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MASTER THESIS

TITLE

Qualitative and quantitative detection of the crayfish plague pathogen *Aphanomyces astaci*

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Abstract

Crayfish plague, causing rapid mortality of infected susceptible crayfish, is the most severe disease afflicting freshwater crayfish and have been responsible for serious devastations throughout Europe. The oomycete *Aphanomyces astaci* has been identified as the etiological agent for the disease.

Other than the highly susceptible European species, the North American crayfish are barely affected, since there immune system is able to restrict the pathogen growth. But this fact makes the North American crayfish to a perfect host and carrier for *A. astaci*. In Europe, the nonindigenous species have therefore been widely spread and the native species are already threatened with extinction.

For this reason it is absolutely important to have a reliable tool for a large scale detection of the pathogen to eradicate the disease. Such modern molecular tools that meet these standards require multiple functionally constraint target sequences. But the methods that have been established up to now do not fulfil these demands. They simply use the ITS, a heterogenous multi-copy cluster, from which the existence of a primary sequence constraint is unknown, as target region.

In this work, a reliable, sensitive and robust qPCR/MCA for qualitative detection and a TaqMan qPCR for absolute quantitative detection have been established. It is shown that this diagnostic tool discriminates *A. astaci* from related species like *A. frigidophilus*, *A. invadans*, *A. laevis*, *A. helicoides*, *A. irregularis* and *Leptolegnia caudata*. These assays are targeting the sequences of the constitutively expressed and functionally constrained members of the glycosyl hydrolase 18 (GH 18) gene family, *CHI2* and *CHI3*. The multiplex qPCR/MCA system uses an additional endogenous control, targeting a region in the *5.8S rRNA* gene that is highly conserved among oomycetes. For these reasons this system minimises the risk to detect false negative results. Moreover, with the quantitative TaqMan qPCR assay it is possible to determine the pathogen load of the sample.

In conclusion, the detection system established in this work has the potential to serve as powerful tool for a reliable, robust and sensitive detection of *A. astaci* in clinical samples.

Zusammenfassung

Krebspest ist für das weitläufige Sterben von Flusskrebsen in Europa verwantwortlich und repräsentiert damit die schwerste Seuche unter Flusskrebsen, da die Krankheit unweigerlich einen schnellen Tod des infizierten Tieres auslöst. Als Erreger dieser tödlichen Krankheit wurde der Oomyzet *Aphanomyces astaci* identifiziert.

Im Gegensatz zu den nordamerikanischen Arten sind die europäischen hoch empfänglich für die Krebspest. Die Tatsache dass das Immunsystem der nordamerikanischen Spezies das Wachstum des Erregers einzudämmen vermag und die Seuche dadurch nur sehr selten ausbricht, macht diese Arten aber zu einem perfekten Wirt und Überträger von *A. astaci*. In Europa breiten sich diese fremden Arten immer weiter aus, wohingegen die einheimischen Krebse durch die Krankheit vom Aussterben bedroht sind.

Deshalb ist es äußerst wichtig ein verlässliches Detektionssystem für den Erreger zu haben. Als Basis für ein derartiges, molekulares Detektionssystem sollten unbedingt multiple, zwangsweise funktionelle DNA Sequenzen dienen. Die bis jetzt etablierten Methoden erfüllen diese Anforderung nicht, da sie lediglich auf die ITS, einem heterogenen multicopy Gencluser von dem nicht bekannt ist ob es zwangsweise funktional ist, basieren.

Die Entwicklung einer modernen, verlässlichen, sensitiven und robusten qPCR/MCA für eine qualitative Detektion und einer TaqMan-qPCR für eine absolute quantitative Detektion war Gegenstand dieser Arbeit.

Es konnte gezeigt werden, dass diese Methode geeignet ist *A. astaci* von anderen nah verwandten Arten (*A. frigidophilus*, *A. invadans*, *A. laevis*, *A. helicoides*, *A. irregularis* and *Leptolegnia caudata*) zu unterscheiden.

Als Basis dieser quantitativen PCR-Methoden dienten die konstitutiv exprimierten, zeitlich unterschiedlich regulierten und daher zwangsweise funktionellen Chitinase Sequenzen der GH18-Genfamilie, *CHI2* und *CHI3*. Zusätzlich wurde in das qPCR/MCA System eine endogene Kontrolle inkludiert, dessen Primer eine Region im *5.8S rRNA* Gen, die in Oomycten am höchsten konserviert ist, erkennen.

So wurde das Risiko falsch negative Ergebnisse zu erhalten beträchtlich minimiert. Zusätzlich ermöglich es die TaqMan-qPCR einen Überblick über die Stärke des Befalls zu bekommen.

Zusammenfassend kann man sagen, dass diese Diagnosemethode die Fähigkeit hat als leistungsfähige, verlässliche, robuste und sensitive Methode zur Detektion von *A. astaci* in klinischen Proben zu dienen.

Contents

1 Introduction

1.1 Crayfish

The Crayfish or *Astacida* is a freshwater invertebrate and belongs to the *Decapoda*, the largest crustacean taxon (Scholtz & Richter, 1995).

There are two superfamilies, the *Astacoidea* and the *Parastacoidea*. The *Astacoidea* consists of two families, the *Astacidae* (Figure 1) and the *Cambaridae* and is restricted in distribution to the Northern Hemisphere, whereas the *Parastacoidea* consist of only one family, the *Parastacidae*, which is limited to the Southern Hemisphere (Hobbs, 1988).

Out of the more than 500 recognised species of freshwater crayfish, adopted to the most widely used taxonomic scheme only five are native to Europe (*Astacus astacus, Astacus leptodactylus, Astacus pachypus, Austropotamobius pallipes and Austropotamobius torrentium*) (Gerhardi & Holdich, 1999), whereas with 384 identified species North America and Mexico have the richest crayfish fauna in the world (Taylor, 2002).

A

B

Figure 1: Specimen of crayfish species belonging to the *Astacidae* family: A. The noble crayfish (*Astacus astacus*) is a species native to Europe. Picture taken from Wikipedia 20 March 2009. Wikimedia Foundation, Inc.. 9 Jan. 2010 <http://de.wikipedia.org/wiki/Edelkrebs>. **B.** The North American signal crayfish (*Pacifastacus leniusculus)* represents an alien species invading into the European crayfish fauna. Picture kindly provided by Gerald Hochwimmer.

Due to competition with the nonindigenous species, range reductions and, most important, the introduction of the pathogen *A. astaci*, which is the causative agent of the crayfish plague (Nybelin, 1936; Schäperclaus, 1935; 1954), European species have been suffering considerable population declines. Since, native crayfish populations are decreasing in that dimension the nonindigenous and probably largely *A. astaci* infected North American species have been widely spread throughout the continent not only unintentionally but also purposely to replace the lost native populations.

Anyway, things look bad for the European species. *A. astacus, A. pallipes* and *A. torrentium* are considered threatened by the International Union for Conservation of Nature and Natural Resources (Baillie *et al.*, 1996) and their exploitation and harvesting are subject to controls since they are listed as protected in Appendix III of the Bern Convention (Council of Europe, 1991). Additionally, *A. pallipes* is cited in Appendix V of the European Habitats Directive (Council of European Communities, 1992), requiring the designation of special areas of conservation for its protection.

1.2 Crayfish plague

In Europe, crayfish plague was first observed in the Po valley in Northern Italy in 1859 (Alderman, 1996; Cornalia, 1860). Since the disease causes rapid mortality of infected susceptible crayfish the plague is deemed to be the most serious disease to affect freshwater crayfish. After Italy the disease spread over France and Germany (Raveret-Wattel, 1885) throughout the whole continent, where the crayfish plague has been responsible for the devastation of numerous crayfish populations during the last century (Alderman, 1996).

Together with the introduction of North American crayfish species (e.g. *Pacifastacus leniusculus, Procambarus clarkii, Orconectes limosus*) it is presumed that *A. astaci* was introduced into Europe (Söderhäll & Cerenius, 1999; Unestam, 1969). The North American species, *Pacifastacus leniusculus, Procambarus clarkii, Orconectes limosus* are considered to serve as perfect long-term host and carrier for the pathogen, because they are able to restrict the pathogen growth to their cuticle and are therefore barely affected by the disease (Dieguez-Uribeondo & Söderhäll, 1993; Unestam, 1969; 1972; 1976; Unestam & Weiss, 1970; Vey *et al.*, 1983). In contrast, species from Australia, New Guinea, Japan and Europe have been shown to be highly susceptible (Unestam, 1969; 1976). Unlike the North American crayfish, the highly susceptible species show only a very weak immune response to *A. astaci* (Cerenius *et al.*, 1988).

It is shown, that highly resistant species produce blood cells that continuously assemble prophenoloxidase (proPO) transcripts. The activity of the enzyme, which catalyses the oxidation of phenols to melanin, leads to the encapsulation of the parasite by a sheath of melanin. However, the number of proPO transcripts in susceptible crayfish species is low (Cerenius *et al.*, 2003).

Thus, the high degree of encapsulation and melanisation of the invading pathogen is considered to be the reason for the inhibition of the outgrowth and invasion of the hyphae into the haemocoel and the inner organs (Unestam, 1976; Unestam & Weiss, 1970). But, *A. astaci* remains viable in the melanin sheath, since host defence and parasite attack are usually balanced. When the host is weakened by immunosuppressive conditions the parasite is able to overtake its host and produce an extensive mycelium (Söderhäll & Cerenius, 1992). In vivo, this may happen during moulting, during attacks by other parasites or during bad environmental conditions. So the susceptibility to the disease varies not only with species but also with stress status. On the contrary, in highly susceptible crayfish species a balance between the host defence and parasite attack is never established and the only partially melanised hyphae grow throughout the crayfish and cause the rapid death of the animal (Cerenius *et al.*, 2003).

1.2.1 Phylogenetic classification of A. astaci

The oomycete *A. astaci* represents the causative agent of the crayfish plague (Nybelin, 1936; Schäperclaus, 1935; 1954), and is therefore regarded as an evident hazard for European crayfish species.

Oomycetes form a diverse group of fungus-like eukaryotic microorganisms, also known as water molds, comprising saprophytes as well as pathogens of plants, insects, crustaceans, fish, vertebrate animals, and various microorganisms (Margulis & Schwartz, 1998).

Latest phylogenetic analysis based on genomic and protein sequence data dedicate oomycetes to the kingdom *Heterokonta*. Before, due to their morphology oomycetes have long been classified as fungi, but this new classification places oomycetes far away from fungi next to photosynthetic algae such as brown algae and diatoms (Baldauf *et al.*, 2000; Lamour *et al.*, 2007).

A. astaci is a member of the order *Saprolegniales*, to which the most important oomycete fish pathogens belong, e.g. *Saprolegnia parasitica* causing ulcerative dermal necrosis (Scott, 1964; Willoughby, 1978) and *A. invadans* causing ulcerative mycosis (Blazer *et al.*, 2000; Blazer *et al.*, 2002). This order also contains plant pathogens like *Phytophthora infestans* and *A. euteiches*, which are the etiological agents of potato late blight (Judelson, 1997) and seedling blight (Gaulin *et al.*, 2008), respectively. Moreover, at least one member of Saprolegniales exhibits zoonotic potential. *Phythium insidiousum* can cause the potentially life-threatening disease, phytiosis insidiosii in human (Mendoza *et al.*, 1993).

1.2.2 The life cycle of *A. astaci*

The parasite propagates via asexual zoospores that measure 8 x 12 µm and that have the ability to swim towards a potential host attracted by chemotaxis (Cerenius & Söderhäll, 1984) targeting the soft parts of the crayfish integument, particularly the joints, the bottom side of the abdomen and the eyestalks (Oidtmann *et al.*, 1999) as well as fresh wounds (Nyhlen & Unestam, 1980). Once the zoospore settles on the crayfish cuticle it discards the flagellae, encysts and an infection plug (germ tube) that penetrates the cuticle and weakens the lipid layer is developed (Svensson, 1978). Soon after the germ tube has penetrated the cuticle a vegetative mycelium develops and the fungal hyphae ramify the cuticle mostly in a direction parallel to the chitin fibrils (Unestam & Weiss, 1970). The hyphae begin to secrete chitinases and proteases (Söderhäll & Unestam, 1975). The different chitinases (Andersson & Cerenius, 2002) cooperatively provide further nutrients and facilitate further growth by degrading the chitin polymers of the endocuticula (Nyhlen & Unestam, 1975). It is shown that *A. astaci* permanently expresses chitinase and that this feature distinguishes *A. astaci* from the other closely related crayfish afflicting oomycetes (Andersson & Cerenius, 2002). Moreover, no other cellolytic or pectinolytic enzymes are expressed (Unestam, 1966).

The growing hyphae may penetrate the ventral nerve cord and in weakened or susceptible crayfish the hyphae further invade deeper tissues and other organs via connective tissues, with the tissue sheaths around nerves and muscles becoming infected before penetration of remaining organ tissue (Alderman & Polglase, 1988; Oidtmann *et al.*, 1999). The latter phase is observed in the last few hours before death when the parasite completely disrupts the internal defence mechanisms of the host (Alderman & Polglase, 1988; Unestam, 1973). Finally, just prior or soon after death of the host, sporulation and release of zoospores takes place when hyphae grow outwards where the primary spores are extruded through the hyphal tip after which they round up, develop a cell wall and cluster around the sporangial orifice to form a spore ball, which consist of primary spores that cyst and are discharged as secondary zoospores, which in turn develop flagella and swim off (Alderman & Polglase, 1986; Cerenius *et al.*, 1988).

As *A. astaci* is highly specialized on crayfish, the survival time outside his host can be depending on environmental conditions and life stage considerably limited (CEFAS, 2000; Oidtmann *et al.*, 2002; Söderhäll & Cerenius, 1999). Anyway, *A. astaci* is able to remain viable on fish scales and in the cuticle of fish gut, what enables the propagation of the pathogen also by fish transfer (Oidtmann *et al.*, 2002).

1.3 The aim of this work

For the effective protection of the native European freshwater crayfish species it is absolutely important to have a rough, sensitive tool that is able to detect the pathogen reliably in clinical samples. With its help you can get an overview of the prevalence of the disease and it allows taking steps against a further spread of *A. astaci*. Such a modern molecular tool that meet these standards requires multiple functionally constraint target sequences. Unfortunately, establishing such a diagnostic tool has not been yet successful. The aim of this work is establishing a detection system that meets these demands.

1.3.1 So far established detection systems

There have been attempts to identify afflicted crayfish by surveying for visible melanised spots (Figure 2) in the cuticle. But these symptoms can also occur after physical injury (Söderhäll & Cerenius, 1998).

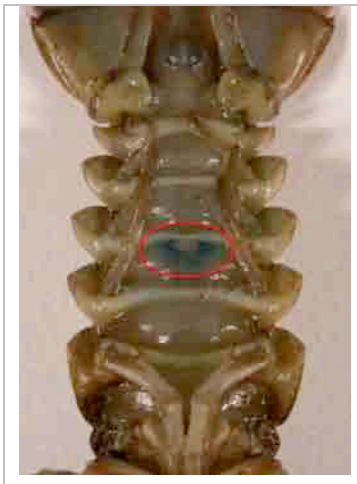

Figure 2: Melanised spot on the abdomen of a plague afflicted crayfish can be a symptom of an aphanomycosis. The melanised spot, which is indicated by the red circle, is regarded as a product of an immune reaction against the invading parasite. Picture kindly provided by Gerald Hochwimmer.

Checking for characteristic *A. astaci* fungal hyphae with direct microscopy turned out to be not sufficient enough for species determination (Alderman & Polglase, 1986; Oidtmann *et al.*, 1999).

Isolation and cultivation of the pathogen combined with following infection experiments on susceptible crayfish is therefore necessary for reliable results. However, such an experiment is very complicated and therefore not always successful. Apart from a time horizon of several weeks, plague-free crayfish stock is also needed for the experiment. Due to the fact that the cuticle of crayfish is usually also secondary affected mainly with saprophytic moulds or bacteria, isolation and identification of the pathogen is complicated enormously. Compared to other oomycetes *A. astaci* has a low grow rate and is therefore easily overgrown by other moulds. Besides, bacteria are shown to inhibit growth of the agent (Oidtmann *et al.*, 1999). The best recovery rate (70 %) reported after an optimisation of the cultivation method by Oidtmann (1999) was achieved with good quality samples from recently dead or moribund samples. Hence an alternative method, which meets the mentioned standards, is required. For this purpose, PCR based methods have already delivered promising results but suffer from cross reactivity with closely related species (Ballesteros *et al.*, 2007; Oidtmann *et al.*, 2006). Although, a recently published assay (Vralstad *et al.*, 2009) promises to overcome this problem, but like the other it also uses primer targeting the internal transcript spacers (ITS). However, a modern molecular pathogen detection system requires multiple functionally constraint target sequences for maximum reliability. But the existence of a primary sequence constraint is unknown in this heterogeneous multi-copy cluster.

1.3.1.1 Internal Transcribed Spacers (ITS)

This nuclear region lies between the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA) and consists of two noncoding spacer regions, ITS1 and ITS2, separated by the *5.8S rRNA* gene (Gardes & Bruns, 1993) (Figure 3).
Eukaryotes typically encode hundreds of copies of this transcription unit and these units are organized in large tandem arrays. Including the *5.8S rRNA* gene, this region is typically about 550 to 900 bp long and can due to highly conserved flanking regions be amplified from various eukaryotes with the universal primers ITS1 and ITS4 or ITS5 and ITS4 (White *et al.*, 1990).

As this gene family undergoes rapid concerted evolution via unequal crossing-over and gene conversion the ITS have great potential for phylogenetic analysis and identifying (Arnheim, 1983; Arnheim *et al.*, 1980; Hillis *et al.*, 1991).

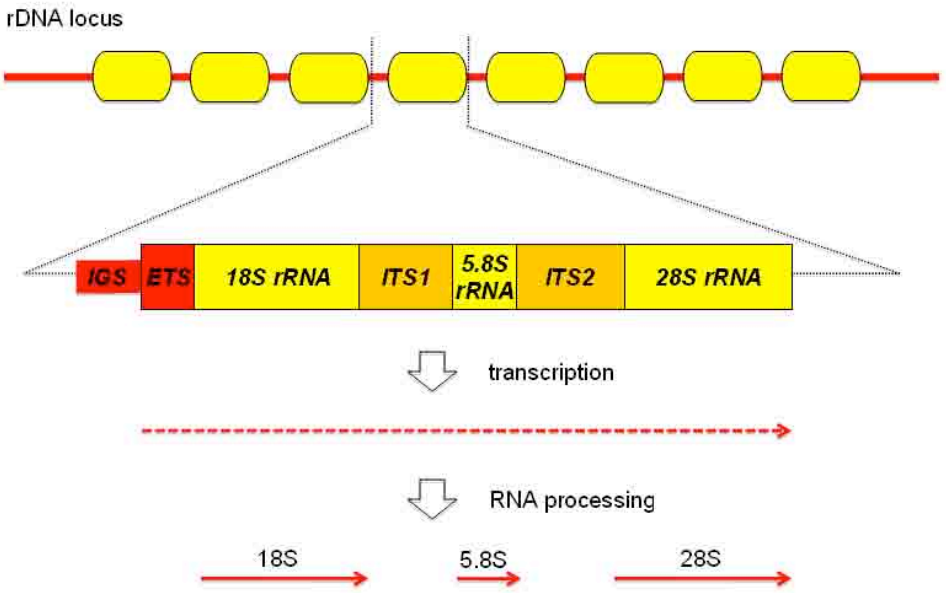

Figure 3: Organisation of the ribosomal RNA (rRNA) genes in eukaryotes. The rRNA genes are organized into tandemly repeated units. One typical unit is shown in detail. The three rRNA genes (18S, 5.8S and 28S) are given in yellow boxes while the other regions, processed in the primary transcript are given in orange (internal transcribed spacer, ITS) or red (external transcribed spacer, ETS), respectively. The transcribed units are linked via IGS (intergenic spacer sequence). The primary transcript is shown as slashed arrow while the final mature rRNAs are indicated by solid arrows.

The ITS region is considered to be used as a standard region for the DNA barcoding approach especially in fungi (Horton & Bruns, 2001). In animals and eukaryotic algae such as diatoms, the mitochondrial *cytochrome oxidase 1* gene (*coxI or COI*) has shown great potential for identifying (Hebert *et al.*, 2003; Hebert *et al.*, 2004a; Hebert *et al.*, 2004b). However, in fungi the mitochondrial genome is not as well qualified for identification purposes (Horton & Bruns, 2001), since amplification can be difficult due to introns that can be variably present within a species (Bruns *et al.*, 1998).

1.3.1.2 DNA Barcoding

DNA barcoding is a technique that uses a relatively short standardised DNA fragment for species-level identification.

The Consortium for the Barcode of Life (CBOL) is the international initiative aiming at developing DNA barcoding as a global standard for the automated and facilitated identification of species (www.barcoding.si.edu).

Proponents of barcoding claim that the level of variation between species (interspecific) is much larger than that within a species (intraspecific) and that this fact causes a barcoding gap which should enable a clear identification of individuals with an insignificant error rate (Hebert *et al.*, 2003; Hebert *et al.*, 2004b). But there are also studies, which argue against the existence of the barcoding gap (Meier *et al.*, 2006; Wiemers & Fiedler, 2007). Additionally, the approach that morphological keys are disregarded and only a single gene sequence should be the primary identifier for species also have been met with opposition, especially by taxonomists (Ebach & Holdrege, 2005; Moritz & Cicero, 2004), although these critics admit that the approach to select reference genes and to develop a comprehensive database of sequences, associated with voucher specimens representing described species, against which sequences from sampled individuals can be compared, may have potential.

1.3.2 QPCR/MCA and TaqMan qPCR Detection System established in this work

The qPCR/MCA and TaqMan assay developed in this work target a sequence coding for the conserved chitin-binding site of the *A. astaci* chitinases *Chi2* and *Chi3*. Since permanent expression and activity of chitinase is a unique feature of *A. astaci* among crayfish-afflicting oomycetes (Andersson & Cerenius, 2002) and because of the functional constraint put on this enzymes (Hochwimmer *et al.*, 2009), these sequences were considered as a very promising region for a reliable pathogen detection. Additionally, a highly conserved region among oomycetes of the *5.8S rRNA* gene was used as target for an endogenous positive control.

1.3.2.1 The Principle of the qPCR/MCA System

This detection system is based on a fluorescent DNA binding dye, which fluoresce upon light excitation when bound to double stranded DNA. The MCA is originally used as presumptive identification of the amplified target (Ririe *et al.*, 1997). After PCR, the amplified DNA is melted at a constant rate and the decrease in fluorescence is monitored as the strands dissociate. Since the energy required to break the hydrogen bonds between the two complementary strands depends on their length and their sequence, this technique enables the identification of one or even more products in one single reaction based on the specific melting temperature (T_m) of the respective product.

Apart from its original usage the field of application for MCA has been extended. For example, the qualitative detection of two or multiple target sequences have already been reported for single-tube SNP genotyping (Wang *et al.*, 2005), sex determination (Chang *et al.*, 2008) or the simultaneous detection of multiple pathogens (Gibellini *et al.*, 2006; Selvapandiyan *et al.*, 2005). Figure 4 illustrates the principle of MCA with DNA binding dyes.

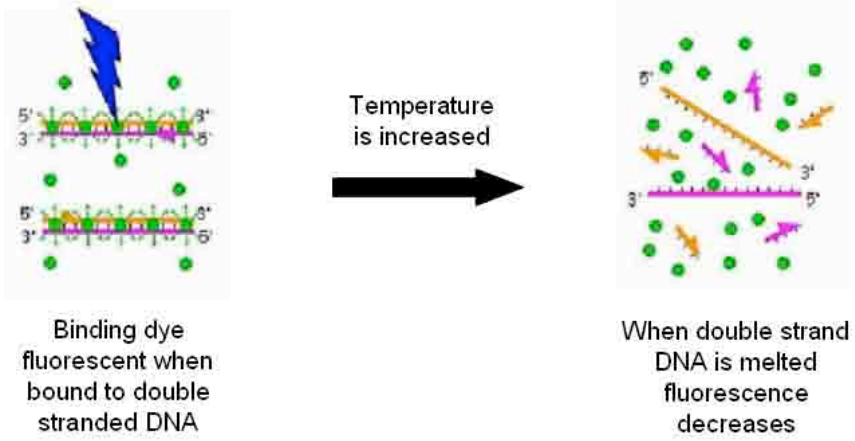

Figure 4: Principle of MCA performed with DNA binding dyes. Figure modified from Wikipedia 7 March 2006. Wikimedia Foundation, Inc.. 9 Jan. 2010 <http://commons.wikimedia.org/wiki/File:PCR_with_SYBR_ green.jpg>.

1.3.2.2 The Principle of the TaqMan qPCR System

The TaqMan system (Heid *et al.*, 1996) uses a fluorescence resonance energy transfer (FRET) probe as reporter dye. Such a probe is a short oligonucleotide that is complementary to one of the DNA strands. The probe is provided with a “reporter” and a “quencher” molecule at the 5’ and 3’ end, respectively. The quencher fluorochrome extinguishes the fluorescence of the reporter, because the two molecules are in a sufficient proximity, but when the polymerase synthesises the new DNA strand, the 5’ to 3’ exonuclease activity of the enzyme degrades the (TaqMan) hydrolysis probe, which is bound to the template DNA. This releases the reporter from the proximity to the quencher and causes fluorescence of the reporter. The accumulation of fluorescent reporter molecules, as a result of target amplification is detected. The mechanism of the TaqMan probe chemistry is indicated in Figure 5.

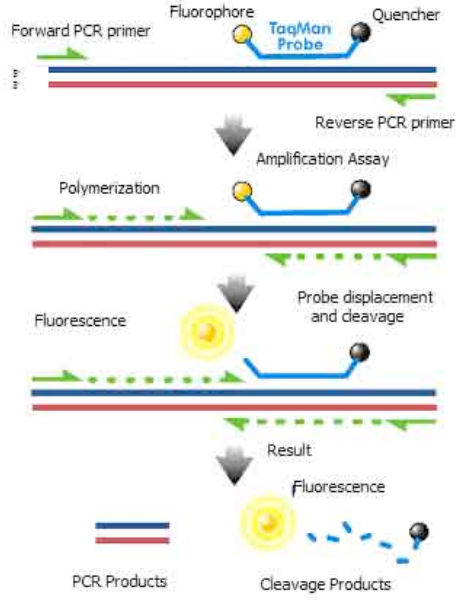

Figure 5: Mechanism of the TaqMan probe chemistry. Picture from Wikipedia May 2009. Wikimedia Foundation, Inc.. 9 Jan. 2010 < http://en.wikipedia.org/wiki/File:Taqman.png>.

1.3.2.3 Positive controls

In molecular diagnostics it is important to eliminate false negative results due to insufficient DNA quality and the presence of PCR inhibitors. Therefore positive controls are absolutely essential for obtaining reliable results.

While endogenous controls use a sequence originally present in the sample, exogenous controls are spiked into the sample. Moreover, it is differentiated between homologues and heterologues positive controls. Homologous or competitive controls use the same primers as the detection assay, whereas heterologous controls apply a separate primer pair.

The coextraction of an internal positive control (IPC) with the clinical samples and coamplification in the qPCR assay is state of the art for internal controls. This method allows accurate control of the entire molecular assay. In case the DNA quality of the template is not sufficient enough, the homologous internal control is amplified and confirms that DNA extraction was successful and PCR was not inhibited. It was shown, that this competitive PCR method did not affect the amplification of the target sequence when the addition of IPC did not increase 100 copies per PCR (Betsou *et al.*, 2003; Gregory *et al.*, 2006).

1.3.2.4 Chitinases

Chitinases (EC 3.2.1.14) are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin.

Chitin is an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc) and is one of the most abundant polysaccharides in nature. Based on their substrate affinity these enzymes can be classified into three groups: (i) endochitinases, which randomly cleave glycosidic linkages, generating free ends and long chitooligosaccharides that are processed by (ii) exochitinases (chitobiosidases), which release diacetylchitobiose (chitobiose) and (iii) N-acetylglucosaminidases (chitobiases), which hydrolyse chitobiose or release N-acetylglucosamine monomer from chitin chains (Chernin *et al.*, 1995; Tews *et al.*, 1996).

On the basis of their amino acid sequence chitinases can be classified into the GH18 or GH19 family (Henrissat, 1991). The two families differ in their sequence similarity and display different 3D protein structures. The catalytic domain of the GH18-family chitinases shows a catalytic barrel structure (Hollis *et al.*, 2000; Perrakis *et al.*, 1994; Robertus & Monzingo, 1999), whereas chitinases of the GH19 family are mainly composed of α-helices forming two lobes (bilobal structure) (Fukamizo; Hahn *et al.*, 2000).

While *Chi1* was the first described chitinase for *A. astaci* (Andersson & Cerenius, 2002), Hochwimmer (2009) identified two novel GH18 family chitinases in *A. astaci* (*Chi2* and *Chi3*). GH18 chitinases can be divided into three clusters, two of which (A and B) differentiated before appearance of the eukaryotic lineage (Karlsson & Stenlid, 2008), thus it can not be used for reconstruction of species phylogeny. Fungal GH18 families comprise between one and twenty genes (Seidl *et al.*, 2005).

2 Results

2.1 Assay development for qualitative and quantitative detection of *A. astaci* in clinical samples based on chitinase gene sequences

In contrast to other crayfish-afflicting oomycetes, *A. astaci* exhibits a permanent chitinase expression and activity (Andersson & Cerenius, 2002; Unestam, 1966). Available data from experiments performed by Hochwimmer (2009) let assume a functional constraint, since significant alterations of temporal gene expression could be observed. For that reason its chitinolytic system was chosen as target for the development of a diagnostic test.

2.1.1 qPCR/MCA

By using degenerated PCR primers, targeting two conserved motifs within the GH18 chitinase domain (Hochwimmer *et al.*, 2009), we were able to amplify and sequence the homologous sequences of the *A. astaci* related *A. frigidophilus* and *A. invadans*. The GenBank accession numbers are given in Figure 6.

Chitinase sequences of *A. astaci*, six further oomycetes species and two fungi, which are known to live in or on proximity of crayfish, have been available from previous work of Hochwimmer (2009). On the basis of these sequences (GenBank accessions in Figure 6) a diagnostic primer pair, producing a 93 bp-amplicon from each of the three related chitinase genes, *CHI2* and *CHI3:* (Hochwimmer *et al.*, 2009), was designed (Figure 6). Its melting temperature of 86.7 °C in MCA was regarded as criterion for assignation to *A. astaci* (Figure 7).

***A. astaci* CHI3** (FJ457089)	TCAAGCAAAAGCAAAAGGCT	TCCATCGCGAGCACGG
***A. astaci* CHI2** (DQ974157)	....	..............A.
A. astaci CHI1 (AY866375)	....	....
A. frigidophilus* SAP472, CHI1 (GQ152605)	..............GCGC..C	GG..............
A. frigidophilus* NJM 9665, CHI1 (GQ152604)	..............GCGC..C	GA..............
A. invadans* WIC, CHI1 (GQ374536)	A.....TC...A.....TTC	GA......CGA.T...
***A. laevis* CHI1** (AY866379)	A.....TC...A.GC..TTC	GG......C.....C.
***A. repetans* CHI1** (EU523123)	A.....TC...A.GC..TTC	GG......C.....C.
***A. helicoides* CHI1** (AY866380)	A.....TC...A.GC..TTC	AG......C.....C.
***Leptolegnia caudata* CHI1** (AY866382)	A....ATG...A.G...TT.	GG.T.T..C.....C.
***Saprolegnia parasitica* CHI1** (AY866378)	A....ATG...A.GGTCCA.	GG.T.T..AGC...C.
***Achlya racemosa* CHI1** (AY866381)	AT...ATG..AA.G...TAC	GGGT.TT.T.......
***Trichosporon cutaneum* CHI1** (AY866377)	A....ATG..AA.G.C.CAC	GG.T.T...GCG..C.
***Fusarium solani* CHI1** (AY866376)	A.CTC.TC...AGGCGCAAC	G.TCC...T.....CC

Figure 6: Qualitative detection of the oomycete *A. astaci*. Diagnostic qPCR/MCA primers (blue arrows) target *A. astaci*-specific sites in the homologous chitinase genes *CHI1*, *CHI2* and *CHI3*, but not homologous sequences of related oomycetes and fungi. Parentheses contain GenBank accession numbers. Dots and letters represent identical and substituted nucleotides compared to the *A. astaci* sequence, respectively. Asterisks denote chitinase sequences determined in this work.

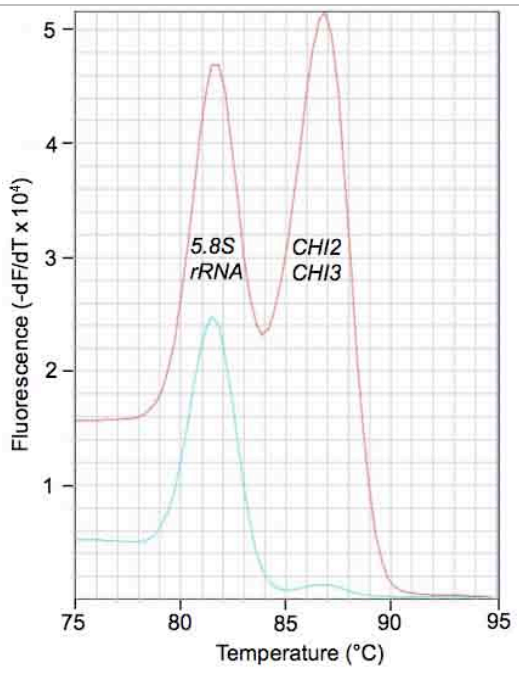

Figure 7: Qualititative detection of *A. astaci* by qPCR/MCA. The left and right peaks are derived from amplification of the endogenous control, and the chitinase genes *CHI2* and *CHI3*, respectively. Red plot: *A. astaci*, blue plot: *A. frigidophilus*.

For assay robustness the chitinase primer pair was multiplexed with primers targeting the *5.8S rRNA* gene as an endogenous control (Figure 8). A peak in MCA at 81.5 to 83.5 °C, depending from the species investigated, is yielded (Table 1, Figure 7).

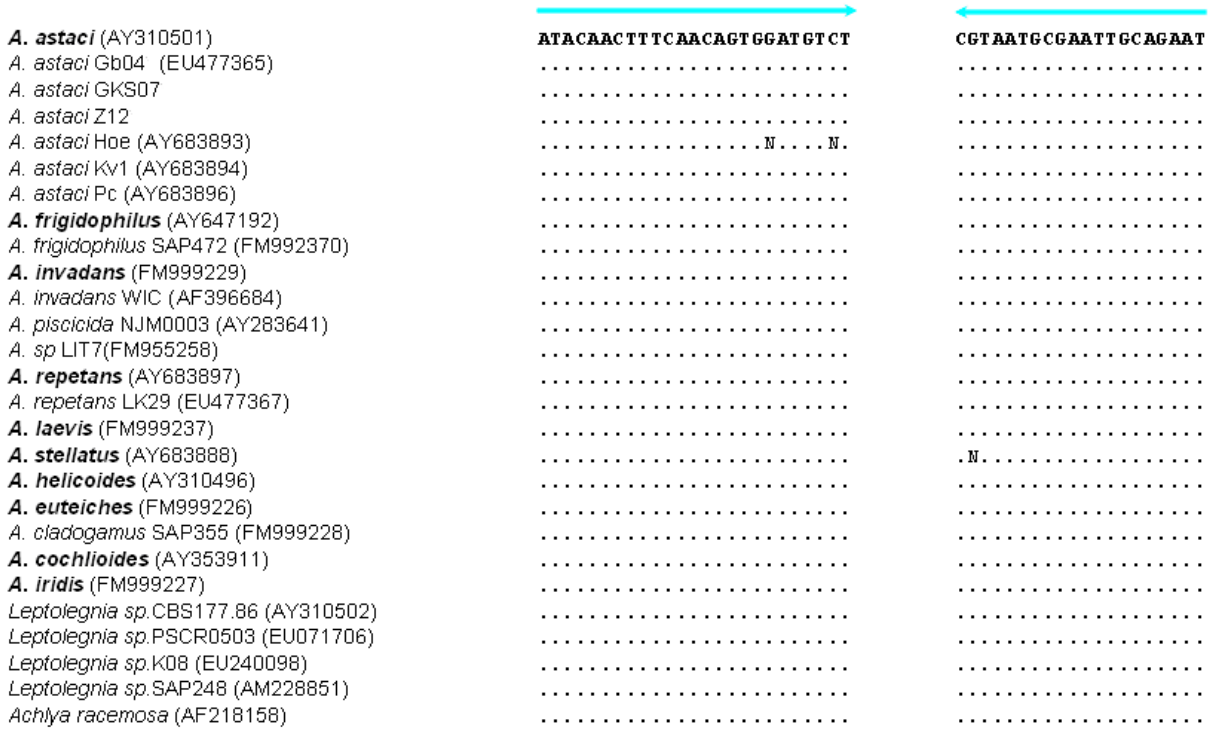

Figure 8: Alignment of primer sites for the endogenous control *5.8S rRNA* amplicon used in qPCR/MCA. Parentheses contain GenBank accessions. Oomycete reference strains (Dieguez-Uribeondo *et al.*, 2009) are given in bold. Dot: identical nucleotide with the *A. astaci*-sequence.

The qPCR/MCA assay was tested for specificity against the oomycetes *A. frigidophilus, A. invadans*, *A. laevis*, *A. helicoides*, *A. irregularis*, and *Leptolegnia caudata*. Only the endogenous control was recorded, but not the *A. astaci*-specific chitinase peak.

Table 1: Results of Melting peaks. Mean T_m determined by qPCR/MCA.

Species	Isolate	T_m* *5.8S rRNA* (°C)	T_m* *CHI* (°C)
A. astaci	L1	81.6	86.7
A. astaci	Gb04	81.5	86.7
A. astaci	Sv	81.5	86.7
A. frigidophilus	NJM 9665	81.6	
A. invadans	WIC	81.5	
A. laevis	CBS 107.52	83.3	
A. helicoides	CBS 210.82	83.3	
A. irregularis	CBS 278.81	81.9	
L. caudata	CBS 680.69	81.8	

* SD ≤ 0.3 °C for duplicates

2.1.2 TaqMan qPCR

For sensitive detection of the pathogen, but also for quantification of agent levels in susceptible crayfish and carrier crayfish, a TaqMan-probe-based qPCR assay was developed. The assay uses the same primers as qPCR/MCA except the additional nucleotide at the very 5' end of the reverse primer. Using amplicon standards with known copy numbers spiked into genomic crayfish DNA, a quantitative detection limit of 25 target sequences was determined (Figure 9).

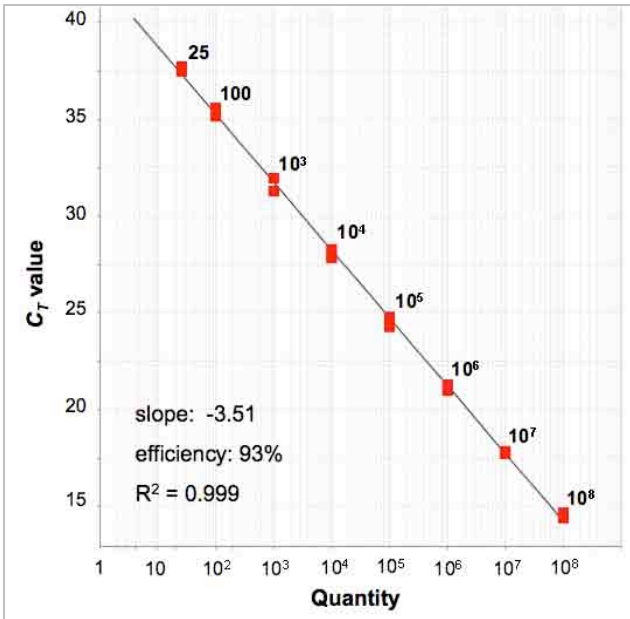

Figure 9: Quantitative detection of *A. astaci* by TaqMan qPCR. The standard curve of the assay demonstrates an optimal high amplification efficiency and with a quantification down to 25 copies a good sensitivity.

No amplification, *i.e.* C_T > 50, was obtained for *A. frigidophilus*, A. *invadans*, *A. leaevis* and *A. irregularis*. In the case of the oomycete species *A. helicoides* and *L. caudata* a cross-amplification signal corresponding to 28 and 45 copies was detected, respectively (Table 2, Figure 10).

Table 2: Results from absolute quantification of tested species. Mean copy numbers calculated from duplicates are given. ND: No signal detected.

Species	Isolate	DNA copy number
A. astaci	*L1*	*5.15×10^4*
A. astaci	*SV*	*2.03×10^4*
A. astaci	*Gb04*	*3.44×10^4*
A. frigidophilus	*NJM 9665*	*ND*
A. invadans	*WIC*	*ND*
A. laevis	*CBS 107.52*	*ND*
A. helicoides	*CBS 210.82*	*28*
A. irregularis	*CBS 278.81*	*ND*
Leptolegnia caudata	*CBS 680.69*	*45*

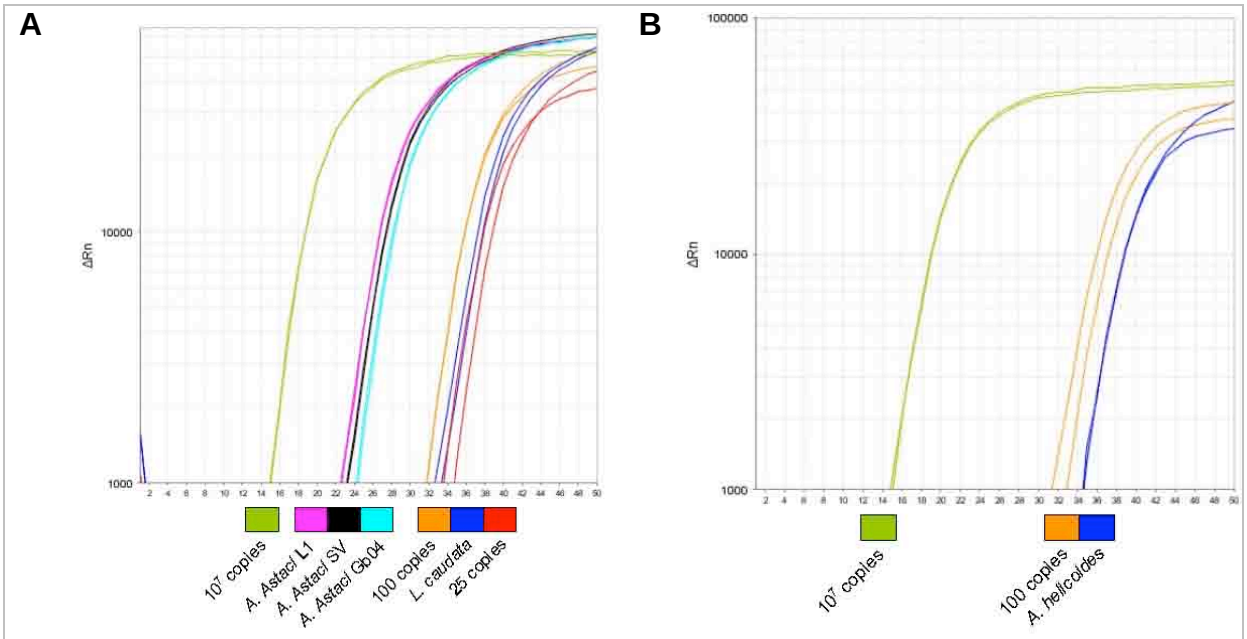

Figure 10: Amplification Plots of TaqMan qPCR. A. The amplification plot shows amplification curves of *A. astaci* L1, SV and Gb04 in comparison with the cross-amplification signal of *L. caudata*. The amplification curves of the copy standards are given for orientation purposes. **B.** The cross-amplification signal of *A. helicoides* is given in the amplification plot together with the amplification curves of the 10^7 and 100 copies standard.

3 Methods

3.1 Biological material

Isolates of oomycetes and related fungi used to validate the molecular assays were obtained from stocks of the Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine, Vienna. *A. helicoides* and *L. caudata* were bought from The Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre (Utrecht, The Netherlands). Javier Diéguez-Uribeondo (Real Jardín Botánico CSIC, Madrid, Spain) provided the *A. frigidophilus* isolate SAP472 (Dieguez-Uribeondo *et al.*, 2009). A DNA aliquot of *A. frigidophilus* NJM 9665 (Kitancharoen & Hatai, 1998; Vandersea *et al.*, 2006) and *A. invadans* WIC (Vandersea *et al.*, 2006) was obtained from Mark W. Vandersea (Center for Coastal Fisheries and Habitat Research, National Ocean Service, National Oceanic Atmospheric Administration, Beaufort, North Carolina, USA).

3.2 DNA isolation from mycelium of oomycetes

The mycelium was transferred to a 2 ml-extraction tube containing 0.7 g Precellys® ceramic beads of 1.4 mm diameter (Peqlab Biotechnology, Erlangen, Germany) and 180 µl buffer ATL, the lysis buffer of the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Samples were homogenised twice for 15 s at 5000 rpm using the MagNA Lyser (Roche). Further isolation was performed according to the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" provided by the manufacturer.

3.3 De novo sequencing of partial GH domain using degenerate PCR primers

Partial GH18 domains of chitinases from *A. frigidophilus* SAP472, *A. frigidophilus* NJM9665 and *A. invadans* WIC were amplified using the primers SEQ685F (5'-CCGGAGACTCGTGGAACGAC) and SEQ1159R (5'- TTGCTCCAGCTGCCCGC). Primers targeting the amino acid motifs DSWND and AGSW, respectively, amplified an approximately 475-bp product by qPCR. The 20-µL reaction consisted of 0.4 × EvaGreen™ dye (Biotium, Hayward, USA), 4 mM $MgCl_2$, 200 µM of each dNTP, 375 nM of each primer, 2 µl template DNA, 1 U GoTaq® DNA polymerase - a proprietary formulation of Taq DNA polymerase (Promega, Madison, USA), and 1 × Colorless GoTaq® Flexi Reaction Buffer (Promega) not containing magnesium. Amplification was performed in the Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) using denaturation for 4 min at 94 °C, amplification for 35 cycles (1 min at 94 °C, 1 min at 63 °C and 1 min at 72 °C), and final elongation of 7 min at 72 °C followed by MCA.

Agarose gel electrophoresis was used to determine amplicon size. The MSB® Spin PCRapace Kit (Invitek, Berlin, Germany) was used for amplicon purification in case of a single band showing the expected length. Multiple bands were excised from the gel and purified with the Xact DNA Cleanup kit (genXpress, Wiener Neudorf, Austria). Sequencing of PCR products was performed at VBC Genomics Bioscience Research GmbH (Vienna, Austria).

3.4 Alignment of nucleotide sequences

All alignments necessary for finding appropriate primer target regions were performed on a Windows station using the software SeaView (Galtier *et al.*, 1996). This software was used with the MUSCLE algorithm (Edgar, 2004). This procedure revealed variable and conserved regions within respective genes.

3.5 Qualitative detection of *A. astaci* using qPCR/MCA

The 20-µl duplex qPCR/MCA contained 2 µl 10 × PCR buffer B (Solis BioDyne, Tartu, Estonia), 200 nM of forward and reverse chitinase gene(s) primers (5'-TCAAGCAAAAGCAAAAGGCT and 5'-CCGTGCTCGCGATGGA, Figure 6), 125 nM of forward and reverse 5.8S rRNA primers (5'- ATACAACTTTCAACAGTGGATGTCT and 5'-ATTCTGCAATTCGCATTACG, Figure 8), 200 µM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.4 × EvaGreen™ (Biotium), 3.0 mM $MgCl_2$, 1 U Taq DNA polymerase chemically modified for "hot start" (Hot FirePol®; Solis BioDyne, Tartu, Estonia) and 10 ng DNA template or water in the case of the no-template control. QPCR/MCA was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) run under the StepOne™ software version 2.0. Polymerase activation (95 °C for 15 min) was followed by amplification for 35 cycles (95 °C for 15 s, 59 °C for 15 s and 72 °C for 10 s). After an initial denaturation step at 95 °C for 15 s, amplicon melting was recorded during a gradual increase of the temperature from 60 °C to 95 °C.

Oligonucleotides (Sigma-Aldrich, Steinheim, Germany) were designed with Primer Express Software Version 2.0 (Applied Biosystems). The difference between amplicon melting temperatures was calculated using the Nearest Neighbor mode implemented in the online oligonucleotide properties calculator OligoCalc (Kibbe, 2007).

3.6 Sensitive detection and quantification of *A. astaci* using TaqMan qPCR

Duplicate TaqMan qPCR was carried out in a total volume of 20 µl containing 2 µl 10 × PCR buffer A2 (Solis BioDyne), 0.2 mM of each dNTP, 4 mM $MgCl_2$, 300 nM of each primer (Chi3-324f20 and AaChi-Tmr), 150 nM TaqMan probe (AaChi-FAM), 1 U HOT FIREPol DNA polymerase (Solis BioDyne), 20 ng template DNA or water in the case of the no-template control. Reactions were amplified in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) under the StepOne™ software version 2.0 using thermal cycling conditions of 15 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 64 °C. A standard curve was generated by plotting the logarithm of the standards copy numbers versus measured C_T values.

3.6.1 Isolation of spike-in DNA for use in serial dilutions

A crayfish sample extracted from the abdomen of *Cherax quadricarinatus* (Australian red-claw crayfish) was transferred to a 2 ml extraction tube containing 0.7 g Precellys® ceramic beads of 1.4 mm diameter (Peqlab Biotechnology, Erlangen, Germany) and 180 µl buffer ATL, the lysis buffer of the DNeasy® Blood & Tissue Kit (Qiagen). The MagNA Lyser (Roche) was used for three mechanical lysis cycles consisting of 30 s at 6,500 rpm followed by 60 s on a cooling block held at 4°C. Further isolation was performed according to the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" provided by the manufacturer. DNA concentration was determined spectrophotometrically using the Hellma® TrayCell (Hellma, Müllheim/Baden, Germany) on the Eppendorf BioPhotometer 6131.

3.6.2 Generation of copy standards

A DNA template stock consisting of *CHI1*, *CHI2* and *CHI3* sequences was generated as follows. Genomic DNA from chitinase sequences was amplified with the primers Chi3-324f20 (5'-TCAAGCAAAAGCAAAAGGCT) and AaChi-Tmr (5'-TCCGTGCTCGCGATGGA). Amplification was evaluated by the signal generated from the TaqMan® probe AaChi-FAM (5'-FAM-TCAACGTCCAC- CCGCCAATGG-BHQ-1). Amplification was performed in a total volume of 20 µl containing 2 µl 10 × PCR buffer A2 (Solis BioDyne), 0.2 mM of each dNTP, 4 mM $MgCl_2$, 250 nM of each primer, 150 nM TaqMan probe, 1 U HOT FIREPol® DNA polymerase (Solis BioDyne) and 20 ng DNA or water in the case of the no-template control. DNA denaturation and enzyme activation were performed for 15 min at 95 °C. DNA was amplified over 50 cycles consisting of 95 °C for 15 s, 60 °C for 1 min. QPCR was run on StepOnePlus™ Real-Time PCR System (Applied Biosystems) under the StepOne™ software version 2.0.

PCR fragments were purified with the MSB® Spin PCRapace Kit (Invitek). The copy number of the target template was determined spectrophotometrically using the Hellma® TrayCell (Hellma) on the Eppendorf BioPhotometer 6131. Serial dilutions of the target sequence (10^8 to 10^2, 50, 25 and 12.5 copies per 2 µl) prepared in 10 ng/µl *C. quadricarinatus* DNA were used to determine the amplification efficiency and the quantitative detection limit.

4 Discussion

Our results show, that the MCA of multiplex qPCR assay is a highly discriminative, robust and reliable diagnostic assay for rapid identification of the crayfish plague pathogen *A. astaci*. Even *A. invadans* and *A. frigidophilus*, which are closest related to *A. astaci* can be discriminated evidently with our qPCR/MCA assay.

Table 1 and Figure 7 show, that the *A. astaci* specific PCR assay has a melting temperature of 86.7 °C and is clearly distinguishable from the melting peak of the endogenous control that lies between 81.5 and 83.3 °C. The higher melting temperature for *A. helicoides* and *A. laevis* are caused by the increased GC content (Figure 11).

Figure 11: Alignment of the endogenous control *5.8S rRNA* amplicon used in qPCR/MCA. Parentheses contain GenBank accessions. Oomycete reference strains (Dieguez-Uribeondo *et al.*, 2009) are given in bold. Dot: identical nucleotide with the *A. astaci*-sequence.

The catalytic region of the GH18 domain was chosen as basis for the diagnostic assay. This region was considered to enable a reliable species identification because a constitutive chitinase gene expression of *A. astaci*, is not observed in the other closely related *Aphanomyces species* (Andersson & Cerenius, 2002; Ballesteros *et al.*, 2006). Furthermore, the work of Hochwimmer (2009) revealed two new members of the GH18 family (*CHI2* and *CHI3*) which gene products are functionally constrained like an expression analysis by Hochwimmer (2009) turned out.

Since *A. astaci* is an asexual organism, it can be subject to a high level of genetic variation (Van Doninck *et al.*, 2004). By simultaneously targeting the novel functionally constrained chitinase sequences the risk of getting false negative results should be reduced considerably.

Additionally to the *A. astaci*-specific assay, a second primer pair, targeting the *5.8S rRNA* gene was used as endogenous control. This sequence turned out to be most conserved among all *Aphanomyces* species found on the NCBI GeneBank DNA database (Figure 8). This intracellular marker should further decrease the risk of false negative results and therewith the robustness of the assay, as it can be proven if the DNA quality is sufficient. Furthermore, because the endogenous control and the chitinase gene targets can be expected to the same range of magnitude (Drouin & de Sa, 1995; Seidl *et al.*, 2005; van Doorn *et al.*, 2007) it can be stated that if non-limited primer concentrations are applied like here, the simultaneous amplification of more than one target in a single PCR leads to competition between multiple targets for a finite number of reagents. This leads to the welcomened side effect of increased specificity.

A further step for enhancing the robustness of the assay is to include an internal positive control. Especially for this assay a homologous (competitive) internal positive control was designed by Prof. Ralf Steinborn (Hochwimmer *et al.*, 2009). This proposed IPC was originally not practically used in the development phase of the assay but was applied by Georg Mair within the scope of testing a set of clinical samples (data not shown).

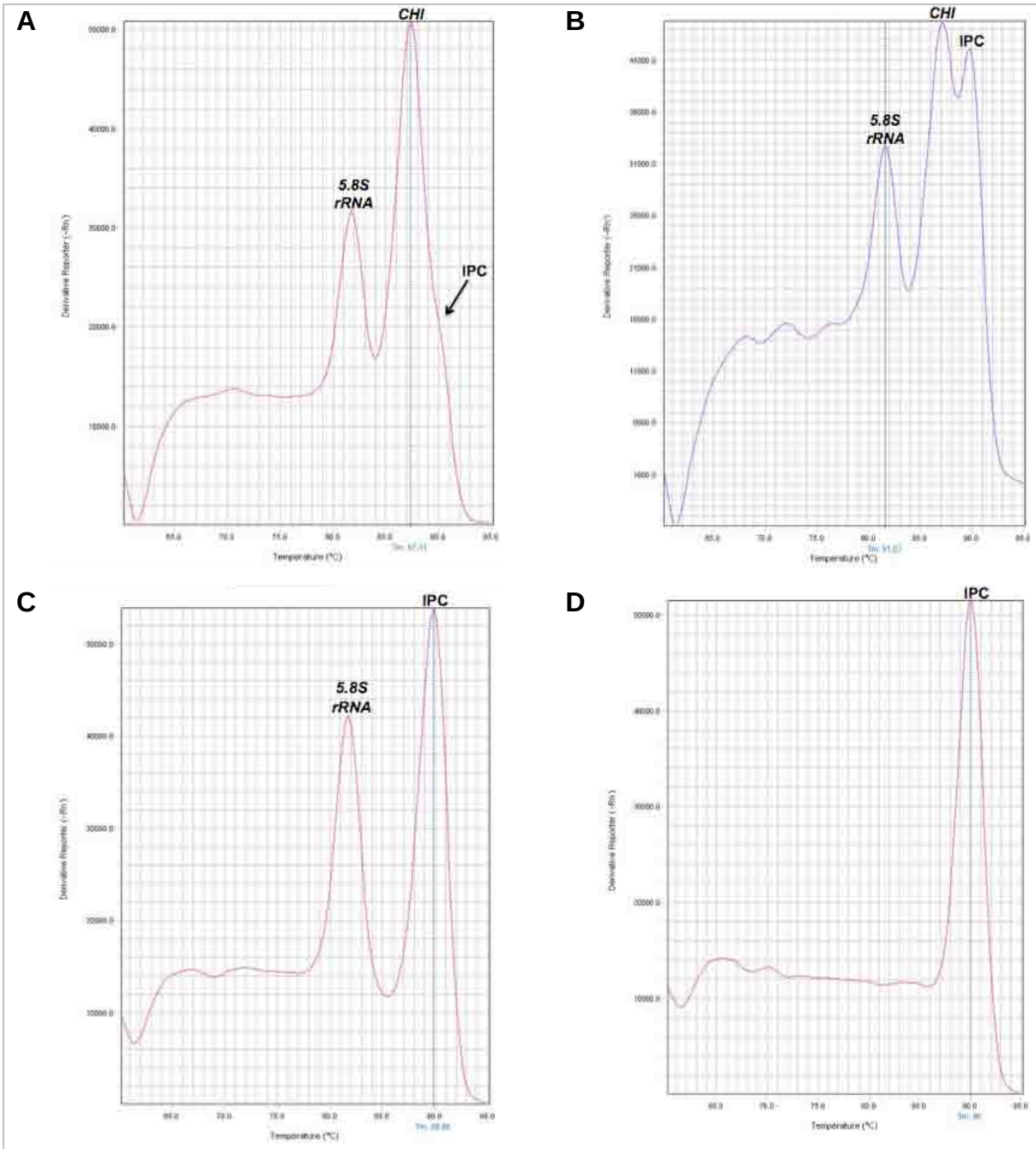

Figure 12: Qualitative qPCR/MCA of clinical samples with IPC. A. A clearly positive result. The two melting peaks derived from the amplification of the endogenous control (*5.8S rRNA*) and the *A. astaci* specific chitinase genes show a clear positive test result. The IPC peak can only be divined. **B.** Critical (low) copy number of DNA target. The number of specific target necessary for detection of the pathogen is close to the detection limit, since the peak indicative for the IPC is also visible. **C.** A clearly negative sample. The melting peak obtained from amplification of the IPC and the peak specific for the endogenous control (*5.8S rRNA*) are indicative for a working PCR and a sufficient DNA quality of the sample. **D.** Only the melting peak of IPC is detected. It can be deduced that the amount of amplifiable DNA targets were to low. Plots were kindly provided by Georg Mair.

As expected, the IPC shows only a signal when the number of chitinase gene targets is low (Figure 12 B, C, D). Since the target that is predominantly present is preferably amplified, as the same primer pair is used for amplification of the IPC and the chitinase genes. The IPC peak is hardly identifiable if enough copies of chitinase genes are available (Figure 12 A). By this means it can be assured that the PCR worked properly and was not inhibited, although no signal for the chitinase genes and the *5.8S rRNA* is obtained (Figure 12 D).

The quantitative TaqMan assay turned out to have the potential to quantify the levels of *A. astaci* in susceptible crayfish and carrier crayfish down to 25 copies. However, the assay shows a cross-amplification signal with *A. helicoides* and *L. caudata*. Anyway, these signals were characterised by late C_T values, corresponding to less than 50 copies. In contrast, the tested *A. astaci* samples revealed between about 20,000 and 51,000 copies (Table 2). But, since this assay is primary intended to enable a reliable absolute quantification in order to estimate the degree of an infection, it is usually performed in combination with the highly specific qualitative qPCR/MCA method. Therefore, the observed cross amplification signals do not pose a problem, anyway.

The so far developed diagnostic PCR based assays for the detection of *A. astaci* use the ITS as target region (Ballesteros *et al.*, 2007; Oidtmann *et al.*, 2006) and they all suffer from cross reactivity with closely related species. A recently published quantitative TaqMan® MGB qPCR assay (Vralstad *et al.*, 2009) promises to overcome the problem of detecting false positives. The use of the special probe seems to increase the specificity in a sufficient degree. Though, like the other PCR assays this method also uses primer targeting the ITS region.

Indeed, the ITS is thought to have such a high interspecific sequence variation that they are recommended for species identification purposes at least in fungi (Horton & Bruns, 2001), but it is found that the internal transcript spacers are not considered for identification purposes in diatoms, because of the problem with intraindividual variation (Alvarez & Wendel, 2003; Alverson & Kolnick, 2005). For example, a study within the diatom *Sellaphora auldreekie* revealed three types of ITS sequences that differed in 48 positions and two indels of 50 and 4 bp within one isolate (Behnke *et al.*, 2004). Furthermore, a consequence of the high copy number of rDNA is the widespread of pseudogenes in eukaryotic organisms (Alvarez & Wendel, 2003; Thornhill *et al.*, 2007; Xu *et al.*, 2009). Also in *Heterokonts*, a study in the diatom *Cyclotella meneghiniana* indicates that some strains probably contain ITS rDNA pseudogenes, because several highly divergent, phylogenetically anomalous ITS rDNA was found (Beszteri *et al.*, 2005).

Anyway, for species identification the approach to use only one single gene is being met with criticism as the existence of the so called barcoding gap, necessary for a reliable identification of unknown species, is being questioned in several studies (Meier *et al.*, 2006; Wiemers & Fiedler, 2007) and may therefore always produce some erroneous results.

A future prospect in this field of molecular species identification is the use of multiple genes (Chase *et al.*, 2005; Dasmahapatra & Mallet, 2006). A study in *Pythium* for example revealed, that some species share the same ITS (Levesque & de Cock, 2004). The multi-gene approach would significantly increase the reliability in the identification of species. The challenge is to identify appropriate regions. In our case, *coxI* could be a candidate for this purpose, since oomycetes and diatoms both belong to the kingdom *Heterokonta*. *CoxI* has already turned out to be better suited for identification purposes in diatoms than the ITS (Evans *et al.*, 2007). In *coxI* intragenomic variation mediated by the occurrence of introns is restricted to sites outside of the diagnostic target region (Supplement 1). Beside of *coxI*, *coxIII* might be a further candidate (R. Steinborn, personal communication, February 2010).

It could be a further prospect to include an assay targeting a mitochondrial gene like *coxI* as additional marker into the existing qPCR/MCA assay. Besides, it should be also possible to use the ITS assay developed by Vralstad et al. (2009) in our qPCR/MCA. Although, according to the online oligonucleotide properties calculator OligoCalc in the Nearest Neighbor mode (Kibbe, 2007) the melting temperature of this amplicon does not vary by more than 1 °C from the *5.8S rRNA* gene and cannot therefore be distinguished from the *5.8S rRNA* specific melting peak, the problem could be overcome by also using the appropriate TaqMan probe. This probe has to be alternatively labelled with a reporter molecule which emission spectrum differs from that of EvaGreen. The amplification of the ITS sequence can be observed by measuring the fluorescence in the corresponding second channel.

5 Conclusion

By using the functionally constrained and constitutively expressed chitinase genes of *A. astaci* as basis, establishing a reliable, robust, and efficient diagnostic tool for a highly specific pathogen detection has been successful. This diagnostic tool adds to the molecular repertoire for *A. astaci* detection.

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7 Supplementary Material

Supplement 1

Alignment of the oomycete cytochrome oxidase 1 (CoxI or COI) nucleotide sequences

Supplement 2

Alignment of the oomycete cytochrome oxidase 3 (CoxIII) nucleotide sequences

Supplement 3

Poster: 14th European Association of Fish Pathologists (EAFP) Conference, Diseases of Fish and Shellfish, September 14-19, 2009, Prague

Supplement 4

Research Article: Hochwimmer et al., 2009

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S.ferax (AY534144)          ATGAACTC-- -AAATTTTTA TAACTGGTCA TCTAGATGGC TTTTTTCAAC AAATCATAAA
P.acanthophoron (EU350529) --------.. .--------- ---------- ---------- ---------- ..........
P.cactorum (AY129174)       .....T.TTC A....A.AA. ...A...G.. A.A....... .......... ..........
P.citricola (FJ237512)      .....T.TTC A....A.AA. ...A..... A.A....... .......... ..........
P.colocasiae (AY129173)     .....T.TTC A....A.AA. ...A..... A.A....... .......... ..........
P.gonapodyides (AY129175)   .....T.TTA A....A.AA. ...A..... A..C...... .......... ..........
P.hibernalis (AY129170)     .....T.TTC A..G.A.AA. ...A..... A.A....... .......... ..........
P.ilicis (AY129172)         .....T.TTC A....A.AA. ...A..... A.A....... .......... ..........
P.nicotianae (AY129169)     .....T.TTC A....A.AA. ...A..... A.A....... .......... ..........
P.phaseoli (AY129168)       .....T.TTC A....A.AA. AC.A..... A.A....... .......... ..........
P.plurivora (FJ237511)      .....T.TTC A....A.AA. ...A..... A.A....... .......... ..........
P.pseudotsugae (AY129167)   .....T.TTC A....A.AA. ...A...G.. A.A....... .......... ..........
[1]Primer-fwd                                             TCA WCWMGATGGC TTTTTTCAAC

S.ferax                     GATATAGGTA CTTTATATAT GATTTTTGGT GCTTTTTCAG GTGTAGTTGG TACAACTTTA
P.acanthophoron             ..C..T.... ........T. A......... ........T. .......... ...T..A...
P.cactorum                  .....T..A. ........T. A......A.. ......G.T. ....T..... ......A...
P.citricola                 .....T..A. ........T. A......A.. ......G.T. ....T..... ......A...
P.colocasiae                .....T..A. ........T. A......A.. ......G.G. ....T..... ......A...
P.gonapodyides              .....T.... ........T. A......A.. ......G.T. ..A.T..... ......C...
P.hibernalis                .....T..A. ........T. A......A.. ......G.T. ..A.T..... ..........
P.ilicis                    .....T.... ........T. A......A.. ......G.T. ....T..A.. ......A...
P.nicotianae                .....T..A. ........T. A......A.. ......G.T. ....T..A.. ......AC..
P.phaseoli                  .....T..A. ........T. A......A.. ......G.T. ....T..... ......A..T
P.plurivora                 .....T..A. ........T. A......A.. ......G.T. ....T..... ......A...
P.pseudotsugae              .....T..A. ........T. A......A.. ......G.T. ....T..... ......A...
[2]Primer FM84                         TTT AATTTTTAGT GCTTTTGC

S.ferax                     TCTGTTTTAA TTCGTATGGA ATTAGCACAA CCTGGAAATC AAATTTTTAT GGGAAATCAT
P.acanthophoron             .......... ..A.A..... .......... .....T.... ........GA A..T......
P.cactorum                  ...C...... ..A.A..... .......... ..G..T.... .......C.. ..........
P.citricola                 ...C...... ..A.A..... G......... .....T.... .......... ..........
P.colocasiae                ...C...... .......... .......... .....T.... .......... ...T......
P.gonapodyides              ..AC...... .C........ .......... ..A..T.... .......... .........C
P.hibernalis                ...C...... ....A..... .......... ..A..T.... .......... ...T......
P.ilicis                    ..AC...... .C..A..... ...G...... ..A..T.... .......... ..........
P.nicotianae                ...C...... ..A.A..... .......... .....T.... .......... ...T......
P.phaseoli                  ...C...... ..A.A..... .......... ..A..T.... .......... ..........
P.plurivora                 ...C...... ..A.A..... G......... ..A..T.... .......... ..........
P.pseudotsugae              ...C...... ..A.A..... .......... ..A..T.... .......C.. ..........

S.ferax                     CAATTATATA ACGTAGTTGT TACTGCACAC GCGTTTATTA TGATTTTCTT TATGGTTATG
P.acanthophoron             ..T....... .T..T..... A..C..T... ..A....... ....C..T.. ..........
P.cactorum                  .......... .T..T..... ...A.....T ..T....... ..G.G..T.. .T.A......
P.citricola                 .......... .T..T..... ...A..... ..T.....A. ..G....... .T.A......
P.colocasiae                .......... .T..T..... ......T..T ..T....... ..G....... .T.A......
P.gonapodyides              .......... .T..T..... .........T ..T....... ..G....... .T.A......
P.hibernalis                .......... .T..T..... ......C..T ..T....... ..G....T.. .T.A......
P.ilicis                    .......... .T..T..... ......G..T ..T....... ..G....T.. .T.A......
P.nicotianae                .......... .T..T..... ...A.....T ..T....... ..G.A..T.. .T.A......
P.phaseoli                  .......... .T..T..... ...C.....T ..T....... ..G....... .T.A......
P.plurivora                 .......... .T..T..... ...A.....T ..T.....A. ..G....... .T.A......
P.pseudotsugae              .......... .T..T..... ...A.....T ..T....... ..G.G..T.. .T.A......

S.ferax                     CCTATTTTAA TTGGAGGTTT TGGTAACTGG TTTATTCCTT TAATGTTAGG TGCTCCTGAT
P.acanthophoron             ...G...... ....T..A.. ......T... ...G.A.... .....A.T.. ...A......
P.cactorum                  ...GC..... ....T..... .......... ...G...... .....A.T.. ..........
P.citricola                 ...GC..... ....T..... ......T... ...G...... .....A.... ...A......
P.colocasiae                ..AGC..... .......... ......T... ...G...... .....A.... ...A......
P.gonapodyides              ...GCA.... ....T..... ...A..T... ...G...... .....A.... ..........
P.hibernalis                ...GC..... ....T..G.. .......... ..CG...... .....A.... G.....A...
P.ilicis                    ..AGC..... .......... ......T... ...G....A. .....A.T.. ...A......
P.nicotianae                ...GC..... ....T..G.. .......... ...G.A.... .....A.... ..........
P.phaseoli                  ...GC..... .C..T..... ......T... ...G...... .....A.... ......G...
P.plurivora                 ...GC..... ....T..... ......T... ...G...... .....A.... ...A......
P.pseudotsugae              ...GC..... .A..T..... .......... ...G...... .....A.T.. ..........
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S.ferax                ATGGCTTTTC CAAGAATGAA TAATATTAGT TTTTGGTTAT TACCTCCTTC ATTATTTTTA
P.acanthophoron        .......... .TC.T..... ...C...... .......... .......A.. ......A...
P.cactorum             .....A.... .TC.T..... ......A... .......... .......AG. T.....A...
P.citricola            .......... ..C.T..... ......A... .......... .......AG. T.....A...
P.colocasiae           .......... .TC.T..... ......A... .......... .......AG. T.....A...
P.gonapodyides         ........C. ..C.T..... .......... .......... ....A..AG. ......A...
P.hibernalis           .......... ..C.T..... .......... .......... .......CG. T.....A...
P.ilicis               ........C. ..C.T..... ......A... .......... .......AG. T.....A...
P.nicotianae           .....A.... .TC.T..... ......A... .......... .......AG. T.....A...
P.phaseoli             .......... .TC.T..... .......... .......... .G........ T.....A...
P.plurivora            .......... ..C.T..... ......A... .......... .......AG. T.....A...
P.pseudotsugae         .....A.... .TC.T..... ......A... .......... .......AG. T.....A...

S.ferax                TTAGTTTCTT CAGCTATTGT TGAATCTGGT GCTGGTACTG GTTGGACTGT ATATCCACCT
P.acanthophoron        .....A.... .......... ......A... .......... .......A.. ......T...
P.cactorum             ........A. .......... ......A... ..A.....A. .......A.. T........A
P.citricola            ........A. .......... A......... ........A. .......... T........A
P.colocasiae           ........A. .......... .......... ........A. .......A.. T........A
P.gonapodyides         .....G..A. .......... C......... .....G.... .......A.. T........A
P.hibernalis           .....A..A. .......... G........A ..A....... .......A.. T.........
P.ilicis               ........A. .......... A.....A... ........A. .......A.. T........A
P.nicotianae           ........A. .......... A......... .......... .......... T.....C..A
P.phaseoli             .......... .......C.. .........G .......... .......A.. T........A
P.plurivora            ........A. .G........ A......... ........A. .......... T.........
P.pseudotsugae         ........A. .......... ......A... ..A....... .......A.. T........A
S.ferax                TTATCTAGTG TACAAGCTCA CTCAGGTCCT TCAGTTGATT TAGCTATTTT TAGTTTACAC
P.acanthophoron        .....A.... .T.....A.. T.....G... ..T..A.... .......... .........T
P.cactorum             .......... .......G.. ......A... .....A.... .......... .........T
P.citricola            .......... .G.....A.. ...T..A... .....A.... .......... .........T
P.colocasiae           .......... .......... .......... ..T..A.... .......... .........T
P.gonapodyides         .....A.... .......A.. ......A... .....A.... .......... .........T
P.hibernalis           .....A.... .......A.. T.....A... .....A.... .......... .........T
P.ilicis               .......... .......A.. ......A... .....A.... .......... .........T
P.nicotianae           .......... .......A.. T......... ..T..A.... ....G..... .........T
P.phaseoli             .......... .T.....A.. T.....A... ..T..A.... .......... .........T
P.plurivora            .......... .G.....A.. ...T..A... .....A.... .......... .........T
P.pseudotsugae         .......... .......A.. ......A... .....A.... .......... .........T

S.ferax                TTAGCAGGTA TTTCTTCATT ATTAGGTTCA ATAAATTTTA TTACTACCAT TTATAATATG
P.acanthophoron        ...T...... .A..A..T.. .......G.T ..T.....CT .AT.A..T.. ..........
P.cactorum             ...A...... .......T.. .......G.T ..T....... ..T.A..T.. A.........
P.citricola            ...A...... .......... .......G.. ..T....... ..T.A..T.. ..........
P.colocasiae           ...A...... .C........ .......G.. ..T....... ..T.A..T.. ..........
P.gonapodyides         ...A.G.... ....A..... ...G...G.. ..T....... ..T.A..T.. ..........
P.hibernalis           ...A...... .......T.. .......G.. ..T....... ..T.A..T.. ..........
P.ilicis               ...A...... .......... .......G.T ..T....... ..T.A..T.. ..........
P.nicotianae           ...T...... .......T.. .......G.T ..T....... ..T.A..T.. ..........
P.phaseoli             ...T...... .......T.. .......G.T ..T....... ..T.A..A.. ..........
P.plurivora            ...A...... .......... .......G.. ..T....... ..T.A..T.. ..........
P.pseudotsugae         ...A...... .......T.. .......G.T ..T....... ..T.A..T.. A..C......

S.ferax                AGATCACCAG GTTTAACTTT CCATAAATTA CCTTTATTTG TATGGTCAGT TTTTATTACA
P.acanthophoron        ...G.T..T. ......G... T....G.... .......... .....G.TA. ..........
P.cactorum             ...G.T..T. .A....G... .....G.... .......... .......TA. A..A.....T
P.citricola            ...G.T.... ......G... T..C.G.... .......... .T.....T.. A..A......
P.colocasiae           ...G.T..T. ......G... T....G.... .......... .T.....T.. A..A......
P.gonapodyides         ...G.T..T. ......G... T..C.G.... ..CC...... .T.....T.. G..A......
P.hibernalis           ...G.T..G. ......G... T....G.... .......... .T.....T.. A..A......
P.ilicis               ...G.C..C. ......G... T....G.... .......... .......T.. A..A.....T
P.nicotianae           ...G.T..T. ......G... T....G.... .........C. .......TA. A..A.....T
P.phaseoli             ...G.T..T. ......G... T....G.... .......... .......TA. A..A.....T
P.plurivora            ...G.T..T. ......G... .....G.... .......... .T.....T.. A..A......
P.pseudotsugae         ...G.T..T. .A....G... .....G.... .......... .......TA. A..A......

S.ferax                GCTTTTTTAT TATTATTAAG TTTACCTGTA TTAGCTGGAG CAATTACTAT GTTATTAACT
P.acanthophoron        .......... .........C .......... .....A..T. .T........ ..........
P.cactorum             ..A...C.T. .........C .......... .......... .C........ ..........
P.citricola            ..A...C.T. .........C .......... ........T. .......... ..........
P.colocasiae           ..A...C.T. .........C .C........ ........T. .......... ..........
P.gonapodyides         ......C.T. .........C A.....C... ........T. .C........ ..........
P.hibernalis           ......C.T. .........C C........T .....A..T. .......... .........A
P.ilicis               ..A...C.T. .........C .......... .....C..T. .......A.. ...G.....C
P.nicotianae           ..A...C.T. .........C .......... .......... .......... .........A
P.phaseoli             ..A...C.T. .........C .......... C.......G. .......... ....C.....
P.plurivora            ..C...C.T. .........C C......... ........T. .......... ..........
P.pseudotsugae         ..A...C.T. .........C .......... .......... .T........ ..........
```

```
S.ferax                 GATAGAAATT TAAATACTTC ATTTTTTGAT CCTTCAGGTG GAGGTGATCC TGTATTATAC
P.acanthophoron         .......... .......A.. T....A.... .......... .T........ ...T.....T
P.cactorum              .......... .......... T....A.... ..A..T..A. .G........ A........T
P.citricola             .......... .......... T....A.... .......... .G........ A........T
P.colocasiae            .......... .......... T....A.... .....T.... .......... A........T
P.gonapodyides          .......... .......... G....AC... ..A..T..A. .......... G........T
P.hibernalis            .......... .......... T....A...C ..C..T..A. .T........ C........T
P.ilicis                .......... .......... .....A.... .....T.... ....A..... .........T
P.nicotianae            .......... .......... T....A.... ..A..T.... .......... A........T
P.phaseoli              .......... .......... .....A.... ..A....... .......... A........T
P.plurivora             .......... .......... T....A.... .......... .......... A........T
P.pseudotsugae          .......... .......... T....A.... ..A..T..A. .......... A........T

S.ferax                 CAACATTTAT TTTGGTTTTT CGGTCACCCA GAAGTTTATA TTTTAATTTT ACCTTCTTTT
P.acanthophoron         .......... .......... T..A..T... .....A...G .......... ...AG..---
P.cactorum              .......... .......... T......... .........G .......... ...AG.....
P.citricola             .......... .......... T.....T... .......... .......... ....GGA...
P.colocasiae            .......... .......... ......T..G .......... .......... ...AGGA...
P.gonapodyides          .......... .......... ......T..T .......... .......... ....G.....
P.hibernalis            .......... .......... T......... .......... .......... ...AG.A...
P.ilicis                .......... .......... T........T .......... .......... ...AG.....
P.nicotianae            .......... .......... T.....T... .........G .A........ ...AG.A...
P.phaseoli              .......... .......... T.....T... ..G......G .......... ...GG.A...
P.plurivora             .......... .......... T.....T... .......... .......... ...AGGA...
P.pseudotsugae          .......... .......... T.....T... .........G .......... ...AG.....
[1]Primer rev                                                                    TTT
[2]Primer FM85                                                                   TTT

S.ferax                 GGTATCATAA GTCAAGTTAT TTCAACTTTT TCTAGAAAAC CAGTTTTTGG TTATTTAGGT
P.acanthophoron         ---------- ---------- ---------- ---------- ---------- ----------
P.cactorum              .....T..T. .........TC .G..G..... G...A....A AT..A..... .........A
P.citricola             .....T..T. .........TC AG..G..... G...A....A AT..A..... .........A
P.colocasiae            .....T..T. .........TC .A.GG..... G...A....A AT..A..... .........A
P.gonapodyides          .....T..T. .........GC CG..G..... G...A....A AT..A..C.. .........A
P.hibernalis            .....T..T. .........TC .G..G..... G...A....A AT..A..... ...C......
P.ilicis                .....T..T. .........TC AG..G..... G...A....A AT..A..... ..........
P.nicotianae            .....T..T. .........TC .G.TG..... G...A....A AT..A..C.. .........A
P.phaseoli              .....T..T. .........TC .G..T..... G.A.A....A AT..A..... ..........
P.plurivora             .....T..T. .........TC AG..G..... G...A....A AT..A..... .........A
P.pseudotsugae          .....T..T. .........TC .G..G..... G...A....A AT..A..... .........A
[1]Primer rev           GGTATRATDA GTCAKGTWHR R
[2]Primer FM85          GGTATTATTA GTCAAGTT
```

Supplement 1

Alignment of the oomycete cytochrome oxidase 1 (*CoxI* or *COI*) nucleotide sequences: Nucleotide sequences from *Phytophtora* and *Saprolegnia* submitted to the GenBank were aligned. The grey boxes indicate primer target sites. Parentheses contain GenBank accessions. Dot: identical nucleotide with the Saprolegnia ferax sequence; gap: deletion. Mismatches in the primer binding sites are given in red. Incompletely specified bases are given in blue: D=G or A or T, H=A or T or C, K=G or T (Keto), M=A or C (AMino), R=A or G (PuRine), W=A or T (Weak 2H bonds).

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[1] Universal primer set (5'-TCAWCWMGATGGCTTTTTTCAA and 5'-RRHWACKTGACTDATRATAC-CAAA) intended to amplify the barcode region of all oomycetes (Robideau et all, 2007. DNA barcoding of oomycetes with the Cytochrome Oxidase I (COI) gene. In BOL Meeting. Guelph).

[2] Primers FM84 (5'-TTTAATTTTTAGTGCTTTTGC) and FM85 (5'-AACTTGACTAATAATACCAAA) are designed for use in *Phytophtora* species (Martin & Tooley, 2003. Mycologia 95: 269-284).

```
S.ferax (NC_005984)    ATG------- ---------- ---------- ---------- ---------- ---------- ---TCTAATA
P.infestans (NC_002387)...AAAAATT ATTATTATAT TAATAAAAAA AATTTACAAA TTGAAACAAC AACAACAAAT GAT.......
P.ramorum (NC_009384)  ...AAAAATT ATTATTATAT TAATAAAAAA AATTTACAAA TTGA...AAC TACTACTAAT GAT.......

S.ferax                ---------- ---------- ---------- ---------- -----AAAAT ATACAAAATT TATATCAACA
P.infestans            TCAATAAATT TCAAAATGAT TTAAAATTTT TAAAAGAAAT CACAC..... .C...G...A C.C.AA.T..
P.ramorum              TCGATAAATT TCAAAATGAT TTTATCTTTT TAAAAGAAAT TTCAA..... .C......C. CTC.AA.T..

S.ferax                TCCATATCAT TTAGTTGATC CAAGTCCTTG GCCTTTTTTC GCATCTTTTG GTTGTTTATT TTTTACTTTC
P.infestans            C..T..C... .....A.... .......A.. .......G.T ATT..A.... .GCT...T.. ...A.....T
P.ramorum              C......... .....A.... .......... .......G.T ATT..A.... .GCT...T.. ...A..A..T

S.ferax                GGAACTGCTA TGTGGTTTCA TGGTTATACT GGAAGTATTT TTTTAGTTTT TACAGG---T TTAATTAGTA
P.infestans            ..TGG..... ...AT..A.. ........TA ..T....A.. .....AC... A.....TTT. .....GGT..
P.ramorum              .......... ...AT..... ...A....T. ..T....A.A ..C....... A..T..ATT. .....GGT..

S.ferax                TATTATTTGT TTTTTATACT TGGTTAAGAG ATGTAGTTCG TGAAGCTGTT TATGAAGGTC AACATACTTT
P.infestans            .T.....-AC .A.G.....A .....TC.T. ..A.....A. A........A .......... .......AAA
P.ramorum              .T.....-AC AA.G..... .....TC.T. ..A.T...A. A........A .......... .......AAA

S.ferax                AAAAGTACAA TTAGGTTTAC GTAACGGAAT GTTAATGTTT ATCATTAGTG AAGTTATGTT CTTTTTAAGT
P.infestans            .C........ .........A .A..T..T.. .C.TT.A... ..TT..... ..T.AC.A.. T...A.T...
P.ramorum              .C....T... .........A .A..T..T.. .C.TT.A... ..TT..... ..T.AT.A.. T...G.T..C

S.ferax                TTTTTTTGGG CATTTTTTCA TTCAGCATTA GCTCCTACCC CTGAAATAGG TTCGGTATGG CCACCTTTAG
P.infestans            .......... .T........ ...T..T... ..A..C.... .A..G..T.. G...T..... .....A....
P.ramorum              .......... .T.....C.. ...G..T... ..A..A..A. .......... ...AT..... ..........

S.ferax                GTATAGAAAC TATAAATGCA TGGGGTATTC CTTTATTAAA TACTGTTATT TTATTAACTT CAGGTGCTAC
P.infestans            ....T..G.. .G.T.....T .......... .......... ....A.A... ......T.A. ....A..A..
P.ramorum              .......... .G.G.....T .......... .......... ...CA.A..A ......T.A. .......A..

S.ferax                CATTACTTGG GCACATCATG CTATTATAGT TGGTTCTAGA AAAAATATTA TTTTAAGTTT AATATTAACT
P.infestans            A.....A... ........CT .....G..T. C...GA.... ......GCA. .......... ...TA.T...
P.ramorum              T......... .....C...T .....G.TT. ....GA.... ......GCA. .......... ...TA.T..A

S.ferax                ATTTCTTTAG CTGTATTTTT TACATTTATT CAAGCATACG AATATATAGA AGCTTCATTT TCAATTTCTG
P.infestans            ....TA.... ..T.T..... .T.T..A... ........T. .......T.. .AG.A.T... ..........
P.ramorum              .....A.... ..T.T..... ......A... ........T. .......T.. .AG.A.T... ..T..A..A.

S.ferax                ATAGTATTTA TGGTACTACT TTCTTTTTAT TAACTGGATT TCACGGAATT CACGTAATTG TAGGTACTAT
P.infestans            .......... .......... ..T....... ....A..T.. ...T..T..A ..T..T.... ..........
P.ramorum              .......... .......... ..T....... ....A..T.. ...T..TG.A ........A. ..........

S.ferax                TTTTATTATC GTAGGTACTA TTAGAGCAGT TAAACATCAT TTTACTAGAC AAAATCATTT TGGTTTTGAA
P.infestans            .........A ...A.....T .A...TT.A. ...T...... .....A.A.. ..C.C..... C.........
P.ramorum              ......AG.A ...A...... ..C..TT.A. ...T..C... .....A.A.. ..C.C..... C.........

S.ferax                GCTGCTGCTT GGTATTGGCA TTTTGTAGAT GTTGTATGGT TATTTTTATT TATTGTTGTT TATTGGTGGG
P.infestans            ........A. .......... ......T... .....T.... .......... .G.A.C.... ..C.......
P.ramorum              .......... .......... .......... .....T.... ....C..... CG.A.C...A ..........

S.ferax                GTGGTCAATA A
P.infestans            .....A.T.. .
P.ramorum              .....A.T.. .
```

Supplement 2

Alignment of the oomycete cytochrome oxidase 3 (*CoxIII*) nucleotide sequences: Nucleotide sequences from *Phytophtora* and *Saprolegnia* submitted to the GenBank were aligned. Parentheses contain GenBank accessions. Dot: identical nucleotide with the Saprolegnia ferax sequence; gap: deletion.

Multiple, Unique and Functionally Constrained Target Sequences Used for Species Typing of the Crayfish Plague Oomycete *Aphanomyces astaci*

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ABSTRACT

The pseudofungus *Aphanomyces astaci* is regarded as the causative agent of crayfish – plague and as an evident hazard for European crayfish species. The bioinvader *Pacifastacus leniusculus* (signal crayfish) is considered to be the most important vector and reservoir for the germ in Europe. Mortalities in affected autochthonous crayfish populations of up to 100% and a manifold propagation necessitate the development of a reliable, robust and efficient test for pathogen detection in clinical samples. Currently, *A. astaci* is being diagnosed by a PCR-based assay that suffers from cross-species amplification of the primers used. Here we present a closed-tube assay alternative which achieves robustness by the simultaneous amplification of multiple functionally constrained genes of the pathogen. The simultaneous detection of multiple sequences by real-time PCR combined with MCA represents a promising way to detect species with elevated levels of genetic variation and/or limited sequence information available. The homogenous closed-tube format, the reduced time for detection, the higher specificity and the considerably reduced chance of false negative detection achieved by targeting multiple genes (*CHI1, CHI2, CHI3* and *5.8S rRNA*) at least two of which are subject to high functional constraint, are the major advantages of this multiplex assay compared to other diagnostic methods.

MATERIAL AND METHODS

Biological material

Validation of assays was conducted using Oomycete isolates and related fungi obtained from The Centraalbureau voor Schimmelcultures (CBS), the German Collection of Microorganisms and Cell Cultures (DSMZ), the American Type Culture Collection (ATCC), or cultured from lesioned tissue by standard methods [1, 2]. The *A. astaci*-types 1 to 4 were purchased from Lage Cerenius (Uppsala University, Uppsala, Sweden). Javier Diéguez-Uribeondo (Real Jardín Botánico CSIC, Madrid, Spain) provided the *A. frigidophilus* isolate SAP472. A DNA aliquot of *A. frigidophilus* NJM 9665 and *A. invadans* WIC was obtained from Mark W. Vandersea (Center for Coastal Fisheries and Habitat Research, National Ocean Service, National Oceanic Atmospheric Administration, Beaufort, North Carolina, USA).
The Austrian *A. astaci* strains GB04, Z12 and the *A. repetans* strain Lk29 were isolated from dissected melanised spots found in the integument of signal crayfish [3]. The *A. astaci* strain GKS07 was grown out of a moribund noble crayfish collected during an acute crayfish-plague outbreak. Melanised necrobiopsies were incubated in PG1 medium [4] for three days at 18°C in a humidified chamber and subcultured every two weeks on PG1 agar medium. The same growth and subculturing conditions were applied to the strains obtained from the culture collections.

Table 1: Biological material used in this work

Species	Isolate: reference	Origin (year, location)	Species	Isolate: reference	Origin (year, location)
A. astaci type 1	L1	*Astacus astacus* (1962, Sweden)	*A. frigidophilus*	SAP472: [30]	*Austropotamobius pallipes* (2008, Spain)
A. astaci type 1	Ra	*A. astacus* (1973, Sweden)	*A. invadans*	WIC: [6]	*Brevoortia tyrannus* (2004, USA)
A. astaci type 1	Sv	*A. astacus* (1970, Sweden)	*A. laevis*	CBS 107.52	unknown (1952, unknown)
A. astaci type 2	H5	*A. astacus* (1974, Sweden)	*A. helicoides*	CBS 210.82	unknown (1982, former USSR)
A. astaci type 2	Ti	*A. astacus* (1970, Sweden)	*A. repetans*	Lk29	*P. leniusculus* (2004, Leithakanal, Austria)
A. astaci type 2	Yx	*A. astacus* (1973, Sweden)	*A. irregularis*	CBS 278.81	pond (1981, The Netherlands)
A. astaci type 3	Kv1	*Pacifastacus leniusculus* (1978, Sweden)	*Achlya racemosa*	CBS 578.67	unknown (1967, Great Britain)
A. astaci type 4	Pc	*Procambarus clarkii* (1992, Sweden)	*Leptolegnia caudata*	CBS 680.69	unknown (1969, Canada)
A. astaci	GB04 (CBS 121.537)	*P. leniusculus* (2004, Ganaubach, Austria)	*Saprolegnia parasitica*	CBS 540.67	fish hatchery (1967, Great Britian)
A. astaci	GKS07 (CBS 121.538)	*A. astacus* (2007, Gleinkersee, Austria)	*Aspergillus sp.*	not assigned	horse food (2004, Vienna, Austria)
A. astaci	Z12 (CBS 117.160)	*P. leniusculus* (2004, Zöbernbach, Austria)	*Fusarium solani*	CBS 181.29	unknown (1929, Germany)
A. frigidophilus	NJM 9665 (ATCC 204464): [6, 61]	egg of *Onchorhynchus masou* (1996, Japan)	*Trichosporon cutaneum*	DSM 70675	sulfite liquor waste

DNA isolation from mycelium of oomycetes

The mycelium was transferred to a 2 ml-extraction tube containing 0.7 g Precellys® ceramic beads of 1.4 mm diameter (Peqlab Biotechnology, Erlangen, Germany) and 180 µl buffer ATL, the lysis buffer of the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Samples were homogenised twice for 15 s at 5000 rpm using the MagNA Lyser (Roche). Further isolation was performed according to the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" provided by the manufacturer.

Qualitative detection of *A. astaci* using qPCR/MCA

The 20-µl duplex qPCR/MCA contained 2 µl 10 x PCR buffer B (Solis BioDyne), 200 nM of forward and reverse chitinase gene(s) primers (5'-TCAAGCAAAAGCAAAAGGCT-3' and 5'-CCGTGCTCGCGATGGA-3'), 125 nM of forward and reverse 5.8S rRNA primers (5'-ATACAACTTTCAACAGTGGATGTCT and 5'-ATTCTGCAATTCGCAATTACG), 200 µM of each dNTP, 0.4 x EvaGreenTM (Biotium), 3.0 mM MgCl2, 1 U Taq DNA hot start polymerase (Hot FirePol®; Solis BioDyne) and 10 ng DNA template or water in the case of the no-template control. QPCR/MCA was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Polymerase activation (95 °C for 15 min) was followed by amplification for 35 cycles (95 °C for 15 s, 59 °C for 15 s and 72 °C for 10 s). After an initial denaturation step at 95 °C for 15 s, amplicon melting was recorded during a gradual increase of the temperature from 60°C to 95°C.

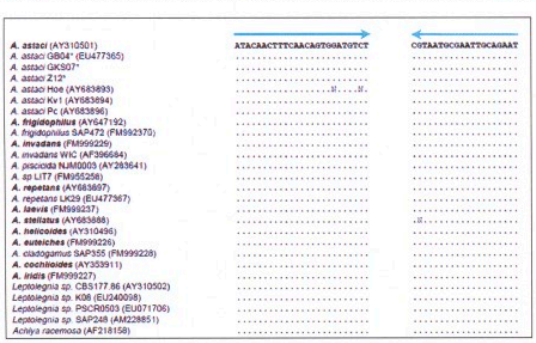

Figure 1. Alignment of primer target sites for the *5.8S rRNA* gene used as endogenous control in qPCR/MCA. The qPCR/MCA primers (blue arrows) target highly conserved sites in the *5.8S rRNA* gene of various oomycete species. Parentheses contain GenBank accession numbers. Dots and letters represent identical and substituted nucleotides compared to the *A. astaci* sequence, respectively.

RESULTS

On the basis of three GH18 chitinase gene sequences (*CHI1*: [5], *CHI2* and *CHI3*: this work) a diagnostic primer pair (Figure 2) producing a 93-bp amplicon from each of the three genes was designed. Its melting temperature of 86.7°C in MCA was regarded as criterion for identification of an *A. astaci* strain. For increasing robustness and specificity of the assay, the chitinase primer pair was multiplexed with primers targeting the *5.8S rRNA* gene as an endogenous control (Figure 1). It yielded peaks in MCA ranging from 81.5 to 83.5 °C depending from the species investigated (Figure 3). The qPCR/MCA assay was tested for specificity against the oomycetes *A. frigidophilus*, *A. invadans*, *A. laevis*, *A. helicoides*, *A. irregularis* and *Leptolegnia caudata*. Only the endogenous control was recorded, but not the *A. astaci*-specific chitinase peak.

The qPCR/MCA-based detection of *A. astaci* was used to elucidate several spontaneous crayfish mortalities in Austrian waterbodies. In detail, *A. astaci* was identified as causative agent of acute crayfish-plague outbreaks among noble crayfish in waterbodies near Hartberg (Styria), Velden/Wörthersee (Carinthia), Roßleithen (Upper Austria) and among a large stone crayfish population near St. Andrä (Carinthia). *A. astaci* was also detected by MCA in necrobiopsy pools each derived from up to five euthanised signal crayfish specimens collected at the streams Ganaubach, Zöbernbach, Strem, Tauchenbach and Güns (all Burgenland).

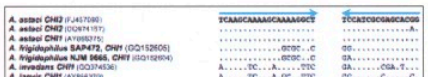

Figure 2. *In-Silico* specificity of the chitinase genes primers. Alignment of diagnostic qPCR/MCA primers (blue arrows) target *A. astaci*-specific sites in the homologous chitinase genes *CHI1*, *CHI2* and *CHI3*, but not homologous sequences of related oomycetes and fungi. Parentheses contain GenBank accession numbers. Dots and letters represent identical and substituted nucleotides compared to the *A. astaci* sequence, respectively.

DISCUSSION

Qualitative detection of two or multiple target sequences by MCA has been reported before. In this work we have used MCA of multiplex qPCR for rapid species identification of the crayfish-plague pathogen *A. astaci* in a closed-tube format. The diagnostic assay for qualitative detection is highly discriminative, robust, inexpensive and reliable.

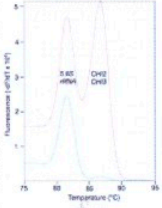

Figure 3. MCA of Qualitative detection of *A. astaci* by qPCR/MCA. The left and right peaks are derived from amplification of the endogenous control, and the chitinase genes CHI2 & CHI3, respectively. Red plot: *A. astaci*, blue plot: *A. frigidophilus*.

High discrimination was aimed at, since new *Aphanomyces ITS* sequences, probably representing new *Aphanomyces* spp. and including sequences closely related to *A. astaci*, were reported [6, 7]. To facilitate unambiguous species identification, we considered the unique feature of constitutive chitinase gene expression of *A. astaci*, not found in closely related *Aphanomyces* species [5].

Assay robustness was another important issue, because it can be assumed that in *A. astaci* a low level of genetic variation is the exception rather than the rule [8]. Targeting the three constrained chitinase gene sequences restricts the genotypic variations allowed. A further level of robustness was achieved by multiplexing with a primer pair targeting the *5.8S rRNA* gene as an endogenous control. This DNA sequence is naturally present at multiple copies [9] and harbours two completely homologous primer target sites in each experimental oomycete species. The simultaneous amplification of this *5.8S rRNA* sequence controlling for the DNA extraction and amplification steps reduces the chance of false negative detection due to insufficient sample quality and allows detection of oomycetes lacking appropriate chitinase-specific primer binding sites (e.g. due to mutations or new closely related species).

Coextraction of an homologous (competitive) internal positive control (IPC) with the clinical samples and coamplification in the qPCR/MCA assays with the same primers used for the target DNA ensures accurate control of the entire molecular assay. An additional welcomed side effect of dye- instead of probe-mediated assays is the cost reduction for screening clinical samples.

CONCLUSION

The identification of two new chitinase genes showing specific patterns of constitutive temporal expression in the absence of substrate has facilitated the development of a discriminative, robust and reliable method for qualitative detection for *A. astaci*.

REFERENCES

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Identification of two GH18 chitinase family genes and their use as targets for detection of the crayfish-plague oomycete *Aphanomyces astaci*

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Abstract

Background: The oomycete *Aphanomyces astaci* is regarded as the causative agent of crayfish plague and represents an evident hazard for European crayfish species. Native crayfish populations infected with this pathogen suffer up to 100% mortality. The existence of multiple transmission paths necessitates the development of a reliable, robust and efficient test to detect the pathogen. Currently, *A. astaci* is diagnosed by a PCR-based assay that suffers from cross-reactivity to other species. We developed an alternative closed-tube assay for *A. astaci*, which achieves robustness through simultaneous amplification of multiple functionally constrained genes.

Results: Two novel constitutively expressed members of the glycosyl hydrolase (GH18) gene family of chitinases were isolated from the *A. astaci* strain Gb04. The primary amino acid sequence of these chitinase genes, termed *CHI2* and *CHI3*, is composed of an N-terminal signal peptide directing the post-translational transport of the protein into the extracellular space, the catalytic GH18 domain, a proline-, serine-, and threonine-rich domain and a C-terminal cysteine-rich putative chitin-binding site. The *A. astaci* mycelium grown in a pepton-glucose medium showed significant temporal changes in steady-state *CHI2* and *CHI3* mRNA amounts indicating functional constraint. Their different temporal occurrence with maxima at 48 and 24 hours of incubation for *CHI2* and *CHI3*, respectively, is in accordance with the multifunctionality of GH18 family members. To identify *A. astaci*-specific primer target sites in these novel genes, we determined the partial sequence homologs in the related oomycetes *A. frigidophilus*, *A. invadans*, *A. helicoides*, *A. laevis*, *A. repetans*, *Achlya racemosa*, *Leptolegnia caudata*, and *Saprolegnia parasitica*, as well as in the relevant fungi *Fusarium solani* and *Trichosporon cutaneum*. An *A. astaci*-specific primer pair targeting the novel genes *CHI2* and *CHI3* as well as *CHI1* - a third GH18 family member - was multiplexed with primers targeting the *5.8S rRNA* used as an endogenous control. A species was typed unambiguously as *A. astaci* if two peaks were concomitantly detected by melting curve analysis (MCA). For sensitive detection of the pathogen, but also for quantification of agent levels in susceptible crayfish and

carrier crayfish, a TaqMan-probe based real-time PCR (qPCR) assay was developed. It targets the same chitinase genes and allows quantification down to 25 target sequences.

Conclusion: The simultaneous qualitative detection of multiple sequences by qPCR/MCA represents a promising approach to detect species with elevated levels of genetic variation and/or limited available sequence information. The homogenous closed-tube format, reduced detection time, higher specificity, and the considerably reduced chance of false negative detection achieved by targeting multiple genes (*CHI1*, *CHI2*, *CHI3*, and the endogenous control) at least two of which are subject to high functional constraint, are the major advantages of this multiplex assay compared to other diagnostic methods.

Sensitive quantification achieved with TaqMan qPCR facilitates to monitor infection status and pathogen distribution in different tissues and can help prevent disease transmission.

Background

Oomycetes are a group of filamentous, unicellular heterokonts. They are fungus-like in their growth form, adsorptive and parasitic lifestyles and formation of spores, but are relatively closely related to photosynthetic algae such as brown algae and diatoms [1]. Among oomycetes, also known as water molds, there are economically important pathogens that comprise severe pests, like *Phytophthora infestans* [2,3] causing potato late blight, *A. euteiches* causing seedling blight or legumes root rot [4], *A. astaci* [5], - the causative agent of crayfish plague, and several fish pathogens from the genera *Aphanomyces* [6], *Achlya* and *Saprolegnia* [7]. There is also at least one species with zoonotic potential, namely *Pythium insidiosum* - the etiologic agent of the human disease pythiosis insidiosii, which can be life-threatening [8]. The oomycetes *A. astaci* and *Phytophthora cinnamomi* are listed among the world's 100 worst invasive species (Global Invasive Species Database: http://www.issg.org/database, alphabetical list as of November 2008).

The crayfish plague, representing the most severe disease among Asian, Australian, and European crayfish species, is caused by the oomycete *A. astaci* (*Saprolegniales*, *Oomycetes*). Crayfish plague-associated die-offs in Austrian waters were first reported in 1879 [9] and in the 1920s [10], and continue sporadically into the present. An estimated 80% of all native Austrian crayfish populations disappeared in the 20th century (Pöckl, personal communication). A high percentage of these die-offs are associated with crayfish plague, which represents one of the major threats to the recovery of populations of native crayfish species in Central Europe [11]. For example, *Astacus astacus*, formerly a very abundant species in Europe, is now considered threatened by the International Union for Conservation of Nature and Natural Resources (IUCN) [12]. In many countries this economically valuable crayfish is on the Red List and its current harvest is probably less than 10% of the harvest rate before introduction of the crayfish-plague pathogen [13,14]. *A. astaci* was introduced from North America, where various species harbour the pathogen without showing clinical signs of infection. Crayfish-plague outbreaks among such populations often occur only under stress conditions. The introduction of resistant North American species like the signal crayfish (*Pacifastacus leniusculus*), the red-swamp crayfish (*Procambarus clarkii*) and the spiny-cheek crayfish (*Orconectes limosus*) http://www.issg.org/database has established a permanent reservoir for the pest in Europe. The transmission of the pathogen occurs via crayfish cadavers, crayfish-feeding fish [15], fish scales [16] and all kinds of equipment, which have been in contact with contaminated water [10]. The adaptive life style, high fecundity, and resistance to the pathogen make introduced crayfish species a potent bioinvador and the most dangerous vector for pathogen transmission.

Biflagellated secondary zoospores, measuring 8 × 12 μm, represent the infective unit of *A. astaci*. They target host tissue by various mechanisms including chemotaxis [17,18] on soft parts of the crayfish integument, especially at the joints, the bottom side of the abdomen and even near the eyestalks [19] as well as fresh wounds [20]. Once zoospores reach the upper lipoprotein-layer of the crayfish cuticle, they discard their flagellae, and develop a penetration peg, that weakens the lipid layer enzymatically [21]. Soon after the germ tube has penetrated the cuticle by mechanical force, the developing hyphae begin to secrete chitinases and proteases [22]. In this phase different chitinases [18] jointly degrade chitin polymers in order to release nutrients and facilitate further growth mainly parallel to the chitin fibrils of the endocuticula [23]. Based on their substrate affinity these enzymes can be classified into three groups: (i) endochitinases, which randomly cleave glycosidic linkages, generating free ends and long chitooligosaccharides that are processed by (ii) exochitinases (chitobiosidases), which release diacetylchitobiose (chitobiose) and (iii) N-acetylglucosaminidases (chitobiases), which hydrolyse chitobiose or release N-acetylglucosamine monomer from chitin chains [24,25].

High-level production of extracellular chitinase in the absence of substrate is one of the most prominent features of the specialised crayfish-parasite *A. astaci* [26,18]. The GH18 family-chitinase Chi1 was the first chitinase described for *A. astaci* [18]. Here we selected two additional members of this gene family as targets for an *A. astaci*-specific diagnostic assay. GH18 chitinases can be divided into three clusters, two of which (A and B) differentiated before the appearance of the eukaryotic lineage [27]. For example, fungal GH18 families comprise between one and twenty genes represented by members of all three clusters [28]. We demonstrate the temporally regulated expression of two novel members of the *A. astaci*-GH18 family. This functional constraint was regarded as a basic criterion for the development of a closed-tube diagnostic method for qualitative and quantitative detection of *A. astaci*. In conclusion, simultaneously targeting multiple chitinase sequences including the novel, functionally constrained chitinase sequences, facilitates a robust analysis of clinical samples with a maximum reduced chance of false-negative detection.

Results

Strain identification

Two putative *A. astaci* strains were recovered from healthy signal crayfish in two small streams in the Austrian province of Burgenland (Gb04 - Ganaubach and Z12 - Zöbernbach). A third strain (GKS07) was isolated from the subabdominal cuticle of a moribund noble crayfish specimen collected during an acute crayfish-plague outbreak in the lake „Gleinkersee" (Austrian province: Upper Austria) in March 2007 (Table 1). *ITS*-sequence data and constitutive chitinase secretion specific for *A. astaci* (Additional file 1) confirm the assumed species assignment for all three strains. The strain Gb04 was used to identify two new chitinase genes, test for their functional constraint and finally to develop the diagnostic assay for *A. astaci*.

The *Aphanomyces* strain LK29 was isolated from a healthy signal crayfish (*Pacifastacus leniusculus*). Physiological and genetic evidence showed that the strain does not fit into any previously identified group of *A. astaci*. It exhibited properties like repeated zoospore emergence and lack of sexual reproduction commonly associated with parasitic species. In contrast to *A. astaci*, the strain LK29 does not express chitinase constitutively during growth or sporulation. Phylogenetic analysis of *ITS* sequences (Additional file 1A) demonstrated clustering within the *A. laevis-repetans* clade [29]. In addition, a Blastn search with the *28SrDNA* sequence of LK29 (GenBank:GQ152606, this work) showed close homology to *A. laevis* (99%, GenBank:AF320584), but clear difference (97% identity) to the *A. astaci* strains Hö, FDL, GB04 and Z12 (AF320583, AF320582, GQ374534, GQ374535, respectively). Until their taxonomic status is fully elucidated the new isolate was assigned to *A. repetans*. This species is not capable of killing crayfish following standardised experimental infection and is characterised by a high growth rate, and germination in response to nutrients [30].

Sequence determination of the novel A. astaci *genes* CHI2 *and* CHI3

Fungal species contain one to twenty GH18 chitinase family genes [28]. In order to develop a robust diagnostic assay for *A. astaci*, we asked whether the chitinolytic system of the pathogen would contain multiple genes of this ancient gene family widely expressed in archea, prokaryotes and eukaryotes [31].

As indicated by the two cross-reacting bands detected in western-blot analysis with antibodies raised against the catalytic GH18 domain, *A. astaci* contains more than one chitinase-like protein (Figure 1). Therefore, we attempted to identify homologous genes using PCR amplification with consensus primers targeting the amino acid motifs DSWND and AGSW (Figure 2). For various *A. astaci* strains representing all four genotype groups described (type A: L1, Sv, Ra; B: Hö, Yx, Ti; C: Kv; D: Pc; [32]) and the Austrian strain Gb04 isolated in this work (Table 1), partial GH18 domain sequences were amplified and subsequently sequenced. Analysis revealed a mixture of sequences derived from two new chitinase genes (*CHI2* and *CHI3*, see below), as concluded by retrospective evaluation. Only synonymous substitutions were found in these genes (data not shown). Starting from the consensus sequence obtained for the "core" of *CHI2* and *CHI3* mRNAs, their complete mRNA sequences were identified by Rapid Amplification of cDNA Ends (RACE)-PCR and submitted to the GenBank (accessions FJ439177 and FJ386997, respectively).

Genomic DNA amplified with gene specific primers designed near the start and stop codons of *CHI2* and *CHI3*, yielded fragments of 1810 bp and 1617 bp for *CHI2* and *CHI3*, respectively. Subsequent sequence analysis performed with a primer-walking strategy (data not shown) confirmed the absence of the consensus sequence for exon-intron junctions (5'-GTRNGT...YAG-3' [33]) and identity of cDNA and genomic sequences (GenBank:DQ974157 and FJ457089 for genomic sequences of *CHI2* and *CHI3*).

Characterization of cDNA and deduced amino acid sequences of CHI2 *and* CHI3

Without the poly(A) tail, the cDNA sequences of *CHI2* and *CHI3* measure 1807 and 1591-bp in length and exhibit a relatively high guanine and cytosine base content of 59.9% and 60.3%, respectively, a typical feature of oomycete genes [34]. *CHI2* and *CHI3* code for open read-

Table 1: Biological material used in this work.

Species	Isolate: reference	Origin (year, location)	Issue addressed
A. astaci type 1	L1	*Astacus astacus* (1962, Sweden)	CHI, MCA, TaqMan
A. astaci type 1	Ra	*A. astacus* (1973, Sweden)	CHI
A. astaci type 1	Sv	*A. astacus* (1970, Sweden)	CHI, MCA, TaqMan
A. astaci type 2	Hö	*A. astacus* (1974, Sweden)	CHI, Chi activity, Western, PCR
A. astaci type 2	Ti	*A. astacus* (1970, Sweden)	CHI
A. astaci type 2	Yx	*A. astacus* (1973, Sweden)	CHI
A. astaci type 3	Kv1	*Pacifastacus leniusculus* (1978, Sweden)	CHI
A. astaci type 4	Pc	*Procambarus clarkii* (1992, Sweden)	CHI
A. astaci	GB04 (CBS 121.537)	*P. leniusculus* (2004, Ganaubach, Austria)	CHI, PHYLO, RACE, GX, MCA, TaqMan
A. astaci	GKS07 (CBS 121.538)	*A. astacus* (2007, Gleinkersee, Austria)	PHYLO
A. astaci	Z12 (CBS 117.160)	*P. leniusculus* (2004, Zöbernbach, Austria)	PHYLO
A. frigidophilus	NJM 9665 (ATCC 204464): [6,61]	egg of *Onchorhynchus masou* (1996, Japan)	CHI, MCA, TaqMan
A. frigidophilus	SAP472: [30]	*Austropotamobius pallipes* (2008, Spain)	CHI
A. invadans	WIC: [6]	*Brevoortia tyrannus* (2004, USA)	CHI, MCA, TaqMan
A. laevis	CBS 107.52	unknown (1952, unknown)	CHI, MCA, TaqMan
A. helicoides	CBS 210.82	unknown (1982, former USSR)	CHI, MCA, TaqMan
A. repetans	LK29	*P. leniusculus* (2004, Leithakanal, Austria)	CHI, PHYLO
A. irregularis	CBS 278.81	pond (1981, The Netherlands)	CHI, MCA, TaqMan
Achlya racemosa	CBS 578.67	unknown (1967, Great Britain)	CHI
Leptolegnia caudata	CBS 680.69	unknown (1969, Canada)	CHI, MCA, TaqMan
Saprolegnia parasitica	CBS 540.67	fish hatchery (1967, Great Britian)	CHI
Aspergillus sp.	not assigned	horse food (2004, Vienna, Austria)	MCA
Fusarium solani	CBS 181.29	unknown (1929, Germany)	CHI
Trichosporon cutaneum	DSM 70675	sulfite liquor waste	CHI

Western: western-blot analysis, *CHI*: partial sequencing of homologous chitinase gene(s), RACE: rapid amplification of cDNA ends, PHYLO: determination of *ITS* nrDNA sequences for phylogenetic analysis, GX: temporal gene expression of *Chi2* and *Chi3*, MCA: qPCR/MCA for qualitative detection of *A. astaci*, TaqMan: TaqMan qPCR

ing frames of 596 and 522 amino acids (Figure 2) with molecular masses of 64.0 kDa and 56.7 kDa and isoelectric points of pH 6.14 and 6.63 predicted for the mature secreted enzymes Chi2 and Chi3 (see below), respectively. The mRNAs possess an identical 42-bp 5' untranslated region (UTR) carrying the major part of the oomycete consensus sequence for the start site of transcription (TATTCAATTTGCCAT, [33]). The 3' UTRs of *CHI2* and *CHI3* contain the polyadenylation signal WAUAAC (W = A or T) [35] (Additional file 2). In both genes the translation start codon is part of the eukaryotic consensus ACCATGA [33]. The enzymes are predicted to be cleaved by signal peptidase between positions A20 and A21 producing a hydrophobic signal peptide of 20 amino acids (Figure 2).

Overall, the deduced amino acid sequences of *CHI2* and *CHI3* are highly homologous with an identity of up to 79.0% (overlapping residues 1 to 596 and 1 to 522,

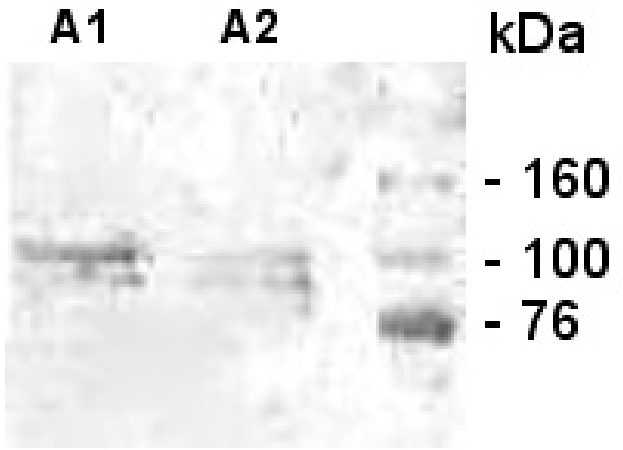

Figure 1
Western-blot analysis of chitinfree PG1-supernatant of a ten-day old *A. astaci* (strain Hö) broth culture. Two bands of about 100 kDa and slightly below this size were detected by antibodies A1 and A2 raised against epitopes in the catalytic domain of the first *A. astaci* GH18 chitinase family member Chi1.

respectively). The proline-, serine-, and threonine-rich domain [36] of Chi2 contains extra residues resulting in an extended amino acid sequence of the whole protein compared to Chi3 (Figure 2). This domain also represents the most heterologous part of the enzymes regarding primary sequence. Chi2 and Chi3 possess an oomycete-type catalytic GH18 domain (A21 to G400/403, Figure 3). It contains a conserved chitin-binding (CB) site [37] (CB site 1 in Figure 2), and an active site consensus [LIVMFY] - [DN] - G - [LIVMF] - [DN] - [LIVMF] - [DN] - x - E (Prosite no. PS01095) being variant at one position (Additional file 3). The catalytic-site residues D154, D156 and E158 are putatively required for catalytic activity [27]. A second putative, highly homologous CB site was identified in the C-terminal part of the chitinases (CB site 2 in Figure 3). It contains four cysteines, instead of the five residues found in a diatom chitinase (GenBank:EED92972) or six in most insect chitinases [38].

Chi2 and Chi3 sequences contain sites for amidation, N-myristoylation or O-linked glycosylation (Additional file 4). The latter type of glycosylation predicted for the C-terminal protein parts occurs often at serine and threonine residues that would otherwise be phosphorylated; one illustration of the complex interplay among eukaryotic post-translational modification systems [39]. N-glycosylation at N165/165 (site: NDS) and N296/298 (site: NFT) was predicted for Chi2/Chi3, respectively. These post-translational modifications may account for the discrepant masses deduced from primary protein sequences and calculated on the basis of the electrophoretic mobility (Figure 1). Putative sites for C-linked glycosylation (C-mannosylation, [39]) were not found. The tripeptide 'RGD' mediating cell adhesion (R81 to D83) was predicted for Chi2. Potential sites for phosphorylation at serine, threonine and tyrosine residues are listed in the Additional file 4.

Temporal mRNA expression analysis for CHI2 *and* CHI3

Next, we verified that target genes selected for the DNA-based diagnostic crayfish-plague assay are subject to functional constraint. This could be assumed if temporal expression of target genes significantly changes during physiological conditions relevant to the infection *in vivo*.

The *CHI2* and *CHI3* mRNA copy numbers expressed in the *A. astaci* mycelium, grown in chitin-free culture were quantified over three days at intervals of twelve hours using one-step qRT-PCR. A partial sequence of the nuclear gene *NDUFV1* encoding the mitochondrial protein NADH dehydrogenase (ubiquinone) flavoprotein 1, which is part of mitochondrial respiratory chain complex I, was identified in this work (data not shown, GenBank:EU500726). We used this sequence as target for an endogenous positive control qRT-PCR assay reporting deviations in extraction, reverse transcription and PCR amplification including mRNA integrity, quality, and quantity. Overall, levels of *NDUFV1* mRNA changed only slightly across the time points studied (< 2.5-fold), including, however, expression changes which were near or below the level of significance ($p = 0.05$) but not matching the temporal expression patterns of the chitinases. In detail, the dynamic growth of the mycelium during the first hours in drop culture (12 to 24 hours, [18]) was reflected by the higher *NDUFV1* expression found after 12 and 24 hours of culture ($P = 0.03$ and 0.07, respectively). Mycelium growth reached its plateau after 72 hours of incubation. The decreasing energy requirement and the beginning of autolytic processes at this stage are reflected by a lower *NDUFV1*-transcript copy number ($P = 0.05$ for expressions at 72 and 24 hours).

The chitinase genes *CHI2* and *CHI3* were both constitutively expressed in mycelium grown in a medium lacking the substrate chitin. However, different mRNA amounts and temporal expression patterns, including the time point at which the maximum level was reached, were observed (Figure 4). Most prominent was the significant maximum in the *CHI2* mRNA level reached after 48 hours ($P = 0.013$).

Analogous to data obtained for *CHI1* [18], we demonstrated, exemplarily for *CHI3*, that neither the amplitude of expression nor its pattern was influenced by substrate

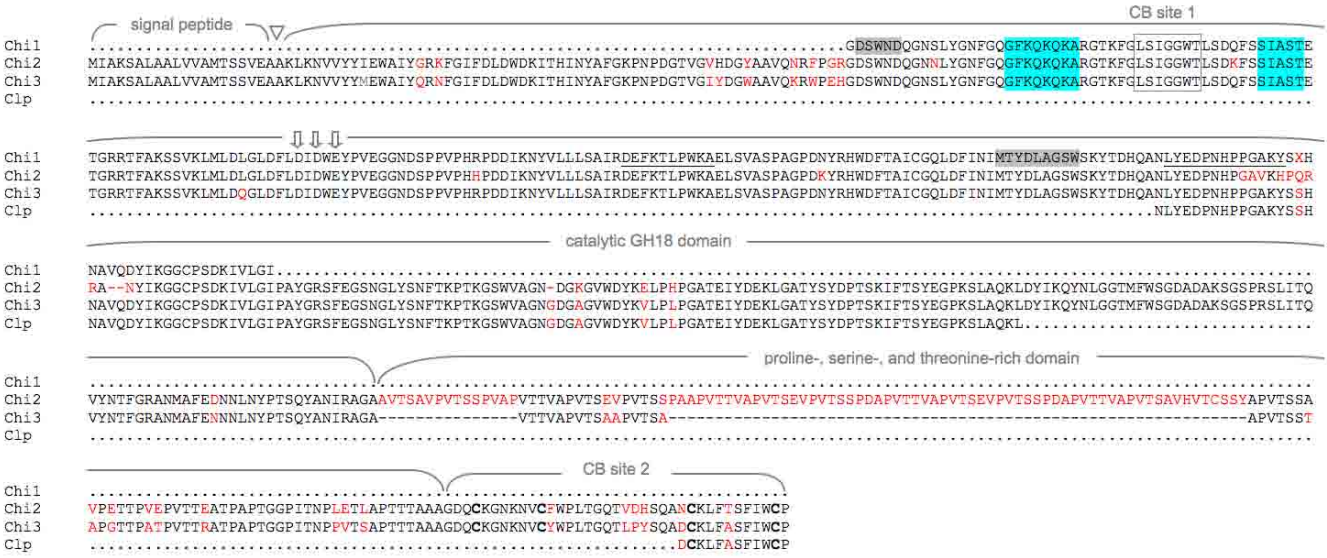

Figure 2
Domains completeley homologous in the novel chitinases *Chi2* and *Chi3* as well as in the first *A. astaci* chitinase (*Chi1*, GenBank:AJ416354, [18]) were selected as primer target sites in the diagnostic assays for *A. astaci*. In blue: primer target sites. Note that only the homologous part of Chi1 is shown. The chitinase-like protein Clp mRNA (GenBank:FJ439176) was amplified from cDNA, but failed to amplify from genomic DNA for unknown reasons (data not shown). Chi1 peptide sequences selected to generate antibodies for Western blot analysis are underlined. Highly conserved motifs in the GH18 domain (grey boxes) were selected as primer target sites to identify the homologous genes of related oomycetes and relevant fungi (see text). Dots indicate missing sequence homology. The triangle marks the signal peptide cleavage site in Chi2 and Chi3. The catalytic-site residues D154, D156 and E158 putatively required for catalytic activity [27] are indicated by vertical arrows. Residues given as red or black letters represent mismatches and conservative changes, respectively. The conserved cysteines in the CB site 2 are highlighted in bold.

addition (0.6% colloidal chitin instead of glucose, data not shown).

Assay development for qualitative and quantitative detection of A. astaci *in clinical samples based on chitinase gene sequences*

Compared to other crayfish-afflicting oomycetes, permanent chitinase expression and activity represents a unique feature of *A. astaci* [18,40]. Due to the assumed functional constraints demonstrated by the significant alterations of temporal gene expression (Figure 4), its chitinolytic system was chosen as a target for the development of a diagnostic test.

qPCR/MCA

A BLASTp search with the deduced amino acid sequence of *CHI1* as query identified two conserved motifs within the GH18 chitinase domain (83-DSWND and 229-MTYDLAGSW, Figure 2). The nucleotide sequences of these motifs were used as target sites for the design of degenerated PCR primers. Using these primers we were able to amplify and sequence the homologous sequences of nine strains from eight oomycete species and two fungi which are known to live on or in proximity of crayfish (species and GenBank accessions in Figure 5a). On the basis of these sequences, we designed a diagnostic primer pair producing a 93 bp-amplicon from each of the three related chitinase genes (*CHI1*: [18], *CHI2* and *CHI3:* this work, Figure 5a). Its melting temperature of 86.7°C in MCA was regarded as criterion for identification of an *A. astaci* strain. For assay robustness the chitinase primer pair was multiplexed with primers targeting the *5.8S rRNA* gene as an endogenous control (Additional file 5) and yielding a peak in MCA at 81.5 to 83.5°C depending from the species investigated.

The qPCR/MCA assay was tested for specificity against the oomycetes *A. frigidophilus*, *A. invadans*, *A. laevis*, *A. helicoides*, *A. irregularis*, and *Leptolegnia caudata*. Only the endogenous control was recorded, but not the *A. astaci*-specific chitinase peak.

qPCR/MCA-based detection of *A. astaci* was used to elucidate several spontanous crayfish mortalities in Austrian waterbodies. In detail, *A. astaci* was identified as causative agent of acute crayfish-plague outbreaks among noble crayfish inhabiting a small unnamed pond-system (Hartberg, district Hartberg, province Styria), in the noble cray-

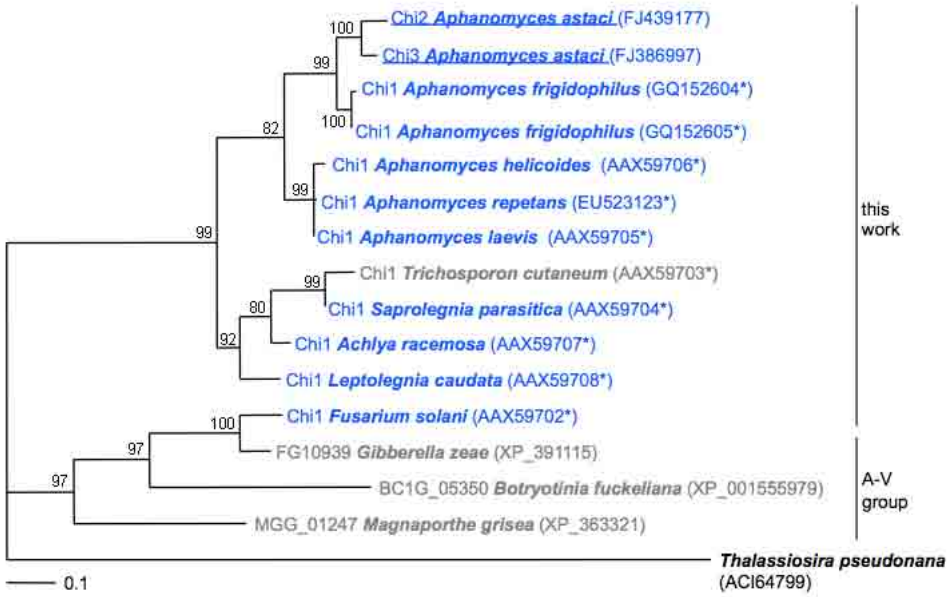

Figure 3

The *A. astaci* chitinases Chi2 and Chi3 possess an oomycete type-GH18 catalytic domain. Maximum likelihood phylogenetic analysis was performed with TreePuzzle using the diatom *Thalassiosira pseudonana* as an outgroup. Oomycete and fungal sequences are given in blue and grey, respectively. GenBank accession numbers of partial or complete amino acid GH18 domain sequences are indicated in parentheses. The scale bar represents 0.1 substitutions per site. The numbers at the nodes are quartet puzzling values indicating the frequencies of occurrence for 1,000 replicate trees and can be interpreted in much the same way as bootstrap values. The group A-V - one of six separate fungal groups classified [27,28] - showing the closest homology to the sequences identified in this work, is represented by two members. An asterisk denotes partial sequences.

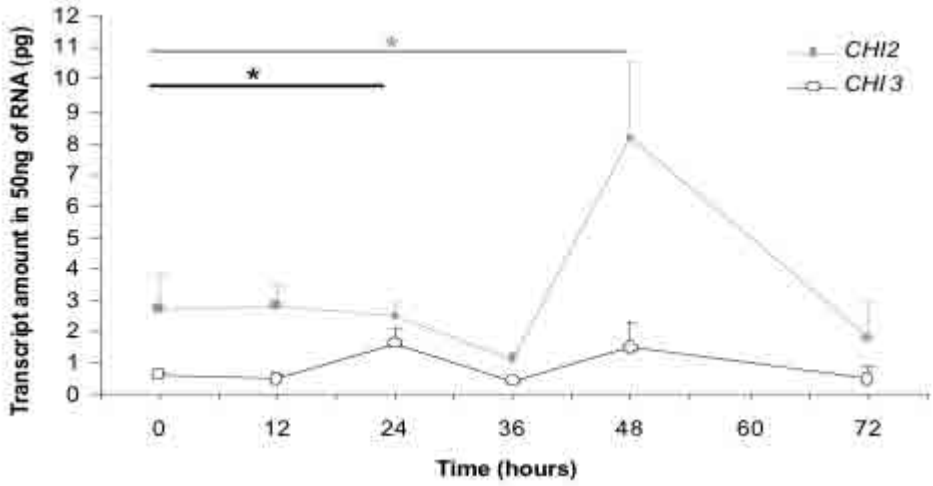

Figure 4

Significant changes of temporal expression of *CHI2* and *CHI3* mRNAs. The transcript abundance changes during 72 hours of growth in chitinless, liquid PG-1 medium. The significant differences in temporal expression indicate functional constraint and are in accordance with the plurifunctionality of GH18 family members, respectively. Error bars (only the positive error bar is shown) represent the standard errors of the mean obtained from three independent time-course experiments. The asterisk designates significance at $p < 0.05$.

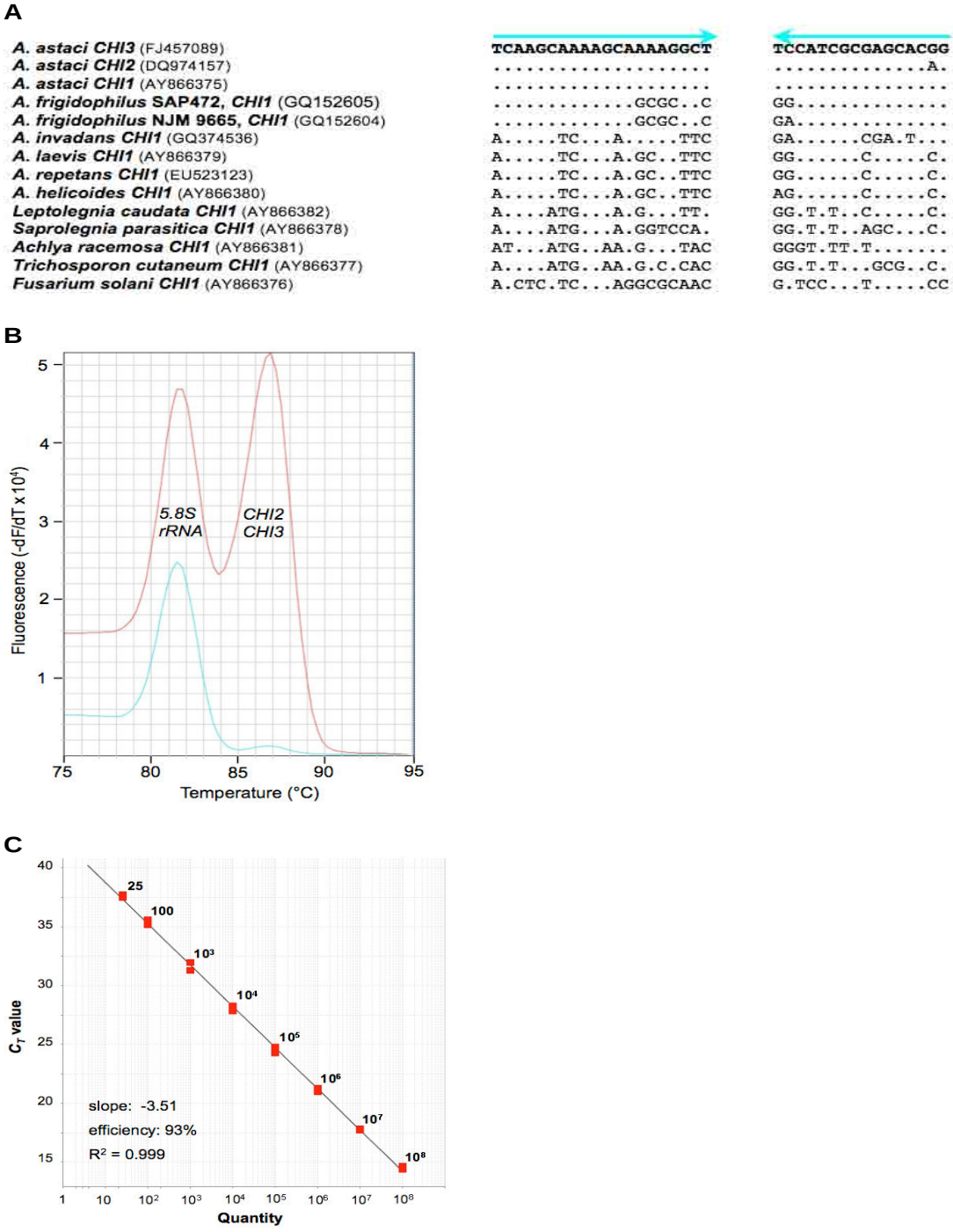

Figure 5

Qualitative and quantitative detection of the oomycete *A. astaci*. A: Diagnostic qPCR/MCA primers (blue arrows) target *A. astaci*-specific sites in the homologous chitinase genes *CHI1*, *CHI2* and *CHI3*, but not homologous sequences of related oomycetes and fungi. Parentheses contain GenBank accession numbers. Dots and letters represent identical and substituted nucleotides compared to the *A. astaci* sequence, respectively. **B:** Qualititative detection of *A. astaci* by qPCR/MCA. The left and right peaks are derived from amplification of the endogenous control, and the chitinase genes *CHI2* &*CHI3*, respectively. Red plot: *A. astaci*, blue plot: *A. frigidophilus*. **C:** Quantitative detection of *A. astaci* by TaqMan qPCR. The standard curve of the assay demonstrates quantification down to 25 copies.

fish-pond Bäckerteich (Velden am Wörthersee, district Villach-Land, province Carinthia), in the brook Hahntrattenbach near St. Andrä (district Wolfsberg, province Carinthia) known for its large stone crayfish population and in a noble crayfish population of the lake Gleinkersee (Roßleithen, district Kirchdorf an der Krems, province Upper Austria). *A. astaci* was also detected by MCA in necrobiopsy pools each derived from up to five euthanised signal crayfish specimens collected at the streams Ganaubach, Zöbernbach, Strem, Tauchenbach and Güns (province Burgenland). Clinical samples tested positive by MCA were subjected to pathogen isolation. In case of isolation failure the qPCR/MCA amplicon was sequenced.

TaqMan qPCR

For sensitive detection of the pathogen, but also for quantification of agent levels in susceptible crayfish and carrier crayfish, a TaqMan-probe-based qPCR assay was developed. TaqMan qPCR uses the same primers as qPCR/MCA except the additional nucleotide at the very 5' end of the reverse primer compared to qPCR/MCA. Using amplicon standards with known copy numbers spiked into genomic crayfish DNA, a quantitative detection limit of 25 target sequences was determined (Figure 5c). No amplication, *i.e.* $C_T > 50$, was obtained for *A. frigidophilus*, A. *invadans*, *A. leaevis* and *A. irregularis*, In the case of the oomycete species *A. helicoides* and *Leptolegnia caudata* a cross-amplification signal corresponding to 28 and 44 copies was detected, respectively.

Discussion

Qualitative detection of two or multiple target sequences by MCA has been reported before. Single-tube SNP genotyping [41], sex determination [42], identification of methylation in promoter sequences [43] or the simultaneous detection of multiple pathogens [44,45] are exemplarily mentioned.

In this work we have used MCA of multiplex qPCR [46] for rapid species identification of the crayfish-plague pathogen *A. astaci* in a closed-tube format. The diagnostic assay for qualitative detection is highly discriminative, robust, inexpensive, and reliable.

High discrimination was aimed at since new *Aphanomyces ITS* sequences, probably representing new *Aphanomyces* spp. and including sequences closely related to *A. astaci* were reported [47,48]. Current molecular techniques for *A. astaci* detection based on *ITS* sequences suffer from a lack of specificity ([47,48], Additional file 6), or are laborious and time-consuming due to agarose electrophoresis and subsequent amplicon sequence analysis [11]. To facilitate unambiguous species identification, we considered the unique feature of constitutive chitinase gene expression of *A. astaci*, not found in closely related *Aphanomyces* species [18,26]. In a search for additional GH18 family members the novel chitinase genes *CHI2* and *CHI3* were identified in this work. The genes differ in their 3' UTRs including variant putative polyadenylation signals. Their temporal mRNA expressions change differently during mycelium growth in chitin-free medium. The deduced extracellular protein sequences are different in proline-, serine-, and threonine-rich domain size, and either possess or lack a putative cell attachement site. This speaks in favour of a joint action during the infection process. Therefore, we regarded *CHI2* and *CHI3* as different members of the GH18 gene family rather than allelic sequences. Altogether, three genes (*CHI1*, *CHI2* and *CHI3*) encoding constitutively expressed GH18 chitinases in the absence of chitin were identified as unique characteristics of *A. astaci* and selected as targets for species-specific detection.

Assay robustness, characterised by a low risk of false negatives related to genotypic variation of pathogenic strains, was another issue for assay design. This was especially important since *A. astaci* belongs to the group of asexual organisms, for which a low level of genetic variation turns out to be the exception rather than the rule [49]. We argued that targeting one or even several functionally constrained sequences would restrict the genotypic variations allowed. The novel chitinase genes *CHI2* and *CHI3* being functionally constrained as concluded from their significant changes in temporal mRNA expression during growth (Figure 4) were regarded to be appropriate candidates to achieve this aim. Together with the first member of the GH18 gene family of *A. astaci* (*CHI1*: [18]) they served as targets in the diagnostic assays based on qPCR/MCA or TaqMan qPCR. In the qPCR/MCA-based assay for qualitative detection, a further level of robustness was achieved by multiplexing with a primer pair targeting the *5.8S rRNA* gene as an endogenous control. This DNA sequence is naturally present at multiple copies [50] and harbours two completely homologous primer target sites in each experimental oomycete species (Figure 5a). The simultaneous amplification of this *5.8S rRNA* sequence controling for the DNA extraction and amplification steps reduces the chance of false negative detection due to insufficient sample quality. The chitinase gene targets and the endogenous control can be considered to be present at comparable copy numbers [50,28]. Therefore, if non-limited primer concentrations are applied like here, the simultaneous amplification of more than one target in a single PCR, *i.e.* multiplexing, leads to competition between multiple targets for a finite number of reagents. Representing a welcomed side effect, this further enhances assay discrimination (see above). Co-amplification of an endogenous control adds another level to assay robustness and represents an improvement compared to the *ITS1*-based TaqMan minor-groove binder qPCR assay for

A. astaci-detection reported recently [51]. Coextraction of an homologous (competitive) internal positive control (IPC) with the clinical samples and coamplification in the qPCR or qPCR/MCA assays with the same primers used for the target DNA ensures accurate control of the entire molecular assay and represents the state of the art for internal controls. It was shown that the addition of an IPC at levels resulting in 100 copies per PCR did not affect the amplification of the target sequence [52,53]. A competitive IPC compatible with the qPCR/MCA and TaqMan qPCR assays developed in this work is presented as Additional file 7.

Another level of diagnostic uncertainty in the assay developed for *A. astaci* detection [51] is added by the use of a synthetic amplicon mimicking one of the closest relatives, *A. frigidophilus*. This approach supposes the intragenomic homogeneity of the ITS regions which has already been rebutted in many organisms [54,55]. The addition of a minor-groove binder to a TaqMan probe in the assay reported by Vralstad *et al*. allows to use shorter probes. However, probe cost increases by about 2.5-fold compared to our conventional TaqMan qPCR designed for quantitative detection. It also elevates the chance of detection failure when varying genotypes are present. Generally, the avoidance of false negatives represents a major challenge in molecular diagnostics. Particularly, in TaqMan qPCR assays the possibility of false-negative testing poses a substantial problem because mutations within the probe-binding site can prevent annealing of the probe and subsequent detection [56,57]. For example, TaqMan qPCR failed to detect any target with more than two mutations at the probe-binding site in contrast to a dye-based assay [56]. The dilemma of false-negative detection due to probe-binding site variation can be overcome, for example, by combining a DNA probe with a fluorescent, double-stranded DNA-binding dye for specific nucleic acid quantification by probe-based qPCR and MCA [58]. In this case the dye would report a detection failure if the probe-binding site of a clinical specimen is mutated. However, "compensation" for mutations in the probe-binding site is no longer an issue if only two instead of three regions of conserved sequence are required for assay design as in the dye-based qPCR/MCA developed in this work. If very limited prior target sequence information exists from a population of interest like in our case, a dye-based detection approach represents a favourable strategy for species confirmation.

A welcomed side effect of dye- instead of probe-mediated monitoring is the cost reduction for screening clinical samples.

Last but not least, the reliability of the diagnostic assay was proven on a set of relevant related pathogens and during an acute crayfish-plague outbreak in the small, noble crayfish (*Astacus astacus*) population inhabiting the lake "Gleinkersee" located at an altitude of about 800 meters above sea level at the foothills of the Austrian Alps. In addition to qPCR/MCA typing (not shown), the presence of the pathogen *A. astaci* was independently confirmed by *ITS*-sequence analysis and testing for constitutive chitinase activity (*A. astaci*-strain GKS07 in Additional file 1). Finally, the *A. astaci* strain GKS07 was isolated on PG-1 agar from an infected noble crayfish. Numerous crayfish individuals were found to be affected but were still alive during the outbreak of late March 2007. At that time the ice of the lake Gleinkersee was melting and the physiological activities of both pathogen and victim would have been expected to be at a minimum. These circumstances strongly indicated the acuteness of the outbreak. The suspicion of a deliberate introduction of the pathogen could not be confirmed by an inquiry led by the local criminal investigation department. Fish stocking performed in autumn 2006 may be the most likely source of disease transmission.

Sensitive quantitative detection of the crayfish-plague pathogen is currently of increasing importance for screening natural non-native crayfish populations or for certifying a pathogen-free status of hatchery fish before introduction into natural habitats or aquaculture facilities. Samples of fish transport water including sediments can be filtered via membrane filters [59] and subsequently screened by TaqMan qPCR (see Results and Additional file 8). This circumvents pathogen transmission via transport water, fish faeces, mucus and scales.

Conclusion

The identification of two new chitinase genes showing specific patterns of constitutive temporal expression in the absence of substrate has facilitated the development of a discriminative, robust and reliable method for qualitative and quantitative detection for *A. astaci*.

Methods

Biological material

Isolates of *Oomycetes* and related fungi used to validate the molecular assays were either obtained from The Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre (Utrecht, The Netherlands), the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany), the American Type Culture Collection (ATCC) or cultured from lesioned tissue by standard methods [60,61]. The *A. astaci*-types 1 to 4 were purchased from Lage Cerenius (Uppsala University, Uppsala, Sweden). Javier Diéguez-Uribeondo (Real Jardín Botánico CSIC, Madrid, Spain) provided the *A. frigidophilus* isolate SAP472 [29]. A DNA aliquot of *A. frigidophilus* NJM 9665 [6,62] and *A. invadans* WIC [6] was

obtained from Mark W. Vandersea (Center for Coastal Fisheries and Habitat Research, National Ocean Service, National Oceanic Atmospheric Administration, Beaufort, North Carolina, USA).

The Austrian *A. astaci* strains Gb04, Z12, and the *A. repetans* strain Lk29 were isolated from dissected melanised spots found in the integument of signal crayfish [19]. The *A. astaci* strain GKS07 was grown out of a moribund noble crayfish collected during an acute crayfish-plague outbreak. Melanised necrobiopsies were incubated in peptone-glucose (PG1) medium (3 g/l glucose, 6 g/l peptone, 0.37 g/l KCl, 0.17 g/l $MgCl_2 \cdot 6H_2O$, 0.15 g/l $CaCl_2 \cdot 2H_2O$, 20 mg/l $FeCl_3 \cdot 6H_2O$, 44 mg/l Na_2EDTA, 13 mM sodium phosphate buffer (pH 6.3); [63]) for three days at 18°C [19] in a humidified chamber and subcultured every two weeks on PG1 agar medium. The same growth and subculturing conditions were applied to the strains obtained from the culture collections.

Fungal contamination of oomycete culture encountered when culturing the *A. astaci* strain Z12 and the *A. repetans* strain LK29 were overcome as follows. A piece of agar culture was incubated for one day at 20°C in autoclaved pond water (pH 6.5 to 7) collected at the central biotop of the University campus. This depletion of nutrients induced the sporulation of the oomycete [64]. Under an inverted microscope the swimm spores were aspired into a 100 μL Gilson pipette and re-cultured on PG1 agar medium.

A fungus isolated from horse food was assigned to *Aspergillus sp*. based on morphological evaluation and added to the strain collection of the Institute of Bacteriology, Mycology and Hygiene (University of Veterinary Medicine, Vienna).

An overview on the biological material used in this work is presented in Table 1.

Species assignment of Austrian Aphanomyces strains

ITS sequences of nuclear rDNA were analysed to allow species assignation of the Austrian *A. astaci* strains GB04, GKS07, and Z12 as well as of the *A. repetans* strain LK29 (Table 1, Additional file 1). For this purpose DNA was extracted from 25 mg drop culture mycelium using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). A DNA fragment of about 1,000 bp was amplified and sequenced using the universal primers V9D (5'-TTACGTCCCTGCCCTTTGTA) [65] and LSU266 (5'-GCATTCCCAAACAACTCGACTC, [66]). Sequences obtained were compared with reference homologs of *Aphanomyces* [29] retrieved from GenBank. For sequence alignment the CodonCode Aligner software (version 3.0.1; CodonCode, Dedham, USA) was used. Molecular phylogenetic relationships were reconstructed using default settings in a program package for quartet-based maximum-likelihood analysis (TREE-PUZZLE, version 5.2 [67]) and TreeView for graphical illustration [68].

Additional evidence for species assignation was obtained from sequence analysis of the large subunit ribosomal RNA gene using the primers nuLSU-5' (5'-CGCTGATTTTTCCAAGCCC) and nuLSU-3' (5'-GAGATAGGGAGGAAGCCATGG) for amplification and sequencing.

Thus far *A. astaci* represents the only species within the genus *Aphanomyces* known to produce significant amounts of chitinase in chitin-free medium [18]. This unique feature was additionally used for species assignment. In detail, chitinase activity accumulated in broth culture supernatant was measured in a reaction volume of 100 μL containing 5 mM sodium-phosphate buffer (pH 7), 180 μM 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside (4-MU-chitotrioside; Sigma-Aldrich, Vienna, Austria) as substrate, and 75 μl of supernatant [18]. Following incubation at room temperature for 10 min, the fluorescence intensity was evaluated under UV light.

DNA isolation from mycelium of oomycetes

The mycelium was transferred to a 2 ml-extraction tube containing 0.7 g Precellys® ceramic beads of 1.4 mm diameter (Peqlab Biotechnology, Erlangen, Germany) and 180 μl buffer ATL, the lysis buffer of the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Samples were homogenised twice for 15 s at 5000 rpm using the MagNA Lyser (Roche). Further isolation was performed according to the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" provided by the manufacturer.

De novo sequencing of partial GH domain using degenerate PCR primers

Partial GH18 domains of chitinases from various *A. astaci* strains representing all four genotype groups described (A: L1, Sv, Ra; B: Hö, Yx, Ti; C: Kv; D: Pc; [32]), the Austrian strain Gb04 isolated in this work and six related oomycete species (*A. laevis*, *A. helicoides*, *A. repetans*, *A. irregularis*, *Saprolegnia parasitica*, *Achlya racemosa*, *Leptolegnia caudata* (Table 1) were amplified using the primers SEQ685F (5'-CCGGAGACTCGTGGAACGAC) and SEQ1159R (5'-TTGCTCCAGCTGCCCGC). Primers targeting the amino acid motifs DSWND and AGSW, respectively, amplified an approximately 475-bp product by qPCR. The 20-μL reaction consisted of 0.4 × EvaGreen™ dye (Biotium, Hayward, USA), 4 mM $MgCl_2$, 200 μM of each dNTP, 375 nM of each primer, 2 μl template DNA, 1 U GoTaq® DNA polymerase - a proprietary formulation of *Taq* DNA polymerase (Promega, Madison, USA), and 1 × Colorless GoTaq® Flexi Reaction Buffer (Promega) not containing

magnesium. Amplification was performed in the Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) using denaturation for 4 min at 94°C, amplification for 35 cycles (1 min at 94°C, 1 min at 63°C and 1 min at 72°C), and final elongation of 7 min at 72°C followed by MCA.

Amplicons from *Fusarium solani* and *Trichosporon cutaneum*, representing fungi, were obtained with the degenerate primer SEQuni-F (5'-CGCCGGAGAYTCTTGGAAYGA, Y = C or T) in combination with the primer SEQuni-R (5'-CCAGCATAGTCGTAGGCCAT) targeting the amino acid motifs xxDSWND and MTDYAG, respectively.

Agarose gel electrophoresis was used to the determine amplicon size. The MSB® Spin PCRapace Kit (Invitek, Berlin, Germany) was used for amplicon purification in case of a single band showing the expected length. Multiple bands were excised from the gel and purified with the Xact DNA Cleanup kit (genXpress, Wiener Neudorf, Austria).

The BigDye® Terminator sequencing chemistry (Applied Biosystems, Foster City, USA) was used for sequence analysis of amplicons performed at VBC Genomics Bioscience Research GmbH (Vienna, Austria).

Identification and phylogenetic analysis of GH18 domains

The GH18 domain in the amino acid sequences of *CHI2* and *CHI3* were identified using the Reversed Position Specific Blast (rpsblast) search modus and the conserved domain database [69]. Domain sequences were aligned to GH18 domain sequences of related species with the ClustalW alignment program implemented in the graphical multiple sequence alignment editor SeaView version 4 [70]. Quartet-based maximum likelihood analysis for aligned amino acid sequences was performed using TreePuzzle with default settings [67]. The graphical display of the phylogram was generated as described above.

Western blot analysis of **A. astaci** *culture supernatant*

The peptides DEFKTLPWKAE and LYEDPNHPPGAKY were selected from the deduced amino acid sequence of the *A. astaci* gene *CHI1* (GenBank:AJ416354). Conjugates of these peptides with bovine serum albumin (BSA) were obtained from PSL GmbH (Heidelberg, Germany). Coupling to BSA was achieved via the SH group of a cysteine residue introduced at the C terminus of the peptide to be synthesised. Conjugates were used for the production of polyclonal rabbit serum antibodies served as primary antibodies. Peroxidase-labelled goat anti-rabbit IgG antibodies (K&P Laboratories, Gaithersburg, USA) were used as secondary antibodies.

Western-immunoblot analysis was performed as follows. The *A. astaci* strain Hö was grown in broth culure. The culture supernatant was boiled for 5 min in a buffer consisting of 25 mM Tris-HCl (pH 6.8), 2.2% sodium dodecyl sulfate (SDS), 15% glycerol and 0.001% bromophenol blue. Insoluble debris was removed by centrifugation. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide Tris-glycine gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, USA) using a tank blot system (Bio-Rad). The Opti-4CN™ substrate detection kit (Bio-Rad) was used for colorimetric detection of secondary antibodies conjugated to horseradish peroxidase.

Determination of complete cDNA- and genomic-DNA sequences for **CHI2** *and* **CHI3**

Mycelium derived from the *A. astaci*-strain Gb04 was grown in liquid PG1 medium for three days and transferred to fresh medium for another 24 h. Total RNA was isolated from mycelium using the Plant and Fungi Protocol provided with the RNeasy Plant Mini Kit (Qiagen). Treatment with DNase I (Promega, Mannheim, Germany) was performed at 37°C for 40 min according to the supplier's instructions. The complete cDNA sequences of *CHI2* and *CHI3* were generated by RACE-PCR using the 5'/3' RACE Kit (Roche Applied Science, Vienna, Austria).

To amplify genomic sequences corresponding to the cDNAs determined, we designed primers in the region of the start and stop codons of *CHI2* and *CHI3*. The common forward primer (Chi5'f: 5'-AGCAAACTGCAACAAGCATG) targeting a region immediately upstream of the start codon of putative *CHI2* and *CHI3* genomic sequences, was combined with a gene-specific reverse primer binding adjacent to the stop codon (Chi2.3'r: 5'-GGGCACCAGATGAACGACGC or Chi3.3'r: 5'-ACTAACATACACAACGAATGCGC for *CHI2* and *CHI3*, respectively). The matching fragment size between cDNA and respective DNA sequences shown by agarose gel electrophoresis, and the identity of genomic and cDNA sequences identified by a primer-walking strategy (data not shown), were considered as experimental demonstration for the absence of intronic sequences within *CHI2* and *CHI3* genes.

In silico analysis of amino acid sequences deduced from **CHI2** *and* **CHI3**

Multiple matching subsegments in two protein sequences were identified with the LALIGN program http://www.ch.embnet.org/software/LALIGN_form.html implementing the algorithm of Huang & Miller [71].

The theoretical isoelectric points for the protein sequences were calculated using the Protein Isoelectric Point menu within the Sequence Manipulation Suite [72].

The presence and location of signal peptide cleavage sites in the amino acid sequences of *CHI2* and *CHI3* were predicted with the SignalP 3.0 Server http://www.cbs.dtu.dk/services/SignalP:[73]). Protein phosphorylation at serine, threonine or tyrosine residues was predicted with the NetPhos 2.0 Server [74]. Putative sites for amidation, N-myristoylation and cell attachment were identified by a protein pattern search against the Prosite database http://www.expasy.org/prosite/; [75]). O-, N-, and C-glycosylated sites were predicted with EnsembleGly - a web server for prediction of O-, N-, and C-linked glycosylation sites with ensemble learning [39].

Transcript quantification by real-time reverse transcription PCR (qRT-PCR)

Propagules of the strain Gb04 were grown in PG1 medium for three days, washed in fresh medium for 2 min and transferred to another portion of fresh medium (time point 0). Twelve, 24, 36, 48 or 72 hours later the mycelium was shortly washed with distilled water, quick-frozen in liquid nitrogen and stored at -80°C. RNA was isolated from three independent samples grown per time point.

For quantification of transcript mass expressed from the chitinase genes *CHI2* and *CHI3* as well as the endogenous positive control *NDUFV1*, sense strand transcript standards were generated by *in vitro* transcription from a PCR product template tailed with the T7 phage promoter sequence. In more detail, for template construction a minimum sequence of 19 bases (5'-TAATACGACTCACTATAGG) required for efficient transcription was selected out of the 23 nt T7 phage promoter sequence and added to the 5' end of the respective PCR primer. *In vitro* transcription was performed with the RNAMaxx™ High Yield Transcription Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions. Transcription was terminated by adding 1 μl DNase I (10 units/μl RQ1 RNase-Free DNase, Promega) and incubation at 37°C for 40 minutes. The amount of the *in vitro* transcript was determined by UV-absorbance measurement performed at 260 nm on a GeneQuantII RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK). Ten-fold serial dilutions were used as absolute concentration standards.

The 10-μl one-step qRT-PCR contained 125 nM of each primer (5'-CCATCACGAACCCCCTTGAG and 5'-GGGCACCAGATGAACGACG for *CHI2*, 5'-GTGGCCCCATCACGAACC and 5'-ACTAACATACACAACGAATGCGC for *CHI3*, 5'-TCGGCTGTCGCACTTCTACA and 5'-ATCCACCCCGTTCCTTCG for *NDUV1*), 75 nM TaqMan probe (Hexachloro-6-carboxyfluorescein (HEX)-5'-CTGCGGCCAATGTACCCCTTGCC black-hole quencher 1 (BHQ1) and 6-carboxyfluorescein (FAM)-5'-TTGTTGCCCTTGCACTGGTCGCC-BHQ1 for *NDUV1* and *CHI2*/*CHI3*, respectively), 0.1 μl of the QuantiTect RT Mix, 5 μl of the 2 × QuantiTect Probe PCR Master Mix (Qiagen) and 50 ng total RNA or 1 μL *in vitro* transcript. In minus RT controls the QuantiTect RT Mix was replaced by water. Reverse transcription of one-step RT-PCR was conducted at 50°C for 30 min followed by a 15 min-activation of the HotStartTaq DNA polymerase at 95°C and amplification for 35 cycles (94°C for 20 s, 60°C for 1 min).

Qualitative detection of **A. astaci** *using qPCR/MCA*

The 20-μl duplex qPCR/MCA contained 2 μl 10 × PCR buffer B (Solis BioDyne, Tartu, Estonia), 200 nM of forward and reverse chitinase gene(s) primers (5'-TCAAGCAAAAGCAAAAGGCT and 5'-CCGTGCTCGCGATGGA), 125 nM of forward and reverse 5.8S rRNA primers (5'-ATACAACTTTCAACAGTGGATGTCT and 5'-ATTCTGCAATTCGCATTACG, Figure 5a), 200 μM of each dNTP (Fermentas, St. Leon-Rot, Germany), 0.4 × EvaGreen™ (Biotium), 3.0 mM $MgCl_2$, 1 U Taq DNA polymerase chemically modified for "hot start" (Hot FirePol®; Solis BioDyne, Tartu, Estonia) and 10 ng DNA template or water in the case of the no-template control. QPCR/MCA was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) run under the StepOne™ software version 2.0. Polymerase activation (95°C for 15 min) was followed by amplification for 35 cycles (95°C for 15 s, 59°C for 15 s and 72°C for 10 s). After an initial denaturation step at 95°C for 15 s, amplicon melting was recorded during a gradual increase of the temperature from 60°C to 95°C.

Oligonucleotides (Sigma-Aldrich, Steinheim, Germany) were designed with Primer Express Software Version 2.0 (Applied Biosystems). The difference between amplicon melting temperatures was calculated using the Nearest Neighbor mode implemented in the online oligonucleotide properties calculator OligoCalc [76].

Sensitive detection and quantification of **A. astaci** *using TaqMan qPCR*

Duplicate TaqMan qPCR was carried out in a total volume of 20 μl containing 2 μl 10 × PCR buffer A2 (Solis BioDyne), 0.2 mM of each dNTP, 4 mM $MgCl_2$, 300 nM of each primer (Chi3-324f20 and AaChi-Tmr), 150 nM TaqMan probe (AaChi-FAM), 1 U HOT FIREPol DNA polymerase (Solis BioDyne), 20 ng template DNA or water in the case of the no-template control.

Reactions were amplified in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) under the StepOne™ software version 2.0 using thermal cycling conditions of 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 64°C. A standard curve was generated by plotting

the logarithm of the standards copy numbers versus measured C_T values.

Isolation of spike-in DNA for use in serial dilutions

A crayfish sample extracted from the abdomen of *Cherax quadricarinatus* (Australian red-claw crayfish) was transferred to a 2 ml-extraction tube containing 0.7 g Precellys® ceramic beads of 1.4 mm diameter (Peqlab Biotechnology, Erlangen, Germany) and 180 µl buffer ATL, the lysis buffer of the DNeasy® Blood & Tissue Kit (Qiagen). The MagNA Lyser (Roche) was used for three mechanical lysis cycles consisting of 30 s at 6,500 rpm followed by 60 s on a cooling block held at 4°C. Further isolation was performed according to the protocol "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)" provided by the manufacturer. DNA concentration was determined spectrophotometrically using the Hellma® TrayCell (Hellma, Müllheim/Baden, Germany) on the Eppendorf BioPhotometer 6131.

Generation of copy standards

A DNA template stock consisting of *CHI1*, *CHI2* and *CHI3* sequences was generated as follows. Genomic DNA from chitinase sequences were amplified with the primers Chi3-324f20 (5'-TCAAGCAAAAGCAAAAGGCT) and AaChi-Tmr (5'-TCCGTGCTCGCGATGGA). Amplification was evaluated by the signal generated from the TaqMan® probe AaChi-FAM (5'-FAM-TCAACGTCCAC-CCGCCAATGG-BHQ-1). Amplification was performed in a total volume of 20 µl containing 2 µl 10 × PCR buffer A2 (Solis BioDyne), 0.2 mM of each dNTP, 4 mM $MgCl_2$, 250 nM of each primer, 150 nM TaqMan probe, 1 U HOT FIREPol® DNA polymerase (Solis BioDyne) and 20 ng DNA or water in the case of the no-template control. DNA denaturation and enzyme activation were performed for 15 min at 95°C. DNA was amplified over 50 cycles consisting of 95°C for 15 s, 60°C for 1 min. QPCR was run on StepOnePlus™ Real-Time PCR System (Applied Biosystems) under the StepOne™ software version 2.0.

PCR fragments were purified with the MSB® Spin PCRapace Kit (Invitek, Berlin, Germany).

The copy number of the target template was determined spectrophotometrically using the Hellma® TrayCell (Hellma, Müllheim/Baden, Germany) on the Eppendorf BioPhotometer 6131. Serial dilutions of the target sequence (10^8 to 10^2, 50, 25 and 12.5 copies per 2 µl) prepared in 10 ng/µl *C. quadricarinatus* DNA were used to determine the amplification efficiency and the quantitative detection limit.

Statistical analysis of expression changes

A univariate one-way analysis of variance (ANOVA) with Scheffè's post-hoc test was used to evaluate the significance of changes in temporal mRNA expression. The dependent variable was the log-transformed mRNA amount. The time was considered a fixed effect. A value of $p < 0.05$ calculated by the Scheffè's post-hoc test was regarded as significant. The normality assumption was tested using the Kolmogoroff-Smirnow test.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GH, RB-R and RS conceived and designed the experiments. RB-R performed Western blot analysis, GH, RT and RS provided the diagnostic assays. GH performed all other experiments. RS supervised the experimental work and the interpretation of data and planned the manuscript. EL provided funding. GH and RS wrote the paper. All authors analysed the data, commented on and approved the manuscript.

Additional material

Additional file 1

Species identification of Austrian* A. astaci *strains Gb04, Z12, and GKS07 based on phylogenetic analysis and constitutive chitinase activity in substrate-free medium. *ITS sequence and chitinase expression in chitin-free medium are criteria to classify a strain as* A. astaci

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[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S1.pdf]

Additional file 2

Sequences of 3' untranslated regions (UTRs) of* CHI2 *and* CHI3 *mRNAs. *Alignment shows differences between 3' UTRs of CHI2 and CHI3 mRNAs*

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[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S2.pdf]

Additional file 3

Amino-acid substitutions in the GH18 catalytic site of oomycete species. *Table lists amino-acid substitutions in the GH18 catalytic site of oomycete species*

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[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S3.pdf]

Additional file 4

O-linked glycosylation and phosphorylation predicted for Chi2 and Chi3. *Predicted O-linked glycosylations and phosporylations at serine and threonine residues for Chi2 and Chi3 are listed in a table*

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[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S4.pdf]

Additional file 5

Alignment of primer target sites for the* 5.8S rRNA *gene used as endogenous control in qPCR/MCA. *Primers target conserved sites in the* 5.8S rRNA *gene of various oomycete species*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S5.pdf]

Additional file 6

A conventional PCR assay for detection of* A. astaci *that may fail to discriminate between closely related species. *Alignment of primer sites for a conventional PCR assay reported for detection of* A. astaci
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S6.pdf]

Additional file 7

Design of a homologous IPC for use in the qPCR/MCA or qPCR assays. *The IPC monitored by a characteristic melting temperature or by an alternatively labeled hydrolysis probe in the qPCR/MCA or qPCR assays, respectively, helps to prevent false-negative detection due to insufficient extraction and/or amplification.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S7.pdf]

Additional file 8

***TaqMan qPCR assay design for sensitive detection and quantification of* A. astaci.** *Primers, but also TaqMan probe facilitate discrimination between* A. astaci *and various related or relevant oomycete species.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-184-S8.pdf]

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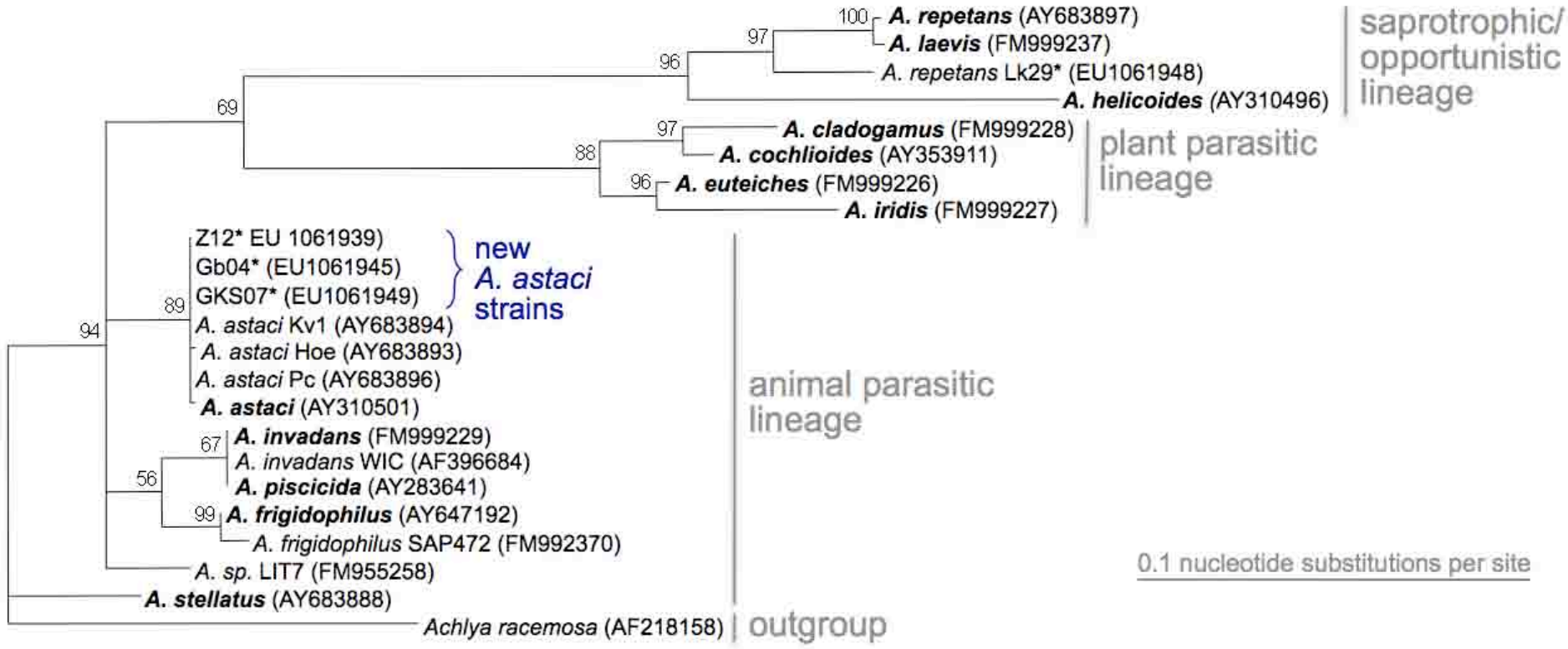
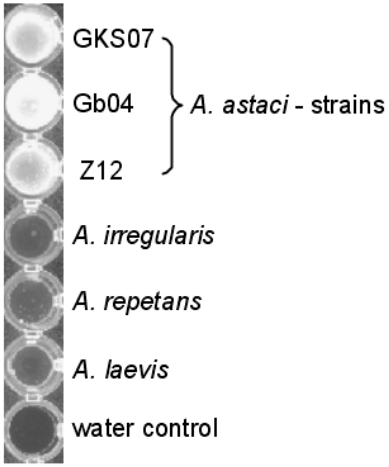
A



B



Additional file 1

Species identification of the Austrian strains isolated in this work based on phylogenetic analysis of *ITS* sequences (A) and constitutive chitinase activity in substrate-free medium (B).

A: Phylogenetic relationships among members of the genus *Aphanomyces* analysed by maximum likelihood of *ITS* sequences. The Austrian strains isolated in this work are denoted by an asterisk (*A. astaci*-strains Gb04, Z12, and GKS07, and *A. repetans* strain LK29). Parentheses contain GenBank accession numbers. Reference strains [1] are given in bold. Note that *A. invadans* is synonymous with *A. piscicida* since species separation is not supported by *ITS*-sequence phylogeny [1].

B: Detection of chitinase activity in chitin-free medium using the 4-methylumbelliferyl caprilate fluorescence test. All three Austrian *A. astaci*-strains developed strong chitinolytic activity in substrate-free medium which is a characteristic feature of this species.

Reference: 1. Dieguez-Uribeondo J, Garcia MA, Cerenius L, Kozubikova E, Ballesteros I, Windels C, Weiland J, Kator H, Soderhall K, Martin MP: **Phylogenetic relationships among plant and animal parasites, and saprotrophs in Aphanomyces (Oomycetes)**. *Fungal Genet Biol* 2009, **46**(5):365-376.

```
CHI2  TAAATGCACATTGAATTCTTTTATATTAGTAACTCATATAACCTCGAATTGACCATTGGGT-TGTG-TGTAAAAAAAAAAAAAAAA
CHI3  TAAAAGCGCATTCG-TT-GTGTATGTTAGT----CGAATAACCTTGAATTGAAGATTCGATGTGAGTTGTGAAAAAAAAAAAAAAA
```

Additional file 2. Sequences of 3' untranslated regions (UTRs) of *CHI2* and *CHI3* mRNAs. Bold letters: stop codon, grey background: sequence identity, dash: deletion, underline: putative polyadenylation signal. Sequences were derived from GenBank:FJ439177 and FJ386997, respectively.

Additional file 3. Amino-acid substitutions in the GH18 catalytic site of oomycete species

Species	Protein	GenBank accession number	Conserved consensus sequence (N-terminal → C-terminal)								
			(LIVMFY)	(DN)	G	(LIVMFY)	(DN)	(LIVMFY)	(DN)	X	E
Aphanomyces astaci	ChiI2	ABK59977			F						
A. astaci	Chi3	ACJ66260			F						
A. helicoides	Chi1	AAX59706			F						
A. laevis	Chi1	AAX59705			F						
A. repetans	Chi1	ACB12070			F						
Achlya racemosa	Chi1	AAX59707			F						
Leptolegnia caudata	Chi1	AAX59708			F						
Saprolegnia parasitica	Chi1	AAX59704			F						
Trichosporon cutaneum	Chi1	AAX59703			F						

blue: oomycetes; grey: fungus

Additional file 4. O-linked glycosylation and phosphorylation predicted for Chi2 and Chi3. O-glycosylation at residues that would otherwise be phosphorylated is one illustration of the complex interplay among eukaryotic post-translational modification systems (Caragea C, Sinapov J, Silvescu A, Dobbs D, Honavar V: Glycosylation site prediction using ensembles of Support Vector Machine classifiers. *BMC Bioinformatics.* 2007 Nov 9; 8:438.)

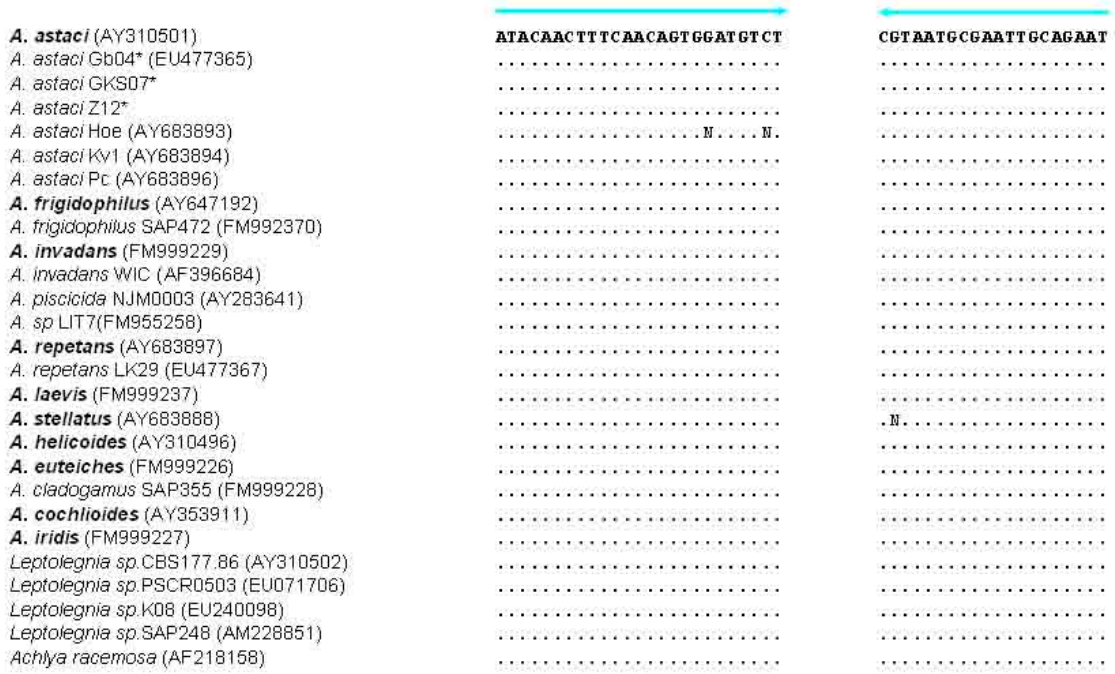
Thresholds of > 0.5 and ≥ 0.4 for potential phosphorylated and O-linked glycosylated sites, respectively.

Sites for which both types of modification were predicted are highlighted in yellow. Bold letters: modified residue.

position in Chi2	site	score	
		phosphorylation	O-glycosylation
15	VVAM**T**SSVE	0.67	no
16	VAMT**S**SVEA	0.54	no
17	AMTS**S**VEAA	0.60	no
71	VHDG**Y**AAVQ	0.85	no
93	GNNL**Y**GNFG	0.85	no
84	GRGD**S**WNDQ	1.00	no
109	KARG**T**KFGL	0.94	no
114	KFGL**S**IGGW	0.79	no
121	GWTL**S**DKFS	0.51	no
125	SDKF**S**SIAS	0.55	no
126	DKFS**S**IAST	0.83	no
129	SSIA**S**TETG	0.98	no
132	ASTE**T**GRRT	0.94	no
140	TFAK**S**SVKL	0.73	no
141	FAKS**S**VKLM	0.85	no
204	LSVA**S**PAGP	0.92	no
236	DLAG**S**WSK	0.57	no
240	SWSK**Y**TDHQ	0.97	no
248	QANL**Y**EDPN	0.94	no
294	SNGL**Y**SNFT	0.96	no
329	ATEI**Y**DEKL	0.76	no
342	SYDP**T**SKIF	0.62	no
343	YDPT**S**KIFT	0.98	no
347	SKIF**T**SYEG	0.76	no
348	KIFT**S**YEGP	0.98	no
361	QKLD**Y**IKQY	0.72	no
381	ADAK**S**GSPR	0.76	no
383	AKSG**S**PRSL	0.99	no
386	GSPR**S**LITQ	0.77	no
414	PTSQ**Y**ANIR	0.91	no
424	GAAV**T**SAVP	no	0.50
425	AAVT**S**AVPV	no	0.73
430	AVPV**T**SSPV	no	0.88
431	VPVT**S**SPVA	no	0.72
438	VAPV**T**TVAP	no	0.48
439	APVT**T**VAPV	0.69	0.75
444	VAPV**T**SEVP	no	0.69
445	APVT**S**EVPV	no	0.97
450	EVPV**T**SSPA	no	0.81

451	VPVT**S**SPAA	no	0.68
458	AAPV**T**TVAP	no	0.65
459	APVT**T**VAPV	0.69	0.81
464	VAPV**T**SEVP	no	0.85
465	APVT**S**EVPV	no	0.97
470	EVPV**T**SSPD	no	0.65
471	VPVT**S**SPDA	0.99	0.50
472	PVTS**S**PDAP	0.58	no
478	DAPV**T**TVAP	no	0.50
479	APVT**T**VAPV	0.69	0.75
484	VAPV**T**TVAP	no	0.77
485	APVT**S**EVPV	no	0.97
490	EVPV**T**SSPD	no	0.65
491	VPVT**S**SPDA	0.99	0.50
492	PVTS**S**PDAP	0.58	no
498	DAPV**T**TVAP	no	0.56
499	APVT**T**VAPV	0.69	0.80
504	VAPV**T**SAVH	no	0.79
505	APVT**S**AVHV	no	0.83
510	AVHV**T**CSSY	no	0.68
514	TCSS**Y**APVT	0.66	no
518	YAPV**T**SSAV	no	0.45
519	APVT**S**SAVP	no	0.53
525	AVPE**T**TPVE	no	0.67
526	VPET**T**PVEP	0.99	0.52
536	TTEA**T**PAPT	0.92	0.76
532	VEPV**T**TEAT	no	0.84
533	EPVT**T**EATP	no	0.75
540	TPAP**T**GGPI	no	0.85
550	NPLE**T**LAPT	0.55	0.67
554	TLAP**T**TTAA	no	0.40
556	APTT**T**AAAG	no	0.48

position in Chi3	site	score	
		phosphorylation	O-glycosylation
15	VVAM**T**SSVE	0.67	no
16	VAMT**S**SVEA	0.54	no
17	AMTS**S**VEAA	0.60	no
84	EHGD**S**WNDQ	1.00	no
93	GNSL**Y**GNFG	0.86	no
109	KARG**T**KFGL	0.94	no
114	KFGL**S**IGGW	0.79	no
129	SSIA**S**TETG	0.98	no
132	ASTE**T**GRRT	0.94	no
140	TFAK**S**SVKL	0.73	no
141	FAKS**S**VKLM	0.85	no
204	LSVA**S**PAGP	0.92	no
236	DLAG**S**WSK	0.57	no
240	SWSK**Y**TDHQ	0.97	no
248	QANL**Y**EDPN	0.94	no
259	PGAK**Y**SSHN	0.76	no
268	AVQD**Y**IKGG	0.52	no
296	SNGL**Y**SNFT	0.96	no
332	ATEI**Y**DEKL	0.76	no
345	SYDP**T**SKIF	0.62	no
346	YDPT**S**KIFT	0.98	no
350	SKIF**T**SYEG	0.76	no
351	KIFT**S**YEGP	0.98	no
364	QKLD**Y**IKQY	0.72	no
384	ADAK**S**GSPR	0.76	no
386	AKSG**S**PRSL	0.99	no
389	GSPR**S**LITQ	0.77	no
417	PTSQ**Y**ANIR	0.91	no
426	AGAV**T**TVAP	no	0.57
427	GAVT**T**VAPV	0.78	0.51
432	VAPV**T**SAAP	no	0.92
433	APVT**S**AAPV	no	0.80
438	AAPV**T**SAAP	no	0.93
439	APVT**S**AAPV	no	0.99
444	AAPV**T**SSTA	no	0.95
445	APVT**S**STAP	no	0.93
446	PVTS**S**TAPG	no	0.58
447	VTSS**T**APGT	no	0.73
451	TAPG**T**TPAT	no	0.90
452	APGT**T**PATP	0.51	0.89
455	TTPA**T**PVTT	0.68	0.88
458	ATPV**T**TRAT	0.80	0.97
459	TPVT**T**RATP	no	0.85
462	TTRA**T**PAPT	0.97	0.84
466	TPAP**T**GGPI	no	0.90
471	GGPI**T**NPPV	no	0.52
476	NPPV**T**SAPT	0.70	0.96
477	PPVT**S**APTT	no	0.41
480	TSAP**T**TTAA	no	0.64
481	SAPT**T**TAAA	0.52	no
482	APTT**T**AAAG	no	0.58
508	TLPY**S**QADC	0.77	no



Additional file 5.

Alignment of primer sites for the endogenous control *5.8S rRNA* amplicon used in qPCR/MCA. Asterisks denote *A. astaci*-strains isolated in this work. Parentheses contain GenBank accessions. Oomycete reference strains [1] are given in bold. Dot: identical nucleotide with the *A. astaci*-sequence.

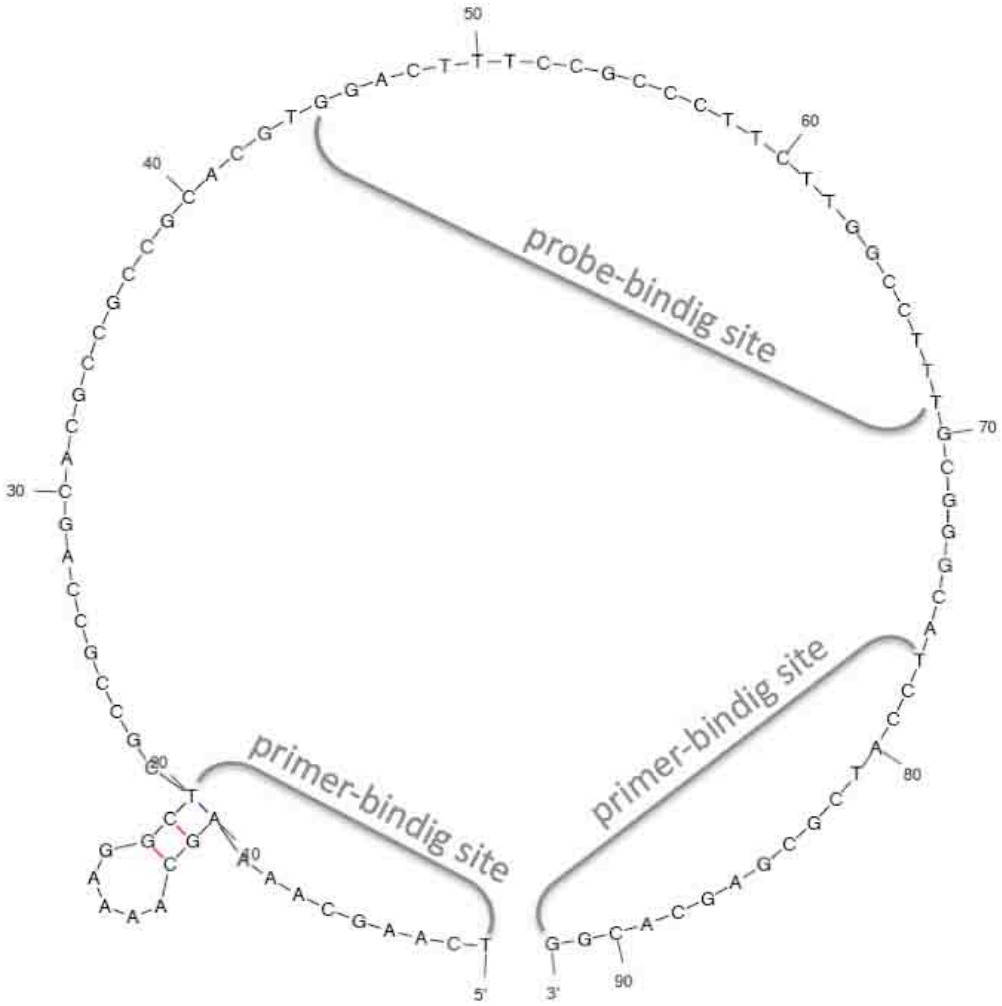
References

1. Dieguez-Uribeondo J, Garcia MA, Cerenius L, Kozubikova E, Ballesteros I, Windels C, Weiland J, Kator H, Soderhall K, Martin MP: **Phylogenetic relationships among plant and animal parasites, and saprotrophs in Aphanomyces (Oomycetes)**. *Fungal Genet Biol* 2009, **46**(5):365-376.

Additional file 6.
A conventional PCR assay for detection of *A. astaci* that may fail to discriminate between closely related species.
Alignment of target sites for the *ITS2*-primers 525 (5'-AAGAAGGCTAAATTGCGGTA) and 640 (5'-CTATCCGACTCCGCATTCTG) from [1]. Oomycete reference strains [2] are given in bold. Dot: identical nucleotide with the *A. astaci* sequence; gap: deletion.

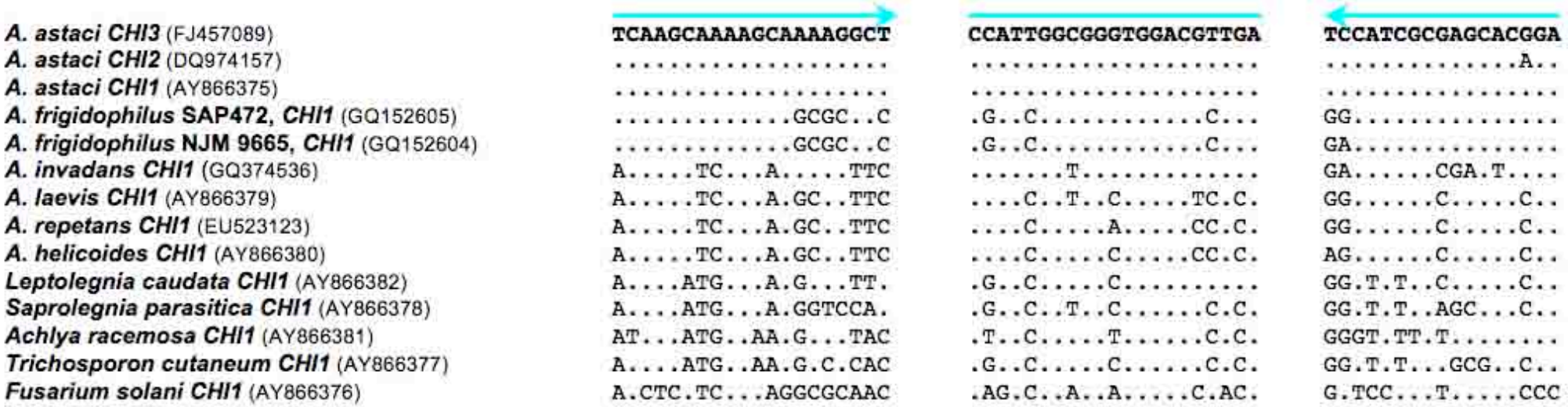
References

1. Oidtmann B, Schaefers N, Cerenius L, Söderhäll K, Hoffmann RW: **Detection of genomic DNA of the crayfish plague fungus Aphanomyces astaci (Oomycete) in clinical samples by PCR**. *Vet Microbiol* 2004, **100**(3-4):269-282.
2. Dieguez-Uribeondo J, Garcia MA, Cerenius L, Kozubikova E, Ballesteros I, Windels C, Weiland J, Kator H, Soderhall K, Martin MP: **Phylogenetic relationships among plant and animal parasites, and saprotrophs in Aphanomyces (Oomycetes)**. *Fungal Genet Biol* 2009, **46**(5):365-376.



Additional file 7:
Secondary structure of a homologous (competitive) IPC which can be used for coextraction and/or coamplification with the clinical samples. The Mfold web server [1] was used for DNA folding prediction. The sequence of the 93 bp-IPC sequence (TCAAGCAAAAGCAAAAGGCTCGCCGCCAGCACGCCGCCGCACGT**GGACTTTCCGCCCT TCTTGGCCTTT**GCGGGCATCCATCGCGAGCACGGA) is compatible with the qPCR/MCA and the TaqMan qPCR assays developed in this work. The target sites of the primers used in the qPCR/MCA and the qPCR assays for *A. astaci*-detection are underlined. Note that the 16 bp-reverse primer used in qPCR/MCA is shortened by a base at its 5' end compared to the reverse primer of TaqMan qPCR. Bold letters denote the site targeted by the hydrolysis probe sequence DYE-AAAGGCCAAGAAGGGCGGAAAGTCC-BHQ to monitor the IPC in the TaqMan qPCR assay designed for *A. astaci*-quantification.
In the dye-based qPCR/MCA assay the IPC can be differentiated by a 3 grd higher melting temperature from the *A. astaci*-specific chitinase peak. This was predicted by the Nearest Neighbor method implemented in the online oligonucleotide properties calculator OligoCalc [2].

References:
1. Zuker M: **Mfold web server for nucleic acid folding and hybridization prediction**. *Nucleic Acids Res* 2003, **31**(13):3406-3415.
2. Kibbe WA: **OligoCalc: an online oligonucleotide properties calculator**. *Nucleic Acids Res* 2007, **35**(Web Server issue):W43-46.



Additional file 8.

TaqMan qPCR assay design for sensitive detection and quantification of *A. astaci*.

Target sites of primers and probe are highlighted by arrows and line, respectively. Parentheses contain GenBank accession numbers. Primers of the TaqMan qPCR and qPCR/MCA assays are identical except the deleted nucleotide at the very 5' end of the reverse primer used in qPCR/MCA (Figure 5a).