



Universidade do Minho
Escola de Ciências

Analysis of functional genes diversity in aquatic fungal species

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**Analysis of functional genes diversity in aquatic fungal
species**

Master Thesis

Master's in Molecular Genetics

Work under the supervision of

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Análise da diversidade de genes funcionais em espécies de fungos aquáticos

Resumo

A folhada que cai nos ribeiros é a fonte principal de energia nestes ecossistemas. Os hifomicetos aquáticos (AQH) são um grupo de decompositores microbianos que dominam o processo de decomposição da folhada devido à sua capacidade de produzir enzimas extracelulares que degradam as moléculas complexas da folhada e de assimilar nutrientes da água, imobilizando-os na folhada em decomposição. A influência de alterações globais, como aumentos na temperatura da água, é particularmente preocupante nestes ecossistemas, podendo provocar perdas de biodiversidade que, no caso de AQH, poderão representar algumas perdas de capacidade enzimática nas suas comunidades. A maioria dos estudos de biodiversidade destes organismos baseia-se em parâmetros filogenéticos. Existem também estudos qualitativos sobre a capacidade de degradação de AQH, que, apesar de informativos, não representam totalmente a diversidade funcional destes organismos. O nosso estudo compreende a primeira análise da diversidade de um conjunto de genes funcionais que codificam enzimas envolvidas na decomposição de folhada num grupo alargado de espécies de AQH. Para tal, foram utilizadas 30 espécies de AQH e sequências parciais de 16 genes funcionais. Essas sequências foram obtidas através de técnicas como amplificação PCR, clonagem e análises *in silico*, sendo a avaliação da diversidade funcional feita através de árvores filogenéticas. Os resultados obtidos apontam para uma correlação entre proximidades taxonómicas e funcionais na maioria dos casos. Além disso, observamos uma abundância de diferenças intra- e interespecíficas entre isolados, bem como casos de divergência entre as sequências de gene funcional e a proteína que codificam. Segundo os nossos resultados, a intensidade das reações enzimáticas de organismos e o seu grau de proximidade em termos de gene funcional não parecem estar relacionadas. As observações aqui compreendidas servirão de base a estudos de avaliação dos impactos das alterações globais na sobrevivência, diversidade funcional e capacidades de degradação das comunidades de AQH e, conseqüentemente, no funcionamento dos ecossistemas ribeirinhos.

Palavras-chave

Hifomicetos aquáticos • Biodiversidade • Decomposição da folhada • Enzimas • Ecossistemas ribeirinhos

Analysis of functional genes diversity in aquatic fungal species

Abstract

Freshwater ecosystems comprise detritus-based food webs, which use allochthonous sources of nonliving organic carbon, such as leaf litter, as their main energy sources. A group of microbial decomposers, known as aquatic hyphomycetes (AQH), have a dominant role in the leaf litter decomposition process. These organisms can produce extracellular enzymes targeting complex molecules involved in this process and are also able to assimilate nutrients from stream water, immobilizing them in the decomposing substrate. However, global alterations, particularly increases in stream water temperatures, are predicted to substantially impact freshwater ecosystems functioning, potentially causing biodiversity losses, which could, in the case of AQH communities, lead to losses of specific enzymatic abilities. AQH diversity has been assessed in some studies, but mostly regarding their taxonomic relationships. Some other studies have assessed degradative potentials of AQH species through qualitative enzymatic assays. Our study is, to the best of our knowledge, the first to evaluate functional gene and protein diversities of an assortment of 16 functional genes involved in leaf litter decomposition in 30 different AQH species. Functional genes assessments were performed resorting to methods such as PCR amplifications, cloning and *in silico* analyses. Functional diversity was assessed through phylogenetic trees. Our results reveal, for most cases, a correlation between taxonomic and functional gene relatedness. Additionally, we have found intra- and interspecific differences between AQH isolates, for both functional gene and protein partial sequences as well as divergences between nucleotide and amino acid sequences for the same organism and functional gene. No correlation was found between the intensity of the degradative reaction and the degree of phylogenetic relatedness. These results are the kickstart to further assessments on functional diversity across AQH species and the impacts of global change alterations in AQH communities' survival, interactions and degradative performances. Studies as such will also allow better predictions on how those alterations will affect nutrient cycling and supplying to higher trophic levels and, subsequently, freshwater ecosystems functioning.

Keywords

Aquatic hyphomycetes • Biodiversity • Enzymes • Freshwater ecosystems • Leaf litter decomposition

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List of abbreviations and acronyms

OM – organic matter

CPOM - coarse particulate organic matter

DOM – dissolved organic matter

FPOM – fine particulate organic matter

AQH – aquatic hyphomycetes

rDNA – ribosomal Deoxyribonucleic acid

gDNA – genomic Deoxyribonucleic acid

PCR – polymerase chain reaction

ITS – internal transcribed spacer

BLAST - basic local alignment search tool

SRABLASTn - sequence read archive nucleotide BLAST

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Chapter 1

Introduction

1.1. Litter breakdown in streams

1.1.1. Description of the process and influencing factors

Small forested streams make up the majority of river basins' water courses in temperate regions (Feio and Ferrreira, 2019). They are extensive, dynamic areas, comprising material interchanges between land, water and atmosphere, all collectively contributing to the ecosystems processes functioning (Graça et al., 2015). These ecosystems are open, meaning their efficient functioning relies mostly on energy inputs from allochthonous sources (Fisher and Likens, 1973; Allan and Castillo, 2007). Said inputs may occur as solar radiation or organic matter (OM), either brought to the stream via the atmosphere (meteorological input), stream flow (geologic input) or *in situ* primary production (biologic input) (Fisher and Likens, 1973; Graça and Canhoto, 2006).

In forested streams, tree canopies provide shade to the stream channels, hindering primary producer's subsistence, as they require light to produce native OM (Hill et al., 1995). This way, the energy fixed by photosynthesis seems to be collectively exceeded by allochthonous sources of nonliving organic carbon (C) such as animal waste products, fragments of organic material and several organic compounds, hence the often attributed nomenclature of detritus-based fluvial food webs (Graça and Canhoto, 2006; Allan and Castillo, 2007; Feio and Ferrreira, 2019). These streams are known to retain large volumes of leaf litter shed from vegetation, which may include branches and twigs of wood, bark, leaves, leaf fragments and other plant parts (Benfield, 1997; Graça et al., 2005). Even though litter constitution varies with climate, location and vegetation type, leaves are usually its biggest component (Abelho, 2001). As leaf-litter-provided allochthonous OM reaches the stream channels, it becomes the dominant energy and carbon source supporting low order headwater streams (Mninishall, 1967; Kaushik and Hynes, 1971; Graça and Canhoto, 2006). These new substrates implement a food web based on its decomposition, thus enabling physical and chemical transformations, as well as recycling of chemical elements at an ecosystem's scale (Benfield, 1997; Berg and Laskowski, 2005; Graça et al., 2015). Leaf litter breakdown in streams generally involves three major subprocesses: leaching, conditioning/colonization and fragmentation. Even though these are generally categorized and analysed separately, they aren't confined to a specific time period; instead, there's an interplay between them, as they can (and usually do) occur at the same time (Gessner et al., 1999). Leaching is characterized by the abiotic removal of soluble substances (Graça et al., 2005). This subprocess is usually fast and induces substantial mass loss. Shortly after leaf litter's immersion in a stream, the action of water leads to

the transformation of coarse particulate organic matter (CPOM) into dissolved organic matter (DOM), causing losses of mostly soluble carbohydrates and polyphenols (Suberkropp et al., 1976; Gessner et al., 1999; Abelho, 2001). Conditioning consists in the colonization and establishment of invertebrates and microbial decomposers (bacteria and fungi), which are able to directly enhance the breakdown process through leaf litter transformation (by enzymatic maceration) into biomass, fine particulate organic matter (FPOM), DOM and nutrients (Gessner et al., 1999; Allan and Castillo, 2007). These microbial communities are also capable of increasing detritus palatability to invertebrate shredders (through the build-up of microbial biomass) (Bärlocher, 1992; Abelho, 2001; Chung and Suberkropp, 2009).

Carbon and nutrients initially present in litter undergo several processes, the last of which known as microbial decomposer mineralization, consisting in the conversion of organic molecules to inorganic carbon dioxide (CO₂) and ammonium (NH₄⁺) (Marks, 2019). Microbial decomposers are crucial in C and nutrient cycling in headwater streams, growing at the expense of OM through the breakdown of plant polysaccharides (and other recalcitrant compounds) and processing, transforming (through mineralization) and storing (through immobilization) C and nutrient products (Suberkropp and Klug, 1980; Cadish and Giller, 1997; Feio and Ferreira, 2019). Many studies, comprising a wide range of streams, point out fungi (aquatic hyphomycetes (AQH) in particular) as the prime microbial decomposers, dominating the early stages of decomposition (Baldy et al., 1995; Hieber and Gessner, 2002; Pascoal and Cássio, 2004). However, bacteria seem to complement fungi in advanced stages of this process, becoming more important to breakdown as the size of the particles gets smaller (Baldy et al., 1995; Gessner, 1997; Abelho, 2001).

Fragmentation can be differentiated depending on the type of driving forces that induce this subprocess. Abiotic fragmentation occurs as a result of physical abrasion and shear stress induced by the flowing water (Gessner et al., 1999). Biotic fragmentation, on the other hand, comprises the conversion (and subsequent release) of leaf detritus to particulate OM, accomplished through fungal mycelial growth and enzymatic activities, as well as by invertebrate shredding (Gessner et al., 1999; Graça, 2001). Shredders also feed on leaf litter, but tend to prefer leaves colonized by fungi; usually softer (due to fungal enzymatic activities) and with increased nitrogen content (due to fungal biomass) (Cummins, 1974; Suberkropp et al., 1983; Graça et al., 1993). These organisms are able to convert CPOM into FPOM, consequently providing food to higher trophic levels (Graça and Canhoto, 2006; Graça, 2001). Assuredly, leaf litter constitutes a heterotrophic

food base, with its decomposition being a necessity for consumers' survival and growth (Cummins, 1974; Webster and Meyer, 1997; Feio and Ferreira, 2019).

Every decomposition stage is affected by a massive number of factors, whether large-scale or intermediate. There are many large-scale factors, such as geology and eutrophication (Graça et al., 2015), but climate is undoubtedly one of the most prominent. The ongoing surface temperature rise and the recurrence and intensification of heat strikes and extreme events, such as droughts or intense rainfalls constitute serious threats to biota and, therefore, ecosystem functioning at a global scale (Pereira et al., 2010; Bellard et al., 2012; IPCC, 2014). Temperature has a fundamental role in regulating biological processes, especially in low order freshwater ecosystems; particularly vulnerable to its effects due to the close relationship between air temperature and water temperature (Carpenter et al., 1992; Dudgeon et al., 2006; Woodward et al., 2010). Temperature variations shape directly and indirectly most aspects regarding leaf litter decomposition (Gonçalves et al., 2013). In addition, the type and quality of litter inputs, along with water chemistry, are considered very relevant factors (Cornwell et al., 2008; Ferreira and Chauvet, 2011). Regarding the former, leaf litter composition is known to vary immensely between ecosystems. Some leaves are very nutrient-rich and/or have high concentrations of labile carbon (e.g. alder leaves). This type of substrate requires little effort to degrade, hence being typically preferred by microbial decomposers and detritivores (Cornwell et al., 2008; Gessner et al., 2010). Other leaves (e.g. oak leaves) have very low nutrient concentrations and/or elevated levels of recalcitrant carbon, which is highly resistant to degradation (Cornwell et al., 2008; Gessner et al., 2010). Temperature rises have the potential to induce modifications in vegetation's dominance and composition, which can subsequently affect freshwater ecosystems community structures and functioning (Woodward et al., 2010; Fernandes et al., 2013).

Water quality depends on aerial and terrestrial inputs and the biogeochemical ways by which they are processed (Murdoch et al., 2000). Water and flow-related factors, namely temperature, pH, alkalinity, nutrient concentration, conductivity, current velocity and turbulence are of great influence in all stages of leaf litter decomposition (Jenkins and Suberkropp, 1995; Suberkropp and Chauvet, 1995; Abelho, 2001). Events like seasonal floods can elevate the streams water levels, boosting the leaching of organic compounds (Coûteaux et al., 1995; Gessner et al., 2010). Global-change-induced stream water temperature rises can lead to higher evapotranspiration rates in low-order streams, potentially intensifying pollutants and nutrient concentrations in these ecosystems (Murdoch et al., 2000). Anthropogenic changes (e.g. heavy metal pollution) share this potential to

increase nutrient and pollutant concentrations (Jørgensen et al., 2013). Modifications in precipitation and seasonality patterns, despite only indirectly related with global alterations, are also able to influence leaf litter breakdown, microbial diversity and, subsequently, ecosystem functioning (Murdoch et al., 2000; Pascoal et al., 2005c). In short, local resources and climate appear to be a strong combined driving force of leaf litter breakdown (Woodward et al., 2010).

1.1.2. Microorganisms involved in litter decomposition

Conditioning, as previously mentioned, comprises colonization, settlement and proliferation of a wide variety of microorganisms on the leaf matrix, simultaneously transforming leaves into FPOM through microbial activities and providing nutrition for invertebrates (Gessner et al., 1999; Abelho, 2001; Feio and Ferreira, 2019). This is a fundamental step in the leaf litter breakdown process and a central part of many stream food webs, as microbial decomposition greatly contributes to nutrient cycling and mineralization in these ecosystems (Elwood et al., 1981; Sinsabaugh and Findlay, 1995; Webster et al., 2009). Microbes enter the water alongside leaf litter or rapidly colonize leaf substrates after stream immersion (either by contact with previous colonized material or by establishment of microbial cells, carried by the water flow), being the first organisms in the ecosystem to have access to energy and nutrient sources in these substrates (Suberkropp and Klug, 1980; Dang et al., 2007). The microbial community in decomposing plant litter is comprised by a diversity of fungi, bacteria and protists, each with different biochemical and physiological abilities, hence distinct roles and influences in the breakdown process (Suberkropp, 1998; Das et al., 2007; Marks, 2019). Protists, the smallest microbial consumers, mostly feed on bacteria, which is typically abundant in decomposing litter (Finlay and Esteban, 1998; Ribblett et al., 2005). In several studies, protists were found to be top-down controls of bacterial abundance and carbon flow, meaning their predation on bacteria affects bacterial biomass accumulation, benefiting, in terms of energy transfer, higher trophic levels (Berninger et al., 1991; Carlough and Meyer, 1991; Finlay and Esteban, 1998). Even though colonization occurs due to bacteria and fungi, protists might play a (still not very well understood) role in decomposition rates in running waters (Suberkropp, 1998; Ribblett et al., 2005). Leaves' submersion in streams is commonly followed by biofilm formation. Biofilms are complexes of auto and heterotrophic microorganisms and OM attached to a substrate (Findlay and Arsuffi, 1989; Lock, 1993; Battin et al., 2007). Bacteria can be found in all stream habitats, including leaf litter (Suberkropp et al., 1976; Findlay

and Arsuffi, 1989; Baldy et al., 1995) and biofilms are believed to have high densities of bacteria (Geesey et al., 1978). Certain bacterial taxa, such as Actinomycetes (gram-positive) have been found in decomposing plant litter and seem to significantly contribute to OM processing (Srinivasan et al., 1991; Wohl and McArthur, 1998). Before leaves are shed, they are usually colonized by an array of terrestrial fungi, thus entering streams already with a fungal community (Kaushik and Hynes, 1971; Bärlocher and Kendrick, 1974; Suberkropp, 1998). Once submerged, leaves are rapidly colonized by stream fungi belonging to an ecological group known as aquatic hyphomycetes (or Ingoldian fungi) (Suberkropp, 1998). Representatives of almost all major groups of fungi, either yeasts, terrestrial (transient from terrestrial ecosystems) or Ingoldian exist in submerged leaf litter and are somewhat related to leaf litter decomposition (Graça and Ferreira, 1995; Sampaio et al., 2007; Seena et al., 2008). Due to fundamental differences between fungal and bacterial life forms these should be expected to have distinct functions and impacts in litter decomposition (Baldy et al., 1995). Even though they coexist in colonized litter as a microbial assemblage, most studies point out that fungal decomposers, rather than bacteria, are the most relevant to this process, as they are more abundant and have much higher biomass and productivity/degradability rates (Gulis and Suberkropp, 2003a; Pascoal et al., 2005a; Krauss et al., 2011). Amongst all types of fungal decomposers, AQH are considered the most important for leaf litter breakdown in streams, dominating the community (Bärlocher, 1992; Suberkropp, 1998; Gessner et al., 2007). AQH produce extracellular enzymes able to degrade complex plant fibre polysaccharides, like celluloses, hemicelluloses, pectin and lignin into smaller molecules that they can assimilate, as well as enzymes that allow nitrogen and phosphorus acquisition (Chamier, 1985). AQH dominate throughout decomposition processes, whilst bacterial decomposers tend to be less at the beginning of the process, but gradually increase after fungal breakdown commences (Suberkropp et al., 1976; Gessner and Chauvet, 1994; Marks, 2019). Fungal decomposers seem to produce a broader range of extracellular enzymes than bacteria, whose prime role is to degrade simpler polymeric compounds (FPOM and DOM) after highly complex polymer decomposition by fungi (Kirk and Farrell, 1987; Romani et al., 2006). Bacterial colonization seems to be facilitated due to AQH enzymatic degradation of recalcitrant compounds, causing leaves' breakdown into smaller particles with higher surface areas (Fenchel, 1970; Romani et al., 2006) and by allowing, through mycelial penetration into leaf tissues, bacteria to reach otherwise inaccessible compounds (Gulis and Suberkropp, 2003b; De Boer et al., 2005; Kohlmeier et al., 2005). AQH have established their place as the most prominent microbial colonizers of submerged leaf litter and the main agents of

OM and nutrient recycling in headwater stream ecosystems (Pascoal and Cássio, 2004). Assessments on their degradative potential and enzymatic functionalities are essential to better understand breakdown processes of plant litter complex compounds and freshwater ecosystems functioning (Chamier, 1985; Chauvet and Suberkropp, 1998; Pascoal and Cássio, 2004).

1.2. Enzymes involved in leaf decomposition

Leaves contain great amounts of energy in the form of carbon and are relevant sources of nutrients such as nitrogen and phosphorus, supporting multiple trophic levels (Wallace et al., 1997; Ferreira et al., 2012). Despite the great physical and chemical differences in leaves' composition, there are structural components common to all, whose role is furnishing the support, strength and protection necessary for plant cell survival, termed plant cell walls (Ochoa-Villarreal et al., 2012). Plant cell walls are constituted by three major layers: the primary wall, the middle lamella and the secondary wall, which structurally and chemically differ from one another (Harris and Stone, 2009; Ochoa-Villarreal et al., 2012). The primary cell wall is generally thin and consists mainly of proteins and polysaccharides such as cellulose microfibrils, hemicellulose and pectin (Bacic et al., 1988). The middle lamella is particularly rich in pectic polysaccharides and, in most cases, lignified (Hall, 1976; Harris and Stone, 2009). The secondary wall consists of cellulose, hemicelluloses, noncellulosic substances and, usually, lignin (Chamier, 1985). Whole plant litter degradation entails combined actions from different microbial decomposers, mostly bacteria and fungi. These organisms play a major role in plant litter decomposition by producing and secreting extracellular degradative enzymes that: (i) break down structurally complex compounds, such as the aforementioned plant cell wall compounds, into smaller assimilable molecules and (ii) mineralise non-carbon nutrients, such as nitrogen and phosphorus (Chróst, 1991; Sinsabaugh et al., 2002).

Cellulases

Cellulose is the major component of the plant cell wall, comprising a structure of three dimensional microfibrils (Rose, 2003; Harris and Stone, 2009). Due to fibre size, most of the cellulose degradation process happens outside the microbial cell. Cellulose is usually arranged in one of two ways: a crystalline form, hard to degrade due to its high level of structural organization and an amorphous form, which is soluble, therefore relatively easier to degrade (Eriksson et al., 1990;

Berg and Mcclaugherty, 2003; Graça et al., 2005). As an inherently insoluble and structurally complex compound, crystalline cellulose is only completely broken down by the combined action of a synergistic enzyme system, comprised by endoglucanases (EC 3.2.1.4), exoglucanases, also termed as cellobiohydrolases (CBHI (EC 3.2.1.176) and CBHII (EC 3.2.1.91)), and β -glucosidases (EC 3.2.1.21) (Chamier, 1985; Mansfield et al., 1999; Lynd et al., 2002).

Hemicellulases

The plant cell wall possesses structurally independent but intertwined biopolymer networks, one of those involving a variety of polysaccharides that coat cellulose microfibrils, termed hemicelluloses (Rose, 2003; Kumar et al., 2008). These polymers are able to hydrogen-bond themselves, therefore becoming less water soluble, which helps stabilizing the cell wall and protecting it against cellulose degradation (Linden et al., 1994; Kumar et al., 2008). Hemicelluloses are a heterogeneous group of polysaccharides composed of both linear and branched heteropolymers, with a varying backbone of 1,4-linked β -D-pyranosyl residues, which can be constituted by xylans, xyloglucans, glucans, mannans, glucomannans, galactans and arabinans (Chamier, 1985; Ochoa-Villarreal et al., 2012; Rytioja et al., 2014). They are usually named after the dominant sugar (e.g. xylan, mannan) or by the most abundant sugars, in case they are present in approximate amounts (e.g. xyloglucan, arabinogalactan) (Bastawde, 1992; Rose, 2003; Gamauf et al., 2006). Hemicelluloses are very chemically diverse and structurally complex, therefore requiring an extensive set of specific hydrolysing enzymes (Eriksson et al., 1990; Gamauf et al., 2006; Harris and Stone, 2009). As elements of the many existing heterogeneous hemicelluloses, xylans are found in the cell walls of almost all plant parts and are the most studied polymers in terms of enzymatic degradation (Whistler and Richards, 1970; Gamauf et al., 2006). Due to their complexity, degradation of xylans requires the co-operative action of a variety of enzymes, termed (as a group) xylanases (Puls and Poutanen, 1989; Linden et al., 1994). The enzymatic system of xylanases is based on two core enzymes, endoxylanases and β -xylosidases (main chain depolymerisers) and other accessory enzymes (responsible for the release of substituents) (Linden et al., 1994; Wood and Wilson, 1995; Gamauf et al., 2006). These seem to be the key components of xylanolytic systems produced by microbial decomposers (Dekker and Richards, 1976; Reilly, 1981; Dekker, 1985). Accessory enzymes are also relevant components of this enzymatic complex, as they are able to cleave substituents, therefore facilitating the action of the core enzymes and contributing to total xylan

mineralisation (Linden et al., 1994; Faulds et al., 1995). This enzymatic subgroup includes enzymes such as α -L-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterases (AXE) (EC 3.1.1.72) and α -D-glucuronidases (EC 3.2.1.139) (Linden et al., 1994; Manin et al., 1994; Wood and Wilson, 1995; Puls, 1997).

Pectinases

Pectins are complex polysaccharides that provide additional cross-links between the cellulose and hemicellulose polymers (Rytioja et al., 2014). They are present in both the primary cell wall and the middle lamellae, granting structural integrity and cohesion (Alkorta et al., 1998; Seymour and Knox, 2002; Pedrolli et al., 2009). These biopolymers are the most readily available for decomposers in non-lignified plant tissues, and their degradation exposes other polysaccharides such as xylans, mannans and cellulose to microbial enzymatic attacks (Darvill et al., 1980; Chamier and Dixon, 1982). Pectins are branched heteropolysaccharides, mainly composed by galacturonic acid units, present as covalently-linked structural domains such as homogalacturonan, xylogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II, with varying degrees of esterification and polymerization (Rexová-Benková and Markovič, 1976; Mohnen, 2008; Chaudhri and Suneetha, 2012). Due to pectin's structural and chemical complexity, its degradation entails a vast group of specific enzymes, responsible for degrading this compound through different combined activities, highly dependent on the substrate's degrees of polymerization (Fogarty and Kelly, 1990; Schols et al., 2009; Lara-Márquez et al., 2011). Based on the mechanism used to attack the galacturonan main chain, this enzymatic group can be further divided into de-esterifying enzymes, depolymerizing enzymes and protopectinases (Gummadi and Panda, 2003; Pedrolli et al., 2009; Chaudhri and Suneetha, 2012). De-esterifying enzymes, commonly called esterases, comprises enzymes such as acetyl esterases (EC 3.1.1.6) and pectin methyl esterases (EC 3.1.1.11) (Pedrolli et al., 2009; Schols et al., 2009; Lara-Márquez et al., 2011). The activities of all these esterases are crucial for the whole degradation of pectin, as they promote the action of depolymerising enzymes (de Vries et al., 2000; de Vries and Visser, 2001; Kashyap et al., 2001) like hydrolases or lyases (Rexová-Benková and Markovič, 1976; Alkorta et al., 1998). The most well-known hydrolases are polygalacturonases (Jayani et al., 2005; Latarullo et al., 2016; Amin et al., 2019) which can be classified as endo- (EC 3.2.1.15) and exopolygalacturonases (EC 3.2.1.67)

(Rexová-Benková and Markovič, 1976; Latarullo et al., 2016). Lyases can be further categorized as pectate- and pectin lyases (Sakai et al., 1993; Jayani et al., 2005; Yadav et al., 2009).

Ligninases

Another biopolymer comprising plant cell walls is lignin, an amorphous and heterogeneous polysaccharide intimately associated with cellulose microfibrils that provides the cell wall with structural support, as well as with protection against chemical and biological attacks (e.g. by microorganisms) (Lu et al., 2017). Lignin is a complex, highly branched polymer with a three-dimensional structure constituted by three standard phenylpropane monomers linked by carbon and ether bonds, taking part in cross-links to other polymers (polysaccharides and proteins) (Moore et al., 2011; Zhou et al., 2013). The linkages between these units are not susceptible to hydrolytic attack, categorizing lignin as highly resistant to degradation, i.e. highly recalcitrant (Bugg et al., 2011). This recalcitrant nature can, depending on the proportion of lignin in the leaf litter, hamper the accessibility to cellulose microfibrils as well as other carbon compounds, generally easier to degrade (Gessner and Chauvet, 1994; Manavalan et al., 2015). Despite this, lignin can still be degraded (through carbon and ether bond cleavage) into low molecular weight aromatics by extracellular oxidative enzymes produced by microbial decomposers (Moore et al., 2011; Wang et al., 2017). Ligninolytic enzymes can be categorized as lignin-modifying enzymes or lignin-degrading auxiliary enzymes (Janusz et al., 2017). These are oxidative enzymes, further classified as phenol oxidases (including laccases, catechol oxidases and tyrosinases) or heme peroxidases (comprising lignin-, manganese-, and versatile peroxidases) (Mayer, 1987; Falade et al., 2016; Janusz et al., 2017).

Macronutrients

Also amongst the processes emerging at the ecosystem level are the cycling of nutrients such as nitrogen (N), phosphorus (P), potassium, calcium, sulphur and magnesium, which can be majorly influenced by several factors, such as geology, climate, vegetation, water chemistry and anthropogenic pressures (Carpenter et al., 1998; Allan and Castillo, 2007; Gessner et al., 2010). In aquatic systems, the demand of nutrients, in particular N and P, is much greater than their supply, thus limiting the biological activity/productivity of the ecosystem (Allan and Castillo, 2007). This is especially true for heterotrophic microbes, the trophic level performing nutrient

incorporation into organic form (Allan and Castillo, 2007). These organisms are able to either accumulate nutrients or transform them (through enzymatic degradation of complex molecules in decaying litter) into soluble inorganic forms, assimilable by other trophic levels (DeAngelis, 1992; Burns and Dick, 2002; Sigee, 2004). These nutrient transfers between distinct trophic levels, termed nutrient cycling, entail a sequence of alterations in the nutrients' chemical states, ending with their release into the water column in their soluble inorganic (bioavailable) form (Sigee, 2004). Microorganisms have lower C:N and C:P ratios than leaf litter and nutrient uptake and assimilation helps them resolve this mismatch (Cross et al., 2003; Marks, 2019). Many studies report that N and P-rich substrates degrade faster (Howarth and Fisher, 1976; Carpenter and Adams, 1979; Cornwell et al., 2008) and that N and P enrichment also stimulates decomposition rates (Triska and Sedell, 1976; Meyer and Johnson, 1983; Ferreira et al., 2014) by increasing microbial decomposers activity (Cheever et al., 2013; Pastor et al., 2014).

Nitrogen

In freshwater ecosystems, nitrogen can be naturally acquired through soils, geology, climate and vegetation, as it is present in membranes, nucleic acids and proteins of newly shed plant litter and occurs in different chemical states, such as ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) (Carpenter et al., 1998; Allan and Castillo, 2007). There are also some anthropogenic inputs elevating nitrogen concentration and availability in streams, such as fertilizers, sewages and atmospheric depositions (Berg and McLaugherty, 2003; Allan and Castillo, 2007). Nitrogen cycling comprises N-attaining and energy-attaining pathways, called assimilatory and dissimilatory uptakes, respectively (Allan and Castillo, 2007). Assimilatory pathways consist of fixation and assimilation of dissolved inorganic nitrogen (NH_4^+ , NO_3^- and NO_2^-) and are performed by both auto- and heterotrophs (Allan and Castillo, 2007). Microbial decomposers are major drivers of nitrate assimilation (through enzymatic degradation of complex molecules) during litter decomposition, using nitrate as their favoured N source, as it often limits their growth (Melillo et al., 1984; Crawford and Glass, 1998; Daniel-Vedele et al., 1998).

Uptake of N compounds depends on the existence of nitrate transporters, which are crucial for the efficiency of this process, as they actively import the molecules to the interior of the cells, setting the assimilatory pathway in motion (Galván and Fernández, 2001; Siverio, 2002). This energy-demanding mechanism comprises two enzymes: nitrate reductase and nitrite reductase. Nitrate

reductase is responsible for initiating the metabolic process through the conversion of nitrate to nitrite, being compulsory for cellular nitrate accumulation in nitrate-assimilating fungi (Unkles et al., 2004; Marcos et al., 2016). Nitrite reductase mediates the reduction of nitrite to ammonium (Unkles et al., 2004; Marcos et al., 2016). Nitrate assimilation regulation in terrestrial fungi is currently well understood and appears to be quite straightforward, as nitrate uptake is repressed by high levels of more appealing nitrogen sources, such as ammonia, and stimulated by low intracellular levels of these preferred sources and by the presence of nitrate or nitrite (Marzluf, 1997; Siverio, 2002; Koon et al., 2008).

Phosphorus

Phosphorus is a crucial nutrient in all freshwater organisms, compulsory in structural molecules (in phospholipids and nucleic acids), storing compounds (mostly polyphosphates) as well as energy-conversion processes (e.g. ATP) (Sigg, 2004). P in streams have several sources, like sewage treatment and soluble inorganic fertilisers from anthropogenic activities, rock weathering, atmospheric inputs and vegetation (Carpenter et al., 1998; Mainstone and Parr, 2002; Manzoni et al., 2010). Due to the multitude of phosphorus sources, P pools in streams comprise several forms and chemical states. The main constituents are dissolved organic phosphorus compounds (mostly phosphate esters), which aren't readily available for microorganisms to take up directly through their cell membranes (Overbeck and Chróst, 1994; Burns and Dick, 2002; Sigg, 2004). Decomposers have the ability to not only uptake soluble phosphorylated P monosaccharides (assimilable P) but also to dephosphorylate dissolved organic P compounds through production and secretion of extracellular hydrolytic enzymes whose activity converts said compounds into bioavailable phosphate, released into the surrounding environment in a process called mineralization (Burns and Dick, 2002; Sigg, 2004; Allan and Castillo, 2007). These processes are extremely important in streams, as they actively increase the substrate pool of assimilable P in freshwater environments (Peters and Lean, 1973; Burns and Dick, 2002).

Assimilable P release occurs due to the action of three groups of extracellular hydrolytic enzymes: (i) nonspecific and partially specific phosphoesterases (such as mono- and diesterases), (ii) nucleotidases (predominantly 5'-nucleotidase) and (iii) endo- and exonucleases (Overbeck and Chróst, 1994; Burns and Dick, 2002). The first group of enzymes comprises phosphatases, which are the most extensively studied out of the three groups (Burns and Dick, 2002). These enzymes

catalyse the hydrolysis of phosphate esters, degrading complex P compounds into biologically available orthophosphate (Jansson et al., 1988). Phosphatases reach maximum hydrolysing capacities at different pH values and are therefore commonly divided into alkaline phosphatases (highest activity between pH 7.6–9.6) and acid phosphatases (pH optimum below pH 7, normally between 4 and 6) (Jansson et al., 1988; Burns and Dick, 2002).

1.3. Aquatic hyphomycetes

Aquatic hyphomycetes (also known as freshwater hyphomycetes, amphibious fungi or Ingoldian fungi) are a polyphyletic group of fungi with the ability to grow and colonize decaying litter in rapidly flowing, well-aerated streams and rivers, where they are most abundant and play an essential role in OM turnover (Bärlocher, 1992; Gessner et al., 2007). Nevertheless, they have also been found in stagnant waters, as well as in some terrestrial habitats (Fisher et al., 1991; Bärlocher, 1992).

Despite the fact that some AQHs were previously described by Hartig (Hartig, 1880), Saccardo (Saccardo, 1880) (from terrestrial habitats) and De Wildeman (De Wildeman, 1893, 1894, 1895) (from ponds), this group of fungi only became renowned much later, when Ingold (Ingold, 1942) identified decaying leaves in streams as their main habitat. While looking for chytrids in foam formed in an alder-lined stream behind his house, Ingold found a large collection of fungal spores, which he was able to connect later to mycelia growing and sporulating in submerged leaves (Ingold, 1942; Bärlocher, 1992). The conidiophores were found to be typically submerged, releasing the asexual spores below water (Ingold, 1942). These spores have two very different basic shapes (tetradiradial and sigmoid) depending on the AQH genera, hinting that these fungi could have originated through a process of parallel evolution (Ingold, 1942). Besides, conidia shapes suggest adaptations to aquatic environments, allowing conidia to act as anchors, efficiently attaching to litter surfaces as well as easing their dispersal in flowing waters (Webster, 1975; Chauvet and Suberkropp, 1998; Descals, 2005a). AQH's adhesion ability, combining with the following characteristics: (i) quick colonization, with production and attachment of a large number of underwater spores in a short amount of time, (ii) capacity to survive, grow and sporulate at very low temperatures, and (iii) production a wide range of extracellular enzymes essential to leaf litter breakdown, are the skill set that makes this group of fungi extremely successful and dominant on

decaying leaves in stream environments (Zemek et al., 1985; Suberkropp, 1998; Gessner et al., 2007). Throughout time, global distribution of AQH has become evident and all sorts of ecological, taxonomic and molecular studies have been developed, providing more information on these organisms' phylogeny, diversity, morphology, physiology and enzymatic abilities, even though there is still a lot unanswered, particularly regarding their genetics (Krauss et al., 2011; Duarte et al., 2016).

1.3.1. Evolution and Biogeography

AQH belong to the kingdom Fungi, subkingdom Dikarya (Hibbett et al., 2007). Conidial fungi with none or few known sexual states are commonly termed Fungi Imperfecti and belong to the Deuteromycetes, a subdivision that comprises three form-classes: Blastomycetes, Coelomycetes and Hyphomycetes (Cole, 1986; Webster and Weber, 2007). Form-class is a type of classification based on morphological resemblances, rather than phylogenetic closeness, between taxa (Cole, 1986).

The kingdom Fungi is estimated (through 18S rDNA based molecular clock techniques) to have emerged around 800 million years ago, with the separation of ascomycetes and basidiomycetes occurring 200 million years later (Berbee and Taylor, 1993). Freshwater taxa origin is believed to have happened approximately 390 million years ago, before Pangaea's split up into several continents (Vijaykrishna et al., 2006; Barlocher, 2010). Even though this separation might have contributed to some extent to AQH dispersal, the presence of similar freshwater communities on recent geological formations, such as the Hawaiian islands, highlights the existence of other dispersal mechanisms (Eldredge and Miller, 1995; Hyde and Goh, 2003; Vijaykrishna et al., 2006). These include: (i) possible endophytic, pathogenic or saprobic interactions with plants followed by collective entrance in aquatic environments, (ii) transportation by litter, rainwater or sediments and (iii) global environmental changes (Bromham and Penny, 2003; Vijaykrishna et al., 2006). Phylogenetically speaking, the majority of AQH are closely related to Ascomycota, with a small minority belonging to Basidiomycota (Ingold, 1959; Descals, 2005b; Shearer et al., 2007). Through assessment of anamorph-teleomorph connections and molecular analyses of several ribosomal DNA (rDNA) genes, a considerable number of ascomycetous AQH have been placed in the subphylum Pezizomycotina in five different classes: Leotiomyces (predominantly),

Orbiliomycetes, Dothideomycetes, Sordariomycetes and Pezizomycetes (Hibbett et al., 2007; Baschien et al., 2013). The basidiomycetous AQH have been placed in two classes: Urediniomycetes and Hymenomycetes (Hibbett et al., 2007; Marvanová, 2007). Phylogenetic data for taxa comprised in this form-class clarify that AQH have several origins, evolving independently through multiple lineages (Shearer, 1993; Vijaykrishna et al., 2006). AQH are believed to share a common ancestor with terrestrial ascomycetes, having evolved and adapted from terrestrial environments to an aquatic lifestyle (Vijaykrishna et al., 2006). Lignicolous fungi lack many survival-necessary adaptations to freshwater ecosystems that AQH possess, such as rapid spore germination at low temperatures, quick dispersal rates, a spore morphology that potentiates efficient attachment to suitable substrates and the ability to degrade submerged substrates, thereby evincing the evolutionary process of convergent evolution they have gone through (Bärlocher, 1992; Dang et al., 2007; Krauss et al., 2011).

Initially, AQH geographical distribution was believed to follow the hypothesis of Baas Becking, which suggested that, due to their size and dispersal capabilities, microbial organisms would have cosmopolitan distribution, being found at suitable environments as “everything is everywhere, but the environment selects” (Baas Becking, 1934). However, posterior studies found that AQH do have a biogeography and most species do not occur everywhere (Jabiol et al., 2013; Duarte et al., 2016). In fact, these fungi seem to follow a biogeographical model of moderate to pronounced endemism, meaning they exhibit a few distinct geographical distribution tendencies (Foissner, 1999; Duarte et al., 2016). Many species are cosmopolitan, with a worldwide distribution, some appear to be restricted to certain climatic regions and others seem to only exist in small geographic areas (Wood-Eggenschwiler and Bärlocher, 1985; Shearer et al., 2007; Jabiol et al., 2013). Several studies argued that AQH occurrence isn't constricted by geological barriers or distance, as similarities in community composition have been found between geographically distant regions with equivalent climatic zones (Wood-Eggenschwiler and Bärlocher, 1985; Duarte et al., 2016). In fact, temperature and its effect on vegetation are considered main drivers of diversity patterns worldwide and the primary predictive factor of AQH geographical distribution (Wood-Eggenschwiler and Bärlocher, 1985; Seena and Monroy, 2016; Seena et al., 2019).

The most widely documented macroecological pattern is based on the increase in species richness from higher (polar regions) to lower latitudes (equatorial regions) (Peay et al., 2016). AQH species richness seems to diverge from this pattern, exhibiting a hump-shaped relationship with latitude, as it declines towards the equator, being its lowest at latitudinal extremes and peaking at mid-

latitudes (temperate regions) (Jabiol et al., 2013; Duarte et al., 2016; Seena et al., 2019). This greater diversity in temperate zones is probably due to a wider diversification of ecological niches, whilst the lower diversity at tropical streams might be attributed to streams' lower turbulence and nutrient concentrations (Shearer et al., 2007; Jabiol et al., 2013; Graça et al., 2016). Besides latitude, altitude also appears to have an influence on AQH community structure (Chauvet, 1991; Hyde et al., 2016). Studies have found AQH communities' distribution to slightly peak at mid-elevations and decline at higher altitudes (e.g. Shearer et al., 2015).

1.3.2. Taxonomy and identification

A substantial part of the knowledge on AQH diversity in freshwater ecosystems was obtained using traditional methods of identification based on morphology and development of conidia (asexually produced spores) (e.g. Gessner et al., 2003; Bärlocher, 2005a; Gulis et al., 2005). Typically, detached conidia present in the water column or in naturally submerged substrata (i.e., leaves or twigs) are collected from freshwater ecosystems and then identified by microscopy (Descals, 2005b). Owing to their characteristic conidial morphology and conformation, identification of detached anamorphic spores in environmental samples at the genus level is reliable (Gulis et al., 2005; Letourneau et al., 2010). Despite this, there are cases where species-level identifications may not be accurate due to a high level of similarities in conidia shape and size between distinct species (Letourneau et al., 2010). Besides, this kind of identification depends on several factors, such as: (I) conidial production ability at a given time, as some mycelia present on leaves might not release conidia under laboratory conditions and (II) trained taxonomists, whose expertise are often needed due to analogous morphology of conidia from different species (e.g. the genus *Tetracladium*, containing seven species with overlapping conidial morphologies (Roldán et al., 1989)). More recently, efforts have been made to develop and improve molecular techniques of fungal identification, through comparison of nucleotide sequences' selected genes (Nikolcheva and Bärlocher, 2004; Letourneau et al., 2010). These methods enable a more precise identification and can be used regardless of fungal life stage and ability to sporulate, therefore bypassing most of the misidentification problems of the traditional approach (Seena et al., 2010; Krauss et al., 2011).

Since their emergence, molecular techniques have been a powerful tool to increase and deepen our knowledge about AQH diversity (Seena et al., 2010; Duarte et al., 2015). The importance of

DNA sequencing in modern mycology is exemplified by the concept of fungal barcoding (Seena et al., 2010; Krauss et al., 2011). Molecular barcodes are short (a few 100 bp), standardized DNA sequences from a particular region of the genome that is individual for each species (Hebert et al., 2003). Whilst coding gene regions, because of their highly conserved nature, appear to be ideal for analysis of higher taxonomic levels (e.g. genera), the internal transcribed spacer (ITS) region of the rDNA, being highly variable, enables lower-level analysis (e.g. species) and was recommended as the most adequate barcode for fungal species identification (Schoch et al., 2012). With a characteristic morphology and a relatively low diversity (over 300 species), the process of AQH barcode libraries generation seems to be somewhat facilitated (Baschien et al., 2013; Duarte et al., 2014). Despite this, there is still a relatively low number of ITS sequences available in genomic databases and the alignment of attained ITS sequences against annotated reference specimen is not always possible (Seena et al., 2010; Duarte et al., 2013b). A combination of molecular and traditional identification methods, allowing the intersection of phenotypic characters analysis with nucleotide sequences comparisons is, therefore, very advantageous to unambiguously determine AQH taxonomy.

A few studies about anamorph-teleomorph connections using DNA sequencing and molecular markers have been connecting anamorph genus to their monophyletic or polyphyletic origins (Baschien et al., 2006; Campbell et al., 2006; Seena et al., 2010). *Tetracladium* anamorph genus appears to be monophyletic (Roldán et al., 1989; Nikolcheva and Bärlocher, 2002). *Tricladium* is the largest anamorph genus in AQH, normally described as a form genus, because of its heterogeneous morphology (Campbell et al., 2009). *Tricladium* anamorphs are clearly polyphyletic, with three of them being already linked to their teleomorphic states: *T. splendens* to *Hymenoscyphus splendens* (Helotiaceae), *T. chaetocladium* to *Hydrocina chaetocladia* (Hyaloscyphaceae) and *Tricladium indicum* to *Cudoniella indica* (Helotiaceae) (Bärlocher, 1992; Sivichai and Jones, 2003; Campbell et al., 2009). *Anguillospora* anamorph genus is also heterogeneous and polyphyletic, with seven connected teleomorphs in four distinct ascomycete orders (Sivichai and Jones, 2003). Only about 10% of described species have been connected to a teleomorph, therefore, there is still much to find out regarding AQH teleomorph-anamorph connections (Bärlocher, 2009).

1.3.3. Fungal biodiversity and ecosystem functioning

At the early stages of decomposition, leaves are colonized by several AQH species, differing in growth rates, life strategies and sporulation abilities (Shearer and Lane, 1983; Laitung et al., 2004). In general, fungal diversity appears to be higher during early breakdown stages comparing to later ones (Barlocher and Corkum, 2003; Treton et al., 2004; Duarte et al., 2006; Pascoal et al., 2010). This might be due to interactions between mycelia and inability of establishment of some spores in the given substrate (Nikolcheva et al., 2003; Laitung et al., 2004; Treton et al., 2004). Even within the same stream, litterfall collected at different breakdown stages or in different seasons typically gathers distinct fungal communities (Suberkropp, 1984; Gessner et al., 1993).

Due to the fact that AQH inhabit freshwaters for most of their life cycle, genetic variation between unconnected populations is expected (Laitung et al., 2004). The findings of interspecific and intraspecific differences in AQH species have opened the gate for several studies on the relevance of species biodiversity for ecosystem functioning (Duarte et al., 2006; Fernandes et al., 2011; Geraldés et al 2012). The majority of said biodiversity assessments underlined the positive effects of AQH species richness leaf litter decomposition (e.g. Barlocher and Corkum, 2003; Duarte et al., 2006, Fernandes et al 2011). Other studies failed to detect effects of fungal diversity on leaf mass loss, pointing to considerable functional redundancy among fungi (Dang et al 2005; Geraldés et al 2012; Andrade et al 2016). Few studies have addressed other biodiversity measures, like intraspecific diversity (Fernandes et al 2011; Duarte et al. 2019) or genetic diversity (Andrade et al 2016). However, these questions deserve attention since intraspecific traits seem to be important to maintenance of ecosystem processes, particularly under stress (Fernandes et al., 2011). That being said, the conservation of specific metabolic pathways (genetic diversity), rather than species richness *per se* might be preferential for microbial communities and ecosystems undergoing environmental changes (Falkowski et al., 2008; Fernandes et al., 2011). It would be important to go beyond taxonomic diversity and address how functional (intra- and interspecific) diversity can impact leaf decomposition in streams. Finally, fungal diversity worldwide can be extremely affected by a number of stressors: temperature shifts due to climate change, hydrological disturbances (such as flood and droughts), over-exploitation, chemical pollution, habitat loss and emergence of invasive species (Allan and Castillo, 2007; Dang et al., 2009; Larned et al., 2010; Pereira et al., 2012). It is therefore important to understand if the species that are able to cope with these stressors have also the ability to maintain ecosystem functioning.

1.3.4. What is known about aquatic hyphomycetes enzymatic abilities?

AQH communities play a major role in freshwater ecosystems by producing a wide range of extracellular enzymes active towards major polysaccharides of leaf litter, promoting its decomposition (Chamier, 1985; Suberkropp, 1991). The efficiency of this very relevant process, however, depends on several individual factors, among which fungal versatility, functional diversity and resilience (Burns and Dick, 2002). Fungal communities can vary tremendously from site to site and are usually constituted by different AQH species, which often exhibit selectivity towards different types of substrate as well as distinct polysaccharidase capabilities from each other (Barlocher, 1982; Thomas et al., 1991; Bärlocher, 1992). This functional diversity among communities potentiates collective contributions to more efficient litter decomposition and nutrient mobilization processes (Burns and Dick, 2002). The strong connection between enzyme activities and OM degradation, as well as the need for a better understanding of which extracellular enzymes are being produced by which AQH species, began to be assessed through solid media enzyme assays, used to qualitatively evaluate enzymatic capabilities in pure cultures (Chamier, 1985; Bärlocher, 1992). These assays involve the incorporation of the desired degradable compound in the media in order to detect enzyme synthesis and secretion, as well as post-production enzymatic activity (Abdel-Raheem and Ali, 2004). A negative result could translate one of three circumstances: (i) the enzyme wasn't produced, (ii) the enzyme was produced but wasn't secreted from the mycelium, and (iii) the enzyme was produced and released, but the medium impeded its detection, meaning that this kind of result does not necessarily prove inability to produce an enzyme (Abdel-Raheem and Ali, 2004). Some early studies have reported that several AQH species are capable of degrading cellulose and other complex leaf litter polymers, such as pectins, hemicelluloses and, in limited cases (due to structural and chemical complexity), lignin-like substrates (Thornton, 1963; Suberkropp and Klug, 1980; Fisher et al., 1983; Chamier, 1985) (Table 1). Besides this, they are also able to utilize nitrate and ammonium as nitrogen sources, as well as other nutrients, like phosphorous (Suberkropp and Jones, 1991).

Considering these assays, it becomes clear that fungal species can have distinct preferences when it comes to nutrient utilization, displaying variability in terms of enzymatic activity. This can potentially be looked at as an indicative of functional gene diversity. Despite the crucial role AQH possess in ecological processes in freshwaters, studies characterizing this functional genetic diversity are scarce (e.g. Solé et al., 2012; Pradhan et al., 2014). Gorfer and collaborators (Gorfer et al., 2011) developed degenerate primers to assess gene pools and expression of fungal nitrate

reductases (through polymerase chain reaction (PCR)) in agricultural soils, which included some species of the genus *Tetracladium*.

Studies have found ascomycetes and litter-decomposing soft-rot fungi to have limited ligninolytic abilities and effective lignin degradation by stream fungi has not been described yet (Rabinovich et al., 2004; Gessner et al., 2007; Fukasawa et al., 2011; Cragg et al., 2015). Despite this, Solé and collaborators (Solé et al., 2008) identified and compared two putative laccase gene fragments in *Clavariopsis aquatica*, assessing their expression. Laccase diversity assessment studies had already been performed in salt marsh (Lyons et al., 2003) as well as terrestrial basidio- and ascomycetes (Hoegger et al., 2006; Tetsch et al., 2006), but weren't accessed in AQH until then. This scenario is common for most functional gene assessments and is likely linked to the fact that, despite their importance, only 6 AQH genomes are available on genetic databases (accessed on December 2020), however not annotated yet. Still, the fact that this kind of studies were carried out in several terrestrial organisms (many times ascomycetes), targeting distinct functional genes (ex: Cellobiohydrolase (Edwards et al., 2008), Endopolygalacturonase (Gacura et al., 2016), Tyrosinase (Kellner and Vandenbol, 2010)) has the potential to aid the characterization of AQH functional genetics, given their shared ancestry.

Table 1. Enzymatic assays to qualitatively assess activity in AQH pure cultures.

AQH Species	Cellulolytic enzymes			Hemicellulolytic enzymes						Pectinolytic enzymes		Lignolytic enzymes		Phosphatidic enzymes		
	Endoglucanase	Exoglucanase	β -glucosidase	Amylase	glucosidase	Arabinosidase	Mannanase	Manosidase	Xylanase	Xylosidase	Endopolygalacturonase	Pectin lyase	Phenoloxidase	Laccase	Acid phosphatase	Alkaline phosphatase
<i>Alatospora acuminata</i>	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Abdullah and Taj-Aldeen, 1989)		(Suberkropp et al., 1983; Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Zemek et al., 1985; Abdullah and Taj-Aldeen, 1989)	(Zemek et al., 1985)	(Suberkropp et al., 1983)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986)	(Abdullah and Taj-Aldeen, 1989; Abdel-Raheem and Ali, 2004)	(Abdel-Raheem, 1997; Abdel-Raheem and Ali, 2004)	(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Anguillospora crassa</i>	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Zemek et al., 1985)	(Abdel-Raheem and Ali, 2004)	(Abdel-Raheem and Ali, 2004)		
<i>Anguillospora filiformis</i>															(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Articulospora tetracladia</i>	(Chamier et al., 1984; Chamier, 1985; Zemek et al., 1985)	(Chamier et al., 1984)	(Thomton, 1963; Chamier, 1985; Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)			(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Clavariopsis aquatica</i>	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988)		(Suberkropp et al., 1983; Zemek et al., 1985)	(Zemek et al., 1985; Zare-Maivan and Shearer, 1988)	(Zemek et al., 1985)	(Suberkropp et al., 1983)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988)	(Suberkropp et al., 1983; Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988)		(Zare-Maivan and Shearer, 1988)		(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Flagellospora curvula</i>	Butler, suber83, canhoto2002 (Suberkropp et al., 1983; Butler and Suberkropp, 1986; Canhoto et al., 2002)		(Suberkropp et al., 1983; Abdel-Raheem and Ali, 2004)			(Suberkropp et al., 1983)			(Suberkropp et al., 1983; Butler and Suberkropp, 1986; Canhoto et al., 2002; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Butler and Suberkropp, 1986; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988)		(Abdel-Raheem, 1997; Abdel-Raheem and Ali, 2004)	(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Flagellospora penicillioides</i>	(Zemek et al., 1985; Chandrashekar and Kaveriappa, 1988, 1991; Canhoto et al., 2002)	(Chandrashekar and Kaveriappa, 1988, 1991)	(Thomton, 1963; Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Zemek et al., 1985; Chandrashekar and Kaveriappa, 1988)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985; Canhoto et al., 2002; Abdel-Raheem and Ali, 2004)	(Thomton, 1963; Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Zemek et al., 1985; Canhoto et al., 2002; Abdel-Raheem and Ali, 2004)	(Canhoto et al., 2002)	(Abdel-Raheem and Ali, 2004)	(Abdel-Raheem, 1997; Abdel-Raheem and Ali, 2004)	(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Heliscus lugdunensis</i>	(Suberkropp et al., 1983; Suberkropp and Arsuuffi, 1984; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988; Abdullah and Taj-Aldeen, 1989)	(Chamier et al., 1984; Chamier, 1985)	(Suberkropp et al., 1983; Chamier, 1985; Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985; Zare-Maivan and Shearer, 1988)	(Zemek et al., 1985)	(Suberkropp et al., 1983)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988; Canhoto et al., 2002)	(Thomton, 1963; Suberkropp et al., 1983; Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988; Canhoto et al., 2002)	(Chamier and Dixon, 1982; Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Zare-Maivan and Shearer, 1988; Canhoto et al., 2002)	(Abdullah and Taj-Aldeen, 1989)	(Zare-Maivan and Shearer, 1988)	(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Lemmoniera aquatica</i>	(Suberkropp et al., 1983; Chamier et al., 1984; Chamier, 1985; Zemek et al., 1985; Butler and Suberkropp, 1986; Abdullah and Taj-Aldeen, 1989; Canhoto et al., 2002)	(Chamier et al., 1984; Chamier, 1985)	(Suberkropp et al., 1983; Chamier, 1985; Zemek et al., 1985)	(Zemek et al., 1985; Abdullah and Taj-Aldeen, 1989)	(Zemek et al., 1985)	(Suberkropp et al., 1983)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Canhoto et al., 2002)	(Suberkropp et al., 1983; Zemek et al., 1985)	(Chamier and Dixon, 1982; Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Canhoto et al., 2002)	(Chamier and Dixon, 1982; Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Canhoto et al., 2002)	(Abdullah and Taj-Aldeen, 1989)		(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Lunulospora curvula</i>	(Singh, 1982; Chandrashekar and Kaveriappa, 1988; Abdullah and Taj-Aldeen, 1989; Chandrashekar and Kaveriappa, 1991; Canhoto et al., 2002)	(Chandrashekar and Kaveriappa, 1988, 1991)	(Abdel-Raheem and Ali, 2004)	(Chandrashekar and Kaveriappa, 1988; Abdullah and Taj-Aldeen, 1989)					(Canhoto et al., 2002; Abdel-Raheem and Ali, 2004)	(Abdel-Raheem and Ali, 2004)	(Canhoto et al., 2002; Abdel-Raheem and Ali, 2004)	(Canhoto et al., 2002)	(Abdullah and Taj-Aldeen, 1989)	(Abdel-Raheem and Ali, 2004)	(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Margaritopsis aquatica</i>	(Singh, 1982; Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)				
<i>Tetracladium marchalianum</i>	(Suberkropp et al., 1983; Chamier et al., 1984; Chamier, 1985; Zemek et al., 1985; Butler and Suberkropp, 1986)	(Chamier et al., 1984; Chamier, 1985)	(Suberkropp et al., 1983; Chamier, 1985; Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Suberkropp et al., 1983)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986)	(Suberkropp et al., 1983; Zemek et al., 1985; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986; Abdel-Raheem and Ali, 2004)	(Suberkropp et al., 1983; Zemek et al., 1985; Butler and Suberkropp, 1986)	(Abdel-Raheem and Ali, 2004)	(Abdel-Raheem, 1997; Abdel-Raheem and Ali, 2004)	(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Tetracladium setigerum</i>	(Chamier, 1985; Zemek et al., 1985; Abdullah and Taj-Aldeen, 1989)	(Chamier, 1985)	(Thomton, 1963; Chamier, 1985; Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985; Abdullah and Taj-Aldeen, 1989)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)	(Abdullah and Taj-Aldeen, 1989)			
<i>Tricladium chaetocladium</i>	(Chamier, 1985)		(Chamier, 1985)												(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Tricladium splendens</i>	(Chamier et al., 1984; Chamier, 1985; Zemek et al., 1985)	(Chamier et al., 1984; Chamier, 1985)	(Thomton, 1963; Chamier, 1985; Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)			(Suberkropp and Jones, 1991)	(Suberkropp and Jones, 1991)
<i>Varicosporium elodeae</i>	(Zemek et al., 1985)		(Thomton, 1963; Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985; Abdullah and Taj-Aldeen, 1989)	(Zemek et al., 1985)		(Zemek et al., 1985)	(Zemek et al., 1985)	(Zemek et al., 1985)	(Thomton, 1963; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)	(Chamier and Dixon, 1982; Zemek et al., 1985)				

1.4. Aim of this work

As key microbial decomposers in freshwaters, AQH are crucial for nutrient cycling in these ecosystems (Pascoal and Cássio, 2004). These fungi are able to produce extracellular enzymes targeting complex molecules involved in plant-litter decomposition and to assimilate nutrients (e.g. nitrogen and phosphorus) from stream water, immobilizing them in decomposing leaf litter, therefore increasing its nutritional value for higher trophic levels (Bärlocher, 2005a). Nevertheless, these interactions are susceptible to many global change-induced environmental factors, amongst them temperature rises and fluctuations (Friberg et al., 2009; Leadley et al., 2010). This might increase the likelihood of critical, prevalent and irreversible consequences for species and ecosystems like species loss, which has been predicted to increase due to species inability to adapt to the new climate change-induced conditions (Keith et al., 2008; Pereira et al., 2010; Bellard et al., 2012). That said, unravelling AQH functional diversity is of extreme importance to better understand the potential consequences of biodiversity loss on freshwater ecosystem functioning.

The aim of this study was to detect and analyse functional genes involved in leaf litter breakdown from several AQH species. First, AQH species identification was assessed by traditional (observation of the reproductive structures under a microscope) and molecular techniques (sequencing of the ITS region of the rDNA). Then, nucleotide sequences of the functional genes were obtained by two methods: i) through PCR amplifications, cloning and sequencing; or ii) *in silico* analyses, by compiling different functional gene sequences acquired from Genbank (<https://www.ncbi.nlm.nih.gov/nucleotide>) and performing BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) comparisons against AQH whole-genome shotgun sequences available on Genbank (www.ncbi.nlm.nih.gov/Genomes/). The attained sequences were then analysed and compared in order to evaluate functional diversity among and within the tested species. We hypothesised that (i) species with closer taxonomic relatedness would exhibit elevated functional proximity, (ii) divergences in nucleotide sequence would be attenuated in the amino acid sequences and (iii) species with similar enzymatic activities would present lower degree of functional gene divergence.

Chapter 2

Materials and Methods

2.1. Fungal strains and growth conditions

Culture collections supply well-defined and taxonomically determined starting material, assuring availability of fungal sources at all times (Graça et al., 2005; Homolka, 2014). AQH species used belong to the fungal collection of the Centre of Molecular and Environmental Biology (CBMA) of the University of Minho (UMB collection). A complete list of fungal strains used throughout this study is given in Table 2. Fungal colonies were grown in 2% Malt Extract Agar media. A piece of agar colony was sub-cultured onto new medium using a sterile loop. Petri dishes were sealed and kept in the dark at 15°C (Graça et al., 2005). Throughout the course of this study, a new subculture was made every month to ensure fungal growth at all times.

Table 2. List of fungal strains utilized in this study. n/a, not attributed. NCBI Genome accession numbers correspond to complete genome sequences. The information comprised on the Class and Order rows was retrieved from Mycobank (<https://www.mycobank.org/>).

Species	Culture collection	Codes	NCBI Genome accession number	Class	Order
<i>Alatospora acuminata</i>	UMB collection	223; 741	n/a	Leotiomycetes	Helotiales
<i>Alatospora pulchella</i>	UMB collection	902; 1115	n/a	Leotiomycetes	Helotiales
<i>Anguillospora crassa</i>	UMB collection	217; 1150	n/a	Dothideomycetes	Pleosporales
<i>Anguillospora filiformis</i>	UMB collection	16; 225; 232	n/a	Dothideomycetes	Pleosporales
<i>Aquanectria penicillioides</i>	UMB collection	304	n/a	Sordariomycetes	Hypocreales
	Nakdonggang National Institute of Biological Resources, South Korea	NNIBRFG19	PYIU00000000.1		
<i>Articulospora tetracladia</i>	UMB collection	61; 72; 712; 719; 1144	n/a	Leotiomycetes	Helotiales
	Nakdonggang National Institute of Biological Resources, South Korea	NNIBRFG329	GCA_003415645.1		
<i>Candelabrum spinulosum</i>	UMB collection	193	n/a	Leotiomycetes	Helotiales
<i>Clavariopsis aquatica</i>	UMB collection	110	n/a	Dothideomycetes	Pleosporales
	Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Germany	WD(A)-00-1	GCA_013620735.1		
<i>Collembolispora barbata</i>	UMB collection	88 ; 200	n/a	Leotiomycetes	Helotiales
<i>Dactylella cylindrospora</i>	Laboratory for Conservation and Utilization of Bio-Resources and Key Laboratory for Microbial Diversity, Southwest China	CBS325.70	GCA_012184295.1	Orbiliomycetes	Orbiliales
<i>Dendrospora erecta</i>	UMB collection	126	n/a	Leotiomycetes	Helotiales
<i>Dendrospora tenella</i>	UMB collection	891; 913	n/a	Leotiomycetes	Helotiales
<i>Dendrosporium lobatum</i>	UMB collection	145	n/a	Sordariomycetes	Hypocreales
<i>Dimorphospora follicola</i>	UMB collection	215; 1119	n/a	Leotiomycetes	Helotiales
<i>Filosporella fuistucella</i>	UMB collection	7	n/a	Leotiomycetes	Helotiales
<i>Flagellospora curvula</i>	UMB collection	39	n/a	Sordariomycetes	Hypocreales
<i>Heliscus lugdunensis</i>	UMB collection	3; 160; 161; 311	n/a	Sordariomycetes	Hypocreales
<i>Heliscus submersus</i>	UMB collection	1; 135	n/a	Sordariomycetes	Hypocreales
<i>Lemonniera aquatica</i>	UMB collection	594; 595	n/a	Leotiomycetes	Helotiales
<i>Lunulospora curvula</i>	UMB collection	108; 498	n/a	Sordariomycetes	Sordariales
<i>Margaritisporea aquatica</i>	Nakdonggang National Institute of Biological Resources, South Korea	NNIBRFG339	GCA_007644065.1	Leotiomycetes	Helotiales
<i>Tetracladium apiense</i>	UMB collection	535	n/a	Leotiomycetes	Helotiales
<i>Tetracladium marchalianum</i>	UMB collection	1028; 1079	n/a	Leotiomycetes	Helotiales
<i>Tetracladium furcatum</i>	UMB collection	736	n/a	Leotiomycetes	Helotiales
<i>Thelonectria rubi</i>	Agricultural Research Service, United States Department of Agriculture	CBS 177.27	GCA_013420875.1	Sordariomycetes	Hypocreales
<i>Tricladium chaetocladium</i>	UMB collection	904; 1116	n/a	Leotiomycetes	Helotiales
<i>Tricladium splendens</i>	UMB collection	100; 414; 1117	n/a	Leotiomycetes	Helotiales
<i>Varicosporium elodeae</i>	UMB collection	310; 713 ; 878	n/a	Leotiomycetes	Helotiales

2.2. Morphological identification by microscopy

Fungal isolates were sub-cultured into a 50-ml Erlenmeyer flask with sterile deionized water and kept in an orbital shaker (200 revolutions per minute (rpm)) at 15°C. Water was renewed weekly. Every other day, routine sporulation examination was performed by collecting 200 µl of water suspension into a microscope slide and letting it dry. Once dried, a drop of 0.1% cotton blue in lactic acid and a cover glass were placed on top of the slide, making it ready for microscope examination (Bärlocher, 2005b). Images were captured using a Leica ICC50 W light microscope at 400x magnification.

For the ones which did not sporulate with the first approach, fragments of alder leaves (chosen because of their chemical composition, which favours colonization by AQH, usually with high breakdown rates (Gulis, 2001)) were added to the flasks to invigorate sporulation and/or aeration was made using air pumps in order to simulate the well-aerated turbulent conditions found in streams (Bärlocher, 2005b). At this stage, the flasks were kept at room temperature, around 20°C.

2.3. DNA extraction from fungal cultures

To perform DNA extraction, 2-3 small agar plugs were removed from an actively growing part of fungal mycelia (grown on 2% Malt Extract Agar for 15-30 days) and placed in a 2 ml Bead Solution Tubes provided by the MO BIO Laboratories' UltraClean® Soil DNA Isolation Kit. Extraction was performed according to manufacturer's instructions, with the exception of the final elution step where only 30 µl (instead of the recommended 50 µl) of Solution S5 were added, to increase the final DNA concentration. DNA was stored at -80°C until used.

2.4. Molecular identification through ITS1-5.8S-ITS2 sequencing

The ITS region of the rDNA of each fungal strain was amplified by PCR as follows: 12.5 µl of Accuzyme mix (2x) (Bioline), 0.4 µM of each primer (ITS1F and ITS4, Table A4) and 2 µl of DNA in a final volume of 25 µl. The amplification programme was performed in an iCycler Thermal Cycler (BioRad) as follows: initial denaturation at 94°C for 2 min, 30 cycles of denaturation (94°C, 45s), annealing (56°C, 45s) and extension (72°C, 90s) and a final extension at 72°C for 7 min.

Negative controls with no DNA template were included in each batch. The PCR products were run on a 2% agarose gel with 3% GreenSafe Premium (NZYtech) at 80V for 45 min, to check the presence of the desired band. Then, the PCR products were cleaned using Invitrogen's PureLink® PCR Purification Kit according to the manufacturer's instructions, with the exception of final elution where 30 µl were used (instead of the recommended 50 µl) to increase the final DNA concentration. DNA concentration and quality were confirmed using Nanodrop (Spectrophotometer ND-1000). DNA sequencing was performed at STABVIDA, using primers ITS1F and ITS4. The sequences were trimmed and aligned against each other in order to establish the consensus ITS sequence for each fungal strain, and subjected to an individual Basic Local Alignment Search Tool (BLAST; <https://BLAST.ncbi.nlm.nih.gov/BLAST.cgi>) to verify identity. The parameters for species name attribution were met when ITS sequences presented $\geq 80\%$ of query coverage and 97-100% of sequence similarity with the results from the BLAST search (Nilsson et al., 2008). Afterwards, a species identification/confirmation table was elaborated (Table A1), combining information from both traditional (sporulation assays) and molecular identification methods.

2.5. Primer selection/design for targeting functional genes

Firstly, a bibliographic search was made for articles targeting fungal genes (mostly from terrestrial fungi) potentially involved in leaf litter breakdown. Due to distinct fields of investigation, as well as the different aims concerning each study, both regular and degenerate primers were found in this search. To compile this information, a document with all retrieved primers was created (if a primer was degenerate, all its possible combinations were written down). Every primer was run using Sequence Read Archive Nucleotide BLAST (SRABLASTn; <https://BLAST.ncbi.nlm.nih.gov/BLAST.cgi>) against the genomes of *Articulospora tetracladia* (SRX4652715), *Margaritipora aquatica* (SRX6454163) and *Aquanectria penicillioides* (SRX5023576), the only available AQH genomes at the date of database accession (November 2019). Primers presenting 100% identity with one or more of the considered genomes were seen as potential candidates for our study, being then selected based on the amount of hits (for both forward and reverse primers). Based on the SRABLASTn results ($\geq 94\%$ of query coverage and $\geq 94\%$ of sequence similarity), some of the primers that obtained fewer hits with 100% of identity were degenerated even further, as an attempt to adapt them to AQH.

The results of the bibliographic search regarding fungal functional genes (mostly from terrestrial fungi) involved in leaf litter breakdown are presented in Table A3. Based on the SRABLASTn hits, several primer sets were excluded due to lack of hits with the AQH genomes. Primers targeting endo- β -1,4-glucanase (Barbi et al., 2014), β -xylosidase (Wegener et al., 1999), acetyl xylan esterase (Kellner and Vandenbol, 2010), endopolygalacturonase (Centis et al., 1996) and laccase (Hirschhäuser and Fröhlich, 2007) obtained few hits of 100% of identity being, therefore, further degenerated. Primers targeting for nitrate reductase (Gorfer et al., 2011), cellobiohydrolase (Edwards et al., 2008), β -glucosidase (Takashima et al., 1999), endopolygalacturonase (Gacura et al., 2016) and tyrosinase (Kellner and Vandenbol, 2010) displayed a considerable number of hits, being therefore selected. Table A4 presents all selected primers for PCR testing. After selection, the primers were ordered from STABVIDA to proceed with PCR amplification.

2.6. PCR amplifications of functional genes

For every functional gene tested, three main conditions were applied: (i) negative controls with no DNA template were included in each batch, (ii) success of PCR was assessed by gel electrophoresis (2% agarose and 3% GreenSafe Premium (NZYtech) run at 80V for 45 min); with any lanes displaying band(s) of the correspondent expected sizes (Table A4) being considered to contain a potential functional gene fragment, and (iii) PCR products containing the band of interest were cleaned using Invitrogen's PureLink® PCR Purification Kit according to manufacturer's protocol, and its concentration was confirmed using nanodrop (Spectrophotometer ND-1000). DNA was kept at -20°C.

2.6.1. Nitrogen assimilation

Nitrate reductase

PCR amplification was performed according to the conditions described in Table 3. A nested PCR was performed using a set of primers targeting a wider region (niaD01F/niaD04R) and the products of this amplification used as DNA template in a following amplification, in which a more target-sequence-specific set of primers was utilized (niaD15F/niaD12R) (Gorfer et al., 2011, Table A4). This was made to test whether nested PCR would improve the reaction yield, i.e. if the

concentration of our potential band of interest would increase (observed as a more intense band in the agarose gel) and the number of non-specific bands would be significantly reduced. Highlighted conditions (considered optimal) were used to test selected genomic DNA (gDNA) fungal templates.

Table 3. Tested conditions for the nitrate reductase gene PCR amplification (adapted from Gorfer et al., 2011).

PCR conditions													Thermocycler conditions			
MIX	1	2	3	4	5	6	7	8	9	10	11	12		Temp	Time	Cycles
Accuzyme (U)	1	1	1	1	1	1	1	1	1	1	1	1	initial denaturation	95°C	2:30 min	35
Primer niaD01F (μM)	0.4	—	0.4	—	0.4	0.4	0.4	2	2	2	—	—	denaturation	94°C	20 s	
Primer niaD04R (μM)	0.4	—	0.4	—	0.4	0.4	0.4	2	2	2	—	—	denaturation	94°C	20 s	
Primer niaD15F (μM)	—	0.4	—	0.4	—	—	—	—	—	—	2	2	annealing	52°C	20 s	
Primer niaD12R (μM)	—	0.4	—	0.4	—	—	—	—	—	—	2	2	annealing	52°C	20 s	
MgCl ₂ (mM)	—	—	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	extension	72°C	1 min	
BSA (μg/μl)	—	—	—	—	10	—	10	10	—	10	10	10	extension	72°C	1 min	
DMSO (%)	—	—	—	—	—	5	5	—	—	5	—	5	Final extension	72°C	5 min	
DNA (μl)	2	2	2	2	2	2	2	2	2	2	2	2	Final extension	72°C	5 min	

2.6.2. Cellulolytic enzymes

Endo-β-1,4-glucanase

PCR amplifications were performed using the primer pair fungGH5-5-F/fungGH5-5-R (Barbi et al., 2014, Table A4) and mixes with different concentrations of primers, MgCl₂, DMSO and gDNA, as described in Table 4.

Table 4. Tested conditions for the Endo-1,4-β-glucanase gene PCR amplification (Mix 1 is adapted from Barbi et al., 2014).

PCR conditions			Thermocycler conditions			
MIX	1	2		Temp (°C)	Time	Cycles
Accuzyme (U)	1	1				
Primer fungGH5-5-F (μM)	0.4	2				
Primer fungGH5-5-R (μM)	0.4	2	initial denaturation	94°C	3 min	
MgCl ₂ (mM)	—	1.25	denaturation	94°C	45 s	45
BSA (μg/μl)	10	10	annealing	50°C	45 s	
DMSO (%)	—	5	extension	72°C	45 s	
DNA (μl)	0.5	2	final extension	72°C	10 min	

Cellobiohydrolase

PCR amplification was performed using the primers FungcbhIF/ FungcbhIR (Edwards et al., 2008, Table A4) as described in Table 5. Two mixes were tested: Mix 1, using the conditions described in Edwards et al., 2008 and Mix 2, where primer concentration was increased and MgCl₂ and DMSO were added.

Table 5. Tested conditions for the cellobiohydrolase PCR amplification. (Mix 1 is adapted from Edwards et al., 2008).

PCR conditions			Thermocycler conditions			
MIX	1	2		Temp (°C)	Time	Cycles
Accuzyme (U)	1	1				
FungcbhIF (μM)	0.5	2				
FungcbhIR	0.5	2	initial denaturation	94°C	2 min	
MgCl ₂ (mM)	—	1.25	denaturation	94°C	45 s	30
BSA (μg/μl)	10	10	annealing	56°C	45 s	
DMSO (%)	—	5	extension	72°C	90 s	
DNA (μl)	2	2	final extension	72°C	7 min	

β-glucosidase

PCR amplifications were performed using the primer pair sense/ antisense (Table A4). As there were no indications regarding PCR conditions in the original article (Takashima et al., 1999), the PCR testing conditions were based on what had worked for other functional gene amplifications (Table 6). Gradient PCR was performed to determine the most suitable annealing temperature. Highlighted conditions (considered optimal) were used to test selected gDNA fungal templates.

Table 6. Tested conditions for the β-glucosidase gene PCR amplification. G-gradient; OC – optimal conditions.

PCR conditions		Thermocycler conditions						
MIX	1	Mix1 - G			Mix1 – OC			
Accuzyme (U)	1		Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycle
Primer sense (μM)	2							
Primer antisense (μM)	2	initial denaturation	94°C	5 min		94°C	5 min	
MgCl ₂ (mM)	1.25	denaturation	94°C	45 s	35	94°C	45 s	35
BSA (μg/μl)	10	annealing	45-57°C	30 s		48°C	30 s	
DMSO (%)	5	extension	72°C	100 s		72°C	100 s	
DNA (μl)	2	final extension	72°C	10		72°C	10 min	

2.6.3. Hemicellulolytic enzymes

Acetyl xylan esterase

PCR amplifications were performed using the primer pair NAXE_155F/ NAXE_250R (Kellner and Vandenberg, 2010, Table A4) and testing different concentrations of primers, MgCl₂, BSA, DMSO and gDNA, as described in Table 7.

Table 7. Tested conditions for the acetyl xylan esterase gene PCR amplification (Mix 1 is adapted from Kellner and Vandenberg, 2010).

PCR conditions			Thermocycler conditions			
MIX	1	2		Temp (°C)	Time	Cycles
Accuzyme (U)	1	1				
Primer NAXE_155F (μM)	0.2	2				
Primer NAXE_250R (μM)	0.2	2	initial denaturation	94°C	5 min	
MgCl ₂ (mM)	—	1.25	denaturation	94°C	45 s	35
BSA (μg/μl)	—	10	annealing	50°C	45 s	
DMSO (%)	—	5	extension	72°C	1:40 min	
DNA (μl)	0.5	2	final extension	72°C	10 min	

β-xylosidase

PCR amplifications were performed using the primer pair NxypF/xypr (Table A4). As there were no indications regarding PCR conditions in the original article (Wegener et al., 1999), the PCR testing conditions were based on what had worked for other functional gene amplifications, as described in Table 8.

Table 8. Tested conditions for the β-xylosidase gene PCR amplification.

PCR conditions		Thermocycler conditions			
Accuzyme (U)	1		Temp (°C)	Time	Cycles
Primer NxypF (μM)	2				
Primer xypr (μM)	2	initial denaturation	94°C	3 min	
MgCl ₂ (mM)	1.25	denaturation	94°C	1 min	35
BSA (μg/μl)	10	annealing	55°C	2 min	
DMSO (%)	5	extension	72°C	3 min	
DNA (μl)	2	final extension	72°C	10 min	

2.6.4. Pectinolytic enzymes

Endopolygalacturonase

Two sets of primers were used to perform PCR amplification of the endopolygalacturonase gene. In the first test, PCR amplification was performed using the primers GH28F-1786F/ GH28F-2089R (Gacura et al., 2016, Table A4), and testing addition of DMSO and MgCl₂, as described in Table 9. Gradient PCR was performed to determine the most suitable annealing temperature. Highlighted conditions (considered optimal) were used to test selected gDNA fungal templates.

Table 9. Tested conditions for the endopolygalacturonase gene PCR amplification (adapted from Gacura et al., 2016). G- gradient; OC – optimal conditions.

PCR conditions				Thermocycler conditions								
MIX	1	2	3									
Accuzyme (U)	1	1	1				Mix3 - G			Mix3 - OC		
Primer GH28F-1786F (μM)	0.4	2	2				Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycles
Primer GH28F-2089R	0.4	2	2	initial denaturation	95°C	3 min	95°C	3 min	35	95°C	3 min	35
MgCl ₂ (mM)	—	—	1.25	denaturation	94°C	30 s	94°C	30 s	35	94°C	30 s	35
BSA (μg/μl)	10	10	10	annealing	64°C	30 s	45-66°C	30 s		52.9°C	30 s	
DMSO (%)		5	5	extension	72°C	90 s	72°C	90 s		72°C	90 s	
DNA (μl)	2	2	2	final extension	72°C	7 min	72°C	7 min		72°C	7 min	

In the second test, PCR amplification was performed using the primers NC1pgF/ NC1pgR (Centis et al., 1996, Table A4). PCR conditions were tested based on what had worked for other functional gene amplifications, as described in Table 10. Gradient PCR was performed to determine the most suitable annealing temperature.

Table 10. Tested conditions for the endopolygalacturonase gene PCR amplification (from Centis et al., 1996).

PCR conditions		Thermocycler conditions			
Accuzyme (U)	1				
Primer NC1pgF (μM)	2		Temp (°C)	Time	Cycles
Primer NC1pgR (μM)	2	initial denaturation	95°C	2:30	
MgCl ₂ (mM)	1.25	denaturation	94°C	20 s	35
BSA (μg/μl)	10	annealing	45-54°C	20 s	
DMSO (%)	5	extension	72°C	1 min	
DNA (μl)	2	final extension	72°C	5 min	

2.6.5. Lignolytic enzymes

Tyrosinase

PCR amplifications were performed using the primer pair Tyr_137F / Tyr_282R (Kellner and Vandenbol, 2010, Table A4) and testing mixes with different concentrations of MgCl₂, BSA and DMSO as described in Table 11. Gradient PCRs were performed to determine the most suitable annealing temperature.

Table 11. Tested conditions for the tyrosinase gene PCR amplification (adapted from Kellner and Vandenbol, 2010). G- gradient.

PCR conditions				Thermocycler conditions									
MIX	1	2	3	Mix1 and 2			Mix3 – G1			Mix3 – G2			
Accuzyme (U)	1	1	1	Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycles	
Primer Tyr_137F (μM)	1.2	2	2	initial denaturation	94°C	5 min	35	94°C	5 min	35	94°C	5 min	
Primer Tyr_282R (μM)	1.2	2	2	denaturation	94°C	45 s		94°C	45 s		94°C	45 s	35
MgCl ₂ (mM)	---	---	1.25	annealing	50°C	45 s		45-50°C	45 s		45-66°C	45 s	
BSA (μg/μl)	---	---	10	extension	72°C	100 s	72°C	100 s	72°C	100 s	72°C	100 s	
DMSO (%)	---	---	5										
DNA (μl)	2	2	2	final extension	72°C	10 min	72°C	10 min	72°C	10 min	72°C	10 min	

Laccase

PCR amplifications were performed using the primer pair Lac2F/ Lac2R (Hirschhäuser and Fröhlich, 2007, Table A4) and testing different Mix with different concentrations of MgCl₂ and DMSO, as described in Table 12. Gradient PCRs were performed to determine the most suitable annealing temperature. Highlighted conditions (considered optimal) were used to test selected gDNA fungal templates.

Table 12. Tested conditions for the laccase gene PCR amplification. G-gradient; OC – optimal conditions.

PCR conditions			Thermocycler conditions									
MIX	1	2	Mix1 and 2			Mix2 – G			Mix2 – OC			
Accuzyme (U)	1	1	Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycles	
Primer Lac2F (μM)	2	2	initial denaturation	95°C	4 min	30	95°C	4 min	30	95°C	4 min	
Primer Lac2R (μM)	2	2	denaturation	94°C	1 min		94°C	1 min		94°C	1 min	30
MgCl ₂ (mM)	---	1.25	annealing	55°C	1 min		45-53.6°C	1 min		53.6°C	1 min	
BSA (μg/μl)	10	10	extension	72°C	2 min	72°C	2 min	72°C	2 min	72°C	2 min	
DMSO (%)	---	5										
DNA (μl)	2	2	final extension	72°C	10 min	72°C	10 min	72°C	10 min	72°C	10 min	

2.7. Cloning

Cloning of the PCR amplicons of interest was performed using NZY-blunt PCR cloning kit (Nzytech) according to manufacturer's protocol. The optimal amount of PCR product required was calculated using the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The recommend 1:10 molar ratio of vector:insert and an initial amount of 20 ng of the linearized pNZY28 vector was used. In cases where the PCR fragment quantity didn't reach the minimum required for the cloning, the PCR amplification was repeated (for 50 µl), in order to increase DNA quantity. A number between 8-20 clone colonies were selected from each cloning plate. Plasmids were extracted using NZYMiniprep (Nzytech) according to manufacturer's protocol. Plasmid DNA was then subjected to restriction digestion to confirm the presence of the insert. Briefly, the purified plasmid DNA was incubated overnight with EcoRI as follows: for each 10 µl reaction, 2 µl of DNA, 1 µl of NZYSpeedyBuffer Colourless and 0.3 µl of Speedy EcoRI was used. The last step was enzyme inactivation at 80°C for 20 min, followed by confirmation of presence of inserts by agarose gel electrophoresis. Samples presenting bands with expected band sizes (Table A4) were sent for sequencing at STABVIDA facilities using primers T7 and U19mer. After receiving and analysing the sequences, in cases where there were very few fungal DNA results in terms of final sequences, restriction digestion was repeated using the restriction enzyme BamHI (same conditions as EcoRI). This was performed for plasmid DNAs that showed no insert within the expected size after digestion with ECORI.

2.8. Sequence analysis and alignment

Sequences were arranged resorting to the softwares BioEdit 7.2.5 (Hall, 1999) and Chromas (<http://technelysium.com.au/wp/chromas/>). Forward and reverse sequences from each sample were firstly trimmed to remove primers' binding sites T7 and U19mer (through its reverse complement). Then, both sequences were subjected to pairwise alignment, with all appearing incongruencies being corrected through chromatogram assessments (and respective nucleotide qualities), in order to establish a primary consensus sequence. This sequence was then

subjected to a second trimming, to remove the EcoR V flanking regions at the beginning and end of the primary consensus sequence. Blunt-end cloning ligates PCR products' blunt ends with a blunt-cut vector (often dephosphorylated) (Chen and Janes, 2002). This way, DNA fragments can be inserted into the vector in both orientations (5'-3' or 3'-5'), as it isn't a directional cloning process (Chen and Janes, 2002). That being said, after the second trimming was completed, there was a need to evaluate the sequences' orientation in order to perform the last trimming, which consists on removing the primers used to perform the PCR amplification for each functional gene. These gene-specific primers always have the same orientation so, if the consensus sequence presented itself in reverse, it would be necessary to obtain its reverse complement, and only then search and remove the primers. After this last trimming, all sequences were subjected to an individual BLASTn search, to evaluate whether they exhibited significant alignments with the targeted functional gene. After this procedure was completed, a multiple alignment was performed to compare these sequences and identify potential differences, creating a final consensus sequence for each fungal isolate.

2.9. *In silico* analysis

Even though cloning after PCR amplifications is a standard procedure for functional gene detection, there are other ways of assessing and analysing functional genes, such as *in silico* analysis. Factors such as the increase of sequence data available in databases, as well as the strong computational biology development throughout the years solidified scientific community acceptance and implementation of virtual investigation methods driven by computational strategies (Murray et al., 2007). As previously mentioned, AQH are poorly represented genomic-wise. To the date of this analysis (database accessed on December 2020), only six AQH complete genomes were available on Genbank (www.ncbi.nlm.nih.gov/Genomes/), from whole genome shotgun sequencing projects: *Articulospora tetracladia* (NNIBRFG329), *Aquanectria penicillioides* (NNIBRFG19), *Clavariopsis aquatica* (WD(A)-00-1), *Dactylella cylindrospora* (CBS325.70), *Margaritispota aquatica* (NNIBRFG339), *Thelonectria rubi* (CBS 177.27). Nevertheless, genomes of these isolates are still very sparsely annotated, highlighting the need for query sequences when searching regions of interest of functional genes.

In order to include data from those genomes in our analysis, we've assessed the ITS sequences through primer (ITS1F and ITS4) search for all the six genomes, aiming to confirm identities and to establish taxonomic phylogenetic affiliations. This was followed by a bibliographic search, complementary to the one made in chapter 2.5 (Table A3) for articles comprising isolation and characterization of extracellular enzymes potentially involved in leaf litter decomposition, with substantial involvement in C, N or P cycling. The partial and complete nucleotide sequences for the different functional genes found in these articles were retrieved and compiled in Table A5. Besides, several NCBI (<https://www.ncbi.nlm.nih.gov/>) nucleotide sequence searches were conducted to acquire partial and complete sequences for other functional genes from multiple published and unpublished sources. After the nucleotide sequences for many of the most relevant litter-degrading extracellular enzymes were gathered, a nucleotide BLAST analysis was performed against all 6 whole-genome shotgun contigs. Most of the retrieved sequences from the bibliographic and NCBI searches belonged to terrestrial fungi, which possess considerable genetic differences from AQH. Therefore, a certain degree of divergence in the BLASTn alignments was expected. With that in mind, sequences presenting $\geq 80\%$ query coverage and $\geq 60\%$ sequence similarity with a retrieved sequence were considered potential hits for the target gene. After acquiring sequences from all the AQH genomes for a target gene, a multiple CLUSTALW alignment was performed with all the previously retrieved sequences for that gene as well as the sequences obtained with BLASTn. Sequence trimming and motif assessment and analysis were also performed in BioEdit 7.2.5. Afterwards, a new file comprising our trimmed sequences was created.

2.10. Translation of gene sequences to protein sequences

Final consensus sequences obtained from both cloning and *in silico* analysis approaches were translated into protein sequences, resorting to the translate tool in the ExPASy portal (<https://web.expasy.org/translate/>) and the protein BLAST (BLASTp) tool from NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) in order to establish the correct reading frame for each protein sequence and detect significant alignments with the target protein sequence.

2.11. Clustering and phylogenetic analysis

Final gene sequences acquired through both cloning and *in silico* analysis approaches, as well as their respective protein sequences, were analysed using MEGA-X (Kumar et al., 2018). Firstly, a multiple alignment was performed resorting to the MUSCLE tool, to identify significant differences among isolates of the same species (intraspecific differences) or between species (interspecific differences). In addition, aligned nucleotide sequences for all functional genes were concatenated into a matrix using MEGA-X (Kumar et al., 2018), to obtain an overall functional gene assessment (adapted from Johnston et al., 2019).

The alignments were then exported and utilized to construct a phylogenetic tree in Mega-X (Kumar et al., 2018). The chosen running options were: 1) Neighbour-joining (NJ) method (Saitou and Nei, 1987); 2) Bootstrap analysis (1000 replicates (Felsenstein, 1985)); 3) Maximum composite likelihood distance model for nucleotide sequences and Poisson model for amino acid sequences; 4) pairwise deletion for gaps/missing data to account for highly variable sites (exclusion of characters absent in one of both compared sequences and inclusion of characters present in both sequences); 5) Uniform rates for rates among sites and 6) Same (Homogeneous) for the Pattern among Lineages. Sequences from terrestrial fungi available in Genbank were used as Outgroup to root the trees in all cases, except for Figure 11, where rooting was not possible due to lack of availability of a single species comprising all functional gene sequences. Assessments on percentual pairwise distances and overall mean distances (number of base substitutions per site from averaging overall sequence pairs) were obtained through MEGA-X, with the following running options: 1) Bootstrap analysis (1000 replicates (Felsenstein, 1985)); 2) Nucleotide substitutions (for nucleotide sequences) and Amino acid substitutions (for amino acid sequences) 3) p-distance model; 5) pairwise deletion for gaps/missing data to account for highly variable sites (exclusion of characters absent in one of both compared sequences and inclusion of characters present in both sequences); 6) Uniform rates for rates among sites and 7) Same (Homogeneous) for the Pattern among Lineages.

Chapter 3

Results

3.1. Morphological identification by microscopy

When spore presence was detected from fungal strains, pictures were taken using a Leica ICC50 W Microscope Camera (Figure A1). In some cases, despite the methodological alterations to induce sporulation (leaves addition, aeration induced by air pumps), a microscopic identification was not possible, as the species didn't produce conidia (Table A1). This was the case for the isolates *Heliscus submersus* UMB1, *Tricladium splendens* UMB100, *Heliscus lugdunensis* UMB161, *Collembolispora barbata* UMB200, *Alatospora acuminata* UMB 223, *Lemonniera aquatica* UMB595, *Varicosporium elodeae* UMB713, *Dendrospora tenella* UMB913 and *Tetracladium marchalianum* UMB1079. Even so, identification of 35 out of the 48 isolates tested was confirmed by conidia morphology.

3.2. Molecular identification through ITS1-5.8S-ITS2 sequencing

Molecular identification of 48 isolates of the UMB fungal collection was performed (Table A1). Final consensus sequences of the ITS region of the fungal isolates obtained by sequencing, together with 6 sequences retrieved through *in silico* analysis from the genomes available at Genbank are listed in Table A2. Most ITS sequences confirmed the previously established species identifications in the UMB collection. However, there were some disparities: (i) UMB7, re-identified as *Filosporella fistucella* (previously named *F. fustiformis*), (ii) UMB16, re-identified as *Anguillospora filiformis* (previously described as *Cylindrocarpon ianthothele*), (iii) UMB902, re-identified as *Alatospora acuminata* (instead of *A. pulchella*) and (iv) UMB595, previously identified as *Lemonniera aquatica* and re-classified as *Phoma sp.* (not AQH), therefore excluded from the study.

3.3. PCR amplifications and cloning of selected-functional genes

Despite the fact that all primer sets indicated in Table A4 were tested, PCR amplifications for Endo- β -1,4-glucanase (Barbi et al., 2014), Cellobiohydrolase (Edwards et al., 2008), Acetyl xylan esterase (Kellner and Vandenbol, 2010) and β -xylosidase (Wegener et al., 1999) fragments were not successful, even after alterations of the PCR mixture and/or the thermocycler protocol.

Successful amplification was obtained for nitrate reductase (56.25%), β -glucosidase (78.26%), endopolygalacturonase (69.57%) and laccase (78.13%) genes (Table 13). In some cases, successful amplifications were obtained for all the species isolates (e.g. *Varicosporium elodeae*, laccase; *Tetracladium marchalianum*, all functional genes in Table 13). There were also some species for which no amplification was obtained for the most part of their isolates (e.g. *Heliscus lugdunensis*, nitrate reductase). Most species (with more than one isolate) revealed intraspecific variability in terms of functional gene amplifications, as successful amplifications were obtained for some isolates and no amplification for others. In the end, 18 amplicons from nitrate reductase and 7 from β -glucosidase were further used for cloning and sequencing (Table 13).

Table 13. Amplification success of the different functional genes. Yes, successful amplification; No, unsuccessful amplification; nt, not tested. Highlighted Yes indicate amplicons used for cloning and sequencing.

Species	UMB code	Nitrate reductase (Gorfer et al., 2011)	β -glucosidase (Takashima et al., 1999)	Endopolygalacturonase (Gacura et al., 2016)	Laccase (Hirschhäuser and Fröhlich, 2007)
<i>Alatospora acuminata</i>	741	Yes	nt	nt	Yes
<i>Alatospora pulchella</i>	1115	Yes	nt	nt	Yes
<i>Anguillospora crassa</i>	217	No	nt	nt	Yes
<i>Anguillospora crassa</i>	1150	Yes	nt	nt	No
<i>Anguillospora filiformis</i>	225	Yes	nt	nt	Yes
<i>Anguillospora filiformis</i>	232	Yes	nt	nt	Yes
<i>Articulospora tetracladia</i>	72	No	No	No	Yes
<i>Articulospora tetracladia</i>	719	Yes	Yes	Yes	No
<i>Articulospora tetracladia</i>	1144	No	Yes	Yes	No
<i>Articulospora tetracladia</i>	712	Yes	Yes	Yes	Yes
<i>Collembolispota barbata</i>	88	Yes	nt	nt	Yes
<i>Dimorphospora foliicola</i>	1119	Yes	nt	nt	No
<i>Flagellospora penicillioides</i>	304	No	nt	nt	Yes
<i>Heliscus lugdunensis</i>	311	No	No	No	Yes
<i>Heliscus lugdunensis</i>	3	No	Yes	Yes	No
<i>Heliscus lugdunensis</i>	161	No	Yes	Yes	No
<i>Heliscus lugdunensis</i>	160	No	Yes	No	Yes
<i>Lemonniera aquatica</i>	594	Yes	Yes	No	Yes
<i>Lunulospora curvula</i>	108	No	No	Yes	Yes
<i>Lunulospora curvula</i>	498	No	Yes	Yes	No
<i>Tetracladium apiense</i>	535	Yes	Yes	Yes	Yes
<i>Tetracladium marchalianum</i>	1028	Yes	Yes	Yes	Yes
<i>Tetracladium marchalianum</i>	1079	Yes	Yes	Yes	Yes
<i>Tetracladium furcatum</i>	736	No	Yes	Yes	Yes
<i>Tricladium chaetocladium</i>	1116	Yes	Yes	Yes	Yes
<i>Tricladium chaetocladium</i>	904	Yes	Yes	No	Yes
<i>Tricladium splendens</i>	414	No	No	Yes	Yes
<i>Tricladium splendens</i>	100	Yes	Yes	No	Yes
<i>Tricladium splendens</i>	1117	Yes	Yes	Yes	Yes
<i>Varicosporium elodeae</i>	310	No	Yes	Yes	Yes
<i>Varicosporium elodeae</i>	878	No	No	No	Yes
<i>Varicosporium elodeae</i>	713	Yes	Yes	Yes	Yes
Amplification success rate (%)		56.25	78.26	69.57	78.13

3.4. Nitrate reductase and β -glucosidase sequences analysis

For nitrate reductase, some sequences had to be discarded due to: i) poor quality results in terms of sequencing, ii) being highly different from the other sample sequences for the same species, and iii) not having relevant identity hits in the BLASTn platform. Table 14 comprises the information regarding the number of plasmid clones for each fungal species used for enzymatic restriction (EcoRI and BamHI), the number of plasmids sent for sequencing, as well as the number of sequences used in the final consensus sequences for each fungal strain. Due to the molecular identification results re-classification of *Lemmoniera aquatica* UMB594 as *Phoma* sp. (not an AQH; probable contamination), this cloning result was excluded. Only 1 out of 7 and 1 out of 4 sequences were used for the final consensus sequences of *Tricladium splendens* UMB100 and *Varicosporium elodeae* UMB713, respectively. For *Collembolispora barbata* UMB88, *Anguillospora filiformis* UMB225 and UMB232 and *Articulospora tetracladia* UMB712, 1 complete sequence was aligned with partial(s) sequence(s). In the cases of *Tetracladium apiense* UMB535 and *Alatospora acuminata* UMB741, all the sent sequences were utilized when generating final consensus sequences. The final consensus sequences for the nitrate reductase gene are presented in appendix Table A6, and the corresponding protein sequences in Table A7.

For β -glucosidase, most of the obtained sequences did not present significant similarities with the target gene fragment in BLASTn searches and were also not similar enough to perform alignments and obtain consensus sequences for fungal species.

Table 14. Number of clone colonies used for enzymatic restriction (EcoRI and BamHI), DNA inserts sent for sequencing and sequences utilized in the alignments of each fungal isolate (using all the sequencing efforts; both digestions with EcoRI and BamHI). nt, not tested.

AQH	UMB code	Number of			
		Clone colonies		Inserts sequenced	Sequences used
		EcoRI	BamHI		
<i>Alatospora acuminata</i>	741	8	Nt	4	4
<i>Alatospora pulchella</i>	1115	8	Nt	6	5
<i>Anguillospora crassa</i>	1150	8	Nt	5	4
<i>Anguillospora filiformis</i>	225	7	6	5	1 (+1 partial)
<i>Anguillospora filiformis</i>	232	8	4	6	1 (+1 partial)
<i>Articulospora tetracladia</i>	719	8	Nt	6	3
<i>Articulospora tetracladia</i>	712	6	5	7	1 (+ 2)
<i>Collembolispora barbata</i>	88	7	5	4	1 (+1 partial)
<i>Dimorphosphora foliicola</i>	1119	8	Nt	5	4
<i>Lemmoniera aquatica</i>	594	8	3	7	3
<i>Tetracladium apiense</i>	535	10	Nt	7	7
<i>Tetracladium marchalianum</i>	1028	8	Nt	5	3
<i>Tetracladium marchalianum</i>	1079	6	Nt	6	6
<i>Tetracladium furcatum</i>	736	8	3	3	2
<i>Tricladium chaetocladium</i>	1116	10	1	4	3
<i>Tricladium chaetocladium</i>	904	8	Nt	8	6
<i>Tricladium splendens</i>	100	8	7	7	1

<i>Tricladium splendens</i>	1117	8	Nt	5	4
<i>Varicosporium elodeae</i>	713	8	5	4	1

3.5. *In silico* analysis

Firstly, ITS sequences were obtained (through primer ITS1F and ITS4) for all six genomes, confirming their identities. Regarding the functional genes, we were able to retrieve sequences implicated in N and P cycling, as well as carbon-degrading extracellular enzymes, involved in pectin, cellulose and hemicellulose breakdown (Table A6-Table A7). Despite the efforts, we weren't able to obtain sequences encoding major lignin modifying enzymes, except for the already published laccase from *Clavariopsis aquatica* (Solé et al., 2008) and an aromatic peroxygenase (involved in oxidative cleavage of non-phenolic lignin dimers) (Kellner and Vandenberg, 2010). Sequences for Nitrate reductase, β -glucosidase/xylan- β -xylosidase (GH3), Cellobiohydrolase (cbhl), Cellobiohydrolase I / endoglucanase (GH7), Cellobiohydrolase II (GH6), Endo- β -xylanase (GH11), Xylanase (xyn2) and Acid phosphatase (phoA) genes were obtained from all six genomes. For the remaining genes (α -glucosidase (GH31), α -glucuronidase (GH67), α -mannosidase (GH92), endo-mannosidase/endoglucanase (GH5), endoglucanase (GH45), pectin esterase, pectate lyase (plyA) and aromatic peroxygenase), sequences were obtained for only some of the fungal species. Nucleotide sequences for endoglucanase (glu) and endopolygalacturonase (enpg) were also found (in 6 and 5 genomes, respectively), however, multiple alignments performed suggested that the obtained sequences belonged to different regions in the gene, being therefore excluded from the phylogenetic analysis.

Overall, *Dactylella cylindrospora* was the species for which we obtained the least amount of functional gene sequences. Regarding the assessed pectinolytic enzymes, *D. cylindrospora* was the only species for which we weren't able to obtain any sequences (Table A6). On the other hand, the majority of functional gene sequences was found for *Articulospora tetracladia* and *Margaritispora aquatica*.

3.6. Phylogenetic analysis

ITS1-5.8S-ITS2

NJ phylogenetic tree for the ITS1-5.8S-ITS2 region of fungal strains for which functional genes sequences were obtained is presented in Figure 1. All isolates of the *Tetracladium* genus showed close phylogenetic relatedness, grouping in their own clade with a bootstrap support of 100%. The same pattern was observed for isolates of the genus *Alatospora* (also with 100% confidence) and for both isolates of *Articulospora tetracladia* (99% confidence). However, species of the genus *Anguillospora* and *Tricladium* presented substantial interspecific variability, grouping in different clades. The average overall sequence divergence was $17.58 \pm 0.96\%$. The evolutionary divergences between AQH species based on ITS-5.8S-ITS2 sequences ranged between $0.00 \pm 0.00\%$ and $27.25 \pm 1.98\%$ (Table A8). Species from the genus *Tricladium* (*T. chaetocladium* and *T. splendens*; $18.13 \pm 1.61\%$ divergence) and *Anguillospora* (*A. filiformis* and *A. crassa*; $16.83 \pm 1.60\%$ divergence), exhibited relevant interspecific divergence. However, this wasn't the case for the genera *Alatospora* (*A. acuminata* and *A. pulchella*), displaying a species divergence of $5.19 \pm 0.92\%$ and *Tetracladium* (*T. apiense*, *T. furcatum* and *T. marchalianum*), with an evolutionary divergence of $1.85 \pm 0.41\%$. The lowest interspecific divergence (0.00%) was found between *Thelonectria rubi* and *Dactyllella cylindrospora* and the higher between *Clavariopsis aquatica* and *Dimorphospora foliicola* ($27.25 \pm 1.98\%$).

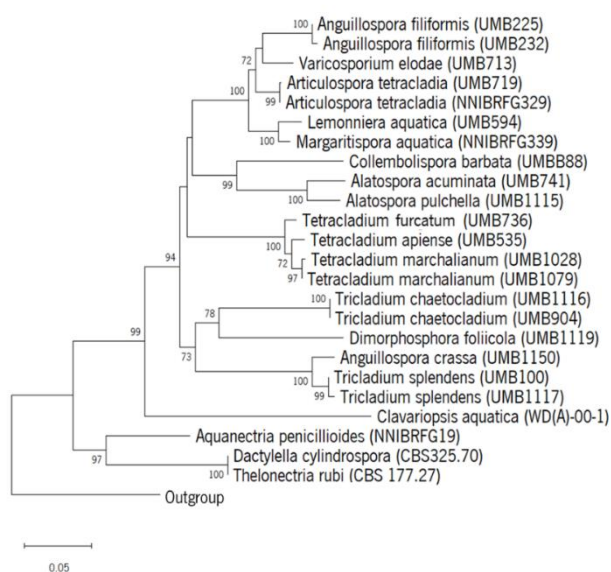


Figure 1. Neighbour joining tree based on ITS1-5.8S-ITS2 nucleotide sequences (Table A2) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full

heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroup: Genbank code MH855715.1.

Nitrate reductase

Isolates from *Anguillospora filiformis* UMB225 and UMB232 possess identical nucleotide (hence amino acid) sequences with a bootstrap support of 100% (Figure 2). *Anguillospora crassa* isolate UMB1150, however, exhibits substantial interspecific differences with both isolates from *A. filiformis* (in both phylogenetic trees), grouping in a different clade. Notwithstanding, interspecific evolutionary divergence is higher for nucleotide ($21.92 \pm 1.39\%$, Table A9) than for amino acid sequences ($11.74 \pm 1.85\%$, Table A10). The isolates *Tetracladium apiense* UMB535 and *Collembolispora barbata* UMB88 share the same nucleotide sequence, which is also the case for *Tricladium splendens* isolates UMB 100 and UMB1117 (0.00% nucleotide sequence divergence). *Tricladium chaetocladium* isolates (UMB904 and UMB1116), however, exhibit a substantial degree of divergence from one another in both nucleotide ($30.01 \pm 1.50\%$) and amino acid ($19.93 \pm 2.31\%$) sequences, stemming from different nodes. Besides, evolutionary divergence between *T. chaetocladium* and *T. splendens* is relevant for both nucleotide and amino acid sequences ($26.49 \pm 1.21\%$ and $15.48 \pm 1.75\%$, respectively). *Articulospora tetracladia* isolates UMB719 and NNIBRFG329 are highly similar (divergences of $2.43 \pm 0.47\%$ in nucleotide and $1.07 \pm 0.57\%$ in amino acid sequences), stemming from the same node in both phylogenetic trees (100% bootstrap support). This is also the case for isolates of *Lemonniera aquatica* UMB594 and *Margaritispora aquatica* NNIBRFG339 isolates (divergences of $4.76 \pm 0.70\%$ in nucleotide and $1.43 \pm 0.69\%$ in amino acid sequences), as well as *Tetracladium marchalianum* isolates UMB1028 and UMB1079 (divergences of $0.95 \pm 0.34\%$ in nucleotide and $0.36 \pm 0.35\%$ in amino acid sequences). In fact, the genus *Tetracladium* exhibits close phylogenetic relatedness, grouping in their own clade in both phylogenetic trees, with 100% confidence (divergences of $10.19 \pm 0.71\%$ in nucleotide and $3.14 \pm 0.79\%$ in amino acid sequences). This group of isolates exhibits a close phylogenetic relatedness with *Collembolispora barbata* UMB88 ($8.83 \pm 0.68\%$ nucleotide and $2.40 \pm 0.62\%$ amino acid sequence divergences). *Aquanectria penicillioides* (CBS325.70) and *Clavariopsis aquatica* (WD(A)-00-1) exhibit the highest degree of divergence in the gene phylogenetic tree ($38.78 \pm 1.63\%$ divergence), but in the protein one the highest degree of divergence was found between *Thelonectria rubi* CBS 177.27 and *Clavariopsis aquatica* (WD(A)-00-1) ($76.10 \pm 2.59\%$). Isolates from *Alatospora acuminata* UMB741 and *Alatospora pulchella* UMB1115 stem from the same

node (100% confidence) with $13.36 \pm 1.11\%$ nucleotide and $4.15 \pm 1.12\%$ amino acid sequence divergences. The average overall mean divergences for nitrate reductase nucleotide and amino acid sequences were $27.20 \pm 0.79\%$ and $22.11 \pm 1.31\%$, respectively.

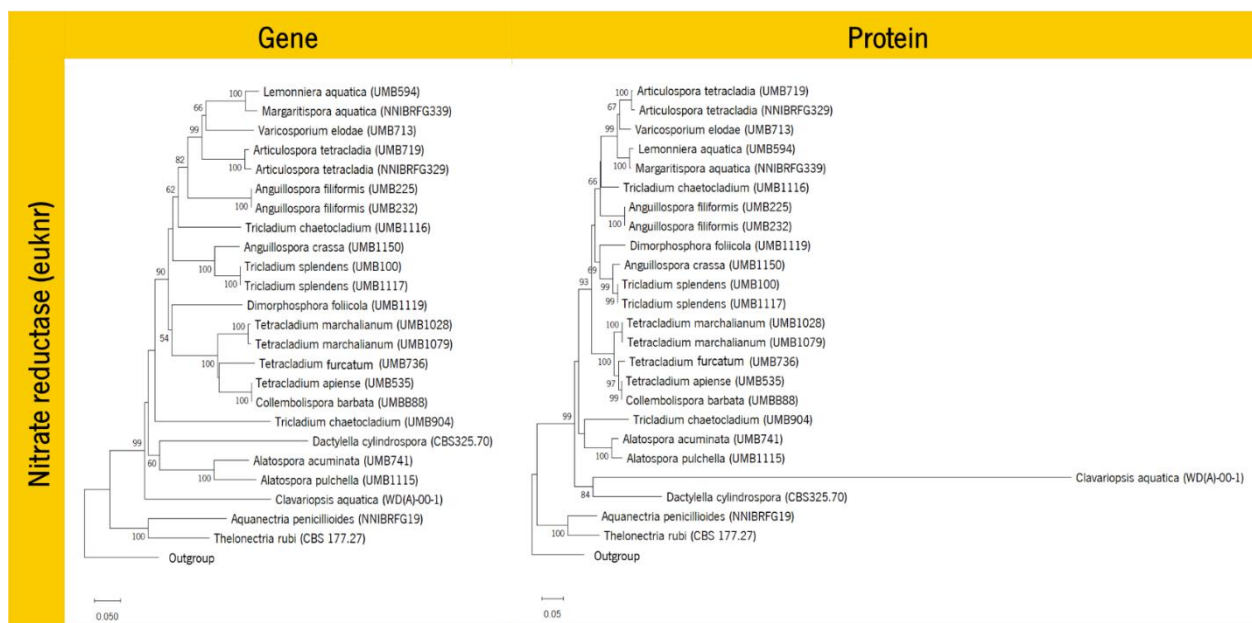


Figure 2. Neighbour joining tree based on nitrate reductase nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroup: Genbank code HQ234814.1.

Aromatic peroxygenase

The two phylogenetic trees are similar (Figure 3), with average overall mean divergences of $24.37 \pm 2.38\%$ for nucleotide and $16.67 \pm 3.52\%$ for amino acid sequences. *Articulospora tetracladia* NNIBRFG329 and *Margaritispora aquatica* NNIBRFG339 exhibit the highest similarities in nucleotide ($20.28 \pm 2.62\%$ divergence) and amino acid ($8.57 \pm 3.44\%$ divergence) sequences, stemming from the same node (bootstrap supports of 85% and 99%, respectively). These isolates and *Dactylella cylindrospora* CBS325.70 exhibit a similar nucleotide sequence divergence ($26.42 \pm 2.66\%$), however, amino acid sequence divergence is considerably higher in these case ($20.71 \pm 4.44\%$).

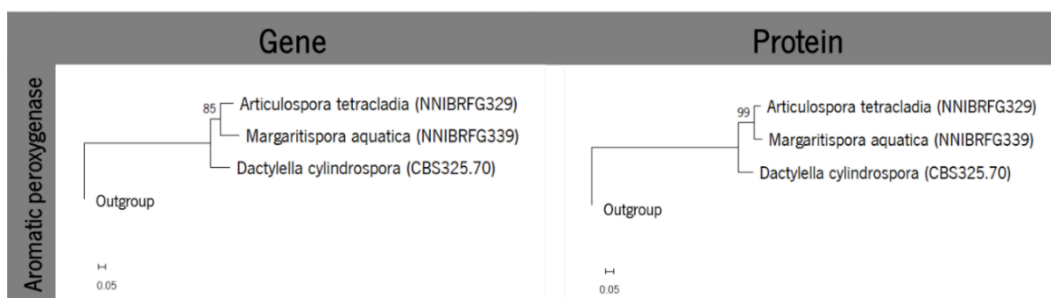


Figure 3. Neighbour joining tree based on aromatic peroxygenase nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroup: Genbank code GU734350.1.

Cellulolytic enzymes

For endoglucanase GH45, both gene and protein phylogenetic trees exhibit a main clade formed by two nodes, one consisting of *Articulospora tetracladia* NNIBRFG329 and *Margaritispora aquatica* NNIBRFG339 (51% and 96% confidence, respectively) and the other formed by *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 (96% and 98% confidence, respectively) (Figure 4). The average overall mean divergences were $38.84 \pm 1.62\%$ and $45.24 \pm 3.21\%$ for nucleotide and amino acid sequences, respectively. Endoglucanase/endo-mannosidase (GH5) trees exhibit a similar conformation, with a clade constituted by *Articulospora tetracladia* NNIBRFG329 and *Margaritispora aquatica* NNIBRFG339, and another of *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 (both with 100% bootstrap support in both trees) (Figure 4). However, the divergence between *Articulospora tetracladia* NNIBRFG329 and *Margaritispora aquatica* NNIBRFG339 was lower in GH5 than in GH45, for both nucleotide ($18.87 \pm 1.85\%$, contrasting with $34.55 \pm 2.34\%$) and amino acid ($3.42 \pm 1.66\%$, contrasting with $29.66 \pm 4.15\%$) partial sequences. This is also the case for *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 (divergence of $18.62 \pm 1.89\%$, contrasting with $31.33 \pm 2.55\%$ for nucleotide and of $13.91 \pm 3.26\%$, contrasting with $33.33 \pm 3.39\%$ for amino acid sequences). The average overall mean divergences for nucleotide and amino acid sequences for GH5 were $30.80 \pm 1.67\%$ and $27.09 \pm 3.01\%$, respectively.

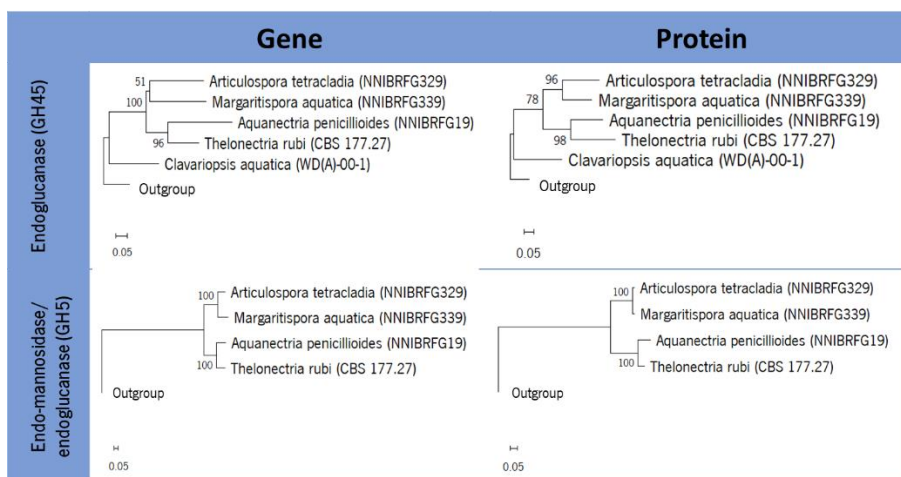


Figure 4. Neighbour joining tree based on the assessed cellulolytic enzymes – endoglucanases nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroups: GH45: Genbank code GU734468.1; GH5: Genbank code GU734400.1; glu: Genbank code HG799540.1.

The two β -glucosidase/Endo- β -xylosidase GH3 phylogenetic trees are similar (Figure 5). The main clade is formed by two nodes, one consisting of *Articulospora tetracladia* NNIBRFG329 and *Margaritispota aquatica* NNIBRFG339 (99% confidence in both trees) and the other formed by *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 (bootstrap support of 98% for gene and 96% for protein phylogenetic trees). These were the pairings with the lowest nucleotide ($14.59 \pm 2.30\%$ for *A. tetracladia* and *M. aquatica*, $18.03 \pm 2.50\%$ for *A. penicillioides* and *T. rubi*) and amino acid ($2.60 \pm 1.74\%$ for *A. tetracladia* and *M. aquatica*, $5.20 \pm 2.50\%$ for *A. penicillioides* and *T. rubi*) sequence divergence. The average overall mean divergences for nucleotide and amino acid sequences were $31.89 \pm 2.01\%$ and $25.86 \pm 3.12\%$, respectively.

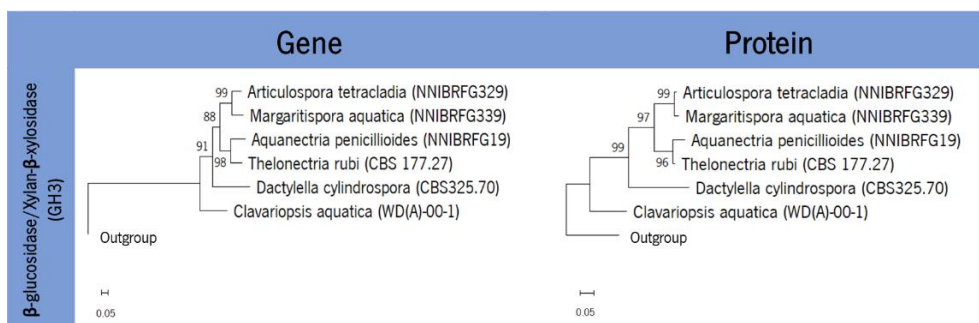


Figure 5. Neighbour joining tree based on the assessed cellulolytic enzymes – β -glucosidase nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroup: Genbank code GU734392.1.

For Cellobiohydrolase I/endoglucanase (GH7), *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 present a closer phylogenetic relatedness, stemming from the same node (100% and 62% confidence in each tree, respectively) (Figure 6). That being said, this pair still exhibits a considerable degree of divergence for both nucleotide ($25.09 \pm 1.74\%$) and amino acid ($32.80 \pm 3.47\%$) sequences. This was the only node in the GH7 gene tree with a bootstrap support above 50% in the functional gene tree, meaning that these results are lacking statistical power in comparison to the majority of the distinct functional gene nucleotide sequences acquired. The average overall mean divergences are $31.83 \pm 1.18\%$ for nucleotide and $38.85 \pm 2.55\%$ for amino acid sequences. For Cellobiohydrolase I (cbhl), *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 grouped together with 100% bootstrap support on both trees (divergences of $20.96 \pm 1.70\%$ for nucleotide and $13.90 \pm 2.42\%$ for amino acid sequences) (Figure 6). *Articulospora tetracladia* NNIBRFG329 and *Clavariopsis aquatica* WD(A)-00-1 display a closer phylogenetic relatedness, stemming from the same branch (84% confidence) in the protein but not in the gene phylogenetic tree. Notwithstanding, this pair exhibits substantial divergence for both nucleotide ($40.93 \pm 2.05\%$) and amino acid ($46.78 \pm 3.82\%$) sequences. The average overall mean nucleotide and amino acid sequence divergences are $34.67 \pm 1.21\%$ and $42.94 \pm 2.39\%$ respectively. For Cellobiohydrolase II (GH6), *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 present the lowest nucleotide ($25.25 \pm 2.19\%$) and amino acid ($28.23 \pm 3.89\%$) sequence divergence, stemming from the same node (confidences of 96 and 91%, respectively) (Figure 6). *Articulospora tetracladia* NNIBRFG329 and *Margaritispota aquatica* NNIBRFG339 exhibit a lower degree of nucleotide divergence ($22.60 \pm 1.92\%$) comparing to their degree of amino acid sequence divergence ($63.43 \pm 4.18\%$). The average overall mean nucleotide and amino acid sequence divergences were $42.33 \pm 1.39\%$ and $63.58 \pm 2.23\%$, respectively.

Considering all assessed cellulases, an overall pattern of lower divergence between *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 is noticeable. *Articulospora tetracladia* NNIBRFG329 and *Margaritispota aquatica* NNIBRFG339 were also amongst the least divergent in endoglucanases (GH45 and GH5) and β -glucosidase (GH3). This wasn't the case for cellobiohydrolases (GH7, cbhl, GH6), where the already relevant nucleotide divergence translated into an even higher amino acid divergence.

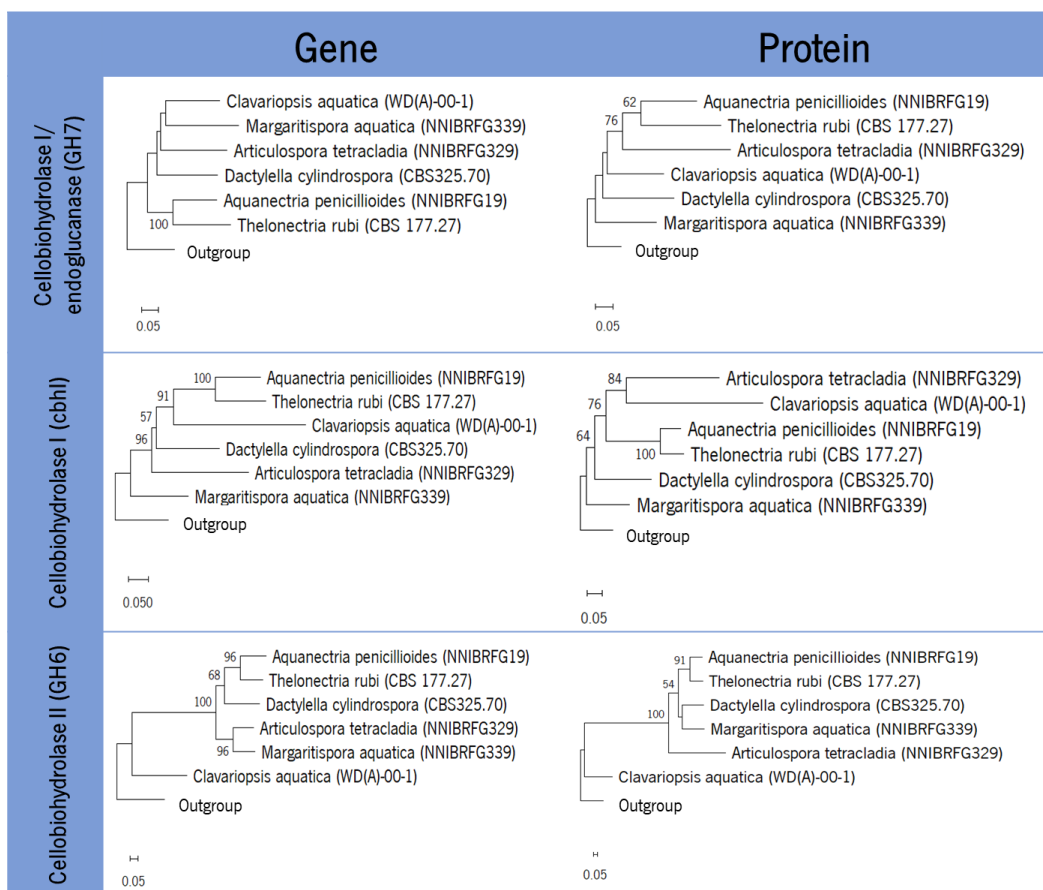


Figure 6. Neighbour joining tree based on the assessed cellulolytic enzymes – exoglucanases nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroups: GH7: Genbank code GU734556.1; cbhl: Genbank code EU345462.1; GH6: Genbank code GU734409.1.

Hemicellulolytic enzymes

Endo- β -xylanase (GH11) phylogenetic trees are similar, with an average overall mean nucleotide and amino acid sequence divergences of $32.42 \pm 1.48\%$ and $29.65 \pm 2.64\%$, respectively (Figure 7). *Auanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 are phylogenetically closer, stemming from the same node (98-99% confidence). This pair exhibited the lowest nucleotide ($21.94 \pm 1.89\%$) and amino acid ($6.92 \pm 2.12\%$) divergences. *Margaritispora aquatica* NNIBRFG339 and *Articulospora tetracladia* NNIBRFG329 display closer phylogenetic relatedness (99%-100% confidence), with $23.33 \pm 2.01\%$ (nucleotide) and $13.18 \pm 2.85\%$ (amino acid) sequence divergences. However, for endo-xylanase (xyn2), this pair exhibited a higher nucleotide divergence ($34.83 \pm 2.02\%$) that translated into an even higher amino acid ($83.70 \pm 3.07\%$) divergence. *Auanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 exhibited a substantial

degree of nucleotide divergence ($30.69 \pm 2.10\%$) for endo-xylanase (*xyn2*), but a lower divergence for amino acid sequences ($6.92\% \pm 2.12\%$). This shift in similarity is also noticeable in the phylogenetic trees, as the isolates group together in the protein tree (99% bootstrap support) but not in the gene tree. The average overall mean nucleotide and amino acid sequence divergences was $32.42 \pm 1.48\%$ and $29.65 \pm 2.64\%$, respectively.

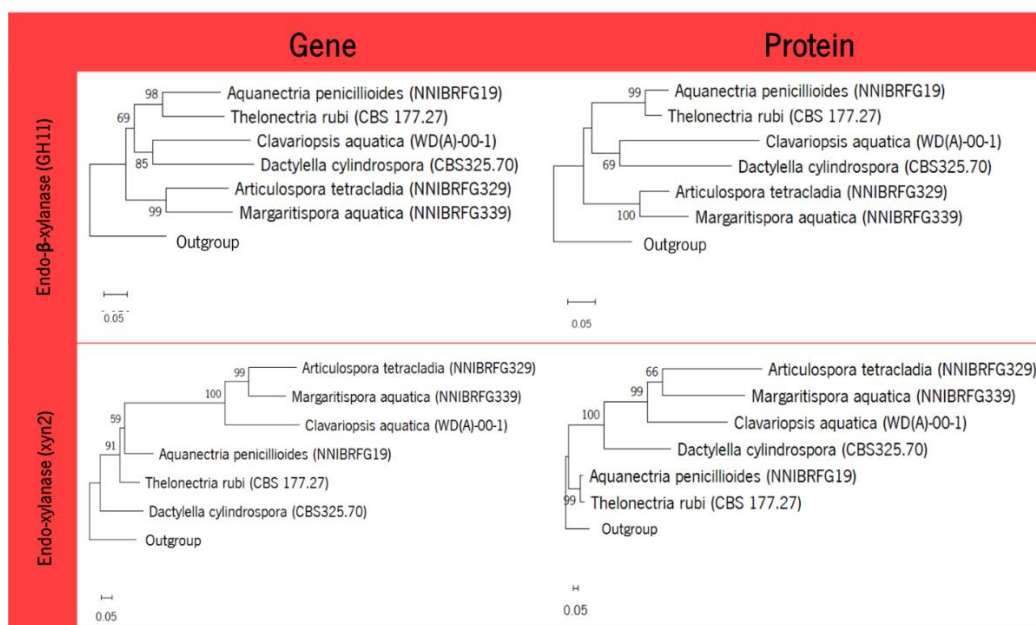


Figure 7. Neighbour joining tree based on the assessed hemicellulolytic enzymes – endo-xylanases nucleotide (left) and amino acid (right) (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroups: GH11: Genbank code GU734415.1; *xyn2*: Genbank code EU532196.1.

In the case of α -glucosidase (GH31), even though *Margaritispora aquatica* NNIBRFG339 and *Articulospora tetracladia* NNIBRFG329 exhibited a nucleotide divergence on the lower end ($21.00 \pm 1.14\%$), it translated into a substantially higher amino acid divergence ($77.67 \pm 2.24\%$). This discrepancy is evinced in their phylogenetic relationship, as they group together in the node in the gene (100% confidence), but not in the protein tree (Figure 8). This scenario of higher amino acid sequence divergence was verified for most of the sequences, with the overall mean distance being $74.59 \pm 1.51\%$, contrasting with $35.06 \pm 1.03\%$ for nucleotide sequences.

This was not the case for α -glucuronidase, where *Margaritispora aquatica* NNIBRFG339 and *Articulospora tetracladia* NNIBRFG329 presented the lowest values of both nucleotide ($15.89 \pm 1.42\%$) and amino acid ($12.37 \pm 2.24\%$) sequence divergence, stemming from the same

nodes in both trees (100% bootstrap support). The average overall mean divergences were $32.92 \pm 1.32\%$ for nucleotide and $38.35 \pm 2.37\%$ for amino acid sequences. For α -mannosidase (GH92), *M. aquatica* and *A. tetracladia* exhibit the highest degree of similarity, stemming from the same node (100% bootstrap support; divergences of $20.85 \pm 2.63\%$ and $25.71 \pm 5.20\%$ for nucleotide and amino acid sequences, respectively) (Figure 8). The average overall mean divergences for nucleotide and amino acid sequences are $35.75 \pm 2.37\%$ and $40.03 \pm 4.79\%$, respectively. *M. aquatica* and *Clavariopsis aquatica* WD(A)-00-1 presented higher divergence for all 3 aforementioned functional genes (varying between 34.27-43.60% for nucleotide and 47.89-77.18% for amino acid sequences).

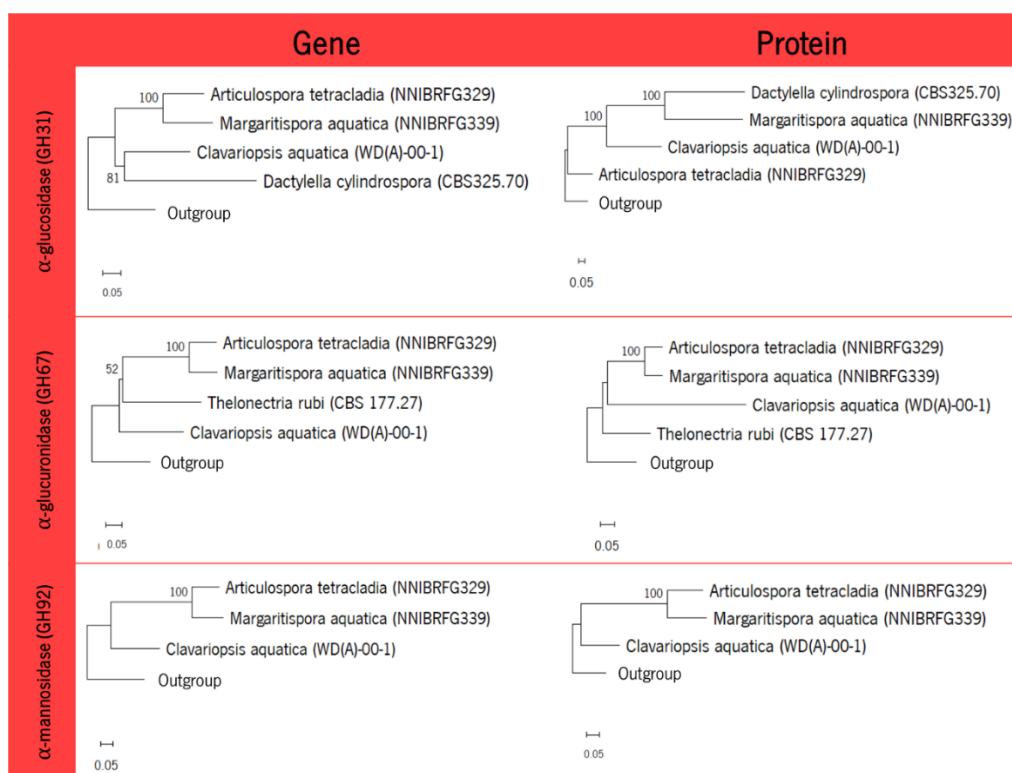


Figure 8. Neighbour joining tree based on the assessed hemicellulolytic enzymes – glucosidases, glucuronidases and mannosidases nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroups: GH31: Genbank code GU734458.1; GH67: Genbank code GU734488.1; GH92: Genbank code GU734500.1.

Pectinolytic enzymes

For Pectate lyase (*plyA*), *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 are highly related, stemming from the same node, which is also the case for *Articulospora*

tetracladia NNIBRFG329 and *Margaritispota aquatica* NNIBRFG339 (both pairs with 100% confidence on both trees) (Figure 9). *A. penicillioides* and *T. rubi* exhibit a low degree of nucleotide ($21.21\pm 1.33\%$) and amino acid ($12.46\pm 1.75\%$) divergence. Even though *Articulospora tetracladia* and *M. aquatica* display an even lower degree of nucleotide divergence ($12.69\pm 1.08\%$), their divergence in terms of amino acid sequences is much higher ($70.36\pm 2.50\%$). This scenario of higher amino acid sequence divergence repeated itself for the remaining pairings. The average overall mean distances were $50.42\pm 1.59\%$ for nucleotide and for $83.39\pm 1.68\%$ amino acid sequences.

For Pectinesterase, *Articulospora tetracladia* NNIBRFG329 and *Margaritispota aquatica* NNIBRFG339 display a closer phylogenetic relatedness in the protein tree, stemming from the same node (96% bootstrap support), than in the gene tree. This resonates with their nucleotide and amino acid sequence divergences ($35.35\pm 1.46\%$ and $30.70\pm 2.41\%$, respectively). *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 group together in the same branch (97% and 98% confidence for each tree) (Figure 9). Despite the phylogenetic relatedness, the degree of divergence for both nucleotide and amino acid sequences was high ($40.78\pm 1.52\%$ and $58.36\pm 2.62\%$, respectively). Once again, this was the case for the remaining pairings. The average overall mean divergences were $42.71\pm 0.99\%$ for nucleotide and $51.71\pm 1.65\%$ for amino acid sequences.

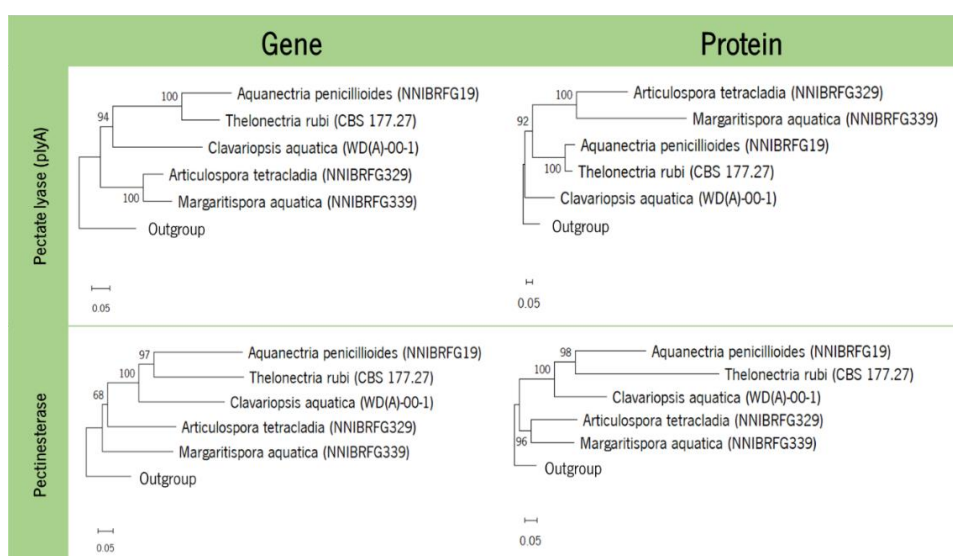


Figure 9. Neighbour joining tree based on the assessed pectinolytic enzymes nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroups: enpg: Genbank code KU664140.1; plyA: Genbank code XM_031151333.1; pectinesterase: Genbank code XM_031149046.1.

Acid phosphatase

The main clade (100% confidence in both trees) presents one branch constituted by *Margaritispora aquatica* NNIBRFG339 and *Articulospora tetracladia* NNIBRFG329 and other branch constituted by *Aquanectria penicillioides* NNIBRFG19 and *Thelonectria rubi* CBS 177.27 (>96 confidence) (Figure 10). Both pairs exhibited a low degree of nucleotide sequence divergence ($17.22\pm 1.44\%$ and $16.60\pm 1.27\%$, respectively), but a noticeably higher degree of amino acid sequence divergence ($50.61\pm 3.08\%$ and $47.32\pm 3.02\%$, respectively). A higher amino acid sequence divergence was also the case for the remaining pairs, with the exception of *Clavariopsis aquatica* WD(A)-00-1 and *Dactylella cylindrospora* CBS325.70 (divergences of $27.80\pm 1.60\%$ for nucleotide and $11.61\pm 1.90\%$ for amino acid sequences). This corroborates their conformation in the phylogenetic trees, as they were more closely related in the protein tree (stemming from the same node with 77% confidence) than in the gene tree. The average overall mean divergences were $40.62\pm 1.01\%$ for nucleotide and $70.26\pm 1.21\%$ for amino acid sequences.

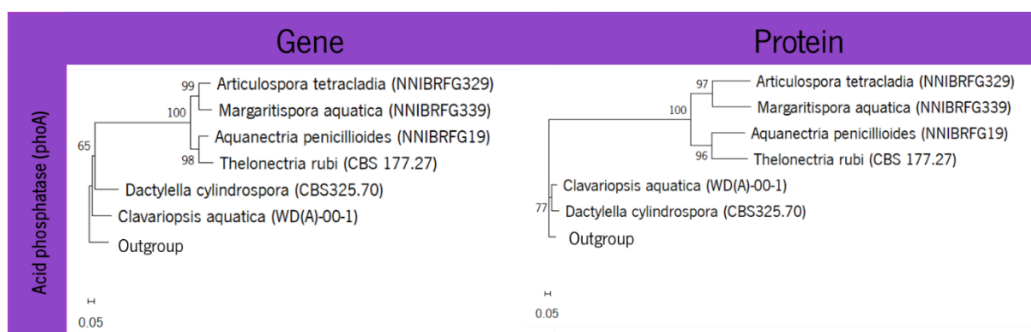


Figure 10. Neighbour joining tree based on Acid phosphatase nucleotide (left) and amino acid (right) sequences (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions. Outgroup: Genbank code XM_014678996.1.

Overall functional gene phylogenetic relatedness

Comparing all *in silico* obtained functional gene nucleotide sequences together (Figure 11), an average overall nucleotide mean divergence of $37.73\pm 0.32\%$ was found. The main clade (with 100% bootstrap support) is constituted by a branch with *Margaritispora aquatica* and *Articulospora tetracladia*, and another one with *Aquanectria penicillioides* and *Thelonectria rubi* (both pairs with 100% bootstrap support). Concordantly, their nucleotide sequence divergences were the lowest ($25.11\pm 0.45\%$ for the first and $24.98\pm 0.38\%$ for the second pair). A pattern of close relatedness

between these groups was noticeable in most of the phylogenetic trees (Figures 3-10). *Clavariopsis aquatica* WD(A)-00-1 and *Aquanectria penicillioides* displayed the highest degree of nucleotide sequence divergence ($42.70 \pm 0.55\%$). Comparing mean distance phylogenetic tree results between all *in silico* assessed genes, there seems to be no pattern between the type of enzymatic activity and the degree of phylogenetic divergence, as the 3 highest observed mean distances comprised genes with cellulytic (Cellobiohydrolase II (GH6), $42.33 \pm 1.39\%$), hemicellulolytic (Xylanase (xyn2), $48.77 \pm 1.25\%$) and phosphoric (acid phosphatase (phoA), $40.62 \pm 1.01\%$) activities.

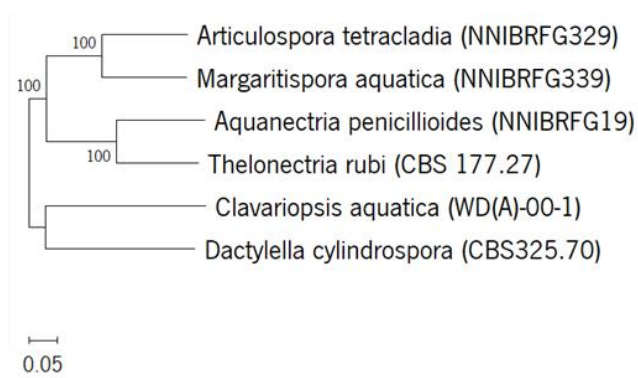


Figure 11. Neighbour joining tree based on the nucleotide sequences of all studied functional genes (Table A6-Table A7) using Maximum composite likelihood distances. Numbers displayed at the nodes represent bootstrap values above 50%, calculated from 1000 full heuristic replicates. Scale bar represents one base change per 100 nucleotide positions.

Chapter 4

Discussion

Discussion

Most studies on detection and biodiversity assessments of functional genes codifying carbon and inorganic nutrient degrading enzymes comprise community analysis of decomposer microorganisms, whether bacteria or fungi, in terrestrial ecosystems (e.g. Hirschhäuser and Fröhlich, 2007; Edwards et al., 2008; Asemoloye et al., 2018; Rykov et al., 2019). For freshwater ecosystems, most studies on bacterial and fungal contribution to the plant litter breakdown process focus on biomass comparisons and production rates estimates (Weyers and Suberkropp, 1996; Baldy et al., 2002; Gulis and Suberkropp, 2003a). Notwithstanding, some studies also evaluate functional parameters like enzymatic performances of fungi and bacteria (e.g. Romani et al., 2006) or the entire microbial community (e.g. Mora-Gómez et al., 2018). AQH have been established as the main microbial decomposers of leaf litter in streams, being able to inhabit and colonize a wide range of decaying leaves (Weyers and Suberkropp, 1996; Pascoal and Cássio, 2004). Regarding their enzymatic potential for leaf litter decomposition, several qualitative assessments on extracellular enzymatic activities have been performed (e.g. Zemek et al., 1985; Suberkropp and Jones, 1991; Abdel-Raheem and Ali, 2004). However, studies on aquatic fungal genetic biodiversity are mostly restricted to their taxonomy (e.g. Nikolcheva and Bärlocher, 2004; Seena et al., 2012; Baschien et al., 2013), with the exception of a recent study assessing genomic and ecological diversity of several *Tetracladium* species, through detection of carbohydrate active enzymes (e.g. pectate lyase) and secondary metabolites (Anderson and Marvanová, 2020). The present study is the first, to the best of our knowledge, to perform an extensive functional gene analysis of leaf-litter-degrading extracellular enzymes of AQH species, aiming to assess their functional biodiversity. To plausibly represent this diversity and assess whether taxonomic phylogenetic affiliation is correlated with functional gene relatedness, 30 species from 22 genera (4 different classes) were studied.

Amplicons potentially containing the gene of interest were obtained for nitrate reductase, β -glucosidase, endopolygalacturonase and laccase enzymes, with amplification success rates of 56.25%, 78.26%, 69.57% and 78.13%, respectively. However, it is relevant to state that the lack of amplification doesn't necessarily mean functional gene absence. Even though all the primer pairs utilized were degenerate, they were designed based on terrestrial fungal species, which might be very distinct from the AQH. A single nucleotide difference (in the fungal gDNA) from the possibilities comprised by that primer would mean a lack of amplification, even though the desired nucleotide sequences could still very well exist. Our results showed that the successful amplicons displayed

multiple bands. Because of that, cloning was performed in order to obtain the sequences of the genes of interest. Nitrate reductase was the first selected gene for cloning due to the following reasons: (I) the agarose gel exhibited the least amount of non-specific bands, and (II) amongst the successful amplified genes, nitrate reductase was the only one previously amplified in AQH species (4 isolates of the *Tetracladium* genus (Gorfer et al., 2011)). Regarding β -glucosidase gene, the obtained sequences presented inconclusive results, as sequences from the same sample did not align or weren't related to any sequences available at NCBI (as revealed by the blastn analysis). This was probably a result of amplification of non-specific amplicons, as multiple bands were observed in the agarose gel. In the original article from which the β -glucosidase gene primers (sense/antisense) were retrieved, there were no indications of the PCR conditions used. We attempted to amplify this gene using reagent concentrations and PCR conditions that worked for other functional gene amplifications and performing a gradient PCR to determine the most suitable annealing temperature. The optimal annealing temperature was 48°C, which is relatively low and might have potentiated the non-specific amplifications. An alternative to optimize this protocol and achieve β -glucosidase gene amplification would be touchdown PCR. This procedure focuses on annealing temperature variations (incremental decreases), rather than attempting distinct combinations of buffer and cycling conditions, being therefore very useful in cases where the degree of primer/template complementarity is not fully known (Hecker and Roux, 1996). Notwithstanding, considering the primers used in this study were developed for other organisms with a considerable degree of genetic divergence from AQH, the design of more specific primers for these organisms would probably increase the success rates of β -glucosidase gene amplification.

The increasing sequence data availability and strong computational biology development throughout the years solidified the acceptance and implementation of virtual investigation methods driven by computational strategies by the scientific community (Murray et al., 2007). The recent publication of 6 AQH whole genomes in public databases opened a world of possibilities to our study, allowing a complementary *in silico* approach to obtain functional genes sequences. Sequences from a total of 16 functional genes, selected based on their relevance to litter breakdown and nutrient cycling processes, and the previous data concerning AQH enzymatic activity, were successfully retrieved from the available genomes.

The 19 species utilized in the taxonomic and initial functional gene assessments, considering nitrate reductase, are comprised in four different classes: Orbiliomycetes, Sordariomycetes,

Dothideomycetes and Leotiomyces (Table 2). Partial functional gene sequences obtained by cloning procedures comprised fungal species belonging to Dothideomycetes and Leotiomyces. *In silico* analyses were a useful resource to access a wider variety of functional gene sequences, while adding biodiversity to the already existing fungal strains pool, by the inclusion of new sequences belonging to the classes Orbiliomyces and Sordariomyces. The majority of the isolates used in this study belongs to Leotiomyces, which are recognized as one of the most diverse classes of Ascomycota (Baral, 2016). Current classification of Leotiomyces is still mostly based on taxa morphology (traditional identification methods), commonly conflicting with the phylogenetic relationships suggested by sequencing studies (Johnston et al., 2019). As phylogenetic relatedness and similarities in functional diversity are expected to be correlated, functional gene analyses can provide new insights on fungal classifications and species role in the ecosystems (Johnston et al., 2019). Several results in our study suggest the possibility of a correlation between phylogenetic relatedness and functional gene similarities. ITS1-5.8S-ITS2 sequence results displayed a clade comprised by *Varicosporium elodeae*, *Articulospora tetracladia*, *Anguillospora filiformis*, *Lemonnieria aquatica* and *Margaritispora aquatica*, which was also observed by Baschien and collaborators, when assessing molecular phylogenies of AQH with affinity to Leotiomyces (Baschien et al., 2013). Nitrate reductase phylogenetic trees also reflect this close phylogenetic relatedness. Additionally, ITS1-5.8S-ITS2 and nitrate reductase phylogenetic trees exhibited relevant divergences between *Anguillospora filiformis* and *Anguillospora crassa*, and close phylogenetic relationship between *A. crassa* and *Tricladium splendens*. Previous phylogenetic assessments support these results (Belliveau and Bärlocher, 2005; Baschien et al., 2006, 2013). The polyphyly of the genus *Anguillospora* is well documented, comprising anamorphs of seven teleomorph genera, distributed among Dothideomycetes, Orbiliomyces and Leotiomyces (Belliveau and Bärlocher, 2005; Baschien et al., 2006, 2013). Besides, all *Tetracladium* isolates (including *T. apiense*, *T. marchalianum* and *T. furcatum*) were comprised in one clade in both ITS1-5.8S-ITS2 and nitrate reductase phylogenetic trees, which is supported by previous phylogenetic (Baschien et al., 2013) and nitrate reductase (Gorfer et al., 2011) assessments. This genus has been described as monophyletic by several studies (Nikolcheva and Bärlocher, 2002; Baschien et al., 2006; Bärlocher et al., 2010). The grouping of Helotiales in a major branch was observed in both ITS1-5.8S-ITS2 and nitrate reductase phylogenetic trees as is concordant with previous phylogenetic (Baschien et al., 2013) and nitrate reductase (Gorfer et al., 2011) assessments.

For most of the cases where the retrieval of functional gene sequences from both *Aquanectria penicillioides* and *Thelonectria rubi* was possible, they exhibited high degrees of phylogenetic relatedness. This pair also displayed a close taxonomic relationship, which could be linked with the fact that these are the only two species belonging to Hypocreales. In the case of Endoglucanase GH5, the results obtained by Barbi and collaborators support this hypothesis, as their isolates belonging to Hypocreales also exhibited high functional gene phylogenetic relatedness (Barbi et al., 2014). However, the observed concordance between taxonomic and functional gene phylogenetic relatedness wasn't always the case. For instance, *Tricladium chaetocladium* isolates UMB904 and UMB1116, despite being highly similar at a taxonomic level (0.0% divergence), displayed a considerably higher degree of divergence for the nitrate reductase gene ($19.93 \pm 2.31\%$). Another example is the substantial divergence between *Margaritispora aquatica* and *Articulospora tetracladia* ($33.39 \pm 1.82\%$) for the Cellobiohydrolase I (GH7) gene, although these species are highly similar taxonomic-wise ($5.46 \pm 0.95\%$ divergence). These results suggest that close taxonomic phylogenetic relatedness does not inherently translate into highly similar functional gene nucleotide sequences. Notwithstanding, results from the phylogenetic tree comprising the compilation of all studied functional genes exhibit a correlation between phylogeny and functional diversity (close relatedness between *A. tetracladia* and *M. aquatica* (Leotiomycetes) and *A. penicillioides* and *T. rubi* (Sordariomycetes)).

When comparing the gene and protein phylogenetic trees we observed that in some cases the divergence was higher in the gene but other times it was higher in the protein. Exemplifying with Cellobiohydrolase II (GH6), the pair *Articulospora tetracladia* NNIBRFG329 and *Margaritispora aquatica* NNIBRFG339, with a relevant divergence in their nucleotide sequences ($22.60 \pm 1.92\%$), exhibited an even higher amino acid divergence ($63.43 \pm 4.18\%$). Due to the fact that a single base difference can be translated into a different codon, interspecific nucleotide divergences that are maintained in amino acid sequences can proportionally result into higher amino acid divergences, as every three bases correspond to only one codon. However, for some other cases like β -glucosidase/ xylan- β -xylosidase (GH3), the already low degree of nucleotide divergence exhibited by the same pair ($14.59 \pm 2.30\%$) translated into an even lower degree of amino acid sequence divergence ($2.60 \pm 1.74\%$). Due to the redundancy of the genetic code, distinct codons might codify to the same amino acids, meaning that interspecific differences in the gene sequences don't always translate into differences in the protein sequences.

Regarding divergence among functional genes, we observed that the average overall mean divergence was considerably variable even within the same type of functional genes (e.g. endoxylanases GH11 and xyn2 ($32.42 \pm 1.48\%$ and $48.77 \pm 1.25\%$, respectively)). These interspecific differences can be an indicator of enzymatic functionality variance, which could possibly lead to differences in enzymatic behaviour or degradative yields. However, it is important to point out that all functional genes were considered using only partial sequences (ranging between approximately 0.2 and 1.2 kb), since we could not obtain the complete gene sequence. For every gene, some regions revealed, as expected, more conserved than others. Therefore, the differences in phylogenetic relatedness/divergences might be a result of using only partial sequences of the genes, where some might present more conserved regions than others. To address this question, it would be important to access the complete functional gene sequences for each of the selected functional gene partial sequences.

Even though AQH are capable of producing a wide range of extracellular enzymes, several studies have reported species-specificity, as well as variations in presence and strength of their enzymatic abilities (e.g. Suberkropp et al., 1983; Zemek et al., 1985; Abdel-Raheem and Ali, 2004) as summarized in Table 1. Distinct substrate-specificity among AQH communities is very likely related to existence/absence of functional genes and, therefore, the degradative extracellular enzymes they codify. To our knowledge, the degradative abilities of *Dactylella cylindrospora* weren't yet assessed, being our results the first assessment enlightening its functional gene potential and consequently its enzymatic capabilities.

In the present study, partial nucleotide sequences were found for all the functional genes codifying for the enzymes with described degradative activities. Our results showed that *Margaritispora aquatica* (not assessed in (Suberkropp and Jones, 1991) possess an acid phosphatase (phoA) gene. Additionally, partial sequences for α -glucuronidase, pectinesterase, nitrate reductase, acid phosphatase and aromatic peroxygenase, not previously evaluated in terms of enzymatic activities in pure AQH cultures, were retrieved for most of the assessed organisms. However, for *Dactylella cylindrospora*, none of the pectinases assessed were found (met the similarity criteria) in its genome. It is important to reiterate that the multiple alignments performed comprised nucleotide sequences from very distinct organisms (mostly terrestrial fungi). That being said, *D. cylindrospora* functional gene sequences can differ greatly from the ones gathered for the alignments of those pectinases. The inability to retrieve partial functional gene sequences doesn't necessarily mean

that an organism doesn't possess functional genes coding for that enzyme. These factors must be taken into consideration before making assumptions about the inexistence of functional genes, and consequently degradative abilities, in any organism. Another inference of our study is that a clear correlation between phylogenetic relatedness and the degree of enzymatic activity/substrate degradation doesn't seem to exist. In particular, *A. tetracladia* and *C. aquatica* were described as having weak phosphatidic activities, while *A. penicillioides* as having strong ones (Suberkropp and Jones, 1991). However, our phylogenetic assessments for acid phosphatase revealed that the degree of nucleotide divergence between *A. tetracladia* and *C. aquatica* ($55.34 \pm 1.83\%$) was considerably higher than the one obtained between *A. tetracladia* and *A. penicillioides* ($25.15 \pm 1.58\%$).

Environmental factors (such as vegetation type and abundance) are known to influence biota's phenology and physiology, having the potential to massively affect litter decomposition, nutrient cycling and stream productivity (Gessner et al., 2010; Krauss et al., 2011). Leaf litter varies immensely in chemical composition depending on the type of vegetation and AQH degradative abilities seem to vary depending on the type of substrate, indicating a certain degree of selectivity (Barlocher, 1982; Thomas et al., 1991; Gulis, 2001). Typically, the bulk of AQH carbon-degrading enzymatic abilities is directed to the degradation of more labile, rather than recalcitrant, carbon compounds (Chamier, 1985; Zemek et al., 1985). Lignin is a particularly recalcitrant compound, for which AQH seem to have a low degradative capacity (Chamier, 1985; Gessner et al., 2007). This might justify the scarcity of functional genes for lignolytic enzymes in our results (despite being one of our most searched enzymes). Aromatic peroxygenase was the only functional gene connected with lignin degradation from which nucleotide sequences were obtained, in 3 out of the 6 AQH genomes. Nevertheless, functional genes for laccases were previously found for *Clavariopsis aquatica* (Solé et al., 2008).

Temperature increases due to global warming are one of the most striking threats to freshwater biodiversity (Geraldes et al., 2012; Martínez et al., 2014). As freshwater ecosystems are particularly susceptible to climate alterations, understanding how species are affected in terms of survival, interactions and ecological functioning is of paramount relevance for aquatic ecologists (Dudgeon et al., 2006; Parmesan, 2006; Duarte et al., 2013a). In these ecosystems, plant litter is usually colonized by a considerable number of AQH (up to 20 sporulating species), with a smaller number of species (1-4) dominating the assemblage through high levels of sporulation (>90% of the

community sporulation capacity) (Pascoal et al., 2005b; Duarte et al., 2009, 2010). Changes in stream water temperature deeply impact AQH assemblages (Bärlocher, 2000; Nikolcheva and Bärlocher, 2005). For instances, AQH winter assemblages are commonly replaced by summer assemblages in response to water temperature increases (Suberkropp, 1984; Bärlocher, 1991). Temperature increases are predicted to stimulate AQH biological activities, such as microbial respiration and enzymatic activity (Bärlocher et al., 2008; Canhoto et al., 2016). The accelerated litter decomposition rates could end up inducing anaerobic conditions in the microhabitats, shortening AQH residence time in the available substrates (Field and Webster, 1983; Bärlocher et al., 2008). This, paired with increases in the stream water temperature, could lead to losses in fungal richness and diversity (Hughes, 2000; Bärlocher et al., 2008). AQH species have distinct thermal profiles and species with lower optimal and maximum growth and sporulation temperatures might be less tolerant to temperature increases (Bärlocher and Kendrick, 1974; Bärlocher, 1992; Sridhar and Bärlocher, 1993). If these sensitive species were to possess functional genes (and, therefore, enzymatic capacities) that aren't shared by other species in the fungal community, their loss could potentially compromise ecosystem functioning. As an example, partial nucleotide sequences for laccase's functional gene were found in *Clavariopsis aquatica* by Solé and collaborators (Solé et al., 2008). In the present study, we were able to retrieve these nucleotide sequences from *C. aquatica* genome, but not from the genomes of any of the other available AQH species. Let's assume that the presence of laccase genes in *C. aquatica* was an indicator of lignolytic activity ability (i.e., the functional genes were expressed, and the extracellular enzymes produced degraded lignin compounds) and that the absence of laccase genes in *Articulospora tetracladia*, *Thelonectria rubi*, *Aquanectria penicillioides*, *Margaritispora aquatica* and *Dactylella cylindrospora* was an indicator of the species inability to produce laccases. In this scenario, the effects of global change (like increases in the stream water temperature) in a fungal community consisting of these six species, could potentially lead to the loss of *Clavariopsis aquatica*, since this species possesses low optimal and maximum growth/sporulation temperatures (10-15°C (Rajashekhar and Kaveriappa, 2000)). If that was the case, this community would no longer be able to degrade lignin compounds, possibly having less access to a part of the labile carbon sources (as lignin intimately associated with cellulose microfibrils) (Chamier, 1985). This could hinder not only fungal assemblages but also higher trophic levels, due to limitations in nutrient cycling and energy transfers (Gessner et al., 2010). Notwithstanding, scenarios where biodiversity loss doesn't translate into losses in function are also possible. Let's keep the hypothesis

of a fungal assemblage comprising *Articulospora tetracladia*, *Thelonectria rubi*, *Aquanectria penicillioides*, *Margaritispora aquatica*, *Clavariopsis aquatica* and *Dactylella cylindrospora*, where lack/presence of functional gene corresponds to lack/presence of enzymatic abilities. Taking endo-xylanases (hemicellulolytic enzymes) as an example, if this community was severely affected by increased temperatures, losing species such as *Clavariopsis aquatica* (likely to be very sensitive to temperature increases due to its lower range of optimal and maximum growth temperatures) and *A. tetracladia* (with documented growth/sporulation inhibition above 25°C (Fernandes et al., 2009; Geraldine et al., 2012; Duarte et al., 2013a)), probably it would not severely compromise the community's xylanolytic abilities. As all AQH species in the assemblage possess xylanolytic activities and (at least) *Margaritispora aquatica* is known to respond well to higher temperatures (optimal mycelial growth between 20-25°C (Koske and Duncan, 1974)), some (if not all) of the remaining species would also be able to degrade xylans, and therefore maintain enzymatic function. That being said, it is important to point out that these kinds of predictions, as they comprise the influence of several joint effects on litter decomposition and fungal communities, are still conjectural. Most studies point to a positive relation between AQH species diversity and leaf mass loss (e.g. Barlocher and Corkum, 2003; Duarte et al., 2006). But, environmental factors can modulate this relation (e.g. Barlocher et al., 2008; Baudoin et al., 2008). For instance, temperature alterations have been shown to affect not only individual AQH performances but also interspecific relationships within communities, potentially leading to shifts in dominance and/or altering species succession patterns (Duarte et al., 2013a) or losses in fungal richness and diversity (Hughes, 2000; Barlocher et al., 2008). Our results cement the need for a better understanding of the AQH functional genes diversity and the extension of global change impacts on enzymatic activities. Further studies would undoubtedly provide very relevant information on nutrient cycling, litter breakdown and energy transfer dynamics as well as on potential ways of recovery/preservation of freshwater biota and ecosystem functions.

Final considerations and future perspectives

The goal of assessing and evaluating functional gene diversity in different AQH species was met. In many cases, phylogenetic similarities at a taxonomic level weren't representative of functional gene phylogenetic relatedness. Even though the number of isolates for each species was low (most of the times with only one, and at best two isolates per AQH species), some cases of functional intraspecific differences were found. Performing this kind of studies with a higher representativity number of isolates per species would be of great relevance to enhance results robustness and infer intraspecific variability in a clearer manner. There were also cases where, due to redundancy in the genetic code, functional gene nucleotide sequence divergence did not translate (in the same degree) into amino acid sequence divergence. Ascomycetous AQH have been placed in the subphylum Pezizomycotina in five different classes: Leotiomycetes (predominantly), Orbiliomycetes, Dothideomycetes, Sordariomycetes and Pezizomycetes. This study comprised organisms belonging to four of these five classes, with the majority belonging to Leotiomycetes, order Helotiales. To better represent AQH biodiversity, more species should be tested, with emphasis on the remaining classes with none or very little representation, as well as with other orders of Leotiomycetes.

Even though AQH are poorly represented genomic-wise, with only 6 complete genomes (sparsely annotated), performing *in silico* analysis for an extensive array of functional genes provided us with very relevant information in terms of AQH functional gene diversity characterization and consequently its potential substrate selectivity and enzymatic abilities. Going further, we would like to retrieve complete functional gene sequences for every assessed partial sequence (through amino acid multiple alignments with available reference sequences, likely from closely related terrestrial fungi), in order to perform a more robust comparative analysis. This would allow to design primers with a higher degree of specificity towards AQH, based on those complete functional gene sequences. These primers could be utilized to measure gene expression (e.g. through qPCR), which coupled with enzymatic activity assays would allow us to support our results and establish connections between the existence of functional genes and presence/absence of the correspondent enzymatic ability. Finally, the design of specific primers could benefit the development of high-throughput sequencing protocols, aiming to evaluate functional diversity in environmental samples (e.g. Pathan et al., 2015; Bergkemper et al., 2016).

Chapter 5

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Chapter 6

Appendix

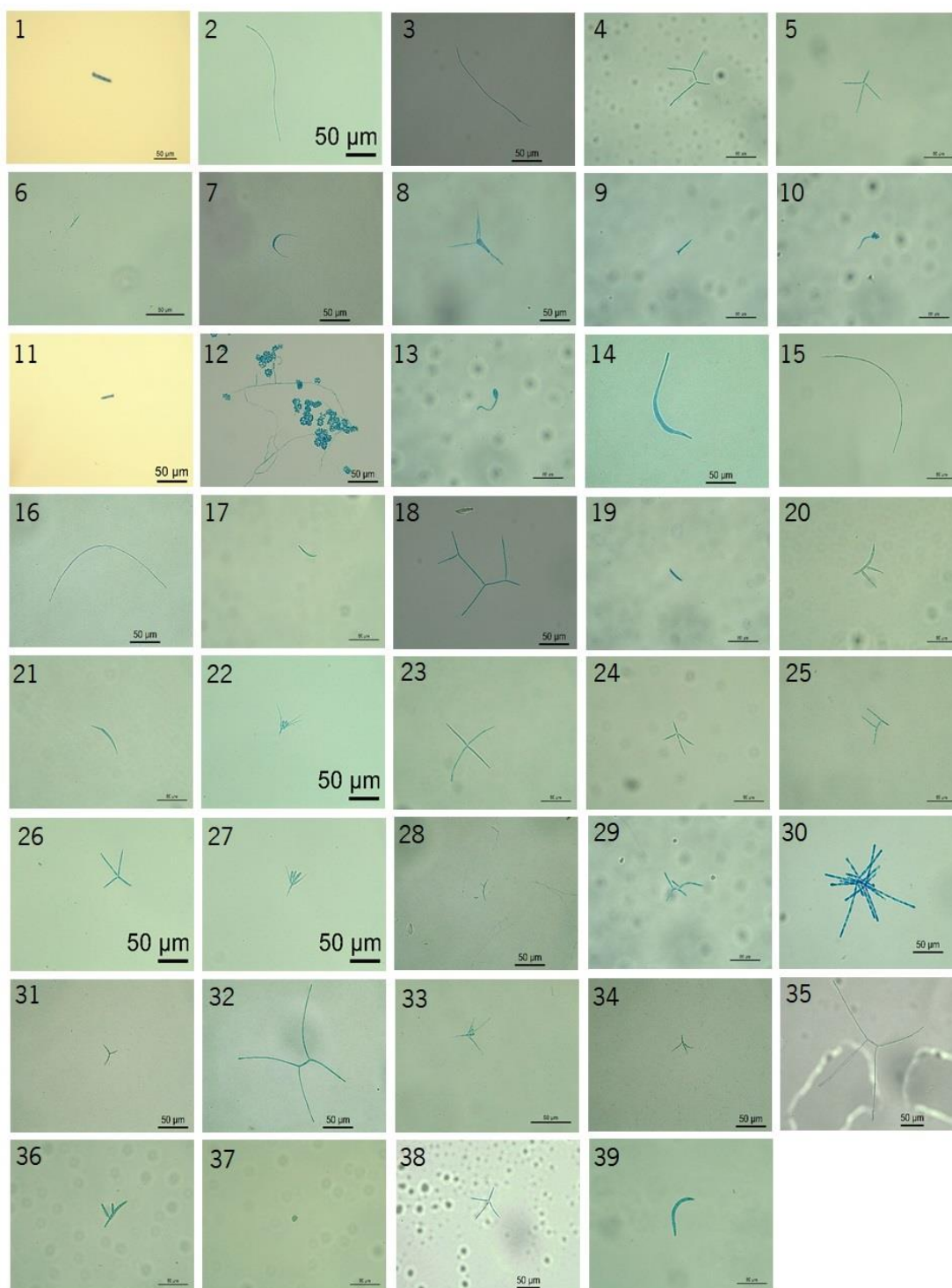


Figure A1. Conidia of AQH. 1- *Heliscus lugdunensis* (UMB3), 2- *Filosporella fistucella* (UMB7), 3- *Anguillospora filiformis* (UMB16), 4- *Articulospora tetracladia* (UMB61), 5- *Articulospora tetracladia* (UMB72), 6- *Collembolispora barbata* (UMB88), 7- *Lunulospora curvula* (UMB108), 8- *Clavariopsis aquatica* (UMB110), 9- *Heliscus submersus* (UMB135), 10- *Dendrosporium lobatum* (UMB145), 11- *Heliscus lugdunensis* (UMB160), 12- *Candelabrum spinulosum* (UMB193), 13- *Dimorphospora foliicola* (UMB215), 14- *Anguillospora crassa* (UMB217), 15- *Anguillospora filiformis* (UMB225), 16- *Anguillospora filiformis* (UMB232), 17- *Flagellospora penicillioides* (UMB304), 18- *Varicosporium elodeae* (UMB310), 19- *Heliscus lugdunensis* (UMB311), 20- *Tricladium splendens* (UMB414), 21- *Lunulospora curvula* (UMB498), 22- *Tetracladium apiense* (UMB535), 23- *Lemonniera aquatica* (UMB594), 24- *Articulospora tetracladia* (UMB712), 25- *Varicosporium elodeae* (UMB713), 26- *Articulospora tetracladia* (UMB719), 27- *Tetracladium furcatum* (UMB736), 28- *Alatospora acuminata* (UMB741), 29- *Varicosporium elodeae* (UMB878),

30- *Dendrospora erecta* (UMB891), 31- *Alatospora acuminata* (UMB902), 32- *Tricladium chaetocladium* (UMB904), 33- *Tetracladium marchalianum* (UMB1028), 34- *Alatospora pulchella* (UMB1115), 35- *Tricladium chaetocladium* (UMB1116), 36- *Tricladium splendens* (UMB1117), 37- *Dimorphospora foliicola* (UMB1119), 38- *Articulospora tetracladia* (UMB1144), 39- *Anguillospora crassa* (UMB1150).

Table A1. Species identification/confirmation for UMB- and genome-available AQH strains.

Species	UMB code	Molecular identification	Sporulation
<i>Alatospora acuminata</i>	223	<i>Alatospora acuminata</i>	No
<i>Alatospora acuminata</i>	741	<i>Alatospora acuminata</i>	No
<i>Alatospora pulchella</i>	902	<i>Alatospora acuminata</i>	Yes
<i>Alatospora pulchella</i>	1115	<i>Alatospora pulchella</i>	Yes
<i>Anguillospora crassa</i>	217	<i>Anguillospora crassa</i>	Yes
<i>Anguillospora crassa</i>	1150	<i>Anguillospora crassa</i>	Yes
<i>Anguillospora filiformis</i>	225	<i>Anguillospora filiformis</i>	Yes
<i>Anguillospora filiformis</i>	232	<i>Anguillospora filiformis</i>	Yes
<i>Articulospora tetracladia</i>	61	<i>Articulospora tetracladia</i>	Yes
<i>Articulospora tetracladia</i>	72	<i>Articulospora tetracladia</i>	Yes
<i>Articulospora tetracladia</i>	712	<i>Articulospora tetracladia</i>	Yes
<i>Articulospora tetracladia</i>	719	<i>Articulospora tetracladia</i>	Yes
<i>Articulospora tetracladia</i>	1144	<i>Articulospora tetracladia</i>	Yes
<i>Candelabrum</i>	193	<i>Candelabrum spinulosum</i>	Yes
<i>Clavariopsis aquatica</i>	110	<i>Clavariopsis aquatica</i>	No
<i>Collembolispora barbata</i>	88	<i>Collembolispora barbata</i>	Yes
<i>Cylindrocarpon</i>	16	<i>Anguillospora filiformis</i>	Yes
<i>Dendrospora tenella</i>	891	<i>Dendrospora tenella</i>	Yes
<i>Dendrospora tenella</i>	913	<i>Dendrospora tenella</i>	No
<i>Dendrosporium lobatum</i>	145	<i>Dendrosporium lobatum</i>	Yes
<i>Dimorphospora foliicola</i>	215	<i>Dimorphospora foliicola</i>	Yes
<i>Dimorphospora foliicola</i>	1119	<i>Dimorphospora foliicola</i>	Yes
<i>Filosporella fustiformis</i>	7	<i>Filosporella fistucella</i>	Yes
<i>Aquanectria</i>	304	<i>Aquanectria penicillioides</i>	Yes
<i>Heliscus lugdunensis</i>	3	<i>Heliscus lugdunensis</i>	Yes
<i>Heliscus lugdunensis</i>	160	<i>Heliscus lugdunensis</i>	Yes
<i>Heliscus lugdunensis</i>	161	<i>Heliscus lugdunensis</i>	Yes
<i>Heliscus lugdunensis</i>	311	<i>Heliscus lugdunensis</i>	Yes
<i>Heliscus submersus</i>	1	<i>Heliscus submersus</i>	No
<i>Heliscus submersus</i>	135	<i>Heliscus submersus</i>	Yes
<i>Lemonniera aquatica</i>	594	<i>Lemonniera aquatica</i>	Yes
<i>Lemonniera aquatica</i>	595	<i>Phoma sp.</i>	No
<i>Lunulospora curvula</i>	108	<i>Lunulospora curvula</i>	Yes
<i>Lunulospora curvula</i>	498	<i>Lunulospora curvula</i>	Yes
<i>Tetracladium</i>	1028	<i>Tetracladium marchalianum</i>	No
<i>Tetracladium</i>	1079	<i>Tetracladium marchalianum</i>	No
<i>Tetracladium apiense</i>	535	<i>Tetracladium apiense</i>	Yes
<i>Tetracladium furcatum</i>	736	<i>Tetracladium furcatum</i>	Yes
<i>Tricladium</i>	904	<i>Tricladium chaetocladium</i>	Yes
<i>Tricladium</i>	1116	<i>Tricladium chaetocladium</i>	Yes
<i>Tricladium splendens</i>	100	<i>Tricladium splendens</i>	No
<i>Tricladium splendens</i>	414	<i>Tricladium splendens</i>	Yes
<i>Tricladium splendens</i>	1117	<i>Tricladium splendens</i>	Yes
<i>Varicosporium elodeae</i>	310	<i>Varicosporium elodeae</i>	Yes
<i>Varicosporium elodeae</i>	713	<i>Varicosporium elodeae</i>	Yes
<i>Varicosporium elodeae</i>	713	<i>Varicosporium elodeae</i>	Yes
<i>Varicosporium elodeae</i>	878	<i>Varicosporium elodeae</i>	Yes

<i>Heliscus lugdunensis</i>	160	GQ411333.1
<i>Heliscus lugdunensis</i>	161	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACCGAGTTTAAACCTCCAAACCCCTGTGAACATACCTATCGTTCGCGGGTCCCGCTCCGGCGCCGCGAGGACCCCAACTTTGTTT TTACACAGTATCTTCTGAGTACACGATTAAATAATCAAACCTTCAACACCGATCTCTGGTCTGGCATCGATGAAGAACGCAGAAATCGGATAAGTGAATTCAGATTCAGTGAATCATCGAATCTTGAAC GCACATTCGCGCCGCGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGGGATCGCGTCCCTCGCGGGCCGCGCAATCTAGTGGGGCTCTCGCTGTA GCTTCCCTGCGTAGTACACCTCGACATGGAAGACGCGCGCCACCGCTTAAACCCCCACTCTGAAAGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Heliscus lugdunensis</i>	311	GQ411336.1
<i>Heliscus submersus</i>	1	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACCGAGTTTAAACCTCCAAACCCCTGTGAACATACCTATCGTTCGCGGGTCCCGCTCCAGCGGGCCGCGAGGACCCAACTCT TGATTTGAATTGAGTCTTCTGAGTACACGATTAAATAAACAACCTTCAACACCGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGATTCAGTGAATTCAGGAAATCA TTGAACGCACATTGGCGCCGCGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGCAACACCGCCCGCGGGGGTTCGCGCCCTCCAAATCTAGT GGCGGTCTCGCTGAGTCTCTCTCGTGAAGTAACTACCTCGCATGGACGCGCGGCCACCGCTTAAACCCCACTCTGAAAGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Heliscus submersus</i>	135	GQ411328.1
<i>Lemonniera aquatica</i>	594	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTACGCTCCAGGGTATCTCCACCCCTGAATATACATCACTAGTTGCTTTGGTAGCCGCTGGAACACTATGGCTCCAGCTCGTA CGTGCCTACCGAAGAAACAACCTCTGTTTCTAGTATGCTGAGTACTATAAATAGTAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGGAAATCA GTGAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCTAACTATGGCGGGT CTATCGAGCTCGAGCGTAGTAAATTTCTCGCTATAGGGTCTCGGTGGTGGCTTCCCAACACCCCACTTTTATCAGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Lunulospora curvula</i>	108	GQ411321.1
<i>Lunulospora curvula</i>	498	JX089526.1
<i>Margaritospira aquatica</i>	NNIBRF339	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTACGCTCCAGGGTATCTCCACCCCTGAATATACATCACTAGTTGCTTTGGTAGCCGCTGGAACACTACGGCTCCGCTCGTAC GTGCTACCGAAGAAACAACCTCTGTTTCTAGTATGCTGAGTACTATAAATAGTAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGGAAATCA GTGAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCTAACTATGGCGGGT ATCGAGCTCGAGCGTAGTAAATTTCTCGCTATAGGGTCTCGGTGGTGGCTTCCCAACACCCCACTTTTATCAGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Tetracladium furcatum</i>	736	KF952740.1
<i>Tetracladium marchalianum</i>	1028	KF952722.1
<i>Tetracladium marchalianum</i>	1079	KF952718.1
<i>Tetracladium setigerum</i>	535	KF952738.1
<i>Thelonectria rubi</i>	CBS 177.27	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTACGCTCCAAACCCCTGTGAACATACCTATCTTCTGCTCGCGGACACCCCTCGCTGACGCGGGGGCCCGCCAGGACCCAAA ACCCAACTTGTCTTTGCTTTCGAAACGAACCTCTGAGTGGATTATAAATCAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGGAAATCA GAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCTAACTATGGCGGGT TCCCCTCCGCGCCGCGCGCCGCTCCCAACACCGTCCGGGCTCACCGCGGGCCCTTTCGCGTGTAGTAACTAACACCTCGCGCGCGGCGCTGTGGTGTGCGTCCAGGAAACCCCGCTTCTCAAAATGACCTC GAATCAGTGAAGTAACTCCGCTGAACCTAA
<i>Tricladium chaetocladium</i>	904	KF952689.1
<i>Tricladium chaetocladium</i>	1116	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTAAACGATTCAACACGAGTGGGGCCGTGGCTCGCGGGCCCTGCGATGTTTTGGTGTGCGTCCCGGCTCGGGTGACGCCGATCACATCA CCCTATGCTACGTACCTTGTGTTGTTGGTGGCCCGGCTCCGCTCGCGGGCCCTGGCTCGCACGTGCCCGCCAGAGAACCCAACTCTGATTTAGTATGCTGAGTACTATAAATAGTAAACTTTCACACCGGAT GGATCTCTGGTCTGCGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGGAAATCGGAAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTC ATGACCACTCAGCTCTGCTGGTCTCGGGTCCGCTGTCCAGCGGGCCCTTAAACCCAGTGGCGGTGCGGTGGCTCTCAGCGTGAAGTAACTATCTCGCTACAGGGTCCGCTCGGTGTGGCGACCAACCCAACTA TTTCTAGTTGACCTCGGATCAGTGAAGTAACTTCTCGCTACAGGATACCCGCTGAACCTAA
<i>Tricladium splendens</i>	100	GQ411347.1
<i>Tricladium splendens</i>	414	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTCCCTGCCCTCGGGGTAGGATCGCACCCCTGATTATTTATGAGTGTGCTTTGGCGGGCCCTCGCGCTGGCGCCCGCGGCTCC GGCGGGGAGCGCCCGCCAGAGGCTTCTCAAAACCTGTGATTAGTGTGCTGAGTACTATAAATAGTAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATGTGA ATTGACAATCAGTGAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCT CAAGGCAAGTGGCGGTACCGCGGGCTCTGAGCGTAGTAAATTTCTCGCTACAGGTTCCCGGGCGGCACTGGCCAGCAACCCCAACTTTCACAGGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Tricladium splendens</i>	1117	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTCCCTGCCCTCGGGGTAGGATCGCACCCCTGATTATTTATGAGTGTGCTTTGGCGGGCCCTCGCGCTGGCCCGCCCGGCTCC GGCGGGGAGCGCCCGCCAGAGGCTTCTCAAAACCTGTGATTAGTGTGCTGAGTACTATAAATAGTAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATGTGA ATTGACAATCAGTGAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCT CAAAAGCAGTGGCGGTACCGCGGGCTCTGAGCGTAGTAAATTTCTCGCTACAGGTTCCCGGGCGGCACTGGCCAGCAACCCCAACTTTCACAGGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Variosporium elodeae</i>	310	GQ411283.1
<i>Variosporium elodeae</i>	713	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTACGCTCCAGGGTATCTCCACCCCTGAATATACATCACTCGTTCGTTGGTAGCCGCTGGAACACTACAGGCTCCGCTGATG CGTGCCTCCAGAGAAACAACCTCTGTTTCTAGTATGCTGAGTACTATAAATAGTAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGGAAATCA GTGAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCTAACTATGGCGGGT CTATTGAGCTCGAGCGTAGTAAATTTCTCGCTATAGGTTCCGTTGGTGGTGGCTTCCCAACACCCCACTTTTATCAGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA
<i>Variosporium elodeae</i>	878	AAGTCGTAACAGGTTCCGTTGGTGAACACAGGAGGATCATTACAGAGTTTCCCTGCCCTCGGGGTAGGATCGCACCCCTGATTATTTATGAGTGTGCTTTGGCGGGCCCTCGCGCTGGCCCGCCCGGCTCC CGTGCCTCCAGAGAAACAACCTCTGTTTCTAGTATGCTGAGTACTATAAATAGTAAACTTTCACACCGGATCTCTGGTCTGGCATCGATGAAGAACGCAGGAAATCGGATAAGTGAATTCAGGAAATCA GTGAATCATCGAATCTTGAACGCACATTGGCGCCCGGAGTATTCTGGCGGGCATGCCCTGGTGGAGGCTATTTCACACCTCAAGCCCGCGGCTTGGTTGGAGATCGGAAATCGCGCTCCAGCTCTAACTATGGCGGGT CTATTGAGCTCGAGCGTAGTAAATTTCTCGCTATAGGTTCCGTTGGTGGTGGCTTCCCAACACCCCACTTTTATCAGTTGACCTCGGATCAGTGAAGTAAACCCGCTGAACCTAA

Table A3. Primer bibliographic search.

Activity	Enzymes	Primer pair (5'-3')	Retrieved from
Nitrogen cycle	Nitrate reductase	niaD01F	GTNTGYCNGGNAA
		niaD04R	GTNGGRTGYTCRAA
		niaD15F	GGNAAYMGNMNGNAARGARCARAA
		niaD12R	AACCANGGRTTRTTCATCATNCC
Nitric oxide reductase	p450nor394F	SCIACITTYGTIGAYATGGA	
	p450nor809R	ATCATGTTIACBAIITIGICIT	
Cellulolytic enzymes	Endo-(1,4)-β-glucanase	F40_gr1	GGMTCSGGMTCCCARITGC
		F130_gr1	AGCGGSGAYTATGARCTSATGATC
		R220_gr1	RCCRGTRAAAGGGYTCAGTMCC
	Endo-β-1,4-glucanases	R130_gr1	GATCATSAGYTCATARTSCCGCT
		fungGH5-5F	GARATGCAYCARTACCTYGA
	Endo-1,4-β-mannosidase/ endoglucanase	fungGH5-5R	CANGGCCRCRCCACCA
		GH5_130F	GGTHTGGGGTYYAAYGA
	Endoglucanase	GH5_245R	GGYTCRTTSGARYTCCCA
		GH45_155F	GTAYTGGGAYTYGYAA
		GH45_280R	CCACCNCNCNCCNGGCAT
		GH74_130F	TTYAARGTGGGNAAYATG
	Endoglucanase/ xyloglucan-specific endo-β-1,4-glucanase	GH74_280R	CCRTCRTAGGCCNNGCNCC
		Glycoside hydrolase family 6 (GH6 family) cellulase genes	cell2F
	cell2R		GAGSGARTCSGGCTCRAT
Cellulohydrolase	fungcbh1F	ACCAAYTGCTAYACIRGYAA	
	fungcbh1R	GCYTCCAIASTRCCATC	
Cellulohydrolase II	GH6_270F	GAYCGCGCAYGCNNGNTGG	
	GH6_400R	TCNCCNCCGGYTTDACCCA	
β-glucosidase	sense	TAYGNATGAAYCAYTA	
	antisense	TTNGCRTARTCNACRTA	

	β -glucosidase	P31 (sense)	GAGGGTTTCGGCTCGG ATCCCTA	(Abdeljalil et al., 2013)
		P32 (sense)	TTCATGTGCTCGTACAACCCAG	
	β -glucosidase	P34 (antisense)	GGCAGGATGCCGTTGTTCTTGAA	
		P36 (antisense)	CAGTCCGAAGCCAACTCGTA	
Hemicellulolytic enzymes	Endo- β -1,4-xylanases	Glc1_155F	GGMGAAYTGGGARGGNTT	(Kellner and Vandenbol, 2010)
		Glc1_235R	AYGCRTCGCRAANGGCCA	
	Endo-1,4- β -xylanase	fungGH11-F	GGVAAGGGTGGAAAYCCNGG	(Barbi et al., 2014)
		fungGH11-R	TGKCGRACGACCARTAYTG	
	Endo-1,4- β -xylanase	GH10_80F	CCGARAAYWSATGAARTGG	(Kellner and Vandenbol, 2010)
		GH10_190R	TARTCRTTATRTAARYTT	
	xylanase	GH11_80F	GGAARGGNTGGAAAYCCNGG	
		GH11_200R	TACCYTCGTGCDACDAT	
	Xylanase 10	XYN1F	AAAAAAAAAAAAAGTNGGNTAYGA	(Salles et al., 2007)
		XYN1R	AAAAAAAAAAAAARTTYTGRAARTA	
		Xyl2-F1	ACNCCNGARAAYTCNATGAAR	(Boonyapakron et al., 2005)
	Xyl2-B2	HATYTCRTTNACNACRTCCCA		
	Xyl2-B1	RTCRTTHATRTANAGYTTNGC		
	Xyloglucanase	F41_gr2	TACAACRAYCTYGGGG	(Rykov et al., 2019)
		F140_gr2	GCYYTGGGCGGYGCKGGHCC	
		R230_gr2	GAAKGGCTCRGTWCCGGCCTG	
	R140_gr2	GGDCCMGCRCGCCARRGC		
	Acetylxylan esterase	AXE_155F	GTHATGGCGCACNTAYCC	(Kellner and Vandenbol, 2010)
		AXE_250R	TACCRAADACCCNGCCCA	
	α -glucuronidase/ xylan α -1, 2-glucuronosidase	GH67_390F	GGCCATHGAYTTYCARGT	
GH67_570R		GCCKGTCCAYTGNCCECA		
α -glucosidase	GH31_350F	CAYCARTGYMGTGGGGNTA		
	GH31_660R	TTRTCCNCCCARTGNCC		
α -L-arabinofuranosidase	GH51_280F	AGNTGGCARTGGAAYGCNAC		
	GH51_350R	ATYTGRTCDATGCTYGYTG		
α -1,2-mannosidase	GH92_350F	ACGGGARAAYCCNYTNTGG		
	GH92_420R	GCRTTSWCCCCYTGNGT		
amylase	amy1F	ACCGCATATGGTGATGCCTAYCAYGGNTAYTGG	(Wu et al., 2006)	
	amy1R	CTTGCCGCTAGTAAATGATNGGDATNCCRTC		
β -xylosidase	xyp1F	GAYATHCCNTGGGCNAC	(Wegener et al., 1999)	
	xyp1R	TTNCCNARNGGYTTRTC		
α -Galactosidase	n/a	CTIGGSTGGAACCTITGGAAYGC	(Similä et al., 2010)	
	n/a	CGICCRATRGGRCGRGTICA		
	n/a	CAYTYGCICTKTGGGCRATSATG		
	n/a	GGATGCMTGGACMGCAAAG		
Pectinolytic enzymes	Endopolygalacturonase	sense	ACNTTYACNGAYGCNGCNGCNGT	(Gao et al., 1996)
		antisense	TCGAASGCRTRCGTGTTRGT	
	Endopolygalacturonase	ClpGF	GTYCTIAACAACATYCC	(Centis et al., 1996)
		ClpGR	GCRASRCAGTCRTCYTGGTT	
Endopolygalacturonase	FPG1	GAYAAYGAYTYRAYCCBATY	(Chimwumombe et al., 2001)	
	FPG2	CANGTRTNGTVGGRTARTTR		
Endopolygalacturonase	GH28F-1786F	TRBTGGGAYGHNWRGG	(Gacura et al., 2016)	
	GH28F-2089R	GCVABRCARTCRCTYGRIT		
Lignolytic enzymes	Tyrosinase	Tyr_137F	CCNTWYTGGAAYTGGGC	(Kellner and Vandenbol, 2010)
		Tyr_282R	TGRTGARRAARAADATGG	
	Intradiol ring cleavage dioxygenase	IRDC_F	TAYCCATHCCCAYGAYGG	(Asemoloye et al., 2018)
		IRDC_R	TTDACCRAADACGCRTC	
	Catalase	cbhi.1u	ACAATGTTCCGCACGTCTACTT	
		cbhi.1d	AGGGTGCCCGCGGAGGTGCC	
		cbhi.2u	CACTCCTCGCATTCACTTGTCT	
		cbhi.2d	CTGCCGGTCTCGGTCCAGTTGC	
		Cbhiiu	CCTCAGCCCTTACTACGC	
		Cbhiiid	CCAATCTACCTCTACAGC	
	Lignin peroxidase	lig1u	GCCGCAATTTCTCTTGCTCTTTCCA	
		lig1d	TACATCGAACCCAGCCACGATGATT	
		lig2u	CATCGCAATTCGCCCGCCATGGAGGCA	
		lig2d	ACCTTCTGAACGAATGGCTTCTGGAGC	
		lig4u	GTGGCCCTGGTTCCCATCTGCGAC	
		lig4d	AATTGGTCTCGATAGTATCGAAGAC	
		lig6u	GACCTGCTCGAACGGCAAGTCTGTC	
		lig6d	CATGATAGAACCATCGGCCTCGC	
	Manganese peroxidase	mnp1u	TCCGGTCAACGGCTTGGTATTCAG	
		mnp1d	GCGATCGTCTTGTTCCGGCGGCCAG	
Laccase	Lac2F	CTTCAATAYGSTGATGGAC	(Hirschhäuser and Fröhlich, 2007)	
	Lac2R	AATCGTGRCCGTGGAGATG		
Laccase	cbs2	ACGTCKTGGTACCACTSGCACTWC	(Tetsch et al., 2005)	
	cbs3	SCCGTGCAGGTGGATSGGGTG		

		5'-int	GGMCAGCGCTACGACGTSATCATC	
		3'-int	GATGATSACGTCGTAGCGCTG(GT)CC	
Laccase		lcc1u	TGGTAYCAYAGTCATTATTC	(Asemoloye et al., 2018)
		lcc1g	ATGTGRCARTGRAASRGCCA	
Laccase		forward	ATTGGCACGGCTTCTTCC	(Collins and Dobson, 1997)
		reverse	GATCTGGATGGAGTCGAC	
Laccase		LAC2FOR	GGIACIWIITGGTAYCAYWSICA	(Lyons et al., 2003)
		LAC3REV	CCRTGIWKRTGIAWIGGRTGIGG	
Laccase		Cu1AF	ACMWCBGTYCAYTGGCAYGG	(Kellner et al., 2007)
		Cu2R	GRCTGTGGTACCAGAANGTNCC	
Laccase		lcc1_for	GGTCCACTGGCACGGTCTTC	(Solé et al., 2008)
		lcc1_rev	CAAGTTCAAAGCCACACAATATGG	
		lcc2_for	GTCGGTGTGACCCAGTGCC	
		lcc2_rev	ACCAGAACGTTCTCTGATTGTCC	
Others	Acidic cutinase	5'AOX1	GACTGGTTCCAATTGACAAGC	(Nyüssölä et al., 2013)
		3'AOX1	GCAAAATGGCATTCTGACATCC	
	Triacylglycerol lipase	n/a	AA(CT)GCICCI(CT)TIAA(CT)GA(AG)TT(CT)(CT)T	(Toida et al., 2000)
		n/a	GCIGGIA(AG)(CT)TG(CT)TCIACIGC(AG)TT	

Table A4. Primers used for amplification of ITS region and functional genes.

Activity	Enzymes	Primer pair (5'-3')		Expected fragment size	Retrieved from	Primer alteration
Fungal ID	ITS1-5.8S-ITS2 region	ITS1F	CTTGGTCATTAGAGGAAGTAA	~ 0,6 Kb		No
		ITS4	TCCTCCGCTTATTGATATGC			No
Nitrogen cycle	Nitrate reductase	niaD01F	GTNTGYGCNGGNA	~ 0,7-1 Kb	(Gorfer et al., 2011)	No
		niaD04R	GTNGGRTGYTCRAA			No
		niaD15F	GGNAAYMGNMGNAARGARCARAA			No
		niaD12R	AACCANGGRTTRTTCATCATNCC			No
Cellulolytic enzymes	Endo- β -1,4-glucanase	NfungGH5-5-F	GARATSCAYCARTAYCTYGA	~ 248 bp	(Barbi et al., 2014)	Yes
		NfungGH5-5-R	CANGSIYMRGVSVGHCCACCA			Yes
	Cellobiohydrolase	fungcbhF	ACCAAYTGCTAYACIRGYAA	~ 500bp	(Edwards et al., 2008)	No
		fungcbhR	GCYTCCCAIATRCCATC			No
	β -glucosidase	sense	TAYGGNATGAAYCAYTA	~ 450 bp	(Takashima et al., 1999)	No
		antisense	TTNGCRTARTCNACRTA			No
Hemicellulolytic enzymes	β -xylosidase	Nxyp1F	GAYABNBCNTGGVCNAC	~ 141 bp	(Wegener et al., 1999)	Yes
		xyp1R	TTNCCNARNGGYTTRTC			No
	Acetyl xylan esterase	NAXE_155F	NTHATGRYIBCIACNHAYCC	~ 290 bp	(Kellner and Vandenbol, 2010)	Yes
NAXE_250R		HAICCRDADABICBNNCCCA	Yes			
Pectinolytic enzymes	Endopolygalacturonase	GH28F-1786F	TRBTGGGAYGGHNWRGG	300-500 bp	(Gacura et al., 2016)	No
		GH28F-2089R	GCVABRCARTCRCTYGRIT			No
		NClpgF	DTYCTIRACAACATYVM	~ 542 bp	(Centis et al., 1996)	Yes
		NClpgR	KCRASRCARTCRCTBTGGTT			Yes
Lignolytic enzymes	Tyrosinase	Tyr_137F	CCNTWYTGGAAYTGGGC	~ 470 bp	(Kellner and Vandenbol, 2010)	No
		Tyr_282R	TGRTGIARRAARAADATIGG			No
	Laccase	NLac2F	STTCAAYAGSTGAWGGMY	~ 446-835 bp (depending on the organism)	(Hirschhäuser and Fröhlich, 2007)	Yes
		NLac2R	VRCGTGRYCKTSGAGRTS			Yes

Table A5. Gene search, selection and accession numbers used in the alignment for in silico assays. For NCBI searches, functional gene partial and complete nucleotide sequences were retrieved from multiple (published and unpublished) sources.

Activity	Enzyme (EC number)	Presumed enzyme function	Referencing	Accession numbers used in alignment
Nitrogen cycle	Nitrato reductase (EC 1.7.1.1; euknr)	Reduction of nitrate to nitrite	Retrieved from (Gorfer et al., 2011)	EEQ88796, EEQ72680, EFE34296, CAQ77148, EAW07550, EED54629, AAL85636, EAW07550, EAL90615, EAA65574, P36858,208048(JGI), CAK45533, BAA08551, AAC49605, EAU39120, EDN30738, AAC02633, EAQ88219, 113444(JGI), CA59336, FOXG_04181.2 (FGI), CAA80270,BAG12905, BAG12906,FVEG_07298.3 (FGI), CAA62232.XP_382123, P36842, EDJ96311, CAA04554, EEQ28849, MGYG_04402.1(FGI), BAB84515, 97565(JGI), 56303(JGI), 111003(JGI), EEU34169, EAW22584, CAA43600, EAA32833,128588(JGI), 124300(JGI), EEH17834, EEH46709, ACI29313, AAB03900, CAP92210, AAP12556, EEA25715, CAA08857, CAA74005, EAT87086, CAA88925, CAP70343, EDN96957, CBI54944, EED21598, 36169(JGI), 128887(JGI), 81955(JGI), 33018(JGI),TEQG_08349.1(FGI), TERG_07816.2(FGI), TESG_00729.1 (FGI), EFE41244, AAN64993, CAZ85382, EEY14658, VDAG_00378(FGI), AAO63560, AAF28059, EAU81300, CAB60010, EDRO2368, 33678(JGI), 136276(JGI), 1,47370(JGI), 34275(JGI),EAK84753, 81522(JGI), P39864, 71442(JGI), 140563(JGI), AAY59538, EEC44781, EED88244
	Nitrite reductase (EC 1.7.1.4; nirK)	Reduction of nitrite to ammonium	NCBI search for nirK gene	GU247810-11, GU144509, KX273057, KT462525-35, KT462537, KT462513, KT462500-01, KT462505-6, KT462518, KT462468-99, AB938217-39, KF481847-51, KF481842-44, KF481826, GU320194, EF600898, MN977819, MG581313-20, LC201969-75, KU556433-59, AB904794-830, GU247812-13, MT277586-619, MT293549, MT250084
Cellulolytic enzymes	GH3: putative β -glucosidase (EC 3.2.1.21) or xylan 1,4- β -xylosidase (EC 3.2.1.37)	Cellulose and xylan backbone degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734375-99
	GH45: endoglucanase (EC 3.2.1.4)	Cellulose degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734468-78
	GH5: putative mannan endo-1,4- β -mannosidase	Mannan & cellulose degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734400-08
	GH5: endoglucanase (EC 3.2.1.4; glu)	Cellulose degradation	Retrieved from (Barbi et al., 2014)	HG799539-69
	GH74: endoglucanase (EC 3.2.1.4) or putative	Cellulose degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734496-99
	GH7: cellobiohydrolase I (EC 3.2.1.-) or endoglucanase	Cellulose degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734555-65
	Cellobiohydrolase I (EC 3.2.1.-; cbhI)	Cellulose degradation	Retrieved from (Edwards et al., 2008)	EU345437 - 72, EU359569-616
GH6: cellulose 1,4- β -cellobiosidase, i.e. cellobiohydrolase II	Cellulose degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734409-11	
Hemicellulolytic enzymes	GH10: endo-1,4- β -xylanase (EC 3.2.1.8)	Xylan backbone degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734412-15
	GH11: endo-1,4- β -xylanase (EC 3.2.1.8)	Xylan backbone degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734415-28
	Endo-1,4- β -xylanase 2 (EC 3.2.1.8; xyn2)	Xylan backbone degradation	NCBI search for xyn2 gene	MN395664, KC893554, AF155594, U24191, EU532196, HG764395, JN010439
	GH31: α -glucosidase (EC 3.2.1.20)	Starch degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734458-67
	GH67: α -glucuronidase (EC 3.2.1.139) or xylan α -1,2-glucuronosidase (EC	Xylan sidechain (glucuronic acid) degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734488-95
	GH92: putative α -1,2-mannosidase (EC 3.2.1.-)	Mannan sidechain degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734500-08
CE1: acetylxylin esterase (EC 3.1.1.72)	Xylan sidechain degradation	Retrieved from (Kellner and Vandenbol, 2010)	GU734536-40	
Pectinolytic enzymes	Endopolygalacturonase (EC 3.2.1.15; enpg)	Degradation of the pectic chain	Retrieved from (Gacura et al., 2016)	KU664125-40
	Pectate lyase (EC 4.2.2.2; plyA)	Eliminative (nonhydrolytic) cleavage of de-esterified pectin backbone	NCBI search for plyA gene	XM_024851886, XM_024833432, XM_025720867, XM_025632050, XM_025597356, AJ276331, XM_037315432, XM_036632029, XM_024814703, XM_032026501, XM_028644910, XM_031151333,
	Pectinesterase (EC 3.1.1.11)	Pectin de-esterification	NCBI search for pectinesterase gene	XM_031149046, XM_020269131, XM_037364718, XM_037354209, XM_037328710, XM_037328279, XM_037326253, XM_036731686, XM_036718615, XM_036644923, XM_036641626, XM_036674912, XM_031199916, XM_035482210, XM_035476491, XM_035476462, XM_035479568, XM_035477907, XM_009652647, XM_032031071, XM_003718361, XM_031177513, XM_031167428, XM_031167429, XM_028654632, XM_025700754, XM_025650498, XM_025664838, XM_025640580
Lignolytic enzymes	Manganese peroxidase (EC 1.11.1.13)	Lignin oxidation and breakdown via Mn ⁺	Retrieved from (Kellner and Vandenbol, 2010)	GU734546-49
	Laccase (EC 1.10.3.2)	Lignin and phenolics oxidation	Retrieved from (Kellner and Vandenbol, 2010)	FJ040222-25 and GU734541-45
	Laccase (EC 1.10.3.2; lcc1)	Lignin and phenolics oxidation	NCBI search for lcc1 gene	EF990894; XM_958985; XM_007819053; AF118267; AY338757; L10664; AY338756; AB055157; XM_003654761; KT802747; KJ420608; FJ940742;
	Laccase (EC 1.10.3.2; lcc2)	Lignin and phenolics oxidation	NCBI search for lcc2 gene	AF118268; BK004112; MK095585-87; KY129773; EU747646; HM461990; KU055622; AB822543; LC026459; XM_009544882; KJ420607; KC991151; KC507936; JX879726; JQ307224; FJ940743; GQ899202; L10663; AB539119; AF243855; EF990895; XM_033553579; XM_002849598; XM_957343; XM_022612347; XM_007825578; XM_007810155; AY338758; AB252575; FJ432085; EF050079-81; FJ040342; FJ040338; FJ040335; FJ040329; FJ040324; FJ040322; FJ040317-19; XM_031150999; XM_031146003;
	Laccase (EC 1.10.3.2; lcc3)	Lignin and phenolics oxidation	NCBI search for lcc3 gene	EF990899; XM_950742; KJ420606; FJ804119;
	Oxalate decarboxylase (EC 4.1.1.2)	Oxalate breakdown	Retrieved from (Kellner and Vandenbol, 2010)	GU734358-74
	Aromatic peroxidase (EC 1.11.2.-), chloroperoxidase (EC 1.11.1.10)	Extracellular oxygenations; non-specific	Retrieved from (Kellner and Vandenbol, 2010)	GU734340-50
	Tyrosinase (EC 1.14.18.1)	Cell-wall-associated oxidation of phenols	Retrieved from (Kellner and Vandenbol, 2010)	GU734550-54
Phosphorus cycle	Intradiol ring cleavage dioxygenase, putative catechol 1,2-dioxygenase (EC 1.13.11.1) or hydroxyquinol 1,2-dioxygenase (EC 1.13.11.37)	Intracellular cleavage of aromatic rings	Retrieved from (Kellner and Vandenbol, 2010)	GU734351-57
	Acid phosphatase (EC 3.1.3.2; phoA)	Removal of phosphate from other molecules at an acidic pH	NCBI search for phoA gene	XM_031147717, FR692330, XM_745014, XM_742838, XM_007293419, AF462065, XM_001265297, XM_035503464, XM_035495335, XM_015556249, XM_025534280, XM_025529375, XM_024851703, XM_024824355, XM_024831610, XM_002150323, XM_002149969, XM_002845867, XM_028629030, XM_025607646, XM_025712483, XM_025709432, XM_025681454, XM_025671402, XM_025694225, XM_025716941, XM_025663669, XM_025657138, XM_025634861, XM_025583365, XM_025584018, XM_025620195, XM_951758, XM_022534294, XM_014678996, XM_014320649, XM_013568423, XM_008088858, XM_008078839, XM_002484221, XM_002483857, XM_001269797, JG697465
Alkaline phosphatase (EC 3.1.3.1; phoD)	Removal of phosphate from other molecules at an alkaline pH	NCBI search for phoD gen	XM_031149735, XM_001936893, L27993, L36230, KP188592-620, MF488670-93	

		<i>Clavariopsis aquatica</i>	WD(A)-00-1	WRKSKGFSWGPAGVSTALFTGVMADVIRRRARLPAKYVMEGADKLVSLDNQKSSLT-GSRMDTGMPLLS-TGSWTPIEESCSPTK-TEKLLHLMVNPYGR-FVKLAEEV-SGLRSLF-PRSRVITGTTFTTGYRSRELSFH-GLKLTWTRWVWQMSQLEIRHGGRTSMVQSTI-TRMPQLPNQPTMNSSSLKMPQRCTQQEVMTAEVAASHEPKQLTKAKHGWDWPTSNMFKIAETMKTNNCLAE-D-TWGGKRRPAGASGASTYQW-NSRTPRICVSRVWTRATRA-TSCLTEICIGLF
		<i>Dactylella cylindrospora</i>	CBS325.70	MVRKTKGFSWGAAGVSTALWTGPMKLDIIRAKPLRRAKYVMEGADKLVSLDNQKSSLT-GSRMDTGMPLLS-TGSWTPIEESCSPTK-TEKLLHLMVNPYGR-FVKLAEEV-SGLRSLF-PRSRVITGTTFTTGYRSRELSFH-GLKLTWTRWVWQMSQLEIRHGGRTSMVQSTI-TRMPQLPNQPTMNSSSLKMPQRCTQQEVMTAEVAASHEPKQLTKAKHGWDWPTSNMFKIAETMKTNNCLAE-D-TWGGKRRPAGASGASTYQW-NSRTPRICVSRVWTRATRA-TSCLTEICIGLF
		<i>Margaritopsis aquatica</i>	NNIBRF339	QVRKSKGFSWGAAGVSTALWTGVAISELLKRAVPLRGAKYVMEGADKLVSLDNQKSSLT-GSRMDTGMPLLS-TGSWTPIEESCSPTK-TEKLLHLMVNPYGR-FVKLAEEV-SGLRSLF-PRSRVITGTTFTTGYRSRELSFH-GLKLTWTRWVWQMSQLEIRHGGRTSMVQSTI-TRMPQLPNQPTMNSSSLKMPQRCTQQEVMTAEVAASHEPKQLTKAKHGWDWPTSNMFKIAETMKTNNCLAE-D-TWGGKRRPAGASGASTYQW-NSRTPRICVSRVWTRATRA-TSCLTEICIGLF
		<i>Thelonectria rubi</i>	CBS 177.27	MVRKTKGFSWGAAGVSTALWTGVAISELLKRAVPLRGAKYVMEGADKLVSLDNQKSSLT-GSRMDTGMPLLS-TGSWTPIEESCSPTK-TEKLLHLMVNPYGR-FVKLAEEV-SGLRSLF-PRSRVITGTTFTTGYRSRELSFH-GLKLTWTRWVWQMSQLEIRHGGRTSMVQSTI-TRMPQLPNQPTMNSSSLKMPQRCTQQEVMTAEVAASHEPKQLTKAKHGWDWPTSNMFKIAETMKTNNCLAE-D-TWGGKRRPAGASGASTYQW-NSRTPRICVSRVWTRATRA-TSCLTEICIGLF
Lignolytic activity	Aromatic peroygenase	<i>Articulospora tetracladia</i>	NNIBRF329	NAMANHGILPHDGNITFKQLNKATRETNFAPSFCCFVFKFAADFLNRSYWKDFLEIEISKHNAIEHD
		<i>Dactylella cylindrospora</i>	CBS325.70	NAMANHGILPHDGNITFKQLNKATRETNFAPSFCCFVFKFAADFLNRSYWKDFLEIEISKHNAIEHD
		<i>Margaritopsis aquatica</i>	NNIBRF339	NAMANHGILPHDGNITFKQLNKATRETNFAPSFCCFVFKFAADFLNRSYWKDFLEIEISKHNAIEHD
Cellulolytic activity	Endoglucanase GH45	<i>Articulospora tetracladia</i>	NNIBRF329	WDCCKASCAPWGLTLASGSPNVTSCDKSDNPTDYNVAVSNGSGSATMCCSSQSPWAVSSTLSYGAATHISGGTEASWCCACYQLTFTSGAVAGKTMIVQATNTGGDLGSGNQFDIAV
		<i>Aquanectria penicillioides</i>	NNIBRF619	WDCCKASCAPWGLTLASGSPNVTSCDKSDNPTDYNVAVSNGSGSATMCCSSQSPWAVSSTLSYGAATHISGGTEASWCCACYQLTFTSGAVAGKTMIVQATNTGGDLGSGNQFDIAV
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	WDCCKASCAPWGLTLASGSPNVTSCDKSDNPTDYNVAVSNGSGSATMCCSSQSPWAVSSTLSYGAATHISGGTEASWCCACYQLTFTSGAVAGKTMIVQATNTGGDLGSGNQFDIAV
		<i>Margaritopsis aquatica</i>	NNIBRF339	WDCCKASCAPWGLTLASGSPNVTSCDKSDNPTDYNVAVSNGSGSATMCCSSQSPWAVSSTLSYGAATHISGGTEASWCCACYQLTFTSGAVAGKTMIVQATNTGGDLGSGNQFDIAV
		<i>Thelonectria rubi</i>	CBS 177.27	WDCCKASCAPWGLTLASGSPNVTSCDKSDNPTDYNVAVSNGSGSATMCCSSQSPWAVSSTLSYGAATHISGGTEASWCCACYQLTFTSGAVAGKTMIVQATNTGGDLGSGNQFDIAV
		<i>Thelonectria rubi</i>	CBS 177.27	WDCCKASCAPWGLTLASGSPNVTSCDKSDNPTDYNVAVSNGSGSATMCCSSQSPWAVSSTLSYGAATHISGGTEASWCCACYQLTFTSGAVAGKTMIVQATNTGGDLGSGNQFDIAV
	Endo-mannosidase/endoglucanase	<i>Articulospora tetracladia</i>	NNIBRF329	VWGFNDVTSFGSGTWYQSFVSGASPVINTGANGQLRDLVYASAEAHGISLINFVNNWTDYGGMAAYCSYGISPVYTWSTLAQTQYKAIQAVSRVYTSKAIFAWELANE
		<i>Aquanectria penicillioides</i>	NNIBRF619	VWGFNDVTSFGSGTWYQSFVSGASPVINTGANGQLRDLVYASAEAHGISLINFVNNWTDYGGMAAYCSYGISPVYTWSTLAQTQYKAIQAVSRVYTSKAIFAWELANE
		<i>Margaritopsis aquatica</i>	NNIBRF339	VWGFNDVTSFGSGTWYQSFVSGASPVINTGANGQLRDLVYASAEAHGISLINFVNNWTDYGGMAAYCSYGISPVYTWSTLAQTQYKAIQAVSRVYTSKAIFAWELANE
	Endoglucanase GH5	<i>Articulospora tetracladia</i>	NNIBRF329	EMHQYLDSDSGTSDACVSTTIGVERVTSATAWLKANGKQIIEGFAAGANSQCMATVGMTDLHLKANSVDVWGLWGGGGP
		<i>Aquanectria penicillioides</i>	NNIBRF619	EMHQYLDSDSGTSDACVSTTIGVERVTSATAWLKANGKQIIEGFAAGANSQCMATVGMTDLHLKANSVDVWGLWGGGGP
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	EMHQYLDSDSGTSDACVSTTIGVERVTSATAWLKANGKQIIEGFAAGANSQCMATVGMTDLHLKANSVDVWGLWGGGGP
		<i>Dactylella cylindrospora</i>	CBS325.70	EMHQYLDSDSGTSDACVSTTIGVERVTSATAWLKANGKQIIEGFAAGANSQCMATVGMTDLHLKANSVDVWGLWGGGGP
		<i>Margaritopsis aquatica</i>	NNIBRF339	EMHQYLDSDSGTSDACVSTTIGVERVTSATAWLKANGKQIIEGFAAGANSQCMATVGMTDLHLKANSVDVWGLWGGGGP
		<i>Thelonectria rubi</i>	CBS 177.27	EMHQYLDSDSGTSDACVSTTIGVERVTSATAWLKANGKQIIEGFAAGANSQCMATVGMTDLHLKANSVDVWGLWGGGGP
	β-glucosidase/xylohydrolase 1,4-β-xylidase GH3	<i>Articulospora tetracladia</i>	NNIBRF329	GRNWEFGADPVLQGIAGSOTVKIQDQGVMTAKHFVIGNEQEHFRQSEWGLPNAMSSNIDDRTHLEMYGWPFGDA
		<i>Aquanectria penicillioides</i>	NNIBRF619	GRNWEFGADPVLQGIAGSOTVKIQDQGVMTAKHFVIGNEQEHFRQSEWGLPNAMSSNIDDRTHLEMYGWPFGDA
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	GRNWEFGADPVLQGIAGSOTVKIQDQGVMTAKHFVIGNEQEHFRQSEWGLPNAMSSNIDDRTHLEMYGWPFGDA
<i>Dactylella cylindrospora</i>		CBS325.70	GRNWEFGADPVLQGIAGSOTVKIQDQGVMTAKHFVIGNEQEHFRQSEWGLPNAMSSNIDDRTHLEMYGWPFGDA	
<i>Margaritopsis aquatica</i>		NNIBRF339	GRNWEFGADPVLQGIAGSOTVKIQDQGVMTAKHFVIGNEQEHFRQSEWGLPNAMSSNIDDRTHLEMYGWPFGDA	
<i>Thelonectria rubi</i>		CBS 177.27	GRNWEFGADPVLQGIAGSOTVKIQDQGVMTAKHFVIGNEQEHFRQSEWGLPNAMSSNIDDRTHLEMYGWPFGDA	
Cellulohydrolase / endoglucanase GH7	<i>Articulospora tetracladia</i>	NNIBRF329	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Aquanectria penicillioides</i>	NNIBRF619	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Clavariopsis aquatica</i>	WD(A)-00-1	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Dactylella cylindrospora</i>	CBS325.70	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Margaritopsis aquatica</i>	NNIBRF339	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Thelonectria rubi</i>	CBS 177.27	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
Cellulohydrolase I (cbhI)	<i>Articulospora tetracladia</i>	NNIBRF329	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Aquanectria penicillioides</i>	NNIBRF619	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Clavariopsis aquatica</i>	WD(A)-00-1	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Dactylella cylindrospora</i>	CBS325.70	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Margaritopsis aquatica</i>	NNIBRF339	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
	<i>Thelonectria rubi</i>	CBS 177.27	TNCTYGTNTWNTACTGCTCAANCALEGAAYASYGITGTHGNALKNFVTVASQTNVGSRYLMAAGSTTAYQMFQLGQEFFDVSNSLPCGLNGALYFAEMSDGGMARFPTNLGAKYGTGYCDAQCPQDIKFINGEVSELDNLLTNKFORPTRSDDGPHPATMSTPVLDNALAVTRWISGR	
Cellulohydrolase II GH6	<i>Articulospora tetracladia</i>	NNIBRF329	GLIQTAKSSALPVAAGVTRPMPVPLTHQSPQAANAVWVGTPLRAMAGMASVNLQSVRQVGGVTKYCL-SGLGIQRQAEQER-YFARRSLHQM-RKW-HSTRYSWRRLWLDL-PQLGIRQCHNCRKVGQPARC-RSIPSTSLRV	
	<i>Aquanectria penicillioides</i>	NNIBRF619	GLIQTAKSSALPVAAGVTRPMPVPLTHQSPQAANAVWVGTPLRAMAGMASVNLQSVRQVGGVTKYCL-SGLGIQRQAEQER-YFARRSLHQM-RKW-HSTRYSWRRLWLDL-PQLGIRQCHNCRKVGQPARC-RSIPSTSLRV	
	<i>Clavariopsis aquatica</i>	WD(A)-00-1	GLIQTAKSSALPVAAGVTRPMPVPLTHQSPQAANAVWVGTPLRAMAGMASVNLQSVRQVGGVTKYCL-SGLGIQRQAEQER-YFARRSLHQM-RKW-HSTRYSWRRLWLDL-PQLGIRQCHNCRKVGQPARC-RSIPSTSLRV	
	<i>Dactylella cylindrospora</i>	CBS325.70	GLIQTAKSSALPVAAGVTRPMPVPLTHQSPQAANAVWVGTPLRAMAGMASVNLQSVRQVGGVTKYCL-SGLGIQRQAEQER-YFARRSLHQM-RKW-HSTRYSWRRLWLDL-PQLGIRQCHNCRKVGQPARC-RSIPSTSLRV	
	<i>Margaritopsis aquatica</i>	NNIBRF339	GLIQTAKSSALPVAAGVTRPMPVPLTHQSPQAANAVWVGTPLRAMAGMASVNLQSVRQVGGVTKYCL-SGLGIQRQAEQER-YFARRSLHQM-RKW-HSTRYSWRRLWLDL-PQLGIRQCHNCRKVGQPARC-RSIPSTSLRV	
	<i>Thelonectria rubi</i>	CBS 177.27	GLIQTAKSSALPVAAGVTRPMPVPLTHQSPQAANAVWVGTPLRAMAGMASVNLQSVRQVGGVTKYCL-SGLGIQRQAEQER-YFARRSLHQM-RKW-HSTRYSWRRLWLDL-PQLGIRQCHNCRKVGQPARC-RSIPSTSLRV	

Hemicellulolytic activity	Endo-β-xylanase GH11	<i>Thelonectria rubi</i>	CBS 177.27	STYQTNASRRLLSVAEGRVPKVPLALHQSPH-DWPVCGCFPERWSPVVKRTSDQPYCSRGLMA-M-DFSS-MELPW-LGAVLRDQAL-LATFVARPRSAAGLPASV-MPAKSSAAGDNLGAGQSPQAWP
		<i>Articulospora tetracladia</i>	NNIBRF329	LVSNITNIPRAITYSGTFSVGNGLVSYGWTTDPLIEYVIESYGYNPSSAATLKGVTSDGGTYDILETRTNQASIEGATFQQYWSVRTSKRTSGVTVTANHFAAWAKLGMTLGTNYQIVATEG
		<i>Aquanectria penicillioidea</i>	NNIBRF619	GKGYWPGSARTINYSFSPNGSNGVYLVYGTWTPSKLIEYVIESYGYNPSSAATLKGVTSDGGTYDILETRTNQASIEGATFQQYWSVRTSKRTSGVTVTANHFAAWAKLGMTLGTNYQIVATEG
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	GKGYWPGSARTINYSFSPNGSNGVYLVYGTWTPSKLIEYVIESYGYNPSSAATLKGVTSDGGTYDILETRTNQASIEGATFQQYWSVRTSKRTSGVTVTANHFAAWAKLGMTLGTNYQIVATEG
		<i>Dactylella cylindrospora</i>	CBS325.70	ARRLR-HQTCRTVYTSYGNPNNGNSLAWYGTWTKNPLIEYVIEFNGTNPSSGASRSLGSLVSDGGTYDILETRTNQASIEGATFQQYWSVRTSKRTSGVTVTANHFAAWAKLGMTLGTNYQIVATEG
		<i>Margaritopsis aquatica</i>	NNIBRF339	VNMWHAQVCRATYSGTFSVGNGLSYGWTTDPLIEYVIESYGYNPSSAATLKGVTSDGGTYDILETRTNQASIEGATFQQYWSVRTSKRTSGVTVTANHFAAWAKLGMTLGTNYQIVATEG
	Endo-xylanase (xyn2)	<i>Thelonectria rubi</i>	CBS 177.27	GKGYWPGSARTINYSFSPNGSNGVYLVYGTWTTDPLIEYVIESYGYNPSSAATLKGVTSDGGTYDILETRTNQASIEGATFQQYWSVRTSKRTSGVTVTANHFAAWAKLGMTLGTNYQIVATEG
		<i>Articulospora tetracladia</i>	NNIBRF329	PSRYRA-TNASHDSDFCNERHRPNFLNFS-SCPSHTMLVECLDGNCRSARTLTLGSHNLWVSAKRHAKLSPGCKVIGSCHCGCAL-GPDRVPLECCSCLNRSLSGSCFCQFNVRSAIAGHRALQCCTRRVCSVALDDWFDQRIGRPSVH-EVAVADRAECRSVVG
		<i>Aquanectria penicillioidea</i>	NNIBRF619	PSRYRA-TNASHDSDFCNERHRPNFLNFS-SCPSHTMLVECLDGNCRSARTLTLGSHNLWVSAKRHAKLSPGCKVIGSCHCGCAL-GPDRVPLECCSCLNRSLSGSCFCQFNVRSAIAGHRALQCCTRRVCSVALDDWFDQRIGRPSVH-EVAVADRAECRSVVG
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	PSIFMLYRSFGAQP-SFTTYNLVAFKSPCLSLAHSQGRPPRFHSHS-SQTRCSDNQ-P-SYNGCSLASHSSKXHQNGFP-YCRQASACAPAKTKDRRSHDQSTAG-PAASDRYHFKRHHSG-FRAGSGRCWYTHSSQRYSTQSDAPWTHRAPPDSSDRRRCRCR-
		<i>Dactylella cylindrospora</i>	CBS325.70	YYSWTDGGSDVYTNLAGGAYVWSSGGNFVGGKGNWPNQGRPRYDQTLVYIC-KASLNTL-NCHLLRYLQSKRKLPCHLRLDQEPETH-VLYC-ELWHLQPIFRRLQELRLH-LWHL-LYLRQHPYQPAIYRNRNLPAI-LVCSQEQANRRYHRKSKPLQRLGRCWPPVGYLGLYDCCD-RLQWQ
		<i>Margaritopsis aquatica</i>	NNIBRF339	QWKFTRKDNSSIKACRFCSVFN-IPSMIEYSIKIHATS-P-QS-P-LSKFNHSLR-PQSG-MWFRFCQ-PKQRNGVQ-S-LYRWFA-CQQTISAGWQCLRLRLRSFVSSRGCHWHHRW-PYPSM-QRLKGMCRSSQCCSTRSGVQWSIRLRDNRRLK-KYQSM-
	α-glucosidase GH31	<i>Thelonectria rubi</i>	CBS 177.27	YYSWTDGGSDVYTNLAGGAYVWSSGGNFVGGKGNWPNQGRPRYDQTLVYIC-KASLNTL-NCHLLRYLQSKRKLPCHLRLDQEPETH-VLYC-ELWHLQPIFRRLQELRLH-LWHL-LYLRQHPYQPAIYRNRNLPAI-LVCSQEQANRRYHRKSKPLQRLGRCWPPVGYLGLYDCCD-RLQWQ
		<i>Articulospora tetracladia</i>	NNIBRF329	HQCRWGANWSQLQDVNDNFRFGIPLIETW-LICPYICMCLNTPRDTIDYMNQYDFENDQTFYFGEAGKLSQLHANGHQYHVPVDSIAYDPENASDAYATSRGIEAFEMMNDPQSDYIYAWPGYVTFDFAGAVLNGTAIDWVNE-MKTYWQVNSFDGIMDSEVSVFCVCSGCSGNLTPNVPFPFLPGEPGNVYGYEFGNITNATEFSSVQAASSSAASASSARA
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	HQCRWGANWSQLQDVNDNFRFGIPLIETW-LICPYICMCLNTPRDTIDYMNQYDFENDQTFYFGEAGKLSQLHANGHQYHVPVDSIAYDPENASDAYATSRGIEAFEMMNDPQSDYIYAWPGYVTFDFAGAVLNGTAIDWVNE-MKTYWQVNSFDGIMDSEVSVFCVCSGCSGNLTPNVPFPFLPGEPGNVYGYEFGNITNATEFSSVQAASSSAASASSARA
		<i>Dactylella cylindrospora</i>	CBS325.70	HQCRWGANWSQLQDVNDNFRFGIPLIETW-LICPYICMCLNTPRDTIDYMNQYDFENDQTFYFGEAGKLSQLHANGHQYHVPVDSIAYDPENASDAYATSRGIEAFEMMNDPQSDYIYAWPGYVTFDFAGAVLNGTAIDWVNE-MKTYWQVNSFDGIMDSEVSVFCVCSGCSGNLTPNVPFPFLPGEPGNVYGYEFGNITNATEFSSVQAASSSAASASSARA
		<i>Margaritopsis aquatica</i>	NNIBRF339	HQCRWGANWSQLQDVNDNFRFGIPLIETW-LICPYICMCLNTPRDTIDYMNQYDFENDQTFYFGEAGKLSQLHANGHQYHVPVDSIAYDPENASDAYATSRGIEAFEMMNDPQSDYIYAWPGYVTFDFAGAVLNGTAIDWVNE-MKTYWQVNSFDGIMDSEVSVFCVCSGCSGNLTPNVPFPFLPGEPGNVYGYEFGNITNATEFSSVQAASSSAASASSARA
		α-glucuronidase GH67	<i>Articulospora tetracladia</i>	NNIBRF329
<i>Clavariopsis aquatica</i>	WD(A)-00-1		GPIDFQVREPSPVPLFTHLRQTGSAAVELQYQTEYQLQQAHLVYAPWMLKELDFDLRVDKSPQLKNIKGGQFGQLGGYAAVNAAGTNTTLWGLSHAMSNLYAGRLAWNPDQSAEDLLESWTKLTFSDHDKRVIINIKSISMISSWASAYENYSGNLGIQTLNLLAHYGNPNASQDGNPWGQWTR	
<i>Margaritopsis aquatica</i>	NNIBRF339		GPIDFQVREPSPVPLFTHLRQTGSAAVELQYQTEYQLQQAHLVYAPWMLKELDFDLRVDKSPQLKNIKGGQFGQLGGYAAVNAAGTNTTLWGLSHAMSNLYAGRLAWNPDQSAEDLLESWTKLTFSDHDKRVIINIKSISMISSWASAYENYSGNLGIQTLNLLAHYGNPNASQDGNPWGQWTR	
<i>Thelonectria rubi</i>	CBS 177.27		GPIDFQVREPSPVPLFTHLRQTGSAAVELQYQTEYQLQQAHLVYAPWMLKELDFDLRVDKSPQLKNIKGGQFGQLGGYAAVNAAGTNTTLWGLSHAMSNLYAGRLAWNPDQSAEDLLESWTKLTFSDHDKRVIINIKSISMISSWASAYENYSGNLGIQTLNLLAHYGNPNASQDGNPWGQWTR	
α-manno-sidase GH92	<i>Articulospora tetracladia</i>		NNIBRF329	TGENPLWKSNEPYSYCIWDSFRSHLPLTLIDPHSQTLVRSIDYRHEGQIFFCF-ISCIS-FLLGKL
	<i>Clavariopsis aquatica</i>		WD(A)-00-1	TGENPKWQSSEPYFDSYCYLWDSFRSRLPFLTLIDPSSLETMRISLINTYHEGWLPCRMTLCKGYTQGGSN
	<i>Margaritopsis aquatica</i>	NNIBRF339	TGENPLWKSNEPYSYCIWDSFRSHLPLTLIDPHSQTLVRSIDYRHEGQIFFCF-ISCIS-FLLGKL	
Endopolygalacturonase (enpg)	<i>Articulospora tetracladia</i>	NNIBRF329	PRSHPGYKRSRQL-LRC-SQYRIHLCCGPEHQSHLPCQWSCR-LSSRSR-C-QPELQFCGADW-W-NQSGCEHKRTRL-LHLLLVHPTM	
	<i>Aquanectria penicillioidea</i>	NNIBRF619	KFADW-R-FVSFQARFPHRRQEDVQLQAHWEH-ELAHSLLLHQRLRPRRQ-PDSGQLWRRAQLQQRWRPRCPQHRRLHLVGHDRHQEHLGPQGR	
	<i>Clavariopsis aquatica</i>	WD(A)-00-1	QDNRPQS-CWRSRW-CRKHQHRKHRYCAH-SHHRPSCRW-CGREPDQ-LS-C-NLQGEYS-HSSSRW-IASWERRRITISLFSKVVRCVW	
	<i>Margaritopsis aquatica</i>	NNIBRF339	YWDGQSGNGDTPKHDFVLKDLVSTISLHQNPWPHCFDVTGCDLMTGLTLDNSAGDAANSASDGAHAAHNSDGFSSDNNVLSNSVYVQDDC	
	<i>Thelonectria rubi</i>	CBS 177.27	RRSRPGCTPWRW-WRQNTSRCSRSPGCRRRRCSRQW-CPSGPASARR-RPRGRAGSQS-SPRWSSRSRGPGRGLASSCRRCRPRPPT	
	Pectinolytic activity	<i>Articulospora tetracladia</i>	NNIBRF329	MKFSGLSGLLAGVAMAAPTPTVNEVNLNRAAATPIGASQNGTGGAGGTTTIVSYAQFTAAGVAGTAKVWVSGPSTAVSQVKVQNSITSLGASSKVVFTGFLGLVKGASVNIIRNIAIAKVAANGDALGVQKSTNWIHDVLDSSVAHDKDYDGLDVRTHLHFAREHDANTYPLAYRPS-
		<i>Aquanectria penicillioidea</i>	NNIBRF619	MRFSQSPVAIIGMAIASPTPTFKQVDRSIAKRATIDACDIYASTNGTGGAGGTTTIVSYAQFTAAGVAGTAKVWVSGPSTAVSQVKVQNSITSLGASSKVVFTGFLGLVKGASVNIIRNIAIAKVAANGDALGVQKSTNWIHDVLDSSVAHDKDYDGLDVRTHLHFAREHDANTYPLAYRPS-
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	MKFSGLSGLLAGVAMAAPTPTVNEVNLNRAAATPIGASQNGTGGAGGTTTIVSYAQFTAAGVAGTAKVWVSGPSTAVSQVKVQNSITSLGASSKVVFTGFLGLVKGASVNIIRNIAIAKVAANGDALGVQKSTNWIHDVLDSSVAHDKDYDGLDVRTHLHFAREHDANTYPLAYRPS-
		<i>Margaritopsis aquatica</i>	NNIBRF339	MKFSGLSGLLAGVAMAAPTPTVNEVNLNRAAATPIGASQNGTGGAGGTTTIVSYAQFTAAGVAGTAKVWVSGPSTAVSQVKVQNSITSLGASSKVVFTGFLGLVKGASVNIIRNIAIAKVAANGDALGVQKSTNWIHDVLDSSVAHDKDYDGLDVRTHLHFAREHDANTYPLAYRPS-
		<i>Thelonectria rubi</i>	CBS 177.27	MKFSGLSGLLAGVAMAAPTPTVNEVNLNRAAATPIGASQNGTGGAGGTTTIVSYAQFTAAGVAGTAKVWVSGPSTAVSQVKVQNSITSLGASSKVVFTGFLGLVKGASVNIIRNIAIAKVAANGDALGVQKSTNWIHDVLDSSVAHDKDYDGLDVRTHLHFAREHDANTYPLAYRPS-
		Pectate lyase (pLVA)	<i>Articulospora tetracladia</i>	NNIBRF329
	<i>Aquanectria penicillioidea</i>		NNIBRF619	YSLFVLSLVAGFAARTSAPASDAIWAQSGDGYTQAAVADSDSDSIVYQAGTYEEQVLDSSVGSGLTYGYTDDQYSKNEVHLTHSGLADEAGNDSSTGLAKNDGLKVVNWNVRSRGEVQALASAYGSEQYGCQFSGYQDVLNENGYHYHFCYEGATDFGQEAIAWFSECTIGSGSYTASGRDSEDFNSVYVINK
<i>Clavariopsis aquatica</i>	WD(A)-00-1		RSFLSLLSATTVAASRTSPGCLVKSASQYSVNTAKLSTSTIAGQIFVYSYGEATIKARSALTYGYTDDQYSKNEVHLTHSGLADEAGNDSSTGLAKNDGLKVVNWNVRSRGEVQALASAYGSEQYGCQFSGYQDVLNENGYHYHFCYEGATDFGQEAIAWFSECTIGSGSYTASGRDSEDFNSVYVINK	
<i>Margaritopsis aquatica</i>	NNIBRF339		DHSSHWCSYWNRRQIFKYCKEL-KL-NLNTNW-VQKADALSKTSTTASQIFVYSYGEATIKARSALTYGYTDDQYSKNEVHLTHSGLADEAGNDSSTGLAKNDGLKVVNWNVRSRGEVQALASAYGSEQYGCQFSGYQDVLNENGYHYHFCYEGATDFGQEAIAWFSECTIGSGSYTASGRDSEDFNSVYVINK	
Pectinesterase	<i>Articulospora tetracladia</i>		NNIBRF329	KSFASLFSFASALATRSAPSAGCTVAPASAVLSLSTSSQAFIQAQIATYEQVLVAPASQVITGATSDSSYGSNTVTITNQLSQANDLSDNETALTRVADKDFRLVYVNSVENSYEGCSQVAVLSAYSDSGYGSFSGFDTLNENGYHYHFCYEGATDFGQEAIAWFSECTIGSGSYTASGRDSEDFNSVYVINK
	<i>Clavariopsis aquatica</i>		WD(A)-00-1	KSFASLFSFASALATRSAPSAGCTVAPASAVLSLSTSSQAFIQAQIATYEQVLVAPASQVITGATSDSSYGSNTVTITNQLSQANDLSDNETALTRVADKDFRLVYVNSVENSYEGCSQVAVLSAYSDSGYGSFSGFDTLNENGYHYHFCYEGATDFGQEAIAWFSECTIGSGSYTASGRDSEDFNSVYVINK
	<i>Thelonectria rubi</i>	CBS 177.27	KSFASLFSFASALATRSAPSAGCTVAPASAVLSLSTSSQAFIQAQIATYEQVLVAPASQVITGATSDSSYGSNTVTITNQLSQANDLSDNETALTRVADKDFRLVYVNSVENSYEGCSQVAVLSAYSDSGYGSFSGFDTLNENGYHYHFCYEGATDFGQEAIAWFSECTIGSGSYTASGRDSEDFNSVYVINK	

Phosphorus assimilation	Acid phosphatase	<i>Articulospora tetracladia</i>	NNIBRF329	TMDVAVHHEHCVTLVLIIEAFFAKDRRCRGRSVAE-LSSLE-HTFGSFACTQSTLKQARCAQMAFF-CGKYERADLHWLQCQGVVWRCTSQP-YIQOTRYQC-YCMSDFREAHVLYTR-QLRWRYR-GEFQTRQEQNLPAEAVWY-LEDFEA-GRDVGHSYGRSCRPRGVA-MI-LVAMPFLDRLGCPICSSDPCPYRKPFA-AYFRCG-HRGG-QCFHSTPSSKSLGWQWVWVCLLPSRGGSP-VLPLQSDVDFDL-T
		<i>Aquanectria penicillioides</i>	NNIBRF619	TMDAERRVH-FATQCAIEAFAGGRCRLDRSREGYSEDPHLITSGM-ACRTRTG-CQARYRWGCP-CEISEQFGRLLFLGREGVR-TSQP-YQSRTRYQCWCKCFYRAEHREHTGTPQRWRK-LGCQRHVPDRNRQASRGPY-AGFGV-GHDARRDFGDRNQLAT-RT-RT-WRVSGHPARHSCPCIASDHHPCTQIRA-AYSRCG-RRVGRQYSRA-APS-AKSRGQWGTGFLPSPDGF-GRQARQSGGYAP-R
		<i>Clavariopsis aquatica</i>	WD(A)-00-1	TGELVALKEIHLDEEGTPTAIREISLMKELKHENLVLDHVIHTENKLMVFEFMDKDLKRYMDSRGRDGLDPTTICSMFYQLRGIAFCHDNRLHRLKPNLNNRQQLKADFLARAGFIPVNTFSNEVTLWYRAPDVLGSRTYNTSIDIWSAGCIAMAYTRPLFGTTNEDLQKIFRLMGTSPERSWPQISQYPEYKGN-FHIFATQDLRLILPQVDQGLNLLNSMLQLRPEMRYASAAALNHPWFNDLPQR
		<i>Dactylella cylindrospora</i>	CBS325.70	TGELVALKEIHLDEEGTPTAIREISLMKELKHENLVLDHVIHTENKLMVFEFMDKDLKRYMDSRGRDGLDPTTICSMFYQLRGIAFCHENRVLHRLKPNLNNRQQLKADFLARAGFIPVNTFSNEVTLWYRAPDVLGSRTYNTSIDIWSAGCIAMAYTRPLFGTTNEDLQKIFRLMGTSPERSWPQISQYPEYRNN-FHYATQDLRLILRQDPVGLDLSMLQLRPEMRYASAAALNHPWFNDLPQR
		<i>Margaritopsis aquatica</i>	NNIBRF339	TMGAAHRVR-FVALASTEFAFFGDQFQ-DRSGVRSPLVHTFESSACIRSTRVFLAKVRMEFP-FGKSEQAGPRWSFLEIMVQCTFLP-CILQTRYRCWYMYMDFQEEHQVLTITLPRWRKC-REFRMLWQDQNLPAEAWC-SEDFV-DRGEEHLDCKSQYPEVDA-TT-WLWLLDRLQYPTYSNCPYRKP-ACSQCG-HRVG-RYSRASVPSSKISPGWL-KYPLPLNDGSPSGQLIHPSVDFDP-R
		<i>Thelonectria rubi</i>	CBS 177.27	TRGAAERRGH-FATRVGAGACAEVGRRCWRGLREGCF-GPWRTFTWACTRGTG-CPATCAPWCEP-CGRFSQSPRF-WFQKQEVDR-TSQP-CNRRTRCRWCSSRYSYAKHREDRDTP-PPRY-RY-RVCRRRGQDRNRNPASAAPCC-ASSGASDLGAARSGGRSFP-EVGT-TI-WWGA-GHPALRCCPCIASDRRPRTRRA-ACSPCV-HRGGQYSRV-R-VPS-KRESPEYWGFLRNPNGSP-ARRARRSDGYGP-R

Table A8. Evolutionary divergence (%) between AQH species based on ITS-5.8S-ITS2 sequences. The number of base substitutions per site from averaging overall sequence pairs and single isolates between the species is shown. Analyses were performed resorting to the maximum composite likelihood-parameter method. The lower half of the matrix contains p-distance values (in percentage) and the upper half values correspond to the standard error for each p-distance.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>Alatospora acuminata</i> (1)		0.92%	1.73%	1.61%	1.66%	1.93%	1.94%	1.54%	1.91%	1.79%	1.66%	1.62%	1.68%	1.63%	1.63%	1.91%	1.68%	1.71%
<i>Alatospora pulchella</i> (2)	5.19%		1.66%	1.60%	1.57%	1.93%	1.88%	1.47%	1.95%	1.73%	1.65%	1.61%	1.64%	1.59%	1.59%	1.95%	1.65%	1.67%
<i>Anguillospora crassa</i> (3)	19.65%	19.15%		1.60%	1.60%	1.92%	1.89%	1.77%	1.91%	1.57%	1.66%	1.65%	1.66%	1.62%	1.60%	1.91%	1.66%	0.68%
<i>Anguillospora filiformis</i> (4)	17.72%	18.25%	16.83%		1.09%	1.78%	1.95%	1.55%	1.92%	1.61%	1.14%	1.13%	1.66%	1.67%	1.57%	1.92%	1.71%	1.56%
<i>Articulospora tetracladia</i> (5)	16.46%	15.88%	16.07%	6.61%		1.68%	1.78%	1.52%	1.81%	1.63%	1.03%	0.95%	1.43%	1.38%	1.37%	1.81%	1.54%	1.54%
<i>Aquanectria penicillioides</i> (6)	24.08%	23.16%	25.16%	21.69%	20.45%		1.90%	1.84%	1.44%	2.01%	1.80%	1.79%	1.87%	1.82%	1.83%	1.44%	1.90%	1.87%
<i>Clavariopsis aquatica</i> (7)	24.58%	24.84%	25.05%	25.05%	22.18%	26.42%		1.90%	1.89%	1.98%	1.82%	1.87%	1.80%	1.74%	1.79%	1.89%	1.86%	1.79%
<i>Collemboispora barbata</i> (8)	14.81%	13.37%	19.69%	16.46%	15.03%	21.33%	25.89%		1.96%	1.80%	1.63%	1.57%	1.67%	1.68%	1.67%	1.96%	1.66%	1.72%
<i>Dactylella cylindrospora</i> (9)	26.81%	25.86%	26.02%	25.30%	23.86%	13.78%	26.13%	24.80%		1.96%	1.84%	1.86%	1.80%	1.81%	1.76%	0.00%	1.85%	1.85%
<i>Dimorphospora foliicola</i> (10)	20.62%	19.42%	17.90%	16.89%	17.07%	24.48%	27.25%	24.03%	26.18%		1.61%	1.64%	1.77%	1.67%	1.69%	1.96%	1.48%	1.45%
<i>Lemonniera aquatica</i> (11)	17.69%	17.69%	18.38%	8.08%	6.13%	21.18%	23.75%	17.44%	23.75%	18.09%		0.67%	1.48%	1.42%	1.46%	1.84%	1.48%	1.59%
<i>Margaritopsis aquatica</i> (12)	16.25%	16.28%	16.93%	7.35%	5.46%	20.91%	23.74%	16.18%	24.03%	17.29%	2.59%		1.45%	1.41%	1.42%	1.86%	1.51%	1.58%
<i>Tetracladium apiense</i> (13)	17.92%	17.73%	18.60%	17.55%	14.40%	22.61%	22.86%	17.32%	22.37%	19.84%	14.58%	14.13%		0.55%	0.55%	1.80%	1.61%	1.63%
<i>Tetracladium marchalianum</i> (14)	17.71%	17.13%	18.36%	17.93%	14.39%	22.78%	23.39%	17.69%	22.30%	18.62%	14.75%	14.30%	1.82%		0.62%	1.81%	1.55%	1.56%
<i>Tetracladium furcatum</i> (15)	17.69%	17.50%	17.99%	16.13%	13.82%	22.76%	23.02%	17.48%	22.75%	18.13%	14.55%	13.91%	2.00%	2.64%		1.76%	1.58%	1.57%
<i>Thelonectria rubi</i> (16)	26.81%	25.86%	26.02%	25.30%	23.86%	13.78%	26.13%	24.80%	0.00%	26.18%	23.75%	24.03%	22.37%	22.30%	22.75%		1.85%	1.85%
<i>Tricladium chaetocladium</i> (17)	18.27%	18.65%	18.64%	18.28%	16.34%	22.07%	24.75%	19.70%	23.17%	15.54%	16.57%	15.61%	17.34%	16.59%	17.68%	23.17%		1.61%
<i>Tricladium splendens</i> (18)	20.15%	19.98%	2.85%	16.61%	16.11%	24.26%	23.37%	20.06%	24.82%	16.80%	17.61%	16.57%	18.48%	17.70%	18.06%	24.82%	18.13%	
<i>Varicosporium elodeae</i> (19)	17.16%	16.93%	17.13%	5.76%	3.80%	21.26%	24.20%	15.35%	25.00%	16.57%	6.82%	6.51%	15.16%	14.55%	14.54%	25.00%	17.52%	16.91%

Table A9. Evolutionary divergence (%) between AQH species based on nitrate reductase nucleotide sequences. The number of base substitutions per site from averaging overall sequence pairs and single isolates between the species is shown. Analyses were performed resorting to the maximum composite likelihood-parameter method. The lower half of the matrix contains p-distance values (in percentage) and the upper half values correspond to the standard error for each p-distance.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>Alatospora acuminata</i> (1)		1.11%	1.47%	1.44%	1.49%	1.64%	1.57%	1.47%	1.52%	1.48%	1.52%	1.51%	1.47%	1.43%	1.51%	1.56%	1.23%	1.44%
<i>Alatospora pulchella</i> (2)	13.36%		1.48%	1.40%	1.49%	1.69%	1.57%	1.53%	1.55%	1.47%	1.55%	1.53%	1.53%	1.55%	1.53%	1.57%	1.32%	1.52%
<i>Anguillospora crassa</i> (3)	26.18%	28.08%		1.39%	1.43%	1.64%	1.57%	1.37%	1.59%	1.36%	1.40%	1.39%	1.37%	1.39%	1.34%	1.62%	1.23%	0.94%

<i>Anguillospora filiformis</i> (4)	29.15%	29.86%	21.92%		1.31%	1.51%	1.48%	1.48%	1.57%	1.40%	1.36%	1.41%	1.48%	1.45%	1.52%	1.53%	1.14%	1.32%
<i>Articulospora tetracladia</i> (5)	30.22%	30.16%	23.91%	19.69%		1.54%	1.49%	1.37%	1.45%	1.35%	1.18%	1.23%	1.37%	1.35%	1.49%	1.58%	1.16%	1.37%
<i>Aquanectria penicillioides</i> (6)	37.46%	37.10%	34.84%	34.60%	33.79%		1.50%	1.52%	1.63%	1.56%	1.58%	1.56%	1.52%	1.53%	1.54%	1.39%	1.38%	1.65%
<i>Clavariopsis aquatica</i> (7)	33.65%	32.70%	31.98%	33.29%	32.24%	36.75%		1.55%	1.55%	1.50%	1.55%	1.56%	1.55%	1.53%	1.46%	1.60%	1.30%	1.56%
<i>Collembolispora barbata</i> (8)	31.28%	32.11%	24.17%	26.42%	24.98%	35.91%	33.17%		1.61%	1.36%	1.46%	1.41%	0.00%	1.03%	1.09%	1.53%	1.24%	1.44%
<i>Dactylella cylindrospora</i> (9)	32.70%	34.49%	33.41%	34.49%	32.66%	38.78%	37.13%	37.11%		1.57%	1.63%	1.53%	1.61%	1.60%	1.66%	1.57%	1.32%	1.61%
<i>Dimorphospora foliicola</i> (10)	28.79%	29.86%	22.16%	25.47%	25.82%	35.79%	31.86%	24.64%	35.92%		1.39%	1.34%	1.36%	1.37%	1.33%	1.49%	1.13%	1.33%
<i>Lemonniera aquatica</i> (11)	30.44%	30.92%	22.95%	22.47%	17.55%	34.96%	32.82%	24.73%	34.13%	26.04%		0.70%	1.46%	1.47%	1.53%	1.58%	1.18%	1.37%
<i>Margaritopsis aquatica</i> (12)	29.37%	29.85%	23.42%	22.47%	17.91%	33.89%	32.46%	24.61%	34.37%	25.68%	4.76%		1.41%	1.49%	1.47%	1.56%	1.21%	1.42%
<i>Tetracladium apiense</i> (13)	31.28%	32.11%	24.17%	26.42%	24.98%	35.91%	33.17%	0.00%	37.11%	24.64%	24.73%	24.61%		1.03%	1.09%	1.53%	1.24%	1.44%
<i>Tetracladium marchalianum</i> (14)	29.92%	32.29%	25.12%	26.60%	25.79%	35.49%	32.76%	11.61%	37.17%	22.87%	26.28%	25.45%	11.61%		1.10%	1.55%	1.18%	1.39%
<i>Tetracladium furcatum</i> (15)	31.75%	32.23%	24.88%	27.25%	26.89%	34.96%	32.10%	12.09%	38.31%	24.41%	26.99%	26.52%	12.09%	12.44%		1.57%	1.26%	1.43%
<i>Thelonectria rubi</i> (16)	34.72%	34.72%	31.99%	32.10%	32.36%	22.35%	34.84%	34.96%	38.07%	33.29%	34.13%	32.70%	34.96%	34.54%	32.94%		1.35%	1.65%
<i>Tricladium chaetocladium</i> (17)	29.87%	30.11%	26.61%	25.96%	25.29%	35.45%	33.85%	27.44%	37.43%	26.55%	26.35%	26.53%	27.44%	26.52%	26.97%	34.38%		1.21%
<i>Tricladium splendens</i> (18)	26.66%	28.32%	9.08%	21.56%	24.75%	35.08%	32.46%	24.64%	32.70%	21.80%	24.02%	24.61%	24.64%	23.82%	25.83%	33.06%	26.49%	
<i>Varicosporium elodeae</i> (19)	29.13%	30.68%	22.83%	21.64%	16.66%	35.08%	31.38%	25.33%	33.89%	25.21%	17.00%	16.65%	25.33%	25.80%	25.92%	33.53%	26.71%	23.07%

Table A10. Evolutionary divergence (%) between AQH species based on nitrate reductase amino acid sequences. The number of base substitutions per site from averaging overall sequence pairs and single isolates between the species is shown. Analyses were performed resorting to the maximum composite likelihood-parameter method. The lower half of the matrix contains p-distance values (in percentage) and the upper half values correspond to the standard error for each p-distance.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>Alatospora acuminata</i> (1)		1.12%	2.26%	2.20%	2.39%	2.51%	2.64%	2.15%	2.52%	2.25%	2.26%	2.23%	2.15%	2.13%	2.14%	2.61%	1.96%	2.20%
<i>Alatospora pulchella</i> (2)	4.15%		2.29%	2.24%	2.43%	2.49%	2.63%	2.25%	2.58%	2.27%	2.29%	2.26%	2.25%	2.24%	2.25%	2.60%	1.97%	2.24%
<i>Anguillospora crassa</i> (3)	18.15%	18.51%		1.85%	2.02%	2.69%	2.62%	1.95%	2.52%	1.78%	1.96%	2.02%	1.95%	2.01%	2.00%	2.64%	1.74%	0.96%
<i>Anguillospora filiformis</i> (4)	18.51%	19.22%	11.74%		1.79%	2.63%	2.61%	2.05%	2.53%	1.86%	1.93%	1.93%	2.05%	2.07%	2.11%	2.64%	1.66%	1.78%
<i>Articulospora tetracladia</i> (5)	21.25%	21.96%	14.11%	11.43%		2.64%	2.65%	2.22%	2.45%	2.13%	1.45%	1.51%	2.22%	2.24%	2.29%	2.60%	1.78%	2.02%
<i>Aquanectria penicillioides</i> (6)	28.93%	28.21%	30.00%	29.64%	28.75%		2.59%	2.66%	2.79%	2.68%	2.60%	2.60%	2.66%	2.62%	2.67%	1.96%	2.44%	2.67%
<i>Clavariopsis aquatica</i> (7)	72.62%	72.62%	72.62%	71.83%	72.31%	75.30%		2.68%	2.67%	2.62%	2.61%	2.64%	2.68%	2.67%	2.70%	2.59%	2.68%	2.65%
<i>Collembolispora barbata</i> (8)	17.08%	18.51%	12.10%	14.59%	16.96%	29.29%	72.22%		2.60%	2.00%	2.03%	2.04%	0.00%	1.11%	0.87%	2.63%	1.69%	1.93%
<i>Dactylella cylindrospora</i> (9)	26.88%	26.88%	26.52%	28.67%	26.80%	35.61%	72.40%	28.67%		2.59%	2.42%	2.43%	2.60%	2.57%	2.56%	2.83%	2.38%	2.50%
<i>Dimorphospora foliicola</i> (10)	17.44%	18.86%	10.32%	12.46%	15.71%	30.00%	72.22%	13.17%	29.39%		2.15%	2.12%	2.00%	1.98%	2.09%	2.64%	1.88%	1.74%
<i>Lemonniera aquatica</i> (11)	20.36%	21.07%	13.21%	13.57%	7.50%	28.57%	73.31%	14.64%	25.54%	16.07%		0.69%	2.03%	2.05%	2.11%	2.61%	1.78%	2.01%
<i>Margaritopsis aquatica</i> (12)	18.93%	19.64%	13.57%	13.21%	7.86%	27.86%	72.91%	14.29%	25.54%	15.00%	1.43%		2.04%	1.99%	2.14%	2.59%	1.79%	1.97%
<i>Tetracladium apiense</i> (13)	17.08%	18.51%	12.10%	14.59%	16.96%	29.29%	72.22%	0.00%	28.67%	13.17%	14.64%	14.29%		1.11%	0.87%	2.63%	1.69%	1.93%
<i>Tetracladium marchalianum</i> (14)	16.19%	17.97%	12.81%	14.95%	17.86%	30.18%	72.62%	3.74%	28.32%	12.81%	15.18%	14.11%	3.74%		1.21%	2.64%	1.80%	1.93%
<i>Tetracladium furcatum</i> (15)	17.08%	18.51%	12.81%	15.66%	18.04%	29.64%	72.62%	2.14%	28.32%	14.23%	15.71%	15.36%	2.14%	4.45%		2.65%	1.73%	2.00%
<i>Thelonectria rubi</i> (16)	30.36%	29.64%	30.71%	29.64%	28.39%	13.57%	76.10%	30.00%	36.33%	30.00%	29.29%	28.57%	30.00%	30.36%	30.36%		2.46%	2.63%
<i>Tricladium chaetocladium</i> (17)	18.15%	17.97%	15.48%	15.12%	15.80%	28.93%	71.83%	15.12%	29.93%	16.90%	16.43%	15.89%	15.12%	16.10%	15.48%	29.11%		1.75%
<i>Tricladium splendens</i> (18)	16.73%	17.79%	2.82%	11.03%	13.75%	29.64%	72.22%	12.10%	25.45%	9.61%	13.57%	13.21%	12.10%	11.74%	12.81%	30.00%	15.48%	
<i>Varicosporium elodeae</i> (19)	19.29%	20.00%	12.86%	11.79%	5.89%	29.29%	72.11%	15.36%	26.26%	14.64%	6.43%	6.07%	15.36%	15.89%	16.43%	30.00%	15.89%	13.21%