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Exploring Fungal Diversity and distribution in Dukan Freshwater Lake Using ITS rDNA-based PCR Cloning/ Sequencing

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Abstract

Fungi represent important trophic inhabitants in aquatic ecosystems, they have crucial roles in nutrient cycling in freshwater ecosystems. In the present study, Internal Transcribed Spacer ITS rDNA-based investigations of fungal communities in Dukan Lake were conducted from two collection sites using large-scale clonal Sanger sequencing. Herein we report for the first time, the composition of fungal communities in water samples of Dukan Lake through cloning/sequencing method. A total of 576 clones were obtained from ITS gene library and resulted in retrieving of 33 fungal operational taxonomic units (OTUs). The majority of clone sequences belonged to Ascomycota while members of other fungal phyla were recovered at much lower frequencies. Molecular diversity analysis in Dukan Lake revealed the existence of fungi belonged to Pleosporales, Hypocreales, Helotiales, Mortierellales, Eurotiales, whereas Capnodiales, Onygenales, Pezizales, and Xylariales were detected in relatively minor abundances in the Dukan Lake ecosystem. In conclusion, molecular techniques using ITS rDNA-based PCR cloning/ sequencing is currently used as an effective tool to investigate the complex fungal communities in the aquatic systems however additional studies using next generation sequencing are needed for descriptive details regarding the occurrence and abundance of fungi in aquatic environments.

Introduction

Fungi are ubiquitous inhabitants n aquatic ecosystems [1]; they have an essential role in both decomposing organic materials and cycling in freshwater ecosystems [1, 2]. Due to the instability in fungal morphological characteristics and presence of intermediate forms, studies of fungal diversity have been hampered by the lack of suitable mycological strategy [3]. Although many efforts have been made in last decades to reveal the diversity of aquatic fungi in freshwater on the basis of culture dependent methods [4, 5], only very few fungi in aquatic environmental samples have been cultured [6, 7]. The main disadvantage of conventional culturing methods is that there are many of fastidious fungi are unable to be cultured normally and many of them have specialized complex nutritional and growth requirements. Hence, the traditional culture dependent approaches cannot be directly applied to explore the fungal diversity in these ecosystems [3, 8]. In the last decade, the introduction of culture independent methods into marine ecology

field has significantly increased our knowledge for elucidating composition of fungal communities residing in various aquatic environments [3, 7, 8, 11]. The application of molecular approaches such as extracting, cloning and amplifying DNA from marine environmental samples, allows us to explore the fungal biodiversity without the need of culturing [9-11]. Several molecular genetic markers have been applied for rapid identification different type of fungi [12, 13] but the ITS region of nuclear ribosomal DNA represents an ideal target for designing DNA barcodes for detection of fungal species [14, 15]. The fungal diversity in Dukan Lake has not been well documented, especially at the molecular level. Therefore, the main objective of the current study is to explore the diversity of aquatic fungi in Dukan Lake using the cloning/sequencing of the complete of the internal transcribed spacer (ITS) as a molecular marker.

Materials and methods

A. Sample collection

Dukan Lake (36°08′N 44°55′E) is located on the Lower Zab River approximately 65km southeast of Sulaimani city. The lake is formed by the Dukan dam, which was built in1959 upstream of the Dukan city [16]. A field cruise was carried out in the lake of Dukan on December 2015; surface water (5, 20 and 40 cm depth) samples were collected from two collection site (35° 57° 42° 3 N, 044° 58° 16° 4 E) and (35° 57° 47° 6 N, 044° 58° 02 °7 E) in the lake according to their proximity to the shore.. Water samples of 1000 mL aliquots were filtered through a 200 µm sieve then passed through a 0.22 µm pore polycarbonate membrane to remove debris. Membranes were kept at 20 °C for further process.

B. DNA extraction and genomic amplification

Using DNA mini kit (Qiamp®, Qiagen, Courtaboeuf, France), DNA was extracted according to previously described protocol [17] except a minor modification that was made to increase the proteinase K incubation time to 2 h at 70°C to improve fungal DNA extraction. Distilled water of 200 µL was used as a negative control for all DNA extractions and extracted DNA samples were stored at -20°C until the next step. A universal fungal primer pairs ITS F (CTTGGTCATTTAGAGGAAGTAA)[14] and ITS-4R (TCCTCCGCTTATTGATATGC) [18] were selected to amplify 380-580 bp of the complete ITS gene of fungi. The PCR amplification was conducted in 50 µL PCR reaction mixture contained 5 µL of dNTPs (2 mM of each nucleotide), 5 µL of DNA polymerase buffer (Qiagen), 2 µLof $MgCl^2$ (25 mM), 0.25 µL HotStarTaq DNA polymerase (1.25 U) (Qiagen), 1 µL of each primer (Eurogentec, Liège, Belgium), and 5 µL of DNA. The PCR was performed with an initial denaturation temperature at 95°C for 15 min, followed by 40 cycles of 95°C for 45 s, an annealing temperature 50°C for 30 s, 72°C for 1 min, and a final extension temperature at 72°C for 5 min. The PCR products were migrated on ethidium bromide containing agarose gel (1.5%) and visualized under UV light in gel documentation cabinet. Using the 96 PCR Kit (NucleoFast®, MACHEREY-NAGEL, Hoerdt, France), positive PCR products were subsequently extracted from agarose gel and purified according to the manufacturer's instructions.

C. Cloning and Sanger sequencing

PCR product was cloned using the pGEM® -T Easy Vector System Kit (Promega, Lyon, France) as described in [17]. The presence of the insert was confirmed by PCR amplification using M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-AGGAAACAGCTATGAC-3') primers (Eurogenetec) and an annealing temperature of 58°C. PCRs were performed as described above. Purified PCR products were sequenced in both directions using the Big Dye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Villebon-sur-Yvette, France) with the M13 forward and M13 reverse primers. These products were run on an ABI PRISM 3130 automated sequencer (Applied Biosystems).The obtained sequences were compared with a BLAST database of pre-assigned sequences in GenBank (available at the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/).

D. Phylogenetic analysis

Fungal ITS sequences of this study and the pre-assigned sequences in GenBank were both aligned by

CLUSTAL-X, version 2.1 and imported into MEGA 6.0.6 [19] to generate Neighbor-joining (NJ) trees. The program parameters used were p-distance with pairwise deletion.

E. Nucleotide sequence accession numbers

All sequences generated from the fungal ITS libraries of this study were registered in GenBank database with the accession numbers from KY615767 to KY615794.

Results

A. Phylogeny of environmental fungal ITS-rDNA sequences

A universal fungal PCR primer sets recognizing the internal transcribed region were applied for testing the surface seawater samples collected from two collection sites with various depth-levels (5, 20 and 40 cm depth). Cloning was performed with a positive PCR amplification with each deep level and a total 576 clones sequence from this library was subjected to sequence analysis (Table 1).

Of the resulting 576 cloned sequences, all of them were found to be fungal, and depending on a 98% sequence identity criterion, a total of 33 operational taxonomic units (OTUs) (Table 1) were identified after clustering. Among all these OTUs, the majority of identified ITS sequences showed a similarity of ≥97% as compared to sequences from their closest relative taxa in GenBank reference data base (Table 1). The results from cloning and sequencing indicates that 91.2 % (525/576) of these recovered ITS sequences belonged to the phyla Ascomycota (Table 1; Fig 1) whereas 5% (22/576) of cloned sequences belonged to the phylum Basidomycota and only 3.8 % (22/576) of cloned sequences referred to the phylum Zygomycota (Table 1; Figure 1).

Moreover, the phylogenetic analyses demonstrated that 33 OTUs obtained in this study were most closely belonging to orders related to filamentous fungi, including order Pleosporales (22 %), order Eurotiales (15.6 %), order Capnodiales (15.6 %), order Hypocreales (14.5 %), order Helotiales (5.3 %), order Entylomatales (5.3 %), order Pezizales (4.8 %), order Mortierellales (3.7 %), order Xylariales (3.7 %) and order Onygenales (3.1 %).

Table – 1: List of fungal OTUs detected in two locations with various deep levels in Dukan Lake using ITS targeting primer.

Fungal OTUs	Closest match by BLAST	% Coverage	% Identity	Location I			Location II		
				5 cm	20 cm	40 cm	5 cm	20 cm	40 cm
1	*Alternaria alternata*	99	99	3	0	0	8	6	0
2	*Tilletiopsis albescens*	98	98	1	3	22	0	3	0
3	*Cadophora sp.*	99	99	2	0	0	0	1	0
4	*Cadophora malorum*	99	99	0	8	0	7	4	0
5	*Cladosporium cladosporioides*	99	99	3	17	0	1	0	0
6	*Davidiella tassiana*	99	98	8	13	0	10	13	2
7	*Davidiella sp.*	99	99	2	0	0	0	0	0
8	*Cladosporium sp.*	99	99	6	0	0	0	2	0
9	*Varicosporium elodeae*	99	99	1	3	0	0	0	0
10	*Fusarium verticillioides*	99	99	4	0	0	2	8	0
11	*Geomyces sp.*	99	99	3	2	6	1	5	0
12	*Tetracladium ellipsoideum*	98	98	8	0	0	4	0	0
13	*Helotiales sp.*	99	99	3	0	0	0	0	0
14	*Microdochium nivale*	98	99	5	4	0	3	0	31
15	*Microdochium phragmitis*	99	98	3	4	12	5	0	0
16	*Mortierella alpina*	99	99	1	1	0	2	5	0

17	*Mortierella sp.*	99	99	2	8	0	1	0	0
18	*Phoma paspali*	99	99	1	0	0	1	0	0
19	*Paraphoma fimeti*	99	99	0	2	3	1	0	0
20	*Penicillium paneum*	99	99	6	0	0	0	0	0
21	*Penicillium sublectaticum*	98	98	12	7	0	0	41	0
22	*Penicillium verrucosum*	99	99	1	0	0	0	0	0
23	*Phaeosphaeria sp.*	99	99	0	1	0	7	6	0
24	*Phoma herbarum*	99	99	6	0	13	0	0	7
25	*Phoma sp.*	99	99	0	6	0	2	2	0
26	*Pleosporales sp.*	99	99	3	0	0	0	0	0
27	*Pseudeurotium sp.*	99	99	0	0	0	2	0	0
28	*Cosmospora viridescens*	99	99	4	0	21	8	0	0
29	*Pseudogymnoascus pannorum*	98	98	2	5	0	9	0	0
30	*Heydenia alpina*	98	97	0	5	0	2	0	19
31	*Aspergillus versicolor*	99	99	4	7	0	13	0	29
32	*Trichothecium roseum*	98	98	1	0	14	5	0	0
33	*Trichoderma longibrachiatum*	99	99	1	0	5	2	0	8

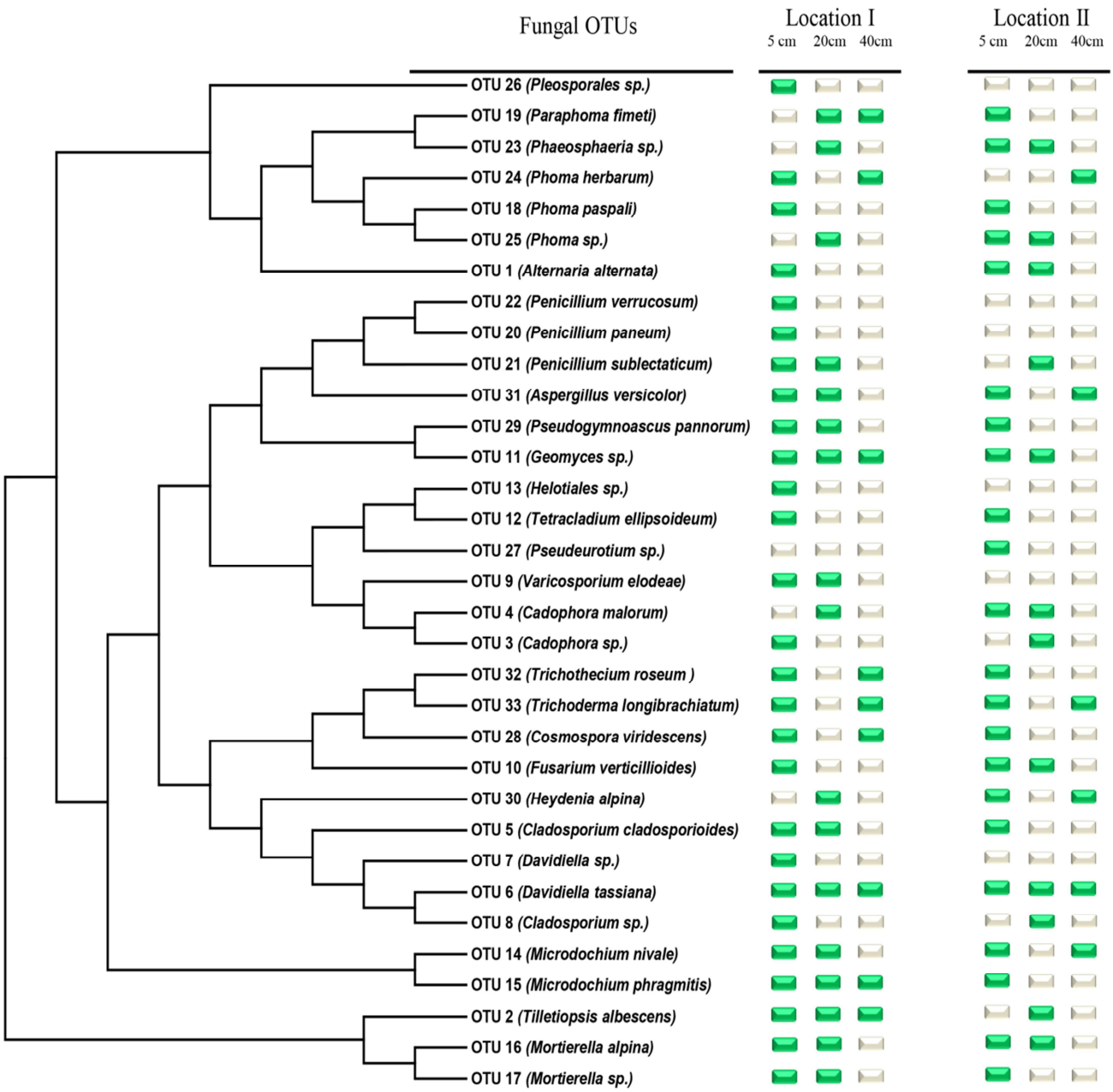

Figure-1: Neighbor-joining phylogenetic tree from analysis of ITS rDNA from 33 fungal OTU sequences in six libraries retrieved from two locations with variousdepth levels in Dukan Lake.

B. Diversity estimation

The Shannon Index (H′) that measure both species numbers and the evenness of their abundance were used to estimate fungal OTU diversity in water samples with various deep levels in the Dukan Lake. The highest fungal diversity value was obtained for surface water sample (5 cm) from the first location (35° 57° 42° 3 N, 044° 58° 16° 4 E) with 27 OTU identified (H′ = 3.05) (Table 2). In contrast, the lowest diversity value was obtained water sample (40 cm) from the second location (35° 57° 47° 6 N, 044° 58° 02 °7 E) with only 6 OTU identified (H′ = 1.526) (Table 2).

Overall, the value of the Shannon Index ranged from 2.592 to 1.917 with recovering 17 and 9 OTU for water sample with (20 and 40 cm) deep in Location 1 respectively (Table2). While it ranged from 1.945 to 1.526 with recovering 12 and 6 OTU for water sample with (20 and 40 cm) deep in Location II respectively (Table 2).

Table – 2: Shannon diversity values calculated for the sampled sites.

Cloning library/site/deep level	Total clone /site	Total species/ site	H′ per site
ITSF-ITS-4R/ L1 /5 cm	96	27	3.050
ITSF/ITS-4R L1 20 cm	96	17	2.592
ITSF/ITS-4R L1 40 cm	96	9	1.917
ITSF/ITS-4R L2 5 cm	96	22	2.794
ITSF/ITS-4R L2 20 cm	96	12	1.945
ITSF/ITS-4R L2 40 cm	96	6	1.526

H′= Shannon index
ITSF-ITS-4R= Cloning library
L=location

Discussion

The present work is the first attempt to get insight into the identification of fungal communities in Dukan Lake using culture independent methods. To the best of our knowledge, there have been no studies addressed the molecular diversity of fungi from different deep-levels in Dukan Lake. Thus, our main aim of current study was to assess the fungal biodiversity in two locations in Dukan Lake with various deep levels using clone libraries and sequencing method. A field cruise was carried out in the lake of Dukan on December 2015, from two collection sites in the lake depending on the proximity to the sea shore. Three different depths (5, 20, 40 cm) were selected to study the fungal diversity.

Herein, we reported the diversity of 33 fungal OTUs in different deep points of water level in Dukan Lake. Most of the retrieved sequences (91.2 %) in this study belonged to the phylum Ascomycota while only a few sequences (5% and 3.5 %) belonged to the both Basidiomycota and Zygomycota respectively. Therefore, these results indicated that the members of phylum Ascomycota might have dominated the fungal communities in Dukan Lake. However, the recovering of relatively very few basidiomycetous and Zygomycetous sequences in all water samples collected from both sites in the Dukan Lake could be due to either inadequate sample size or scanty amount of DNA resulting from bias in the DNA extraction, amplification and cloning process. In our study, we also found out that the fungal abundance were more at the depth of 5 cm than other depth points. This consistency in fungal richness might belong to many factors temperature and accessibility to the nutrient and light.

Even though that PCR dependent cloning and sequencing method are useful for evaluating the diversity of fungi in aquatic environment [20, 21], several confrontations and constraints should be encountered when utilizing this method. The PCR artifacts and biased amplification of a single sequence type are regarded as a potential drawback of this method. In this study, in order to explore the fungal diversity in Dukan lake, we used ITSF/ITS-4R primer set which shows a possible amplification biases towards amplification of Basidiomycetes [22], but we do not think that the ITSF/ITS-4R primer set was quite unable to amplify these fungal taxa because the ITSF/ITS-4R has been shown as a primer set capable to amplify a wide range of Basidiomycota from intestinal tract of human and non-human primates [17, 23, 24]. Determining correct sequence cutoff for assigning OTUs that corresponding species was also another limitation in this study [21] .

Conclusions

As we have no previous data on exact fungal diversity in Dukan Lake, therefore, it is important to conduct further investigations using a larger number of samples collected at diverse collection site s during each season of the year. Moreover, expanded sequencing analysis using a combination of PCR based cloning and sequencing, and high-throughput sequencing may expand our knowledge about the diversity of the fungal community in Dukan Lake in future.

Disclosure

The authors declare no conflicts of interest. All the experiments were undertaken in this study comply with the current laws of the country where they were performed.

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