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Sequence-based Detection and Identification Biodiversity of Uncultivated Fungi in Soils.

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ABSTRACT

Detection and identification biodiversity of uncultured agriculture soil is not an easy one of the major problems that participate to this difficulty is the fastidious nature of the majority of unculturable soil fungal species .In this study we have need for identification of uncultivated soil groups, fundamentally rely on isolation of DNA from soil with a polymerase chain reaction and sequencing to diagnose and classify uncultivated soil fungal isolates .In the present study, two fungal-specific primer sets (NSIF/LRIFr and NSIF/ITS4r) were used to amplify partial sequences of fungal rRNA gene included ITS sequences. Five partial sequences of five fungal species were aligned through the BLASTN phylogenetic analysis against NCBI database which revealed higher identities with *Fungal sp., uncultured fungus clone, Fusarium oxysporum, Clonostachys rosea and uncultured fungal clone.* The blast tree showed that fungal species are neighbor joined, these results suggest that the uncultivated soil fungi is complex and diverse. Distinctly beneficial in ways and integration of various molecular methods (DNA-based) has shed light on these facets but a synthesis of both techniques is necessary for a finer image and a deeper understanding. Therefore, the taxonomic complexity of unculturable communities of fungal organisms needs to be unraveled.

Keywords: unculturable soil fungi, biodiversity, DNA extraction, 18s rRNA gene.

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INTRODUCTION

Soil fungal biodiversity can be collectively measured as the microbial biomass in the soil. The biodiversity of soil microbes is vital for the survival of fertile soil as these microbes are involved in several diverse functions such as essential cycles of C, N, P, soil texture and the removal of toxins (1). Previous research on the production of microbial communities included the isolation of such microbes from the soil sample using culture-independent techniques accompanied by a set of detection tests and their detection (2). Moreover, owing to the unculturability of several species, the fungal estimation experiments performed in soil is largely biased. Microbial groups required a more comprehensive and predictive understanding of these critical factors and how they react to environmental stress and changes (3). However, all soil microbe studies are disserted by the heterogeneity of soil texture, molecular taxon identification is primarily focused on marker gene sequencing whose responsiveness, resolution, and throughput are regulated by the marker gene and sequencing platform selection. Though Sanger-sequencing is the standard for the identification of single taxa (4). Fungal genes have ranged in length between various fungal groups, phylogenetic tree, amount of assembly sequences available and sufficient primer sets and essential methods effective for identification of different RNA microbes or soil DNA samples using fluorescent in situ hybridization(FISH) have been introduced to soil with minimal success in order to avoid the cultivation of fungi in the laboratory due to the direct decomposition of soil biochemical markers. (5,6).

A large database of the small subunit 18s rRNA gene sequence offers genus, species and less extreme level classification of diverse soil fungi, although others are still unknown as uncultivated (unculturable) fungi. (7).

Knowledge on the genomic population of soil fungi is insufficient to decide how this issue affects the assessment of the fungal habitat structure based on rRNA genes. While small subunit 18s rRNA is the best molecular identification for microbial molecular systematics since the rRNA gene progresses gradually, it provides a basis for sequence appropriation to genera and species for microbial community diversity research (8,9).

Therefore, PCR amplified soil DNA using specific primers for fungal sequences, availability of PCR primers is a significant factor concerning uncultivated fungal group analysis (10).

In the last decade, PCR-based technique has revolutionized the study of soil fungal groups for detection and identification, so that the whole diversity organism groups, including uncultivated soil fungi (11). An additional benefit is that the estimation of fungal biodiversity requires only a limited volume of soil sampled. In the present study, DNA extraction methods and using the i-genomic soil DNA Extraction Mini Kit was used to isolate DNA directly from the soil, and PCR technique estimated the efficacy of these methods. The aims of study are identification uncultivated fungal species by DNA –based through amplification of partial rRNA gene sequences, including internal transcribed space (ITS), and then, their sequences used as queries in BLASTN search to test their identity with those sequences in the database and their potential affiliation to fungal groups to ascertain the validity of the method. Direct soil DNA extraction, along with polymerase chain reaction amplification and population sampling techniques, has been effective in uncultured soil fungal ecology studies, it shows great potential to explain the taxonomic and functional features of uncultured soil fungal populations and provides new and unexpected forms of study. Several questions remain unanswered, but ongoing developments in biochemical techniques and field laboratory studies suggest a promising future for uncultured fungal ecology.

MATERIALS AND METHODS

Collection of Sampling

Agricultural soil samples were collected from a garden in the University of Sulaimani randomly. Samples were taken from the superficial layer within a depth not exceeded 30 cm, mixed thoroughly and collected in sterile containers, sealed and carefully placed in a sterile polyethylene bag, and brought to the Botany laboratory. Samples were mixed and sieved twice to remove large stones and debris and tested immediately (12).

Molecular methods

DNA Extraction

DNA was from 0.5 g of agricultural soil samples using the i-genomic soil DNA Extraction Mini Kit according the manufacturer's instructions. Amplification partial genomic sequences of rRNA gene between 18s rRNA and 28 rRNA implicated internal transcribed spacer region (ITS) sequences was done using Maxime premix Kit(i-Taq). PCR products were visualized on 1% agarose gel electrophoresis in 1X TBE buffer (9Mm Tris-borate,0.2mM EDTA) at 5 vol/cm for 1.15 hour and staining with Red stain.

Bioinformatics and Phylogenetic Analysis

The amplification of partial genomic sequences of rRNA genes between 18SrRNA and 28SrRNA included sequences of ITS region allowed use of Maxime PreMix Kit (i-Taq). Two pairs of fungal specific primers namely, (LRIr) 5′-GGTTGGTTCTTTTCCT-3′ and (NSIF) 5′-GTAGTCATATGCTTGTCTC-3′. Forward and reverse (amplicons) were sequenced at macrogen company (Korea) using Applied Biosystemes 3730Mxl automated DNA sequences (13). Then sequences were submitted to Mega6 and BLASTN for pair wise alignment against sequences available at GenBank database (http://WWW.ncbi.nlm.nih.gov) and for phylogenetic analyses. Sequence of isolates were finalized on the basis of the blast searches by the best hit and the different best hit that could match the sequence from different taxonomic. The phylogenetic tree was figured and drawn using Neighbor – joining, phylogenetic analysis which used relying on the data accessibility in the gene data base NCBI GenBank ((http:// www.ncbi.nlm.nih.gov/genbank)). Alignment between the sequences of the isolates obtained in this study was also achieved as multiple queries against each other to identify the phylogenetic relatedness between them.

RESULT

In the current analysis, the soil DNA extraction kit is easy and reliable, requiring no correct equipment and yielding DNA of good quality suitable for studying fungal genes. The PCR targeted the sequences between 18SrRNA and 28SrRNA gene sequences as expected. Band length of isolates detected more than 1000 bp; sequencing of the amplicons revealed that the amplicon of that amplified with (LRIr) is 1300 bp (Fig. 1) and that with (NSIF) is 1200 bp. Amplicon of C1, C2, C3, C4 and C5 that amplified (Fig. 2). When the sequences of the two amplicons of five isolates were used as BLASTN queries against the GenBank database. In table(1) the results showed multiple alignments between the two sequences of five isolates revealed that NSIF/ITS4r amplicon of C1, C2, C3, C4 and C5 were in 96% identity with its analog five isolates, whereas the NSIF/LRIr, amplicon of C1 showed higher identity (97%) with the partial rRNA sequence of *Fungal sp.* (acc.No.KT582248.1), C2 showed identify (94%) with the partial sequence of *Uncultured fungus clone* (acc.No.JF421681.1), C3 showed higher identify (98%) with the partial sequence of *Fusarium oxysporum* (acc.No.KP027007.1), C4 showed identify(95%) with the partial sequence of *Clonostachy rosea* (acc.No.MH031706.1) and C5 showed higher identify (97%)with the partial sequence of *Fusarium culmorum* (acc.No. KJ855139.1), NSIF/LRIr amplicon of C1 showed identify (94%) with the partial sequence of *Aspergillus sp.* (acc.No.DQ810192.1), C2 showed higher identify (98%) with the partial sequence of *Clonostachys rosea* (acc.No.MH031706.1), C3 showed higher identify (98%) with the partial sequence of *Fusarium oxysporum* (acc.No.MK500621.1), C4 showed less identify (90%) with the partial sequence of *Clonostachys rosea* (unclassified) (acc.No.MH031706.1) and C5showed identify (94%) with the partial sequence of *Uncultured fungus clone* (acc.No.KC670813.1).The phylogenetic tree of the (NSIF/ITS4r, NSIF/LRIr) amplicons RNA gene sequence of isolates revealed closely neighbor joining Fig (3).

Table 1: The BLASTN Matching Result of rDNA Fragment Sequences of Five Fungal Isolates against Sequences available in the Genbank database.

isolates	source	Amplicon	species	Acc.no.	Identify (%)
C1	soil	NSIF/LRIr	*Aspergillus sp.*	DQ810192.1	94%
		NSIF/ITS4r	*Fungal sp.*	KT582248.1	97%
C2	soil	NSIF/LRIr	*Clonostachys rosea*	MH031706.1	98%
		NSIF/ITS4r	*Uncultured fungus*	JF421681.1	94%
C3	soil	NSIF/LRIr	*Fusarium oxysporum*	MK500621.1	98%
		NSIF/ITS4r	*Fusarium oxysporum*	KP027007.1	98%
C4	soil	NSIF/LRIr	*Clonostachys rosea*	MH031706.1	90%
		NSIF/ITS4r	*Clonostachys rosea*	MH031706.1	94%
C5	soil	NSIF/LRIr	*Uncultured fungus*	KC670813.1	94%
		NSIF/ITS4r	*Fusarium culmorum*	KJ855139.1	97%

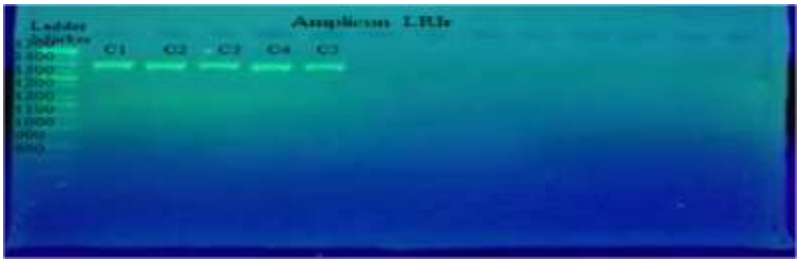

Figure 1: Amplification products the rRNAgene for the five isolate (C1, C2, C3, C4 andC5) using LRIr for each isolate. Ladder Marker (100bp.).

Figure 2: Amplification products the rRNA gene for the five (C1, C2, C3, C4 andC5) isolates, using NSIF for each isolate. Ladder Marker (100bp.).

Figure 3: The Phylogenetic tree of uncultivated soil fungi among identified Taxa and other Sequences based on ITS and 18s rRNA gene regions.

DISCUSSION

This research discusses the probability of isolating DNA from agricultural soil, which raises the amount of hops in biodiversity to detect and classify unculturable fungi studies as well as new avenues for surveying the production of culture independent methods of unculturable soil fungi diagnosis and identification. Implication for fungal diversity, we find a large number of species from only a few grams of soil insistent, but this is just a fraction of the total affluence of uncultivated fungal species total at 1-5 million ITS-based species–estimates ranging from 3.5-5.1 m are almost certainly underestimated as the riches numbers continue to increase with that sampling and due to the conservative 97% ITS similarity clustering used to classify it. Fungi reflect a significant quantity of previously uncharted uncultivated fungal diversity; true extent of fungal diversity may be higher than previously proposed. DNA-based surveillance technique to efficiently fulfill soil diversity studies across types of taxa habitats is a huge challenge, this method supports a wide variety of soil microbes such as fungi (14,15,16)

Soil DNA is isolated or adsorbed from soil molecules inside diverse cell walls and may be co-with a varying volume of humic substances, a multi-habitat that is a significant source for genetic variation in fungi. Soil DNA extraction techniques typically have two big issues, i.e. improper cell breakup, humic material toxicity, and DNA decline. Thus, large molecular weight DNA extraction which can prevent PCR amplification (17). Many laboratory protocols or trading kits were revised to optimize the performance of extracted DNA and PCR (18). Such procedures have been designed to quantify microbial diversity, though kits often focus on sample quantities that vary representatively from 0.25 to 1 g of wet soil and is less reliable and characteristic of local microbial communities than larger sample sizes. This sampling bias will be much greater to select larger species because it is not feasible to reproduce (19).

We discuss two methods used in the analysis of fungal diversity to classify uncultivated soil fungi, including DNA approach using the ITS region and DNA taxonomy utilizing one or more sequence similarity genes and phylogenetic tree. (20,21).

In DNA barcoding, Table (1) contrasts an uncertain sequence against a sequence database (Genebank, NCBI) and distinguishes organisms based on similarities of sequences. Alternatively, DNA taxonomy attempts to classify unknown

organisms by using phylogenetic method to position the sequence of certain homologues in an evolutionary context Fig (3). It is necessary to note that certain barcodes are therefore higher of their rapid evolution thus high rates of taxonomic complexity species and genus (22).

The ITS region shows the largest variability in ITS phylogeny in the uncultured genus according to categorized fungi in to five isolates Table (1). The related sequence database and taxonomy (family, order, class and phyla) and permitted fully resolved taxonomic sequence data assignment; there was no substantial difference in the number of bands which appeared in each sample primer sets in the current analysis Fig (1,2). The whole soil fungal region ITS can be effectively amplified for all soil fungal by means of primer pairs that both target the end of the 18s rRNA gene (23,24,25).

CONCLUSION

Knowledge of soil fungi abundance, identification and dissemination and soil ecology dynamics is only limited. Improvement of techniques and the introduction of various molecular approaches (DNA-based) have certainly shed light on these issues but for a simpler vision and a deeper understanding, a convergence of both techniques is important. The taxonomic complexity of fungal species classes continues to be unravelled, Molecular methods can be used to determine their genotypical richness in soil. Fungal soil diversity studies have previously been performed predominantly in agricultural soil, especially those covering plant roots, so potential studies will target specific ecosystems, such as freshwater, estuarine or coastal environments, and therefore high taxonomic levels (species and genus).

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