetic assignment in soils. Equal proportions of Asocomycota and

Table 1. Sequence assignment and diversity estimates

	Asco[a]	Basidio	tChytridio	Glomero	tZygo	Total
Sequence reads	2,708	6,837	66	3	84	9,698
Assignment to known taxa	182	172	8	3	7	372
Phylotypes at 1% difference	1,202	2,024	50	3	55	3,334
Phylotypes at 3% difference	311	362	31	3	29	736
Phylotypes at 5% difference	101	140	22	3	20	286

[a] Asco-Ascomycota, Basidio-Basidiomycota, tChytridio-traditional Chytridiomycota, Glomero-Glomeromycota, tZygo-traditional Zygomycota

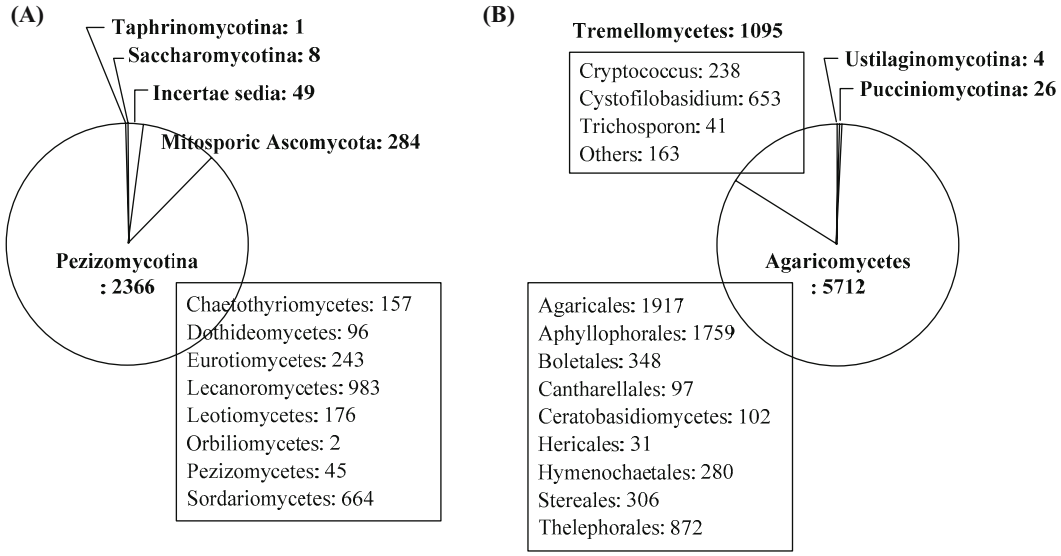

Fig. 2. Assignment of Ascomycota (A) and Basidiomycota (B) sequences. The number of sequence reads is presented after the names of the class or order.

Basidiomycota sequences were found in forest soil in both ITS and SSU clone libraries (O'Brien *et al.*, 2005), but there were more Basidiomycota sequences recovered in the present study. The main Ascomycota detected in this study have been described as lichen-forming fungi, litter/wood decomposers, plant parasites, endophytes, and saprotrophs (Table 2). Basidiomycetes included ectomycorrhizae and decay organisms of plant residues. Ectomycorrhizal fungi colonize plant roots and help plants obtain nutrients, such as phosphorus, from soil (Li *et al.*, 2006). Decay fungi are essential to the decomposition of hard woody organic matter and convert organic matter into fungal biomass, carbon dioxide, and organic acids. It was interesting that several decay fungi were detected in the soil samples. These might take essential nutrients such as nitrogen from the soil in order to decompose woody carbon-rich residues that are low in nitrogen (Lindahl *et al.*, 2007). Compared to the numbers of sequences in the Ascomycota and Basidiomycota, however, fewer sequences of Zygomycota, Chytridiomycota, and Glomeromycota were recovered. Similar results were reported in forest soil (Zygomycota relative frequency, 1.5% ITS, 4% SSU; Glomeromycota, 0.5% ITS, 1% SSU; and Chytridiomycota,

Table 2. The 20 most dominant Ascomycota and Basidiomycota taxa found in the study soil

No[a]	Ascomycota	Classification	No[a]	Basidiomycota	Classification
544	*Peltigera neopolydactyla* (X89218)	Lecanoromycetes	757	*Typhula phacorrhiza* (AF026630)	Thelephorales
505	*Paecilomyces* sp. (DQ401104)	Sordariomycetes	653	*Cystofilobasidium infirmominiatum* (AB072226)	Heterobasidiomycetes
254	*Phacopsis huuskonenii* (AF450289)	Lecanoromycetes	552	*Punctularia strigosozonata* (AF518586)	Aphyllophorales
161	*Raffaelea hennebertii* (U44476)	Mitosporic Ascomycota	263	*Hymenochaete corrugata* (AF518579)	Hymenochaetales
97	*Hymenoscyphus ericae* (AY524847)	Leotiomycetes	259	*Stephanospora caroticolor* (AF518591)	Boletales
90	*Geomyces pannorum* (AY129548)	Eurotiomycetes	258	*Coprinopsis atramentaria* (DQ115781)	Agaricales
82	*Leuconeurospora pulcherrima* (AF096178)	Sordariomycetes	226	*Cortinarius iodes* (AF026633)	Agaricales
60	*Goniopila monticola* (AY357277)	Mitosporic Ascomycota	221	*Gloeoporus taxicola* (AF082682)	Aphyllophorales
60	Melanized limestone (AY559390)	Chaetothyriomycetes	211	*Anthracophyllum archeri* (DQ092915)	Agaricales
47	*Phaeophyscia hispidula* (AY648103)	Lecanoromycetes	156	*Steccherinum fimbriatum* (AF518590)	Stereales
44	*Rhynchostoma minutum* (AF242270)	Chaetothyriomycetes	152	*Halocyphina villosa* (AF426951)	Aphyllophorales
34	*Exophiala oligosperma* (AY554287)	Chaetothyriomycetes	146	*Mythicomyces corneipes* (DQ092917)	Agaricales
28	*Anguillospora rosea* (AY357265)	Mitosporic Ascomycota	134	*Hygrophorus sordidus* (AF287834)	Agaricales
27	*Oidiodendron tenuissimum* (AB015787)	Incertae sedia	133	*Cryptococcus terreus* (AB032649)	Heterobasidiomycetes
27	*Tornabea scutellifera* (AJ549810)	Lecanoromycetes	133	*Amauroderma* sp. (AF255199)	Aphyllophorales
22	*Uncinocarpus queenslandicus* (AJ315175)	Eurotiomycetes	116	*Leucopaxillus albissimus* (AF287839)	Agaricales
22	*Psora testacea* (AY548823)	Lecanoromycetes	112	*Marasmius* sp. (AY916710)	Agaricales
22	*Arachnopeziza aurata* (U67427)	Leotiomycetes	112	*Tomentella* sp. (DQ092920)	Thelephorales
20	*Pilophorus acicularis* (AF085469)	Lecanoromycetes	102	*Lycoperdon* sp. (AF026619)	Agaricales
19	Fungal endophyte (AF503564)	Dothideomycetes	96	*Macrocystidia cucumis* (DQ089014)	Agaricales

[a] number of sequences read

0% ITS, 4% SSU, O'Brien *et al.*, 2005). Most sequences in Zygomycota and Chytridiomycota matched *Mortierella alpina* and *Spizellomyces* spp., respectively, which have been reported as common fungal species in soils (Booth, 1971; Smit *et al.*, 1999; De Bellis *et al.*, 2007). The former mineralizes readily available dissolved organic substrates rather than breaking down soil litter polymers (Schmidt *et al.*, 2008), while the latter has been reported to infect spores of arbuscular mycorrhizal fungi (Ross and Ruttencutter, 1977; Daniels, 1981). Despite the important roles of members of Chytridiomycota in the soil environment (Lozupone and Klein, 1999, 2002), little is known about their ecology or abundance in nature.

Most filamentous fungi form an extensive network of hyphae that is very persistent or survival structures in soil or on plant residues. In addition to increasing nutrient uptake and decomposing woody organic matter, fungal hyphae play an important role in improving soil structure. The sheer size and mass of fungal hyphae in soil help decrease plant susceptibility to pests, diseases, and drought. Fungal hyphae bind soil particles together to create water-stable aggregates, which, in turn, create pore spaces in the soil and enhance water retention and drainage (Tisdall, 1991). Some pathogenic fungi, such as *Rhizoctonia*, *Typhula*, and *Verticillium*, were also detected. The highest number of sequences (757 reads) matched *Typhula phacorrhiza*, a known pathogen of grasses and cereals (Burpee *et al.*, 1987). Therefore, *T. phacorrhiza* appears to be dominant in the island soils. Pathogenic fungi are often dominant in soils but are generally suppressed by high fungal biodiversity (Brussaard *et al.*, 2007).

Pyrosequencing is faster and approximately 20- to 30-times less expensive than Sanger sequencing, and does not require cloning for environmental samples. It also eliminates many of the problems associated with cloning techniques (Edwards and Rohwer, 2005). Therefore, we exploited pyrosequencing of the partial 18S rDNA region in order to assess the extensive fungal diversity in soils. By applying high-throughput pyrosequencing, we observed a high diversity of Ascomycota and Basidiomycota in soils of the three study islands. With this technique we were also able to detect taxa that are only present at low levels in the soil. Since a skewed fungal diversity can arise from the steps involved in DNA extraction and PCR amplification, and the choice of primers also influences the results, careful interpretation is required. The up-graded pyrosequencing technique provides individual sequencing reads with an improved length of 400-500 base pairs, enabling large-scale sequencing and accurate identification to the species level that can be applied to estimating fungal diversity in the environment.

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