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Development of a Real-Time PCR assay for quantitative assessment of uncultured freshwater 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 is missing. We have developed a quantitative PCR (qPCR) assay for the quantification of fungal zoospores in lakes. Specific primers were designed and qPCR conditions were optimized using a range of target and non-target plasmids obtained from previous freshwater environmental 18S rDNA surveys. When optimal DNA extraction protocol and qPCR conditions were applied, the qPCR assay developed in this study demonstrated high specificity and sensitivity, with as low as 100 18S rDNA copies per reaction detected. Although the present work focuses on the design and optimization of a new qPCR assay, its application to natural samples indicated that qPCR offers a promising tool for quantitative assessment of fungal zoospores in natural environments. We conclude that this will contribute to a better understanding of the ecological significance of zoosporic fungi in microbial food webs of pelagic ecosystems.

1. Introduction

Recently, molecular environmental surveys of the eukaryotic microbial community in the pelagic zone of lakes have revealed an unexpectedly high diversity of undescribed zoosporic fungi, i.e. chytrids (Lefranc et al., 2005; Lefèvre et al., 2007; 2008; Chen et al., 2008; Lepère et al., 2008). Although zoosporic fungi are commonly observed in freshwater systems as saprobes on various organic substrates (Czeczuga et al., 2002, 2005; Czeczuga and Muszynska, 2000; Kiziewickz and Kurzatkowska, 2004; Kiziewickz and Nalepa, 2008), and as parasites on various phytoplanktonic species (see review by Kagami et al., 2007a), they are totally neglected in microbial food web studies. Recently, Kagami et al. (2004, 2007b) showed that fungal zoospores (i.e. flagellated fungal spores) could be efficiently grazed upon by filter feeder zooplankters. Hence, zoosporic fungi, via the consumption of their zoospores, might transfer energy from large inedible algae and particulate organic material to higher trophic levels (Kagami et al., 2004, 2007a,b; Lefèvre et al., 2008). However, due to their small size (2–5 μm) and their lack of distinctive morphological features, traditional microscopic methods are not sensitive enough to detect fungal zoospores among a mixed assemblage of microorganisms. Recently, it has been suggested that fungal zoospores may have been misidentified as bacterivorous heterotrophic flagellates (HF) (Kagami et al., 2007a; Lefèvre et al., 2007, 2008). Indeed, the proportion of 'small unidentified HF' in lakes is unusually high, ranging from 10 to 90% of the total HF abundance (Carrias et al., 1998; Cleven and Weisse, 2001; Domaizon et al., 2003; Comte et al., 2004; Sonntag et al., 2006). As suggested by Lefèvre et al. (2008), if a portion of these unidentified HF corresponded to fungal zoospores, saprobes and parasitic fungi could be quantitatively important in pelagic systems. Unfortunately, because specific methodology for their detection is not available, quantitative data on fungal zoospores are missing.

Molecular approaches have profoundly changed our view of eukaryotic microbial diversity, providing new perspectives for future ecological studies. Among these perspectives, linking cell identity to

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abundance and biomass estimates is highly important for studies on carbon flows and the related biogeochemical cycles in pelagic systems. Historically, taxonomic identification and estimation of *in situ* abundances of small aquatic 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 (Lim, 1996; Lim et al., 1993, 1996, 1999; Amann et al., 1995; Massana et al., 2002; Lefèvre et al., 2005). However, this technique is limited because of (i) the relatively low number of samples that can be processed at a time, and (ii) its relatively low sensitivity due to the potentially low number of target rRNA molecules per cell in natural environments (Moter and Göbel, 2000). In contrast, quantitative PCR (qPCR), which has been widely used to estimate prokaryotic and eukaryotic population abundances in marine systems (Audemard et al., 2004; Skovhus et al., 2004; Zhu et al., 2005; Coutway and Caron, 2006; Sub et al., 2006; Park et al., 2007), allows the simultaneous analysis of a high number of samples with a high degree of sensitivity (Klein, 2002).

The objective of this study was to develop a qPCR assay for the quantitative assessment of uncultured fungal zoospores in natural environments. Our qPCR assay was based on the detection of the SYBR Green dye (Walker, 2002). However, because SYBR Green binds to specific and non-specific PCR products, PCR conditions and primers specificity were carefully optimized. In addition, because inhibitory factors co-extracted along with DNA during extraction are known to reduce the sensitivity of PCR (Wilson, 1997; Martin-Laurent et al., 2001; Park and Crowley, 2005), extraction needed to be optimized. Finally, the method was validated on lake water samples in which the targeted zoosporic fungi were previously detected.

2. Materials and methods

2.1. Primer design

Primers were designed using a database containing about a hundred 18S rDNA environmental sequences recovered from surveys conducted in seven different lakes (Lefranc et al., 2005; Slapeta et al., 2005; Lefèvre et al., 2007, 2008; Lepère et al., 2008; Chen et al., 2008) and sequences belonging to described fungi. Sequences were aligned using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999) 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 and R-Chyt were designed in order to fulfill three requirements: (i) a GC content between 40 and 70%, (ii) a melting temperature (T_m) similar for both primers and close to 60 °C, and (iii) PCR products below 500 bp (Edwards, 2004; Table 1). Potential complementarities (hairpins and dimers) were checked using Netprimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html).

2.2. Optimization of DNA extraction

In order to assess the most appropriate DNA extraction method suitable to maximize genomic DNA quality, and thus enhance the downstream PCR efficiency, combinations of several cell lysis and DNA purification techniques were tested. Since the aim of this study was to quantify fungal zoospores, optimization of genomic DNA extraction was performed on the zoosporic life stage of *Zygorhizidium planktonicum* (clone FMS34600; Kagami et al., 2004), a chytrid parasite on *Asterionella formosa*. Zoospores were collected on 0.6 µm pore-size polycarbonate filters (24 replicates) after removal of the algal host by prefiltration.

For cell disruption, four methods were tested: (i) an enzymatic digestion with proteinase K: 6 filters were incubated in 560 µl of a buffer containing 1% SDS (sodium dodecyl sulfate) and 1 mg ml^{-1} proteinase K and TE (Tris–EDTA, 1 mM EDTA and 10 mM Tris–HCl) for 1 h at 37 °C. (ii) An enzymatic digestion with lyticase (Sigma) and proteinase K: 6 filters were incubated overnight at 30 °C in 500 µl of a sorbitol based buffer containing 400 units of lyticase (Karakousis et al., 2006), followed by the proteinase K digestion as previously described. (iii) A physical cell disruption by sonication: 6 filters were recovered with 500 µl of TE buffer and sonicated three times on ice, for 30 s at 20 kHz with a power output of 30 W (model VC 50–220V, Bioblock Scientific). (iv) A physical cell disruption procedure by thermal shocks: 6 filters were recovered with 500 µl of TE buffer, frozen in liquid nitrogen and thawed three times at 37 °C, and finally incubated in boiled water for 10 min.

For all the cell lysis protocols described, two purification methods were applied: (i) a phenol–chloroform extraction method previously used by Lefèvre et al. (2007, 2008), and (ii) a purification step using silica-membrane columns provided with the NucleoSpin Plant kit® (Macherey-Nagel). Integrity and yield of the extracted genomic DNA were visualized in a 1% agarose gel stained with 0.3 µg ml^{-1} of ethidium bromide solution (Sigma). DNA extracts were run with dilutions of calf thymus DNA (Sigma) and concentrations calculated using a standard curve of 5 to 100 ng of calf thymus DNA versus band intensity. A two way analysis of variance, ANOVA (factor 1: cell lysis procedures; factor 2: DNA purification procedures) was performed to determine significant differences in the DNA concentration obtained (at 95% confidence limit).

2.3. Material used as template for qPCR assays

As none of the chytrids targeted in this study were available in culture, the qPCR assay was optimized using (i) DNA extracted from 29 strains representative of different non-target phylogenetic groups (Table 2), (ii) 18S rDNA-containing plasmids obtained from one of our previous environmental DNA surveys on Lake Pavin, France (Lefèvre et al., 2007; Table 3), and (iii) 20 environmental samples collected during our previous environmental DNA surveys in Lake Pavin (Lefèvre et al., 2007; 2008). Except for one date (12/1/04), water samples were collected during spring and autumn thermal stratifications. Oxygen and temperature profiles were measured for every sampling dates.

Table 1
Properties of the primers used in this study.

Name	Sequence (5′→3′)	Length (bp)	Tm[a] (°C)	GC (%)	Max. number of mismatches to target sequences	Position in PFB11AU2004 sequence	Amplicon size (bp)
F-Chyt	GCAGGCTTACGCTTGAATAC	20	57.69	50	1	773	304 to 313
(F-Chyt′[b])	(GCAGGC**ATTT**GCT**C**GAATA−)	(19)	(59)	(47.37)	(0)		(308)
R-Chyt	CATAAGGTGCCGAACAAGTC	20	58.23	50	1 (2)	1076	

[a] Tm was calculated using Primer3 software.
[b] The forward primer F-Chyt′, modified from F-Chyt (modified nucleotides in bold), was used with R-Chyt to amplify *Z. planktonicum*.