A decade's perspective on the impact of DNA sequencing on aquatic hyphomycete research

Sofia Duarte[a*], Sahadevan Seena[a], Felix Bärlocher[b], Cláudia Pascoal[a] and Fernanda Cássio[a]

[a]Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Sofia Duarte: sduarte@bio.uminho.pt

Sahadevan Seena: ssahadevan@bio.uminho.pt

Cláudia Pascoal: cpascoal@bio.uminho.pt

Fernanda Cássio: fcassio@bio.uminho.pt

[b]Department of Biology, Mount Allison University, Sackville, New Brunswick, E4L 1G7, Canada

Felix Bärlocher: fbaerloc@mta.ca

*corresponding author: Sofia Duarte

e-mail: sduarte@bio.uminho.pt

Telephone: +351 253601526

Fax: +351 253678980

Abstract

A decade has passed since the first DNA sequences of aquatic hyphomycete species have become available. They have illuminated aspects of their phylogeny and evolution that were inaccessible by conventional methods. Here we present examples of how the resulting information has modified our knowledge of aquatic hyphomycetes. Generating more and better DNA sequence data will continue to expand the range of questions we can investigate concerning the evolution and ecology of aquatic hyphomycetes. We highlight the importance of moving forward with next generation sequencing technologies to more accurately determine the true diversity and composition of fungal communities on environmental samples. In addition, sequences targeting functional genes will offer further insights into the roles of aquatic fungi in ecosystem processes.

Keywords: Aquatic hyphomycetes; DNA sequences; Phylogeny; Evolution; Barcoding; Community-level techniques

1. Introduction

Until the recognition of the crucial role that fungi (Kaushik and Hynes, 1971), and specifically aquatic hyphomycetes (Bärlocher and Kendrick, 1974; Suberkropp and Klug, 1976), play in plant litter decomposition in streams, limnologists largely ignored these organisms. The predominantly multiradiate (often tetraradiate) and sigmoid spores of aquatic hyphomycetes were very early assumed to be due to convergent evolution and thus providing limited information on phylogenetic relationships. Taxonomy and phylogenetic speculation concerning aquatic hyphomycetes began when Ingold (1942) first discovered and identified members of this group growing and sporulating on allochthonous leaf litter submerged in a stream. Conventional identification of aquatic hyphomycetes has been based on the morphology and development of conidia (mitospores) produced by anamorphic genera, and spore similarity was interpreted as indicator of close phylogenetic relationship. More reliable information was provided by the simultaneous appearance of anamorphic (asexual, mitosporic) and teleomorphic (sexual, meiosporic) reproduction in pure cultures (Webster, 1992). This traditional methodology provided important clues about the relatedness of various aquatic hyphomycete species and showed that an overwhelming majority has strong affinities to various Ascomycota, with a minority belonging to Basidiomycota (Ranzoni, 1956; Ingold, 1959; Webster, 1992). Unfortunately, its success is strongly tied to the availability of pure cultures that produce reproductive structures. Isolating pure cultures from single conidia is tedious and time-consuming. Not surprisingly, the number of aquatic hyphomycetes species in culture collections is low. On the other hand, DNA is ubiquitous and present in all stages of the fungal life cycle. Carefully chosen sequences are reliable indicators of evolutionary history and therefore phylogenetic relationships. Their use circumvents most of the hurdles associated with the conventional culture-

dependent and microscopic techniques (Berbee and Taylor, 2001). In particular, sections of nuclear rDNA are considered to be ideal for the study of evolutionary relationships among fungi and many other taxa. Of particular significance are the small subunit ribosomal DNA (SSU-rDNA or 18S rDNA), the internal transcribed spacer ITS (ITS1 and ITS2), and the large subunit ribosomal DNA (28S rDNA). Ribosomal DNA is found in all fungi, allowing a universal phylogeny (though its suitability for phylogenetic reconstruction has recently been questioned; Dupuis et al. 2012; Taylor and Harris 2012). Lateral transfer of rDNA is rare, ensuring that the evolutionary history of rDNA reflects the evolutionary history of the organism. Finally, rDNA contains a mixture of highly conserved regions and moderately and highly variable regions. Highly conserved regions (18S and 28S rDNA regions) are convenient sites for annealing of universal primers for analyses of higher-level phylogenetic classification (genera and higher), while moderately (e.g. domain D1/D2 on 18S rDNA) and highly variable (ITS) regions are suitable targets for lower level analyses (species and lower; Head et al., 1998). Anderson and Shearer (2011) used partial β-tubulin sequences to examine variation within one (morpho)species. For more accurate and reproducible results, comparisons of several loci with multiple alleles are recommended (Taylor et al. 2006).

As summarized in this review, the last ten years have been marked by substantial advances in our understanding of the evolution, phylogeny and molecular identification of aquatic hyphomycetes through DNA sequence analysis (Fig. 1). Nevertheless, their representation in genomic databases is still poor. To date, over 300 aquatic hyphomycete species have been described (Belliveau and Bärlocher, 2005; Letourneau et al., 2010; Shearer et al., 2007), but only ca. 72 species are represented by sequences in the National Center for Biotechnology Information (NCBI)

(http://www.ncbi.nlm.nih.gov/). We advocate implementing next-generation sequencing techniques as a tool for detecting and identifying fungi in environmental samples to unravel the phylogenetic and genealogical histories and community ecology of aquatic hyphomycetes.

2. Phylogeny of aquatic hyphomycetes

Phylogenetic studies of aquatic hyphomycetes using DNA sequences go back to the study of Nikolcheva and Bärlocher (2002), in which they sequenced complete 18S rDNA regions of 5 representative species of the genus *Tetracladium*. This study resulted in well resolved and statistically supported conclusions that *Tetracladium* species are part of a monophyletic group as suggested by traditional, morphology-based taxonomy (Roldán et al., 1989). Three years later, partial 18S rDNA sequences were obtained from 22 additional species of aquatic hyphomycetes in order to evaluate existing taxonomic systems and phylogenetic affinities that had been based mainly on morphological features (Belliveau and Bärlocher, 2005). Evidence from this study put an end to the debate as to whether aquatic hyphomycetes were monophyletic or polyphyletic. Molecular phylogenies of members belonging to the genus *Anguillospora* and *Tricladium* showed that aquatic hyphomycetes formed mixed groups in phylogenetic trees and were dispersed among different orders (Fig. 2). The polyphyletic origin of aquatic hyphomycetes has been reinforced by further studies (Baschien et al., 2006; Campbell et al., 2006, 2009). These studies gave consistent classifications for the majority of the species, with the exception of *Goniopila monticola*. Partial 18S rDNA placed this species within the Leotiomycetes (Belliveau and Bärlocher, 2005), while partial 28S rDNA placed it among the Dothideomycetes (Campbell et al., 2006) (Fig. 2).

DNA sequences also helped connect anamorphic to teleomorphic states. For example, the analysis of partial 18S rDNA sequences showed that the anamorph *Jaculispora submersa* was connected with the teleomorph *Classicula fluitans* (Bauer et al., 2003). Furthermore, without DNA sequencing the link between aquatic and endophytic phases of *Dwayaangam* would have gone unnoticed (Sokolski et al., 2006). ITS sequences of *Tetracladium* isolated from endophytic and aquatic environments were interspersed in phylogenetic trees, indicating the amphibious nature of aquatic hyphomycetes (Selosse et al., 2008). DNA sequencing can provide presumptive evidence for a species' occurrence in multiple ecological niches, and suggests potential pathways of how aquatic hyphomycetes evolved from a terrestrial to an aquatic way of life (Selosse et al., 2008).

3. Molecular identification of aquatic hyphomycetes

Identification through molecular barcoding has only recently been applied to aquatic hyphomycetes (Letourneau et al., 2010; Seena et al., 2010). Barcoding identifies biological specimens and assesses biodiversity through the use of short (a few 100 bp) DNA sequences or barcodes (Hebert et al., 2003). Barcodes are obtained in a single amplification and flanked by conserved regions allowing annealing by universal primers (Letourneau et al., 2010). Ideally, they are of low intraspecific and sufficient interspecific variability. Only a few studies have compared the suitability of different regions for discriminating among aquatic hyphomycete species (Letourneau et al., 2010). Among published sequences, the ITS region has received most of the attention, with 305 records belonging to 42 species. This corresponds to ca. 57% of all aquatic hyphomycete sequences published in NCBI (http://www.ncbi.nlm.nih.gov/) (Fig. 3). The ITS region is also by far the most commonly sequenced region for systematic and

taxonomic queries of fungi in general at and below the genus level (Nilsson et al., 2009). Considerable ITS variation between species was shown in initial studies, reinforcing the usefulness of the ITS region as a potential barcode for aquatic hyphomycetes (Letouneau et al., 2010; Seena et al., 2010). Based on a 72 % identification success rate, the ITS region was formally chosen and declared the best barcode marker for fungi at two conferences in 2011 ("Fungi DNA Barcoding Symposium" in Amsterdam, Holland and "Fourth International Barcode of Life Conference" in Adelaide, Australia; http://www.ecbol.org). Very recently, ITS barcodes were also successfully used to assess the intraspecific variation within *Articulospora tetracladia* (Seena et al., 2012) and to assess for the first time the phylogeography of 6 aquatic hyphomycete species (*Anguillospora filiformis*, *Flagellospora penicillioides*, *Geniculospora grandis*, *Lunulospora curvula*, *Tetrachaetum elegans* and *Tricladium chaetocladium*), collected from streams of Southwest Europe and East Australia (Duarte et al., 2012). While these studies provided valuable insights into the geographical distribution of different phylotypes, the reliance on a single sequence can be problematic (Taylor and Harris 2012). NGS approaches may provide a solution.

We emphasize that a sustained effort is needed to provide public DNA databases with additional ITS and other sequences from aquatic hyphomycetes. Having access to larger DNA datasets will provide greater opportunities to address phylogeographical questions with multiple species, and facilitate insights into the nearest terrestrial relatives of aquatic hyphomycetes and the timeline of their transition to the aquatic environment.

4. Community-level sequence data

Traditional microscopy based techniques are not adequate to fully document the aquatic fungal diversity on plant-litter decomposing in streams (Nikolcheva et al., 2003; Seena

et al., 2008; Duarte et al., 2008, 2010). The extraction of whole-community DNA, followed by amplification with fungal-specific primers and the establishment of ribosomal clone gene libraries, showed that the majority of the generated sequences from environmental substrates had high affinities to uncultured Ascomycota, which may or may not be related to aquatic hyphomycetes (Seena et al., 2008). Other sequences were close to Chytridiomycota, Oomycota and Basidiomycota. Although creating cloning libraries of aquatic fungi has increased our understanding of the diversity of uncultured fungi in environmental samples (Bärlocher et al., 2008; Seena et al., 2008), the generation of clone libraries is very time consuming, and cloned sequences can rarely be assigned to known species.

To avoid the tedious detour over clone libraries, we recommend embracing next-generation sequencing techniques (which, however, does not negate the need to add to the databases of reference sequences established from pure cultures). Next-generation sequencing offers platforms for directly recovering all the genetic material present in an environmental sample (Edwards et al., 2006; Medinger et al., 2010) and is a promising molecular approach to assess aquatic fungal diversity. It is now possible to sequence entire microbial communities at a lower cost than sequencing 1,000 individual specimens a decade ago (Nilsson et al., 2011). Several pyrosequencing efforts have reported a great diversity of fungi, but have generally failed to connect sequences to known species, genera and orders of fungi (Buée et al., 2009; Jumpponen et al., 2009; Ghannoum et al., 2010). To date less than 1% of the estimated fungal species has been sequenced (Nilsson et al., 2011), which emphasizes the need of greatly expanding reference sequences libraries. The ITS region has been suggested as a prime target for pyrosequencing environmental samples of fungi (Nilsson et al., 2009). Next-generation sequencing techniques have not yet been applied to fungal communities in streams;

their application promises further insights into the diversity of aquatic hyphomycetes, as well as contributions of other fungal groups (e.g., zoosporic fungi, Bärlocher et al., 2012).

5. Functional aspects and conclusions

Determining identities and frequencies of occurrence of species is an important first step in ecological research, but offers limited insight into the roles of the documented species and their participation in ecosystem processes. At best, it reveals some broad patterns of species (OTU) replacement along environmental gradients (with aquatic hyphomycetes, Bärlocher et al., 2008; Duarte et al., 2009; with ectomycorrhizae, Jumpponen et al., 2010). Information on the metabolic potential of the microbial community can be attained by comparing the pyrosequening dataset against a library of modules from an enzyme database (e.g., sequences associated with enzymes involved in carbohydrate metabolism in a tropical peat swamp; Kanokratana et al., 2011). Or, relevant sequences can be directly targeted and amplified from environmental samples (e.g., sequences associated with glycosyl hydrolases, Jacobson et al., 2005). It is also possible to characterize currently expressed genes by extracting environmental mRNA and reverse transcribing it into complementary cDNA, which is then amplified by PCR (reverse transcriptase PCR). The diversity of metabolically active fungi can be characterized by RT-PCR of precursor rRNA molecules, which still contain the ITS region (Anderson and Parkin 2007). Combining DNA and RNA diversity estimates from soil samples showed that the most common fungi (based on diversity of amplified DNA) are not necessarily the most active ones (based on diversity of amplified rRNA) (Baldrian et al. 2012). A further complication is the weak or missing correlation between mRNA levels and synthesis level of the corresponding protein (Gygi et al.

1999). Nevertheless, at least in principle, taxonomic composition and metabolic pathways in an environmental sample can be connected to determine which taxa are present, what metabolic potential they possess, and which of their genes are currently transcribed. Evaluation of the enormous amount of data generated by a combination of such approaches will present significant challenges (DeLong, 2009; Bärlocher, 2010; Purdy et al., 2010). To our knowledge, only one study of aquatic hyphomycetes has been done at the transcript level. Solé et al. (2008) investigated the expression of two laccase gene fragments in *Clavariopsis aquatica*.

Understanding the potential and actual participation in decomposition processes by aquatic hyphomycetes is clearly of paramount importance for stream ecologists and it is useful when assessing the integrity of aquatic ecosystems. Metabolic capabilities circumscribe the fungi's "nutritional niche", but are insufficient to fully explain the actual presence or absence of a given species in a habitat. Their interactions with other fungi, bacteria and invertebrates, as well as preference for, or tolerance of, abiotic conditions have to be considered as well.

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Figure legends

Fig. 1. Timeline representing major achievements in sequencing of aquatic hyphomycete species during the last decade (2002-2012).

Fig. 2. Phylogenetic relationships among aquatic hyphomycetes based on molecular studies (18S and 28S rDNA) from 2002 to 2009.

Fig. 3. Percentage of aquatic hyphomycete DNA sequences present in NCBI until December 2011. Only sequences linked to species were included in the analysis. "Other" includes partial sequences of the following genes: cythocrome oxidase subunit I (*COX*I), β-actin, β-tubulin, laccase, cuticle-degrading serine protease, mitogen-activated protein kinase, elongation factor 1-α, RNA polymerase II subunits, gluthatione synthetase, ATP citrate lyase, nitrate reductase and microsatellites sequences.

Sofia Duarte got her graduation in Applied Biology in 2002 and completed her PhD degree in Sciences (Area of knowledge Biology) at University of Minho (Braga, Portugal), in 2008. During her doctoral studies, S. Duarte focused on the effects of anthropogenic stress, namely eutrophication and metals, on the diversity and activity of microbial decomposers of plant-litter in freshwaters. Since July 2009 that S. Duarte has been developing post-doctoral studies about barcoding and biogeography of aquatic fungi, at University of Minho (Braga, Portugal), in collaboration with the University of Mount Allison (Sackville, Canada).

Seena Sahadevan did her doctoral research (2002-2005) in Biosciences, Mangalore University India. She was a postdoctoral fellow (2006-2008) in Dr. Felix Bärlocher's lab at Mount Allison University, Canada. Currently, she is working as an assistant researcher (2008-present) at the Centre of Molecular and Environmental Biology (CBMA), University of Minho, Portugal. Her research interests include, but not limited to the following: (1) Estimating the diversity of aquatic fungi both by microscope-based conventional and DNA/RNA-based molecular techniques; (2) Evaluating the role of microbial decomposers (aquatic hyphomycetes and bacteria) and invertebrates in leaf litter breakdown; (3) Examining the impacts of anthropogenic pollution to leaf litter decomposition and related biodiversity concerns; (4) Understanding the potential threats of nanoparticles to aquatic food webs.

Felix Bärlocher did his first degree at the ETH in Zürich, Switzerland, on the cytology of gall midges. In 1973, he completed a Ph.D. thesis in Waterloo, Canada, on the involvement of aquatic hyphomycetes in the detritus food chain of streams. He has continued work on the ecology, taxonomy and evolution of aquatic fungi, including some work on freshwater and salt marshes, first in Basel, Switzerland, and since 1983 at Mt. Allison University in Sackville, Canada. Since 2001, he has adapted various molecular methods to the study of aquatic fungal ecology. His recent interests have included connections between fungal diversity and their ecological functions, fungal responses to anthropogenic change and barcoding and biogeography of aquatic hyphomycetes.

Cláudia Pascoal is an Assistant Professor at the Department of Biology of the University of Minho (Portugal). She did her Graduation in Biology at the University of Coimbra (Portugal) and concluded the PhD in Aquatic Ecology at the University of Minho by 2004. Her research interests are related to the impacts of global change on biodiversity of benthic organisms and ecosystem functioning with emphasis on decomposition of organic matter in streams. She has given particular attention to the ecology of aquatic hyphomycetes and to the application of molecular tools to assess microbial biodiversity and activity.

Fernanda Cássio is Associate Professor at the Biology Department of the University of Minho, Portugal. She received her degree in Biology from the University of Porto (Portugal). She concluded her Ph.D. in Microbiology at the University of Minho (Braga, Portugal) in 1994. She has been working in aquatic microbial ecology and ecotoxicology.

Particular attention has been paid to the impacts of persistent and emergent contaminants on microbial decomposers in freshwaters. Over the last few years, she has been developing molecular barcodes to provide a better knowledge on the biodiversity and distribution of aquatic hyphomycetes.