

Development of a Real-Time Quantitative PCR Assay for the Assessment of Uncultured Zoosporic Fungi

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Abstract

Recently, molecular environmental surveys of the eukaryotic microbial community in lakes have revealed a high diversity of sequences belonging to uncultured zoosporic fungi. Although they are known as saprobes and algal parasites in freshwater systems, zoosporic fungi have been neglected in microbial food web studies. Recently, it has been suggested that zoosporic fungi, via the consumption of their zoospores by zooplankters, could transfer energy from large inedible algae and particulate organic material to higher trophic levels. However, because of their small size and their lack of distinctive morphological features, traditional microscopy does not allow the detection of fungal zoospores in the field. Hence, quantitative data on fungal zoospores in natural environments are missing. We provide a simplified step-by-step real-time quantitative PCR laboratory protocol, for the assessment of uncultured zoosporic fungi and other zoosporic microbial eukaryotes in natural samples.

Keywords

Fungi • Spores • DNA extraction • Real-time qPCR • Environmental samples

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Introduction

Recent molecular surveys of microbial eukaryotes have revealed overlooked uncultured environmental fungi with novel putative functions [1–3], among which zoosporic forms (i.e., chytrids) are the most important in terms of diversity, abundance, and functional roles, primarily as infective parasites of phytoplankton [4, 5] and as valuable food sources for zooplankton via massive zoospore production, particularly in freshwater lakes [6–8]. However, due to their small size (2–5 μm), their lack of distinctive morphological features, and their phylogenetic position, traditional microscopic methods are not sensitive enough to detect fungal zoospores among a mixed assemblage of microorganisms. Most chytrids occupy the most basal branch of the kingdom Fungi, a finding consistent with choanoflagellate-like ancestors [9]. These reasons may help explain why both infective (i.e., sporanges) and disseminating (i.e., zoospores) life stages of chytrids have been misidentified in previous studies as phagotrophic sessile flagellates (e.g., choanoflagellates) and “small underdetermined” cells, respectively. These cells often dominate the abundance of free-living heterotrophic nanoflagellates (HNFs), and are considered the main bacterivores in aquatic microbial foodwebs [2, 10]. Their contribution ranges from 10 to 90% of the total abundance of HNFs in pelagic systems (see review in reference [11]). Preliminary data have provided that up to 60% of these unidentified HNFs can correspond to fungal zoospores [12], establishing HNF compartment as a black box in the context of microbial food web dynamics [4]. A recent simulation analysis based on Lake Biwa (Japan) inverse model indicated that the presence of zoosporic fungi leads to (1) an enhancement of the trophic efficiency index, (2) a decrease of the ratio detritivory/herbivory, (3) a decrease of the percentage of carbon flowing in cyclic pathways, and (4) an increase in the relative ascendancy (indicates trophic pathways more specialized and less redundant) of the system [13]. Unfortunately, because specific methodology for their detection

is not available, quantitative data on zoosporic fungi are missing.

Molecular approaches have profoundly changed our view of eukaryotic microbial diversity, providing new perspectives for future ecological studies [3]. Among these perspectives, linking cell identity to abundance and biomass estimates is highly important for studies on carbon flows and the related biogeochemical cycles in natural ecosystems [11]. Historically, taxonomic identification and estimation of *in situ* abundances of small microorganisms have been difficult. In this context, our inability to identify and count many of these small species in the natural environment limits our understanding of their ecological significance. Thus, new tools that combine both identification and quantification need to be developed. Fluorescent *in situ* hybridization (FISH) has been an assay of choice for simultaneous identification and quantification of specific microbial populations in natural environments [14, 15]. However, this technique is limited because of (1) the relatively low number of samples that can be processed at a time, and (2) its relatively low sensitivity due to background noise and the potentially low number of target rRNA molecules per cell in natural environments [16]. In contrast, real-time quantitative PCR (qPCR), which has been widely used to estimate prokaryotic and eukaryotic population abundances in natural ecosystems, allows the simultaneous analysis of a high number of samples with a high degree of sensitivity [15].

The main objective of this chapter is to provide, in a simplified step-by-step format, a qPCR assay for the quantitative assessment of uncultured zoosporic fungi and other zoosporic microbial eukaryotes in natural environments [15], together with practical advice on how to apply the method. QPCR was recently used to estimate fungal biomass in a stream during leaf decomposition [17] and in biological soil crusts [18]. The interpretation of the semi-quantitative data obtained in these studies was relatively difficult because the whole fungal community was targeted (including unicellular, multicellular, and multinuclear fungal species). Thus, an estimation of fungal density or even fungal biomass was not

possible. In the following protocol, the primary targets are zoospores in liquid suspensions. Because zoospores are unicellular, qPCR data could be directly converted into cell density estimates (i.e., by multiplying semi-quantitative data by a number of rDNA copies per cells). Moreover, we designed primers targeting Rhizophidiales taxon to limit quantification bias generated by the variability in the number of rDNA copies within eukaryotic ribosomal operon.

Materials

1. Gloves (should be worn when manipulating most of the following materials).
2. Sterile distilled water.
3. 0.6 μm pore size polycarbonate filters.
4. Filtration columns.
5. Sodium dodecyl sulfate (SDS).
6. Proteinase K.
7. TE buffer—10 mM Tris-HCl pH 7.5 (25 °C), 1 mM EDTA.
8. NucleoSpin Plant kit® (Macherey-Nagel, Bethlehem, PA) with silica-membrane columns and the materials for running the provided protocol from the manufacturer.
9. Molecular-biology-grade agarose.
10. Ethidium bromide—because suspected as a mutagen, particular care should be taken when handling (consult safety data sheet).
11. Calf thymus DNA (Sigma-Aldrich, St. Louis, MO).
12. Oligonucleotidic primers resuspended in sterile distilled water and stored at -20 °C (see Note 1).
13. SYBR Green (Sigma-Aldrich).
14. dNTPs—a mixture of dATP, dCTP, dGTP, and dTTP (10 mM of each), stored at -20 °C.
15. Thermostable DNA polymerase and reaction buffer supplied by manufacturer. To avoid nonspecific amplicon, use hot-start (e.g., HotStarTaq, Qiagen, Valencia, CA).
16. Vortexer.
17. Centrifuge.
18. Water bath.
19. Horizontal electrophoresis machine.
20. TBE buffer: 50 mM Tris, 50 mM boric acid, 1 mM EDTA, diluted when needed from a 50 \times stock solution.
21. Spectrophotometer—the authors use NanoDrop (NanoDrop Technologies, Inc, Wilmington, DE).
22. Disposable conical tubes (1.5 mL); PCR tube strips or plate with adhesive film and cap adapted for real-time quantitative PCR assay.
23. Thermal cycler—we use Mastercycler ep realplex detection system (Eppendorf).
24. UV transilluminator equipped with a camera suitable for photographing agarose gels

Methods

DNA Extraction and Purification

Collect zoosporic organisms onto 0.6 μm pore size polycarbonate filters (after removal of the algal host by prefiltrations when only zoospores are targeted) (see Note 2).

1. For cell disruption, incubate the filters in 560 μL of a buffer containing 1% SDS and 1 mg/mL proteinase K in TE buffer for 1 h at 37 °C in a water bath (see Notes 3 and 4).
2. For DNA purification, use the silica-membrane columns provided with the NucleoSpin Plant kit® (Macherey-Nagel), following the instructions from the manufacturer (see Note 4).
3. Visualize the integrity and yield of the extracted genomic DNA in a 1% agarose gel stained with 0.3 $\mu\text{g}/\text{mL}$ of ethidium bromide solution (Sigma-Aldrich), using UV transilluminator and photograph. For this (1) heat (45 s using a microwave oven) a mixture of agarose in 1 \times TBE buffer; (2) leave it to cool on the bench for 5 min down to about 60 °C before adding ethidium bromide (i.e., to avoid vapor formation); (3) mix and pour into suitable gel gray with comb and leave to set for at least 30 min; (4) remove the comb and submerge the gel to 2–5 mm depth in electrophoresis tank containing 1 \times TBE buffer; (5) transfer DNA sample aliquots (i.e., 2 volumes of sample and 1 volume of loading buffer), marker,

- and the serial dilution of 5–10 ng of calf thymus DNA (Sigma-Aldrich-Aldrich) to the wells of the agarose gel; and (6) start the electrophoresis migration for about 30 min at 100 V.
- Calculate DNA extract concentrations from dilutions of calf thymus DNA (Sigma-Aldrich), using a standard curve of calf thymus DNA vs. band intensity.

Real-Time qPCR Assays

- PCR mix contained SYBR Green (Sigma-Aldrich), 200 μM of each dNTPs, 10 pM of each primer, 2.5 units of *Taq* DNA polymerase, the PCR buffer supplied with the enzyme, and 1.5 mM MgCl_2 . Vortex briefly (less than 10 s) and centrifuge the mix before distributing aliquots in suitable PCR tubes (strips or plates) and place on ice.
- Add variable quantity of DNA (we used 5 ng for our environmental freshwater samples, and 10 ng for DNA from appropriate PCR negative control strains) used as template in a final volume of 25 μL (see Note 5).
- Standard curve of C_i (see Note 4) vs. DNA copy number required to calculate target copy numbers (see Note 6) in each reaction is generated using triplicates of PCR reactions of tenfold dilutions of linear plasmid (containing Rhizophidiales 18S rDNA insert; PFB11AU2004) ranging from 100 to 1×10^8 copy/ μL (see Note 7). This number of copies was calculated using the equation: molecules/ $\mu\text{L} = a/(b \times 660) \times 6.022 \times 10^{23}$, where a is the plasmid DNA concentration (g/ μL), b the plasmid length in bp, including the vector and the inserted 18S rDNA fragment, 660 the average molecular weight of one base pair, and 6.022×10^{23} the Avogadro constant [15, 19].
- Place all tubes (i.e., samples, controls, and standards) in the real-time qPCR cyclers and run the appropriate cycling program: initial HotStarTaq activation at 95 °C for 15 min, 35 cycles with denaturation at 95 °C for 1 min, annealing at 63.3 °C for 30 s with Fchyt/Rchyt primers pair (see Note 1), elongation at 72 °C for 1 min, and a final additional elongation step at 72 °C for 10 min.
- Using SYBR Green molecule, melting curves analysis can be performed immediately following each qPCR assay to check specificity of amplification products (to confirm the absence of primer dimers or unspecific PCR products) by increasing the incubation temperature from 50 to 95 °C for 20 min.
- Analyze the real-time PCR result with the suitable software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot (see Note 5) before calculating the initial concentration of the targeted uncultured fungal 18S rDNA (copies/mL) in the environmental genomic DNA (see Note 6).

Notes

- Consensus (universal) primers can be used to amplify regions of fungal ribosomal RNA gene. For natural waters, we have designed primers specific to chytrids using a database containing about a hundred 18S rDNA environmental sequences recovered from surveys conducted in different lakes and sequences belonging to described fungi [15]. Sequences were aligned using BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and the resulting alignment was corrected manually. A great proportion of the environmental chytrid sequences recovered from lakes was closely affiliated to the Rhizophidiales. Thus, Rhizophidiales-specific primers F-Chyt (sequence 5'>3': GCAGGCTT ACGCTTGAATAC) and R-Chyt (sequence 5'>3': CATAAGGTGCCGAACAAGTC) were designed in order to fulfill three requirements: (1) a GC content between 40 and 70%, (2) a melting temperature (T_m) similar for both primers and close to 60 °C, and (3) PCR products below 500 bp (i.e., between 304 and 313 bp depending on the species considered). The absence of potential complementarities (hairpins and dimers) was checked using Netprimer (<http://www.premierbiosoft.com/>

- netprimer/netprlaunch/netprlaunch.html), and confirmed by inspection of the melting curve following the qPCR assay.
- For targeting uncultured zoospore fungi, zoospores are discarded from other environmental microorganisms by successive prefiltrations through 150, 80, 50, 25, 10, and 5 μm filters before collected them onto 0.6- μm polycarbonate filters. Filters can be conserved at $-80\text{ }^{\circ}\text{C}$ until DNA extraction in appropriate tubes (2 mL).
 - Other enzymes such as lyticase can be used for cell disruption, with no significant difference compared to proteinase K. However, the one-step proteinase K yields higher amount of genomic DNA than the lyticase method and has a better reproducibility. Physical disruption procedures such as sonication or thermal shocks (i.e., freezing in liquid nitrogen and thawing) are to be avoided because of the possible degradation of DNA.
 - A standard phenol–chloroform purification procedure can also be used but when the genomic DNA extracts are used as template in PCR reactions, the DNA purification method using the commercial kit gave significantly better results (based on C_t , the threshold cycle during PCR when the level of fluorescence gives signal over the background and is in the linear portion of the amplified curve) than the phenol–chloroform method. Consequently, the DNA extraction method using Proteinase K and the commercial kit was selected and considered the best overall.
 - In the case of novel designed primers (see Note 1) for uncultured fungi, DNA from both positive and negative plasmids and different mixtures (e.g., 5, 10, 25, and 50% of the positive plasmids) will be used for the optimization of the conditions (annealing temperature, cycling), cross-reactivity, the detection limit (using serial tenfold dilutions of the positive plasmids; see Note 7), and the amplification efficiency of the qPCR essays, which should be at least 90%. Poor primer quality is the leading cause for poor PCR efficiency. In this case, the PCR amplification curve usually reaches plateau early and the final fluorescence intensity is significantly lower than that of most other PCRs. This problem may be solved with re-synthesized primers.
 - The initial concentration of target 18S rDNA (copies/mL) in environmental samples can be calculated using the formula $[(a/b) \times c]/d$, where a is the 18S rDNA copy number estimated by qPCR, b is the volume of environmental genomic DNA added in the qPCR reaction, c is the volume into which the environmental genomic DNA was resuspended at the end of the DNA extraction, and d is the volume of sample filtered from which environmental DNA was extracted.
 - In the absence of cultures, plasmids used in qPCR to construct standard curves and to optimize qPCR reactions come from genetic libraries constructed during previous environmental surveys [2]. Briefly, the complete 18S rRNA gene was amplified from environmental genomic DNA extracts using the universal eukaryote primers 1f and 1520r. An aliquot of PCR products was cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Plasmid containing the insert of interest was extracted with NucleoSpin® plasmid extraction kit (Macherey-Nagel) following the manufacturer's recommendations. The 18S rRNA gene was sequenced from plasmid products by the MWG Biotech services using M13 universal primers (M13rev (–29) and M13uni (–21)). Phylogenetic affiliation of sequences acquired was established using Neighbor-Joining and Bayesian methods. In our case, positive plasmids contain insert affiliated to target chytrid (i.e. Rhizophidiales species) and displaying less than two mismatches with primers F-Chyt and R-Chyt sequences (see Note 1). The plasmid PFB11AU2004 (Genbank accession number DQ244014) was selected to construct the standard curve required for qPCR. Linearized plasmids were produced from supercoiled plasmids by digestion with restriction endonuclease one-time cutting into the vector sequence. Linear plasmid DNA concentration can be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer.

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References

1. Jobard M, Rasconi S, Sime-Ngando T (2010) Diversity and functions of microscopic fungi: a missing component in pelagic food webs. *Aquat Sci* 72:255–268
2. Lefèvre E, Bardot C, Noël C, Carrias JF, Viscogliosi E, Amblard C et al (2007) Unveiling fungal zooflagellates as members of freshwater picoeukaryotes: evidence from a molecular diversity study in a deep meromictic lake. *Environ Microbiol* 9:61–71
3. Monchy S, Jobard M, Sancier G, Rasconi S, Gerphagnon M, Chabe M et al (2011) Exploring and quantifying fungal diversity in freshwater lake ecosystems using rDNA cloning/sequencing and SSU tag pyrosequencing. *Environ Microbiol* 13(6):1433–1453. doi:10.1111/j.1462-2920.2011.02444.x
4. Gachon C, Sime-Ngando T, Strittmatter M, Chambouvet A, Hoon KG (2010) Algal diseases: spotlight on a black box. *Trends Plant Sci* 15:633–640
5. Rasconi S, Jobard M, Sime-Ngando T (2011) Parasitic fungi of phytoplankton: Ecological roles and implications for microbial food webs. *Aquat Microb Ecol* 62:123–137
6. Gleason FH, Kagami M, Marano AV, Sime-Ngando T (2009) Fungal zoospores are valuable food resources in aquatic ecosystems. *Mycologia* 60:1–3
7. Kagami M, Von Elert R, Ibelings BW, de Bruin A, Van Donk E (2007) The parasitic chytrid, *Zygorhizidium*, facilitates the growth of the cladoceran zooplankton, *Daphnia*, in cultures of the inedible alga. *Asterionella* Proc R Soc B 274:1561–1566
8. Kagami M, Helmsing NR, Van Donk E (2011) Parasitic chytrids could promote copepod survival by mediating material transfer from inedible diatoms. In: Sime-Ngando T, Niquil N (eds) *Disregarded microbial diversity and ecological potentials in aquatic systems*. Springer, Heidelberg, pp 49–54
9. James TY, Letcher PM, Longcore JE, Mozley-Standridge SE, Porter D, Powell MJ et al (2006) A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). *Mycologia* 98:860–871
10. Lefèvre E, Roussel B, Amblard C, Sime-Ngando T (2008) The molecular diversity of freshwater picoeukaryotes reveals high occurrence of putative parasitoids in the plankton. *PlosOne* 3:2324
11. Sime-Ngando T, Lefèvre E, Gleason FH (2011) Hidden diversity among aquatic heterotrophic flagellates: ecological potentials of zoospore fungi. In: Sime-Ngando T, Niquil N (eds) *Disregarded microbial diversity and ecological potentials in aquatic systems*. Springer, Heidelberg, pp 5–22
12. Jobard M, Rasconi S, Sime-Ngando T (2010) Fluorescence *in situ* hybridization of uncultured zoospore fungi: testing with clone-FISH and application to freshwater samples using CARD-FISH. *J Microbiol Methods* 83:236–243
13. Niquil N, Kagami M, Urabe J, Christaki U, Viscogliosi E, Sime-Ngando T (2011) Potential role of fungi in plankton food web functioning and stability: a simulation analysis based on Lake Biwa inverse model. In: Sime-Ngando T, Niquil N (eds) *Disregarded microbial diversity and ecological potentials in aquatic systems*. Springer, Heidelberg, pp 65–79
14. Lefèvre E, Carrias J-F, Bardot C, Amblard C, Sime-Ngando T (2005) A preliminary study of heterotrophic picoflagellates using oligonucleotide probes in Lake Pavin. *Hydrobiologia* 55:61–67
15. Lefèvre E, Jobard M, Venisse JS, Bec A, Kagami M, Amblard C et al (2010) Development of a real-time PCR assay for quantitative assessment of uncultured freshwater zoospore fungi. *J Microbiol Methods* 81:69–76
16. Moter A, Göbel UB (2000) Fluorescence *in situ* hybridization (FISH) for direct visualization of microorganisms. *J Microbiol Methods* 41:85–112
17. Mayura AM, Seena S, Barlocher F (2008) Q-RT-PCR for assessing *Archaea*, *Bacteria*, and *Fungi* during leaf decomposition in a stream. *Microb Ecol* 56:467–473
18. Bates ST, Garcia-Pichel F (2009) A culture-independent study of free-living fungi in biological soil crusts of the Colorado plateau: their diversity and relative contribution to microbial biomass. *Environ Microbiol* 11:56–67
19. Zhu F, Massana R, Not F, Marie D, Vaulot D (2005) Mapping of picoeukaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. *FEMS Microbiol Ecol* 52:79–92